PHD

The biotransformation of diosgenin and its precursors extracted from Trigonella foenumgraecum L. seed.

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THE BIOTRANSFORMATION OF DIOSGENIN AND ITS PRECURSORS EXTRACTED FROM *TRIGONELLA FOENUMGRAECUM* L. SEED

Submitted by Roger Saunders
for the degree of Doctor of Philosophy
of the University of Bath

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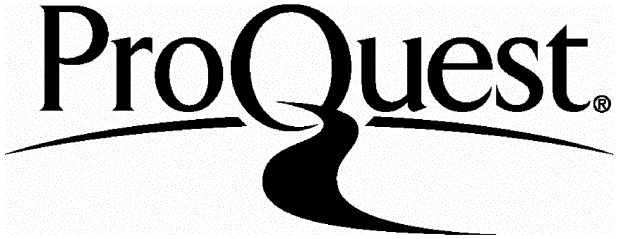

SUMMARY

In the introduction, the sources and use of diosgenin and other steroid raw materials in the Pharmaceutical Industry are reviewed. The biotransformations of steroids, and in particular diosgenin, are described.

Microorganisms, selected from natural sources and from culture collections, were screened for activity against diosgenin. Species were found which had not been reported to attack the steroid. Biotransformation products of diosgenin were isolated and their structures elucidated. The major products were diosgenone and 1-dehydrodiosgenone; androstenes were also formed.

The formation of diosgenone from diosgenin was shown to be mediated by a cholesterol oxidase enzyme which was inducible in growing cells.

Methods were investigated to prevent the total degradation of the diosgenin steroid nucleus by Mycobacterium phlei. Addition of the chelating agent, α, α'-bipyridyl, resulted in a 7% yield of androstenes. However, mutagenesis of M. phlei with N-methyl-N'-nitro-N-nitrosoguanidine was shown to be of more promise.

To optimise the biotransformation process, the effects of the physical form of the diosgenin and the physiological condition of the microorganisms (including their immobilisation in calcium alginate) were studied. Addition of an ethanolic solution of diosgenin to stationary phase cells in a rich medium, was found to give the best rate of biotransformation.

Methods were investigated to increase the solubility of diosgenin in the fermentation medium. Water-immiscible organic solvents allowed an

increased initial biotransformation activity with Nocardia rhodochrous and M. phlei. The use of crude water-soluble glycosidic precursors of diosgenin resulted in a different pattern of biotransformations. Fusarium solani produced diosgenin (84% efficiency), thus avoiding an acid hydrolysis step.

Silastic resin was used in microbial fermentations. It provided a novel and convenient method for the collection of hydrophobic products from biotransformations. Diosgenin, produced from its glycosidic precursors by F. solani, was collected by the resin and transferred in it to a culture of M. phlei which further transformed the diosgenin to androstenes. These remained within the resin and could be readily recovered from it with solvents. Silastic resin offers considerable advantages in its retention of intermediate and then final product, in a sequence of biotransformations with successive organisms.

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CHAPTER 1

INTRODUCTION

SECTION A: Diosgenin - sources, biosynthesis and uses.

Diosgenin [(25R)-spirost-5-en-3β-ol] is a steroidal plant sapogenin of molecular weight 414.6 g. It has a melting point of 204 - 207°C a $[\alpha]_D^{25}$ - 129° (Merck Index, 1968) and forms white, needle shaped crystals from ethanol.

Diosgenin was first isolated from the tuber of Japanese Dioscorea (D. tokoro) by Tsukamoto and Ueno (1936) and later characterized (Tsukamoto et al., 1936; Huzii and Matukawa, 1937). It is of considerable importance to the pharmaceutical industry as a major precursor for synthesis of steroidal drugs, including corticosteroids and contraceptives. The structure is given in Figure 1. Frequently the word diosgenin is also used for the mixture of diosgenin and yamogenin [the (25S)-epimer] as isolated from natural sources (Hardman, 1975).

Major Sources of Diosgenin-Dioscorea

Species of the genus Dioscorea (yams) are the most important and the only commercially exploited source of diosgenin (Coppen, 1979). Many of them contain diosgenin but few have the 3% (w/v) content generally considered to be the lower limit for economic exploitation. A number of species of Dioscorea have been listed by Coursey (1967) and Martin (1969). Those used commercially at present are D. composita Hemsl., 'barbasco'; D. floribunda Mart and Gal; D. deltoidea Wall; D. prazeri Prain and Burk., with the first two species occurring naturally in Mexico and Guatemala and the others in the temperate Himalayan regions of India, Nepal and China (Coursey, 1967; Coppen, 1979). The tubers average

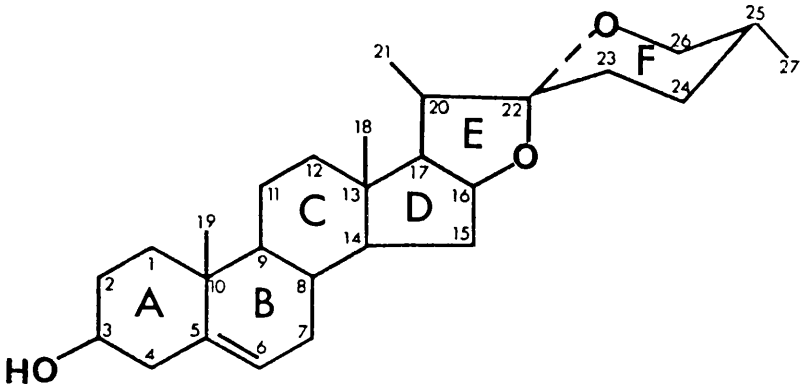


Figure 1. Diosgenin (25R)-spirost-5-en-3$_\beta$-ol

between 4% and 6% diosgenin on a moisture free basis (Hardman, 1969).

In general the wild plants are harvested by hand after a minimum of 3-4 years growth and processed in the country of origin. Although this procedure seems to be inefficient, it had been considered, until recently, that cultivation of dioscorea was economically impractical because of its labour intensive nature. Manual labour is required for weeding and harvesting the tubers, and also for building and maintaining the supporting framework necessary for the cultivated dioscorea vines. At least 3-4 years growth was thought necessary before harvest to achieve reasonable diosgenin yields. For a discussion of the economic and agronomic problems see Blunden *et al.* (1975). Cultivation studies have been carried out in India (Bammi, 1972) and in Puerto Rico and Florida (Martin and Gaskins, 1968). Despite the problems Ciba-Geigy are now cultivating *D. floribunda* and *D. composita* on a commercial scale in Goa, India, harvesting after 3 years growth (Nair, 1979), and yields of 520 kg diosgenin per hectare after 2 years growth have been achieved with experimental plantations (Coppen, 1979). This makes cultivation of dioscoreas a feasible source of diosgenin supply, especially when coupled with the low labour costs in India.

Other Sources of Diosgenin

An extensive survey of over 400 plant species in the southern United States and Mexico was carried out by Russell Marker looking for sapogenins. The results were first published as brief notes and later in a full paper (Marker *et al.*, 1947). A further survey of 6,000 plants was later conducted by the United States Department

of Agriculture (Wall et al., 1961). Of the many plants surveyed only a few contained sufficient diosgenin to be of potential commercial interest and none except the dioscoreas are at present being used industrially for diosgenin (COppen, 1979).

One potential source, currently under investigation at the University of Bath is the fenugreek plant Trigonella foenumgraecum, L. This is an annual legume occurring naturally in some mediterranean, middle and far eastern countries such as Morocco, Egypt, Saudi Arabia, Pakistan and India (Fazli and Hardman, 1968). The seed of varieties under cultivation at Bath generally yield about 1 - 1.4% diosgenin on a moisture-free basis (Hardman and Cornish, 1981). The interest in fenugreek derives from its ability to be cultivated in temperate countries. Furthermore the crop can be planted and harvested in a single season. Although the diosgenin content of the seed is lower than that of the Dioscorea tuber the crop is made more economically attractive by its potential for use as forage and the seed as a source of fixed oil, protein and mucilage. After root nodulation with Rhizobium meliloti, it will fix atmospheric nitrogen and thus enrich the land (Hardman, 1975). A breeding programme to isolate high-yielding varieties is being undertaken at the University of Bath and agricultural trials are taking place throughout Britain this year (1981).

The fruits of various wild species of Balanites found in Africa, Burma and India contain 4-8% diosgenin in their outer parts and rather less in the seed (0.5-3%) (Hardman and Wood, 1971; Hardman and Sofowora, 1972). This source has been employed for

diosgenin by the British Drug Houses (now part of the Glaxo Group, Hardman, 1975). Glaxo are at present investigating another alternative source, solasodine, a nitrogen analogue of diosgenin. This is extracted from species of Solanum, in particular S. khasianum Clarke, grown in India.

A further potential plant source of diosgenin is Costus speciosum Sm. which yields 0.6-2.6% diosgenin in its rhizomes (Sarin et al., 1974) when grown in India. Work is in progress towards the large scale introduction of the plant with a view to commercial utilization.

Recent reviews about plants as sources of sapogenins include Blunden et al, 1975; Hardman, 1969 and 1975. Plant tissue culture has also been considered as a diosgenin source. It has been shown that species of Solanum and Dioscorea could be grown on solid nutrient media as callus tissue with some steroid contents and also that these could be propagated in a fermenter by submerged culture. However, calculations by Kieslich (1980) show that to be economical yields of 2-4 kg diosgenin per cubic metre per 24 hours incubation are necessary. The optimum rate of formation of diosgenin at present is 0.05-0.07 kg per cubic metre in 24h (Kieslich, 1980).

Biosynthesis of Diosgenin

The biosynthetic route for the formation of sapogenins and in particular diosgenin is now well established. The pathway is common to all major phytosterols and sapogenins up to the formation of cycloartenol (4,4,14α-trimethyl-9β,19β-cyclo-5α-lanost-24-en-3β-ol). This compound forms the branch point at which cholesterol biosynthesis

separates from the other phytosterols (Figure 2). Sapogenins are synthesised by plants mainly from cholesterol (Heftmann, 1967,1968), although evidence exists of a route from sitosterol (Stohs et al., 1974). Hardman and Fazli (1972) have confirmed the former pathway operates in Trigonella foenumgraecum. There are a number of recent detailed reviews of the biosynthetic process (Heftmann, 1973; Grunwald, 1975, 1980).

In plants the sapogenins occur in the form of their glycosides, the saponins. Generally it is the hydroxyl group at C-3 which is glycosylated. An example of this is dioscin, isolated from Dioscorea tokoro Makino (Fieser and Fieser, 1959) (Figure 3). The biosynthesis of saponin from sterol follows closure of the side chain resulting in formation of saponin rings E and F. However Marker and Lopez (1947) suggested that saponins may occur in the open side chain form. Direct evidence of this came later with the isolation of compounds in which ring E was closed but ring F was open (Heftmann, 1967; Tschesche et al., 1967; Joly et al., 1969a). These compounds are named furostanols; those with closed side chain (e.g. dioscin), spirostanols. In all cases of furostanol isolated so far the F ring is kept open by a 26β D-glucose unit. For example furost-5-en-3β,22α,26βtriol 3β chacotrioside 26β-D-glucopyranoside (see Figure 3) isolated by Joly et al.(1969a) from Dioscorea floribunda and later by Kawasaki et al. (1974) from Dioscorea gracillima. This compound is obviously a precursor of dioscin and can be converted to it by plant tissue extracts (Joly et al., 1969b). It could be hypothesized that the furostanol is the naturally occurring form and that the spirostanol is an artefact of the extraction process.

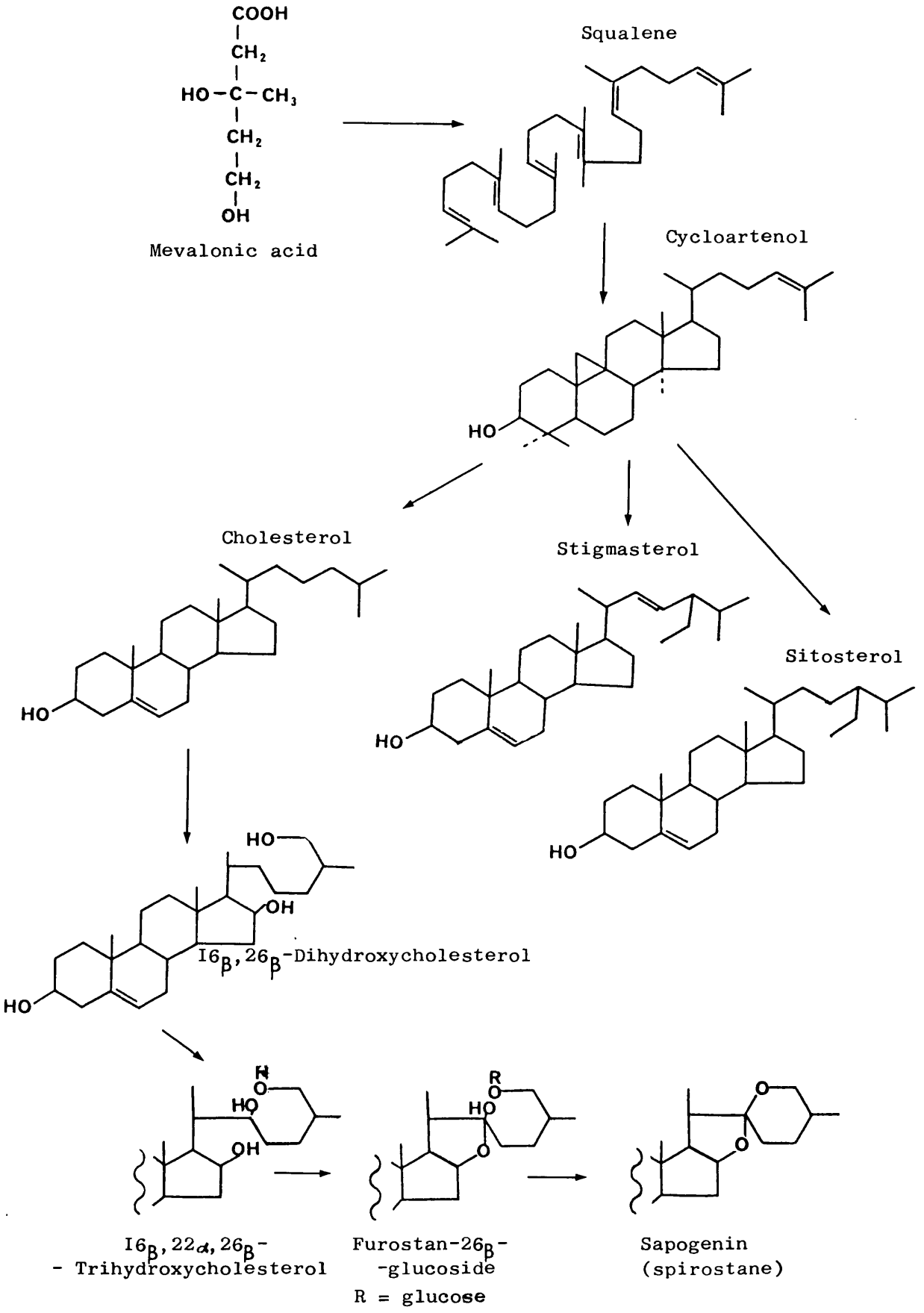


Figure 2. The biosynthesis of diosgenin

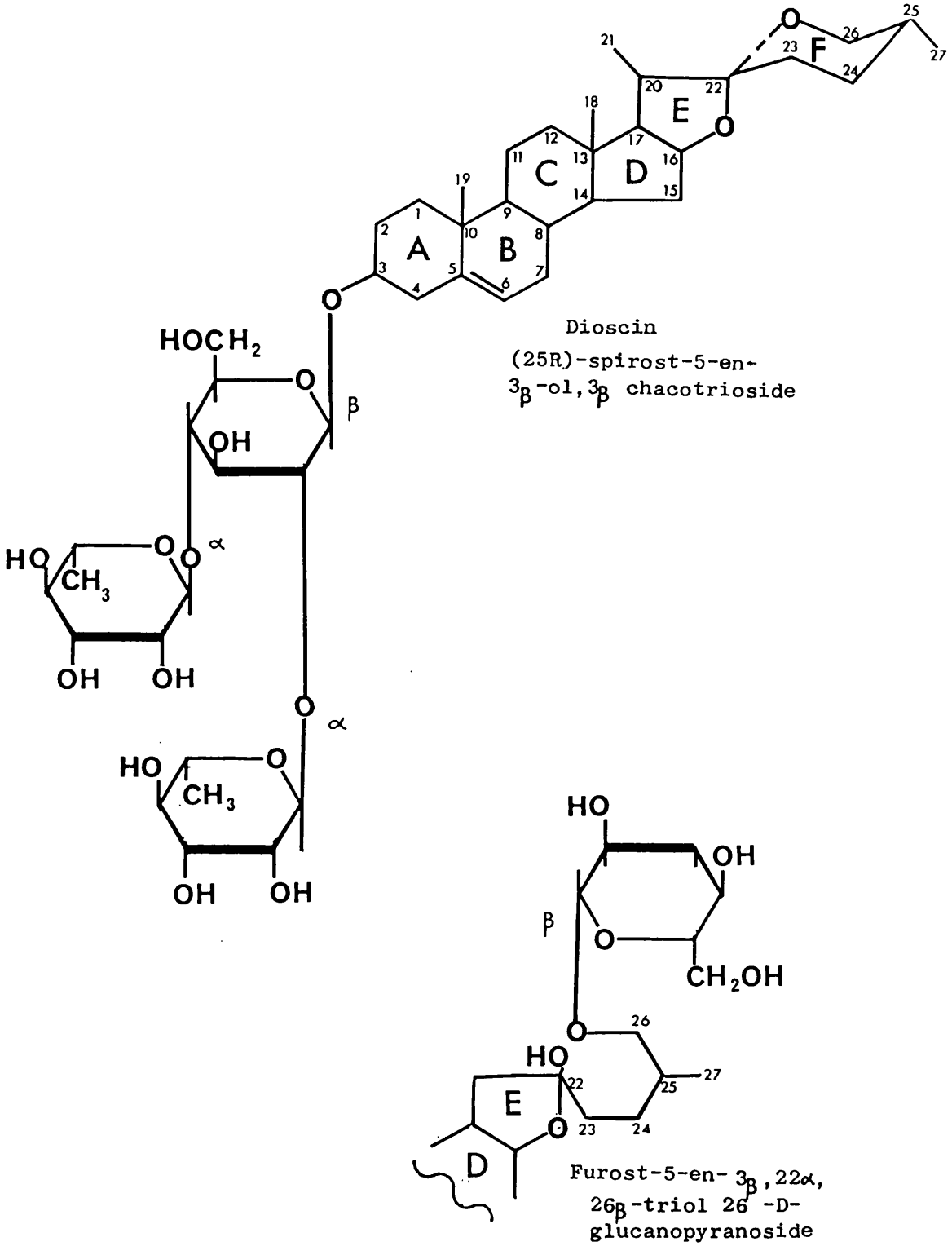


Figure 3.Spirostanol and furostanol glycosides.

Most workers however suggest that both co-exist *in vivo*.

Furostanols have been isolated from *Trigonella foenumgraecum* seed including the furostanol mentioned above, named Trigonelloside C, by Bogacheva *et al.* (1976). A different furostanol which has the trisaccharide of L-rhamnosyl (1-2)-[D-glucopyranosyl (1-3)]-D-glucanopyranoside at the C-3 position of 22-methoxy-5α-furostan-3β,26 diol has also been isolated from fenugreek seed using methanol. Treatment with boiling water gives the 22-hydroxy compound, which is probably its naturally occurring form (Hardman *et al.*, 1980). Another glycoside has been reported (Gangrade and Taushal, 1979) with glucose, rhamnose and arabinose sugars at the C-3. The authors do not specify whether the compound was a furostanol or a spirostanol.

One of the important physical properties of all these glycosides is their solubility in aqueous solvents, which sapogenins lack. The commonest solvents used in the extraction process are ethanol (Marker *et al.*, 1947; Wall *et al.*, 1952) and methanol. However this type of extract contains a complex mixture from which the saponins are hard to purify. Prior acid hydrolysis of the glycosides in the plant tissue converts them into sapogenins which can be extracted by a suitable solvent in a much purer form. This is the commercial method of diosgenin production.

Processing for Diosgenin

On an industrial scale the yams are broken up and dried, this material is then acid hydrolysed and the acid insoluble matter collected and neutralised. Extraction with heptane by soxhlet

apparatus yields sapogenin (Hardman, 1969). A similar process is used at Bath for the extraction of diosgenin from fenugreek seed (see Materials and Methods, Chapter 2).

The diosgenin collected is subjected to chemical degradation according to a process developed by Marker (Marker et al., 1940) and changed only in detail since. It involves the elimination of a six-carbon fragment from the side chain to leave a pregnane (Marker et al., 1947). When diosgenin, as its acetate derivative is heated with acetic anhydride at 200° ring F is cleaved with the introduction of a 20,22 double bond to give the pseudosapogenin or furostadiene derivative. Chromic acid oxidation converts this into the keto ester diacetate, which on cleavage by boiling acetic acid gives 16-dehydropregnenolone (3β-hydroxypregna-5,16-dien-20-one) acetate. This is converted by selective hydrogenation into pregnenolone acetate (Figure 4). This can then be converted to progesterone by Oppenauer oxidation, a process which involves alkoxide exchange between the alcohol oxidised and the aluminium alkoxide present $\left[Al(OC_3H_7)_3\right]$. At first yields from the process were 33% of the initial weight of diosgenin (Fieser and Fieser, 1959). However, changes in the reagents and conditions have brought the yield of 16-dehydropregnenolone up to 80% (Hardman, 1975). It is from Marker's end product that a whole range of important steroidal compounds are manufactured including the corticosteroids and oral contraceptives (see Figures 5 and 6).

Despite its efficiency the chemical degradation is expensive as the process involves using both high temperatures and pressures.

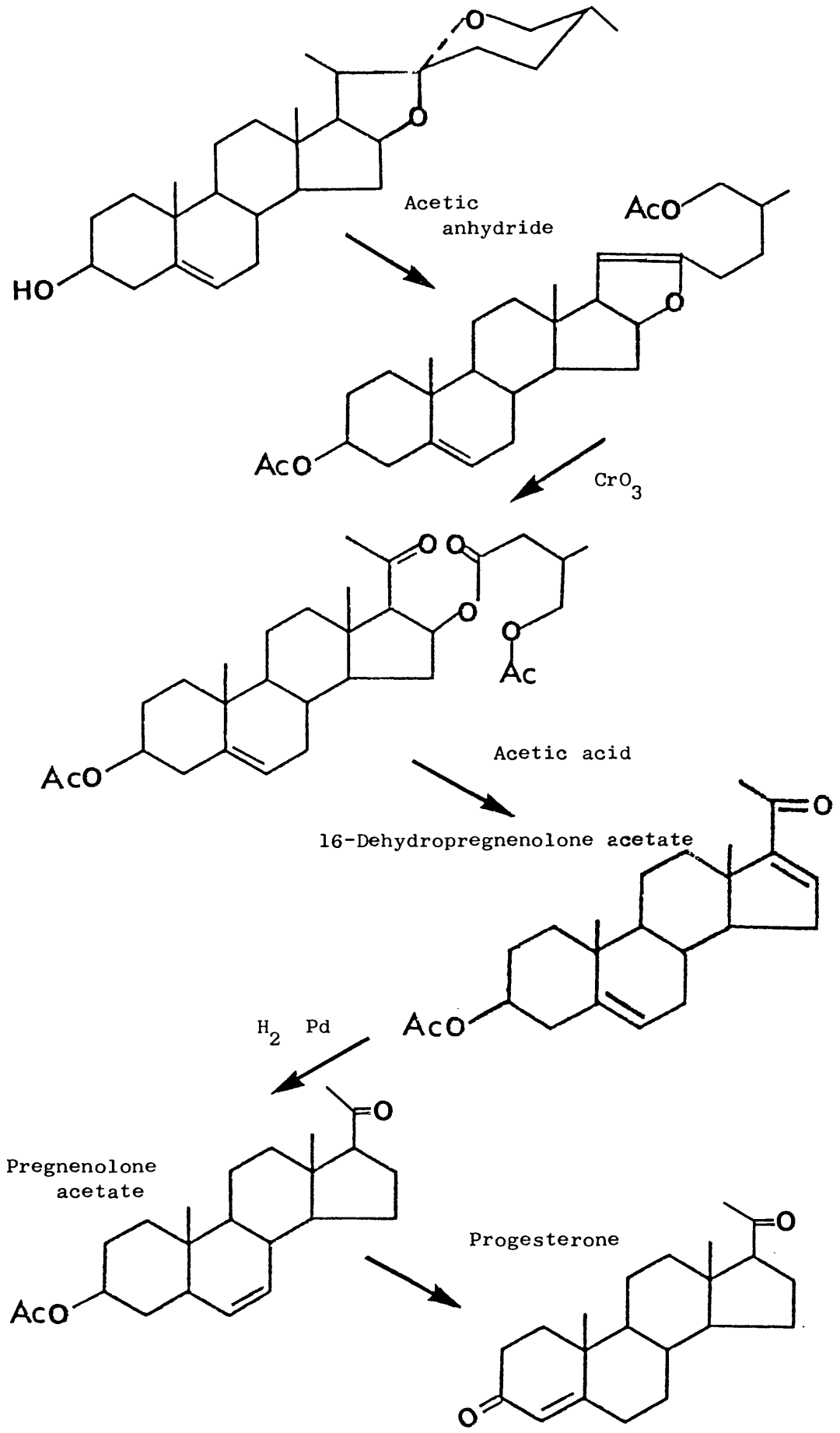


Figure 4.Marker's degradation of diosgenin.

Diosgenin in the Pharmaceutical Industry

In 1944 Marker established the company Syntex in Mexico to produce progesterone from locally extracted diosgenin. At this time there was not a large market for the product. The discovery in 1949 of the ability of cortisone (17α,21-dihydroxypregn-4-ene-3,11,20-trione) to suppress the symptoms of rheumatoid arthritis (Hench et al., 1950) stimulated a much greater demand. In 1952 it was found that certain microorganisms were capable of introducing an 11-oxygen function into steroid structures, so allowing corticosteroids as well as the sex hormones to be produced from the same precursor - diosgenin (Peterson et al., 1952). It was also in the 1950's that Djerassi, working on 19-nor steroids, introduced the idea of oral contraception, thus firmly establishing a very large market for steroidal drugs and diosgenin as the preferred starting point.Since this beginning the consumption of steroids has steadily risen. In 1976 (latestfigures available) 1025 tonnes of diosgenin equivalent (tde)were used in corticosteroid production, 235 tde for contraceptives, 162 tde for oestrogens, androgens etc. and 199 tde for spironolactone (a diuretic),giving a total consumption of 1621 tde and a world wide market of over $300 million per year (Coppen, 1979; Kieslich, 1980). The relative proportions of the products have stayed almost constant since 1963, although only 500 tde were consumed at that time.

Diosgenin retained its pre-eminent position as the major raw material until the 1970's. Soon after the establishment of Syntex the Mexican government prohibited export of diosgenin and its early transformation products, making the country an industrial processing

centre. In 1975 the government took direct control over the collection and sale of the diosgenin yielding yams, later considerably increasing their price (Applezweig, 1976). This caused the multinational drug companies to pursue vigorously alternative sources of raw materials for the production of steroidal drugs. These efforts have been successful and in 1978 it was estimated that Mexican diosgenin accounted for only 11% of the market compared with 75% in 1963 and 42% in 1973 (Coppen, 1979).

Alternatives - Total Synthesis

Steroids are essentially hydrocarbons and their total synthesis offers independence from natural raw materials. A major problem in steroid synthesis is formation of the 'correct' isomer with natural active configuration. Processes have been developed for certain syntheses which include a microbiological step to aid formation of the correct isomer, for example those of Schering and Roussel (Kieslich, 1980).

Although total synthesis is used in only a few cases and is not feasible for corticosteroids (Applezweig, 1976) two of the most widely used contraceptive components, norethisterone and norgestrel, are manufactured this way. Total synthesis accounted for 11% of steroid manufacture in 1976 (Coppen, 1979).

Alternative Raw Materials to Diosgenin

Sterols: Sterols are the most widely distributed group of steroids in the plant kingdom, commonly occurring as mixtures of sitosterol, campesterol and stigmasterol. The chemical transformation of soyabean

stigmasterol to progesterone was developed in the 1950's (Poulos et al., 1961; Applezweig, 1976). Stigma sterol is still the compound most used industrially for chemical degradation to useful intermediates. Sources are available comparatively cheaply as waste products.

The use of these plant sterols and also cholesterol from animal sources has been the area of greatest change recently in the steroid industry. A number are now used in industrial processes (Figures 5 and 6). The principal biotransformation of commercial interest is the removal of sterol C-17 side chain to yield an androstane nucleus which can then be used as a precursor for steroidal drug manufacture. In an elegant series of experiments Sih and his collaborators (1967a, b; 1968, a, b) completely elucidated the pathway of side chain degradation. They found that microorganisms of the genera Nocardia, Arthrobacter, Mycobacterium and Corynebacterium appear to shorten the side chain by a mechanism similar to β-oxidation of fatty acids (Figure 7). Unfortunately at the same time as degrading the C-17 side chain these organisms also cleaved the steroidal ring structure. The various methods of preventing this are reviewed later (Chapter 5).

Cholesterol and sitosterol can be transformed to androst-4-ene-3,17-dione (AD) or androsta-1,4-diene-3,17-dione (ADD) in a single step microbiological reaction (Arima et al.,,1968).Sterol residues accumulated from processing soya-derived stigmasterol and cholesterol derived from wood grease, both waste products, can now be utilized. Searle started commercial production of AD from sitosterol in 1976

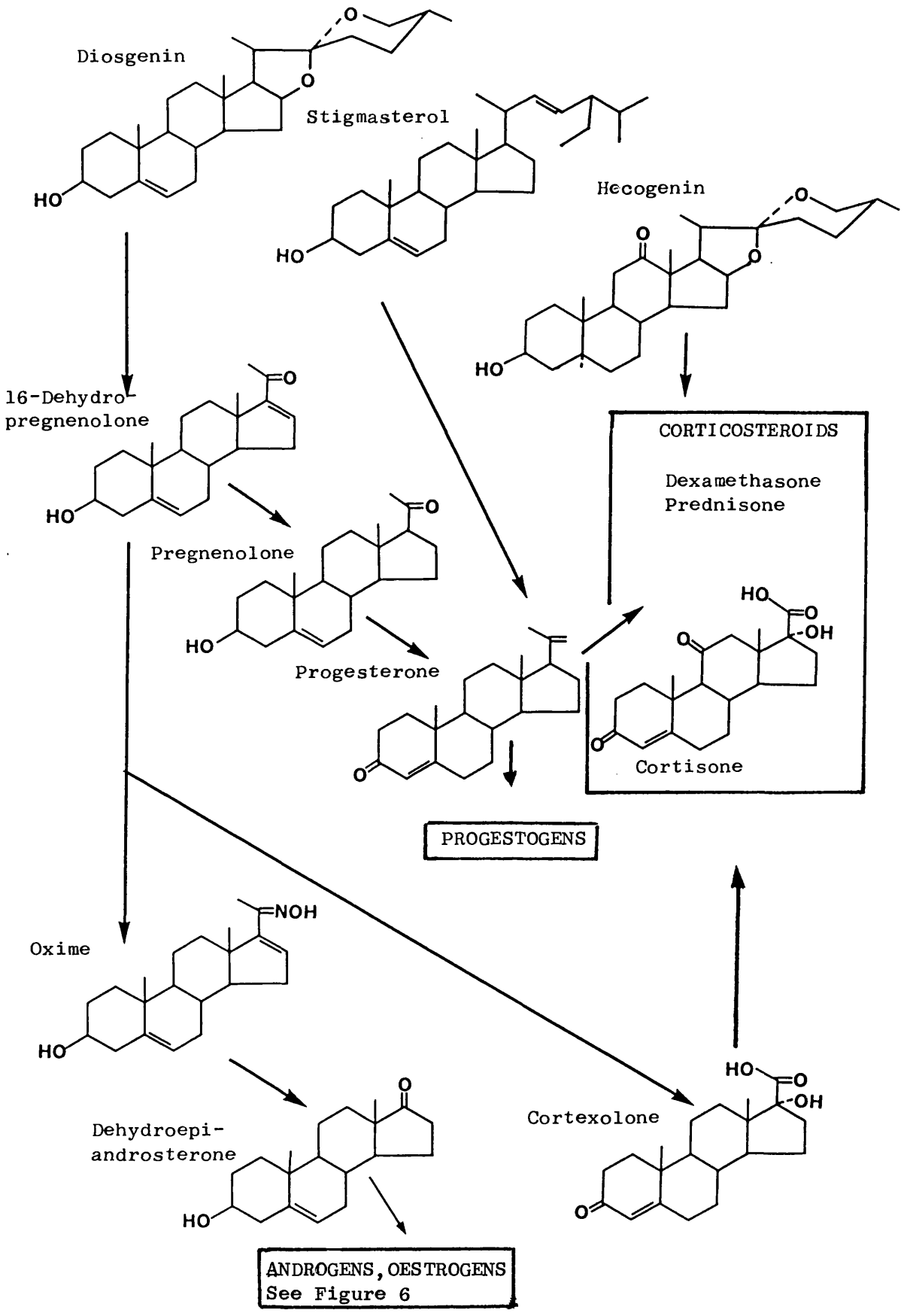


Figure 5.Main routes in the production of steroid drugs (i)

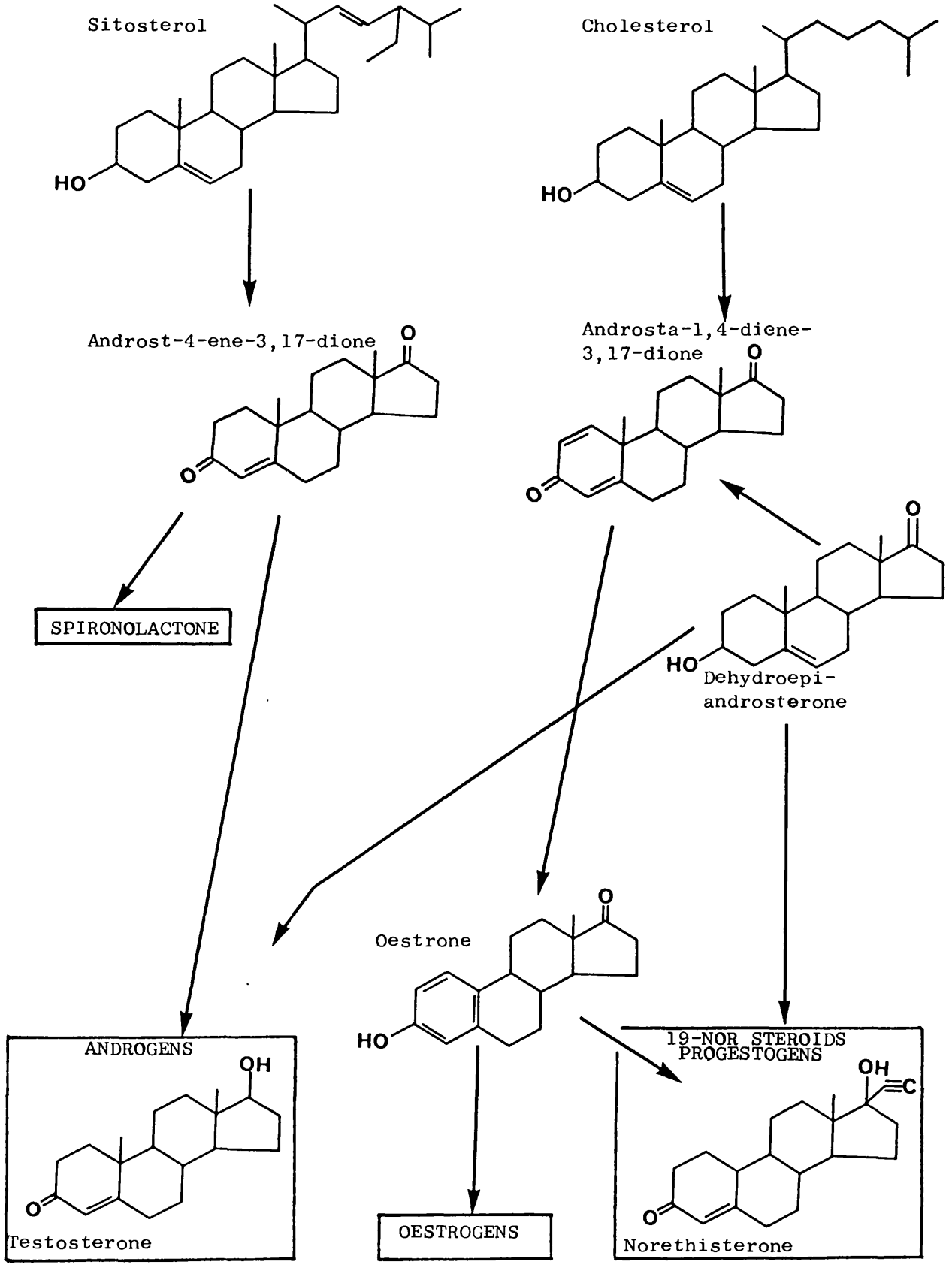


Figure 6.Main routes in the production of steroid drugs (ii).

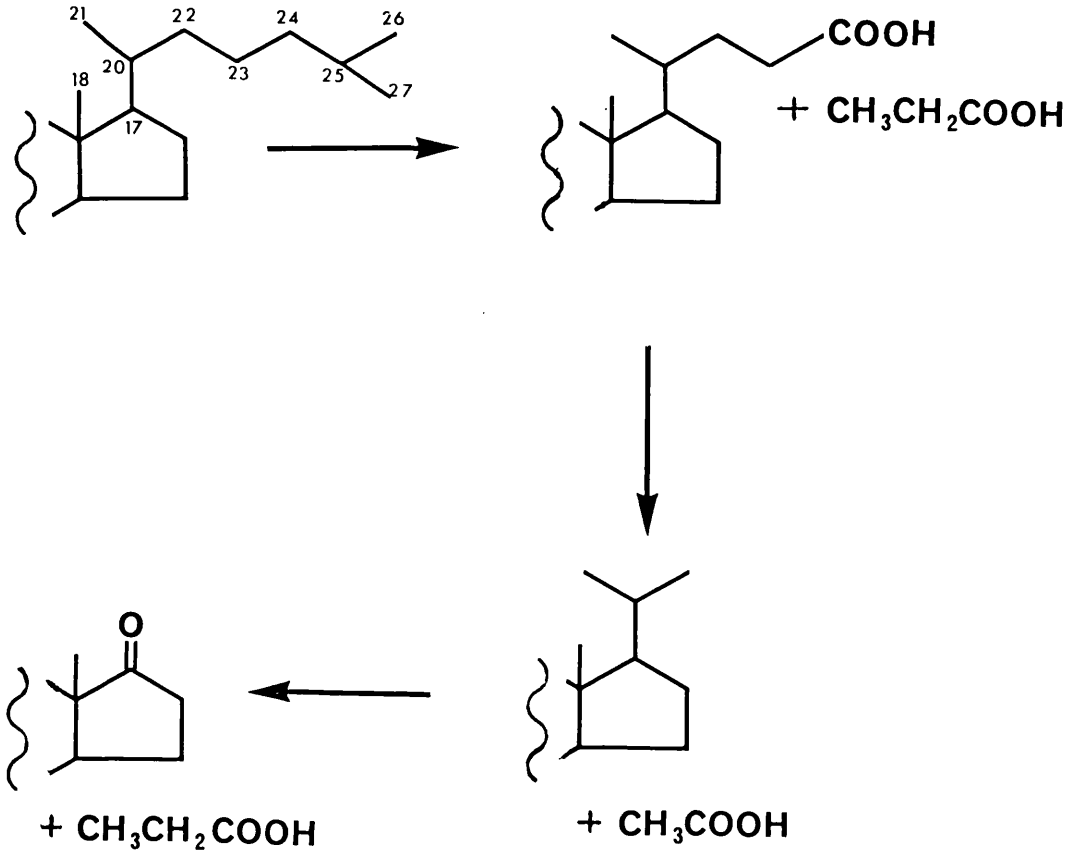


Figure 7. Microbial side chain cleavage of sterols.

(Anon, 1975) and Mitsubishi have recently started a similar process using cholesterol (Coppen, 1979). This completes a cycle from the use of cholesterol as a precursor using chemical degradation (Schering A.G. 1935-1956) which was later abandoned as uneconomic (Kieslich, 1980). A number of other manufacturers (Gist-Brocades, Schering, Upjohn) also have methods based on the microbiological transformation of sitosterol and stigmasterol from seed oils (e.g. soya bean and sunflower). Workers at Searle first reported the microbial formation of androstanes from stigmasterol (Marscheck et al., 1972) and later patented it (Marscheck and Kraychy, 1973a, 1973b; Kraychy et al., 1972).

Upjohn have a patented microbiological method which produces 9α-hydroxyandrosta-4-ene-3,17 dione from sitosterol (Wovcha, 1977). This can readily be converted to a 9α-halo-11β-hydroxyandrostane. The presence of the 11 oxygen function obviates the need for its introduction by a second microbiological step and makes it particularly suitable for conversion to corticosteroids.

Conner et al. (1976) described a process using tall oil (a by product of the Kraft pulping of pinewood chips) as a source of phytosterols. The oil contains 3% steroid compounds of which 85% is sitosterol and campesterol. They estimated that 20,000 tons of tall oil phytosterols were available per year in the United States. Using Mycobacterium mutants 52% sterol conversion to ADD was achieved in 6 days. However, because of the large variation in sterol content of the substrate, this process is not used industrially.

Another process, using the sterol residue remaining from the distillation of soyabean and sunflower fatty acids, produces 20-carboxypregna-1,4-diene-3-one with a Corynebacterium species (Struve et al., 1981). The process is made more interesting by the survival of a pregnane nucleus without the further degradation to an androstane as commonly occurs.

Recent reviews on the microbial transformation of sterols include Martin, 1977; Schömer and Martin, 1980.

Hecogenin

This sapogenin is used by Glaxo for corticosteroid production (Figure 5). It is obtained from the leaf of Agave sisalana Perrine. Experiments have been carried out on its large scale production in Haiti (Applezweig, 1976). The 12-oxygen is removed to the 11-position (necessary for corticosteroid activity) by chemical means.

Bile acids

Bile acids are 5β saturated steroids of C_{24} or C_{27} and C_{28}. They are frequently hydroxylated in the steroid nucleus and always have a terminal carboxyl on the side chain. The most frequently occurring bile acids are hydroxylated derivatives of 5β-cholanic acid, usually having a 3α-OH group.

Bile acids occupy an important place as a precursor of corticosteroids accounting for 10% of total steroid production in 1976. Conversion is by purely chemical means.

Although diosgenin is still an important raw material

for the steroid industry it is no longer pre-eminent. In some processes, notably in the production of 19-nor contraceptives, oestrogens and diuretics it is no longer competitive. It seems likely that sterols are going to become of increasing importance in the steroid industry with the improved microbiological conversion techniques now available.

SECTION B: Analytical Techniques:

A number of analytical procedures have been used to assay the sapogenin content of plants and in particular their diosgenin content. Gravimetric (Rothrock et al., 1957a), colourimetric (Yamagishi and Nakumura, 1958; Slack and Mader, 1961) ultraviolet spectrometric (Walens et al., 1954) and thin layer chromatographic (Heftmann, 1975) methods have all been used but later abandoned as inaccurate and/or time-consuming.

Infrared analysis was first developed for sapogenins by Wall and co-workers (Wall, et al., 1952) and later applied in a modified form to fenugreek extracts (Jefferies and Hardman, 1972). It still remains a useful technique, especially for determining the ratio of R- and S-isomers present (e.g. diosgenin and yamogenin).

As a routine quantitative procedure gas-liquid chromatography is frequently used. Cooke (1970) describes a method for the determination of diosgenin in crude extracts of Dioscorea tubers using SE-30 as the stationary phase.

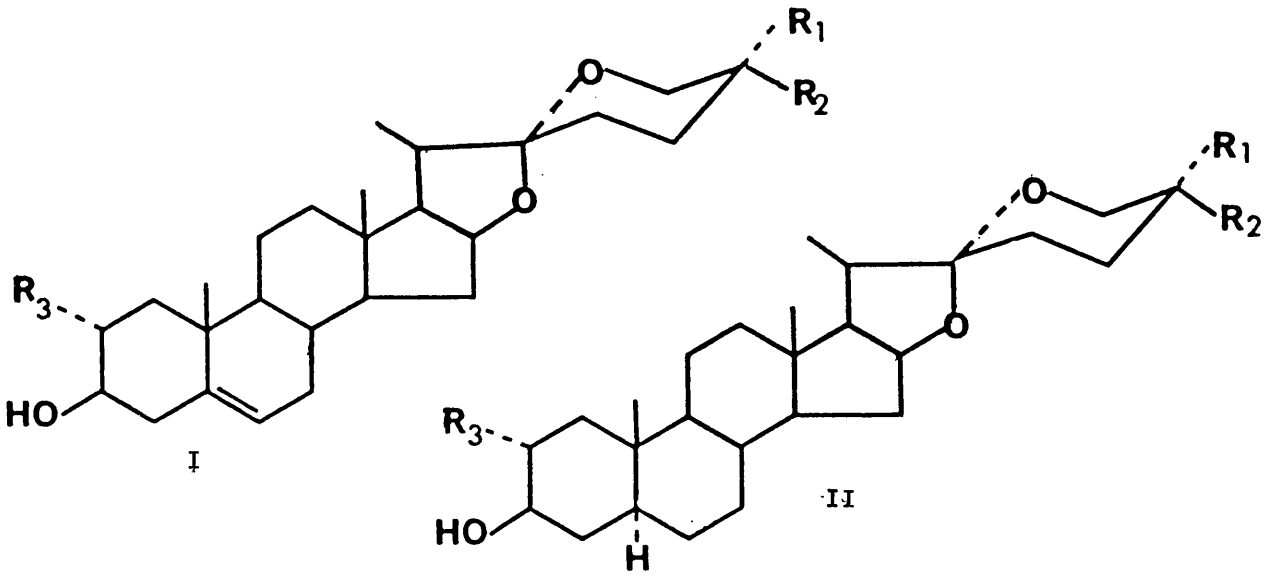
This method was later simplified by Rozanski (1972). However the impurities contained in crude extracts of acid hydrolysed

fenugreek seed, namely fixed oil, sterols, sterol esters, spirostadienes and dihydroxysapogenins reduce the suitability of his method. It also fails to separate diosgenin from yamogenin, tigogenin and neotigogenin giving a single peak for these monohydroxysapogenins (See Figure 8 for structures).

A g.l.c. procedure using OV-17 as stationary phase and the sapogenins as trimethylsilyl derivatives has been developed by Jefferies (1978) and gives satisfactory results for fenugreek extracts by separating monohydroxysapogenins from dihydroxysapogenins, sterols and sterol esters. This is essentially the method used in this thesis (see Materials and Methods, Chapter 2). It still fails to resolve the individual monohydroxysapogenins but this was taken into account by using carbon-13 nuclear magnetic resonance spectroscopy as a further analytical tool.

Knight (1977) achieved some separation of the various component sapogenins present in fenugreek using 3% QF-1 as the stationary phase, and by using the sapogenins as their tri-fluoro acetate derivatives. Further refinements to this method and fine tuning should improve the separation sufficiently to allow for precise quantification of the individual sapogenins by g.l.c. for the first time. Due to the long retention times (45 minutes compared to 15 minutes using the OV-17 method) the method was not used for the work in this thesis, which required large numbers of assays to be performed.

Several other methods for the g.l.c. determination of diosgenin have been published recently in rather inaccessible form (Kabanov



I

R_1	R_2	R_3	
H	CH_3	H	Diosgenin
CH_3	H	H	Yamogenin
H	CH_3	OH	Yuccagenin
CH_3	H	OH	Lilagenin

II

R_1	R_2	R_3	
H	CH_3	H	Tigogenin
CH_3	H	H	Neotigogenin
H	CH_3	OH	Gitogenin
CH_3	H	OH	Neogitogenin

Figure 8. Steroidal sapogenins occurring in fenugreek seed extracts.

et al., 1980; Reichelt and Cizek, 1978), including one using fenugreek extracts (Gu et al., 1980).

A method which has not yet been explored for sapogenin analysis is the use of capillary columns in g.l.c. These are of much higher resolving power and therefore likely to give better separations. Examples of their use with steroids are given by Jennings (1980), Madani and Chambaz (1979) and Sandra et al., (1979).

High-performance liquid chromatography (h.p.l.c.) offers the prospect of a highly efficient, non-destructive method of sapogenin analysis. However, little work in this area has been published to date. In his review on the h.p.l.c. of steroids Heftmann (1979) could only find one paper - that of Higgins (1976). Higgins quantified the sapogenins (including diosgenin) of Agave and other plant genera in the form of their benzoate esters, using reversed phase techniques. Recently Mahato et al. (1981) have published a method for diosgenin determination using underivatized samples detected by a refractometer. Neither of these papers have considered the separation of diosgenin from yamogenin.

Carbon-13 nuclear magnetic resonance (^{13}C n.m.r.) has only recently been extensively applied to steroids (Blunt and Stothers, 1977; Smith, 1978). The method is a very powerful tool in structural analysis and can be used both quantitatively and qualitatively. Eggert and Djerassi (1975) demonstrated that the R- and S- isomers of sapogenins give rise to slightly different spectra. Batta et al. (1980) used ^{13}C-NMR to differentiate between the 25R-and 25S- isomers of 5β-cholestan-3α,7α,26-triol. The method was used during my

experiments as a convenient way of determining the presence of 25R-, 25S- and saturated sapogenins (i.e. diosgenin, yamogenin, and tigogenin) in partially purified samples. This was found to be a more rapid and effective procedure than the chromatographic methods employed previously (Dawidar et al., 1973).

SECTION C: Microbiological Transformation of Steroids

History

The first scientific investigations into chemical transformations by microorganisms were not carried out until the 19th century when Pasteur studied the conversion of sugar to lactic acid and ethanol. He demonstrated that individual microbial species were responsible for discrete chemical alterations of selected substrates.

The first steroid transformation experiments were published in 1937, when it was shown that fermenting yeast could reduce 17-oxosteroids to 17β-hydroxysteroids (Mamoli and Vercellone, 1937). Mamoli and Vercellone also discovered sequential oxidation-isomerisation and reduction reactions (1938 a and b) - for example the conversion of 3β,21-dihydroxypregn-5-ene-20-one-21 acetate to 21-hydroxypregn-4-ene-3,20-dione (deoxycorticosterone) by Corynebacterium mediolanum. A similar reaction was used for a time by Schering to manufacture cortexolone (17α,21-dihydroxypregn-4-ene-3,20-dione) (Charney and Herzog, 1967).

Studies by Turfitt (1943, 1944 a, 1944b ,1946, 1948) revealed that cholest-4-en-3-one and cholan-4-en-3-one-24-oic acid were transformed into androst-4-en-3-one-17-oic acid by Proactinomyces

erythropolis, although only to a very limited extent. This evidence suggested that cholesterol could be microbially transformed into steroids of lower molecular weight, an important concept at the time as cholesterol was the major starting material for steroid hormone synthesis. Towards the end of the 1940's cholesterol was replaced by diosgenin.

Investigations of microbial transformations received further impetus when it was discovered that cortisone-21-acetate was a useful palliative in rheumatoid arthritis (Hench et al., 1950) which consequently led to an enormous increase in demand for the compound. Synthesis, especially the introduction of the 11 oxygen atom into the steroid nucleus was a very difficult chemical task, the result being low yield and high cost (Fieser and Fieser, 1959).
In 1950 Peterson and Murray achieved the 11α-hydroxylation of progesterone (pregn-4-ene-3,20-dione) using Rhizopus arrhizus (Murray and Peterson ,1952) . This important discovery caused the development of a new technology for the manufacture of adrenocortical hormones and their synthetic analogues by the use of microbiological transformation. Many of the large pharmaceutical companies with interests in steroid chemistry (Lederle, Merck, Pfizer, Schering, Syntex and Upjohn) became involved.

