Novel Membrane Separations In Biotechnology.

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Moray James Campbell

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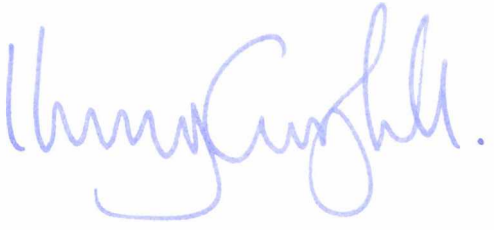
No Part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning.



Moray J. Campbell.

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Contents.

Abbreviations.

acid dissociation constant, minus log concentration	pKa
amino acid concentration	[A]
applied pressure	P
aqueous	aq.
acquired immune deficiency syndrome	AIDS
aspartic acid	asp
average	Av
boundary layer thickness	δ
bovine serum albumin	BSA
carrier transport factor	CTF
celsius, degrees	°C
centimetre	cm
channel radius (pore diameter)	r
clean water flux	CWF
coefficient	coeff.
concentration	concn.
crossflow filtration	CFF
Daltons	Da
deoxyribonucleic acid	DNA
di(-2-ethylhexyl) phosphoric acid	D2EHPA
diameter	diam
diffusion coefficient for the species	D
double distilled water (filtered)	DDWF
estimated	est.
et alia	et al
ethylene-tetrafluorehylene	ETFE
extraction equilibrium constant	K_{ext}

flux	J
fraction of total liquid flowing through pores large enough to pass solute molecules	σ
gram	g
high-pressure liquid chromatography	HPLC
hours	h
hydrogen ion, minus log concentration	pH
hyperfiltration	HF
in situ product recovery	ISPR
initial amino acid concentration	A^o
initial carrier concentration	C^o
initial chloride concentration	X^o
kilo Daltons	kDa
kilo Pascals	kPa
liquid emulsion membrane	LEM
litres	l
lysine	lys
mass transfer coefficient	k
maximum velocity	V_{max}
membrane resistance	R_m
metres	m
Michaelis constant (half maximum velocity)	K_m
microfiltration	MF
micromolar	μM
micron	μm
milligrams	mg
millilitres	ml
millimetres	mm
millimolar	mM
minutes	min.
molecular weight	mol. wt.

molar	M
nanometres	nm
nominal molecular weight cut-off	NMWCO
O-phthaladehyde	OPA
organic phase	org
per cent	%
permeate protein concentration	C_p
permeation coefficient	P
phenylalanine	phe
polysuphone	PS
recombinant deoxyribonucleic acid	rDNA
recombinant tissue plasminogen activator	rt-PA
retentate protein concentration	C_r
retention coefficient	R
"skin" thickness	x
substrate concentration in the bulk phase	C_B
solute concentration at the upstream surface	C_s
solute flux	J_s
solution	soln.
source phase	s
substrate	S
substrate concentration in the gel at the membrane surface	C_G
sum of the carrier (L) present as free carrier in the organic phase	L_{total}
supported liquid membrane	SLM
surface porosity	Σ
stirred cell filtration	SCF
tetraoctylammonium bromide	TOAB
transport path length through the membrane	l
tricaprylylammonium chloride	TCAC
ultrafiltration	UF
ultraviolet	uv
units of enzyme activity	U

viscosity of fluid	μ
volume by volume	v/v
water flux	J_w
weight by volume	w/v

Dedication.

I would like to acknowledge the great support of my parents, June and Jim, and my brothers, Ross and Stuart, who have all helped me at every stage of my career.

This thesis is dedicated to Mareile and our new born son, Cameron.

Abstract.

Membrane processes have great potential in biotechnology. This study was an attempt to investigate ways in which greater novelty could be achieved.

One of the advantages of ultrafiltration for the separation of proteins is the comparatively mild conditions used. Various parameters were considered when operating a crossflow filtration system containing polysulphone membranes. A key operational parameter in such a system is the temperature utilised for the process. There are conflicting demands when selecting an operating temperature between the fluid dynamics and the biochemical properties of the system. An analysis of this conflict, in order to establish the optimal temperature, involved a model crossflow filtration system, where shear rate, volume of protein containing solution and protein concentration can be kept constant and only the temperature varied. In such studies denaturation of the permeate was observed and an optimal operating temperature was observed between 30 and 40 °C.

A supported liquid membrane system was investigated for the carrier mediated transport of phenylalanine to more fully understand the contradictory effects, described in the literature, of chloride ion concentration in the aqueous phases on the stability of the system. Various parameters were considered to optimise transport and stability. The role of the organic phase and its interaction with carrier and support material was also considered. The carrier mediated transport was comparable to an enzyme mediated process. Kinetic studies were undertaken and the data interpreted in a manner appropriate to biological transport processes to consider the transport process at a molecular level. The system was shown to deviate from a direct 1 : 1 exchange process between phenylalanine and chloride and had a high degree of selectivity with respect to phenylalanine.

Now the whole earth had one language and few words. And as men migrated.... they said to one another.... "Come let us build a city, and a tower with its top in the heavens, and let us make a name for ourselves, lest we be scattered abroad upon the face of the whole earth."

And the Lord came down to see the city and the tower, which the sons of men had built. And the Lord said "Behold, they are one people, and they have all one language; and this is only the beginning of what they will do; and nothing that they propose to do will now be impossible for them. Come, let us go down, and there confuse their language, that they may not understand one another's speech." So the Lord scattered them abroad from over the face of all the earth, and they left off building the city.

Genesis 11:0, The Old Testament.

I found them witless and gave them the use of their wits and made them masters of their minds.... men at first had eyes but saw to no purpose; they had ears but did not hear.... So let the curling tendril of the fire from the lightening bolt be sent against me: let the air be stirred with thunderclaps, the winds in savage blasts convulsing all the world. Let earth to her foundations shake, yes to her root, before the quivering storm: let it confuse the paths of heavenly stars and the sea's waves in a wild surging torrent: this my body let Him raise up on high and dash it down into black Tartarus with rigorous compulsive eddies: death he cannot give me.

Prometheus Bound *by* Aeschylus.

Biology is as important as the sciences of lifeless matter; and biotechnology will in the long run be more important than mechanical and chemical engineering.

Julian Huxley, 1933.

1. Introduction.

1.1. Separation processes in biotechnology.

1.1.1. The importance of separation processes.

1.1.1.1. The biotechnology sector.

Biotechnology is a rapidly growing industry which has been compared to the electronics industry in the 1960's and the nuclear industry in the 1970's in terms of its potential extent and impact. However measurements of this nebulous sector, and its growth rate are not readily undertaken. This is partly because it is multidisciplinary, and also there are many disparate applications. Further the development is often masked by the need to protect intellectual propriety rights in industrial institutions.

However, various assessments of the size of the sector, and its projected growth have been made. Modern biotechnology companies, for example in the US, which specialise in recombinant protein and monoclonal antibody-based products had a revenue in 1993 of $ 8.1 billion (Ernst & Young, 1993), which represents less than 10% of the total pharmaceutical market. The traditional pharmaceutical companies, who produce drugs by both biological and chemical technologies are separate, although often intimately interacting with the biotechnology sector either through research, or marketing and sales.

By June of 1993 the Food and Drug Administration (FDA, Bethesda, MD) licensing body in the US had given approval to a total of 27 biotechnology derived therapeutics and over 600 diagnostics products. Furthermore there were 270 therapeutics in clinical trials and over 2000 in preliminary development. It is interesting to note that in 1992 the top 10 biotechnology derived therapeutics resulted in world wide sales of $ 4.5 billion and the highest selling drug, erythroprotein, had total sales of $ 1.1 billion, therefore there is an exceedingly large potential for growth. Spalding (1993) reports that spending in 109 of the worlds top biopharmaceutical companies increased by 71% from 1992 to 1993. Coupled with this, is a predicted annual growth rate in double figures. Biotechnology derived research has or is addressing many major disease including AIDS and other autoimmune disorders, allergies, asthma, cardiovascular disorders, genetic diseases, bacterial and viral infectious diseases and neurological disorders.

Against this optimism are certain constraints; the cost of development of a novel biologic is approximately $ 125 million and a novel pharmaceutical is approximately $ 230 million. Further difficulty exists in obtaining approval by government licensing bodies for therapeutic agents that are often aimed at unpredictable markets. This is aggravated by public opinion, which is often overwhelmed by the power of this technology, and reluctant to allow biotechnology to have an impact beyond novel therapeutics. In particular the agrobiotech industry faces vociferous opposition to the development and enhancement of foodstuffs.

For the bio-pharmaceutical industries one of the greatest threats involves the many health reforms that are underway in many developed economies. For instance in the US, under Hillary Clinton, and in the UK the National Health Service, drugs

bills are to be reduced, voluntarily under government suggestion, in the case of the UK, by £ 85 million over the next five years. Similar reforms exist in Germany and Italy and other European countries threaten to squeeze profit margins further. Interestingly the leaner, more adaptable and efficient biotechnology industry may survive reform better than traditional pharmaceutical industries.

The modern industrial applications of biological systems, underpinned and promoted by molecular biology, can be termed as modern biotechnology. Many of the technologies have been reserved to industrial processes where high profit margins can support financially intensive research and production techniques. However these are now becoming cheaper, for instance recombinant DNA technology that during the 1980's was reserved exclusively for the production of therapeutics, such as interferon, can now be applied to the production of products such as foods, for example "Flavr Savr" Tomatoe (Calgene Inc., Davis CA.). Thus technologies that were exclusively used in the profitable bio-pharmaceutical industries have been applied to the mass production of high volume industries such as the agricultural, food and beverage industries. The variety of approach and application is ever increasing from high value cancer drugs and diagnostic kits to artificial sweeteners and plastic substitutes. This transfer of technology and the predicted growth of non-therapeutic or agrobiotech biotechnology has been reviewed by Bains (1993). The further establishment of biotechnology, and its continued application to successful large scale production of substances depends upon increased efficiency. This development will allow high value added products, with high profit margin, to become near commodity products, thereby firmly establishing the industry.

The production of biotechnology derived products can be enhanced by the genetic manipulation of microorganisms or extracts from them (enzymes) to enable more efficient production. Alternatively process development to support production can be enhanced by techniques such as immobilisation and continuous processing, or the product is isolated with greater efficiency and at less cost.

1.1.1.2. The demands of industrial separations for biotechnology.

Many therapeutics and diagnostics are produced in fermentation processes which yield broths containing the desired product, but also an often larger concentration of other solutes and colloids. The whole broth tends to be dilute and have a low specific gravity. Product concentrations are variable depending on the extent of genetic manipulation of the producing organism, and the enhancement of fermentation processes. Therefore in process streams the product is at a concentration as high as 10 % or as low as 0.01% (w/v). The product itself is often labile, being sensitive to temperature, pH, solvents, ionic strength and physico-chemical factors. Also fermentation broths have a great tendency to foul all surfaces they come into contact with.

Isolation of labile products from a complex, interactive broth is very difficult. They are also required to be very pure, for example injectable therapeutics and food grade substances. The required purity is often beyond that of the chemical industry, which has served as a traditional source of separation processes. Often the scaling up of laboratory separations to process scale is impractical and, coupled with the high sensitivity and purity requirement, has enabled novel technologies to be developed, for example membrane processes. Present requirements within many industries are aimed

at reducing staff levels. Hence separation processes need to be self contained and relatively easily supervised.

The inherent advantages of membrane processes, such as the continuous, cheap and mild nature of the process coupled with a high degree of scalability and the applicability of operating in very dilute solutions has created a great potential for these processes in biotechnology.

Porter (1990) described the importance of separation technologies and concluded that the commercial success of recombinant DNA (rDNA) technologies is dependent on advances in bio-process engineering. This view is supported by Dale (1987) who cites more efficient production and downstream processes as being responsible for a decrease in the cost of penicillin by 300% between 1945 and 1955 and thus allowed a specialist product to be produced on a near commodity scale.

Flaschel, Wandrey & Kula (1983), pointed out that the main cost of production in many biotechnology products is the downstream processing. Bioprocess engineering is critical to improve downstream processing, for example Belfort (1989) has indicated that efficient bio-processing will reduce the costs most significantly for high value-added low-volume products such as many of the modern therapeutics and that this could most significantly be achieved by applying membrane processes. Freeman, Woodley & Lilly (1993) have reviewed *in situ* product removal (ISPR) as a method for increased product yield and illustrated the benefits of immediate product recovery from the site of production. They concluded that the role of membrane processes are integral to the development of this approach to process development.

Separation processes are not only important for product recovery, but also to remove impurities or waste products. The increase in awareness of environmental

protection and tighter legislation coupled with increased resources to alleviate the problem, for example the Super-fund in the US, has led to an increase in the cost of pollution and is securing industrial responsibility for it. Therefore many processes that were developed to solve industrial, and in particular biotechnology separation problems, can be applied to environmental problems and has resulted in the rapid development of environmental biotechnology.

1.1.2. The application of membrane systems to biotechnology.

1.1.2.1. The development of membrane systems.

In common with the development of biotechnology, membrane technology has followed a similar route from an art to a science. Filtration devices have been used for many centuries in the traditional biotechnology processes of beer, wine and cheese production. However the transition to a more scientific approach can be traced back to the Age of Enlightenment, where the roots of much of inductive science can be found. In 1748 Abbé Nollet observed the swelling of an animal bladder, containing alcohol, when immersed in water, and in doing so initiated the development of membrane science.

The first synthetic semi-permeable membrane was prepared by Fick from nitrocellulose in 1865. Following this the term ultrafiltration (UF) is found in the colloid chemical literature towards the end of the nineteenth century. However, it was limited to a laboratory curiosity, of little industrial application, as permeate flux levels

were impractically low. During the next 50 years there was further development in the preparation of membranes, plus progress in the thermodynamic theory of solutions. This culminated in manufacture of the first synthetic membrane by Sartorius in 1927. During the 1930's progress was made in the manufacture of cellulose acetate membranes which permitted their application to various industrial processes in the 1940's such as the removal of microbes from industrial streams.

After the end of the Second World War the US Department of the Interior set up an ongoing research initiative into the desalination of salt and brackish waters. The programme ran continuously from 1950 to 1973 and the membrane research involved has resulted in the USA becoming, and remaining, a world-leader in these technologies. In 1988 the world market for membranes was approximately $ 1 billion and divided between the USA (35 %), Europe (30 %), Japan (10 %) and Rest of the World (25 %), but the USA produced over 50 % of the membranes.

The principal limitation, to the successful application of membrane technology, during the 1950's, was the manufacturing process which could either produce high fluxes but poor rejection, or good rejection abilities but with very low flux rate through the membrane. Leob and Sourirajan in 1963 were the first to solve this problem through pore size restriction of cellulose acetate membranes, by heating, to generate the first real UF membranes that were genuinely anisotropic. This asymmetry allowed high tensile strength, and flux, yet retained good rejection characteristics and therefore initiated large scale application of UF membranes.

The industrial impact of these systems was exploited by Amicon, founded by Prof. A.S. Michaels in 1962, and other companies, and the first modern type UF membranes, and ancillary equipment was placed on the market during the 1960's. This

This resulted in the development of thin membranes with high flux and therefore greater efficiencies. New and novel polymers were used for production of UF membranes in an attempt to produce membranes for the large scale removal of colloidal and macromolecular impurities from secondary sewage sludge; a goal which has largely still to be realised with membranes. During the 1970's these specialist membrane preparation techniques enabled the application of UF to laboratory scale processes especially in the expanding field of molecular biology.

Research activity in the 1960's enabled the controlled manufacture of the membrane pore size and hence the three general groups of membrane were produced: hyperfiltration (HF), where all components except water are rejected by the membrane (also known as reverse osmosis), UF, where macromolecules within the range 1 - 500 kDa are rejected by the pores which are of average diameter 0.001 - 0.01 μm, and microfiltration (MF) where particles are rejected which are of greater diameter than the average pore diameter of 0.01 - 10 μm are rejected.

Of all the pressure driven membrane processes, it is UF that has the greatest number of existing, and potential, applications in biotechnology because it is applied primarily to protein isolation. This is the essence of downstream processing in modern biotechnology. Therefore in this thesis UF has been considered as a typical pressure driven membrane process and aspects of its performance have been examined in detail to illuminate the potential for development.

In its broadest definition a membrane is any phase, gas, liquid or solid separating two other phases. Further it prevents mass movement but allows restricted and/or regulated passage of one or more species through it.

With the development of coupled transport and carrier chemistry, attempts have been made to mimic the specific, carrier mediated transport of substances across biological membranes. One method to reproduce this involves copying the classical cellular membrane structure with a non-polar membrane phase separating two aqueous polar ones. A carrier molecule is contained within an organic liquid phase, which separates two aqueous phases. A species in one of the aqueous phases combines to the carrier and is transported across the liquid membrane phase and released into the other aqueous phase.

One of the first studies to postulate transport by carriers in membranes was by Pfeffer in 1890, although the majority of the initial research to construct analogues of biological cell membranes was undertaken in the 1950's. One such study, by Shean & Sollner (1966), described the use of an inverted U-tube to construct a bulk membrane as a crude approximation of a cellular membrane. About the same time, various workers began to consider the first real applications of this research, in the form of mineral recovery of uranium ions. This involved the use of polyvinyl chloride and phosphate esters cast in a paper support. Fluxes were prohibitively low and therefore commercial realisation was not possible.

After this period two other approaches were instigated, firstly the work of Li (1968) who described the preparation and use of liquid emulsion membranes (LEM). Li, and coworkers at Exxon, applied this type of separation process to pilot plant studies in 1979 for hydrometallurgical separations. The second involved the immobilisation, by capillarity and hydrophobic interactions, of the organic phase within the pores of a microporous support with aqueous phases on either side of the support. However the instability problems of this system prevented pilot plant scale

applications of the system being achieved until the 1980's. Isolation of the liquid membrane phase within a support can be prepared in several forms, for example hollow fibres and flat sheet membranes to form a supported liquid membrane (SLM).

It is within the last category that the potential for commercial development is greatest due to the ease of preparation, continuous nature and readily analyzed geometry. Therefore in this thesis certain aspects of this process have been considered in detail, for the separation of small molecular weight ions, such as amino acids.

Liquid membrane technologies are at an emergent stage of development, compared to the established processes of pressure driven membrane processes. Both pressure driven and liquid membrane processes, and other separation techniques, are compared in Figure 1.1. on the basis of the separating principle and the size of the species to be separated.

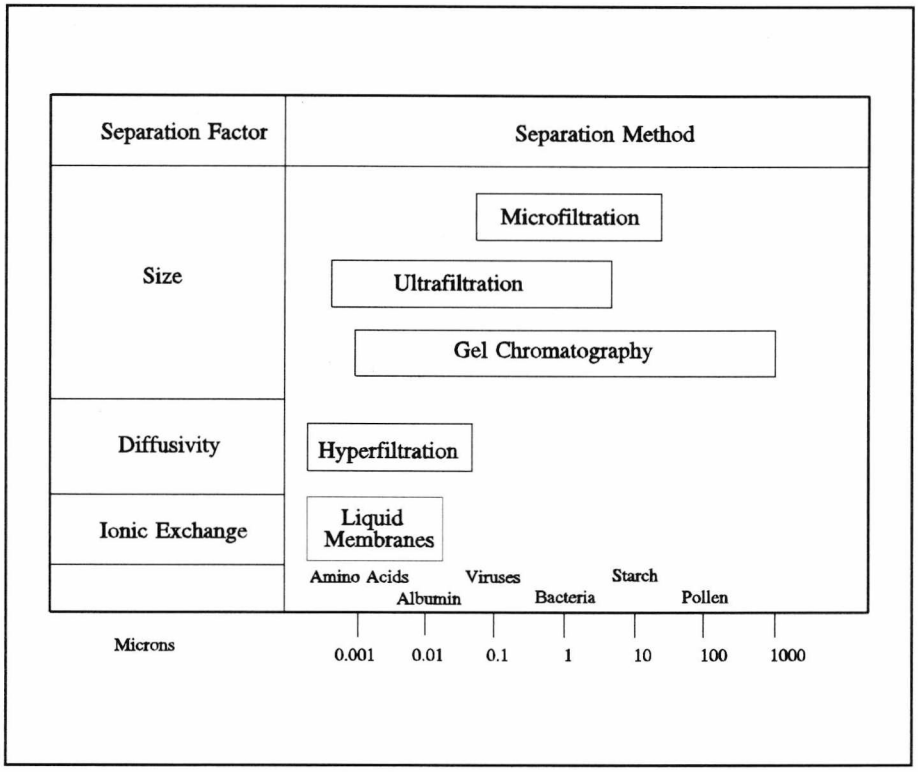


Figure 1.1. Comparison of separation technologies on the basis of size of particles and principle of separation.

1.1.2.2. The role of membrane separation processes in biotechnology.

The predicted growth for membrane separations, that was expected in the 1970's, has still not fully been realised. This unfulfilled potential was examined by Porter (1990) in an analysis of the development of membrane technology. He concluded that the rapid developments in membrane structure and application at the laboratory scale were made at the cost of large scale applications, and hence the basic biochemical engineering required did not progress to such an extent. This lack of large scale expertise has impeded the application of membranes to biotechnology.

However in the past ten years there has been renewed optimism about the resurgent growth of these processes in biotechnology. Increasingly it is ultrafiltration, because of its specific application to proteins, that is most prevalent in biotechnology industry.

The continued successful applications of UF technology on an industrial scale requires membranes to have good rigidity, stability, high flux and allow the passage of solutes in the range 0.5 to 500 kDa. They must be manufactured on a large continuous industrial scale with control over the pore size distribution and be able to withstand *in situ* sterilising.

It is especially ironic that successful applications of UF were found initially in the pharmaceutical industries only because ion-exchange, demineralisation, lyophilization and selective precipitation processes were, by comparison, more inefficient than UF, itself a relatively inefficient process. This enabled the biochemical engineering principles involved in this process to be developed and thus support applications of this technology in other, less profitable, and more efficient, industries.

The widespread existing applications of UF have been reviewed by Tutunjian (1983) who indicated large cost savings could be made from these processes. Other surveys (Strathmann, 1985) have reviewed the many existing applications in biotechnology at all stages of production. For example, reliable sterilisation of bioreactor feed streams, both liquid and gaseous, can routinely occur with pressure membrane processes. Within the fermentation chamber immobilisation of the biocatalyst may be achieved with membranes to enable continuous operation. Also on-line monitoring of bioreactor constituents via biosensors involves enzyme immobilisation, often on membranes.

Downstream, membrane processing is applied to the fermentation medium both for product recovery, and for treatment of spent broth. The choice of regime for separation is largely dependent on the molecular weight of the product. Essentially it is either a macromolecule such as a therapeutic protein or low molecular weight product such as a secondary metabolite. These small molecular weight products readily pass through UF membranes and therefore other strategies have to be developed, although these may involve membranes, for example SLM systems. In this case the carrier induces specificity to the transport process and separation and concentration can occur in a single step.

The separation of a large molecular weight intracellular rDNA therapeutic is shown in Figure 1.2.

Traditional applications of UF membranes are comparatively established, but the second generation of applications such as those for enzyme bioreactors, to undertake biotransformations, mammalian cell culture devices, immobilised cells, large scale affinity separations and biosensors are all rapidly developing.

A theoretical expansion of these applications is of membranes in enzyme bioreactors for concentrated continuous processes. For example, enzymes can be immobilised upon UF membranes and selective transport of products and substrates can occur. This can be further enhanced by the application of affinity membranes, to enable high specificity separations, and the use of liquid membranes to enable both specificity and utilisation of organic solvents as a buffer for reaction. The ultimate development of this process would involve compartmentalisation with several membranes of different permeabilities to produce an analogue of a biological cell.

restriction could be coupled with co-factor regeneration and applied to the generation of complex chiral therapeutics in one process step.

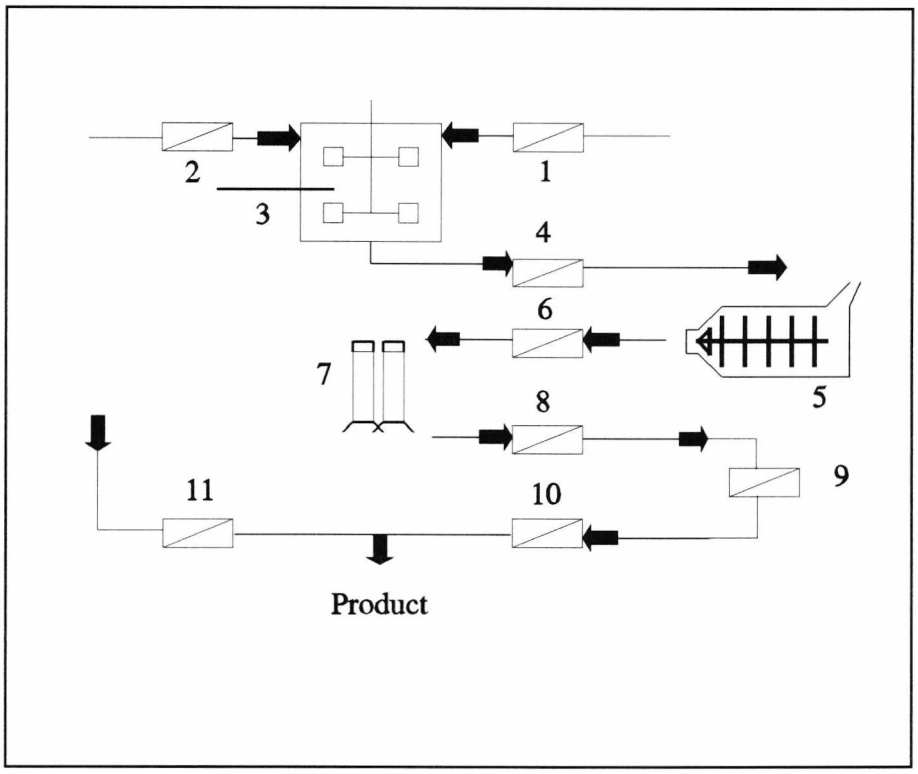


Figure 1.2. Idealised separation process for a large molecular weight intracellular protein. The fermentation media and gaseous feed streams are sterilised by membrane (1,2). In the fermentation chamber on-line monitoring occurs by membrane biosensors (3). The cells are harvested by membrane (4) before being homogenised (5). Cell debris is removed by membrane (6) before entering ionic exchange columns (7). The product is eluted and the buffer is changed by membrane (8) before passing to an affinity membrane system for final purification (9). The protein is concentrated further (10) and membrane de-pyrogenated water (11) is added to it to form the final product.

1.1.2.3. The economics of separations.

One of the main rival processes for ultrafiltration is centrifugation. UF has advantages compared to centrifugation for certain sized particles. Bjurstrom (1985) concluded that the separation costs for UF are independent of size whereas those for centrifugation increase dramatically with decreasing size. Figure 1.3. illustrates the different cost dependency patterns for the two separation processes.

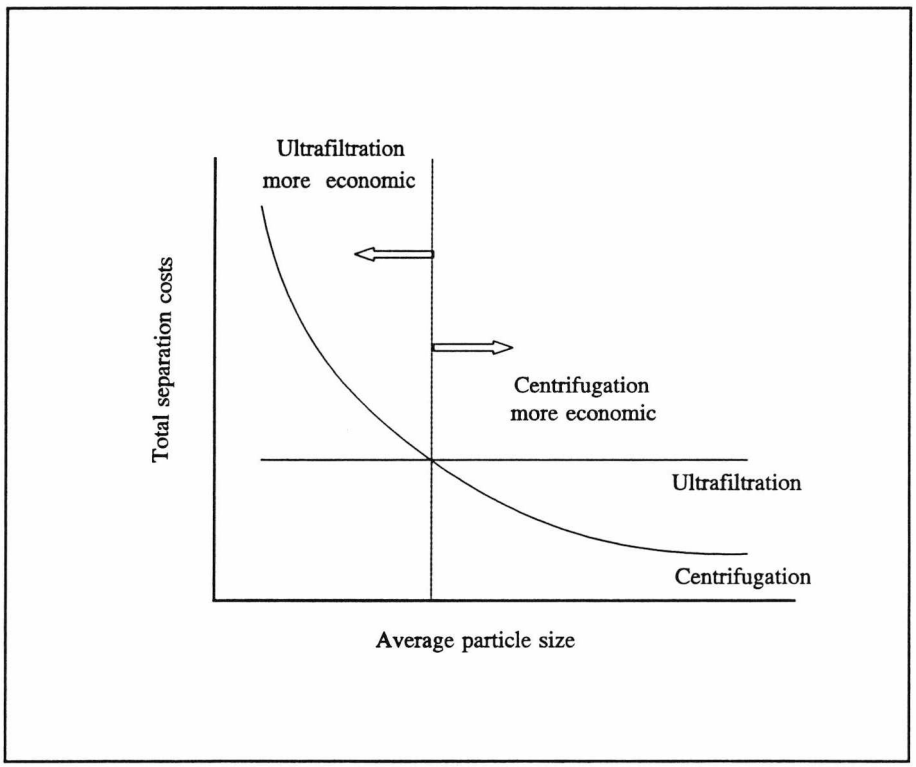


Figure 1.3. Comparison of separation costs with increasing particle size for centrifugation and ultrafiltration.

The exact cross-over point is difficult to predict and is dependent on many factors. For example, Devereux, Hoare & Dunhill (1986) have indicated the level at which centrifugation becomes uneconomical with soya protein precipitates is due to long run times at high speed and approximates to particles of about 0.5 μm diameter

and less. Tutunjan (1983) also described the cross over point at which UF becomes more economical; small scale UF for most size particles and large scale UF for small particles. Further, he cites a personal communication from D'Agostino (University Micro Reference Laboratory, Ann Arbor, MI, USA) for a comparison between UF and centrifugation for the industrial concentration of cells and showed that UF generated a one third cost saving.

UF also provides an economical alternative especially when labile components, such as products of mammalian cell culture, are to be separated as the high shear forces associated with centrifugation cannot be tolerated. Centrifuges often have associated noise and vibration problems, complex cleaning and sterilising regimes and aerosol generation presents a biohazard, especially significant when products are being derived from genetically modified organisms.

Eyal & Bressler (1993) have considered the process economics of liquid membrane technologies. The initial applications in biotechnology have been on compounds that are of small molecular weight and tend to be bulk commodities, for example organic and amino acids. These molecules have a market value of approximately $2000 per ton, therefore the separation cost, to be economical, needs to be approximatley $2-3 hundred per ton but can be up to 50 % of production costs.

The fermentations to produce these compounds often produces very similar by-products, for example isocitric acid is a by-product of citric acid production. Therefore purification often requires seven or eight separation stages to purify adequately. However these products cannot support very expensive separations, but can support novel medium priced technologies. One such technology which involves ambient temperatures, no phase changes and requires only small amounts of the active carrier

reagent is SLM systems and therefore these systems have received much research attention.

1.1.2.4. Definitions of membrane processes.

Ultrafiltration and supported liquid membranes represent two examples of the application of membrane systems, and can be classified on more than one basis. Firstly, on the size of the species separated and secondly, on the mode of separation. Separations in biotechnology can be considered on two arbitrary levels, either for macromolecules where the molecular weight is greater than 10 kDa, for example therapeutics, cells, cell components and sewage particulates or small molecular weight molecules with a molecular weight of less than 1 kDa for example antibiotics, amino acids, vitamins and other secondary metabolites. Both are highly lucrative markets and the distinction in size is a real division for separations. As such UF is a technology for large molecules and SLM is for small molecular weight molecules.

Secondly they can be considered on the basis of the nature of the separation process. For example, the transport across either type of membrane can be described as either saturating, or non-saturating. Transport in a SLM system, via the carrier, is saturating and will reach a maximum value. However a pressure driven process has no carrier and therefore transport will not be saturating. However the observed phenomena does not support this; the transport rate in UF is not proportional to substrate concentration because of concentration polarization of the membrane (see Section 1.2.2.3.) and hence this distinction is misleading.

Instead a broader definition can be used to decide whether the process is facilitated or passive. Separation across a UF membrane is permeation, driven by a

pressure gradient, and does not have a specific component to facilitate the process. However SLM systems, by virtue of the carrier, have a much greater specificity and, by virtue of the counter ion exchange, can be operated against the substrate concentration gradient. It is within this broad definition that SLM systems are considered as a facilitated process and UF systems a passive process.

These definitions are not exclusive and certain processes will fall between the two. For example Molinari et al (1990) used a UF membrane coupled to an affinity process and achieved a facilitated separation on a pressure driven process.

1.2. Passive membrane separation processes.

1.2.1. Ultrafiltration as a passive membrane process for the separation of macromolecules.

1.2.1.1. Structure of ultrafiltration membranes.

UF membranes are a composite with a thin skin, of approximately 1 μm thickness, which is the selective barrier. This is supported by a much thicker more open structure, of approximately 125μm thickness, which allows high hydraulic permeability and gives mechanical strength.

The manufacture of membranes is via patented industrial processes and therefore not in the public domain. However Lloyd & Meluch (1985), in a survey of the industry, indicated that at least 80 polymers were used and the choice often depended on availability, in-house expertise, production capability and industrial legislation.

Several categories of polymer used in membrane manufacture can be identified. The first group are those derived from cellulose acetate. Cellulose gives a high degree of tensile strength, and therefore high flux rates, but is also susceptible to extremes of pH and organic solvents and, being derived from a natural polymer, it is biodegradable.

Polysulphone membranes were developed to overcome the instability problems of cellulose acetate. This is achieved by a backbone of phenyl rings which give a high degree of steric hindrance, and electronic resonance, to enable a strong and stable membrane which can operate at temperatures up to 75°C and between pH 1 - 13. The control of the manufacturing process enables a wide pore size distribution to allow passage of molecules between 1 and 500 kDa. However the flux rates are traditionally not as high as those with cellulose acetate and hence pressure resistance is a significant drawback. This has led to the application of other polymers (polyvinyl chloride, polyacrlonitrile and polycarbonate) coupled with novel side chain modifications.

Finally, as an alternative to the above polymer based designs, ceramic based membranes have been developed. Formation of these involves the deposition of inorganic solutes onto microporous supports. These membranes are highly resistant to environmental factors but to date their manufacture has been prohibitively costly.

1.2.1.2. Flow regimes for ultrafiltration membranes.

UF membrane systems are all pressure driven but vary in their level of sophistication and the method used to remove concentration polarization at the membrane surface. The first devices were operated in a dead end mode with no attempt made to reduce concentration polarisation. Such a device quickly becomes fouled and inoperative, and is therefore limited to laboratory applications. Attempts have been made to overcome this by having a mechanism of agitation at the membrane surface. This led to the development of the stirred cell filtration (SCF) unit

where a magnetic stirrer operates above the membrane surface, however filtration is still dead end.

The restrictions of this system, due to its batch operation and fouling problems, has also limited this application to the laboratory scale. To overcome these severe restrictions, crossflow filtration (CFF) systems have been developed. A comparison between dead end and cross flow filtration are shown in Figure 1.4.

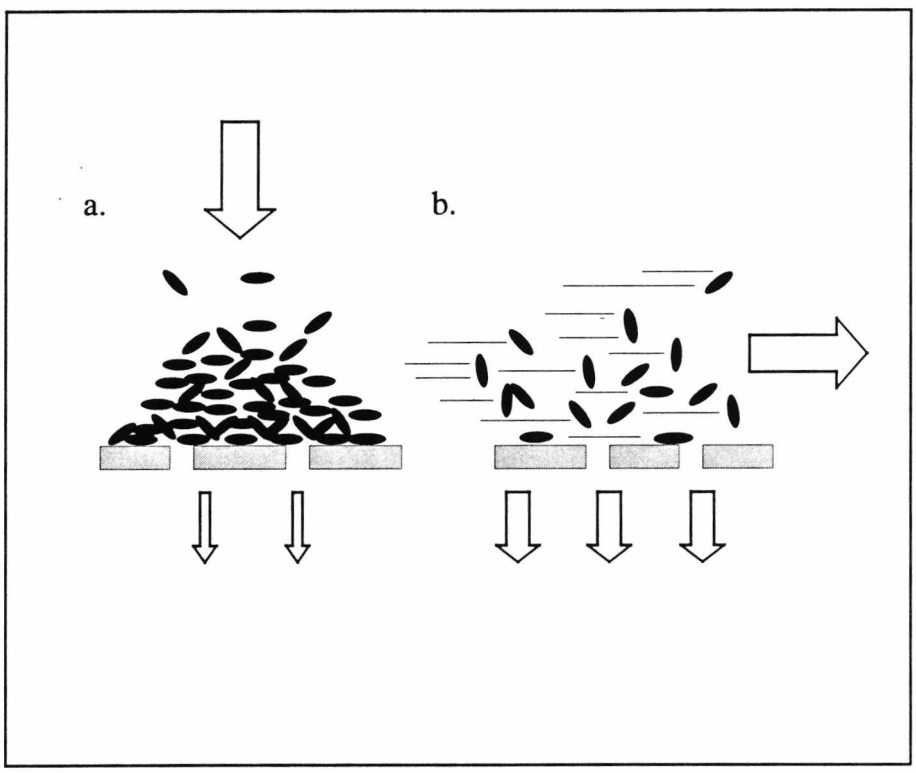


Figure 1.4. Comparison of dead end and crossflow filtration modes. a) Dead end filtration quickly results in concentration polarization and therefore poor flux results. b) Crossflow filtration reduces the tendency for concentration polarization and therefore flux is greater.

Many membrane designs have addressed the issue of concentration polarization and its effects on selectivity and flux. The designs involve membranes formed into tubes, hollow fibres, and pleated and flat sheets. These various cross flow filtration arrangements have been reviewed extensively by Krohner et al (1984), Brown & Kavangh (1987) and Murkes & Carlsson (1988). Attempts to reduce concentration polarization through design are essential, but can have deleterious effects. For instance, rapid flow thin channels promote higher mass transfer and flux but result in a greater tendency to foul and increases turbidity and stress effects. Therefore novel designs must consider other factors, including ease of cleaning, operation, manufacture and suitability for processing target molecules.

The choice of a pump to drive the membrane process is also highly significant for efficient operation. The pump must allow for viscosity changes, increase in back pressure, and be able to withstand sterilising regimes. For the production of therapeutics and food grade materials all surfaces that are in contact with the liquid should be 316 stainless steel and sterile.

1.2.1.3. Modes of operation for a crossflow filtration system.

Various modes of separation can be achieved, with the same cross flow filtration unit, depending whether the required product is able to permeate the membrane. This flexibility is highly advantages. The same unit can be operated in each of three ways as shown in Figure 1.5.

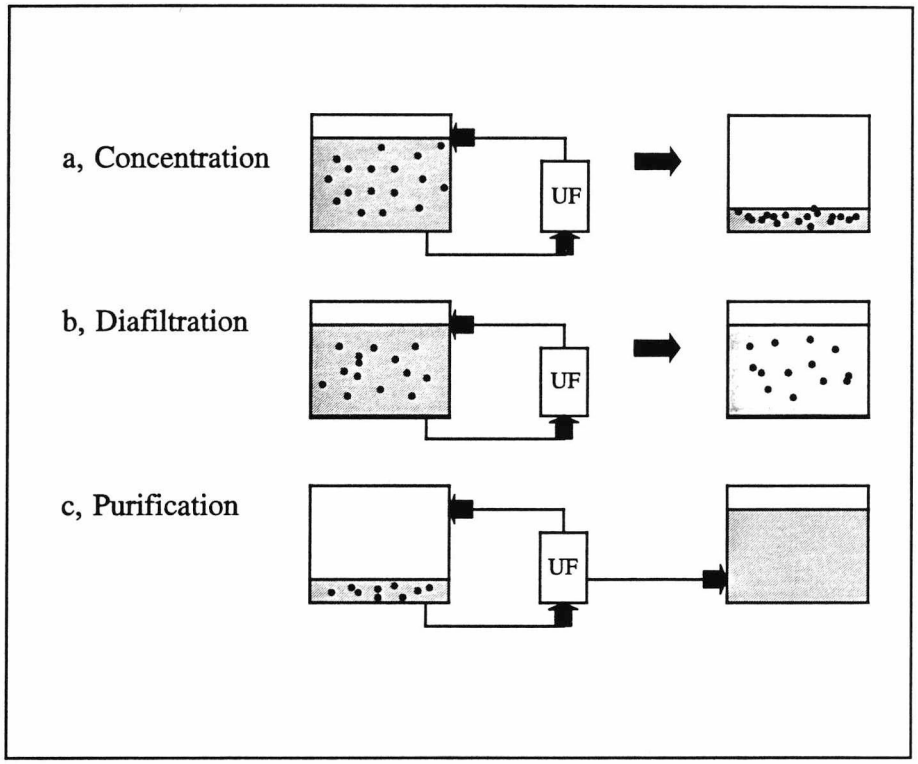


Figure 1.5. Modes of operation of a crossflow filtration unit.
a) Concentration - the product is not able to permeate the membrane and is therefore retained above it; the buffer is removed and hence product concentration is increased.
b) Diafiltration - the product is retained and an alternative buffer is replaced into the unit as the original buffer is filtered out, and therefore constant product concentration is maintained but purified of contaminants.
c) Purification - the product, and other species of the similar or smaller molecular weight, permeate the membrane thus purifying the product of larger molecular weight impurities and debris.

1.2.2. Theoretical description of transport across ultrafiltration membranes.

1.2.2.1. Aims of modelling of ultrafiltration.

The very large body of work reported in the literature, aimed at modelling UF processes, is indicative of the complex phenomena involved. The greatest need is to explain the fouling problems that result in lowered flux, decreased fractionational ability and reduced permeability. The difficulty in explaining this system is intimately linked with the properties of proteins.

Classical filtration theory predicts a S-shaped sieving curve for solute rejection, as a function of the solute molecular diameter and the membrane mean pore size. Data obtained with nonionic water-soluble polydisperce linear polymers, for example dextrans, supports this. These findings lead to the predicted ability of UF membranes to fractionate mixtures of proteins. UF systems have not yet satisfactorily met these requirements, when processing proteins, and this represents a serious impediment to its wide-scale application.

Proteins are highly reactive molecules that undertake many interactions in membrane systems. These interactions include inter-protein, protein-water, protein-membrane and protein-protein associated with the membrane. Therefore models must consider the properties of protein, and other solutes, the membrane structure and the operating variables.

Modelling of all these interactions is extremely demanding and therefore often empirical evaluations are necessary with only qualitative theoretical guidance. The inherent complexity of UF systems explains the wide variety of data observed in these systems.

1.2.2.2. Pressure controlled transport.

An examination of why flux loss occurs during UF, by Michaels (1980), combined classical fluid mechanics and filtration theory. Most applications are very dilute solutions, and therefore at the simplest level transport across the membrane can be considered as the flux of water. This is described as a modified Kozeny-Carmen equation;

$$J = \frac{P}{R_m} \qquad \textbf{(1.1.)}$$

where J = permeate flux, P = applied pressure and R_m = membrane resistance.

Another model for the basic flux of permeate species observed across the membrane is the Pore Flow model;

$$J_s = \sigma J_w C_s \qquad \textbf{(1.2.)}$$

where J_s = solute flux, σ = fraction of total liquid flowing through pores large enough to pass solute molecules, J_w = water flux and C_s = solute concentration at the upstream surface.

Qualitative interpretations of equations **1.1.** and **1.2.** reveal that the permeate flux will increase with the applied pressure, solute concentration and increasing porosity.

The Hagen-Poiseuille Law is a more complex model for the description of solvent through ideal cylindrical pores in a membrane. It relates flux to pressure change, viscosity, density of the fluid and the channel dimensions of the module;

$$J = \frac{\Sigma r^2 P}{8 \mu x} \qquad \textbf{(1.3.)}$$

where J = Permeate flux, Σ = surface porosity, r = channel radius (pore diameter), P = applied pressure, μ = viscosity of fluid and x = "skin" thickness.

This equation describes an ideal situation with a uniform distribution of evenly sized pores, where negligible concentration polarisation occurs and the membrane is assumed to be homogenously permeable. The fluid is Newtonian in nature and has a constant density, flowing in a laminar manner.

From **1.3.** it can be seen that higher pressure, wider pores, increased porosity coupled with lower solution viscosity and thinner membranes will all increase flux. Flux is directly proportional to applied pressure and inversely proportional to viscosity. Any increase in temperature or pressure will lead to an increase in flux.

1.2.2.3. Pressure independent flux.

However these predictions are not observed for all operating conditions of pressure driven systems. The typical flux pattern for changes in pressure are shown in Figure 1.6. During ultrafiltration large molecules, such as proteins, form a boundary layer when the rate of back diffusion is relatively slow compared to the force driving them towards the membrane. This leads to a concentration profile at the membrane surface.

