Studies of Alkaloid Metabolism in *Papaver somniferum* L.

Thesis presented by

Thi Danh Thom Pham

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Department of Pharmacognosy
The School of Pharmacy
University of London
29-39 Brunswick Square
London WC1N 1AX

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ABSTRACT

Six *P. somniferum* L. cultivars were investigated for quantitative analysis of their alkaloid contents during plant development in the attempt to screen plants with high yields in noscapine and to determine what losses in alkaloid occurred as a result of harvesting the capsule plus 8" stem rather than the whole plant. High temperature sum and sunshine level resulted in high alkaloid content. Two minor alkaloids, oripavine and N- methylnarceine, were successfully isolated during the investigation.

Feeding experiments showed tetrahydroberberine, hydrastine, and to a lesser extent berberine, improved noscapine yield.

The latex contains a large number of acidic vacuoles in which the alkaloids are found. The vacuoles were separated into two discrete fractions: the 900xg and the 1100xg vacuoles. The two vacuolar populations were assayed over the three-week period of capsule maturation. The 900xg vacuoles contain most of the alkaloids, dopamine and the acids (meconate, sulphate and malate) of the latex. In addition, estimations of the cations K^+ , Na^+ , Mg^{2+} , Ca^{2+} and Cl^- were also made.

Uptake by the latex vacuoles was specific for the native alkaloids. Both the 900xg and 1100xg vacuolar fractions take up morphine rapidly and effectively. Morphine uptake by the 1100xg vacuoles was more dependent on exogenous ATP and, after uptake of large amounts of alkaloids, both populations were stimulated by exogenous ATP to take up further amounts of alkaloid.

Uptake of [^{35}S]-sulphate and L-[U-^{14}C]-malate was continuous and the rate of uptake was much lower than that of morphine and other alkaloids.

A correlation between alkaloids and organic acids in the 900xg vacuoles supports the "ion- trap" mechanism proposed for alkaloid sequestration in the poppy latex vacuoles. The young vacuoles (1100xg fraction) which do not contain sufficient organic acids are more dependent on the ATPase-proton pump for the specific and rapid movement of native alkaloids across the vacuolar membrane .

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Section 1

INTRODUCTION

Alkaloids were first defined as the alkali-like compounds found in the plant by the pharmacist W. Meissner in 1819. Winterstein and Trier (1910) referred to alkaloids as the basic nitrogenous substances produced by the plant and in some animals; and the terms "true alkaloid" or "alkaloid-proper" were used to indicate the nitrogen-containing compounds, with basic character and complex molecular structure derived from amino acids. Southon and Buckingham (1988) have defined alkaloids as the naturally occurring, nitrogen-containing secondary metabolites of plant, microbial or animal origin which exhibit a pronounced physiological activity.

The role of alkaloids in plants has received much consideration and it has been suggested that they could be the end products of metabolism or waste products, growth regulators, inhibitors, protective agents for the plant against attack by predators, and storage reservoirs of nitrogen (Waller and Nowacki 1978).

Alkaloids constitute a vast class of naturally occurring compounds exhibiting various structural types. Of the several thousands known alkaloids, about 30 of them have been used clinically for a wide range of pharmacological effects. Despite considerable progress in organic synthesis and in plant biotechnology, many pharmaceutically useful alkaloids are still obtained from plants.

Papaver somniferum L. of the Papaveraceae produces morphine, codeine, thebaine, papaverine and noscapine as major alkaloids which are all medicinally important, especially morphine. More than 50 minor alkaloids have been isolated so far from this plant. Opium, the dried exuded latex of the poppy, contains at least 25 alkaloids which occur as salts of meconic and sulphuric acids and complexes with phenolic acids.

1.1. Taxonomy and Chemotaxonomy

1.1.1. Alkaloids in the plant kingdom

According to Mabry and Mears (1970) and Schultes (1972), alkaloids have been found distributed mainly in the division Angiosperms (the flowering plants) of the Spermatophytes. The other division of the Spermatophytes is the Gymnosperms with 700 species in 65 genera and rarely contain alkaloids. The Angiosperms comprises 200,000-250,000 species in 300 families and 10,500 genera. This vast group of the plant kingdom is subdivided into two subgroups: the Dicotyledons comprises around 165,000 species and the Monocotyledons about 50,000 species. According to Cronquist (1977), the Angiosperms is divided into 6 subclasses (one of these is the Magnoliidae) that make up the Dicotyledons. The subclass Magnoliidae, according to Dahlgren (1980), is much broader and includes all the Dicotyledons. The families Papaveraceae and Fumariaceae of the Magnoliidae are well known as taxonomic groups and both are very rich in medicinal alkaloids. The taxonomic delineation of the Papaveraceae is not uniform . Attempts have been made by both botanists and chemists to find a correlation between chemical properties and botanical features.

1.1.2. Chemotaxonomy of benzylisoquinoline alkaloids and related compounds

Chemotaxonomy is defined as the characterisation and classification of plants on the basis of their chemical constituents (Swain 1963).

Simple isoquinoline alkaloids which contain only one aromatic nucleus and no other cyclic structure except a methylene substituent are distributed throughout the plant kingdom (Bisset 1985). Within the division Angiosperms of the

Spermatophytes (Mabry and Mears,1970), complex phenylalanine-derived alkaloids (benzylisoquinolines and others) are present in about 40 families (Bisset 1985). The main distribution of the benzylisoquinolines in the subclass Magnoliidae, Dicotyledons (according to Dahlgren 1980) is shown in Table 1.1 (Bisset 1985) .

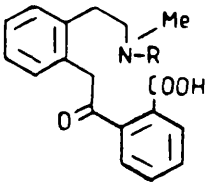
1.1.3. Chemotaxonomy of the Papaveraceae and the genus *Papaver*

Based on botanical characteristics, the order Papaverales is divided into families and subfamilies as shown in Fig. 1.1 , according to Engler (1909). The Papaveraceae of the order Papaverales has been divided into 23 genera, and includes about 430 species, whereas 7 genera with about 350 species were characterised in the Fumariaceae (Preininger 1986). Alkaloids are found in about 168 species of the Papaveraceae and in about 90 species of the Fumariaceae (Preininger 1986). Tetrahydroisoquinoline alkaloids and their derivatives are a characteristic feature of these two families. The chemotaxonomic significance for both families is the presence of alkaloid types: quaternary protoberberines and benzophenanthridines, protopines and aporphines. In contrast, the distribution of some alkaloids are limited to only one of the two families. The promorphinans, morphinans, pavine, isopavine, retroprotoberberine, rhoeadane and their N-methyl derivatives, and papaverrubines are only found in the Papaveraceae. The spirobenzylisoquinoline and indenobenzazepine alkaloids are present only in the Fumariaceae, while the phthalideisoquinoline and secophthalideisoquinoline alkaloids are mainly, but not exclusively found in the same family (Table 1.2). The presence of meconic acid is a distinctive taxonomic feature of the Papaveraceae (Fairbairn and Williamson 1978). Quaternary highly polar alkaloids are also normally present

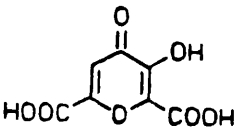
in the Papaveraceae (Preininger 1985, 1986). Special emphasis is given to the genus *Papaver* because of the relevance to the present work (Figures A&B).

Papaver is the largest and most studied genus. It was originally divided into five sections by Bernhardi (1833). Sections of the genus *Papaver* were further introduced as shown in Table 1.3A. The division of the genus *Papaver* into nine sections described by Fedde (1909) is still used by many authors.

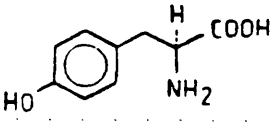
Approximately 120 species were found in the genus *Papaver*, of which about 75 species are known to contain about 170 alkaloids (Preininger 1986). Each *Papaver* species in these sections yields various alkaloid types. Most of the alkaloids found in the genus *Papaver* are biosynthesised from the amino acids phenylalanine, tyrosine and 3,4 - dihydroxyphenylalanine. The occurrence of alkaloid types found in the genus *Papaver* is shown in Table 1.3B .



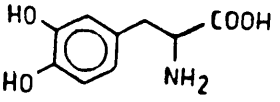
secophthalideisoquinoline group



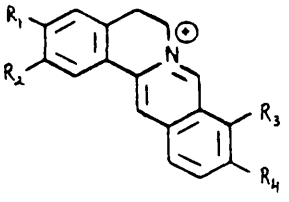
meconic acid



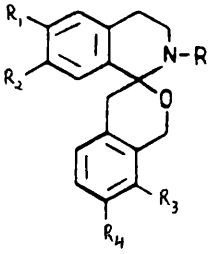
L-tyrosine



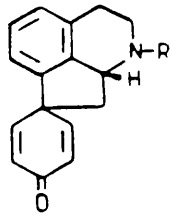
3,4-dihydroxyphenylalanine (DOPA)



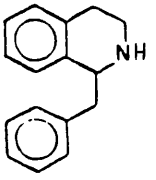
berberine group



secoberberine group



proaporphine group



benzylisoquinoline

Figure B : Alkaloid types and compounds mentioned in Section 1.1.3

Table 1.1 Distribution of benzylisoquinoline alkaloids in the subclass Magnoliidae

(classified according to Dahlgren 1980)

Superorder	Order	Family
Magnoliiflorae	Annonales	Annonaceae, Eupomatiaceae , Canellaceae
	Aristolochiales	Aristolochiaceae
	Magnoliales	Magnoliaceae
	Laurales	Monimiaceae , Lauraceae , Hernandiaceae
	Nelumbonales	Nelumbonaceae
Nymphaeiflorae	Piperales	Piperaceae
Ranuncuiiflorae	Ranunculales	Menispermaceae , Ranunculaceae, Berberidaceae , Nandinaceae
	Papaverales	Papaveraceae , Fumariaceae

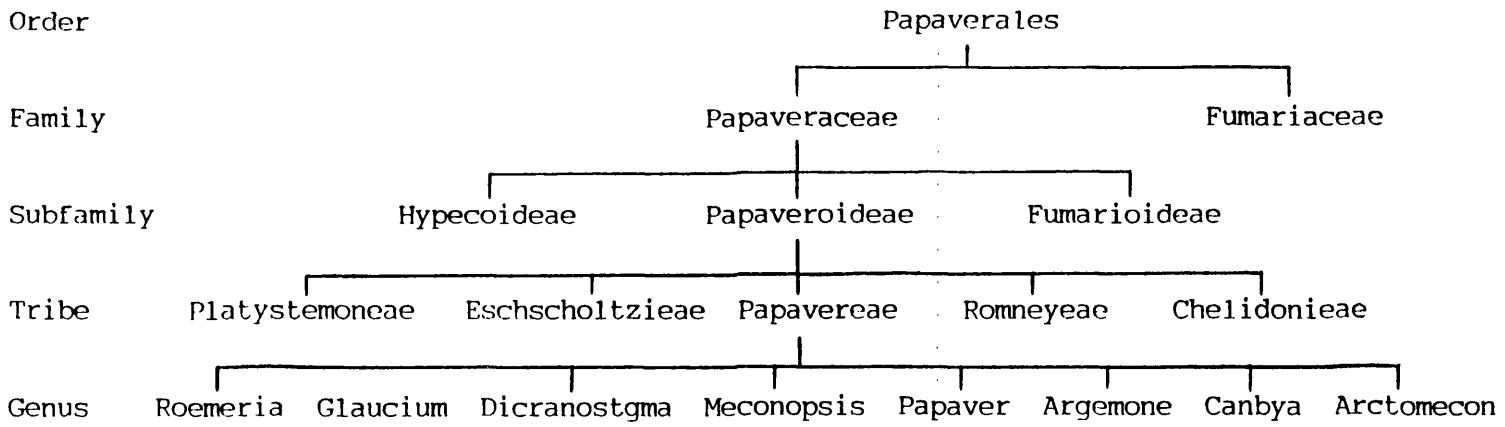


Figure 1.1 Taxonomy of the order Papaverales (Engler 1909)

Table 1.2 Distribution of alkaloid types in the order Papaverales

(classified according to Preininger 1985)

Family	Genus	Alkaloid type
Papaveraceae	Papaver	promorphinan,morphinan, protoberberine berberine , 1-benzylisoquinoline , 1-benzyltetrahydroisoquinoline , phthalideisoquinoline , protopine, benzophenanthridine , aporphine , proaporphine ,rhoeadine , isopavine , papaverrubine
	Eschscholtzia	benzophenanthridine , aporphine , benzylisoquinoline , pavine
	Bocconia	rhoeadine , papaverrubine
	Glaucium	aporphine , protopine , protoberberine benzophenanthridine
	Meconopsis	protopine , protoberberine , aporphine benzophenanthridine , rhoeadine , proaporphine , promorphinan
	Argemone	benzophenanthridine , pavine , protopine , berberine , protoberberine
	Hypecoum	secoberberine , protoberberine , protopine , benzophenanthridine
Fumariaceae	7 genera	phthalideisoquinoline , secoberberine, secophthalideisoquinoline , spirobenzylisoquinoline , indenobenzazepine

Bernhardi (1833)	Elkan (1839)	Prantl (1889)	Fedde (1909)	Gunther (1975)	Preininger et al (1981)
Lasiotrachyphylla	Scapiflora	Lasiotrachyphylla	Scapiflora	Lasiotrachyphylla	Meconella
Oxytona	Macrantha	Macrantha	Macrantha	Oxytona	Macrantha
		Pilosa	Pilosa	Pilosa	Pilosa
Miltantha	Pyramistigmata	Miltantha	Miltantha	Miltantha	Miltantha
Rhoeades	Rhoeades	Rhoeades	Argemonorhoeades	Argemonidium	Argemonidium
			Carinatae	Carinatae	Carinatae
			Orthorhoeades	Rhoeades	Rhoeadium
Mecones	Mecones	Mecones	Mecones	Papaver	Papaver
					Glauca
	Horrida	Horrida	Horrida	Horrida	Horrida
				Roemeria	Roemeria
				Pseudo-pilosa	

Table 1.3 A Sections of the genus Papaver

Table 1.3 B Distribution of alkaloid types in the genus Papaver

Genus	Section	Alkaloid type	Examples of species
Papaver	Orthorhoeades	rhoeadine, papaverrubine, protopine, isorhoeadine, aporphine, (benzylisoquinoline), (morphinan), (proaporphine), (protoberberine), (benzophinanthridine)	P. rhoeas P. dubium
	Argemonorhoeades	protopine, rhoeadine, papaverrubine, benzophenanthridine, protoberberine	P. argemone P. pavonium
	Carinatae	protopine, rhoeadine, papaverrubine, benzophenanthridine	P. macrostonium
	Mecones (Papaver)	benzylisoquinoline, promorphinan, morphinan, phthalideisoquinoline, secophthalideisoquinoline, protopine aporphine, protoberberine, benzophenanthridine, (rhoeadine), (papaverrubine)	P. somniferum P. setigerum P. glaucum P. gracile P. decaisnei
	Horrida	rhoeadine, papaverrubine, (aporphine) (benzophenanthridine), (morphinan), (benzylisoquinoline), (phthalideisoquinoline)	P. aculeatum
	Pilosa	rhoeadine, papaverrubine, aporphine, (protopine), (promorphinan), (benzophenanthridine)	P. pilosum P. oreophilum
	Miltantha	protopine, 1-benzylisoquinoline, 1-benzyltetrahydroisoquinoline, promorphinan, morphinan, proaporphine aporphine, benzophenanthridine, tetrahydroprotoberberine, berberine, phthalideisoquinoline, rhoeadane, isopavine	P. fugax P. armeniacum
	Macrantha	morphinan, promorphinan, aporphine, proaporphine, benzophenanthridine, tetrahydroprotoberberine, protopine, berberine, secoberberine, rhoeadane, 1-benzylisoquinoline	P. bracteatum P. orientale P. pseudo-orientale
	Scapiflora	isopavine, rhoeadine, promorphinan, protopine, papaverrubine	P. nudicaule

Note: _____ major alkaloid type

( ) occasionally occurred

1.2. The Genus *Papaver* and its Significance

1.2.1. Utilisation of *Papaver*

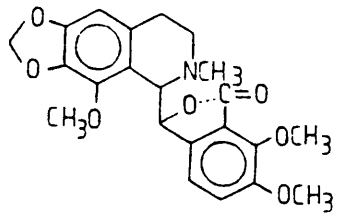
Papaver has provided Man with antibiotics, antidiarrhoeals, antimalarials, analgesics, sedatives, tranquillisers, cough remedies, goitre treatments, baby rattles, ant food, bird food, fuel, copper and molybdenum indicators, hypnotics, opiates, poppy seeds, poppy seed oil, poppy cakes, poppy straws, poppy flour (Duke 1973).

Although all members of the Papaveraceae are more or less poisonous. It is *Papaver somniferum* (opium poppy) that has proved to be the most medicinally useful plant (Popov 1970).

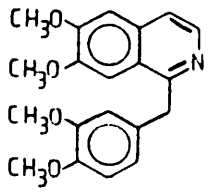
1.2.2. *Papaver somniferum* L. and the discovery of its pharmacologically active constituents

It is recognised that the capsules of *P. somniferum* L. were employed as early as 2,500 B.C. (Bisset 1985). The therapeutic use of poppy capsule latex was recorded in the third Century B.C. (Hort 1916). The different uses of both latex and extracts of whole plants for the curative properties of opium poppy were described in A.D. 77 (Bisset 1985). However, it was only at the beginning of the 19th Century A.D. that the active principle of opium isolated from the dried exuded latex of the poppy was discovered by Derosne in 1803. As a result of more detailed work, morphine was first isolated as a pure compound by Serturner in 1817 (Lockemann 1951). Codeine was later isolated in 1832 by Robiquet, thebaine in 1835 by Thiboumery, and papaverine in 1848 by Merck (Trease and Evans 1978).

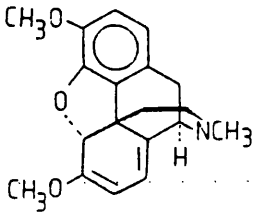
The isolation of morphine, codeine, thebaine, papaverine



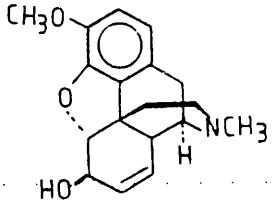
noscapine



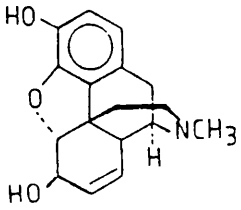
papaverine



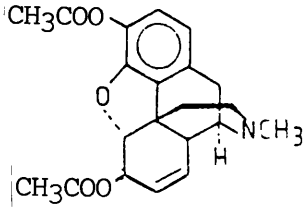
thebaine



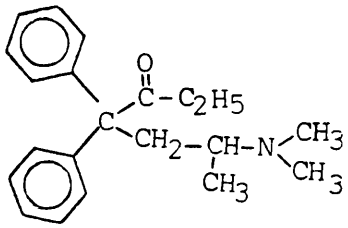
codeine



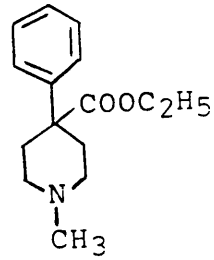
morphine



heroin



methadone



meperidine
(pethidine)

Figure C : Major alkaloids in P. somniferum and synthetic analgesics

and noscapine from *P. somniferum* plants, together with the diacetylation of morphine to produce heroin has proved beneficial in medicine. Morphine and its derivative heroin have euphoric effects in addition to their useful analgesic properties. Codeine is antitussive and antidiarrhoeal, besides being a moderate analgesic. Thebaine is also useful as an analgesic , and papaverine is used as a bronchodilator and for the relaxation of smooth muscle. Noscapine is also an antitussive and used as a cough suppressant. However, it is also recognised that the physical and psychological dependence on morphine and heroin has become one of the major international social and criminal problems. Attempts have been made to eliminate the addictive properties of morphine and heroin. Thousands of synthetic substitutes of morphine were synthesised and tested but only about thirty of them have been used and brought under international control (Husain and Sharma 1983). Pethidine (meperidine), methadone, phenazocine are useful synthetic analgesics, but unfortunately they are also addictive (Figure C).

The discovery of morphine, codeine, thebaine, papaverine and noscapine in *P. somniferum* was the starting point for the vast field of alkaloid chemistry and phytochemistry, and the Papaveraceae has been screened for alkaloids of potential medicinal use. A wide range of alkaloids have been found throughout the family (Table 1.2). The medicinally valuable morphinan alkaloids (morphine and codeine), have been found in four out of nine sections of the genus *Papaver*, including Mecones (syn. Papaver), Miltantha, Horrida, and Macrantha (Table 1.3B). The promorphinans are also present in sections Miltantha, Mecones, Pilosa, Macrantha and Scapiflora. The species *Papaver somniferum* L. of the section Mecones contains morphine, codeine, thebaine, papaverine and noscapine as major alkaloids, and has so far proved to be the most medicinally useful plant.

1.2.3. The production of *P. somniferum* and world requirement

Seeds of the opium poppy have also been of interest owing to their edibility and high oil content (45-52%) (Yarosh and Megorskaya 1975). Despite the worldwide problem of drug abuse, there is still considerable demand for licit opium production. More than 1 tonne of legal opium was required for the year 1986 (Anonymous 1985). Because of its narcotic properties, the opium lends itself to frequent illicit transactions. The legal exportation of opium has been controlled since 1953 by the Protocol of United Nations Opium Conference for countries Bulgaria, Greece, India, Iran, Turkey, USSR and Yugoslavia. Opium has been legitimately cultivated in Yugoslavia, USSR, Turkey, India, Australia, China, Egypt, France, Holland, Hungary, Greece, Spain, Portugal, Italy (Ramanathan and Ramchandran 1977, Veselovskaya 1976). Turkey and USSR, however, have stopped poppy cultivation for opium since 1972 and 1974,respectively; though they still grow opium poppy for extraction of alkaloids from straw. Japan and Bulgaria also grow opium poppy on a very small scale (INCB 1981). Australia, France, Spain, Romania, etc. have changed their choice from opium to poppy straw for alkaloid extraction. Some other countries grow poppy for seed and/or oil, eg. Austria, Czechoslovakia, West and East Germany, Hungary, Netherlands, Poland, Turkey, etc. .However, only India continues to produce opium for licit purposes .

The illicit transactions and illegal use of opium, and the easy conversion of morphine to heroin has long been a problem associated with the cultivation of *P. somniferum* on a large scale (e.g. in the Golden Triangle and Golden Cresent). The severity of the problem is demonstrated by the U.N. Division of Narcotic Drugs' approval and financing various research projects to promote the use of *P. bracteatum*

as a source of thebaine, which may be readily converted to codeine (Weller and Rapoport 1976) for which there is a major world requirement. Codeine is also produced by methylation of morphine (Anonymous 1976). Since no case of thebaine abuse has been reported and thebaine is the only major alkaloid in *P. bracteatum*, this plant has been regarded as a promising solution to the production of codeine. The further development of *P. bracteatum* as a medicinal plant is currently limited by practical conditions of the cost involved the agricultural production, the conversion of thebaine to codeine in industry and the influence of political considerations.

1.3. Chemical Synthesis of Morphinan Alkaloids

As previously mentioned, many alkaloids are clinically important and the recognised physical and psychological dependence on morphine and its derivative heroin has become an international social and criminal problem, it is desirable to establish a synthetic route that provides production of the morphinans which is independent of the natural sources.

The correct structure of morphine was shown by Gulland and Robinson in 1925, and attempts were made to synthesise the morphinan alkaloids by Grewe and Mondon (1948) who reported a novel detailed synthesis of the morphinan structure. However, the first total synthesis of morphine was demonstrated by Gates and Tschudi (1952). The stereochemistry of morphine was proposed by Stork (1952), and Rapoport and Levine (1953); which was then confirmed by Kalvoda et al (1955) and Goto (1964). The practical total synthesis of morphinan alkaloids (morphine, codeine and thebaine) was finally shown at the Universities, Pharmaceutical Industries and Government Research Laboratories of the NIH (Rice 1985) (Fig. 1.2). However, chemical synthesis of morphine is not at present considered to be commercially viable .

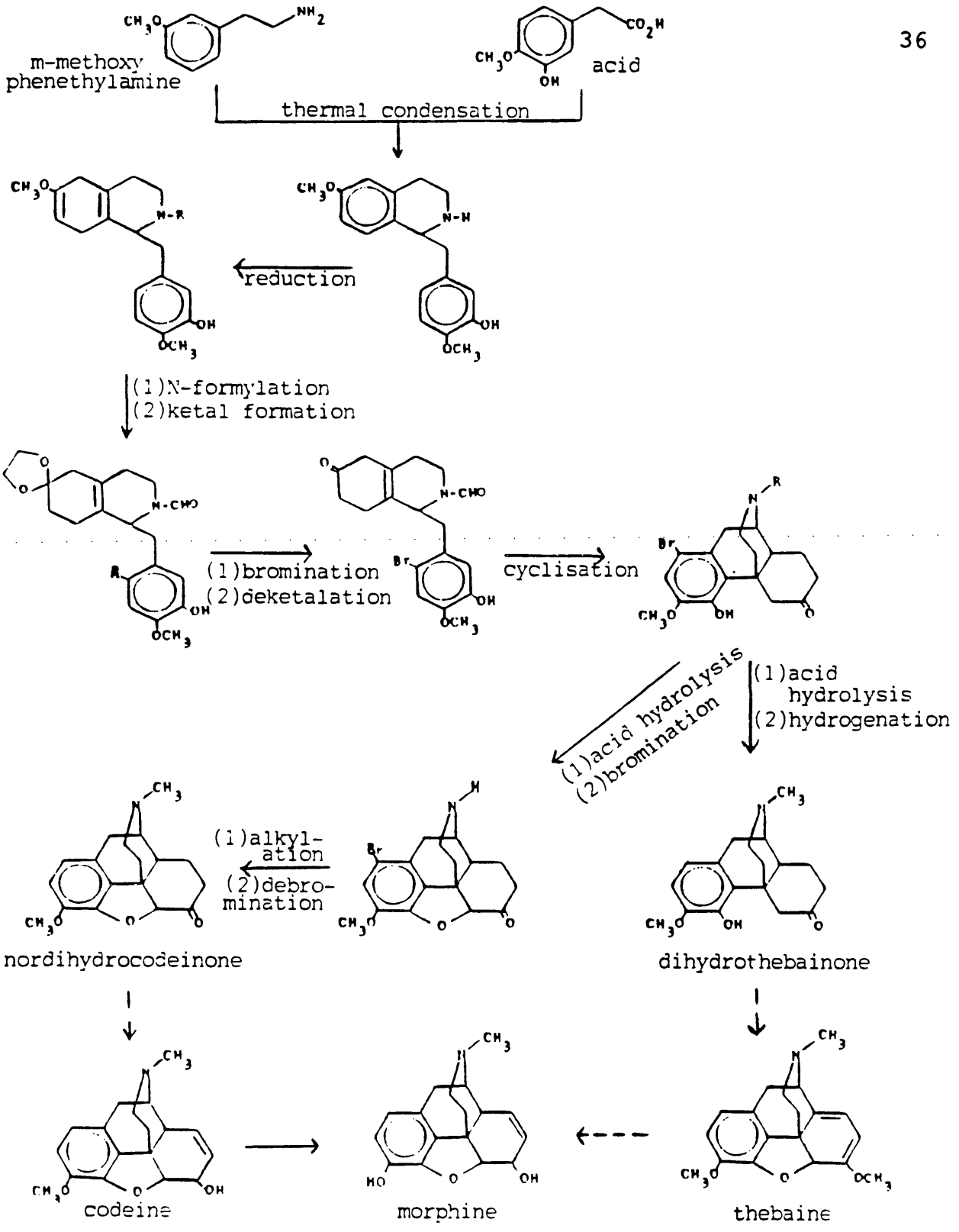


Figure 1.2 Chemical synthesis of morphinan alkaloids

(Rice 1985)

1.4. Alkaloids from *P. somniferum* L. Plants

1.4.1. Alkaloids isolated from *P. somniferum* plants

In addition to the five main alkaloids of *P. somniferum* L., about 52 minor alkaloids have been isolated so far from this plant (Santavy 1970, Preininger 1986, Brochmann-Hanssen 1984, Nielson et al 1983 and Chaudhuri and Thakur 1989). They are shown in Table 1.4 , according to their structural types.

1.4.2. Factors affecting alkaloid formation in *Papaver* plants

1.4.2.1. Effect of nutrients and other factors

Kuzminska (1973b) reported that nitrogen given along with phosphorus stimulated both the alkaloid production and capsule yield, but potassium slightly depressed morphine content in *P. somniferum*. Nitrogen application increased the formation of phenylalanine and hence increased alkaloid content in plants (Nowacki et al 1976). Magnesium and calcium also boosted morphine yield in opium poppy (Kuzminska 1973a). In addition, magnesium deficiency brought about a marked elongation of stem and early flowering without changing morphine content, while calcium deficiency decreased alkaloid level in *P. somniferum* (Costes et al 1976). Sodium favoured the development of flowers and capsules and therefore increased morphine production (Husain and Sharma 1983). Application of salts like sodium chloride and sodium sulphate

Table 1.4 Alkaloids isolated from P. somniferum L. plants

Alkaloid type	Alkaloid name
Benzylisoquinolines	reticuline , orientaline , papaverine , papaveraldine , papaveramine , codamine , tetrahydropapaverine , laudanine , laudanidine, laudanosine
Simple isoquinoline	hydrocotarnine
Morphinans	morphine , normorphine , morphine N-oxide , pseudomorphine , morphinone ,neopine ,oripavine, codeine , codeine N-oxide , 10-hydroxycodeine , codeinone , 6-methylcodeine , thebaine , thebaine N-oxide , 16-hydroxythebaine
Promorphinan	salutaridine
Phthalideiso-quinolines	noscapine , narcotoline , narceine ,nornarceine, narceine imide , narceinone
Protoberberines/ Berberine	scoulerine , coptisine ,canadine , berberine , tetrahydrocolumbamine ,coreximine
Aporphines	magnoflorine , isoboldine , corytuberine
Benzophenanthridines	dihydrosanguinarine , sanguinarine , oxysanguinarine , norsanguinarine , 6-acetonyldihydrosanguinarine
Protopines	dihydroprotopine,protopine, 13-oxycryptopine, cryptopine , allocryptopine
Rhoeadine/ Papaverrubine	glaudine , papaverrubine C and D

also increased morphine production (Spasenovaski 1980).

Chemical fertilisers have been used in poppy cultivation for the last two decades. Both phosphatic and nitrogenous fertilisers besides organic manures have a dominant role in poppy cultivation (Gupta et al 1978). Irrigation is also essential for successful cultivation of opium poppy, and irrigated plants contained more alkaloids than un-irrigated ones (Husain and Sharma 1983). The pH=7 was found to be best for poppy growth (Kinoshita et al 1962).

1.4.2.2. Influence of environment

Environment has significant effect on alkaloid formation in the plant. *P. somniferum* plants produced high alkaloid content in moderately dry fields in the tropics (Il'inskaya and Yosifova 1956). Voseurusa (1960), Tetenyi et al (1961) and Vagujfalvi (1963a,b; 1964) demonstrated the influence of the variation of climatic conditions within a cultivation year, in addition to the geographical location, on alkaloid production in *P. somniferum* plants, especially during the ripening period. Rain can cause the washing out of alkaloids from the capsules (Poethke and Arnold, 1951; Hofman and Menary 1979). Germination of seeds was found to be best in darkness from 8°c to 35°c and the maximum rate occurred between 13°c and 33°c (Bare et al 1978). They also showed that germination was not inhibited or enhanced by intermittent or continuous exposure to Far Red (700-750 nm) or Red (600-680 nm) light. Poppies grown in a controlled environment were generally smaller than the same variety grown in the field (Tookey et al 1976). The metabolic breakdown of alkaloids by endogenous enzymes in the capsule wall (Bunting 1963; Laughlin 1977) and by fungi (Laughlin 1977) were suggested for the losses of morphine and codeine contents in the capsule of *P. somniferum* during moist periods. Hofman and Menary (1979) showed that persistent rain

was associated with a decrease in the capsule morphine and codeine content, and a marked increase in fungal colonization and discoloration of the capsule.

1.4.2.3. Genetic studies of morphinan formation in some *Papaver* species

It is known that alkaloids are formed in plant by the transformation of primary metabolites, especially amino acids. Moreover, the skeleton of alkaloids is determined by the structure of these amino acid units. It is assumed that the formation of alkaloids in plants is genetically controlled (Bohm 1983). Experiments concerning genetic aspects of alkaloid metabolism have been done and most results were based on cross-pollination of different plant species.

When mutants isolated from *P. somniferum* cv. Indra, which contains low morphine content, was cross-pollinated with the normal *P. somniferum* types; the F_1 hybrids showed a 3:1 segregation, with high-morphine hybrid being predominant. Furthermore, the low morphine level was caused by one recessive gene (Nyman 1978, 1980). However, the attempt of combining a low morphine level and a relatively high concentration of papaverine and noscapine in the same *P. somniferum* type was unsuccessful (Nyman, 1980). From these results, it was suggested by Nyman that the low level of morphine in those *P. somniferum* mutants was not due to the inhibition of a specific step of morphine biosynthesis, but caused by the restricted formation of the precursor from which various biosynthetic pathways start.

P. bracteatum contains thebaine as the major morphinan alkaloid. Traces of codeine were detected in some varieties of *P. bracteatum*, but the presence of morphine has not been reported. Crosses between *P. bracteatum* and *P. somniferum* resulted in the F_1 plants with morphine as the main alkaloid (Bohm 1965b,1983). Bohm (1983) also demonstrated that when

the species contains thebaine and no morphine (eg. *P. orientale* or *P. pseudo-orientale*) was crossed with *P. somniferum*, all the F_1 plants could produce morphine. Similarly, *P. orientale* has the ability to form oripavine from thebaine (Fig. 1.14, Section 1.6.10.2), the F_1 descendants of the cross between *P. bracteatum* and *P. orientale* showed the presence of oripavine (Bohm 1983).

1.5. *Papaver* Alkaloids from Plant Cell and Tissue Cultures

Plant cell cultures have been investigated as a source of the *Papaver* alkaloids particularly the morphinans, noscapine and papaverine.

Plant cell and tissue culture techniques for the production of alkaloids offer certain advantages over traditional methods of cultivation for :

1/ minimising the danger of opium abuse;
2/ elimination of plant diseases, geographical influence and environment factors such as climatic and seasonal variation;
3/ more consistent product yield at comparatively stable prices; and
4/ easy cultivation under special control for short growth cycles.

However, plant cell cultures offer disadvantages over traditional cultivation : (1) low yield, (2) high energy costs and requirement, (3) massive economic and social displacement of growers and dependent industries, and (4) consequent political problems .

1.5.1. Plant cell and tissue cultures as alternative methods for alkaloid production

A variety of alkaloid types have been detected and isolated from plant cell cultures. In other research areas, alkaloid yields in general have been low using these techniques. However, a number of high-yielding cultures have been developed which produce alkaloids at levels higher than those of the parent plant eg. *Catharanthus roseus* (ajmalicine and serpentine) and *Coptis japonica* (berberine), (Zenk et al 1977, and Sato and Yamada 1984).

Although plants of the genus *Papaver* normally contain morphinan alkaloids, which are the major components of *P. somniferum* and *P. setigerum*, these compounds are not usually produced at a significant level in the derived callus cultures. The main alkaloids found in callus tissues of *Papaver* species are the benzophenanthridines, protopines and aporphines. However, the attempts to produce the morphinans, especially morphine and codeine from *P. somniferum* cultures have proved to be extremely difficult. In poppy plants, alkaloids are primarily accumulated in vacuoles of the latex which in turn is contained in specialised cells, laticifers (Roberts et al 1983 and references therein). Callus and cell suspension cultures of opium poppy have not been seen to develop typical laticifers. The absence of those specialised cells was often cited as the reason for failure to produce morphine and codeine by poppy cell cultures, since differentiated tissue cultures of *P. bracteatum* tend to produce more thebaine than do comparable batches of undifferentiated calluses (Kamimura and Nishikawa, 1976) and, most frequently, morphinan occurrence coincided with shoot and plantlet formation (Schuchmann and Wellmann 1983, Kutchan et al 1983). Other reason may be the lack of some of enzymes necessary for the biosynthesis of morphine and codeine in *P. somniferum* cultures. Alkaloids detected in cell and tissue cultures from *Papaver* species are shown in Table 1.5 .

1.5.1.1. Morphinan alkaloids from *Papaver* cell and tissue cultures

Thebaine is the only morphinan alkaloid found in cell cultures derived from *P. bracteatum*. In contrast to *P. somniferum* plants, tissue cultures from *P. somniferum* produce only small amounts of codeine in some strains, in addition to the occurrence of thebaine (Khanna et al 1978, Hodges and Rapoport 1982a). However, their concentrations were much

lower than those found in opium.

The use of 2,4-dichlorophenoxyacetic acid promoted codeine formation in cell suspension cultures of *P. somniferum* cv. Marianne (Tam et al 1980). Cytokinins (benzyladenine and kinetin) promoted codeine production in tissue and callus cultures of *P. somniferum* (Staba et al 1982, Hodges and Rapoport 1982a). The addition of tyrosine and ascorbic acid as media supplements were beneficial for the formation of thebaine in suspension cultures of *P. bracteatum* and of major opium alkaloids in *P. somniferum* (Kamimura et al 1976, Khanna et al 1978). In *P. somniferum*, plantlet formed through rooting of callus-borne shoots could produce regenerants which contained codeine as the main alkaloid (Yoshikawa and Furuya, 1983). The formation of embryoids by transferring *P. somniferum* callus on solid or in liquid medium to media without hormones was reported to increase thebaine production (Schuchmann and Wellmann 1983). It was observed that the capacity to produce alkaloids was gradually lost after subculturing (Hodges and Rapoport 1982a, Kamimura et al 1976).

The influence of physiological factors might also be another possibility for improving morphinan production. Stress caused by exposure of cells to 5°C for a period of 3 days prior to harvesting was beneficial to thebaine accumulation (Lockwood 1984). It is known that plant growth regulators are effective triggers of secondary metabolism in vivo (Bohm 1980). The production of thebaine by suspension cultures of *P. bracteatum* was improved by the addition of indole-3-acetic acid, an auxin type, to the medium rather than napthaleneacetic acid or 2,4-dichlorophenoxyacetic acid (Kamimura and Nishikawa, 1976). Protein synthesis inhibitors, actinomycin and puromycin, stimulated morphinan alkaloid accumulation in poppy cell cultures (Hsu 1981). Plants, which had been regenerated from embryogenic callus cultures of *P. bracteatum* and then transplanted to soil, produced thebaine contents comparable to those in seed-grown plants

(Day et al 1986). (-) Codeinone was reduced to (-) codeine by immobilised cells of *P. somniferum* in both shake flask and columnn bioreactor methods (Furuya et al 1984) or by cells immobilised in reticulate polyurethane foam (Corchete and Yeoman 1989) suggesting that at least some of the enzymes of the biosynthetic pathway to morphine were present. ^{14}C-labelled codeine added to the cultured cells of *P. somniferum* was converted into N-oxide products along with the formation of minor amounts of morphine (Hsu and Pack 1989).

It is known that in *P. somniferum* and *P. bracteatum* the morphinan alkaloids accumulate in specialised cells, the laticifers, and that morphine may constitute as much as 10 to 20% of the dried *P. somniferum* latex. The absence of these specialised cells has been suggested as the reason for failure to produce morphine by poppy cell cultures. Kutchan et al (1985,1986) have conclusively shown that the presence and accumulation of thebaine in cell cultures of *P. bracteatum* is associated with cytodifferentiation and the development of laticifer-like cells. For commercially viable production of the morphinan in tissue culture, the development of laticifer-like cells to store the alkaloids without significant metabolic degradation may be essential. However, it is possible that alkaloid biosynthesis may not require a laticiferous system, but the large scale accumulation of the alkaloids may require the development of an artifactual equivalent, together with the use of the media as a lytic compartment as suggested by Wink (1984).

1.5.1.2. Other alkaloid types from *Papaver* cell and tissue cultures

Alkaloid types found in the cultures of *Papaver* species are shown in Table 1.5. Alkaloid extracts from *Papaver* cell and tissue cultures have been shown to contain mixtures of alkaloids with sanguinarine as the major component in *P. bracteatum* (Kutchan et al 1983), and in *P.*

somniferum and *P. orientale* (Schuchmann and Wellmann 1983). The predominance of the benzophenanthridine, protopine and aporphine alkaloids is common in cultured tissues of the Papaveraceae, despite their restricted occurrence in intact plants of this family (Ikuta et al 1974, Kettenes-Van Den Bosch 1981, Phillipson 1983) .

The search for optimal morphinan production by the cell and tissue cultures of *Papaver* species has led to the investigation of the unusual formation of sanguinarine by these cultures. Sanguinarine is a main alkaloid found in *Chelidonium majus* and *Sanguinaria canadensis*, and is stored in laticifers and specialised cells, respectively, in these plants (Jans 1974, Matile 1976, and Neumann and Muller 1972). However in *P. bracteatum* cell cultures, sanguinarine was found in vacuoles which were of a different density than those containing thebaine and dopamine (Kutchan et al 1985, 1986). In contrast to thebaine, cytodifferentiation was not required for sanguinarine accumulation (Eilert and Constabel 1985). Cline and Coscia (1989) found sanguinarine in electron-dense deposits which occur on the tonoplast and as freely floating bodies in vacuoles. Sanguinarine was found localised extracellularly in elicited *P. bracteatum* cell cultures in a 100xg pellet (Cline and Coscia 1988).

In *P. somniferum* and *P. bracteatum* suspension cultures, fungal elicitor induced sanguinarine production by cells with excretion into the medium over a short period of time (Eilert et al 1985a, Eilert and Constabel 1986, and Cline and Coscia 1988). The rapid accumulation of alkaloid was shown to be both elicitor-dose and time dependent, and hormonal deprivation has a synergistic effect on fungal-elicited sanguinarine accumulation (Cline and Coscia 1988).

Since sanguinarine has considerable market potential in oral hygiene products (Southard et al 1984, Anonymous 1985), the production of the benzophenanthridines by the cell and tissue cultures of *Papaver* species may have some commercial consideration.

Table 1.5 Alkaloids from *Papaver* cell cultures

Alkaloid type	Alkaloid name	Source	Reference
Morphinans	Thebaine	*P. bracteatum*	Kamimura & Nishikawa (1976)
			Kamimura et al (1976)
			Zito & Staba (1982)
			Day et al (1986)
		P. rhoeas	Khanna & Sharma (1977)
		P. somniferum	Hsu & Pack (1989)
	Codeine	*P. somniferum*	Corchete & Yeoman (1989)
			Furuya et al (1971)
			Tam et al (1980)
			Hodges & Rapoport (1982)
			Hsu & Pack (1989)
		P. rhoeas	Khanna & Sharma (1977)
	Morphine	*P. rhoeas*	Khanna & Sharma (1977)
		P. somniferum	Furuya (1981)
			Hsu (1981)
			Hsu & Pack (1989)
Benzyliso-quinoline	Orientalidine	*P. bracteatum*	Lockwood (1984)
Phthalide-isoquinoline	Noscapine	*P. rhoeas*	Khanna & Sharma (1977)
Benzophenan-thridines	Sanguinarine	*P. somniferum* *P. setigerum* *P. bracteatum* *P. orientale* *P. rhoeas*	Ikuta et al (1974)
	Oxosanguinarine	*P. somniferum* *P. setigerum* *P. bracteatum* *P. orientale* *P. rhoeas*	Ikuta et al(1974)
	Chelirubine	*P. bracteatum*	Ikuta et al (1974)

Alkaloid type	Alkaloid name	Source	Reference
	Norsanguinarine	P. somniferum P. setigerum P. bracteatum P. rhoeas P. orientale	Ikuta et al(1974)
	Dihydrosanguinarine	P. somniferum P. setigerum P. bracteatum P. rhoeas P. orientale	Ikuta et al(1974)
Protopines	Cryptopine	P. somniferum	Furuya et al (1971)
	Protopine	P. somniferum	Furuya et al (1971)
		P. bracteatum P. setigerum P. orientale P. rhoeas	Ikuta et al(1974)
Protoberberine	Stylopine	P. bracteatum	Kimimura et al (1976)
Aporphines	Magnoflorine	P. somniferum P. setigerum P. bracteatum P. orientale P. rhoeas	Ikuta et al(1974)
	Isothebaine	P. bracteatum	Lockwood (1984)

Table 1.5 (cont.)

1.5.2. Plant cell and tissue cultures for the studies of biosynthetic pathways

Although plant cell cultures may not produce the same secondary metabolites as the parent plant, they are valuable for the investigation of biosynthetic pathways leading to established or novel products in plants (Dougal 1981, Overton and Picken 1977)

As described by Anderson et al (1987), the advantages of using cell cultures over the whole plants for biosynthetic studies may be :

- Cultures are generally less complex in organisation and can be grown for short growth cycles under standard conditions, and are not subject to seasonal variation.

- Cell cultures enable purified enzymes and active cell-free systems to be prepared more easily.

However, the disadvantages in using plant cell cultures for biosynthetic investigations include the need for working under aseptic conditions, and the inability of those cultures to produce the same secondary metabolites as the parent plant since the biosynthesis of some secondary metabolites is associated with cell differentiation.

1.6. The Biosynthesis of *Papaver* Alkaloids

Since the discovery of the major opium alkaloids, a large number of alkaloids have been isolated from *Papaver* plants. Attention has been focussed on the ways by which these natural products were formed in the living plant, and the proposed pathways were developed on the basis of known chemical reactions. After the Second World War, radioisotopes for tracer experiments were available and the developed liquid scintillation counter has allowed the accurate measurement of ^{14}C and ^{3}H activities. The validity of the hypotheses for isoquinoline alkaloid formation could therefore be tested, using radiolabelled compounds in in-vivo experiments.

Winterstein and Trier (1910) first suggested that the benzylisoquinoline structure is built up from two units derived from 3,4-dihydroxyphenylalanine (DOPA). Norlaudanosoline was postulated to act as an intermediate in the isoquinoline biosynthetic pathway (Robinson 1917). Gulland and Robinson (1925) proposed that morphine and related compounds are formed in plants by oxidative coupling of a suitable benzyltetrahydroisoquinoline precursor such as norlaudanosoline. Based on Pummerer's work on the oxidation of phenols to produce relatively stable radicals (Pummerer et al 1925), Barton and Cohen (1957) suggested the biosynthesis of alkaloidal ring system is the result of intramolecular coupling of phenol radicals.

General ideas about the biosynthetic pathways leading to the formation of the *Papaver* alkaloids were based on the results from feeding experiments, using proposed radiolabelled precursors. The use of tissue and cell suspension cultures to produce the desired secondary products (Zenk 1978) and to provide a source of enzyme systems (Stockigt 1980) has revealed the actual sequence of the

biosynthetic process in detail. As a result, the pathways especially to the formation of the tetrahydroisoquinolines have been revised at the enzyme level. Of the alkaloid types found in *Papaver* plants (Table 1.3B), only the biosynthetic pathways to the protopines, benzophenanthridines and berberine have been extensively studied at the enzyme level.

1.6.1. The elucidation of the major pathways using radiolabelled precursors and intermediates in the pathway to the benzylisoquinolines

1.6.1.1. General pathways to the *Papaver* alkaloids

The suggested involvement of DOPA and norlaudanosoline in the biosynthesis of the benzylisoquinoline nucleus was tested when radiolabelled compounds became available. Since DOPA, suggested by Winterstein and Trier (1910) as the basic unit for the formation of the benzylisoquinolines, is the 3-hydroxylated form of tyrosine, the early investigations involved feeding experiments with radiolabelled tyrosine and its related compounds dopamine, phenylalanine and DOPA. Based on the results from tracer studies, the general biosynthetic pathways to *Papaver* alkaloids were proposed as shown in Figure 1.3 .

1.6.1.2. Routes to the benzylisoquinoline molecule

Battersby and Harper (1958) showed the formation of radioactive morphine from ^{14}C-labelled (DL)-tyrosine. In addition, (DL)-phenylalanine was also incorporated into morphine (Leete 1959). It was found that half of the radioactivity detected in isolated morphine was located at position C-16 when these precursors were labelled at position C-2 on the molecules (Leete 1959, Battersby et al 1960). L-

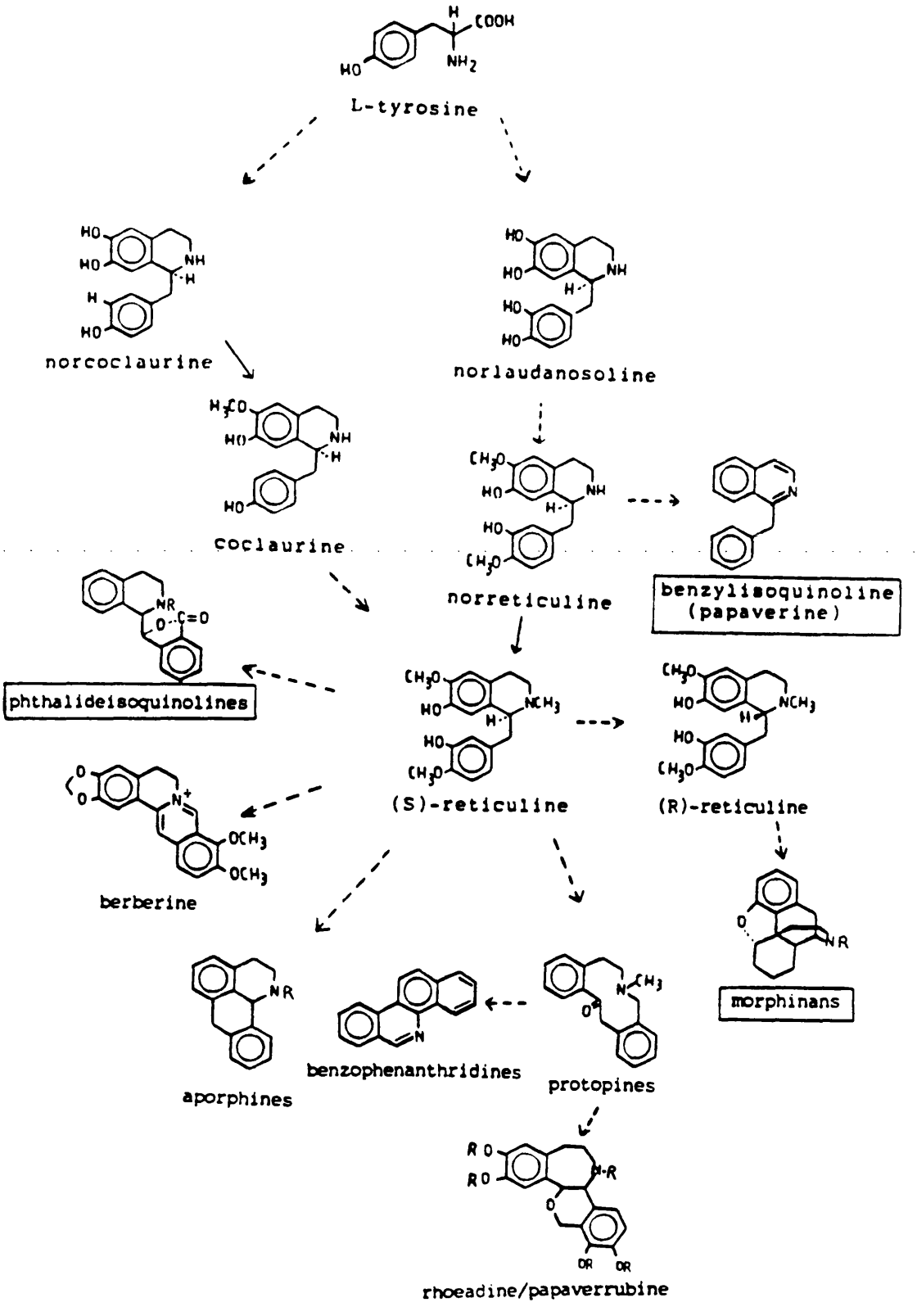


Figure 1.3 General biosynthetic pathways to Papaver alkaloids

Tyrosine and L-DOPA were good precursors of morphine in both the in-vitro and in-vivo experiments with *Papaver* latex (Fairbairn et al 1968 b,c; Fairbairn and Djote 1970).

Chemical degradation of the benzylisoquinoline nucleus showed two molecules of tyrosine form the tetrahydroisoquinoline and benzyl portions of this molecule (Holland et al 1979). Wilson and Coscia (1975) demonstrated that the benzyl moiety was derived from DOPA via 3,4-dihydroxyphenylacetaldehyde, and subsequently norlaudanosoline carboxylic acid was proposed to be an intermediate prior to the formation of norlaudanosoline (Fig. 1.4). The results were in agreement with those shown by in-vivo (Battersby et al 1975b) and in-vitro experiments (Scott et al 1978 a,b). In contrast, Holland et al (1979) showed DOPA underwent decarboxylation to dopamine which was in turn incorporated exclusively into the isoquinoline moiety of the benzylisoquinoline molecule. Holland et al (1979) also suggested norlaudanosoline was formed by the condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde (Fig. 1.4). Tyramine, which is a decarboxylated form of tyrosine, was incorporated into the tetrahydroisoquinoline moiety of the morphinans (Roberts et al 1987a) and of the bisbenzylisoquinoline berbamunine (Stadler et al 1988). However, a revised pathway based on the results from feeding experiments in *Annona reticulata* leaves was proposed. Stadler et al (1987) showed the conversion of L-tyrosine to (S)-coclaurine as an intermediate for the formation of (S)-reticuline, and norlaudanosoline was not involved in the biosynthetic pathway as had been suggested (Fig. 1.5). This proposed pathway and the evidence for it will now be discussed in some details.

