Permeation of Dipeptides and Phosphono Dipeptides through Liquid Emulsion Membranes; Stereoselectivity of the Process [1]

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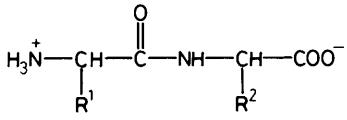
Dipeptides and their analogues are readily transported through liquid emulsion membranes. They rapidly permeate the membrane during the first ten minutes of the process and then the permeation velocity achieves a constant value. The permeation rate strongly depends on the concentration of Rokwin 60, a commercially available optically active surfactant used to stabilize the emulsion, while the influence of macrocyclic carriers on the transport efficiency was less significant. A membrane composed of 8% w/w Rokwin 60 solution in carbon tetrachloride showed marked chiral discrimination, i.e. LD-dipeptides were transported faster than their LL-counterparts. The degree of stereoselectivity of the permeation process varied from 2.13 to 1.10 depending on both the peptides structure and the permeation time.

The isolation and purification of isomers is one of the more interesting areas of the science of separation. Membrane-mediated separation methods modified versions of the conventional two-phase solvent extraction technique, are attractive as both models of biological transport and as possible preparative methods.[2]

A typical membrane system consists of a hydrophobic liquid phase separating two water phases. Chemical species pass through the membrane from one aqueous phase (source phase) to another (receiving phase). Several mechanisms of this transport have been demonstrated, of which transport mediated by mobile carriers dissolved in the membrane phase is one of the most widely studied.[2] Such systems have been successfully applied to the separation of metal ions, organic acids, phenols, and some drugs. Surprisingly, the literature contains only a few examples of the carrier-mediated permeation of amino acids and their derivatives (including crown ether-mediated transportation of potassium salts of *N*-blocked peptides and quarternary-ammonium-salt-mediated transportation of di-peptides),[3,4] and, to our knowledge, there are no reports on the macrocyclic-ligand-facilitated transportation of peptides through liquid membranes.

In this paper we report studies on the stereoselectivity of transport of dipeptides (**1**) through thin (1–10 μm) membranes, these latter being formed by an emulsion-treating technique developed by Li.[5] These membranes more closely resemble biological membranes than the bulk membranes which are usually used in this type of work, and offer the potential of moving significant amounts of compounds from source to receiving phase in a short time.

For several years we have been engaged in the synthesis of phosphono peptides (**2**), a new class of promising antibacterials and plant growth regulators, and their separation into diastereoisomers.[6] These diastereoisomeric peptides are indispensable for biological studies, and consequently we have also focussed our interest on their transport.



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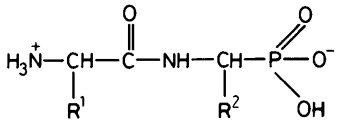

(**2**)

Results and Discussion

The liquid-emulsion membranes used in this study consisted of emulsions of two immiscible phases, *i.e.* an aqueous solution of the peptide and carbon tetrachloride, dispersed in distilled water saturated with carbon tetrachloride (continuous phase). This resulted in the dispersed interior source phase being separated from the exterior continuous (receiving) phase by a thin carbon tetrachloride membrane between the two aqueous phases (Figure 1).

In order to ensure the stability of the emulsion during the separation process, the membrane phase also contained Rokwin 60, a commercially available non-ionic surfactant, which is composed of esters of long-chain fatty acids with D-sorbitol. Thus, the surfactant used was optically active.

Since the peptides are ionic species they cannot favourably be partitioned into the carbon tetrachloride phase without a carrier. Thus, for the transport to occur a water-insoluble agent which can complex the peptide and carry it across the membrane is required. Such a carrier is a vital component in the liquid-membrane formulation.

More detailed studies on the role of the carrier used and the influence of surfactant on transport kinetics were carried out using a diastereoisomeric mixture of 1-(*N*-L-valylamino)ethylphosphonic acid [**2**; $R^1 = (CH_3)_2CH$, $R^2 = CH_3$], the phosphonic acid analogue of L-valyl-DL-alanine. This phos-

