STUDIES ON THE BIOTRANSFORMATION OF CODEINONE TO CODEINE BY CELL CULTURES OF PAPAVER SOMNIFERUM

by

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A THESIS PRESENTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY, UNIVERSITY OF EDINBURGH, 1991
Acknowledgements

Firstly, I would like to thank my supervisor, Professor M.M.Yeoman, for his invaluable advice and guidance throughout the preparation of this thesis. I would also like to thank all those, who during the past four years, have worked and studied in the Department of Botany (especially Lab.205) for their friendship and generosity. In particular, I am very grateful to Drs. M.A.Holden, P.R.Holden and M.B.W.Miedzybrodzka for their day to day help with experimental work. Most credit for my "lasting the course", however, must go to Rachel whose love and support have been unwavering.

A research studentship award from the Biotechnology Directorate of the Science and Engineering Research Council is also acknowledged.

Finally, I would like to say just how much I am indebted to my parents, Mary and Graham, and my sister, Johanna. This thesis is dedicated to the three of them, in grateful recognition of their support, encouragement and above all tolerance, of its author.
Crimson flames tied through my ears
Rollin' high and mighty traps
Pounced with fire on flaming roads
Using ideas as my maps
"We'll meet on edges soon," said I
Proud 'neath heated brow.
Ah, but I was so much older then,
I'm younger than that now.

<table>
<thead>
<tr>
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<tr>
<td>A</td>
<td>y-intercept of regression line</td>
</tr>
<tr>
<td>AuFs</td>
<td>attenuated units full scale</td>
</tr>
<tr>
<td>B</td>
<td>slope of regression line</td>
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<tr>
<td>ca.</td>
<td>approximately</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
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<tr>
<td>Cd=O</td>
<td>standard codeinone</td>
</tr>
<tr>
<td>Cd-OH</td>
<td>standard codeine</td>
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<tr>
<td>conc.</td>
<td>concentrated</td>
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<td>[]</td>
<td>concentration</td>
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<td>d</td>
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<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
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<tr>
<td>dwt</td>
<td>dry weight</td>
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<td>eg.</td>
<td>for example</td>
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<td>et al.</td>
<td>et alia</td>
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<td>Fig.</td>
<td>figure</td>
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<td>fresh weight</td>
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<td>gramme(s)</td>
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<tr>
<td>gdwt</td>
<td>gramme(s) dry weight</td>
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<td>h</td>
<td>hour(s)</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>ie.</td>
<td>that is</td>
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<tr>
<td>K</td>
<td>kinetin</td>
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<td>l</td>
<td>litre(s)</td>
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<tr>
<td>log</td>
<td>logarithmic</td>
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<td>m</td>
<td>metre(s)</td>
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<td>M</td>
<td>molar</td>
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<td>min</td>
<td>minute(s)</td>
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<tr>
<td>mix</td>
<td>mixture of standard alkaloids</td>
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<tr>
<td>µg</td>
<td>microgramme(s)</td>
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<tr>
<td>µl</td>
<td>microlitre(s)</td>
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<td>µm</td>
<td>micrometre(s)</td>
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<td>milligramme(s)</td>
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<td>millilitre(s)</td>
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<td>mm</td>
<td>millimetre(s)</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>n</td>
<td>number of replicates</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>N°</td>
<td>number</td>
</tr>
<tr>
<td>P.b</td>
<td>plant or suspension culture of <em>Papaver bracteatum</em></td>
</tr>
<tr>
<td>pH</td>
<td>negative log of hydrogen ion concentration</td>
</tr>
<tr>
<td>±</td>
<td>plus or minus</td>
</tr>
<tr>
<td>P.o</td>
<td>plant or suspension culture of <em>Papaver orientale</em></td>
</tr>
<tr>
<td>P.s</td>
<td>plant or suspension culture of <em>Papaver somniferum</em></td>
</tr>
</tbody>
</table>
Definitions

Biotransformation capacity - this is a measure of the potential of a particular culture to convert codeinone to codeine and may be expressed either as the amount of product formed per specified volume or the amount of product formed per unit biomass (\( \text{unit} = \text{either} \, \mu\text{g}.\text{flask}^{-1} \text{ or } \mu\text{g}.\text{gdwt}^{-1} \)).

Biotransformation ratio - this is the percentage of codeinone added to a culture converted to codeine (\( \text{unit} = \% \)).

Where relevant, the time taken to achieve a biotransformation was also included, by defining the rate of a particular reaction (\( \text{unit} = \mu\text{g}.\text{h}^{-1} \)).
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Abstract

The aim of this project was to examine the process by which cultured cells of *Papaver somniferum* and related species, biotransform codeinone to codeine.

It was confirmed in this study that plants of *P. somniferum* produce the morphinan alkaloids thebaine, codeine and morphine and therefore, possess the enzyme involved in the biotransformation of codeinone to codeine. However, plants of *P. bracteatum* and *P. orientale* only accumulate thebaine, apparently lacking one or more of the enzymes necessary to convert thebaine to morphine. Suspension cultures derived from *P. somniferum*, *P. bracteatum* and *P. orientale* all produced the morphinan alkaloid(s) common to the parent plant but in significantly lower amounts. *P. somniferum* suspension cultures converted a maximum of 35.3% of added codeinone to codeine. However, "meristemoid" cultures of *P. somniferum*, which displayed a high state of differentiation and consisted of densely cytoplasmic cells organised in recognisable morphological structures, converted up to 60.0% of added codeinone to codeine. By contrast, suspended cells of *P. bracteatum* and *P. orientale* biotransformed less than 5.0% of added codeinone to codeine. This strongly suggests the existence of a relationship between biotransformation capacity (amount of product produced per specified volume or unit of biomass) and culture origin. Immobilisation increased the biotransformation capacity of *P. somniferum* suspension cultures but not of *P. somniferum* "meristemoid" cultures.

Suspension cultures of *P. somniferum*, at different stages of development, converted approximately equal quantities of codeinone to codeine over a 72h period. However, the rate at which codeine was produced in these cultures varied considerably and was fastest in cultures entering the stationary phase. This also corresponded to the stage at which the concentration of endogenous alkaloids in the cells was highest.

"Meristemoid" and suspension cultures of *P. somniferum* converted codeinone to codeine faster than immobilised cells. However, as the concentration of substrate was increased the rate of codeine production in suspended cultures slowed down. In immobilised cultures, although the conversion rate was lower, the period over which the biotransformation took place was extended.

"Bound" forms of codeinone were detected in the media of all cultures but only in
very small quantities in suspension and "meristemoid" cultures of *P.somniferum*. At no point was "free" codeinone detected in the cells of any culture type. However, both "free" and "bound" forms of codeine were present in the media and cells of suspended and immobilised cultures of *P.somniferum* and *P.somniferum* "meristemoids". At no time did the total amount of codeine detected in cells account for more than 15% of the codeinone added to the cultures.

The relationship of culture origin, stage of development and state of differentiation with the ability of cultured poppy cells to biotransform codeinone to codeine is discussed.
Chapter 1.
INTRODUCTION
1.1. Commercial importance of the opium poppy

1.1.1. Historical background

The opium poppy (*Papaver somniferum*) is, as its name suggests, the source of the pharmacologically active compound opium, the sun-dried latex exuded from incised capsules, used for many centuries as a pain relieving agent. There is some controversy about the first recorded use of opium with various forms of evidence available including linguistic, botanical and archaeological (Bisset, 1985). However, extracts of opium are known to have been used by man for at least 3,500 years. This was established by the discovery in the tomb of the Egyptian royal architect Cha (who died between 1417 - 1379 BC) of seven small alabaster vases containing a product which comprised vegetable fat, iron and morphine, indicating the presence of opium (Muzio, 1925; Schiaparelli, 1927). Other potential samples have been recovered from sites in Egypt, Crete and Cyprus but the identification of opium in these cases is not entirely free of doubt (Germer, 1979).

1.1.2. The genus *Papaver*

In 1909, Fedde divided the genus *Papaver* into nine sections (Table 1.1.1). Although this classification is still widely in use today, more recent chemotaxonomic studies by Günther (1975) and Preininger et al. (1981) are felt by many authors to warrant a new classification (Table 1.1.1). Of the twelve sections proposed those with most relevance to this thesis are sections *Papaver* (syn. Mecones Bernh.), *Glaucan* and *Macrantha*.

1.1.2.1. Sections *Papaver* (syn. Mecones Bernh.) and *Glaucan*

Detailed investigation of the alkaloid content of the Papaveraceae has shown that the chemical composition of the section *Papaver* (syn. Mecones Bernh.) is relatively varied. For example, the alkaloids present in *P. somniferum* L. and *P. setigerum* DC. differ markedly from those in *P. glaucum* Boiss. et Hausskn., *P. gracile* Auch. and *P. decaisnei* Hoehst et Steud. These differences are summarised in Table 1.1.2.
Table 1.1.1

Sections of the genus *Papaver* L.

Fedde (1909) | Present state (Günther, 1975; Preininger *et al.*, 1981)
---|---
*Orthorhoeades* Fedde | *Rhoeadium* Spach.
*Argemonorhoeades* Fedde | *Argemonidium* Spach.
*Carinaeae* Fedde | *Carinaeae* Fedde
*Mecones* Bernh. | *Papaver*

*Pseudo-pilos* M.Pop. | |

*Horrida* Elk. | *Roemeria* (Medic.) Günther

(after Preininger, 1985)

Thebaine is found in a wide variety of plants belonging to the sections *Orthorhoeades, Pilosa, Miltantha* and *Macrantha*. By contrast codeine and especially morphine are thought to be unique to *P.somniferum* and *P.setigerum* with the exception of morphine as a minor alkaloid in poppy heads of *P.decaisnei*. The formation of the characteristic 4,5-epoxybridge of thebaine (Fig.1.2.1) appears to be a relatively common reaction, whilst the ability to demethylate the methoxy groups, necessary for codeine and morphine synthesis, appears to be a much rarer characteristic.

It was as a result of these findings that the original section *Papaver* (syn. *Mecones* Bernh.) was divided into the section *Papaver* which contains *P.somniferum* and *P.setigerum* and the new section *Glaucac J.Nov. et V.Prein*. which includes *P.glaucac, P.gracile* and *P.decaisnei* as members. Studies of morphological features and basic
chromosome number have substantiated these categorisations (Novák and Preininger, 1980). For example, species of section *Papaver* have a chromosome number of n=11 whereas for section *Glauca* n=7. Individual plants of *P.somniferum* and *P.setigerum* are mostly diploid (2n=22) although tetraploid (4n=44) forms do occur. By contrast, all species of the section *Glauca* are diploid (2n=14). Plants of the new section *Glauca* differ in their habitat from species of the section *Orthorhoea* but do contain similar alkaloids and some authors consider these plants to be very closely related (Stermitz, 1968). The other section of particular interest to this project is the section *Macrantha*, which includes *P.bracteatum* and *P.orientale*.

| Plant |
| Characteristic alkaloid types |
| Example(s) |

### Table 1.1.2

**Characteristic alkaloid types of various species of sections**

*Papaver* (syn. *Mecones Bernh.*) and *Glauca*

<table>
<thead>
<tr>
<th>Plant</th>
<th>Characteristic alkaloid types</th>
<th>Example(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P.somniferum</em> and <em>P.setigerum</em></td>
<td>morphinan</td>
<td>thebaine, codeine, morphine</td>
</tr>
<tr>
<td></td>
<td>secophthalide-isoquinoline</td>
<td>narceine, nomarceine</td>
</tr>
<tr>
<td></td>
<td>phthalideisoquinoline</td>
<td>narcotine, narcotoline</td>
</tr>
<tr>
<td><em>P.glaucum</em>, <em>P.gracile</em> and <em>P.decaisnei</em></td>
<td>rhoeadine/</td>
<td>rhoeadine</td>
</tr>
<tr>
<td></td>
<td>papaverrubine</td>
<td>glaucaamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>papaverrubine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-A,B,C and D</td>
</tr>
</tbody>
</table>

NB. Traces of rhoeadine type alkaloids have been found in *P.somniferum* and *P.setigerum* but no morphinan type alkaloids have been found in *P.glaucum* or *P.gracile*. Indeed, the only report of morphine in a species other than *P.somniferum* or *P.setigerum* was as a minor alkaloid from the poppy heads of *P.decaisnei* (Slavík, 1980).

### 1.1.2.2. Section *Macrantha*

During the last 20 years, plants belonging to the section *Macrantha* have been the subject of fairly intensive research. This is mainly attributable to the considerable yields of thebaine found in certain varieties of *P.bracteatum*. Thebaine is of
commercial interest because it represents a potential source of codeine. Due to the massive, worldwide, sociological problems associated with opium, it is believed by some to be preferable to ban the farming of *P.somniferum* and replace it with *P.bracteatum*. Thebaine, harvested from *P.bracteatum* could then become the source of codeine for pharmaceutical companies. Since it is significantly harder to produce heroin (diacetylmorphine) from thebaine than from morphine this might also reduce illegal drug traffic. However, the commercialisation of *P.bracteatum* as an economically viable source of thebaine (and codeine) awaits political rather than commercial developments (Bryant, 1988). Another plant of this section is *P.oorientale*, which like *P.bracteatum*, accumulates thebaine. However, the major alkaloid of *P.oorientale* plants tends to be oripavine (Shafflee et al., 1977), although the exact alkaloid composition of many species of the genus *Papaver* varies with temperature, day length and light intensity (Bernáth and Tétényi, 1979.a, 1979.b, 1981).

Having briefly introduced the genus *Papaver*, the next section describes how and why, the opium poppy, is harvested and then utilised by pharmaceutical companies.

**1.1.3. The harvesting and processing of opium**

So, what exactly is opium? Opium is the sun-dried latex exuded from the incised capsules of *P.somniferum* and is the major source of morphine. Approximately, 80d after planting the flower petals drop, leaving the green capsule, which contains the developing seeds. The capsule is incised with a special tool, often in the late afternoon, releasing the white latex which rapidly dries and darkens on exposure to air. This fresh opium is collected early the next morning and stored in large open air tanks where it is allowed to dry down to *ca.* 15% moisture content. The resultant black, semi-solid substance is the opium of commerce. A second commercial source of morphine is opium straw, which is the term given to the dried capsules, once freed of their seed and milled. It is important to note that the economics of growing opium poppies dictates that seeds must be suitable for the food industry since the value of the seed is roughly equivalent to the value of morphine. However, opium straw still only represents *ca.* 10% of the total worldwide source of morphine, although this figure is rising slowly (Bryant, 1988).

About 45% of the world’s morphine requirements is derived from Indian opium, the composition of which is shown in Fig.1.1.1
Fig. 1.1.1 Composition of Indian opium (after Bryant, 1988)
1.1.4. Pharmaceutical products obtained from opium

The pharmaceuticals obtained from opium fall into three main therapeutic categories:

1.1.4.1. Analgesics

The best known use of alkaloids obtained from opium is in the relief of pain. Table 1.1.3 summarises the particular usage(s) of various opiates and the relative potency of each compound, as compared with morphine.

<table>
<thead>
<tr>
<th>Opiate Drug</th>
<th>Relative Potency</th>
<th>Pharmacological Useage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>1</td>
<td>Control of severe pain, antidiarrhoeal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and sedative</td>
</tr>
<tr>
<td>Codeine</td>
<td>0.1</td>
<td>Mild analgesic</td>
</tr>
<tr>
<td>Dihydrocodeine</td>
<td>0.15</td>
<td>Moderate analgesic</td>
</tr>
<tr>
<td>Diamorphine</td>
<td>2.5</td>
<td>Supression of severe pain</td>
</tr>
<tr>
<td>(or heroin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydromorphone</td>
<td>4.4</td>
<td>&quot;ditto&quot;</td>
</tr>
<tr>
<td>Oxymorphone</td>
<td>10</td>
<td>&quot;ditto&quot;</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>38</td>
<td>&quot;ditto&quot;</td>
</tr>
<tr>
<td>Etorphine</td>
<td>10,000</td>
<td>Powerful veterinary sedative</td>
</tr>
</tbody>
</table>

1.1.4.2. Antitussives

Codeine, pholcodine and ethyl morphine are the major opiate drugs used in the supression of coughs. Narcotine, not strictly an opiate despite its name, is also used as an antitussive and its lack of addictive properties makes it more acceptable in some areas of the world (eg. Moslem countries).
1.1.4.3. Narcotic antagonists

The most undesirable side effects of opiates during medicinal usage is respiratory depression or sedation. Opiate antagonists naloxone and naltrexone are used as an antidote to suppress this problem. Diprenorphine is a very powerful antagonist used to revive animals sedated with etorphine (see Table 1.1.3).

1.1.5. Extraction of alkaloids from opium

In order to manufacture the range of pharmaceutical products listed above, it is necessary to isolate morphine, codeine, thebaine and noscapine from opium, whilst only the first three are extracted from opium straw. Since the nineteenth century, when opium processing began in earnest, several different extraction methodologies have been used. Today the majority of morphine recovered is converted to codeine, which despite being a less effective analgesic is also less addictive. Fig.1.1.2 shows a simplified version of the extraction procedure currently used by Macfarlan Smith Ltd., Edinburgh; as well as the method used to convert morphine to codeine.

Having presented general background information on *P. somniferum*, the next section examines the biosynthesis of opium and its constituent alkaloids in plants.
1. Opium is dissolved in hot water, basified, filtered and washed - the main alkaloids remaining in solution.

2. The alkaline liquors are extracted with xylene and the xylene extract washed with sulphuric acid - the phenolic alkaloids remain in the alkaline liquors while the non-phenolic alkaloids are transferred to the acid washes.

3. The pH of the alkaline liquors is adjusted - morphine is precipitated, purified and the majority converted to codeine.

4. The acid washes from 2. are concentrated to low bulk and allowed to cool - crude codeine sulphate crystallises out and is then purified or converted to other codeine salts.

5. Morphine collected from 3. is converted to codeine by reaction with phenyltrimethylammonium hydroxide.

6. The reaction solution from 5. is extracted with aqueous sodium hydroxide - removes unreacted morphine and other impurities.

7. Codeine is extracted into hot sulphuric acid solution and then charcoaled and filtered.

8. The aqueous solution is then allowed to cool - codeine sulphate precipitates.

9. The crude codeine sulphate is then purified or converted to other salts.
1.2. The biosynthesis of opium and its constituent alkaloids

1.2.1. Latex and laticifers

Latex is a term used by plant anatomists to describe fluids which are usually of a milky white appearance although, other colours such as yellow, orange, red and green are known. The milky appearance of latex is due to the suspension of many small particles in a liquid of a different refractive index. The cells or series of fused cells that contain latex are known as laticifers and permeate various tissues of the plant body. Laticifers are grouped into two major classes, on the basis of their structure:

1. Articulated - these are compound in origin and consist of longitudinal chains of cells in which the walls separating the individual cells either remain intact, become perforated or are completely removed.

2. Non-articulated - these originate from single cells, which through continued growth develop into tube like structures, often branched, but do not tend to fuse with other similar cells.

Two subdivisions of the articulated laticifers have been established and reflect the occurrence or absence of lateral connections between the longitudinal chains of cells. Those that do form chains are called articulated anastomosing laticifers and those that do not, are referred to as articulated non-anastomosing laticifers. Non-articulated laticifers are also subdivided into two groups, but this time on the basis of whether the laticiferous cell branches. Therefore, the two types recognised are, non-articulated unbranched laticifers and non-articulated branched laticifers.

*P.somniferum* contains articulated anastomosing laticifers, the development of which have been studied by several authors (Felklova and Babkova, 1958; Fairbairn and Kapoor, 1960). In addition Nessler and Mahlberg (1978) investigated the stages involved in the resorption of the end cell walls of individual cells and also studied the ontogeny and cytochemistry of alkaloidal vesicles in laticifers. It became apparent from this work that it can sometimes be difficult to distinguish between laticifers and sieve-tube elements, because latex containing cells can also possess sieve plates. One possible way to discriminate between these two cell types is that young laticifers, unlike young sieve-tube cells, do not develop a central vacuole at an early stage but instead the cytosol within laticifers remains entire until numerous smaller, then larger vacuoles are formed (Sárkány et al., 1964). In *P.bracteatum*, laticifer cells appear as
early as a few days after germination and contain many vesicles with electron dense caps (Nessler and Mahlberg, 1978; Rush et al., 1985). Similar vesicles are also found in *P. somniferum* cells when ground and centrifuged at 1000g for 0.5 to 1h and the vesicles were found to contain 95-99% of all the alkaloids present in the pellet (Fairbairn et al., 1974). There was also evidence to suggest that the alkaloids were present in the sap of the vesicle rather than being membrane bound.

1.2.2. Site of alkaloid synthesis

Latex contains millimolar concentrations of dopamine (see Fig.1.2.1), a fraction of which is also present in vesicles that are rich in alkaloids (Roberts et al., 1983). Dopamine (as will be seen later) is an essential intermediate in the synthesis of most alkaloids found in the *Papaveraceae* (Spenser, 1968; Shamma and Moniot, 1978). Dopamine itself, is produced from tyrosine and condenses with a number of carbonyl derivatives to form various tetrahydroisoquinolines which in turn give rise to a large array of alkaloids. The chirality of the tetrahydroisoquinolines appears to greatly influence their subsequent transformation. For example, morphinan alkaloids are formed from (R)-reticuline, whereas most other alkaloids, including sanguinarine, are derived from (S)-reticuline (Battersby et al., 1965.a, 1975.a and 1975.b; Wilson and Coscia, 1975; Borkowski et al., 1978; Brossi, 1982). Whether the actual sites of morphinan alkaloid synthesis are laticiferous cells is somewhat controversial (Kutchan et al., 1985). Latex, isolated from *P. somniferum* converts radio-labelled tyrosine and L-DOPA (precursor of dopamine synthesis) to morphine (Fairbairn et al., 1968; Fairbairn and Steele, 1981). Enzymes that can convert tyrosine to dopamine have also been detected in *P. somniferum* latex (Roberts, 1974; Roberts and Antoun, 1978). In addition, latex isolated from *P. orientale*, exhibited biosynthetic activity for several of the early steps of alkaloid synthesis (Wilson and Coscia, 1975). However, it has been argued that the biosynthetic activity present in latex is not high enough to account for the large quantity of alkaloids produced by plants of *Papaver* spp. (Böhm et al., 1972). As a consequence these authors believe that synthesis actually occurs in cells surrounding laticifers and latex simply represents a storage site for alkaloids (Böhm and Franke, 1982; Franke and Böhm, 1982).
1.2.3. Biosynthesis of the morphinan alkaloids in *Papaver somniferum*

1.2.3.1. Biosynthesis of thebaine

Morphine was the first alkaloid isolated (Serturner, 1806) and by the beginning of the twentieth century a large number of alkaloids had been identified, ≥40 of them from opium (Lindner, 1985). Due to this vast array of different compounds, researchers began to investigate the ways in which alkaloids were synthesised in plants. Winterstein and Trier (1910) suggested that the benzylisoquinoline structure was built up from two units derived from 3,4-dihydroxyphenylalanine (DOPA), which was known to be derived from tyrosine. Gulland and Robinson (1925) were first to put forward the correct structure of morphine and also proposed that morphine (and related alkaloids) were produced in the plant via a suitable benzyltetrahydroisoquinoline precursor such as norlaudanosoline (Brochmann-Hanssen, 1985). After World War II suitable radio-isotopes became available, enabling tracer studies to be made to test the validity of these and other hypotheses. The first investigations involved feeding radio-labelled tyrosine, dopamine (3,4-dihydrophenylethylamine) and norlaudanosoline then isolating radioactive thebaine, codeine and morphine (Battersby and Harper, 1958; Kleinschmidt and Mothes, 1959; Leete, 1959; Leete and Murril, 1964). As a result of experiments like these, the major steps involved in the biosynthesis of morphine have now been established (Fig.1.2.1 and Fig.1.2.2). The 1-benzyltetrahydroisoquinoline skeleton (Fig.1.2.1) is formed from two molecules of tyrosine. One of these is utilised via dopamine and the other via 3,4-dihydroxyphenylacetaldehyde or 4-hydroxyphenylacetaldehyde (Zenk, 1985). This latter point was only recently established and led to the discovery of a new enzyme, named (S)-norlaudanosoline synthase, which has been purified and characterised (Rueffer *et al.*, 1981; Schumacher *et al.*, 1983; Zenk, 1985). Previously, 3,4-dihydroxyphenylpyruvate was thought to be one of the substrates for this enzyme but was found to be inactive when assayed. The (S)-norlaudanosoline formed as a result of this reaction is next methylated in three positions to form reticuline. The three enzymes responsible have again been purified and characterised by Zenk (1985) and vary in the degree of specificity they show for their respective substrate.
Fig. 1.2.1 Biosynthetic pathway leading to the first of the morphinan alkaloids, thebaine.
Reticuline: an important branch point in alkaloid synthesis

Tracer experiments in the early 1960's had shown that thebaine is the first of the morphinan alkaloids produced by *P. somniferum* and was subsequently converted to codeine and morphine (Battersby and Harper, 1960; Rapoport et al., 1960; Stermitz and Rapoport, 1961). However, it was suspected that a precursor closer to thebaine than norlaudanosoline existed. Like thebaine this precursor would have to possess an N-methyl and two O-methyl groups and a new benzylisoquinoline alkaloid isolated from *Annona reticulata*, called reticuline, fulfilled these requirements (Gopinath et al., 1959). Feeding experiments with racemic mixtures of radio-labelled reticuline gave better incorporation into thebaine, codeine and morphine than had previously been achieved (Battersby et al., 1964; Barton et al., 1965). However, it was soon realised that S-(+)-reticuline isolated from *A. reticulata* possessed the wrong stereochemistry as regards the morphinan alkaloids (Battersby et al., 1965). These same authors decided to feed optical isomers, S-(+)- and R-(-)-reticuline, which carried $^3$H label at the asymmetric C$_1$ and $^{14}$C labels at three other positions. From the results (see Brochmann-Hanssen, 1985), it was proposed that for an inversion in configuration to occur at C1 an intermediate called the 1,2-dehydroreticulinium ion had to be formed (Fig.1.2.1). The natural occurrence of this compound was later demonstrated in *P. somniferum* (Borkowski et al., 1978). It is interesting to note that reticuline is present in opium in both forms, but with an excess of the S-(+)- form over the R(-)- form (Brochmann-Hanssen and Furuya, 1964; Brochmann-Hanssen and Nielsen, 1965). Salutaridine, isolated from *Croton salutaris* (Barnes, 1964 in Barton et al., 1965), was thought to be the intermediate between reticuline and thebaine. This proved correct, when salutaridine was shown to be present in *P. somniferum* (Barton et al., 1965) and the conversion of reticuline to salutaridine was carried out in cell-free systems of *P. somniferum* ( Hodges and Rapoport, 1982). The effects of structural modification to reticuline upon the conversion to salutaridine have also been studied (Brochmann-Hanssen et al., 1982). Prior to the formation of the characteristic 4,5-epoxy bridge of thebaine, salutaridine is believed to be reduced to a dienol. Two possible epimers were synthesised, the one shown in Fig.1.2.1 proving to have the correct stereochemistry at C$_7$ (Barton et al., 1965 and 1967).

The conversion of thebaine to morphine

The conversion of thebaine to codeine was believed to proceed via 6-O-demethylation to neopinone and a subsequent isomerisation to codeinone (Barton
and Cohen, 1957). This hypothesis proved to be correct when (i) neopinone and codeinone were found to be excellent precursors of codeine and morphine and (ii) neopinone and codeinone were detected in *P.somniferum* plants by carrier dilution after exposure to $^{14}$CO$_2$ (Battersby et al., 1967; Blaschke et al., 1967; Parker et al., 1972). The intermediate, neopinone, also provided a possible explanation for the presence of neopine in opium (Hohmeyer and Shilling, 1947). Thus, the 6-O-demethylation (of the enol ether) of thebaine leads to neopinone which isomerises to codeinone which in turn is reduced to codeine. This reduction is the reaction to be studied in this thesis using cell cultures of *P.somniferum, P.bracteatum* and *P.orientale*. It is the only enzymatic step between salutaridine (Fig.1.2.1) and morphine (Fig.1.2.2) to have been firmly established. Using cell-free preparations of *P.somniferum* callus cultures and whole plants, the reaction has been shown to require NADH as a co-factor and be stereospecific (Furuya et al., 1978; Hodges and Rapoport, 1980). However, codeinone reductase has still not been studied in a pure form. More information about this enzymatic step can be found in the next section (1.3).

The 6-O-demethylation of thebaine and the 3-O-demethylation of codeine are believed to proceed by the same oxidative mechanism (Horn et al., 1978), although they require different enzymes which demonstrate little substrate specificity. Indeed, the efficiency of both demethylations appears to be unaffected by major structural changes in thebaine or codeine (Kirby et al., 1972; Brochmann-Hanssen and Okamoto, 1980; Brochmann-Hanssen and Cheng, 1982). The lack in specificity of the two enzymes led to speculation about the possible presence of oripavine in *P.somniferum*, which would be formed if thebaine were initially 3-O-demethylated instead of 6-O-demethylated. This alkaloid was duly isolated from Tasmanian varieties by Nielson *et al.* (1983), indicating that the further metabolism of thebaine can follow one of two pathways. Additionally, when radio-labelled oripavine was fed to plants of *P.somniferum*, high incorporations into morphinone and morphine were obtained (Brochmann-Hanssen, 1984). The oripavine recovered was high in total activity, but lower in specific activity than the oripavine added, indicating the presence of a natural pool of oripavine. As the thebaine and codeine recovered were non-radioactive it would appear that the 3-O-demethylation of thebaine is "irreversible". The final conclusion of this experiment is that a second possible pathway for the conversion of thebaine to morphine exists (Fig.1.2.2) and that both pathways would appear to be operating simultaneously.
Fig. 1.2.2 Biosynthetic pathway showing the interconversions of the morphinan alkaloids.
1.2.3.4. Biosynthesis of morphinan alkaloids by other *Papaver* spp.

The biosynthesis of thebaine by *P. bracteatum* has been shown to follow the same pathway as that described above for *P. somniferum* (Hodges *et al.*, 1977; Brochmann-Hanssen and Wunderly, 1978). Indeed, many *Papaver* spp. are able to produce thebaine but only *P. somniferum* and *P. setigerum* are able to synthesise significant quantities of morphine. It would appear that other thebaine producing plants lack the (active) enzyme required for the 6-O-demethylation of thebaine. However, several of the non-morphine producing species are capable of the 3-O-demethylation of thebaine to oripavine. Indeed, in contrast to earlier studies, more recent work has revealed that *P. bracteatum* is in fact capable of synthesising trace amounts of codeine and neopine as well as oripavine (Küppers *et al.*, 1976; Phillipson *et al.*, 1976; Theuns *et al.*, 1977). Therefore, it is possible that *P. bracteatum* does possess the two enzymes necessary for the O-demethylation of thebaine and codeine, but that they are extremely "sluggish" by comparison with the corresponding enzymes in *P. somniferum* and therefore, thebaine accumulates.

From this section it is clear that a great deal is known about the likely morphine biosynthetic pathway. However, much work remains to be done before a full understanding of the pathway at the cellular and sub-cellular levels is achieved. Most of the studies described thus far have been made using radio-labelled precursors fed to whole plants. This can lead to problems such as low radio-label incorporation into product, channelling of intermediates into other pathways, low levels of enzymes involved in secondary metabolism and seasonal/daily fluctuations in enzymatic activity. As a result the use of callus and suspension cultures of *P. somniferum* have increasingly been used as relatively homogeneous sources of enzymes involved in secondary product synthesis. The next section looks at plant tissue culture of *Papaver* spp. and in particular the production and biotransformation of morphinan alkaloids by cultures of *P. somniferum*. 
1.3. Morphinan alkaloid production by callus and suspension cultures of *Papaver* spp.

Interest in cell culture of *P. somniferum* and other *Papaver* spp. has increased recently for two main reasons. Firstly, the possible development of morphinan alkaloid production on an industrial scale using plant cell culture. This is not because morphinan alkaloids are particularly rare or expensive, but due to restrictions in the harvest and movement of opium and sociological and political problems. The second reason is the use of plant cell cultures as a source of enzymic activity for the study of the synthesis and biotransformation of secondary metabolites. Although cultures often produce fewer secondary products in lesser quantities than whole plants, cultures accumulate secondary metabolites much faster than plants and remain unaffected by conditions such as seasonal variation.

Analysis of extracts obtained from cell cultures of *P. bracteatum* and *P. somniferum* has revealed that thebaine is the major alkaloid accumulated by the former whilst thebaine, codeine and to a lesser extent morphine, are accumulated by the latter. Table 1.3.1 and Table 1.3.2 show some of the reported instances of morphinan alkaloid accumulation in cultures of *P. bracteatum* and *P. somniferum*, respectively. As stated in 1.2, morphinan alkaloid production is reliant upon various factors such as ctyodifferentiation and the absence or presence of certain key enzymatic steps and can vary considerably (Petiard *et al.*, 1985). Therefore, studies tend to have concentrated on optimising the levels of various media components to promote those conditions that enhance morphinan alkaloid production. Media components studied include plant growth regulators, the carbon source and various inorganic constituents such as the concentration of phosphate, nitrate and ammonium ions. However, the results of many of these experiments have proved inconclusive. For example, concentrations of 2,4-D $\leq 1\text{mg}\cdot\text{l}^{-1}$ have sometimes been reported to inhibit morphinan alkaloid production (Morris and Fowler, 1980; Nessler, 1982), whilst this was not the case with codeine accumulation noted by Tam *et al.* (1980). The presence of cytokinins in the growth medium appear, in general, to be favourable to morphinan alkaloid accumulation ( Hodges and Rapoport, 1982; Staba *et al.*, 1982) as does the addition of ascorbic acid and the precursor tyrosine (Kamimura *et al.*, 1976; Khanna *et al.*, 1978).
Table 1.3.1

Thebaine accumulation in *Papaver bracteatum* cell cultures

<table>
<thead>
<tr>
<th>Source of Alkaloids</th>
<th>Amount of Thebaine</th>
<th>Separation Technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callus (1st subculture) (ca. 1 year old)</td>
<td>60μg.gdwt⁻¹ 130μg.gdwt⁻¹</td>
<td>TLC and GLC</td>
<td>Kamimura <em>et al.</em>, 1976</td>
</tr>
<tr>
<td>Cell suspension (cv.Arya II)</td>
<td>+++</td>
<td>TLC</td>
<td>Shafiee <em>et al.</em>, 1976</td>
</tr>
<tr>
<td>Callus with shoots (cv.Arya II)</td>
<td>70 μg.gdwt⁻¹</td>
<td>TLC and HPLC</td>
<td>Staba <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>Cell suspension (cv.Arya II)</td>
<td>0.03% (fwt)</td>
<td>TLC and HPLC</td>
<td>Zito and Staba, 1982</td>
</tr>
<tr>
<td>Callus with shoots and meristemoids (cv. Arya II)</td>
<td>0.87 μg.gfwt⁻¹</td>
<td>HPLC</td>
<td>Kutchan <em>et al.</em>, 1983</td>
</tr>
</tbody>
</table>

Key: +++ = thebaine present but at unknown level

More recently, production of codeine and morphine by suspension cultures of *P.somniferum* was reported to be enhanced by removal of the plant growth regulators, 2,4-D and kinetin (Siah and Doran, 1991). In these experiments cultures were said to have "developed hormone self-sufficiency without organogenesis or development of meristemoids" and the "enhanced synthesis of morphinan alkaloids was not dependant upon formation of shoots, roots or embryos." In contrast, other reports have stated that upon transfer of *P.somniferum* callus to solid or liquid medium, without plant growth regulators, embryoids were formed which unlike seed embryos accumulated up to 0.2% (dwt) thebaine (Schuchmann and Wellmann, 1983). Despite all of this work the amount of morphinan alkaloids produced by cultures remains low in comparison to the plant, being at best approximately equal to the levels found in leaf tissue (ca. 0.14% dwt), but always much less than in opium (ca. 10-20%).
Table 1.3.2

Morphinan alkaloid accumulation in *Papaver somniferum* cell cultures

<table>
<thead>
<tr>
<th>Source of Alkaloids</th>
<th>Amount of Thebaine</th>
<th>Amount of Codeine</th>
<th>Amount of Morphine</th>
<th>Separation Technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell suspension</td>
<td>1.49% (dwt)</td>
<td>0.34% (dwt)</td>
<td>1.31% (dwt)</td>
<td>TLC</td>
<td>Khanna <em>et al.</em>, 1978</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>---</td>
<td>0.15% (dwt)</td>
<td>---</td>
<td>TLC and GLC</td>
<td>Tam <em>et al.</em>, 1980</td>
</tr>
<tr>
<td>Callus</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>TLC</td>
<td>Hsu, 1981</td>
</tr>
<tr>
<td>Callus with roots</td>
<td>+++</td>
<td>+++</td>
<td>---</td>
<td>TLC and HPLC</td>
<td>Staba <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>Callus</td>
<td>12.7μg.gfw t⁻¹</td>
<td>34.3μg.gfw t⁻¹</td>
<td>1.4μg.gfw t⁻¹</td>
<td>RIA and HPLC</td>
<td>Hodges and Rapoport, 1982.a</td>
</tr>
<tr>
<td>Callus</td>
<td>0.1μg.gdw t⁻¹</td>
<td>33.0μg.gdw t⁻¹</td>
<td>---</td>
<td>TLC and GLC</td>
<td>Kamo <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>Suspensions with shoot buds</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>TLC and GLC</td>
<td>Yoshikawa and Furuya, 1982</td>
</tr>
<tr>
<td>Embryoids</td>
<td>0.2% (dwt)</td>
<td>---</td>
<td>---</td>
<td>TLC</td>
<td>Schuchmann and Wellman, 1983</td>
</tr>
<tr>
<td>Cell suspension (cv. 14-14)</td>
<td>---</td>
<td>3.0mg.gdw t⁻¹</td>
<td>2.5mg.gdw t⁻¹</td>
<td>HPLC</td>
<td>Siah and Doran, 1991</td>
</tr>
</tbody>
</table>

Key: +++ = alkaloid present but at unknown level; --- = alkaloid not detectable

Although many of the studies on cultures of *P. somniferum* and *P. bracteatum* have given fairly inconclusive results some ideas about the optimal conditions for morphinan alkaloid accumulation have been gained. For example, the production of thebaine, codeine and to a lesser extent morphine does not wholly depend on the presence of laticiferous cells. It would seem obvious however, that the formation of latex vessels would substantially enhance the capacity of cultures to produce these alkaloids. The presence of laticifers in somatic embryos (Nessler, 1982), would seem to indicate that the induction of mass-embryogenesis in the final stages of the culture...
cycle might lead to a significant increase in yields (Constable, 1985). As morphinan alkaloids, like other alkaloids (Lindsey and Yeoman, 1983), tend to be accumulated towards the end of the culture cycle, the use of culture conditions that induce slow growth and increase the state of differentiation (e.g. immobilisation) would also appear to advantageous (Lindsey et al., 1983; Yeoman, 1986). Another approach to enhance yields might be to elicit alkaloid production perhaps by use of temperature stress (Lockwood, 1984) or with fungal preparations (Cline and Coscia, 1983; Eilert et al., 1985; Tyler et al., 1989). Indeed, the semi-continuous production of sanguinarine and dihydrosanguinarine by suspension cultures of P. somniferum treated with a fungal homogenate has also been reported (Tyler et al., 1988). However, actual commercial production of morphinan alkaloids by plant cell cultures of Papaver spp. is still some way off, due mainly to the low yields of product.

1.4. Biotransformations of added precursors by plant cell cultures

Whilst the capacity of plant cell cultures to produce secondary products is of considerable commercial interest, only two processes have progressed to an industrial stage (shikonin and berberine production by Mitsui Petrochemical Company, Japan). The major barrier to production of other commercially desirable metabolites is the generally low yield obtained in plant cell cultures. Therefore, a great deal of attention has recently been focused on the ability of cultured plant cells to convert added precursors to desired products (for recent reviews see Suga and Hirata, 1990; Yeoman et al., 1990). The conversion rates achieved are generally rapid and efficient and can often be run (semi-)continuously, making commercial exploitation an attainable goal. In the examples published to date, there are no apparent problems of insufficient yield or product retention. Indeed, in the vast majority of cases the product is almost entirely present in the culture medium, therefore, reducing downstream processing costs. The use of "biological organisms" to transform added precursors, has another advantage, namely, that conversions are usually stereospecific and do not require high temperatures and pressures. Additionally, using plant cell cultures could also be perceived as being "natural", an important factor, especially in the food industry.

There are however, two major problems to be overcome. Firstly, the value added factor must be very high and secondly the product may be further metabolised to a less valuable substance(s). Single-step biotransformations are probably more attractive commercially than multi-step conversions, because the greater the number of steps between precursor and product, then the lower the yield that can be expected. Single-step conversions involving compounds of pharmaceutical interest have
probably received the most attention (eg. Reinhard and Alfermann, 1980; Wichers et al., 1985), although some interest has also been shown in substances associated with the food, fragrance and flavouring industries (eg. Butcher, 1977). Another advantage of studying one-step biotransformation systems is that, as they are controlled by one enzyme, the factors which regulate the conversion are more easily determined.

Biotransformations may be defined as "the conversion of a substance by living cultures, permeabilised cells or entrapped enzymes into a chemically different product" (Yeoman et al., 1990). Steck and Constabel (1974), in the first real review of biotransformations by plant cell cultures listed three requirements for a successful conversion, to which a fourth was later added by Yeoman et al. (1990). These requirements will now be considered in order.

1. The culture must have the enzymes necessary for the transformation of precursor to product

This requirement is probably the most essential, because if the biotransformation is to proceed, then the necessary enzymic activity must be present in the cultured cells in, an unsaturated form and an intracellular location that allows the added substrate to be converted successfully. The importance of selecting the correct species for a particular biotransformation is demonstrated by cultures of *Papaver* spp. As previously stated (1.3), cultures of *P. somniferum* can produce thebaine, codeine and morphine, whilst cultures of *P. bracteatum* only accumulate thebaine. Therefore, although it is not clear whether cultures of *P. bracteatum* lack one or more of the necessary enzyme(s), it can be seen, that the ability of certain plant cell cultures to perform specific conversions must always be considered.

Variations in the biotransforming ability of different cell lines from the same plant have also been demonstrated (Holden et al., 1988). The authors found that the products obtained when immobilised cultures of *Capsicum frutescens* were fed ferulic acid varied significantly. Whilst some cell lines preferentially accumulated vanillin, in others, the major product was *p*-coumaric acid, a precursor of ferulic acid. It was concluded therefore, that the two cell lines differed in their pattern of enzymic activity. This variability in enzyme activity could present a major problem to commercial processes where the maintenance of high biotransformation activity would be essential. One possible solution to this problem is to use fungal elicitors to induce enzyme activity and hence increase product yield (see 1.3).

It has been seen that the endogenous enzyme activity of many cultures can be used to convert added precursors to desirable products. If care is taken in selecting the
culture of the correct cell line or plant spp., then the first requirement for a successful biotransformation can be met.

2. The product must be formed faster than it is further metabolised

Problems can occur when the desired product is not at the end of the biosynthetic pathway but is instead further metabolised. One solution to this problem is to continuously remove the desired product, as it is formed. Another possibility is to select cell lines which do not degrade the product. An example of a product being further metabolised is seen with the conversion of tyrosine to L-DOPA by cultures of *Mucuna pruriens* (Wichers *et al.*, 1984). These authors found that the pharmacologically useful drug, L-DOPA, was further converted to dopaquinone and then to dopachrome, which is of no pharmacological use. Therefore, conditions that reduce the metabolism of L-DOPA, such as the addition of ascorbate, which helps prevent oxidation of the product, need to be developed. However, more detailed studies of secondary product synthesis must first be made, if the true extent of problems presented by degradative reactions are to be fully understood.

