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TRANSLOCATION OF PROTEINS THROUGH BIOLOGICAL MEMBRANES

A CRITICAL VIEW

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I. Introduction

Proteins have to cross biological membranes in many instances during processes such as membrane turnover, turnover of the mitochondrial and chloroplastic matrix, shedding of IgM and secretion and active transport. Various hypotheses have been put forward to account for these phenomena.

Although our initial purpose was to write an extensive review on the subject, the very fast progress in the field has limited us to a critical analysis of some of the contemporary data, part of which will be referred to the excellent reviews that were published in the field. It is hoped that this approach might raise more intriguing questions and perhaps lead to a different image of the membrane than that currently accepted.

At this point, it is of prime importance to evaluate the advantages and the limitations of the membrane model which guided both the experimental approaches and the interpretation of most of the results presently in the literature.

What kind of information is contained in the current model? The existence of a lipid bilayer as the foundation of the membrane superimposed upon fragmentary knowledge of protein topography, general morphology, quantitative and qualitative analysis of constituents and lateral diffusion of lipids in the plane of a membrane led to the conceptualization of a biological membrane in a fluid mosaic which is largely accepted today [1–6].

This model, which was built on the premise of static analysis and on the intrinsic dynamic properties of a membrane in the steady state, accounts for such dynamic properties as 'capping' and 'patching' observed on lymphocytes stimulated by mutagenic agents such as concanavalin A and lipopolysaccharides or antibodies [7].

Starting from this concept, it was also possible to delineate a sequence of events allowing integration, at least partially, of the vesicular aspect of macromolecular secretion [8–10].

However, it does not seem surprising that a model established on the principle of a hydrophobic barrier, impermeable to macromolecules, cannot account for most of the phenomena requiring precisely such a permeability, which is the case for the protein transmembrane translocation phenomena already mentioned.

To reconcile these opposing requirements and to explain the insertion of proteins into and their eventual translocation through the biological membrane, diverse triggering mechanisms have been proposed. These different hypotheses will be examined in this review.

From the current knowledge of membranes, it appears that in the absence of covalent binding, it is the interactions of the electrostatic, electromagnetic or van der Waals' type that control and ensure the cohesion of the membrane [11,12]. These interactions not only allow for a great degree of motional freedom of the different components and a considerable three-dimensional plasticity of the entire structure, but also constitute the basis of the essentially bimodal language used as a reciprocal vector of communication and information between membrane and environment. The 'syntax' of this language is encoded within the forces of interaction, thus forces in $1/d^2$ for the electrostatic interactions and of the order of $1/d^6$ for the van der Waals'-London types.

The consequence of this is that the spatio-temporal organization of the membrane will be coupled to the molecular requirements of the environment, one corollary of which will be the variation of the hydration state of the membrane and hence new solubility characteristics of its constituents.

The above considerations guided the steps of this critical review. For reasons of clarity, the results and hypotheses will be presented in order of growing conceptual complexity, rather than that of chronology or apparent popularity. Accordingly, the work of our laboratory is introduced first.

II. Translocation via environmental induction: reversible-large amplitude protein movement

The present picture of biological membranes rests mostly on the conceptual scheme of the 'fluid mosaic'. This general description attributes to the membrane a mosaic structure in which proteins and lipid bilayers coexist. The charged heads of the lipids are turned towards the hydrophilic areas of the environment, whereas their hydrophobic tails are orientated towards the internal part of the membrane. The proteins are localized either peripherally or intrinsically in the membrane, as a consequence of their amphiphatic interactions with the lipids.

In this current view, the major emphasis has been given to the hydrophobic interactions between protein and lipids privileging the role of membrane lipids and leading to the impossibility of macromolecules crossing the hydrophobic membrane barrier [1]. However, proteins do cross membranes in many instances. Thus, an alternative conceptual scheme must be proposed to account for these phenomena.

In our view, membranes should be considered as spatio-temporal structures forming a dynamic continuum with their environment. The mere fact that membrane cohesion is ensured by electrostatic and van der Waals' type forces interrelates membrane and environment, with the consequence that any change in the environment will correspond to

new spatial segregation of the membrane components. Within this context, we have examined the possibility that in the course of such reorganization, some proteins are released from or directly externalized and internalized through the biological membrane.