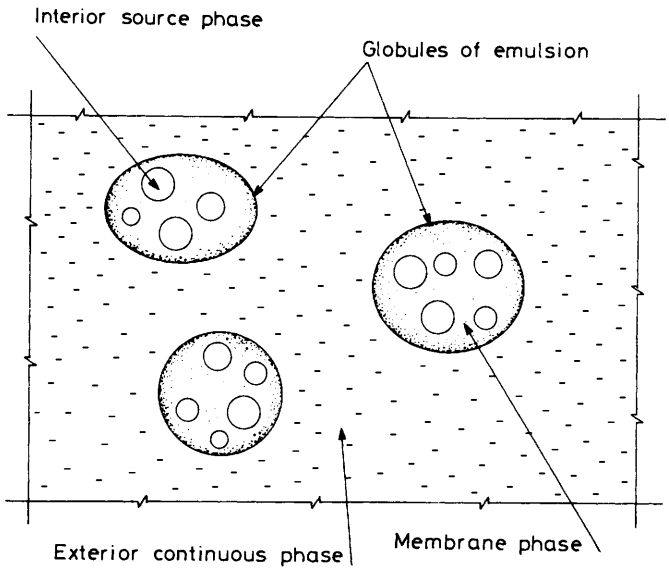

Figure 1. Schematic diagram of liquid-emulsion membrane system.

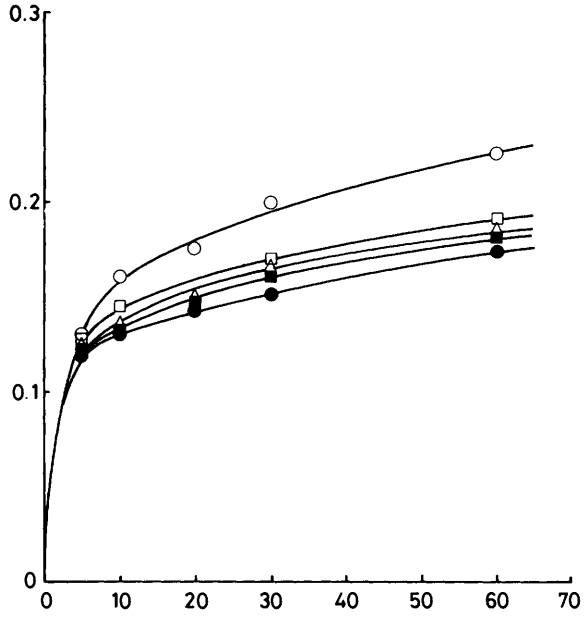

Figure 2. The influence of the carrier used on the efficiency of the permeation, given as the concentration of 1-(*N*-L-valylamino)ethylphosphonic acid in the receiving phase during the process. Data for Rokwin 60 concentration 4% w/w. Kryptofix 5, 0.83 mmol dm^{-3} (○); Kryptofix 5, 0.02 mmol dm^{-3} (□); 18-crown-6, 1.80 mmol dm^{-3} (△); 18-crown-6, 0.02 mmol dm^{-3} (■); without carrier (●). Membrane thickness ½ (for explanation see the text).

phono peptide rapidly permeated through the membrane during the first 10 minutes of reaction (Figure 2) and then the permeation velocity achieved a constant value, thus showing a standard time-dependence of permeation. Quite surprisingly, the permeation rates strongly depended on the Rokwin 60 concentration, while the dependence on the type of macrocyclic ligand used was less significant (Figure 2). We attribute this result to the existence of a combined-type transport mechanism *i.e.* transport mediated by the reversed-micelles formed in the membrane phase and transport mediated by the presence of a macrocyclic carrier (Figure 3). The transport mediated by reversed micelles is a well-known process and has been

