Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 143–147, January 1994
Applied Biological Sciences

Detergent-enabled transport of proteins and nucleic acids through hydrophobic solvents

(membrane transport/biological barriers/insulin/lipophilization of proteins/drug delivery)

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Communicated by Robert Langer, October 4, 1993 (received for review August 23, 1993)

ABSTRACT **It is demonstrated that proteins and nucleic acids can be transported through hydrophobic organic solvents (liquid membranes) via nonspecific complex formation with detergents, whereas no macromolecule transport is observed without the latter. A protein (or a nucleic acid) first interacts with an oppositely charged detergent due to hydrophobic ion pairing in the aqueous feed phase. The resultant hydrophobic complex readily partitions into an organic solvent and then into the aqueous receiver phase, where it dissociates. Experiments with (*i*) different detergent/protein molar ratios, (*ii*) a range of unrelated organic solvents as liquid membranes, and (*iii*) homologous detergents with hydrophobic tails of varying lengths indicate that the protein flux through the membrane directly correlates with the partitioning of the protein–detergent complexes from the aqueous feed into the organic phase. Very little protein transport was detected at detergent concentrations above the critical micelle concentration, suggesting that individual detergent molecules, rather than micelles, play the key role. The rate of the detergent-enabled protein transport is not a function of the protein molecular weight, provided that enough detergent molecules bind to make the complex sufficiently hydrophobic; e.g., bovine serum albumin can be transported faster than insulin, which is less than 1/10th of its size.**

The advent of modern biotechnology has afforded the ready availability of a wide range of recombinant proteins (as well as nucleic acids) as potential pharmaceutical agents. A major hurdle that must be overcome in order to realize this potential is a poor bioavailability of proteins which stems from their inability to effectively cross cellular membranes and other biological barriers, such as the skin, the gastrointestinal mucosa, and the blood–brain barrier (1–3).

Several strategies have been examined to assist peptides and proteins in crossing biological barriers, including the use of liposomes, microparticulates, penetration enhancers (e.g., chelating agents and bile acids), iontophoresis, and chemical modification to form hydrophobic prodrugs (4, 5). Nevertheless, additional approaches are highly desirable due to the limited success achieved thus far.

The transport of low molecular weight ionic drugs through liquid membranes can be markedly enhanced by forming ion pairs with hydrophobic counterions, such as detergents (6, 7). Proteins constitute a formidable challenge to this hydrophobic ion-pairing approach because of their large size and amphiphilicity. As a step toward addressing this problem, in the present work we have explored protein transport through hydrophobic organic solvents. It has been found that ionic detergents enable facile penetration of various proteins (and nucleic acids) of the opposite charge across such membrane-mimicking media. Fundamentals of this phenomenon are mechanistically investigated.

MATERIALS AND METHODS

Bovine pancreatic Zn-insulin (27 units/mg), hen egg-white lysozyme (52,000 enzyme units/mg of protein), soybean trypsin inhibitor (type I-S), bovine pancreatic chymotrypsinogen A (type II, 49 potential chymotrypsin units/mg of solid), bovine serum albumin (essentially fatty acid-free), pepsin from porcine stomach mucosa (3900 enzyme units/mg of protein), and Tyr-Pro-Leu-Gly-NH_2 (the 6–9 tetrapeptide fragment of [Tyr^6]oxytocin, 97% pure by HPLC) were purchased from Sigma. *Torula utilis* RNA (M_r 5000–8000) and herring low molecular weight DNA were from Fluka. Molecular weight of the DNA was estimated by polyacrylamide gel electrophoresis using a 1-kb DNA ladder (BRL Life Technologies) as an ethidium bromide-stainable marker (8); the main fraction was found to have a molecular weight of 50,000.

Detergents used in this work were obtained as follows: sodium dodecyl sulfate (SDS) (ultra pure), from International Biotechnologies; sodium salts of dodecyl, decyl, octyl, hexyl, and butyl sulfonates, from American Tokyo Kasei; dodecyltrimethylammonium bromide (DTMAB) (99% pure) and octyl β-D-glucopyranoside, from Aldrich. All other chemicals and organic solvents used were obtained from commercial suppliers and were of analytical grade or purer.