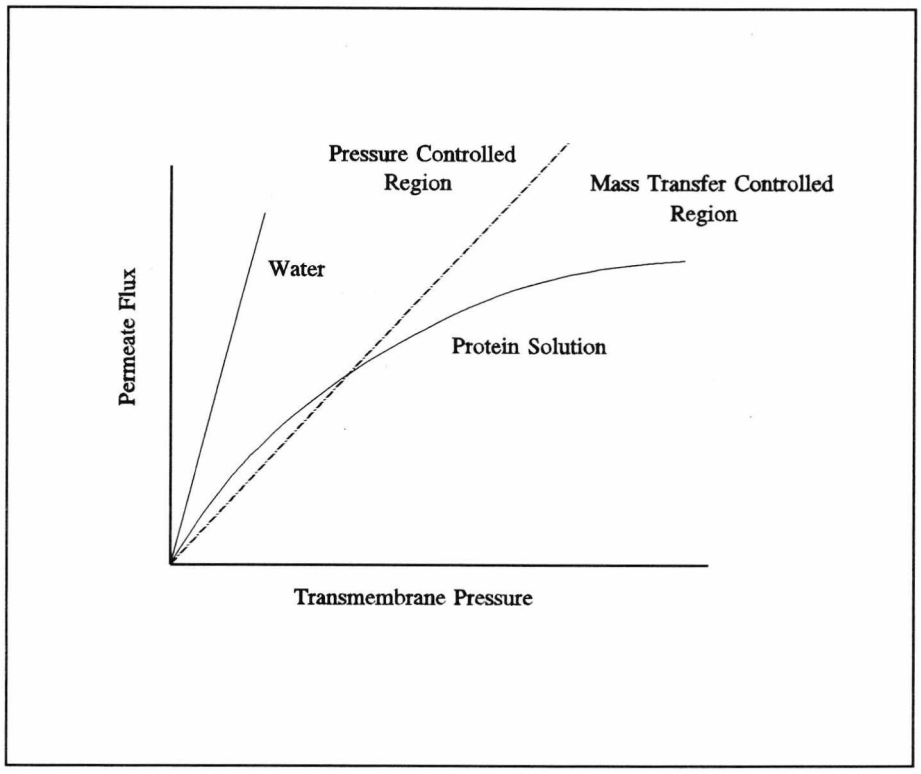


Figure 1.6. Typical data for the effect of increased pressure on permeate flux during ultrafiltration. Transmembrane pressure increases the permeate flux of a protein solution to a point and then levels off.

This accumulation of rejected species constitutes a secondary membrane and is highly influential on the behaviour of the system. Such dynamic membranes have been reviewed by Tanny (1978). Interestingly, in certain circumstances, the dynamic membrane can be used to control filtration in a positive way by being designed into the system.

Above a certain limit the system operates independently of applied pressure. This is ironic as the system is a pressure driven one but behaves as if independent of

pressure. Under these conditions, the models expressed in equations **1.1.**, **1.2.** and **1.3.** all cease to be accurate representations of observed behaviour.

Recently developed anisotropic UF membranes permit higher flux, and operating pressures, and are more resistant to internal clogging. However this underlines the advances that have been made in membrane technology at the expense of modelling and unit design. The new generation of membranes increase the effects of concentration polarization and in turn the effect this has upon solute flux and fractionation.

The exact point at which concentration polarization occurs is difficult to model and hence predict the rate of flux. The modelling of the boundary layer build up is essential to enable the design of more efficient systems. Many workers have modelled these processes, one of the earliest, and most comprehensive attempts to do this was by Blatt et al (1970) and more recently Merin & Cheryan (1980) and Baker et al (1985).

The accumulation of rejected species at the membrane surface forms a boundary layer. The concentration at the wall, within this boundary layer, increases with time and may achieve its maximal value, where it forms a gel. The gel concentration (C_G) is the maximum concentration at the wall, the build up of which is shown in Figure 1.7.

One approach to model this describes the balance of forces to and away from the membrane surface, as represented by the following equation;

$$J = k \ln (C_G / C_B) \qquad \textbf{(1.4.)}$$

where J = permeate flux, k = mass transfer coefficient (= D/δ = diffusivity / boundary layer thickness), C_G = the substrate concentration in the gel at the membrane surface and C_B = the substrate concentration in the bulk phase.

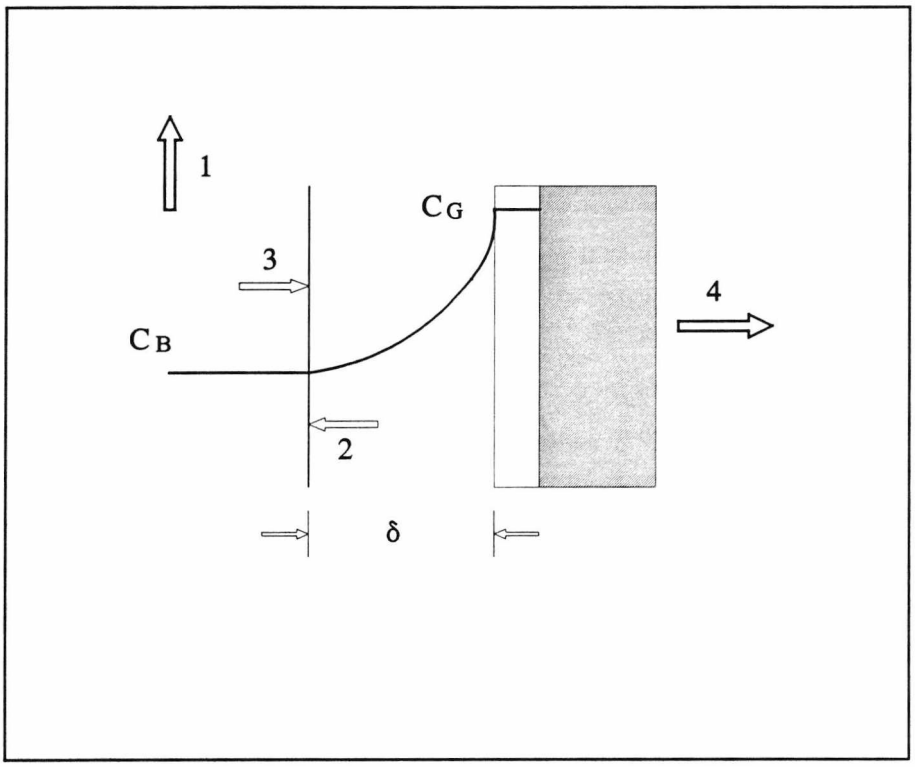


Figure 1.7. Concentration polarization at the membrane surface. Flow across the membrane (1) enhances the diffusion of rejected species away from the membrane surface (2) and into the bulk. Pressure drives solute to the wall (3). A balance between these forces results in a concentration profile between the concentration in the bulk (C_B), across the boundary layer (δ), to the membrane surface gel concentration (C_G). This balance represents the driving force that results in the permeate flux (4).

1.2.2.4. Interpretation and criticisms of the concentration polarization model.

A qualitative interpretation of the relationship given in equation **1.4.** reveals that as the bulk concentration (C_B) increases to the gel concentration (C_G), the permeate flux (J) decreases to zero. That is, as the concentration gradient reduces, with increasing bulk concentration, there will be a corresponding decrease in back diffusion and reduction in flux. However it is not experimentally possible for C_B ever to attain C_G. Another interpretation indicates that if the boundary layer thickness (δ) is reduced, the mass transfer coefficient (k) will increase and in turn increase the permeate flux (J).

Significantly expression **1.4.** has no pressure term and therefore is applicable when the system is operating in a pressure independent manner. Increases in pressure alone will result in an increase in the gel concentration, and in turn a greater rate of diffusion from the gel to the bulk, but no increase in flux. The relationship C_G / C_B is determined by the physico-chemical properties of the system and is therefore fixed. To enhance flux, the mass transfer coefficient (k) must be increased, for example by reducing the boundary layer using crossflow filtration or higher stirrer speeds. Augmented cross flow effects will reduce polarization, for example centrifugal forces (Robertson, Olieman & Farkas, 1981) perpendicular to membrane and opposite to flux. Also electric fields cause charged species to migrate away from the membrane (Robinson et al, 1993). Another approach is to use pulsatile transmembrane pressure in a positive (Bauser et al 1986) and a negative direction (Rodgers & Sparks 1991). Diffusivity can be increased, and therefore the mass transfer coefficient (k), by using a turbulent flow regime. Porter (1981) has reviewed the fluid management of

filtration systems to reduce concentration polarization and enhance transport processes.

Mass transfer coefficients (k), which are analogous to mass transfer-heat processes in chemical engineering, are pivotal in any modelling of UF processes. Under laminar flow conditions, k increases by increasing shear rate at membrane surface and flux increases as the cube root of the wall shear rate per unit channel length. Under a turbulent flow regime flux increases with increasing channel flow rate but is independent of channel length as the profile is fully developed upon entrance.

These relationships, when combined with diffusivity data for the retained species, have contributed to the successful prediction of flux in some real applications.

Porter (1990) have found that experimentally determined values of C_G are reasonable and occur at concentrations of approximately 25% to 35 % (w/v). Furthermore Porter (1990) reports better correlation between theory and experimental results for macromelecular solutions than colloidal ones, for example skimmed milk. This is probably because higher rates of back diffusion due to the tubular pinch effect are possible with colloidal solutions. This effect describes the tendency for particles, flowing down a tube, to migrate across the velocity gradient toward the region of maximum velocity. The possibility exists for the use of colloidal particles to induce a turbulence pinch effect and thus increase flux. The significnce of this effect in other sytems is not known.

Discrepancies exist between the predicted and observed behaviour of many UF systems and therefore the full application of theory is limited. The model given in **1.4.** assumes that fluxes are constant for the same hydrodynamic conditions and gel concentration independent of membrane type, permeability and flow regime. Blatt et al

(1970) have not found this to be the case and that C_G differs with differing membranes. The fact that, after the establishment of gel polarization, constant flux irrespective of the membrane type is not observed highlights one of the major deficiencies of this model.

Furthermore, concentration polarization and the critical C_G concentration has been demonstrated to be dependent on the viscosity, pH, ionic strength, turbulence, density and other physicochemical factors, often specific to the macromolecule under consideration, and not considered in model **1.4.**. For instance, Aimar (1988) studied pseudoplastic fluids (many biological solutions are pseudoplastic) and has shown that lower viscosity exists at the membrane wall because shear rate is greater there. This in turn allows higher mass transfer coefficients.

In general, the gel polarization theory is successful in that it predicts the effect of changing bulk concentration, the advantage of reducing the boundary layer by stirring, crossflow filtration and other techniques, and the presence of a pressure independent flux plateau. However there are several criticisms of it and it appears it doesn't completely explain membrane surface phenomena for all applications.

The accumulation of species neglects axial concentration variations, assumes the process is at a steady state, and that the osmotic potential of the boundary layer is negligible because of the high molecular weight of the rejected species. Finally, all these relationships require total flux to be small compared to the retentate flow through channel. The relationship breaks down with systems that have larger permeate flux values.

It is experimentally difficult to observe the resumption of pressure dependent operation because it is not clear how long it takes to restore. Also it is often not clear if pressure irreversibly affects the membrane structure.

1.2.2.5. Alternative transport models.

The sum of the individual resistances of concentration polarization induced gel layer (if present), surface fouling, pore occlusion, and the resistance of the membrane have been combined to form a model. This has been described by several workers including Grund, Robinson, & Glick (1992).

In reverse osmosis the effect of rejected salts on hydraulic pressure is very significant. The extent to which the osmotic pressure of the gel layer reduces the driving force during UF is not clear, although it has received significant attention (Van Den Berg, Hanemaaijer & Smoulders, 1987 and Ko & Pellegrino, 1992). Wales (1981) has concluded that osmotic pressure forces are very significant and that UF can be viewed as a special case of reverse osmosis. Osmotic pressure has been demonstrated to significantly reduce applied pressure at concentrations below those required to generate gel formation. Hence C_G becomes the membrane protein concentration that is sufficient to generate an osmotic potential such that applied pressure is reduced and countered. This may explain why experimentally determined C_G values are often found to be below the known solubility of the protein. However Grund et al (1992) have estimated that the reduction in driving hydraulic pressure due to the osmotic pressure of the gel layer, for UF of proteins, is between 1 and 5 %.

The osmotic pressure model and the gel-layer model are two parameter models where the effects of concentration polarization and solute adsorption are modelled onto

one parameter, for example the gel layer. Instead it is potentially more illuminating to consider three parameter models. Ko et al (1992) have used an osmotic pressure - adsorption model where the concentration profile is modified to allow for a concentration profile across the membrane and into the receiving phase. The equation to predict flux includes components for pressure, osmotic potential, fouling resistance and membrane resistance.

1.2.2.6. Fouling.

Fouling problems limit the wider application of UF technology. It leads to reduced permeate flux, increased downtime, loss of fractionation and increased cost of the system both in terms of regeneration, and the design factors required to compensate for lower flux.

Much of the ambiguity which exists in predicting the gel concentration value with different membranes is linked with the extent of irreversible adsorption. It is important to differentiate between concentration polarization, which is independent of membrane type, and the membrane specific phenomena of protein adsorption. Concentration polarization is a function of the hydrodynamic conditions in the system, theoretically independent of the membrane, whereas fouling is membrane deposition and therefore specific to the particular membrane type. Concentration polarization, which is reversible, allows the build up of materials which in turn foul the membrane, an irreversible phenomena, although these two processes occur in an inter-related and simultaneous manner.

The interaction of these processes has been reviewed by Nilsson, (1990) and Marshal, Munro & Trägårdh (1993). Fouling dictates membrane performance, with both surface and internal fouling occurring.

Flux reduction occurs routinely to 10 - 20 % of the initial value. It is not clear if the observed reduction in flux is due to concentration polarization *per se*, or concentration polarization inducing increased adsorption at the membrane surface. This is a pertinent question as it has been found in the present study and others (Swaminathan, Chaudhuri & Sirkar, 1980) that the times required to attain steady flux and retentivity can differ significantly, with flux achieving a steady state value first. This indicates that changes in resistance to solute transport continue to occur even though concentration polarization, and therefore overall flux, is established.

Empirical examinations, summerised by Marshall et al (1993), identified various components in the process. Initially pore diameter restrictions occur due to solute adsorption onto the wall or even complete blockage of single pores by individual proteins; known as pore occlusion (Howell & Velicangil, 1980). This is followed by surface adsorption which is consolidated by pressure and forms a dense, partially denatured, layer with low water permeability of approximatley 100 nm depth. The formation of this hydrogel of concentrated protein then dominates membrane performance. Finally over a longer period of time, the polarized layer extends into the bulk phase and has a degree of osmotic activity. The first two components, pore and surface adsorption, are determined by membrane properties and hence are reducible by design of membrane surface interactions.

Due to the complexity of fouling processes, many of the modifications to the flux and fouling models tend to be system specific. For example, Fane, Fell & Waters

(1983) have shown that at the isoelectric point fouling occurred only at the surface, but at other pH values it occurred within the membrane. Also the shape of the molecule, depending whether it is linear or globular, and the surface hydrophobicity. Merin et al (1980) have shown that small molecules, such as salts, are significant in fouling possibly by acting as a salt bridge between proteins to form agglomerates, and also to induce conformational change.

To avoid formation of a dynamic gel layer it is desirable for runs to be at very dilute concentration. However this is often impractical for industrial processes. Both physical and chemical approaches to alleviate the problem of fouling have been reviewed by Flaschel et al (1983) for many industrial applications.

1.2.3. The analysis and operation of ultrafiltration systems.

1.2.3.1 The advantages and disadvantages of ultrafiltration.

Ultrafiltration has various advantages and disadvantages compared with other separation systems.

Advantages:

i. No phase change is required.

ii. Small molecules are able to pass through the membrane and hence there are no changes in pH or ionic concentration.

iii High purity supernatant is generated which can be successfully integrated into other processes which are otherwise intolerant of the presence of debris, for example chromatography.

iv. No restriction in location of energy supply coupled with potentially large energy savings.

v. Ambient temperatures can be used and the temperature can be controlled.

Disadvantages;

i. Membrane fouling leads to flux decline which in turn results in a requirement for an inbuilt design of greater membrane area, increased pumping costs and increased downtime to clean. Fouling also results in loss of fractionational ability and a fouled membrane may result in microbial contamination.

ii. The complexity of the system cannot be modelled completely and published manufacturers data are rarely good guidelines to performance.

iii Reduction of water content is limited to approximately 35 %.

iv Glycerine and other detergents may be released from new unprepared membranes

v. Detergents and other process chemicals can deleteriously affect membrane structure.

1.2.3.2. Assessment of ultrafiltration membrane characteristics.

Assessment of UF membranes can be undertaken by various methods. For example, use of contact angle measurements coupled with pore size and distribution measurements (Gekas & Zhang, 1989 and Gekas et al, 1990), the use of electron

microscopy (Lee, Miranda & Merson, 1975 and Fane, Fell & Waters 1981), other novel techniques including gas adsorption/desorption (Smoulders & Vugteveen 1985), and to examine the membrane surface. However these methods do not give a direct analysis of membrane performance. Instead they are physical examinations of the membrane surface, which ultimately have only limited implications for membrane performance.

These limitations can be overcome by an *in situ* assessment of UF membrane performance using dextrans or proteins (Hancher & Ryon, 1973). This work has been extended to the use of polydisperse polymers of known molecular weight distribution (Cooper & Van Derveer, 1979, Adachi et al, 1986, and Tkacik & Michaels, 1991) to give a fuller rejection profile of the membrane. However, this approach is also potentially inaccurate due to the various factors which influence protein separation across UF membranes.

Bottino et al (1984) in an assessment of operating conditions upon membrane performance has concluded that estimation of nominal molecular weight cut-off (NMWCO) is system dependent and should be undertaken using a solute with good chemical and conformational activity to avoid solute-membrane interactions. These include gel polarization and dynamic membrane formation as well as a consideration of protein biochemistry (Sirkar & Pravasd 1986). Consequently a full description of a UF membrane should include a physical description as well as performance-related data.

However despite the great consideration that has been given to assessment and characterisation of UF membranes, there remain many problems in predicting flux data for membrane systems. Many of these inaccuracies arise as a result of laboratory

assessment of membranes using dead-end SCF systems. Such units can be operated in one of two modes; concentration mode, where the feed volume is reduced under pressure, or diafiltration mode, where constant feed volume is maintained via use of a buffer reservoir operated under pressure.

Operation in a concentration mode, although simple, does not maintain constant conditions throughout the experiment. Instead the reduction in feed volume, during the course of the experiment, affects many other parameters that are volume dependent, for example stirring, shear rate and, especially, protein concentration and gel layer thickness. Alternatively, if run in a diafiltration mode, although the feed volume is maintained at a constant level, there is membrane polarization and fouling is likely to occur. Further the concentration of the protein may decrease if it is able to permeate the membrane. Separation efficiency cannot be directly attributed to membrane performance. Therefore assessment of membranes is preferentially performed in a CFF unit where concentration polarization is reduced and recycling of permeate and retentate to a reservoir allow constant protein concentration to be maintained.

1.2.3.3. Operating temperature.

Of the many parameters that are considered in the operation of an ultrafiltration system such as pressure and concentration, temperature is critical. Considerations of the physicochemistry of the system indicate that it must be operated at as high a temperature as possible for two reasons. Firstly, in the pressure dependent regime flux doubles for every 10°C rise in temperature. In the pressure independent regime the flux is controlled by mass transfer coefficient, which is influenced by the diffusivity. This in turn is characterised by the Stokes-Einstein equation where a 3% rise in

diffusivity occur per °C. Secondly, the viscosity of the solution decreases with temperature. For instance the viscosity of water decreases by 2.5% every °C, and hence flux will, theoretically, increase.

Porter (1990) indicated that flux rises with temperature but concluded that temperature affects many variables and that it is often unique for the particular solute and membrane system. For example the desire to increase the operating temperature, to improve membrane performance, can affect protein conformation and its relationship to function.

Proteins, as solutes, do not have a fixed conformation, but are in a dynamic state where conformation and activity are a compromise between flexibility of structure and stability of the molecule. This has been reviewed by Jaenicke & Zavodszky (1990) and Jaenicke (1991). It has been demonstrated that protein structure and folding is controlled by the free energy of stabilisation, which is a result of the difference between stabilising and destabilising forces, and is approximately 50 kJ mol^{-1}. It is attributable to a small number of hydrogen bonds, ion pairs or patches of hydrophobic interactions. The energy required to destabilise such a structure can be supplied in many different forms such as thermal, pressure or shear forces, pH extremes, ionic strength, number and type of proteins and the presence of lipids and carbohydrates (Robinson et al 1993).

Meirles, Aimar & Sanchez (1991), who examined a model ultrafiltration system with respect to the relationship between increased temperature, decreased stability of protein conformation and membrane fouling, have shown that an increase in temperature may result in exposure of the protein's inner hydrophobic core to the environment. Such a denatured protein may have a tendency to either aggregate by

crosslinking, via intermolecular disulphide bridges, or adsorb to suitable surfaces rather than remain in solution. The membrane can provide a surface to which the protein may adsorb, leading to an increase in fouling (Dillman & Miller, 1973, Ingham et al, 1980, Wahlgren & Arnebrant, 1991).

Lower operating temperatures are therefore preferable in order to maintain protein conformation and importantly to avoid microbial contamination. Kerkof (1988, 1989) has examined the conflicting demands in choosing a particular operating temperature, and presented rudimentary models for predicting flux coupled with enzyme activity.

1.2.4. Applications of ultrafiltration systems in biotechnology.

1.2.4.1. Traditional biotechnology.

Ultrafiltration is extensively used in traditional biotechnology and has been reviewed by Cooper (1980) and McGregor (1986a). The initial driving force for this technology was to design systems for applications to the sewage and waste water industries. The present day applications are now very diverse and include; the dairy industry, for cheese whey protein recovery; the food industry, for soy whey dewatering without denaturing proteins therefore resulting in cheaper transport and processing costs; wine clarification to remove tanins, colloids, carbohydrate, proteins and cold sterilisation; and beer clarification and alcohol free production by diafiltration.

Finally, the largest application of biotechnology is the biological treatment of sewage where membranes have found applications, principally for dewatering. Industrial waste treatment has attracted novel developments in anaerobic and aerobic effluent treatment and ultrafiltration processes can be essential in these processes. Indeed they may be designed to allow both the retentate and the permeate to be of value and therefore recoverable.

1.2.4.2. Bio-pharmaceutical applications.

Membrane UF systems are also found in the modern bio-pharmaceutical industries and have also been examined extensively by Le & Howell (1985). For example, a case study for the separation, using a UF system, of recombinant tissue type plasminogen activator (rt-PA) on an industrial scale has been reported by Reis et al (1991). It is within the bio-pharmaceutical industries that UF has one of the greatest commercial potentials. Essentially these processes are either to remove dissolved macromolecules or cells.

For example, for concentration of yeast cells (Pritchard, Scott & Howell, 1988) UF is preferable to MF because anisotropic membranes have less fouling. Cellular products are separated by UF from whole cell and disrupted broths (Kroner et al, 1984, Brown et al, 1987, Tejayadi & Cheryan, 1988) during many processes, for instance to obtain hormones, cytokines eg. interferons, biosurfactants from very dilute fermentation broths (Mulligan & Gibbs, 1989), lipases and other enzymes (Sztajer & Bryjak 1989), blood plasma and other proteins. Concentration of viruses, such as rubella, by up to 600 fold using a 80 kDa NMWCO membrane, is also a highly profitable application. Clarification of fermentation broths for further purification to obtain small molecular weight products such as antibiotics and alcohol also occurs.

Novel applications of membranes as supports for immobilised enzymes, include immobilisation of RNase to remove RNA from solution, (Rucka & Trkiewicz, 1989). Usage of UF membranes have also been applied to bioreactors, for example as a support for animal cell cultures. The application of membranes for the entrapment of cells has been further reviewed by Mazid (1993).

Finally ultra-pure water can be produced by UF using a 10 kDa NMWCO membrane. This is essential for the production of injectable therapeutics and requires the removal of pyrogens (lipopolysaccharides from gram negative bacteria).

1.2.5. Aims of investigation.

The principal aim of the work on UF in this thesis has been to illustrate the causes of flux decline, in UF systems, and relate this ultimately to membrane structure. Several workers have considered the relationship between membrane structure and performance and found it to be ambiguous. Kim et al (1992) considered factors such as membrane material, NMWCO, porosity, pore diameter, hyrophobicity, hydraulic permeability, surface roughness, and that for two very similar membranes, differing only in this last component, very different fouling behaviour occurred. Nakatsuka & Michaels (1992) have concluded that transport is strongly dependent upon chemical composition of the membrane, whereas formation of a gel and its diffusion back to the bulk will always occur to an extent.

The essence of membrane design has been addressed by many workers, however the aim was succinctly expressed by Ingham et al (1979):

"A solution to the problem of protein adsorption requires the availability of a synthetic material which has little affinity for proteins and which can also be fashioned into thin porous sheets capable of withstanding varying degrees of mechanical stress. Such a material should be hydrophilic and bear very little net charge..."

Therefore, the present study was designed to limit the potential vagaries of ultrafiltration by developing a model system, using a crossflow regime in a recycle

mode of operation. This involves the permeate and retentate being reunited, before entering a reservoir and then being recycled across the membrane, thus maintaining as much homogeneity as possible above the membrane.

A CFF system was used to ensure control of the shear rate. An examination of temperature effects was initiated after localised temperature increases had been observed around a gear pump head which had been used to drive a CFF unit in a recycle mode for extended periods of time.

If these were not taken into consideration, and corrected, they could have considerable influence upon fouling and flux. Use of a reservoir enabled accurate temperature control and hence a detailed examination of temperature effects. This thesis therefore describes an assessment of the conflicting effects of variations in operating temperature for an idealised ultrafiltration system.

Therefore, having established a model system, the role of some operating parameters were considered. This was an attempt to gain an understanding of membrane-macrosolute interactions and what is required in membrane design, and what could realistically be achieved in a rational membrane design programme and a subsequent dedicated manufacturing process.

1.3. Facilitated membrane systems.

1.3.1. Modes of facilitated membrane separation processes.

1.3.1.1. Liquid membrane devices.

The basic device for the investigation of this technology is a bulk separator that contains three phases, two aqueous phases separated by an organic phase. Such a set-up is a crude laboratory approximation, but useful to assess extraction coefficients, investigate carriers and the system viability.

Much of the development work on liquid membranes has been undertaken using liquid emulsion membrane (LEM) systems. These are an emulsion of an organic phase containing an internal aqueous phase, which is suspended in an external aqueous phase. This system has a very large surface area to volume ratio and very thin membranes, thus allowing high flux rates. However LEM systems have several difficulties associated with the instability of the emulsion, due to breakage and swelling, and difficulty in product recovery, hence they are usually limited to usage on a laboratory scale.

Despite this, the large surface area to volume ratio and high flux rates make this technology attractive. Hence a limited commercial potential has been realised in

Austria for the treatment of waste waters. Bulk and LEM systems are shown in Figure 1.8.

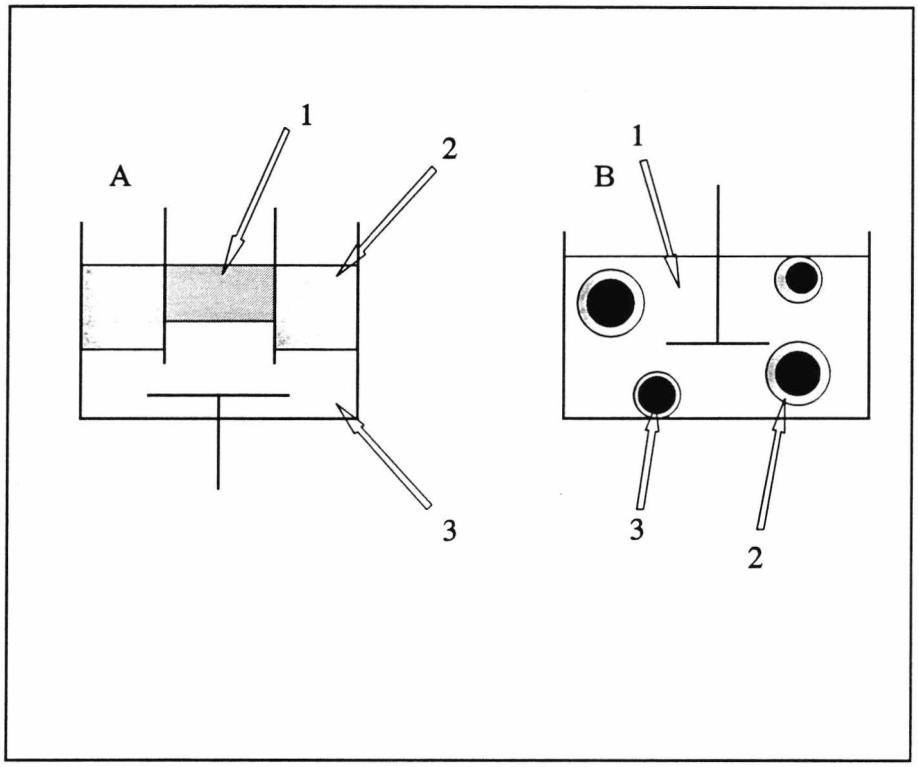


Figure 1.8. Unsupported liquid membrane devices. A. Bulk liquid membrane - a source aqueous phase (1) is separated from the receiving phase (2) by an organic phase (3), which is stirred. B. Liquid emulsion membrane - a source aqueous phase (1) contains an emulsion of organic particles (2) each of which contains the aqueous receiving phase (3).

The disadvantages of LEM systems have been overcome by immobilising the organic liquid membrane layer in a polymeric support, typically a microporous membrane, in a flat sheet, hollow fibre or tubular configuration to form a supported liquid membrane (SLM) (see Figure 1.9.)

One consequence of SLM systems is that the membrane phase is stagnant and as such diffusion is often the rate limiting step. One method of overcoming this has

been to develop membrane contactors (Boyadzhiev, 1990, Lazarova & Boyadzhiev, 1993) in which the feed and stripping phase flow across solid supports which are themselves immersed in the membrane phase flowing co or counter-current (see Figure 1.9). Although the liquid membrane is of the order of a few millimetres thick its effective thickness for diffusion, due to the flow, is of the order of microns.

1.3.1.2. Other systems.

Another method of achieving facilitated separations is achieved by using a membrane in conjunction with an affinity system. In this case the established methodology applied to affinity chromatography is utilised in a membrane system in one of two modes. Either an affinity ligand is immobilised on the membrane or it is in solution above the membrane. In either case specific binding to the target molecule results in it being captured on the membrane surface, or in solution where the affinity ligand-target molecule complex is impervious to the membrane. Controlled release of the target molecule is initiated by elution.

The advantages of this system result from compressing the column length to the width of the membrane and hence elution band broadening is reduced. Also recycling allows for even higher degrees of concentration. The advantages of membrane processes, such as scalability and ease of operation, are utilised and operated in a continuous or plug flow manner.

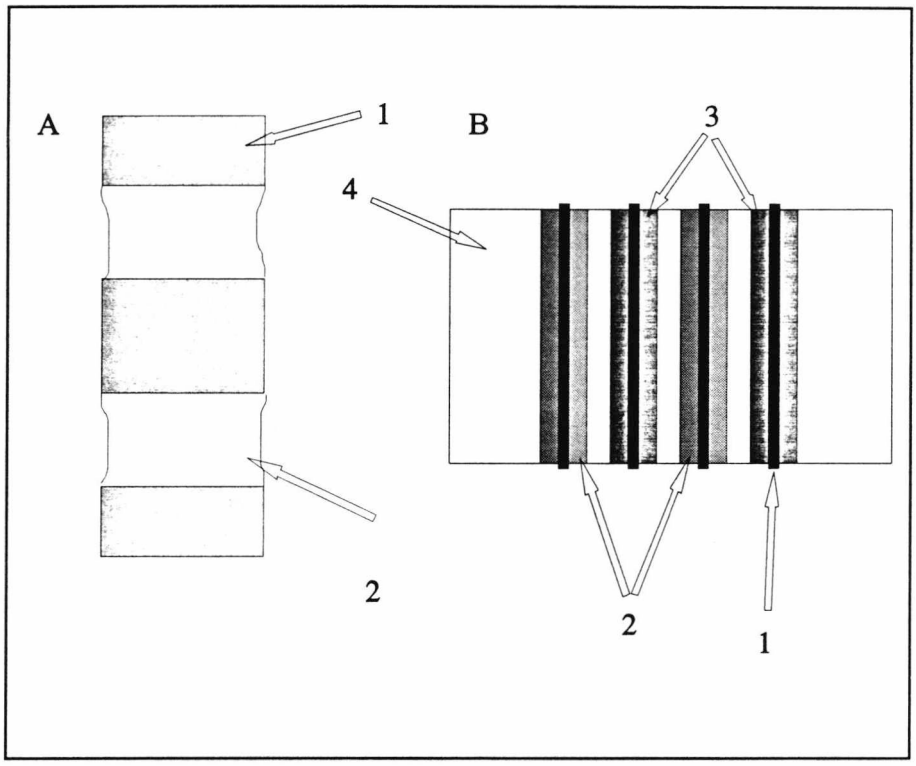


Figure 1.9. Supported liquid membrane devices. A. Supported liquid membrane - a microporous support (1) contains the organic phase (2). This structure separates the two aqueous phases on either side. B. Flowing supported liquid membrane - a solid support (1) has the aqueous source phase (2) and receiving phase (3) flowing down them with the organic phase (4) flowing around all supports.

1.3.2. Supported liquid membranes as a facilitated separation process for small molecular weight molecules.

A SLM system has advantages over other liquid membrane systems because it has the most readily defined geometry. Also access and manipulation of both aqueous phases can occur throughout extraction, for instance, for sampling or product recovery.

The diffusion of species through the liquid membrane phase, typically organic, is enhanced or, in the case of charged species made possible, by the use of a carrier which adds selectivity to the process. Hence these membranes can offer selectivity on the basis of size and, more importantly, carrier-induced specificity.

The movement of the carrier can be coupled to that of another species to drive transport, against a concentration gradient. In this situation, the carrier mediated transport of a species can be either counter-transport, where the transported species is exchanged for another ion at the receiving phase, or, co-transport where the transported species combines with the carrier and another ion from the source phase, via ion-pairing.

Amino acids can be separated with SLM system and their transport can be with either a cationic or an anionic carrier, as a result of the zwitterionic nature of these molecules. In either case, their transport can be achieved in a counter-transport system. The charged substrate species cannot enter the organic phase on its own and therefore, once it has been transported, it is contained within the receiving phase. An idealised

exchange process across a SLM for an anionic amino acid, such as phenylalanine, under alkaline conditions, across a SLM is shown in Figure 1.10.

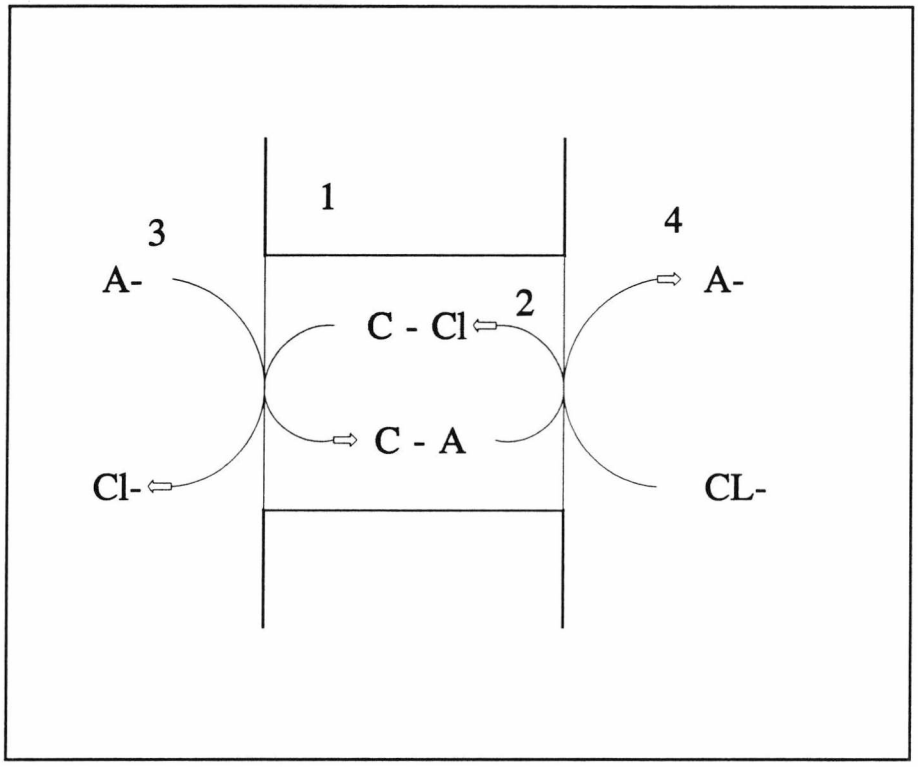


Figure 1.10. Generalised transport mechanism for the carrier facilitated movement of an amino acid across a supported liquid membrane. The polymeric membrane support (1) contains the organic membrane phase (2) and separates the aqueous source phase (3) and aqueous receiving phase (4); A = amino acid; C = carrier; Cl = chloride ion.

1.3.3. Theoretical description of transport across a supported liquid membrane.

1.3.3.1. Diffusion limited model

Immobilisation in a polymeric support to give a SLM, reduces the available area for transport, but overcomes some of these drawbacks by applying the advantages of crossflow filtration systems. The influence of the support has been reviewed by various workers (Douglas Way, Noble & Bateman, 1985, Takigawa, 1992) who indicated the requirements such as short non-deviating pores to reduce path length for diffusion. It should be less than 100 μm thick with greater than 50 % porosity and pore diameters of less than 0.1 μm. Movement across a liquid membrane is a complex phenomenon consisting of a mixture of diffusion of the substrate carrier complex between, and reaction steps at, two independent interfaces. The goal of modelling the process is to identify, and enhance, the rate limiting process.

Transport limited by exchange diffusion of two substrates, involving a counter-ion, was used as a model in this study. Many workers have reviewed similar systems (Behr, Kirch & Lehn, 1985, Haensal, Halwachs & Schügerl, 1986, Izatt et al, 1989, Baker & Blume, 1990 and Inoues, 1990).

Fick's first law of diffusion contributes to a description of transport, assuming that linear concentration profiles exist within the organic phase. Prediction of flux of the substrate across the liquid membrane is complex; one of the more illustrative models was presented by Izatt et al (1989). In their model factors are included for membrane geometry, solute diffusion coefficient in the membrane, initial aqueous

phase concentrations, and an equilibrium constant for the extraction coefficient for the substrate. This model assumes that the chemical reactions occurring at the aqueous-organic interface are at equilibrium and are not rate limiting. Further it is assumed that an equimolar exchange process occurs, co-transport of buffer is ignored, equal affinities for the separate substrates and equal diffusional rates occur for the different carrier-substrate complexes. Also the aqueous phases are homogenous except in the unstirred layer (Nernst Layers) at the membrane surface,

This model also predicts that the fastest transport rate occurs when the carrier has the same affinity for the two substrates, and that transport is impeded when there is carrier saturation. It also considers the distribution of the carrier/substrate complex, and its diffusivity and equilibrium constants.

This is simplified by assuming that the charged species are confined to the aqueous phases, non-charged ones to the organic phase and also that the non-carrier mediated transport of species is negligible. For the diffusion of the substrate-carrier complex from the source phase to the receiving phase, assuming negligible substrate in the receiving phase and re-extraction of the substrate out of the receiving phase, the equation for flux can be written as;

$$J = \frac{D_{SL} K_{ext} [L_{total}]^{org} [S]^{s}}{l\,[1 + K_{ext}(S)^{s}]} \qquad \textbf{(1.5.)}$$

where J = flux of the substrate, D is the diffusion coefficient for the species, K_{ext} = extraction equilibrium constant, L_{total} = the sum of the carrier (L) present as free carrier in the organic phase, S = the substrate, l = the transport path length through the membrane and the superscripts s and org represent the source and receiving phase.

1.3.3.2. Interpretation and criticism of the diffusion limited model.

Several qualitative predictions are possible from the model in **1.5.**. Firstly, decreased thickness of the membrane, increased diffusivity of the substrate-carrier complex, increased concentration of substrate and carrier will all increase flux.

Interfacial activity is not limiting, but the position of the equilibrium constant can be moved to favour greater transport by decreasing the concentration of substrate in the receiving phase, for example, by removing it by increased flow rates. Furthermore transport will only occur if substrate concentration in the source phase is greater than the extraction equilibrium constant and continues whilst carrier saturation at the receiving phase remains lower than the extraction equilibrium constant.

A development of the diffusional model by various workers (Yamguchi et al, 1988, Yoshikawa et al, 1990 and Mutihac, Mutihac & Luca,1991) has been to obtain extraction and permeation coefficients to characterise the affinity, and the rate of diffusion, of the carrier and substrate. Interpretation of the model in this manner is comparable to the carrier-mediated transport of a species across biological cell membranes and is characterised in terms of saturating enzyme kinetics.

All of these models assume that interfacial rate effects are not limiting, although in reality there are three scenarios as shown in Figure 1.11.

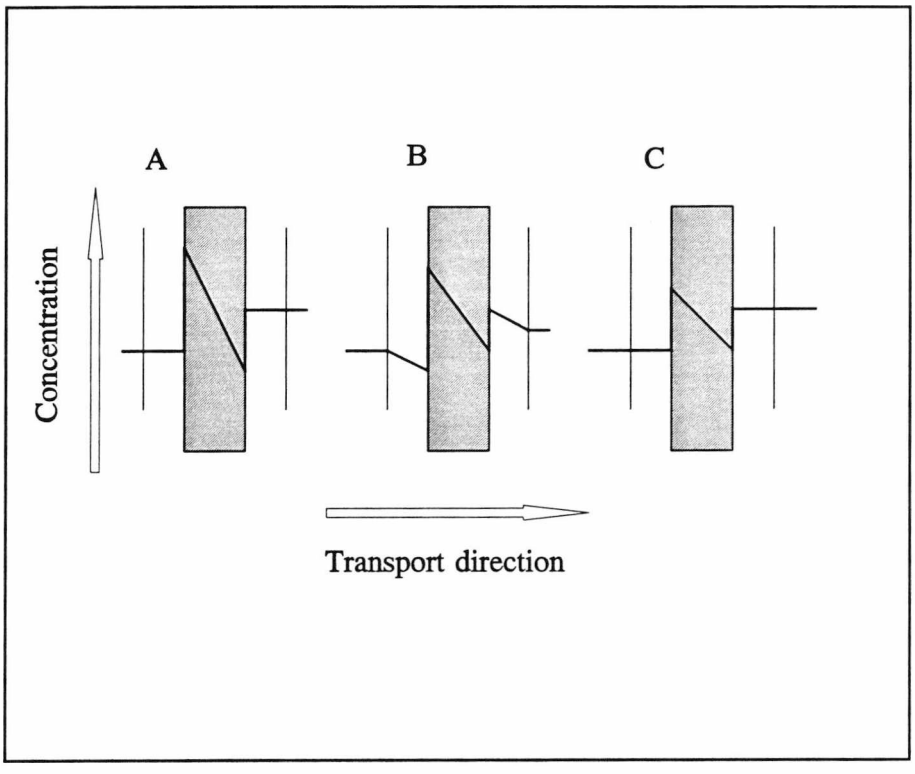


Figure 1.11. Different limiting conditions in transport across a supported liquid membrane. A. Diffusion across organic phase is limiting. B. Diffusion across the boundary layer is limiting. C. The interfacial exchange reaction is limiting.

Observed phenomena are an interplay of these mechanisms and therefore there is ambiguity as to the nature of the limiting factor, whether it is a diffusional or interfacial limited reaction. Many different systems have been examined with varying conclusions as to the rate limiting step. Uddin et al (1990) and Chan & Wang (1993) have described a liquid membrane stirred transfer cell system for the extraction of phenylalanine by trioctylmethylammonium chloride and concluded that an interfacial reaction mechanism is limiting, whereas Haensel et al (1986), using the same carrier, but in a SLM format, have shown that phenylalanine extraction is diffusion limited.

Therefore it appears that the limiting reaction can be system dependent. Possibly the much thinner membranes in LEM systems do not cause diffusion to be the limiting factor whereas in the comparatively thick SLM systems, with unstirred aqueous boundary layers, may be diffusion limited.

Several authors have noted the complexity of the exchange process and indicated that the aqueous film diffusion, interfacial chemical reaction and diffusion through the membrane are all significant (Mohapatra, Kanungo & Sarma, 1992). The exchange process and thickness of the unstirred aqueous boundary layer will be determined by the flow rate across the membrane surface in a SLM system. Tangential flow strategies from traditional membrane processes can be used to manipulate this and control any concentration polarization at the membrane surface.

Shiau (1992) concluded that the interfacial processes for the transport of copper are not in equilibrium, and the rate limiting step changes between the exchange processes and the diffusion rate as the process continues. A similar study by Lazarova et al (1993) on the extraction of copper ions across a SLM system has concluded that transport across the two different aqueous organic interfaces is at a different rate. Two steps are thought to interact. Firstly, transfer of the substrate through the bulk organic phase is diffusion controlled, whereas the second step involving the extraction out of the organic phase is controlled by the rate of the chemical reaction.

This problem is further compounded by the nature of the system with instability effects often making long-term quantitative modelling difficult.

1.3.4. The analysis and operation of supported liquid membrane systems.

1.3.4.1. Advantages and disadvantages of supported liquid membrane systems.

Advantages:

i. Separation has a potential for a high degree of specificity.

ii. The system, as defined in Section 1.1.2.4. is a facilitated one and can therefore be used to concentrate the selected species, against its own concentration gradient.

iii. The process can be applied to a continuous separation regime.

iv. Like other membrane processes, SLM systems are readily scalable and thus enable a transition from laboratory development to process assessment.

v. The system requires no temperature changes, but can be operated at whatever temperature is required.

vi. The potentially expensive carrier, and even the organic phase, are limited to small amounts and reusable, therefore reducing costs.

vii. SLM systems have been shown to have greater operational ease of use and lower energy consumption than LEM systems.

viii. Crossflow processes allow the reduction or elimination of unstirred layers at the aqueous organic interphace.

Disadvantages:

i. Macromolecules with a complex tertiary structure, such as proteins, can be damaged by movement across the interface, and interaction with the organic phase may also be damaging.

ii. These systems often show instability, with breakdown of organic phase allowing direct contact between the two aqueous phases. Furthermore if these separations are to be applied to food and pharmaceutical manufacture processes it must be demonstrated that neither the carrier nor the organic phase leak out.

iii. Some carrier compounds and organic phases are potentially toxic to microorganisms. For instance quaternary ammonium compounds can cause damaging reactions with microbial cytoplasmic membranes.

iv. Interaction with the carrier may be irreversible and result in loss of substrate.

v. Hydrophobic molecules, which are not target molecules, may also diffuse across and reduce the purity of the product.

