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Transport of proteins dissolved in organic solvents across biomimetic membranes

(transdermal drug delivery/solubility of proteins/methanol/ethanol/lipidized membranes)

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ABSTRACT **Using lipid-impregnated porous cellulose membranes as biomimetic barriers, we tested the hypothesis that to afford effective transmembrane transfer of proteins and nucleic acids, the vehicle solvent should be able to dissolve both the biopolymers and the lipids. While the majority of solvents dissolve one or the other, ethanol and methanol were found to dissolve both, especially if the protein had been lyophilized from an aqueous solution of a pH remote from the protein's isoelectric point. A number of proteins, as well as RNA and DNA, dissolved in these alcohols readily crossed the lipidized membranes, whereas the same biopolymers placed in nondissolving solvents (e.g., hexane and ethyl acetate) or in those unable to dissolve lipids (e.g., water and dimethyl sulfoxide) exhibited little transmembrane transport. The solubility of biopolymers in ethanol and methanol was further enhanced by complexation with detergents and poly(ethylene glycol); significant protein and nucleic acid transport through the lipidized membranes was observed from these solvents but not from water.**

The utility of proteins and other biopolymers as pharmaceutical agents is critically dependent on their bioavailability. Because of difficulties with the oral delivery of proteins due to proteolytic destruction in the stomach, nonoral modes of administration should be considered (1).

The transdermal route potentially provides a safe and noninvasive path for protein drug delivery (2). The major source of resistance to penetration through the skin is the stratum corneum, a 15- to 20-μm thick membrane, which primarily consists of blocks of cytoplasmic keratin matrices embedded in extracellular lipid (3). Because of the stratum corneum's impermeability to proteins and other biopolymers, the transdermal mode exhibits a much greater resistance to the absorption of these molecules compared with the mucosal route (4). Of the two possible approaches to overcome this resistance, iontophoresis (5, 6) and the use of penetration enhancers (7–9), the former has been ineffective with proteins unless the integrity of the stratum corneum has been severely compromised (10, 11), and the latter has not been explored with proteins.

Since the rate-limiting step of transdermal drug transport is the passage through the structured lipids residing in the intercellular channels of the stratum corneum (8), effective penetration enhancers (vehicles) should be able to solubilize lipids. At the same time, protein penetration enhancers must also dissolve proteins to afford their monomolecular, rather than polymolecular, dispersions. Finally, an acceptable penetration vehicle should have a history of successful pharmaceutical applications to allay regulatory concerns.

In the present study, using a model system, we have addressed the question of whether it is possible to attain simultaneously all three aforementioned requirements. It has been found that many proteins (and nucleic acids), including therapeutically active ones, can be dissolved in a widely pharmaceutically used solvent such as ethanol and in another United States Pharmacopeia solvent, methanol (12); this solubility can be increased even further by using certain benign additives. Moreover, proteins and nucleic acids dissolved in ethanol and methanol have been found to readily cross biomimetic membranes, while no significant transport has been observed with solvents that dissolve only either lipids or proteins.

MATERIALS AND METHODS

Bovine pancreatic Zn^{2+}–insulin (27 units/mg), hen egg-white lysozyme (52,000 enzyme units per mg of protein), myoglobin from horse heart (essentially salt free), bovine serum albumin (essentially fatty acid free), bovine pancreatic ribonuclease A (type III-A), bovine pancreatic chymotrypsinogen A (type II; 49 potential chymotrypsin units per mg of solid), soybean trypsin inhibitor (type I-S), cytochrome *c* from horse heart (95% pure), egg yolk L-α-phosphatidylcholine (type X-E), and cholesterol (>99% pure) were purchased from Sigma. Recombinant human insulin (Na^+–insulin), biosynthetic human growth hormone, and porcine glucagon were a generous gift from Eli Lilly & Co. *Torula utilis* RNA (M_r of 5000–8000) and herring low molecular weight DNA [M_r of the main fraction of 50,000 (13)] were purchased from Fluka. PEG was obtained from Serva and Aldrich (M_r of 40,000 and 3400, respectively). SDS (ultra pure), dodecyltrimethylammonium bromide (DTMAB) (99% pure), and absolute ethanol and methanol (analytical grade) were purchased from International Biotechnologies, Aldrich, Pharmco Products (Bayonne, NJ), and Mallinckrodt, respectively. All other chemicals and organic solvents used were obtained from commercial suppliers and were of analytical grade or purer.

