A STUDY OF THE FACTORS INFLUENCING THE STABILITY AND OPERATION OF CELLULAR BIOCATALYSTS IN THE PRESENCE OF ORGANIC SOLVENTS.

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by

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ABSTRACT

The microbial conversion of compounds that have a low solubility in aqueous systems is typified by the fungal hydroxylation of steroids. In systems of this type the substrate of the reaction is of very low water solubility causing mass transfer limitations. Incorporation of a second organic liquid phase, in which the substrate has a far greater solubility, may be used to overcome such problems.

Using the 11α-hydroxylation of the steroid progesterone by the fungus Rhizopus nigricans as a model system, a packed-bed design of bioreactor is assessed with respect to its suitability for use in conjunction with an organic-aqueous two liquid-phase system. The fungal biocatalyst was not immobilised but packed as whole cells, either filamentous or pelletted, into the column reactor. It was considered that the packed-bed might act as a static mixing device so maintaining an emulsion on the bed. Results show the unsuitability of operation of the reactor with an emulsion or an alternative single organic phase. However, use of a methanol/aqueous cosolvent is shown to produce a comparatively stable bed from which 11α-hydroxylase activity data was obtained.

A detailed study is presented of the causes for the loss of 11α-hydroxylase activity by cells of R.nigricans in systems containing an organic liquid phase. It is shown that a strong correlation exists between the loss of 11α-hydroxylase activity and the concentration of organic solvent that partitions into the cell membranes. The concentration of organic solvent present in the membranes may be

calculated from a modification of a Collander type equation. For R.nigricans in the particular aqueous buffer system employed this becomes

$$[solvent_{membrane}] = 0.19 \times P_{octanol}^{0.84} \times [solvent_{aqueous}]$$

where $[solvent_{membrane}]$ is the concentration of organic solvent partitioning into the membranes, $[solvent_{aqueous}]$ is the concentration of organic solvent dissolved in the aqueous phase and $P_{octanol}$ is the partition coefficient for the organic solvent in a standard octanol/aqueous system.

Total loss of activity is shown to occur at a single critical membrane solvent concentration irrespective of the organic solvent type. Organic solvents unable to achieve this critical membrane concentration are shown to allow the retention of hydroxylase activity at saturating aqueous phase concentrations. However, with the development of a discrete second organic liquid phase loss of activity occurs through so termed 'phase' effects.

Design parameters are given, based on the above criteria, that will aid the rational selection of organic solvents for future two liquid-phase reactor systems.

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CHAPTER 1

1 INTRODUCTION

Organic molecules such as steroids, fats and hydrocarbons are generally hydrophobic in nature and consequently have low solubilities in water or aqueous solutions. Their solubility in organic solvents however, tends to be far higher. As an example the steroid, progesterone, has a solubility of 11µg/ml (30^0C) in water (Madan and Cadwallader 1973) compared to 630mg/ml (28^0C) in dichloromethane (Ceen *et al* 1987a).

Biotransformation of such organic molecules rather than chemical transformation allows stereo and regio-specific reactions to be exploited. Chemical conversions can employ organic solvents in order to obtain high reactant concentrations. However, for a long time it was thought that the presence of an organic solvent environment would be incompatible for use with biological catalysts.

Much work has now been performed with cosolvent systems in an attempt to increase reactant concentrations (Butler 1979). Here the organic and aqueous phases are totally miscible, giving a homogeneous reaction system. Although at low concentrations of organic solvent the stability and activity of the biocatalyst may be hardly affected or even improved (Kim *et al* 1980) (Asakura *et al* 1978) (Pesheck and Lovrien 1977) at higher concentrations, denaturation, inhibition and loss of specificity occur (Sonomoto *et al* 1982, 1983) (Singer 1962).

Immobilisation has been employed in an attempt to stabilise the biocatalyst from the effects of the solvent. Omata *et al* (1981) have managed to increase the half-life of the menthyl succinate hydrolysis reaction of Rhodotorula minuta cells in the presence of water saturated n-heptane from 2 days to an estimated 63 days by immobilisation. Fukui and Tanaka (1985) found that not only could increased stability be achieved but that by using immobilisation matrixes of varying degrees of hydrophobicity the activity of the biocatalyst may also be improved.

Alternative methods of obtaining high reactant concentrations have also been investigated. Micronisation of progesterone particles to create

'smooths' (Weaver 1962) allowed higher reactor loadings, and proved to be less damaging to the cells of Rhizopus nigricans as compared to the 'raw' progesterone. Merkle (1980) reported that stable supersaturated aqueous hydrocortisone solutions of up to 3g/L can be prepared by using hydrocortisone-poly(vinylpyrrolidone) co-precipitates. Kloosterman and Lilly (1984) have shown how this method may be applied to the Δ-1 dehydrogenation of hydrocortisone by free and immobilised Arthrobacter simplex. Substrate inhibition, however, was found to occur beyond 0.3g/L of hydrocortisone. Recently a method of co-immobilisation of substrate and biocatalyst was proposed (Kaul *et al* 1986) again for the Δ-1 dehydrogenation of hydrocortisone by A.simplex. Immobilisation in agarose proved to be superior to the normal free cell/substrate system because of reduced diffusion distances and allowed easy re-use of the biocatalyst.

1.0.1 Two Liquid-Phase Reaction Systems

A two liquid-phase system consists of an aqueous phase containing the biocatalyst and an immiscible organic phase (Carrea 1984) (Lilly 1982). The organic phase may itself be the reactant or may have the reactant dissolved in it. Two liquid-phase systems have many advantages over the reaction systems mentioned so far (Lilly and Woodley 1985). These are summarised below for an ideal system, although in many real cases compromises between various factors have to be made.

Perhaps the most fundamental attribute of a two liquid-phase reaction system is its capacity to dissolve large amounts of both substrate and product of the reaction. This can lead to substantially reduced reactor volumes as compared with the purely aqueous reaction where the reactant solubilities are extremely low. Using organic solvents with high partition coefficients for substrate and/or reactant, biocatalyst inhibition can be reduced owing to the lower concentration of the inhibiting substance in the aqueous phase.

The separation of biocatalyst from the product, even when the former is not immobilised, may be far easier since the biocatalyst remains in the aqueous phase while the product is present in the immiscible organic phase.

Another major benefit of the two liquid-phase system is expected when compared to the cosolvent system. In the latter, high concentrations of organic solvents in the aqueous phase lead to poor biocatalytic activities and stabilities. With the two liquid-phase system high retention of both activity and stability may be achieved owing to the immiscibility of the organic phase with the aqueous phase containing the biocatalyst.

The high capacity that organic solvents have for hydrophobic compounds such as steroids, fats and hydrocarbons has already been mentioned. Organic solvents however, also have large solubilising capacities for certain other inorganic molecules. In this respect, perhaps the most interesting is that of oxygen which may be required in high concentrations by certain oxidative and hydroxylating reactions. The solubility of oxygen as a mole fraction in water is 0.231×10^{-4} as compared to 19.3×10^{-4} for hexane, both measured at $25^{0}C$ and one atmosphere pressure of gas (Clever and Battino 1976).

Finally, but not of least importance, certain thermodynamically unfavourable reactions such as interesterification and peptide bond formation can be achieved if the amount of water is restricted to a large degree. The low availability of water leads to a reduction of hydrolysis and to a reversal of the normal reaction direction (Yokozeki *et al* 1982) (Klibanov *et al* 1977).

Certain terminology which appears in the literature associated with two liquid-phase systems requires explanation. The phase ratio of a reaction mixture has been defined as the ratio of organic phase volume to aqueous phase plus biocatalyst volume. This is a rather difficult ratio to deal with and when employed graphically gives emphasis to high organic volumes (figure 1.0.1a). Ceen (1986) defined a new term, the relative oil/organic volume (ROV), as being the ratio of the organic phase volume to the total volume in the reactor (liquid plus biocatalyst). The ROV is a far simpler ratio to work with since multiplying the value by 100 gives the organic phase volume as a percentage of the total volume. Its linear nature also lends equal weight to each region of organic volume when plotted graphically (figure 1.0.1b). The ROV or phase ratio will, along with other factors, determine whether a discrete aqueous phase exists in the case of

Figure 1.0.1a Graphical representation of the relationship between organic phase volume and the 'phase ratio' of an organic/aqueous two liquid-phase system. Total volume of the two phases is equal to 100ml. The non-linear nature of this parameter places a disproportionate emphasis on higher organic volumes. Organic/aqueous volume ratios 50:50 or less are confined to the region of phase ratio less than 1.

$$\text{Phase Ratio} = \frac{\text{Volume of organic phase}}{\text{Volume of aqueous phase + biocatalyst}}$$

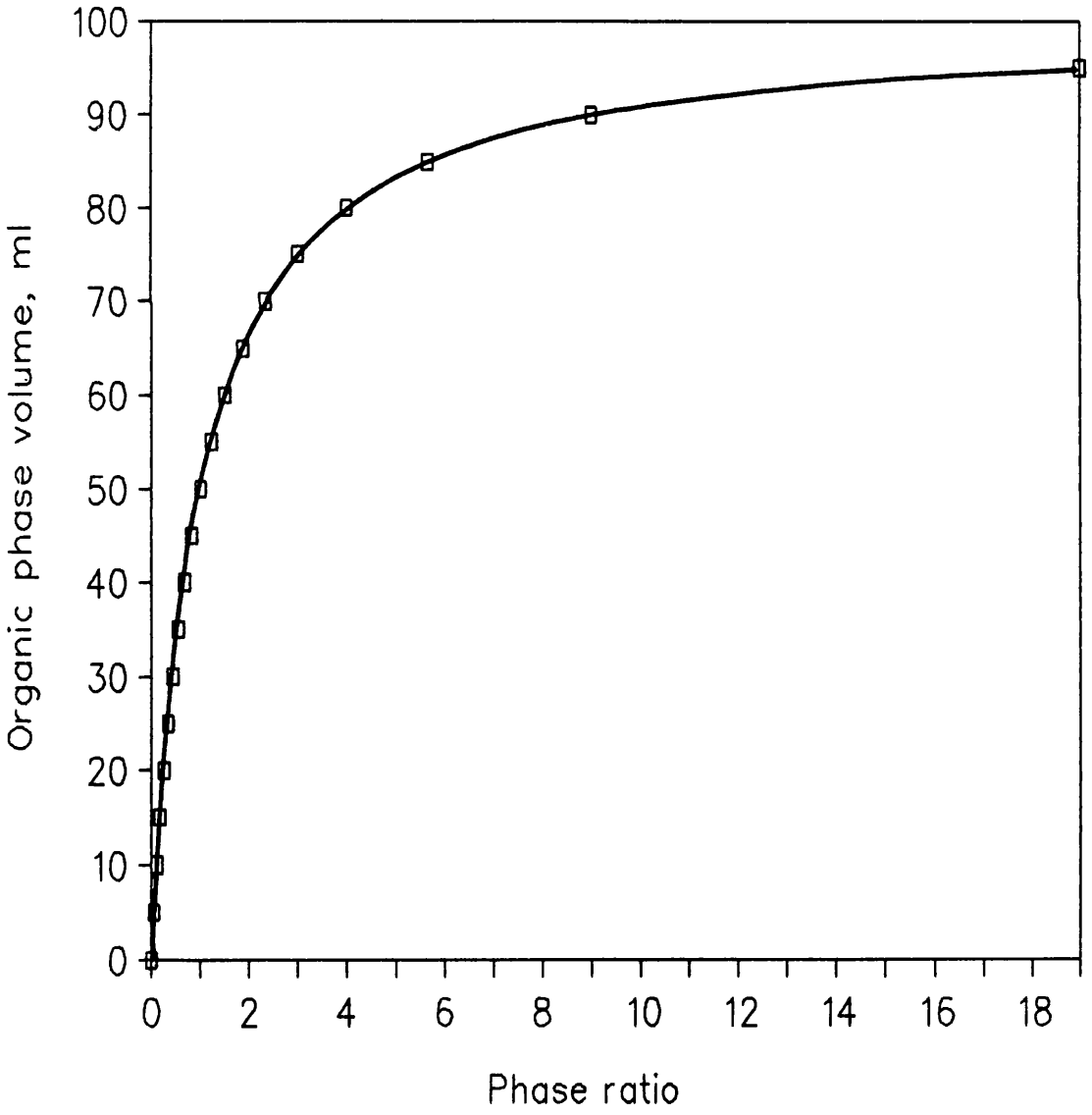
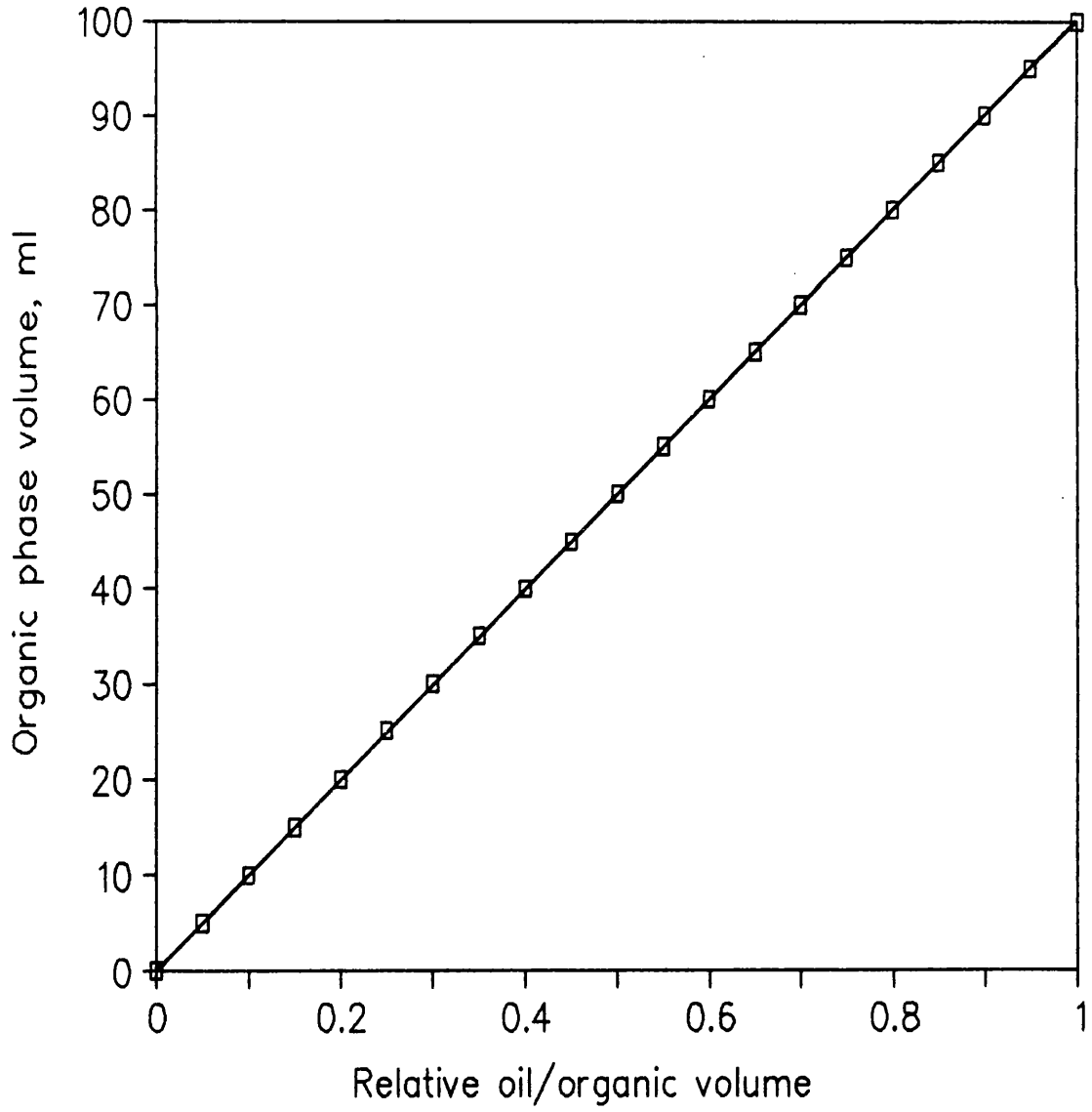


Figure 1.0.1b Graphical representation of the relationship between oil/organic phase volume and the 'Relative oil/organic volume' (ROV) of an organic/aqueous two liquid-phase system. Total volume of the two phases is equal to 100ml. The linear nature of this parameter gives an equal emphasis to each region of the graph.

$$\text{Relative organic volume} = \frac{\text{Volume of organic phase}}{\text{Total liquid volume in the reactor}}$$



insoluble biocatalysts and also which of the phases is continuous/discontinuous (a discontinuous phase will be present as droplets dispersed in the continuous phase). If a two liquid-phase system forms a semi-stable dispersion of one phase in the other then the system may be called an emulsion. Emulsions are formed when the surface tension between the phases is low. This can be enhanced by extraneous material or one of the two liquid phases exhibiting surfactant properties due to the amphipathic nature of the molecules.

1.1 Types of Two Liquid-Phase Reaction System

A two liquid-phase reaction system may be classified according to a number of criteria. Lilly (1982, 1983) describes four criteria which are given, along with others, in the following list.

1. The type of biocatalyst employed. This may be whole cells, sub-cellular components (eg. organelles) or purified enzymes.
2. State of the biocatalyst. Either free or immobilised.
3. Nature of the reactant. Either present as the organic phase or dissolved in the organic phase.
4. The liquid phase ratio. This will influence whether the aqueous or organic phase is the continuous phase.
5. The presence or absence of a discrete aqueous phase.
6. The site of reaction. In the aqueous phase or at the aqueous-organic interface.
7. The type of reactor used. This may be a stirred tank, fluidised bed, bubble column, packed bed or membrane reactor.

Most of the above combinations of criteria are in theory obtainable, and many of them have been described in the literature. A brief review of some of the two liquid-phase systems that have been employed will be presented in the following sections.

1.1.1 Examples of Two Liquid-Phase Reaction Systems

The early seventies saw the first major interest in biocatalytic two liquid-phase reaction systems. Since that time workers have described a variety of configurations including the use of free/immobilised, enzymes/cells, reversed micelles and poly(ethylene) glycol modified enzymes. These forms of biocatalyst have been employed in a range of reactors; batch stirred tank, fluidised bed, bubble column, fixed bed and membrane reactors. Within the past nine years numerous reviews have appeared describing the types of two liquid-phase system that have been used (Halling 1987) (Fukui and Tanaka 1985) (Carrea 1984) (Antonini *et al* 1981) and defining criteria by which they may be categorised (Lilly and Woodley 1985) (Lilly 1983, 1982). The following sections give a brief account of some of the work that has appeared concerning two liquid-phase biocatalysis.

1.1.1.1 Free Enzymes as Biocatalysts

Perhaps the most extensively studied two liquid-phase reaction system to date is that of free enzymes in shake flask or stirred tank reactors.

The oxidation of oestradiol by lacasse A from the fungus Polyporus versicolor was described by Lugaro *et al* (1973). Equal volumes of the various solvents and aqueous buffer were used as the reaction medium. They found the best solvents to be ethyl acetate, diethyl ether, butyl acetate and methyl ethyl ketone, while those of greater polarity led to greater enzyme inactivation. The enzyme showed stability and retention of activity for several days in the presence of most of the solvents investigated.

The dehydrogenation of testosterone by β-hydroxysteroid dehydrogenase (β-HSDH) from Pseudomonas testosteroni was shown to work with butyl or ethyl acetate as the second liquid-phase (Cremonesi *et al* 1973). The rate of reaction increased with agitation up to 100 oscillations per minute but further agitation caused denaturation of the enzyme with resultant loss of activity. The activity of the enzyme and the kinetic parameters of the reaction were found to be similar to those of the

purely aqueous system. Other enzyme catalysed steroid reactions were also carried out in organic/aqueous two-phase systems, examples being the use of δ^{4-5} steroid isomerase, 3α20β-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase. In all cases similar results were obtained to those described for β-HSDH showing the general applicability of the two liquid-phase system to enzymatic steroid conversions.

In an almost identical system to that described above, β-HSDH was shown to have significant activity even after 72 hours (Cremonesi *et al* 1974). The suitability of such a system for preparative purposes was achieved by employing a second enzyme, lactate dehydrogenase, in the two liquid-phase system (Carrea *et al* 1974) so that the required cofactor, NAD^+, could be regenerated. The presence of an organic phase allowed a more than 100 fold reduction in the reaction volume as compared to the solely aqueous reaction.

More recently Singh and Thomas (1985) have described the conversion of hydroquinone to *p*-benzoquinone in a water-chloroform system. The amount of purified *p*-benzoquinone increased by 29 percent as compared to the aqueous system. Formation of side-products of the reaction was reduced significantly due to the partitioning of the *p*-benzoquinone away from the aqueous phase such that further conversion could not occur.

The kinetics of lipid hydrolysis in a biphasic organic-aqueous system has been studied in detail (Mukataka *et al* 1985). The apparent Michaelis constant, K_m, was seen to change with the solvent used for solubilisation of the lipid, whereas the maximum velocity of the reaction, V_{max}, remained constant. The apparent Michaelis constant also varied with phase ratio and interfacial area and equations have been proposed to correlate these parameters.

1.1.1.2 Free Cells as Biocatalysts

The use of cells as biocatalysts in two liquid-phase systems has been prompted by the fact that many of the biotransformations of use to the biological and chemical industries involve reactions that require energy in the form of cofactors. In order that such reactions can

continue over meaningful periods of time, cofactor regeneration must be employed since the cofactors themselves are expensive and would, if required in large quantities, make the processes commercially unviable. Cells are generally able to achieve this regeneration internally, so obviating the need to incorporate secondary enzymatic processes into a reaction system.

Nocardia rhodocrous has been used extensively in two liquid-phase systems due to its marked degree of resistance to organic solvent attack. This has allowed the various engineering parameters of two liquid-phase systems to be explored. However, the fact that the organism is highly resistant to organic solvents means that it has limited use as a model system from which the compatibility of solvent types with different microorganisms may be assessed.

The production of cholest-4-ene-3-one from cholesterol by free cells of N.rhodocrous was investigated in the presence of several organic solvents (Buckland *et al* 1975). Carbon tetrachloride was selected for further study on the basis of non-flammability though toluene and hexadecane gave equally good conversions. Experiments used a thawed cell paste in batch stirred tank reactors. The reaction rate was found to be independent of stirrer speed except for the lowest speed employed. While thawed cells showed high levels of activity over a range of phase ratios, other results using freeze-dried N.rhodocrous demonstrated a minimum requirement for intracellular water in order to obtain expression of activity. Maximum activity was found to be possible only when an aqueous environment for the cells was present.

Free Nocardia corallina cells have been used for the formation of epoxides from C_2-C_{18} alk-1-enes and styrene (Furuhashi *et al* 1986). Hexadecane was employed as the second phase and its presence reduced the toxic nature of the lower hydrocarbons by partitioning them away from the aqueous phase (Foster 1963). The second liquid-phase also acted as a product reservoir removing the inhibitive effects of the epoxides.

1.1.1.3 Immobilised Biocatalysts

The immiscibility of the organic solvents employed in a two liquid-phase system should overcome the disruptive and denaturing effects on the biocatalyst as compared with a cosolvent system. Unfortunately, however, the solvents usually still have a small but finite solubility in the aqueous phase. This means that biocatalyst inactivation may occur over extended time periods. Many workers have used immobilisation of the enzyme or cell in an attempt to increase the stability of the biocatalyst, as well as improving its recovery (Soda 1983).

The synthesis of n-acetyl-*l*-tryptophan ester from the corresponding acid and alcohol has been described (Klibanov *et al* 1977). Chymotrypsin, the enzyme catalysing this reaction, was immobilised on porous glass via covalent linkage. With the organic phase exceeding 99 percent of the reaction volume, the presence of a reduced aqueous phase meant that the thermodynamically unfavourable synthesis of an ester bond was possible. Almost 100 percent conversion was obtained after overnight stirring in the presence of chloroform whereas a theoretical maximal conversion in a purely aqueous reaction is about 0.01 percent.

Yokozeki *et al* (1982) have described the use of several different types of immobilisation procedure for the enzyme lipase obtained from Rhizopus delmar. Matrixes included 1.) entrapment in various hydrophilic and hydrophobic resins, in some cases following adsorption onto celite, 2.) entrapment of the lipase in polyurethane foams and 3.) immobilisation of the enzyme onto porous silica beads by adsorption, ionic binding or covalent bonding. The lipase was used for the interesterification of olive oil with stearic acid in a hexane saturated water system, the oil and the fatty acid acting as the second liquid phase. Celite adsorbed, hydrophobic resin entrapped lipase retained greater than 90 percent of its activity after 12 days compared to just celite adsorbed lipase which lost approximately half its activity in only 5 days.

Immobilisation of cells has been shown to significantly increase the stability of the biocatalyst (Omata *et al* 1981). In this work the stereo-selective hydrolysis of *dl*-menthyl succinate by gel-entrapped Rhodotorula minuta cells was performed in an aqueous-heptane two

liquid-phase system. Three types of immobilisation matrix were used and half-lives for the activity of the biocatalyst increased from 50 hours for the free cells to a maximum of 1,520 hours when a hydrophobic polyurethane gel was employed. Optical selectivity towards the *dl*-menthyl ester was completely maintained even after 200 hours, making this reaction highly suitable for large scale production of *l*-menthol.

Duarte (1982) used Nocardia rhodocrous for the conversion of cholesterol to cholestenone. By entrapping the cells in two different types of support he was able to show that the biocatalyst could be used for up to 138 hours in the presence of 1,1,1-trichloroethane, enabling the degradation of cholesterol to go beyond cholestenone to androst-4-ene-3,17-dione.

1.1.1.4 Alternative Biocatalytic Systems

Several other techniques have received close attention in recent years. Reverse micelles have been used for stabilising biocatalysts in organic solvents (Martinek *et al* 1981). The structure of these systems is such that amphipathic surfactant molecules arrange themselves at the aqueous/organic interface with their hydrophobic 'tail' regions in the organic phase and their polar 'heads' directed towards the aqueous phase. By manipulative techniques such as agitation or sonication the two phases may be dispersed with the aqueous phase becoming sequestered as tiny droplets within a monolayer membrane structure of the surfactant.

Lipase from Candida rugosa has been shown to catalyse the hydrolysis of olive oil when solubilised in organic solvents in the presence of both surfactant (Aerosol OT) and water. Of the eight organic solvents tested, isooctane was found to be the most effective (Han and Rhee 1986). Lipase activity was shown to be dependent on the ratio of water to surfactant (AOT), and this was attributed to changes in the water structure of the hydration shell surrounding the enzyme molecules (Martinek *et al* 1981).

The incorporation of bacterial cells in to reverse micelles has been investigated (Haring *et al* 1985). Escherichia coli cells exhibited

β-galactosidase activity over extended periods in a Tween 85-isopropyl-palmitate reversed micellar system. The use of other solvents such as hexanol and cyclohexane caused rapid death of the bacteria. It was noted that immediately following incorporation, isolated bacteria were observed in the micelles but that with time these tended to aggregate.

The recovery of biocatalyst, design and scale up of reactors for reversed phase micellar systems has received little attention and is likely to prove a major obstacle to further developments. Some of the factors involved have been reviewed (Luisi and Laane 1986) but they conclude that it would be several years before it was known whether the realisation of expectations in this area are justifiable.

To complete this section it is worth mentioning the use of poly(ethylene) glycol-modified enzymes in two liquid-phase systems (Inada *et al* 1986). The modified enzymes are soluble in organic solvents such as benzene, toluene and chlorinated hydrocarbons and exhibit high enzymatic activities. Trace amounts of water have been shown to be necessary for the expression of activity (Takahashi *et al* 1984) as is the case with reversed micelles. The applicability of this method to those enzymes requiring ATP, cofactors or metal ions has not as yet been investigated.

1.1.2 Choice of Organic Solvent - General Considerations

The choice of organic solvent for use in a two liquid-phase system is dependent on several factors. Perhaps the most important are those of biocatalyst activity and stability in the presence of a given solvent and this is discussed in the following section (1.1.3). Other factors that should also be considered are given below.

As has already been discussed, there is a requirement that the organic solvent has a high solubilising capacity for at least the substrate of the reaction. The extent to which a compound is soluble will be dependent on the hydrophobic/philic nature of the molecule and of the molecules making up the solvent. Hydrogen bonding, Van der Waals forces and dipolar moments will all contribute to the standard free-energy change that occurs on transferring a non-polar molecule into a liquid

solvent. This can be expressed as

$$U_0 = G_c + G_i$$

where U_0 is the standard free-energy change, G_c is the work of forming a cavity in the solvent and G_i is the work done by attractive forces between the solvent and solute molecules.

For a given solute it can be shown that the term G_i is similar for all solvents including water (Pierroti 1963) and is therefore unlikely to contribute to the marked differences in solubility. G_c however, is related to the change of entropy on solvation, which is itself effectively a function of the number of solvent molecules surrounding the solute molecule (Lee 1985). The latter value will depend primarily on two geometrical properties of the solvent, its molecular size and the average packing density, both of which vary greatly between solvents. Conversely, for a given solvent, G_c will remain almost constant and the solubility of various solutes will depend to a great extent on the attractive forces between solvent and solute molecules, G_i.

In general hydrophobic solutes have a high solubility in relatively non-polar solvents, whereas hydrophilic molecules are more soluble in polar solvents. In many instances biocatalyst action will enhance polarity of the product. A non-polar solvent with high solubilising capacity for the reactant may therefore have low capacity for the product. This emphasises the need for caution when choosing a solvent and usually means that one of a partially polar nature may be most suitable.

The partitioning of reactant and product between the aqueous and organic phases is also an important consideration (Carrea 1984) and will depend on the organic solvent used. In an ideal situation the product should partition entirely into the organic phase easing its complete separation and recovery. With regard to the reactant several factors have to be considered. If substrate inhibition of the biocatalyst occurs, solvents with high partition coefficients for the substrate should be used to reduce the concentration of substrate in the aqueous phase. The reverse is true when employing biocatalysts with

a high Michaelis constant, K_m value, for the substrate. Compromises between all the above mentioned factors have to be made. However, other considerations should not be overlooked.

It has been shown that the type of organic solvent (Omata *et al* 1980) and the hydrophobicity of the immobilisation matrix (Omata *et al* 1979) greatly influence the partitioning behaviour of the substrate, and therefore the resulting activities of the immobilised cells. Carrea *et al* (1979) have also shown that certain solvents may tend to shrink immobilisation matrixes such as Sepharose.

In some instances the ultimate decision may be based on the safety aspects of a given two liquid-phase system. Many solvents of potential use are highly flammable and/or potentially toxic. The requirement for specialised containment facilities may then become prohibitive.

1.1.3 Choice of Organic Solvent - Biocatalyst Activity and Stability

Until recently the relationship between biocatalyst activity and stability, and the type of solvent used as the organic phase had only been described in qualitative terms. In general, experimental evidence indicated that small polar solvent molecules having a higher solubility in water caused more immediate and extensive inactivation of biocatalysts than larger apolar solvent molecules. However, a quantitative relationship between some physical parameter of the solvent and its disruptive effects on the biocatalyst was required if more rapid optimisation of the choice of solvent was to be achieved.

A comprehensive study on the microbial epoxidation of propene and 1-butene by an immobilised strain of Mycobacterium in a two liquid-phase system (Brink and Tramper 1985) showed that the retention of activity could be related to a physical parameter of the organic solvent. The Hildebrand solubility parameter, δ $(j/cm^3)^{0.5}$, is given as the square root of the cohesive energy density of a solvent (Hildebrand *et al* 1970) and can be represented as follows

$$\delta = (-E/v)^{0.5} = (-H_v-RT/v)^{0.5} = -(p(H_v-RT)/M)^{0.5}$$

where E is the molar cohesive energy, v is the molar volume, H_v is the molar heat of vaporisation, R is the gas constant, T is the absolute temperature, p is the density and M is the molecular weight of the solvent.

The solubility parameter is suited to describing intermolecular forces and gives a semi-quantitative measure of the polar nature of an organic solvent. Values for the molar heat of vaporisation, H_v, are readily available in the chemical literature so that values for the solubility parameter of most solvents can be easily calculated. This contrasts with the limited availability of data on other physical parameters like the dielectric constant, dipole moment and polarisability.

The correlation between retention of biocatalytic activity and solubility parameter was weak, but confirmed the qualitative results of other workers in that solvents of low polarity (low δ) tended to give a higher retention of activity.

Other easily determinable parameters were looked at in an attempt to obtain a better correlation. It was found that on a plot of solubility parameter (δ) against molecular weight (M), those solvents causing most disruption fell in an area of high polarity and low molecular weight ($\delta>8$ and $M<200$). The majority of solvents outside these limits proved to have far less effect on the biocatalyst with resultant high retention of activity. Use of molecular volume and the logarithm of the solvent solubility in water, in place of molecular weight, gave similar results. Brink and Tramper concluded that of 150 solvents for which values of δ and M could be obtained, many would cause strong inactivation of the immobilised cells, since they fell in the area $\delta>8$ and $M<200$.

By plotting the results of Playne *et al* (1983) who used free cells of an anaerobic bacteria in a similar set of solvents, the general applicability of this relationship was demonstrated. Perhaps most interesting of all, however, was the seeming lack of change in boundary conditions ($\delta>8$ and $M<200$). This seemed to indicate that the immobilisation of cells in calcium alginate used by Brink and Tramper gave little additional protection to the cells from the effects of the solvents.

As an extension of the work, the correlation between solubility parameter and oxygen solubility (expressed as mole fractions) in various organic solvents was investigated. A strong negative relationship was shown to exist with high oxygen solubilities occurring in solvents of low polarity, as previously found by Hildebrand *et al* (1970).

Following this innovative work, Laane *et al* (1985) realised that the Hildebrand solubility parameter was a rather poor indicator of solvent polarity, particularly for relatively apolar solvents. They suggested that a more explicit parameter is that of $LogP_{octanol}$, which is defined as the logarithm of the partition coefficient of a given compound in a standard octanol-water two-phase system. $LogP_{octanol}$ values may be determined experimentally, or calculated from hydrophobic fragmental constants according to principles set out by Rekker (1977).

By replotting the data of Brink and Tramper, Laane *et al* showed that a significant correlation existed between $LogP_{octanol}$ and immobilised cell activity retention, whereas that between the Hildebrand solubility parameter and activity retention was weak. Similar observations were made when the results of Playne *et al* (1983) were replotted. The sigmoidal shape of the $LogP_{octanol}$-activity retention plots indicated that the biocatalyst was strongly inactivated by solvents of high polarity ($LogP_{octanol}<2$) and that inactivation was minimal with solvents of low polarity ($LogP_{octanol}>4$).

Laane *et al* (1985) described three sets of solvents on the basis of $LogP_{octanol}$ values.

1. Solvents having $LogP_{octanol} < 2$. These were deemed least suitable in biocatalytic systems.
2. Solvents having $LogP_{octanol}$ between 2 and 4. These were considered to be of potential use in some instances but inactivation could be expected.
3. Solvents having $LogP_{octanol} > 4$. These were thought to be applicable to most biocatalytic systems, with high retention of activity resulting from their use.

The use of $LogP_{octanol}$ was justified by Laane et *al* on the basis that it gave a better reflection of the polarity of the more apolar solvents. Other inferences from the results using $LogP_{octanol}$ were not discussed but may actually give us an understanding of the mechanisms by which the organic solvents cause loss of biocatalytic activity. These are dealt with in section 1.4.5.

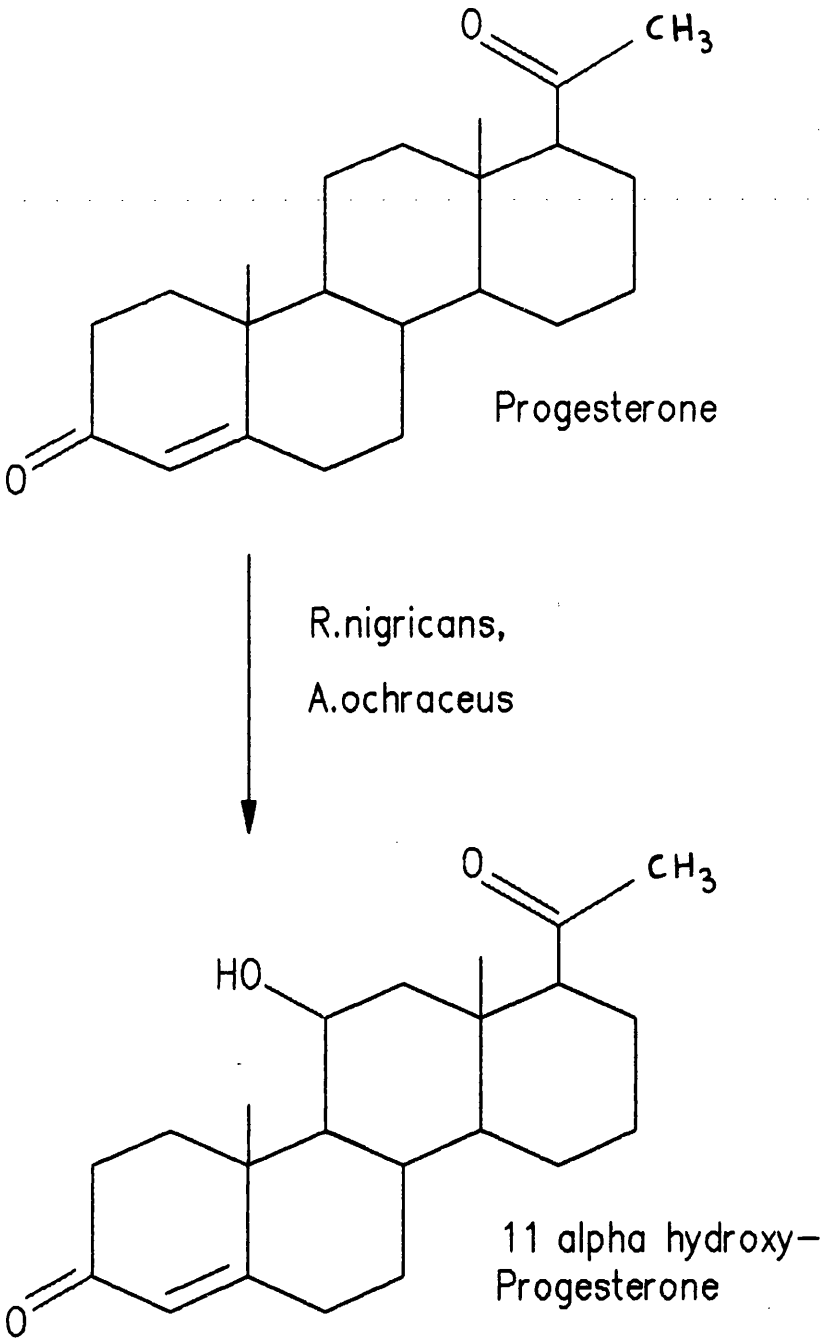
1.2 The 11α-Hydroxylase System

The conversion of progesterone to 11α-hydroxyprogesterone is one of the major steps involved in the production of corticosteroid drugs (Fieser and Fieser 1959). The similar reactivity of the unactivated carbon atoms of the steroid nucleus, figure 1.2, makes stereo and regio-specific addition of functional groups difficult and expensive by chemical methods. Murray and Peterson (1952) reported that this conversion could be achieved using the fungus Rhizopus nigricans. Since then it has been shown that other microbial systems including Rhizopus, Aspergillus, Cunninghamella and Curvularia species also perform this important reaction.

Considerable information is now available regarding mammalian and bacterial hydroxylating systems. However, knowledge concerning the steroid-hydroxylating enzymes of the various filamentous fungi is still extremely limited and care should be taken in making inferences that relate one system to another.

Fungal hydroxylase enzymes are generally inducible and occur at very low constitutive levels within the cell. Using cycloheximide the inducible nature of the systems in Aspergillus (Ghosh and Samanta 1981) and Rhizopus (Breskavar and Hudnik-Plevnik 1978) has been conclusively demonstrated. Cycloheximide acts as an inhibitor of eukaryotic protein synthesis by ribosomes free in the cytosol and attached to the endoplasmic reticulum. Hydroxylase activity was only observed in cultures induced with progesterone prior to the addition of the inhibitor, no activity being present if the cycloheximide was added before induction. Chloramphenicol which is an inhibitor of mitochondrial protein synthesis was used in similar experiments. No significant reduction in 11α-hydroxylase activity was observed with

Figure 1.2 Conversion of progesterone to 11α-hydroxyprogesterone can be achieved using many different microorganisms including R.nigricans and A.ochraceus. The stereo and regio-specific nature of the reactions performed make these organisms of particular industrial importance in competing with chemical conversion processes.



chloramphenicol added either pre or post induction, indicating that the enzyme system was not mitochondrial in nature.

The conditions for maximal induction of hydroxylase activity have also been investigated. Aspergillus ochraceus has been shown to give good conversion after a three hour induction period (Shibahara *et al* 1970). Ten hours was chosen as an optimum since extension of the induction period to sixty hours meant that induction of the unwanted 6β-hydroxylase also occurred. Conversion in this instance led to complete formation of the 6β11α-dihydroxylated product.

Induction of Rhizopus nigricans has been more systematically studied (Hanisch 1978). It was suggested that induction could be carried out at any stage of growth with 0.3g/L progesterone giving maximal hydroxylase enzyme. The dissolved oxygen tension (DOT) was shown to have a considerable influence on both induction and expressed activity. Dissolved oxygen tensions of 10-15 percent and 30 percent were given as optimum levels respectively. Since the fungal cells were filamentous, high agitation rates in the fermenter were required to obtain adequate mixing and DOT. An impeller tip speed of 3.1m/s promoted good hydroxylation but agitations in excess of this led to mycelial damage and so a drop in activity. Recent work (Breskavar and Hudnik-Plevnik 1981) has shown that the synthesis of the NADPH cytochrome P450 reductase component of the enzyme lags that of the cytochrome P450 and acts as the limiting rate to inducibility of the hydroxylase system.

1.2.1 Intracellular Location of the Hydroxylase System

Work on cell free extracts of Aspergillus ochraceus has indicated that the 11α-hydroxylase activity is solely associated with the microsomal membrane fraction sedimenting at 105000g (Madyastha *et al* 1984). This also happens to be the fraction containing the highest NADPH-cytochrome-C reductase activity. Addition of cytosolic supernatant to the microsomes did not enhance the hydroxylase activity while non-induced cells were found to contain no hydroxylase activity in either microsomal or cytosolic fraction.

In similar earlier experiments using A.ochraceus (Ghosh and Samanta 1981) and Rhizopus nigricans (Breskavar and Hudnik-Plevnik 1977), activity was detected in the post mitochondrial supernatant. However, when this was further separated into cytosolic and microsomal fractions no activity was found. Only by recombining the supernatant and microsomal pellet could hydroxylase activity be recovered. It was concluded that in these cases the enzyme system was distributed between the membrane and the cytosol.

If one or more components of the hydroxylase system are loosely bound to the membrane fraction then variations in disruption techniques, homogenisation media and fractionation procedures may lead to release of the components into the cytosolic fraction. This may well account for the observed differences in the localisation of the hydroxylase system.

Involvement of cytochrome P450 in the reaction has been demonstrated in many studies. Characteristics of the enzyme were reported early on (Singh 1962) and have been used to show its presence in the microsomal fraction. A reduced carbon monoxide difference spectra, stimulation by cyanide and inhibition by metapyrone have all been used to indicate the involvement of cytochrome P450 (Madyastha *et al* 1984) (Jayanthi *et al* 1982) (Ghosh and Samanta 1981).

1.2.2 Mechanism of Biocatalytic Activity

The 11α-hydroxylase system is a cytochrome P450 mono-oxygenase which functions with the stoicheiometry

$$R\text{-}H + NADPH + O_2 \longrightarrow R\text{-}OH + NADP^+ + {}^-OH$$

Incorporation of one atom of molecular oxygen into an organic substrate occurs with the other being reduced to water (Holland 1982). Despite much of the mechanistic work being performed on enzymes from bacterial and mammalian sources, available evidence (Sato 1978) suggests that all cytochrome P450 dependent mono-oxygenases function in a similar manner.

A general scheme for the reaction is shown, figure 1.2.2 (Holland 1982) (White and Coon 1980) (Estabrook 1978). Before oxygen can bind to the iron centre of cytochrome P450 the substrate must first bind to the apoprotein in close proximity to the cofactor. The two reducing equivalents are provided by NADPH, NADH being far less efficient (Madyastha *et al* 1984). The electrons are transferred to the cofactor via a flavin nucleotide and cytochrome b5, firstly reducing the ferric iron molecule to a ferrous state and then activating the bound oxygen.

It is now generally recognised that four proteins make up the 11α-hydroxylase system.

1. Cytochrome P450
2. NADPH-cytochrome P450 reductase
3. Cytochrome b5
4. Cytochrome b5 reductase

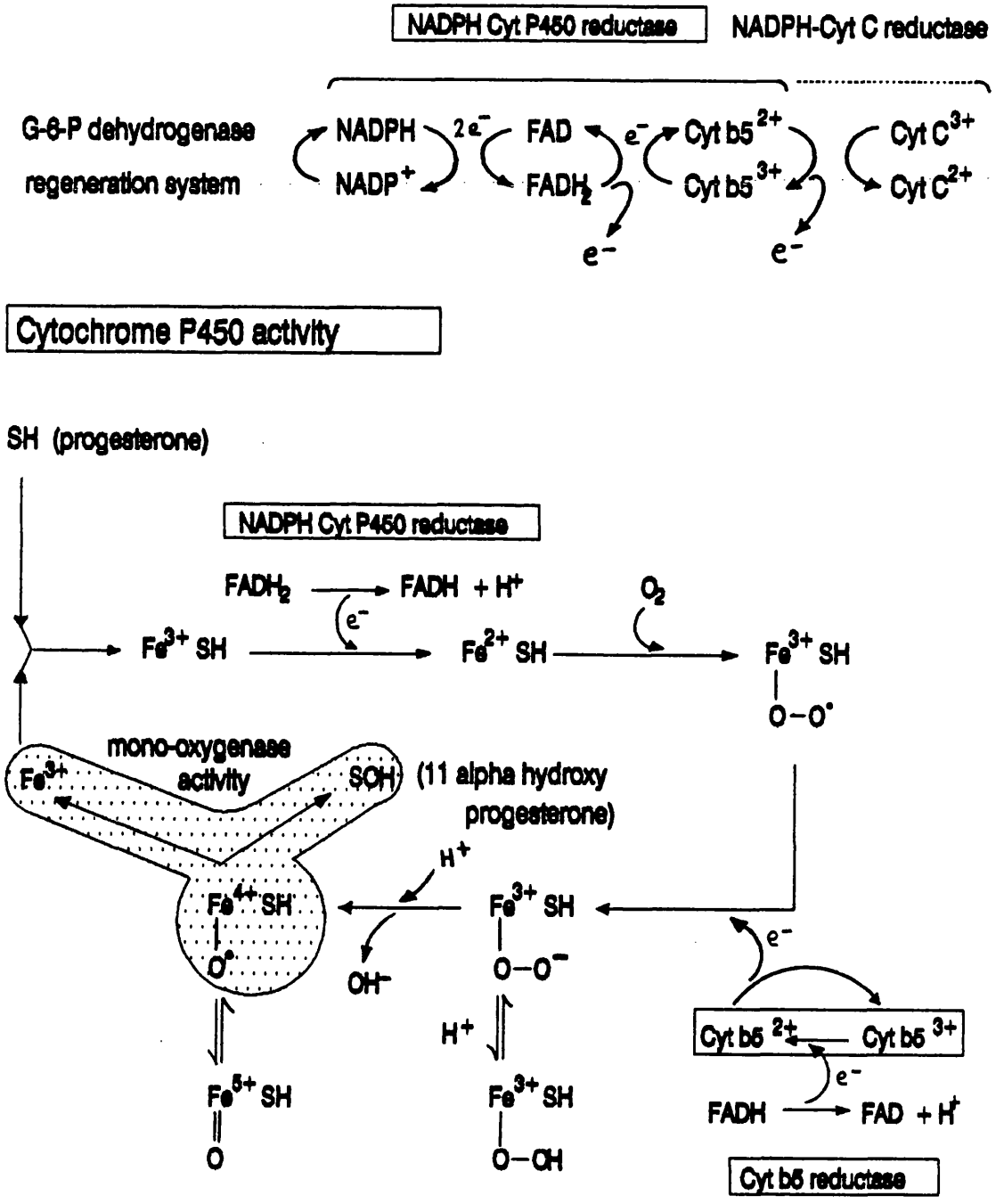
All have been shown to be located on the cytoplasmic side of the endoplasmic reticulum but the dynamic relationship and interaction between each has not been fully defined. One proposal is that all four exist as a single structured complex in the membrane but little evidence exists to confirm this. A second theory for which greater experimental evidence exists (section 1.2.3) is one in which the separate proteins are freely mobile in the fluid membrane. Interactions occur by random collision forming dimer, trimer or even tetramer complexes of short life time during which transferal of electrons occur.

1.2.3 Orientation and Interaction of Proteins

The structure of three of the four proteins making up the hydroxylase system have been characterised, although not directly from the fungal source.

Cytochrome b5 and cytochrome b5 reductase are both monotopic proteins in that they are only partially buried within the lipid bilayer but do not completely penetrate through it (De Pierre and Dallner 1975). Hydrophobic domains on each serve to anchor the proteins in the

Figure 1.2.2 A general scheme of the reaction mechanism for the hydroxylation of progesterone. The four proteins; NADPH-Cyt P450 reductase, Cytochrome b5, NADPH-Cyt b5 reductase and the Cytochrome P450 mono-oxygenase that constitute the hydroxylase system are shown boxed. An expanded reaction pathway for NADPH-Cyt P450 reductase is given at the top of the page.



membrane and so effectively increase their local concentration by restricting their diffusional mobility to the plane of the membrane. This also leads to an optimisation of their relative orientation (Enoch *et al* 1977). Larger hydrophilic domains protrude into the cytosol and contain the catalytic sites as shown by loss of activity following mild proteolytic digestion (Spatz and Strittmatter 1973, 1971).

Cytochrome P450 unlike the above two proteins has hydrophobic domains that traverse the lipid bilayer (De Pierre and Ernster 1980). It has been shown that the substrate binding site of membrane bound cytochrome P450 directly faces the vertical plane of the lipid bilayer (Taniguchi *et al* 1984). This is important since most substrates are lipophilic and will partition into the membranes giving a higher local concentration than in the surrounding cytosol.

The spatial relationship between the proteins has been the subject of much experimental work. Strittmatter and Rogers (1975) showed that cytochrome b5 and its reductase interact as a result of collisions between the proteins as they diffuse within the lipid bilayer. A similar model (Trudell and Bösterling 1983) was proposed for the interaction of cytochrome P450 with NADPH-cytochrome P450 reductase. A comparatively long-lived complex was required so that orientation of the catalytic sites by rotational diffusion would allow electron transfer from the flavin of the reductase to the porphyrin of cytochrome P450. Formation of an ionic bond between the two seems likely and may also play a part in the interaction between cytochrome b5 and cytochrome P450.

Diffusion in the two-dimensional surface of the membrane is a much more rapid process than diffusion in the three dimensions of the cytosol. Collision-coupling to form transient dimers and even ternary complexes is therefore a particularly attractive mechanism for membrane protein interactions as it also offers a unique opportunity for flexibility in response since the concentration of only one component need be altered to regulate overall activity.

1.3 Solvent Effects on Microbial Biocatalysts

The complex organisation present in cells means that it is extremely difficult to determine the mechanism(s) by which organic solvents act to cause loss of biocatalytic activity. In many cases it is likely that several separate sites of action may be affected. Each may have its own 'rate constant' for inactivation and extent to which it will contribute to the overall loss in activity. In some instances loss of activity may be a reversible phenomena but in most cases where an organic solvent with small polar molecules is used, inactivation is seemingly rapid and non-reversible.

The extent to which organic solvents cause loss of activity has been discussed in section 1.1.3. The following sections describe the possible sites at which organic solvents may act, although the contribution of each to the overall loss of activity will depend on many factors including

1. Type of microorganism.
2. Membrane composition.
3. Mechanism of reaction.
4. Enzyme location (cytosolic or membrane bound).
5. Cofactor requirement.
6. Presence of an antagonist.

1.3.1 Permeabilisation of Membranes

Various agents including organic solvents can cause permeabilisation of cells by attacking the outer membrane of the organism. The resulting effect is to make the cell more permeable and so increase the transfer rate of species into and out of the cell. Many observations on permeabilisation by organic solvents have been reported which indicate the effects that occur to whole cell structure and metabolism (Felix 1982).

Toluene has been used extensively as a permeabilising agent. The effect on cells of *Escherichia coli* was found to be dependent on concentration and the presence or absence of magnesium ions (De smet et al 1978). At

low concentrations, 1 percent toluene, release of phospholipids, protein and lipopolysaccharide was observed. Increasing the concentration to 10 percent caused complete disorganisation of the cytoplasmic membrane. The presence of magnesium ions antagonised the disruptive effects of toluene though release of phospholipid was still observed. The outer cell wall consisting of lipopolysaccharide, protein and some phospholipid was much less susceptible to toluene treatment.

Permeabilisation of the cells led to release of certain specific intracellular proteins. Malate dehydrogenase was particularly susceptible to release, unlike similar cytoplasmic proteins such as glucose-6-phosphate dehydrogenase and glutamic dehydrogenase. A possible explanation put forward to account for the differences was that the cell wall and permeabilised plasma membrane acted as a molecular sieve letting through only those proteins with a small enough molecular weight. The permeability of pyridine nucleotides was shown to be mainly dependent on the integrity of the membrane and the presence of EDTA which acts to destabilise the latter. The effect of EDTA may be due to its ability to chelate divalent cations such as magnesium which are known to stabilise the membrane.

Fungal cells of Cephalosporium acremonium have been permeabilised using ether with retention of activity of certain enzymes (Felix *et al* 1980). Both hexokinase and glucose-6-phosphate dehydrogenase were fully active after 90 minutes. Entry of the substrates, $NADP^+$, glucose, ATP and glucose-6-phosphate after permeabilisation was found to be not rate limiting as compared to cell free extracts. However, the enzyme RNA polymerase is far less stable to permeabilisation procedures, an increase in the amount of RNA only occurring during the first 5 minutes. This does however, represent the sum of both RNA synthesis and degradation reactions.

Immobilised plant cells of Catharanthus roseus have similarly been permeabilised with ether or dimethyl sulphoxide (DMSO) allowing entry of substrates which are not permeable to untreated cells (Felix *et al* 1981). Five enzymes of the primary metabolic pathways and one of secondary metabolism were shown to be active following permeabilisation. Retention of viability of the cells could be achieved using up to 10 percent DMSO for 30 minutes exposure (Brodelius and

Nilsson 1983). Other plant species required higher concentrations to achieve full permeabilisation an observation accounted for by either a difference in the morphology or composition of the plasma membrane/ cell wall.

Permeabilisation by organic solvents causes disorganisation of the plasma membrane. Biocatalyst inactivation as a direct result will only usually occur if the activity of plasma membrane bound enzymes are required for the particular biocatalytic reaction. In most cases however, loss of activity is due to some secondary process associated with permeabilisation. Leakage of cofactors necessary for energy requiring reactions is perhaps the major problem although release of enzymes from the cell has been shown to occur in harsher conditions. It should no be forgotten however, that perturbations in the cytosolic composition with respect to pH and ionic strength may have far wider influences on the general metabolic state of the cell.

1.3.2 Microbial Toxicity of Organic Solvents

The terms bacteriotoxic and fungitoxic in respect of organic solvents has mainly been applied to n-alkanes, alkenes and their derivatives (fatty acids and alcohols).

The possible use of fatty acids as anti-mycotic agents was tested using the organisms Trichophyton interdigitale and Aspergillus niger (Wyss et al 1945). The study concluded that the fungicidal and fungistatic activity of the fatty acids increased (ie. the concentration required to cause death or cessation of growth decreased) with increasing carbon chain length and was greater in the case of unsaturated alcohols. The results were difficult to interpret since with increasing chain length the solubility of the fatty acid in water decreases. The toxic effect will still therefore exist but may not be expressed due to insufficient fatty acid being dissolved in the aqueous phase. Only increasingly sensitive organisms will then be affected by the highly insoluble longer chain fatty acids as borne out by the use of T.purpureum. Whereas A.niger demonstrated maximum toxic effect at undecanoic acid, T.purpureum being far more sensitive was inhibited up to tetradecanoic acid.

The pH was also found to have a marked effect on the fungicidal properties of the fatty acids. Inhibition concentrations using A.niger increased from 0.03 to 0.15 percent for octanoic acid with a pH increase from 4.5 to 7.5. This effect of pH was greatest for short chain fatty acids, no measurable differences being observed above dodecanoic acid.

Morisaki (1984) indicated that the bacteriostatic activity of certain hydrocarbons may be related to the surface tension of the organic phase. Using Escherichia coli as the organism it was shown that dodecane, tridecane and tetradecane depressed the rate of glucose uptake but enhanced the respiratory activity as measured by oxygen uptake. More surprisingly hexane and octane showed far less effect. Correlating this to surface tension, the greatest effects were observed at between 24-26 dynes/cm corresponding to the alkanes dodecane and tridecane.

The results of earlier workers are now thought to be incorrect as to the degree of fungitoxicity of fatty acids. This has been attributed to excessive manipulative techniques including the use of other organic solvents to incorporate the fatty acids into the aqueous media. In a recent study an ultrasonicator was used to incorporate the fatty acids as a stable emulsion into the culture media (Garg *et al* 1986). Nine keratinophilic fungi were tested against nine fatty acids of which three were unsaturated. It was found that short chain fatty acids (up to $C_{12:0}$) were more toxic than long chain fatty acids with the corresponding unsaturated fatty acid exhibiting even greater toxicity. The degree of fungitoxicity of unsaturated fatty acid was also observed to increase with the degree of unsaturation.

Nieman (1954) has suggested that the toxic and inhibitory action of the organic solvents may be the result of similar effects to those occurring in permeabilisation. The generally reversible nature of metabolic and growth inhibition, however, indicates that loss of cellular components only occurs in certain extreme cases.

Calcium, magnesium, ergocalciferol and cholesterol have been used as antagonists to the action of the fatty acids (Galbraith *et al* 1971). The divalent cations may act by stabilising the cytoplasmic membrane,

as was observed in permeabilisation experiments (section 1.3.1). However, it is thought that the other antagonists, which also include serum albumin and starch, compete for adsorption to the cell wall thereby blocking further entry by the fatty acid molecules. Alternatively they may combine with the fatty acid to form a complex which is inactive as an inhibitor.

In a few cases the higher alkanes and fatty acids can stimulate growth. For fatty acids at least this has been attributed to their possible action as a biotin substitute (Skeggs 1944). Some species of yeast are known to grow well on higher alkanes as demonstrated by hydrocarbon fermentations (Fukui and Tanaka 1981). In such cases smaller hydrocarbon molecules are inevitably also present giving possible toxic side effects. From experimental investigations it was concluded that the smaller toxic hydrocarbons partitioned into the bulk hydrocarbon phase reducing their effective concentration in the aqueous phase to a non-toxic level, so allowing growth of the microorganisms.

1.3.3 Solvent Effects on Proteins

Most of the work performed relating to organic solvent effects on proteins has used enzymes whose in vivo environment is the cytosolic milieu. Little information is available as to the direct effects on membrane bound proteins though a wealth of information is present on indirect effects transmitted through the membrane itself (section 1.4.5). Since some of the components of the 11α-hydroxylase system have hydrophilic domains that protrude into the cytosol it is still worth considering the general nature of organic solvent-protein interactions.

The effects of organic solvents on protein structure are likely to be the sum of the individual effects on the various interactions that normally contribute to protein conformation in aqueous solution (Singer 1962). These interactions include hydrogen bonds between peptide linkages, hydrophobic and electrostatic forces.

Hydrogen bonds within proteins result from the interaction of a strong proton acceptor (C=O) with a weak proton acceptor (N-H) from different

peptide linkages. It is rare that organic solvents act by displacing the carboxyl group from the amine since few other functional groups are stronger proton acceptors. It is therefore the proton donating capacity of a solvent which is of importance in assessing its effects on hydrogen bonding in a protein. By comparing the proton donating ability of various solvents containing hydroxyl groups it was suggested (Pimental and McClellan 1960) that the ability to disrupt peptide hydrogen bonds would increase in the order alcohols, phenols, carboxylic acids, substituted carboxylic acids (containing other electronegative groups).

In an aqueous environment it is likely that the non-polar hydrophobic side chains of amino acids such as leucine, valine, and phenylalanine will be grouped together in a manner so as to minimise their water exposed surfaces (Kauzmann *et al* 1959). In many instances they will be orientated to the interior of the protein giving it a hydrophobic core. On transfer to a non-polar environment destabilisation of the protein may occur with a tendency for the sequestered interior to disperse and sometimes invert (Tanford 1978).

Electrostatic interactions include attractive forces over short distances between fixed charges to form ion pairs or salt linkages. Repulsive forces are able to act over longer distances and are due to the net charge present on various regions of the protein molecule. In conditions of extreme pH the repulsive forces will tend to destabilise the normal conformation. Similarly if an organic solvent is present the charges may alter depending on the pKa's of the amino acid side chains. The dielectric constant of the solvent and its proton donating ability will therefore be important.

Qualitative relationships can be used to determine the effects of dielectric, dipole moment and protonic activity on protein structure. However, the selection of a solvent strictly on the basis of these physical properties is not usually feasible and experimental determinations of enzyme activity and stability in the presence of the organic solvent are still generally required (Butler 1979).

1.3.4 Solvent Coating of Cellular Biocatalysts

When solvent is in excess of that required to saturate the aqueous phase, accumulation of solvent molecules on the organisms surface may occur as it makes contact with the organic-aqueous interface. This has been seen to occur in hydrocarbon fermentations. The surface of the yeast Candida lipolytica was found to be covered in a thin layer of hydrocarbon which penetrated the cell wall and was concentrated at the surface of the cytoplasmic membrane (Ludvik *et al* 1968). In this case the reactant is the hydrocarbon itself and little detrimental effect was observed. However, if the substrate is to be dissolved in the organic solvent then problems may arise.

Bar (1986) has described the phenomena of 'phase' toxicity and relates the effects to excesses of solvent above that required to saturate the aqueous phase, effectively the presence of a bulk organic phase. Coating of the organism was viewed as being a likely result. Cell wall disruption through extraction of outer cellular components and blockage of the diffusion of polar nutrients and cofactors from the aqueous phase to the cells would therefore be direct consequences. A point omitted, however, was that of mass transfer of reactant.

In cases where the reactant is dissolved in the organic solvent a thin film of the latter around the cells may act as a major limitation to mass transfer once the substrate initially dissolved in it had been exhausted. In such a film the mixing would be low and as a result a stagnant layer could be produced adding a further boundary in which diffusion of the reactant may be reaction rate limiting.

1.4 Membranes and Membrane Bound Enzymes

The fluid mosaic model for the structure of biological membranes was first proposed by Singer and Nicholson (1972). In this model most of the lipids are present in the form of a bilayer with proteins either bound to the charged surfaces (peripheral) or embedded to varying degrees within the membrane (integral), figure 1.4. Alternative models (Rothfield and Finkelstein 1968) (Davson and Danielli 1943) could not meet the thermodynamic criteria that the structure should have minimum

free energy. This requires that the amphipathic lipid molecules be orientated such that their polar head groups face the aqueous phase and their non-polar hydrocarbon tails are sequestered in the hydrophobic interior (Tanford 1980).

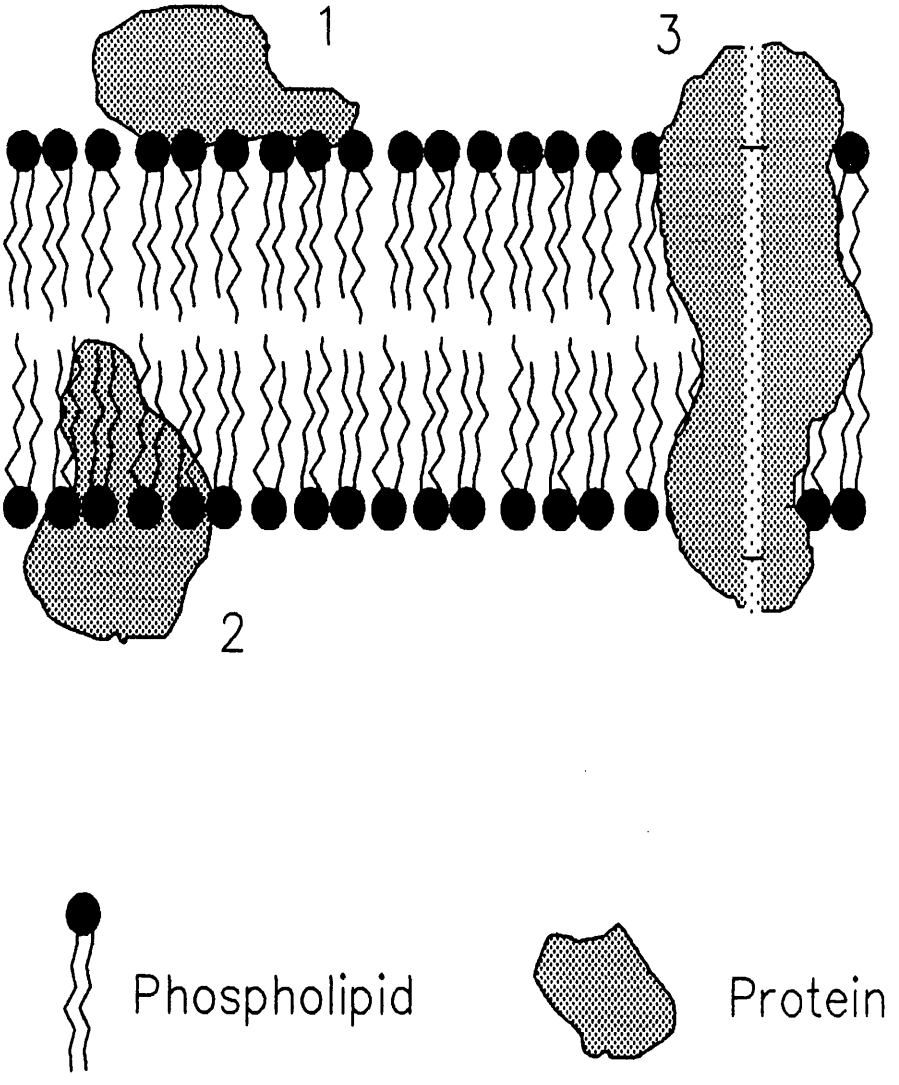
To achieve the requisite polar and non-polar domains, membrane proteins either have long sequences with a high content of hydrophobic amino acids (Nakashima and Konisberg 1974) or create the required domains by special folding of chains of 'normal' amino acid composition (Brock and Tanner 1982). In many instances considerable numbers of the carboxyl groups are amidated in order to increase hydrophobicity. In the hydrophobic interior of the membrane the protein can only make hydrogen bonds with itself and so in order to maximise the hydrogen bonding in this region there is likely to be a great deal of secondary structure (Kennedy 1978). This may include the possible existence of beta-helices in addition to alpha-helices and beta pleated sheets.

