SOLVENT SELECTION FOR WHOLE CELL BIOTRANSFORMATIONS IN ORGANIC MEDIA

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1. Abstract.

Although they were used historically as antimicrobial agents, there is a modern requirement to devise organic solvent systems for exploitation in the biotransformation by intact cells of substrates that are poorly soluble in water. Water-immiscible solvents are normally less cytotoxic than are water-miscible ones. Whilst a unitary mechanism is excluded, damage to the membrane remains the likeliest major mechanism of cytotoxicity, and may be conveniently assessed using an electronic biomass probe. Studies designed to account for the mechanism of actions of general anæsthetics and of uncouplers parallel those designed to account for the cytotoxicity of organic solvents. Although there are hundreds of potential physical descriptors of solvent properties many are broadly similar to each other, such that the intrinsic dimensionality of solvent space is relatively small (<10). This opens up the possibility of providing a rational biophysical basis for the optimisation of the solvents used for biotransformations. The widely used descriptor of solvent behaviour log P (the octanol:water partition coefficient) is a composite of more fundamental molecular descriptors; this explains why there are rarely good correlations between cytotoxicity and log P when a wide variety of solvents is studied. Although the intrinsic dimensionality of solvent space is relatively small, pure solvents still populate it rather sparsely. Thus, mixtures of solvents can and do provide the opportunity of obtaining a solvent optimal for a biotransformation of interest.

2. Introduction and scope.

There is much current interest in the performance of biotransformations using or aided by organic solvents (Brink & Tramper 1985, Adlercreutz & Mattiasson 1987, Laane *et al*. 1987a,b, Brink *et al*. 1988, Khmelnitsky *et al*. 1988, Zaks & Klibanov 1988, Zaks & Russell 1988, Waks 1989, Anderson & Hahn-Hägerdahl 1990, Arnold

1990, Halling 1990, Lilly *et al*. 1990, Osborne *et al*. 1990, Mattiasson & Adlercreutz 1991, Woodley *et al* 1991a&b, Dordick 1992a, Gupta, 1992, Tramper *et al.* 1992, Sonsbeek *et al* 1993, Halling 1994, Heipieper *et al* 1994, Sikkema *et al* 1994). This is because the organic solvent itself may be of interest as a substrate, or it may be needed to shift the equilibrium composition in a favourable direction, and in particular because many of the substrates of interest in modern biotransformations are essentially insoluble in aqueous media, and/or are highly toxic to whole cells.

While the use of organic solvents with enzymes is becoming particularly widespread, their use with viable (or at least intact) cells has received much less attention, despite the fact that most of the advantages for enzyme systems also hold true for intact cell systems. This is almost certainly due to the perceived difficulties of maintaining viable cells within solvent systems as compared to the comparative ease with which enzymes may be dispersed. Most of the systems described using enzymes are two-phase systems, in which the volume of the aqueous phase is smaller than that of the organic one and in which the system, upon stirring, forms a water-in-oil emulsion. Both this and the alternative oil-in-water emulsions obtained when the aqueous phase is the more voluminous, are thermodynamically unstable arrangements, and upon standing will undergo phase separation. This means that such systems must be agitated to facilitate mass-transfer. Alternatively the biocatalyst may be immobilised, allowing the aqueous phase to be greatly reduced and non-continuous; in the extreme this may result in water being present only in a bound form, attached to the biocatalyst and the support. Thermodynamically stable arrangements also exist, in the form of micro emulsions and reverse micelles. While in theory such systems could be used as a vehicle for viable cells, they have been largely ignored to date.

The most significant problem with using organic solvents with viable cells is lies not with the system or reactors employed, but rather in the choice of solvents. As is well known (e.g. Hugo 1971), a great number of solvents are highly cytotoxic or

inhibitory. Those that are non-toxic are typically highly apolar and as such have a rather restricted solvating power and are consequently of limited use as a solvent for the substrates and products that are usually of interest. This problem is compounded by the fact that different cell types, lines, or indeed individual strains may vary considerably in their response to a given solvent, even under the same physiological conditions.

While this review will indeed deal generally with whole cell biotransformations that have been aided by organic media, special emphasis will be placed on the attempts that have been made to account for the mechanisms, or biophysical basis, underlying solvent toxicity, and in particular on the use of appropriate indicators and predictors for solvent selection.

3. Historical

3.1 Why use organic media anyway?

The main advantages of using organic solvents to aid biotransformations using viable cells are given in Table 1.

The advantages given in Table 1 are all *potential* advantages, of a general nature. In reality, one would try and prioritise why the use of solvents may benefit a particular process, and maximise those advantages that are quantitatively most important for the process of interest, a principle similar to that operating in the optimization of *metabolic* fluxes (e.g. Kell *et al*. 1989). This will in all probability result in those aspects that have less importance actually becoming less facile through the use of organic solvents. At all events, when using organic solvents, especially with viable cells, there are a number of potential *dis*advantages to consider; these include those tabulated in Table 2.

Even with these difficulties, the use of organic solvents does, at least potentially, permit many biotransformations that would be very difficult to achieve within conventional aqueous systems, and for this reason alone interest will continue.

3.2 Organic solvents as "classical" antimicrobial agents:

Phenolic compounds and aromatic alcohols have a long history as agents of antimicrobial activity. Phenols contained within natural oils and balsams have been used by man from the earliest antiquity as deodorants, preservatives and as constituents of aromatic vapours (incenses), although their use as antimicrobials was serendipitous and arose because of the pleasant aroma of these products and their ability to counteract malodour (Hugo, 1978). Bacterial contamination and its control in medical science was not widely known until the time of Dr Jules Lemaire (who published a comprehensive work of 754 pages on phenolic (carbolic) acid in 1863, with a 2nd edition in 1865 (titled "De l'acide phenique. De son action sur les vegetaux, les animaux, les ferments, les venins, les virus, les miasmas et de ses applications à l'industrie, à l'hygiene, aux sciences anatomiques et à la therapeutique" (Kelly, 1901))). When Pasteur's evidence that organic matter was not inherently putrefiable was accepted, Lister (1867), seemingly independently, was also using carbolic acid in a liquid form during this period ("the addition of a few drops of water to a considerable quantity of the crystallised acid induces it to assume permanently the liquid form") to treat suppuration caused by "minute organisms floating in the air" (Miles, 1967).

From the late eighteenth century, coal carbonisation made available a wider range of phenolic compounds, including both the volatiles and coal tar itself, the active ingredient being phenol. Distillation of coal tar produced the heavy oil creosote (= "flesh saver") which contains *o*-, *m*-, and *p*-methylphenols or cresols, the six dimethylphenols or xylenols and ethylphenol (Hugo, 1978), all of which are toxic to

micro-organisms, 360 mg/l of *p*-cresol being sufficient to reduce the growth rate of *Escherichia coli* in suspension by 50% (calculated from Heipieper *et al*, 1991). The concentrations of phenolic compounds which inhibited the growth of the cells also increased the permeability of the membranes, the efflux rate correlating well with the phenol concentration used (Heipieper *et al*, 1991).

Lucchini *et al.* (1990) studied a range of phenolics and aromatic alcohols and found that all were able to alter membranes, especially in Gram-negative bacteria. Whilst such general antibacterial modes of action would seem to be common to just about all toxic organic solvents, Corre *et al* (1990) also demonstrated a more specific toxicity. Treatment of *E. coli* cells in the mid-logarithmic phase of growth by phenethyl alcohol (PEA) or benzyl alcohol led to a rapid reduction in living bacteria, but this effect was stopped by the addition of chloramphenicol or chlortetracycline, suggesting a role for protein synthesis in the bactericidal activity of PEA. The inhibition of RNA synthesis by rifampicin did not interfere with the bactericidal effects of PEA or benzyl alcohol.

While many more selective and active compounds are available today to combat microorganisms, especially bacteria, organic liquids are still heavily relied on as general biocides, with many still being based on those of historical importance above.

3.3 Permeabilization.

Living cells control solute uptake primarily by means of the plasma membrane. The permeability of this membrane can be damaged without the total destruction of cell integrity, a treatment commonly known as permeabilisation (e.g. Felix 1982, Flores *et al*, 1994). Thus enzymes and enzyme systems in permeabilised cells can be studied *in situ*, and the enzymes do not have to be isolated and reconstituted *in vitro*. In this

way it is hoped that the enzymes can be experimented under conditions which more closely resemble those *in vivo* (Mendes *et al*. 1995).

The use of organic solvents to effect cell permeabilisation has been prevalent since at least the early 1960s. Thus it has been known for a considerable period that at least some solvents induce gross damage to cell membranes, even if the molecular mode of action was not known. Gram-negative and -positive bacteria, yeast, plant and mammalian cells have been successfully permeabilised using organic solvents (Naglak *et al,* 1990), and Felix (1982) provides an excellent review of many of the chemical agents that have been employed.

Of the organic solvents available, toluene is perhaps the most frequently used for this purpose in microbiology. The effectiveness of the permeabilisation of *E. coli* cells by toluene has been studied by Deutscher (1974), who found the time of exposure, temperature, pH and growth stage all to be of importance. Using *E. coli* it was demonstrated that the amount of protein released depended on the concentration of the toluene and the temperature (DeSmet *et al*, 1978; Jackson and DeMoss, 1965), and that toluene-treated cells were no longer viable. The presence of Mg^{2+} was also found to be important; when it was present at a higher concentration then normal, the amounts of protein, phospholipid or polysaccharides in *E. coli* removed by toluene were reduced significantly (DeSmet *et al*, 1978) (as was end product inhibition/toxicity towards *S. cerevisiae* (Dasari *et al*, 1990)). Examination of freeze-fracture electron micrographs showed that toluene caused much damage to the cytoplasmic membrane. Ether acts in the same manner as does toluene (Felix *et al*, 1980), whilst phenethyl alcohol has a less disruptive effect (Woldringh, 1973).

3.4 Two-phase systems and enzymes.

The use of enzymes with two-phase systems has received considerably more attention than has the use of viable cells, and numerous reviews and texts are available (e.g. Butler 1979, Lilly and Woodley 1985, Zaks and Klibanov 1985, Aldercreutz and Mattiasson 1987, Halling 1987a and b, Semenov *et al* 1987, Hwang and Arnold 1991, Deetz and Rozzel 1991, Dordick 1991, Valivety *et al* 1991, Zaks 1991, Dordick 1992b, Zaks 1992, Narayan and Klibanov 1993 and Halling 1994). These texts cover such topics as the criteria for solvent selection, kinetic effects, the use of solvents as substrates, immobilised enzymes, and reactions specific to enzyme/two-phase systems. Unfortunately much of this information is not directly applicable to whole cell systems and thus falls outside of the scope of this review.

4. Organic media

4.1 Single-phase.

True single-phase systems are produced when water-miscible co-solvents are added to the medium to improve the solubility of compounds that are relatively insoluble in aqueous systems. This can considerably reduce mass-transfer limitations resulting in more rapid reaction rates.

Careful consideration of the choice and concentration of co-solvent is required as many miscible solvents are cytotoxic at ostensibly quite modest concentrations. Regardless of the intrinsic toxicity of such co-solvents, mass action consideration alone mean that miscible solvents are potentially likely to be present at high molar concentrations. Thus if viable cells are required to perform the biotransformation the choice between two types of system is forced upon the user. The first option is to use the substrate/product and co-solvent at very low (non-toxic) concentrations. This allows the use of continuous processes or repeated batch production using the same cells. Volumetric productivities, while higher than those for a comparable aqueous

system, are low. Some protection from the co-solvent may be gained by cell immobilisation (Fukui *et al* 1987, Kawabata and Nakagawa 1991). Alternatively, one may seek to use the substrate/product and co-solvent at toxic concentrations. This dictates that batch systems are used, with cell death/inactivation hopefully being at a rate sufficiently low to allow high yields before activity is essentially lost, and where repeated batches will rapidly result in reduced activities of the biocatalyst (e.g. Christen and Crout 1987, Freeman and Lilly 1987). Volumetric productivities may be improved by this method but biomass demands are correspondingly high.