Proteins and nucleic acids were lyophilized prior to use from solutions or suspensions of 5 mg/ml in deionized water adjusted to the desired pH. Protein concentrations were determined with the bicinchoninic acid (BCA) assay (14). RNA and DNA were assayed by measuring the absorbance of their aqueous solutions at 260 nm. The lysozyme activity was measured spectrophotometrically on the basis of the enzymatic lysis of the dried cells of *Micrococcus lysodeikticus*, as described by Shugar (15).

The solubility of a protein or a nucleic acid in organic solvents was measured by placing it in a screw-cap vial, followed by addition of the solvent. The resultant suspension (1 or 5 mg/ml) was shaken at 30°C for 16 hr and then centrifuged at 30,000 × *g* and 30°C for 0.5 hr. The undissolved residue was removed, the supernatant was evaporated to dryness under vacuum, the resultant solid was redissolved in 1 ml of phosphate-buffered physiological saline (PBS), the sample was briefly sonicated, and the protein concentration was determined as described above.

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Abbreviation: DTMAB, dodecyltrimethylammonium bromide.

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Two-compartment glass diffusion cells (Crown Glass, Somerville, NJ) were used for the membrane transport experiments. The compartments had a volume of 3.4 ml each and were separated by a composite membrane (see below) with a working area of 0.5 cm^2. Both chambers were stirred by Teflon-covered magnetic stir bars to minimize the boundary layer effect. The *feed* and *receiver* chambers were initially filled with the mixture containing the species to be transported and PBS (pH 7.4), respectively. At certain time intervals, samples were withdrawn from the receiver chamber and, after the removal of turbidity (caused by minor stripping of the lipids from the membrane) by centrifugation (30,000 × *g*, 0.5 hr), were analyzed for the protein or nucleic acid content as described above. All transport experiments were conducted at 37°C.

A composite membrane of a total thickness of 0.6 mm was made of 589 Black Ribbon cellulose filter paper (Schleicher & Schuell) with a pore diameter of 20–30 μm, impregnated with phosphatidylcholine or cholesterol, and sandwiched between two identical nonimpregnated membranes. For the impregnation, a disk of the filter paper (440 ± 5 mg, 9 cm in diameter) was immersed in 20 ml of a solution of the lipid (100 g/liter) in diethyl ether and placed in a flat-bottom beaker, and the ether was allowed to evaporate overnight at 23°C under a flow of air (16, 17). The weight gain of the membrane after impregnation and drying was measured to be 220% ± 5%.

RESULTS AND DISCUSSION

The conventional notion is that proteins are soluble only in very few organic solvents, such as dimethyl sulfoxide, formamide, and ethylene glycol (18). Recently, using the representative hydrophilic protein hen egg-white lysozyme as a model, we revisited this issue (19). As a result, the list of protein-dissolving organic solvents was greatly expanded to include many common, protic, hydrophilic solvents. A key parameter affecting lysozyme's solubility in organic solvents was found to be the pH of the aqueous solution from which the protein was lyophilized prior to placement in the solvent: the farther away this pH was from protein's isoelectric point, the higher was the protein's solubility in the solvent (19).

As mentioned in the Introduction, ethanol and methanol might be acceptable vehicles for transdermal delivery of proteins [in fact, ethanol has been successfully used to increase the flux of low molecular weight drugs, such as nitroglycerol, across the skin (20)]. Therefore, armed with a new insight into protein solubility in organic solvents, we embarked on a systematic and quantitative investigation of the solubility of various proteins, as well as RNA and DNA, in these two alcohols.