Protein concentrations in the absence of detergents and in the presence of all anionic detergents were determined with the Lowry assay (9). When the cationic detergent DTMAB or the nonionic detergent octyl β-D-glucopyranoside was present, it was removed prior to the Lowry assay by using the Bio-Rad detergent-compatible procedure. The tetrapeptide Tyr-Pro-Leu-Gly-NH_2, RNA, and DNA were assayed by measuring the absorbance of their aqueous solutions at 275, 260, and 260 nm, respectively.

The rates of transport through hydrophobic (water-immiscible) solvents were measured using glass U-tubes (1.5-cm internal diameter). A U-tube was charged by adding first 17 ml of the solvent (methylene chloride in most experiments) and then 17 ml of an aqueous solution (usually 55 mM citrate buffer, pH 2.5) containing a protein and a detergent to the left arm of the tube and 17 ml of another aqueous solution (usually 0.1 M HCl) to the right arm. The two aqueous solutions, henceforth referred to as *feed* and *receiver* phases, respectively, were separated by the organic solvent (all the organic solvents used had a higher density than water and thus stayed at the bottom) and had no immediate contact with each other apart from through the solvent. The organic layer was stirred with a magnetic stir bar and an electromagnetic stirrer; the rate of stirring was kept constant (unless stated otherwise). The feed and receiver phases were stirred as well, with variable-speed mechanical stirrers equipped with pro-

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Abbreviations: DTMAB, dodecyltrimethylammonium bromide; cmc, critical micelle concentration.

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pellers. For all three stirrers, the rate of stirring was not sufficient to generate mechanical transfer of aqueous solution from one arm to another. The concentration of a protein or a nucleic acid in the receiver phase was measured as described above; the initial rate of their accumulation was used to calculate the flux J.

Partition coefficients for the insulin–SDS complex between organic solvents and water were determined as follows. Appropriate volumes of a water-immiscible organic solvent and 55 mM aqueous citrate buffer (pH 2.5), containing 0.16 mM insulin and 1.0 mM SDS, were brought into contact and shaken at 23°C for 1 hr. After filtration through a glass filter, the resultant transparent biphasic mixture was shaken at 200 rpm and 23°C for 6 hr (it was shown that this period of time was sufficient to attain the equilibrium concentrations of the insulin–SDS complex in both phases). Then the phases were separated in a separatory funnel. The organic phase was evaporated to dryness under vacuum, 0.5 ml of 0.1 M HCl in water was added, the sample was briefly sonicated, and dissolved protein was determined as described above. The aqueous phase was concentrated in an Amicon Centricon-3 microconcentrator, and the protein concentration in the organic phase was divided by that in the aqueous phase.

The enzymatic activity of lysozyme was measured spectrophotometrically on the basis of the lysis of the dried cells of *Micrococcus lysodeikticus* as described by Shugar (10).

RESULTS AND DISCUSSION

Okahata and Ijiro (11, 12) reported that several lipases formed complexes with different detergents; in contrast to the original enzymes, these complexes were insoluble in water but soluble in certain organic solvents. The same approach, which is based on hydrophobic ion pairing, was used by Matsuura *et al.* (13) to solubilize insulin in octanol and some other nonaqueous solvents. Here we investigate the use of such protein–detergent complexes to transport various proteins through hydrophobic organic layers (liquid membranes) which model biological membranes.

The rates of protein transport were measured in a U-tube where two aqueous solutions were separated from each other by a water-immiscible organic solvent. All three phases (feed, organic, and receiver) were stirred at 23°C, and protein (in most cases insulin) accumulation in the receiver phase was measured as a function of time. Our initial feed-phase composition was similar to the conditions employed by Matsuura *et al.* (13): 0.16 mM insulin, 1.0 mM SDS, and 55 mM citrate buffer (pH 2.5). At this pH, the insulin molecule has a net positive charge of 6 (due to two histidine, one lysine, one arginine, and two N-terminal amino acid residues; the acidic and C-terminal amino acid residues are presumably essentially protonated at pH 2.5) (14, 15). Therefore, the 6:1 molar ratio of the detergent to the protein ensured the minimal stoichiometry in order for each of the protein's positively charged groups to form a hydrophobic ion pair with a molecule of SDS. It should be pointed out that when SDS was added to the insulin solution, a white precipitate of the protein–detergent complex immediately formed.