1.3.4.2. Breakdown of membrane integrity due to instability.

To maximize the commercial application of supported liquid membrane technology it is essential that the SLM maintains its integrity. The required lifetime of the system is dictated by the value of the product, hence only high value added products can support an efficient but short lived separation technology. Two phenomena are particularly significant, leakage and instability. The model shown in Figure 1.10 is not the only possible route for the transport of substrates such as phenylalanine. Possible routes are shown in Figure 1.12;

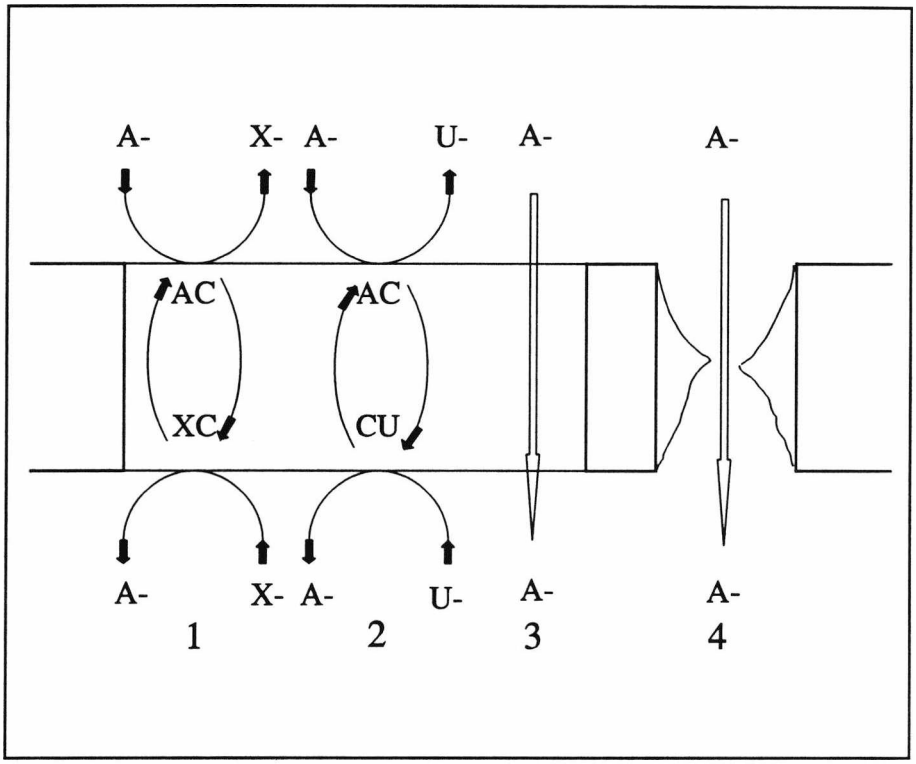


Figure 1.12: Possible routes for amino acid transport across a supported liquid membrane. Carrier mediated exchange with chloride ions (1), carrier mediated transport with other ions (2), non-carrier mediated transport (3), diffusion by direct contact of source and receiving phase (4). A = amino acid; C = carrier; X = chloride ions; U = hydroxyl or buffer ions. Processes 2, 3 and 4 are leakage termed leakage and 4 is also a special case in that it is due to the instability of the membrane.

Processes 2,3, and 4 (Figure 1.12) are defined as leakage, and process (4) is specifically termed instability, and concerns containment of the membrane phase and carrier within the polymeric support matrix. This relationship is influenced by the relative solubilities of the components and the interfacial tensions between the aqueous and organic phases and intersurface tensions between the organic phase and the

support. These processes are not mutually exclusive and part of the present study is an attempt to examine the roles the various components play in the process.

Many groups have addressed the causes of instability-induced leakage (4 in Figure 1.12.) which represents one of the most serious barriers to the widespread application of this technology. Although Zha et al (1992) attributed instability to the presence of pressure differentials across the membrane, it is thought that generally this is not significant. Instead Neplenbroek, Bargeman & Smoulders (1990, 1992a) concluded that the principal cause of leakage is a propensity to emulsion formation of the organic layer due to either lateral shear forces or membrane vibrations.

The principal cause was shown to be a propensity to emulsion formation due to lateral shear forces. These can be described by "Kelvin - Helmholz instabilities" and arise when two phases move parallel to one another at different velocities - an effect that is comparable to the formation of waves upon water when wind moves across it. (see Figure 1.13.)

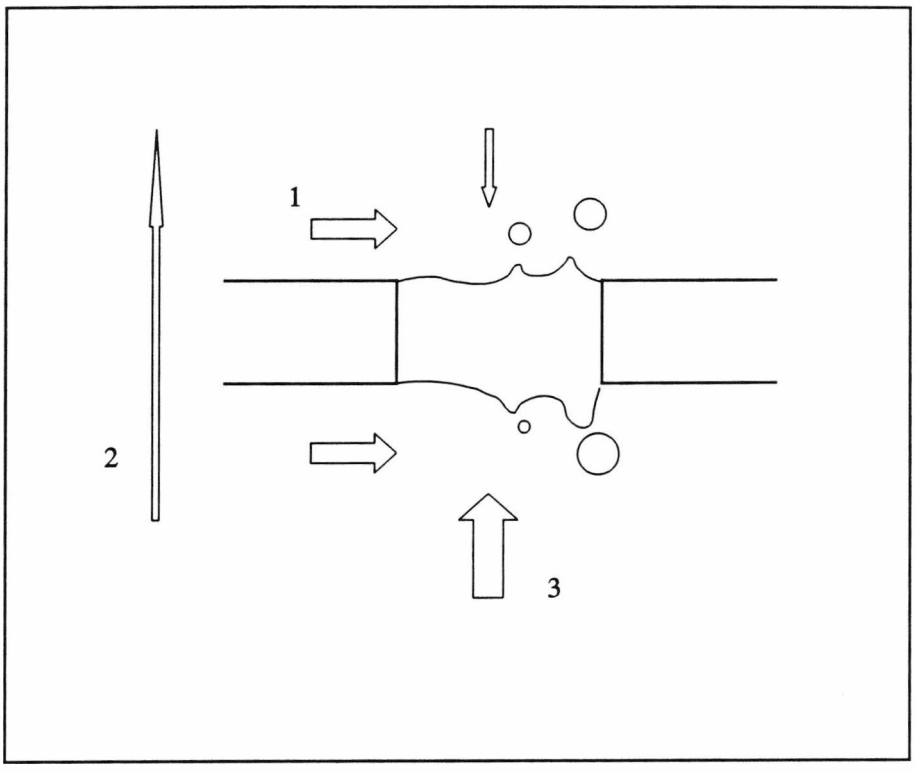


Figure 1.13. Mechanisms of instability. Flow across the membrane surface results in emulsification processes (1), osmotic potentials induce water movement (2) and pressure differentials may displace the membrane (3).

An increase in ionic strength of the aqueous phase can act to reduce these effects by increasing the instability of colloidal substances and therefore increase the sedimentation properties of emulsified organic phase. Therefore these particles are more likely to be reunited with the main body of the organic phase. Ionic strength also increases the interfacial tension (Binks, 1993) and therefore decreases the probability of initial emulsion formation.

Other groups (Danesi, Reichly-Yinger & Rickert, 1987 and Ramaseder, Marr & Bart, 1993) have attributed instability to the presence of osmotic forces across the membrane related to the presence of a carrier. The carriers may either hydrogen bond

to water molecules and repel the organic solvent, causing instability, or form reverse micelles to solubilise water.

1.3.4.3. Prevention of instability.

Stability is primarily concerned with the containment of the membrane phase, and carrier, within the support. This relationship is naturally determined by the relative solubilities of the components, and the interfacial and intersurface tensions of the phases.

Preparation of the system and the interaction of the organic phase, carrier and support have been found to be critical in preparing stable membranes. Bryjak et al (1991) described various conditioning steps for the instalment of the liquid phase. Essentially these include the addition of ethanol to the aqueous phase to decrease the surface tension resulting in a fuller contact between the two phases. The use of modifiers is a methodology well established in two phase liquid extraction and has also been applied by Friesen et al (1991) who used long chain alcohols in the membrane phase to promote transfer across the interface.

Stolwijk, Sudhölter & Reinhoudt (1989a) have concluded that membrane stability is positively related to hydrophobicity of the carrier molecule. Nijenhuis et al (1991) have shown that partitioning of the carrier into the aqueous phase can be overcome by using extremely lipophilic compounds, such as calixarenes, as carriers. This result was mirrored by the work of Chang et al (1991) who considered the transport of amino acid esters and found that transport efficiency was strongly correlated to the hydrophobicity of the permeating species.

The required surface activity of the carrier can induce instability. Takeuchi, Takahashi & Goto (1987) concluded that to overcome the reduction in the interfacial tension caused by the carrier aliphatic organic solvents with higher boiling points should be used. Chiarizia (1991) also described the influence of carrier chemistry on liquid membrane stability for a series of aliphatic amine carriers and demonstrated that an order exists of carriers that result in greater stability. Tertiary amines are better than secondary which are in turn better than primary. This reflects the relative solubilities in water and the propensity to lower interfacial surface tension; tertiary amines are the least soluble and affect interfacial surface tension the least. This work also described the requirements of the two phases which must have a high interfacial tension between themselves but the liquid membrane phase must have a lower surface tension than that of the support. The support properties are critical in maintaining membrane integrity for example wettability and the intersurface tension, interfacial tension and pore size.

Nakatsuji et al (1992) suggested that the presence of a fluorinated alcohol (as compared to an unfluorinated alcohol) in the organic phase was able to solvate the counter-ion to a far greater extent. This enables novel modifications to the system, for example manipulation of organic phase interactions with the support.

An increase in the stability of an SLM can be achieved by coupling the carrier to a carbon chain backbone (Wienk et al, 1990) or immobilising it on the support (Cussler, Aris & Bhown, 1989 and Noble, 1990 & 1992). Other physical methods of containment include attempts to prevent meniscus deformation include gelling the membrane phase with polymers, such as polyvinyl chloride, either on both or only one side (Bromberg, Levin & Kedam, 1992, and Neplenbroek, Bargeman & Smoulders 1992b). The latter case results in a membrane with a comparable flux to the non-gelled

version but has a far higher stability. Alternatively the SLM can be placed between two dialysis membranes which also results in a suppressed but constant lower rate of flux (Saito, 1992).

1.3.4.4. Carriers.

Carriers, by undertaking a reversible specific reaction with the permeating species, enable transport across the membrane along a diffusion gradient of the substrate carrier complex. Various workers (Thien, Hatton & Wang, 1988 and Itoh et al, 1990) considered the optimum concentration of carrier and concluded it to be approximately 10% (v/v) above which membrane instability can result and below which there is only a low transport rate. The transport of neutral substances such as urea, is highly desirable, but is problematic, owing to the normal requirement for reversible bonding of the oppositely charged double ion pair to the carrier and has only recently been reported (Straaten-Nijenhuis et al, 1993, Paugam & Smith, 1993). Charged moieties have received more attention. The transport of the substrate can be further enhanced by the presence of complexing agents in the receiving phase to move the position of the extraction equilibrium coefficient to promote release of the substrate into the receiving phase (Matsukata et al, 1993, Kojima, Shimokoshi, & Matsukata, 1993).

The choice of a carrier for a SLM system, like many other emergent technologies, has often not been made on purely logical grounds but has instead been swayed by historical applications. The carriers chosen are those initially developed in the chemical industries for metal extraction and are principally metal chelating agents. For example, quaternary ammonium salts have been used for the separation of metals

and their complexes by Sato et al (1984) and Hiranti & Taguchi (1990). Other compounds, for example crown ethers (Shukla, Kumar & Singh, 1992), have also made the transition from metal chelating processes. Pellegrino & Noble (1990), in an extensive review of liquid membrane systems have indicated the diversity of the choice of carriers.

Crown ethers have received much attention. Izatt, Lamb & Bruening (1988), in a review of liquid membrane processes, reported different selectivities for these compounds as carriers. Stolwijk et al (1986, 1989b) further investigated these compounds and modelled a transport process where the maximum selectivity was obtained by precisely controlling the number of carbon atoms in the ring to induce a precise fit with the target molecule.

In an attempt to reproduce transport processes that have greater specificity for biologically derived molecules various synthetic carriers have been considered. Tsukuke (1990) considered the selectivity and molecular design of synthetic carriers. Alternatively instead of designing novel molecules naturally occurring carriers have been considered as a basis for modification. For instance, lanthanide complexes (Willner et al, 1991) and natural and derived ionophores.

Ionophores naturally transport metal species across cell membranes, by convergent binding to present a hydrophobic exterior, and therefore have limited human therapeutic potential as the antibiotics nonactin and valinomycin. To enhance binding and transport processes these molecules have been synthesised by various workers (Maruyama, Sohmiya & Tsukube, 1989, Chia et al, 1991, Armstrong and Still, 1992, Kumar, Saini & Singh, 1992, and Wang et al, 1992) who have investigated the naturally occurring ionophore, lasalocid A and its potential for chiral separations.

Similar work has been reported by using a vitamin B_{12} complex for application in ion selective electrodes (Palet et al 1993). Another major group of molecules to be researched as separating complexes are the cyclodextrins (Armstrong & Jin 1987).

Although the biological transport of small charged molecules occurs in a very similar carrier mediated manner to the model in Figure 1.10. the exact mechanism for the direct transport of macromolecules across the cell membrane is not clear. The design of systems that mimic macromolecular cell transport systems remains a highly desirable goal and rational design processes for transport process molecules has been undertaken by various workers (Section 1.3.4.4.1.). Design can also decrease water solubility, and hence increase stability.

1.3.4.4.1. Molecular recognition

The coupling of two or more species by means of certain physical-chemical affinities without formation of a covalent bond is referred to as molecular recognition, and includes forces such as electrostatic attraction, hydrogen bonding and hydrophobic interactions. In the field of carrier chemistry molecular recognition has become significant to obtain a more fundamental understanding of many processes (Suckling 1991). For example, host-guest interactions are a useful tool for the analysis of biochemical systems as they can often display specificity, previously thought exclusive to larger biological molecules, in a much simpler format.

Researchers in this field have considered existing specific host-guest interactions to gain a greater understanding of substrate-receptor and enzyme catalysis biochemistry, for example a biotin-streptavidin model system has been considered by many workers (Hoffmann et al 1992). Others have attempted to model nucleotide

interactions, as these can only occur in non-aqueous solvents where there is little interaction between binding species and buffer (Furuta, Magda & Sessler, 1991) although limited binding can occur in reversed micelles with non-polar microenvironments (Nowick & Chen 1992).

The steric and electrical interactions of carriers and permeate species, which are the molecular basis of nucleotide interactions (eg. H-bonding, Watson-Crick and Hoogsteen base pairing) have been investigated and used as an impetus to create molecules with clefts, incorporating charged, hydrophobic and aromatic domains, to transport amino acids with corresponding domains. This convergent binding has resulted in a high degree of specificity (Hamilton & Kazanjian, 1985, Rebek, 1987, Rebek et al, 1987a,b,).

1.3.5. Applications of liquid membranes in biotechnology.

1.3.5.1. Separation of amino acids, organic acids and other small molecular weight products.

Many applications are based upon the separation of fermentation products either as part of a downstream protocol or an extractive fermentation process. For example Matsumara, Takehara & Katoka (1992) have outlined an extractive fermentation process for the continuous production of butanol.

Amino acids are one of the largest bulk fermentation products. Some of the major amino acids, such as phenylalanine, are produced in bulk (up to 500 000

tons/yr). Consequently, much research has centred around the separation of these molecules by both cationic and anionic exchange processes using coupled transport across SLM systems (Deblay, Minier & Renon, 1990, Bryjak et al 1991, Molinari, De Bartolo & Drioli 1992) and also across LEM systems (Thien et al, 1988, Teramoto et al, 1991, Hong, Choi & Nam, 1992).

The extraction of organic acids from fermentations is useful in its own right as a source of such products as citric acid. It is also advantageous to remove these often inhibitory byproducts to promote the fermentation process and hence increase yields. Citric acid is a very large commodity product and is only produced by fermentations. Therefore there has been much research to refine its extraction. Various workers (Kertes and King 1986, Boey, Garcia del Cerro & Pyle, 1987 and Schöller, Chaudhuri & Pyle, 1993) considered the application of LEM systems for this process. Friesen et al (1991) considered the use of coupled transport in SLM using tertiary amines as the carrier. Other small molecular weight molecules to be considered for separation across liquid membranes are penicillin G (Lee & Lee, 1992, Lee et al, 1993), diltiazem, a cardiac drug, (Basu & Sirkar, 1992) and alcohol for the production of low alcohol beer as examined by Etuk and Murray (1990).

Recently elucidation of intra and inter-cellular signalling has resulted in the isolation and cloning of receptor and effector molecules, for example ciliary neurotrophic factor. These molecules, and other similar factors, intervene at a fundamental cellular level and promise to deliver treatment for diseases such as Alzheimer's syndrome, many cancers and other degenerative diseases (Edgington, 1993). These molecules tend to be small and their potentially high value would

support the development of very specific carriers for use in liquid membrane separations.

1.3.5.2. Separation of large molecular weight molecules and other processes.

Thien & Hatton (1988), in an extensive review of LEM technology and its applications to biotechnology, have concluded that much of the work developed for liquid membranes can be applied to all charged moieties that don't have a tertiary structure. They also noted that the transition across the interphase would be detrimental to the structure of proteins and hence deleterious to activity. Therefore this system is limited in its extraction of large molecules. Few reports of this exist, for instance Yurtov et al (1987) utilised a membrane of mineral oil with an aqueous receiving phase with digitonin for the selective concentration of cholesterol from rabbits blood.

To overcome the interfacial transport problems of large molecules the application of reversed micelles can be applied for the selective extraction of proteins. This field has received much attention (Woll, Hatton, & Yarmush, 1989 and Dungan et al, 1991). Few researchers have attempted to utilise this technology in a membrane configuration to combine the selectivity of reversed micellar with the efficiency of membranes. Armstrong and Li (1988) combined these processes and demonstrated high efficiency and selectivity by manipulation of pH, ionic strength and a consideration of protein pI which resulted in little loss in specific activity. Whereas Derouiche & Tondre (1989) and Ismael & Tondre (1992) both considered the application of revesed micelles to transport potassium picrate across liquid membranes.

Other important applications of this technology include chemical synthesis (van Eikenen et al, 1990), photoresponsive membrane processes (Sunamoto et al, 1982, Willner, Sussan, & Rubin, 1992) and ion selective electrodes (Dinten et al, 1991). These systems also have potential applications as a technique to prepare and enrich samples for analytical measurement (Jönsson & Mathiasson, 1992 and Jönsson, Lövkvist, Audunsson and Nilvé, 1993) in applications such as amines in urine and herbicides in water.

Another area where movement across apolar liquid membranes is highly illustrative is the transport processes studied for the development of epidermal and other drug delivery systems (Barker, Hadgraft & Wotton, 1984 and Florence, 1993). For instance, AZT is highly charged and therefore cannot cross cell membranes. Carriers for molecules like this would be highly advantageous to transport drugs into cell.

1.3.5.3. Chiral separations.

One area of application which takes particular advantage of the chemical specificity of these types of separations is that of chiral selectivity. Van Eikenen et al (1990) examined the records of drugs given approval in the USA in 1985 and found that of 1805 drugs released, 518 were chiral and of these only 12% were single enantiomers, the rest being racemates. This trend towards chiral drugs is increasing, with an increase in demand for large scale chiral separations. Burke (1994) has concluded that chiral products will constitute 80 % of the pharmaceutical market, equal to approximatley $ 300 billion, by 2000. Industrial resolution is often performed using liquid chromatography techniques which tend to have a low-capacity, high-cost

and require batch separations. Therefore large scale continuous preparative separations are greatly in demand.

Much of the work on chiral separations has developed from resolution techniques initially devised for the isolation of chiral metal complexes. These involve the use of carriers which express some form of isomerism. Many workers have investigated this and utilised crown ether carriers to achieve separation of isomers of amino acids (Yamaguchi et al 1985, Yamaguchi et al 1988, Pietraszkiewicz & Kozbial 1992). Alternatively Armstrong et al (1987) have developed the novel approach of an aqueous membrane separating two organic phases. The carrier in the aqueous phase was cyclodextrin.

1.3.5.4. Enzyme processes.

A final but potentially very promising area of applications is the use of enzyme coupled transport and biotransformations in liquid membranes. The use of enzymes in organic solvents has already been shown to have many advantages, including enhanced stability, reversal of the aqueous phase hydrolysis into a synthetic process, isolation from proteases and the ability to co-immobilize important co-factors.

Scheper et al (1987), Ha & Hong (1992) and Ricks et al (1992) have all investigated the selective conversion of D,L phenylalanine methyl ester to L-phenylalanine and D-ester by chymotrypsinogen, which is of great potential commercial importance, in liquid membrane systems. In all of the systems the efficiency of enzyme immobilisation and the selectivity of liquid membranes were shown to be highly advantageous.

Rethwisch et al (1990) combined enzymes and membranes to result in a separation process that united both selectivity and concentration in one step. Lipases immobilised in an organic phase were used for the facultative transport of organic acids. This reaction occurred seventy times faster with the enzyme as the carrier. It was also shown to be selective in its choice of substrate.

1.3.6. Aims of investigation.

The discussion of stability in Section 1.3.4.2. highlights two areas, identified as leading to membrane instability, which are directly contradictory. Emulsification processes, which are reduced by the presence of chloride ions, in the aqueous phases also increase the osmotic potential across the membrane resulting in leakage. These two models predict exactly opposite effects caused by an increase in ionic strength on one side of the membrane.

A model system for the transport of phenylalanine across a SLM was established and the components were examined to illustrate their interplay, and used in an attempt to resolve this contradiction. The models of transport, including those of biological transport, formed the basis for an examination of the exchange process used in the present study.

In the field of carrier chemistry molecular recognition is becoming significant to obtain a more fundamental understanding of many processes. Therefore these studies formed a basis for considering SLM transport processes, using amino acids as the transported molecules. The ultimate goal of this research is to establish a stable selective transport process. In the present study this has been tested by the application

of a selection of amino acids at different pH's, whose charges are different at different pH values.

2. Experimental.

All reagents were of analytical grade and supplied by Sigma Ltd (Poole, UK) or Fluka Ltd. (Gillingham, UK) unless otherwise stated.

2.1. Ultrafiltration Membranes.

2.1.1. Assessment of the nominal molecular weight cut-off of ultrafiltration membranes.

2.1.1.1. Assessment in a stirred cell filtration unit.

The most basic method of ultrafiltration (UF) is based on dead-end filtration in a stirred cell filtration (SCF) unit. In the present study an Amicon 8050 SCF unit (43 mm diam.) (Amicon, Beverly, MA) was used and shown in Figure 2.1. To assess the nominal molecular weight cut off (NMWCO) of an UF membrane a series of passage markers (either protein or polysaccharide), selected from those in Table 2.1, were used to challenge a flat sheet Millipore (Bedford, MA) PTTK 100 kDa NMWCO membrane of 43 mm diam.

Before each clean, fresh membrane was challenged with a passage marker it was prepared for experimental use by soaking in 5% w/v NaCl solution for 30 min prior to use. This procedure removed any substances, present as impurities, that may have ultraviolet (UV) interfering properties. The membrane was then placed in the SCF unit and rinsed by successive aliquots of double distilled water that had been previously filtered through a 0.2 μm membrane (DDWF).

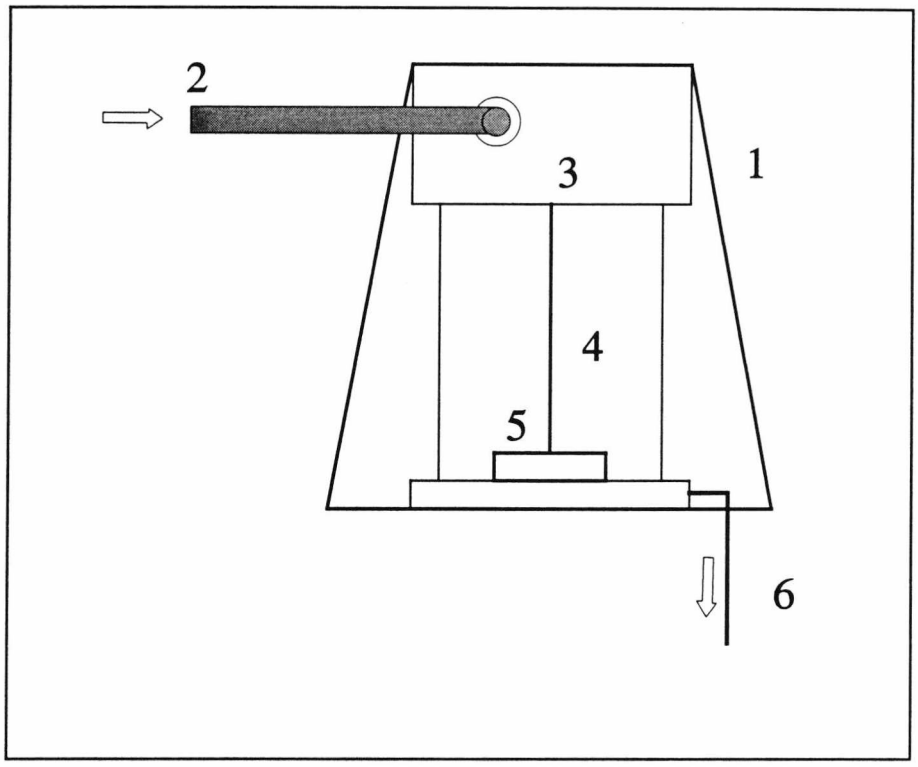


Figure 2.1. A stirred cell filtration unit. The unit is contained within a metal clamp **(1)** allowing the system to be pressurised by compressed air **(2)**. The unit **(4)** is clear perspex with an air tight cap **(3)** and a magnetic stirrer **(5)**. The permeate leaves the unit from the base and drains into an exit tube **(6)**.

The time required to collect the permeate aliquot was recorded as the clean water flux (CWF) in l m^{-2} h^{-1}. Once this parameter reached a constant value, the membrane was fully prepared for the measured movement of a molecular weight marker.

In the present study the method developed by Cheryan (1986) was used as a basis for the NMWCO assessments of UF membranes in a SCF unit. A 50 ml solution of the passage marker (either protein or polysaccharide) at 0.1% w/v concentration in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7) was filtered at 25^{o}C and 3.5 x 10^2 kPa. The stir

rate is a critical factor in this type of filtration. Although a maximal rate of stirring is preferential, it was maintained at a rate sufficient to create a vortex of less than or equal to one third of the depth of the liquid above the membrane (Amicon recommendation) and hence was adjusted during the course of the experiment. The ratio of the initial volume of solution above the membrane (50 ml) to the membrane test area (14.5 cm^2) was modified to 3.5 ml/cm^2 due to volume limitation of the stirred cell unit. The percentage of the permeate volume removed was increased to 40% of the initial volume to enable greater accuracy in measurement of the protein concentration in the permeate.

Determination of protein concentration was by either the Lowry or Coomassie blue methods, depending on the concentration present. The carbohydrate concentration was measured by the Anthrone reagent method. These methods are described in Section 2.1.1.1.1.

From measurements of the passage marker concentration (either protein or polysaccharide) in the permeate and the retentate solutions it is possible to determine the retention coefficient of the membrane for the passage marker using equation **(2.1.)**.

$$R = 1 - C_p / C_r \qquad \textbf{(2.1.)}$$

where R = retention coefficient, C_p = permeate protein concentration, C_r = retentate protein concentration.

These data can be plotted against the respective molecular weight of the passage marker to obtain a graph of retention coefficient against molecular weight. The NMWCO value was obtained by interpolation from a retention coefficient value of 0.9 as adopted by the European Society of Membrane Science and Technology.

This assessment was routinely performed in duplicate.

PASSAGE MARKER	TYPE	EST. PURITY %	MOL. WT.
Bacitracin	Protein	97	1440
Inulin	Polysacch.	97	5500
Cytochrome-c	Protein	96	12700
Lysozyme	" "	95	14800
α-Chymotripsinogen	" "	95	25100
Pepsin	" "	70	35000
Egg Albumin	" "	97	45000
Bovine Serum Albumin	" "	97	67500
Hexokinase	" "	76	99000
ß Galactosidase	" "	95	116000
Aldolase	" "	99	161000

Table 2.1. Molecular weight markers used to challenge ultrafiltration membranes. The markers (either protein or polysaccharide) were used to establish the nominal molecular weight cut-off (NMWCO) of the membrane.

2.1.1.1.1. Analysis of molecular weight marker concentration.

i. Lowry Assay.

Four reagent solutions were prepared as follows;

Soln. A = 2% w/v Na_2CO_3 in NaOH

Soln. B = 0.5% w/v $CuSO_4.5H_2O$ in 1 % Na Tartrate

Soln. C = 50 ml Soln. A and 1 ml Soln. B

Soln. D = Folin Ciocalteu reagent diluted 1 : 1 in H_2O .

A series of dilutions of the protein under consideration were prepared in the range 100 - 300 μg / ml and 0.2ml of each solution was pipetted into each of two tubes. To each of these tubes 1 ml of solution C was added. The solution was then mixed and left for 10 min. Solution D (0.1 ml) was then added to each of these tubes and the solution was mixed. The absorbance was measured at 750 nm using a 1 cm cuvette. Bovine serum albumin (BSA) was used as a standard.

ii. Coomassie Blue Assay.

The reagent solution was prepared as follows;

Coomassie brilliant blue G (100 mg) was dissolved in 50 ml of 95 % (w/v) ethanol. To this was added 100 ml of 85 % (w/v) phosphoric acid, and the solution diluted to 1 litre.

Estimation of dilute protein solutions (5 - 20 μg / ml) was undertaken by placing 0.8 ml of each sample into each of two tubes. To each tube 0.2 ml of reagent was added, and the tubes incubated at room temperature for 5 min. The

absorbance was read at 595 nm using a 1 cm cuvette. The results were compared to a range of BSA standards (5 - 20 μg / ml).

iii. Anthrone Reagent Assay

The anthrone reagent (2 g/l) was dissolved in conc. H_2SO_4

Estimation of carbohydrate concentration involves the preparation of a standard curve using the carbohydrate under examination (inulin in the present study). A range of dilutions of the carbohydrate under consideration were prepared (100 - 300 μg / ml), and 0.5 ml of each was pipetted into each of two tubes. To these 2 ml of anthrone reagent was added. The standards and samples were incubated in a boiling water bath for 10 min. with a glass marble placed on the top of each tube. The tubes were cooled and the absorbance was read at 620 nm using a 1 cm cuvette.

2.1.1.2. Assessment in a crossflow filtration unit.

A more advanced filtration unit is the crossflow filtration (CFF) system where flow is parallel to the membrane surface thereby reducing fouling and concentration polarization. Assessment of NMWCO was in a CFF UF membrane unit obtained from Life Science Laboratories (LSL, Luton UK) (Midas CFF module). For each molecular weight marker (either protein or polysaccharide), a new clean flat sheet LSL Iris polysulphone (PS) UF Membrane 20 kDa NMWCO with an area of 0.01 m^2 was used. Prior to challenging the membrane with a molecular weight marker establishment of CWF was undertaken. The membrane was placed *in situ* and two successive 1 litre aliquots of DDWF were passed through the unit and across a new membrane.

This was performed in a "recycle mode" where the retentate and permeate, after passage through the membrane, are reunited before returning to the reservoir. (Figure 2.2.)

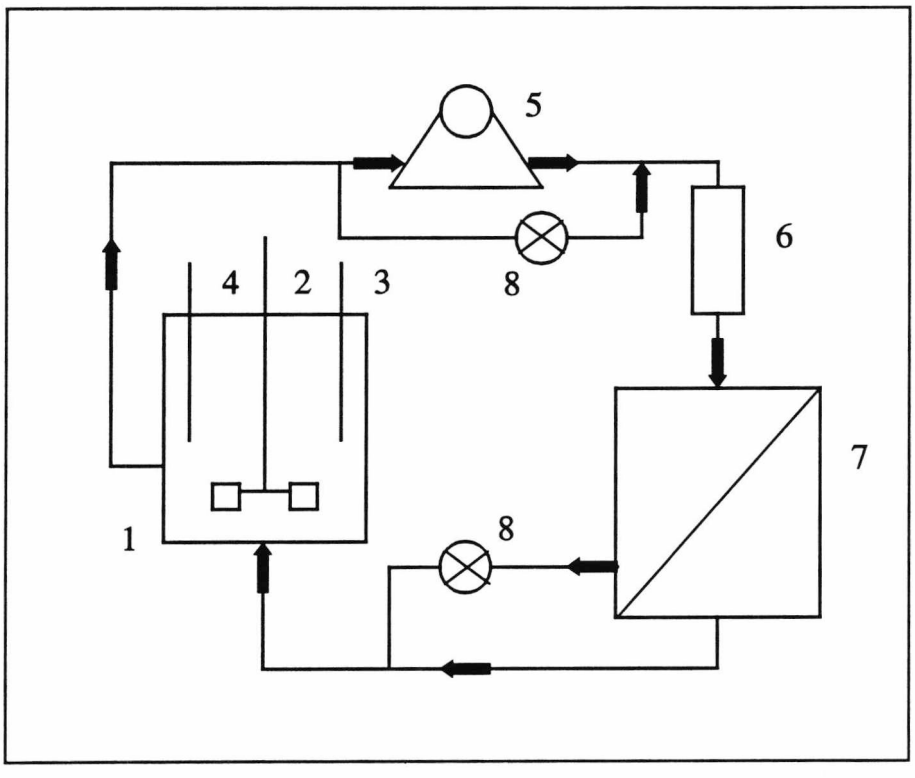


Figure 2.2. Cross flow filtration (CFF) unit operated in a recycle mode. The reservoir **(1)** has a temperature probe **(2)**, a heat element **(3)** and an impeller **(4)** to maintain homogeneity. The buffered molecular weight marker (either protein or polysaccharide) solution is pumped around the system by a gear pump **(5)** and passes through a flow meter **(6)** before entering the CFF unit **(7)** and across the membrane. A valve **(8)** controls the back pressure and the flow rate. The permeate tube was reunited with the retentate tube thus allowing molecular weight marker molecules (either protein or polysaccharide) that had permeated the membrane to re-enter the reservoir.

The back pressure specified by the manufacturer was achieved by manipulating a valve on the retentate line. Each 1 litre aliquot was filtered for approximately 15 min and then drained from the system. The permeate flux after the two litres of DDWF had passed through the membrane was recorded as CWF ($l\ m^{-2}\ hr^{-1}$).

The final DDWF aliquot was drained from the system and a concentrated solution of the molecular weight marker was introduced into the buffer in the reservoir, to give a final concentration of 0.01 % (w/v) in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7) and a volume of 200 ml. Prior to the UF experiments the system was run, with no back pressure, at room temperature, to give an homogenous solution.

A back pressure of 2.5×10^2 kPa was then applied to run the system in a "concentration mode" (Figure 2.3). The flow rate was held at 1500ml min^{-1}, which is equal to a tangential velocity of 2.3 m sec^{-1}. The retentate line valve was adjusted throughout the experiment to maintain a constant level of back pressure. The system was then filtered until 60 ml of permeate (30 % of the initial system volume) had been collected. The final concentrations of permeate and retentate were estimated and the retention coefficient calculated according to the equation (**2.1.**). This assessment was routinely performed in duplicate.

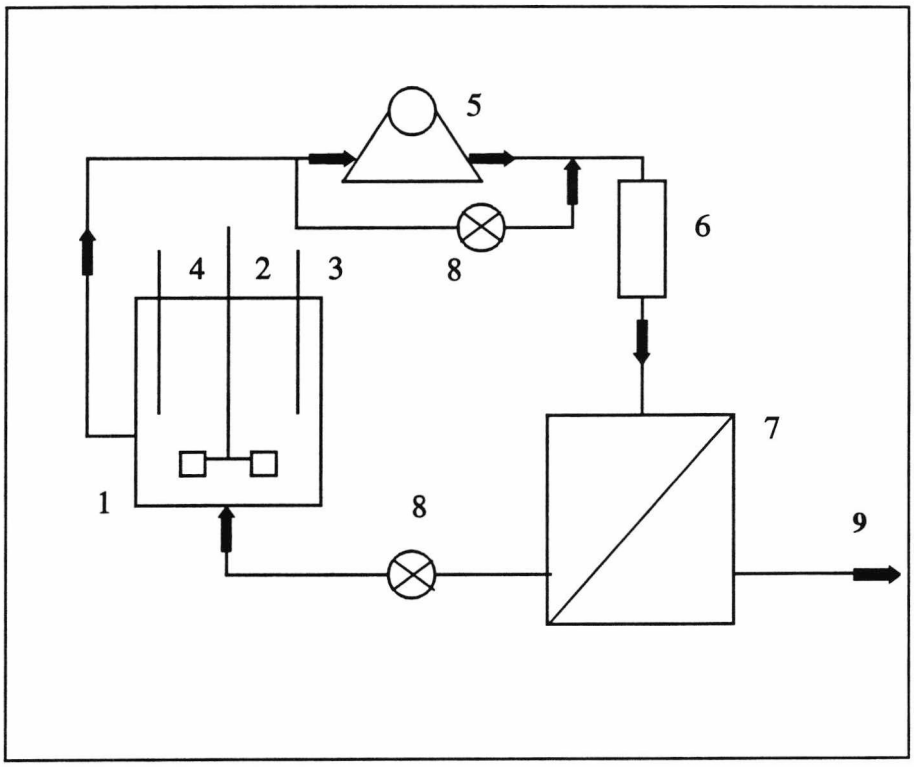


Figure 2.3. Crossflow filtration (CFF) unit operated in a concentration mode. Conditions were the same as Figure 2.2 except the permeate is collected **(9)** and only the retentate is returned to the reservoir.

2.1.1.3. Use and storage of a cross flow filtration unit.

A washing procedure for the CFF unit was instigated after the membrane had been removed and discarded. The gear pump used to drive the system could not be run dry and therefore a cleaning procedure was applied *in situ*, chosen from the those recommended in the LSL manual. The system was flushed with 0.01 M NaOH for 30 min. in a recycle mode to solubilise any remaining protein which adhered to the module, tubing, valves and pump. This solution was drained out and five successive

1 litre aliquots of DDWF were flushed through the system. The system cannot be completely bled and therefore a dead volume of liquid was present which would could act as an ideal breeding ground for microorganisms. Hence after cleaning the system was flushed with a 20 % (v/v) methanol solution and stored with solution *in situ* and bled from the system prior to re-usage.

2.1.1.4. Comparison of membranes in stirred cell and cross flow filtration units.

A LSL 100 kDa NMWCO PS UF membrane was assessed in a SCF unit (see Section 2.1.1.1.) and a CFF units (see Section 2.1.2.) by challenging it with a 0.01 % (w/v) solution of egg albumin in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7). In the SCF unit the system was operated under 3.7 x 10^2 kPa applied pressure and in the CFF unit the system was operated under 1.75 x 10^2 kPa applied pressure. A comparison was provided by challenging a Millipore 100 kDa PTHK (PS) UF NMWCO membrane with the same marker protein, at a operating pressure of 3.7 x 10^2 kPa in the SCF unit. All assessments were performed in duplicate.

2.1.2. Analysis of the operating parameters of a cross flow filtration unit.

2.1.2.1. Concentration effects.

The CFF unit, with a LSL 100 kDa NMWCO PS UF membrane was challenged (see Section 2.1.1.2.) with 200 ml of two different concentrations of egg albumin (1% and 0.01 % w/v) in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7) to determine the effects of concentration polarization at the membrane surface.

This assessment was performed in duplicate.

2.1.2.2. Pressure effects.

The CFF unit, with a Scimat (Scimat Ltd., Swindon UK) 100 kDa NMWCO PS UF membrane, was challenged (see Section 2.1.1.2.) with a 0.01% (w/v) solution of lysozyme in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7) and operated in a recycle mode (Figure 2.2.). This protein, which is relatively permeable to the membrane, is thus able to illustrate the transition to concentration polarization. The volume of the solution in the reservoir was increased to 400 ml to reduce the effect of the sampling procedure on the volume of protein solution. The back pressure increased with time, and at each sample point the period required to collect the permeate aliquot was noted and recorded as the flux in l m^{-2} hr^{-1}. The retention coefficient was calculated at each sample time.

This assessment was performed in duplicate.

2.1.2.3. Temperature effects.

The CFF unit was operated with a Scimat PS UF membrane. These membranes were estimated to have a 60 kD NMWCO by the *in situ* assessment method for estimation of NMWCO (see Section 2.1.1.2.). To investigate the effect of temperature a single molecular weight marker was used, α-amylase, a protein which is derived from Bacillus cereus. This protein has a molecular weight of 49 kDa and is thermostable up to 40°C, and then gradually looses activity until it is completely denatured and inactive, at approximately 70°C.

Ultrafiltration experiments were performed at a range of temperatures and a back pressure of 1.75 x 10^2 kPa. The reservoir contained 0.05% (w/v) α-amylase in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7) in a final volume of 400 ml.

Prior to the addition of protein to the solution, the flux, with buffer only, was measured for each membrane. After addition of protein to the reservoir an initial sample (5ml) was taken from the 400 ml reservoir and divided into two: one aliquot was stored at 4°C, and the other aliquot maintained in a water bath at the same temperature as the reservoir for the duration of the experiment, to act as a control. Aliquots (5ml) were taken at 5, 15, 30 and 45 min. from the permeate, retentate and reservoir, respectively, and stored at 4°C. The experiment was run at five different temperatures 12, 25, 30, 38, 50 and 60°C. At each sample time the period required to collect the permeate aliquot was noted and recorded as the flux in l m^{-2} hr^{-1}. The initial flux was also measured at the start of the experiment as the time taken to collect a 5 ml aliquot from the initial flow through the previously drained permeate tube. The average of these values was plotted against temperature to establish a permeate flux profile.

All samples that had been stored in the fridge after sampling were allowed to equilibrate at room temperature prior to simultaneous analysis of total protein content (to yield the retention coefficient of the protein at the experimental temperature), enzyme activity and conformational changes to the protein.

This study was performed in triplicate except the analyses of enzyme activity and conformational change studies which, although performed in duplicate, were undertaken on one set of data collected at the five experimental temperatures.

2.1.2.3.1. Protein analysis

Total protein concentration was measured using Sigma kit number P5656, which was a rapid Lowry assay. Specific activity of the α-amylase was measured using Sigma kit number 577-3, a colorimetric method which involved a quantifiable interaction between α-amylase and its substrate.

Changes in conformation were measured using fluorescence spectroscopy. The aromatic amino acids of proteins fluoresce at various wavelengths when excited. The application of fluorescence spectroscopy to characterisation of protein confirmation has been described previously (Pace, Shirley & Thomson, 1990). Aromatic amino acids are hydrophobic and tend to be buried in the core of the protein. Hence, when denaturation occurs they may be exposed to the environment and a shift in the emission spectra between the native and denatured state is observed. In this study fluorescence spectroscopy was performed at 25^{o}C by exciting the protein molecules at 280 nm and then scanning the emission spectra between 280 and 450 nm using a Perkin-Elmer LS-5B Luminescence Spectrophotometer. Samples were diluted to the correct range by the addition of 20 mM KH_2PO_4/Na_2HPO_4 (pH 7).

2.2. Supported liquid membranes.

2.2.1. Transport rate of phenylalanine and chloride ions.

Separations using a supported liquid membrane were based on the method of Thien et al (1988), who considered a liquid emulsion membrane (LEM) system for the transport of phenylalanine ions, using a chloride ion gradient to drive an ion-exchange process at the aqueous-organic interfaces. This enabled carrier mediated facilitated transport of phenylalanine ions to occur from the source phase into the receiving phase (see Section 1.3.2.).

The supported liquid membrane (SLM) system consists of a cationic carrier, tricaprylylammonium chloride (TCAC) (Sato et al, 1984, Deblay et al, 1990) mixed with decanol, as the organic phase, at a concentration of 10% (v/v) of TCAC. The mixture was soaked into a polymeric support for 5 minutes. The support used was a Scimat ethylene-tetrafluoroethylene (ETFE) membrane of 110 μm thickness, 0.2μm pore size and 65 % porosity cut, to an area of 0.01 m^2. The membrane was removed from the organic phase and blotted dry before being placed in a Scimat designed unit as shown in Figure 2.4.