First, we tested the generality of the above-mentioned pH effect. As one can see in Table 1, solubility of hen egg-white lysozyme in both methanol and ethanol indeed rises orders of magnitude when the lyophilization pH is moved away from the pI of the protein. The same phenomenon was observed for other proteins: hexameric Zn^{2+}–insulin, monomeric Na^{+}–insulin, and human growth hormone (Table 1). The magnitude of this effect is quite striking: e.g., the solubility in methanol of Zn^{2+}–insulin lyophilized from pH 5.3 (the pI of the protein) is <2% of that lyophilized from pH 3.0. Therefore, all other proteins examined were lyophilized from aqueous solutions of a pH remote from the corresponding pI values.

Inspection of Table 1 leads to several additional conclusions. It is seen that the solubility of proteins (although not of nucleic acids) in almost all cases is much higher in methanol than in the more hydrophobic ethanol. Also, there is a strong dependence—spanning up to 3 orders of magnitude—of the solubility in either solvent on the nature of the protein. Finally, one can see that in many instances protein solubility in both

Table 1. Solubility of proteins and nucleic acids in methanol and ethanol

Biopolymer	pI*	pH†	Solubility,‡ μg/ml	
			Methanol	Ethanol
Lysozyme	11	11	11 ± 4	9.1 ± 0.1
		6.0	>5000	200 ± 5
		3.0	>5000	250 ± 11
Zn^{2+}–insulin	5.3	7.4	240 ± 1	130 ± 1
		5.3	78 ± 2	37 ± 1
		3.0	>5000	320 ± 7
Na^{+}–insulin	5.3	7.4	160 ± 2	<5
		5.3	120 ± 4	<5
		3.0	1100 ± 10	14 ± 2
Growth hormone	5.2	5.2	ND	260 ± 6
		3.0	ND	2400 ± 40
Myoglobin	7.0	3.0	>5000	590 ± 11
BSA	4.9	9.0	80 ± 2	<5
RNase A	9.6	3.0	62 ± 2	<5
Chy A	9.5	3.0	53 ± 1	<5
Trypsin inhibitor	4.5	9.0	47 ± 3	<5
Cytochrome *c*	10.6	5.0	1400 ± 20	<5
Glucagon	7.5–8.5	3.0	ND	1100 ± 5
T. utilis RNA	—	7.4	14 ± 1	8.8 ± 2.8
Herring DNA	—	7.4	7.4 ± 0.6	7.5 ± 1.3

Proteins and nucleic acids, lyophilized from the pH values indicated, were placed in methanol or ethanol at 5 mg/ml, and their solubilities were determined as described in *Materials and Methods*. BSA, bovine serum albumin; Chy A, chymotrypsinogen A.

*The isoelectric points for most of the proteins listed were taken from ref. 21. For glucagon and growth hormone, the pI values were from ref. 22.

†Lyophilized from the pH value indicated.

‡All solubility experiments were carried out in duplicate; the solubilities listed are the mean values. ND, not determined. Solubilities of >5000 μg/ml indicate that the entire amount of the protein added (5 mg/ml) dissolved in methanol. The sensitivity limit of our protein solubility measurements was 5 μg/ml.

alcohols is very high—milligrams per milliliter—and seems sufficient for meaningful transport studies (see below).

Since the predominant route of the transdermal transport is through intercellular stratum corneum lipids (23, 24), the skin discriminates among permeants on the basis of both their size and their lipophilicity. Hence realistic biomimetic barriers should do the same. Lipid-impregnated polymeric filters meet these requirements, and indeed they have been used to mimic the barrier properties of the skin and other biological membranes (16, 25–27). We have selected this approach for the present investigation as well.

