77. Micellar Solubilization of Proteins in Aprotic Solvents and their Spectroscopic Characterisation

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Summary[2])

The quaternary ammonium salt methyl-trioctylammonium chloride enables the transfer of α-chymotrypsin, trypsin, pepsin and glucagone from water to cyclohexane. Reversed micelles, whose polar core solubilizes both protein and water, are probably formed in the apolar phase. The influence of various parameters on the phase transfer (concentration, pH, solvent, temperature, *etc.*) has been investigated.

Absorption, fluorescence and circular dichroism studies of the biopolymers in the cyclohexane system have been carried out. For trypsin and chymotrypsin, the CD. signal in the 200 nm region is very similar in water and in cyclohexane, which suggests that the polypeptide folding is not substantially different in the two phases. The fluorescence quantum yield is always much larger in the cyclohexane phase than in water. The longer wavelength region of the UV. absorption spectrum is slightly red-shifted relative to water, and a band at 225 nm, probably arising from the aromatic chromophore, is apparent in the organic phase. Reasons for these spectral perturbations are discussed. The enzymes transferred from water into cyclohexane phases can be continuously retransferred into a second water phase. The possible relevance of this 'double transfer' as a model for the vectorial transport of biopolymers or a separation technique is discussed.

Introduction. – Several authors have investigated the interaction between proteins and organic solvents or mixed water-organic solvents [1–13]. For example, *Timasheff & Inoue* [1], and *Inoue* [2] have considered the preferential binding of solvent components to proteins in mixed water/organic-solvent systems; *Kaminsky & Davison* have studied the effects of organic solvents on cytochrome-C [3]; and *Curtis* evaluated the physical properties of some mithocondrial membrane proteins dissolved in chloroform/methanol [4]. There have also been attempts to measure enzymatic activity in organic solvents [9] [10]. In a new approach to the reconstitution of biological membranes, *Gitler & Montal* have developed a technique for extracting proteolipids into *n*-decane [12] [13]. In particular, they investigated the case of cytochrome-C complexed with phospholipids.

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More recently, *Martinek et al.* [14] have been able to solubilize α-chymotrypsin and peroxidase in *n*-octane using sodium di-(2-ethylhexyl)-sulfosuccinate (Aerosol OT) as a surfactant. According to the authors, the enzymes so solubilized in the aqueous polar core of the reversed micelles are capable of displaying catalytic activity. Neither spectroscopic properties nor physicochemical characterization are reported.

We have recently shown that α-chymotrypsin can be transferred from water into aprotic solvents with the help of methyltrioctylammonium chloride (from now on simply indicated as $NR_4^+Cl^-$) [15] [16]. The organic phase, thus obtained, is a clear solution, which permits reliable spectroscopic and conformational studies; moreover, the protein does not appear to have undergone gross structural alterations, as judged from UV. absorption [15] and circular dichroic [16] studies.

The present work considers in more detail the various factors influencing phase transfer of biomolecules from water into cyclohexane, referring mainly to α-chymotrypsin and trypsin, but also to pepsin, glucagone, L-tryptophan and L-tryptophylglycine. The spectroscopic properties of these biomolecules in the apolar phase are also considered, in order to understand the possible structural changes accompanying the phase transfer. Data are discussed in terms of the formation of 'reversed micelles' [17–19] in the apolar phase. Finally, we consider to what extent the enzyme in the cyclohexane solution can be transferred again into a water solution. Problems of reactivity of enzymes in the apolar phase will be discussed in a subsequent paper.

Experimental

Materials. – Methyl-trioctylammonium chloride, a *Serva* product, was purified as previously reported [16]. Elementary analysis after purification: Found C 74.92 H 13.38 N 3.04%, Calc. for $C_{25}H_{54}ClN$, C 74.29 H 13.41 N 3.42%. The solid purified ammonium salt is rather hygroscopic and precautions were required when preparing solutions to use as dry samples. Sodium-di(2-ethylhexyl)-sulfosuccinate (Aerosol OT) was a gift from Prof. *H.F. Eicke*, University of Basel. Cyclohexane and the other solvents were UV. spectroscopy grade products from *Fluka*. Deuteriated cyclohexane, used for NMR. experiments, was obtained from *Ciba-Geigy AG* (Isotopic purity > 99.5%). The enzymes α-chymotrypsin, trypsin (*puriss.*, salt free products) and pepsin (3× cryst.) were obtained from *Fluka*. Chymotrypsinogen, glucagone, L-tryptophan and L-tryptophylglycine were *Serva* products. Cytochrome-C, haemoglobin and myoglobin were obtained from *Fluka*. Ascorbate oxydase was a gift from Dr. *A. Marchesini*, University of Milano.