Curve a in Fig. 1*A* depicts the time course of insulin transport from the feed aqueous phase through a layer of methylene chloride into the receiver aqueous phase (0.1 M HCl). One can see that a substantial transfer of the protein through the organic layer took place. After 9 days, approximately one-fifth of all insulin was transferred from the feed into the receiver. Importantly, under identical conditions but in the absence of SDS, no insulin transport was observed.

We found that a 2-fold increase or decrease in the rate of stirring had no appreciable affect on the rate of the SDS-enabled transport of insulin through methylene chloride. Likewise, the rate of insulin transport was unaffected by changes in the ratio of the volumes of feed to organic to receiver phases from 1:4:1 (8.5, 34, and 8.5 ml) to 1:1:1 (all 17 ml) to 2:1:2 (20, 10, 20 ml). These findings indicate that the overall insulin transport is not limited by the transfer of the protein across the bulk of the aqueous or organic phases.

Note that the receiver phase does not have to be acidic: when 0.1 M HCl was replaced with phosphate-buffered physiological saline (pH 7.4), the rate of insulin transport remained virtually unchanged. It seems that the complex dissociates in the receiver phase mainly due to the mass action law, since the concentrations of both the protein and the SDS are drastically diminished compared with those in the feed phase, especially during the initial stages of the transport (studied herein).

To elucidate the mechanism of the detergent-enabled insulin transfer through the liquid membrane, we examined the dependence of the rate of this process on the concentration ratio of SDS to insulin in the feed: the latter was fixed at 0.1 mM, and the SDS concentration was varied 1000-fold from 0.1 to 100 mM. The data obtained are presented in Fig. 1*A*. In order to see the trend more clearly, for each SDS/insulin ratio we calculated the flux of insulin defined (16) as