Cellulose filter membranes (0.6 mm thick, pore diameter of 20–30 μm) were impregnated with either phosphatidylcholine or cholesterol [both are components of natural membranes that have been used in studies of transport of low molecular weight permeants (16, 17, 25)]. To minimize physical stripping of the lipids from the impregnated membrane (leading to formation of fine suspensions), the latter was sandwiched between two identical nonimpregnated membranes. The resultant composite three-membrane disk (nonimpregnated/impregnated/nonimpregnated filters) was used to separate the feed from the receiver chamber (the latter always contained aqueous PBS in this work) in a stirred, temperature-controlled diffusion cell.

The main goal of all subsequent experiments was to verify the following hypothesis: for a solvent to be an effective vehicle in transporting proteins through this composite membrane, it should be able to *simultaneously* dissolve proteins and lipids. If the solvent dissolves only the proteins but not the lipids (e.g., water and dimethyl sulfoxide), the membrane will remain impermeable because the proteins will be unable to cross the

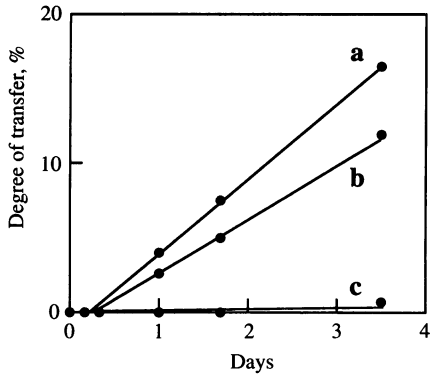

FIG. 1. Kinetics of transport of Zn^{2+}–insulin through composite porous membranes impregnated with phosphatidylcholine. The feed phase was insulin at 0.3 mg/ml in ethanol (line a), insulin at 5 mg/ml in methanol (line b), or insulin at 5 mg/ml in aqueous PBS (line c); the receiver phase was aqueous PBS in all instances. All transport experiments were carried out at 37°C with Zn^{2+}–insulin lyophilized from pH 3.0 as described in *Materials and Methods.*

lipid layer. Likewise, if the solvent dissolves only the lipids but not the proteins (e.g., hexane and most other organic solvents), the membrane again will remain essentially impermeable because the filter pores (as the keratinous network of the stratum corneum) will not allow passage of large protein agglomerates from their suspension. Since methanol and ethanol dissolve both many proteins (Table 1) and lipids, they should be effective penetration vehicles.

Fig. 1 depicts the time course of the transport of Zn^{2+}–insulin dissolved in ethanol or methanol (lines a and b, respectively) through the composite membrane (henceforth referred to as the lipidized membrane). One can see that a significant transfer of the protein occurs: after 3.5 days, 33% and 24% of the maximal transfer (which corresponds to the total equilibration between the feed and receiver chambers) are observed. In contrast, <1% of insulin was transferred through the lipidized membrane after the same time period from an aqueous solution—either PBS (Fig. 1, line c) or pH 3.0 (data not shown). The fact that no insulin transport took place from the aqueous solvent was unequivocally due to the impregnation with phosphatidylcholine; when the middle filter was replaced with an otherwise identical nonimpregnated one, complete equilibration of insulin, dissolved in aqueous PBS, between the chambers was observed in <1 day.

To test our hypothesis further, several other organic solvents were selected as protein permeation vehicles: glycerol, dimethyl sulfoxide, hexane, ethyl acetate, and acetone. All were examined with respect to their ability to dissolve phosphatidylcholine and cholesterol at 3 mg/ml (the concentration that would have resulted from dissolution of all the lipid contained in the impregnated filter in the volume of the solvent in the feed chamber). At 37°C, only hexane and ethyl acetate dissolved both lipids (as did ethanol and methanol); acetone dissolved only cholesterol. However, the solubility of Zn^{2+}–insulin (lyophilized from pH 3) in these three solvents was <5 μg/ml. On the other hand, glycerol and dimethyl sulfoxide dissolved >5 mg of Zn^{2+}–insulin per ml, although both failed to dissolve the lipids. Therefore our hypothesis would predict that all these solvents should be very poor vehicles for insulin transfer through the lipidized membrane because they can dissolve only the lipid or the protein but not both.