11β-Hydroxylation followed quickly (Colingsworth et al., 1952). In 1955 Schering found that the clinical effectiveness of hydrocortisone (11β, 17α,21-trihydroxypregn-4-ene-3,20-dione) was improved by introduction of a 1-2 double bond to form the orally active compound prednisolone. A micro-organism was used to carry

out the reaction (Nobile, 1958); the same organism (Corynebacterium simplex) is still used today by Schering for this reaction (Sebek and Perlman, 1979).

At the present time, although a very wide range of microbiological transformations are known (it is possible to microbially hydroxylate every position on the steroid skeleton) relatively few microbial transformations are used industrially (Murray, 1976). The most important of these include 11α-hydroxylation which has acquired a sophisticated level of development. Using progesterone or related structures at concentrations of up to 50 g l^{-1} the hydroxylation gives yields of more than 80% in two to three days. Rhizopus nigricans (ATCC 6227b) is the microorganism currently used (Murray, 1976).

11β-Hydroxylation is used by Pfizer in the production of hydrocortisone from cortexolone using Curvularia lunata.

16α-Hydroxyhydrocortisone is produced by 16α-hydroxylation using Streptomyces roseochromogenus (ATCC 3347) by Squibb and Sons (Murray, 1976).

Microbiological Transformation of Diosgenin

Hydroxylation

Early attempts to hydroxylate diosgenin using a wide variety of microorganisms failed to produce positive results (Mininger et al., 1956). This led the authors to conclude that steroidal sapogenins are not readily hydroxylated by microorganisms. Although since that

time a number of transformations have been reported, including hydroxylations, diosgenin tends to be a more difficult substrate to transform than many other steroids. Brooks and Smith (1975) found it a poor substrate for cholesterol oxidase compared to other substances tested. Nagasawa et al. (1970a) failed to achieve any conversion to androstane compounds using Arthrobacter simplex although the organism was successful with many other steroidal compounds. None of the organisms tested by Mininger et al.,(1956) were subsequently shown to be active against diosgenin.

The first transformation of diosgenin reported was oxygenation at the 7β and 11α positions (7β,11α-dihydroxydiosgenin, 10-15% yield) and the formation of 11α-hydroxy-7-oxo-diosgenin (5-10% yield) (Hayakawa and Sato, 1962; 1963). The fungus Helicostylum piriforme ATCC 8992 was used in a complex media, but with only a low level of diosgenin (10mg/100 mls medium). A similar transformation but with even lower yields was discovered by Kaneko et al.(1969).They were able to identify three products from diosgenin after incubation with Cunninghamella blakesleeana in complex media for 7 days; 7β-hydroxydiosgenin (2.5% yield), 7β,11α-dihydroxydiosgenin (1.2% yield) and 7β, 12β-dihydroxydiosgenin (0.1% yield).

Ring A Oxidation

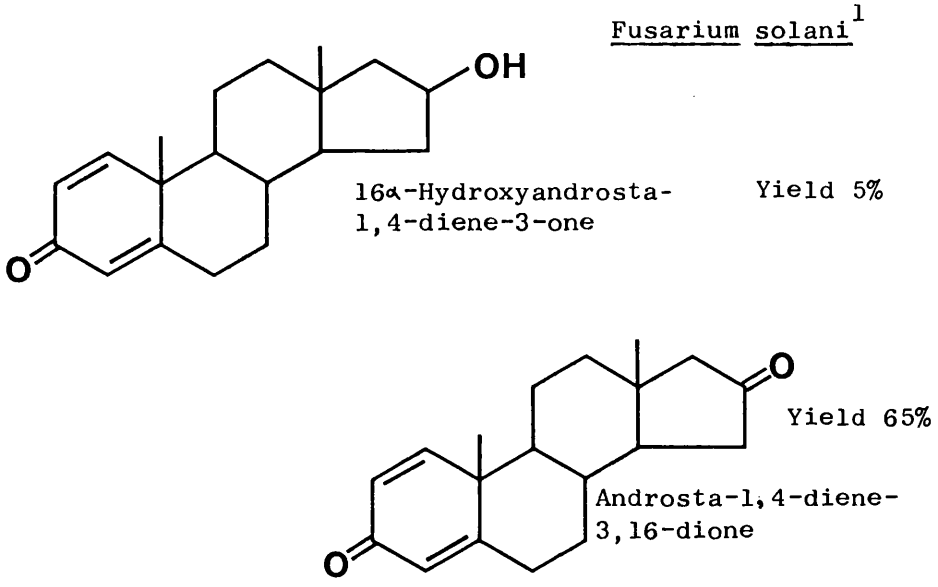
U.S. patent 3,134,718 (Nobile, 1964) gives the first mention of the formation of diosgenone [(25R)-spirost-4-en-3-one] and 1-dehydrodiosgenone [(25R)-spirosta-1,4-dien-3-one] from diosgenin, using Corynebacterium simplex. No yield figure is given. Iizuka and

Iwafuji (1967) patented a method for the production of 1-dehydrodiosgenone giving a 27% yield after 96 hours using Brevibacterium maris. The concentration of diosgenin added to the fermentation (30 mg/100mls) was relatively low.

Removal of rings E and F, and Total Degradation

An important advance, the removal of rings E and F of diosgenin was accomplished in 1966 by Kondo and Mitsugi. Using Fusarium solani 101, androsta-1,4-diene-3,16-dione, 16α-hydroxyandrosta-1,4-dien-3-one and 16β-hydroxyandrosta-1,4-dien-3-one were produced from diosgenin, but only in very low yields. When diosgenone was used as substrate much higher yields (e.g. 65% androsta-1,4-diene-3,16-dione) were achieved (Figure 9). The authors further studied the transformation and suggested a probable pathway for the reaction (Figure 10), with 20α-hydroxypregnan-3,16-dione as an intermediate. This scheme was later shown to be largely correct by the same authors (1973) when they isolated pregnanes from fermentations of diosgenone with Verticillium theobromae and Stachylidium bicolor (Figure 9) in resting cell suspensions. A patent for this process later appeared (Kondo and Mitsugi, 1975). However, once again diosgenin itself did not prove to be an effective substrate (and was not included in the patent).

An alternative pathway to the formation of androstanes from diosgenin was discovered by Ambrus and Buki (1969). Culturing Mycobacterium phlei in the presence of 8-hydroxyquinoline, four steroid products were obtained after 48 hours: diosgenone (6% yield), 1-dehydrodiosgenone (16% yield), androst-4-ene-3,17-dione (0.5%



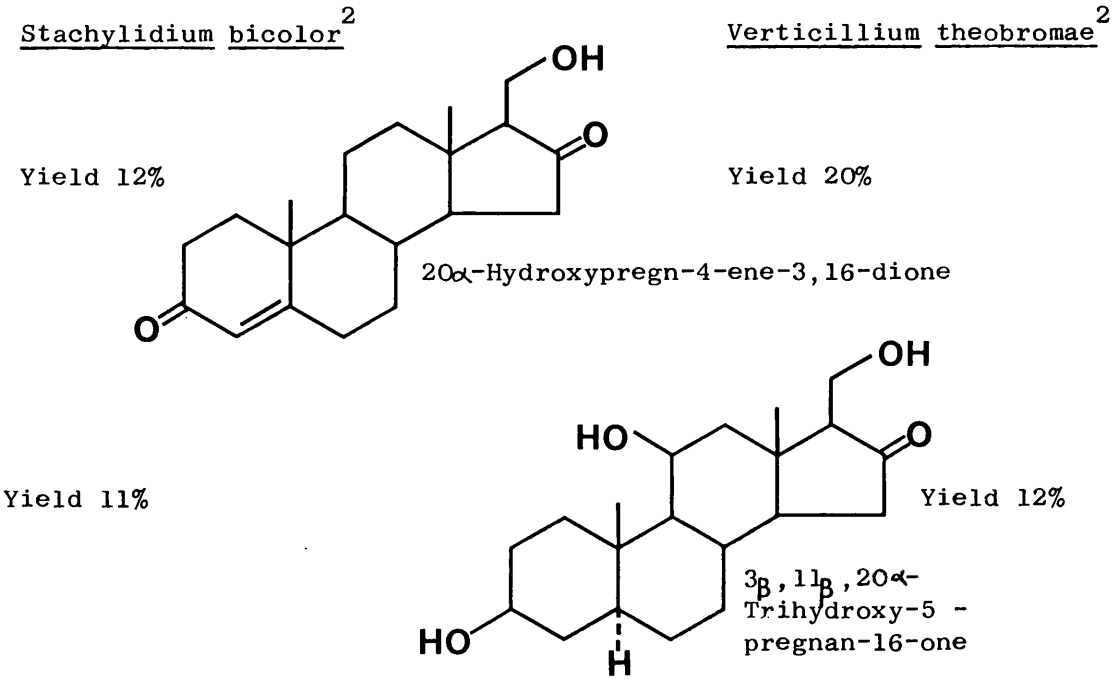


Figure 9.Products of ring E and F degradation of diosgenone.

1 Kondo & Mitsugi,1966. 2 Kondo & Mitsugi,1973.

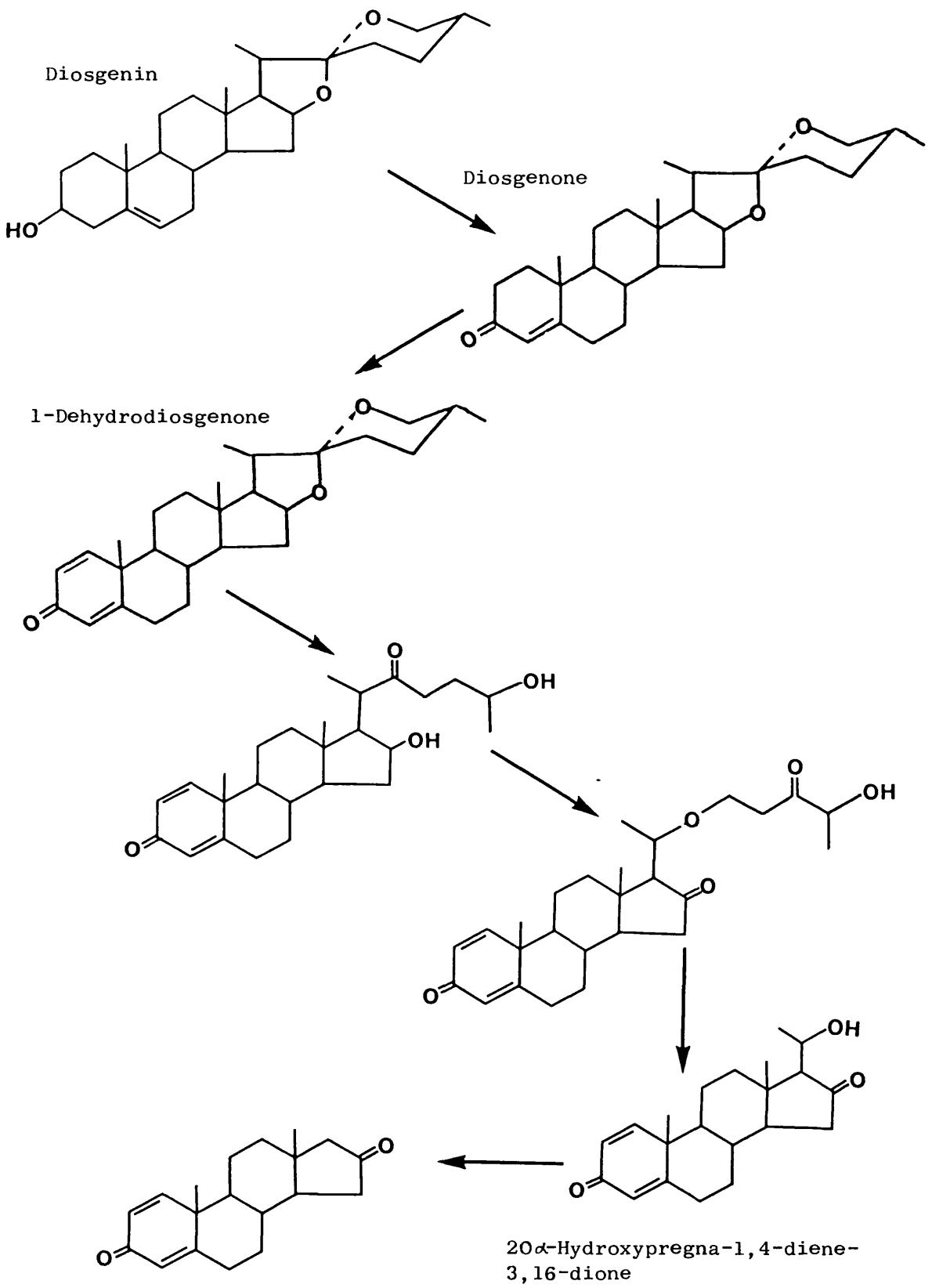


Figure 10. Suggested pathway for the transformation of diosgenin by F.solani.

yield) and androsta-1,4-diene-3,17-dione (3% yield). Whereas Kondo and Mitsugi (1966, 1973) found that Fusarium, Stachylidium and Verticillium species oxidised the sapogenin at the 16-position the Mycobacterium oxidised it at the 17-position (as is more commonly found). This suggests two different mechanisms of ring degradation. Ambrus and Buki (1969) found that in the absence of 8-hydroxyquinoline Mycobacterium phlei completely degraded diosgenin within 9 hours; the only remaining products were traces of diosgenone and 1-dehydrodiosgenone. A yield of between 2.5% and 4% total androstane was achieved using the saturated (5α) sapogenins tigogenin and neotigogenin as substrates.

The degradation of androstanes by M. phlei is likely to follow the common pathway in the breakdown of the sterol nucleus shown by microorganisms of the genera Nocardia, Mycobacterium, Pseudomonas, Bacterium and Corynebacterium (Dodson and Muir, 1961a, 1961b; Schübert et al., 1961; Sih and Wang, 1963; Nagasawa et al., 1969). All these organisms oxidise the sterol at the 17 position to form androst-4-ene-3,17-dione, followed by 9α-hydroxylation. Then the 9-10 bond is broken producing 9,10-seco-3-hydroxyandrosta-1,3,5(10)-triene-9,17 dione. This is further metabolised to carbon dioxide and water (Figure 11).

Removal of Rings A and B

Howe et al.(1973), using an unidentified bacterium (I.C.I. reference number ACC 3600) formed 9-oxode-AB-spirostane-8α-propionic acid via diosgenone and 1-dehydrodiosgenone (Figure 12).This is the first report of the isolation of products from diosgenin

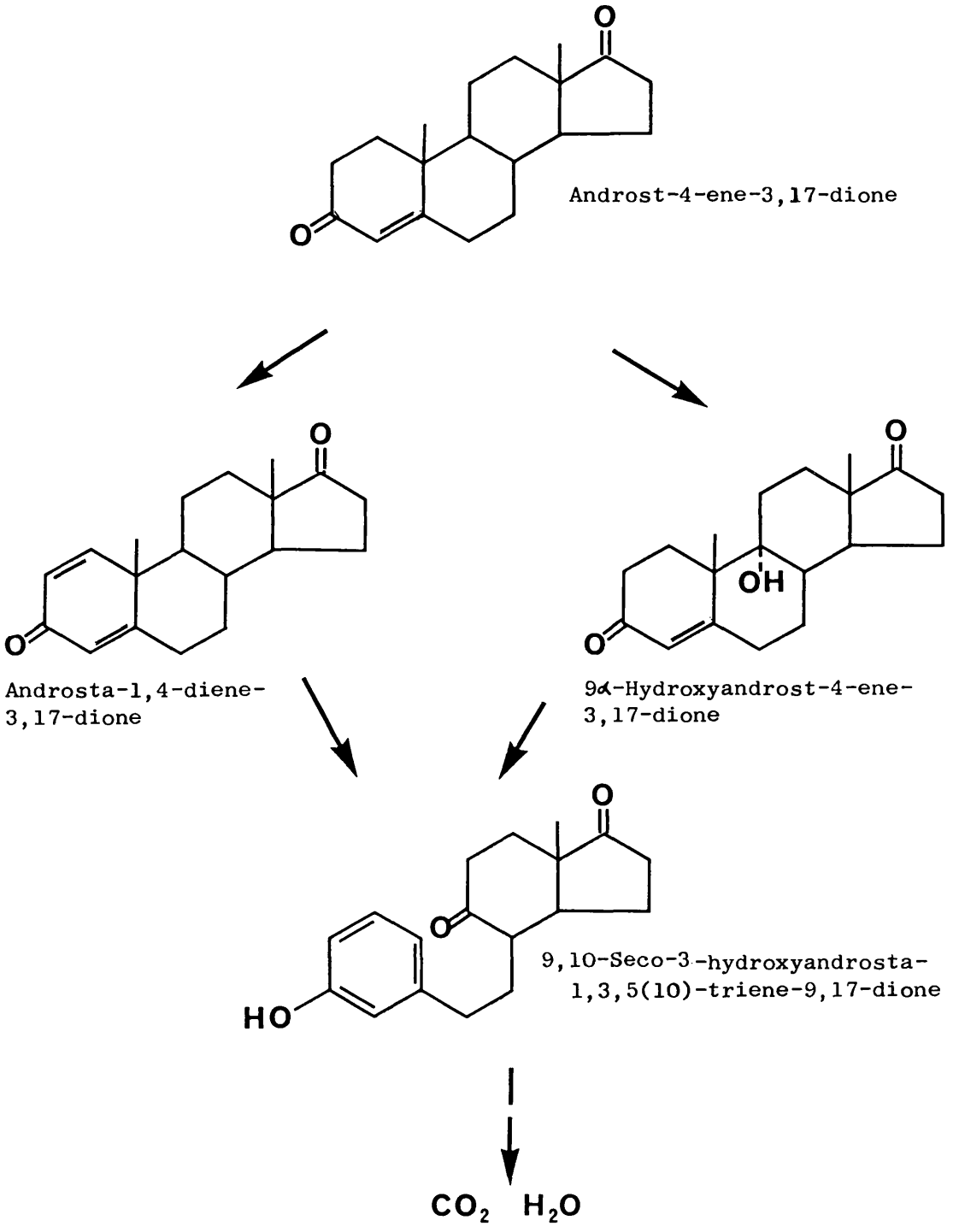


Figure 11. Steroid nucleus degradation.

with the rings A and B removed. They were also able to produce the same oxo acid and its 17β-hydroxy derivative (as a minor product) from diosgenin using Nocardia globerula NCIB 9158. Addition of inhibitors of steroid nucleus degradation (e.g. α,α'-bipyridyl) led to the accumulation of diosgenone and 1-dehydrodiosgenone. A similar pathway was observed with hecogenin.

This removal of ring A is unusual, ring B is generally attacked whilst the A ring remains intact.

Thus to date neither high yields of pregnanes or androstanes have been achieved when diosgenin was used as a substrate, although many organisms have been screened for the purpose. Kondo and Mitsugi (1966, 1973) have managed to obtain satisfactory yields from diosgenone with three fungal species. Several different degradation pathways seem to exist, with enzymes attacking rings A, B, E and F of diosgenin.

Saponin hydrolysis

Another relevant transformation to be considered is the hydrolysis of spirostanol and furostanol glycosides to give the aglycones (e.g. diosgenin, hecogenin).

Krider et al.(1954),found certain microorganisms, especially those of the genera Aspergillus and Penicillium were able to hydrolyse saponins from Agave leaves, forming sapogenins (mainly hecogenin). Yields of up to 80% of those achieved by acid hydrolysis were obtained and the method was later patented (Krider et al., 1957).

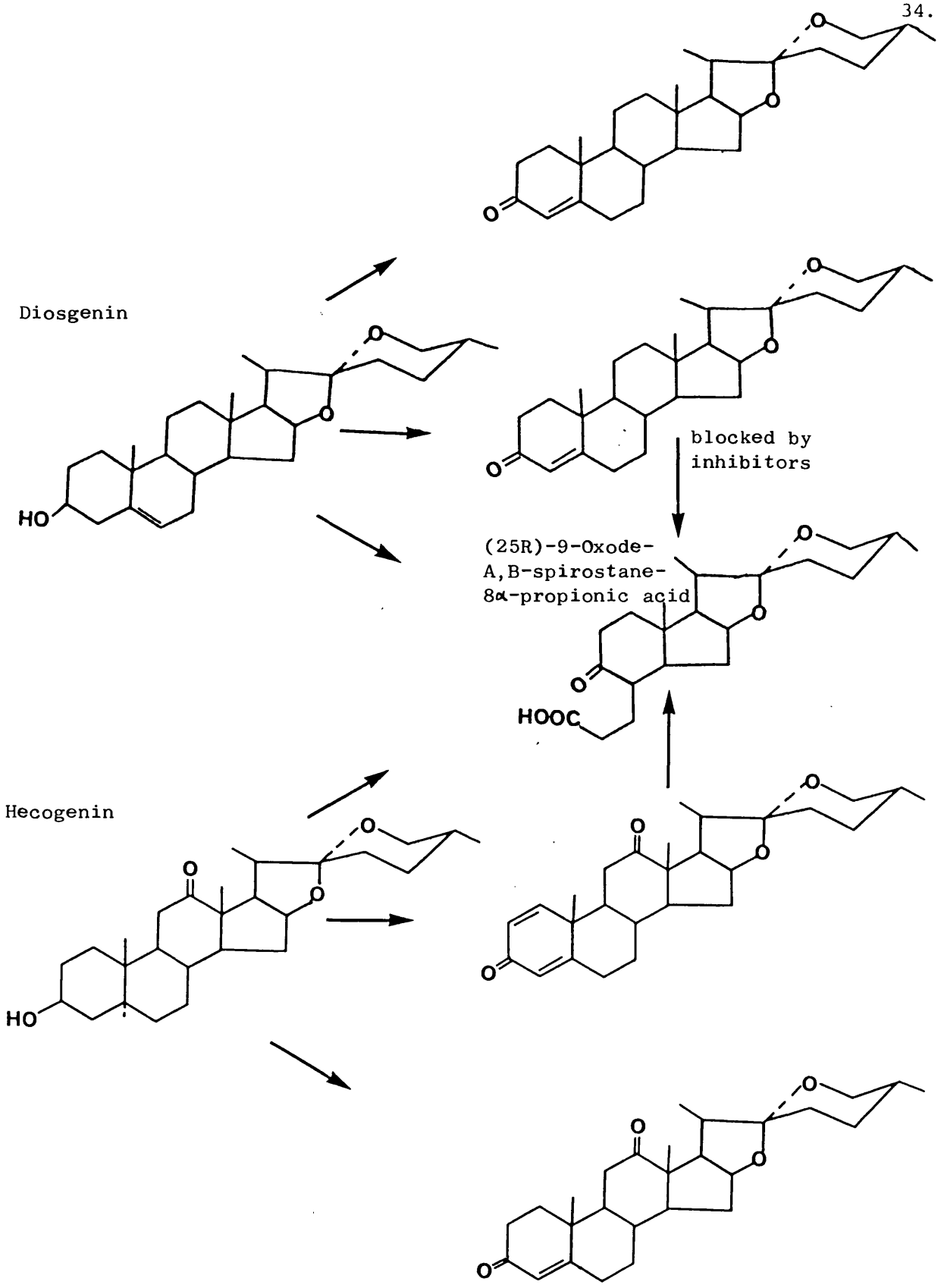


Figure 12.Products of ring A and B degradation of diosgenin and hecogenin.

Rothrock et al. (1957b) isolated diosgenin after the fermentation of Dioscorea tubers using both pure cultures (Penicillium and Aspergillus species) and mixed cultures. After 6 days incubation of Aspergillus terreus with pulverised tuber a yield of 58% of that obtained by acid hydrolysis was achieved.

Using non-autoclaved tuber and undefined mixed cultures yields of up to 90% of the potential diosgenin could be reached. However, they found that there was a danger of further degradation of diosgenin occurring in these conditions, especially at 37°C.

Aims

The aim of this thesis has been to study the microbiological transformation of diosgenin and its furostanol precursors derived from fenugreek seed. Organisms capable of transforming diosgenin were to be isolated and methods to promote the genin's efficient transformation investigated, together with the possibility of producing commercially useful metabolites, such as androstanes or pregnanes. The utilization of microbiological rather than chemical methods to obtain diosgenin from seed crude extracts was also to be considered.

CHAPTER 2

General Materials and Methods

Extraction of Diosgenin from Fenugreek Seed

Whole, commercial Moroccan seed (obtained from the London Spice Market) was sorted to remove extraneous material and hydrolysed by refluxing for 2 hours in 2N hydrochloric acid (250 g seed per 1 litre acid). After rapid cooling the acid insoluble residue was collected (on a Whatman No. 1 paper using buchner vacuum filtration apparatus) and then made alkaline with 10% v/v ammonia solution. The material collected was dried for 24 hours at 60°C in a fan oven before being ground into a coarse powder with a Glen Creston mill. The powder was packed into a soxhlet thimble and partially defatted by soaking in cold, light petroleum (b.p. 40°-60°C) for 6 hours. This extract was discarded and the powder extracted with fresh solvent in a soxhlet for 24 hours (60°C). The bulk of the solvent was removed by evaporation (Buchi rotary evaporator) and sapogenin allowed to crystallize out from the remaining solution. Several crops of crystals were collected by repeated filtration and concentration steps. All the harvested 'diosgenin' (monohydroxysapogenin) was then purified by recrystallisation from acetone and its composition checked by thin-layer chromatography. Only material which gave a single spot, namely monohydroxysapogenin, was used as 'diosgenin' in the fermentation experiments.

Quantitative Analysis by gas-liquid Chromatography

A Sigma 4 gas-liquid chromatograph (Perkin-Elmer Ltd.) fitted with a flame ionisation detector and a model 023 chart recorder (Perkin-Elmer) were used for all g.l.c analyses. The column used was of 1 metre length, glass, internal diameter 1.75 mm, packed with

Chromosorb-G, 80-100 mesh size, acid washed DMCS coated with 2½% OV-17 stationary phase. Before use this column was preconditioned at 325°C for 18 hours unconnected to the detector and then treated with repeated injections of Bistrimethylsilyl-trifluoro acetamide (BSTFA) + 1% Trimethyl chlorosilane (TMCS) reagent at 150°C.

In use the oven temperature was 277°C and the injector/detector at 300°C. Nitrogen flow rate was 20 ml min^{-1}, hydrogen 23 p.s.i. and air 26 p.s.i. A chart speed of 30 cm h^{-1} was used.

Samples were prepared by evaporating to dryness a measured quantity of chloroform extract with a chloroform solution of internal standard (5α-cholestan-3β-ol was routinely used) in a vacuum oven (80°, 250 mm Hg). The residue was dissolved in 150 µl tetrahydrofuran and 100 µl BSTFA was added. The samples were heated for 2 hours in a sealed vial to allow the BSTFA reagent to silylate the hydroxyl groups, i.e.:-

$$-\overset{|}{\underset{|}{C}}\text{-OH} + CF_3\overset{O}{\overset{\|}{C}}N\left[Si(CH_3)_3\right]_2 \rightarrow -\overset{|}{\underset{|}{C}}\text{-O-}Si(CH_3)_3 + CF_3\overset{O}{\overset{\|}{C}}NHSi(CH_3)_3$$

Samples (2 µl) were then analysed by g.l.c. using a 5 µl S.G.E. syringe, 7 cm needle with direct on column injection.

Oven temperature and nitrogen flow rates were optimised empirically by using plate number calculations (Table 1). When the plate number had decreased by 25% the column was repacked and recalibrated.

A calibration curve was calculated using 5α-cholestan-3β-ol as internal standard with silylated diosgenin (monohydroxysapogenin) (Table 2, Figure 13). The line was fitted using the method of least squares and the correlation coefficient calculated to be 0.999.

Diosgenone and 1-Dehydrodiosgenone Calibrations

To quantify the amounts of diosgenone present in the fermentations a calibration was carried out using purified, bacterially produced diosgenone. 5α-Cholestan-3β-ol was used as an internal standard (silylated as described above). The result is given in Table 3.

As insufficient pure 1-dehydrodiosgenone was available to carry out a similar full calibration its presence in extracts was estimated assuming a similar response to that given by diosgenone. This was found to give accurate values for two prepared standard samples.

Qualitative Analysis of Diosgenin and Products by Thin Layer Chromatography

Pre-spread Kieselgel 60F 254 plates were used for all routine t.l.c.analyses. The standard solvent system was toluene, 66: ethyl acetate, 33: formic acid, 1. The solvent front was allowed to run a distance of 150 mm (time - 40 minutes) before the plates were removed and assayed by both fluorescence at 254 nm and acid charring (10% sulphuric acid spray, 140°C, 10 minutes).

Table 1. Optimisation of gas-liquid chromatographic performance for diosgenin analysis

N_2 Flow rate ml min^{-1}	Column temp. ^oC	t_R	$W_{\frac{1}{2}}$	n
20	265	271	26.4	584
20	270	252	22.6	689
20	275	209	18.6	699
20	280	176	16	670
20	285	147	13.5	648
20	290	122	11.7	602
20	275	209	18.6	699
20	276	204	18.1	703
20	277	196	17.4	703
20	278	184	16.6	680
18	277	203	18.1	697
20	277	196	17.4	703
22	277	190	17.0	692
24	277	180	16.8	636

$n = 5.54 \left(\frac{t_R}{W_{\frac{1}{2}}} \right)$ = theoretical plate number

t_R = distance from point of injection to peak maximum (mm)

$W_{\frac{1}{2}}$ = width of peak at half height (mm)

Table 2. Diosgenin Calibration

Sample	µl diosgenin	Diosgenin (mg) per mg internal standard	Mean peak height ratio (diosgenin/internal standard)
1	250	5.0	1.9746
2	100	2.0	0.7655
3	75	1.5	0.5519
4	50	1.0	0.3705
5	5	0.1	0.0393

Internal standard 5α-cholestan-3β-ol; 50 µl, 5 mg ml^{-1} used per sample

Diosgenin stock solution 5 mg ml^{-1} (monohydroxysapogenin mixture as defined in the text)

For the equation $y = mx + c$

m (slope) = 0.3977

c (intercept) = -0.023

Correlation coefficient = 0.9997

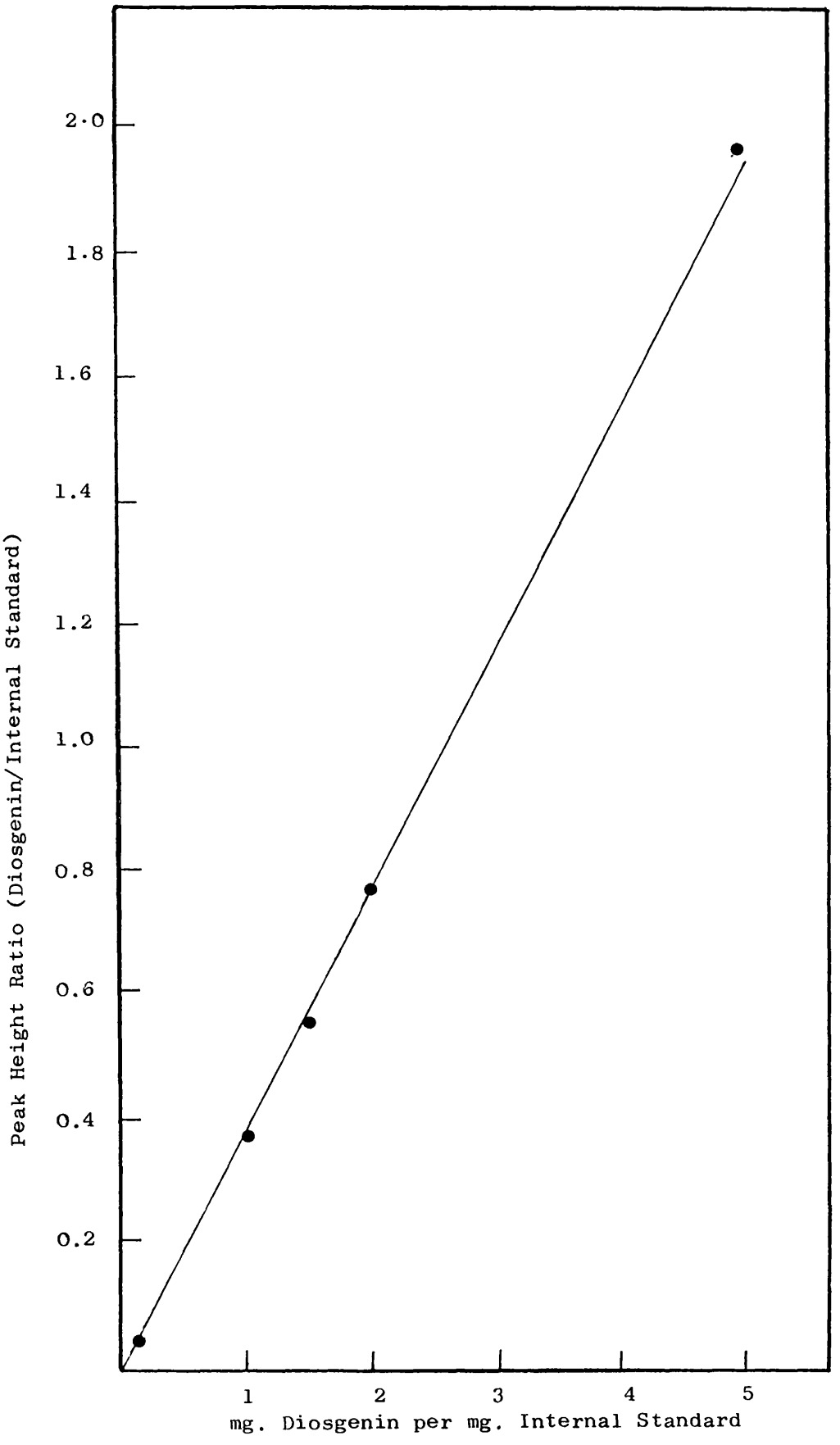


Figure 13. Diosgenin calibration curve.

Table 3. Diosgenone calibration

Sample	Diosgenone (mg) per mg internal standard	Mean peak height ratio (Diosgenone/Internal Standard)
1	2.37	0.305
2	1.185	0.125
3	0.595	0.058
4	0.395	0.033

By least squares linear regression. Slope (m) = 0.138

intercept (y) = -0.0265

Correlation Coefficient = 0.998

Microbiological Materials and Methods

Pipettes - Oxford pipettes with disposable tips were used throughout the work, in particular the Oxford Macro Set (0 - 5 ml volume) and Oxford sampler micropipette (0.2, 0.5, 1.0 ml volume). Prior to use the pipettes were calibrated by measuring the weight of distilled water pipetted onto a tarred dish using a balance accurate to 5 decimal places (Stanton Instruments Ltd.). The pipettes were checked every 6 weeks (see Table 4) .

Glassware was washed with the non-ionic detergent Decon 90, rinsed three times in running tap water and three times in distilled water before drying in a hot air oven (160^{o}C). New glassware was soaked in 1% hydrochloric acid for 24 hours before washing and use.

Culture Maintenance

After reconstitution from a freeze dried state the micro-organisms were purified twice by streaking onto suitable solid media before use. Cultures were stored as slopes (in universal bottles, 4^{o}C) reculturing every 4 months and in vials under liquid nitrogen. All cultures were regularly checked for contamination on the basis of their gram-stained appearance (using the method of Preston and Morrell, 1962) and colony morphology.

Extraction of Steroids after Fermentation

After fermentation the steroids were extracted from the media in a separating funnel three times with ethyl acetate. The organic extract was then dried with magnesium sulphate, filtered and evaporated to dryness on a Büchi rotary evaporator (60^{o}C). Using

Table 4. Weights of water delivered by Oxford pipettes.

Volume setting	micropipette			'Macro Set'
	0.2 ml	0.5 ml	1.0 ml	2.5 ml
Replicate No.				
1	0.1996	0.4961	0.9925	2.4768
2	0.2007	0.4981	0.9843	2.4471
3	0.2007	0.5055	0.9764	2.4434
4	0.1999	0.5031	0.9854	2.4599
5	0.1991	0.5009	0.9853	2.4645
6	0.2037	0.4920	0.9782	2.4674
7	0.2001	0.4939	0.9838	2.4599
8	0.2001	0.4981	0.9710	2.4494
9	0.2018	0.5002	0.9963	2.4573
10	0.2006	0.4962	0.9946	2.4660
Mean weight	0.2006	0.4984	0.9848	2.4592
Standard deviation	±0.00132	±0.0041	±0.0086	±0.010
Coefficient of variation	±0.66%	±0.82%	±0.87%	±0.41%

$$\text{Standard deviation } (s) = \sqrt{\frac{\left(\sum_{i=1}^{n} x_i^2\right) - nx^{-2}}{n-1}}$$

$$\text{Coefficient of variation} = \frac{s}{\bar{x}}$$

redistilled chloroform the residue was quantitatively transferred to a 5 ml volumetric flask and made up to volume with solvent. Samples for analysis were stored at $4^{o}C$ in solvent proof screw capped specimen tubes.

The efficiency of extraction and sampling error were ascertained. The former was calculated from extractions of 5 cultures containing Nocardia rhodochrous NCIB 10554 cells , each spiked with an identical amount of diosgenin immediately before extraction. Efficiency was found to be 98% (Table 5). Sampling error was assayed by taking six, 10 ml aliquots from a single culture and extracting each separately. The diosgenin content was calculated from g.l.c. analysis of each sample in duplicate. The coefficient of variation was found to be 2.28% (Table 6).

Table 5. The efficiency of extraction of diosgenin from microbiological cultures (Culture samples spiked with 60 mg diosgenin immediately prior to extraction.

Sample	Diosgenin (mg)	Diosgenin recovery (%)
1	58.9	98.2
2	57.6	96
3	60.0	100
4	59.8	99
5	58.0	96.7
Mean	58.8	98%
Standard deviation	= ±1.06	
Coefficient of variation	= ±1.80%	

Table 6. An assay of sampling error in extraction procedures. Six samples were taken from one culture and extracted separately.

Sample	mg Diosgenin
1	13.5
2	12.65
3	12.95
4	13.23
5	13.03
6	12.85
Mean	13.03
Standard deviation	±0.297
Coefficient of Variation =	±2.28%

Culture Media

Medium 1 (Non defined)

	g l^{-1}
Lab Lemco Powder (Oxoid L29)	1
Yeast extract (Oxoid L21)	7
Peptone (Oxoid L37)	5
Sodium chloride	5

pH adjusted to 7.4 with NaOH before sterilisation.

Medium 2 (defined)

	Stock conc. (mg l^{-1})	Final conc. (mg l^{-1})
Solution A		
$CuSO_4.5H_2O$	80	0.08
$MnSO_4.4H_2O$	800	0.8
$ZnSO_4.7H_2O$	800	0.8
$Na_2MoO_4,4H_2O$	370	0.37
H_3BO_3	50	0.05
KI	200	0.2
Solution B		
$MgSO_4,7H_2O$	20.5	0.205
$CaCl_2.2H_2O$	2.7	0.027
$NaNO_3$	51	0.51
Solution C		
KH_2PO_4	4.6	0.46
Na_2HPO_4	22	2.2
Solution D		
EDTA-Na_2	2.0	0.01
Urea	72	0.36
$FeSO_4.7H_2O$	2.4	0.012
Solution E		
CH_3COONa	82.04 (1M)	10 mM

Solutions A, B, C and E were steam sterilised (121^{o}C, 15 minutes) and solution D was filter sterilised in 200 ml volumes using a 0.2 μ, 2.5 mm diameter filter in a swinnex holder (Millipore Ltd.). To make 1 litre of medium the following solutions were mixed aseptically:- A, 1 ml; B, 10 ml; C, 100 ml; D, 5 ml; E, 10 ml and made up to 1 litre with glass distilled sterile water.

Medium 3 (for Verticillium and Stachylidium spp.)

	Final conc. (g l^{-1})
Glucose	20
Yeast Extract	15
Peptone	0.5
KH_2PO_4	1.5
$MgSO_4$	0.5
$CaCl_2$	0.01

(McCoy et al., 1972)

Medium 4 For agar plates with a limited carbon source:

	final conc. (g l^{-1})
KH_2PO_4	2.0
$(NH_4)SO_4$	1.0
$MgSO_4.7H_2O$	0.4
Minor elements concentrate	1.0 (see below)
Agar	15

After the sterilisation of the medium and the subsequent pouring of the agar plates diosgenin was added to the still liquid media as an ethanolic solution to give a diosgenin concentration of 20 mg per plate.

Minor elements concentrate	g l^{-1}
$FeSO_4.7H_2O$	1.0
$CuSO_4.5H_2O$	0.15
$ZnSO_4.7H_2O$	1.0
$K_2MoO_4,4H_2O$	0.1

(Brian et al., 1946)

Isolation of Organisms using Media with a Limited Carbon Source

Two sets of media were made, one as listed above (Medium 4) and the other without diosgenin. Soil samples were taken from a number of locations, shaken in phosphate buffered saline solution (Oxoid BR14a) and then streaked onto the agar plates containing diosgenin. After 7 days incubation (20°C) colonies were picked off and purified on nutrient agar (Oxoid CM3). The purified colonies were then plated in duplicate on the agar with and without diosgenin present. Colonies showing much better growth after 7 days in the presence of diosgenin than in its absence (non autotrophs) were screened in liquid media.

Growth and Transformation Experiments - General Methods

For most of the organisms studied a complex medium based on that of Buckland et al. (1976) was found to produce favourable growth and transformation rates. Its composition is given below.

Medium 5

	Final Conc. (g l^{-1})
$(NH_4)_2SO_4$	2
$CaCl_2.2H_2O$	0.001
$FeSO_4.7H_2O$	0.01

K_2HPO_4	2
$MgSO_4.7H_2O$	0.1
Glycerol	6
Yeast extract (Oxoid L21)	12

Initial pH = 7.3

Unless otherwise stated cultures were grown in 500 ml indented shake flasks (A. Gallenkamp Ltd.)using a Gallenkamp orbital incubator at 28°C, 175 r.p.m. Diosgenin was generally added after dissolution in ethanol to give a final concentration of 1 mg ml^{-1} (2.4 μmoles), and an ethanol concentration of 5%. The levels of diosgenin and metabolites in the fermentations were monitored by g.l.c. and t.l.c.

Cell Dry Weight Determinations

Cell dry weights were measured using Whatman GF/F glass fibre filters which give 98% retention for particles down to 0.7 μm. A 7 cm Hartley funnel was used. Portions (5 ml) of medium were removed throughout the period of a culture's incubation. These samples were then filtered and washed (under vacuum) through a pre-weighed GF/F filter which was subsequently dried (60°C, 24 hours) and reweighed. Background readings were taken into account. For organisms not giving evenly dispersed growth (e.g. M.phlei, F. solani) whole cultures were filtered at each measurement time.

Viable Count Procedure

Viable count determinations were carried out using phosphate buffered saline (Oxoid BR14a) as diluent. Aliquots (0.5 ml) were removed from the culture and serially diluted (0.5 ml to 4.5 ml)

into sterile capped test tubes, vortexing with a Whirlimix (Fisons Ltd.) at each step. Triplicate 0.2 ml samples were then spread onto predried (1 hour, 37°C) nutrient agar plates. After allowing 20 minutes for absorbtion the plates were inverted, incubated at 30°C for 3 days and the colonies counted.

Collection, Purification and Structural Determination of Products

Microorganisms found to be forming products from diosgenin were grown on a 1.5 l scale (15 x 100 ml cultures), extracted as before and all the extracts bulked together. These extracts were then purified and analysed as described below.

Separation and Purification of Products

i. Separation by column chromatography

A 1 cm diameter glass column was packed with 8 g Woehlm silica gel at activity grade II (10% distilled water added), suspended in hexane:ethyl acetate (9:1). The silica was compacted and any air bubbles were dislodged by mechanical vibration. The sample to be chromatographed was evaporated to dryness and re-dissolved in a minimum quantity of hexane:ethyl acetate (9:1 mixture) before layering on top of the silica gel. The flow rate was adjusted to 1 ml min^{-1} and a reservoir of the above solvent mixture placed over the column to maintain a constant head (and therefore a constant flow rate) throughout the experiment.

Fractions were collected at 5 minute intervals using an L.K.B. Ultrarac 7000 fraction collector and assayed by t.l.c. after concentration to 1 ml. All samples which showed the same product

in pure form by t.l.c. were bulked and rechecked for purity by both t.l.c. and capillary column g.l.c. (Perkin-Elmer F-33 gas chromatograph, F.I.D. detector, 30 m WCOT SE30 column; temperature programme 200°C to 260°C, 3° min^{-1}, no initial holding time. Helium carrier gas, 40 ml min^{-1}) The results are given in Chapter 3.

ii Separation by preparative thin layer chromatography

A slurry of silica gel (PF 254/366) was prepared by shaking 80 g silica with 182 ml distilled water. A 1 mm layer of silica was spread onto glass chromatography plates (40 x 20 cm) which were then air dried and conditioned (110°C, 2 hours) before use. To achieve good separation of products the effect of different running times in standard and continuous elution chromatography tanks using solvent system; toluene 66 : ethyl acetate 33 : formic acid 1 and other solvent systems were tried. The results are given in Chapter 3.

After development the bands on the preparative plates were visualised by fluorescence (254 nm) and marked with a mounting needle. The positions of bands which did not fluoresce under ultra-violet were estimated by comparison with small test plates run in the same tanks and then acid charred. The separated bands were removed by scalpel and extracted for 24 hours with chloroform (HPLC grade) in soxhlet apparatus. The thimbles and their cotton wool closures had been cleaned for 24 hours in the soxhlet with chloroform prior to use . The product-containing chloroform extracts were evaporated to dryness, redissolved in chloroform,

filtered through millipore filters (0.45 μ pore size) to remove any silica, assayed for purity by t.l.c. and then weighed. The pure samples were used for structure determination.

Spectroscopic Methods

Ultraviolet spectroscopy

A Perkin-Elmer 550s spectrophotometer and chart recorder were used, with matched quartz cuvettes. Samples were dissolved in hexane.

Infra-red spectroscopy

Samples were analysed in chloroform solution in sodium chloride cells and also as solid incorporated into potassium bromide discs. The instrument used was a Unicam SP 200 spectrophotometer.

Nuclear Magnetic Resonance Spectroscopy

i) ^{13}Carbon - was carried out on a Jeol FX 90Q instrument. Samples were dissolved in deuterochloroform with tetramethylsilane (TMS) as an internal standard.

ii) Proton (^{1}H) was carried out on a Jeol PS-100 also with TMS as standard and $CDCl_3$ as solvent.

Mass spectrometry

A VG MN 70-70 mass spectrometer was used at 70 eV, by direct introduction of the sample into the source by probe. The ionization chamber temperature was 275^{o}C.

Gas chromatographic mass spectrometry

Crude steroidal samples were run on an A.E.I. MS12 instrument, g.l.c. was on a Pye Unicam 201 with a 5 ft S.E. 30 column. Oven temperature was 270^{o} and the flow rate of carrier gas (helium) 30 ml min^{-1}.

CHAPTER 3

Screening of microorganisms and the structural determination of products from diosgenin.

Introduction

Screening Techniques

In a screening programme carried out by Arima et al. (1969) 1,589 named strains of microorganisms were tested for their ability to decompose cholesterol. Eighteen strains were found to be capable of at least 50% cholesterol decomposition in 7 days - about a 1% success rate. A further 286 strains (18% of those tested) were able to oxidise 20% or more of the added cholesterol. The authors concluded that there was no close correlation apparent between taxonomical characteristics and cholesterol decomposing activity. This conclusion has generally been reached with other screening programmes.

To have the greatest chance of success a screen must be designed to be rapid, simple and highly selective. Selection can take place at two levels: selection by the experimentalist and selection by enrichment culture. The former usually follows a literature search to identify organisms which carry out the desired transformations on similar substrates, or which are known to have a wide range of catabolic pathways. This approach often gives a good yield of positive microorganisms but, by its nature, rarely yields novel pathways or organisms.

It is possible, by applying harsh selective pressures upon a mixed bacterial population, to eliminate the majority of organisms which do not possess the capacity to carry out a particular function. This artificially enriches the population for a particular type of organism. This method (enrichment culture), using

natural isolates of microorganisms gives a better chance of producing novel transforming microorganisms and products than a literature survey method. However it is also likely to produce a much higher proportion of failures,as well as difficulties in the isolation of pure cultures and their subsequent identification. Both approaches were used in this research. Enrichment selection was based upon the ability of soil organisms to utilize diosgenin as a carbon source on an otherwise carbon-depleted solid medium. Any promising isolates were then tested in the standard screening media.

Formulation of the growth medium is an important aspect of the planning of any screening programme. Ideally the growth medium should be capable of supporting good growth, allow maximum expression of transformation ability, be well defined, not subject to batch variation and be cheap to use on an industrial scale.

Unfortunately these qualifications are to an extent mutually exclusive. To achieve rapid growth and high cell density an easily accessible source or carbon and energy is generally required (Bridson and Brecker, 1970). However, whilst the inhibition, repression or inactivation of enzymes in a fermentation by a readily used carbon source does not influence the yield of primary metabolites, there is little doubt that some sort of catabolite control affects secondary metabolism (Demain, 1972). Enzyme repression is most intense when the requirements for both catabolism and anabolism are minimal (Paigen and Williams, 1970). It is probable that such repression also affects the transformation of xenobiotic substances, such as diosgenin. As a result, after years of empirical

development, many fermentations are conducted with sources of carbon and energy other than glucose (Demain, 1972), thus limiting or slowing down growth related functions in the hope of causing the accumulation of secondary products.

The form in which nitrogen is supplied to a culture medium is important. Media based on corn-steep liquor are commonly used industrially (Calam, 1969). The liquor contains amino acids as well as inorganic salts and imparts a significant buffering capacity to the growth medium. However corn-steep liquor is subject to considerable batch variation. Casein hydrolysate is an alternative nitrogen source which is rather better defined but is generally less stimulatory to growth. An alternative completely defined source of nitrogen is urea. Whilst supporting growth it does not provide such a rapidly assimable form of nitrogen and lacks the buffering effect of amino acids. The pH control of simple synthetic media is difficult since buffering power is low and therefore the final pH depends greatly upon the form of carbon utilized by the microorganisms. Whilst inorganic buffers are used in these synthetic media their concentration is limited by the ionic-strength tolerance of the microorganisms. Furthermore the supply of the many vitamins, cofactors and other growth factors in a defined medium to stimulate good growth would be both very time-consuming and expensive.

It was decided to use two different screening media: one of a chemically defined nature allowing moderate growth but less likely to lead to repression of xenobiotic transformations, and a rich,

non defined medium which would allow maximal growth of a wide range of microorganisms.

Results

Screening in Liquid Media

All organisms used were screened in both defined and undefined media (Media 1 and 2). Single colonies of the organisms to be screened were picked off solid media and inoculated into the two screening media (50 ml volume). These cultures were incubated at 28^{o}C, 175 r.p.m. in an orbital incubator for a minimum of four days (and afterwards until good growth was seen) before diosgenin dissolved in ethanol was added to give a final diosgenin content of 1 mg ml^{-1} culture. After a further 7 days incubation the cultures were extracted by the standard method and assayed for both diosgenin remaining (g.l.c) and products formed (t.l.c.).

The results are listed in Table 7. It can be seen that a number of bacteria are capable of transforming diosgenin to a considerable degree. In some cases metabolites were accumulated (usually diosgenone and 1-dehydrodiosgenone). Few of the natural isolates (which were selected from a medium where diosgenin was sole carbon source) showed good activity, the exception being isolate 5 (100% diosgenin transformation in 7 days).

The organisms most active in transforming diosgenin were:- Bacillus sphaericus, Corynebacterium mediolanum, Mycobacterium fortuitum, M. phlei, Nocardia rhodochrous, Fusarium solani, Helicostylum piriforme, Stachylidium bicolor and natural isolate 5.