Methods. – *Spectroscopic measurements.* UV. absorption measurements were carried out with an *Acta* M VI spectrophotometer. Cyclohexane solutions of the ammonium salt occasionally gave some scattering in the spectrophotometric studies. Filtering through a Millipore filter (type FH 0.5 μ) somewhat reduced this scattering. Spectroscopic studies in the aprotic solvent were recorded against a reference cell containing the organic solvent with the same concentration of ammonium salt as in the protein solution. Circular dichroism studies were carried out with a *Jasco* J40 AS recording spectropolarimeter. In the near UV. range (250–320 nm), we used solutions having an optical density at 280 nm between 0.5–0.9 O.D. units in 1 cm cells. The same solutions were used in thinner cells for recording the spectra in the range 185–240 nm. For a first qualitative evaluation of the spectroscopic integrity of the proteins in the organic phase, we used the parameter R ($\lambda_{max}/\lambda_{min}$), *i.e.* the ratio of the absorbance at the maximum (around 280 nm) to that at the minimum (around 250 nm). For the native protein in aqueous solution in the pH range 7–9, this absorbance ratio is about 2.63 for chymotrypsin, 2.84 for trypsin and 2.33 for pepsin. It generally drops considerably upon denaturation. Corrected fluorescence spectra were carried out with an *Aminco*-1000, and quantum yield values were evaluated relative to tryptophan in water (quantum yield 0.146) directly from the integrated areas. Cyclohexane solutions were not deaerated for fluorescence studies, except for some

experiments with glucagone. Correction for refractive index has been carried out according to the literature [26]. All spectroscopic investigations were carried out at room temperature, unless otherwise specified. NMR. data were collected with a *Bruker* HXS-360 instrument, or with a *Bruker* WH-90. The typical resonance of water protons appeared at about 4.55 ppm, whereas the main resonance peaks of the ammonium salt appear at *ca.* 3.07, 1.56, 1.38, 1.79 and 0.90 ppm. The relative amount of water could be evaluated from the ratio of the integrated signal of the two water protons with respect to the signal either of the 9 protons of the three terminal methyl groups (at 0.90 ppm) or of the 9 protons belonging to the 4 carbon atoms in α-position to nitrogen (at 3.07 ppm). The enzyme concentration in the apolar phase was determined, in first approximation, using the same extinction coefficient in water and cyclohexane solution, *i.e.* 50,000 cm^{-1} M^{-1} at 280 nm (283 nm in cyclohexane) for α-chymotrypsin [20], 33,680 cm^{-1} M^{-1} calculated from [21] for trypsin at 278 nm (282 nm in cyclohexane), 50,900 cm^{-1} M^{-1} at 278 nm for pepsin [22]. For L-tryptophylglycine and glucagone, we used extinction coefficients at 278 nm (281 nm in cyclohexane) of 5610 cm^{-1} M^{-1} [23], and 8310 cm^{-1} M^{-1} [24], respectively; for L-tryptophan 5550 cm^{-1} M^{-1} at 280 nm [25] (284 nm in cyclohexane). The same extinction coefficients at the absorption maximum in both cyclohexane and water were used since for a series of tryptophan-containing peptides, whose precise extinction coefficients have been determined in cyclohexane/NR_4^+ [15], the differences with respect to water solution were negligible.

Phase transfer. In most of the present work, the transfer process was carried out in closed 10 ml vials ($\varnothing = 1.5$ cm) under nitrogen or argon atmosphere, in order to minimize the CO_2 absorption and the corresponding pH changes (nevertheless, there were some small pH changes following the phase transfer). The pH of the water solution of the protein was adjusted using 0.1N NaOH or 0.1N HCl (no buffer, unless otherwise specified); the concentration of the quaternary ammonium salt in the supernatant cyclohexane solution was determined by weight. The volume of the water and of the cyclohexane solutions was typically 4 ml. Unless otherwise specified, the transfer was carried out without stirring by simply leaving the two phases in contact for one to several days. The supernatant cyclohexane phase was analyzed from time to time to determine the amount of transferred biomolecules. Kinetic experiments were carried out directly in closed quartz spectrophotometric cells (1 cm × 1 cm), using 1.5 ml of each phase. Some experiments were carried out with mild shaking and others without shaking. Reproducibility in the percent transfer is no better than 10%, perhaps because of pH oscillations in the aqueous unbuffered phase.

In most of the experiments, (see Figs. 1–3), we have allowed enough time for the transfer to reach or to closely approach a time independent plateau, indicating the establishment of a chemical equilibrium. For the re-extraction of the protein from the cyclohexane to a water phase, two 10 ml vials were connected *via* a supernatant cyclohexane layer as shown in the insert of *Figure 3*. The first vial contained the water solution of the enzyme (α-chymotrypsin or trypsin, no buffer), the second one a buffer, *e.g.* 0.2M acetate buffer pH 5.5 or 0.2M phosphate buffer pH 6.2. The double transfer apparatus was mildly shaken continuously. The shaking was only interrupted for analyzing the content of B, this aliquot being then re-added to B (see insert of *Figure 3*). In all the double transfer experiments, reproducibility of the amount of transferred protein is no better than ± 10%. All experiments were carried out at room temperature, except for glucagone (at 37°).

Results. – *General features of the phase transfer.* Several factors influence the transfer of biomolecules from the water into the organic phase. Let us consider first the effect of pH of the water phase, making reference first to low molecular weight compounds. *Figure 1* shows the case for L-tryptophylglycine. Clearly, the pH-profile of the percent transfer is determined by the ionization of the amino group: the pK_a values obtained directly from the two curves are 9.7 for L-tryptophan and 7.6 for the dipeptide, which correspond rather closely to literature values [28] [29]. In the case of proteins, results are not as easily interpretable. We have tested chymotrypsin and pepsin, having isoelectric points of 8.3 and below 1.1 [30] respectively. The same featureless pH-profile is obtained, indicating that other factors, together with the isoelectric point, influence the phase transfer of proteins.