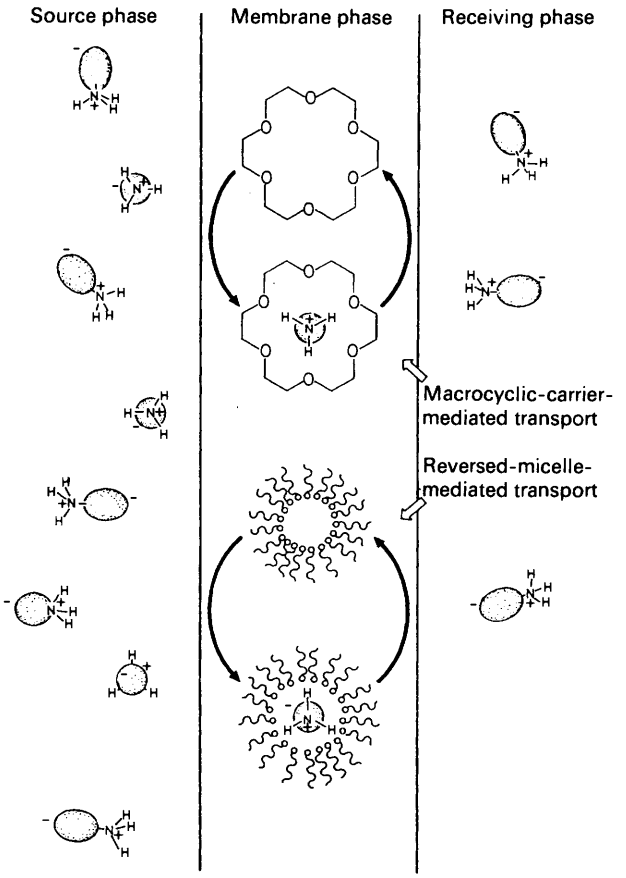

Figure 3. Schematic diagram of the combined-type transport of peptides through a carbon tetrachloride membrane containing 18-crown-6 as the macrocyclic carrier.

described in the papers devoted to the separation of hydrocarbons and proteins.[7] The transportation abilities of the macrocyclic ligands used in this work were as follows: Kryptofix 5 > Kryptofix 222 > Kryptofix 22 > 18-crown-6, and are in good agreement with those reported in the literature for compounds containing NH_3^+ moieties.[2]

In order to eliminate the transport mediated by reversed-micelles, we have used as emulsion stabilizers those surfactants which do not form reversed micelles in organic media,[8] *e.g.* block co-polymers of propylene and ethylene oxides. Unfortunately, in this case, the phosphonic acid analogue of L-valyl-DL-alanine was efficiently transported through carbon tetrachloride membrane in the absence of a macrocyclic carrier (data not shown). This arises from the ability of these surfactants to serve as the carriers by complexing the ammonium group of the peptides in a manner similar to that of crown ethers, since the structural building blocks of copolymers and crown ethers are the same.

Other dipeptides were readily transported across the membrane in a similar manner to DL-1-(*N*-L-valylamino)ethylphosphonic acid (Table 1) and the steady state was usually reached within 15–30 minutes. With the exception of DL-[(*N*-L-alanylamino(4-methoxyphenyl)]methylphosphonic acid and L-alanyldehydrophenylalanine the rates of slow (2 h) permeation of the peptides through the membrane containing Kryptofix 5 were very close to each other, while for transport in the absence of the carrier marked differences in the rates were observed. The data shown in Table 1 together with those given below are not sufficient for a deduction of the structure–transport ability relationship. Evidently there is no strict dependence on the hydrophobicity of the transported molecule as was observed by Thien *et al.*[3] for permeation of amino acids.

The main objective of this work was to examine the stereoselectivity of dipeptide transport. Thus, we have studied in some detail the kinetics of permeation of diastereoisomeric pairs of alanylalanine, its phosphonic acid analogue, alanylleucine, leucylphenylalanine, valylalanine and the phosphonic acid analogue of leucylalanine. The preliminary studies clearly showed that although the presence of the macrocyclic ligand in the membrane phase accelerated the transport, it simultaneously decreased the stereoselectivity. Therefore, we used a carbon tetrachloride membrane simply containing Rokwin 60, where the surfactant acted as both an agent stabilizing the membrane and a carrier. Since the limits to carrier concentration for surfactants are typically around 10% v/v, we used an 8% w/w Rokwin 60 solution as the membrane. The concentration of the surfactant is vital for the permeation process since the increase in peptide transport rates is concomitant with the increase of Rokwin 60 concentration, and this represents the ability of the peptide to permeate through the membrane *via* a surfactant/peptide complex. We have also found (data not shown) that the increase of Rokwin 60 concentration had no influence on the stereoselectivity of the process.

Our system showed a significant preference towards LD-dipeptides, which were transported faster than their LL-isomers (Table 2). The degree of stereoselectivity of the process, defined as the ratio of LD-to LL-dipeptide permeation rates, varied from 2.13 to 1.10, depending on both the structure of the peptide and the extent of the reaction. The ratio was usually highest after the first 5 minutes of the process and gradually decreased to a constant value. However, in experiments with diastereoisomers of valyl-leucine and valylalanine an opposite effect was observed, *i.e.* a significant increase of stereoselectivity with time. The values of the degree of stereoselectivity after 1 h permeation of the peptides across the carbon tetrachloride/Rokwin 60 membrane (Table 3) indicate that this system is of potential value as a technique for the separation of diastereoisomeric dipeptides and phosphono dipeptides.