Table 7. Results of the screening programme

Organism	Medium 1 (complex)		Medium 2 (defined)	
	% diosgenin transformed (time of fermentation)	Products observed/ identified	% diosgenin transformed (time of fermentation)	Products observed/ identified
Bacillus megaterium MUCOB 127	1(*)	none	0 (*)	-
Bacillus sphaericus	100 (48h)	D.DHD	10	none
Bacillus subtilis MUCOB 284	6	none	2	none
Corynebacterium mediolanum NCIB 7206	100 (36h)	D, DHD	100	D, DHD
Corynebacterium xerosis MUCOB 255	0	-	0	-
Mycobacterium fortuitum NTCC 1542	100 (120h)	none (a) D,DHD,AD,ADD(b)	63	none (a) D,DHD,AD,ADD(b)
Mycobacterium fortuitum NTCC 10394	100 (120h)	none (a) D,DHD,AD,ADD(b)	69	none (a) D,DHD,AD,ADD(b)
Mycobacterium phlei NCIB8573; MUCOB 346	100 (96h)	none (a) D,DHD,AD,ADD(b)	70	none (a) D,DHD,AD,ADD (b)

(*) Incubation time was 7 days unless indicated otherwise.

Table 7 (contd.)

Organism	Medium 1 (complex)		Medium 2 (defined)	
	% diosgenin transformed (time of fermentation)	Products observed/ identified	% diosgenin transformed (time of fermentation)	Products observed/ identified
Nocardia graminis MUCOB 139	0	-	0	-
Nocardia restricta NCIB 10027	0	-	0	-
Nocardia rhodochrous NCIB 10554	100 (48h)	D,DHD	100 (5 days)	D,DHD
Pseudomonas aeruginosa NCIB 950	0	-	0	-
Pseudomonas viscosa MUCOB 151	0	-	0	-
Streptococcus faecalis MUCOB 37	0	-	No growth	-
Streptococcus mutans MUCOB 263	0	-	No growth	-
Streptomyces albus MUCOB396	10	none	2	none

Table 7 contd.

Organism	Medium 1 (complex) % diosgenin transformed (time of fermentation)	Medium 1 (complex) Products observed/ identified	Medium 2 (defined) % diosgenin transformed (time of fermentation)	Medium 2 (defined) Products observed/ identified
Streptomyces griseus NCIB 8506	4	none	5	none
Streptomyces lincolnensis ATCC 25466	3	none	0	-
Streptomyces mimosus NCIB 8229	1	none	8	none
Streptomyces paucis-porogenes NCIB 8790	15	none	8	none
Streptomyces platensis NCIB 9607	6	none	10	none
Streptomyces scabies MUCOB 128	0	-	0	-
Cunninghamella blakesleeana CMI 63877	23	none	0	-
Cunninghamella blakesleeana CMI 53585	24	none	0	-

Table 7 (contd.)

Organism	Medium 1 (complex) % diosgenin transformed (time of fermentation)	Medium 1 (complex) Products observed/ identified	Medium 2 (defined) % diosgenin transformed (time of fermentation)	Medium 2 (defined) Products observed/ identified
Cunninghamella elegans CMI 54601	0	-	0	-
Fusarium solani CMI 129056	9	none	85	none
Helicostylum piriforme CMI 147995	56	none	10	none
Penicillium notatum MUCOB 418	0	-	4	none
Stachylidium bicolor ATCC 126722	49[c]	one; unidentified	No growth	-
Verticillium theobromae CMI 172699	27[c]	none	No growth	-
Natural isolate 1	0	-	0	-
Natural isolate 2	0	-	0	-
Natural isolate 3	0	-	4	none
" " 4	0	-	0	-
" " 5	100 (30h)	D,DHD	11	D,DHD

Table 7 (contd.)

Organism	Medium 1 (complex) % diosgenin transformed (time of fermentation)	Medium 1 (complex) Products observed/ identified	Medium 2 (defined) % diosgenin transformed (time of fermentation)	Medium 2 (defined) Products observed/ identified
Natural isolate 6	0	-	15	none
" " 8	0	-	16	none
" " 9	0	-	0	none
" " 10	0	-	7	none
" " 18	No growth	-	10	none
" " 20	0	-	0	-
" " 21a	2	none	8	none
" " 23	6	none	2	none
" " 26	9	none	4	none
" " 31	2	none	15	none
" " 35	8	none	No growth	-
" " 165	2	none	1	none
" " 210	2	none	0	-

Table 7 (concluded)

Organism	Medium 1 (complex) % diosgenin transformed (time of fermentation)	Medium 1 (complex) Products observed/ identified	Medium 2 (defined) % diosgenin transformed (time of fermentation)	Medium 2 (defined) Products observed/ identified
Natural isolate 211	0	-	2	none
" " 213	0	-	11	none

Notes a - without α,ά-bipyridyl present

b - with α,ά-bipyridyl present

c - medium 3

D = diosgenone; DHD = 1-dehydrodiosgenone; AD = androst-4-ene-3,17 dione

ADD = androsta-1,4 diene-3,17 dione

Identification of an unknown bacterium

In the initial cultures of Streptomyces albus MUCOB 396, a contaminant was detected which was later found to be able to transform diosgenin in the screening programme. It was a gram-positive rod with terminal spores causing slight swelling and capable of good growth on Tryptone Soya Agar (Oxoid CM 131). On the basis of biochemical tests (Table 8) the organism was identified as Bacillus sphaericus.

Table 8. The identification of an unknown bacterium: Results of biochemical tests

Vogues Proskaur	-
Urease	+8 and 24 hours
Methyl red	-
Indole	-
Growth in 5% NaCl	+
Growth in 7% NaCl	-
Growth at 65°C	-
Hydrolysis of casein	-
Hydrolysis of starch	-
Acid from mannitol	+
Acid from glucose	slight ±
Acid from xylose	-

Tests - according to the methods listed by Cowan and Steel, 1974.

Separation of Transformation Products of Diosgenin

i) Column chromatography

The results of these experiments (Table 9, Figure 14) showed that the column was able to separate products 2, 3 and diosgenin adequately. However, most of product 1 co-eluted with diosgenin. A number of different eluent mixtures, flow rates and loading capacities were tried, but failed to yield pure product 1.

ii) Preparative plate thin layer chromatography

The results of the preliminary experiments showed that a continuous elution tank system gave a better band separation than a standard tank (Figure 15). A time of 2½ hours gave the best separation and was used in the product collection procedure. However product 1 was still contaminated with diosgenin so several alternative solvent systems were tested (Figure 16).

Table 9. Separation of diosgenin and products by column chromatography

Column: 1 cm diameter, 8 g silica gel G, activity grade 2.

Solvent: hexane 9:ethyl acetate 1. Flow rate 1 ml min^{-1}

Fraction	Eluate content
0 - 31 ml	nothing
32 - 55 ml	product 3
56 - 62 ml	nothing
63 - 104 ml	product 2
105 - 137 ml	diosgenin
138 - 144 ml	diosgenin + product 1
145 - 200 ml	nothing

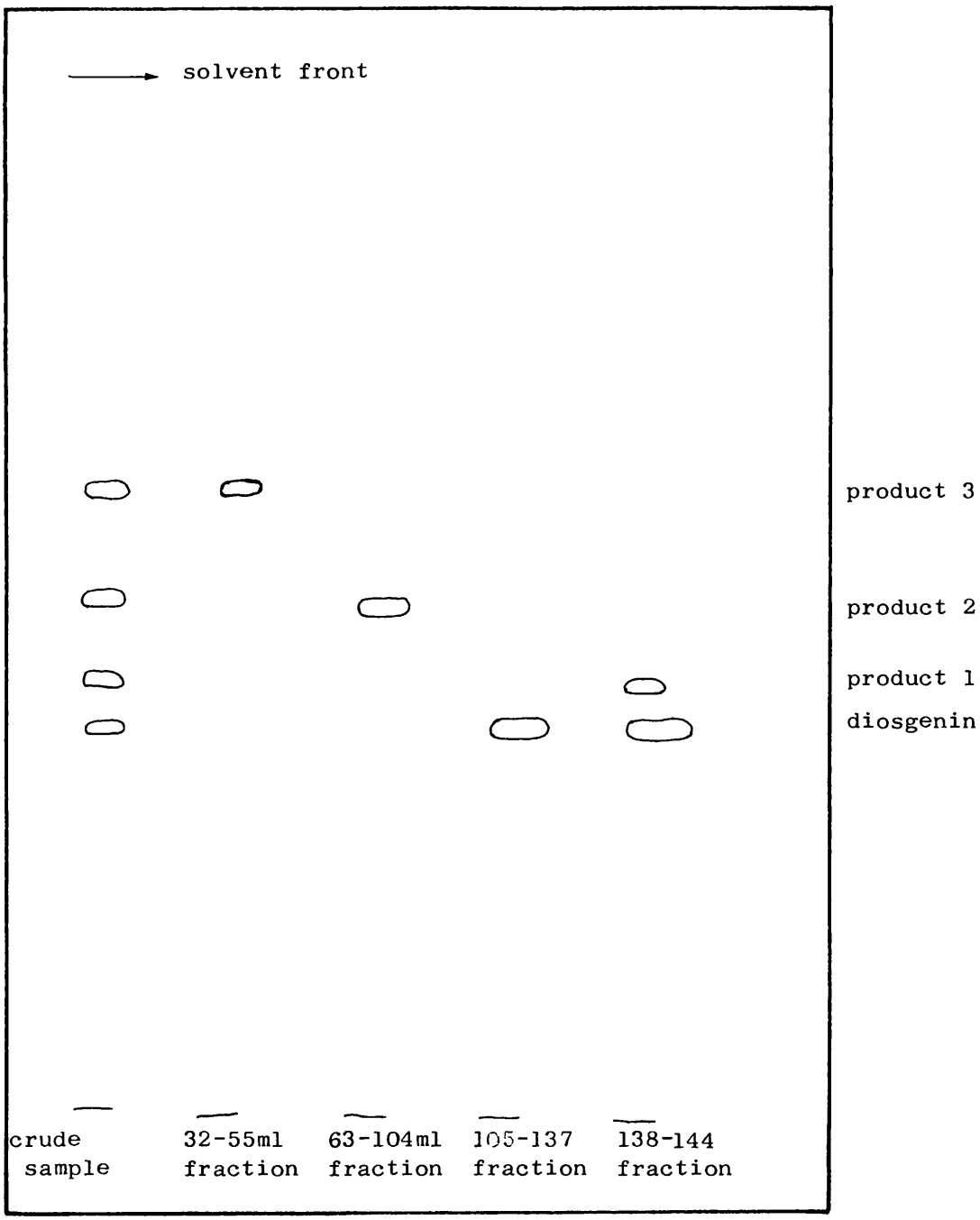


Figure 14.T.l.c. of column fractions of diosgenin and products. Prespread Kieselgel 60F plate.Solvent system:- toluene 66;ethyl acetate 33;formic acid 1.

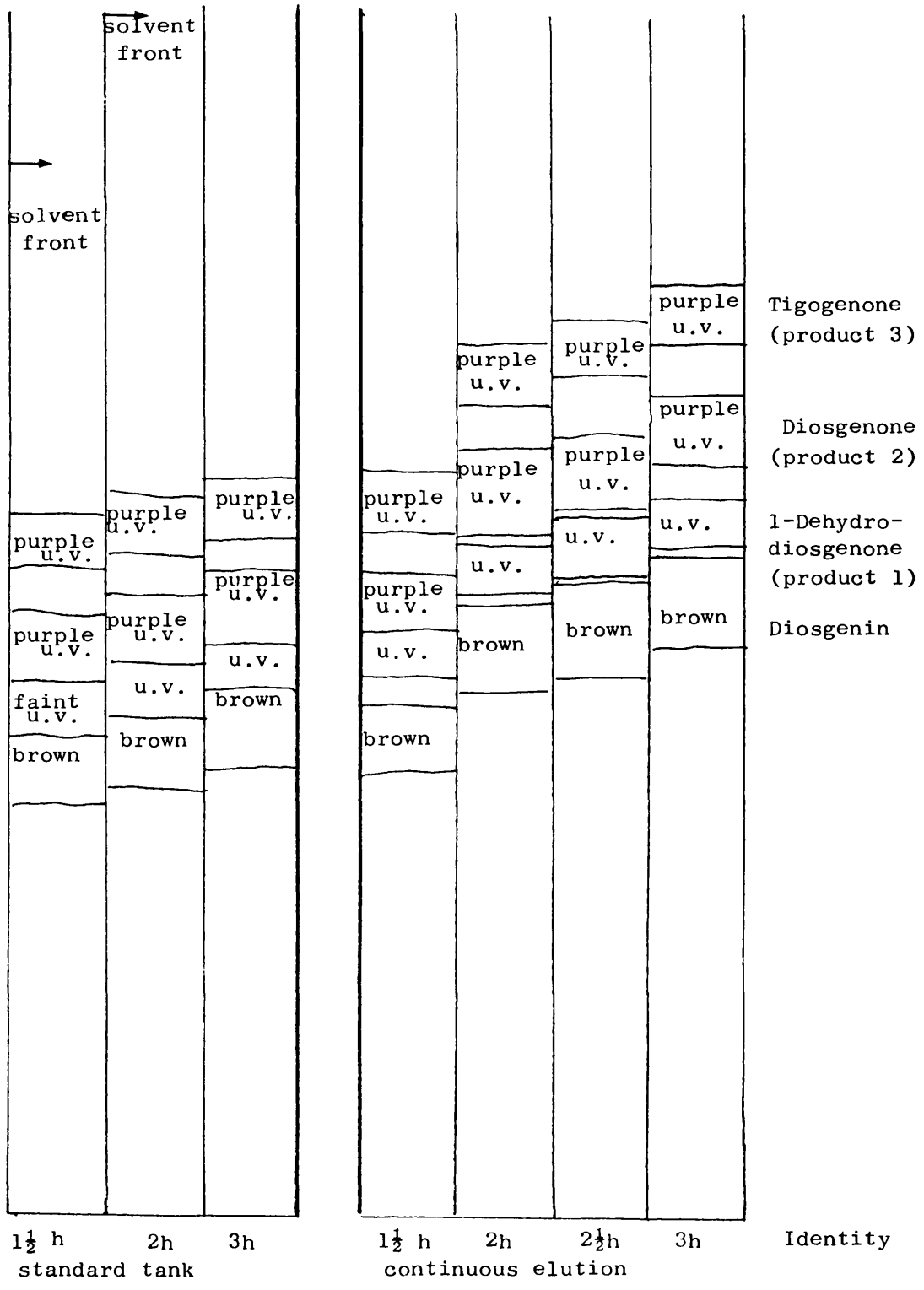
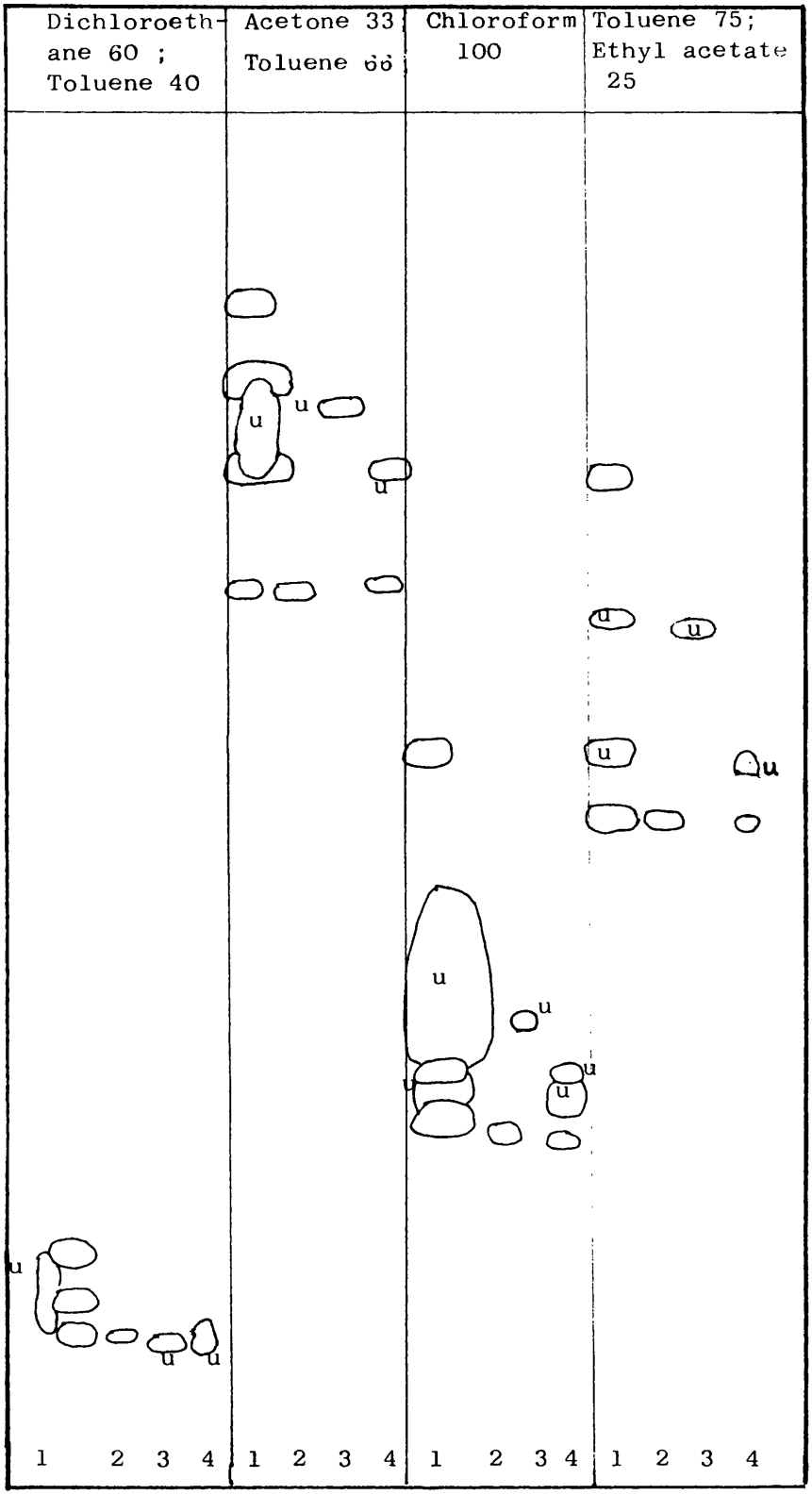


Figure 15.Preparative t.l.c. of diosgenin and products from Bacillus sphaericus.Effect of time on separation. Solvent system:toluene 66;ethyl acetate 33;formic acid 1.



1=crude sample;2=diosgenin;3=product 2;4=impure product 1.
u=fluorescence,u.v.254nm.

All samples run for 2½ hours,continuous elution.

Figure 16.Preparative t.l.c. of diosgenin and products from Bacillus sphaericus.Effect of different solvent systems on separation.

Acetone 33: toluene 66 (2½ hours, continuous elution) was found to give good separation of the components in the impure product 1 mixture and was used for its further purification.

Structural analysis of products

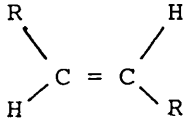
Product 1 - (25R) and (25S)-Spirosta-1,4 diene-3-one (1-dehydrodiosgenone)

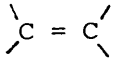
Product 1 (40 mg) was obtained by preparative t.l.c. of crude material from an experiment with N. rhodochrous (1.5 l)

i) Infrared spectrum

Many of the absorption bands in the finger print region of the spectrum (650 cm^{-1} - 1350 cm^{-1}) are the same as those observed in the spectrum from the crude diosgenin used, including those of the spiroketal side chain which occur at 865, 900, 920, 980 cm^{-1}, (25R); 850, 900, 920 and 990 cm^{-1} (25S), (Wall et al., 1952). This shows that in the experiment with N. rhodochrous the spiroketal ring remained intact.

However, a number of strong absorptions occurred which were not given by diosgenin namely: (assignments from Williams and Fleming, 1980).

990 cm^{-1}  out of plane deformation, shifted from 960-970 due to presence of a carbonyl group.

1610 cm^{-1}  conjugated with C=O(range 1590-1650)

1650 cm^{-1} unsaturated ketone (range 1660-1670 less 10-20 for liquid film)

ii) Ultraviolet spectrum

A concentration of 1 mg in 50 ml hexane was used. The major absorbance was at λ = 231 nm ($\log_{10} I_o/I$ = 0.628). Assuming a mass of 410 (see mass spectrum, below) this gives an ε of 12,874. Dusza et al. (1963) state that $\Delta^{1,4}$-3-one steroids have λ_{max} 235-250, with ε range 9,700 - 19,000. Correcting the experimental λ value for the solvent used (hexane) gives a value of 242 - well within the range quoted.

iii) Mass spectrum

Budzikiewicz et al.(1962) studied the mass spectra of a number of steroidal sapogenins including diosgenin. They list the important high mass fragments and suggest structures for them (Figure 17). My samples of fenugreek diosgenin gave a matching spectrum.

A similar series of fragments is shown by product 1, but with each fragment 4 mass units less than that of diosgenin. The other difference in the two spectra (Table 10) is a lack of fragments corresponding to structures a, b and c less 18 units as a result of losing the hydroxyl group of diosgenin at C-3 as water. This suggests that product 1 no longer has such an hydroxyl group. A carbonyl group, which seems probable from I.R. and U.V. data would be resistant to water loss.

iv) Proton n.m.r.

Considering the spectral evidence above a $\Delta^{1,4}$ diene-3-one structure is likely (Figure 18). This has 3 vinylic protons which will appear downfield on an ^1H nmr spectrum. The values quoted for

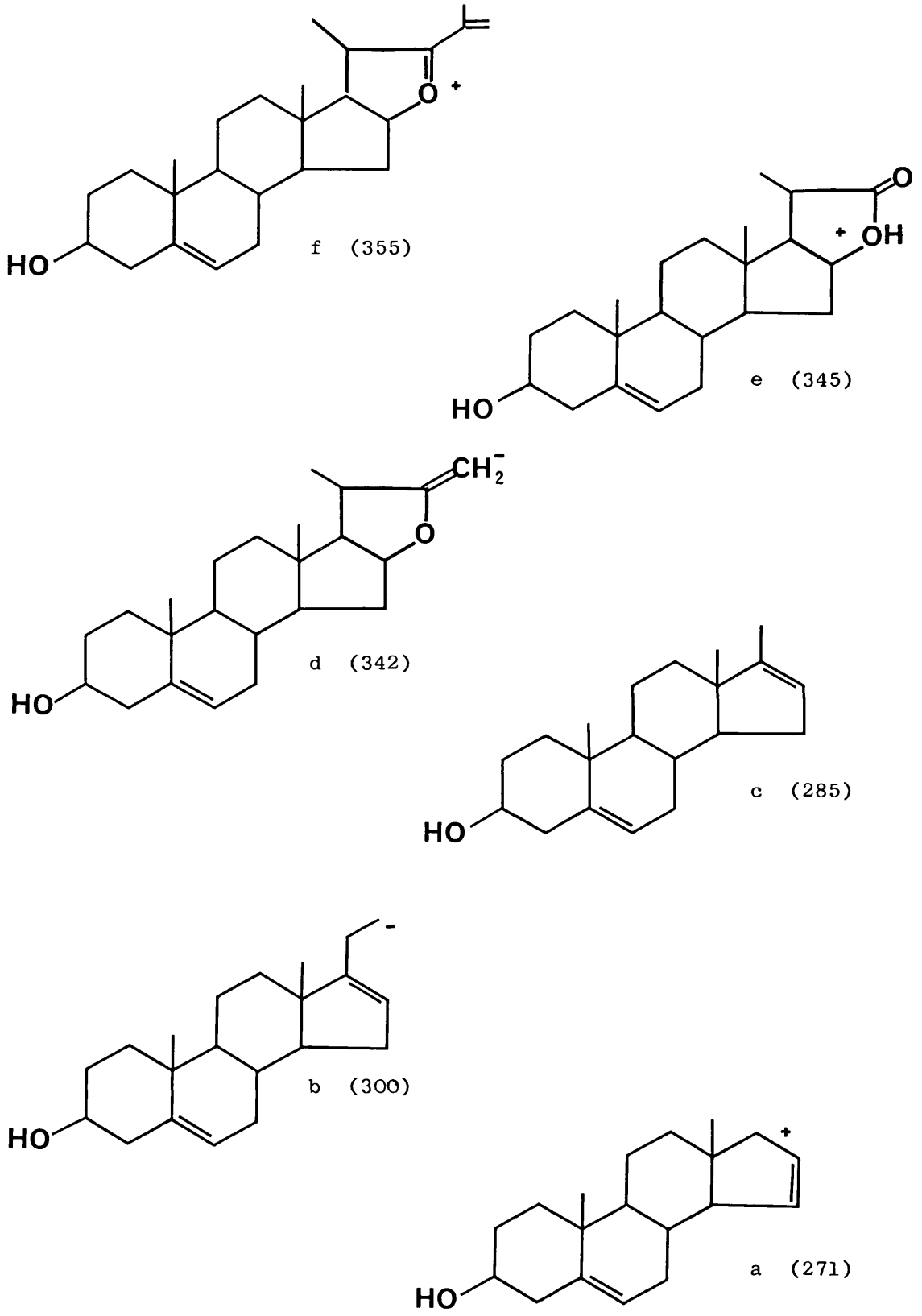


Figure 17.Mass fragments of diosgenin proposed by Budzikiewicz et al.,(1962).

Table 10 Mass spectral assignments of product 1 structure

Product 1	m/z	Diosgenin	m/z	structure assignment (see Figure 17)
Molecular ion	410	Molecular ion	414	
major fragments		major fragments		
	351		355	f
	341		345	e
	338		342	d
	296		300	b
	281		285	c
	absent		282	b-H_2O
	267		271	a
	absent		267	c-H_2O
	absent		253	a-H_2O
base peak	139		139	

an unsaturated ketone (i.e. protons a and b, Figure 18) by Williams and Fleming (1980) are δ6.5 - 8 for a and δ5.8 - 6.7 for b. Protons a and b are coupled and will therefore give doublets whilst c will be a singlet with a shift value similar to proton b. The actual nmr spectrum of the product (Figure 19) has two doublets and one singlet as required for the $\Delta^{1,4}$-diene-3-one structure. The integral values are 1:1:1 indicating 3 protons. The singlet falls within the expected range of 6.04 ppm, one doublet (6.2 ppm) is within the range predicted for proton b, and the other at 7 ppm where the proton a would be expected. As they are in a cyclohexane type ring the a and b doublets show spin-spin splitting patterns. The vicinal protons produce a coupling constant (J) of 10 hz. The placement of protons a and b is further confirmed by the slight splitting of the b doublet caused by long range coupling with c across the π bonds. (The peak at 7.25 ppm is due to impurities in the deuterochloroform).

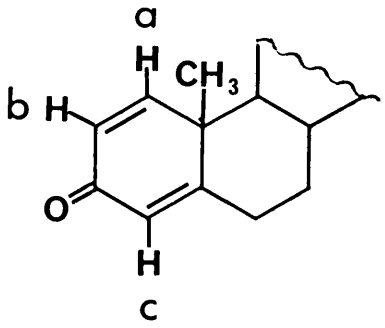


Figure 18. Rings A and B of product 1 with A ring protons labelled.

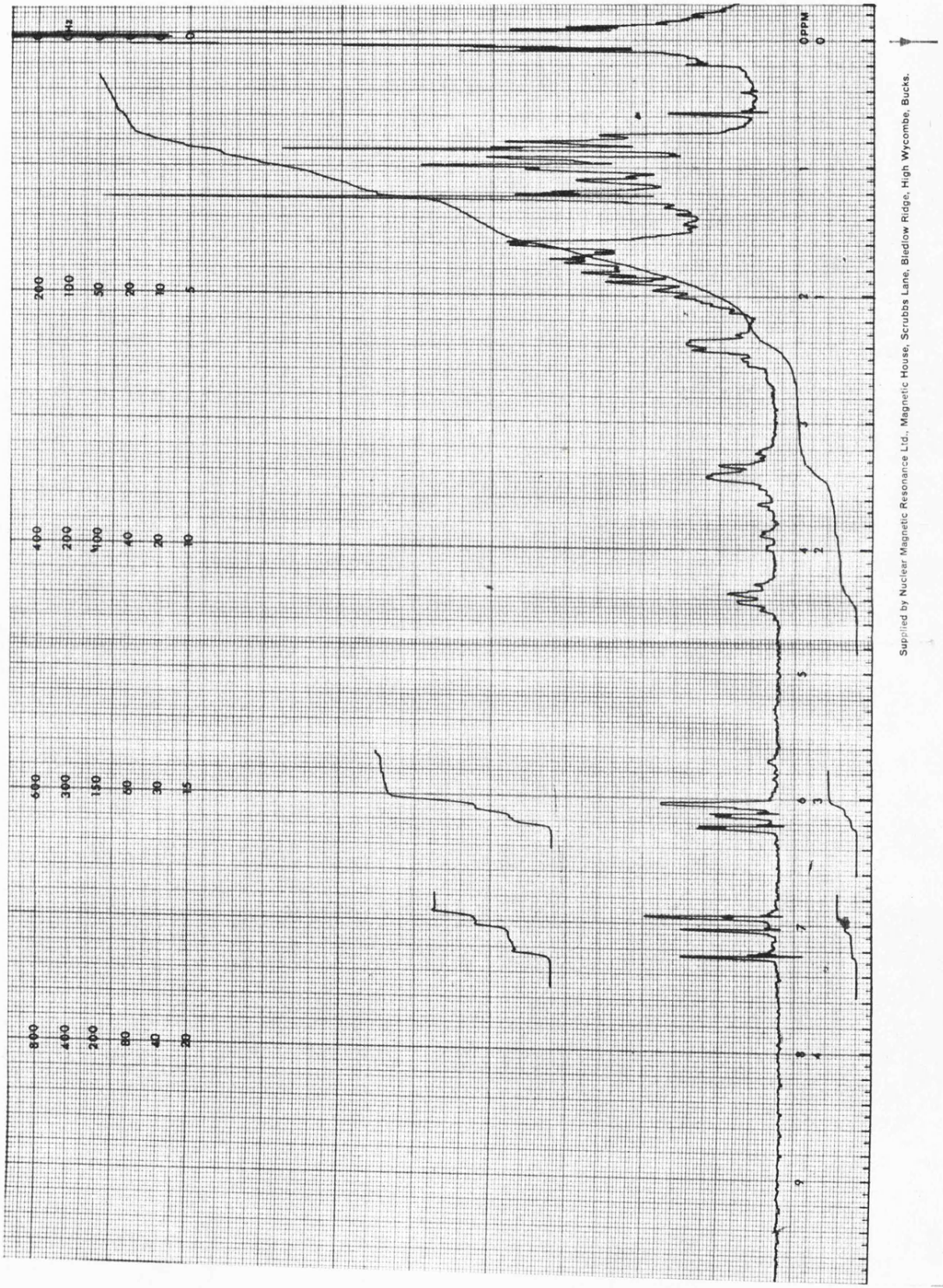


Figure 19. ^{1}H N.M.R. Product 1.

v) Carbon-13 nmr

The analytical evidence so far described indicated the structure of product 1 to be (25ξ)-spirosta-1,4-diene-3-one. Such a structure will give very similar chemical shift values to those of other Δ1,4-diene-3-one's for rings A and B, e.g. androsta-1,4-diene-3,17-dione. The carbon shifts for this compound are readily available from the literature (Hanson and Siverns, 1975). When considering the ^{13}C spectrum it must be remembered that the substrate added to the fermentation is not pure diosgenin but predominantly a mixture of diosgenin and yamogenin. Eggert and Djerassi (1975) have shown that 25R and 25S epimers of sapogenins produce different shift values for carbons 20 - 27, although they have not studied yamogenin itself. A 13-C nmr spectrum of the diosgenin/yamogenin mixture used in these experiments (Table 11) matches well with the data for diosgenin given by Eggert and Djerassi (1975), with 7 extra values which match those that would be expected for the 25S epimer, yamogenin, also present.

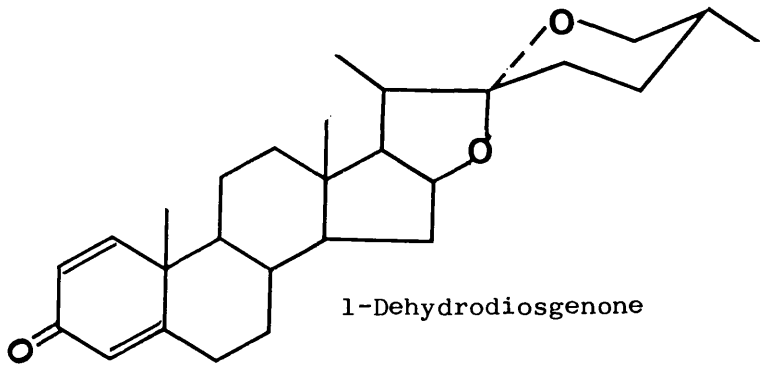
Product 1 has a spectrum (Table 11) which is almost identical to androsta-1,4-diene-3,17-dione for carbon atoms 1 - 11 (rings A and B), and to diosgenin and yamogenin for carbon atoms 12 - 27 (rings D, E and F). The structure of the A ring is further confirmed by off-resonance decoupling which shows carbons 1, 2 and 4 to each have one proton (producing doublets) and carbons 3 and 5 to lack protons (singlets).

Product 1 therefore can be confirmed as a mixture of (25R) and (25S)-spirosta-1,4-diene-3-one, the trivial name of the (25R)-epimer is used in this thesis; 1-dehydrodiosgenone (Figure 20).

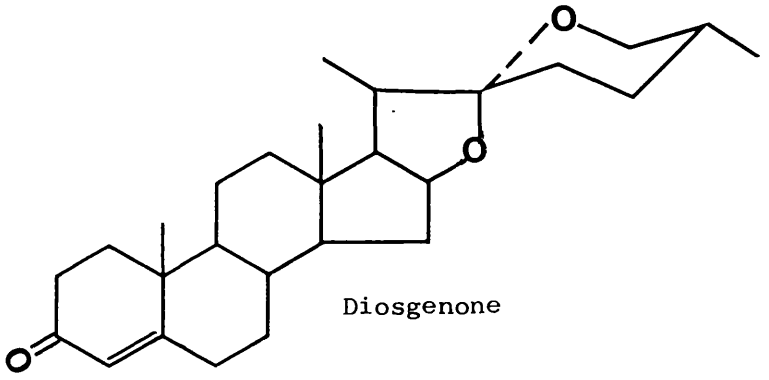
Table 11. Carbon-13 nmr shift values of diosgenin and product 1.

Carbon	androsta-1,4-diene-3,17-dione[1]	Product 1	Diosgenin/yamogenin substrate mixture[2]	
1	155.2 D	155.4 D	37.2	
2	127.6 D	127.6 D	31.6	
3	186.0 S	185.8 S	71.5	
4	124.0 D	124.0 D	42.2	
5	168.2 S	168.6 S	140.8	
6	32.3	32.8	121.3	
7	31.2	31.5	32.0	
8	35.0	35.3	31.4	
9	52.3	52.6	50.1	
10	43.4	43.6	36.6	
11	22.0	22.8	20.9	
12	32.5	39.7	39.8	
13	47.6	40.7	40.2	
14	50.4	55.4	56.5	
15	21.8	32	31.8	
16	35.5	80.6	80.7	
17	219.6	62.1	62.1	
18	13.8	16.4	16.3	
19	18.7	18.9	19.4	
20	-	41.8	41.6	
			(25R) DIOS	(25S) YAMO
21	-	14.5	14.5	14.3
22	-	+109.2	109.1	109.5
23	-	31.5,27.2	31.4	27.2
24	-	28.9,25.9	28.8	25.8
25	-	30.3,26.1	30.3	26.0
26	-	66.9,65.2	66.7	65.0
27	-	17.1,16.1	17.1	16.1

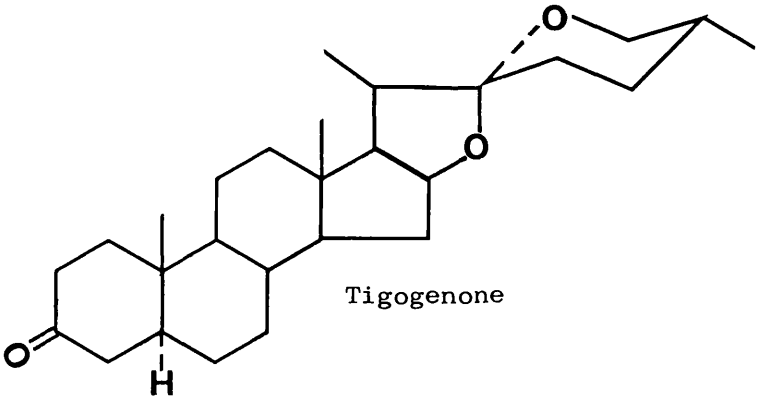
Notes: 1 - Hanson and Siverns, 1975; 2 - Own data, shifts based on Eggert and Djerassi, 1975.



1-Dehydrodiosgenone



Diosgenone



Tigogenone

Figure 20. Structures of the (25 R)- epimers of the major products obtained from the microbial transformation of crude diosgenin.

Product 2: (25R) and (25S)-Spirost-4-en-3-one (Diosgenone)

Determination of Structure

Product 2, (98 mg) was obtained by preparative t.l.c. of products from an experiment with 1.5 l of N.rhodochrous.

i) Infrared spectrum

As with product 1 many of the absorption bands in the finger-print region correspond to those of diosgenin.

The only major difference in the I.R. spectra of diosgenin and product 2 is the presence of a strong absorption at 1670 cm^{-1}. This is characteristic for the presence of an α,β-unsaturated ketone (Boul et al., 1971; Williams and Fleming, 1980); simple ketones give values above 1700 cm^{-1}.

ii) Ultraviolet spectrum

The major absorbance is at λ = 230 nm, $\log_{10} {}^{I_o}/_{I}$ = 0.845. A concentration of 1 mg in 50 ml hexane was used. Assuming a mass of 412 (see mass spectrum, below) this gives an ε of 17,407. Correcting the λ_{max} for the solvent used (+11, hexane) gives a λ_{max} of 241 nm. Applying the Fieser Woodward rules (Williams and Fleming, 1980) to the α,β-unsaturated carbonyl suggested by infrared spectroscopy gives a value of 244 nm - close to the experimental value. Dusza et al. (1963) give a λ_{max} of 241 nm for such a structure in a steroid. The ε value at 17,407 falls well within the range (10,000 - 20,000) that would be expected for a conjugated system.

iii) Mass spectrum

The mass spectrum of this product has a molecular ion of 412 and major fragments: 353, 343, 342, 340, 298, 283, 269 with a base peak of 139. These correspond to the series of fragments obtained from diosgenin less 2 mass units (Budzikiewicz et al., 1962). Again fragments arising from water loss from the molecule (which would occur at 280, 265 and 253) are not present in the spectrum, implying that the C-3 hydroxyl group has changed - presumably to a carbonyl.

iv) Proton nmr

A Δ4-ene-3-one steroidal structure will have one proton which will be considerably down field. This is clearly shown in practice with a singlet appearing at 5.66 ppm.

v) Carbon-13 nmr

Considering the preceding analytical data a spirost-4-ene-3-one structure seemed likely for this product. The compound should have a spectrum with the carbons of rings C, D, E and F giving chemical shift values similar to diosgenin with different values for rings A and B. Several steroidal compounds with a Δ4-ene-3-one structure will have similar shift values for the carbons of rings A and B. The spectra obtained from crude diosgenin, the product and an androst-4-ene-3-one (Holland et al., 1978) are listed in Table 12. The results confirm that the metabolite has the structure (25R) and (25S)-spirost-4-en-3-one (diosgenone) (Figure 20).

Table 12. Carbon-13 nmr shift values of product 2.

Carbon	Androst-4-ene-3-one	Product 2		Diosgenin/ yamogenin	
1	35.8	35.8		37.3	
2	34.0	34.0		31.6	
3	199.3	199.1		71.5	
4	124.1	124.0		42.2	
5	170.8	170.9		140.8	
6	32.7	32.8		121.3	
7	31.6	32.2		32.0	
8	35.5	35.4		31.4	
9	53.8	53.9		50.1	
10	38.7	38.7		36.6	
11			20.9	20.9	
12			39.8	39.8	
13			40.5	40.2	
14			55.8	56.5	
15			31.8	31.8	
16			80.7	80.7	
17			62.1	62.1	
18			16.4	16.3	
19	17.5	17.4		19.4	
20			41.8	41.6	
				(25R) DIOS	(25S) YAMO
21		14.5	+	14.5	14.3
22		109.2	+	109.1	109.5
23		31.5	27.1	31.4	27.2
24		28.9	25.9	28.8	25.8
25		30.3	25.9	30.3	26.0
26		66.9	65.2	66.7	65.0
27		17.1	16.1	17.1	16.1

Product 3: (25R) and (25S)-5α-Spirostan-3-one (Tigogenone)

Determination of Structure

Product 3, (46 mg) was obtained by preparative t.l.c. of the crude products from an experiment involving a 1.5 l culture of N. rhodochrous.

i) Infrared spectrum

The 'fingerprint' region of this product was again very similar to diosgenin, with the characteristic absorption bands of a spiroketal side chain showing the presence of a complete F ring. The only major difference in the two spectra is the presence of a strong absorbance at 1702 cm^{-1} in the product's spectrum. This is characteristic of an unconjugated carbonyl group (Williams and Fleming, 1980; Boul et al., 1971)

ii) Ultraviolet spectrum

No strong absorbances of ultraviolet light occurred. This indicated that the molecule does not have any conjugated systems.

iii) Mass spectrum

The molecular ion of the product was 414 and the major mass fragments 355, 345, 344, 342, 300, 285, 271, with a base peak of 139.

These masses were identical to the fragments obtained from diosgenin with the exception of those arising from loss of water (m/z 282, 267, 253). This suggested some change to the hydroxyl at C-3 or in the'A' ring structure.

iv) Carbon-13 nmr

On the basis of the infrared, ultraviolet and mass spectrometric evidence it seemed likely that this compound had a ketone group at C-3 and a saturated 'A' ring. Eggert and Djerassi (1973) give assignments for a compound with this structure for the A and B rings (3-androstanone). Using their data it was possible to assign all the 13-carbon nmr values obtained (Table 13) giving the structure suggested by the other spectroscopic evidence, namely (25R)- and (25S)-5α-spirostan-3-one (tigogenone and neotigogenone, Figure 20).

The source of this compound will be the tigogenin and neotigogenin impurities present in the crude diosgenin used in these experiments.

Other products - Androstanes

In later experiments with the Mycobacteria (Chapter 5), two more products were formed from diosgenin, but only in low yields (the maximum yield being less than 5%). As this made their isolation difficult and they were likely to be androstanes (Ambrus and Buki, 1969) identification was made by comparison with authentic reference samples.

The products were found to have the same g.l.c. retention times and t.l.c. r.f. values (in two different solvent systems) as androsta-1,4-diene-3,17-dione and androst-4-ene-3,17-dione. Although androsta-3,16-diones give the same r.f. values and U.V. absorbance on the t.l.c. systems used as androsta-3,17-diones, they can be differentiated on the basis of spot colour after the

Table 13. Carbon-13 nmr shift values of product 3

Carbon	3-Androstanone	Product 3		Diosgenin/yamogenin	
1	38.7	38.7		37.2	
2	38.1	38.1		31.6	
3	211.0	210.7		71.5	
4	44.6	45.0		42.2	
5	46.7	47.0		140.8	
6	29.0	29.9		121.3	
7	32.1	32.2		32.0	
8	35.7	35.9		31.4	
9	54.1	54.1		50.1	
10	35.7	35.3		36.6	
11	21.5	21.4		20.9	
12	38.8		40.2	39.8	
13	40.8		40.8	40.2	
14	54.3		56.3	56.5	
15	25.5		32.0	31.8	
16	20.5		80.8	80.7	
17	40.3		62.2	62.1	
18	17.5		16.6	16.3	
19	11.4	11.5		19.4	
20			41.9	41.6 (25R) DIOS	(25S) YAMO
21			14.6 14.3	14.5	14.3
22			109.1 109.6	109.1	109.5
23			31.7 27.4	31.4	27.2
24			29.1 26.0	28.8	25.8
25			30.5 26.2	30.3	26.0
26			66.8 65.0	66.7	65.0
27			17.2 16.3	17.1	16.1

10% sulphuric acid spray (Heftmann, 1975). Androsta-3,16-dione forms a pale green spot changing to grey after 10 minutes, androsta 3,17-dione forms a blue green spot changing to emerald green after 10 minutes. My products clearly correspond to the androsta-3,17-dione configuration.

Discussion

General Techniques

The extraction and sampling methods fall well within acceptable error limits, with an extraction efficiency of 98%. Routine analyses by g.l.c. and t.l.c. were found to be rapid and accurate methods of measuring steroid concentrations. A non-selective t.l.c. spray was used (10% H_2SO_4) so that any compounds present in the extracted crude material would be visualised.

Screening

The media chosen for the selection of microorganisms with steroid transforming activity appeared to fulfil their purpose. The defined medium (Medium 2) had urea as a nitrogen source and acetate as an initial source of carbon and energy. EDTA was present to prevent the formation of insoluble ionic complexes. This medium, being totally defined, should be free of any batch to batch variation. This was further ensured by making it from concentrated stock solutions. The medium was capable of supporting the growth of nearly all of the microorganisms screened although growth was generally much slower and the final cell density less than that obtained with the complex medium, 1. One of the reasons for the lower cell densities achieved was due to buffering problems

caused by the use of acetate as a carbon source. Other buffers were tried but were no more effective, nor were other carbon sources (e.g. succinate). Only by changing the carbon source to a carbohydrate could the pH be held at around 7 instead of climbing to pH 9 during the fermentation. However, this would have defeated the purpose of the growth medium and so it was not used. The complex medium (Medium 1) was a simple rich mixture in which most organisms were capable of heavy growth. The special medium (Medium 3) was used for Verticillium theobromae and Stachylidium bicolor on the recommendation of the Commonwealth Mycological Institute, Kew.

Differences in the steroid transformation rates occurred in the defined and complex media. In most cases better transformation was obtained in the complex medium (1) and this was probably due to the better growth most organisms achieved in this medium. However, several microorganisms had higher transformation rates in Medium 2 (defined) than in Medium 1, in particular Fusarium solani (85% transformation, Medium 2 and 9% in Medium 1) which is probably due to some form of repression occurring in the richer medium.

A number of isolates were made of organisms apparently capable of using diosgenin as a carbon source. When tested under screening conditions only one, a gram-positive coccus (Isolate 5) was highly active against diosgenin and accumulated products. A possible improvement to the selective plates, which was not attempted, would have been the addition of a broad spectrum fungicide to the

medium, thus preventing fungal overgrowth and aiding the isolation of bacteria.

Cunninghamella blakesleeana and Helicostylum piriforme although both metabolising diosgenin did not, as has been reported in the literature (Kaneko et al., 1969; Hayakawa and Sato, 1963) accumulate hydroxylated compounds. Possible reasons are strain differences or the unsuitability of the culture medium. The problem was not further pursued as the reported products were not of immediate interest.

The two commonest products obtained from the microbiological degradation of diosgenin were also the ones obtained in highest yield, namely diosgenone and 1-dehydrodiosgenone. This is in accordance with the limited previous reports indicating the 3β-hydroxyl group of diosgenin to be the initial point of attack.

Although the ability of microorganisms to transform diosgenin cannot be described as common, on the basis of the screen it cannot be described as very rare.

Amongst the microorganisms screened the Mycobacteria were noticeable for their ability to transform the 27 carbon diosgenin molecule to a 19 carbon androstane. This genus seems particularly active against a wide range of steroidal compounds and is currently a topic of much industrial interest.

The most interesting organisms selected through the screening programme were then investigated further. Details are given in the next chapter.

CHAPTER 4

The optimization of the microbiological transformation of diosgenin: the effects of varying the physical form of the reactants and the physiological state of the cells.

Introduction

Modes of Substrate Addition

Several different methods exist for the addition of steroids to fermentation media. Each has its advantages and disadvantages. Generally they involve the addition of fermentable substrate as a solution, as a dry powder or as a powder in aqueous suspension. The most widely used form is addition as a solution in a water-miscible organic solvent such as methanol or ethanol (Stadtman et al., 1954; Herzog et al., 1962; Hanson et al., 1953; Mamoli and Vercellone, 1937). Other water-miscible organic solvents are also used, for example N,N-dimethyl-formamide and dimethylsulphoxide (Dulaney and Stapley, 1959; Hanson, 1964). The major problem with all these solvents is their toxicity to microorganisms. The concentration of the organic solvent must be kept low if the viable cell population is not to be adversely affected and as a consequence the amount of substrate which may be added to the fermentation is also limited, generally to a level of 2 g l^{-1} or less. This is probably an important factor in limiting the rate of steroid biotransformation.

This problem can be overcome by addition of steroid in organic solvent followed by removal of the solvent, leaving finely divided microcrystalline substrate. Tak (1942) added cholesterol to his fermentation medium in acetone which was then removed by boiling, prior to inoculation with microorganisms. Loomeijer, (1958) used the same method. A similar process but using heptane: ethylene dichloride or methylene chloride as solvent has been patented more recently (Beaton, 1978). This patent claims to allow substrate concentrations as high as 200 g l^{-1}. The major disadvantages of the method are the

troublesome and expensive removal of organic solvent and the limitation of substrate addition to a time prior to inoculation with micro-organisms.

The addition of steroid to fermentation medium in a finely powdered form dates back to the earliest work on steroid transformation. The method was used by Mamoli and Vercellone (1937) and also by Turfitt (1944b, 1948) in his studies on cholesterol metabolism. Weaver and co-workers (1960, 1961) showed that micronisation of progesterone with a grinding mill before addition to a culture of Aspergillus ochraceus resulted in increased yields of the 11α-hydroxyl derivative. However, Charney (1966) states that the addition of steroid as a dry powder considerably slows its rate of transformation.

A frequent supplement to such micronised powders (which are usually generated by sonication or by mechanical grinding), is a detergent such as Tween 80 which facilitates wetting and therefore aids steroid dispersion and its subsequent bioconversion (Martin and Wagner, 1976).

The choice of method depends upon the transformation to be undertaken. For example Gabinskaya et al. (1980) found that ethanol or methylene chloride inhibited hydroxylation of androst-4-ene-3,17-dione by Rhizopus nigricans whilst dimethyl formamide (1% solution) did not do so.

A recent development has been the addition of steroid to fermentations in very high concentrations of water-immiscible solvent. This is considered in Chapter 7.

Cell Immobilisation

Immobilisation provides a convenient way of partitioning cells from fermentation media, aids product extraction and generally protects the cells from physical denaturation. Immobilised cells are being increasingly considered for transformation reactions because of their ease of handling and capability for re-use. Currently the two most frequently used immobilisation materials are polyacrylamide and calcium alginate. Polyacrylamide immobilisation is rapid and the structures formed are stable. However the presence of free radicals, toxic monomers and the heat generated by the polymerisation reaction leads to a considerable impairment of cell integrity and enzyme action (Cheetham, 1979a).

Calcium alginate gels are block co-polymers composed of β-D-mannuronate and α-L-guluronate residues gelled by the presence of calcium ions. They are moderately strong, resistant to fragmentation and compression and suitable for large scale column applications (Cheetham, 1979a; Cheetham et al., 1979). The method is very gentle, not causing cell or enzyme damage, with the cells actually being entrapped in the interstitial spaces of the gel. The major disadvantage of alginate gels is their disintegration in the presence of phosphates or other calcium chelating agents. Attempts have been made to prevent this by cross-linking agents (Birnbaum et al., 1981) but with only moderate success, the agents either being too expensive for large scale use or too toxic to the cells.