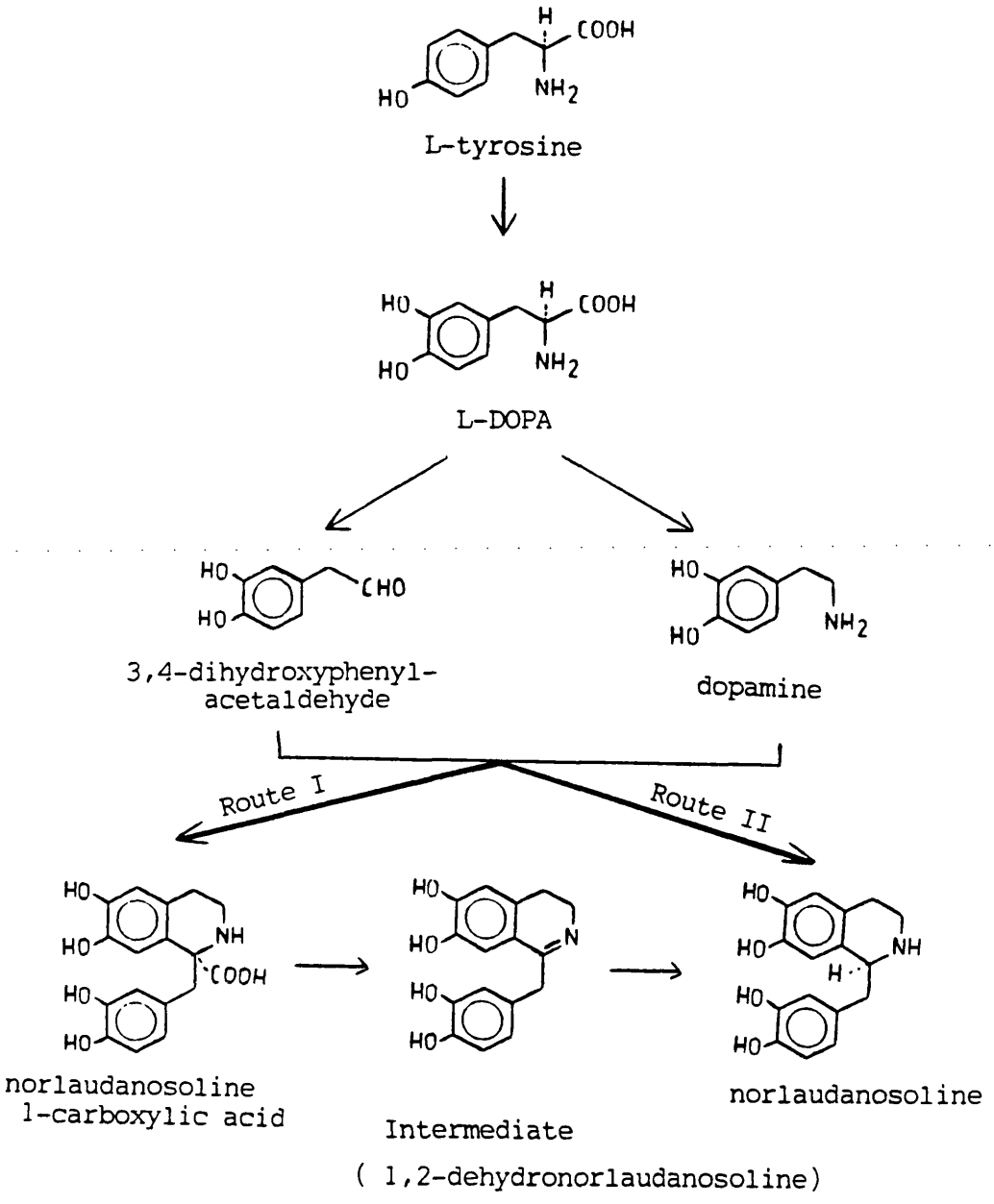
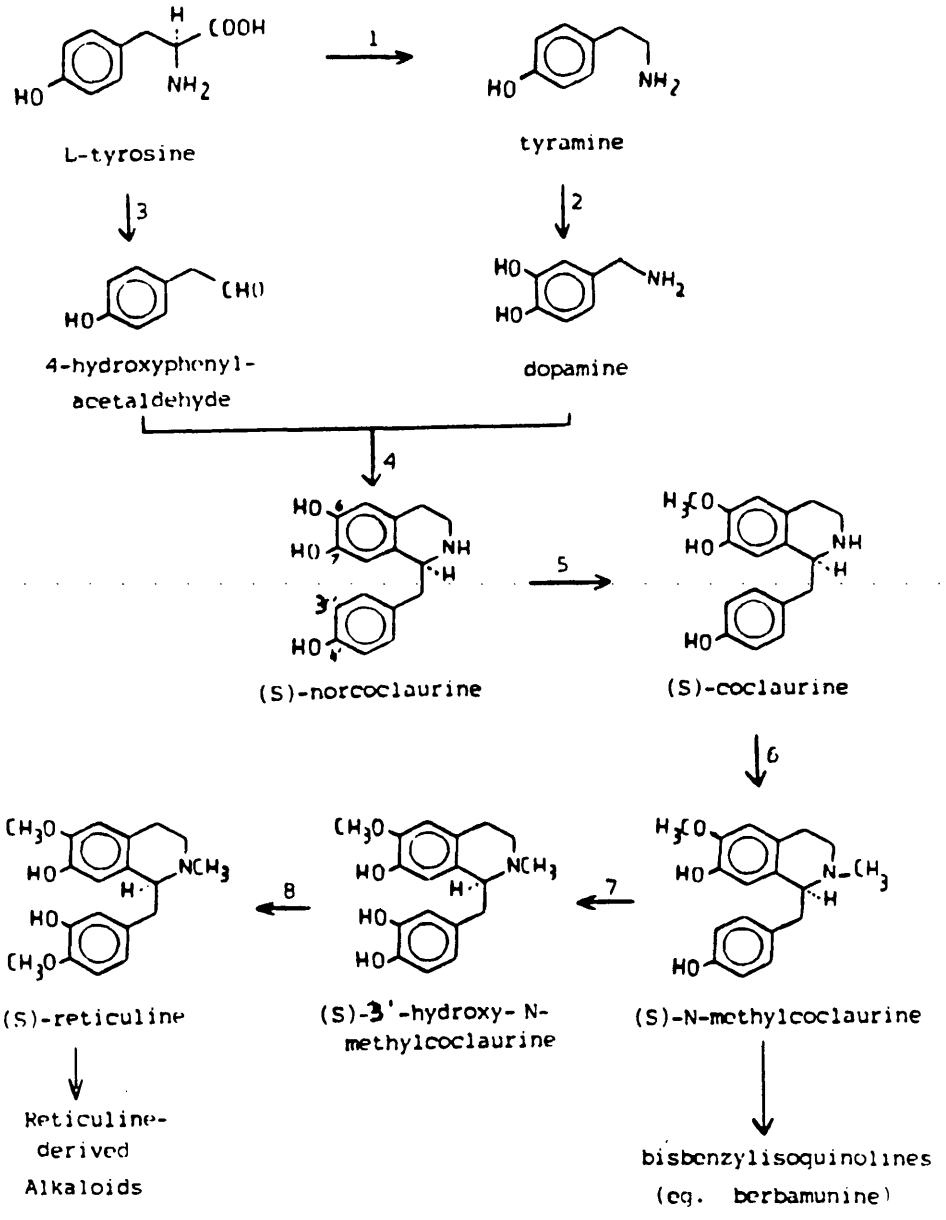


Figure 1.4 Proposed biosynthetic pathways from L-tyrosine to norlaudanosoline



enzyme involved:

1. Tyrosine decarboxylase
2. Phenolase
3. Amineoxidase / transaminase
4. Norcoclaurine synthase
5. Norcoclaurine - 6 - O -methyltransferase
6. Coclaurine-N-methyltransferase
7. N-methylcoclaurine-3'-hydroxylase (a phenolase type)
8. (S)-3'-hydroxy-N-methylcoclaurine-4'-O-methyltransferase

Figure 1.5 Revised biosynthetic pathway from L-tyrosine to (S)-reticuline

1.6.2. The revisions to the pathways of benzylisoquinoline biosynthesis resulting from the isolation of the enzymes required for the biosynthetic sequences

1.6.2.1. Identification of first alkaloid in the biosynthetic sequence

Recently, enzymes involved in the early steps of alkaloid biosynthesis have been investigated by Rueffer and Zenk (1987). These enzymes included tyrosine/DOPA decarboxylase, phenolase, transaminase, p-hydroxyphenylpyruvate decarboxylase, amineoxidase and ATP: L-methionine-S-adenosyltransferase. The activities of the enzymes investigated suggested that the most likely routes from tyrosine to the benzylisoquinoline alkaloids were the conversion of tyrosine to tyramine by tyrosine decarboxylase followed by the conversion of tyramine to dopamine via hydroxylation. L-Tyrosine can also be converted to 4-hydroxyphenylacetaldehyde by an amineoxidase or transaminase (Fig. 1.5). The activity of the various enzymes involved in tyrosine metabolism is likely to vary not only with the plant species used but also with the developmental stage of the plant. Similarly, differences between activities in the plant and in cell cultures might also be expected. These considerations no doubt account for the variations observed in the labelling of the benzyl and isoquinoline moieties of these alkaloids with radiolabelled tyrosine (Spenser 1968).

The recent experiments of Stadler et al (1987) with leaves of *Annona reticulata* indicated that L-tyrosine was principally incorporated into (S)-coclaurine and (S)-reticuline, and this therefore casts doubt on the role of norlaudanosoline as an intermediate in benzylisoquinoline biosynthesis. Furthermore, Stadler et al (1987) and Loeffler

et al (1987) clearly showed that only (S)-coclaurine and not the (R)-isomer was incorporated into protoberberines, benzophenanthridines and the morphinan thebaine.

More recent work on the enzyme norlaudanosoline synthase which catalysed the condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde revealed that this enzyme would also catalyse the condensation of dopamine and 4-hydroxyphenylacetaldehyde with greater efficiency to give (S)-norcoclaurine (Rueffer et al 1981, Rueffer and Zenk 1987), which is then methylated to give (S)-coclaurine and by a series of further methylations and hydroxylation to (S)-reticuline (Stadler and Zenk 1990). (S)-Coclaurine and (S)-N-methylcoclaurine were also found incorporated into the bisbenzylisoquinolines (eg. berbamunine) (Stadler et al 1988). This revised pathway from L-tyrosine to (S)-reticuline and the enzymes involved are given in Fig. 1.5 .

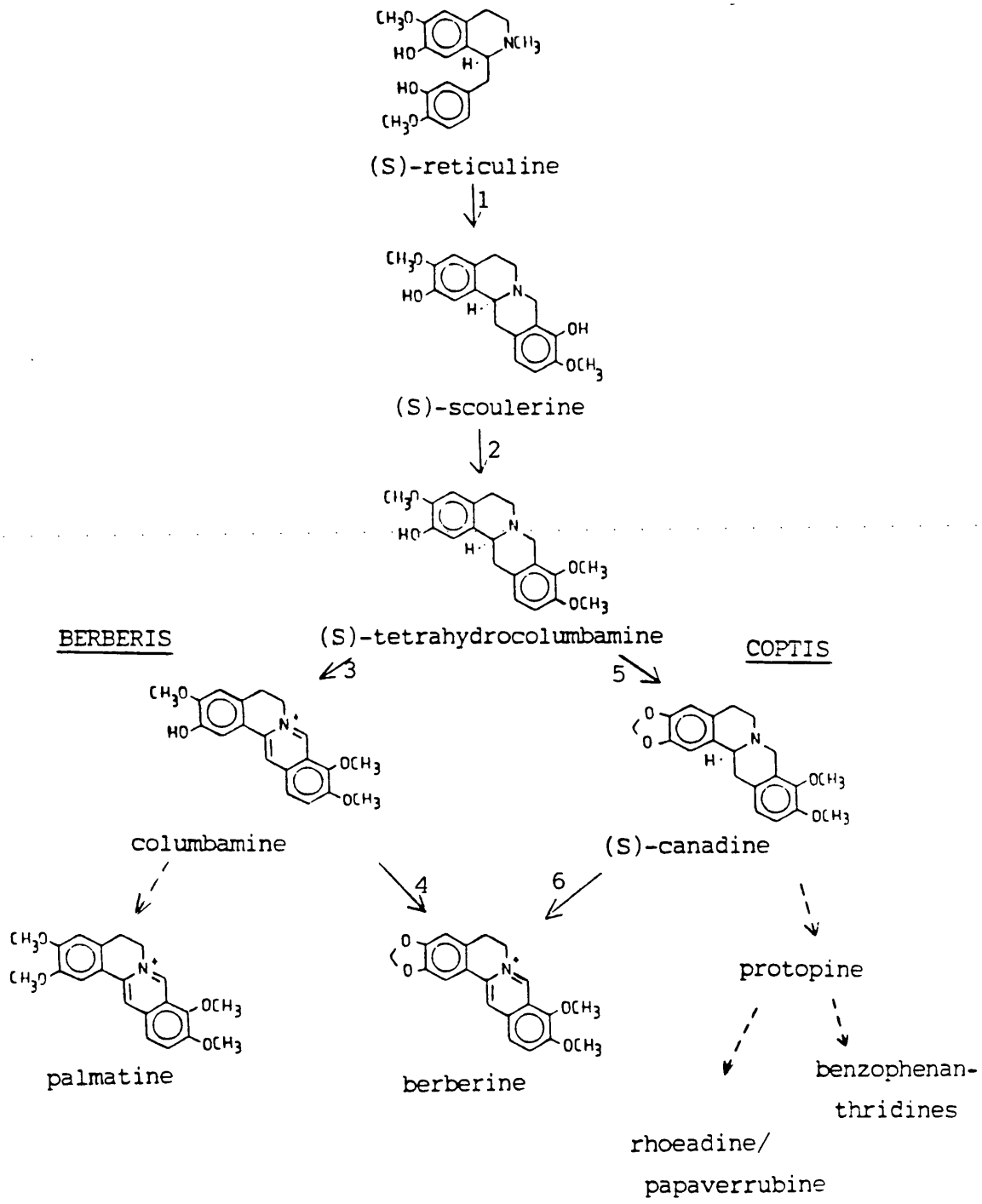
1.6.2.2. Role of reticuline

(S)-Reticuline is an intermediate in the biosynthetic pathway leading to the formation of many different alkaloid types found in the plant. In *Papaver* species, (S)-reticuline is required for the formation of the benzophenanthridines, protoberberines, phthalideisoquinolines, rhoeadine/papaverrubine and aporphines. The biosynthesis of the morphinans, in contrast, proceeds via (R)-reticuline, and 1,2-dehydroreticuline was shown to act as an intermediate between the two isomers of reticuline (Battersby et al 1965b, Borkowski et al 1978) (Section 1.6.10.1). Zenk (1985) showed the presence of the enzyme that converted (S)-reticuline to 1,2-dehydroreticuline in the biosynthetic pathways leading to the formation of morphinan alkaloids. The general biosynthetic pathways to the mentioned alkaloid types derived from (S)-reticuline were previously shown in Fig. 1.3.

1.6.3. Route to berberine

Berberine is used as an antibacterial agent, and its biosynthesis is known in detail. All the enzymes of this biosynthetic pathway have been isolated and characterised.

(S)-Reticuline is converted to (S)-scoulerine by the berberine-bridge enzyme (Steffens et al 1984, 1985). Muemmler et al (1985) showed the presence of the enzyme called (S)-adenosyl-L-methionine: (S)-scoulerine-9-O-methyltransferase which catalysed the formation of (S)-tetrahydrocolumbamine from (S)-scoulerine (Fig. 1.6). The terminal steps involved in the biosynthesis of berberine were found to be different at the biochemical level in the *Berberis* and *Coptis* species (Galneder et al 1988). In *Berberis* cell cultures, the enzyme (S)-tetrahydroprotoberberine oxidase (STOX) converts (S)-tetrahydrocolumbamine to columbamine which in turn serves as a substrate for berberine synthase to form berberine (Amann et al 1984, and Rueffer and Zenk 1985). Columbamine was also found to be converted to palmatine by a specific methyltransferase in *Berberis* cell cultures (Rueffer and Zenk 1986). In contrast, the conversion of (S)-tetrahydrocolumbamine to (S)-canadine (a tetrahydroberberine) was catalysed by (S)-canadine synthase in *Coptis japonica* (Galneder et al 1988). The formation of berberine from (S)-canadine was completed by an oxidase enzyme (Galneder et al 1988, and Okada et al 1988). (S)-Canadine was also found to act as an intermediate for the biosynthesis of the protopine, benzophenanthridine and rhoeadine alkaloids (Cordell 1981). The *Berberis* oxidase enzyme can also dehydrogenate (S)-norreticuline, (S)-scoulerine and (S)-canadine ; while the *Coptis* enzyme only catalyses the oxidation of (S)-canadine in the biosynthetic pathways to berberine (Galneder et al 1988).



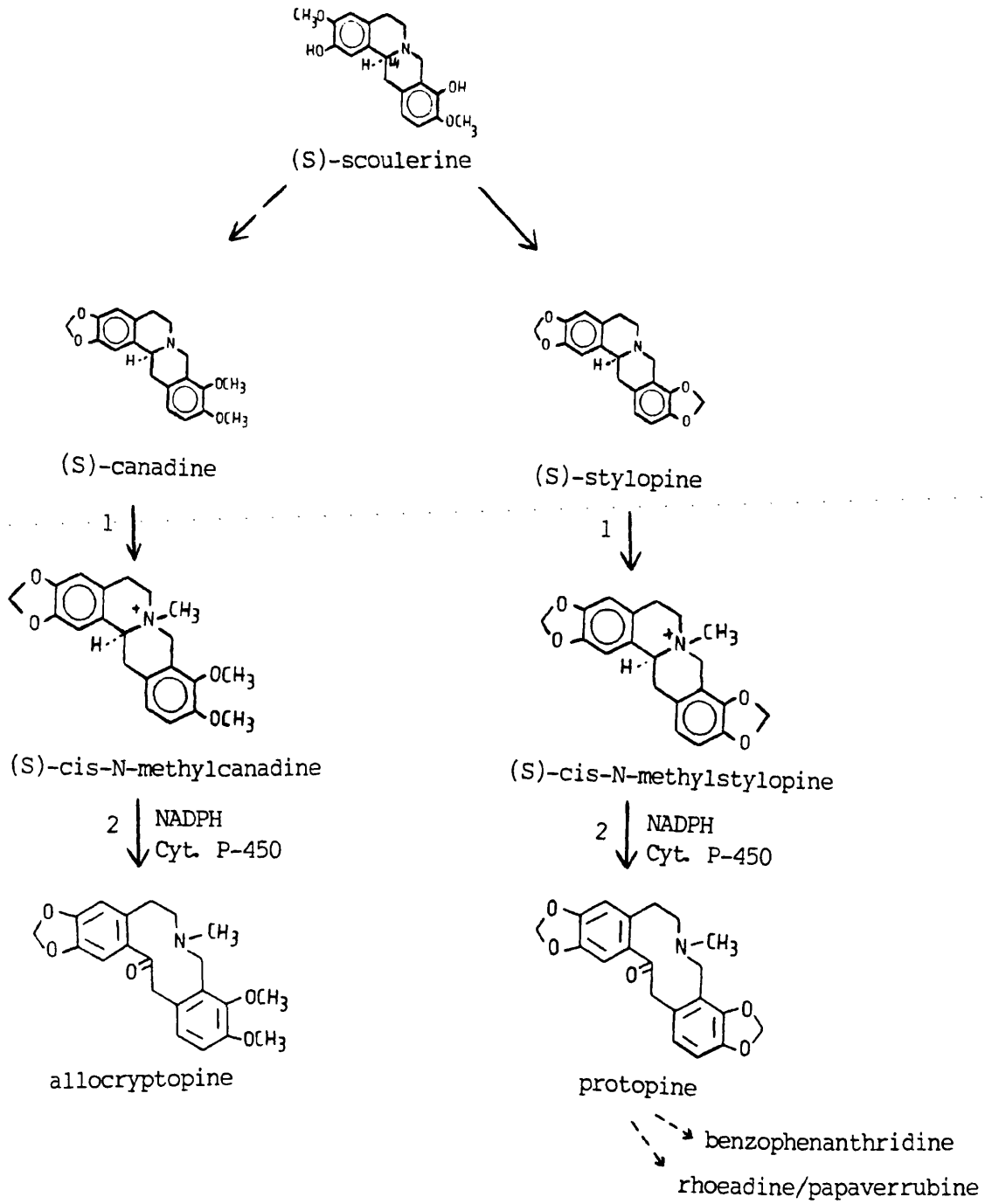
Enzyme involved:

1. Berberine bridge enzyme
2. (S)-Scoulerine-9-O-methyltransferase
3. (S)-Tetrahydroprotoberberine oxidase
4. Berberine synthase
5. (S)-Canadine synthase
6. (S)-Canadine oxidase

Figure 1.6 Biosynthetic pathways from (S)-reticuline to berberine

1.6.4. Route to protopine alkaloids

The protopines are characterised by the presence of a ketone group at position C-14 of the molecules. As previously shown in Fig. 1.6 , (S)-tetrahydroprotoberberine alkaloids can either be transformed into the protoberberines by enzymatic oxidation of ring C (Amann et al 1988), or N-methylated and serve as precursors of the protopine, rhoeadine/papaverrubine and benzophenanthridine alkaloids, etc. (Cordell 1981). Radiolabelled tetrahydroprotoberberines and their N-methyl derivatives were good precursors of protopines in feeding experiment with differentiated plants (Batterby et al 1975a) and with callus cultures (Takao et al 1983).The hydrogen atom at C-14 of a tetrahydroprotoberberine such as stylopine was completely lost during the oxygenation process (Battersby et al 1975a). Bauer and Zenk (1989) isolated the microsomal bound enzymes which converted (S)-scoulerine to (S)- stylopine by the formation of two methylenedioxy-bridges (Fig. 1.7). N- Methylation of (S)-tetrahydroprotoberberines such as (S)-canadine and (S)-stylopine is the first committed step in the biosynthesis of the protopines, benzophenanthridines, tetrahydrobenzazepines (rhoeadine) and spirobenzylisoquinolines, etc. (Cordell 1981). The N- methylation of tetrahydroprotoberberines occurs prior to oxygenation was shown by in-vivo experiment (Takao et al 1983) and enzyme preparation (Rueffer and Zenk 1986b). This N- methyltransferase, named S-adenosyl-L-methionine:(S)-tetrahydroberberine-cis-N-methyltransferase, has been isolated from cell cultures of a variety of plants containing benzylisoquinolines (Rueffer et al 1990). The N-methylated tetrahydroprotoberberines are not aromatised in ring C by the specific oxidase enzyme utilised for the formation of berberine from tetrahydroberberine (Amann et al 1988). The route to protopine therefore occurs as a result of hydroxylation at position 14 of the N-



Enzyme involved:

1. S-Adenosyl-L-methionine: (S)-tetrahydroprotoberberine-cis-N-methyltransferase
2. Microsomal cytochrome P-450

Figure 1.7 Biosynthesis of the protopines from (S)-tetrahydroprotoberberines

methyltetrahydroprotoberberine by a microsomal cytochrome P_{450} system (Rueffer and Zenk 1987a).

1.6.5. Route to the benzophenanthridine alkaloids

Robinson (1955) suggested the involvement of the protoberberines in the biosynthesis of benzophenanthridine alkaloids. This hypothesis was supported by the results from feeding experiments, which showed the incorporation of scoulerine and stylopine into chelidonine and sanguinarine (Leete and Murrill 1964, and Battersby et al 1965c, 1967b, 1975a,d). The benzophenanthridine alkaloids were shown to arise from protoberberine precursors by cleavage of the N-C^6 bond and a new bond between C-6 and C-13 was formed (Mc Donald 1981).

Takao et al (1983) showed the direct involvement of protopines in the biosynthesis of the benzophenanthridine nucleus. Tanahashi and Zenk (1990) succeeded in demonstrating a cytochrome P_{450} linked microsomal enzyme which hydroxylates protopine at the C-6 position, thus provoking the resultant 6-hydroxyprotopine molecule to rearrange into the benzophenanthridine skeleton (Fig. 1.8). Schumacher and Zenk (1988) characterised a dihydrobenzophenanthridine oxidase from cell suspension cultures of *Eschscholtzia californica*. This enzyme converted dihydrosanguinarine, dihydrochelirubine and dihydromaccarpine to sanguinarine, chelirubine and maccarpine, respectively. This biosynthetic route is based on the fact that the acid salts of protopines have the structure as indicated in Fig. 1.8 (Sharma 1972). Hence the route to the benzophenanthridine alkaloids is now clearly defined.

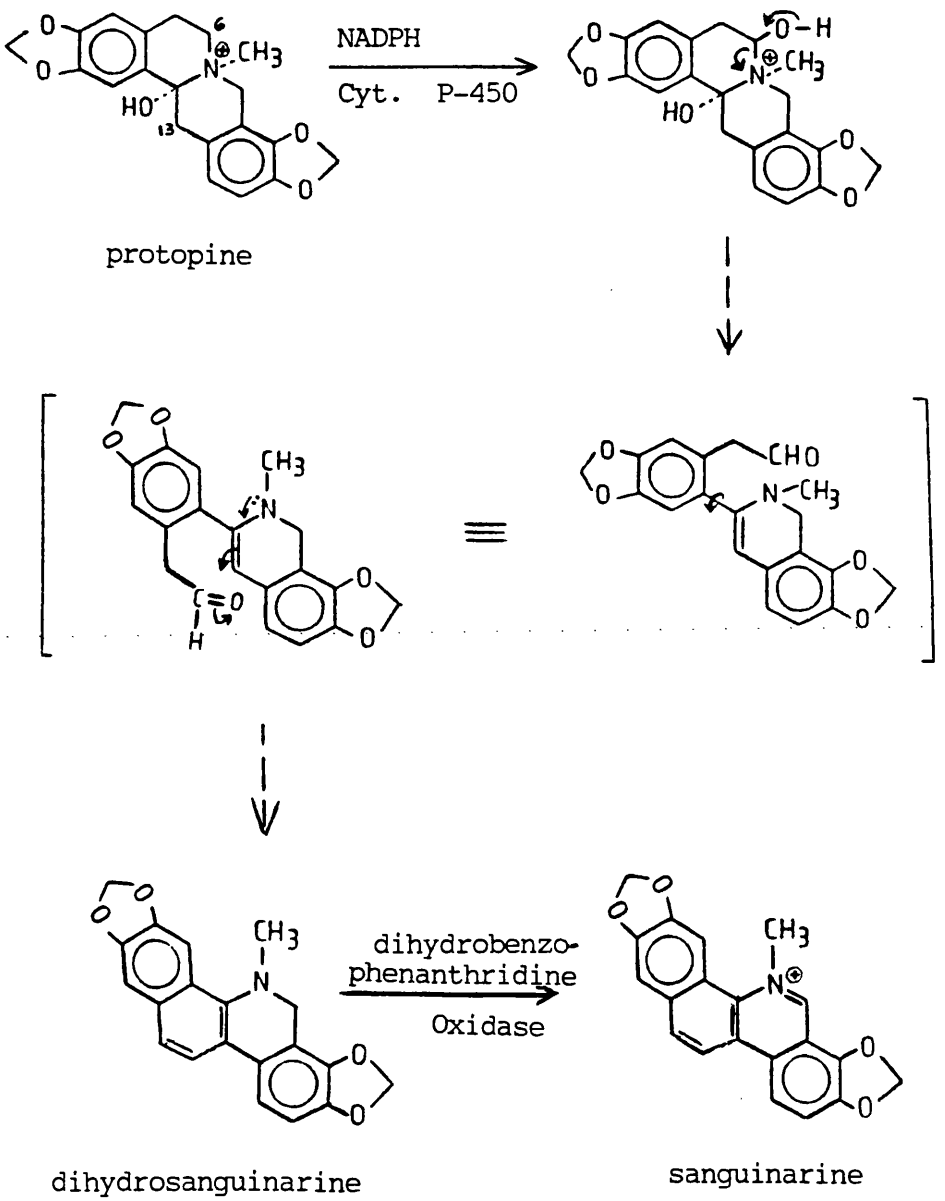


Figure 1.8 Biosynthesis of the benzophenanthridines from protopine

1.6.6. Route to the rhoeadine and papaverrubine alkaloids

Rhoeadine present in *P. rhoeas* was shown to arise by extensive modification of scoulerine to form stylopine on the pathway to rhoeadine (Battersby and Staunton 1974b). Radiolabelled protopine was found incorporated into rhoeadine in *P. rhoeas* (Shamma and Moniot 1978, Tani and Tagahara 1977). Alpinigenine isolated from *P. bracteatum* was shown to be biosynthesised via tetrahydroprotoberberine (tetrahydropalmatine and its methiodide) and protopine-type intermediates (Roensch 1977) (Fig. 1.9). The papaverrubine-type alkaloid was derived from (-)hydrastine by a route similar to that used in the synthesis of (+)rhoeadine (Hohlbrugger and Klotzer 1974). These biosynthetic routes have yet to be clearly defined by the isolation of the appropriate enzymes.

1.6.7. Route to the aporphine alkaloids

The characteristic feature of the aporphine alkaloids is the presence of a single bond between two aromatic rings. Herbert (1980) suggested that this bond is formed by the coupling of two phenolic radicals. Coupling of the two aromatic nuclei then occurs at position ortho or para to each of the hydroxy groups.

In *P. somniferum*, isoboldine was found to be derived from (S)-reticuline (Brochmann-Hanssen et al 1971, 1973b). Isothebaine was formed from (+)-orientaline (Battersby et al 1965). (-) Orientalinone which is a natural constituent of *P. orientale* was also found to be involved in the biosynthesis of isothebaine (Brochmann-Hanssen et al 1972 and Bhakuni et al 1980) (Fig. 1.10). Rueffer (1990) showed the membrane preparations from *Berberis stolonifera* cell cultures could transform (S)-reticuline to corytuberine. The biosynthetic

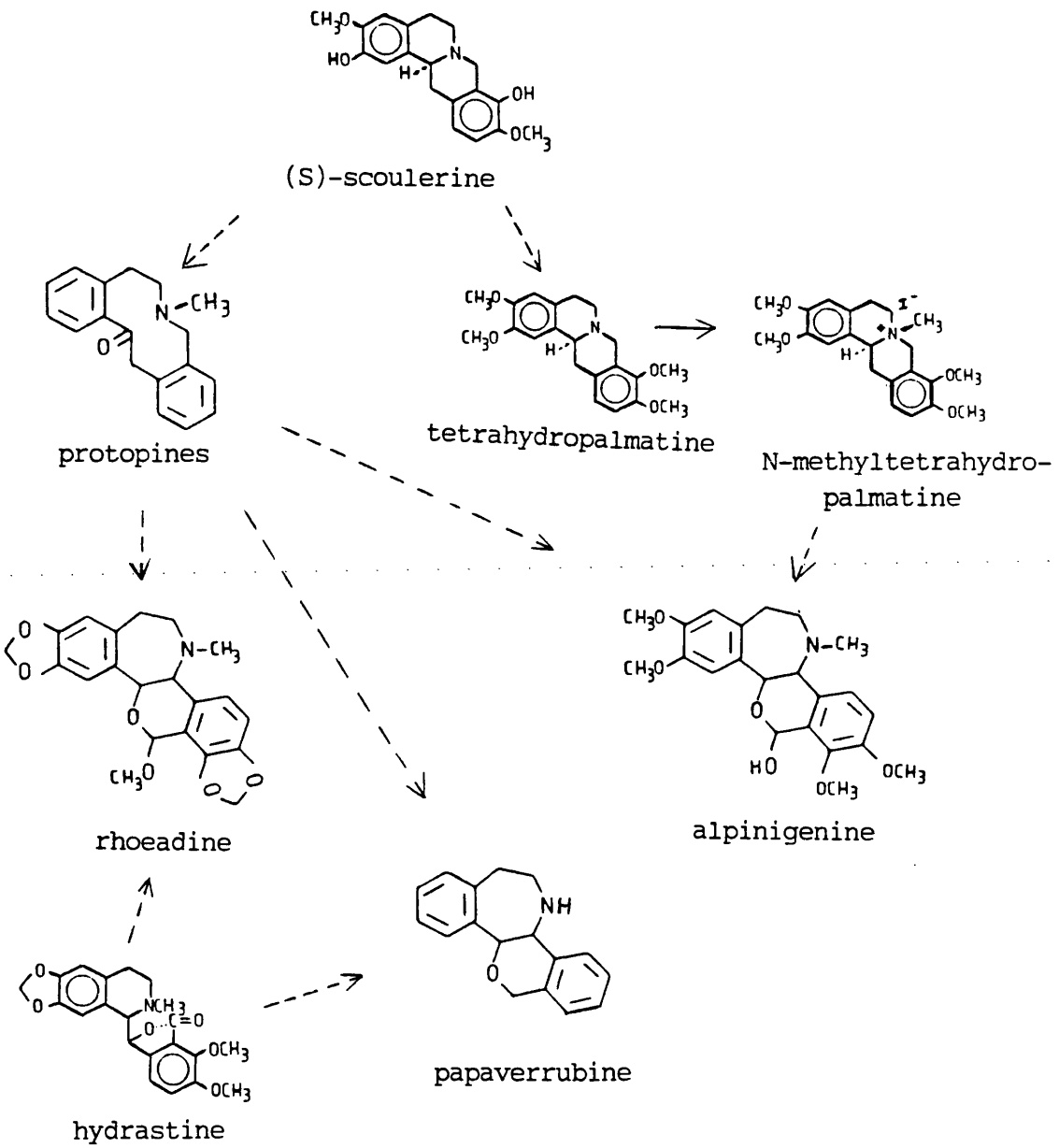


Figure 1.9 Biosynthetic pathways from (S)-scoulerine to the rhoeadine/papaverrubine alkaloids

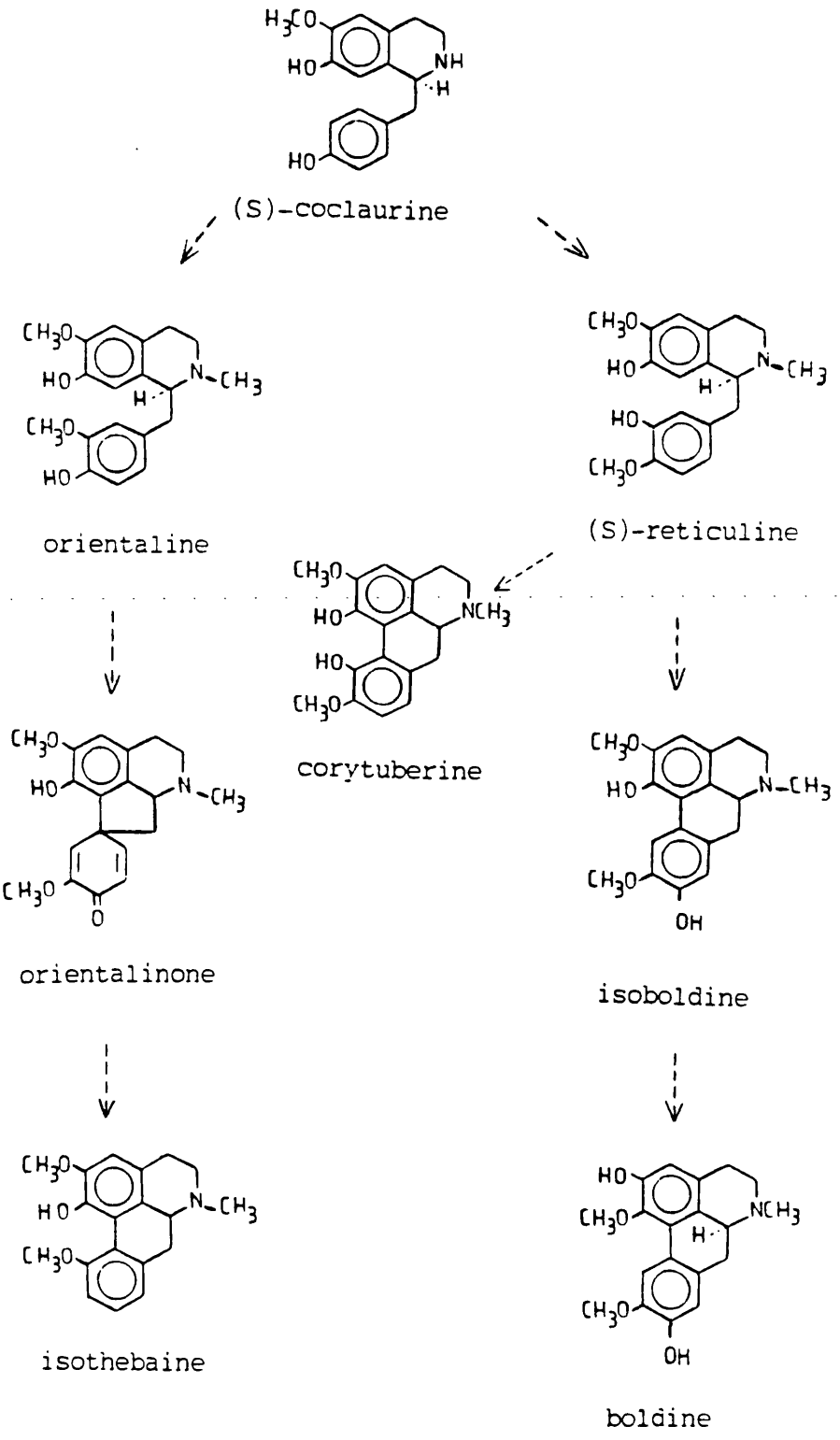


Figure 1.10 Biosynthetic pathways from (S)-coclaurine to the aporphine alkaloids

sequences to the aporphine alkaloids have yet to be clearly defined by the isolation of the appropriate enzymes .

1.6.8. Route to the phthalideisoquinoline alkaloids

Phthalideisoquinolines are mainly found in *P. somniferum*. Robinson (1955) suggested that the phthalideisoquinoline skeleton arises from tetrahydroprotoberberine by oxidation process, and the carbonyl group of noscapine is probably derived from the S-methyl group of methionine as are the carbons of the methylenedioxy group in ring A, the N-methyl of ring B and the two O-methyl groups of ring D (Fig. 1.11).

Battersby et al (1968b) confirmed Robinson's hypothesis by using 2-^{14}C-(±)-tyrosine in feeding experiment, and found the radioactivity equally occurring at C-1 and C-3 of noscapine. 1-^{14}C-(±)-Norlaudanosoline was also incorporated into noscapine , with the label found at C-1. Both the (+) and (-) isomers of reticuline were good precursors for the biosynthesis of noscapine, but the (-) enantiomer was less efficient than the (+) form which corresponds to the absolute stereochemistry of noscapine.

The incorporation of the (-) isomer was proposed to proceed via 1,2-dehydroreticuline. The formation of ring C (berberine bridge) was shown to occur via oxidation cyclisation of an N-methyl group (Barton et al 1963a), which is derived from the S-methyl group of methionine (Battersby et al 1963). The phthalideisoquinoline scoulerine was isolated from opium (Brochmann-Hanssen and Nielsen 1966), and its (-) isomer which corresponds in absolute configuration to noscapine was found to be a much more effective precursor of noscapine than the (+) form. The enzyme which catalyses the formation of (S)-scoulerine from (S)-reticuline was isolated from *Berberis beaniana* cell suspension cultures, and named the berberine bridge-forming enzyme (Zenk 1985). The

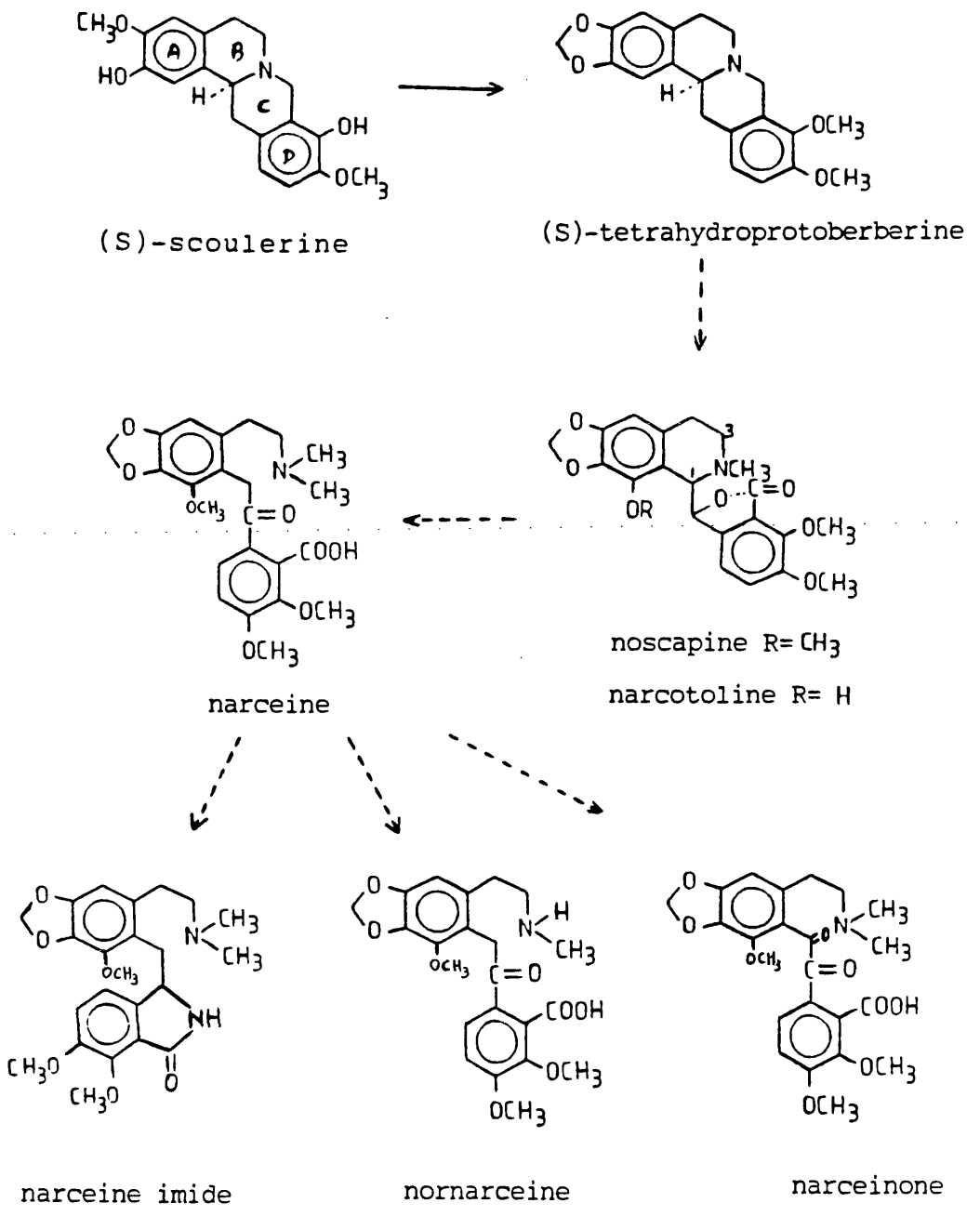


Figure 1.11 Biosynthetic pathway from (S)-scoulerine to the phthalideisoquinoline alkaloids

methylenedioxy group is formed by ring closure of the O-methoxyphenol system to form a tetrahydroprotoberberine system (Barton et al 1963b). Suitable O- and N-methylation of the tetrahydroprotoberberine skeleton followed by several oxidative steps to complete the formation of noscapine (Battersby et al 1968b).

Narcotoline, which is a demethylated form of noscapine at position C-8, was isolated from *P. somniferum* plants (Wrede 1937; Miram and Pfeifer 1958, 1959; Williams and Ellis 1989). Narceine probably formed by ring-cleavage of the noscapine molecule was also isolated from *P. somniferum* (Holubek and Strouf 1965, Khanna and Khanna 1977). The presence of other related alkaloids narceine imide , nornarceine and narceinone were also reported (Kametani et al 1977, Shamma 1972, and Chaudhuri and Thakur 1989, respectively) (Fig. 1.11) .

1.6.9. Route to papaverine (benzylisoquinoline)

Two Ar-C-C- units derivable from tyrosine were found to form the skeleton of papaverine (Battersby and Harper 1962). Radiolabelled tyrosine and norreticuline were incorporated into papaverine (Battersby et al 1962, 1964). Brochmann-Hanssen et al (1975) and Uprety et al (1975) showed tetrahydropapaverine was the main intermediate in the biosynthesis of papaverine in *P. somniferum*, and tetrahydropapaverine was found naturally occurred in this plant. Nororientaline was also biotransformed to papaverine in *P. somniferum* (Brochmann-Hanssen et al 1975). The lack of incorporation of 1,2-dehydronorreticuline into papaverine suggested that the dehydrogenation process does not take place at the norreticuline stage (Brochmann-Hanssen et al 1975). The bioconversion to papaverine was stereospecific, and only the (S)-isomers of norreticuline and tetrahydropapaverine could act as precursors of papaverine formation (Battersby et al 1977). The dehydrogenation of tetrahydropapaverine to form papaverine was catalysed by a

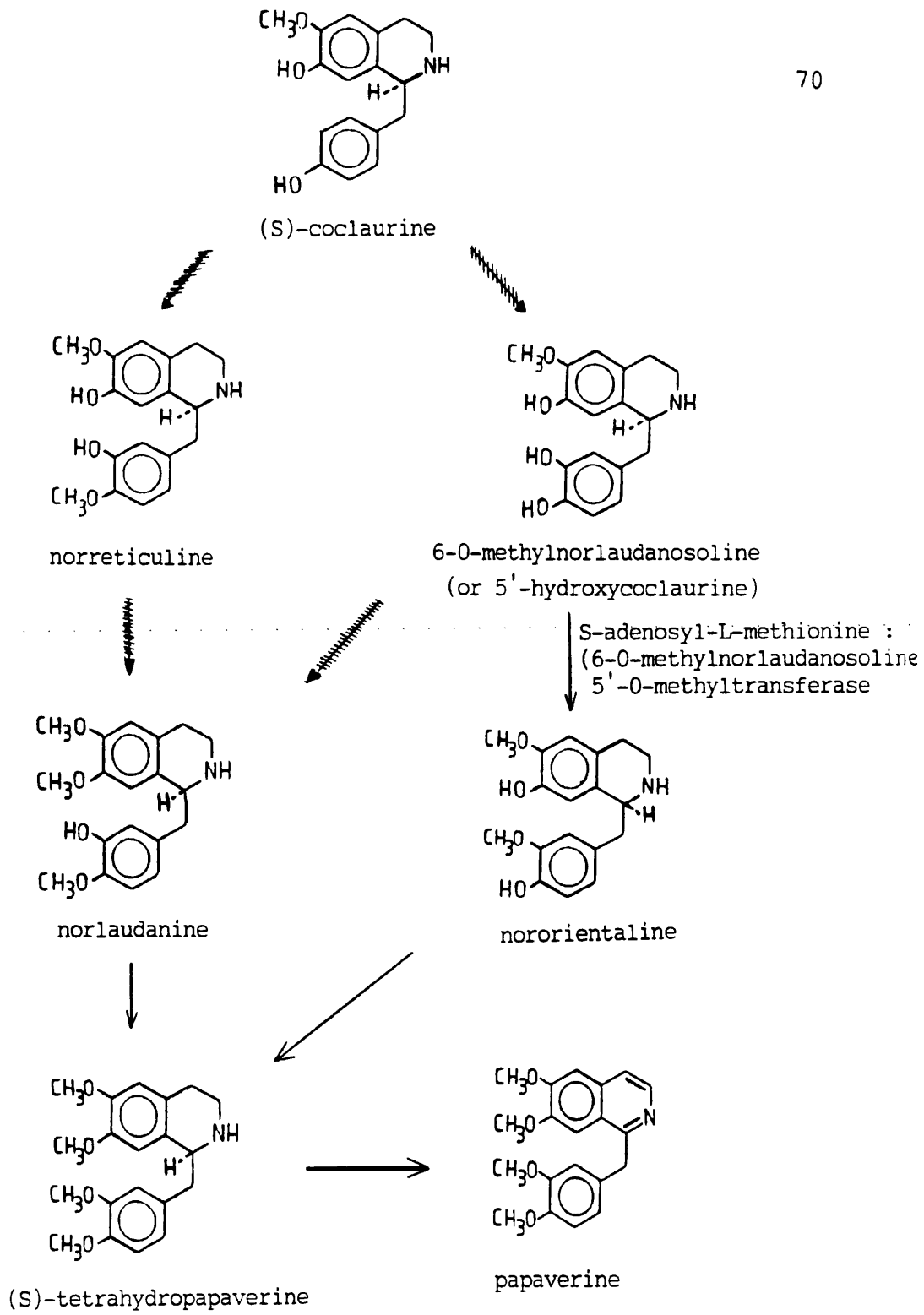


Figure 1.12 Biosynthetic pathways from (S)-coclaurine to papaverine

dehydrogenase which can also catalyse the dehydrogenation of a number of other benzylisoquinolines (Amann et al 1985). However, based on the revised pathway (Fig. 1.5) and the isolation of the enzyme (S)-adenosyl-L-methionine:(6-O-methylnorlaudanosoline)-5-O-methyltransferase which converts 6-O-methylnorlaudanosoline to nororientaline (Rueffer et al 1983c), and the observation that norlaudanine was also incorporated into papaverine, the biosynthesis of papaverine is suggested as shown in Fig. 1.12 .

1.6.10. Routes to the morphinan alkaloids

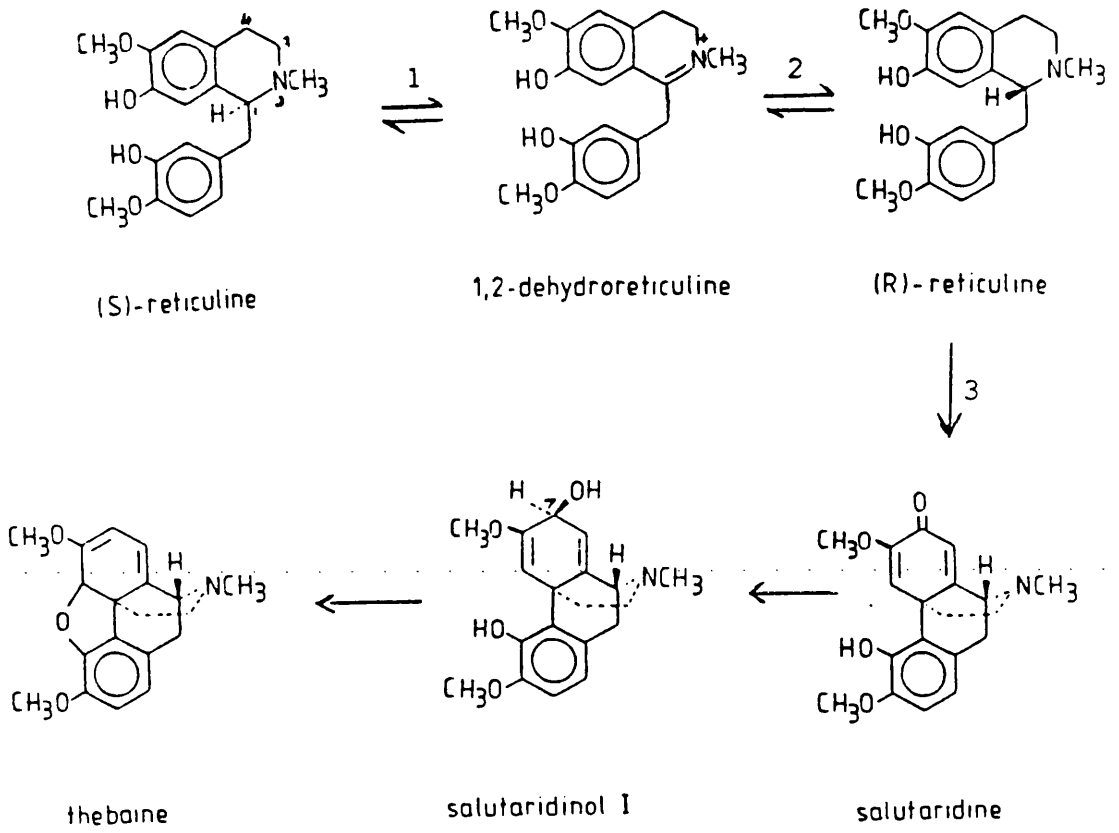
1.6.10.1. Role of (R)-reticuline

The role of reticuline as an intermediary in the biosynthesis of morphinan alkaloids was demonstrated by the isolation of both the (S)- and (R)-isomers of reticuline from opium poppy (Martin et al 1964, Brochmann-Hanssen and Furuya 1964, Brochmann-Hanssen and Nielsen 1965). An excess of the (S)-reticuline over the (R)-isomer was found in opium, in contrast to roughly equal amounts of these two isomers in poppy seedlings (Brochmann-Hanssen and Nielsen 1965, Battersby et al 1965b, Martin et al 1967). Both isomers of reticuline were incorporated into morphine in feeding experiments, but the (R)-form was a slightly more efficient precursor than the (S)-enantiomer (Battersby et al 1965b). Feeding experiments with (S)-reticuline labelled with ^{3}H at C-1 and ^{14}C at the N-methyl group showed the incorporation into thebaine was accompanied by a considerable loss of tritium while ^{14}C was retained (Battersby et al 1965a). 1,2-Dehydroreticuline is an intermediate between (S)- and (R)-reticuline in the biosynthetic pathway to morphinan alkaloids (Battersby et al 1965a, Borkowski et al 1978). The C-1 hydrogen atom of (S)-reticuline is lost while that of (R)-reticuline is retained during the incorporation of both precursors into thebaine in *P. somniferum* plants (Loeffler et

al 1990). Radiolabelled 1,2-dehydroreticuline was detected as a natural compound in *P. somniferum* (Borkowski et al 1978). Using label ^{3}H at C-1 of the reticuline molecule, Amann et al (1985) isolated the enzyme dehydrogenase, which catalyses the removal of tritium from 1-^{3}H-reticuline, from *Berberis* and *P. somniferum*. The enzyme also catalyses the dehydrogenation of a number of benzylisoquinolines including tetrahydropapaverine. The enzyme was found to be identical to the (S)-tetrahydroprotoberberine oxidase, and is specific for the substrate with (S)-configuration; the (R)-isomer can not be a substrate. 1,2-Dehydroreticuline has recently been found to be converted to (R)-reticuline by a stereospecific $NADPH_2$ -dependent cytosolic enzyme, which was first isolated from seedlings of *P. somniferum* and is also found in *P. bracteatum* but was not found in cell cultures of *Papaver* species (De-Eknamkul and Zenk 1990).

The intramolecular condensation of (R)-reticuline to salutaridine, a morphinandienone, was detected by tracer-feeding studies (Barton et al 1963). The natural occurrence of salutaridine was confirmed by the isolation of this compound from cell-free extract of opium poppy after feeding experiment (Hodges and Rapoport 1982b). The results also showed high yields of [^{3}H]-salutaridine from [3-^{3}H]-reticuline. The biochemical formation of salutaridine from (R)-reticuline is stereospecific, and attempted chemical syntheses resulted in low yields (Szantay et al 1982). The formation of salutaridine from (R)-reticuline in *P. somniferum* was found to be catalysed by a highly selective microsomal bound cytochrome P-450 enzyme (Zenk et al 1989, Gerardy and Zenk 1990).