3. The culture must tolerate the added precursor and the product

The efficiency of a biotransformation can be reduced when the added precursor is toxic to cells or inhibits the enzymes involved in secondary metabolism. For example, the addition of cinnamic acid to *Capsicum frutescens* cultures, at high concentrations results in the loss of cell viability. Even low levels of this compound reduce phenylalanine ammonia lyase activity. Therefore, this compound is unlikely to be a suitable precursor of the phenylpropanoid branch of the capsaicin biosynthetic pathway (Holden *et al.*, 1988). In some cases, the product formed as a result of a biotransformation can actually be more toxic to cells than the added precursor. An example is the methoxylation of cinchonichine and cinchonine by cultures of *Cinchona ledgeriana* (Robins *et al.*, 1986).

As a result, unless the product is stored in the vacuole or released into the medium, cultured cells must tolerate both the added precursor and the product.

4. The substrate must be able to enter the cell and the product will preferably be released into the medium

The vast majority of biotransformations mediated by plant cell cultures appear to take place intracellularly. Therefore, the added substrate must be able to enter the cell and
the compartment in which the enzyme is situated, if a successful biotransformation is to occur.

Vanillylamine, which is a close precursor to capsaicin, has a very slow solubility in culture medium. This compound is also highly ionised at near neutral pH's and as a consequence, uptake into cells is very low. Therefore, although alteration of pH can increase uptake of vanillylamine, it remains a poor precursor of capsaicin synthesis (Yeoman et al., 1990).

The biotransformation of L-tyrosine to L-DOPA by cultures of *Mucuna pruriens*, is catalysed by tyrosinase (Huizing et al., 1985). The two activities of this enzyme have been found to preside in most subcellular fractions of the cultured cells. However, upon immobilisation of the cells, the proportion of the activity in the cell wall fraction increased. Thus the requirement for the substrate to enter the cells will be reduced (Yeoman et al., 1990).

Uptake of precursors is in fact, rarely a problem, although product release can be a restraint. Destructive and non-destructive means for releasing such products into the medium have been studied, and include the use of solvents, antibiotics and detergents. Immobilisation can also increase product release (Holden et al., 1988), as well as enhancing the conversion efficiency (Furuya et al., 1984; Corchete and Yeoman, 1990). As a result immobilisation is likely to become increasingly used in the exploitation of biotransformations.

**Immobilisation and biotransformations**

Immobilisation encourages cells to grow in a multicellular and partially organised state. This can lead to physical and chemical gradients becoming established, as well as conditions that resemble the situation *in vivo*. These and other advantages, which particularly apply to biotransformation systems have been well documented (Brodelius et al., 1979; Brodelius and Mosbach, 1982; Lindsey et al., 1983; Brodelius, 1985; Lindsey and Yeoman, 1986; Holden and Yeoman, 1987; Yeoman et al., 1990). In particular, so long as the product is removed from the medium, the immobilisation allows a biotransformation process to be run (semi-)continuously. It also means that, substances can be added rapidly to the circulating medium, facilitating, efficient use of relatively small amounts of biomass.
Types of biotransformations reported to date

The conversions reported in plant cell cultures can be divided into six broad groups (Suga and Hirata, 1990). These are:


Biotransformations of morphinan alkaloids by cultures of *Papaver spp.*

Of particular interest to this project are the reactions involving oxidation-reduction between alcohols and ketones as this includes the conversion of codeinone to codeine. Indeed, quite a number of biotransformations involving the reduction of a C=O group have been reported (including: Hirata et al., 1982; Furuya et al., 1978 and 1984; Lappin et al., 1987; Hamada et al., 1988; Corchete and Yeoman, 1990). The biotransformation studied by Furuya et al. (1978 and 1984) and Corchete and Yeoman (1990), was the same as that studied here, namely the reduction of codeinone to codeine by cell suspensions of *P.somniferum*. Indeed, several different biotransformations using cells and cell-free extracts of *P.somniferum* have been observed (Table 1.4.1). Only on one occasion has a multi-step biotransformation of any real economic significance been reported (Hsu, 1981, see Table 1.4.1). In this experiment, radio-labelled tyrosine was fed to suspension cultures of *P.somniferum* and converted to various alkaloids, including thebaine, codeine and morphine. However, when other authors have added thebaine to cultures they have failed to detect any conversion to codeine or morphine (Furuya et al., 1978; Morris and Gibbs, 1986), this despite thebaine being a far closer precursor to codeine and morphine than tyrosine. Indeed, Tam et al., (1982), reported thebaine as being converted to neopine (see Table 1.4.1) instead of codeine and morphine. These findings confirm what was stated earlier, namely that the biotransforming ability of different cell lines
from the same plant spp. can vary significantly and therefore, the cultures must differ in their pattern of enzymic activity.

However, it is the conversion of codeinone to codeine that has received most attention, particularly due to the high percentage of substrate that is converted to product (60.8% - Furuya et al., 1984; 57.0% - Corchete and Yeoman, 1990). Both sets of authors also found that immobilisation, either in calcium alginate or reticulate polyurethane foam respectively, increased the percentage of codeinone reduced to codeine (70.4% - Furuya et al., 1984; 79.0% - Corchete and Yeoman, 1990). Hodges and Rapoport, (1980), were able to obtain codeinone reductase activity from cell-free extracts of P. somniferum suspension cultures and showed that NADH was a co-factor for the reaction. It is also known that during biotransformation the vast majority of the product is released into the medium, which might prove to be of particular economic significance in reducing downstream processing costs.

Despite the many one-step and multi-step biotransformations that have been reported, few workers have studied the relationship of biotransformation capacity with culture origin, stage of development (ie. culture age) and state of differentiation. Therefore, in this study the one-step biotransformation of codeinone to codeine, which is not commercially relevant, has been used as a "model system" to study these relationships.

Numerous different terms have been used to describe the amount of product produced during biotransformations and the activity present in a particular culture type. In this thesis the two terms used to describe and compare the conversion activity of different cultures of Papaver spp. are biotransformation capacity and biotransformation ratio, definitions of which can be found in the abbreviations.

The next section outlines the aims and objectives of the studies described in this thesis.
### Table 1.4.1

**Biotransformations with cells or cell-free extracts of *P.somniferum* cultures**

<table>
<thead>
<tr>
<th>Biotransformation Reported</th>
<th>System Used</th>
<th>Biotransformation Ratio (%)</th>
<th>Reaction Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeinone to Codeine</td>
<td>Immobilised/ Suspended Cells</td>
<td>70.4/60.8</td>
<td>Reduction of C=O</td>
<td>Furuya <em>et al.</em>, 1978 and 1984</td>
</tr>
<tr>
<td>Codeinone to Codeine</td>
<td>Immobilised/ Suspended Cells</td>
<td>79.0/57.0</td>
<td>Reduction of C=O</td>
<td>Corchete and Yeoman, 1990</td>
</tr>
<tr>
<td>Codeinone to Codeine</td>
<td>Cell-free Extract</td>
<td>26.0</td>
<td>Reduction to C=O</td>
<td>Hodges and Rapoport, 1980</td>
</tr>
<tr>
<td>Reticuline to Scoulerine and Cheilanthifoline</td>
<td>Suspended Cells</td>
<td>14.7/0.5</td>
<td>Berberine Bridge Formation</td>
<td>Furuya <em>et al.</em>, 1978</td>
</tr>
<tr>
<td>Reticuline to Salutaridine</td>
<td>Cell-free Extract</td>
<td>80.0</td>
<td>Oxidative Coupling Formation</td>
<td>Hodges and Rapoport, 1982.b</td>
</tr>
<tr>
<td>Tyrosine to Morphine</td>
<td>Suspended Cells</td>
<td>----</td>
<td>Numerous</td>
<td>Hsu, 1981</td>
</tr>
<tr>
<td>Codeine to Morphine</td>
<td>Isolated Capsules</td>
<td>----</td>
<td>Demethylation</td>
<td>Hsu <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>Thebaine to Neopine</td>
<td>Suspended Cells</td>
<td>3.9</td>
<td>Several</td>
<td>Tam <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>Morphine, Codeine and Thebaine to N-Oxides</td>
<td>Crude Enzyme Extract</td>
<td>----</td>
<td>Several?</td>
<td>Vagujfalvi and Petz-Stifter, 1982</td>
</tr>
</tbody>
</table>

**Key:** ---- = biotransformation ratio unknown
1.5. Aims and objectives

The aim of the studies detailed in this thesis was to examine the process by which cell cultures of *Papaver somniferum* and related species, biotransform codeinone to codeine. To achieve this aim the following set of objectives were defined:

1. To study the alkaloid content of plants and cell cultures of several *Papaver* spp. and establish the relationship with the biotransformation capacity of each culture type.

2. To determine the relationship between the stage of development of a culture (ie. culture age) and biotransformation capacity.

3. To establish the relationship between the state of differentiation and the biotransformation capacity of each culture type.

4. To make detailed kinetic studies of the biotransformation so that the rates of conversion in cultures of different origins can be compared.

5. To use the detailed kinetic studies of the biotransformation to compare the nature of the reaction in suspended and immobilised culture systems.
Chapter 2.
MATERIALS AND METHODS
2.1. Plant material

Seeds of the following plants were obtained from McNair, Portobello, Edinburgh:-

*Papaver somniferum*

*Papaver orientale* (scarlet)

*Papaver bracteatum*

Seeds were sown in 10cm pots in an equal mixture of Fisons Levington potting compost and perlite. Germination took place in the Department of Botany greenhouse under these conditions:

- 16h day
- 400Wt mercury vapour light source
- 16-25°C night temperature depending on seasonal variations

After six weeks the seedlings were transferred to compost in 17.5cm pots and grown to maturity.

2.2. Tissue and cell culture

2.2.1. Preparation of culture medium and growth regulators

A supplemented Murashige and Skoog basal salts medium (MS) was used as the standard growth medium for callus, suspension and immobilised cultures of the three species studied. The medium was prepared by dissolving 4.71g.l⁻¹ of a powdered MS basal salts mixture (Imperial Laboratories) in distilled water. This basal medium was supplemented with 30g.l⁻¹ sucrose as a carbon source (BDH Ltd., Poole, Dorset, U.K.), 1mg.l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2mg.l⁻¹ kinetin (both Sigma Chemical Co.). The pH of the medium was then adjusted to pH 5.8 using N potassium hydroxide (BDH Ltd.). In addition to the above supplements, a solidified MS medium containing 8g.l⁻¹ agar (Bacteriological Oxoid No.3, Oxoid Ltd.) was used for callus cultures. The exact composition of the growth medium (MS) is given in Table 2.2.1.
Constituents of supplemented Murashige and Skoog (MS) medium used throughout this investigation

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount in Media (MS), mg.l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂.2H₂O</td>
<td>440</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>FeNaEDTA</td>
<td>36.7</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>370</td>
</tr>
<tr>
<td>MnSO₄.4H₂O</td>
<td>22.3</td>
</tr>
<tr>
<td>NaMoO₄.2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>8.6</td>
</tr>
<tr>
<td>myo inositol</td>
<td>100</td>
</tr>
<tr>
<td>nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>thiamine HCl</td>
<td>0.1</td>
</tr>
<tr>
<td>pyridoxine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>glycine</td>
<td>2.0</td>
</tr>
<tr>
<td>2,4-dichlorophenoxyacetic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>kinetin</td>
<td>0.2</td>
</tr>
<tr>
<td>sucrose</td>
<td>30,000</td>
</tr>
<tr>
<td>pH of medium</td>
<td>pH5.8</td>
</tr>
</tbody>
</table>
2.2.1.1. Germination medium

Sterile seeds were germinated on a half strength (ie. 2.355g.l⁻¹) Murashige and Skoog basal salts medium containing 8g.l⁻¹ agar and 15g.l⁻¹ sucrose. No growth regulators were added to this medium.

2.2.1.2. Stock growth regulator solutions

Stock solutions of the following two growth regulators were used to prepare the standard growth medium (MS).

- kinetin (6-furfurylaminopurine)
- 2,4-D (2,4-dichlorophenoxyacetic acid)

Stock solutions were made, by dissolving either 20mg kinetin in 5ml of 0.1N KOH or 100mg 2,4-D in 5ml ethanol, then slowly adding distilled water to each solution up to a total volume of 100ml. These stock solutions were stored at 4°C and were routinely replaced every two months.

2.2.2. Sterilisation techniques

2.2.2.1. Sterilisation by heat

All glassware, instruments, pretreated polyurethane blocks, distilled water and growth media were sterilised by autoclaving at 121°C for 20min. at a steam pressure of 15 p.s.i.

2.2.2.2. Sterilisation with hypochlorite

Seeds, contained in small muslin bags, were surface sterilised by a rapid pre-sterilisation in 95% (v/v) ethanol for 15sec., then immersed in 10% (v/v) sodium hypochlorite (1.5% available chlorine) for 20min. The seeds were then washed (3 times) in sterile distilled water, prior to being plated out on germination medium (2.2.1.1).
2.2.2.3. Sterilisation with ethanol

All cell culture manipulations were carried out on the bench of a laminar air flow cabinet, over which a continuous stream of sterile air was passed. Internal surfaces were sprayed, then swabbed with ethanol prior to use. Any instruments used for culture manipulations were stored in 95% ethanol (v/v) and flamed immediately before being used.

2.2.3. Initiation and maintenance of cultures

2.2.3.1. Culture conditions

Unless otherwise stated in the text, all cell cultures were grown in a culture room under the following conditions:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>24±°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photon Flux Density/</td>
<td>25μmol.m⁻².sec⁻¹/1050lux</td>
</tr>
<tr>
<td>Illuminance</td>
<td></td>
</tr>
<tr>
<td>Light Source</td>
<td>Compton Warmwhite Fluorescent</td>
</tr>
<tr>
<td>Liquid Culture Agitation</td>
<td>Continuous Rotation in Horizontal Plane Orbital Diameter 1.5cm, 98 r.p.m.</td>
</tr>
</tbody>
</table>

2.2.3.2. Callus cultures

Sterilised seeds were transferred onto 20-25ml solid germination medium (2.2.1.1) contained in polystyrene Petri dishes (Sterilin Ltd., Hounslow, U.K.). The Petri-dishes were double sealed with parafilm (American Can Co., Greenwich, U.S.A.) to exclude micro-organisms and to prevent desiccation. Nine to ten days after germination, whole seedlings or excised hypocotyls were transferred aseptically to Petri dishes containing 20-25ml of the growth medium (MS). Within three weeks pale brown, friable callus formed on the cut ends of hypocotyls or around roots of whole seedlings. The callus was separated from hypocotyl or seedling and aseptically transferred to fresh growth medium (MS). Stock callus cultures were maintained by aseptic transfer to fresh, solid, growth medium every three to four weeks. All Petri dishes were placed on shelves in the culture room under the standard culture
conditions (2.2.3.1).

2.2.3.3. Suspension cultures

Suspension cultures were initiated by transferring 3-5g of friable callus into 250ml conical (Erlenmeyer) flasks containing 50ml of the growth medium. The flasks were covered with a triple layer of aluminium foil to exclude contaminating organisms and placed on a rotary shaker under the standard culture conditions. Subcultures were made every 12-15 days, depending on the growth rate of the cultures. This involved filtering the cultures through sterilised nylon mesh (pore size 64μm) and then using a perforated spoon spatula to transfer 3-4g of cells into 50ml of fresh liquid growth medium.

2.2.3.4. *P. somniferum* "meristemoid" cultures

On initiation of *P. somniferum* callus cultures, small white spheres (0.5-2mm in diameter) of tissue developed on the surface of the callus. These compact masses of cells or "meristemoids" (Nessler and Mahlberg, 1979) were isolated from the main body of callus and maintained on solid growth medium. The "meristemoid" callus cultures were used to initiate "meristemoid" suspension cultures (1-1.5g tissue/flask). Subcultures of the "meristemoid" callus cultures were made every 3-4 weeks but every 18-21 days for the suspension cultures. "Meristemoid" cultures (callus and suspension) were maintained under the same conditions as the other cultures.

2.2.4. Immobilisation of suspended cells

Cell suspension cultures of all *Papaver* spp. (including "meristemoid" cultures) were immobilised in blocks of polyurethane foam, first described by Lindsey *et al.* (1983). The polyether-type polyurethane foam which contained 18 pores.cm⁻¹ (Declon Ltd., Corby, U.K.) was cut into cm³ blocks using a sharp scalpel. Before the blocks could be used for immobilisation work it was necessary to pre-treat them, as described in the next section.
2.2.4.1. Pre-treatment of foam blocks

Prior to experimental use all foam blocks were heat sterilised in a large volume of distilled water as described in 2.2.2.1. The blocks were then soaked in ethanol for 30 min. and finally rinsed (three times) with large quantities of distilled water. Between five and eight blocks were added to the liquid growth medium in each 250 ml flask. The blocks were then heat sterilised along with the liquid medium.

2.2.4.2. Immobilisation procedure

To fully submerge sterile foam blocks in the liquid medium it was necessary to agitate the flasks vigorously, immediately prior to subculturing. Filtered cells (2.2.3.3) from 12-15d old suspension cultures were transferred to the liquid medium (3-4 g flask⁻¹), and the flasks placed on an orbital shaker (2.2.3.1). Within two weeks cells had become entrapped in the foam matrix where they proceeded to grow and divide. After 2-3 weeks, the blocks were transferred, using long sterile forceps, back onto solid growth medium in Petri-dishes. This allowed the blocks to become densely packed with cells prior to being transferred back into fresh liquid growth medium.

2.2.5. Biotransformation experiments

Codeinone, dissolved in 100 μl methanol, was administered to suspension cultures of all types using a 100 μl glass syringe (Scientific Glass Engineering), inserted through the foil top. The resulting puncture mark was then sealed with autoclave tape and the culture gently shaken to ensure complete mixing of culture medium and methanol. Control flasks were administered 100 μl methanol at the same time as their corresponding experimental flasks received methanol containing codeinone.

2.3. Characterisation of culture growth and viability

2.3.1. Determination of fresh weight

The fresh weight of suspended and immobilised cells was determined after separating the cells from their culture medium. Filtration was through two pieces of damp Whatman No.1 filter paper (Whatman International Ltd., Maidstone U.K.), using a Büchner funnel and flask. Cells were then washed three times with distilled water.
(total volume ca. 30ml) to remove any alkaloids from cell surface. All cell types were filtered until there appeared to be no more freely separable medium. The fresh weight of immobilised cells was determined by weighing the foam block containing cells, and subtracting the mean weight of foam blocks which had been saturated in medium then filtered as above.

2.3.2. Determination of dry weight

The dry weight of cells was measured by freeze drying cells, separated from their culture medium (2.3.1). The freeze drier used was an LSL Secfroid, Lyolab A. The dry weight of immobilised cells was determined by weighing the freeze dried block and cells together, then subtracting the mean weight of freeze dried foam blocks.

2.3.3. Determination of percentage dry weight

The percentage dry weight of a culture was calculated by dividing its dry weight (2.3.2) by its known fresh weight (2.3.1) and then multiplying by 100.

2.3.4. Determination of cell number

The cell number of suspended and immobilised cultures was estimated using a Hawksley Crystallite Haemocytometer (Grid Volume 1.8µl). Two ml of a thoroughly mixed suspension culture or one foam block was incubated in 10% (w/v) chromium trioxide solution at room temperature overnight (ca. 16h). Prior to counting, cell aggregates were dispersed by being rapidly agitated up and down a Pasteur pipette. Six grids were counted for each sample, the mean of which was used to estimate the density of the suspended cell population or the immobilised cell number.

2.3.5. Determination of cell viability

Determination of cell viability was by the method of Widholm (1972). It relies upon the ability of living cells to cleave fluorescein diacetate molecules to produce free fluorescein. This is detectable by its bright yellow/green fluorescence under U.V. light.

To prepare a stock solution, 25mg fluorescein diacetate was dissolved in 5ml acetone. This solution was stored at 4°C and routinely replaced at monthly intervals.
Immediately before analysis the stock solution was added dropwise to 5ml of culture medium until a slight turbidity persisted. One drop of this solution was then mixed with one drop of thoroughly mixed cell suspension on a microscope slide and covered with a coverslip. The cells were observed 10min. later under visible and U.V. light using a Vickers Photoplan U.V. Microscope. The number of cells with fluorescent (yellow/green) protoplasm was determined in a random sample of ca. 500 cells. Results have been expressed as percentages of the total cell number.

2.3.6. Determination of packed cell volume

Packed cell volume was determined by centrifuging 50ml of a suspension culture at 3,000xg for 5min. and measuring the volume of the pellet, which was then expressed as a percentage of the total volume.

2.4. Extraction and chemical analysis of the morphinan alkaloids

2.4.1. Extraction of the morphinan alkaloids

2.4.1.1. Extraction from whole plants

Morphinan alkaloids were extracted from plants using a modified method of Brochmann-Hanssen and Cheng (1982). Fresh or frozen plant stems and leaves were cut up into 2/3cm pieces. These pieces were then homogenised with methanol in a high speed blender (ca. 100ml per 40g plant matter). The resulting slurry was filtered through miracloth (Calbiochem, San Diego, California) and then washed several times with methanol. The filtrate was concentrated under reduced pressure and transferred to a separation funnel. It was extracted with five small portions (ca. 20ml each) of ethyl acetate. This removed chlorophyll from the sample. Ethyl acetate fractions were combined and extracted three times with 50ml 0.01N HCl to recover any alkaloids that had passed into the ethyl acetate. Next, the HCl fractions were added to the concentrated methanol fraction and the pH adjusted to pH 9.0 using concentrated NH₄OH. The solution was transferred to a separation funnel and then extracted three times (1min. each) with a chloroform:propan-2-ol (3:1) mixture (total volume 100ml). The three fractions were combined and dissolved water removed by passing the solution through anhydrous sodium sulphate (ca. 10g) in a Büchner funnel. The solution was then transferred to a round bottomed flask and evaporated to dryness under reduced pressure. The morphinan alkaloids present were then taken
2.4.2.4. Alkaloid location reagents for thin layer chromatography

(i) Dragendorff's Reagent

Two stock solutions were prepared as follows:

Solution A; 0.85g bismuth oxynitrate was dissolved in 10ml glacial acetic acid and 40ml d.H₂O added.

Solution B; 16g potassium iodide dissolved in 40ml d.H₂O.

Both solutions were kept at 4°C for up to three months.

Immediately before use, Dragendorff's reagent was prepared by mixing 2.5ml each of Solutions A and B, then adding 25ml 20% (v/v) aqueous acetic acid. The reagent stained morphinan alkaloids deep orange and the background yellow.

(ii) Iodoplatinate Reagent

This reagent was prepared by adding 10ml 5% (w/v) aqueous platinum chloride and 5ml concentrated hydrochloric acid to 240ml 2% (w/v) aqueous potassium iodide. This solution was then filtered through Whatman filter paper No.1 and stored at 4°C until required. Morphinan alkaloids appeared as dark violet spots with the exception of codeinone which appeared as a paler brown-violet spot. The background was a pale pink colour. The solution was kept for up to 6 months.

2.4.3. Analysis of alkaloids by high performance liquid chromatography

High performance liquid chromatography (HPLC) was used for accurate quantitative analysis of extracts.

2.4.3.1. High performance liquid chromatography system

HPLC was performed using a Gilson 302 liquid chromatograph fitted with a C18 Nova-Pak reverse-phase column (Waters 150mm x 3.9mm) and a Gilson 111B U.V. detector. The system was controlled by an IBM PS2 model 50 microcomputer using the Gilson HPLC system controller 714V1.2. All data handling and processing were performed using the same software. Samples were loaded onto the column by
means of a Rheodyne sample injector 7125 fitted with a 100μl sample loop.

2.4.3.2. Preparation of mobile phases

All solvents used, were of HPLC grade or equivalent. Prior to use, all mobile phases were filtered under reduced pressure through 0.45μm Nylon-66 membrane filters (Rainin Instrument Co.) to remove any particles that might block or damage the HPLC system. Immediately before use, all mobile phases were degassed for 30min. by passing a steady stream of helium (BOC) through them via a sparger. This was to prevent air bubbles forming within the HPLC system as a result of the mobile phase being subjected to large pressure differences. Mobile phase reservoirs were sealed with Parafilm to reduce the build up of dissolved gases and prevent the entry of dust particles.

2.4.3.3. Preparation of samples for high performance liquid chromatography analysis

Samples were extracted as described in sections 2.4.1.1 and 2.4.1.2. Samples for analysis using HPLC were finally taken up in 1ml acetonitrile. These solutions were then filtered using a Bioanalytical Systems Microfilter fitted with a 0.45μm Nylon-66 membrane filter (Rainin Instrument Co.) and centrifuged at 1000xg for 5min. The filtrate was transferred to a 1.5ml crimp top rubber sealed sample vial (Chromacol, London). Any samples not analysed immediately were stored at 4°C for a maximum of three weeks prior to analysis.

2.4.3.4. Operating conditions for the separation of codeine and codeinone

The mobile phase used for the separation of codeine and codeinone consisted of 25mM sodium dihydrogen orthophosphate, 10% (v/v) acetonitrile and 0.1% (w/v) triethylamine in water. The pH was adjusted to pH3.5±0.1 using powdered citric acid. This mobile phase was pumped isocratically at a flow rate of 1.5ml.min⁻¹. Alkaloids were detected by their U.V. absorption at 254nm, sensitivity being set at 0.1 Aufs. Prior to each analysis run, mixed external standards of codeine and codeinone were injected onto the column at three levels 50μg, 25μg and 10μg (amount on column), each level being duplicated. This enabled the system software to calculate directly the quantity of codeine or codeinone present in samples from the area under each peak. A linear regression analysis (2.5.2) was performed on the peak
areas given by standards during each analysis run. This allowed an assessment to be made of both the linear nature of the standard curves and of the overall column performance. Where discrepancies occurred, then internal standards were also used. The run time for each sample was 15min., which enabled the baseline to equilibrate between each sample injection. The mean retention times of codeine and codeinone were 3.8min. and 7.8min. respectively. The retention times of both compounds varied due to deteriorating column performance and fluctuations in the ambient temperature. Fig.2.4.3 shows the HPLC traces obtained for a codeine/codeinone mixture when injected onto a column equilibrated to operating conditions. Fig.2.4.4 meanwhile, shows the corresponding standard curve obtained from the same run with the appropriate regression line fitted (section 2.5.2).
Fig. 2.4.3 HPLC traces obtained for a codeine/codeinone mixture (two repeats at three levels), separated using conditions stated in 2.4.3.4. Alkaloids were detected by their U.V. absorption at 254nm. Peak 1 corresponds to codeine whilst Peak 2 corresponds to codeinone.
Fig.2.4.4 Peak areas obtained for a codeine/codeinone mixture (two repeats at three levels), separated by HPLC using conditions stated in 2.4.3.4. The plotted line is a "best-fit" regression line (2.5.2). Correlation coefficient \( p \), was 0.998 for codeine and 0.999 for codeinone.
2.5. Mathematical analysis of data

2.5.1. Calculation of standard error of a mean

In most experiments, three replicates (n) were used for each treatment. The mean (\( \bar{x} \)) of these three values was calculated (using equation 1.), the standard error (S.E) of the mean was then determined (equation 3.) having first calculated the standard deviation (S.D., equation 2.)

\[
\bar{x} = \frac{\Sigma x}{n} \quad \text{Equation 1.}
\]

\[
S.D. = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}} \quad \text{Equation 2.}
\]

\[
S.E. = \frac{S.D.}{\sqrt{n-1}} \quad \text{Equation 3.}
\]

The mean value obtained for a particular treatment (± its S.E. where shown) was used in all subsequent figures and tables. This provided an indication as to the amount of variation, both within that particular treatment and between it and other treatments.

2.5.2. Linear regression analysis of high performance liquid chromatography standard curves

To determine the relationship between two variables x and y, in order to predict the value of one variable from that of the other, requires the determination of the regression line. The two variables being considered here were the amount of standard alkaloid (x) and peak area obtained for that alkaloid (y) during HPLC analysis. The best fitting straight line (equation 4.) through the values obtained was defined as the line from which the sum of the squares of deviation of the predicted y-values from the observed values was a minimum (Hayslett, 1983). The nature of this line was determined (equations 5 and 6.) and then plotted through the data points obtained. The correlation coefficient (\( \rho \)) was also calculated (equation 7.) for each regression line. This always has a value of between +1 and -1. A value of +1 indicates a perfect, positive relationship between x and y, a value near zero indicates no linear
relationship and a value of -1 indicates a perfect, negative linear relationship. All values obtained for $\rho$ were between +0.995 and +1.000.

$$y = A + B.x$$  \hspace{1cm} \text{Equation 4.}$$

$$B = \frac{n.\Sigma x.y - \Sigma x.\Sigma y}{n.\Sigma x^2 - (\Sigma x)^2}$$  \hspace{1cm} \text{Equation 5.}$$

$$A = \frac{\Sigma y - B.\Sigma x}{n}$$  \hspace{1cm} \text{Equation 6.}$$

$$\rho = \frac{n.\Sigma x.y - \Sigma x.\Sigma y}{\sqrt{(n.\Sigma x^2 - (\Sigma x)^2).(n.\Sigma y^2 - (\Sigma y)^2)}}$$  \hspace{1cm} \text{Equation 7.}$$

where:

$A =$ y-intercept of regression line

$B =$ slope of regression line

$\rho =$ correlation coefficient

2.5.3. Calculation of the mean relative growth rate, $\bar{r}$

The relative growth rate of a culture (RGR) is defined as the increase in plant material per unit of material per unit of time (Hunt, 1979). The mean relative growth rate of a culture ($\bar{r}$) is simply the mean of all the instantaneous (theoretical) RGR's between two defined time points and was calculated using equation 8.

$$\frac{\log_{e.2}W - \log_{e.1}W}{2T - 1T}$$  \hspace{1cm} \text{Equation 8.}$$

where

$1.2\bar{r} =$ Mean RGR between Timepoints 1 and 2

$1T =$ Initial Time Point (d)

$2T =$ Final Time Point (d)

$1W =$ Initial Dry Weight of Culture (g)

$2W =$ Final Dry Weight (g)

The calculation of $\bar{r}$ enabled the growth rates of different types of suspension culture to be compared, having taken into account the initial dry weight of each culture.
Chapter 3.
RESULTS
3.1. Distribution of morphinan alkaloids in plants of *Papaver* spp.

The aim of this set of experiments was to study the distribution of morphinan alkaloids in the plants of *Papaver* spp. used in this study. The plants under investigation were *Papaver somniferum* (P.s), *P.bracteatum* (P.b) and *P.orientale* var."Scarlet" (P.o). Many species of *Papaver* exist in chemical races (Phillipson, 1983 and references therein) so it was important to screen the plants for their alkaloid content prior to initiating callus cultures. This would enable comparisons to be made between the alkaloid content of the parent plant and the cultures derived from them and also indicate which of the poppy cultures are most likely to show activity for the biotransformation of codeinone to codeine. As a consequence it should be possible to establish the nature of the relationship between the biotransformation capacity of a culture and the plant chemotype from which it was derived. The alkaloids of particular interest were the morphinan alkaloids thebaine, codeinone, codeine and morphine.

3.1.1. Morphinan alkaloids in plants of *Papaver somniferum*

The aim of this experiment was to screen plants of *P.s* for the presence of morphinan alkaloids. The morphinan alkaloids thebaine, codeine and especially morphine are characteristic of *P.s* and *P.setigerum* (P.stg). Indeed, some workers regard *P.stg* to be a sub-species of *P.s*. There has only been one report of morphine from a species other than these two plants which was as a minor alkaloid from poppy heads of *P.decaisnei* (Slavík, 1980). However, thebaine is found in a whole series of plants from the sections Orthorhoeadas, Pilosa, Milantha and Macrantha (see Introduction). Morphine and codeine appear to be unique to *P.s* and *P.stg*.

In addition to numerous garden hybrids the following varieties of *P.s* are recognised:

(i) *P.somniferum* var. *glabrum* Boiss - cultivated in Turkey
(ii) *P.somniferum* var. *album* D.C. - cultivated in India
(iii) *P.somniferum* var. *nigrum* D.C. - cultivated in Europe
(iv) *P.somniferum* var. *setigerum* D.C. - truly wild form found in southern Europe

There is some variation between species in colour and shape of leaves, seeds and flowers. However, all are believed to produce morphinan type alkaloids though the
proportions do vary from species to species. The photograph in Fig.3.1.1 shows some plants of *P.s* used during this work. The exact variety is unknown but the petal (Fig.3.1.2.a) and seed (Fig.3.1.2.b) colour are consistent with *P.somniferum* var. *nigrum* D.C. or a closely related garden hybrid.

Plants of *P.s* were grown in the Department of Botany greenhouse under the conditions stated in 2.1.1. Three or four days after petal fall portions of stem and capsule were removed and extracted for morphinan alkaloids as described in 2.4.1.1.

The extract was run on a TLC plate using solvent system T2 (2.4.2.2) and visualised with Dragendorff’s reagent (Fig.3.1.3). It shows the presence of six Dragendorff positive spots two of which (spots c and f) cochromatograph with standard codeine (spot A) and thebaine (spot C) respectively. However, some spots (a/b, c/d and h/i) appeared to contain more than one compound. Therefore to see if any other Dragendorff positive spots could be identified, a sample of the same extract was separated by 2D TLC using solvent systems T1 and T2 (2.4.2.2). The plate obtained was visualised with Dragendorff’s reagent and is shown in Fig.3.1.4. This produced nine Dragendorff positive spots as compared to the six obtained in Fig.3.1.3. Spots c and f cochromatograph with standard codeine (spot A) and thebaine (spot C) respectively in both dimensions. Spot a, was suspected of being morphine due to its size and low mobility in these two solvent systems. To confirm this suspicion, a second plate was run under the same conditions as in Fig.3.1.3. Instead of visualising this plate with Dragendorff’s reagent the silica gel was scraped from the plate in the region corresponding to spot a. Any substances present were then eluted by stirring for 4h in 10ml of methanol. This extract was concentrated under reduced pressure to ca. 1ml, 100μl of which was then run on TLC plates using solvent systems TA, TB and TC as described in 2.4.2.3 (Fig.3.1.5). The single Dragendorff positive spot obtained in all three TLC systems had Rf values which corresponded with those published in Clarke’s: Isolation and Identification of Drugs (2.4.2.3). This provides strong evidence that the unknown alkaloid was indeed morphine. Similar extracts were obtained from the spots suspected of being codeine and thebaine. The presence of these two alkaloids was then confirmed by running these two extracts under the same conditions as described in Fig.3.1.5 (results not shown). Further proof for the presence of morphine was obtained by studying the UV spectra of the compound in strong acid and strong alkali. This is because a peak shift from 289nm to 298nm occurs for morphine under these extreme conditions (Seagers *et al.*, 1952). The UV spectra obtained from the alkaloid spot are presented in Fig.3.1.6 and show that this characteristic peak shift occurred. It should be noted that closely related alkaloids such as normorphine also demonstrate this UV shift but with the additional evidence
shown in Fig. 3.1.5 it was concluded that spot a was morphine. The total amount of alkaloid present in these plants was semi-quantitatively determined (2.4.2.1) and found to ca. 0.2% (w/w).

Morphine is synthesised in P.s plants by the demethylation of thebaine and codeine, though a second pathway from thebaine via oripavine and morphinone has also been shown to occur (Brochmann-Hanssen, 1984). The predominance of one pathway over another may depend upon the relative activities of the 3-O-methyloxidase and the 6-O-methyloxidase acting on thebaine (Brochmann-Hanssen, 1985). The presence of codeine in the plant extract means that the first pathway to morphine synthesis does operate in the plants and that as a consequence the codeinone to codeine step must also prevail. This is because in plants codeine cannot be formed from morphine and the synthesis of codeinone from thebaine via neopinone is generally regarded as being "irreversible" (Brochmann-Hanssen, 1984). Therefore, the codeine accumulated must have been produced via the first pathway. A more detailed description of the pathway can be found in the introduction.

Having established the presence of the morphinan alkaloids thebaine, codeine and morphine in plants of P.s, the next section describes the results obtained when plants of P.b were screened for the presence of these same alkaloids. If plants of P.b do not contain thebaine, codeine or morphine then it would be possible to establish the relationship between the biotransformation capacity of P.s and P.b cultures and the alkaloid profile of the parent plant.
Fig. 3.1.1 Plants of *Papaver somniferum* grown to maturity in the Department of Botany greenhouse under the conditions stated in 2.1.1. On average the plants grew to between 0.9 and 1.3m in height.
Fig. 3. Flower, seeds and dried capsules of mature *Papaver somniferum* plants grown in the Department of Botany greenhouse under the conditions stated in 2.1.1. Bar = 1cm.
Fig. 3.1.3 Alkaloids extracted from plants of *Papaver somniferum* and separated by one dimensional TLC using solvent system T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the plate was visualised with Dragendorff’s reagent (2.4.2.4). Key: Cd-OH = standard codeine (A), Cd=O = standard codeinone (B), The = standard thebaine (C), Mix = mixture of standard alkaloids and Ext = Extract of *Papaver somniferum*. 
Fig. 3.1.4 Alkaloids extracted from plants of *Papaver somniferum* and separated by two dimensional TLC using solvent systems T1 and T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff's reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [A], codeinone [B] and thebaine [C]) and Ext = extract of *Papaver somniferum*. 
Fig. 3.1.5 Identification of morphine extracted from plants of *Papaver somniferum* and separated by one dimensional TLC using solvent systems TA, TB or TC (2.4.2.3). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff’s reagent (2.4.2.4). Key: Cd-OH = standard codeine (A), Cd=O = standard codeinone (B), The = standard thebaine (C) and Ukwn = unknown alkaloid (a).
Fig. 3.1.6 U.V. spectra of morphine extracted from plants of *Papaver somniferum* in 1M HCl and 1M NH₄OH indicating characteristic peak shift (Seagers *et al.*, 1952).
The aim of this experiment was to screen the plants of P. bracteatum used in this study for the presence of morphinan alkaloids. The previous experiment had shown that plants of P. somniferum do contain the morphinan alkaloids thebaine, codeine and morphine. Therefore, if P. bracteatum plants have a different alkaloid profile from P. somniferum plants, it would be possible to establish the relationship between the biotransformation capacity of cultures derived from both these plants and the parent plant chemotype.

P. bracteatum is a tall (60-90cm) perennial with whitish, bristly stems. The occurrence of the morphinan alkaloid thebaine in P. bracteatum is a characteristic shared with many other plants (Preininger, 1985). The biosynthesis of thebaine in P. bracteatum has been shown to follow the same pathway as in P. somniferum (Hodges et al., 1977; Brochmann-Hanssen and Wunderly, 1978) and P. bracteatum was thought not to accumulate other morphinan alkaloids due to the lack of active O-methyloxidases. However, detailed studies have revealed that P. bracteatum does contain small amounts of codeine, neopine and oripavine as well as 14-β-hydroxycodeinone, 14-β-hydroxycodeine and both isomers of thebaine-N-oxide (Kuppers et al., 1976; Phillipson et al., 1976; Theuns et al., 1977; Meshulam and Lavie, 1980). Thus it appears that P. bracteatum does possess O-methyloxidases but that these enzymes are comparatively inactive with respect to the corresponding enzymes in P. somniferum and as a consequence the major alkaloid accumulated by P. bracteatum is thebaine.

Plants of P. bracteatum were grown in the Department of Botany greenhouse (Fig. 3.1.7) under the conditions stated in 2.1.1. Unfortunately, it was not possible to induce the plants to flower and therefore no capsules or seeds were produced. This was due to the plants being susceptible to low winter temperatures in the greenhouse and whitefly. However, alkaloids would still be present in the vegetative structures of the plant as the site of alkaloid accumulation (and possibly biosynthesis) is the latex found in laticiferous cells and not the capsules or seeds. Further details of alkaloid accumulation in plants of Papaver spp. can be found in the introduction. It has been shown that laticiferous cells appear as early as a few days after germination (Nessler and Mahlberg, 1978). Therefore, it is reasonable to assume that these individuals would contain alkaloids though not necessarily in the proportions found in more mature plants. Due to the lack of poppy capsules, leaves and roots of the plant were extracted for morphinan alkaloids. In plants of P. bracteatum that have flowered 60% of the total thebaine produced is reported to occur in the roots whilst the remaining 40% resides in the aerial portions. The extraction procedure used was that described in 2.4.1.1 and the samples were run on a TLC plate using solvent system T2 (2.4.2.2).
The resultant TLC plate (Fig.3.1.8) showed the presence of four Dragendorff positive spots in the extract of leaves and roots. One of these (spot 4) cochromatographs with standard thebaine (spot C). However, due to their shape it appeared that two of the spots (1/2 and 5/6) might contain more than one alkaloid. Therefore, the extract was separated by 2DTLC using solvent systems T1 and T2 (2.4.2.2) to see whether other Dragendorff positive spots could be isolated. The resulting plate (Fig.3.1.9) showed six alkaloid spots compared to the four that were obtained by 1DTLC (Fig.3.1.8). One of these spots (spot 4) cochromatographed with standard thebaine (C) in both dimensions but none of the others corresponded to standard codeine (spot A) or codeinone (spot B). Additionally, there was no spot at the Rf value attributable to morphine. To confirm the presence of thebaine a second plate was run under the same conditions as in Fig.3.1.9. However, this plate was not sprayed with Dragendorff’s reagent. Instead, the silica gel was scraped from the same region as spot 4 and extracted by stirring for four hours in 10ml of methanol. This extract was filtered and concentrated under reduced pressure to ca. 1ml, 100μl spotted onto TLC plates and run using solvent systems TA, TB and TC (Fig.3.1.10). The single Dragendorff positive spot obtained in each of the three systems cochromatographed with standard thebaine (spot C) and all had Rf’s that corresponded with those published in Clarke’s Identification of Drugs (2.4.2.3). This provides strong evidence that the compound present was thebaine. The other alkaloids present were not formally identified due to the lack of appropriate standards. However, some 25 or so alkaloids have been isolated from P.b plants. The fact that only six alkaloids were extracted from the plants being studied here is probably due to the pH at which extractions were performed. The pH used (pH9.0) was optimal for phenolic alkaloids (see Bryant, 1988) but not necessarily for any other alkaloids present. It is also likely that other alkaloids were present in the extract but at concentrations too low to be detected using the procedures described here.