There have been a number of reports of the use of immobilised cells in the transformation of steroids. Skryaban et al. (1974)

report (in abstract form) the Δ1-dehydrogenation, C-20 ketone reduction, 11α- and 11β-hydroxylation of 4-ene-3-oxo-steroids using immobilised cells of Mycobacterium globiforme or Tieghemella orchedis. Voishvillo et al., (1976) used polyacrylamide immobilised M. globiforme and C. simplex cells to produce 1,4-diene-3-oxo steroids from 5-ene-3β-hydroxy and 5-ene-3β-acetoxy steroids with 70 - 80% yield. Yang and Studebaker (1978) succeeded in Δ1-dehydrogenating cortexolone (17α,21-dihydroxy-pregn-4-ene-3,20-dione) with polyacrylamide immobilised Pseudomonas testosteroni. They found that the cells had a half life of 103 hours, compared to 10 hours when not immobilised.

The most thoroughly studied immoblised cell system for the transformation of steroids concerns the production of prednisolone from cortexolone - the process is now in commercial use (Cheetham, 1980). The system is documented in a series of papers (Mosbach and Larsson, 1970; Larsson et al., 1976; 1978; 1979; Ohlson et al.,1978; 1979; 1980). The reaction is a two step one where cortexolone is converted to hydrocortisone (11β-hydroxylation) with Curvularia lunata, and the hydrocortisone to prednisolone (Δ1-dehydrogenation) with Corynebacterium simplex. The second step was initially carried out with polyacrylamide immobilised cells - which retained about 40% of their original activity. The group later switched to using alginate, where 80 - 90% of original activity was retained after immobilisation. The activity could be increased further (up to 9 fold) by incubation of the immobilised cells in a peptone-glucose media. This "activation" was primarily due to an increase in the cell population inside the calcium alginate pellets. Even after 40 days most of this increased activity was retained. Initial attempts to immobilise C. lunata were less successful, with

only low activity being retained. However by immobilising the fungal spores in alginate pellets and then allowing them to germinate *in situ* good activity was achieved, similar to that of free cells. Interestingly the same approach with polyacrylamide yielded only very low transformation rates. Maddox *et al.* (1981) also found 11α-hydroxylation activity by *Rhizopus nigricans* was very sensitive to entrapment; all activity was lost in polyacrylamide but some was retained in alginate, this activity could then be increased by an activation step (1% glucose).

Several recent reviews cover the field thoroughly including Wiseman 1975; Chibata *et al.*, 1976; Wang *et al.*, 1979; Cheetham, 1980. Current commercial usage is reviewed by Bucke and Wiseman, 1981.

Pseudo-crystallofermentation

An interesting discovery of Kondo and Masuo (1961) was the process of pseudo-crystallofermentation. Whilst investigating the conversion of hydrocortisone to prednisolone by *Corynebacterium simplex*, they found that very high concentrations of hydrocortisone (up to 500 g l^{-1}) could be transformed simply by adding it in a finely powdered form to the fermentation broth. As the transformation proceeded the crystals of hydrocortisone disappeared and the product crystallised out as needles. More than 90% of the prednisolone could be recovered after 120 hours from 100 ml of broth when 50 g of hydrocortisone was added as the starting material. Presumably the following reaction occurs

$$\underset{\text{(solid)}}{\text{Hydrocortisone}} \rightleftharpoons \underset{\text{(soln.)}}{\text{Hydrocortisone}} \rightleftharpoons \underset{\text{(soln.)}}{\text{Prednisolone}} \rightleftharpoons \underset{\text{(solid)}}{\text{Prednisolone}}$$

Although Kondo and Masuo tried many other microorganisms and steroidal

substrates this was the only combination they found where the pseudo-crystallofermentation took place. Subsequently one other report of a similar process has been published (Fa, Y-H et al.,1978) in the preparation of 13β-ethyl-3 methoxy-8,14 seco-1,3,5(10), 9(11)-estra-tetraene-17β-ol-14 one with Saccharomyces cerevisiae.

The unusual properties of the hydrocortisone to prednisolone reaction have been exploited by Mosbach's group (e.g. Larsson et al., 1976; Ohlson et al., 1979).

Constantinides (1980) further studied the system using collagen immobilised Corynebacterium simplex cells. He found that the initial rate of reaction increased with bulk steroid concentration up to a maximum of 40 mg ml^{-1} steroid. This is well above the maximum solubility of steroid (< 5 mg ml^{-1}), upon which reaction rate is usually dependent. Product inhibition was also observed: at low bulk concentrations of substrate (10 mg ml^{-1} hydrocortisone) the inhibition was severe with the reaction rate dropping to 25% of the rate obtained in the control (no product present) when in the presence of 4% (of the total steroid bulk concentration) product. At higher substrate concentrations (25 mg ml^{-1}) the reaction rate was first stimulated up to 1½ times normal (in the presence of 15% product) and then inhibited. However, even when 32% product was present the reaction rate was only reduced to 80% of the control (no product present). It is clearly this unusual characteristic which enables pseudo-crystallo-fermentation to take place.

Specific Materials and Methods

Sonication of diosgenin particles and measurement of particle size

An Ultrasonics Ltd. Rapidis 300 sonicator with 10 mm probe (20K frequency) was used. Sonication of diosgenin was carried out in a 25 ml beaker, containing 6 ml filtered (0.2 μ pore size) water, 45 μl (10%) Triton-X-100 and 0.3 g diosgenin. The beaker was placed in an ice bath. After sonication 1 ml from the resulting suspension was added to 50 ml prefiltered medium (0.2 μ pore size, Medium 5).

The steroid particle size in the medium was measured using a Fleming Instruments Ltd. Particle size micrometer and analyser (type 526) with a Watson microscope. Pictures of the crystals were taken through an Amplival microscope (Carl Zeiss, Jena) using phase contrast and Kodak Technical Pan film 2415 which was then developed in HC 110 (8 minutes, 1+9 dilution).

Whole Cell Immobilisation with Calcium Alginate

Microbial cells to be immobilised were harvested from early stationary phase cultures by centrifugation (23,000 g, M.S.E. High Speed 18 centrifuge). Cell immobilisation was carried out according to the methods of Kierstan and Bucke (1977), with some modifications to their concentrations.

Sodium alginate solution (2.5%, w/v) was made with rapid stirring and autoclaved before use. A solution (2 x 1 l) of 0.2 M calcium chloride was also prepared and steam sterilised prior to use. The cells were thoroughly mixed with alginate solution in the centrifuge buckets to give a cell concentration of 20% (w/v) and therefore an

alginate concentration of 2%. This slurry was pipetted dropwise into stirred calcium chloride solution from a height of 10 cm. The drops immediately polymerised into roughly spherical calcium alginate pellets with an approximate diameter of 2.5 mm. The pellets were left for 1 hour in the stirred calcium chloride before use to ensure complete polymerisation.

Results

Rates of diosgenin transformation by several microorganisms

Microorganisms found to be active against diosgenin in the screening programme (Chapter 3), were further studied.

All cultures were grown at 28°C in 500 ml baffled flasks. Medium 2, supplemented with 20 mM acetate, and Medium 5 were used (100 ml volumes). The fermentations were inoculated (0.5 ml) with starter culture in the early stationary phase. Throughout the experiment culture purity was checked by gram staining and colony morphology on nutrient agar (malt extract agar, Oxoid CM59, was used for *F. solani*). When the cultures reached early stationary phase diosgenin was added (after dissolution in ethanol) to give a final concentration of 1 mg ml^{-1} in the culture and an ethanol concentration of 5%.

The results are given in Table 14, Figures 21 and 22. The micro-organisms studied show a wide range of rates of growth and degradation. *N. rhodochrous* gave the highest rates both for the total degradation of diosgenin (Figure 21) and for maximum catabolic activity per hour (35 μmoles g^{-1} h^{-1}, Medium 5, Table 14).

Table 14. Rates of diosgenin transformation by several microorganisms

Organism	Medium Number	Diosgenin degradation		Product Formation[B]	
		μmoles h^{-1} (maximum)	μmoles $g^{-1}h^{-1}$ (maximum)	Diosgenone μmoles $g^{-1}h^{-1}$ (max)[A]	1-Dehydrodiosgenone μmoles $g^{-1}h^{-1}$ (max)[A]
B. sphaericus	5	3.07	6.8	2.24	1.63
	2	0.12	0.98	1.64	0
C. mediolanum	5	7.05	16.0	3.27	0
	2	0.37	2.87	1.06	0
M. phlei	5	1.67	4.82	0[C]	0[C]
	2	0.75	2.99	0[C]	0[C]
M. fortuitum	5	1.59	4.61	0[C]	0[C]
Natural isolate 5	5	7.44	13.2	2.23	0
	2	0.44	2.73	1.59	0
N. rhodochrous	5	22.45	35.08	2.02	1.52
	2	0.57	5.15	2.04	0
F. solani	5	0.45	0.62	0	0
	2	1.71	5.9	0	0

Medium 5 complex; Medium 2 defined

[A] Cell weights measured as dry cell weight (g)

[B] No other products detected

[C] No inhibitor of steroid nucleus degradation used.

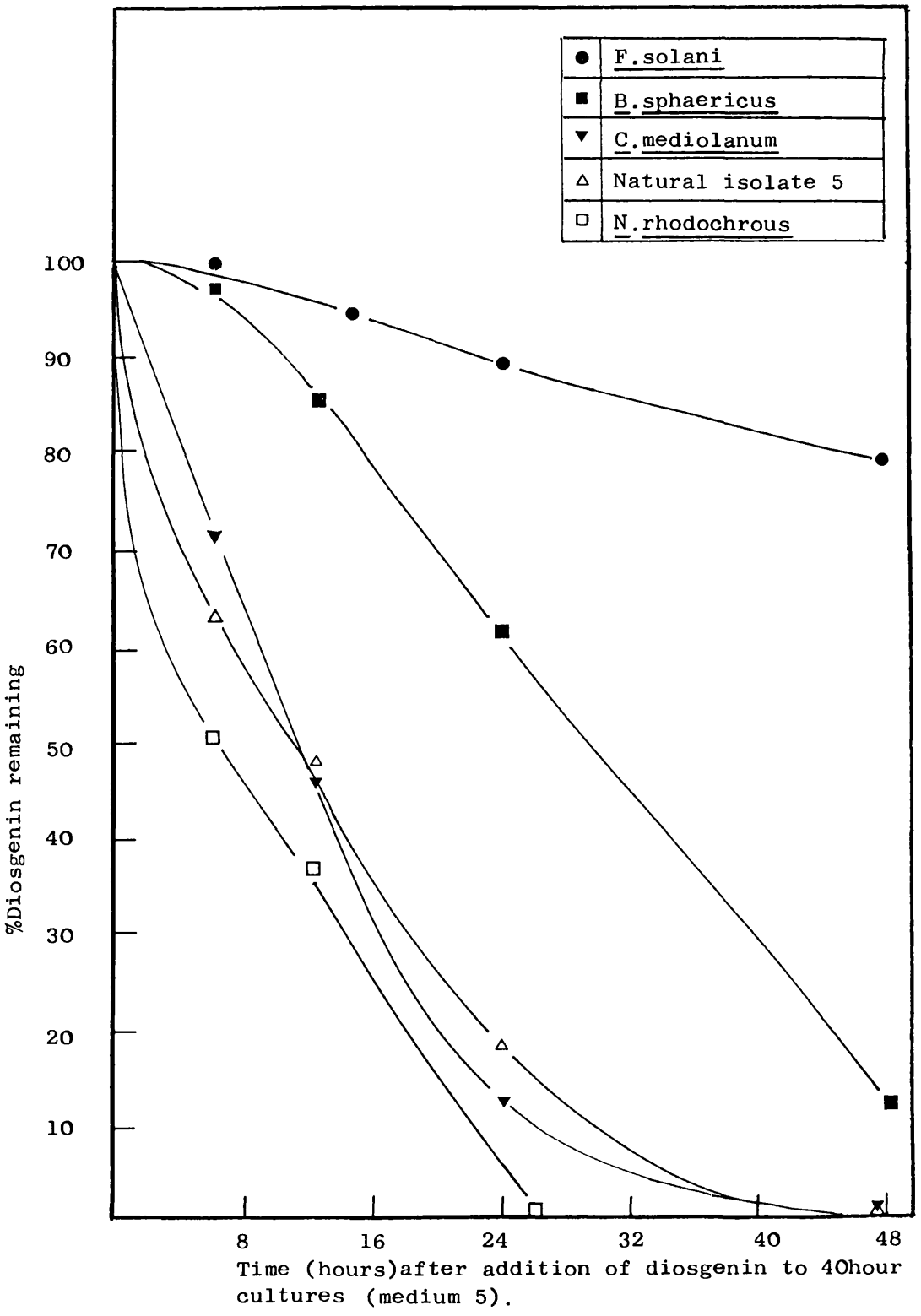


Figure 21.Rates of diosgenin transformation by several microorganisms.

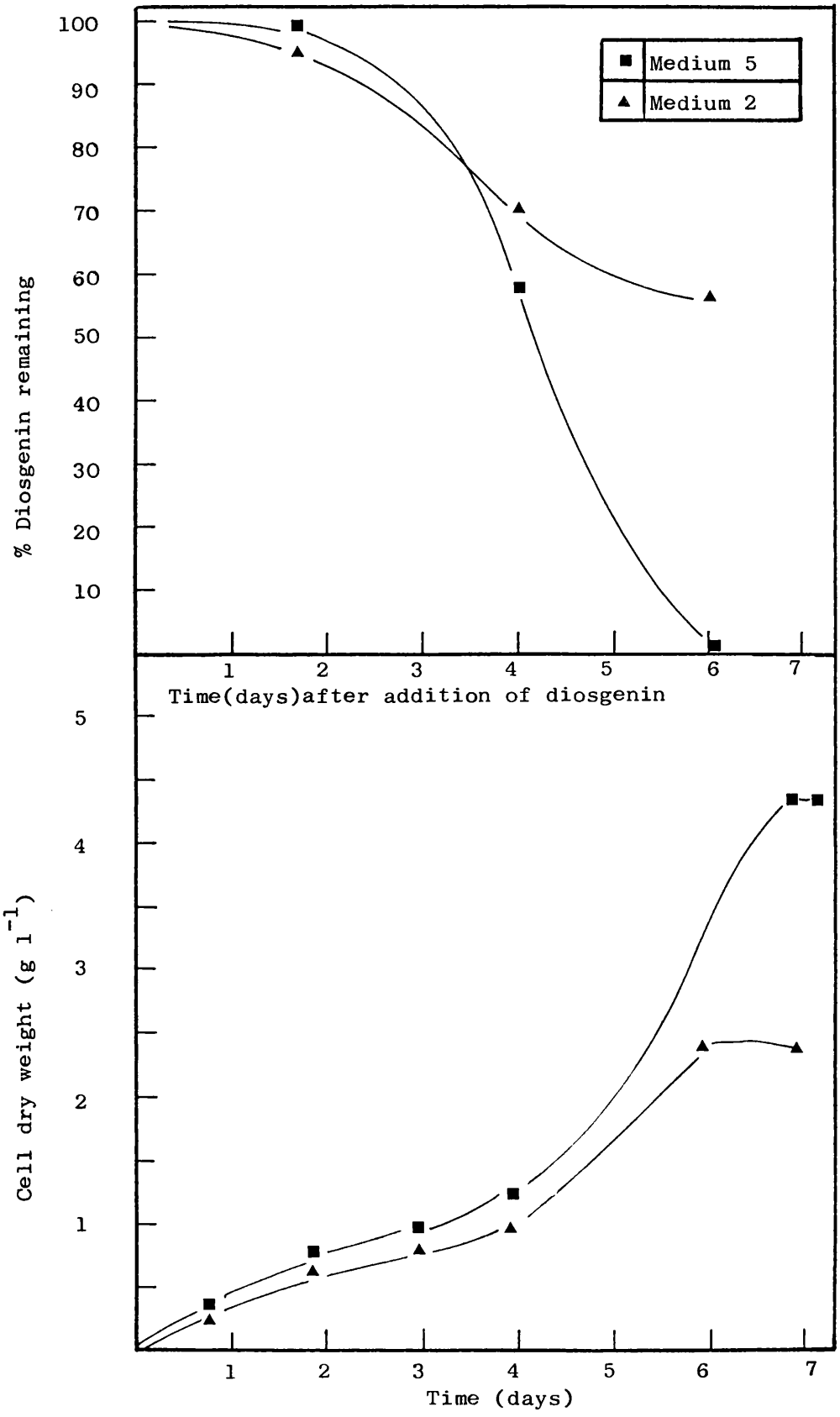


Figure 22. Mycobacterium phlei, growth and transformation

C. mediolanum and natural isolate 5 had comparable but lower maximum rates (16 μmoles $g^{-1}h^{-1}$ and 13.2 μmoles $g^{-1}h^{-1}$, respectively) whilst B. sphaericus, F. solani, M. phlei and M. fortuitum were considerably slower in degrading diosgenin (rates 6.8, 5.9, 4.8 and 4.6 μmoles $g^{-1}h^{-1}$).

All microorganisms which accumulated products (B. sphaericus, C. mediolanum ,N. rhodochrous, natural isolate 5) formed the same ones - diosgenone and 1-dehydrodiosgenone. C. mediolanum gave the highest rate of diosgenone formation (rate 3.2 μmoles $g^{-1}h^{-1}$) closely followed by B. sphaericus (2.2 μmoles $g^{-1}h^{-1}$), natural isolate 5 (2.2 μmoles $g^{-1}h^{-1}$) and N. rhodochrous (2.0 μmoles $g^{-1}h^{-1}$).

The Mycobacteria studied (M. phlei and M. fortuitum) catabolised all the diosgenin without accumulation of metabolites. M.phlei was used in later experiments as it lacks the pathogenic properties associated with M. fortuitum.

F. solani failed to break down all the diosgenin present over the period of the experiment (6 days) and did not accumulate any products in the medium.

The differences between the defined and complex media were clearly shown, with the undefined medium (5) allowing both heavier cell growth and also more activity per gram cells. The only exception to this was F. solani which, although growing to a higher cell density in the complex medium had less transforming ability in that medium.

Modes of Diosgenin Addition

The various common methods of addition of steroids to fermentation systems are discussed in the introduction. The effects of several methods of steroid addition to N. rhodochrous fermentations were tested.

i) The effect of ethanol concentration on N. rhodochrous viable cell count.

Twelve flasks containing Medium 5 were inoculated from a starter culture of N.rhodochrous and allowed to grow for 48 hours. Absolute ethanol was then added to duplicate flasks to give final concentrations of 20%, 15%, 10%, 5%, 2.5% and 0% ethanol. After a further 24 hours incubation viable counts from all cultures were taken. The results are illustrated in Figure 23.

No change in the viable cell count was observed up to the 5% ethanol level, the population remaining at 8×10^8. At 10% ethanol the population decreased by half to 4×10^8. At higher concentrations of ethanol the cell population declined at a very rapid rate and at concentrations of over 15% no viable cells could be detected.

ii) The effect of addition of diosgenin in solution and in suspension to N. rhodochrous cultures.

N. rhodochrous cells were grown for 52 hours in 100 ml Medium 5 before diosgenin (100 mg) was added in various forms. After a further 18 hours incubation the cultures' dry weights, viable cell counts and diosgenin contents were determined. The results are given in Table 15.

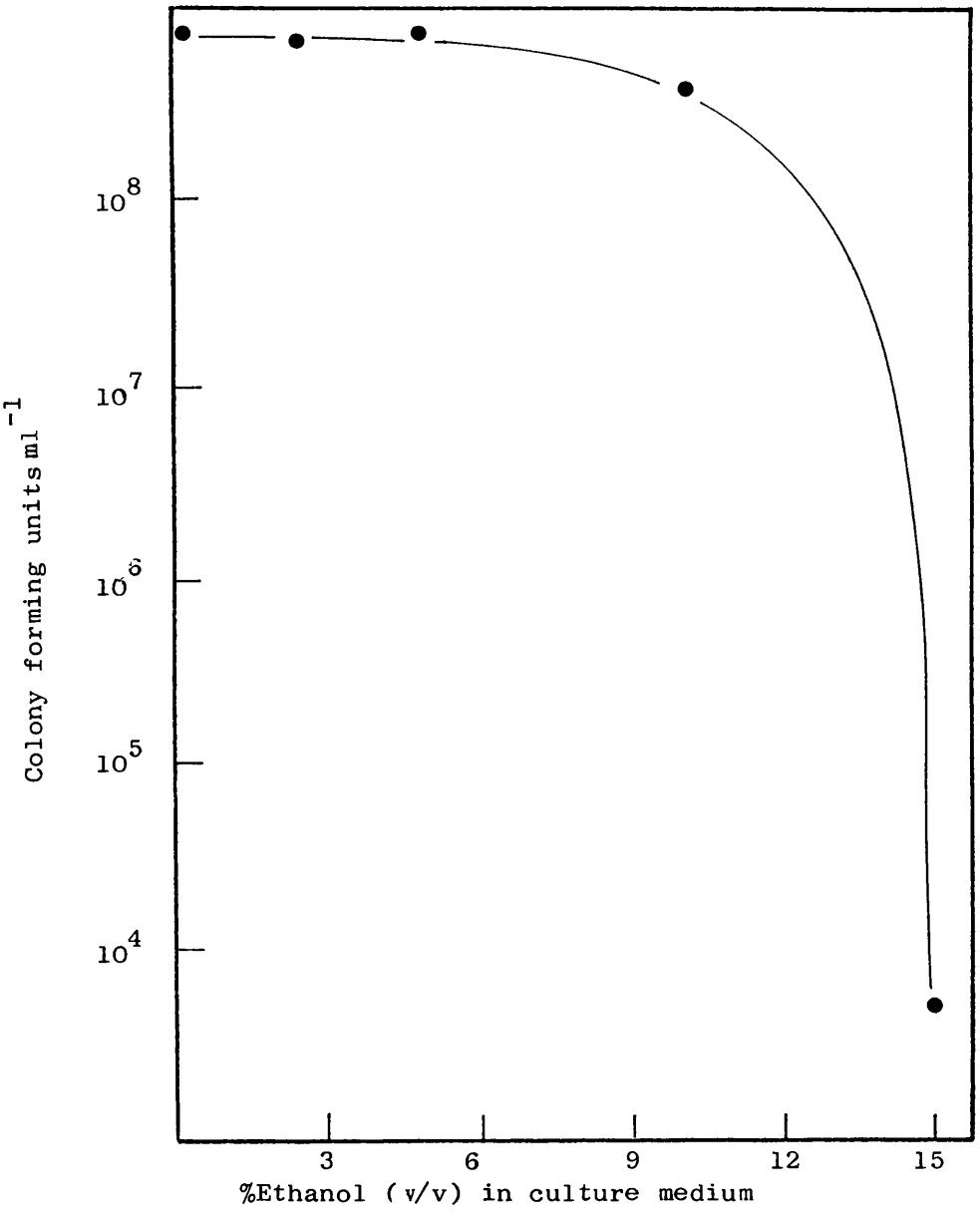


Figure 23. The effect of ethanol concentration on N. rhodochrous viable cell count.

N. rhodochrous cells were grown for 48 hours in Medium 5 before the addition of ethanol, viable counts were taken 24 hours later.

Table 15. The effect of addition of diosgenin in solution and in suspension to N. rhodochrous cultures

Form	Dry cell weight, $g\ l^{-1}$, 18h post addition	Viable count 18 h post addition	% Diosgenin remaining	Transformation rate ($\mu moles\ g^{-1} h^{-1}$)
DMF[1]	4.1	1.2×10^9	27.5	23.7
Ethanol[2]	4.1	7.7×10^8	34.0	21.6
Triton[3]	3.8	1.4×10^9	77.4	9.6
Crude[4]	4.1	1.4×10^9	100.0	0

Notes

1. 100 mg diosgenin dissolved in 2.5 ml dimethylformamide added to 100 ml culture
2. 100 mg diosgenin dissolved in 5 ml ethanol added to 100 ml culture
3. 100 mg diosgenin in 2 ml distilled water + 15 µl 10% Triton-X-100 sonicated for 5 minutes (50 watts) and then added to 100 ml culture.
4. 100 mg coarsely powdered diosgenin added to culture without any other treatment.

When diosgenin was added as a crude dry powder without any treatment no transformation took place over the 18 hours of the experiment. Cell dry weight and viable count were similar to control values.

The addition of diosgenin in solid form, but as a sonicated suspension dispersed in Triton-X-100 was more effective, a transformation rate of 9.6 μmoles $g^{-1}h^{-1}$ being achieved. A slight decrease in dry cell weight occurred, from 4.1 g l^{-1} to 3.8 g l^{-1}.

The most effective form of diosgenin addition was as a solution in either ethanol or N,N-dimethylformamide (DMF). Even though the steroid was precipitated immediately the solvent and fermentation broth were mixed this method gave the most rapid rates of diosgenin transformation, more than twice that achieved by sonication. DMF (23.7 μmoles $g^{-1}h^{-1}$) allowed a slightly higher rate than ethanol (21.6 μmoles $g^{-1}h^{-1}$) and a slightly higher viable cell count (1×10^9 compared to 8×10^8). With either solvent the diosgenin concentration used was approaching saturation point.

Microscopic examination of the diosgenin particles in the fermentation medium resulting from sonication in Triton or dissolution in ethanol (Figure 24) showed that they had very different physical forms. The sonicated samples were composed of small fragmented prisms. In contrast the particles precipitated from ethanolic solution were amorphous, diffuse and irregular, forming thin flakes.

iii) The effect of sonication on diosgenin crystal size and its transformation by N. rhodochrous.

In an attempt to improve the rate of transformation of the Triton

Diosgenin after sonication (5 minutes, 50 watts)
Magnification x180

Diosgenin after precipitation from ethanolic solution.
Magnification x180

Figure 24. The physical form of diosgenin after addition to culture medium from solution or suspension.

suspended diosgenin the effect of sonication on both particle size and the subsequent transformation were investigated.

Microscopic examination of the crystals showed that the time of sonication had an effect on their size (Figure 25). This was investigated in more detail using a particle size micrometer (see Materials and Methods Section, this chapter). Figure 26 shows the effect of various sonication treatments on diosgenin crystal size. As the length of sonication was increased the proportion of crystals with a small particle size increased. With 5 minutes sonication, 50 watts power, 70% of crystals fell in the particle size range 0 - 2.5 μ; with 20 minutes sonication, 200 watts power nearly 90% of crystals were in this size range.

To determine whether this had any effect on the rate of steroid transformation samples of the sonicated diosgenin were added to 48 hour cultures of N. rhodochrous (Medium 5) and incubated for a further 24 hours before extraction. The results are given in Table 16. Clearly the degree of sonication does have an effect on the rate of diosgenin degradation by N. rhodochrous, the rate increased from 9.0 μmoles $g^{-1}h^{-1}$ (5 minutes, 50 watts) to 12 μmoles $g^{-1}h^{-1}$ (20 minutes, 200 watts). However this rate was still considerably lower than that achieved by adding diosgenin in solution to fermentations.

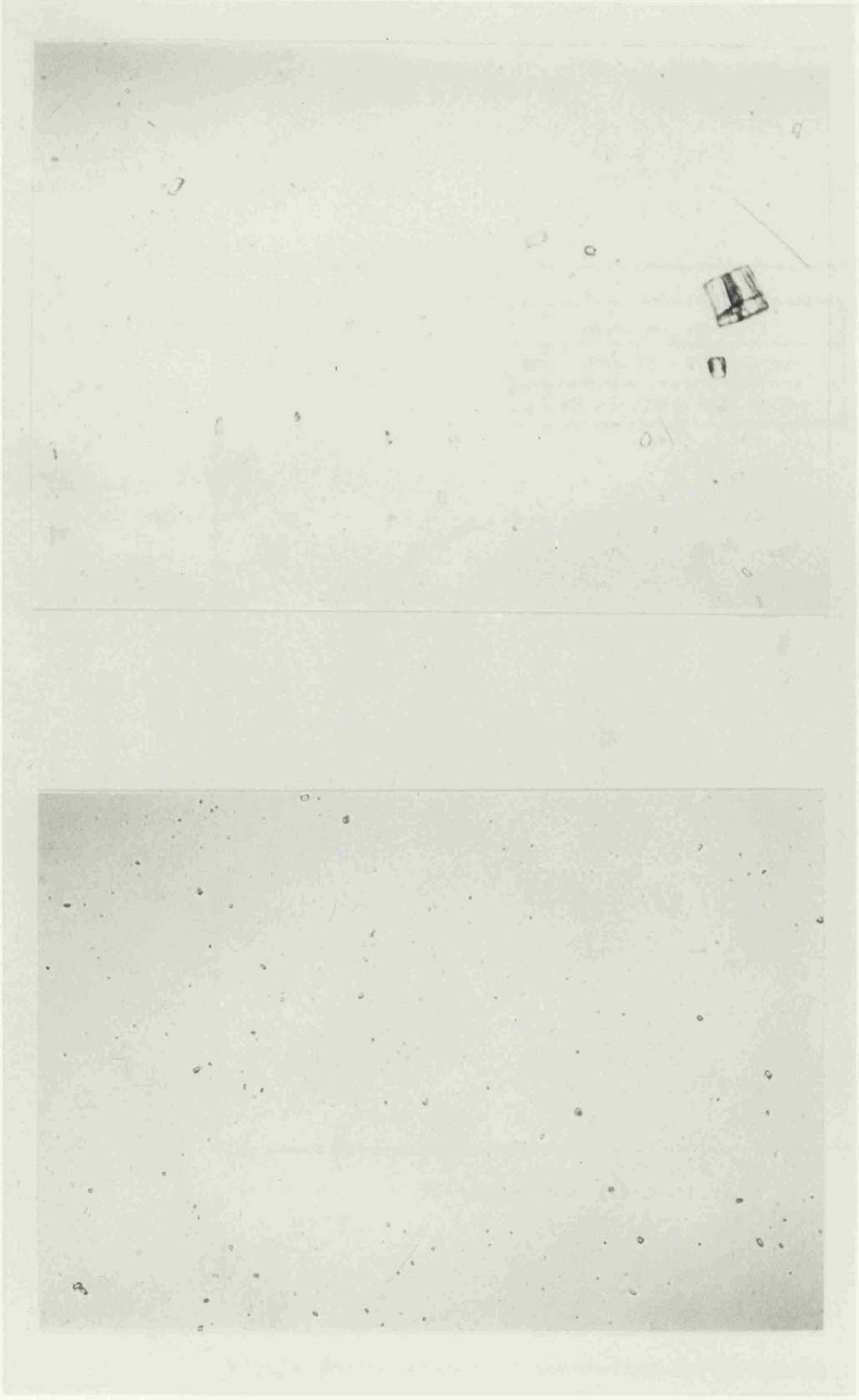
Transformation of diosgenin by growing, resting and calcium alginate immobilised cells

This experiment was carried out to determine in which environment N. rhodochrous and M.phlei cells were most active - as growing cells

Diosgenin after sonication (5 minutes,200 watts)
Magnification x180

Diosgenin after sonication (20 minutes,200 watts)

Figure 25.The effect of time of sonication on diosgenin particle size.



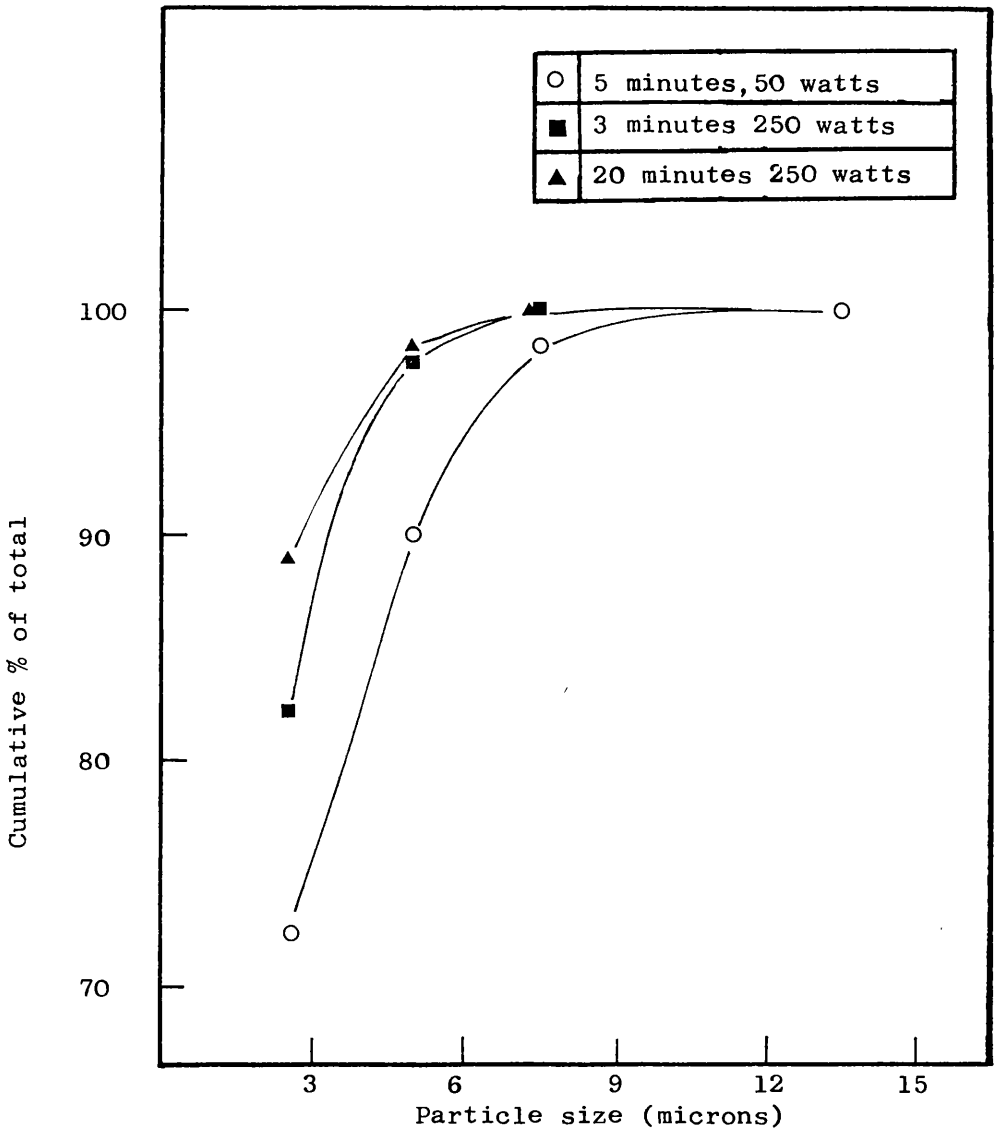


Figure 26. The effect of sonication on diosgenin crystal size.
Sonication was carried out with an Ultrasonics rapidis 3000(10mm probe).

Table 16. The effect of sonication on the rate of diosgenin transformation by N. rhodochrous

Diosgenin sample	Rate of transformation (N. rhodochrous)
	μmoles $g^{-1}h^{-1}$
No sonication	0
5 minutes, 50 w	9.0
5 minutes, 200w	12.1
20 minutes, 200w	12.0

in a medium containing a rich carbon source or as resting cells in a mineral salts solution with diosgenin as a carbon source. The effect of immobilisation of the cells in calcium alginate pellets was also determined (both as resting cells and with nutrients present in the suspension medium). Cultures (100 ml) were grown in Medium 5 for 42 hours (N. rhodochrous) and 7 days (M. phlei). The cells were then collected by centrifugation (23,000 g), the wet weight of cells per culture was measured by using tared centrifuge buckets. After resuspension of the cells in 50 ml of the appropriate medium, diosgenin was added as an ethanolic suspension to give a final concentration of 1 mg ml^{-1} (2.4 μmoles ml^{-1}). Cultures were incubated for 72 hours (N. rhodochrous) and 144 hours (M. phlei) before analysis by standard procedures. Calcium alginate immobilised cells were prepared as described in the Materials and Methods section of this chapter and treated as above. At the end of this experiment, in order to determine the steroid content of the immobilised cell pellets, they were washed with water and left (for 1 hour) to dissolve in saturated dihydrogen

potassium orthophosphate solution before ethyl acetate extraction. The results are listed in Table 17.

Both M. phlei and N. rhodochrous had higher diosgenin degradation rates in growing cultures than in the resting cell state. N. rhodochrous was ten fold more active in the growing state, with an activity of 4.08 μmoles $g^{-1}h^{-1}$ growing compared to a maximum of 0.34 μmoles $g^{-1}h^{-1}$ when suspended in 0.7% sodium chloride solution. However the resting cells were more efficient in accumulating products, 74% of transformed diosgenin was present as product in the resting cell suspension (N. rhodochrous) compared to less than 50% when in the growing environment. Presumably growing cells were able to carry out further degradative steps although no other products have been detected.

M. phlei showed a much smaller difference between the transformation rates of growing and resting cells. Growing cells were about 20% more active than resting cells. Once again no products were detected.

Attempts to transform diosgenin using calcium alginate entrapped cells were not very successful (Table 17). N. rhodochrous cells showed little ability either to degrade diosgenin or to accumulate products when in immobilised form, with only 19% of the transformation activity shown (after 72 hours) by the same cells when unimmobilised. This activity was not increased in the presence of peptone but the transformation rate was increased in the presence of 1% glucose to 50% of the resting free cell activity, although product accumulation was still minimal. Some diosgenin succeeded in penetrating the calcium alginate pellets as 5.6 μmoles (about 5% of the total added) and a trace of diosgenone were found after dissolution of the washed calcium alginate

Table 17. Transformation of diosgenin by growing, resting and calcium alginate immobilised cells

Medium	State of cells	Diosgenin[A] metabolised µmoles $g^{-1}h^{-1}$	Product formation[B] Diosgenone µmoles $g^{-1}h^{-1}$	1-Dehydrodiosgenone µmoles $g^{-1}h^{-1}$
N. rhodochrous				
5	Growing	4.08	0.38	0.29
0.7% NaCl	Resting	0.34	0.252	trace only
0.7% NaCl	Immobilised	0.065	0	0
0.7% NaCl + 1% glucose	Immobilised, growing	0.17	0	0
0.7% NaCl + 0.5% peptone	Immobilised, growing	0.07	0	0
" "	Immobilised pellet contents 5.6 µmoles		0.02	
M. phlei				
5	Growing	1.01	0	0
0.7% NaCl	Resting	0.81	0	0
0.7% NaCl	Immobilised	0.16	0	0
Nutrient broth	Immobilised, growing	0.26	0	0

A Rates (total transformation time) per gram wet cell weight

B No other products were detected

cell pellets. The rate of diosgenone formation inside the pellets was 0.02 μmoles $g^{-1}h^{-1}$.

M. phlei showed a considerable reduction in transformation activity; resting immobilised cells being only about 20% as active as free cells. As with N. rhodochrous this activity could be increased if nutrients were added to the cell suspension medium. In the case of N. rhodochrous transformation activity increased from 0.065 μmoles diosgenin metabolised $h^{-1}g^{-1}$ to 0.17 μmoles $h^{-1}g^{-1}$ when 1% glucose was present. With M. phlei the increase was from 0.16 μmoles $h^{-1}g^{-1}$ to 0.26 μmoles $h^{-1}g^{-1}$ (in nutrient broth)which is approximately 30% of the activity of non-immobilised cells.

The effects of media, pH and culture age on the transformation of diosgenin by Corynebacterium mediolanum

As differences in growth media and pH often have considerable effects on the metabolic activity of microorganisms several different growth media and a range of pH conditions were investigated with C. mediolanum. This microorganism was chosen because it had the highest rate of product formation (Table 14)of the organisms tested.

Growth Medium 1, nutrient broth and brain-heart infusion broth (Oxoid CM 225) were used. The initial pH of nutrient broth was adjusted by diluting double strength nutrient broth with appropriate buffer (Dawson et al., 1969)

pH 5.3 - sodium maleate buffer

pH 6, 7.12 and 7.95 - phosphate buffer (Gomori, 1955).

All pH measurements were taken with a pre-calibrated Cambridge digital pH meter.

For the growth medium experiments duplicate 100 ml cultures were inoculated (0.5 ml) from a 24 hour C. mediolanum culture. Diosgenin (1 mg ml^{-1}) was added after 30 hours growth and the fermentation continued for a further 48 hours before extraction and analysis. The results are given in Table 18i.

To determine the effect of pH, media at pH 5.3, 6.0, 7.12 and 7.95 were inoculated with C. mediolanum, diosgenin, as ethanolic solution, was added at 30 hours and after a further 48 hours the cultures were extracted with ethyl acetate. Viable count determinations were made at regular intervals. The results are given in Table 18ii.

There are clear but not extreme differences of growth and transformational activity in the three media tested (Table 18i). Brain-heart infusion broth gave the heaviest growth (4.34 g l^{-1}) but Medium 1 (the screening medium) supported both the fastest rate of diosgenin degradation (5.03 µmoles h^{-1}) and the fastest rate per hour per gram dry cell weight (12.5 µmoles $h^{-1}g^{-1}$). Nutrient broth gave the lowest dry cell weight yield (3.6 g l^{-1}) but an intermediate rate of diosgenin degradation (11.5 µmoles $g^{-1}h^{-1}$).

Changing the pH of the growth medium also had an effect on growth and transformation activity (Table 18ii). The optimum for growth and transformation was at pH 7.12. Between the limits tested (pH 5.3 - 7.95) there was a clear progression, both growth and transformation increased to a maximum at pH 7.12 and then declined again as the pH was further increased. However even at the extremes of the range the differences are not great, only a reduction of 20% from the maximum transformation activity was seen.

Table 18. The effects of media and pH on the transformation of diosgenin by *Corynebacterium mediolanum*

i. Growth media

	Media		
	Brain Heart Infusion	Medium 1	Nutrient broth
Dry cell weight, 30 h (g l^{-1})	4.34	4.03	3.60
Rate of diosgenin degradation over 48 h (μmoles h^{-1})	4.32	5.03	4.13
Rate of degradation (μmoles $g^{-1}h^{-1}$)	9.97	12.5	11.5

ii. pH (medium - Nutrient broth)

	Viable counts (ml^{-1})			
Time (h)	pH 5.3	pH 6.0	pH 7.12	pH 7.95
0	8.5×10^3	8.5×10^3	8.5×10^3	8.5×10^3
1	4.5×10^4	5.7×10^4	6.0×10^4	4.7×10^4
2	8.95×10^4	1.42×10^5	1.2×10^5	9.7×10^4
3.25	1.4×10^5	6.0×10^5	2.5×10^5	2.2×10^5
4.16	1.2×10^5	1.75×10^5	3.8×10^5	4.0×10^5
5	4.5×10^5	4×10^5	5.05×10^5	1.0×10^5
7	8.25×10^5	1.75×10^6	1.27×10^6	1.2×10^6
23	6.5×10^7	5.3×10^8	7.75×10^8	1.2×10^8
Dry cell weight 30 h (g l^{-1})	3.0	3.5	3.8	3.6
Rate of diosgenin degradation over 48 h (μm.h^{-1})	2.9	3.7	4.27	3.3
Rate of degradation (μm.$g^{-1}h^{-1}$)	9.7	10.6	11.25	9.20

To investigate the effect of culture age a 0.5 ml, 24 hour, inoculum of C. mediolanum in brain heart infusion broth (BHI) was added to duplicate flasks containing the same medium. Diosgenin was then added at the following times (post inoculation):- 0, 8, 32, and 56 hours. After incubation the amounts of diosgenin transformed in 24 hours and in 6 days were determined. The results are listed in Table 19. The time of diosgenin addition to C. mediolanum cultures had a very marked effect on its rate of transformation (Table 19). No transformation of diosgenin was seen and a severe reduction in the number of viable cells occurred when the steroid was added before 32 hours growth had taken place. When diosgenin was added at 32 hours a normal viable cell count was observed (10^9 Cells), transformation of diosgenin took place and products (diosgenone and 1-dehydrodiosgenone) were accumulated. When diosgenin was added to a 56 hour culture no change in the cell viable count was observed but the transforming activity increased from 2.38 μmoles h^{-1} (32 hours) to 3.42 μmoles h^{-1}.

At both the 32 hour and 56 hour addition times the rate of transformation was much more rapid over the initial 24 hours after diosgenin addition. The rate of transformation declined over the following 5 days to about 60% of the original rate. The only product detected after 24 hours was diosgenone, later both diosgenone and 1-dehydrodiosgenone were detected.

The effect of culture age on the diosgenin transforming ability of N. rhodochrous

Complex Medium 5 was used throughout the experiment. Duplicate 50 ml flasks of medium were inoculated with 0.5 ml, 48 hour (stationary

Table 19. The effect of culture age on the diosgenin transforming ability of *C. mediolanum* in brain heart infusion broth.

Culture age at diosgenin addition time	Viable count, at time of addition (cells ml^{-1})	Diosgenin transformed after 24 hours (µmoles h^{-1})	Products formed, 24 hours	Viable count after 6 days fermentation (cells ml^{-1})	Diosgenin transformed after 6 days (µmoles h^{-1})	Products formed, 6 days
0h	$< 2.5 \times 10^5$	0	0	$< 5 \times 10^6$	0.47	0
8h	3.7×10^6	0.60	0	6×10^3	0.78	0
32h	2.5×10^9	2.38	D	5.5×10^8	1.53	D, DHD
56h	4×10^8	3.42	D	2.6×10^7	1.92	D, DHD
No diosgenin added	6×10^9, 32 h	-	-	1.8×10^8	-	-

D - diosgenone

DHD - 1-dehydrodiosgenone

phase) N. rhodochrous culture. After 0, 6, 18, 42 and 60 hours diosgenin was added to give a final concentration of 1 mg ml^{-1}. After a further 8 hours incubation the cultures were analysed for diosgenin content. The results are given in Figure 27.

Clearly N. rhodochrous exhibits similar behaviour to C. mediolanum with respect to cell age. No transformation of diosgenin took place when it was added at the time of bacterial inoculation. Transformation was still much reduced (60% of maximum) when diosgenin was added to 8 hour cultures (log phase). The maximum rate of transformation (35 µmoles $g^{-1}h^{-1}$) was obtained between 18 hours and 40 hours. After this there was a slight decline in activity to 32.5 µmoles $h^{-1}g^{-1}$ at the diosgenin addition time of 60 hours.

Transformation of diosgenin by N. rhodochrous in a defined medium

Medium 2 (defined) supplemented with 2% (w/v) D-glucose was used. Four flasks were inoculated from a 4 day (stationary phase) N. rhodochrous culture in the same medium. After 94 hours incubation (i.e. at stationary phase) diosgenin was added to give a concentration of 1 mg ml^{-1}. Duplicate 10 ml samples were removed at various times for steroid analysis. The results are given in Figure 28.

This experiment was carried out in a defined medium to allow a longer time scale from the start of transformation to achieving 100% conversion of diosgenin than would be possible with a complex medium. The results showed a constant rate of diosgenin degradation over the first three days, during which time 80% of the substrate was transformed. The rate then decreased for the degradation of the

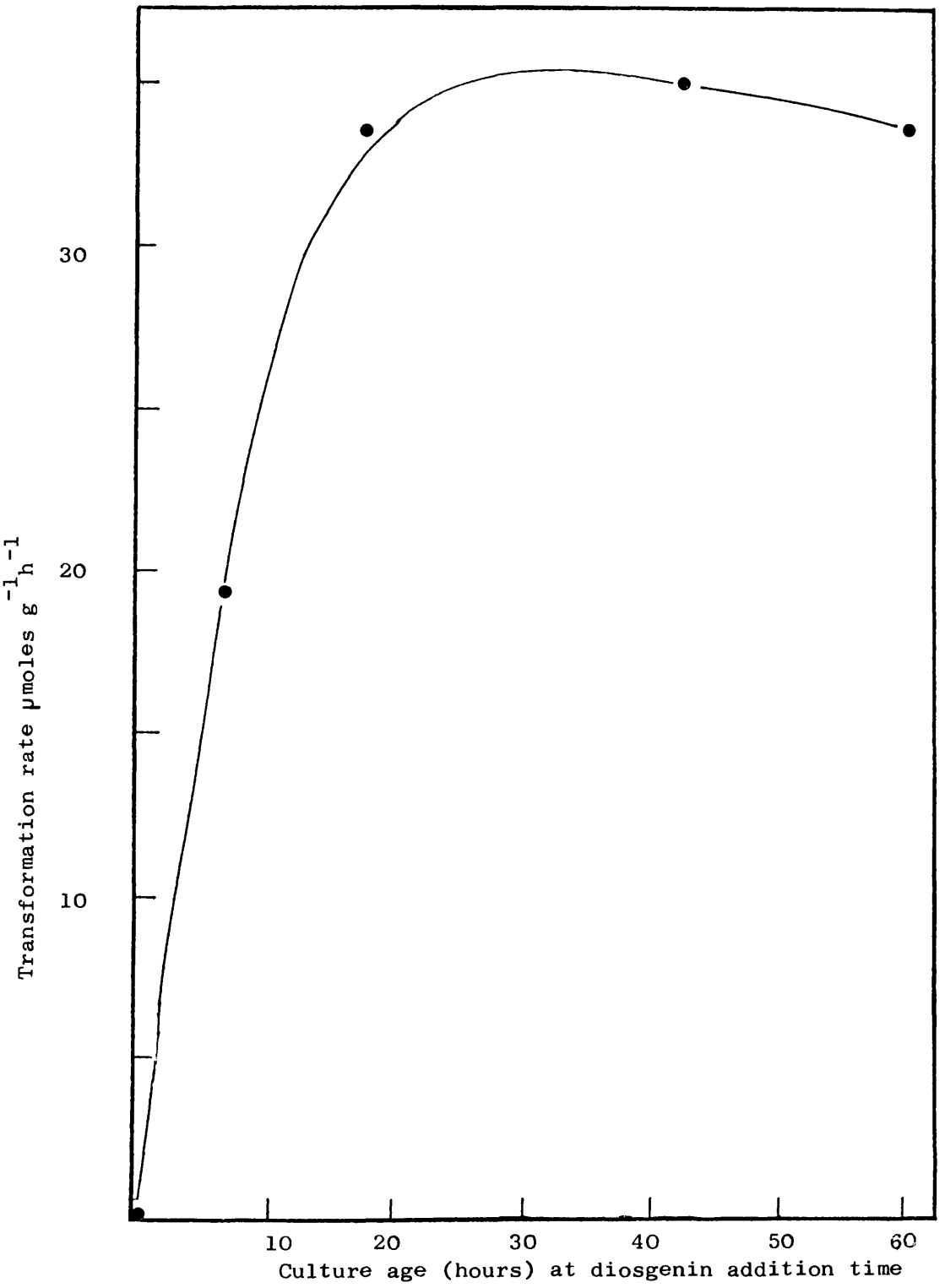


Figure 27. The effect of culture age on the diosgenin transforming ability of N. rhodochrous.