1.4.1 Membrane Fluidity in Biological Membranes

Early studies concerning membrane fluidity emphasised the extent of molecular mobility associated with the phospholipid molecules (Chapman *et al* 1967) (Byrne and Chapman 1964). This is particularly true when a certain critical transition temperature, T_c, is exceeded. On the basis of calorimetric studies Chapman *et al* (1969) suggested that the fluid character of the bilayer is different to that of a simple paraffinic melt. This contrasted with the previously held view that the interior hydrocarbon chains were in a random chaotic state (Luzzati 1968). Experiments showed that the changes in energy, δH, associated with the gel to liquid-crystalline transitions of lipid bilayers are much lower than the δH values from the melting of pure hydrocarbons, indicating that the chains are more ordered in the membrane structure.

Despite this ordering of the membrane, quantitative studies of lipid diffusion in model bilayers have demonstrated the marked fluidity that still exists (Devaux and McConnel 1972) (Trauble and Sackmann 1972). Diffusivities of the order $D=1.6\times10^{-8}$ to 1.8×10^{-8} cm^2 s^{-1} have been reported for measurements on lipid molecules above the transition

Figure 1.4 Schematic diagram of a biological membrane according to the fluid mosaic model of Singer and Nicholson (1972). Several types of protein/enzyme are represented. Type 1 is a peripheral protein only loosely associated with the membrane. Types 2 and 3 are integral membrane proteins having hydrophobic domains that are sequestered within the membrane. Cytochrome b5 and its reductase are of type 2 proteins. Type 3 is more specifically a transmembrane protein of which cytochrome P450 is an example.



temperature, dropping to $1x10^{-10}$ cm^2 s^{-1} below T_c.

Similarly with the lipid providing a fluid matrix, integral proteins would be expected to diffuse in the plane of the membrane at rates comparable but slightly lower than those of lipids, due to their greater molecular size. From the mixing of antigenic determinant proteins on the surface of human and mouse cells, lateral diffusion rates of $5x10^{-11}$ cm^2 s^{-1} were calculated (Singer and Nicholson 1972). From these measurements of diffusion rates it is possible to estimate the collision frequency between proteins (Lee 1975). Values will inevitably be dependent on the concentration of the protein in the membrane but orders of $1x10^5$ to $1x10^6$ s^{-1} have been determined for the rhodopsin protein molecule which are high enough to account for the observed reaction rates that exist between other integral membrane proteins (Raison 1980).

1.4.2 Membrane Fluidity and Enzyme Activity

To study the behaviour of membrane enzymes as a function of membrane fluidity it is necessary to be able to modulate the fluidity in a predictable manner. Several possibilities are available including alteration of the phospholipid composition (Kimelberg and Papahadjopoulos 1974) and/or the cholesterol content present in the membrane (Jain 1975). Temperature, however, is a convenient means of manipulating the fluidity providing that the enzyme is not inactivated in the range to be studied.

Defined synthetic phospholipids, when formed into liposomes exhibit distinct, highly co-operative 'lipid phase transitions' where all of the lipid passes from a gel-fluid to a liquid-crystalline state at a particular temperature, T_c. In contrast biological membranes contain complex mixtures of lipids which do not go from a fluid to solid state during a single co-operative event. Instead a decrease in temperature can induce certain species of the bulk lipid pool to be preferentially segregated out and form clusters of semi-crystallised lipid within the bulk fluid pool. This occurs at a particular point called the 'lipid phase separation temperature', T_s, and can result in a change in the composition and physical properties of the fluid lipid pool.

The activity of certain integral proteins, which as freeze-fracture electron microscopy shows are excluded from solid lipid domains, can be affected to a marked extent by the altered physico-chemical properties of the remaining fluid lipid, following phase separation.

By using an Arrhenius plot it is relatively easy to obtain information on the sensitivity of functional proteins to lipid phase separations occurring within membrane bilayers. The Arrhenius equation is given by

$$Ln(k) = Ln(KT/h) - (\delta H/RT) + (\delta S/R)$$

where k is the rate constant for the reaction, K is Boltzmann's constant, h is Plank's constant, R is the gas constant, T is the absolute temperature, δH is the enthalpy of activation and δS is the entropy of activation. This can be reduced to

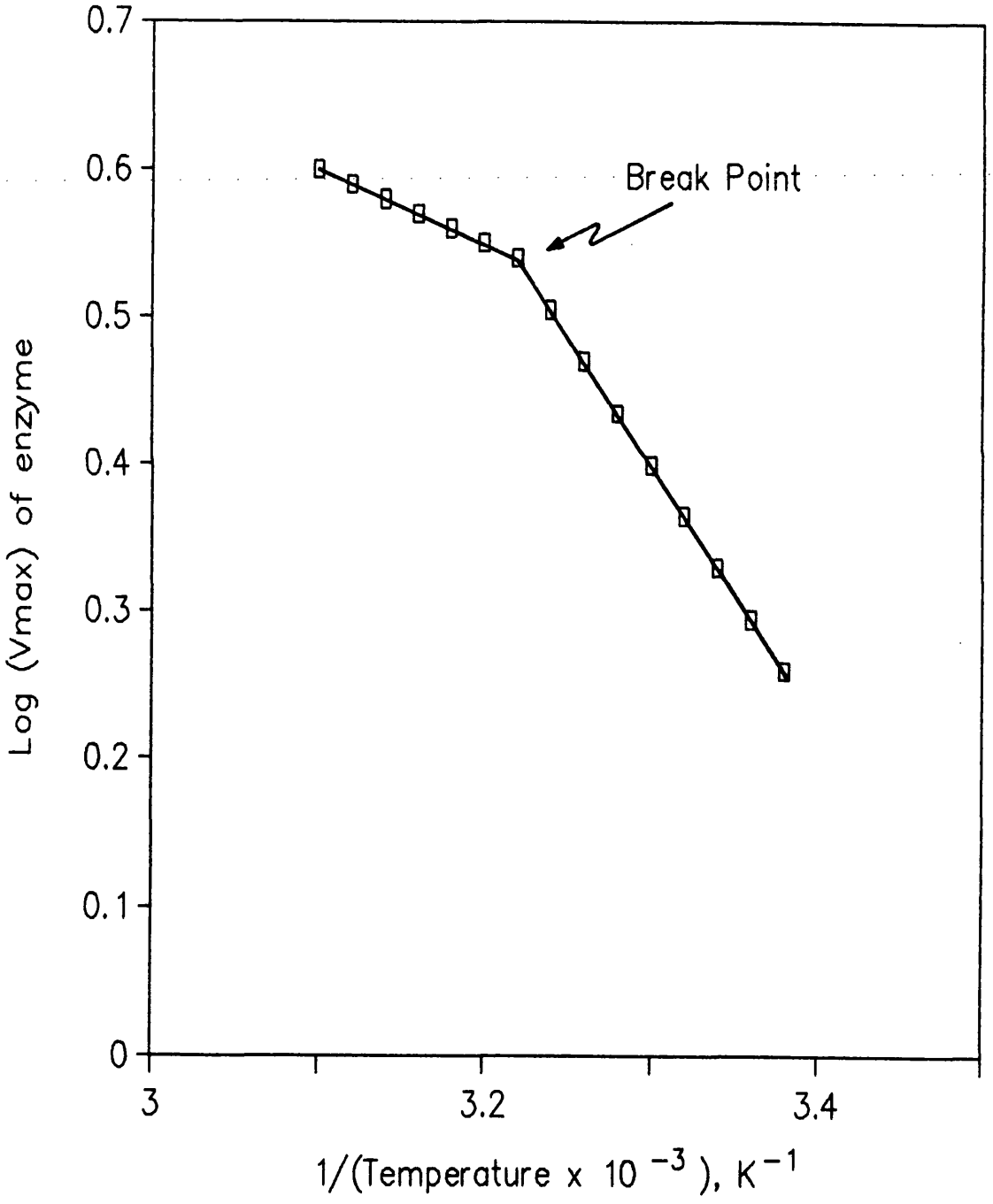
$$Ln(k) = (-E_a/RT) + Ln(A)$$

where E_a is the energy of activation and A is a constant called the pre-exponential term. A plot of the logarithm of the reaction rate constant against the reciprocal of the absolute temperature should therefore give a straight line.

The Arrhenius plot of the activity of many membrane-bound enzymes exhibit abrupt changes in slope occurring at a particular temperature known as the 'break' point, figure 1.4.2. In many cases the temperature at which the break point occurs corresponds closely to the lipid phase separation temperature of the membrane in which the enzyme is integrated (Stier and Sackmann 1973). Occasionally Arrhenius plots of some transmembrane proteins exhibit two distinct breaks corresponding to distinct lipid phase separations in each half of the bilayer. In many studies enzymes have been incorporated into artificial membranes of single phospholipid composition (Strittmatter and Rogers 1975). In these instances the breaks in the Arrhenius plot are associated with phase transitions rather than separations.

Interpretation of break points in Arrhenius plots requires a thorough understanding of the kinetics and reaction mechanism of the system

Figure 1.4.2 The activity of many membrane-bound enzymes when presented as an Arrhenius plot show abrupt changes known as the 'break point'. This corresponds to lipid phase separations in the membrane occurring at a particular temperature. The graph shows the break point for a generalised membrane-bound enzyme.



being studied as many other factors can give rise to similar effects (Raison 1980). The fact that biological membranes are totally heterogeneous in nature means that numerous break points may actually occur with different lipid components aggregating and separating out as an almost continuous function of decreasing temperature. Arrhenius plots in these instances will be curves.

A second type of phase separation can occur or is naturally present above the temperature, T_s. This involves the fluid/fluid phase separation of certain molecules due to their limited co-miscibility, a particular example being that of phospholipids and non-hydrating lipids like triglycerides and cholesterol esters.

1.4.3 Lipid Requirement of Membrane Bound Enzymes

The preceding section discusses the modulation of enzymatic activity by changes in the physical property of the gross membrane structure, namely fluidity. In many instances it can be shown that membrane enzymes are inactive if certain specific lipid-protein interactions are absent. The classical way to demonstrate the lipid dependence of an enzyme has been the loss of activity when the lipids are removed during purification and restoration of activity when reconstituted with lipid (Fourcans and Jain 1974). It has since been found that these criteria are not always sufficient proof for an obligatory lipid requirement. For some membrane enzymes a lipid requirement is only observed for reactions involving non-polar substrates (Hatefi and Stiggall 1976). The lipid acts as a form of 'cofactor' allowing the substrate to dissolve and so pass to the catalytic site of the protein. The general response of integral proteins to lipids, however, arises from lipid-induced stabilisation of the catalytic conformation.

It was originally thought that there was a strict lipid species specificity for the restoration of activity of purified enzymes. However, it has since been shown that membrane lipids activate more efficiently than a single lipid type (Sandermann 1978). Only in a few instances has there been found an obligatory requirement for a particular lipid type. Gazzoti *et al* (1975) have shown this to be true for the mitochondrial β-hydroxybutyrate dehydrogenase, the activity

only being fully restored by unsaturated diacyl phosphatidylcholines.

Chapman (1982) has defined several types of lipid that may be of importance to membrane bound enzymes. Purification by solvent extraction may leave residual lipid associated with the protein which may be tightly attached and can be termed 'bound lipid'. On removal of lipid from a membrane system there is a point reached, 'minimum lipid', where on removal of further lipid a marked decrease in activity occurs. As has already been discussed, 'specific lipids' may be necessary for retaining a protein in a certain conformation, without which loss of activity occurs.

The term annular lipid has been applied to the ring of lipids immediately surrounding a protein molecule (Jost and Griffith 1982). The protein itself will modify the degree of order of the acyl chains of annular phospholipids and depress or abolish the phase transition of the annular lipid. By virtue of their interaction with integral proteins, annular lipids exchange with their nearest neighbours in the bulk lipid pool at least an order of magnitude slower than that of normal bulk lipid ($V_{ex}>10^{-6}$ compared to $V_{ex}=10^{-7}$ s) but still far quicker than the turnover rate of most membrane bound proteins. Lipids in the annular region are therefore not static and will change several times during the turnover of an enzyme.

The amount of lipid that constitutes an annulus must by definition be sufficient to form a ring around the hydrophobic domain of the protein. The type of lipid present, however, can be influenced by electrostatic and Van der Waal's interactions between particular protein and lipid species. In such a situation the equilibrium composition of lipids in the annular domain need not reflect that of the bulk pool.

Beta hydroxybutyrate, as has already been mentioned, segregates out the highly fluid dioleoyl phosphatidylcholine molecules from bulk mitochondrial lipids so influencing the environment in which it functions. This is reflected in the extremely low rates of exchange of annular phosphatidylcholine with the bulk lipid, $V_{ex}<10^{-3}$ s. There is evidence suggesting that certain proteins can order phospholipids well beyond the nearest neighbours (Longmuir *et al* 1977). Such boundary lipid has distinctive properties in that it is not readily extracted

and does not exchange freely with bulk lipids of the membrane. The environment (polarity, motional freedom, packing) sensed by boundary lipids is considerably different to that experienced by bulk lipid. They experience greater polarity, less regular packing but a greater degree of immobilisation.

The functional significance of boundary lipids and of separate lipid domains in biomembranes is far reaching (Jain and White 1977). It implies that; different lipids may modulate the functioning of different proteins, minor lipid components may have a major effect on membrane properties since they can modulate the functions of specific components, the overall phase characteristics of the membrane arise from the coexistence of separate phases rather than from a homogeneous phase of several lipid species, and separate phases and proteins therein may be perturbed independently by specific lipid-soluble molecular species (eg. organic solvents).

1.4.4 Influences of Lipid on the Cytochrome P450 Mono-oxygenase System

Perhaps the earliest and most striking evidence for the influence of phospholipids on protein function has been the demonstration that in the absence of phospholipids or detergents, cytochrome P450 is unable to function. In recent studies far more detailed information has been obtained on the specific interactions between lipids and the various components of the cytochrome P450 mono-oxygenase system (Trudell and Bösterling 1983).

The presence of cytochrome P450 and NADPH-cytochrome P450 reductase in membranes has been shown to give measurable decreases in membrane fluidity indicating the restriction of movement of the lipid molecules. By using artificially constructed vesicles containing known mixtures of different phospholipids with defined phase separations Bösterling *et al* (1981) were able to show that cytochrome P450 had a preferential interaction with negatively charged lipids like phosphatidic acid which were concentrated in the region surrounding the protein. Strong interactions were also demonstrated with phosphatidyl-choline and phosphatidyl-ethanolamine.

Cytochrome b5 like cytochrome b5 reductase has both a hydrophobic and hydrophilic domain. The former anchors the protein to the membrane while the latter protrudes into the cytosol and contains the active site. The molecular structure of cytochrome b5 has been visualised in the form of a 'mushroom'. Insertion of the hydrophobic domain into the membrane is thought to cause reorganisation of the protein-phospholipid interactions in the bilayer, with the hydrophilic domain acting as a cap (Mathews *et al* 1972). The charged groups on the caps under-surface will attract the polar head groups of certain phospholipids, so restricting the movements of specific boundary lipids (Dehlinger *et al* 1974). A defined micro-environment different to that of the bulk lipid phase will therefore be present in which functioning of the protein is optimal. A similar form of boundary lipid has been observed for cytochrome P450 reductase (Stier and Sackmann 1973).

There is a wealth of further information indicating the lipid requirements of the various components of the cytochrome P450 mono-oxygenase system (Nisomoto and Lambeth 1985) (Muller-Enoch *et al* 1984) (Strobel *et al* 1970). It is however, generally recognised that the following interactions all play some role in the functioning of the cytochrome P450 mono-oxygenase system as well as in many other multiple enzyme systems.

1. The lipids in the form of a membrane, solubilise the individual protein components restricting their movement to a two dimensional plane. This increases their relative concentration and collision frequency; orients the proteins both with respect to the membrane and to each other allowing precise interaction of the catalytic sites.
2. The hydrophobic interior of membranes may partition non-polar substrates giving higher concentrations than in the cytosol and serve as a route by which non-polar substrates can travel to and enter the catalytic site of certain integral membrane proteins.
3. Liquid/liquid phase separation of phospholipids may provide different optimal micro-environments for the functioning of proteins and cause non-random distribution of protein in the membrane, with some having greater preference for interaction with a particular lipid species.

4. Annulus and boundary lipid which can be formed from short and long distance protein-lipid interactions may similarly act as defined environments in which the enzymes can act. The annular lipid more specifically may serve to stabilise the catalytic conformation of a protein.

1.4.5 Solvent Influence on Lipids and Membranes - Anaesthetic Action

Anaesthetics incorporate a group of chemically and structurally diverse molecules that if strictly defined, produce a reversible loss of consciousness in animals having a nervous system (Seeman 1972). However, it has long been known that anaesthetics affect a great variety of cellular functions including cell motility, cell division, the light emission of certain animals and bacteria, photosynthesis, oxidative metabolism and the excitability of nerve cells.

Examples of anaesthetics include many conventional organic solvents; n-alkanes and alcohols, benzyl alcohol, acetone, fatty acids, ethers, nitro, chloro and fluoro compounds along with other agents such as xenon, nitrous oxide, steroids, detergents and vitamin A. Their precise mode of action is still undetermined, however, either directly or indirectly they influence the functioning of specific membrane bound proteins.

Two lines of reasoning have been proposed as to the site of action of anaesthetics. The first of the theories for which least experimental evidence is available, suggests that anaesthetics interact directly with membrane bound proteins. Franks and Lieb (1978) believe that the lipid solubility hypothesis (see later) points to hydrophobic regions within protein molecules as being the site of action and perhaps more specifically, binding to the catalytic site which in many instances is hydrophobic in nature. Although anaesthetics have been shown to interact with these regions (Balasubramanian and Wetlaufer 1966) (Wetlaufer and Lovrien 1964) none of the models proposed to date provide good correlations with general anaesthetic potency. An alternative proposal suggests that because of the enormous structural diversity of anaesthetics, direct interaction with a single protein site is improbable. It is therefore required that different structural

classes of anaesthetic have different binding sites which may even be on different proteins, but that bring about the same overall response (Halsey *et al* 1980). Unfortunately this mechanism is in a real sense not applicable to testing.

The second set of theories consider that anaesthetics act by dissolving in the membrane lipid bilayer, so perturbing the environment in which the proteins function. Far more experimental evidence exists to support this mode of action but it is still recognised that in some instances, particularly at high concentrations, both protein and membrane theories may apply.

The anaesthetic potency, or aqueous concentration of a molecular species required to achieve a set response, bears a strong correlation to its oil/aqueous partitioning behaviour (Meyer 1937). The original thinking was that the oil might model the lipid part of the membrane which was imagined to be present in cells. This has been termed the 'lipid solubility hypothesis'. The model has since been refined by using octanol, lipid bilayers and biomembranes (**figure 1.4.5a**) instead of the bulk oil (Seeman 1972). By manipulating the equation for the membrane/aqueous partition coefficient the following equations are obtained.

$$P_{membrane} = [solvent_{memb.}] \; / \; [solvent_{aq.}]$$

$$Log(P_{membrane}) = Log(1/[solvent_{aq.}]) + Log([solvent_{memb.}])$$

The latter is the equation that describes, **figure 1.4.5a**. The linearity of the graph indicates that the product term

$$P_{membrane} \; x \; [solvent_{aq.}] = [solvent_{memb.}]$$

is a constant for a given response. This concentration may be found from the regression intercept value and is calculated to be ED_{100}=175mM. The inference then is that irrespective of the compound used, anaesthesia occurs when a critical concentration is achieved in the membranes.

Figure 1.4.5a Correlation of the anaesthetic potency of a compound with its membrane/aqueous partition coefficient (data of Seeman 1972). The anaesthetic potency in this instance represents the minimum concentration (moles/litre) of a given compound required to inhibit the conduction of a nerve impulse in a frog sciatic nerve.

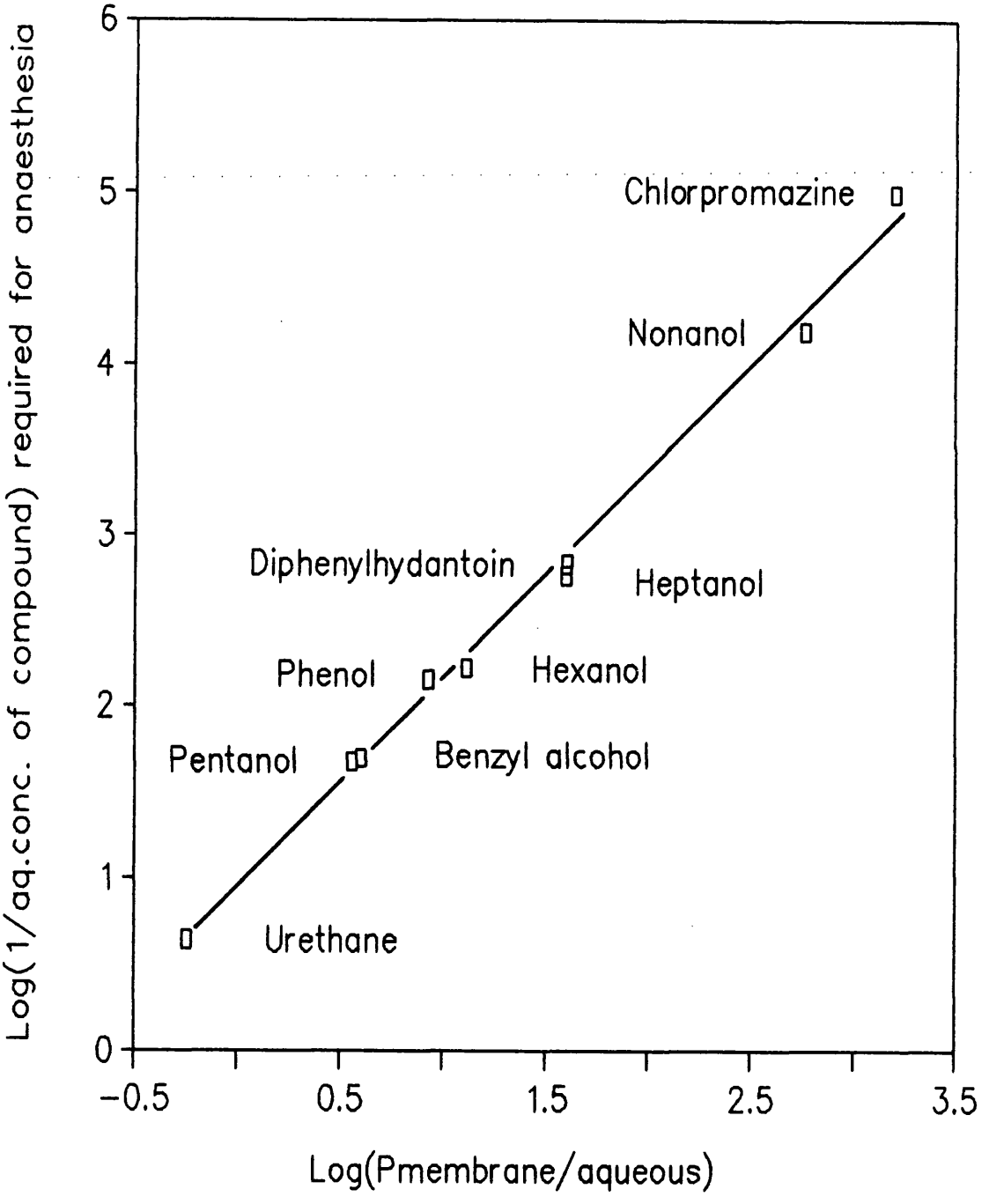
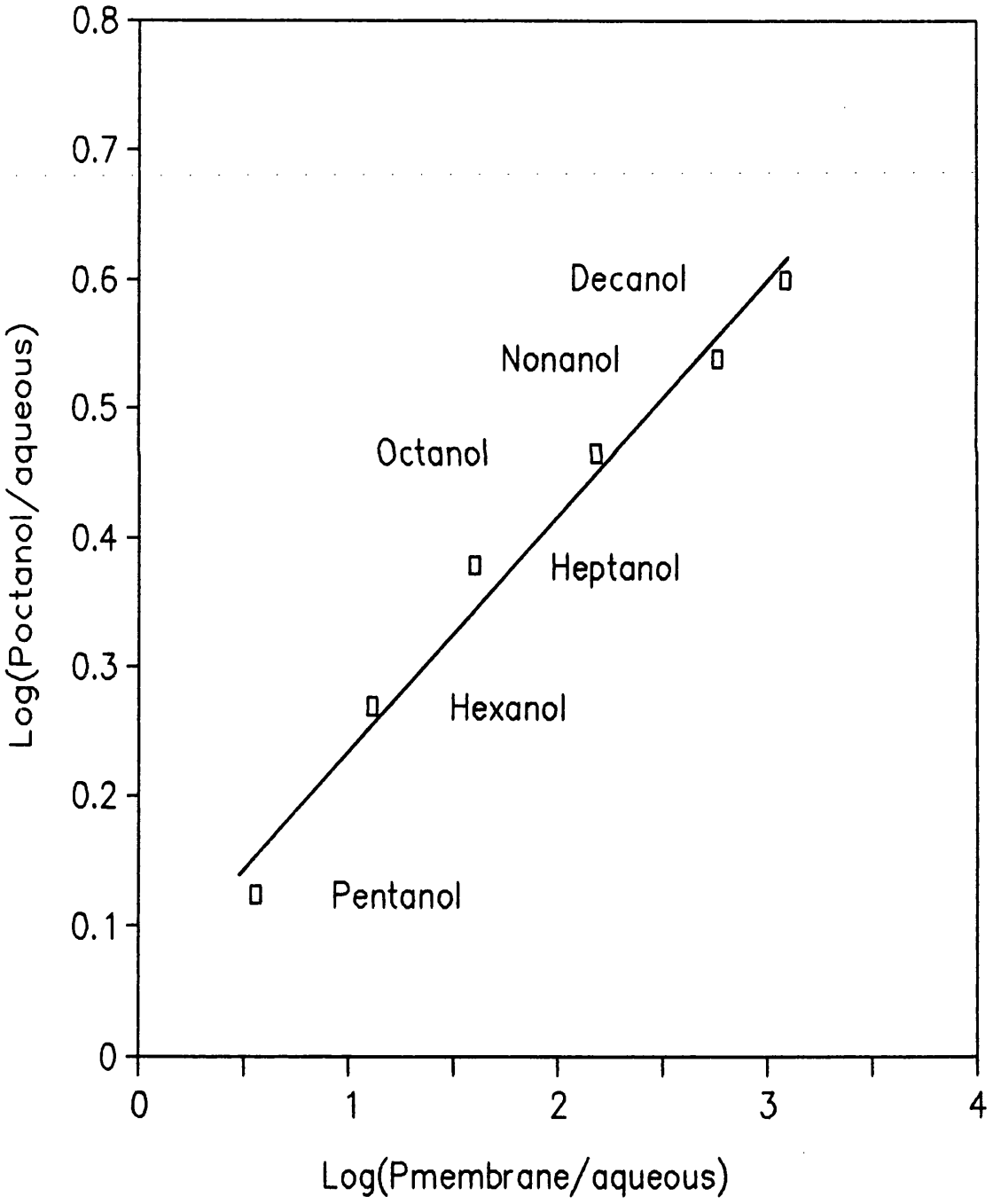


Figure 1.4.5b The relationship between the partition coefficient of a compound in an octanol/aqueous system and a biomembrane/aqueous system. The biomembranes used in this case were erythrocyte ghost membranes. However, a similar relationship can been shown to exist with other membrane types, synaptosomal and sarcoplasmic reticulum (Seeman 1972).



Seeman also shows that a good correlation exists between a compounds partition coefficient in biomembrane/water systems and its partition coefficient in an octanol/water system, figure 1.4.5b. The choice by Laane *et al* (1985) of the octanol/water system solely as a measure of a compounds polarity, section 1.1.3, can now be seen differently as representing to a high degree the environment present in biological membranes. It is also significant that the membrane partition coefficients of compounds in several types of biological membrane; synaptosomal, sarcoplasmic reticulum and erythrocyte, have extremely similar values.

Pringle *et al* (1981) have shown that under saturating aqueous conditions the maximum concentration of the n-alcohols partitioning into membranes decreases slightly with increasing chain length, up to dodecanol. This reflects the sum of two opposing physical factors; aqueous solubility falling rapidly with increasing chain length but being counteracted by an increasing membrane/buffer partition coefficient. Beyond dodecanol the membrane partition coefficient falls precipitously reflecting some effect of the alcohol-membrane interaction. It is suggested that at this point entropy effects become dominant as the alcohols begin to align in the bilayer so losing some degrees of motional freedom upon dissolution. At this same point, alcohols C_{13}-C_{14}, the concentration of anaesthetic achievable in the membrane becomes less than that required for anaesthesia, ED_{50}=10-30mM, figure 1.4.5c. This then accounts for the previously unexplained phenomenon of a 'cut off' in anaesthetic potency of the alcohols above dodecanol. The explanations given here have, however, been disputed (Franks and Lieb 1986).

Anaesthetics may perturb membranes in a number of different ways. Gordon *et al* (1980) have shown a good relationship between benzyl alcohol concentration and the fluidity of liver plasma membranes, figure 1.4.5d. Other experiments showed an increase in the activity of many of the membrane bound enzymes at low benzyl alcohol concentrations. Using various controls, it was concluded that the increase in activity was due to the increase in the membrane fluidity and not through direct interaction of the anaesthetic with the protein.

Several models for anaesthetic action have been proposed on the basis of the anaesthetics causing changes in the lipid phase separations, section 1.4.2 (Lee 1976) (Trudell *et al* 1975). The first envisages that just as temperature can cause phase separations of the lipids in membranes, the presence of other compounds or organic solvents may act in a similar fashion. The proteins may then move to patches of lipids for which they have a greater affinity but that alter the conformational structure of the protein. This has been observed experimentally (Poste *et al* 1975), with changes in the lipid packing causing aggregation of previously dispersed membrane bound proteins.

Lee's theory (Lee 1976) envisages that the anaesthetics fluidise and disrupt annular and boundary layer lipid associated with each protein, so causing loss of conformational integrity. In this respect, the dissociation of the bacterio-rhodopsin assembly has been shown to occur in the presence of small hydrophobic anaesthetics (Stelzer and Gordon 1986). Many proteins require the particular environment provided by annular and boundary lipid to actually remain solubilised in the membrane. It can therefore be imagined that perturbations of these regions may in extreme cases lead to the release of the protein from the membrane.

Charged anaesthetics are able to partition selectively and fluidise one or other half of a biological membrane since, in the majority of cases, the acidic (negatively charged) phospholipids predominate in the cytosolic facing half. This may cause particular problems when attempting to correlate the anaesthetising concentrations of charged compounds with partitioning data. A further characteristic is that they may also interact strongly with charged membrane protein moieties so displacing the annular and boundary lipid, as has already been mentioned.

Figure 1.4.5c The maximum achievable concentration (mM) of aliphatic alcohols in biomembranes, □ , decreases with increasing carbon chain length. The concentrations required for anaesthesia, + , and, ◊ , remain approximately constant (ED_{50}=10-30mM shown by area between dashed lines, - - -). Alcohols of carbon chain length greater than tridecanol (C_{13}-ol) cannot achieve this concentration in the membrane and are therefore not active as anaesthetics. Sources for the data are given, Pringle *et al* 1981.

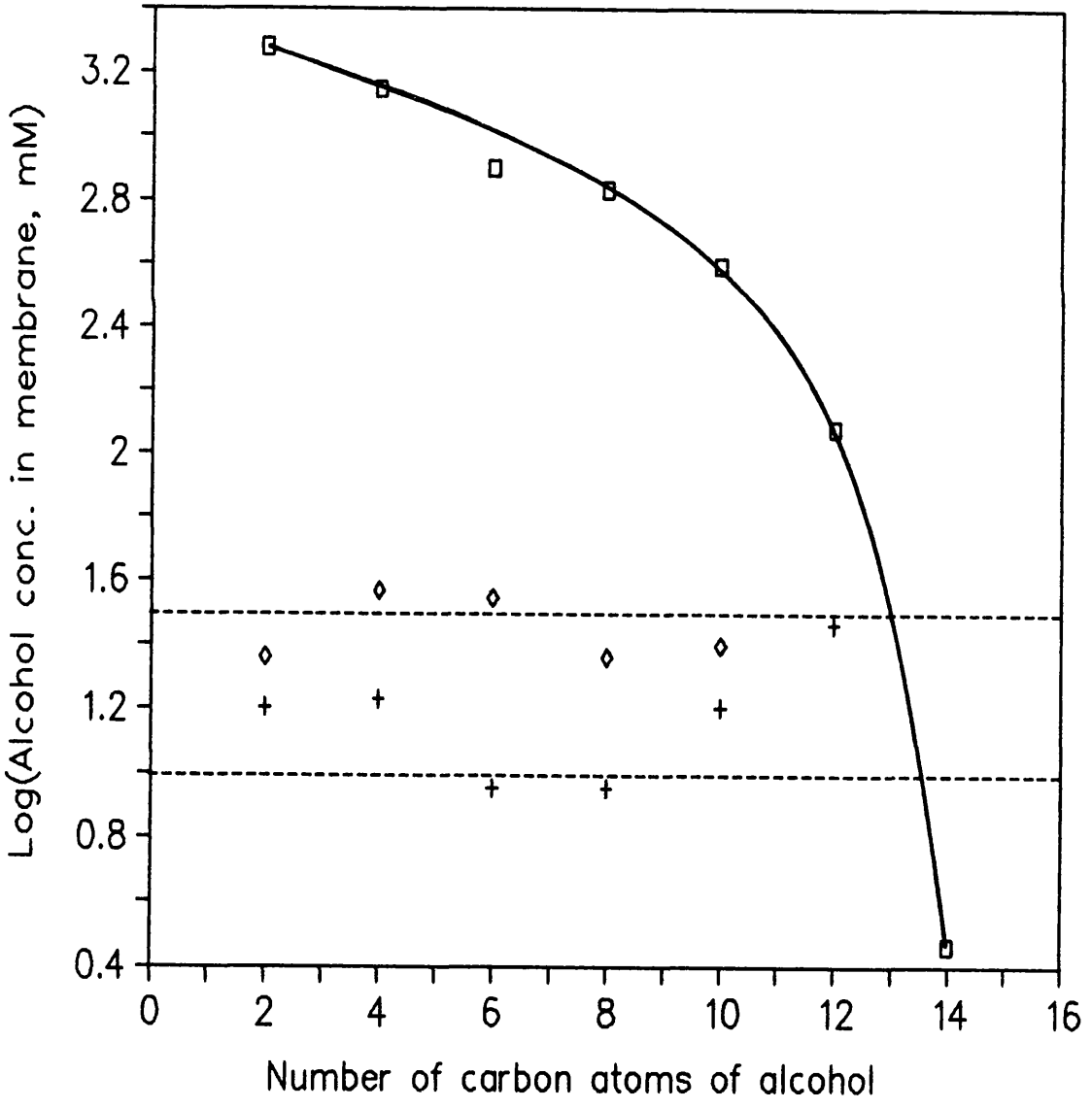
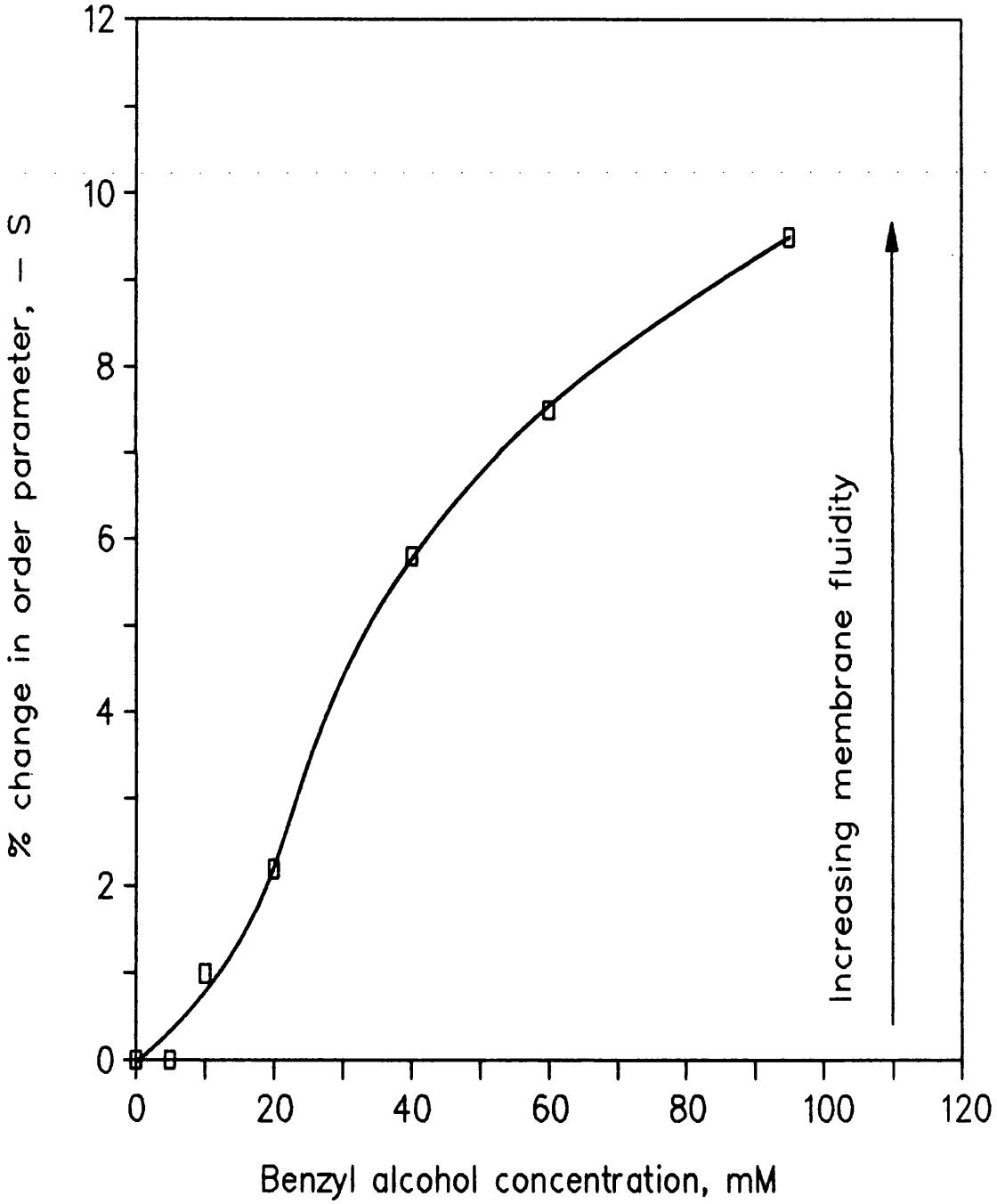


Figure 1.4.5d Anaesthetics (organic solvents) may affect biological membranes by perturbing the semi-crystalline structure of the lipids (decrease in the order parameter). This may lead to an increase in the fluidity of the membrane as is shown by the effect of benzyl alcohol on liver plasma membranes. Data is from Gordon *et al* 1980.



1.5 Reactor Options for use with Two Liquid-Phase Systems

The type of reactor used for an organic-aqueous two liquid-phase process need not differ fundamentally from one for the design of a conventional biocatalytic process. A requirement for high levels of agitation in order to maintain the two-phase dispersion may in certain circumstances limit the choice of reactor. This factor as well as simplicity of use has meant that shake flasks and batch stirred tank reactors have been most commonly employed in two liquid-phase reaction experiments.

In designing a process, one of the first decisions to be made is whether the reactor will operate batchwise or continuously. This one factor will greatly influence both capital investment and labour costs. Batchwise processes have the advantage of broad flexibility in operation whereas reactors for continuous use usually have to be specifically dedicated to a particular process. Kinetic considerations play an important part in the choice of reactor for a continuous process. In general, packed-bed reactors have intrinsic kinetic advantages over stirred-tank reactors for most reaction types. In a continuous stirred-tank reactor, the average reaction rate is lower than in a packed-bed due to the different operational concentration of substrate. An advantage of the CSTR, however, will arise in cases of a substrate inhibited reactions (Lilly and Dunnill 1972).

The form and characteristics of the biocatalyst must also be considered. In a stirred tank reactor there is a high risk of loss of biocatalytic activity due to shear inactivation of enzymes, cells or disintegration of immobilisation matrixes through mechanical shear. On the other hand, preparations of immobilised biocatalyst on very small particles can result in unacceptably high pressure drops and blinding problems when used in a packed-bed reactor. In this case the complications can be overcome by using a fluidised bed (Lieberman 1975), which provides a degree of mixing between that of a CSTR and a plug flow reactor. Operational requirements will also be a factor influencing the choice of reactor. For instance a batch reactor or CSTR is more suitable for reactions that require high oxygen transfer or the manipulation of the biocatalysts environment, in terms of pH and temperature control.

The production of 11α-hydroxyprogesterone by Rhizopus nigricans in a two liquid-phase system would in particular require the following to be considered when choosing the type of reactor to be employed. Firstly there should be sufficient mixing to maintain the two-phase emulsion in a highly dispersed form so as to reduce mass transfer limitations. Secondly there is a need for oxygen in the system since the 11α-hydroxylase reaction is oxygen dependent. Oxygen may also be required for the cells basal metabolic rate or even growth if new biocatalyst is to be generated. Finally low shear is preferable. This is important as R.nigricans is a non-septate fungus and needs to be structurally intact to perform the hydroxylase reaction. Breakage of the shear-sensitive filamentous mycelia results in loss of large amounts of the cytosolic interior including cofactors of the reaction and the endoplasmic reticulum to which the enzyme is bound.

A number of conventional reactor systems of possible use in two liquid-phase reactions have been mentioned; batch stirred tank, CSTR, fluidised bed, bubble column and packed-bed. Recently there has been interest in a novel form of membrane reactor. Here the organic and aqueous phases are separated by a membrane which can be a microporous polypropylene structure or material similar to that used in hollow fibre technology. The membrane will not necessarily restrict dissolved solvent from partitioning across the membrane, but will prevent droplets of emulsified solvent reaching the catalyst. Cho and Shuler (1986) have integrated the membrane system into an extractive fermentation. Four layers, gas, cells, nutrients and organic solvent are all separated by membranes of varying hydrophobicity. Using tri-butylphosphate as the organic phase it was shown that the solvent dissolved in the aqueous phase had no toxic effect on Saccharomyces cerevisiae cells, or on the yield of ethanol produced in the fermentation.

1.5.1 Packed-bed Reactors - Design Considerations

Packed bed reactors are generally employed in a continuous mode with immobilised enzymes or cells as the biocatalyst. This has many advantages over batch methods including; simple automatic control and operation, reduction of labour costs and stabilisation of operating

conditions preferable to downstream processing. When using packed bed reactors there are several important factors to consider.

Perhaps the most important is that of pressure drop across the packed bed. In order to determine the pressure drop of a packed bed, the experimental equation of Kozeny and Carman may be applied (Carman 1937).

$$dP = \frac{k \times (1-e)^2 \times \mu u L}{e^3 \times (q/D)^{-2} \times g_c} \qquad [E1.5.1a]$$

Here, dP is the pressure drop, k is a constant, e is the voidage, q is the shape factor, D is the particle diameter, μ is the viscosity, u is the linear flow velocity, L is the column length and g_c is the gravity coefficient. For many cases this can be simplified to

$$dP = KuL \qquad [E1.5.1b]$$

If K is a constant for a specific packed bed, the pressure drop of the column becomes proportional to the flow rate through the column and the column length. This relationship has been shown to be true for non-compressible beds such as a DEAE Sepharose immobilised aminoacylase column (Tosa *et al* 1971). The pressure drop was, however, also dependent on the temperature largely due to its effect on decreasing viscosity of the substrate solution. A far smaller pressure drop was observed when immobilised cells of Escherichia coli, having a larger mean particle diameter of 3mm were used (Tosa *et al* 1973). This is in agreement with the first equation where pressure drop is inversely proportional to the square of the diameter, D, though this is also compounded by an increase in the voidage, e.

The above equation does not hold true for compressible particles, because the pressure drop over the first part of the bed compresses the following layers of particles, giving decreasing permeability, G. To take this into account the following equation has been proposed and verified on pilot and industrial scale glucose isomerase columns (Norsker *et al* 1979).

$$dP = 1/G \times dL \times u \qquad [E1.5.1c]$$

Here dP is the differential pressure drop over a small section of the bed of length, dL. The formula is similar to that of equation [E1.5.1b] but the permeability (reciprocal of K) varies through the bed. By integrating this equation the pressure drop across the whole column can be obtained. The permeability is also a function of time and this effect may be accounted for using extrapolative techniques. Based upon pressure drop data from pilot scale work, Norsker *et al* (1979) recommend that pressure drops exceeding 1 bar should be avoided in industrial reactors. For design purposes it is useful to know the maximum value of the product of bed height and linear flow velocity that can be used before exceeding this 1 bar limit.

To overcome the problem of compaction of 'soft' beds having a high deformation modulus, Furui and Yamashita (1985) have described the use of horizontal baffles in columns containing particles of k-carrageenan. The void fraction in the columns with baffles was only slightly affected by superficial liquid velocity as compared to the control which showed a linear decrease in void fraction with increasing superficial liquid velocity. The pressure drop was smaller and, even more remarkable, the liquid motion was closer to plug flow in the column with baffles.

For industrial applications the liquid direction of flow is important. In many cases downward flow causes greater compression of the beds so upward flow is generally preferred. The direction of liquid flow in two liquid-phase systems can be critical to their operation. In most instances the organic phase has a lower specific gravity than the aqueous phase and will float above the aqueous phase when the emulsion breaks up. If separation of the phases were to occur on a column then an upward flow of liquid would drive off the organic phase from the reactor. Alternatively, if downward flow was used the organic phase would start to collect at the top of the column and might not be removed unless the liquid flow rate was very high.

1.5.2 Packed-bed Reactors - as Static Mixing Devices

Many models have been proposed to characterise the non-ideal flow that occurs within vessels. The dispersion models draw an analogy between

mixing in actual flow and diffusional processes. Models vary in complexity but ones containing a single functional parameter can adequately represent the mixing that occurs in packed-beds. Deviations from plug flow are thought to occur due to the interstitial spaces between the bed particles acting as miniature mixing chambers.

The backmixing of fluid flowing in a direction, x (perpendicular to the cross-sectional area), can be described by a form of equation similar to that used in Flick's law for molecular diffusion (Levenspiel 1972).

$$\frac{dC}{dt} = D \cdot \frac{d^2C}{dx^2}$$

where dC is the change in concentration (response) over a distance dx and during time dt from a residence time distribution plot. D is a parameter called the longitudinal or axial dispersion coefficient and characterises the degree of back mixing during flow. If t' is the mean residence time, L the total reactor length and u the linear flow velocity then the dimensionless variables z and θ may be defined as

$$z = \frac{x}{L} \qquad \text{and} \qquad \theta = \frac{t}{t'} = \frac{tu}{L}$$

giving

$$\frac{dC}{d\theta} = \left[\frac{D}{uL} \times \frac{d^2C}{dz^2} \right] - \frac{dC}{dz}$$

where the dimensionless group (D/uL), called the dispersion number, is the parameter that measures the extent of axial dispersion for a vessel. Therefore, D/uL tends to 0 as a consequence of lower dispersion resulting from plug flow and D/uL tends to infinity with increasingly mixed flow. This model will only be satisfactory for representing fluid movement that is close to plug flow.

With increasing flow rate through a packed bed the dispersion number also increases and the residence time distribution curves become increasingly skewed, figure 1.5.2. Concomitant with large extents of backmixing it becomes more unlikely that the real system will be satisfied by the dispersion model. Experimental curves should be

examined and compared with predictions of the model in order to assess how well the model fits. The mean, m, and variance, s, of the curves are found to be

$$m_t' = \frac{t_c'}{t'} = 1$$

$$s_\theta^2 = \frac{s^2}{t'^2} = \frac{2\,D}{uL} - [\,2\,(D/uL)^2 \times (1-e^{-uL/D})\,]$$

Furui and Yamashita (1985) have described the use of the Bodenstein number to measure the degree of mixing in a packed bed of k-carrageenan particles. The Bodenstein number, B_0, is derived from the dispersion number by the equation

$$B_0 = (uL/D) \times E_{av}$$

The value varies in a reciprocal manner to that of the dispersion number. It takes into account the void fraction, E_{av}, present in the packed bed, defined as

$$E_{av} = 1 - (\text{volume of particles} / \text{volume of column})$$

which may be determined experimentally using high molecular weight markers such as blue dextran.

1.5.3 Mass Transfer Requirements

The presence of a second liquid phase will present a further boundary for the transfer of a reactant species to the biocatalyst via a discrete aqueous phase. Assuming perfect mixing within the bulk phases the concentration profiles shown in figure 1.5.3 will apply (Lilly and Woodley 1985). Partitioning at the aqueous/biocatalyst surface may occur due to the relatively hydrophobic nature of microbial cell surfaces or the material used for immobilisation. The reactant concentration profile within the biocatalyst will depend to a great extent on the porosity of the structure, location and degree of the catalytic activity (Harbron et *al* 1985). The transfer of reactant from

Figure 1.5.2 Residence time distribution profiles from a pulsed input injection to a closed vessel as predicted by the dispersion model. As the extent of backmixing, dispersion number (D/uL), rises the curves become increasingly skewed. The C-curves for closed vessels are obtained by numerical methods and cannot be represented by analytical expressions (Levenspiel 1972).

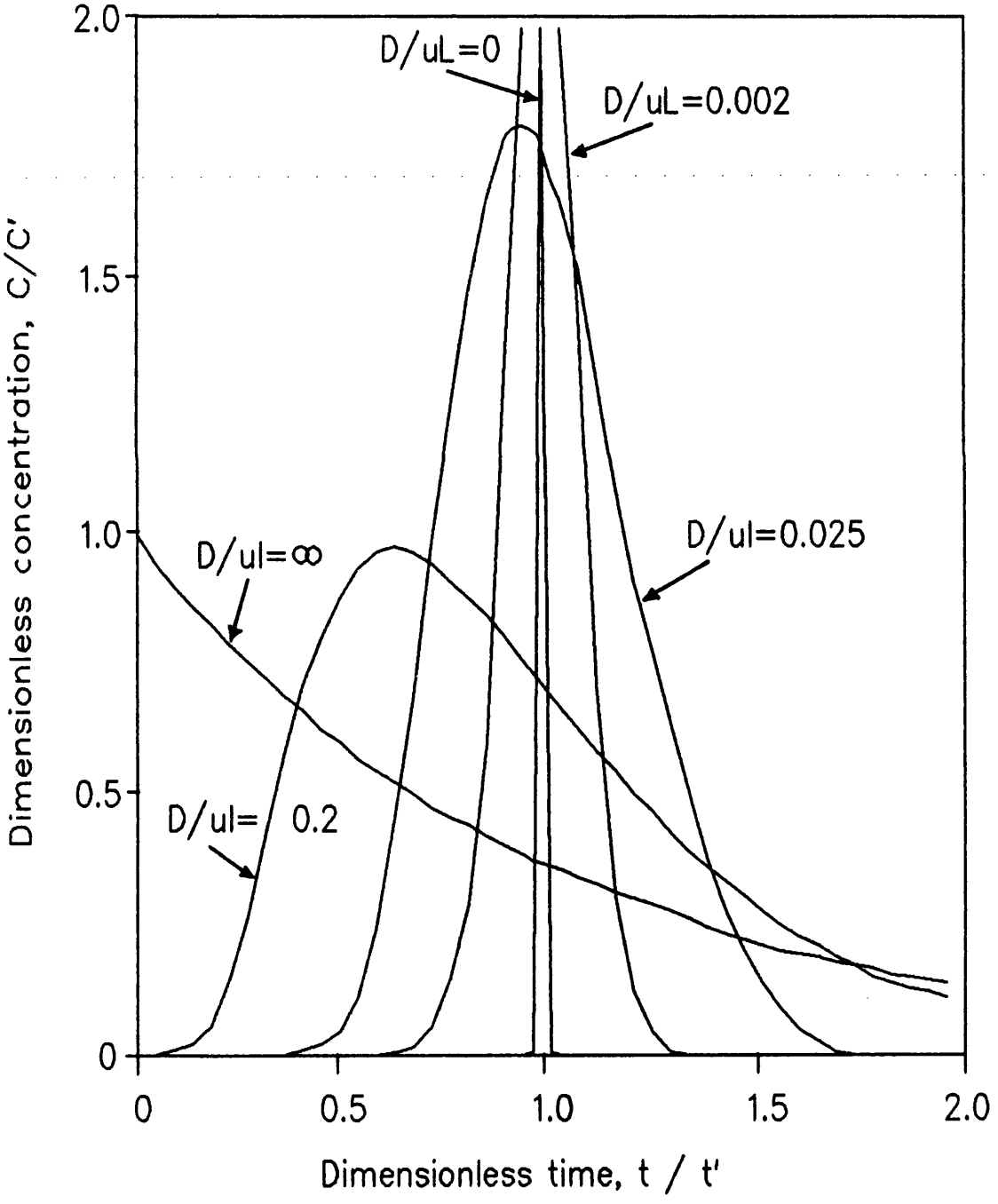
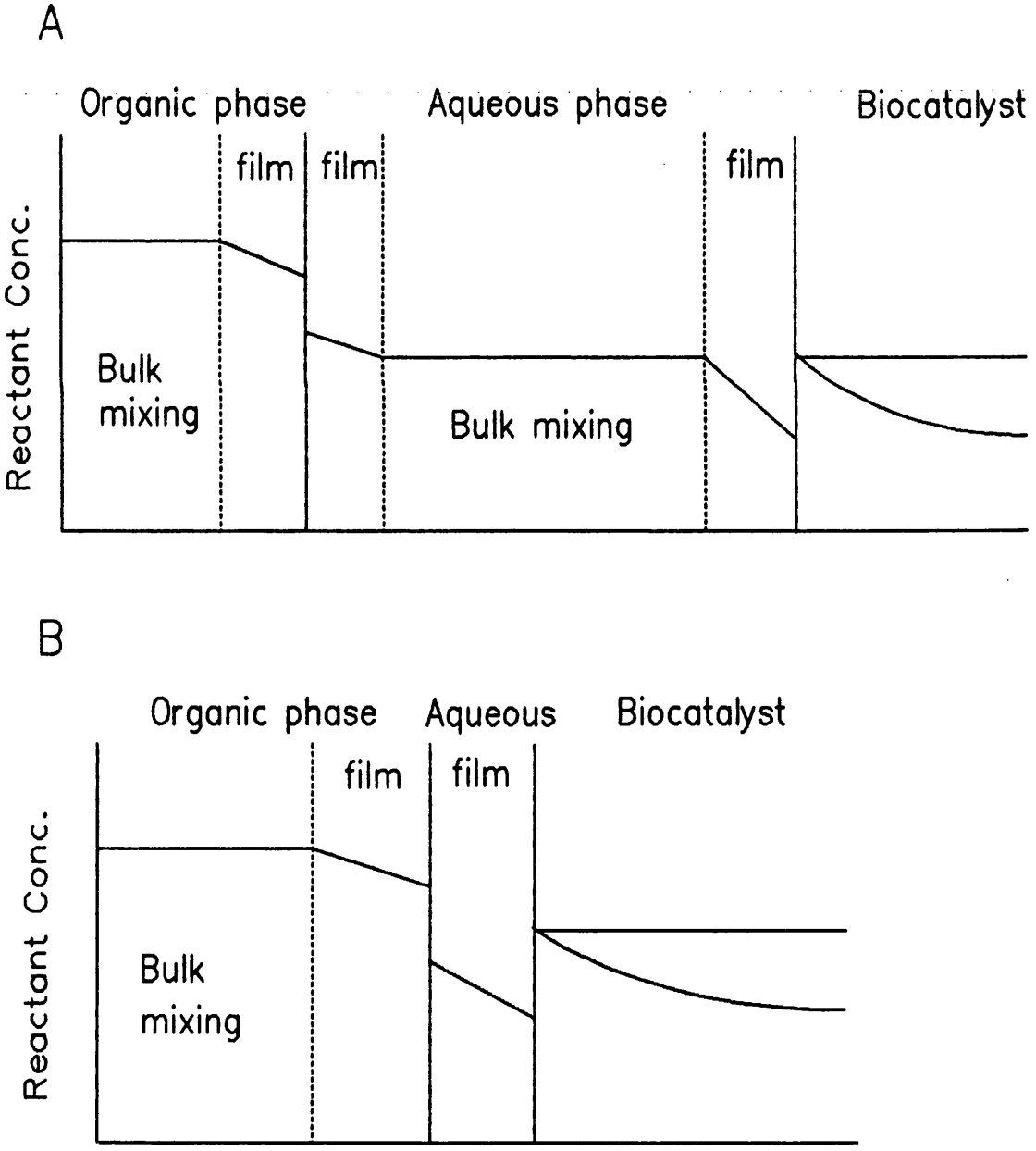


Figure 1.5.3 The concentration profiles for substrates in organic/aqueous two liquid-phase systems will depend on the presence (A) or absence (B) of a discrete aqueous phase (Lilly and Woodley 1985). The concentration profile within the biocatalyst (cell, immobilisation matrix etc.) will depend on porosity of the structure, diffusivity of the substrate, location and degree of biocatalytic activity.



the organic phase to the biocatalyst will be altered if surfactant is secreted by cells.

Mass transfer of a species between two fluid phases will depend on the physical properties of the two phases, the concentration difference of the species, the interfacial area and the degree of turbulence. Usually turbulence is maintained at a high level in an attempt to obtain perfect mixing in the bulk phases. In general if the two phases are not physically separated by some form of membrane, then higher levels of mixing will also cause the break up of the dispersed phase into smaller droplets so producing a larger surface area across which transfer can take place. In biological systems an optimum agitation level may need to be determined since shear inactivation can occur at high degrees of turbulence.

The transfer of a species across the boundary between two perfectly mixed fluid phases has been given by Whitman (1923) as

$$N_a = D/L\ (C_0-C_a) = k\ (C_0-C_a)$$

where N_a is the rate of transfer per unit area, D is the molecular diffusivity of the species, L is the film thickness and C_0, C_a are the concentrations of the species in the organic and aqueous phases respectively. The thickness of the stationary film that is assumed to exist between two phases is not generally known. D and L are therefore combined to give k, the mass transfer coefficient.

The simple model (film theory) from which this equation is derived assumes that the turbulence present in the two phases dies out close to the interface so that a stationary film occurs through which the transfer of matter is by random movement of molecules. Other more complex surface renewal theories have been proposed and some combine features of both types of theory (Dankwerts 1951) (Higbie 1935). Recent studies however, have shown that the presence of small quantities of impurities, which would normally occur in most practical systems, can completely change the interfacial behaviour. The impurities concentrate at the interface and cause fluid streaming and rapid flows, due to the complex action of surface tension forces. This is known as the Marangoni effect and generally significantly lowers the mass transfer

coefficient (Levenspiel 1972). In two liquid-phase processes it is considered that the resistance to transfer of a species across the interface will be negligible as compared to the diffusional resistance in each of the two phase (Scholtens and Bijsterbosch 1976).

As has already been mentioned the size and structure of the biocatalyst will affect the transfer of species within it. Using different techniques and various matrixes these parameters are relatively easily altered for most immobilised biocatalysts. When considering whole free cells and in particular fungal organisms this is not quite so simple.

Schugerl *et al* (1983) have, however, described the controlled production of Penicillium chrysogenum in pellet form. Similar investigations by Metz and Kossen (1977) and Whitaker and Long (1973) have indicated that three major variables seem to influence pellet structure; inoculum (spore) concentration, presence of polymer additives and the shear forces experienced during growth. The form of pellet can be varied in size and the degree of porosity of the intermeshed filaments. The ability to alter the biocatalyst structure is also of importance when considering the static mixing effects in packed beds (section 1.5.2).

Radovich (1985) has reviewed the area of mass transfer effects in fermentations using immobilised whole cells, and defines an 'effectiveness' factor for both external and internal mass transfer limitations. This information may equally be applied to fungal pellet fermentations, however, in both immobilised and pellet fermentations the presence of a second liquid phase will significantly alter the transfer kinetics making analysis of the system far more difficult.

1.5.4 Oxygen Supply to the Biocatalyst

In the production of 11α-hydroxyprogesterone, oxygen is required by the reaction itself (section 1.2.2) and by the fungal cells of Rhizopus nigricans in order to maintain a basal metabolic rate or even for growth. In packed beds, oxygen cannot be supplied as a gas since bubble formation causes increased pressure drops across the column. Supply must therefore be in the form of dissolved oxygen. The capacity of

aqueous solutions to carry oxygen is not particularly high, 0.231×10^{-4} as a mole fraction in water, and may not be sufficient to meet the demands throughout the whole length of the reactor. It is fortunate therefore that the organic solvents used in two liquid-phase systems generally have a greater capacity to dissolve oxygen. For example hexane will dissolve approximately 83 times more oxygen per unit volume than water at 25°C (Clever and Battino 1976).

For some solvents whose ability to dissolve oxygen is not as high, there may still be insufficient oxygen for the cells needs. In these cases several methods for increasing oxygen supply have been studied. The decomposition of hydrogen peroxide to produce oxygen has been investigated using the enzyme catalase present in cells of Gluconobacter oxydans (Holst et al 1985). Strict control of the hydrogen peroxide concentration is necessary in order to minimise the deleterious effects that occur. Many microorganisms could utilise oxygen produced in this manner by their own catalase enzymes, however, the tolerance of organisms towards hydrogen peroxide is extremely variable and much work is still required in this area.

Adlercreutz and Mattiasson (1982a,b) have described the use of perfluoro-chemicals (completely fluorinated organic compounds), as a means of carrying oxygen to immobilised cells. By saturating these liquids with pure oxygen, having a partial pressure about 5 times that of air, a 25 percent emulsion of perfluoro-chemical and buffer was found to have an oxygen dissolving capacity 17 times that of the buffer solution saturated with air. The main advantage of using these chemicals, as opposed to other organic liquids with high oxygen capacity is that perfluoro-chemicals are biologically inert. This means that biocatalyst toxicity and inactivation is apparently not a problem.

1.6 The Production of 11α-Hydroxyprogesterone in Organic/Aqueous Two Liquid-Phase Systems

The following sections give a brief description of the work performed by Ceen (1986) on the production of 11α-hydroxyprogesterone in organic/aqueous two liquid-phase systems. The study used whole cells of

the filamentous fungus Aspergillus ochraceus NRRL 405 to perform the microbial hydroxylation of progesterone. The cells are septate and therefore more resistant to shear inactivation than corresponding non-septate fungi like Rhizopus nigricans.

Before immiscible organic phases could be used in the system, optimisation of the purely aqueous reaction had to be performed. Cells were grown for 21 hours prior to induction of the hydroxylase system using 2g/L progesterone. The induction period was 3 hours after which time the cells were harvested, washed and resuspended in a defined reaction buffer. Phosphate buffer, 50mM and pH 7.4, along with 0.25 percent glucose were found to give optimal rates of reaction. Production of the unwanted 6β11α di-hydroxyprogesterone was also minimal under these conditions.

1.6.1 The Use of Conventional Organic Solvents

Solubility measurements of progesterone and 11α-hydroxyprogesterone were made for a range of immiscible conventional organic solvents. Values which were orders of magnitude greater than that for water were obtained indicating the possible suitability of these solvents for use as a second liquid phase. Solubilities of progesterone varied from 3.3mg/ml for hexane to 630mg/ml for dichloromethane, whereas solubilities for the hydroxylated product were always far lower due its more polar nature.

To assess the stability of the organism in the presence of some of the solvents, 'contacting' experiments were performed. The cells were contacted with particular solvents by agitation in shake flasks for given periods of time, washed with buffer, and then assayed for activity in an aqueous reaction system. For all organic solvents tested a rapid loss of activity occurred. Total loss of hydroxylase activity was observed following only 10 minutes of contact with the solvents. Hexane was an apparent exception and still retained about 25 percent activity as compared to a control set of cells. Apart from carbon tetrachloride the other solvents caused increasing biocatalyst inactivation with increasing polarity (as indicated by their

solubilities in water).

Immobilisation of the cells was considered as a means of protecting the cells from the deleterious effects of the solvents. Two types of immobilisation matrix were studied, Hypol 2000 (a polyurethane foam) and calcium alginate. Neither method seemed to significantly improve biocatalyst stability. Contacting experiments similar to those described above showed that calcium alginate immobilisation slowed the rate of activity loss slightly. However for hexane, the best solvent, still less than 25 percent of activity remained after just 40 minutes exposure.