Having established the presence of the morphinan alkaloid thebaine, but not codeine or morphine, in plants of P.b it was concluded that cultures derived from these plants could be used to investigate the relationship between the biotransformation capacity of a culture and the plant chemotype from which it was derived (3.5.5). The next section describes the results obtained when plants of P.o were screened for the presence of these same alkaloids. The total number of alkaloids detected in plants of P.o is only about one half of those detected in P.b (Šántavý, 1979). However, plants of P.o do accumulate thebaine but unlike P.b, codeine has never been reported. This implies that no suitable O-methyloxidases exist in P.o or if they do then they are inactive. Thus, cultures of P.o could be used to help establish the relationship
between biotransformation capacity and plant chemotype.
Fig.3.1.7 Three month old plants of *Papaver bracteatum* grown in the Department of Botany greenhouse under the conditions stated in 2.1.1. On average plants grew to between 0.3 and 0.5m in height.
Fig.3.1.8 Alkaloids extracted from plants of *Papaver bracteatum* and separated by one dimensional TLC using solvent system T1 (2.4.2.2). The amount of standard alkaloid applied was 20µg and the plate visualised with Dragendorff's reagent. Key: Cd-OH = standard codeine (A), Cd=O = standard codeinone (B), The = standard thebaine (C) and Ext = extract of *Papaver bracteatum*. 
Fig. 3.1.9 Alkaloids extracted from plants of *Papaver bracteatum* and separated by two dimensional TLC using solvent systems T1 and T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff's reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [A], codeinone [B] and thebaine [C]) and Ext = extract of *Papaver bracteatum*. 
Fig.3.1.10 Identification of thebaine extracted from plants of *Papaver bracteatum* and separated by one dimensional TLC using solvent systems TA, TB or TC (2.4.2.3). The amount of standard alkaloid applied was 20\(\mu\)g and the plate visualised with Dragendorff’s reagent (2.4.2.4). Key: Cd-OH = standard codeine (A), Cd=O = standard codeinone (B), The = standard thebaine (C) and Ukwn = unknown alkaloid (4).
3.1.3. Morphinan alkaloids in plants of *Papaver orientale* var."Scarlet"

The aim of this experiment was to determine which of the morphinan alkaloids were present in plants of *P.o*. The results from the previous experiment (3.1.2) have shown that plants of *P.b* do contain thebaine but lacked detectable quantities of codeine and morphine. However, the presence of trace amounts of codeine has been reported in *P.b* (Kuppers *et al.*, 1976) which implies that O-methyloxidases do exist in *P.b* but that they are relatively inactive in comparison to the corresponding enzymes in *P.s*. It might also suggest that cultures derived from *P.b* possess a limited biotransformation capacity for converting codeinone to codeine. On the other hand plants of *P.o* have never been shown to produce even trace amounts of codeine. Assuming that the synthesis of codeine follows the same pathway as in *P.s* this would imply that *P.o* lacks active O-methyloxidases. Consequently, by determining whether cultures derived from *P.o* are able to convert codeinone to codeine it would be possible to establish the relationship between the biotransforming activity and the parent plant chemotype.

*P.o* is an erect perennial (2 to 3ft high) with an unbranched, sparsely leafed stem. Unfortunately, it was not possible to induce *P.o* plants to flower because the plants were susceptible to low winter temperatures in the greenhouse and whitefly. Due to the lack of poppy capsules only leaves and roots were extracted for morphinan alkaloids. The procedure used was that described 2.4.1.1. The extract obtained was run on a TLC plate using solvent system T2 (2.4.2.2) a copy of which is shown in Fig.3.1.11. This shows the presence of three Dragendorff positive spots one of which (spot ii) cochromatographs with standard thebaine (spot C). However, due to its shape spot iii/iv might contain more than one alkaloid. Therefore, the plant extract was separated by 2DTLC using solvent systems T1 and T2 (2.4.2.2) to see whether other Dragendorff positive spots could be identified. The resulting plate (Fig.3.1.12) showed four alkaloid spots compared to the three obtained by 1DTLC (Fig.3.1.11). One of these (spot ii) cochromatographed with standard thebaine (spot C) in both dimensions but none of the others corresponded to standard codeine (spot A) or codeinone (spot B). However, two of the spots (iii and iv) did possess the same Rf values as two of the spots (5 and 6) obtained from *P.b* (Fig.3.1.9). Apart from thebaine the only alkaloids reported in both *P.o* and *P.b* are bracteoline, isothebaine, mecambridine, papaverrubine B,D and E and protopine (Šantavý, 1979). Therefore, the two spots (5 and 6 on Fig.3.1.9; iii and iv on Fig.3.1.11) could well correspond to one or more of these alkaloids, though a more detailed investigation would be required to establish this properly. To confirm the presence of thebaine a second
plate was run under the same conditions as in Fig. 3.1.12. However, this plate was not visualised with Dragendorff’s reagent but instead the silica gel was scraped from the same region as spot ii and extracted by stirring for four hours in 10ml methanol. This extract was then filtered and concentrated under reduced pressure to about 1ml, 100µl of which was then run on TLC plates using solvent systems TA, TB and TC (Fig. 3.1.13). The single Dragendorff spot obtained in all three systems cochromatographed with standard thebaine and all had Rf values which corresponded with those published in Clarke’s Identification of Drugs (2.4.2.3). This provides strong evidence that the compound present was thebaine.

Having established that plants of P. o do contain thebaine but not codeine or morphine it was concluded that cultures derived from these plants could be used to investigate the relationship between the biotransformation capacity of a culture and the plant chemotype from which it was derived (3.5.5).

To study the biotransformation capacity of cultures it was first necessary to obtain callus which could then be used to initiate suspension cultures of the three species under investigation. The next section describes the procedures used to establish callus cultures of P. s. A latin-square experiment was devised to determine the concentrations of plant growth regulators necessary to give both good growth and friable callus, suitable for the initiation of suspension cultures. The calli were also screened for the presence of alkaloids so that those with high levels of alkaloid accumulation could be identified. The optimum concentrations of plant growth regulators used in the growth medium would then be used to establish calli of P. b and P. o. This ensured that direct comparisons could be made between the various cultures used to study the biotransformation of codeinone to codeine.
Fig.3.1.11 Alkaloids extracted from plants of *Papaver orientale* var."Scarlet" and separated by one dimensional TLC using solvent system T1 (2.4.2.2). The amount of standard alkaloid applied was $\mu$g and the plate visualised with Dragendorff's reagent (2.4.2.4). Key: Cd-OH = standard codeine (A), Cd=O = standard codeinone (B), The = standard thebaine (C), Mix = mixture of standard alkaloids and Ext = extract of *Papaver orientale*. 
Fig.3.1.12 Alkaloids extracted from plants of *Papaver orientale* var."Scarlet" and separated by two dimensional TLC using solvent systems T1 and T2 (2.4.2.2). The amount of standard alkaloid applied was μg and the plate visualised with Dragendorff's reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [A], codeinone [B] and thebaine [C]) and Ext = extract of *Papaver orientale.*
Fig.3.1.13 Identification of thebaine extracted from plants of *Papaver orientale* var. "Scarlet" and separated by one dimensional TLC using solvent systems TA, TB or TC (2.4.2.3). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff’s reagent (2.4.2.4). Key: Cd-OH = standard codeine (A), Cd=O = standard codeinone (B), The = standard thebaine (C) and Ukwn = unknown alkaloid (ii).
3.2. Optimisation of plant growth regulator concentration for callus initiation and growth

The aim of this experiment was to optimise the amount of auxin and cytokinin added to the growth medium, for the initiation and maintenance of callus cultures from sterile seedlings of *P. somniferum*. Suspension cultures of *P.s*, derived from these callus cultures, would then be used to study the biotransformation of codeinone to codeine. The optimum concentrations of plant growth regulators would also be used to establish callus and suspension cultures of *P.b* and *P.o*, which would then be studied for any biotransforming activity. Callus cultures of *P.s* were also analysed, semi-quantitatively (2.4.2.1), for the presence of morphinan alkaloids.

Most studies conducted on PGR's in cultures of *Papaver* spp. have varied PGR concentration as a means to optimise alkaloid production. The results of such evaluations have not been entirely successful, with no clear indication of any superior medium. However, concentrations of 2,4-D in excess of 1mg.l⁻¹ were often found to prevent morphinan alkaloid accumulation (Constabel, 1985), with the exception of codeine accumulation observed by Tam *et al.* (1980). Alternatively the presence of the cytokinins, benzyladenine and kinetin is reported to be beneficial for codeine production in cultures (Staba *et al.*, 1982; Hodges and Rapoport, 1982).

Cultures of *Papaver* spp. have been initiated from a variety of sources including seedling hypocotyls (Hsu, 1981; Corchete and Yeoman, 1990), whole seedlings (Furuya *et al.*, 1984; Day *et al.*, 1986), and also capsules of *P. somniferum* (Furuya *et al.*, 1978). In preliminary experiments to initiate callus, excised hypocotyls from sterile seedlings were used as the source of plant tissue. These were relatively unsuccessful with only limited callus formation. The sterile excision of hypocotyls also proved to be quite time consuming. However, plating out whole sterile seedlings proved to be both less time consuming and also less prone to contamination.

A number of different plant growth regulators (PGR) have been used in the culture medium of *Papaver somniferum* and *P. bracteatum*. Auxins used include 2,4-D, IBA and NAA whilst cytokinins used include kinetin, BA, zeatin and 2iP (Constabel, 1985). The auxin chosen for this experiment was 2,4-D, as its successful use has been reported over a fairly wide range (0.02-4.0mg.l⁻¹). The cytokinin chosen was kinetin, again because its successful use is well documented but in the lower range of 0.03-1.0mg.l⁻¹. A Latin-Square experiment was devised using various levels of 2,4-D (0.5, 1.0, 2.0 and 4mg.l⁻¹) and kinetin (0.05, 0.1, 0.2 and 0.4mg.l⁻¹) in the medium. This gave a total of 16 different treatments which were numbered as indicated in
Table 3.2.1. Six replicates were set up for each individual treatment.

Table 3.2.1

Numbers assigned to each different Plant Growth Regulator treatment

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<thead>
<tr>
<th>Amount of Kinetin in Medium (mg.l⁻¹)</th>
<th>0.40</th>
<th>0.20</th>
<th>0.10</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 1.0 2.0 4.0</td>
<td>13</td>
<td>9</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>14   10   6    2</td>
<td>15</td>
<td>11</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>16   12   8    4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ten day old sterile seedlings of *P. somniferum* (2.2.3.2) were individually placed on 20-25ml of MS medium (2.2.1) in 9cm Petri dishes containing the different PGR regimes. After 21d incubation under the standard conditions (2.2.3.1), the seedlings were assessed for any callus produced. Results were recorded separately for the radicle, hypocotyl and cotyledon/epicotyl regions of each seedling. Any callus initiated was weighed and transferred onto fresh medium at the same PGR levels, then incubated under the standard conditions for 21d. If the callus grew successfully it was reweighed and its RGR calculated. Several calli from these successful regimes were then pooled and assessed for their alkaloid content (2.4.1.2 and 2.4.2.1). Concentrated samples were loaded onto TLC plates and separated using solvent system T2 (2.4.2.2). Alkaloids were semi-quantified by comparing the visualised spots of extracts with those obtained from known quantities of standard alkaloids. Photographs of representative samples from each of the 16 different treatments used, are shown on the following four pages (Fig.3.2.1 to Fig.3.2.4). The overall results are summarized in Table 3.2.2.
Fig. 3.2.1 Photographs of seedlings plated onto medium containing different amounts of 2,4-D and kinetin (No. 1-4) for the initiation of callus. Quantities of each PGR are expressed in mg l⁻¹.
Fig. 3.2.2 Photographs of seedlings plated onto medium containing different amounts of 2,4-D and kinetin (No. 5-8) for the initiation of callus. Quantities of each PGR are expressed in mg.l⁻¹.
Fig. 3.2.3 Photographs of seedlings plated onto medium containing different amounts of 2,4-D and kinetin (No. 9-12) for the initiation of callus. Quantities of each PGR are expressed in mg l⁻¹.
Fig. 3.2.4 Photographs of seedlings plated onto medium containing different amounts of 2,4-D and kinetin (No.13-16) for the initiation of callus. Quantities of each PGR are expressed in mg.l⁻¹.
Table 3.2.2

Effect of different 2,4-D and kinetin levels upon initiation and maintenance of callus from sterile seedlings of *Papaver somniferum*

<table>
<thead>
<tr>
<th>Treatment No.</th>
<th>2,4-D/kinetin</th>
<th>Radicle</th>
<th>Hypocotyl</th>
<th>Epicotyl/ Cotyledon</th>
<th>Alkaloid Content</th>
<th>f (g.g⁻¹).day⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5/0.05</td>
<td>A Lb M</td>
<td>NVC</td>
<td>NVC El</td>
<td>Th</td>
<td>0.019</td>
</tr>
<tr>
<td>2</td>
<td>1.0/0.05</td>
<td>A Lb M</td>
<td>NVC</td>
<td>NVC</td>
<td>-</td>
<td>0.020</td>
</tr>
<tr>
<td>3</td>
<td>2.0/0.05</td>
<td>NVC</td>
<td>F Db</td>
<td>F Lb</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>4.0/0.05</td>
<td>NVC</td>
<td>W Db</td>
<td>W Db</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>0.5/0.1</td>
<td>A Lb M</td>
<td>NVC</td>
<td>W Db</td>
<td>Th</td>
<td>0.024</td>
</tr>
<tr>
<td>6</td>
<td>1.0/0.1</td>
<td>NVC</td>
<td>NVC</td>
<td>W Db</td>
<td>Th</td>
<td>0.040</td>
</tr>
<tr>
<td>7</td>
<td>2.0/0.1</td>
<td>NVC</td>
<td>F Db</td>
<td>W Db</td>
<td>-</td>
<td>0.023</td>
</tr>
<tr>
<td>8</td>
<td>4.0/0.1</td>
<td>NVC</td>
<td>W Lb</td>
<td>W Lb</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>0.5/0.2</td>
<td>F Lb</td>
<td>NVC</td>
<td>El</td>
<td>Th Cd</td>
<td>0.050</td>
</tr>
<tr>
<td><em>10</em></td>
<td>1.0/0.2</td>
<td>F Lb M</td>
<td>F Db</td>
<td>F Lb</td>
<td>Th Cd Mo</td>
<td>0.053</td>
</tr>
<tr>
<td>11</td>
<td>2.0/0.2</td>
<td>NVC</td>
<td>W Db</td>
<td>W Db</td>
<td>-</td>
<td>0.011</td>
</tr>
<tr>
<td>12</td>
<td>4.0/0.2</td>
<td>M</td>
<td>NVC</td>
<td>W Db</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>0.5/0.4</td>
<td>NVC</td>
<td>F Lb</td>
<td>NVC</td>
<td>Th</td>
<td>0.043</td>
</tr>
<tr>
<td>14</td>
<td>1.0/0.4</td>
<td>NVC</td>
<td>NVC</td>
<td>W Db</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>2.0/0.4</td>
<td>NVC</td>
<td>NVC</td>
<td>NVC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>4.0/0.4</td>
<td>NVC</td>
<td>NVC</td>
<td>NVC</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Plant Growth Regulator Concentrations expressed in mg.l⁻¹
Alkaloids were present at ca. 1 to 10µg.gdw⁻¹

Abbreviations:

- **Callus Type**
  - NVC = no viable callus
  - A = aggregated callus
  - F = friable callus
  - M = meristemoids
  - * * = callus chosen
  - W = wet callus
  - El = extra leaves formed

- **Callus Colour**
  - Lb = light brown
  - Db = dark brown

- **Alkaloids Detected**
  - Th = thebaine
  - Cd = codeine
  - Mo = morphine
  - = none detected
Levels of 2,4-D greater than 1.0mg.l⁻¹ appeared to be detrimental to both callus initiation and alkaloid accumulation. Any callus established at these high levels of 2,4-D grew very poorly upon transfer to fresh medium. Increasing kinetin levels from 0.05 to 0.2mg.l⁻¹ was beneficial for alkaloid accumulation though at levels above this, alkaloid production was reduced. The optimal levels for initiation and maintenance of callus were 1.0mg.l⁻¹ 2,4-D and 0.2mg.l⁻¹ kinetin. Callus initiated under this regime also produced thebaine, codeine and morphine as identified by TLC. At the same PGR levels mentioned above it was also possible to initiate callus from sterile seedlings of *P.orientale* var.Scarlet and *P.bracteatum*. However, the percentage of seedlings which produced callus was less than obtained with *P.s* seedlings, whilst the only alkaloid detected in *P.b* and *P.o* calli was thebaine. Seedlings of *P.orientale* var.Allegro failed to produce any callus at all.

The next section describes the results obtained when suspension cultures of *P.s*, *P.b* and *P.o* were characterised for their growth and alkaloid content. The suspension cultures had been initiated from calli established in this section and were to be used for biotransformation experiments. Therefore, it was important to establish whether endogenous alkaloids were present, prior to quantifying the biotransformation capacities of the various culture types. It would also be possible to determine whether the procedures used in biotransformation experiments affected the culture cycle of the cultures.
3.3. Characterisation of growth and alkaloid content of suspension cultures of *Papaver* spp.

The aim of these experiments was to study the growth and morphinan alkaloid content of suspension cultures of *Papaver* spp. These suspension cultures had been derived from callus cultures of *Papaver somniferum* (P.s), *P.somniferum* "meristemoids" (P.s.m), *P.bracteatum* (P.b) and *P.orientale* var."Scarlet" (P.o). The medium used for initiating calli of the species listed above was that identified as giving optimal P.s callus growth in 3.2. The studies in 3.2 had also shown that callus cultures of P.s accumulated alkaloids common to the parent plant and it was now intended to establish whether suspension cultures derived from these calli retained the ability to synthesise morphinan alkaloids.

The suspension cultures characterised here were to be used for subsequent biotransformation studies so it was important to first screen the cultures for the presence of endogenous alkaloids. This would enable comparisons to be made between the alkaloid content of the parent plant and the cultures derived from them, as well as indicating which of the poppy cultures were most likely to show activity for the biotransformation of codeinone to codeine. The alkaloids of particular interest were thebaine, codeine and morphine. These experiments would also make it possible to determine at a later date whether the procedures used in the biotransformation experiments adversely affected the "normal" culture cycle of the cultures.

Three flasks were harvested for each timepoint in each of the various growth cycles. Small aliquots (1-2ml) of known volume were taken from each culture and the cell number (cell N, 2.3.4) and cell viability (%v, 2.3.5) calculated. The cultures were then centrifuged in 50ml centrifuge tubes so as to determine packed cell volume (pcv, 2.3.6). The cells were separated from the culture medium and the fresh weight calculated (fwt, 2.3.1) and the filtered cells frozen at -40°C and freeze-dried to determine dry weight (dwt, 2.3.2). These latter two calculations took into account the small aliquots removed earlier in the experiment. Having determined these growth parameters the freeze-dried cells from the three flasks were pooled and extracted for morphinan alkaloids (2.4.1.2). The spent medium obtained from the three flasks was also pooled and the alkaloids extracted. The reason for pooling samples was to maximise the chances of detecting any alkaloids present.
3.3.1. Growth and alkaloid content of suspension cultures of *Papaver somniferum*

The aim of this experiment was to characterise the growth and alkaloid content of *P. s* suspension cultures. The growth cycle was studied over a period of 20d, with three flasks being harvested every second day. It was shown in 3.2 that callus cultures of *P. s* were capable of accumulating morphinan alkaloids common to the plant from which they were derived. Therefore, this experiment would establish whether this biosynthetic capability was retained in suspension cultures derived from these calli. The results presented in this section actually arise from two different growth cycles. This was because a decision was made part-way through these studies to subculture younger cells than previously (Corchete and Yeoman, 1990). This practice slightly altered the growth characteristics of the suspension cultures and as a consequence both sets of data are presented.

3.3.1.1. First culture cycle

The fwt (Fig.3.3.1.a) and dwt (Fig.3.3.1.b) data indicate that the cells underwent a lag phase for ca. five days, followed by a growth phase reaching a maximum fwt of 15.125g and a maximum dwt of 0.518g, both after 19d. The cells then entered a stationary phase during which the medium pH (Fig.3.3.1.d) rose to a maximum of pH7.0, 29d after subculturing. The %dwt (Fig.3.3.1.c) of the cultures after an initial rise to 7.12% (3d) fell to ca. 3.5% by 8d after which it remained fairly constant.

The results presented in Fig.3.3.2 show that the cell N° (Fig.3.3.2.a) increased in parallel with fwt (Fig.3.3.1.a) and dwt (Fig.3.3.1.b). The %v (Fig.3.3.2.b) initially fell to 75% (3d) during the lag phase, rose to a maximum of 87% (15d) during the growth phase and then fell steadily during the stationary phase to 71% (29d).
Fig. 3.3.1 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during first growth cycle of *Papaver somniferum* suspension cultures. Each point represents the mean of three replicates ± standard error (where shown).
Fig. 3.3.2 Changes in cell number (a), percentage viability (b) and packed cell volume (c) during first growth cycle of *Papaver somniferum* suspension cultures. Each point represents the mean of three replicates.
Table 3.3.1 shows which of the morphinan alkaloids were detected in the \textit{P.a} cultures during the growth cycle. Also shown is the mean relative growth rate ($r$) of the cultures between timepoints, calculated as described in 2.5.3. These results show that the \textit{P.a} cultures did contain thebaine and codeine at various timepoints during the growth cycle but that no morphine was detected. The $r$ of the cultures reached a maximum during the growth period from 11-13d after which both thebaine and codeine were detected. The presence of thebaine and codeine in 1d cultures was probably due to a carry-over from the 15d cultures used to set up this experiment. No alkaloids were detected in the spent medium at any time.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Culture Age (days) & Alkaloid Content & Mean Relative Growth Rate $r$ (g.g$^{-1}$.day$^{-1}$) \\
\hline
1 & The and Cd-OH & 0.073 \\
3 & Cd-OH & 0.000 \\
5 & N.D. & 0.134 \\
8 & N.D. & 0.139 \\
11 & N.D. & 0.263 \\
13 & The & 0.061 \\
15 & The and Cd-OH & 0.072 \\
17 & The and Cd-OH & 0.074 \\
19 & Cd-OH & 0.083 \\
20 & N.D. & -0.096 \\
22 & N.D. & 0.002 \\
25 & N.D. & -0.022 \\
29 & N.D. & \\
\hline
\end{tabular}
\caption{Mean relative growth rates and morphinan alkaloid content of cells of \textit{Papaver somniferum} suspension cultures during the first growth cycle.}
\end{table}

Key: The = thebaine, Cd-OH = codeine, Mor = morphine and N.D. = none detected
3.3.1.2. Second culture cycle

The results presented in Fig.3.3.3 and Fig.3.3.4 correspond to the second growth cycle. These cultures were set up using 12d cultures and as a consequence grew differently from those of the first growth cycle. The fwt (Fig.3.3.3.a) and dwt (Fig.3.3.3.b) curves show that the cells underwent a lag phase of ca. five days, followed by a growth phase which reached a maximum fwt of 15.482g and a maximum dwt of 0.570g after 16d. These figures are similar to those achieved during the first growth cycle (Fig.3.3.1.a and Fig.3.3.1.b) but were both achieved three days earlier than previously observed. The %dwt (Fig.3.3.3.c) and medium pH (Fig.3.3.4.d) both changed during the growth cycle in a similar manner to the first culture type (Fig.3.3.1.c and Fig.3.3.1.d). However, the maximum cell No (Fig.3.3.4.a) appeared to have been increased by this earlier subculturing and was reflected in higher values for pcv (Fig.3.3.4.c). The major difference between the two cultures was in the %v (Fig.3.3.4.b), where the percentage of viable cells was between 5% and 12% higher than in comparable cells from the first growth cycle (Fig.3.3.2.b). In general, the cultures appeared "healthier", paler coloured cells. Photographs of P.s cultures from the second growth cycle are shown in Fig.3.3.5.

The morphinan alkaloid content of the cells from the second growth cycle are shown in Table 3.3.2 along with the values of F between timepoints. The alkaloid content appeared to be unaffected by the slightly faster growth of these cultures, indeed, the presence of a small quantity of morphine was detected in 12d cultures (Fig.3.3.6). The levels of alkaloid found during both growth cycles were similar being ca. 10μg.gdwt⁻¹ which was similar to the levels found in P.s callus (Table 3.2.2). Morphine levels did appear to be lower than in the callus cultures but this was difficult to establish due to the semi-quantitative nature of the method. As noted in Table 3.3.1 the alkaloids were accumulated after F had reached a maximum value and had begun to fall. This period corresponded to the late growth phase and the early stationary phase of the growth cycle. Apart from thebaine (spot 3), codeine (spot 2) and morphine (spot 1, all Fig.3.3.6) two other alkaloids were isolated from 12d cultures by 2DTLC (Fig.3.3.6). One of these (spot 4, Fig.3.3.6) corresponded to an alkaloid extracted from the parent plant (spot h, Fig.3.1.4) whilst the other (spot 5, Fig.3.3.6) appeared to be unique to the culture. No alkaloids were found in the spent medium at any time during the growth cycle.
Fig. 3.3.3 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during second growth cycle of *Papaver somniferum* suspension cultures. Each point represents the mean of three replicates ± standard error (where shown).
Fig. 3.3.4 Changes in cell number (a), percentage viability (b) and packed cell volume (c) during second growth cycle of *Papaver somniferum* suspension cultures. Each point represents the mean of three replicates.
Fig. 3.3.5 Suspension culture (a) and cultured cells (b) from second growth cycle of *Papaver somniferum* (10d) grown under the conditions described in 2.1.1.
Fig. 3.3.6 Alkaloids extracted from cells of *Papaver somniferum* suspension cultures and separated by two dimensional TLC using solvent systems T1 and T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff’s reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [X], codeinone [Y] and thebaine [Z]) and Ext = extract of *Papaver somniferum*. 
The results of the experiment show that suspension cultures of \textit{P.s} retained the ability to synthesise some of the morphinan alkaloids previously identified in the parent plant (Fig.3.1.4). This implies that the cultures might possess some activity for the biotransformation of codeinone to codeine. However, the levels of alkaloid present were significantly lower than in the plant (3.1.1). The next experiment was set up to investigate whether suspension cultures of \textit{P.b} would also retain the ability to produce some of the morphinan alkaloids present in the parent plant (3.1.2). The growth of these cultures was characterised to establish the relationship between $\bar{r}$ and any alkaloid production.

### 3.3.2. Growth and alkaloid content of suspension cultures of \textit{Papaver bracteatum}

The aim of this experiment was to characterise the growth and alkaloid content of suspension cultures of \textit{P.b}. The growth cycle was studied over a period of 20d with three cultures harvested every second day. The \textit{P.b} suspensions were set up from

<table>
<thead>
<tr>
<th>Culture Age (days)</th>
<th>Alkaloid Content</th>
<th>Mean Relative Growth Rate $\bar{r}$ (g.g$^{-1}$.day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The and Cd-OH</td>
<td>-0.034</td>
</tr>
<tr>
<td>2</td>
<td>N.D.</td>
<td>0.048</td>
</tr>
<tr>
<td>4</td>
<td>N.D.</td>
<td>0.092</td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
<td>0.219</td>
</tr>
<tr>
<td>8</td>
<td>N.D.</td>
<td>0.284</td>
</tr>
<tr>
<td>10</td>
<td>N.D.</td>
<td>0.104</td>
</tr>
<tr>
<td>12</td>
<td>The, Cd-OH and Mor</td>
<td>0.074</td>
</tr>
<tr>
<td>14</td>
<td>The and Cd-OH</td>
<td>0.055</td>
</tr>
<tr>
<td>16</td>
<td>Cd-OH</td>
<td>-0.034</td>
</tr>
<tr>
<td>18</td>
<td>Cd-OH</td>
<td>-0.002</td>
</tr>
<tr>
<td>20</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Key: The = thebaine, Cd-OH = codeine, Mor = morphine and N.D. = none detected
callus initiated as described in 2.2.3 using the same medium as optimised for P.s cultures (3.2).

The fwt (Fig.3.3.7.a) and dwt (Fig.3.3.7.b) curves show that the cells underwent a lag phase of ca. four days, then entered a growth phase where the fwt reached a maximum of 18.057g and the dwt a maximum of 0.598g, both after 16d. The fwt and dwt both dropped slightly between 16d and 20d as the cells entered a stationary phase. The initial %dwt (8.47%, Fig.3.3.7.c) of the cultures was slightly higher than noted in P.s cultures (6.73%, Fig.3.3.3.c) but did fall during the growth cycle to ca. 3.5%, a similar level to that observed in P.s. The medium pH (Fig.3.3.7.d) fluctuated in much the same way as it did in P.s cultures (Fig.3.3.3.d) initially dropping to pH4.9 (6d) then rising during the rest of the growth cycle eventually peaking at pH6.4 (20d).

The cell N° (Fig.3.3.8.a) increased in parallel with the fwt (Fig.3.3.7.a) and the dwt (Fig.3.3.7.b) reaching a maximum density of 3.21x10^6 after 14d. This was a slightly higher value than noted with P.s suspensions (Fig.3.3.2.a and Fig.3.3.4.a) and was reflected in a higher maximum pcv of 65% (Fig.3.3.8.c) at the same timepoint. The %v of the P.b cultures (Fig.3.3.8.b) was as high as that of P.s cultures in the second growth cycle (Fig.3.3.4.b) and was consistent with the "healthy" appearance of the light brown cells (Fig.3.3.9).

Table 3.3.3 shows the morphinan alkaloid content of the cultures during the growth cycle. Thebaine (spot a, Fig.3.3.10) was the only morphinan alkaloid positively identified (at a maximum of ca. 10μg.gdwt⁻¹) in 0d, 12d, 14d and 16d cultures. As in P.s cultures (Table 3.3.1 and Table 3.3.2) alkaloid accumulation occurred after t had reached a maximum value. The presence of thebaine in 0d cultures is probably due to a carry over from the 12d cultures used to set up this experiment. One other alkaloid was isolated from these cultures (spot b, Fig.3.3.10) and this cochromatographed with one of the unknown alkaloids (spot 6, Fig.3.1.9) isolated from the parent plant. No alkaloids were detected in the spent culture medium at any time during the growth cycle.
Fig. 3.3.7 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during growth cycle of *Papaver bracteatum* suspension cultures. Each point represents the mean of three replicates ± standard error (where shown).
Fig. 3.3.8 Changes in cell number (a), percentage viability (b) and packed cell volume (c) during growth cycle of *Papaver bracteatum* suspension cultures. Each point represents the mean of three replicates.
Fig. 3.3.9 Suspension culture (a) and cultured cells (b) from growth curve of *Papaver bracteatum* (10d) grown under the conditions described in 2.1.1.
Fig. 3.3.10 Alkaloids extracted from cells (12d) of *Papaver bracteatum* suspension cultures and separated by two dimensional TLC using solvent systems T1 and T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff’s reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [X], codeinone [Y] and thebaine [Z]) and Ext = extract of *Papaver bracteatum*. 
Table 3.3.3

Mean relative growth rates and morphinan alkaloid content of cells of *Papaver bracteatum* suspension cultures during the growth cycle

<table>
<thead>
<tr>
<th>Culture Age (days)</th>
<th>Alkaloid Content</th>
<th>Mean Relative Growth Rate $f$ (g.g$^{-1}$).day$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The</td>
<td>-0.041</td>
</tr>
<tr>
<td>2</td>
<td>N.D.</td>
<td>0.030</td>
</tr>
<tr>
<td>4</td>
<td>N.D.</td>
<td>0.106</td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
<td>0.193</td>
</tr>
<tr>
<td>8</td>
<td>N.D.</td>
<td>0.197</td>
</tr>
<tr>
<td>10</td>
<td>N.D.</td>
<td>0.111</td>
</tr>
<tr>
<td>12</td>
<td>The</td>
<td>0.106</td>
</tr>
<tr>
<td>14</td>
<td>The</td>
<td>0.028</td>
</tr>
<tr>
<td>16</td>
<td>The</td>
<td>-0.050</td>
</tr>
<tr>
<td>18</td>
<td>N.D.</td>
<td>-0.062</td>
</tr>
<tr>
<td>20</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Key: The = thebaine, Cd-OH = codeine, Mor = morphine and N.D. = none detected

This experiment has shown that suspension cultures of *P.b* retained the ability to synthesise thebaine previously identified in the parent plant (3.1.2) but that the amount accumulated was significantly less than occurred in the plant (on a.gdw$^{-1}$ basis). The absence of either codeine or morphine implies that these cultures might not be active in the biotransformation of codeinone to codeine. The next experiment was set up to investigate whether suspension cultures of *P.o* also retained the ability to produce thebaine. The growth of these cultures was also characterised to establish the nature of the relationship between the $f$ of the cultures during the growth cycle and any alkaloid accumulation.

3.3.3. Growth and alkaloid content of suspension cultures of *Papaver orientale* var."Scarlet"

The aim of this experiment was to characterise the growth and alkaloid content of suspension cultures of *P.o*. The growth cycle was studied over a period of 20d with
three flasks harvested every two days. The P_o cultures were set up from callus initiated as described in 2.2.3 and grown on the medium optimised in 3.2. The fwt (Fig.3.3.11.a) and dwt (Fig.3.3.11.b) curves show that the cells underwent a lag phase for ca. six days followed by a growth phase where fwt reached a maximum of 17.699g and dwt a maximum of 0.602g, both after 16d. The cells then entered a stationary phase where the fwt and dwt values fell slowly. The initial %dwt (Fig.3.3.11.c) of the cultures was 7.92% and as with P_s (Fig.3.3.3.c) and P_b (Fig.3.3.7.c) cultures this decreased during the growth cycle to ca. 3.5% by 20d. The medium pH (Fig.3.3.11.d) also followed a similar trend to that observed in P_s (Fig.3.3.3.d) and P_b (Fig.3.3.7.d) cultures falling during the lag phase to pH4.4 then rising during the rest of the growth cycle to pH6.4 by 20d. The cell N (Fig.3.3.12.a) rose during the growth cycle to a maximum of 3.24x10^6 cells.ml^-1 by 14d. This was similar to the values obtained for P_s (Fig.3.3.4.a) and P_b (Fig.3.3.8.a) which the cultures closely resembled. Unfortunately, no photographs of the P_o cultures are available. The %v (Fig.3.3.12.b) of the P_o cultures was also high with 93% of the cells viable during the late growth phase. This viability coupled with the increases in fwt and dwt was reflected in the high pcv (Fig.3.3.12.c) which peaked at 68% after 14d.
Fig. 3.3.11 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during growth cycle of *Papaver orientale* suspension cultures. Each point represents the mean of three replicates ± standard error (where shown).
Fig.3.3.12 Changes in cell number (a), percentage viability (b) and packed cell volume (c) during growth cycle of *Papaver orientale* suspension cultures. Each point represents the mean of three replicates.
Fig. 3.3.13 Alkaloids extracted from cells (12d) of *Papaver orientale* suspension cultures and separated by two dimensional TLC using solvent systems T1 and T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff's reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [X], codeinone [Y] and thebaine [Z]) and Ext = extract of *Papaver orientale*.
Table 3.3.4 shows the morphinan alkaloid content of *P. o.* cultures throughout the growth cycle. Thebaine (spot a, Fig.3.3.13) was the only morphinan alkaloid positively identified in 0d, 12d and 14d cultures. The presence of thebaine in 0d cultures was probably due to a carry over from the 12d cultures used to set up this experiment. Alkaloid accumulation once more appeared to occur after t had reached a maximum value. One other alkaloid (spot b, Fig.3.3.13) was isolated from these cultures and it cochromatographed with one of the unknown alkaloids found in the parent plant (spot iv, Fig.3.1.12). This compound was also found in *P.b* plants (spot 6, Fig.3.1.9) and suspension cultures (spot b, Fig.3.3.10) but was not successfully identified. No alkaloids were found in the spent medium at any time during the growth cycle.

Table 3.3.4

<table>
<thead>
<tr>
<th>Culture Age (days)</th>
<th>Alkaloid Content</th>
<th>Mean Relative Growth Rate ( \tau ) (g.g(^{-1}).day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The</td>
<td>-0.148</td>
</tr>
<tr>
<td>2</td>
<td>N.D.</td>
<td>0.045</td>
</tr>
<tr>
<td>4</td>
<td>N.D.</td>
<td>0.075</td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
<td>0.349</td>
</tr>
<tr>
<td>8</td>
<td>N.D.</td>
<td>0.170</td>
</tr>
<tr>
<td>10</td>
<td>N.D.</td>
<td>0.173</td>
</tr>
<tr>
<td>12</td>
<td>The</td>
<td>0.106</td>
</tr>
<tr>
<td>14</td>
<td>The</td>
<td>0.025</td>
</tr>
<tr>
<td>16</td>
<td>N.D.</td>
<td>-0.029</td>
</tr>
<tr>
<td>18</td>
<td>N.D.</td>
<td>-0.033</td>
</tr>
<tr>
<td>20</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Key: The = thebaine, Cd-OH = codeine, Mor = morphine and N.D. = none detected

This experiment has shown that suspension cultures of *P.o* retain the ability to synthesise thebaine, previously found in the parent plant (Fig.3.1.12). The absence of either codeine or morphine implies that these cultures might not be active in the
biotransformation of codeinone to codeine. However, the amount of alkaloid produced was significantly lower (on a gdwt⁻¹ basis) than in the plant.

The next experiment was set up to investigate whether suspension cultures of *Papaver somniferum* "meristemoids" retain the ability to produce any morphinan alkaloids. *P.s.m* callus cultures have been shown to be more highly differentiated than "normal" *P.s* callus cultures (Nessler and Mahlberg, 1979: Nessler, 1982) and this was also expected to be the case with suspension cultures. An increase in the differentiated state of a culture (eg. by immobilisation) has previously been shown to increase the biotransformation capacity of poppy cells (Furuya *et al*., 1984; Corchete and Yeoman, 1990). Therefore, the possibility that *P.s.m* suspension cultures might have an increased capacity to produce/accumulate morphinan alkaloids is explored in the next experiment. The growth of these cultures was also characterised to establish the nature of the relationship between the f of the cultures during the growth cycle and any alkaloid production.

3.3.4. Growth and alkaloid content of suspension cultures of *Papaver somniferum* "meristemoids"

The aim of this experiment was to characterise the growth and alkaloid content of *P.s.m* suspension cultures. The results in 3.3.1 to 3.3.3 have shown that cultures of *P.s, P.b* and *P.o* all retained a limited capacity to accumulate at least one of the morphinan alkaloids common to the plant from which they were derived. Nessler and Mahlberg (1979) have demonstrated that callus of *P.s* are more highly differentiated than "normal" *P.s* calli. Therefore, by studying the ability of *P.s.m* suspension cultures to produce morphinan alkaloids, it will be possible to establish if the increase in differentiated state enhances the amount of alkaloid accumulated.

"Meristemoids", first named by Nessler and Mahlberg (1979) appeared as white spheres of tissue (0.5-2mm in diameter) that developed, apparently at random, on the surface of *P.s* callus grown under the conditions described in 2.2.3.2. The white spheres were removed using a small spatula and grown on callus medium for two or three passages prior to being transferred to liquid medium. Due to the slow growth of these cultures the growth cycle was studied over a period of 30d, with three flasks harvested every third day. The fwt (Fig.3.3.14.a) and dwt (Fig.3.3.14.b) curves show that the cells did not appear to undergo a lag phase but instead grew steadily from 0d onwards reaching a maximum fwt of 3.478g and a maximum dwt of 0.308g, both after 24d. This was in contrast to the large increases in fwt and dwt noted in *P.s*
cultures (Fig.3.3.3.a and Fig.3.3.3.b). The %dwt (Fig.3.3.14.c) of P.s.m cultures was ca. twice that of comparable P.s cultures (Fig.3.3.3.c) throughout the course of the growth cycle, reaching a maximum of 14.98% at 3d. The medium pH (Fig.3.3.14.d) underwent similar fluctuations to those observed in P.s cultures (Fig.3.3.3.d) though it did peak somewhat higher at pH7.4 after 30d. The data for cell N° (Fig.3.3.15.a) should be treated with some caution because of the very compact nature of the "meristemoid" cultures (Fig.3.3.16.b), which were difficult to disperse efficiently. Therefore, it is likely that the values for cell N° should be higher than those indicated. With the reduced levels of fwt (Fig.3.3.14.a) and pcv (Fig.3.3.15.c) for P.s.m cultures this implies that the P.s.m cells were significantly smaller and denser than the P.s cultures. This was in fact the case as studies presented in show. The %v (Fig.3.3.15.b) of the cultures was high although due to the compact nature of the P.s.m aggregates it was difficult to determine the viability of those cells in the middle of an aggregate.

Table 3.3.5 shows the morphinan alkaloid content of P.s.m cultures during the growth cycle as well as the T of the cultures between timepoints. Morphinan alkaloids were found throughout a greater proportion of the P.s.m growth cycle than during the growth cycle of P.s cultures (Table 3.3.1 and Table 3.3.2). Morphine was found at two timepoints (12d and 15d), codeine at seven (0d, 12d, 15d, 18d, 21d, 24d and 27d) and thebaine at four (0d, 9d, 15d and 18d). The levels of codeine and thebaine were approximately equal to those found in P.s cultures (ca. 10μg.gdwt⁻¹) whilst the level of morphine appeared to be slightly higher than in P.s cultures. The T of the cultures was greatest over the first three timepoints and then appeared to level off. It was during this period of slower growth that morphinan alkaloid accumulation was at its highest. The presence of thebaine and codeine in 0d cultures was probably due to a carry over from the 15d cultures used to set up this experiment. Apart from thebaine (spot 3), codeine (spot 2) and morphine (spot 1, all Fig.3.3.17) two other alkaloids were separated by 2D TLC (Fig.3.3.17). Both of these cochromatographed with alkaloids isolated from P.s cultures (spot 4 and spot 5, Fig.3.3.6), spot 4 corresponding to an alkaloid in the parent plant (spot h, Fig.3.1.4) whilst the other (spot 5) appeared to be unique to cultured P.s or P.s.m cells.
Fig. 3.3.14 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during growth cycle of *Papaver somniferum* "meristemoid" suspension cultures. Each point represents the mean of three replicates ± standard error (where shown).
Fig. 3.3.15 Changes in cell number (a), percentage viability (b) and packed cell volume (c) during growth cycle of *Papaver somniferum* "meristemoid" suspension cultures. Each point represents the mean of three replicates.
Fig. 3.3.16 Suspension culture (a) and cultured cells (b) from growth curve of *Papaver somniferum* "meristemoids" (15d) grown under the conditions described in 2.1.1.
Fig. 3.3.17 Alkaloids extracted from cells (15d) of *Papaver somniferum* "meristemoid" suspension cultures and separated by two dimensional TLC using solvent systems T1 and T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff's reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [X], codeinone [Y] and thebaine [Z]) and Ext = extract of *Papaver somniferum* "meristemoids".
Table 3.3.5

Mean relative growth rates and morphinan alkaloid content of cells of *Papaver somniferum* "meristemoid" suspension cultures during the growth cycle

<table>
<thead>
<tr>
<th>Culture Age (days)</th>
<th>Alkaloid Content</th>
<th>Mean Relative Growth Rate ( f ) (g.g(^{-1}).day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The and Cd-OH</td>
<td>0.261</td>
</tr>
<tr>
<td>3</td>
<td>N.D.</td>
<td>0.106</td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
<td>0.098</td>
</tr>
<tr>
<td>9</td>
<td>The</td>
<td>0.054</td>
</tr>
<tr>
<td>12</td>
<td>Cd-OH and Mor</td>
<td>0.036</td>
</tr>
<tr>
<td>15</td>
<td>The, Cd-OH and Mor</td>
<td>0.062</td>
</tr>
<tr>
<td>18</td>
<td>The and Cd-OH</td>
<td>0.032</td>
</tr>
<tr>
<td>21</td>
<td>Cd-OH</td>
<td>0.025</td>
</tr>
<tr>
<td>24</td>
<td>Cd-OH</td>
<td>-0.053</td>
</tr>
<tr>
<td>27</td>
<td>Cd-OH</td>
<td>-0.124</td>
</tr>
<tr>
<td>30</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Key: The = thebaine, Cd-OH = codeine, Mor = morphine and N.D. = none detected

This experiment has shown that suspension cultures of *P.s.m* retained the ability to synthesise some of the alkaloids previously identified in the parent plant (Fig.3.1.4). The amount of alkaloid produced in *P.s.m* cultures was probably slightly higher than in *P.s* cultures but was still significantly lower than in the parent plant (on a gdwt\(^{-1}\) basis). The fact that *P.s.m* cultures produced alkaloids during more of their growth cycle than *P.s* cultures was probably due to their slower growth and more highly differentiated state. Slow growth has previously been seen to increase biotransformation capacity in immobilised poppy cells (Furuya *et al.*, 1984; Corchete and Yeoman, 1990) and so *P.s.m* cultures might be expected to show a higher activity for the conversion of codeinone to codeine than *P.s* cultures. The next two experiments were set up to establish whether the immobilisation of *P.s* and *P.s.m* cultures increased the level of endogenous alkaloid production. The growth characteristics of these two culture types were also studied to establish the relationship between the \( f \) of the cultures during the growth cycle and any alkaloid production.
3.3.5. Growth and alkaloid content of immobilised cultures of *Papaver somniferum*

Cells of *P.s* were immobilised (*P.s.i*) in foam blocks as described in 2.2.4.2, transferred to solid growth medium for three weeks and then put back into fresh liquid growth medium (2.2.1). The growth cycle was studied over a period of 20d, with three flasks harvested every two days. The fwt (Fig.3.3.18.a) and dwt (Fig.3.3.18.b) curves show that the cells underwent a lag phase of ca. four days prior to a growth phase which levelled off into a stationary phase after 12d or 14d. The maximum fwt observed was 9.480g (20d) which was substantially less than achieved in *P.s* cultures (Fig.3.3.3.a). Meanwhile, the dwt reached a maximum of 0.579g after 16d, which was a higher value than recorded with *P.s* cultures (Fig.3.3.3.b). These two facts were reflected in the higher %dwt (Fig.3.3.18.c) of the *P.s.i* cultures as compared to those measured in cultures of *P.s* (Fig.3.3.3.c). The medium pH (Fig.3.3.18.d) changed throughout the growth cycle in much the same way as it did in *P.s* cultures (Fig.3.3.3.d), though the fluctuations were not quite as large. It should be noted that much of the growth was attributable to suspended cells which had been released from the surface layers of the foam blocks (Fig.3.3.1). The extent of this growth varied substantially from flask to flask and was partly responsible for the relatively large SE bars obtained with these cultures. Due to the nature of immobilised growth it was not possible to obtain consistent results for cell N°, %v or pcv. However, the cell viability appeared to be ca. 75% throughout the growth cycle which was slightly lower than observed in suspended *P.s* cultures (Fig.3.3.4.b).