The highest stereoselectivity (4.7) of the permeation of optically active isomers through liquid membrane was achieved by Yamaguchi *et al.*,[9] who studied the transport of enantiomers of phenylglycine across a chloroform membrane immobilized in a porous polymeric film, using a chiral crown ether as a carrier. It is noteworthy that in the experiments where the mixture of enantiomers of phenylglycine was applied in the source phase, the stereoselectivity of the permeation increased up to 22.7. This effect was attributed to the competitive binding of enantiomers by the chiral macrocyclic ligand during the process. Rokwin 60 is also optically active (derived from D-sorbitol), however, the mechanism of transport in this case seems to be more complex. The stereoselectivity of the process may result from both differences in physico-chemical properties of the diastereoisomers and chiral discrimination in their uptake into the chiral reversed-micelles.

The influence of the membrane thickness on transport efficiency seems to be obvious, *i.e.* an increase in thickness should result in the decrease of solute flux across the membrane. Since this parameter of liquid emulsion membranes is difficult to measure, it is defined as the ratio of the volumes of membrane to interior phase. We have studied the systems in which the membrane thicknesses were $\frac{1}{2}$ and $\frac{1}{3}$, and thus the differences between them were quite small. The efforts to obtain thinner membranes were unsuccessful because when the membrane-to-

Table 1. Concentration (mmol dm^{-3}) of the peptides in the receiving phase after 2 h of permeation process.

Peptide	With Kryptofix 5[a] [Rokwin 60] = 4	2	Without carrier 4	2%
H-Val-NH-CH(CH_3)-PO_3H_2[b]	0.625	0.625	0.255	0.155
H-Ala-NH-CH(CH_2H_5)-PO_3H_2[b]	0.625	0.495	0.505	0.370
H-Leu-NH-CH($CH^2C_6H_5$)-PO_3H_2[b]	0.510	0.430	0.235	0.155
H-Ala-NH-CH(C_6H_4-(*p*-OMe))-PO_3H_2[b]	0.070	0.000	0.030	0.000
HCl·H-GlyHisOH	0.430	0.420	0.250	0.160
H-Ala-NH-C(=CH-C_6H_5)-COOH	0.060	0.052	0.036	0.032

[a] In the membrane phase at a concentration of 0.83 mmol dm^{-3}. [b] Mixture of diastereoisomers, (L,DL)-dipeptide.

Table 2. Changes of concentration (mmol dm^{-3}) of dipeptides in receiving phase during their permeation across carbon tetrachloride/Rokwin 60 (8% w/w) emulsion membrane.

Peptide	Membrane thickness[a] $\frac{1}{2}$ $t_{Perm.}$ = 5	15	30	60	$\frac{1}{3}$ 5	15	30	60 min
L-Ala-D-Ala[b]	0.340	0.430	0.470	0.500	0.400	0.500	0.540	0.570
L-Ala-L-Ala	0.180	0.260	0.310	0.340	0.250	0.330	0.380	0.400
L-Ala-D-Leu	0.230	0.300	0.350	0.375	0.260	0.340	0.395	0.425
L-Ala-L-Leu	0.210	0.260	0.280	0.280	0.270	0.290	0.300	0.305
L-Val-D-Ala	0.200	0.270	0.300	0.310	0.260	0.340	0.360	0.360
L-Val-L-Ala	0.150	0.175	0.180	0.180	0.210	0.260	0.275	0.275
L-Leu-D-Phe	0.120	0.165	0.200	0.210	0.145	0.210	0.250	0.275
L-Leu-L-Phe	0.075	0.140	0.180	0.190	0.085	0.180	0.220	0.230
L-Ala-D-Ala*P*[c]	0.320	0.400	0.435	0.450	0.330	0.390	0.430	0.455
L-Ala-L-Ala*P*	0.150	0.235	0.290	0.310	0.160	0.240	0.300	0.320
L-Leu-D-Ala*P*	0.225	0.305	0.350	0.370	0.230	0.320	0.345	0.380
L-Leu-L-Ala*P*	0.130	0.185	0.200	0.205	0.125	0.175	0.205	0.215

[a] Defined as the ratio of the volumes of membrane phase to interior phase. [b] The standard IUPAC three-letter code was used for the description of amino acids. [c] The code for the phosphonic acid analogue of amino acid was formed by addition of letter *P* to the three letter code of the parent amino acid.