The inner mitochondrial membrane is a particularly suitable system for such an approach. It is rich in protein, endowed with considerable plasticity and easy to isolate in a structurally intact form [13]. Moreover, most mitochondrial proteins are coded by nuclear genes, synthesized outside the mitochondria on cytoplasmic ribosomes and then imported [14]. These imported proteins have to be translocated across the mitochondrial membrane.

Starting from the above premise, it was possible to observe and describe an intramitochondrial large-amplitude protein movement which included such phenomena as release and binding of proteins and protein translocation through the inner mitochondrial membrane [15,17]. It is characterized as follows:

(1) it is specific with regard to both the changes in the environment and the nature of the proteins involved in the movement;

(2) it is reversible and transmembranal for some of the proteins;

(3) it is dependent both on the lipid composition and the temperature and

(4) it can be demonstrated in whole fully metabolically active mitochondria.

IIA. Specificity

When mitochondria are incubated in the presence of movement-effector molecules (succinate, fumarate, aspartate, pyruvate or those molecules which trigger the above phenomenon) or some of their structural analogs, some of the intramitochondrial proteins and enzymes (aspartate aminotransferase, malate dehydrogenase) show a change in their repartition between the different submitochondrial compartments [17,18].

Thus, in the presence of 20 mM succinate, the enzyme aspartate aminotransferase is localized 40% in the intermembrane space, 25% in the inner membrane and 35% in the matrix. In the presence of fumarate, these figures become, respectively, 79, 14 and 7%,

TABLE I

INFLUENCE OF THE NATURE OF MOVEMENT EFFECTOR ON THE SUBMITOCHONDRIAL LOCALIZATION OF ASPARTATE AMINOTRANSFERASE

Results are expressed as percent of total aspartate aminotransferase activity. Total aspartate aminotransferase activity was constant to within ±5%. Sucrose was 0.25 M. Effectors were at 2 μmol per mg of mitochondrial protein.

Movement effector	Percent of total aspartate aminotransferase activity in different submitochondrial compartments		
	Intermembrane space	Inner membrane	Matrix
Sucrose	9	79	12
Phosphate	19	68	13
Acetate	20.5	72	7.5
Succinate	39	25	36
Fumarate	79	6	15
Oxaloacetate	77.5	11.5	11
Citrate	59.5	1.5	39

whereas in the presence of sucrose alone, phosphate or acetate, most of the enzyme (70%) is associated on the inner face of the inner membrane [19]. The results (Table I) suggest the specific influence of these environments and also imply that succinate and fumarate provoke the translocation of the enzyme from the inner face of the inner membrane, where it is localized in sucrose towards the intermembranal space where it is to be found when succinate or fumarate are present. Under such conditions, it can also be shown by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis that the proteins released into the intermembranal space vary with the nature of the movement effector used [20].

IIB. Reversibility

Rebinding of released enzyme to inner mitochondrial membrane was observed on removal by dialysis of the movement effector responsible for release [17,18]. Internalization of the rebound enzyme, aspartate aminotransferase, was demonstrated in both mitoplast and inside-out submitochondrial particles, and by measuring on both sides of these vesicular structures either the accessibility of the rebound activity to non-permeable substrate or to specific antibodies, or the accessibility of ^{125}I-labelled aspartate aminotransferase to protease digestion (Table II).

TABLE II

BINDING AND INTERNALIZATION OF ASPARTATE AMINOTRANSFERASE WITH MITOPLASTS AND SUBMITOCHONDRIAL (INVERTED) PARTICLES

Binding is expressed as percent of total enzymatic activity or immunoactivity associated with mitoplasts after internalization, reincubation and isotonic washing. Activity was measured after Lubrol treatment in 0.25 M sucrose. Immunoprecipitation was performed after 3 min sonication of the vesicles. Internalization is expressed in terms of accessibility to aspartate aminotransferase either on the outer side of the intact mitoplast or on the outer side of the corresponding inside-out vesicle. Aspartate aminotransferase activity is measured by the coupled method described in Ref. 15 using NADH as non-permeating cofactor. Immunoprecipitation was performed as in Ref. 16. Mild protease digestion was performed as in Ref. 16 after internalization of ^{125}I-labelled aspartate aminotransferase.