Table 3.3.6 shows the morphinan alkaloid content of *P.s.i* cultures during the growth cycle as well as the f of the cultures between each timepoint. Alkaloids were detected in more timepoints of the *P.s.i* growth cycle than during the *P.s* growth cycle (Table 3.3.2) from which they had been derived (Table 3.3.2). This was probably a result of a reduction in f across the growth cycle. At their highest (12d and 14d), the levels of thebaine (spot 3), codeine (spot 2) and morphine (spot 1, all Fig.3.3.20) were ca. 20μg.gdw⁻¹, which was approximately twice that detected in *P.s* cultures. This implies that *P.s.i* cultures might show an enhanced level of activity for the biotransformation of codeinone to codeine and this has in fact been demonstrated on two previous occasions (Furuya *et al.*, 1984; Corchete and Yeoman, 1990). However, the quantity of alkaloids accumulated by *P.s.i* cultures was still significantly less than found in the parent plant (3.1.1). The presence of alkaloids was highest when the value of f was low (12d and 14d), the period corresponding to the late growth phase and early stationary phase of the growth cycle. Apart from thebaine, codeine and
morphine, two other alkaloids (spot 4 and spot 5, Fig.3.3.20) were isolated from 12d cultures by 2DTLC (Fig.3.3.20). Both of these corresponded to alkaloids extracted from P.s cultures (spot 4 and spot 5, Fig.3.3.6), spot 4 occurring in the parent plant (spot h, Fig.3.1.4) and spot 5 appearing to be unique to P.s cultures of all types. No alkaloids were found in the spent medium at any time during the growth cycle.

Table 3.3.6

Mean relative growth rates and morphinan alkaloid content of cells of *Papaver somniferum* immobilised cultures during the growth cycle

<table>
<thead>
<tr>
<th>Culture Age (days)</th>
<th>Alkaloid Content</th>
<th>Mean Relative Growth Rate $r$ (g.g$^{-1}$).day$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The and Cd-OH</td>
<td>-0.019</td>
</tr>
<tr>
<td>2</td>
<td>Cd-OH</td>
<td>-0.092</td>
</tr>
<tr>
<td>4</td>
<td>N.D.</td>
<td>0.138</td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
<td>0.101</td>
</tr>
<tr>
<td>8</td>
<td>N.D.</td>
<td>0.040</td>
</tr>
<tr>
<td>10</td>
<td>The and Cd-OH</td>
<td>0.051</td>
</tr>
<tr>
<td>12</td>
<td>The, Cd-OH and Mor</td>
<td>0.013</td>
</tr>
<tr>
<td>14</td>
<td>Cd-OH and Mor</td>
<td>0.010</td>
</tr>
<tr>
<td>16</td>
<td>Cd-OH</td>
<td>-0.013</td>
</tr>
<tr>
<td>18</td>
<td>Cd-OH</td>
<td>-0.003</td>
</tr>
<tr>
<td>20</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Key: The = thebaine, Cd-OH = codeine, Mor = morphine and N.D. = none detected
Fig. 3.3.18 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during growth cycle of *Papaver somniferum* immobilised cultures. Each point represents the mean of three replicates ± standard error (where shown).
Fig. 3.3.19 Immobilised culture (a) and blocks of cells (b) from growth curve of *Papaver somniferum* (10d) grown under the conditions described in 2.1.1 and 2.2.4.
Fig.3.3.20 Alkaloids extracted from cells (12d) of *Papaver somniferum* immobilised cultures and separated by two dimensional TLC using solvent systems T1 and T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff's reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [X], codeinone [Y] and thebaine [Z]) and Ext = extract of immobilised *Papaver somniferum.*
This experiment has shown that \textit{P.s.i} cultures retained the ability to synthesise some of the morphinan alkaloids previously identified in the parent plant (Fig.3.1.4). Although the levels of alkaloid present were significantly lower than in the plant, the reduced growth of these cultures caused by the immobilisation of suspended cells did increase the quantity of each alkaloid accumulated. \textit{P.s.m} cultures were also shown to produce alkaloids at more points during their growth cycle than the less differentiated \textit{P.s} cultures (3.3.4). Therefore, the next experiment was set up to investigate whether the immobilisation of \textit{P.s.m} cultures further increased endogenous alkaloid production. The growth characteristics of these cultures was also studied to determine the relationship between the \( r \) of the cultures during the growth cycle and any alkaloid production.

### 3.3.6. Growth and alkaloid content of immobilised cultures of \textit{Papaver somniferum} "meristemoids"

Cells of \textit{P.s.m} were immobilised (\textit{P.s.m.i}) in foam blocks as described in 2.2.4.2 and then transferred into fresh liquid growth medium (2.2.1). The growth cycle was studied over a period of 30d with three flasks harvested every third day. The fwt (Fig.3.3.21.a) and dwt (Fig.3.3.21.b) curves show that the cells underwent very little growth over the course of the experiment. The maximum fwt was 4.289g and the maximum dwt was 0.356g, both after 24d. These were similar to those levels measured in \textit{P.s.m} cultures (Fig.3.3.14.a and Fig.3.3.14.b) although the actual amount of growth during the growth cycle was substantially less. The \%dwt (Fig.3.3.21.c) of the \textit{P.s.m.i} cultures was relatively level during the experiment being 9.73\% at its highest (3d) and 7.81\% at its lowest (27d). This was equivalent to the \%dwt of suspended \textit{P.s.m} cells in their stationary phase (Fig.3.3.14.c). The medium pH (Fig.3.3.21.d) also remained fairly constant during the first half of the growth cycle though it did rise slowly to pH 6.2 after 30d. Due to the nature of the immobilised growth it was not possible to obtain consistent results for cell N\(^0\), \%v or pcv. However, the \%v appeared to be >90\% throughout the growth cycle.

The morphinan alkaloid content of the \textit{P.s.m.i} cells is shown in Table 3.3.7 along with the \( r \) of the cultures between each timepoint. This shows that cells from all but one timepoint contained detectable quantities of one or more of the morphinan alkaloids, the most common being codeine. At their highest, the levels of thebaine (spot 3), codeine (spot 2) and morphine (spot 1, all Fig.3.3.23) in \textit{P.s.m.i} cells (Fig.3.3.23) were approximately twice those detected in \textit{P.s.m} cells (Fig.3.3.17) being ca. 20\(\mu\)g.gdw\(^{-1}\). This was probably due to the very slow growth of the immobilised
cells. However, the quantity of alkaloids present was still significantly less than in the parent plant. Apart from thebaine, codeine and morphine two other alkaloids (spot 4 and spot 5, Fig. 3.3.23) were detected in 18d P.s.m.i cultures. Both of these corresponded to alkaloids isolated from all the other P.s culture types (P.s, 3.3.1; P.s.m, 3.3.4; P.s.i, 3.3.5 and P.s.m.i, 3.3.6). Spot 4 corresponded to an alkaloid found in the parent P.s plant (spot h, Fig. 3.1.4) whilst spot 5 appeared to be unique to P.s.m.i cells and the cultures listed above. No alkaloids were detectable in the culture medium at any point during the growth cycle.
Fig.3.3.21 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during the growth cycle of immobilised *Papaver somniferum* "meristemoid" cultures. Each point represents the mean of three replicates ± standard error (where shown).
Fig. 3.3.22 Immobilised culture (a) and blocks of cells (b) from growth cycle of *Papaver somniferum* "meristemoids" (15d) grown under the conditions described in 2.1.1 and 2.2.4.
Fig. 3.3.23 Alkaloids extracted from cells (18d) of *Papaver somniferum* "meristemoid" immobilised cultures and separated by two dimensional TLC using solvent systems T1 and T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the plate visualised with Dragendorff’s reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [X], codeinone [Y] and thebaine [Z]) and Ext = extract of immobilised *Papaver somniferum* "meristemoids".
Mean relative growth rates and morphinan alkaloid content of immobilised cells of *Papaver somniferum* "meristemoid" cultures during the growth cycle

<table>
<thead>
<tr>
<th>Culture Age (days)</th>
<th>Alkaloid Content</th>
<th>Mean Relative Growth Rate $r$ (g.g⁻¹).day⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The and Cd-OH</td>
<td>0.023</td>
</tr>
<tr>
<td>3</td>
<td>N.D.</td>
<td>0.051</td>
</tr>
<tr>
<td>6</td>
<td>The</td>
<td>0.048</td>
</tr>
<tr>
<td>9</td>
<td>Cd-OH</td>
<td>0.009</td>
</tr>
<tr>
<td>12</td>
<td>The and Cd-OH</td>
<td>0.018</td>
</tr>
<tr>
<td>15</td>
<td>The, Cd-OH and Mor</td>
<td>0.038</td>
</tr>
<tr>
<td>18</td>
<td>The, Cd-OH and Mor</td>
<td>0.040</td>
</tr>
<tr>
<td>21</td>
<td>Cd-OH and Mor</td>
<td>0.027</td>
</tr>
<tr>
<td>24</td>
<td>Cd-OH</td>
<td>-0.016</td>
</tr>
<tr>
<td>27</td>
<td>Cd-OH</td>
<td>-0.025</td>
</tr>
<tr>
<td>30</td>
<td>Cd-OH</td>
<td></td>
</tr>
</tbody>
</table>

Key: The = thebaine, Cd-OH = codeine, Mor = morphine and N.D. = none detected

This experiment has shown that P.s.m.i cultures retained the ability to synthesise some of the morphinan alkaloids previously identified in the parent plant (Fig.3.1.4). The quantity of each alkaloid accumulated was higher than in the suspended P.s.m cultures presumably due to the very slow growth of these cultures. This implies that cultures of P.s.m.i might show a relatively high level of activity for the conversion of codeinone to codeine. However, the amount of alkaloid actually accumulated by the P.s.m.i cultures was still significantly lower than the amount accumulated in the plant.

The next section briefly summarise the results obtained from the experiments undertaken in 3.3.
3.3.7. Summary

In these experiments the growth cycles of P.s, P.b, P.o, P.s.m, P.s.i and P.s.m.i cultures were characterised. P.s, P.b and P.o cultures all exhibited "classic" sigmoidal growth curves whilst cultures of P.s.m, P.s.i and P.s.m.i grew very much more slowly. These differences in growth were reflected in the ability of the various culture types to accumulate morphinan alkaloids. Those cultures which grew more slowly accumulating ca. twice as much of thebaine, codeine or morphine as the faster growing cultures. Alkaloid production was at its highest in all cultures when the value of t was low. However, the level of alkaloids present in all cultures was significantly less (on a gdwt⁻¹ basis) than in the parent plants. The alkaloids produced by the cultures were all common to the plant chemotype from which they were derived, with the exception of one isolated from cultures of P.s, P.s.m, P.s.i and P.s.m.i. Neither, plants of P.b and P.o or cultures derived from them accumulate codeine or morphine, under the conditions employed. This implied that these cultures might show little activity for the biotransformation of codeinone to codeine. However, P.s plants which do synthesise codeine and morphine produced cultures that accumulated both of these alkaloids and hence might be expected to convert codeinone to codeine. Cultures of P.s.m were probably more highly differentiated than P.s cultures and produced alkaloids at more timepoints during their growth cycle than the less differentiated P.s cultures. At no time were any alkaloids found in the filtered medium of any of the culture types.

The next section describes experiments undertaken to establish the effect of culture age on the biotransformation capacity of suspended cells. The results from this section will also help to determine whether the biotransformation capacity of a given cell type depends upon the plant chemotype from which it was derived.
3.4. Effect of culture age on the biotransformation capacity of suspended cells

The aim of these experiments was to establish whether a relationship exists between culture age and biotransformation capacity. Cultures identified with an enhanced biotransformation capacity would then be used to study the biotransformation in more detail (3.5). The cultures under investigation were suspensions of *P.somniferum* (P.s), *P.somniferum* "meristemoids" (P.s.m), *P.bracteatum* (P.b) and *P.orientale* (P.o). The first set of experiments (3.4.1 to 3.4.3) used P.s suspension cultures to biotransform codeinone to codeine. Having established an appropriate procedure with these cultures it was then repeated in 3.4.4 and 3.4.5 to identify cells of P.s.m, P.b and P.o with an enhanced capacity for the conversion.

The ability of plant cell cultures to produce secondary metabolites has been extensively researched with particular emphasis on possible biotechnological applications. However, in the majority of species studied the formation and accumulation of secondary metabolites does not occur readily. Recently there has been a shift in emphasis towards studying the ability of cells to biotransform substances administered to cultures. Plant cells that perform either "added value" or stereo-specific reactions are of particular interest.

Many of the papers which describe biotransformations simply report the occurrence of the conversion. Papers that include more detailed studies are Drawert *et al.* (1984) and Aviv and Galun (1981). The former paper reports that the biotransformation rates for the conversion of valencene to nootkatone varied depending upon which *Citrus* spp. was used. They also found that rapidly growing cells had the highest conversion activity. The product nootkatone appeared within six hours and was then further metabolised. Aviv and Galun (1981), showed that the reduction of (-)-menthone to (+)-neomenthol was stereospecific with respect to both the precursor and the product. They also established a relationship between the biotransformation capacity of a given cell line and the major oil constituents of the plant chemotype from which it was derived.

The reduction of (-)-codeinone to (-)-codeine by suspension cultures of P.s has previously been reported (Furuya *et al.* 1978 and 1984; Corchete and Yeoman, 1990). These papers describe the use of immobilised cultures (in calcium alginate or polyurethane foam blocks) to increase the overall biotransformation yield. Little is known however, about the age at which cultures are best able to perform the conversion. In the experiments described here the biotransformation capacity of the cultures was either expressed as total product produced (μg.flask⁻¹) or product...
produced per unit weight of biomass present (μg.gdwr⁻¹). A combination of these two parameters was used to identify cultures with an enhanced biotransformation capacity.

3.4.1. Biotransformation with a 72 hour incubation period using *P. somniferum* suspension cultures of different ages

The first biotransformation experiments reported here are based upon the results of experiments described by Furuya *et al.* (1978 and 1984) and Corchete and Yeoman (1990). In their experiments, codeinone was added to *P.s* suspension cultures and incubated for periods up to 24h and then extracted for morphinan alkaloids. Furuya *et al.* (1984) and Corchete and Yeoman (1990) report a maximal conversion after three days and assume that the conversion is relatively linear. Other papers also report biotransformations that take days rather than hours to complete (Drawert *et al.*, 1984; Suga, 1988). Here again the conversions also appear to be linear over the first two or three days. An incubation period of 72h was used in this preliminary experiment based upon the reported linearity of other biotransformations using plant suspension cultures. The main objective of this first experiment was to determine whether any conversion actually took place, hence the long incubation period. However, if the conversion under investigation is not linear then cells which biotransform at a relatively slow velocity could still achieve the same overall biotransformation ratio as more efficient cells which have completed the conversion well within the three day period. Consequently, it would not be possible to distinguish (after 72h) between the initially more efficient cells and those cells that proceed at a slower velocity.

Fourteen day old suspension cultures of *P.s* were filtered through 64 μm nylon mesh (2.2.3.1). The filtered cells were then separated into 3.00±0.05g fractions and transferred to fresh medium. Six flasks were used for each timepoint, three controls and three experimental. (-)-Codeinone, 800μg dissolved in 100μl methanol, was added to the cultures by injection using a 100μl glass syringe. The tiny puncture mark made in the foil cap was then resealed with autoclave tape. Codeinone was added 0, 4, 8, 10, 12 and 16d after subculture and the flasks harvested 72h later (2.3.1). Codeinone was added to 0h flasks just prior to the subculture of cells into the medium. Methanol (100μl) was added to control flasks at the same time as the corresponding experimental flasks received codeinone dissolved in methanol. The cells and medium of each flask were separated as described in 2.3.1. Alkaloids were extracted from the culture medium immediately whilst the cells were frozen at -40°C,
freeze-dried (2.3.2) and then extracted as described in 2.4.1.2.

The addition of codeinone had no discernible effect on the fresh weight (fwt), dry weight (dwt), percentage dry weight (%dwt) or medium pH during the growth cycle (Figs.3.4.1.a-d). By comparing the results in Fig.3.3.1 with those in Fig.3.4.1 the presence of methanol can be seen to have had no effect upon the growth of P.s cultures. The levels of codeine shown in Figs.3.4.2.a-c are expressed as μg/flask⁻¹ and the data given in Figs.3.4.2.a and 3.4.2.b show that most of the codeine produced was present in the medium and not in the cells. The total amount found in cells (Fig.3.4.2.b) did increase with age, probably due to the increase in the amount of biomass. Fig.3.4.2.c shows both the total codeine detected in each flask and the corresponding dwt of cultures at each timepoint. It shows that the biotransformation capacity after 72h is fairly constant during the growth phase, but with slightly reduced activities at timepoints corresponding to the lag and stationary phases of the growth cycle. Codeinone was not detected, in cells or medium, during the course of this experiment. As a maximum of only 35.3% (8d) of the 800μg codeinone was converted to codeine (Table 3.4.1) the whereabouts of ca. 510μg of codeinone added to the cultures is unknown. Possibilities include that codeinone is converted into an alkaloid(s) other than codeine or that either codeinone or codeine is conjugated with another molecule, forming a polar compound, which remained in the aqueous fraction after extraction. The presence of other alkaloid products seems unlikely as no alkaloids other than codeine were detected when the appropriate samples were analysed by 2DTLC (solvent systems, T1 and T2). However, alkaloids bound to polar compounds have been reported in plants (Wold, 1978; Fairbairn and Steele, 1980) and cultures (Morris and Gibbs, 1986). The efficiency with which codeinone and codeine were extracted from cultures (cells and medium) was subsequently determined in 3.4.6 and the possible presence of bound forms of morphinan alkaloids investigated further in 3.5.

Table 3.4.1 shows the mean relative growth rates (f) of the cultures throughout the experiment and the corresponding biotransformation ratios achieved. The timepoints do not overlap but start or finish on 0h of the day quoted. A maximum biotransformation of 35.3% was achieved using 8d cultures (harvested at 11d) with >90% of the product in the medium. This maximum ratio occurred at the timepoint immediately before the culture reached a maximum. Codeine was not detected by HPLC in control cultures at any point during the experiment (results not shown).
Table 3.4.1

Relative growth rates and biotransformation ratios of cultures at different timepoints during the culture cycle

<table>
<thead>
<tr>
<th>Growth Period (Days)</th>
<th>$\tilde{r}$ (g.g$^{-1}$).day$^{-1}$</th>
<th>Exposure to Codeinone (Days)</th>
<th>Biotransformation Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 3</td>
<td>0.029</td>
<td>0 to 3</td>
<td>27.0</td>
</tr>
<tr>
<td>3 to 7</td>
<td>0.111</td>
<td>4 to 7</td>
<td>35.2</td>
</tr>
<tr>
<td>7 to 11</td>
<td>0.118</td>
<td>8 to 11</td>
<td>35.3</td>
</tr>
<tr>
<td>11 to 13</td>
<td>0.163</td>
<td>10 to 13</td>
<td>31.6</td>
</tr>
<tr>
<td>13 to 15</td>
<td>0.086</td>
<td>12 to 15</td>
<td>34.0</td>
</tr>
<tr>
<td>15 to 19</td>
<td>0.073</td>
<td>16 to 19</td>
<td>27.3</td>
</tr>
</tbody>
</table>

NB. These timepoints do not overlap.

When expressed as $\mu$g.gdwt$^{-1}$ (Fig.3.4.3) the biotransformation capacity of cultures at each timepoint reflects the increase in dwt observed in Fig.3.4.1.b. Cells from the first two timepoints corresponding to the lag and early growth phases of the cultures are ca. twice as productive as the rest of the cultures.

It is clear from this experiment that cultures of all ages were able to convert codeinone to codeine, reaching a similar biotransformation ratio after 72h. However, if the conversion had taken place at different rates in the various ages of cultures studied, then initially more efficient cells would be indistinguishable (after 72h) from those cells that had proceeded at a slower velocity. To observe these differences in rates of conversion, two modifications could be made to the experimental procedure. The first would involve reducing the amount of time that cells are exposed to codeinone prior to harvesting. The second would be to increase the amount of codeinone added to cultures, to a level sufficient to ensure codeinone is present in excess throughout the biotransformation period. It was decided to use the first of these two options as the maximum amount of codeinone that can be dissolved in 100\mu{l} methanol is only ca. 1200\mu{g}. This would not necessarily be sufficient to guarantee the excess substrate required by the second option. The next section (3.4.2) describes the full experimental procedure employed and the results obtained when P.s cells were incubated with codeinone for 24h. This incubation period was decided upon so as to enable the biotransformation to proceed far enough for detectable quantities of product to be formed whilst being able to observe the differences in the initial conversion activities of the various ages of culture used.
Fig.3.4.1 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during growth curve of *Papaver somniferum* suspension cultures. Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; △ = experimental flask
Fig. 3.4.2 Amount of codeine produced (μg. flask\(^{-1}\)) in medium (a), cells (b) and in total (c) by *Papaver somniferum* suspension cultures after 72h exposure to codeinone. Each bar represents the mean of three replicates ± standard error. Control flask data not shown.
Fig. 3.4.3 Amount of codeine produced (μg.gdw⁻¹) in medium (a), cells (b) and in total (c) by *Papaver somniferum* suspension cultures after 72h exposure to codeinone. Each bar represents the mean of three replicates ± standard error. Control flask data not shown.
3.4.2. Biotransformation with a 24 hour incubation period using *P. somniferum* suspension cultures of different ages

The results from experiment 3.4.1 showed that *P.s* cells of all ages tested, exhibited a similar biotransformation capacity after 72h incubation with codeinone. However, it was not possible to determine whether the cells of the different ages had performed the conversion at different rates. Consequently, to study the initial biotransformation activities of the cells it was concluded that the codeinone incubation period should be reduced.

The procedures used in this experiment were the same as those in Experiment 3.4.1, but with two modifications. Firstly, the flasks were harvested after 24h rather than 72h and secondly an additional timepoint (20d) was added to ensure that the complete growth cycle was covered during the experiment.

As in the previous experiment, the addition of codeinone to suspension cultures of *P.s* was found to have no discernible effect upon the fresh weight (fwt), dry weight (dwt), percentage dry weight (%dwt) or medium pH during the growth cycle (Figs.3.4.4.a-d). However, by comparison with the results in Fig.3.4.1 it is apparent that the cultures grew more quickly and to a greater extent than in Experiment 3.4.1. This was probably due to the use of younger cells (12d) for the initiation of cultures than in the previous experiment. Younger cells were used because they produced "healthier" cultures and showed increased viability (3.3.1). As in 3.4.1 methanol had no discernible effect on the growth of the cultures (by comparison with results in 3.3.1).

The data presented in Figs.3.4.5.a and Fig.3.4.5.b show, as before (Fig.3.4.2.a and Fig.3.4.2.b), that almost all of the codeine formed was present in the medium and not in the cells. The maximum biotransformation of 33.6% occurred using 10d suspension cultures (Table 3.4.2). The biotransformation capacity was similar for cells during the growth phase, but a distinct tailing off in activity was noted in cultures corresponding to the lag and stationary phases of the culture cycle (Fig.3.4.5.c). The only flasks in which codeinone was detected in the medium after 24h, were at timepoints 0d and 4d. The fact that codeinone is not present in the medium of older cultures after a 24h period, suggests that, as in 3.4.1, the codeinone may have been converted into some substance (undetected) other than codeine. This is because a maximum of only 33.6% of the codeinone added to the cultures can be accounted for as codeine. Consequently, the fate of *ca. 530μg* codeinone is unknown. As stated in 3.4.1 the unknown product could be another alkaloid or a
conjugate of an alkaloid and a polar substance which would remain in the polar fraction after non-polar compounds had been extracted. TLC analysis of pooled culture samples showed the presence of trace amounts (ca. 10μg.gdwt⁻¹) of morphine and thebaine in experimental cultures (cells and medium). The estimate for morphine assumes a similar sensitivity to Dragendorff’s reagent as observed for standard alkaloids actually possessed (ie. thebaine, codeine and codeinone). Codeine produced by biotransformation was also present in these cultures, but at levels ca. 50 to 100 times, those of morphine and thebaine. The morphine was probably synthesised by the demethylation of codeine. However, morphine was not present in sufficient quantities to account for all of the codeinone not converted to codeine. Control flasks were found to contain only codeine and thebaine at trace levels (ca. 10μg.gdwt⁻¹), similar to those in Experiment 3.3.1. This indicates that the morphine detected in experimental flasks was probably formed from the added codeinone via codeine.

Table 3.4.2

<table>
<thead>
<tr>
<th>Growth Period (Days)</th>
<th>f (g.g⁻¹).day⁻¹</th>
<th>Exposure to Codeinone (Days)</th>
<th>Biotransformation Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 1</td>
<td>0.025</td>
<td>0 to 1</td>
<td>24.2</td>
</tr>
<tr>
<td>1 to 5</td>
<td>0.043</td>
<td>4 to 5</td>
<td>9.9</td>
</tr>
<tr>
<td>5 to 9</td>
<td>0.189</td>
<td>8 to 9</td>
<td>27.0</td>
</tr>
<tr>
<td>9 to 11</td>
<td>0.207</td>
<td>10 to 11</td>
<td>33.6</td>
</tr>
<tr>
<td>11 to 13</td>
<td>0.100</td>
<td>12 to 13</td>
<td>24.8</td>
</tr>
<tr>
<td>13 to 17</td>
<td>0.066</td>
<td>16 to 17</td>
<td>12.3</td>
</tr>
<tr>
<td>17 to 21</td>
<td>(-)0.026</td>
<td>20 to 21</td>
<td>10.9</td>
</tr>
</tbody>
</table>

NB. Timepoints do not overlap

When codeine production is expressed as μg.gdwt⁻¹ (Fig.3.4.6) the histograms show that cells of the earliest timepoint (0d) are more active than any other culture age. However, 8d cultures were marginally better than 4d cultures (Fig.3.4.6.c) which was not the case previously (Fig.3.4.3.c). The level of codeine in cells (Fig.3.4.6.b) was again fairly constant along the growth curve, indicating that the codeine content of cells was related to biomass. The high biotransformation potential of 0d cultures may be regarded as an anomaly, representing a carry over of activity from cells (12d) used to inoculate the cultures. The maximal biotransformation of 33.6% was achieved by 10d cells when f was at a maximum and just prior to a large fall in its value (Table 3.4.2.). This conversion of 33.6% was very close to the maximal figure.
obtained in the previous experiment (Table 3.4.1). This implies that codeine production in the previous experiment was not linear over the 72h incubation period. Indeed, in this experiment cells from four timepoints (0, 8, 10 and 12d) had achieved levels of conversion, after 24h incubation with codeinone, approaching those achieved after 72h. Therefore, it was concluded that the time cells were incubated with codeinone should again be reduced. This would then enable initially more efficient cells to be distinguished from those that proceed at a slower velocity. Experiment 3.4.3 describes the results obtained when P.s cells of different ages were incubated with codeinone for 8h prior to harvesting. This was one third of the previous incubation time and was consistent with the original reduction in incubation period of 72h to 24h.
Fig. 3.4.4 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during growth curve of *Papaver somniferum* suspension cultures. Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; △ = experimental flask
Fig. 3.4.5 Amount of codeine produced (µg/flask⁻¹) in medium (a), cells (b) and in total (c) by *Papaver somniferum* suspension cultures after 24h exposure to codeinone. Each bar represents the mean of three replicates ± standard error. Numbers in brackets show levels of codeinone in medium after 24h (µg/flask⁻¹). Control flask data not shown.
Fig. 3.4.6 Amount of codeine produced (μg.gdw⁻¹) in medium (a), cells (b) and in total (c) by *Papaver somniferum* suspension cultures after 24h exposure to codeinone. Each bar represents the mean of three replicates ± standard error. Control flask data not shown.
3.4.3. Biotransformation with an 8 hour incubation period using *P. somniferum* suspension cultures of different ages

The results presented in Experiment 3.4.2 show that 10d *P.s* cells reached a maximal biotransformation of 33.6% after 24h. This conversion ratio was similar to that obtained by equivalent cells (after 72h) in Experiment 3.4.1 and implies that the cells in 3.4.1 had reached their maximal biotransformation ratio 24h after the addition of codeinone. It was concluded that the time cells were incubated with codeinone should be reduced. This would enable cells which are initially more efficient at converting codeinone to be distinguished from those that proceed at a slower velocity. Therefore in this experiment the same experimental procedure as described in 3.4.2 was used except that flasks were harvested 8h after the addition of codeinone.

The results presented in Fig.3.4.7 again show that the addition of codeinone at the levels under investigation had no measurable affect upon culture growth. By comparing these results with those shown in 3.3.1 it can also be seen that the addition of 100μl methanol had no discernible effect upon the growth of *P.s* cultures.

As in the previous experiments (3.4.1 and 3.4.2) the codeine formed was mostly present in the medium (Fig.3.4.8.a and Fig.3.4.8.b). Codeinone was still present in the medium of younger cultures (0d and 4d), but had disappeared from the medium of all other cultures after 8h incubation. This means that the fate of substantial amounts of codeinone has still to be established. The possibility that codeinone had been converted to an alkaloid other than codeine was investigated by 2DTLC analysis. As in 3.4.2, the presence of morphine was noted in experimental and not in control flasks. However, the quantity present was not sufficient to account for all of the missing codeinone. The possible presence of bound forms of morphinan alkaloids occurring during biotransformation experiments was subsequently investigated in 3.5. A maximum biotransformation of 20.6% was obtained with 12d cultures (Table 3.4.3). By comparison, the initial biotransformation capacities of the cultures at the beginning and end of the culture cycle were greatly reduced. Only 10d cultures achieved a conversion similar (19.8%) to that of 12d cultures after 8h (Table 3.4.3). The reason that the cultures did not achieve maximal biotransformation ratios as high as those in Experiments 3.4.1 and 3.4.2 was because the cultures were only exposed to codeinone for 8h. Therefore, in this experiment an incomplete conversion was being observed and not the maximal biotransformation ratio possible. Fig.3.4.8.c shows that the highest conversion ratio occurred during the growth phase of the cultures. However, the results in Table 3.4.3 show that this ratio also coincided with
the first timepoint after it had reached a maximum value. This suggests that cultured cells switching from the growth to the stationary phase of their culture cycle have the greatest biotransformation capacity.

Table 3.4.3

<table>
<thead>
<tr>
<th>Growth Period</th>
<th>Expos. to</th>
<th>Biotransformation Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Days+Hours)</td>
<td>Codeinone</td>
<td>(Days+Hours)</td>
</tr>
<tr>
<td></td>
<td>(g.g⁻¹).day⁻¹</td>
<td></td>
</tr>
<tr>
<td>0 to 0+8h</td>
<td>0.019</td>
<td>0 to 0+8h</td>
</tr>
<tr>
<td>0+8h to 4+8h</td>
<td>0.070</td>
<td>4 to 4+8h</td>
</tr>
<tr>
<td>4+8h to 8+8h</td>
<td>0.203</td>
<td>8 to 8+8h</td>
</tr>
<tr>
<td>8+8h to 10+8h</td>
<td>0.313</td>
<td>10 to 10+8h</td>
</tr>
<tr>
<td>10+8h to 12+8h</td>
<td>0.247</td>
<td>12 to 12+8h</td>
</tr>
<tr>
<td>12+8h to 16+8h</td>
<td>(-)0.010</td>
<td>16 to 16+8h</td>
</tr>
<tr>
<td>16+8h to 20+8h</td>
<td>(-)0.173</td>
<td>20 to 20+8h</td>
</tr>
</tbody>
</table>

NB. Timepoints do not overlap

When codeine production is expressed as µg.gdw⁻¹ the initial timepoint (0d) again shows the highest activity (Fig 3.4.9.c). Cultures at 8d and 10d achieve the next best biotransformation capacities but these are only one third of the figure achieved by 0d cultures. However, 8d and 10d cultures produce twice as much codeine per flask by comparison with the 0d cultures. The apparent superiority of 0d cultures could be explained by a carry over effect from cells (12d P.s stock) used to inoculate the 0d cultures. When selecting cells for biotransformations of potentially economic importance it is desirable to have significant levels of product. Thus it was decided that 10d P.s cultures would be used for the detailed kinetic study of the conversion of codeinone to codeine as they had high biotransformation capacities when expressed as both µg.flask⁻¹ and µg.gdw⁻¹ (3.5.1.1).

One of the initial objectives of this set of experiments was to identify cultures of other Papaver spp. and types capable of the biotransformation of codeinone to codeine. Cultures with increased levels of differentiation (ie. immobilised cells) have previously been shown to possess enhanced biotransformation capacities (Furuya et al., 1984; Corchete and Yeoman, 1990). In the next section cultures of P.s.m were studied to establish if the differentiated state of these cultures (3.3.4) enhanced their biotransformation potential. P.s.m cells having enhanced activities would then be
used to make more detailed kinetic studies of the conversion (3.5.1.3) and enable comparisons to be made with the $P_s$ cells selected in this section. The experimental procedure used was the same as that in 3.4.3.
Fig. 3.4.7 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during growth curve of *Papaver somniferum* suspension cultures. Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; △ = experimental flask
Fig. 3.4.8 Amount of codeine produced (μg flask⁻¹) in medium (a), cells (b) and in total (c) by *Papaver somniferum* suspension cultures after 8h exposure to codeinone. Each bar represents the mean of three replicates ± standard error. Numbers in brackets show levels of codeinone in medium after 8h (μg flask⁻¹). Control flask data not shown.
Fig. 3.4.9 Amount of codeine produced (µg.gdw⁻¹) in medium (a), cells (b) and in total (c) by *Papaver somniferum* suspension cultures after 8h exposure to codeinone. Each bar represents the mean of three replicates ± standard error. Control flask data not shown.
3.4.4. Biotransformation with an 8 hour incubation period using *P. somniferum*
"meristemoid" suspension cultures of different ages

The aim of this experiment was to establish the relationship between the age of *P. s.m* suspension cultures and biotransformation capacity. It was seen in 3.4.1 that *P.s* cells of all ages achieved a similar biotransformation ratio after 72h incubation with codeinone. However, in 3.4.3 it was shown that by studying the cultures before they reached a maximal conversion (ie. 8h incubation) it was possible to distinguish between the initially more efficient cells and those cells that proceeded at a slower velocity. This enabled cells with the greatest biotransformation capacity to be identified and subsequently used for detailed kinetic studies of the biotransformation process (3.5.1.1). The two parameters used to identify these cells were total product produced (µg/flask⁻¹) and product produced per unit weight of biomass present (µg/gdwt⁻¹). The main objective of this experiment was, to employ the experimental procedure developed in 3.4.1 to 3.4.3, to identify cultures of *P.s.m* that had an enhanced biotransformation capacity. These cells would subsequently be used for detailed kinetic studies of the conversion (3.5.1.3) and as a means of comparing *P.s* and the more highly differentiated *P.s.m* cultures.

"Meristemoid" cultures, first described by Nessler and Mahlberg (1979), have been shown to form embryoids and redifferentiate into *P.s* plants, regaining their ability to synthesise and accumulate morphinan alkaloids. (Nessler, 1982; Yoshikawa and Furuya, 1984). An increase in the differentiated state of a culture (eg. by immobilisation) has been shown to increase the biotransformation capacity of poppy cells (Furuya *et al.*, 1984; Corchete and Yeoman, 1990). Therefore, cultures of *P.s.m* may also have an enhanced biotransformation capacity. This possibility was explored in this experiment.

The design used for this experiment was the same as that described in 3.4.3, but with two modifications. Firstly, only 1.5g (fwt) of cells were added to each flask at the beginning of the growth cycle. This was because the percentage dry weight of *P.s.m* cultures was ca. twice that of *P.s* cultures. This meant that the dry weight of cells transferred to each flask at the beginning of the growth cycle was approximately equal to that used in Experiments 3.4.1 to 3.4.3. Secondly, due to the extended length of the *P.s.m* growth curve (3.3.4) it was decided to alter the timepoints at which codeinone was added to the cultures. Consequently, codeinone was added to flasks 0, 6, 10, 15, 20, 25 and 30d after subculturing. Cultures were harvested in the usual manner (2.3.1).
The results presented in Fig.3.4.10 show that the growth curve of \textit{P.s.m} cultures was very different from that of the \textit{P.s} cultures previously seen in Fig.3.4.7.a-d. Very little increase in fwt (Fig.3.4.10.a) was observed throughout the growth curve, peaking at 3.4g in experimental flasks after 30 days. This is \textit{ca}. one quarter that seen in \textit{P.s} cultures. This is partly due to the essentially non-vacuolated culture state of the "meristemoid" cells throughout the growth cycle (see Appendix). The dwt (Fig.3.4.10.b) of experimental flasks peaked at 0.290g (20d and 25d) as compared to the average 0.570g for \textit{P.s} cultures. The percentage dry weight of "meristemoid" cultures dropped more rapidly than in the \textit{P.s} cultures (Fig.3.4.10.c). This may be partly attributable to the increased difficulty in filtering meristemoid cultures as they age, due to an increase in the viscosity of the medium. The pH of the medium did not undergo the characteristic drop after subculture (Fig.3.4.10.d) observed in \textit{P.s} (Fig.3.4.7.d), and the final pH reached was also higher.

As noted in the \textit{P.s} cultures (Fig.3.4.8.a and Fig.3.4.8.b), most of the codeine formed was present in the medium and not in the cells (Fig.3.4.11.a and Fig.3.4.11.b). The amount of codeine produced by \textit{P.s.m} cultures after 8h, was much greater than by \textit{P.s} cultures (Table 3.4.3). A maximum biotransformation of 60.0% was achieved by 15d cultures with \textit{ca}. 97% of the product in the medium. No codeinone was detected in cells from any timepoint and only a small amount in the medium of 0d cultures (Fig.3.4.11.a). As noted in biotransformations using \textit{P.s} cultures (3.4.1 to 3.4.3) the fate of some codeinone is unknown. This is because only 60.0% of the codeinone added can be accounted for as codeine. It is feasible that some of the codeinone or codeine became conjugated with a polar molecule and remained in the aqueous phase after the non-polar compounds have been extracted. This possibility was subsequently investigated in 3.5.3. The biotransformation capacities of cells from timepoints at the beginning and the end of the growth cycle were significantly less than those achieved by 15d cultures (Fig.3.4.11.c). Table 3.4.4 shows that the maximal biotransformation ratio coincided with a large drop in \( \tau \) as previously seen in 3.4.3. This implies that like \textit{P.s} cultures (3.4.1 to 3.4.3) the cells have a maximal biotransformation capacity when they are switching from the growth to the stationary phase of the culture cycle.
Table 3.4.4

Relative growth rates and biotransformation ratios of cultures at different timepoints during the culture cycle

<table>
<thead>
<tr>
<th>Growth Period (Days+Hours)</th>
<th>$r$ (g.g$^{-1}$).day$^{-1}$</th>
<th>Exposure to Codeinone (Days+Hours)</th>
<th>Biotransformation Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 0+8h</td>
<td>0.035</td>
<td>0 to 0+8h</td>
<td>22.4</td>
</tr>
<tr>
<td>0+8h to 6+8h</td>
<td>0.097</td>
<td>6 to 6+8h</td>
<td>27.0</td>
</tr>
<tr>
<td>6+8h to 10+8h</td>
<td>0.072</td>
<td>10 to 10+8h</td>
<td>45.2</td>
</tr>
<tr>
<td>10+8h to 15+8h</td>
<td>0.034</td>
<td>15 to 15+8h</td>
<td>60.0</td>
</tr>
<tr>
<td>15+8h to 20+8h</td>
<td>0.032</td>
<td>20 to 20+8h</td>
<td>44.0</td>
</tr>
<tr>
<td>20+8h to 25+8h</td>
<td>0.000</td>
<td>25 to 25+8h</td>
<td>27.1</td>
</tr>
<tr>
<td>25+8h to 30+8h</td>
<td>(-)0.067</td>
<td>30 to 30+8h</td>
<td>19.4</td>
</tr>
</tbody>
</table>

NB. Timepoints do not overlap

When expressed as µg.gdwr$^{-1}$ the cells of the 0d timepoint appear, as in previous experiments (3.4.2 and 3.4.3), to be the most productive (Fig.3.4.12.a to Fig.3.4.12.c). However, this result must be treated with some caution as a significantly reduced amount (2.5 x less) of codeine was produced per flask. The next most productive cells were those of the 15d cultures. These cells also had a far higher biotransformation ratio after 8h than did 0d cultures (60.0% compared to 22.4%). Consequently 15d cultures were used for the detailed kinetic studies of the biotransformation made in 3.5.1.3.

The results presented in Fig.3.4.13 show the clear superiority of P.s.m cultures over P.s suspensions, presumably due to their increased level of differentiation. The results are calculated in terms of µg.gdwr$^{-1}$.h$^{-1}$ and represent the average rate at which cells produced codeine over the period they were exposed to codeinone. When expressed in these terms the most productive P.s.m cultures (15d) are ca. twice as active as the most productive of the P.s cultures (0d).