At pH values higher than 13, the percentage of transfer decreases sharply for both the proteins and tryptophan (*e.g.* at pH 13.5 it is around 2–5%). This is probably due to competition with OH^- ions. Water phase denaturation makes the analysis in the high alkaline pH range difficult for proteins. This is particularly apparent for pepsin.

By way of example other features of the phase transfer are shown in *Table 1* for *α*-chymotrypsin. As shown in the first column, the concentration of either $NR_4^+Cl^-$ or protein affects the transfer yield. It should be noted that a lower transfer efficiency is usually attended by lower R values, *i.e.* by an apparent loss of the structural integrity of the protein. Analogous results are obtained for trypsin and pepsin. This correspondance between optimal transfer yield and optimal spectroscopic properties is also apparent in the second column of *Table 1*.

Competition between the biomolecules and ions present in the water solution is also present in the case of proteins. In fact, the presence of buffer in the

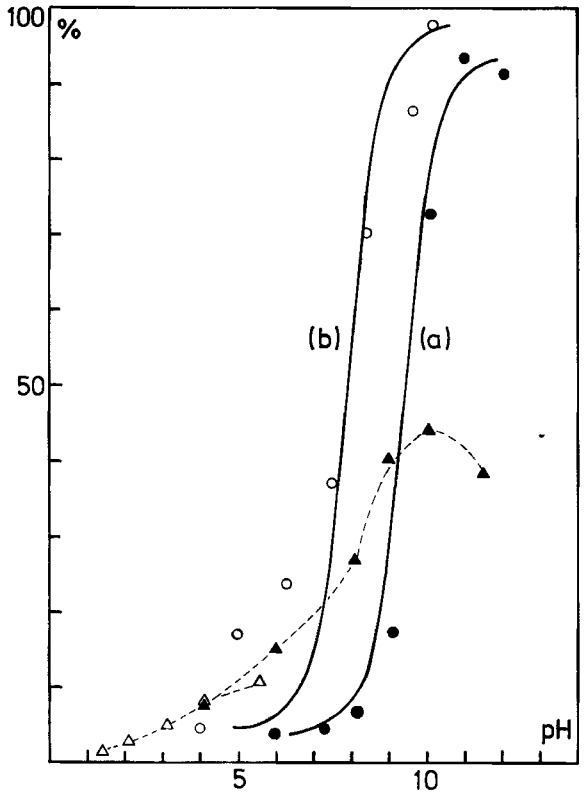

Fig. 1. *Influence of pH on the phase transfer of* L-*tryptophan* (curve a), L-*tryptophylglycine* (curve b), *α-chymotrypsin* (▲) *and pepsin* (△) (curves calculated with literature p*K* values), *from a water solution* (pH adjusted with 0,1 N HCl or NaOH) *to a cyclohexane/*$NR_4^+Cl^-$ *solution.*
Ammonium salt concentration 12 mM, initial L-tryptophan (●) and L-tryptophylglycine (○) concentrations in the water phase 360 μM. Initial concentration of *α*-chymotrypsin (▲) and pepsin (△) in the water phase was 70 μM. Concentration in the organic phase measured in all cases after 130 h

Table 1. *Influence of Experimental Parameters on the Transfer of α-Chymotrypsin from water to aprotic solvents*[a])

Influence of concentration[b])				Influence of pH and buffer[b])[c])			Influence of solvent[c])		
Protein μM	NR_4^+ mM	R[d])	Percent transfer	pH and buffer	R[d])	Percent transfer	Solvent	R[d])	Percent transfer
80	12	2.7	30	13	1	2	Cyclohexane	2.7	30
40	12	1.7	21				Diethylether	~8	20
20	12	~1	12	10.8	3.2	27	Amyl alcohol	-	0
80	4	1.7	10	7	2.1	18	*n*-Hexane	3.1	20
80	2	-	~ 0	5.5	1.6	7	Methyl	3	16
				10 (borate, 0.1M)	2.0	11	cyclohexane		
80	50	2.2	31	10 (glycine/ NaOH, 0.1M)	2.2	14	Chloroform	-	8
200	50	2.5	34	9 (pyroph 0.1M)	1.4	7			

a) The ammonium chloride concentration in the organic solvent ~ 12 mM, transfer time around 100 h; the volume ratio of the two phases (4 ml each) is 1:1, the temperature 25°, the pH of the water phase is ~ 9.0 (adjusted with 0.1N NaOH).

b) Transfer into cyclohexane.

c) The concentration of the protein in the aqueous phase was around 80 μM.

d) R is the ratio between the absorbances at the maximum (around 280 nm) and at the trough (about 250 nm).