The use of co-solvents may work well when substrates/products are merely sparingly soluble within an aqueous system. Indeed, it is possible that the addition of co-solvents may considerably increase the solubility of the substrate but not that of the product, enabling *in situ* product recovery (Silbiger and Freeman 1991). However, when the compound of interest is really insoluble in water the addition of water-miscible co-solvents usually does little to improve solubility unless they are added at very high concentrations. At the concentration typically required (often near 100%) biocatalyst inhibition/denaturation occurs. In addition, if the substrate/product of interest is cytotoxic, the use of single-phase systems will enhance this toxicity, both in terms of the basic cytotoxicity of the system and the speed of action. Thus, a quite modest concentration of ethanol (6% (v/v)) added in an attempt to improve the solubility of 6-methyl-5-hepten-2-one (which is cytotoxic) greatly increases the toxicity of the latter (figure 1).

4.2 Two-phase.

Two-phase systems consist of a continuous and a discontinuous phase, formed by two (or more) immiscible liquids. Either phase may be organic or aqueous, but considerations of interfacial area normally demand that an effective mixing regime is in place (see later). The aqueous phase will contain the biocatalyst either dissolved or in

colloidal or insoluble form (quite possibly immobilised); hence it is sometimes known as the "Biophase". In addition it will generally contain all the nutrients and other compounds the biocatalyst will require to function. Organic solvents will also be present, including all of those within the non-aqueous phase, including the substrate/product, typically in low concentrations. Other congeners which are miscible in one or both phases may also be added.

The organic phase in its simplest form could consist of the substrate and/or product only (see e.g. Walter *et al*. 1989). More typically the substrate/product is carried within another organic solvent, or a mixture. The ratio of the aqueous-phase to the organic-phase can vary considerably, with the organic-phase being present as only as a very small partial volume and being highly dispersed. At the other extreme, the aqueous phase may be present only in bound form on the biocatalyst and the support. The reaction site may be within the aqueous-phase or at the liquid-liquid interface.

A number of good general texts and reviews have been produced on biocatalysis using two-phase systems, including Lilly 1982, Brink & Tramper 1985, Laane *et al* 1985, Adlercreutz & Mattiasson 1987, Halling 1987a, Laane *et al*. 1987a,b, and c, Legoy *et al* 1987, Lilly *et al* 1987, Reslow 1987, Brink *et al*. 1988, Khmelnitsky *et al*. 1988, Zaks & Klibanov 1988, Zaks & Russell 1988, Waks 1989, Anderson & Hahn-Hägerdahl 1990, Arnold 1990, Halling 1990, Lilly *et al*. 1990, Osborne *et al*. 1990, Mattiasson & Adlercreutz 1991, Khmelnitsky *et al* 1991, Woodley *et al* 1991a&b, Dordick 1992a, Gupta, 1992, Tramper *et al.* 1992, Woodley and Lilly 1992, Sonsbeek *et al* 1993, and Halling 1994.

4.3 Reversed micelles.

Luisi and his colleagues (e.g. Luisi & Laane 1986, Luisi *et al*. 1988, Hochkoeppler & Luisi 1989) and others (see e.g. Martinek *et al*. 1987, Laane *et al*

1988, Pileni 1989) have pioneered the exploitation of *microemulsions*, often called "reverse micelles". These are thermodynamically stable, "single-phase" systems in which the addition of an appropriate amphiphile or detergent (surfactant) permits the single-phase coexistence of otherwise mutually insoluble aqueous and organic media. (Notwithstanding, the microemulsions *do* consist of small aqueous agglomerates surrounded by a surfactant shell, dispersed in the organic phase.) Surfactants such as AOT (bis-(2-ethylhexyl)- sulfosuccinate) (Fendler 1982), and indeed phospholipids (Garza-Ramos *et al*. 1989) seem to be suitable for forming such microemulsions, and do not greatly affect the stability of the biocatalysts (enzymes) employed. Their use however with whole cell systems is not greatly favoured, perhaps because of the difficulties encountered in larger scale product separation when surfactants/detergents are involved.

Investigations of respiratory chain electron transfer activity in asolectin-isooctane reverse micelles (Escobar and Escamilla, 1992), and of oxygen consumption by yeast in Tween-isopropyl palmitate reverse micelles (Hochköppler *et al* 1989), would tend to suggest, however, that such systems are indeed compatible with viable cells.

5. Measurement and prediction of solvent toxicity

5.1 Physiological effects

5.1.1 Access to membrane.

The ease of access to the cell membrane is important in determining how toxic a solvent may be. Clearly for water-soluble organic solvents in a single-phase system direct access is possible, and thus, any toxic effects are rapidly seen. For two-phase systems, if cell contact with the organic phase can be prevented, the solubilty of the

organic phase within the aqueous phase will determine the rate of mass transfer into the cell membrane; it will not however affect the equilibrium position. This mass transfer rate may be altered by the addition of various compounds to the aqueous phase, for example, the addition of ethanol will normally increase the solubility of the organic phase (within the aqueous) and thus increase mass transfer rates, this in turn will result in cytotoxic concentrations being attained more rapidly. The membrane concentration of an (aqueous) insoluble toxic organic solvent at equilibrium may be lowered when used within a mixed-solvent two phase system.

5.1.2 Membrane effects

It is well known that modest concentrations of ethanol and other alcohols lead to reduced fermentation and growth rates of those organisms which produce them, and that high concentrations are cytotoxic. While much research has been carried out (Ingram and Buttke, 1985, Lovitt *et al*, 1988), the methods by which these organic solvents affect the cell are poorly documented; in many cases they are simply cited as being multi-target or non-specific in their action. It is however generally agreed that the cell membrane is one of, if not "the", primary target for organic solvents.

Carlson *et al* (1991) demonstrated clearly that increasing concentrations of 6 alcohols inhibit anaerobic fermentation (as carried out by *Saccharomyces cerevisiae*); a correlation with increased partition coefficients into a hydrophobic milieu was also evident. This would tend to suggest that the action of these alcohols is primarily located at a hydrophobic site, possibly at the membrane. In this study of the effect of n-alcohols on the ATP-dependent generation of ΔpH and $\Delta\psi$ across plasma membrane vesicles of *Saccharomyces cerevisiae*, the alcohols were shown to collapse ΔpH and $\Delta\psi$ in the order $C_2<C_3<C_4<C_5\leq C_6\geq C_7>C_8>C_{11}$, i.e. that there was an optimal chain length for cytotoxicity. Inhibition of the plasma membrane H^+-ATPase was insignificant (Petrov and Okorokov, 1990). Using pH-stat measurements Stevens

and Hofmeyer (1993) have shown that quite modest concentrations of both octanoic and decanoic acid, in the presence of ethanol, increase the rate of passive H^+ influx across the plasma membrane. It has been suggested that toxicity is a result of the increased anion and proton permeability of the plasma membrane, de-energising it and ultimately blocking the secondary transport systems (Petrov and Okorokov, 1990, Sikkema *et al*, 1992, Stevens and Hofmeyr, 1993). This increase in permeability may be due to the increase in the fluidity of the membrane lipids (Petrov and Okorokov, 1990), or to the solvent partitioning into the lipids causing membrane expansion (Seeman, 1972); such membrane expansion can be clearly demonstrated by fluorescence selfquenching techniques (Sikkema *et al* 1992) and by dielectric spectroscopy (Stoicheva *et al*. 1989, Salter & Kell 1992, Davey *et al*, 1993)). It has also been suggested that cytotoxicity is exerted mainly when a "critical", solvent-independent membrane concentration is reached (Osborne *et al*. 1990).

In an investigation of respiratory electron transfer activity in an asolecithin-isooctane reverse micellar system, it was suggested that in the organic medium electron transfer from NADH to O_2 is arrested at the terminal oxidase step (Escobar and Escamilla, 1992). Given that respiratory electron transfer requires the diffusional motions of various membranous complexes (see e.g. Anthony 1988, Westerhoff *et al*. 1988), this is perhaps not surprising. However, results from other work in which the electron transport chain was replaced with artificial electron acceptors such as phenazine methosulphate ((PMS) Fukui *et al* 1980, Hocknull and Lilly 1987, Freeman and Lilly 1987) or menadione (Pinheiro and Cabral 1991) suggest that organic solvents can exert significant effects on the rate of electron transfer directly.

n-Alkanes (especially the larger ones) are commonly regarded as being non-cytotoxic towards a whole range of micro-organisms. It has been reported however that the growth of various yeasts on, or in the presence of these compounds results in changes in the fatty acid composition of the cell, varying according to the chain length

of the alkane involved (Thorpe and Ratledge, 1972). Fractionation of the lipids from yeasts grown on various alkanes showed that although triglycerides still constituted the major fraction, their relative proportion was less than when glucose was the growth substrate. Loss of triglycerides was compensated for by a corresponding increase in the phospholipids (Thorpe and Ratledge, 1972).

In a similar investigation Vollherbst-Schneck *et al* (1984) showed that butanol, at sub-growth inhibitory levels had a significant fluidizing effect on the bulk lipid regions of *E. coli* and *Clostridium acetobutylicum*. When grown in the presence of butanol, *C. acetobutylicum* synthesised increased levels of saturated acyl chains at the expense of unsaturated chains. This has also been shown to occur in yeast (under aerobic conditions); interestingly, immobilised cells, which are known to have a higher ethanol tolerance, also have significantly more saturated fatty acyl residues than do free cells (Hilge-Rotmann and Rehm, 1991). The ratio of unsaturated to saturated fatty acids has long been known to control membrane fluidity, with increases in unsaturation leading to greater membrane fluidity, such that cells subjected to temperature changes adjust the degree of saturation to maintain a constant fluidity, a mechanism knows as homeoviscous adaptation. On this basis it would seem logical, if solvents increase the fluidity, that increasing the ratio of saturated fatty acids would reduce this effect, and restore the "normal" fluidity of the membrane. Sterols also play an important role in the regulation of membrane fluidity. Agudo (1992) showed that the presence of ergosterol was important for increased ethanol tolerance in *Saccharomyces cerevisiae*, along with a shift to shorter chain lengths of the fatty acids (C_{12}-C_{14}). Finally, Heipieper *et al*. (1994) summarise the evidence that in cells tolerant to organic solvents a major fraction of the *cis*-unsaturated is converted to the corresponding *trans*-fatty acids.

5.1.3 General anæsthesia/narcosis

Many organic solvents that may be of interest in two-phase systems, either as carriers for substrates, or as substrates themselves, such as alcohols, ketones, ethers, and alkanes also have general anæsthetic properties (Richards *et al* 1978, Pringle *et al* 1981, Janoff and Miller 1982, Franks and Lieb 1981, 1985a, b, 1986, 1987, Chiou *et al* 1990, Curry *et al* 1990, Janes *et al* 1992, Messaoudi *et al* 1992, Dickinson *et al* 1993 and Slater *et al* 1993). Thus much of the work carried out in an attempt to understand the mechanism of general anæsthesia/narcosis may also be relevant for toxicity studies of organic solvents generally, especially as such anæsthetics are known to be toxic at high concentrations. Indeed, the literature of narcosis (and also that of uncouplers; Kell & Westerhoff, 1985) provides a rich hunting ground for hypotheses designed to uncover the mechanisms by which organic molecules of diverse structure can affect biological activities.

General anæsthesia can be induced by a wide variety of structurally dissimilar molecules. Consequently, the mechanism must involve some rather non-specific interactions at the target site(s), often held to be within the membrane (Bowman *et al*. 1968). Membrane expansion due to organic solvents and general anæsthetics is now well documented, and Seeman and co-workers in particular have developed the idea that the anæsthetic action of alcohols may be mediated by a critical amount of membrane expansion and associated protein conformational changes (Seeman 1972). Such an hypothesis (generally known as the critical volume theory) is supported by the observation that anæsthesia is reversed by pressure (Miller et al 1973). Kita and Miller (1982) suggest that the bilayer must expand anisotropically, experiencing a decrease in thickness with increasing volume, or that conformational changes in membrane-associated proteins can occur at constant volume with an increase in membrane area. A volume change of 0.15% in red cell ghost membranes has been found to be associated with anæsthesia. Other theories put forward to explain the role of membrane expansion include the decoupled bilayer theory, where the inner and outer bilayer become free to move laterally in relation to each other, allowing changes in surface morphology (Bull

et al 1980). Sandorfy (1980) suggested that anæsthetic action was due to the breaking and formation of hydrogen bonds to produce changes in lipid and/or protein conformations. This idea is supported by the work of Shibata *et al* (1991) who have shown that volatile anæsthetics disrupted the hydrogen bonds of poly(*L*- lysine) resulting in the rearrangement of α-helix backbones into β-sheet conformations. The effect was reversible when the system was purged with nitrogen gas.