In an attempt to produce the 4,5 - epoxy bridge from salutaridine, the reduction reaction was carried out in vitro using sodium borohydride; and as predicted, a mixture of two epimers named salutaridinol I and salutaridinol II were formed (Barton et al 1965, 1967). Both epimers were easily converted to thebaine in vitro but only salutaridinol I had



Enzyme involved:

1. Dehydrogenase
2. 1,2-dehydroreticuline reductase
3. Microsomal cytochrome P-450 enzyme

Figure 1.13 Biosynthetic pathway from (S)-reticuline to thebaine

correct stereochemistry at C-7 for the in-vivo conversion to thebaine (Fig. 1.13).

1.6.10.2. Biotransformation of thebaine to morphine

The biotransformation of thebaine to morphine requires the O-demethylations of thebaine at positions C-3 and C-6 (Fig. 1.14). Neopinone and codeinone were shown to be good precursors of codeine and morphine (Battersby et al 1967, Blaschke et al 1967 and Parker et al 1972). Neopine, which is a reduced form of neopinone, was isolated from opium (Hohmeyer and Shilling 1947). The structural modifications of codeine (Kirby et al 1972), oripavine (Brochmann-Hanssen and Okamoto 1980) and thebaine (Brochmann-Hanssen and Okamoto 1980, and Brochmann-Hassen and Cheng 1982) did not affect the O-demethylation processes to form morphine. Since codeine and morphine are absent in *P. bracteatum* and *P. orientale* plants, attempt was made to produce morphine by feeding codeinone to the *P. bracteatum* plant (Hodges et al 1977). The results show codeinone was reduced to codeine but the plant could not undergo demethylation of codeine to morphine. In addition, Hodges et al (1977) also demonstrated that the *P. bracteatum* plant could not O-demethylate thebaine to form codeinone. In *P. bracteatum*, the biosynthetic pathway to thebaine is the same as in *P. somniferum* (Hodges et al 1977, Brochmann-Hanssen and Wunderly 1978). *P. bracteatum* and other *Papaver* species which produce thebaine as the only morphinan alkaloid may lack the enzymes necessary for converting thebaine to codeine and then to morphine.

Oripavine was found in some *P. somniferum* strains (Kirby et al 1972, Brochmann-Hanssen and Okamoto 1980, and Neilsen et al 1983). Feeding experiment with *P. somniferum* plants showed the formation of morphine from oripavine, and morphinone was an intermediate in the pathway (Brochmann-Hanssen 1984) (Fig. 1.14). It was suggested that the two pathways leading to morphine may be operating simultaneously,

and the predominance of one pathway to the other may depend on the relative activities of the 3-O- and 6-O-oxidases acting on the thebaine molecule (Brochmann-Hanssen 1984).

Of all the enzymatic steps involved in the biosynthesis of morphine from salutaridine, only the conversion of (-) codeinone to (-) codeine has been characterised. The crude enzyme which reduced (-) codeinone to codeine in cell-free extracts from *P. somniferum* was obtained (Furuya et al 1978, Hodges and Rapoport 1980).

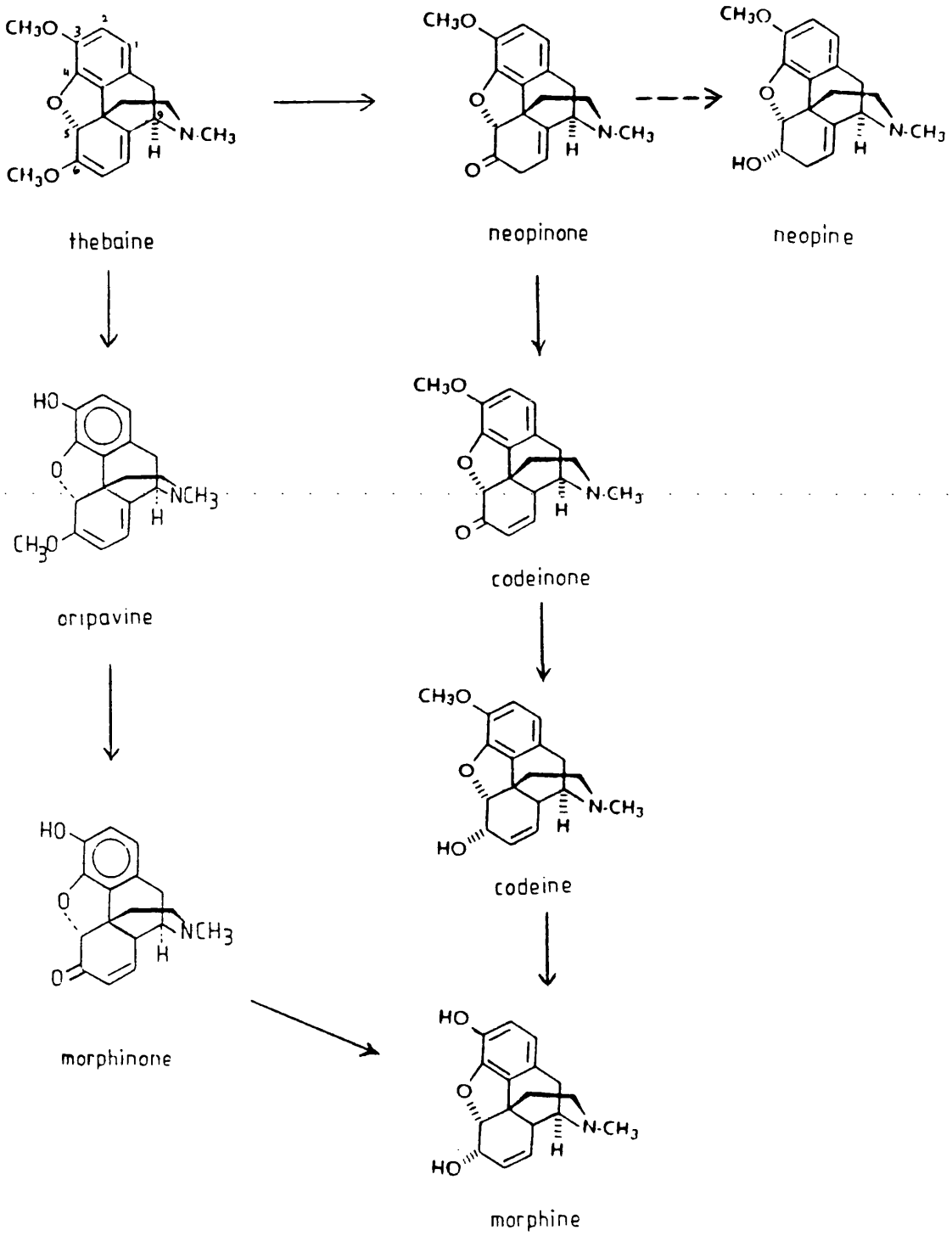


Figure 1.14 Biosynthetic pathways from thebaine to morphine

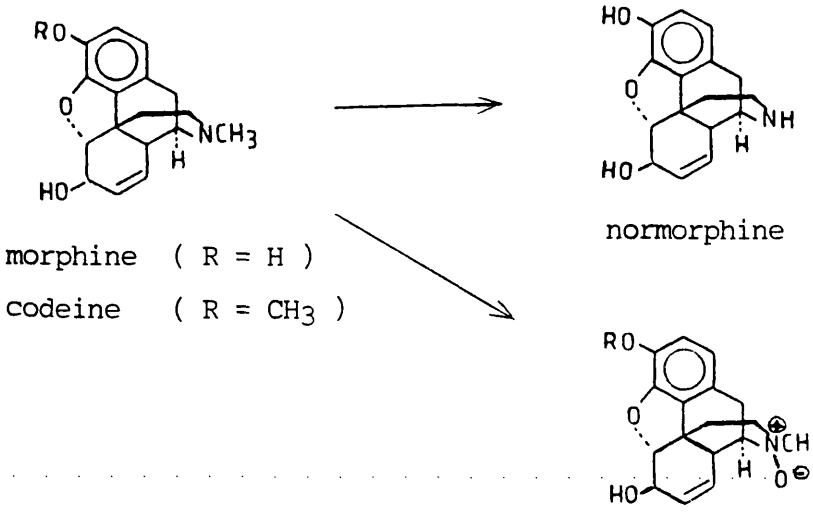
1.7. Metabolism of the Major Alkaloids in *P. somniferum* L.

1.7.1. Metabolism of morphinan alkaloids

Levels of morphinan alkaloids were shown to fluctuate during the day (Pfeiffer and Heydenrich 1962a). Significant variations of morphinan contents at intervals of 1 to 2 hours in the same day were observed (Fairbairn and Wassel 1964a, Fairbairn and Helliwell 1977). Some morphine metabolites described as methanol-soluble, methanol-insoluble, water-soluble or water-insoluble compounds were detected (Fairbairn and Paterson 1966; Fairbairn and El Masry 1967, 1968; Fairbairn and Djote 1970; Fairbairn et al 1974). The bound forms of morphine and codeine were found in the polysaccharide fraction of the capsule (Wold 1978), and of the pericarp and seed (Fairbairn and Steele 1980). These bound forms were suggested to represent transitional forms in the metabolism and translocation of morphine from latex to seed (Fairbairn and Steele 1980). However, only normorphine, N-oxides of the morphinans and N-methylthebaine have been identified as morphinan metabolites.

1.7.1.1. Normorphine

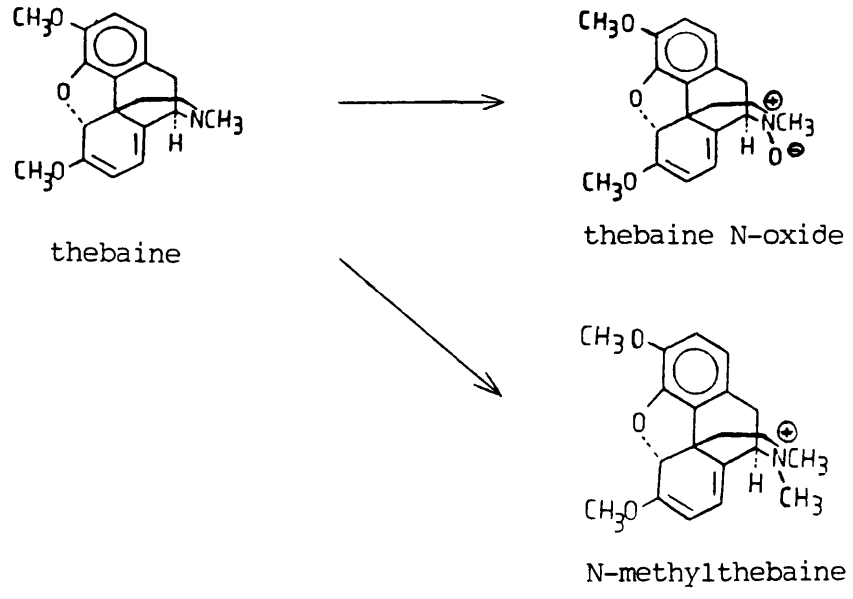
Normorphine was found as a natural compound in opium, and its presence was detected throughout the life cycle of the plant (Miller et al 1973). Normorphine constituted about 5% of the total radioactivity detected when radiolabelled morphine was fed to *P. somniferum* plants. The dying plants, in contrast, had no normorphine although level of morphine in the plant was still high. The N-demethylation of morphine to normorphine was found irreversible, and the morphinan alkaloids were suggested to play an active



morphine (R = H)
codeine (R = CH_3)

normorphine

morphine N-oxide (R = H)
codeine N-oxide (R = CH_3)



thebaine

thebaine N-oxide

N-methylthebaine

Figure 1.15 Morphinan metabolites in Papaver plants

metabolic role, perhaps as specific methylating agents (Miller et al 1973) (Fig. 1.15) .

1.7.1.2. Morphinan N-oxides

Morphine N-oxide was identified in man (Woo et al 1968) and in animals (Ziegler et al 1969, Heimans et al 1971). Phillipson et al (1976) showed the natural occurrence of codeine N-oxide and the two isomers of morphine N-oxide in *P. somniferum* plants. Radioactive morphine N-oxide was also detected in in-vitro experiment with the poppy latex (Fairbairn et al 1978). In additiion, two N-oxides of thebaine were isolated from *P. bracteatum* plants (Phillipson et al 1976). The low yields of the morphinan N-oxides suggested that they do not accummulate but are either transferred into other metabolites or return to the corresponding tertiary bases (Phillipson et al 1976). Morphine N-oxide was suggested to be involved in the N-demethylation of morphine to normorphine (Fairbairn et al 1978) (Fig. 1.15).

1.7.1.3. N-methylthebaine

N-methylthebaine was isolated from the crude extract of *P. cylindricum* (a Turkish *Papaver* species) (Sariyar et al 1990) (Fig. 1.15).

1.7.2. Metabolism of noscapine

The isolation of narceine, narceine imide, nornarceine and narceinone from *P. somniferum* plants (Section 1.6.8) shows that noscapine is further metabolised in the plant. In addition, N-methylnoscapine was recently isolated from *P. cylindricum* (Sariyar et al 1990).

In animals, oral administration of noscapine to male

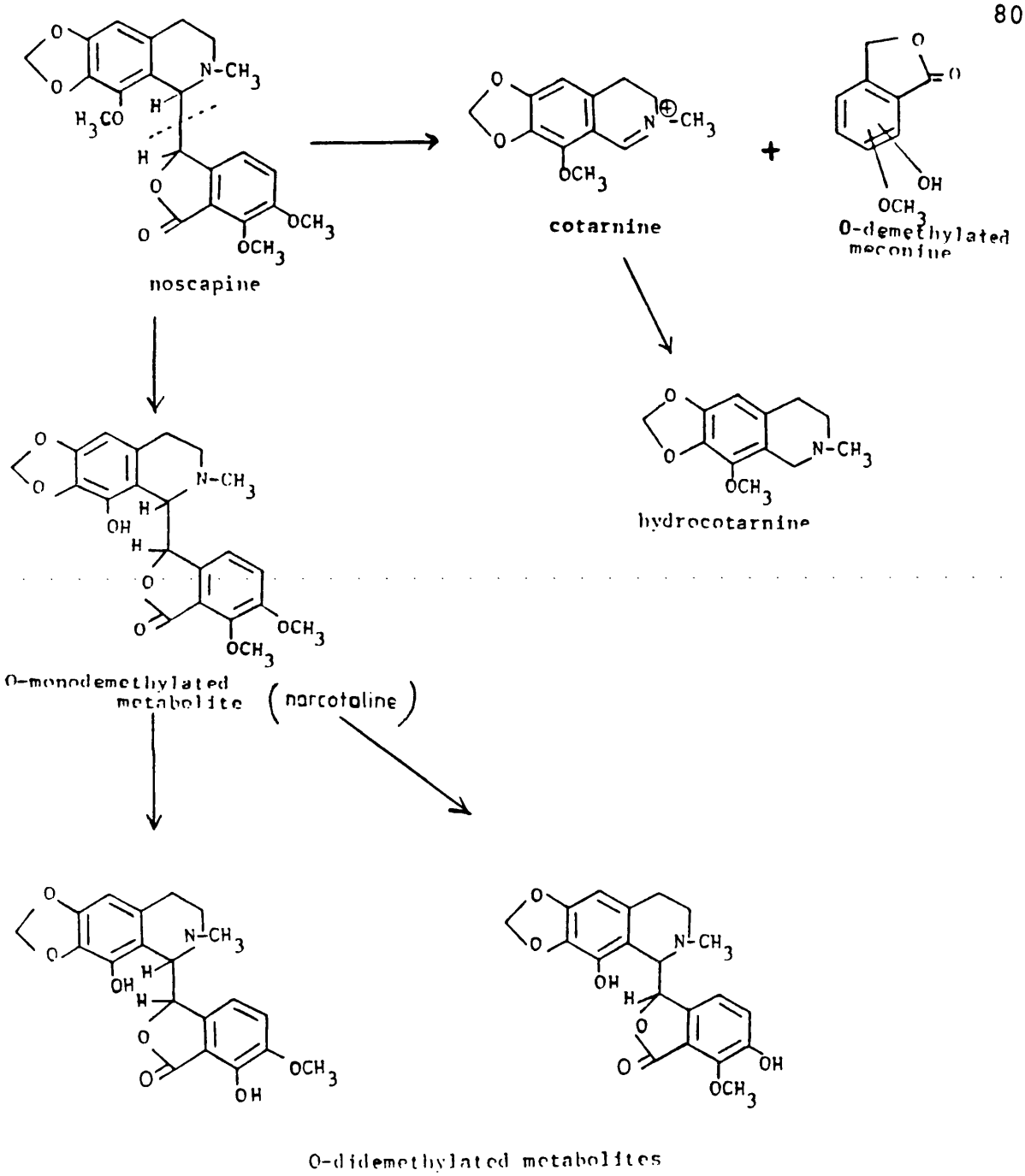


Figure 1.16 Possible metabolites of noscapine in animals

rabbits and rats showed the degradation of noscapine to O-demethylated meconine and cotarnine, and the formation of the demethylated forms of noscapine, including narcotoline, in the 24 hours urine (Al-Yalya and Hassan 1982) (Fig. 1.16).

1.7.3. Metabolism of papaverine

Papaveraldine was isolated from *P. somniferum* plants (Preininger 1986). In other plant species, papaverine was found further monodemethylated at the 6 and 4′ positions to form 6-monodemethylpapaverine and 4′-monodemethylpapaverine. The 6-demethyl derivative of papaverine was found in *Aspergillus alliaceus* and the 4′-demethylpapaverine in *Cunninghamella echinulata* (Rosazza et al 1977). These two derivatives of papaverine were also found in *Silene alba* cell suspension when papaverine hydrochloride was added to the medium (Verdell et al 1985) (Fig. 1.17).

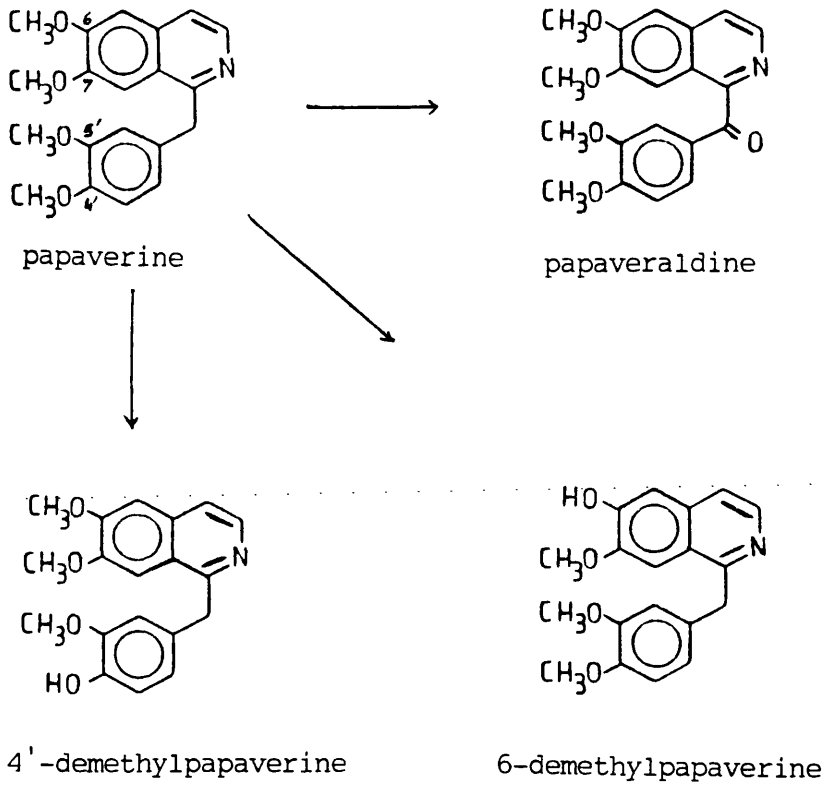


Figure 1.17 Papaverine metabolites in plants

1.8. Alkaloid Localisation and Accumulation in *P. somniferum* L.

1.8.1. Location of alkaloids in plants of the Papaveraceae

Alkaloids of the Papaveraceae are accumulated in particular cells, laticifers (Yoder and Mahlberg 1976, Nessler and Mahlberg 1977). This family is characterised by the presence of laticiferous vessels containing the creamy liquid of cytoplasmic origin called latex. The latex occurs under positive pressure in laticiferous vessels throughout the plant, and is easily obtained by cutting off the capsule and collecting the exuded latex. The latex contains a large number of acidic vacuoles in which significantly high quantities of alkaloids are found.

1.8.2. Latex from *Papaver* plants

Latex, first defined as a suspension of minute organic particles in a liquid matrix (Esau 1964), generally occurs in about 12,500 species covering 20 families (Metcalfe and Chalk 1950).

The presence of laticifers under the phloem tissue in *P. somniferum* plant was first recognised by Trecul (1865) which he termed "lacteal ducts". Alkaloids were first found in latex in 1889 when Clautriau observed the absence of laticifers and alkaloids in seeds, while young seedlings contained both of them. Microscopic study by Thureson-Klein (1970) showed latex is a multitude of particles suspended in a large central vacuole. Poppy latex consists of whole cytoplasm exuded from a series of cells whose adjacent walls

dissolve to form continuous tubes called latex vessels (Nessler and Mahlberg 1977, 1981). Fragments of nuclei, mitochondria, endoplasmic reticulum and spherical bodies were referred to as the 1000 x g vesicles by Dickenson and Fairbairn (1975) and subsequently referred to as vacuoles (Pham and Roberts 1991). Nessler and Mahlberg (1977) investigating the development of alkaloidal vesicles in laticifer cells of *P. somniferum* suggested that laticifer cell initially contained abundant endoplasmic reticulum throughout their dense cytoplasm and that during differentiation the endoplasmic reticulum organised into long folded sheets that were parallel to the longitudinal walls along the periphery of the cell. It was suggested that the latex vesicles appeared to be derived from dilation of the endoplasmic reticulum. Subsequently Griffing and Nessler (1989) has suggested a new model for laticifer vesiculation in which it is suggested that many vesicles arise as fragmentation of the large central vacuole; and this process gives rise to the highly vesiculated cytoplasm observed in the mature latex vessel.

The latex found in many species of the Papaveraceae is a remarkable biological fluid not only because of its organic contents but also because of its unusual storage capacity. The Papaveraceae is characterised by the presence of alkaloids in the articulated laticiferous vessels with some anastomosing, in contrast to the non-anastomosing systems in *Chelidonium* plants (Metcalfe 1966). In mature laticifers, alkaloids were found in a vacuolar population which was different from the vacuolar subpopulation containing the major latex proteins (MLP's) (Griffing and Nessler 1989 , Nessler and Vonder Haar 1990). Laticifers of *P. bracteatum* were found to be similar in structure and distribution to those of *P. somniferum* but they were usually more closely packed and anastomose more frequently (Fairbairn and Williamson 1978b). The appearance of laticifers in developing seedlings was closely paralleled by the development of the

capacity to biosynthesise and store alkaloids (Griffing et al 1989).

1.8.3. Metabolic activity of isolated *Papaver* latex

Meissner and Mothes (1964) and Meissner (1966 a,b) showed the presence of ribosomes and a high rate of gaseous exchange not due to microorganisms in the expelled latex of 29 plant species. Fairbairn et al (1974) demonstrated that stem latex was usually more biosynthetically efficent than capsule latex, and that the variation in the biosynthetic capacity of isolated latex could be due to upsurges of latex from the stem into capsule depending on climatic conditions.

Some enzymes of the tricarboxylic, glycolysis cycles; three enzymes associated with lysosomes, glyoxysomes and peroxisomes; and some enzymes involved in the early stages of benzylisoquinoline biosynthesis were found in *P. somniferum* (Roberts and Antoun 1978 and references therein). Alkaloids were mainly found in the 1000 x g vacuoles of the *P. somniferum* latex (Fairbairn and Steele 1981), together with most of the catecholase and dopamine found in the latex (Roberts et al 1983).

It was found that most biosynthesis occurs in cells surrounding laticifers that serve as storage sites (Bohm and Franke 1982, Franke and Bohm 1982). However, the incorporation of precursors was found to be better with latex isolated for the experiment (Wilson and Coscia 1975).

1.8.4. Mechanism of alkaloid accumulation in *P. somniferum* L.

Papaver alkaloids accumulate in the latex vacuoles which remained acidic despite their capacity of accumulating large quantities of alkaloids (Homeyer and Roberts 1984a). The translocation of alkaloids across the vacuole tonoplast

with the build up of a large concentration gradient across the membrane remains open to hypothesis. The occurrence of a specific, active transport system has been suggested for alkaloid sequestration in *Lupinus* epidermal cells (Mende and Wink 1987) and in *Catharanthus roseus* (Deus-Neumann and Zenk 1984, 1986). Other studies with *Macleaya* protoplasts and *Catharanthus* protoplasts and vacuoles have suggested simple diffusion across the vacuole tonoplast with ion-trapping responsible for the accumulation of large amounts of alkaloids (Muller 1976, Renaudin and Guern 1982, Neumann et al 1983 and Matile 1984). In particular this latter mechanism appears to account for the sequestration of alkaloids in *Chelidonium majus* latex vacuoles (Matile 1984). It has been shown that the uptake of morphinan alkaloids and papaverine in the vacuoles of *P. somniferum* latex did not show an absolute requirement for ATP/Mg^{2+} (Homeyer and Roberts 1984a), as also demonstrated for the rapid accumulation of sanguinarine in the latex of *Chelidonium majus* and of ajmalicine in cell vacuoles of *Catharanthus roseus* (Matile et al 1970, Neumann et al 1983, Renaudin and Guern 1987). As early as 1946 Dawson suggested that alkaloids are trapped as a result of protonation as the organellar membrane is only slightly permeable to the protonated form of the alkaloid.

1.9. Aims of the Present Work

The objectives of the present work were two fold :-

(1) To investigate new varieties of *Papaver somniferum* to identify plants with high yields in noscapine which could be used in commercial breeding programmes.

This part of the project was designed to investigate alkaloid levels in the young, five week old seedlings and to determine whether a correlation existed with the alkaloid levels found in the mature plant. The proposal was to use seedling screening to isolate cultivars high yielding in noscapine. Mature plants grown in the UK were to be investigated to determine optimal harvest time for maximum yield and to determine what losses in alkaloid occur as a result of harvesting the capsule + 8" stem (general commercial practice) rather than the whole plant.

Finally factors which might increase noscapine content or be responsible for the further metabolism of noscapine were to be investigated.

(2) A further investigation of the sequestration of alkaloids in *Papaver somniferum* was to be made in order to define more clearly the mechanisms of movements of alkaloids across the latex vacuole membrane (tonoplast) and their sequestration within the vacuole.

Two theories have been postulated for vacuolar sequestration of alkaloids :

(i) The alkaloids pass through the tonoplast by simple diffusion with entrappment in the acidic vacuolar compartment by protonation and the formation of non-diffusable salts.

(ii) Alkaloid uptake into the vacuole may occur by specific catalysed transport.

Previous studies with *P. somniferum* latex vacuoles had

suggested a third possibility, the rapid and specific transport of morphine into vacuoles not influenced by ATP/Mg^{2+} or by temperature suggesting the existence of a channel protein. This third alternative required a more detailed investigation.

Section 2

MATERIALS AND METHODS

2.1. Plant Materials and Methods for Analysis of Alkaloids

2.1.1. Plant sources

Seed samples from some *P. somniferum* cultivars, coded CV6, CV7, UL9, UL10 and UL11 , were harvested from plants grown in research trials by The Research and Development Department of Glaxo, Australia. They were sown in the School of Pharmacy Medicinal Plant Garden in the Summers of 1987, 1988 and 1989 for quantitative analysis of their alkaloid contents.

2.1.2. Cultivating and harvesting plants

2.1.2.1. Young seedlings

Seed samples were sown in half pans under similar conditions to those used for the plants grown to maturity in seed pans (6" x 9" size) and provided with fertilisers. Harvesting was done by taking the whole seedlings, each with 4-5 leaves. The fresh seedlings were washed and dried with paper tissues and then weighed before storing in deep freeze in sealed plastic bags.

2.1.2.2. Plants grown to maturity

Seed samples were field grown, and the plants were collected at some or all of the following stages: young plant (20-25 cm high), bud, flower and green capsule. Because of poor weather, plants were unable to reach ripe capsule stage. *P. somniferum* cv. Halle seeds were also grown and the plants were collected for comparison.

P. somniferum cv. CV6 and CV7 seeds were sown in the Spring of 1987. Twelve plants from each of CV6 and CV7 cultivars were collected at young plant, bud, flower and green capsule stages. They were dried at room temperature in the laboratory, weighed and stored in deep freeze, in sealed plastic bags for 2-3 months before analysis.

Seed samples UL9, UL10 and UL11 were sown in the Summer of 1988 and harvested as random samples of twelve plants at all of the mentioned stages of plant development. They were dried in the oven at 40^{0}C, weighed and stored in deep freeze for 2-3 months, in sealed plastic bags.

The UL9, UL10 and UL11 samples sown in the Summer of 1989 were collected at the bud and young capsule stages. The plants were dried, weighed and stored as described for those collected in the Summer of 1988. A random sample of 6 plants at each stage was collected for quantitative analysis.

2.1.3. Isolation of alkaloids

The plant was ground, and one gram of sample was weighed out and exhaustively extracted with 5% acetic acid. After filtration, the filtrate was transferred to a round flask for evaporation of the acid. The residue was dissolved in 2% H_2SO_4 and then basified with NH_4OH to pH 9.5. This solution was placed onto an Extrelut column (Merck, D-6100 Darmstadt). The column was allowed to stand for 45 minutes. The alkaloids were eluted from the column with chloroform : isopropanol (85:15). The eluent was dried with anhydrous Na_2SO_4 if necessary and then vacuumed down to dryness at 40^{0}C . The residue was dissolved in MeOH for HPLC analysis.

2.1.4. Quantitative analysis of alkaloids by HPLC

Alkaloids were quantified by one of the following HPLC systems, using a mixed standard alkaloid solution of known concentrations.

The instrument was either the Altex Model 110A with Altex Module 153 Analytical UV detector or Beckman 110B Solvent Delivery Module with UV detector and NEC Controller Model 424

2.1.4.1. Normal-phase HPLC

The method is given in the " United Nations Document ST/SOA/SER.J/No.33" , as follows:

Chart Recorder: Kipp and Zonen IBD8-1 , 0.5-100 mV
System:
Column: HiChrom S5W 5µ Spherisorb 250 x 4.9 mm
Mobile phase: 30 ml MeOH + 10 ml $CHCl_3$ + 0.1 ml Et_2N, of which 37.5 ml is mixed with 290 ml n-hexane.
Chart speed: 5 mm/min.
Flow rate: 1.0 ml/min.
Wavelength: 280 nm

2.1.4.2. Isolation of minor alkaloid constituents by HPLC

The minor alkaloid constituents were isolated by normal-phase HPLC as given in Section 2.1.4.1 , except that a semiprep column (250 x 8 mm) was used.

2.1.4.3. Reverse-phase HPLC

The method is given in the " European Pharmacopoeia Commission PA/PH/Exp.13/T(84) 2 COM " , as follows:

Chart Recorder: Kipp and Zonen IBD8-1, 0.5-100 mV
System:
Column: Altex 5µ Ultrasphere octyl (octyl silylated silica gel) 250 x 4.6 mm
Mobile phase: 1.0 g sodium heptane sulphonate in

	420 ml distilled water adjusted to pH 3.2 with orthophosphoric acid and add 180 ml acetonitrile
Chart speed:	10 mm/min.
Flow rate:	1.0 ml/min.
Wavelength:	280 nm

2.1.5. Qualitative analysis of alkaloids by TLC

The five main alkaloids of *P. somniferum* L. can be separated by the following TLC solvent systems:

System number	Solvent system	Adsorbent
1	Acetone:toluene:ethanol:0.88 ammonia (40 : 40 : 12: 2.5)	Silica Gel GF_{254}
2	Acetone:toluene:ethanol:0.88 ammonia (80 : 80 : 24 : 2.5)	Silica Gel GF_{254}
3	Benzene : methanol (90 : 10)	Silica Gel GF_{254}
4	Chloroform : isopropanol (80 : 20)	Silica Gel GF_{254}
5	Chloroform : methanol (90 : 10)	Silica Gel GF_{254}
6	Acetone:water:0.88 ammonia (80:15:5)	Silica Gel GF_{254}

The alkaloids were visualised in UV light and with Dragendorff's reagent, which changes colour from yellow to orange in the presence of alkaloid(s).

2.1.6. Authentication of alkaloids

The main alkaloids of the *P. somniferum* L. plants were identified by comparing their relative retention time (R_t - R_0) with those from reference standard alkaloids under the same HPLC conditions. Thin layer chromatograms were run to qualitatively assess the minor components in the alkaloid extracts. The minor compounds, shown by the analytical HPLC, were isolated by the normal-phase HPLC with a semiprep column (Section 2.1.4.2). The pure alkaloid isolated from normal-phase HPLC was then run in the reverse-phase HPLC system for comparison of its relative retention time with those from standard alkaloids under exactly the same HPLC conditions. The alkaloid with similar (R_t - R_0) value was used as the standard for the identification of the unknown by UV, NMR and MS . UV spectra were obtained on a Perkin Elmer 402 Ultraviolet Spectrophotometer.

^{1}H-NMR spectra were recorded at 250 MHz with TMS as internal standard on a Bruker NM 250 instrument.

Mass spectra were obtained on a VG Analytical LTD ZAB - IF Mass Spectrometer .

2.2. Materials and Methods for the Studies of Alkaloidal Storage in Latex Vacuoles

P. somniferum cv. Halle was cultivated at The School of Pharmacy Medicinal Plant Garden. Plants were grown in sequential batches so that flowering occurred from late June until the end of September each year. The latex was collected at weeks 0,1,2 and 3 after petal opening.

2.2.1. Isolation of the 900xg and 1100xg vacuoles from *P. somniferum* cv. Halle latex

The capsules were cut with a sharp knife to allow latex to be collected from both the stem and capsule. Latex was collected in 700 mM mannitol to a final concentration of 50% latex. Vacuoles were sedimented by centrifuging the collected latex at 900xg for 30 minutes and the supernatant of that by centrifugation at 1100xg for 30 minutes. The vacuoles were washed twice, resedimented and finally resuspended in a buffer of 700 mM mannitol, 100 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) adjusted to pH 6.8 with triethanol amine ; this buffer was used in all subsequent experiments.

2.2.2. Determination of vacuolar volumes of the 900xg and 1100xg vacuoles

The determination of vacuolar volume was based on the method described by Rottenberg et al (1972).

The intravacuolar volumes of the 900xg or 1100xg vacuolar samples were determined by incubating a 200 µl aliquot of isolated and resuspended vacuoles with a 30 µl aliquot of ^3H_2O

(185 MBq ml^{-1})and a 10 µl aliquot of a [^{14}COOH]-dextran-carboxyl solution (18.5-74.0 MBq g^{-1}) for 30 minutes at the room temperature (approximately 24.5°C). The sample was centrifuged in an Eppendorf tube, at 900 x g or 1100xg as appropriate, for 15 minutes and a sample of the supernatant counted for ^{3}H and ^{14}C in a Packard Tri-Carb Liquid Scintillation Spectrometer using Aquasol LSC cocktail. The pelleted vacuoles were suspended in 500 µl of 0.1 N HCl in methanol, digested for 30 minutes at the room temperature, centrifuged at 2000xg for 15 minutes and the entire supernatant counted for ^{3}H and ^{14}C. Dividing ^{3}H counts per µl of supernatant into the ^{3}H counts from the pellet provided the total sample void volume trapped in the pellet; dividing the ^{14}C counts per µl of supernatant into the ^{14}C counts from the pellet provided the trapped extravacuolar void volume; and subtracting the trapped extravacuolar void volume from the total sample void volume left the intravacuolar volume. The vacuolar volumes for the 900xg and 1100xg vacuoles were very consistent for all similarly aged latex samples in a given season.

2.2.3. Isolation and quantitation of alkaloids in the latex vacuoles

The 900xg and 1100xg vacuoles were isolated as described in Section 2.2.1 , and plasmolysed in 0.025N HCl/MeOH for 30 minutes. The samples were then centrifuged at 1300xg to remove insoluble material. The supernatant was collected and brought to pH 9.5 with NH_4OH and then pipetted onto an Extrelut column. The column was treated as described in Section 2.1.3 for the isolation of alkaloids. The alkaloids were quantified by normal-phase HPLC (Section 2.1.4.1).

2.2.4. Isolation and quantitation of dopamine in the latex vacuoles

The 900xg and 1100xg vacuoles were isolated from the *P. somniferum* cv. Halle by the method given in Section 2.2.1. They were plasmolysed in 3M Tris pH 8.6 and rapidly pipetted onto an alumina column previously equilibrated with 3M Tris pH 8.6. The column was then washed with 5 volumes of 6 mM Tris pH 8.6, followed by 5 volumes of distilled water. Dopamine was eluted with 5 volumes of 1N HCl. Since dopamine is rapidly oxidised by air, nitrogen gas was used to speed up the procedure.

Dopamine in HCl was quantified by reverse-phase HPLC using the following conditions:

Instrument:	Altex Model 110A with Altex Model 153 Analytical UV detector.
Chart Recorder:	Kipp and Zonen IBD 8-1 , 0.5-100 mV.
Column:	HiChrom, LiChrosorb RP18 , 10 µ, 25 cm x 4.9 mm
Mobile phase:	H_2O : EtOH : HOAc (95:5:1) containing 2 mM n-heptanesulphonate sodium salt pH 4.5
Chart speed:	5 mm/min.
Flow rate:	1 ml/min.
Wavelength:	280 nm

2.2.5. Isolation and quantitation of meconic acid in the latex vacuoles

The 900xg and 1100xg vacuoles isolated by the method described in Section 2.2.1 were plasmolysed in 0.025N HCl/MeOH for 30 minutes. The samples were then centrifuged at 1300xg to remove insoluble material. The supernatant was pipetted onto a cation-exchange column

(Dowex-50W, H^+ form, 8% crosslinking), which was previously equilibrated in 1N HCl overnight and then thoroughly washed with distilled water. Meconic acid was eluted with distilled water (30 ml water for 1 ml of latex buffer) .

Meconic acid in distilled water was quantified by reverse-phase HPLC with modified conditions from those described by Fairbairn and Steele (1981b) as follows:

Instrument:	Altex Model 110A with Altex Model 153 Analytical UV detector.
Chart recorder:	Kipp and Zonen BD40.
Column:	HiChrom , Lichrosorb RP 18 , 10 µ, 25 x 4.9 mm
Mobile phase:	MeOH: 0.05% Atropine sulphate (17:83)
Chart speed:	5 mm/min.
Flow rate:	1.0 ml/min.
Wavelength:	280 nm

2.2.6. Isolation and quantitation of sulphate in the latex vacuoles

The 900xg and 1100xg vacuoles isolated by the method given in Section 2.2.1 was plasmolysed in 0.025 N HCl/MeOH and centrifuged to remove insoluble material.

The 900xg sample was pipetted onto a cation exchange column (Dowex 50W , 8% cross-linking) which was previously equilibrated with 1N HCl and thoroughly washed with distilled water. Distilled water was then used to elute the unbound components from the column (30 ml of water for 1 ml of latex). $Ba(OH)_2$ solution was added to the eluate until the liquid became basic. It was then centrifuged, and the supernatant was pipetted off. The pellet was washed twice with ice water followed by centrifugation each time. 1N HCl was then added to dissolve barium meconate. The adding of 1N

HCl was repeated two or three times followed by centrifugation to remove all traces of barium meconate. The solid was washed with distilled water twice followed by centrifugation to remove traces of HCl . Methanol was then used to dissolve methanol soluble material, followed by chloroform with centrifugation between them. The solid $BaSO_4$ was dried with nitrogen gas and weighed.

The chloride and sulphate in the 1100xg vacuole samples were quantified by Ion Chromatography carried out by Butterworth Laboratories Ltd., Middlesex, UK ; with the following conditions:

Instrument: Dionex Model 16 Ion Chromatograph with an HPIC Anion Separator, ASI ;
Eluent: 0.008 M sodium carbonate .

2.2.7. Isolation, identification and quantitation of malic acid in the latex vacuoles

2.2.7.1. Isolation of malic acid

Malic acid was isolated from the 900xg and 1100xg vacuoles by the same method as for meconic acid (Section 2.2.5) .

2.2.7.2. Identification of malic acid by paper chromatography

Ascending paper chromatography, with solvent system 1-propanol : 1M NH_4OH (70:30) and spraying reagent bromophenol blue in diluted NaOH, was used to visualise the spots (yellow spots).

2.2.7.3. Quantitation of malic acid by enzymatic method

Since malic acid does not absorb UV light, the quantitation of L-malic acid was carried out by the use of malate dehydrogenase and APAD (3-acetylpyridine adenine dinucleotide) given by Irene Witt (1974).

L-Malate dehydrogenase from bovin heart suspended in 3M $(NH_4)_2SO_4$ 0.01M KH_2PO_4 solution pH 7.3, 1 mg protein /ml. Standard 0.2 mM L-malic acid in distilled water was freshly made for reference.

2.2.8. Assay for total phenolics in the latex vacuoles

The measurement of total phenolics was made using vacuole samples (50-500 µl) which had been processed for the determination of sulphate and meconate: the method used was that of Roberts et al (1983). Owing to interference by meconic acid a calibration curve for this acid was constructed and, using the HPLC calibration for meconic acid, an adjustment for its presence in samples was made in calculating the total phenolics present in each sample. Results were estimated on the basis of tannic acid (MW = ca. 1550) for the purpose of estimating millimolar concentrations in the vacuole (tannic acid $E^{1\%/1cm}$ = 1010 ; meconic acid $E^{1\%/1cm}$ = 531).

2.2.9. Isolation and quantitation of cations and inorganic phosphate in the latex vacuoles

2.2.9.1. Isolation of cations and inorganic phosphate

The 900xg and 1100xg vacuoles, isolated by the method given in Section 2.2.1 , were plasmolysed in 20 mM phthalic acid for 30 minutes. The insoluble material was removed from the sample by centrifugation at 1300xg . The supernatant was then prepared for atomic absorption spectroscopy or ultraviolet spectroscopy.

2.2.9.2. Quantitation of cations by atomic absorption spectroscopy

The supernatant was diluted 1 in 20 for atomic absorption spectroscopy, using a Perkin-Elmer 280 Atomic Absorption Spectrophotometer and with the following conditions:

Ca^{2+} : 422.7 nm , split width = 0.7 nm
Mg^{2+} : 285.2 nm , split width = 0.7 nm
K^{+} : 766.5 nm , split width = 2.0 nm
Na^{+} : 589.0 nm , split width = 0.7 nm

2.2.9.3. Quantitation of inorganic phosphate

The method is given by Ohnishi et al (1975), as follows:

Reagents :

Molybdate preservative contained 0.016M tetrasodium - ethylenediaminetetraacetate (EDTA-Na_4) solution. Ammonium molybdate was dissolved in this solution (to produce ammonium

molybdate reagent) to reduce turbidity in the acid phase. Reductant catalyst contained 0.172M hydroxylamine in 0.001M phosphorus-free polyvinylpyrrolidone (PVP), with 0.086-0.75M sulphuric acid. The solution was stable indefinitely at room temperature. Colour developer contained 6.47M sodium hydroxide with 0.05M total carbonate concentration. This was also stable indefinitely at room temperature, but it must be kept from prolonged exposure to air to avoid formation of insoluble carbonate. Molybdate reagent contained 4% ammonium molybdate in molybdate preservative.

Assay procedure :

The fresh latex in mannitol, HEPES buffer was centrifuged as described in Section 2.2.1 to give the 900xg , 1100xg and supernatant (> 1100xg) populations. These sediments were plasmolysed in 20 mM phthalic acid. After centrifugation to remove insoluble material, the liquid (1 ml) was added to the assay mixture (5 ml of assay mixture containing water, ammonium molybdate reagent and reductant catalyst in a 1:2:3 ratio). After a waiting time of 2 minutes, 0.5 ml of colour developer was added while the solution was mixed on a vortex mixer.The absorbance at 720 nm was then read after 30 minutes.

2.3. Materials and Methods for the Uptake Experiments with *P. somniferum* cv. Halle Latex

P. somniferum cv. Halle was cultivated at The School of Pharmacy Medicinal Plant Garden. Plants were grown in sequential batches so that flowering occurred from late June until the end of October.

2.3.1. Determination of intravacuolar pH

The distribution of the membrane permeable base, methylamine, has been successfully used for the determination of intracellular and intravacuolar pH values (Waddell et al 1969 and Rottenberg et al 1972). The method is based on the assumption that uncharged compounds can easily pass across a membrane whereas charged forms are impermeable. Since the ratio of charged to uncharged compound is governed by the pH value, that value can be deduced. These assumptions have proved correct (Smith and Raven 1979).

In the present studies, intravacuolar pH was determined by incubating the vacuoles with 21 µM [^{14}C]-methylamine (3.7 x 10^2 Bq) for 30 minutes and subsequent separation of supernatant and pellet, and counting as described above in the determination of intravacuolar volume. The calculation was based on the equilibrium of charged methylamine across cell membrane and the impermeability of membrane to charged methylamine (Rottenberg 1975). The pH gradient (pH_{in} - pH_{out}) was assumed to be equal to the $\log_{10}$ of the ratio of the proton concentration on either side of the membrane, which was equal to the $\log_{10}$ of the ratio of the probe concentrations.

2.3.2. Uptake of [^{35}S]-sulphate and L-[U-^{14}C]-malate by the latex vacuoles

2.3.2.1. Materials and solutions

[^{35}S]-H_2SO_4 (44.4-51.8 TBq mM^{-1}) was purchased from Du Pont (UK) Ltd. as a solution of H_2SO_4 in H_2O (43 Ci/mg, 5 mCi/ml). It was diluted to give 10 µCi (2.37 x 10^{-6} µmoles H_2SO_4) in 10 µl input for the experiment.

L-[U-^{14}C] malic acid (1.5-2.2 GBq mM^{-1}) was purchased from Amersham International UK (200 µCi/ml, 53 mCi/mmol). It was then diluted to give 0.2 µCi (ca. 3.77 x 10^{-3} µmoles) in 10 µl input in each sample.

The 900xg and 1100xg vacuoles of the latex were isolated by the method described in Section 2.2.1 . These vacuolar fractions were washed twice with 700 mM mannitol, 100 mM HEPES buffer pH 6.8. The vacuoles were resuspended in 700 mM mannitol 100 mM HEPES, 100 µM ammonium molybdate pH 6.8 , to equal the original latex: buffer (1:1) concentration.

1.2 M KCl and 125 mM ATP/$MgSO_4$ were freshly made to give the final concentrations of 50 mM and 5 mM, respectively, in each sample. The 20% and 16% (w/w) sucrose solutions were prepared for the 900xg and 1100xg vacuoles, respectively.

2.3.2.2. Experimental method

The experiment was carried out in Eppendorf tubes. Each tube contained 200 µl latex vacuoles in buffer, 10 µl KCl solution and 10 µl ATP/Mg^{2+} (or 10 µl buffer). 10 µl of diluted ^{14}C-malic acid solution was finally added to each tube to give 4, 8, 12, 24 and 48 minute incubation. They were then immediately transferred to small centrifuge tubes containing 20% or 16% sucrose gradient for the 900xg and 1100xg vacuoles, respectively. Only the 900xg vacuoles were

successfully sedimented at this stage. The supernatant was poured off and the tube was gently washed with the appropriate sucrose solution to remove traces of the unwanted radiolabelled compound. The pellet was plasmolysed in 500 µl of 0.025 N HCl/MeOH. A vortex mixer was used to speed up the plasmolysis process. Each sample was left to stand for at least 30 minutes and then centrifuged at 2000xg to remove insoluble material. The supernatant was counted in a Packard Tri-Carb Liquid Scintillation Spectrometer using Aquasol LSC cocktail.

In each individual experiment, duplicate samples were used and the results are averages.

2.3.3. Uptake of alkaloids by the latex vacuoles

2.3.3.1. Experimental methods

The solutions were freshly made as described for the uptake experiments with acid (Section 2.3.2), except that the following alkaloid concentrations were used:

32.2 µmoles of [^{14}C]-labelled morphine / 200 µl latex buffer.

0.2 µmoles of [^{14}C]-labelled methylamine / 200 µl latex buffer.

10 µmoles of 1-methoxycanthin-6-one / 200 µl latex buffer.

7.7 µmoles of caffeine / 200 µl latex buffer.

10.2 µmoles of noscapine / 200 µl latex buffer.

200 µmoles of nicotine / 200 µl latex buffer.

0.5 µmoles of (+) or (-) [C^3H_3O]-codeine / 200 µl latex buffer.

The 900xg vacuoles were isolated as described in Section 2.2.1. They were then washed twice with 700 mM mannitol, 100 mM Hepes buffer pH 6.8. The vacuoles were

finally resuspended in 700 mM mannitol 100 mM Hepes, 100 µM ammonium molybdate buffer pH 6.8. The isolation of the 1100xg vacuoles was unsuccessful in this experiment so only the 900xg vacuoles were available.

The experiment was carried out in Eppendorf tubes as described for the uptake of L-malic acid (Section 2.3.2.2); except that the incubation time was 30 minutes , and the pellet after the experiment was plasmolysed in 500 µl of 0.1 N HCl/H_2O with 1 drop of MeOH added for the samples fed caffeine and 1-methoxycanthin-6-one and of 0.025 N HCl/MeOH for the other alkaloid samples (nicotine and noscapine). A vortex mixer was used to speed up the plasmolysis process. The samples were left to stand for at least 30 minutes and then centrifuged to remove insoluble material.

2.3.3.2. Extraction and quantitation of caffeine and 1-methoxycanthin-6-one

2.3.3.2.1. Extraction of alkaloids

The supernatant sample (Section 2.3.3.1) was transferred to a small separating funnel. The alkaloids were extracted by chloroform four times. The chloroform extracts were combined, and the solvent was removed in a rotary evaporator. The residue was made up in a standard volume of MeOH for HPLC analysis.

2.3.3.2.2. Quantitation of caffeine

Caffeine was quantified by reverse-phase HPLC described in Section 2.1.4.3 , except that the mobile phase was 20% MeOH/H_2O , and with flow rate 1.5 ml/minute .

2.3.3.2.3. Quantitation of 1-methoxycanthin-6-one

1-Methoxycanthin-6-one was quantified by both normal- and reverse-phase HPLC as follows:

(i) Normal-phase HPLC as described in Section 2.1.4.1, except that the mobile phase was hexane : ethyl acetate : NH_4OH (70:30:0.1)

(ii) Reverse-phase HPLC as described in Section 2.1.4.3 and the mobile phase was 70% MeOH/H_2O and flow rate of 1.5 ml/min. .

2.3.3.3. Extraction and quantitation of noscapine and nicotine

2.3.3.3.1. Extraction of alkaloids

The supernatant samples obtained by the method described in Section 2.3.3.1 were basified to pH 9.5 with NH_4OH. The alkaloids were extracted with chloroform four times. The chloroform extracts from each sample were combined and the solvent was evaporated in a rotary evaporator. The residue was made up in a standard volume of MeOH for HPLC analysis.

2.3.3.3.2. Quantitation of noscapine

Noscapine was quantified by normal-phase HPLC as described in Section 2.1.4.1 .

2.3.3.3.3. Quantitation of nicotine

Nicotine was quantified by reverse-phase HPLC as described for 1-methoxycanthin-6-one (Section 2.3.3.2.3).

2.3.4. Conditions affecting morphine uptake by the latex vacuoles

The experiments were carried out as described for the uptake of acids and alkaloids, except the following concentrations and conditions were used, as follows:-

2.3.4.1. Effect of ATP/Mg^{2+}

Low [^{14}C]-morphine concentration (4.5 µM , 1.0Bq) and 10 mM ATP/Mg^{2+} were added to latex vacuole suspensions in the experiment to study the effect of ATP/Mg^{2+} on the uptake of [^{14}C]-morphine by the 900xg and the 1100xg vacuoles.

In the experiment, in which high concentrations of [^{14}C]-morphine (10 $mg.ml^{-1}$ for 900 x g vacuoles, 1 $mg.ml^{-1}$ for 1100xg vacuoles) were used to preload isolated latex vacuoles, 10 mM ATP/Mg^{2+} was added to some samples 8 minutes after the start of incubation with morphine.

2.3.4.2. Effect of temperature

[$^{14}CH_3$]-morphine, final concentration 85 µM, 3 Bq, was incubated with latex vacuole suspensions for 4 minutes.

2.3.4.3. Effect of inhibitors

The inhibitors KNO_3 (50 X 10^{-4} M), orthovanadate (50 x 10^{-4} M), FCCP [carbonylcyanide 4-(trifluoromethoxy) phenylhydrazone] (5 x 10^{-4} M) and DCCD (N,N'-dicyclohexyl carbodiimide) (5 x 10^{-4} M) were separately added to the latex vacuole suspensions 20 minutes prior to the incubation of 4.5 µM (1.0 Bq) [$^{14}CH_3$]-morphine. The uptake of radiolabelled morphine was measured 4 minutes after incubation.

2.4. Feeding Experiment on the Biosynthesis of Noscapine from its proposed Precursors

2.4.1. Materials and methods

2.4.1.1. Precursors

Unlabelled tetrahydrocolumbamine, tetrahydroberberine were provided by Professor Jack Beal, Ohio State University, Columbus, Ohio. Berberine and hydrastine were of commercial origin.

One milligram of each precursor was weighed out and fed to each plant (random sample of 3 plants).

2.4.1.2. Feeding experiment

P. somniferum cv. Halle used in the experiment were grown in The School of Pharmacy Medicinal Plant Garden.

The method of administration consisted of direct application of unlabelled precursors, in 10 mM potassium phosphate buffer pH 6.5 , to the stem surface of intact plants at the bud stage after removal of the cuticle. The technique used was a development of that used by Fairbairn (Fairbairn and Steele 1980 , Roberts et al 1987a). A cup, consisting of a beeswax base with lygon tubing walls and holding a total volume of 400 µl , was placed at the site of cuticle removal (5-10 cm below the bud).

The stem and capsule directly above the feeding cup were harvested. They were stored in sealed plastic bags and in deep freeze for three months before analysis.

2.4.1.3. Isolation and quantitation of alkaloids after the feeding experiment

The capsules plus 8" stems were exhaustively extracted with 5% acetic acid. The alkaloids were isolated by the method given in Section 2.1.3 and then quantified by normal-phase HPLC described in Section 2.1.4 .

Section 3

RESULTS AND DISCUSSION

3.1. Characteristics of the Alkaloids used as Standards for the Investigation of *P. somniferum* L.

3.1.1. High performance liquid chromatography (HPLC)

The relative retention time (R_t-R_0) of standard alkaloids in both normal- and reverse-phase HPLC systems, described in Section 2.1.4 , are given in Figs. 3.1 A&B .