A possible cause of loss of activity was the release of cofactors from the cells following permeabilisation. A regeneration system consisting of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase was employed in an attempt to restore the loss of activity. Stability of the cells increased slightly with up to 10 minutes of solvent contact, but the regeneration system had no observable effect over longer periods of contact.

The stability of the 11α-hydroxylase system was still found to be insufficient to allow the hydroxylation of the large amounts of reactant that could be dissolved in the two-phase system. This meant that the process was impractical for scale-up and could not be used to study the fundamental physical interactions of engineering importance.

1.6.2 Oils and Fatty Acids as the Second Liquid Phase

Using free or immobilised cells the poor stability of the 11α-hydroxylase system in the presence of conventional organic solvents indicated the need for an alternative solvent type to be found for use as the second liquid phase. Oils and fatty acids were chosen as it was felt that these would not interact strongly with the cellular lipids of the fungal cell.

The oils and fatty acids had a generally lower capacity to dissolve progesterone and 11α-hydroxyprogesterone than most but not all of the conventional organic solvents assayed. The solubility of progesterone,

however, was still some 600 to 700 times greater than its solubility in water, and twice that of the commonly used solvent, hexane.

To test the stability of the hydroxylase system in the presence of these oils and fatty acids, the cells were contacted for 3 hours, washed and then assayed for activity in an aqueous reaction buffer (section 1.6.1). Except for octanoic acid (a relatively small polar molecule) all the other six oils retained greater than 70 percent activity, glycerol trioleate having almost 90 percent retention of activity. From shake flask experiments using oleic acid as the organic phase, at constant agitation, a number of results were obtained that are summarised on the following page. The relative oil volume (ROV) was defined as the ratio of the oil phase volume to the total reaction volume (oil plus aqueous plus cell).

1. At constant cell mass and progesterone loading per reactor, optimum hydroxylation activity occurred between 0.4 and 0.6 ROV where interfacial area for mass transfer is greatest.
2. At constant cell mass in the aqueous phase this optimum was extended to 0.7 ROV, indicating that mass transfer limitations may occur in the aqueous phase due to the dense mycelial mass that is present when the water phase volume is low.
3. With increasing oil volume, greater amounts of 11α-hydroxyprogesterone are partitioned away from the catalyst, decreasing the production of the unwanted dihydroxylated product.

1.6.3 Batch Stirred Tank Reactor Studies

By using batch stirred tank reactors Ceen (1986) was able to demonstrate the effects of agitation as well as relative oil volume, cell density and progesterone loading on the production of 11α-hydroxyprogesterone by Aspergillus ochraceus.

Other physical parameters were also studied. The point at which phase inversion occurs (an oil in water turns to a water in oil emulsion), was determined by measuring the conductivity of the system. Inversion occurred between 0.3 and 0.4 ROV in the absence of cells but shifted to between 0.6 and 0.7 if cells were introduced at a concentration of 3mg

dry weight per millilitre of aqueous phase. Various phase ratios and agitations were used and the 30 percent saturation recommended for hydroxylation in a purely aqueous reaction by Hanisch (1978) was exceeded at agitations above 800 rpm for emulsions having ROV's between 0.3 and 0.7.

The production of 11α-hydroxyprogesterone was dependent on a number of interlinked parameters. With low cell concentrations in the aqueous phase the production (per g dry weight of cells) increased dramatically with increased agitation, illustrating the effect of increased surface area for mass transfer across the phase interface. At higher concentrations of cells in the aqueous phase (higher ROV's at constant cell mass per reactor) production was limited by mass transfer of progesterone in the increasingly dense and viscous cell mass. At constant progesterone loading per reactor, three compounding effects led to extremely low productions of 11α-hydroxyprogesterone when high phase ratios were used. Firstly phase inversion occurs and the interfacial area is low. Secondly the concentration of progesterone in the organic phase will be low, providing little driving force for transfer across the limited interface. Finally, the aqueous cell concentration will be high, leading to mass transfer limitations within this phase.

1.6.4 Long Term Stability of the Biocatalyst

The reactor studies described in the previous section (1.6.3) were performed over an 18 hour period, after which time the organic and aqueous phases were assayed for product. This is an extremely long period of time as compared to the stability of the cells contacted by conventional organic solvents, however, possible re-use of the cells would make a given process far more economically viable. An experiment was performed where the cells were harvested following 21 hours of a two-phase reaction, washed, and resuspended in fresh buffer, oleic acid and progesterone. Control cells not resuspended in fresh medium, showed a marked decline in the rate of product formation at about 21 hours. Cells resuspended at 21 hours, however, showed production rates comparable to the initial activities of the cells though this lasted for a shorter period of time, 15 hours.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Materials

Progesterone was a gift from J.P.R Herrmann (Glaxo Group Research Ltd, Greenford, Middlesex). 11α-hydroxyprogesterone and bovine serum albumin were obtained from Sigma Ltd (Poole, Dorset). Potato dextrose agar, Sabouraud agar and lactalbumin hydrolysate were obtained from Oxoid Ltd (Basingstoke, Hants.), yeast extract from Bovril Ltd (Burton-on-Trent, Staffs.), dextrose monohydrate from Tunnel Refineries (Greenwich, London) and corn steep liquor was from Garton Ltd (London). Precipitated calcium carbonate was obtained from Fisons (Loughborough, Leics.). Polypropylene glycol 2025 was from BDH Ltd (Poole, Dorset) and Dow Corning Siliconising fluid from Hopkin and Williams (Romford, Essex). Methanol-resistant filters were obtained from Millipore Ltd (Harrow, Middlesex).

2.1.1 Organic Solvents

The organic solvents used are shown in table 2.2 on the following page along with the supplier and the purity of the solvent.

2.2 The Microorganisms

Rhizopus nigricans (stolonifer) ATCC 6227b and Aspergillus ochraceus NRRL 405 were gifts from M.K. Turner (Glaxo Group Research Ltd, Greenford, Middlesex). Spores were stored at 4^0C in a freeze-dried state with 20 percent (w/v) skimmed milk as the carrier and at a concentration of $5x10^7$ spores/vial. Vials of lyophilised cultures were resuspended in 5ml of sterile deionised water which was then used to inoculate two agar slopes.

Each agar slope was comprised of 2.78g potato dextrose and 1.16g Sabouraud agar dissolved in 100ml deionised water and placed in 500ml

Table 2.2 Organic solvents used, their purity and the supplier.

Solvent	Purity (%)	Supplier
Benzene	> 99	Fisons
Butanol	> 99	Fisons
Butyl acetate	> 98	Fisons
Carbon tetrachloride	> 99	BDH
Chlorobenzene	> 99	Fisons
Cyclohexane	> 99	Fisons
Cyclohexanol	> 95	Fisons
p-Cymene	technical	Fisons
Dimethyl sulphoxide	> 99	BDH
Decanol	> 98	Sigma
Dodecanol	> 99	Sigma
Ethanol	> 99	Fisons
Ethyl acetate	> 99	Fisons
Ethyl benzoate	> 99	Fisons
Heptanol	> 99	Sigma
Hexane	> 99	Fisons
Hexanoic acid	> 99	Sigma
Hexanol	> 95	Fisons
Methanol	> 99	Fisons
Methyl acetate	> 98	Fisons
2-Methyl butan-2-ol	> 98	BDH
Methyl cyclohexane	> 95	Fisons
4-Methyl Pentan-2-one	> 99	Fisons
Nitrobenzene	> 99	Fisons
Nonanol	> 98	Sigma
Octanol	> 99	Sigma
Octanoic acid	> 99	BDH
Octyl acetate	> 99	Sigma
Oleic acid	technical (65-70)	BDH
Oleic acid	> 92	BDH
Pentanol	> 99	Sigma
Pentyl acetate	> 95	Fisons
Propanol	> 99	Fisons
Propyl acetate	> 95	Fisons
Toluene	> 99	BDH
Undecanol	> 99	Sigma
m-Xylene	technical	Fisons
o-Xylene	technical	Fisons
p-Xylene	technical	Fisons

Fisons = Fisons, Loughborough, Leics.

Sigma = Sigma Ltd, Poole, Dorset.

BDH = BDH Chemicals Ltd, Poole, Dorset.

medical flat bottles fitted with foam bungs. The contents were autoclaved at 121°C for 15 minutes and stored at 4°C until required for use.

Inoculated slopes were incubated at 28°C for 7-14 days by which time heavy sporulation had occurred. Spores were then harvested from the slopes into 100ml of sterile deionised water and stored for up to 14 days at 4°C. Spore concentration was estimated using a haemocytometer (Gallenkamp, London) viewed at times 400 magnification using a light microscope (Olympus).

2.3 Growth Media for Shake Flasks and Fermenters

Rhizopus nigricans ATCC 6227b was grown in a medium consisting of 25g dextrose monohydrate, 20g yeast extract and 1ml of trace mineral solution all dissolved in 1 litre of deionised water. The trace mineral solution was made according to the Herbert formula (Elsworth et al 1968) but with the solids content reduced by a factor of ten. Growth medium for fermenters was supplemented by the addition of 0.1ml/L polypropylene glycol 2025 for foam control.

Aspergillus ochraceus NRRL 405 was grown in a medium consisting of 20g dextrose monohydrate, 10g lactalbumin hydrolysate, 5g corn steep liquor and 5g of precipitated calcium carbonate (to reduce pelleting, Ceen et al 1987a) all dissolved in 1 litre of deionised water.

The pH of all media was adjusted to 4.5 using concentrated phosphoric acid prior to sterilising at 121°C for 15 minutes.

2.4 Growth in Shake Flasks

2.4.1 Shake Flask Siliconisation

Previously reported fungal wall growth was reduced by siliconisation of glassware (Hanisch 1978). Cleaned, two litre Erlenmeyer flasks were treated with 30 percent (v/v) siliconising fluid in acetone. The solution was allowed to coat the inside of the flask and then drained

off. The acetone was left to evaporate and the flasks baked at 190°C for one hour followed by autoclaving at 121°C for 15 minutes. Successful siliconisation was tested by the absence of a meniscus. Before use the flasks were washed thoroughly and rinsed with deionised water.

2.4.2 Growth of *Rhizopus nigricans* ATCC 6227b

2 litre Erlenmeyer flasks each containing 250ml of growth media were inoculated with the required volume of spore suspension. This was equivalent to $5x10^8$ spores for filamentous growth or $1.7x10^8$ spores for pelletted growth. Cultures were grown for 12 hours if required as the inocula for larger fermentations. Cultures needed for direct experimentation were grown for 15 or 18 hours followed by a 3 hour induction period (section 2.6). The flasks were maintained at 28°C in a New Brunswick reciprocating shaker (Edison, N.J. USA) with a 50mm throw set at 100 strokes/min.

2.4.3 Growth of Aspergillus ochraceus NRRL 405

2 litre Erlenmeyer flasks each containing 250ml of growth media were inoculated with $2.5x10^9$ spores. Cultures were grown for 18 hours followed by a 3 hour induction period (section 2.6). The flasks were maintained at 28°C in a New Brunswick orbital shaker (Edison, N.J. USA) and agitated at 200 revolutions/min.

2.5 Fermentation Procedure for *R.nigricans*

Fermentations of Rhizopus nigricans were carried out in a 20 litre LH fermenter (LH Engineering, Slough, Bucks.), baffled and operated at 14.75 to 15 litre volume capacity. All components of the media were sterilised together, in situ, for 15 minutes at 121°C. The pH and DOT electrodes fitted were Ingold steam sterilisable (Life Science Labs. Luton, Beds.). Instrumentation was through both 2000 series 1 and I3000 series 1 controllers (LH Engineering, Slough, Bucks.) connected to a VG MM8-80 mass spectrometer (VG Gas analysis, Middlewich, Cheshire) for

exit gas analysis. All automatically measured variables were channelled to a developmental on-line analysis data storage computer system (Bio-I, Biotechnology Computer Systems, Chiswick).

2.5.1 Growth Conditions

Filamentous growth was obtained by inoculating the fermenter with 1000ml of culture (4 shake flasks, $5x10^8$ spore dosage, at 12 hours growth). Aeration was maintained at 0.14 vvm and tip speed at 2.0 m/s.

Pelletted growth was achieved by inoculating the fermenter with 750ml of culture (3 shake flasks, $1.7x10^8$ spore dosage, at 12 hours growth). Aeration was similarly maintained at 0.14vvm but the tip speed was increased to 3.1 m/s. The temperature in both types of fermentation was kept constant at 28^0C.

2.6 Enzyme Induction

Induction of the 11α-hydroxylase system was carried out, for both shake flasks and fermenters, 3 hours prior to harvesting by the addition of 2g/L micronised progesterone. The addition stage and subsequent handling operations necessitated the wearing of protective gloves and respirator masks (3M, type 9913).

2.7 Harvesting of Cells

Shake flask and large scale fermentations were harvested through nylon mesh (Mary Quant, London). The cell mass was then washed with two volumes of wash buffer (section 2.7.1) at 4^0C. The cell mass was lightly squeezed to express excess liquid. A representative sample of the cell cake was then weighed, dried at 80^0C for 24 hours, and reweighed.

Cell cake that was not used immediately was kept for up to 24 hours resuspended in reaction buffer A (section 2.7.1) at 4^0C. In such instances the cell material was not used for activity measurements but only for packed-bed flow-dispersion characterisation.

2.7.1 Suspension Buffers

Wash buffer and 50mM phosphate buffer consisted of 7.21g $Na_2HPO_4.2H_2O$ and 1.74g $NaH_2PO_4.2H_2O$ made up to 1 litre with deionised water and the pH adjusted to 7.4. Reaction buffer A was the same as wash buffer but with the addition of 2.5g/L glucose. Reaction buffer B was the same as reaction buffer A but with the further addition of magnesium chloride to a concentration of 1.5mM.

2.8 Progesterone 11α-Hydroxylase Activity Following Organic Solvent Contacting

25ml aliquots of induced filamentous cell suspension (R.nigricans, 20g wet weight/500ml) were contacted with an equal volume of organic solvent for 3, 5 and 10 minutes in 250ml glass stoppered conical flasks agitated on an orbital shaker at 200rpm and 28°C. After the required time the cells were filtered through nylon mesh and washed with phosphate buffer. The cell cake obtained was resuspended in 25ml of reaction buffer A and 0.1g of progesterone added. Flasks were agitated, 200rpm at 28°C, for 18hr and assayed for 11α-hydroxyprogesterone. Activities were compared with aqueous controls which had not been contacted with organic solvent.

2.9 Growth and Hydroxylase Activity of *Rhizopus nigricans* in the Presence of Oleic Acid

2 litre Erlenmeyer flasks each containing 250ml of growth media were inoculated with $5x10^8$ spores. The spores were grown and induced under normal conditions (section 2.4.2) except that 30min after inoculation a set volume of oleic acid was added to the flasks. Control flasks having no added oleic acid were also cultured. Cells from each type of flask were then harvested, washed and a proportion of the cells removed for dry weight analysis. A fixed mass of the remaining cells were then assayed for 11α-hydroxylase activity in the presence and absence of oleic acid.

2.10 Batch Stirred Tank Reactor Hydroxylations

The reactors used were the same as those described by Ceen (1986) and were constructed from the following components.

250ml round bottom flask	-	Quickfit FR 250F
Vessel cover	-	Quickfit MAF 1/75
Vessel Seal	-	Quickfit JC 100F

Four equidistant vertical baffles (70mm x 7mm) were constructed from stainless steel. Mixing was achieved by a 45^0 two-bladed propeller placed 2cm from the base of the vessel and powered by a 0-6000rpm Citenco Varilab motor (Citenco Ltd, Boreham Wood, Herts.). Speed of rotation was measured using a digital optical tachometer (Compact Instruments Ltd, Barnet, Herts.). Temperature of the vessel was maintained at 28^0C using a water bath.

2.10.1 Hydroxylation in the Presence of Oleic Acid

The required volume of oleic acid (36ml=0.3 ROV, 60ml=0.5 ROV) was transferred to the batch tank reactor and 300mg of progesterone added. This was allowed to equilibrate with gentle agitation at 28^0C for 2hr. Addition of the required volume of cell suspension was then made to give 120ml final working volume. Agitation was set at the required level and checked at intervals. After an 18hr reaction period 20ml samples were removed and the emulsion broken by centrifugation (Centaur, MSE, Crawley, West Sussex) at 4000rpm. Each phase was then assayed separately as described in section 2.15.

2.10.2 Hydroxylation in an Aqueous System at 1200rpm

100ml of filamentous *Rhizopus nigricans* cell suspension was placed in the reactor. 20ml of 0.01% Tween 80 in which 1g of progesterone had been slurried was then added. Agitation was set at 1200rpm and the temperature maintained at 28^0C. Samples, 3ml, were taken at set time intervals and assayed as described in section 2.15.1.

2.11 Packed-Bed Reactor System

2.11.1 Design and Operation

Minor modifications to the design and operation of the packed-bed reactor setup were made continually as the system was developed. The major components were a chromatography column (Amicon Ltd, Stonehouse, Gloucs.) with fluid feed from a peristaltic pump (Watson-Marlow Ltd., Falmouth, Cornwall). Operating setup :

Column type (model 87040)	:	70mm x 250mm, organic solvent resistant borosilicate glass. Pressure rating - 3bar
End piece support cells	:	Polypropylene
Bed support meshes	:	Stainless Steel, 10μm porosity
Seals / Tubing	:	Ethylene propylene / Polyethylene
Connectors	:	Nylon (polyamide)
Pump type (model 501U)	:	Variable speed peristaltic with hard springs, flow rates of 0-200ml/min

A schematic diagram of the system is given in figure 2.11.1. The feed reservoir tank was kept in a water bath at 28°C. Agitation of the feed, if required, was provided by a four-bladed turbine impeller powered by a 0-6000rpm Citenco Varilab motor (Citenco Ltd, Boreham Wood, Herts.). The speed of rotation was monitored using a digital optical tachometer (Compact Instruments Ltd, Barnet, Herts.)

2.11.1.1 Column Packing

Cells of either filamentous or pelletted *Rhizopus nigricans* were resuspended in phosphate buffer or reaction buffer at concentrations of about 250g wet weight per litre.

500ml of reaction buffer was poured into the column. Air bubbles which became trapped around the sides of the support net were removed by blowing jets of buffer from a long needled syringe. Buffer was then pumped, using the peristaltic, from the base outlet at a high flow rate

to ensure the removal of air from the base end piece support. Leaving about 100ml of buffer in the column, the exit tap was closed and a packing reservoir (model 87240, Amicon Ltd, Stonehouse, Gloucs.) attached to the top of the column.

Cell suspension was then poured into the column. Although care was taken at this stage, small bubbles still became entrained in the cell mass and had to be removed. The column and attached reservoir were filled to the top with more buffer and a long stainless steel rod (1mmx1m) used to gently stir the cells allowing the bubbles to rise to the surface. The height adjustable top end piece was then attached making sure that no bubbles were present in the system. With the buffer reservoir tank at a height above the column, buffer was pumped from the bottom exit. Flow rate of buffer through the column was varied according to the cell packing density required. Once the bed height had fallen below the bottom of the column reservoir this was removed and the adjustable top end piece attached directly to the column.

2.11.1.2 Dye Injection and Monitoring of the Outlet Stream

Tracer dyes were injected manually into the feed stream of the column through an hypodermic syringe. The design of the injector port is shown in figure 2.11.1.2.

The outlet stream from the column was monitored on occasions for steroid, tracer dyes and protein. A fraction collector (Ultrorac type 7000, LKB, Stockholm, Sweden) was employed and the samples obtained analysed separately. In cases where emulsions were passed through the column the collected fractions were either left to stand or centrifuged to separate the phases which were then analysed individually. The steroids, progesterone and 11α-hydroxy-progesterone, were assayed in the usual manner (section 2.15). The absorbence of tracer dyes was measured using a spectrophotometer (model 552, Perkin Elmer, Beaconsfield, Bucks.) at their absorbence maxima. Dyes used were; aqueous soluble, naphthol green-B (710nm) and blue dextran (620nm) both from Sigma Ltd, Poole, Dorset and oil soluble Bieberich scarlet-R (514nm) from Hopkin and Williams Ltd, Chadwell Heath, Essex. Protein concentrations were measured using Pierce protein assay reagent

Figure 2.11.1 A schematic diagram of the experimental set up for the packed-bed column studies. The in-line static mixer was only used in a few of the experiments. The rotameter was placed prior to the dye injection point, so as to minimise mixing, in those experiments where residence time distribution profiles of tracer dyes were monitored.

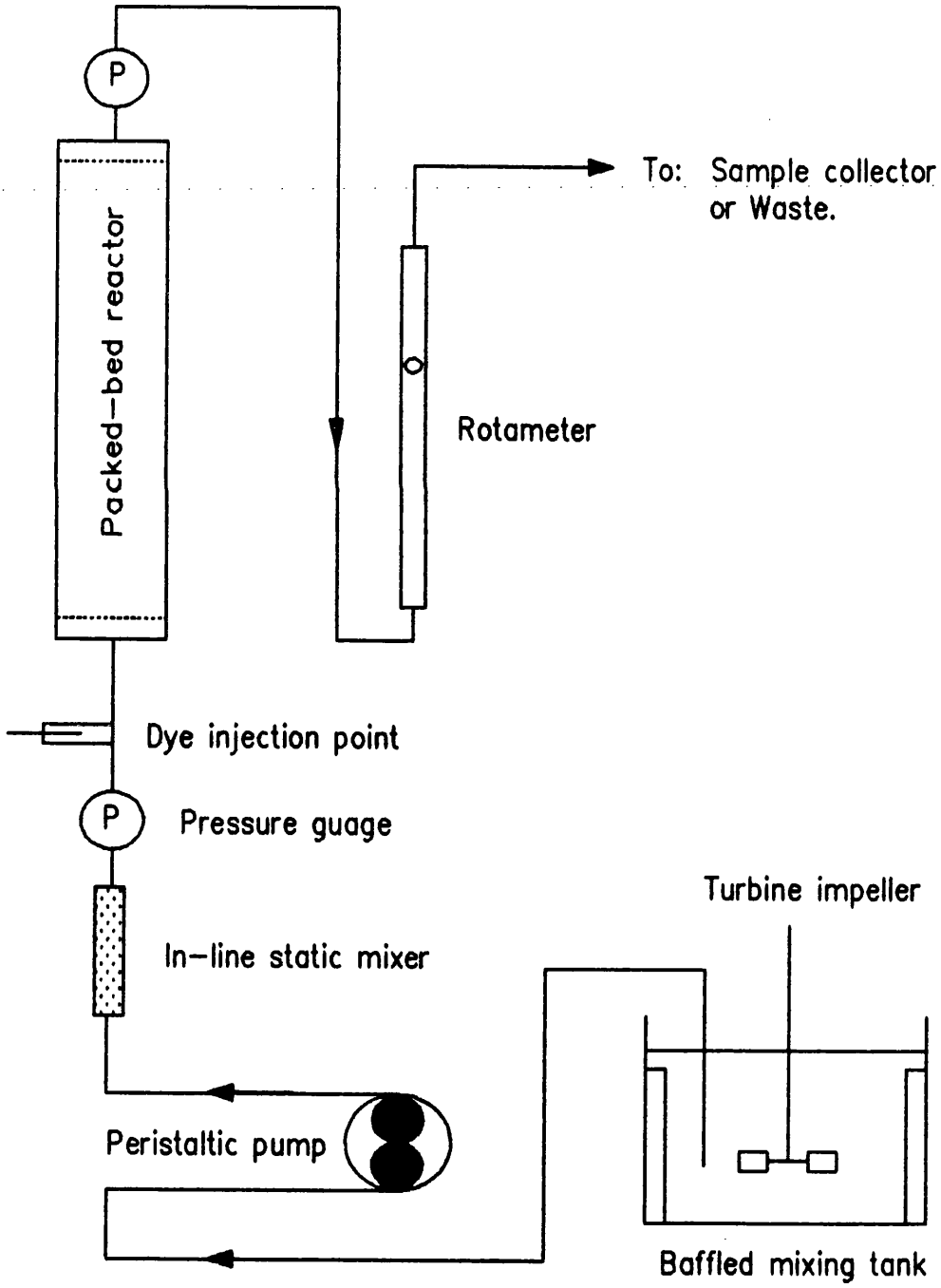
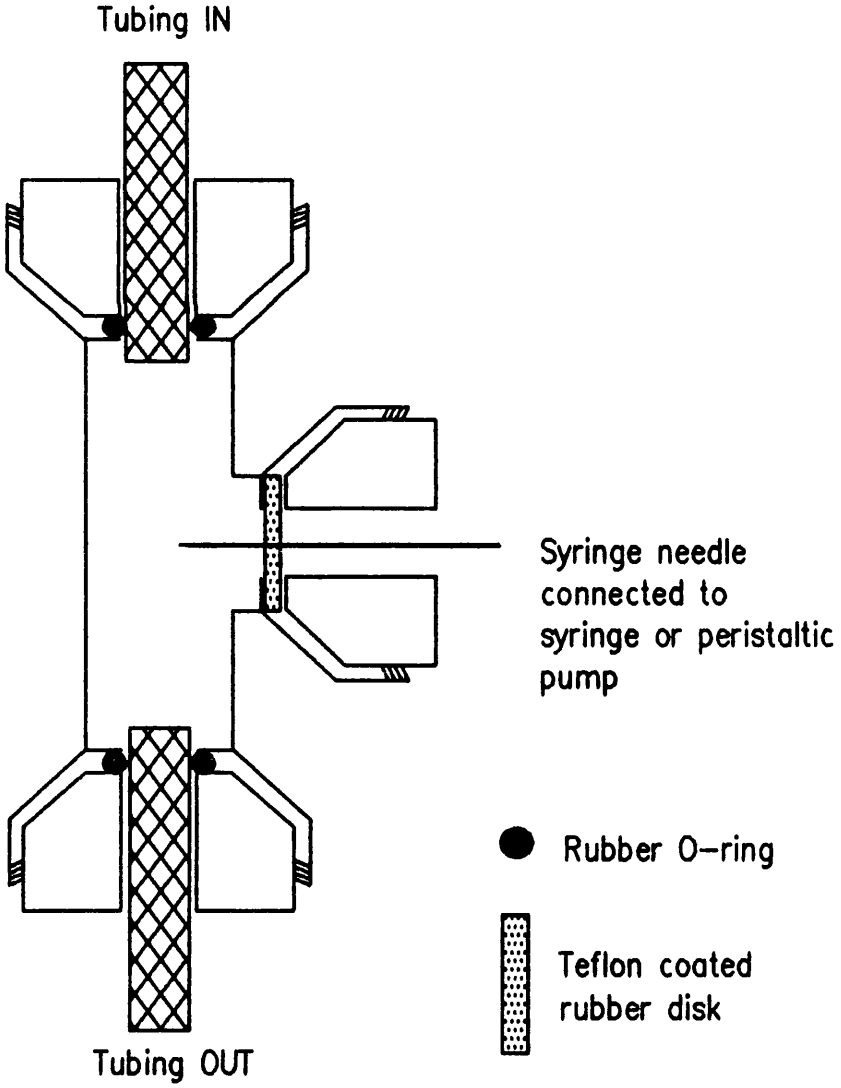


Figure 2.11.1.2 Diagram showing the design of the dye injection port and of a second similar injection port used for the introduction of aqueous droplets to the top of the packed bed (section 2.11.3.1).



(Pierce, Cambridge, Cambs.) with bovine serum albumin used for the calibration curves.

2.11.2 Flow of Emulsions

Oleic acid/aqueous buffer emulsions were prepared in a baffled tank. Addition of the oleic acid to the highly agitated buffer was achieved by pumping the oil through a syringe needle at a point close to the impeller region. Entrained air that occurred either deliberately when attempting to oxygenate the emulsion or accidentally when this was not necessary was removed by passing the emulsion through a bubble trap of short residence time before it was pumped to the column. Flow of emulsions through the column was generally attempted in an upward direction.

2.11.2.1 Modelling of Emulsion Breakup

Ballotini beads, 400-800 mesh (Potters ballotini, Barnsley, S.Yorks.) were used in place of the packed-bed of cells of R.nigricans. An emulsion of 0.3 relative oil volume (definition of ROV in section 1.6.2), formed as in section 2.11.2, was passed through the column for 20 minutes after which time the relative oil volume of the exiting emulsion was measured. An emulsion of oil/aqueous tracer dye, 1ml, was then injected into the feed stream just prior to entry into the column. The absorbence of oil and aqueous phases exiting from the column were then measured from collected fractions.

2.11.3 Flow of a Single Oleic Acid Phase

Cells were packed into the column in the normal aqueous suspension as described in section 2.11.1.1. Oleic acid was then pumped at low flow rates in a downward direction through the column, displacing the aqueous phase from the cell-bed. In instances where the biocatalytic activity of the cells was studied it was necessary to oxygenate the oil. The oleic acid was agitated vigorously under vortex for several hours followed by a standing period in which the larger entrained air

bubbles were allowed to escape. The oleic acid was then doped with progesterone.

2.11.3.1 Addition of Aqueous Droplets To the Bed

In some of the later column runs involving a single oleic acid phase, aqueous reaction buffer was added as droplets into the feed stream via a syringe needle attached to a micro peristaltic pump (Varioperpex type 12000, LKB, Stockholm, Sweden). The injection port used for this is similar to that shown in figure 2.11.1.2.

2.11.4 Methanol/Aqueous Cosolvent System

Harvested cells of R.nigricans were washed in copious quantities of reaction buffer B containing 5% v/v methanol. They were then resuspended in reaction buffer B and packed in the column in the normal manner. The cosolvent feed material comprised reaction buffer B plus 10% v/v methanol and 30mg/L progesterone. Collected fractions, 10ml, were placed in a vacuum oven at 40^{0}C for 2hr to remove the methanol that might complicate the extraction process prior to steroid analysis.

2.12 Solvent Extraction of Membrane Lipids

Glassware was rigorously cleaned, rinsed with deionised water and allowed to dry. It was then further rinsed with acetone and again allowed to dry.

Filamentous cells of Rhizopus nigricans, 130g wet weight, were resuspended in 1L of phosphate buffer. 50ml aliquots of cell suspension were transferred to 250ml Quickfit glass stoppered conical flasks (Fisons, Loughborough, Leics.) containing 50ml of the required organic solvent. Control flasks contained 50ml of cell suspension plus a further 50ml of phosphate buffer. The flasks were then placed on an orbital shaker for 1hr at 250rpm and 28^{0}C. The contents of each flask were filtered and the phases collected in 100ml stoppered refluxing

tubes (Tecam, Techne Cambridge Ltd, UK). The tubes were left to stand for 24hr to allow the phases to separate. Using a long needled syringe volumes of both organic and aqueous phases were carefully removed to leave 30ml of each phase on either side of the interface. The removed phases were then jointly transferred to 100ml round bottomed flasks and placed in a vacuum oven (Townsen and Mercer Ltd, Croydon). The temperature was increased gradually over several days until all the organic solvent and aqueous phase had evaporated. The resulting residue was then extracted into 12ml of hexane of which 10ml was transferred to small glass vials and again evaporated under vacuum. The resulting lipids were then redissolved in methanol and stored at 4°C.

2.12.1 Assay of Lipid Fatty Acid Moieties

The methanol extracts were evaporated to dryness under vacuum and resuspended in 2ml of methanol containing 0.5M NaOH. Samples, 400µl, were hydrolysed at 100°C for 3min converting the lipids to their fatty acid moieties. After cooling, 400µl boron trifluoride (12.5% in methanol) was added and allowed to reflux at 100°C for a further 3min. Again the mixture was allowed to cool before extracting into 400µl of hexane, washing with 400µl of deionised water and drying with a few grains of sodium sulphate. Determination of the quantities of the lipid fatty acid moieties was performed by gas chromatography (Perkin Elmer, Beaconsfield, Bucks.).

The operating conditions were:

GC type	:	Perkin Elmer 8310
Column type	:	2mx3mm glass, 10% polybutane-diol succinate on 80:100 mesh Chromosorb W
Oven temp	:	190°C
Injector temp	:	220°C
Detector temp	:	230°C
Carrier gas	:	Helium, 40ml/min
Sample volume	:	2µl
Separation time	:	27min

Fatty acid standards (Sigma Ltd, Poole, Dorset) were prepared and analysed under the same conditions and the peak areas or heights compared with those of the experimental samples.

2.13 Effect of Organic Solvents on Hydroxylase Activity

Harvested cells of Rhizopus nigricans were resuspended in 1.5L of reaction media B. Samples, 5ml, were placed in vials containing a further 5ml of reaction media B, micronised progesterone 25.5mg (±0.5mg) and organic solvent. The amount of progesterone used was in excess of the amount required to saturate the solvents (ie. undissolved progesterone was present in the vials). A possible exception occurred using larger volumes of ethyl benzoate where particulate progesterone was not observed in the vials. Solvents were equilibrated in a water bath at 25°C for 2hr before the required volume was added to the vials. At all stages the vials were capped quickly to minimise the loss of volatile solvents through evaporation. Control vials were used to account for steroid that might have been transferred from the induction process. These contained no solvent but instead 1ml of phosphoric acid to stop the reaction. Samples of cell suspension were also taken at regular intervals for dry weight analysis to ensure that variations in cell density were not significant.

The vials were agitated on a whirlimixer (Fisons, Loughborough, Leics.) to resuspend the progesterone and then placed on a reciprocal shaker at 25°C and 120 strokes per minute for 24hr. Hydroxylase activity was stopped by the addition of 1ml of 50% phosphoric acid to each of the vials. Those vials containing greater than 50µl of solvent were then placed in a vacuum oven (Townsen and Mercer Ltd, Croydon) at 40°C and 1.3KPa for 3hr, to remove excess solvent that would complicate the partition of steroid in the extraction procedure. 11α-hydroxyprogesterone levels were then analysed (section 2.15.1).

2.14 Fungal Wet/Dry Weight Ratio Determination

2.14.1 Percoll Gradient Determination of Fungal Density

Cell densities were measured by density gradient centrifugation. Gradients were composed of 10 times strength reaction buffer B, Percoll solution (Pharmacia Ltd, Milton Keynes, Bucks.) and deionised water. The required volumes of each were calculated using the following equation to give an isotonic solution that had a density close to that of the organism.

$$V_0 = \frac{V \times (P - \{0.1 \times P_{10}\} - 900)}{P_0 - 1000} \qquad [E2.14.1]$$

where V_0 = volume of Percoll required, L

V = final volume of working solution, L

P = desired density of final solution, Kg/m^3

P_0 = density of neat Percoll, Kg/m^3

P_{10} = density of 10x strength reaction buffer, Kg/m^3

The density of the cells was estimated to be around 1080 Kg/m^3, while the density of the 10 times strength reaction buffer B was measured (1062 Kg/m^3) using an hydrometer. Having made up the Percoll solution 10ml aliquots were placed into graduated centrifuge tubes along with one drop of cell suspension and Pharmacia density beads (Pharmacia Ltd, Milton Keynes, Bucks.). Gradients were formed in situ by centrifugation at 10000g at 10^0C for 30min (Mistral 24, MSE Ltd., Crawley, West Sussex). The density of the cells was then determined from the relative position of the cells and the marker density beads.

2.14.2 Density Bottle Determination of Fungal Wet/Dry Weight Ratio

The wet to dry weight ratio of fungal cells may be calculated from the equations below using density bottles if the density of the suspending buffer and the cells are known.

$$M_1 = M_b + (V_b \times P_a) \quad [E2.14.2a]$$

$$M_2 = M_b + P_a (V_b - V_c) + P_c \times V_c \quad [E2.14.2b]$$

$$V_c = (M_2 - M_1) / (P_c - P_a) \quad [E2.14.2c]$$

Wet weight of cells = $V_c \times P_c$

where M_1, M_2 are the masses (Kg) of the density bottle, M_b, plus buffer suspension and cell suspension respectively. V_b and V_c are the volumes (m^3) of the density bottle and wet cells respectively. P_a and P_c are the densities (Kg/m^3) of the buffer suspension and cells respectively. P_a may be calculated from equation [E2.14.2a] or measured using an hydrometer. P_c is measured using density gradient centrifugation, section 2.14.1.

The dry weight of cells is obtained by filtering the cells used to obtain the mass, M_2. This was achieved by Buchner filtration through Whatman grade 1 filter paper (Whatman Ltd, Maidstone, Kent). The cells were then washed with deionised water, dried at 80°C for 24hr and weighed

2.14.3 Wet/Dry Weight Ratio Determination by Filtration

Two types of filtration method were used. Both used as material filamentous cells of Rhizopus nigricans resuspended in phosphate buffer with 0.001% Tween 80 (BDH Ltd, Poole, Dorset) as surfactant to minimise the amount of extra-cellular water remaining at the end of filtration.

In the first of the methods a range of cell suspension volumes were filtered on a Buchner funnel through Whatman grade 1 filter paper (Whatman Ltd, Maidstone, Kent) for 3min. Any cell cakes that cracked during this period were discarded. The resulting cakes were weighed, dried at 80°C for 24hr and reweighed.

The second method used centrifugal filtration as a means of dewatering the cells. Two centrifugal filter units (figure 2.14.3) were filled with 20ml volumes of cell suspension and spun at 3900rpm in a bench top centrifuge (Centaur 2, MSE, Crawley, West Sussex) for 5min. The cell cakes were weighed, dried at 80°C for 24hr and reweighed. The filter

membrane used in the filter unit was a Terylene cloth (type J/SYN/32, Johnson-Progress Ltd., London).

2.14.4 Dye Exclusion Method for Wet/Dry Weight Ratio Determination

Water soluble dyes were made up in solutions of reaction buffer B and their absorbences measured at the extinction maxima. Dyes used were naphthol green B (710nm) and blue dextran (620nm) both from Sigma Ltd, Poole, Dorset. A fixed volume, 10ml, of dye solution was mixed with different volumes of cell suspension. The cells were filtered and washed with a further 20ml of deionised water to remove excess dye solution. This filtrate was added to the original filtrate and the absorbence measured in a spectrophotometer (model 552, Perkin Elmer, Beaconsfield, Bucks.). The dewatered cell mass was removed, dried at 80^{0}C for 24hr and weighed.

2.15 Assay Procedures for Steroids

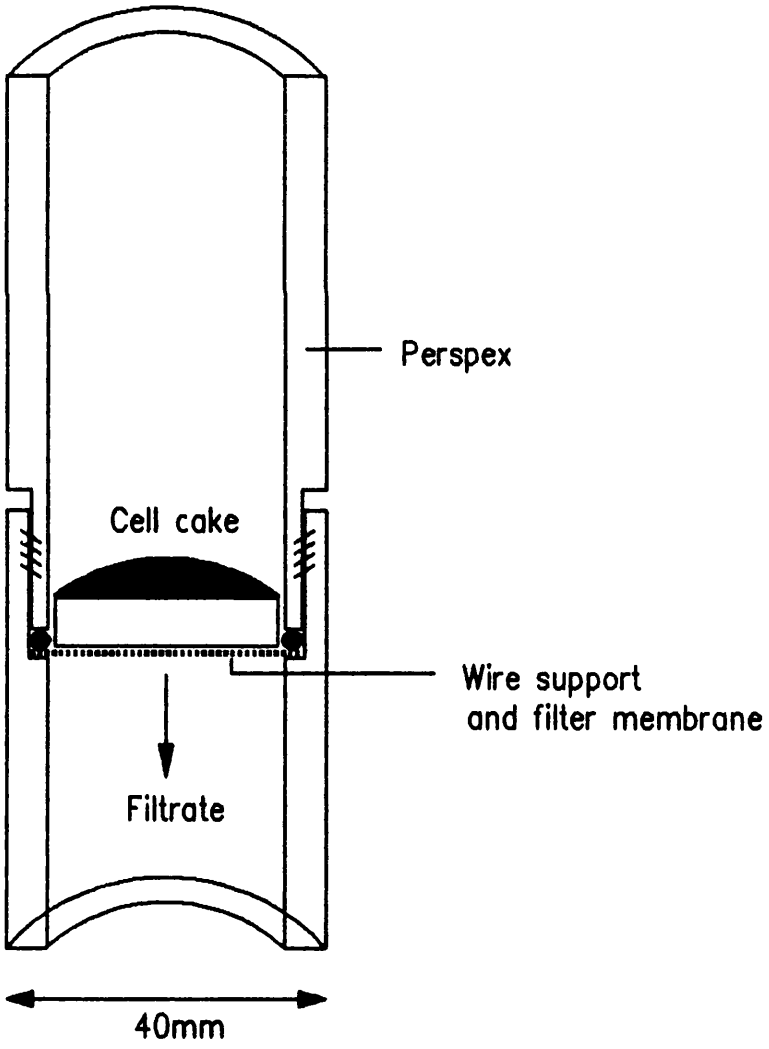
2.15.1 Extraction and Treatment of Aqueous Phase Samples

A known volume (5-10ml) of aqueous phase, inclusive of cells, was shaken with 1ml of 50 percent phosphoric acid to stop the reaction. 7ml of ethyl acetate was added and the phases mixed thoroughly using a whirlimixer (Fisons, Loughborough, Leics.). The resulting emulsion was broken by centrifugation at 4000rpm in an MSE Centaur (MSE, Crawley, West Sussex). 4.5ml of the upper organic phase was removed and evaporated to dryness in a vacuum oven at 40^{0}C and 1.3KPa.

2.15.2 Extraction and Treatment of Oleic Acid Phase Samples

The extraction procedure described was modified from a method used for the determination of pesticide residues in fat tissue (De Faubert Maunder 1964). 5ml of acetonitrile was added to 3ml of oleic acid and mixed thoroughly (Whirlimixer, Fisons, Loughborough, Leics.). The

Figure 2.14.3 Diagram (to scale) of the centrifugal filter unit used in the wet/dry weight ratio determination of R.nigricans. The unit was spun at 3900rpm for 5min in a bench top centrifuge (Centaur 2, MSE, Crawley, West Sussex).



emulsion formed was separated by centrifugation (Centaur, MSE, Crawley, West Sussex) at 4000rpm. 3ml of the acetonitrile phase was removed and added to 3ml of hexane and the phases thoroughly mixed. The phases were allowed to separate and the top hexane phase discarded by removing with a pasteur pipette. 2ml of the acetonitrile solvent was then removed and evaporated to dryness in a vacuum oven at 40°C and 1.3KPa.

2.15.3 Steroid Assay by HPLC

Steroid residue (obtained as described in sections 2.15.1 and 2.15.2) was dissolved in 1ml of 100 percent methanol and filtered into HPLC sample bottles through 0.45µ Durapore filters (Millipore, London). Samples were assayed using high pressure liquid chromatography (LDC, Stone, Staffs. and Perkin Elmer, Beaconsfield, Bucks.). The operating conditions for the HPLC were as follows;

Conditions for the HPLC were as follows with a Perkin Elmer LC-75 Spectrophotometric Detector used.

Pressure	:	20-21 MPa
Flow rate	:	1.25 ml/min
Mobile phase	:	Methanol:water (80:20 v/v)
Column	:	Spherisorb $C_8$5µ (LDC, Stone, Staffs.)
Loop volume	:	20µl
Separation time	:	7min

Peak areas or heights were compared with progesterone and 11α-hydroxyprogesterone standards.

CHAPTER 3

3 PRELIMINARY EXPERIMENTAL RESULTS

3.1 Fermentation Results

3.1.1 Shake Flask Growth of *Rhizopus nigricans* and *Aspergillus ochraceus*

Protocols for the shake flask growth of Rhizopus nigricans and Aspergillus ochraceus were developed from those of Thomas (1988) and Ceen (1986) respectively. These workers allowed the growth of the organisms to continue for 24hr after inoculation with spores so that optimal biomass was obtained. It was felt however, that the cells by this stage were either nearing the end of exponential growth or entering the stationary phase and that some autolytic processes may be occurring. This is known to take place when cells are stressed (Trinci and Righelato 1970). Since optimal biomass production was not a requirement of this work it was decided to reduce the growth period to 18hr or in some cases 15hr so that the cells were harvested in mid-exponential phase. By this method it was hoped that the effects of autolytic processes, which might significantly affect the state of the cell membranes and enzyme turnover, could be minimised. The time of enzyme induction was kept the same, at 3hr prior to the harvesting of cell biomass. Biomass production was very consistent and by 18hr thick voluminous suspensions were produced.

3.1.2 Fermentation of *Rhizopus nigricans*

Growth of R.nigricans in the 20L LH fermenter was very reproducible and could be monitored using the on-line analysis system. Characteristic fermentation data profiles for filamentous and pelletted growth are shown in figures 3.1.2a, 3.1.2b. One aspect of the fermentations that showed variability was the presence or absence of a diauxic lag phase. This occurs as a result of the cells switching utilisation of carbon sources from those in the yeast extract to that of the glucose present (Hanisch 1978). When such diauxic growth took place the lag period,

which was of about 1hr duration, generally occurred at between 3 and 6 hours during filamentous growth and between 8 and 10 hours during pelletted growth.

Cells were harvested at around 14 to 15 hours after inoculation of the fermenter. This corresponded to late exponential phase or occasionally to early stationary phase if cell growth was particularly rapid. Although it would have been preferable to harvest the cells at a slightly earlier stage, for the reasons mentioned in section 3.1.1, working hour arrangements made this impractical.

Morphological characterisation of filamentous fermentations was not addressed but the size of pellets resulting from pelletted fermentations was investigated using an image analyser (Magiscan, Joyce Loebl Ltd., UK.). Several parameters are generated using this technique including pellet diameter which is the average of four diameters taken at 45^0 angles to each other, and area of the pellet obscured by fungal hyphae. Figure 3.1.2c shows the distribution of pellet size harvested from a single fermentation and figure 3.1.2d shows how the corresponding hyphal packing density within the pellets is almost independent of the pellet size. The hyphal packing density is defined here as the area of the pellet obscured by hyphae divided by the circular area of the pellet calculated from the diameter.

Cells not used immediately following harvesting of the fermenter were stored for up to 24hr. It was found that cells stored as a dewatered cell cake were extremely difficult to resuspend. This was attributed to continued growth of the hyphae with the result that closely packed cell aggregates or pellets became cross linked. This problem was overcome by storing harvested cells as a suspension in either wash buffer or reaction buffer at 4^0C. Hyphal growth was still expected to take place but the greater separation of the cell aggregates or pellets in the suspension was thought to minimise the amount of cross linking that could occur. Following storage for 24hr the cells were stirred briefly using a paddle. The resulting suspensions appeared to have very similar rheological and packing properties as compared to the fresh cells and were therefore considered suitable for use in packed-bed liquid-flow experiments but not for biocatalysis purposes.

Figure 3.1.1a Fermentation profile for a filamentous fermentation of Rhizopus nigricans in a 20L LH vessel (15L working volume). Aeration, 0.14vvm, tip speed, 2.0m/s, temperature, 28°C and pH 4.5. A diauxic lag phase occurs at 4.5hr.

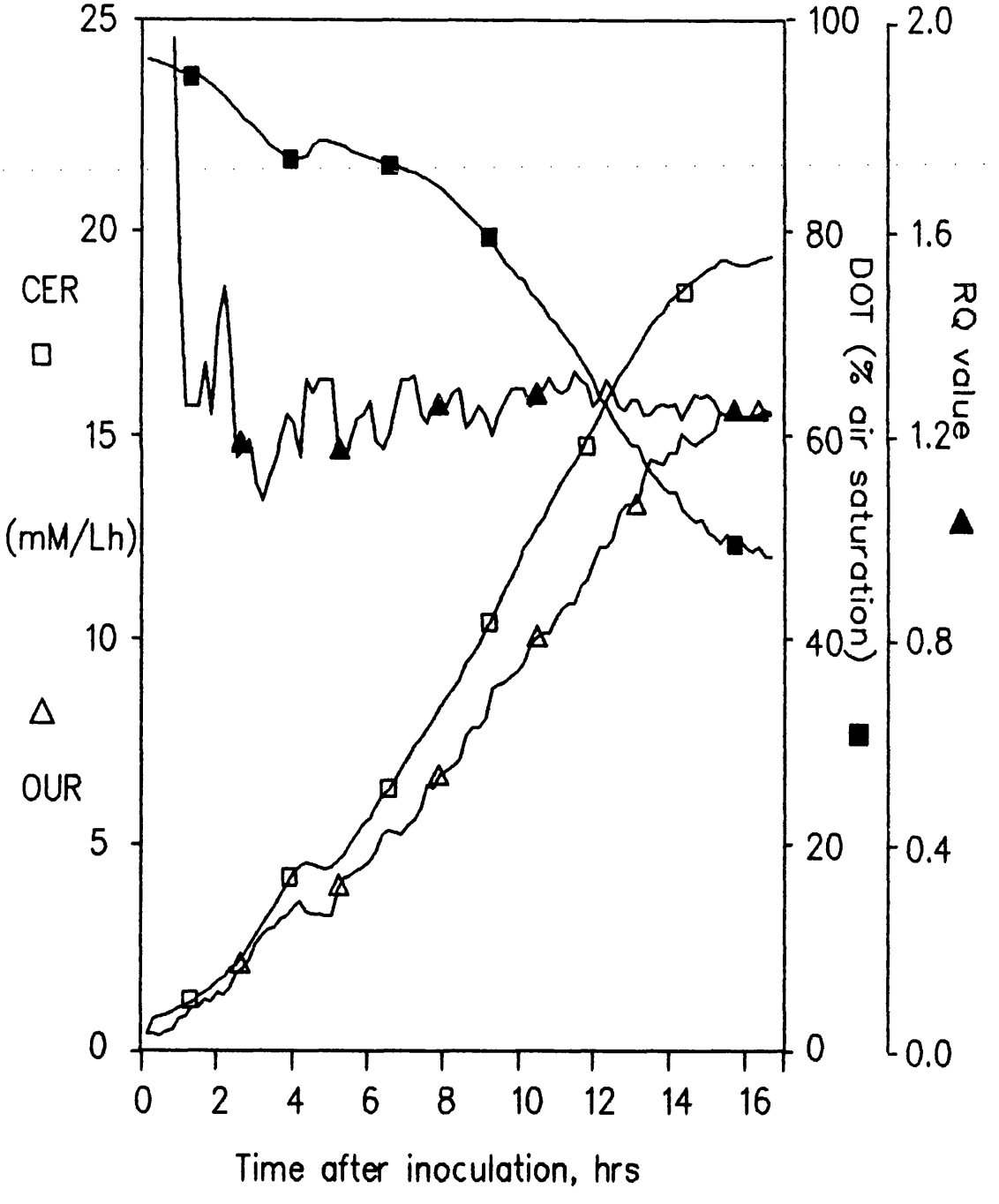


Figure 3.1.1b Fermentation profile for a pelletted fermentation of Rhizopus nigricans in a 20L LH vessel (14.75L working volume). Aeration, 0.14vvm, tip speed, 3.1m/s, temperature, 28°C and pH 4.5. A diauxic lag phase occurs at 9hr.

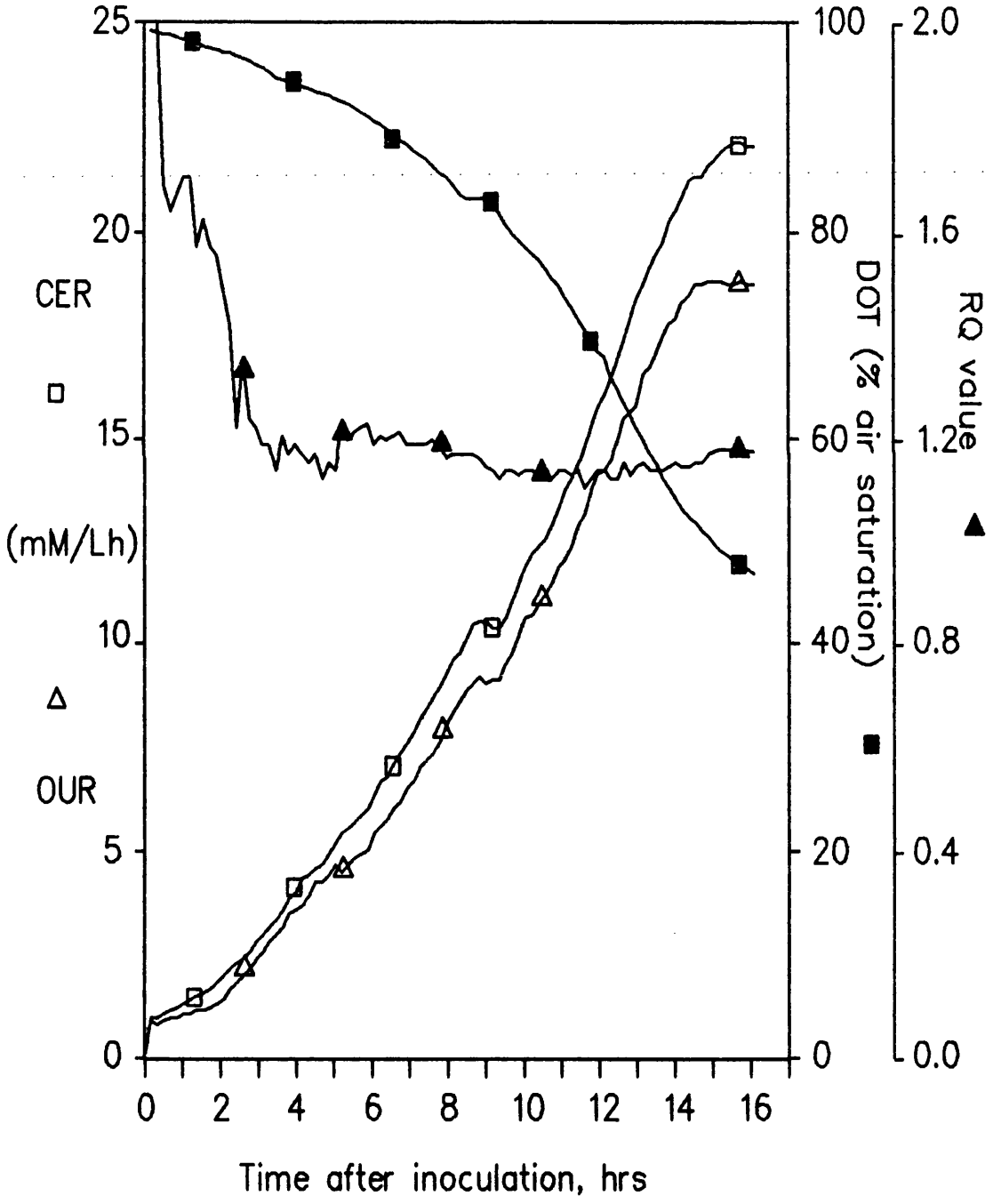


Figure 3.1.1c Histogram showing the distribution of pellet sizes obtained from a single fermentation of Rhizopus nigricans. The bars show the percent of pellets falling in the size range from the value of the previous bar to that of the labelled bar.

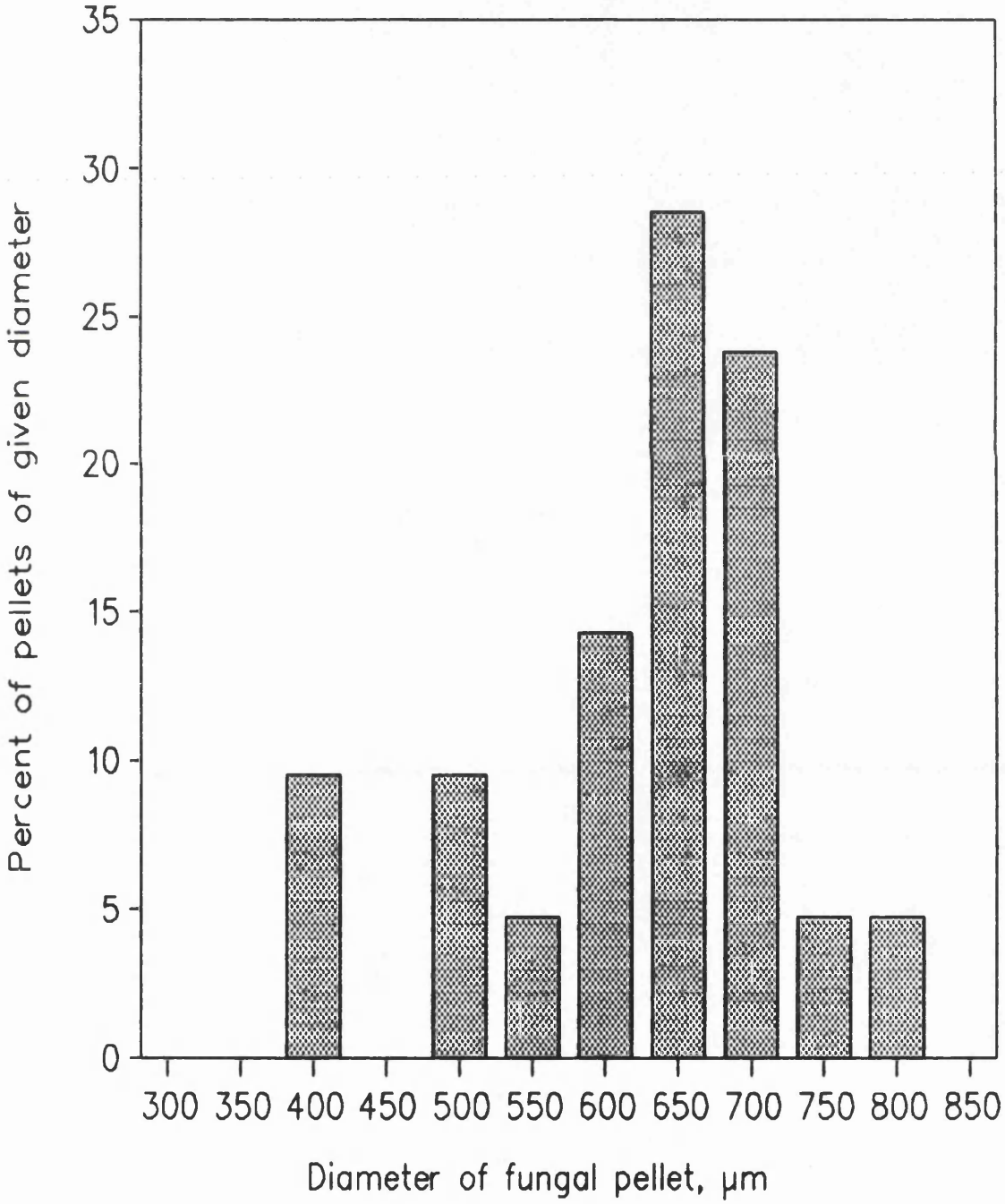
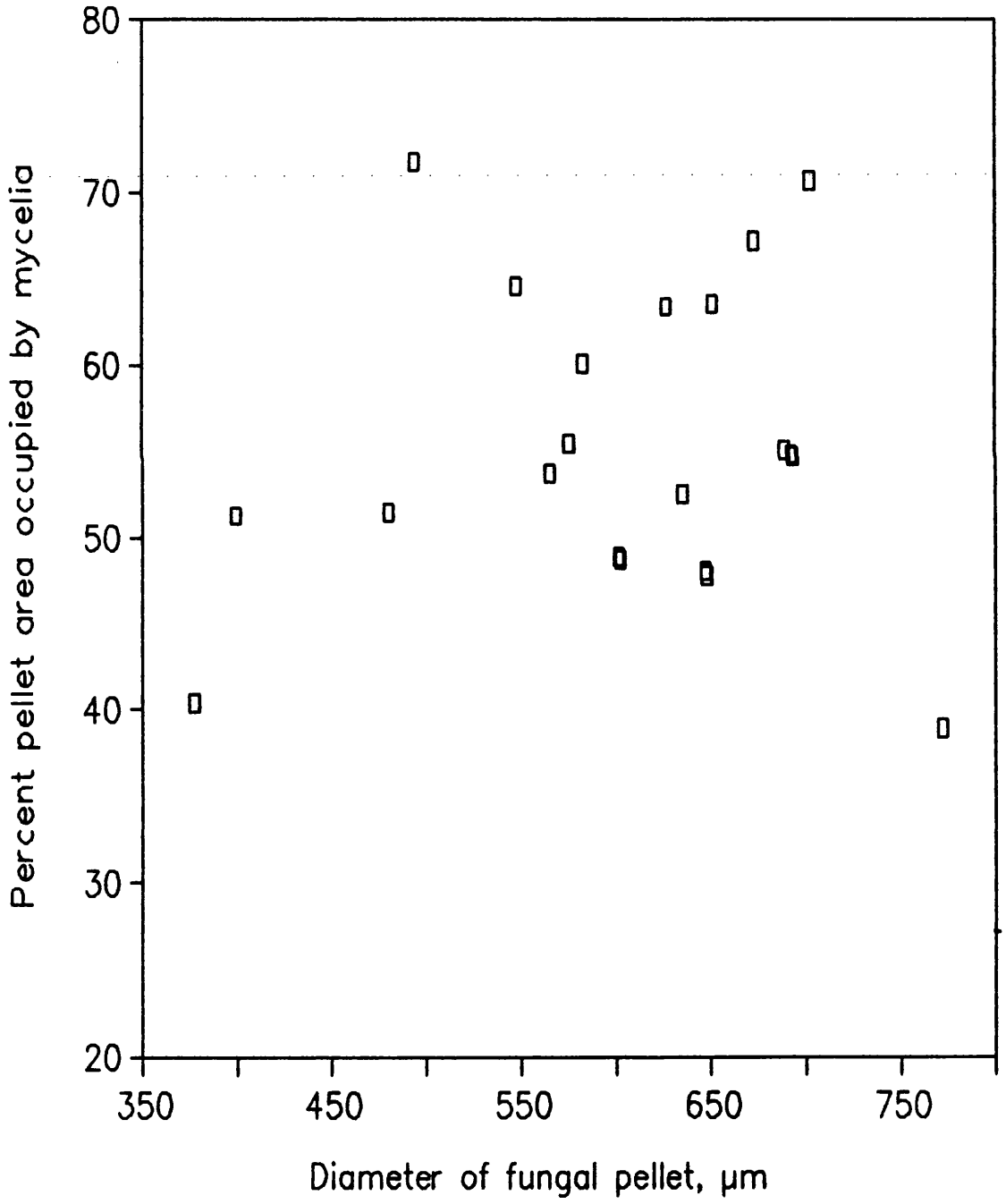


Figure 3.1.2d Graph showing the hyphal density (percent of pellet area occupied by mycelia) within the cell pellets as a function of the pellet size. Hyphal density is calculated from image analysis measurements as follows;

$$\frac{\text{area of pellet obscured by hyphae}}{\text{calculated area of a pellet of given diameter}} \times 100$$



3.2 Fungal Wet/Dry Weight Ratio Determination

The wet/dry weight ratio of cells of Rhizopus nigricans was required so that the volume fraction of cells present in the packed-bed column experiments could be determined and liquid superficial linear flow velocities calculated. Several of the methods employed required that the density of the cells be known.

3.2.1 Percoll Gradient Determination of Fungal Density

The density of cells of R.nigricans was found by density gradient centrifugation using a Percoll gradient (Pharmacia Ltd, Milton Keynes, Bucks.). The methodology (section 2.14.1) was simple to follow and gave the results presented in figure 3.2.1. The cells formed a narrow band between 4.2 and 4.3cm from the bottom of the centrifuge tube. Reading from figure 3.2.1 gives values for the density of the cells between 1096 and 1097Kg/m^3. The value used for further calculations was taken as the average of the two at 1096.5Kg/m^3.

3.2.2 Density Bottle Determination of Fungal Wet/Dry Weight Ratio

The nature of filamentous suspensions is such that cell volume fractions greater than about 0.07 are not sufficiently fluid to allow the use of normal density bottles. Also at these higher volume fractions entrainment of small air bubbles occurs within the suspension. To obtain sufficiently accurate dry weights, high cell masses were needed which required the use of 0.5L volumetric flasks in place of the normal density bottles. Suspensions were also degassed by an applied vacuum to remove both dissolved air and entrapped air bubbles. Results of the wet/dry weight ratio obtained in this manner are shown in table 3.2.2.

Figure 3.2.1 Gradient centrifugation data for the density determination of cells of Rhizopus nigricans. The fungal cells formed a narrow band at between 4.2 and 4.3cm from the base of the centrifuge tube corresponding to an average density of 1096.5 Kg/m^3.