Alternatively it has been suggested that anæsthetics interact with macromolecules (lipids and/or proteins) to release water bound at the interface (Ueda 1989, Shibata 91). When the macromolecular structures are freed from the hydrogen-bonded network of water molecules, they become disordered and undergo conformational changes (Shibata 1991) or expansion (Ueda 1989) into a non-functional form. Whatever the precise mechanism, while bulk water may not be not crucial for enzymic activity, tightly bound structural water plays an important role in enzymatic catalysis (Rupley *et al*. 1983, Gorman and Dordick 1991). Such disorder would lead to an increase in the fluidity of the cell membrane (Ueda 1991). Janes *et al* (1992) point out that all processes that require a defined lipid architecture are liable to disutrbance, and that it should be considered that that anæsthetics act entropically, equivalent to the generation of a mosaic of local heating and cooling, which alters the energetic balance and architecture. This may explain why cells exposed to organic solvents have a much lower tolerance to temperature increases (van Uden 1984). This theory that anæsthetics act on proteins or the protein-lipid interface also gains support from other groups (e.g. Richards *et al* 1978 and Slater *et al* 1993). Trudell (1991) argues that the increase in membrane fluidity is an indicator of an important change in the protein-lipid interactions, but in itself, is not the direct cause of anæsthesia.

Franks and Lieb (1981) make the point that if the critical volume/membrane expansion theory were applied to whole membranes, than a temperature rise of only a few degrees should also cause anæsthesia (Franks and Lieb 1981, 1985, 1994). This is

not seen in poikilothermic animals, and they therefore conclude that the expansion of whole membranes or lipid-bilayers is not relevant to the mechanism of anæsthesia. Instead they argue that specialised regions of the membranes (Franks and Lieb 1981) constitute the target sites of narcotic agents, and that these are probably particularly sensitive proteins (Franks and Lieb 1982), the simplest model being one in which a single anæsthetic molecule binds to the site and inactivates it, a suitable site being a ligand-gated (rather than a voltage-gated) ion channel (Franks and Lieb 1994).

Much evidence which lends support to the general view that proteins rather than lipids are the major site of action of narcotics has come from the study of the enzyme luciferase, which is extremely sensitive to the action of these molecules (Franks and Lieb 1985a and b, 1986, Curry *at al* 1990, Dickinson *et al* 1993). It was found that the concentrations of a diverse range of anæsthetic agents (such as halothane, methoxyflurane, chloroform, diethylether, aliphatic and aromatic alcohols, ketones and alkanes) that were needed to inhibit luciferase activity by 50% were essentially identical to those needed to induce general anæsthesia (Franks and Lieb 1985b). From the results it was suggested that anæsthetic molecules may bind within the same pocket as the substrate (luciferin), or within another hydrophobic pocket, inducing an inactive conformation of the enzyme (Franks and Lieb 1985b). This inhibition was found to be competitive in nature (Curry *at al* 1990, Dickinson *et al* 1993). Finally, the observation of stereoselectivity between enantiomers of chiral anæsthetics is probably the strongest evidence that general anæsthetics bind directly to proteins (Dickinson *et al*. 1994, Franks and Lieb 1994).

Within the literature on narcotics, much has previously been made of the phenomenon of cut-off, in which for an homologous series of agents anæsthetic potency increases with chain length up to a point, beyond which further increases in chain length give a substance which does not have an anæsthetic effect. This has some similarity to the toxic effects of organic solvents in yeast, where, for example, alcohols

show increasing potency upto C_{6-8} where activity plateaus until it is lost suddenly at around C_{11-13} (Carlson *et al* 1991, Salter and Kell, unpublished results). The "lipid school" holds that cut-off occurs simply because as the MW of the homologues increases they becoming increasingly less soluble in the membrane, cut-off occurring at a point at which the solubility required is insufficient for the membrane expansion necessary for narcosis. The theory that cut-off in anæsthetic potency is due to a corresponding cut-off in partitioning into lipid bilayers (e.g. Pringle *et al* 1981) looks increasingly unlikely, however, since the potency of *n*- alcohols levels off at about C_{11} and disappears by C_{14}. Franks and Lieb (1985a and 1986, 1994) have shown that there is no corresponding cut-off in partitioning into lipid bilayers, and use this same phenomenon to strengthen their own theory for specific proteins being the site of action. If the binding of anæsthetics takes place in a pocket or cleft of amphiphilic nature with circumscribed dimensions, there could clearly be a point at which size would prevent the larger anæsthetic molecules from binding.

Finally, Chiou *et al* (1990) have demonstrated *via* FTIR (Fourier transform infrared spectroscopy) that the ability of a 1-alkanol series to break hydrogen bonds and release bound D_2O from dipalmitoyl-L-α-phosphatidylcholine (DPPC) was linearly related to the anæsthetic properties towards brine shrimps, and agreed with the cut-off point at C_{10}, lending strength to Ueda's arguments above.

On this basis, whilst the current view of organic solvent cytotoxicity focuses on the interaction of these molecules with membrane lipids, we should recognise that the bacterial cytoplasmic membrane has a protein:lipid ratio exceeding 2:1 by weight (see Kell 1988). It is entirely plausible that we may see a shift in our view of the importance of proteins as the targets of organic solvent in intact cells, much as has occurred in the fields of narcosis and uncouplers (Kell & Westerhoff 1985) reviewed above. Notwithstanding, most methods for estimating cytotoxicity to date are based on measurements of the relatively gross disruption of the plasma membrane.

5.2 Determination of toxicity

5.2.1 Biomass and viability determination.

Over an extensive period of time many methods for the estimation of microbial biomass have been developed (see e.g. Harris and Kell 1985, Sonnleitner *et al*. 1992). These range from the traditional methods of plating, and counting chambers such as the hæmocytometer, to more recent innovations such as the "Coulter counter" and flow cytometry.

However, these methods are typically aimed at determining hygiene, sterility or suspended biomass, and very few are applicable to the monitoring of biomass *in situ* and in real time (see Clarke *et al*. 1986, Harris & Kell 1985, Kell *et al*. 1990, Kennedy *et al*. 1992, Sonnleitner *et al*. 1992, Junker *et al*. 1994). The special problems encountered in real-time measurements in fermentors, and possible methods for their solution, have been summarised by Kell *et al* (1990). Two-phase systems, especially if cells are immobilised, represent a much more complex system, and very few methods of biomass calculation can even be applied to such systems (Kennedy *et al*. 1992), let alone be carried out *in situ* and real-time. While cell growth, numbers or viability in the presence of organic solvents may be measured by optical density (e.g. Boeren *et al* 1987, Preusting *et al* 1991, Cruden *et al* 1992, Favre-Bulle *et al* 1993), dry weights (e.g. Ho *et al* 1990, Bassetti and Tramper, 1992, Cruden *et al* 1992), and plating techniques (e.g. Preusting *et al* 1991, Cruden *et al* 1992, Favre-Bulle *et al* 1993), such measurements are often ignored in favour simply of determining the rate of the desired activity or final yield (e.g. Fukui *et al* 1980, Hocknull and Lilly 1987 Steinert *et al*, 1987, Nakamura *et al* 1988, Harrop *et al* 1989, Sode *et al* 1989, Hocknull and Lilly 1990, Takeuchi *et al* 1990, Kawabata and Nakagawa 1991). This may be a satisfactory approach when assaying biotransformations, but a far from perfect approach as an aid

to understanding solvent effects on cells as a whole. However, a high viability does not automatically imply a high activity (Boeren *et al* 1987), nor *vice versa*.

It is obvious that any method with the potential to determine biomass within a two-phase reactor in real-time (especially if immobilised biomass is used) would offer a tremendous improvment over the commonly employed methods.

5.2.2 Biomass probe.

As stressed above, the question arises as to how one might conveniently assess the cytotoxicity of organic solvents to microbial cells. Although one cannot exclude that individual solvents (or substrates for biotransformations) might in some cases be cytotoxic by a specific mechanism, in general the non-specific cytotoxicity is to be expected to be due to the ability of solvents to interact with and solvate the cytoplasmic membranes of cells (e.g. Seeman 1972, Tanford, 1980). Thus an assay based on the assessment of membrane damage is indicated.

Dielectric spectroscopy is a technique which, at the appropriate frequencies, provides a signal that is relatively specific for the intactness of biological membranes or, equivalently in many cases, for the amount of biomass (Harris *et al*. 1987, Kell *et al*. 1987, 1990). This is because the so-called β-dispersion (Schwan 1957, Grant *et al.*, 1978; Pethig, 1979; Kell & Harris 1985, Pethig & Kell 1987, Davey & Kell 1995), which typically occurs at low radio-frequencies, is caused overwhelmingly by the charging of the cell membrane capacitance and may be observed as the macroscopic capacitance (at such frequencies) of cell suspensions. Destroying the integrity of cellular membranes, e.g. with detergents (Asami et al, 1977), thus leads to a decrease in capacitance which will thus reflect the ability of any such added molecule to destroy the intactness of cellular membranes and thereby to be cytotoxic (Figure 2). We and others have shown using this approach that this is indeed true for organic solvents

generally (Stoicheva *et al*. 1989, Salter & Kell 1992, Woodley & Lilly 1992, Davey *et al*. 1993). The particular strength of this approach, in contrast to staining methods described above, is that a continuous readout is obtained, and thus the kinetics of cytotoxicity may be examined in detail, for example, the effects of agitation (Figure 3) or the addition of a second solvent to a toxic two-phase system (Figure 1).

5.3 Toxicity in fermentors and reactors

As well as the more obvious products from fermentations such as ethanol, butanol, acetone etc. that are well known to be toxic (Ingram and Buttke, 1985, Lovitt *et al*, 1988), a wide variety of products such as octanoic and decanoic acids are produced at lower concentration. These compounds are also cytotoxic to *Saccharomyces cerevisiae* (Stevens and Hofmeyer 1993), and at the concentrations found in fermentors, even CO_2 has been found to be inhibitory to growth (De Mattos *et al* 1984, Dixon & Kell 1989). With increasing process temperatures some of these effects may become more severe (Brown and Oliver 1982, van Uden, 1984) and the toxicity of mixtures may be synergistic.

At concentrations of a solvent much lower than those necesssary to exert gross toxicity, marked physiological changes occur within an organism. The optimum temperature for the growth of *S. cerevisiae* is decreased by about 12ºC from about 37ºC in the absence of ethanol to about 25ºC in the presence of 6% (w/v) ethanol (van Uden, 1984). The temperature at which thermal death occurs is similarly depressed. These changes may occur wholly, partially, or not at all, depending on the alcohol tolerance of the strain, the final ethanol concentration, and the process temperature. In high-temperature processes, such as may occur in red wine fermentations, so-called "heat sticking" due to the events described above may stop the fermentation prematurely (van Uden, 1984).

Methods for the removal of these toxic fermentation products have long been sought, and many methods are now available, at least at the laboratory scale (for a review of the main methods available see Groot *et al* 1992)**.** One of the more popular methods is solvent extraction or extractive fermentations. This is a process where metabolites can be extracted by contacting the fermentation broth with a suitable organic solvent which is insoluble in water, forming a two-phase system. Products partitioning into the solvent can be recovered by distillation or back extraction.

