

IMMOBILISATION OF ENZYMES FOR ALKALOID PRODUCTION

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ABSTRACT

The thesis is concerned with the condensing enzyme systems of the plant *Catharanthus roseus* which are involved in the synthesis of dimeric indole alkaloids and their monomeric precursors, in particular vindoline and catharanthine.

The antineoplastic dimers are synthesised in plants in very low quantities and there is considerable incentive to produce them more efficiently. Most efforts in this direction have utilised both chemical and enzyme techniques with varied success.

This work deals with synthesis of alkaloids using a *C. roseus* cell free system immobilised on tressyl activated agarose. The report describes the isolation and purification of the enzyme system and the extraction and synthesis of the precursor alkaloids, using liquid chromatography techniques, as well as the immobilisation procedure used. The condensing reaction catalysed by the enzymes was studied using radiotracer methods and the results critically compared with the behaviour of the free enzyme system.

It is concluded that immobilisation may be a useful technique for the production of alkaloids, and that it may be useful as a model system for the study of plant cell reactions.

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LIST OF ABBREVIATIONS

approx.	:	approximately
av.	:	average
cat	:	catheranthine
C. roseus	:	Catheranthus roseus
3,4,dehydroVLB	:	3,4, dehydrovinblastine
dpm	:	disintegrations per minute
FAD	:	Flavine Adenine Dinucleotide
FPLC	:	Fast Protein Liquid Chromatography
HPLC	:	High Performance Liquid Chromatography
I.A.	:	Indole Alkaloids
NADPH	:	Nicotine Adenine Dinucleotide Phosphate (reduced)
PVP	:	Polyvinylpyrrolidone
rel.	:	relative
RIA	:	Radioimmunoassay
Rf	:	Ratio of fronts
TLC	:	Thin Layer Chromatography
Trp	:	Tryptamine
vin	:	vindoline
VLB	:	Vinblastine
vol.	:	volume

1. INTRODUCTION

A great many natural products isolated from higher plants are utilised in the food and pharmaceutical industries. Most of them are secondary metabolites, usually alkaloids, many of which occur only as minor components in plants, making isolation tedious and costly (Ref. 8).

One alternative lies in the use of tissue and cell suspension cultures derived from alkaloid containing species to produce these compounds.

But, as shown in 1.1, low productivity has made this method of limited applicability at present (Ref. 3). Other attempts involve chemical synthesis as a replacement for alkaloid isolation but with varied success (Ref. 18).

Synthetic studies, in vivo feeding experiments and in vitro enzyme work suggest an enzymatic process derived from the plant as a third approach to the production of alkaloids. This would involve the biosynthesis of a medically valuable alkaloid from its precursors using an enzyme system isolated from the plant. A suitable candidate for study was the alkaloid vincalcalcin (VLC), valuable as an anti-cancer agent (Ref. 37), which occurs in the well investigated plant *Catharanthus roseus*, known for its ease of cultivation. It appears at very low levels in the plant and not at all in tissue culture, so that isolation is tedious. It is a dimeric indole alkaloid formed from monomeric precursors, catharanthine and vindoline, both found in and isolated from the plant. This process may be followed by radioactive tracer incorporation studies (Ref. 13).

The application of enzymes to alkaloid production has not been sufficiently studied before. Unfortunately, the commercial application of any enzyme is hampered by its often low stability and high cost. Methods facilitating reutilisation and stabilisation are thus very

desirable. Immobilising the enzymes on a support is one method of accomplishing this. For industrial processes a further advantage of immobilisation lies in the removal of enzymes from solution without contamination of the reaction mixture and decreasing the possibility of enzyme washout (Ref. 42).

While much work has been done on indole alkaloid biosynthesis with plants, cultured cells and enzyme systems (Refs. 3, 11, 32), and also on immobilised plant cells (Ref. 7), there is a dearth in the literature of immobilised enzyme systems as applied to the biosynthesis of indole alkaloids. This paucity is interesting in that there is an indication that immobilised systems can also be used as models for the study of enzymes in a simulated natural environment as many enzymes are thought to be attached to supports, such as membranes, in the cell. For instance the enzymes of the urea cycle have been co-immobilised to supports to study their sequential actions in cells (Ref. 34).

This work reports the application of immobilised enzymes in the biosynthesis of indole alkaloids from *Catharanthus roseus*. As this process is being tried out for the first time there are likely to be many difficulties not envisaged at the beginning but turning up in the course of the investigation. These will be dealt with as they occur and also at the conclusion of the report. The study thus offers a brief introduction to the alkaloids and their parent plant as well as to the biosynthetic and immobilisation procedures involved. This is followed by a description and presentation of the practical work performed and the results obtained. Discussion of the results is concluded with suggestions for further research.

1.1 *Catheranthus roseus* G. Don (*C. roseus*)

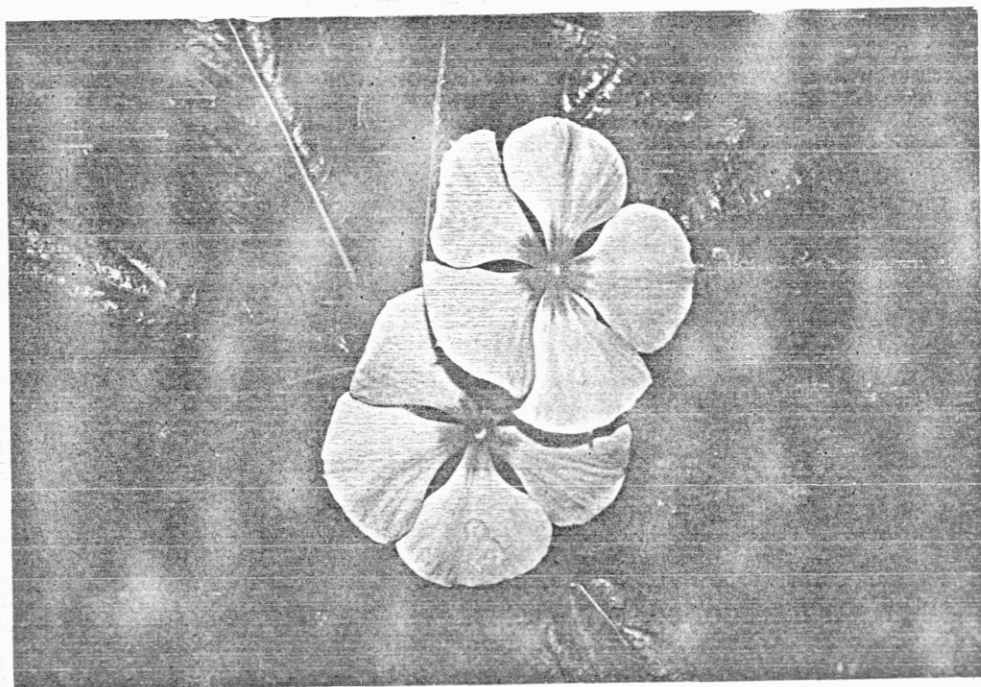
Initial studies on the Madagascar periwinkle or *Catheranthus roseus* G. Don of the botanical family Apocynaceae were based on its reported folkloric medicinal usage, which led to the isolation of more than 100 indole alkaloids with all 3 major classes being represented. While catheranthine and vindoline are major isolation products, interest lies in the dimeric compounds like vincaleucoblastine (VLB) which are the most valuable antineoplastic drugs available. *C. roseus* is used in most biosynthetic studies on alkaloids because of the ease with which it can be cultivated and handled and also its relatively high alkaloid content. It is an erect, everblooming shrub, 30 to 60 cm high, with two colour varieties, pink and white, that appear to show no differences in average alkaloid content. Originally native to Madagascar, it is pantropical in occurrence (Ref. 37). It is illustrated in fig. 1.1.

The importance of periwinkle alkaloids has led to the study of plant cell cultures as a relatively homogenous, reproducible, controllable and easily manipulated source for the industrial scale isolation of these compounds (Ref. 3). So far there have been large differences in the concentration of alkaloids produced by different strains and clonal variation in response to growth conditions. The evidence for the occurrence of various alkaloids has been tentative, based on TLC analysis rather than characterisation of isolated compounds (Ref. 19). Generally, callous tissue has a lower alkaloid content than the whole plant; the average vindoline content in plants is approx. 0.4% dry weight, that of calli is 0.002% (Ref. 3). Selection of high producing cell lines could overcome this difficulty, but the technique is still in its infancy and the number of such lines is limited (Ref. 7), so that most studies have been done on whole plant systems.

Fig. 1.1 Catheranthus roseus G. Don.



Detail of leaves and flowers.



1.2. The Alkaloids

Alkaloids are a group of naturally occurring compounds containing heterocyclic nitrogen atoms. They are secondary metabolites, i.e. they are not directly required for life functions (Ref. 11). Several thousands of them are known, usually by trivial names based on the plants in which they occur, e.g. catheranthine from the genus catheranthus). Valuable information on structure and occurrence may be found in Glasby (Ref. 14).

1.2.1. Classification

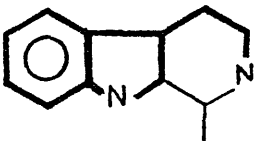
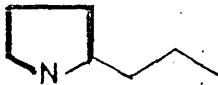
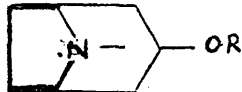
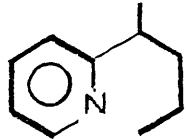
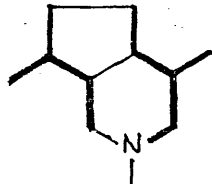
Alkaloids are classified chemically according to their carbon- nitrogen skeleton and also on their biogenesis, both of which are given in table 1.1. However the definition of alkaloids offered in 1.2. would exclude aliphatic nitrogen containing simple compounds that resemble more complex ones in function. The term "Proto- alkaloid" to define these also indicates their connection with complex alkaloids in that as decarboxylation and hydroxylation products of amino acids, they serve as alkaloid precursors. The alkaloids can thus be subdivided, depending on their biogenetic precursors, into derivatives of various amino acids like lysine, ornithine and tryptophan (Ref. 12).

1.2.2. Occurrence

Probably approximately 10 to 20% of all higher plants contain alkaloids. They abound in dicotyledons, in particular the family Apocynaceae. A few alkaloid-like compounds can be found in lower plants, fungi and animals ; e.g. Salamander alkaloids (Ref 32). A listing of plants and their alkaloids has been made by Willaman and Li (Ref. 41).

Within plants the incidence of alkaloids varies notably between tissues, leaves and roots being important sources. Changes can also be seen during the course of ontogeny, the alkaloid content generally increasing at cell enlargement and decreasing at senescence. Several external

Table 1.1. The most important classes of alkaloids and their precursors (precursors are indicated by heavy lines)

Class of alkaloid	structural type	main biogenetic precursor	alkaloid and plant of extraction
Indole		tryptophan	catheranthine C. roseus.
Pyrrolidine		ornithine and acetate	hygrine Erythroxyton truxillence
Tropane		ornithine and acetate	cocaine Erythroxyton coca
Quinolizine		lysine	lupinin Lupinus luteus
Terpene		mevalonic acid	connessine Holarrhena species

Data from Dalton (Ref. 12)

factors like light and nutrition influence alkaloid content in that those affecting growth also tend to favour alkaloid formation (Ref. 27).

A general theory to explain alkaloid distribution in plants, based on their defensive function, has been proposed by McKey (Ref. 21). Thus alkaloids must be inactive in the plant but readily available in case of herbivorous attack. This would concentrate them in the outer layers of the plant and in structures like vacuoles where they would be isolated from sensitive tissue. These hypotheses are born out by many studies which show that due to their polar, basic nature, most alkaloids form salts with organic acids or phenols present in cellsap, especially in vacuoles (Ref. 29).

1.2.3. Biological and Economic Significance

Research on alkaloids has been concerned mainly with biosynthesis and not on the physiology of the formed product, so that there are few theories involving useful functions of alkaloids. The role of alkaloids as detoxification products seems plausible in instances where they remove such active entities as nicotinic acid from sites where they could unbalance metabolism (Ref. 15). Alkaloids have been termed waste products analogous to urea in animals, but nitrogen is scarce in plants and would tend to be reutilised (Ref. 27). Defence of plants from attack by herbivores has also been proposed as an alkaloidal function (Ref. 21). It has been shown that alkaloids have an effect on growth of other types of plants; e.g. barley produces gramine which has an inhibitory effect on *Stellaria* species. This has implied a role for alkaloids in the competition between plants for survival (Ref. 27). Because information about katabolism and the transport of alkaloids within tissues is still wanting, and also because of the great diversity of alkaloids, these theories cannot be generally applicable.

In contrast to alkaloid metabolism in plants, in other organisms it has been studied because of the pharmacological effects of alkaloids. Most work has been done on mammalian pathways as being immediately relevant. It is asserted that administered drugs may be converted to real physically active substances inside the organism. This process of detoxification, a major site of which is the liver, involves oxidations, including dehydrogenations, hydroxylations and demethylations or transformations to less toxic compounds by conjugation to other molecules, or a combination of both; e.g. arecoline from *Areca catechu* is metabolised to its N-oxide and to a glucoromide (Ref. 32).

Alkaloids and their detoxification products act at low concentrations on many biochemical processes. A general explanation for drug effects may lie in the structural changes caused in macromolecules by the adsorption or displacement of alkaloids. Complementarity between alkaloid and its site of action or receptor must exist and bonding in the form of covalent, ionic, hydrogen and metal ligands has been proposed (Ref. 22). Such associations would then account for the wide area of alkaloid effects; on mechanisms of growth, enzyme regulation and activity, membrane mediated transport and nervous processes.

The economic importance of alkaloidal drugs cannot be undervalued. Ways of increasing alkaloid yield by selective feeding of plants and cell cultures, genetic manipulation of alkaloid containing organisms and improving synthetic techniques are being continually carried out (refs. 3, 19, 43). Their success is probably only a matter of time.

1.2.4. Biogenesis

Robinson first suggested that alkaloids could be made up of amino acids and other small components derived from primary metabolism (Ref. 31). Only a few types of reactions would be involved.

These include redox reactions, decarboxylations, aldol and amine condensations, all of which are fundamental to organic chemistry and are illustrated in table 1.2. (Ref. 12). The Robinson proposals were based on analogy with chemical reactions and structures rather than on direct biochemical evidence. However this 'Biogenetic' approach enables the construction of different compounds on rational chemical grounds, using a few building blocks and simple bonding reactions. In plants these reactions would be mediated by enzymes and associated cofactors. Thus the complex structures of alkaloids are seen as the sum of their components rather than a fortuitous collection of atoms and bonds.

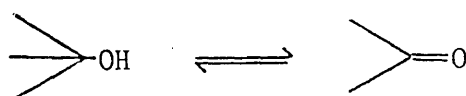
Many of the pathways predicted by this approach have been experimentally confirmed by in vivo work involving the feeding of isotopically labelled materials to plants, then isolating the alkaloids formed to determine the label incorporation. More complex reactions at the enzyme level were studied using cell free extracts containing plant enzymes and incubating them with radiolabelled precursors (Ref. 5,33,36,40). However, little is known about the enzyme systems involved, or how they evolved. It has been proposed that many may be of low specificity which otherwise catalyse other metabolic processes. Some steps may occur without the benefit of enzymes, if the right conditions of pH, temperature etc. are met. Plants that do not synthesise alkaloids may have potential alkaloid synthetic pathways which require only a single mutation to be activated.

There is some evidence in the case of Leguminosae, for an alkaloid bearing ancestor whose biosynthetic mechanism was lost in certain species such as beans and peas, but retained in others such as lupins (Ref. 32). A general survey of alkaloid synthesis may be found in Dalton (Ref. 12).

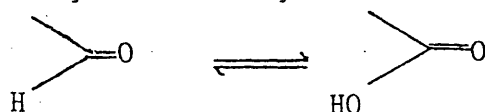
Table 1.2. Key initiating reactions in alkaloid synthesis

Redox reactions :

alcohol to ketone (or aldehyde)

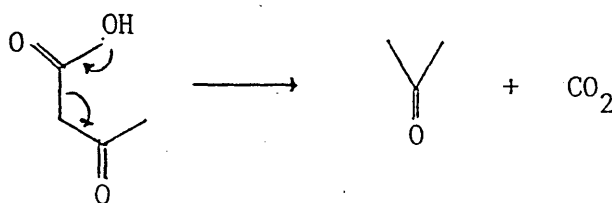


aldehyde to carboxylic acid



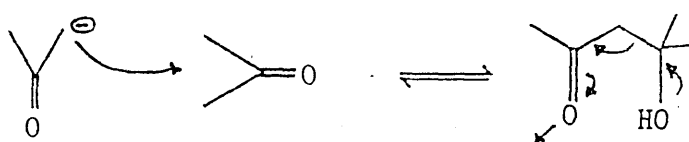
Decarboxylation :

B- keto acids to ketones

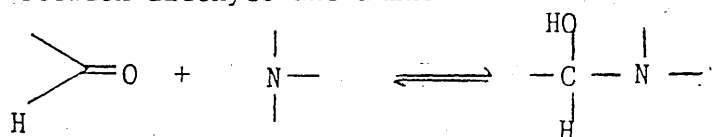


Aldol condensation :

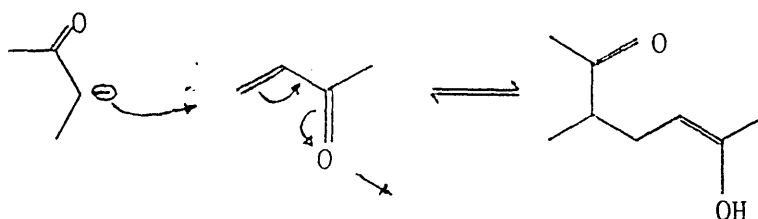
between ketones



between aldehyde and amine



between carbonyl compounds with double bonds
interposed between reaction site and leaving group
(A vinylogous analogue)



1.3. The Indole Alkaloids

Indole alkaloids (I.A.) are defined as natural products containing an indole nucleus (fig.1.2) or a derivative such as N-acyl-indole (Ref. 18). Given the complexity of these systems, and the inherent potential for rearrangement of the elaborated skeleton, it is not surprising that the number of currently known structures amounts to about 1200 of which 80% are monomeric (Ref.32). There are also about 100 "bis-indole" or dimeric I.A.s derived from the monomers (Ref. 12).

1.3.1. Classification

I.A.s may be classified according to their plant sources as many plant genera contain characteristic alkaloid structural forms as shown in table 1.3. They may also be divided into simple and complex compounds. The latter can be further classified by the carbon backbone of the non-indole moiety as illustrated in fig.1.3. The alkaloids of interest to this study fall into the I.A.s derived from loganin where three major classes are distinguished; Corynanthe (e.g. ajmaline, ajmalicine), Aspidosperma (vindoline) and Iboga (catheranthine), as shown in fig.1.4 (Ref. 9). The bisindoles are grouped into two categories, namely, those where both "halves" are composed of related or identical groupings (e.g. calycanthine) and those where the halves, though ultimately derived from tryptophan, are different (vinblastine). These are shown in fig. 1.5.(Ref. 12).

1.3.2. Occurrence and Economic Significance

The majority of the I.A.s have been isolated from the plant families Apocynaceae, Loganiaceae and Rubiaceae with the genera Catheranthus and Rauwolfia being specially prolific.(Ref. 32).

I.A.s are pharmaceutically useful. Harmine and related alkaloids are used as a tapeworm remedy, dimeric alkaloids from Catheranthus in the

Fig. 1.2. The Indole Nucleus

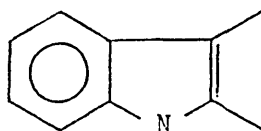


Table 1.3. The classification of the Indole Alkaloids

structural type	alkaloid type	structure
carboline	harman alkaloids	harman The structure of harman consists of an indole nucleus fused to a six-membered ring containing a nitrogen atom and a double bond. A methyl group is attached to the carbon atom adjacent to the nitrogen in the six-membered ring.
ergoline	ergot alkaloids	lysergic acid The structure of lysergic acid is a complex pentacyclic system. It features an indole nucleus fused to a six-membered ring, which is further fused to a seven-membered ring containing a nitrogen atom. A carboxylic acid group (HO₂C) and a hydrogen atom are attached to the seven-membered ring, and a methyl group is attached to the nitrogen atom.
iridoid indoles	rauwolfia alkaloids calabash curare " catheranthus " cinchona "	vindoline (see fig. 1.4.)

Fig. 1.3. Classification of Indole Alkaloids by
Structure and Biogenesis

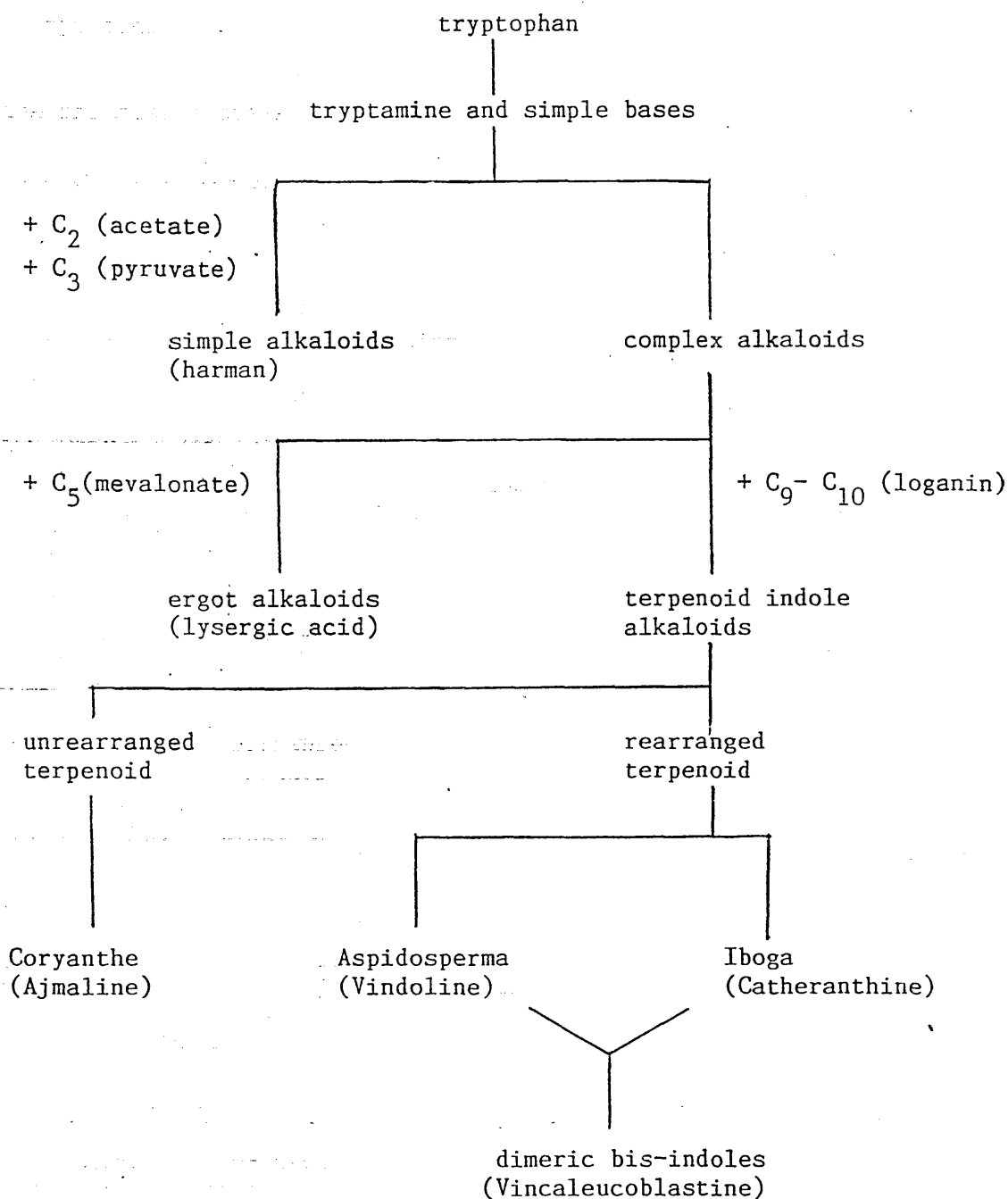
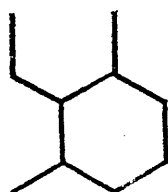
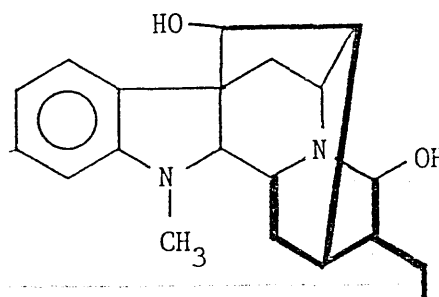


Fig. 1.4. Three major classes of Indole Alkaloids and their representatives (The monoterpenoid moiety is shown with heavy lines)

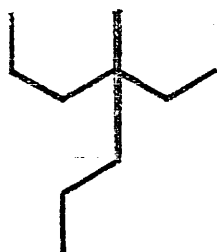
Corynanthe type :



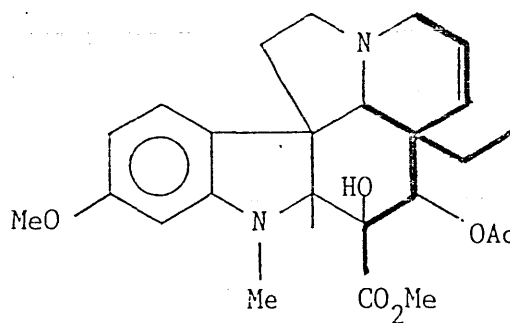
Ajmaline



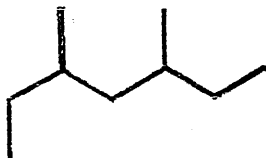
Aspidosperma type :



Vindoline



Iboga type :



Catheranthine

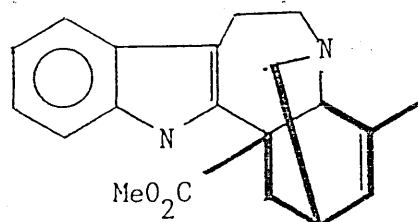
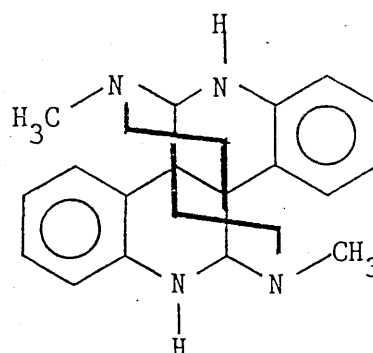


Fig. 1.5. Classification of Dimeric (Bis-) Indole Alkal

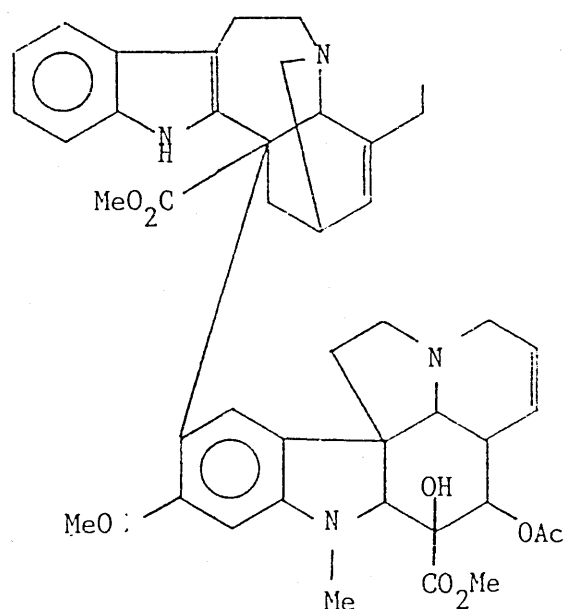
1. Both sub-units derived from identical compounds

Calycanthine :



2. Each sub-unit derived from different indole compound

Vinblastine :



treatment of Hodgkins' disease and various carcinomas. The catheranthus alkaloids appear to exert their effects through the inhibition of the mitiotic apparatus of the cell and thus on cell division. The clinical use of these compounds is reviewed in a survey (Ref. 13).