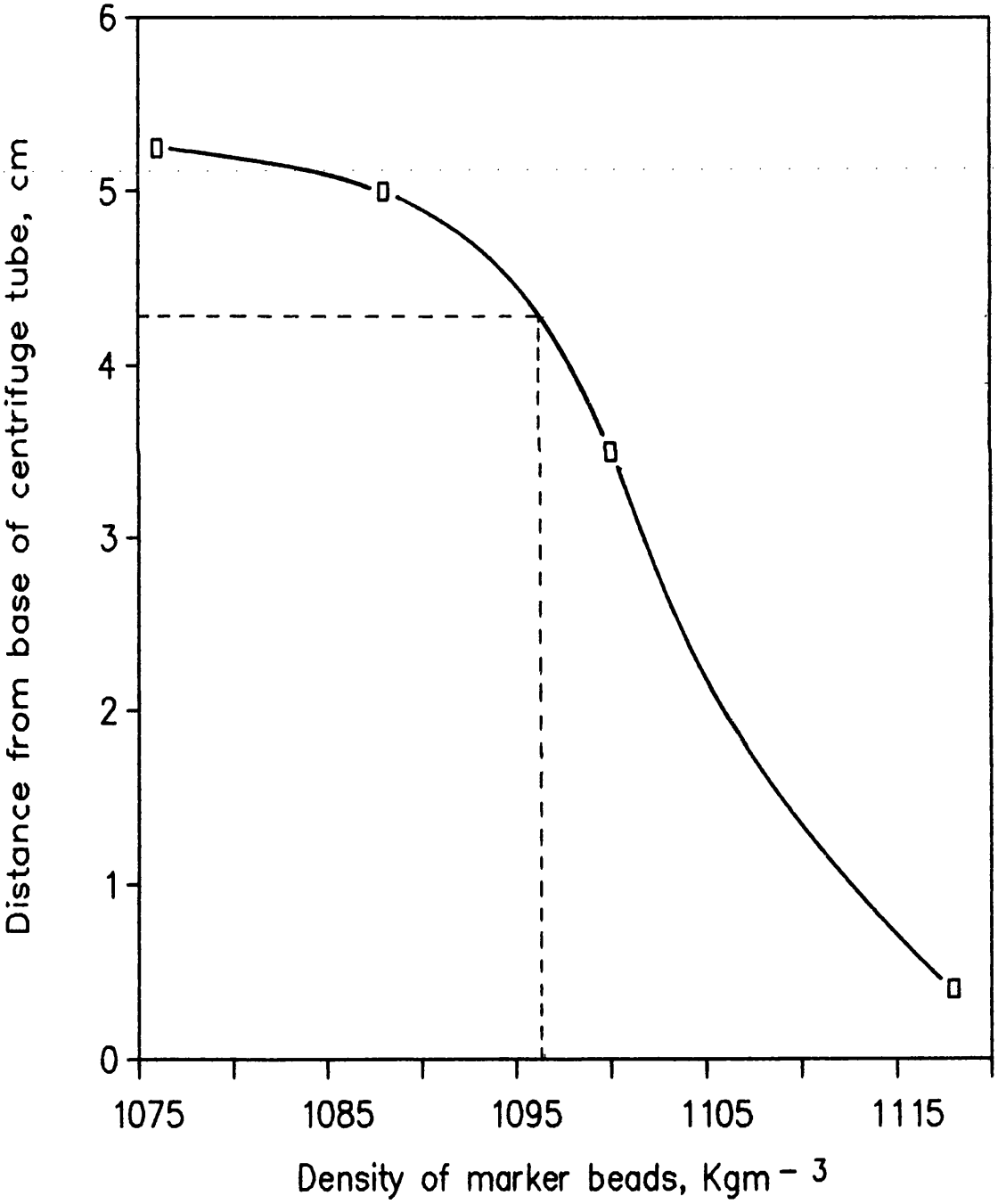


Table 3.2.2

Wet to dry weight ratio for cells of R.nigricans		
	Density bottle method	Centrifugal filtration
	3.67 3.64 3.56 3.52	3.55 3.58
Average	3.60	3.57

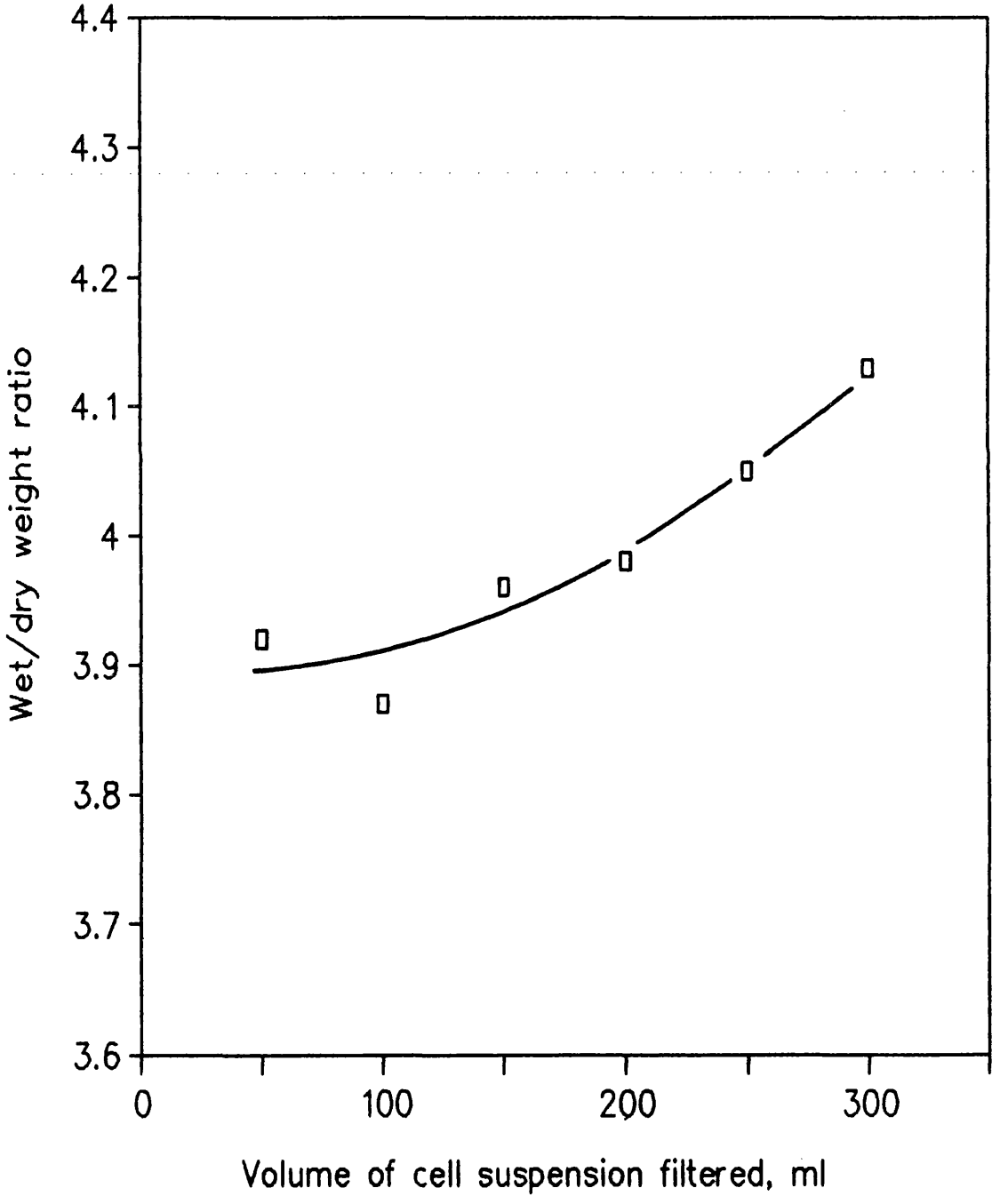
3.2.3 Wet/Dry Weight Ratio Determination by Filtration

Two filtration methods, vacuum and centrifugal, were also employed to try and determine the wet/dry weight ratio of R.nigricans. Vacuum filtration of a range of suspension volumes gave the results shown in figure 3.2.3a. The slight increase in wet/dry weight ratio with increasing volume of cell suspension used would tend to imply that the efficiency of the vacuum filter to remove all of the interstitial buffer decreases as the cell cake becomes thicker. The lower values for wet/dry weight ratio are therefore likely to be more accurate. These values however, may still be too high due to excess water and traces of surfactant that cannot be removed by this method.

Only two results were obtained from the centrifugal filtration of cell suspensions of R.nigricans. These values of wet/dry weight ratio, table 3.2.2, are very similar to those obtained by the density bottle method.

To explain the differences in the wet/dry weight ratio values obtained by the two filtration methods one has to consider how each method works. In the centrifugal filter, irrespective of whether the cell cake cracks or not, the buffer trapped within the cake has a continual applied force trying to remove it from the cell mass. However, in the

Figure 3.2.3 The effect of cell suspension volume on the wet/dry weight ratio of R.nigricans obtained by vacuum filtration.



vacuum filter method if areas of the cake dry out, or cracks appear which allow the easy passage of air, the pulling force of the vacuum on the remaining cake is considerably reduced and may prevent the complete removal of buffer from these areas. This then increases the apparent wet weight of the cells and so the overall wet/dry weight ratio.

3.2.4 Wet/Dry Weight Ratio Determination by a Dye Exclusion Method

The principle of this technique is explained fully in appendix 1. Several values for the cell wet to dry weight ratio were obtained from the dye exclusion method (appendix 1, table A1.3) depending on whether constrained or unconstrained regression fits to the data points were applied (also explained in appendix 1). Of these the most realistic value obtained was equal to a ratio of 4.27. However, in performing the calculations it was realised that due to the nature of the equations, very small changes in the gradient of the graph could have very profound effects on the value of the wet/dry weight ratio. As an example, a 1 percent change in the gradient produces a 39 percent change in the value of the wet/dry weight ratio. Small inaccuracies in the measurement of volumes, which are almost inevitable when trying to pipette fungal suspensions, make the method inherently unreliable. It is suggested therefore, that the use of this method should be limited to those instances when only a very approximate value is required.

3.3 Assay Procedures for the Determination of Steroid Concentrations

Methodologies for the extraction of steroids from aqueous, organic solvent and oil phases were adapted from the procedures detailed by Ceen 1986. The major alteration to the procedures was the use of larger volumes of extraction solvents, 7ml compared to 4ml ethyl acetate and 5ml compared to 4ml of acetonitrile. With the phases separated following centrifugation, the removal of a quantity of the extraction solvent without disturbance of the interface was made considerably easier. This meant that the samples were far less contaminated with extraneous material (fungal cells, debris etc.). In some instances it

Figure 3.3a Calibration curve for the assay of progesterone by HPLC. Peak areas are compared with the peak area of a reference standard (1.0mg/ml).

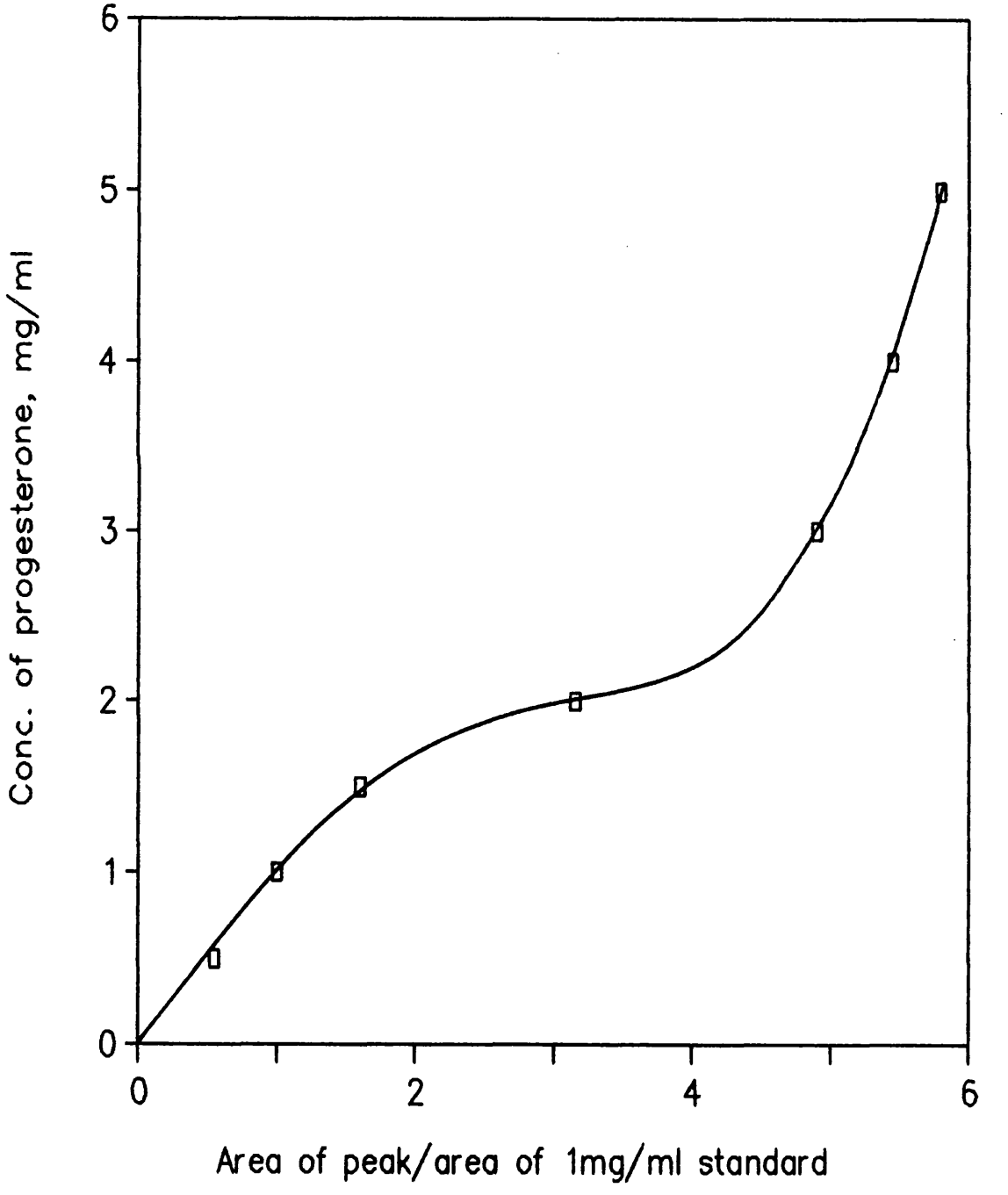


Figure 3.3b Calibration curve for the assay of 11α-hydroxy progesterone by HPLC. Peak areas are compared with the peak area of a reference standard (1.0mg/ml).

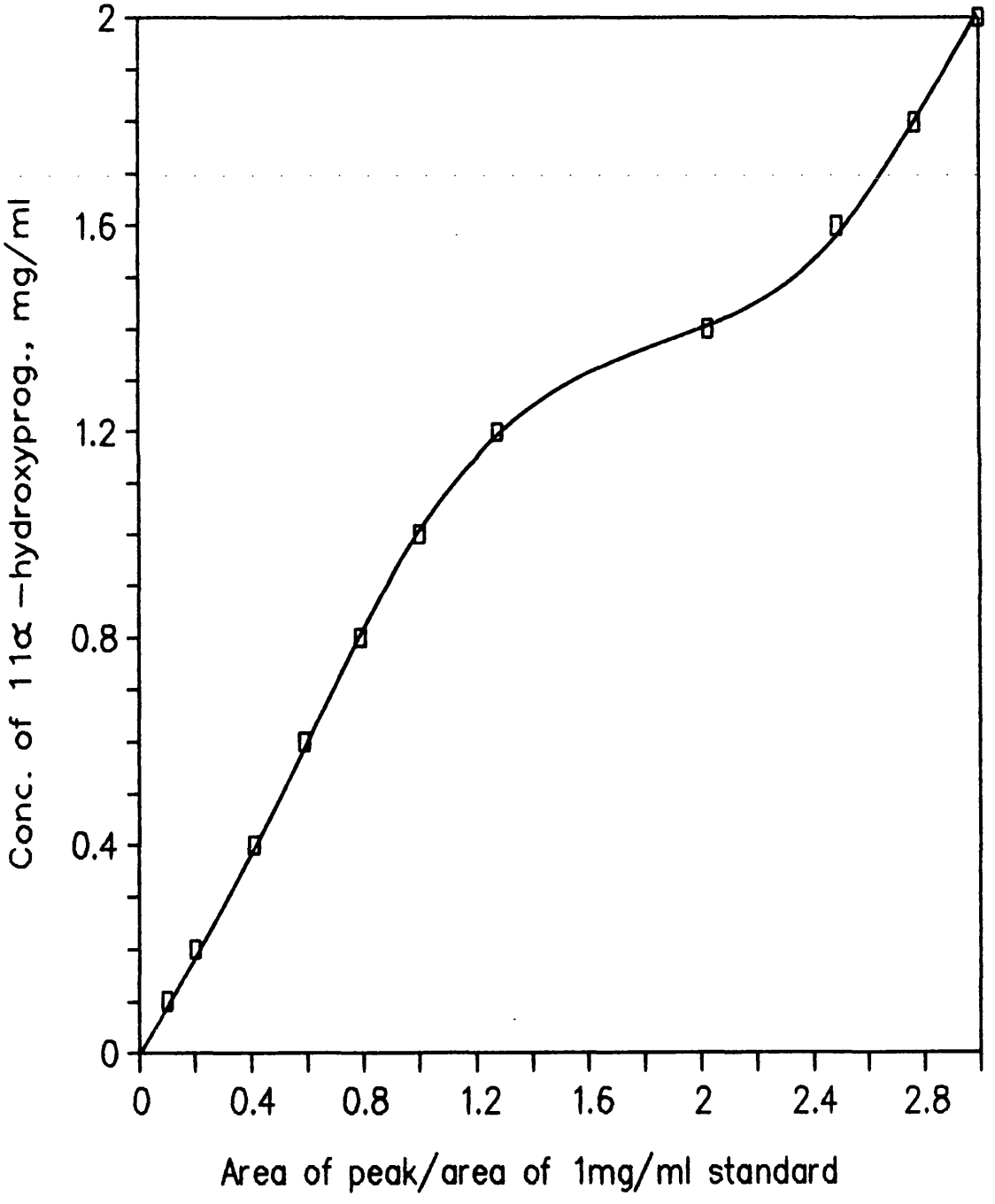


Figure 3.3c Calibration curve for the assay of 11α-hydroxy progesterone by HPLC at low final methanol concentrations. Peak areas, + , and peak heights, □ , are compared with the peak area/height of a reference standard (0.1mg/ml).

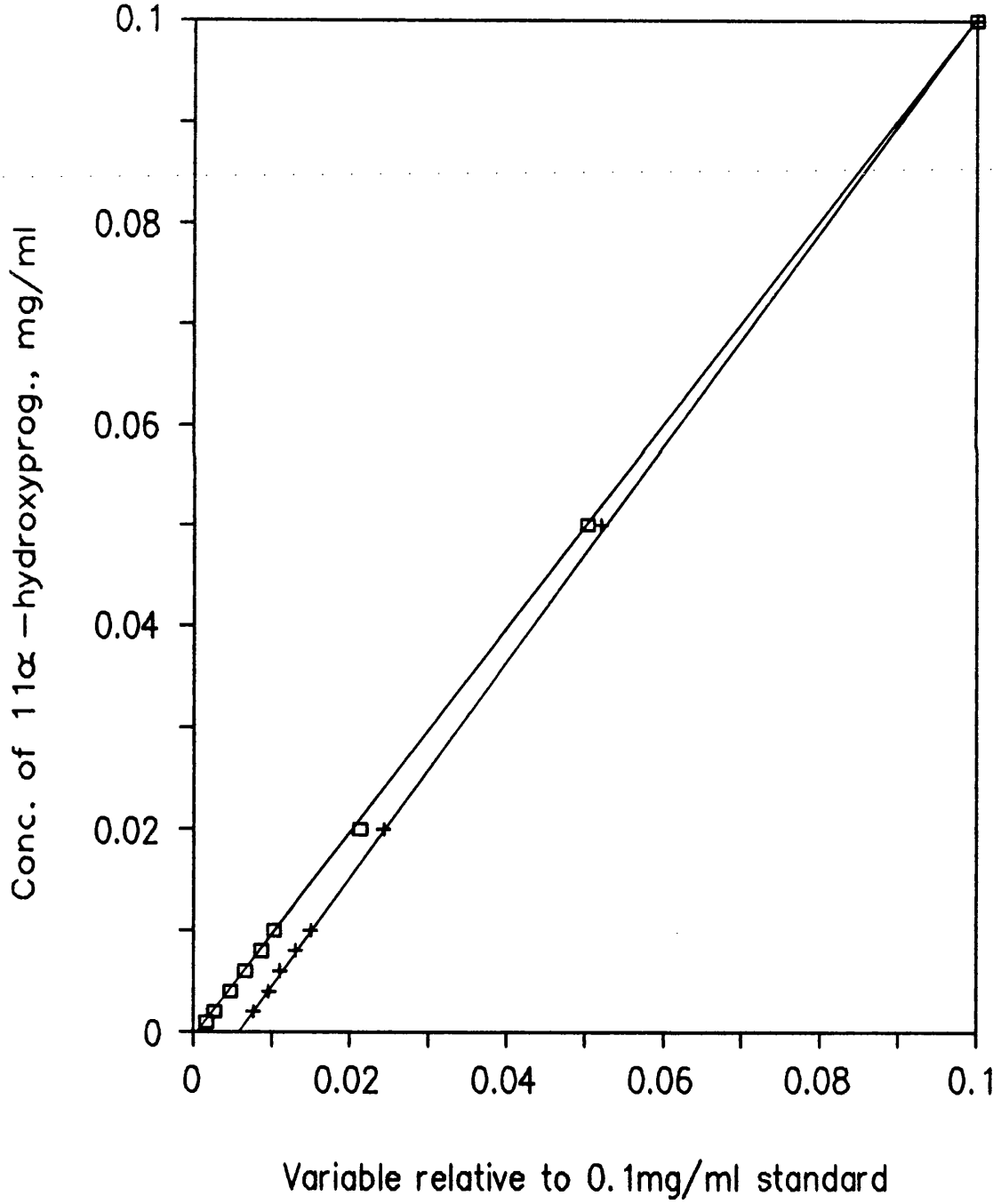
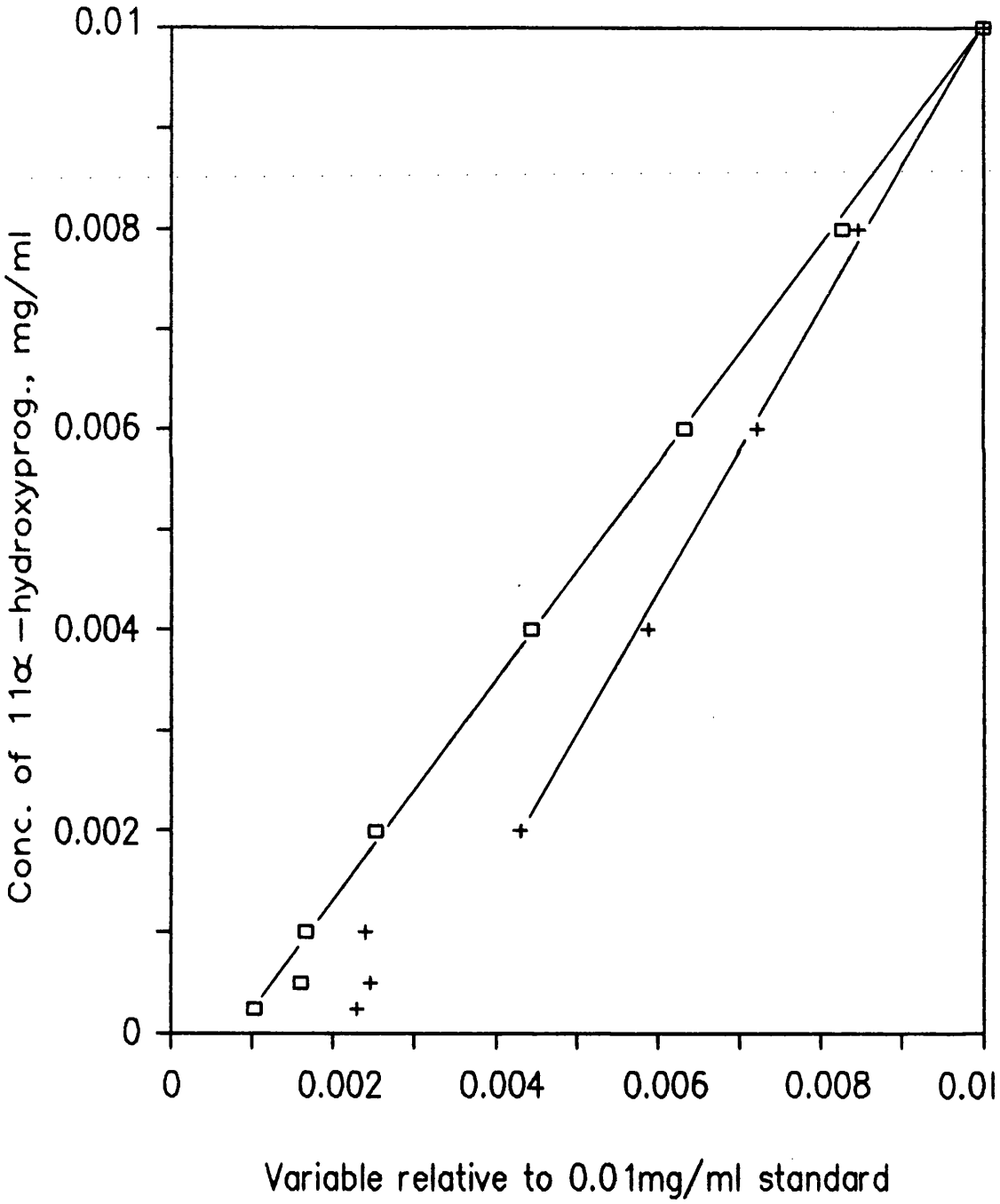


Figure 3.3d Calibration curve for the assay of 11α-hydroxy progesterone by HPLC at very low final methanol concentrations. Peak areas, + , and peak heights, □ , are compared with the peak area/height of a reference standard (0.01mg/ml).



was possible to load the final methanol samples directly onto the HPLC without the requirement of filtering. Calibration curves for both progesterone (figure 3.3a) and 11α-hydroxyprogesterone (figure 3.3b) show non-linearity of the peak area data above a concentration in the final methanol of 1.0mg/ml. Experimental assay samples were therefore diluted with methanol, if necessary, to bring them into the concentration range 0.2-1.0mg/ml. The assay samples produced from the cosolvent column experiments were of extremely low steroid concentrations. The linearity of the calibration curves of 11α-hydroxyprogesterone below 0.1mg/ml were therefore checked. Figures 3.3c and 3.3d show that at these lower concentrations use of peak height as the reference variable is far more accurate. With extremely clean samples, producing little background noise, concentrations in the final methanol of 0.001mg/ml can be practically measured.

3.4 Preliminary Experiments to Assess the Suitability of Using *Rhizopus nigricans* in Two Liquid-Phase Systems

Using the filamentous fungus Aspergillus ochraceus as the biocatalyst and the 11α-hydroxylation of progesterone as the reaction, Ceen 1986 demonstrated the unsuitability of a range of conventional organic solvents for use in two liquid-phase systems. Loss of biocatalytic activity was found to be extremely rapid with these solvents which prevented their long-term use in reactor studies. With oils and fats as the second liquid-phase, retention of biocatalytic activity was shown to be greatly increased. After 3hr of contact with these solvents up to 89% of hydroxylase activity still remained as compared with control samples.

An option existed to continue using A.ochraceus as a model system for the two liquid-phase experiments to be studied. This had the advantage that little additional work would have to be performed to define the basic operating conditions and solvent resistance of the organism. There were, however, several reasons that justified the change to Rhizopus nigricans, a fungal microorganism that performs the same 11α-hydroxylase reaction. The reasons for changing to R.nigricans were as follows.

1.) Conditions for growth and induction of the organism were already well defined at the 20L fermenter scale (Hanisch 1978) (Thomas 1988).

2.) Alteration of the organisms morphology from filamentous to pelletted was similarly well defined and reproducible (Thomas 1988).

3.) The ability to isolate microsomes of the hydroxylase complex was present if required (Talboys 1985a,b) (Thomas 1988).

4.) A.ochraceus, unlike R.nigricans, produces ochratoxins that could complicate the large scale production and recovery of catalytic biomass.

Having made the decision to change to R.nigricans it was necessary to perform a set of preliminary experiments that would assess the organisms suitability for use in the presence of organic solvents and show whether it differed significantly in its responses as compared to A.ochraceus.

3.4.1 The Effect of Organic Solvents on the Hydroxylating Ability of *Rhizopus nigricans*

Aqueous cell suspensions of R.nigricans were contacted with an equal volume of the organic solvent for set time periods (3, 5 and 10min). The cells were then harvested, washed and assayed for 11α-hydroxylase activity (section 2.8). The results, figure 3.4.1a, show that for all the conventional organic solvents except hexane, loss of biocatalytic activity is rapid and virtually complete within a contact period of only 10min. Hexane is the least denaturing of the solvents causing a 50 percent loss of hydroxylase activity after 10min contact of the organism with the solvent. The results are very similar to those given for A.ochraceus (Ceen et *al* 1987a).

In a similar experiment the conventional organic solvents were replaced by oleic acid as the second liquid-phase. The contact times were extended to 1, 2 and 5hr followed by harvesting, washing and assaying as above. Loss of activity was rapid over the first hour but appeared to be little affected by the contact time during the following 4hrs,

figure 3.4.1b. The activity remained at approximately 77% of the aqueous control. The high retention of biocatalytic activity in this system, which was similarly shown by Ceen 1987a for A.ochraceus, indicated the potential for using oleic acid as a second liquid-phase over extended reactor times.

3.4.2 The Effect of Oleic Acid on the Growth and Hydroxylase Activity of *Rhizopus nigricans*

The possibility of regenerating fungal biomass in an organic/aqueous two liquid-phase system was investigated by attempting to grow R.nigricans in the presence of oleic acid. In situ growth of the organism and regeneration of lost biocatalytic activity, was viewed as a favourable additional criteria for an organic solvent which was to be used in a continuous reactor configuration over extended time periods. Table 3.4.2 shows the effect of oleic acid on the growth from spores of R.nigricans and the resulting 11α-hydroxylase activity of the biomass produced. The addition of oleic acid to the shake flask cultures is seen to only slightly decrease the amount of biomass produced. This effect may be due to one or more factors. Firstly the oleic acid may be exhibiting a direct fungistatic and/or fungitoxic activity as discussed in section 1.3.2. Alternatively the organic phase, which under the agitation conditions of the experiment was not well dispersed and formed a semi-continuous layer on top of the aqueous media phase, may act indirectly. In this way it may reduce the rate of oxygen transfer to the cells and so act to limit the growth rate of the organism. The actual reasons for the reduced biomass production were not investigated further.

Further growth of the organism is of little use if the resulting biomass does not perform the required biocatalytic conversion. However, table 3.4.2 shows that appreciable levels of 11α-hydroxylase activity are present in cells of R.nigricans grown and induced in the presence of oleic acid. From these results it would appear that the regeneration of biocatalytic biomass within a two liquid-phase reactor system is possible using a suitable organic solvent as the second phase.

Figure 3.4.1a Loss of 11α-hydroxylase activity by cells of R.nigricans in an aqueous assay as a function of the exposure time to five conventional organic solvents in shake flask two liquid-phase systems.

	Symbol	$LogP_{octanol}$
Ethyl Acetate	+	0.64
4-methyl pentan-2-one	◊	1.17
2-methyl butan-2-ol	△	1.30
Carbon tetrachloride	X	2.98
Hexane	□	3.52

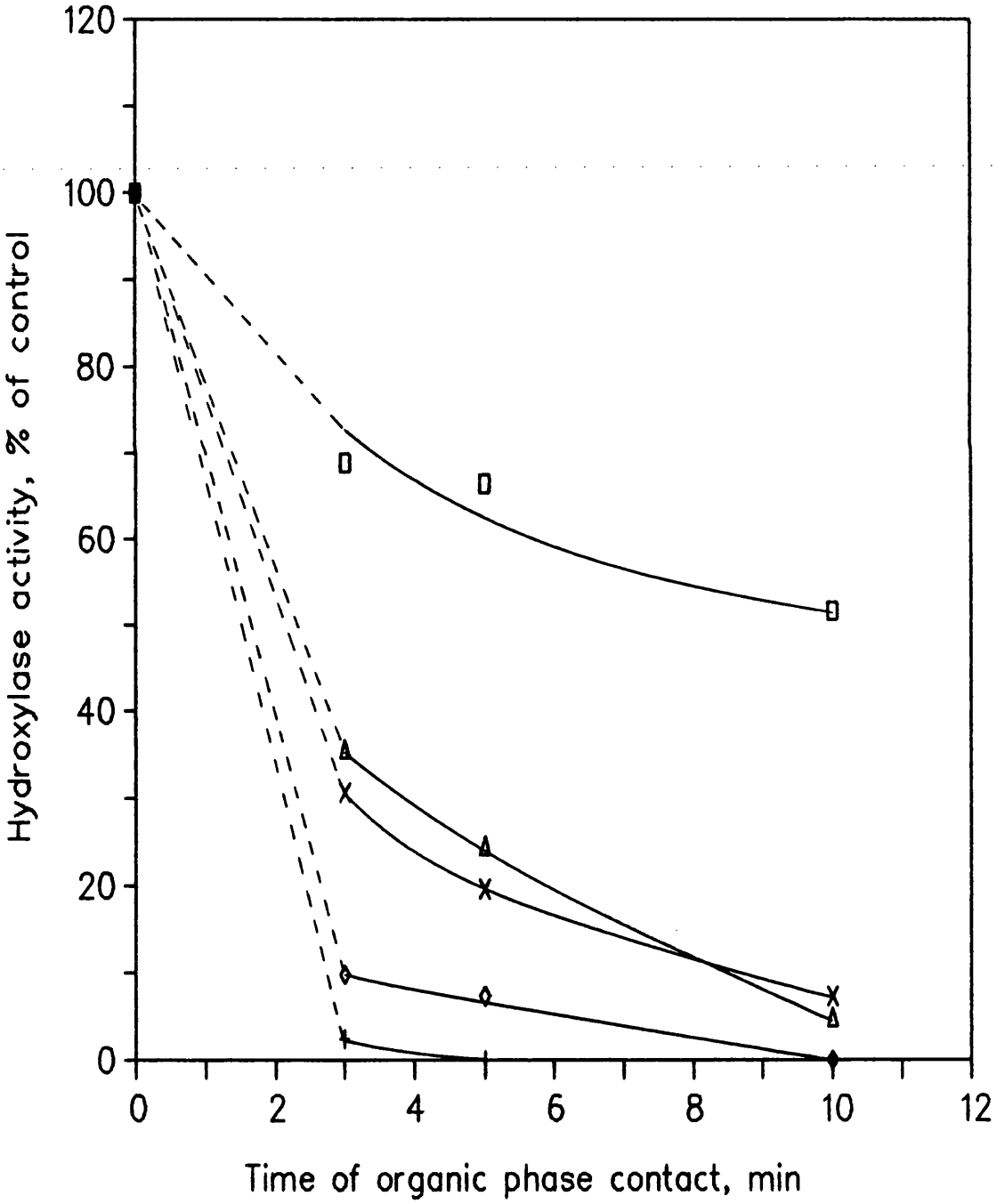


Figure 3.4.1b Loss of 11α-hydroxylase activity by cells of R.nigricans in an aqueous assay as a function of the exposure time to oleic acid in a shake flask two liquid-phase system.

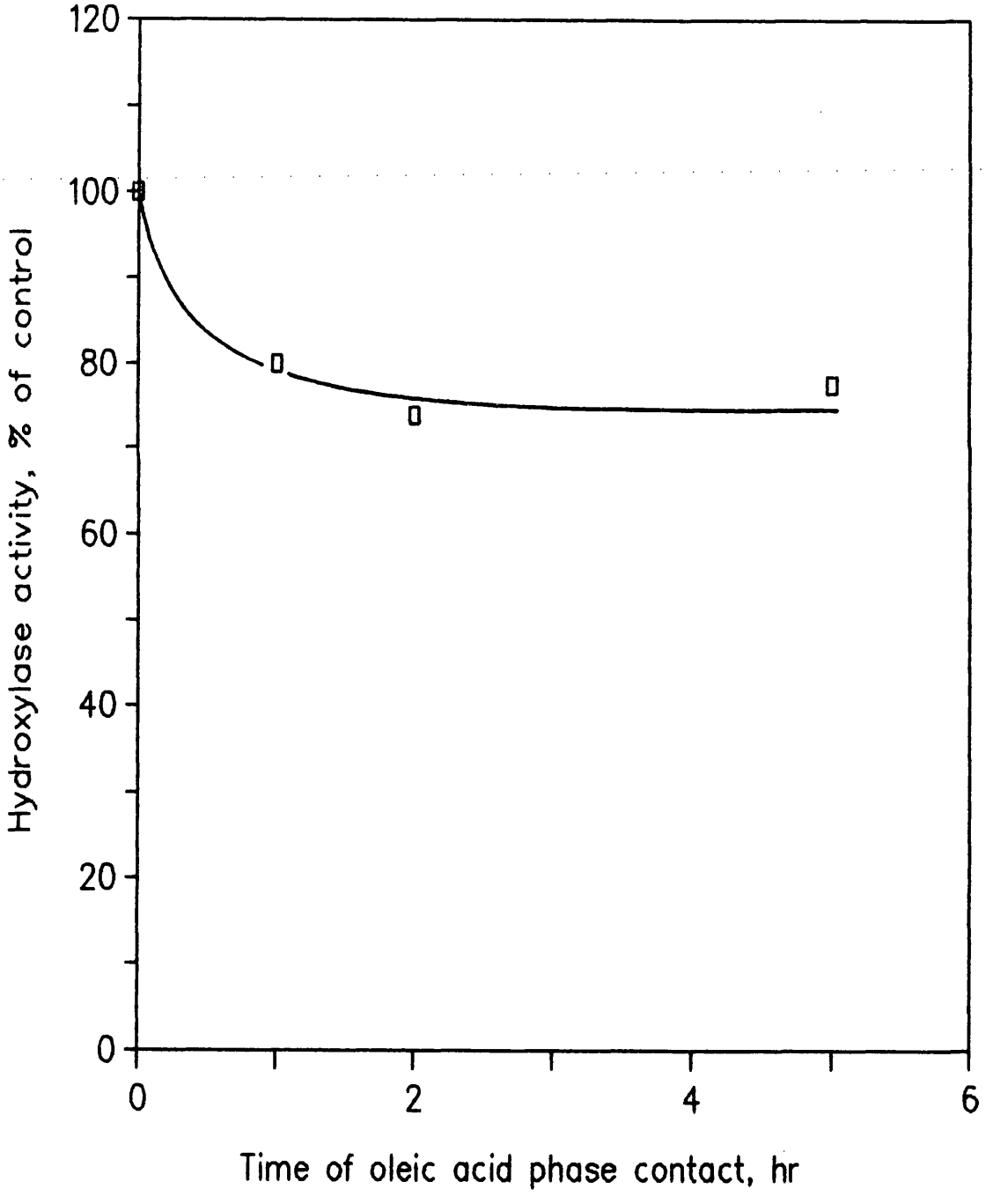


Table 3.4.2 The effect of oleic acid on the growth of R.nigricans and induction of the 11α-hydroxylase enzyme

	Growth conditions for R.nigricans		
	Control	+25ml Oleic A.	+50ml Oleic A.
Biomass Produced (g dry wt)	1.67	1.45	1.39
Assay conditions	Hydroxylase activity (mg/g dry wt/18hr)		
50ml Aqueous	78.9	66.7	78.6
25ml Aqueous + 25ml Oleic acid	84.1	62.4	62.9

3.4.3 The Effect of Agitation on the Hydroxylase Activity of *R.nigricans* and *A.ochraceus* in Oleic Acid/Aqueous Two-Liquid Phase Stirred Tank Reactors

Batch stirred tank reactor studies were performed to further validate the use of R.nigricans in an oleic acid/aqueous two liquid-phase system. The performance of these reactor studies were compared to repeat experiments of the type performed by Ceen 1986 on A.ochraceus. Figure 3.4.3 shows the production of 11α-hydroxyprogesterone after 18hr for both the above mentioned organisms. Although the data for A.ochraceus at a relative oil volume of 0.3 is very similar to that presented by Ceen, the production of 11α-hydroxyprogesterone at 0.5 ROV is significantly higher. This is possibly attributable to a lower cell density within the aqueous phase (5.0mg dry wt/ml) as compared to the experiment of Ceen (6.7mg dry wt/ml) with the result that substrate diffusional limitations within the cell mass may be reduced.

The results for R.nigricans at a ROV of 0.3 are very different to those for A.ochraceus. A possible explanation is the higher aqueous cell densities present in the reactors containing R.nigricans (5.4mg dry wt/ml) as compared to those containing A.ochraceus (4.5mg dry wt/ml). The higher cell densities might counter the expected rises in 11α-hydroxyprogesterone production at higher agitation rates by limiting

Figure 3.4.3 Variation in the production of 11α-hydroxy-progesterone with the degree of agitation in a two liquid-phase batch stirred tank reactor. Oleic acid used as the organic phase.

Organism	ROV	Symbol
R.nigricans	0.3	□
A.ochraceus	0.3	◊
A.ochraceus	0.5	+

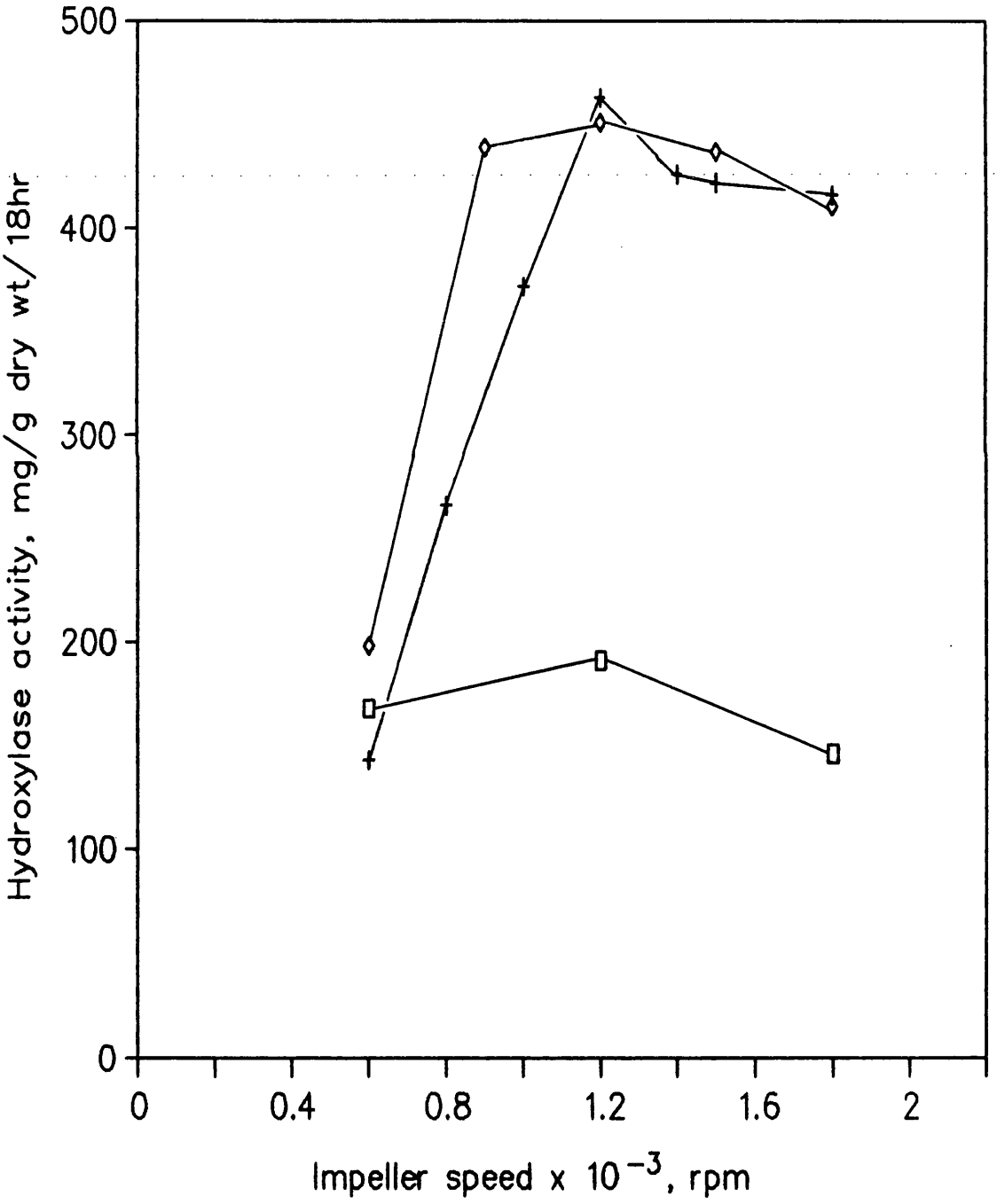
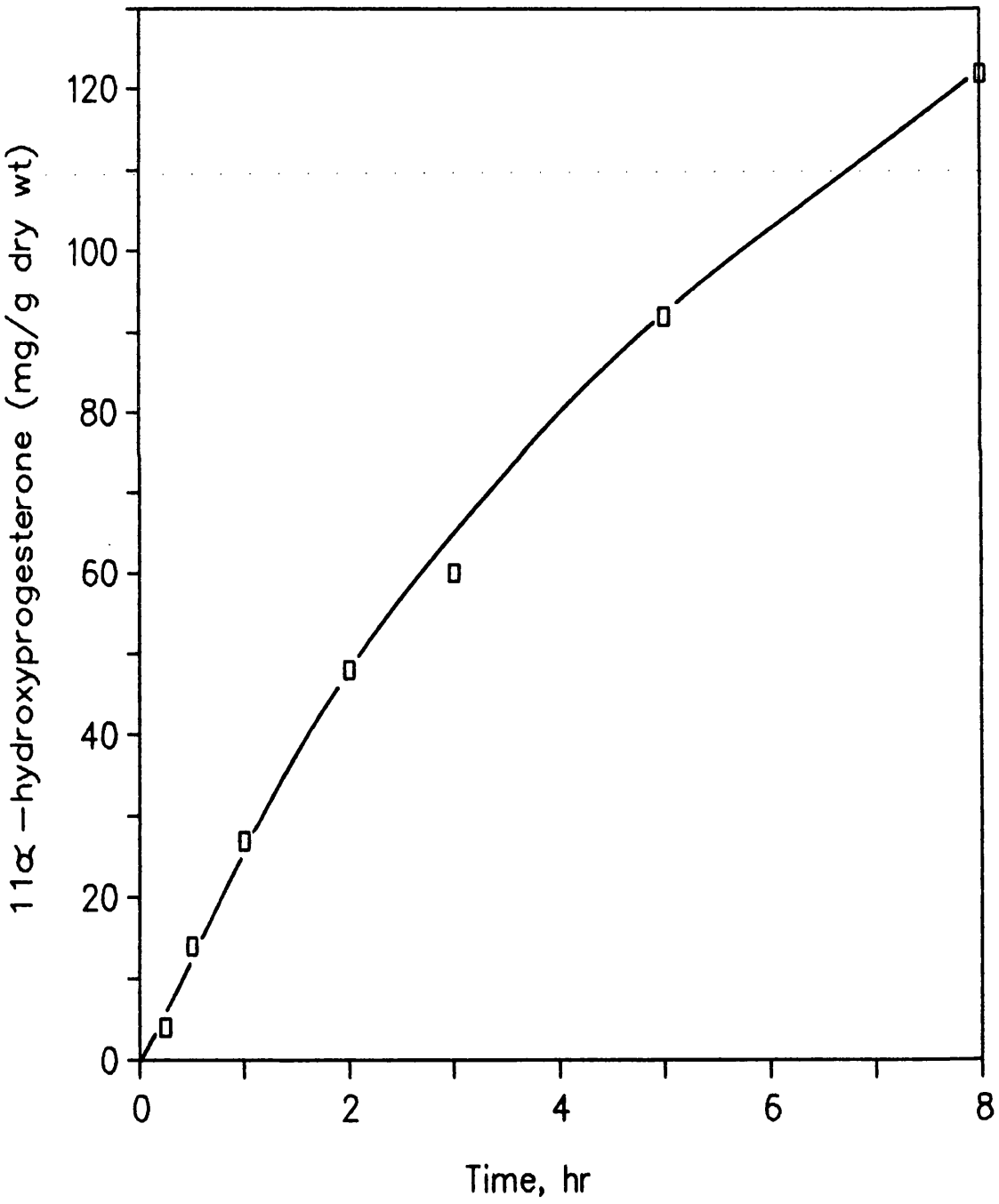


Figure 3.4.4 The rate of production of 11α-hydroxy-progesterone by cells of R.nigricans in an aqueous batch stirred tank reactor. Agitation set at 1200rpm and progesterone loading of 1.0g.



the diffusion of substrate within the cell mass. Alternatively, or additionally, increases in agitation may cause greater damage to R.nigricans as compared to A.ochraceus and so decrease the biocatalytic activity of the organism. The latter fungus is septate and shear damage would cause the release of cytoplasmic components only from individual cells. However, R.nigricans is a non-septate organism so that shear damage to a single portion of a filament may lead to a gross loss of intracellular material from a significant amount of biomass.

This effect is also seen in the data for A.ochraceus at both 0.3 and 0.5 ROV but occurs at higher agitation rates. Increases in 11α-hydroxyprogesterone production are observed with agitation rates up to 1200rpm due to increases in the surface area available for mass transfer of substrate. However, beyond 1200rpm a loss of production is observed probably due to the shear breakage of the cells.

3.4.4 Reaction-Time Course for the Hydroxylase Activity of *R.nigricans* in an Aqueous Stirred Tank Reactor

Figure 3.4.4 shows how the progesterone 11α-hydroxylase activity of R.nigricans varies with time in a batch stirred tank reactor at an agitation rate of 1200rpm. The reaction rate appears to be linear up to about 1hr after which there is a gradual decrease in activity. The loss of activity may result from; a disruption of the metabolic state of the cell (levels of cofactors etc.), proteolysis of the hydroxylase components or gradual shear breakage of the cells.

CHAPTER 4

4 ORGANIC/AQUEOUS PACKED-BED REACTORS

The use of packed-bed reactors in conjunction with organic/aqueous two liquid-phase reaction media has given rise to only a limited amount of literature. Many of the reported systems involve the hydrolysis of fats where the triglyceride substrate is dissolved in a second organic liquid (Bell *et al* 1981) (Macrae 1983) (Gancet and Guignard 1987). The amount of aqueous phase is generally limited to less than one half of one percent weight by volume making it totally soluble in the triglyceride/organic solvent mix. The fluid feed to the column is therefore homogeneous and will have flow characteristics that can be modelled in a similar manner to those of fully aqueous systems.

Brink and Tramper (1985) used a similar approach to study the epoxidation kinetics of immobilised Mycobacterium cells in an organic aqueous two liquid-phase system. The organic phase fluid feed, n-hexadecane, carried the dissolved propene substrate to the column while the aqueous phase was confined to the calcium alginate immobilisation matrix. To obtain the required high liquid/solid contacting efficiency it was found necessary to dilute the biocatalyst bed with small glass beads which reduced the biocatalytic density in the reactor to about 3.4×10^{-3}g dry weight/ml.

Leaver *et al* (1987) provide some details, in one of the few reported studies, of a reaction system using a non-homogeneous organic/aqueous fluid feed in conjunction with a packed-bed reactor. The stereospecific reduction of bicycloheptenones was catalysed by 3α20β-hydroxysteroid dehydrogenase which was coimmobilised with yeast alcohol dehydrogenase within Eupergit beads. It was found that droplets of micellar ketone substrate, pumped as an aqueous dispersion to the column, soon coalesced and did not pass through the column. To try and prevent this high flow rates and vigorous mixing were employed with no success. It was found, however, that by using a carrier solvent such as octanol the substrate could be effectively transported to the bed.

In the present study two objectives were considered. The first was concerned with using cells of the fungus R.nigricans as biomass in a packed-bed reactor system without the requirement for an immobilisation matrix. In this way the catalytic density of the reactor may be increased significantly. The second objective was to explore the possibilities of using the packed-bed of fungal cells as a static mixing device whereby an organic/aqueous fluid feed would be maintained as an emulsion on the column.

4.1 Preliminary Packed-Bed Reactor Experiments

Before experiments could be performed to study the flow of fluids through the packed-beds, methods for packing the cells of R.nigricans had to be examined and refined. The final techniques which proved to be the most reliable and easy to perform are detailed in section 2.11.1.1. The most difficult part of the set-up operation was the removal of small gas bubbles that became entrapped in the fungal suspensions. The elimination of these bubbles was particularly laborious when packing filamentous as compared to pelletted cells. The time taken would, under circumstances where the packed-bed was to be used in a biocatalytic reactor operation, be a major contributing cause to the overall loss of catalytic activity. Another problem associated with the use of filamentous cells was the difficulty in achieving a uniformity of cell packing throughout the bed. No measurements were made to confirm packing uniformity, however, observations of the filamentous beds through the glass walls of the column showed visible striations marking the interfaces between areas of bed having different cell packing densities.

All the preliminary experiments were performed with an aqueous (reaction buffer A) fluid feed to the column. The compressible nature of the packed-beds with both filamentous and pelletted morphologies of the fungus can be seen in figures 4.1a and 4.1b. Standard equations which relate the linear flow velocity u, to the voidage e, and pressure drop dP (see section 1.5.1) are not applicable when considering these highly deformable packed-beds. The apparent linear flow velocity, U, of fluid through the bed is therefore defined here by the following equations and calculated knowing the cell dry weight in the column, M_c,

and the volumetric flow rate, F.

$$U = \frac{F}{\pi r^2 (1-\phi_c)}$$

where r is the radius of the column and ϕ_c, the cell volume fraction is given by

$$\phi_c = \frac{(M_c \times R_{w/d} / P_c)}{\pi r^2 \times L}$$

$R_{w/d}$ is the cell wet to dry weight ratio (sections 3.2.2 to 3.2.4), P_c is the cell density (section 3.2.1) and L is the height of the packed-bed.

The apparent linear flow velocity calculated in this manner will be less than the actual linear flow velocity owing to the tortuous nature of fluid flow around the filaments/particles and ultimately through the bed (Carmen 1937). In effect the length that a fluid molecule may travel, L_e will be significantly greater than the depth of the bed, L. The apparent linear flow velocity therefore only provides a means of comparing similar systems.

A feature of compressible packed-beds at a constant volumetric flow rate is the increase in apparent linear flow velocity as the cell volume fraction increases (bed height decreases). Figure 4.1c shows how at high volume fractions a precipitous rise in the apparent linear flow velocity occurs. This relationship provides some explanation for the long time periods (seen in figure 4.1b) over which the beds continue compressing at constant volumetric flow rates.

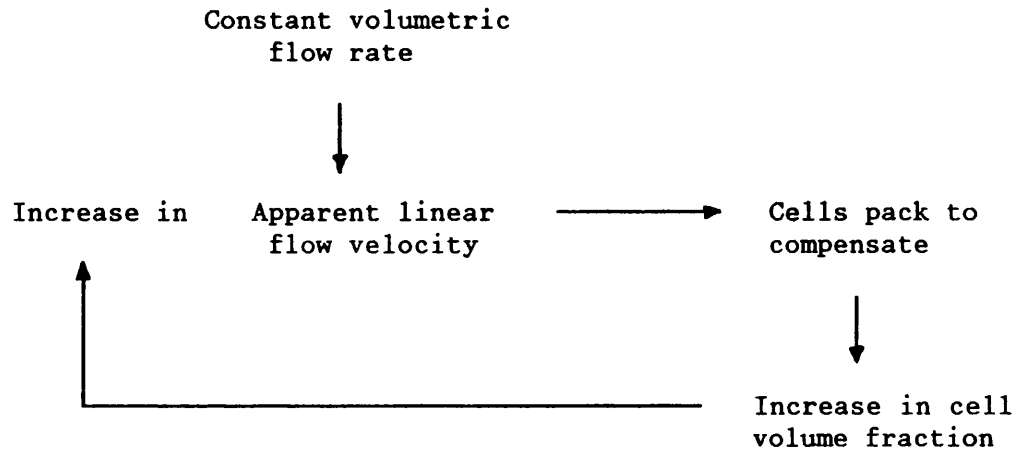


Figure 4.1a Filamentous, □ , and pelletted, △ , cells exhibit a high degree of compressibility as is shown by the increase in cell volume fraction (related to a decrease in packed-bed height) with increasing apparent linear flow velocity through the beds. Measurements of the bed heights were taken five minutes after the flow rates were altered. Pressure drops across the beds were all less than 0.1 bar.

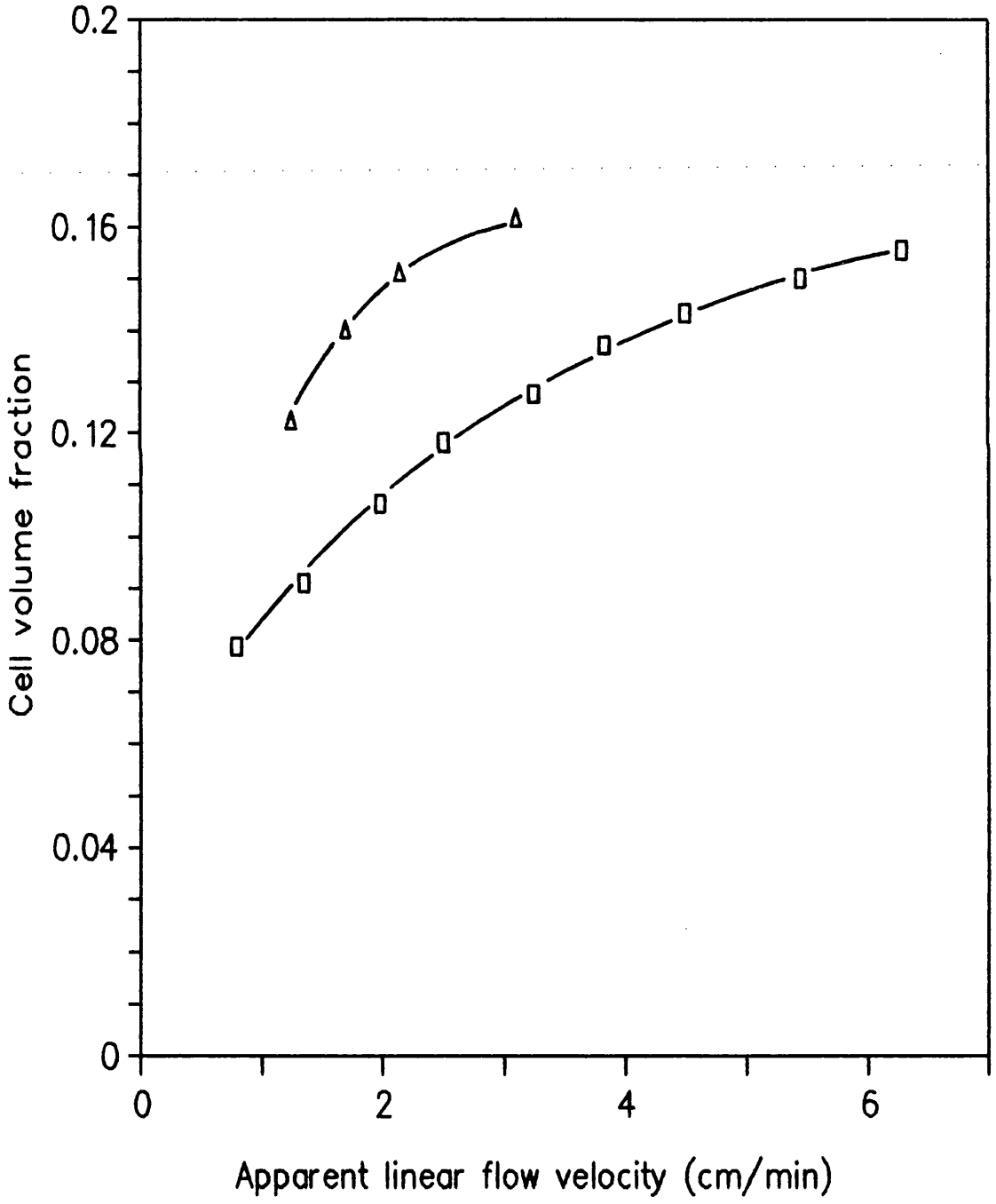


Figure 4.1b At constant volumetric flow rate through a packed-bed of pelletted cells the degree of bed compression is very much time dependent. It should be noted that although the volumetric flow rate remains constant the apparent linear flow velocity through the bed increases with increasing cell volume fraction. Volumetric flow rates of 70ml/min, □ , and 110ml/min, △ , are shown.

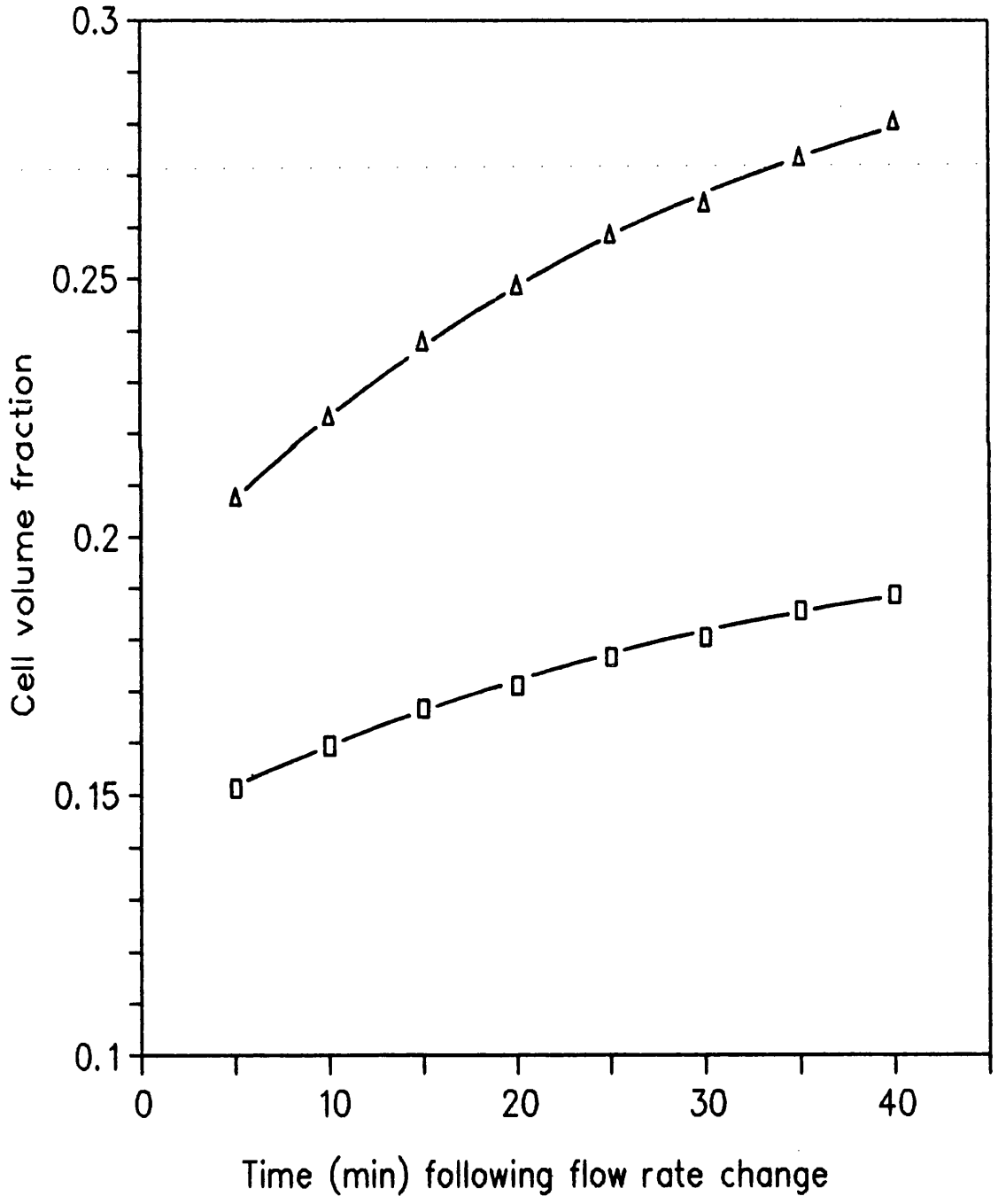
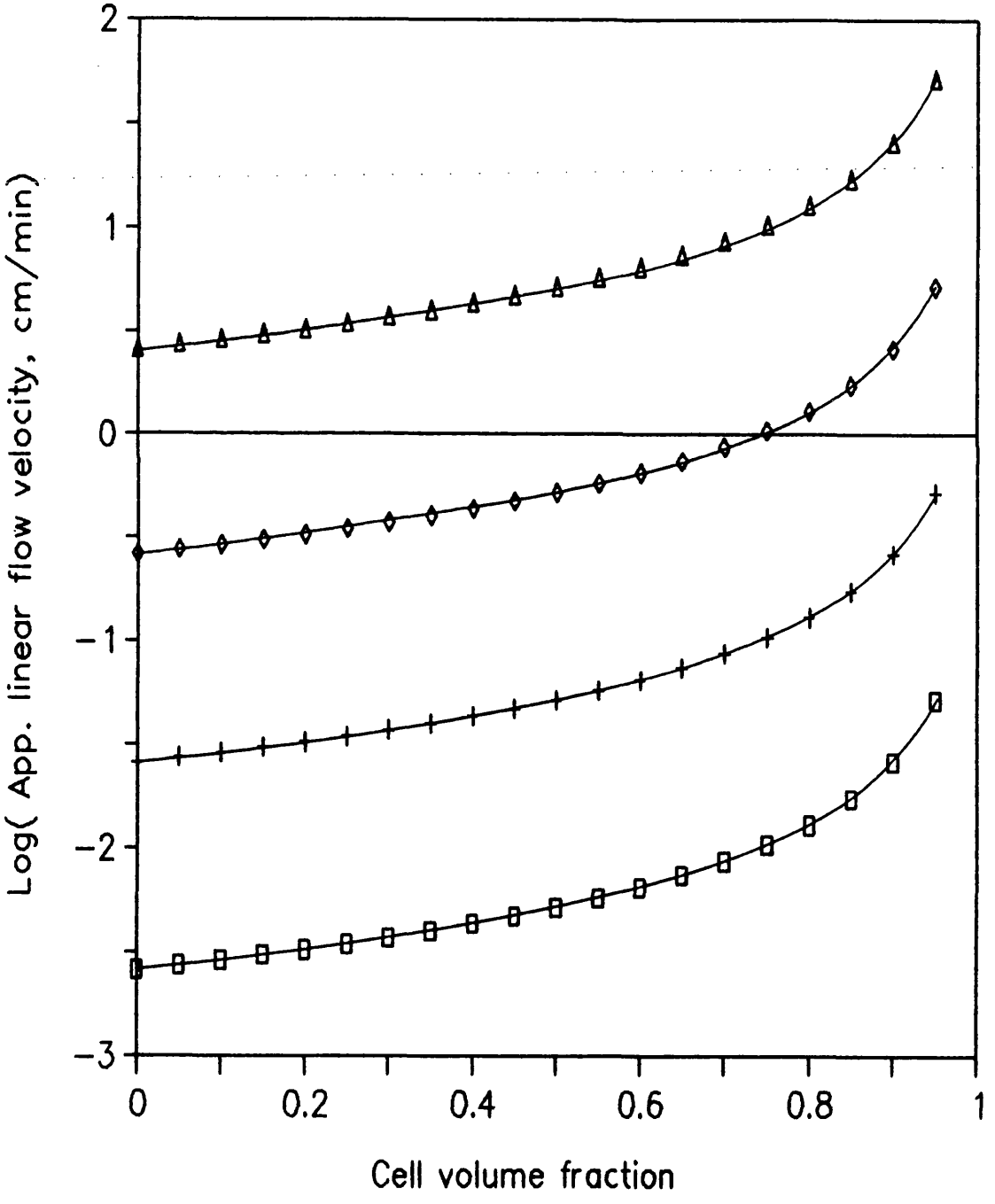


Figure 4.1c At constant volumetric flow rate, the theoretical apparent linear flow velocity through a packed-bed increases as the cells compact (higher cell volume fractions). A theoretical cell volume fraction equal to unity would have a packed cell density of 0.304 gdry wt/ml. Shown are volumetric flow rates (ml/min) of; 0.1, □ , 1.0, + , 10, ◊ , and 100, △ .