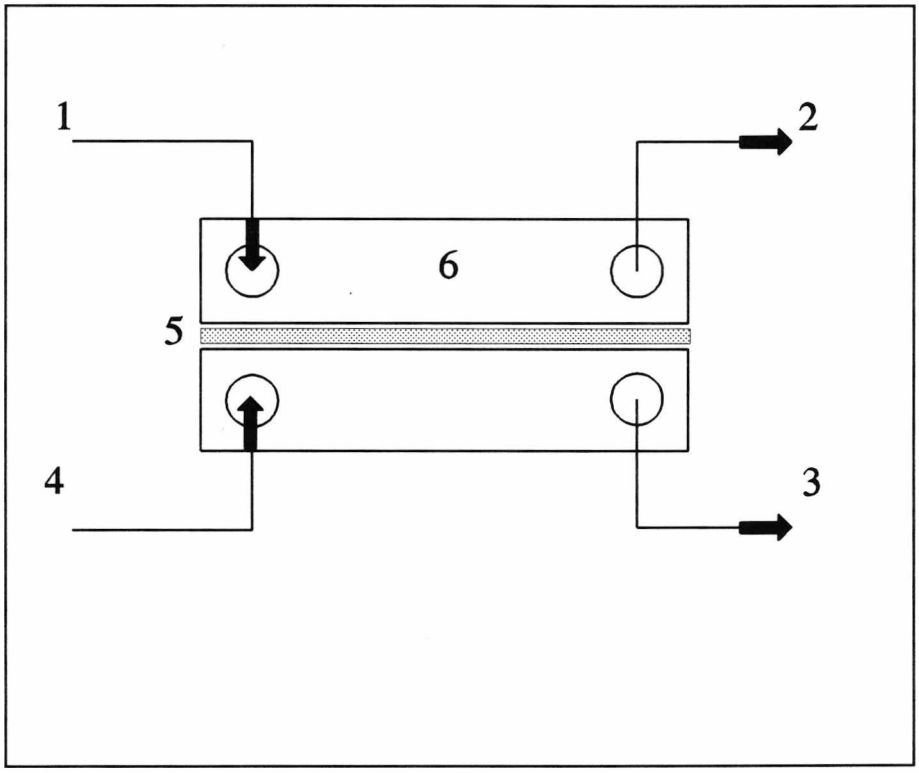


Figure 2.4. Scimat supported liquid membrane (SLM) unit. The source phase consisted of 12.1 mM phenylalanine in 50 mM Na_2HPO_4 (pH 11) and was pumped into the unit (**1**), across the membrane surface and out of the unit (**2**). The receiving phase consisted of 0.5 M NaCl in 50 mM Na_2HPO_4 (pH 11) and was pumped into the unit (**4**), across the membrane surface and out of the unit (**3**). The SLM (**5**) consisted of the cationic carrier, tricaprylylammonium chloride (TCAC) mixed with decanol, as the organic phase, at a concentration of 10% (v/v) of TCAC. The support used was a Scimat ethylene-tetrafluoroethylene (ETFE) membrane cut to an area of 0.01 m^2 and held in a modified cross flow unit (**6**).

Two different phases were used to drive the carrier-mediated transport of the amino acid across the membrane. The source phase consisted of 12.1 mM phenylalanine in 50 mM Na_2HPO_4 adjusted to pH 11 with 1M NaOH. The receiving phase consisted of 0.5 M NaCl in 50 mM Na_2HPO_4 adjusted to pH 11 with 1M

NaOH. The two phases were maintained in separate reservoirs, each of which was immersed in a constant temperature water-bath at 35°C (Figure 2.5.).

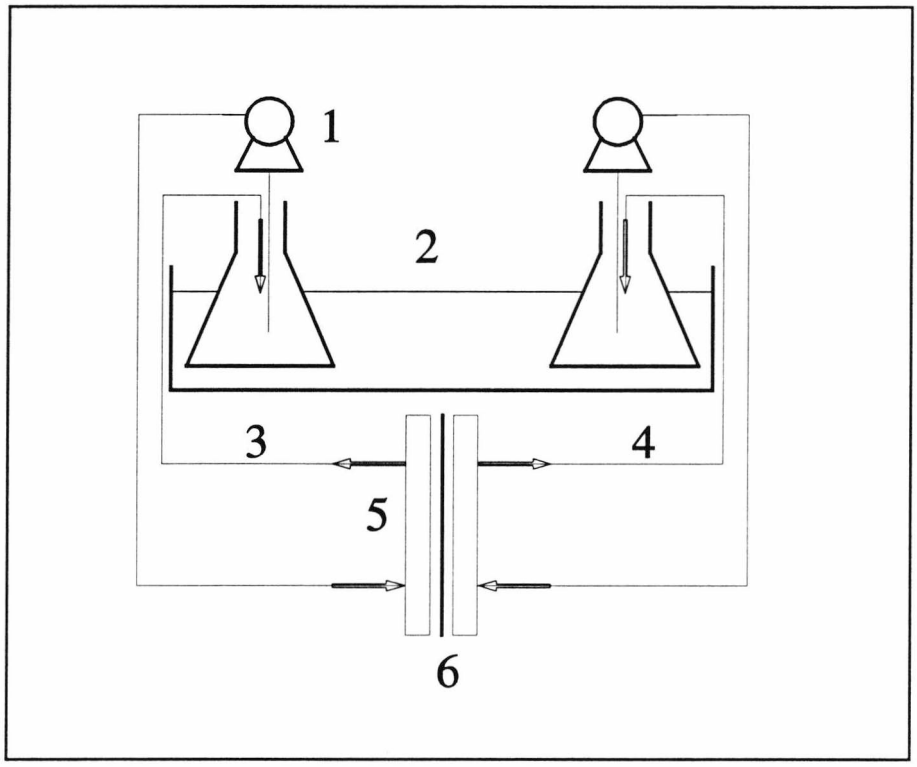


Figure 2.5. Experimental apparatus for investigation of a supported liquid membrane (SLM). The two aqueous phases were pumped by a dual head peristaltic pump **(1)** from the waterbath **(2)**, where the source phase **(3)** and receiving phase **(4)** were held at the operating temperature, to the Scimat SLM unit **(5)** which contained the membrane **(6)**.

The solutions were pumped round the system, (50 ml/min.), parallel to the membrane surfaces (crossflow mode) before being returned to the reservoirs . Samples were taken from the receiving reservoir, and phenylalanine ion concentrations were measured by the absorbance at 257 nm using a 1 cm cuvette.

This investigation was performed in duplicate.

2.2.1.1. The effect of the components on the rate of carrier mediated transport.

The system was run as described in the above section except that each of the components listed below were selectively removed and the effects on the rate of transport recorded.

i. The receiving phase was used without added chloride ions, and the effects on the transport rate of phenylalanine ions to the receiving phase and on the pH, in both the source and receiving phase, were measured.

ii. The source phase was used without added phenylalanine ions, and the effect on the transport rate of chloride ions to the source phase and on the pH in both the source and receiving phase, were measured.

iii. The organic phase was used without added carrier molecules and the effects on the phenylalanine and chloride ion concentrations and on the pH in both the source and receiving phase were measured.

Chloride concentration was measured by using a Corning 926 Chloride Analyzer. Sodium concentration was measured by a Perkin Elmer 1100 B atomic adsorption spectrophotometer.

2.2.1.2. The effect of a substituted potassium buffer.

A buffer based on potassium, not sodium, (K_2HPO_4 buffer) was substituted for the Na_2HPO_4 buffer that was routinely used to study the effect of the sodium ions, which are present as the salt NaCl, more closely. Phenylalanine and chloride ion concentrations in both the source and receiving phase and the pH were measured.

2.2.1.3. Comparison of carrier molecules.

The system described above (Section 2.2.1.) is based on an anionic carrier exchange process. Various carrier molecules can be utilised to facilitate such transport. Instead of TCAC, other carrier molecules, such as tetraoctylammonum bromide (TOAB) can be used. It is also possible to operate a SLM system with a cationic carrier due to the zwitterionic nature of amino acids. For the latter, in the present study, di(-2-ethylhexyl) phosphoric acid (D2EHPA) was used in an exchange with hydrogen ions as the counter ions. This method is based on that described by Itoh et al (1990) who investigated the transport of amino acids across a LEM system by D2EHPA. The present method differed from the method of the above authors by applying the system to a SLM format.

The carrier was used at the same concentration as the TCAC (0.2 M) and the hydrogen ion concentration, in the form of HCl, was at a 1 M concentration.

2.2.1.4. Comparison of different counter-ions.

The system described above (Section 2.2.1.) was routinely operated with a chloride ion gradient as the driving counter ion. It was also used with different counter ions, nitrate and sulphate; the counter ion was present only in the receiving phase at a concentration of 0.5 M.

2.2.2. Transport rate studies.

Many factors, both physical and chemical are able to influence the rate of carrier mediated transport of phenylalanine ions across the SLM.

2.2.2.1. Physical factors.

The system described above (Section 2.2.1.) was run at two different temperatures, 25°C and 45°C, and at three different flow rates; 50, 100 and 300 ml/min.

2.2.2.2. Physicochemical factors.

The system described above (Section 2.2.1.) was operated with a range of chloride, phenylalanine, carrier concentrations and at a range of pH values.

TCAC does not have a specific molecular weight, having variable length carbon chains. Tetraoctylammonium bromide (TOAB), which has a symmetrical structure and a defined molecular weight, was also used as a carrier (Neplenbroek et al, 1992c) . TCAC is a synthetic molecule with an approximate molecular weight of

438 Da and hence the estimated concentration was 0.2 M. Therefore, TOAB was also used at a concentration of 0.2 M.

2.2.3. Stability Studies

2.2.3.1. Organic phase effects.

Methods to measure concentration of the carrier in the organic phase are not readily available and tend to be destructive in their methodology. In the present study concentration of the carrier (TCAC) in the organic phase was estimated by extracting it with solvent, and performing a colorimetric reaction (A. Kemperman, University of Twente, personal communication).

As a standard, the membrane was prepared as usual with the organic solvent and carrier using $25cm^2$ of support membrane. This was placed in a glass flask containing 20 ml chlorobenzene and shaken for approximately 10 min. to extract the organic phase. A solution of the monosodium salt of 4-(2-pyridylazo)resorcinol (PAR^-) was prepared by dissolving a solution of 1 mM PAR^- in 0.1M $NH_4)_2SO_4$ (pH 10). In a glass stoppered bottle, 9ml of chlorobenzene, 1ml of PAR^- and a range of volumes of the chlorobenzene-extracted carrier (0 to 500 µl) were added. The total volume was made up to 10.5 ml with chlorobenzene. The bottle was shaken vigorously for several minutes and excess deionised water added to wash the contents which were then allowed to settle for 10 min. The bottom organic layer was sampled with a micropipette and centrifuged for 10 min. The colour was measured at 395nm using a 1 cm cuvette against a chlorobenzene blank.

Samples of membrane, of the same area as the standard, were tested after a degree of artificial disturbance. To investigate stability of decanol a standard curve was prepared with this organic phase as well as three other solvents, 2-nitrophenyl octyl ether (NPOE), 1,3 diethylbenzene and 1,3,5 triethyl-benzenol (1,3,5, TEB).

Each set of four samples of membrane was impregnated with one of the four organic phases. One of the membrane samples was assayed immediately for the carrier content. The other three samples were placed, in three separate 1 litre conical flasks, each containing 800 ml of 50 mM Na_2HPO_4 buffer adjusted to pH 11 with 1M NaOH. These were then placed on a magnetic stirrer, to create shear forces, in order to simulate those that are created in situ but to a greater extent. Over an eight hour period the three remaining membrane samples were measured for their carrier content.

2.2.3.2. Carrier effects.

A comparison was made between TCAC and the more regular structure TOAB by examining transport in the abscence of a driving chloride gradient.

2.2.3.3. Chloride ion effects.

The effects of chloride ions were investigated in three ways;

i. The level of chloride ions in the receiving phase were reduced to 0.1 M, and secondly removed completely to reveal the effects on the transport rate of phenylalanine ions.

ii. Two concentrations of chloride ions (0.1 and 1.0 M) were added to both aqueous phases to remove the chloride ion gradient, but retain the presence of

chloride ions to reveal if there was an added stability effect caused by the presence of chloride ions on both sides of the membrane.

2.2.3.4. Comparison of decanol and NPOE as organic phases

A comparison was made between decanol and NPOE, in the organic phase, both with a 0.1 M chloride ion gradient as well as with 0.1 M chloride ion in both aqueous phases.

2.2.4. Specificity studies

2.2.4.1. Molecular exchange processes.

Neplenbroek et al (1992d) have shown that a direct one-to-one exchange process occurs at the organic-aqueous interphase for the transport of nitrate ions in exchange for chloride ions. This can be quantified by measuring the molar exchange rate of the components of the system. The carrier transport factor (CTF) is defined as follows;

$$CTF = M(Cl)_{Feed} / M(Phe)_{Receiving} \qquad \textbf{(2.2.)}$$

where M = molar concentration, Cl = Chloride ions and Phe = Phenylalanine.

In the present study this factor was calculated at each sample time to describe the exchange process for the system described in Section 2.2.1.

2.2.4.2. Transport of mixtures of amino acids.

Three different amino acids were chosen for transport and the experiments were run in the same manner as above except the pH was corrected to either pH 5.48 or 11. The effect of the pH changes on the molecular charge of the amino acids are shown in Table 2.2.

Each amino acid was used at a 10 mM concentration. Analysis of the amino acid mixtures was performed at ambient temperatures using a Jones Chromatography (Mid Glamorgan, UK) Apex Cyano RP 5U HPLC reverse phase C18 column (25 cm x 4.6 i.d.) at a flow rate of 0.8 ml/min. The mobile phase was methanol/10 mM orthophosphoric acid (6:4, v/v) and detection was in conjunction with *O*-phthaladehyde (OPA) derivitisation (Gardner & Miller III 1980).

This assessment was performed in duplicate.

Amino acid	pH	pKa Carboxyl Group	pKa Amino Group	Charges present	Overall charge
Phe	5.48	1.83	9.13	1+, 1-	0
Lys	5.48	2.20	8.90	2+, 1-	1+
Asp	5.48	1.88	9.60	2-, 1+	1-
Phe	11	1.83	9.13	1-	1-
Lys	11	2.20	8.90	1-	1-
Asp	11	1.88	9.60	2-	2-

Table 2.2. The effects of pH on the overall charge and charges present on aspartic acid, lysine and phenylalanine.

3. Results

3.1. Ultrafiltration membranes.

3.1.1. Assessment of nominal molecular weight cut-off of ultrafiltration membranes.

A key parameter for characterisation of ultrafiltration (UF) membranes is the nominal molecular weight cut-off (NMWCO). This series of experiments was an investigation of the assessment protocols to establish the NMWCO of UF membranes. Implicit within this was a consideration of the validity of the manufacturer's published data.

3.1.1.1. Assessment in a stirred cell filtration unit.

Figure 3.1 shows typical retention coefficient data obtained for a Millipore 100 kDa NMWCO PTHK UF membrane in a stirred cell filtration (SCF) unit. The NMWCO was obtained by interpolation at a retention coefficient of 0.9 to yield a value of 105 kDa, which is a good correlation with the manufacturer's published data.

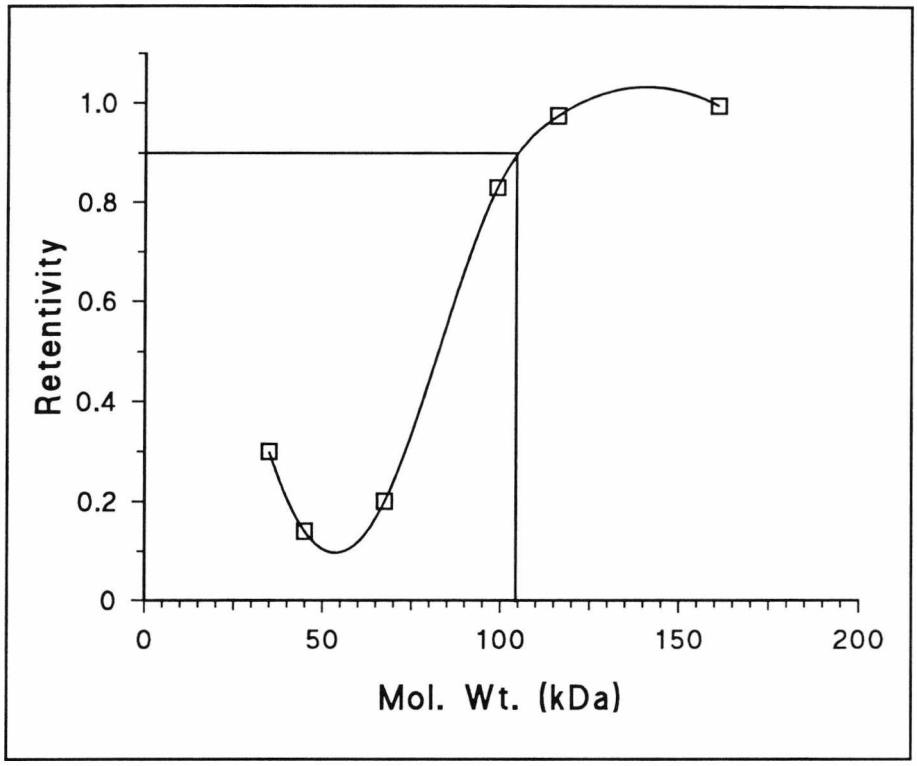


Figure 3.1. Graph of molecular weight marker against a retention coefficient. A Millipore 100 kDa PTHK NMWCO UF membrane was assessed in a 43 mm diameter Amicon stirred cell filtration unit. A marker (either protein or polysaccharide) solution of 0.01% w/v concentration in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7) was filtered at an operating of temperature of 25^oC and a pressure of 3.5×10^2 kPa.

3.1.1.2. Assessment in a crossflow filtration unit.

Figure 3.2. also shows typical retention coefficient data for assessment of a LSL 20 kDa NMWCO Polysulphone (PS) UF membrane in a crossflow filtration (CFF) unit. The NMWCO was obtained by interpolation to yield a value of 12.5 kDa. This does not represent a good correlation with the manufacturer's published data.

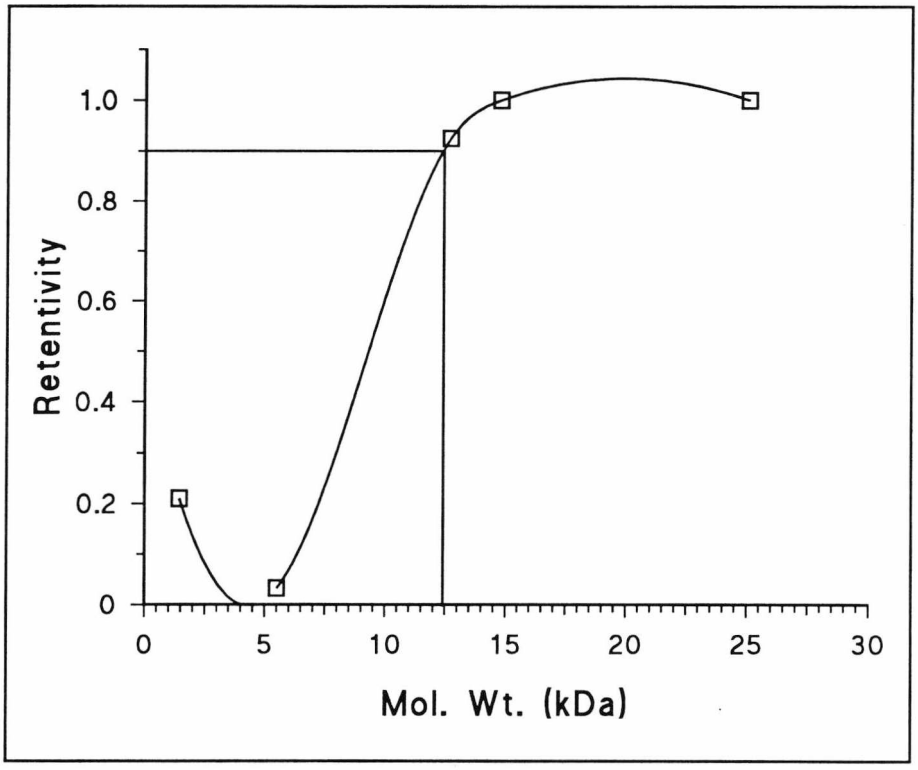


Figure 3.2. Graph of molecular weight marker against a retention coefficient. A LSL 20 kDa PS NMWCO UF membrane was assessed in a LSL crossflow filtration unit. A marker solution of 0.01 % (w/v) in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7) and a final volume of 200 ml, and operated at 25^{o}C and a back pressure of 1.75 x 10^2 kPa.

3.1.1.3. Comparison of membrane assessment in a stirred cell and a crossflow filtration units.

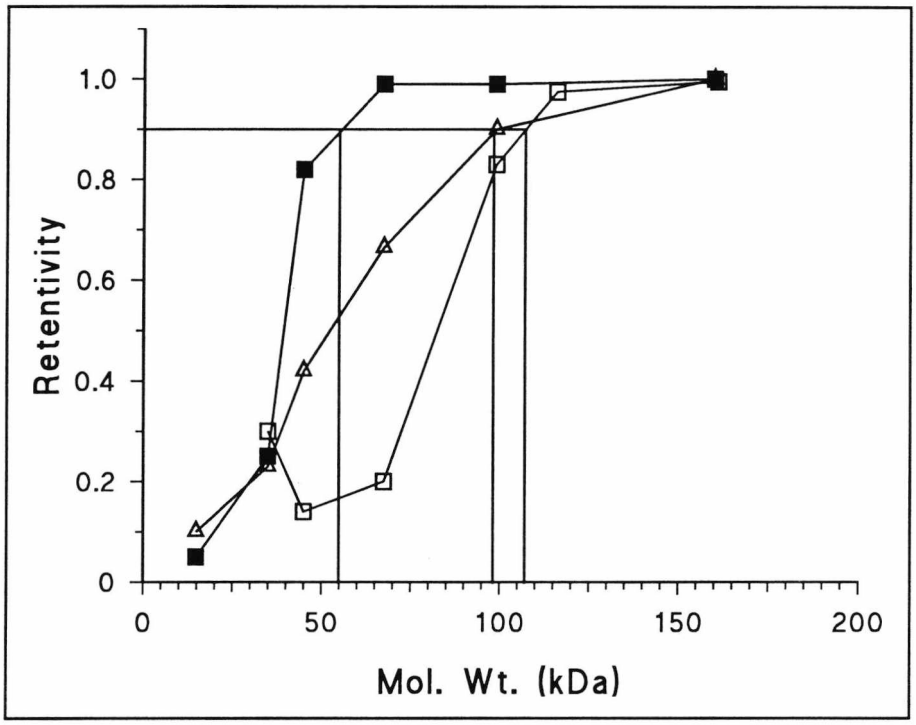


Figure 3.3. Comparison of estimated nominal molecular weight cut-off (NMWCO) of membranes in a stirred cell filtration (SCF) and a crossflow filtration (CFF) units. A LSL 100 kDa PS NMWCO UF membrane (■) and a Millipore 100 kDa PTHK NMWCO UF membrane (□) were assessed in a SCF unit. A LSL 100 kDa PS NMWCO UF membrane was also estimated in a CFF unit (▵). The membranes in both the CFF and SCF units were challenged with a 0.01 % (w/v) solution of each molecular weight marker in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7). In the SCF unit the system was run under a pressure of 3.5 x 10^2kPa, and in the CFF unit the pressure was 1.75 x 10^2kPa, both were operated at 25^{o}C.

A LSL 100 kDa NMWCO PS UF membrane was assessed under two different regimes for comparative analysis, using a SCF and a CFF unit. For further comparison a Millipore 100 kDa NMWCO PTHK UF membrane was also assessed in a SCF unit. (Figure 3.3). The LSL 100 kDa NMWCO membrane gave an apparent NMWCO of 55 kDa, in a SCF unit, but the NMWCO was 95 kDa in a CFF unit, whereas a Millipore 100 kDa NMWCO had a value of 105 kDa in a SCF unit.

3.1.2. Analysis of the operating parameters of a crossflow filtration unit.

Ultrafiltration systems, either CFF or SCF, have many potential variables. An investigation was undertaken on an CFF unit in an attempt to assess the observed behaviour.

3.1.2.1. Concentration effects.

The initial concentration of solute, usually protein, above the membrane surface is critical to the membranes performance. The significance of this dependency was examined by operating a CFF system in a concentration mode, as outlined in Section 2.1.1.2., with two initial concentrations of buffered egg albumin solution (0.1 % and 0.01 % w/v). Table 3.1. outlines these effects and shows that there is a significant change in retention coefficient. Egg albumin has a molecular weight of 45 kDa.

Membrane	LSL 100 kDa NMWCO	LSL 100 kDa NMWCO
Pressure (kPa x 10^2)	1.75	1.75
Protein concn. % (w/v)	1.0	0.01
Retention coeff.	0.98	0.3

Table 3.1. The effect of different concentrations on the retention coefficient assessed in a crossflow filtration (CFF) unit. Clean, fresh LSL 100 kDa PS UF membranes were challenged in a CFF system operated in a concentration mode with 0.01 % or 0.1 % (w/v) solution of egg albumin in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7) with a final volume of 200 ml and operated under a pressure of 1.75 x 10^2 kPa and at a temperature of 25°C.

3.1.2.2. Pressure effects.

Pressure is a key parameter in the operation of ultrafiltration systems because of its pivotal role in controlling concentration polarization at the membrane surface. The effect of increased pressure on the retention coefficient and permeate flux was measured with a 0.01 % (w/v) lysozyme solution using a CFF unit operated in a recycle mode. Lysozyme has a molecular weight of 14.8 kDa. The increase of pressure resulted in a linear increase in permeate flux, whereas the retention coefficient had an initial rapid decrease and then tended to a constant value of 0.32 (Figure 3.4.).

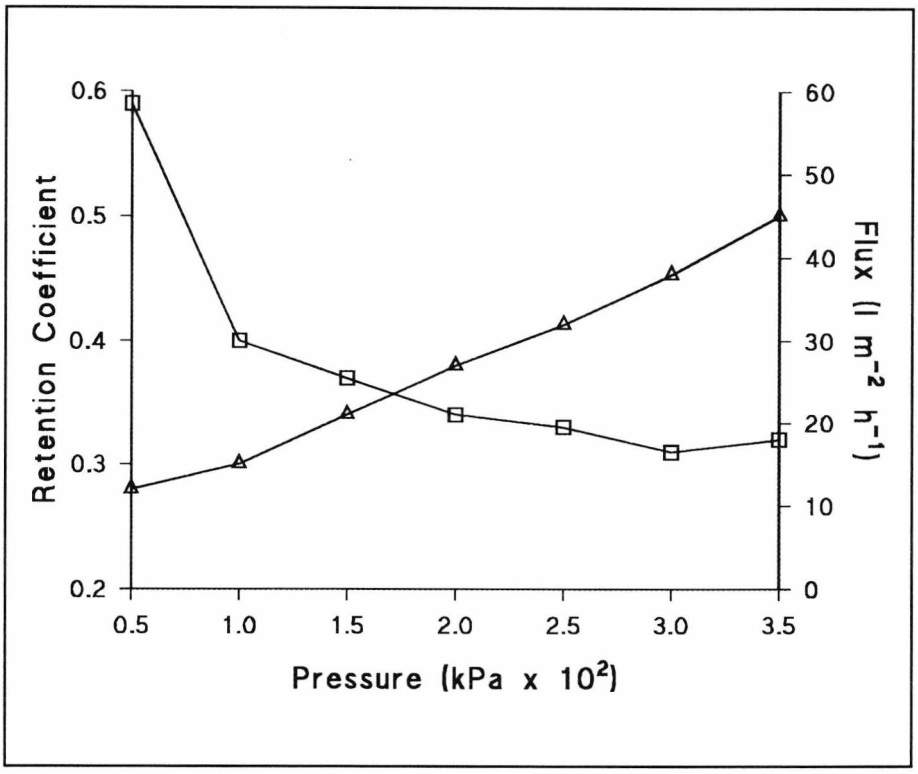


Figure 3.4. The effect of increasing pressure on the retention coefficient and permeate flux. A 0.01% (w/v) solution of lysozyme in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7), final volume of 400 ml, was filtered in a recycle mode with a Scimat 100 kDa NMWCO PS UF membrane. At each sample point the permeate flux was measured (△) and the retentivity calculated (□).

3.1.2.3. Temperature effects.

Temperature has a significant effect on physical properties of the system such as viscosity and diffusion. These factors in turn have a very influential effect on the concentration polarization at the membrane surface. However temperature can also have a strong influence upon other physicochemical components of the system such as the stability of proteins. Thus temperature is a link between many inter-related phenomena.

3.1.2.3.1. Effect of temperature on flux.

Theory predicts that higher operating temperatures will lead to reduced concentration polarization at the membrane surface which in turn will allow higher permeate flux. Figure 3.5. shows the effect of temperature on permeate flux and reveals that a maximum flux rate occurs at approximately $30^{o}C$ which represents the interplay of several factors.

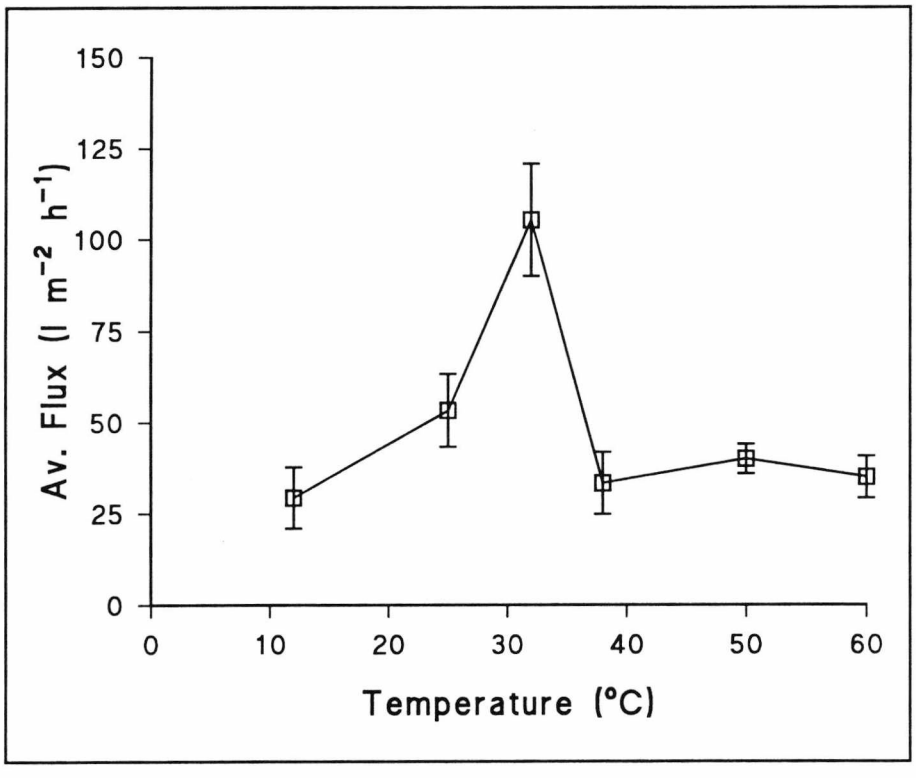


Figure 3.5. Effect of temperature on the average flux. The permeate flux was measured over the experimental period of 45 min. The experiment was performed in triplicate. The graph shows the average value with error bars which represent the standard deviation. Ultrafiltration was undertaken on a 0.05% (w/v) solution of α-amylase (molecular weight of approximately 50 to 55 kDa) in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7), final volume of 400 ml, filtered across a Scimat 60 kDa NMWCO PS UF membrane in a CFF system operated in a recycle mode operated under a pressure of 1.75 x 10^2 kPa.

3.1.2.3.2. Effect of temperature on the retention coefficient.

The graph in Figure 3.6 reveals that retention coefficient decreases with increasing temperature.

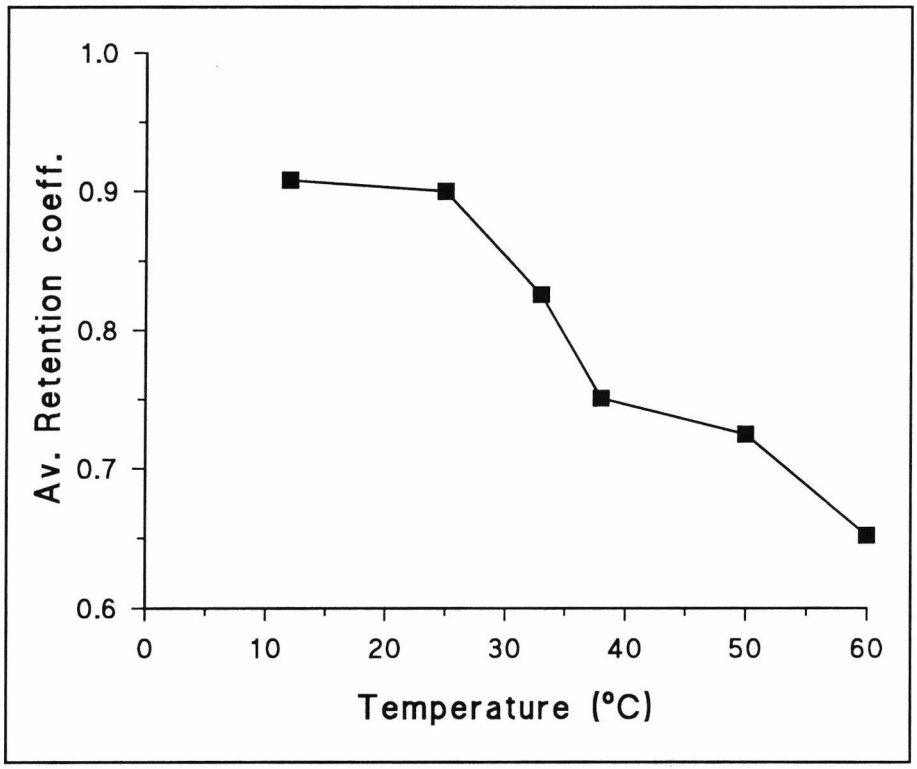
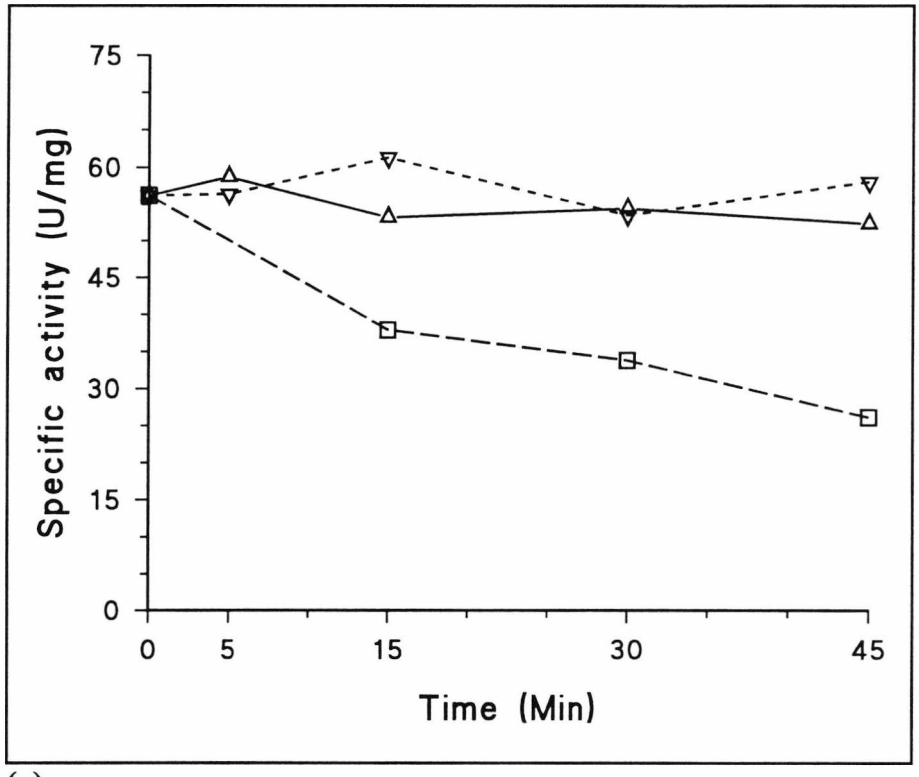


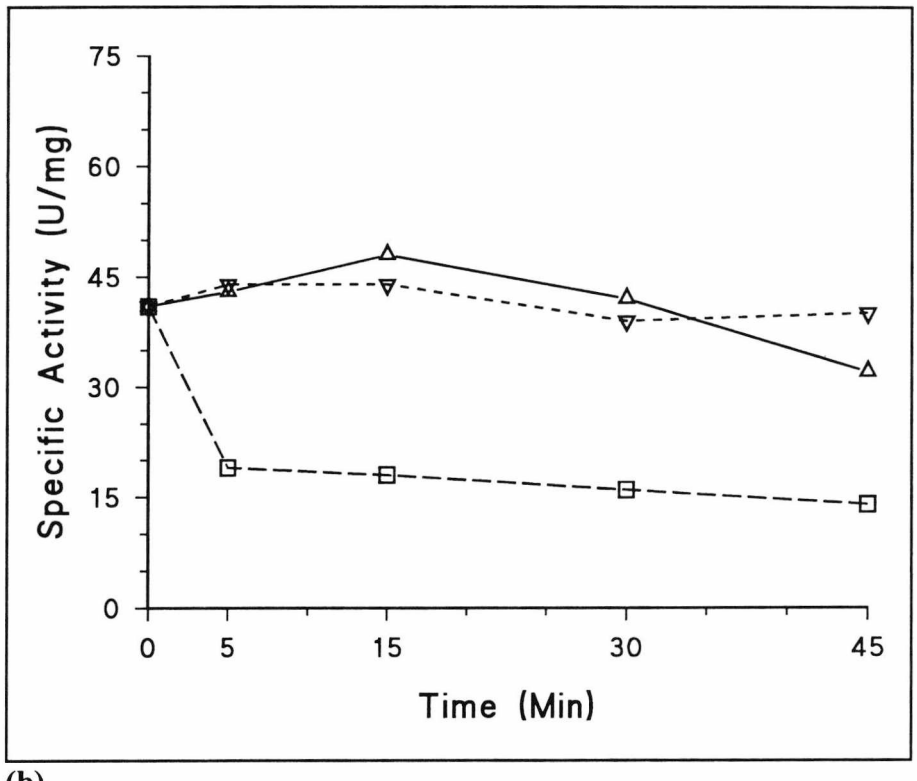
Figure 3.6. Effect of temperature on the average retention coefficient. The retentivity was measured over the experimental period of 45 min. Initial conditions were the same as those in Figure 3.5.

3.1.2.3.3. Effect of temperature on the specific activity of protein in the retentate, permeate and reservoir.

Although temperature is influential in the reduction of concentration polarization at the membrane surface it can also be detrimental to the tertiary structure of the α-amylase (mol. wt. 49 kDa) used as the marker protein. The specific activity of the protein is indicative of the tertiary structure of the molecule. Figure 3.7 a & b. reveal that protein sampled from the permeate has the lowest activity, and the degree of activity reduction is proportional to the increase in temperature.



(a)

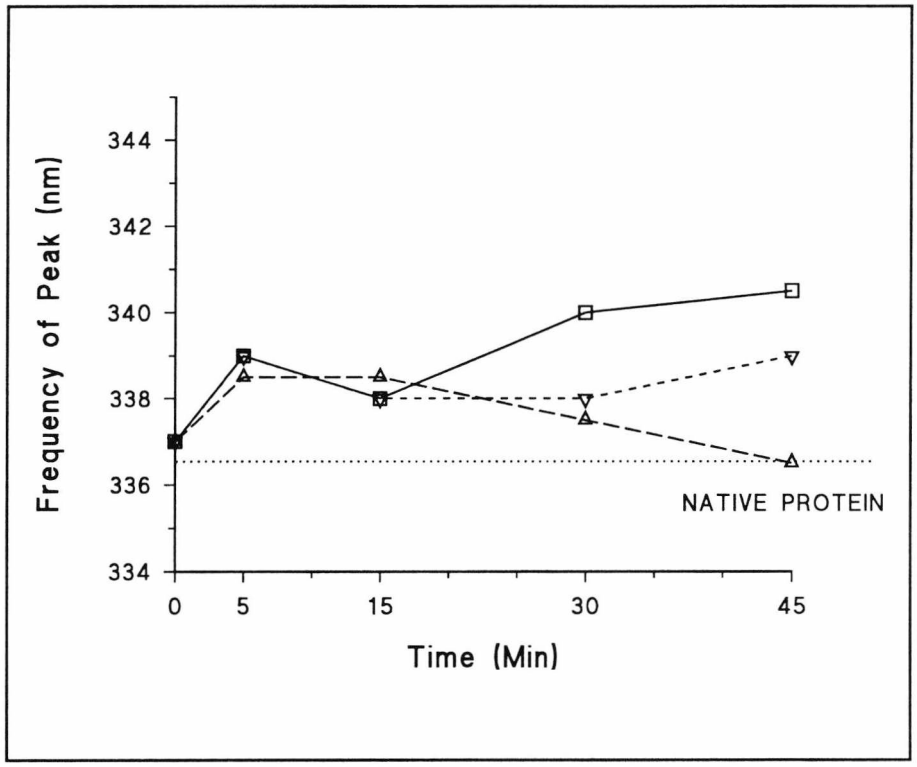


(b)

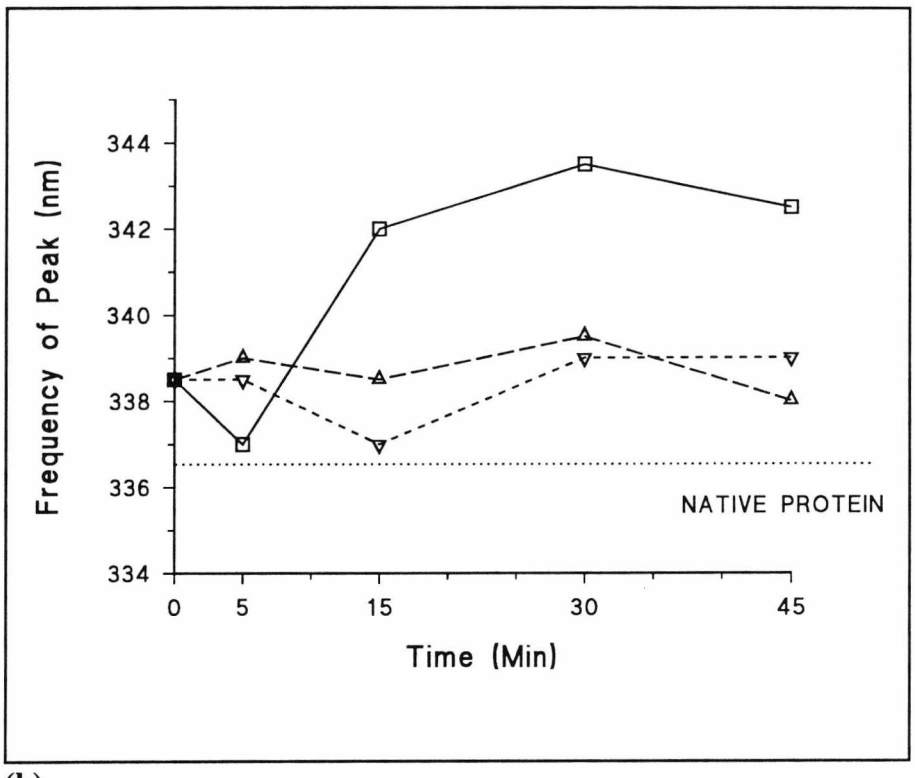
Figure 3.7. Specific activity of 0.05 % (w/v) α-amylase during ultrafiltration in a crossflow filtration (CFF) unit. Samples of permeate (□), retentate (△) and the reservoir (▽) during CFF at (a) 25°C and (b) 60°C were assayed for specific activity. Initial conditions were the same as those in Figure 3.5. Similar data was obtained at 12, 30, 38 and 50°C.

3.1.2.3.4. Effect of temperature on the fluorescence emission.

Fluorescence emission spectra are characteristic of the tertiary structure of protein molecules. An example of the shift in fluorescence spectra is shown in Figure 3.8. a & b. The data reflect the results obtained for the specific activities. Protein samples from the permeate show a greater shift in the spectrum, especially for samples obtained at the higher temperatures.



(a)



(b)

Figure 3.8. The shift in fluorescence emission spectra of a 0.05 % (w/v) concentration α-amylase during ultrafiltration in a crossflow filtration (CFF) unit. The fluorescence emission spectra was measured for aliquots sampled from the permeate (□), retentate (△) and reservoir (▽) during CFF at (a) 25°C and (b) 60°C. Initial conditions were the same as those in Figure 3.5. Similar data was obtained at 12, 30, 38, and 50°C.

3.1.2.3.5. Effect of temperature on control samples.

Samples were taken from the reservoir and maintained at the operating temperature of the CFF unit, for the duration of the experiment, without undergoing ultrafiltration, to act as a control for the analysis. The protein was stable until 40°C and then began to lose activity at higher temperatures (Figure 3.9).

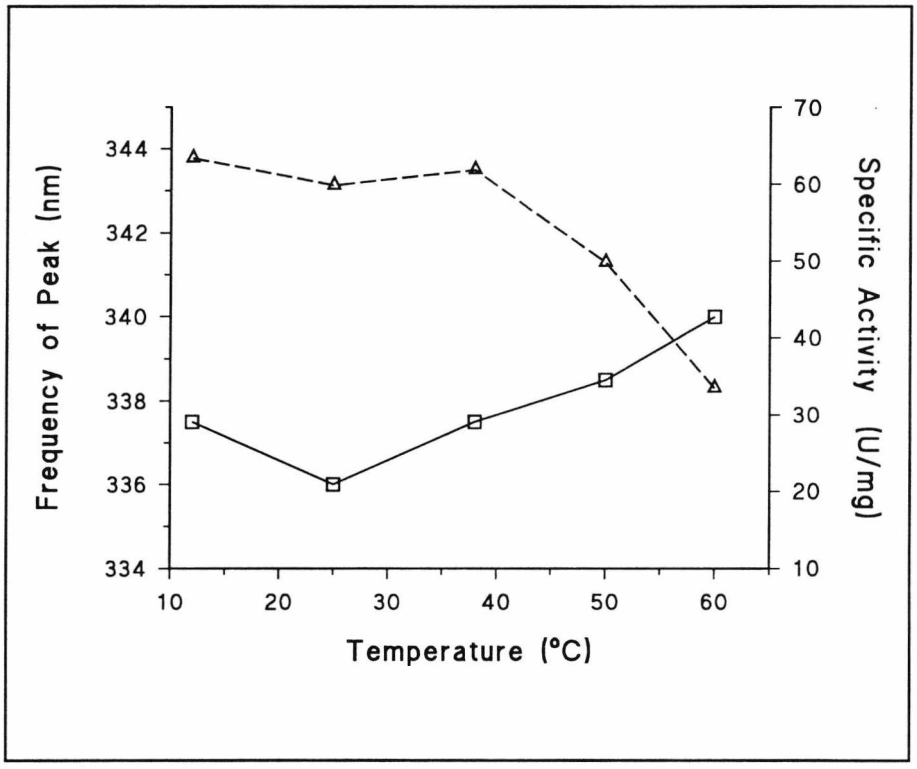


Figure 3.9. Temperature effects on control samples of α-amylase. Variations in specific activity (△) and protein conformation from fluorescence spectra (□) were measured for samples of 0.05% (w/v) α-amylase in 20 mM KH_2PO_4/Na_2HPO_4 (pH 7) held at the operating temperatures for duration of experiment.