Initially much of the work was aimed at improving the acetone-butanol-ethanol (ABE) fermentation productivities. In the search for superior organic solvents, alkanes were quickly identified as being rather nontoxic to many cell lines and therefore a possible extractive agent (Finn 1966). Playne and Smith (1983) screened the effects of thirty organic solvents to be used for ethanol and volatile fatty acid extraction produced by anaerobic, acid-producing bacteria (*Bacillus, Corynebacterium, Pseudomonas aeroginosa,* and *Lactobacillus plantarum*) and found thirteen to be nontoxic (as judged by gas evolution), including alkanes (C_6-C_{12}), phthalates, organophosphorus compounds, freon 113, aliquat 336, di-isoamyl ether and trioctylamine. Yet Roffer *et al* (1984) showed that aliquat 336 and tributylphthalate are both toxic to *Lactobacillus delbrueckii* (the latter being wholly nontoxic to yeast; Salter & Kell, *in preparation*). Clearly the toxicity of a particular solvent is highly dependent on the microbe in question, making it essential to screen organic solvents with the specific microorganisms to be used.

Most of the early screening studies have not been systematic, or have investigated only a few solvent types; nevertheless, some success has been achieved, especially in the improved production of ethanol. Minier and Goma (1982) have used 1-decanol, while Kang *et al* (1990) and Malinowski & Daugulis (1993) have used oleyl alcohol or dibutylphthalate as the extractants within reactors designed specifically for extractive fermentations. Improved productivities have also been demonstrated using

extractants within less specialised reactors to produce ethanol (Matsumara and Märkl 1984 and Barros *et al* 1987) and long chain unsaturated hydrocarbons (Frenz *et al* 1989). Interestingly, Matsumara and Märkl (1984), in investigating the protective potential of cell immobilisation against extractive solvents found that Porapack Q was an effective barrier. Ethanol production by immobilised cells remained unchanged after eight batches in medium saturated with octan-2-ol, the most toxic solvent (to free cells) studied. The use of mixed solvent systems as extractants has also been studied (Mitchell *et al* 1987 and Evans and Wang 1988), suggesting that a mixed solvent system may have more suitable extractive properties than a pure solvent, although prediction of the behaviour of the solvent mixture based on interpolation between the pure solvents is difficult (Mitchell *et al* 1987, Munson and King 1984).

A more systematic approach to solvent screening has been initiated by the use of a database containing some 1500 solvents and a computer program that uses the UNIFAC and UNIQUAC models to calculate multicomponent liquid-liquid equilibria (Kollerup and Daugulis 1985 and 1986). Subsequent experimental analysis of some 70 of these solvents has identified 19 as being appropriate for use in a continuous extractive fermentation process. A further computer program has been developed to predict the behaviour of virtually any product in any solvent/aqueous system. It has been demonstrated that a biocompatible yet poor solvent can be mixed with a toxic solvent that has better extractant properties to yield a mixture with improved solvent characteristics that is still biocompatible (Bruce and Daugulis 1991, Salter & Kell 1992).

The use of water-immiscible solvents for extraction often results in the formation of rather stable emulsions which cause problems during the process. Lennie *et al* (1990) report that these may be destabilised by the use of small-molecule surfactants such as cetyl pyridinium bromide (CPB) or sodium dodecyl sulphate (SDS).

The use of solvent extraction to recover products such as alcohols, penicillin and other antibiotics is now becoming widespread (Schügerl 1994), and it would appear that continuing progress in this technology will ensure that the study of solvent properties will continue to be of crucial importance in the downstream processing side of biochemical engineering.

5.4 Methods for devising solvent systems with reduced toxicity.

5.4.1 Methods based on Log P and similar properties

Using dielectric spectroscopy to assess cytotoxicity it was found that the majority of organic solvents tested demonstrated a "threshold effect" (Figure 4), in that there is a quite specific concentration at which the solvent became toxic. Small increases in the concentration above this threshold had a marked effect on cell viability. This effect may be explained in terms of the requirement for either a critical swelling of the membrane to produce leakiness (since solvents are known to cause an increase in membrane area) or a critical number of solvent molecules to extract sufficient phospholipid to make the membranes leaky. In this sense a threshold before the compound's toxic effects can be seen is inevitable. In addition, concentrations of organic solvent lower than that needed to have a toxic effect could have an adverse effect on the physiology/metabolic activity of the cells. As the concentration was increased the effects would become more and more pronounced, eventually leading to cell death. The dielectric spectroscopic method is unable to see these effects though, as it is really only capable of detecting the rupture of cell membranes (since there is only a weak dependence of the dielectric increment on the membrane conductance when the latter is reasonably low). Thus, while the increase in concentration of a toxic organic solvent may in reality show a gradual increase in damage incurred by the cells, dielectric spectroscopy would detect only the last stages, those of membrane destruction. This would also contribute to the appearance that the solvent is toxic over

a very narrow concentration range. Finally, such macroscopic spectroscopic methods are unable to determine the distribution of cytotoxic effects between the different cells in a suspension (Kell 1988); for assesssing this, methods, such as flow cytometry (Kell *et al*. 1991, Kaprelyants and Kell 1992), which measure individual cells are required.

The toxicity of an organic solvent will depend both on its intrinsic toxicity and on its effective concentration at its site of action. This in turn will depend on its total concentration and on its solubility in the aqueous, non-aqueous and biological phases. Thus, in two-phase systems the solubility of the solvent in the aqueous phase is largely irrelevant, and it is the solubility within the cell wall/membrane that is important. The only effect that the aqueous solubility can have is on the rate of mass transfer between the organic phase and the cells. It has been argued that if the toxicity of an aliphatic compound is dependent upon its concentration in aqueous solution, then, in accordance with Henry's and Raoult's Laws, the addition of a non-toxic compound with which it is miscible would relieve the toxic effect by decreasing the vapour pressure of the toxic solvent and hence its concentration in the aqueous phase (Gill and Ratledge, 1972). Indeed, it has been clearly demonstrated that the addition of the correct non-toxic immiscible solvent will indeed reduce or negate the effects of a toxic solvent present in the system (Gill and Ratledge, 1972, Rezessy-Szabó *et al* 1987, Bruce & Daugulis 1991, Salter and Kell, 1992). This however is not proof that it is the aqueous solubility/concentration that is important in determining an organic solvent's toxicity; rather it shows the importance of the partitioning coefficient of the toxic solvent into the membrane. The introduction of a second, immiscible solvent, if it is to confer any protection, must be of one into which the toxic solvent will partition preferentially (e.g. Figure 1).

With regard to the nature of the organic solvent employed (Bruce & Daugulis 1991), a widespread consensus has emerged, based on many studies with single solvents, that, to a reasonable approximation, biocatalyst stability with respect to log

P, the octanol-water partition coefficient of the solvent, decreases as log P increases, reaching a minimum at log P values of 0-2 for enzymes and 2-4 for micro-organisms, after which increasing log P of the solvent (or, for that matter, substrate) results in increased biocatalyst stability (e.g. Laane *et al* 1985, Halling 1987a, Hocknull and Lilly 1987, Laane *et al*. 1987b and c, Harrop *et al* 1989, Mozhaev *et al* 1989, Hocknull and Lilly 1990, Osborne *et al*. 1990, Inoue and Horikoshi, 1991, Stevenson and Storer 1991, Cruden *et al* 1992, Harrop *et al*. 1992, Osborne *et al* 1992, MacNaughtan and Daugulis 1993), i.e. that biocatalysts are more stable in less polar solvents. If true, this is arguably because (i) such solvents are so insoluble in water that they do not gain access, via the aqueous phase, to the apolar enzyme interior or the cell plasma membrane where they would be able to effect denaturation, and (ii) that the less polar solvents cannot disturb the mono- or bimolecular water layer surrounding biomolecules which is essential for their activity (Gorman & Dordick 1992a, Zaks 1992). The transition from toxic to nontoxic solvents typically occurs between log P 3 and 5, and depends on the homologous series (Vermüe *et al*, 1993). However, this picture is not without both technical and intellectual problems.

The major technical problems are (i) that many substrates of interest are not in fact particularly soluble in the very apolar solvents (e.g. Reslow *et al*. 1987, Cassells and Halling 1990), and (ii) that the rates of reaction in favoured (bio-compatible) solvents are often low (Cassells and Halling 1990). The intellectual problems stem, for instance, from the facts that (i) there are potentially hundreds of physical properties which may be expected to influence both the solvating power of different organic solvents (e.g. Carlson *et al* 1985, Hampe 1986, Prausnitz *et al*. 1986, Isaacs 1987, Kaliszan 1987, Reichardt 1990, Horvath 1992) and indeed any quantitative structure-activity relationship (e.g. Hansch & Leo 1979, Shorter 1982, Hansch & Klein 1986, Mager 1988, Devillers & Karcher 1991, Dunn 1989, Hansch 1993, Miyashita *et al*. 1993) such as their inhibitory activity towards biocatalysts, (ii) the partition coefficient of any molecule between water and an organic solvent depends on the organic solvent

chosen, and solvents such as cyclohexane may be better models than octanol for the interior part of a lipid bilayer (e.g. Rich & Harper 1990), and (iii) a more critical inspection of published data (e.g. Snijder-Lambers *et al* 1987, Zaks & Klibanov 1988, Fitzpatrick & Klibanov 1991, Schneider 1991, Valivety *et al*. 1991, Barros *et al* 1992, Bassetti and Tramper 1992, Laroute and Willemot 1992, Bonneau *et al* 1993, Narayan & Klibanov 1993, MacNaughtan and Daugulis 1993, Vermüe *et al*, 1993) shows that properties such as catalytic rate, stability or cytotoxicity are often not in fact especially well correlated (in the statistical sense) with log P alone (see also Figure 5). Whilst correlations between biocatalytic activities and single "established" criteria or indicators, such as dielectric constant, electrostatic properties (Bonneau *et al* 1983) or solubility (Laroute and Willemot 1992) have been largely discounted for a number of years (although may still remain useful for specific applications or solvent classes), log P is still widely held to have some utility in this regard (e.g. Figure 6)

We have argued previously, and shown in certain cases, that one solution of these problems lies in the use of *mixed organic solvents* as the organic phase (Salter & Kell 1992), since it is well known from work with chromatographic methods such as TLC and HPLC (e.g. Glajch *et al.* 1980, Hamoir & Massart 1993) that mixtures of solvents have a much greater range of properties, including solvating power (e.g. Yalkowsky *et al*. 1976), than do single solvents. In particular, we have shown (Salter & Kell 1992) that mixtures of a highly apolar solvent (such as hexadecane or iso-octane) with a more polar one (such as a medium-chain alcohol) which is itself poorly water-soluble can combine the desirable properties of each: the more polar, but still organic, solvent will allow the solvation of (or be used as) the substrate of interest, whilst both the substrate and the more polar organic solvent will themselves be dissolved in the (generally) more biocompatible, highly apolar solvent (see also Gill and Ratledge, 1972 and de Smet *et al*. 1983). Phillips *et al*. (1990) did find (apparently inadvertently!) that *mixtures* of alcohols provided a much better matrix for amino acid *esterification* than did single alcohols alone. As a prelude to accounting in detail for

the performance of these mixed solvents, it is therefore of interest to seek to understand exactly which biophysical properties of suitable individual solvents are responsible for biocompatibility

We therefore next draw attention to the work of Schneider (1991), who has shown that enzymatic activity in both miscible and immiscible aqueous/non-aqueous systems is much better correlated with the 3-dimensional Hansen (1967a,b, Hansen & Skaarup 1967) solubility parameters (consisting of dispersive, polar and hydrogen-bonding interactions) than with log P alone. This adds further weight to the view that optimising the organic solvent used for a particular biotransformation is best achieved by using more than one solvent, since the Hansen parameters of a mixture can be varied independently of each other.

As mentioned above, there are potentially hundreds of possible physical properties which one might seek to include in a multivariate statistical analysis of organic solvent behaviour (Prausnitz *et al*. 1986, Reichardt 1990, Horvath 1992). However, two types of reasoning lead to the general view that a much more restricted number of composite parameters may generally be appropriate, i.e. that the intrinsic dimensionality of solvent space is actually rather low (say between 3 and 10). The first point is there are a variety of individual constants for describing say the polarity of a solvent, including (in this case) permittivity, dipole moment, Coulombic and dispersive interactions; many of these will vary collinearly with each other, such that a composite "polarity" parameter might be expected to give the best *general* description of this property, since sparse or 'parsimonious' models are known always to be preferably in multivariate analyses of this type (Seasholtz & Kowalski 1993). A similar conclusion for other properties such as basicity comes from a statistical analysis of the variance of a variety of different properties between known solvents.