The cyclical process represented in the flow diagram only gradually diminishes as the internal mechanical strength of the bed particles begins to resist the deformation.

4.2 Two Liquid-Phase Packed-Bed Experiments

For the packed-bed systems being studied to be developed and run as biocatalytic reactors there was a need for a minimal loss of catalytic activity to occur. To meet this requirement pelletted cells were used for all further experiments as the time taken to pack these was far less than for filamentous cells. The organic phase to be used in combination with the packed-bed system was selected on the basis of previous work (sections 3.4 to 3.4.4) which showed that oleic acid could allow the retention of high levels of biocatalytic activity in R.nigricans and over prolonged reactor times (Ceen 1986). A particular disadvantage of using oleic acid is its high viscosity (24.6cP at 28^{0}C, obtained from interpolation of data from Riddick *et al*, 1986) and the direct effect that this has on the pressure drop across a packed-bed (equation [E1.5.1a]). The emulsion, formed in a stirred baffled tank, was passed to the column with the minimum possible length of piping in an attempt to avoid emulsion separation prior to delivery onto the column.

The following list gives a summarised account of the methods employed to try and maintain an emulsion on the packed-beds of pelletted R.nigricans cells. Although both downward and upward flow of emulsion was tried, the latter proved slightly more effective with any coalesced oleic acid (having a lower specific gravity) being driven off the top of the column.

1.) Cells were packed to a high cell density to avoid further bed compaction (volume fraction = 0.23). An emulsion of relative oil volume equal to 0.3 was passed directly (100ml/min) to a normally packed column of cells. The base of the column where the inlet of emulsion occurred turned a milky white which slowly passed up the bed. After several minutes small beads of oil appeared at the base of the column which then formed finger like protrusions around the outside of the

bed, figure 4.2a. These fingers of oil then channelled to the top of the column totally by-passing the bed.

2.) Cells were packed to a similar volume fraction as above. The relative oil volume of the emulsion that was passed to the column was gradually increased from 0 to 0.05. This was achieved by slowly pumping oleic acid into the baffled stirred tank through a syringe, continually pumping the resulting emulsion to the column at a volumetric flow rate of 180ml/min (apparent linear flow velocity = 6.1cm/min) and recirculating the exit stream of the column to the tank.
Similar results to those described above were observed though it required some 20 minutes before channelling of the oil around the bed occurred.

3.) It was thought that the emulsion might be coalescing prior to reaching the base of the packed-bed. To try and reduce this effect two mechanisms of in-line static mixing device were employed, figure 4.2b. The first consisted of a small length of glass tube, filled with ballotini beads (400-800 mesh), at the inlet to the column. This had no significant beneficial effects. The second type consisted of filling the column to a height of approximately 1cm with the same ballotini beads before packing the cells of R.nigricans into the column. This again appeared to have little beneficial effect.

4.) Cells were suspended in an emulsion of low relative oil volume (< 0.05) prior to packing using a fluid feed of low relative oil volume. Uniform packing of the cells was made extremely difficult and separation of the phases was worse than with the previous methods.

5.) An attempt was made to mix the cells of R.nigricans with ballotini beads prior to packing. This gave a very uneven bed distribution with the beads tending to fall to the lower portion of the bed. Again separation of the phases was worse than using methods 1 or 2.

As a result of observations from all the above methods it was realised that the packed-beds of cells were acting as a form of filter coalescer causing the separation of the two phases followed by channelling of the oil phase through and around the beds.

Figure 4.2a Schematic diagram showing the result of flowing an emulsion onto a packed-bed of pelletted cells of R.nigricans.

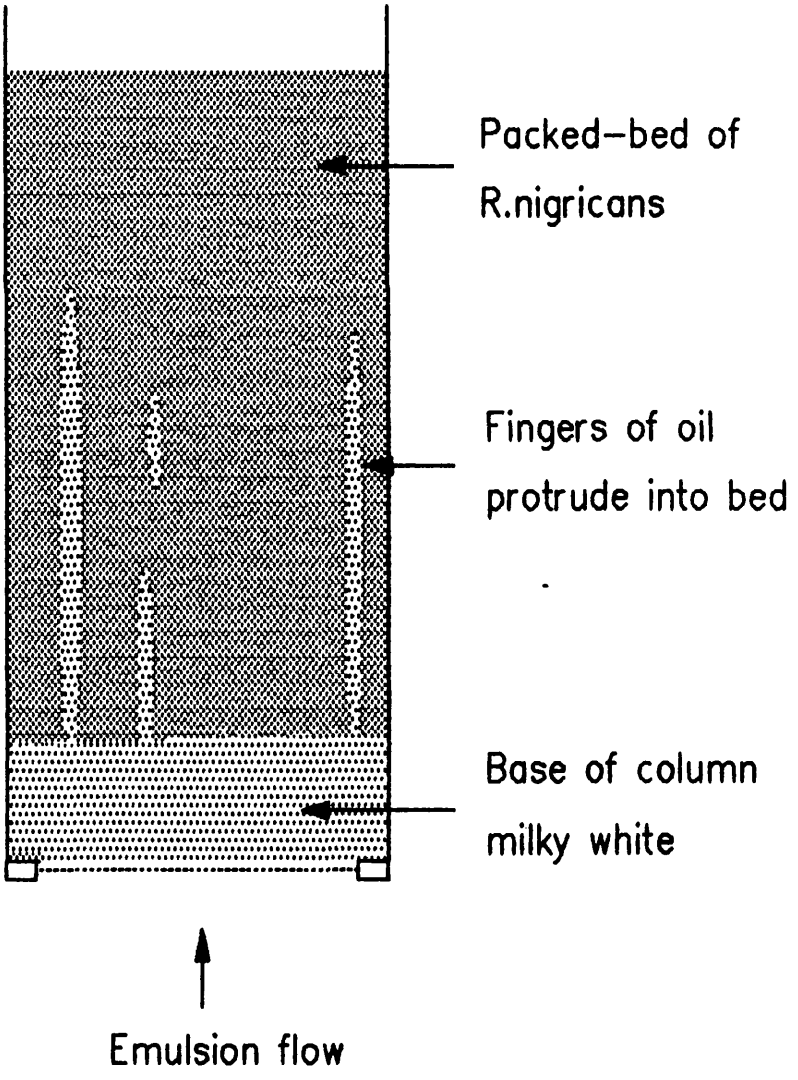
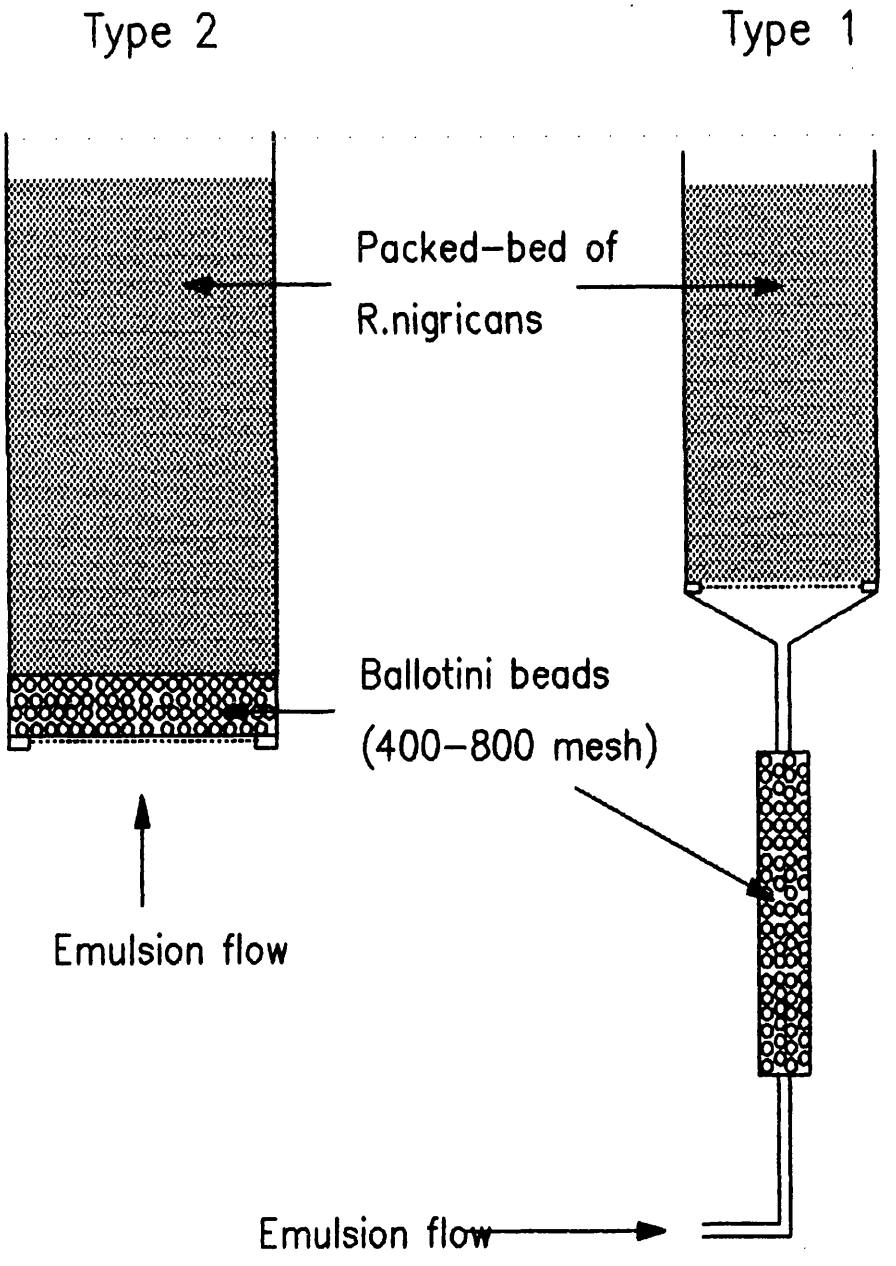


Figure 4.2b Schematic diagram illustrating the configurations in which ballotini beads were used as static-mixing devices in an attempt to re-emulsify any oleic acid that had coalesced prior to entering onto the packed-bed.



4.3 Oleic Acid Coalescence in a Model Packed-Bed Reactor

To further understand what happens in the packed-beds, a model system was employed which used ballotini beads (400-800 mesh) in place of the cells of R.nigricans. The emulsion that was passed through the column exited as large droplets of oleic acid suspended in a comparatively clear aqueous phase. This exit stream was then recirculated to the stirred tank. The data for the system is summarised below. After 20 minutes the exit emulsion stream from the column had reached a constant relative oil volume (ROV). A pulse of mixed dye emulsion was then injected prior to the column and the absorbences of the aqueous and oleic acid phases monitored (section 2.11.1.2). Figure 4.3 shows the absorbence profiles of the exit stream.

Details of the model system

At start of experiment ;

Total recirculating fluid volume = 1000ml (ROV of system = 0.2)

Volumetric flow rate = 200ml/min

Void volume of dye injection point to detection point = 126ml

Void volume on column = 70ml

After 20mins (at equilibrium) ;

ROV of exit stream from column = 0.175

The change in ROV of the emulsion in the system (from 0.2 to an apparent 0.175) during the 20 minutes suggested that 25ml of oleic acid had been lost from the emulsion. Some of this loss of oleic acid may have been through droplets coalescing and adhering to the inside surfaces of the piping but it is likely that the vast majority is due to the oleic acid coating the high surface area of the ballotini beads within the column.

From figure 4.3, and taking account of the volume of fluid in the piping to and from the column, it is possible to perform a second calculation to determine the volume of oleic acid retained on the column. This is done by comparing the theoretical volume of liquid at which the aqueous peak absorbence should occur (assuming plug flow), with the measured experimental volume, table 4.3. The difference in values implies that there is an extra 26.4ml volume on the column from which the aqueous phase is being excluded. The conclusion therefore is

that this is due to oleic acid being retained on the column, the volume agreeing closely with that obtained from the difference in ROV's as calculated above.

Table 4.3 Theoretical and experimental fluid volumes in column of ballotini beads.

	Theoretical (ml)	Experimental (ml)
Total fluid vol.	70	38
Aqueous phase vol.	57.75	31.35

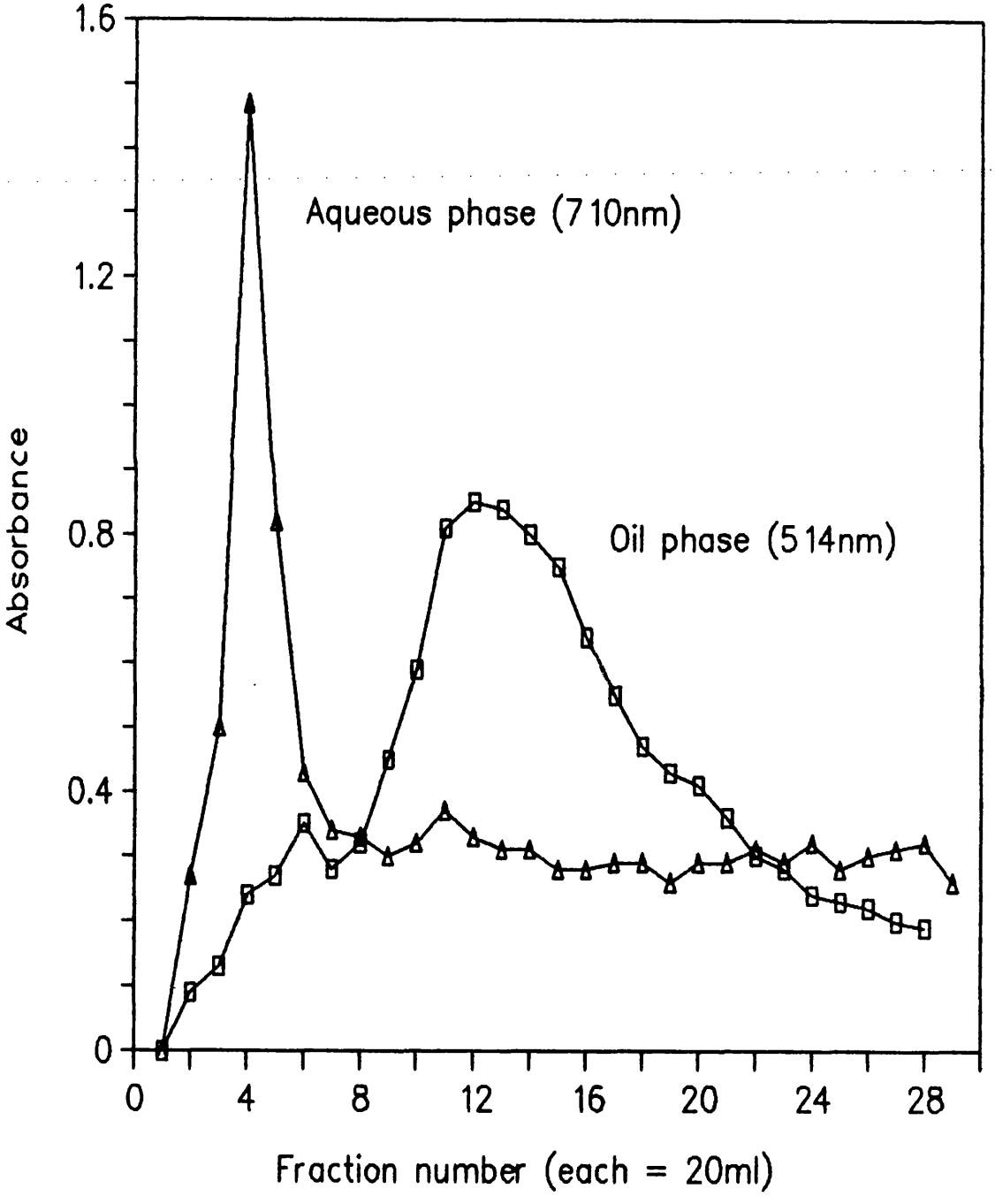
Theoretically the oil phase peak should occur at the same time as the aqueous phase peak. However, figure 4.3 shows that the peak occurs much later than that of the aqueous phase and is considerably broader. This can be explained if the majority of the oleic acid entering the column coalesces with the comparatively stagnant film of oleic acid surrounding the beads and only slowly passes through the column. The exiting oleic acid will therefore be large droplets as was observed experimentally. The simple model system of ballotini beads as a packed-bed would therefore appear to provide a good representation of the experimental results observed using pelletted cells of R.nigricans.

4.4 Dispersion Characteristics of Compressible Packed-Beds

It was originally intended that the packed-beds of pelletted cells were to act so as to maintain an emulsion on the column. For this to occur there would have to be a high linear flow velocity of the emulsion through the column so that the interstitial spaces between the pelletted cells behave as miniature mixing chambers and for the bed as a whole to act as a static mixing device.

The amount of mixing that occurs in the beds can be quantified by monitoring the output response from the column to a pulsed input of dye. With the extreme difficulties of operating the columns in

Figure 4.3 Exit stream absorbence profiles for aqueous and oleic acid phases following a pulsed input of dye to a model packed-bed consisting of ballotini beads (400-800 mesh). Fraction 0 had a volume of 14ml.



conjunction with emulsions it was found necessary to return to operation of the packed-beds with a purely aqueous fluid stream. Figure 4.4a shows the response profiles to a pulsed input of dye. The same pelletted cells were compacted to three different bed-heights to produce different cell volume fractions and so alter the apparent linear flow velocity (ALFV) at a constant volumetric flow rate (40ml/min). The results are summarised in table 4.4 (calculation of the vessel dispersion number (D/uL) is given in appendix 4).

Table 4.4

	Bed height (cm)		
	20	15	10
Cell volume fraction	0.075	0.10	0.150
ALFV (cm/min)	1.124	1.150	1.223
Dispersion no. (D/uL)	0.009	0.0139	0.0251

Figure 4.4b shows that the vessel dispersion number increases almost linearly with increases in the cell volume fraction over the range 0.075-0.150. For an emulsion to be maintained on the packed-bed it is likely that the required mixing would correspond to dispersion numbers having values several orders of magnitude greater. Further increases in cell volume fraction alone would not be sufficient to achieve this mixing. An alternative is to significantly increase the linear flow velocity through the bed, however, this would undoubtedly result in particularly high pressure drops across the column.

4.5 Single Phase Oleic Acid Flow through Packed-Beds of *R.nigricans*

Limited progress in the use of packed-beds of *R.nigricans* as static-mixing devices to maintain emulsions on the column meant that alternative systems might be considered as potentially more suitable. One possible system that was evaluated involved flowing a single phase of oleic acid across the beds of pelletted cells. The oleic acid was

Figure 4.4a Exit stream concentration profiles (C-curves) for a pulsed input of aqueous dye to the same column of pelletted R.nigricans, packed to three different bed heights. ◊ , 20cm; △ , 15cm; □ , and 10cm. The dotted line represents an artificial base-line used for a set of dispersion number calculations (see appendix 4).

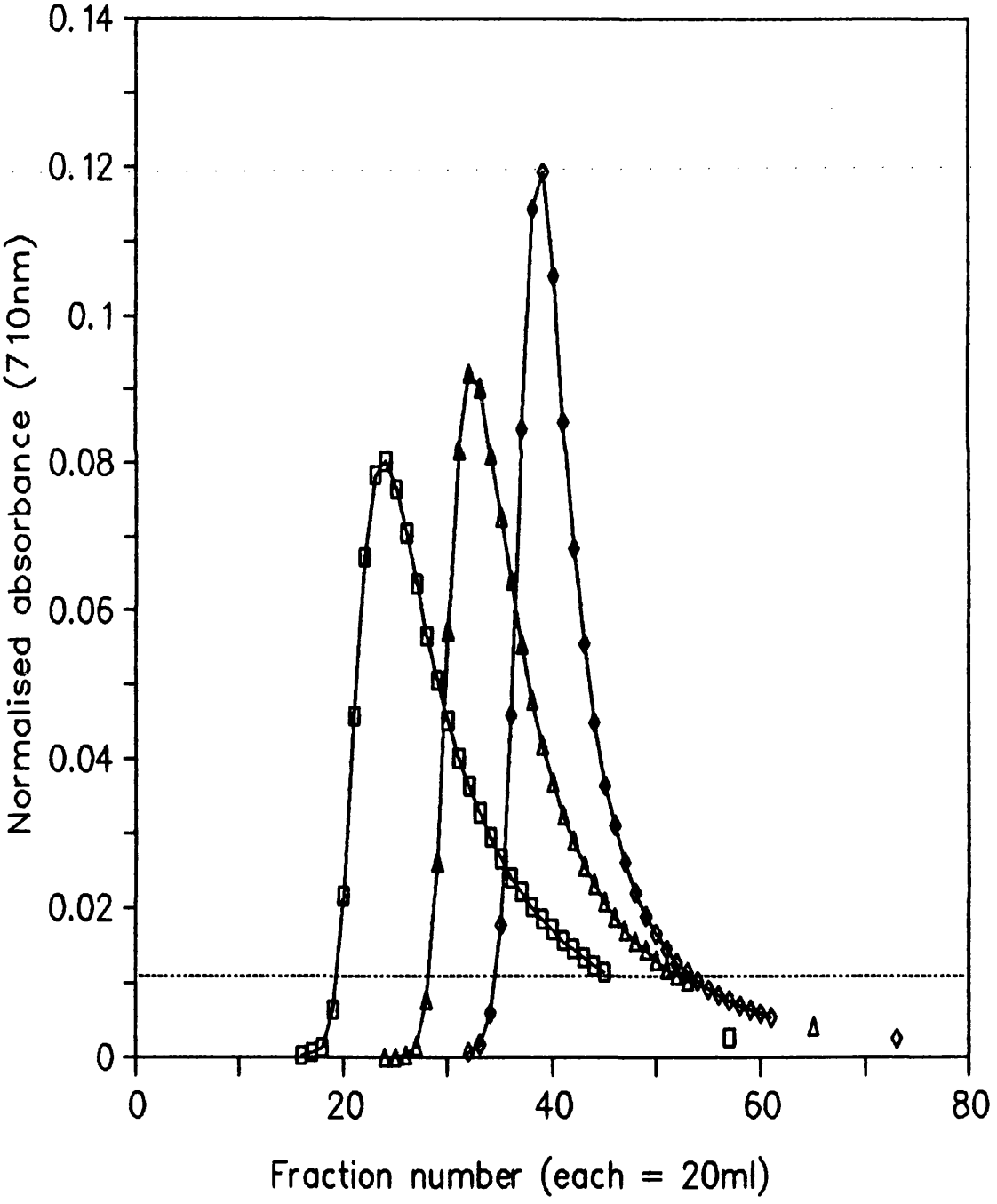
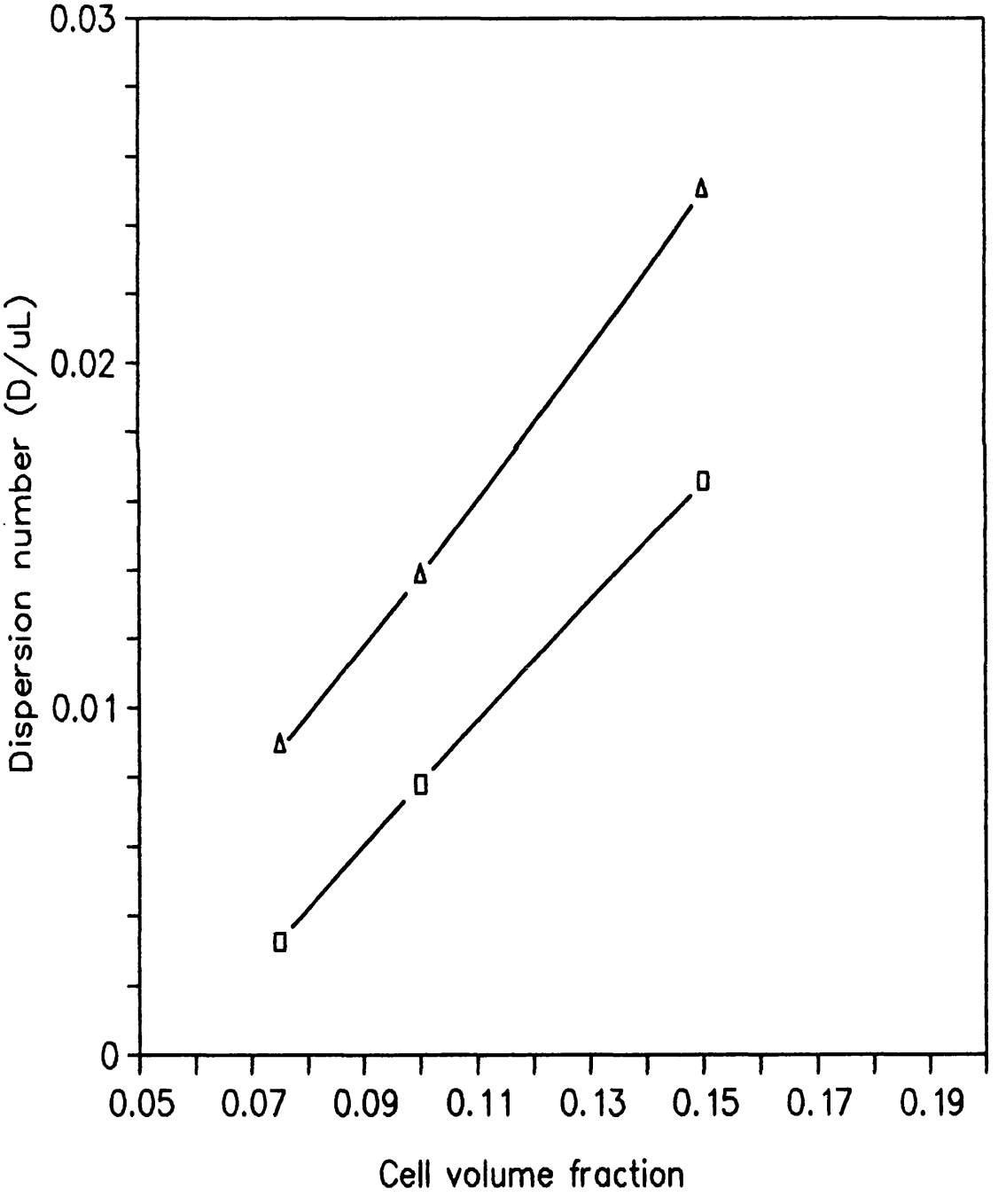


Figure 4.4b The increase in vessel dispersion number with increasing cell volume fraction. Calculated from, △ , complete unmodified C-curves and □ , C-curves using artificial base-line (shown in figure 4.4a).



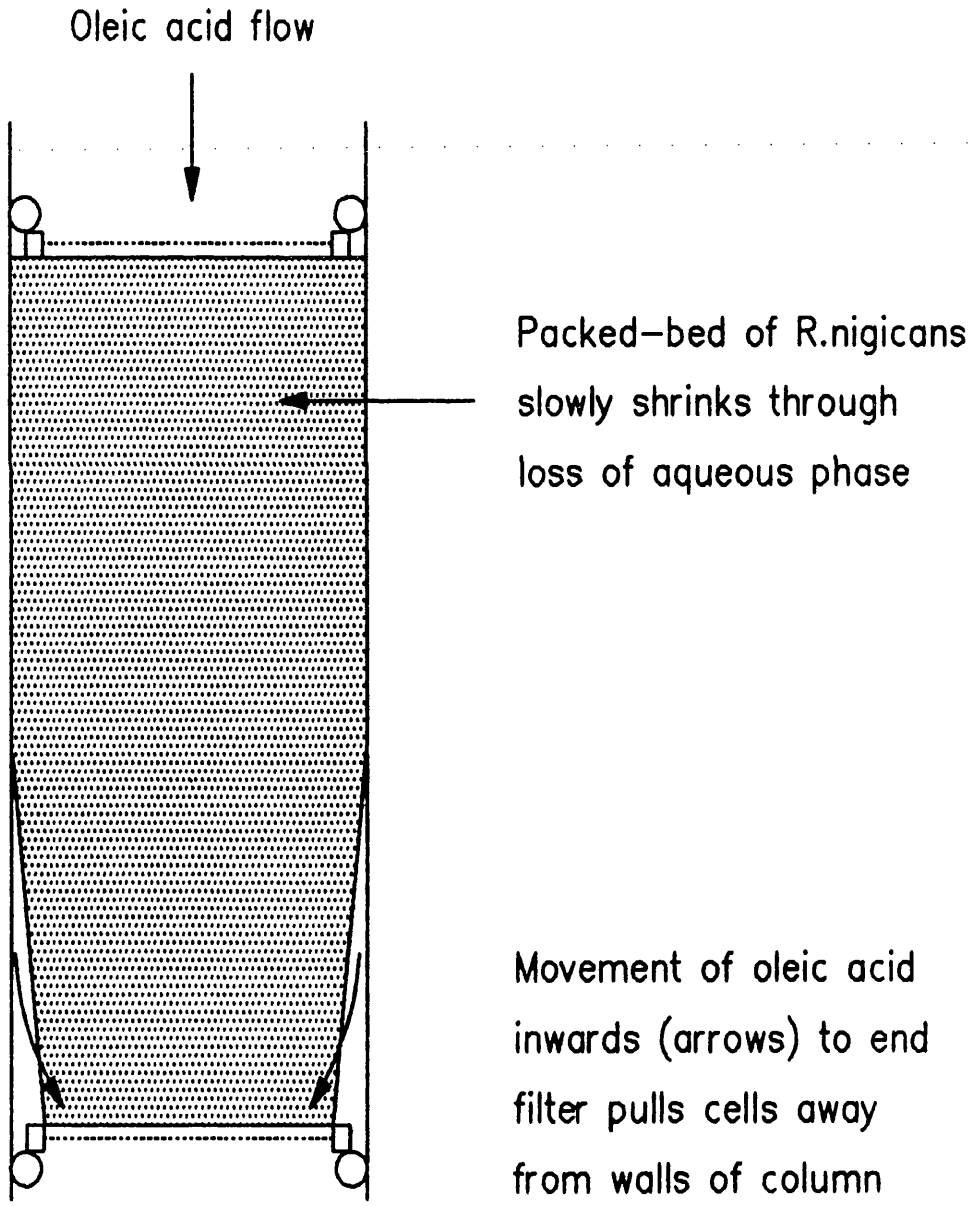
passed to the column at very low volumetric flow rates (<15ml/min) in a downward direction to slowly displace the interstitial aqueous phase from the bed. If high flow rates were used then the oil phase was found to channel around the column walls. After an hour of oleic acid flow through the column small droplets of aqueous phase were still seen to be present in the exit stream and the bed was seen to slowly contract away from the walls at the base of the column, figure 4.5. Two explanations for this latter behaviour are proposed. Firstly the oleic acid will not be moving in an entirely plug flow manner close to the base of the column owing to the design of the end piece filter (see figure 4.5). The oleic acid is made to flow inwards and with its high viscosity will have a tendency to pull the cells away from the walls. This is coupled with the gradual shrinkage of the cell mass through loss of the aqueous phase from the pellets, as demonstrated by the continued presence of aqueous droplets in the exit stream.

4.5.1 Aqueous Phase Droplet Addition to the Packed-Bed

An attempt was made to reduce the shrinkage of the packed-bed that was occurring through loss of aqueous phase from the cell pellets. Buffer was pumped to the bed (2-5ml/min) using a micro-peristaltic, the aqueous phase emerging as droplets from a needle injection port (figure 2.11.1.2), placed before the top of the column. This had the effect of increasing the length of time over which the column could be operated by about a further hour.

Several experiments were run on this type of column to try and determine the biocatalytic activity of the cells. The oleic acid was doped with progesterone and the product, 11α-hydroxyprogesterone, was detected in the exit stream from the column. However, it was discovered from mass balances of steroid material, that both progesterone and 11α-hydroxyprogesterone were present in the exit stream in excess amounts to those that could be expected from the concentration of steroid in the feed material. This meant that steroid material from the induction period had remained attached to the catalytic biomass despite copious washing with aqueous buffer and was released by dissolving in the more hydrophobic oleic acid as it passed through the column. A further experiment was performed in which clean oleic acid (not doped

Figure 4.5 Schematic diagram of the flow of a single oleic acid phase through a packed-bed of pelletted cells of R.nigricans.



with steroid) was run initially through the column to remove steroid transferred from the induction process, followed by oleic acid doped with progesterone. The length of time required to perform this sequence resulted in the packed-bed collapsing before meaningful results could be obtained.

4.6 A Methanol/Aqueous Cosolvent System used in Conjunction with a Packed-Bed of *R.nigricans*

In order that some biocatalytic measurements might be made of the cells in the packed-bed reactor system it was decided to use a methanol/cosolvent (10 percent v/v) fluid feed to the column. This ensured that the column could be operated for far longer periods (up to 20hrs) without the problems of channelling that were present in the emulsion and single oleic acid phase systems. In an attempt to minimise the amount of steroid transferred to the column reactor the cells were washed after harvesting from the fermenter in two volumes of wash buffer and a further volume of 5 percent (v/v) methanol in wash buffer.

4.6.1 11α-Hydroxylase Activity of Cells of *R.nigricans* in a Packed-Bed Reactor

Figures 4.6.1a and 4.6.1b show the results of the column run. The total amount of steroid (progesterone, 11α- and 11α-6β-hydroxyprogesterone) exiting from the column shows a marked increase at 30 minutes following the start of flow of the cosolvent fluid through the bed. This corresponds to the time at which the first bed void volume ends (aqueous phase) and the start of the second bed void volume (cosolvent). It illustrates that despite the extra washing step, steroid is still attached to the cells from the induction process and can be further removed by the increased hydrophobicity of the higher cosolvent concentration. By 100 minutes the total steroid level in the exit stream has stabilised suggesting that the excess steroid has been washed from the column. 11α-hydroxylase activities calculated before this point are therefore a combination of steroid carried over from the

induction and product formed by the biocatalytic activity of the cells in the column. It is notable that the total steroid level calculated to be in the exit stream is less than that measured in the feed stream. It is possible, therefore that the cells are using the progesterone as an energy source, though glucose is also present in the feed stream, or that it is being converted to other steroid compounds which were not detected. Table 4.6.1 gives the average 11α-hydroxylase activities of the cells at the different flow rates once a bed void volume of cosolvent has passed through.

Table 4.6.1 11α-hydroxylase activities of R.nigricans.

	11α-activity (mg/g dry wt/min)
Flow rate (≈102ml/min)	0.0115
Flow rate (≈38.5ml/min)	0.00557
Shake flask cells (Metoh/Aq.)	0.024 *

* calculated on the basis of a single point 18hr assay

The 11α-hydroxylase activities of cells in the packed-bed reactor system are markedly lower than the average activity of the same cells in a shake flask reactor. Several explanations for this difference are listed below.

1.) Substrate (progesterone) concentrations in the systems are considerably different (0.03mg/ml progesterone in the packed-bed as compared to 4mg/ml in the shake flasks).

2.) Mass transfer rates of the substrate to the cells may be lower in the packed-bed column system.

3.) Channelling of the cosolvent could be occurring so that the cell mass is not being used efficiently.

4.) Depletion of oxygen along the length of the reactor may act to limit the rate of the reaction. Preliminary calculations, however, showed that sufficient oxygen should be present for the 11α-hydroxylase reaction throughout the reactor but oxygen required for basal metabolic

functions was not incorporated into the calculations.

Following the stoppage of flow for the 960min period (overnight) it was expected that a high concentration of 11α-hydroxy-progesterone would be present in the exit stream from the column. However, the results suggest that a significant amount is further converted to the 6β-11α-dihydroxylated product, as a result of the build up of high levels of 11α-hydroxyprogesterone inducing the second enzyme.

Figure 4.6.1a 11α and 6β-11α-hydroxylase activities of pelletted cells of R.nigricans (56.3g dry weight) in a packed-bed reactor with a methanol/aqueous (10% v/v) cosolvent fluid feed. The feed stream to the column was switched off at 140 and 185mins, for 35 and 960mins respectively. The concentration of progesterone in the feed stream is shown by the dotted lines (- - -). Open squares, □ ,indicate where the first bed void volume ends following a change in the flow rate.

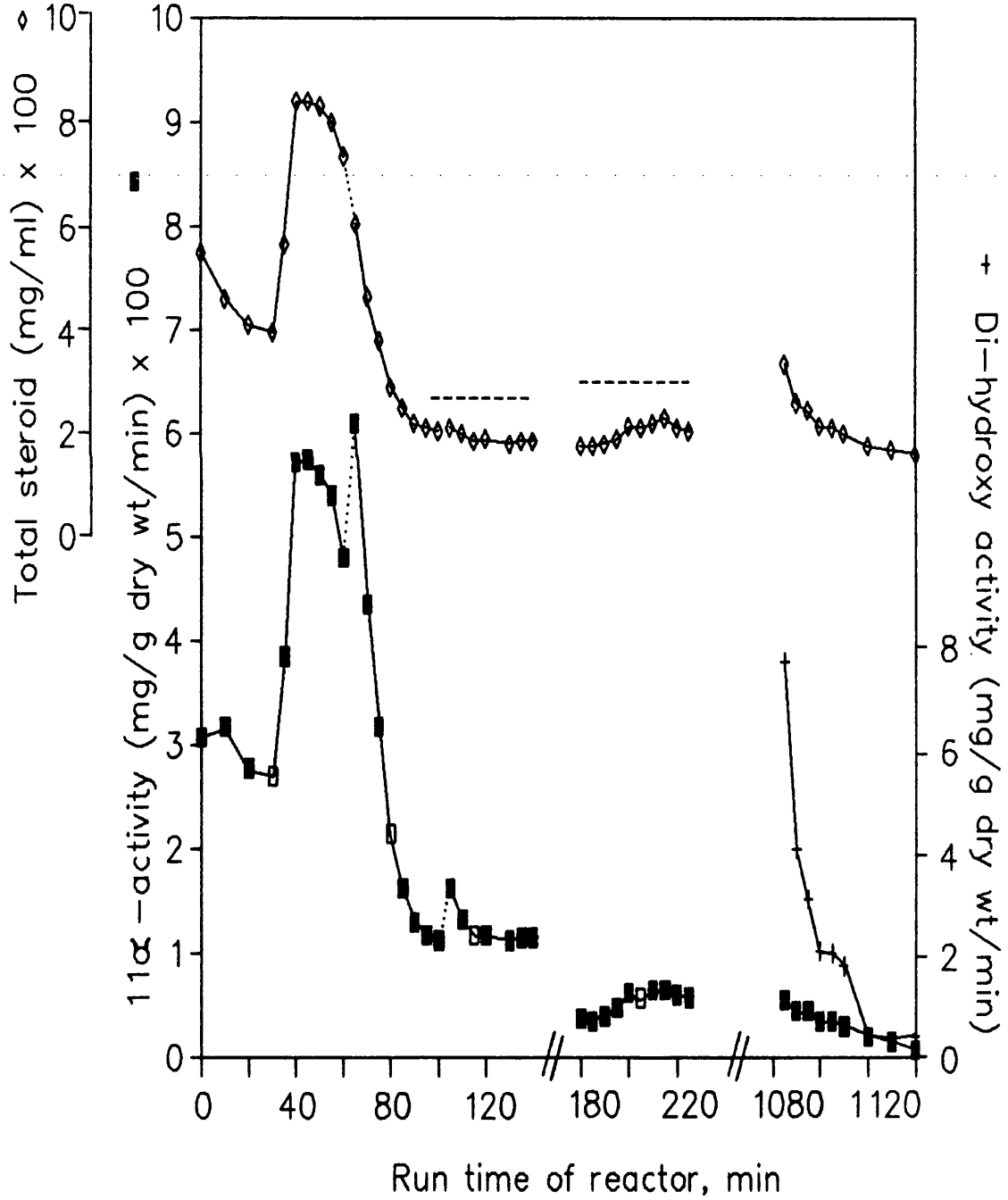
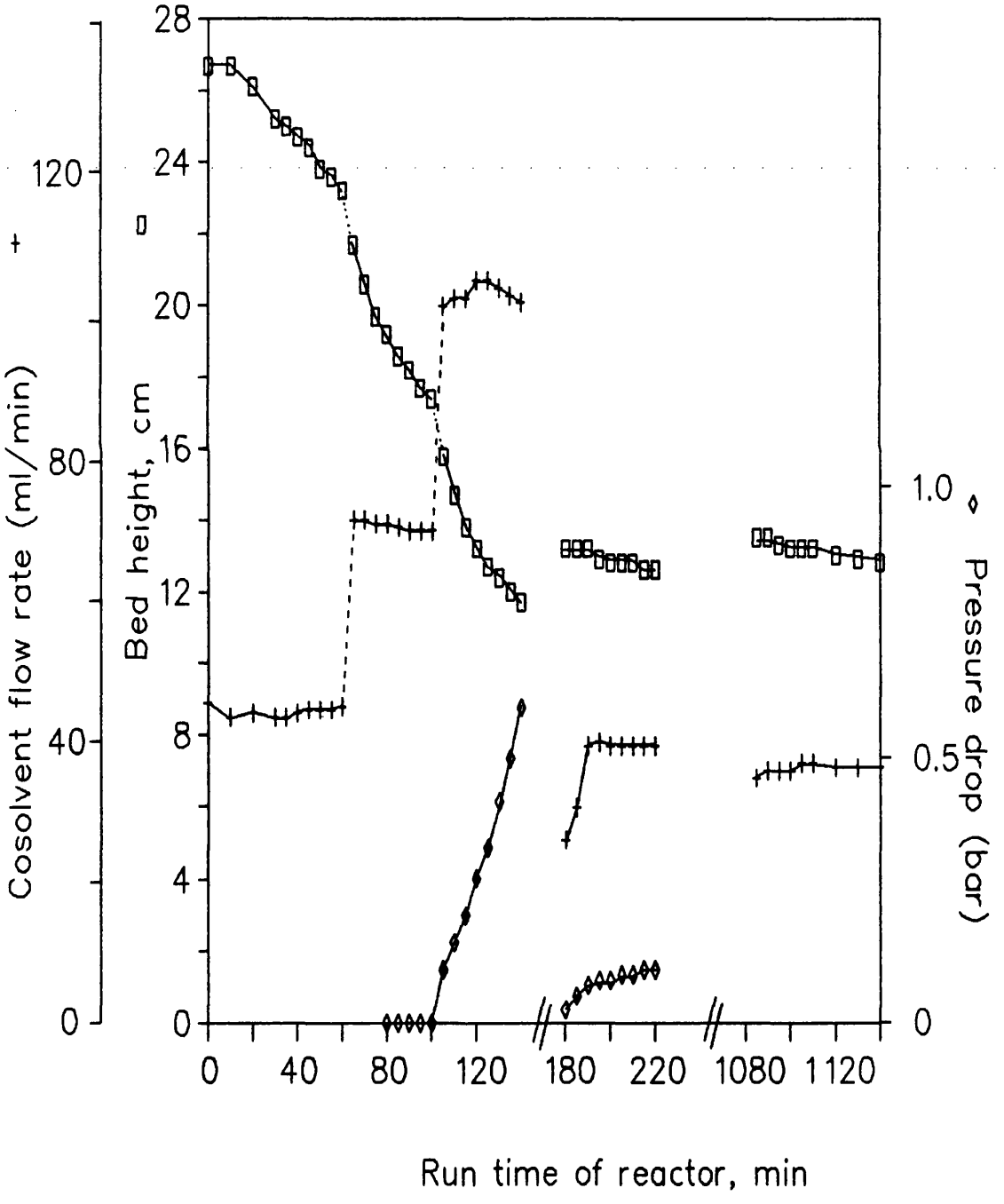


Figure 4.6.1b Bed-height, volumetric flow rate and pressure drop data for the packed-bed column of R.nigricans whose hydroxylase activity data is presented in figure 4.6.1a.



CHAPTER 5

5 RESULTS OF EXPERIMENTS ON THE INTERACTION OF ORGANIC SOLVENTS WITH MEMBRANES

5.1 Extraction of Membrane Lipids by Organic Solvents - Correlation with $LogP_{octanol}$

It was initially considered that one of the main causes for loss of 11α-hydroxylase activity in Rhizopus nigricans in a two liquid-phase system might be permeabilisation of the cytoplasmic membrane (section 1.3.1). This could allow the release of cytosolic constituents such as cofactors, in particular NADPH which is required for the hydroxylase reaction, and produce a general disruption of the chemical state of the cell (eg. pH and ionic strength).

The precise method by which permeabilisation occurs is not fully understood. However, it is thought that organic solvents act by solubilising membrane lipids so causing a disorganisation of the membrane structure (De Smet et al 1978). By this process lipids may be released into the organic phase and then partition between the organic and aqueous phases. To quantify the amount of lipid released in this manner it was necessary to hydrolyse the various lipid types (phospholipid, sphingolipid etc.) to give the corresponding fatty acid moieties of which they are made up.

Figure 5.1a shows how the total amount of higher molecular weight lipids (having fatty acid moieties of carbon length C_{16}-C_{18}) released from R.nigricans varies with the $LogP_{octanol}$ value of the organic solvent used to contact the cells in the two liquid-phase system. The percentage release of individual fatty acid moieties is shown in figure 5.1b and is compared with the results of Erwin (1973) for the composition of lipids in R.nigricans.

The weight of fatty acid moieties released per gram dry weight of cells is given in table 5.1 along with the $LogP_{octanol}$ of the respective organic solvent. The total amount of lipid present in fungal cells will depend on many factors including the type of organism, the growth conditions,

Figure 5.1a Relationship between the amount of lipids released from R.nigricans in a two liquid-phase system and the $LogP_{octanol}$ value of the organic solvent used in the reactor. Numbers refer to the solvents listed in table 5.1.

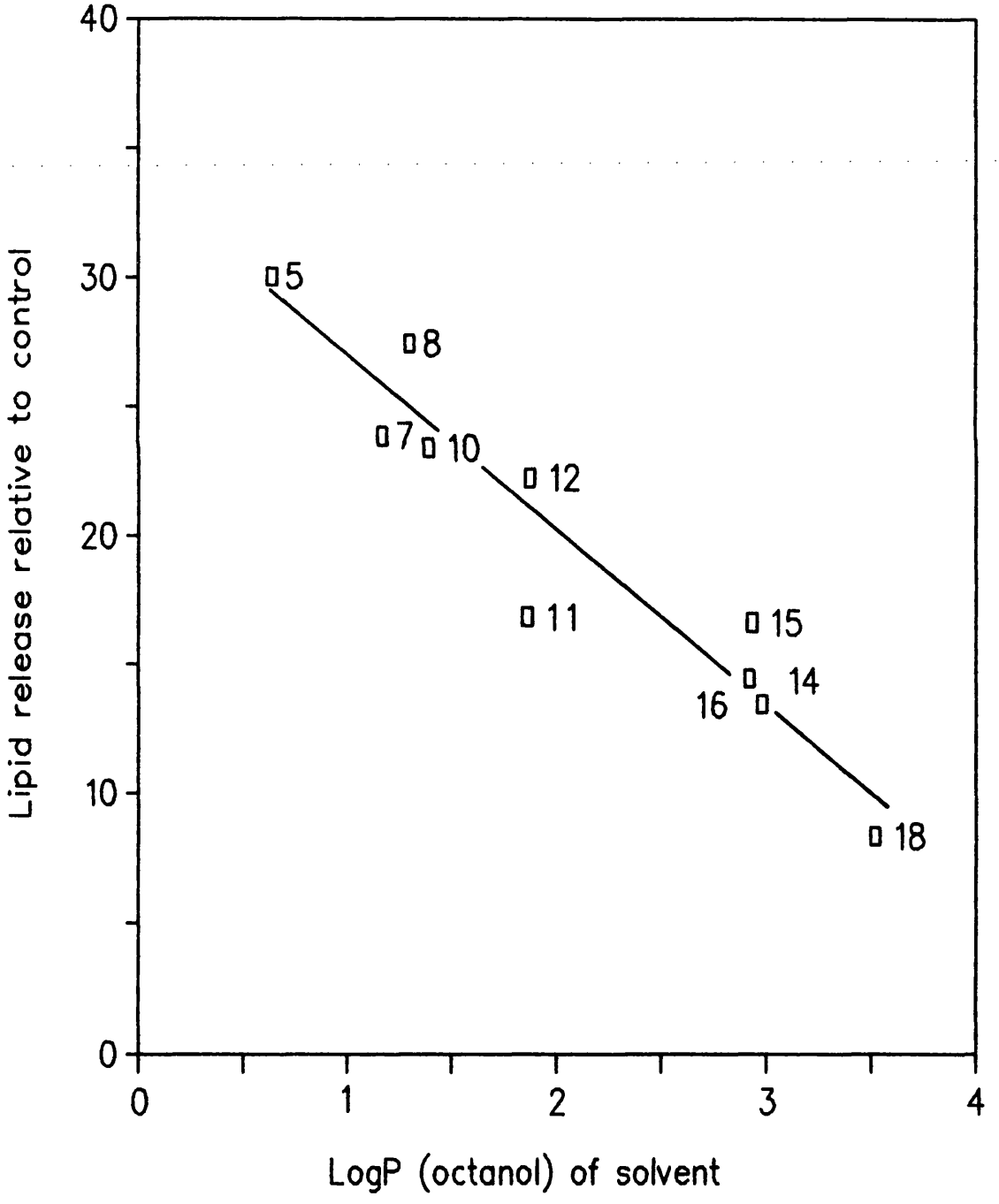
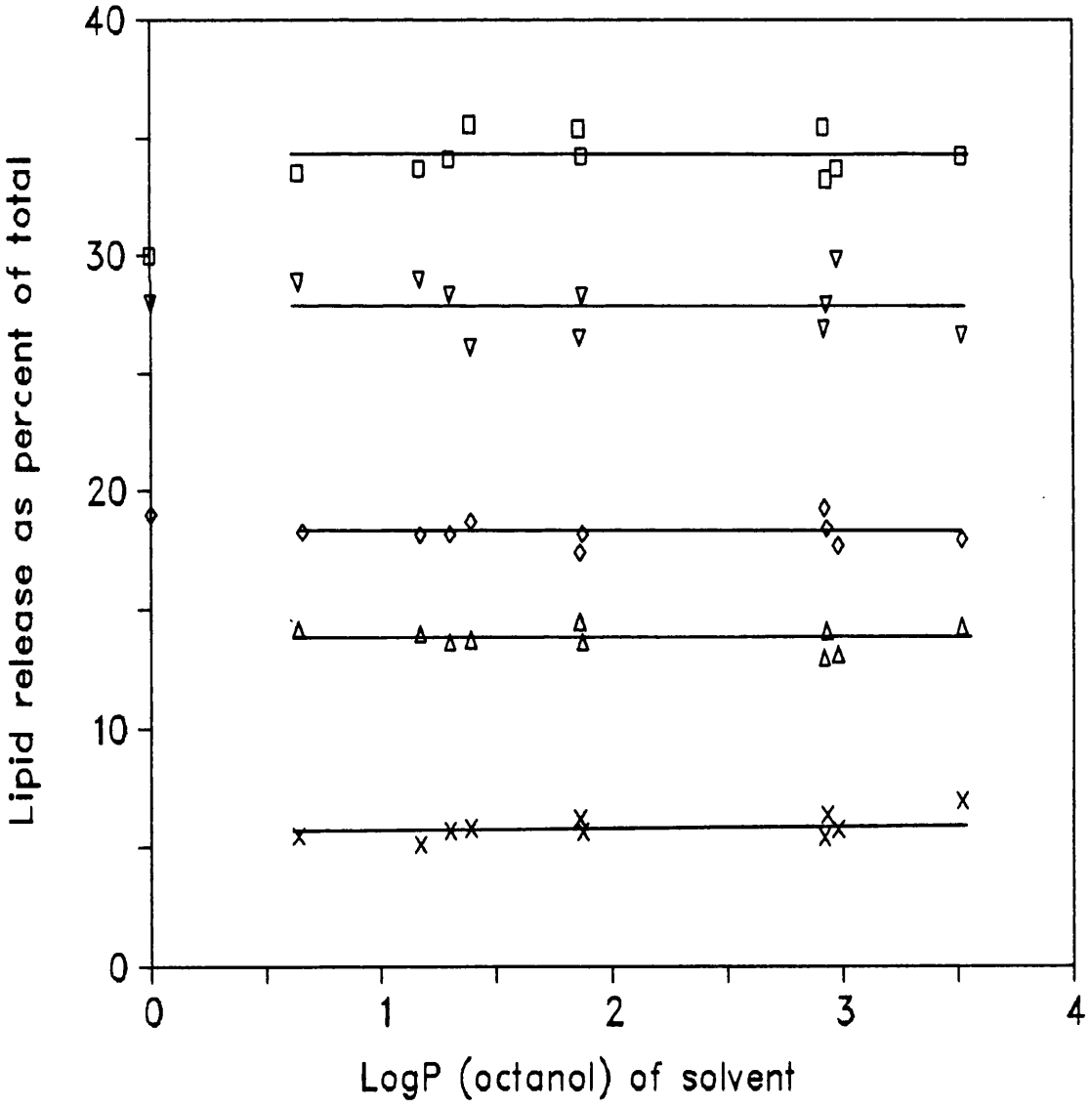


Figure 5.1b Percentage release of the individual fatty acid moieties of which the lipids of the membranes of R.nigricans are composed correlated with the $LogP_{octanol}$ of the organic solvent used in the two liquid-phase systems (table 5.1). Symbols refer to the chain length and position of unsaturation of the fatty acids released;
$C_{16:0}$ ◊ , $C_{18:0}$ + , $C_{18:1}$ □ , $C_{18:2}$ △ , $C_{18:3}$ ▽ .
The results of Erwin (1973) are presented on the Y-axis with the additional 19% fatty acids making up the 100% total consisting of mainly $C_{16:0}$ with some $C_{18:0}$ and $C_{14:0}$.



and the stage of growth. Erwin (1973) has given general values for the total amount of lipid of between 30-40 percent of fungal biomass dry weight. More specifically the lipid content of two species of Rhizopus have been given as 32 percent for R.oryzae (Suzuki 1980) and 49 percent for R.arrhizus (Ratledge 1982).

Table 5.1

Solvent	LogP octanol	Lipid Released (mg/g dry wt cells)
Ethyl acetate	0.64	6.28
4-Met. Pentan-2-one	1.17	5.00
2-Met. Butan-2-ol	1.30	5.73
Cyclohexanol	1.39	4.89
Hexanol	1.86	3.52
Hexanoic acid	1.87	4.65
Octanol	2.92	3.02
Octanoic acid	2.93	3.47
Tetrachloromethane	2.98	2.81
Hexane	3.52	1.75
Control (aqueous)		0.21
Total lipid present at 30-40% of biomass		300 - 400

The total amounts of lipid released are calculated from the fraction of solvent used for analysis as compared with the amount used for extraction. It should be noted that the experimental methodology adopted did not sample the interfacial solvent of the two liquid-phases. A significant proportion of the released lipid might be present at the interface owing to their amphipathic nature so that values presented in table 5.1 are likely to underestimate the true amounts of lipid released. Despite this inaccuracy, it can be clearly seen that only a few percent of the total cellular lipid available for extraction is actually released in the two liquid-phase systems. However, a high proportion of the released lipid is probably extracted solely from the cytosolic membrane. This may lead to severe disruption of the latter and consequently permeabilisation of the cells.

5.1.1 Extraction of Membrane Lipids by Organic Solvents - Correlation with other Solvent Parameters

In the two liquid-phase systems studied, the release of lipids from R.nigricans decreases with increasing $LogP_{octanol}$ of the organic solvent used as the second liquid phase. If lipid release is also associated with permeabilisation then the results tend to support the now generally recognised trend (Laane *et al* 1985) that organic solvents having higher $LogP_{octanol}$ values cause less damage to the biocatalyst and so allow greater retention of catalytic activity.

For organic solvents to cause permeabilisation of the cytoplasmic membrane, molecules of the solvent must, by some means, reach the cell and pass through the cell wall. Two obvious processes by which this may occur are 1) solvation of organic solvent molecules in the aqueous phase which then partition between the membrane and the aqueous phase and 2) direct contact of the cells with the organic phase followed by movement of the organic solvent molecules through the cell wall by some form of capillary action. The ability of lower $LogP_{octanol}$ solvents to cause greater release of lipid may be argued on the basis of either process. Firstly, it may reflect their generally increased capacity to dissolve in the aqueous phase and partition to give higher concentrations in the membranes (see sections 5.2.2 to 5.2.3.1 and table 5.2.8). Alternatively, it is possible that the generally lower surface tension and viscosity of these solvents (figures 5.1.1a, 5.1.1b) allows the formation of more dispersed systems, giving a higher surface area with which the cells may come in contact. In addition, the lower surface tension may increase the ability of these solvents to 'wet' (Muncaster, 1981) the organism and so come into contact with the cell wall.

If the solvent/cell direct contact process of permeabilisation was dominating then the amount of lipid released might be expected to decrease with either increasing surface tension and/or viscosity. As is shown in figures 5.1.1c, 5.1.1d there does appear to be some correlation between lipid release and the surface tension of the organic solvent used in contacting the cells. However, the solvent hexane is particularly anomalous in that it has the lowest values for both surface tension and viscosity but causes the least amount of lipid

Figure 5.1.1a Relationship between the surface tension of a set of organic solvents and their $LogP_{octanol}$ values. Numbers refer to the solvents listed in table 5.1.

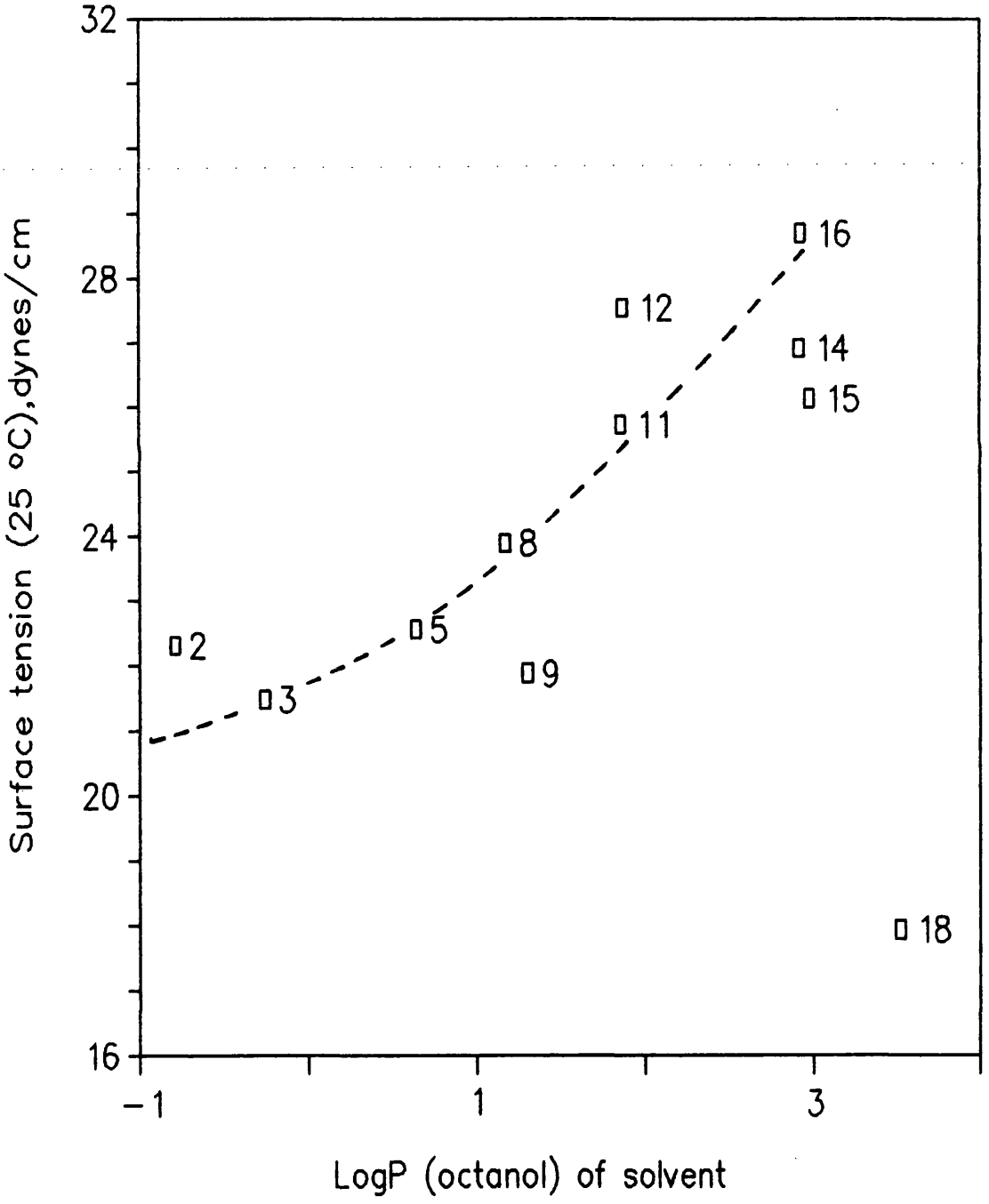


Figure 5.1.1b Relationship between the viscosity of a set of organic solvents and their $LogP_{octanol}$ values. Numbers refer to the solvents listed in table 5.1.

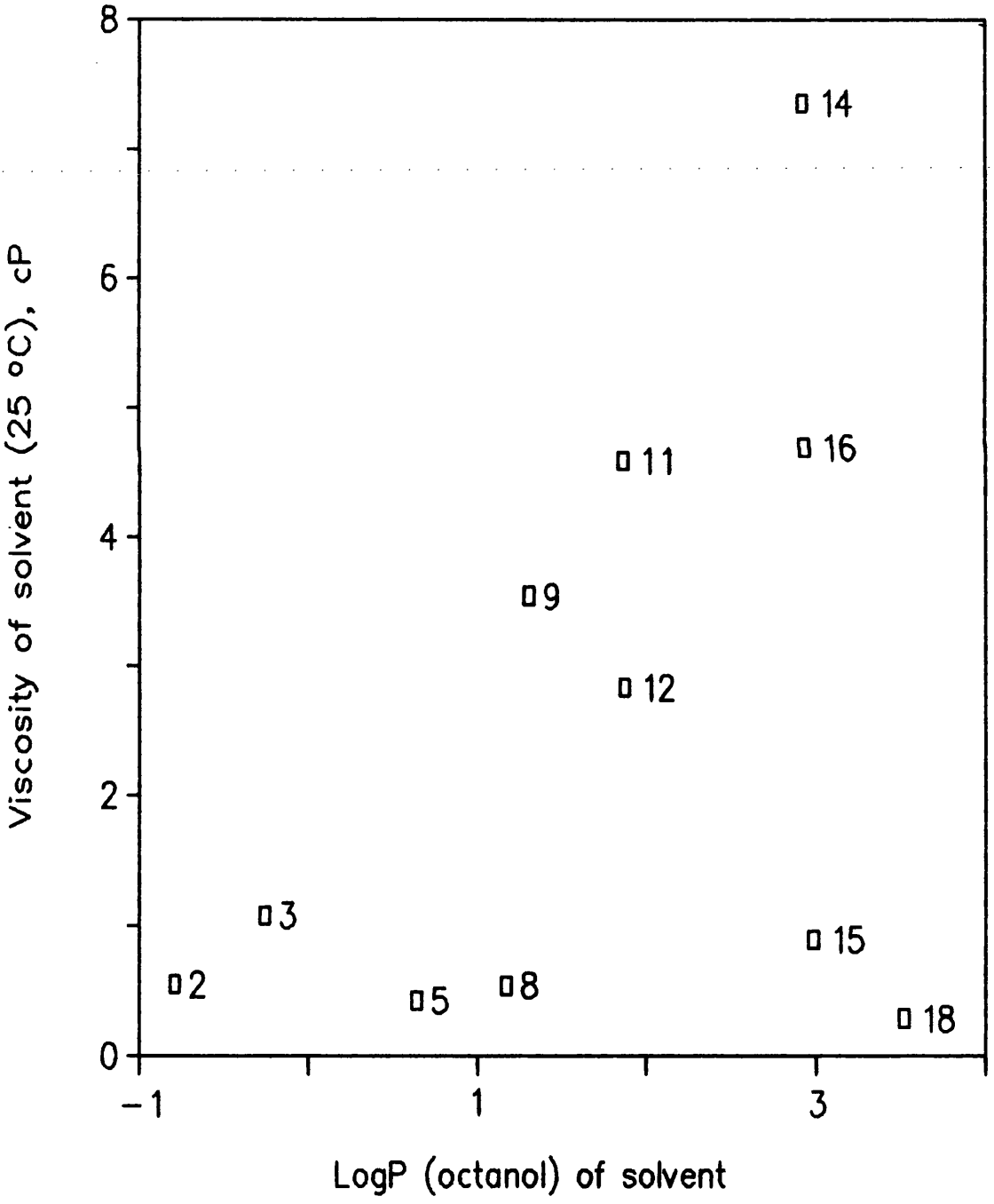


Figure 5.1.1c Relationship between the amount of lipid released from the membranes of R.nigricans and the surface tension of the organic solvents used in the two liquid-phase systems. Numbers refer to the solvents listed in table 5.1.