Fig. 2 shows the degree of Zn^{2+}–insulin transfer after 3.5 days through the composite membrane impregnated with phosphatidylcholine (Fig. 2*A*) or cholesterol (Fig. 2*B*) as a function of the solvent in the feed chamber. It is seen that a significant fraction of insulin crossed the lipidized membrane in both instances when ethanol or methanol was used as a vehicle (Fig. 2, bars a and b). In contrast, as expected, very little transport was observed with all the other solvents (Fig. 2, bars c–h).

Encouraged by these results, we examined the transport of other proteins dissolved in the alcohols through the lipidized membranes. As shown in Table 2, lysozyme, myoglobin, cytochrome *c*, growth hormone, and glucagon all exhibited substantial transfer from their alcohol feed solutions through both phosphatidylcholine- and cholesterol-impregnated membranes. Very high transport rates of the pharmaceutically important protein hormones, human growth hormone and glucagon, were observed from their solutions in ethanol: nearly half of the equilibrium concentrations were reached in the receiver chamber after 3.5 days.

It was essential to determine whether proteins suffer any irreversible deterioration upon dissolution in ethanol or methanol and subsequent transfer through the lipidized membranes. This question was addressed with lysozyme, whose enzymatic activity provides a sensitive measure of its conformational integrity. After the experiments presented in Table 2, the lysozyme activity in the receiver phase was assayed. The specific activity in all cases was found to be the same, within experimental error, as that of the native enzyme, thus ruling out appreciable irreversible damage.

Analysis of the data in Table 1 suggests that the concept of transmembrane protein transport from alcoholic solutions will be somewhat restricted in scope by the fact that the solubility of some proteins, as well as of RNA and DNA, is rather low, particularly in ethanol. This would limit the amount of biopoly-

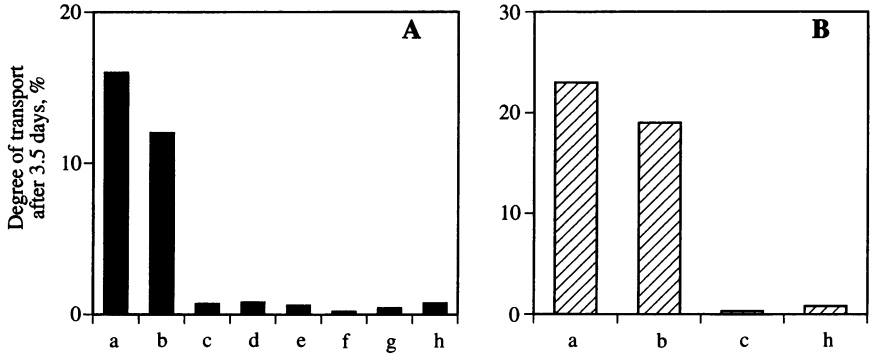

FIG. 2. Transport of Zn^{2+}–insulin through composite porous membranes impregnated with phosphatidylcholine (*A*) or cholesterol (*B*) as a function of the solvent in the feed chamber. Solvents: bar a, ethanol; bar b, methanol; bar c, aqueous PBS; bar d, dimethyl sulfoxide; bar e, glycerol; bar f, acetone; bar g, hexane; bar h, ethyl acetate. The receiver chamber always contained aqueous PBS. The concentration of insulin (always lyophilized from pH 3.0) was 0.3 mg/ml in ethanol and 5 mg/ml in all other solvents. At these concentrations, the protein was completely soluble in ethanol, methanol, PBS, dimethyl sulfoxide, and glycerol; it was insoluble in acetone, hexane, and ethyl acetate. For other experimental conditions, see *Materials and Methods.*

Table 2. Protein transport through the lipidized membranes from different solvents