Thus, Snyder (1974, 1978; see also Glajch *et al*. 1980) used 3 quantitative descriptors based on "polarity" (dipolar interaction) and on the ability to donate or to accept protons, to describe the ability of different solvents to affect the chromatogaphic behaviour of 3 test solutes. It was found that all solvents studied could be classified into 8 self-consistent groups, some of which in fact overlapped. Svoboda *et al*. (1983) used factor analysis to classify 85 solvents on the basis of 35 physicochemical constants; the 4 major latent variables accounting for the bulk of the variance in the data were related to the Kirkwood function of polarity ($(\varepsilon - 1)/(2\varepsilon + 1)$, where ε is the permittivity), polarisability (based on the refractive index function $(n^2 - 1)/(n^2 + 1)$), Lewis acidity and Lewis basicity. Chastrette *et al*. (1985) chose 8 composite functions of physical properties as the inputs for a principal components analysis of solvents; their inputs were the Kirkwood function, the molecular refraction, the molecular dipole moment, Hildebrand's solubility parameter δ, the boiling point, and the energies of the highest occupied and lowest unoccupied molecular orbitals. The first three principal components accounted for most of the variance, and could be interpreted respectively largely in terms of the polarizability, the polarity and the Lewis acidity of the solvents. The solvents formed 9 clusters in the 3D space. However, as Reichardt (1990) points out, some of the clusters seemed far from natural, and one group was merely named "miscellaneous". Finally, it is worth pointing out that analyses of this type aim to use a sufficient number of solvents and solvent types that any 'specific' effects of particular solvents on particular cells are not weighted unreasonably, such that the trends that are observed are real - 'specific' effects then show up as deviations or outliers.

Perhaps the most persuasive analysis for the present purpose is that derived by Abboud, Abraham, Doherty, Kamlet, Poole, Taft and coworkers, and referred to as Linear Solvation Energy Relationships (see e.g. Kamlet & Taft 1985, Taft *et al*. 1985a,b, Kamlet *et al*. 1986a,b, 1987a,b, 1988a,b, El-Tayar *et al*. 1991, Grate & Abraham 1991, Abraham *et al*. 1990, 1991a,b, Wilson & Famini 1991, Abraham &

Whiting 1992, Famini *et al*. 1992, Poole *et al*. 1992, Abraham 1993a,b,c, Abraham *et al*. 1993, 1994, Cramer *et al*. 1993, Poole & Kollie 1993, Larrivee & Poole 1994). These workers point out that the process of solution consists essentially of 3 energy-dependent steps: a cavity must be formed in the solvent into which the solute can enter, a single solute molecule must be separated from the bulk liquid solute and deposited in that cavity, and attractive forces must exist between the solvent and the solute to keep the solute in the cavity. The first step is usually endoergic, the latter two exoergic. This leads to the view that solvation should depend fundamentally on a cavity (size) term, dipolar terms, and hydrogen bonding terms, and it is found (e.g. Abraham *et al*. 1991, 1994) that a suitable multiple linear regression equation can be used to predict solubility with high accuracy.

The terms ("descriptors" or "explanatory variables") of this multiple linear regression equation are R_2, π_2^H, α_2^H, β_2^H and V_x, which are respectively an excess molar refraction term which may be obtained from refractive index measurements (Abraham *et al*. 1990), a dipolar/polarizability term which may be obtained gas chromatographically (Abraham *et al*. 1991b, 1992, 1993a), an effective hydrogen-bond donor (acidity) term which is actually a summation of terms $\Sigma\alpha_2^H$ and which may also be obtained by GLC, an effective hydrogen-bond acceptor (basicity) summation term (again obtainable by GLC, Abraham 1993b) and the volume, specifically McGowan's characteristic volume, of the solute molecule, which may be calculated from the molecular structure (Abraham & McGowan 1987). Since solvation is but one general *type* of chemical interaction, it is clear that such an equation might also be applied to the prediction (in the sense of Quantitative Structure Activity Relationships) of any type of pharmacological effect, such as the potency of different general anaesthetics; this indeed turns out to be the case (e.g. Kamlet *et al*. 1986c, 1987b, 1988a,b, Abraham *et al*. 1991).

Whilst soft modelling chemometric approaches such as partial least squares may have slightly better predictive abilities (Sjöström & Wold 1981, Wold & Sjöström 1986, Carlson & Lundstedt 1987), the advantage of the multiple linear regression analysis (MLR) is that each of the factors whose importance we wish to establish has a precise physical meaning (Kamlet & Taft 1985, Kamlet *et al*. 1987a, Abraham *et al*. 1991), and a corollary of the applicability of MLR is that the descriptors used necessarily represent appropriate combinations of perhaps more numerous but simpler descriptors which may be available. Another substantive point about the MLR approach is that because the equation represents a straight line, it will extrapolate, once an equation has been appropriately parameterised, such that it may be interrogated with solvents which have not been incorporated into the training or calibration data and which might produce a (desirable) biological activity more potent than those seen with the compounds on which the model was formed. In other words, MLR methods can extrapolate well. Finally, one should also point out that the above 5 parameters are sol*ute* descriptors, whilst we are nominally here studying these compounds as sol*vents*; however, in this case the pertinent effect is logged via the solution of the solvents *by the membrane*. In this sense, then, it is indeed legitimate to consider the solvents from the LSER point of view.

Thus in the LSER approach we seek to relate a property of interest, e.g. solvent cytotoxicity, to the above terms by means of an equation of the following type:

$$\log \text{cytotoxicity} = c + rR_2 + s\pi_2^H + a\alpha_2^H + b\beta_2^H + vV_x \qquad \text{(Eq. 1)}$$

Since one can use these parameters to predict solubility and hence log P they are thus more "fundamental" and could therefore at least in principle explain data which both do and do not fit log P; indeed, on this basis log P *per se* is not therefore expected to correlate with toxicity if all the LSER relations hold. In fact, the single parameter V_x (Mc Gowan's characteristic volume) shows a slight improvement over

log P (although still a poor fit) in explaining cytotoxicity (cf. Figs 5 and 7). Equation 1 has been fitted to potency data of the type shown in Figs 5 and 7, but thus far only with poor results (Salter and Kell, unpublished data). Since this equation broadly assumes a unitary site of action, however, this would support the physical evidence that more than one site is involved in determining cytotoxicty.

It has been demonstrated above that it is highly unlikely that a single characteristic will be able to separate/predict toxic and nontoxic solvents. The use of two characteristics however, while still far from ideal, does show a very considerable improvement (e.g. Figure 8). It is thus probable that the further addition of only a limited number of carefully chosen characteristics will be able to improve the system yet further, a view consistent with the low intrinsic dimensionality of solvent space and offering the likelihood of a simple set of descriptors of wide applicability. However, it is worth mentioning here that whilst the data in Figure 8 show that the classes toxic/nontoxic are separable they are far from being *linearly* separable in this 2-dimensional space. Finally, we would comment that our present experience is that the analysis, modelling and visualisation of such large datasets is beyond the capabilities of many standard graphics packages popular with personal computers, and often requires the use of rather recondite or specialised systems or software, particularly for adequate visualisation.

5.4.2 Immobilisation/MATH

The introduction of a water-immiscible organic solvent phase to a planktonic (free) salt cell suspension can give rise to some undesirable phenomena, such as inactivation by the solvent, clotting of the biomass, and the aggregation of cells at the liquid-liquid interface (Brink and Tramper, 1985). The immobilisation of viable cells within hydrogels would appear to offer a convenient means of reducing/eliminating these problems (Salter & Kell 1991). It is unfortunate that with the exception of

calcium alginate (Brink and Tramper, 1985, Hocknull and Lilly, 1990, Hertzberg *et al* 1992) hydrogels have been claimed to be unstable when used with organic solvents (Smidsrød and Skjåk-Bræk, 1990), although even calcium alginate may fail with some solvent combinations (Hertzberg *et al* 1992)

However, gel entrapment did not seem to provide any additional protection against solvents in a study carried out by Brink and Tramper (1985), while Ceen *et al* (1987) reported that the protection conferred *via* calcium alginate immobilisation was only modest for polar solvents. In addition, mass-transfer has also been identified as a more general problem with entrapped-cell systems and organic solvents (Pinheiro and Cabral 1992). However in studies involving essentially single-phase systems, immobilisation within polymeric matrices would seem to confer some protection. It has been repeatedly shown that tolerance to phenol is increased for a variety of micro-organisms, such as *Pseudomonas* spp. (Bettman and Rehm 1984 & 1985, Keweloh *et al* 1990), *Escherichia coli* and *Staphylococcus aureus* (Keweloh *et al* 1990). Increased tolerance has also been shown towards 4-chlorophenol (Keweloh *et al* 1990), cresols (Bettman and Rehm 1985), acetophenone, 1-hexanol, hexane (Wu *et al.* 1991), ethanol and ethyleneglycol (Dror *et al*, 1988), but not for other solvents such as methanol, formamide and dimethyl-formamide (Dror *et al*, 1988). It is not so clear whether tolerance is due to reduced access/diffusion or the physiological state of the cells induced by non-growing conditions (Kaprelyants *et al* 1993).

Clearly the option exists to immobilise cells and use non-toxic solvents, such as hexadecane, to aid substrate/product delivery/removal, or even to supply gaseous components, such as oxygen to reduce O_2 limitation during aerobic processes (Brink and Tramper 1987a and b). For some organic solvents, polymeric matrices other than calcium alginate have been shown to be stable, at least for short periods. These include glass beads coated with gelatin cross-linked with glutaraldehyde (Wu *et al*, 1991), polyacrylamide-hydrazide ((PAAH) Bettmann and Rehm 1984 and 85, Dror *et al*

1988), polyurethane foams (Pinheiro and Cabral 1992), photo-crosslinkable resin prepolymers, urethane prepolymers (Omata *et al* 1980) and a hydrophobic silicone polymer (Kawakami *et al* 1992).

It has been suggested that organic solvents can exert toxicity towards cells *via* two types of mechanism: a), at the molecular level or b), at the phase level. Molecular toxicity represents the effects caused by organic solvents that are dissolved within the aqueous phase and include enzyme inhibition, protein denaturation and membrane modifications such as membrane expansion, structure disorders and permeability changes. Phase toxicity effects include the extraction of nutrients, disruption of the cell wall (extraction of outer cellular components), and the limited access to nutrients caused by cell attraction to interfaces, the formation of emulsions and the coating of cells (Bar 87, Hocknull and Lilly 1990). It would be expected that where toxicity is caused at the phase level, immobilisation by entrapment would confer protection to the cells. Where organic solvents exert molecular toxicity, immobilisation can have little effect other than to produce diffusion gradients across the matrices, and thus may confer some protection towards a few compounds over a relatively small concentration range. Wu *et al* (1991) suggest this toxicity may be used to prevent cell overgrowth often associated with immobilised cell reactors, thereby increasing column longevity. In theory at least, immobilised cell reactors should prove very useful in maintaining viable cells where the mechanism of toxicity is contact with the liquid-liquid interface.

It has already been mentioned that microorganisms can partition/accumulate at the liquid-liquid interface when two-phase systems are used. Rosenburg *et al* (1980) demonstrated that various bacterial strains thought to possess hydrophobic surface characteristics adhered to liquid hydrocarbons such as hexadecane, octane and xylene. This is now the basis of the MATH (microbial adhesion to hydrocarbons, formerly BATH, the bacterial adhesion to hydrocarbons) assay, a test for measuring cell-surface hydrophobicity (Rosenburg *et al* 1980, Rosenburg and Kjelleberg 1986, Sharon *et al*

1986, Rosenburg and Doyle 1990, Rosenburg 1991). This ability of microorganisms to adhere to hydrocarbons is not limited to those compounds that may be metabolised, or indeed to hydrocarbon degrading microorganisms (Rosenburg *et al* 1980).