$$J = \frac{V}{S} \cdot \frac{dc}{dt}, \qquad [1]$$

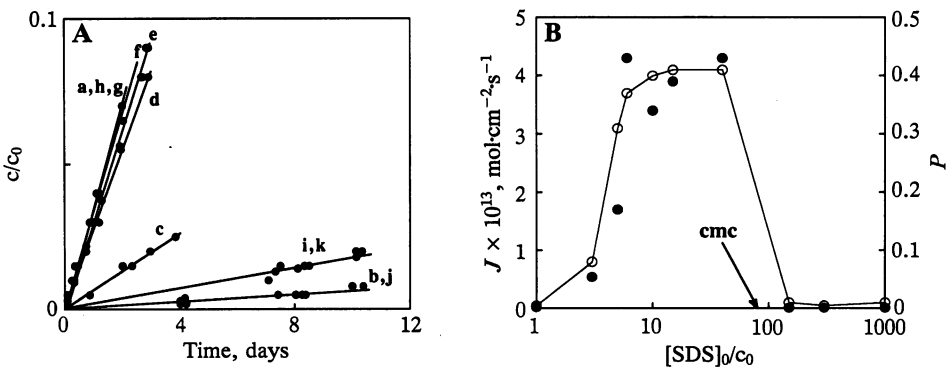

FIG. 1. Dependence of the SDS-enabled insulin transport through the methylene chloride liquid membrane on the concentration of the detergent. (*A*) Time courses of the insulin transport at 1.0 mM (curves a and f), 0.1 mM (b), 0.3 mM (c), 0.5 mM (d), 0.6 mM (e), 1.5 mM (g), 4.0 mM (h), 15 mM (i), 30 mM (j), and 100 mM (k) SDS; insulin concentration was 0.16 mM for curve a and 0.10 mM in all other instances. (*B*) The insulin flux (J) calculated from the data in *A* (open circles) and the methylene chloride-to-feed partition coefficient (P) (closed circles) as a function of the SDS/insulin molar ratios at 0.1 mM insulin. Conditions: 17 ml of the aqueous feed phase consisting of the above-mentioned concentrations of insulin and SDS dissolved in 55 mM citrate buffer (pH 2.5); 17 ml of methylene chloride; 17 ml of the aqueous receiver phase consisting of 0.1 M HCl; constant stirring at 23°C; for other experimental details, see *Materials and Methods*. c_0 and c are insulin concentrations in the feed phase at $t = 0$ and in the receiver phase, respectively; cmc is the critical micelle concentration of SDS under the conditions used.

where V is the volume of the receiver phase, S is the aqueous/organic interface area, and c is the insulin concentration in the receiver phase. Open circles in Fig. 1*B* depict the dependence of the flux on the SDS/insulin molar ratio. One can see that J first sharply increases with SDS/insulin, then reaches a plateau, and finally drops precipitously to nearly zero. Significantly, the drop occurs at the concentration of SDS in the vicinity of the detergent's cmc, 8 mM, under these conditions (17). Therefore, only individual SDS molecules, but not their micelles, enable insulin to penetrate through the methylene chloride layer.

For the transport of insulin through the methylene chloride layer to occur, the insulin–SDS complex must first partition from the feed aqueous phase into the organic phase. To ascertain how this partitioning depends on the SDS/insulin molar ratio, we experimentally determined the methylene chloride-to-feed partition coefficients, P, at each ratio value. The resultant dependence is represented by closed circles in Fig. 1*B*. The striking resemblance of the curves for J and P is suggestive of a common mechanism. Specifically, it appears that upon raising the SDS/insulin ratio at SDS concentration below the cmc, the protein becomes increasingly hydrophobic due to a greater number of SDS molecules bound per insulin molecule. Consequently, partitioning of the complex from the aqueous feed into the methylene chloride phase becomes more favorable, thereby elevating the complex concentration in methylene chloride and thus accelerating the insulin transport. Above the cmc, the micelles of SDS solubilize the protein–detergent complexes (indeed, in agreement with ref. 13, the complex precipitate is no longer observed), thus making their partitioning form the aqueous into the methylene chloride phase unfavorable and hence the transport slow. In all subsequent experiments, detergent concentrations were always below the cmc.

Replacement of the sulfate head in SDS with the sulfonate resulted in only a minor change in the detergent's ability to transport insulin through the methylene chloride membrane (the first two rows in Table 1). However, a change in the length of the hydrophobic tail in the series of sodium alkyl sulfonates had a marked effect on the insulin transport (Table 1): for dodecyl and decyl, the flux values were comparable to those for SDS; for octyl, a much slower insulin transport was observed; and for hexyl and butyl, the J values were close to zero. Formation of the insulin–detergent precipitate in the feed phase correlated with the flux values, as did the literature cmc values (Table 1).

It is well established (19) that shortening the hydrophobic tail of a detergent lowers the detergent's affinity for proteins (and for itself, as reflected by the cmc values). Therefore, in our case one would expect fewer detergent molecules bound per insulin molecule for short-chain compared with long-chain detergents. In addition, the former are less hydrophobic. Hence insulin–detergent complexes for long-chain detergents should be much more hydrophobic than those for their short-chain counterparts. This explains the observed (Table 1) solubility differences in the feed aqueous phase, as well as those in the rates of transport through the organic layer, since, as follows from Fig. 1*B*, the flux values positively correlate with the complexes' hydrophobicity.

Table 1. Insulin transport through a methylene chloride liquid membrane mediated by various anionic detergents

Detergent	$J \times 10^{13}$, mol·cm^{-2}·s^{-1}	Precipitate	cmc,* mM
Sodium dodecyl sulfate	4.0	Yes	8
Sodium dodecyl sulfonate	3.2	Yes	10
Sodium decyl sulfonate	3.0	Yes	44
Sodium octyl sulfonate	0.8	Minor	160
Sodium hexyl sulfonate	0.1	No	460
Sodium butyl sulfonate	†	No	1600

Conditions: 0.1 mM insulin, 1 mM detergent; others are the same as in Fig. 1.