3.2. Supported liquid membranes.

3.2.1. Transport studies.

3.2.1.1. Phenylalanine and chloride ion transport.

The transport rate across the membrane, in the presence of all the system components, was calculated for the movement of phenylalanine ions from the source phase to the receiving phase, and for movement of chloride ions to the source phase from the receiving phase. Figure 3.10 reveals that the transport rates are linear over the experimental period.

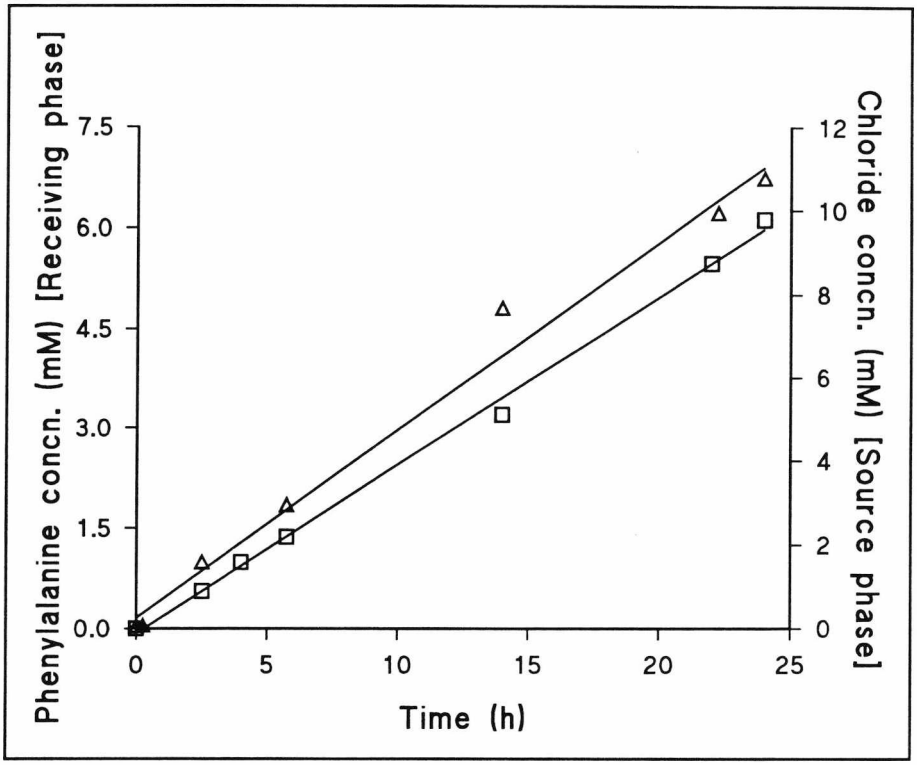


Figure 3.10. The transport of phenylalanine and chloride ions into the receiving and source phase respectively. The initial conditions were 12.1 mM phenylalanine in 50 mM Na_2HPO_4 adjusted to pH 11 with 1M NaOH, in the source phase, and 0.5 M NaCl in 50 mM Na_2HPO_4 adjusted to pH 11 with 1M NaOH in the receiving phase. The carrier, tricaprylylammonium chloride (TCAC), was present in decanol as the organic phase at a concentration of 10% (v/v), and contained in a microporous ethylene-tetrafluoroethylene (ETFE) membrane of 110 μm thickness and 0.2μm pore size, with a 65 % porosity. The membrane was cut to an area of 0.01 m^2. The two phases were maintained at 35 ^oC and the flow rate was 50 ml/min parallel to the membrane surface. The transport rate for the movement of phenylalanine to the receiving phase (□) was 0.25 mM/h and for chloride into the source phase (△) was 0.45 mM/h.

3.2.1.1.1. The effect of the components on the rate of carrier mediated transport.

The model given in Figure 1.10 (Section 1.3.2.) indicates the potential role each component plays in the transport of phenylalanine across the membrane. To investigate the validity of this model the substrates (phenylalanine and chloride) and the carrier were systematically removed to investigate the effect on the rates of transport of phenylalanine and the counter ion, chloride, as compared to transport when all components were present.

The pH of both phases remained constant throughout all experiments indicating that there wasn't any significant movement of these ions.

The results are summarised in Table 3.2., which shows the rates of phenylalanine and chloride transport under certain modified conditions.

Component removed	Transport rate of phenylalanine (mM/h)	Transport rate of chloride (mM/h)
Phenylalanine	-	0.306
Chloride	0.098	-
Carrier	0.0009	0.033
[All components present]	0.25	0.45

Table 3.2. Transport rates of phenylalanine and chloride in the absence of individual components. Transport was measured for a SLM system under the same conditions as described in Figure 3.10 except where indicated.

3.2.1.1.2. The effect of a substituted potassium buffer.

Sodium ions were supplied in the form of NaCl, and were therefore present at a 0.5 M concentration in the receiving phase, and could possibly interfere with the transport process. A potassium based phosphate buffer was used in place of the sodium-based buffer, and the sodium concentration was measured in the source phase by atomic adsorption spectrophotometry. Figure 3.11 indicates the very small change in concentration of potassium ions in the receiving phase, compared to other ions.

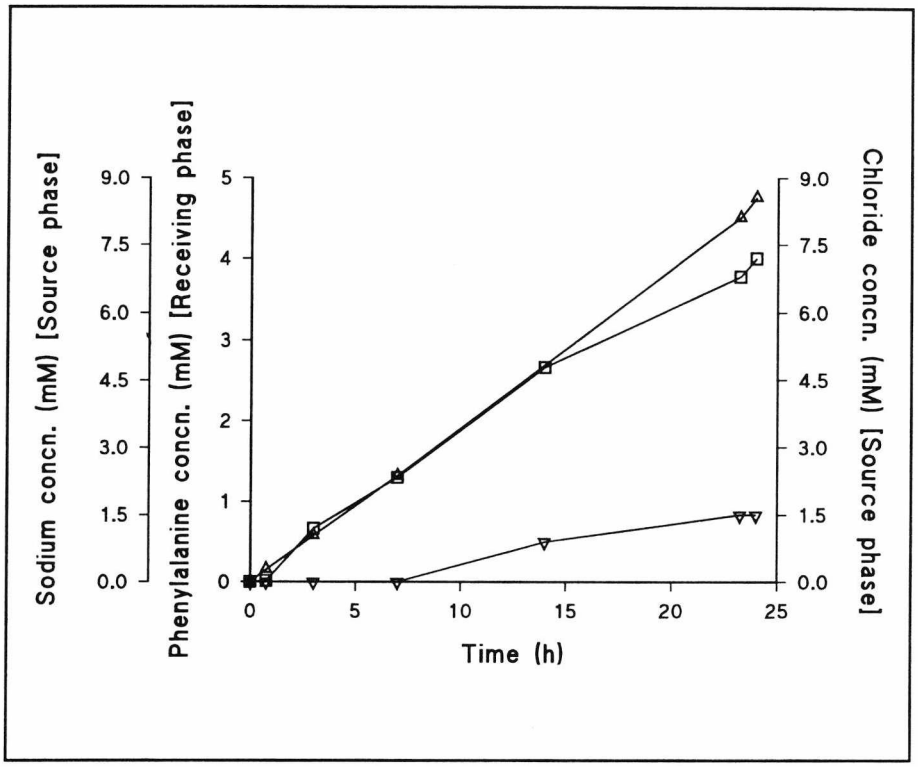


Figure 3.11. The movement of sodium ions during the transport of phenylalanine ions. Conditions were the same as Figure 3.10 except the 50 mM Na_2HPO_4 buffer was replaced by 50 mM K_2HPO_4 buffer in the aqueous phases. Phenylalanine ions concentration in the receiving phase (□), chloride concentration in the source phase (△) and sodium ion concentration in the source phase (▽) were all measured.

3.2.1.1.3. Comparison of different carrier molecules on the transport of phenylalanine ions.

The use of various carrier molecules has been cited in the literature, for the transport of both anionic and cationic substrates. Of these, several were considered in the present study. Figure 3.12 demonstrates that the cationic carrier di(-2-ethylhexyl) phosphoric acid (D2EHPA) is considerably less effective than either of the anionic carriers (tricaprylylammonium chloride (TCAC) and tetraoctylammonum bromide (TOAB)) considered for the transport of phenylalanine.

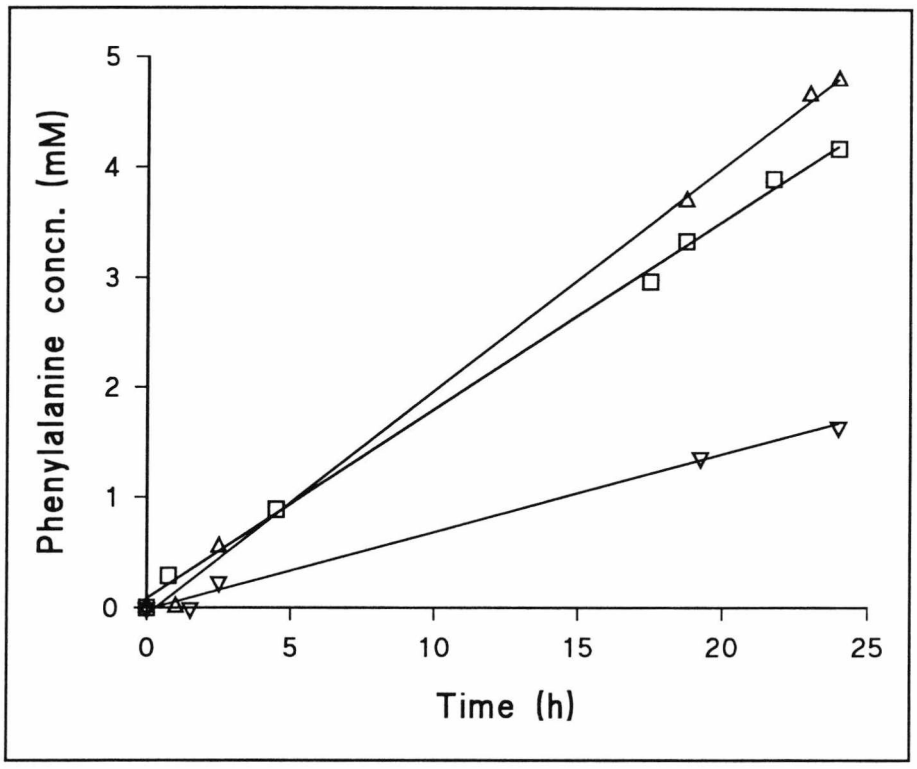


Figure 3.12. The effect of different carrier molecules on the transport of phenylalanine ions. The conditions were the same as Figure 3.10, with 12 mM phenylalanine and 0.5 M NaCl (□), and also with tetraoctylammonium bromide (TOAB) (△) or with the carrier Di(-2-ethylhexyl) phosphoric acid (D2EHPA) (▽) instead of TCMA. D2EHPA tranports anions, with protons (H^+) as the counter ions, in the form of 1 M Hcl in the receiving phase.

3.2.1.1.4. Comparison of different counter-ions.

The rate of phenylalanine transport was measured using different counter ions, as a gradient initially present only in the receiving phase. It can be seen from Table 3.3 that sulphate ions transported phenylalanine at approximately half the rate of the other of the other counter-ions.

Counter ion	Transport rate (mM/h)
Chloride	0.254
Nitrate	0.203
Sulphate	0.120

Table 3.3. Comparison of phenylalanine transport rates for different counter ions. Initial conditions were the same as Figure 3.10 except the counter ion, present in the receiving phase, was either chloride, nitrate or sulphate.

3.2.2. Transport rate studies.

The specific transport rate when all the components are present is dictated by several physical and physicochemical factors. The physical factors could readily be assessed, and their is influence described below.

3.2.2.1. Physical factors.

3.2.2.1.1. Temperature.

The graph in Figure 3.13 shows that the rates of diffusional transport processes across the organic phase are influenced by the temperature. An increase in temperature resulted in an increase in the rate of transport of phenylalanine ions. This supports the idea that the limiting step in the transport of the carrier complex across the organic phase is a diffusional process, characterised by the Stokes-Einstein equation which relates diffusional processes to temperature and viscosity.

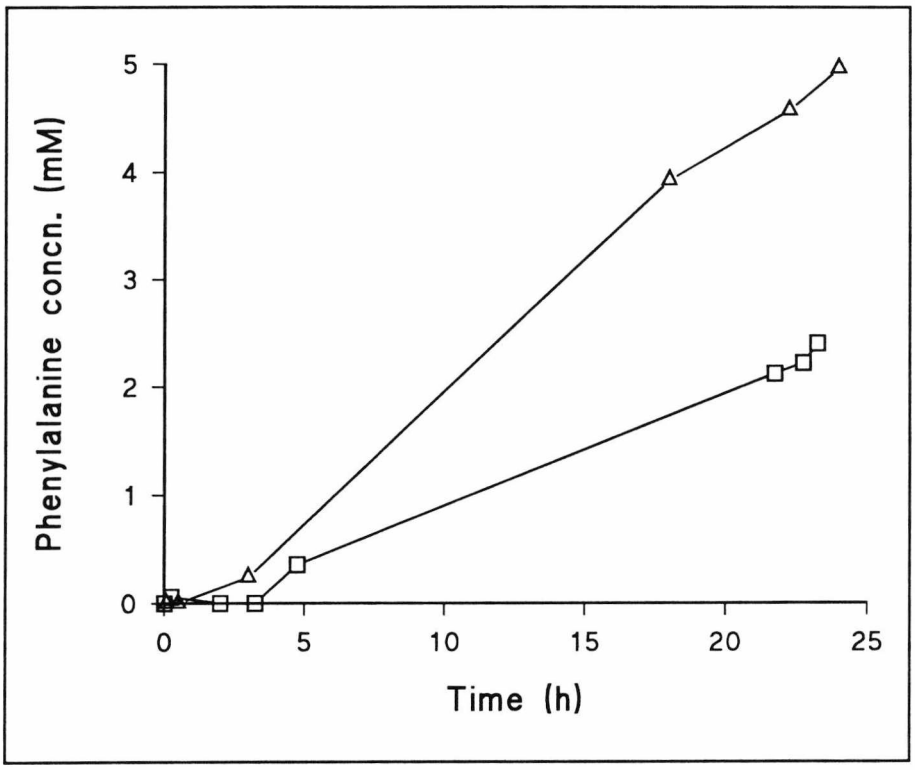
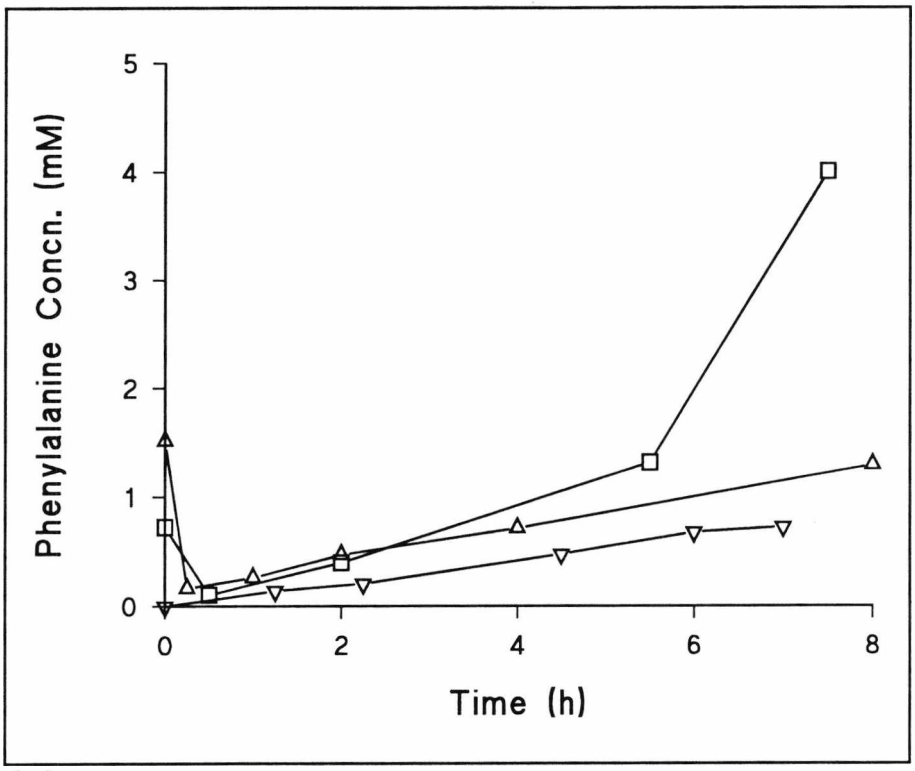


Figure 3.13. Effect of temperature on phenylalanine transport across a supported liquid membrane. Conditions were the same as Figure 3.10 and the two phases were maintained at 25 ^{o}C (□) or 45 ^{o}C (△). The receiving phase contained 0.1 M NaCl as the gradient.

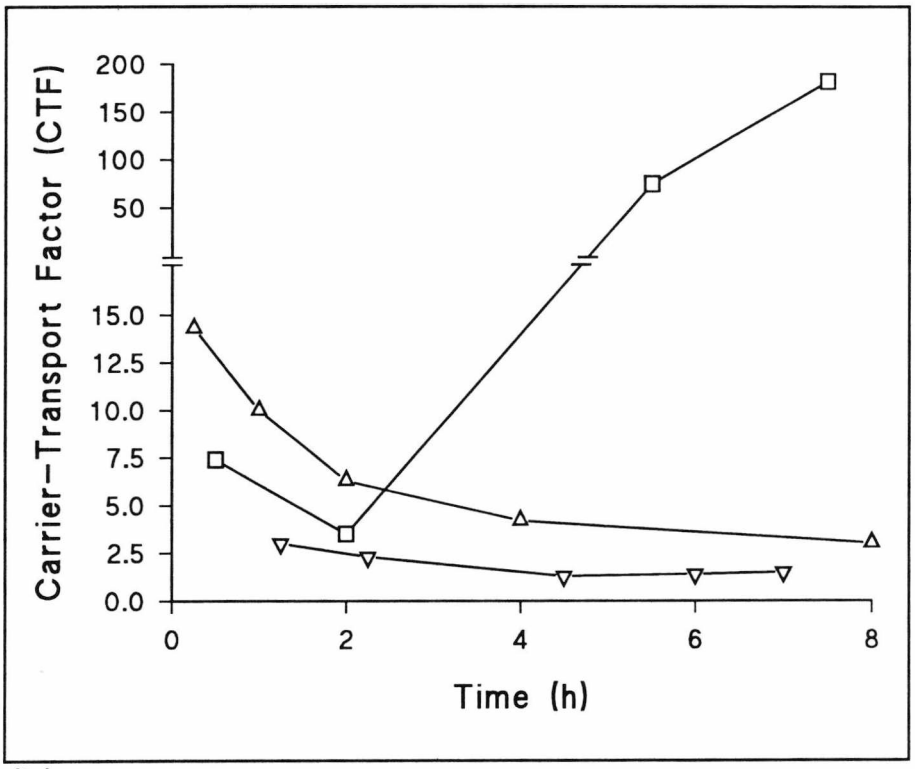
3.2.2.1.2. Flow rate.

An increase in flow rate, as shown in Figure 3.14a, leads to an increase in the rate of phenylalanine transport into the receiving phase. This is predicted by theory as the resistance due to unstirred boundary layers at the membrane surface is reduced by an increase in flow rate and has been reported elsewhere (Uddin et al 1990). However Figure 3.14b reveals that by using the carrier transport factor (CTF)

(Experimental Section 2.2.4.1.), not all of the increase in phenylalanine concentration in the receiving phase was due to increased carrier mediated transport, but occurred by an increase in leakage across the membrane.



(a)



(b)

Figure 3.14. Effect of flow rate on phenylalanine transport across a supported liquid membrane. Initial conditions were the same as Figure 3.10, with 12 mM phenylalanine and 0.5 M NaCl, except the flow rate was 50 ml/min (▽), 100 ml/min (△), or 300 ml/min (□) parallel to the membrane surface. Transport was measured by the increase in phenylalanine concentration in the receiving phase (a), and the effect of flow rate on the carrier transport factor as a measure of the stoichiometry of the system (b).

3.2.2.2. Physicochemical factors.

Figures 3.15 and 3.16 show the effect of changing the substrate concentration on the rate of transport of phenylalanine into the receiving phase. The data is typical of a carrier-facilitated transport process, for example an enzyme mediated transport process across a biological cell membrane ie. they are saturating rather than linear with increasing concentration of the transported species (phenylalanine and chloride).

3.2.2.2.1. Phenylalanine concentration.

To investigate the extent of saturation limitation of the carrier on the transport of phenylalanine ions the system was operated at two carrier concentrations (Figure 3.15).

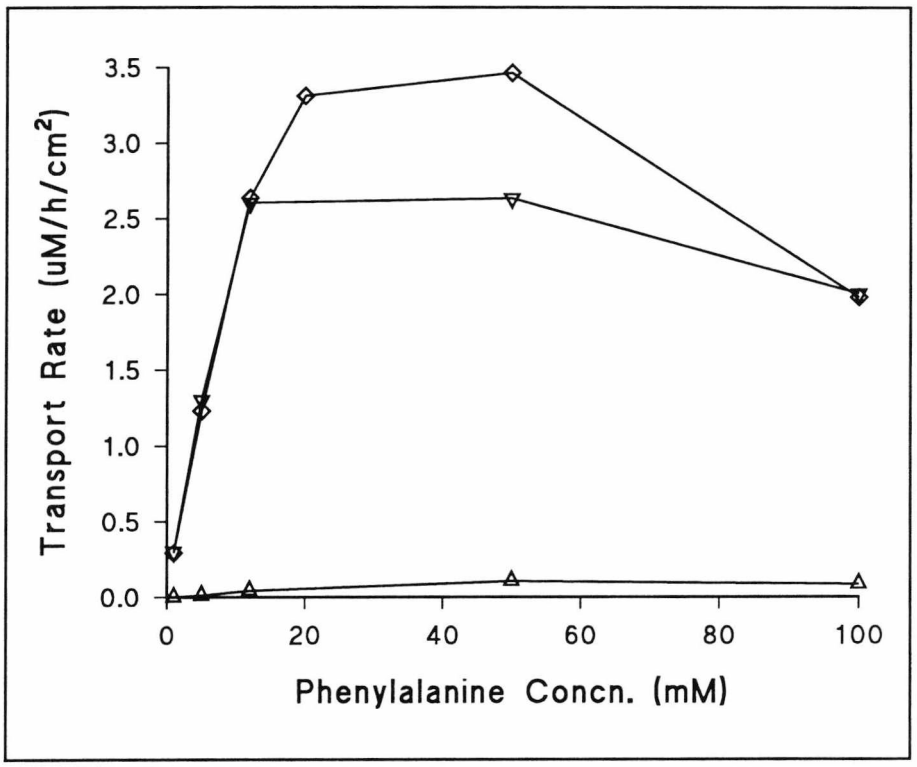


Figure 3.15. Effect of increasing phenylalanine ion concentration in the source phase on the carrier and non-carrier mediated transport rates of phenylalanine into the receiving phase. Initial conditions were the same as Figure 3.10, with 0.5 M NaCl chloride in the receiving phase, except the phenylalanine concentrations was varied from 5 to 100 mM. The carrier, (TCAC), was mixed with decanol at a concentration of 10% (v/v) (▽) and 20% (v/v) (◊) or the TCAC was absent, to detect non-carrier mediated transport (△).

3.2.2.2.2. Chloride concentration.

The graph in Figure 3.16. shows the effect of increasing chloride concentration on the carrier and non-carrier mediated transport of phenylalanine ions across the SLM

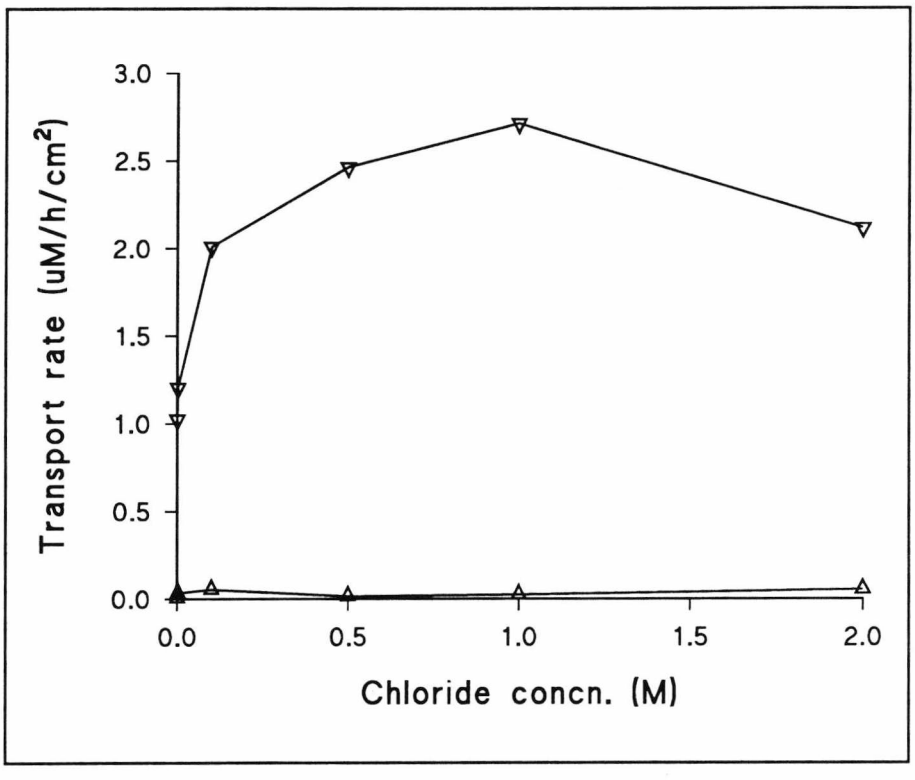


Figure 3.16. Effect of increasing chloride ion concentration in the receiving phase on the carrier and non-carrier mediated transport rates of phenylalanine into the receiving phase. Initial conditions were the same as Figure 3.10 with 12 mM phenylalanine in the source phase, except the chloride ion concentrations were varied from 0 to 2 M in the receiving phase. Carrier present (▽) and absent (△).

The analogy with enzymic processes can be expanded in a useful manner. The maximum transport rate, V_{max}, is the same for variations in both chloride (fixed phenylalanine concentration of 12 mM) and phenylalanine concentrations (fixed chloride concentration of 0.5 M), approximately 2.5 μM/h/cm^2 of membrane. However, this was achieved at very different concentrations of either anion. In the case of phenylalanine ions the saturating concentration was about 12 mM whereas for chloride it was about 1.0 M, indicating that they have very different K_m values; where the K_m is the substrate concentration that indicates when half the carrier sites are filled.

3.2.2.2.3. Analysis of kinetic data.

Yamaguchi et al (1988) presented an equation for flux by carrier mediated systems, which assumes the same affinity by the carrier molecule for either substrate and hence is a form of competitive transport. This equation describes the one-way carrier mediated flux of an amino acid [A] across a liquid membrane. However in their study the system was an ion pair exchange, but the principle of the exchange species being a substrate is equally applicable to the present study. It is also analogous to the Michaelis-Menton equation for enzyme mechanisms and for carrier mediated transport across biological cell membranes, the expression is presented below.

$$J = \frac{P\,[C^o]\,K_{ext}}{1 + K_{ext}\,[A^o]\,[X^o]} \qquad \textbf{(3.1.)}$$

Where J = Flux, P = Permeation Coefficient, C^o = Initial carrier concentration, K_{ext} = Extraction equilibrium constant, A^o = Initial amino acid concentration, X^o = Initial chloride concentration

A plot of 1/J against $1/[A^o][X^o]$, where J = permeate flux, A^o = initial amino acid concentration and X^o = initial chloride concentration, is a plot of rate against substrate concentration and the intercepts with abscissa and ordinate give the extraction equilibrium constant (K_{ext}) and permeation coefficient (P) respectively. Yamaguchi et al (1988) reported that variations in substrate concentration, either amino acid or counter ion, fit a single straight line. However, in the present study variations in phenylalanine concentration at a constant chloride concentration, and variations in chloride concentration at a constant phenylalanine concentration did not fit a single straight line. Instead they form two lines, yielding two sets of data (Figure 3.17).

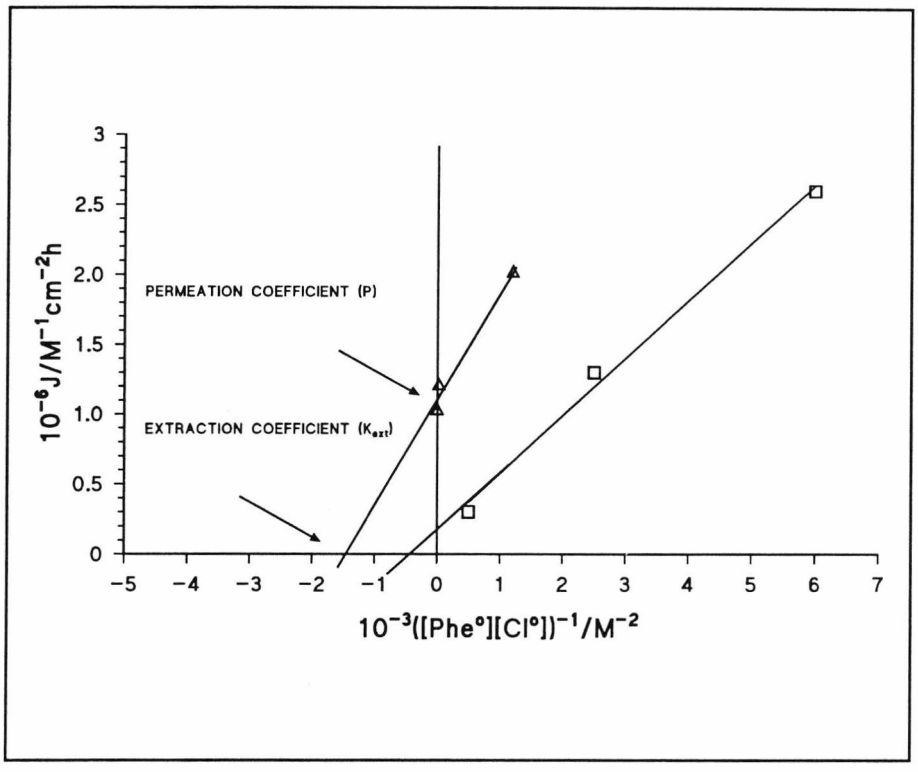


Figure 3.17. Double reciprocal plot of phenylalanine flux (J) against the product of initial amino acid and chloride ion concentration. Initial conditions were the same as Figures 3.18 and 3.19. Experimental data was obtained by measuring the flux at various phenylalanine ion concentrations (□) and chloride ion concentrations (△). The intercept with the ordinate yields the Permeation Coefficient (*P*), for phenylalanine it was $0.2 \times 10^{-6}M^{-1}cm^{-2}h$ and for chloride it was $1.1 \times 10^{-6}M^{-1}cm^{-2}h$ and the intercept with the abscissa yields the Extraction Coefficient (K_{ext}), for phenylalanine it was $0.5 \times 10^{-3}([Phe^o][Cl^o])^{-1}/M^{-2}$ and for chloride it was $1.5 \times 10^{-3}([Phe^o][Cl^o])^{-1}/M^{-2}$.

3.2.2.2.4. The effect of substrate concentration on non-carrier mediated transport.

To further investigate the transport of chloride and phenylalanine in the absence of the carrier, the rate of transport was measured using different substrate concentrations. Figures 3.18 & 3.19 reveal that an increase in the concentration of either substrate resulted in an increase in the rate of non-carrier mediated transport, but that this rate was very much lower than the carrier mediated rates (Figure 3.15 and 3.16).

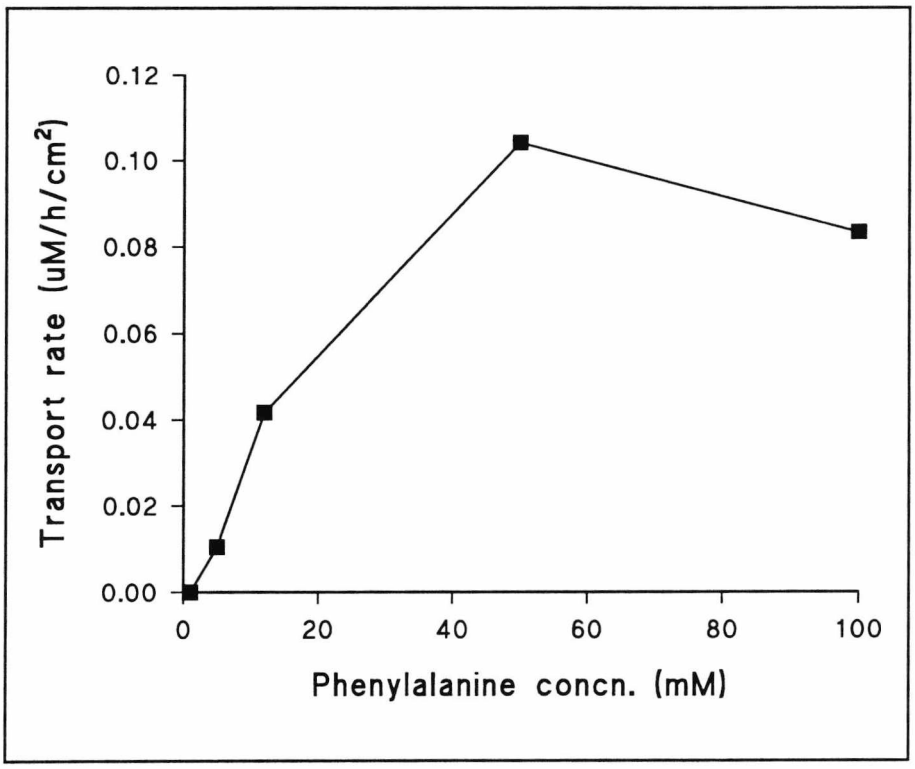


Figure 3.18. The effect of removing the carrier on transport of phenylalanine. Initial conditions were the same as Figure 3.10 except the carrier was absent and phenylalanine ion concentration ranged from 5 to 100 mM with 0.5 M NaCl

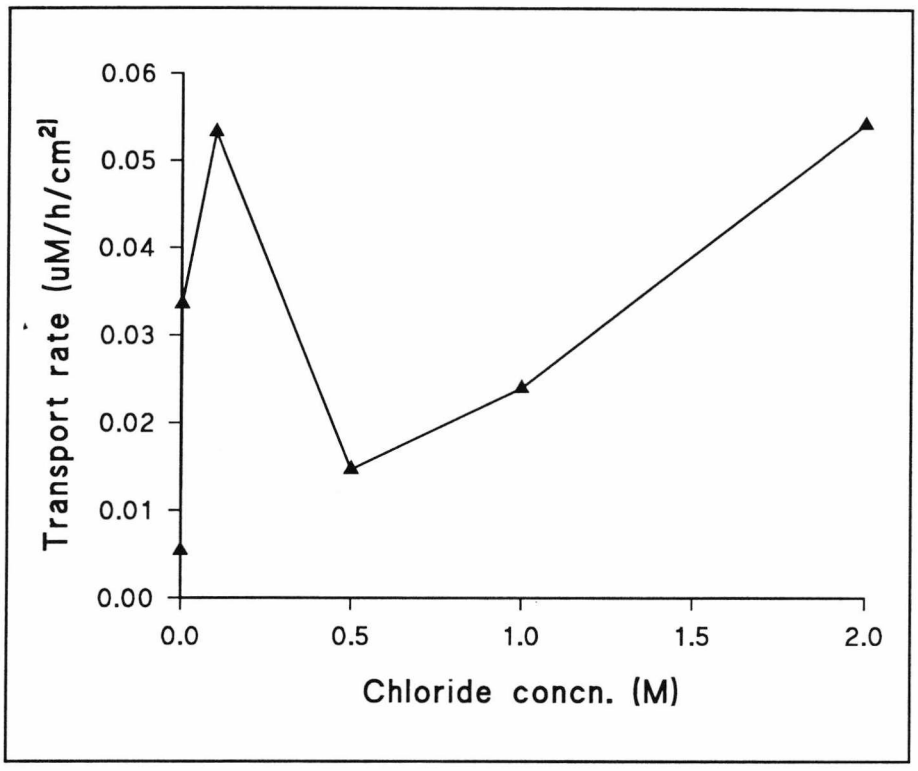


Figure 3.19. The effect of removing the carrier on transport of chloride ions. Initial conditions were the same as Figure 3.10 except the carrier was absent and chloride ion concentration ranged from 0 to 2 M with 12 mM phenylalanine.

3.2.2.2.5. Carrier concentration.

Figure 3.20 shows the interesting data obtained on the rate of phenylalanine ion transport using various carrier concentrations was in two phases; ther was an initial very slow increase in transport rate and a second more rapid increase at higher carrier concentrations, giving an "S" shaped responce curve.

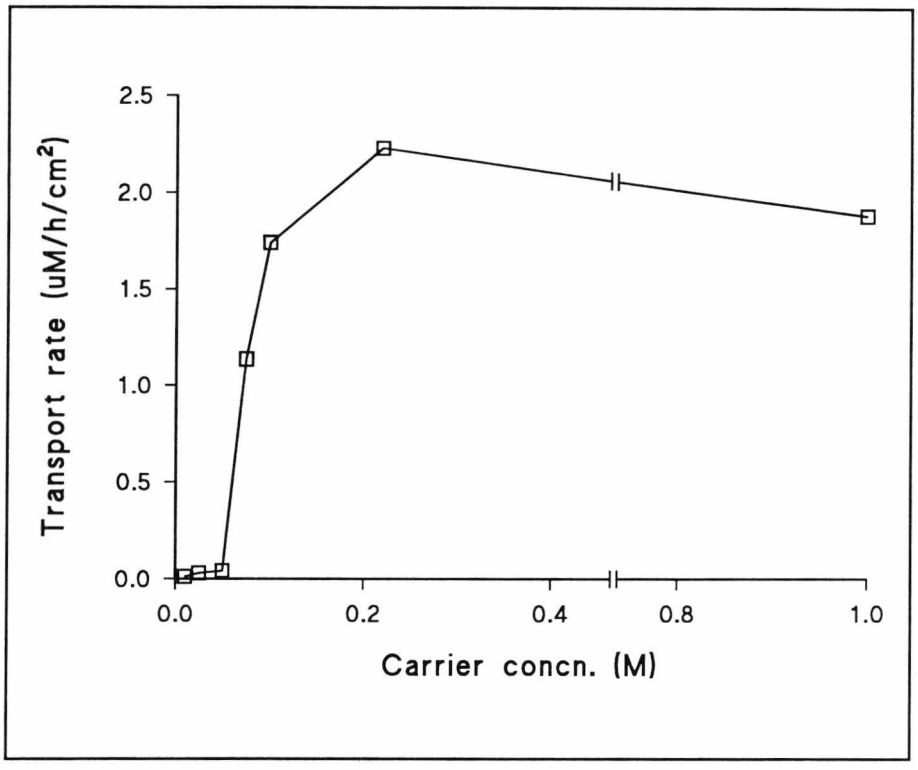


Figure 3.20. The effect of increasing carrier concentration on the rate of transport of phenylalanine into the receiving phase. Initial conditions were the same as Figure 3.10 except the carrier was and tetraoctylammonium bromide (TOAB) and was mixed with decanol at a range of concentrations from 0.01 - 1.0 M (□).

3.2.2.2.6. pH effects.

An increase in the rate of transport of phenylalanine ions with an increase in pH might be expected as the pKa of the amino group of phenylalanine is 9.13, hence above this value the overall charge of the amino acid is negative (Table 2.2) and it could therefore be more receptive to transport by interaction with the cationic carrier (Figure 3.21). Above pH 11 the rate of transport was approximately constant.

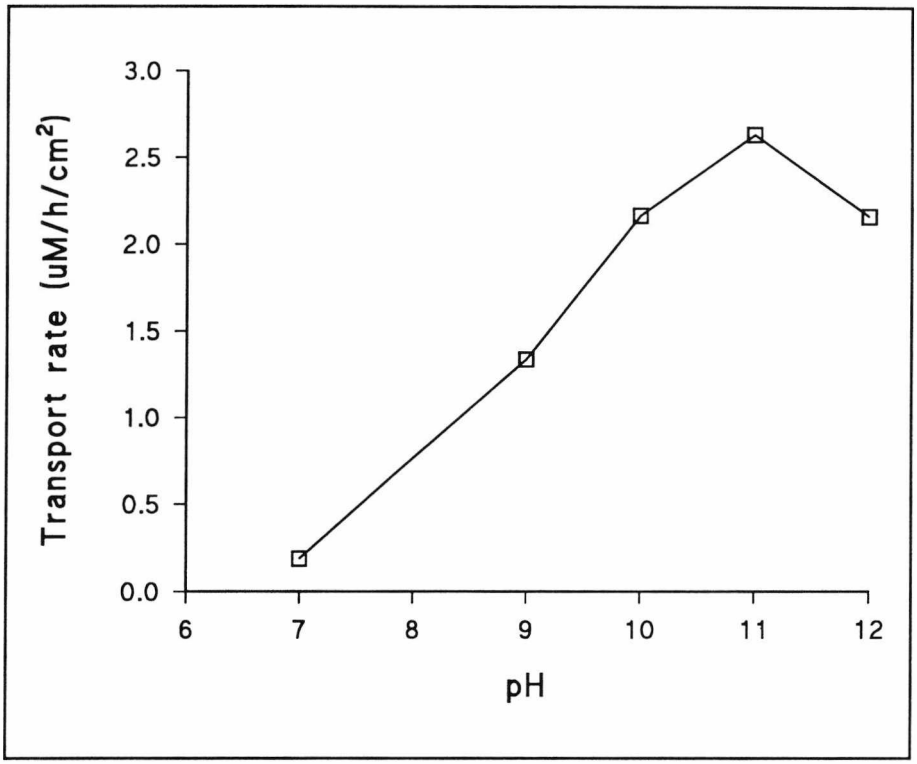


Figure 3.21. The effect of the pH of both aqueous phases on the transport rate of phenylalanine ions into the receiving phase. Initial conditions were the same as Figure 3.10, with 12 mM phenylalanine in the source phase and 0.5 M NaCl in the receiving phase, except the pH values were adjusted to the required pH with 1 M NaOH and 1 M HCl.

3.2.3. Stability Studies.

3.2.3.1. Organic phase effects.

Stability of the transport process using different organic phases was assessed by measurement of retention of the carrier after exposure to shear forces by the somewhat crude method given in Section 2.2.6.1. The extracted carrier was expressed as a percentage of the concentration of the carrier present in the initial sample.

Interpolation of the data, after six hours, revealed that the decanol membrane had retained 55 % of the initial carrier present, the 1,3 DEB membrane retained 73 % of the initial carrier present, the NPOE membrane retained 93 % of the initial carrier present and the 1,3,5 TEB membrane retained 95 % of the initial carrier present.

The graphs presented in Figure 3.22, confirm the results predicted by other groups (Neplenbroek et al, 1992a, and Itoh et al, 1990) that 2-Nitrophenyl octyl ether (NPOE) and 1,3,5 Triethyl-benzneol (1,3,5,TEB) are the more stable organic solvents. This can also be predicted by a consideration of the ratio of the partition values of the organic solvents into water compared to their partition into octanol (the Log P values) as described by Laane et al (1987). In their paper, a hydrophobic fragmental constant was assigned to each functional group on a compound and hence the Log P value can be determined for an organic solvent. The Log P values, calculated by this method are 4, 4.4 and 5.3 for decanol, NPOE and 1,3,5,TEB respectively. Decanol has the lowest Log P value and has therefore is relatively more hydrophilic.

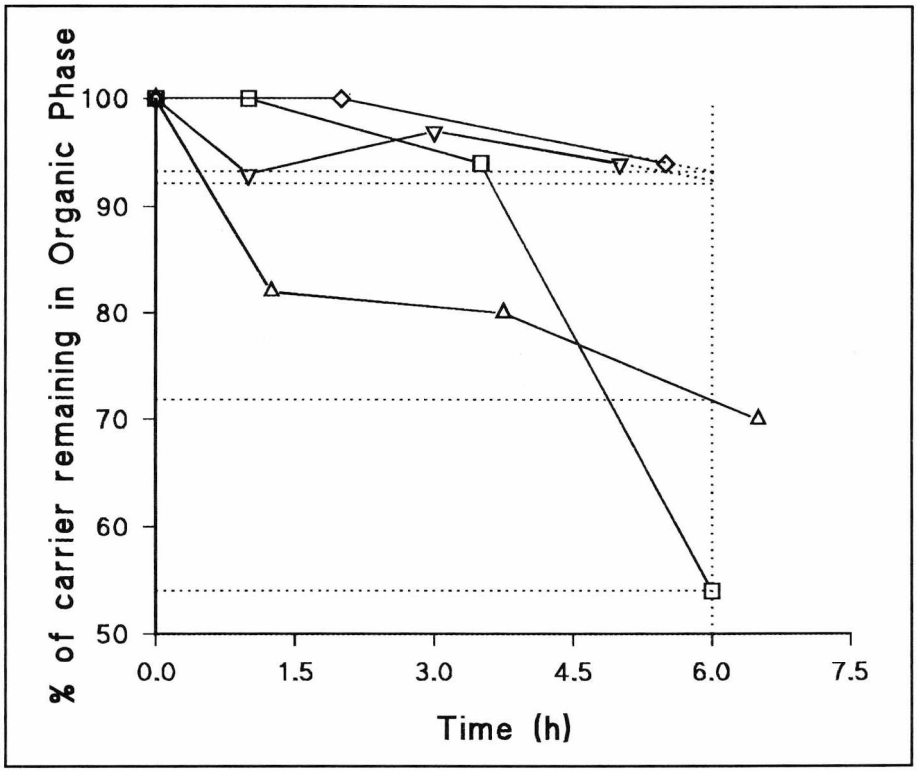


Figure 3.22. The effect of shear forces on the removal of carrier from the organic phase. Membranes were prepared with four different organic phases (decanol (□) 2-nitrophenyl octyl ether (▽), 1,3, diethylbenzene (△) and 1,3,5 triethyl-benzenol (◇)). They were immersed in 1 litre conical flasks containing 50 mM Na_2HPO_4 adjusted to pH 11 with 1M NaOH with 0.5 M NaCl present. Each flask was magnetically stirred to cause shear stress and samples of membrane were assayed at intervals for the concentration of the carrier as indicated in the Experimental Section 2.2.6.1.