Table 2. *Absorption and fluorescence properties of glucagone and proteins in water*[a]) *and in the cyclohexane/NR_4^+ phase*[b])

System	UV. Absorption properties		Fluorescence properties			
	λ_{max} nm (R)[c])		λ_{max} nm[d])		Quantum yield[e])	
	in H_2O	in C_6H_{12}/NR_4^+[f])	in H_2O	in C_6H_{12}/NR_4^+	in H_2O	in C_6H_{12}/NR_4^+
Glucagone	277 (2.67)	281 (2.29)	348	340 (331)[g])	0.10	0.24 (0.31)[g])
	220 sh	225				
α-Chymotrypsin	281 (2.63)	283 (3.25)	334	346	0.09	0.36
	n.d.	n.d.				
Trypsin	278 (2.84)	282 (3.89)	350	347	0.07	0.34
	220 sh	227				
Pepsin	276 (2.33)	281 (2.58)	346	340	0.12	0.24
	220 sh	225				

a) Unbuffered solution, pH 8–9.

b) The phase-transfer has been carried out under the conditions indicated for *Table 1*, footnote a.

c) ± 1 nm above 250 nm, ± 2 nm below. R defined as in *Table 1*; sh = shoulder.

d) ± 2 nm.

e) The uncertainty of the quantum yield values is around 10%.

f) C_6H_{12} = cyclohexane.

g) For deaerated cyclohexane solutions.

initial water solution decreases the percentage transfer of the protein, as shown by the experiment in which glycine/NaOH buffer or pyrophosphate are used. This competition has already been observed in our previous studies on the transfer of amino acids and peptides [31]. The third column of *Table 1* shows that the transfer is somewhat less efficient in methyl-cyclohexane or chloroform than in cyclohexane. This effect cannot be easily explained at this stage of the investigation. Transfer is successful when aromatic solvents such as benzene or toluene are used, but these are not suitable for spectroscopic investigations of proteins. Temperature markedly affects the transfer. In the case of α-chymotrypsin in $CHCl_3$, increasing the temperature from 25 to 40° results in *ca.* 50% higher transfer yield. In the case of glucagone, no appreciable transfer takes place at room temperature, whereas at 37° the transfer is practically quantitative within a few days.

Figure 2 shows the time progress of the phase transfer of three different proteins under the same conditions. These experiments have been carried out with mild shaking of the reaction vials, the transfer being much slower when the two phases are left in contact at rest. Much faster transfer reactions than those of *Figure 2* can be effected by vigorous stirring, but in this case there is usually a larger scattering in the aprotic phase (which makes spectroscopic measurements difficult), and some protein denaturation. It is clear that the velocity of the transfer depends, in addition to chemical factors and the extent of stirring, on the geometrical parameters of the reaction vial, primarily the contact surface

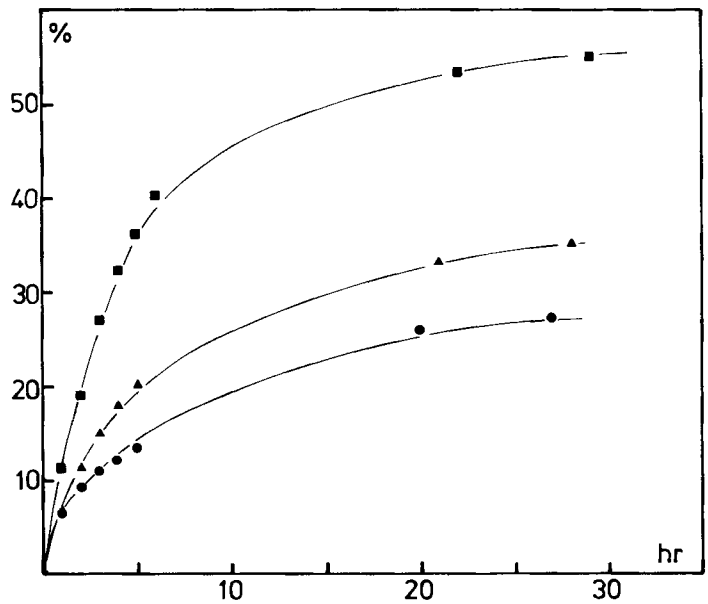

Fig. 2. *Time progress of the phase transfer for proteins,* (●) *pepsin,* (▲) *α-chymotrypsin and* (■) *trypsin.* Experiments were carried out in 3 ml spectrophotometric cells which were mildly and continuously shaken, except during spectrophotometric readings. The initial protein concentration in the water phase (pH 8) was 70 μM, the ammonium salt concentration 12 mM.

between the two phases. All these variables make it difficult, at least at this point, to analytically interpret the progress curves (which are not of a simple first order kinetics). Despite these interpretation difficulties, *Figure 2* shows that under the same conditions, different proteins are transferred with different velocities, and suggests that different chemical distribution equilibriums are approached.

The amount of water transferred under the present conditions into the cyclohexane solution can be readily determined by NMR. spectroscopy (see experimental part).

In the case of the tryptophan solution, the amount of tryptophan relative to H_2O and NR_4^+ can be evaluated based on the integration of the signal of the 5 aromatic protons (this is in good agreement with direct calculation based on spectroscopy of the amino acid in cyclohexane). The concentration of water present in the organic phase was similar (25–35 mM) for the various systems investigated (α-chymotrypsin, glucagone, tryptophan and their blanks). This corresponds to two-three water molecules for each ammonium salt molecule (in the case of transfers from pH 8.0 water solutions). The present data do not show any regular trend, and no significance can be attributed to the small differences observed in the various systems. The content of water of the 'anhydrous' ammonium salt in the solution (a cyclohexane solution of ammonium salt which