The procedure established in 3.4.3 had been successfully used to identify cultures of P.s.m with enhanced biotransformation capacities. The same experimental design was next used to establish whether suspension cultures of P.b and P.o are capable of converting codeinone to codeine, despite the inability of their respective parent plants to synthesise codeine. Using cultures of a different biochemical nature from that of P.s it should be possible to establish whether there is a relationship between the biotransformation capacity of a given Papaver spp. culture and the plant chemotype from which it was derived. However, it has been reported that limited enzyme
activity for the conversion of codeinone to codeine exists in cell-free extracts derived from P.b plants (Hodges and Rapoport, 1980). Thus by studying the biotransformation in P.b suspension cultures it will be possible to establish if this enzymic activity is present in cells in their "undifferentiated" state. If cultures of P.b and P.o show a similar activity to that observed in P.s cultures this will imply that the conversion is relatively unspecific and conceivably that the enzyme(s) responsible for the biotransformation is not the same as that operating in mature P.s plants. These possibilities are investigated in the next section.
Fig. 3.4.10 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during growth curve of *Papaver somniferum* "meristemoid" suspension cultures. Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; Δ = experimental flask
Fig. 3.4.11 Amount of codeine produced (μg/flask⁻¹) in medium (a), cells (b) and in total (c) by *Papaver somniferum* "meristemoid" suspension cultures after 8h exposure to codeinone. Each bar represents the mean of three replicates ± standard error. Numbers in brackets show levels of codeinone in medium after 8h. Control flask data not shown.
Fig. 3.4.12 Amount of codeine produced (µg·gdwt⁻¹) in medium (a), cells (b) and in total (c) by *Papaver somniferum* "meristemoid" suspension cultures after 8h exposure to codeinone. Each bar represents the mean of three replicates ± standard error. Control flask data not shown.
Fig. 3.4.13

Amount of codeine produced (µg.gdwt⁻¹.h⁻¹) by *Papaver somniferum* and *P. somniferum* "meristemoid" suspension cultures after 8, 24 or 72h exposure to codeinone. Each point represents the mean of three replicates. Control flask data not shown.
3.4.5. Biotransformation with a 24 hour incubation period using *P. bracteatum* and *P. orientale* suspension cultures of different ages

The aim of this experiment was to establish whether suspension cultures of *P. b* or *P. o* are able to biotransform codeinone to codeine. Any cultures identified as having significant biotransformation capacities would then be used to study the biotransformation in more detail. Plants (3.1.2 and 3.1.3) and cultures (3.3.2 and 3.3.3 respectively) of these two species had previously been found not to accumulate codeine or morphine. However, there is a report that cell-free extracts obtained from *P. b* plants show a limited ability to convert codeinone to codeine (Hodges and Rapoport, 1980). Consequently by using cultures of *P. b* and *P. o* it might be possible to establish whether there is a relationship between the biotransformation capacity of a *Papaver* spp. culture and the plant chemotype from which it was derived. If the cultures of *P. b* and *P. o* show a level of conversion activity similar to that observed in *P.s* cultures this will imply that the conversion is relatively unspecific. It will also give an indication as to whether the enzyme(s) responsible for the biotransformation are the same as those operating in mature *P.s* plants.

The design used in this experiment was the same as that described in 3.4.2. Cells were incubated with codeinone for 24h rather than 8h (as in 3.4.3) because any biotransformation activity present was likely to be lower than in *P.s* and *P.s.m* cultures (Hodges and Rapoport, 1980).

The results presented in Fig.3.4.14 and Fig.3.4.15 show that codeinone had no discernible effect upon the fresh weight (Fig.3.4.14.a and Fig.3.4.15.a), dry weight (Fig.3.4.14.b and Fig.3.4.15.b), percentage dry weight (Fig.3.4.14.c and Fig.3.4.15.c) or medium pH (Fig.3.4.14.d and Fig.3.4.15.d) for either *P.b* cultures or *P.o* cultures. Comparing the above data with that presented in 3.3.2 and 3.3.3 shows that the 100μl of methanol added to each culture also had no detrimental effect upon culture growth in either species.

During the course of Experiment 3.4.5 no codeine or codeinone was found in the cells of either species. However, small quantities of codeine were found in the medium of 0d and 20d *P.b* cultures (Fig.3.4.16.a) and 0d, 8d, 10d and 20d *P.o* cultures (Fig.3.4.16.c). This is consistent with the results of Hodges and Rapoport (1980) and suggests that the parent plant lacks significant enzyme activity for the conversion of codeinone to codeine. Codeinone was still detectable after 24h in 0d *P.b* cultures and 0d and 4d *P.o* cultures. The low levels or absence of codeinone coupled with the absence of significant levels of codeine suggests that codeinone has been converted to
a product other than codeine. 2DTLC analysis of the chloroform:iso-propanol extract (2.4.1.2) using solvent systems T1 and T2 (2.4.2.2) did not reveal any other alkaloids when developed with Dragendorff's reagent (2.4.2.4). However, a Dragendorff positive spot was obtained from the aqueous fraction which suggests that the alkaloid present had become conjugated to form a polar compound. This aqueous fraction was obtained by evaporating the spent and extracted medium to dryness, under reduced pressure, and then redissolving the residue in 2ml of 80% methanol. Next, 200μl of this solution was spotted onto a plate and run using TLC systems T2 (2.4.2.2) and TA (2.4.2.3). With T2 the alkaloid positive spot had an Rf of 0.00 whilst with TA it had an Rf of 0.05. This low motility could either be as a result of the high salt concentration interfering with the running of the unknown substance or due to the addition of a relatively large polar compound to the suspected alkaloid. The possible presence of bound forms of morphinan alkaloids was subsequently investigated in 3.5.3.

The maximum biotransformations for P.b of 2.9% (Table 3.4.5.a) and P.o of 4.4% (Table 3.4.5.b) were much less than the 24.2% obtained using the equivalent Od P.s cultures (Table 3.4.2). Therefore, it would appear that there is a relationship between the biotransformation capacity of a Papaver spp. culture and the plant chemotype from which it was derived.

Table 3.4.5.a

Relative growth rates and biotransformation ratios of P.b cultures at different timepoints during the culture cycle

<table>
<thead>
<tr>
<th>Growth Period (Days)</th>
<th>r (g.g(^{-1}).day(^{-1}))</th>
<th>Exposure to Codeinone (Days)</th>
<th>Biotransformation Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 1</td>
<td>0.092</td>
<td>0 to 1</td>
<td>2.9</td>
</tr>
<tr>
<td>1 to 5</td>
<td>0.118</td>
<td>4 to 5</td>
<td>0</td>
</tr>
<tr>
<td>5 to 9</td>
<td>0.197</td>
<td>8 to 9</td>
<td>0</td>
</tr>
<tr>
<td>9 to 11</td>
<td>0.152</td>
<td>10 to 11</td>
<td>0</td>
</tr>
<tr>
<td>11 to 13</td>
<td>0.136</td>
<td>12 to 13</td>
<td>0</td>
</tr>
<tr>
<td>13 to 17</td>
<td>0.060</td>
<td>16 to 17</td>
<td>0</td>
</tr>
<tr>
<td>17 to 21</td>
<td>(-)0.037</td>
<td>20 to 21</td>
<td>1.6</td>
</tr>
</tbody>
</table>

NB. Timepoints do not overlap
### Table 3.4.5.b

Relative growth rates and biotransformation ratios of \( P_o \) cultures at different timepoints during the culture cycle

<table>
<thead>
<tr>
<th>Growth Period (Days)</th>
<th>( r ) (g.g(^{-1}).day(^{-1}))</th>
<th>Exposure to Codeinone (Days)</th>
<th>Biotransformation Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 1</td>
<td>0.087</td>
<td>0 to 1</td>
<td>4.4</td>
</tr>
<tr>
<td>1 to 5</td>
<td>0.119</td>
<td>4 to 5</td>
<td>0</td>
</tr>
<tr>
<td>5 to 9</td>
<td>0.163</td>
<td>8 to 9</td>
<td>2.7</td>
</tr>
<tr>
<td>9 to 11</td>
<td>0.155</td>
<td>10 to 11</td>
<td>3.4</td>
</tr>
<tr>
<td>11 to 13</td>
<td>0.158</td>
<td>12 to 13</td>
<td>0</td>
</tr>
<tr>
<td>13 to 17</td>
<td>0.105</td>
<td>16 to 17</td>
<td>0</td>
</tr>
<tr>
<td>17 to 21</td>
<td>(-)0.020</td>
<td>20 to 21</td>
<td>2.4</td>
</tr>
</tbody>
</table>

NB. Timepoints do not overlap

When expressed as \( \mu g.gdwt^{-1} \) the results presented in Fig.3.4.16.b and Fig.3.4.16.d also show that the biotransformation capacity of cells of \( P_b \) and \( P_o \) are far lower than in equivalent \( P_s \) cultures (Fig.3.4.6.c). Highest activity was shown by 0d cultures of both species. Although low, this activity cannot be explained by a carry over effect, as no activity was detected in the 12d cultures used for subculturing. It is possible that this represents a low level of relatively non-specific conversion activity present in the cultures of \( Papaver \) spp. studied.

The biotransformation ratios observed in 3.4.1 were significantly lower than reported by Furuya \textit{et al.} (1978 and 1984) and Corchete and Yeoman (1990). This could be due to the known variability of plant cell cultures or as a result of reduced extraction efficiencies of both codeinone and codeine. Therefore the next experiment was set up to quantify the efficiency with which codeinone and codeine were extracted (2.4.1.2) from either spent medium or cultured cells.
Fig. 3.4.14 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during *Papaver bracteatum* growth cycle. Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; △ = experimental flask
Fig. 3.4.15 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during *Papaver orientale* growth cycle. Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; △ = experimental flask
Fig. 3.4.16 Total amount of codeine produced in μg.flask\(^{-1}\) (a and c) or μg.gdw\(^{-1}\) (b and d) by *Papaver bracteatum* (a and b) and *P. orientale* (c and d) suspension cultures after 24h exposure to codeinone. Each bar represents the mean of three replicates ± standard error. Numbers in brackets show levels of codeinone in medium after 24h (μg.flask\(^{-1}\)). Control flask data not shown.
3.4.6. Determination of extraction efficiency

The aim of this experiment was to determine the efficiency with which alkaloids added to suspension cultures of *Papaver* spp. were recovered using the extraction method described in 2.4.1.2. It was seen in 3.4.1 to 3.4.5 that when codeinone was added to cultures of *Papaver somniferum* (P.s), *P.somniferum* "meristemoids" (P.s.m), *P.bracteatum* (P.b) and *P.orientale* (P.o) the whereabouts of between 40 and 60% remained unknown. Therefore, it was important to calculate the efficiency of the extraction procedure used. The extraction efficiency calculated took into account all of the various culture types being studied. Should any consistent differences be noted between cultures then individual extraction efficiencies would have to be calculated for each culture type. During the biotransformation studies the quantity of alkaloids present in the cells and the culture medium was separately determined. Therefore, the efficiency with which alkaloids were extracted from cells or medium were determined independently.

The cultures used for this experiment were those previously shown (3.3) to have the highest alkaloid content because it was important to establish whether or not the alkaloids present in the cultures could be detected by the HPLC system described in 2.4.3. If they could then the presence of these alkaloids would have to be taken into account when determining the extraction efficiency of added alkaloids.

Two flasks (one control and one experimental) of each culture type were taken and the cells separated from the medium as described in 2.3.1. The cells were frozen at -40°C and then freeze-dried (2.3.2). Immediately prior to extraction, codeinone or codeine (100, 400 or 800μg) dissolved in 100μl methanol, was added to the filtered medium using a 100μl glass syringe. The flasks were shaken to ensure complete mixing and then extracted according to the procedure described in 2.4.1.2. The amount of alkaloid added to the cells or medium was representative of the levels expected during the biotransformation studies. The freeze-dried cells were placed in a mortar and 100μl of methanol containing 100, 400 or 800μg of either codeinone or codeine added whilst the cells were stirred using a spatula to ensure a relatively homogenous mixture. The cells were then left in a fume cupboard for 30min to allow the methanol to completely evaporate. When completely dry the cells were extracted as described in 2.4.1.2. Control flasks were treated in exactly the same way as experimental flasks except that the 100μl methanol added to the medium or freeze-dried cells contained no alkaloids. The same experimental procedure was also used on fresh autoclaved growth medium (2.2.1) except that three experimental flasks
were used for each level of alkaloid added to the medium. If the alkaloids were extracted more efficiently from the fresh than the used medium then this would imply that some factor associated with cultured plant cells was affecting the extraction procedure.

The results of this experiment are summarised in Tables 3.4.6 to 3.4.11. Table 3.4.6 shows the amount of codeine recovered from freeze-dried cells of *P.s*, *P.s.m*, *P.b* and *P.o* whilst Table 3.4.7 shows the amount of codeine recovered from the culture medium of the same cultures. The overall efficiency with which codeine was extracted from the cells and medium of all the culture types was 60.6%. There was some variation in the amount of codeine extracted from the different cells and media but there was no clear indication that the extraction was more efficient with any one particular culture type. There also appeared to be little difference in the efficiency with which codeine was extracted from either freeze-dried cells or culture medium.

Therefore, any calculations which took into account the reduced extraction efficiency of codeine were made using 60.6% as the extraction rate for both cells and medium of all the culture types studied. The whereabouts of the codeine not extracted from the cultures is discussed later. Table 3.4.8 shows the amount of codeinone recovered from freeze-dried cells of *P.s*, *P.s.m*, *P.b* and *P.o* whilst Table 3.4.9 shows the amount of codeinone recovered from the culture medium of these same cultures. The overall efficiency with which codeinone was extracted from the cells and medium of all the culture types was 50.4%. There was some variation in the amount of codeinone recovered from the different cultures but as was the case with codeine (Tables 3.4.6 and 3.4.7) there was no conclusive proof that codeinone was extracted more efficiently from any one particular culture type. There was also little difference between the extraction efficiency from freeze-dried cells compared to that from culture medium.

Therefore, any calculations which took into account the reduced extraction efficiency of codeinone were made using 50.4% as the extraction rate for both cells and medium of all the culture types studied. No codeine or codeinone were detectable in either the cells or medium of control flasks despite the known presence of codeine in these same cultures. This was due to the fact that less biomass (dwt) was being extracted per sample than had been the case in 3.3.

The whereabouts of *ca.* 50% of the codeinone and *ca.* 40% of the codeine added to the cells or medium is unknown, though several feasible explanations exist. The first explanation concerns the possible conjugation of the alkaloids with a polar compound(s) during the experiment. If formed these compounds would reduce the solubility of codeine and codeinone in the chloroform:iso-propanol mixture used during the extraction procedure and consequently reduce the amount of alkaloid
recovered from the cultures. Such "bound-forms" have previously been reported in plants (Wold, 1977; Fairbairn and Steele, 1980) and cultures (Morris and Gibbs, 1986) of P.s and P.b and this possibility was further investigated in 3.5.3. A second possible explanation is that the pH used for the extraction was sub-optimal, again reducing the solubility of the alkaloids in the non-polar extraction solvents. The third possibility involves the boundary layer which forms between the aqueous and the non-aqueous phases during extraction. Although this layer was broken up as much as possible using a Pasteur pipette it is still possible that a substantial quantity of alkaloid remained after the extraction was complete. This layer was not included in the extracts because it interfered with the HPLC analysis.

Table 3.4.10 shows the amount of codeine recovered from the fresh culture medium. The overall extraction efficiency was 77.3% compared to the 60.8% obtained from used medium (Table 3.4.7). This implies that some factor associated with cultured cells remains in the medium after filtration and adversely affects the extraction of codeine. Table 3.4.11 shows the amount of codeinone recovered from the fresh culture medium. The overall extraction efficiency was 73.2% compared to the 49.8% obtained from used medium (Table 3.5.9). As noted with the extraction of codeine this implies that after filtration some factor remained in the medium which reduced the efficiency with which codeinone was extracted from cultures.

The results in this section have shown that there is a significant problem encountered when extracting the morphinan alkaloids codeine and codeinone from cell cultures of Papaver spp. The reduction in extraction efficiency was similar in both extractions made from freeze-dried cells or culture medium and was also independent of the different levels of alkaloid added to the cultures. These levels represent the range in amounts of both alkaloids measured during the biotransformation experiments. Therefore, the extraction efficiencies calculated for codeine and codeinone were applied universally to freeze-dried cells and filtered medium for all culture types. The fact that more codeine and codeinone were recoverable from fresh culture medium than from spent medium implies that cultured cells release some compound that is partially responsible for the reduced extraction efficiencies. The possibility that some polar compound forms a conjugated product with the alkaloids, reducing their solubility in non-polar solvents is investigated further in 3.5.3. The next section summarises the results obtained in 3.4.1 to 3.4.5 taking into account the reduced extraction efficiencies calculated in this section. As a result it might be possible to determine more accurately the fate of that codeinone that is unaccounted for as "free" codeine.
### Table 3.4.6

**Amount of codeine recovered from freeze-dried cells of *Papaver spp.* suspension cultures**

<table>
<thead>
<tr>
<th>Culture Type, Fraction and Age (days)</th>
<th>Amount of Codeine Added/Recovered (μg)</th>
<th>Total Recovered (μg)</th>
<th>Percentage Recovered (Cell Type)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 400 800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P.s cells (14d)</td>
<td>58.3 260.0 462.7</td>
<td>781.0</td>
<td>60.0%</td>
</tr>
<tr>
<td>P.s.m cells (20d)</td>
<td>58.6 232.1 501.4</td>
<td>792.1</td>
<td>60.9%</td>
</tr>
<tr>
<td>P.p cells (12d)</td>
<td>61.8 238.9 486.3</td>
<td>787.0</td>
<td>60.5%</td>
</tr>
<tr>
<td>P.o cells (12d)</td>
<td>61.7 245.6 475.8</td>
<td>783.1</td>
<td>60.2%</td>
</tr>
<tr>
<td>Mean±S.E.</td>
<td>60.1±1.0 244.2±6.0 481.6±8.2</td>
<td>785.8±2.4</td>
<td></td>
</tr>
<tr>
<td>Total Recovered (μg)</td>
<td>240.4 976.6 1926.2</td>
<td>3143.2</td>
<td></td>
</tr>
<tr>
<td>Percentage Recovered</td>
<td>60.1% 61.0% 60.2%</td>
<td>60.4%</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.4.7

**Amount of codeine recovered from filtered medium of *Papaver spp.* suspension cultures**

<table>
<thead>
<tr>
<th>Culture Type, Fraction and Age (days)</th>
<th>Amount of Codeine Added/Recovered (μg)</th>
<th>Total Recovered (μg)</th>
<th>Percentage Recovered (Cell Type)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 400 800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P.s medium (14d)</td>
<td>61.7 237.6 466.6</td>
<td>765.9</td>
<td>58.9%</td>
</tr>
<tr>
<td>P.s.m medium (20d)</td>
<td>63.5 221.7 507.9</td>
<td>793.1</td>
<td>61.0%</td>
</tr>
<tr>
<td>P.p medium (12d)</td>
<td>66.8 258.1 483.1</td>
<td>808.0</td>
<td>62.2%</td>
</tr>
<tr>
<td>P.o medium (12d)</td>
<td>55.3 242.2 492.5</td>
<td>790.0</td>
<td>60.8%</td>
</tr>
<tr>
<td>Mean±S.E.</td>
<td>61.8±2.4 239.9±7.5 487.5±8.6</td>
<td>789.2±8.7</td>
<td></td>
</tr>
<tr>
<td>Total Recovered (μg)</td>
<td>247.3 959.6 1952.1</td>
<td>3159.0</td>
<td></td>
</tr>
<tr>
<td>Percentage Recovered</td>
<td>61.8% 60.0% 60.9%</td>
<td>60.8%</td>
<td></td>
</tr>
</tbody>
</table>

Total Codeine Extracted = 6302.2μg

Total Codeine Added = 7800.0μg

Overall Extraction Efficiency = 60.6%
Table 3.4.8

Amount of codeinone recovered from freeze-dried cells of *Papaver* spp. suspension cultures

<table>
<thead>
<tr>
<th>Culture Type, Fraction and Age (days)</th>
<th>Amount of Codeinone Added/Recovered (μg)</th>
<th>Total Recovered (μg)</th>
<th>Percentage Recovered (Cell Type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.s cells (14d)</td>
<td>46.3</td>
<td>209.2</td>
<td>390.6</td>
</tr>
<tr>
<td>P.s.m cells (20d)</td>
<td>57.5</td>
<td>200.4</td>
<td>422.4</td>
</tr>
<tr>
<td>P.b cells (12d)</td>
<td>43.2</td>
<td>207.9</td>
<td>409.2</td>
</tr>
<tr>
<td>P.o cells (12d)</td>
<td>54.6</td>
<td>216.2</td>
<td>393.7</td>
</tr>
</tbody>
</table>

Mean±S.E.: 50.4±3.4 208.4±3.2 404.0±7.4 662.8±7.0

Total Recovered (μg): 201.6 833.7 1615.9 2651.2

Percentage Recovered: 50.4% 52.1% 50.5% 51.0%

Table 3.4.9

Amount of codeinone recovered from filtered medium of *Papaver* spp. suspension cultures

<table>
<thead>
<tr>
<th>Culture Type, Fraction and Age (days)</th>
<th>Amount of Codeinone Added/Recovered (μg)</th>
<th>Total Recovered (μg)</th>
<th>Percentage Recovered (Cell Type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.s medium (14d)</td>
<td>56.9</td>
<td>196.8</td>
<td>392.0</td>
</tr>
<tr>
<td>P.s.m medium (20d)</td>
<td>44.5</td>
<td>203.2</td>
<td>385.2</td>
</tr>
<tr>
<td>P.b medium (12d)</td>
<td>46.8</td>
<td>209.3</td>
<td>402.6</td>
</tr>
<tr>
<td>P.o medium (12d)</td>
<td>49.5</td>
<td>195.5</td>
<td>408.3</td>
</tr>
</tbody>
</table>

Mean±S.E.: 49.4±2.7 201.2±3.2 397.0±5.2 647.6±5.6

Total Recovered (μg): 197.7 804.8 1588.1 2590.6

Percentage Recovered: 49.4% 50.3% 49.6% 49.8%

Total Codeine Extracted = 5241.8μg
Total Codeine Added = 7800.0μg
Overall Extraction Efficiency = 50.4%
Table 3.4.10

Amount of codeine recovered from fresh growth medium of *Papaver spp.* suspension cultures

| Flask No. | Amount of Codeine Added/Recovered (μg) |  
|---|---|---|
| 100 | 400 | 800 |
| 1. | 76.4 | 315.4 | 631.2 |
| 2. | 75.4 | 307.6 | 617.8 |
| 3. | 71.1 | 302.3 | 618.5 |
| Mean±S.E. | | | |
| 74.3±1.6 | 308.4±3.8 | 622.5±4.4 |
| Total Recovered (μg) | | | |
| 222.9 | 925.3 | 1867.5 |
| Percentage Recovered | | | |
| 74.3% | 77.1% | 77.8% |

Total Codeine Extracted = 3015.7μg
Total Codeine Added = 3900.0μg
Overall Extraction Efficiency = 77.3%

Table 3.4.11

Amount of codeinone recovered from fresh growth medium of *Papaver spp.* suspension cultures

| Flask No. | Amount of Codeinone Added/Recovered (μg) |  
|---|---|---|
| 100 | 400 | 800 |
| 1. | 75.2 | 297.8 | 591.0 |
| 2. | 78.2 | 281.3 | 587.0 |
| 3. | 77.3 | 286.2 | 580.3 |
| Mean±S.E. | | | |
| 76.9±0.9 | 288.4±4.9 | 586.1±3.1 |
| Total Recovered (μg) | | | |
| 230.7 | 865.3 | 1758.3 |
| Percentage Recovered | | | |
| 76.9% | 72.1% | 73.3% |

Total Codeinone Extracted = 2854.3μg
Total Codeinone Added = 3900.0μg
Overall Extraction Efficiency = 73.2%
3.4.7. Summary of experiments 3.4.1 to 3.4.6

The results presented in 3.4.1 to 3.4.5 have established that there is a relationship between culture age and biotransformation capacity. Cultures with enhanced biotransformation capacities appear to be switching from the growth to the stationary phase of the growth cycle (Tables 3.4.1 to 3.4.4). There is also a relationship between the ability of the *Papaver* spp. suspension cultures studied to biotransform codeinone and the plant chemotype from which it was derived. The maximum biotransformation of 35.3% achieved by *P.s* cultures (Table 3.4.3) was significantly lower than the figures achieved in similar experiments by Furuya *et al.* (60.8%, 1984) and Corchete and Yeoman (57.0%, 1990). This could be due to culture variation or the possible presence of products other than codeine. However, the results obtained from 3.4.5 showed that the extraction of codeine and codeinone from cells or medium was not 100% efficient. Indeed, extraction of codeine is 60.6% efficient and codeinone only 50.4% efficient. Table 3.4.12 shows the culture age for experiments 3.4.1 to 3.4.5 which gave the maximum biotransformation ratio. It also indicates the age of culture used and the length of exposure to codeinone. The last two columns show firstly, the biotransformation ratios as recorded and secondly, a corrected figure based upon the extraction efficiencies as calculated in 3.4.5. This means that the biotransformation ratio as calculated for 3.4.1 (35.3%) would become 58.3%. This value is approximately equal with those achieved by Furuya *et al.* (1984) and Corchete and Yeoman (1990) and is another possible explanation of the differences noted between the cultures. However, this assumes that biotransformation ratios reported in these two papers compensated for extraction efficiencies and still does not account for ca. 450μg of codeinone added to the cultures. By contrast the corrected biotransformation ratio achieved by *P.s.m* cultures is almost 100% (Table 3.4.12). This would imply that after 8h incubation with 800μg codeinone, 15d cultures of *P.s.m* had converted virtually all of the substrate to codeine.

This results section has described the procedures to identify suspension cultures of *Papaver* spp. with enhanced biotransformation capacities. It has been shown that cultures of all ages appear to reach a similar maximal biotransformation ratio if incubated with the substrate for a sufficient period (3.4.1). However, the velocity at which the various ages of culture perform the biotransformation appears to differ (3.4.1 to 3.4.3). Thus, it was decided to make detailed kinetic studies of the conversion using cultures identified as having enhanced biotransformation capacities (10d *P.s* and 15d *P.s.m*). These experiments will involve following the biotransformation as it occurs and enable the location of codeine and codeinone to be
determined throughout the reaction. Hopefully, these experiments will indicate the advantage differentiation confers upon the biotransformation capacity of cells. It might also be possible to establish the fate of that codeinone added to the medium which is not converted to codeine. Another experiment will determine whether the presence of *Papaver* spp. cells is necessary for the biotransformation to occur.

Table 3.4.12

**Maximum biotransformation ratios obtained using cultures of various *Papaver* spp.**

<table>
<thead>
<tr>
<th>Culture Type</th>
<th>Age (days)</th>
<th>Exposure to Codeinone (h)</th>
<th>Biotransformation Ratio (%)</th>
<th>Corrected Biotransformation Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P.somniferum</em></td>
<td>8</td>
<td>72</td>
<td>35.3</td>
<td>58.3</td>
</tr>
<tr>
<td><em>P.somniferum</em></td>
<td>10</td>
<td>24</td>
<td>33.6</td>
<td>55.4</td>
</tr>
<tr>
<td><em>P.somniferum</em></td>
<td>12</td>
<td>8</td>
<td>20.6</td>
<td>34.0</td>
</tr>
<tr>
<td><em>P.somniferum</em> &quot;meristemoids&quot;</td>
<td>15</td>
<td>8</td>
<td>60.0</td>
<td>99.0</td>
</tr>
<tr>
<td><em>P.bracteatum</em></td>
<td>0</td>
<td>24</td>
<td>2.9</td>
<td>4.79</td>
</tr>
<tr>
<td><em>P.orientale</em></td>
<td>0</td>
<td>24</td>
<td>4.42</td>
<td>7.29</td>
</tr>
</tbody>
</table>
3.5. Detailed studies on the biotransformation of codeinone to codeine

The aim of this set of experiments was to make detailed kinetic studies of the biotransformation of codeinone to codeine. The cultures under investigation in this section were suspensions of \textit{P. somniferum} (\textit{P.s}), \textit{P. bracteatum} (\textit{P.b}) \textit{P. somniferum} "meristemoids" (\textit{P.s.m}) and immobilised cultures of \textit{P. somniferum} (\textit{P.s.i}) and \textit{P. somniferum} "meristemoids" (\textit{P.s.m.i}). Some of the experiments involved following the biotransformation as it occurs, enabling the location of codeine and codeinone to be determined throughout the conversion. Attempts were made to establish the fate of that codeinone not converted to codeine. Another experiment determined whether the conversion activity remained in the medium after the cells of \textit{Papaver} spp. had been removed. An attempt was also made to quantify the enzymic activity present in each cell type and to relate this to the degree to which a culture is differentiated, the plant chemotype from which it was derived and the immobilised or suspended state of that culture. In the final experiment a crude enzyme extract was used to study the reduction in a cell free system and as a first step to further purification of the enzyme(s) involved.

3.5.1. Biotransformation time course studies

The relationship between culture age and biotransformation capacity was established for each cell type in 3.4. In that section biotransformation capacity was expressed as, either total product produced (\mu g.\text{flask}^{-1}), or product produced per unit weight of biomass (\mu g.\text{gdwt}^{-1}) A combination of these two parameters was then used to identify the age of a particular culture type that demonstrated the highest level of conversion activity. These are the cultures used in this section.

3.5.1.1. Biotransformation time course experiment using 10d suspension cultures of \textit{P. somniferum}

Suspension cultures of \textit{P.s} were initiated by transferring 3.00\pm0.05g of filtered 12d suspension cultures into experimental and control flasks. Ten days after initiation. 800\mu g of codeinone dissolved in 100\mu l of methanol, was added to experimental flasks by injection using a 100\mu l glass syringe. The tiny puncture mark made in the foil cap was then resealed with autoclave tape. Six flasks (three control and three experimental) were used for each timepoint on the timecourse. Control flasks were treated in exactly the same way as experimental flasks except that the 100\mu l of
methanol added to these cultures contained no alkaloid. Methanol (with or without codeinone) was added to flasks from each timepoint within a 1min interval. Cultures were incubated for the appropriate length of time and the cells then separated from the medium as described in 2.3.1. Alkaloids were extracted from the spent culture medium immediately whereas the filtered cells were frozen at -40°C and then freeze-dried (2.3.2). Any alkaloids present in the cells were extracted from the freeze-dried cells as described in 2.4.1.2. Flasks were harvested 0, 1, 3, 6, 12, 24, 48, 72 and 100h after the addition of methanol (with or without codeinone). The length of time between the addition of methanol and the separation of cells and medium for 0h flasks was only ca. 15s.

The fwt (Fig.3.5.1.a) and dwt (Fig.3.5.1.b) curves show that the cultures underwent a small amount of growth during the course of the experiment as would be expected from cells of this age (3.3.1). However, unlike previous experiments (3.4.1 to 3.4.3) there appeared to be some difference between the experimental and control flasks. This was most noticeable in the dwt data where after 24h the difference between the two means was 0.152g. After this timepoint the fwt and dwt curves converged. The difference was reflected by the higher %dwt (Fig.3.5.1.c) and medium pH (Fig.3.5.1.d) of the experimental flasks as compared to the results obtained for the control flasks. At the time of the experiment several attempts were made to try and repeat this peculiar result but could not be repeated (results not shown).

The total amount of codeinone and codeine detected in the cultures from each timepoint is shown in Fig.3.5.2. Codeinone levels decreased from ca. 400μg at 0h to 0μg after just 12h. No codeinone was found in the cells at any time during the experiment. Over the same period the codeine level increased, reaching a maximum of 317.8μg after 48h. In fact, the level of codeine is constant from 12h onwards. It should be noted that the maximum amount of codeinone detected (0h) accounts for only one half of the precursor added to the cultures. This implies that some product(s) as well as codeine is formed either during the experiment or during the extraction procedure. This reduced extraction efficiency was reported in 3.4.1 to 3.4.3 and is further explored in 3.5.2 and 3.5.3. The HPLC traces of extracts obtained from representative media samples are depicted in Fig.3.5.3 and show the decrease in codeinone and increase in codeine levels as the biotransformation proceeded. Codeine was only detected in cells of experimental cultures 100h after the addition of codeine and then only at <10% of the total codeine produced. At no point was codeinone or codeine detected in cells of control cultures despite the known presence of codeine in this type of culture (2.3.1). This was because in the previous study less biomass (dwt) was extracted per sample.
The next experiment describes the results obtained when \( P_{th} \) suspensions were used for time course studies on the biotransformation. These cultures had previously been shown to have a very poor biotransformation capacity (3.4.5). However, the cultures had only been exposed to codeinone for 24h, so by harvesting cultures up to 100h after the addition of codeinone it would be possible to establish whether these cultures were slower or less effective than \( P_{ps} \) cultures.
Fig. 3.5.1 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during a biotransformation time course study using 10d suspension cultures of *Papaver somniferum*. Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; △ = experimental flask
Fig. 3.5.2 Amount of codeinone and codeine detected in cultures during a biotransformation time course study using 10d suspension cultures of *Papaver somniferum*. Each point represents the mean of three replicates ± standard error.
Fig. 3.5.3 HPLC traces showing conversion of codeinone to codeine during a biotransformation time course study using 10d suspension cultures of *Papaver somniferum*.
3.5.1.2. Biotransformation time course experiment using 0d suspension cultures of *P. bracteatum*

The aim of this experiment was to determine the biotransformation capacity of *P. bracteatum* cultures. It was shown in 3.4.5 that *P. bracteatum* cultures converted very little codeinone to codeine. However, codeinone was not present in the medium after 24h and so must have been converted to another product. By studying the fate of codeinone closely it will be possible to establish the quantity of codeinone that is not accounted for as codeine. In 3.4.5 *P. bracteatum* cultures were exposed to codeinone for a maximum of 24h, therefore, by exposing the cultures to substrate for up to 100h it will be possible to determine whether the cultures produce more codeine in this period than previously.

The experimental procedure used in this experiment was the same as that described in 3.5.1.1 but with two modifications. Firstly, 0d *P. bracteatum* cultures were used for the time course study since these had previously been shown to have the maximum (if very limited) biotransformation capacity of all *P. bracteatum* culture ages (3.4.5). Secondly, the 800 μg of codeinone dissolved in 100 μl of methanol was added immediately before the initiation of the 0d cultures. The same volume of methanol was added to control flasks but contained none of the alkaloid.

The fwt (Fig.3.5.4.a) and dwt (Fig.3.5.4.b) curves show that the cultures underwent no growth during the course of the experiment, which was expected, because the experiment took place over what corresponded to the lag phase of the *P. bracteatum* growth curve (3.3.2). No differences were noted between the results obtained for experimental and control flasks. The %dwt (Fig.3.5.4.c) also remained fairly constant throughout the time course though the medium pH (Fig.3.5.4.d) did drop slowly from pH 5.6 to pH 4.9 in experimental cultures.

The total amount of codeinone and codeine detected in the cultures at each timepoint is shown in Fig.3.5.5. Codeinone levels fell rapidly from ca. 400 μg at 0h to 0 μg after 24h. No codeinone was detected in the cells at any time. However, there was only very limited codeine production which reached a maximum value of 24.18 μg after 24h. As seen in 3.5.1.1 the maximum amount of codeinone detected at any time corresponded to only half of that added to the cultures (0h). This fact, coupled with the disappearance of codeinone detected at 0h, without the formation of codeine, implies that the precursor is being converted to some product(s) other than codeine. The nature of these other reactions is reported in 3.5.2 and 3.5.3.

The next experiment describes the results obtained when *P. s. m* suspensions were used
for the time course studies. These cultures had been shown to have a substantially enhanced biotransformation capacity over less differentiated cells (eg. P.s and P.b). Therefore, by making a detailed study of the conversion it should be possible to establish the relationship between biotransformation capacity of a culture and its differentiated state.
Fig.3.5.4 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during a biotransformation time course study using 0d suspension cultures of *Papaver bracteatum*. Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; △ = experimental flask
Fig. 3.5.5 Amount of codeinone and codeine detected in cultures during a biotransformation time course study using 0d suspension cultures of *Papaver bracteatum*. Each point represents the mean of three replicates ± standard error.
3.5.1.3. Biotransformation time course experiment using 15d suspension cultures of *P. somniferum* "meristemoids"

The aim of this experiment was to quantify the enhanced biotransformation capacity of *P.s.m* cultures. The experiment was also designed to establish the relationship between the enhanced conversion activity and the more highly differentiated state of these cultures.

The experimental procedure used was as described in 3.5.1.1 with two modifications. Firstly, 15d *P.s.m* cultures were used, as these had previously been identified as having the highest biotransformation capacity (3.4.4). Secondly, the cultures were initiated by transferring 1.50±0.05g of filtered "meristemoids" because the %dwt of *P.s.m* cultures was ca. twice that of *P.s* cultures. Therefore, using half the weight of cells ensured that approximately the same amount of biomass (dwt) was present.

The fwt (Fig.3.5.6.a) and dwt (Fig.3.5.6.b) curves show that the cells underwent no growth during the time course and that there was no difference between the experimental and control cultures. The %dwt (Fig.3.5.6.c) and medium pH (Fig.3.5.6.d) of the cultures also stayed constant throughout the experiment with no difference between experimental and control flasks.

The total amount of codeinone and codeine detected in the cultures at each timepoint is shown in Fig.3.5.7. Codeinone levels dropped more rapidly than in *P.s* (Fig.3.5.2) or *P.b* (Fig.3.5.5) cultures. Indeed, from a maximum of 359.1μg at 0h no codeinone was detected just 1h after the addition of the precursor. No codeinone was detected in the cells at any time. The rapid disappearance of codeinone coincided with the rapid appearance of codeine in the culture medium. Codeine levels reached a maximum of 534.3μg after just 6h and remained level until 50h. This was substantially higher than the 317.8μg detected after 48h in *P.s* cultures (Fig.3.5.2).

As seen in previous experiments (3.5.1.1 and 3.5.1.2) the maximum amount of codeinone detected in 0h cultures still only accounts for approximately half of the codeinone added to the cultures. The possibility that some product other than codeine was present in the cultures has been investigated (see 3.5.2 and 3.5.3). Codeine was present in "meristemoid" cells from 1h through to 50h after the addition of codeinone. The level of codeine was between 8% and 12% of the total detected and was 61.02pg (12.03%) after 50h. This was in contrast to *P.s* cultures (3.5.1.1) where codeine was only present in cells 100h after codeinone was added to the cultures. No codeinone or codeine were detected in the cells or medium of control cultures at any time despite the known presence of codeine in these cultures (3.3.4). This was because in
the previous study replicates from each timepoint were pooled, and therefore, a
greater amount of biomass was extracted.

The next experiment describes the results obtained when cultures of \( P. s. i \) were used. Immobilisation of poppy cells has previously been reported to enhance the biotransformation capacity of cultures (Furuya et al., 1984; Corchete and Yeoman, 1990). Therefore, by closely studying the biotransformation it might be possible to determine why immobilised cultures have superior biotransformation capacities to suspended cultures. It will also establish if there is a difference in the rate at which the conversion takes place in \( P. s \) and \( P. s. i \) cultures.
Fig.3.5.6 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during a biotransformation time course study using 15d suspension cultures of *Papaver somniferum* "meristemoids". Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; △ = experimental flask
Fig. 3.5.7 Amount of codeinone and codeine detected in cultures during a biotransformation time course study using 15d suspension cultures of *Papaver somniferum* "meristemoids". Each point represents the mean of three replicates ± standard error.
3.5.1.4. Biotransformation time course experiment using 10d immobilised cultures of *P.somniferum*

The aim of this experiment was to establish whether cultures of *P.s.i* possessed an enhanced biotransformation capacity. By closely following the conversion it should be possible to compare the rate at which the reaction occurs with the rate determined for *P.s* suspension cultures (3.5.1.1). The experiment was also designed to determine whether codeinone and codeine were distributed in the cells and medium in the same proportion as noted in suspended *P.s* cultures (3.5.1.1). Therefore, it might be possible to establish the relationship between immobilisation of a culture and enhanced biotransformation activity.

The experimental procedure was as described in 3.5.1.1 with one modification, which was that *P.s.i* cultures were initiated by transferring 3.00±0.05g of filtered cells from 12d *P.s* suspension cultures and after 12d growth the foam blocks filled with cells transferred to fresh medium. These cultures were then allowed to grow for 10d prior to use in the time course experiment. This was because 10d *P.s* suspension cultures were known to have the highest overall biotransformation capacity (3.4.3).

The fwt (Fig.3.5.8.a) and dwt (Fig.3.5.8.b) data show that the cells did not grow during the experiment. The dwt results show more variation than observed with other culture types (3.5.1.1 to 3.5.1.3) due to the variable extent with which cells left the surface of the foam blocks (3.3.5). The %dwt (Fig.3.5.8.c) and medium pH (Fig.3.5.8.d) also remained constant throughout the experiment with no distinguishable difference between the results from the experimental and control flasks.

The total amount of codeinone and codeine detected in the cultures at each timepoint is shown in Fig.3.5.9. Codeinone levels dropped fairly rapidly and none was detectable after 24h. Also, no codeinone was present in cells at any time. Codeine levels rose relatively slowly from 0.5h to reach a maximum of 430.2μg at 72h. This was 180.3μg higher than had been found with the *P.s* suspension cultures (Fig.3.5.2) but 104.1μg lower than with *P.s.m* cultures (Fig.3.5.7). The maximum amount of codeinone detected in 0h cultures of 358.9μg accounted for approximately one half of the codeinone added to the cultures. This had been seen previously (3.4.6 and 3.5.1.1 to 3.5.1.3) and the possibility that some of the codeinone had been converted to a product(s) other than codeine during either the experiment or the extraction procedure is explored further in 3.5.2 and 3.5.3. Codeine was found in cells at all timepoints after 6h, in a range of between 8-15% of the total detected. This was in contrast to
P.s cultures (3.5.1.1), where codeine was only present in cells 100h after the addition of codeinone. Control cultures were not found to contain codeine or codeinone at any time, despite the fact that these cultures were known to contain very small amounts of codeine (3.5.5). This was because in the previous study replicates were pooled and this provided more biomass (dwt) per sample than in this experiment.

The next section describes the results obtained when cultures of P.s.m.i were used for the time course studies. These cultures had previously been shown to contain higher levels of codeine than P.s suspension cultures and were expected to have an enhanced biotransformation capacity. By studying the conversion in detail it should be possible to determine if the rate of product formation differed from that seen in P.s.m suspension cultures. It might also be possible to establish if the distribution of codeinone and codeine in cells and medium differs between culture types.
Fig.3.5.8 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during a biotransformation time course study using 10d immobilised cultures of *Papaver somniferum*. Each point represents the mean of three replicates ± standard error (where shown).

○ = control flask; △ = experimental flask
Fig. 3.5.9 Amount of codeinone and codeine detected in cultures during a biotransformation time course study using 10d immobilised cultures of *Papaver somniferum*. Each point represents the mean of three replicates ± standard error.
3.5.1.5. Biotransformation time course experiment using 15d immobilised cultures of *P.somniferum* "meristemoids"

The aim of this experiment was to establish whether cultures of *P.s.m.i* possessed an enhanced biotransformation capacity. The rate at which the conversion occurred and the distribution of codeinone and codeine in cells and medium determined. The results obtained could then be compared to those reported in other types of culture (3.5.1.1 to 3.5.1.4).

The experimental procedure was as described in 3.5.1.1 with one change. *P.s.m.i* cultures were initiated by transferring 1.50±0.05g of filtered cells from 15d *P.s.m* suspensions into fresh growth medium containing five foam blocks. After 15d growth, the foam blocks filled with cells were transferred to fresh growth medium and incubated for 15d under the conditions described in 2.2.3.1, prior to use in the time course experiment.