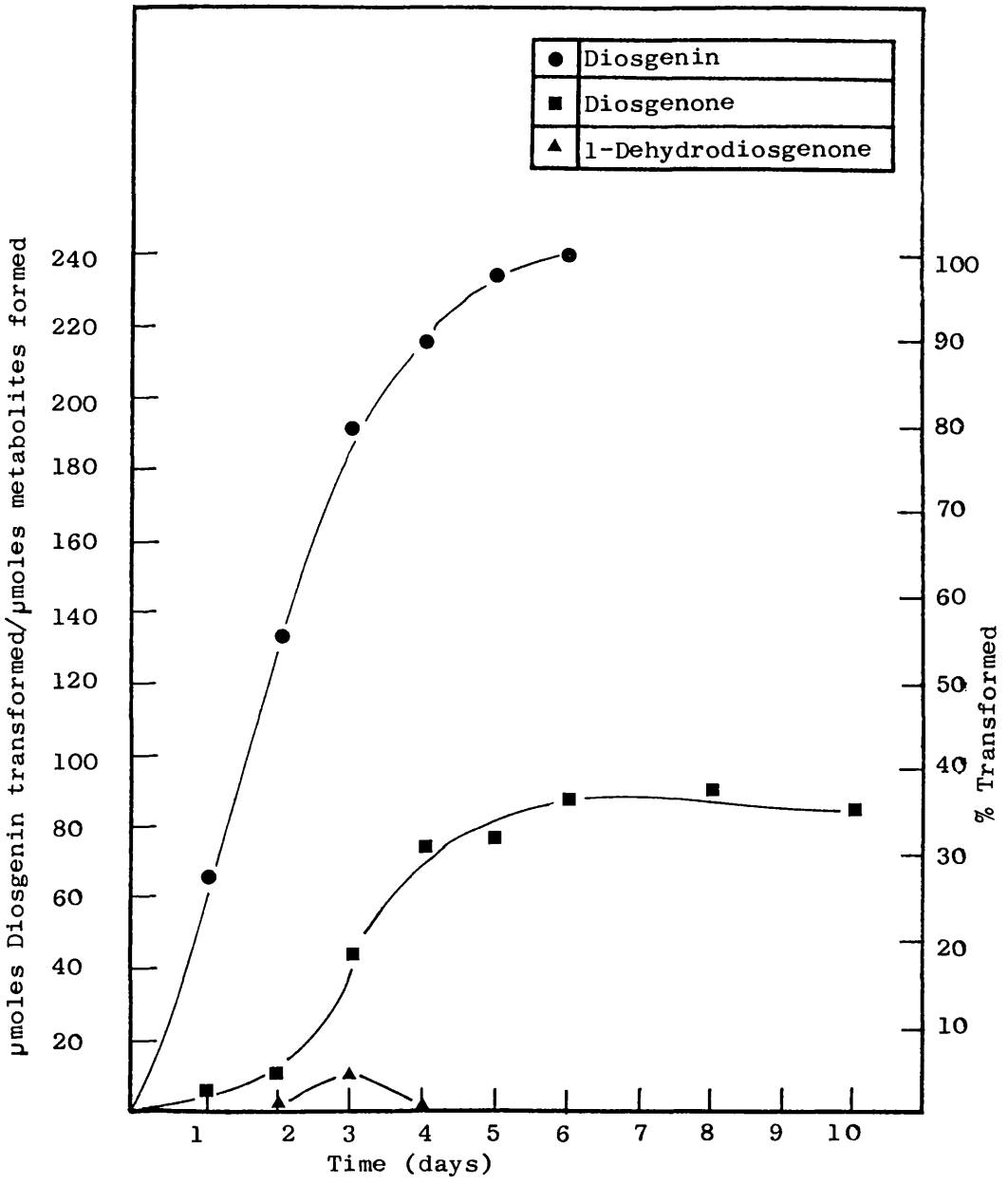


Figure 28. Transformation of diosgenin by N. rhodochrous in a defined medium.
Medium 2 supplemented with 2%w/v D-glucose, diosgenin added after 94 hours growth.

remaining 20% diosgenin with complete transformation taking 6 days.

There was a lag after the addition of diosgenin before any appreciable quantity of products were formed, over 50% of the diosgenin had been degraded before levels of diosgenone started to rise. The concentration of diosgenone formed reached a peak (90 μmoles per 100 ml culture) when all the diosgenin had been transformed and declined only slightly over the next four days (to 80 μmoles). The levels of 1-dehydrodiosgenone formed were much lower with a peak of 10 μmoles per 100 ml culture at 3 days. After this all 1-dehydrodiosgenone disappeared rapidly. No other products were detected.

On completion of the experiment only 40% of the metabolised diosgenin could be accounted for as product.

The effect of carbon source on the rate of transformation of diosgenin by N. rhodochrous

This experiment was carried out to determine what effect, if any, a variety of carbon sources (including the presence and absence of acetate) and an enrichment of the nitrogen content had on the rate of degradation of diosgenin by N. rhodochrous.

The basic medium used was the defined Medium 2. The supplements employed were as follows:- Casein hydrolysate (acid) (Oxoid L41) as an extra source of nitrogenous material and amino acids, the sterile filtrate was used at a concentration of 1% (w/v). Sodium acetate and sodium succinate were used as additional carbon sources at a level of 20 mM. Various carbohydrates were added to the medium as solutions (filter sterilised) to give final (w/v) concentrations of ½%, 1% and 5%.

A stationary phase (48 hour) culture in complex medium was used as inoculum. After 48 hours growth diosgenin was added to the cultures and after a further 48 hours they were extracted and analysed. The results are given in Table 20.

In the absence of any carbon source except either 20 mM acetate or succinate little diosgenin transformation took place (1.9 µmoles $g^{-1}h^{-1}$) and there was little cell growth. The addition of casein hydrolysate increased diosgenin degradation considerably, to 11.1 µmoles $g^{-1}h^{-1}$, and also gave a higher cell yield (2.3 g l^{-1} compared to 1.7 g l^{-1}). In both cases the final pH of the culture medium was high(pH 8.9 - pH 9.1).

Mannitol: This carbohydrate allowed the highest rate of diosgenin transformation both in terms of µmoles diosgenin transformed and µmoles diosgenin transformed per gram cells, with a maximum of 25 µmoles $g^{-1}h^{-1}$. It is interesting that the activity of the cells considerably decreased, to 4 µmoles $g^{-1}h^{-1}$, at the highest concentration of mannitol tested, when the pH was also lower (pH 4.5 - 4.7) than at other mannitol concentrations (pH 6.8 - 8.4). In the cultures where no acetate was present the transformational activity of the cells was low (5 - 8 µmoles $g^{-1}h^{-1}$) although growth remained good (average 2.9 g l^{-1}).

Glucose: Both cell growth and the rate of transformation were lower than with mannitol. The highest transformation rate achieved was 13.5 µmoles $g^{-1}h^{-1}$. A small variation in activity occurred through the concentration range of glucose tested, with transformational activity decreasing slightly as the glucose concentration increased. The

Table 20. The effect of carbon source on the rate of transformation of diosgenin by N.rhodochrous.

Carbon Source (% w/v) ± Casein hydrolysate (CA.)	+ 20 mM acetate in medium			No acetate in medium		
	Dry cell weight ($g\ l^{-1}$)	Final pH	Transformation rate ($\mu moles\ g^{-1}h^{-1}$)	Dry cell weight ($g\ l^{-1}$)	Final pH	Transformation rate ($\mu moles\ g^{-1}h^{-1}$)
½ Mannitol	1.6	8.4	23.2	2.4	6.8	8.1
1 Mannitol	2.9	NT	24.2	2.7	6.9	6.0
5 Mannitol	3.1	4.5	3.7	3.4	4.7	4.1
1 + 1% CA	3.0	NT	25.1	2.9	7.9	8.4
5 + 1% CA	2.73	8.3	5.5	3.2	NT	6.3
½ Glucose	2.0	NT	13.5	2.0		7.5
1 Glucose	2.3	NT	13.0	2.3	7.4	7.5
5 Glucose	2.3	8.4	12.6	2.8	6.8	6.0
1 + 1% CA	2.4	NT	13.4	2.8	8.3	13.8
5 + 1% CA	2.3	8.6	12.1	2.7	8.3	6.8
½ Sucrose	1.6	NT	11.4	NT	NT	NT
1 Sucrose	1.8	NT	11.2	2.6	5.5	15.7
5 Sucrose	1.4	8.4	20.8	3.7	6.6	6.5
1 + 1% CA	2.4	NT	11.5	2.9	8.1	16.9
5 + 1% CA	2.9	8.3	11.1	3.2	NT	7.2
½ Xylose	1.4	NT	2.2	NT	NT	NT
1 Xylose	1.5	NT	2.4	1.5	7.6	4.8
5 Xylose	1.5	8.7	11.5	2.9	6.7	4.3
1 + 1% CA	2.1	NT	3.8	2.4	8.3	8.4
5 + 1% CA	2.1	8.7	13.5	NT	NT	NT

Table 20 continued

Carbon Source (% w/v) ± Casein hydrolysate (CA)	+ 20 mM acetate in medium			No acetate in medium		
	Dry cell weight $g\ l^{-1}$	Final pH	Transformation rate (μmoles $g^{-1}h^{-1}$)	Dry Cell weight $g\ l^{-1}$	Final pH	Transformation rate (μmoles $g^{-1}h^{-1}$)
½ Lactose	1.2	NT	1.2	NT	NT	NT
1 Lactose	1.3	NT	1.9	NT	NT	NT
5 Lactose	1.4	9.2	3.1	NT	NT	NT
1 + 1% CA	2.1	NT	4.3	NT	NT	NT
5 + 1% CA	2.1	NT	4.8	NT	NT	NT
(1)	1.6	9.0	1.9	-	-	-
" + 1% CA	2.3	9.1	11.1	-	-	-
Succinate (20mM) (2)	-	-	-	1.8	9.1	1.9
+ 1% CA	-	-	-	2.2	8.9	11.1

Notes 1 - No additional carbon source

2 - Succinate replaced acetate

NT - Not tested

final pH was similar to that of mannitol, as was the greatly reduced activity (6.8 - 8.4 μmoles $g^{-1}h^{-1}$) in cultures without acetate present.

Sucrose: Transformation rate and cell growth were further reduced on average with typical values (in the presence of 20 mM acetate) of 11 - 11.5 μmoles $g^{-1}h^{-1}$ for transformation and 1.6 - 2.4 g l^{-1} for dry cell weight. Growth was particularly poor at the lower concentrations of sucrose in the presence of acetate. Unusually, and with the exception of 5% sucrose, transformation rates were better in the media lacking acetate (15.7 - 16.9 μmoles $g^{-1}h^{-1}$). In these cases sucrose gave the highest rates for the transformation of diosgenin in media without acetate. Cell dry weight was also higher in these media.

Xylose : The use of this sugar as a carbon source resulted in poor growth and transformation rates, although the latter did increase (to 11.5 μmoles $g^{-1}h^{-1}$) at the 5% xylose level. Growth remained below average. In the absence of acetate transformation tended to further decrease.

Lactose: The lowest growth and transformation rates in the experiment resulted from the use of lactose as a carbon source. In cultures without casein hydrolysate the highest transformational activity was 3.1 μmoles $g^{-1}h^{-1}$ and the greatest cell weight 1.4 g l^{-1}. The final pH of the growth medium was very high (pH 9.2) suggesting an increased utilisation of acetate by the bacteria.

The presence of casein hydrolysate in the medium generally encouraged a slight increase in both cell growth and the transformation

activity per gram cells. This increase became large in cultures where acetate or succinate were the only other carbon source available to the growing cells. In the case of 20 mM acetate the increase was from 1.9 μmoles $g^{-1}h^{-1}$ to 11.1 μmoles $g^{-1}h^{-1}$. The cell dry weight (g l^{-1}) also increased in these cultures. A further general effect of the casein hydrolysate was to impart a slight buffering effect, due to the zwitterionic properties of the amino acids present.

In general the presence of acetate as a supplementary carbon source had a stimulatory effect on the rate of transformation of diosgenin by N. rhodochrous but little effect on growth.

The effect of diosgenin concentration on its rate of transformation by N. rhodochrous and M. phlei.

N. rhodochrous stationary phase cultures (48 hour) in Medium 5 were inoculated with diosgenin dissolved in ethanol to give final diosgenin concentrations of 0.2, 0.6, 0.8, 1.0, 1.4 and 2.0 mg ml^{-1} culture (24, 72, 96, 120, 168 and 240 μmoles ml^{-1} respectively). The total ethanol concentration of all the cultures was adjusted to 5%.

One hour after diosgenin addition the cultures initially containing 0.2 mg ml^{-1} diosgenin were harvested. After three hours the remaining cultures were also extracted. M. phlei cultures (5 day, Medium 5) were similarly treated. Both sets of results are given in Figure 29.

At the lowest concentrations of diosgenin used (24, 72 μmoles) the transformational activity of N. rhodochrous was considerably

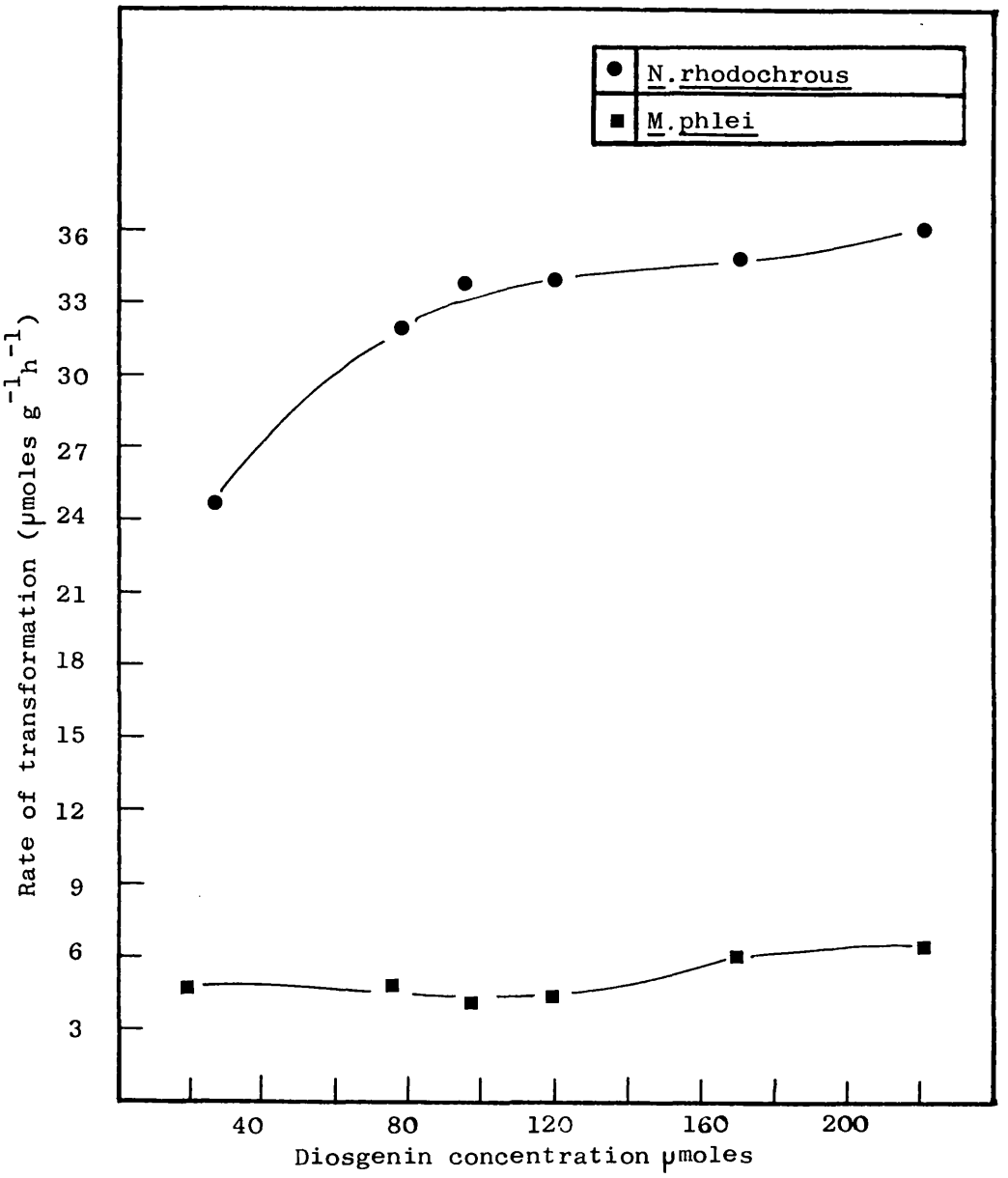


Figure 29. The effect of diosgenin concentration on its rate of transformation by M.phlei and N.rhodochrous
Diosgenin was added dissolved in ethanol to stationary phase cultures in Medium 5. The total ethanol concentration was adjusted to 5%.

below the optimum. The rate of transformation increased as the steroid was increased, to 120 µmoles. However above this concentration there was only a small further increase in activity. The concentration used in other experiments in this thesis was equivalent to the 120 µmoles level, i.e. near the optimum.

M. phlei had a lower but more constant level of activity through the range tested. Again the lowest levels of diosgenin resulted in lower rates of transformation (4.5 µmoles $g^{-1}h^{-1}$) however this reduction from the optimum was much less marked than with N. rhodochrous. Even at the highest diosgenin concentration the activity had only increased to 6 µmoles $g^{-1}h^{-1}$.

The 240 µmolar level (equivalent to 2 mg diosgenin per ml culture) was the highest concentration possible of diosgenin to be added dissolved in ethanol and the ethanol concentration in the culture not to exceed 5%.

Discussion

Rates of Diosgenin Transformation by Several Microorganisms

These experiments were carried out to determine which organisms were the most efficient at transforming diosgenin into isolatable products. Those of interest were then selected for further study in the subsequent experiments in this chapter. N. rhodochrous gave the fastest rate of diosgenin degradation, C. mediolanum the highest rate of product accumulation (Table 14). The Mycobacteria tested were interesting because of their ability to totally decompose all diosgenin present in the culture medium whilst not accumulating any products.

Those organisms which did accumulate products formed the same ones, diosgenone and 1-dehydrodiosgenone. This suggests that, at least in the initial stages, the transformation of diosgenin follows a common pathway.

Of the seven organisms tested only F. solani showed a greater ability to transform diosgenin in defined Medium 2 rather than in the richer, undefined Medium 5. This must be due to some constituent of the undefined culture medium having a repressive effect on the F. solani degradative enzymes; growth was not inhibited.

Modes of Diosgenin Addition

i) The effect of ethanol concentration on N. rhodochrous viable cell count

As the results showed (Figure 23) concentrations of ethanol up to and including the 5% level have no effect on the N. rhodochrous viable cell number. This gives good justification for its continued use in the standard experimental procedure.

The rapidly lethal effect observed at ethanol concentrations over 10% suggested that its use in cultures at such concentrations would not result in good yields, although it must be noted that viable cells are not always necessary for biotransformational activity. Indeed the use of high concentrations of organic solvents as a cell suspension medium were studied and are described in Chapter 7.

ii) The effect of addition of diosgenin in solution and in suspension to N. rhodochrous cultures

The lack of transformation of diosgenin which occurred when it

was added to the fermentation as a powder (Table 15) was not altogether surprising. Charney (1966) had found that the addition of steroid as a dry powder, to a Corynebacterium fermentation, considerably slowed the rate of reaction. The effect observed with diosgenin was presumably due to the large particle size and the low solubility in water of the sapogenin, leading to poor contact with the cells or their enzymes. Relatively little work has been published on the effect of the physical form of steroid addition on its biotransformation. The most relevant (and also the most recent) paper is by Lee et al. (1970) who were studying the transformation of 16α-hydroxycortexolone-16,17-acetonide to its 1-dehydro-11β-hydroxy product in a mixed culture of Corynebacterium simplex and Curvularia lunata. They compared the addition of steroid in ethanolic solution, DMF, or 0.1% (w/v) Tween 80 to give a final steroid concentration of 1 mg ml^{-1}. No mention of sonication is made. They found that steroid dissolved in ethanol and DMF was the most rapidly transformed whilst ethanol in which the steroid was incompletely dissolved allowed lower rates of transformation. The lowest rates of transformation were achieved with the Tween 80 suspensions. Their conclusion was that the differences in transformation rate were due to solubility differences of the steroid preparations. The solubility was measured by assaying the amount of steroid in the supernatant after centrifugation.With this method there is a danger that the smallest particles of steroid are not precipitated, giving a misleading result.

The results obtained from N. rhodochrous with diosgenin are similar to those above. However the difference observed between the results obtained with the sonicated crystals of diosgenin and the diosgenin precipitated from ethanol (Figure 24) is likely to be due to the difference in form of the diosgenin.

The precipitated particles were much less dense than the sonicated ones, thus providing a much larger surface area to interact with the microorganisms, which is in turn likely to promote an enhanced rate of reaction.

The best mode of diosgenin addition up to culture concentrations of 1 mg ml^{-1} is by addition in solution. Whilst DMF was slightly more effective than ethanol (Table 15) the former was not chosen because of its toxic properties to humans. Therefore diosgenin dissolved in ethanol was used in most further experiments in this thesis. The main disadvantage of this mode of steroid addition is when concentrations much higher than 1 mg ml^{-1} are required. In such cases it is more practical to use sonicated suspensions to avoid ethanol toxicity problems, as illustrated by Buckland et al., 1976.

iii) The effect of sonication on diosgenin crystal size and its transformation by N. rhodochrous.

Whilst having an effect on particle size (Figure 26) the small differences in transformation rate observed (Table 16) give further weight to the hypothesis (see above) that it is particle form which is important in determining the rate of transformation rather than small differences in size or solubility of the steroid particles.

Transformation of diosgenin by growing, resting and calcium alginate immobilised cells

The majority of commercial fermentations are carried out with growing cells, which were also used in the early experiments reported in this thesis. This method is the most straightforward as growth

and transformation take place in the same medium. Recently the use of resting cells has received further attention. Aharonowitz and Demain (1980) have investigated the production of cephalosporins by Streptomyces clavuligerus. In normal batch cultures growth did not cease until 40 hours and no cephalosporin was produced until 48 hours. However by transferring cells from a growing culture to a simple buffer solution they found that the most active antibiotic producing cells came from 12 - 16 hour cultures. They concluded that the reason for the late appearance of antibiotic in the growing culture was due to inhibition of the cephalosporin producing enzymes by a form of catabolite repression.

Resting cells have also been found useful in the transformation of steroids. Lee et al. (1971) found that resting cells of Aspergillus ochraceus were much more efficient in the 11α-hydroxylation of 16-hydroxycortexolone than growing cells. They achieved 100% conversion in 8 hours with resting cells, compared to the 48 hours needed by growing cells. McGregor et al. (1972) found that Sepedonium ampullosporum was able to 16α-hydroxylate 9β,10α-pregna-4,6-dien-3,20-dione in 50 - 70% of the time and with increased stability as resting cells rather than in a batch fermentation.

However the results (Table 17) clearly showed that both M.phlei and N. rhodochrous were considerably more active in transforming diosgenin to diosgenone and 1-dehydrodiosgenone when in a growing culture rather than in the resting state. The advantages of being in a nutritious culture medium must outweigh the potential disadvantages of catabolite repression.

As the growing cells were not only more active against diosgenin but also more convenient to handle, this was the state in which they were used for most of the further experiments in this thesis.

Immobilised cells

Alginate was chosen as the method of immobilisation for the experiments described in this section because of its gentle nature, causing only minimal damage to cells. (Kierstan and Bucke, 1977).

There have been no reports in the literature of the immobilisation of whole Nocardia rhodochrous cells in aqueous media. However the cholesterol oxidase from it has been immobilised onto Amberlite IRC-50 resin, Amberlite XAD-7 and Sepharose 4B, retaining some activity (Lilly et al., 1978; Cheetham, 1979b).

Mycobacterium cells have been immobilised (Skryaban et al., 1974; Voishvillo et al., 1976; see Introduction to this Chapter) and retained their activity against steroids. Atrat et al. (1981) have immobilised M. phlei cells in a polyacrylamide gel and still retained 50 - 70% of steroid side chain degrading activity (substrate: 4-cholesten-[3-O-carboxymethyl]-oxime; product 4-androsten-17-one-3-[O-carboxymethyl]-oxime).

In the experiments carried out in this thesis it has not been possible to demonstrate the retention of such high activities with immobilised M. phlei or N. rhodochrous cells against diosgenin when the cells were entrapped in calcium alginate. Although the diosgenin degrading activity of immobilised N. rhodochrous cells did increase

from 0.065 μmoles $g^{-1}h^{-1}$ to 0.17 μmoles $g^{-1}h^{-1}$ in the presence of 1% glucose (although not in 0.5% peptone) the transformation rate was only 5% of that of free growing cells and no products were detected in the medium. An increase in transformation was also shown by immobilised M. phlei cells when nutrients were added to the suspension medium (from 16% to 26% of free growing cell activity). It is possible that these increases came from the growth of free cells released into the medium (some were detected) but an activation mechanism as suggested by Larsson et al., (1976, 1978) is at least equally likely.

There are a number of possible reasons for the reduction in activity occurring after immobilisation. The cells were not induced prior to immobilisation to avoid complicating the experiment. However this is unlikely to be a major cause of reduced activity as the resting cells, with much higher activity, were not induced either. Ohlson et al. (1979) have shown that cells immobilised in calcium alginate can be induced in situ by the addition of a suitable substrate.

The loss of activity is more probably due to severe diffusional problems caused by the very hydrophobic nature of diosgenin (more so than the 4-cholestan-oxime of Atrat et al., 1981). Although large molecules, for example inulin (mol.wt. 6000), are able to diffuse easily through calcium alginate (Kierstan and Bucke, 1971) the diosgenin precipitated from ethanol would be less able to do so. However a certain amount of diosgenin did penetrate the pellets (5.6 μmoles, 4.5% of the total diosgenin present). Presumably some of the smallest particles of diosgenin entered the pellet whilst the larger particles remained outside. Once inside the pellets a certain

amount of biotransformation was able to take place as small quantities of diosgenone were detected. This was not released into the aqueous medium and only detected after dissolution of the pellets.

The conclusion of this experiment was that calcium alginate immobilisation of growing cells in an aqueous medium did not benefit the transformation of diosgenin.

The effects of media and pH on the transformation of diosgenin by Corynebacterium mediolanum

The effects caused by differences in growth media or changes in the initial pH of the cultures (Table 18) in this experiment were relatively small.

The decreased rate of diosgenin transformation in brain-heart infusion broth was presumably due to a component of the medium which was inhibitory to the diosgenin transforming system of C. mediolanum. As brain-heart infusion broth is a rich medium designed for the cultivation of fastidious organisms this result was not surprising.

The relative lack of effect that the pH variation caused (Table 18ii) suggested that the enzymes concerned were not particularly pH sensitive or, because of their association within the bacterial cells, they were shielded from the bulk pH. Therefore precise pH control of the initial fermentation medium, whilst desirable was not essential.

The effect of culture age on the diosgenin transforming ability of C. mediolanum and N. rhodochrous

The age of a microbiological culture often has a critical bearing on its transformational abilities. Although generally expressed in terms of chronological age frequently the differences observed are due to a function of nutritional regulation (Aharonowitz and Demain, 1980). Rapid growth (exponential phase) takes place when there are forms of carbon in the growth medium which can be readily assimilated. When these are exhausted the growth phase ceases and the organism reaches stationary phase. At this time many transformation rates are maximal, either due to the cessation of growth or the removal of catabolite repression because of exhaustion of the carbon source.

In the case of C. mediolanum addition of steroid itself had a very deleterious effect on both the viable cell count and the steroid transformation rates when added before 32 hours (Table 19). The young, rapidly growing cells were greatly inhibited by the presence of 5 ml ethanolic solution of diosgenin. This sensitivity was probably due to the ethanol at a concentration of 5% in the culture medium. Cells at or reaching stationary phase (32 hours and 56 hours) appeared to be largely resistant to this effect as their viable cell number was only slightly affected. Further evidence for this came from Figure 23, where the viable cell population of 48 hour cells was unaffected by 5% ethanol.

After 32 hours the steroid transforming activity of the C. mediolanum cells (as μmoles h^{-1}) continued to increase although there was no parallel increase in the cell viable count. In this case the increase

was probably due to either cessation of growth or the exhaustion of substances in the growth medium which were previously inhibiting maximal transformational activity by a mechanism such as that described by Aharonowitz and Demain (1980).

A similar activity profile was shown by N. rhodochrous cells, although in this case the maximum rate of transformation occurred at 40 hours. Buckland et al. (1976), also using N. rhodochrous cells, reported a comparable effect. They found that for the maximal induction of cholesterol oxidase enzymes in N. rhodochrous the time of inducer (cholesterol) addition was critical. If the inducer was added at the start of growth enzyme activity was markedly reduced. The highest enzyme activity occurred if the inducer was added when the cell density was at 2.8 g dry weight l^{-1} in a fermenter culture. They concluded that the effects were due to catabolite repression.

Transformation of diosgenin by N. rhodochrous in a defined medium

The use of a defined medium allowed a clear picture of the degradation of diosgenin and the formation of products to be seen (Figure 28).

As would be expected diosgenin degradation rapidly reached its maximum rate, which then remained constant until the majority of the steroid had been transformed. Why there was a lag in product accumulation, without any significant accumulation occurring until 50% of the substrate had been degraded, was difficult to explain. The lag could be due to a form of product inhibition, possibly the presence of a certain threshold level of diosgenone in the culture prevented

the diosgenone's further degradation. The transformation of diosgenone to 1-dehydrodiosgenone appears to be a rate-limiting step resulting in the accumulation of the former product. Complete degradation accounted for the disappearance of 60% of the steroid content of the culture.

The effect of carbon source on the rate of transformation of diosgenin by N. rhodochrous

It is known that the use of different carbon sources for the production of a given metabolite results in different rates of growth and product yields. An important practical aspect of any developmental programme for the industrial production of a new compound by fermentation is the assessment of the best carbon source to use. However, the mechanisms by which a given carbon source will affect the production of a metabolite are not clearly understood (Demain et al., 1979).

The results (Table 20) showed that mannitol supported the best rate of growth and the highest diosgenin transformational activity of the sugars tested. The sharp decline in activity and pH at the 5% mannitol concentration could be linked together by a single hypothesis; at the highest concentration there was sufficient mannitol present for it to be used as the sole carbon source, resulting in good cell growth and a lowering of the pH caused by the production of acid. At lower mannitol concentrations, after an initial period of cell growth the mannitol was exhausted. Hence the bacteria were then forced to use acetate, causing an incidental increase in pH. At the same time this slowing down of growth and the exhaustion of the mannitol supply freed the culture from catabolite repression caused by the presence of

sugar, thus resulting in the increased rate of transformation observed.

A similar mechanism could explain the generally higher pH levels and catabolic activity found in all the cultures supplemented by acetate when compared to cultures containing a sugar as sole carbon source. When used on their own the acetate or succinate were insufficiently easily assimilated to support satisfactory cell growth.

The effect of diosgenin concentration on its rate of transformation by N. rhodochrous and M. phlei.

This experiment was carried out to determine if the transformation of diosgenin by N.rhodochrous or M. phlei was a substrate limited reaction. At the lowest concentrations of diosgenin (24, 72 μmoles) used with N. rhodochrous this appeared to be the case (Figure 29). However at the concentrations used in most of the experiments in this thesis substrate limitation would not have occurred.

When very high concentrations of diosgenin were added (incompletely dissolved in ethanol) no further increase in transformation rate was seen. There was no evidence for the occurrence of a pseudo-crystallo-fermentation process. M. phlei was little affected by the concentration of diosgenin present in the culture medium.

CHAPTER 5

Methods to inhibit the degradation of the diosgenin steroidal nucleus.

Introduction

The inhibition of steroid nucleus degradation

A number of microorganisms with the potential to carry out useful steroid transformations do not yield products. Instead they break down the steroid completely. As mentioned in the general introduction (Chapter 1) this degradation appears to follow a common pathway (Figure 11). The initial steps are the transformation of the 3β-hydroxy-5-ene structure to a 3-oxo-4-ene compound followed by 9α-hydroxylation and C1(2)-dehydrogenation. The resulting metabolite undergoes simultaneous aromatization with cleavage of ring B via a non-enzymic reverse aldol type reaction to produce a 9, 10-secophenol derivative (Martin, 1977). This is then further metabolised as a source of carbon.

Three approaches have been developed to prevent this:-

i) The transformation is carried out in the presence of compounds which inhibit complete enzymic degradation of the steroid.

ii) the steroid is structurally modified by chemical means

iii) the microorganism is mutated.

i) Use of enzyme inhibitors

The substances most used for this purpose are chelating agents. Their action was first discovered in 1964 when Whitmarsh found that the complete oxidation of cholesterol by a Nocardia species could be inhibited by the addition of the chelating agent 8-hydroxyquinoline. The result was the accumulation of cholanic acids and androstanes (e.g. 3-oxo-23,24-dinorcholan-4-ene-22-oicacid and androst-4-ene-3,17-dione). Wix et al. (1968) also found that chelating agents could inhibit the enzymic oxidation of the steroid nucleus of cholesterol. The group showed that

androst-4-ene-3,17-dione remained unchanged by cultures of M. phlei in the presence of 8-hydroxyquinoline but 9α-hydroxy androst-4-ene-3,17-dione was decomposed. They concluded that the iron chelating agent inhibited the introduction of the 9α-hydroxyl group. The group also found a number of other chemicals with similar properties (all are chelating agents) - 1,10 phenanthroline, 5 nitro-1,10-phenanthroline, cupferron and α,α'-bipyridyl. In a more extensive survey Nagasawa et al.(1970b) studied 167 compounds and found that 23 were effective in causing the accumulation of androsta-1,4-diene from the microbiological degradation of cholesterol. The most efficient substances were the chelating agents α,α'-bipyridyl, 1,10-phenanthroline, 8-hydroxyquinoline and the metal compounds cobalt and nickel sulphate. The authors suggested that a metal was probably concerned in the active centre of the degrading enzyme system. The high inhibitory activity of Co^{+2} or Ni^{+2} was likely to be due to their displacement of such an ion from the active site. The most probable ions with a similar ionic radius are Fe^{+2} and Zn^{+2}. Van der Waard et al. (1968) had previously shown nickel sulphate to be effective in preventing the further degradation of androsta-1,4-diene-3,17-dione by Mycobacterium sp. KNGSF 70. The inhibitory effects of the nickel sulphate could be counteracted by the addition of much smaller amounts of ferrous sulphate. In fact the enzyme responsible for 9α-hydroxylation was subsequently found to be a mono-oxygenase consisting of several proteins forming an electron transfer chain containing iron (Martin, 1977).

ii) Structural modification of steroid

As described above, two enzymic processes are essential for the degradation of the steroid nucleus, C1(2)-dehydrogenation and 9α-hydroxylation.

The order of these two reactions depending on the microorganism involved. By blocking one of these chemically, nucleus degradation should be prevented, without affecting side-chain degrading activity.

Dodson and Muir (1961a, 1961b) and Sih and Rahim (1963) showed that 19-hydroxy-4-androstene-3,17-dione (Figure 30) could be converted by Pseudomonas and Nocardia species into oestrone without any further metabolism taking place. Following this work Sih and Wang (1965) showed that it was possible to aromatize a 19-nor or 19-hydroxy sterol with the concomitant degradation of the side chain to a 17-ketone but without any further degradation taking place. In both cases the modification of the C19 methyl prevents 9α-hydroxylation. Sih et al. concluded (1965) that an ideal substrate for microbial conversion to oestrone would be 3β-acetoxy-19-hydroxy-5-cholestene (Figure 30), the 19-hydroxyl group preventing 9α-hydroxylation. Using this compound they were able to obtain a 72% yield of oestrone after 96 hours.

Van der Waard (1970) showed that C1(2)-dehydrogenation could also be prevented by structural modification of the steroid. He reported that 3α,5α-cyclocholestan-6β-ol was converted by cultures of Mycobacterium sp. KNGSF 70 into 3α,5α-cycloandrostan-6,17-dione and 3α,5α-cycloandrostan-6β-ol-17-one in high yields (Figure 30).

iii) Mutation techniques

The most recent approach to the prevention of steroid nucleus degradation has been the use of mutation techniques. Marsheck et al. (1972) used ultra-violet light to form a mutant Mycobacterium species (NRRL B-3683) which accumulated good yields of androstanes from various

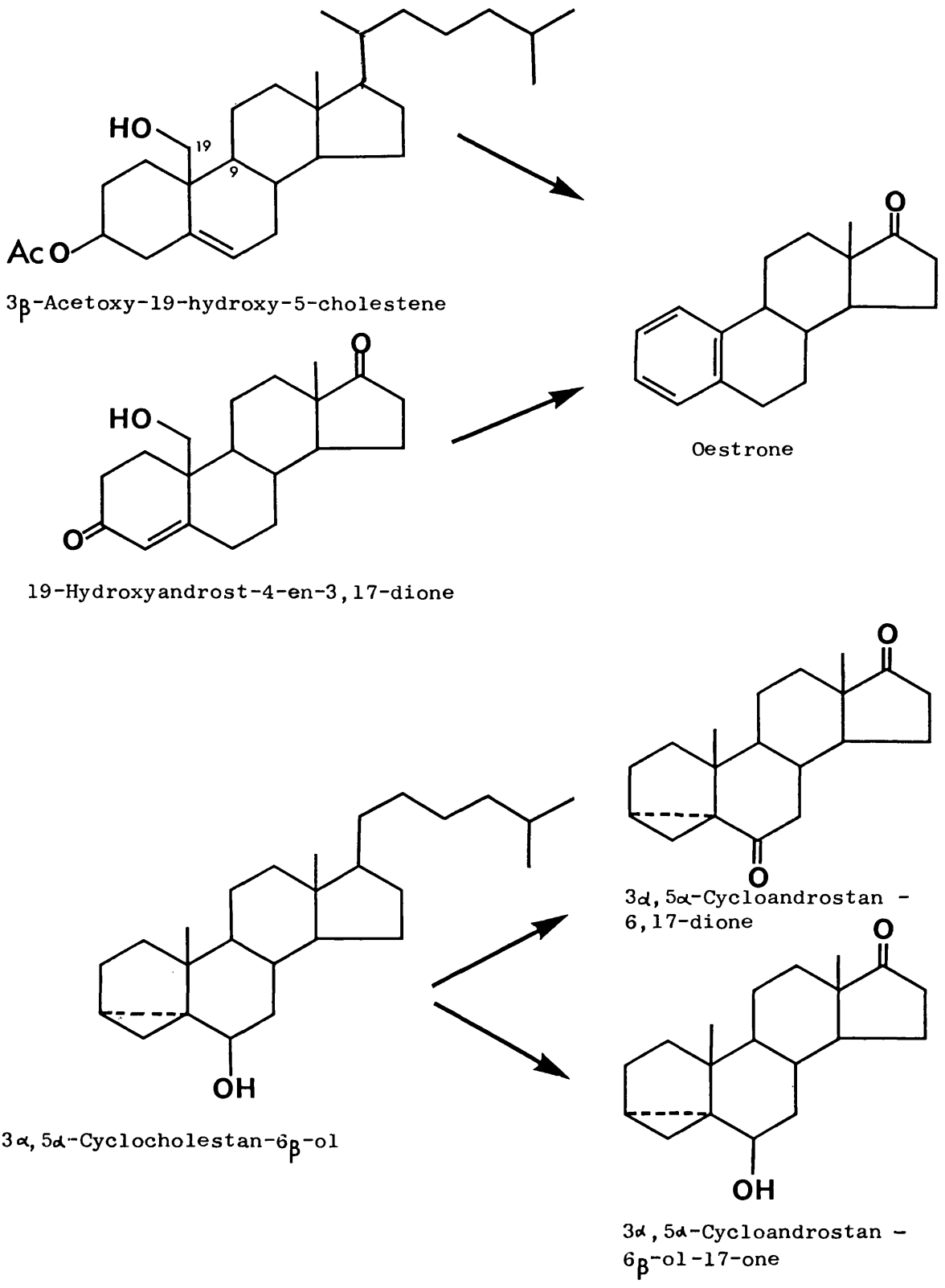


Figure 30. Chemical modification of steroid structure to prevent nucleus degradation.

sterol substrates in the absence of chelating agents or other inhibitors. The process is the subject of U.S. patent 3,684,657 (Kraychy et al., 1972).

Similar mutants were developed by Cargile and McChesney (1974) using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as the mutagenic agent. A maximum yield of 78% androsta-1,4-diene-3,17-dione from cholesterol was achieved with a mutant Mycobacterium strain. The formula of MNNG is $CH_3N(NO)C(=NH)NHNO_2$.

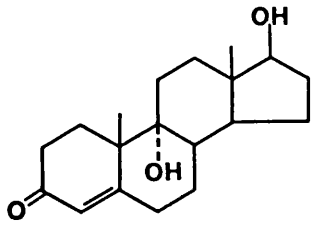
Numerous other reports of mutant strains capable of causing the accumulation of steroid intermediates, generally Mycobacterium species and frequently M. fortuitum, have since been published, usually in patent form. The mutagen most commonly used to generate the strains is MNNG. This is an alkylating agent which causes transition mutations. It is thought to alkylate the base guanine such that it pairs with thymine rather than cy tosine (Davis and Dulbecco, 1973).

Some of the more interesting products accumulated from fermentations of steroids using mutated microorganisms are illustrated in Figure 31. One of these is a 9α-hydroxylated androstane. The accumulation of this compound must be the result of a mutation affecting an enzyme later in the degradation pathway than the monoxygenase which is inhibited by the chelating agents discussed above.

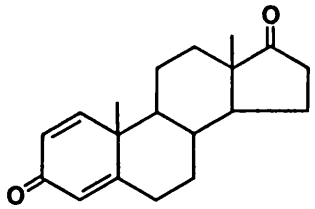
Specific Materials and Methods

N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis

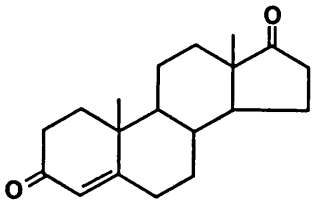
The mutation method used was based on that developed by the Upjohn Company (Kalamazoo, Michigan) and disclosed in U.S. patents 4,097,335



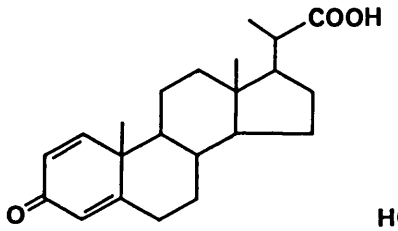
Mycobacterium fortuitum
NRRL B-8119
Wovcha et al.,1979



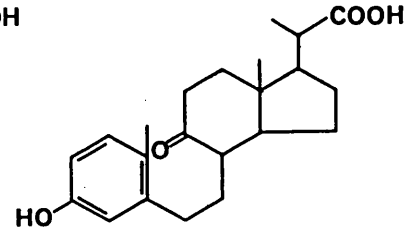
Mycobacterium
NRRL B-3683
Kraychy et al.,
1972.



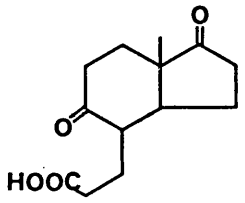
Mycobacterium
NRRL B-3805
Marscheck & Kraychy,
1973a,1973b.



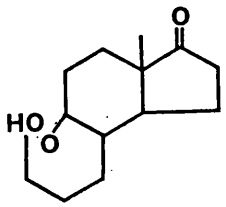
Nocardia sp.M29
Martin & Wagner,
1976.



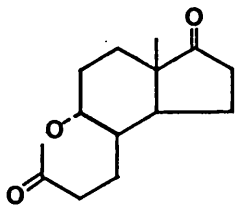
Arthrobacter simplex
MCI 0808
Imada & Teranishi,
1980.



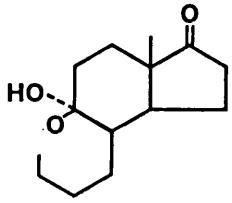
M.fortuitum B-8129
Biggs et al.,1977.



M.fortuitum B-8128
Knight & Wovcha,
1979.



M.fortuitum B-8128
Knight & Wovcha,
1979.



M.fortuitum B-8129
Pyke & Salmond,
1978 .

Figure 31.Products of the transformation of sterols accumulated by mutated microorganisms.

(Pyke and Salmond, 1978); 4,175,006 (Wovcha et al., 1979); 4,176,123 (Knight and Wovcha, 1979) and European patent 8,214 (Wovcha and Brooks, 1980). However the growth and selection media have been altered in these experiments.

A six day (stationary phase) culture of Mycobacterium phlei was used for mutagenesis. Aliquots (4.5 ml) of the culture were added to sterile plastic screw capped test tubes (Sterilin Ltd.)and centrifuged (M.S.E. bench centrifuge) for 5 minutes to pellet the cells. The cell pellet was washed with citrate buffer of pH 5.6, pelleted and finally resuspended in 4.5 ml citrate buffer. N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) had been previously prepared in stock solutions of 500 $\mu g\ ml^{-1}$ and 1 $mgml^{-1}$ in screw capped bijou bottles and stored frozen, protected from the light. Thawed MNNG was added to the suspended cells to give final concentrations of 0, 20, 50, 250 $\mu g\ ml^{-1}$ (triplicate samples in each case). The MNNG containing cultures were incubated at $37^{o}C$ for 25 minutes in a shaking water bath, spun down as before and resuspended in phosphate buffer (pH7). After serial dilutions in phosphate buffered saline (Oxoid BR14a) the cultures were plated out on Medium 2, supplemented with 2% glucose and 1% (w/v) agar. They were incubated ($37^{o}C$) for 5 days to allow growth to take place. Growth on this medium eliminates most auxotrophs produced by the mutagenesis procedure, as vitamins and growth factors are absent. The colonies were then replicated onto test plates containing androst-4-ene-3,17-dione as the sole source of carbon. Colonies which grew on the control plates (Medium 2 + 2% glucose) but were unable to utilize androst-4-ene-3,17-dione as a carbon source were purified by streaking on nutrient agar and then retested on the selective plates. Purified isolates

which still exhibited a phenotype different from the parental culture were evaluated in shake flasks. The selective plates consisted of Medium 2 (without a carbon source) plus 1% (w/v) agar (Oxoid L11). After autoclaving (121^{o}C, 20 minutes) the medium was dispensed into 100 ml volumes and 0.1 g androst-4-ene-3,17-dione added. This mixture was vigorously stirred using a pre-sterilised M.S.E. homogeniser in a laminar flow cabinet for 2 minutes to disperse the steroid. The medium was then poured (20 ml volumes) into petri dishes and allowed to cool. The steroid could be seen as evenly dispersed small white crystals in the agar. This method for the dispersion of steroid in agar plates was first described by Peterson et al. (1962). Shake flask evaluation was carried out in both Medium 2 + 1% glucose and Medium 5. Cultures were grown for 7 days at 30^{o}C before the addition of diosgenin. After a further 6 days the cultures were extracted and the results analysed by t.l.c. and g.l.c.

Results

i) The use of enzyme inhibitors

The effect of α,α'-bipyridyl on diosgenin degrading microorganisms

Organisms apparently capable of completely degrading diosgenin were cultured in the presence of α,α'-bipyridyl in an attempt to accumulate products. Organisms were grown initially as follows: Mycobacterium phlei, Medium 2 + 2% glucose, 6 days; Mycobacterium fortuitum, Medium 2 + 2% glucose, 6 days; Fusarium solani, Medium 2, + 20 mM acetate, 6 days; Bacillus sphaericus, Nocardia rhodochrous, Corynebacterium mediolanum, Medium 5, 48 hours. After the addition of diosgenin and α,α'-bipyridyl a further incubation period followed before the cultures were extracted and assayed. The results are listed

in Table 21. Of the organisms tested only the Mycobacteria accumulated androstanes. Because of their genotypic similarity it seems highly probable that M. fortuitum and M. phlei follow the same steroid degradation pathway. The inhibitor did not make any difference in the pattern of products accumulated in the other cultures, although it did reduce their rate of formation and also the rate of diosgenin degradation. Some complete metabolism of diosgenin still occurred.

The effect of α,α'-bipyridyl on the transformation of diosgenin by M. phlei.

Culture Medium 2 (volume 100 ml) supplemented with 2% w/v glucose was used. M. phlei was grown for 6 days before diosgenin and α,α'-bipyridyl (both dissolved in ethanol) were added, the concentration of α,α'-bipyridyl varying from 0.05 - 15 mM (α,α'-bipyridyl is sufficiently soluble to remain in solution). After a further 7 days the cultures were extracted and analysed. In a second experiment diosgenin and α,α'-bipyridyl were added after 4 days growth, other conditions were as above. The results are given in Table 22, Figure 32. As the concentration of α,α'-bipyridyl increased a clear progression of effects was seen. At low concentrations the viable cell count was little affected and complete degradation of diosgenin with no product accumulation occurred. Once the α,α'-bipyridyl concentration reached 0.3 mM and above, the viable cell count decreased considerably from the control value of 2×10^9 to 3×10^7 (0.3 mM) and 7×10^6 (3.0 mM). At the same time products started to accumulate, mainly diosgenone and 1-dehydrodiosgenone. However small amounts of androstanes (up to 6.5% of total steroid) were also found at the 0.3 mM and 0.6 mM α,α'-bipyridyl concentrations. As the concentration of α,α'-bipyridyl further increased

Table 21. The effect of α,α'-bipyridyl on diosgenin degrading micro-organisms.

Organism	Diosgenin fermentation	α,α'-bipyridyl concentration (mM)	Products accumulated
F. solani	6 days	0.3 - 1.0	none
B. sphaericus	5 days	1.2	D, DHD
C. mediolanum	4 days	0.3 - 0.6	D, DHD
N. rhodochrous	4 days	0.3 - 1.2	D, DHD
M. fortuitum	6 days	0.3	D, DHD, AD, ADD
M. phlei	6 days	0.3	D, DHD, AD, ADD

notes: D = diosgenone

DHD = 1-dehydrodiosgenone

AD = androst-4-ene,3,17-dione

ADD = androst-1,4-diene-3,17-dione

Table 22. The effect of α,α'-bipyridyl on the transformation of diosgenin by *M. phlei*

α,α'-Bipyridyl concentration (mM)	Viable cell count at harvest (cells ml^{-1})	Diosgenin remaining after 7 days (%)	Products Expressed as % of diosgenin			
			D	DHD	AD	ADD
i) Diosgenin and inhibitor added after 6 days growth						
0	2×10^9	0	0	0	0	0
0.05	1×10^9	0	0	0	0	0
0.09	-	0	0	0	0	0
0.3	3×10^7	0	29	50	2	4.5
0.6	-	0	28	40	1	2
0.9	2×10^7	4	51	35	0	0
3.0	7×10^6	32	61	0	0	0
7.5	-	66	24	0	0	0
15	-	80	12	0	0	0
ii) Diosgenin and inhibitor added after 4 days growth						
0	-	18	6	4	0	0
0.05	-	35	18	6	0	0
0.1	-	48	17	9	0	0
0.3	-	72	14	11	0	0

D diosgenone

DHD 1-dehydrodiosgenone

AD androst-4-ene-3,17-dione

ADD androsta-1,4-diene-3,17-dione

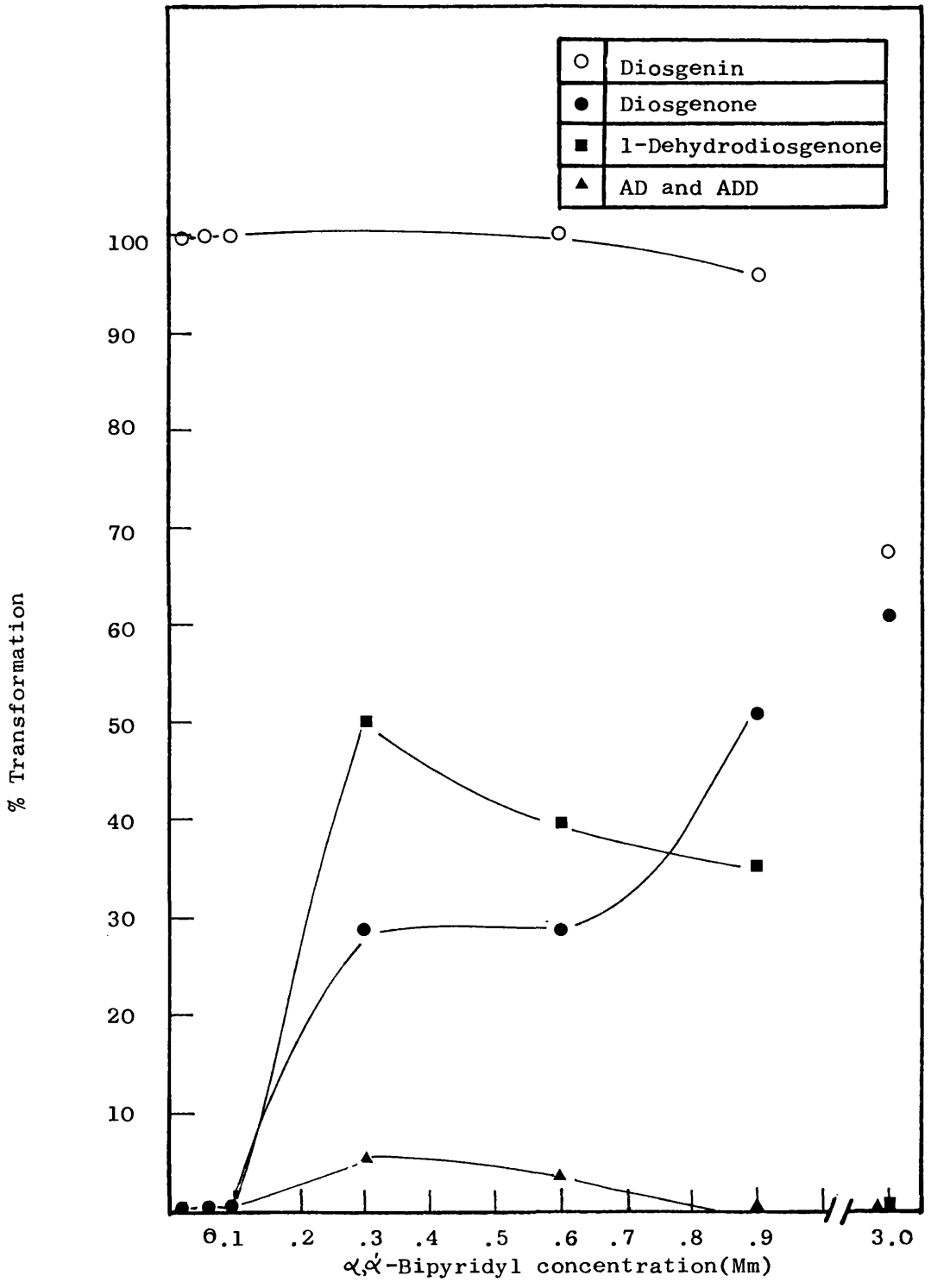


Figure 32. The effect of α,α'-bipyridyl on the transformation of diosgenin by M.phlei.

androstane accumulation was prevented but increasing amounts of diosgenone and 1-dehydrodiosgenone were formed.