	Mitoplast	Submitochondrial particles	
Binding			
Immunoprecipitation	78% (succinate treated 46%)	–	total enzyme (intact mitoplast is 100%)
Lubrol fractionation	69%		% of reassociated enzyme recovered in the membranal fraction
Internalization			
Latency	31% (1)	100% (3.2)	non-latent reassociated enzyme (ratio)
Protease treatment	11% (1)	50% (4.6)	accessible reassociated enzyme (ratio)
Antibody binding	1	4.4	ratio of binding

After internalization, about 60% of the rebound enzyme became inaccessible on the outer face of the mitoplast, whereas it appeared on its inner face (outer face of the corresponding submitochondrial particles) [21].

These results clearly show that some proteins (aspartate aminotransferase, malate dehydrogenase) are able to cross the inner mitochondrial membrane reversibly in response to changes of the environment. In this case, it is the disappearance of the exogenous effector signal which is the cause of the internalization of the previously externalized

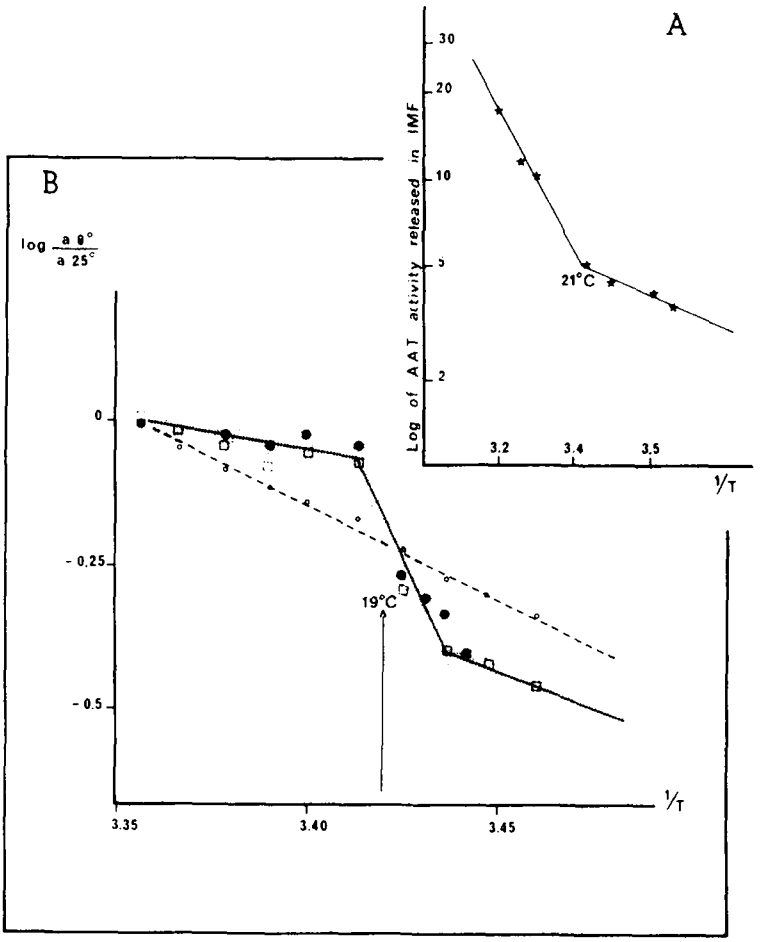


Fig. 1. Influence of varying temperature on the intramitochondrial (IMF) localization of aspartate aminotransferase (AAT) in the presence of movement effector. A, influence of temperature on the intermembranal release of aspartate aminotransferase. Arrhenius plot of aspartate aminotransferase release in the presence of succinate as a function of $1/T$. Control in the absence of succinate shows no release. B, reversible loss of aspartate aminotransferase latency in whole mitochondria as a function of temperature. Enzyme activity was measured by a coupled method using NADH, which does not permeate the inner membrane. Temperature increase, 0.5°C per min; temperature decrease, 0.2°C per min. (- - - - -) Temperature-dependent activation of solubilized aspartate aminotransferase; (———) aspartate aminotransferase activity as measured on whole mitochondria under isotonic conditions. (□) Increasing temperature; (●) decreasing temperature. Activity is expressed as log of activity at temperature (θ) compared to standard activity at 25°C.

molecule. This might be in relation to the new membrane asymmetry recognized by the enzyme prior to its internalization.