1.3.3. Biogenesis

Their very diversity has endowed the I.A.s with more hypothetical metabolic pathways than any other alkaloid group. However, it was suggested at the turn of the century by Pictet and confirmed later by *in vivo* studies that the indole nucleus of I.A.s is derived from tryptamine (Trp) formed from the decarboxylation of the amino acid tryptophan (Ref. 5). The conversion presumably involves a pyridoxal-bond intermediate and is shown in fig. 1.6.a.

The condensation between Trp and an aldehyde to form B-carboline derivatives is depicted in fig. 1.7.a. Such reactions are thought to form harman and other simple alkaloids (Ref. 32). Additional structural or functional groups on the aldehyde portion lead to more elaborate alkaloids. Thus Trp can combine with C_2 (acetate) or C_5 (pyruvate) to yield B-carboline and ergoline alkaloids respectively (Fig. 1.4.).

The non-indole unit of I.A.s with which this report is concerned was shown to be a C_9 - C_{10} fragment later identified as the monoterpeneoid Secologanin, found to derive from Loganin through an as yet uncharacterised multienzyme pathway as depicted in fig. 1.6.b. (Ref. 5).

For many alkaloids, the initial steps are the cleavage of Loganin to Secologanin and the condensation of the latter with Trp (Fig. 1.7.b.), a reaction which resembles that shown in fig. 1.7.a, except for the aldehyde component. By this Pictet-Spengler type reaction, a tetrahydro-B-carboline is formed with the elimination of the H atom at position C-2 of the indole ring (Ref. 39). The formation of two

Fig. 1.6.a. The conversion of Tryptophan to Tryptamine

(The structures in parentheses indicate hypothetical intermediates)

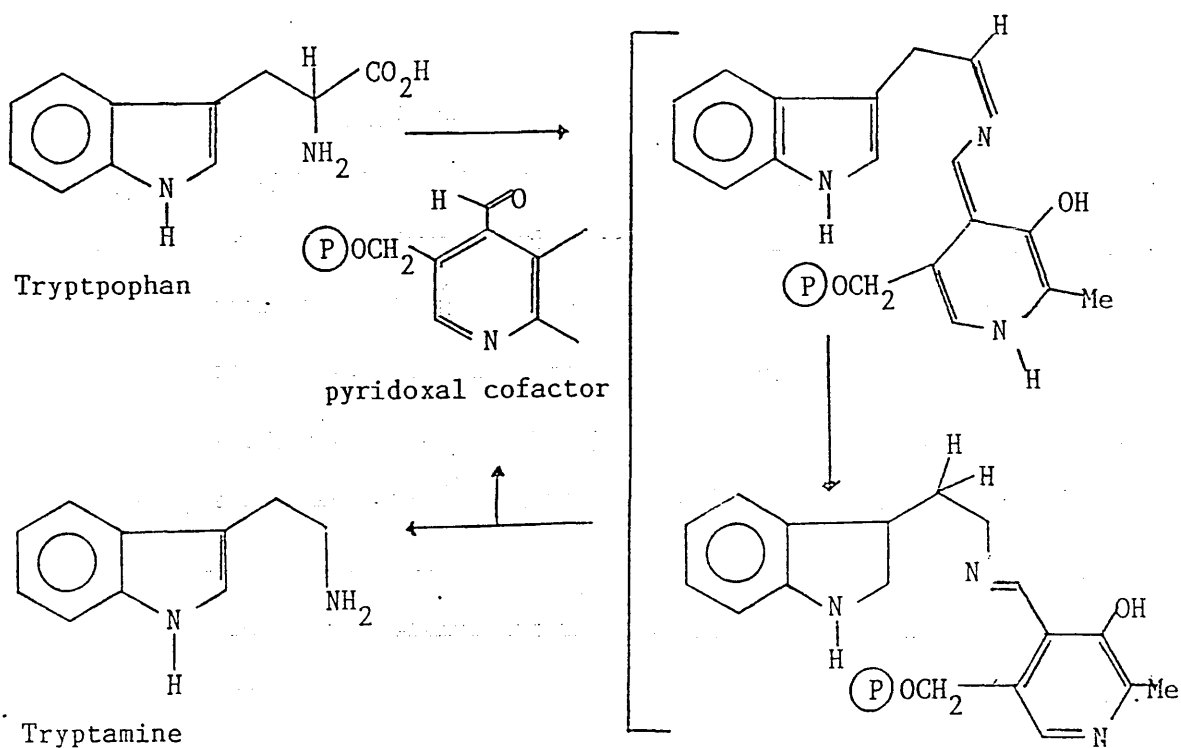


Fig. 1.6.b. The conversion of Loganin to Secologanin

(The structures in parentheses indicate hypothetical intermediates)

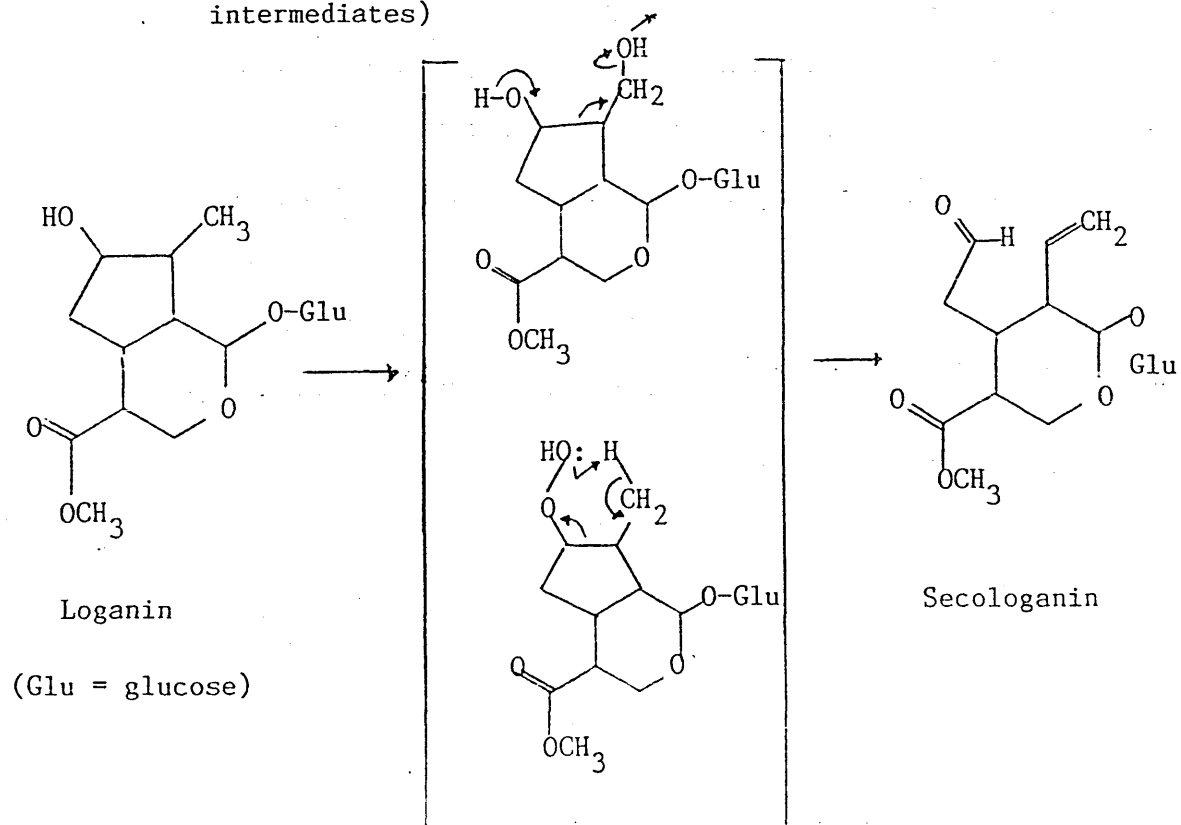


Fig. 1.7.a. The condensation between Tryptamine and Aldehyde

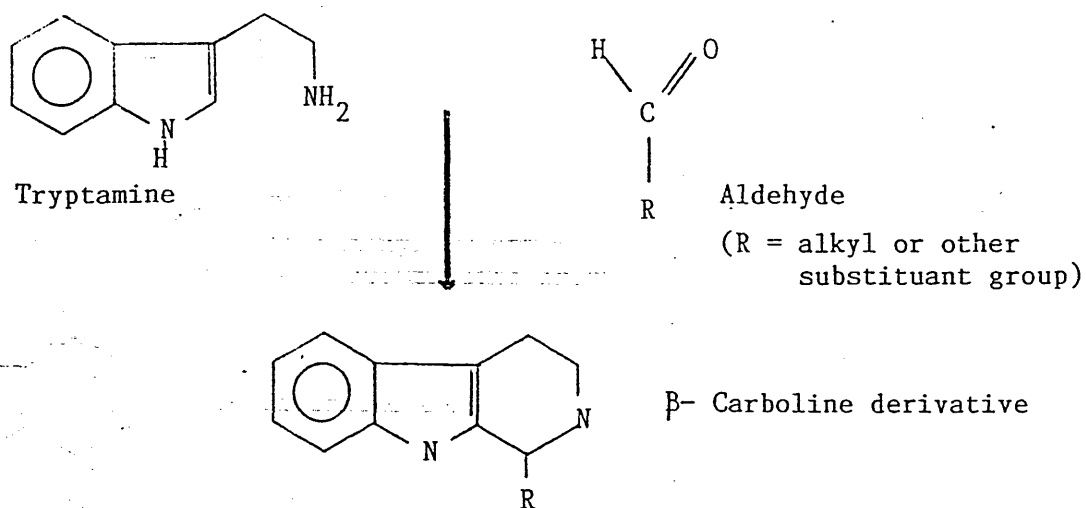
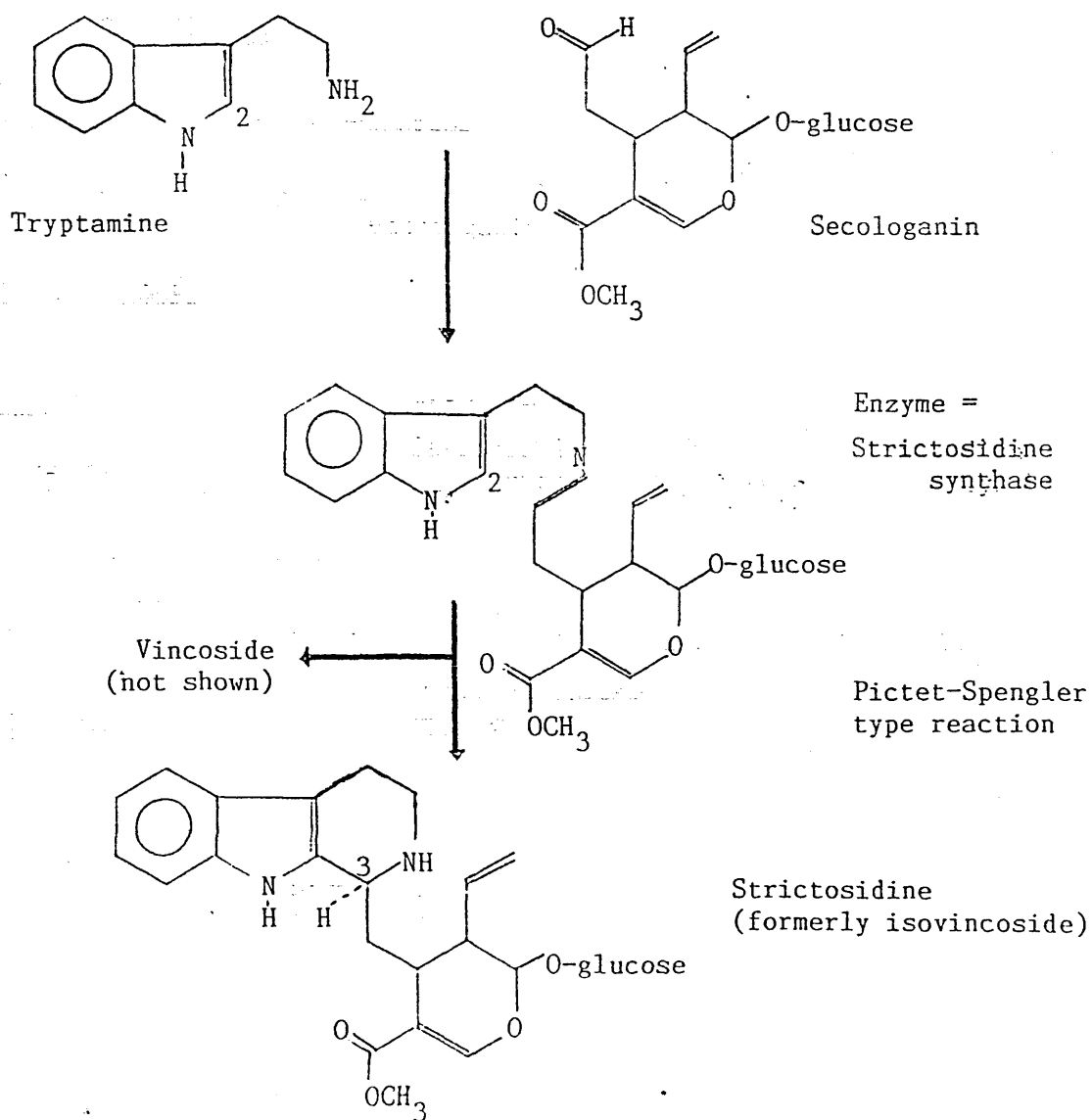


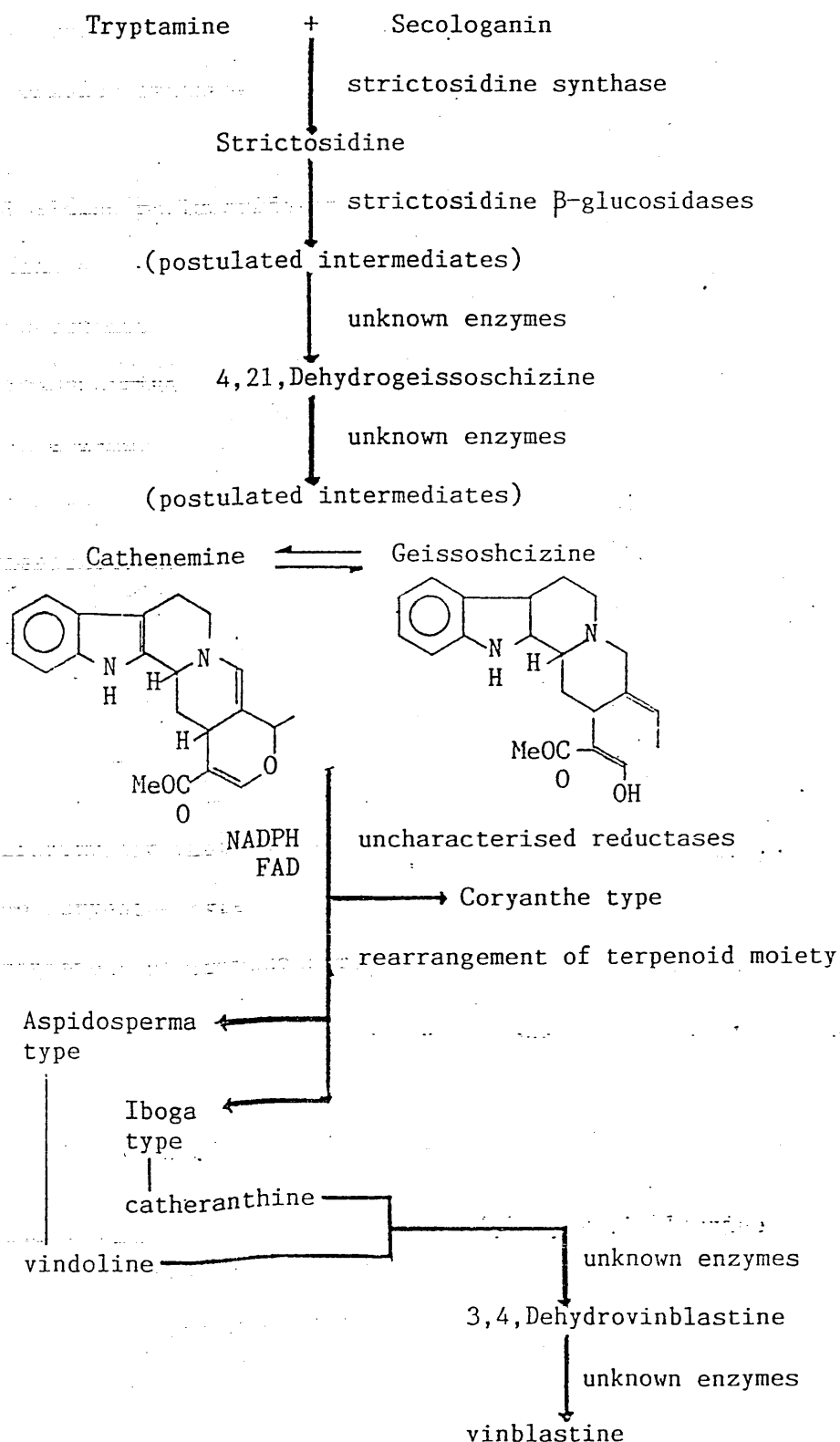
Fig. 1.7.b. The condensation between Tryptamine and Secologanin



intermediates, vincoside and isovincoside, was demonstrated. At first, the key was thought to lie in vincoside, which required a subsequent inversion at position C-3 to proceed (Ref. 5). Later cell free work showed it to be isovincoside (renamed strictosidine), the isomer with the proper $3\alpha(S)$ configuration (Ref. 44). The enzyme responsible for the reaction, strictosidine synthase, has been characterised (Ref. 39). A β -glucosidase system has been discovered that subsequently splits off the glucose moiety on strictosidine to yield an intermediate 4,21,dehydrogeissoschizine, which rearranges to form, most probably, two interconvertible compounds, cathenemine and geissoschizine (fig. 1.8) either of which could give rise to the three major classes of I.A. It has been assumed that the relatively unspecific, cofactor dependent reductases act at this point, but they have not been isolated and characterised (Ref. 44). Many of the intermediates also remain to be isolated, though cell free systems have shown the conversion of Trp and secologanin to ajmaline and vindoline requires cofactors NADPH and FAD (Ref. 19,33). Additionally, the isolation of the alkaloids at different times after germination demonstrate the sequential formation of Coryanthe, Aspidosperma and Iboga types as illustrated in fig. 1.8. (Ref. 18).

The oxidation of monomeric I.A.s, followed by self coupling and cyclisation, appear to generate symmetrical dimeric alkaloids. The structures of the asymmetric dimeric alkaloids relevant to this report strongly implicate catharanthine and vindoline as possible precursors. This was confirmed by both in vitro and in vivo studies (Ref. 14). However, the incorporation of these compounds is very low, due either to a low rate of dimer biosynthesis, or the inefficient transport of precursors to the biosynthetic site (Ref. 18). Success in the development of routes to the partial synthesis of the dimers led to the discovery and isola-

Fig.1.8. The Biosynthetic Pathway of Indole Alkaloids from Tryptamine and Secologanin, indicating the involved enzymes and cofactors.



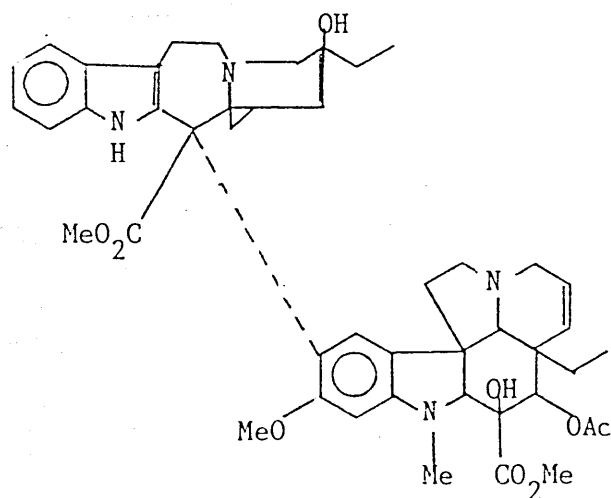
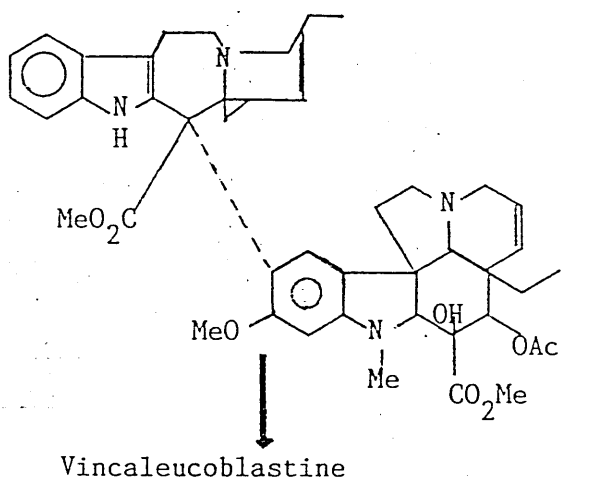
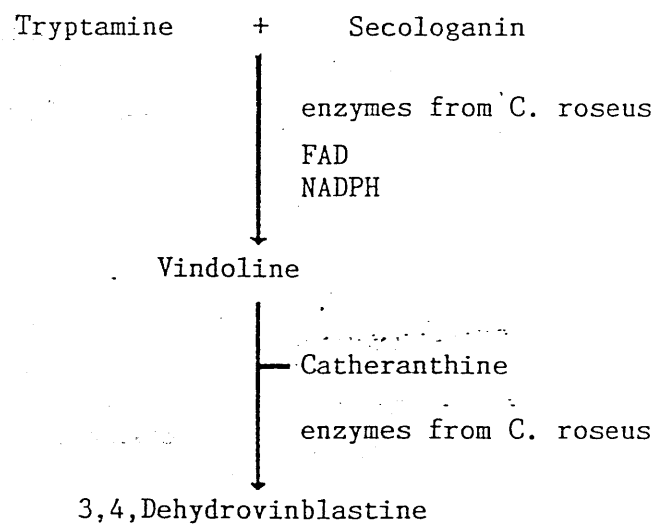
the formation of 3,4,dehydroVLB, the condensation product of catheranthine and vindoline N-oxide and vindoline under reductive conditions (Ref.30). It was also shown to be important in *C.roseus* cell free systems (Ref.19). The reaction is illustrated in figs. 1.8. and 1.9. Most of the work on structure and pathway elucidation has been done on *C. roseus* but complementary studies are available on other plant systems such as *Rauwolfia verticillata* (Ref. 12). More work remains to be done and the future appears to point to cell free systems as the main tool in the detailed study of the enzymes involved.

1.4. Alkaloids, Reactions and Enzymes Relevant to Report.

The aim of the study, as explained in the general introduction, was to apply enzyme immobilisation techniques to improve the yield of low incidence, valuable alkaloids. This means the isolation of the alkaloid synthesising system in question and the further isolation and immobilisation of relevant enzymes. Kutney (Ref. 19) has demonstrated the existence of a system synthesising Vincalencoblastine (Vinblastine, VLB) a minor component (about $3 \times 10^{-4}\%$ by dry weight) of *C.roseus*, from its precursors catheranthine and vindoline (Figs. 1.8, 1.9). The system consisted of a cell free extract of *C.roseus* containing the relevant condensing enzymes, to which the required alkaloids and cofactors had to be added. This system was also able to form vindoline from Trp and secologanin (Fig.1.9). It was this system that was chosen for the work presented in this report. Thus the plant selected was *C.roseus*, which was fortunate as it is also an easily cultivated plant, with a high content of catheranthine and vindoline (about 0.48% by dry weight), whose structures are shown in fig. 1.4. (Ref. 43).

The individual components of the enzyme system are unknown, though Kutney has tried further isolations (Ref. 19). In the work now being presented, similar extractions were attempted, though not to a very

Fig. 1.9. The condensation of Vindoline and Catheranthine
to form Vincaleucoblastine (Vinblastine)



high degree of purity ; it was hoped that a relatively crude immobilised enzyme preparation would be synthetically active.

The process of immobilisation is outlined in the following section.

1.5. Immobilisation of Enzymes.

Enzymes are biological catalysts that, unlike chemical ones, operate under mild conditions such as normal temperature and pressure, neutral aqueous solution and with high specificity. They have been used since ancient times in a variety of fields including brewing, tanning, textiles, food preparation and medicine. The enzyme market today supports several multibillion dollar industries. The requirements of enzymes, while efficient in organisms, poses problems for practical industrial applications. From an operations point of view the most important property of an enzyme is its solubility. Usually, enzyme reactions are carried out in batch processes with a mixture of the enzyme and substrate in solution. The enzyme is either destroyed during the reaction or discharged with the effluent, thus requiring the removal of enzyme impurities from the final product. Specialised separation steps may be uneconomical for the cheap, impure enzymes most used in industry, but for costly or purified ones they may be essential.

To keep costs down, the retention of enzyme within the reactor appears to be a prerequisite for continuous operation and reuse of the enzyme.

The modification of enzymes by immobilisation is an attempt to solve the problems inherent in an enzyme system in solution. In the process

valuable plant and animal sources of enzymes can be conserved. There are other advantages of immobilisation including the stabilisation of some labile enzymes through bonding or enclosure in a protective environment.

Immobilised enzymes are used in industry today to improve yield, product, improve control and reduce costs. The field is expanding rapidly since the first commercialised immobilised enzyme process was patented

in 1967 (Ref. 1, 4.).

The immobilisation of an enzyme is considered to be the modification of enzymes so that they are physically confined to a support with the retention of their activities and so that they can be reused (Ref.10).

The terms carrier, support and matrix are used interchangeably to mean the material used for enzyme immobilisation.