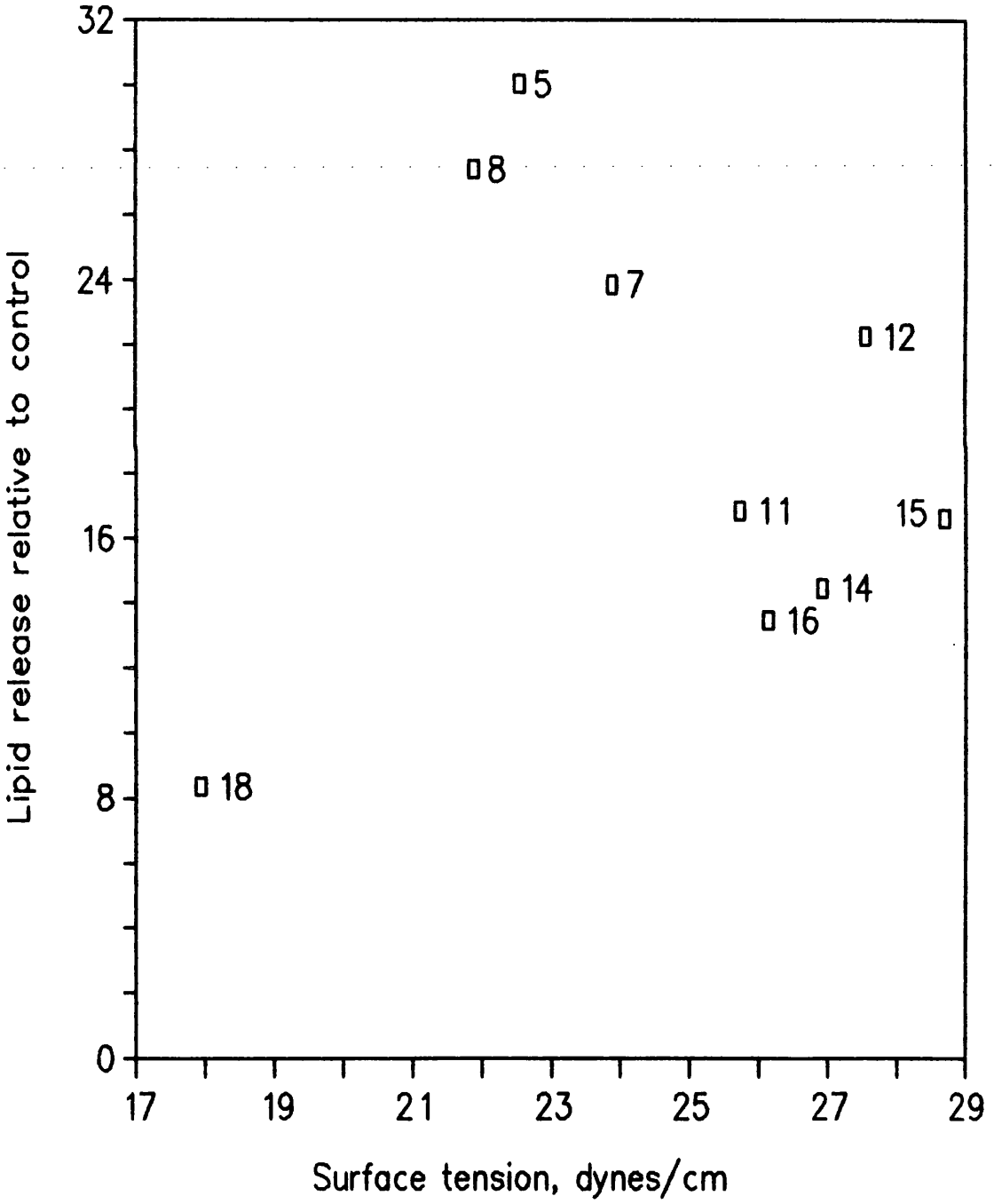


Figure 5.1.1d Relationship between the amount of lipid released from the membranes of R.nigricans and the viscosity of the organic solvents used in the two liquid-phase systems. Numbers refer to the solvents listed in table 5.1.

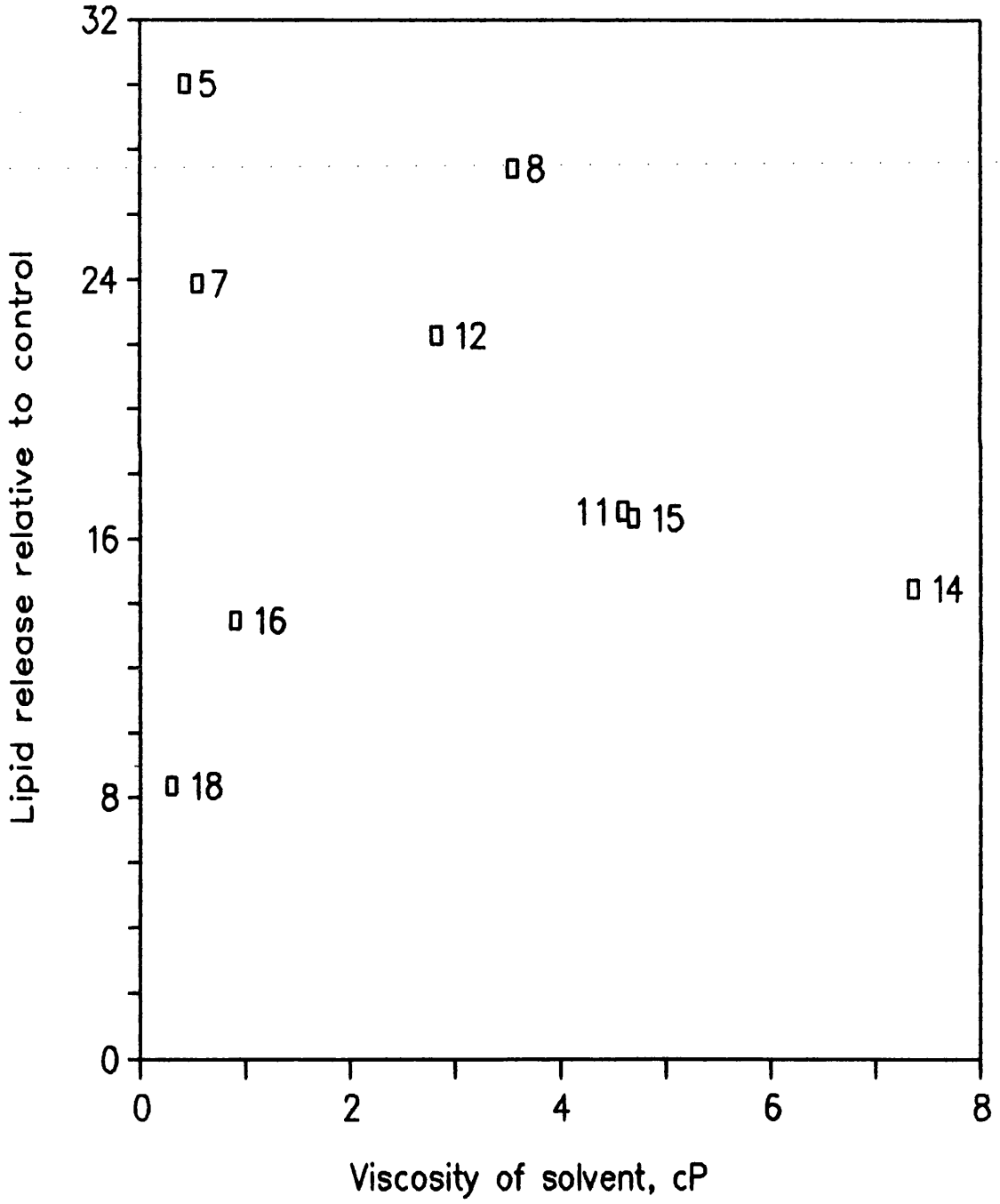
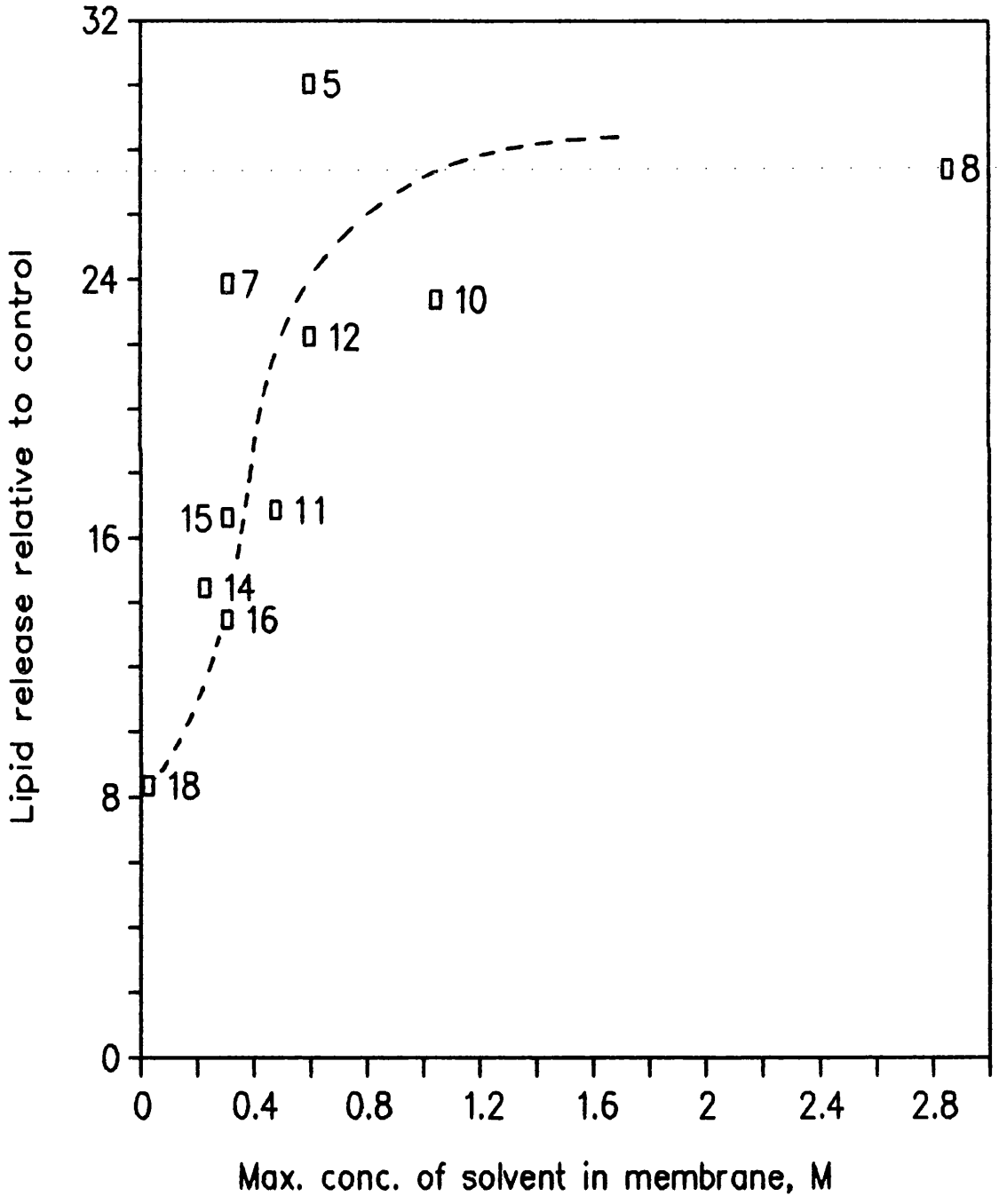


Figure 5.1.1e Relationship between the amount of lipid released from the membranes of R.nigricans and the equilibrium concentrations of the organic solvents (see sections 5.2.2 to 5.2.3.1) calculated to be present in the membranes of the organism. Numbers refer to the solvents listed in table 5.1.



release.

A good correlation, which accounts for all the solvents studied, is achieved if lipid release is plotted against the equilibrium solvent concentration calculated to be present in the membranes, figure 5.1.1e (see section 5.2.2 for an explanation of solvents partitioning into membranes). This would tend to support the hypothesis that the main way in which permeabilisation occurs is through solvent dissolving in the aqueous phase, partitioning into the membrane and then solubilising the lipids causing their release.

The results presented appear to indicate that for the two liquid-phase systems studied permeabilisation occurs through partitioning of the organic solvents from the aqueous phase into the membrane so causing release of lipids. However, with changes in operating conditions such as increased agitation to give highly dispersed systems and higher phase ratios, the direct contact of cells with the organic solvent may prove to be of greater importance as a cause of permeabilisation. A further discussion of these results in the light of other findings is presented in section 6.3.

5.2 Experimental Procedure 1 : Loss of 11α-Hydroxylase Activity by Cells of *Rhizopus nigricans* in Two Liquid-Phase Systems

Many researchers have sought to relate the loss of biocatalytic activity of organisms in two liquid-phase systems with physico-chemical properties of the organic solvents used in the systems (Brink and Tramper 1985) (Laane *et al* 1985). Although correlations of this sort have provided a less empirical means of selecting organic solvents they have contributed little to understanding the mechanisms involved by which solvents act to cause loss of biocatalytic activity.

By definition two liquid-phase systems should have a discrete second phase of organic solvent. The presence of a liquid-liquid interface in such systems does not allow the necessary separation of effects caused by solvent dissolved in the aqueous phase and 'phase' phenomena as described by Bar 1986, 1987 (section 1.3.4). To study the effects of

organic solvent dissolved in the aqueous phase on biocatalytic activity it is necessary to reduce the amount of organic solvent in the systems to a point where no second liquid phase is present. Unsaturated aqueous phases of this sort can then be regarded as a form of cosolvent system.

True cosolvents comprise systems where the organic solvent is totally miscible with the aqueous phase. It has been found that biocatalytic activity may be retained if the concentration of solvent in the aqueous phase is kept low (Sonomoto 1982, 1983). However, the complete absence of 'phase' effects in such systems means that the loss of activity which is observed at higher solvent concentrations can be directly attributed to the presence of dissolved organic solvent in the aqueous phase. If dissolved solvent can be the sole cause of solvent-related loss of activity in these systems, it is possible that it also accounts for the loss of activity in two liquid-phase systems where the aqueous phase is saturated with organic solvent.

5.2.1 Relationship Between 11α-Hydroxylase Activity, Organic Solvent Concentration in the Aqueous Phase and Formation of a Liquid-Liquid Interface

The 11α-hydroxylase activity of R.nigricans was studied in seventeen organic-aqueous two liquid-phase systems and four cosolvent systems. For each solvent system a range of solvent volumes was studied. For the two liquid-phase systems this corresponded to aqueous phase concentrations less than and greater than that required to saturate the aqueous phase.

From physical data in the literature (Riddick *et al* 1986) and other calculated data (appendix 2.1) the aqueous phase concentrations of the organic solvents were determined.

$$C_{aq} = \frac{V_0 \times P_0}{M_0} \times \frac{1000}{V_{aq}} \qquad [E5.2.1]$$

where C_{aq} is the aqueous phase concentration of organic solvent, V_0 is the volume of organic solvent, P_0 is the density of the organic solvent,

M_0 is the molecular weight of the organic solvent and V_{aq} is the volume of aqueous phase (ml). The latter value, V_{aq}, is calculated from the volume of cell suspension added (V_{sus}), taking into account the volume of the cells present.

$$V_{aq} = V_{sus} - (M_t \times R_{w/d} \times P_c)$$

where M_t, the dry mass of cells in suspension volume V_{sus}, is obtained from dry weight analysis of a known volume of cell suspension. P_c the cell density is obtained by density gradient centrifugation (section 3.2.1) and $R_{w/d}$ the measured wet/dry weight ratio (sections 3.2.2 to 3.2.4).

Figures 5.2.1a and 5.2.1b show how the aqueous phase solvent concentration at which all hydroxylase activity is lost covers greater than three orders of magnitude, dependent on the organic solvent type. This clearly indicates that there is no direct correlation between loss of biocatalytic activity and the concentration of solvents in the aqueous phase.

Knowing the maximum solubility of each solvent in the aqueous phase (Riddick *et al* 1986) it is possible to relate the 11α-hydroxylase activity to the presence of a liquid-liquid interface. The parameter 'multiples of maximum aqueous phase solvent concentration' (MMAPSC) is here defined as:

$$\text{MMAPSC} = \frac{\text{Volume of organic solvent in the reactor}}{\text{Volume of organic solvent required to saturate aqueous phase}}$$

The formation of a liquid-liquid interface in the system under study should therefore occur theoretically when this parameter is greater than unity. Figure 5.2.1c shows how the 11α-hydroxylase activity is related to this parameter and that for the twelve solvents displayed loss of biocatalytic activity occurs well before the theoretical appearance of an interface.

Figure 5.2.1a 11α-hydroxylase activity of *R.nigricans* in eight primary alcohol-aqueous co-solvent and two-liquid phase systems as a function of the logarithm of the solvent concentration in the aqueous phase. C1, C2 etc. refer to the number of carbon atoms of the alcohol.

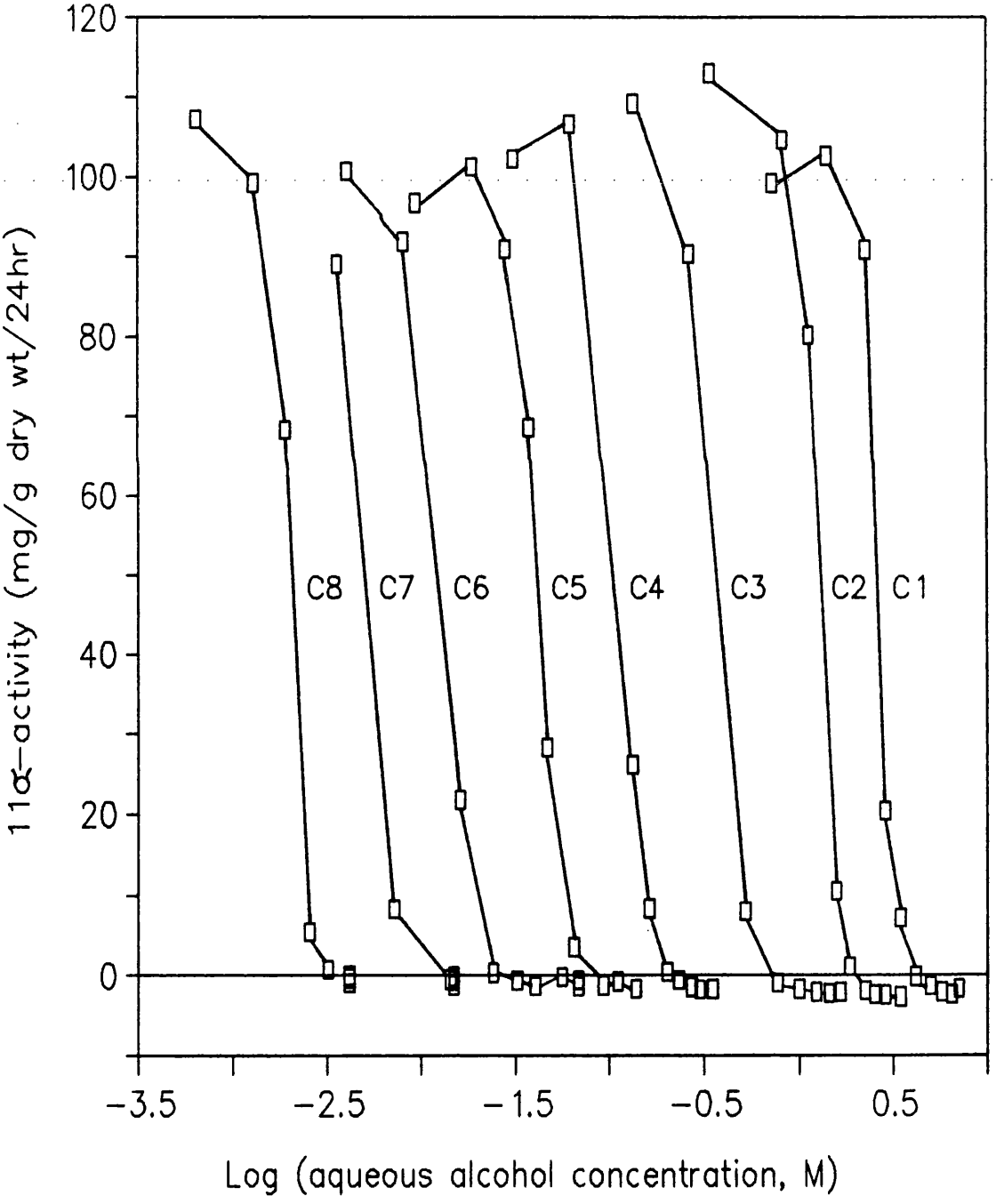


Figure 5.2.1b 11α-hydroxylase activity of *R.nigricans* in four organic-aqueous two-liquid phase systems as a function of the logarithm of the solvent concentration in the aqueous phase.

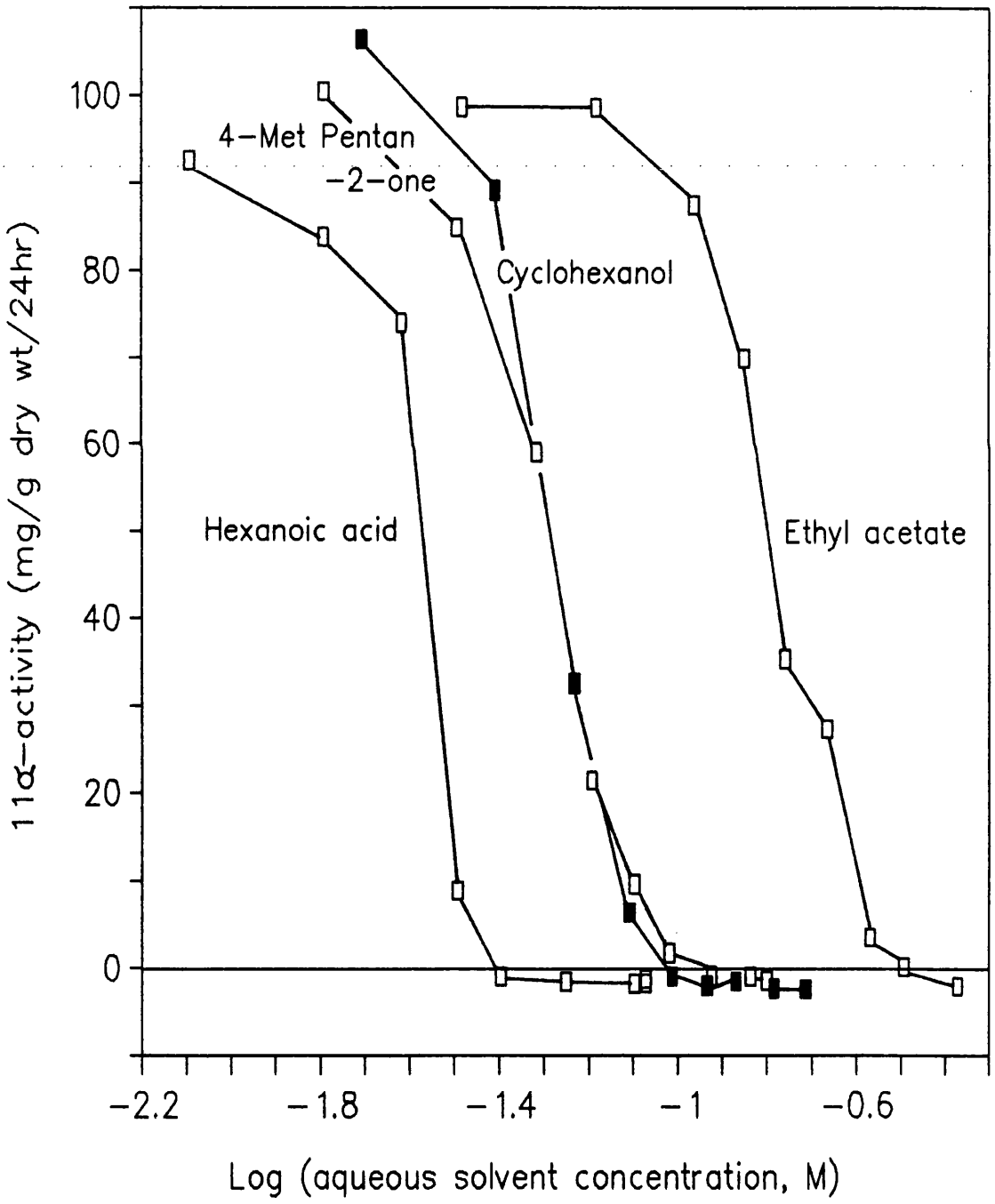
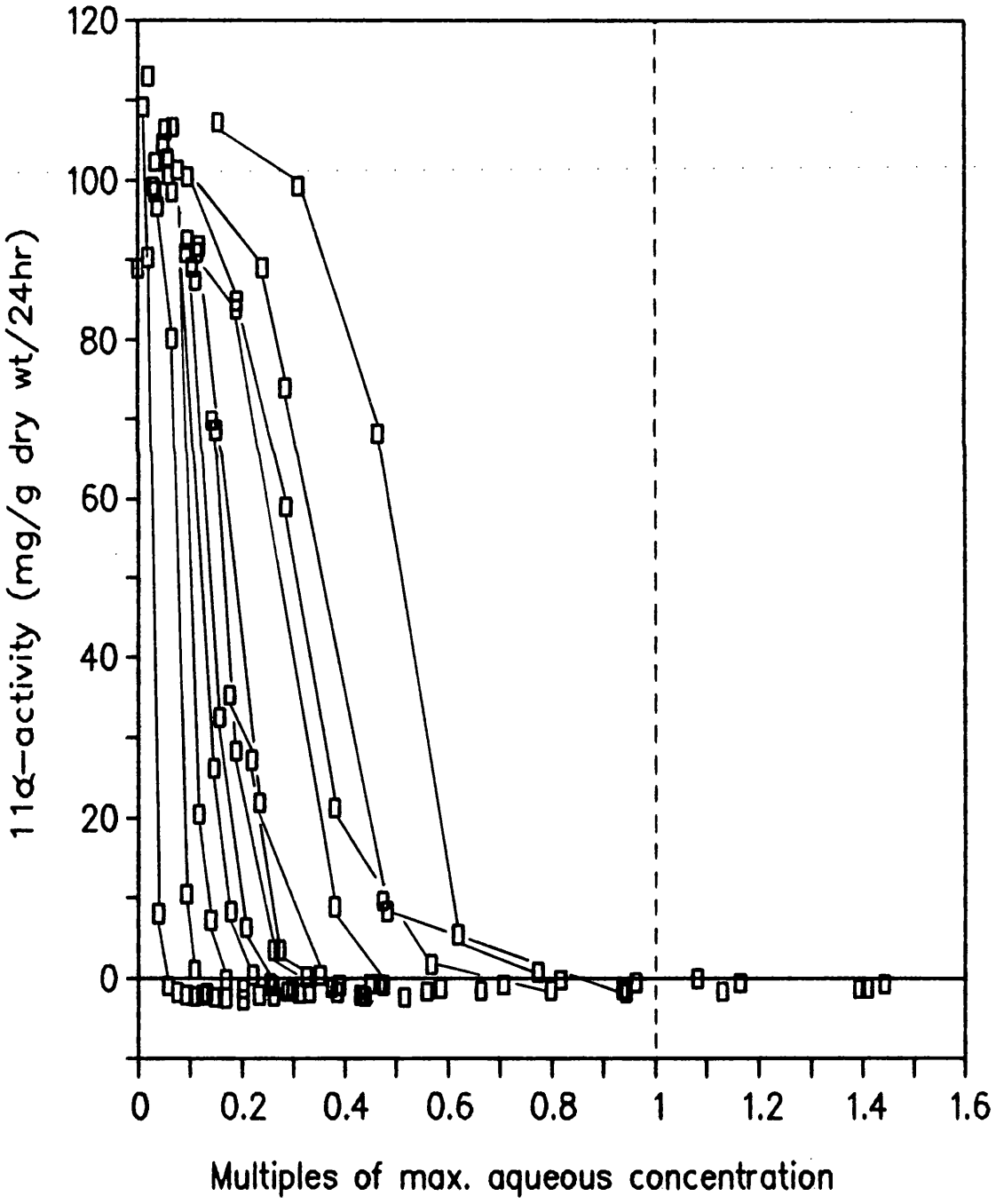


Figure 5.2.1c The relationship between loss of 11α-hydroxylase activity of R.nigricans and the formation of a second liquid-phase (interface) for 12 organic-aqueous solvent systems. Solvents are those referred to in figures 5.2.1a and 5.2.1b. See text for the definition of the X-axis.



Given that the loss of activity does not correlate with either the aqueous phase solvent concentration or the point at which a solvent interface is formed it is necessary to seek other physical or physiological effects which may be acting.

5.2.2 Partitioning Theory and Anaesthetic Action

Molecular species when placed in a system containing two immiscible solvents will partition to give concentrations in each of the phases that are related by a simple partition equation. As an example the standard octanol/aqueous system will have a partition coefficient for any organic solvent given by:

$$P_{octanol} = \frac{[solvent_{oct.}]}{[solvent_{aq.}]} \qquad [E5.2.2a]$$

where $[solvent_{oct.}]$ is the concentration of solvent in the octanol phase and $[solvent_{aq.}]$ is the concentration of solvent in the aqueous phase. Similarly the partition coefficient, $P_{membrane}$, of an organic solvent in a membrane/aqueous system will be given by:

$$P_{membrane} = \frac{[solvent_{memb.}]}{[solvent_{aq.}]} \qquad [E5.2.2b]$$

The partition coefficients of a molecular species in two different partitioning systems may be related, provided that the two systems are not too dissimilar (ie. with respect to polarity). Collander 1950, 1951 was the first to describe this type of relationship which can be given for the systems above and has the form:

$$P_{membrane} = X \times P_{octanol}^{Y} \qquad [E5.2.2c]$$

or

$$Log(P_{membrane}) = Y \times Log(P_{octanol}) + Log(X) \qquad [E5.2.2d]$$

Anaesthetics comprise many of the organic solvents that are studied in the area of two liquid-phase biocatalysis; alcohols, hydrocarbons,

ethers, phenols, nitro, chloro and fluoro compounds. Work by Seeman 1972 on anaesthetic action showed that the Collander relationship holds for several types of membrane; erythrocyte, synaptosomal and sarcoplasmic reticulum. However, the equation proposed for the correlation between $P_{membrane}$ and $P_{octanol}$ was semi-empirical and had the constant for X (in equation [E5.2.2c]) equal to 0.2 and that for Y equal to unity. By applying regression analysis to the data of Seeman 1972 the actual equation becomes:

$$P_{membrane} = 0.19 \times P_{octanol}^{0.98} \qquad [E5.2.2e]$$

By substituting equation [E5.2.2b] we can obtain the following equation that relates the concentration of solvent present in the membrane to both the $P_{octanol}$ of the partitioning solvent and the solvent concentration in the aqueous phase.

$$[solvent_{memb.}] = 0.19 \times P_{octanol}^{0.98} \times [solvent_{aq.}] \qquad [E5.2.2f]$$

or rearranging and taking logarithms

$$Log[solvent_{aq.}] = 0.98 \times Log(1/P_{octanol}) + Log([solvent_{memb.}]/0.19) \qquad [E5.2.2g]$$

5.2.3 Correlating Loss of Hydroxylase Activity with the Concentration of Solvent in the Membranes of *R.nigricans*

The critical aqueous phase concentration of solvent at which complete loss of 11α-hydroxylase activity occurs may be denoted by $[solvent_{aq.C}]$ and its corresponding critical concentration in the membrane, $[solvent_{memb.C}]$. By substituting in equation [E5.2.2g] the following equation is obtained:

$$Log[solvent_{aq.C}] = 0.98 \times Log(1/P_{octanol}) + Log([solvent_{memb.C}]/0.19) \qquad [E5.2.3a]$$

Values for $Log[solvent_{aq.C}]$ can be read directly from figures 5.2.1a, 5.2.1b or more accurately calculated from plots similar to

figure 5.2.3a. $LogP_{octanol}$ values (table 5.2.3a) may be taken from the literature (Leo et *al* 1971) or calculated from hydrophobic fragmental constants according to Rekker 1977. By plotting $Log[solvent_{aq.C}]$ against $Log(1/P_{octanol})$ of the solvents, figure 5.2.3b, a straight line is obtained. The significance of this correlation comes from the linearity which gives rise to a single Y-intercept value equal to $Log([solvent_{memb.C}]/0.19)$. This implies that the concentration of solvent in the membrane at which total loss of 11α-hydroxylase activity occurs is constant, irrespective of the solvent type. Table 5.2.3b gives the regression values and shows that for the best correlation the critical concentration of solvent in the membrane is 198mM.

Table 5.2.3a Solvents and their calculated $LogP_{octanol}$ values (Rekker 1977).

Number	Solvent	$LogP_{octanol}$
1	Dimethyl sulfoxide	-1.35
2	Methanol	-0.79
3	Ethanol	-0.26
4	Propanol	0.27
5	Ethyl acetate	0.64
6	Butanol	0.80
7	4-Methyl pentan-2-one	1.17
8	2-Methyl butan-2-ol	1.30
9	Pentanol	1.33
10	Cyclohexanol	1.39
11	Hexanol	1.86
12	Hexanoic acid	1.87
13	Heptanol	2.39
14	Octanol	2.92
15	Octanoic acid	2.93
16	Carbon tetrachloride	2.98
17	Nonanol	3.45
18	Hexane	3.52
19	Decanol	3.98
20	Undecanol	4.51
21	Dodecanol	5.04

Figure 5.2.3a 11α-hydroxylase activity of R.nigricans in three organic-aqueous reaction systems as a function of the solvent concentration in the aqueous phase. Points a, b, c are the critical aqueous phase solvent concentrations for pentanol, butanol and propanol respectively.

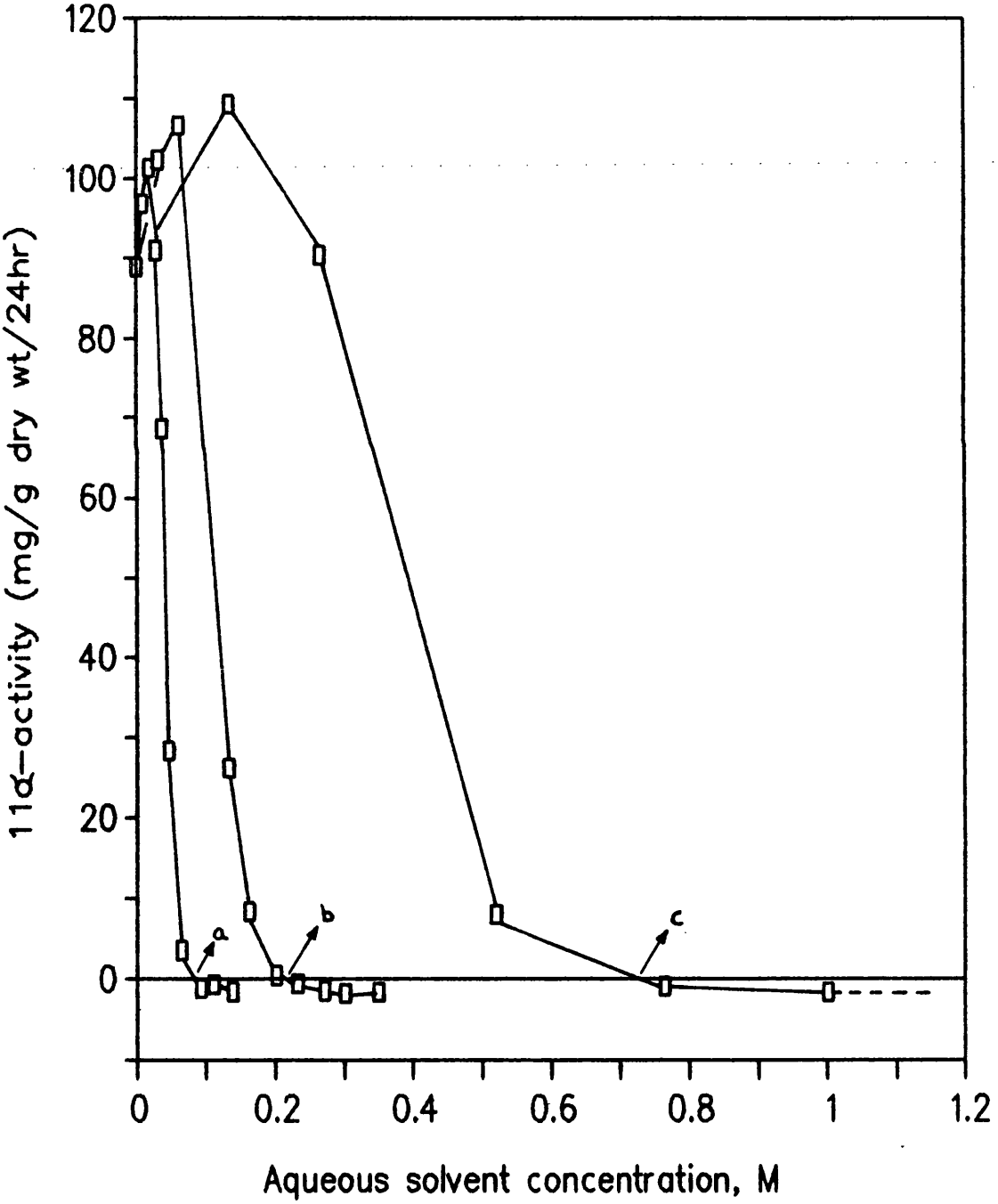


Figure 5.2.3b Relationship between the aqueous solvent concentration at which all 11α-hydroxylase activity is lost and the octanol partition coefficient of the solvent. Numbers refer to the solvents listed in table 5.2.3a.

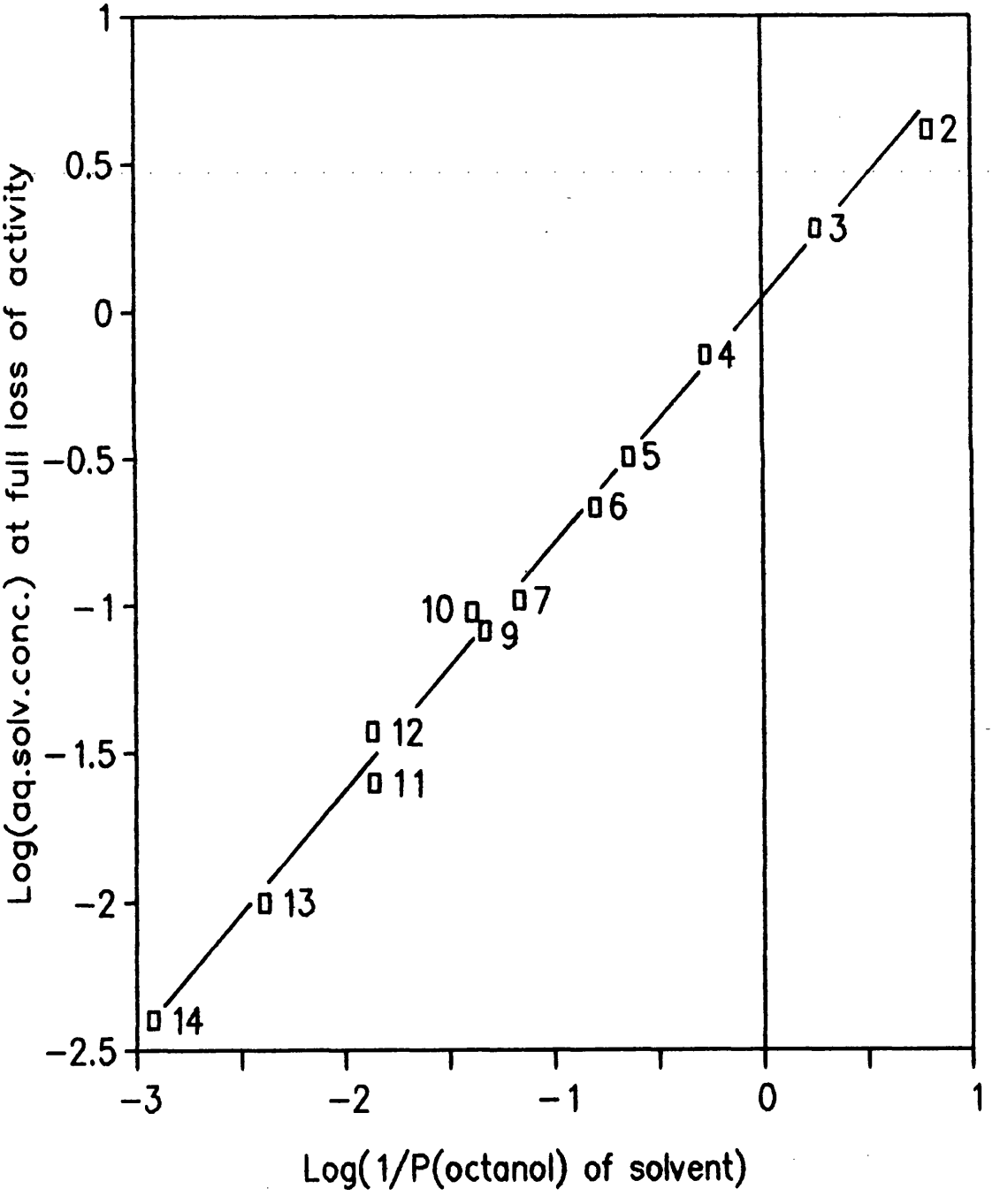


Table 5.2.3b Regression fits to the data presented in figure 5.2.3b.

	All solvent data	Alcohol data only
Gradient Y-intercept Corr. Coeff.	0.83 0.0289 0.997	0.84 0.0173 0.999
[$solvent_{memb.C}$], M	0.203	0.198

5.2.3.1 Adapting the Collander Equation of Seeman to the Microbial System of *R.nigricans*

Equation, [E5.2.3a], obtained from the partitioning data of Seeman 1972 suggests that the gradient of the line should have a value of 0.98. The regression results of table 5.2.3b gives this value for the data obtained with R.nigricans as 0.84 (alcohol data used as it provides the best correlation). The difference between the two values probably reflects differences in the nature of the two systems including; the structure and composition of the membranes (mammalian erythrocyte compared to microbial), the sensitivity of the membranes to alterations in the hydrophobic nature of the solvents, and perhaps less importantly the chemical composition of the aqueous phases (50mM sodium phosphate buffer pH 7.4, 1.5mM $MgCl_2$, 0.25% glucose in this study as compared to 0.9% NaCl for the study of Seeman 1972). Equation [E5.2.2f] can therefore be changed for the microbial system of R.nigricans to:

$$[solvent_{memb.}] = 0.19 \times P_{octanol}^{0.84} \times [solvent_{aq.}] \qquad [E5.2.3.1]$$

Using this equation it is now possible to calculate the concentration of solvent in the membranes of R.nigricans for all the aqueous phase solvent concentrations presented in figures 5.2.1a, 5.2.1b. The results, figure 5.2.3.1a, clearly show how loss of 11α-hydroxylase

Figure 5.2.3.1a The 11α-hydroxylase activity of R.nigricans in twelve organic-aqueous reaction systems as a function of the calculated solvent concentration in the cell membranes. Solvents are those referred to in figures 5.2.1a and 5.2.1b.

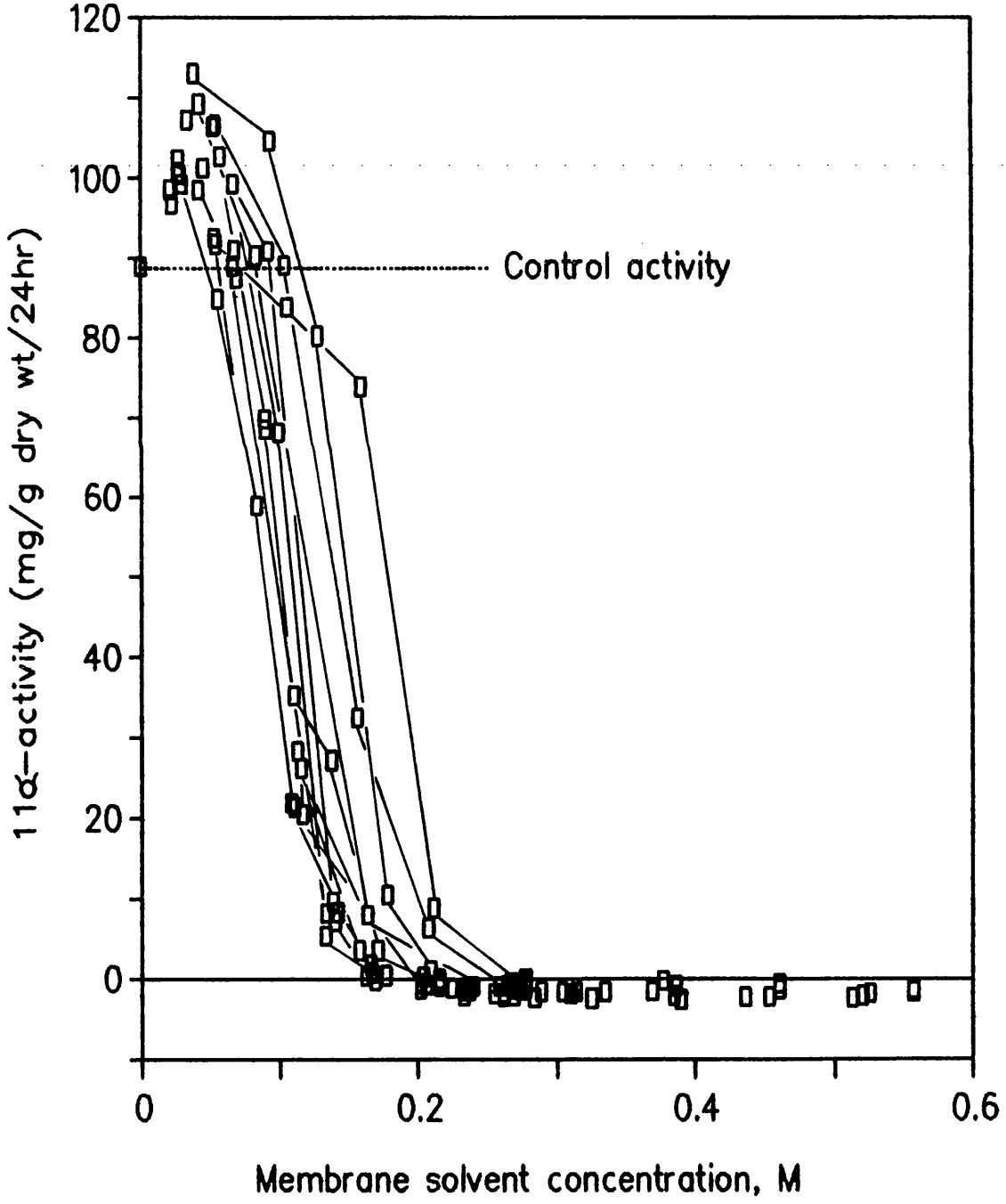
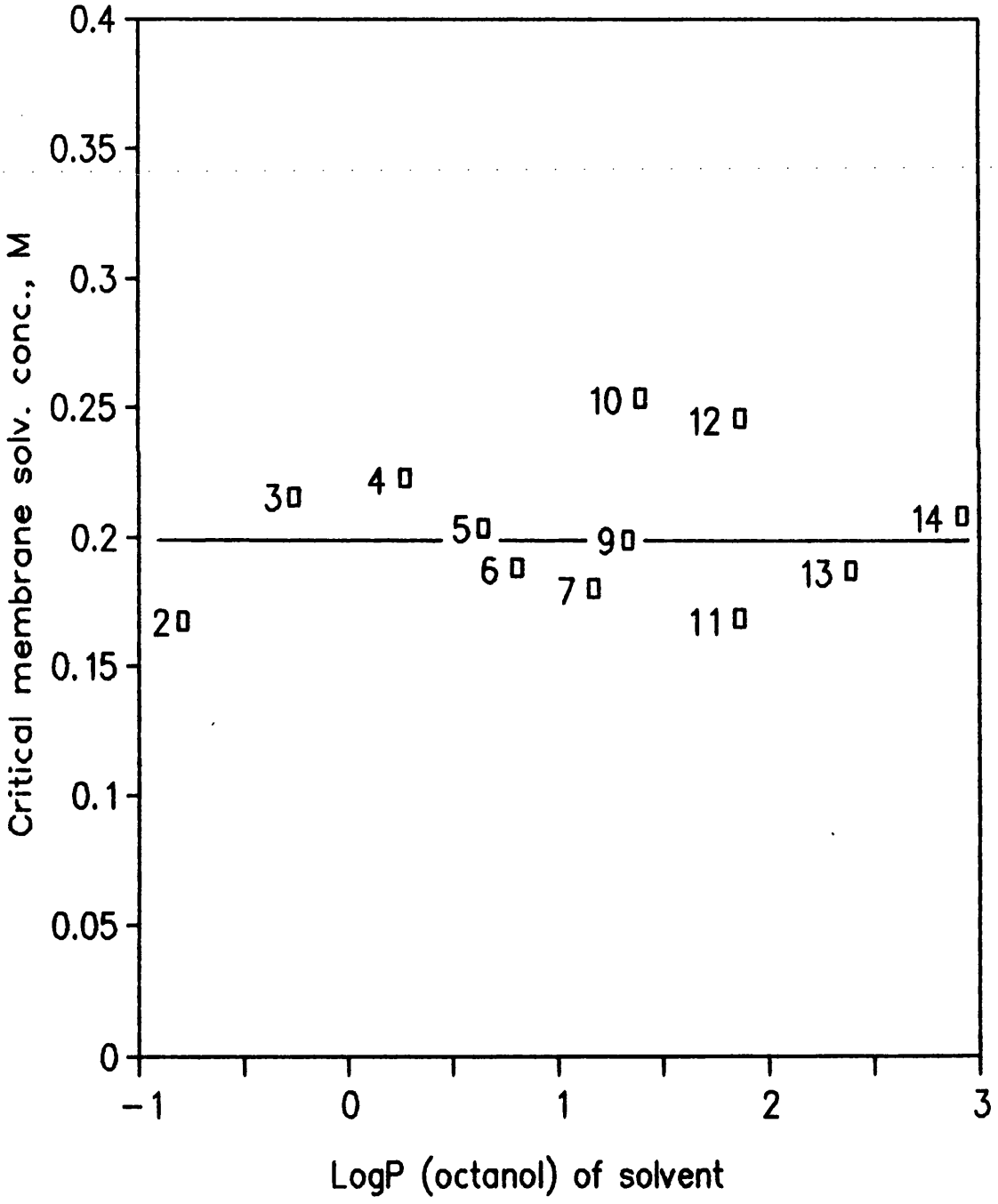


Figure 5.2.3.1b Relationship between solvent concentration in the membrane at which all 11α-hydroxylase activity is lost, 'critical membrane solvent concentration', and the logarithm of the octanol partition coefficient of the solvent. The solid line denotes the critical membrane solvent concentration taken from the regression fit of figure 5.2.3b, 198mM. Numbers refer to the solvents listed in table 5.2.3a.



activity correlates well with the solvent concentration calculated to be present in the membranes. However, the membrane solvent concentration at which all biocatalytic activity is lost is not precisely the same for each solvent type (this is also implicit from the deviations in the data points from the fitted regression line, figure 5.2.3b).

It has been suggested by Mullins 1954 that it is not a critical concentration of solvent but a critical volume of solvent in the membrane that induces the response. This theory would suggest that smaller molecules (related to lower $LogP_{octanol}$ value solvents) should require greater concentrations in the membrane as compared to larger molecules (higher $LogP_{octanol}$). By plotting the critical membrane solvent concentrations against the $LogP_{octanol}$ value for the solvent, figure 5.2.3.1b, it is possible to test this hypothesis. A negative correlation coefficient would be expected if Mullins theory were correct. This is not observed, the points being approximately randomly distributed around the fitted line of critical membrane solvent concentration. The implication is that a single critical membrane solvent does indeed exist at which all 11α-hydroxylase activity and that the observed differences are due to either experimental error or other factors such as solvent impurities.

5.2.3.2 Organic Solvents that Do Not Cause Total Loss of 11α-hydroxylase Activity

Hexane, a commonly used conventional organic solvent, has an extremely low solubility in aqueous systems (0.143mM in water). Calculating its maximum achievable concentration in the cell membranes from equation [E5.2.3.1] ($LogP_{octanol}$=3.52) a value for [$solvent_{memb.}$] of 25mM is obtained. This is considerably lower than the critical concentration of 198mM required to cause total loss of 11-hydroxylase activity. As figure 5.2.3.2 illustrates, activity only begins to be lost when 160 times the volume of hexane required to saturate the aqueous phase is present. Loss of hydroxylase activity must therefore be due to some gross 'phase' effect and not to dissolved solvent in the aqueous phase.

Similarly, at saturating aqueous phase concentrations, undecanol and dodecanol can only achieve maximum membrane solvent concentrations of 81mM and 63mM respectively. Figure 5.2.3.2 shows that like hexane, undecanol and dodecanol cause loss of activity through 'phase' effects only, since respectively 4 and 20 times the volume of solvent required to saturate the aqueous phase is achieved before any loss of activity is observed. In all three cases some residual activity is always present which would seem to indicate that there is still a functioning 11α-hydroxylase system although its activity is limited as a result of 'phase' effects.

5.2.3.3 Solvents that Appear Not to Obey the General Trends

Four solvents examined in this study were found to not fit into either of the two categories of organic solvent described above (sections 5.2.3.1 and 5.2.3.2). Dimethyl sulfoxide is an exceptionally polar molecule ($LogP_{octanol}$=-1.35) that has a calculated maximum achievable concentration in the membranes of 46mM. This is well below the critical concentration (198mM) expected to be required for complete loss of 11α-hydroxylase activity. However, as figure 5.2.3.3a shows complete loss of activity does occur and at a solvent concentration in the membranes close to the expected maximum. The maximum in the membranes also corresponds to a maximum in the aqueous phase solvent concentration (ie. saturation) so that total loss of activity in this case may be associated with the appearance of a liquid-liquid interface. This does not, however, explain the gradual loss of activity at increasing aqueous phase solvent concentrations below the saturation concentration.

The solvent 2-methyl butan-2-ol ($LogP_{octanol}$=1.3) has the capacity to reach a concentration in the membranes of 1215mM, well in excess of the critical concentration and therefore should be able to cause complete loss of 11α-hydroxylase activity. This is achieved, figure 5.2.3.3b, but at a concentration in the membranes (>600mM) far higher than the expected critical concentration of (198mM). A possible explanation is that the value of $LogP_{octanol}$ for 2-methyl butan-2-ol calculated from hydrophobic fragmental constants does not truly represent the hydrophobicity of the solvent. This hypothesis is

Figure 5.2.3.2 The relationship between loss of 11α-hydroxylase activity and the formation of a second liquid-phase (interface) for the solvents hexane, undecanol and dodecanol.

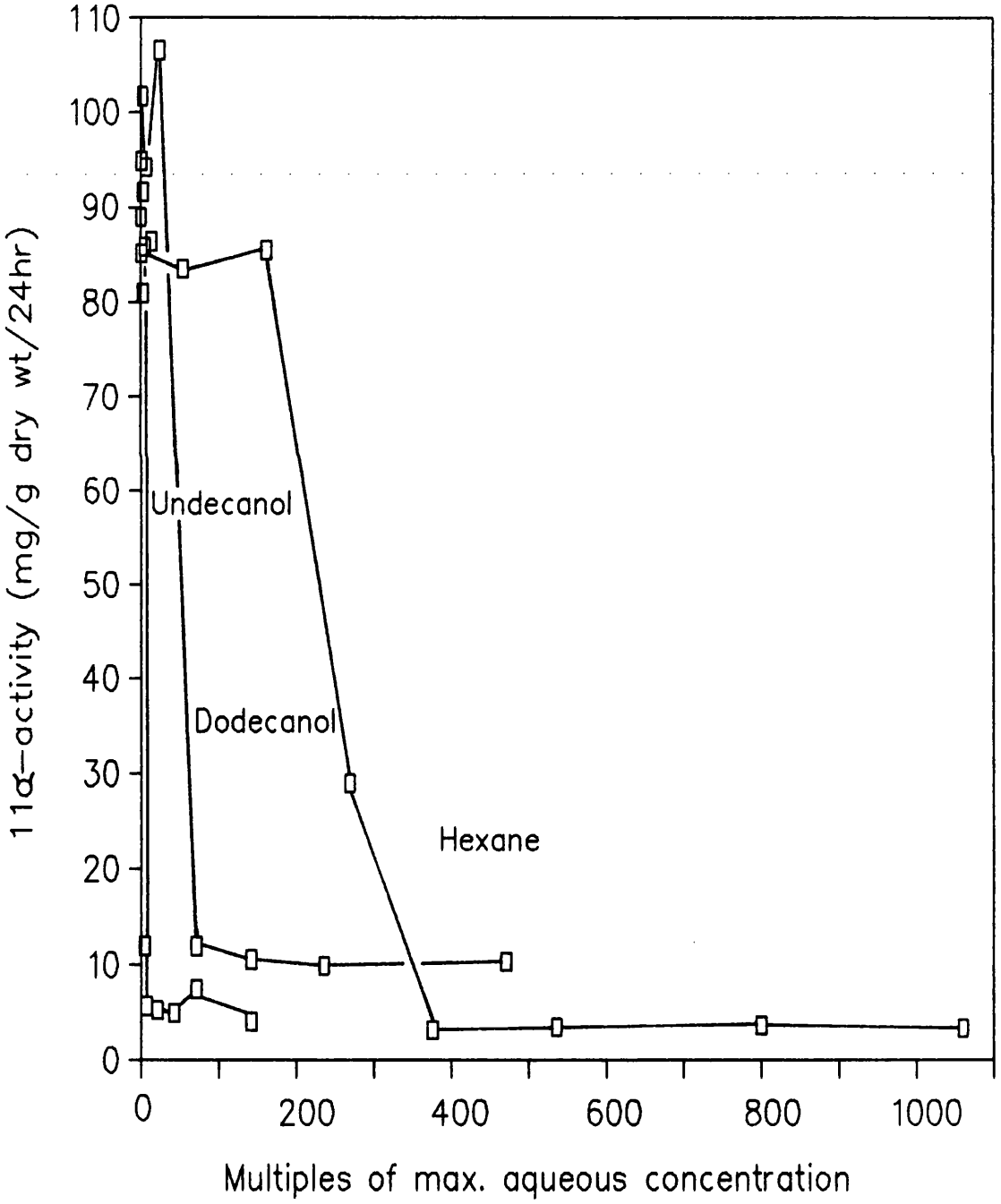


Figure 5.2.3.3a The relationship between loss of 11α-hydroxylase activity by cells of R.nigricans and the calculated membrane solvent concentration of dimethyl sulfoxide.

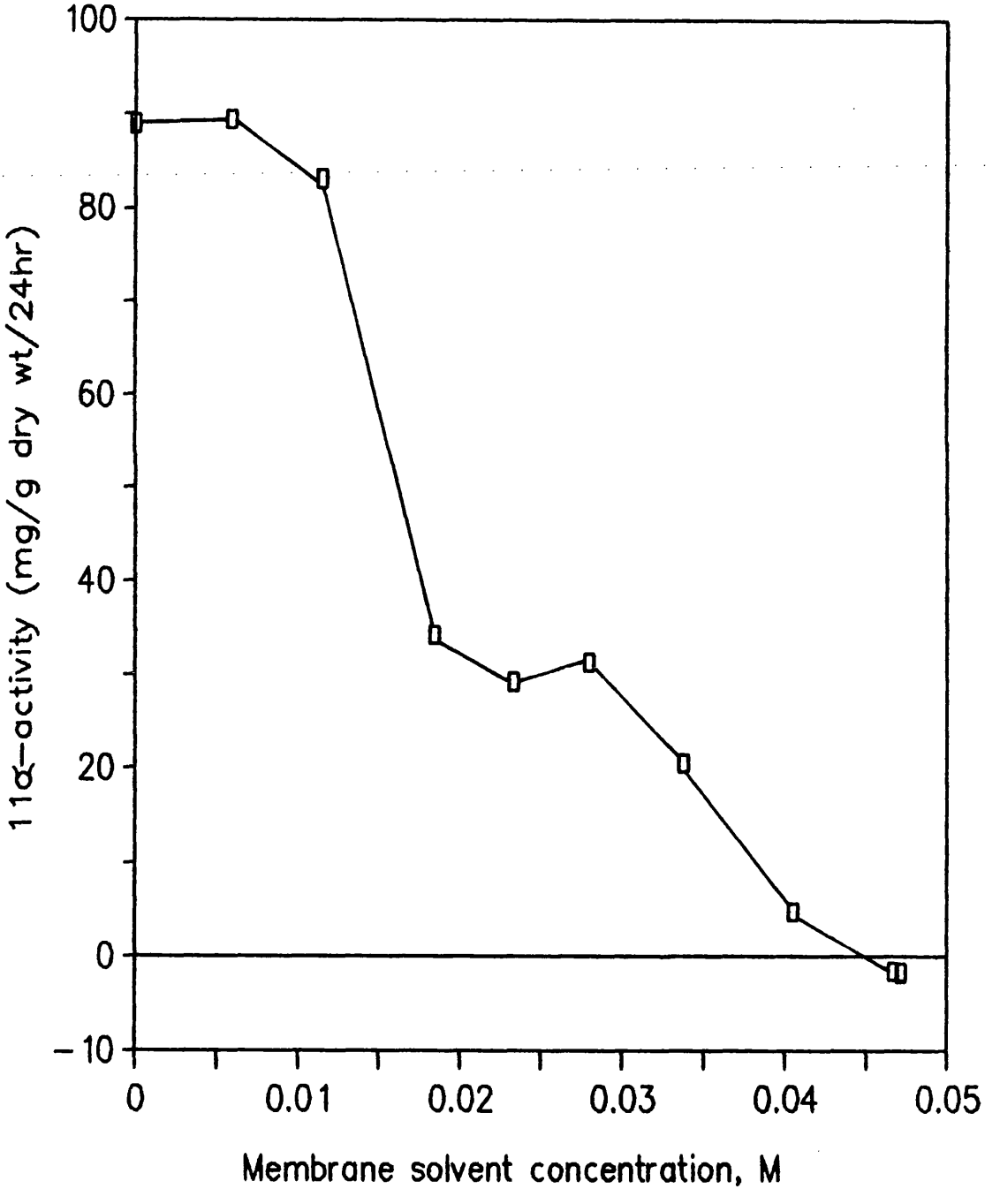


Figure 5.2.3.3b The relationship between loss of 11α-hydroxylase activity by cells of R.nigricans and the calculated membrane solvent concentration of 2-methyl butan-2-ol.