IIC. Lipid dependence

Membrane fluidity is dependent, amongst other factors, upon the nature and the degree of polyunsaturation of its fatty acids. In the case of environmentally induced translocation of aspartate aminotransferase and malate dehydrogenase, it was shown that the more fluid the membrane in terms of lipids the lower the temperature of the breaking point of the Arrhenius curve describing the externalization event [22,23]. This has been tested on four systems:

(a) normal rat liver mitochondria;

(b) rat liver mitochondria enriched in saturated fatty acids as a consequence of a 'lipid-free' diet;

(c) liver mitochondria from *Cricetus cricetus* at various periods of its annual cycle;

(d) two types of rat brain mitochondria showing a natural difference in their composition of polyunsaturated fatty acids, which is the case for cell body and synaptosomal mitochondria.

In all these cases, a correlation can be established between the lipid fluidity of the membrane and the externalization phenomenon [22,23].

In the particular case of the hibernator, *C. cricetus*, the results suggest that a preferential lipid microenvironment might be required in the membrane area where externalization of proteins occurs. The Arrhenius plot also showed that after internalization, protein interacted with a lipoprotein environment comparable to that with which it interacted before initial externalization.

Using the property of preferential outer mitochondrial membrane permeability to NADH, it was possible to demonstrate that both malate dehydrogenase and aspartate aminotransferase are translocated through the inner mitochondrial membrane towards the intermembranal space in whole functional mitochondria [18,24]. Reversibility of aspartate aminotransferase movement was shown using the temperature dependence of this phenomenon [21] (Fig. 1).

All the above results indicate that a protein (aspartate aminotransferase) is able to cross a membrane reversibly without permanent modification of its polypeptide chain and as a response to changes in the environment.

III. Other examples of protein translocation without permanent modification of the polypeptide chain

Examples of transmembrane translocation such as rat liver mitochondrial aspartate aminotransferase have been seldom explored. Nevertheless, some can be found in the literature [25–39].

5′-Nucleotidase is an enzyme present in all cellular membranes of the rat hepatocyte. Its localization in Golgi cisternae and secretion vacuoles has been studied by the cytochemical techniques of enzymatic latency, binding of specific antibody and of concanavalin A [25]. Results of these studies led the authors to conclude that the enzyme is translocated from the cytoplasmic side of Golgi cisternae membranes to the inside of secretion vacuoles. Although the mechanism of this translocation was not established, the authors proposed that orientation of the enzyme might be controlled by other membrane pro-

teins and a possible minor modification of the polypeptide chain such as phosphorylation, acetylation or glycosylation.

Studying the biogenesis of cytochrome *c* in *Neurospora crassa*, Korb and Neupert [26] showed that, in an in vitro system, apocytochrome *c* is synthesized on cytoplasmic ribosomes and released free into the supernatant. Apocytochrome *c*, but not holocytochrome *c*, is incorporated into mitochondria. The heme group is linked to the holoprotein in the inner mitochondrial membrane. The authors propose that this change of conformation leads to trapping of holocytochrome *c* in the membrane. It is noteworthy that no concomitant change in molecular weight of the apoprotein could be shown.

With regard to the problem of incorporation of cytoplasmically derived proteins into mitochondria, Marra et al. [27,28] demonstrated that rat liver mitochondria are capable of taking up in vitro purified mitochondrial aspartate aminotransferase but not the cytosolic isoenzyme. The techniques used were measurement of enzyme activity in whole mitochondria by a fluorescence method [27], determination of enzyme latency and direct measurement of association of ^{3}H-labelled enzyme with mitochondria [28]. Binding and actual incorporation to mitochondria were distinguished only from enzymatic results [28]. In a more recent paper [29], these authors studied the effect of sulfhydryl-group reagents on the translocation of aspartate aminotransferase and found that it was possible to inhibit the translocation of the enzyme by blocking a single sulfhydryl group per monomer of the enzyme. The blockage does not inhibit the enzyme activity. This stresses the importance of the spatial structure of the protein for the translocation.