3.1.2. Thin layer chromatography (TLC)

R_f values of the five main alkaloids of *P. somniferum L.* (morphine, codeine, thebaine, papaverine and noscapine) are shown in Table 3.1A .

3.1.3. Ultraviolet spectroscopy (UV)

The wavelengths of maximal UV absorption of standard alkaloids are given in Table 3.1B .

Table 3.1 A R_f values of the five major alkaloids in TLC systems

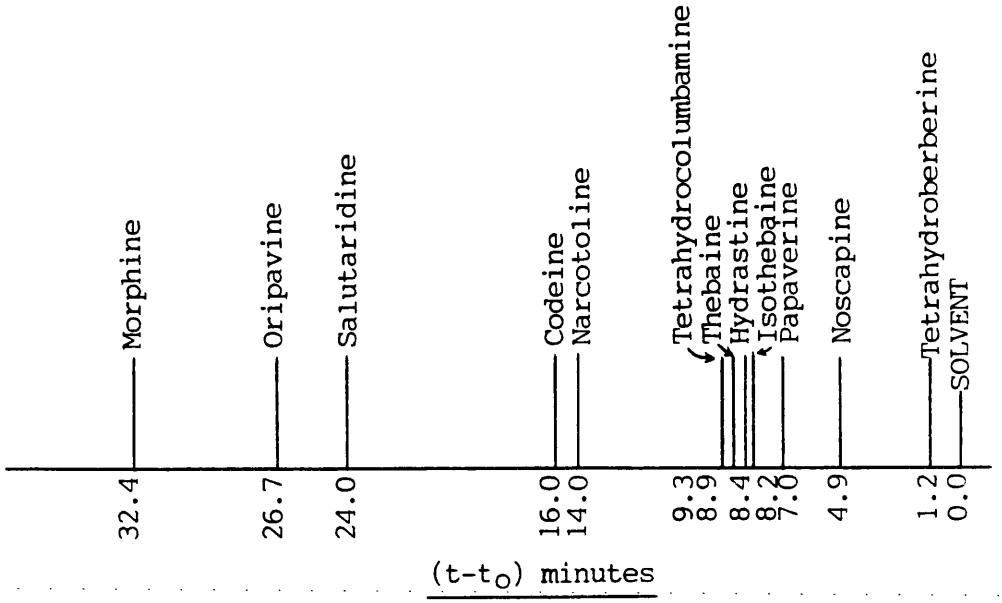
Solvent system number	Morphine	Codeine	Thebaine	Papaverine	Noscapine
1	0.28	0.50	0.70	0.79	0.90
2	0.14	0.27	0.47	0.57	0.67
3	0.11	0.14	0.18	0.39	0.62
4	0.08	0.16	0.21	0.69	0.73
5	0.14	0.21	0.41	0.72	0.76
6	0.74	0.81	0.92	0.94	0.96

Note : Solvent systems are shown in Section 2.1.5

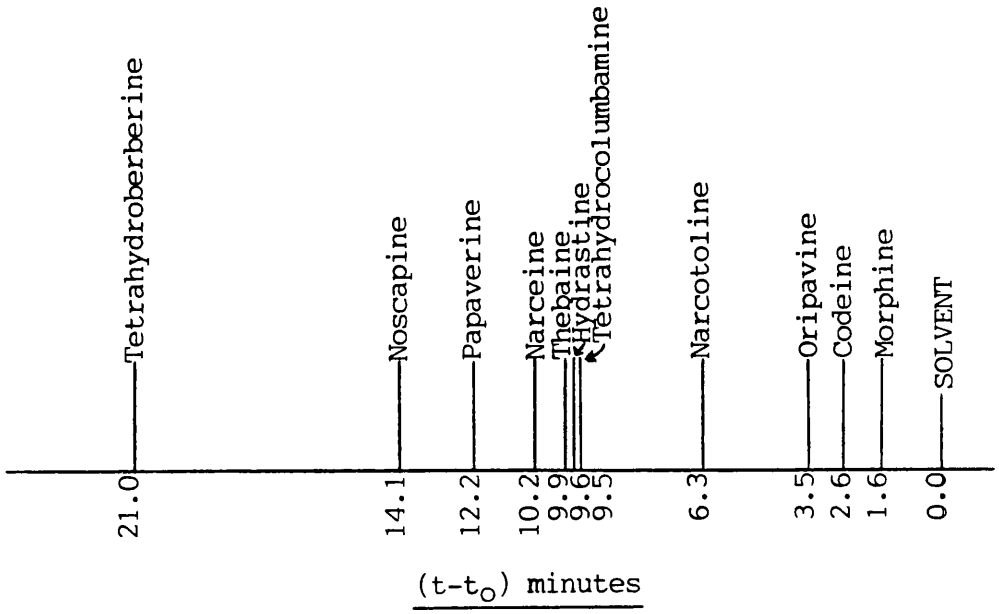
Table 3.1 B UV absorption of standard alkaloids

Alkaloid	λ (nm)			
Noscapine	310	290		
Papaverine	242	280	314	327
Thebaine	287			
Codeine	287			
Morphine	285			
Oripavine	288			
Isothebaine	270	294		
Salutaridine	240	280		
(-) Reticuline	285			
(-) Reticuline	285			
(±) Reticuline	285			
Narcotoline	310			
Narceine	277			

A. Normal phase HPLC



B. Reverse phase HPLC



Figures 3.1 A & B Positions of standard alkaloids in HPLC elution chromatograms

3.2. Identification of Minor Alkaloid Constituents from *P. somniferum* Plants

HPLC elution chromatograms of crude alkaloid extracts from *P. somniferum* plants investigated in the present studies showed the presence of three unknown peaks between codeine and morphine in the described normal-phase system (Section 2.1.4.1 and Fig. 3.1A) . The isolation of these unknown compounds was carried out by using normal-phase HPLC with a semiprep column (Section 2.1.4.2) . Two of these three unknowns, one from the cultivars UL9-11 and the other one from the cultivar Halle , were successfully isolated from *P. somniferum* plants.

3.2.1. Minor alkaloid C.2.M from *P. somniferum* cultivars UL9-11

3.2.1.1. Chemical characteristics of the isolated alkaloid

The crude alkaloid extract isolated from *P. somniferum* cv. UL9-11 plants at green capsule stage was found to contain a minor alkaloid which was not readily identifiable from the information available for a range of alkaloids known to occur in *P. somniferum* . This alkaloid was isolated from the extract using normal-phase HPLC described in Section 2.1.4.2 . The isolated alkaloid had the following characteristics :

(i) Dragendorff's test : positive

(ii) HPLC (Section 2.1.4)
normal-phase HPLC system: R_t-R_o = 30 minutes
reverse-phase HPLC system: R_t-R_o = 10.1 min.

(iii) TLC (system 1 , Section 2.1.5), with R_f = 0.65

(iv) MS (Figs. 3.2 A&B)

(EI) : m/e 58(100%) , 427(18%), 234(19%) 151(10%) .

(MNOBA matrix,FAB) :

m/e 55(100%) , 461$[M+1]^+$(39%) 413(21%) , 329(20%) , 241(13%) 223(12%) , 176(90%), 149(31%), 136(28%) , 91(38%) , 81(43%) , 69(86%) .

(v) ^{1}H-NMR (in CD_3OD + TMS) (Fig. 3.2C)

δ 2.56 (S , 6H , CH_3-N-CH_3) , 2.76 (S, 3H, NCH_3) , 3.83 (S , 3H , OCH_3) , 3.84 (S , 2H) , 3.87 (S , 3H , OCH_3), 3.98 (S , 3H , OCH_3) , 5.91 (S , 2H , CH_2 dioxy bridge) , 6.55 (S , 1H) , 7.49 (d , 1H , aromatic) , 8.15 (d , 1H, aromatic) .

The isolated alkaloid C.2.M and narceine gave similar fragmentations with MS(EI) (Fig. 3.2A) and relative retention time ($t-t_o$=10.1 minutes) in the described reverse-phase HPLC (Section 2.1.4.3) . In addition, the ^{1}H-NMR and MS(FAB) data suggested a compound similar to narceine but with an extra -CH_3 group . The ^{1}H-NMR and MS(FAB) of narceine were therefore recorded for comparision (Figs. 3.2 D&E). From the data described and comparing them with those for narceine, the minor alkaloid C.2.M from *P. somniferum* cv. UL9-11 was suggested to be the N-methylated form of narceine.

3.2.1.2. Synthesis of N-methylnarceine from narceine

Narceine was N-methylated by methyliodide using the method described by Roberts et al (1987a) . The methylated alkaloid was separated from the unreacted narceine by preparative TLC using solvent system acetone:toluene:0.88 ammonia (40 : 40 : 12 : 2.5). In this solvent system,

narceine remained on the baseline while the methylated alkaloid gave two spots with R_f=0.65 and 0.66 which was equivalent to that of the minor alkaloid C.2.M . The mass spectrum of this product revealed a molecular ion of 460 which was also identical to that of the isolated alkaloid C.2.M from *P. somniferum* cv. UL9-11 plants (Fig. 3.2F) .

3.2.1.3. Identification of the minor alkaloid C.2.M

The minor alkaloid C.2.M isolated from *P. somniferum* cv. UL9-11 plants at green capsule stage was identified as N-methylnarceine .

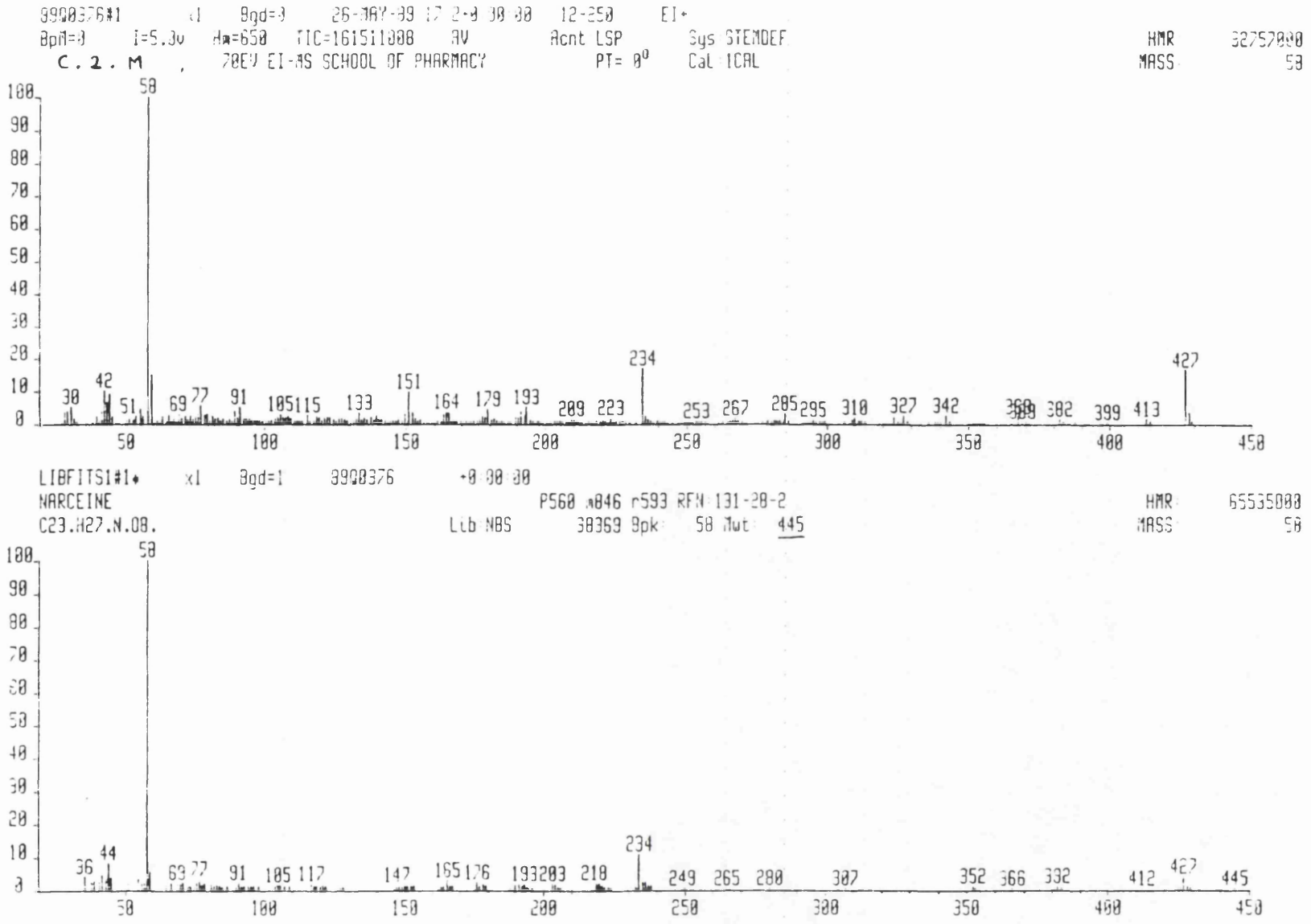


Figure 3.2A EI-MS of the minor alkaloid C.2.M isolated from *P. somniferum* cv. UL9-11 compared to standard narceine

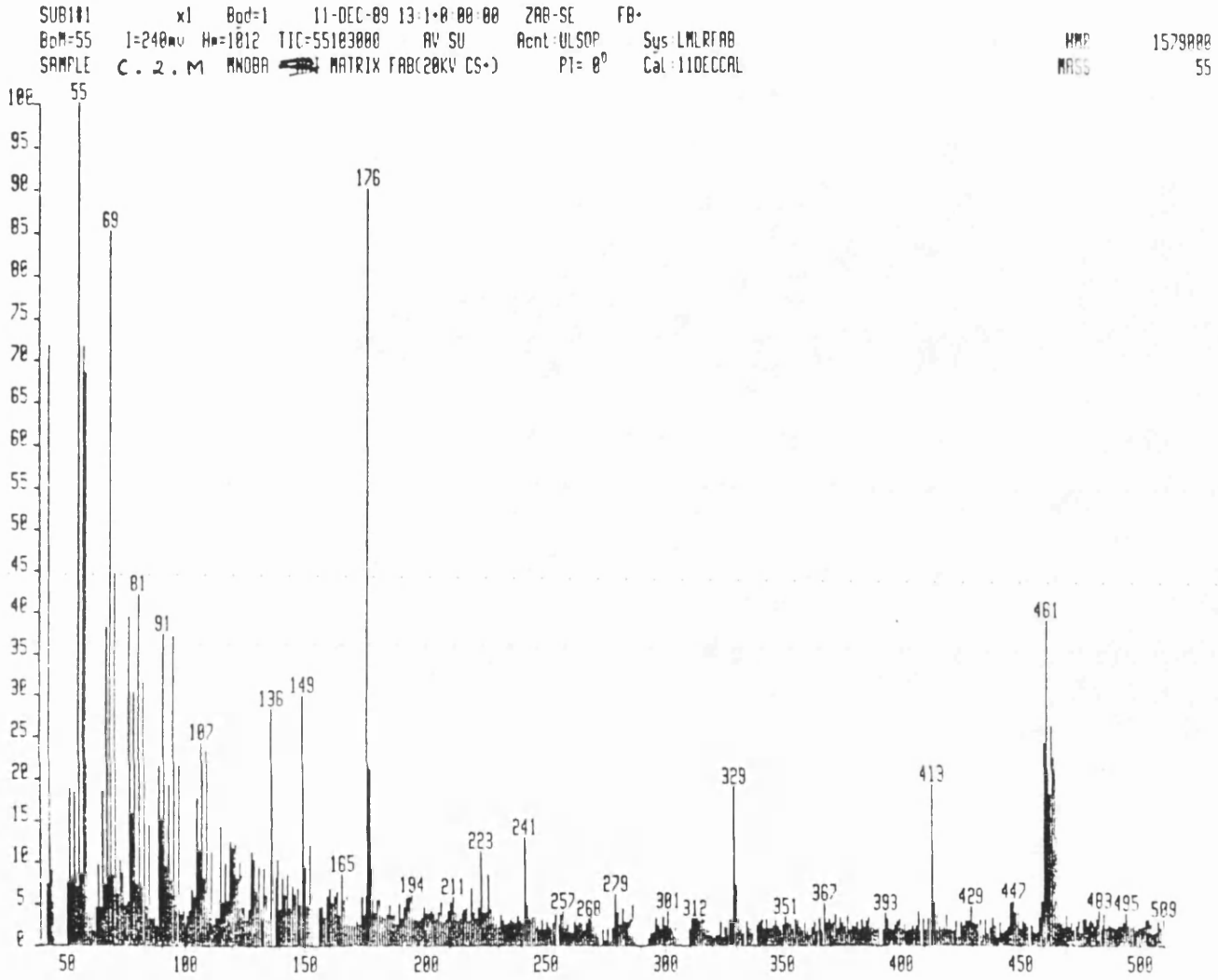


Figure 3.2 B MS(FAB) of the minor alkaloid C.2.M from P. somniferum cv. UL9-11 plants

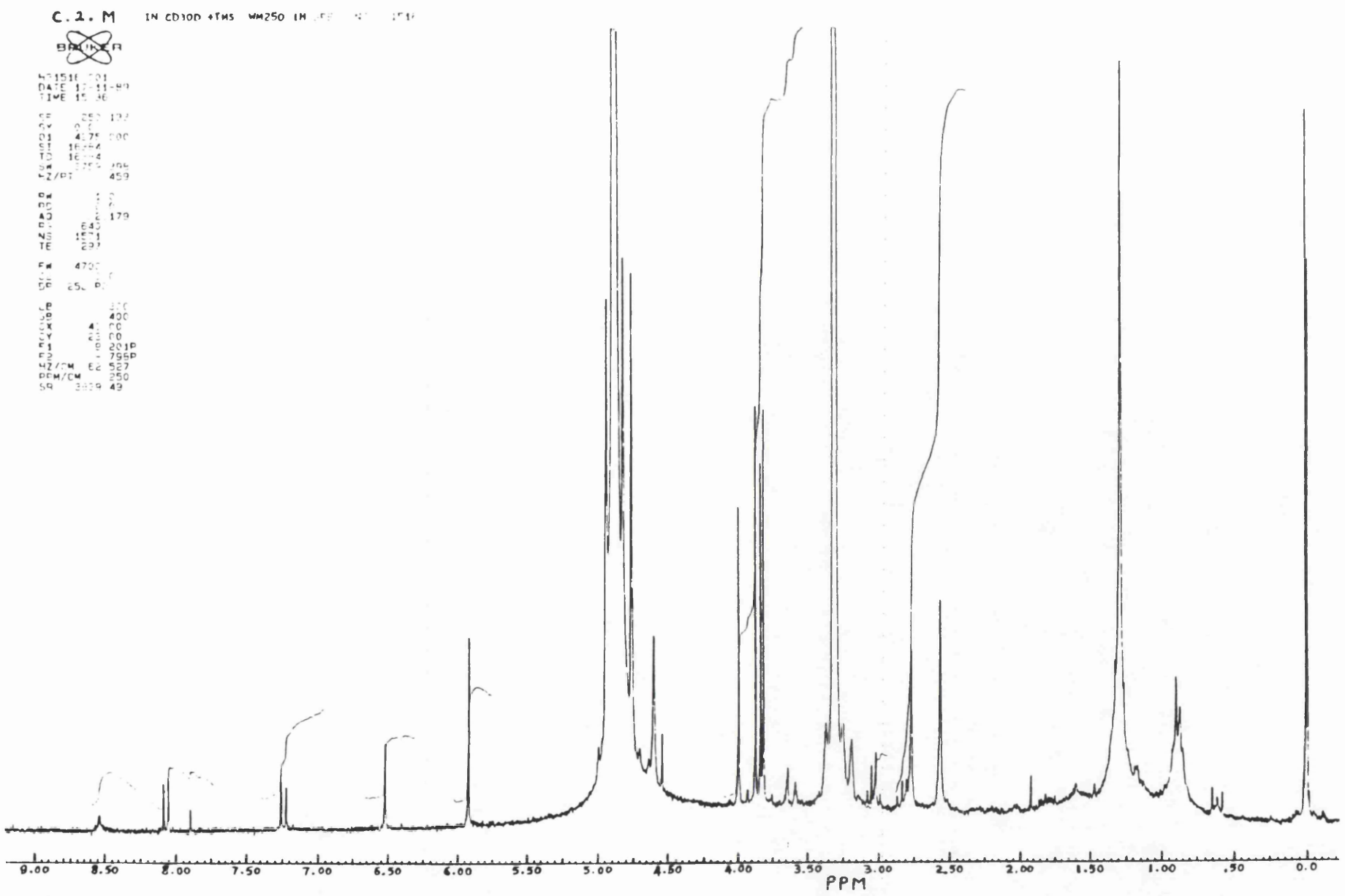


Figure 3.2 C ^{1}H-NMR of the minor alkaloid C.2.M from P. somniferum cv. UL9-11 plants

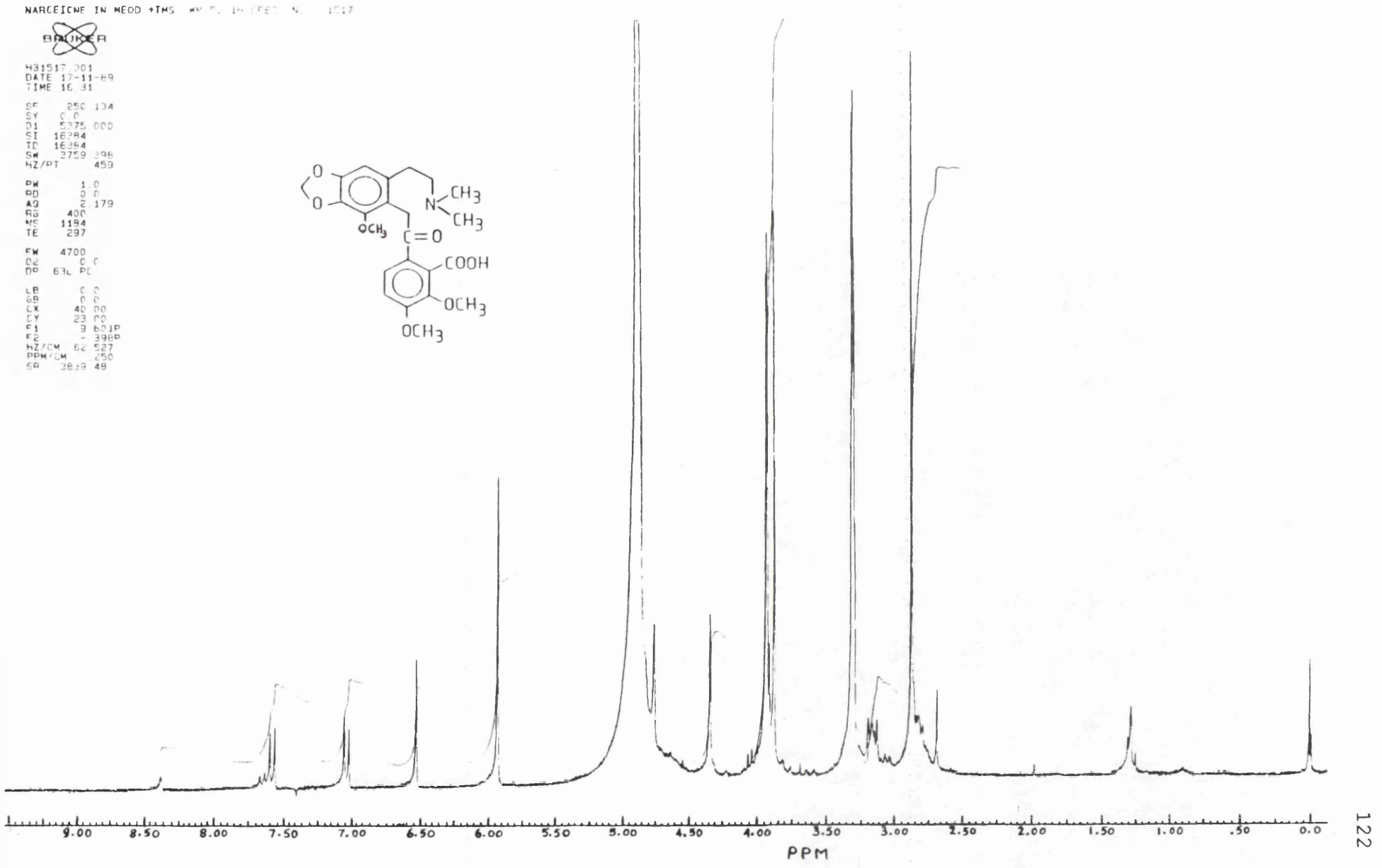


Figure 3.2 D ^{1}H-NMR of narceine

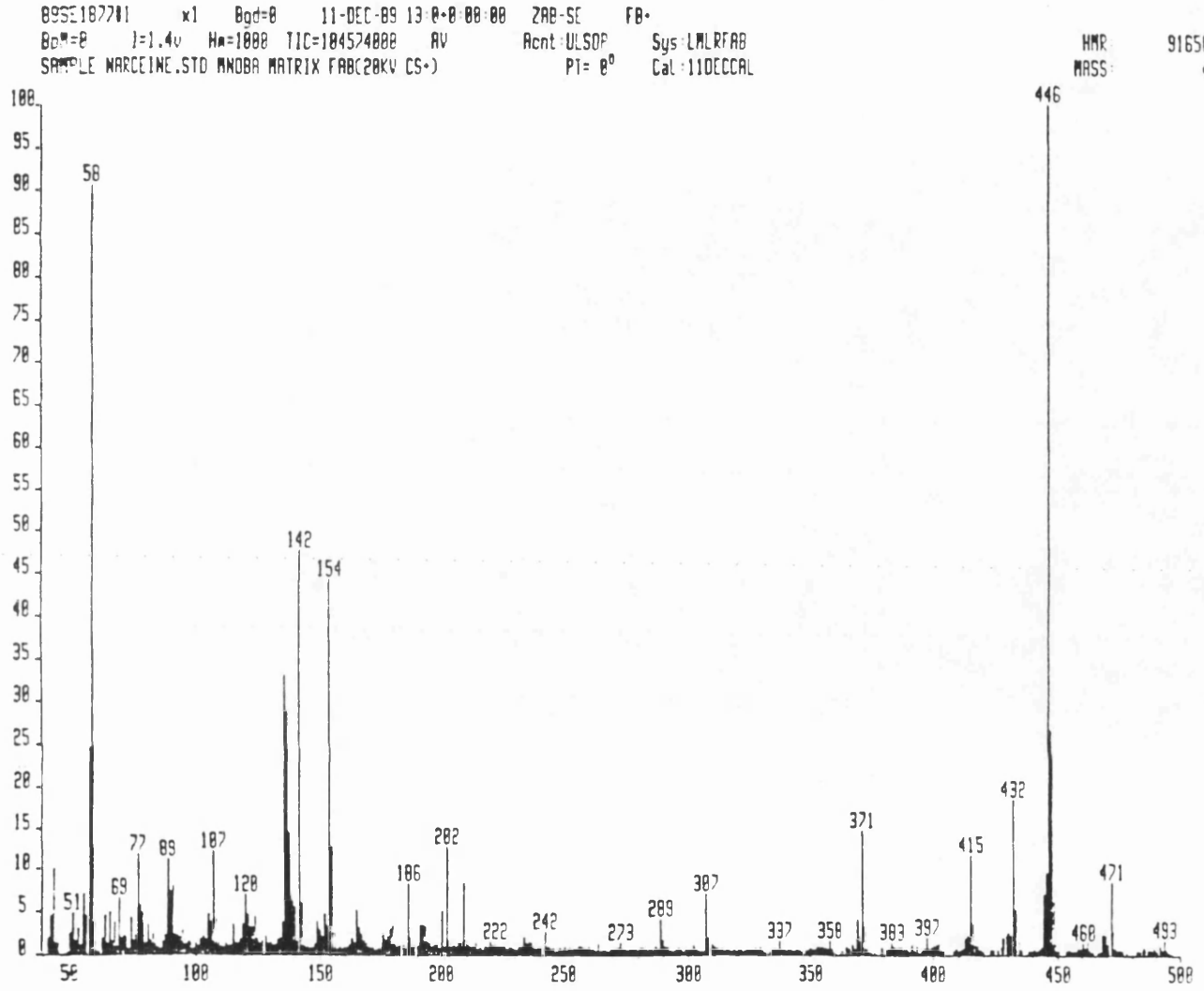
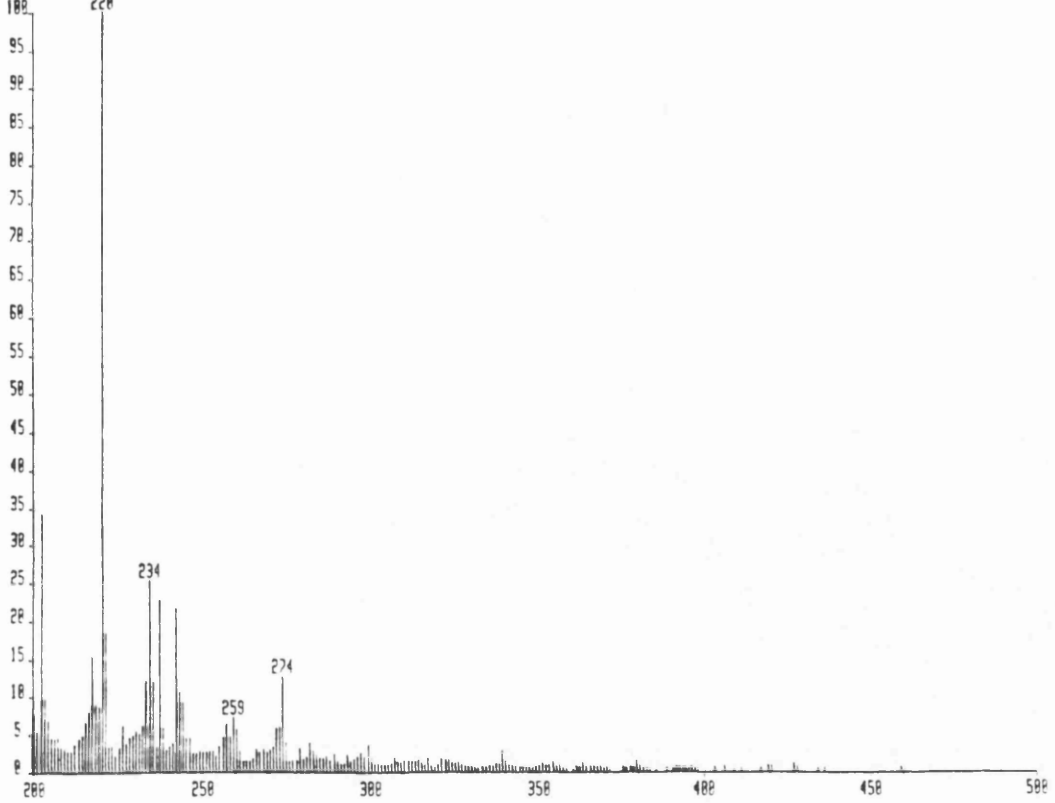


Figure 3.2 E MS(FAB) of narceine



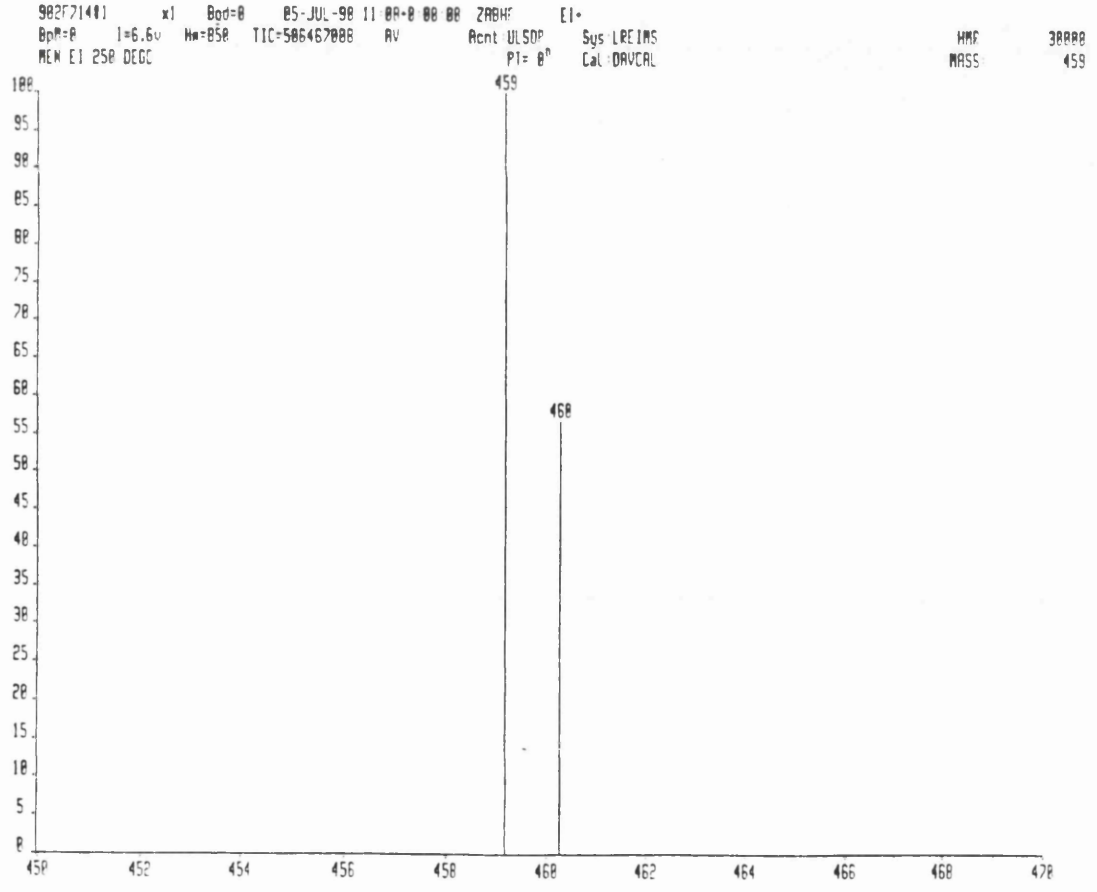


Figure 3.2 F MS(EI) of the alkaloid product from N-methylation of standard narceine

3.2.2. Minor alkaloid C.1.M from *P. somniferum* cultivar Halle

The minor alkaloid C.1.M was isolated from *P. somniferum* cv. Halle plants at young capsule stage by the same method as described in Section 3.2.1 for the minor alkaloid C.2.M from *P. somniferum* cv. UL9-11.

3.2.2.1. Chemical characteristics of the isolated alkaloid

(i) Dragendorff's test : positive

(ii) HPLC :

normal-phase system : R_t-R_o= 26.6 minutes

reverse-phase system : R_t-R_o= 3.55 minutes

(iii) TLC (system 1 , Section 2.1.6), R_f=0.60

(iv) UV = 286 nm

(v) MS (EI) (Fig. 3.3A)

m/e 297 (76%) , 282 (13%) , 240 (14%) , 149 (33%) , 91 (22%) , 71 (37%) , 57 (84%) , 43 (100%) .

(vi) ^{1}H-NMR (in CD_3OD + TMS) (Fig. 3.3B)

δ 6.55 (2H , q , aromatic) , 5.65 (1H , d) , 5.27 (1H , s), 5.17 (1H , d) , 3.77 (2H , d), 3.63 (3H , s , $-OCH_3$) , 2.53 (3H , s , $N-CH_3$) .

3.2.2.2. Identification of the minor alkaloid C.1.M

Since the minor alkaloid C.1.M isolated from *P. somniferum* cv. Halle and oripavine had the same molecular ion of 297 and similar R_f value and relative retention time in both normal- and reverse-phase HPLC systems, the ^{1}H-NMR and EI-MS of oripavine were recorded for comparison

(Figures 3.3 C & D). The minor alkaloid C.1.M from *P. somniferum* cv. Halle was identified as oripavine.

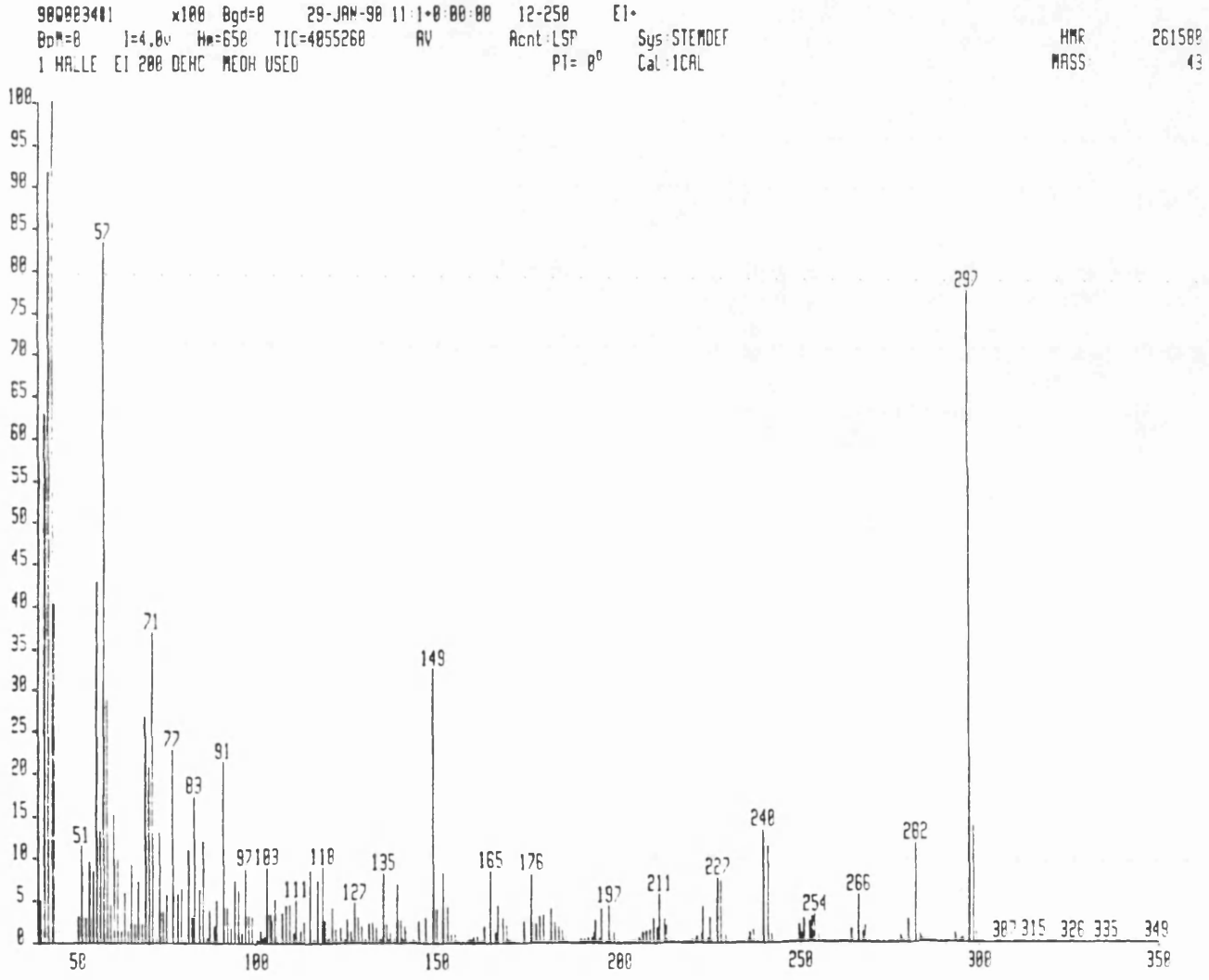


Figure 3.3 A MS(EI) of the minor alkakoid C.1.M from P. somniferum cv. Hallé plants

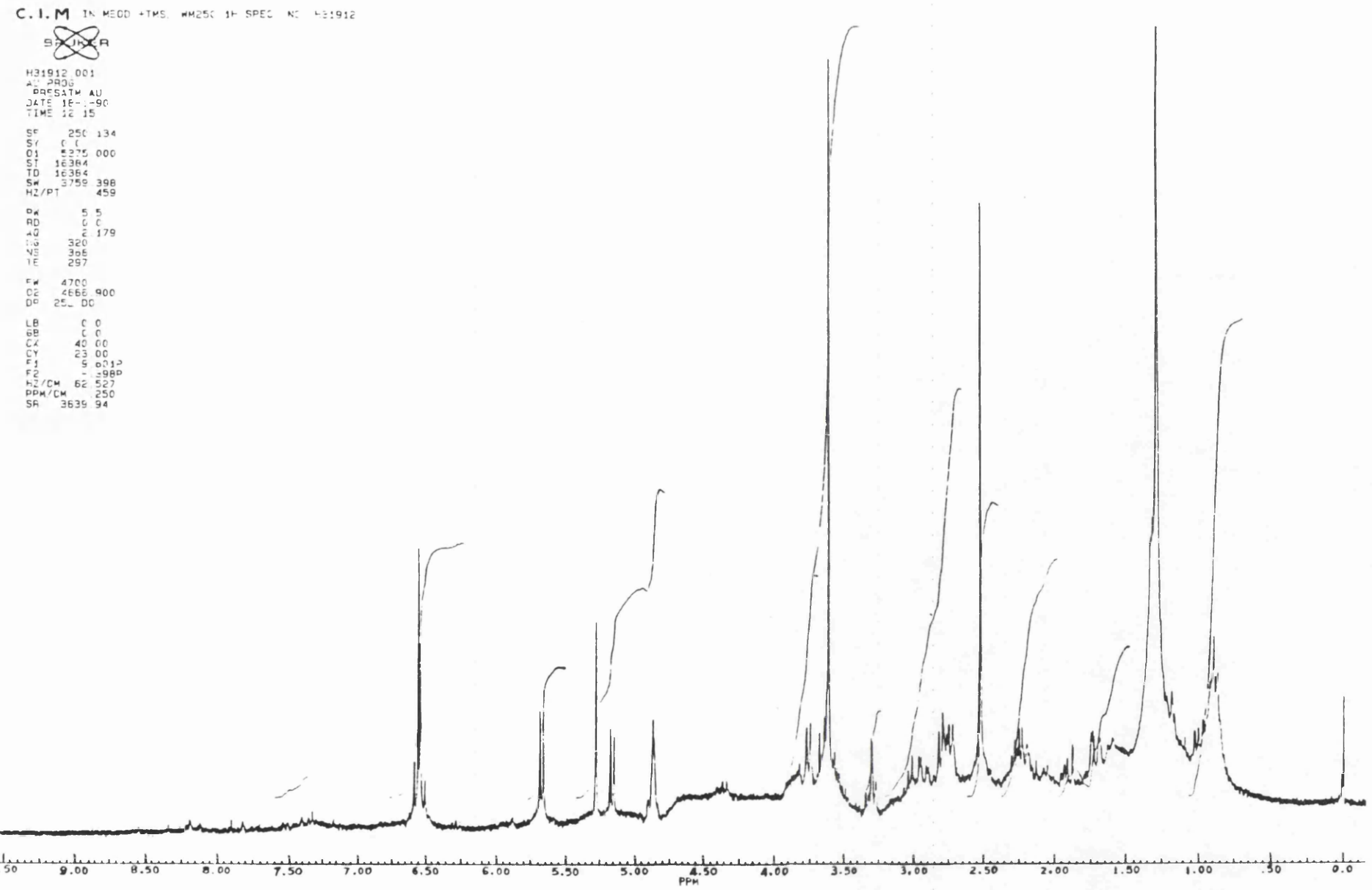


Figure 3.3 B ^{1}H-NMR of the minor alkaloid C.1.M from P. somniferum cv. Hallé plant

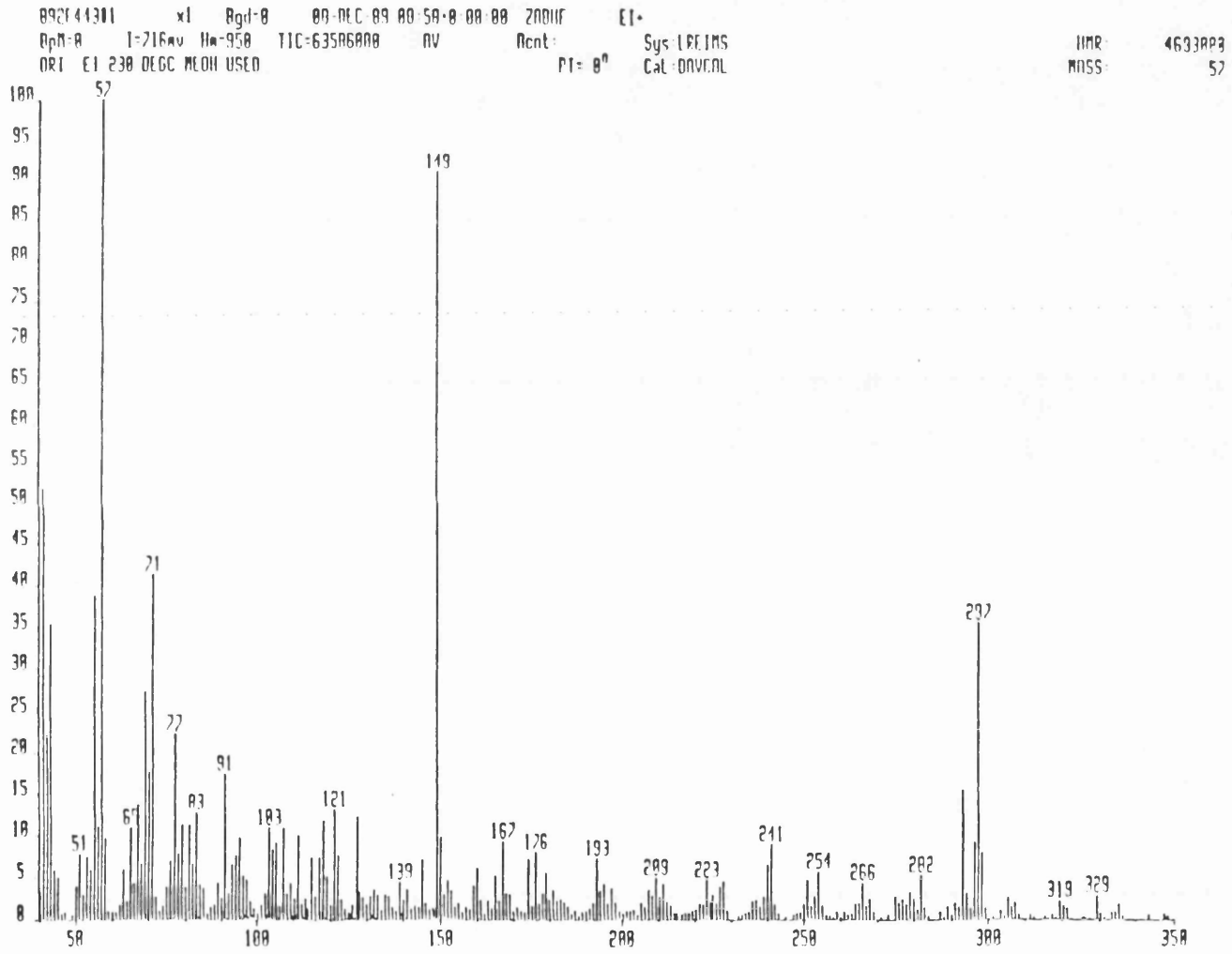


Figure 3.3 C MS(EI) of standard oripavine

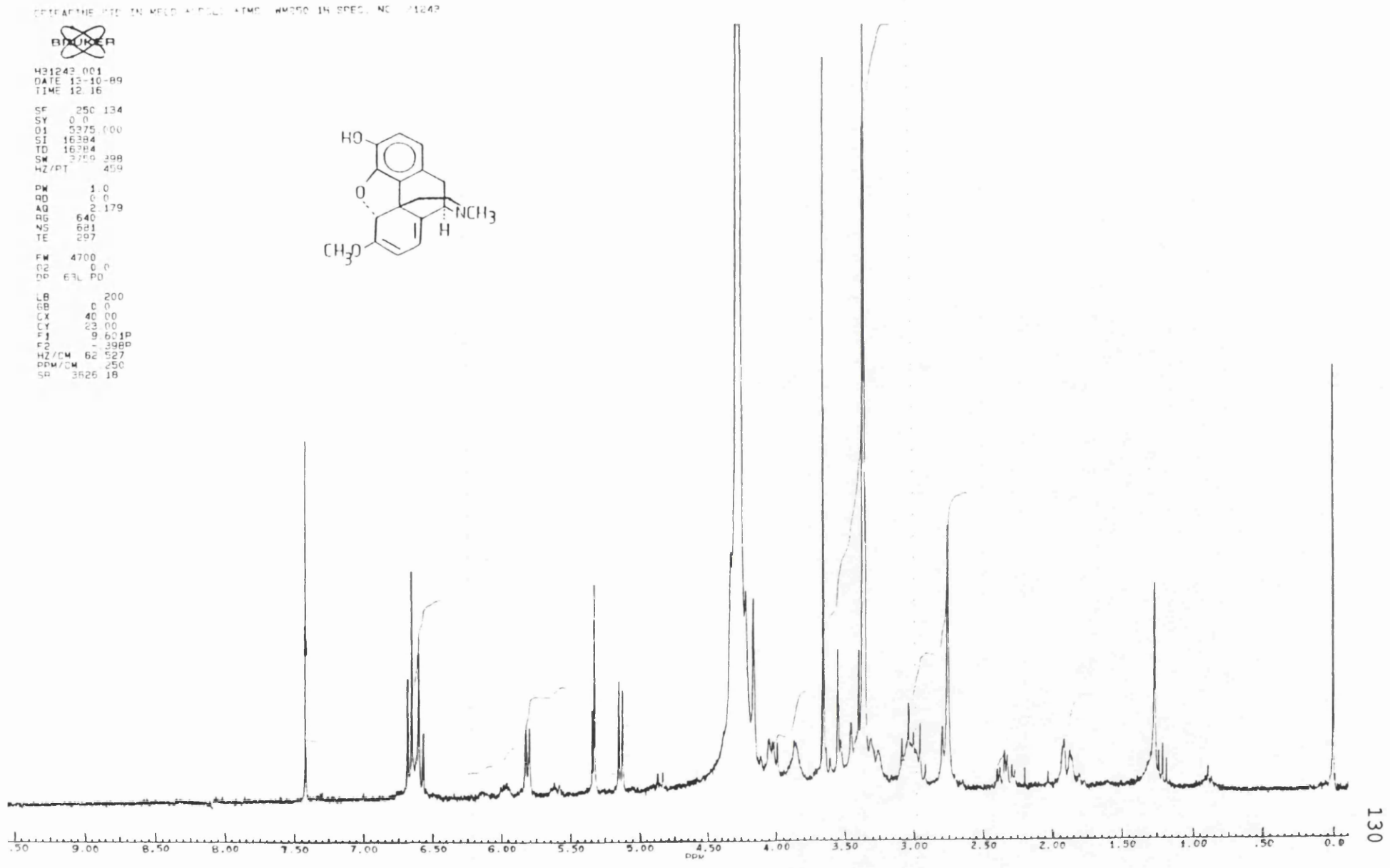


Figure 3.3 D ^{1}H- NMR of oripavine

3.3. Variations of Alkaloid Contents during Plant Development

In this series of experiments, the cultivars Halle, CV6 and CV7 of *P. somniferum* L. were investigated . The work on these particular cultivars resulted from some early results where a series of CV samples were grown as seedlings in the UK for comparison with the mature plant grown in Tasmania, Australia. In the present excercise, a selected numbers of these cultivars were grown as seedlings and to maturity in the UK for further comparison with Tasmania grown material in an effort to determine whether useful consistent correlation between alkaloid content in the seedling and the mature plant could be used as a first screen prior to growing new cultivar in the field .

3.3.1. Results for *P. somniferum* cultivars Halle , CV6 and CV7 grown in the summer of 1987

Seed samples coded Halle, CV6 and CV7 were grown in the summer of 1987 as described in Section 2.1.2 . The plants were unable to reach the ripe capsule stage by the end of summer period (June to September). Plants were collected at the following stages: young seedling (with 4-5 leaves), young plant (20-25 cm high), plant with bud, flower and green capsule for quantitative analysis of their alkaloid contents.

Morphinan alkaloids (morphine, codeine and thebaine) were detected in seedlings of the CV6 and CV7 cultivars (ca. 26 and 27 µg /g fresh weight, respectively). Noscapine and papaverine were also found at much lower levels in the seedlings (between 0.01 and 1.75 µg /g fresh weight (Table 3.2). As the plant developed, morphinan contents rapidly increased up to about 1.2 mg/g dry weight in the whole plants of both the CV6 and CV7 cultivars at maturity.

Papaverine and noscapine concentrations in both of these two plant cultivars, however, slightly increased throughout the growing season (up to ca. 10 µg and 39 µg /g dry weight for papaverine and noscapine, respectively) .

The *P. somniferum* cv. Halle, which is known for its high morphinan production, was also grown under the same experimental conditions for comparision. Alkaloid contents in the plant of this cultivar also increased as the plant developed to maturity, and the rapid increase was also observed for the morphinan alkaloids (Table 3.2). Most of the alkaloids extracted from the plants of these three cultivars were the morphinans, which constituted ca. 85-98% of the total five major alkaloids. Papaverine production by these plants was least significant compared to the other four main alkaloids of *P. somniferum* L. . The cultivars CV6 and CV7 could produce more morphinan alkaloids. Papaverine and noscapine contents in the CV6 and CV7 plants, in contrast, were lower than that in the cultivar Halle .