At the highest concentrations tested the metabolism of diosgenin became severely inhibited. At 7.5 mM α,α'-bipyridyl 66% of the original diosgenin remained unmetabolised and at the 15 mM concentration 80% remained unmetabolised with the only product being diosgenone.

The relative proportions of diosgenone and 1-dehydrodiosgenone were also dependent on the α,α'-bipyridyl concentration; more 1-dehydrodiosgenone accumulated at the lower concentrations (0.3 - 0.6 mM) and more diosgenone accumulated at the higher concentrations (0.9 mM and above).

When α,α'-bipyridyl was added to M. phlei cells before they reached stationary phase (Table 22ii) its effects were even more inhibitory. Androstanes were not accumulated at any of the α,α'-bipyridyl concentrations tested and diosgenin metabolism was inhibited even at the 0.05 mM level (35% diosgenin remaining after 7 days). The levels of diosgenone and 1-dehydrodiosgenone accumulated were also much lower - the combined maximum being 26% of the added diosgenin (0.1 mM) compared to 86% achieved if the inhibitor was added after 6 days (0.9 mM). This suggested that the α,α'-bipyridyl was more toxic to growing cells than those in stationary phase even after allowing for the potentially toxic effect of the ethanol added.

The effect of cell density on α,α'-bipyridyl with M. phlei

M. phlei cells were grown in 2 L of culture Medium 2 + 2% w/v glucose

for 6 days, harvested by centrifugation (23,000 g) and redistributed into sterile flasks as indicated in Table 23. The supernatant resulting from the centrifugation was used as the resuspending medium. Diosgenin and α,α'-bipyridyl were added and the cultures extracted after a further 6 days incubation. The results are listed in Table 23. The dry weight of cells was monitored throughout the experiment but little increase was seen in any cultures during the experiment. As the initial dry weight of cells was increased, with the α,α'-bipyridyl level remaining constant, its inhibitory effects were lessened. The effects were very similar to those observed in the previous experiment. Increasing metabolism of diosgenin occurred as the effective concentration of α,α'-bipyridyl per gram cells dropped. The connection between dry cell weight and α,α'-bipyridyl concentration was clearly demonstrated (see lower part of Table 23). As long as the ratio between α α'-bipyridyl and cell dry weight remained constant the quantities and proportions of products accumulated also remained constant. Although the concentration of α,α'-bipyridyl was increased from 0.3 mM to 0.9 mM the amount of diosgenone (23 - 24%) and androstanes (6.5%) remained almost constant whilst the α,α'-bipyridyl concentration was 1.25 mM g^{-1} cells.

ii) Structural modification of steroid

The effect of structural modification to diosgenin on its transformation rate by three microorganisms

Diosgenin acetate was prepared by mixing 4 g diosgenin, 4 ml acetic anhydride and 4 ml pyridine. This mixture was left overnight and then 160 ml cooled water added and allowed to stand for 2 hours. The residue was collected by filtration, washed with water and dried in a vacuum oven at 80°C. The acetate was then recrystallized from acetone. T.l.c.

Table 23. The effect of cell density on α,α'-bipyridyl with M. phlei.

α,α'-Bipyridyl concentration (mM)	Dry cell wt per 100 mls	α,α'-Bipyridyl mM g cells^{-1}	Diosgenin remaining after 7 days (%)	Products Expressed as % of diosgenin D	DHD	AD	ADD
0.3	0.05	6	75	15	0	0	0
0.3	0.1	3	25	33	30	Trace	Trace
0.3	0.24[1]	1.25	0	23	43	2	4.5
0.3	0.5	0.6	0	0	0	0	0
0.3	0.24	1.25	0	23	43	2	4.5
0.6	0.5	1.25	0	18	41	1	2
0.9	0.72	1.25	0	24	35	2	4.5

1 Corresponds to cell weight used in previous experiment (Table 22)

D diosgenone

DHD 1-dehydrodiosgenone

AD androst-4-ene-3,17-dione

ADD androst-1,4-diene-3,17-dione

of the product showed it to contain only trace amounts of residual diosgenin and its melting point agreed with the published value (198°C, Merck Index 1968). A calibration curve prepared by g.l.c assay had a slope of 0.319, an intercept (y axis) of -0.05 and a correlation coefficient of 0.999.

Diosgenin benzoate was prepared from 2 g of diosgenin mixed with 10 ml pyridine and 3 ml benzoyl chloride. This was left overnight at room temperature and then poured into iced water, left for 2 hours and filtered. The crystals collected were then recrystallized from acetone. The melting point was in accordance with the published value (Merck Index, 1968). The molecule was too large to be assayed by g.l.c. and U.V. spectroscopy proved unsatisfactory. Hence it was assayed by t.l.c.

The rates of transformation of the two compounds by M. phlei, M. fortuitum and N. rhodochrous were determined. Diosgenin acetate and benzoate dissolved in ethanol were separately added to 6 day (stationary phase) cultures of both M. phlei and M. fortuitum in Medium 5. After a further 6 days the cultures were extracted and assayed for products. The compounds were also added to 48 hour (stationary phase) N. rhodochrous cultures in Medium 5 and incubated a further 48 hours before extraction and assay. The results are given in Table 24.

The addition of an acetate or benzoate group to the 3-position of diosgenin completely inhibited its transformation by N. rhodochrous.

The benzoate group also prevented degradation of the compound by the two species of Mycobacteria tested. However, they did show some

Table 24. The effect of structural modification on diosgenin transformation by three microorganisms

Organism	Rate of transformation (μmoles $g^{-1} h^{-1}$)[A]		
	Diosgenin	Diosgenin acetate	Diosgenin benzoate
N. rhodochrous	33	0	0
M. phlei	5.02	0.48	0
M. fortuitum	5.13	0.75	0

No products were detected

A weight as dry cell weight

slight activity with the acetate, about 10% of that achieved with diosgenin. No products were detected.

iii) Mutagenesis experiments - M. phlei

Initial experiments of serially culturing M. phlei in the absence of any mutagenic agents yielded a mutant strain which grew in liquid media in a dispersed form rather than in the large visible flakes of the wild type. An explanation of this type of growth is given in Chapter 7, discussion. As this mutation was unstable, reverting back to wild-type growth if cultured on solid media, it was used directly from liquid culture for nitrosoguanidine mutation in mutation run 1. A wild type organism was used in run 2. The mutation method is fully described in the specific materials and methods section of this chapter. The results are given in Tables 25, 26, 27; Figure 33.

Table 25 and Figure 33 show the killing characteristics of MNNG. It can be seen that the effect was linear on a log plot up to MNNG

Table 25. *M. phlei* mutagenesis experiments

i) MNNG toxicity curve

MNNG concentration $\mu g\ ml^{-1}$	Viable count (after 30 minute exposure)
0	5×10^6
20	1.4×10^5
50	1.5×10^4
250	6×10^3

ii) Percentage of colonies not capable of growth on selective media

MNNG	250 µg	50 µg	20 µg	Total (%)
Run 1	8	3	30	7.9
Run 2	12.5	14	11	12

iii) Mutation rate - mutants per 10^6 survivors

MNNG	250	50	20
Run 1	8×10^4	3×10^4	3×10^5
Run 2	1.25×10^5	1.36×10^5	1.14×10^5

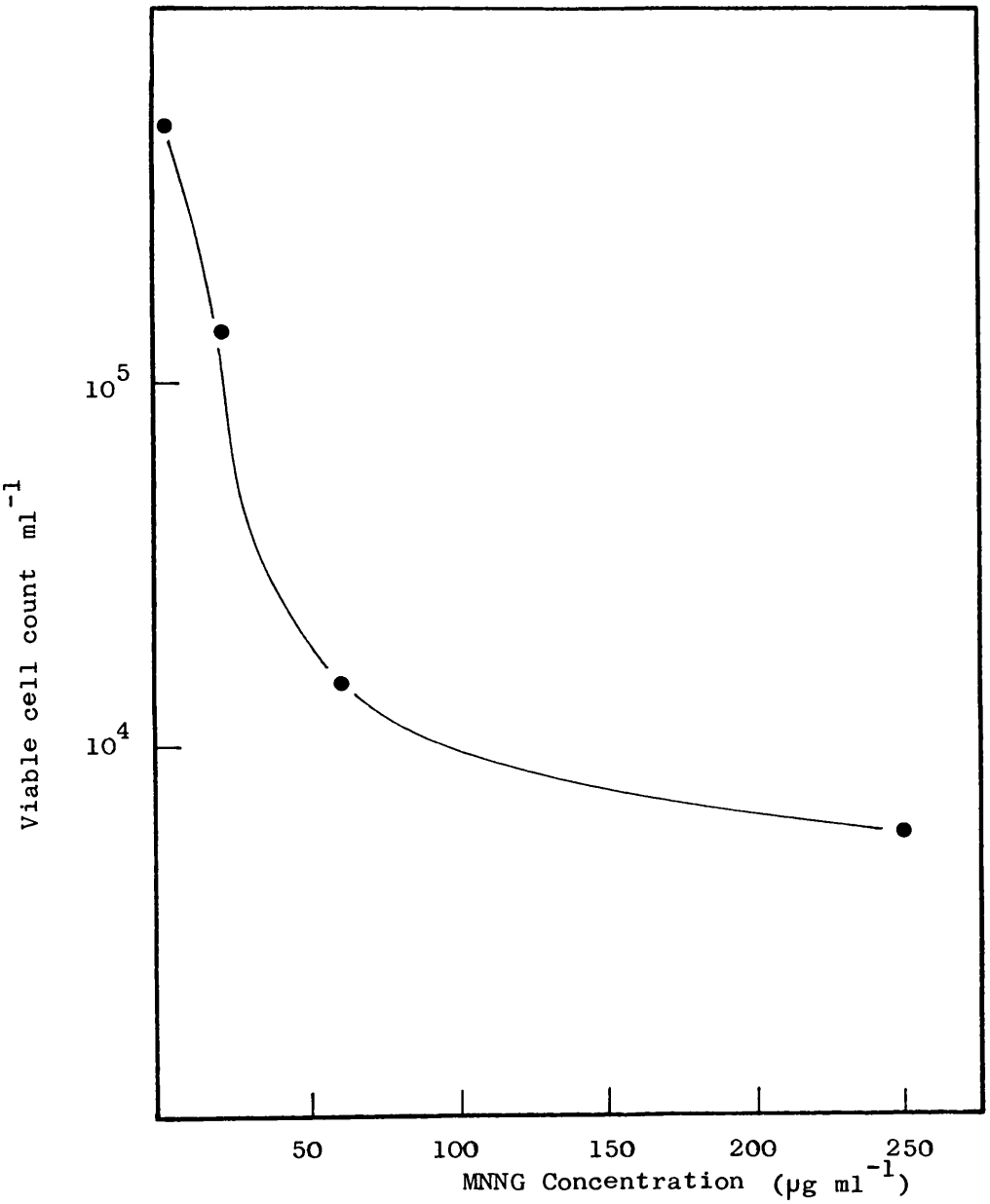


Figure 33. *M. phlei* viable cell count after exposure to N-methyl-N'-nitro-N-nitrosoguanidine.

concentrations of 50 μg ml^{-1}. A considerable tailing effect occurred at the 250 μg ml^{-1} concentration. Some differences were seen in the mutants produced by the different concentrations of mutagen (Table 25ii and iii). In the first run more useful mutants (30%) and a higher mutation rate was achieved at the lowest mutagen level tested (20 μg ml^{-1}) compared to the other levels. In the second run there was little difference between the results obtained at the three MNNG levels used. In both runs however a very high number of mutants were incapable of growth using androst-4-ene-3,17-dione as the sole carbon source, indicating that both the selection procedure and mutation treatments were effective. Most M. phlei colonies that retained the ability to degrade the androstane were surrounded by a zone of clearing (radius 5 - 7 mm) on the selective plates. This suggests that some of the steroid degrading enzymes operate extracellularly, at least on solid media.

A number of the mutants, when tested in shake flask, (Tables 26 and 27) showed a marked reduction in diosgenin degrading activity compared to the wild-type. In complex media (Table 26) only 2 of the 66 clones tested accumulated products. One of these, mutant 20.2.6.3 accumulated almost all the diosgenin it transformed as diosgenone and 1-dehydro-diosgenone. The other mutant (20.2.6.5) formed several other products, some with similar r.f.values to androstanes. However, on a large scale culture (3 L) this mutant proved to be unstable and no products were accumulated.

Using the defined medium (Table 27) a number of the mutants were actually more active in degrading diosgenin than the non-mutated control (about 30% of those tested). Those organisms with low degradative activity

Table 26. *M. phlei* mutants screening - complex Medium 5

Mutant	% Diosgenin metabolised	Products	Mutant	% Diosgenin metabolised	Products
20.2.6.3	25	1-dehydro-diosgenone	250.1.51.4	100	none
20.1.21.5	32	none	250.1.62.2	"	"
50.2.11.1	36	"	250.1.38.1	"	"
20.1.45.6	66	"	250.1.66.2	"	"
20.2.73.4	68	"	250.1.72.4	"	"
20.2.87.2	69	"	250.1.80.3	"	"
20.2.6.5	70	several unidentified	250.1.87.6	"	"
250.1.22.1	80	trace diosgenone	250.1.87.6	"	"
250.2.4.1	80	none	250.1.94.5	"	"
20.1.54.4	86	"	250.1.97.6	"	"
20.2.12.6	87	"	50.1.40.3	"	"
20.2.96.1	87	"	50.1.36.3	"	"
20.1.87.3	87	"	50.1.78.1	"	"
250.1.74.6	89	"	50.2.2.6	"	"
50.1.83.2	89	"	50.2.69.2	"	"
20.1.90.2	89	"	50.2.71.3	"	"
20.1.95.4	90	"	50.2.86.5	"	"
250.2.78.1	90	"	20.2.4.5	"	"
20.2.1.6	91	"	20.2.11.3	"	"
20.2.88.1	91	"	20.2.6.4	"	"
20.2.2.6	92	"	20.2.6.6	"	"
20.1.17.1	94	"	20.2.6.1	"	"
20.1.100.2	94	"	20.2.6.2	"	"
20.1.34.2	94	"	20.2.70.1	"	"
20.1.42.3	96	"	20.2.77.2	"	"
20.1.70.4	96	"	20.2.79.6	"	"
50.1.32.5	96	"	20.2.93.1	"	"
20.2.72.5	96	"	20.1.52.2.6	"	"
50.1.68.1	96	"	20.1.34.5	"	"
20.1.17.6	97	"	20.1.72.5	"	"
250.1.1.5	97	trace diosgenone	20.1.88.3	"	"
20.1.99.1	97	none	250.1.68.6	"	"
250.1.83.6	97	"	250.1.3.3	"	"
250.1.50.4	100	"	Control	"	"

Table 27 M. phlei mutants screening - defined Medium 2

Mutant	% Diosgenin metabolised	Products	Mutant	% diosgenin metabolised	Products
20.2.4.5	21	trace diosgenone	50.2.69.2	40	"
			50.2.71.3	41	"
20.1.52.2	22	"	20.1.70.4	41	"
20.2.70.1	24	"	250.1.62.2	42	"
20.1.72.5	25	"	250.1.50.4	44	"
250.1.38.1	27	"	250.1.94.5	44	"
50.1.40.3	28	"	250.1.87.6	44	"
20.1.17.1	32	"	250.2.41	44	"
250.1.97.6	33	"	250.2.78.1	44	trace diosgenone
250.1.80.3	34	"	20.1.17.6	44	"
50.1.78.1	34	"	CONTROL (NON MUTATED)	45	"
20.1.87.3	36	"			
20.2.6.5	36	"	250.1.51.4	46	"
20.2.1.6	37	"	20.2.6.6	49	"
20.2.12.6	38	"	20.1.45.6	50	"
20.2.88.1	38	"	20.1.54.4	52	+ 1 other
20.2.77.2	38	"	250.1.74.6	51	trace diosgenone
50.1.36.3	38	diosgenone	250.1.83.6	51	"
20.2.87.2	39	trace diosgenone	20.1.90.2	51	"
50.1.68.1	39	"	20.2.73.4	52	"
50.2.2.6	38	"	20.1.95.4	52	"
20.1.34.2	39	"	20.1.21.5	52	"
20.1.42.3	40	"	20.2.11.3	52	"
50.2.11.1	40	"	50.1.32.5	60	"
20.1.00.2	40	"			

in one medium did not necessarily correspond to those with similar activity in the other medium. Once again few of the mutants screened in the defined medium yielded significant quantities of products. Mutant 50.1.36.3 however produced 23% diosgenone although no other products were detected from that fermentation.

Discussion

i) The use of enzyme inhibitors

Most of the organisms tested failed to accumulate androstanes (Table 21) although by experiments previously described they have been shown to be capable of completely degrading diosgenin (especially *N. rhodochrous*). Reasons for this can be suggested. The organisms could possess a 9α-hydroxylase enzyme which was insensitive to α,α'-bipyridyl or the organisms degraded the diosgenin by a pathway not involving 9-hydroxylation. In either case product would not be accumulated.

The only organisms which did accumulate androstanes were the *Mycobacteria*. The phenomenon was further investigated. *M. phlei* showed that an optimum α,α'-bipyridyl concentration existed for the accumulation of androstanes. At the 0.3 mM level the 9α-hydroxylase enzyme was inhibited resulting in formation of androstanes (Table 22). However the chelating agent clearly has an inhibitory effect on other enzymes in the diosgenin transformation pathway as diosgenone and 1-dehydrodiosgenone also accumulated in increasing quantities.

Similar effects have been reported by Martin and Wagner (1976), and also by Wix *et al.* (1968) who studied the transformation of cholest-4-ene-3-one by *M. phlei*. At low inhibitor concentrations cleavage of

the androstane nucleus was not prevented whilst at higher concentrations both this and side chain degradation were inhibited. They found 0.67 mM 8-hydroxyquinoline and 0.32 mM α,α'-bipyridyl to be the optimal concentrations for the accumulation of androstanes. Nagasawa et al. (1970b) found 1.0 mM α,α'-bipyridyl to be optimal for the accumulation of androstanes during the biotransformation of cholesterol by Corynebacterium simplex.

The results showed that the levels of α,α'-bipyridyl necessary to completely inhibit the 9α-hydroxylase enzyme also had an adverse effect on the enzymes necessary for the formation of androstanes. It is difficult to see how higher yields of androstanes from diosgenin could be obtained using M. phlei with an inhibitory agent such as α,α'-bipyridyl. In fact the accumulation of androstanes achieved in these experiments was slightly higher than the only other reported yield from diosgenin when M. phlei was also used. (Ambrus and Buki, 1969).

The final experiment studied the effect of different M. phlei cell densities on α,α'-bipyridyl action (Table 23). This demonstrated that the concentration of inhibitor per gram of cells in stationary phase was the important factor determining inhibitor activity. Even allowing for the decreased cell weight in some cultures the diosgenin degrading activity was further inhibited by the presence of relatively more α,α'-bipyridyl per gram cells, although the aqueous concentration of α,α'-bipyridyl remained constant.

ii) Structural modification of steroid

The results (Table 24) show the importance of the 3β-hydroxyl group

of diosgenin in its biotransformation. The replacement of the hydroxyl group by the relatively small acetate group had a considerable effect. Although satisfying the aim of prevention of steroid nucleus degradation, the structural modification as acetate or benzoate did not enhance the degradation of rings E and F of diosgenin.

It might be possible to exploit this property in a future screening programme to select for organisms which attack the molecule at sites other than the 3β-hydroxyl group. However the experiment demonstrated that this type of structural modification to diosgenin was not an effective method for increasing the yield of androstanes using the microorganisms which were available at this time.

iii) Mutagenesis experiments - M. phlei.

As neither the use of inhibitors nor structural modification of diosgenin yielded a satisfactory level of androstane production, mutagenesis was tried as an alternative method.

MNNG is an alkylating agent and one of the most potent mutagens known (Hopwood, 1970). As the optimum physical conditions, such as pH and time of exposure, have been shown to vary amongst the microorganisms tested (Delic et al., 1970), a mutation regime was chosen which has yielded many interesting mutants with the closely related species M. fortuitum (Pyke and Salmond, 1978 ; Wovcha et al., 1979; Knight and Wovcha, 1979; see also Figure 31).

The initial results showed that the mutation technique was producing a high proportion of clones that were incapable of degrading androst-4-ene-

3,17-dione on solid media. Although a few of these did accumulate metabolites when screened in shake flask culture the most interesting lacked stability. By using other mutagenic agents it may be possible to increase clone stability. However, as the many recent patents attest, MNNG can successfully produce stable changes in the steroid transformation abilities of microorganisms. A more rigorous clone purification procedure involving more platings-out on solid media might yield improved results. Even so the method described and carried out should have been thorough enough to be effective.

Whilst this experiment failed to produce stable M. phlei mutants capable of accumulating androstane compounds from diosgenin it demonstrated that the technique could eventually succeed. Chemical mutagenesis with MNNG is an essentially random process. Thus further mutation experiments could still yield the desired clone. Of the three approaches studied in these experiments to prevent the degradation of the steroid nucleus of diosgenin it was considered that mutagenesis offered the best chance of success in any future work.

CHAPTER 6

The role of surfactants and cholesterol oxidase enzyme in the biotransformation of diosgenin.

Surfactants as stimulants of transformation rate

There are several reports in the literature that surfactants can act as stimulants of enzyme production or catabolic activity with micro-organisms.

Takahashi et al. (1960) reported a 10 - 40% increase in amylase production by Aspergillus niger in the presence of non-ionic surface active agents. Similarly Reese and Maguire (1969) found that by using Tween-80 they could increase the production of some fungal enzymes greatly (200 - 400%).

Nagasawa et al. (1971) increased yields of androsta-1,4 -diene-3,17-dione from cholesterol at least two-fold in the presence of sorbitan monolaurate.

More recently a group of workers in Germany have studied the effect of Tweens and lipophilic organic adsorbents (XAD resins) on transformations by microorganisms (Martin and Wagner, 1976; Schömer et al., 1978; Sauerbaum et al., 1978; Schömer and Wagner, 1980). They used Nocardia sp. M29 to transform β-sitosterol to C-17 oxosteroids. In their experimental system α,α'-bipyridyl was present to prevent 9α-hydroxylase activity. However they encountered the same problems that were discussed in the previous chapter. At low concentrations of inhibitor there was little or no accumulation of product and at high concentrations both steroid nucleus and side chain degradation were blocked. In the presence of uncharged lipophilic resins (in particular Amberlite XAD-2) and a Tween surfactant they were able to increase product accumulation two to four fold. They postulated a dual mechanism for this effect.

The lipophilic sterols, with the aid of the Tween present, aggregated into micelles and were therefore more easily available to the bacteria for transformation. The toxicity of the inhibitor was lowered due to its adsorption onto the XAD-resin, whilst the products of side chain cleavage were selectively trapped by the resin. This product adsorption would also prevent any feedback inhibition (for example Sih et al., 1968b).

As one of the organisms found here to be active against diosgenin is a Nocardia species attempts to stimulate the rate of transformation by using XAD-2 and Tween were made in this thesis.

Cholesterol Oxidase

One of the most frequent transformations of 3β-hydroxysteroids is the formation of 4-en-3-one compounds. Some of these reactions have commercial importance in the production of cortexolone (17α,21-dihydroxypregn-4-ene-3,20-dione) and 17β-hydroxy-17α-methyl androsta-1,4-dien-3-one (Smith, 1974). A Japanese group (Nagasawa et al., 1969; Arima et al., 1969) was able to demonstrate the production of cholest-4-ene-3-one from cholesterol using species of the genera Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Microbacterium, Mycobacterium, Nocardia, Protaminobacter Serratia and Streptomyces.

The same type of reaction has been reported with diosgenin leading to the formation of diosgenone (Mycobacterium phlei, Ambrus and Buki, 1969; Nocardia globerula, Howe et al., 1973; Corynebacterium simplex, Nobile, 1964; Brevibacterium maris; Iizuka and Iwafuji, 1967). In this work (Table 7) a number of other bacteria capable of forming diosgenone have been found.

The reaction can be considered a two step one involving oxidation of the 3β-hydroxyl to a ketone and the isomerisation of the Δ5 double bond to the Δ4 position. (Figure 34). A number of enzymes have been isolated from bacteria that are capable of performing this reaction *in vitro*. The best characterised system is that of *Pseudomonas testostoneri* where the two steps are catalyzed by separate enzymes, a 3β-hydroxysteroid dehydrogenase (EC 1.1.1.51) and a $\Delta^5$3-ketosteroid isomerase (EC 5.3.3.1) (Talalay and Wang, 1955).

Cholesterol oxidases (EC 1.1.3.6), capable of carrying out the same reaction, have been isolated from a number of sources - *Brevibacterium sterolicum* (Uwajima *et al.*, 1973); *Streptomyces* sp. (Fukuda *et al.*, 1973; Kerenyi *et al.*, 1975); *Schizophyllum* (Noma and Nakayama, 1976); *Bacillus sphaericus* (Hayashi *et al.*, 1979); *Nocardia erythropolis* (Turfitt, 1944b) and *Nocardia rhodochrous* (Richmond, 1973). In none of these has it been possible to separate the oxidation and isomerisation activities,but the presence of the Δ5-ketone intermediate has been demonstrated (Smith and Brooks, 1977; Cheetham, 1979b). The *Nocardia rhodochrous* isomerisation reaction was found to have a rate twice that of the oxidation reaction (Cheetham, 1979b), explaining the non-accumulation of cholest-5-en-3-one. All the cholesterol oxidases studied have been shown to be relatively non-specific, attacking a wide range of 3β-hydroxysteroids (Uwajima *et al.*, 1974; Fukuda *et al.*, 1973; Richmond, 1973; Smith and Brooks, 1974, 1975, 1977; Brooks and Smith, 1975).

It has been shown that *N. rhodochrous* NCIB 10554 is a high producer of cholesterol oxidase (Richmond, 1973; Buckland *et al.*, 1974, 1976). The aim of the experiments in this chapter was to determine whether

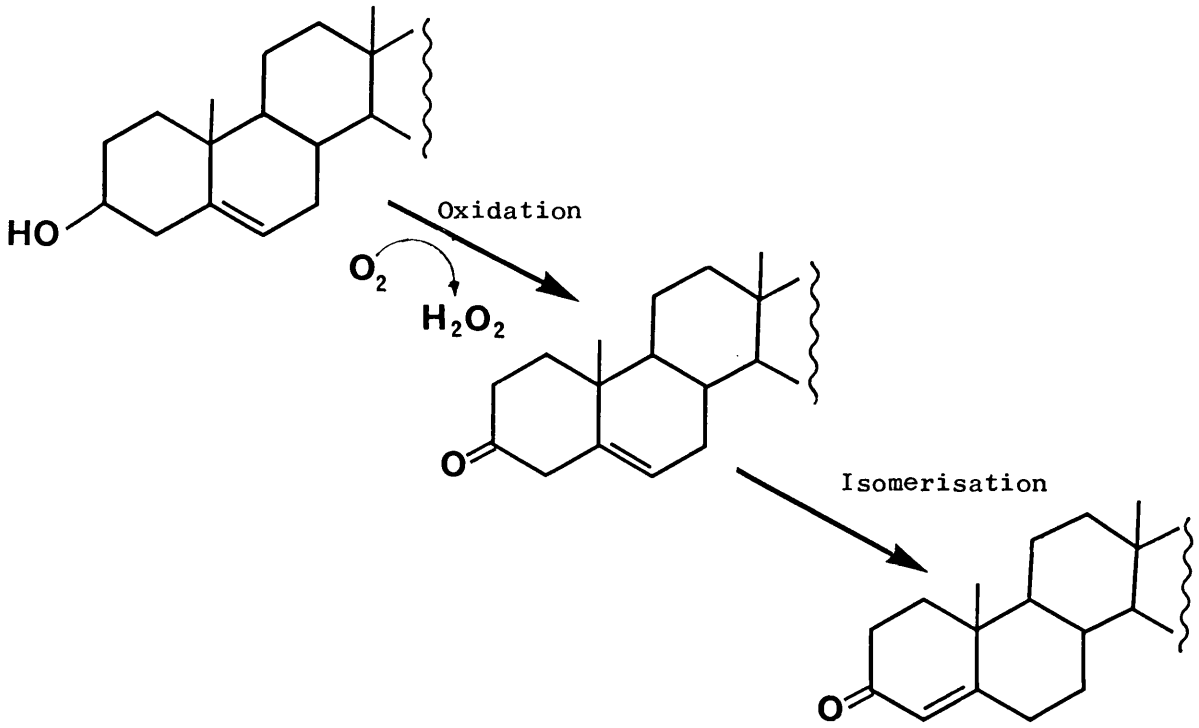


Figure 34.The oxidation/isomerisation reaction of 3_β-hydroxysteroids by cholesterol oxidase.

commercially available cholesterol oxidase enzyme was capable of transforming diosgenin extracted from fenugreek, to determine whether free or cell bound enzyme from N. rhodochrous was more effective in this transformation and whether higher levels of N. rhodochrous cholesterol oxidase could be induced.

The efficient production of diosgenone is of potential importance. Kondo and Mitsugi (1966, 1973) have found it to be a more amenable substrate for bioconversion to androstanes and pregnanes than diosgenin.

Results

The effects of Tween 20 and XAD-2 resin on N. rhodochrous fermentations

Diosgenin, Tween 20 (Polyoxyethylene sorbitanmonolaurate), α,α'-bipyridyl and Amberlite XAD-2 resin were added to N. rhodochrous cultures which had been grown in nutrient broth for 24 hours. The quantities used per 100 ml culture were:- XAD resin 0.5 g; Tween 20 0.7% , α,α'-bipyridyl 0.3 mM. The combinations used and results obtained are listed in Table 28.

Three different aspects of the results can be considered; the effects on the viable cell population, diosgenin degradation and product accumulation.

The addition of inhibitor (α,α'-bipyridyl) had a significant effect on cell viability; the cell population without inhibitor (cultures 1 and 6) was between 3×10^8 and 6×10^8 cells ml^{-1}, with inhibitor it fell to 2.5×10^6 ml^{-1} (culture 2) after 192 hours. The presence of XAD-2 resin and inhibitor (without Tween-20) resulted in an increased

Table 28 The effects of Tween 20 and XAD-2 resin on N. rhodochrous fermentations in the presence of α,α'-bipyridyl

Culture	Viable cell count, 48 hours fermentation	Viable cell count, 192 hours fermentation	Diosgenin transformed, 192 hours, μmoles	Products
1 Control (no diosgenin, Tween, XAD-2)	1.04×10^9	8×10^8	-	-
2 Diosgenin + inhibitor	3×10^8	2.5×10^6	193	D,DHD
3 Diosgenin + inhibitor + 0.5 g XAD-2	4×10^8	1.2×10^8	200	D,DHD
4 Diosgenin + inhibitor + 0.7% Tween 20	6.7×10^8	$< 5 \times 10^2$	216	D,DHD
5 Diosgenin + inhibitor + 0.5 g XAD-2 + 0.7% Tween 20	9.2×10^8	2.95×10^8	227	D,DHD
6 Diosgenin + 0.5 g XAD-2 + 0.7% Tween 20	1.04×10^9	3×10^8	226	D,DHD

D = diosgenone DHD - 1-dehydrodiosgenone

diosgenin transformation rate, although the increase was small (200 μmoles transformed, culture 3 compared to 193 μmoles in the absence of resin, culture 2). There was also a considerably higher cell population in the presence of XAD-2 resin and α,α'-bipyridyl compared to α,α'-bipyridyl alone (1.2×10^8 and 2.5×10^6 cells ml^{-1} in cultures 3 and 2 respectively). The presence of the XAD-2 resin presumably decreased the toxicity of the inhibitor to the cells. Little difference was seen in the quantities of products accumulated by the two cultures.

Tween-20 encouraged a further increase in the amount of steroid metabolised (to 216 μmoles in culture 4) but the pattern and quantity of products remained the same, chiefly diosgenone and 1-dehydrodiosgenone. Tween also caused considerable cell mortality. At the end of the fermentation period less than 5×10^2 viable cells ml^{-1} remained, lower than any other culture by a factor of 10^4.

When both XAD-2 resin and Tween 20 were used together (cultures 5 and 6) the highest overall diosgenin transformation was achieved, a total of 226 μmoles. Similar values were obtained both in the presence and in the absence of α,α'-bipyridyl, and high cell viable counts were retained (comparable with the control figures) in both cases.

Discussion

The effects of Tween 20 and XAD-2 resin on N. rhodochrous fermentations

The most striking result of this experiment was when Tween 20 was present alone. There was considerable cell mortality coupled with a transformation rate actually higher than the control cultures.

Buckland et al. (1974) and Cheetham et al. (1980) used the non-ionic detergent Triton-X-100 to extract cholesterol oxidase from N. rhodochrous. They found that a 60% release of enzyme could be achieved at concentrations of 0.5% and 1.0%; in this experiment (as in those of Martin and Wagner, 1976) 0.7% Tween-20 was present. In view of the high cell mortality and high diosgenin transformation rate it would seem that Tween was disrupting the microbial cell membrane sufficiently to cause cell death. At the same time the enzyme(s) responsible for the formation of diosgenone and 1-dehydrodiosgenone were being released into the supernatant but retaining their activity.

When Tween and XAD-2 resin were both present, although diosgenin transformation was further stimulated, little cell death occurred. This suggests that Tween was not having such a disruptive action on the cell membrane. Using the same resin, Cheetham (1979c) found that it adsorbed Triton-X-100 with a maximum capacity of 0.475 mM g^{-1} dry weight. Presumably it is this adsorption which resulted in the increased cell viable count. If similar figures are assumed for Tween-20 then about half of the surfactant will have been adsorbed by the resin.

The stimulation of reaction rate (17%) achieved in this experiment by the use of XAD resin and Tween was considerably less than that achieved by Martin et al. (see introduction, this chapter). The adsorption of Tween onto the XAD resin also raised doubts about part of their proposed mechanism for the increase in rate achieved (micelle formation). It could instead be that the resin is adsorbing Tween, inhibitor (α'α-bipyridyl) and diosgenin. In this condition the resin might behave as an active site making the diosgenin more accessible to the cells' enzymes.

This experiment strongly suggested that the transformation of diosgenin to diosgenone was mediated by an enzyme very similar to cholesterol oxidase. Experiments were therefore carried out to establish whether the enzyme was cholesterol oxidase and, if so, whether higher levels of enzyme could be induced.

Cholesterol Oxidase Experiments

i) The use of commercially extracted cholesterol oxidase enzyme to transform diosgenin.

Nocardia erythropolis cholesterol oxidase (Sigma Ltd.) yielding 1 mg protein ml^{-1}, 20 I.U., 16 units ml^{-1} was used. The cholesterol was biochemical standard grade (supplied by BDH Ltd.). The substrate (diosgenin or cholesterol) was dissolved in isopropanol and used from a stock concentration of 0.015 M. Experiments were carried out in cuvettes each containing (unless otherwise specified) 3 ml buffer, 0.1% Triton-X-100 and 10 µL of a 1 in 10 dilution of enzyme. The amount of diosgenone formed was calculated from a calibration curve (Figure 35) prepared with bacterially produced diosgenone from earlier experiments. The amount of cholestenone formed (µmoles) was calculated assuming an ε value of 12,200 (Smith and Brooks, 1977). The results of the experiment are shown in Figure 36. N. erythropolis enzyme was capable of transforming diosgenin extracted from fenugreek. An initial diosgenin transformation rate of 1.1 µmoles $min^{-1}mg^{-1}$ was achieved. Cholesterol was much more rapidly degraded by the same enzyme with a maximal rate of 7.0 µmoles $min^{-1}mg^{-1}$. This was expected as cholesterol has generally been found to be a better substrate for bioconversion.

Neither diosgenone nor cholestenone were further degraded by the

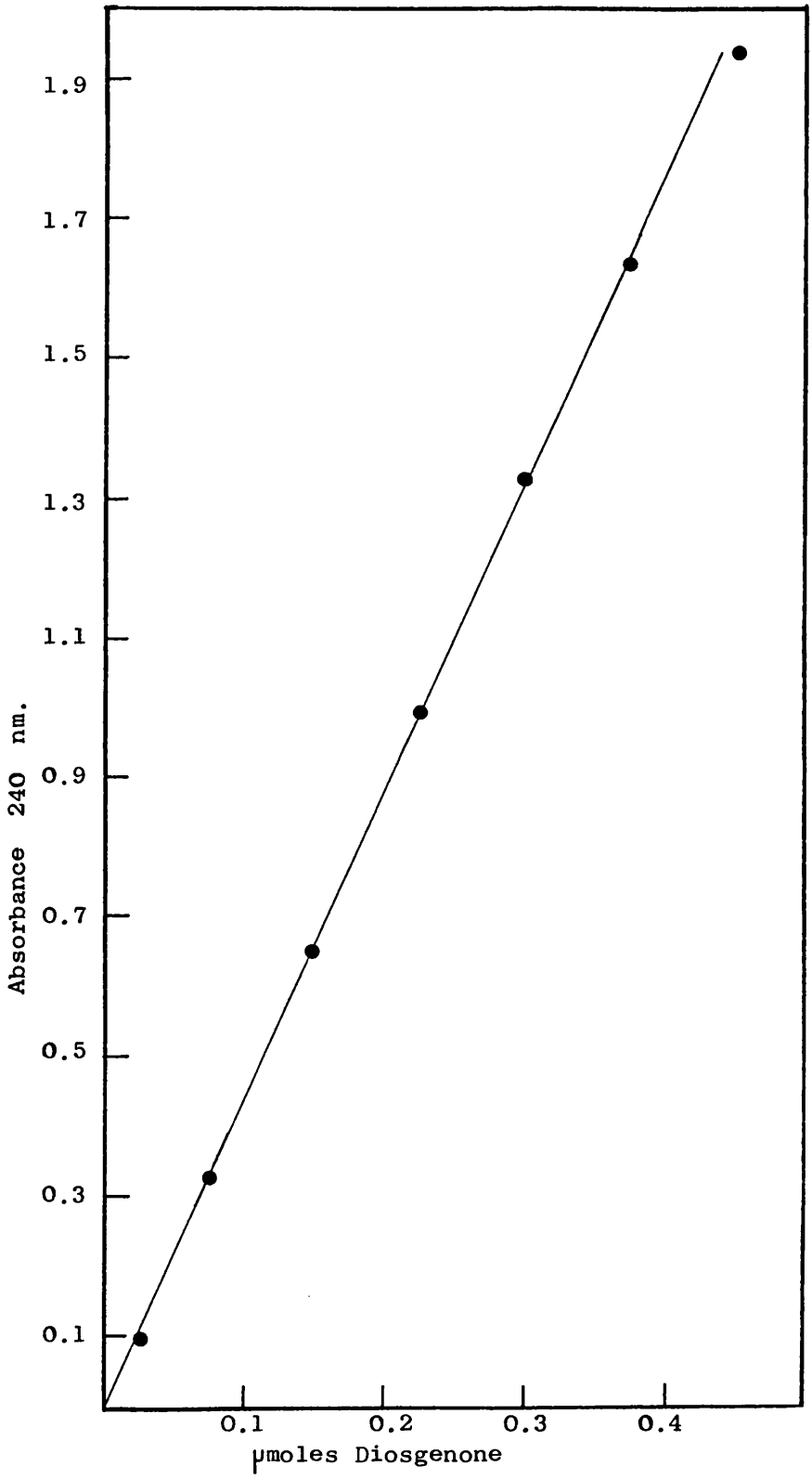


Figure 35. Diosgenone calibration curve.
Slope 4.35 Intercept 0.0
Correlation coefficient 0.999

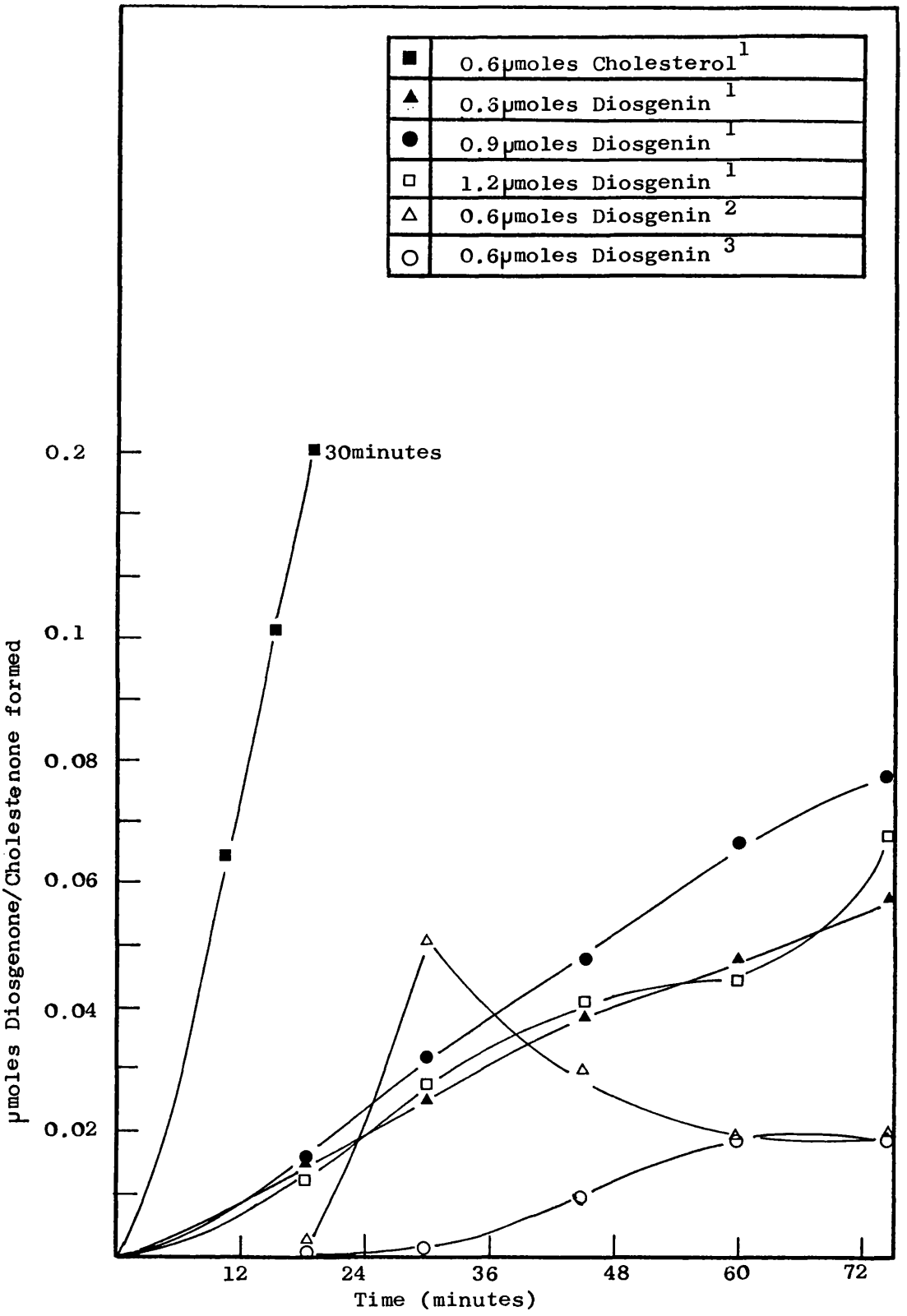


Figure 36. The use of commercially extracted cholesterol oxidase enzyme to transform diosgenin and cholesterol. Notes:1 +Triton-X-100 2 no Triton-X-100. 3 +Tween 20.

enzyme mixture over the time of the experiment; preparations containing enzyme plus product gave constant absorbance readings.

In the absence of surfactant little transformation took place (less than 30% of the maximum activity obtained). Small clumps of steroid were visible in these cuvettes, unlike the even dispersions obtained in the presence of Triton-X-100. A similarly low level of activity occurred when Tween 20 was substituted for Triton-X-100 although no clumps of steroid were seen in this case.

ii) Cholesterol oxidase activity in the cell-free supernatant of *N. rhodochrous* cultures.

N. rhodochrous cells were incubated for 32 hours in several media, some containing surfactants, before diosgenin was added. After a further 8 hours incubation a 20 ml sample was taken from each broth, prefiltered through a GF/F filter and then filtered through a sterile Millipore (0.2 µ) membrane to yield a cell free supernatant. Aliquots (5 ml) of this supernatant were mixed with an equal volume of phosphate buffer and either diosgenin or cholesterol (both 1.2 µmoles dissolved in iso-propanol) added. After 17 hours incubation the mixture was extracted with chloroform (HPLC grade), the organic extract evaporated to dryness, redissolved in cyclohexane and its absorbance at 240 nm determined. The amount of diosgenone and cholestenone could then be calculated using the calibrations from the previous experiment . T.l.c. showed that no other products were formed. The results of the experiment are given in Table 29.

Large differences in cell growth and activity were observed in the

Table 29. Cholesterol oxidase activity in the cell free supernatant of *N. rhodochrous* cultures

Culture medium	Medium 5	Nutrient broth	Nutrient broth + 0.7% v/v Tween-20	Nutrient broth + 0.7% Triton-X-100 (v/v)
Dry cell weight ($g\ L^{-1}$)	3.4	1.02	1.01	0.92
% diosgenin transformed in culture	69	34	20	13
Products present in culture	D,DHD	D,DHD(trace amounts)	None	D,DHD (trace amounts)
Cell-free supernatant activity				
Diosgenone formed $\mu moles\ g^{-1}h^{-1}$	0	0	0.098	0.23
Cholestenone formed $\mu moles\ g^{-1}h^{-1}$	0.08	0	0.58	7.54

D - diosgenone

DHD - 1-dehydrodiosgenone

four media tested. The rich medium (5) supported much better growth than nutrient broth (3.4 g L^{-1} dry weight compared to 1.02 g L^{-1} dry weight) and also gave a higher transformation of diosgenin (69% in 8 hours compared to 34%). The same products, diosgenone and 1-dehydro-diosgenone were formed in both. In the absence of surfactant little or no cell-free enzyme activity was detected against diosgenin or cholesterol.

If surfactant was present differences were observed (Table 29). Cell yield was not affected by Tween-20 but was slightly depressed (from 1.02 g L^{-1} to 0.92 g L^{-1}) by Triton-X-100. In both instances diosgenin transformation was considerably inhibited. In the case of Tween 20 transformation was reduced to 20% and no products were detected. With Triton-X-100 present transformation was even lower (13%), but trace amounts of diosgenone and 1-dehydrodiosgenone were detected. However in both cultures enzyme activity was found in the cell free suspensions. The medium containing Triton-X-100 was especially active giving a rate of 7.54 µmoles cholestenone formed $g^{-1}h^{-1}$. It was rather less active in forming diosgenone (0.23 µmoles $g^{-1}h^{-1}$).

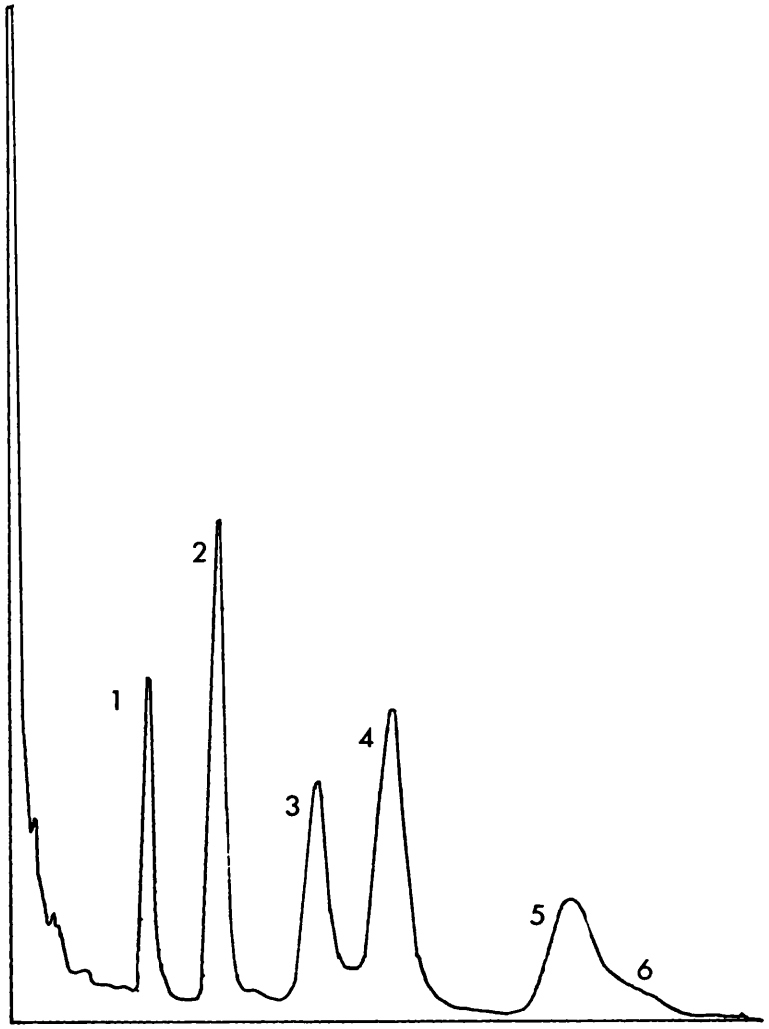
iii) Attempted induction/cometabolism of N.rhodochrous diosgenin transformation activity

Two different techniques were used in an attempt to increase the rate of transformation of diosgenin to diosgenone. Cholesterol was added to the cultures several hours before diosgenin to act as an enzyme inducer or at the same time as the diosgenin to encourage its cometabolism. The precise culture conditions (in duplicate cultures) were as follows:

N. rhodochrous cells were grown for 18 hours and 42 hours in Medium 5 (100 ml). Cholesterol (240 μmoles) was added to a pair of cultures at each time to act as an inducer. After 6 hours (to allow maximum induction) diosgenin (240 μmoles) was added and the fermentation continued a further 8 hours before extraction. Simultaneously with these cultures, cholesterol + diosgenin (240 μmoles, for cometabolism); cholesterol only and diosgenin only (to give control values) were added to further duplicate cultures. All were incubated for 8 hours before extraction. Dry cell weights were measured prior to the addition of steroid.