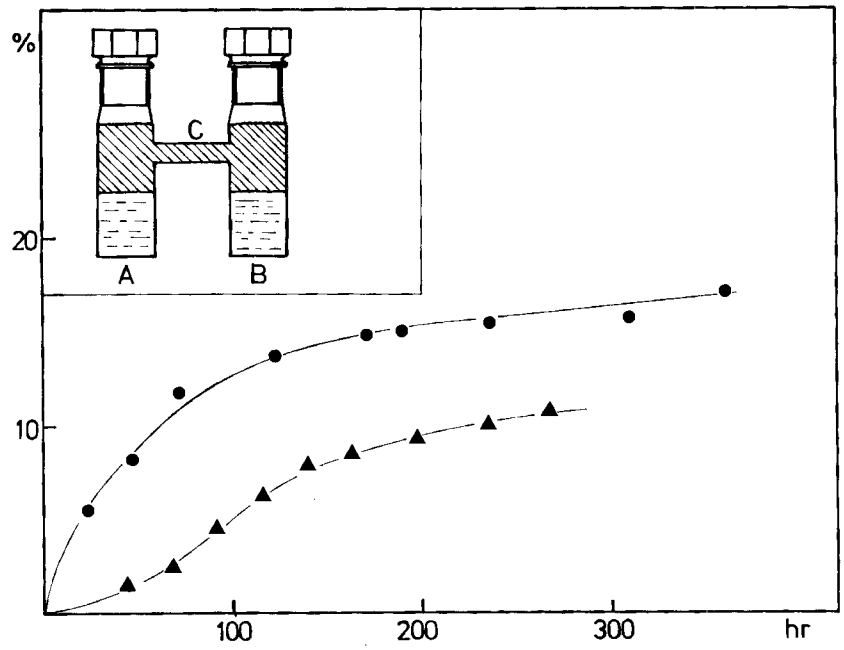

Fig. 3. *Double-transfer experiments with trypsin* (●) *and α-chymotrypsin* (▲) *carried out with the system shown in the insert.*

Initially the protein is in the first water phase (A in the insert) at a concentration of 70 μM, pH 8.0. Reported in the ordinate is the percentage of protein transferred into the second water phase B (0.2M phosphate buffer, pH 8.0). The cyclohexane bridge C contains 12 mM $NR_4^+Cl^-$. The system was mildly shaken.

had not been in contact with the water phase) was 4–5 times lower (resulting from water absorbed by the hygroscopic ammonium salt).

We have attempted to transfer other proteins into cyclohexane over a wide pH range, with the same conditions as those used for the transfer of α-chymotrypsin. Under conditions of maximum extraction of α-chymotrypsin, less than 5% of lysozyme was transferred into the cyclohexane, while peroxidase was not transferred at all. Haemoglobin, yeast and horse liver alcohol dehydrogenase, myoglobin, ascorbate oxidase were transferred less than 5–10% into cyclohexane or chloroform and appeared completely denatured. Cytochrome C could be transferred by *ca.* 5% into cyclohexane and spectral properties indicate a substantial maintenance of the structural integrity.

Thus, the solubilization of proteins into an organic solvent, with the help of NR_4^+, is a very selective process (at least under our present conditions). This selectivity is best illustrated by the striking observation that chymotrypsinogen is not transferred at all at pH 8.0, and not more than 1% at pH 9.0, under the same conditions which give 30% transfer of α-chymotrypsin.

The transfer into cyclohexane was also carried out using an anionic surfactant, *i.e.* sodium-di-(2-ethylhexyl)sulfosuccinate (Aerosol OT). The transfer takes place at low pH (~ 4) in the presence of Ca^{++} ions (0.02 M).

As already mentioned [16], other types of biomolecules can be transferred into apolar solvents with the help of $NR_4^+Cl^-$ in addition to proteins, *e.g.* dinucleotides such as NADH can be readily transferred into benzene.

The protein solubilized in cyclohexane can also be reextracted into water. This can be done by shaking the cyclohexane solution with strongly buffered water solutions, (*e.g.* 0.2 M acetate phosphate or pyrophosphate buffer pH 6–9), or continuously, the transfer into the second water phase being due to an exchange between the buffer-anion and the protein across the second boundary. *Figure 3* shows the time course of typical double transfer experiments in the case of α-chymotrypsin and trypsin. The double vial shown in the insert of *Figure 3* was used, with the protein in water solution in A and a strong buffered solution in B, connected by a cyclohexane bridge C.

The experiment reported in *Figure 3* is similar to that described by *Behr & Lehn,* who showed that amino acids and peptides could be transported from one water solution to another across a toluene barrier [32].

Spectroscopic studies. – The UV. spectrum of α-chymotrypsin in the cyclohexane phase in the 240–330 nm region has already been reported [15]. *Figure 4* shows the case of trypsin in the cyclohexane/NR_4^+ phase in the 190–330 nm absorption region. *Table 2* summarizes the data obtained for the different systems. In the near UV., the red shift with respect to the water solution and the larger $\lambda_{max}/\lambda_{min}$ ratio (except for glucagone) may be noted. These properties did not change when the NR_4^+ concentration was increased ten times by adding solid surfactant directly to the cyclohexane solution. It is known that the absorption bands of indole are sensitive to the environment [33–35], and the effect found in our case may indicate a more pronounced engagement of some of the aromatic acids (most likely those on the surface of the protein) in dipole-dipole interactions.