The construction of any immobilised-enzyme system must consider the characteristics of the carrier and type of attachment, the properties of the enzyme and the configuration of the reactor. These factors are interrelated; a reactor form under consideration or the pH of the reaction may determine the choice of carrier and hence the method of attachment. However, they can be dealt with separately.

1.5.1. Methods of Attachment.

Since an immobilised enzyme can be defined as one whose free diffusional mobility is restricted, the term covers any enzyme that is constrained through any of the techniques described below, as well as any combination of these (Ref. 10). These methods can be categorised as follows (Ref. 46).

Physical methods : Adsorption on water-insoluble support

Entrapment within water-insoluble polymer
lattice

Encapsulation within a semipermeable membrane

Chemical methods : Covalent attachment to water-insoluble support

Crosslinking

All of these methods are practicable for a simple enzymatic reaction. With a more complex system involving a number of enzymes, it is perhaps unlikely that the same technique would be suitable for each enzyme, though mechanical entrapment appears feasible in all cases except where

low molecular weight coenzymes are attached to the enzyme (Ref. 4).

The usefulness of an immobilised enzyme preparation will depend on its applications in industry, medicine or as a model enzyme system. The major techniques are discussed below and illustrated in fig. 1.10.

1.5.1.1. Adsorption.

This is the simplest form of attachment where the enzyme adheres to the surface of a carrier through weak bonds such as hydrogen or ionic bonds. It appears to be the most economical procedure for immobilising an enzyme and the most advanced enzyme reactors are based on ionic adsorption to Sephadex ion-exchangers, developed in Japan (Ref. 10).

Inorganic supports are also used. Theoretically, it is a reversible process, so that both enzyme and support may be regenerated. In practice it depends on the ionic strength, temperature of the reaction medium and other factors. Disadvantages to this method lie in the possible desorption of enzyme to the surrounding solution and the possible adsorption of the reaction products (Ref. 29).

1.5.1.2. Entrapment.

The enzyme is incorporated into a polymer lattice that prevents it from diffusing into the reaction medium but allowing access of reactants to the catalyst. Entrapment is accomplished either by polymerisation to form a gel or membrane around an enzyme or by introducing the latter into a performed polymer and 'trapping' it by generating a crosslinked network. The matrix would then be reduced to suitably sized particles. This method is primarily used to entrap whole cells, where seaweed derivatives like Alginic acid are employed. For enzymes, polyacrylamide gels are used though polymerisation required for entrapment may inactivate the enzyme. Theoretically, the tertiary structure of the enzyme is retained but the specific activity is affected by the pH etc. of the

surrounding medium. Enzyme leakage from carriers with variation in pore size is also a drawback (Ref. 1, 17).

1.5.1.3. Encapsulation.

This is a mild method where whole droplets of enzyme are included in a semipermeable membrane such as nylon. Mechanical shear of the enzyme is avoided and external substrates can dialyse freely across the membrane. The thickness and permeability of the membrane depends on the material used, the solvent and the time taken for encapsulation to occur. The enzyme is not modified during the process, but its activity varies with its milieu. There are also problems of leakage and enzyme instability is not uncommon. The applications of this method are at the present, mainly therapeutic and future developments appear to be in the field of artificial cells consisting of encapsulated enzyme systems or cell extracts (Refs. 1, 8).

1.5.1.4. Crosslinking.

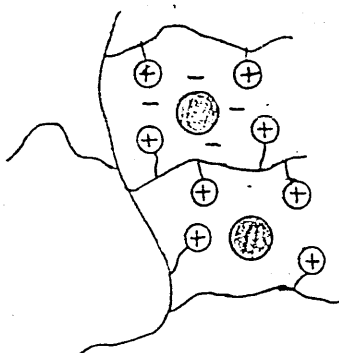
The enzyme is insolubilised by intermolecular crosslinking with a multifunctional reagent like polyacrylamide that can both bind enzymes and react with themselves to form polymers. Thus it resembles entrapment except that enzyme leakage is reduced by fixing enzyme in carrier pores. However, large losses in enzyme activity during immobilisation limit the applicability of the method (Ref. 25).

1.5.1.5. Covalent Attachment.

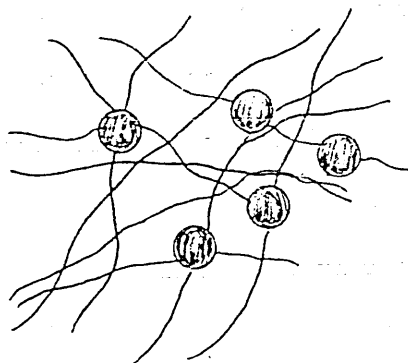
The method of immobilisation that is most extensively researched is covalent attachment. Enzymes are bound to water-insoluble carriers by relatively strong, stable covalent bonds. Binding occurs via the functional groups of amino acids located on the enzyme surface. If factors like relative concentration, hydrophobicity and reactivity are considered, then lysine, cysteine and tyrosine would react best with

Fig. 1.10. Schematic Representation of Immobilisation Methods.

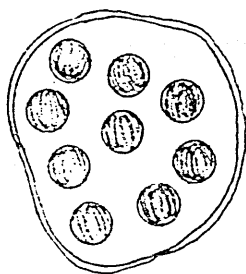
Adsorption



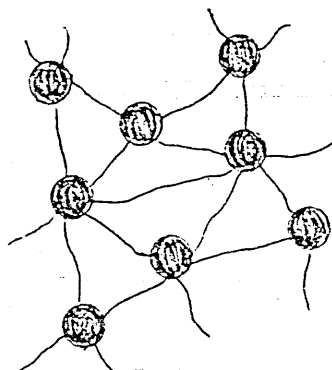
Entrapment



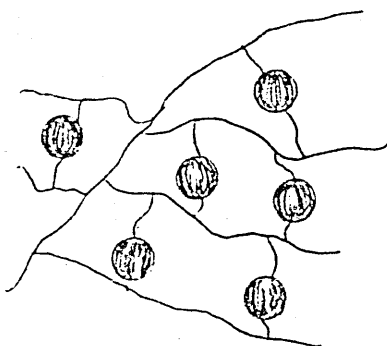
Encapsulation



Crosslinking



Covalent Attachment



 = Enzyme

their amino ($-\text{NH}_2$), hydroxyl ($-\text{OH}$), and carboxyl ($-\text{COOH}$) groups being the most reactive residues. Binding these groups to the carrier is a two step process:

1. The introduction of reactive electrophilic groups onto the support (activation).
2. The reaction of enzyme nucleophilic groups with the activated support (coupling).

Numerous techniques of covalent binding are to be found but the most common ones are shown in fig. 1.11 and are as follows :

1. Cyanogen-bromide activation - An extensively used method is based on the reaction between cyanogen bromide (CNBr) and hydroxyls on the matrix to give intermediates which form isourea linkages with primary amino residues of the enzyme. Multipoint attachment ensures that enzyme does not hydrolyse from the support and crosslinking of the latter enhances chemical stability of the enzyme (Refs. 2,29).

2. Carbodiimide activation - Carbodiimides promote the condensation of a free amino with a free carboxyl group to form a peptide linkage. Thus supports with amino groups can react with carboxyls on enzymes and carboxyl containing carriers with amino groups on enzymes (Ref. 38).

The most recent addition to covalent binding methods is the activation of carrier by organic sulfonyl chlorides :

3. Sulfonyl chloride activation - Organic sulfonyl chlorides such as p-toluene sulfonyl chloride (tosyl chloride) and 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) can be used to activate hydroxyls to sulfonates that react with enzyme residues to form stable linkages. Thiols and

primary amino groups react most readily with sulfonate esters on gels. Amino acid analyses of bound proteins indicate that hydroxyls on tyrosine and imidazoles are also reactive. This broad range of binding possibilities may stabilise the bound enzyme. Tresyl chloride appears to activate the support more readily than tosyl chloride and is used more often. The activation and coupling steps are thought to occur as shown in fig. 1.11. (Ref. 24). The efficiency of coupling is equal to or better than that for CNBr. The advantage of this method is that water-soluble polymers can also be activated and perhaps used for biochemical preparations (Refs. 23, 25, 34).

Applications of enzyme immobilisation by various attachment methods are illustrated in table 1.4.

1.5.2. The Carrier.

Choosing the carrier for immobilisation means that the conditions of operation (pH), enzyme, reactor design and type of enzyme attachment must be considered. Matrix composition determines the type of attachment and the durability of the system. A chemically inert material ensures the latter and also product purity. A diversity of support materials can be found in the literature, including glass, sand, alumina, ion exchange resins, polyacrylamide gels, polysaccharides etc. In reactors, inorganics like glass can be damaged by abrasion while with the softer, more compressible organics, pressure drop and plugging can occur (Ref. 10). Hydrophilicity or the tendency to incorporate water into their structure appears to be an important criterion for the preservation of high enzyme activity after immobilisation to supports (Ref. 29). Thus hydrophilic gels like cellulose, dextran, starch and agar become attractive as carriers. However, the first two materials have irregular macroporous structure and are susceptible to microbial

attack, so that crosslinked dextran and agarose gels appear to have the widest applicability (Ref. 2, 14, 29).

Immobilised supports may be in the form of sheets, blocks, membranes, fibres, tubes or particles. Carriers are usually porous to provide greater available surface area for enzyme attachment. Pore structure then becomes important. Whatever the form of the matrix, the total surface area is inversely proportional to pore size. The latter must be large enough to permit easy entry of enzyme for immobilisation and of substrate for reaction. Very large pores would lower total surface area and thus the enzyme loading (amount of enzyme bound to support), which would increase process costs (Ref. 42). In a reactor, broad pore distribution and irregular particle shape tend to decrease bed volume and drastically increase pressure drop. Most particulate matrices come in a range of sizes so that the trade-off is between diffusional resistance with larger particles or a higher pressure drop, bed compaction and plugging with smaller ones (Refs. 1, 4, 10).

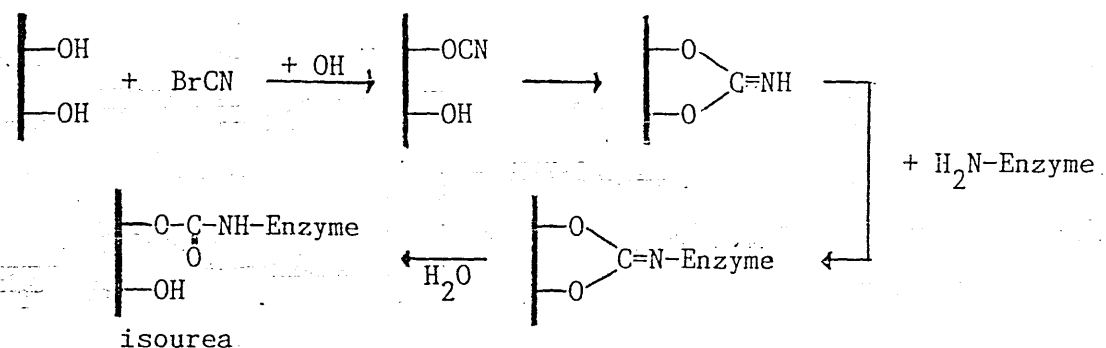
1.5.3. The Enzyme.

When enzymes are immobilised, the enzyme loading, stability and kinetic behaviour must be taken into account. For economic operation, high specific activity per unit weight of immobilised preparation is important. Loss of enzyme may lower this value as can binding of proteins other than that desired.

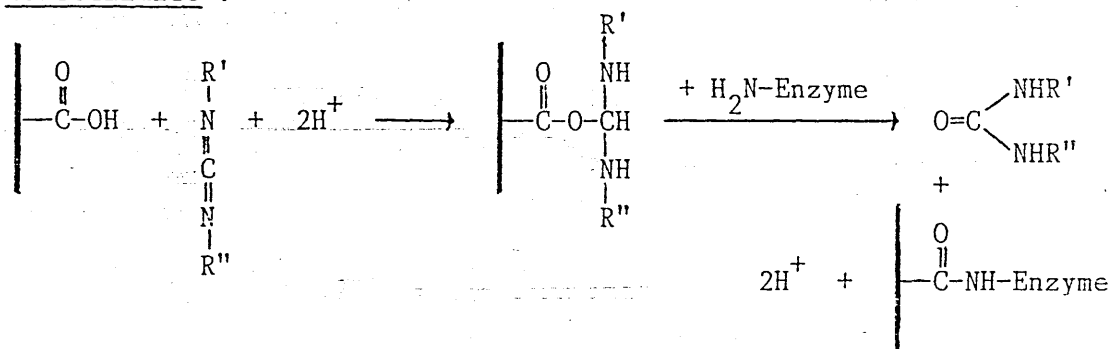
Today, most immobilised enzyme systems in use consist of cells, cell extracts and partially purified enzymes. The degree of isolation is a cost trade-off where less purification means low loading, a large bulk of bound enzyme and more space occupied by the system in the reactor (Ref. 42). More costly purified enzymes mean higher product yield with less contamination from cell debris etc. At present, immobilised enzymes

Fig. 1.11. Methods of Covalent Attachment of Enzyme to Support.

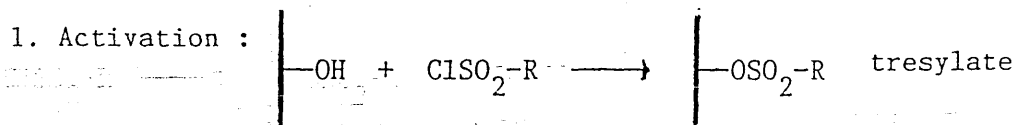
Cyanogen bromide :



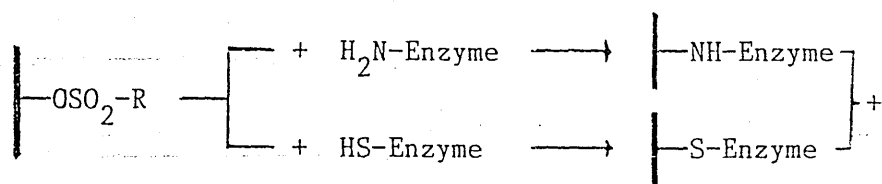
Carbodiimide :



Sulfonyl chlorides :



2. Coupling :



HOSO_2R

$\text{R} = \text{CH}_2\text{CF}_3$ (Tresyl chloride) or $\text{C}_6\text{H}_4\text{CH}_3$ (Tosyl chloride)

$\text{R}', \text{R}'' = \text{different alkyl groups}$

can be reused upto ten times in batch processes while bound cells have been used for shorter periods (Ref. 20).

The stability of bound enzyme in use and in storage is important where long operating life and enzyme reuse is critical. In general, stability is temperature dependent so that highest total productivity is achieved at lower temperatures. Also, high temperatures lead to enzyme denaturation (Ref. 10).

The kinetic behaviour of bound enzymes can vary radically from that of free ones. The observed differences may be due to electrostatic or hydrophobic interactions between support and medium components. Thus the shift of 1 to 2 units in pH optima observed on immobilisation of enzymes is attributed to the change induced by the charged matrix, in positively charged ion $[H^+]$ concentration in the immediate vicinity of the enzyme (Ref. 26). In bound systems, the substrates must diffuse from bulk solution to the site of enzyme reaction. When the rate of diffusion is slower than that of reaction, then the apparent rate of reaction would be lower than that of free enzyme. In this case, the immobilised enzyme would be exposed to local concentrations of substrate etc. different from those in bulk solution. These diffusional or mass transfer effects are greater in the case of large particle size and fast enzyme reaction (Ref. 1,4). For enzymes bound within porous or semipermeable membranes, limitations from internal mass transfer or pore diffusion are more frequent than from external mass transfer of substrate from bulk solution to carrier surface (Ref. 20).

Complete immobilisation of enzyme may not always be obtained. Conformational changes arising from strain in the tertiary structure of protein upon immobilisation have been put forward to explain the low specific activity seen with some bound systems. It may also account for the

reported thermal stability of many bound enzymes. Less stable configurations may cause a decrease in stability (Refs. 22, 29).

Steric limitations on the penetration of substrate may also explain the low activities seen with immobilised high molecular weight enzymes (Refs. 38, 29). The data available are fragmentary and these effects are the least well known. Despite the large number of methods reported in the literature, information concerning the influence of chosen conditions on the amount of enzyme bound, its retention of activity and stability is limited, specially for covalent binding where the number of bonds between enzyme and support may be expected to have an effect.

1.5.4. The Reactor.

Finally the configuration of the reactor to be used must be optimised. Many different types of reactor have been employed with immobilised enzymes. They are illustrated in fig. 1.12.

The simplest type is the batch stirred tank reactor where enzyme and reactants are placed in a container and the reaction proceeds to a desired conversion level. The continuous stirred tank reactor (CSTR) operates at a steady state with continuous feeding of substrate and removal of product. Another continuous flow system is the plug-flow or tubular type, of which an example is the fluidised bed where the particulate-bound enzyme is fluidised by the upward flow of substrate solution. The most widely used type, specially on a large scale is the fixed-bed reactor, the most common configuration being a packed bed of particulate-bound enzyme. Additionally, reactors with porous sheets, blocks or membranes have been tried (Ref. 1, 10, 20).

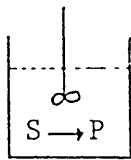
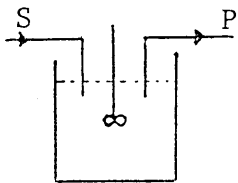
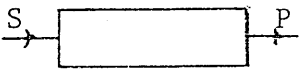
Packed beds tend to be cheaper and have higher enzyme loading per unit volume reactor (which is important in determining reactor size) than stirred tanks. Thus, in the latter, the emphasis is on high enzyme

Table 1.4. Selected Applications of Immobilised Enzymes

Enzyme	Attachment to Carrier	Application
Amino acylase	Adsorption DEAE Sephadex	L-amino acid separation from racemic mixtures
β -galactosidase	Fibre Entrapped	Lactose reduction
Glucose oxidase	Entrapment Polyacrylamide	Glucose electrode
Glucose isomerase	Crosslinking Cell homogenates with glutaraldehyde	High fructose syrups
Alkaline phosphatase	Microencapsulation Collodion membrane	Synthetic enzyme membrane
Catalase	Covalent, glass beads	Enzyme thermistors
Acylase	Covalent, CNBR activated Sepharose	Synthetic penicillin
Chymotrypsin	Covalent, Tresyl activated Sepharose	Peptide synthesis
T ₄ -DNA ligase	Covalent, Tresyl activated Sepharose	Joining of DNA fragments
Concavalin A	Covalent, Tresyl activated Sepharose	Peroxidase purification

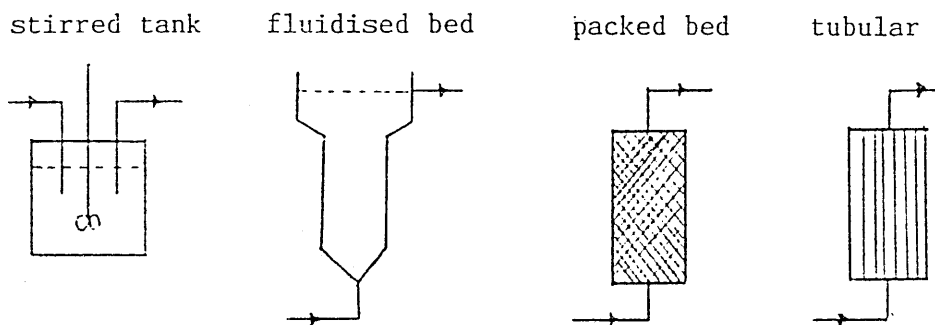
Data from Methods in Enzymology Vol. XLIV 'Immobilised Enzymes'
Ed. Mosbach K., Academic Press (1976).

Fig. 1.12. Diagrammatic Representation of Reactor Configurations.

type of reactor	flow pattern in reactor	diagrammatic representation
Batch	well-mixed	
CSTR	well-mixed	
Tubular	plug-flow	

S = substrate
P = product

Examples of continuous-flow systems :



After Lilly (Ref. 20)

activity per volume of support. Diffusional effects have been observed in both systems. The higher flow rates or smaller particles employed to overcome these problems in packed beds may give rise to high pressure drop or plugging respectively. In fluidised beds, high fluid velocities may mean inadequate residence times for optimal conversion. The presence of back mixing in tubular reactors or poor mixing in stirred tanks can drastically reduce output when flow patterns deviate from the ideal (Ref. 20). Many enzymes are inhibited by excess substrate or product. This effect is most pronounced in batch systems but may be reduced by semi-continuous operation where substrate is fed and product removed at intervals. Catalyst regeneration is also easier in such a reactor whereas most continuous processes must be stopped for enzyme replacement (Ref. 42). Continuous systems may offer constant reaction conditions with ease of automatic control but it may also involve special process design, and be uneconomic where output is small (Ref. 20). For free enzymes, a completely mixed batch reactor is a relatively cheap and flexible system where temperature and pH are readily controlled. This could take the form of a simple vessel where agitation assists in the uniform distribution of enzyme and moderates variations in concentration, pH and temperature. For an immobilised system, a similar reactor may be adequate, agitation being a prerequisite if the bound enzyme is to be kept in suspension (Ref. 1).

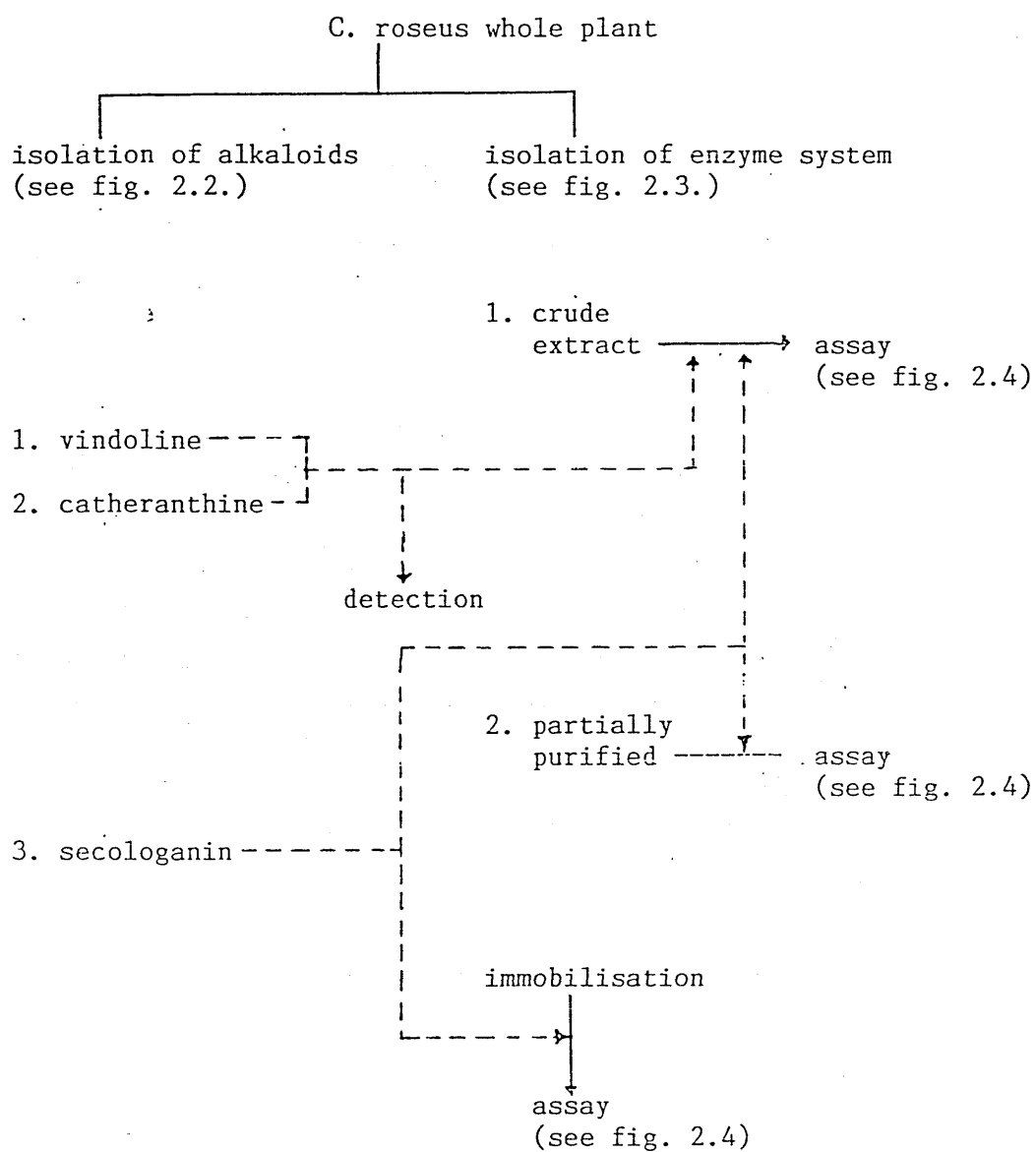
The problems that may be anticipated with this immobilised system are outlined in the following section .

1.6. Conclusion.

The aim of this study was the use of immobilised enzymes in the biosynthesis of clinically important indole alkaloids. There are references to antibiotic production from immobilised microbial enzymes and cells

(Refs. 10, 20, 42). but no equivalent studies on plant enzymes and alkaloids exist, perhaps because of the emphasis on plant cell tissue culture. Immobilised whole plant cells, though not enzymes, have been used to produce a few secondary products like digitoxin from *Digitalis* species (Ref. 7). Thus the very novelty of the present study indicates its likely difficulty. An outline of the work is schematically presented in fig. 2.1. The relevant enzyme system had to be isolated, immobilised and assayed. The isolation requires an assay based on a recognisable biological effect which allows the purification process to be followed. In the case of VLB, this involves the incorporation of precursors, catheranthine and vindoline, into its structure. The most immediate obstacle is then the quantitative detection of nonpigmented alkaloids present at very low concentration in a reaction medium containing many potentially interfering substances. The use of radiolabelled precursors, useful for minute amounts of reactants, also poses special problems in handling and detection. In addition, the time involved in analysing large numbers of samples may also limit process efficiency. Decision on the purity of enzyme used and the method of immobilisation should be influenced by safety criteria. Though the use of immobilised enzymes is usually safer than using many other chemical or biochemical methods, reagents for covalent attachment are predominantly protein binding compounds and must be treated with respect. Other problems to be expected are in the efficiency of enzyme binding to support and the retention of enzyme activity by the immobilised preparation. There may be difficulties in the assay of this system. Detailed discussion of these aspects of the work can be found in the following chapters.

Fig. 2.1. Schematic Representation of Extractions from C. roseus



2. MATERIALS AND METHODS

The scheme in fig. 2.1. shows the relationship between the various experimental sections of this work.

The dimeric alkaloid VLB is formed from monomeric precursors, i.e. vindoline (vin) and catheranthine (cat). Because only a few milligrams of these monomers, sufficient for analytic studies but not for synthetic work, were obtained from Kutneys group in Canada, the compounds had to be isolated from *C. roseus* in the laboratory.

The growth of *C. roseus* plants is described first, followed by the alkaloid extraction methods, the analytical techniques, isolation of the enzyme system, its assay and immobilisation.