The fwt (Fig.3.5.10.a) and dwt (Fig.3.5.10.b) data show that cells did not grow during the experiment. The %dwt (Fig.3.5.10.c) and medium pH (Fig.3.5.10.d) also remained constant throughout the experiment with no distinguishable difference between the results of experimental and control cultures.

The total amount of codeinone and codeine detected in the cultures at each timepoint is shown in Fig.3.5.11. Codeinone levels dropped rapidly and none was detectable after 3h. No codeinone was found in cells at any time. The amount of codeine in the cells rose relatively slowly from 0.5h to reach a maximum of 527.1μg at 72h. This was 209.3μg higher than with the highest codeine producing *P.s* suspension culture (Fig.3.5.2). However, the amount of codeine present in the 72h *P.s.m.i* cultures was approximately equal to that in the highest codeine producing *P.s.m* cultures (6h, Fig.3.5.7) but was achieved some 66h later than in the suspension cultures. The maximum amount of codeinone detected in 0h cultures was 354.9μg and accounted for only one half of the codeinone added to the cultures. This was noted in the previous time course experiments (3.5.1.1 to 3.5.1.4) and the fate of the codeinone not converted to codeine is reported in 3.5.2 and 3.5.3. The codeine detected in cells of all timepoints after 1h was between 8-13% of the total codeine produced. This was a similar result to that observed in *P.s.m* (3.5.1.3) and *P.s.i* (3.5.1.4) cultures but not in *P.s* (3.5.1.1) cultures. No codeinone or codeine was detected in any control cultures despite the fact that these cultures were known to contain codeine (3.3.6). This was because in the previous study replicates were pooled and consequently more biomass (dwt) was extracted per sample than was the case here.
These experiments have established that cultures of *Papaver* spp. biotransform codeinone to codeine at different rates. The vast majority of the product was present in the medium whilst cells of *P.s* were seen to contain less codeine than cells from *P.s.m*, *P.s.i* and *P.s.m.i* cultures. The next section describes an experiment which investigated whether any conversion activity remained in the culture medium after the cells had been removed. This might also help establish the fate of that codeinone which is not converted to codeine.
Fig. 3.5.10

Fig. 3.5.10.a

Fig. 3.5.10.b

Fig. 3.5.10.c

Fig. 3.5.10.d

Fig. 3.5.10 Changes in fresh weight (a), dry weight (b), percentage dry weight (c) and medium pH (d) during biotransformation time course study using 15d immobilised cultures of *Papaver somniferum* "meristemoids". Each point represents the mean of three replicates ± standard error (where shown)

○ = control flask; △ = experimental flask
Fig.3.5.11 Amount of codeinone and codeine detected in cultures during a biotransformation time course study using 15d immobilised cultures of *Papaver somniferum* "meristemoids". Each point represents the mean of three replicates ± standard error.
3.5.2. Biotransformation experiment using spent medium of Papaver spp. cultures with the cells removed

The aim of this experiment was to determine whether any biotransformation activity remained in the culture medium after the cells had been removed. If the amount of codeinone recovered from fresh medium is more than with spent medium then this will indicate that cultured cells release some compound into the medium which interferes with the extraction procedure. It might be possible to determine how much codeinone is "lost" and may also explain the fate of that codeinone not converted to codeine during previous biotransformation experiments (3.4.1 to 3.4.6 and 3.5.1.1 to 3.5.1.5).

The spent medium used in this experiment came from suspensions of P. somniferum (P.s), P. somniferum "meristemoids" (P.s.m) and P. bracteatum (P.b) and immobilised cultures of P. somniferum (P.s.i) and P. somniferum "meristemoids" (P.s.m.i). Autoclaved fresh medium (2.2.1) was used as a control to establish the extent to which poppy cells had affected any conversions that occurred. Cultures of P.s, P.b, P.s.m, P.s.i and P.s.m.i were initiated and grown for the same period of time as described in 3.5.1.1 to 3.5.1.5, respectively. Cells were then separated from their culture medium by sterile filtration through a Millipore Sterifil Aseptic System (Millipore [U.K.] Ltd, Watford, Herts, U.K.) fitted with a 0.2μm cellulose acetate filter. The spent medium was transferred to clean sterile flasks and then placed under the culture conditions stated in 2.2.3.1 to reach ambient temperature. As previously, 800μg of codeinone, dissolved in 100μl of methanol, was added to flasks by injection using a 100μl glass syringe and the foil cap resealed with autoclave tape. Flasks containing fresh autoclaved medium were treated in the same way as described above but were unfiltered. Cultures were incubated for 0, 24 or 48h, with three flasks being harvested at each timepoint. The length of time between the addition of methanol containing codeinone and the start of extraction for 0h cultures was ca. 15s. The flask contents were extracted at the appropriate timepoint and then analysed for the presence of codeine and codeinone by HPLC.

The total amount of codeinone detected in the flasks at each timepoint is shown in Fig.3.5.12. More codeinone was extracted from fresh culture medium (Fig.3.5.12.f) than from the spent medium in any of the other culture flasks (Fig.3.5.12.a to 3.5.12.e). This implies that some compound associated with cultured cells remained in the medium after filtration and adversely affected the efficiency with which codeinone was recovered from the spent medium. The amount of codeinone
recovered from fresh medium remained constant over the 48h incubation period. This was in contrast to the spent medium of \textit{P.s} (Fig.3.5.12.a), \textit{P.b} (Fig.3.5.12.b), \textit{P.s.m} (Fig.3.5.12.c), \textit{P.s.i} (Fig.3.5.12.d) and \textit{P.s.m.i} (Fig.3.5.12.e) cultures where the amount of codeinone detected dropped over the course of the experiment. This was especially so with \textit{P.b} culture medium where after 48h only 103.9\textmu g of codeinone was detectable. The amount of codeinone extracted from fresh medium at 0h was ca. 200\textmu g higher than from spent medium and this rose to between 350 and 400\textmu g after 48h. However, in none of the flasks was any codeine detected indicating that some other product(s) must have been formed during the time course. This also implies that some factor associated with poppy cells remains in the medium and slowly reduces the amount of codeinone present in its free form. It also confirms that cells of \textit{P.s}, \textit{P.s.m}, \textit{P.s.i} and \textit{P.s.m.i} are necessary for the biotransformation of codeinone to codeine.

The next section describes the attempts made to identify the presence of various bound forms of morphinan alkaloids in poppy cultures. Such compounds have been reported before in both plants (Wold, 1978; Fairbairn and Steele, 1980) and cultures (Morris and Gibbs, 1986) of \textit{P.somniferum} and their presence in cultures used for biotransformation experiments could explain the fate of that codeinone not transformed to codeine.
Fig. 3.5.12 Amount of codeinone detected in cell free medium of cultures of P.s (a), P.b (b), P.s.m (c), P.s.i (d), P.s.m.i (e) and fresh medium (f) during a biotransformation time course study. Each point represents the mean of three replicates ± standard error.
3.5.3. Identification of bound forms of morphinan alkaloids in cultures of *Papaver* spp.

The aim of this experiment was to investigate the possible presence of bound forms of morphinan alkaloids in cultures of *Papaver* spp. Such compounds have previously been described, both in plants (Wold, 1978; Fairbairn and Steele, 1980) and suspension cultures (Morris and Gibbs, 1986) of *P.somniferum*. Fairbairn and Steele (1980) reported bound forms of morphine and codeine in the polysaccharide fraction of the pericarp and suggested that "these substances represent transitional forms in the metabolism and translocation of morphine from latex to seed". If bound forms of morphinan alkaloids were formed during the biotransformation experiments described in 3.4 and 3.5.1.1 to 3.5.2, then this could explain the fate of codeinone not converted to free codeine.

Suspension cultures of *P.somniferum* (P.s), *P.bracteatum* (P.b) and *P.somniferum* "meristemoids" (P.s.m) and immobilised cultures of *P.somniferum* (P.s.i) and *P.somniferum* "meristemoids" (P.s.m.i) were initiated as described in 3.5.1.1 to 3.5.1.5, respectively. Six flasks (three control and three experimental) were used for each culture type and in addition, six flasks of fresh, autoclaved medium (2.2.1) were also studied. Using a 100μl glass syringe, 800μg of codeinone, dissolved in 100μl of methanol, was added to P.s, P.b and P.s.i experimental cultures 10d after initiation and 15d after the initiation of P.s.m and P.s.m.i experimental cultures. Codeinone was added to fresh, autoclaved medium once it had reached ambient temperature (2.4.1.2). The foil caps were resealed with autoclave tape and the cultures then incubated under the conditions stated in 2.2.3.1. Control cultures were treated in exactly the same way as experimental cultures except that the methanol added to control cultures contained no codeinone. All flasks were harvested 12h after the addition of codeinone and the medium and cells separated by filtration (2.3.1). The cells and medium were then extracted and hydrolysed as depicted in Fig.3.5.13. This yielded six samples per flask C1, C2 and C3 from the cells and M1, M2 and M3 from the medium. The numbers corresponded to an increasing degree of hydrolysis (i.e. 1 = no hydrolysis, 2 = mild hydrolysis and 3 = vigorous hydrolysis). All samples were analysed by 1DTLC (solvent system T2, 2.4.2.2) and any alkaloids present located using Dragendorff's reagent (2.4.2.4).
Experimental protocol to study bound forms of codeinone and codeine in cultures of *Papaver* spp.

Addition of codeinone to experimental and control cultures (3.5.3)
Harvest cultures 12h after the addition of codeinone
Separation of cells and medium (2.3.1)

**MEDIUM**
- Extraction of morphinan alkaloids → M1 at pH 9.0 (2.4.1.2)
- Mild hydrolysis (1M NH₄Cl, 1h, 100°C)
- Extraction of morphinan alkaloids → M2 at pH9.0 (2.4.1.2)
- Vigorous hydrolysis (2M HCl, 1h, 100°C)
- Extraction of morphinan alkaloids → M3 at pH9.0 (2.4.1.2)

**CELLS**
- Freeze cells at -40°C
- Freeze dry cells
- Extract cells in methanol, evaporate to dryness under reduced pressure and take up in 0.01M HCl (2.4.1.2)
- Extraction of morphinan alkaloids → C1 at pH9.0 (2.4.1.2)
- Mild hydrolysis (1M NH₄Cl, 1h, 100°C)
- Extraction of morphinan alkaloids → C2 at pH9.0 (2.4.1.2)
- Vigorous hydrolysis (2M HCl, 1h, 100°C)
- Extraction of morphinan alkaloids → C3 at pH9.0 (2.4.1.2)

Fig.3.5.13 Experimental procedure used in 3.5.3 for the hydrolysis and subsequent extraction of bound forms of morphinan alkaloids in cultures of *Papaver* spp. (after Fairbairn and Steele, 1980). 1DTLC analysis was performed on samples M1, M2, M3, C1, C2 and C3 obtained from all cultures (solvent system T2, 2.4.2.2).
Table 3.5.1 shows which of the C1 and M1 samples were found to contain codeine or codeinone. The results show that codeinone was converted to codeine in P.s, P.b, P.s.m, P.s.i and P.s.m.i cultures and are consistent with those recorded in the biotransformation experiments described in 3.5.1.1 to 3.5.1.5. Codeine was present in the medium of all culture types except the fresh medium, although the amount present in P.b cultures was very much less than detected in P.s, P.s.m, P.s.i and P.s.m.i cultures. It was also possible that very small amounts of morphine were present in these latter cultures. However, the alkaloid spots were very small and reacted poorly with Dragendorff’s reagent (2.4.2.4).

### Table 3.5.1

**Presence of free codeinone and codeine in cultures of Papaver spp.**

<table>
<thead>
<tr>
<th>Culture Type</th>
<th>CELLS</th>
<th>MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
<td>M1</td>
</tr>
<tr>
<td></td>
<td>Cd=O</td>
<td>Cd-OH</td>
</tr>
<tr>
<td></td>
<td>Cd=O</td>
<td>Cd-OH</td>
</tr>
<tr>
<td>P.s</td>
<td>E</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>P.b</td>
<td>E</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>P.s.m</td>
<td>E</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>P.s.i</td>
<td>E</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>P.s.m.i</td>
<td>E</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>Fresh Medium</td>
<td>E</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**KEY:**
- + = alkaloid detected
- - = alkaloid undetected
- ++ = alkaloid present at higher level than in control flask
- n/a = not applicable
- E = experimental flask
- C = control flask
- C1+M1 = samples obtained prior to hydrolysis
- Cd=O = codeinone
- Cd-OH = codeine
Table 3.5.2 summarises the results obtained from TLC analyses of samples C2, C3, M2 and M3. The results for codeinone and codeine are described separately in the two paragraphs below.

**Codeinone**

Mild hydrolysis yielded codeinone from the medium samples (M2) of all experimental culture types, including the fresh medium. This suggests that when codeinone is added to the cultures some of it becomes ionically bound to a polar compound(s) in the medium. This would reduce the solubility of codeinone in the chloroform:iso-propanol mixture used during the extraction procedure and consequently reduce the amount of alkaloid recovered from the medium. However, mild hydrolysis did not release substantial quantities from the cells (C2) of any experimental culture. It is possible that codeinone was present in these samples but the alkaloid spots detected were both very small and faint. Such observations could be the result of ionically bound codeinone not being completely washed from the cell surfaces during the separation of medium and cells. All samples obtained from the medium and cells of experimental flasks subjected to a vigorous hydrolysis (C3 and M3) were found to contain codeinone. This was with the exception of samples obtained from fresh medium, where no strongly bound codeinone was detected. It was difficult to ascertain with some samples whether codeinone was actually present due to "streaking" effects on the TLC plates. However, for each culture type, at least one of the three replicates clearly demonstrated the presence of codeinone. This is the first time during the project that codeinone has been detected, in any form, in samples obtained from cultured cells. The amounts present were higher than in samples subjected to a mild hydrolysis so it seems unlikely that the presence of codeinone in C3 samples was due to incomplete washing of cells. However, the amount of codeinone present was still small and probably only accounted for ca. 10μg per flask. The release of codeinone after vigorous hydrolysis of cell and media samples of all culture types other than fresh medium would suggest that some factor(s) associated with cultured poppy cells, covalently binds to codeinone. This would account for the fact that less codeinone was extracted from filtered spent medium (0h) than from fresh autoclaved medium (0h) in the experiments described in 3.4.6 and 3.5.2. At no point was codeinone released by either mild or vigorous hydrolysis of cell and medium samples obtained from control cultures.
Codeine

Mild hydrolysis released codeine from cell and medium samples of all experimental cultures except those of P.b and fresh medium. Codeine was also released by mild hydrolysis of cell and medium samples of P.s, P.s.m, P.s.i and P.s.m.i control cultures. However, in these four cases the amount of alkaloid released was less than had been detected in the corresponding experimental flasks. This implies that some of the codeine produced during the biotransformation of codeinone, became bound ionically to a polar compound(s) present in both cells and medium. The reduction in the solubility of codeine in the chloroform:iso-propanol mixture, used to extract the alkaloids, would consequently reduce the amount of codeine recovered from the cultures. As with mild hydrolysis, vigorous hydrolysis released codeine from cell and medium samples of all experimental cultures except those of P.b and fresh medium. Control cultures of P.s, P.s.m, P.s.i and P.s.m.i were also found to yield codeine after vigorous hydrolysis though to a lesser extent than in experimental cultures. This implies that some of the codeine produced from the biotransformation of codeinone became covalently bound to some compound(s) associated with cultured poppy cells reducing the amount of codeine recovered from the cultures. The ratio of bound codeine found in cells and medium appeared to be approximately one (i.e. 1:1) whilst the ratio of free codeine in cells and medium was ca. 1:9 (3.4 and 3.5.1.1 to 3.5.1.5).

In cell and media samples obtained from control and experimental cultures of P.s, P.s.m, P.s.i and P.s.m.i, there was evidence to suggest that some bound forms of morphine were also present. However, the appropriate alkaloid spots were small and reacted very faintly with Dragendorff's reagent.

During the mild, and especially the vigorous hydrolysis of samples, some degradation of the alkaloids occurred which produced "streaking" effects on the TLC plates. Unfortunately, quantification of the alkaloids present by HPLC proved impossible due to the broad and overlapping peaks obtained for both codeinone and codeine.

This section has shown that bound forms of both codeinone and codeine occurred during biotransformation experiments, adversely affecting the extraction of both alkaloids. In the next section the rate at which P.s, P.s.m, P.s.i and P.s.m.i cultures convert codeinone to codeine at different substrate concentrations is studied. This might help establish why the biotransformation capacities of immobilised cultures are greater than those of suspension cultures.
**Table 3.5.2**

Presence of bound forms of codeinone or codeine in cultures of *Papaver* spp.

<table>
<thead>
<tr>
<th>Culture Type</th>
<th>CELLS</th>
<th>MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C2</td>
<td>C3</td>
</tr>
<tr>
<td></td>
<td>Cd=O</td>
<td>Cd-OH</td>
</tr>
<tr>
<td><strong>P.s</strong></td>
<td>E</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td><strong>P.b</strong></td>
<td>E</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td><strong>P.s.m</strong></td>
<td>E</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td><strong>P.s.i</strong></td>
<td>E</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td><strong>P.s.m.i</strong></td>
<td>E</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>Fresh</td>
<td>E</td>
<td>n/a</td>
</tr>
<tr>
<td>Medium</td>
<td>C</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**KEY:**

- + = alkaloid detected
- - = alkaloid undetected
- ++ = alkaloid present at higher level than in control flask
- n/a = not applicable
- ? = "streaked" spot

E = experimental flask
C = control flask
C2+M2 = samples obtained after mild hydrolysis
C3+M3 = samples obtained after vigorous hydrolysis
Cd=O = codeinone
Cd-OH = codeine
3.5.4. Rate of biotransformation in cultures of *Papaver* spp. at different substrate concentrations

The aim of this experiment was to determine the rate at which suspension cultures of *P. somniferum* (P.s), *P. somniferum* "meristemoids" (P.s.m) and immobilised cultures of *P. somniferum* (P.s.i) and *P. somniferum* "meristemoids" (P.s.m.i) converted codeinone to codeine at different substrate concentrations. If the rate of reaction in suspended and immobilised cultures is different at the same substrate concentration then it might be possible to establish a reason for the enhanced biotransformation capacity of immobilised cultures (3.5.1.4). A comparison, with the rate of reaction in the more highly differentiated "meristemoid" cultures could also provide an explanation for the high level of conversion activity reported in these cultures (3.4.4 and 3.5.1.3).

Cultures of P.s, P.s.m, P.s.i and P.s.m.i were initiated as described in 3.5.1.1, 3.5.1.3, 3.5.1.4 and 3.5.1.5 respectively. Codeinone, dissolved in 100μl of methanol, was added 10d after the initiation of P.s and P.s.i cultures and 15d after the initiation of P.s.m and P.s.m.i cultures using a 100μl glass syringe. As previously described the punctured foil cap was resealed with autoclave tape. Codeinone was added in the following amounts 0, 100, 200, 400, 800 and 1200μg/flask^{-1}. Two replicate flasks of each culture type were used at each substrate level. It had already been established in 3.5.1.1 (P.s), 2.5.1.3 (P.s.m), 3.5.1.4 (P.s.i) and 3.5.1.5 (P.s.m.i) that the rate of codeine production was linear over the first 6h of incubation for each culture type. Therefore, during this experiment, all cultures were incubated with codeinone for 6h under the conditions stated in 2.2.3.1. Cells were then separated from the culture medium (2.3.1) and the cells frozen at -40°C and freeze-dried (2.3.2). Alkaloids were extracted from the spent culture medium immediately, but extracted from the cells after they had been freeze-dried (2.4.1.2). The amount of each alkaloid present in each sample was then quantified by HPLC analysis (2.4.3).

The total amount of codeine present in cultures after 6h is shown in Table 3.5.3 (P.s), Table 3.5.4 (P.s.m), Table 3.5.5 (P.s.i) and Table 3.5.6 (P.s.m.i). In most cases the amount of codeine produced increased with substrate concentration. However, at substrate levels of 800 and 1200μg/flask^{-1}, there appeared to be some limitation to the amount of codeine produced in suspended cultures (P.s and P.s.m). This was not the case in immobilised cultures (P.s.i and P.s.m.i). Suspended and immobilised "meristemoid" cultures (P.s.m and P.s.m.i) showed greater codeine production than the corresponding "normal" cultures (P.s and P.s.i). This confirms the earlier finding.
that "meristemoid" cultures have an enhanced biotransformation capacity. The rate of conversion (over 6h) was higher than in immobilised cultures at all levels of substrate. This was also reported in 3.5.1 and could be due to suspended cultures having easier access to the substrate and fewer mass transfer problems than immobilised cultures. At higher levels of codeinone the limitation to codeine production might occur for two possible reasons. Firstly the "active sites" of the suspended cultures might have become saturated, thereby reducing the amount of product produced. Secondly, some product inhibition of the conversion could have occurred. The percentage of codeine recovered from cultures to which 100, 200 and 400μg of codeinone had been added was greater than the calculated extraction efficiency (61.0%, Table 3.4.7). This could mean that codeine production was "elicited" by the presence of codeinone. However, the extraction efficiency was calculated using 20d P.s.m cultures which have a slightly more viscous medium than 15d cultures. This could have affected the extraction procedure by causing a boundary layer to form between the aqueous and non-aqueous fractions.

The results in this section have shown that the rate at which immobilised and suspended cultures convert codeinone to codeine differ. They have also shown that $P.s.m$ and $P.s.m.i$ cultures have higher conversion rates than the corresponding $P.s$ and $P.s.i$ cultures. The next section describes an attempt made to use a crude enzyme extract of $P.s$ cultures to biotransform codeinone to codeine. This simple experiment will establish whether the enzymic activity is present in a cell-free system.
### Table 3.5.3

**Amount and rate of codeine production by *P. somniferum* suspension cultures at different codeinone concentrations**

<table>
<thead>
<tr>
<th>Amount of codeinone added (µg.flask⁻¹)</th>
<th>Amount of codeine produced (µg.flask⁻¹±S.E.)</th>
<th>Biotransformation ratio (%)</th>
<th>Velocity of reaction (µg.h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>58.56±2.6</td>
<td>58.6</td>
<td>9.76</td>
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<tr>
<td>200</td>
<td>124.8±3.7</td>
<td>62.4</td>
<td>20.8</td>
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<tr>
<td>400</td>
<td>255.0±2.9</td>
<td>63.8</td>
<td>42.5</td>
</tr>
<tr>
<td>800</td>
<td>320.4±5.6</td>
<td>40.0</td>
<td>53.4</td>
</tr>
<tr>
<td>1200</td>
<td>343.2±4.3</td>
<td>28.6</td>
<td>57.2</td>
</tr>
</tbody>
</table>

### Table 3.5.4

**Amount and rate of codeine production by *P. somniferum* "meristemoid" suspension cultures at different codeinone concentrations**

<table>
<thead>
<tr>
<th>Amount of codeinone added (µg.flask⁻¹)</th>
<th>Amount of codeine produced (µg.flask⁻¹±S.E.)</th>
<th>Biotransformation ratio (%)</th>
<th>Velocity of reaction (µg.h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>539.6±3.8</td>
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### Table 3.5.5

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<tr>
<th>Amount of codeinone added (µg./flask⁻¹)</th>
<th>Amount of codeine produced (µg./flask⁻¹±S.E.)</th>
<th>Biotransformation ratio (%)</th>
<th>Velocity of reaction (µg.h⁻¹)</th>
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<td>0</td>
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<td>137.7±2.6</td>
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### Table 3.5.6

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<th>Amount of codeine produced (µg./flask⁻¹±S.E.)</th>
<th>Biotransformation ratio (%)</th>
<th>Velocity of reaction (µg.h⁻¹)</th>
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<td>800</td>
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<td>1200</td>
<td>250.8±2.8</td>
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<td>41.8</td>
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3.5.5 Biotransformation using a crude enzyme extract of P.somniferum cultures

The aim of this experiment was to demonstrate the presence of enzymic activity for the conversion of codeinone to codeine in a crude extract of P.somniferum suspension cultures (P.s). This might establish whether the conversion by Papaver spp. cultures occurred in the cell or culture medium.

The use of crude enzyme preparations from plant (Hodges and Rapoport, 1980) and callus (Furuya et al., 1978) tissue of P.somniferum to convert codeinone to codeine has previously been reported. Furuya et al. (1978) ground up callus cells in a borate buffer, centrifuged the extract, then used the pellet resuspended in fresh borate buffer as the source of enzyme. However, Hodges and Rapoport (1980), homogenised plant tissue in 150mM Tris-HCl buffer (1.3ml.g⁻¹ tissue) at pH 7.5, the homogenate being filtered through Miracloth, and the filtrate then used as the source of the enzyme. The method used in this experiment was after Hodges and Rapoport (1980) except that suspension cultures of P.s were extracted in 150mM Tris-HCl buffer (2.0ml.g⁻¹ tissue) instead of whole plants. These cultures had been initiated as described in 3.5.1.1 and after 10d growth the cells and culture medium were separated (2.3.1).

4ml portions of the enzyme preparation were placed in 15ml test-tubes at 24°C and 37°C and allowed to equilibrate. Just prior to starting the reaction, 100μl of extraction buffer containing 2μmol of NADH was added with a 100μl glass syringe. In a separate injection, 40μl of methanol containing 50μg of codeinone was added to both control and experimental test-tube. The reaction was stopped by the addition of 50μl of 85%H₃PO₄ and then cooled over ice. Six tubes (three control and three experimental) were removed for assay at 0, 1, 3, 6 and 24h after the addition of codeinone. Control tubes contained 4ml of enzyme preparation previously denatured in a boiling waterbath (100°C for 1h). All samples were extracted as previously described (2.4.1.2) except that the smaller volumes sampled were extracted three times with 5ml of the solvent mixture in 25ml Erlenmeyer flasks. The solvent layer was removed using a Pasteur pipette, the solvent fractions combined, evaporated to dryness under reduced pressure and then taken up in 1ml of methanol. Analysis of the alkaloid content was made by 1DTLC (solvent system T2, 2.4.2.2) and the alkaloids localised with Dragendorff’s reagent (2.4.2.4).

Fig.3.5.13 shows a TLC separation of the alkaloids present in the extracts at different timepoints during the course of the reaction at 24°C. The samples from 1, 3 and 6h after the addition of codeinone can be seen to contain several alkaloids with codeine present in all three. 0h samples contained only codeinone and possibly some codeine
(endogenous), whilst after 24h virtually all the alkaloids have disappeared. Low amounts of codeine were present in the controls similar to the quantities found at 0h in experimental tubes. No alkaloids other than codeinone (0 and 1h experimental tubes) were detected in the experiment carried out at 37°C. The relatively large number of alkaloids found in the 6h samples at 24°C suggests that enzymes other than the one responsible for the biotransformation of codeinone to codeine are also present. This was not surprising given the crude nature of the enzyme preparation.

The next section contains a summary of all the results presented in this thesis.
Fig. 3.5.13 Alkaloids extracted from enzyme assay tubes (24°C) 0, 1, 3, 6 and 24 after the addition of codeinone and separated by 1D TLC using solvent system T2 (2.4.2.2). The amount of standard alkaloid applied was 20μg and the spots were visualised with Dragendorff’s reagent (2.4.2.4). Key: Mix = mixture of standard alkaloids (codeine [A], codeinone [B] and thebaine [C])
3.6. Summary of results

1. Plants of P.s, P.h and P.o vary in their ability to synthesise and accumulate morphinan alkaloids.

2. Suspension cultures of P.s, P.s.m, P.h and P.o are able to synthesise and accumulate one or more of the morphinan alkaloids common to the parent plant.

3. The higher state of differentiation of P.s.i, P.s.m and P.s.m.i cultures appears to allow larger quantities of morphinan alkaloids to be accumulated than in "normal" P.s suspensions.

4. The quantity of alkaloids present in cultures of all types was significantly less than found in the parent plants.

5. The ability of cultures derived from plants of P.s, P.h and P.o to biotransform codeinone to codeine, was related to the ability of the parent plant to perform this same conversion in vivo.

6. The maximum biotransformation ratio achieved by P.s cultures was relatively constant across the growth cycle. However, the rate of product formation was fastest in cultures entering the stationary phase.

7. Cultures of P.s.i, P.s.m and P.s.m.i were more highly differentiated and achieved higher maximum biotransformation ratios than cultures of P.s.

8. "Bound" forms of codeinone were found in the media of all culture types but were present in P.s cells in only very minor amounts.

9. "Bound" forms of codeine were present in cells and media of all P.s culture types, at much higher levels than codeinone.

10. The rate of product formation was faster in suspension cultures than in immobilised cultures. However, immobilisation of P.s cultures did increase the maximum biotransformation ratio achieved.
Chapter 4.
DISCUSSION
Introduction

The biochemical potential of plants is extremely wide and they are a source of many secondary metabolites utilised in the pharmaceutical, food, fragrance and agrochemical industries (Collin, 1987; Phillipson, 1990). However, in culture most plant cells tend to accumulate very small quantities of secondary products, both common and foreign to the parent plant and this low yield is a major barrier to commercial production (Yeoman et al., 1990). In addition, cultured cells possess the ability to convert added substances, both natural and synthetic, to commercially desirable products. Such conversions, or biotransformations, are potentially useful for changing cheap and plentiful substances into more expensive and rarer compounds. A biotransformation may be defined as "the conversion of a substance by living cultures, permeabilised cells or entrapped enzymes into a chemically different product" (Yeoman et al., 1990). Many studies have been conducted on these biotransformations (for a recent review see Suga and Hirata, 1990), most of which focus on the ability of cultured cells to perform a particular, usually stereospecific, chemical reaction. However, few detailed accounts on the relationship of biotransformation kinetics with culture origin, stage of development and state of differentiation have been published. This discussion concentrates on these relationships, bearing in mind their possible application to commercially viable processes.

Four requirements for a successful biotransformation have already been defined (Steck and Constabel, 1974; Yeoman et al., 1990). These are:

1. The culture must have the enzymes necessary for the transformation of precursor to product.
2. The product must be formed faster than it is further metabolised.
3. The culture must tolerate the added precursor and the product.
4. The substrate must be able to enter the cell and the product will preferably be released into the medium.

During the discussion attempts will be made to establish whether these criteria have been fulfilled, though not necessarily in the order presented above. As already pointed out in the introduction to this thesis, the biotransformation of codeinone to codeine is, in commercial terms, "trivial" because the reduction can be carried out quantitatively and stereospecifically by borohydride (Gates, 1953). The problem is
compounded by the fact that codeinone is produced exclusively from codeine and is not readily available commercially. However, this reaction does provide a good "model system" to study a one-step biotransformation which, being controlled by only one enzyme, means that factors involved in the regulation of the reaction can be clearly determined. Other advantages of studying this particular biotransformation are that the product is accumulated rapidly without significant further conversion and, like the vast majority of reported biotransformations, the product is released into the medium. The system also provides a means to study the relationship between differentiation and enhanced biotransformation capacity, as P.s cultures randomly produce "meristemoids", in which the cells are less vacuolated and slower growing than the cells in P.s suspensions.

The discussion is divided into six sections, the first three of which examine the relationship of biotransformation capacity with culture origin, stage of development and state of differentiation, respectively. The fourth section looks at the problems of the further metabolism of the product and the efficiency with which alkaloids are extracted from the cultures. The fifth section examines aspects of the kinetics of the conversion paying particular attention to the reaction rate at different substrate concentrations. The final section summarises the results that have been discussed and looks at the implications for commercially viable biotransformations.

4.1. Relationship between biotransformation capacity and culture origin

Alkaloid content of plants

The plants used during these studies Papaver somniferum (P.s), P.bracteatum (P.b) and P.orientale var. "Scarlet" (P.o) vary considerably in their alkaloid content (3.1). Plants of P.s were found to contain thebaine, codeine and morphine as well as six other unidentified alkaloids. Plants of P.b and P.o accumulated thebaine but not codeine or morphine. In all, P.b contained six alkaloids whilst only four were detected in P.o. Two alkaloids appeared to be common to these latter two plants, having the same Rf values after 2DTLC. Apart from thebaine, the only alkaloids reported in both P.b and P.o are bracteoline, isothebaine, mecambidine, papaverrubine (B,D and E) and protopine (Šantavý, 1979). Therefore, it is possible that the two alkaloids detected in both P.b and P.o during these experiments, could correspond to one or more of those listed above. However, a more detailed investigation would be required to establish this with certainty.

The differences in alkaloid content of the three plants were not unexpected, and have
previously been reported by several authors (including Šantavý, 1979; Phillipson, 1983). The implication of these findings is that the enzyme responsible for the 6-O-demethylation of thebaine (the first step to codeine and morphine synthesis) is either not present or inactive in plants of P.b and P.o. This assumes that codeine and morphine biosynthesis follow the same pathway as seen in P.s. It has also been shown that the synthesis of thebaine in P.b proceeds by the same route as in P.s (Hodges et al., 1977; Brochmann-Hanssen and Wunderly, 1978). However, in contrast to early studies, the attainment of lower detection limits has enabled the presence of very small amounts of codeine to be located in P.b (Kuppers et al., 1976). This implies that the enzyme is present in P.b but relatively inactive compared to the enzyme in P.s. Therefore, cultures of P.s derived from morphine producing plants might be expected to accumulate thebaine, codeine and morphine and demonstrate considerably more activity for the biotransformation of codeinone to codeine than cultures of P.b and P.o.

Alkaloid content of suspension cultures

The biochemical profile of callus or suspension cultures is seldom the same as that of the parent plant and some cultures even accumulate novel compounds not common to the plant from which they were derived. All cultures initiated during this study were found to accumulate fewer alkaloids than the parent plant and these were also present at lower levels (3.3). P.s suspension cultures were found to contain thebaine, codeine and traces of morphine, plus two other alkaloids. One of these latter alkaloids corresponded to an alkaloid extracted from the plant whilst the other appeared to be unique to the culture. The quantity of alkaloids present, both individually and collectively, was significantly less than in the plant. By contrast, the only morphinan alkaloid accumulated by P.b and P.o suspension cultures was thebaine. One other alkaloid, detected in both P.b and P.o suspensions, cochromatographed with one of the unknown alkaloids isolated from the parent plants. These findings show that P.s (both types), P.b and P.o cultures retained the ability to synthesise the morphinan alkaloid(s) common to the respective parent plant. Therefore, P.s cultures would seem to fulfil the first of the four criteria listed earlier for a successful biotransformation. That is, the cultures have the enzyme necessary for the biotransformation of codeinone to codeine. This also implies that P.s cultures might show significantly higher activity for the conversion than P.b and P.o cultures.
Conversion activity of different culture types

The first set of biotransformation experiments (3.4) set out to establish the relationship between culture age and biotransformation capacity. The experiments were also designed to identify which cultures could reduce codeinone to codeine and establish the amount of substrate and product present in the cells and medium after incubation with codeinone for 72, 24 or 8 h. It is the findings of this aspect of the experiments that are discussed here. In 3.3 two different types of culture were derived from *P. s.* seedlings. These were cell suspension cultures (*P. s.*) and "meristemoid" (Nessler and Mahlberg, 1979) suspension cultures (*P. s. m.*). Both *P. s.* and *P. s. m.* cultures were able to reduce codeinone to codeine whilst *P. b.* and *P. o.* showed little activity for the conversion. This strongly suggests that not only do suspensions of *P. s.* and *P. s. m.* contain the necessary enzyme but that they are also able to act on exogenous substrate. It is interesting to note that intact plants of *P. b.* (Hodges *et al.*, 1977; Brochmann-Hanssen and Wunderly, 1978) and extracts of them (Hodges and Rapoport, 1980) can reduce added codeinone to codeine, although the activity is low compared to *P. s.* This is in agreement with the results presented in 3.4 and lends weight to the argument that *P. b.* plants do possess an enzyme capable of reducing codeinone but which is relatively inactive when compared with the corresponding enzyme in *P. s.*

Presence of product in the medium

It has been shown that in *P. s.* and *P. s. m.* cultures >90% of the product was present in the medium. Combined with the fact that the enzyme was able to act on exogenous substrate this suggests that the fourth requirement for a successful biotransformation has been satisfied, namely that the substrate must be able to enter the cell and the product preferably be released into the medium. To recover an extracellular product from culture medium costs significantly less than recovery of a product that is intracellular. Since the recovery costs of microbial products can vary from 20% to 60% of the total manufacturing cost (Aiba *et al.*, 1973; Swartz, 1979; Pace and Smith, 1981; Atkinson and Sainter, 1982), it is clear that the financial viability of a plant biotransformation system could depend on the cost of downstream processing. Although plant biotransformation activity can be located at the outer surface of cultured cells and even in the culture medium (Smith and Fry, 1991), the majority of biotransformations are intracellular (Suga and Hirata, 1990). This means that the added substrate must be able to enter the cell and compartment where the enzyme is located (Yeoman *et al.*, 1990). However, at no time was "free" codeinone detected in *P. s.* cells, so it is not possible to say for certain whether the substrate entered the cells.
The absence of codeinone in *P.s* cells could be due to the relatively rapid rate of the reaction or that the conversion occurred at or near the cell surface. Some of the codeinone present in the culture medium will be in an ionic form which could hinder uptake into the cells. This in turn might reduce the amount of codeinone available for conversion to codeine. Additionally, since the presence of codeinone and codeine in *P.s* cultures did not affect the growth cycle of these cultures it would appear that *P.s* cells are able to tolerate both precursor and product and therefore, meet the third of the four requirements listed earlier.

**Relationship between biotransformation capacity and culture origin reported by other authors**

The most important implication of these results is that a relationship exists between the biotransformation capacity of *Papaver* spp. cultures and the plant from which they were derived. Other authors have made similar observations, for example, Aviv and Galun (1981) reported a relationship between the major essential oil constituent of cultured *Mentha* cell lines and the biotransformation capacity of the cell. Drawert et al. (1984), showed that different *Citrus* spp. varied in their ability to convert valencene to nootkatone but did not relate this directly to the biosynthetic ability of the parent plant. As stated earlier, some cultures accumulate secondary metabolites not common to the plant from which they were derived. This might imply that some cultures would be capable of performing biotransformations that do not occur in the parent plant. Therefore, the relationship between biotransformation capacity and culture origin established for *P.s* cultures should not be taken as being universal for all culture systems but should properly be established for each conversion under investigation.

Having identified which cultures were able to reduce significant quantities of codeinone to codeine it was important to establish at what stage of development the conversion activity of a culture was greatest. This was done by determining the culture age that had the highest biotransformation capacity. Since the relationship between morphinan alkaloid accumulation and culture age was established in 3.3, it will also be possible to determine if cultures capable of accumulating morphinan alkaloids have enhanced biotransformation capacities. The results obtained are discussed in the next section.
4.2. Relationship between biotransformation capacity and stage of development

Biotransformation capacity and rate of growth

In the last section the relationship between culture origin and biotransformation capacity of the culture was established. However, it is also possible to determine from these biotransformation experiments the relationship between biotransformation capacity and culture age. The rate of culture growth during the growth cycle was estimated using the mean relative growth rate ($r$; for formula see 2.5.3), as defined by Hunt (1979). The value obtained is a measure of the amount of growth (dwt) between two timepoints, taking into account the quantity of biomass present at the first of these two timepoints and the time interval over which the increase has occurred. In all experiments, the biotransformation capacity was expressed as either $\mu g$/flask$^{-1}$ or $\mu g$/gdwt$^{-1}$. This was because for commercial processes it is important to maximise both the amount of product formed per volume (ie. $\mu g$/flask$^{-1}$) as well as the activity per unit biomass (ie. $\mu g$/gdwt$^{-1}$).

It has been shown that the biotransformation of codeinone to codeine is enhanced in slow growing cultures, however, this is not the the only factor affecting conversion activity. For instance, P.s suspension cultures from the period just after maximum growth (ie. downturn in culture growth) have the highest biotransformation capacity, whilst slow growing cultures from the lag phase of the culture cycle have a reduced biotransformation capacity. It is important to realise that cultures are a heterogeneous mixture of cells at different stages of their own "individual growth cycles" and it is the sum total of these "individual growth cycles" that constitutes the culture growth cycle. During the culture cycle, cells will range in age from those that are newly formed to those that have just lysed. This makes it difficult to ascertain exactly which cell type is responsible for the enhanced biotransformation capacity of the culture as a whole. The use of synchronous cultures could provide a solution but these are not readily available in the amounts necessary for a successful industrial process. So the important question is at what age is it best to add substrate to the cultures to achieve the most effective conversion?

Mean relative growth rate ($r$) vs "growth index"

Drawert et al. (1984), concluded that the biotransformation capacity of cultured Citrus spp. cells "ran parallel with growth up to the middle of the logarithmic phase and remained constant until the carbon source is completely exhausted". However, the growth indicator used by these authors was a "growth index" defined in the paper.
as the "dwt of cells (at given timepoint) divided by the dwt of the inoculum". Unlike \( T \), this "growth index" is not a measure of the rate of growth, because it does not take into account the amount of growth prior to a selected timepoint or the time interval over which any increase in dwt occurred. This means that growth in cultures, said by Drawert et al. (1984) to be in the "logarithmic phase", might in fact have begun to decelerate and casts some doubt on the conclusion stated above. Another problem presented by the data of Drawert et al. (1984), is that cultures were harvested when the biotransformation ratio (\% of substrate converted to product) was at a maximum. It has been shown in this thesis (3.4 and later in this section) that when product formation reaches a maximum it is not always possible to distinguish between cultures which convert substrate at a fast rate and cultures where the product is formed less rapidly. This is compounded by the fact that the biotransformation studied by Drawert et al., (1984) was not a one-step reaction and the product nootkatone was further metabolised. Therefore, the biotransformation capacity (\( \mu g.\text{flask}^{-1} \)) measured by Drawert et al. (1984), is not necessarily a correct reflection of the actual conversion activity present.

The data obtained from the biotransformation experiment described in 3.4.3 are presented in Table 4.2.1 to show the relationship between culture age, biotransformation ratio, "growth index", growth period and \( T \).

Table 4.2.1

<table>
<thead>
<tr>
<th>Culture Age (d)</th>
<th>&quot;Growth Index&quot;</th>
<th>Growth Period (days+hours)</th>
<th>( T ) (g.g(^{-1}).day(^{-1}))</th>
<th>Biotransformation Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>0 to 0+8h</td>
<td>0.019</td>
<td>11.0</td>
</tr>
<tr>
<td>4</td>
<td>1.325</td>
<td>0+8h to 4+8h</td>
<td>0.070</td>
<td>0.3</td>
</tr>
<tr>
<td>8</td>
<td>2.988</td>
<td>4+8h to 8+8h</td>
<td>0.203</td>
<td>11.8</td>
</tr>
<tr>
<td>10</td>
<td>5.588</td>
<td>8+8h to 10+8h</td>
<td>0.313</td>
<td>19.8</td>
</tr>
<tr>
<td>12</td>
<td>7.150</td>
<td>10+8h to 12+8h</td>
<td>0.247</td>
<td>20.6</td>
</tr>
<tr>
<td>16</td>
<td>6.863</td>
<td>12+8h to 16+8h</td>
<td>(-0.010)</td>
<td>7.2</td>
</tr>
<tr>
<td>20</td>
<td>6.325</td>
<td>16+8h to 20+8h</td>
<td>(-0.173)</td>
<td>2.2</td>
</tr>
</tbody>
</table>

In Table 4.2.1 the following definitions apply:

- "Growth Index" is defined as the dwt of cells at a given timepoint divided
by the dwt of the inoculum (after Drawert et al., 1984).