The most striking feature of *Figure 4* is the band appearing in the cyclohexane solution around 225 nm while the aqueous solution of proteins presents in this region only a shoulder. This band can be ascribed to the B-bands of the aromatic residues, as already noted in our first work [31]. In the apolar phase this band is much more in evidence than in water because it is considerably red-shifted, and therefore better separated from the large absorption bands at lower wavelength.

Fluorescence properties have also been investigated. *Figure 5* shows the corrected emission spectra of glucagone in the cyclohexane phase compared to water. There is a remarkable increase in the quantum yield in the organic solvent attended by a blue shift. These effects are enhanced when the organic solution is degassed. This is consistent with the accepted view that oxygen has a pronounced effect on a fluorophor dissolved in organic solvents [26]. In turn, this effect indicates that some tryptophyl residues are perhaps in contact with the organic medium. *Table 2* shows in all cases that the quantum yield in the organic phase is in the range 0.24–0.36, regardless of the value of the quantum yield in water. A similar observation was made for a series of tryptophan-containing peptides, which, although having quite different quantum yields in water solutions, showed a similar quantum yield (in the range 0.24–0.29) in the cyclohexane/NR_4^+ phase [31].

Concerning λ_{max} of emission, values in the cyclohexane/NR_4^+ system are blue-shifted with respect to the values in aqueous (unbuffered) solution, except for α-chymotrypsin. We found that fluorescence properties, contrary to UV. absorption properties, are affected strongly by several parameters, *viz.* pH variations, the

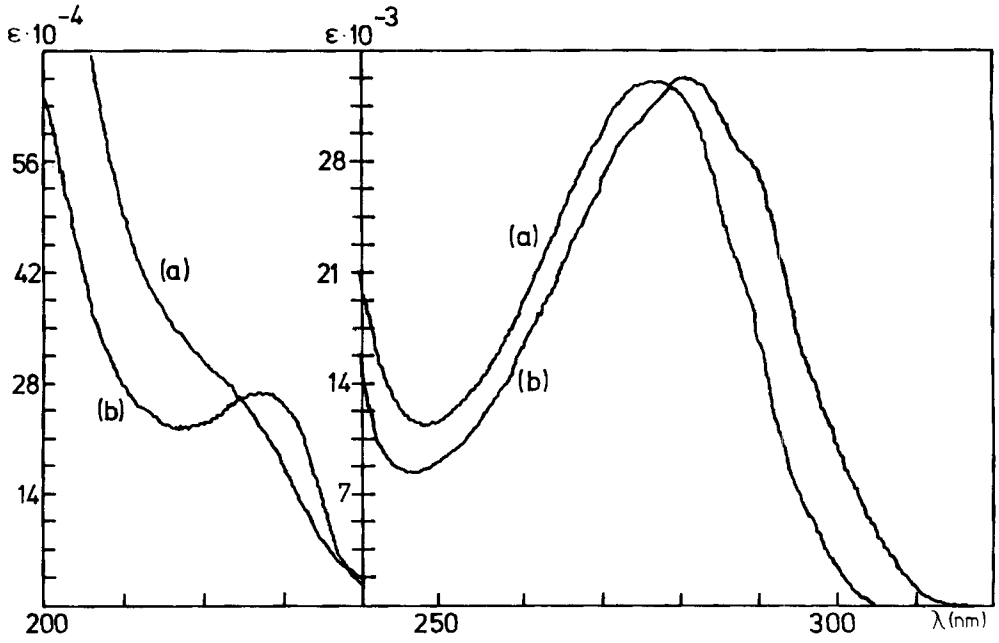

Fig.4. *UV. Absorption spectra of trypsin in H_2O, pH 7.0* (curve a) *and cyclohexane/$NR_4^+Cl^-$* (curve b). The concentration of the ammonium salt in the organic phase is 12 mM.

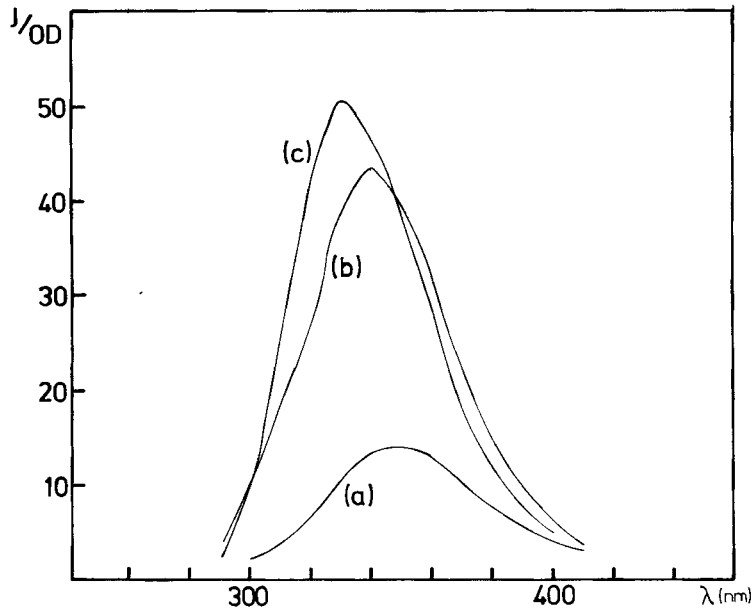

Fig. 5. *Corrected fluorescence spectra of glucagone in water, pH 8.0* (curve a), *in cyclohexane/$NR_4^+Cl^-$* (curve b), *and the same as* (b) *but deaerated with a flow of nitrogen* (curbe c).
Excitation at 280 nm. Concentration of the ammonium salt is 12 mM. In all cases, the optical density at the excitation wavelength was 0.2.

amount of oxygen present in the organic phase, the temperature, and the history of the sample. This explains the rather large experimental uncertainty reported in *Table 2*, and the deviation of some of the chymotrypsin values of *Table 2* with respect to previously published preliminary data [15] [16].