Protein	Impregnating lipid	Solvent	Degree of transport after 3.5 days, %
Growth hormone	Phosphatidylcholine	Ethanol	18
	Cholesterol	Ethanol	24
	Cholesterol	PBS	<1
Glucagon	Cholesterol	Ethanol	26
	Phosphatidylcholine	Ethanol	19
	Phosphatidylcholine	PBS	<1
Lysozyme	Cholesterol	Methanol	24
	Cholesterol	Ethanol	19
	Phosphatidylcholine	Ethanol	17
	Phosphatidylcholine	PBS	<1
Myoglobin	Cholesterol	Ethanol	19
Cytochrome *c*	Cholesterol	Methanol	24

Protein transport through the composite membranes, where the middle filter was impregnated with phosphatidylcholine or cholesterol, was measured at 37°C as described in *Materials and Methods*. Initial dissolved protein concentrations in the feed phase were as follows: 2 mg/ml for growth hormone; 1 mg/ml for glucagon; 5 mg/ml and 0.2 mg/ml for lysozyme in methanol and in the other solvents, respectively; 0.5 mg/ml for myoglobin; and 1 mg/ml for cytochrome *c*.

mer that could be delivered from a reasonable volume, such as that of a transdermal patch. Therefore we decided to explore whether it is feasible to increase the biopolymer solubility in ethanol by means of additives.

Recently, it was demonstrated (13, 28–30) that protein solubility in organic solvents can be greatly enhanced by hydrophobic ion pairing with detergents (importantly, the concentrations of the latter should be below their critical micelle concentration values). Herein we examined whether this approach could enhance the solubility in ethanol of biopolymers that are poorly soluble in this alcohol: chymotrypsinogen, Na^+–insulin, *T. utilis* RNA, and herring DNA (Table 1). Since the rationale of the hydrophobic ion pairing requires that the biopolymer and detergent molecules be oppositely charged (13), we employed SDS with chymotrypsinogen and insulin (both cationic at pH 3.0; Table 1) and DTMAB with RNA and DNA (both anionic at pH 7.0).

To aqueous solutions of chymotrypsinogen, Na^+–insulin, RNA, and DNA, optimal concentrations (13) of the detergents were added, and the mixtures were stirred. In the case of the proteins, the ensuing precipitates were removed by filtration and dried under air. In the case of the nucleic acids, where no stable precipitates formed, the mixtures were evaporated under vacuum to dryness. The solubility of all the complexes in ethanol was then determined at 30°C as described in *Materials and Methods*, and the values obtained are depicted in the last column in Table 3. Comparison of these data with the solubilities of the same biopolymers without detergents (the middle column in Table 3) shows that complex formation markedly raises the solubility in ethanol. For example, the solubilities of Na^+–insulin and RNA increase by factors of 55 and 36, respectively.

Table 3. The solubility in ethanol of various biopolymers and their complexes with detergents

Biopolymer	Solubility, μg/ml	
	Without detergent	In a complex with detergent
Chymotrypsinogen A	<5	21 ± 1
Na^+–insulin	14 ± 2	830 ± 5
RNA	8.8 ± 2.8	240 ± 4
DNA	7.5 ± 1.3	90 ± 2

Biopolymer solubilities without detergents were determined as described in the legend to Table 1 except that the concentration of the added biopolymer was 1 mg/ml; Na^+–insulin was lyophilized from pH 3.0. Complexes of biopolymers with detergents—SDS for the proteins and DTMAB for RNA and DNA—were formed as follows. To solutions of Na^+–insulin or chymotrypsinogen at 1 mg/ml in distilled water (pH 3.0), 1.7 mM and 0.86 mM SDS, respectively, were added (13). The resultant precipitates were filtered, dried, and suspended in ethanol at 1 mg/ml. To solutions of RNA or DNA (50 μg/ml) in distilled water (pH 7.4), 1 mM DTMAB was added (13). The resultant mixtures were subjected to rotary evaporation, and the solid residues were suspended in ethanol at 1 mg/ml. The solubilities were measured as described in *Materials and Methods*; separate experiments demonstrated that no interference from the detergents occurred.