- Biotransformation ratio is defined as the percentage of codeinone added to cultures that is converted to codeine.

- The formula for $f$ is shown in 2.5.3.

- The growth period is the age (days + hours) of culture over which the value for $f$ corresponds.

It can be seen from Table 4.2.1 that as the "growth index" increases so does the biotransformation ratio, which implies that there is indeed a relationship between growth and biotransformation capacity. However, as previously stated, the "growth index", unlike $f$, is not a measure of the rate of growth. Therefore, although dwt reaches a maximum after 12d, the fastest rate of growth actually took place between 8d+8h and 10d+8h (Table 4.2.1). Indeed, towards the end of this period the growth rate is in fact falling. Consequently, it is cultures entering the stationary phase (ie. downturn in culture growth) that achieve the highest biotransformation ratio, 8h after the addition of codeinone. From these observations it would seem preferable to:

1. Use $f$ rather than "growth index" as a measure of culture growth.

2. Only incubate substrate with the cultures long enough for significant product formation to occur, but before the maximum biotransformation ratio is achieved.

**Importance of length of incubation period in determining biotransformation capacity**

$P_s$ suspension cultures of all ages, achieved similar biotransformation ratios if incubated with codeinone for 72h. However, in these experiments it was not possible to determine whether the cultures were performing this conversion at the same rate throughout the 72h period. It is possible that the codeine produced was further metabolised or that the capacity of the cells was reduced by the presence of the product, these points are discussed later in 4.4 and 4.5. Yet another possibility is that the substrate levels were "sub-optimal", in which case cultures with an enhanced biotransformation capacity might not be discernibly different from those of a lesser capacity, when viewed over a 72h period. Increasing the amount of codeinone added to $P_s$ cultures could have resolved this problem. However, some difficulty was
encountered in dissolving a sufficient quantity of codeinone in the small amount of methanol (100μl) added to the cultures. It was shown that the addition of 100μl of methanol had no effect upon culture growth but an increase in the volume of methanol, necessary to dissolve the increased amount of codeinone was likely to prove toxic to the cells. The problem was solved by reducing the incubation period from 72h to 24h and then subsequently to 8h. Biotransformation over an 8h period was short enough to allow the different rates of product formation in cultures of various ages to be determined. The rates of codeinone reduction in P.s.m cultures of different ages exposed to substrate for 8h also varied. Fifteen day old P.s.m cultures showed the highest activity and achieved significantly higher biotransformation ratios than the best P.s cultures. This was partly attributed to the slow growth of the former cultures but also to an increase in the state of differentiation. This latter point is discussed further in 4.3.

Conversion activity and synthesis of endogenous morphinan alkaloids

The majority of secondary metabolites are reported to accumulate in plant suspension cultures during the stationary phase of the culture cycle (Yeoman et al., 1980; Morris et al., 1986 and references therein). Consequently, to increase product yield it has been found necessary to manipulate culture conditions to prolong this phase of the growth cycle thereby ensuring that the appropriate biosynthetic pathway is expressed and operative for as long as possible. This requires some degree of biochemical differentiation between cells involving an increase in the proportion of cells capable of producing and accumulating the metabolite in question. Therefore, it is not surprising that the cultures identified in this thesis as having enhanced biotransformation capacities were those entering the stationary phase. Cell division in such cultures is in decline and accumulation of secondary metabolites has begun. Indeed, the endogenous levels of morphinan alkaloids in P.s suspensions were highest in cultures of 12 and 14d old. By contrast the highest level of alkaloid accumulation in P.s.m suspensions was at 15 and 18d. Presumably the biochemical pathway is most active just prior to these times, which corresponds to the culture ages that demonstrated the highest biotransformation capacities.

It has been shown that whilst slow growth is an important factor in achieving high biotransformation ratios, it does not in itself guarantee a high level of conversion activity. This can be seen by comparing the rate of reduction in cultures from the lag phase where growth is slow, with the amount of product formed in older, faster growing, cultures. Therefore, the enhanced biotransformation capacity of the older cultures must be due to some factor other than slow growth. It is possible that the
state of differentiation of the cells is important. In this discussion differentiation refers to changes in the shape, structure and function of individual cells (i.e. cytodifferentiation; Lindsey and Yeoman, 1986) and not necessarily differentiation where cells become organised into morphologically recognisable structures. Cytodifferentiation, results in changes to the rate and type of cell-division which subsequently alter both the sub-cellular organisation and metabolism of cells. The relationship between the state of differentiation of cells and biotransformation capacity is discussed in the next section.

4.3. Relationship between biotransformation capacity and state of differentiation

Variation in state of differentiation of culture types

The last section discussed the affect of culture age upon biotransformation capacity. Although slow growth appeared to be important, it was the differentiated state of some slow growing cultures that seemed to be the key factor. Suspension cultures of P.s.m and immobilised cultures of P.s.i and P.s.m.i are more highly differentiated than P.s suspension cultures. The three former cell types have been shown to grow more slowly than cell suspensions of P.s and have higher %dwt, which may give an indication of the degree of differentiation in each culture. This is supported by the results presented in the Appendix, which show light and electron micrographs of P.s, P.b and P.s.m suspension cultures. The age of the cells shown in these micrographs is that which gave the maximum biotransformation capacity for each culture type. The smaller size of P.s.m cells means that their surface area to volume ratio will be greater than that of P.s cells. This might mean that the substrate has easier access to the enzyme in "meristemoid" cultures than in P.s cultures. However, P.s.m cells did grow in sizeable aggregates, which might adversely affect the transport of substrate and product, but as the cells of P.s and P.b were very similar in size and cytoplasm content (see Appendix) yet differed greatly in biotransformation capacity, then substrate transport is probably a minor consideration (see Furusaki et al., 1985).

A positive correlation between the state of differentiation of cultures and secondary metabolite production has been reported by several authors (Yeoman et al., 1980; Hagiomori et al., 1982; Kamo et al., 1982; Lindsey and Yeoman, 1983; Kutchan et al., 1985). Kamo et al. (1982), showed that morphinan alkaloids were present in greater quantity in differentiated organs of P.s than in other less organised cultured tissues. Kutchan et al. (1985), reported that thebaine and sanguinarine were accumulated in different cellular compartments and different cell types of P.b
cultures. However, the major site of thebaine accumulation in these cultures was in relatively dense vesicles of laticifer cells whilst "sanguinarine was mostly present in other cellular and sub-cellular loci." This clearly demonstrates the importance of cytodifferentiation in poppy alkaloid synthesis and accumulation.

**Immobilisation and secondary metabolite production**

Immobilisation in foam blocks has previously been shown to increase the differentiated state and cell to cell contact of cultured plant cells, as well as promoting slow growth (Lindsey and Yeoman, 1983). The **P.s.i** and **P.s.m.i** cultures studied here both possessed a higher %dwt than **P.s** cultures implying that immobilised cells had smaller vacuoles, denser cytoplasm and were more highly differentiated than cells in **P.s** suspension cultures.

Although the study of endogenous morphinan alkaloids present in **P.s**, **P.s.m**, **P.s.i** and **P.s.m.i** cultures was semi-quantitative, the latter three cultures did appear to accumulate larger amounts of thebaine, codeine and morphine than the less differentiated **P.s** suspensions. It is possible that increasing the state of differentiation of *Papaver* spp. cultures leads to a greater proportion of cells involved in morphinan alkaloid production which in turn might result in an enhanced biotransformation capacity. In fact, immobilisation has previously been shown to increase the biotransformation capacity of cultured **P.s** cells (Furuya *et al.*, 1984; Corchete and Yeoman, 1990) which was also the case in this present study. Immobilisation did not however, increase the maximum biotransformation ratio of "meristemoid" cultures, possibly because the **P.s.m** suspension cultures were already aggregated or due to a limitation in substrate availability. There was also a considerable difference in the rate at which suspended and immobilised cultures reduced codeinone to codeine, the implications of which are discussed in 4.5.

Throughout this thesis, the biotransformation ratios obsevered in **P.s** and **P.s.i** cultures were less than previously reported by Furuya *et al.* (1984) and Corchete and Yeoman (1990). This could simply be due to differences between culture lines although other factors appear to be involved. For example, there was the possibility that the product was further metabolised and that the extraction efficiencies of codeinone and codeine were not 100%. These and other points are discussed in the next section which explores the fate of codeinone and codeine during the course of biotransformation experiments.
4.4. Fate of codeinone and codeine during biotransformation

Fate of added codeinone

At the start of a biotransformation the maximum amount of codeinone detected was only ca. 50% of that added. The time that elapsed between the addition of substrate and the point at which extraction commenced was ca. 15s., whilst complete extraction of each flask using a chloroform:iso-propanol mixture took ca. ten minutes. Therefore, it is unlikely that significant conversion of codeinone to codeine had occurred in this short period. As the extraction efficiency of codeinone from spent medium was 50.4%, whilst from fresh autoclaved medium it was 73.2% it would appear that some substance(s) associated with cultured Papaver spp. cells adversely affects the extraction of codeinone. In addition there would also seem to be some compound present in fresh medium which reduces the extraction efficiency. It is not known whether this compound continues to have an effect after cells have been subcultured into the medium. Both mild and vigorous hydrolysis of the spent medium released codeinone but vigorous hydrolysis was necessary to release codeinone from cells and then only in small amounts. Indeed, the amount of codeinone released by mild hydrolysis of both fresh and spent medium was approximately equal, which might imply that the compound reducing the extraction efficiency of codeinone from fresh medium might still be present after the introduction of cultured cells.

Codeinone was recovered from fresh medium with the same efficiency over a 48h period which implies that codeinone was not subject to chemical alteration during incubation. By contrast, the amount of codeinone recovered from spent medium decreased over the same 48h period. Because no codeine was detected during the experiment this shows that the biotransformations previously observed are dependant upon the presence of cultured P.s cells. Even at 0h, the efficiency with which codeinone was extracted from spent medium was lower than that from fresh medium. It is possible that the factor which caused this decrease in extraction efficiency at 0h, was also involved in the apparent loss of codeinone in spent medium during the course of the 48h incubation. Unfortunately, it is not known whether that codeinone which became converted during the incubation would have been released by a mild or vigorous hydrolysis of the spent medium.

Some of the compounds that might reduce the efficiency with which codeinone can be extracted from fresh or spent culture medium are discussed later in this section, after the fate of codeine has been examined.
Fate of codeine

The efficiency with which codeine can be recovered from spent medium (60.8%) was lower than the rate of recovery from fresh medium (77.3%). As codeinone and codeine are structurally very similar it is possible that whatever factor was affecting codeinone extraction might also be reducing the recovery of codeine. During the course of biotransformation, codeine is produced by the one-step reduction of codeinone and once formed, there was little evidence from the time course studies (3.5.1) to suggest that codeine was degraded to any great extent. In plants the degradative pathway for codeine is via morphine and although some morphine was detected in cultures (cells and medium) of P.s, P.s.m, P.s.i and P.s.m.i the amount present was not sufficient to account for the reduced levels of codeine or codeinone. However, codeine was released by mild and vigorous hydrolysis of both medium and cells which implies that some codeine is involved in other reactions.

So far, the fate of codeinone and codeine in cultures during biotransformation experiments has been discussed, now some possible explanations for the reduced extraction efficiencies of these two alkaloids will be explored.

Possible explanations for reduced extraction efficiencies of codeinone and codeine

Two questions have been raised and require to be answered to explain the fate of codeinone and codeine in cultures during biotransformation. Firstly, the cause of the different recovery rates of codeinone and codeine from fresh and spent medium? Secondly, are there "bound" forms of these two alkaloids in the cultures?

Problems associated with liquid/liquid extraction

(i) During the liquid/liquid extraction of products from the medium, sucrose, extracellular polysaccharides and cell debris are known to reduce interfacial tension. This "results in the formation of stable emulsions which make product recovery difficult and can lead to substantial loss of that product" (Weatherley, 1987). In the experiments reported in this thesis the "product" referred to by Weatherley could be either codeinone or codeine, as both are extracellular. The presence of such an emulsion was noted at the liquid/liquid interface during extraction of both cells and medium. This emulsion was almost completely dispersed after mild hydrolysis and was not detected after vigorous hydrolysis. This suggests that in part, the problem was due to the presence of polysaccharides, as these would be broken down by vigorous hydrolysis. Since codeinone and codeine are released from spent medium
after mild hydrolysis it might explain the reduction in the efficiencies with which both alkaloids can be extracted from fresh medium. The problem could also be compounded in spent medium due to the presence of cell debris and various extracellular polysaccharides not present in fresh medium.

Possible presence of "bound" forms of alkaloids during biotransformation

(ii) The second possibility is that polar conjugates of codeinone and codeine are formed with cell and medium constituents. The increased polarity of these compounds would reduce solubility in the chloroform:iso-propanol mixture used during the extraction procedure and hence lower the recovery rate. Compounds that are capable of forming conjugates with alkaloids include glucuronic acid, sulphate ions, amino acids and glutathione. Indeed, in human metabolism both morphine and codeine are excreted by means of glucuronide formation at either the 3-O- (morphine) or 6-O- (morphine and codeine) groups (Clarke, 1985). Obviously in plant cell cultures the acceptor molecule is unlikely to be glucuronic acid but it does demonstrate the fact that these two morphinan alkaloids can form ether linkages with organic acids. Such bonds, would be covalent and only broken by vigorous hydrolysis. Indeed, it is significant that both codeinone and codeine were released from spent medium after hydrolysis with 2M HCl at 100°C for 1h. Codeine was also released from cells after this treatment, together with a very small amount of codeinone. Other forms of bond could also occur between morphinan alkaloids and cell and medium constituents in these cultures. For example, Fairbairn and Steele (1980) reported "bound" forms of morphinan alkaloids in both P.s and P.b plants. They describe the presence of both "weakly" bound and "strongly" bound forms in the polysaccharide fraction of the pericarp and seed. Now, since codeinone, unlike codeine and morphine, does not have any hydroxyl groups it cannot form glycosidic bonds. It is also unlikely to form a -C-C bond but it is possible that an ionic association may form between the basic nitrogen and the acid groups of the polysaccharides present, as suggested by Wold (1978). However, what degree of hydrolysis would be required to break this bond is not known. "Bound" forms of morphinan alkaloids have been reported in cultures of P.s (Hutin et al., 1983; Morris and Gibbs, 1986) so it is not unreasonable to speculate about their presence during these biotransformation experiments. It is not clear whether the "bound" form(s) of codeinone were available to the enzyme for reduction to codeine. However, the maximum amount of codeinone converted to codeine was 60.0% (P.s.m cultures) which suggests that at least a small amount of "bound" substrate might be available to the enzyme, given that the maximum amount of "free" codeinone detected at any timepoint was 50.4%. Another explanation for this discrepancy is that the amount of
"free" codeinone present was higher than that measured but appeared to be lower due to the emulsion at the extraction interface.

In Fig.4.4.1 an overall summary of the possible fate(s) of codeinone, once added to P.s and P.s.m suspension cultures is attempted. It does not necessarily include every possibility but does indicate the proportion of codeinone present in each fraction 12h after the addition of codeinone. As P.s.m cultures converted more codeinone to codeine than cultures of P.s and no codeinone remained in the medium, some of the substrate must have been converted to other compounds in P.s cultures. It appears from the semi-quantitative studies, that the fate of the non-reduced codeinone in P.s cultures is to be conjugated to one of the "bound" forms of the alkaloid. During biotransformations by P.s and P.s.m cultures >90% of the "free" codeine produced was found in the medium. However, "bound" forms of codeine ("strong" and "weak") were present in medium and cells in approximately equal amounts. It is possible that "bound" codeine in the cell fraction was associated with the outer surfaces of the cell since very little "free" codeine was found in the cells. It also possible that any "free" codeine in cells was converted to "bound" forms within the plasmalemma, perhaps as a detoxification step. Given that codeine was possibly conjugated with sugars and/or polysaccharides the former of these two explanations might be the more likely. As indicated in Fig.4.4.1 there was some evidence to suggest that "free" and "bound" forms of morphine were also present in P.s and P.s.m cultures. However, the quantities were insufficient to account for the reduced recovery rates reported for both codeinone and codeine.

In the introduction to this discussion it was stated that the second requirement for a successful biotransformation is "the product must be formed faster than it is further metabolised." From the results discussed here it would seem that codeinone might be involved in reactions other than reduction to codeine. Therefore, perhaps the second requirement should read: "The product must be formed faster than it is further metabolised and ideally the substrate will not be converted into an alternative product(s)". Although the problem of alternative products being formed was not too great in this particular system, the presence of "bound" forms of substrate/product might increase downstream processing costs in an industrial process.

Throughout the work in this thesis no evidence was found to suggest that the conversion of codeinone to codeine was anything but a "single-step" reduction. However, it is clear from the results discussed above that both codeinone and codeine were involved in other reactions. This is not surprising given the relatively complex nature of a cell culture compared with an isolated enzyme system. The relative rates
of the two (or more) reactions competing for "free" codeinone (Fig.4.4.1) might determine how much codeine is produced. For example, if the rate of reduction to codeine is faster than the conjugation to a "bound" form then this could lead to an excess of codeine and vice versa. Therefore, although the enzyme(s) was not in an isolated form, some insight into the conversion in different culture types might be obtained by studying the biotransformation system using Michaelis-Menten kinetics. The next section discusses the results obtained when the rate of biotransformation was studied at different substrate concentrations.
Fig. 4.4.1 Possible fate of codeinone added to *Papaver somniferum* cell cultures and *P. somniferum* "meristemoid" cultures. Figures in brackets (P.s/P.s.m) are the approximate percentages of codeinone that was detected in each fraction 12h after the addition of the substrate. These values were estimated semi-quantitatively using TLC.
4.5. Rate of biotransformation by cultures at different substrate concentrations

Conversion rates in suspension and immobilised cultures

From the discussion so far it has become clear that events other than the "single-step" conversion of codeinone to codeine occur during the biotransformation. Significant variation in the conversion activity of different culture types has also been observed. By studying the rate of biotransformation in the various cultures at different substrate concentrations it was hoped to establish and explain the differences in conversion activity intrinsic to the particular culture system being used i.e. suspended vs immobilised.

The rate of conversion of codeinone to codeine in suspension cultures (P.s, Fig.4.5.1.a; P.s.m, Fig.4.5.2.a) is faster at all substrate levels than in immobilised cultures (P.s.i, Fig.4.5.3.a; P.s.m.i, Fig.4.5.4.a). Possible reasons for this include:

1. Easier access of substrate to enzyme in suspension cultures.
2. Greater product inhibition of enzyme in immobilised cultures.
3. Suspension cultures contain more enzymic sites than immobilised cultures.
4. Rate at which codeinone is conjugated to "bound" form(s) is greater in immobilised cultures.
5. Rate of regeneration of NADH is faster in suspension cultures (see Fig.4.5.5).

These five possible explanations will now be considered in the order shown here.

Easier access of substrate to enzyme in suspension cultures

Due to the free movement of cells growing in small aggregates in suspension cultures it is possible that access to substrate is easier than for immobilised cells growing in 1cm³ foam blocks. However, it should be remembered that foam blocks filled with cells do contain significant amounts of media and these cells must have reasonable access to substrate given the large quantities of codeinone converted to codeine in cultures of P.s.i and P.s.m.i. Another fact to be considered is that 10d after subculturing, relatively large amounts of biomass are present in P.s suspension
cultures. This could prevent a homogeneous mix of cells and medium (and substrate) forming in cultures of P.s but would be unlikely to cause a problem in immobilised cultures.

Greater product inhibition of enzyme in immobilised cultures

If the transport of substrate to cells is limited in immobilised cultures (as above) then it is also possible that the transport of product away from cells is also limited. This could result in higher levels of codeine accumulating around immobilised cells and in turn lead to product inhibition. As stated above, it is important to note that immobilised cultures actually produce more codeine in the long term than suspension cultures and that mixing of cells and media in suspension cultures is not always complete. Therefore, problems presented by substrate and product transport are not as clear cut as they might first appear.

Suspension cultures contain more enzymic sites than immobilised cultures

The faster rate of conversion of codeinone to codeine in suspension cultures could be explained by the presence of a greater number of enzymic sites than occur in immobilised cultures. This might be due to a higher proportion of cells actually reducing codeinone or a greater number of enzymic sites in these cells. If either of these were correct then suspension cultures might be expected to produce more codeine in total than immobilised cultures. However, immobilised cultures actually converted more codeinone to codeine than P.s cultures, even though the time taken to achieve the maximum level of product was greater in cultures of P.s.i and P.s.m.i.

Rate at which codeinone is conjugated to "bound" form(s) is greater in immobilised cultures

In Fig.4.4.1, codeinone can be seen to have two general fates. The first is to be reduced to codeine and the second is to be conjugated to one of the "bound" form(s). It is possible that the rate at which the second reaction(s) takes place differs in each culture type. If "bound" forms of codeinone were formed more rapidly in immobilised cultures then this might decrease the amount of codeinone available for biotransformation to codeine. If this is correct, and given that immobilised cultures produce large quantities of codeine, it is possible that some "bound" codeinone would have to disassociate, thereby releasing "free" codeinone for reduction to codeine.
Fig. 4.5.1 (a) Total amount of codeine detected in suspension cultures of *P. somniferum* 6h after the addition of different amounts of codeinone. (b) Lineweaver-Burk plot of data presented in (a) allowing values for "$K_m$" and "$V_{max}$" to be determined. Points are the means of two replicates ± S.E. (where shown).
Fig. 4.5.2 (a) Total amount of codeine detected in suspension cultures of *P. somniferum* "meristemoids" 6h after the addition of different amounts of codeinone. (b) Lineweaver-Burk plot of data presented in (a) allowing values for "K_m" and "V_max" to be determined. Points are the means of two replicates ± S.E. (where shown).
Fig.4.5.3 (a) Total amount of codeine detected in immobilised cultures of *P. somniferum* 6h after the addition of different amounts of codeinone. (b) Lineweaver-Burk plot of data presented in (a) allowing values for "K_m" and "V_max" to be determined. Points are the mean of two replicates ± S.E. (where shown).
Fig. 4.5.4 (a) Total amount of codeine detected in immobilised cultures of *P. somniferum* "meristemoids" 6h after the addition of different amounts of codeinone.

(b) Lineweaver-Burk plot of data presented in (a) allowing values for "$K_m$" and "$V_{max}$" to be determined. Points are the means of two replicates ± S.E. (where shown).
Rate of regeneration of NADH faster in suspension cultures

The reduction of codeinone to codeine (Fig.4.5.5) has been shown to require NADH as a co-factor (Furuya et al., 1978; Hodges and Rapoport, 1980).

Fig.4.5.5

Reduction of codeinone to codeine

It is important to note that the quantity of nucleotides in a cell is small compared with the amounts of different substrates that pass through NAD$^+$ or NADH mediated reactions (McGilvery, 1970; Brown and Thorpe, 1980). Consequently, reactions utilising particular forms of the nucleotides would quickly exhaust the supply if there were no reactions regenerating the required nucleotide. In this case, NAD$^+$ must itself be reduced back to NADH, before participating in further reduction of codeinone. NAD$^+$ is reduced to NADH as a result of several primary metabolic pathways, including glycolysis and the tricarboxylic acid cycle. As the rate of growth in 10d P.s and 15d P.s.m cultures (ages optimal for codeinone biotransformation) is faster than in immobilised cultures of the same age it follows that primary metabolic processes will also be faster in suspension cultures. This suggests that the rate at which NADH is regenerated in suspension cultures would be greater than in immobilised cultures. Therefore, the cells reducing codeinone in cultures of P.s and P.s.m would be less likely to experience low levels of NADH resulting in faster conversion of codeinone to codeine in these cultures.
Limitation of conversion activity in suspension cultures

Whilst Fig.4.5.1.a and Fig.4.5.2.a show that the rate of conversion of codeinone to codeine in suspension cultures is faster than that in immobilised cultures (Fig.4.5.3.a and Fig.4.5.4.a), there is also evidence to suggest that the rate of reduction in suspension cultures is limited by some factor(s) at the higher substrate concentrations studied. In order to try and clarify this situation "Lineweaver-Burk" plots have been made for each culture system (P.s, Fig.4.5.1.b; P.s.m, Fig.4.5.2.b; P.s.i, Fig.4.5.3.b; P.s.m.i, Fig.4.5.4.b) and from these, values for \( K_m \) and \( V_{max} \) calculated. It is realised that such an approach, normally applied to enzymes in isolation, is of limited importance but could provide some insight into the kinetics of the biotransformation in each culture system. For example, the \( K_m \) values determined for suspension cultures (Fig.4.5.1.b and Fig.4.5.2.b) are higher than the \( K_m \) values obtained for the immobilised cultures (Fig.4.5.3.b and Fig.4.5.4.b). This reflects the fact that the rate of codeine production in suspension cultures was faster than in immobilised cultures, the possible reasons for which have already been discussed. However, the rate of reduction of codeinone to codeine that could be expected in P.s and P.s.m cultures, at a substrate concentration equal to "\( K_m \)" is unlikely to be half of "\( V_{max} \)" (Fig.4.5.1.b and Fig.4.5.2.b). This suggests that the rate of codeine production in suspension cultures is indeed limited by some other factor(s). Possibilities include:

1. The enzyme in cells of suspension cultures has become saturated at 1200μg.flask⁻¹, therefore restricting the amount of codeine produced.

2. Product remaining in close contact with the enzyme inhibits conversion in suspension cultures, thus preventing further reduction of substrate.

The first of these explanations could be easily tested by measuring the rate of conversion at higher substrate concentrations. However, it is not as easy to establish whether product transport and inhibition presents more of a problem for suspension or immobilised cultures.

By contrast, the rate of reaction in cultures of P.s.i and P.s.m.i that might be expected at a substrate concentration equal to "\( K_m \)" is approximately half the value of "\( V_{max} \)". Therefore, the conversion in immobilised cultures would appear to follow a more "typical enzyme catalysed reaction", though as already stressed, these "enzyme" experiments were performed using biological systems far more complex than...
buffered proteins.

Another point, possibly clarified by these kinetic studies, is the fact that immobilised cells (P.s.i) eventually convert a higher percentage of codeinone to codeine than suspension cultures of P.s, despite P.s.i cultures proceeding at a slower rate. Two possible reasons for this are that firstly the increased state of differentiation of P.s.i cells leads to a higher number of enzymic sites per culture and secondly, slow growth induced by immobilisation leads to an extended period of viability for each cell. If the first of these two explanations is correct then the rate of reduction of codeinone in immobilised cultures might be expected to be faster than that in suspension cultures, however, this was not the case. Therefore, although the rate of codeine production in immobilised cultures was slower than in suspension cultures, the extended viability of these cultures might be the factor that enables higher levels of product to be accumulated by immobilised cultures.

The results and discussion presented in this thesis have shown that, what appeared initially to be a relatively straightforward biotransformation is in fact quite complex. Therefore, in the next section an assessment is made, based upon the findings established in the laboratory, of the commercial criteria that must be met if an economically viable biotransformation is to become a reality.

4.6. Commercial implications of the results

All four of the requirements for a successful biotransformation as stated in the introduction to this discussion have, in the main, been fulfilled. However, the major commercial requirement that the biotransformation must be economically viable has not been met. This does not matter in this case as the biotransformation is a "model system" set up to determine the relationship of biotransformation capacity to culture origin, culture age and state of differentiation.

From the preceding discussion it is evident that the relatively complex biotransformation system of cell cultures is not under strict control and this can allow commercially undesirable side products to form. This necessitates the rapid removal of product from the medium on formation, before any further metabolism or complexing can take place. In other systems it may prove necessary to inhibit the conversion of substrate into alternative products. This might be achieved by selection of suitable cell-lines or the use of inhibitors specific to enzyme(s) governing any undesirable side reactions. It might also prove necessary to combine the biotransformation activities of two different cultures to achieve a conversion that is
economically viable. This might be achieved by either fusing protoplasts of the two cultures or genetic manipulation. Other commercial considerations might involve the attainment of a compromise between the amount of product produced per volume (eg. \( \mu g.\text{flask}^{-1} \)) and the amount of product produced per unit of biomass (eg. \( \mu g.\text{gdwt}^{-1} \)). This is because the conversion activity per unit biomass of some culture ages can be extremely high whilst the amount of product formed is extremely low. Another factor is the length of time over which the enzyme involved in the biotransformation remains active. Whilst some cultures show enhanced biotransformation capacities over a relatively short period, in commercial terms it must be advantageous to keep a culture of lesser enzymic activity over an extended period in a semi-continuous process.

The techniques adopted for increasing the efficiency of a biotransformation system are very similar to the methods devised for enhancing secondary metabolite accumulation in cultured cells. These include the manipulation of culture conditions to increase the number and proportion of active cells as well as the use of growth regulators and nutrient deprivation to promote cytodifferentiation and tissue organisation. However, the largely empirical nature of this approach means that progress is often slow and the biochemical basis of the advantage gained little understood. That is why it is vital that the biotransformation kinetics of a particular culture are fully understood in terms of the culture origin, culture age and the state of differentiation. The results presented in this thesis have shown that such considerations are very important in indicating where future research should be focussed whilst also providing a biochemical understanding of biotransformation systems. Only then will the main aim of a commercially viable biotransformation governed by cultured plant cells become a reality.

The next chapter explores some possible avenues for future research based upon the findings made in the laboratory.
Chapter 5.
Future Work
The work presented in this thesis has pointed to four main avenues of possible future work. These are:

1. Enzyme purification
2. Biochemical studies
3. Cytological investigations.
4. Industrial strategies.

Enzyme purification

The biotransformation studies and the crude enzyme assay described in the results show that cell cultures of P._s could be a good source of codeinone reductase activity. One main objective for the future should be the purification of this enzyme. To do this a sensitive assay for the enzyme must first be developed. Possible ways of measuring the activity of the enzyme include:

1. Monitoring the disappearance of the substrate, codeinone.
2. Monitoring the appearance of the product, codeine.
3. Measuring the rate at which the co-factor NADH is utilised.

The first two of these options require (i) the efficient extraction of the alkaloids concerned and (ii) a quantitative analysis sensitive enough to detect low levels of alkaloid. The extraction methodology and HPLC system described in this thesis are unlikely to meet these criteria, given that the amount of substrate added to a test-tube for an enzyme assay is very much less than that added to a cell culture during a biotransformation experiment. It is possible that the extraction methodology and HPLC system might be improved, but this could be both time consuming and ineffective. It would also be unwise to monitor the disappearance of codeinone for this particular enzyme assay, due to problems of the substrate being metabolised or complexed to alternative products. Almost complete (98%) extraction of codeine from blood (Tebbett, 1987) is possible using solid phase extraction but the columns are expensive and would not however, solve the problem of low detection limits faced by HPLC analysis. Therefore, probably the best option for assaying the reduction of codeinone to codeine would be to measure the levels of NADH and NAD⁺ during the reaction. This could simply be achieved by monitoring the change
in absorbance at 340nm because NAD\textsuperscript{+} has a single U.V. peak with a maximum at 260nm whilst NADH gives two peaks, one at 260nm and a broader peak with a maximum at 340nm. Such an assay would also negate the necessity to develop a very sensitive HPLC methodology and the amount of codeinone and codeine in assay samples could be semi-quantitatively determined using TLC, which has much lower detection limits. However, because the initial experiments will be performed using a crude enzyme extract, it will first be necessary to monitor the fluctuations in the level of NADH, in the absence of codeinone. This will allow the amount of NADH being used in reactions other than the reduction of codeinone to codeine to be determined. As a consequence it should then be possible to accurately measure the activity of any codeinone reductase in the extract.

The procedures to follow for the successful purification of an enzyme are well documented and include ammonium sulphate precipitation, gel-permeation chromatography, ion-exchange chromatography and fast protein liquid chromatography. Once purified the enzyme should be characterised in terms of pH optimum, molecular weight, temperature optimum and isoelectric point. Having established all this information, it will then be possible to compare the enzymes recovered from different plants and cultures of \textit{P.s, P.b} and \textit{P.o}. This could help to establish the reason for the enhanced biotransformation capacity of immobilised and "meristemoid" cultures. In the longer term it might also prove possible to sequence the protein and use this information to make a detailed study of the control and expression of the gene(s) involved, in plants and cultures (see Nessler, 1988).

\textbf{Biochemical studies}

During biotransformation experiments the exact site of the conversion of codeinone to codeine was not clear. Therefore, using the enzyme assay established above, it would be useful to study the sub-cellular distribution of the enzyme. These fractions could be obtained by homogenising cell cultures of \textit{P.somniferum}, filtering and centrifuging the homogenate (eg. X1000g), removing the pellet and re-centrifuging the supernatant (eg. X13,000g). If the subsequent pellet was removed and the supernatant centrifuged again (eg.X100,000g), this would yield four sub-cellular fractions in which the level of codeinone reductase activity could be determined. By comparing the activity of the enzyme in fractions obtained from suspended and immobilised cultures, it may also prove possible to explain the differences in biotransformation capacity of these two culture systems.
Cytological investigation

Cytodifferentiation has been shown to be important in morphinan alkaloid accumulation. It is also believed to play a significant role in the biotransformation activity of cells. By making light and transmission electron microscopy studies of both plants and cultures, it should be possible to demonstrate the presence or absence of laticifer type cells in cultures. It might also be possible to locate codeinone and codeine within these cells by use of radio-labelled substrate or raising antibodies to both alkaloids and then detecting them by fluorescence. Studies such as these might also help determine whether the actual site of morphinan alkaloid synthesis in plants is laticiferous cells or the cells surrounding laticiferous vessels (see Introduction).

Industrial strategies

The results in this thesis have shown that many factors are involved in the regulation of the biotransformation of codeinone to codeine by cell cultures of P.s. However, if the biotransformation of morphinan alkaloids is to become commercially viable then several requirements still have to be met. For example, the biotransformation studied here is not economically viable in that codeinone is actually more expensive than codeine. Therefore, it will be necessary to produce cultures capable of multi-step biotransformations such as thebaine to codeine. This would enable plants of P.b to grown and harvested in place of P.s. Thebaine extracted from P.b plants could then be converted to codeine for use in pharmaceutical products, as well as possibly helping to reduce illegal traffic in opium and heroin. To obtain cultures capable of such multi-step biotransformations it might be necessary to combine the biosynthetic potential of two different cell types which in turn could be achieved by either genetic engineering or protoplast fusion. On a larger scale, different fermenter configurations, probably operating on a semi-continuous basis, would also need to be developed. Therefore, it is clear that much work is still to be done if a commercially viable biotransformation, mediated by Papaver spp. cell cultures, is to become a reality.
Chapter 6.
APPENDIX
6.1. Light micrographs of cell cultures of *Papaver* spp.

**Protocol for dehydration and fixation of material for light microscopy**

**Stock solutions:**
- **Solution A:** 0.1M KH$_2$PO$_4$ (keep refrigerated)
- **Solution B:** 0.1M K$_2$HPO$_4$
- **Solution PB:** Mix 51ml of solution A with 49ml of solution B to make 0.1M phosphate buffer with pH 6.8
- **Solution FS:** Add 5ml of 25% glutaraldehyde to 95ml of solution PB to make fixative solution.

**DAY1:** Incubate samples in solution FS overnight (ca. 16h)

**DAY2:**
- (i) Rinse samples three times in solution PB
- (ii) Incubate samples in 1% OsO$_4$, for 2h
- (iii) Step dehydration of sample into ethanol
  - 10% (v/v) for 10min, 20% (v/v) for 10min, 30% (v/v) for 10min etc. until absolute ethanol, four times (10min, 30min, 30min then overnight)

**DAY3:**
- (i) Transfer sample from ethanol into PPO
  - Ratio 1:3 (10min), 1:2 (10min), 1:1 (10min), 2:1 (10min), 3:1 (10min) and absolute PPO (10min).
- (ii) Transfer sample from PPO into Spurr's resin
  - Ratio 1:3 (15min), 1:2 (30min), 1:1 (60min), 2:1 (60min) and (overnight)

**DAY4:** Infiltration of sample by transferring into pure Spurr's resin for 3h, 5h, then overnight

**DAY5:** Continue to infiltrate sample by transferring into fresh Spurr's resin twice daily for minimum of one week

**DAY12:** After 7-10d of infiltration transfer sample into fresh Spurr's resin three more times (2h each), place in bean capsules and polymerise at 70°C overnight

**DAY13:** Section samples and stain with 1% toluidine blue in 1% borax solution
Fig.6.1.1 Light micrograph of 10d *Papaver somniferum* suspension culture. For details of embedding, sectioning and staining see p235. Bar = 110µm.
Fig. 6.1.2 Light micrograph of *Od Papaver bracteatum* suspension culture. For details of embedding, sectioning and staining see p235. Bar = 110 \( \mu \text{m} \).
Fig. 6.1.3 Light micrograph of 15d *Papaver somniferum* "meristemoid" suspension culture. For details of embedding, sectioning and staining see p235. Bar = 110μm.
6.2. Electron micrographs of cell cultures of *Papaver* spp.

Freshly filtered culture biomass (2.3.1) was placed onto an electron microscope stub, immediately submerged into liquid nitrogen then gold coated prior to viewing. The electron microscope used was a Cambridge Instruments S250 Mk. 2. The cryopreservation unit was an EMscope 2000.
Fig. 6.2.1 Electron micrographs of 10d *Papaver somniferum* suspension culture. For details of electron microscope see p239. Bar = as indicated.
Fig. 6.2.2 Electron micrographs of 0d *Papaver bracteatum* suspension culture. For details of electron microscope see p239. Bar = as indicated.
Fig. 6.2.3 Electron micrographs of \( ^{15} \text{A} \) \( \text{Papaver somniferum} \) "meristemoid" suspension culture. For details of electron microscope see p239. Bar = as indicated.
Chapter 7.
REFERENCES


BATTERSBY,A.R., J.STAUNTON, H.R.WILTSHIRE, R.J.FRANCIS and R.SOUTHGATE (1975.a). The origin of chelidonine and of other alkaloids derived


BORKOWSKI,P.R., J.S.HORN and H.RAPOPORT (1978). Role of
1,2-dehydroreticulinium ion in the biosynthetic conversion of reticuline to thebaine. J.Am.Chem.Soc. 100: pp276-281.


BROSSI,A. (1982). Mammalian TIQ's: products of condensation with aldehydes or pyruvic acids? In: Progress in clinical and biochemical research: Beta-carbolines and

246


KUTCHAN,T.M., S.AYABE and C.J.COSCIA (1985). Cytodifferentiation and


MORRIS, P. and S. GIBBS (1986). Uptake, binding and metabolism of morphinan


ROBINS, R.J., J. PAYNE and M. J. C. RHODES (1986). Cell suspension cultures of
Cinchona ledgeriana. I. Growth and quinoline alkaloid production. Planta Medica 3,
pp22-26.

(S)-Norlaudanosoline synthase: The first enzyme in the benzylisoquinolone

RUSH, M. D., T. M. KUTCHAN and C. J. COSCIA (1985). Correlation of
the appearance of morphinan alkaloids and laticifer cells in germinating Papaver


SÁRKÁNY, S. et al. (1964). Studien über die feinstruktur der jungen milchröhren,
pp161-162.

SCHIAPIARELLI, E. (1927). La tomba intatta dell’architetto Cha nella necropoli di
Tebe. In: Relazione sui lavori della missione archeologica italiana in Egitto (anni

cultures of Papaver somniferum and it’s relationship to alkaloid and lipid

Partial purification and properties of (S)-norlaudanosoline synthase from

SEAGERS, W. J., J. D. NEUSS and W. J. MADER (1952). The identification and
41: pp640-642.

SERTÜRNER, (F. W. A. F.) (1806). Darstellung der reinen Mohnsäure (Opiumsäure)
nebst einer chemischen Untersuchung des Opiums mit vorzüglicher Hinsicht auf
einen darin neu entdeckten Stoff und die dahin gehörigen Bemerkungen.


WICHERS,H.J., R.WIJNSMA, J.F.VISSIER, Th.M.MALINGRE and H.HUIZING
(1985). Production of L-DOPA by cell suspension cultures of Mucuna pruriens. II. Effect of environmental factors on the production of L-DOPA. Plant, Cell, Tissue and Organ Culture 4: pp75-82.


ZITO, S.W. and E.J. STABA (1982). Thebaine from root cultures of Papaver
Chapter 8.
PUBLICATIONS
Details of publications

1. Abstract from poster presented at VIIth International Congress of the International Association of Plant Tissue Culture (IAPTC), Amsterdam, Holland.

Abstracts VIIIth International Congress on Plant Tissue and Cell Culture
THE BIOTRANSFORMATION OF (-)-CODEINONE TO (-)-CODEINE IN CULTURES OF PAPAVER SOMNIFERUM

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The Opium poppy, Papaver somniferum, is commercially exploited as the source of the pharmacologically active morphinan alkaloids. The biotransformation of (-)-codeinone to (-)-codeine was achieved in cell suspension cultures of P. somniferum with over 90% of the product being in the culture medium. No (-)-codeinone was detectable in the medium after 12 hours. (-)-codeine levels peaked 12 hours after the start of the biotransformation and were maintained until 100 hours. Cells immobilised in reticulate-polyurethane foam converted (-)-codeinone to (-)-codeine more efficiently than suspended cells.

Meristemoids derived from callus of P. somniferum and inoculated into liquid medium were assessed for their biotransformation capacity. Suspension cultures of P. bracteatum and P. orientale are currently being investigated for their ability to biotransform (-)-codeinone.

Cell free extracts of P. somniferum reduced (-)-codeinone to (-)-codeine with other alkaloids also being formed. Extracts from the different species are presently being used to study the enzymatic basis of the reaction.
8 Exploitation of disorganized plant cultures for the production of secondary metabolites

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Introduction

Plants exhibit a wide biosynthetic repertoire, indeed many of the materials used in the food, flavouring, fragrance, and pharmaceutical industries are extracted from plants (Collin 1987, Phillipson, this volume). However, most plant cells in culture tend to accumulate only small amounts of the compounds typical of the species from which the cultures were isolated. Indeed the spectrum of compounds produced is often quite different from that in the plant. However, it is the low yield of product which is the major barrier to the commercial production of useful secondary metabolites by plant cells in vitro. There is, however, one commercial process in operation, the production of shikonin, a reddish-purple pigment with mildly antiseptic properties (Mitsui Petrochemical Company, Japan), with the prospect of another, berberine production, by the same company.