When the reproductive parts plus 8" stems of these plants were taken for analysis of their alkaloid contents, it was found that alkaloid levels in the reproductive parts also increased as the plants developed to maturity (Figs. 3.4 , 3.5 and 3.6). Morphinan contents rapidly increased up to 2.7 mg , 4.0 mg and 2.5 mg per green capsule (or 0.25%, 0.23%. and 0.28% dry weight) in the cultivars CV6, CV7 and Halle, respectively. Papaverine and noscapine levels also gradually increased in the reproductive parts of these three cultivars throughout the growing season. As the plant matured, most of the available alkaloids were found in the capsule of *P. somniferum* cv. Halle plant, i.e. capsule contained approximately 85% of the total alkaloids found in the whole plant (Fig. 3.9). *P. somniferum* cv. CV6 and CV7 plants could produce more alkaloids both in the whole plants and in the capsules. However, slightly more than half of the available alkaloids were still found in the plant remainders (i.e. plants minus capsules) (Figs. 3.7 and 3.8). In

Table 3.2 Yields of major alkaloids from P. somniferum cv. CV6 and CV7 seedlings cultivated during the summer of 1987

Plant cultivar	Alkaloid	µg/g fresh weight
CV6	Morphine	10.87
	Codeine	9.43
	Thebaine	6.06
	Papaverine	0.01
	Noscapine	1.75
CV7	Morphine	11.30
	Codeine	11.59
	Thebaine	4.33
	Papaverine	0.02
	Noscapine	0.68

Table 3.3 Major alkaloids in P. somniferum cultivars CV6 , CV7 and Hallé during plant development

Plant cultivar	Sample	Dry weight per whole plant (grams)	µg / g dry weight (whole plant)					
			morphine	codeine	thebaine	papaverine	noscapine	morphinans
CV6	young plant	0.95	736.5	177.6	1.8	0.4	19.3	915.9
	plant with bud	2.69	837.2	179.3	5.4	3.2	27.2	1021.9
	plant with flower	3.05	899.1	182.9	12.9	5.7	29.6	1094.9
	plant with capsule	4.27	1064.9	129.9	20.8	9.3	32.3	1215.6
CV7	young plant	1.01	702.4	46.3	1.6	1.7	4.3	750.3
	plant with bud	3.75	745.7	60.5	5.3	2.6	18.7	811.5
	plant with flower	5.12	830.5	105.6	10.6	3.8	25.2	946.7
	plant with capsule	7.28	941.5	211.7	20.7	5.2	38.6	1173.9
Hallé	young plant	0.45	483.0	100.7	1.7	8.6	32.3	585.4
	plant with flower	1.39	597.3	99.2	27.3	12.9	69.2	723.8
	plant with capsule	2.65	838.7	106.6	73.5	34.4	131.3	1018.8

Note: Random samples of 6 plants for analysis .

One gram of ground sample was extracted for alkaloid content .

The results are averages of duplicate determinations .

addition, less noscapine and papaverine were found in the capsules of the CV6 and CV7 plants than those in the same part of the cv. Halle cultivar (Fig. 3.10) ; and this pattern of alkaloid content was also observed with the whole plant samples (Table 3.3) .

3.3.2. Discussion

Alkaloid contents of the five major alkaloids increased as the plants developed from young seedlings to maturity in all the investigated cultivars. The dry weight of the plant steadily rose during plant development. Maximum alkaloid accumulation occurred during the period the reproductive organs developed and the increase was most rapid between flowering and capsule stages. The accumulation of alkaloids in *P. somniferum* plant, especially the morphinans, was roughly parallel with the growth of its reproductive part.

The five main alkaloids were found in the early stage of plant development, i.e. young seedlings. Thebaine constituted ca. 23% and 16% of the total morphinans in the young seedlings of the cultivars CV6 and CV7, respectively. As the plant developed, the concentrations of the three morphinan alkaloids increased. Thebaine constituted only about 2% of the total morphinan contents in the green capsules of both the CV6 and CV7 plants, and codeine levels were about 8% and 22% , respectively. In contrast, about 70-80% of the total five main alkaloids found in the matured plants was morphine. The results indicated that as the plant developed to maturity, more thebaine and codeine were converted to morphine. It is established that thebaine is transformed to morphine by two O-demethylation processes at C-3 and C-6 positions, and codeine is O- demethylated at C-3 to form morphine (Fig. 3.14 , Section 1.6.10.2). The results also suggested that the enzymes which catalyse these two O-demethylation reactions were not very active at the early

stage of plant development; as a result, more than 50% of the total morphinan found in young seedlings were thebaine and codeine. At maturity, almost all of thebaine formed was biotransformed to codeine and then to morphine so that only about 2% of the total morphinans was thebaine .

Noscapine and papaverine, in contrast, contributed only small fractions to the total alkaloid content. Noscapine was more abundant than papaverine, and noscapine concentration increased more rapidly than papaverine. However, as the plant matured more noscapine was found in the plant remaiders than in the capsules of the CV6 and CV7 plants. Papaverine levels in these plants were very low in both the reproductive parts and the lower parts of the plants, so the differences in its contents were negligible. Similar to noscapine, more morphinan alkaloids were found in the plant remainders of CV6 and CV7 plants at maturity. *P. somniferum* cv. Halle is known for its high morphinan and low noscapine and papaverine contents. At maturity, the cv. Halle plant contained more alkaloids in its capsule than in the rest of the plant. The CV6 and CV7 cultivars were able to produce more morphinan alkaloids than the cv. Halle. This ability is parallel with the increases in plant size and hence the bigger capsules (Footnotes in Figs. 3.7, 3.8 and 3.9). The CV6 and CV7 plants, in contrast, contained much less noscapine and papaverine than the cultivar Halle. The results also show that the plant cultivar with high morphinan content produced less noscapine and papaverine both in the whole plant and in the capsule. It has been established that (S)-reticuline is the branching point for the formation of noscapine (phthalideisoquinoline) and morphinan alkaloids in *P. somniferum* L. . The results suggested that the enzyme dehydrogenase which converts (S)-reticuline to 1,2-dehydroreticuline, prior to the formation of (R)-reticuline required for the biosynthesis of the morphinans, is more active in *P. somniferum* than in other *Papaver* species. As a result, more morphinan alkaloids than noscapine were produced

by this plant. Since more work is still required to establish the actual steps in the biosynthesis of papaverine, it is assumed that (S)-coclaurine is the branching point for the formation of papaverine (benzylisoquinoline) and other alkaloid types found in *P. somniferum* (Figs. 1.5 and 1.12) . It is therefore also suggested that the conversion of (S)-coclaurine to (S)- N-methylcoclaurine by coclaurine N-methyltransferase in the biosynthetic pathway to (S)-reticuline is predominant; as a result, only small amounts of papaverine were formed in the plant .

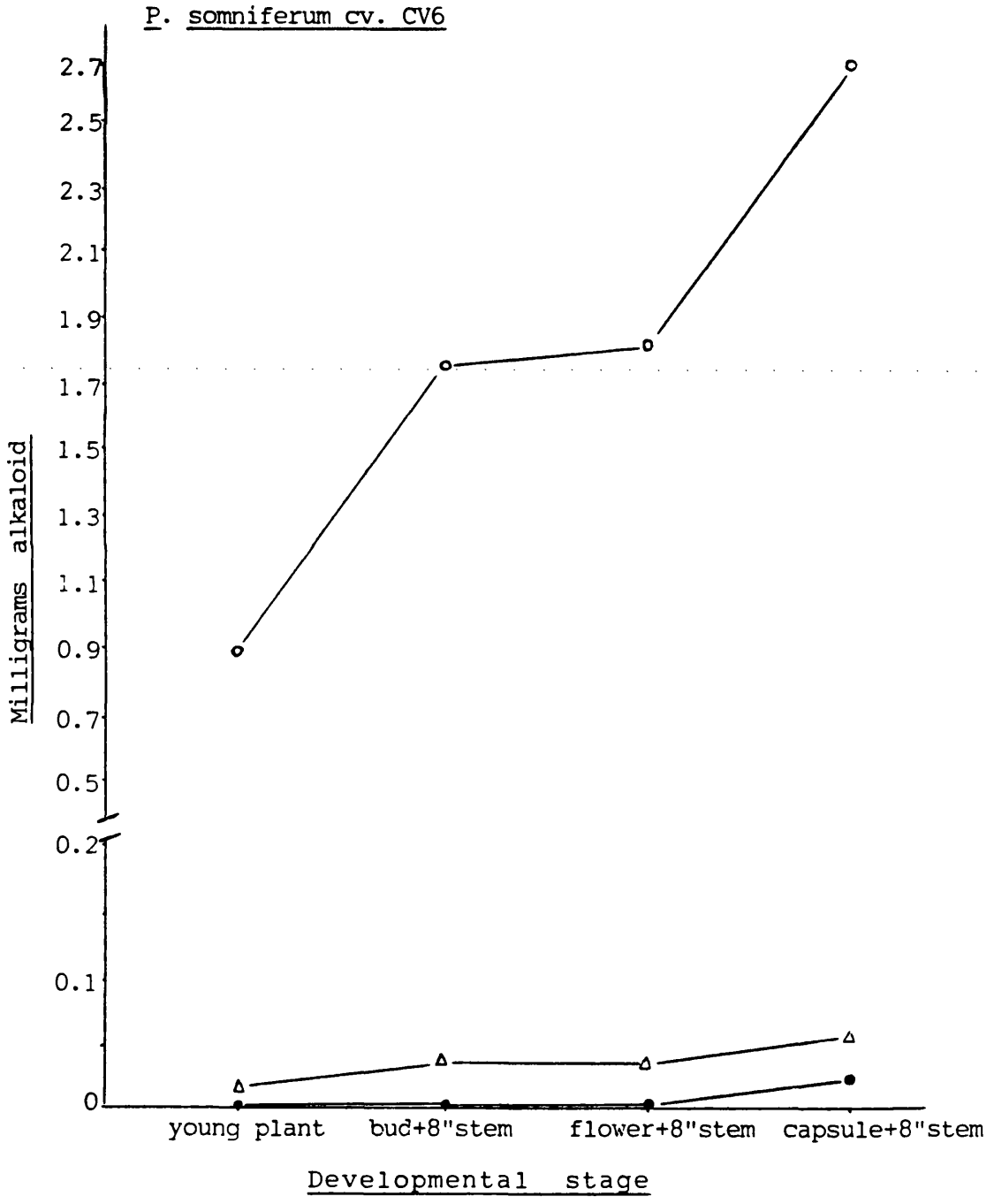
Of the three high morphinan yielding cultivars investigated in the present studies, the CV6 was best for morphinan production. This capacity could be seen at each of the stages from young plant to maturity (green capsule). Since the morphinan contents in the young seedlings of the CV6 and CV7 cultivars were not significantly different, morphinan production by the plant could only be screened at the young plant stage (20-25 cm high). The noscapine level in the young, five week old seedlings seemed to show this correlation with that found in the mature plant. The seedling screening to isolate cultivar high yielding in noscapine might be practical, but it would not reflect the potential morphinan production by the plant at the early stage of plant development. Radioimmunoassay was used for the detection of morphinan alkaloids and as a method for screening of *Papaver* plants (Wieczorek et al 1986). However, the results found in the present studies only suggested the use of young plants in screening of *P. somniferum* cultivars for alkaloid content, especially the morphinans.

For the determination of what losses in alkaloid occurs as a result of harvesting only the capsule, it was found that that slightly more than half of the available alkaloids was still in the plant remainders of the CV6 and CV7 cultivars at green capsule stage. The CV6 and CV7 cultivars were capable of producing significantly high morphinan contents. These plants, however, were not able to reach full maturity due to

poor weather. Harvesting the capsule plus 8" stem rather than the whole plant resulted in a loss of about 50% of the total alkaloid content for the investigated CV6 and CV7 cultivars at green capsule stage.

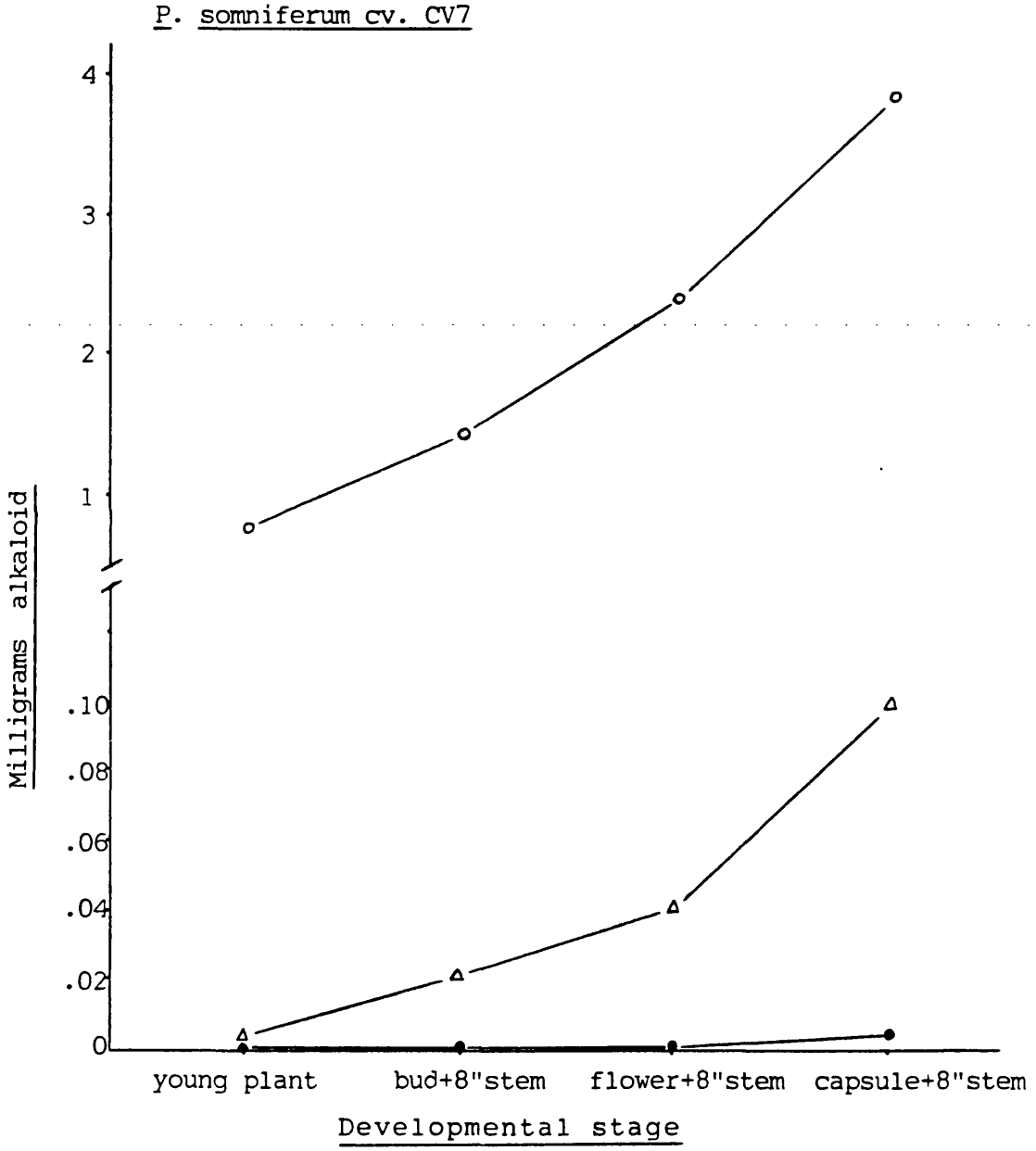
In the present studies, random samples of 6 plants were taken at each stage from flowering to maturity for investigation and the results were averages. The individual alkaloids accumulate in the capsules at markedly different rates. The capsule of the opium poppy is the main organ richest in alkaloids. The best time to pick the poppy for extraction purposes is the stage of biological maturity if the capsules are used.

Figure 3.4 Variation of alkaloid content in P. somniferum cv. CV6 during plant development (summer 1987)



o—o total morphinans , Δ—Δ noscapine , ●—● papaverine
1 gram of ground dried material taken from a random sample of 6 plants for quantitative analysis . Results are averages of duplicate determinations .

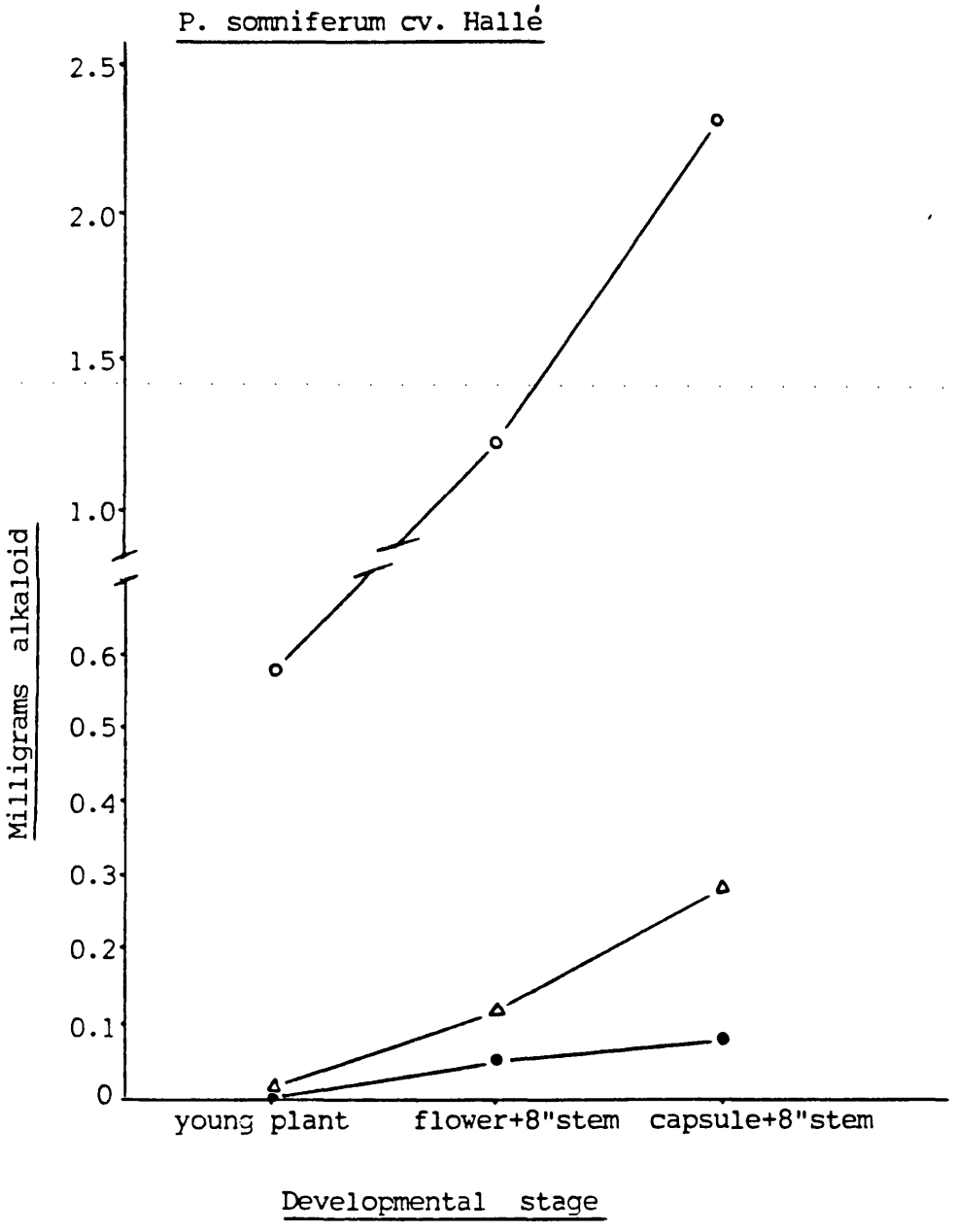
Figure 3.5 Variation of alkaloid content in P. somniferum cv. CV7 during plant development (summer 1987)



o—o total morphinans , Δ—Δ noscapine , ●—● papaverine

1 gram of ground dried material was taken from a random sample of 6 plants for quantitative analysis . Results are averages of duplicate determinations .

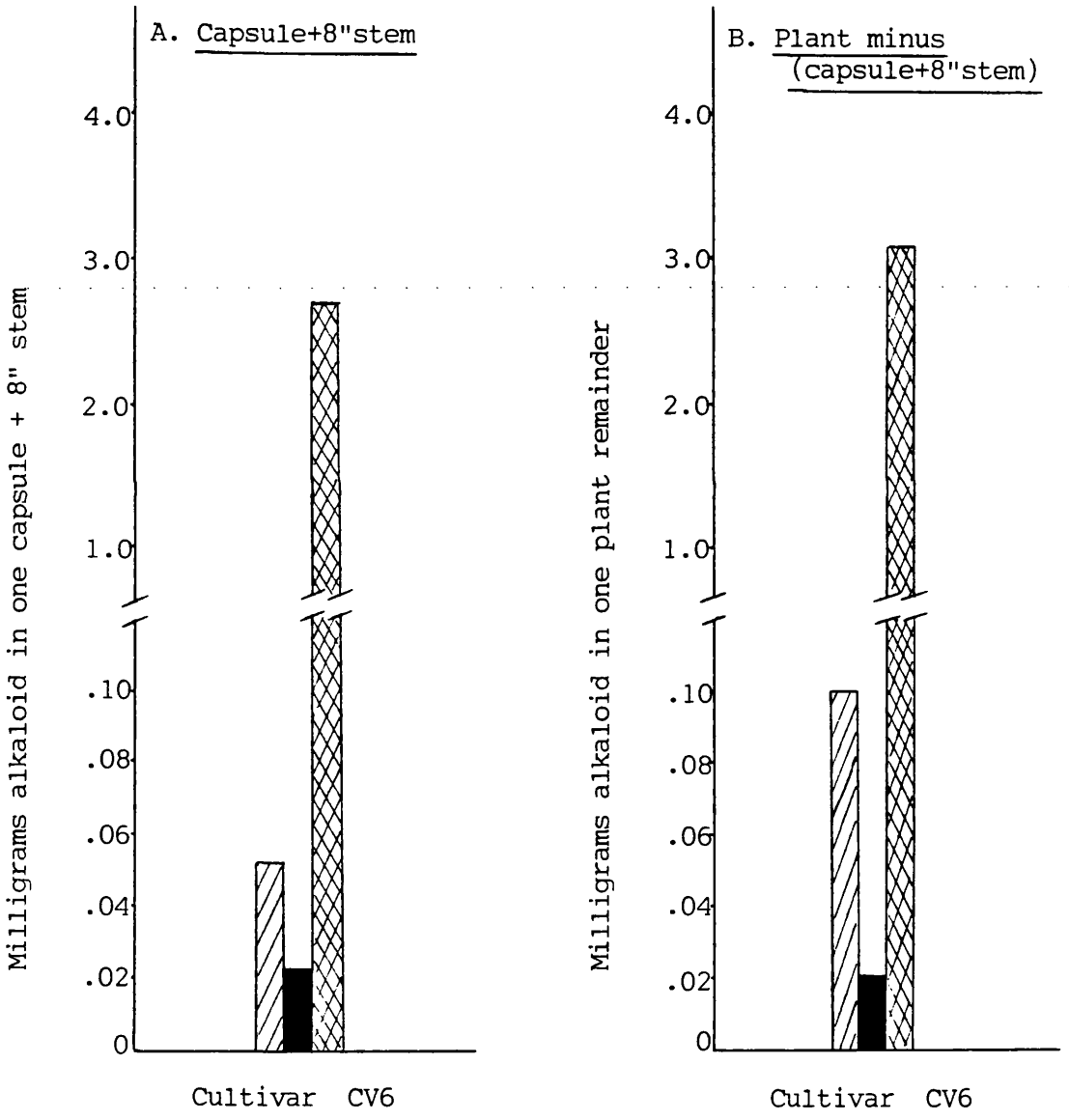
Figure 3.6 Variation of alkaloid content in P. somniferum cv. Hallé during plant development (summer 1987)



o—o total morphinans , Δ—Δ noscapine , ●—● papaverine

1 gram of ground dried material was taken from a random sample of 6 plants for quantitative analysis . Results are averages of duplicate determinations .

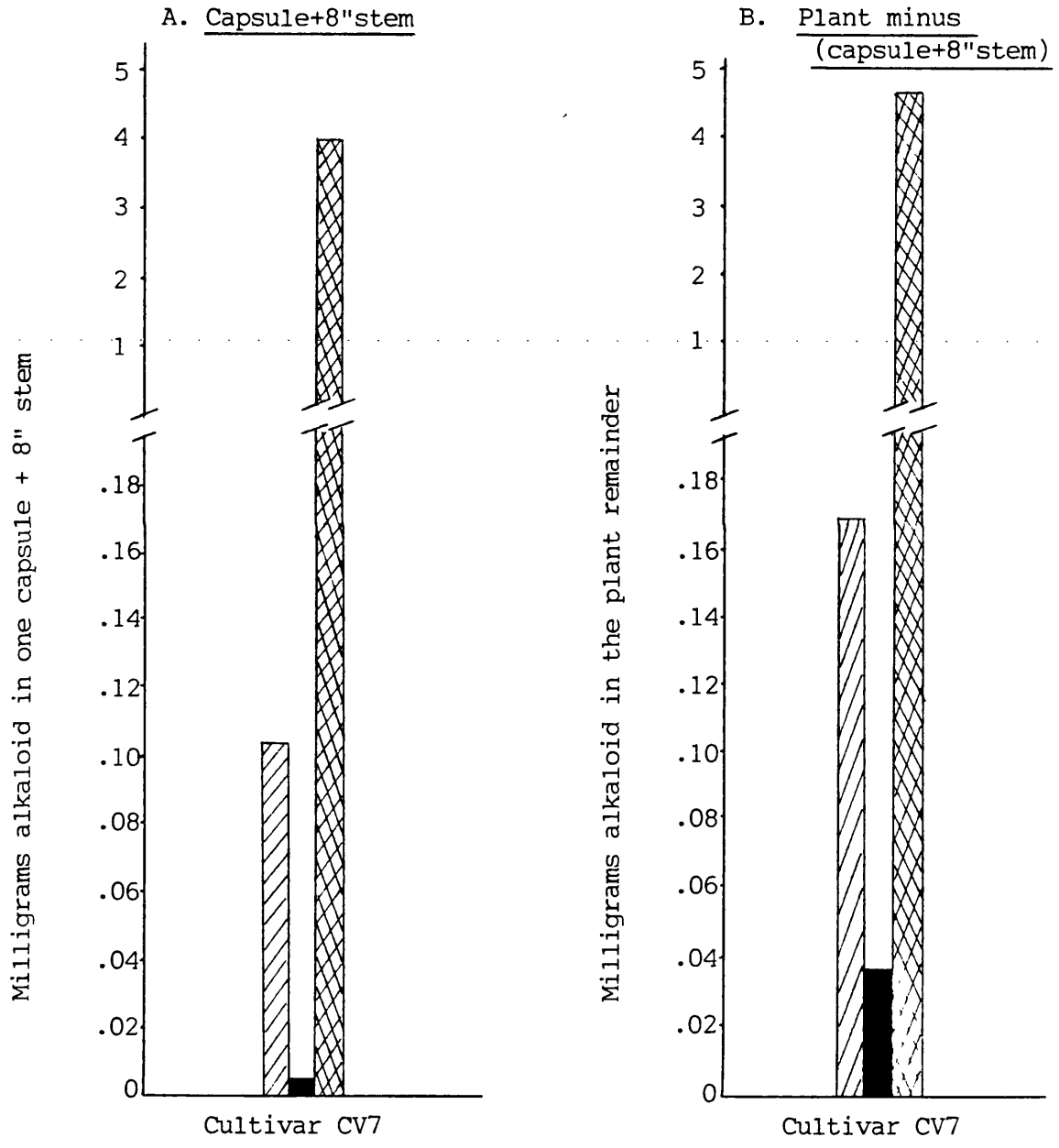
Figure 3.7 Comparison of alkaloid contents in the green capsule plus 8" stem and in the rest of the plant of *P. somniferum* cv. CV6



▨ noscapine , ■ papaverine , ▩ total morphinans

1 gram of ground dried material was taken from a random sample of 6 plants for quantitative analysis . Results are averages of duplicate determinations .

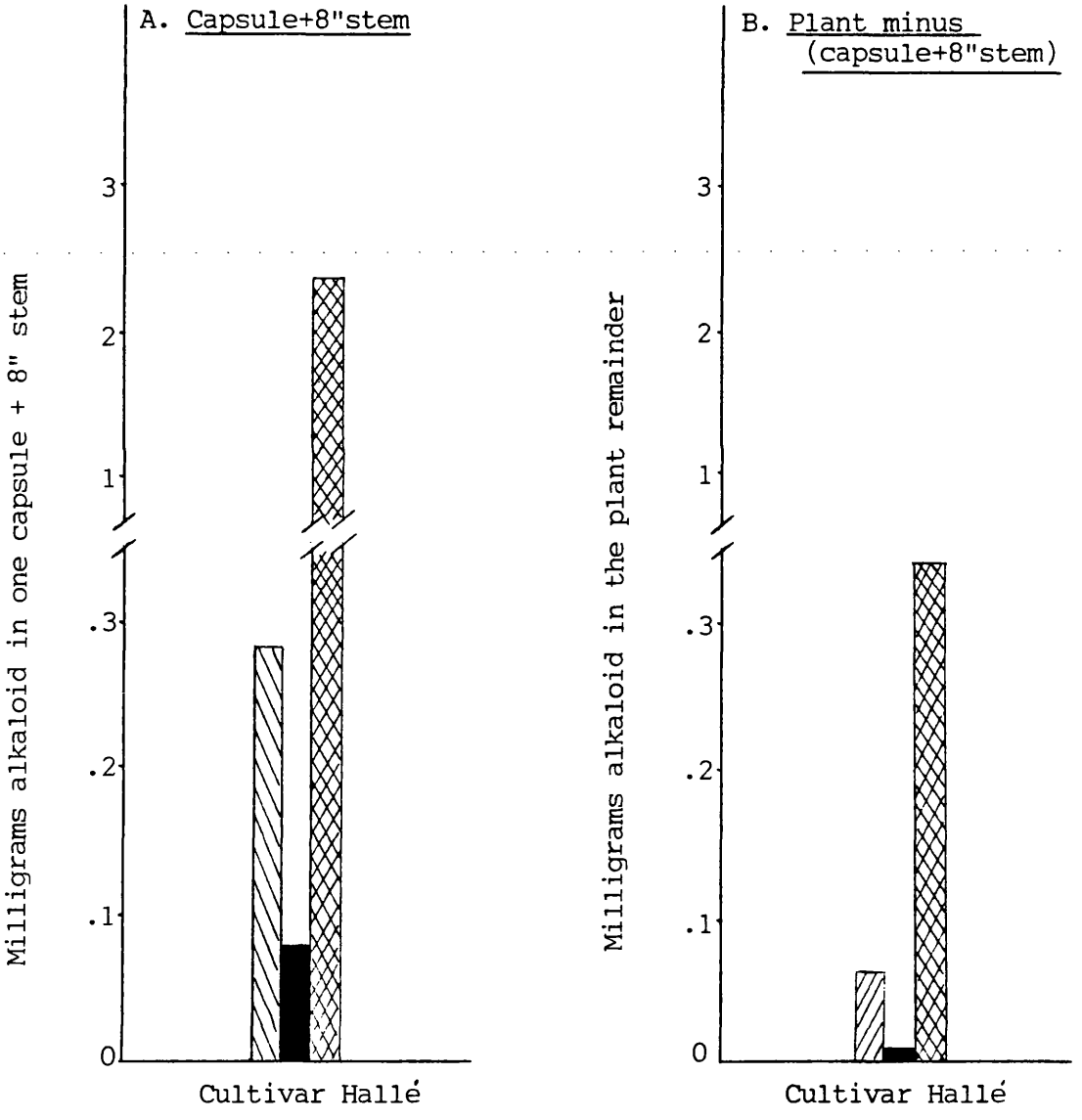
Figure 3.8 Comparison of alkaloid contents in the green capsule plus 8" stem and in the plant remainder of P. somniferum cv. CV7



▨ noscapine , ■ papaverine , ▩ total morphinans

1 gram of ground dried material was taken from a random sample of 6 plants for quantitative analysis . Results are averages of duplicate determinations .

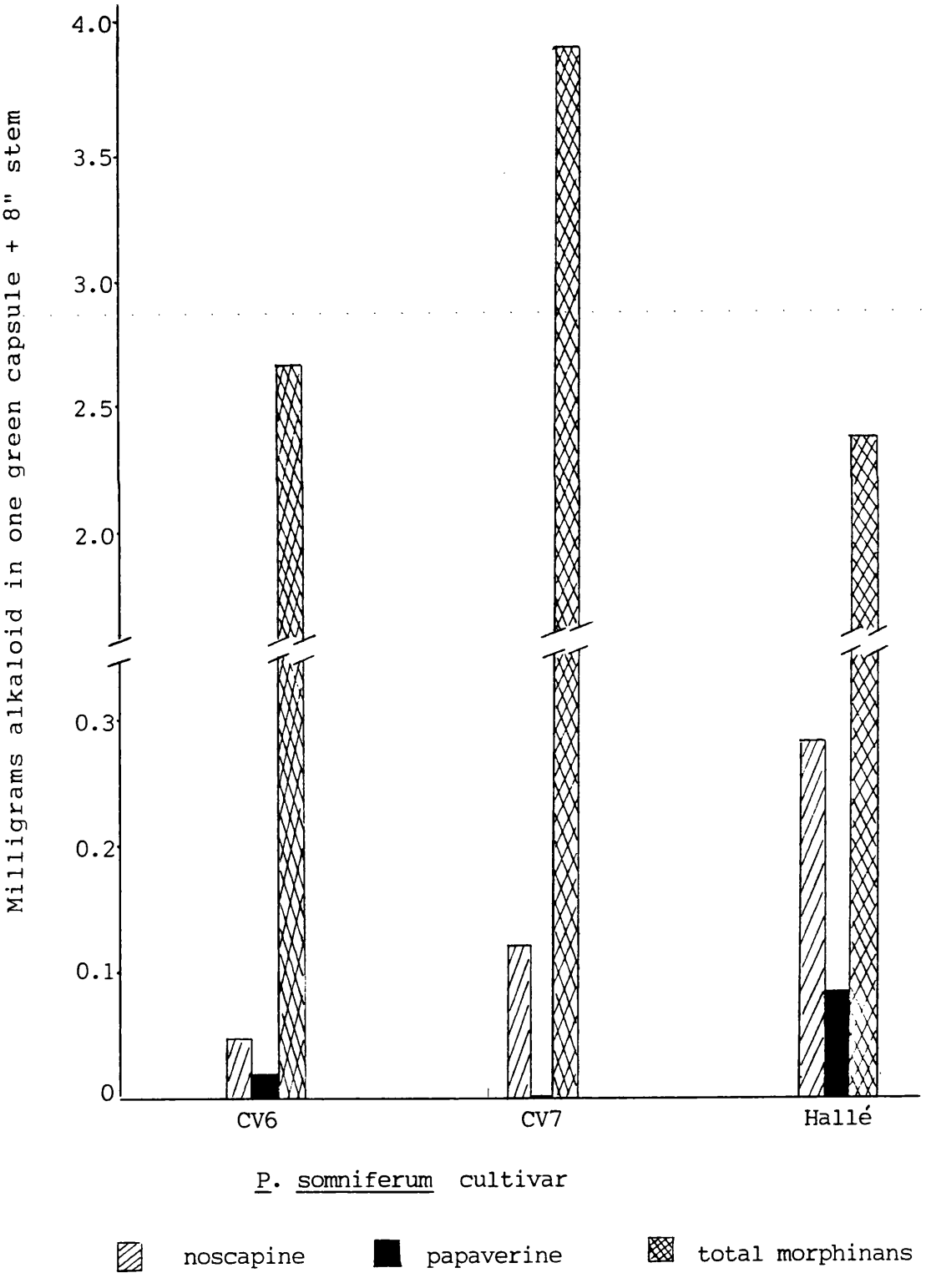
Figure 3.9 Comparison of alkaloid contents in the green capsule plus 8" stem and in the plant remainder of P. somniferum cv. Hallé



▨ noscapine , ■ papaverine , ▩ total morphinans

1 gram of ground dried material was taken from a random sample of 6 plants for quantitative analysis . Results are averages of duplicate determinations .

Figure 3.10 Comparison of alkaloid contents in the green capsules plus 8" stems of P. somniferum L. cv. CV6 , CV7 and Hallé plants



3.4. Seasonal Variation in Alkaloid Content in the Summers of 1988 and 1989 for *P. somniferum* Cultivars UL9, UL10 and UL11

Further investigation were made in the summers of 1988 and 1989 for the studies of seasonal variation in alkaloid content in *P. somniferum* using cultivars UL9, UL10 and UL11.

Seed samples coded UL9-11 were grown as described in Section 2.1.2 . The plants were unable to reach the ripe capsule stage. These plants were collected at the young plant, bud, flower and green capsule stages in the summer of 1988 for quantitative analysis of their alkaloid contents. The plants grown in the summer of 1989 were collected at the bud and young capsule stages for HPLC analysis. The weather data recorded in these two summers are given in Table 3.4 .

Alkaloid contents in the three *P. somniferum* cultivars UL9-11 increased as the plants developed to maturity in the summer of 1988 (Figs. 3.11, 3.12 and 3.13). The morphinan content rapidly increased whereas noscapine and papaverine levels were slowly built up as the plant developed. All these three cultivars produced more morphinan alkaloids than noscapine, and papaverine was the least significance. For example, approximately 0.2 mg total morphinans were found in a young UL9 plant whereas about 3.7 mg was detected in a whole plant at green capsule stage (Fig. 3.11). The increase in morphinans was rapid during the period in which the reproductive parts developed in all these three cultivars .

The investigated plant cultivars UL9-11 could produce high noscapine contents (ca. 0.25 - 0.39% in the capsules plus 8" stems) compared with less than 0.1% found in the same part of the high morphinan-producing cultivars CV6 and CV7 previously shown in Section 3.3 . The weather data given in Table 3.4 shows higher rainfall, and lower temperature sum and sunshine hours were observed in May and July of 1988. The UL9-11 plants collected in the summer of 1988 were larger in

size than those grown in the same period of 1989 (Table 3.5). The plants grown in the hot and sunny weather of 1989 produced more alkaloids in their capsules, on the dry weight basis, than those collected in the summer of 1988 (Table 3.6). High sunshine level and high temperature sum during the period the reproductive organ developed may have resulted in high alkaloid yield in the plant. High rainfall level, in contrast, only stimulated plant growth and hence less alkaloid in each unit weight of the plant (i.e. % dry weight). The marked increases in alkaloid contents in these investigated cultivars were the result of a hot but not very humid weather especially during the periods of flowering and capsule maturation. These results were also in line with those reported by Kopp (1957) and Kopp et al (1961). If the weather had allowed the plant to reach full maturity (ripe capsule), higher alkaloid content would have been expected in the capsule, as observed in these plant cultivars grown in Tasmania (unpublished work). Hofman and Menary (1979) showed rain can cause the washing out of alkaloids from the capsules. In the present excercise, the capsules were unable to reach ripe capsule stage due to poor weather. High rainfall levels were observed in May and July of 1988 . The green capsules collected in the summer of 1988 contained lower alkaloid contents than those in the hot and sunny weather of 1989, but it was not clear whether the wet weather of 1988 directly resulted in low alkaloid contents in those capsules. High sunshine level and high temperature sum throughout the summer season of 1989, however, may have more effect on alkaloid production by the plant .

Table 3.4 Weather data recorded in the summers of 1988 & 1989 (obtained from The Meteorological Office, Bracknell, Surrey and The School of Pharmacy Medicinal Plant Garden , Enfield , Middlesex)

Year	Growing season									
	summer 1988					summer 1989				
Period	April	May	June	July	August	April	May	June	July	August
Rainfall (cubic inches)	2.60	2.53	1.62	4.65	1.07	4.48	0.36	1.77	1.83	2.13
Temperature sum (°C)	904	2277	3866	5670	7487	799	2370	4016	6130	8310
Sunshine (hours/week)	147	210	143	148	198	168	296	251	243	228
Solar radiation ($MJ/m^2/day$)	47.4	75.5	60.4	59.6	62.5	54.6	86.5	80.3	76.9	67.8

Note:

Temperature sum = sum of the mean daily air temperatures

Solar radiation was estimated from the sun hours , with error ± 3-5%

Table 3.5 Dry weight of P. somniferum cultivars UL9-11 grown in the Summers of 1988 and 1989

Plant cultivar	Developmental stage	Average dry weight of one plant (grams)	
		summer 1988	summer 1989
UL 9	plant with bud	1.36	0.34
	plant with capsule	2.42	0.91
UL 10	plant with bud	1.84	0.48
	plant with capsule	2.86	0.95
UL 11	plant with bud	2.56	0.72
	plant with capsule	3.50	1.37

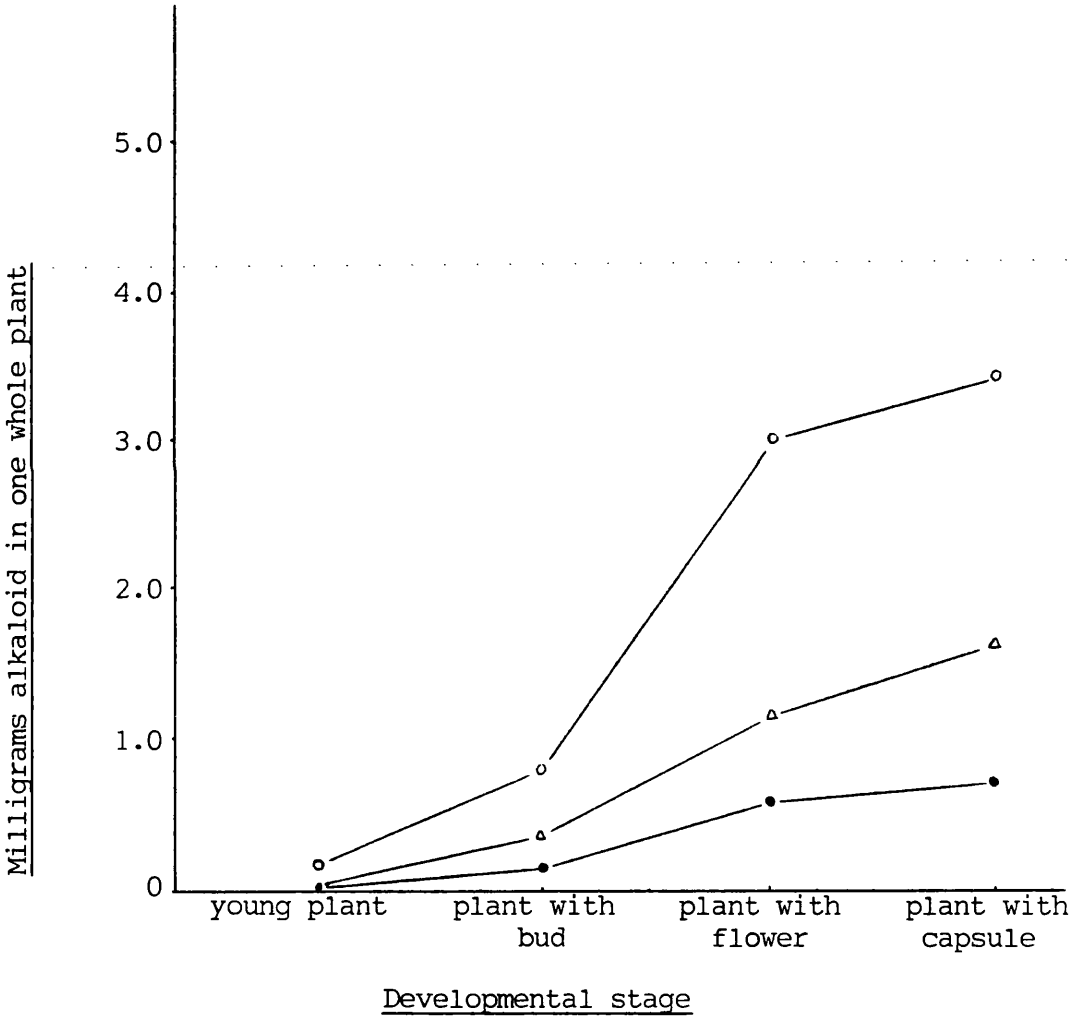
Note: Plants were dried in the oven at 40°C overnight.
A random sample of 6 plants at each stage.
Results are averages from duplicate determinations.

Table 3.6 Alkaloid contents in the capsules of P. somniferum cv. UL9-11 plants grown in the Summers of 1988 and 1989

Sample	Alkaloid	% dry weight in one capsule + 8" stem	
		summer 1988	summer 1989
UL9 capsule	Morphinans	0.34	0.43
	Noscapine	0.17	0.34
	Papaverine	0.06	0.09
UL10 capsule	Morphinans	0.38	0.47
	Noscapine	0.14	0.25
	Papaverine	0.03	0.05
UL11 capsule	Morphinans	0.49	0.59
	Noscapine	0.29	0.39
	Papaverine	0.11	0.18

Note: Plants were dried in the oven at 40°C overnight.
A random sample of 6 capsules.
Results are averages from duplicate determinations.

Figure 3.11 Variation of alkaloid content in P. somniferum cv. UL9 during plant development (Summer 1988)



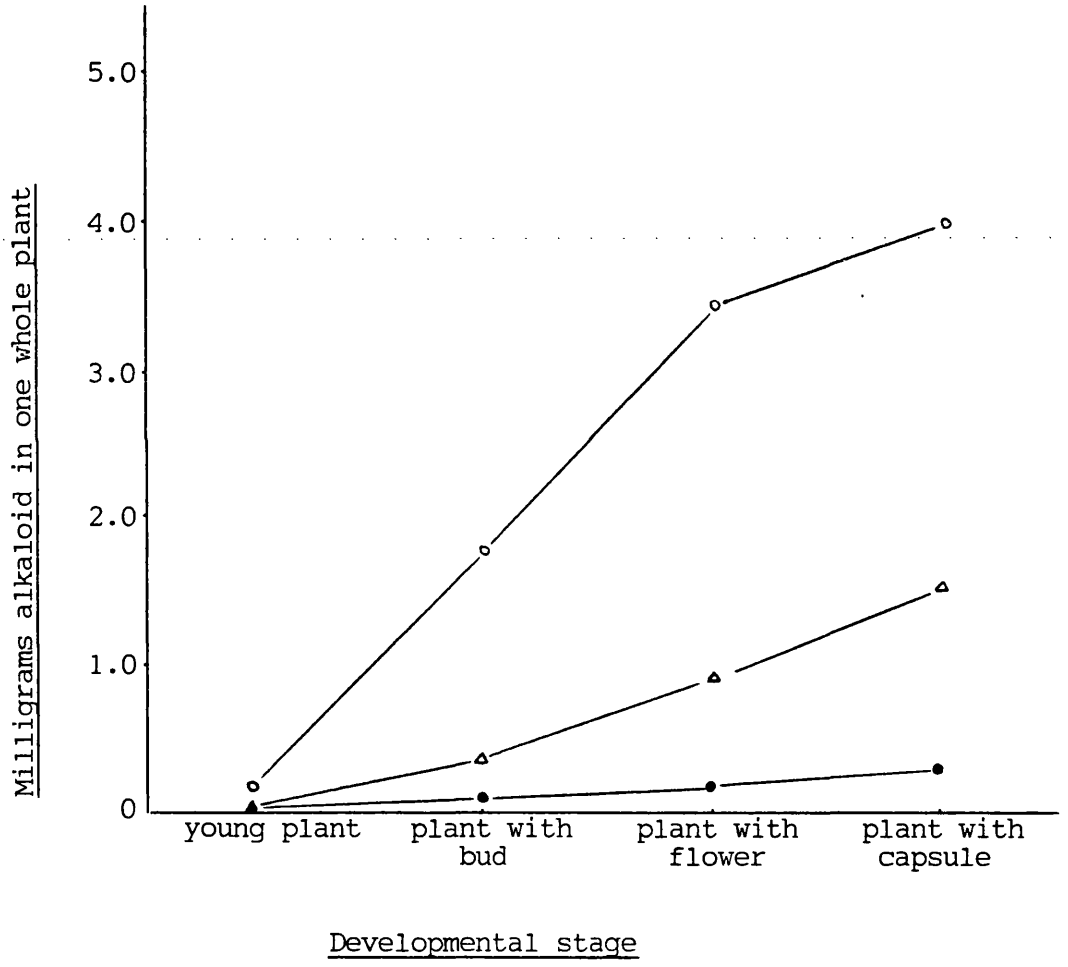
Key: o——o total morphinan, Δ——Δ noscapine, ●——● papaverine

A random sample of 6 plants at each stage.

Average dry weight of one plant: young plant(0.24g) , plant with bud(1.36g) , plant with flower(1.58g) , plant with capsule(2.41g).

Results are averages of duplicate determinations.

Figure 3.12 Variation of alkaloid content in P. somniferum cv. UL10 during plant development (Summer 1988)



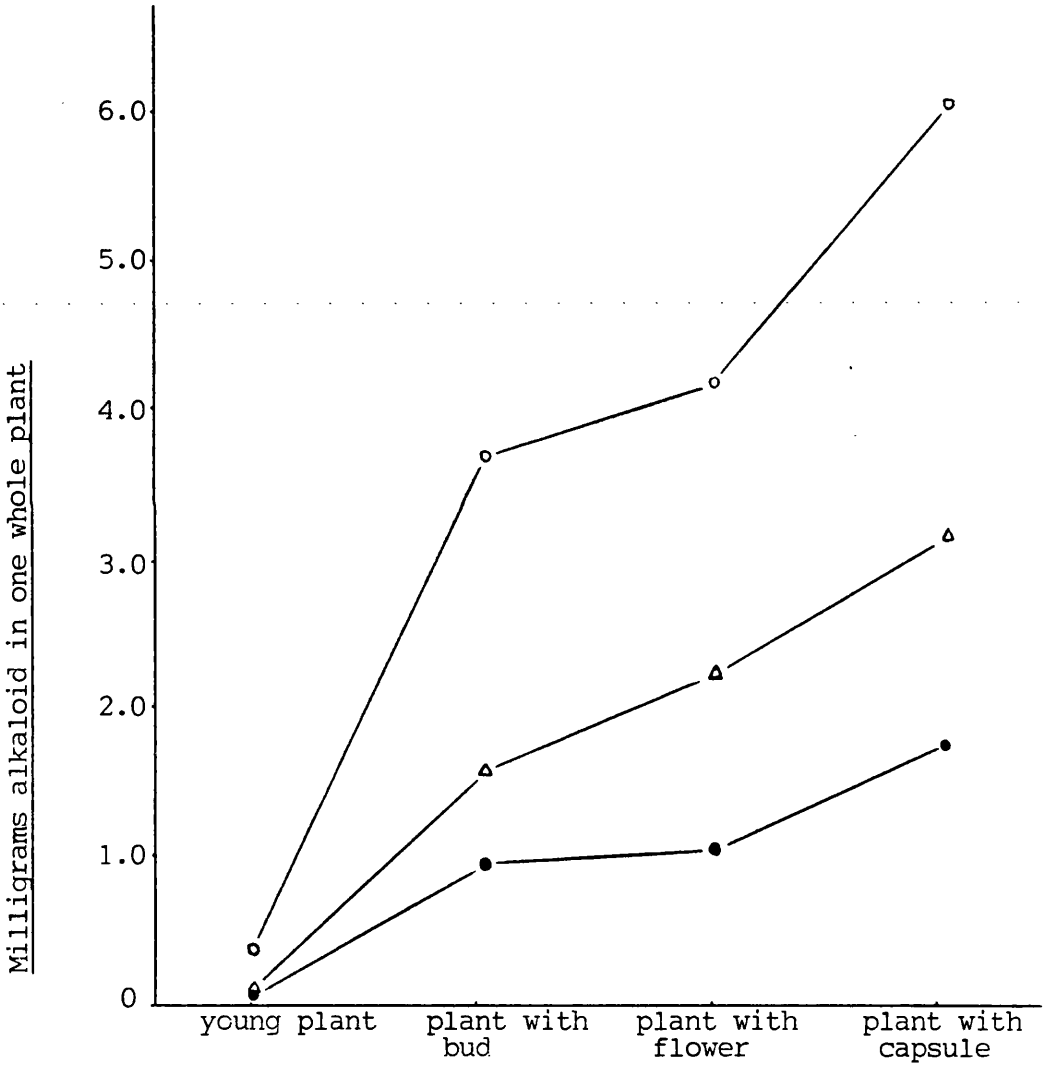
Key: o——o total morphinan , Δ——Δ noscapine , ●——● papaverine

A random sample of 6 plants at each stage.

Average dry weight of one plant: young plant(0.33g) , plant with bud(1.84g), plant with flower(1.95g) , plant with capsule(2.86g).

Results are averages of duplicate determinations.

Figure 3.13 Variation of alkaloid content in P. somniferum cv. UL11 during plant development (Summer 1988)



Key: o——o total morphinan , Δ——Δ noscapine , ●——● papaverine

A random sample of 6 plants at each stage.

Average dry weight of one plant: young plant(0.31g) , plant with bud(2.56g) , plant with flower(2.60g) , plant with capsule (3.51g) . Results are averages of duplicate determinations.

3.5. Investigation of Precursor Feeding as a Method of boosting Noscapine Content

3.5.1. Alkaloid contents in the plant capsules after feeding the proposed precursors

The unlabelled tetrahydrocolumbamine , tetrahydroberberine, berberine and hydrastine were separately fed to the *P. somniferum* cv. Halle plants by the method described in Section 2.4.1.2 . The alkaloids were extracted from the capsules plus 8" stems (random sample of 3 plants for each precursor) as described in Section 2.1.3, and then quantitatively analysed by normal-phase HPLC (Section 2.1.4.1). The results are given in Table 3.7 .

Only trace amounts of noscapine and papaverine were detected in control sample (less than 0.001 mg per one capsule). In contrast, significantly high morphinan content (ca. 15 mg/capsule) was found in the control case. The plants produced high levels of noscapine (1.1 - 1.6 mg/ capsule) when fed with hydrastine, tetrahydroberberine and berberine. Tetrahydrocolumbamine, however, did not have significant effect on noscapine production by the plant. It was also observed that more papaverine and slightly less morphinan alkaloids were found when the plants produced more noscapine, as seen in the samples fed with tetrahydroberberine, hydrastine and berberine (Table 3.7) .

3.5.2. Fate of non-utilised precursors

HPLC data for the precursors, previously shown in Fig. 3.1A (Section 3.1.1), indicates that tetrahydroberberine appears just after the solvent peak in the normal-phase HPLC elution chromatogram, and hydrastine and tetrahydrocolumbamine are seen just before and after thebaine, respectively . HPLC traces (Fig. 3.14) show less

Table 3.7 Alkaloid content in the capsule of P. somniferum cv. Hallé plant fed with the proposed precursor

Alkaloid	Milligrams alkaloid in one capsule + 8" stem				
	Control	Precursor			
		Tetrahydro-berberine	Tetrahydro-columbamine	Hydrastine	Berberine
Noscapine	$\simeq 10^{-3}$	1.68	0.08	1.31	1.14
Papaverine	$\simeq 10^{-3}$	0.15	0.13	0.56	0.24
Thebaine	2.40	0.84	1.38	0.65	2.07
Codeine	0.78	0.84	0.77	0.83	0.83
Morphine	11.95	9.35	11.26	11.26	10.00
Total morphinans	15.13	11.03	13.41	12.74	12.90

Note: A random sample of 3 plants in each case. The top part (capsule + 8" stem) was collected for HPLC analysis. Results are averages of duplicate determinations.

thebaine was formed in the plants fed with the proposed precursors, and no tetrahydroberberine was detected in all alkaloid extracts. Tetrahydroberberine, hydrastine and tetrahydrocolumbamine were not present in the alkaloid extracts after the feeding experiments, using the extraction methods described in Section 2.1.3 .