Assay. In the standard g.l.c. assay it was found that the internal standard (5α-cholestan-3β-ol) and cholesterol had the same retention time, while diosgenin and cholest-4-en-3-one (a likely transformation product of cholesterol) also had identical retention times. The use of a different internal standard (androsta-1,4-diene-,3,17-dione) and the omission of silylation allowed separation of diosgenin, diosgenone, 1-dehydrodiosgenone, cholesterol and cholest-4-en-3-one (Figure 37). The calibration is given in Table 30. The experimental results are listed in Table 31.

Contrary to expectations derived from the previous experiments, diosgenin was actually transformed more rapidly than cholesterol (when they were present singly) by the 42 hour cells. The rates were 60 μmoles $h^{-1}g^{-1}$ (diosgenin) and 40 μmoles $h^{-1}g^{-1}$ (cholesterol). The activity of the 18 hour cells for the two single compounds was approximately equal (33 - 34 μmoles $h^{-1}g^{-1}$). In general the 42 hour cells were more active metabolically than the 18 hour cultures. The total activity per



1 Androsta-1,4-diene-3,17-dione (Internal Standard)
2 Cholesterol
3 Diosgenin
4 Cholest-4-en-3-one
5 Diosgenone
6 1-Dehydrodiosgenone

Figure 37.Gas liquid chromatograph of diosgenin, cholesterol and biotransformation products.

Table 30. Calibration by g.l.c. of diosgenin and cholesterol using androsta-1,4-diene-3,17-dione as an internal standard

Sample	Diosgenin (mg ml^{-1} internal standard)	Mean Peak ht ratio (diosgenin/ I.S.)	Cholesterol (mg ml^{-1} internal standard)	Mean Peak ht ratio (chol. /I.S.)
a	10	2.471	0.6	0.395
b	4	0.924	1	0.615
c	3	0.657	2	1.214
d	2	0.439	4	2.52
e	1	0.202	6	3.793

Diosgenin; slope 0.258; y intercept - 0.11; Correlation coefficient 0.999

Cholesterol; slope 0.633; y intercept - 0.015; Correlation coefficient 0.999

Table 31. Attempted induction/cometabolism of N. rhodochrous diosgenin transformation activity

Culture	Diosgenin transformation rate (μmoles $g^{-1} h^{-1}$)	Diosgenin transformed μmoles	Cholesterol transformation rate (μmoles $g^{-1} h^{-1}$)	Cholesterol transformed μmoles	Diosgenone amount formed μmoles	Combined activity transformation rate (μmoles $g^{-1} h^{-1}$)	Combined activity total steroid transformed μmoles
i. 18 hour cells							
diosgenin only	33.1	73.9	-	-	54	33	74
cholesterol only	-	-	34.4	75.9	-	34	76
diosgenin + cholesterol (cometabolism)	26.9	60.6	32.9	73.9	42.3	60	135
cholesterol induced + diosgenin	38.7	87	40.7	161.6	67	79	249
ii 42 hour cells							
diosgenin only	59.5	173.7	-	-	119	60	174
cholesterol only	-	-	39.8	116.4	-	40	117
diosgenin + cholesterol (cometabolism)	31	90	40	117.5	62	71	208
cholesterol induced + diosgenin	48.2	140	29.7	153.5	101.7	78	294

culture was also higher at 42 hours than at 18 hours, this was partly but not wholly due to the increased cell density. This higher activity agrees well with experiments from Chapter 4 on steroid addition time (Figure 27). In all the cultures containing diosgenin, formation of diosgenone was at approximately 70% of the total diosgenin metabolised.

When cholesterol was added as a cometabolite to the 18 hour cells the total steroid transformed was almost double that in cultures where either steroid was present alone (135 µmoles compared to 74 µmoles and 76 µmoles). However in this cometabolism culture the individual rates for diosgenin and cholesterol metabolism were slightly decreased (diosgenin 26.9 µmoles $g^{-1}h^{-1}$, cholesterol 32.9 µmoles $g^{-1}h^{-1}$) when compared to the controls (diosgenin 33.1 µmoles $g^{-1}h^{-1}$, cholesterol 34.4 µmoles $g^{-1}h^{-1}$).

When cholesterol was added as an inducer to 18 hour cells the overall cell activity was considerably enhanced. The total steroid transformed was 249 µmoles compared to 135 µmoles (cometabolism culture) and 74 µmoles and 76 µmoles for the single steroids. The rate of transformation was also higher when individual steroids were compared. When inducer was present diosgenin was transformed at a rate of 39 µmoles $g^{-1}h^{-1}$ (without induction the rate was 33 µmoles $g^{-1}h^{-1}$) more diosgenone was formed (67 µmoles) than in any of the other 18 hour cultures.

The 42 hour culture with diosgenin only, showed a higher rate of diosgenin transformation (60 µmoles $g^{-1}h^{-1}$) and also a higher total of diosgenin transformed (174 µmoles) than any of the18 hour cultures. This culture also gave the highest yield of diosgenone (119 µmoles). Similar results were obtained from the culture with cholesterol only.

Both cometabolism and induction gave increased total steroid transformation over these controls. Induction yielded a higher diosgenin transformation rate (48 μmoles $g^{-1}h^{-1}$) than cometabolism (31 μmoles $g^{-1}h^{-1}$). However the diosgenin transformation rate was still lower than when the single steroid alone was present (60 μmoles $g^{-1}h^{-1}$).

The presence of cholesterol as a cometabolite had little effect on its rate of degradation when compared to the control, both giving a rate of 40 μmoles $g^{-1}h^{-1}$. Induction actually reduced the rate of cholesterol metabolism to 30 μmoles $g^{-1}h^{-1}$.

Discussion

Cholesterol Oxidase Experiments

i) The use of commercially extracted cholesterol oxidase enzyme to transform diosgenin

Previously Brooks and Smith (1975) have shown that *N. erythropolis* cholesterol oxidase enzyme is able to transform diosgenin extracted from yams. The results (Figure 36) showed that the enzyme was also active against 'diosgenin' extracted from fenugreek. The transformation rate (1.1 μmoles $min^{-1}mg^{-1}$) achieved was comparable to the published value (Brooks and Smith, 1975) of 0.6 μmoles $min^{-1}mg^{-1}$.

The presence of Triton-X-100 was clearly important in allowing maximum dispersion of steroid and maximum enzyme action. Tween 20 was however inhibitory to enzyme action. Since cholesterol oxidase enzyme was capable of forming diosgenone, further experiments were carried out to determine whether it was released from *N. rhodochrous* cells in normal culture conditions and whether the free enzyme gave a

higher transformation rate than cell bound enzyme.

ii) Cholesterol oxidase activity in the cell-free supernatant of *N. rhodochrous* cultures

The results (Table 29) showed that in the standard media and fermentation conditions almost all steroid transforming enzyme activity remained associated with the *N. rhodochrous* cells.

Although the presence of surfactant was able to cause considerable release of this cell bound enzyme, the transformational ability of the cultures was depressed and only very low levels of products accumulated. This contrasts with the result of a previous experiment (Table 28) where a small increase in transformation activity occurred in the presence of Tween-20. However surfactant was added at different times in the two experiments (at time of inoculation in this experiment, after 24 hours growth in the preceding one) and so the results are not directly comparable.

In conclusion there would appear to be little advantage in the addition of surfactant to the culture medium. A more fruitful method of increasing the transformation of diosgenin might be by inducing the enzymes thought to be active against it (see below).

iii) Attempted induction/cometabolism of *N. rhodochrous* diosgenin transformation activity

It has been shown (Lee *et al.*, 1971) that the rates of many steroid conversions by microorganisms are greatly improved by growing the cultures in the presence of suitable enzyme inducers. Martin (1977), in a review,

reported that the enzymes responsible for primary attack and further breakdown of cholesterol were inducible. Only in a few strains have constitutive formation of these enzymes been reported. Buckland et al. (1976) found that addition of cholesterol to growing cultures of N. rhodochrous cells resulted in a substantial increase in cholesterol oxidase activity - up to 15 fold was the maximum reported.

A variant of induction is cometabolism, where the inducer is a prime enzyme substrate and is added together with the substrate of interest. The prime substrate is rapidly exhausted. However its presence is sufficient to stimulate degradative enzymes. These enzymes are often capable of attacking other substrates which, on their own, might not stimulate enzyme induction. Cholesterol is a candidate for induction of enzymes with activity against diosgenin because of the structural similarities of these two steroids and the widespread degradation of cholesterol in the microbial world.

The results (Table 31) showed that cholesterol can act both as an inducer of diosgenin degradation and as a cometabolite. The 18 hour cultures were the most receptive to induction of steroid transforming enzymes although less metabolically active than the 42 hour cultures. The 42 hour cultures could also be induced but the effects were smaller than with the 18 hour cultures.

At no time was an increase comparable to that of Buckland et al. (1976) achieved. This was presumably due to the batch growth of the cultures in contrast to the continuous culture methods of Buckland's group. The culture medium used in these experiments was the same as that used by Buckland et al.

These experiments have shown that if stationary phase cells are required, an induction step does not increase the yield of diosgenone. However if rapidly growing cells are required an induction step can considerably increase their steroid transforming capability.

CHAPTER 7

Methods to increase steroid solubility in fermentations.

Methods to increase steroid solubility in fermentations

A factor contributing to the difficulty of microbially transforming diosgenin is its very low solubility in water. This limits the concentration of diosgenin to which cellular enzymes can be exposed. If the solubility of diosgenin in the fermentation system was increased the rate of transformation should benefit and additional steps in the transformation pathway beyond 1-dehydrodiosgenone might proceed.

i) The use of organic solvents to increase the rate of steroid biotransformation

By the use of organic solvents, in which steroids are highly soluble, it is possible to considerably increase the concentration of dissolved steroid substrate. This in turn should displace the transformation reaction equilibrium towards product formation. Water-miscible solvents have been used for this purpose (Larsson et al., 1976; Ohlson et al., 1978; Mosbach and Larsson, 1970; Weetall and Vann, 1976). However, in many cases the use of free enzymes in a water/water miscible organic solvent system has been unsuccessful. Often the catalytic activity of the enzymes decrease dramatically and substrate specificity disappears (Klibanov, 1979; Tanford, 1968). This effect can be reduced by immobilizing the enzyme to enhance its stability against solvent denaturation and conformational change. The mechanisms of this technique have been fully discussed by Klibanov (1979).

The problem may be minimised if a water-immiscible organic solvent was used, as then the enzymes would be expected to remain in the aqueous phase. When a mixture of water-immiscible organic solvent and water are present the organic phase acts as a reservoir of substrate. The

substrate can diffuse into the water layer and product diffuse back, thus preventing inhibition of the reaction by product accumulation. The choice of organic solvent is dependent upon parameters such as capacity for steroid, the partition coefficient of steroid between the two phases and any inhibiting or denaturing effects of the organic solvent on the enzymes.

A number of workers have investigated the technique using both enzyme and cell systems. Cremonesi et al. (1973, 1974, 1975) and Carrea et al. (1979), studied the effect of water-immiscible organic solvents on hydroxysteroid dehydrogenase (HSDH) enzymes. Using a 50% water:50% organic solvent mixture they achieved conversion of testosterone (17β-hydroxyandrost-4-ene-3-one) into androst-4-ene-3,17-dione and cortisone (17α,21-dihydroxypregn-4-en-3,11,20-trione) to 17α,20β,21-trihydroxypregn-4-en-3,11-dione. They found that the rate of conversion was dependent on the nature of the organic solvent used. Although enzyme activity decreased as the polarity of the solvent increased this effect was counteracted by the increase in steroid solubility. Hexane and carbon tetrachloride gave low conversions of substrate (< 5%) when compared to ethyl acetate (90%) and butyl acetate (100%). The stability of the enzymes in the presence of organic solvent was greatly improved by immobilising the enzymes on cyanogen-bromide activated sepharose. When so immobilised 20β-HSDH retained 80% of its original activity in phosphate buffer after 15 days. After 2 months continuous re-use in water/ethyl acetate some samples of β-HSDH retained about 60% of their original activity. They found the addition of a co-factor (NAD^+) greatly increased enzyme activity.

Klibanov et al (1977) used chymotrypsin covalently bound to porous

glass. The enzyme synthesized N-acetyl-L-tryptophan ethyl ester from the respective alcohol and acid in 100% chloroform. They studied the influence of the chloroform on the equilibrium position of the reaction and found that the solvent caused it to be dramatically shifted in favour of ester production. A reversal of the normal reaction.

Buckland et al. (1975) investigated the use of non-immobilised whole microbial cells in water-immiscible organic systems of low polarity. They found that several such solvents allowed a much higher rate of conversion from cholesterol to cholest-4-en-3-one by Nocardia rhodochrous when compared with an aqueous suspension. After 30 minutes 24% and 21% conversion was achieved with toluene and carbon tetrachloride respectively, and 3% or less in water, acetone, isopropanol and ethanol. They demonstrated that re-use of the same cells up to 7 times (69 hours elapsed time) was possible in a stirred tank reactor using carbon tetrachloride. The final reaction rate was 52% of the initial rate. Duarte and Lilly (1980) found this could be improved to 80% retention of activity after 150 hours by immobilisation of the cells in polyacrylamide or alginate gels.

Following this work Fukui and coworkers studied the effects, on several transformations, of organic solvents and immobilization in hydrophobic and hydrophilic gels. The most straightforward reaction considered was the transformation of 3β-hydroxy-5-ene-steroids to 3-oxo-4-ene steroids; no exogenous cofactors were required and substrate inhibition was not observed. In contrast to the system of Cremonesi et al., Fukui found that the highest initial transformation activity with free cells (N. rhodochrous) was obtained in non-polar solvents. Carbon tetrachloride, benzene and toluene were most effective although

they significantly deteriorated 3β-HSDH activity in the longer term. Transformation decreased in semipolar solvents (e.g. chloroform), decreasing still further in polar solvents (ethyl acetate, methylene chloride) with no activity in extremely polar solvents (methanol, acetone and ethanol) (Omata et al., 1979; 1980).

In order to allow solubilisation of steroid but maintain the 3β-HSDH activity a benzene:n-heptane (1:1 v/v) mixture was used as solvent (Omata et al., 1979, 1980; Fukui et al., 1980a,b). This unfortunately means that the system has very little applicability to potential commercial users due to the severely toxic nature of benzene. They found that cells entrapped in hydrophobic gels had comparable cholesterol transforming activity to free cells, whilst the cells in hydrophilic gels did not catalyze the reaction at all (Omata et al., 1979). When using a less hydrophobic compound, dehydroepi-androsterone (3β-hydroxyandrost-5-en-17-one), the difference in activity between the two gels was less, with some activity being observed in the hydrophilic gel. This pattern was also shown by other compounds bearing a hydrophilic group at C-17 e.g. androst-4-ene-3,17-dione and hydrocortisone (11β-17α,21-trihydroxypregn-4-ene-3,20-dione) (Sonomota et al., 1979; Yamane et al., 1979; Fukui et al., 1980a). In general they found that the activities of the entrapped cells increased as the partition coefficient between gel and solvent increased. Their experiments have shown that the entrapment of microbial cells and use in organic solvents can be a practical method for steroid transformation. Although in most cases the initial rate of transformation decreased when compared to free cells, the stability and reusability of the cells is much higher (Sonomota et al., 1979, 1980; Omata et al., 1979). Patents now

cover several aspects of the use of organic solvents/immobilised cells for steroid transformation - U.K. 1,555,004 (Dunnill and Lilly, 1979) Japanese Kokai Tokkyo Koho 80,15,760 (Fukui, et al., 1980b).

By washing cells with organic solvents it might be possible to increase cell wall and membrane permeability. This could lead to faster rates of transport of substrates and products. A well known example is the use of toluene in the assay of β-galactosidase (Monod et al., 1951). The approach has been tried more recently in enzyme studies by Weitzmann (1973) and also with immobilised Arthrobacter simplex using methanol, butanol and toluene (Ohlson et al., 1978; Larsson et al., 1978). A 20 minute treatment with 0.1% butanol gave the best results, a 33% increase in the transforming activity of the cells 3-oxosteroid Δ1-dehydrogenase enzyme compared to untreated cells. Toluene rapidly destroyed the cells dehydrogenating activity although this could be fully reversed by addition of menadione. However methods which disrupt cell membranes are unlikely to result in stable, reusable cell systems, which are the most economic to use in industry.

ii) Modification of steroid structure to increase its water solubility

This approach is well demonstrated by Lee et al. (1971) who were studying the transformation of 16α-hydroxycortexolone to its 1-dehydro-11α-hydroxy derivatives. The substrate had a solubility of less than 10 μg ml^{-1} in the fermentation broth. However, by forming a borate complex of substrate (Figure 38) solubility was greatly improved, with concentrations of more than 1 mg ml^{-1} in the fermentation broth possible. Use of this complex increased the rate of transformation 3 fold and enabled quantitative conversion to be obtained; less than

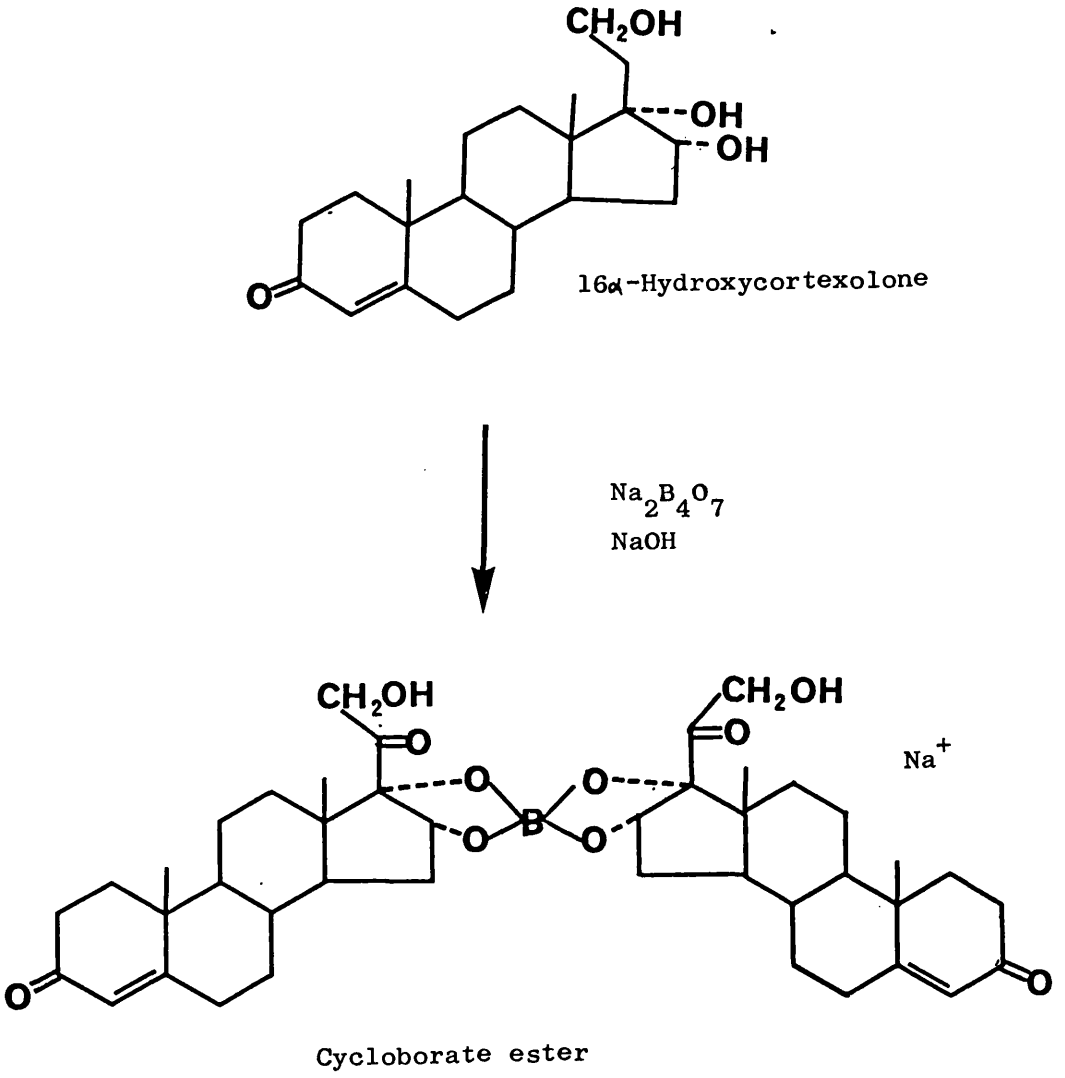


Figure 38. Modification of steroid structure to increase its water solubility. Cycloborate ester formation.

40% conversion was achieved with unmodified substrate. At the end of the reaction acidification of the culture broth to pH 3.9 was sufficient to break the borate bridge and yield free steroid.

In U.S. Patent 3,770,586 (Kominek, 1973) a method is described where 21-hydroxy steroids are added to the fermentation medium in the form of 21-hydrocarbon dicarboxylic acid ester alkali metal salts. These salts have increased water solubility and therefore higher effective substrate concentrations (2 - 15 g L^{-1}) were achieved.

iii) Use of solubilizing agents

It is known that rates of dissociation of drugs increase appreciably in the presence of polymers (Simonelli et al., 1969). They also tend to stabilize solutions by inhibition of crystal growth (Simonelli et al., 1970). Polyvinyl pyrrolidones are widely used for these purposes as they are non-toxic, highly soluble in aqueous media and increase the solubility of organic compounds by their ability to engage in hydrophobic bonding. Chien and Rosazza (1980) were able to considerably increase the water solubility of the aromatic compound ellipticine (from 2% to 22%) by the use of polyvinyl pyrrolidones and in doing so doubled the rate of its hydroxylation by Aspergillus alliaceus.

Results

i) The use of organic solvents to increase the rate of steroid biotransformation

Frozen N. rhodochrous cells induced to a high cholesterol oxidase activity were provided by Tate and Lyle Ltd. M. phlei was pretreated as follows to maximise activity. Cultures were grown for 7 days (Medium

5), diosgenin was added (0.5 mg ml^{-1}) and the culture incubated a further 48 hours before the cells were harvested by centrifugation (23,000 g, 30 minutes). These cells were then used immediately for the organic solvent experiments.

Diosgenin (87 μmoles) and 6 ml of solvent were added to 20 ml screw capped glass vials. The organic component of the solvent was varied from 0 - 100%. A purely aqueous control sample contained 15 μL, 10% Triton-X-100 in phosphate buffer (pH 7). To initiate the reaction 0.1 g frozen Nocardia cells or 0.2 g M. phlei cells with 0.3 mMoles α,α'-bipyridyl were added, the mixture vortexed and time 0 samples taken (0.2 ml volume). The vials were placed in an orbital incubator (30°C, 250 r.p.m.) and samples taken at regular intervals. The results are given in Tables 32, 33 and Figures 39, 40, 41, 42.

Three different classes of water-immiscible solvent were chosen for use: carbon tetrachloride - a chlorinated compound of low polarity (E^{o} 0.18), toluene - an aromatic with slightly increased polarity (E^{o} 0.29) and ethyl acetate - an aliphatic ether of high polarity (E^{o} 0.58).

All three yielded a higher initial diosgenin transformation rate than the 100% aqueous suspension. However all the solvents also caused enzyme activity to decline rapidly over the initial 6 hour experimental period. The different proportions of organic solvent to aqueous mixture had an effect on transformation rate. The highest rates of diosgenin metabolism were generally achieved with 50% or 75% organic solvent; at 100% organic solvent the rate was much reduced.

The only products detected in organic solvent with either microorganism were diosgenone and 1-dehydrodiosgenone.

N. rhodochrous gave its highest initial activity with toluene (at the 50% level) with a diosgenin transformation rate of 25 μmol $g^{-1}h^{-1}$ at 2 hours, declining to 18 μmol $g^{-1}h^{-1}$ at 6hours. After 6 hours almost all degradative enzyme activity had ceased. The suspensions with 25%, 75% and 100% toluene transformed 1% or less diosgenin in the 6 - 24 hour period. The 50% sample transformed 1.6% in the same time, compared to 12.4% in the first 6 hours (11% in the first 4 hours).

Ethyl acetate allowed the second highest rate of diosgenin transformation by N. rhodochrous (75% organic solvent, rate at 2 hours 23.4 μmoles $g^{-1}h^{-1}$). Again a rapid decrease in enzyme activity occurred, only 1% of the diosgenin was transformed in the period 6 hours to 18 hours. The qualitative effects were very similar to those of toluene with the exception of the 25% organic solvent suspension. This actually showed a small increase over the initial activity in the 2 hours - 4 hours period (from 12.6 μmoles $g^{-1}h^{-1}$ to 14.4 μmoles $g^{-1}h^{-1}$). However after this the rate declined rapidly.

Carbon tetrachloride gave a similar pattern (maximum activity 18 μmoles $g^{-1}h^{-1}$ with 75% organic solvent) with the fairly high initial rates declining rapidly. As with ethyl acetate no decrease in activity occurred between 2 hours and 4 hours with the 25% organic solvent sample (14.4 μmoles $h^{-1}g^{-1}$, initial activity 15 μmoles $h^{-1}g^{-1}$ 2 - 4 hours).

Table 32 Diosgenin transformation in organic solvent - N. rhodochrous

	Time (hours)	TOLUENE % Diosgenin transformed	TOLUENE Rate $\mu mol\, g^{-1} h^{-1}$	ETHYL ACETATE % Diosgenin transformed	ETHYL ACETATE Rate $\mu mol\, g^{-1} h^{-1}$	CARBON TETRACHLORIDE % Diosgenin transformed	CARBON TETRACHLORIDE Rate $\mu mol\, g^{-1} h^{-1}$	100% AQUEOUS % Diosgenin transformed	100% AQUEOUS Rate $\mu mol\, g^{-1} h^{-1}$
100% organic solvent	2	2	7.2	3	14.4	1	6	1.5	6.6
	4	4	8.1	5	12	1	2.88	3.5	7.2
	6	5	6.6	5	7.2	1	1.92	5.5	7.8
	24	6	2.2	6	2.4	2.5	0.9	20	7.2
75% organic solvent	2	4	18	5	23.4	4	18	1.5	6.6
	4	7	16.2	10	21	7	16	3.5	7.2
	6	8	12	10	14.7	9	13.8	5.5	7.8
	24	9	3.2	11.5	4.2	12	42	20	7.2
50% organic solvent	2	6	25.2	3	11.4	3	13.8	1.5	6.6
	4	11	23.4	5	11.4	5.5	11.4	3.5	7.2
	6	12.4	18	10	13.8	6	9	5.5	7.8
	24	14	5.4	11	4.2	7.5	2.4	20	7.2
25% organic solvent	2	5	21	3	12.6	3	14.4	1.5	6.6
	4	8	18.6	7	14.4	7	15	3.5	7.2
	6	11	15.6	8	10.8	9	13.2	5.5	7.8
	24	11	3.6	9	3.0	10	3.6	20	7.2

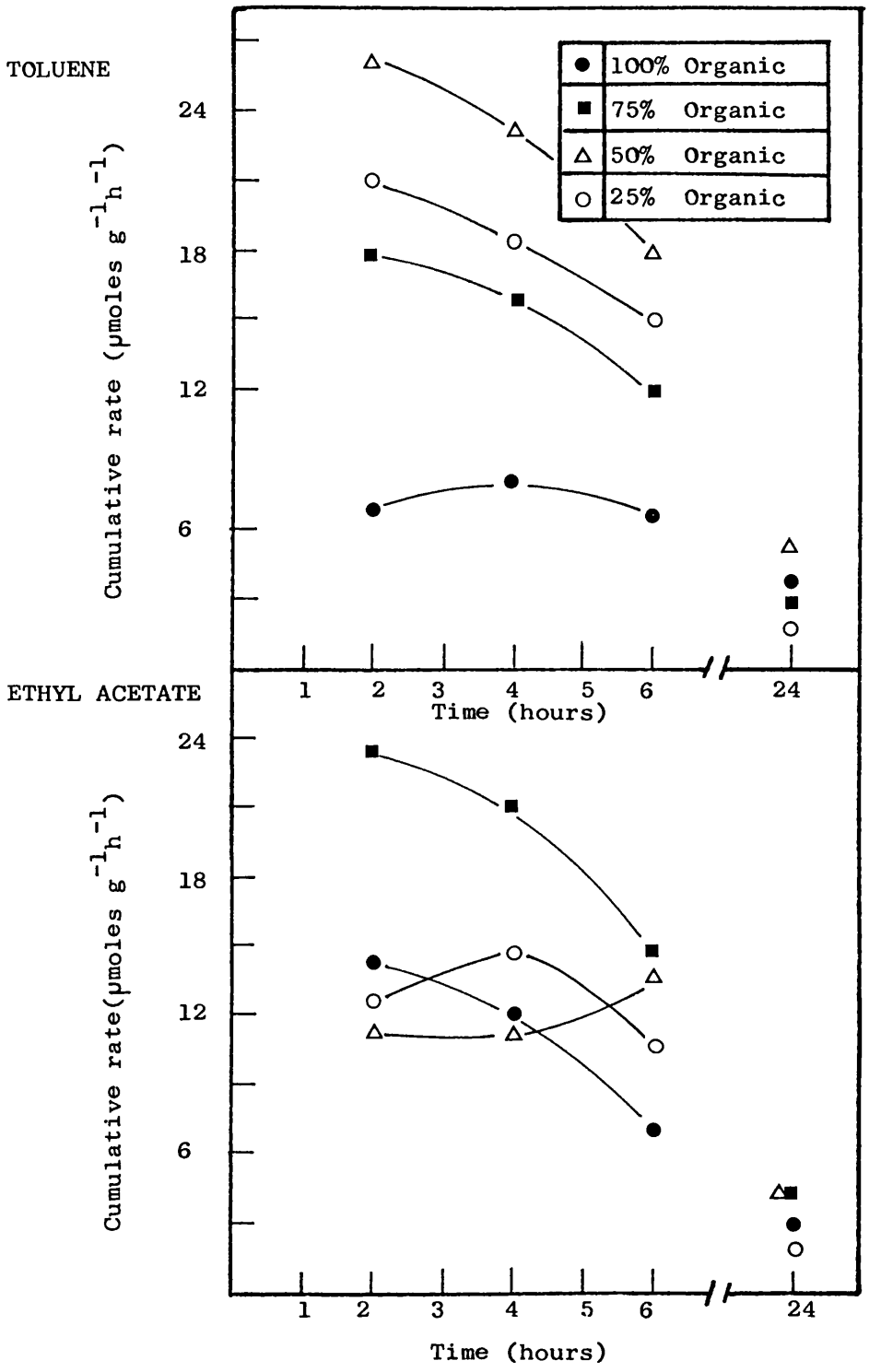


Figure 39.Transformation in organic solvent
N.rhodochrous (i)

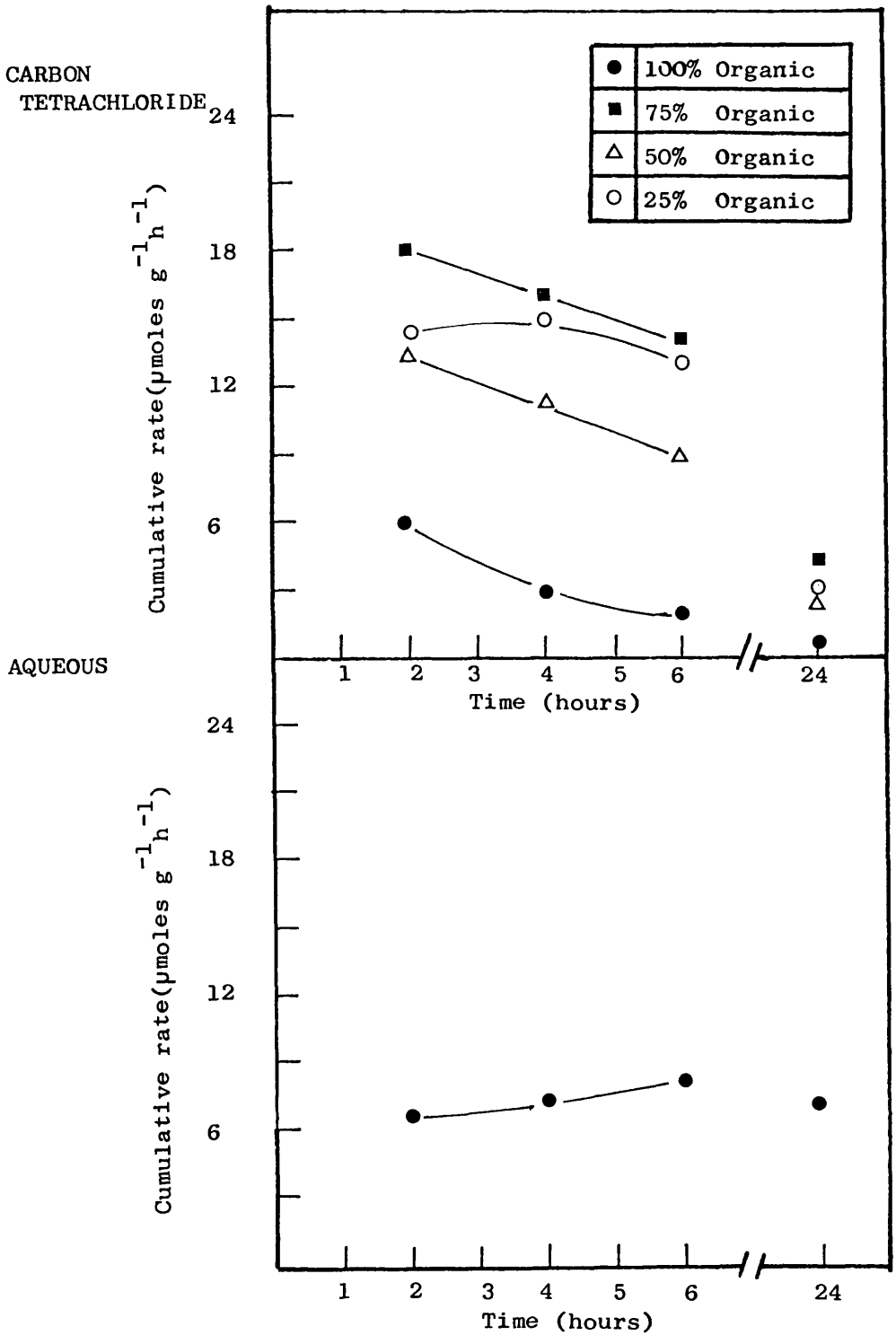


Figure 40.Transformation in organic solvent N.rhodochrous. (ii)

In purely aqueous suspension (phosphate buffered saline, pH 7) activity increased over the initial 6 hours from 6.6 μmoles $g^{-1}h^{-1}$ to 7.8 μmoles $g^{-1}h^{-1}$ and then declined only slightly over the next 18 hours to 7.2 μmoles $g^{-1}h^{-1}$. This stability resulted in the highest degree of transformation achieved over 24 hours (20%).

Organic solvents also stimulated the initial diosgenin degrading activities of M. phlei. The differences between solvents were greater and the rate of reaction generally higher than with N. rhodochrous (Table 33, Figures 41 and 42).

The least polar solvent, carbon tetrachloride, supported the highest rate of transformation. With 50% carbon tetrachloride the transformation rate was 57 μmoles $h^{-1}g^{-1}$ at 1 hour declining to 35 μmoles $g^{-1}h^{-1}$ (4 hours) at which time all diosgenin transformation ceased. Similar activity occurred with 100% carbon tetrachloride (initial rate 54 μmoles $h^{-1}g^{-1}$) with rather lower activity at 75% (32 μmoles $h^{-1}g^{-1}$) and 25% (35 μmoles $h^{-1}g^{-1}$). In all cases diosgenin transformation had ceased at 4 hours.

Toluene, which is of intermediate polarity, gave the second highest rate of diosgenin transformation. With 50% toluene the initial activity was 30 μmoles $g^{-1}h^{-1}$, considerably lower than that achieved with the same concentration of carbon tetrachloride. However with toluene the enzyme activity appeared to decline more slowly than with carbon tetrachloride. A small amount of activity was detected after 4 hours; between 0.5 - 1% diosgenin was transformed over the next 20 hours.

The most polar solvent tested, ethyl acetate, supported the least

Table 33 Diosgenin transformation in organic solvent - M. phlei

	Time (hours)	TOLUENE		ETHYL ACETATE		CARBON TETRACHLORIDE		AQUEOUS - 100%	
		% Diosgenin transformed	Rate µmol $g^{-1}h^{-1}$	% Diosgenin transformed	Rate µmol $g^{-1}h^{-1}$	% Diosgenin transformed	Rate µmol $g^{-1}h^{-1}$	% Diosgenin transformed	Rate µmol $g^{-1}h^{-1}$
	1	3	12.3	0.2	1.1	12	53.5	4.5	17.4
100%	2	5	11.5	5	10.8	17	38	10.7	20.7
organic	3	12	17.4	8	11.7	19	27	11.5	14.7
solvent	4	13.5	14.9	10.1	11.1	20	22	12	11.7
	24	14	2.7	11	1.8	20	3.6	18	3.0
	1	5.6	24.6	4.9	21	7.3	31.8	4.5	17.4
75%	2	10.3	22.8	8.6	18.6	16.2	35.4	10.7	20.7
organic	3	13.9	20.4	12.9	18.6	23	33.3	11.5	14.7
solvent	4	14.9	16.5	15.9	17.1	27.3	29.7	12	11.7
	24	15.4	2.7	15.6	2.7	27.6	5.1	18	3.0
	1	7	30.3	1.3	5.7	13	57.3	4.5	17.4
50%	2	13.4	29.1	7.1	15.6	21.4	47.1	10.7	20.7
organic	3	16.1	23.1	13.7	20.1	28	41.1	11.5	14.7
solvent	4	18.1	19.5	17.3	18.9	32.2	35.4	12	11.7
	24	18.6	3.3	16.7	3.0	32.6	6	18	3
	1	5.6	24.6	5.6	24.6	8	35.4	4.5	17.4
25%	2	10.2	22.8	10.7	23.7	14.5	32.1	10.7	20.7
organic	3	11.7	17.4	12.7	18.6	20.4	30	11.5	14.7
solvent	4	12.7	14.1	14	15.3	25.3	28.2	12	11.7
	24	13.8	2.7	14.3	2.7	25.3	4.5	18	3.0

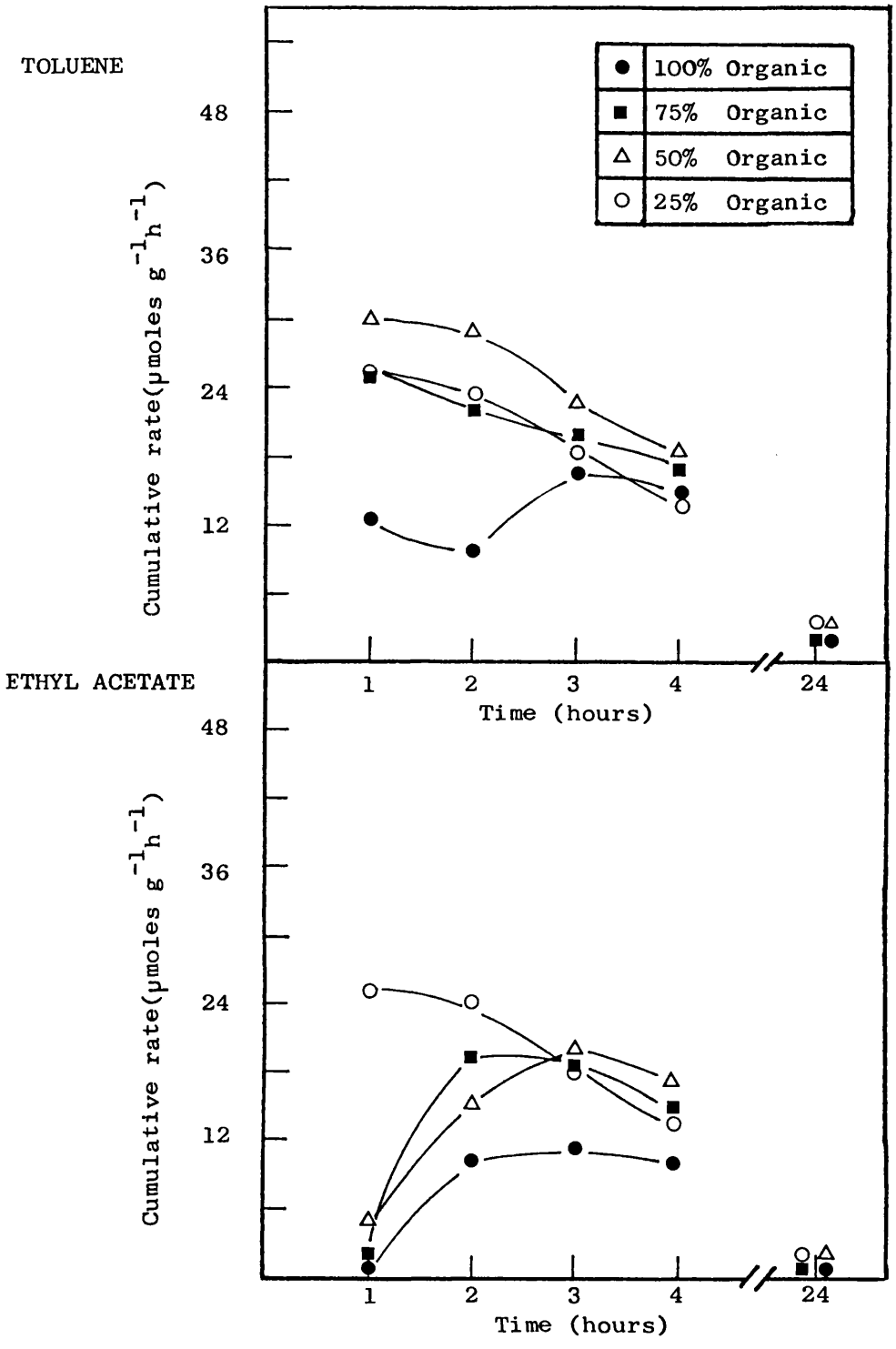


Figure 41.Transformation in organic solvent
M.phlei (i)

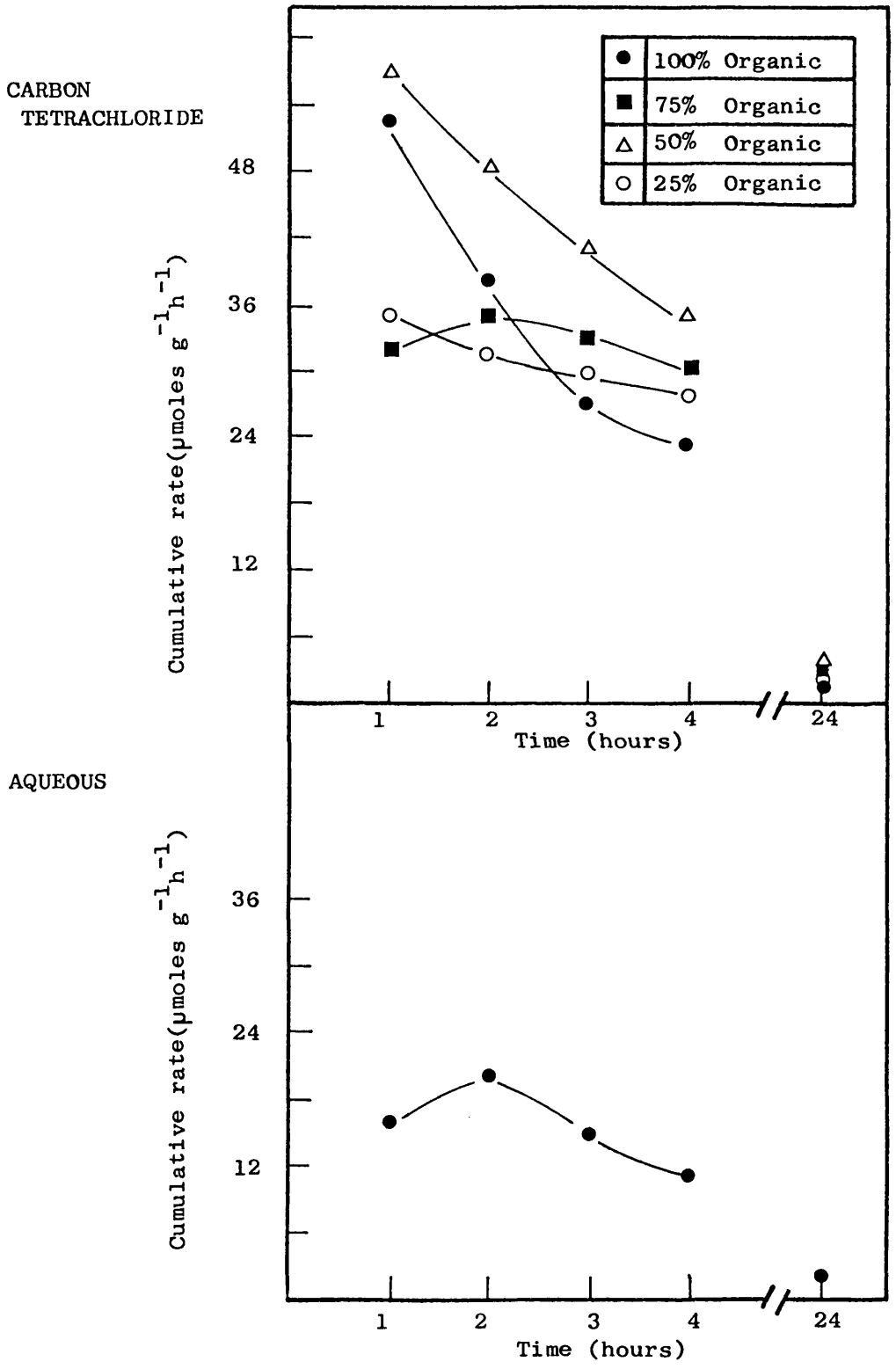


Figure 42.Transformation in organic solvent
M.phlei (ii)

enzyme activity. Indeed three out of the four solvent mixtures tested (100%, 75% and 50% ethyl acetate) gave lower transformation activities than the cells in 100% aqueous solution. This solvent also caused the least enzyme degradation, activity actually increased over the first three hours (with 50%, 75% and 100% organic solvent) from less than 5 μmoles $g^{-1}h^{-1}$ to between 10 and 20 μmoles $g^{-1}h^{-1}$. After 4 hours enzyme activity declined again and almost no steroid was metabolised between 4 hours and 24 hours.

In 100% aqueous solvent M. phlei showed an initial activity of 17 μmoles $g^{-1}h^{-1}$ increasing to 20.7 μmoles $g^{-1}h^{-1}$ at 2 hours and then declining. In the 4 - 24 hours period a further 6% of the diosgenin was transformed.

Little difference in transformation rate occurred if α,α'-bipyridyl was omitted from the experiments with organic solvents, diosgenin and 1-dehydrodiosgenone were still accumulated.

ii) Modifications to increase steroid aqueous solubility

Fenugreek seed methanol extract of crude furostanol glycosides

As previously discussed (Chapter 1) diosgenin itself is not found in fenugreek seed. Only its precursors occur naturally, mainly as furostanol glycosides but probably also with small amounts of furostanol peptide ester.

Due to the presence of sugar units at positions C-3 and C-26 these furostanols are much more water-soluble than diosgenin. Therefore the use of furostanols can be considered within the scope of this chapter.

An effective microbiological transformation to diosgenin (by removal of the sugars) could provide a more energy efficient process than the chemical method at present employed.

Furostanol glycosides are generally obtained from fenugreek seed by methanol extraction of the defatted seed. Such an extract will contain the precursors of diosgenin, yamogenin, tigogenin, neotigogenin, gitogenin, neogitogenin and sterols (mainly sitosterol but also campesterol with traces of stigmasterol and cholesterol). Also present will be many other non steroidal seed components.

Extraction

One kilogram of commercial Moroccan fenugreek seed was powdered and soxhlet extracted with petroleum spirit of boiling point 40° - $60^{\circ}C$ for 18 hours to remove most of the fixed oils present. The residue was then further extracted in a soxhlet with 6 litres of methanol for 48 hours. The filtered extract was concentrated by rotary evaporation ($60^{\circ}C$) to yield 160 g of a brown viscous gum.

Time of hydrolysis and yield of diosgenin

To assay the potential diosgenin content in the methanol extract it is necessary to cleave the glycosidic bonds by acid hydrolysis. If the hydrolysis is continued too long some of the diosgenin formed is converted to its 1,4-diene. Therefore to ensure the accuracy of experimental results an assay of the methanol extract was carried out. The extract (2.3 g) was refluxed with 2N hydrochloric acid for 20, 40, 60, 90 and 120 minutes, cooled, filtered and the acid insoluble material collected after being made alkaline with 10% ammonia. This

material was dried and the diosgenin in it collected by soxhlet extraction. The results are given in Table 34.

The optimum time of hydrolysis to yield maximum quantities of diosgenin was between 40 and 60 minutes. A time of 50 minutes was used in all future work.

Table 34. The use of fenugreek seed methanol extract: time of hydrolysis and yield of diosgenin

Time of hydrolysis (refluxing in 2N acid) (minutes)	mg diosgenin after extraction	Sterol (arbitrary units)
20	69.2	0.193
40	70.1	0.234
60	69.4	0.23
90	66.9	0.23
120	65.5	0.25

Maximum yield of diosgenin from methanol extract = 3%.

The transformation of fenugreek seed methanol extract by several microorganisms

Three grams of methanol extract (previously sterilised by autoclaving) was added to each of the following cultures:-

Fusarium solani, Medium 2 + 10 mM acetate, 96 hours incubation; *Verticillium theobromae*, *Stachylidium bicolor*, Medium 3, 96 hours incubation; *Helicostylum piriforme*, *Mycobacterium phlei*, *Mycobacterium*

fortuitum, Nocardia rhodochrous Medium 5, 96 hours incubation. After a further 7 days incubation the cultures were divided into two fractions. One was extracted directly to determine the amount of free steroid. The other fraction was acid-hydrolysed prior to extraction to determine the total diosgenin present. The results are given in Table 35 and Figure 43.

The methanol extract did not inhibit the growth of the 7 microorganisms tested. The three organisms most active against diosgenin, N. rhodochrous, M. phlei and M. fortuitum, were virtually inactive in transforming the diosgenin precursors present in the extract.

A number of other organisms screened were active. Helicostylum piriforme had little effect on the diosgenin precursors or sterol content of the extract but it did yield several products after hydrolysis of the extract (Figure 43). These unidentified products must arise from components in the extract other than the diosgenin precursors.

Stachylidium bicolor was capable of hydrolysing the furostanol glycosidic bonds and produced 37% of the potential diosgenin content in the non-hydrolysed extract. It did not metabolise the diosgenin produced any further as, after acid hydrolysis, all the potential diosgenin was present. This organism was also very active against the sterols present in the extract, only 27% of the control value remained at the time of extraction.