*Literature values (17) were taken for all detergents but the last one, for which the cmc value was calculated by using Tschernikov's equation, as described by Sowada (18).

†Not detectable.

It was essential to establish whether detergents could enable insulin to penetrate through liquid membranes other than methylene chloride. To this end, we replaced methylene chloride with eight unrelated water-immiscible solvents. As can be seen in Fig. 2*A*, for each of them a significant transport of insulin was observed; importantly, none was detected without SDS.

Inspection of the data in Fig. 2*A* reveals that the rates of SDS-enabled transport greatly depend on the solvent and cover almost a 50-fold range from $J = 13.9 \times 10^{-13}$ mol·cm^{-2}·s^{-1} in dimethyl phthalate to $J = 0.3 \times 10^{-13}$ mol·cm^{-2}·s^{-1} in perfluoro(methylcyclohexane). We determined earlier that the partitioning of the insulin–SDS complex from the aqueous phase into methylene chloride, which correlated with the complex's hydrophobicity, was the defining event in the overall insulin transport. Therefore it was reasonable to presume the same for other solvents and expect a correlation between the rate of insulin transport and the partition coefficient for the complex between the solvent and the aqueous (feed) phase: the more the complex partitions into the organic phase, the faster the transport.

To test this hypothesis, we separately measured such partition coefficients for all the solvents where it was experimentally feasible and then plotted against them the J values calculated from Fig. 2*A*. As seen in Fig. 2*B*, not only is there a correlation between J and P but the resultant plot is quite linear. This finding confirms the importance of the partitioning event and allows one to predict the value of J if P has been determined.

Next, we explored the SDS-enabled transport of proteins other than insulin through an organic liquid membrane. The oxytocin tetrapeptide Tyr-Pro-Leu-Gly-NH_2, hen egg-white lysozyme, soybean trypsin inhibitor, bovine pancreatic chymotrypsinogen A, and bovine serum albumin (covering a >100-fold range of molecular weights: 448, 14,300, 21,500, 25,000, and 66,000, respectively) all penetrated through a methylene chloride membrane (Fig. 3); again, no transport was detected in the absence of detergent.

Two alternative explanations come to mind in order to rationalize the wide disparity among the rates for different proteins depicted in Fig. 3. The first is that large proteins simply diffuse slower than smaller ones (indeed, it is clear from Fig. 3 that, in general, the smaller the protein the faster its transport through methylene chloride). The second explanation is that, since in all instances 0.1 mM protein and 1 mM SDS were employed, there was not enough detergent for larger proteins to neutralize all the positive charges present and thus to make the protein maximally hydrophobic. To distinguish between these possibilities, we increased the SDS/protein ratio for the slowest (and the largest) protein examined, bovine serum albumin, from 10:1 to 100:1 (by lowering the protein concentration to 10 μM at the same concentration of SDS). As a result, the transfer of albumin accelerated >10-fold and became approximately twice as fast as that for insulin. This observation rules out the first, and supports the second, of the aforementioned two explanations. It also indicates that even very large proteins can be transferred through liquid membranes, provided that there is enough detergent present (but less than the cmc; see Fig. 1*B*).

The rationale of our detergent-enabled protein transport through liquid membranes requires that the detergent and the protein be oppositely charged—i.e., in the case of SDS that the protein be positively charged. Consequently, one would