Godinot and Lardy [30] and Kawajiri et al. [31] studied the biosynthesis of rat liver glutamate dehydrogenase, another mitochondrial enzyme, of which the synthesis is directed by the nuclear genome. These authors found that glutamate dehydrogenase is a product of protein synthesis by reticulum-bound ribosomes. In pulse-labelling experiments, glutamate dehydrogenase is at first found associated with microsomes before being transferred to mitochondria. Accessibility of this microsomal enzyme to proteases and antibody demonstrates that the glutamate dehydrogenase-synthesizing polysomes discharge their nascent chains in the cytoplasm [9]. Studies of the interactions of glutamate dehydrogenase and cardiolipin, a potent inhibitor of the enzyme and a characteristic component of the mitochondrial inner membrane, led Godinot [32] to propose that their association could favor insertion of glutamate dehydrogenase through the mitochondrial membrane. Furthermore, the fact that substrates of the glutamate dehydrogenase, glutamate and NADH, have an antagonist effect on association with cardiolipin indicated that this phenomenon could play a role in the regulation of glutamate dehydrogenase activity via variable interactions with the membranes of mitochondria.

Ohashi and Sinohara [33] presented evidence that the ^{125}I-labelled cytosolic form of δ-aminolevulinate synthase is incorporated into rat liver mitochondria in vitro. The cytosolic form of this enzyme is a complex made up of three subunits, one of which is catalytically active. When incubated alone in the presence of mitochondria, this active subunit is not taken up, suggesting that the two inactive components of the enzyme play an important role in its incorporation. Catalase and uricase are two peroxisomal enzymes of rat liver which are synthesized in the cytoplasm on free ribosomes and subsequently transferred into the organelles. The peroxisomal and cytosolic forms of the two enzymes are identical in molecular weight [34].

Another approach to investigate the mode of penetration of a cytoplasmically synthesized protein through the mitochondrial membrane is to study the interactions of such enzymes with artificial liposomes. Mitochondrial cytochrome *c*, aspartate aminotrans-

ferase [35] and malate dehydrogenase [36] have all been demonstrated to interact strongly and even to become latent when associated with negatively charged liposomes. Furthermore, in the case of mitochondrial malate dehydrogenase, a small conformational change occurs when the dimeric enzyme dissociates into its subunits, the latter only being able to interact with phospholipid vesicles in a way indicating incorporation [32]. Other techniques, such as fluorescence-labelling [33] and freeze-fracture electron microscopy [34,35] have illustrated the possibility of transmembrane movements for membrane proteins. Some of these movements are related to changes in the membrane fluidity [33,34].

IV. Translocation via subunit dissociation (toxic peptides)

Other proteins that have been shown to cross membranes are certain toxins of plant and microbial origin. These include diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, colicins E_2 and E_3, cholera toxin, *Escherichia coli* enterotoxin (bacterial toxins), modeccin, abrin and ricin (plant toxins) [40–45]. They all have to cross the membrane of susceptible cells to exert their lethal effects.

The best known of these toxins is diphtheria toxin [41], for which a transport model has been proposed [43]. As a complete bibliography is to be found in Refs. 40, 41 and 45, we shall only summarize here the significant data on the transmembrane crossing of these peptides. We shall focus first on the diphtheria toxin and its postulated mode of entry into cells and then examine the common features of the different toxins and their relationship to a possible general mechanism of uptake.