Figure 3.23 shows the transport rate in the different organic phases. The results show that the previously determined most stable organic phases do not show the greatest rates of transport. The transport rates, over a 24 h test period, for decanol, 1,3,5 TEB and NPOE were 2.5 μm/h/cm^2, 1.17 μm/h/cm^2 and 0.6 μm/h/cm^2 respectively. Therefore transport across the organic phase is influenced by factors

other than the stability of the organic phase. Similar observations were reported by Molinari et al (1992).

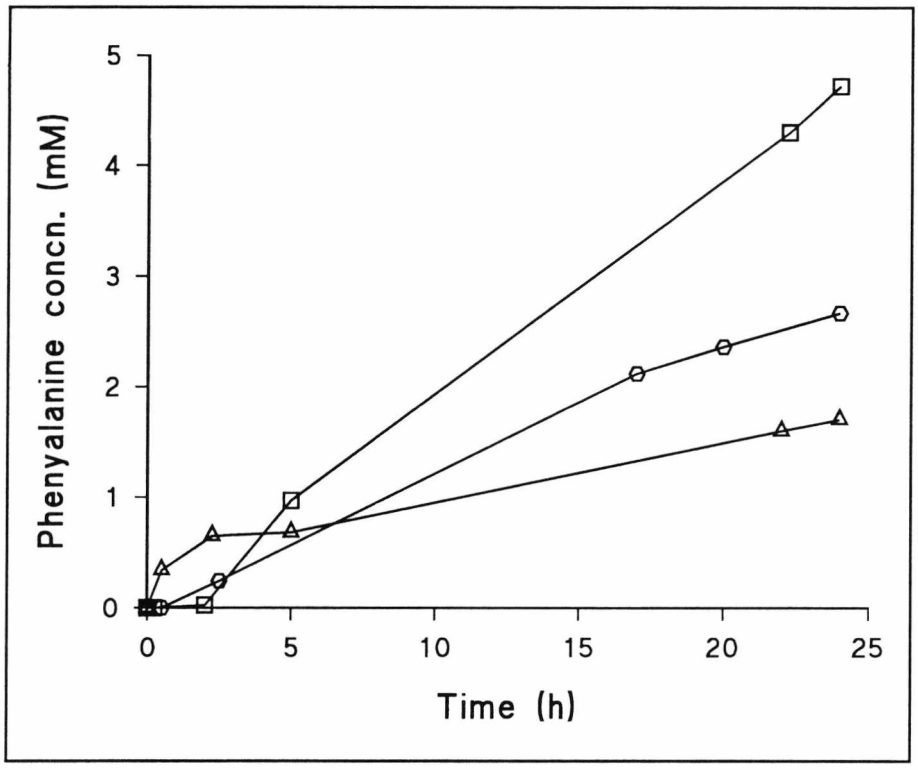


Figure 3.23. The effect of different organic phases on transport of phenylalanine ions into the receiving phase. Initial conditions were the same as Figure 3.10 except carrier, tricaprylylammonium chloride (TCAC), was mixed with decanol (□), 2-nitrophenyl octyl ether (NPOE) (△), or 1,3,5 triethyl-benzenol (◊) each at a concentration of 10% (v/v).

3.2.3.2. Carrier effects.

The rate of TCAC mediated, or induced, transport in the absence of chloride was 0.7 μm/h/cm^2, compared to 2.5 μm/h/cm^2 in the presence of a 0.5 M Cl gradient. TOAB yielded a similar transport rate (0.55 μm/h/cm^2) in the absence of a chloride gradient.

3.2.3.3. Chloride ion effects.

Figure 3.24 shows that in the absence of chloride on either side of the membrane amino acid transport was approximately 0.7 μM/h/cm^2 indicating that there is significant carrier mediated leakage in the absence of a chloride gradient. This has also been reported by Haensel et al, (1986) and Yoshikawa et al (1990).

The reduction in transport (leakage) when equal concentrations of chloride ions are present on both sides of the membrane could be due to greater membrane stability or to greater competition for transport sites on the carrier in the organic phase. This is pertinant as the lowest chloride ion concentration in the feed phase was greater than the initial phenylalanine ion concentration in the source phase (12 mM) and could possibly compete with the exchange process at the source phase interface.

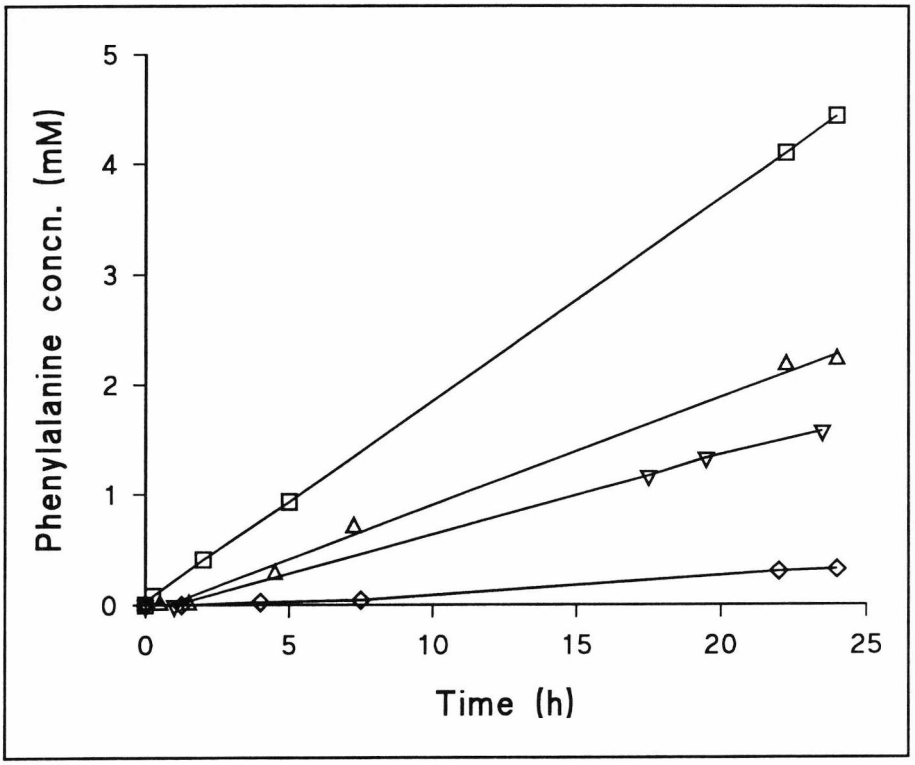


Figure 3.24. The effect of different chloride ion concentrations on the transport of phenylalanine. Initial conditions were the same as Figure 3.10, with 12 mM phenylalanine present in the source phase, except the chloride ion gradient was 0.1 M (no chloride ions in the feed and 0.1 M chloride ion in the receiving phase) (□), zero chloride ion gradient (no chloride ions present) (△), zero chloride ion gradient (0.1 M chloride ion present in both aqueous phases) (▽), and zero chloride ion gradient (1.0 M chloride ion present in both aqueous phases) (◇).

3.2.3.4. Comparison of decanol and NPOE as organic phases.

The aim of this series of experiments was to investigate how one of the more stable of the organic phases (NPOE) compared to decanol under different chloride conditions.

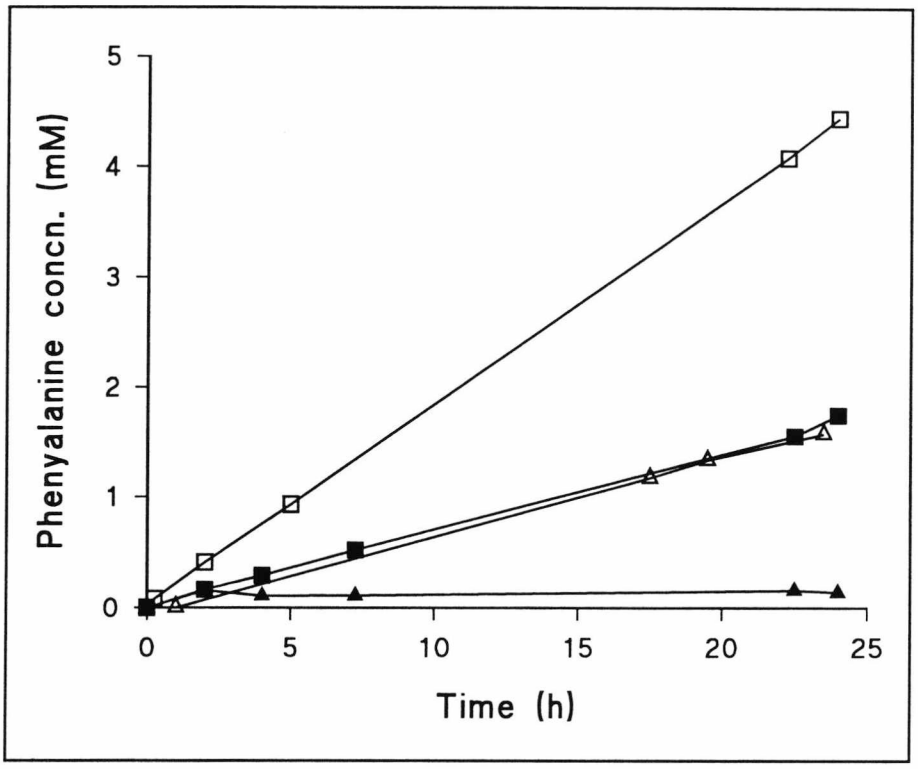


Figure 3.25. The effect of different organic phases, with 0.1 M chloride ion gradient and ubiquitous chloride ion concentration in the aqueous phases, on the transport of phenylalanine ions into the receiving phase. Initial conditions were the same as Figure 3.10 except the carrier, tricaprylylammonium chloride (TCAC), was mixed with decanol (□), or 2-nitrophenyl octylether (NPOE) (■) and driven by a 0.1 M chloride ion gradient (0.1 M chloride ion in the receiving and no chloride ions in the feed), and with zero gradient (0.1 M chloride in both phases) (decanol (△) and NPOE (▲)).

Figure 3.25 shows that with TCAC as the carrier and NPOE as the organic phase there is a decreased rate of transport with a 0.1 M chloride ion gradient, and virtually negligible leakage when equal concentrations of chloride ions were present on both sides of the membrane.

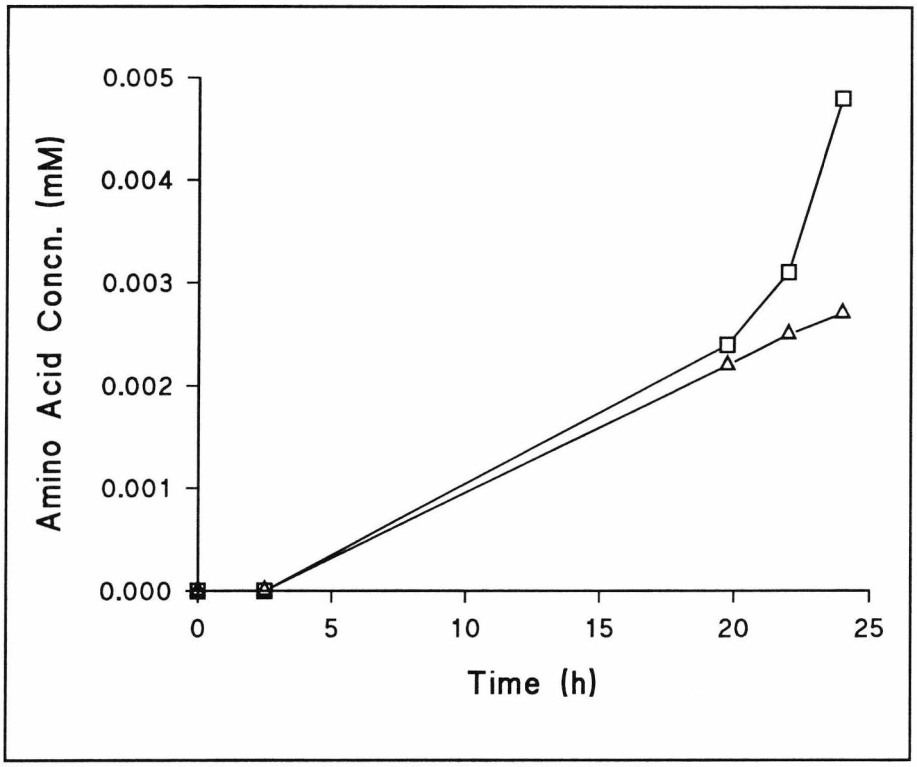
3.2.4. Specificity Studies.

3.2.4.1. Molecular exchange processes.

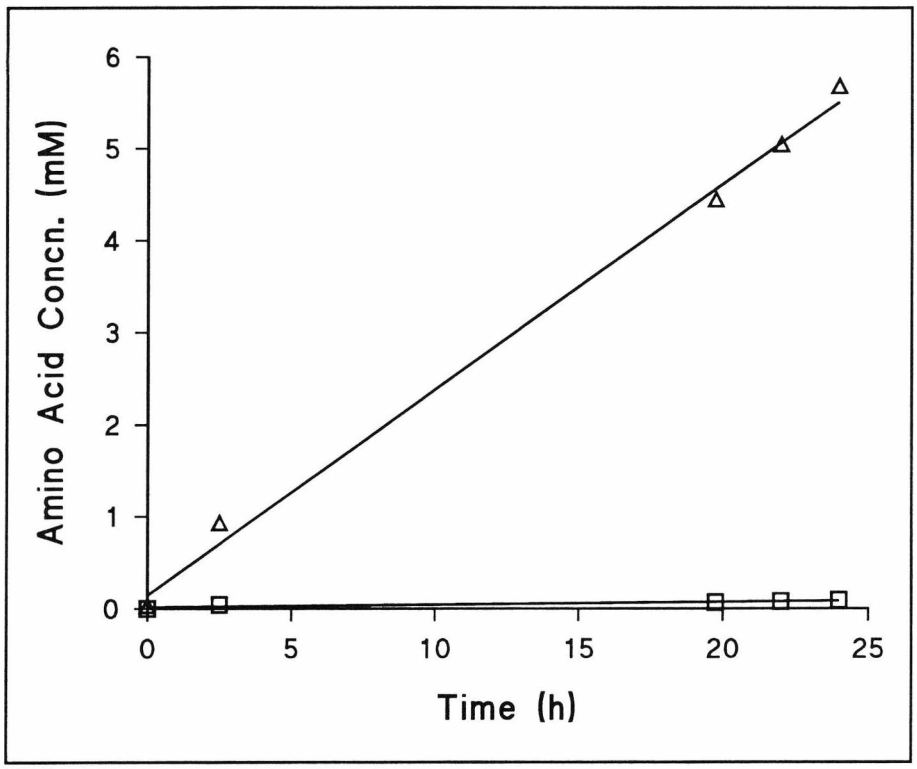
The carrier transport factor (CTF) (Section 2.2.4.1.) for the transport of phenylalanine ions into the receiving phase, across a decanol/TCAC membrane, under standard conditions (Section 2.2.1.) has been shown to have a value higher than the value of 1 predicted by the model in Figure 1.10. (Section 1.3.2.). In fact, it was found to be a constant value of approximately 2 over repeated 24 hour periods of investigation which indicates that each phenylalanine molecule was exchanged for two chloride ions.

3.2.4.2. Transport of mixtures of amino acids.

The relative rates of transport of a mixture of amino acids at different pH values and hence different prevailing potential charges (Experimental Section Table 2.2.) were studied. The results obtained were not those expected (Figure 3.26a and 3.26b). Instead at pH 11 only phenylalanine and aspartic acid were transported to any significant degree, and at pH 5.48 only trace amounts of these amino acids were found in the receiving phase. Lysine was not transported at either pH value. The transport rates are summarised in Table 3.3.


Amino Acid Concn. (mM)
0.005
0.004
0.003
0.002
0.001
0.000
0
5
10
15
20
25
Time (h)

(a)



(b)

Figure 3.26. Transport of amino acid mixtures across a supported liquid membrane. Initial conditions were the same as Figure 3.10 except the initial concentrations of amino acid in the source phase were 10 mM phenylalanine (△), aspartic acid (□) or lysine. The system was operated with both aqueous phases at pH 5.48 (a) or pH 11 (b). Lysine was not detected in the receiving phase in either system.

Transport rate (μM/h/cm^{-2})	pH 11	pH 5.48
phe	2.230	0.0019
asp	0.028	0.0017
lys	0	0

Table 3.4. Transport rates of different amino acids at different pH values. The amino acids were present in equimolar amounts (10 mM) and challenged a SLM as described in Figure 3.10. Selective transport rates were measured over a 24 hour period.

4. Discussion

4.1. Ultrafiltration membranes.

4.1.1. Membrane assessment.

4.1.1.1. Assessment of nominal molecular weight cut-off of ultrafiltration membranes.

The assessed nominal molecular weight cut-off (NMWCO) of two different types of ultrafiltration (UF) membrane are shown in Figures 3.1 and 3.2.. These graphs are indicative of the difficulty in reproducing manufacturer's NMWCO data. Partly this is because NMWCO is an arbitrary value that is influenced by many factors, not only composition of the membrane, but also by the method of assessment. The difficulty in reproducing manufacturer's data has been discussed by many other workers. For example, McGregor (1986b) examined 20 commercially available membranes and found that only 25% approximated to the manufacturer's claim.

Interestingly Figure 3.1. indicates that the NMWCO assessment in a stirred cell filtration (SCF) unit is closer to that of the manufacturers stated data than the values obtained in the crossflow filtration (CFF) system, as given in Figure 3.2. The under-estimation of NMWCO in the case of the latter may be a direct result of the assessment mode where the solute concentration at the membrane is reduced by cross flow effects and hence reduces rejection. The manufacturer of the membrane in Figure 3.2. (LSL, Luton, UK) appears to include concentration polarization effects in the membrane performance. Therefore assessment of the NMWCO in a mode where concentration polarization effects are reduced will result in different values. This was

confirmed in a personal communication with LSL enquiring about assessment methodology. The manufacturers indicated that membrane protein saturation is essential for successfully achieving the stated NMWCO value.

usage.

It is therefore important to take into account that the NMWCO value should be considered only in terms of the system it is obtained on, whether SCF or CFF, and in conjunction with other, physical, data. This might include, for example, data on membrane hydrophobicity, surface roughness and the distribution of pore sizes. This latter component is critical as a few large pores in a membrane can radically alter its performance. Fane et al (1981) noted that in some membranes 50% of flux occurs through approximately 25% of the pores.

Membranes are supplied by the manufacturers only with an arbitrary NMWCO value and little other data. It is interesting to note that manufacturers often use highly idealised conditions to obtain the stated NMWCO values. For example, in a personal communication with LSL (Luton, UK) (see above), it was also revealed that a log scale was used on the abscissa of the assessment graph. Clearly this greatly reduces the accuracy of the graph and also increases the apparent sharpness of the cut off. Trägårdh & Ölund (1986) have considered the vagaries of assessment methodology and proposed certain standard conditions for undertaking transport examination, for example using defined dextrans as molecular weight markers and a CFF unit of defined dimensions. Bottino (1984) also discussed assessment methodology and concluded that to successfully produce reliable NMWCO data there should be no concentration polarization, as any polarised layer above the membrane will influence the original membrane properties. Therefore low pressure should be used combined

with a high recirculation rate and turbulent flow, and low solute concentration. The solute should be of non-interactive nature and maintain conformational stability.

The difficulty in meeting these requirements, either in assessment or usage, is one of the reasons that membrane users often remain dissatisfied and probably why this technology has not received a wider acceptance.

4.1.1.2. Comparison of membrane performance in stirred cell and crossflow filtration units.

Theory predicts (Section 1.2.2.3.) that the protein concentration above the membrane will be highly influential on membrane performance. In a CFF system there is reduced concentration polarization, less interference from rejected solutes and therefore higher permeability resulting in a reduced retention coefficient for the same membrane and molecular weight marker. Various parameters will a have significant effect upon concentration polarization, and the retention coefficient of a particular molecular weight marker. An examination of the significance of operation modes, either CFF or SCF, is shown in Figure 3.3. for the same LSL membrane assessed in different systems. The principal difference is the gradient of the line. A more efficient membrane system will have a steep gradient and therefore a sharp cut-off. This is more readily obtained by a CFF system, as might be expected, due to reduced concentration polarization, and therefore it is desirable that both assessment and application is undertaken in CFF apparatus.

4.1.2. Analysis of the operating parameters of a crossflow filtration unit.

Of the many factors that affect concentration polarization, and by implication fouling, concentration and pressure were considered, as well as temperature because of its effects upon protein conformation.

4.1.2.1. Concentration effects.

During assessment of NMWCO, and general usage, protein concentration must be considered in system design. Generally concentration polarization theory predicts that flux reduces with the logarithm of concentration. Daufin et al (1991) concluded that with increased concentration irreversible fouling remained constant, and reduced permeate flux was due to an increase in reversible fouling, principally concentration polarization. This indicates that membrane resistance does not increase with increased concentration and therefore retention remains constant.

The values in Table 3.1. reveal that an increase in concentration by a factor of a hundred had a highly significant effect upon the retention coefficient. Hence at the high concentration there was a significant increase in irreversible fouling.

4.1.2.2. Pressure effects.

Within the levels of the pressure examined in this study the classical pressure independent flux behaviour, described in Section 1.2.2.3., was not observed. The point at which pressure independence occurs is system dependent and influenced by the

balance of forces driving solute to the membrane and its diffusion away. In this case, the driving force, due to pressure, was not sufficient to create a controlling dynamic membrane, above the UF membrane. Instead, a degree of concentration polarization, and possible membrane fouling and compaction, result in a stable retention, and increased pressure resulted only in greater concentration polarization, not fouling, and the retention coefficient remained constant and flux increases. Marshall et al (1993) has reviewed the pressure effects in UF systems and concluded that generally concentration polarization is not significant below 4×10^2 kPa and therefore pressure independent flux is not observed below this level.

4.1.2.3. Temperature effects.

4.1.2.3.1. Effect of temperature on permeate flux.

Workers who have considered the effects of temperature on UF systems have principally examined the relationship between flux, viscosity and diffusivity (Scott, 1988). Surprisingly few studies have considered the effect of temperature as a denaturing force on proteins and its implications for membrane performance. Meirles et al (1991) considered a model system for a polysulphone membrane using a bovine serum albumin solution. Potential denaturation during UF was measured by increased turbidity and gel chromatography, however their system was only examined at temperatures up to 30 ^{o}C. It is perhaps more illuminating to consider the activity and conformation of the protein.

Despite a high degree of membrane variability the effect of temperature on flux is shown in Figure 3.5. It is interesting that the highest level of flux was at approximately 30^{o}C which may represent the optimum operating temperature,

allowing maximum diffusivity of rejected species away from the membrane surface, and yet resulting in minimum denaturation of the protein and hence minimum adsorption to, and fouling of, the membrane.

Furthermore, it was noted that although flux declined during the experimental period from the initial to a steady state value. The extent of the decrease was not greater at the higher temperatures considered.

4.1.2.3.2. Effect of temperature on the retention coefficient.

Inherent variability between membranes has previously been demonstrated to be significant, with often different permeabilites for different samples of the same membrane. The significance of this is reduced when considering the retention coefficient, thus making this parameter potentially more representative of membrane system interactions. This occurs because this characteristic is a ratio of performance related values instead of a direct measurement, such as flux which is therefore more highly influenced by membrane variability.

The value of the retention coefficient showed three distinct stages. Initially there was little change up to 25^{o}C, but between 25 and 40 ^{o}C there was a rapid decline in retention coefficient indicating the membrane was more permeable to α-amylase. This probably occurred because the greater diffusivity of the protein allowed reduction of the concentration polarization layer and greater passage through the membrane. However above 40 ^{o}C the protein began to experience denaturation and therefore had a greater tendency to aggregate and bind to the membrane. Thus, although diffusivity continued to increase, and reduce concentration polarization, it

was countered, to an extent, by the greater tendency of the protein to aggregate and adsorb to the membrane, hence permeability increased but at a slower rate.

The data given in Figure 3.6 show that increasing the temperature led to greater diffusivity, but a decrease in retention coefficient. Therefore, at higher temperatures there was greater membrane permeability for α-amylase. The flux data (Figure 3.5.) was interpreted in terms of temperature leading to increased denaturation, and therefore adsorption to the membrane, hence leading to decreased flux.

Although greater diffusivity, prior to protein denaturation, was the basis of the interpretation for both the retention coefficient and the flux data, above this point the two sets of data are contradictory. The retention coefficient data reveals that diffusivity remains the dominating factor, although reduced by denaturation, whereas the flux data shows denaturation to be the dominating factor. The cross-over point at which this occurs is approximately 30 ^{o}C for the flux data whereas it was 40 ^{o}C for the retention coefficient data. Stability data (Figure 3.9.) reveals that the onset of denaturation occurs between these two temperatures.

A unifying interpretation is that denatured forms of the protein, which were demonstrated to be present, were adsorbing to the membrane and also aggregating in solution. Calculation of the retention coefficient requires estimation of protein content in the permeate and retentate (Section 2.1.1.1., equation **2.1.**). Aggregated and adsorbed denatured protein molecules would not readily undertake such an estimation thereby indicating a lower concentration of protein in the retentate, and hence yielding a lower retention coefficient when in reality no greater permeability was observed.

These observations support those of various workers who have indicated that the adsorbing potential of polysulphone membranes is significant. For example,

Rodgers & Sparks (1991) indicated that adsorption of rejected protein had the greater influence upon performance of the polysulphone and polyvinylidene difluoride membranes investigated in their studies.

4.1.2.3.3. Effect of temperature on the specific activity of protein in the retentate, permeate and reservoir.

The specific activity of the α-amylase was measured at various times at each temperature (Figure 3.7). It was found that the protein in the permeate had reduced specific activity compared to the retentate. Although this inactivation was enhanced at the higher temperatures, there was a noticeable effect at all the temperatures. Clearly temperature was not the major denaturing effect although it probably increased protein susceptibility to other denaturing effects. Passage through the membrane appeared to have a denaturing effect.

4.1.2.3.4. Effect of temperature on the fluorescence emission.

The potential effect of crossflow filtration on protein structure was measured by changes in the emittance spectrum of α-amylase in the permeate, retentate and reservoir. Figure 3.8 shows the emittance peak position for native protein in phosphate buffer, and the changes on filtration.

In confirmation of the data on specific activity, temperature did not appear to be the major denaturing factor although it appeared to enhance other factors affecting protein conformation. Proteins are dynamic structures that respond to their environment by changes in conformation. Once a denaturing effect has been removed they

will often rapidly reconfigurate to obtain their native structure. Storage of the part denatured proteins at 4°C was an attempt to overcome this problem. However, such storage maybe significant for protein that had been subjected to the higher temperatures where, reducing the operating temperature to 4°C and then back to 25°C, for measurement of fluorescence emittance, represented a potential denaturing force. However this does not negate the results within each experiment i.e. in each case it was the permeate that was most denatured. The fluorescence patterns obtained did not represent the maximum deflection that could have been measured. Instead they are probably due to the protein being partially denatured and then reconfigurating. This could produce agglomerations between proteins which in turn may add significantly to fouling. The fluorescence peaks therefore represent average values of the various denatured and/or agglomerated forms of the molecule.

4.1.2.3.5. Effect of temperature on control samples.

Samples of α-amylase which had not been subjected to cross flow filtration were maintained in a water bath at the desired temperature for the duration of the experiment to yield control data on the temperature effects upon the protein (Figure 3.9.). The measurements of α-amylase activity and fluorescence peak position supported the predicted pattern of data for this enzyme, that is, it was stable until 40°C and then began to lose activity. Although the data for fluorescence peak postion indicates that structural changes occur continuously above 25°C, they result in no loss of activity until over 40°C. Interestingly, the data for the loss of protein specific activity at a particular temperature was slightly less than the loss of specific activity

of the permeate at the same temperature, again supporting the idea of other denaturing forces acting on the protein during cross flow filtration.

4.1.2.3.6. Denaturation in ultrafiltration.

Narendranathan & Dunhill (1982) found that shear forces were not significant in protein denaturation, instead adsorption and interfacial activity associated with solid-liquid and gas-liquid interfaces were more significant. This is pertinent to industrial applications were often high pumping rates introduce gas bubbles. However physiological studies of proteins using *in vitro* studies have shown that shear stress is highly significant and is used as an *in vivo* mechanism for regulation of clotting proteins (Charm & Wong 1970). Charm & Wong (1981) have also shown that shear forces resulting from turbulent flow, pumping and mixing of proteins are significant. Others noted severe losses in activity of enzymes which were not attributable to any obvious forces (Wang, Sinskey & Butterworth 1970). In addition, the effect of the number of passes through the membrane on protein structure as a result of shear forces in and around the pore has been shown to be influential (Denis et al,1990, Bowen & Gan 1992), hence indicating a role of the membrane in denaturation.

Protein that is adsorbed to the membrane acts as a potentially denaturing force as proteins pass through this concentration polarization layer. The adsorbed protein may interact with the functional groups (which may be hydrophobic and usually buried within the protein), which in turn leads to further denaturation. This may in part explain the time dependent nature of the shift in specific activity and fluorescence spectra of the permeate samples since the concentration profile is time dependent.

In the present study it would seem likely that the denatured species which are found in the permeate arise from passage through the membrane. Reed & Sheldon (1988) have shown, by electron microscopy, that BSA is partially denatured during passage through the polysulphone membranes.

This may be due to either the tortuosity of the pores inducing shear forces, or a surface interaction between the protein molecule and the membrane pore. If surface interaction on the membrane is actually causing the denaturation then it was not clear why, at this feed rate and reservoir volume, and within the experimental period, more of the protein was not denatured. For example, α-amylase at 30°C has a stability comparable to that at 25°C. However, flux at the higher temperature is significantly higher and hence would be expected to result in a greater degree of denaturation. The volume of the feed from the 400 ml reservoir that permeated the membrane was approximately 200 ml at 25°C, compared to the volume at 30°C which was approximately 750 ml. This difference in volume of the feed that actually permeates the membrane could be detected by the extent to which the protein was denatured. The specific activity and the fluorescence spectroscopy data did not show any greater extent of denaturation of the protein in the reservoir at 30°C.

The protein content of the permeate samples is significantly lower than the retentate, or reservoir. The inability to detect the denatured form in the permeate may be due to the relative insensitivity of these methods to detect the comparatively dilute denatured form in the presence of the more common native form.

The inability to detect the denatured protein may also have been due to reconfiguration of the protein after removal of the denaturing force. Goldberg, Rudolph & Jaenicke (1991) have shown that reconfiguration is highly concentration

dependent and therefore more concentrated streams would not be as able to assume the native form. This system could be viewed as one in which there are two protein species, the native and denatured forms of the protein. As a recycle system is operating they must both be present in the reservoir. However the concentration of the denatured species, when returned to the reservoir, is actually decreased with respect to the protein concentration in the permeate samples. Thus it is possible for it to reconfigurate more readily than the permeate samples, which contain solely denatured proteins.

An alternative possibility is that the membrane could have been selecting the denatured form of the protein; it may be that a protein that has a partially denatured structure is not so resistant to passage through a pore. If this was the case it was not obvious how it occurred and does not explain the nature of the denaturing force. If there was a ubiquitous denaturing force then there would be more denatured protein and hence it would possible be more detectable in the retentate and reservoir.

This hypothesis cannot be dismissed without further investigation, as there is a tendency for the relatively hydrophobic membrane to act as a selective separating process for part denatured proteins to adsorb to. As with many complex phenomena many processes are occurring simultaneously and this alternative hypothesis possibly contributes to the overall process. For instance a small degree of denaturation may occur at the pump head, by shear and interfacial forces. The membrane favours part denatured proteins, as a surface to adsorb to, and acts as a denaturing force in its own right.

A summary of these possible denaturing effects is shown in Figure 4.1.

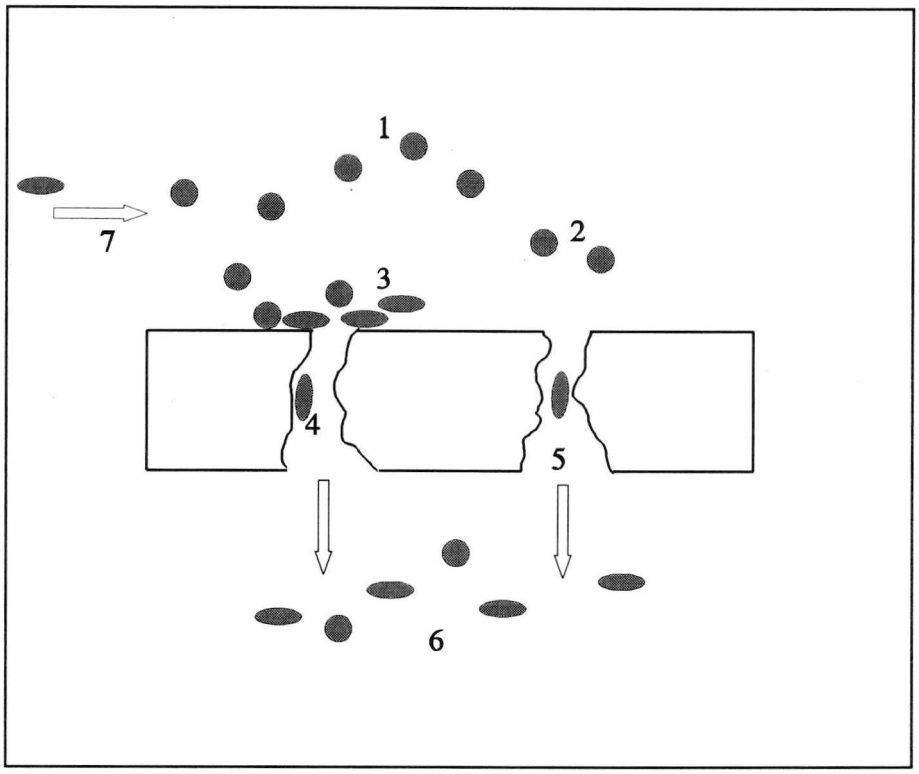


Figure 4.1. Proposed interaction of proteins and membrane. The majority of protein is in a stable configuration with full activity (1), and is driven towards the membrane (2) where part denatured proteins, either as a result of the denaturing force occurring above the membrane (pump head and interfacial effects) or by adsorption to the membrane surface, may interact in a denaturing manner (3). Proteins that pass through the membrane may be denatured by the hydrophobic wall surface of the pore (4) or by the tortuosity of the pore generating high shear effects (5). This results in a significant degree of denaturation of the permeate sample (6) whereas denatured protein that is recycled to the reservoir (7) is diluted and undergoes reconfiguration.

4.1.3. Future work.

Detection of the denatured species in the retentate and reservoir is one area in which greater clarity could be gained. This would possibly be achieved by gel electrophoresis or gel chromatography. If this could be demonstrated not to be present it would support the idea that rapid reconfiguration occurs within the reservoir. Furthermore it might help to resolve the apparent contradiction between the flux and the retention coefficient data.

It would be illuminating to perform experiments for extended periods to investigate the effect of membrane interactions over a longer period of time. It would then be possible to measure extent of adsorbed protein with temperature. (Ko et al 1993)

This work could also be extended to characterise the membrane in terms of surface roughness, surface charge and porosity. Similar work has been undertaken by Ko et al (1992) who investigated membrane types in terms of hyrdophilicity and other factors and showed that they were significant in influencing the type of fouling, and whether indeed osmotic forces were influential. For instance hydrophilic membranes have little protein association and therefore osmotic forces are most significant in flux reduction whereas hydrophobic membranes have greater association and therefore aggregation and adsorbed layer resistance is a greater influence on reduction in flux. This adsorbed layer is more influenced by pH and ionic charge modifications. To more fully understand membrane protein interactions it is essential to be aware of the charge of the protein as determined by its isoelectric point, and therefore it is necessary to determine this.

Temperature influences many factors, and to illuminate these interactions it would be helpful to isolate these factors. For instance sucrose can be added to the solution to maintain viscosity at higher temperatures, or a chemical denaturing agent could be added to cause denaturation at lower temperatures, to examine whether greater transport of protein occurs and therefore whether the membrane is selecting denatured protein.

4.2. Supported liquid membranes.

4.2.1. Transport studies.

4.2.1.1. Phenylalanine and chloride ion transport.

When all components are present the transport of phenylalanine was linear over the experimental range (Figure 3.10). However when individual components of the system were systematically removed lower but variable rates were obtained (Table 3.2.). These rates were all considerably lower than when all components of the system were present. When the carrier was not present, there was insignificant non-carrier mediated transport.

More interestingly, the carrier mediated transport of phenylalanine, when no chloride was present, was approximately 40% of its value when chloride was present. Correspondingly, the carrier mediated transport of chloride, when no phenylalanine was present, was 70% of its value when phenylalanine was present.

A possible interpretation of this is that not only is chloride more readily transported (the rate of chloride transport being greater than phenylalanine transport) but also that it more readily undergoes exchange reactions at the interface, with molecules other than phenylalanine, than phenylalanine is able to do at the opposite interface. This is indicative of the greater affinity of the carrier for phenylalanine.

4.2.1.2. The effect of a substituted potassium buffer.

Limited sodium transport occurred, indicating that this ion is not significantly involved in the transport processes (Figure 3.11.). It cannot be concluded this movement is carrier mediated and may have been due to leakage. Likewise hydrogen and hydroxyl ions were shown not to move and therefore did not take part in any exchange processes.

4.2.1.3. Comparison of different carrier molecules on the transport of phenylalanine ions.

Significantly, the carriers that transport phenylalanine in the anionic form (tricaprylylammonium chloride (TCAC) and tetraoctylammonum bromide (TOAB)) are more effective than those that transport in the cationic form (di(-2-ethylhexyl) phosphoric acid (D2EHPA)) (Figure 3.12.). The reasons for this are not clear as the hydrogen gradient for phenylalanine transport with D2EHPA was greater in concentration than the chloride ion gradient used with TCMA. Also other workers (Teramoto et al, 1991 and Hong et al, 1992) have used this carrier and reported reasonable transport rates. However no workers have published data for the comparable transport of phenylalanine in a cationic and anionic form in otherwise similar conditions. The lower rate of transport with D2EHPA is probably a result of its lower affinity for cationic phenylalanine and hydrogen ions than TCMA has for anionic phenylalanine and chloride ions.

4.2.1.4. Comparison of different counter-ions on the rate of transport of phenylalanine.

The model of transport in Figure 1.12. involves a single negative charge, on the counter ion, which therefore accounts for the comparable phenylalanine transport rates driven by either nitrate or chloride ions. These two ions (chloride and nitrate) have formed the basis of a carrier mechanism, being exchanged for each other using TCAC (Neplenbroek et al, 1992c) for the removal of nitrate from water.

Ironically, the lower transport rate for nitrate is a reflection of the greater selectivity for this ion by TCAC (Itoh, Kobayashi & Ueno, 1979). Therefore the carrier more readily undergoes an interaction with this ion than with chloride, however it is not as readily released and therefore exchanged for phenylalanine, at the other organic-aqueous interface. This results in a lower transport rate for phenylalanine in exchange for nitrate than chloride. This was reflected in the study of Neplenbroek et al (1992c) who found that release of nitrate in exchange for chloride was a limiting factor in transport, and a large excess of chloride ions was required to shift the equilibrium constant to favour release. Therefore the choice of counter-ion must reflect the balance between the need to readily undertake both complexation and dissociation ion-exchange reactions at the organic-aqueous interfaces.

The sulphate ion, having a double negative charge, is not readily transported because it requires two carrier molecules for each molecule of sulphate to maintain electrical neutrality and hence allow solubility of this carrier-substrate moiety. The reduced transport with this ion reflects the relatively low probability for this occurring (Sato et al 1984).

4.2.2. Transport rate studies.

4.2.2.1. Physical factors.

4.2.2.1.1. Temperature.

Figure 3.13 shows that an increase in temperature resulted in an increase in the rate of transport of phenylalanine. This supports the idea that the exchange process is a diffusional one, characterised by the Stokes-Einstein equation where diffusion is proportional to temperature and inversely proportional to the molecular radius of the permeating species and the viscosity.

The model presented in Figure 1.11. indicates that there are several possible limiting factors. Molinari et al (1992), in an investigation of SLM systems, indicated that it is a combination of diffusivity and equilibrium constant that combine to give the transport rate. However diffusion at the unstirred aqueous layers of the aqueous - organic interfaces will also be elevated at higher temperatures and it is unclear from this study, and the literature as indicated in Section 1.3.3.2., whether the rate limiting diffusional process is across these layers or within the organic phase.

Uddin et al (1990) have examined the mass transfer processes occurring at the interface during phenylalanine transport across an LEM system carried by TCAC. They reported that transport across the unstirred film to be the limiting factor in the transport process. Chan & Wang (1993) studied a similar system and successfully modelled it with the limiting case based on an interfacial chemical reaction. However other workers (Behr et al, 1985, Izatt et al, 1989, and Mohapatra et al, 1992) have

reviewed published data and shown that a organic phase diffusion-limited model is most appropriate.

4.2.2.1.2. Flow rate.

An increase in flow rate, as shown in Figure 3.14a, leads to an increase in the rate of phenylalanine transport into the receiving phase. This is predicted by theory as the resistance due to unstirred boundary layers is reduced and has been reported elsewhere (Neplenbroek et al 1992c).

However Figure 3.14b reveals that using the carrier transport factor (CTF) described in the Section 2.2.4.1. not all of the increase in phenylalanine concentration in the receiving phase was due to increased carrier mediated transport, but partially occurred by an increase in leakage across the membrane. This is indicative of instability of the membrane organic phase as a result of shear induced emulsification processes at the higher flow rates, and hence it is difficult to conclude that the flow rate increases transport solely by reducing unstirred boundary layer resistance and instead is associated with leakage.

4.2.2.2.Physicochemical factors.

4.2.2.2.1.Substrate concentration.

The effect of changing the operating parameters on the rate of transport of phenylalanine is shown in Figures 3.15 and 3.16 which show a typical facilitated transport process, for example an enzyme mediated transport process across a biological cell membrane, i.e. the rate is saturating rather than linear with increasing

concentration of the transported species (phenylalanine or chloride). If transport was occurring by a passive route a linear increase in rate would be expected. Similar data by Straaten-Nijenhuis et al (1993) has been interpreted and successfully predicted in terms of a diffusion-limited carrier mediated transport model.

Similar data was reported for the carrier mediated transport of nitrate by TCAC in exchange for chloride ions (Neplenbroek et al, 1992d). The limiting factors were attributed to concentration polarization in the source phase and an interfacial exchange limiting factor in the receiving phase; the role of the carrier was not determined to be pivotal. Whilst these factors maybe significant, the role of the carrier is also important. The significance of the interplay of these factors is shown, in the present study, by the level of transport when the carrier concentration is doubled (Figure 3.15). This results in a higher maximum rate of transport but has the same final level of phenylalanine transported since the concentration polarization in the feed phase becomes significant at higher phenylalanine concentration.

Molinari et al (1992) also reported similar data for the transport of amino acids, across a SLM system, in exchange with chloride ions. Variations in flux with increasing chloride concentration in the receiving phase were observed. They concluded that the levelling off (and decline) of transport rate was characterised by a decrease in the partition coefficient (K_d) of phenylalanine due to the increasing concentration of chloride in the source phase. Lee et al (1992) considered the carrier mediated transport of penicillin G across an SLM and suggested that at initial low substrate concentrations the transport is limited by diffusion through the unstirred aqueous boundary layers, but at higher substrate concentrations transport was limited by the diffusion of the carrier in the organic phase.

Figure 3.15 and 3.16 also show the rates of leakage which occur when no carrier is present. The levels of carrier-mediated transport are very much higher than the leakage rates, indicating that non-carrier mediated transport is not a significant phenomenon under these conditions.

The difference in the K_m values obtained from the graphs has two potential interpretations, either the carrier has different affinities for the two substrates, phenylalanine and chloride, or the carrier acts in such a manner that there are two different carrier sites. The carrier for the transport of phenylalanine has either a higher affinity for the amino acid than chloride, or has fewer sites for its transport. This is possibly the limiting factor for transport and hence could explain why a similar maximum transport rate was achieved. However the limitation may also be a form of inhibition at high substrate concentration levels, as higher concentrations of either ion (phenylalanine or chloride) at the interface, would lead to increased interfacial tensions and hence resist the movement of substrate into the organic phase.

4.2.2.2.2. Analysis of kinetic data.

As shown in Section 3.2.2.2.3., a plot of 1/J against $1/[A^o][X^o]$, is a plot of rate against substrate concentration and the intercepts with abscissa and ordinate yield the Extraction constant (K_{ext}) and Permeation Coefficient (P) respectively.