While the MATH assay depends on samples being vortexed to promote cell adhesion, it has also been demonstrated that a number of compounds, such as ammonium sulphate (Rosenburg 1984) and cetylpyridinium chloride (Goldberg *et al* 1990) promote cell adhesion to very high levels (near 100%) for normally non-adherent cells, such as *E. coli*. Adhesion is generally strongly dependant on the water:hydrocarbon ratio employed (Sharon *et al* 1986) and the amount of surface area created (van Loosdrecht 1987). Indeed for cells using hydrocarbons as their carbon source, growth may be totally dependent upon physical contact with the hydrocarbon (Mc Lee and Davies 1972). Several bacterial strains have the ability to adhere so strongly as to form stable emulsions, especially *Micrococcus luteus* (van Loosdrecht 1987). The addition of propan-2-ol to a final concentration of 5% ((v/v) Rosenburg *et al* 1980), or the freezing and thawing of the hydrocarbon phase (Rosenburg and Kjelleburg 1986) results in breakage of the emulsion and release of cells back into the aqueous phase.

Thus there is clearly a possibility of using a two-phase systems where the cells are immobilised/adhered *directly onto the organic/hydrocarbon phase*, which in turn can carry the substrate of interest. For such a system to be successful clearly both the organic solvents/hydrocarbons used would need to be nontoxic. We have shown (Salter, Morris and Kell, unpublished work) that both an *Arthrobacter* sp. and a *Saccharomyces cerevisiae* mutant (selected by growth in a chemostat whose dilution rate based on the aqueous phase volume exceeded the growth rate of planktonic cells of the organism) were capable of adhesion to hexadecane droplets within a bubble reactor, growth continuing normally over an extended period. Finally, an *Arthrobacter* sp. has been shown to attach to a 2,2,4,4,6,8,8-heptamethylnonane-water interface and

mineralise *n*-hexadecane within the solvent phase. The larger the solvent phase present, the higher the degree of mineralisation, whilst the addition of Triton-100 prevented cell adhesion and mineralisation (Efroymson and Alexander (1991).

6. Applications/Biotransformations:

Steroids are poorly soluble in aqueous systems and as such are good candidates for use with organic solvents. When the enzyme responsible for the desired conversion has to be induced, it is essential that the micro-organisms remain viable in the presence of the organic solvent, since proteins are normally rapidly degraded within dead cells (Boeren *et al* 1987). While some work has been carried out using co-solvent systems, with the solubility of hydrocortisone increased some fivefold by the use of 5% (v/v) triethyleneglycol (Silbiger and Freeman 1991) and methanol successfully being used for the conversion of cortisol to prednisolone (Kawabata and Nakagawa 1991), the main interest has been in two-phase systems using a wide variety of solvents. Conversions carried out include Δ^1-dehydrogenation by *Arthrobacter simplex* in planktonic (Hocknull and Lilly 1987, 1990, Pinheiro and Cabral 1991) and immobilised states (Hocknull and Lilly 1990, Pinheiro and Cabral 1992), and by immobilised *Nocardia rhodocrous* (Fukui *et al* 1980), 11α–hydroxylation of progesterone by free and immobilised *Aspergillus ochraceus* (Ceen *et al* 1987), steroid side chain cleavage with immobilised mycobacteria (Steinert *et al* 1987), and deacylation, oxidation and isomerization by *Flavobacterium dehydrogenans* (Boeren *et al* 1987).

Interest in stereospecific reactions has increased greatly in recent years, and a number of examples are to be found using two-phase systems. These include asymmetric reductions by baker's yeast of α-keto esters to α-hydroxyesters in benzene (Nakamura *et al* 1991) and hexane (Nakamura *et al* 1988 and Naoshima *et al* 1992), and the production of stereospecific propene oxide by *Mycobacterium*, showing the choice of solvent to be critical (Brink and Tramper 1987b). Chiral reductions are of

particular interest in whole-cell biotransformations, since the regeneration of reductant is typically effected much more easily *in vivo* than in purely enzymatic systems.

Although oxidation/reduction reactions are amongst the most common intact cell biotransformations in aqueous systems, their use in two-phase systems is much less common. Some examples do however exist. *Pseudomonas oleovorans* is able to grow on *n*-alkanes and 1-alkenes, cells grown continuously on *n*-octane were found to accumulate the intracellular polyester poly(β-hydroxyalkanoate) (Preusting *et al* 1991). The *alk* genes from the catabolic OCT plasmid of *P. oleovorans*, encoding for the oxidation of *n*-alkanes to carboxylic acids can be introduced into *E. coli*. The resulting recombinant converts *n*-octane in a two-liquid phase medium into octanoic acid (Favre-Bulle *et al* 1991, 1992 and 1993). Nikolova and Ward (1992) have demonstrated that *Saccharomyces cerevisiae* will reduce benzaldehyde to benzyl alcohol in organic solvents, the highest rates being observed when hexane was used. Unfortunately the conversion rates observed in aqueous media were some two to three times higher than those using hexane. Using six derivatives of benzaldehyde it was later found that the catalytic activity could be related to a number of descriptors for the solvent employed, including log P, Hildebrand solubility parameter and the dielectric constant (Nikolova and Ward 1994).

While it is not uncommon to find that a biotransformation performed with the aid of organic solvents can be performed "better" within an aqueous system, the reverse is probably the more common. For example, the rate of conversion β-ionone into a mixture of products was found to be more than doubled by the use of isooctane (Sode *et al* 1988). Creuly *et al* (1992) also showed that the bioconversion of fatty acids into methyl ketones using *Penicillium roquefortii* within a tetradecane/aqueous system offered considerable advantages over doing the reaction in an aqueous system alone. Interestingly, a strain of *Rhodococcus* that *cis*-desaturated a variety of long-chain alkanes, haloalkanes and acyl fatty acids demonstrated higher productivities

when the two-phase system was changed from an oil-in-water to a water-in-oil emulsion (Tekeuchi *et al* 1990).

Other biotransformations using two-phase systems include the epoxidation of liquid alkenes such as 1-octene by *Nocardia corallina* (Kawakami *et al* 1992), and of 1,7,-octadiene by *Pseudomonas putida* (Harbron *et al* 1986), and the production of L-tryptophan by *E. coli* (Ribeiro *et al* 1987). Numerous examples using a variety of organisms and organic solvents such as the hydrolysis of 2-ethylhexyl acetate and the oxidation of 2-octanol are given by Oda and Ohta (1992). All the biotransformations are carried out at the interface between hydrophilic carriers (usually agar) and hydrophobic organic solvents with little or no free water being present.

While the vast majority of examples use liquid organic solvents to carry liquids (or solids) dissolved within, examples may also be found in which organic solvents are used to carry dissolved gases. Thus, Ho *et al* (1990) have shown that hexadecane can be used to increase oxygen availability and thus enhance penicillin fermentations; both cell growth and penicillin production were significantly increased. Brink and Tramper (1987a,b) also used hexadecane to supply both oxygen and the gaseous substrate propene to calcium alginate-immobilised cells.

7. Future prospects

Whole cells have received much less attention than have enzyme systems as catalysts for carrying out biotransformations in organic media . Even so, interest in their use has expanded greatly in recent years, indicating the potential that such an approach may hold for the future. One of the most obvious area that holds particular potential in the immediate future is extractive fermentation, while the bioremediation and removal of pollutants from wastewaters and contaminated terrestrial sites is an area of increasing environmental and political interest. The costs, while considerable,

will eventually have to be met and the lessons learnt in developing this technology are applicable in many other areas such as biotransformations using exotic or cytotoxic compounds. However, only when the rather disparate and interdisciplinary aspects of using organic solvents with viable cells systems are brought together, as we have tried to do here, will further meaningful progress be made.

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Table 1. Potential advantages of the use of organic solvents in whole-cell biotransformations

1. They can increase the concentration of poorly water-soluble substrates/products.
2. They can reduce product and/or substrate inhibition.
3. They can prevent hydrolysis of substrates/products.
4. Many organic solvents are themselves of interest as substrates.
5. There may be a reduction of mass-transfer limitations.
6. They may alter the partitioning of the substrate/product.
7. They may improve the stereoselectivity of a biotransformation.
8. They may improve the ease of product recovery.
9. Their use may allow a better integration with chemical steps/processes.

Table 1. Potential disadvantages of the use of organic solvents in whole-cell biotransformations

1. The solvent may be cytotoxic/inhibitory to the biocatalyst (and to other life forms).
2. Non-toxic compounds tend to be highly apolar and have poor solvation properties and low reaction rates.
3. Reactant complexes that are poorly soluble in both aqueous and organic-phase may precipitate out at the interface.
4. There is an increase in system complexity, which is always undesirable.
5. Costs will increase to ensure safety, both within the reactor and downstream.
6. There is necessarily a problem of waste disposal, or at least of recycling.
7. Very little real experience exists to draw upon, especially at a large scale.
8. Product recovery may be problematic, especially if surfactants are used and/or emulsions are formed.

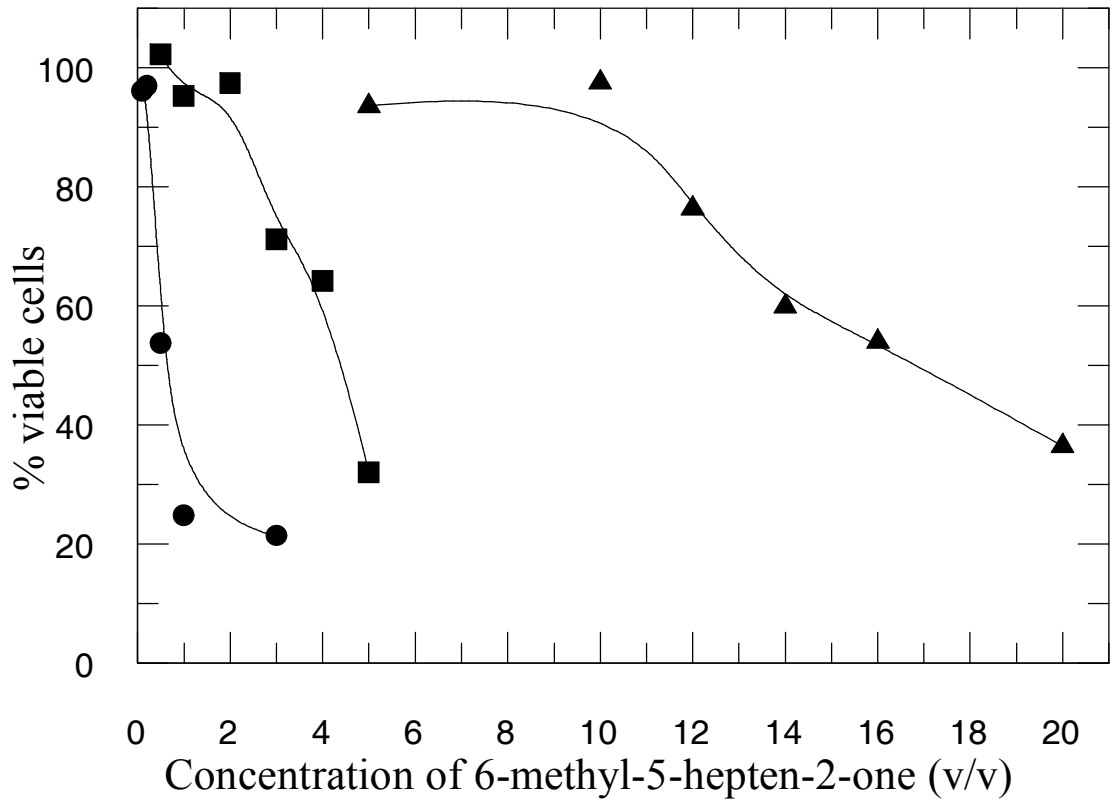

Figure 1.

The effect of a water-miscible solvent and a highly apolar solvent on the toxicity demonstrated by 6-methyl-5-hepten-2-one (sulcatone) towards *S. cerevisiae*. *S. cerevisiae* in pressed form was resuspended in a 20mM solution of KH_2PO_4 to give a cell concentration of 90 g/l (wet weight of cells) in the final 50ml sample. The cell suspension was placed in a 100ml container, fitted with a standard 4-terminal gold Biomass Monitor probe (Salter and Kell 1992) inserted laterally. Agitation was provided by a magnetic follower, the formation of a central vortex being prevented by a large centrally positioned baffle. Cell viability was monitored *via* dielectric spectroscopy at two frequencies (0.3 and 9.5 MHz) and plotted as % viable cells after 120 minutes (as compared to the differential capacitance at time zero, prior to the addition of any organic solvents). The toxicity of 6-methyl-5-hepten-2-one (squares) is considerably enhanced by the addition of ethanol at 6% ((v/v) circles), whilst the combined toxicity of 6-methyl-5-hepten-2-one and ethanol is in turn reduced by the addition of the highly apolar ester dioctylphthalate at 10% ((v/v) triangles).