Table 3. Stereoselectivities of the one-hour permeation of the dipeptides through a carbon tetrachloride/Rokwin 60 (8% w/w) membrane.

Peptide	Stereoselectivity	
	Membrane thickness = $\frac{1}{2}$	Membrane thickness = $\frac{1}{3}$
AlaAla	1.47	1.42
HBr·AlaAla	1.72	1.73
AlaLeu	1.34	1.40
ValAla	1.72	1.31
LeuPhe	1.10	1.09
AlaAla*P*	1.45	1.42
LeuAla*P*	1.80	1.80

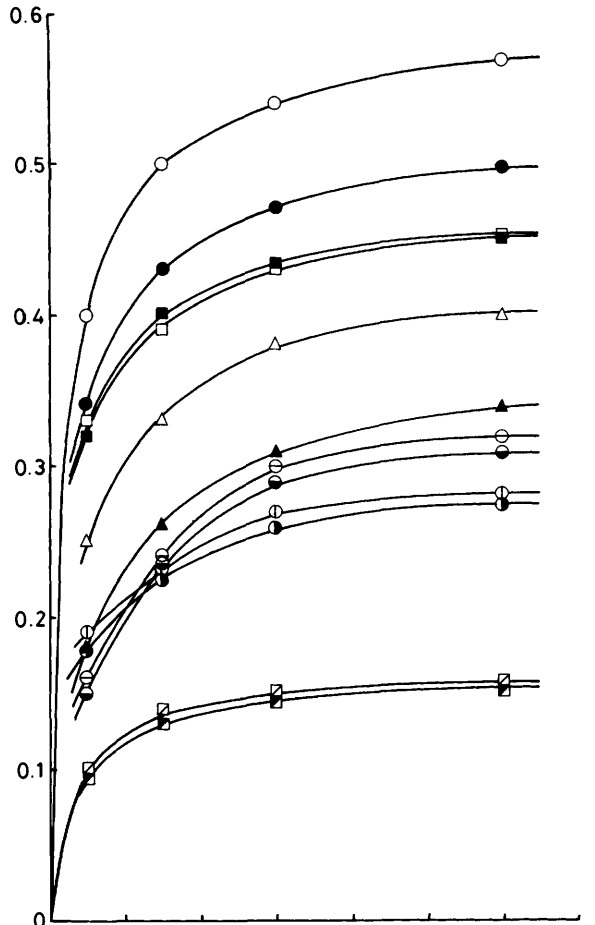

Figure 4. Kinetics of the transport of alanylalanine, its hydrobromide and phosphonic acid analogue through a carbon tetrachloride/Rokwin 60 (8% w/w) liquid emulsion membrane. L-Alanyl-D-alanine, membrane thickness $\frac{1}{2}$ (●) and $\frac{1}{3}$ (○); L-alanyl-L-alanine, membrane thickness $\frac{1}{2}$ (▲) and $\frac{1}{3}$ (△); phosphonic acid analogue of L-alanyl-D-alanine, membrane thickness $\frac{1}{2}$ (■) and $\frac{1}{3}$ (□); phosphonic analogue of L-alanyl-L-alanine, membrane thickness $\frac{1}{2}$ (◒) and $\frac{1}{3}$ (⊖); L-alanyl-D-alanine hydrobromide, membrane thickness $\frac{1}{2}$ (◑) and $\frac{1}{3}$ (◐); L-alanyl-L-alanine hydrobromide, membrane thickness $\frac{1}{2}$ (◩) and $\frac{1}{3}$ (◪).

interior-phase ratio was 1:5, the emulsion formed appeared to be too viscous to be dispersed in the continuous phase. It is seen from Table 3 that the membrane thickness had no influence on the stereoselectivity of the permeation process. Its influence on the transport rates was, however, complex and a representative example is shown in Figure 4. Generally, dipeptides were transported faster through a thinner membrane, while the permeation of their hydrobromides and phosphonic acid analogues was independent of the membrane thickness. We speculate that the more acidic hydrobromides and phosphono peptides were less efficiently uptaken by the reversed-micelles than zwitterionic, nearly neutral dipeptides. This speculation is additionally supported by the observation that the transportation efficiency of our system decreased with the increase of acidic character of the permeating compound. Thus, alanylalanine was transported significantly faster than its phosphonic analogue which, in turn, permeated faster than alanylalanine hydrobromide. Transport of zwitterionic, nearly neutral dipeptides seems to be governed mainly by the mobility of the peptide–micelle complexes in the membrane phase, while the transport of the more acidic species is probably limited by the rates of formation of these complexes.