2.1. Sources of Chemicals

Sephadex G-25, DEAE Sepharose 6CL, Tresyl activated Sepharose 4B were from Pharmacia (Uppsala, Sweden). PVP, NADPH, FAD were from Sigma (St. Louis, USA). [2-¹⁴C]Tryptamine bisuccinate from New England Nuclear (USA). Biorad protein assay from Biorad (California, USA). *C. roseus* seed and Secologanin was a gift from P. Brodelius (ETH-Zürich, Switzerland). Chromatographic reagents were of the highest purity available, all other chemicals being of analytical grade.

2.2. Growth of *C. roseus*

Seeds of *C. roseus* G. Don were germinated on cotton wool in glass flasks. After a month, the two leaved seedlings were transplanted into small plastic pots with loamy soil (24 % sand, 29% clay, 47% silt) and kept for another month. The plants were then transferred to 30 cm diameter pots containing same soil type. Plants were fed every two weeks with a commercial houseplant fertiliser. Plants were maintained in a greenhouse at 22°C, 70% humidity and photoperiod of 16 hours.

Many studies on the total alkaloid content of *C. roseus* indicate that the maximum yield of alkaloids is obtained from the leaves, followed by the stems and roots while flowers and seeds trail far behind (Refs. 9, 13, 19, 37, 43). The alkaloids accumulate at a higher rate in young plants. The average alkaloid content in leaves increase with age but a drastic drop in concentration occurs in older ones. For instance, the average concentration of Vindoline in dry weight is about $3 \times 10^{-4}\%$ for seeds, 0.4% for young leaves, 7.0% for just mature leaves and 0.2% for older leaves. The optimal significant yield of alkaloids is thus obtained in just fully matured, non-senescent leaves, usually during the flowering stage, after 10 weeks (Ref. 43).

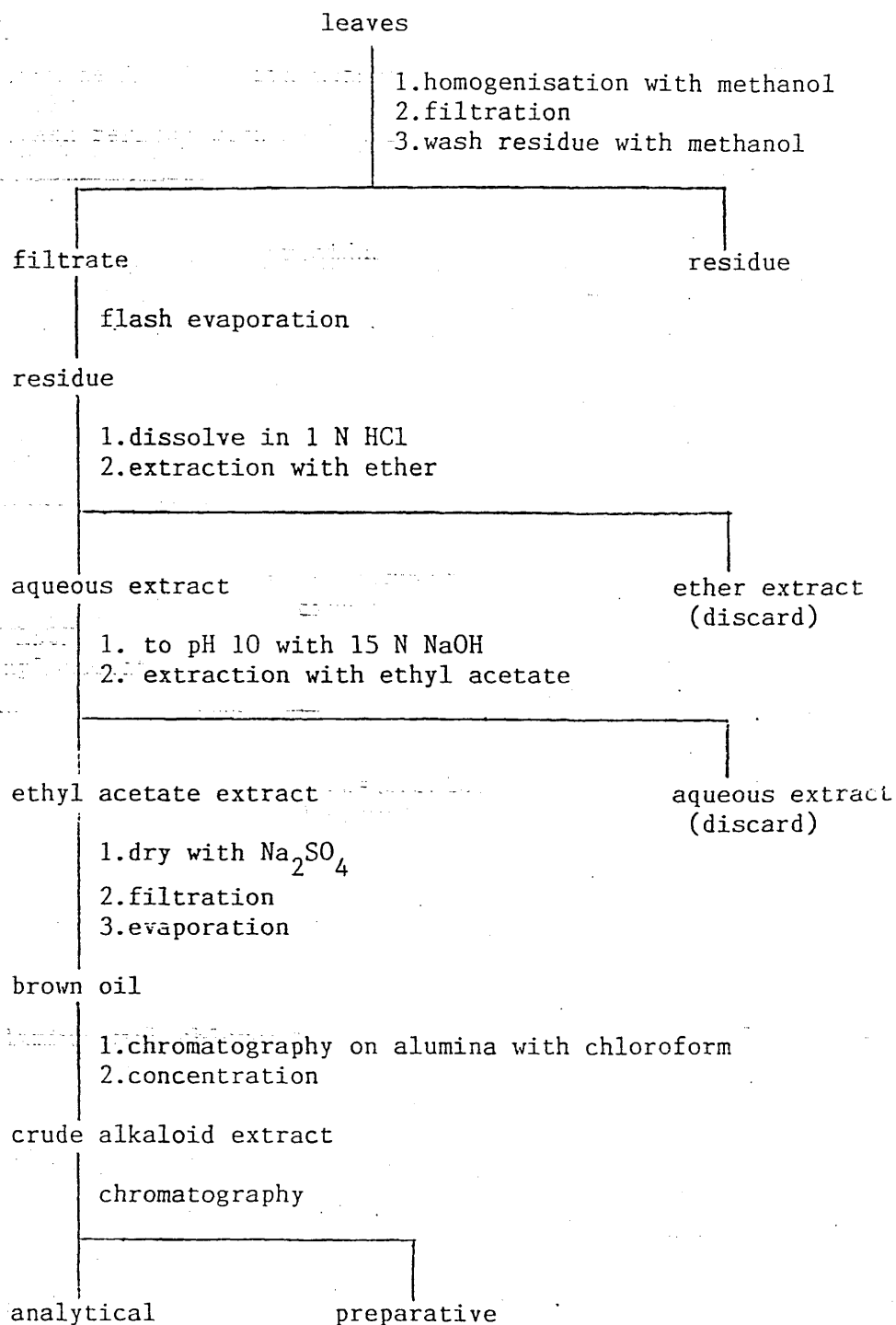
2.3. Isolation of Alkaloids from *C. roseus*

Extraction methods have not changed markedly since the beginnings of alkaloid research. They all depend on the physical characteristics of the compounds, i.e. solubility in polar solvents at low pH and in non-polar ones at high pH, usually immiscible with water. Thus separation of alkaloids from non-alkaloids can be achieved by solvent-solvent extractions. Chromatographic techniques are widely used in alkaloid studies, both at preparative and analytical levels.

With all types of solvents, except hydrocarbons, artifacts may occur (Ref. 18). They were therefore distilled before use. Hydrocarbons such as toluene were preferred as solvents to benzene, even though the latter has a lower boiling point, because they are less toxic. The scheme for alkaloid extraction is given in fig 2.2.

500g young mature leaves were homogenised with methanol in a waring blender and filtered through muslin. The residue was washed with methanol to a final volume of 1 L, until colourless, and then discarded. The combined filtrate was evaporated in vacuo at 35°C and the residue

Fig. 2.2. General Scheme of Alkaloid Extraction from *C. roseus*.



taken up in 50 ml 1 N HCL, the flask being rinsed out with two more volumes of acid, which binds the alkaloid bases in the extract (Ref.37). This aqueous solution was repeatedly extracted with ether to remove chlorophyll, fats and other impurities, until the latter was quite light in colour. Then the aqueous layer was made basic to pH 10 with 15 N NH_4OH , as the required alkaloids are found at this pH (Ref. 37). The alkaloid bases were extracted with a total of 450 ml ethyl acetate. This layer was removed and dried with Na_2SO_4 , filtered and evaporated to afford a brown oil. This crude alkaloidal concentrate was eluted with chloroform through a short column (1 x 5 cm) of alumina (Woelm III) to remove polar components and then concentrated by in vacuo evaporation. The yellow residue obtained was taken up in various solvents and subjected to analytical and preparative chromatography.

2.4. The Analysis of Alkaloids.

The properties of chromatographic methods such as their sensitivity and ease of quantitation make them particularly suitable for the analysis of alkaloids. Gas-liquid methods, though very sensitive, are limited by the instability of some alkaloids at the temperatures required for volatilisation. With high Performance Liquid Chromatography (HPLC), even unstable alkaloids can be measured.

2.4.1. Analysis of alkaloids by HPLC.

The HPLC system used was a Kontron System 600 incorporating a Uvicord spectrophotometer and using reversed-phase column Lichrosorb: RP-18 (0.7 x 4 cm) from Merck (Damstadt, GDR). The crude alkaloid residue was dissolved in methanol, aliquots injected onto the column and eluted with phosphate buffer (0.01 M, pH 7) containing 60% methanol. The elution profile was monitored by absorbance at 254 nm with authentic samples of catheranthine and vindoline as references. Quantitation was

by peak area using an integrator on the recorder.

In terms of sensitivity, HPLC is very variable, depending on the extinction coefficients of the alkaloids of interest which can result in equal amounts of compounds giving different peak areas. Only prepurified extracts can be successfully used and the number of samples which can be processed in a working day is limited.

A relatively tedious but practical method is Thin Layer Chromatography (TLC). Of the three chromatographic techniques, it is the least sensitive, with detection in the 10-100 ng range. Its separation power is compatible with that of HPLC. Like HPLC, the analysed compounds may be recovered, in this case by their elution from the plate, thus enabling quantitative analyses to be done. The great advantage of TLC over HPLC lies in the large number of samples that can be analysed under the same conditions at the same time. As a TLC plate is used only once, it does not interfere with subsequent analyses. Also, after detection the compounds are visible.

2.4.2. Analysis of Alkaloids by TLC

Samples were spotted onto silica gel 60 F-254 plates (Merck) and developed with chloroform-methanol (9:1) with authentic reference alkaloids. The chromatograms were analysed at 254 nm using a TLC scanner (Camag).

2.5. Preparative Chromatography of Alkaloids

2.5.1. Preparative HPLC of Alkaloids

The same HPLC system as in section 2.4.1. was used except that the column was a Lichroprep RP-8 (1 x 24 cm) from Merck. The fractions corresponding to elution peaks obtained by preparative HPLC were further characterised by TLC as described in section 2.4.2. Vindoline containing fractions were evaporated and recrystallised from ether while those with catheranthine were recrystallised from methanol.

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2.5.2. Preparative TLC of Alkaloids.

This was carried out similarly to the analytic method except that the plates used were preparative. The spots were eluted with ethyl acetate-methanol (95 : 1) and analysed by TLC as in section 2.4.2.

2.5.3. Preparative Liquid Chromatography of Alkaloids.

Because of the problems associated with the HPLC separations, i.e. the small amounts of crude alkaloidal extract that could be run at one time (about 500-1000 μ l), and the high back pressure resulting from the presence of impurities clogging the column, thus making cleaning impossible, classical methods of separation were tried.

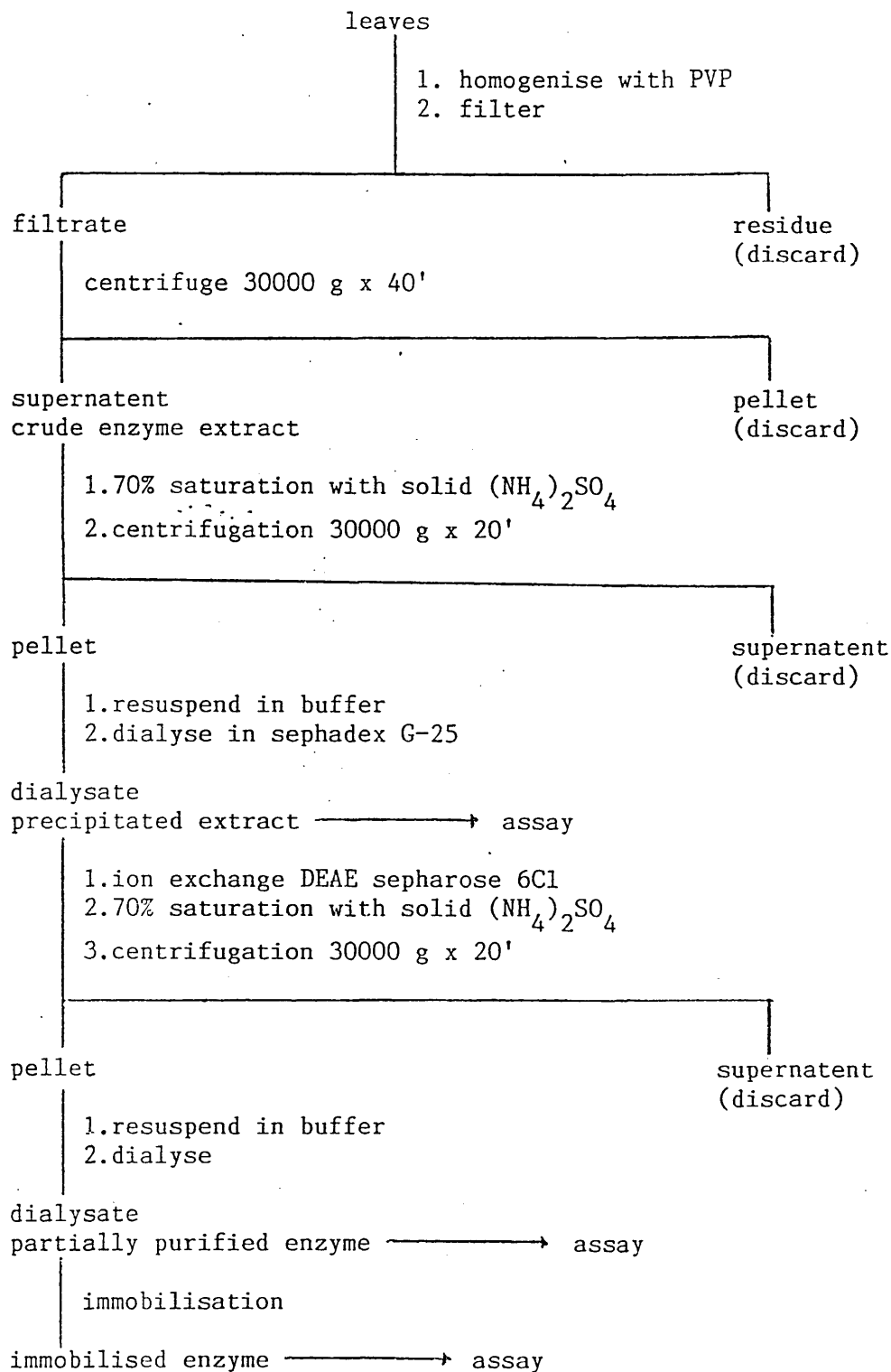
The crude alkaloid mixture was dissolved in benzene and chromatographed on silica gel 60 (Merck) with chloroform-methanol (9 : 1) as eluant, or on alumina (Woelm III) eluted successively with petroleum ether (40 -60), petroleum ether-benzene (1 : 1), benzene, benzene-chloroform (9 : 1), chloroform and methanol, after the method of Wigfield (Ref. 40). Every second fraction was checked by TLC as in section 2.4.2. Identical fractions were pooled and run on preparative TLC. The spots were eluted with chloroform and analysed by TLC.

2.6. Preparation of cell free extracts from *C. roseus*.

Extracts of this plant contain a number of enzymes that catalyse both the biosynthesis of vin and cat and also that of the dimeric alkaloids (Ref. 19). The extraction scheme is presented in fig. 2.3. The speed of operation is important as enzymes can lose activity during purification. Therefore all operations were performed at 4°C.

Leaves (5 g) from mature *C. roseus* plants were homogenised in a waring blender with phosphate buffer (50 ml, 0.1 M, pH 6.3) containing 0.02 M β -mercaptoethanol to stabilise protein. PVP (5 g) previously wetted with the same buffer was added gradually during the process. This binds

Fig. 2.3. General Scheme of Enzyme Isolation from *C. roseus*



phenols that could inactivate the preparation. The homogenate was filtered through cheesecloth and centrifuged (Beckman JS 21) at 30,000 g for 40 minutes. The supernatant (25 ml) was used as cell free extract. This supernatant was brought to 70% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and centrifuged at 30,000 g for 20 minutes to concentrate the proteins and was desalted by resuspending the precipitated proteins in minimum amount of buffer (0.02 M phosphate, pH 6.8, with 0.02 β -mercaptoethanol) and dialysis on a column of Sephadex G-25 (2.5 x 16 cm) equilibrated with the same buffer, at an elution rate of 1 ml/min with monitoring at 280nm. This was the precipitated extract.

The diasylate (ca.15 ml) was applied onto an ion-exchange column of DEAE-Sepharose 6 CL (1.5 x 3 cm) previously equilibrated with the above buffer which was also used to wash the column until only traces of protein remained. Subsequently, a linear gradient from 0.0 M to 0.5 M NaCl in the same buffer was applied at a flow rate of 0.25 ml/min. The 1 ml fractions collected were monitored by absorbance at 280 nm. The fractions were pooled and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and desalting as above. This was the partially purified extract.

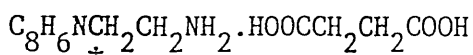
The activity and protein content of the fractions were determined as described in sections 2.7. and 2.9.

The enzyme preparations were stored at -20°C .

2.7. Assay of Cell Free Preparations.

The assay developed by Kutney (Ref. 19) was used as it could be easily modified to assay immobilised enzymes. The activity of the crude enzyme extract was measured by the incorporation of $[2\text{-}^{14}\text{C}]$ labelled Trp in the form of its bisuccinate salt into the alkaloids after reaction with secologanin and cofactors. Aliquots of the reaction mixture were removed spotted onto TLC plates, resolved and analysed for the presence of label

by liquid scintillation counting. The condensing reaction between monomeric alkaloids to form dimers was performed with cold secologanin and Trp which had been radioactively labelled by biosynthesis from labelled Trp. Cat was obtained from alkaloid extraction: these reactions are depicted in fig 2.4. [2-¹⁴C]Trp bisuccinate has the formula:



The asterisk indicates ^{14}C atom. Thus one mole of reactant will be incorporated into one mole of vindoline if the reaction scheme and structures in fig 1.8 are considered.

$$\% \text{ incorporation of label into product} = \frac{\text{product radioactivity}}{\text{precursor radioactivity}} \times 100$$

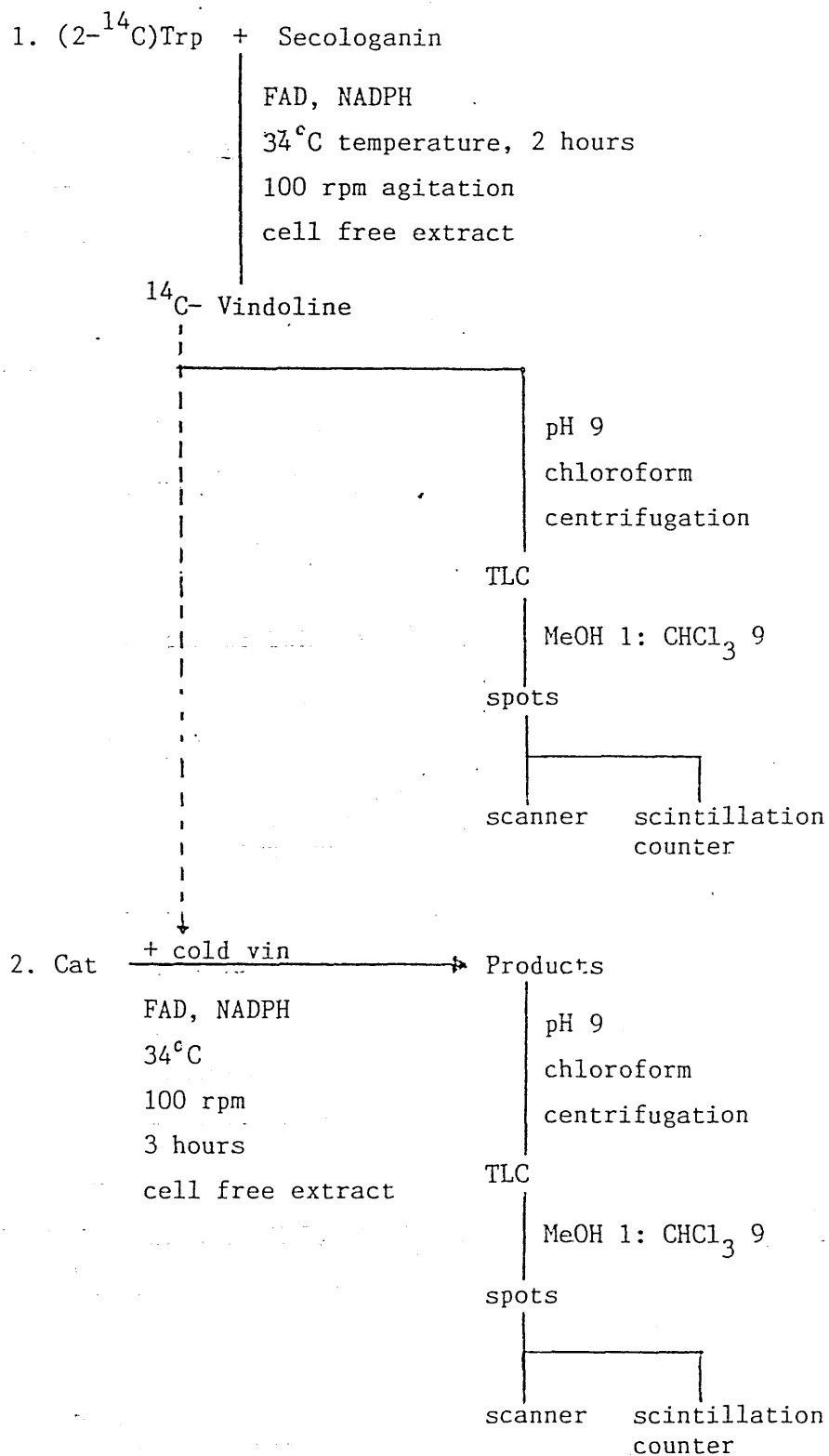
The radioactivity of the precursor (in μCi) is known, as a given amount is added to the reaction mixture. The radioactivity of the product is measured by scintillation counter and is given by :

$$\frac{\text{product spot dpm on TLC plate}}{\text{product spot volume}} \times \text{total reaction mixture volume}$$

(dpm : disintegrations per min. Conversion to μCi : $1 \mu\text{Ci} = 2.2 \times 10^6$ dpm). In some cases the total volume, spot volume and precursor radio-activity being constant, the activity of enzyme is given by the dpm of spot. Each experiment was performed in duplicate and repeated. Boiled extracts were used as controls.

To the extract (25 ml) were added (2-¹⁴C)Trp bisuccinate (2.5 μCi, 0.53 μmol) and secologanin (0.1 mg) and incubated for two hours at 34°C with NADPH (0.5 mg). Alkaloidal products were obtained by adjusting the solution to pH 9 with ammonia and extracting with chloroform. Aliquots were spotted onto silica gel TLC plates backed with aluminium foil (Merck), and run as described in section 2.4.2. The spots were then cut out, put into scintillation vials and the alkaloid eluted with

Fig. 2.4. Scheme of Assays of Cell Free Extractions



0.5 - 1.0 ml water at 40°C for 3 hours. 5 - 10 ml scintillation liquid (Beckman EP) was added and the samples counted in a scintillation counter (Beckman LS 1800) at 95% efficiency.

2.7.1. Assay of Condensing Reaction of Crude Extract

A 5 x 25 ml incubation was made and labelled vin isolated by preparative TLC as in section 2.5.2.

To the extract (25 ml) were added NADPH (0.5 mg), FAD (0.5 mg), cat (1.0 mg), vin (1.0 mg, 0.02 μ Ci with 1.0 mg cold vin) and incubated at room temperature for 3 hours. The alkaloid products were extracted and analysed by TLC as in section 2.4.2. The incorporation of label into the products was determined as in section 2.7. In later experiments the precipitated extract was used as this had negligible background levels of precursor alkaloids.

2.7.2. Assay of Partially Purified Extract

Activity was measured by the formation of labelled vindoline. The standard assay consisted of 50 μ l each of NADPH (10 mg/ml), FAD (10 mg/ml), secologanin (2 mg/ml), 5 - 10 μ l [2-¹⁴C]Trp bisuccinate (0.5 - 1.0 μ Ci), Trp chloride (5 - 10 μ M), enzyme preparation (0.5 - 1.0 mg) and phosphate buffer (0.02 M, pH 6.8) made up to a total of 1.0 ml with buffer. After two hours, 100 - 200 μ l aliquots were removed, brought to pH 9 with ammonia, 200 μ l chloroform added, centrifuged at 1000 g for 5 mins to separate the phases and 50 - 100 μ l aliquots spotted onto TLC plates as before. The plates were handled as described in section 2.7.1.

These Assays, while perhaps adequate for preparative work, are cumbersome and time consuming for analytical work. The disadvantage lies in the time required to spot many samples carefully onto the plates. About 1 - 2 days are needed for one experiment. However, the large

number of samples that can be processed per day should outweigh the drawbacks.

2.8. Immobilisation of Enzyme Extract

Covalent attachment of enzyme to support was chosen mainly because the incidence of enzyme leakage from support by this method seems to be lower than by other ones (Ref. 10)

CNBr, Carbodiimide and Tresyl chloride activated supports, principally Sepharose gels, are commercially available (Pharmacia, Sweden) thus minimising the risks involved in dealing with harmful activating agents.

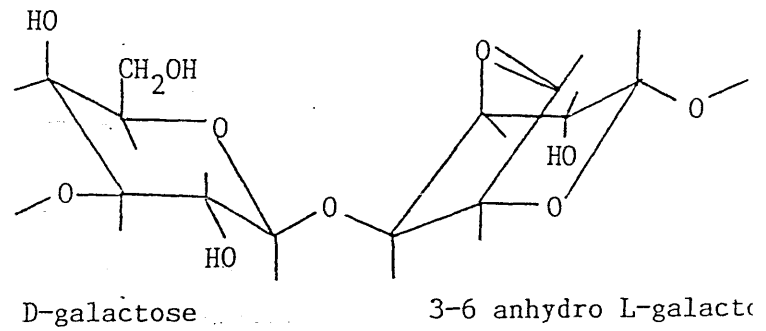
Sepharose is a bead-like agarose gel made from agar by removal of charged species to leave neutral polysaccharides. The gel may be represented as an aggregate of double helical polysaccharide chains made up of repeating units of sugar residues as shown in fig. 2.5.

(Refs. 28,23).

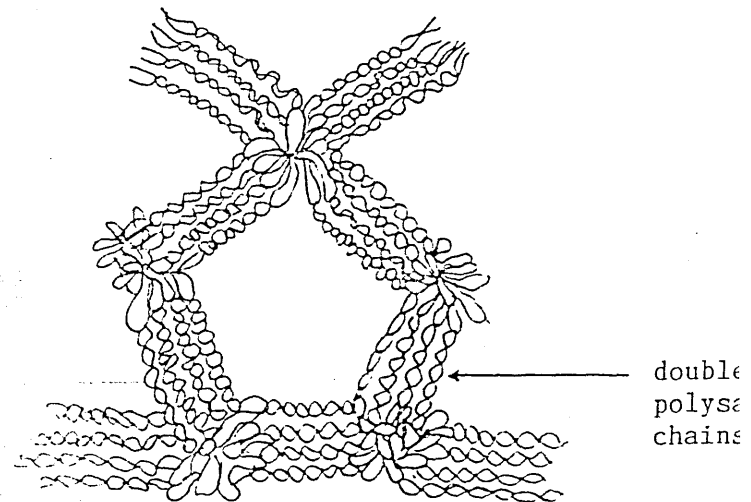
These gels come closest to being the ideal solid matrix for binding proteins. The hydroxyls on the sugar residues can be easily activated for covalent attachment of an enzyme. They are rigid, durable gels whose mechanical strength increases with increasing agarose concentration (usually between 2 and 6%). They are also stable under a broad range of experimental conditions such as high and low pH, detergents, dissociation media and bacterial attack. They appear to interfere less with the normal catalytic function of immobilised enzymes than do most other types of support. The bead form of the gel ensures good flow characteristics in reactors. The pore structure gives a good relationship between permeability and matrix density (Refs. 29, 38.).