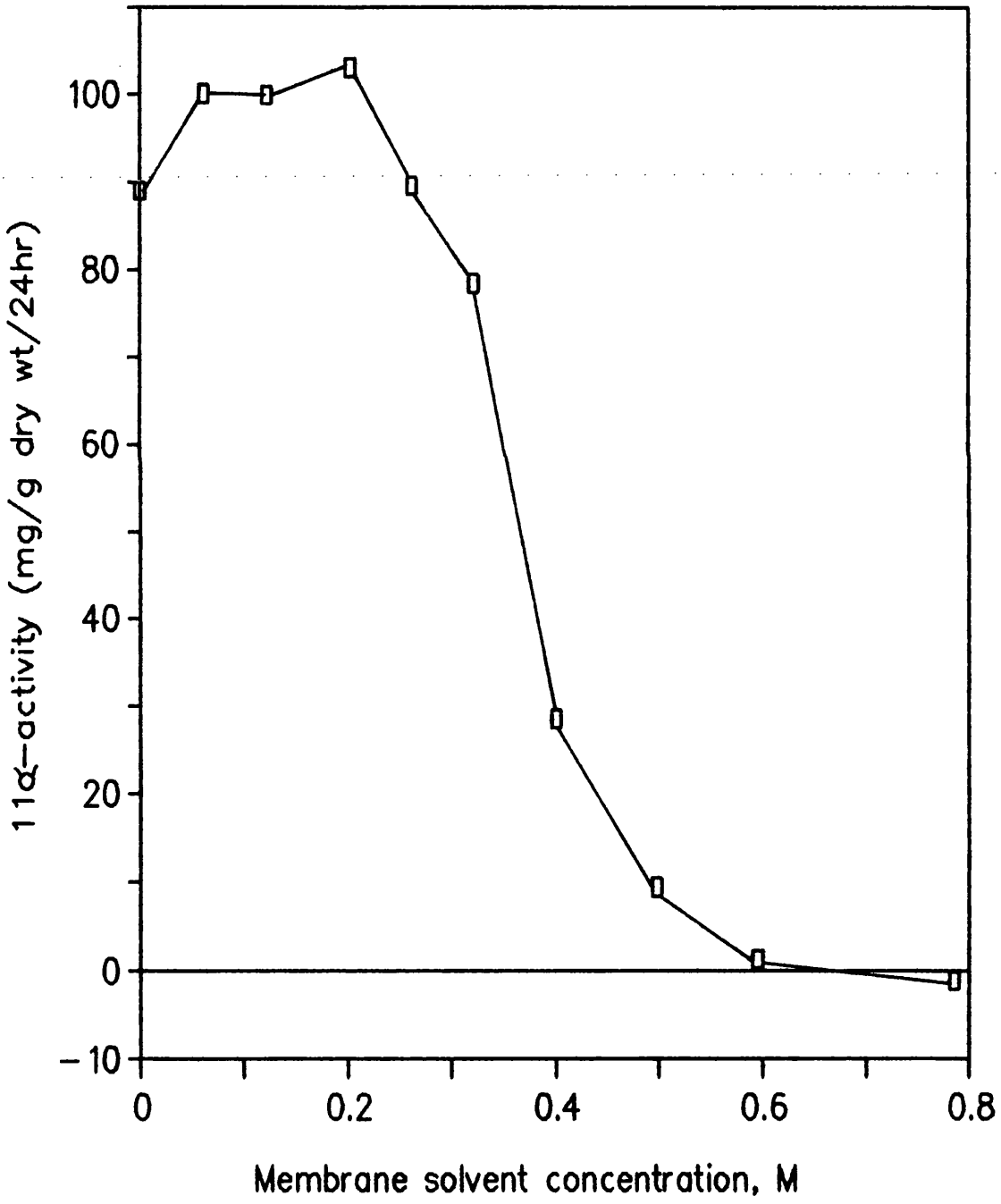
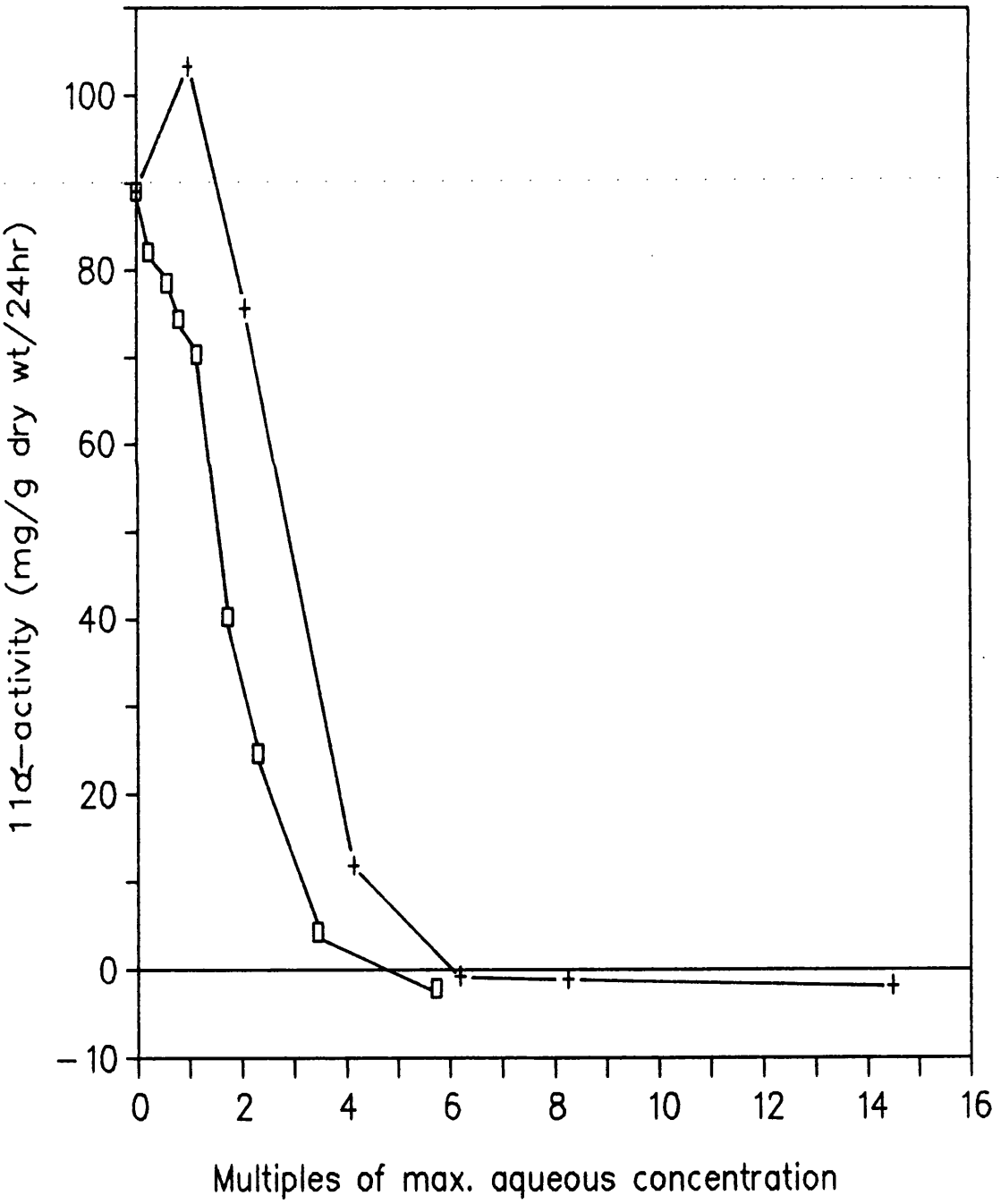


Figure 5.2.3.3c The relationship between loss of 11α-hydroxylase activity and the formation of a second liquid-phase (interface) for the solvents carbon tetrachloride, + , and octanoic acid, □ .



possibly justified by the apparently high solubility in aqueous solution of 2-methyl butan-2-ol as compared to other solvents of similarly calculated hydrophobicity (table 5.2.8). The solvent, therefore, may be far more polar than its $LogP_{octanol}$ value suggests and a reduction in the $LogP_{octanol}$ value of 2-methyl butan-2-ol to 0.64 would make the calculated critical membrane solvent concentration similar to that of other solvents.

Octanoic acid and carbon tetrachloride are two solvents which in theory should be able to achieve the critical membrane solvent concentration of 198mM (table 5.2.8) and therefore cause complete loss of activity at sub-saturating aqueous phase concentrations. However, figure 5.2.3.3c shows that both solvents cause total loss of 11α-hydroxylase activity at solvent volumes of approximately 5 and 6 times that needed to saturate the aqueous phase for octanoic acid and carbon tetrachloride respectively. The results tend to suggest that the solvents are in some way prevented from reaching the necessary concentration in the membranes so that activity loss does not occur until 'phase' effects dominate. Theories that explain why the partitioning behaviour of these solvents might be restricted are not known.

5.2.4 The Results Explained in Terms of the Interaction of Solvents with the Membranes of *R.nigricans*

The use by other workers (section 5.2) of the physico-chemical parameters of solvents to help in the selection of suitable second liquid phases has provided little information as to the site or mode of action by which solvents act to cause loss of biocatalytic activity. However, sections 5.2.2 to 5.2.3.1 show that loss of 11α-hydroxylase activity in R.nigricans is closely correlated with the concentration of organic solvents partitioning into the membranes. The strong implication is that the membranes are the site of action of the organic solvents in causing loss of activity.

At this stage it is worth briefly recalling the nature of the biocatalytic system under study (sections 1.2 to 1.2.3). The progesterone 11α-hydroxylase system of R.nigricans consists of four proteins that are located in the endoplasmic reticular membrane of the

organism. The protein molecules diffuse in the plane of the membrane and interact by collision-coupling to form transient complexes. Theories to account for the action of anaesthetics (section 1.4.5) may also be applicable to the R.nigricans membrane system being studied. These theories include; alterations of membrane fluidity, lipid phase separations, direct solvent-protein interactions and membrane permeabilisation. Each of these factors may contribute to some extent to the overall loss of biocatalytic activity, particularly at high membrane solvent concentrations. However, further interpretation of the experimental data provides some evidence that alterations of membrane fluidity may be the primary cause for loss of activity.

Figure 5.2.3.1a shows that at low membrane solvent concentrations (<75mM) there is an increase in the 11α-hydroxylase activity of R.nigricans for the twelve solvents studied. Sonomoto *et al* 1983 attributed such enhancements of activity to an increased solubility of the substrate in the aqueous phase due to the presence of dissolved organic solvent. However, the aqueous phase concentrations of solvent corresponding to a membrane solvent concentration of 75mM range from 1819mM for methanol to 1.4mM for octanol (calculated using equation [E5.2.3.1]). The greater than 1000 fold range in aqueous phase organic solvent concentrations might be expected to give appreciable differences in dissolved aqueous phase substrate concentrations, but enhancements in the hydroxylase activity are remarkably similar (12-30 percent).

The hydroxylase activity of R.nigricans is known to fall during the 24hrs of the reaction period (figure 3.4.4). Therefore to make interpretations of the data for the partial inhibition and stimulation of activity it is necessary to make the assumption that; both the concentration of different solvents in the membranes and the different types of solvent used have exactly the same effect on the time dependent decay kinetics of the enzyme activity (eg 1st, 2nd or 3rd order).

With the above proviso, a possible explanation for the observed increases in activity comes from the work of Gordon *et al* 1980. This study showed that similar to the solvent effects observed on the 11α-hydroxylase system, benzyl alcohol at membrane concentrations of

20-100mM causes increases in the activity of seven different membrane bound enzyme systems. They then proceeded to correlate the increases in enzyme activity to small increases in the fluidity of the membranes (figure 1.4.5d). In a similar way the short chain primary alcohols ($<C_{10}$, of which C_1 to C_8 alcohols are represented in this study) have also been shown to increase lipid bilayer fluidity (Chapman and Benga 1984).

It is therefore possible that at low membrane solvent concentrations in the endoplasmic reticulum, increased membrane fluidity may allow more rapid diffusion of the 11α-hydroxylase proteins resulting in higher collision frequencies and greater observed activity. Subsequent loss of activity at higher membrane solvent concentrations may be due to too high a membrane fluidity interfering with the maintenance of protein complexes. The latter occur in the interaction of cytochrome P450 with NADPH-cytochrome P450 reductase which is known to require the formation of a comparatively long-lived complex so that orientation of the catalytic sites by rotational diffusion allows electron transfer (section 1.2.3). An alternative explanation for the loss of activity at higher membrane solvent concentrations is that the solvents cause the conversion of cytochrome P450 to an inactive form, P420, so effectively uncoupling the reaction (Mason *et al* 1965). However, such a conversion is thought unlikely to occur at the same concentration irrespective of solvent type and this is supported by experimental results (Ichikawa and Yamano 1967a,b).

Loss of activity in this or different membrane bound enzyme systems may also be the result of other perturbations in the physico-chemical properties of the membrane. The presence of partitioned organic solvent may; cause lipid phase separations to occur disturbing the particular environment in which the enzymes are required to operate, disrupt critical interactions between protein and protein or protein and lipid moieties, cause the release of protein from the membrane by altering the solubility of the protein in the lipid bilayer (see sections 1.4.1 to 1.4.4).

5.2.5 Why some Solvents are Unable to Cause Total Loss of 11α-Hydroxylase Activity

Section 5.2.3.2 describes certain solvents that allow almost full retention of 11α-hydroxylase activity even at saturating aqueous phase concentrations. This is attributed to their inability to reach a particular critical concentration in the membranes. Figure 5.2.5 illustrates why the longer chain alcohols, undecanol and dodecanol, permit the full expression of 11α-hydroxylase activity at saturating aqueous phase concentrations. With increasing carbon chain length beyond butanol, the maximum solubility of the alcohols in the aqueous phase decreases. This is partially counteracted by an increasing membrane/aqueous partition coefficient so that the maximum membrane solvent concentration achievable by successive alcohols decreases only gradually. The alcohols up to and including octanol are able to achieve membrane solvent concentrations greater than that required to cause total loss of activity. However, 'cross-over' occurs at about nonanol and the higher alcohols are no longer able to achieve the critical membrane solvent concentration and so cause no loss of activity until 'phase' effects become dominant.

It is likely that many homologous series will behave in a similar, though not identical manner. This is illustrated by the aliphatic acetates whose maximum membrane solvent concentrations (calculated from equation [E5.2.3.1]) also fall off but more precipitously (figure 5.2.5).

5.2.6 Constructing a Simple Algorithm to Predict which Solvents are likely to Cause Loss of 11α-Hydroxylase Activity. Elucidation of the Sigmoidal $LogP_{octanol}$ - Activity Curves

An algorithm, based on whether a particular solvent is or is not able to achieve a critical concentration in the membrane has been formulated and is shown on the following page (table 5.2.6a). [$solvent_{membmax}$] is the maximum membrane solvent concentration calculated from equation [E5.2.3.1] and [$solvent_{membcrit}$] is the critical membrane solvent concentration at which all 11α-hydroxylase activity is lost.

Figure 5.2.5 The maximum achievable membrane solvent concentration, □ , ■ , for the alcohols and acetates respectively, decreases with increasing $LogP_{octanol}$ of the solvents. The critical membrane solvent concentration (198mM), —— , is the average of 12 values, + . A point is reached where the higher LogP solvents can no longer reach this critical concentration in the membrane. The symbols, Δ , ▲ , denote the expected percent retained activity as predicted from a simple algorithm (section 5.2.6).

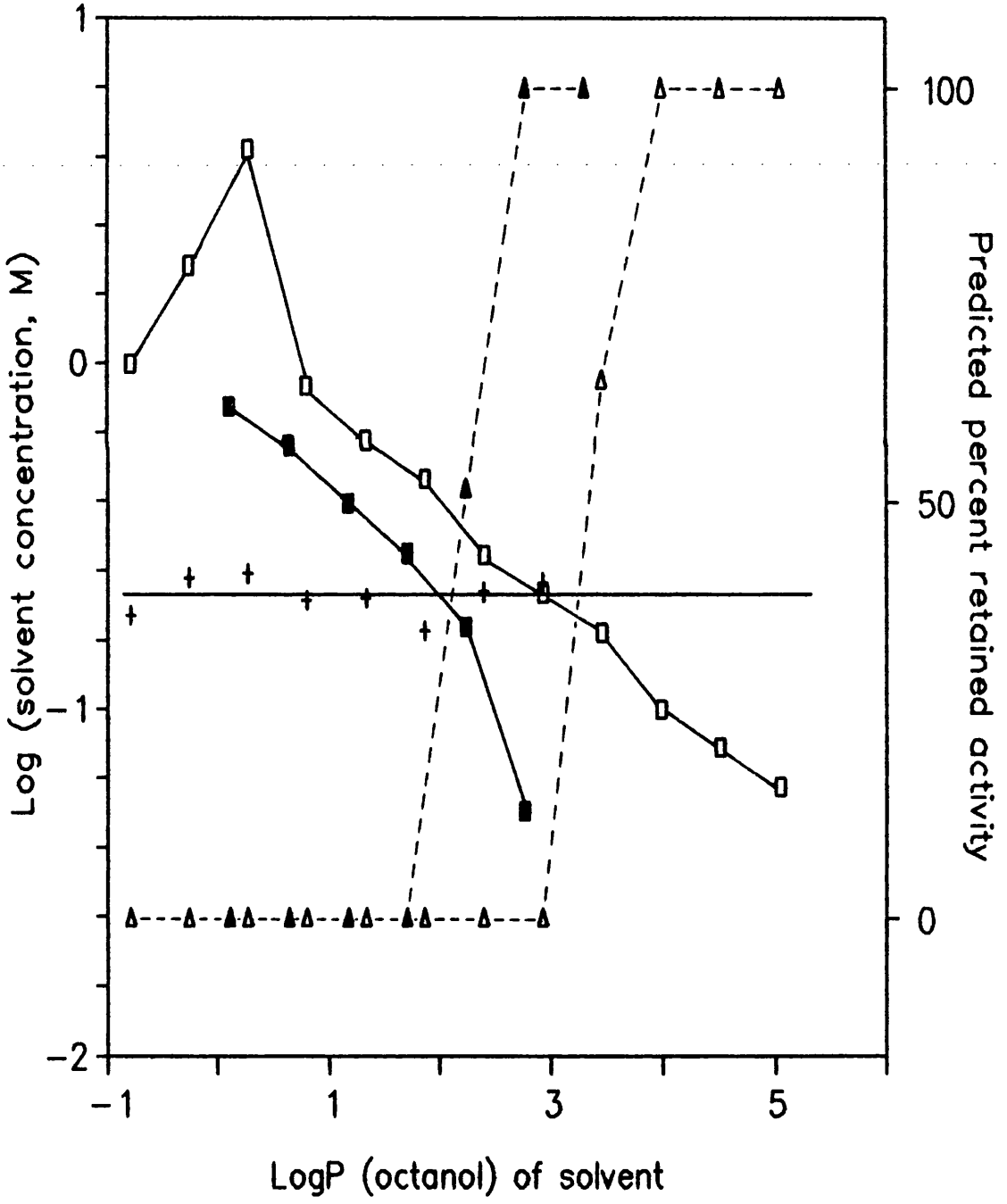


Table 5.2.6a

If		
1.	$[solvent_{membmax}] > [solvent_{membcrit}]$	then Activity = 0%
2.	$[solvent_{membmax}] < [solvent_{membcrit}]$	then Activity = 100%
3.	$[solvent_{membcrit}] > [solvent_{membmax}] > 0.75 \times [solvent_{membcrit}]$	then Activity (0-100%) is proportional to the difference in $[solvent_{membcrit}] - [solvent_{membmax}]$.

The result of applying such an algorithm to the data of the primary alcohols and acetates is shown in figure 5.2.5. Two sigmoidal curves of the type described by Laane et *al* 1985 are produced. If more physical data were available for other homologous solvent groups a whole series of curves would be expected, with the approximate conditions set by Laane et *al* 1985 (table 5.2.6b).

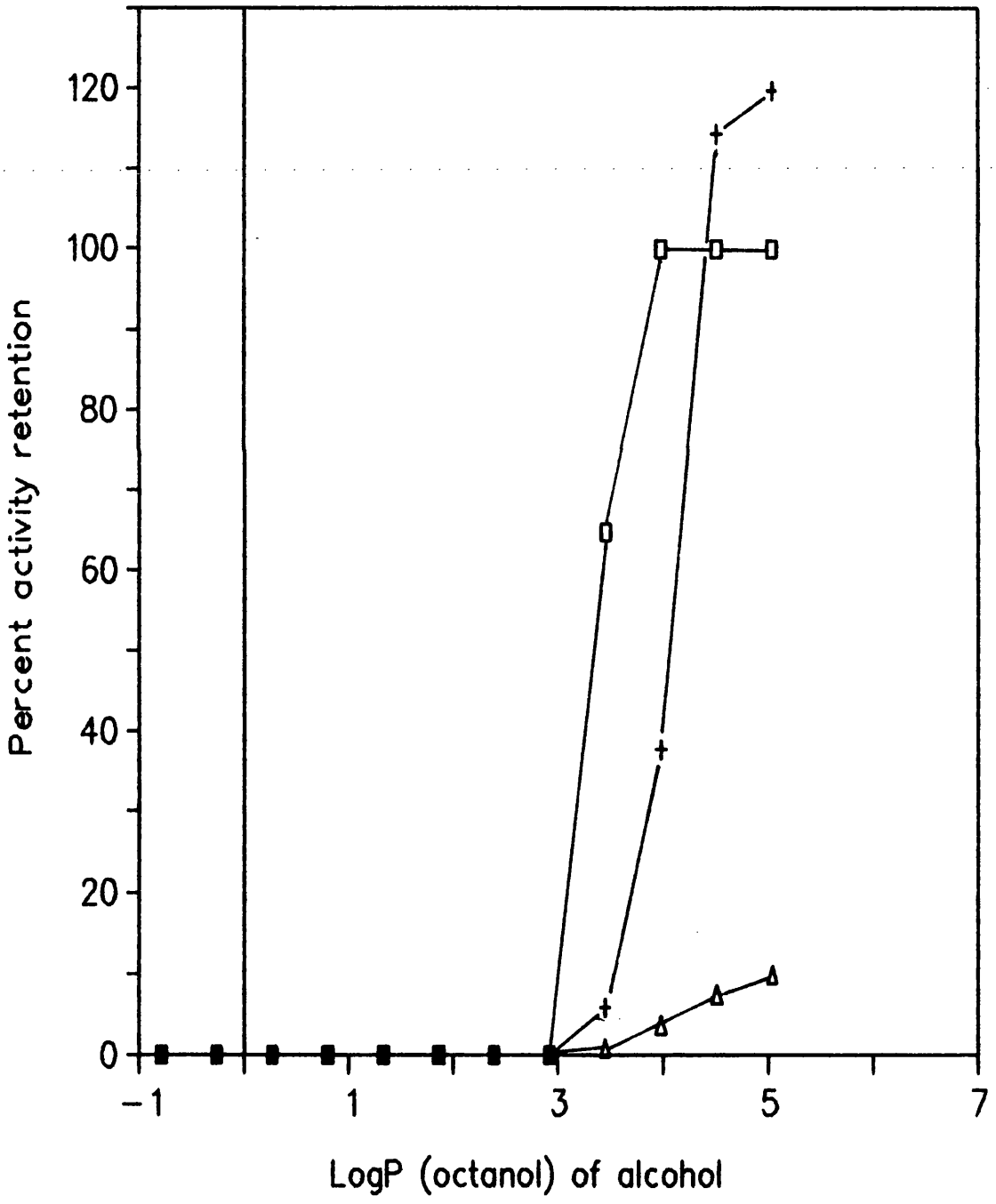
Table 5.2.6b

	$LogP_{octanol} < 2$	No observed activity.
$4 > LogP_{octanol} > 2$		Activity dependent on solvent.
	$LogP_{octanol} > 4$	Full activity observed.

By plotting the experimentally observed activity data for the primary alcohols and the activity retention predicted by the above algorithm, figure 5.2.6, it can be seen that the model fits well under the stated conditions (multiple of maximum aqueous phase solvent conc^n. = 1). If, however, the 'phase' effects are taken into account for the higher alcohols a compressed sigmoidal curve results.

Several of the systems for which Laane et *al* 1985 showed the sigmoidal $LogP_{octanol}$-activity retention correlations resemble those presented in this study; microbial whole cell systems performing a conversion using a membrane associated enzyme. The explanations for these observed

Figure 5.2.6 Relationship between $LogP_{octanol}$ of the primary alcohols and percent activity retention: + observed results, □ predicted results at saturating aqueous phase concentrations of solvent and △, observed results with second liquid-phase present.



results are therefore likely to be similar to those presented for the R.nigricans system. However, it should be realised that the same explanations relating to perturbations of fluidity cannot justifiably be employed for the sigmoidal correlations shown by systems using aqueous soluble or immobilised enzymes.

5.2.7 The Collander Equation of the *R.nigricans* System - Possible Variations

In section 5.2.3.1 the Collander equation derived from the system studied by Seeman 1972 has been adapted to the R.nigricans system of the present study by applying regression fitted results of experimental data to determine the value of a particular term within the equation (namely the exponential value = 0.84). The equation obtained is shown below.

$$[solvent_{memb.}] = 0.19 \times P_{octanol}^{0.84} \times [solvent_{aq.}] \qquad [E5.2.7]$$

So far an assumption has been made that the value of the product term (0.19) is a constant independent of the membrane system studied or the partitioning solvent. Seeman 1972 showed that this was apparently true for three types of mammalian membrane system and several species of partitioning solvents. However, the product term can only be calculated when experimentally measured data for partitioning membrane solvent concentrations are available. To substantiate the value for the R.nigricans system would therefore require direct measurement of the minute solvent concentrations within the membranes of the organism. However, examination of the experimental data already available provides some simple evidence that the product term value of 0.19 is likely to be very similar for the microbial system of R.nigricans. In section 5.2.4 it was shown how the membrane solvent concentrations at which 11α-hydroxylase activity is observed to increase correspond to a similar range of concentrations in the study of Gordon *et al* 1980 on the enhancement of activity of seven other membrane bound enzyme systems. If the product term of 0.19 used to calculate the membrane solvent concentrations in this study were to vary considerably then the range of solvent concentrations at which

11α-hydroxylase activity is seen to increase would fall outside the range (20-100mM) and so not correspond to those experimentally observed by Gordon *et al* 1980.

It is, however, possible that for some solvents the value of 0.19 may be significantly different reflecting some hypo or hyper discriminative power of the membranes towards certain type of molecule. Carbon tetrachloride, a highly symmetrical non-polar molecule, and dimethyl sulfoxide, a highly polar molecule, may well be examples and this could explain the apparently anomalous partitioning behaviour of these solvents (section 5.2.3.3).

5.2.8 Selection of Organic Solvents for Two Liquid-Phase Systems

To aid the rational selection of organic solvents for future two liquid-phase systems it is necessary to formulate criteria based on the experimental results obtained. However, it should be remembered that the application of such criteria is likely to be restricted to those systems in which a biocatalytic conversion involves the use of a membrane associated enzyme. Apart from a few solvents that appear not to obey the general trends, organic solvents may be divided into two groups.

Group 1 Solvents

These solvents are able to attain a critical concentration in the membrane and therefore cause complete loss of activity at sub-saturating aqueous phase concentrations. They will have limited use in two liquid-phase systems. Exceptions may occur when the cells/isolated membranes are immobilised in very hydrophilic matrixes. The solvents may also be useful as cosolvents at low aqueous phase concentrations (equivalent to membrane solvent concentrations <75mM) where stimulatory effects on enzyme activity are observed.

These cause no, or limited, loss in biocatalytic activity at saturating aqueous phase concentrations as they are unable to attain the required critical membrane solvent concentration. Loss of activity is observed when gross 'phase' effects dominate. Immobilisation of the biocatalyst or retention behind some form of membrane would be possible methods of overcoming such effects.

Knowing the maximum aqueous solubility of any organic solvent and its $LogP_{octanol}$ value allows its theoretical maximum solvent membrane concentration to be calculated according to equation [E5.2.7]. If this value is greater than the critical membrane solvent concentration for the system under study then the solvent will be of little use as a two liquid-phase organic solvent. **Figure 5.2.8a** shows a design graph based on the data presented in **table 5.2.8** for the selection of suitable two liquid-phase solvents for the R.nigricans 11α-hydroxylase system. Unfortunately the aqueous phase solubility data of higher $LogP_{octanol}$ solvents are frequently unavailable limiting the number of solvents covered to 81. **Figure 5.2.8b** shows how by applying the algorithm described in section 5.2.6 to the data of **table 5.2.8** a general sigmoidal $LogP_{octanol}$-activity retention curve is obtained that approximates to the conditions of Laane *et al* 1985.

5.3 Experimental Procedure 2 : Solvent Selection, Membrane Partitioning Data - Further Experimental Evidence

The results presented so far (sections 5.2 to 5.2.8) were obtained from an experimental procedure in which all the fungal biomass of Rhizopus nigricans was obtained from a single fermentation and used immediately without storage. This was expected to provide a minimum of variation in the physical and chemical properties of the membranes of the organism exposed to the various two liquid-phase systems. In this way it was hoped that the data from each system could be compared directly to the other systems with the knowledge that the membrane properties were as identical as could be reasonably achieved.

Figure 5.2.8a Relationship between the logarithm of the octanol partition coefficient of a solvent and its maximum achievable membrane concentration (numbers refer to the solvents listed in table 5.2.8). The dashed line shows the critical membrane solvent concentration of 198mM for the R.nigricans system. Solvents that fall on or below the line are those most likely to be suitable for two-liquid phase studies.

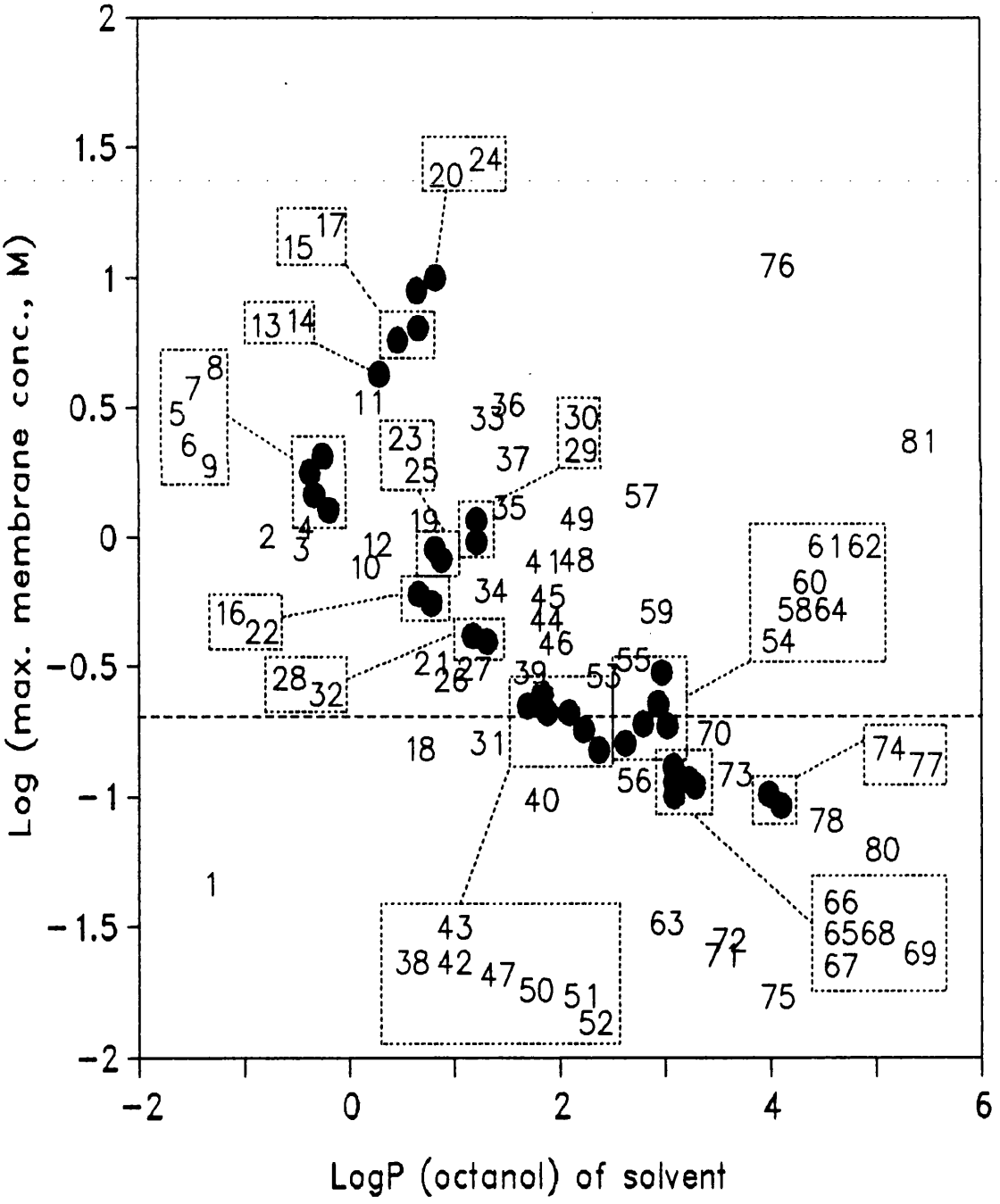


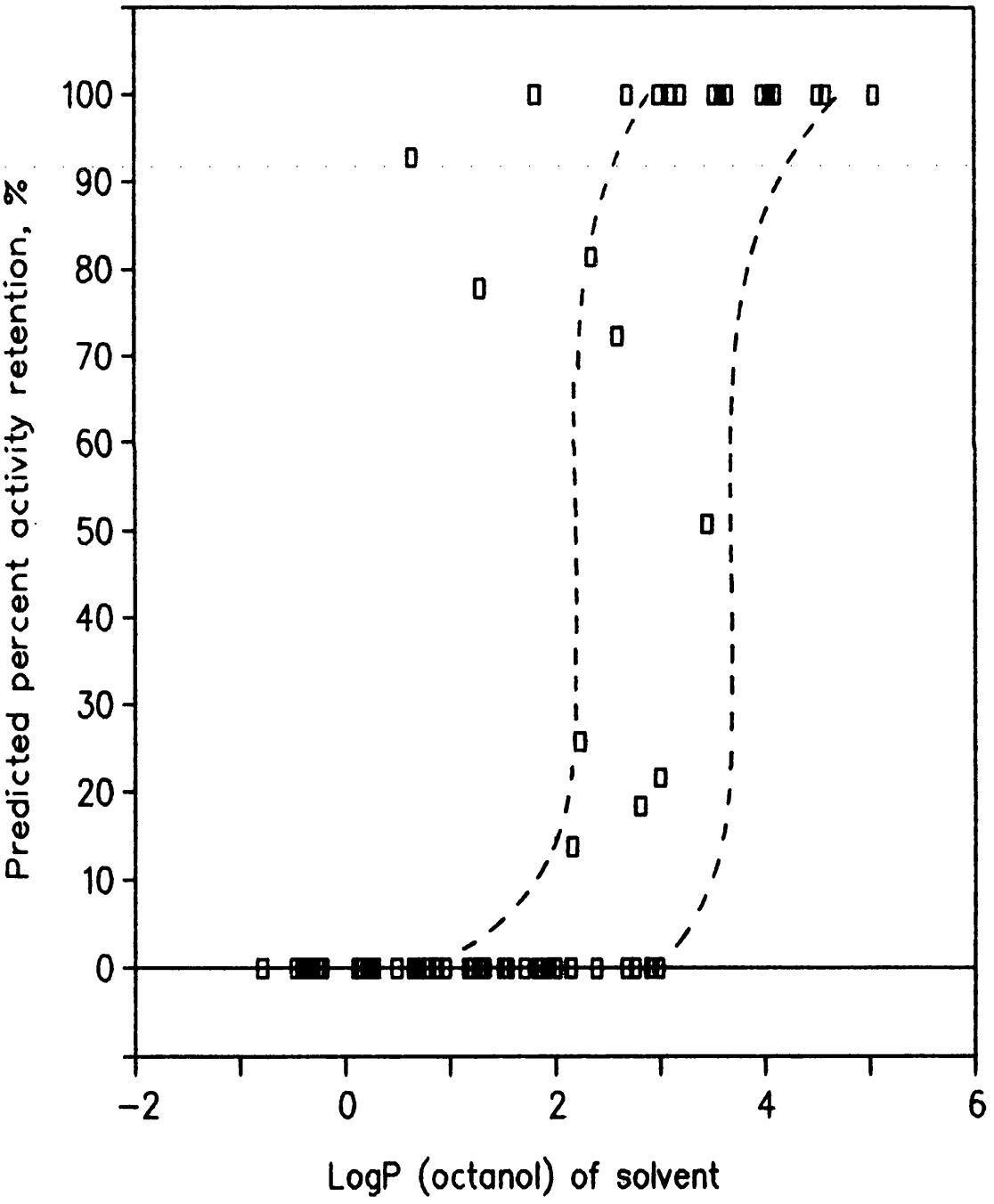
Table 5.2.8 Solvents are shown with their respective $LogP_{octanol}$ values (Rekker 1977), maximum aqueous phase solubilities (Riddick *et al* 1986) and maximum membrane solvent concentrations (calculated according to equation [E5.2.3.1]). Solvent numbers refer to the design graph, figure 5.2.8a. Solvents marked with an asterisk are those predicted to be most suitable for use in two-liquid phase systems. The critical membrane solvent concentration is taken to be 198mM but solvents within ten percent of this value are also marked with an asterisk. A few solvents are shown experimentally not to obey the general predictions (section 5.2.3.3).

Solvent	$LogP_{oct.}$	Maximum Aq. Phase Conc. (mM)	Maximum Membrane Conc.(mM)
1 **Dimethyl-sulfoxide ***	-1.35	3311	46.2
2 Methanol	-0.79	24540	1010
3 Dioxane	-0.47	11670	893
4 N,N-dimethyl-formamide	-0.42	12910	1090
5 Acetonitrile	-0.36	18910	1790
6 Acetone	-0.30	13510	1440
7 Ethanol	-0.26	17040	1960
8 Acetic acid	-0.25	17380	2040
9 Ethoxyethanol	-0.22	10270	1280
10 Methyl acetate	0.11	3245	763
11 2-Propanol	0.15	13000	3300
12 Butanone	0.23	3140	931
13 1-Propanol	0.27	13310	4260
14 Propionic acid	0.28	13340	4360
15 Tetrahydrofuran	0.49	12110	5940
16 Ethyl acetate	0.64	908.4	595
17 Diethylamine	0.64	9593	6290
18 **Methyl propionate ***	0.64	232.1	152
19 2-Butanol	0.68	1636	1160
20 Pyridine	0.71	12370	9280
21 Pentan-3-one	0.76	391.6	324
22 Pentan-2-one	0.76	680.8	563
23 1-Butanol	0.80	987.4	882
24 Butyric acid	0.81	10820	9850
25 Diethyl ether	0.88	795.1	829
26 Cyclohexanone	0.95	234.0	279
27 4-Methyl pentan-2-one	1.17	169.0	309
28 Propyl acetate	1.17	224.5	410
29 2-Pentanol	1.21	500.6	988
30 3-Pentanol	1.21	577.5	1140
31 **Ethyl chloride ***	1.29	69.25	160

Table 5.2.8 continued.

Solvent	$LogP_{oct.}$	Maximum Aq. Phase Conc. (mM)	Maximum Memb. Conc.(mM)
32 Hexanone	1.29	174.0	401
33 2-Methyl butan-2-ol	1.3	1215	2850
34 1-Pentanol	1.33	247.2	615
35 Cyclohexanol	1.50	373.9	1290
36 Phenol	1.50	929.6	3210
37 Triethyl amine	1.54	532.3	1990
38 2-Chloropropane	1.70	43.52	222
39 Butyl acetate	1.70	58.48	298
40 Nitrobenzene *	1.81	15.44	97.2
41 3-Heptanone	1.82	124.8	801
42 1-Chloropropane	1.82	34.49	221
43 2-Heptanone	1.82	37.62	242
44 Hexanol	1.86	68.99	479
45 Hexanoic acid	1.87	82.41	583
46 Dipropyl ether	1.94	47.87	388
47 Benzene *	2.00	22.92	209
48 Chloroform	2.14	68.45	816
49 Methoxybenzene	2.15	96.16	1170
50 Methyl benzoate *	2.16	15.43	191
51 Pentyl acetate *	2.23	13.05	185
52 2-Octanone *	2.35	8.811	158
53 Heptanol	2.39	14.85	287
54 Toluene *	2.60	5.589	162
55 Ethoxybenzene	2.68	9.822	333
56 Ethyl benzoate *	2.69	3.329	115
57 Dibutyl amine	2.76	36.31	1440
58 Chloro benzene *	2.81	4.336	189
59 2-Octanol	2.90	9.834	510
60 1-Octanol	2.92	4.131	223
61 Octanoic acid	2.93	5.470	301
62 Carbon Tetrachloride	2.98	5.007	303
63 Pentane *	2.99	0.5267	32.5
64 Styrene *	3.00	2.976	187
65 p-Xylene *	3.10	1.469	112
66 o-Xylene *	3.10	1.648	126
67 m-Xylene *	3.10	1.375	105
68 Ethyl benzene *	3.12	1.432	114
69 Cyclohexane *	3.20	1.188	110
70 Nonanol *	3.45	1.151	173
71 Hexane *	3.52	0.1427	24.6
72 Methyl cyclohexane *	3.59	0.1426	28.1
73 Propyl benzene *	3.65	0.5433	120
74 Decanol *	3.98	0.2480	104
75 Heptane *	4.05	0.03563	17.1
76 Diphenyl ether	4.06	22.92	1120
77 p-Cymene *	4.10	0.1743	92.1
78 Undecanol *	4.51	0.06905	80.6
79 Octane *	4.58	57.78×10^{-6}	0.0722
80 Dodecanol *	5.04	0.01922	62.5
81 Dibutyl phthalate	5.40	0.3593	2350

Figure 5.2.8b Predicted activity retention for the solvents presented in figure 5.2.8a and table 5.2.8 according to the algorithm described in section 5.2.6. Three solvents that do not fit the sigmoidal pattern have been omitted; dimethyl sulfoxide, diphenyl ether and dibutyl phthalate.



A second experimental procedure was performed, using a range of solvents selected from figure 5.2.8a, to further test the prediction that organic solvents which are unable to achieve a critical concentration in the membranes permit the retention of biocatalytic activity (section 5.2.8). Although an attempt was made to keep the fermentation conditions identical to those in the first experimental procedure it was realised that slight differences might occur. In fact the growth temperature dropped by 2^0C to 26^0C for about 14hr during the night. It was known that slight changes in the growth conditions of microbial organisms could alter the physiology of the cell membranes (Wieslander and Rilfors 1977, Wieslander *et al* 1980). Supplementary experiments were therefore performed to see if the membranes of R.nigricans resulting from this second fermentation showed similar properties to those of R.nigricans derived from the first experimental procedure.

5.3.1 Reproducibility of Membrane Partitioning Data Between Cells of *R.nigricans* Obtained from Different Fermentations

By using the methods presented in sections 5.2.3 and 5.2.3.1 the critical aqueous phase concentrations at which no 11α-hydroxylase activity is observed, $[solvent_{aq.C}]$, were calculated for the primary alcohols, methanol to octanol, and the acetates, methyl acetate to butyl acetate. The logarithm of these values were then plotted against the logarithm of the reciprocal of $P_{octanol}$ of the solvent, figure 5.3.1. Again the significance of the plot derives from its linearity and the single Y-intercept, implying that a critical solvent concentration in the membranes causes total loss of 11α-hydroxylase activity. Regression fits, table 5.3.1, were obtained for the data of; all the solvents, only the primary alcohols and only the acetates. The regression fits of all the solvent data and the primary alcohols were then compared statistically (appendix 3) with the regression fits obtained from the first experimental procedure (table 5.2.3b).

Figure 5.3.1 Relationship between the aqueous solvent concentration at which all 11α-hydroxylase activity is lost and the octanol partition coefficient of the solvent (alcohols, □ , and acetates, △). Numbers refer to the solvents listed in table 5.3.3a.

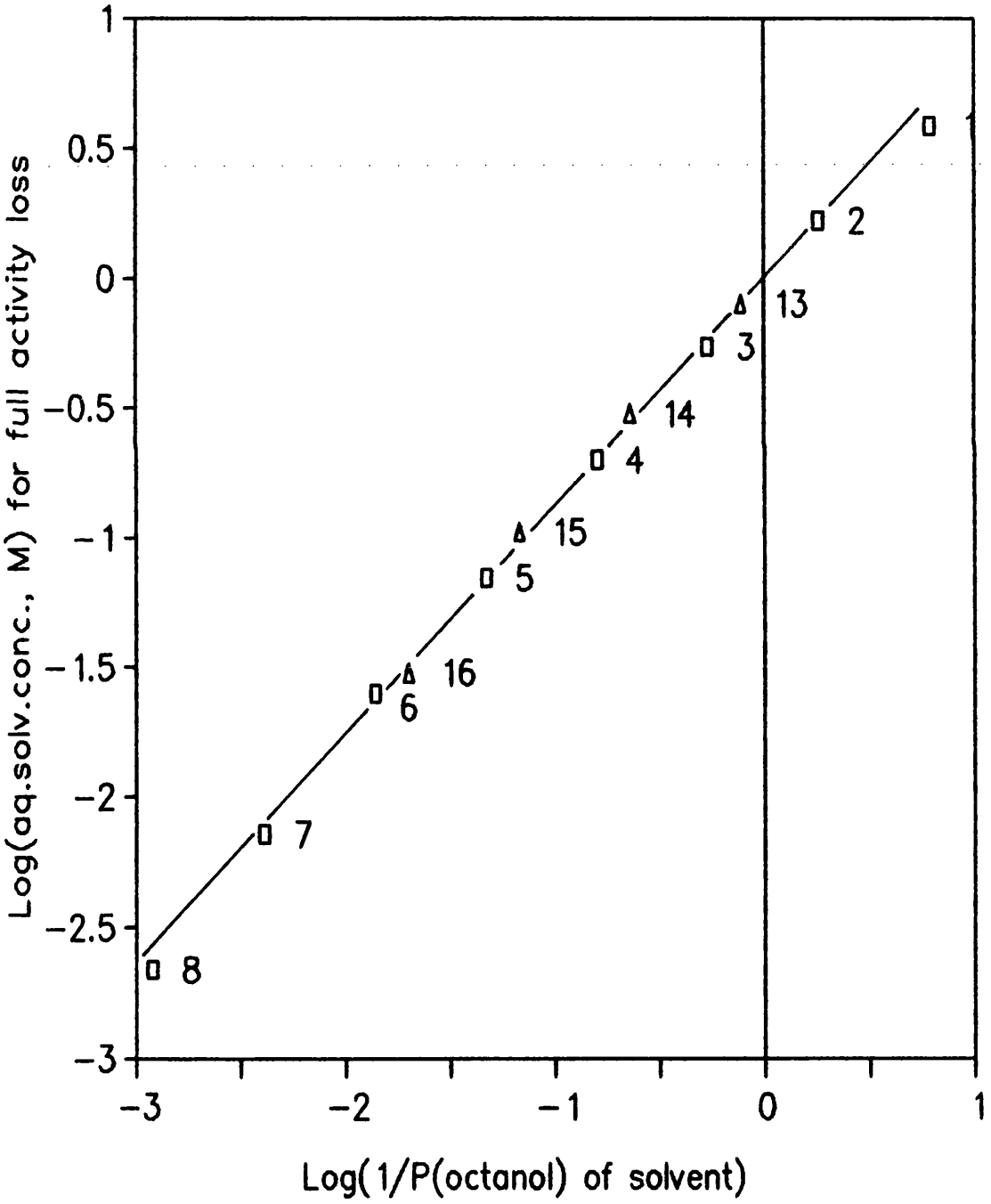


Table 5.3.1 Regression fits to the data presented in figure 5.3.1.

	All solvent data	Alcohol data only	Acetate data only
Gradient	0.881	0.877	0.894
Y-intercept	-0.0105	-0.0278	0.0298
Corr. Coeff.	0.999	0.999	0.998
$[solvent_{memb.C}]$, M	0.185	0.178	0.203

The statistical tests showed that comparing the experimental results derived from the two R.nigricans fermentations;

1. The solvents display a significantly similar dependence on partitioning into the cell membranes with changing $LogP_{octanol}$ of the solvent (ie. the gradients of the correlations are not significantly different).

2. There is a significant similarity in the critical concentration of solvent required in the membranes to cause total loss of 11α-hydroxylase activity (ie. the Y-intercept values are not significantly different).

It may therefore be concluded that the differences in fermentation conditions produced no statistically significant difference in the physico-chemical properties of the membranes of R.nigricans which are associated with the partitioning behaviour of organic solvents. This means that, providing the fungal biomass of R.nigricans is grown in a similar manner each time; the critical membrane solvent concentration need not be recalculated and that a solvent selection graph of the type given in figure 5.2.8a should be applicable to any similar system.

5.3.2 The Collander Equation for H-Donor and H-Acceptor Solvents

In section 5.2.2 the partitioning of any molecular species between two immiscible solvents was described by a single partition coefficient, the octanol partition coefficient being given as an example

$$P_{octanol} = \frac{[solvent_{oct.}]}{[solvent_{aq.}]}$$

However, Leo and Hansch 1971, Leo et *al* 1971 describe two groups of molecular species, H-donors and H-acceptors (table 5.3.2) which have different Collander equations suggesting that their partitioning behaviour may also be different. The naming of the groups relates to the ability of the solutes to donate or accept hydrogen bonds when in a partitioning system. To determine whether such a difference in the partitioning behaviour of solvents might exist for the membrane/aqueous system of R.nigricans the experimental data for the primary alcohols (H-donors) was compared to that of the acetates (H-acceptors). The regression fits (table 5.3.1) were compared statistically (appendix 3) and the results showed that;

1. Both the alcohols and acetates show the same dependence on partitioning into the membranes of R.nigricans with changing $LogP_{octanol}$ of the solvent (ie. the gradients of the correlations are not significantly different).

2. There is a significant similarity in the critical concentration of solvent (alcohol or acetate) required in the membranes to cause total loss of 11α-hydroxylase activity (ie. the Y-intercept values are not significantly different).

The statistical tests suggest that the partitioning behaviour of the two solvent types towards the membranes of R.nigricans is not significantly different and justifies the pooling of the data to obtain a general regression fit of all the data (table 5.3.1). The Collander equation that describes the partitioning of the solvents in the membrane/aqueous system of R.nigricans of this experiment is therefore

given by

$$[solvent_{memb.}] = 0.19 \times P_{octanol}^{0.88} \times [solvent_{aq.}] \qquad [E5.3.2]$$

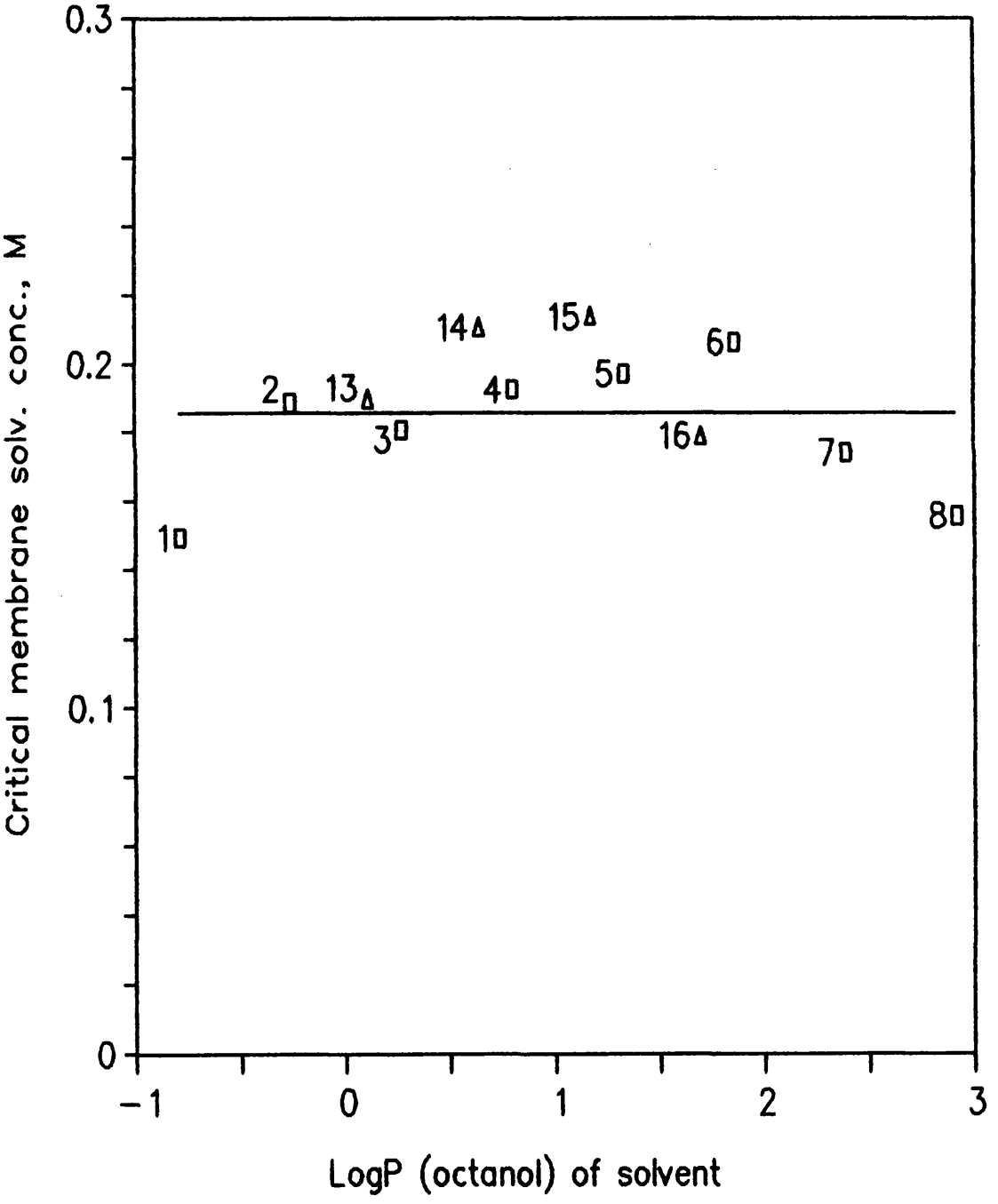
A single critical membrane solvent concentration is predicted (185mM) above which a total loss of 11α-hydroxylase activity is expected to occur. However, when the critical membrane solvent concentration is calculated independently for each solvent using equation [E5.3.2] slight differences in value are observed, figure 5.3.2. Possible causes for these differences have already been examined in section 5.2.3.1.

Table 5.3.2 H-donor and acceptor solutes. See Leo and Hansch 1971 for two liquid-phase systems that may reverse the donor, acceptor rule.

H-donors	1.	Acids
	2.	Phenols
	3.	Barbiturates
	4.	Alcohols
	5.	Amides (neg. substituted, but not di-N-substituted)
	6.	Sulfonamides
	7.	Nitriles
	8.	Imides
	9.	Amides
H-acceptors	10.	Aromatic amines (not di-N-sub.)
	11.	Aromatic hydrocarbons
	12.	Intramolecular H bonds
	13.	Ethers
	15.	Esters
	16.	Ketones
	17.	Aliphatic amines and imines
	18.	Tertiary amine (including ring compounds)

In section 5.2.7 the possibility that the product term (0.19), in equation [5.3.2], might take other values was discussed. Evidence was also presented that suggested that this term should be, if not a constant, very similar in value for all solvents. However, it is worth noting that if the product term were to vary between solvent classes, then there would still be no statistical difference in the partitioning

Figure 5.3.2 The critical membrane solvent concentration at which all 11α-hydroxylase activity is lost for the alcohols, □ , and acetates, △ , as a function of the logarithm of the octanol partition coefficient of the solvent. The solid line denotes the critical membrane solvent concentration, 185mM, taken from the regression fit (table 5.3.1) of all the data presented in figure 5.3.1. Numbers refer to the solvents listed in table 5.3.3a.



behaviour of the alcohols and acetates with respect to changes in $Log P_{octanol}$ values, but the calculated critical membrane solvent concentrations would be altered and might prove to be not statistically similar.

5.3.3 Solvents Predicted to be Suitable for Use in Two Liquid-Phase Systems

Organic solvents which are unable to achieve the critical membrane solvent concentration of 185mM are expected to allow the retention of 11α-hydroxylase activity at sub-saturating aqueous phase concentrations of the solvent. From the design graph, figure 5.2.8a, 18 solvents were 'selected' on the basis that their predicted maximum membrane solvent concentrations fell below or close to the critical membrane solvent concentration. These solvents are given in table 5.3.3a with their maximum membrane solvent concentrations recalculated according to equation [E5.3.2].

All of the selected solvents show some retention of 11α-hydroxylase activity at sub-saturating aqueous phase solvent concentrations. This is surprising since solvents such as benzene, chlorobenzene and toluene have predicted maximum membrane solvent concentrations slightly in excess of the critical membrane solvent concentration (185mM) and therefore should in theory cause total loss of activity.

The 11α-hydroxylase activity profiles of R.nigricans in two liquid-phase systems with 'selected' solvents as the second phase fall into three categories (table 5.3.3b)

Category 1 solvents These systems show an increase in activity at sub-saturating aqueous phase concentrations of solvent followed by a loss of activity at higher solvent volumes in the system. The activity then appears to level off to a constant residual value. Activity profiles for the higher alcohols are presented in figure 5.3.3a.

Figure 5.3.3a 11α-hydroxylase activity profile with increasing volume of solvent in the reactor for 'category 1' solvents. Increased activity is observed at sub-saturating aqueous phase solvent concentrations. Loss of activity then occurs at higher volumes of solvent in the reactor and levels out to a constant value.

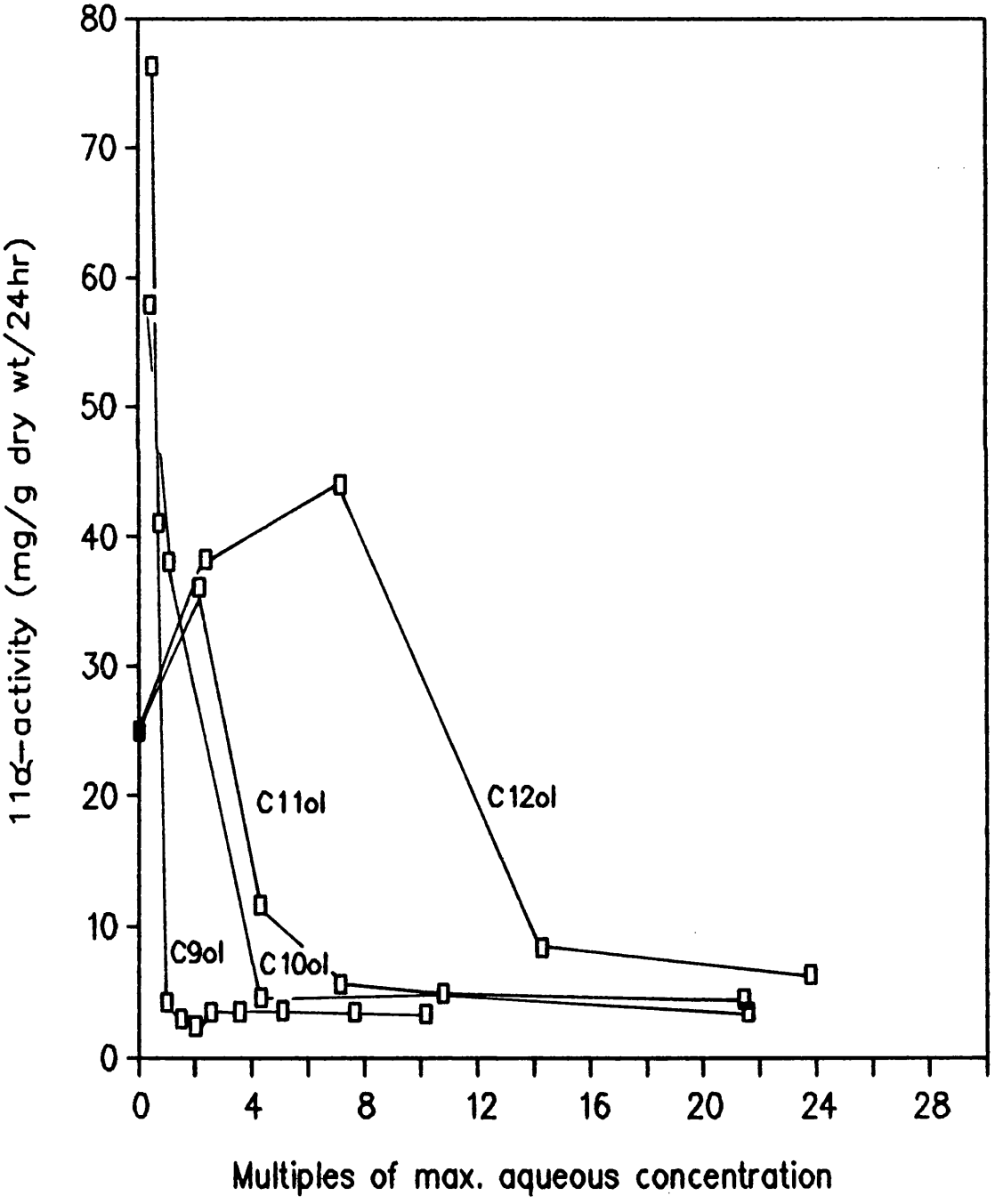
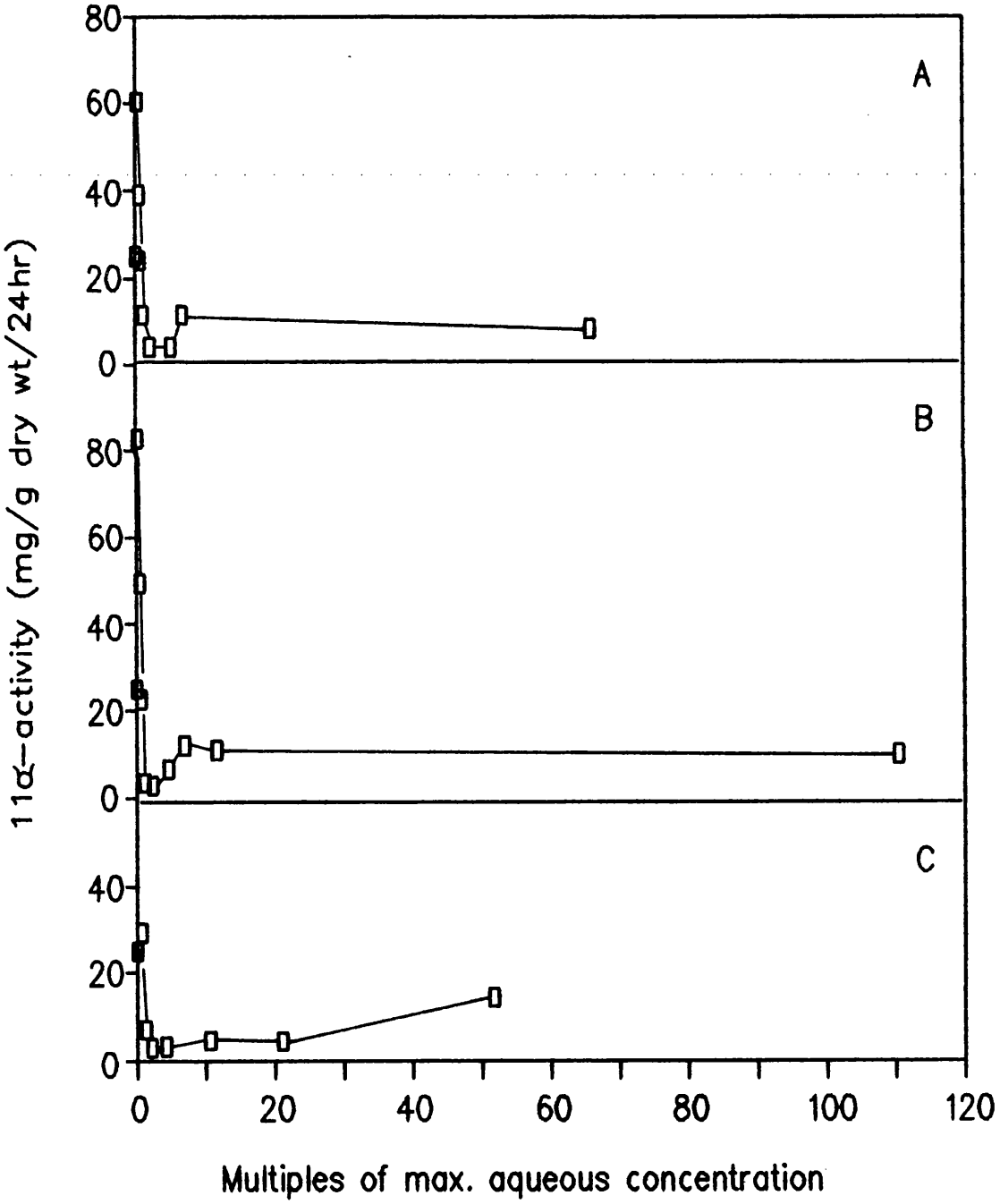


Figure 5.3.3b 11α-hydroxylase activity profiles with increasing volume of solvent in the reactor for 'category 2' solvents. (A) toluene, (B) chlorobenzene, (C) ethyl benzoate. The profiles are similar to 'category 1' profiles (figure 5.3.3a) except that activity is seen to increase with higher volumes of solvent present in the reactor.



Category 2 solvents The profiles are similar to those of category 1 solvents except that loss of activity is followed by an observed increase in activity with even greater volumes of solvent in the system, figure 5.3.3b.

Category 3 solvents These systems show an increased activity at sub-saturating aqueous phase solvent concentrations. This is followed by a region of consistently high activity where the solvent volume in the reactor is up to 10 times that required to saturate the aqueous phase. Loss of activity then only occurs when much higher volumes of solvent are present in the system. Figures 5.3.3c to 5.3.3e.

Table 5.3.3b Categories of solvents based on the activity profiles of the *R.nigricans* 11α-hydroxylase systems in which they are employed as a second liquid phase.

Category 1	Category 2	Category 3
Nonanol	Ethyl benzoate	Hexane
Decanol	Chlorobenzene	Cyclohexane
Undecanol	Toluene	Methyl cyclohexane
Dodecanol		
Pentyl acetate		
Octyl acetate		
Nitrobenzene		
Benzene		
m, o, p-Xylenes		
Cymene		

Figure 5.3.3c The 11α-hydroxylase activity profile with increasing volume of solvent in the reactor for 'category 3' solvent, cyclohexane. Inset is an enlarged section of the activity profile at low volumes of solvent in the reactor.