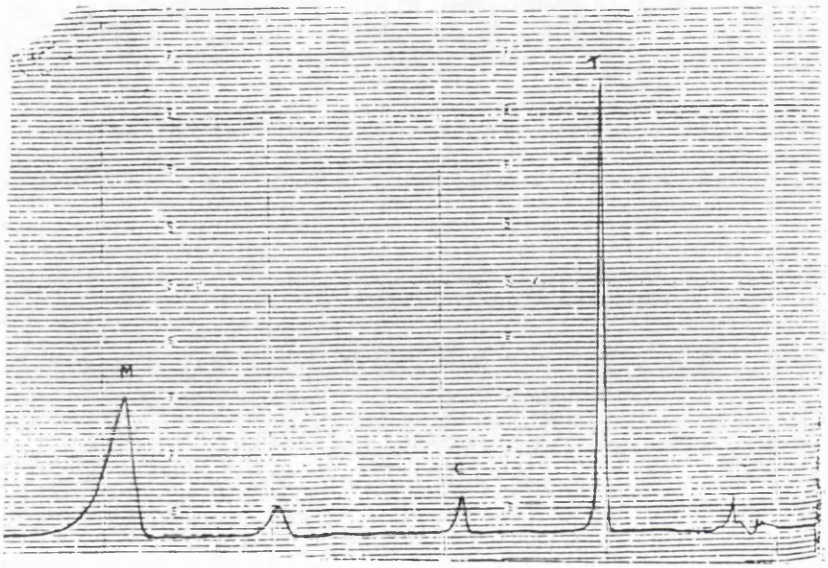
Berberine, however, was not resolved by both normal and reverse-phase systems described in Section 2.1.4 . Since berberine was tightly bound to the column material in the normal-phase and eluted together with the solvent in the described reverse-phase HPLC system (Fig. 3.1B) . The unused berberine therefore cannot be detected by the methods shown in the present studies .

3.5.3. Suggested involvement of hydrastine , tetrahydroberberine and berberine in the biosynthesis of noscapine

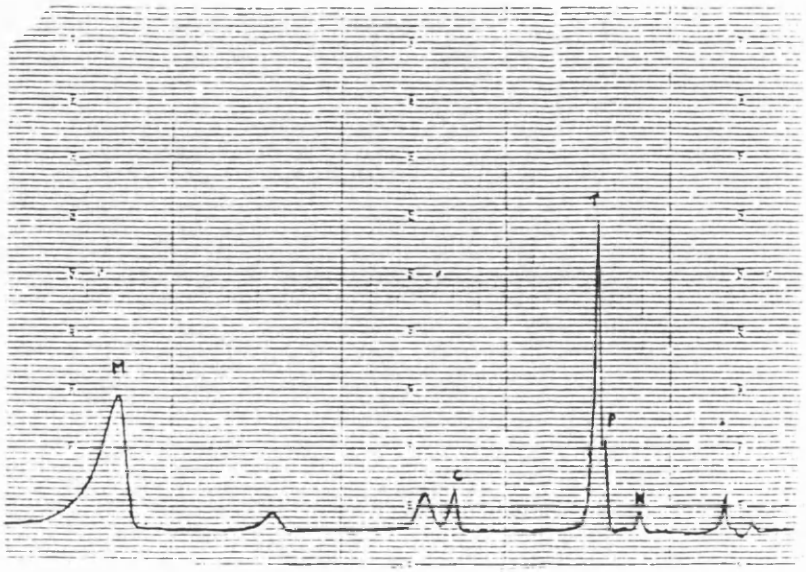
The results from this series of experiments suggest that hydrastine, tetrahydroberberine and berberine may be biotransformed to noscapine when they were fed to the plant. It has been established that the protoberberine alkaloid scoulerine, is a precursor of noscapine in *P. somniferum* (Fig. 1.11 , Section 1.6.8) . In the present studies, tetrahydroberberine was the best precursor for improving noscapine yield in *P. somniferum* plants . Hydrastine, which is an 8-demethoxylated form of noscapine, was also a good precursor for improving noscapine production. Better result was observed with tetrahydroberberine than with hydrastine, and this could be presumably due to the hindrance of the substitution C-8 position by the rings C and D in the hydrastine molecule . The results also suggested that berberine could be biotransformed to noscapine, which suggested that the reduction of ring C of berberine to tetrahydroberberine may be required in the biosynthetic sequence to noscapine formation .

Figure 3.14 HPLC elution chromatograms of alkaloid extracts after feeding experiment

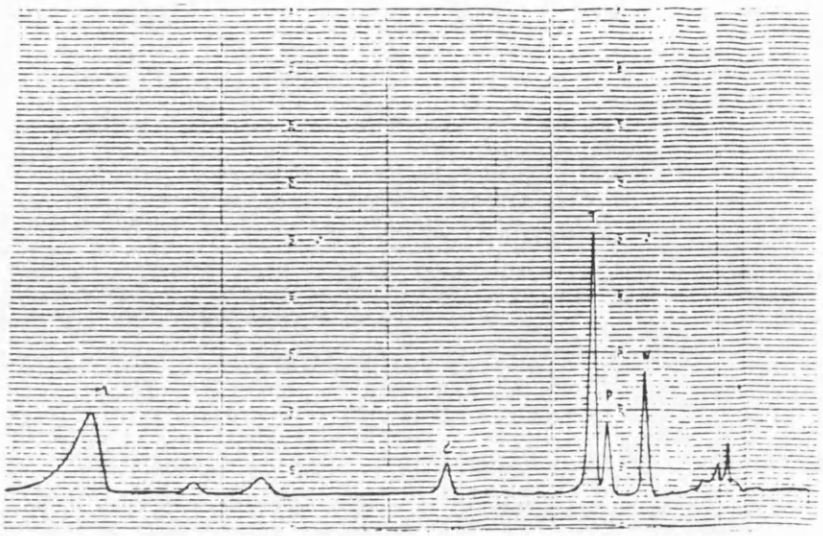
A. Control



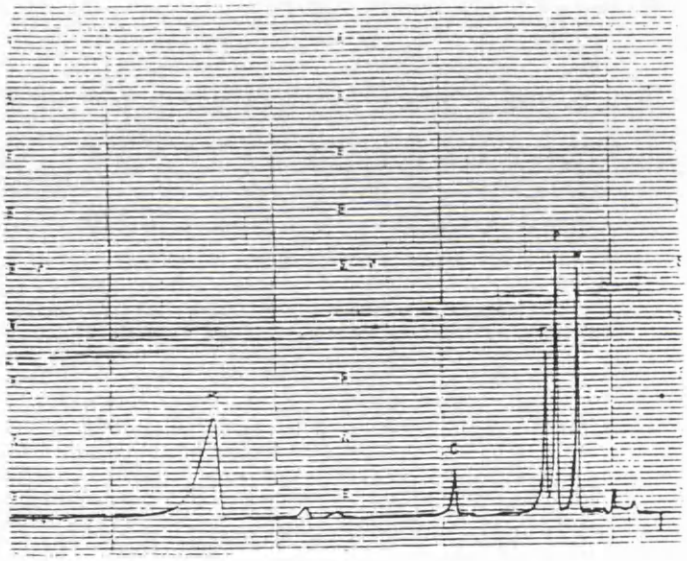
B. Tetrahydro-columbamine



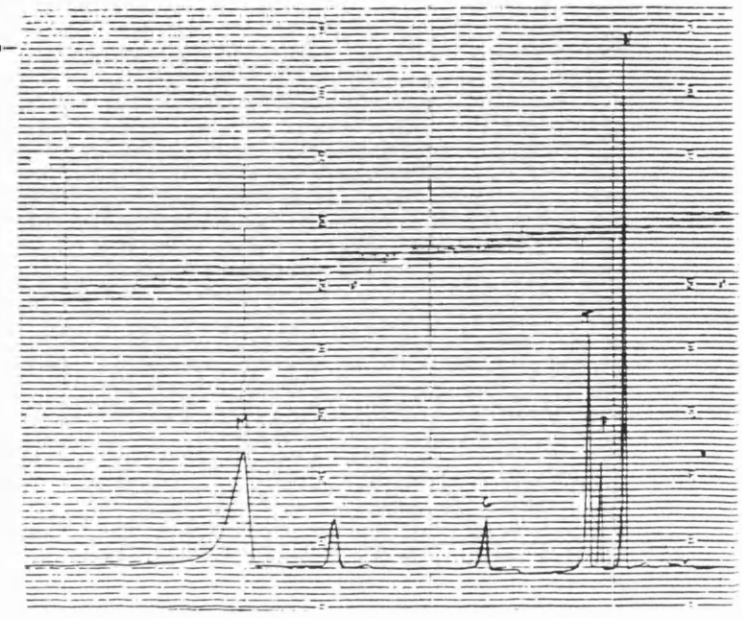
C. Berberine



D. Hydrastine



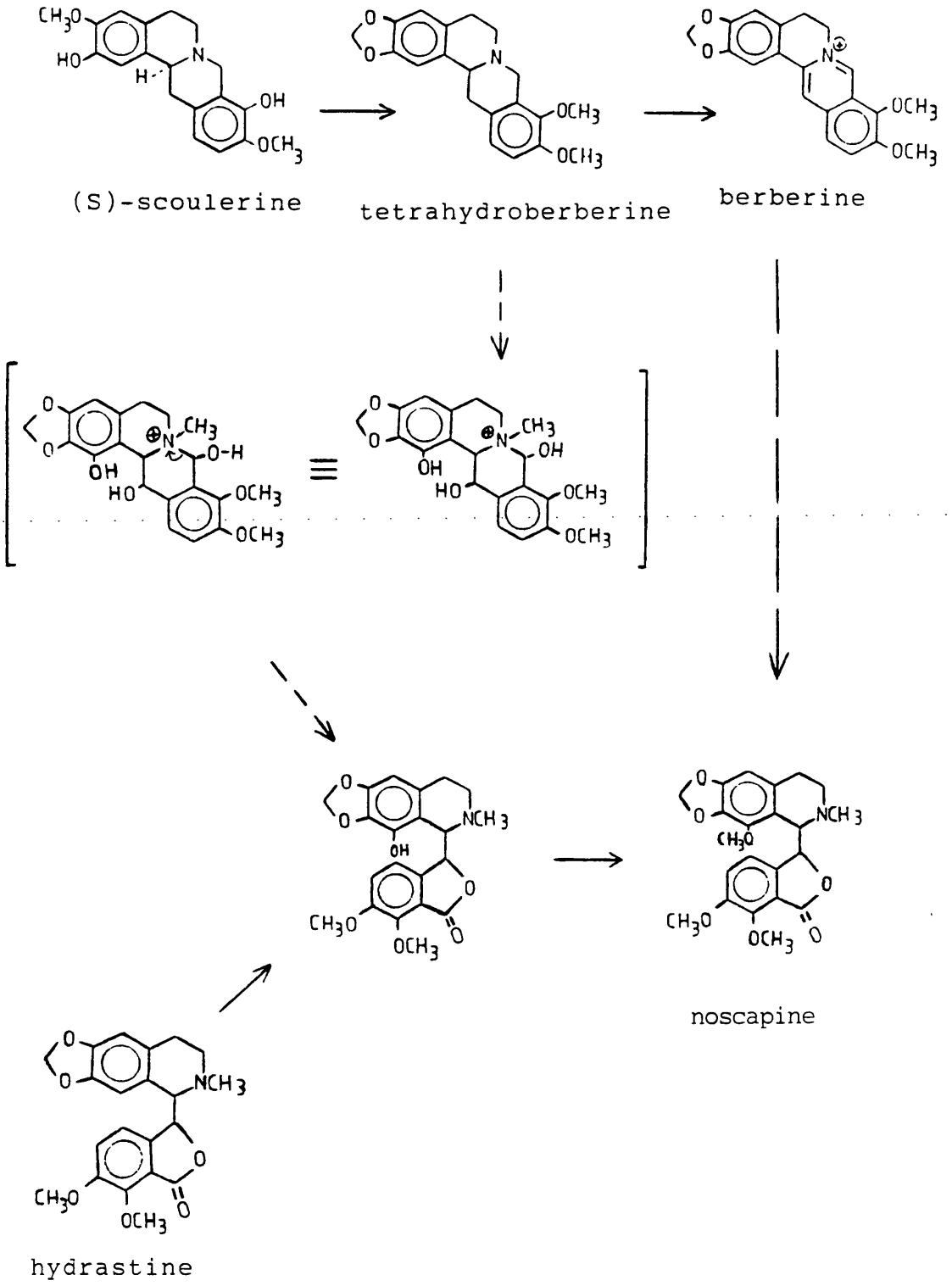
E. Tetrahydro-
berberine



Tetrahydrocolumbamine, as a potential precursor of noscapine, had negligible effect on noscapine yield. In *Coptis* species, tetrahydrocolumbamine was converted to a tetrahydroberberine (canadine) by canadine synthase prior to the formation of berberine (Galneder et al 1988 , Okada et al 1988). The results from the present studies suggested that the enzyme which converts tetrahydrocolumbamine to tetrahydroberberine, by forming a methylene-dioxy bridge on ring A, was not very active in *P. somniferum*. As a result, only trace amount of noscapine was found when the plant was fed tetrahydrocolumbamine, while tetrahydroberberine markedly improved the yield of noscapine .

The high incorporation of hydrastine into noscapine also suggested the N-methylation of tetrahydroberberine is one of the steps required for the biotransformation of tetrahydroberberine to noscapine. Its improvement of noscapine yield would also suggest that the methoxylation at C-8 of the isoquinoline moiety of noscapine occurs at a late stage in the biosynthetic pathway. As mentioned in Sections 1.6.3 and 1.6.4 that tetrahydroberberine (canadine) is the branching point for the formation of berberine and protopine which is further biotransformed to benzophenanthridines and rhoeadine/papaverrubine. The results in the present studies suggested another pathway from tetrahydroberberine to noscapine (phthalideisoquinoline) in which the N-methylation of tetrahydroberberine was required in the biosynthetic sequences to noscapine .

Further studies are required to establish the actual sequence in the biosynthesis of noscapine, by the isolation of the appropriate enzymes. However, based on the results from feeding experiments in the present studies, the steps involved in the biosynthesis of noscapine are suggested as follows :



Suggested steps involved in the biosynthesis of noscapine from (S)-scoulerine and from hydrastine

3.6. Studies of Sequestration of Alkaloids in *P. somniferum* L.

The latex is easily obtained by cutting off the capsule and harvesting the exuded latex.

In this series of experiments, the latex collectd from the capsule has been found to contain two distinct groups of alkaloid-containing vacuoles. These vacuoles were isolated by fractionation and their volumes were determined by the method given in Sections 2.2.1 and 2.2.2 . These fractionated vacuoles were designated the 900xg and 1100xg vacuoles. The remaining part of the latex was referred to as the latex supernatant. The latex vacuoles were investigated over the first three weeks of capsule development after petal opening (week 0). Quantitative analysis of alkaloids, dopamine, acids, cations and anions was undertaken to investigate the relationship between alkaloid accumulation and the occurrence of organic acids in the latex vacuoles. An attempt has also been made to determine whether the alkaloids penetrate the latex vacuolar membrane by diffusion with entrapment in the acidic vacuolar compartment by protonation and formation of non-diffusable salts or whether alkaloid uptake across the vacuolar membrane is the result of catalysed transport. The mechanism of alkaloid sequestration in the latex vacuoles was investigated by studying the uptake of acids and alkaloids by the 900xg and 1100xg vacuoles of the latex, and the effects of ATP/Mg^{2+}, ATPase inhibitors and temperature on morphine uptake by the latex vacuoles. Morphine was especially used for the studies of alkaloid sequestration in *P. somniferum* latex vacuoles because of the availability of ^{14}C- labelled morphine and also because it is the most abundant alkaloid in this plant .

3.6.1. Vacuolar storage in *P. somniferum* latex

3.6.1.1. Comparison of alkaloid levels in the 900xg and 1100xg vacuoles during capsule maturation

The latex was collected at 0, 1, 2 and 3 weeks after petal opening. The latex vacuoles were isolated by the method given in Section 2.2.1 . The alkaloids were extracted and quantified as described in Section 2.2.3 . The alkaloid contents in the 900xg and 1100xg vacuoles are given in Table 3.8 . The variations of alkaloid levels in these vacuoles during the period from 0 to 3 weeks after flowering are shown in Figs. 3.15A and 3.15B . In contrast, alkaloids were not detected in the supernatant fraction of the latex under the same experimental conditions.

The results shown in Table 3.8 clearly demonstrate that the levels of the five main alkaloids (morphine, codeine, thebaine, papaverine and noscapine) in both the 900xg and 1100xg vacuoles of the latex increased as the capsule developed during the first three weeks after petal opening. Increases in alkaloid content, especially morphine are most significant in the 900xg vacuoles which contain the major part of the capsule alkaloid content. Morphine constituted about 66-80% of the total five main alkaloids in the 900xg latex vacuoles. Similarly, approximately 70-82% of the total five major alkaloids in the 1100xg fraction was morphine. Papaverine, in contrast, was the last significant alkaloid, and its level reached only about 16 mM compared to ca. 420 mM of morphine in the 900xg vacuoles at 3 weeks after petal fall. Noscapine concentration was also low (34 mM) in the 900xg fraction at maturity. In the 900xg vacuoles, alkaloid levels doubled from week 0 to week 3 during capsule maturation, whereas those in the 1100xg population remained fairly constant. These results are in keeping with those found previously by Fairbairn and Steele (1981 and referenes therein). Of particular interest however is the fact that

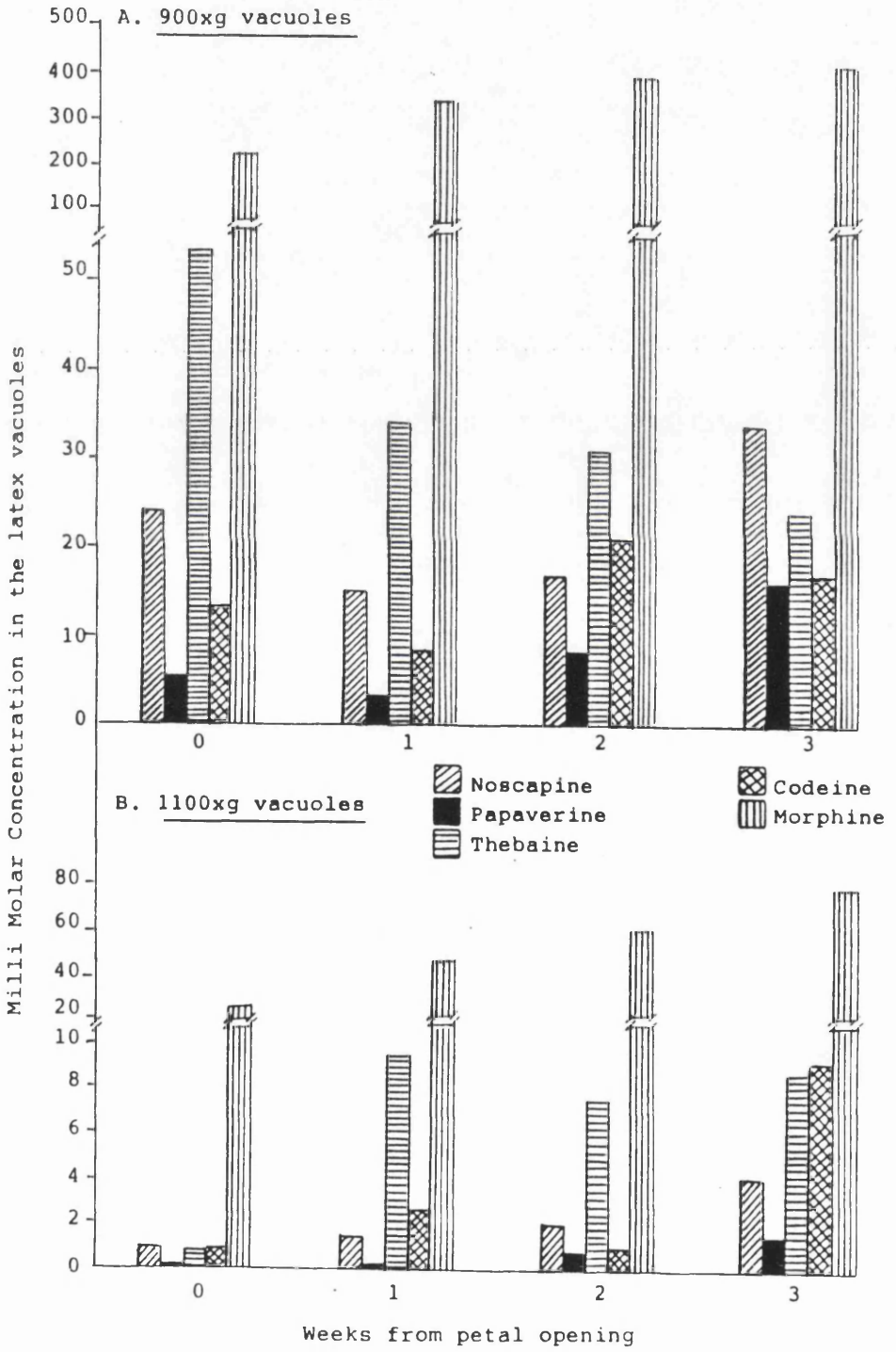
within a unit amount of latex (200 µl latex in buffer, 1:1 ratio), vacuolar volume increased substantially for the 900xg vacuole fraction; whereas a slight reduction in vacuolar volume for the 1100xg vacuoles was observed (Table 3.8). The increase in concentration of alkaloids in the 1100xg fraction is possibly due to a decrease in vacuolar volume. In contrast, the increase in the alkaloid concentration (220-429 mM for morphine) within the 900xg vacuoles is parallel with the increase in vacuolar volume during capsule maturation. Whether the increase in vacuolar volume per unit volume of latex is related to an increase in volume of the existing vacuoles or whether it relates to an increase in the number of vacuoles per unit volume of latex cannot be determined by the present methods.

Table 3.8 A comparison of alkaloid contents in the latex vacuoles with changes in vacuolar volume during capsule maturation

	milligrams alkaloid/ ml latex							
	900 x g vacuoles				1100 x g vacuoles			
week / Alkaloid	0	1	2	3	0	1	2	3
Morphine	34.25	56.44	78.73	96.71	2.25	4.08	3.92	4.22
Codeine	2.27	1.59	4.11	4.23	0.09	0.25	0.07	0.08
Thebaine	9.15	6.31	6.79	6.23	0.80	0.90	0.52	0.50
Papaverine	0.90	0.74	2.07	4.45	0.02	0.05	0.07	0.11
Noscapine	5.29	3.61	4.95	11.76	0.11	0.20	0.19	0.32
Vacuolar Volume ($\mu l/ml^{-1}$ latex)	541	586	701	817	320	310	229	188

Note: The vacuoles were isolated from randomly collected latex. Vacuolar volumes were determined as given in Section 2.2.2 . Results are averages of duplicate determinations.

Figure 3.15 Changes in concentration of alkaloids in *P. somniferum* latex vacuoles during capsule maturation



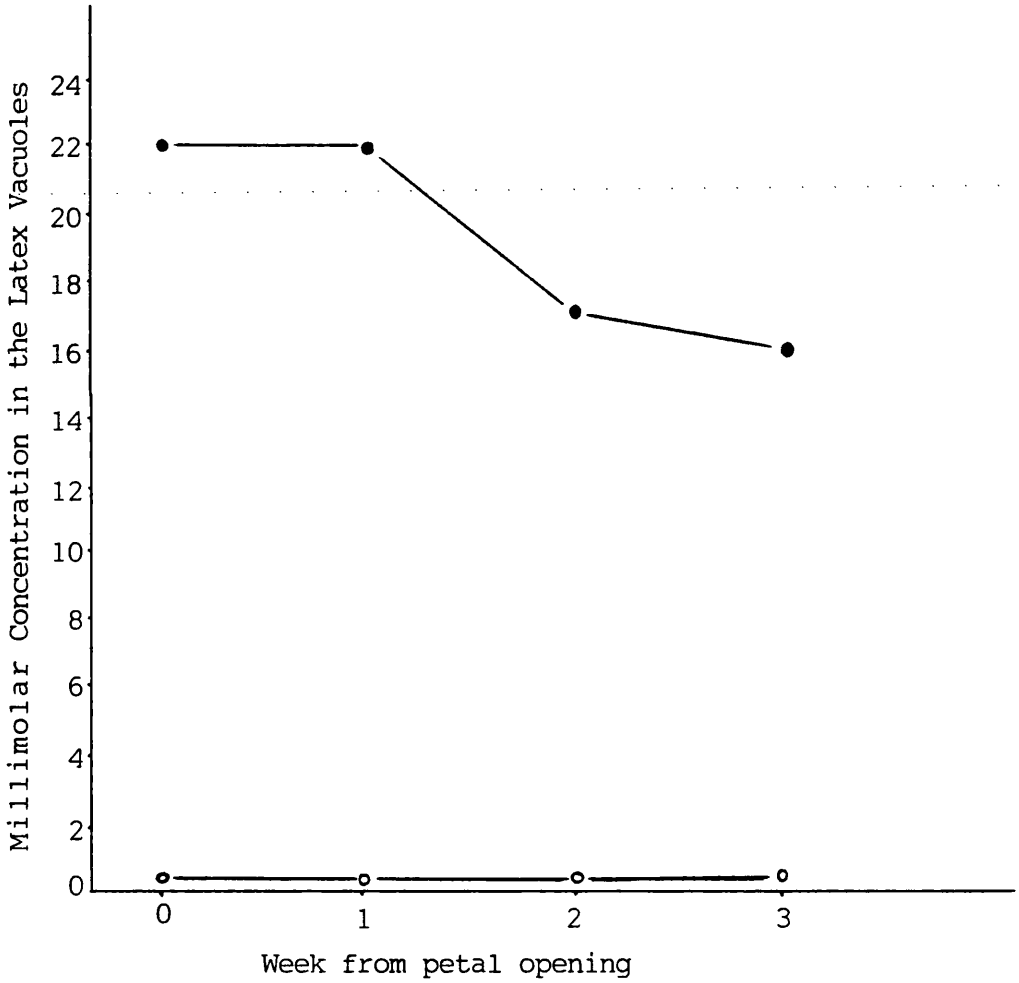
These figures have been calculated using the results shown in Table 3.8 .

3.6.1.2. Dopamine in the latex vacuoles

The 900xg and 1100xg vacuoles from *P. somniferum* latex were isolated as previously described (Section 2.2.1). Dopamine was isolated and quantified by the method given in Section 2.2.4 . Dopamine content in the 900xg population of the latex, collected at weeks 0, 1, 2 and 3 after flowering, is shown in Fig. 3.16 .

Dopamine concentration in the 900xg vacuoles of the latex slightly decreased from ca. 22 mM to ca. 16 mM from week 1 to 3 after petal fall, whereas the alkaloid content increased with capsule maturity in the same vacuole fraction. Only trace amount of dopamine (ca. 0.1-0.5 mM) was found in the 1100xg population. In contrast, dopamine was not detected in the latex supernatant under the same experimental conditions.

Figure 3.16 Variation of dopamine level in the latex vacuoles during capsule maturation



Key:

●——● 900 x g vacuoles

○——○ 1100 x g vacuoles

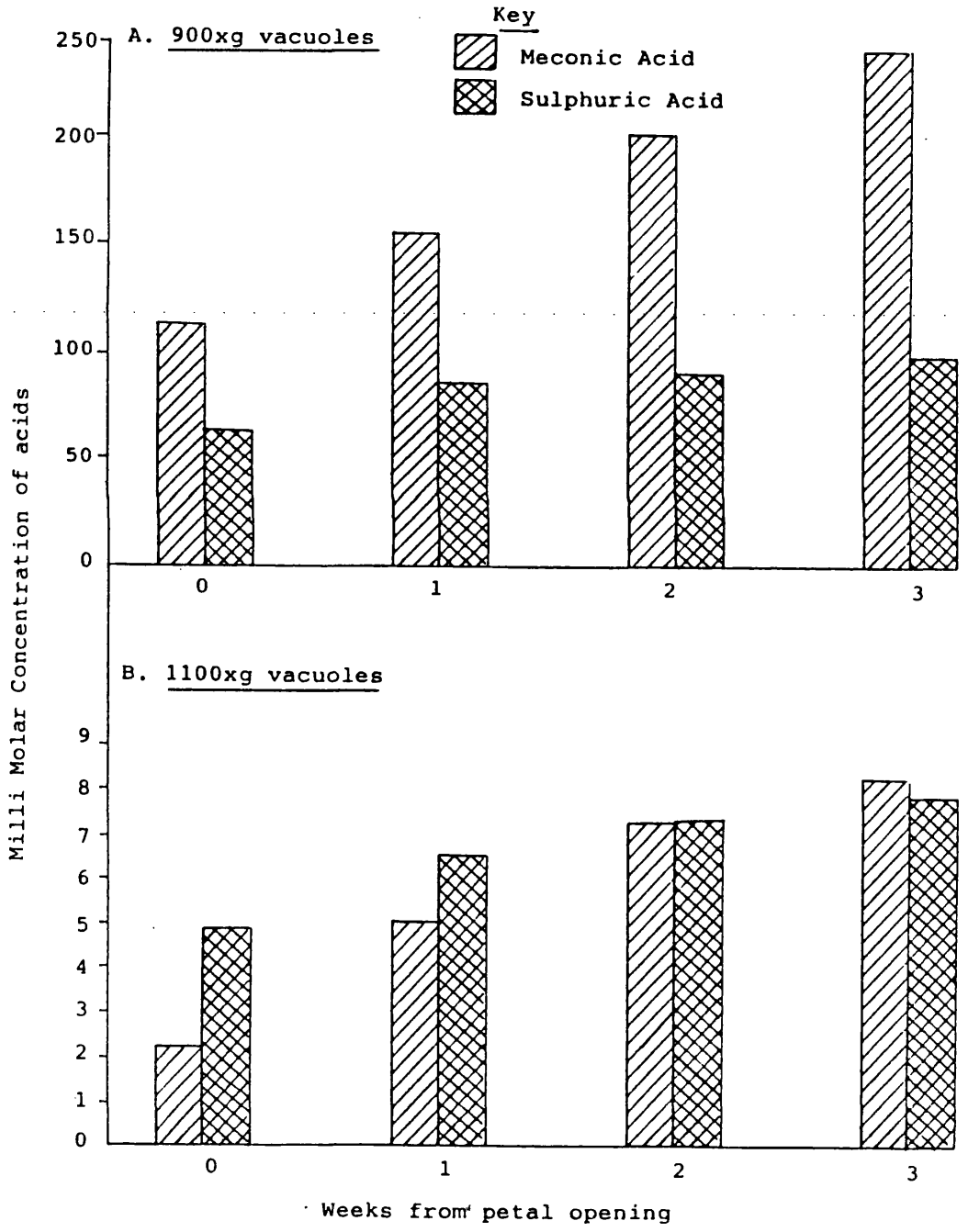
The results are averages from duplicate determinations. Latex were randomly collected at each stage and the same samples of latex as were used for Table 3.8 .

3.6.1.3. Meconic and sulphuric acid in the latex vacuoles

Meconic acid was isolated from the 900xg and 1100xg vacuoles by the method described in Section 2.2.5 . Levels of meconic acid found in these latex vacuoles after petal opening are shown in Figs. 3.17 A&B . The latex supernatant, in contrast, did not contain meconate under the same experimental conditions.

Previous researches have indicated that meconate and sulphate constitute 60-80% of the acid content, and meconic acid occurs to the extent of 5-7% in opium (Miyamoto and Brochmann-Hanssen 1962). Results given in Figs. 3.17 A&B show that meconate and sulphate occurred almost exclusively in the 900xg vacuoles of the latex although small amounts were also found in the 1100xg fraction. In both vacuole populations, levels of meconate and sulphate increased with the maturation of the poppy capsule. Sulphate content in the 900xg vacuoles was approximately half of meconate level, whereas more sulphate than meconate was found in the 1100xg population as the plant reached maturity (3 weeks after petal fall).

Figure 3.17 Relative concentration of major acids in the latex vacuoles during capsule maturation



These results were obtained from the same samples of latex as were used for Table 3.8 .

3.6.1.4. Malic acid in the latex vacuoles

Malic acid was isolated from the latex vacuoles by the method given in Section 2.2.7.1 and identified by paper chromatography (Section 2.2.7.2). The quantitation of malic acid was carried out by enzymatic method (Section 2.2.7.3). Extract from the 900xg vacuoles was found to contain meconate (R_f=0.35) and malate (R_f=0.30). The quantitation of malic acid was then carried out by enzymatic method described in Section 2.2.7.3 . Approximately 10 mM malic acid was found in the 900xg vacuoles from the latex 2-3 weeks after petal fall. No malic acid was detected in the 1100xg population and in the latex supernatant under the same experimental conditions used for the acid extract from the 900xg pellet. In contrast to meconate and sulphate, malate was found to be a minor acid in the latex vacuoles.

3.6.1.5. Total phenolics in the latex vacuoles

Organic acids were separated from the alkaloids by the method described in Section 2.2.5 . The assay for total phenolics was carried out as given in Section 2.2.8 after an adjustment for meconate which interferes in the test had been made.

Using the Folin-Ciocalteu method for the assay of total phenolics it could be calculated on the basis of tannic acid that low levels of phenolic substances existed in the 900xg vacuoles. The concentrations within the vacuoles at weeks 0-3 of capsule development were shown in Table 3.9 . Similar estimations using the 1100xg vacuoles did not yield positive results after the necessary adjustments for meconic acid had been made. Phenolic acids appear to be a minor component of the *Papaver* latex vacuoles as compared with the occurrence of meconic acid and sulphate.

Table 3.9 Phenolate content in the 900xg vacuoles of *P. somniferum* latex

week after petal fall	millimolar concentration in the 900xg vacuoles
1	1.6
2	2.9
3	4.6
4	6.4

3.6.1.6. Inorganic cations in the latex

Inorganic cations K^+, Na^+, Ca^{2+} and Mg^{2+} were measured in both vacuole fractions and in the latex supernatant. The results given in Table 3.10 show there was no significant difference in the levels of K^+ among the three populations of the latex. The concentrations of Na^+ and Ca^{2+} were significantly high inside the vacuoles. A very high level of Na^+ in the 900xg vacuoles (about 100 times of that in the supernatant) decreased from nearly 400 mM to about 250 mM during the three week maturation of the capsule. Na^+ content in the 900xg fraction was over twice that of the 1100xg vacuoles at petal opening (week 0) and then declining to near the same concentration at week 3. Ca^{2+} concentration in the 900xg vacuoles was 2-3 times those found in the 1100xg fraction and about 30-60 times those in the supernatant.

In *Catharanthus rosus*, malic and citric acid were the main organic acids, and Ca^{2+} was found almost exclusively in the cell vacuoles with about 68% of the K^+ and Mg^{2+} (Renaudin et al 1986). K^+ concentration in the *Beta vulgaris* vacuole was also high and only about 50% of the K^+ was located in the vacuole (Leigh and Tomos 1983). Na^+ level in the vacuoles of *Beta vulgaris* was also high (Leigh and Tomos 1983), and Ca^{2+} concentrations were maintained at low concentration in the cytosol as a part of the role of calcium as an intracellular signal (Bush and Sze 1986). Since in many higher plant cells the large central vacuole enables the accumulation and storage of osmotica which would otherwise interfere with the cytoplasmic processes, it would not be supprising if the latex vacuoles had a similar function with particular specialisation for alkaloid storage. It is not uncommon for both Na^+ and Ca^{2+} to accumulate in the central vacuole of the cell (Poole and Blumwald 1986). The results found in the present studies were also in line with those shown by these groups but whether there is any correlation

Table 3.10 Concentrations of inorganic cations in *P. somniferum* latex

Fraction	Week from Petal Opening	Millimolar Concentration			
		K^+	Na^+	Mg^{2+}	Ca^{2+}
900 x g vacuoles	0	56.0	388.7	10.0	53.3
	1	90.7	367.3	7.3	43.3
	2	58.0	266.7	10.0	65.3
	3	55.0	254.7	8.0	51.7
1100 x g vacuoles	0	39.1	151.3	5.0	10.9
	1	27.0	181.7	3.9	17.4
	2	40.9	159.1	2.9	14.8
	3	60.9	187.0	5.1	16.5
Supernatant	0	29 ↓ 58	1.2	1.7	0.6
	1		2.4	1.3	0.9
	2		2.2	1.6	0.8
	3		3.7	1.4	1.8

Samples were taken from the randomly collected latex used for the experiments shown in Table 3.8 .

The results are from duplicate samples at any one week .

between any losses of inorganic cations from the latex vacuole and alkaloid accumulation has yet to be determined.

3.6.1.7. Correlation between acidic and basic compounds in the latex vacuoles

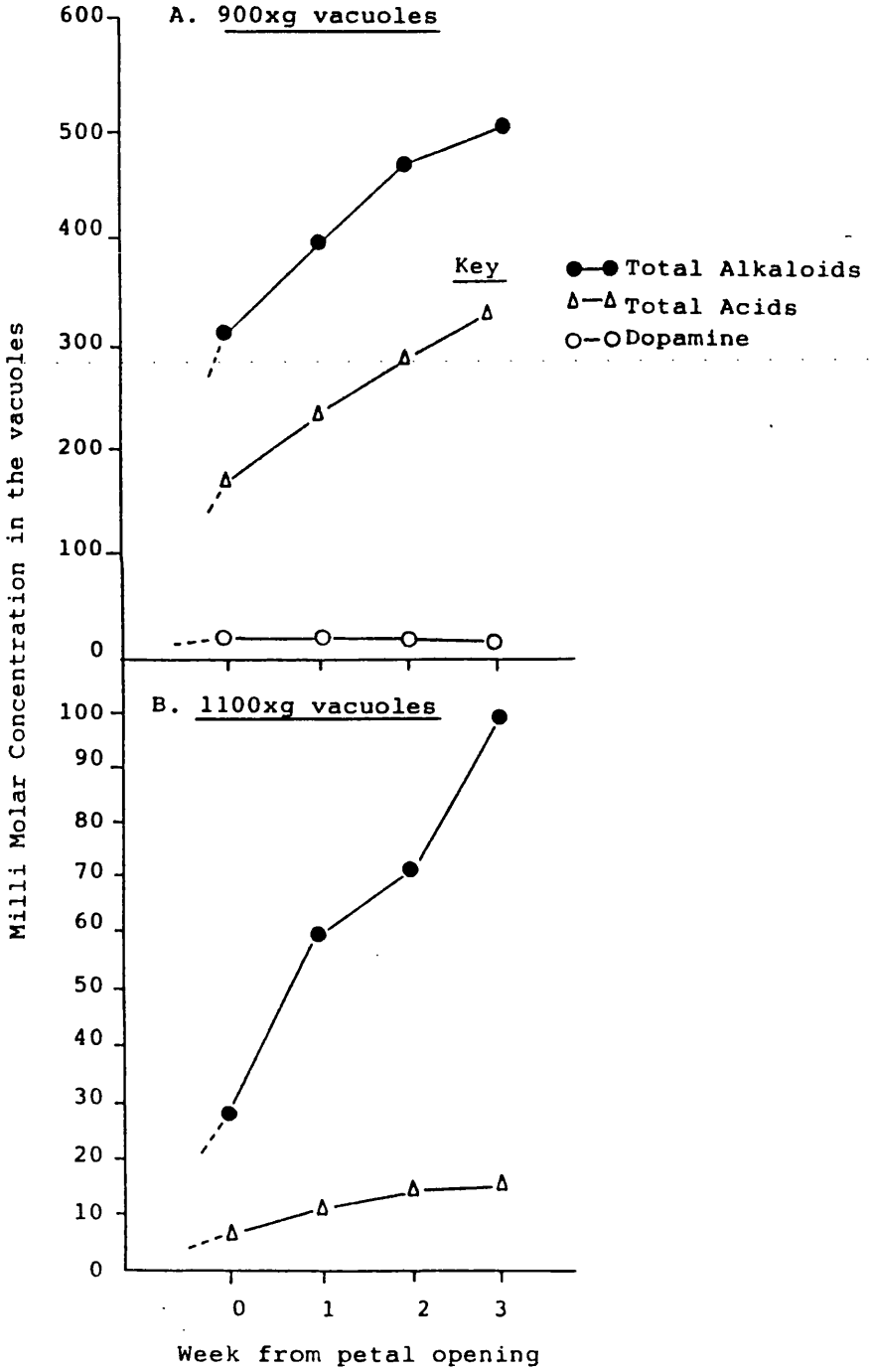
Analysis of the latex vacuoles showed most of the alkaloids, dopamine and acids found in the latex were stored in the 900xg vacuoles (Figs. 3.28 A&B). Phenolic acids appear to be a minor component of the *Papaver* latex vacuoles (1.6-6.4 mM), in contrast to the high levels (621 mM) observed in *Chelidonium* latex (Matile 1976,1984). The levels of alkaloids and acids increased as the capsule matured, whereas dopamine contents in both 900xg and 1100xg fractions remained relatively stable throughout the maturing period (0 to 3 weeks after flowering). For the 900xg vacuoles, the concentration of dibasic acids (meconate and sulphate) was sufficient to protonate all the available alkaloids and to maintain the acidity of the vacuole (pH=5-6). In contrast, the acid content in the 1100xg population was low (ca. 15 mM) compared to ca. 100 mM of the alkaloids at maturity (3 weeks after petal fall). The sequestration of alkaloids in the 1100xg vacuoles therefore would require an additional or a different mechanism to sustain the accumulation of high concentrations of alkaloids and to maintain the vacuolar acidity. The apparent shortfall in anions to equilibrate the inorganic cations in the latex vacuoles could be accounted for by chloride, given the levels (133 mM and 154 mM) in the 900xg samples measured at 2 and 3 weeks from petal opening by the ion chromatography method described for sulphate determination (Section 2.2.6).

Dopamine, a principal precursor of the benzylisoquinoline skeleton of many alkaloids found in *Papaver* species, was not detected in the latex supernatant of *P. somniferum* . The occurrence of dopamine in the latex vacuoles, mainly in the 900xg population, and the extent to which dopamine is

involved in alkaloid biosynthesis requires further investigation. Dopamine was found taken up by the 1000xg fraction of the latex, and the accumulation process was relatively slow compared to the rapid accumulation of alkaloids (Homeyer and Roberts 1984a and 1984b). Dopamine concentration remained relatively stable at about 20 mM in the 900xg vacuoles and ca. 0.3 mM in the 1100xg population, whereas the alkaloid contents increased rapidly with capsule maturity. The role of dopamine inside the latex vacuole and especially in relation to alkaloid accumulation has yet to be determined.

The two main acids, sulphate and meconate, occurred almost exclusively in the 900xg vacuoles of the latex, and the total concentration of these two dibasic acids in the 900xg population was such that all the alkaloids could be trapped in an association with these two acids. In the 1100xg vacuoles, levels of meconate and sulphate were such that another mechanism was required presumably a proton pumping ATPase in order to explain the accumulation of alkaloids and to maintain the acidity of the vacuole.

Figure 3.18 A comparison of levels of bases and acids in the latex vacuoles of P. somniferum L. during capsule maturation



3.6.2. Uptake of acids and alkaloids by the vacuoles of *P. somniferum* latex

The uptake of meconic, suphuric and malic acids into the latex vacuole was investigated. Because of the unavailability of radiolabelled meconate, unlabelled meconic acid was used in the uptake experiment. Uptake of *P. somniferum* alkaloids morphine, codeine and noscapine, and non-*Papaver* alkaloids nicotine, 1-methoxycanthin-6-one and caffeine were investigated.

3.6.2.1. Uptake of ^{14}C-malic acid, ^{35}S-sulphuric acid and unlabelled meconic acid by the 900xg vacuoles

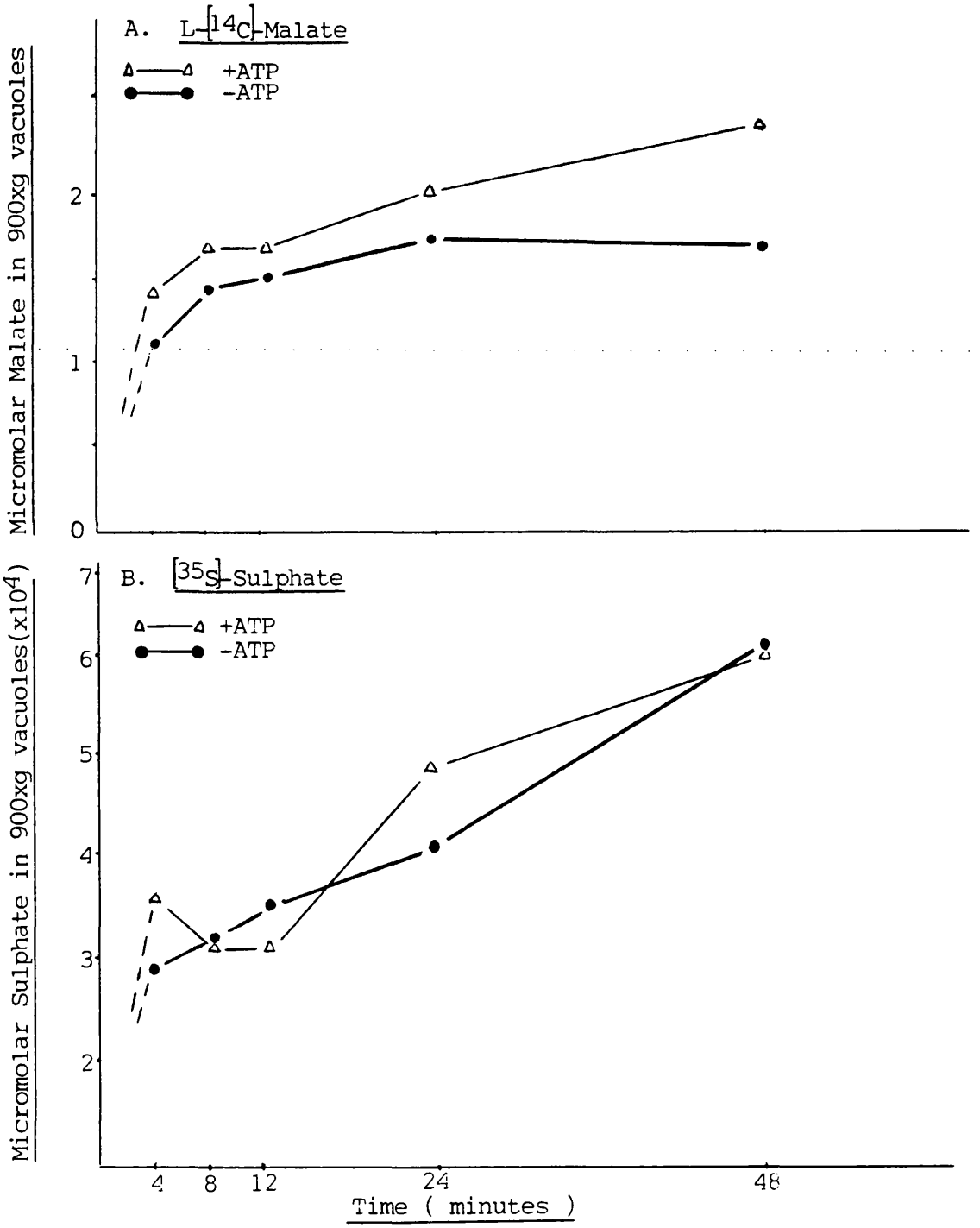
Both malic and sulphuric acids were taken up by the 900xg vacuoles of *P. somniferum* latex (Fig. 3.19). Approximately 1 µM of radiolabelled malic acid was found in the vacuole in 4 minutes. The amount of malic acid taken up by the vacuole increased with the incubation time, but it did not exceed 2 µM in 48 minutes. ATP/Mg^{2+} added to the latex vacuoles appeared to stimulate the uptake of malic acid into the vacuoles. The effect of ATP/Mg^{2+} was clearly seen at 48 minutes when the increase in malate content was about 40% in the presence of exogenous ATP/Mg^{2+}.

Sulphuric acid was also taken up by the 900xg vacuoles. In the absence of added ATP/Mg^{2+}, about $3x10^{-4}$ µM [^{35}S]-H_2SO_4 was added after 4 minute incubation. Its level gradually increased with time and reached $6x10^{-4}$ µM in 48 minutes. The presence of ATP/Mg^{2+} appeared to stimulate the uptake of H_2SO_4 in 4 minutes. However, the sulphate level slowly dropped after 4 minutes to ca. $3x10^{-4}$ µM in 12 minutes and then rapidly increased up to $6x10^{-4}$ µM after 48 minutes. The addition of ATP/Mg^{2+} to the latex vacuoles did not clearly show the stimulation of sulphate uptake into the vacuoles. In

barley seedlings, sulphate was found in the vacuoles of mesophyll cells; and external tracer sulphate, however, was taken up by isolated vacuoles more rapidly in the presence than in the absence of ATP (Kaiser et al 1989) .

In experiments in which unlabelled meconic acid was introduced to 900xg vacuoles, no detection of uptake by the vacuoles was observed. However, with the high levels of meconate already within the vacuole it was thought that the HPLC method used did not have the sensitivity to detect small changes in meconic acid against this high background and therefore radiolabelled meconic acid would be required to determine any uptake into the vacuoles. Preliminary investigations shown in Fig. 3.19 have demonstrated that both radiolabelled sulphate and malate are taken up by the 900xg vacuoles. The uptake of malate was stimulated in the presence of ATP/Mg^{2+}, however, similar use of ATP/Mg^{2+} during sulphate uptake gave equivocal results. The rate and level of uptake of both acids are low compared with the uptake of alkaloids, especially of morphine. This is probably related to ionisation in the bathing medium which is such (pH=6.2-6.8) that, for example, only about 2% of the malic acid would not be ionised and consequently available for uptake in the molecular form .

Figure 3.19 Uptake of [^{14}C-U-] -malic acid and [^{35}S] -sulfuric acid by the 900 x g vacuoles of P. somniferum L. latex



A. L-[^{14}C]-malic acid (final concentration 16.4 µM , 0.2 µCi) was added to 900xg latex vacuoles ≡ 29 µl vacuolar volume in total 200 µl of the buffer.

B. [^{35}S] -H_2SO_4 (final concentration 16 µM , 3.2 µCi) was added to 900xg latex vacuoles ≡ 26 µl vacuolar volume in total 200 µl of the buffer.

3.6.2.2. Specificity of alkaloid uptake by the 900xg vacuoles

The uptake experiments were carried out as described in Section 2.3.4. The amounts of methylamine and alkaloids taken up by a unit sample of latex (200 µl latex buffer, 1:1 ratio) in the absence of ATP/Mg^{2+} are given in Table 3.11A and 3.11B .

The 900xg latex vacuoles could take up large amounts of morphine (ca. 29 µmoles / 100 µl latex) and noscapine (6 µmoles / 100 µl latex). However, only trace amounts of non-*Papaver* alkaloids caffeine and 1-methoxycanthin-6-one were found inside the 900xg vacuoles after the experiments. In contrast, no nicotine was detected under the same experimental conditions applied for all the investigated alkaloids .

Uptake of morphine, codeine, thebaine and papaverine by the 1000xg vacuoles of *P. somniferum* latex was shown to be a rapid process (Homeyer and Roberts 1984a). In the present studies, the *P. somniferum* vacuoles also appeared to be more specific to the alkaloids produced by this plant, especially morphine, when the latex sample was left for 30 minutes. The 900xg vacuoles of the latex could also take up small amounts of other non-*Papaver* alkaloids and methylamine. These results did not suggest the non-specific and simple diffusion of alkaloids into the latex vacuole, since more *Papaver* alkaloids were allowed to cross the vacuolar membrane than the non-*Papaver* alkaloids investigated in the present studies.

In the absence of exogenous ATP/Mg^{2+}, large amounts of morphine and noscapine were taken up by the 900xg vacuoles. However, the involvement of membrane ATPase in the uptake of alkaloids and other compounds could not be ruled out, as more noscapine was found inside the 900xg vacuoles after the

experiment in which ATP/Mg^{2+} was added to the isolated vacuoles (Table 3.11B). In a separate experiment in which both the 900xg and 1100xg vacuole fractions were isolated, ^{14}C-morphine was rapidly taken up by both types of vacuoles and ATP/Mg^{2+} stimulated further accumulation of morphine after 8 minutes (Fig. 3.20). The results shown in Table 3.11A also indicated that little distinction was made between the (-) and (+) isomers of codeine except that uptake of the (-) isomer in all experiments was marginally better than the (+) form. The presence of high levels of meconate and sulphate inside the latex vacuoles, especially in the 900xg fraction (Section 3.6.1.3), suggest that the membrane ATPase was not primarily responsible for the proton gradient across the vacuolar membrane and for the uptake of large quantities of alkaloids in the latex vacuoles .