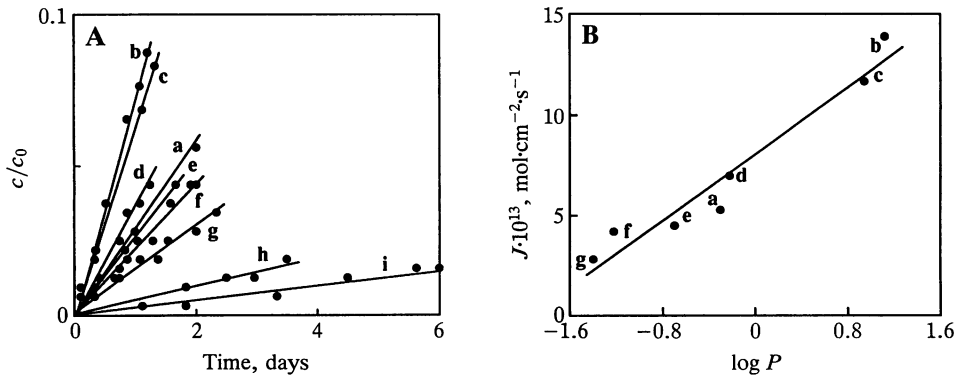

FIG. 2. Kinetics of the SDS-enabled insulin transport through various water-immiscible organic solvents. (*A*) Kinetics of the insulin transport through methylene chloride (curve a), dimethyl phthalate (b), triacetin (glycerol triacetate) (c), nitrobenzene (d), tributyrin (glycerol tributyrate) (e), carbon disulfide (f), carbon tetrachloride (g), silicone oil (h), and perfluoro(methylcyclohexane) (i). (*B*) Dependence of the insulin flux (*J*) calculated from the data in *A* on the solvent-to-feed partition coefficients (*P*) for insulin–SDS complexes, shown in semilogarithmic coordinates. For silicone oil and perfluoro(methylcyclohexane), *P* could not be measured. Conditions: 0.16 mM insulin, 1 mM SDS; others are the same as in Fig. 1.

expect that when the pH is raised (thus lowering the protein's net positive charge), the transport should diminish and come to a halt near the isoelectric point of the protein. This prediction was confirmed experimentally with bovine serum albumin, whose isoelectric point is 4.9 (20). When the pH in the feed phase was increased from 2.5 to 3.5, the flux of the SDS-enabled transport (1 mM SDS, 10 μM protein) through methylene chloride dropped from 1.1×10^{-13} to 0.6×10^{-13} mol·cm^{-2}·s^{-1}. When the pH was increased further to 4.9, no albumin transport was detected. In agreement with these findings, the precipitated albumin–SDS complex was observed at pH 2.5 and 3.5, but not at pH 4.9.

In additional support of the foregoing concept, we found that pepsin from porcine stomach mucosa, which with its isoelectric point of 1.0 (20) has a net negative charge at pH 2.5, was not transferred through the methylene chloride layer by SDS. However, when 10 μM pepsin was mixed in the aqueous feed phase with a cationic detergent, 1 mM DTMAB, a precipitate formed and a transport with $J = 0.6 \times 10^{-13}$ mol·cm^{-2}·s^{-1} was observed (curve a in Fig. 4).

Thus far, all the macromolecules transported through liquid membranes via hydrophobic ion pairing with detergents were proteins. We endeavored to broaden the scope of this approach to other biopolymers, namely nucleic acids. *T. utilis* RNA was dissolved in the aqueous feed phase at pH 7.0 (it is unstable at pH 2.5, which was used in most previous experiments). Since RNA is negatively charged at this pH, it was mixed with the cationic detergent DTMAB. The resultant complex penetrated through methylene chloride, as well as through 1,2-dichloroethane (curves b and c, respectively, in Fig. 4); no transport was observed without detergent or in the presence of (negatively charged) SDS. Similarly, herring DNA, when complexed with DTMAB (but not without it), penetrated through methylene chloride (curve d in Fig. 4).

Of potential utility for drug delivery, it was found that a protein–detergent complex can be isolated and stored without appreciable damage to its membrane-transport ability. For example, a complex formed between 0.16 mM insulin and 1 mM SDS in aqueous citrate buffer (pH 2.5) was filtered, dried, and stored at 23°C overnight. Following subsequent resuspension in the aqueous feed, a transport with $J = 5.0 \times 10^{-13}$ mol·cm^{-2}·s^{-1} was observed through a methylene chloride layer, compared with $J = 5.3 \times 10^{-13}$ mol·cm^{-2}·s^{-1} for the freshly prepared complex.