Excitation spectra in the cyclohexane phase corresponded in all cases to the UV. absorption spectra.

The circular dichroic properties in the cyclohexane/NR_4^+ phase may now be considered. We have discussed previously the case of *α*-chymotrypsin [16] and presented the argument that the conformation of the main chain should not be too different in cyclohexane with respect to water. This statement was based on the fact that in the 190–200 nm region, which is dominated by the peptide chromophore and very sensitive to the overall macromolecule conformation, the two CD. spectra were similar in the two solvents.

Figure 6 shows the CD. spectra of trypsin. In the whole wavelength region the ellipticity in the organic phase is smaller, and this is particularly so in the near UV. In the far UV., it should be noticed, that the position and the intensity of the extrema are very close in the two cases. Thus, it appears that the main chain conformation of trypsin is not substantially altered on going from the water to the organic phase. This statement is not inconsistent with local conformational changes or other perturbations around the external aromatic residues. This may explain the difference in the near UV. region. The CD. properties of

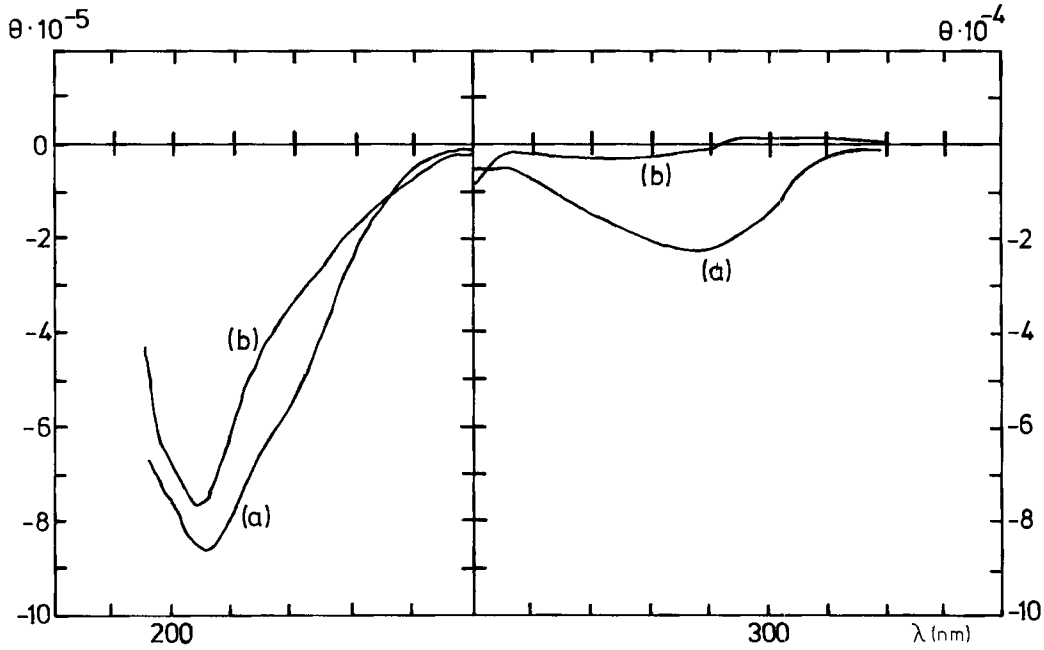

Fig. 6. *Circular dichroic spectra of trypsin in water, pH 8.0* (curve a) *and cyclohexane/$NR_4^+Cl^-$* (curve b). The ammonium salt concentration is 12 mM. θ is the molar ellipticity, referred to the whole molecule.

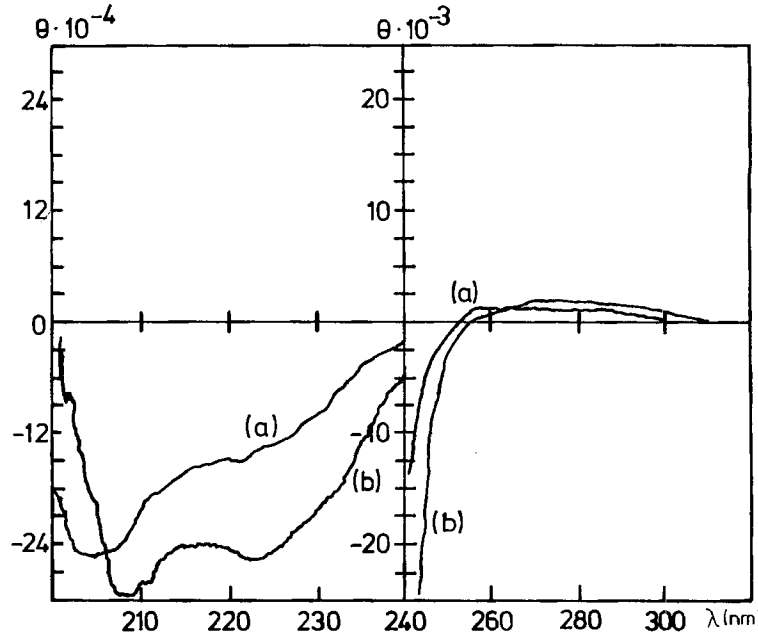

Fig. 7. *Circular dichroic spectra of glucagone in water, pH 8.0* (curve a) *and cyclohexane/$NR_4^+Cl^-$* (curve b).
The ammonium salt concentration is 12 mM. θ is the molar ellipticity, referred to the whole molecule.

trypsin in water, determined in the present work, substantially agree with the literature [36].