The activation of support with Tresyl chloride appears to be a facile one with advantages over the widely used CNBr methods. Like the latter it is a mild method where protein denaturation is avoided. However,

Fig. 2.5. The Structure of Sepharose



Structure of the repeating sub-unit of agarose polysaccharide chains.



Structure of final agarose gel.

Data from Ref. 28.

It is better for coupling pH sensitive proteins with high yields, even at neutral pH and low temperature and with high retention of activity (Ref. 24); an important property for this study where the enzyme system was not well characterised. In contrast to CNBr, the degree of activation of gel by Tresyl Chloride is predictable and reproducible. Highly activated supports may be stored for weeks without significant loss of coupling activity, thus obviating the need to use up all prepared gel at once as is usual with other matrices (Ref. 25,28). As discussed in section 1.5.1.5., binding by Tresyl chloride activated gels could stabilise enzymes through thiol and amino groups, whereas CNBr supports react mainly with the latter. Tresyl chloride activated Sepharose 4B is the only form of this matrix available commercially. Non-specific binding effects that are usual with macroporous matrices have been minimised during agarose purification. Its open pore structure makes the gel interior accessible to proteins in the molecular weight range 60×10^3 to 20×10^6 daltons, so that even large enzymes may be bound (Ref. 23,28). The particle size will also have a bearing on eventual activity of the bound enzyme. For an average enzyme such as alcohol dehydrogenase, almost 95% activity relative to free enzyme was retained when bound to 10 μ m particles of Tresyl activated silica at high enzyme loading. The larger size of Sepharose 4B beads, 60 to 140 μ m in diameter, indicates a degree of diffusional control. Efficient stirring may reduce these effects (Ref. 10,20,28).

The properties of Tresyl activated Sepharose are shown in table 2.1.

2.8.1. Preparation of Tresyl sepharose

2 g amounts of freeze dried gel supplied by the manufacturer was suspended and swollen in 0.1 mM HCL, then washed with 400 ml of the same solution for 1 hour on a sintered glass filter. This preparation could

be stored in 1 mM HCL (which preserves the activity of the reactive groups that would hydrolyse at high pH) for 1 month.

2.8.2. Coupling of Enzyme Extract

The pooled, concentrated fractions from the ion exchange step were changed to coupling buffer (NaHCO_3 , 0.1 M with 0.5 M NaCl) by exchange on Sephadex G-25 (PD-10). Buffers with strong nucleophiles (tris, β -mercaptoethanol) were avoided as they react with the activated support and compete with the enzyme (Ref. 28). The gel was washed with the same buffer and directly transferred to a solution of the enzyme in a stoppered test tube. This was rotated end on end for 16, 24 and 36 hours at 4°C to test the binding capacity of the gel. The amount of protein bound was determined as in 2.8. Excess enzyme was removed by centrifugation at 1000g for 5 min. and washing gel with coupling buffer. Any remaining active groups were blocked with tris-HCL buffer (0.1 M, pH 8) being added to the gel and rotating end on end for 4 hours at 4°C . The product was washed with three cycles of alternating pH (acetate buffer 0.1 M, pH 4 and Tris 0.1 M, pH 8 containing 0.5 M NaCl) to remove free protein, then filtered on a sintered glass filter and weighed.

The immobilised preparations were assayed as in section 2.8.4. and the protein content of the supernatant determined as in section 2.9. Thus

$$P_{\text{gel}} = P_{\text{tot}} - P_{\text{sup}} \quad (P_{\text{gel}} : \text{protein bound to gel, } P_{\text{tot}} : \text{total protein used in coupling, } P_{\text{sup}} : \text{protein determined after centrifugation in supernatant and washings}).$$

The coupled gel was stored at $4^\circ - 8^\circ\text{C}$.

2.8.3. The Reactor

Immobilised enzymes may be used either in solution or packed into columns. In the latter, a concentration gradient arises because bound enzymes are in contact with pure substance at the influent end and mainly with product at the effluent end. Columns containing multistep

enzyme systems may suffer from such heterogeneity, though at low percentage conversions it may be negligible (Ref. 23). Therefore, the reactor chosen was of the simplest batch type, consisting of an open ended test tube of length 10 cm, diameter 10 mm. It was placed in a water bath at 34°C with shaking at 100 rpm to keep the immobilised particles in suspension so that the substrate concentration was kept uniform around all the immobilised enzyme beads. This gave sufficient contact between reactants and enzyme without causing excessive shear forces or substrate diffusion hindrances. Analysis of products was carried out as described in section 2.8.4. The reactor is illustrated in fig. 2.6.

2.8.4. Assay of Immobilised Preparation

Where discontinuous measurements can be made, as in the reactor type chosen here, aliquots may be removed at intervals and analysed, after the removal of bound enzyme, by conventional methods. Thus the immobilised enzymes were assayed as for free extract. The equivalent amount of protein, but bound, as that in the free preparation was used for comparative studies with the free enzyme systems. The bound preparation was centrifuged at 1000g for 5 min. and the supernatant assayed for the presence of radioactivity as in 2.7.2.

Relative activity yields are calculated as the ratio of the determined activity of immobilised enzyme preparation to the same aliquot of initial active free system. This takes into account both the chemical yield of the immobilisation and the specific activity of the immobilised enzyme system. It is expressed as follows:

$$\text{Relative activity (\%)} = \frac{\text{activity of immobilised enzyme}}{\text{activity of free enzyme}} \times 100$$

As explained in section 2.7. The activity of enzyme is given by the radioactivity (in dpm) of the desired product on the TLC plate.

2.9. Determination of Protein

The protein content of all enzyme preparations was determined using the Biorad protein assay, which is based on the differential binding of dye by various concentrations of protein (Ref. 6). This method is rapid and sensitive and interference from non-protein components is negligible.

5 ml diluted dye reagent was added to protein in 0.1 ml phosphate buffer (0.02 M, pH 6.8), mixed well and the O.D. read at 595 nm. A standard curve was constructed with bovine γ -globulin whose response was linear from 50 to 120 μ g. Usually, 10-50 μ l aliquots of solutions with unknown concentrations of proteins were added to buffer to make a total of 0.1 ml. The supernatants from the centrifugation of bound preparations were similarly treated.

2.10. Gel Filtration of enzyme Extract

The molecular weight range of the enzyme preparations was roughly determined by gel filtration on a FPLC system (Pharmacia) using a Superose 12 column (Pharmacia) and phosphate buffer (0.05 M, pH 7.2 with 0.15 M NaCl) as eluant at a flow rate of 0.5 ml/min. Elution was monitored at 280 nm. The column was calibrated with Catalase, Aldolase Horseradish peroxidase and cytochrome c.

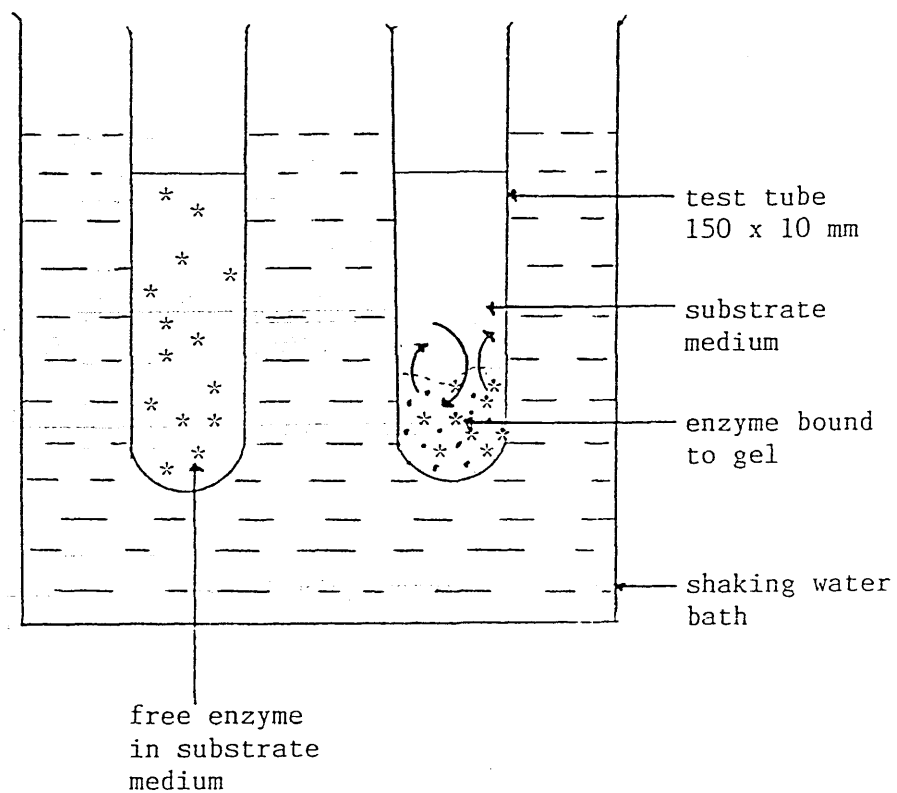
Table 2.1. Characteristics of the Coupling gel

Tresyl activated Sepharose 4 B.

Composition of swollen gel	- 4% agarose
Wet bead diameter of gel	- 60 to 140 μ m
Molecular weight exclusion limit for protein binding	- 60×10^3 to 20×10^6 daltons
Coupling capacity for typical protein	- 8 mg chymotrypsin / ml swollen gel when coupled in NaHCO_3 buffer (0.1 M, pH 8.0) for 4 hours at 25°C
Standard coupling conditions	- 2 to 4 hours at 25°C in NaHCO_3 buffer (0.1 M, pH 8.0) with 0.5 M NaCl
Coupling temperature	- 2 to 4 hours at 25°C and pH 8.0 16 hours or more at 4°C
Maximum operating pressure when used in columns	- 80 cm water
Approximate maximum flow rate in columns	- 0.96 ml/min

Data from Pharmacia (Ref. 28).

Fig. 2.6. Representation of the Reactor used
for the study of the Immobilised Enzyme System.



3. RESULTS

3.1. Analysis of Crude Alkaloids by HPLC.

The presence of alkaloids vin and cat in the ethyl acetate extract of *C. roseus* was confirmed by HPLC analysis as depicted in fig. 3.1.

Average retention times were 4.68 min for vin and 7.48 min for cat.

As shown in fig. 3.2., the crude alkaloid extract from three isolations were pooled and the total amount of alkaloid present in 1 ml concentrate weighing 1.13 g was determined as 26.5 mg vin and 12.5 mg cat.

Information on alkaloid yields from fresh leaves, as opposed to dried, is scarce, so that determinations had to be based on the crude alkaloid extract. Therefore the values obtained in this study, 1.15% for cat and 2.3% for vin are low compared to 2.5% and 8.2% for cat and vin respectively given in the literature (Ref. 37). Alkaloid content is high in leaves that are just mature (Ref. 44); thus the observed values probably reflect the presence of young and old leaves and stems in the starting plant material as mentioned in section 2.2.

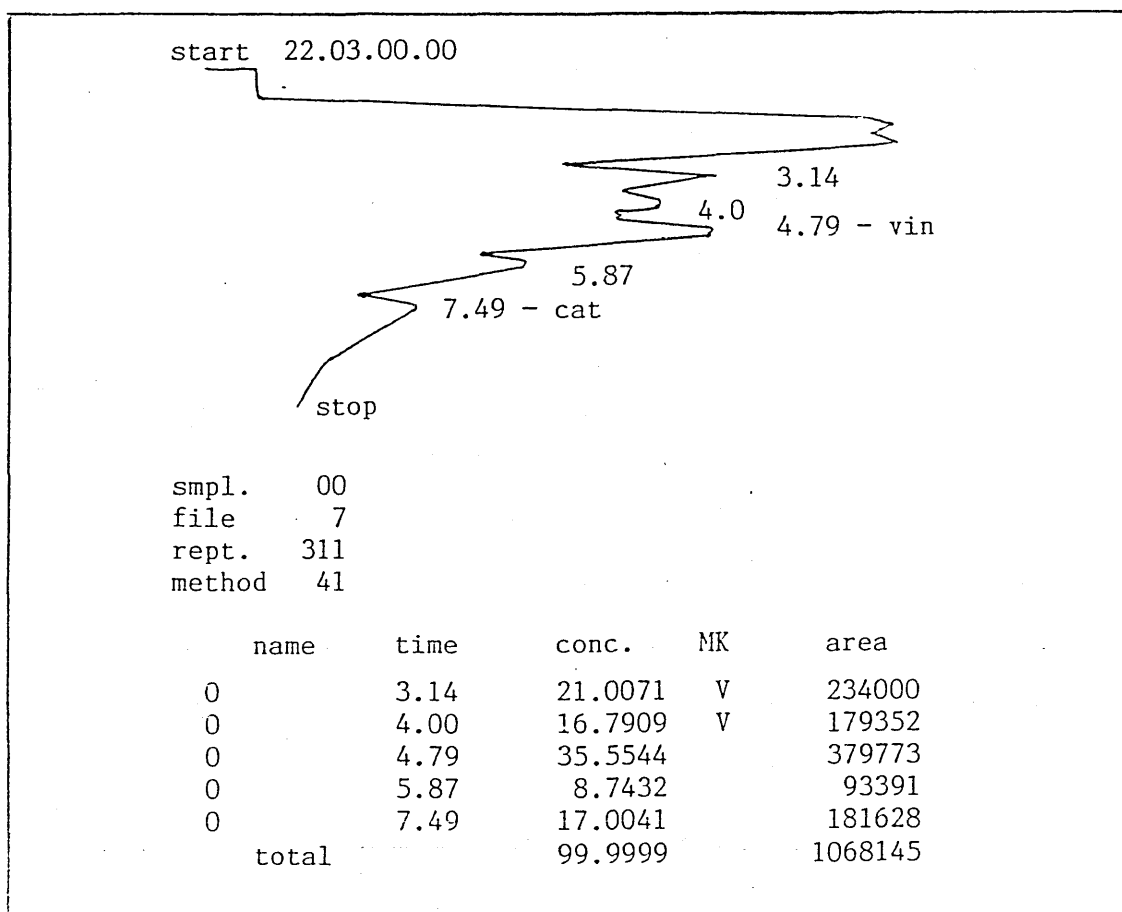
3.2 Analysis of Crude Alkaloids by TLC.

The TLC analysis of the ethyl acetate fraction also showed the presence of the two important alkaloids. A typical chromatogram is shown in fig. 3.3. The R_f (ratio of fronts) values were 0.50 and 0.66 for cat and vin respectively, which agree with those obtained in other studies (Ref. 9). Thus they display a discernible resolution of the alkaloids and demonstrate the suitability of TLC for alkaloid identification.

3.3. Preparative HPLC of Crude Alkaloids.

An example of a typical preparative separation of alkaloids as described in 2.5.1. is shown in fig. 3.4. Again the sample was a crude alkaloid extract pooled from three isolations. It weighed 1.03 g and was

Fig. 3.1 HPLC analysis of the crude alkaloidal products
in the Ethyl Acetate fraction



sample : 1 µl from 1L ethyl acetate fraction

column : Lichrosorb RP-18 (0.7 x 4 cm)

buffer : 0.01 M phosphate buffer pH 7 with 60 % methanol

flow rate : 1 ml / minute

detection : 254 nm absorbance

Fig. 3.2. Isolation of Alkaloids from C. roseus.

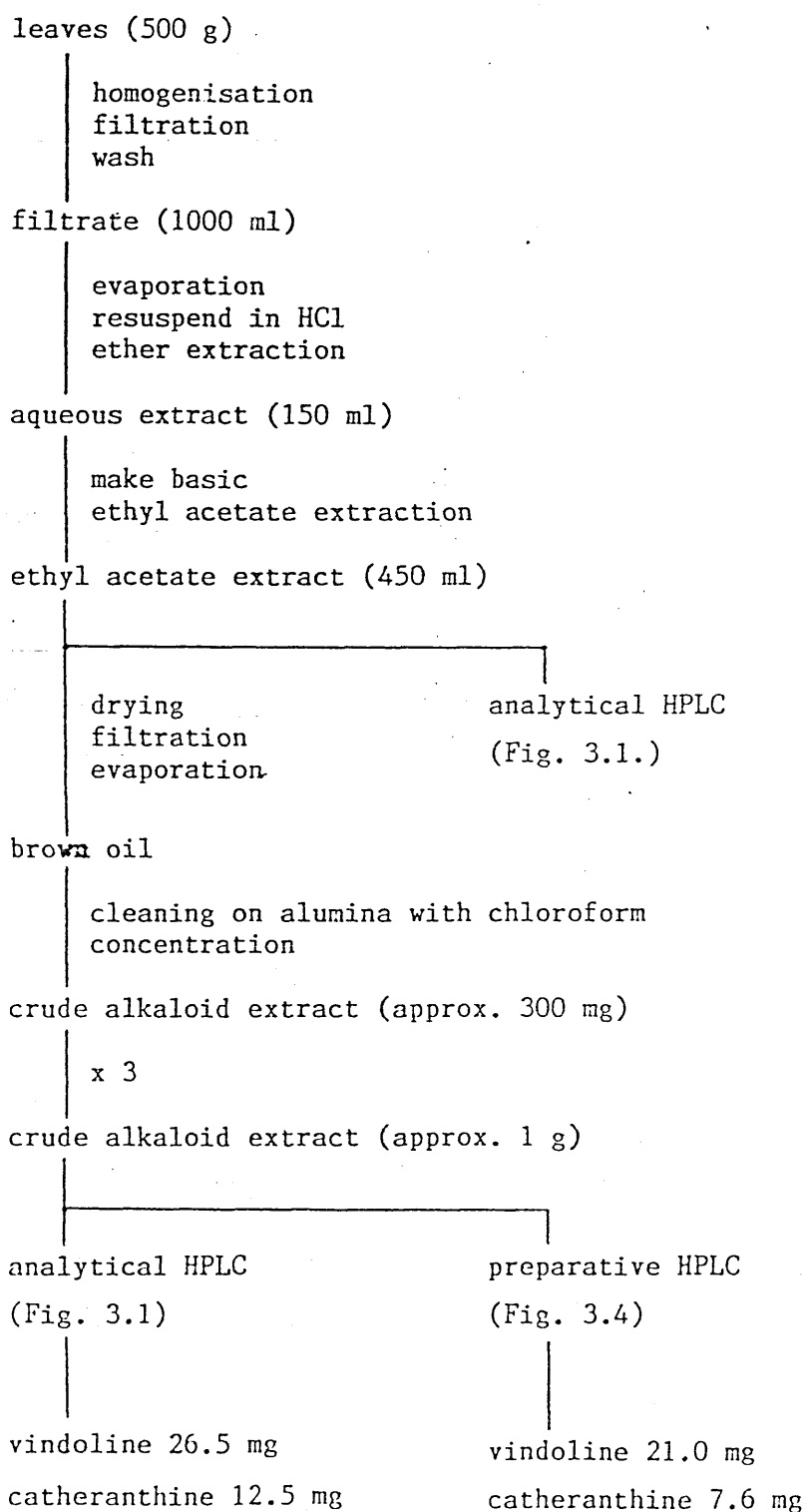
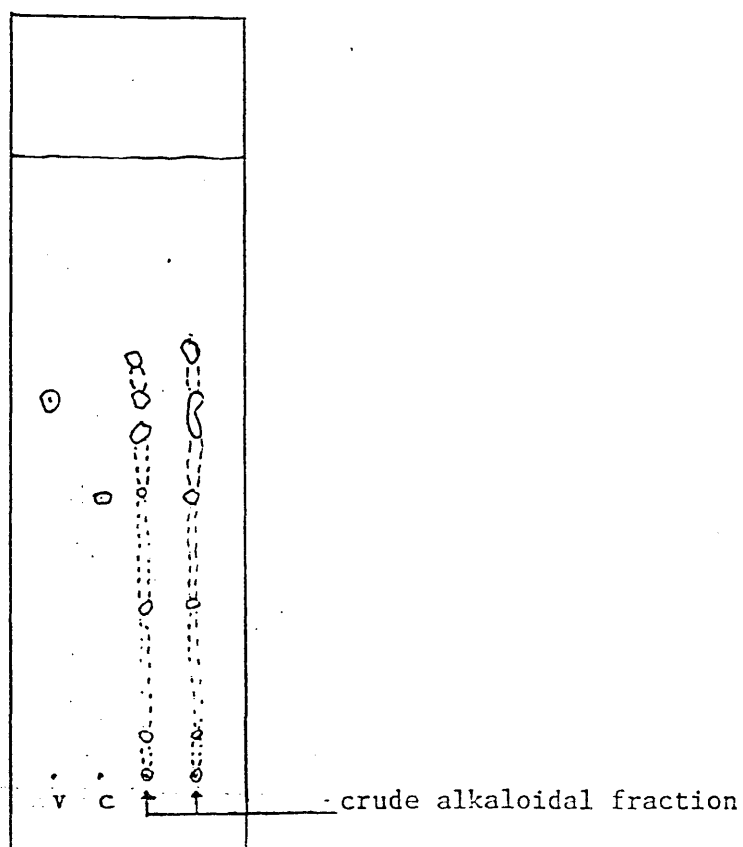


Fig. 3.3. TLC analysis of Crude Alkaloid fraction from ethyl acetate



sample : 1 μ l. from a 5 ml concentrate

reference : 2 μ l cat (c)

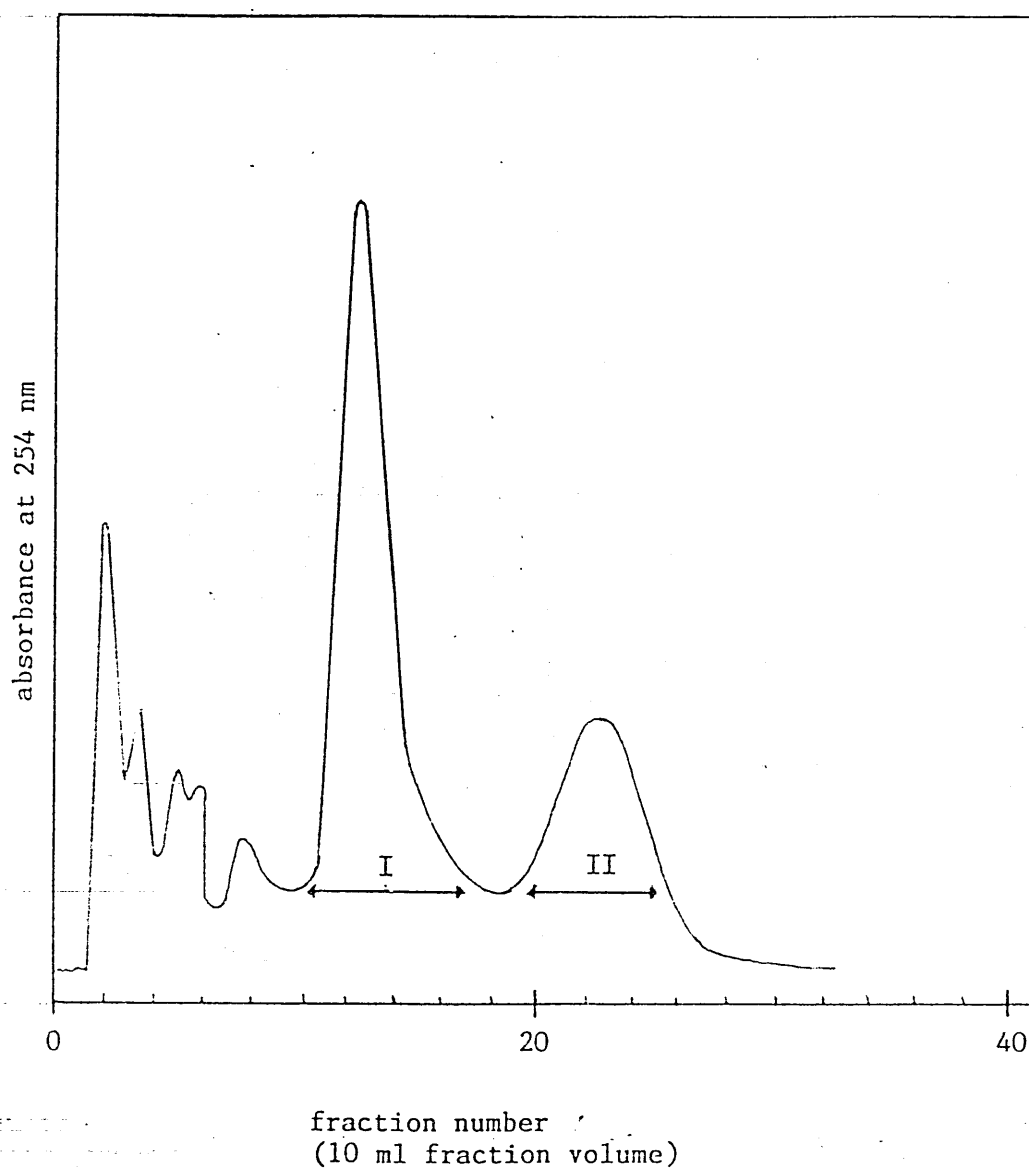
2 μ l vin (v)

solvent : chloroform-methanol 9:1

plates : silica gel

detection : scanning at 254 nm

Fig. 3.4. Elution Profile of a typical HPLC separation of
Crude Alkaloidal fraction.



sample : 1 ml of a 5 ml (1.03 g) concentrate

column : Lichrosorb RP-8 (1 x 24 cm)