Verticillium theobromae was active in forming diosgenin, 23% of the potential content was present in the non-hydrolysed cultures. Alone

Table 35 The transformation of fenugreek seed methanol extract by several microorganisms

i) Without acid hydrolysis

Organism	Diosgenin % of hydrolysed control	Diosgenin mg ml^{-1}	Sterol % of hydrolysed control
Control (no organism)	-	0	0
Helicostylum piriforme	-	0	0
Verticillium theobromae	23	0.21	20
Stachylidium bicolor	37	0.34	0
Fusarium solani	84	0.77	0
Mycobacterium phlei	-	0	0
Mycobacterium fortuitum	-	0	0
Nocardia rhodochrous	-	0	0

ii) With acid hydrolysis

Control (no organism)	100	0.92	100
Helicostylum piriforme	104	0.96	98
Verticillium theobromae	64	0.59	18
Stachylidium bicolor	105	0.97	27
Fusarium solani	111	1.0	31
Mycobacterium phlei	95	0.87	96
Mycobacterium fortuitum	87	0.80	92
Nocardia rhodochrous	101	0.80	96

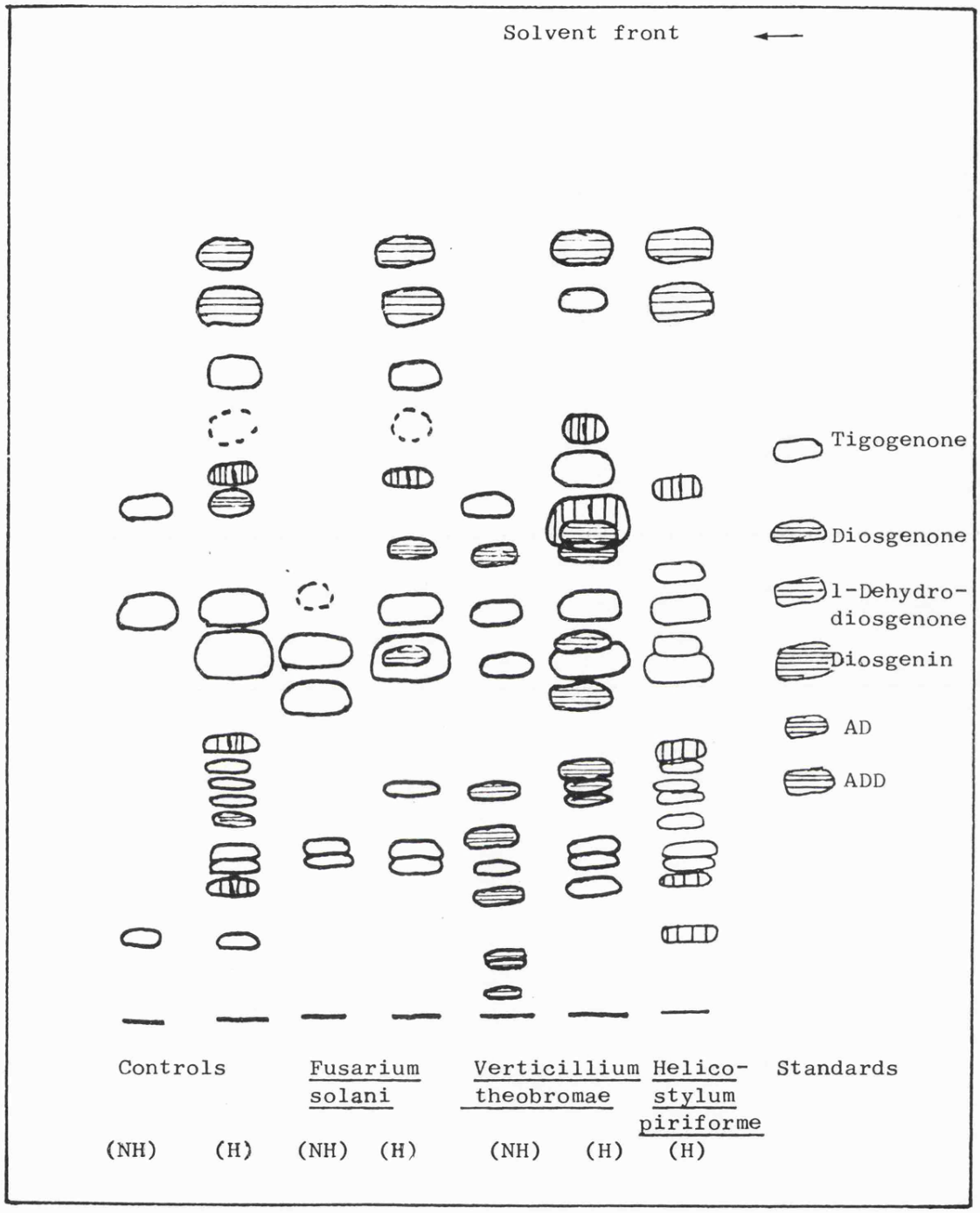


Figure 43. The transformation of fenugreek seed methanol extract by several microorganisms. Thin layer chromatograph of both hydrolysed and non hydrolysed samples.

amongst the microorganisms screened it also released sterol into the non-hydrolysed culture (20% of the hydrolysed control value had accumulated at the time of extraction). The organism can further metabolise diosgenin, at the time of extraction 36% of the potential diosgenin had been transformed. The sterol content had also been greatly reduced (to approximately 20% of the hydrolysed control value.) A number of products were seen on the t.l.c. plates. A minor product was identified as androsta-1,4-diene-3,17-dione on the basis of r.f. values and colour reaction from t.l.c. analysis. The major products did not correspond to the previously detected products from diosgenin, nor to any of the pregnane or androstane standards available.

Fusarium solani was the most active microorganism tested for producing free diosgenin. Over 80% of the control diosgenin value was present in the medium at the end of the fermentation period. It does not significantly metabolise diosgenin as all the diosgenin theoretically present at the start could be recovered after acid hydrolysis of the culture. The organism did degrade sterol, only 31% remained at the end of the fermentation.

The effect of solubilising agents on diosgenin

The following solutions were each prepared in 250 ml Erlenmeyer flasks (100 ml volume).

5% (w/v) Polyvinyl pyrrolidone

20% (w/v) Polyvinyl pyrrolidone

5% (w/v) Polyvinyl alcohol

1% (w/v) Hydroxypropylmethyl cellulose

5% Ethanol

Diosgenin (50 mg) was added to each, the mixtures heated (60°C) and stirred for an hour at 60°C (except the 5% ethanol which was kept at room temperature) before being centrifuged (20,000 g, 30 minutes) and filtered (0.45 μ pore size). The resulting solutions were extracted with ethyl acetate and assayed for dissolved diosgenin by g.l.c. The results are given in Table 36.

No significant increase in diosgenin solubility was achieved compared to the control sample (5% ethanol). Indeed diosgenin solubility remained below the level of detection (2.05 mg L^{-1}) in all cases. In view of this no fermentation experiments were attempted.

Table 36

The effect of solubilising agents on diosgenin

Solution	Diosgenin solubility
5% Polyvinyl pyrrolidone	less than 2.05 mg L^{-1}
20% Polyvinyl pyrrolidone	" " 2.05 mg L^{-1}
5% Polyvinyl alcohol	" " 2.05 mg L^{-1}
1% Hydroxypropylmethyl cellulose	" " 2.05 mg L^{-1}
5 % Ethanol	" " 2.05 mg L^{-1}

Discussion

i) The use of organic solvents to increase the rate of steroid biotransformation

All of the organic solvents tested increased the initial rate of diosgenin degradation by N. rhodochrous (Table 32). The differences between the solvents were small. This suggests that the one essential factor for the increased initial enzyme activity was dissolution of diosgenin, with the nature of the solvent being relatively unimportant.

Buckland et al. (1975) found that N. rhodochrous required at least intracellular water to be active in organic solvents. The results from this experiment (Table 32) show that the presence of more water can further stimulate the rate of reaction. Too much water (e.g. 75% water, 25% organic solvent) reduced the rate. At this level the organic fraction was saturated with diosgenin and some diosgenin remained undissolved. Almost all the transformation of diosgenin observed in the presence of organic solvent occurred over the first six hours. After this time enzyme activity was lost, presumably through denaturation.

The effects of immobilisation on N. rhodochrous in the presence of non-aqueous solvents were investigated by both Duarte and Lilly (1980) and Yamane et al. (1979). Although they used different immobilisation materials (calcium alginate, polyacrylamide and polyethylene glycol derivatives) the trend of their results was the same. Immobilisation led to a decline in transformation capability. With calcium alginate immobilised N. rhodochrous cells the decrease in cholesterol oxidase activity was 10 fold (Duarte and Lilly, 1980). Once immobilised the cells did remain more stable for batch re-use. In view of this decrease in activity it was decided not to use immobilised cells in the presence of organic solvent.

The 100% aqueous samples showed higher activity than previously recorded in the present work for N. rhodochrous as resting cells (Table 17). The use of induced cells in the present experiment and a much shorter period before an initial activity reading (4 hours instead of 72 hours) explain this apparent discrepancy.

The maximum rate of diosgenin degradation achieved by N. rhodochrous cells in organic solvent (25 μmoles $g^{-1}h^{-1}$) was still lower than the best value from cells in purely aqueous media (35 μmoles $g^{-1}h^{-1}$, Table 14).

M. phlei showed much greater transformation rates in organic solvent than N. rhodochrous. Possibly even more surprising was the high activity (maximum 20.7 μmoles $g^{-1}h^{-1}$) of M. phlei cells in purely aqueous suspension, higher than the activity of N. rhodochrous (maximum 7.8 μmoles $g^{-1}h^{-1}$). The reverse would be expected from Table 14 where N. rhodochrous cells were several times more active than M. phlei. Three factors contribute to this high activity with M. phlei; the very rapid mixing by the orbital incubator, the presence of Triton-X-100 (15 μL, 10% solution) and the short time interval over which measurements were taken. In the present experiment a considerable reduction in activity was seen over 24 hours; the previously quoted transformation value for M. phlei resting cells (0.81 μmoles $g^{-1}h^{-1}$, Table 17) was averaged over 144 hours. The results from the use of solvents with different properties correspond with the findings of Fukui et al. (see introduction to this chapter) in that the highest initial rate of activity and the most rapid enzyme denaturation occurred in the least polar solvent.

The most striking feature of the Mycobacteria is their extraordinarily high lipid content, which amounts to between 20% and 40% of the dry cell weight. In particular 60% of the lipid content is found in the cell wall. This accounts for the hydrophobic clumping characteristics of the organism, its resistance to many external factors and the slowness of growth (McCarty, 1973). The interaction of this unusual cell wall with a water immiscible solvent could result in a greatly increased rate of transport of diosgenin into the cell.

This might explain the high transformation rates observed with toluene, carbon tetrachloride and ethyl acetate. Although the presence of extracellular enzyme has been observed (Chapter 5), it is not known what proportion this represents of the total enzyme present.

Whilst the rateof diosgenin transformation by M. phlei was considerably stimulated by the presence of water immiscible organic solvents the only products accumulated were diosgenone and 1-dehydrodiosgenone. These accounted for over 90% of the diosgenin transformed. In none of the cultures (including 100% aqueous) were androstanes detected. It seems probable that the cholesterol oxidase and 1-dehydrogenase enzymes are more resistant to denaturation than the other enzymes in the degradation pathway.

The lack of other products in the aqueous sample could be due to the unusual physical conditions, including increased aeration because of the vigorous mixing. There was evidence that further degradation including steroid nucleus breakdown, was taking place under these conditions because lower yields of diosgenone and 1-dehydrodiosgenone were obtained.

The general conclusion from this experiment was that whilst water immiscible organic solvents gave an increase in the initial reaction rate these solvents would be unable to give an increase in overall product yield.

ii) Modifications to increase steroid aqueous solubility - Fenugreek seed methanol extract of crude furostanol glycosides.

The results (Table 35, Figure 43) indicated that the microorganisms found to be most active against diosgenin, the bacteria, were ineffective against the furostanol precursors of diosgenin. In contrast most of the fungi screened showed good activity.

One of the most active fungi (Verticillium theobromae) has previously been reported to form 16-ketopregnanes from diosgenone, although being inactive against diosgenin (Kondo and Mitsugi, 1973). The products formed in this experiment did not correspond to those of Kondo and Mitsugi. Because of the complex mixture of substances present in the extract purification would prove difficult and any products isolated would not necessarily be derived from the diosgenin precursors. The androstane formed by Verticillium theobromae may have arisen from sterols present in the methanol extract, although the seed was powdered and defatted before methanol extraction.

One of the most interesting results was the highly efficient formation of diosgenin by F. solani (84% efficiency). This compares well with the work of Rothrock et al. (1957b) who only achieved higher yields of diosgenin (from yams) by the use of unidentified mixed cultures. Furthermore they found that there was a danger of complete

degradation of diosgenin in such circumstances. Although previous work in this thesis (Table 14) has shown that F. solani is capable of transforming diosgenin, it has also shown that the reaction was greatly inhibited in rich media. In the controlled conditions of the present experiment the methanol extract in the growth medium provided sufficient nutrients to inhibit the diosgenin degradation. This had led to a highly efficient microbial process for the formation of diosgenin which could compare favourably with the chemical methods of hydrolysis at present used.

The increased diosgenin yield obtained after acid hydrolysis (111% of control) may be caused by an analogous mechanism to that occurring when powdered fenugreek seed is incubated with certain enzyme preparations, which also results in increased yields (Elujoba, 1980).

The presentation of diosgenin in the form of a crude extract of furostanol glycosides caused considerable differences in its transformation. Its high water solubility is an advantage not yet exploited. Higher concentrations could be used in the medium. If the transforming enzymes are substrate limited this would lead to an increased rate of product formation.

iii) The effect of solubilising agents on diosgenin

As the solubilising agents (polyvinyl pyrrolidone, polyvinyl alcohol and hydroxypropylmethyl cellulose) caused no significant increase in diosgenin solubility this line of investigation was not pursued further.

CHAPTER 8

The use of silastic resin as a steroid reservoir

Introduction

Silastic resin reservoirs

Bhasin et al. (1976) used the novel method of substrate immobilisation in an attempt to improve the method of steroid addition to fermentations.

Addition of steroids in an immobilised form which maintained a high substrate concentration in the aqueous phase could offer advantages over other methods such as organic solvents, with their concomitant effects of enzyme denaturation. A further potential advantage of the method would be the ease of separation of hydrophobic products and steroidsubstrate at the end of the fermentation. For the method to be successful the immobilising material must be hydrophobic and the steroid solute highly soluble within it. The kinetic mobility of the polymer chains should permit rapid diffusion of solute molecules. The polymer must also be non-toxic to the enzyme systems and reasonably stable.

The polymer selected by Bhasin and co-workers as most likely to fulfill the above requirements was based on polydimethyl siloxane (PDMS). The group found that the stability of 3(17)β-hydroxysteroid dehydrogenase from Pseudomonas testosteroni was similar in systems containing PDMS to purely aqueous controls, and in both cases higher than those containing butyl or ethyl acetate. Similarly over a 50 hour period the enzyme/PDMS system yielded more product than the enzyme/ organic solvent system.

Although this method apparently has good potential it has not been widely used because of the poor reservoir capacity of the PDMS resin for steroids. Also, unless low enzyme concentrations are employed,

the rate of transfer between water and the polymer is usually low enough to become rate limiting.

Specific Materials and Methods

i) Production of silastic resin cubes

RVT 3110 resin (67.5 g, Dow Corning Ltd.) was thoroughly mixed with 7.5 g Type S catalyst (Dow Corning Ltd.) until homogeneous. The mixture was then layered onto a flat stainless steel plate to a depth of 2.5 mm and left for 60 hours at room temperature to cure. The polymerised sheet was cut into cubes (approximately 2.5 mm square) with a scalpel and dried under vacuum for 3 days (30°C). The cubes were extracted with ethyl acetate (soxhlet, 72 hours) and then dried at room temperature for 18 hours before use.

ii) Silastic resin as a substrate reservoir

This method is essentially that of Bhasin *et al.* (1976) but using diosgenin instead of testosterone (17β-hydroxyandrost-4-en-3-one). Silastic resin cubes (50 g) were added to ethyl acetate (500 ml) saturated with diosgenin. They were left for 6 hours, filtered from the ethyl acetate and dried at room temperature for 24 hours before use.

Results

Density measurement of cubes

Ten grams of the resin cubes were placed in a 25 ml volumetric flask and the volume of water they displaced was accurately measured. From this their density was calculated to be 1.114 kg/dm^3 (0.114 kg/m^3).

Diosgenin accumulation by silastic resin cubes and its extraction from them.

Diosgenin (50 mg) dissolved in 2.5 ml ethanol was added to 50 ml of Medium 1 containing 10 g of silastic resin cubes (prepared as described in Specific Materials and Methods, i). After shaking for 3 hours (150 r.p.m.) the cubes were removed, washed with water, dried at 50°C and extracted with ethyl acetate using either soxhlet (24 hours) or separating funnel methods. The extracts were assayed for diosgenin content, the results are given in Table 37(a).

The experiment confirmed that the silastic resin cubes were capable of sequestering diosgenin from an aqueous suspension. It also showed that soxhlet extraction was more efficient than the standard separating funnel method, giving a 21% higher yield of diosgenin. The soxhlet extraction method was used in all further experiments.

The effect of the hydrophobicity of steroids on their accumulation by silastic resin

Ten grams of silastic resin cubes were placed in a 250 ml baffled flask containing 100 ml distilled water. Cholesterol (0.125mmoles) and 3β-hydroxy-5α-androstan-17-one (0.125mmoles), both dissolved in a total of 4 ml ethanol, were added and the mixture was shaken for 6 hours (28°C). The cubes were removed, washed three times with water, dried, and extracted with ethyl acetate. The extract was assayed for both cholesterol and androstane content by g.l.c. The g.l.c. conditions and calibration results are given in Table 38. The experimental results are given in Table 37(b). The results showed that cholesterol was preferentially accumulated by the resin compared to 3β-hydroxy-5α-androstan-17-one by a ratio of 3 to 1. The total amounts adsorbed were determined by

Table 37. General properties of silastic resin cubes

a) Diosgenin accumulation by silastic resin cubes and its extraction from them.

Method of extraction	μmoles diosgenin g^{-1} resin
Separating funnel	1.4
Soxhlet (24 hours)	1.7

b) The effect of the hydrophobicity of steroids on their accumulation by silastic resin

Steroid mixture (equimolar)	Steroid accumulated (μmoles)g^{-1} resin	Steroid accumulation ratio
Cholesterol	2.78	2.99
3β-hydroxy-5α-androstan-17-one	0.93	1

Table 38 Calibration of OV-17 column for cholesterol and 3β-hydroxy-5α-androstan-17-one

G.l.c. conditions as previously defined, column 1 m OV-17. Samples were silylated prior to injection. Cholesterol and androstane stock solutions were 5 mg ml^{-1}

Sample	μL cholesterol	μL androstane	Mean peak height ratio (cholesterol/androstane)
1	250	150	0.5665
2	200	150	0.449
3	100	150	0.214
4	50	150	0.096
5	25	150	0.048

Slope 0.3485

Intercept - 0.0454

Correlation coefficient 0.9998

taking the mean of 5 injections of the sample and 5 injections of a suitable standard.

The use of silastic resin as a substrate reservoir

Silastic resin cubes containing diosgenin were prepared as described in the Specific Materials and Methods (section ii) of this chapter. The cubes were added in 10 g batches to stationary phase cultures of M. phlei and N. rhodochrous, (both in Medium 5), incubated for a further 96 hours and 72 hours respectively, and the culture contents analysed. The aqueous fraction was extracted separately from the silastic resin cubes. Control cultures had diosgenin added as an ethanolic solution but did not contain any silastic resin. The results are given in Table 39.

The addition of diosgenin by impregnated silastic resin cubes had a very marked effect on its rate of transformation. N. rhodochrous transformed all the diosgenin present in cultures lacking silastic resin (control flasks). However no transformational activity was detected in the cultures where diosgenin was added in the silastic resin. This was despite the detection of a considerable amount of diosgenin (87 - 123 μmoles) "free" in the aqueous fraction at the end of the experiment.

Similar results were observed with M. phlei; little or no transformation took place in the presence of silastic resin. In all cases where diosgenin was added by the silastic resin method considerably more diosgenin could be detected in the aqueous fraction than in the resin on completion of the experiment.

Table 39. The use of silastic resin as a substrate reservoir

Organism and culture conditions	Resin content (µmoles) Diosgenin	D	DHD	Aqueous content (µmoles) Diosgenin	D	DHD
M. phlei, growing cells + 10g resin containing diosgenin 96 hours fermentation	35	trace	0	131	0	0
M. phlei, resting cells (PBS) + 10g resin containing diosgenin 96 hours fermentation	9.5	0	0	138	0	0
M. phlei, growing cells + ethanolic diosgenin solution 96 hours fermentation	-	-	-	9.9	0	0
10g Resin containing diosgenin only, 24 hours in sterile medium	58	0	0	87	0	0
N. rhodochrous, growing cells, + 10g resin containing diosgenin 72 hours fermentation	14.5	trace	0	123	0	0
N. rhodochrous, growing cells + ethanolic diosgenin solution 72 hours fermentation	-	-	-	0	81	15

Notes: D - diosgenone

DHD- 1-dehydrodiosgenone

The use of silastic resin for product collection from N. rhodochrous fermentations.

Steroid free silastic resin cubes and diosgenin (120 μmoles in 2.5 ml ethanol) were added to 48 hour N. rhodochrous cultures (Medium 5). After a further 48 hours incubation the cubes were separated from the medium and both were analysed for steroid content. The results are given in Table 40.

The addition of diosgenin dissolved in ethanol, separately from the silastic resin, resulted in a much higher rate of transformation (Table 40) than when the diosgenin was added entrapped in the resin (Table 39). This improved rate was still slightly lower than the transformation rate achieved in the complete absence of resin.

The silastic resin preferentially accumulated the products of diosgenin transformation, especially at the highest concentrations of resin used. Between 55% (10g resin present) and 100% (20 g resin present) of the diosgenone and 1-dehydrodiosgenone formed were found in the resin although some diosgenin still remained in the aqueous fraction. As the weight of silastic resin per culture was increased so did the total amount of steroid that it accumulated. When the resin content was doubled from 5 g to 10 g the amount of steroid accumulated by the resin also doubled. The increase was slightly less than a further 100% when the amount of resin present was increased from 10 g to 20 g.

The amount of diosgenin which was transformed decreased slightly when more resin was present (with 5 g resin 7.1 μmoles diosgenin remained unchanged; with 10 g resin 12.9 μmoles diosgenin remained). However

Table 40. The use of silastic resin for product collection from N. rhodochrous fermentations

Culture conditions[1]	Resin content µmoles			Aqueous content µmoles			Total, µmoles		
	Diosgenin	D	DHD	Diosgenin	D	DHD	Diosgenin	D	DHD
5 g resin, 48 hours fermentation	5	17	13.4	2.1	61	12	7.1	78	25.4
10 g resin, 48 hours fermentation	10.6	35.2	26.8	2.3	42.5	7.3	12.9	77.7	34.1
20 g resin, 45 hours fermentation [2]	20	32.3	39	28	0	0	48	32	39
No resin, 48 hours fermentation	-	-	-	8.3	89.9	13.4	8.3	89.9	13.4

Notes: D diosgenone DHD 1-dehydrodiosgenone

1. All cultures contained 120 µmoles diosgenin added in 2.5 ml ethanol

2. Culture from separate batch.

the effect was only pronounced in the culture with 20 g resin, when 48 μmoles diosgenin was recovered. As this culture originated from a different batch of cells, treated under slightly different conditions the result is not strictly comparable with the others in this experiment.

The presence of silastic resin had an effect on the pattern of metabolites produced during the transformation process. As the amount of resin per culture was increased so did the ratio of 1-dehydrodiosgenone to diosgenone. With 5 g resin the ratio was 1(1-dehydrodiosgenone) to 3 (diosgenone); with 10 g resin 1:2; with 20 g resin 1:1 and in the absence of resin 1:7.

The effect of silastic resin during a fermentation of diosgenin by N. rhodochrous

To continue the study on the influence of silastic resin on N. rhodochrous fermentations a time-course experiment was carried out. The transformation process was studied over a 45 hour period in the presence and absence of silastic resin. Diosgenin (130 μmoles) was added (in ethanol) to eight 48 hour N. rhodochrous cultures (Medium 5) to give a final concentration of 2.4 μmoles ml^{-1}. Steroid free silastic resin cubes (20 g) were added to four of the cultures. Samples, 10 ml from the aqueous fraction and 5 g(6 g wet weight) from the cubes, were taken from pairs of flasks alternately over the 45 hour period. The results are given in Figure 44.

Differences could be seen in the pattern of transformation between the cultures with and without silastic resin present. Principally the rate of transformation was lower in the cultures containing silastic resin. This was particularly noticeable during the later stages of the

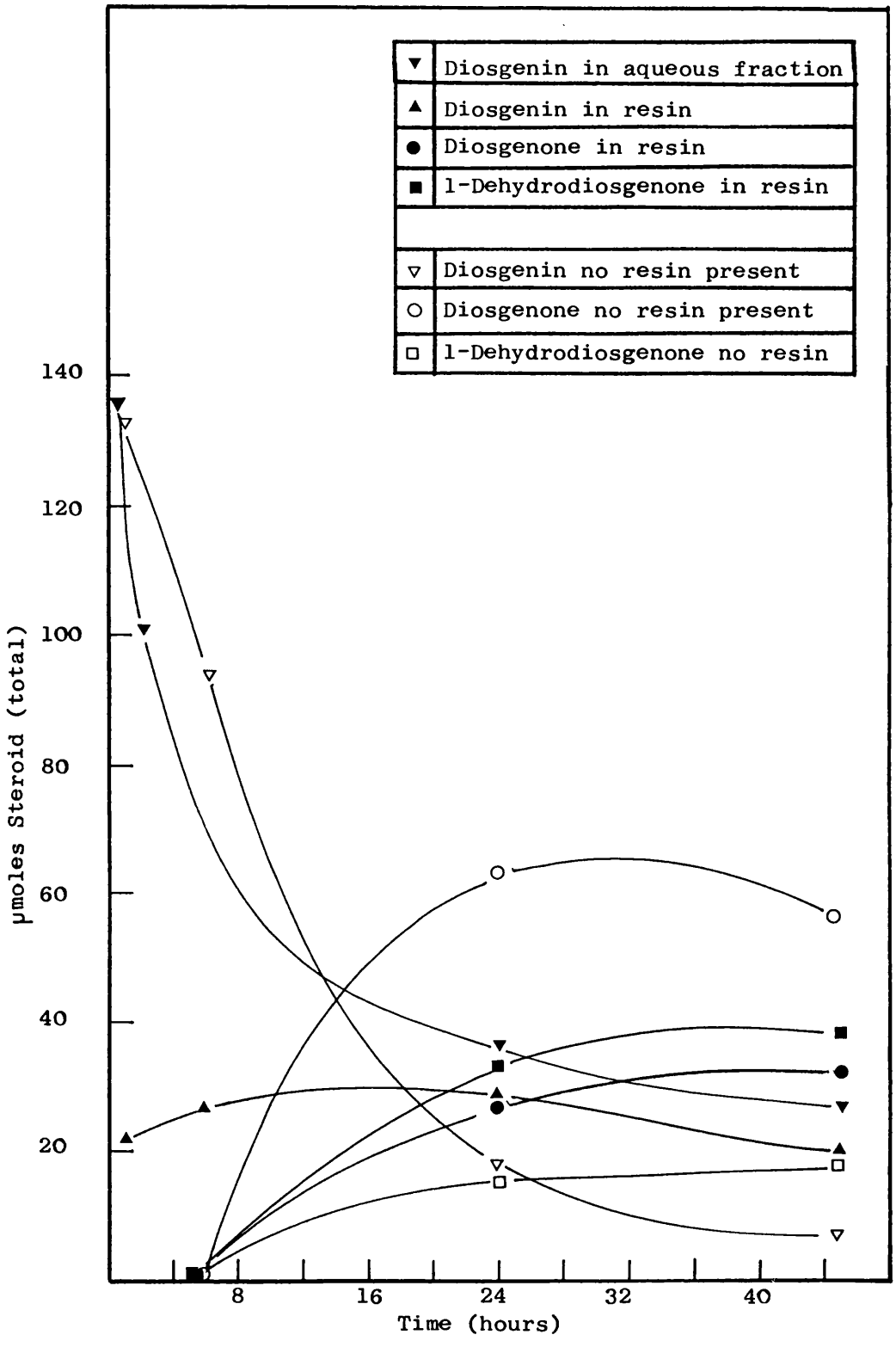


Figure 44. The effect of silastic resin during a fermentation of diosgenin by N.rhodochrous.

fermentation. At 24 hours less than 20 µmoles diosgenin was present in cultures lacking resin, compared to 65 µmoles diosgenin (total) and 35 µmoles diosgenin (in the aqueous fraction) when resin was present. The corresponding values at 45 hours were 8 µmoles (no resin), 48 µmoles (total) and 28 µmoles (in aqueous fraction of resin cultures). The diosgenin content of the silastic resin remained almost constant for most of the experiment, declining slightly at the 45 hour sample. In the same cultures the diosgenin content of the aqueous fraction decreased steadily from the start.

The other major difference between the cultures was in the pattern of metabolites produced. In the presence of resin considerably more 1-dehydrodiosgenone and less diosgenone were produced; all product was contained in the resin with none found in the aqueous fraction. In both fermentations the total amount of product accumulated was similar.

At the end of the experiment over 80% of the total steroid added could be accounted for in cultures containing silastic resin, compared to 62% in the absence of resin.

The use of silastic resin as a transportable product reservoir

An important potential use for silastic resin would be as a method of transferring the product of one microbiological transformation reaction to a second culture, without the need for product extraction and isolation steps.

i) Initial substrate methanol extract

Three grams of methanol extract from fenugreek seed and 20 g silastic

resin cubes were added to 4 day F. solani cultures (Medium 2 + 10 mM acetate). The cultures were incubated for 7 days. A 5 g sample of resin was then removed for sampling and the remainder of the resin transferred to 7 day (Medium 5) M. phlei cultures. α,α'-Bipyridyl (0.45 μM) was also added to the M. phlei cultures at the same time. These were incubated a further 7 days before extraction. The results are given in Table 41.

F. solani was able to form diosgenin from fenugreek seed methanol extract in the presence of silastic resin. In fact the presence of resin appears to have very little effect on the efficiency of formation of diosgenin. A yield of 77% was achieved compared to the previous value of 84% in the absence of resin (Chapter 7, Table 35). Some of the diosgenin formed (21%) was accumulated by the silastic resin which, on transference to a culture of M. phlei, resulted in the formation of diosgenone, 1-dehydrodiosgenone, androsta-1,4-diene-3,17-dione and androst-4-ene-3,17-dione. All these products were found in the silastic resin at the time of extraction.

The other substance accumulated by the silastic resin was tentatively identified as an oil on the basis of t.l.c. studies. The presence of the oil decreased the resin's carrying capacity for diosgenin by as much as 50% (2.06 μmoles g^{-1}) compared to a maximum steroid capacity of 4.66 μmoles g^{-1}, Table 42(3)).

ii) Initial substrate diosgenin

A 48 hour N. rhodochrous culture (Medium 5) was used for the initial transformation of diosgenin (120 μmoles). Twenty grams of steroid free

Table 41. The use of silastic resin as a transportable product reservoir

i) initial substrate methanol extract

Sample	Diosgenin µmoles and (µmoles g^{-1} resin)	D (µmoles) and (µmoles g^{-1} resin)	DHD (µmoles) and (µmoles g^{-1} resin)	Sterol	Other Compounds
1) Resin after F. solani fermentation (7 days, methanol extract)	33.5 (1.6)	0	0	0	Oil
2) Aqueous fraction from (1) non-hydrolysed	128.3	0	0	0	Oil
3) Aqueous fraction from (1) acid hydrolysed	171.4	0	0	21	Many
4) Resin, from (1) further incubated, M. phlei, 7 days	3.6 (.18)	21.7 (1.09)	15.9 (0.8)	0	AD, ADD, (trace only) Oil
5) Aqueous fraction from (4)	0.7	0	0	0	0
6) Methanol extract-no organism control, acid hydrolysed	209	0	0	20.5	Many

Notes D - diosgenone

DHD - 1-dehydrodiosgenone

silastic resin cubes were added with the diosgenin (in ethanol) and the cubes transferred 48 hours later to 4 day F. solani cultures (Medium 2 + 10 mM acetate). After a further 6 days the cubes were removed and their steroid content analysed.

For comparative purposes a fermentation was carried out in mixed culture without silastic resin: after 48 hours fermentation of diosgenin by N. rhodochrous (50 ml) a 4 day F. solani culture (50 ml) was added and the combined culture incubated a further 6 days before extraction. All the results are listed in Table 42.

N. rhodochrous produced diosgenone (47 µmoles) and 1-dehydrodiosgenone (42.5 µmoles) to give a total yield of 90 µmoles of which 79% was contained in the silastic resin. This resin was then transferred to a F. solani fermentation where its steroid content remained unchanged after a 6 day incubation period. No other products were detected and little steroid (about 8%) was released from the resin into the aqueous phase .

In a resin free mixed culture experiment carried out at the same time a large amount of diosgenone (105 µmoles) and a smaller quantity of 1-dehydrodiosgenone (12 µmoles) were formed. No other products were detected.

Discussion

Manufacture of silastic resin

Bhasin et al. (1976) dispersed the mixed resin and catalyst in a solution of glycerine and Tween 80 using a magnetic stirrer. This

Table 42. The use of silastic resin as a transportable product reservoir

ii) initial substrate diosgenin

Sample	Diosgenin remaining µmoles and (µmoles g^{-1} resin)	D (µmoles) and (µmoles g^{-1} resin)	DHD (µmoles) and (µmoles g^{-1} resin)	Other products (µmoles)
1) Resin after 48 h with *N. rhodochrous*	17.4 (0.87)	37.4 (1.87)	33.7 (1.7)	None
2) Aqueous fraction from (1)	21.5	9.9	8.8	None
3) Resin, from (1) after a further 6 days, with *F.solani*	17.3 (0.86)	43.7 (2.2)	31.2 (1.6)	None
4) Aqueous fraction from (3)	0.97	2.9	3.2	None
5) Mixed culture, no resin added, *N. rhodochrous*, 48 hours then *F. solani* added for a further 6 days	0	105.1	12.2	None

Notes: D - diosgenone

DHD - 1-dehydrodiosgenone

resulted in the formation of a non-homogeneous mixture of silastic resin in the form of spheres. These were sorted and collected by sieving.

It was not possible to repeat this process at Bath as magnetic stirrers of sufficient power to disperse the resin were not available. Instead, as described in the Materials and Methods section, the resin was manually cut up into small cubes.

It is probable that these cubes have different properties to those of the spheres. They were larger and so had a smaller surface area to volume ratio and had cut edges, rather than the naturally formed surface of the spherical particles.

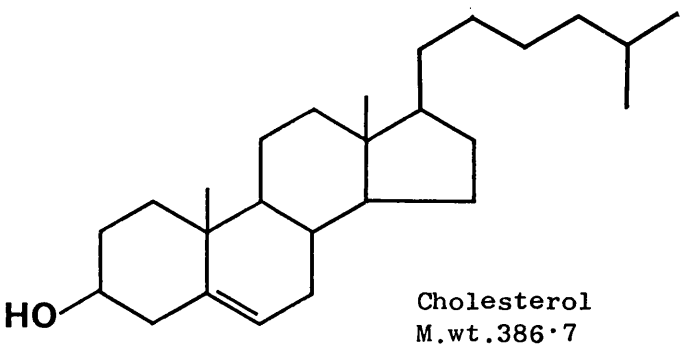
General properties of silastic resin cubes.

Diosgenin accumulation by silastic resin cubes and its extraction from them.

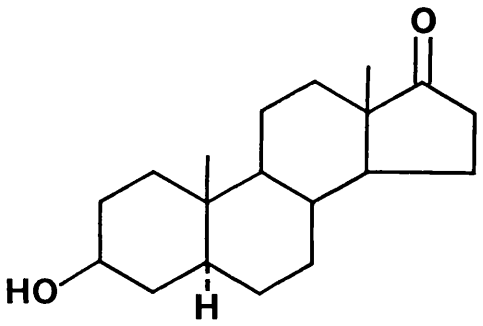
The results from this experiment (Table 37(a)) demonstrated the necessity of a thorough extraction procedure to ensure complete removal of steroid from the silastic resin. To aid this process the silastic resin cubes were dried at 60°C after removal from the steroid containing solution but before extraction with ethyl acetate.

The effect of the hydrophobicity of steroids on their accumulation by silastic resin

A principal difference in structure between the two steroids tested in this experiment (Figure 45) is the presence of a hydrophobic side chain on the cholesterol molecule, which the androstane lacks. This, increasing the hydrophobicity of cholesterol, must have caused its



Cholesterol
M.wt.386·7



3_{β}-Hydroxy-5α-androstan-I7-one
M.wt. 290·5

Figure 45. Steroid structures.

proportionally increased accumulation by the resin (Table 37b) despite the molecule's larger size. This preferential accumulation of cholesterol is reasonable considering hydrophobic nature of the resin. The result suggests the important potential for preferential accumulation by silastic resin of certain compounds from fermentations.

The use of silastic resin as a substrate reservoir

As the results show (Table 39) silastic resin was not an effective vehicle for the addition of diosgenin to fermentations.

During the impregnation of the silastic resin with diosgenin it was inevitable that a certain amount of sapogenin formed deposits on the surface of the cubes. Addition to the bacterial culture and the subsequent mechanical shaking caused these crystalline deposits to flake off into the growth medium. A previous experiment (Table 15) has already demonstrated that large lumps of diosgenin such as these are not readily metabolised by N. rhodochrous. Therefore this was considered to be the cause of the lack of biotransformation.

It has been shown (Table 15) that the most efficient method of diosgenin addition is by prior dissolution in solvent. Furthermore if the limited steroid carrying capacity of the silastic resin is taken into account, then for maximal transformation of diosgenin to occur it is preferable to add the sapogenin separately from the silastic resin.

The use of silastic resin for product collection from N. rhodochrous fermentations

A much higher rate of diosgenin transformation was achieved in this

experiment (Table 40) when compared to the previous experiment (Table 39). The difference was shown to be due to the form in which the diosgenin was added rather than the presence or absence of silastic resin.

The resin accumulated considerably more products than diosgenin from the growth medium. When 20 g of resin was present all the transformation products were found in the resin and none in the aqueous fraction. This is probably because the 3-oxo groups of the products are more hydrophobic than the 3-hydroxyl of diosgenin. The use of silastic resin for product collection, rather than as a reservoir for substrate has not been studied previously.

The use of different quantities of silastic resin showed that the amount of steroid accumulated was governed by the silastic resin's carrying capacity up to quite high concentrations of resin, although the increase in steroid accumulated was not linear at the highest level of resin tested (20 g).

The possibility that silastic resin might have an effect on the pattern of products was more fully explored in the next experiment.

The effect of silastic resin during a fermentation of diosgenin by N. rhodochrous

The reason for the slower transformation of diosgenin by N. rhodochrous in the presence of silastic resin (Figure 44)was unclear. It was unlikely to be due to the accumulation of diosgenin by the resin because, until the later stages, only a small proportion of the total diosgenin

present was contained in the resin. It could have been that the presence of the large amount of resin (20 g in 50 ml culture) hindered the even mixing of steroid and bacteria. Decreased activity was not evident when less resin was present (e.g. 5 g in 50 ml culture, Table 40).

The decline in the diosgenin content of the resin towards the end of the experiment was probably due to the displacement of diosgenin by the more hydrophobic products formed as the resin reached its maximum carrying capacity.

The presence of silastic resin had a stimulatory effect on the formation of 1-dehydrodiosgenone at the expense of diosgenone. This could have been due to product inhibition - the removal of 1-dehydrodiosgenone from the fermentation by the silastic resin preventing such inhibition and causing a greater accumulation than was observed in purely aqueous cultures. Whatever the cause the silastic resin did alter the pattern of transformation of diosgenin by selective removal of products.

The use of silastic resin as a transportable product reservoir

i) Initial substrate methanol extract

Previous experiments have shown that silastic resin is particularly useful in situations where the product is more hydrophobic than the substrate. Therefore the resin was considered to be well suited for use in a fermentation of fenugreek seed methanol extract where the substrate is a complex, hydrophilic mixture and the desired first product (diosgenin) a hydrophobic compound.

In an earlier experiment (Table 35) it has been shown that F. solani is efficient in producing diosgenin from fenugreek seed methanol extract with very few other hydrophobic products. M. phlei is capable of transforming diosgenin and accumulating androstanes in the presence of α,α'-bipyridyl (Table 22) but is inactive against methanol extract (Table 35). Therefore by the use of silastic resin to transfer the diosgenin produced by F. solani to M. phlei cultures the two transformations could be coupled , producing androstanes from the methanol extract.

The experiment was successful: the formation of diosgenin was not impaired by the presence of the silastic resin cubes and transference of the diosgenin-holding cubes to M. phlei resulted in the formation of androstanes (Table 41). However the presence of an oil-like substance absorbed by the resin greatly limited its carrying capacity for diosgenin. Consequently, the majority of the diosgenin formed remained in the aqueous phase of the culture. If the efficiency of transfer could be improved, either by using a larger quantity of resin, or by improving its carrying capacity, the use of silastic resin would offer substantial advantages compared to the traditional extraction and isolation methods. A comparison of the two processes is made in Figure 46. The use of F. solani with silastic resin is more straightforward and energy efficient, no high temperatures, pressures or strong acids being required.

Thus in general the silastic resin could be used to transfer many hydrophobic intermediates between microbiological fermentations, obviating the need for their chemical extraction or the use of mixed microbiological cultures.

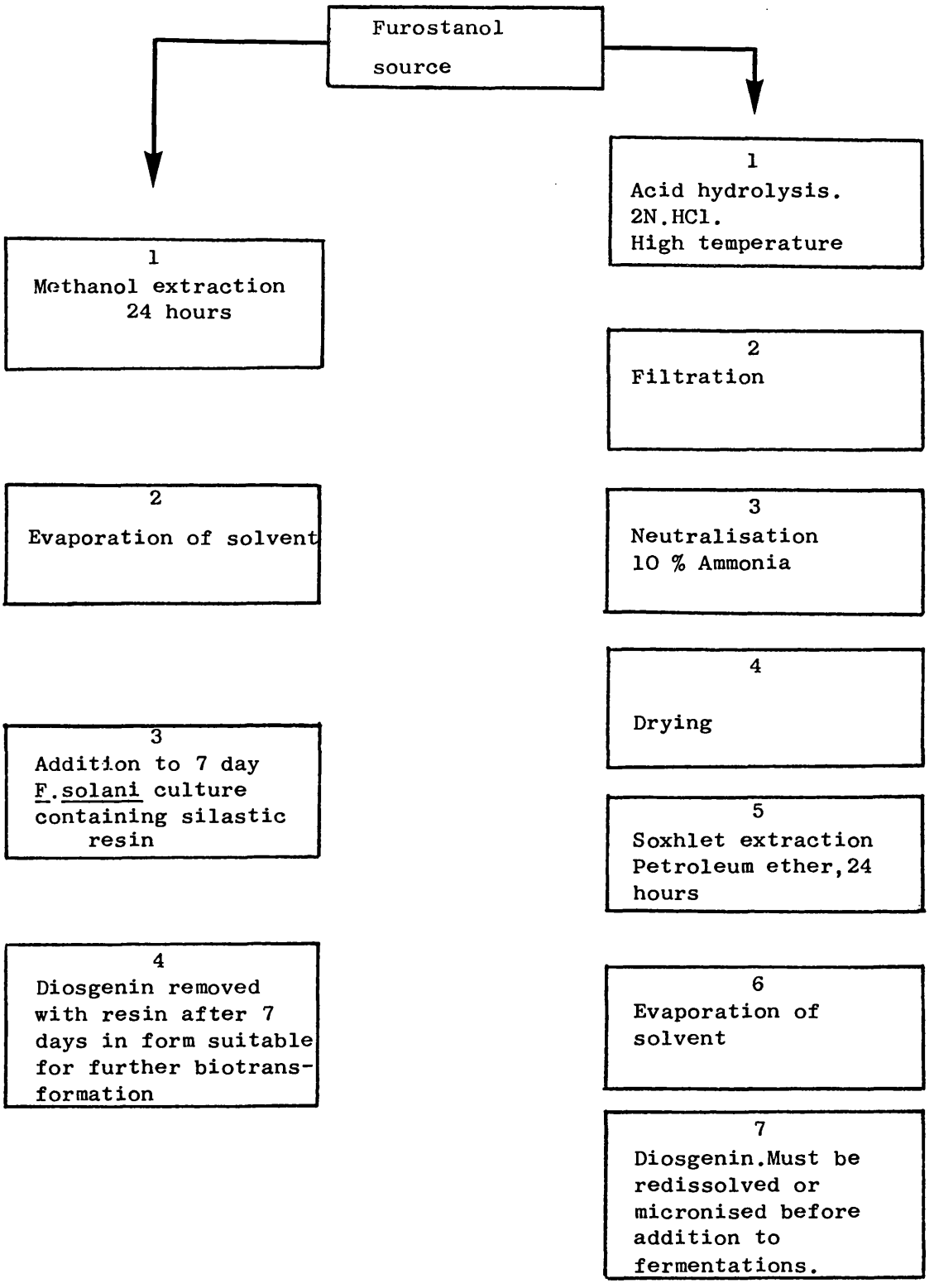


Figure 46. The potential use of silastic resin and F.solani for the formation and collection of diosgenin. A comparison with traditional techniques.

ii) Initial substrate diosgenin

The results from this experiment (Table 42) confirm the results from an earlier experiment, which showed that the presence of silastic resin increased the proportion of diosgenone formed compared to 1-dehydrodiosgenone.

Contrary to the report of Kondo and Mitsugi (1966) my strain of F. solani had little transforming activity against diosgenone. This failure has been noted in earlier experiments and was confirmed as no change occurred in the steroid content of the silastic resin during the 6 days incubation with F. solani.

In a few cases mixed cultures have been found useful in the transformation of steroids. Lee et al.(1969) transformed 9α-fluorohydrocortisone to Δ1-dehydro-16αhydroxy-9α-fluorohydrocortisone (triamcinolone), with a mixed culture of Corynebacterium simplex and Streptomyces roseochromogenus. Yoshida et al. (1981) showed that the success of the system is probably due to the inhibition of an undesirable side reaction of C. simplex by a product from S. roseochromogenus.

However mixed cultures pose the problems of pH and media incompatibility and the overgrowth of one or other organism. They are not in general use for steroid biotransformations. In the mixed fermentation attempted in this work (Table 42) F. solani appeared to have little effect. This was either because of its suppression by N. rhodochrous or, as suggested by the other results in the table, that F. solani was inactive against diosgenone. Thus both in the presence and in the absence of silastic resin, the only products obtained were diosgenone and 1-dehydrodiosgenone. These products were formed from diosgenin by

the N. rhodochrous cultures. Again there was a marked increase in the formation of 1-dehydrodiosgenone in the presence of silastic resin, compared to the yield obtained with the mixed culture.

CHAPTER 9

General Discussion and Conclusions

The studies in this thesis have led to a number of conclusions, many of which apply to more than a single microorganism or the steroid (diosgenin) under consideration.

The screening experiments (Chapter 3) demonstrated that a single screening medium (Medium 1) was capable of supporting the growth of a wide variety of microorganisms and also allowing the expression of their transformational ability. Although it has been reported that diosgenin is a poor substrate for transformation compared to other steroids (Nagasawa et al., 1970a; Mininger et al., 1956; Brooks and Smith, 1975), a number of species were found which have not previously been reported to attack the steroid (Chapter 3, Table 7). There is no doubt that if screening was continued further species would be found.

The results of the screening programme and the structural analysis of transformation products show that the commonest route of transformation of diosgenin proceeds via a 3-oxo-4-ene compound. This pathway is similar to that of many other steroids (e.g. cholesterol) which have the same structure in rings A and B. The E and F rings of diosgenin are less readily attacked. Hence the formation and accumulation of products such as androstanes has been difficult.

The importance of the structural features of rings A and B, particularly the 3β-hydroxyl group was demonstrated. When the group was replaced by benzoate or acetate groups (Chapter 5) attack of the molecule was completely inhibited in most instances. When the group was replaced by sugar residues (Chapter 7) i.e. furostanol and spirostanol compounds, the pattern of active organisms changed. Those active against diosgenin

had little activity against the new structure, but a number of other microorganisms were found to be active. The importance of a 3β-hydroxyl group is also illustrated in the literature. Bell et al. (1972) found that 5α-androstane, a compound without any oxygen function on rings A and B, was very resistant to biotransformation. However transformation of cholestanes can be achieved; Mulheirn and Van Eyk (1981) found 16 organisms which accumulated transformation products from 5α-cholestane. Some of the most active strains found were from M. phlei and various Nocardia species. None of their transformation products have been identified. A potential use of diosgenin acetate or benzoate, because of their resistance to ring A attack, would be as substrates in a screening programme to select organisms more likely to have biotransformational activity against rings E and F.

Many of the problems encountered in the biotransformation of steroids are due to their poor solubility in water. Diosgenin is a particularly hydrophobic compound. In most of the examples in the literature transformation of steroids by intact microorganisms is said to occur within the cell and not in the medium surrounding the cell (Charney and Herzog, 1967). To enter the cell the steroid being transformed must dissolve to some extent in the medium so that it can diffuse through the cell wall. Hence the solubility and the rate of steroid diffusion may become rate-limiting factors for transformation. Therefore the physical form of the steroid substrate and its method of addition to the microbial culture is likely to have an important influence on the rate of its biotransformation. This has been shown to be the case (Chapter 4).Although little work has been published previously on the form of steroid addition it is clear that the highest

rate of transformation is achieved when the steroid substrate is in a diffuse form (Table 15). The final choice of steroid addition method is governed by the concentration of steroid required for a reasonable rate of transformation and the potential toxicity of any solvent employed.

Whilst dissolution of diosgenin in ethanol prior to addition to the cultures was the method of choice for the majority of experiments, other possibilities were explored (Chapter 7). The use of high concentrations of water-immiscible organic solvents (first reported by Buckland et al., 1975) caused a much increased initial rate of diosgenin transformation by free cells (Tables 32 and 33). However enzyme activity was quickly lost and for this reason the method was not suitable for periods of use longer than a few hours.

The culture medium chosen and the time of steroid addition have also been found to exert a profound effect on the rate of diosgenin transformation (Chapter 4); the precise conditions depend upon the microorganisms used. For the majority of organisms studied in this thesis a rich medium allowing heavy growth, with steroid addition at stationary phase, has given the highest quantity of steroid transformation. The use of resting cells was less effective (Table 17).

The transformation of diosgenin to diosgenone was studied in Chapter 6. This is of importance as Kondo and Mitsugi (1966,1973) claim that diosgenone is much more readily transformed to androstanes and pregnanes (by Fusarium solani, Stachylidium bicolor and Verticillium theobromae) than diosgenin. The formation of diosgenone was shown in

this thesis to be mediated by a cholesterol oxidase enzyme which was inducible in growing cells. Experiments using diosgenone as a substrate with F. solani (Chapter 8, Table 42) failed to confirm Kondo and Mitsugi's results.

The work using α,α'-bipyridyl to prevent steroid nucleus degradation (Chapter 5) has confirmed and extended the earlier work of Ambrus and Buki (1969). Although slightly higher levels of androstanes were obtained than they reported, the total yield was still low (6.5%). Mutation of diosgenin degrading microorganisms using MNNG appeared to be the most promising approach for preventing steroid nucleus degradation (Chapter 5). A schedule has been developed which produces large numbers of mutants and continuation of this work is likely to lead to Mycobacterium strains capable of accumulating androstanes and possibly even pregnanes.

The use of microorganisms to form diosgenin from very crude methanol extracts of fenugreek seed containing furostanol glycosides was successful. This is a potential diosgenin source which would obviate the need for an initial acid hydrolysis step. Although Rothrock et al. (1957b) have previously reported the formation of diosgenin from crude fermentations of yams they did not achieve high yields from pure cultures and had problems caused by the further complete degradation of diosgenin. It is shown (Table 35, Chapter 7) that F. solani is capable of producing a high yield of diosgenin (84%) from a crude methanol extract. Such a process could have useful industrial applications.

Silastic resin has been found to provide a novel and practical material for the collection of steroid products from microbial fermentations

(Chapter 8). The resin preferentially accumulated compounds on the basis of their hydrophobicity. The steroid accumulated was then easily transferable to other fermentations in a form still readily available for further biotransformation (Table 41). In the reactions studied the resin had an effect on the product ratio, possibly by preventing product inhibition. Silastic resin would appear to offer considerable advantages over the use of mixed cultures for carrying out two or more separate but sequential microbiological transformation steps.

Methods to increase the carrying capacity of the resin require further study to maximise its usefulness. This might be done by increasing the surface area/volume ratio of the cubes and by investigating different hydrophobic polymers for the production of cubes.

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