In the present study the transport data can be interpreted in the same manner. However it is worth noting that the data for this interpretation was obtained from the initial portion of the graphs of Figures 3.15 and 3.16, before the rates were saturating, i.e. at the initial, maximal reaction rates.

The variations in phenylalanine concentration reveal a transport reaction with lower *P* and K_{ext} values than those for chloride. Although this is not an ion-pair exchange process, the application of this model supports the idea that there are different rates of transport for each of the two species and that the carrier probably has different affinities for each substrate.

The lower *P* and K_{ext} for transport of phenylalanine can be interpreted as a lower transport to, and release of, this ion to the reciving phase. This is reflected by the K_m values which showed that the carrier had a higher affinity for this phenylalanine than chloride. This higher affinity presumably reduces the probability of the dissociation reaction, which is probably the limiting reaction.

4.2.2.2.3. The effect of substrate concentration on non-carrier mediated transport.

Leakage rates, although relatively insignificant when the carrier is not present, do increase with increasing concentration of either phenylalanine or chloride. This suggests that osmotic potential is a minor cause of instability in the absence of the carrier (Figure 3.18. and 3.19.). However it is not possible to quantify the significance of this phenomena when the carrier is present. Other groups (Danesi et al, 1987, Ramaseder et al, 1993) have also indicated that osmotic potential is influential in causing membrane instability. If the sharp rise in transport rate followed by a secondary rise with variation in chloride gradient is genuine this could represent the interplay of two phenomena. Initially the osmotic potential of the chloride is sufficient to cause leakage. As the chloride concentration increases the emulsion sedimentation properties and increased interfacial tension become significant, which results in a

decrease in the rate of leakage followed by a slower increase in leakage rate with increase in chloride concentration, representing a balance between the two opposite phenomena.

4.2.2.2.4. Carrier concentration.

The rate of phenylalanine transport using various carrier concentrations showed two phases (Figure 3.20), an initial very slow increase in rate with an increase in carrier concentration and a second more rapid increase at higher carrier concentrations. This has been reported previously by Kalachev et al (1992) who suggested a "jumping" mechanism which can only be triggered above a certain carrier concentration.

A similar phenomena has been described by Cussler et al (1989) and Noble (1992) who used a "chained" carrier system. In this model distribution of carrier molecules immobilised on the membrane support, within the organic phase is essential; if they are too far apart they are not able to contact and transfer permeate species. The concentration above which this occurs, the percolation threshold, has a faster rate of transport since simple diffusion of the carrier across the organic phase is not a rate limiting component of the transport process, instead carriers make direct contact with one another and a relatively fast exchange process occurs.

However, if this jumping mechanism occurs, it negates the interpretation of data in terms of a diffusional process, and hence the above models would not be appropriate. Although this is not a reason for dismissal of this hypothesis, it seems an improbable scenario because at the transition concentration (approximately 50 mM carrier in 5.5 M decanol) the molecules of carrier are present at only a 1 % molar

concentration in the decanol phase. The molecular sizes, compared to monatomic carbon, of the carrier, diluent (decanol) and substrate are approximately C 16, C 10 and C 8 respectively. Hence the carrier molecules, if homogenously dispersed and in an open conformation, in the decanol, are approximately 1 unit in 60 apart and presumably too far for the amino acid to "jump" between.

Noble (1992) has postulated a diffusional model for when carriers are too far apart. However this requires the substrate to be neutral. Therefore for phenylalanine to be able to jump to another carrier, it must complex with cations, for example either hydrogen or sodium. Only limited amount of sodium leakage occurs across the membrane and therefore these ions are potentially available to undertake such a reaction. No changes in hydrogen ion concentration were detectable across the membrane. The small magnitude of available cations therefore make this type of transport unlikely.

Another possible explanation for the carrier concentration effect is that at the critical carrier concentration there is sufficient carrier, not participating in the exchange processes, to lower the interfacial tension and thereby to allow a greater transport rate. This could increase to a point that is limited by the viscosity of the phase, the availability of phenylalanine molecules and the local solubility of the carrier at the interface. This supports the idea of transport being limited by various factors, depending on concentration. At low carrier concentration it is an interfacial limitation, at higher concentrations (normally used in transport in this study) it is a diffusional limitation.

The levelling off of rate is to be expected as at high concentrations of carrier the organic phase becomes more viscous. Sirman, Pyle & Grandison (1991) and

Shukla et al, (1992) have reported similar phenomena for the transport of plutonium across a SLM. They concluded that the saturation in the rate of transport was also due to insufficient stripping molecules, whereas Molinari et al (1992) included the aggregation of excess carrier molecules to explain the observed effects at high carrier concentration.

4.2.2.2.5. pH effects.

An increase in the rate of transport of phenylalanine with pH might be expected as the pKa of the amino group of phenylalanine is at 9.13, hence above this value the overall charge of the amino acid is negative (Table 2.2) and it could therefore be more receptive to transport by interaction with the carrier (Figure 3.21). Below this pH value, although the carboxyl group is negatively charged it is affected by the positive charge of the amino group.

4.2.3.Stability Studies.

4.2.3.1. Organic phase effects.

Stability of the organic phase was assessed by measurement of retention of the carrier after exposure to shear forces. The extracted carrier was expressed as a percentage of the concentration of the carrier present in the initial sample (Figure 3.22). Decanol has the lowest Log P value and hence the greatest capacity to partition into the aqueous phase. Danesi et al (1987) also indicated that decanol gave the

highest level of water solubility in their study and hence allowed osmotic forces to be influential in the transport process.

Figure 3.23 shows the transport rate in the different organic phases and that the rate of transport is influenced by more than the stability of the organic phase.

There are at least two possible interpretations of these results. Firstly, diffusion of the carrier across the organic phase is the limiting step, which in turn is influenced by the viscosity of the solution. NPOE is a more viscous solution than 1,3,5, TEB and hence a higher transport rate in the latter might be expected. Nakatsuji et al (1992) have shown that NPOE SLM systems are very stable but have low transport rates. However decanol and 1,3,5, TEB have similar viscosities yet different transport rates.

This could be due to the slightly more hydrophillic nature of decanol, which imparts poor stability to the liquid membrane, under extreme shear forces, but can also allow greater diffusivity. Under extreme conditions this could cause membrane instability and allow direct contact between the two aqueous phases. Further, NPOE and 1,3,5 TEB have aromatic domains which may interact with phenylalanine and hence retard the diffusion of the carrier-substrate complex across the membrane.

Previous results have shown that without the carrier present there is only very limited transport and thus it is assumed that the membrane integrity is maintained at the experimental flow rates. The less extreme case of the breakdown in membrane integrity, is that the interfacial tension between the two phases is reduced and therefore allows greater exchange rates. Furthermore, throughout the course of the experiment, a degree of emulsification may lead to intact but thinner membranes.

Alternatively the contact angle of the decanol with the support may be lower; when the membrane is initially prepared the decanol may associate more closely and

therefore form thinner membranes. The ability to form a stable SLM with decanol is as much an attribute of the support chosen; in the present study an ETFE polymer was supplied by Scimat Ltd. (Section 2.2.1.). The nature of the support is often not considered in designing a SLM system. To develop a stable system the critical surface tension of the support and the relationship with the hydrophobicity of the organic phase must be considered. The interplay of these related factors has been investigated by Barnes & van Staden, (1992) to optimise stability and transport rate.

These descriptions are obviously qualitative, although it may be possible to describe them quantitatively in order to correlate transport rates with the Log P values and viscosity measurements of the organic phase, support hydrophobicity and critical surface tension to enable further optimisation of the system.

Measurements of the physical properties of the interactions of NPOE and decanol with the carrier (Neplenbroek et al, 1992d) indicate that the viscosity of the two solvents are approximately the same. However NPOE, despite the presence of the carrier, forms a solution with a much higher interfacial tension and a much lower solvation potential for water than decanol plus carrier. A higher interfacial tension will result in a reduced ability to extract and transport amino acids.

4.2.3.2. Carrier effects.

The rates of carrier mediated or induced transport in the absence of chloride for TCAC and TOAB were equal. Thien et al (1988) and Itoh et al (1990) have concluded that carriers, by virtue of their active nature, associate with interfaces and in turn reduce stability. TCAC has an irregular geometry and therefore has a greater tendency to associate at interfaces and undergo emulsification processes than carrier

molecules with regular structures, such as TOAB. The use of TOAB yielded a similar transport rate in the absence of a chloride gradient and has shown that emulsification processes facilitated by TCAC, were not significant in the leakage of phenylalanine across the membrane. Therefore this must be occurring by either an osmotic potential route or uncoupled carrier mediated transport.

This finding supports the results given above for the effect of different organic phases. The reduction in the rate of transport of the system when using NPOE and 1,3,5, TEB is not due to a reduction in emulsification processes, but instead is due to an increased interfacial tension preventing the necessary exchange processes occurring.

4.2.3.3. Chloride ion effects.

The aim of this series of experiments was to investigate how one of the more stable of the organic phases (NPOE), which has reduced exchange processes, compared to decanol under different chloride conditions. Figure 3.24 shows that significant carrier mediated transport in the absence of a chloride gradient occurs.

The presence of chloride ions, at the aqueous-organic interfaces, increases the interfacial tension and could therefore reduce leakage occurring by preventing instability of the organic phase. Therefore by having chloride ions in both aqueous phases, at an equal concentration, could lead to greater stability. This would theoretically reduce the transport of phenylalanine to the levels observed when no carrier is present i.e. virtually zero. This did not occur although transport was reduced.

The reduction in transport (leakage) when equal concentrations of chloride ions are present on both sides could be due to greater membrane stability or to greater competition for transport sites on the carrier in the organic phase. This is pertinant as

the lowest chloride ion concentration in the feed phase was greater than the phenylalanine concentration and could possibly compete with the exchange process at the source phase interface.

Although Figure 3.24 showed the expected qualitative effect, the levels of transport with 1.0M chloride in both phases were higher than those for transport in the absence of the carrier, indicating that transport occurs, and the presence of the carrier molecule is required for this type of leakage. The carrier, which is interfacially active, can theoretically increase emulsification processes and hence allow water to enter the organic phase, or it could induce an osmotic potential across the membrane. In the present study NPOE was shown to be more stable than decanol due not to a reduction in emulsification processes, but a reduction in carrier exchange processes and that emulsification induced instability was not significant. Therefore the carrier could function despite the lack of chloride gradient, randomly exchanging with any anions present.

4.2.3.4. Comparison of decanol and NPOE as organic phases.

A comparison, shown in Figure 3.25, for TCAC as the carrier, with the NPOE or decanol as the organic phase, shows decreased transport with a 0.1 M chloride gradient for NPOE and virtually negligible leakage when equal concentration of chloride ions were present on both sides. The greater transport rates with decanol, in this study, have been attributed to greater interfacial activity by the carrier, and hence the reduction in transport with NPOE, in the presence of chloride ions in both phases, is presumably due to the increased interfacial tension impeding transport by the

carrier. This indicates that the leakage associated with decanol/TCAC is due to greater interfacial activity by the carrier via random exchange processes.

However when transport of phenylalanine occurs, in the presence of a chloride ion gradient, it is not possible to conclude that the same degree of uncoupled transport occurs. This is because the carrier has a preference for chloride as the counter ion over other anions. Palet et al (1993) also observed non-specific coupled transport with hydroxyl ions but also concluded that the carrier molecule in their study (a vitamin B_{12} complex) had a preference for chloride.

4.2.4. Specificity Studies.

4.2.4.1. Exchange processes.

The carrier transport factor (CTF) was found to be a constant value of slightly greater than 2 for chloride : phenylalanine exchange over the 24 hours period investigated.

This is highly significant as it demonstrates that any leakage is constant throughout the test period and does not become greater in the later stages of the experiment, which might be expected if emulsification gave rise to instability. Instead random exchange processes, due to high interfacial activity, occurred at a constant rate throughout the test period are present.

This result was obtained from a system with a demonstrated stability and only low carrier mediated leakage. The value of 2 : 1 for chloride : phenylalanine, differs from the value obtained by Neplenbroek et al (1992d) for the transport of nitrate by TCAC in exchange for chloride ions. The exchange ratio, for the carrier mediated

transport exchange of chloride for phenylalanine, was possibly even greater in the present study because anions other than chloride could have been involved in interfacial exchange processes. This supports the possibility of even higher ratio exchange processes.

Evidence for this has emerged from other areas of study, for instance affinity studies revealed different K_m values for phenylalanine and chloride, and which, using the model of Yamaguchi et al (1988), yielded lower P and K_{ext} values for the movement of phenylalanine as would be expected if more than one form of the carrier was required in the transport. Sato et al (1984) used TCAC as a carrier for the transport of divalent metal ions and reported a 2 : 1 exchange ratio with the counter ion. Teramoto et al (1991) developed a LEM system for the cationic exchange transport process of phenylalanine, in exchange for protons, and reported a 2 : 1 exchange process where the carrier acted in a dimeric form when transporting the amino acid. Similarly Wienk et al (1990) reported the same exchange ratio for crown ether mediated cation exchange processes.

Rebek et al (1987b) developed a convergent synthetic carrier for amino acids and found that phenylalanine associated in a 2 : 1 ratio and was transported through a bulk liquid membrane down a diffusion gradient of the amino acid. The synthetic carrier used had various domains including an aromatic one and a highly polar microenviroment in a cleft which, despite the lack of solubility of the carrier in an aqueous phase, enabled interactions with the polar regions of the transported amino acids. Therefore the zwitterionic region of one molecule of phenylalanine associated with the polar cleft of one carrier via ion attraction and the aromatic region associated with another the aromatic domain of another carrier. The aromatic domain of the

amino acid was highly significant enabling phenylalanine to be transported at a faster rate than other, more hydrophobic, amino acids such as isoleucine.

4.2.4.1.1.Proposed mechanism of transport.

Molecular recognition studies by Tong et al (1992) have indicated that many weak forces act to induce receptor - substrate bonding eg. dipole - dipole, electrostatic, Van der Waals and hydrogen bonding and hence lead to a high degree of specificity from a relatively simple model. In this case it is proposed that in the organic phase phenylalanine bonds to two carriers by electrostatic interactions and/or hydrogen bonds. Therefore it is proposed that the TCAC associates in a similar manner to the carrier used by Rebek et al (1987b), (Figure 4.2.). The necessity of using two carrier molecules to transport each molecule of phenylalanine results in the there being fewer active sites for transport. This reflects the conclusion of Section 4.2.2.2.1. that the K_m of the carrier for phenylalanine transport is lower.

The substrate - carrier complex is larger and therefore will diffuse more slowly. Furthermore the affinity of phenylalanine for this form of the carrier is sufficiently high to limit transport as the dissociation reaction is not as readily undertaken as the dissociation of chloride from the other form of the carrier. This is reflected by the kinetic data of Section 4.2.2.2.2. where a lower P and K_{ext} is found for the transport of phenylalanine than chloride.

The joining of these two substances in the proposed manner results in some residual charge on the substrate - carrier complex, however this is shielded by the relatively large aliphatic groups of the carrier. This is shown in Figure 4.2. Finally it

is also possible that there is some agglomeration between substrate - carrier complexes to generate even larger species.

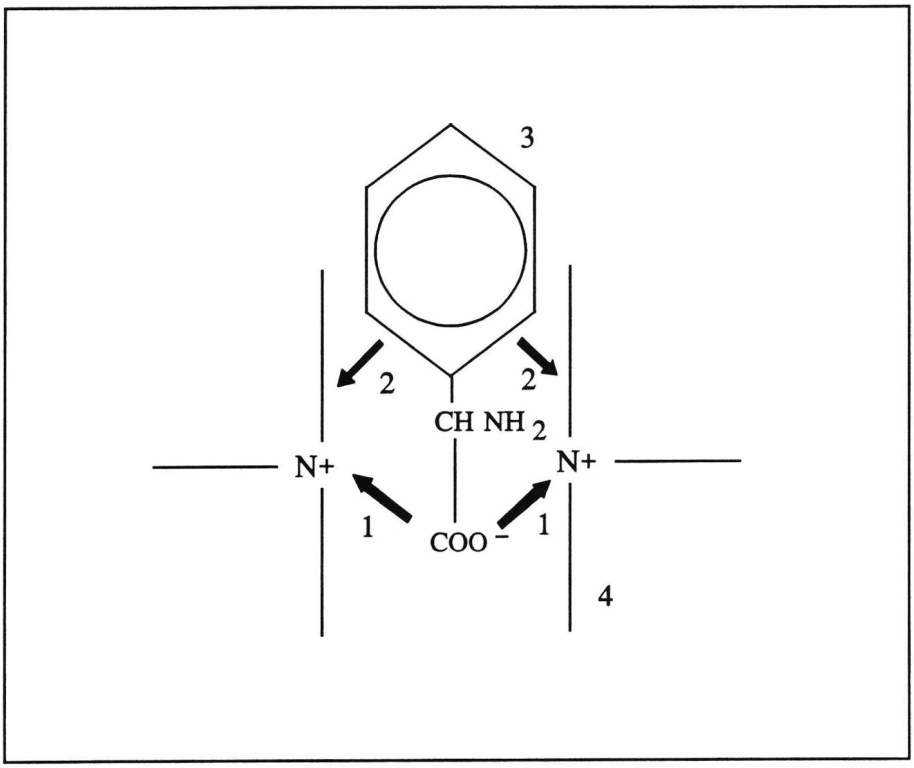


Figure 4.2. Proposed phenylalanine - carrier molecular recognition. The joining forces are electrostatic attraction (1) between the charged domains and hydrogen bonding (2) between the aromatic and aliphatic domains. The substrate (phenylalanine) (3) is surrounded by the carrier (tricaprylylammonium chloride) (4).

4.2.4.2. Rates of transport for mixtures of amino acids.

The relative rates of transport of a mixture of amino acids at different pH values, and hence different prevailing potential charges (see Table 2.2.), were studied. The results obtained were not those expected (Figure 3.26a and 3.26b). Instead at pH 11 only phenylalanine and aspartic acid were transported to any significant degree, and at pH 5.48 only trace amounts of these amino acids were found in the receiving phase. Lysine was not transported at either pH value.

Obviously the transport of amino acids is more involved than suggested by the above relatively simplistic approach based solely on the overall charge. At pH 11 it is not obvious why all three amino acids are not transported to a similar extent. Possibly this relates not to the overall charge on the molecule, but to the individual charges of the carboxyl and amino groups that are present.

For any amino acid-carrier moiety to be soluble, and hence diffuse across the membrane there must be no overall charge present on the surface. In the case of phenylalanine at pH 11, it only has one negative charge which can associate, via electrostatic interactions, with a carrier molecule and be transported. Aspartic acid has two negative charges and hence a higher charge density which could make it unattractive for an exchange process. Itoh et al (1979) in two-phase ion extraction studies with TCAC, has shown that TCAC favours bigger anions with a lower charge density. The charge density and steric hindrance probably prevents aspartic acid associating in a 2 : 1 maner similar to phenylalanine at pH 11. Also lysine has only one negative charge at pH 11, however the pKa of the R Group is approximately 10.6 and therefore at pH 11 there is relatively

more residual positive charge, which is sufficient to make bonding to the carrier less attractive.

At pH 5.48, there was a much lower rate of transport of phenylalanine and aspartic acid. This could be due to the presence of opposite charges on the carboxyl and amino groups which cause only very weak binding of the zwitterionic form of phenylalanine by reducing binding as the amino group acts to make the carboxylate group less attractive.

However it is not obvious why phenylalanine should be transported at this pH, and aspartic acid was not transported to a greater extent. Again this is possibly related to the individual charges present. At pH 5.48 aspartic acid has a higher charge density than at pH 11 and hence has less ability for solvation in the organic phase. Phenylalanine, at pH 5.48, is also in an energetically unattractive form for transport. However it is transported more readily than aspartic acid due to the presence of the aromatic ring which is more soluble in the organic phase and allows hydrogen bonding with the carrier.

Selectivity of quaternary ammonium salts is by virtue of hydration energy of the target ion and therefore selects more hydrophobic ions. Hence the presence of a hydrophobic side chain is critical to transport.(Yamaguchi et al 1988, Rebek et al 1987). The significance of side chains for selectivity in transport processes has been investigated by various workers. For instance phenylalanine has been reported to be transported much more rapidly than alanine, another non-polar amino acid, in an LEM system (Behr & Lehn 1973). Thien et al (1988) investigated the carrier mediated transport of phenylalanine by TCAC and found a high degree of specificity was found for phenylalanine against other inorganic ions. Part of the basis for this selectivity was

based upon the hydrophobic nature of this amino acid; a result that has been found by other groups.(Behr et al, 1973, Wienk et al, 1990, Palet et al, 1993).

Rebek et al (1987b) also noted that lysine was not transported. This was attributed to the lack of an aromatic side chain which is very important in recognition and transport processes.

In this study assessment of the transport rates, at pH 11, of the amino acids revealed that the transport rate for phenylalanine was 2.33 μM/h/cm^2 whereas the rate for aspartic acid was 0.03 μM/h/cm^2, approximately seventy times lower. Lysine was not transported. This significant novel finding underlines the commercial potential of supported liquid membranes for selective separations.

4.2.5. Future work.

To examine the potential for selectivity inherent within this system it is desirable to model the process more precisely. This would entail investigating the structure of the carrier in the organic phase, what conformation it assumes and how combining with amino acids affects this. Computer modelling of the structure and examining the most stable, least energetically demanding form of the molecule would illuminate this. Combined with this would be a consideration of what bond energies represent the optimum for a counter ion

This system also has significant potential for developing into a chiral separation system by using a carrier with a chiral centre. Either commercially available, or novel synthetic compounds could be utilised for a chiral process.

Finally the present system is a model system. There could be substantial commercial benefits from developing such a system. To further develop it is necessary to investigate the effect other interfering substances have on transport of the target species. For instance the system could be applied to a fermentation broth containing the target amino acid.

4.3. Conclusions.

The membrane systems examined in this thesis have several features in common. In both areas of membrane technology surprisingly little research has addressed the relationship between the properties of the membrane, to the performance of the system; this is especially true for SLM systems. However both areas would benefit from rational membrane design processes related to system parameters such as low fouling and defined pore structure for UF membranes and good retention characteristics for the SLM membrane support. This thesis was partly an attempt to address this through the study of UF membranes. However time did not permit full realisation of this due more to commercial reasons associated with the industrial financial support of the project.

The analysis of the both systems is intimately linked to parameters such as diffusivity, viscosity and concentration gradients. Further electrostatic, hydrophobic and hydrogen bonding forces underpin both studies as molecular recognition in both protein structure and carrier - substrate interactions.

4.3.1. Ultrafiltration systems.

This study has illustrated some of the potential areas of inaccuracy in assessment of UF membranes, such as mode of use of membrane, concentration and pressure. It has also attempted to demonstrate the extent to which temperature effects are influential in terms of operating efficiency and protein conformation.

Adsorption of protein, especially when denatured, to membranes resulting in

has been described previously and demonstrated to be an influential factor in this study and that at higher temperatures it overcomes the benefits of greater diffusivity.

Furthermore variations in temperature have not been demonstrated to be an ubiquitous denaturing effect as permeate samples had more significant changes in tertiary structure, than samples from the retentate and reservoir. Instead temperture acts to increase susceptability to other denaturing forces. Although the specific nature of the denaturing force(s) is not clear, it seems probable that this is linked to passage through the membrane.

4.3.2. Supported liquid membrane systems.

The transport of phenylalanine across a decanol\ETFE SLM system has been examined in terms of an ion-exchange, carrier mediated diffusional model and the role of the various components has been examined. It was found that the carrier, TCAC, has different affinities, permeabilities, and extraction coefficients for the two substrates and exhibits optimum substrate concentrations for the transport process.

The organic phase has been shown previously, and in this study, to be susceptible to shear force-induced disruption. However with the Scimat ETFE support membrane and at the flow rate used, emulsification processes were not significant in causing leakage and indicates the potential of this support material for SLM applications. Osmotic forces were also not found to be significnt in any observed leakage. Instead the lower interfacial tension of decanol allows higher transport rates than with

leakage. Instead the lower interfacial tension of decanol allows higher transport rates than with organic phases which are less susceptible to shear forces. However there is a degree of uncoupled carrier mediated transport and a possible route for osmotic potential induced leakage of phenylalanine.

The carrier mediated transport process results in a 2 : 1 exchange process between chloride ions and phenylalanine. This exchange ratio was constant over a 24 hour period, and supports the above conclusions that the system was stable over the test period with uncoupled transport occurring at only a small but constant rate.

When challenged by a series of amino acids it was found that the transport rate for phenylalanine was approximately seventy times greater than that for aspartic acid at pH 11 and that lysine was not transported. The SLM system may therefore have commercial potential for selective separations of amino acids or other molecules.

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Effect of temperature on protein conformation and activity during ultrafiltration

Moray J. Campbell[a,*], Robert P. Walter[a], Robert McLoughlin[b] and Christopher J. Knowles[a]
[a]*Biological Laboratory, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ (United Kingdom)*
[b]*Scimat Ltd., Techno Trading Estate, Bramble Road, Swindon SN2 6EZ (United Kingdom)*

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Abstract

One of the advantages of ultrafiltration for the bioseparation of proteins is the comparatively mild conditions used. A key operational parameter in such a system is the temperature utilised for the process. There are conflicting demands when selecting an operating temperature between the fluid dynamics and the biochemical properties of the system. An analysis of this conflict, in order to establish the optimal temperature, involved a model crossflow filtration system, where shear rate, volume of protein containing solution and protein concentration can be kept constant and only the temperature varied. In such studies denaturation of the permeate was observed whereas the processing efficiency did not appear to have a temperature optimum.

Keywords: ultrafiltration; temperature; protein denaturation; protein conformation; retentivity; fouling

Introduction

Assessment of ultrafiltration (UF) membranes can be undertaken by various methods. For example, use of contact angle measurements coupled with pore size and distribution measurements [1,2], the use of electron microscopy [4,5] and other novel techniques including gas adsorption/desorption [3], and to examine the membrane surface. However these methods do not give a direct analysis of membrane performance. Instead they are physical examinations of the membrane surface, which ultimately have only limited implications for membrane performance.

These limitations can be overcome by an *in situ* assessment of UF membrane performance using proteins [6] or dextrans [7]. This work was extended to the use of polydisperse polymers of known molecular weight distribution [8–10] to give a fuller rejection profile of the membrane. However, this approach is also potentially inaccurate due to the various factors which influence protein separation across UF membranes. These include gel polarization and dynamic membrane formation as well as a consideration of protein biochemistry [11]. Consequently a full description of a UF membrane should include a physical description as well as performance related data.

Many of these inaccuracies arise as a result of assessment of membranes using dead-end stirred separation units. Such units can be operated in one of two modes; concentration mode,

*To whom correspondence should be addressed.

where the feed volume is reduced under pressure, or diafiltration mode, where constant feed volume is maintained via use of a buffer reservoir operated under pressure. Operation in a concentration mode, although simple, does not maintain constant conditions throughout the experiment. Instead, the reduction in feed volume during the course of the experiment affects many other parameters that are volume dependent, for example stirring, shear rate and, especially, protein concentration and gel layer thickness. Alternatively, if run in a diafiltration mode, although the feed volume is maintained at a constant level, the protein concentration above the membrane increases and separation efficiency cannot be directly attributed to membrane performance.

This situation is further complicated by the use of proteins as solutes to measure passage through a membrane. These compounds do not have a fixed conformation, but are in a dynamic state where conformation and activity are a compromise between flexibility of structure and stability of the molecule. This has been reviewed [12] and it has been demonstrated that protein structure and folding is controlled by the free energy of stabilisation, which is a result of the difference between stabilising and destabilising forces, and is approximately 50 kJ-mol^{-1}. It is attributable to a small number of hydrogen bonds, ion pairs or patches of hydrophobic interactions. The energy required to destabilise such a structure can be supplied in many different forms such as thermal, pressure or shear forces, and concentration or chemical interactions.

Temperature, which is highly influential in determining protein conformation, is a key factor in the operation of an ultrafiltration system and its effects upon protein conformation have been examined extensively [13,14] to reveal the conflicting demands in choosing a particular operating temperature. Considerations of the physicochemistry of the system indicate that it must be operated at as high a temperature as possible for two reasons. Firstly, diffusivity is increased hence reducing concentration polarisation at the membrane surface. Secondly, the viscosity of the solution decreases and hence flux will, theoretically, increase.

However the desire to increase the operating temperature, to improve membrane performance, can affect protein conformation and its relationship to function. Studies of a model ultrafiltration system with respect to the relationship between increased temperature, decreased stability of protein conformation and membrane fouling [15], have shown that an increase in temperature may result in exposure of the protein's inner hydrophobic core to the environment. Such a denatured protein may have a tendency to either aggregate or adsorb to suitable surfaces rather than remain in solution. The membrane can provide a surface to which the protein may adsorb, leading to an increase in fouling [16,17]. Lower operating temperatures are therefore preferable in order to maintain protein conformation. It has been observed in our laboratory that retentivity often increases with time, which in turn is influenced by temperature; this is consistent with the occurrence of fouling.

An examination of UF systems was initiated after we had observed that localised temperature increases occurred around a gear pump head which had been used to drive a crossflow filtration unit in a recycle mode for extended periods of time. If these were not taken into consideration, and corrected, they could have a great influence upon flux and fouling. Therefore, the present study was designed to limit the potential vagaries of the system by adopting a recycle mode of operation using a reservoir to maintain constant protein concentration and volume above the membrane. A crossflow system was used to ensure control of the shear rate. Use of a reservoir enabled accurate temperature control and hence a detailed examination

of temperature effects. This paper therefore describes an assessment of the conflicting effects of variations in operating temperature for an idealised ultrafiltration system.

Experimental

The protein used was α-amylase from *Bacillus cereus* supplied by Sigma Ltd., which has a molecular weight of 50 to 55 kDa. This protein has a known temperature sensitivity profile, being heat stable up to 40°C, then gradually losing activity until complete loss of activity at approximately 70°C. It was filtered in a crossflow filtration unit supplied by LSL Ltd., Sedgewick Rd., Luton, Beds., containing planar polysulphone UF membranes, of 50 cm^2 area, which were supplied by Scimat Ltd. These membranes had a formulation as follows: 600 ml dimethylformamide, 120 g polyether sulphone (Grade 4100p supplied by ICI), and 60 g polyethylene glycol (average molecular weight 600). The polyether sulphone was slowly added to continuously stirred dimethylformamide until dissolved. Stirring continued for a further 30 min at which time the liquid polyethylene glycol was added. After a further 30 min of continuous stirring the solution was allowed to stand for 2.5 hr. The solution was spread on to sheets of polyester film, 125 μm thick, mounted on a Werner Mathis Type SV laboratory casting, the spreader knife gap being set at 400 micrometer. The cast films were immediately quenched in deionised water and after 10 min removed from the polyester. This was washed by immersing in a water bath for 24 hr prior to drying in a air circulating oven, set at 70°C for 24 hr. These membranes were estimated to have a 60 kDa nominal molecular weight cut off by the *in situ* assessment of retentivity of various solutions of known concentration of specific proteins.

Ultrafiltration experiments were performed at a range of temperatures using a flow rate of 1500 ml-min^{-1}, which is equal to a tangential velocity of 2.3 m-sec^{-1}, and a back pressure of 1.75 bar. The reservoir contained 0.05% (w/v) α-amylase in 400 ml of 0.02 *M* phosphate buffer (pH 7). The experimental setup is shown in Fig. 1 and illustrates that the permeate and retentate tubes were combined, after passage through the membrane, before recycling to repass through the membrane.

Prior to the addition of the protein to the solution, the flux with buffer only was measured for each membrane. After addition of the protein an initial sample (5 ml) was taken from the 400 ml reservoir and was divided into two: one aliquot was stored at 4°C, and the other aliquot maintained in a water bath at the same temperature as the reservoir for the duration of the experiment, to act as a control. Aliquots (5 ml) were taken at 5, 15, 30 and 45 min from the permeate, retentate and the reservoir, respectively, and stored at 4°C. At each sample time the period required to collect the permeate aliquot was noted and recorded as the flux in l-m^{-2}-hr^{-1}. The flux was also measured at the

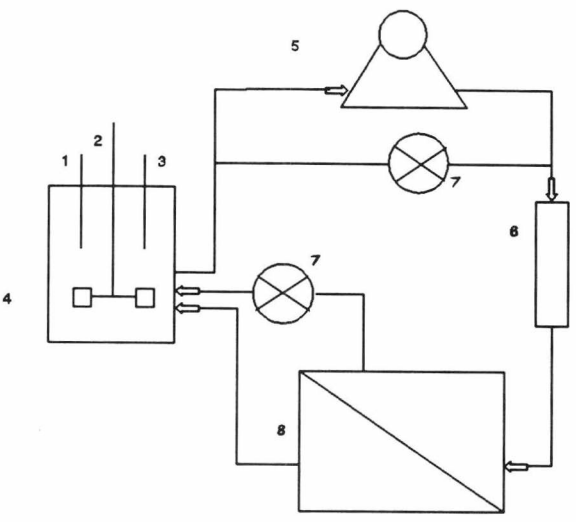

Fig. 1. Diagram of crossflow filtration (CFF) unit operating in a recycle mode. (1) Temperature probe, (2) impellor, (3) heat element, (4) reservoir, (5) gear pump, (6) flow meter, (7) control valve, (8) CFF unit.

start of the experiment as the time taken to collect a 5 ml aliquot from the first flow through the previously drained permeate tube. The experiment was run at five different temperatures: 12, 25, 38, 50 and 60 °C.

All samples that had been stored in the fridge after sampling were allowed to equilibrate at room temperature prior to simultaneous analysis of total protein content, enzyme activity and conformational changes to the protein.

Total protein concentration and specific activity were measured using Sigma kits numbers P5656 and 577-3 respectively. Changes in conformation were measured using fluorescence spectroscopy. The aromatic amino acids of proteins fluoresce at various wavelengths when excited. The application of fluorescence spectroscopy to characterisation of protein confirmation has been described previously [18,19]. The aromatic amino acids are hydrophobic and tend to be buried in the core of the protein. Hence when denaturation occurs they may be exposed to the environment and a shift in the emission spectra between the native and denatured state is observed. Fluorescence spectroscopy was performed at 25 °C by exciting the molecules at 280 nm and then scanning the emission spectra between 280 and 450 nm.

The factors influencing flux decline during ultrafiltration have been modelled as function of the volume of protein permeate [20]. However this approach is only useful for a system operated in a concentration mode and not applicable in a recycling mode. However the relationship, as investigated by Merin and Cheryan [20], between the amount of protein solution passed through the membrane and the degree of fouling was the basis for modelling flux decline during filtration caused by fouling. Plotting flux against time enables a linear regression analysis to yield a gradient factor (b) which is the fouling coefficient, and which is proportional to the rate of flux decline. A more fouled membrane may theoretically have a faster rate of flux decline as revealed by the fouling coefficient.

Results and discussion

The buffer flux, without the presence of proteins, was dependent on the inherent variations of the membrane and the operating temperature, and was found to be approximately 120 l-m^{-2}-hr^{-1}. The permeate flux data were derived as described in the text to obtain the fouling coefficients (b) as given in Fig. 2. At each of the temperatures tested there was no obvious decrease in flux with time as revealed by the relatively constant fouling coefficients; the variations that were detectable were smaller than the inherent variability of samples taken from a given batch of the membrane. A separate experiment with a sample from a separate batch of the membrane revealed the same variability in the initial fluxes with no pattern of change in flux with temperature.

It is interesting that the highest level of flux was at 25 °C which may represent the optimum operating temperature, allowing maximum diffusivity of rejected species away from the membrane surface, and yet resulting in minimum denaturation of the protein and hence minimum adsorption to, and fouling of, the mem-

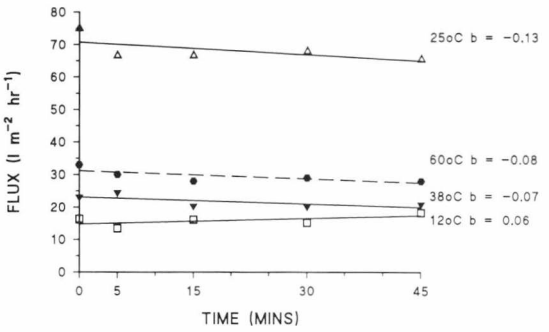

Fig. 2. Change in flux measured as a linear regression of permeate flux against time, to yield the fouling coefficient (b), for crossflow filtration of α-amylase; 12 °C (□), 25 °C (△), 38 °C (▼) and 60 °C (●).

brane. Measurement of protein concentration in the permeate and retentate enabled calculation of retentivity, which is defined as follows and shown in Fig. 3:

Retention coefficient =

$$1 - [\text{protein}]_{\text{permeate}} / [\text{protein}]_{\text{retentate}}$$

Inherent variability between membranes has been demonstrated to be significant, however this is reduced when considering the retention coefficient thus making this parameter potentially more representative of membrane system interactions. This occurs because this characteristic is a ratio of performance related values instead of a direct measurement, such as flux which is therefore more highly influenced by membrane variability.

The data show that increasing the temperature led to a decrease in retentivity. These results indicated that there was not an optimum for retentivity at 25 °C but instead showed that at higher temperatures there was greater membrane permeability for α-amylase. A possible interpretation is that denatured forms of the protein, which were demonstrated to be present, were not adsorbing to a significant degree, and hence fouling of the membrane was low, unlike was initially felt probable. Instead diffusivity away from the membrane was the greater influencing factor and resulted in a lower level of concentration polarisation and lower retentivity at higher temperatures. This also implies that the variations in the flux behaviour are essentially random, the level being dependent upon membrane properties and not the temperature or protein behaviour.

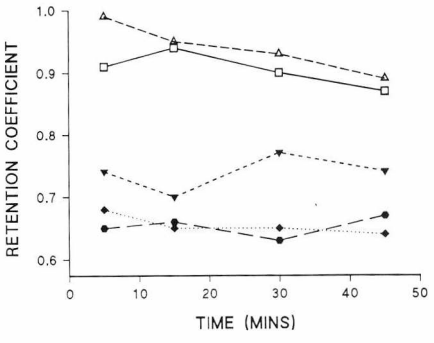

Fig. 3. Variations in retention coefficient against time for crossflow filtration of α-amylase; 12 °C (□), 25 °C (△), 38 °C (▼), 50 °C (♦), and 60 °C (●).

These observations differ from previous data [21] which indicated that adsorption of rejected protein had the greater influence upon performance of the polysulphone and polyvinylidene difluoride membranes investigated in the previous studies. This contradiction is indicative of the paramount role that membrane interactions have upon performance of the system and also suggests a low fouling capacity for the polysulphone membranes used in our study.

The specific activity of the α-amylase was measured at various times at each temperature (Fig. 4). It was found that the permeate had reduced specific activity compared to the retentate. Although the inactivation was somewhat enhanced at the higher temperatures, there was a noticeable effect at all the temperatures. Clearly temperature was not the major denaturing effect although it probably increased protein susceptibility to other denaturing effects. Although the α-amylase was partially denatured at the higher temperatures, it did not significantly affect performance of the crossflow filtration system; this again may in part have been due to the low fouling capacity of the polysulphone membrane used.

The potential effect of crossflow filtration on protein structure was measured by changes in the emittance spectrum of α-amylase in the permeate, retentate and reservoir. Figure 5 shows the emittance peak position for native protein in phosphate buffer, and the changes on filtration.

In confirmation of the data on specific activity, temperature did not appear to be the major denaturing factor although it appeared to enhance other factors affecting protein conformation. Proteins are dynamic structures that

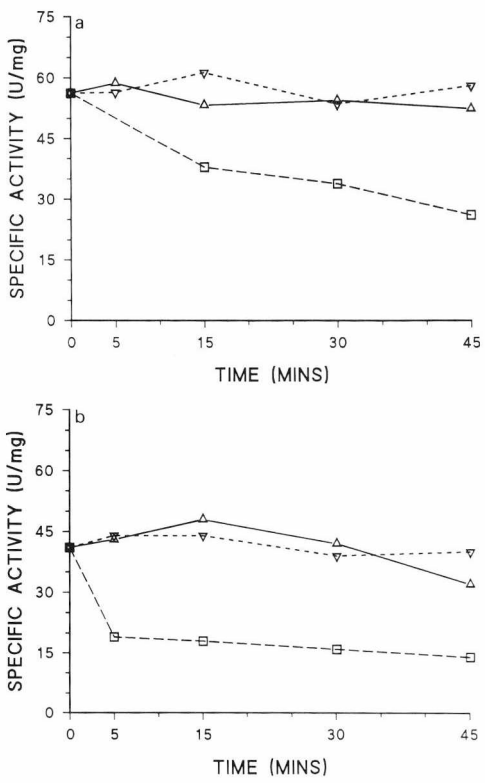

Fig. 4. Specific activity of α-amylase in samples of the permeate (□), retentate (△) and the reservoir (▽) during crossflow filtration of α-amylase at (a) 25 °C and (b) 60 °C. Similar data were obtained at 12, 38 and 50 °C.

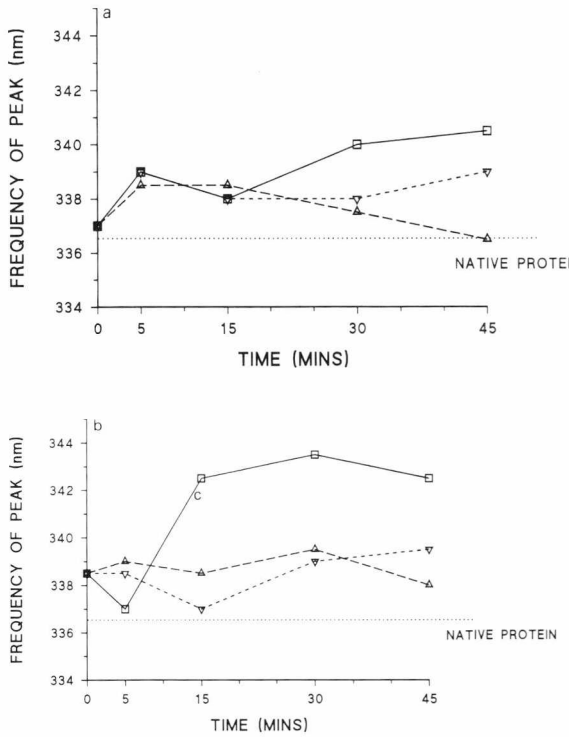

Fig. 5. The shift in fluorescence emission spectra for aliquots sampled from the permeate (□), retentate (△) and the reservoir (▽) during crossflow filtration of α-amylase at (a) 25 °C and (b) 60 °C. Similar data were obtained at 12, 38 and 50 °C.

respond to their environment by changes in conformation. Once a denaturing effect has been removed they will often rapidly reconfigurate to obtain their native structure. Storage of the partly denatured proteins at 4 °C was an attempt to overcome this problem. Such storage maybe significant for proteins that had been subjected to the higher temperatures where, reducing the operating temperature to 4 °C and then back to 25 °C, for measurement of fluorescence emittance, represented a potential denaturing force. However this does not negate the results within each experiment, i.e. in each case it was the permeate that was most denatured. The fluorescence patterns obtained did not represent the maximum deflection that could have been measured. Instead they are probably due to the protein being partially denatured and then reconfigurating. However such a series of events could produce agglomerations between proteins which in turn may add significantly to fouling. The fluorescence peaks therefore represent average values of the various denatured and/or agglomerated forms of the molecule.

Samples of α-amylase which had not been subjected to crossflow filtration were maintained in a water bath at the desired temperature for the duration of the experiment to yield control data on the temperature effects upon the protein (Fig. 6). The measurements of α-amylase activity and fluorescence peak position supported the predicted pattern of data for

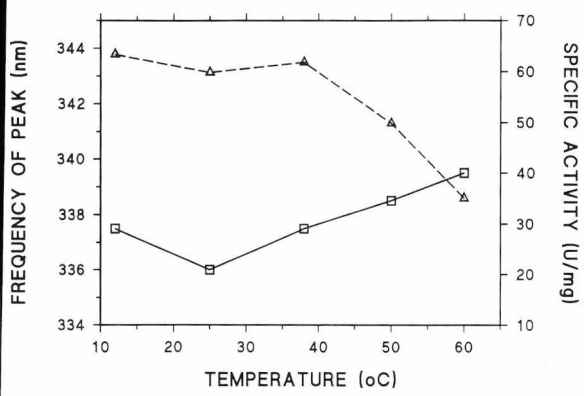

Fig. 6. Variation in specific activity (△) and protein conformation (□) against temperature for control samples held at operating temperature for duration of experiment.

this enzyme; i.e. it was stable until 40°C and then began to lose activity. Interestingly, the data for the loss of protein specific activity at a particular temperature was less than the loss of specific activity of the permeate at the same temperature, again supporting the idea of other denaturing forces acting on the protein during crossflow filtration.

Denaturing of proteins in UF systems has been investigated extensively by several groups and shear forces resulting from turbulent flow, pumping and mixing of proteins have been shown to be significant [22,23], whereas others noted severe losses in activity of enzymes which were not attributable to any obvious forces [24]. In addition, the effect of the number of passes through the membrane on protein structure as a result of shear forces in and around the pore has been shown to be influential [25,26], hence indicating a role of the membrane in denaturation.