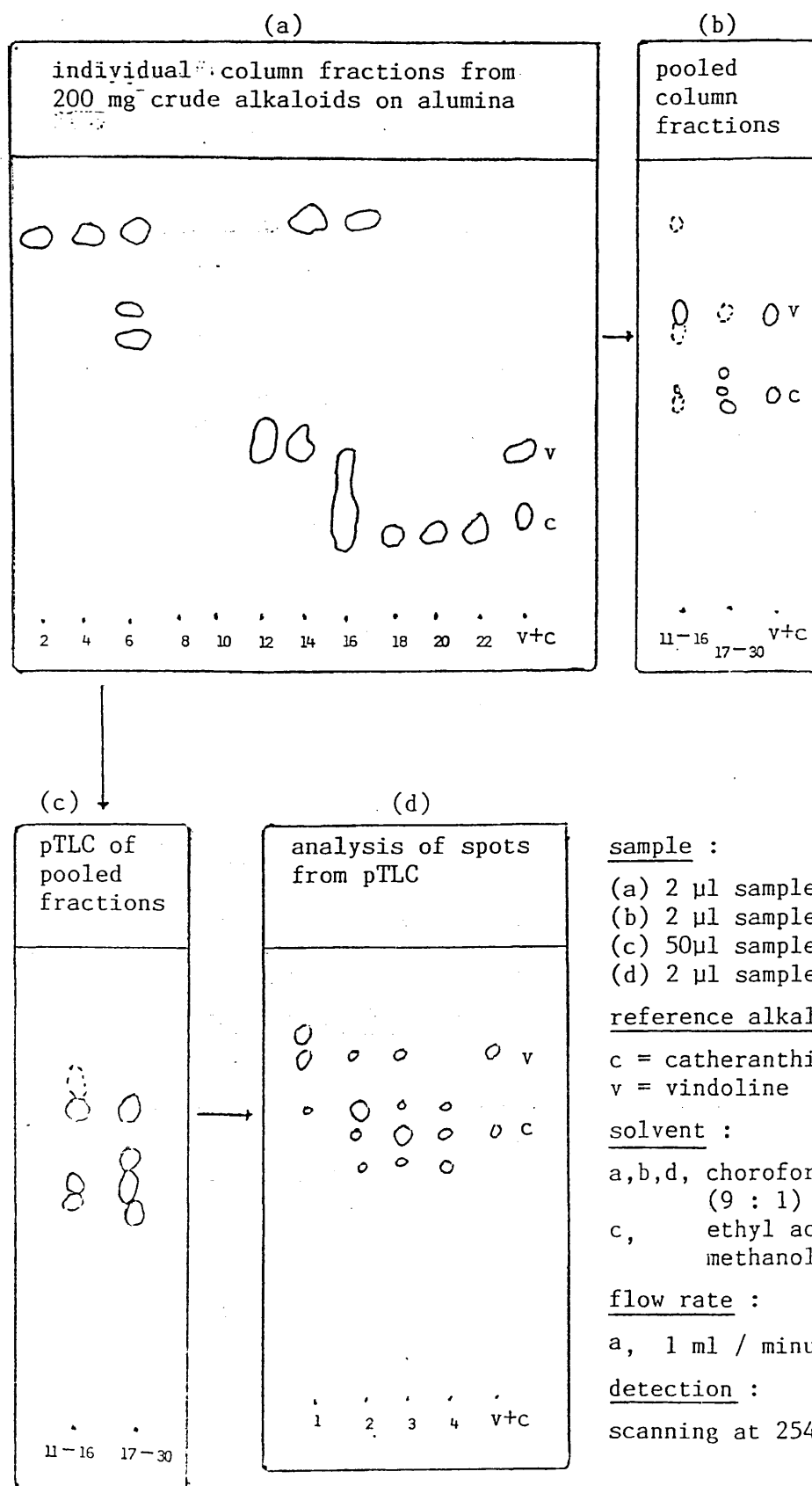
buffer : 0.01 M phosphate pH 7.0 with 60% methanol

flow rate : 1 ml / minute

detection : absorbance at 254 nm

suspended in 5 ml methanol of which 1 ml was subject to HPLC as in fig. 3.4. Peak forming fractions were pooled and analysed by HPLC as in section 2.4.1. and shown to be non-homogeneous. Fractions I and II were shown by TLC to be vin and cat respectively. The separation by preparative HPLC afforded 7.6 mg cat and 21.0 mg. Based on crude alkaloidal fraction, yields of 0.7% for cat and 1.6% for vin were obtained. Again, these values are very low compared to those found in the literature as depicted in fig. 3.2. (Ref. 37). To produce the intermediates for the condensing reaction in figs. 1.8 and 1.9. chemically pure alkaloids are required. It was found that only 500 to 1000 μ l could be run at one time without high back pressure generated by the clogging of the column from gunge-like impurities present in the sample. These problems compounded by the low amounts of alkaloid obtained led to the decision to employ conventional liquid chromatography on alumina or silica gel for preparation as in section 2.5.3. A typical analysis of column fractions by TLC (fig. 3.5a) confirmed the presence of cat and vin in the original extract while demonstrating their separation by liquid chromatography. However, identical fractions showed a degree of heterogeneity on pooling that appeared to intensify on subjection to preparative (p)TLC as in section 2.5.2., even when the same chloroform-methanol eluant that gave good resolution at the analytical level was used (fig. 3.5 c and d). The R_f values (which indicate the distance relative to the eluting solvent front travelled by each substance spotted on a plate) for cat and vin were within the optimal resolution range of 0.3 to 0.7 (Ref. 18). There was no material left at the origin by the inability of the solvent to 'move' the compound so that overloading of plates with sample could be dismissed as the cause of impurities. What appears likely is the decomposition of the material due

Fig. 3.5. TLC Analysis of Liquid Chromatography Fractions.



to successive applications and dryings of substances on plate before runs. The close proximity of spots in fig. 3.5b and d seem to bear this out.

There are other reasons for the failure of the liquid chromatography system. The length of the separation means that artifact formation during the process is enhanced. Though all solvents were distilled, there may have been reactive impurities present that could oxidise the alkaloids. It has also been reported that crystallisation from column fractions in which methanol was used is extremely difficult (Refs. 18, 37). The lack of pure compounds from the isolation meant that chemical methods of radioactive labelling precursors was difficult and normal procedures employing tritiation by vacuum distillation (Ref. 19)., had to be abandoned. Instead one of the precursor alkaloids was enzymatically biosynthesised as described in 2.7.1. Thus the condensing reaction between monomers was followed by labelled vin and cold cat from the separations (Fig. 2.4).

3.4. Activity of the Crude Enzyme Extract.

The homogenisation and centrifugation of *C. roseus* leaves yielded a crude cell free extract that catalysed the conversion of [2-¹⁴C] Trp and secologanin to vin after two hours (table 3.1., fig. 3.6 a,b). Thus the presence of an enzyme system appears to be confirmed. An average incorporation of radioactive Trp into vin of 1.3% of total label added to reaction compares favourably with values of 1.1 - 1.4% found by Kutney. This indicates that the assay fulfils his conditions and can be used for further experiments (Ref. 19).

There is no documented difference in either alkaloid content or catalytic activity between plants bearing flowers of different colour. Thus, there was no attempt to study this aspect in this report, especially

Table 3.1. Comparison of Activities of Boiled and Untreated
Cell Free Extract from C. roseus.

Sample	activity in dpm			average activity
Boiled Extract	5.3	30.5	15.3	17.0
Untreated Extract	1376.2	1430.3	1485.1	1430.6

Crude enzyme extract = 25 ml

[2-¹⁴C]Trp bisuccinate = 2.5 µCi, 0.53 µmol

Secologanin = 0.1 mg

NADPH_i = 0.5 mg

FAD = 0.5 mg

Incubation : 2 hours at 34°C

Assay for vin : TLC on silica gel plates backed with foil

Sample size : 5 µl

Eluant : chloroform-methanol (9 : 1)

Table 3.2. Comparision of Fresh and Frozen Crude Enzyme
Extracts from C. roseus.

condition of leaves	condition of enzyme extr. from leaves	activity as av. dpm	activity as % incorporation of label
fresh	fresh	1445.2	1.35
fresh	frozen	1326.6	1.20
frozen	fresh	1430.1	1.30
frozen	frozen	1162.1	1. 06

frozen leaves and extracts were kept at -20°C for 24 hours before use.

Assay was performed as described in legend to table 3.1.

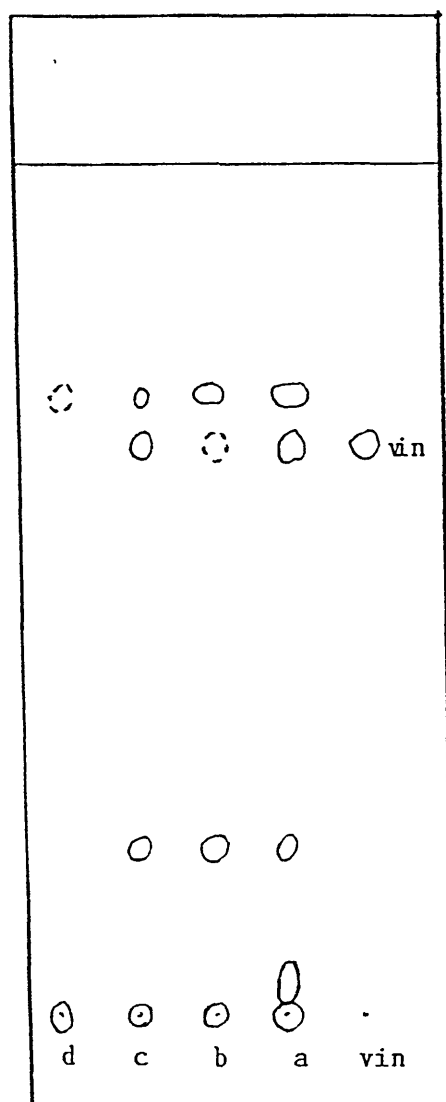
as the majority of plants tended to be of the white variety.

When fresh and frozen leaves were compared with respect to the catalytic activity of their extracts, a progression from totally frozen to totally fresh was discernible, though not marked enough to be significant (table 3.2). This may only mean that the frozen preparations were not stored long enough for a reduction in activity to occur. It appears however, that the freshness of the extract is more important than that of the leaves. The majority of the subsequent experiments were performed with fresh extracts.

3.5. Activity of Precipitated extracts.

The ammonium sulphate precipitated enzyme preparation showed a higher incorporation of label and therefore greater activity than the crude extracts (table 3.3). This may be due to the higher concentration of enzyme system (compared to the crude) in the smaller volume of 3.5 ml obtained after desalting the extract. The removal of other proteins that affect its activity may also be a reason. The crude preparation also appeared to have a pool of endogenous alkaloids which were present in the leaves and were not removed on extraction (Fig. 3.6). They could interfere in the condensation between added cat and labelled vin. Therefore the results may not reflect the true reaction as considerable dilution with unlabelled products may occur. To avoid this effect, henceforth precipitated extracts were used in preference to crude ones. To carry out the condensation reaction, [2-¹⁴C]vin was prepared by a 5 x 25 ml incubation of [2-¹⁴C]Trp and secologanin as in 2.7.1., and the amount of radioactivity it contained estimated at 0.116 μ Ci. This was reduced to 0.064 μ Ci on isolation by pTLC as in section 2.5.2. Of this, 0.02 μ Ci was used in timed incubations, where it was hoped that an increase in radioactivity would occur in some of the new spots

Fig. 3.6. TLC analysis of Assay of Crude and Precipitated
Cell Free Extract from C. roseus.



- a = fresh crude extract
- b = boiled crude extract
- c = fresh precipitated extract
- d = boiled precipitated extract

Incubation : 25 ml crude extract or 3.5 ml precipitated extract
incubated with [2-¹⁴C]Trp bisuccinate and secologanin
for 2 hours at 34°C in presence of cofactors

Analysis : 5 µl samples spotted onto TLC plates and run in
chloroform-methanol (9 : 1)

Table 3.3. Comparison of the Activity of Crude and Ammonium Sulphate
precipitated Extracts from C. roseus

Sample	Activity as av. dpm	Activity as % incorporation of label
crude extract	1405.2	1.28
boiled crude extract	23.8	-
prec. extract	2486.5	2.26
boiled prec. extract	18.6	-

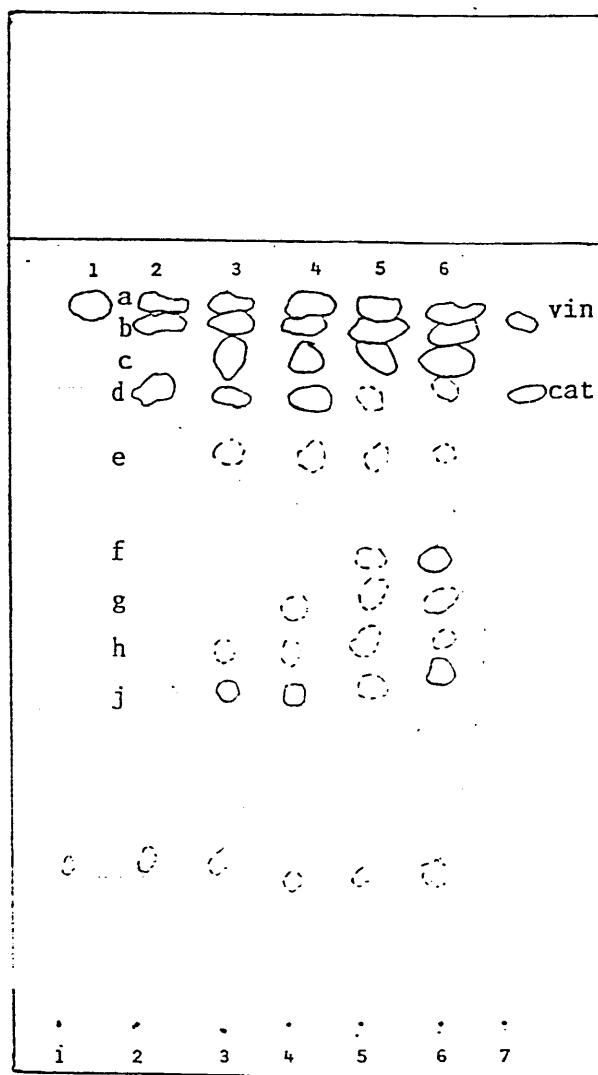
Assay was performed as in legend to fig 3.6.

formed with time and a corresponding decline in the radioactivity of vin would take place. This reaction is schematically illustrated in figs. 1.9 and 2.4. A typical result is depicted in figs. 3.7, 3.8, and 3.9. Spot 1 shows that interference from alkaloids in the cell free extract itself was negligible. The TLC analysis of the reaction (fig. 3.7) and scan at 254 nm (fig. 3.9) reveal a reduction in cat (spot d in fig. 3.7) with time. When the dpm values are considered, vin (spot b in figs. 3.7 and 3.8) also decreases with time. However, scanning of these spots showed no reduction in the corresponding peak. Furthermore, spot c appeared to be labelled vin as well, thus implying that the applied vin suffered decomposition during either drying of spotted sample or sample elution. There appeared to be no noticeable increase in any one spot so that products could not be identified or isolated. Because the original interest in the system lay in the reaction between monomers to yield dimeric intermediates in the pathway from VLB to VLB, the inconclusive results obtained forced a reconsideration of the system under study. The condensing enzyme system catalysing the biosynthesis of labelled vin (fig. 2.4) was employed as an assay because then the reactants and products could be better identified and separated. The cell free extracts were concentrated by further purification so as to increase the efficiency of the reaction as was demonstrated in fig. 3.6 and table 3.3.

3.6. Partial Purification and Assay of Extracts

Ion exchange chromatography of the precipitated extract gave two fractions of which the second was more active on the basis of the vin forming assay (fig. 3.10). The partially purified fraction was more active than the precipitated one either because the enzyme system needed for the reactions was more concentrated in this preparation or because

Fig. 3.7. TLC Analysis of the Products of the Timed Reaction
between Vindoline and Catheranthine to form Dimers.

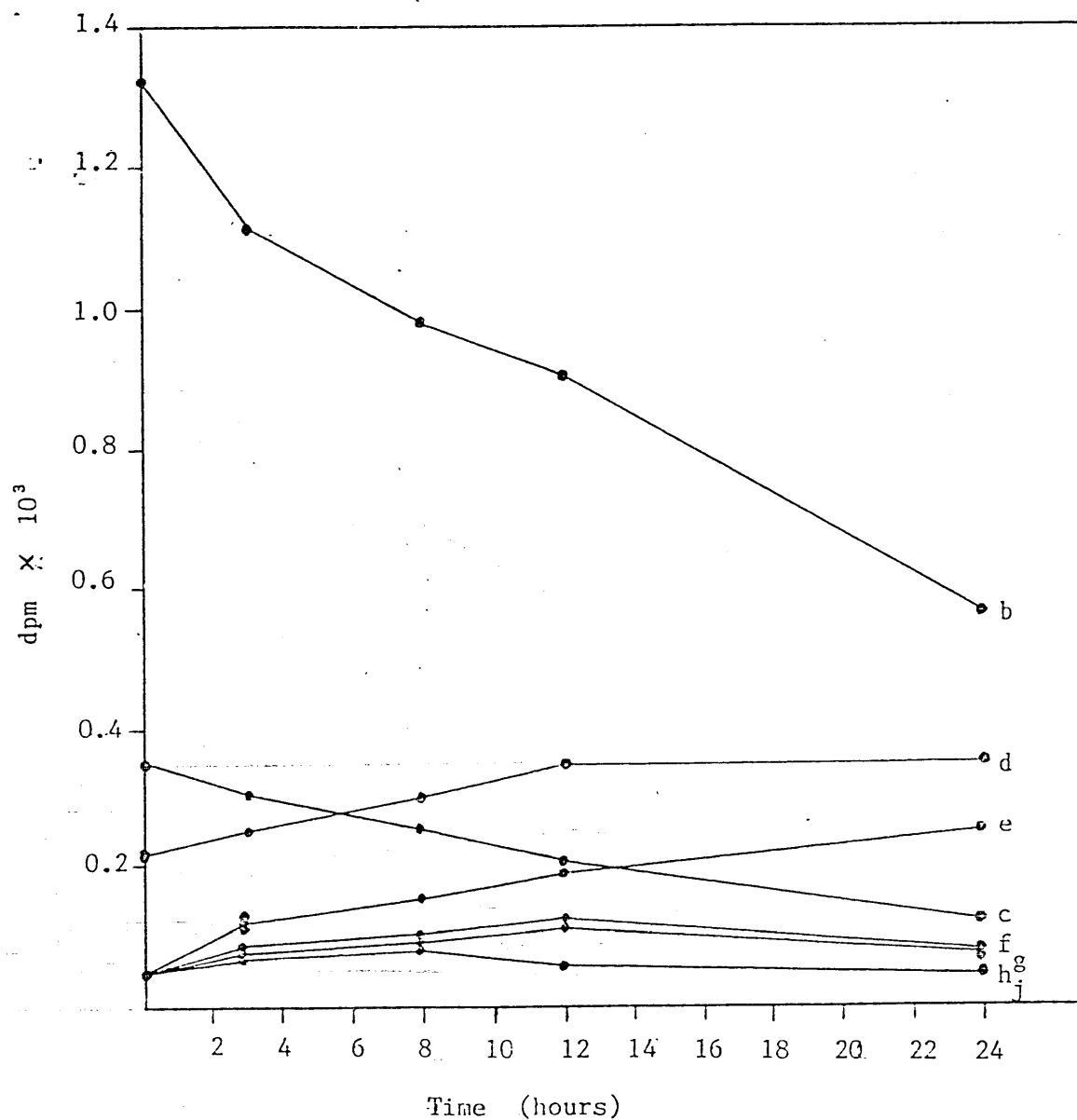


enzyme preparation : precipitated extract

incubation : 3 hours at 34°C with cofactors as in section 2.7.1.

- | | |
|-------------------------|-----------------|
| 1 = boiled extract | 2 = at 0 hours |
| 3 = at 3 hours | 4 = at 7 hours |
| 5 = at 12.5 hours | 6 = at 24 hours |
| 7 = reference alkaloids | |

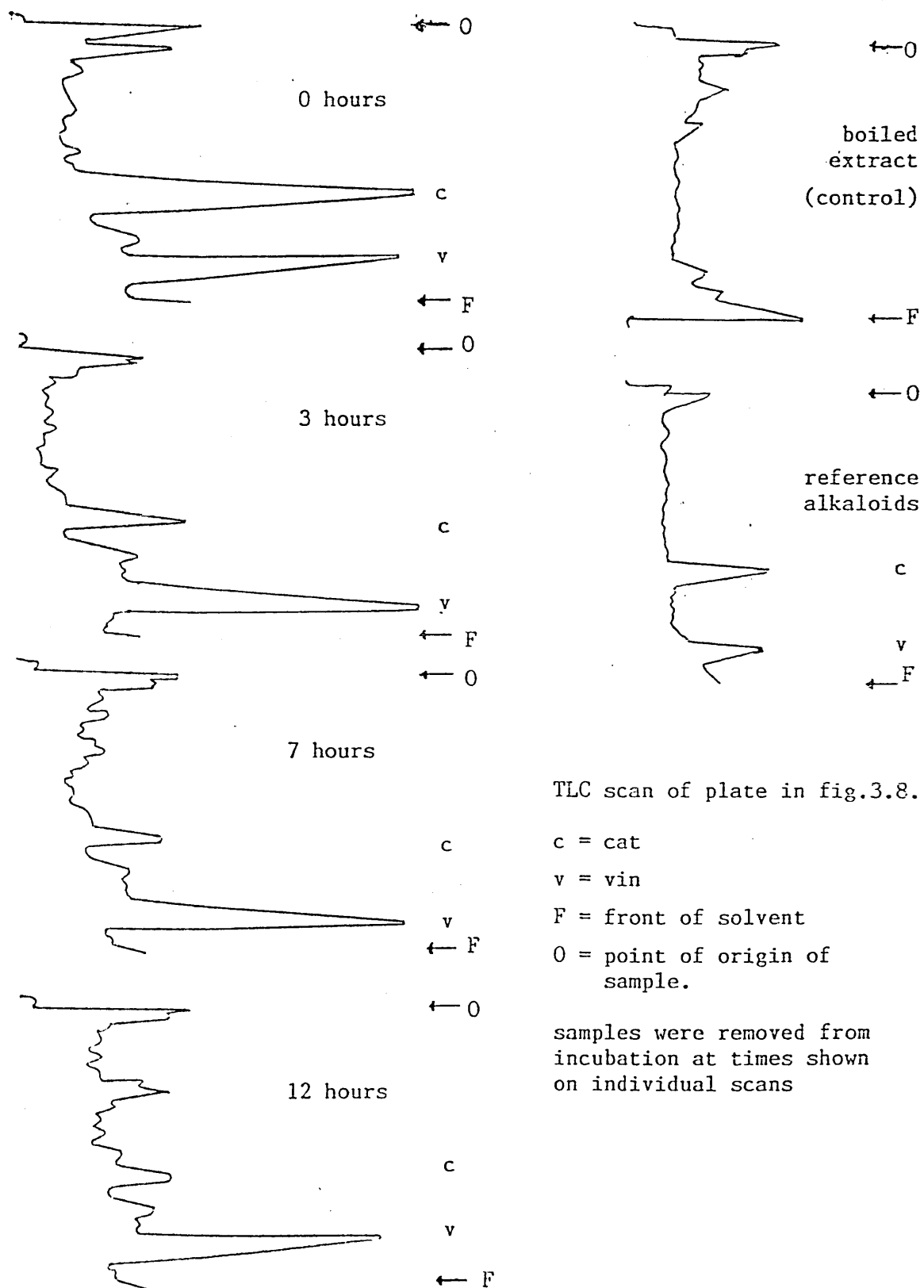
Fig. 3.8. Time course for the Condensing reaction
between Vindoline and Catheranthine



incubation conditions : 0.2 μ Ci vin and 1 mg cat reacted with
cofactors as in section 2.7.1., using
3.5 ml precipitated extract as catalyst.

(the letters refer to the spots on TLC plate in fig. 3.7.)

Fig. 3.9. TLC Scan of Timed Reaction between Catheranthine and Vindoline using Precipitated Extract



potential inhibitors had been removed (table 3.4).

The average protein content of the preparations after the method given in section 2.9 were as follows:

Precipitated enzyme	-	15.4	mg/ml
Fraction 1	-	2.6	mg/ml
Fraction 2	-	7.0	mg/ml

Gel filtration of fraction 2 showed a mixture of enzymes with a molecular weight range between 25,000 & 450,000 daltons (fig. 3.11). This is a fairly wide spectrum so that further purification of the preparation may be necessary. However, the latter was not attempted because, as explained in section 1.4, a high degree of purity was not desired where immobilisation was concerned. It was thought that the stability of the extract could also be compromised.

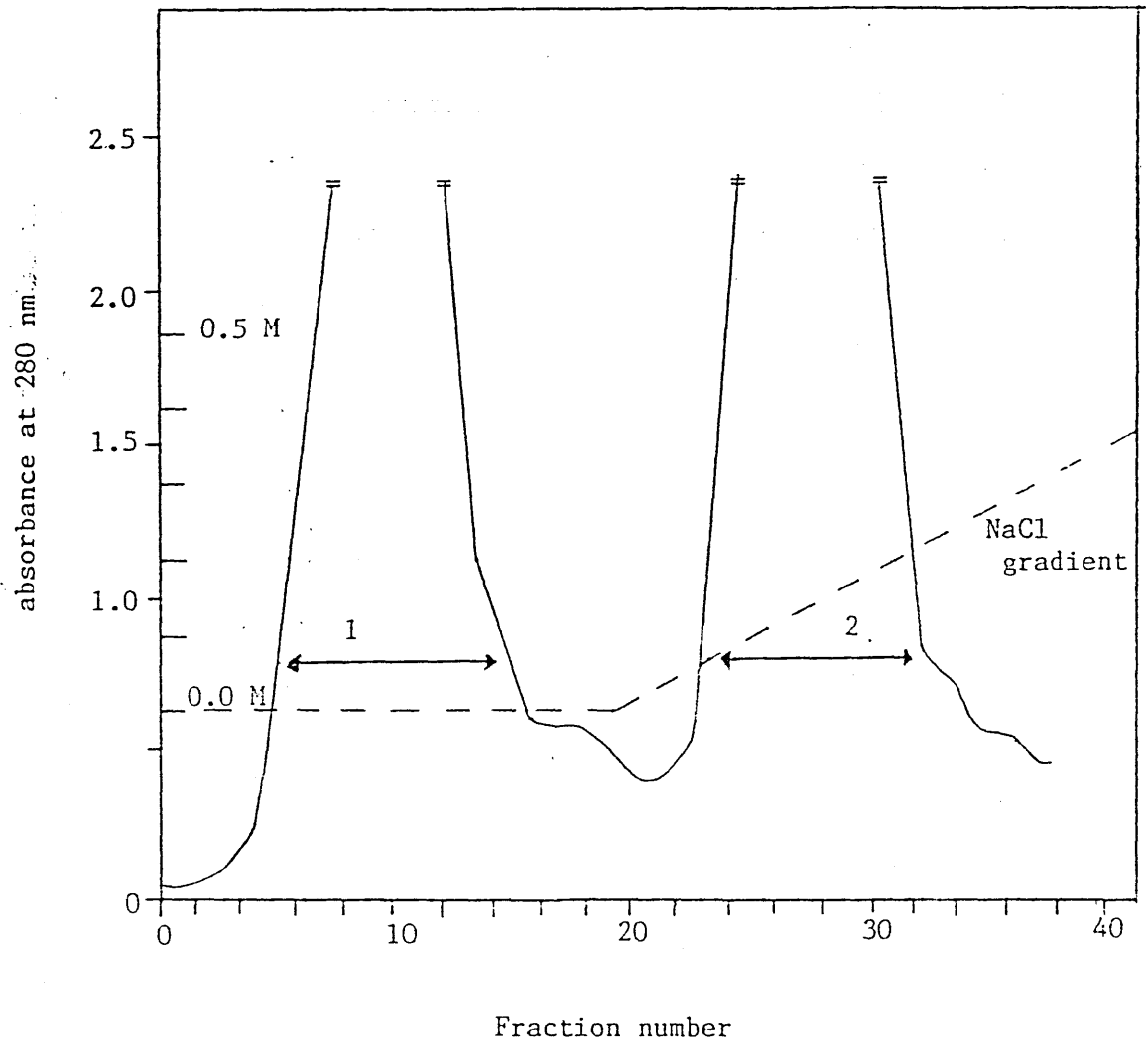
The effect of protein amount on the reaction was studied so as to determine a working concentration of enzyme system for subsequent experiments. Fig. 3.12 shows that saturation with protein under standard conditions occurred after 3 mg/ml protein in reaction volume. A value of 1 mg/ml enzyme extract was chosen as this gave reasonable dpm values for assay without using up too much of the enzyme preparations and expensive reactants.