Finally, we addressed the question of whether any irreversible deterioration would occur in a protein as a result of its transport through, and hence a long-term intimate contact with, a hydrophobic solvent. Lysozyme was selected for such an experiment because its enzymatic activity would be a sensitive indicator of deleterious processes. After a 3-day transport under the same conditions as in Fig. 3, the specific activity of lysozyme in the receiver phase was found to be the same, within the error of the measurement, as in the control

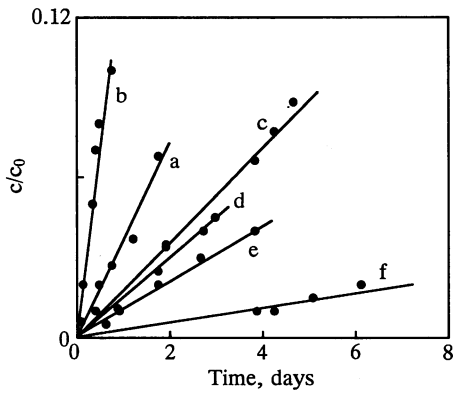

FIG. 3. Kinetics of the SDS-enabled transport of various proteins through the methylene chloride liquid membranes. Curves: a, insulin; b, Tyr-Pro-Leu-Gly-NH_2; c, soybean trypsin inhibitor; d, lysozyme; e, chymotrypsinogen; f, bovine serum albumin. Conditions: 0.1 mM protein, 1 mM SDS; others are the same as in Fig. 1.

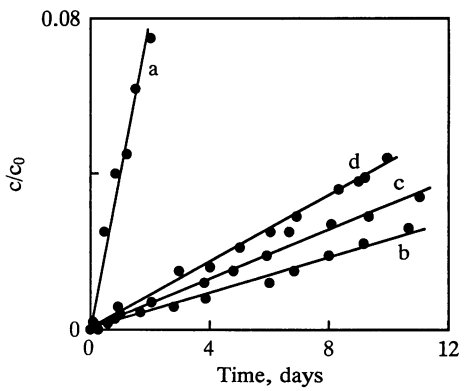

FIG. 4. Kinetics of the DTMAB-enabled transport of pepsin through methylene chloride (curve a), *T. utilis* RNA through methylene chloride (curve b) and 1,2-dichloroethane (curve c), and herring DNA through methylene chloride (curve d). Conditions: 10 μM biopolymer, 1 mM detergent; feed phase was 55 mM citrate buffer at pH 5.0 (curve a) or 10 mM Tris·HCl at pH 7.0 (curves b–d); others are the same as in Fig. 1.

(i.e., of the lysozyme solution of the same protein content). This observation is in agreement with a significant retention of catalytic activity in experiments where enzymes were extracted into organic solvents containing reversed micelles of detergents, followed by reextraction into the aqueous phase (21).

In summary, we have demonstrated detergent-enabled transport of biopolymers through hydrophobic organic solvents (liquid membranes). The presence of a detergent is critical, since without it even insulin (the most hydrophobic of the biopolymers tested) fails to penetrate through the methylene chloride layer not only at pH 2.5, but also at the pH coinciding with the isoelectric point (pH 5.3), where the protein is most hydrophobic because its net charge is zero (22). Moreover, the presence of a nonionic detergent, 1 mM octyl β-D-glucopyranoside, does not lead to any detectable transport. Since so many diverse biomacromolecules were transferred through organic solvents by detergents and various ionic detergents were successfully used, biopolymer–detergent interactions must be of a nonspecific nature. Therefore, it appears that the detergent-enabled transport through liquid membranes should be a rather general phenomenon, effective as long as the biopolyelectrolyte and detergent have opposite charges (and the detergent's concentration is below the cmc). The next test of this approach should be with biological membranes. Literature (23) data on detergent-enhanced rectal absorption of insulin in rabbits give reasons for cautious optimism.

This work was funded by the Lederle-Praxis Biologicals Division of American Cyanamid Co. and by the Biotechnology Process Engineering Center at the Massachusetts Institute of Technology.

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