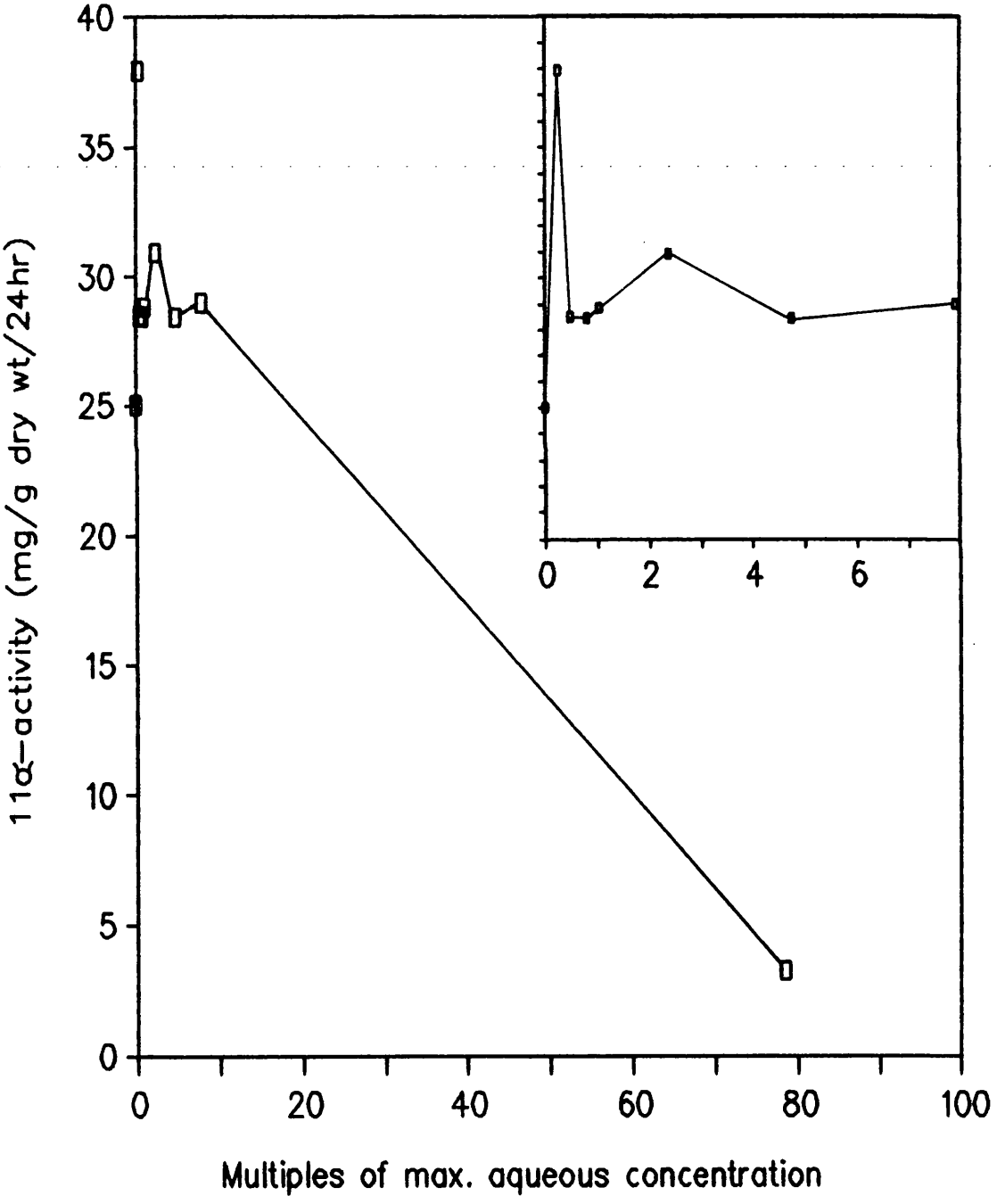


Figure 5.3.3d The 11α-hydroxylase activity profile with increasing volume of solvent in the reactor for 'category 3' solvent, hexane. Inset is an enlarged section of the activity profile at low volumes of solvent in the reactor.

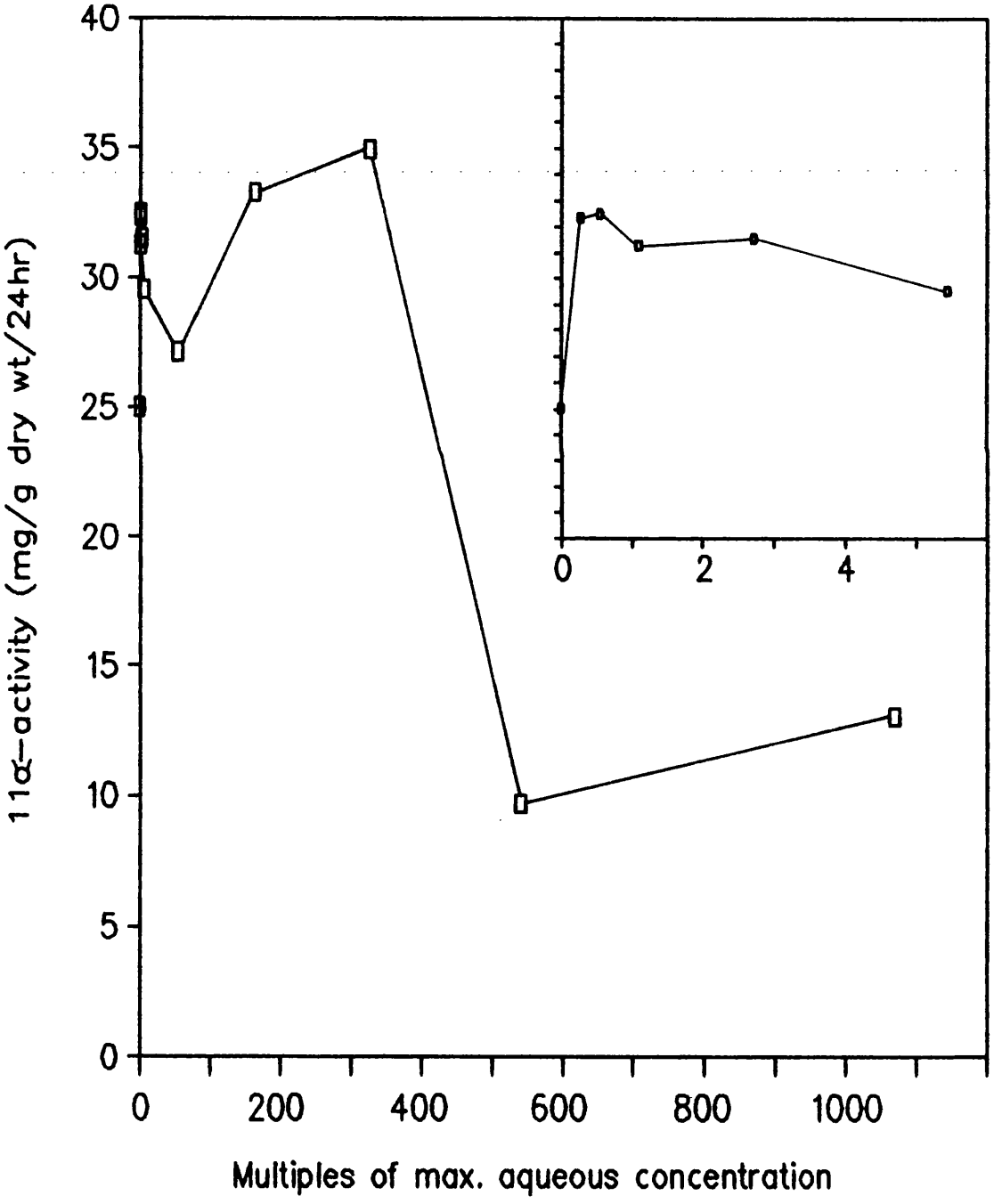


Figure 5.3.3e The 11α-hydroxylase activity profile with increasing volume of solvent in the reactor for 'category 3' solvent, methyl cyclohexane. Inset is an enlarged section of the activity profile at low volumes of solvent in the reactor.

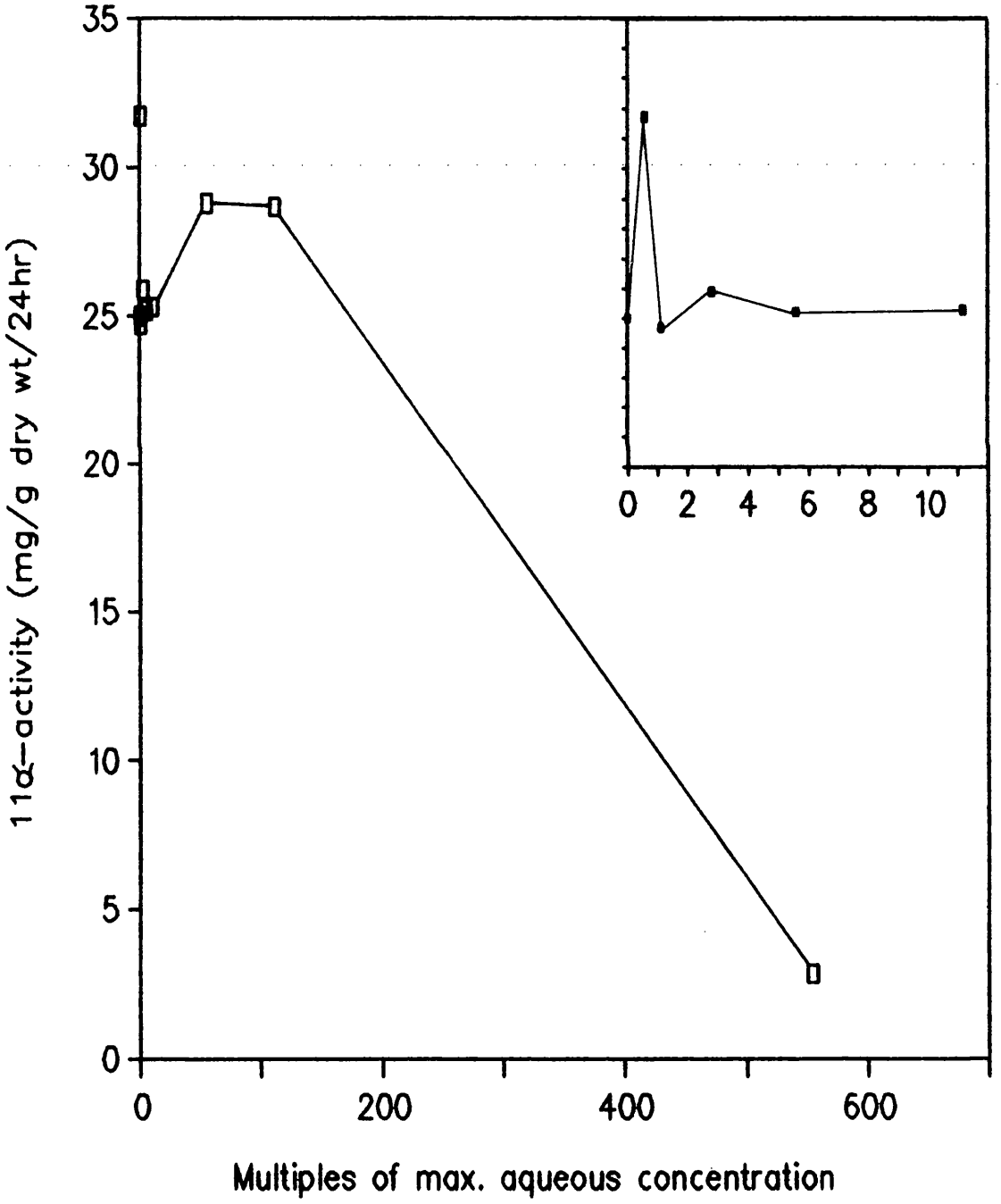


Table 5.3.3a Solvents with their calculated $LogP_{octanol}$ values (Rekker 1977), maximum membrane solvent concentrations (calculated according to equation [E5.3.2]) and the volume (-1-) of organic solvent required to saturate one litre of water (Riddick *et al* 1986). Solvents marked with an asterisk are those 'selected' from figure 5.2.8a.

	Solvent	$LogP_{oct.}$	Maximum Membrane Conc. (mM)	Volume -1- of Organic Solvent, ml
1	Methanol	-0.79	940	100% miscible
2	Ethanol	-0.26	1911	100% miscible
3	Propanol	0.27	4371	100% miscible
4	Butanol	0.80	950	90.8
5	Pentanol	1.33	697	26.9
6	Hexanol	1.86	569	8.65
7	Heptanol	2.39	359	2.11
8	Octanol	2.92	293	0.655
9	Nonanol *	3.45	239	0.201
10	Decanol *	3.98	151	0.0474
11	Undecanol *	4.51	123	0.0143
12	Dodecanol *	5.04	100	0.00430
13	Methyl acetate	0.11	771	259
14	Ethyl acetate	0.64	632	89.5
15	Propyl acetate	1.17	457	26.0
16	Butyl acetate	1.70	349	7.75
17	Pentyl acetate *	2.23	228	1.95
18	Octyl acetate *	2.82	16.3	0.00744
19	Nitrobenzene *	1.81	115	1.59
20	Benzene *	2.00	251	2.05
21	Toluene *	2.60	207	0.597
22	Ethyl benzoate *	2.69	148	0.482
23	Chlorobenzene *	2.81	246	0.441
24	p-Xylene *	3.10	150	0.182
25	o-Xylene *	3.10	168	0.200
26	m-Xylene *	3.10	140	0.170
27	Cyclohexane *	3.20	148	0.129
28	Hexane *	3.52	34.1	0.0188
29	Methyl cyclohexane *	3.59	39.3	0.0183
30	p-Cymene *	4.10	135	0.0274

5.3.4 The Partitioning Behaviour of 'Selected' Solvents at Sub-Saturating Aqueous Phase Concentrations of Solvent

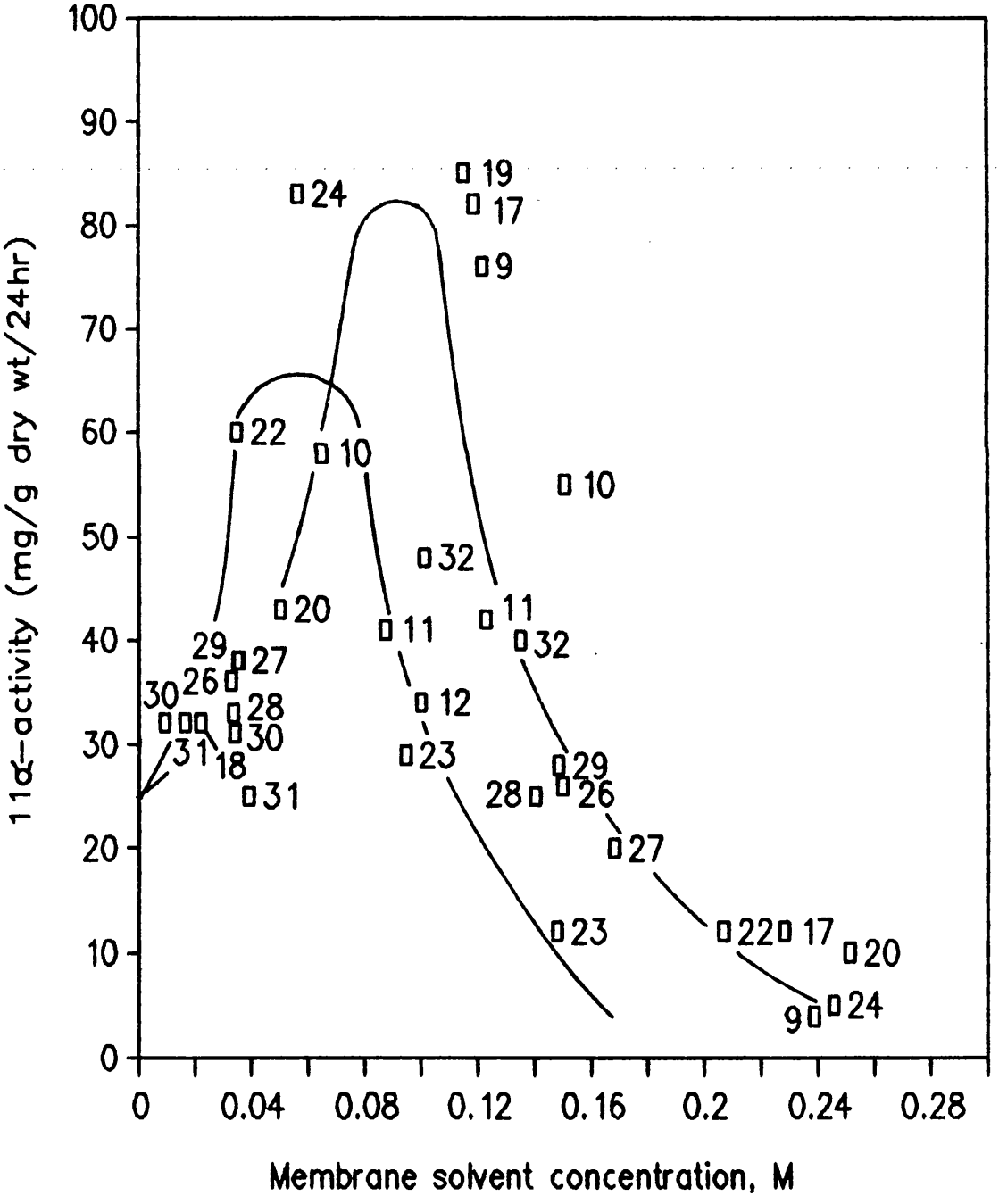
In section 5.2.4 various methods are discussed by which solvents might cause increased biocatalytic activity at low membrane solvent concentrations and loss of activity at higher membrane solvent concentrations. The results presented so far strongly suggest that interaction of the solvents with the membranes of the organism is not related to their physico-chemical functionality but is a non-specific interaction relying solely on the concentration of solvent present in the membranes. Other evidence indicates that this interaction causes changes in the fluidity of the membranes.

It might be expected that the 'selected' solvents would have similar non-specific membrane interactions at sub-saturating aqueous phase concentrations. These would then produce 11α-hydroxylase activities similar to those of the alcohols and acetates (left hand line of figure 5.3.4) at equivalent membrane solvent concentrations. By combining all the activity data for the 'selected' solvents, obtained at sub-saturating aqueous phase solvent concentrations, and calculating the corresponding membrane concentrations (equation [E5.3.2]) a combined 11α-hydroxylase activity profile is obtained (right hand line of figure 5.3.4). Although there is a considerable degree of scatter of the points around this line which also is displaced towards higher membrane concentrations, some similarity to the expected profile appears to exist.

5.3.5 Explanations for the Activity Profiles of the 'Selected' Solvents

Several of the 'selected' solvents should in theory cause total loss of 11α-hydroxylase activity since they are predicted to be able to attain the critical membrane solvent concentration. The fact that they do not cause total loss of activity suggests that they have not managed to partition sufficiently into the membranes. A possible explanation is that the calculated $Log P_{octanol}$ values of these solvents do not properly reflect their true hydrophobicity and lower values might be required. Alternatively the maximum aqueous solubilities of the solvents in the

Figure 5.3.4 The general form of the 11α-hydroxylase activity profile (left hand curve, ——) is shown for the lower alcohols and acetates with increasing concentration of solvent in the membranes. This compares with the combined activity profile (right hand curve) of other 'selected' solvents when their concentration in the aqueous phase is sub-saturating. Numbers refer to the solvents listed in table 5.3.3a).



experimental system may be lower than those given in the literature (Riddick *et al* 1986). This could be explained by the more polar nature of the experimental aqueous system, with its dissolved salts, as compared to the ideal pure water systems used for solubility measurements.

To explain the different types of activity profile obtained undoubtedly requires a greater understanding of how the 'selected' solvents act at greater than saturating aqueous phase concentrations. Factors that need to be studied are droplet size distribution (affected by surface tension and viscosity), ease of coating of the organism by the solvent and solubility properties of the solvent for both the substrate and product of the reaction. However, several points do arise from the profiles. Loss of activity in all cases is likely to be the result of gross 'phase' effects since it occurs when the volume of solvent present in the systems is several multiples of that required to saturate the aqueous phase. 'Category 2' solvent two liquid-phase systems show an increase in activity at higher solvent volumes in the reactor. This might be explained by an increased mass transfer of substrate to the partially active 11α-hydroxylase complex because of the greater interfacial surface area provided by the larger solvent volumes. It is also possible that 'category 1' solvent systems would show similar increases in activity at very high solvent volumes but that the solubility of the substrate in these solvents is too low for the mass transfer effects to be seen at the solvent volumes employed.

CHAPTER 6

6 DISCUSSION

6.1 Preliminary Studies Using the Organism *Rhizopus nigricans* in Two Liquid-Phase Systems

Several conventional organic solvents were tested for their suitability for use as a second liquid-phase with Rhizopus nigricans as the biocatalyst. The results obtained are similar to those of Ceen 1986, who employed Aspergillus ochraceus as the biocatalyst for the same reaction, the conversion of progesterone to 11α-hydroxyprogesterone. Loss of 11α-hydroxylase activity is very rapid and almost complete within 10 minutes of contacting with the conventional organic solvents (figure 3.4.1a). Hexane is a notable exception, R.nigricans retaining about 50 percent of its 11α-hydroxylase activity after 10 minutes as compared to the control.

Oleic acid was similarly tested for its compatibility as a second liquid-phase with the organism R.nigricans following the success of Ceen (1986) in using this solvent with A.ochraceus. The results indicate that although loss of biocatalytic activity is rapid during the first hour of contacting, the activity remains comparatively stable at about 77 percent of the control over the following four hours (figure 3.4.1b). The use of oleic acid as the second liquid-phase is also shown to allow the growth of R.nigricans and induction of the 11α-hydroxylase enzyme in the presence of this solvent (table 3.4.2). This is considered a distinct advantage if there is a need to generate new catalytic biomass during some form of long-term continuous reactor process.

In batch stirred tank reactor studies using oleic acid as the second liquid-phase, R.nigricans proved to be a less suitable biocatalyst as compared to A.ochraceus (figure 3.4.3). The latter organism is able to take advantage of the higher interfacial surface areas for mass transfer of substrate that occur with the increasing agitation rates in the reactor. The production of 11α-hydroxyprogesterone by R.nigricans, however, remains fairly constant. A possible explanation is that the

11α-hydroxylase enzyme is already working at its maximum rate. However, the lack of increase in the production of 11α-hydroxyprogesterone is more likely to be governed by two other opposing factors. First, the 11α-hydroxylase activity of active biomass is increasing with higher agitation rates in the reactor, as occurs with A.ochraceus, but that this is counteracted by a decrease in the amount of active biomass present due to shear inactivation of the organism. R.nigricans unlike A.ochraceus is a non-septate organism and is far more sensitive to shear forces; cell damage causing the release of cytosolic components from substantial portions of the biomass as opposed to single cells. The application of reactor systems in which there are reduced shear forces (eg. packed-bed, airlift reactors) may well prove beneficial when using such shear sensitive organisms.

6.2 Packed-Bed Reactor Studies

The ease with which cells of R.nigricans can be packed into a column reactor is dependent to a large extent on the morphology of the organism. Filamentous cells are more difficult to handle and give rise to a visible non-uniformity of packing along the axis of the bed. With the exception of having to remove small entrapped air bubbles from the mycelial biomass in the column, pelletted cells, can be packed with comparative ease. The resulting beds although highly compressible (figures 4.1a, 4.1b) are stable under the flow of aqueous buffers. Uniformity of packing and lack of channelling is demonstrated by the dispersion characteristics of the beds (figure 4.4a), with the peak dye absorbences corresponding to one bed void volume.

The direct packing of cells of R.nigricans without the requirement for an immobilisation matrix has meant that mycelial volume fractions within the column reactor of 0.33 or greater have been obtained, equivalent to 0.1g dry wt/ml. Other reported packed-bed cell reactor systems tend to have far lower cell densities of the order 10^{-3}g dry wt/ml (Brink and Tramper 1985), the matrixes used for immobilisation of the organisms constituting the major volume fraction within the reactor. The high biocatalytic cell densities achievable using this 'direct' packing method would allow the significant

reduction of column reactor size.

Operation of the packed-bed reactor with an oleic acid/aqueous two liquid-phase emulsion has proved extremely difficult. The mycelial bed appears to act as a form of filter coalescer, with the emulsion breaking up and organic phase channelling through and around the bed. A subsequent study using a model system of glass beads in place of the mycelia shows how the organic phase volume present on the bed increases from that of the starting relative oil volume of the feed emulsion (figure 4.3). It is thought that this retention of organic phase within the bed is due to oil coating the surfaces of the glass bead particles and that a similar process might be happening in the presence of mycelial cells.

Single phase oleic acid flow through the packed-bed of mycelia also proved difficult to achieve. Operation of the reactors at high volumetric flow rates produces rapid channelling of the oil around the bed and high pressure drops across the column. Displacement of aqueous phase by the oleic acid at lower volumetric flow rates eventually causes bed shrinkage away from the column walls with resulting channelling of the organic phase around the bed. This latter effect has been partially overcome by the dropwise addition of aqueous buffer to the feed stream. The packed-bed remains in a more swollen state which allows the reactor to be operated for several hours. Relevant biocatalytic activity data has not been obtained for this type of reactor as the bed stability is still not sufficient to allow the reactor to be run for a long enough period of time to remove excess progesterone carried over from the fermentation induction process.

Use of an aqueous/organic cosolvent feed to the column produced a comparatively stable bed which was operated as a biocatalytic reactor over a period of 20 hours with occasional stopages (figures 4.6.1a, 4.6.1b). The specific 11α-hydroxylase activity (mg 11α-/g dry wt) of the cells within the column reactor was significantly lower than that achieved in shake flask reactions (table 4.6.1). However, with further optimisation of the system (section 6.4), conversion rates equivalent to or higher than those of the batch reactors may be achievable.

6.3 Loss of 11α-hydroxylase Activity by *R.nigricans* in Systems Containing an Organic Solvent

A strong correlation is shown to exist between loss of progesterone 11α-hydroxylase activity from cells of *R.nigricans* and the concentration of organic solvent partitioning into the membranes of the organism (figure 5.2.3.1a) as calculated from a modified Collander equation;

$$[solvent_{membrane}] = 0.19 \times P_{octanol}^{0.84} \times [solvent_{aqueous}]$$

where $[solvent_{membrane}]$ is the concentration of organic solvent partitioning into the membranes, $[solvent_{aqueous}]$ is the concentration of organic solvent dissolved in the aqueous phase and $P_{octanol}$ is the partition coefficient for the organic solvent in a standard octanol/aqueous system.

Total loss of activity is shown to occur at a single critical membrane solvent concentration irrespective of the organic solvent type. Indirect evidence points to perturbations in membrane fluidity as being the primary cause for loss of biocatalytic activity when considering membrane bound/associated enzymes. Organic solvents unable to achieve the critical membrane concentration are shown to allow the retention of hydroxylase activity at saturating aqueous phase concentrations. However, with the development of a discrete second organic liquid phase loss of activity occurs through so termed 'phase' effects.

The discovery of the relationship between biocatalytic activity loss and membrane solvent concentration also serves to explain the sigmoidal nature of the activity retention-$LogP_{octanol}$ plots described by Laane *et al* (1985). A single critical membrane solvent concentration, which is closely related to the $LogP_{octanol}$ of the organic solvent, acts as a 'cut off'. Organic solvents able to achieve this concentration in the membranes cause complete loss of activity when a second liquid phase is present. Those organic solvents unable to reach the concentration in the membranes allow the retention of biocatalytic activity until 'phase' effects dominate. It should be remembered that other explanations will have to be found to describe the correlations obtained by Laane *et al* (1985) where the enzyme system in question is

not membrane bound.

The rational selection of organic solvents for any two liquid-phase systems where the reaction of a membrane bound enzyme is being utilised, can now be performed if the critical membrane solvent concentration is obtained. Referral of this value to an organic solvent selection graph of the type figure 5.2.8a then allows the elimination of a large number of solvents expected to cause complete loss of biocatalytic activity.

Although the available evidence points to alterations in membrane fluidity as the cause of loss of biocatalytic activity in these systems, it has also been shown that the release of membrane lipids correlates closely with both $LogP_{octanol}$ (figure 5.1a) and therefore the corresponding membrane solvent concentration (figure 5.1.1e). It is possible that the critical membrane solvent concentration corresponds to a point where lipid moieties are solubilised from the membrane causing its disorganisation and therefore the loss of biocatalytic activity. However, the experiments were performed with a large volume of second liquid-phase present. It is therefore more likely that the result indicates the ability of the organic solvents to solubilise lipids by direct contact with the cell surface rather than through the partitioning of dissolved organic solvent from the aqueous phase into the membranes.

6.4 Future Work

1. Maintaining an emulsion on the packed-bed of cells of R.nigricans was unsuccessful using oleic acid as the second liquid-phase. Other immiscible organic solvents, however, may prove more suitable. In particular those solvents having lower viscosities and surface tensions could allow higher flow rates across the bed to be employed without the consequent high pressure drops. The packed-bed may then act as a static-mixing device to prevent the break up of the emulsion.

2. Horizontal baffles within the column (Furui and Yamashita 1985) may be beneficial in reducing the large degree of bed compaction that occurred in all the reactor configurations studied. The lowered pressure drop that is likely to result could then allow for further increases in volumetric flow rates that will be required if an emulsion is to be maintained on the column.

3. Use of an aqueous/organic cosolvent feed has made possible the operation of the packed-bed reactor for longer time periods. This has allowed the measurement of 11α-hydroxylase activities for cells of R.nigricans in a column configuration. Kinetic studies at different bed heights and cell densities should be performed to reveal whether oxygen limitation is occurring along the axis of the bed and further optimisation of conditions carried out.

4. The correlation between loss of biocatalytic activity and the concentration of organic solvent partitioning into the membranes which has been demonstrated for the progesterone 11α-hydroxylase enzyme of Rhizopus nigricans should be verified for other membrane bound enzyme systems and other microbial organisms.

5. Measurements of actual membrane organic solvent concentrations should be performed to see if they correspond to those predicted by the Collander equation. This would also allow one to determine the real value for the product term (taken to be 0.19) associated with the membrane type in question.

6. Studies on the variation of membrane fluidity with increasing membrane solvent concentrations may determine whether it is alterations in this gross physical property that is affecting the activity of the 11α-hydroxylase complex or whether more specific lipid-protein interactions are being perturbed.

7. The release of membrane lipids at sub-saturating aqueous phase concentrations of organic solvent should be measured to see if there is a correlation with membrane solvent concentrations.

8. For those organic solvents which have been shown to allow the retention of biocatalytic activity at membrane solvent concentrations equal to the critical membrane solvent concentration, experiments should be performed to determine what gross 'phase' effects are occurring to cause loss of activity at saturating aqueous phase concentrations of solvent. Parameters of interest are surface tension and viscosity which both interact to determine other factors such as droplet size distribution and the ability of a solvent to coat a (cell) surface.

9. If it is conclusively shown that organic solvents do act to cause loss of biocatalytic activity for membrane bound enzymes by causing perturbations in either, gross properties (fluidity) or more specific properties (protein-lipid interactions) of the membranes, then additional studies could involve the selection of new strains/organisms whose membranes/bound enzymes are more resistant to perturbations caused by the presence of the organic solvents.

APPENDIX

APPENDIX

Appendix 1 Calculations for the Determination of Wet/Dry Ratio by a Dye Exclusion Method

Appendix 1.1

When a volume (V_d) of marker dye solution is added to a volume (V_s) of cell suspension the dye concentration of the final solution (C_{fs}) after filtering will be dependent on the volume fraction of cells in the original suspension (ϕ). The mass balance for the dye is given by the following equations.

Dye in = Dye out

Dye in = | Dye conc. in marker solution | x | volume of marker solution |

Dye out = | Dye conc. in final solution | x | volume of marker solution, V_d + cell suspension, V_s + wash buffer, V_w - fungal cells, V_c |

$$\text{Dye in} = C_d \times V_d \qquad [A1.1.1]$$

$$\text{Dye out} = C_{fs} \times \{ V_d + V_s + V_w - V_c \} \qquad [A1.1.2]$$

but V_c, the volume of cells can be calculated from the volume fraction of cells in the original suspension (ϕ).

$$V_c = (V_s \times \phi) \qquad [A1.1.3]$$

this gives

$$C_d \times V_d = C_{fs} \times \{ V_d + V_s + V_w - (V_s \times \phi)\} \qquad [A1.1.4]$$

and rearranging gives

$$\frac{1}{C_{fs}} = V_s \times \frac{(1 - \phi)}{(C_d \times V_d)} + \frac{(V_d + V_w)}{(C_d \times V_d)} \qquad [A1.1.5]$$

cf. $Y = m X + C$

If the volume of marker dye solution and wash buffer used are kept constant then the volume fraction of cells in the original solution can be obtained from the gradient of a plot of the reciprocal of the absorbence of the final filtrate solution (equivalent to $1/C_{fs}$, see figure A1.1a) against the volume of cell suspension added (V_s), figure A1.1b.

The wet weight concentration of cells may then be calculated knowing the density (P_c) of the cells (section 3.2.1).

wet weight conc. of cells (W_{WC}) $= \phi \times P_c$

The corresponding dry weight concentration of cells (W_{DC}) can be calculated from the gradient of a plot of cell dry weight against volume of suspension, figure A1.1c. The wet to dry weight ratio of cells is therefore given by

$$\text{Wet weight / dry weight ratio} = \frac{W_{WC}}{W_{DC}}$$

Appendix 1.2

Equation [A1.1.1] for the mass balance of dye leaving the system makes the assumption that no dye is adsorbed to either (a) the cells themselves and/or (b) the filter paper when cells are separated from the dye solution. Alternative mass balances based on these other factors are easy to produce, with the amount of adsorbed dye being proportional to variables such as the amount of cells and the final concentration of the dye. However, separating ϕ, the cell volume

Figure A1.1a Relationship between the absorbence of naphthol green-B (710nm) and its concentration. Linearity extends to an absorbence of 2.80.

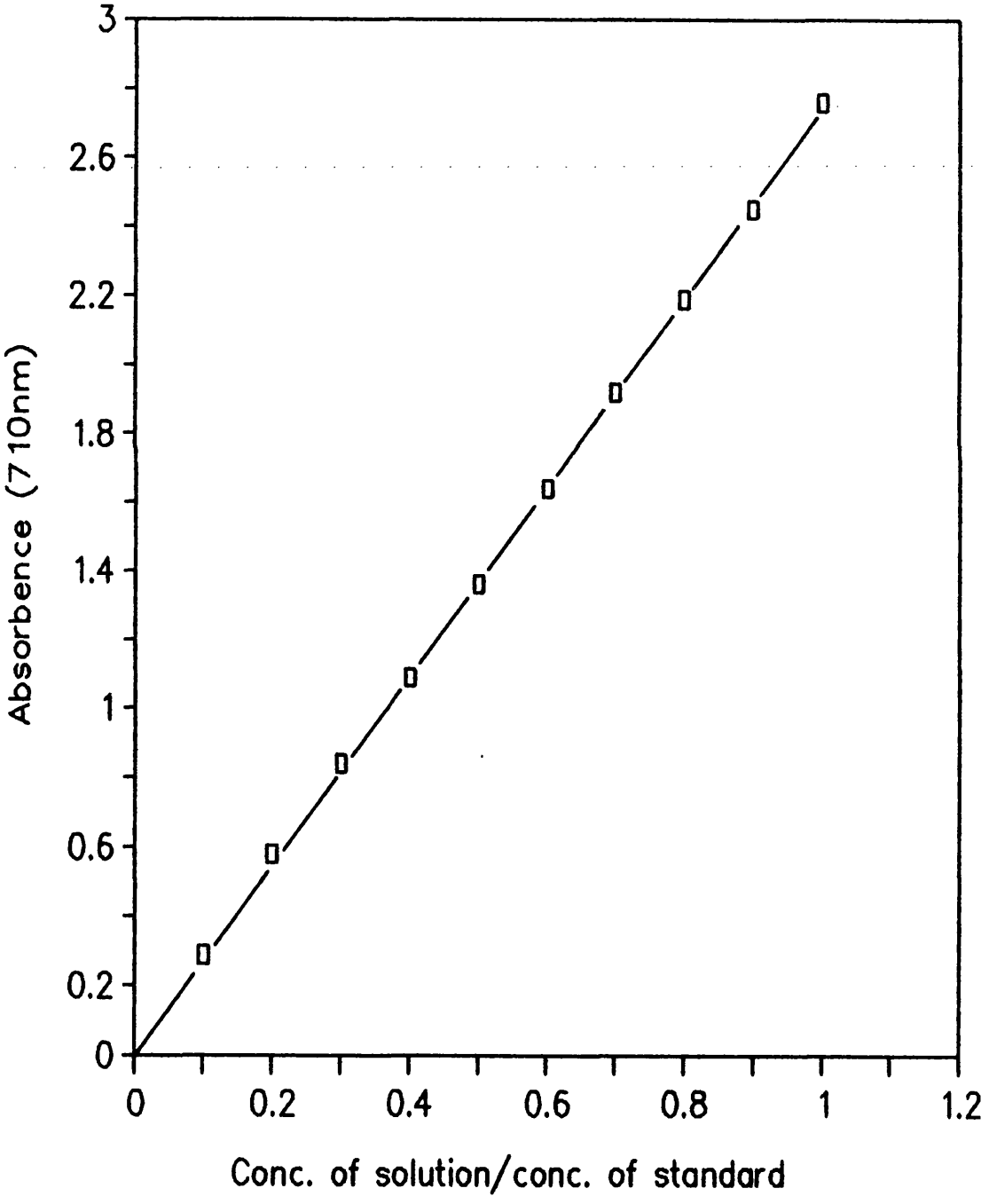


Figure A1.1b Relationship between the absorbence of the final filtrate solution and the volume of cell suspension added. The volume fraction of cells in the original cell suspension is calculated from the gradient of the line.

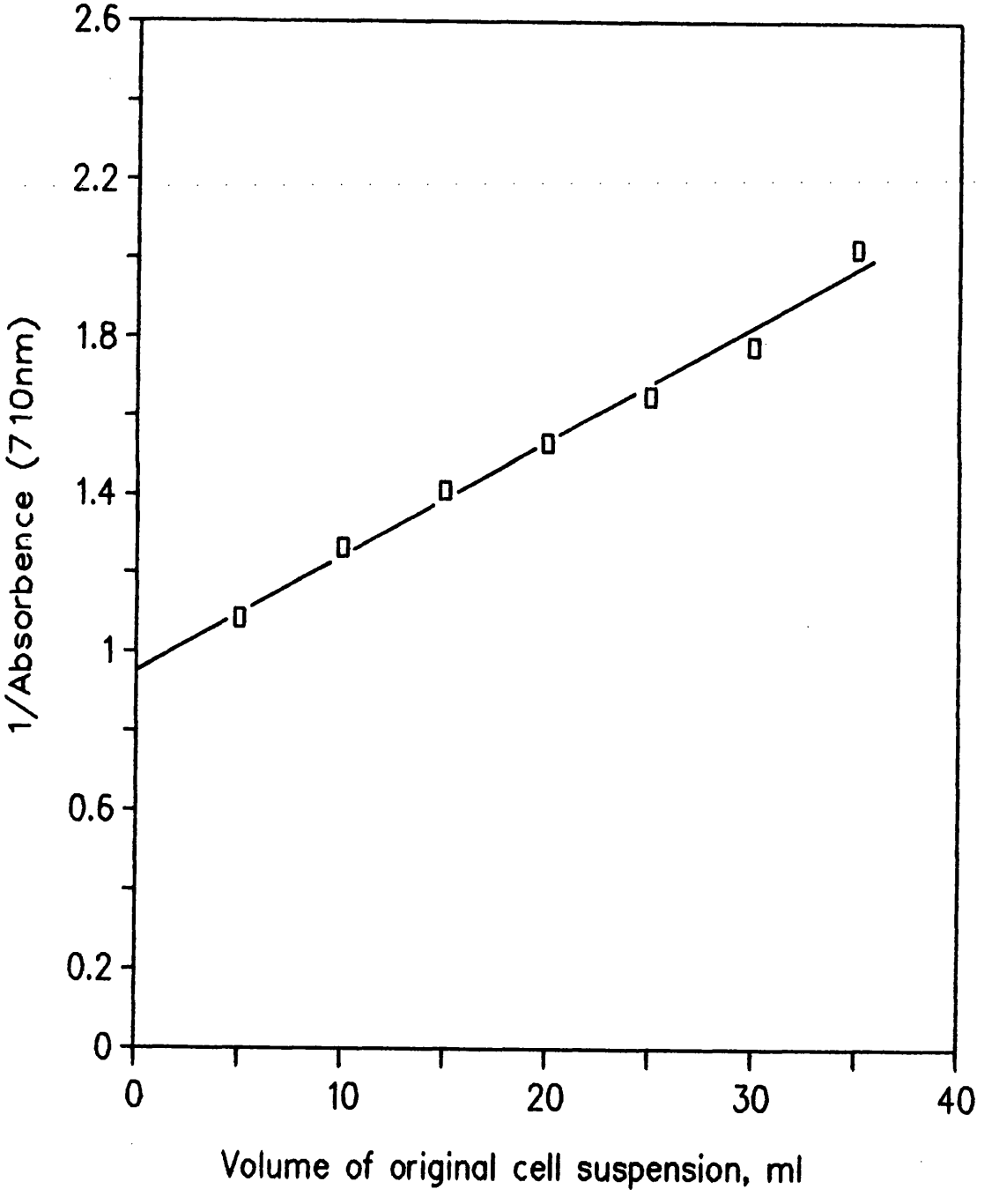
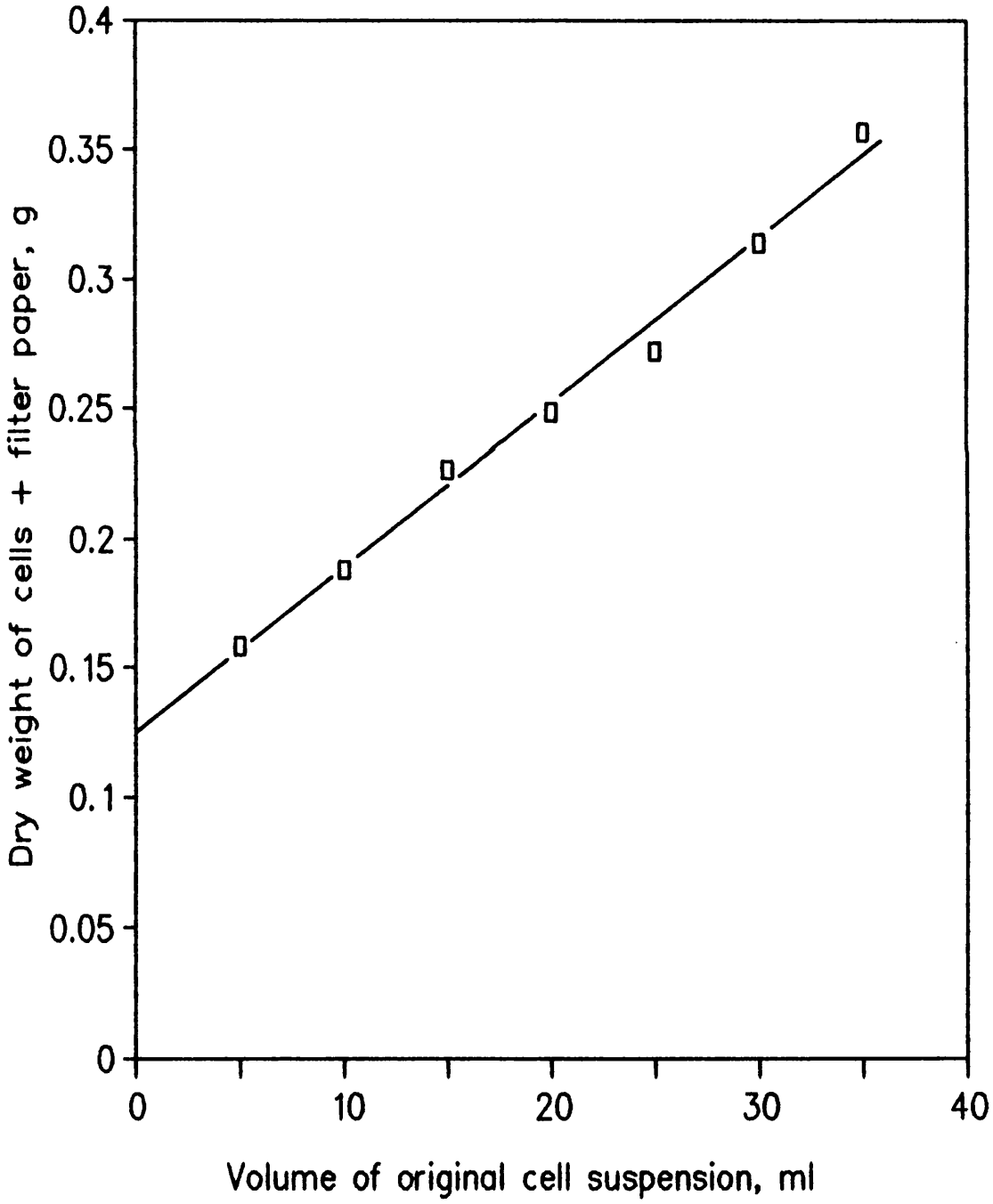


Figure A1.1c Relationship between cell dry weight and the volume of original cell suspension filtered. The Y-axis intercept value is equivalent to the weight of the filter papers used.



fraction, from these other variables then requires more complex experiments to be performed.

A simpler modification of equation [A1.1.1], which requires no additional experiments to be performed, is to assume that a constant amount of dye (K_f) is adsorbed to the filter. Equation [A1.1.1] for the mass balance of dye then becomes

$$\text{Dye out} = C_{fs} \times \{ V_d + V_s + V_w - V_c \} + K_f \qquad [A1.2.1]$$

which when rearranged gives in place of equation [A1.1.5]

$$\frac{1}{C_{fs}} = V_s \times \frac{(1 - \phi)}{(C_d \times V_d)+K_f} + \frac{(V_d + V_w)}{(C_d \times V_d)+K_f} \qquad [A1.2.2]$$

Appendix 1.3

In performing the experiment to obtain the data presented in figure A1.1b, the concentration of the dye in the original solution (C_d=3.23) was measured using a spectrophotometer. The value of C_d may also be calculated from the intercept of the unconstrained regression fit of the data, and need not necessarily be precisely the same as the measured value (C_d=3.155). Alternatively the regression fit may be forced through the intercept (constrained fit) where C_d is equal to the measured value. Table A1.3 shows the values for cell volume fraction (ϕ) and wet/dry weight ratio calculated by the various methods and using both equations [A1.1.5] and [A1.2.2].

Table A1.3

Type of fit	Value of C_d	Value of K_f	ϕ g/ml	wet/dry ratio
Constrained	3.23	0	0.0248	4.27
Unconstrained	3.155	0	0.0753	12.95
Unconstrained	3.23	0	0.0533	9.17
Unconstrained	3.155	0.075	0.0555	9.54

Appendix 2 Calculation of Aqueous Solubility Data

Appendix 2.1

The interpolative and extrapolative methods for determining the aqueous solubilities of the alcohols, heptanol, nonanol, decanol, undecanol and dodecanol were adapted from the work described by Skau and Boucher 1954, Skau and Bailey 1959 and Hoerr *et al* 1944. The 'isotherm method' of Skau *et al* 1954 provides a simple correlation between the solubility of the homolog species (expressed as a mole fraction, X) and the number of carbon atoms, n, in the homolog. The linear equation is derived from the freezing point lowering equation by adapting the known empirical relationships of carbon number and heats/entropies of fusion.

$$\text{Log}(X) = a + (b \times n)$$

where a and b are constants dependent on the temperature and the system involved. The $\text{LogP}_{\text{octanol}}$ of a series of homologs is linearly dependent on the carbon number, n, and the molarity (M) of a solution is linearly dependent on the mole fraction of solute. In this way the equation may be re-written as

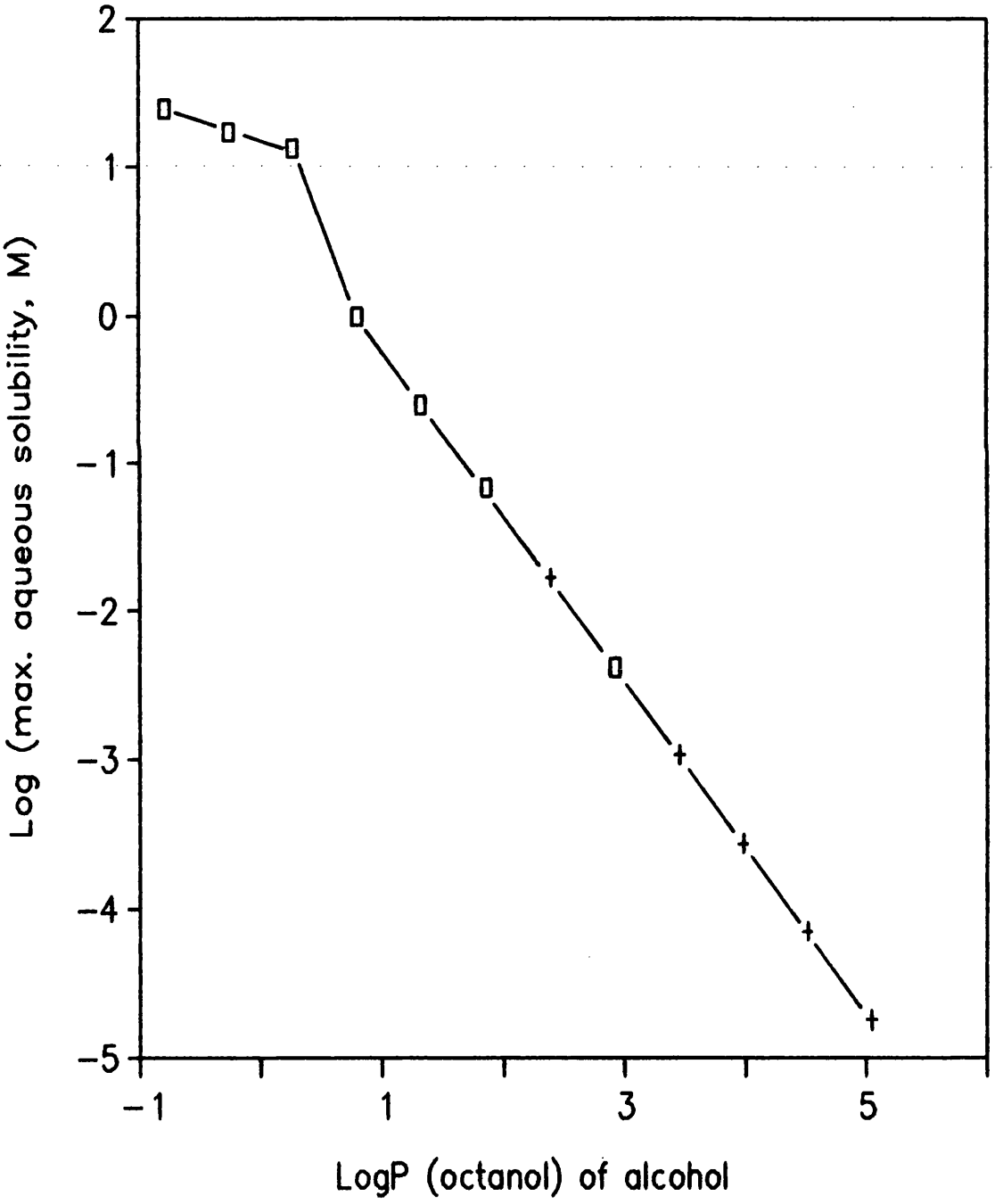
$$\text{Log}(M) = c + (d \times \text{LogP}_{\text{octanol}})$$

Methanol, ethanol and propanol are all totally miscible in aqueous solution and as a result show large deviations from the expected linearity. Regression analysis was therefore only applied to the alcohols, C_4 to C_6 and C_8, for which accurate solubility data were available (Riddick *et al* 1986). The regression results are given in table A2.1 and shown graphically in figure A2.1.

Table A2.1

Correlation coefficient	0.999
Degrees of freedom	2
Constant (c)	0.894
Gradient (d)	-1.119

Figure A2.1 The logarithm of the aqueous solubility of the primary alcohols as a function of their $LogP_{octanol}$ values. Available data (Riddick *et al* 1986) are shown, □ , and calculated data (alcohols C_7 and C_9 to C_{12}) from regression fitting (table A2.1) are shown by, + .



Appendix 2.2

In a similar manner to that described in appendix 2.1, the maximum aqueous solubility of octyl acetate was calculated from a non-linear regression fit ($Y=X^2+X+C$) of the available physical data for the primary acetates, methyl acetate to hexyl acetate (Riddick *et al* 1986). The results are shown in **figure A2.2a**. In addition the density of octyl acetate had to be determined, in order that solvent volumes could be calculated, and this was achieved by a linear regression fit (**table A2.2**) of the respective density data, **figure A2.2b**.

Table A2.2

Correlation coefficient	0.999
Degrees of freedom	4
Constant (c)	0.887
Gradient (d)	-0.043

Appendix 3 Statistical Comparison of Regression Fit Data

Appendix 3.1

All the statistical tests employed were applied according to the principles given by Moroney 1978. Linear regression fits to the various sets of experimental data were obtained (**tables 5.2.3b and 5.3.1**). The independent estimates of correlation coefficient, R, were then tested for similarity by calculating 'z' values for each and referring their difference to the standard error of their differences, where N_1, N_2 are the number of data values in the regression correlations.

Fischer's 'z' transformation

$$z = 1.15 \times \mathrm{Log}_{10}\{ (1+R)/(1-R) \}$$

Figure A2.2a The logarithm of the aqueous solubility of the primary acetates as a function of their $LogP_{octanol}$ values. Available data (Riddick et al 1986) are shown, □ , and calculated data (octyl acetate) from non-linear regression fitting is shown by, + .

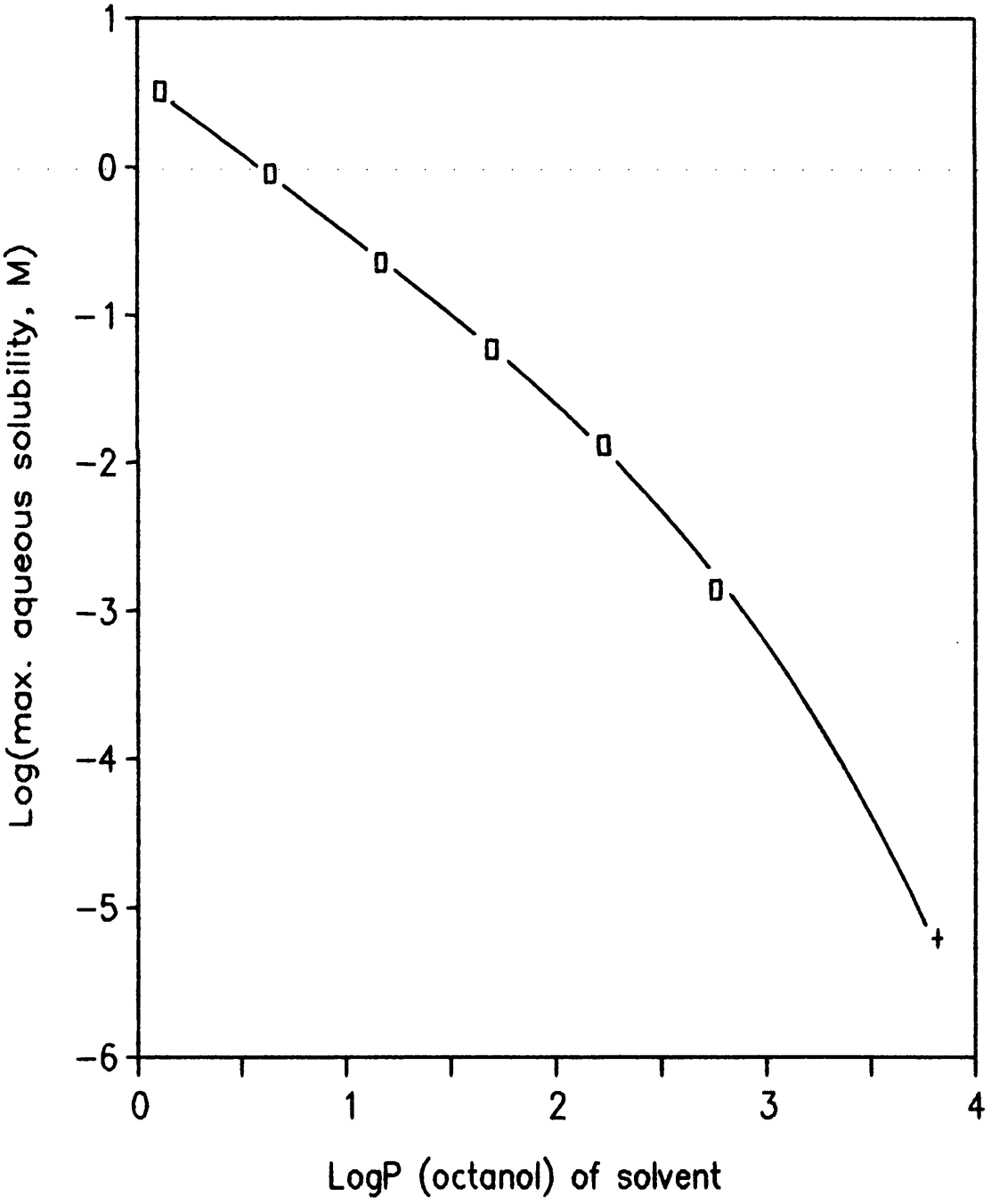
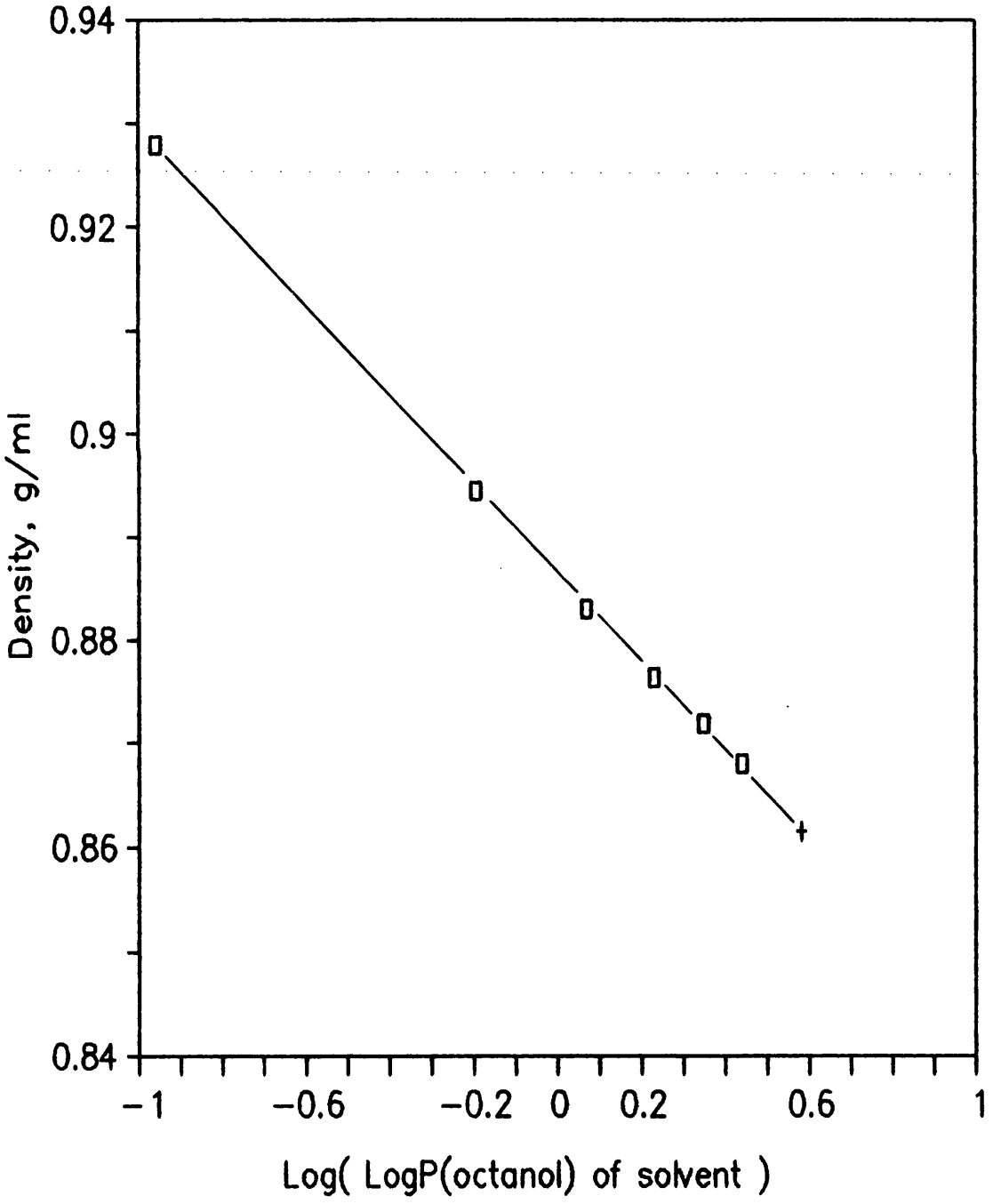


Figure A2.2b Density of the primary acetates plotted against the Logarithm of the $LogP_{octanol}$ of the solvents. Available data (Riddick et al 1986) are shown, □ , and calculated data (octyl acetate) regression fitting (table A2.2) is shown by, + .



Standard error of difference (S.E.D)

$$S.E.D = \sqrt{\left\{ \frac{1}{(N_1-3)} + \frac{1}{(N_2-3)} \right\}}$$

If the difference in 'z' values is less than the standard error of difference then the correlation coefficients are not statistically significantly different (table A3.1a).

Table A3.1a Superscript ^{a1,a2} etc. denotes the data being compared.

	No. N_1, N_2	Regress. coeff.	z value	S.E.D	Signif. differ.
Expt. 1					
Alcohols^{a1}	8	0.999	3.849	$0.632^{a1,a2}$	No
All solvents^{s1}	12	0.998	3.358	$0.408^{s1,s2}$	No
Expt. 2					
Alcohols^{a2}	8	0.999	3.849		
Acetates^{c2}	4	0.998	3.531	$1.095^{a2,c2}$	No
All solvents^{s2}	12	0.999	3.665		

A second test shows whether the values for the critical membrane solvent concentrations calculated using the following equation are of a similar magnitude.

$$[solvent_{memb.C}] = 0.19 \times P_{octanol}^{grad} \times [solvent_{aq.C}]$$

where grad is the gradient of the respective correlation (tables 5.2.3b and 5.3.1).

The student 't' test may then be applied to the groups of values, with Bessel's correction made for small sample numbers.

Student 't' test with (N_1+N_2-2) degrees of freedom.

$$t = (X_1-X_2) \Big/ \left[\sqrt{\frac{(N_1S_1^2 + N_2S_2^2)}{N_1+N_2-2}} \times \sqrt{\left(\frac{1}{N_1} + \frac{1}{N_2}\right)} \right]$$

where X_1, X_2 are the mean values for samples 1, 2 and S_1, S_2 are the respective sample variations. A prior test, the Variance ratio test, is used to determine whether the sample variances are sufficiently alike to warrant assuming that they are independent estimates of the same population variance.

Variance ratio test (applying Bessel's correction) with N_1-1, N_2-1 degrees of freedom for the greater and lesser variance respectively.

$$F = \frac{S_1^2 \times N_1/(N_1-1)}{S_2^2 \times N_2/(N_2-1)}$$

Values for both the variance ratio test and the student 't' test are referred to statistical tables (Moroney 1978) at the 1% confidence limit to determine their statistical significance (table A3.1b).

Table A3.1b Superscript ^{a1,a2} etc. denotes the data being compared.

	Sample variance	F ratio	Signif. differ.	Student 't' test	Signif. differ.
Expt. 1					
Alcohols^{a1}	0.000389	1.145^{a1,a2}	No	1.912	No
All solvents^{s1}	0.000740	1.704^{s1,s2}	No	1.953	No
Expt. 2					
Alcohols^{a2}	0.000340				
Acetates^{c2}	0.000213	0.729^{a2,c2}	No	2.144	No
All solvents^{s2}	0.000435				

Appendix 4 Calculation of Vessel Dispersion Numbers (D/uL)

Figure 4.4a shows the concentration-time profiles (C-curves) for pulsed inputs of dye to the same column of pelleted cells packed to three different volume fractions. To calculate the vessel dispersion number (D/ul) that characterises the amount of axial (longitudinal) mixing it is first necessary to assume that the dispersion is too large to regard the C-curves as being of the simple gaussian (normal) form. The variance of these curves (Levenspiel 1972) is then given by

$$s_\theta^2 = \frac{s^2}{t'^2} = \frac{2D}{uL} - [\, 2\,(D/uL)^2 \times (1-e^{-uL/D}) \,] \qquad [A4.1a]$$

where s_θ^2 is the dimensionless variance, s^2 is the true variance and t' is the mean residence time (reactor holding time). The variance of a continuous distribution of concentrations (C_i) measured at a finite number of equidistant times (t_i) is given by

$$s^2 = \frac{\Sigma\, t_i^2\, C_i}{\Sigma\, C_i} - t'^2 = \frac{\Sigma\, t_i^2\, C_i}{\Sigma\, C_i} - (\,\Sigma\, t_i\, C_i / \Sigma\, C_i\,)^2 \qquad [A4.1b]$$

Ignoring the second term on the right of equation [A4.1a] we have an approximation from which the vessel dispersion number can be estimated.

$$\frac{s^2}{t'^2} \approx \frac{\dfrac{\Sigma\, t_i^2\, C_i}{\Sigma\, C_i} - (\,\Sigma\, t_i\, C_i / \Sigma\, C_i\,)^2}{(\,\Sigma\, t_i\, C_i / \Sigma\, C_i\,)^2} \approx 2\,\frac{D}{uL} \qquad [A4.1c]$$

The approximated value of the dispersion number calculated from equation [A4.1c] is greater than the real value by an amount equal to the right hand term of equation [A4.1a]. However, it should also be noted that this increased amount may, in part, offset the under-estimation of the value that is incurred because the C-curves of figure 4.4a are not totally continuous and do not fully return to the base-line. The values obtained are therefore useful only as a rough guide to the amounts of axial dispersion (mixing) that is taking place in the columns.

A better comparison of the relative (but not actual) amounts of dispersion may be obtained if an artificial base-line is assumed such that all the C-curves return to the base-line. As would be expected, the vessel dispersion numbers obtained are smaller than those for the whole C-curve profiles, **figure 4.4b.**

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