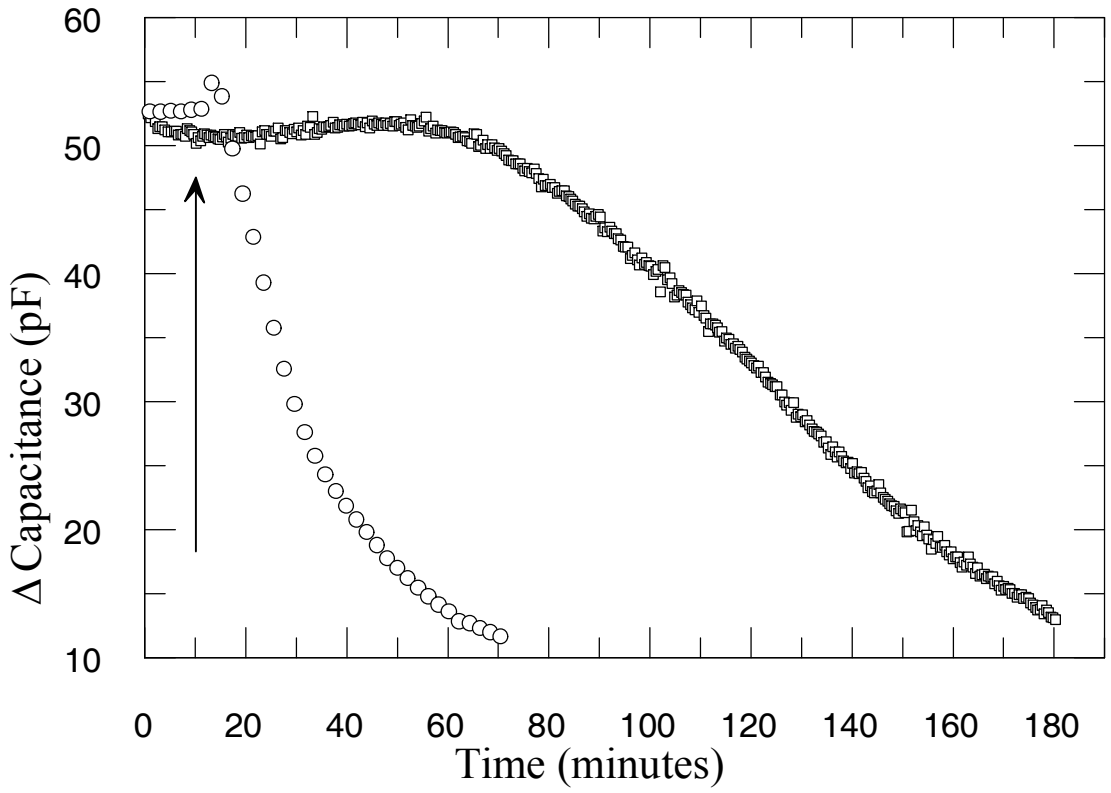

Figure 2.

Effect of sulcatone (6-methyl-5-hepten-2-one) and *n*-octanol on the capacitance of a cell suspension of *S. cerevisiae*. *S. cerevisiae* in pressed form was resuspended in a 20mM solution of KH_2PO_4 to give a cell concentration of 90 g/l (wet weight of cells) in the final 50ml sample. The cell suspension was placed in a 100ml container, fitted with a standard 4-terminal gold Biomass probe (Salter and Kell 1992) inserted laterally. Agitation was provided by a magnetic follower, the formation of a central vortex being prevented by a large centrally positioned baffle. Cell viability was monitored *via* dielectric spectroscopy at two frequency (0.3 and 9.5 MHz). After 10 minutes (at the arrow) sulcatone ((2% (v/v)) squares) or *n*-octanol ((1% (v/v)) circles) were added to the cell suspension.

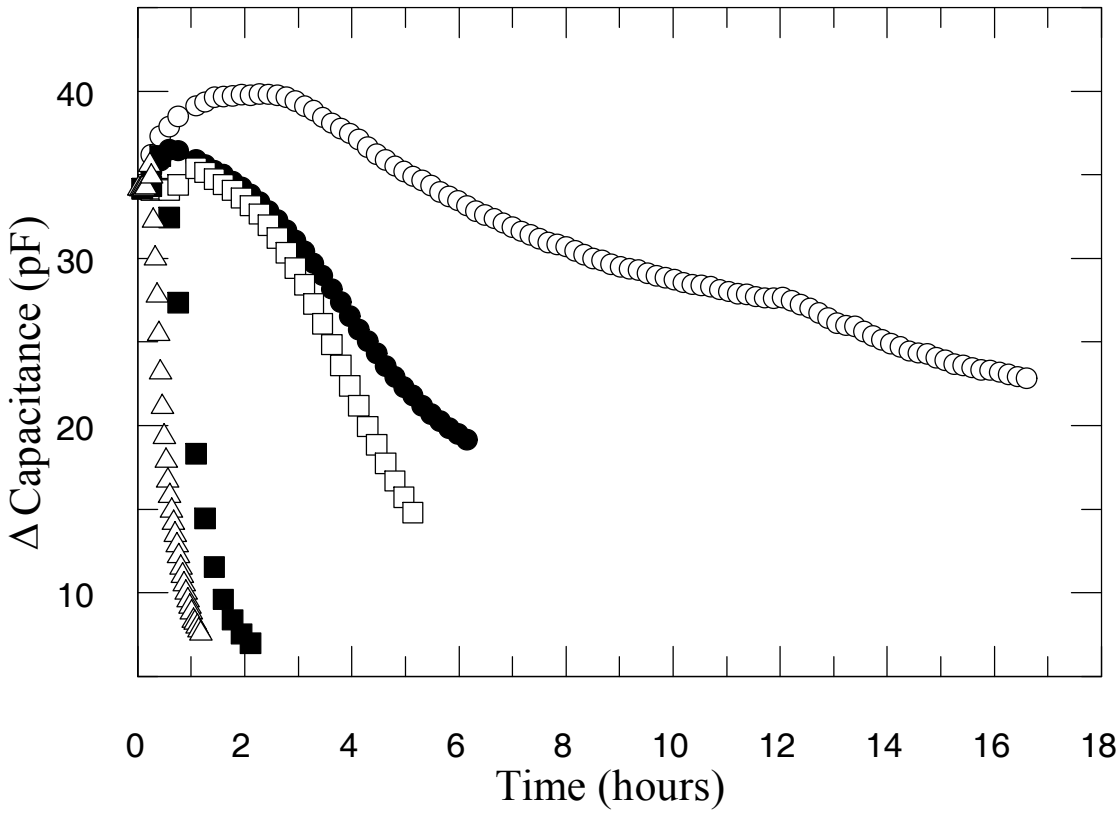

Figure 3.

Effect of agitation rate on the speed at which cell death occures when exposed to a cytotoxic organic solvent. Δ capacitance was determined as described in the legend to Figure 2. The test solvent for all runs was *n*-octanol at a concentration of 1% (v/v). Agitation rates used were 150 rpm (open circles), 350 rpm (closed circles), 450 rpm (open squares), 650 rpm (closed squares), and 750 rpm (triangles). There was no significant change in capacitance over the experimental period at the agitation rates shown if the octanol was omitted.

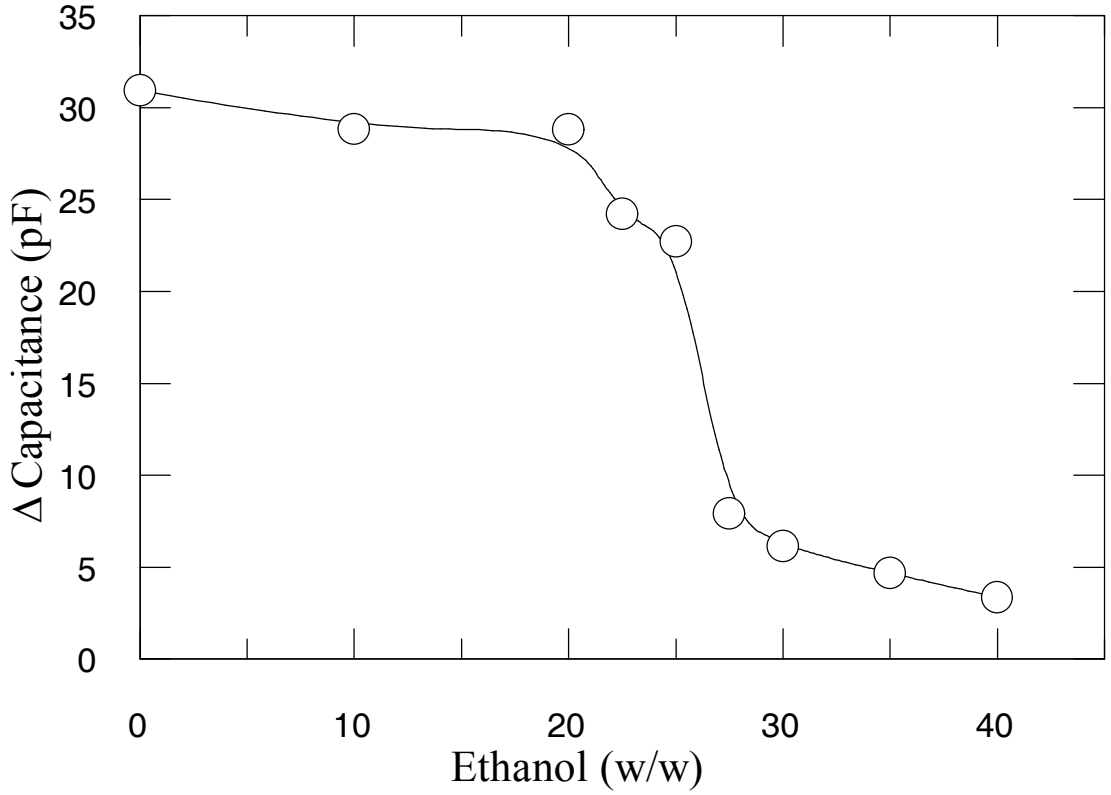

Figure 4.

The exposure of *S. cerevisiae* to different concentrations of ethanol and the effect on cell viability, demonstrating a "threshold effect". *S. cerevisiae* in pressed form was resuspended in a 20mM solution of KH_2PO_4 to give a cell concentration of 90 g/l (wet weight of cells) in the final 50ml sample. The cell suspension was placed in a 100ml container, fitted with a standard 4-terminal gold Biomass probe (Salter and Kell 1992) inserted laterally. Agitation was provided by a magnetic follower, the formation of a central vortex being prevented by a large centrally positioned baffle. Cell viability was monitored *via* dielectric spectroscopy at two frequency (0.3 and 9.5 MHz) and plotted as the Δ capacitance of the cells after 60 minutes (as compared to the differential capacitance at time zero, prior to the addition of any organic solvents). Increasing concentrations of ethanol had little effect until a threshold concentration was reached; at this point relatively small increases in the concentration had a markedly deleterious effect on the capacitance (cell viability).