In an effort to find out if our system may be useful for the separation of diastereoisomers of dipeptides we have studied the permeation of diastereoisomeric mixtures of alanylalanine and 1-(*N*-L-valylamino)ethylphosphonic acid. Thin-layer examination of the receiving phase in various time intervals showed that the LD-isomers were transported faster than the LL-dipeptides. Our system, however, is unsuitable for preparative purposes since the ratio of the volume of the interior (source) phase to the volume of the exterior (receiving) phase was 1:3. Thus, upon equilibration only a quarter of the total volume of starting compound remained in the source phase while the remaining three-quarters permeated into the receiving phase. That meant that the existing concentration gradient facilitated the diffusion of both isomers. Our efforts to design a system with equal volumes of the source and receiving phases were presumed to fail, because the breakage of the membrane was observed. Studies on further modifications of this system are in progress.

The data presented in this paper demonstrate that liquid emulsion membrane systems are of interest as a potential technique for the separation of diastereoisomeric mixtures. Their use in preparative chemistry, however, requires further work.

Experimental

Materials.—Dipeptides were synthesized from L-(*N*-benzyloxycarbonyl)amino acids and methyl esters of amino acids by means of mixed anhydride procedure using the recently recommended modification of the standard protocol.[10] The structures and purity of the product dipeptides were confirmed by infrared and proton NMR spectroscopy, and by thin-layer chromatography. Phosphono peptides were available from previous studies.[6] L-Alanyldehydrophenylalanine was a gift from Dr. M. Makowski.[11] Rokwin 60 (the mixture of esters of higher fatty acids with D-sorbitol) was purchased from Nadodrzanskie Zaklady Przemyslu Organicznego, *Rokita*, (Brzeg Dolny, Poland). Block copolymers of propylene and ethylene oxides *i.e.* Pluroniks L62, L64, and 2600/200, and Tetronik T702 were obtained from Institute of Organic Technology and Polymer Science, Technical University of Wrocław (Poland). Macrocyclic carriers were a generous gift from Merck (Darmstadt, FRG).

Analytical Methods.—The concentration of the peptides in the receiving phase was monitored spectrophotometrically (Carl Zeiss VSU2 spectrophotometer) by following the appearance of the peptide bond absorption (between 215 and 250 nm) for each compound. The composition of the permeate was studied by thin-layer chromatography using HP TLC plates (Merck, Darmstadt) precoated with $60F_{254}$ silica gel. The chromatographs were developed using butanol–acetic acid–water (12:3:5) as the solvent and the spots were developed with ninhydrin spray reagent (Merck, Darmstadt).

Liquid-emulsion Membrane.—The emulsion was obtained by stirring 2.5 mmol dm^{-3} aqueous solution of the peptide (30 cm^3, source phase) with 2–8% w/w solution of Rokwin 60 in carbon tetrachloride (10 or 15 cm^3, membrane phase) for 3 min at 5 000 rpm (83.33 s^{-1}). In some experiments the membrane phase also contained Kryptofix 5, Kryptofix 222, Kryptox 22, or 18-crown-6 at concentrations of 0.02, 0.83, or 1.80 mmol dm^{-3}. The emulsion formed was dispersed in distilled water saturated with carbon tetrachloride (90 cm^3, continuous, receiving phase) by continuous stirring at 200 rpm (33.3 s^{-1}). Aliquots were removed at certain intervals of time (5 min–3.5 h); the samples of permeate were centrifuged at 1 200 rpm to remove small droplets of emulsion and the concentration of the peptide in each sample was determined spectrophotometrically. The values presented in the Tables and Figures are the mean values of three or four experiments.

Stability of the Membrane.—The emulsion produced was left at room temperature for 5 h, and there was no separation of the membrane from the source phase. In order to check whether the emulsion was subject to breakage during the permeation process the experiment was carried out using 0.01 mol dm^{-3} of sodium hydroxide as a source phase (with the absence of macrocyclic ligand). Aliquots of the permeate were removed at certain time intervals and the samples treated with phenolphthalein; the lack of purple colour indicated the stability of the system.

Acknowledgements

The financial support from CPBP 01.13 programme gratefully acknowledged.

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Paper 9/02818H
Received 3rd *July* 1989
Accepted 17th *October* 1989