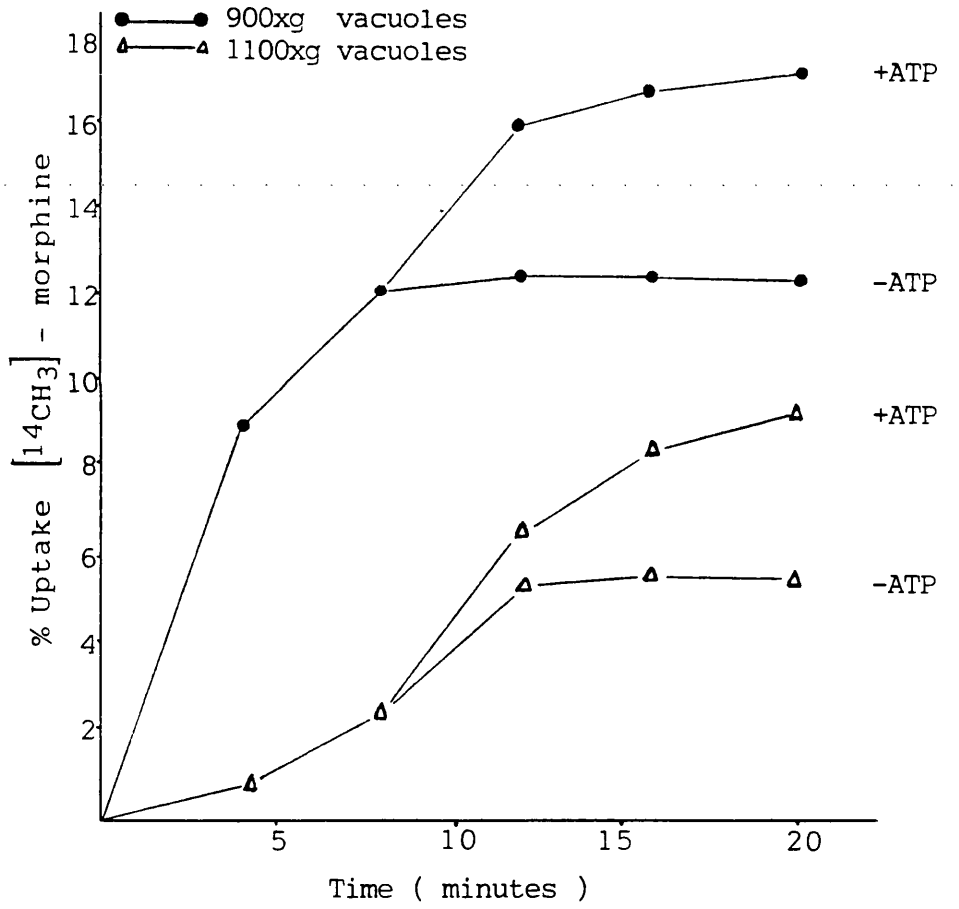
Table 3.11 A Alkaloid uptake by the 900 x g vacuoles of P. somniferum cv. Hallé latex

	µmoles alkaloid input/200 µl sample	µmoles alkaloid uptake/200 µl sample	% Uptake
[^{14}C]-morphine	32.20	29.00	90
(-)[C^{3}H$_{3}$O]-codeine	0.50	0.49	98
(+)[C^{3}H$_{3}$O]-codeine	0.50	0.45	90
caffeine	7.70	1.40	18
noscapine	10.20	6.30	62
nicotine	200.00	0.00	0
1-methoxycanthin-6-one	10.00	0.40	4
[^{14}C]-methylamine	0.20	0.05	25

Table 3.11 B Effect of ATP/Mg^{2+} on noscapine uptake by the 900 x g latex vacuoles

	ATP/Mg^{2+}	µmoles alkaloid input/200 µl sample	µmoles alkaloid uptake/200 µl sample	%Uptake
noscapine	-ATP	10.2	6.3	62
noscapine	+ATP	10.2	8.3	82

Figure 3.20 The accumulation of high concentrations of [$^{14}CH_3$]-morphine in 900 x g and 1100 x g vacuoles in the presence and absence of ATP/Mg^{2+}



High concentrations of morphine (2.0 mg / 300 µl for 900xg and 0.2 mg / 300 µl for 1100xg vacuoles) were used to load the isolated vacuoles to the extent that uptake was terminated . Under these conditions , addition of ATP/Mg^{2+} (10 mM) at 8 minutes stimulated further accumulation of morphine by the vacuoles .

3.6.3. Factors affecting morphine uptake by the 900xg and 1100xg vacuoles of *P. somniferum* latex

Since morphine has previously been shown to be rapidly taken up in large amounts by the 900xg and 1100xg vacuoles of *P. somniferum* latex (Section 3.6.2). This series of experiments was designed to assess the effects of ATP/Mg^{2+}, ATPase inhibitors and temperature on morphine uptake by the latex vacuoles.

3.6.3.1. Effects of ATP/Mg^{2+} on morphine uptake

Both the 900xg and the 1100xg vacuoles contained the major alkaloids found in *P. somniferum*: morphine, codeine, thebaine, papaverine and noscapine (Section 3.6.1.1). Vacuoles fed [$^{14}CH_3$]-morphine in the present studies showed a capacity for the sequestration of high concentrations (up to 415 mM) of the alkaloid .

From the results shown in Fig. 3.21 , suspending the 900xg and 1100xg vacuoles in a bathing medium 4.5 mM with radiolabelled morphine resulted in uptake by the 900xg vacuoles of over 90% of morphine offered in 2 minutes. It should be noted that the presence of ATP/Mg^{2+} had no effect on the rate or amount of uptake by these vacuoles; ATP/Mg^{2+} did, however, stimulate the 1100xg uptake from 12% to about 17% of the morphine offered. These experiments suggested that with the 900xg vacuoles, the proton gradient (ΔpH) across the membrane is sufficient to allow uptake and sequestration of the morphine offered; however, with the 1100xg vacuoles, the ΔpH requires regeneration which, from the stimulation observed with ATP/Mg^{2+}, must occur through a tonoplast ATPase powered proton pump .

In the time course study shown in Fig. 3.20 , high concentrations of morphine were fed to the 900xg vacuoles (2 mg to 60 µl vacuolar volume, ca. 33 mM) and the 1100xg

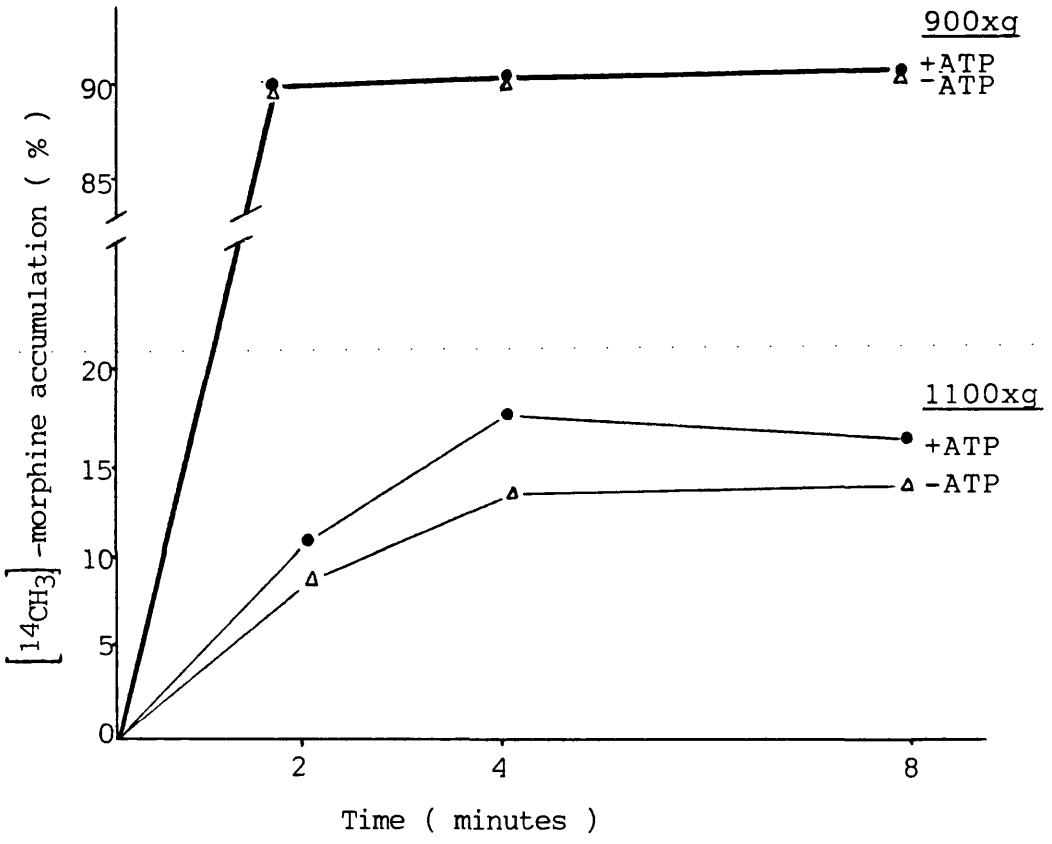
fraction (0.2 mg to 23 µl vacuolar volume, ca. 8.6 mM). The uptake of morphine was rapid and complete after 8 minutes with the vacuolar populations taking up approximately 12% (for 900xg) and 5% (for 1100xg) of the introduced alkaloid. When the addition of ATP/Mg^{2+} was made at 8 minutes, there was a distinct stimulation of morphine uptake suggesting that the high levels of morphine taken up by both vacuole populations dissipated the ΔpH across the membrane which was partially restored as a result of tonoplast ATPase activity when ATP/Mg^{2+} was added after apparent saturation.

Despite the differences in the amount of alkaloid fed to the two vacuole populations and in vacuolar volume used, the 900xg vacuoles proved to have superior capacity to sequester alkaloids (Fig. 3.20).

3.6.3.2. Efflux of alkaloids from latex vacuoles

The 900xg and 1100xg vacuole populations, fed ^{14}C-labelled morphine prior to isolation, were washed twice to remove contaminating [$^{14}CH_3$]-morphine. These vacuoles were suspended in buffer and fed with further large amounts of the unlabelled morphine (2.0 mg to a 200 µl sample of the 900xg vacuoles with a 60 µl vacuolar volume, and 0.2 mg to a 200 µl sample of the 1100xg vacuoles with a 23 µl vacuolar volume). Vacuole samples were incubated at room temperature and harvested after 30, 60 and 120 minutes. There was no significant movement of ^{14}C-labelled morphine out of the vacuoles into the buffer. The alkaloid appeared to be tightly retained within the vacuole and was only released on plasmolysis of the vacuoles. In this respect there was apparently no difference between the 900xg and 1100xg vacuoles .

Figure 3.21 The effect of ATP/Mg^{2+} on the uptake of $[^{14}CH_3]$-morphine by the 900xg and 1100xg vacuoles of P. somniferum L. latex



$[^{14}CH_3]$-morphine (final concentration 4.5 μM , 0.03 μCi) was added to latex vacuoles in buffer .

Vacuoles : 900 x g ≡ 60 μl vacuolar volume

1100 x g ≡ 20 μl vacuolar volume

3.6.3.3. Effects of ATPase inhibitors on morphine uptake

A number of inhibitors designed to affect the proton gradient across the vacuole membrane were used. At the concentrations given in Table 3.12 most of the ATPase inhibitors used had little effect on reducing the uptake of [$^{14}CH_3$]-morphine by either the 900xg or the 1100xg vacuoles, suggesting a stable environment in which the proton gradient was not readily perturbed by the conditions used. However, the 1100xg vacuoles showed reduced uptake (83% of the control) with nitrate, an inhibitor of tonoplast ATPase; and in the presence of ATP/Mg^{2+}, a slight stimulation with FCCP and DCCD . In the absence of ATP/Mg^{2+} the latter two reagents resulted in a slight inhibition of uptake .

3.6.3.4. Effect of temperature on morphine uptake

It was found that the differences in uptake of [$^{14}CH_3$]-morphine by the 900xg and 1100xg vacuoles were observed if the uptake into the vacuoles was measured after 4 minutes and these results are shown in Fig. 3.22 . It was observed that temperature more radically affected the uptake of morphine into the 1100xg vacuoles, where an increase in temperature from 0 to 30^{0}C resulted in an 8 fold increase; uptake of morphine by the 900xg vacuoles over the same temperature range increased by only 2 fold .

Table 3.12 Effect of inhibitors on $[^{14}CH_3]$-morphine accumulation by the 900 x g and 1100 x g vacuoles of P. somniferum latex in the presence of 5 mM ATP/Mg^{2+}

Inhibitor	Concentration (M x 10^{-4})	$[^{14}CH_3]$-morphine uptake % Control		
		900 x g	1100 x g	1100 x g
		±ATP	+ATP	-ATP
Control		100	100	100
FCCP	5	97	122	86
DCCD	5	97	102	79
KNO_3	50	96	83	99
Orthovanadate	50	97	99	99

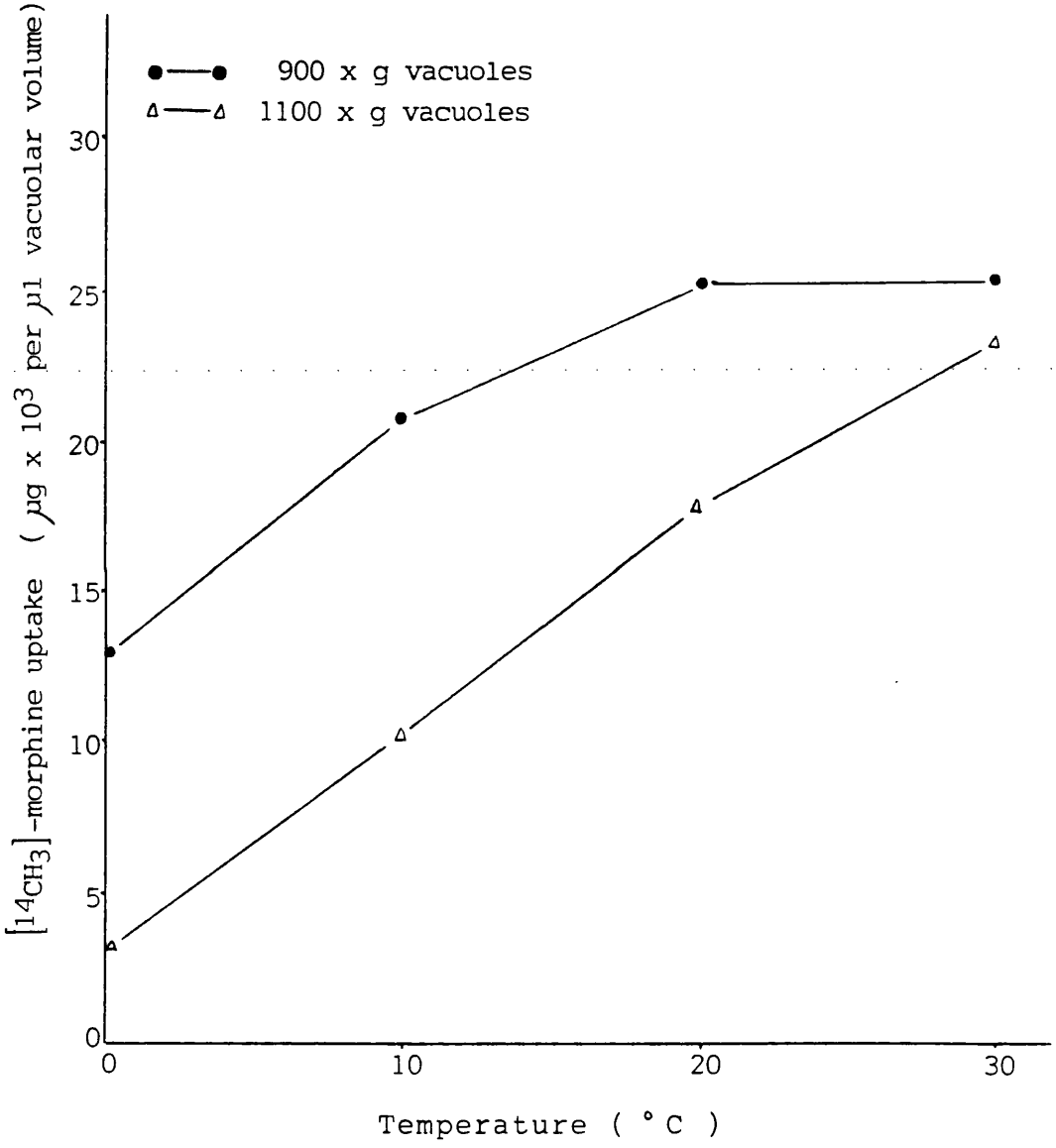
200 µl sample of vacuoles in buffer

Vacuoles : 900 x g , 60 µl vacuolar volume ;
1100 x g , 20 µl vacuolar volume

$[^{14}CH_3]$-morphine : 4.5 µmolar (1.0 Bq per assay) final concentration .

Uptake was measured after 4 minutes after preincubation with inhibitor for 20 minutes .

Figure 3.22 The effect of temperature on alkaloid uptake by the 900 x g and 1100 x g vacuoles from P. somniferum L. latex



[$^{14}CH_3$]-morphine (final concentration 85 µM , 3 Bq) was incubated with latex vacuole suspensions for 4 minutes .
Vacuoles: 900xg ≡ 60 µl vacuolar volume/ 200 µl vacuole sample
1100xg = 20 µl vacuolar volume/ 200 µl vacuole sample
Vacuoles in total 200 µl buffer for each sample.

3.6.4. Discussion

3.6.4.1. Role of organic acids in the 900xg vacuoles of *P. somniferum* latex

In mature poppy latex, proteins were found in a subpopulation of the latex vacuoles which was different from the site of alkaloid accumulation (Griffing and Nessler 1989 and Nessler et al 1985). In contrast, proteins and alkaloids were found in the central vacuole of young latex (Griffing and Nessler 1989). These results would suggest the role of these proteins as enzymes in alkaloid biosynthesis (Griffing and Nessler 1989). In the present studies, separation of *P. somniferum* vacuoles into two populations, the 900xg and 1100xg vacuoles, has shown that the acidic components occurred almost exclusively within the 900xg fraction of the latex at significantly high levels. The concentrations of meconate and sulphate increased during the development of the plant as do the alkaloids. At three weeks after flowering, the level of alkaloids was about 2-5 fold higher than week 0 , as was the meconic acid .

Recent chromatographic evidence and data from experiments using an assay for malic acid with malate dehydrogenase also showed the presence of this acidic compound originally observed by paper chromatography. The levels of malic acid were low (around 10 mM) compared to those of meconate and sulphate (110-250 mM and 60-100 mM , respectively) in the 900xg latex vacuoles. At the pH of the vacuolar interior (pH=5.5), sulphate (pK_1=-3 , pK_2=1.9), meconate (pK_1=2.1) and malate (pK_1=3.4 , pK_2=5.1) will be fully dissociated. Levels of these acids are sufficient to trap the alkaloids inside the vacuole and to assist in the maintenance of vacuolar acidity .

3.6.4.2. Role of ATPase proton pump in *P. somniferum* latex vacuoles

The correlation between acid and alkaloid content throughout maturation of the capsule for the 900xg vacuoles was not observable for the 1100xg fraction although these latter vacuoles still contained significant amounts of alkaloids. The role of organic acids in the maintenance of the acidity of the vacuole is less apparent for the 1100xg vacuoles. Contribution to the pH difference across the vacuolar membrane is also made by the functioning of an ATPase proton pump which appears to be the major source of proton for the protonation and hence trapping of alkaloids in the 1100xg vacuoles. It is thought from these results that the 1100xg vacuoles are the younger versions of the 900xg vacuoles since in older latex they do not exist to the same extent as in latex taken at petal opening. Electron micrographs of latex from *P. somniferum* cv. Soma where only 1100xg vacuoles exist in the latex suggests that small vacuoles coalesce with larger vacuoles [Roberts et al unpublished work], which was also observed with electron micrographs of latex vacuoles from *P. somniferum* cv. Halle (Roberts et al 1983 and unpublished work). It is also likely that the uptake of alkaloids and salts into the vacuoles cause osmotic swelling to form large phase-lucent vacuoles (Poole and Ohkuma 1981). Whilst these findings would support the view that the vacuoles arise from the dilation of the endoplasmic reticulum (Nessler and Mahlberg 1977) they do not support the idea that the vacuoles arise from vesiculation of a central vacuole (Griffing and Nessler 1989). This latter suggestion would not be consistent with the differences in alkaloid and acid compliment of the 900xg and 1100xg vacuoles. It is however possible that vesiculation could occur in the very early stages of alkaloid biosynthesis and sequestration.

Time course studies of the uptake of [$^{14}CH_3$]-morphine by

the latex 900xg and 1100xg vacuoles suggest that both groups of vacuoles take up morphine rapidly, with equilibrium attained in 2-4 minutes. At low morphine concentration (ca. 4.5 μM), exogenous ATP/Mg^{2+} did not show any stimulation of morphine uptake in the 900xg fraction; whereas the 1100xg vacuoles showed very slight stimulation and these 1100xg vacuoles were also much more sensitive to temperature. These results would suggest a simple diffusion process for the rapid accumulation of alkaloids in the latex vacuoles. In consideration of the theory of simple diffusion, the hydrogen ion concentration of the external medium would be expected to influence uptake. The investigation of vacuolar internal pH using ^{14}C-methylamine as probe showed that a difference of approximately 1 pH unit was maintained when the external pH was that of the latex (i.e. between 6.2 and 6.8) (Roberts 1987b). Ammonium chloride is known for its ability to produce dose-dependent decreases in ΔpH (Johnson and Scarpa 1979). The reductions in ΔpH brought about by NH_4Cl in previous studies by Roberts (1987b) showed a reduction in morphine uptake by the 900xg vacuoles. The uptake of large amounts of morphine by both the 900xg and the 1100xg vacuoles resulted in a reduced rate of further morphine uptake, this reduced rate could be improved by the addition of ATP/Mg^{2+}. The effect of ATP/Mg^{2+} on morphine uptake was only observed on a saturated system; and this effect together with the direct correlation between ΔpH and morphine uptake (Roberts 1987b) seems to demonstrate that the function of ATPase is not to power the diffusion process, but to power a proton pump which restores the ΔpH accross the vacuolar membrane. It was also observed that only with latex collected from young capsules (2-3 days after flowering), ATP/Mg^{2+} stimulated alkaloid uptake by the 900xg vacuoles; presumably, the older vacuoles contained enough cations (sulphate, meconate and malate) to stabilise a sufficient quantity of protons to maintain an ΔpH adequate for uptake and

sequestration. Attempts were made either to inhibit ATPase or dissipate ΔpH , by using ATPase inhibitors were relatively unsuccessful in perturbing the system of the 900xg latex vacuoles, presumably because of the high levels of cations and native alkaloids already present which stabilise the protons present within the vacuoles. It has been shown in the present studies that alkaloid concentration increases as the plant develops to maturity, and the vacuolar concentration of these cations is increasing at a rate which allows for the maintenance of a tonoplast ΔpH in addition to establishing a correlation between dibasic acids and alkaloids which would support protonation and complex salt formation as a method of permanent sequestration (ion-trap mechanism). The results from the present studies also show that the correlation between acids and alkaloids was not apparent in the 1100xg population of the latex and as previously mentioned, exogenous ATP/Mg^{2+} stimulated morphine uptake by these vacuoles. The 1100xg vacuoles are presumably the younger form of the 900xg vacuoles; and it was assumed that at this stage of vacuole development, the tonoplast ATPase plays a more important role in the maintenance of ΔpH than it does in the 900xg vacuoles. ATPase inhibitor DCCD and the protonophore FCCP showed a slight inhibition of alkaloid uptake in the 1100xg vacuoles. The transtonoplast proton gradient is therefore important in the regulation of alkaloid uptake into the vacuole. The alkaloids, however, are as tightly held within the 1100xg vacuoles as they are in the 900xg fraction and no alkaloid efflux was observed under the conditions used for the experiments. The significant amounts of cations sulphate, meconate and malate found inside the 900xg vacuoles must play a role in the ion-trapping of alkaloids. If the 1100xg vacuoles may be considered as the less mature forms of the 900xg vacuoles, the results would suggest that the initial development of the tonoplast ΔpH is dependent on a ATPase proton pump. As vacuoles mature, this activity appears

to have less influence on tonoplast ΔpH than does the sequestration of meconate and sulphate. These two acids therefore not only help to maintain tonoplast ΔpH but are also instrumental in trapping the alkaloids within the vacuole .

3.6.4.3. Mechanism of alkaloid translocation in *P. somniferum* latex vacuoles

Experiment with unlabelled meconic acid failed to show uptake of this acid into the latex vacuoles. However, uptake of ^{35}S-sulphate and L-[U-^{14}C]-malate into the *P. somniferum* vacuoles was relatively slow and only marginally stimulated by the addition of ATP/Mg^{2+} . The levels of sulphate taken up would not readily be detected without the use of radiolabelled material, and the same could be true for meconic acid since the vacuoles contain high concentrations against which small changes due to uptake were being measured. Cells of higher plants may accumulate large quantities of malic acid and this accumulation is generally a reversible process (Marigo et al 1985). In *P. somniferum* latex vacuoles malic acid is a minor constituent compared to meconate and sulphate. In *Chelidonium majus*, alkaloids are also exclusively stored in vacuoles of the latex, and vacuolar levels of chelidonic acid and phenolic compounds far exceed the levels of alkaloids (Matile et al 1970, Matile 1976). However, uptake of unlabelled chelidonic acid into the vacuoles of *C. majus* was not observed, and the ion-trap mechanism is also suggested for alkaloid accumulation in *C. majus* latex (Matile 1976) .

It has also been shown in the present studies that the opium alkaloids are specially taken up by the latex vacuoles with great efficiency; caffeine and 1-methoxycanthin-6-one can pass through the vacuolar membrane to a small extent; whereas nicotine was not taken up by the vacuoles. The

accumulation of bases was suggested to be dependent on pK values (Bonysson et al 1987). However, some alkaloids of similar pK values did not show similar degree of specificity for their passage across the vacuolar membrane. For example, the rapid uptake was observed for morphine (pK_1=7.87 , pK_2=9.85), codeine (pK=7.95) and noscapine (pK=6.18). Other alkaloids such as reserpine (pK=6.6), atropine (pK=4.35), quinine (pK_1=5.07 , pK_2=9.7) and cysteine (pK_1=6.11 , pK_2=13.08) did not pass through the latex vacuolar membrane (Homeyer and Roberts 1984a). In the present investigation, nicotine was not detected in the alkaloid extract, and caffeine (pK=10.4) and 1-methoxycanthin-6-one were taken up to a small extent. The results indicate that there is no clear correlation between pK values and the accumulation process for the selectivity and specificity of alkaloid movement across the vacuolar membrane. These results, however, do not support the simple diffusion theory since the alkaloids entered the vacuolar membrane against concentration gradient and the high degree of specificity was observed for the uptake of a limited range of alkaloids as opposed to others of similiar pK and lipophilicity. In addition, the relatively large amounts of *P. somniferum* alkaloids which entered the vacuoles in the absence of exogenous ATP/Mg^{2+} suggested that other factors were also important. In simple diffusion process, the rate of movement of molecules through membranes is proportional to concentration and increases with concentration in an essentially linear fashion. The rapid movement of morphine into the latex vacuoles also revealed another characteristic: At successively higher concentrations the enhancement of uptake slightly dropped off until at some point further increases caused no further rise (Figs. 3.20 and 3.21). This characteristic resembles the behaviour of enzymes in catalysing biochemical reactions. The drop-off noted for morphine uptake suggest that the membrane sites acting on the transported molecules are presumably proteins. The specificity of alkaloid movement

across the vacuolar membrane also suggests that the carrier molecules are proteins since only proteins could "recognise" the transported molecules with the required degree of specificity. The proteins carrying out the diffusion process could act as either mobile carriers or channel formers.

Alkaloids were suggested to have their own specific channel proteins (Homeyer and Roberts 1984a). The use of valinomycin as a mobile carrier and gramicidin as a pore or channel former in alkaloid uptake experiments could support the hypothesis. However, ATPase may also have some role in the translocation of organic substances in the latex vacuoles against their concentration gradient. The effect of exogenous ATP/Mg^{2+} on morphine uptake was only observed on a saturated system in the 900xg vacuoles, whereas the tonoplast ATPase plays a more important role in the 1100xg population. In addition, no absolute requirement of exogenous ATP/Mg^{2+} for the uptake of low concentrations of malic and sulphuric acid in the latex vacuoles was also observed. Similar to alkaloids, these acids moved into the vacuole against their concentration gradient.

The results shown in the present studies would not satisfactorily support either the simple/facilitated diffusion theory or the active transport of alkaloids across the vacuolar membrane. It was shown that chloroplast membranes contain transport proteins specific in their activity for dicarboxylic acids including malic acid (Heldt and Rapley 1970 , Wang and Nobel 1971). The membrane of *P. somniferum* latex vacuole presumably also contains transport proteins specific for native alkaloids and their closely related compounds. Since the accumulation of malic acid is generally a reversible process (Marigo et al 1985) , it is assumed that the accumulation of other organic acids in the latex vacuole was also reversible. The proton-linked cotransport might also in consideration to assist the rapid movement of alkaloids across the vacuolar membrane against concentration gradient. However, alkaloids seem to enter the

vacuole through channels and are protonated within the vacuole. These channels would contain proteins recognising the native alkaloids and their structure related compounds. The idea of specific channel involving the protein and the lipid component of the membrane may also be another consideration .

3.7 CONCLUSION

The medicinal value of *P. somniferum* alkaloids and the laborious methods of extracting alkaloids from this plant has led research workers to look for the better ways of obtaining these alkaloids. It is known that alkaloids occur not only in the opium but also in the poppy plant and alkaloid production increases as the plant develops to maturity. The site of alkaloid biosynthesis is still controversial, but it is likely that the alkaloids are formed in different parts of the plant and are translocated into the capsule at maturity. Each plant cultivar has its own ability to produce alkaloids at different levels, and the pattern of alkaloid production might be screened at the early stage of plant development. The screening procedure to isolate cultivars high yielding in noscapine might be applied at the very young stage (ca. 5 week old seedling), but the high morphinan-yielding cultivars could only be screened from older plant (ie. young plant). There is no doubt that the climatic conditions play an important role in allowing the plant to reach full maturity and hence maximal alkaloid accumulation in its ripe capsule. It has been shown that between 45-80% of the total five major alkaloids in the whole plant was found in the green capsule plus 8" stem depending on plant cultivar and on the weather especially during the period the reproductive organ develops.

Although the outlines of the biosynthetic pathways leading to the alkaloids of *P. somniferum* have been established, the steps involved in the formation of the major alkaloids noscapine and papaverine are still unclear and little is known about the overall regulation of alkaloid biosynthesis in this plant. However, it is likely that the pathway to the predominant morphinan alkaloids suppresses the production of other alkaloids. Quantitative analysis of the five major alkaloids in different *P. somniferum* cultivars showed plant cultivars with high morphinan contents contained

low levels of noscapine. In addition, the investigation of precursor feeding as a method of boosting noscapine content also showed less morphine was found when more noscapine was detected after the experiment. Although noscapine is one of the major alkaloids of *P. somniferum* , it is likely that the plant can only store some small amount of noscapine and papaverine, whereas significantly high morphinan content is always found in this plant. Attempts were made to produce cultivars high yielding in noscapine, but noscapine is further metabolised in these plants. A noscapine metabolite , N-methylnarceine , was isolated and noscapine concentrations of up to 0.4% dry weight were detected. In contrast, the *P. somniferum* plant has the ability to supplement morphinan production by converting thebaine to morphine via oripavine, and this minor alkaloid was also isolated from the high morphinan-producing cultivar Halle in the present investigation. The overall regulation of alkaloid production in this plant has yet to be clarified.

The site of alkaloid accumulation within the plant is the latex. It is likely that the 1100xg vacuoles are the younger form which will in time mature into the 900xg vacuoles since there is frequently a greatly reduced 1100xg fraction from latex taken from more mature plant. The 900xg vacuoles of the latex contains most of the alkaloids and acids found in the latex, whereas only small amounts of these substances are present in the 1100xg population. The levels of organic acids in the 900xg vacuoles are such that the alkaloids are trapped inside the vacuoles as the result of protonation and complex formation with phenolic acids. At the pH of the vacuole interior (pH 5.5-6.5), the acids are dissociated and the protons stabilised through electrophilic attraction. At the pH of the media, however, the alkaloids are not dissociated and this is one factor which contributes to their rapid translocation into the latex vacuoles. The low rate of uptake of both sulphuric and malic acid is probably related to the ionisation in the bathing medium at pH 6.8.

The 1100xg vacuoles of the latex are stimulated by exogenous ATP/Mg^{2+} to give an increase in morphine uptake into the vacuole. These vacuoles lacking stored acids are more dependent on a pump (ATP/Mg^{2+}) for protons and consequently are sensitive to DCCD to a higher degree than the 900xg vacuoles. The 900xg vacuoles are not normally stimulated by exogenous ATP/Mg^{2+} to increase morphine uptake but sequester large amounts of morphine without such stimulation. These vacuoles containing a large concentration of acid with relatively labile protons have a sufficient supply of protons to cope with even high concentrations of alkaloids whilst maintaining vacuolar acidity.

In addition, the effects of age, environment and maturation on the individual capsules on a plant may also contribute to the behaviour of the latex. In younger latex the concentration of stored acids is low, an ATPase is active, and saturating amounts of alkaloid apparently induce activity by an ATPase probably through the reduction in the transmembrane proton gradient. The 1100xg vacuoles do not seem to have the concentrations of acid necessary to permit uptake and proton trapping of alkaloids without the assistance of an ATPase powered proton pump. As latex gets older, the 1100xg vacuoles may develop into 900xg vacuoles. The 900xg vacuoles apparently contain higher concentrations of the acids and the uptake of alkaloids in these vacuoles is through protonation with existing stocks of protons, stabilised by sequestered acids, rather than acquisition of protons through an ATPase powered proton pump.

The vacuolar membrane specifically allows the native alkaloids to pass through with great extent, whereas other non-*Papaver* alkaloids are limitedly taken up by the vacuole. The mechanism of alkaloid translocation into the latex vacuole remains open to conjecture.

3.8. SUGGESTION FOR FURTHER WORK

Although the present studies have investigated the role of organic acids, especially meconate, sulphate and malate, in trapping the alkaloids within the latex vacuole and also in maintaining the transtonoplast proton gradient for alkaloid movement across the vacuolar membrane, and the ATPase proton pump has a less important role in the sequestration process; a number of questions still remain:

(i) Further work is still required to establish the origin of meconic acid found in the latex vacuole. Is it formed within the vacuole or accumulated as are the alkaloids?

Since sulphate and malate were shown to pass the vacuolar membrane with no absolute requirement of exogenous ATP/Mg^{2+}, radiolabelled meconic acid should be made available for the uptake experiment to see whether meconic acid is also accumulated as are the alkaloids, sulphate and malate.

One suggestion is that meconic acid could be formed from malic acid in tropical plants, especially those with both the C_3 and C_4 cycles. Plants with C_4 cycle have the ability to supply CO_2 to the C_3 pathway. It is assumed that the formation of meconic acid from two molecules of malic acid could also provide additional CO_2 to the C_3 cycle. It was shown that fumarate or pyruvate were not incorporated into meconic acid in the isolated latex from *P. somniferum* plant (Steele 1981). In contrast, very low level of radiolabelled meconic acid was detected in feeding experiment with fumarate (Steele 1981). Since the Krebs cycle occurs in chloroplast, it is also assumed that high incorporation of the radiolabelled precursors fumarate and pyruvate into malate and meconate is unlikely happened.

(ii) The mechanism of alkaloid uptake in *P. somniferum* plant still required further work in order to explain the

selectivity and specificity of the vacuolar membrane and the rapid uptake of *P. somniferum* alkaloids which was not influenced by exogenous ATP/Mg^{2+} , as observed in the present studies .

(iii) The biosynthetic pathway to papaverine has yet to be clarified by the isolation of the appropriate enzymes .

(iv) The steps involved between (S)-scoulerine and noscapine in the biosynthesis of noscapine to give a detailed knowledge of the blocks in the biosynthesis from (S)-reticuline so that the possibility of engineering plants which produce high yields of noscapine could be assessed as a practical possibility .

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Further Studies of Sequestration of Alkaloids in *Papaver somniferum* L. Latex Vacuoles

Margaret F. Roberts, B. C. Homeyer, and Thi D. T. Pham

The Department of Pharmacognosy, The School of Pharmacy, London University, 29/39 Brunswick Square, London WC1N 1AX

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Papaver somniferum, Latex Vacuoles, Alkaloid, Sequestration

The uptake and sequestration of alkaloids by latex vacuoles of *P. somniferum* sedimenting at 900 × *g* and 1100 × *g* were studied; both populations take up morphine effectively. Morphine uptake by the 1100 × *g* vacuoles was stimulated by exogenous ATP and, after uptake of large amounts of alkaloid, both populations were stimulated by exogenous ATP to take up further morphine; this effect was no longer present in 900 × *g* vacuoles isolated from the latex of more mature capsules. Uptake by 1100 × *g* vacuoles was more sensitive to temperature than that of 900 × *g* vacuoles. Determination of the proton gradient across the vacuolar membrane (ΔpH), controlled dissipation of ΔpH through the use of NH_4Cl, and correlation with morphine uptake demonstrated that morphine uptake was strongly dependant on the maintenance of ΔpH. Inhibitors designed to produce loss of protons from the vacuole had, however, little effect on the system. Nitrate, as an inhibitor of tonoplast ATPase, had an inhibitory effect on morphine uptake by the 1100 × *g* vacuoles.

Uptake of meconate, [^{35}S]sulphate and L-[U^{14}C]malate, important acid constituents of the 900 × *g* vacuoles was investigated. Uptake of sulphate and malate was continuous and saturation was not reached; the rate of uptake of sulphate and malate was much lower than that of morphine and other alkaloids, and uptake of meconate by these vacuoles was not detected.

Studies on specificity of alkaloid uptake with the 900 × *g* vacuoles indicated no absolute preference for either the (+)- or the (−)-isomer of codeine. Noscapine uptake was stimulated by ATP under conditions where ATP had no effect on morphine uptake. Nicotine was not taken up, but low levels of caffeine and 1-methoxycanthin-6-one were taken up, but less effectively than morphine or noscapine.

Uptake of alkaloids by *P. somniferum* latex vacuoles is dependant on the maintenance of tonoplast ΔpH and an ATPase generates this ΔpH. Sequestration appears to involve protonation and anion-cation stabilization involving meconate and sulphate. The specificity of alkaloid uptake suggested no clear correlation with pK or lipophilicity, and some sort of channel mechanism, more related to alkaloid shape is suggested.

Introduction

In higher plants the cell vacuole has been shown in many instances to be the storage compartment for alkaloids [1–6]. Previous work on alkaloid storage in *Papaver somniferum* has shown that alkaloids are exclusively stored in vacuoles contained within the latex [7, 8] and in this respect alkaloid sequestration in *Papaver somniferum* is similar to that found in *Chelidonium majus* [9, 10]. Like other plant vacuoles, *P. somniferum* latex vacuoles have an internal pH lower than that of their cytoplasmic environment, for the latex this is normally at pH 6.2–6.8 [3]. These vacuoles also accumulate low levels of dopamine [11] and have been shown to contain most of the meconic acid found in the latex [12].

Two distinct mechanisms for alkaloid transport into vacuoles have been proposed: 1. The lipophilic alkaloids can penetrate membranes by diffusion and are trapped in the acidic vacuolar compartment by protonation and salt formation [2, 4, 13–15]. This has been referred to as the ion trap mechanism. 2. Alkaloid uptake across the vacuolar tonoplast may occur *via* catalyzed transport and some highly specific tonoplast transport systems have been reported in a variety of plants [5, 6, 16]. It has been reported that alkaloid uptake into vacuoles isolated from *Fumaria* protoplasts could be stimulated by the addition of MgATP indicating that an ATPase activity was necessary for effi-

Abbreviations: HEPES, N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid; FCCP, carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone; DCCD, N,N′-dicyclohexyl carbodiimide; MgATP, magnesium + adenosyltriphosphate; ΔpH, transmembrane proton gradient.

Reprint requests to Dr. M. F. Roberts.

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cient transport. A similar mechanism is required for lupanine sequestration in vacuoles isolated from *Lupinus* [6] and for senecionine-N-oxide in *Senecio vulgaris* [17]. The vacuoles of *P. somniferum* latex may be considered as specialist vacuoles due to their size, resistance to external measures designed to reduce the ΔpH and capacity to sequester millimolar amounts of alkaloid. The rapid, specific uptake of the major opium alkaloids has no absolute requirement for MgATP, does not show saturation kinetics and is largely insensitive to temperature [3].

FCCP causes some reduction in alkaloid uptake suggesting that a proton gradient across the vacuole tonoplast is essential for continued alkaloid accumulation [3] which in turn implies the existence of a proton pump. The mechanism of sequestration of alkaloids in *P. somniferum* therefore did not clearly fit either hypothesis 1 or 2. In an attempt to resolve some of the conflicting results previously obtained, further investigations of the mechanism of uptake and sequestration of morphinan alkaloids by the vacuoles of *P. somniferum* latex were undertaken.

Experimental

Chemicals

Unless otherwise stated, chemicals were purchased from Sigma, U.K. or The British Drug Houses, U.K. [^{14}C]methylamine hydrochloride (1.48–2.22 GBq mM^{-1}), $H_2{}^{35}SO_4$ (44.4–51.8 TBq mM^{-1}) and ^{14}COOH-dextran-carboxyl (18.5–74.0 MBq g^{-1}) were purchased from New England Nuclear, U.S.A.; ^3H_2O (185 MBq ml^{-1}), L-[U^{14}C]-malic acid (1.5–2.2 GBq mM^{-1}), [$^{14}CH_3$]morphine (2.07 GBq mM^{-1}) was purchased from Amersham International, U.K.; and ATP was purchased from Boehringer, F.R.G.

Plant material

Papaver somniferum cv. Halle was cultivated at The School of Pharmacy Medicinal Plant Garden. Plants were grown in sequential batches so that flowering occurred from late June until end of October.

Isolation of latex vacuoles

Latex was collected into 700 mM mannitol to a final concentration of 50% latex. Vacuoles were sedimented by centrifuging the collected latex at 900 × *g* for 30 min and the supernatant of that centrifugation at 1100 × *g* for 30 min. The vacuoles were washed twice, and finally suspended to the original volume of buffer plus latex in a buffer of 700 mM mannitol, 100 mM HEPES adjusted to pH 6.8 with triethanolamine. This suspension was equivalent to 40 to 60 µl (900 × *g* vacuoles) and 15 to 20 µl (1100 × *g* vacuoles) intravacuolar volume for each 200 µl assay sample.

Determination of vacuolar volume

The intravacuolar volume of the 900 × *g* vacuole sample was determined by incubating a 200 µl aliquot of isolated and resuspended vacuoles with a 30 µl aliquot of tritiated water (1.11×10^3 Bq) and a 10 µl aliquot of a ^{14}COOH-dextran-carboxyl solution (3.7×10^2 Bq) for 30 min at room temperature (approximately 24.5 °C). In experiments testing the effect of substances on the transmembrane proton gradient the substances were added during this incubation. The sample was centrifuged in an Eppendorf tube at 900 × *g* for 15 min and a sample of the supernatant counted for ^{3}H and ^{14}C in a Packard Tri-Carb Liquid Scintillation Spectrometer using Aquasol LSC cocktail. The pellet was suspended in 500 µl of 0.1 N HCl in methanol, digested for 30 min at room temperature, centrifuged at 2000 × *g* for 15 min and the entire supernatant counted for ^{3}H and ^{14}C. Counts were corrected for quenching, counting efficiency, and channel overlap. The concentration of tritiated water was assumed to be constant throughout the sample and the dextran restricted to the extravacuolar space. Dividing ^{3}H counts per µl of supernatant into the ^{3}H counts from the pellet provided the total sample void volume trapped in the pellet; dividing the ^{14}C counts per µl of supernatant into the ^{14}C counts from the pellet provided the trapped extravacuolar void volume; subtracting the trapped extravacuolar void volume from the total sample void volume left the intravacuolar volume [20]. This volume was very consistent for all similarly aged latex samples in a given season.

Determination of intravacuolar pH

The distribution of the membrane permeable base, methylamine, has been successfully used for

the determination of intracellular and intravacuolar pH values [19, 20]. The method is based on the assumption that uncharged compounds can easily pass across a membrane whereas charged forms are impermeant. Since the ratio of charged to uncharged compound is governed by the pH value, that value can be deduced. For most thoroughly studied situations, these assumptions have proved correct [21]. The possibility of the methylamine binding to the membrane must be considered carefully; in a vacuolar system, the possibility of differing effects of a transplasmalemma and a transtonoplast potential does not occur [22].

Intravacuolar pH was determined by incubating the vacuoles with 21 μM [^{14}C]methylamine (3.7×10^2 Bq) for 30 min and subsequent separation of supernatant and pellet and counting as described above in the determination of intravacuolar volume. The calculation was based on the equilibrium of uncharged methylamine across cell membranes and the impermeability of membranes to charged methylamine [23]. The pH gradient (pH_{in}-pH_{out}) was assumed to be equal to the $\log_{10}$ of the ratio of the proton concentration on either side of the membrane, which was equal to the $\log_{10}$ of the ratio of the probe concentrations.

Assay procedure for [$^{14}CH_3$]morphine, (+)-, (−)-[C^3H_3O]codeine, [^{35}S]sulphate and L-[U^{14}C]malate uptake by 900 × g and 1100 × g latex vacuoles

The method used was essentially that given in [3] except that 900 × *g* vacuoles were layered on a 20% sucrose gradient and 1100 × *g* vacuoles on a 16% sucrose gradient and centrifuged at 1000 × *g* and 1300 × *g* respectively to terminate the experiment. The resulting pellets were digested in 500 μl of .025 N HCl in methanol for 30 min centrifuged at 2000 × *g* for 15 min and 400 μl of the supernatant counted in a Packard Tri-Carb Liquid Scintillation Spectrometer using Aquasol LSC cocktail. Counts were corrected for quenching and counting efficiency. In experiments with inhibitors, the vacuole sample was preincubated with the inhibitor at 25 degrees for 20 min. In each individual experiment, duplicate samples were used and the results given are averages of at least two separate experiments.

Isolation of unlabelled alkaloids in uptake specificity experiments

1. Noscapine and nicotine

P. somniferum latex vacuole samples, 900 × *g* and 1100 × *g* were plasmolyzed in 0.025 N HCl in MeOH. After centrifugation the supernatant from each sample was evaporated to dryness and the residue taken up in a small volume of 2% sulfuric acid. The pH was adjusted to 9.5 with NH_4OH the sample placed on a column of Extrelut (Merck, Darmstadt) and the alkaloids isolated by elution with chloroform. The residue after evaporation was made up in a standard volume of MeOH for HPLC analysis.

2. Caffeine and 1-methoxycanthin-6-one

Plasmolysis of the vacuoles was carried out in a similar manner to that for the noscapine and nicotine experiments except that 0.1 N HCl was used. After centrifugation, the supernatant was extracted four times with $CHCl_3$. These extracts were combined and after evaporation the residue was made up in a standard volume of MeOH for HPLC analysis.

In all experiments with non-*Papaver* alkaloids and noscapine comparisons were made with control experiments since the vacuoles contained considerable amounts of the native alkaloids.

Quantitation of alkaloids

1. Noscapine

HPLC analysis: column, Hichrom S5W 5 μ Spherisorb (250 × 4.9 mm); mobile phase, MeOH (30 ml):$CHCl_3$ (10 ml):Et_2N (0.1 ml) of which 37.5 ml was mixed with *n*-hexane (290 ml).

2. Nicotine and 1-methoxycanthin-6-one

HPLC analysis: column, Hichrom S5W 5 μ Spherisorb (250 × 4.9 mm); mobile phase, *n*-hexane:ethyl acetate:NH_4OH (70:30:0.1).

3. Caffeine

HPLC analysis: column, Altex RP 18, 5 μ, ultrasphere octyl (250 × 4.6 mm); mobile phase: 20% MeOH in H_2O.

In all cases the alkaloids were separated from the native alkaloids except for noscapine where %

uptake was determined by difference as compared with control samples.

Isolation of meconic acid

900 × *g* and 1100 × *g* vacuole samples were plasmolyzed in 0.025 N HCl in MeOH and centrifuged at 1000 × *g* for 30 min. The supernatant was placed on a cation exchange column (Dowex – 50 W H^+ form), which had been previously equilibrated with 1 N HCl and washed with distilled water. The acids were eluted with distilled water. After evaporation at reduced pressure the residue was made up in a standard volume. Meconic acid was determined by HPLC on a Hichrom RP18 10 µ (250 × 4.9 mm) column with MeOH:H_2O (17:83) containing 0.05% atropine sulphate. The column was calibrated using a standard sample of meconic acid.

Results

Separation of *P. somniferum* latex vacuoles using discontinuous sucrose gradients suggested that these vacuoles could be separated into two populations. Subsequently it was found that these two populations of vacuoles could be isolated by centrifugation at 900 × *g* and 1100 × *g* as detailed in the Experimental. This simple method of isolation yielded a higher percentage of intact vacuoles as measured using α-mannosidase as a vacuolar marker [8]. The buffer used helped to prevent aggregation of the vacuoles during their isolation and use in the experiments. This method was therefore used to produce the 900 × *g* and 1100 × *g* vacuoles used in the foregoing experiments.

Comparison of morphine uptake by the 900 × g and 1100 × g vacuoles

Both the 900 × *g* and the 1100 × *g* latex vacuoles contained the major alkaloids found in *P. somniferum:* morphine, codeine, thebaine, papaverine and noscapine. Vacuoles fed [$^{14}CH_3$]morphine showed a capacity for the sequestration of high concentrations (up to 415 mM) of alkaloid.

In the experiment shown in Fig. 1, suspending 900 × *g* and 1100 × *g* vacuoles in a bathing medium 4.5 mM with radiolabelled morphine resulted in uptake by the 900 × *g* vacuoles of over 90% of morphine offered in 2 min. It should be noted that the presence of MgATP had no effect on the rate or amount of uptake by these vacuoles; MgATP did, however, stimulate the 1100 × *g* uptake from 12% to about 17% of the morphine offered. These experiments suggested that with the 900 × *g* vacuoles, the proton gradient (ΔpH) across the membrane is sufficient to allow uptake and sequestration of all the morphine offered; however, with the 1100 × *g* vacuoles, the ΔpH requires regeneration which, from the stimulation observed with

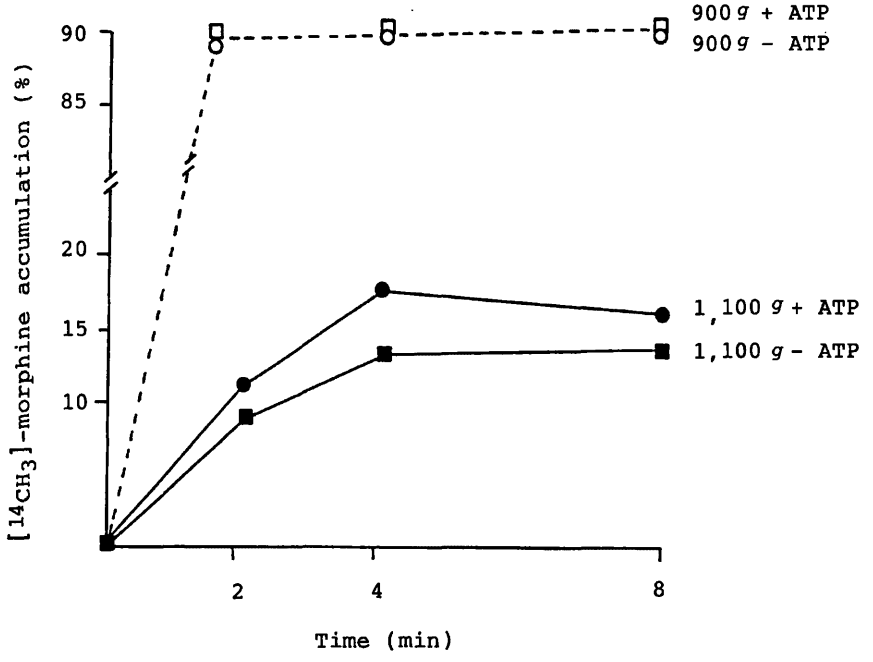


Fig. 1. The effect of MgATP on the uptake of [$^{14}CH_3$]morphine by the 900 × *g* and the 1100 × *g* vacuoles of *P. somniferum* latex. [$^{14}CH_3$]morphine (final concentration 4.5 µM, 1.0 Bq) was added to latex vacuole suspensions. Vacuolar volume: 900 × *g* = 60 µl, 1100 × *g* = 20 µl in 200 µl 100 mM HEPES, 700 mM mannitol, 100 µM molybdate, pH 6.8. MgATP: 10 mM.

MgATP, must occur through a tonoplast ATPase powered proton pump.

In the time course study shown in Fig. 2, high concentrations of morphine were fed to 900 × *g* vacuoles (2.0 mg to 60 µl vacuolar volume, ~33 mM) and 1100 × *g* vacuoles (0.2 mg to 23 µl vacuolar volume, ~3.3 mM). The uptake of morphine was rapid and complete after 8 min with the vacuole populations taking up approximately 12% (900 × *g*) and 5% (1100 × *g*) of the introduced alkaloid. When the addition of MgATP was made at 8 min, there was a distinct stimulation of morphine uptake suggesting that the high levels of morphine taken up by both vacuole populations dissipated the ΔpH across the membrane which was partially restored as a result of tonoplast ATPase activity when MgATP was added after apparent saturation.

Despite differences in the amount of alkaloid fed to the two vacuole populations and the differences in vacuolar volume used, the 900 × *g* vacuoles proved to have superior capacity to sequester alkaloids (Fig. 2).

Efflux of alkaloids from latex vacuoles

Previous experiments [3] have shown that [$^{14}CH_3$]morphine is retained in the vacuoles after uptake even if the pH of the buffer suspending the vacuoles is lowered to 5.5–5.6. Vacuole populations, 900 × *g* and 1100 × *g*, fed ^{14}C-labelled morphine prior to isolation were washed twice to remove contaminating [$^{14}CH_3$]morphine. These vacuoles were suspended in buffer and fed with further large amounts of unlabelled morphine (2.0 mg to a 200 µl sample of the 900 × *g* vacuoles with a 60 µl vacuolar volume and 0.2 mg to a 200 µl sample of the 1100 × *g* vacuoles with a 23 µl vacuolar volume). Vacuole samples were incubated at room temperature and harvested after 30, 60, and 120 min. There was no significant movement of ^{14}C-labelled morphine out of the vacuoles into the buffer. The alkaloid appeared to be tightly retained within the vacuole and was only released on plasmolysis of the vacuoles. In this respect there was apparently no difference between the 900 × *g* and the 1100 × *g* vacuoles.