In the present study it would seem likely that the denatured species which are found in the permeate arise from passage through the membrane. This may be due to either the tortuosity of the pores inducing shear forces, or a surface interaction between the protein molecule and the membrane pore. However if surface interaction on the membrane is actually causing the denaturation then it was not clear why, at this feed rate and reservoir volume, and within the experimental period, more of the protein was not denatured. For example, α-amylase at 38°C has a comparable stability to that at 25°C. However flux at the lower temperature is significantly higher and hence would be expected to result in a greater degree of denaturation. The volume of the feed from the 400 ml reservoir that permeated the membrane was approximately 250 ml at 25°C, compared to the volume at 38°C which was approximately 75 ml. This difference in volume of the feed that actually permeates the membrane could be detected by the extent to which the protein was denatured. However the specific activity and the fluorescence spectroscopy data did not show any greater extent of denaturation of the protein in the reservoir at the former temperature. This may be due to the relative insensitivity of these methods to detect the comparatively less dilute denatured form in the presence of the more common native form.

The inability to detect the denatured protein may have been due to reconfiguration of the protein after removal of the denaturing force. Previous studies [27] have shown that reconfiguration is highly concentration dependent and therefore more concentrated streams would not be as able to assume the native form. This system could be viewed as one in which there are two protein species, the native and denatured forms of the protein. As a recycle system is operating they must both be present in the reservoir. However the concentration of the denatured species, when returned to the reservoir, is actually decreased with respect to the protein concentration in the permeate samples. Thus it is possible for it to reconfigurate more readily than the permeate samples, which contain solely denatured proteins.

Alternatively the membrane could have been selecting the denatured form of the protein; it

may be that a protein that has a partially denatured structure is not so resistant to passage through a pore. If this was the case it was not obvious how it occurred and does not explain the nature of the denaturing force. If there was an ubiquitous denaturing force then, as the protein moved away from the area of influence, or was extracted via the sampling procedure, there would have been a tendency for reconfiguration.

Conclusions

This study has attempted to demonstrate the extent to which temperature effects are influential upon an ultrafiltration system both in terms of operating efficiency and protein conformation. Although adsorption of protein, especially when denatured, to membranes resulting in impairment of operation has been described previously, it was shown not to be as influential a factor in this study. Instead it was found that the greater diffusivity of the proteins at higher temperatures enabled a reduction in concentration polarisation at the membrane surface, thereby increasing permeability. This implies a low fouling capacity for these membranes. Furthermore variations in temperature have not been demonstrated to be an ubiquitous denaturing effect, but instead they act to increase susceptability to other denaturing forces. Although the specific nature of the denaturing force(s) is not yet clear it seems probable that this is linked to passage through the membrane.

Acknowledgement

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Investigation of the Stability and Selectivity of Phenylalanine Transport Across a Supported Liquid Membrane

Moray J. Campbell,[a]* Robert P. Walter,[a] Ray Singleton[b] & Christopher J. Knowles[a]

[a] Biological Laboratory, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ, UK
[b] Scimat Ltd, Techno Trading Estate, Bramble Road, Swindon SN2 6EZ, UK

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Abstract: A supported liquid membrane system was investigated for the carrier mediated transport of phenylalanine to more fully understand the contradictory effects, described in the literature, of chloride ion concentration in the aqueous phases on the stability of the system. The role of the organic phase and its interaction with carrier and support material was considered. The carrier mediated transport was comparable to an enzyme mediated process. Kinetic studies were undertaken and the data interpreted in a manner appropriate to biological transport processes to consider the transport process at a molecular level. The system was shown to deviate from a direct 1:1 exchange process between phenylalanine and chloride and had a high degree of selectivity with respect to phenylalanine.

Key words: supported liquid membranes, phenylalanine, amino acid, stability, transport rate.

1 INTRODUCTION

Liquid membrane technology has received much attention despite the paucity of commercially viable industrial examples. Potential applications of liquid membranes are based upon the separation of fermentation products either as part of a downstream protocol or as an extractive fermentation process.[1] These include extraction of amino acids[2–6] and their enantiomeric resolution,[7,8] the extraction of organic acids,[9–12] penicillin G[13,14] and alcohol (for low alcohol beer production).[15] These systems also have potential applications in biotransformations,[16–21] chemical synthesis,[22] photoresponsive membrane processes,[23,24] ion selective electrodes[25] and the development of epidermal drug delivery systems.[26]

Of the various modes of application of liquid membrane technology, supported liquid membranes (SLM) have the greatest ease of use and therefore the greatest potential for commercial application. To maximise the commercial application of SLM technology it is essential that the system maintains its integrity. Two phenomena are particularly significant, leakage and instability. Carrier mediated transport of substrate is not the only possible route for the transport of substrates such as phenylalanine. Possible routes are shown in Fig. 1 and are summarised below:

1. Carrier mediated transport coupled to ionic exchange with chloride ions.
2. Carrier mediated tranport coupled to ionic exchange with other ions, e.g. buffer ions or hydroxyl ions.
3. Non-carrier mediated transport through the organic phase; for example due to the aromatic ring of phenylalanine, when it is the transported species, causing a degree of solubility of the amino acid in the decanol organic phase.
4. Non-carrier mediated transport through the membrane as a result of breakage of the organic phase and direct contact of the two aqueous phases.

Processes (2) (3) and (4) are defined as leakage, and process (4) is termed instability, and concerns

* To whom correspondence should be addressed.

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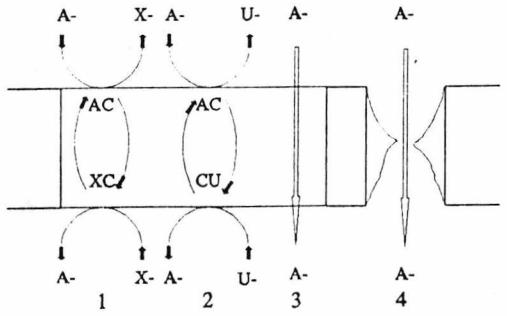

Fig. 1. Possible routes for amino acid transport across a supported liquid membrane. 1 = Carrier mediated exchange with chloride ions; 2 = carrier mediated transport with other ions; 3 = non-carrier mediated transport; 4 = diffusion by direct contact of source and receiving phase; A = amino acid; C = carrier; X = chloride ions; U = hydroxyl or buffer ions. Processes 2, 3 and 4 are termed leakage and 4 is also a special case in that it is due to the instability of the membrane.

containment of the organic phase and carrier within the polymeric support matrix. This relationship is influenced by the relative solubilities of the components, the interfacial tensions between the aqueous and organic phases and intersurface tensions between the organic phase and the support. These processes are not mutually exclusive and part of the present study is an attempt to examine the roles these components play in the process.

Many groups have addressed the causes of instability-induced leakage ((4) in Fig. 1) which represents one of the most serious barriers to the widespread application of this technology. Neplenbroek *et al.*[27,28] concluded that the principal cause of leakage is a propensity to emulsion formation of the organic layer due to either lateral shear forces or membrane vibrations.

An increase in ionic strength of the aqueous phase can act to reduce these effects by increasing the instability of colloidal substances and therefore increase the sedimentation properties of the emulsified organic phase, which is likely to be reunited with the main body of the organic phase. Ionic strength also increases the interfacial tension thus preventing initial emulsification.

Other groups[29,30] have attributed instability to the presence of an osmotic potential across the membrane related to the presence of the carrier. The carriers may hydrogen bond to water molecules and allow them to cross the membrane and hence repel the organic solvent, causing instability, or the carriers may cause formation of reverse micelles to solubilise water.

These two areas, identified as leading to membrane instability, are directly contradictory. Emulsification processes are reduced by the presence of chloride ions which increase the osmotic potential across the membrane possibly resulting in leakage. Therefore these two models predict exactly opposite effects caused by an increase in ionic strength on one side of the membrane. This study was an attempt to resolve this contradiction.

The initial impetus for the development of liquid membranes was to model biological cell membranes. Although detailed models exist for the transport of a species by a carrier across biological cell membranes, their application to SLM systems, which are potentially highly analogous, has only been investigated by a few workers.[22,31,32] These models therefore form the basis for an examination of the exchange process examined in the present study.

TABLE 1
The Effects of pH on the Overall Charge and Charges Present on Aspartic Acid, Lysine and Phenylalanine

Amino acid	*pH*	*pK_a (carboxyl group)*	*pK_a (amino group)*	*Charges present*	*Overall charge*
Phenylalanine	5·48	1·83	9·13	1+, 1−	0
Lysine	5·48	2·20	8·90	2+, 1−	1+
Aspartic acid	5·48	1·88	9·60	2−, 1+	1−
Phenylalanine	11	1·83	9·13	1−	1−
Lysine	11	2·20	8·90	1−	1−
Aspartic acid	11	1·88	9·60	2−	2−

In the field of carrier chemistry molecular recognition is becoming significant in obtaining a more fundamental understanding of many processes.[33] The field of host–guest interactions is a useful tool for the analysis of biochemical systems as they can often display specificity, previously thought exclusive to larger biological molecules, in a much more simple format. Several workers have attempted to design certain carriers for specific target molecules using liquid membranes.[34–36]

The present study therefore formed a basis for considering SLM transport processes using amino acids as the transported molecules. The ultimate goal of our research is to establish a stable selective transport process. In this study this has been tested by the application of a selection of amino acids at different pH values, whose charges are different at different pH values (Table 1).

2 EXPERIMENTAL

2.1 Experimental set-up

The SLM system is based on the method of Thien *et al.*[5] who considered a liquid emulsion membrane (LEM) system for the transport of phenylalanine, using a chloride ion gradient to drive an ion-exchange process at the aqueous–organic interfaces.

The SLM consists of the cationic carrier, tricaprylylammonium chloride (TCAC)[3,37] mixed with decanol, as the organic phase, at a concentration of 10% (v/v) of TCAC. The support used was a Scimat Ltd (Swindon, UK) ethylene–tetrafluoroethylene (ETFE) membrane, 110 μm thick, with 0·2 μm pore size and a 65% porosity cut with an area of 0·01 m^2.

Two different phases were used to drive amino acid transport. The source phase was 12.1 mmol dm^{-3} phenylalanine in 50 mmol dm^{-3} Na_2HPO_4 adjusted to pH 11 with 1 mol dm^{-3}. NaOH and the receiving phase was 0·5 mol dm^{-3} NaCl in 50 mmol dm^{-3} Na_2HPO_2 adjusted to pH 11 with 1 mol dm^{-3} NaOH. The two phases were maintained in separate reservoirs which were immersed in a constant temperature water-bath at 35°C. The solutions were pumped around the system, (50 cm^3/min^{-1}), parallel to the membrane surfaces (cross flow) before being returned to the reservoirs.

Samples were taken from the receiving reservoir and phenylalanine concentrations were measured by absorbance at 257 nm.

2.2 Rate investigations

The experiments were performed using a range of chloride ion, phenylalanine ion and carrier concentrations.

The carrier TCAC does not have a specific molecular weight, having variable length carbon chains. Therefore, tetraoctylammonium bromide (TOAB), which has a similar structure but has a defined molecular weight was also used as a carrier.[38] TCAC is a synthetic molecule with an approximate molecular weight of 438 and a calculated concentration was 0·2 mol dm^{-3}. Hence TOAB was also used as a concentration of 0·2 mol dm^{-1}.

2.3 Stability studies

2.3.1 The effects of the organic phase

Methods to measure the concentrations of the organic phase and the carrier are not readily available. The concentration of the carrier (TCAC) in the organic phase was estimated by extracting it with solvent, and performing a colorimetric reaction (A. Kemperman, University of Twente, personal communication).

As a standard the membrane was prepared with the organic solvent and carrier using 25 cm^2 of support membrane. The concentration of the carrier was measured by extraction with chlorobenzene and reaction with a 1 mmol dm^{-3} solution of the monosodium salt of 4-(2-pyridylazo)resorcinol (PAR^-) in 0·1 mol dm^{-3} $(NH_4)_2SO_4$ (pH 10). In a glass stoppered bottle, 9 cm^3 of chlorobenzene, 1 cm^3 of PAR^- and a range of volumes of the chlorobenzene-extracted carrier (0 to 500 mm^3) were added. The total volume was made up to 10·5 cm^3 with chlorobenzene. The contents of the bottle were shaken vigorously for several minutes, washed with excess deionised water and allowed to settle for 10 min. The bottom organic layer was sampled with a micropipette and centrifuged for 10 min. The colour was measured at 395 nm against a chlorobenzene blank.

Samples of membrane, of the same area as the standard, were tested after a degree of artificial disturbance. To investigate the stability of various organic phases a standard curve was prepared with four organic solvents, decanol, 2-nitrophenyl octyl ether (NPOE), 1,3 diethylbenzene (1,2, DEB) and 1,3,5 triethyl-benzenol (1,3,5 TEB).

Each set of four samples of membrane was impregnated with one of the four organic phases. One of the membrane samples was assayed immediately for the carrier content. The other three samples were placed in three separate 1 dm^3 conical flasks containing 800 cm^3 of the same phosphate buffer as used in the transport experiments (50 mmol dm^{-3} adjusted to pH 11). These were then placed on a magnetic stirrer, to create shear forces, in order to mimic those that are created *in situ* but to a greater extent. Over an eight hour period the membranes were sampled for their carrier content.

2.3.2 The effects of chloride ions

The effects of chloride ions were investigated in three ways: (i) use of a low concentration of chloride ions (0·1 mol dm^{-3}) in the feed phase, whilst maintaining a 1·0 molar ion gradient (1·1 mol dm^3 chloride in the receiving phase), to reveal if there was an added stability effect caused by the presence of chloride ions on both sides of the membrane, (ii) use of a 1·0 mol dm^{-3} gradient with no chloride ions in the feed phase and 1·0 mol dm^{-3} chloride in the receiving phase, and (iii) as a comparison between decanol and NPOE as the organic phase, using a 0·1 mol dm^{-3} chloride gradient (no chloride in the feed phase and 0·1 mol dm^{-3} chloride in the receiving phase) as well as with 0·1 mol dm^{-3} chloride in both aqueous phases.

2.3.3 The effects of the carrier

To investigate the significance of any interface activity of the carrier a study was undertaken on a previously determined unstable system in the presence of TCAC as the carrier, or in the presence of another carrier (TOAB).

2.4 Selectivity studies

2.4.1 Investigation of molecular interactions

Neplenbroek *et al.*[39] have shown that a direct one-to-one exchange process occurs between nitrate and chloride ions at the organic–aqueous interphase which can be quantified by measuring the molar exchange rate of the components of the system. The carrier transport factor (CTF) is defined as follows;

$$CTF = M(Cl)_{Feed}/M(Phen)_{Receiving} \quad (1)$$

where M = molar concentration, Cl = chloride ions and Phen = phenylalanine ions.

In the present study this factor was calculated at each sample time to describe the exchange process with 12·1 m mol dm^{-3} L-phenylalanine in the source phase and 0·5 mol dm^{-3} chloride in the receiving phase. The chloride concentration was measured in the source phase using a Corning 926 Chloride Analyzer.

2.4.2 Investigation of selectivity

Three different amino acids were chosen for transport and the experiments were run in the same manner as above except that the pH was corrected to either 5·48 or 11. The effects of the pH changes on the molecular charge of the amino acids are shown in Table 1. Each amino acid was used at a 10 mmol dm^{-3} concentration. Analysis of the amino acid mixtures was performed using an HPLC reverse phase C18 column at 0·8 cm^3/min^{-1} in conjunction with OPA derivatisation.[40]

3 RESULTS AND DISCUSSION

3.1 Effect of variations of phenylalanine and chloride concentrations

Figures 2a and 2b show that transport of phenylalanine and chloride across the SLM is a facilitated transport system, resembling enzyme mediated transport processes across a biological cell membrane, i.e. they are saturating rather than linear with increasing concentration of the transported species (phenylalanine and chloride ions). Similar data[41] have been interpreted and successfully predicted in terms of a diffusion-limited carrier mediated transport model.

The analogy with enzymic processes can be expanded in a useful manner. The maximum transport rate, V_{max}, is the same for variations in both chloride and phenylalanine ion concentrations, approximately

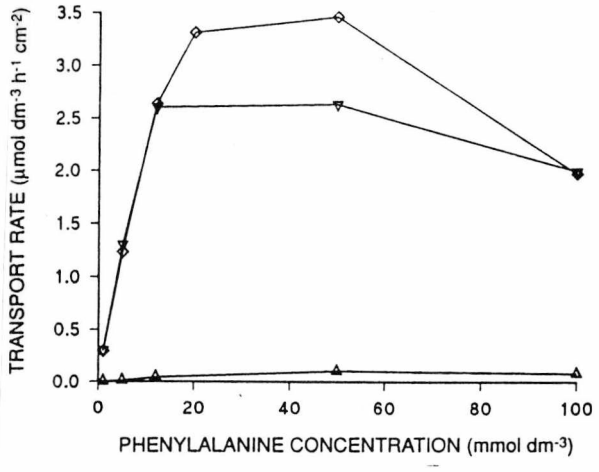

Fig. 2a. Effect of increasing phenylalanine concentration in the source phase on the carrier and non-carrier mediated transport rates of phenylalanine into the receiving phase. Initial phenylalanine concentrations were varied from 5 to 100 mmol dm^{-3} in 50 mmol dm^{-3} Na_2HPO_4 adjusted to pH 11 with 1 mol dm^{-3} NaOH in the source phase, and 0·5 mol dm^{-3} NaCl in 50 mmol dm^{-3} Na_2HPO_4 adjusted to pH 11 with 1 mol dm^{-3} NaOH in the receiving phase. The carrier, tricaprylylammonium chloride (TCAC), was mixed with decanol at a concentration of 10% (v/v) (▽) and 20% (v/v) (□) or TCAC was absent to detect non-carrier mediated transport (△). The flow rate was 50 cm^3/min^{-1} parallel to the membrane surface and the two phases were maintained at 35°C.

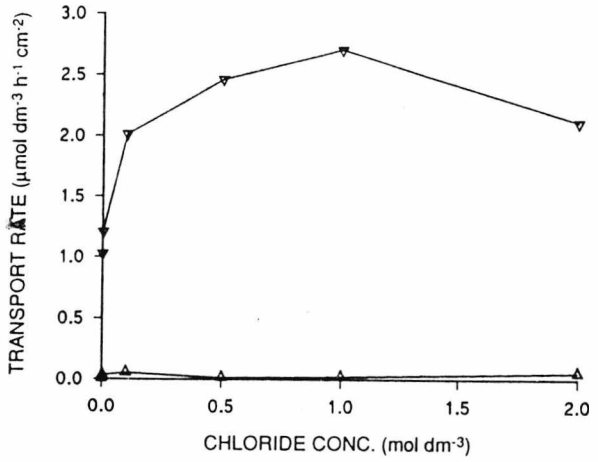

Fig. 2b. Effect of increasing chloride concentration in the source phase on the carrier and non-carrier mediated transport rates of phenylalanine into the receiving phase. Initial conditions were the same as Fig. 2a except the initial chloride ion concentrations were 0 to 2 mol dm^{-3}, the phenylalanine concentration was 12·1 mmol dm^{-3}, and the carrier was tricaprylylammonium chloride (TCAC) mixed with decanol at a concentration of 10% (v/v) (▽) or the carrier was absent to detect non-carrier mediated transport (△).

2·5 μmol dm^{-3} h^{-1} cm^{-2} of membrane. However, this is achieved at very different concentrations of either anion. In the case of phenylalanine the saturating concentration is about 12 mmol dm^{-3} whereas for chloride it is about 1·0 mol dm^{-3}, indicating that they have very different K_m values, where K_m is the substrate concentration that indicates when half the carrier sites are filled.

The difference in the K_m values has two potential interpretations, either the carrier for the transport of phenylalanine ions has a higher affinity for the amino acid than chloride, or it has fewer sites for its transport. This is possibly the limiting factor for transport and hence could explain why a similar maximum transport rate was achieved in both cases. However the limitation may also be a form of concentration inhibition at high concentration levels, as higher concentrations would lead to increased interfacial tensions and hence resist the movement of either substrate into the organic phase.

Yamaguchi *et al.*[42] presented a flux equation for competitive transport by carrier mediated systems, which assumes the same affinity, by the carrier molecule, for either substrate. This equation describes the one-way carrier mediated flux of an amino acid, [A], across a liquid membrane. It is equally applicable to the present study using data from the initial portion of the graphs of Figs 2a and 2b, before the plateau was reached. It is also analogous to the Michaelis–Menton equation for enzyme mechanisms and for carrier mediated transport across biological cell membranes, the expression is presented below.

$$J = P[C^\circ]K_{ext}/1 + K_{ext}[A^\circ][X^\circ] \qquad (2)$$

Where J = flux, P = permeation coefficient, $[C^\circ]$ = initial

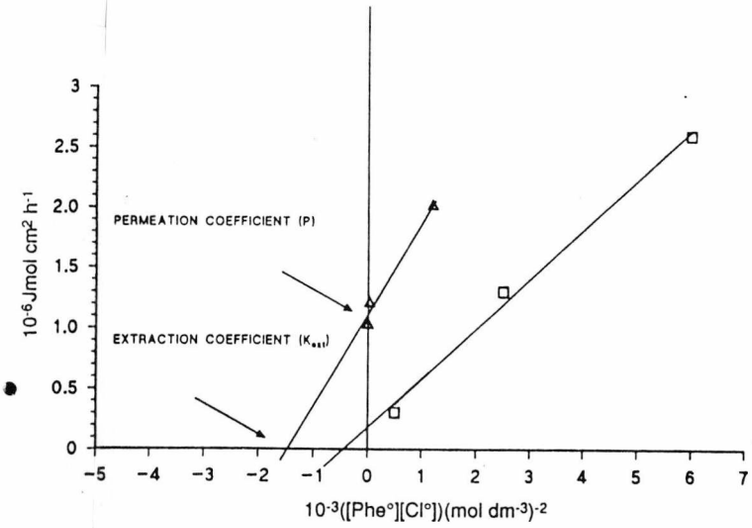

Fig. 3. Double reciprocal plot of phenylalanine flux against the product of the initial amino acid and chloride ion concentrations. Experimental data were obtained by measuring the flux at various phenylalanine (□) and chloride concentrations (△). The intercept with the ordinate yields the Permeation Coefficient (P) and the intercept with the abscissa yields the Extraction Coefficient (K_{ext}).

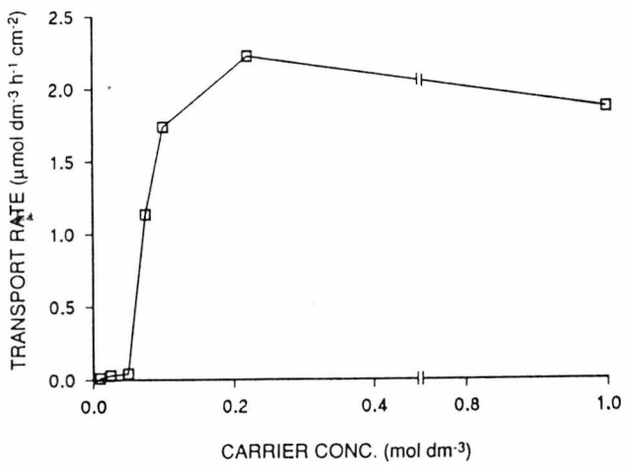

Fig. 4. The effect of increasing carrier concentration on the transport rate of phenylalanine into the receiving phase. Initial conditions were the same as Fig. 2a except phenylalanine concentration was constant at 12·1 mmol dm^{-3} and the carrier, tetraoctylammonium bromide (TOAB), was mixed with decanol at a range of concentrations from 0·01 to 1·0 mol dm^{-3} (□).

carrier concentration, K_{ext} = extraction constant, [A°] = initial amino acid concentration, [X°] = initial chloride concentration.

A plot of $1/J$ against $1/[A°][X°]$ is a plot of rate against substrate concentration and the intercepts with abscissa and ordinate yield the extraction constant (K_{ext}) and permeation coefficient (P) respectively. Yamaguchi *et al.*[42] reported that the variations in concentration of the substrates fit a single straight line. In the present study the variations of phenylalanine concentration with a constant chloride concentration, and variations in chloride concentration with a constant phenylalanine concentration did not fit on a single line, but formed two lines to yield two sets of data (Fig. 3). The variations in phenylalanine concentration reveal a transport reaction with lower P and K_{ext} values than those for chloride. The application of this model supports the idea that there are different rates of transport for each of the two species and that the carrier probably has different affinities for each substrate.

Figures 2a and 2b also show the rates of leakage which occurred when no carrier is present. The levels of carrier mediated transport were very much higher than the leakage rate, indicating that non-carrier mediated transport was not a significant phenomenon under these conditions.

3.2 Variations in carrier concentration

The rate of phenylalanine transport using various carrier concentrations showed two phases (Fig. 4), an initial very slow increase in rate with an increase in carrier concentration, and a second more rapid increase at higher carrier concentrations. This has been reported previously by Kalachev *et al.*[43] who suggested a 'jumping' mechanism which can only be triggered above a certain carrier concentration. A similar phenomenon has been described by Cussler *et al.*[44] and Noble[45] who proposed a 'chained' carrier system.

However, if the 'jumping' mechanism occurs, it negates interpretation of the data in terms of a diffusional process, and hence the above models would not be appropriate. Although this is not a reason for dismissal of this hypothesis, it seems an improbable scenario because at the transition concentration (approximately 50 mmol dm^{-3} carrier in 5·5 mol dm^{-3} decanol) the molecules of carrier are present at only a 1% molar concentration in the decanol phase. The molecular sizes, compared with monatomic carbon, of the carrier, diluent (decanol) and substrate are approximately C_{16}, C_{10} and C_8 respectively. Hence the carrier molecules, if homogenously dispersed and in an open conformation, in the decanol, are approximately 1 unit in 60 apart and presumably too far for the amino acid to 'jump' between.

Another possible explanation for the carrier concentration effect is that at the critical carrier concentration there is sufficient carrier, not participating in the exchange processes, to lower the interfacial tension and thereby to allow a greater transport rate. This could increase to a point that is limited by the viscosity of the phase, the availability of phenylalanine molecules and the local solubility of the carrier at the interface.

A levelling off of the transport rate is to be expected at high concentrations of carrier as the organic phase becomes more viscous. Various workers[46,47] have concluded that the saturation in the rate of transport was also due to insufficient stripping molecules, whereas others[48,49] included the aggregation of excess carrier

molecules to explain the observed effects at high carrier concentration.

3.3 Stability studies

3.3.1 Organic phase

Stability of the organic phase was assessed by measurement of retention of the carrier after exposure to shear forces. The extracted carrier was expressed as a percentage of the concentration of the carrier present in the initial sample. Interpolation of the data, after 6 h, revealed that the decanol membrane had retained 55% of the initial carrier present, the 1,3 DEB membrane retained 73% of the initial carrier present, the NPOE membrane retained 93% of the initial carrier present and the 1,3,5 TEB membrane retained 95% of the initial carrier present. These findings confirm the results predicted by other groups[38,50] that NPOE and 1,3,5 TEB are the more stable organic solvents. This can also be predicted from the ratio of the partition of the organic solvents into water and octanol (log *P* values). Decanol has the lowest value and hence the greater capacity to partition into the aqueous phase. Danesi *et al.*[29] also indicated that decanol had the highest water solubility and allowed osmotic forces to be influential in the transport process.

The transport rates, over a 24 h test period, for decanol, 1,3,5 TEB and NPOE were 2·5 μmol dm^{-3} h^{-1} cm^{-2}, 1·17 μmol dm^{-3} h^{-1} cm^{-2} and 0·6 μmol dm^{-3} h^{-1} cm^{-2} respectively. The results show that the rate of transport is influenced by more than the stability of the organic phase.

There are at least two possible interpretations of these results. Firstly, a diffusion-limited model of carrier mediated transport predicts that the viscosity of the solution is inversely proportional to diffusivity. NPOE is more viscous than 1,3,5, TEB and hence might be expected to have a higher transport rate.[51] However, decanol and 1,3,5, TEB have similar viscosities yet different transport rates. This could be due to the slightly more hydrophilic nature of decanol, which imparts poor stability to the liquid membrane under extreme shear forces, but can also allow greater diffusivity. Under extreme conditions this could cause membrane instability and allow direct contact between the two aqueous phases. However, the previous results have shown that without the carrier present there is only limited transport, making this unlikely at the experimental flow rates. The less extreme case of this interaction is that the interfacial tension between the two phases is reduced, allowing greater exchange rates. Furthermore, throughout the course of the experiment, a degree of emulsification may lead to intact but thinner membranes. Alternatively the contact angle of the decanol with the support may be lower; when the membrane is initially prepared the decanol may associate more closely and therefore form thinner membranes.

The polymeric support is also important when producing a stable SLM, which is a factor rarely considered in designing an SLM system. This is possibly due to the limited range of suitable supports that are available.

Measurements of the physical properties of the interactions of NPOE and decanol with the carrier[38] indicate that the viscosity of the two solvents are approximately the same. However NPOE, despite the presence of the carrier, forms a solution with a much higher interfacial tension and a much lower solvation potential for water than decanol plus carrier. A higher interfacial tension will result in a reduced ability to extract amino acids.

3.3.2 Carrier

The rate of carrier mediated transport in the absence of chloride was 0·7 μmol dm^{-3} h^{-1} cm^{-2}, compared with 2·5 μmol dm^{-3} h^{-1} cm^{-2} in the presence of a 0·5 mol dm^{-3} Cl gradient. Carriers, by virtue of their active nature, associate with interfaces and in turn reduce stability.[5,52] TCAC has an irregular geometry and therefore probably has a greater tendency to associate at interfaces and undergo emulsification processes than carrier molecules with regular structures, such as TOAB. The use of TOAB yielded a similar transport rate in the absence of a chloride gradient and hence emulsification processes facilitated by TCAC were not significant in the leakage of phenylalanine across the membrane, which must occur by either an osmotic potential route or uncoupled carrier mediated transport.

This finding supports the results given above for the effect of different organic phases. The reduction in the rate of transport of the system when using NPOE and 1,3,5, TEB is not due to a reduction in emulsification processes, but instead is due to an increased interfacial tension preventing the necessary exchange processes.

Leakage rates, although relatively insignificant when the carrier is not present, do increase with increasing concentration of either phenylalanine or chloride. This suggests that osmotic potential is a minor cause of instability in the absence of the carrier (Figs 5a and 5b). However it was not possible, in the present study, to quantify the significance of this phenomenon when the carrier was present.

The sharp rise in transport rate followed by a secondary rise as the chloride gradient is increased could represent an interplay of two phenomena. Initially the osmotic potential of the chloride is sufficient to cause leakage. As the chloride concentration increases, the increased emulsion sedimentation properties and the interfacial tension decrease the rate of leakage. The slower increase in leakage rate with increase in chloride concentration could represent a balance between these opposing phenomena.

3.3.3 Chloride

The transport rates for varying concentrations of chloride ion in the aqueous phases. With a chloride gradient of

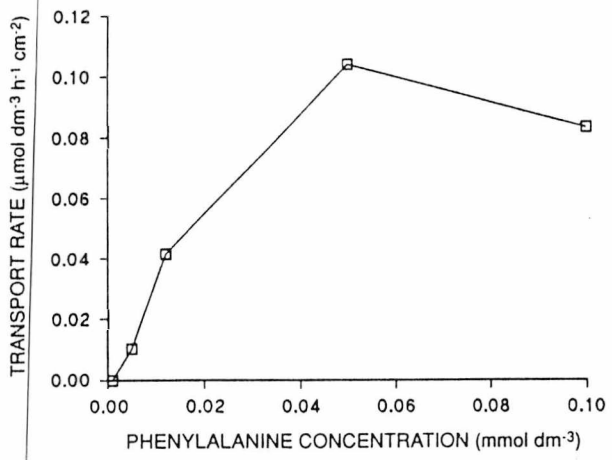

Fig. 5a. The effect of increasing phenylalanine concentration on the non-carrier mediated transport rate of phenylalanine into the receiving phase. Initial conditions were the same as Fig. 2a except the carrier was absent from the organic phase, and the phenylalanine concentration was varied from 5 to 100 mmol dm^{-3} with a constant chloride concentration and the carrier, tricaprylylammonium chloride (TCAC), was mixed with decanol at a concentration of 10% (v/v).

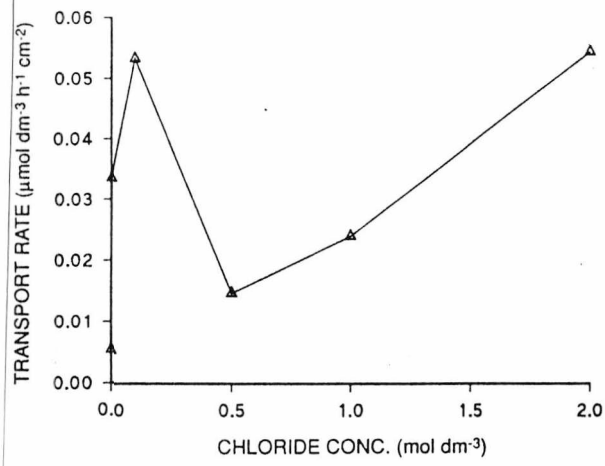

Fig. 5b. The effect of increasing chloride concentration on the non-carrier mediated transport rate of phenylalanine into the receiving phase. Initial conditions were the same as Fig. 7a except the chloride concentration was varied from 0 to 2 mol dm^{-3} with a constant phenylalanine concentration.

0·1 mol dm^{-3} with no chloride in the feed phase the transport rate was 1·8 μmol dm^{-3} h^{-1} cm^{-2}, whereas with no chloride gradient (no added chloride present) the transport rate was 0·98 μmol dm^{-3} h^{-1} cm^{-2}, but with zero chloride gradient and with 0·1 mol dm^{-3} chloride present in both aqueous phases the transport rate was 0·67 μmol dm^{-3} h^{-1} cm^{-2}, and with 1·0 mol dm^{-3} chloride present in both aqueous phases the transport rate was 0·14 μmol dm^{-3} h^{-1} cm^{-2}. Therefore, in the absence of chloride ions on either side of the membrane, the background rate of transport indicates that there is significant carrier mediated leakage without a chloride gradient.[31,53]

Theoretically, the presence of chloride ions at the aqueous–organic interfaces increases the interfacial tension and reduces the leakage that occurs by emulsification. Therefore, having chloride ions in both aqueous phases, at an equal concentration, might lead to greater stability without increasing the osmotic potential across the membrane. This would theoretically reduce the transport of phenylalanine to the levels observed when no carrier is present, i.e. virtually zero. This did not occur although the transport rate was reduced. However, the levels of transport with 1·0 mol dm^{-3} chloride in both phases were higher than those for transport in the absence of the carrier, indicating that transport occurred, and the presence of the carrier molecule was required for this type of leakage.

The reduction in transport (leakage) when equal concentrations of chloride ions were present on both sides could have been due to greater membrane stability or to greater competition for transport sites on the carrier in the organic phase.

In the present study, interfacial tension has been shown to be significant in determining carrier exchange processes, whereas emulsification-induced instability was not significant. The interfacial activity of the carrier was higher in decanol. Therefore the carrier, when present in decanol, could function despite the lack of a chloride gradient, randomly exchanging with other anions.

With TCAC as the carrier in the NPOE system, there was decreased transport with a 0·1 mol dm^{-3} chloride gradient and virtually negligible leakage when equal concentrations of chloride ions were present on both sides (Fig. 6). This reduction in transport with NPOE is presumably due to the increased interfacial tension impeding transport. However, when transport of

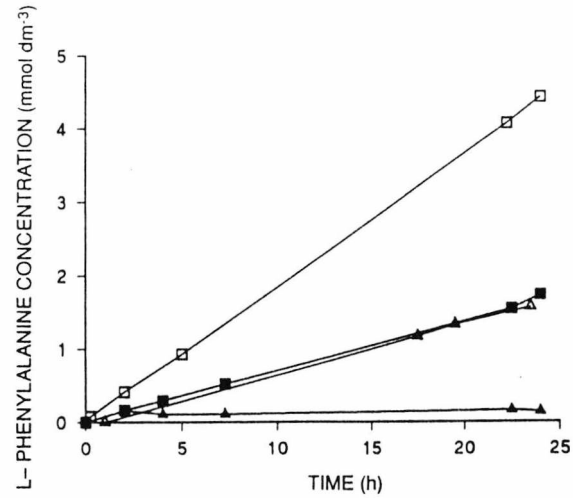

Fig. 6. The effect of different organic phases, with 0·1 mol dm^{-3} gradient and ubiquitous chloride, on transport as measured by the concentration of phenylalanine in the receiving phase. Initial conditions were the same as Fig. 2a except phenylalanine concentration was constant at 12·1 mmol dm^{-3} and the carrier, tricaprylylammonium chloride (TCAC), was mixed with decanol (□), or 2-nitrophenyl octyl ether (NPOE) (■) and driven by a 0·1 mol dm^{-3} chloride gradient, also with zero gradient and 0·1 mol dm^{-3} chloride in both phases (decanol (△) and NPOE (▲)).

phenylalanine occurs across a decanol liquid membrane, in the presence of a chloride ion gradient, it is not possible to conclude that the same degree of uncoupled transport occurs. This is because the carrier has a preference for chloride as the counter ion over other anions. Palet *et al.*[54] also observed non-specific coupled transport with hydroxyl ions and concluded that the carrier molecule in their study (a viamin B_{12} complex) had a preference for chloride.

3.4 Specificity studies

3.4.1 Exchange processes

The carrier transport factor (CTF) for the transport of phenylalanine across a decanol/TCAC membrane under standard conditions had a value higher than one, which is the value if there is an equimolar substrate exchange. In fact, it had a value of slightly greater than 2 for chloride:phenylalanine exchange over the 24 h period investigated. This result confirms that any leakage is constant throughout the test period, and that leakage does not become more significant in the later stages of the experiment, which might be expected if emulsification gave rise to instability. Instead random exchange processes, due to high interfacial activity, occurred at a constant rate throughout the test period.

This result was obtained from a system with a demonstrated stability and only low carrier mediated leakage. The value of 2:1 for chloride:phenylalanine exchange, differs from the value of 1 obtained by Neplenbroek *et al.*[39] for the transport of nitrate by TCAC in exchange for chloride ions. The exchange ratio, for the carrier mediated transport exchange of chloride for phenylalanine, was possibly greater in the present study as anions other than chloride could have been involved in interfacial exchange processes. This supports the possibility of even higher ratio exchange processes.

Evidence for this has remerged from other areas of study. For instance the carrier-affinity studies, using the model of Yamaguchi *et al.*,[42] revealed different K_m values for the extraction equilibrium constant for phenylalanine and chloride. This might be expected if more than one carrier was required in the transport process. Sato *et al.*[37] used TCAC as a carrier for the transport of divalent metal ions and reported a 2:1 exchange ratio with the counter ion. Similarly Teramoto *et al.*[4] developed an LEM system for the cationic exchange transport process of phenylalanine and reported a 2:1 exchange process where the carrier acted in a dimeric form. Furthermore Rebek *et al.*[55] developed a convergent synthetic carrier for amino acids and found that the carrier associated with phenylalanine had a 2:1 ratio due to ionic attraction and hydrophobic interaction with the aromatic ring. Therefore it is probable that the carrier is acting in a dimeric form, for amino acid transport, with both ionic and hydrophobic interactions.

3.4.2 Rates of transport for mixtures of amino acids

The relative rates of transport of a mixture of amino acids at different pH values and hence different prevailing potential charges (see Table 1) were studied. The results obtained were not those expected (Figs 7a and 7b). At pH 11 only phenylalanine and aspartic acid were transported to any significant degree, and at pH 5·48 only trace amounts of these amino acids were found in the receiving phase. Lysine was not transported at either pH value.

Obviously the transport of amino acids is more involved than suggested by the relatively simplistic approach based

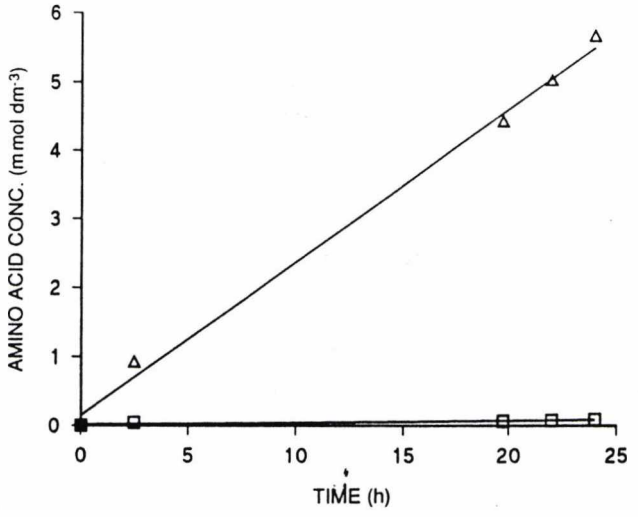

Fig. 7a. Transport of amino acids at pH 11 as measured by the increase in concentration in the receiving phase. Initial conditions were the same as Fig. 2a and the carrier, tricaprylylammonium chloride (TCAC) was mixed with decanol at a concentration of 10% (v/v). The initial concentrations of amino acid in the source phase were 10 mmol dm^{-3} L-phenylalanine (△), aspartic acid (□) or lysine. Lysine was not detected in the receiving phase.

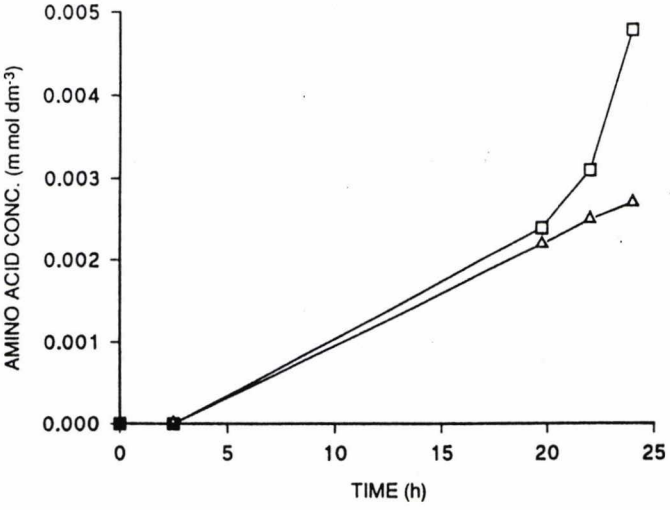

Fig. 7b. Transport of amino acids at pH 5.48 as measured by the increase in concentration in the receiving phase. Initial conditions were the same as Fig. 10a except the aqueous phases were adjusted to pH 5·48 with 1 mol dm^{-3} H_2SO_4. Lysine was not detected in the receiving phase.

solely on the overall charge. At pH 11 it is not obvious why all three amino acids are not transported to a similar extent. Possibly this relates not to the overall charge on the molecule, but to the individual charges of the carboxyl and amino groups that are present.

For any amino acid–carrier moiety to be soluble in the organic phase, and hence diffuse across the membrane there must be no overall charge present. In the case of phenylalanine at pH 11, it has one negative charge which can associate with a carrier molecule and be transported. Aspartic acid, however, has two negative charges and hence a higher charge density which could make it unattractive for an exchange process. Various workers[56,57] have found that TCAC favours bigger anions with a lower charge density and greater hydrophobicity. Lysine has only one negative charge at pH 11, however the pK_a of the alpha amine group is aproximately 10·6 and therefore at pH 11 there is relatively more residual positive charge, which could be sufficient to make bonding to the carrier less attractive. Rebek *et al.*[55] also noted that lysine was not transported. They attributed this to the lack of an aromatic side chain which is important in recognition via hydrophobic interactions.

At pH 5·48, there was a much lower rate of transport of phenylalanine and aspartic acid. This could be due to the presence of opposite charges on both the carboxyl and amino groups which caused only weak binding of the zwitterionic form of phenylalanine to the carrier.

However it is not obvious why phenylalanine should be transported at this pH value, and aspartic acid was not transported to a greater extent. Again this is possibly related to the individual charges present. At pH 5·48 aspartic acid has a higher charge density than at pH 11 and hence has less ability for solvation in the organic phase. Phenylalanine is in an energetically unattractive form for transport. However, it is transported more readily than aspartic acid because of the presence of the aromatic ring which is more soluble in the organic phase. Hence the presence of a hydrophobic side chain is critical to transport.[42,54,55]

Other studies,[5,58] on the carrier mediated transport of phenylalanine have shown a high degree of specificity for this amino acid against other inorganic ions and non-polar amino acids.

Molecular recognition studied[59] have indicated that many weak forces act to induce receptor–substrate bonding, e.g. dipole–dipole, electrostatic, Van der Waals and hydrogen bonding and hence lead to a high degree of specificity from a relatively simple model.

In this study assessment of the transport rates, at pH 11, of the amino acids revealed that the transport rate for phenylalanine was 2·33 μmol dm^{-3} h^{-1} cm^{-2} whereas the rate for aspartic acid was 0·03 μmol dm^{-3} h^{-1} cm^{-2}, approximately 70 times lower. Lysine was not transported. This significant novel finding underlines the commercial potential of supported liquid membranes for selective separations.

4 CONCLUSION

The present study for the transport of phenylalanine across a decanol/ETFE SLM system has found that the carrier, TCAC, has different affinities, permeabilities, and extraction coefficients for the two substrates and exhibits optimum substrate concentrations for the transport process.

The organic phase has been shown previously, and in this study, to be susceptible to shear force-induced disruption. However with the Scimat ETFE support membrane and at the flow rate used, emulsification processes were not significant in causing leakage and indicates the potential of this support material for SLM applications. Instead the lower interfacial tension of decanol allows higher transport rates than with organic phases which are less susceptible to shear forces. However, there is a degree of uncoupled carrier mediated transport and a possible route for osmotic potential-induced leakage of phenylalanine.

The carrier mediated transport process results in a 2:1 exchange process between chloride ions and phenylalanine. This exchange ratio was constant over a 24 h period, and supports the above conclusions that the system was stable over the test period with uncoupled transport occurring at only a small but constant rate.

When challenged by a series of amino acids it was found that the transport rate for phenylalanine was approximately 70 times greater than that for aspartic acid at pH 11 and that lysine was not transported. The SLM system may therefore have commercial potential for selective separations of amino acids or other molecules.

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