3.7. Immobilisation of Fraction 2

Enzyme immobilisation onto Tresyl activated Sepharose was followed by thorough washing to remove all enzyme not bound to matrix; otherwise enzyme leakage into surrounding solution could have given incorrect values, due to the presence of free as well as bound enzyme.

The weight of 1 ml swollen gel = 0.5 mg when drained by suction.

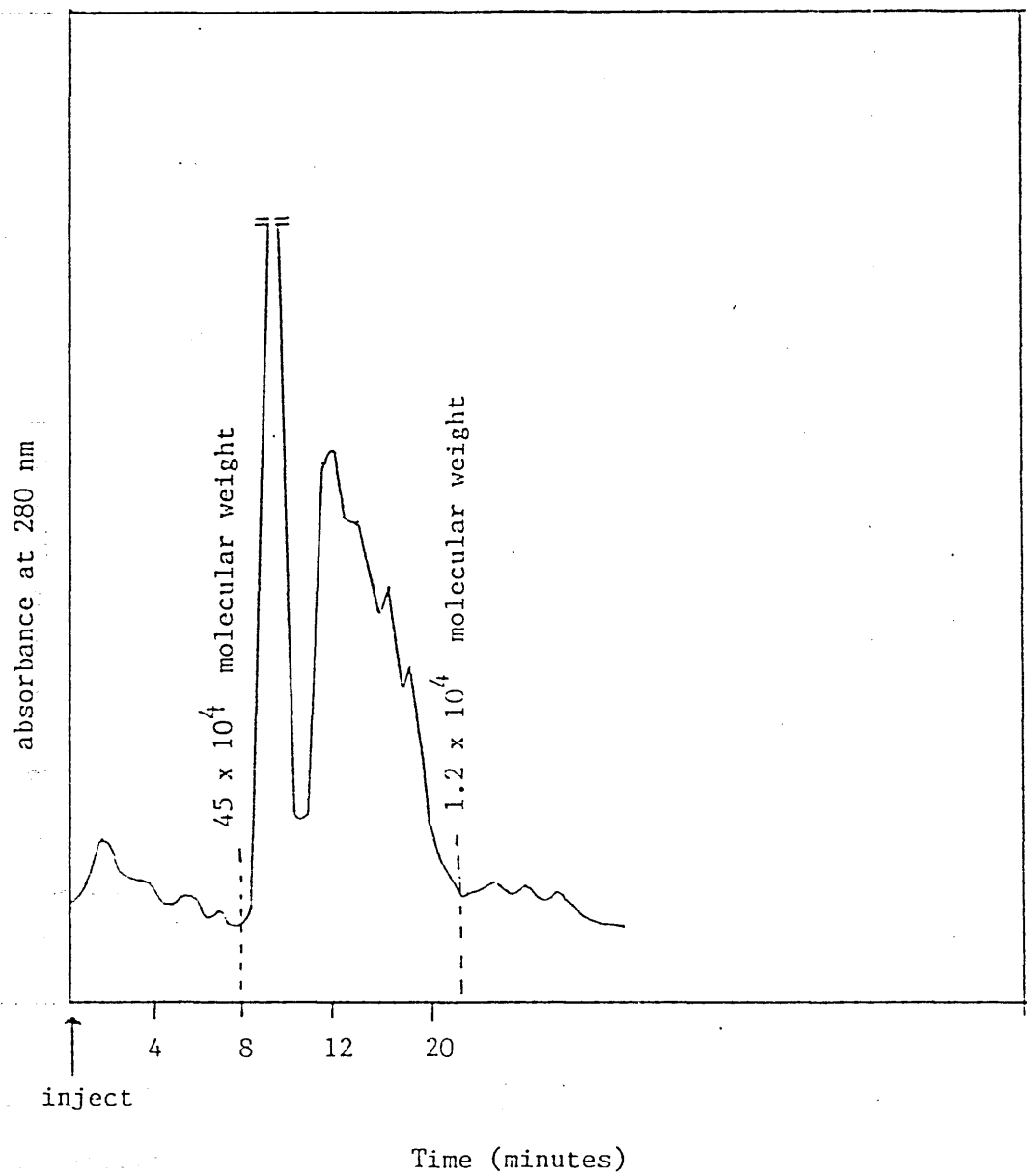
Fig. 3.10. Elution Profile of the Precipitated Extracts on
DEAE Sepharose.



fraction 2 = partially purified extract

conditions : section 2.6.

Fig. 3. 11. Gel Filtration Profile of Fraction 2 by
FPLC on Superose 12 Column



conditions : section 2.10.

Table 3.4. Comparison of Activities of Precipitated and Partially Purified Extracts from C. roseus

Sample		Activity as dpm	Activity as % incorporation of label
Precipitated extract		1689.2	0.76
Partially purified Extract	Fraction 1	298.5	0.14
	Fraction 2	3198.0	2.80

1 mg enzyme was incubated with 1 μ Ci [2- ^{14} C]Trp in 1 ml total incubation volume under standard conditions as in section 2.7.2.

vol of incubation medium assayed on TLC plates :

precipitated extract - 100 μ l

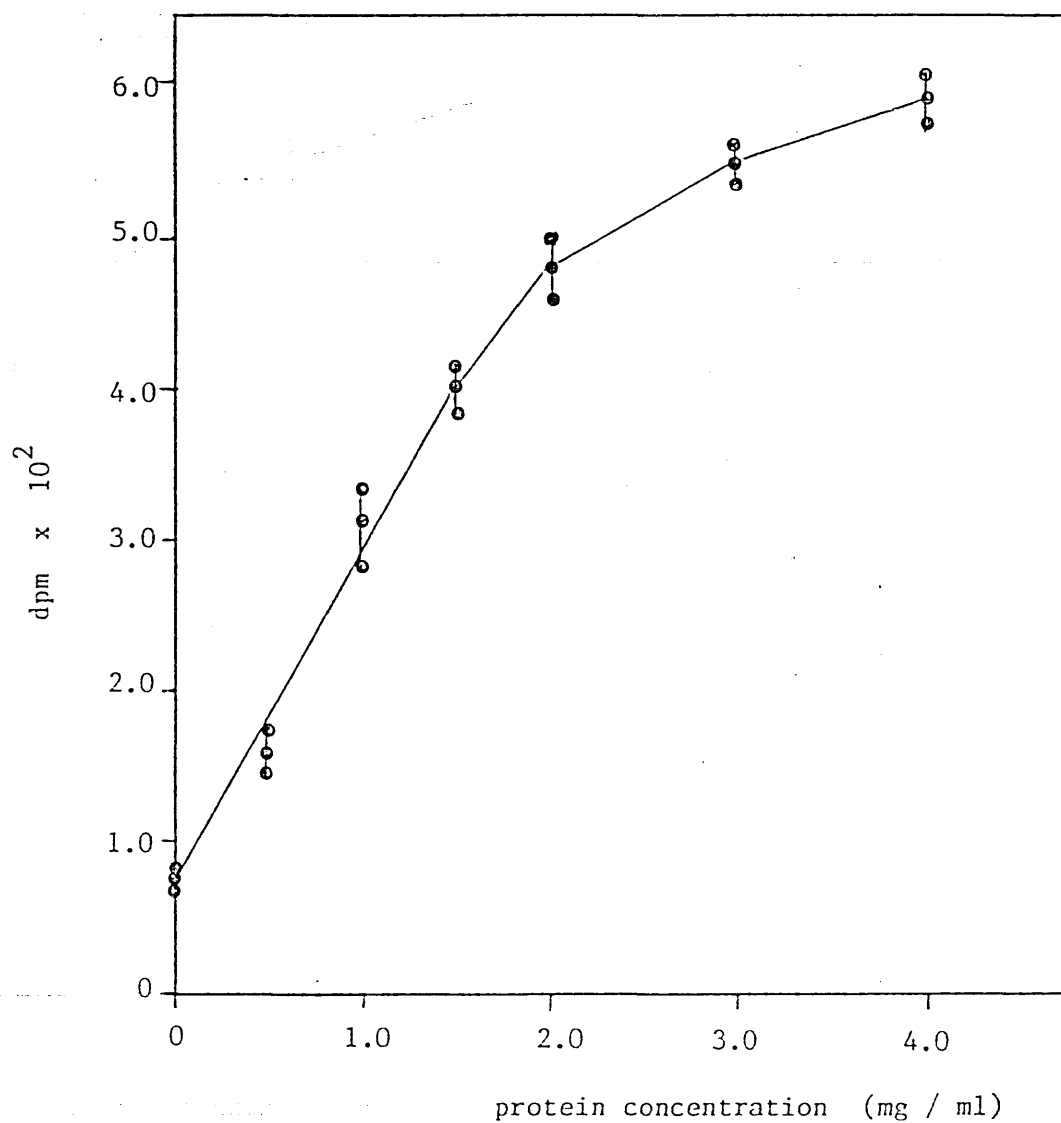
fraction 2 - 50 μ l

specific activity of preparations as % incorporation of Trp :

precipitated extract - 0.76% per mg extract

fraction 2 - 2.80% per mg extract

Fig. 3.12. The Effect of Protein Concentration on the Condensing
Reaction between Tryptamine and Secologanin



incubation conditions : standard conditions as in
section 2.7.2.

Table 3.5 shows the yield of protein bound with time during the coupling process. This was determined by adding fraction 2 to the Tresyl-activated gel and testing the supernatant for protein as in sections 2.8.1 and 2.9., but after the separation of enzyme bound matrix from bulk solution by centrifugation. At 4°C there was no change in protein yield at coupling times greater than the standard 16 hours.

The effect of protein load on the coupling of fraction 2 to the activated gel and its retention of activity was investigated as shown in table 3.6. The amount of protein bound to gel did not exceed 50% of total protein added during coupling. This value is in keeping with the 50% - 90% coupling yields generally encountered in studies on other enzymes immobilised to Tresyl-activated Sepharose. The optimum protein load seemed to lie between 7 and 10 mg/ml or 14 - 20 mg/g drained gel, which compares favourably with the values of 5 - 10 mg/ml in the literature (Refs. 23, 28). The optimum catalytic activity also lay in this protein range. Therefore a load of 15 mg/g drained gel was chosen for subsequent immobilisations.

The activity of the immobilised enzyme system was compared to that of the free one by the incorporation of radiolabelled Trp into vin under standard conditions as in section 2.8.4. As shown in table 3.7., the amount of label incorporated and hence of alkaloid synthesised, appeared to be much less with the immobilised system as compared to the free one. The reasons for this apparent lack of catalytic activity are many. One explanation was that the time taken by the substrate to reach the enzyme could have been greater in the bound system than in the system where the enzyme was free in solution; in other words, mass transfer restrictions. An experiment was then carried out to follow the activity of the extracts as in section 2.7.2. but with removal of aliquots from

the reaction mixture at different time intervals for assay. In general, bound preparations showed very low levels of activity relative to free systems (Fig. 3.13), indicating inactivation of the enzyme preparation, mass transfer problems or even a tendency for inhibition with time.

These observations will be discussed in the next chapter.

3.8. pH Dependence of Immobilised Extracts

The low activity of the bound preparations might be due to the prevalence of adverse conditions in the immediate environment of the carrier.

The most important of these is the pH of the solution. Immobilised enzymes frequently function better at pH values that are different from those for free systems. Charges present on the carrier may attract or repel H^+ ions in solution, thus causing either an accumulation or reduction of protons in the vicinity of the enzyme. This in turn may lead to the exposure of the bound enzyme to either lower or higher pH than in bulk solution so that an apparent shift in pH is discerned (Ref. 16). pH optima for Sepharose immobilised enzyme systems are usually

shifted to alkaline values (Ref. 2). Therefore the pH profile of the immobilised preparation was investigated under standard conditions as in 2.7.2. and 2.8.4. Over the pH range 3 - 9 the following buffers were used: 3 - 7 citrate phosphate, 7 - 8 phosphate, 8 - 9 tris HCl. As shown in fig. 3.14, free enzyme displayed broad pH tolerance with an optimum around pH 7. However, the bound system showed no change or shift in activity with pH after the standard two hours reaction time. Possible explanations are put forward in the discussion.

3.9. Stability of the Enzyme Extracts

The stability of a bound enzyme in use or storage might be one of the most important factors in the design and economics of an immobilised enzyme process. When the bound preparation was stored at 4°C for 30

Table 3.5. Protein Binding in Partially Purified Extract to Gel with
Time

Time hrs	Protein added (mg/g)	Protein Bound (mg/g)
16	15.2	7.20
24	15.3	7.65
36	16.2	7.60

Coupling was carried out at 4°C as in section 2.8.3.

Bound protein was determined as in section 2.9.

Table 3.6. The Effect of Protein Load Immobilised on Gel on the Coupling
Activity of Immobilised & Free Partially Purified Extract

protein added		protein bound		bound pro- tein yield %	av. dpm	activity dpm/ml of gel
mg/g	mg/ml	mg/g	mg/ml			
22.19	11.1	7.6	3.53	31	198.7	7096.4
19.85	9.95	9.44	4.72	47	208.8	9855.0
14.40	7.20	7.40	3.70	51	253.6	9392.8
4.80	2.4	1.00	0.50	20	78.5	391.0

1 mg protein bound to gel was incubated under standard conditions as in section 2.8.4 and samples assayed after 1 hr.

1 mg free protein was used as control.

Fig. 3.13. Comparison between the Activities of Free and Immobilised Enzyme Preparations

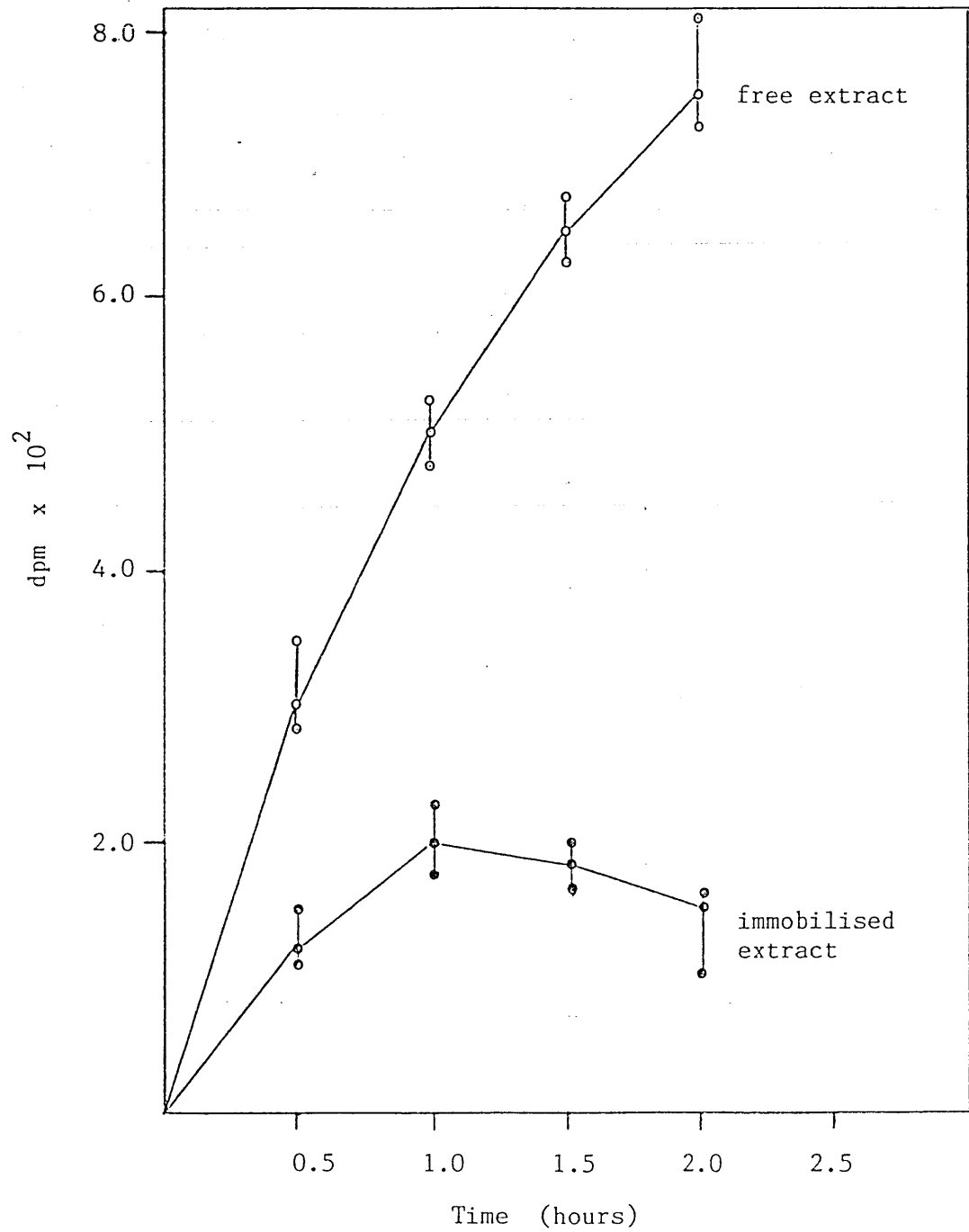
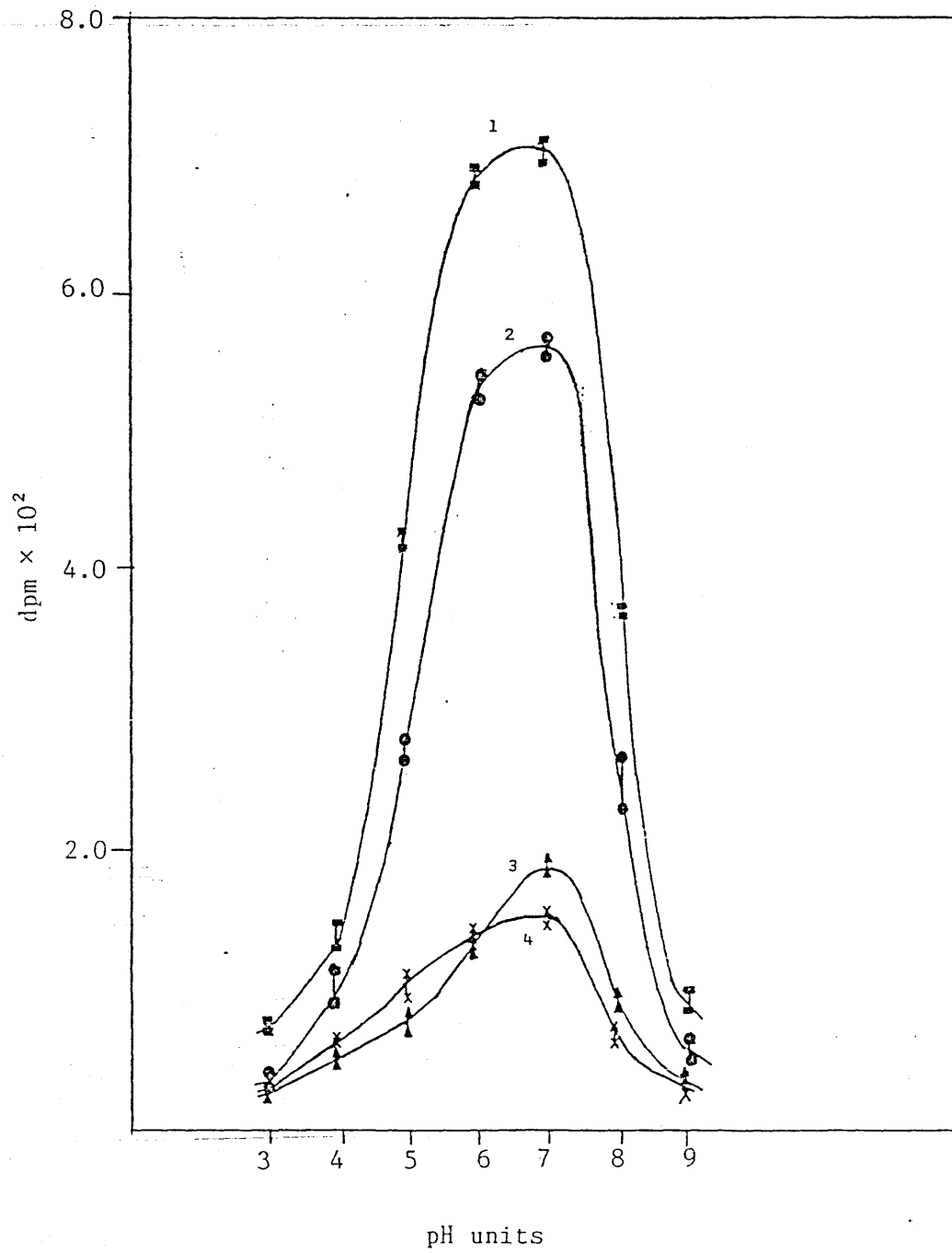


Table 3.7. Comparison of Activity of Free and Immobilised Partially Purified Enzyme

Sample	incubation time (hr)	av. dpm	rel. activity of extract (%)
free extract	1.0	503.95	100
	2.0	846.90	100
bound extract	1.0	215.34	42
	2.0	101.70	12

Incubation conditions: standard conditions as in section 2.7.2 & 2.8.4.

Fig. pH Dependence of the Activity of Free and Immobilised
Enzyme Preparations at Different Incubation Times



- 1 : free at 2 hrs
- 2 : free at 1 hr
- 3 : bound at 2 hrs
- 4 : bound at 1 hr

Table 3.8. Comparision of Stability with Time of Free and Bound Partially
Purified Protein from C. roseus

time (in days)	0	7	14	30
bound extract (dpm)	229.8	224.2	221.1	210.6
bound extract rel. activity %	100.0	100.0	100.0	100.0
free extract (dpm)	763.8	733.5	622.4	534.4
free extract rel. activity %	100.0	86.0	87.0	69.0

Incubation conditions: standard conditions as in section 2.7.2. & 2.8.4.

30 days and assayed at different times as in Table 3.8., it showed practically the same original activity while free enzyme systems lost about 30% of theirs over the same period. Thus immobilisation does appear to confer stability on partially purified cell free extracts from *C. roseus*.

While the results otherwise indicated general trends in the behaviour of the immobilised cell free system from *C. roseus* they were too inconclusive to carry out any more detailed kinetic experiments. The possible significance of deviations from expected behaviour will be discussed in the following chapter.

3.9. Summary of Results

1. yield of alkaloids from HPLC - Cat. 1.15%; Vin 2.30%

Yield of alkaloids from TLC - Cat 0.07%; Vin 1.60%.

2. Activity of Enzyme Extracts - see fig. 3.15.

3. Immobilisation of Fraction 2:

Time for coupling - 16 hrs at 4°C

Optimum protein load - 7 to 10 mg/ml swollen gel

14 to 20 mg/g drained gel

Load chosen for Experiments - 15 mg/ml.

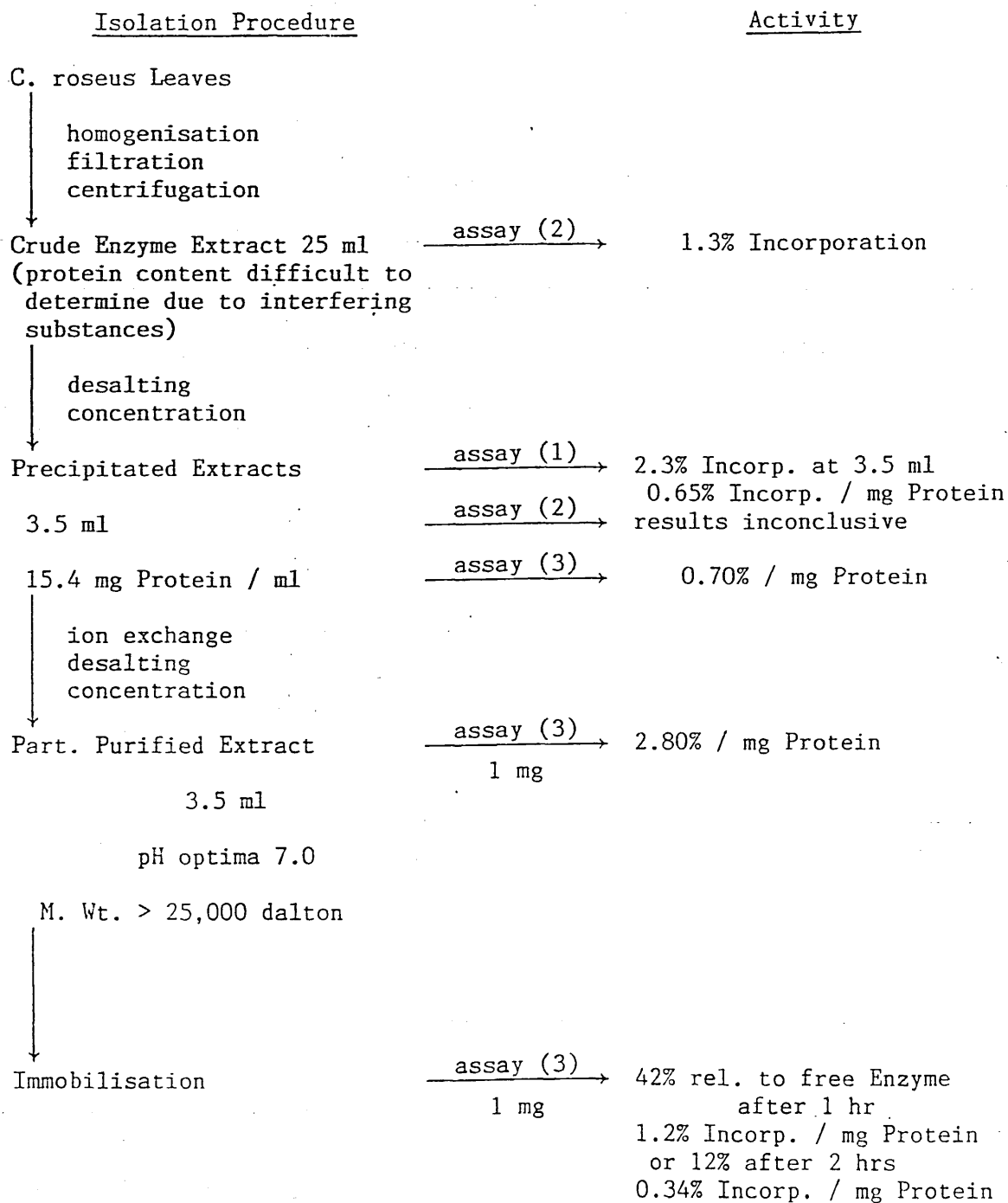
4. Activity of bound fraction 2 - see fig. 3.15.