Having attained much higher solubilities in ethanol, we then examined the transfer of biopolymer–detergent complexes from this solvent through the lipidized membranes. As shown in Fig. 3, bars a–c, in all instances significant transmembrane transport was observed. In contrast, no transport was observed from aqueous PBS.

We also examined an alternative approach to increasing protein solubility in, as well as transport from, ethanol based on a recent finding that proteins can be made soluble in organic solvents by complexation with certain neutral polymers (31, 32). Among these polymers, PEG is attractive from the drug delivery standpoint because it is nontoxic, has been used in pharmaceutical preparations, and is readily excretable

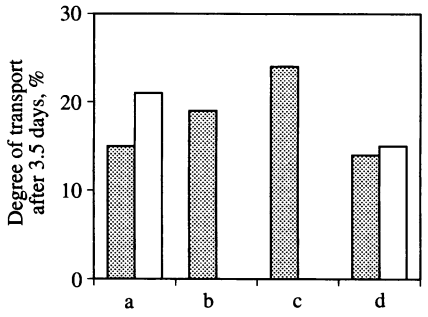

FIG. 3. Transport of complexes of proteins and nucleic acids with solubility enhancing additives through the composite porous membranes impregnated with phosphatidylcholine (stippled bars) or cholesterol (open bars). Feed chambers contained the following solutions in ethanol: Na^+–insulin at 0.8 mg/ml complexed with SDS at a molar ratio of 1:10 at pH 3.0 (bars a); *T. utilis* RNA at 0.24 mg/ml (bar b) and herring DNA at 90 μg/ml (bar c) both complexed with DTMAB at a molar ratio of 1:6 at pH 7.4; and Zn^{2+}–insulin at 0.2 mg/ml complexed with PEG at a weight ratio of 1:100 at pH 5.3 (bars d). The receiver chamber always contained aqueous PBS. When aqueous PBS was employed instead of ethanol as a solvent in the feed chamber, very low degrees of transport were observed: 1.5% (bar a; impregnated with cholesterol), <1.5% (bar b), <4.5% (bar c), and <2% (bar d; impregnated with phosphatidylcholine). For other experimental conditions, see *Materials and Methods*.

through the urine (33). Consequently, we attempted to use complex formation with PEG to enhance the solubility in ethanol of Zn^{2+}–insulin lyophilized from pH 5.3 [this pH affords the lowest solubility, 37 μg/ml (Table 1)]. A mixture of 1 g of PEG (M_r of 40,000) and 10 mg of Zn^{2+}–insulin in 2 ml of water (pH 5.3) was lyophilized, 100 mg of the resultant solid complex was suspended in 1 ml of ethanol, and the suspension was shaken at 30°C for 16 hr. The resultant solubility of insulin was 150 μg/ml—i.e., 4 times above that of the noncomplexed protein (the solubility was even higher, 220 μg/ml, at 37°C). (Note that the molecular weight of PEG is important for its insulin-solubilizing power: no increase in Zn^{2+}–insulin solubility in ethanol was observed under the same conditions with the polymer of M_r of 3400.) Fig. 3, bars d, shows that the Zn^{2+}–insulin–PEG complex dissolved in ethanol indeed penetrates the composite membrane impregnated with either phosphatidylcholine or cholesterol; no appreciable transport of the complex was observed from aqueous PBS.

In closing, we have demonstrated in this study that many proteins are quite soluble in ethanol or methanol when lyophilized from aqueous solutions of optimal pH values. Proteins dissolved (by themselves or with the aid of enhancers, such as detergents or PEG) in ethanol and methanol can readily cross porous membranes impregnated with lipids, whereas solvents that dissolve either proteins or lipids, but not both, were ineffective. The next step is to test this delivery strategy with the skin and other biological barriers.

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