A distinction can be drawn between systems in which cultured cells synthesize and accumulate a particular product when grown on a basic medium containing a carbon and nitrogen source, with other essential mineral elements and growth regulators, and systems in which the cultures are grown on this basic medium together with an added precursor to the desired product. For the purposes of this review the enzymic conversions of exogenous precursors by cultured cells will be termed 'biotransformations'. These biotransformations can be either single-step (mediated by the action of one enzyme) or multi-step (mediated by two or more enzymes) and have been shown to occur in callus, suspension, immobilized, and protoplast cultures. The substrates which are added to cultures can be natural or synthetic and the products formed can be novel or compounds already known to occur in plants. Enzymic conversions which have been reported in plant tissue cultures include esterifications, epoxidations, hydroxylations, glycosylations, hydrolyses, methylations, demethylations, isomerizations, dehydrogenations, and oxidations (see Table 8.1).
### Table 8.1 Biotransformations which can be achieved by plant cell cultures

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Substrate</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction</td>
<td>C=O</td>
<td>CH$_2$—CH$_2$</td>
</tr>
<tr>
<td></td>
<td>C=O—CH</td>
<td>CH$_2$—CH$_2$—CO or</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH$_2$—CH$_2$—CHOH</td>
</tr>
<tr>
<td>Oxidation</td>
<td>CH$_3$</td>
<td>CH$_2$OH or COOH</td>
</tr>
<tr>
<td></td>
<td>CH$_2$OH</td>
<td>CHO</td>
</tr>
<tr>
<td></td>
<td>CHO</td>
<td>CO</td>
</tr>
<tr>
<td>Hydroxylation</td>
<td>CH</td>
<td>OH</td>
</tr>
<tr>
<td></td>
<td>CH$_2$</td>
<td>OH</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>CO$_2$—OH</td>
<td>H$_2$O$_2$</td>
</tr>
<tr>
<td>Epoxidation</td>
<td>CH=CH</td>
<td>OH—CHO—CH$_2$</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>OH—C</td>
<td>O—glucose</td>
</tr>
<tr>
<td>Esterification</td>
<td>OH—CH</td>
<td>O—palmitate</td>
</tr>
<tr>
<td></td>
<td>COOH</td>
<td>COO—malate</td>
</tr>
<tr>
<td></td>
<td>COOH</td>
<td>COO—glucose</td>
</tr>
<tr>
<td>Methyltion and</td>
<td>NH—CH$_3$</td>
<td>O—CH$_3$</td>
</tr>
<tr>
<td>demethylation</td>
<td></td>
<td>N—CH$_3$</td>
</tr>
<tr>
<td>Isomerization</td>
<td>N—CH$_3$</td>
<td>=NH</td>
</tr>
<tr>
<td></td>
<td>cis</td>
<td>laevo-rotation</td>
</tr>
</tbody>
</table>


The rationale for precursor feeding to boost product accumulation is that the intermediates to the product may be absent or present at very low concentrations in the cells or spatially separated from the enzyme. In this article attention will be focused on:

1. how disorganized cultures may be persuaded to produce significant amounts of secondary metabolites from cultures de novo, and
2. the biotransformation of added precursors in the production of commercially useful compounds.

### Environmental manipulation of plant cells to induce and enhance the accumulation of useful metabolites

A survey of the literature shows that most secondary metabolites are accumulated late in the culture cycle after the cessation of cell division and are associated with the process of cell differentiation (see Yeoman *et al.* 1980; Lindsey and Yeoman 1985). From this it follows that in order to increase product yield it is necessary to manipulate the cultures so that the biosynthetic pathways leading to the substances concerned are expressed and operative, i.e., the cells are biochemically differentiated. Limitations to product yield are complex but relate directly to the biosynthetic capability of the culture and involve not only the performance of individual cells but also the proportion of cells engaged in synthesis and accumulation. This aspect is perhaps seen best in cultures which accumulate coloured secondary products such as anthocyanin where the number of cells accumulating the pigment and the concentration of pigment in each cell can be measured directly (Hall and Yeoman 1987). It is clear from this research that both the concentration in the individual cells and the proportion of cells synthesizing the pigment are important. Therefore, manipulation of the culture environment must be effective in both increasing the amount of product accumulated in each cell. Central to the solution of this problem is a basic understanding of how metabolism is regulated and active steps are being taken in many laboratories to engage this problem. (Dixon, this volume). However, this is a major task and results are slow to emerge so that an additional approach is necessary in order to effect progress. Such an approach is basically empirical in nature but has proved useful and is twofold:

1. the production of high yielding cell lines (genetic selection and manipulation);
2. manipulation of the environment in which the plant cells are cultured.

It is the second of these approaches which will be addressed here.

### Development of systems to facilitate culture manipulation

A major problem in the use of conventional air-lift or stirred bioreactors is the time taken to produce sufficient living cells to synthesize the designated substance, and this is important for both multi-step and one-step processes. Inevitably, because of the intrinsic slow growth rates of plant cells, several weeks are necessary to generate the biomass. This growth period is then followed by a much shorter period during which the metabolite is accumulated. As the time spent in the bioreactor largely determines the
cost of the product the process will be uneconomic unless the substance produced is very valuable and the yield is high. There are broadly two solutions:

(1) increase product yield in batch or fed batch processes by employing selected cultures;
(2) adopt a continuous rather than a batch process in which the product is accumulated throughout the life of the culture.

Of course, whenever possible it is advantageous to employ high yielding cultures in a continuous process but the availability and instability of high yielding lines present real practical problems. The compromise then is to employ cultures which accumulate the product at a reasonable level and to work a relatively small (compared with the large amounts necessary for batch and fed-batch processes) amount of biomass over a prolonged period in a continuous mode. In this way it should be possible to produce a wide range of relatively inexpensive products economically (Lindsey and Yeoman 1985; Yeoman 1987; Holden and Yeoman 1988). The standard means of culturing micro-organisms continuously is by the use of chemostats and turbidostats. Indeed, plant cells have been grown in such systems (King and Street 1977). However, such an approach has not proved effective with plant cell cultures, which are generally not sufficiently dispersed for use in systems with complex plumbing. Also it is often the highly aggregated cultures which are the most productive. Therefore, it would seem that the only other approach to the development of a continuous or semi-continuous process is to exploit immobilized cell systems.

Although the concept of immobilizing plant cells is a relatively recent one (Brodelius and Mosbach 1982; Rhodes and Kir sop 1982; Lindsey and Yeoman 1986) the immobilization of microbial and animal cells is a technique which has been performed for a number of years (see Hall et al. 1988). Immobilization can be regarded as the most natural way to culture plant cells because as a result of immobilization the cells are encouraged to grow together in a multicellular, partially organized condition. In this state the cells can be maintained physically stationary and an environment provided in which physical and chemical gradients are established and the conditions most closely resemble the situation in vivo. Therefore it is a technique which allows the sequential manipulation of cells derived from a suspension culture and permits some structural and biochemical differentiation of those cells. It is these advantages which are the strength of immobilization techniques, and which can be exploited most readily and effectively in the field of secondary metabolite production. No attempt will be made here to describe and compare the various techniques that have been published for the immobilization of cultured plants. There are already several reviews on plant cell immobilization especially in relation to the production of secondary plant substances and these should be consulted for further details (see Lindsey and Yeoman 1986; Hall et al. 1988). A summary of the advantages of immobilized plant cell systems is set out in Table 8.2. The emphasis in this article will be on the general advantages of immobilization systems for the manipulation of plant cells particularly in biotransformations, both multi- and one-step.

### Table 8.2 Advantages of immobilized plant cell systems

<table>
<thead>
<tr>
<th>Advantage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Process can be run continuously as long as the metabolite is released from the cells and is removed from the medium</td>
<td></td>
</tr>
<tr>
<td>2. Rapid changes can be made to the medium circulating around the immobilized biomass, substances can be added or removed quickly and easily</td>
<td></td>
</tr>
<tr>
<td>3. The biomass can be rejuvenated (in some systems) in situ by perfusing the cells at intervals with growth medium</td>
<td></td>
</tr>
<tr>
<td>4. The proportion of time spent growing cells is low compared to the time spent in the production phase</td>
<td></td>
</tr>
<tr>
<td>5. Efficient use of relatively small amounts of biomass</td>
<td></td>
</tr>
<tr>
<td>6. Cells can be immobilized easily, either passively or actively</td>
<td></td>
</tr>
<tr>
<td>7. Structural and biochemical differentiation can be achieved within the inert matrix</td>
<td></td>
</tr>
</tbody>
</table>

### Use of nutrient limitation

There have been many studies on the effects of supplied nutrients on the synthesis and accumulation of secondary metabolites (Dougal 1980, for reviews see Staba 1980 and Collin 1987). Because there is often an observed inverse relationship between growth and product yield and products tend to be accumulated towards the end of the growth phase, the general approach has been to slow down or restrict growth by limiting the supply of sugar, nitrogen, or phosphorus. It is found that cultural conditions which promote a high rate of cell division are commonly not conducive to a maximum rate of secondary product formation (e.g. Bhandary et al. 1969; Konishima et al. 1976; Lindsey and Yeoman 1983). Nettleship and Slaytor (1974) have shown that a reduced level of phosphate supplied to callus cultures of *Peganum harmala* induced an increase in the formation of a variety of secondary products. Knobloch et al. (1981) have studied the effect of phosphate on the accumulation of cinnamoyl putrescines by tobacco cultures and have shown an inverse relationship between the concentration of intracellular phosphate and culture growth rate on the one hand, and the production of the secondary
metabolites on the other. Phillips and Henshaw (1977) working with cultures of sycamore have demonstrated that, when protein synthesis was experimentally enhanced, phenolic synthesis was reduced. Lindsey (1985), in a study on the manipulation by nutrient limitation of capsaicin synthesis by immobilized cells of the chilli pepper, has observed a relationship between the intracellular nitrate concentration, the culture growth index, and the incorporation of [14C]phenylalanine into soluble protein; each of these factors was inversely related to the incorporation of label into capsaicin and the total capsaicin content of the cultures. These observations from a variety of laboratories working with several different species further support the concept of an inverse relationship between protein synthesis and the synthesis of secondary metabolites, based on the differential and antagonistic utilization of common precursors. From this it seems clear that the manipulation of the rate of cell division and associated metabolic activity in cell cultures is a useful approach to the production of valuable secondary metabolites and that cell immobilization facilitates this end.

Use of growth regulators

Anyone who has ever put a plant into culture knows the importance of using the appropriate level of an auxin and usually a cytokinin to induce proliferative growth and callus formation (Reinert and Yeoman 1982). The effects of auxin and cytokinin and the balance between them on inducing regeneration from callus is well documented. Plant growth regulators have been used effectively in the development of production media for alkaloid accumulation from Catharanthus roseus (Deus-Neumann and Zenk 1984), Thalictrum minus (Nakagawa et al. 1986), and Rauwolfia serpentina (Yamamoto and Yamada 1987). Generally, treatments which encourage structural differentiation, e.g. shoots or roots from callus, also change the biochemical profile. For example, the regeneration of roots from callus of several species tends to be accompanied by a sharp rise in alkaloid content (Tabata et al. 1972; Hashimoto and Yamada 1983; Endo and Yamada 1985). It has also been shown that levels of growth regulators which support high rates of growth do not induce the accumulation of significant amounts of secondary metabolites.

Removal of product

It may be advantageous to remove the product from the medium around the cells to prevent any possibility of inhibition of synthesis. The simple fact that cultured cells of Erythrorhizon lithospermum can tolerate substantial concentrations of shikonin in the cells and in the medium suggests that end-product inhibition is not significant in this case. However, separation of secondary metabolite from the site of synthesis usually by storage in the vacuole, sometimes of specialized cells, is a commonly observed phenomenon which suggests that end-product inhibition is the norm. Lindsey (1986) has demonstrated that the incorporation of [14C]phenylalanine into capsaicin in immobilized cells of the chilli pepper is inhibited by the presence of capsaicin in the medium around the cells and that as the concentration of capsaicin is increased so does the extent of inhibition. It would therefore appear that where the product is released it would be advantageous to continuously remove it from the medium.

Use of elicitors

Elicitors derived from micro-organisms have been shown to cause increased synthesis and accumulation of secondary metabolites in many plant cell cultures (for review see Eilert 1987). Funk et al. (1987) have demonstrated that a carbohydrate fraction derived from yeast induces glyceollin isomer synthesis in cells of Glycine max and increases berberine biosynthesis in cells of Thalictrum rugosum. This elicitor as well as an elicitor derived from Gliocladium deliquesens is effective in stimulating the production of the phytoalexin capsidiol (Brooks et al. 1986) and the non-phytoalexin capsaicin (Holden et al. 1986). Where the action of elicitors on cultures has been investigated the induction of specific enzymes has been observed (Hahlbrock and Grisebach 1979; Dixon et al. 1986). In the case of elicited cultures of Capsicum an increase in the activity of phenylalanine ammonia lyase, the first enzyme in the phenylpropanoid pathway, is observed. Where increased activity of specific enzymes results in increased product formation it can be assumed that enzyme activity and not substrate availability is limiting to synthesis.

Clearly culture manipulation can increase product yield and taken together with the use of cell lines in which the balance between primary and secondary metabolism has been altered either genetically or epigenetically can lead to enhanced yields of a commercially useful product. Here a better understanding of the complexities of metabolic regulation will help to prescribe more effective means of manipulating the biosynthesis of the chosen metabolite(s). It is here that the use of immobilized cell systems may prove valuable.

Precursor feeding

The feeding to plant cell cultures of precursors distant from the product has generally had relatively little effect on the final product yield. Generally,
the greater the number of steps between precursor and product the lower the yield. However, as will become evident, when single step biotransformations are considered conversions can be very efficient often approaching 100 per cent. Lindsey and Yeoman (1984a,b) have shown that a significant yield of capsaicin can be achieved by feeding iso-capric acid which is only a few steps away from the product; on the other hand the addition of phenylalanine many steps distant from capsaicin also increases the product yield but to a smaller extent (Lindsey and Yeoman 1984b). However, with precursors such as phenylalanine which are distant from the product the general strategy must be to divert the precursor away from the major pathways of consumption (protein and lignin synthesis) to the appropriate sequence of reactions leading to the designated product.

There are, however, other difficulties which impair the effectiveness of precursor feeding apart from competition between metabolic pathways. In many instances, the precursor is not taken up or does not arrive at the appropriate location within the cells. For example, highly ionized molecules are not taken up at the near neutral pHs used in the culture medium; ionization can be reduced, however, by altering the pH, but that is of limited value because of the narrow pH sensitivity of the cells. Precursor molecules can be chemically modified, i.e. acids esterified to enable entry, but this is expensive and usually not very effective. Also the precursors added may be toxic to the cells even at low concentrations and this can reduce their usefulness. However, by the study of biosynthetic pathways more directed approaches can be adopted.

Biotransformations

Biotransformations are an attractive proposition to the Plant Biotechnologist. Generally conversion rates are rapid and efficient so that if the precursor is relatively cheap compared to the product and if the process can be run continuously, commercial exploitation is an attainable goal. There are, however, two problems, firstly the value added factor must be very high, i.e. the product must be substantially more valuable than the precursor, and secondly the product may be further metabolized to less valuable substances. So far most of the research on single-step biotransformations in plant cell cultures has been restricted to products of pharmaceutical interest where the focus has been on single specified compounds (e.g. Reinhard and Allermann 1980; Huizing et al. 1985; Wichers et al. 1985a). However, some attention has been afforded to compounds of interest to the food, flavouring, and fragrance industries including biotransformations of terpenoids (Butcher 1977) and diterpenoids, e.g. stevioside to steviol which is used as a sweetening agent.

A detailed consideration will now be made of the research already performed and the prospects for single step biotransformations.

A definition of biotransformation

The conversion of a substance by living cultures, permeabilized cells, or entrapped enzymes into a chemically different product. This may involve a single step or many steps. So far the biotransformations reported for plants have been achieved with living cells either in the whole organism or as organ cultures, callus, suspension cultures, protoplasts, or with immobilized cells. The substances fed to the cells for conversion can be natural products or unnatural synthetic compounds.

A wide range of biotransformations have already been reported and many recently reported examples of these are presented in Table 8.3 and examples of several of these will be described in greater detail below.

Requirements for successful biotransformation

In 1974 Steck and Constabel in a review paper on 'Biotransformations in plant cell cultures' listed three requirements for the successful biotransformation of a precursor into a designated compound. These were:

1. The culture must have the enzymes necessary for the transformation of precursor to product.
2. The product must be formed faster than it is further metabolized.
3. The culture must tolerate the added precursor and the product.

These requirements are as valid today as they were fifteen years ago but now with the improvements and innovations in culture techniques, mainly through the development of immobilized systems, many of them are easier to achieve.

Let us now consider these requirements in turn, and include one other.

The enzymes must be present in the culture

If the addition of precursors to cell cultures is to be employed the assumption is made that the enzyme must be present, unsaturated and in an intracellular location where supplied substrate can be transformed successfully.

It is a frequent observation that cultures produce much lower amounts of product than the intact plant (Curtin 1983). The absence or lack of activity of the key enzyme(s) to perform specific reactions has been suggested as a possible cause of this failure to synthesize the desired products.
Table 8.3 Some examples of biotransformations in plant cell cultures

<table>
<thead>
<tr>
<th>Species</th>
<th>Source of enzyme*</th>
<th>Method of analysis†</th>
<th>Biotransformation substrate to product</th>
<th>Type of reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisodus tangaricus</td>
<td>S</td>
<td>MS</td>
<td>hyoscyamine to hydroxyhyoscyamine</td>
<td>hydroxylation</td>
<td>Cheng et al. 1987</td>
</tr>
<tr>
<td>Cannabis saliva</td>
<td>S</td>
<td>MS</td>
<td>cannabidiol to cannabicyclol to cannabicyclol to cannabicyclol</td>
<td>multi-step</td>
<td>Braemer and Paris 1987</td>
</tr>
<tr>
<td>Cannabis sativa</td>
<td>S</td>
<td>MS</td>
<td>cannabidiol to cannabicyclol to cannabicyclol to cannabicyclol</td>
<td>multi-step</td>
<td>Hartsel et al. 1983</td>
</tr>
<tr>
<td>Catharanthus roseus</td>
<td>CFE(S)</td>
<td>TLC</td>
<td>anhydrovinblastine to vinblastine</td>
<td>multi-step</td>
<td>Endo et al. 1987</td>
</tr>
<tr>
<td>Catharanthus roseus</td>
<td>S</td>
<td>PMR</td>
<td>hydroxygeraniol to various</td>
<td>multi-step</td>
<td>Baisevich 1987</td>
</tr>
<tr>
<td>Choisya ternata</td>
<td>C</td>
<td>NMR</td>
<td>ellipticine to formyklottipticine</td>
<td>multi-step</td>
<td>Kousidlo et al. 1984</td>
</tr>
<tr>
<td>Citrus spp.</td>
<td>S</td>
<td>NMR</td>
<td>genipin to nootkatone</td>
<td>multi-step</td>
<td>Drawert et al. 1984</td>
</tr>
<tr>
<td>Coffea arabica</td>
<td>S</td>
<td>NMR</td>
<td>diosgenin to diosgenin</td>
<td>multi-step</td>
<td>Furuya et al. 1988b</td>
</tr>
<tr>
<td>Cymbidium spp.</td>
<td>S</td>
<td>—</td>
<td>methyl acetate to menthol</td>
<td>hydrolysis</td>
<td>Mironowicz et al. 1987</td>
</tr>
<tr>
<td>Datura innoxia</td>
<td>S</td>
<td>NMR</td>
<td>umbelliferone to glucoside</td>
<td>glucosylation</td>
<td>Tabata et al. 1988</td>
</tr>
<tr>
<td>Datura innoxia</td>
<td>S</td>
<td>—</td>
<td>menthol to menthol</td>
<td>hydroxylation</td>
<td>Suzuki et al. 1987</td>
</tr>
<tr>
<td>Digitalis lanata</td>
<td>S and F</td>
<td>HPLC</td>
<td>methylidigoxin to methylidigoxin</td>
<td>multi-step</td>
<td>Kreis and Reinhard 1988</td>
</tr>
<tr>
<td>Digitalis lanata</td>
<td>S and I</td>
<td>—</td>
<td>digoxigenin to digoxin</td>
<td>multi-step</td>
<td>Seitz et al. 1983</td>
</tr>
<tr>
<td>Glycyrrhiza glabra</td>
<td>S</td>
<td>NMR</td>
<td>papaverine to various</td>
<td>oxidation and demethylation</td>
<td>Alfermann et al. 1980</td>
</tr>
<tr>
<td>Lactuca sativa</td>
<td>S</td>
<td>TLC</td>
<td>chlortoluron to various</td>
<td>multi-step</td>
<td>Dorisse et al. 1988</td>
</tr>
<tr>
<td>Lavandula angustifolia</td>
<td>S</td>
<td>GC-MS</td>
<td>monoterpenoid aldehydes to alcohols</td>
<td>reduction and hydroxylation</td>
<td>Cole and Owen 1988</td>
</tr>
<tr>
<td>Medicago saliva</td>
<td>S</td>
<td>NMR</td>
<td>cyclohex-en-one to cyclohexanone</td>
<td>reduction</td>
<td>Kergomard et al. 1988</td>
</tr>
<tr>
<td>Mentha spp.</td>
<td>S, D and I</td>
<td>GLC</td>
<td>menthone to neomenthon</td>
<td>reduction</td>
<td>Galun et al. 1985</td>
</tr>
<tr>
<td>Mentha spp.</td>
<td>S</td>
<td>NMR</td>
<td>isopropylidene-cyclohexanone to isopropylidene-cyclohexanone</td>
<td>reduction</td>
<td>Aviv et al. 1983</td>
</tr>
<tr>
<td>Mentha spp.</td>
<td>S</td>
<td>NMR</td>
<td>menthone to neomenthone</td>
<td>reduction</td>
<td>Aviv et al. 1981</td>
</tr>
<tr>
<td>Mentha spp.</td>
<td>S</td>
<td>MS</td>
<td>pulegone to isomenthone</td>
<td>hydroxylation</td>
<td>Aviv and Galun 1978</td>
</tr>
<tr>
<td>Mucuna pruriens</td>
<td>I</td>
<td>MS</td>
<td>monophenolics to catechols</td>
<td>hydroxylation</td>
<td>Pras et al. 1988</td>
</tr>
<tr>
<td>Mucuna pruriens</td>
<td>I and S</td>
<td>—</td>
<td>tyroxine to L-dopa</td>
<td>hydroxylation</td>
<td>Wichers and Pras 1984</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>S</td>
<td>NMR</td>
<td>phenylpropanic acid to various</td>
<td>glycosylation</td>
<td>Furuya et al. 1987</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>S</td>
<td>NMR</td>
<td>oxomethane derivatives to various</td>
<td>hydroxylation and reduction</td>
<td>Suga and Hirata 1988</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>S</td>
<td>GLC</td>
<td>carboxime to ketones</td>
<td>hydrolysis</td>
<td>Suga et al. 1984</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>S</td>
<td>GC-MS</td>
<td>nicotine to normoncine</td>
<td>demethylation</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Papaver somniferum</td>
<td>I</td>
<td>MS</td>
<td>codeinone to codeine</td>
<td>hydroxylation</td>
<td>Furuya et al. 1984</td>
</tr>
<tr>
<td>Papaver somniferum</td>
<td>S</td>
<td>GC-MS</td>
<td>codeinone to codeine</td>
<td>hydroxylation and demethylation</td>
<td>Tam et al. 1982</td>
</tr>
<tr>
<td>Papaver somniferum</td>
<td>S</td>
<td>MS</td>
<td>thebaine to neopine</td>
<td>demethylation</td>
<td>Furuya et al. 1978</td>
</tr>
<tr>
<td>Peganum harmala</td>
<td>S</td>
<td>HPLC</td>
<td>reticuline to scoulerine</td>
<td>berberine bridge</td>
<td>Courtois et al. 1988</td>
</tr>
<tr>
<td>Silene alba</td>
<td>S</td>
<td>GC-MS</td>
<td>trypamine to serotonine</td>
<td>hydroxylation</td>
<td>Christiak et al. 1987</td>
</tr>
<tr>
<td>Strophanthus amboinensis</td>
<td>S</td>
<td>NMR</td>
<td>digitoxigenin to various</td>
<td>multi-step</td>
<td>Kawaguchi et al. 1988</td>
</tr>
<tr>
<td>Strophanthus amboinensis</td>
<td>C and S</td>
<td>MS</td>
<td>digitoxigenin to purpureaglycoside</td>
<td>glycosylation</td>
<td>Dohnal et al. 1986</td>
</tr>
<tr>
<td>Strophanthus gratus</td>
<td>S</td>
<td>HPLC</td>
<td>digitoxigenin to various</td>
<td>hydroxylation</td>
<td>Furuya et al. 1988a</td>
</tr>
</tbody>
</table>

* C, callus culture; CFE, cell-free extract; D, division arrested cells; F, fermenter; I, immobilized culture; S, suspension culture.
† GC, gas chromatography; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance spectrometry; PMR, proton magnetic resonance spectrometry; TLC, thin layer chromatography.
In all of the biotransformations listed in Table 8.3 the enzyme responsible for each of the reactions must be expressed in the particular cell line used. Cultures of different *Papaver* species demonstrate the presence and absence (or separation from substrate) of specific enzymes of secondary pathways. Cultures of *P. somniferum*, *P. setigerum*, *P. bracteatum*, and *P. orientale* are all capable of the biosynthesis of thebaine from tyrosine and therefore all of the enzymic steps shown in Fig. 8.1 are present. However, only cultures of *P. somniferum* and *P. setigerum* are capable of subsequent biotransformations (Furuya et al. 1984) which yield codeinone, codeine, and morphine (Fig. 8.2). Whether the cultures of the other species lack the necessary enzymes or whether enzymes and substrate are separated is not known. This highlights the importance of the choice of species for particular biotransformations.

Different cell lines derived from a single plant and cultured under the same conditions can also show differences in biotransformation activity. The results presented in Table 8.4 shows the effects of the addition of 50 μmoles of ferulic acid to immobilized cells of *Capsicum frutescens* cultured on a production medium. It is apparent that the products of the biotransformation vary. Table 8.4 shows that cell line 122 preferentially accumulates vanillin (the product of the forward reaction) while another cell line (121) accumulates more p-coumarate than vanillin which is the direct precursor to ferulic acid. Therefore it may be concluded that these two cell lines differ in their pattern of enzyme activity. Furthermore, other cell lines will produce high levels of capsaicin when fed with ferulic acid and in others this multi-step biotransformation does not occur (Holden et al. 1988a).

This variability in the metabolic performance of cell lines can be a problem for the maintenance of cultures with high biotransformation activity, but the presence of cultures with different enzymic activities can be exploited. Thus cell lines which preferentially biotransform ferulic acid into vanillin and do not produce capsaicin can be selected. Vanillin, which is conventionally extracted from the pod of *Vanilla planifolia*, the vanilla orchid, is an important flavouring in the food industry for which there is high demand. Thus by cell line selection and manipulation of cultural conditions, including precursor feeding, a product which is not normally accumulated to any great extent by a plant species can be synthesized and accumulated by cultures. Similarly, cell lines which possess the enzyme activities for steps later in the pathway can be utilized for capsaicin biosynthesis (Lindsey and Yeoman 1984a,b).

The study of the enzymology of biotransformation has been undertaken in only a limited number of systems (Wichers et al. 1984; Wichers et al. 1985b). One-step biotransformations are amenable to manipulation as they are controlled by one enzyme and regulating factors can be easily

Fig. 8.1. Biosynthetic pathway leading to the formation of the first morphinan alkaloid, thebaine.
Table 8.4 Conversion of 50 μmoles of ferulic acid into vanillin, coumaric acid, cinnamic acid, and capsaicin in immobilized cell cultures of five cell lines of *Capsicum frutescens*.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ferulic acid μmoles remaining (% used up)</th>
<th>Vanillin μmoles formed (% conversion)</th>
<th>Coumaric acid μmoles formed (% conversion)</th>
<th>Cinnamic acid μmoles formed (% conversion)</th>
<th>Capsaicin μmoles formed (% conversion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td>19.5 (61)</td>
<td>5.0 (10)</td>
<td>10.8 (22)</td>
<td>1.25 (3)</td>
<td>5.9 (12)</td>
</tr>
<tr>
<td>213</td>
<td>19.2 (62)</td>
<td>7.5 (15)</td>
<td>5.6 (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>5.0 (90)</td>
<td>27.0 (54)</td>
<td>14.5 (29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>233</td>
<td>1.25 (99)</td>
<td>1.8 (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>331</td>
<td>22.9 (54)</td>
<td>2.3 (5)</td>
<td>3.0 (6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
determined. For example the formation of cinnamic acid from phenylalanine, by the action of phenylalanine ammonia-lyase (PAL), has been extensively investigated. It has been clearly demonstrated that the activity of this enzyme can be regulated by a number of factors including dilution of culture, light regime, temperature, and the addition of fungal elicitors (for a review see Jones 1984). Elicitation has been shown to increase PAL activity in *Capsicum* cultures (Holden et al. 1988a). Figure 8.3 shows the effect of the addition of sterile spores of *Gliocladium deliquescens* at different stages of the culture cycle on the activity of this enzyme. It is apparent that elicitation at days 10 and 15 leads to a maximal stimulation of enzyme activity over the control which in turn results in a different pattern of incorporation of phenylalanine into enzyme product. The use of fungal elicitors in the induction of enzyme activity has been shown to increase product yield in many systems. In studies on PAL, however, direct relationships between enzyme activity and product formation are not universal (see Jones 1984) and it seems probable that the level of substrate may also be critical in determining product yield. It has been demonstrated that the supply of phenylalanine is limiting to capsaicin production under certain cultural conditions (Lindsey and Yeoman 1984b). This finding has been reported in other systems and makes the utilization of one-step biotransformations an attractive proposition. If feedback inhibition of enzyme activity by the product is to be avoided, continuous removal may be necessary and this is possible in the medium-scale bioreactors designed for use with foam immobilized cells (Mavituna et al. 1988). However, this process may not be necessary as the end products of biosynthesis may be spatially separated from the enzymes.

Another single-step biotransformation where the enzymology of the conversion has been investigated is the B-hydroxylation of B-methylidigitoxin to B-methylidigoxin, a valuable cardiac tonic in cultures of *Digitalis lanata*. Other unrelated species, e.g. *Strophanthus* spp. (Dohnal et al. 1986) and *Daucus carota* (Jones and Veliky 1981) can also perform similar cardenolide biotransformations. Therefore the enzyme necessary for a particular reaction may be present in other species. Aftermann et al. (1980) have demonstrated how the 12B-hydroxylation takes place in cultures of both freely-suspended cells and cells immobilized in beads of calcium alginate gel. In the immobilized cell cultures, the biotransformation activity was reduced compared with the suspension cultures although in both systems 90 per cent of the product was found in the medium rather than in the cells. Similar results have been obtained by Jones and Veliky (1981) for the C-5 biotransformation but they observed that by permeabilizing alginate-entrapped carrot cells with methanol or chloroform, biotransformation activity could be enhanced significantly.

Thus in many systems endogenous enzyme activity is able to transform added substrate into the product. In circumstances where activity is limiting to production, fungal elicitors may be employed to induce increased activity in a limited number of situations.

The product must be formed faster than it is further metabolized

Where the desired product of a metabolic reaction is not the end of a biosynthetic chain but is further metabolized, the problem of degradation is important. This can be solved by continuous product removal or by selection of cell lines which do not degrade the product formed. Tyrosine is converted to the drug L-dopa in cultures of *Mucuna pruriens* but further conversion results in the formation of dopaquinone and then dopachrome which is not a valuable product (Wichers et al. 1984). Therefore, conditions have to be designed to reduce the further metabolism of the desired product L-dopa. For example ascorbate can be added to the medium to maintain reducing conditions and prevent oxidation of the product.
In some cases, for example capsaicin, the desired product is an end product of a biochemical pathway and further metabolism is not a problem. Where the secondary product is released into the medium a beneficial separation of the product and degradative enzymes may occur. Treatments which encourage release (i.e. immobilization and permeabilization) may prove powerful techniques in the exploitation of biotransformations. Much more work involving detailed studies on the regulation of secondary product enzymes is necessary if we are to determine the extent to which the low yields observed in some cultures are due to degradative reactions.

**The culture must tolerate the added precursor and the product formed**

When the added precursor is toxic to the cells or inhibitory to enzymes involved in secondary pathways biotransformation efficiency is reduced. The addition of high concentrations of cinnamic acid to Capsicum cells results in a rapid loss in cell viability. Low levels of this compound have an inhibitory effect on PAL, the enzyme that leads to the synthesis of cinnamic acid. Thus, the addition of even low levels of cinnamic acid will lead to the inhibition of the endogenous synthesis of cinnamic acid. Therefore, this phenolic compound is probably not a suitable precursor for capsaicin synthesis. In this system a large proportion of the capsaicin which is synthesized is released into the medium.

Nicotine which normally accumulates in the tobacco plant can also be synthesized from added nicotinic acid in hairy root cultures (Robins et al. 1987). However, under certain circumstances cultures will perform the further biotransformation to nornicotine (Barz et al. 1978). Table 8.5 shows the distribution of nicotine and the formation of nornicotine which is released into the medium (the data have been acquired from studies in Edinburgh). Lockwood and Essa (1984) added precursors of nicotine to suspension cultures of *N. tabacum* and showed an inverse correlation between nicotine content and content of one of its breakdown products, myosine.

When the biotransformation of interest occurs at the outer surface of the cell, the uptake of substrate and release of product causes fewer problems. The formation of L-dopa from L-tyrosine in cultures of *Capsicum* fed with nicotinic acid biotransformed it into nicotine which is substantially less toxic to the cells (Robins et al. 1987). This has been used as a selection pressure in an attempt to select cultures with a high biosynthetic activity.

There are instances, however, where the product formed from added substrate is more toxic to the cultured cells. The methoxylation of cinchonichine and cinchonine to quinine and quinidine by cultures of *Cinchona ledgeriana* yields more toxic compounds (Robins et al. 1986).

Therefore, unless the product is sequestered to the vacuole or released to the medium, the cells must be tolerant of the product as well as of the substrate.

**The substrate must be able to enter the cell and the product will preferably be released to the medium**

Although in a few instances plant biotransformations can take place at the outer surface of cultured cells the majority of biotransformations take place intracellularly. The added substrate therefore has to enter the cell and the compartment in which the enzyme is localized. Vanillylamine is a close precursor of capsaicin. However, the molecule has very low solubility in the culture medium, is highly ionized at near neutral pHs, and uptake into the cells is very low. Alteration of the pH can increase uptake by suppressing ionization but the incorporation of ferulic acid into capsaicin (which is more water soluble) is always greater than the incorporation of vanillylamine. Therefore ferulic acid which is further from the product than vanillylamine acts as a better precursor for capsaicin synthesis. In this system a large proportion of the capsaicin which is synthesized is released into the medium.

Table 8.5: The distribution of added nicotine and synthesized nornicotine between cells and medium in tobacco cultures

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Nicotine (%)</th>
<th>Nornicotine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
<td>Medium</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>84</td>
</tr>
<tr>
<td>24</td>
<td>21</td>
<td>79</td>
</tr>
<tr>
<td>36</td>
<td>24</td>
<td>76</td>
</tr>
</tbody>
</table>

2.5 mM nicotine was added to cultures in the exponential phase of the growth cycle.
The destructive or non-destructive permeabilization of cultures has been studied as a method of causing release of products to the medium. Treatments which have been employed for permeabilization include the use of solvents, antibiotics, detergents, and a miscellany of other treatments.

Immobilization has also been demonstrated to result in increased product release in many cultures (Hall et al. 1988) and biotransformations have been reported in immobilized cells of several species. The combination of precursor feeding and cell immobilization will therefore be increasingly employed in the exploitation of biotransformations.

**Future prospects for the use of biotransformations in tissue culture**

Biotransformations may be exploited in the synthesis of products which are normally extracted from the plant (all of the examples discussed above) or in the formation of novel products. These are metabolites which have not previously been reported and which are synthesized by cell cultures grown in the presence of natural and synthetic precursors. When suspensions of *Ruta graveolens* were grown in the presence of chemically synthesized derivatives of the furanocoumarin precursor 7-hydroxy eoumarin a number of novel compounds were synthesized. The structures of the novel compounds synthesized depend on which analogue was supplied (Brown et al. 1970; Austin and Brown 1973).

Similarly, the addition of synthetic o-methylcinnamic acid to cells of tobacco results in the formation of two esters which are both unnatural analogues of chlorogenic acid (Steck and Constabel 1974). Although many enzymes show very great specificity for their substrate a large number including peroxidases, some dehydrogenases, and methyl-transferases are relatively non-specific. The potential for the formation of analogues of pharmaceutical drugs is therefore an attractive possibility.

Whether the product formed is natural or novel, any commercial biotransformation requires the successful cultivation of plant cells on a large scale. Relatively recently, bioreactors have been developed for the medium scale culture of cells for subsequent use either as suspensions or in immobilized systems (e.g. for review see Fowler 1986; Mavituna et al. 1988) Cells cultured in such bioreactors grow at a much slower rate than microbial cells. This therefore dictates that batch growth followed by killing the cells for product removal, which is the system usually employed with microbial cultures, is not suitable for exploiting biotransformation in plant cell cultures. A semi-continuous system involving continuous product removal is more suited to plant cultures but this process requires that the product is released into the medium. Where the product of interest is retained within the cells, destructive or non-destructive methods of permeabilization can be considered (Felix 1982). Thus from a chemical engineering point of view the exploitation of plant cell cultures for biotransformations is possible.

The recent advances in recombinant DNA technology make these methods particularly suitable for the manipulation of biotransformations in culture. These techniques are particularly suitable for secondary product genes in cultures for two main reasons:

1. Whole plants do not have to be regenerated from transformed cells. Difficulties in regeneration have limited progress in a number of transformed crop plants.
2. As the genes of interest are involved in secondary metabolism any alteration in regulation of expression is unlikely to seriously affect primary metabolism.

The use of anti-sense RNA can be envisaged in order to block the expression of unwanted native mRNAs. This technique can be considered in the context of biotransformations, as a possible method to block the formation of an enzyme degradative to the desired product. The activities of enzymes which compete for the added precursor could also be inhibited by use of antisense RNA.

There is some evidence (Potrykus et al. 1985) that the copy number of genes can control the activities of certain enzymes. By identification, cloning, and insertion of multiple copies of regulatory genes it may prove possible to increase biotransformation activity. Similarly, if substrate

---

**Table 8.6 Comparison of the subcellular distribution of oxidase and hydroxylase activity in cultured cells of *Mucuna pruriens***

<table>
<thead>
<tr>
<th>Activity</th>
<th>Callus</th>
<th>Suspended cells</th>
<th>Immobilized cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxidase activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble</td>
<td>81.4</td>
<td>68.3</td>
<td>65.6</td>
</tr>
<tr>
<td>Cell wall</td>
<td>5.3</td>
<td>9.8</td>
<td>18.6</td>
</tr>
<tr>
<td>Intermediate</td>
<td>5.1</td>
<td>13.1</td>
<td>13.4</td>
</tr>
<tr>
<td>Microsomal</td>
<td>7.9</td>
<td>8.6</td>
<td>4.2</td>
</tr>
<tr>
<td><strong>Hydroxylase activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble</td>
<td>81.2</td>
<td>68.7</td>
<td>61.9</td>
</tr>
<tr>
<td>Cell wall</td>
<td>18.7</td>
<td>7.9</td>
<td>18.8</td>
</tr>
<tr>
<td>Intermediate</td>
<td>—</td>
<td>15.9</td>
<td>14.0</td>
</tr>
<tr>
<td>Microsomal</td>
<td>—</td>
<td>7.2</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Results are expressed as the percentage of activity detected in the cultures.
concentration is found to be limiting to product formation in culture we can envisage the incorporation into cells of multiple copies of genes that control substrate synthesis and thus increase product formation.

Many secondary products are developmentally regulated in whole plants and are synthesized at only one stage of the growth cycle in culture. In some cases this may be regulated at the level of substrate supply. Therefore it may be possible to engineer genes for secondary products so that they contain a constitutionally expressed promoter so that product is continuously synthesized.

The targeting of proteins to specific sites within cells is due to the presence of signal sequences (Ellis and Robinson 1987). As the compartmental separation of substrate and enzyme can limit product synthesis in certain instances, the possibility of engineering genes so that they ultimately lead to the formation of an enzyme which can be specifically targeted to the location of the substrate can be considered.

Indeed, it is even possible to envisage the insertion of genes responsible for plant biotransformations into microbial cells which are much easier to grow and harvest in large-scale culture. Similarly, it may be possible to transform whole plants with regulatory DNA that controls the formation of substrates to the enzyme of interest so that no additional exogenous precursor is required and the need for culture systems is therefore redundant.

Although all of these possibilities are hypothetical and their exploitation lies in the future, discussion of which (if any) of these possibilities should be pursued is required. It seems probable that depending on the characteristics of the biotransformation concerned, a different set of approaches will be dictated in each case in the ultimate production of a range of plant secondary products.

Acknowledgements

The authors are grateful to Glaxo Research Ltd. and Rothman International for financial support. We also wish to thank Mrs E. Raeburn and Mrs J. Summers for typing this manuscript.

References


digitoxin by strophanthus amboensis and strophanthus intermedius tissue
cultures. Herba Hungarica, 25, 87-95.
Papaverine biotransformation in plant cell suspension cultures. Journal of
Natural Products, 51, 532-6.
formation of valencene in cell suspension cultures of citrus spp. Plant Cell Reports,
3, 37-40.
Research, 14, 1-24.
of anhydrovinblastine to vinblastine by a cell-free extract of Catharanthus
roseus cell suspension cultures. Phytochemistry, 26, 3233-4.
formation in plant cell suspension cultures after treatment with a yeast
Carbohydrate preparation (elicitor). Phytochemistry, 26, 401-5.
reticuline and morphinan alkaloids by cell cultures of Papaver somniferum.
Phytochemistry, 17, 891-3.
to codeine by immobilized cells of Papaver somniferum. Phytochemistry, 23,
999-1001.
2-phenylpropionic acid and its ethyl ester in suspension cultures of N. tabacum,
Dioscoreophyllum cumminisii and Aconitum japonicum. Phytochemistry, 26,
2983-9.
by cell suspension cultures of Strophanthus gratus. Phytochemistry, 27,
2129-33.
Biotransformation of phenylactic acid and 2-phenylpropionic acid in suspension
cultures of Coffee arabica. Phytochemistry, 27, 803.
division-arrested and immobilized plant cells: bioconversion of monoterpenes
by gamma-irradiated, suspended and entrapped cells of Menha and Nicotiana.
cultures and redifferentiated roots of Hyoscyamus niger. Planta Medica, 47,
194-9.
secondary metabolism in culture (ed. R. J. Robins and M. J. C. Rhodes),
secondary metabolism in culture (ed. R. J. Robins and M. J. C. Rhodes),
(1985). Production of L-DOPA by cell suspension cultures of Mucuna pruriens.
I. Initiation and maintenance of cell suspension cultures of Mucuna pruriens
and identification of L-DOPA. Plant Cell Tissue and Organ Culture, 4,
61-74.
Jones, A. and Velicky, I. A. (1981). Examination of parameters affecting the 5b-
hydroxylation of digitoxigenin by immobilized cells of Daucus carota. European
Journal of Applied Microbiology and Biotechnology, 13, 84-9.
digitoxigenin by cell suspension cultures of Strophanthus amboensis. Phyto-
chemistry, 27, 3475-9.
Kergomard, A., Renard, M. F., Veschambre, H., Courtois, D., and Petiard, V.
King, P. J. and Street, H. E. (1977). In Plant tissue and cell culture (ed. H. E.
phosphate on growth and formation of cinnamoyl putrescines in medium induced
cell suspension cultures of Nicotiana tabacum. Planta, 153,
582-5.
Biotransformation of euciliptine into 5-formyl eucilipetine by Choisya ternata
cultured Digitalis lanata cells. Production of deacetyl-lanataside C using a two-
Hall, R. D. and Yeoman, M. M. (1987). Intercellular and intercultural hetero-
genicity in secondary metabolite accumulation in cultures of Catharanthus roseus
of cannabidiol to cannabichromene by suspension cultures of Cannabis sativa and
cultures and redifferentiated roots of Hyoscyamus niger. Planta Medica, 47,
194-9.
secondary metabolism in culture (ed. R. J. Robins and M. J. C. Rhodes),
secondary metabolism in culture (ed. R. J. Robins and M. J. C. Rhodes),
Biotransformation of euciliptine into 5-formyl eucilipetine by Choisya ternata
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