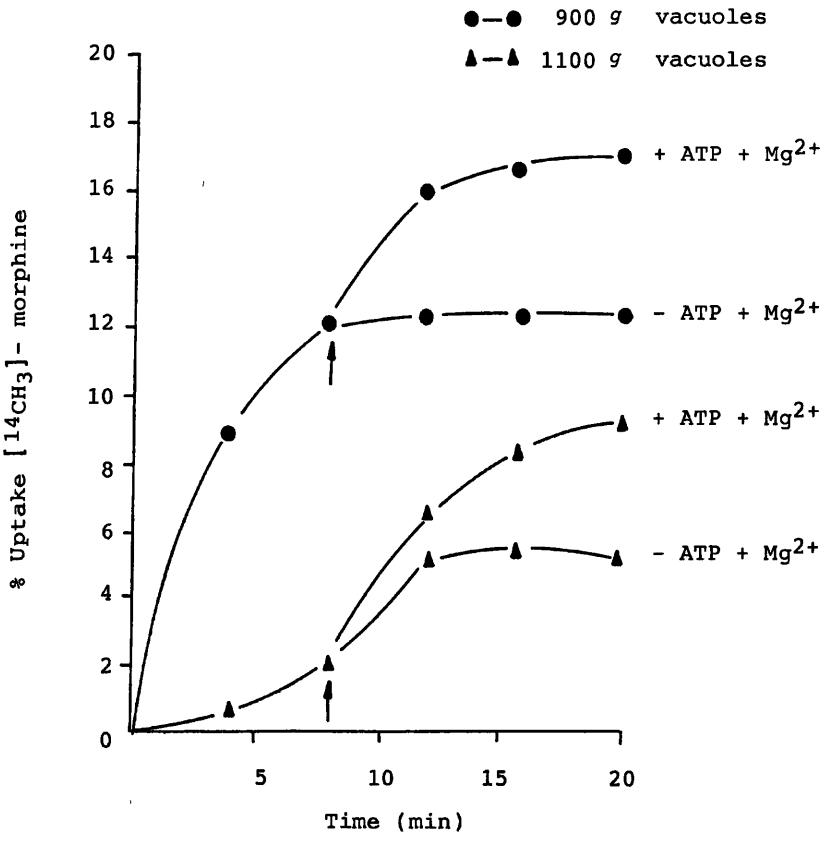


Fig. 2. The accumulation of high concentrations of [$^{14}CH_3$]morphine in 900 × *g* and 1100 × *g* *P. somniferum* latex vacuoles in the presence and absence of MgATP. High concentrations of [$^{14}CH_3$]morphine (10 mg ml^{-1} for 900 × *g* vacuoles, 1.0 mg ml^{-1} for 1100 × *g* vacuoles) were used to preload isolated latex vacuoles. Vacuolar volume: 900 × *g* = 60 µl, 1100 × *g* = 23 µl in 200 µl 100 mM HEPES, 700 mM mannitol, 100 µM molybdate, pH 6.8. MgATP (10 mM) was added to some samples 8 min after the start of incubation with morphine.

Studies using ATPase inhibitors and ionophores

A number of inhibitors designed to affect the proton gradient across the vacuole membrane or inhibit tonoplast or plasmalemma ATPases were used. At the concentrations given in Table I, most of the ionophores and ATPase inhibitors used had little effect on reducing the uptake of [$^{14}CH_3$]-morphine by either the 900 × *g* or the 1100 × *g* vacuoles, suggesting a stable environment in which the proton gradient was not readily perturbed by the conditions used. However, the 1100 × *g* vacuoles showed reduced uptake (83% of control) with nitrate, an inhibitor of tonoplast ATPase, and in the presence of MgATP, a slight stimulation with FCCP and DCCD. In the absence of MgATP these latter two reagents resulted in a slight inhibition of uptake.

The effects of temperature on the 900 × g and 1100 × g vacuoles

Although previous experiments [3] carried out on vacuoles which sedimented at 1000 × *g*, and were therefore rich in the 900 × *g* vacuoles of the present experiments, had suggested that morphine uptake was largely independent of temperature, close inspection suggested that differences in uptake of [$^{14}CH_3$]morphine were observable if uptake into the vacuoles was measured after 4 min and these results are given in Fig. 3. It was observed that temperature more radically affected the uptake of morphine into the 1100 × *g* vacuoles, where an increase in temperature from 0 °C to 30 °C resulted in an 8-fold increase; uptake of morphine by 900 × *g* vacuoles over the same temperature range increased by only 2-fold. The calculated activation energy (E_a) of morphine uptake for the 900 × *g* vacuoles is 22.18 kJ·mol^{-1}; that for the 1100 × *g* vacuoles is 58.08 kJ·mol^{-1}. The value for the 900 × *g* vacuoles lies at the upper limit of the accepted values for passive diffusion, while that for the 1100 × *g* vacuoles is well into the range associated with facilitated uptake [6].

The effect of changes in the membrane proton gradient (ΔpH) on alkaloid uptake by latex 900 × g vacuoles

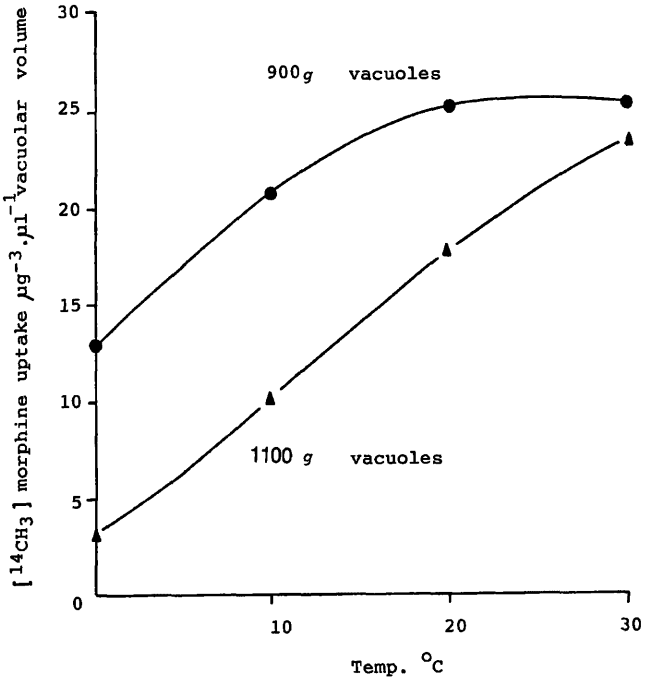
The measurement of ΔpH

A time course of uptake of methylamine by *P. somniferum* latex vacuoles indicated a very rapid penetration by the probe into the intravacuolar space followed by a constant ratio of methylamine inside/outside the vacuoles for at least 30 min, Fig. 4. The successful use of the reagent depends on the lack of binding to macromolecules within the intravacuolar space or to the membrane itself. Osmotic rupture of the vacuoles accomplished liberation of over 95% of the methylamine in every instance. Most investigations were carried out at the natural pH of the latex, pH 6.8, and the ratio of the concentration of intravacuolar methylamine to that outside remained constant over a range of 100 to 750 μM.

Table I. The effect of inhibitors on [$^{14}CH_3$]morphine accumulation by the 900 × *g* and 1100 × *g* vacuoles of *P. somniferum* latex in the presence of 5 mM MgATP. Buffer: 100 mM HEPES, 700 mM mannitol, pH 6.2 – 200 μl. Vacuoles: 900 × *g*, 60 μl vacuolar volume; 1100 × *g*, 20 μl vacuolar volume. [$^{14}CH_3$]morphine: 4.5 μmolar (1.0 Bq per assay) final concentration. Uptake was measured after 4 min after preincubation with inhibitors for 20 min.

Inhibitor	Conc. [M × 10^{-4}]	[$^{14}CH_3$]morphine uptake % Control 900 × *g* ±MgATP	1100 × *g* +MgATP	1100 × *g* −MgATP
Control		100	100	100
FCCP	5	97	122	86
DCCD	5	97	102	79
KNO_3	50	96	83	99
Orthovanadate	50	97	99	99

A



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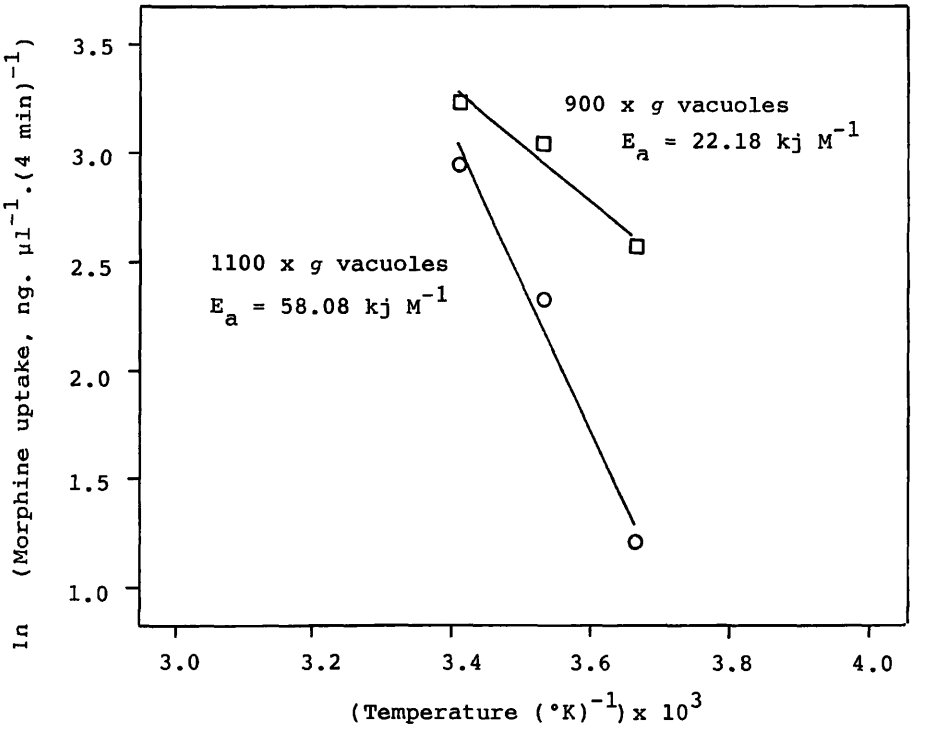

Fig. 3. The effect of temperature on alkaloid uptake by 900 × *g* and 1100 × *g* vacuoles from *P. somniferum* latex. [$^{14}CH_3$]morphine, final concentration 85 µM, 3 Bq, was incubated with latex vacuole suspensions for 4 min. Vacuolar volume: 900 × *g* = 60 µl, 1100 × *g* = 20 µl in 200 µl 100 mM HEPES, 700 mM mannitol, 100 µM molybdate buffer, pH 6.8. Fig. A: orthographic plot; Fig. B: Arrhenius plot.

Using similar techniques it was possible to calculate that at the natural pH of the latex which seasonally varies between 6.2 and 6.8 the internal pH of the vacuoles was always approximately one pH unit lower than the external pH.

The effect of changes in ΔpH on morphine uptake

The effect of varying ΔpH was investigated using ammonium chloride as described in [24]. Ammonia, as ammonium chloride (1–50 mM), added to the highly buffered 900 × *g* vacuole sus-

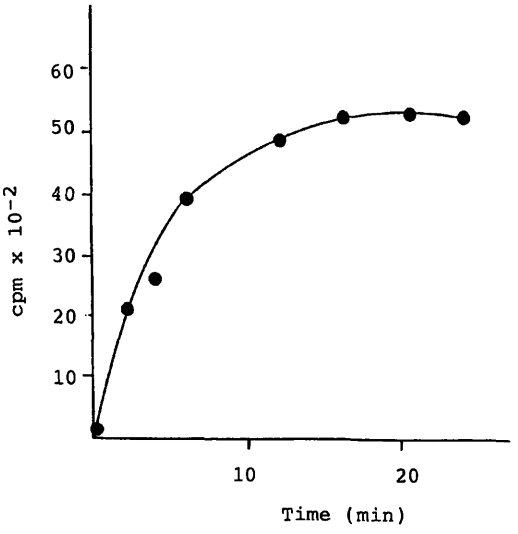

Fig. 4. Time course for [^{14}C]methylamine uptake by *P. somniferum* latex 900 × *g* vacuoles. The 900 × *g* vacuoles (vacuolar volume 43 µl) in 200 µl 100 mM HEPES, 700 mM mannitol, 100 µM molybdate buffer, pH 6.8 with 21 µM [^{14}C]methylamine (7.4×10^2 Bq). The ratio of [^{14}C]methylamine inside/outside the vacuole was linear within a range of 0–200 µM.

pensions, showed a dose dependant decrease in the ΔpH. Addition of morphine at the concentrations used in this series of experiments (4.5 µM) had no significant effect on ΔpH measurements. A reduction in the uptake of [$^{14}CH_3$]morphine paralleled in the reduction in ΔpH (Fig. 5).

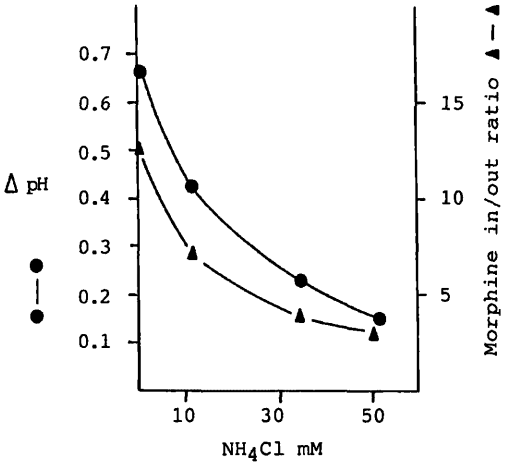

Fig. 5. The effect on morphine uptake by 900 × *g* vacuoles of perturbation of ΔpH using ammonium ions. Vacuolar volume: 43 µl in 200 µl 200 mM HEPES, 500 mM mannitol, pH 6.8. Probes: For ΔpH, 21 µM [^{14}C]methylamine (7.4×10^2 Bq); for [$^{14}CH_3$]morphine uptake, a concentration of 270 µM, 3.7×10^2 Bq was used.

Specificity of alkaloid uptake

The latex vacuoles of *P. somniferum* sequester alkaloids of three basic structural types: the morphinanes: morphine (pK_1 7.8, pK_2 9.85), codeine (pK 7.95), and thebaine (pK 7.95); phthalideisoquinolines: noscapine (pK 6.18); and benzylisoquinolines: papaverine (pK 5.90).

Previous investigation indicated that latex vacuoles show a remarkable degree of specificity for these alkaloids [3]. To extend this study a further group of alkaloids was investigated. In Table II a comparison of the uptake of [$^{14}CH_3$]morphine with (+)-[C^3H_3O]codeine and (−)-[C^3H_3O]codeine at 25 °C by 900 × *g* vacuoles confirms the efficiency of uptake of morphine and indicates that little distinction is made between the natural, (−), and the unnatural, (+), isomers of codeine except that uptake of the (−)-isomer in all experiments was marginally better than the (+)-isomer.

Surprisingly, while uptake of noscapine was marginally less efficient than uptake of morphine, noscapine uptake in the presence of MgATP was enhanced. This enhancement with MgATP is not observed with morphine in the 900 × *g* vacuoles under similar conditions. Nicotine at pH 6.8 was not taken up by the 900 × *g* vacuoles, whereas caffeine and 1-methoxycanthin-6-one were taken up to a small extent (18% and 4% respectively). Uptake of these alkaloids was less efficient than that of [^{14}C]methylamine (24%) or morphine (98%).

Table II. Specificity of alkaloid uptake by the 900 × *g* latex vacuoles. Buffer: 100 mM HEPES, 700 mM mannitol, pH 6.8 (200 µl). Vacuoles: 900 × *g*, 63 µl vacuolar volume. Incubation was for 20 min at 25 °C followed by vacuole isolation and processing as given in the Experimental.

Alkaloid	µmol per 200 µl latex Input	Uptake	[%] Uptake
[$^{14}CH_3$]morphine	0.25	0.245	98
	32.2	29.0	90
(−)-[C^3H_3O]codeine	0.5	0.49	98
(+)-[C^3H_3O]codeine	0.5	0.45	90
Noscapine (−MgATP)	10.2	6.3	62
(+MgATP)	10.2	8.3	82
Nicotine	200	0	0
Caffeine	7.7	1.4	18
1-Methoxycanthin-6-one	10.0	0.4	4
[^{14}C]methylamine	0.2	0.048	24

The uptake of meconic acid, sulphate and malate by 900 × g vacuoles

In experiments in which unlabelled meconic acid was introduced to 900 × *g* vacuoles, no detection of uptake by the vacuoles was observed. However with the high levels of meconate already within the vacuole it was thought that the HPLC method used did not have the sensitivity to detect small changes in meconic acid against this high background and therefore radiolabelled meconic acid would be required to determine any uptake into the vacuoles. Preliminary investigations shown in Fig. 6 have demonstrated that both radiolabelled sulphate and malate are taken up by the 900 × *g* vacuoles. The uptake of malate in the presence of MgATP was stimulated, however, similar use of MgATP during sulphate uptake gave equivocal results. The rate and level of uptake of both acids is low compared with the uptake of morphine. This is probably related to ionization in the bathing medium which is such (pH 6.2–6.8) that, for example, only about 2% of the malate would not be ionized and consequently available for uptake in the molecular form.

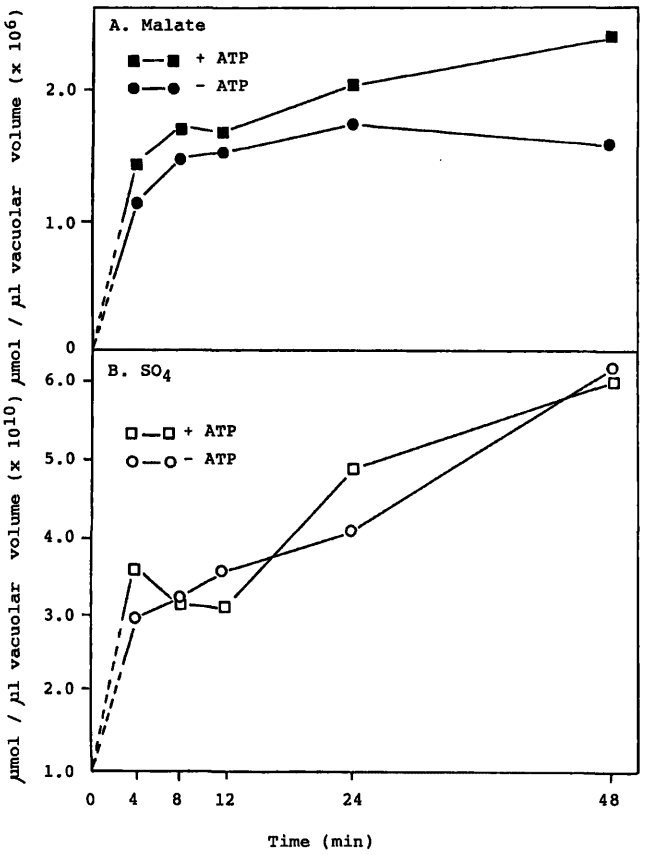

Fig. 6. The uptake of L-[U^{14}C]malate and [^{35}S]sulphate by the 900 × *g* latex vacuoles of *P. somniferum*. A. L-[U^{14}C]-malic acid at a final concentration of 16.4 µM, 3.6 Bq was added to a vacuolar suspension sample, vacuolar volume (900 × *g* vacuoles) 29 µl, in 200 µl 100 mM HEPES, 700 mM mannitol, 100 µM molybdate, pH 6.8. MgATP additions were made for a final concentration of 10 mM. B. [^{35}S]H_2SO_4 at a final concentration of 16 µM (10.0 Bq) was added to an identical vacuolar sample, as in 6A.

Discussion

In the foregoing experiments and those detailed in [3], and in [18], an attempt has been made to determine whether the alkaloids penetrate the latex vacuole tonoplast by diffusion with entrapment in the acidic vacuolar compartment by protonation and formation of non-diffusable salts [2, 3, 13–15] or whether alkaloid uptake across the vacuolar membrane results from catalyzed transport [3, 5, 6, 16]. In considering these possibilities, it is important to distinguish between two processes, the passage of molecules through the tonoplast (diffusion or by transport protein) and the driving force for alkaloid accumulation in the vacuole against a concentration gradient (membrane energization or ion trap mechanism).

Time course studies of the uptake of [$^{14}CH_3$]-morphine by latex 900 × *g* and 1100 × *g* vacuoles (Fig. 1) suggest that both groups of vacuoles take up morphine rapidly *via* a diffusion process with equilibrium attained in 2–4 min.

Previous results with a vacuole population sedimented at 1000 × *g* [3] have shown that saturation kinetics were not attainable and that there was no absolute requirement for MgATP. Furthermore, uptake of morphine was not particularly sensitive to temperature.

All these conditions would support a theory postulating simple diffusion, however, in the present experiments, while the 900 × *g* vacuoles showed absolutely no stimulation of uptake in the presence of MgATP, the 1100 × *g* vacuoles showed very slight stimulation and these 1100 × *g* vacuoles were also much more sensitive to temperature (Fig. 3). The 1100 × *g* vacuoles exhibited an activation energy (E_a) for morphine uptake of 58.08 kJ·M^{-1} (Fig. 3B), while the 900 × *g* vacuoles had an E_a = 22.18 kJ·M^{-1}. The 900 × *g* vacuoles are at the upper limit of activation energies associated with diffusion and the 1100 × *g* vacuoles are well

above the minimum activation energy level associated with facilitated uptake as discussed by Mende and Wink [6]. Closer inspection of the two vacuole populations [8, 18] showed that the 900 × *g* vacuoles contained most of the meconic acid, sulphate, malate and phenolic acids of the latex with only a very small amount present in the 1100 × *g* vacuoles. Investigation of these acids in the vacuoles during capsule maturation [18] revealed a strong correlation between acid and alkaloid content throughout maturation of the capsule for the 900 × *g* vacuoles which was not observable for the 1100 × *g* vacuoles, although these vacuoles still contained significant amounts of alkaloid [18]. In the 900 × *g* vacuoles therefore the acid content of the vacuole plays a major role in the maintenance of the acidity of the vacuole, a factor which is less apparent for the 1100 × *g* vacuoles.

In considering the theory of simple diffusion, the hydrogen ion concentration of the external medium would be expected to influence uptake. Measurements of morphine uptake were low at a pH similar to that of the vacuole internal pH (5.2–5.8) rising to a maximum at pH 8.0 with a 20% fall off at more alkaline pH [3]. The present investigation of vacuole internal pH (pH_{in}) using [^{14}C]methylamine as probe showed that a difference of $\simeq$ one pH unit was maintained when the external pH (pH_{out}) was that of the latex (*i.e.*, between 6.2 and 6.8).

The transtonoplast pH gradient (ΔpH) has been shown to be important in the regulation of alkaloid uptake into the vacuole, since reductions in ΔpH by NH_4Cl (Fig. 5) brought about a reduction in morphine uptake by the 900 × *g* vacuoles. The data in Fig. 2 shows that the uptake of large amounts of morphine by both the 900 × *g* and the 1100 × *g* vacuoles resulted in a reduced rate of uptake, this reduced rate could be improved by the addition of MgATP. This phenomenon was observable with both vacuole populations. The direct correlation between ΔpH and morphine uptake and the effect of MgATP on a saturated system seems to demonstrate that the function of an ATPase is to power a proton pump which restores the ΔpH. The stimulation of alkaloid uptake by MgATP in the 900 × *g* vacuoles was only observable with latex collected from young capsules (day 2–3 after petal opening) collected early in the season; presumably, the older vacuoles contained enough anions (sulphate, meconate and malate) [18] to stabilize a sufficient quantity of protons to maintain a ΔpH adequate for uptake and sequestration. Inhibitors (Table I) designed either to dissipate ΔpH or inhibit tonoplast ATPase were relatively unsuccessful in perturbing the system, presumably because of the high levels of anions and native alkaloids already present which stabilize the protons present within the vacuoles. Previous work [18] showed that during capsule maturation the vacuolar concentration of these anions is increasing at a rate which allows for the maintenance of a tonoplast ΔpH in addition to establishing a correlation between dibasic acids and alkaloids which would support protonation and complex salt formation (ion trap) as a method of permanent sequestration. Results in [18] suggested that the 1100 × *g* vacuoles are probably a younger form of the 900 × *g* vacuoles and since in these vacuoles a correlation between acid and alkaloid was not apparent, it was assumed that at this stage in vacuole development the tonoplast ATPase plays a more important role in the maintenance of ΔpH than it does in the 900 × *g* vacuoles. The distinctly different activation energies of the two vacuolar populations, discussed above, lends support to this hypothesis. The alkaloids, however, are as tightly held within the 1100 × *g* vacuoles as they are in the 900 × *g* and no alkaloid efflux was observed under the conditions used for these experiments. This result was not expected because of the apparent lack of correlation between alkaloid content and acid content, however, it is possible that other anions, *i.e.*, chloride, are important counterbalance ions [18]. In *Chelidonium majus* latex, vacuolar levels of chelidonic acid and phenolic compounds far exceed the levels of alkaloids and these, too, appear to be important components of an ion trap mechanism [25]. In *P. somniferum* latex vacuoles, similar significant amounts of phenolic material, other than morphine, are also found [8, 18] and must also play a role in the ion trap mechanism along with the anions L-malate, meconate and sulphate.

Important to an ion exchange mechanism of accumulation is the uptake of these materials into the vacuole or their synthesis as is suggested for the phenolic material present in *Chelidonium majus* [25]. Matile [25] failed to show uptake of chelidonic acid into the vacuoles of *C. majus* latex and similar problems were found in our experiments with

meconic acid. However, experiments with [^{35}S]sulphate and L-[U^{14}C]malate showed that uptake into the *P. somniferum* vacuoles was relatively slow and only marginally stimulated by the addition of MgATP (Fig. 5). The levels of sulphate taken up in 30–60 min would not readily be detected without the use of labelled material and the same could well be true for meconic acid, since the vacuoles contain large concentrations against which small changes due to uptake were being measured. The biosynthetic pathway and the site of biosynthesis of this unusual acid (pK_2 2.3) remains to be elucidated and so the question of its origins in the vacuoles remains open.

One problem remains which is not totally resolved by the present experiments. Previous results [3] suggested that the opium alkaloids were specifically taken up by the latex vacuoles. If movement across the vacuolar membrane is the result of simple diffusion as has been assumed for methylamine (pK 10.6) then low specificity and selectivity for the passage of alkaloids across the vacuolar membrane might be expected. While morphine (pK_1 6.13, pK_2 9.85), codeine (pK 6.05), thebaine (pK 6.05), papaverine (pK 8.07) and noscapine (pK 6.18) are readily taken up by the latex vacuoles, other alkaloids such as reserpine (pK 6.6), atropine (pK 4.35), quinine (pK_1 5.07, pK_2 9.7), cytisine (pK_1 6.11, pK_2 13.08) [3] and nicotine (pK_1 6.16, pK_2 10.96) do not pass through the latex vacuolar membrane. Present experiments, Table II, reveal however that caffeine (pK 14.0) and 1-methoxycanthin-6-one are taken up to a small extent. It has been suggested that "ion trapping" may be limited to alkaloids with relatively low pK values with the more basic alkaloids, *e.g.*, lupinine (pK 9.1), requiring active transport across the membrane [6]. Other groups [26–28] have suggested that the accumulation of bases is dependant on pK; the more alkaline the pK the higher the accumulation ratio. The present results and those given in [3], however, indicate that there is no clear correlation between pK and accumulation values, nor does there appear to be a correlation with lipophilicity since morphine, one of the least lipophilic of the group is taken up by the latex vacuoles with maximum efficiency. The results seem to suggest that molecular shape is important, although no distinction is made between (+)-codeine and (−)-codeine.

A recent paper by Hauser and Wink [29] reporting on the latex of *Chelidonium majus,* reports low selectivity for the latex vacuoles and a decrease in uptake of alkaloid with exogenous ATP. We, on the other hand, have found specificity of uptake with *P. somniferum* latex vacuoles (Table I), and have demonstrated that ATP does have an effect on morphine uptake by the $1100 \times g$ vacuoles, although it does not increase uptake in mature, $900 \times g$, vacuoles unless they have been exposed to an exceedingly high level of morphine (Fig. 2).

None of the evidence presented based on our present experiments or in [3] would support a requirement for a carrier protein to effect transport across the membrane and the high degree of specificity observed for the uptake of a limited range of alkaloid as opposed to others of similar pK and lipophilicity would not support the simple diffusion theory. The idea of specific channels involving the protein and/or the lipid component of the membrane has to be a consideration.

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Quantitative Characterization of the Contents of *Papaver somniferum* Latex Vacuoles

Thi D. T. Pham and Margaret F. Roberts*

Department of Pharmacognosy, The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK

***Papaver somniferum* latex vacuoles were separated into two discrete fractions—the 900×g and the 1100×g vacuoles. The two vacuolar populations were assayed over the three-week period of capsule maturation. Estimations of the anions sulphate, meconate and chloride, as well as the inorganic cations K^+,Na^+, Mg^{2+} and Ca^{2+}, were made, together with measurements of the alkaloids morphine, codeine, thebaine, papaverine and noscapine. A correlation between alkaloids and organic acids has been established which supports the proposed "ion trap" mechanism proposed for alkaloid sequestration in poppy latex vacuoles.**

Keywords: Papaver somniferum; latex vacuoles; alkaloids; sulphate; meconate; phenolics; inorganic cations.

INTRODUCTION

Alkaloids in *Papaver somniferum* are found almost exclusively within the anastomosing laticiferous system which has been well characterized (Nessler and Mahlberg, 1977; Nessler, 1982). The laticifer system has been shown to contain many of the enzymes associated with normal cellular metabolism (Roberts, 1971, 1974; Antoun and Roberts, 1975a, b) and within the latex is a discrete population of alkaloid-storing vacuoles (Fairbairn and Steele, 1981; Roberts *et al.*, 1983; Homeyer and Roberts, 1984a). The dynamic behaviour of secondary metabolites in plants is now well recognized (Luckner, 1980; Barz and Koster, 1981) but the mechanisms of their transport within cells and tissues is less well understood.

Alkaloids are normally found within cell vacuoles but their translocation across the tonoplast, with the build-up of a large concentration gradient across the membrane, remains open to hypothesis (Matile, 1976, 1984; Boller and Wiemken, 1986; Renaudin, 1989). In some instances an ion trapping mechanism has been suggested to account for the accumulation of large amounts of alkaloid (Muller, 1976; Renaudin and Guern, 1982; Neumann *et al.*, 1983; Matile, 1984). Other studies have suggested the occurrence of a very specific active transport system (Deus-Neumann and Zenk, 1984, 1986; Mende and Wink, 1987).

Since the uptake of alkaloids into the *P. somniferum* latex vacuoles does not have an absolute requirement for energy (Homeyer and Roberts, 1984a) and may involve an "ion trap" mechanism to sustain the accumulation of high concentrations of alkaloid observed, it is important that the occurrence of other anions and cations pertinent to the sequestration of alkaloids within the latex vacuoles be considered. Other investigators have previously reported that the levels of meconic acid and sulphate were high in *P. somniferum* latex (Miyamoto and Brochmann-Hanssen, 1962) and it has been subsequently show that meconic acid occurred in the latex vacuoles (Fairbairn and Williamson, 1978; Fairbairn and Steele, 1981). Renaudin *et al.*, (1986) have investigated the alkaloids, mineral ions and organic acids present in *Catharanthus roseus*. This group showed that malic and citric acids were the principal organic acids present. Ca^{2+} and inorganic phosphate were found almost exclusively in the cell vacuoles with 68% of the K^+ and Mg^{2+}. These results are basically in agreement with previous observations of Yamaki (1984) for organic acids and Lin *et al.*, (1977) for inorganic ions. Nitrate was also found to be principally stored in the vacuole (Renaudin *et al.*, 1986), but other researchers have found that nitrate levels in the vacuole may vary depending on the state of the plant (Martinoia *et al.*, 1981) and this could be expected to be true for other ions.

In the present paper we describe methods that have been developed in order to investigate the variations in inorganic anions and cations, together with the organic acids which occur as the poppy capsule reaches maturity and the alkaloid content increases.

EXPERIMENTAL

Chemicals. Unless otherwise stated chemicals were purchased from Sigma (UK) or British Drug Houses (UK). [^{14}COOH]-Dextrancarboxyl (18.5–74.0 MBq/g) was purchased from New England Nuclear (USA); ^3H_2O (185 MBq/mL) and [$^{14}CH_3$]-morphine HCl (2.07 GBq/mM) were purchased from Amersham International (UK).

Plant material. *Papaver somniferum* c.v. Halle was cultivated at the Medicinal Plant Garden of the School of Pharmacy, London. Plants were grown in sequential batches so that flowering occurred from late June until the end of September.

Isolation of latex vacuoles. Latex was collected from both the stem and capsule of the plant following the removal of the capsule by cutting with a sharp knife. Latex was collected into 700 mM mannitol to a final concentration of 50% latex.

* Author to whom correspondence should be addressed.

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Vacuoles were sedimented by centrifuging the latex at 900 × g for 30 min and subsequent centrifugation of the resultant supernatant at 1100 × g for 30 min. The vacuolar samples were washed twice, resedimented and finally resuspended in a buffer of 700 mM mannitol, 100 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulphonic acid (HEPES) adjusted to pH 6.8 with triethanolamine; this buffer was used in all subsequent operations. On average, vacuoles in buffer (200 μL) were equivalent to 40–60 μL (900 × g vacuoles) and 20 μL (1100 × g vacuoles) vacuolar volume for each assay sample.

Determination of vacuolar sample. The intravacuolar volume of the 900 × g or 1100 × g vacuolar samples was determined by incubating a 200 μL aliquot of isolated and resuspended vacuoles with a 30 μL aliquot of tritiated water (1.11×10^3 Bq) and a 10 μL aliquot of a [^{14}COOH]-dextrancarboxyl solution (3.7×10^2 Bq) for 30 min at room temperature (approximately 24.5 °C). The sample was centrifuged in an Eppendorf tube, at 900 × g or 1100 × g as appropriate, for 15 min and a sample of the supernatant assayed for ^{3}H- and ^{14}C-activities in a Packard Tri-Carb Liquid Scintillation Spectrometer using Aquasol LSC cocktail. The pelleted vacuoles were suspended in 500 μL of 0.1 M HCl in methanol, digested for 30 min at room temperature, centrifuged at 2000 × g for 15 min and the entire supernatant assayed for ^{3}H- and ^{14}C-activities. Counts were corrected for quenching, counting efficiency and channel overlap. It was assumed that the concentration of tritiated water was constant throughout the sample and that the dextran was restricted to the extravacuolar space. Dividing ^{3}H counters per μL of supernatant into the ^{3}H counts from the pellet provided the total sample void volume trapped in the pellet; dividing the ^{14}C counts per μL of supernatant into the ^{14}C counts from the pellet provided the trapped extravacuolar void volume; subtracting the trapped extravacuolar void volume from the total sample void volume left the intravacuolar volume (Rottenberg *et al.*, 1972). The vacuolar volumes for the 900 × g and 1100 × g vacuoles were very consistent for all similarly aged latex samples in a given season.

Isolation of alkaloids. Latex vacuole samples, 900 × g and 1100 × g, were plasmolysed in 0.025 M HCl in MeOH. After centrifugation, the supernatant from each sample was evaporated to dryness and the residue taken up in small volume of 2% H_2SO_4. The pH was adjusted to 9.5 with NH_4OH, the sample placed on a column of Extrelut (Merck, Darmstadt, Germany) and the alkaloids isolated by elution with $CHCl_3$: *i*-PrOH (85:15) (Roberts *et al.*, 1983). The residue on evaporation was made up in a standard volume and used for high performance liquid chromatographic (HPLC) analysis.

Isolation of dopamine. Latex vacuole samples were plasmolysed in 3 M Tris (pH 8.6) and the resultant mixture centrifuged at 1000 × g; the supernatant was applied to an alumina column using the method described by Roberts *et al.*, (1983). Where possible, analysis was carried out in an inert atmosphere (N_2) to reduce the oxidation of dopamine.

Isolation of acids. Latex vacuole samples were plasmolysed in 0.025 M NH_4Cl in MeOH and centrifuged at 1000 × g. The supernatant was placed on a cation exchange column (Dowex-50 W, H^+ form 7 × 100 mm) which had been previously equilibrated with 1 M HCl and washed with distilled water. The acids were eluted with distilled water. Following evaporation, the residue was made up in a standard volume and used for analysis for malate, meconic acid and sulphate.

Quantitative analysis of alkaloids, dopamine, malate, meconic acid and sulphate. Alkaloids and dopamine were analysed using the HPLC methods described in Roberts *et al.*, (1983). The analysis of meconic acid employed HPLC using an Hichrom RP18, 10 μ (25 cm × 4.9 mm) column eluted with MeOH:H_2O (17:83) containing 0.05% atropine sulphate. Sulphate was determined by gravimetric analysis using the method of Miyamoto and Brochmann-Hanssen (1962). In the case of the 1100 × g vacuoles the very low levels of sulphate were determined by ion chromatography (by Butterworth Laboratories Ltd., Teddington, Middlesex, UK) as were chloride determinations made on the 900 × g vacuoles. The instrument was a Dionex Model 16 Ion Chromatograph with an HIPC Anion Separator ASI and 0.008 M $NaHCO_3$ as eluent. Malic acid was determined enzymatically using the method of Witt (1974).

Assay for total phenolics. The measurement of total phenolics was made using vacuole samples (50–500 μL) which had been processed for the determination of sulphate and meconate: the method used was that of Roberts *et al.*, (1983). Owing to interference by meconic acid a calibration curve for this acid was constructed and, using the HPLC calculation for meconic acid, an adjustment for its presence in samples was made in calculating the total phenolics present in each sample. Results were estimated on the basis of tannic acid (MW 1550) for the purpose of estimating millimolar concentrations in the vacuole (tannic acid $E^{1\%/1\,cm} = 1010$; meconic acid $E^{1\%/1\,cm} = 531$).

Determination of cations using atomic absorption spectroscopy. Cation concentrations were measured by standard methods of atomic absorption (AA) spectroscopy using a Perkin-Elmer 280 AA spectrophotometer. Samples of the 900 × g and 1100 × g vacuoles, prepared as previously described, were plasmolysed in 20 mM phthalic acid and centrifuged at 12,000 × g for 15 min to remove insoluble material. The supernatant was removed and diluted 20-fold prior to the determination of cations by AA spectroscopy using standard solutions of the cations (prepared in glass-distilled water) to calibrate the equipment. The conditions used to determine the cation levels in the solution were as follows—Ca^{2+}: wavelength 422.7 nm, slit width 0.7 nm; Mg^{2+}: 285.2 nm, 0.7 nm; K^+: 766.5 nm, 2.0 nm; Na^+: 589 nm, 0.7 nm.

RESULTS AND DISCUSSION

P. somniferum contains a system of laticifers which ramify throughout the plant. The latex is collected by removal of the capsule and has been found to contain two distinct groups of alkaloid-containing vacuoles. These vacuoles were isolated and their volume determined for a given unit of latex using the methods given in the Experimental Section. They were designated as the 900 × g and the 1100 × g vacuoles. The remaining part of the latex was designated the latex supernatant. Although the methods of isolation could be expected to cause some osmotic swelling, the buffer mix used helped to prevent aggregation of the 900 × g vacuoles. Early investigations of the latex (Roberts, 1987, and references therein) suggested that the vacuoles constituted a large proportion of the total volume of the latex. More than 90% of the alkloid content of the latex was found contained within the two vacuole fractions,

Table 1. A comparison of alkaloid levels in the latex with changes in vacuolar volume during capsule maturation[a]

Fraction	Week from petal opening	mg Alkaloid/mL latex					Vacuolar volume
		Morphine	Codeine	Thebaine	Papaverine	Noscapine	μL/mL latex
900 × g	0	34.25	2.27	9.15	0.90	5.29	541
vacuoles	1	56.44	1.59	6.31	0.74	3.61	586
	2	78.73	4.11	6.79	2.07	4.95	701
	3	96.71	4.23	6.23	4.45	11.76	817
1100 × g	0	2.25	0.09	0.80	0.02	0.11	320
vacuoles	1	4.08	0.25	0.90	0.05	0.20	310
	2	3.92	0.07	0.52	0.07	0.19	229
	3	4.22	0.08	0.50	0.11	0.32	188

[a] The results are from duplicate samples from randomly collected latex and are representative of experiments performed over several seasons. Vacuolar volumes were determined as described in the Experimental Section.

apparently sequestered by an ion trap mechanism (Homeyer and Roberts, 1984a).

In the present paper the latex vacuoles were investigated over the first three weeks of capsule development after petal opening (week 0) to determine the relationship between alkaloid accumulation and the accumulation of cations and anions.

Comparison of the alkaloid levels in the latex of 900 × g and 1100 × g vacuoles during capsule development

In these experiments the five major alkaloids, morphine, codeine, thebaine, papaverine and noscapine, were measured using normal phase HPLC. Table 1 clearly indicates that the levels of these alkaloids in the total latex increase as the capsule develops during the first three weeks after petal opening. Subsequently, however, as the capsule dries and the latex structure breaks down, losses of up to 10% of the alkaloid content can occur (Roberts, unpublished results). Increases in alkaloid content, particularly morphine, are most marked in the 900 × g vacuoles, which contain the major part of the capsule alkaloids. In the 1100 × g vacuoles, alkaloid levels remain fairly constant without the doubling in alkaloid content observed for the 900 × g vacuoles. For example, whereas morphine content rises from 34.25 mg/mL of latex to 96.71 mg/mL in the 900 × g vacuoles, the content in the 1100 × g vacuoles remains around 4.0 mg/mL of latex.

These results are in keeping with those found previously by Fairbairn and Steele (1981, and references therein). Of particular interest, however, is the fact that within a unit amount of latex the vacuolar volume increases substantially for the 900 × g vacuole fraction whereas it remains relatively constant for the 1100 × g vacuole fraction (Table 1). In older latex (more than three weeks after petal opening) the 1100 × g vacuolar fraction largely disappears (Roberts, unpublished results). This is reflected in the reduction in vacuolar volume per unit of latex for the 1100 × g vacuoles from week 0 to week 3. Whether the increase in vacuolar volume per unit of latex for the 900 × g vacuolar fraction is related to an increase in the number of vacuoles per unit volume of latex, or whether it relates to an increase in volume of the existing vacuoles, cannot be determined with the present methods.

Analogy with investigations on lysosomes (de Duve *et al.*, 1974; Poole and Ohkuma, 1981) and microscopic inspection suggests that the vacuoles of the 900 × g latex fraction increase in size as a result of the uptake of bases which, when the concentration becomes sufficiently high, cause osmotic swelling to form large phase-lucent vacuoles. The larger vacuoles are readily observable with the phase contrast microscope and in older latex seem to aggregate. Electron micrographs of latex vacuoles from the *P. somniferum* cultivar Soma suggest that the smaller vacuoles coalesce with the larger. It is thought that these two processes contribute to the increase in vacuolar volume observed for the 900 × g vacuoles.

Important to the ion trap mechanism for base sequestration, first put forward by de Duve *et al.*, (1974) and subsequently utilized by Matile (1976) to explain the sequestration of alkaloids in *Chelidonium majus* latex vacuoles, is a consideration of the concentrations of anions and cations within the vacuoles. Figure 1 therefore shows the alkaloids within the two vacuolar populations expressed as millimolar concentrations. It will be noted that the increase in the alkaloid concentration (220–360 mM for morphine) within the 900 × g vacuoles is affected by the increase in vacuolar volume during capsule maturation. The greater increase in concentration in the 1100 × g vacuoles on the other hand is due partly to a decrease in vacuolar volume. It should be noted that the dopamine previously found to occur in the latex vacuoles is found only to a significant extent in the 900 × g vacuoles and this confirms our earlier studies (Homeyer and Roberts, 1984b; Roberts *et al.*, 1983). A comparison of alkaloid and dopamine content for the 900 × g vacuoles is given in Fig. 3(A). The dopamine concentration remains stable at 25 mM whereas the alkaloid concentration increases with capsule maturity.

Inorganic cations in *P. somniferum* latex

In order to complete the investigation of cations within the latex vacuoles, the common inorganic cations, K^+, Na^+, Mg^{2+} and Ca^{2+}, were measured in both vacuolar fractions and in the latex supernatant. The results given in Table 2 show that the concentration of Mg^{2+} was 10 mM or less wherever assayed, with marginally higher

levels in the vacuoles. There was no significant differnece in the levels of K^+ found among the three elements of the latex. A very high concentration of Na^+ in the vacuoles (about 100 times that in the supernatant) decreases in the 900 × g vacuoles from nearly 400 mM to slightly over 250 mM during the three-week maturation of the capsule. Na^+ concentration in the 900 × g vacuoles was initially (at petal opening) over twice that of the 1100 × g vacuoles, declining to nearly the same concentration at week 3. Similar high levels of Na^+ are found in the vacuoles of *Beta vulgaris* where K^+ levels were also high. However in *B. vulgaris* only 50% of the K^+ was located in the vacuole (Leigh and Deri-Tomos 1983).

In *P. somniferum* latex, Ca^{2+} concentrations in the 900 × g vacuoles were 30–60 times those in the supernatant and 2–3 times those found in the 1100 × g vacuoles. Concentrations of Ca^{2+} are usually maintained at low levels in the cytosol as a part of the role of calcium as an intracellular signal, and therefore Ca^{2+} is usually sequestered in the vacuole (Bush and Sze, 1986).

It has been shown by a number of investigators that vacuoles may be isolated with the retention of ions so that analysis of their contents *in vitro* may give meaningful information about the compartmentation of ions *in vivo* (Lin *et al.*, 1977; Martinoia *et al.*, 1981; Moskowitz and Hrazdina, 1981; Leigh *et al.*, 1981). Whether there is any true correlation between losses of inorganic cations from the latex vacuole and alkaloid accumulation has yet to be determined.

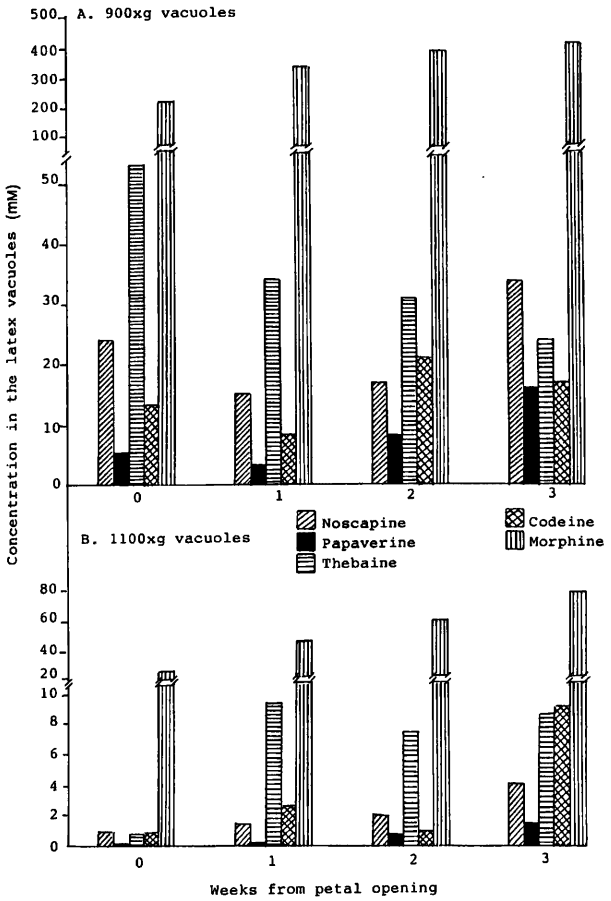

Figure 1. The changes in concentration of alkaloids in *P. somniferum* latex vacuoles during the maturation of the capsule. (These figures have been calculated from the results detailed in Table 1.)

Table 2. Concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} in *P. somniferum* latex[a]

Fraction	Week from petal opening	Concentrations (mM) K^+	Na^+	Mg^{2+}	Ca^{2+}
900 × g vacuoles	0	56	388.7	10.0	53.3
	1	90.7	367.3	7.3	43.3
	2	58.0	266.7	10.0	65.3
	3	55.0	254.7	8.0	51.7
1100 × g vacuoles	0	39.1	151.3	5.0	10.9
	1	27.0	181.7	3.9	17.4
	2	40.9	159.1	2.9	14.8
	3	60.9	187.0	5.1	16.5
Supernatant	0		1.2	1.7	0.6
	1	29	2.4	1.3	0.9
	2	58	2.2	1.6	0.8
	3		3.7	1.4	1.8

[a]Samples were taken from the randomly collected latex used for the experiments in Table 1 and result from duplicate sampling in any one week.

The occurrence of the anions meconate, sulphate and chloride and their accumulation in the 900 × g and 1100 × g vacuoles during capsule maturation

Previous studies have indicated that meconic acid and sulphate constitute 60–80% of the acid content of opium, the dried latex of *P. somniferum* (Miyamoto and Brochmann-Hanssen, 1962). Figure 2 shows that sulphate and meconate occur almost exclusively in the 900 × g vacuoles of the latex although small amounts are also found in the 1100 × g vacuoles. These two acids

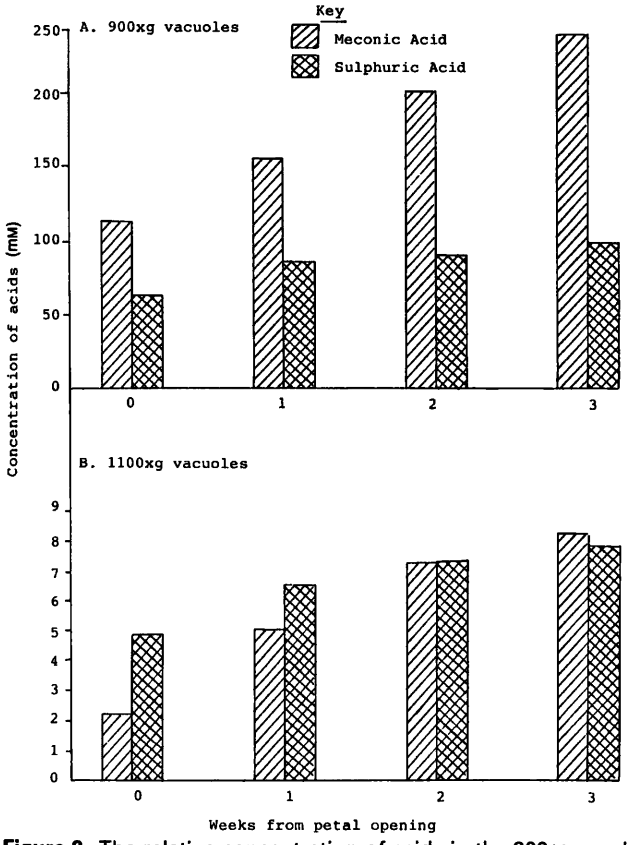

Figure 2. The relative concentration of acids in the 900 × g and 1100 × g vacuoles of *P. somniferum* latex. (These results were obtained from the same samples of latex as were used for Table 1. The levels of meconate and sulphate were calculated using the methodology given in the Experimental Section.)

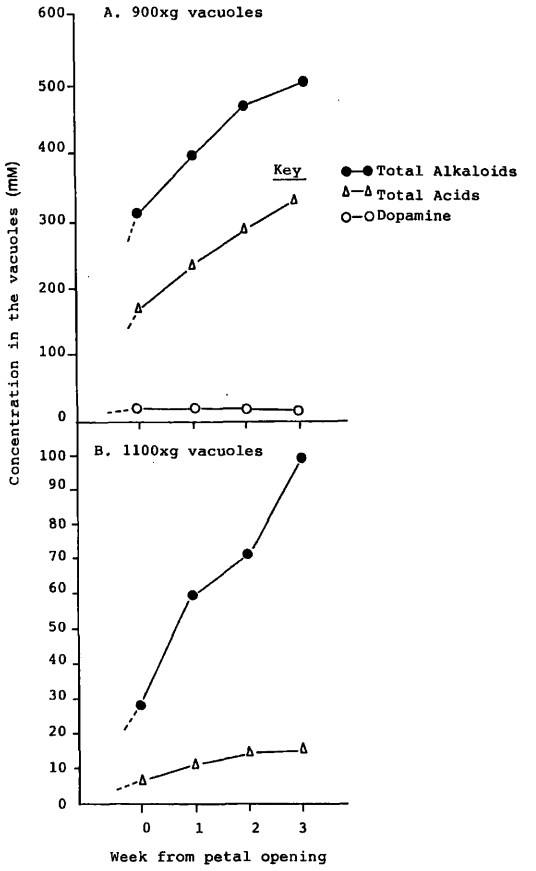

Figure 3. A comparison of bases and acids in the 900 × g and 1100 × g vacuoles of *P. somniferum* latex.

constitute the major anions found in the latex vacuoles as investigated using ion chromatography. Of note is the detection of very low concentrations of malic acid (18 mM) in the 900 × g vacuoles, but the apparent absence of citrate. In both the 900 × g and the 1100 × g vacuolar populations, meconate and sulphate increase with the maturation of the poppy capsule. Cells of higher plants may accumulate large quantities of malic acid and this accumulation is generally a reversible process (Marigo *et al.*, 1985). In *P. somniferum* latex vacuoles malic acid is a minor constituent compared with meconic acid and sulphate.

Chloride determinations on samples of latex 900 × g vacuoles harvested at 2 and 3 weeks from petal opening were similar with a chloride concentration of 133 mM and 154 mM, respectively. This suggested that chloride was one of the major ions involved in equilibrating the positive charges of alkaloids and mineral cations. Chloride may therefore have a significant role to play in alkaloid sequestration.

Presence of phenolic acids

Using the Folin–Ciocalteu method for the assay of total phenolics it could be calculated on the basis of tannic acid that low levels of phenolic substances existed in the 900 × g vacuoles. The concentrations within the vacuoles at weeks 0–3 of capsule development were calculated as 1.6, 2.9, 4.6 and 6.4 mM, respectively. Similar estimations using the 1100 × g vacuoles did not yield positive results after the necessary adjustments for meconic acid had been made. Phenolic acids appear to be a minor component of the *Papaver* latex vacuoles as compared with the occurrence of meconic acid and sulphate, and this is somewhat different from the high levels (621 mM) observed in *Chelidonium* latex (Matile, 1976, 1984).

Correlation between alkaloid and acid concentrations within the latex vacuoles

In Fig. 3(A) a comparison has been made between total alkaloid, dopamine and the total acid concentration. For the 900 × g vacuoles, the concentration of dibasic acid is such that all of the alkaloid could be trapped in an association with these two acids which would involve retention of the base in the environment of the vacuole, which is usually acidic compared with the supernatant by one pH unit (Roberts, 1987). The apparent shortfall in anions to equilibrate the inorganic cations in the 900 × g vacuoles could be accounted for by chloride, given the levels (133 mM and 154 mM) in the samples measured at 2 and 3 weeks from petal opening. In the 1100 × g vacuoles levels of meconate and sulphate are such that another mechanism is required. However, a proton pumping ATPase has been detected which helps to maintain a membrane ΔpH which allows for continued uptake and sequestration of alkaloid in excess of the availability of meconate and sulphate (Pham *et al.*, 1990).

If the 1100 × g vacuoles may be considered as less mature forms of the 900 × g vacuoles, the results would suggest that the initial development of the tonoplast ΔpH is dependent on a ATPase proton pump. As vesicles mature, this activity appears to have less influence on tonoplast ΔpH than does the sequestration of meconate and sulphate. These two acids therefore not only help to maintain tonoplast ΔpH but are also instrumental in trapping the alkaloids within the vacuole.

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