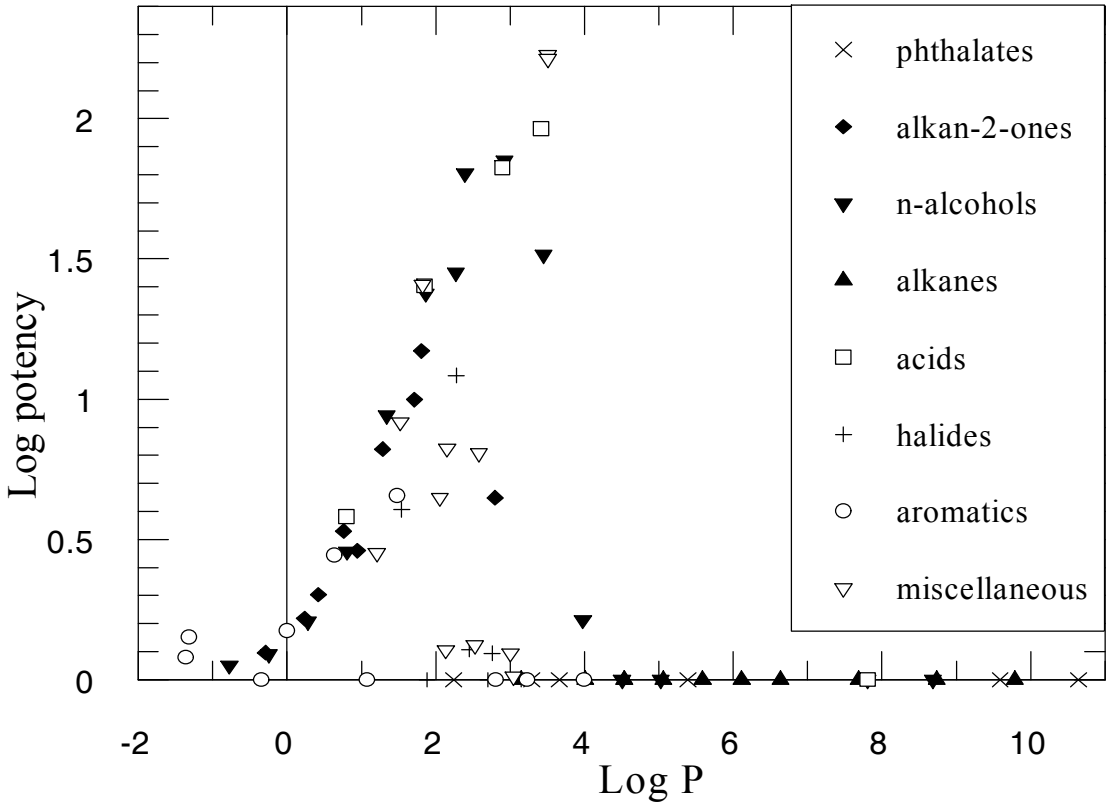

Figure 5.

Solvent toxicity (potency) towards *Saccharomyces cerevisiae* as related to log P for 75 organic solvents. All measurements were carried out using a Biomass Monitor (Salter and Kell 1992). Solvent toxicity, determined as the loss of integrity of cellular membranes, was studied using a standard Biomass Monitor electrode inserted laterally into the base of a 100 ml polypropylene container and sealed with epoxy resin. A tight-fitting lid was placed on the container, onto which a large centrally positioned baffle was positioned. Agitation was achieved by the use of a magnetic follower inserted into the container, the arrangment being placed on a stirrer. All sample volumes were 50 ml.

Cells of *S. cerevisiae* at known concentrations were suspended in 20 mM KH_2PO_4, to which the organic solvent was added. The system was emulsified by agitation at 1000 rpm in all cases. The effects of various solvents were followed over time with the Biomass Monitor, using external control, and two-frequency measurements (0.3 and 9.5 MHz). Potency was calcuated as the reciprocal of the molarity of solvent needed to

reduce the numbers of viable cells (as determined *via* dielectric spectroscopy (as in Fig. 3)) by 50% after 1 hour.

The spread of data points shows how little relation there is between the cytotoxic potency and log P amongst a large variety of solvents when considered as a whole.

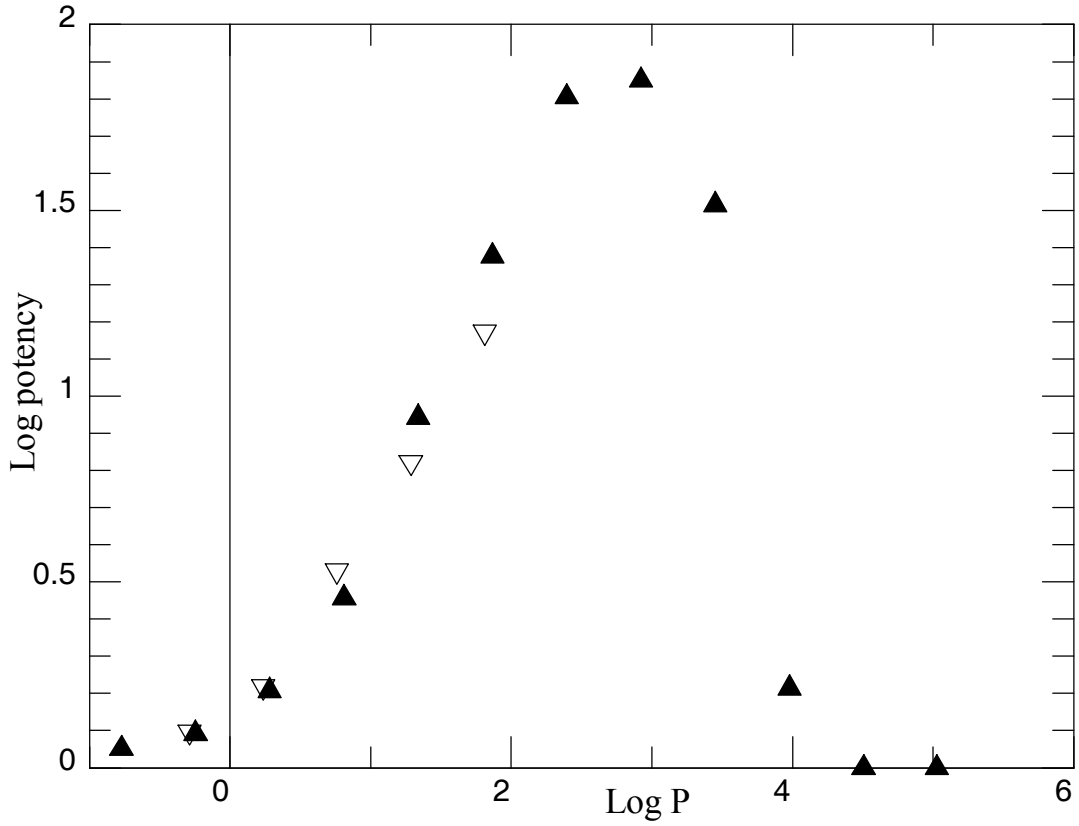

Figure 6.

Solvent toxicity (potency) towards *Saccharomyces cerevisiae* as measured by dielectric spectroscopy (see fig 4 for methods) for a series on *n*-alcohols (methanol to dodecanol (closed triangles)) and alkan-2-ones (propan-2-one to hexan-2-one (open triangles)). These data shows that for selected solvent series log P can reliably predict/explain toxicity data and thus could have some limited use for predictions.

Potency was calcuated as the reciprocal of the molarity of solvent needed to reduce the numbers of viable cells (as determined *via* dielectric spectroscopy as in Fig. 3) by 50% after 1 hour.

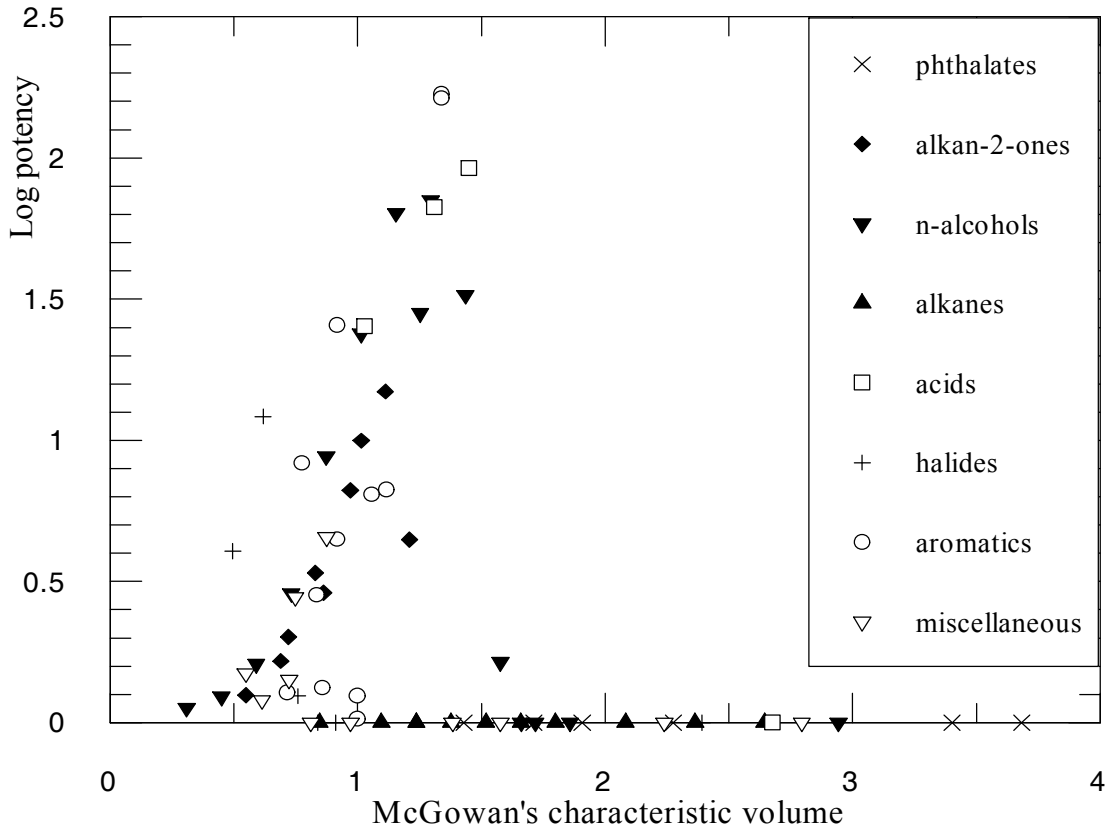

Figure 7.

Solvent toxicity (potency) towards *Saccharomyces cerevisiae* as related to McGowan's characteristic volume. All measurements were carried out as described in Figure 5.

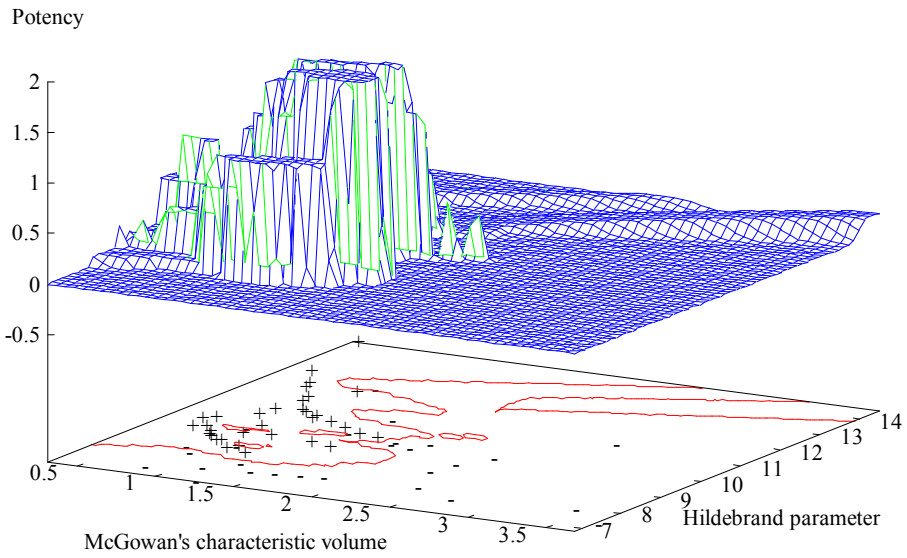

Figure 8.

A 3D chart showing the clustering of cytotoxic solvents when plotted against two characteristics, the Hildebrand parameter (Riddick *et al* 1986) and the characteristic volume of McGowan (Abraham *et al* 1994). All measurements were carried out as described in Figure 5.

The lower chart represents a contour plot of the upper chart. The contour line is set at a value (potency) of 0.05. Non-toxic organic solvents can be seen to be scattered over the base plane (-) while the toxic solvents (i.e. those having a potency value greater than 0.05 (+)) form a distinct cluster.