5. pH optimum for fraction - free & bound enzyme pH 7

6. Stability of Enzyme - bound enzyme stable 30 days.

- free enzyme lost 30% activity.

Fig. 3.15. Schematic Representation of Results from Cell Free Extracts



Assay (1) : Section 2.7

Assay (2) : Section 2.7.1

Assay (3) : Section 2.7.2.

4. DISCUSSION

4.1. Isolation of Alkaloids from *C. roseus*

To investigate the reaction of monomeric I.A.'s to dimers, substantial amounts of pure alkaloids are required. For instance, Kutney (Ref. 19) uses about 220 mg pure alkaloid for chemical labelling of catheranthine. The ease of isolation of these alkaloids will therefore determine the practicability of the project.

The isolation of cat and vin from a mixture of crude alkaloids from *C. roseus* was fraught with difficulty. With both conventional and HPLC methods the extraction of precursor cat and vin was too low and impure to be of extensive use. However, the low values obtained may also be caused by the method of detection or identification of the alkaloids and will be dealt with in section 4.2.

HPLC techniques usually require relatively pure preparations to begin with. However, even with pre-cleaning on alumina with chloroform as eluant, the crude alkaloids gave rise to column clogging and severe back pressure. It appears that concentration of ethyl acetate fractions, cleaning and reconcentration (Fig. 2.2) could all lead to alkaloid decomposition or artifact formation through oxidation, heat and other factors (Ref. 18). The quantity of pure alkaloid obtained (Fig. 3.2), was not sufficient to perform experiments where purity is important, like those in which radioactive label is introduced into monomers chemically.

Conventional liquid chromatography was employed as an alternative method of isolation as in section 2.5.3. Unfortunately, it was almost impossible to obtain adequate separation of alkaloids, even when followed by preparative TLC (fig. 3.5). It is possible that condensation

products were formed between ketones present in the crude alkaloidal fractions and ammonia used in the extraction process. Great care must also be exercised when using chloroform in the isolation process as artifact formation is enhanced during long separations (Ref.18).

It has been shown (Ref. 37) that the crystallisation of alkaloids from separations using methanol is problematic. This may explain the low values from both HPLC and chemical methods. The reason for this has not been given.

Instead of time consuming solvent extraction, it might be worthwhile precipitating the alkaloids from aqueous extracts using agents such as Mayer's reagent, and then converting them to salts by ion exchange (Ref 18). Unfortunately, time constraints prevented the testing of this method.

4.2. Catalytic Activity of Cell Free Extracts

The condensation reaction of economic importance would have been that between cat and vin to form the intermediate dimer 3,4 dehydroVLB and the subsequent conversion to VLB (fig. 1.8 & section 2.7.1.). However, the reaction could only be followed with difficulty. TLC resolution of products was extremely poor.(fig. 3.7.,3.8.,3.9.). The presence of impurities could arise in a number of ways to interfere with the catalytic activity of the extracts or with the assay of reaction products.

During the synthesis of labelled vin from Trp and secologanin, radioactive by-products could be formed by incomplete conversion of intermediates or starting materials, by an 'overrun' of the reaction to form dimers, or by decomposition during handling and purification as described below.

Impurities in a radioactively labelled compound are sometimes different from those expected in the corresponding non-radioactive one. These also

depend on the method of labelling, so that the products obtained in labelling on *vin* by biosynthesis might vary considerably from those found in systems using chemical exchange methods of alkaloid labelling (Ref. 18,19,31). The latter technique should also be attempted. However, to do this, isolation methods should be improved.

When the condensation reaction between Trp and secologanin was used as an assay as in section 2.7.2, the TLC analysis again proved deficient. The fault probably lies in the technique itself.

TLC is one of the most convenient and versatile methods for assaying small quantities of radioactive materials. The distribution of the radioactivity on the chromatogram is easily measured since no volatilisation occurs. However there are drawbacks to this method.

Most of the difficulties in the chromatography of radiolabelled compounds arise at the sample application stage. Unappreciated variations and mistakes in sample handling can account for many apparently inexplicable results, even when separation systems with well known characteristics and behaviour are used. Sample loading and decomposition are critical at this stage, usually resulting in falsely low values for radiolabelling. The solvent chosen may be the cause of decomposition.

The process of drying the applied spots, enables reagents such as atmospheric oxygen to be potentially detrimental to the compound being analysed. For example, the spot C in fig. 3.7. & 3.8. appears to be a decomposition product of vindoline. Long periods of time (upto 1 hr) are necessary for the spotting of several samples onto one plate. The exposure to light during this time and the heating involved, of the sample spots, can also aggravate decomposition.

Degradation may be reduced by performing TLC at lower temperatures. However, the composition of solvents can vary with temperature so that

the genuine impurities present under normal conditions may not be resolved at the lower temperatures. Changes in the composition of eluants containing alcohol or volatile components occur with time. This may explain the inconsistent chromatography observed in the TLC system, though freshly made solutions were used whenever possible. There are also rare variations in support media, such as binding agents and water content, which affect resolution or give rise to artifacts. In this study, the temperature (20°C), support and solvents were standardised as far as possible, so that the problems encountered with resolution, particularly in pTLC, would have their roots in the sample applications, development and measurement techniques (Ref. 31).

After elution, the chromatogram was dried for development. Here too heat may play a role in sample degradation in the case of pTLC, resulting in non-homogenous products.

Spots were visualised under UV light. They were either scanned, when only their intensity was required (fig. 3.9), or marked for identification. The spots were either cut out or scraped off with the adsorbent material and subjected to liquid scintillation counting. A number of problems arose at this juncture. The biggest source of error probably lay in the loss of sample during the removal of spots. Silica gel forms brittle areas, which were easily lost during the dissection of the chromatogram by scraping or cutting.

As the samples are largely insoluble in scintillant fluid, they had to be eluted from silica gel into vial. This is time consuming, but is essential as quench correction cannot compensate for the fact that different pieces of chromatogram are eluted with varying efficiencies on their own, resulting in misleading values (Ref. 31).

Scanning chromatograms by ionisation counter can circumvent problems

encountered with dissection and scintillation counting. It is a quick method with the retention of chromatogram for further study. However, low count rates, generated by the small areas measured at any instant lead to poor statistics. In addition poor peak-to-background ratios are obtained, making scans 'noisy'. In contrast, the counting period in scintillation counting can be lengthened for increased accuracy.

4.3. Alternatives to TLC

Improvements in measuring techniques are of no avail if the chromatographic method itself is at fault. It may thus be worthwhile developing better assay methods for the detection of both monomeric and dimeric products. When separation of compounds is difficult, as in this study, they may be converted to volatile derivatives and separated by gas-liquid chromatography, which has greater resolving powers than TLC (Ref. 18). But these methods are less versatile than the latter. Material may also not elute through the column, so that recoveries can be variable. As mentioned in section 2.4., the number of samples that can be processed is also limited.

The most interesting and potentially useful development in the field of alkaloid assay has been the development of a radioimmunoassay (RIA) for vindoline (Ref. 43). RIA's are competitive binding assays where a radioactive compound or 'tracer' competes with unlabelled counterpart for limited binding sites on antibodies (Ref. 22). Antisera are produced against conjugates between vindoline derivatives and γ -globulin fractions of bovine serum. These antibodies showed high affinity for vindoline and negligible cross reactivity with other naturally occurring and semi-synthetic alkaloids. A standard curve is constructed by competition between varying concentrations of inactive alkaloid, so that the amount of the latter in unknown samples can be determined. The assay

method is schematically depicted in fig. 4.1.

The RIA for vindoline is very sensitive, measuring in a range from 0.5 to 100 pmol (0.2 - 45 ng of vindoline whereas TLC works best around 100 ng. The detection limit is 110 fmol or 50×10^{10} g, and reproducibility of assays is high.

Setting up a RIA is tedious. For example, test rabbits might respond individually to immunisation with vindoline- γ -globulin conjugates so that production of vindoline antibodies becomes variable. But on the whole, the precision, ease and suitability for automation may outweigh the disadvantages of this assay. The high specificity of the assay means that crude extracts can be used for experiments without fear of interfering substances. Several hundred quantitative assays can be performed in one day which is a considerable advantage over the 2 or 3 days required for the TLC evaluation of each experiment.

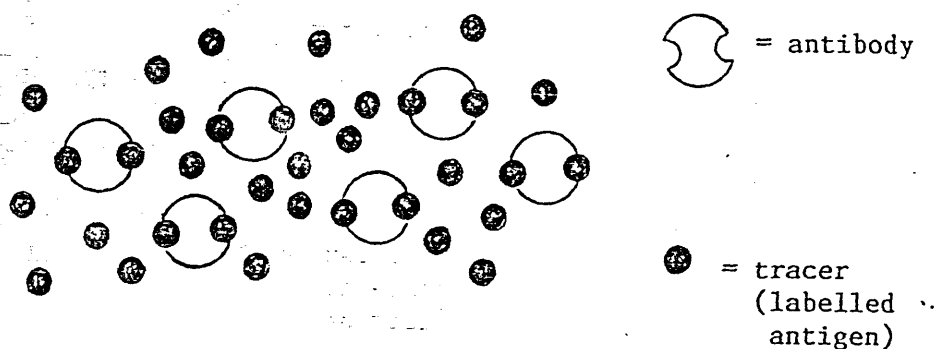
4.4 Cell Free Systems

Intact plants contain higher levels of phenolics and other enzymes inactivators than cell suspension cultures, so that enzymes isolated from the latter could be potentially more active. Large-scale production of homogeneous plant material as an enzyme source may also be possible. Cell free systems from plant cell cultures have been used to study the synthesis of Coryanthe alkaloids but not that of Aspidosperma and Iboga types (Refs. 35, 39, 44).

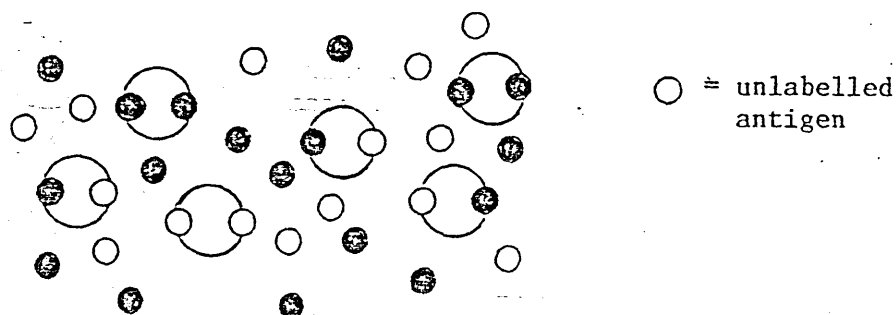
An alternative to cell free systems themselves lies in the use of whole cells for the de novo synthesis of alkaloids. In cells, the stability of enzymes may be enhanced by their retention in natural surroundings. In addition, enzyme and cofactor may be arranged in an optimal way within the cell. Regeneration machinery present in the cell might make the external supply of cofactors unnecessary (Refs. 7, 17, 34, 45)

Fig. 4.1. Principle of Radioimmunoassay (RIA)

1. A limiting and constant amount of antibody is incubated with a constant concentration of tracer such that 30 - 50% of the tracer is bound by the antibody.



2. Competition occurs between labelled and unlabelled antigen for the limited number of binding sites on the antibody.



3. The amount of tracer remaining bound to antibody can be determined after the separation of bound and unbound fractions. The amount of tracer bound will be high when no competing unlabelled antigen is present. Less tracer is bound to antibody when competing antigen is present.

Data from Amersham (Ref. 31)

Problems encountered with cell culture from *C. roseus* are outlined in section 1.1.. Foremost among these is the low level of alkaloid production. More efficient synthesis may be achieved by feeding precursors to cultured cells. Careful selection of variant strains may yield cell lines with alkaloid contents equal to or surpassing those within the original plant. The genetic stability of these strains, as is the case with *C. roseus*, may prove to be a problem, and constant clonal selection of high yielding strains might be necessary (Refs. 44, 45).

4.5. Immobilisation of Partially Purified Extract

The immobilisation of the enzyme system from *C. roseus* on Tresyl activated Sepharose to increase efficiency of the condensing reaction between Trp and secologanin gave very inconclusive results. Although seemingly stable to storage (table 3.8), and more active than crude or precipitated free preparations (fig. 3.15), the activity of the bound preparation was very much less than that of its free counterpart (fig. 3.13).

There are many reasons for the low activity of the bound preparation.

Inactivation can occur during the actual immobilisation process. Supports can have profound influences on the retention of enzyme activity.

The amino acid residues at active sites of enzymes are very reactive and interaction with activated gel may occur to a certain extent. If active sites are on enzyme surface, then this interaction would depend on the actual size of the site relative to the whole protein (Refs. 4, 10, 29). Generally, active sites are recessed in the enzyme molecule and access to them depends upon the size, charge etc. of the attacking group (Ref. 22). Though contact with support can denature the enzyme, active site modification appears to be marginal for CNBr or Tresyl activated gels (Refs. 2, 23, 28). The addition of substrate or competitive inhibitor to the coupling mixture will protect the active site against-

loss of activity and may be used to test the mildness of the immobilisation procedure (Refs. 10, 16, 24, 29).

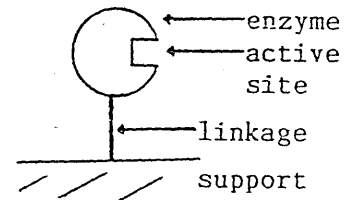
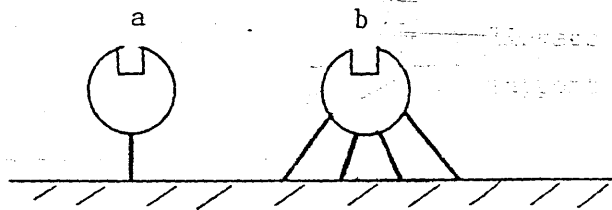
More stress has been laid on conformational and steric effects as being behind altered enzyme activity. There are no covalent methods which enable specific orientation of enzyme on matrix surface. Therefore it may be bound with its active site facing towards bulk solution or partly or totally obscured by other proteins or the matrix. Blocking of active centres by support so that formation of enzyme-substrate complex is prevented has been demonstrated but appears to be less pronounced in agarose gels than in most other types (Ref. 29). Some enzyme molecules may have only one bond to carrier while others such as Tressyl activated Sepharose with both amino and thiol residues, may have multiple ones. The latter can restrict the mobility of enzymes on support and thus result in lower activity relative to the free enzyme. These effects lead to heterogeneous preparations which show complex reaction kinetics even if the enzyme used is highly purified (Refs. 2, 16, 29). Heterogeneity is intensified by the use of irregular surfaces such as fibrous or porous matrices (fig. 4.2). Partially purified extract would, by definition, contain proteins that do not participate in the desired reaction so that the resulting bound extract would exhibit a high degree of non-homogeneity.

A determining factor in the eventual activity of the bound enzyme is the enzyme load or quantity of enzyme bound to carrier. The binding capacity of support for enzyme is influenced by many factors. An increased number of reactive groups on matrix binds larger amounts of protein but may lead to preparations with multiple linkages and lower activity. High enzyme concentrations in solution during the immobilisation process leads to greater amounts of protein bound. However, it has been observed that overloading carrier with protein results in internal diff-

Fig. 4.2. Possible Configurations of Enzymes after Covalent Binding to Support

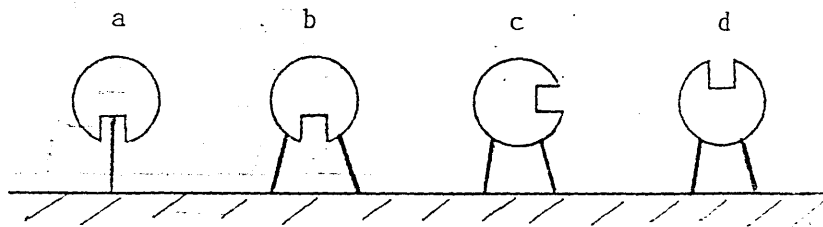
1. Linkages between Enzyme and Support :

- (a) binding through one bond
- (b) binding through multiple bonds



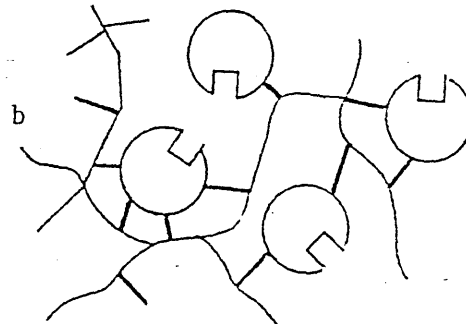
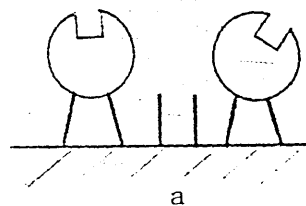
2. Orientation of Enzyme bound to Support :

- (a) active site bound to support
- (b) active site totally obscured by support
- (c) active site partially obscured by support
- (d) active site free to bulk solution



3. Uniformity of Support :

- (a) flat regular surface
- (b) porous irregular surface



From Methods in Enzymology Vol. XLIV 'Immobilised Enzymes'
Ed. Mosbach K., Academic Press (1976).

usion limitations as mentioned in section 1.5 and below, as only those proteins at matrix surface are exposed to substrate. Thus the efficiency of enzyme utilisation is decreased when not all bound enzyme is available for reaction. For kinetic analyses at least, enzyme loadings must be a compromise (Refs. 4, 10, 20).

Enzyme loading is also effected by the surface area of the carrier.

Though the latter may be large, its availability for attachment to the enzyme is restricted by pore size. As the relationship between the two is an inverse one, the lowest possible pore size giving optimal surface area and enzyme loading is generally chosen (Ref. 2, 10). Tresyl activated Sepharose 4B binds proteins between 6×10^3 and 20×10^6 daltons in molecular weight. If the protein system required for the condensing reaction tends towards the lower value, then the pore size may be too large for optimum loading. The exclusion pore size is determined by the coupling of enzyme preparation to supports of varying pore diameter to obtain a critical value at which the entrance of enzyme or substrate is sterically hindered and the activity drops sharply. Unfortunately, there are no other commercially available Tresyl activated gels. Thus other gels such as Sepharose 2B and 6B with 2% and 6% agarose respectively (Refs. 2, 38), will have to be individually activated and coupled to the enzyme systems to determine this parameter (Ref. 24).

Higher binding capacity is also attained by employing smaller support particles which provide better access of matrix for enzymes and substrates (Ref. 20). The gel beads used in this study are between 60 and 140 μm in diameter, which is large compared to the 10 μm activated silica particles that gave over 95% retention of activity (Ref. 23). This may also lead to diffusional limitations on enzyme activity.

The most probable cause of the low level of activity of bound partial-

ly purified extract may be the binding of proteins other than the system required for the condensing reaction. This may be reflected in the decrease of activity of the bound system with increasing protein load. (fig. 3.6). If the system required is diluted by other enzymes, then the chances of large quantities of desired protein being preferentially bound are small. It has been shown that an excess of protein to be immobilised is needed to obtain high levels of binding and that high concentrations in contact with gel for short times give better coupling than low levels for long times (Ref. 26). Therefore modification of the existing procedure using a large excess of partially purified extract might improve the bound enzyme yield and hence the overall activity. Of course further purification of the extract would be the obvious solution to reduce the effect of non-enzyme protein on the maximum loading value, but then problems with stability and cost arise.

The stagnation or decrease in incorporation of $[2-^{14}\text{C}]\text{Trp}$ with time seen in the immobilised system may be due to electrostatic or hydrophobic interactions between support and molecules or ions in bulk solution, so that the environment in the latter differs from that around bound enzyme. This may lead to partitioning of substrate, products etc. between these two phases with an accompanying concentration dependence of kinetic parameters (Ref. 16). With CNBr methods, the activation procedure appears to introduce cationic groups onto matrix which may take part in non-specific adsorption reactions (Ref. 26). Though this effect seems to be less evident with Tresyl activation and the agarose purification process tends to minimise these electrostatic groups (Refs. 23, 28, 29), the large internal surface of the porous gels might still give rise to non-specific adsorption. As shown in fig. 3.14, the bound preparation displayed a small, if any, shift towards the expected alkaline pH

optimum (Ref. 2 and section 1.5.3), demonstrating that the H^+ ion concentration is not adversely affected by immobilisation. However, there may be non-specific adsorption of reactants or products onto matrix which might account for the levelling-off of the reaction with time as in fig 3.13.. Inhibition of enzymes by adsorbed end products could also occur (Ref. 26).

Immobilisation can influence the kinetic behaviour of an enzyme through diffusional or mass transfer effects as mentioned in section 1.5.3.

High matrix density and large particle size lead to external diffusion limitations where transport of substrate through bulk solution to matrix surface is hampered. This may be offset by increasing stirring rate and hence the flow around particles. Internal diffusion plays a greater role in porous matrices where substrate must also reach the enzymes bound within pores. In practice, diffusion and reaction occur in parallel, resulting in non-linear substrate concentration gradients within the particle. These can be reduced and diffusion limitation overcome so that enzyme is saturated by substrate if enzyme loading and carrier bead diameter are reduced or substrate concentration is increased to highest levels practicable (Refs. 1, 4).

Diffusional effects may be linked to steric hindrance of substrate access to enzyme as mentioned above. The pH profile of the bound partially purified extract appeared to be slightly broader than that of the free system which might indicate overloading so that only enzymes at surface are active. When pH in bulk solution is changed, these enzymes become inactive and substrate diffuses into pores to react with 'latent' enzymes that have not been exposed to changes. Thus pH shows a diffusion dependence resulting in a broader profile (Refs. 16, 20).

The stability of enzymes depends upon a number of factors. The part-

ially purified bound extract is more stable than free one (table 3.8).

This might arise from multiple linkages between enzyme and both thiol and amino groups on the support, so that tertiary structure of the enzyme is stabilised. Alternatively, stability may be another aspect of diffusional control. Thus a reduction by denaturing of enzyme activity at surface of carrier will be countered by diffusion of substrate to react with enzyme bound inside pores. In effect, the catalytic activity will appear to decrease little, if at all, with time (Ref. 16).

The effect of temperature on the activity and stability of the extract should be tested.

4.6. Alternatives to Immobilised Cell Extracts

The use of immobilised whole cells eliminates the tedious and expensive steps involved in isolating and purifying intracellular enzymes. The stability of the enzymes are enhanced by being in their natural environment. Where cofactors are necessary, as in this work, the regeneration processes inside the cell may render externally supplied cofactors superfluous. Immobilisation allows the reuse of biocatalyst and permits continuous processing, an important characteristic for reactors. Usually plant cells are bound by adsorption onto safe, mild carriers such as the seaweed derived alginate or carrageenan gels (Refs. 7, 8, 17). Cells from *C. roseus* have been immobilised to investigate Corynanthe alkaloid production, with promising results. A number of model studies have shown that immobilised whole plant cells can carry out synthesis from precursors at almost the same rate as freely suspended cells (Ref. 7). Alkaloid synthesis in *C. roseus* is dependent on the factors mentioned in section 4.4.. Advances in this area must be made before immobilised *C. roseus* cells can be catalytically useful.

5. CONCLUSION

A partially purified enzyme extract was used to improve the efficiency of the condensing reactions which synthesised alkaloids from precursors. The study was a preliminary one outlining the general characteristics of the system and its associated problems. A few general conclusions may be reached.

The most obvious is the difficulty and tediousness of isolation of precursor and product alkaloids from the plant and their identification. TLC methods are not optimal for detection of small quantities of alkaloids from reaction mixtures. HPLC methods are more sensitive, but of limited applicability, where large numbers of highly impure samples must be processed. The isolation of cell free extracts from *C. roseus* was relatively straightforward. The activity of the extracts appeared to increase with the degree of purification on the basis of radiolabelled Tryptamine incorporation into Vindoline. However the condensing reaction between Vindoline and Catheranthine, catalysed by cell free extracts gave inconclusive results, due perhaps to problems associated with TLC methods of detection.

Immobilisation of partially purified extracts was easily accomplished using Tresyl activated Sepharose supports. The yield of protein bound compared favourably with that found in the literature. However the activity of the extracts is very low compared to that of equal amounts of free enzyme. This is probably due to the presence in the bound preparation of high levels of proteins that do not participate in the desired condensing reaction. Other conformational, environmental and diffusional effects might also contribute to low activity levels.

The positive aspect of the immobilised system appears to be its stabi-

lity. Thus its activity was retained, while that of free enzyme was lost by 30% over the same period of time. The long term stability of the system might outweigh its other drawbacks for future applications in alkaloid biosynthesis.

5.1. Suggestions for Further Work

The immobilised system may have potential only if many modifications are made. Foremost among these is the adoption of an improved method of product detection and analysis. That uncertainty about TLC analysis impedes the full characterisation and further development of the system cannot be overemphasised. The most promising method alternative seems to be the radioimmunoassay, which apart from being extremely sensitive and specific, allows large numbers of samples to be processed in fairly short times (Ref. 43). Only then can the various kinetic parameters for both free and bound enzyme preparations be critically compared.

The isolation of alkaloids from the plant may be carried out by precipitation from aqueous extracts and conversion to salts by ion exchange, instead of tedious liquid chromatography methods that give rise to artifacts (Ref. 18).

The activity of the bound enzyme may be increased by the use of a suitable inhibitor to block the active site during the coupling reaction so as to rule out the possibility of support reacting with active site. From the literature, glucosidase or peroxidase inhibitors are the most likely candidates (Refs. 24, 39).

The conformational effects of immobilisation on the protein should also be investigated. Spectroscopic and fluorescence methods are widely used in determining physical characteristics of bound enzymes. Measurements of fluorescence lifetimes and polarisation provides information on environmental constraint etc (Ref. 29).

The enzyme system could be further purified before immobilisation to reduce the effect of undesired protein on the optimal loading rate. The simplest way of assessing the quantity of protein coupled to matrix to measure the disappearance of protein from the coupling mixture as carried out in this study. However, other methods such as amino acid analysis can also be used.

The influence of the support on the immobilisation process could also be investigated. This means varying the density of the gel, the number of active groups on activation, the size of the particle etc. Diffusion effects may be examined by testing the influence of mixing or stirring rates on the reaction. The stability of extracts with temperature is also open to examination.

Removal of products as in a continuously operated reactor might yield higher activity levels for the immobilised system. A packed column may also give better results than a stirred type reactor as the latter requires a higher enzyme loading than the former (Ref. 20).

Alternatives to the use of whole plants lie in suspension culture extracts as a source of condensing enzymes. Immobilised whole cells may also play a bigger role in the future of alkaloid synthesis from precursors (Ref. 7).

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