Figure 7 shows the CD. spectra of glucagone in water and in the cyclohexane phase. In both cases, the near UV. region is not very structured and a comparison is difficult, contrary to the previous cases. The ellipticity in the lower wavelength region is somewhat larger in the organic solvent. In particular, the relative intensity of the 225 nm band is greater in the organic solvent. Recognizing that this band probably originates from secondary structure, in particular from helical regions, it may be suggested that glucagone has a more ordered structure in the cyclohexane phase than in water solution. In this regard, one should recall that for glucagone, contrary to high molecular weight proteins, the conformational equilibrium in diluted water solution can be described in terms of a random coil [37]. Our data and interpretation are in agreement with the work by *Schneider & Edelhoch* [38]. These authors have shown that glucagone, when bound to lysolecithin micelles (formed in water solution), is immobilized in such a way that some residues form α-helices. It is interesting to observe that the CD. spectrum of glucagone has extrema in the same positions (208–209 and 222 and 223 nm respectively) and the same ellipticity values (when the different scales are taken into account) in both types of systems, *viz.* in our reversed micelles and the 'normal' micelles of *Schneider & Edelhoch.*

There are also some other reports in the literature showing that surfactants added to solutions of unordered polypeptide chains bring about a certain degree of conformational order [39] [40].

Conclusions. – By which mechanism does the solubilization of proteins in aprotic solvents proceed under our present experimental conditions? It is known that quaternary ammonium salts in organic solvents aggregate to form micelles, or more specifically 'reversed micelles' [17] [19] [27] [41]. In the last few years, reversed micelles formed by quaternary ammonium salts have been studied in terms of their capability of solubilizing water, and other polar molecules, in organic solvents [17] [19] [42–44]. On the basis of this wealth of data, it appears reasonable to assume that reversed micelles are also formed in our case, the macromolecule, together with water molecules, being contained in the polar core.

A micellar structure would explain the relatively small structural changes of the main chain of proteins in the organic phase, as water molecules and surfactant molecules would shield the biopolymer from the organic solvent. Our spectroscopic studies show that the gross features of the enzyme structure (including the main chain folding) are very similar in the organic milieu and in water. However, certain aromatic residues of the macromolecules (as shown by UV., CD. and fluorescence spectra) experience a different environment and most likely a different conformation.

Another interesting property of the phase transfer process presented in this work is its selectivity towards certain proteins. In this regard, the special nature of the inverted micelles may play an important role. It is known in fact, that micelles in apolar solvents, in contrast to 'normal' micelles in aqueous media, are composed of a rather small number of surfactant molecules [18], and they are monodisperse.

Thus, only proteins with certain dimensions may fit in the apolar core of the reversed micelles.

Another factor which may effect the transfer is the relative hydrophobicity of the protein. We have shown that, contrary to the case of amino acids and simple peptides, factors other than the isoelectric point must play a role in the transfer process of proteins. All these arguments, however, could hardly explain the lack of solubilization of chymotrypsinogen *vis-à-vis* the data of α-chymotrypsin. Thus, some other factor must be operative, which is still escaping our understanding. On the other hand, it should be noted that the high selectivity of the reversed micelles towards proteins, while restricting the applicability of the solubilization method, may turn out to be interesting as a separation method for biopolymers. Studies in this direction are in progress in our group.

The transfer with NR_4^+ proceeds more successfully in the alkaline pH range in all cases investigated. This result differs from those of the method of *Gitler & Montal* [12] [13], who could extract proteolipids in *n*-decane only below pH 4. In our case, it is possible that the phase transfer is initiated at the interface by ion-pair formation between the positively charged surfactant and the negatively charged biopolymer. At lower pH, this initiation step would be hindered, as the biopolymer possesses too many $-\overset{+}{N}H_3$ groups.

Other aspects of this work may be of interest. The double-transfer technique may lend itself to a model for vectorial transport of biopolymers from one to another aqueous solution through a lipophylic barrier. The system of *Figure 3* loosely simulates the transport of a protein from a water phase to another one across a membrane, where the driving force is constituted by a salt gradient. The high selectivity associated with this phase transfer increases the similarity with biological transport processes.

There are several important questions which remain unanswered at this stage. However, the most interesting one concerns the enzymatic activity in the apolar phase. As already mentioned, a recent report is available, which seems to answer this question positively [14]. We can confirm the result of the Russian authors concerning the activity of α-chymotrypsin in the AOT/octane system, however with significant differences in the data and their interpretation. We will consider in detail the question of the activity of enzymes solubilized in reversed micelles in our next paper.

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