Diphtheria toxin is produced by strains of *Corynebacterium diphtheriae*, lysogenic for phages, of which the genomes carry the structural gene for the toxin. The toxin molecule is synthesized and released as a single polypeptide chain of 62 000 daltons. By cleavage of a single peptide bond and of a disulfide linkage, the molecule is split into two fragments. The NH_2 terminal fragment (A) is enzymically active and is a specific inhibitor of eukaryotic protein synthesis. Its action is to catalyse in the cytoplasm the transfer of the ADP-ribosyl moiety of NAD^+ to elongation factor EF-2, thus inactivating it. The COOH-terminal portion of the toxin molecule (B) is responsible for the binding of the toxin to cell membranes, presumably to a specific cell receptor which may be a glycoprotein. Fragment B does not enter the cell. In the absence of fragment B, fragment A cannot cross the cell membrane, and is thus non-toxic when administered alone. The isolation of mutant strains that are non-toxic, either because they lack peptide segments at the N-terminal or at the C-terminal, has been very valuable in ascertaining the different roles of the two fragments. The complete amino acid composition of the molecule and its partial sequence are known. The toxin molecule is very sensitive to proteases which fragment it into its two subunits A and B, still held together by a disulfide bridge. In turn, this disulfide bridge is easy to reduce with thiols and the molecule is thus cleaved into the two fragments. The fragment B is unstable and is composed of a hydrophobic area at its N-terminal and a hydrophilic C-terminal region which is involved in receptor binding. On the contrary, fragment A is very stable, its activity resists boiling and exposure to acids and alkalis, and it is able to refold readily in an active form after exposure to denaturing agents. The kinetic characteristics of the intoxication are as follows. There is a lag between administration of the toxin to cultured cells, variable with the type of cells, and dependent upon the temperature and pH. This lag corresponds to the entry time of fragment A plus the time needed to inactivate enough EF-2 to cause a decrease in protein synthesis. At the beginning of the intoxication, there is an eclipse phase, during which cell-associated toxin can no longer be entirely removed by washing with antisera or proteolytic

enzymes, but before which any fragment A has catalysed transformation of EF-2 in the cytoplasm. This eclipse of the toxin molecule represents its 'transit' through the membrane.

Among the possible means of entry of diphtheria toxin into cells, non-specific diffusion and endocytosis have been ruled out [40,41]. As little as 10^{-10} M toxin is sufficient to provoke inhibition of protein synthesis in susceptible cells, which makes insignificant the aspecific passing observed on resistant cells (of rat and mouse origin) with 1000–10000-fold greater concentrations. For the same reason, endocytosis is not a convenient way to internalize so few molecules. Furthermore, endocytotic uptake would lead to lysosomal degradation of the toxin, and leave it one membrane away from its site of action. In fact, it has been shown that agents known to enhance pinocytotic activity (polyornithine and DEAE-cellulose) actually inhibit the entry of toxin into sensitive cells.

From their studies of binding of Triton X-100 to diphtheria toxin, mutated gene products and fragments, Boquet et al. [43] have proposed a model for the transport of fragment A across the cell membrane to the cytoplasm. When the toxin molecule approaches the cell membrane, the hydrophilic area at the C-terminal of fragment B reacts with the membrane receptor, the hydrophobic region of B (which inserts readily into detergent micelles) is brought into close proximity with the phospholipid bilayer, into which it becomes inserted. In association with a part of the receptor molecule or another integral membrane protein, or by itself, this hydrophobic part of B forms a 'channel' through the membrane. Since fragment A is attached to the hydrophobic region of B, it is drawn through the 'channel' until the disulfide bridge and the sequence that links the two fragments become exposed to the cytoplasmic face of the membrane. There, fragmenting of the polypeptide chain and reduction of the disulfide bridge take place and A enters the cytoplasm. Because fragment A readily reforms after even drastic denaturation conditions, any channel through the membrane need only be large enough to accommodate unfolded A chain.

Other toxins known to penetrate the plasma membranes of cells to exert their effects possess many structural and activity properties very similar to those of diphtheria toxin [40]. Most of these toxins consist of two functionally distinct parts, one active enzymatically (the A component) and one which binds to surface receptors on cells. Like diphtheria toxin, the A component enters the cell to catalyse some intracellular damage and the B part does not cross the membrane but is responsible for the interaction of the toxin with the cell surface, via a receptor. Separated fragment A is unable to enter the cell and isolated fragment B can block the action of the toxin. Insertion of A into the cell cytoplasm requires its separation from B and may involve important conformational changes.

Well studied examples of such toxins are cholera toxin, *Ps. aeruginosa* exotoxin, abrin, ricin and colicin E_3. Cholera toxin has a more complex structure than diphtheria toxin. It is composed of seven peptides: A_1, A_2 and five identical B-subunits. A_1 is the active fragment and is connected to A_2 by non-covalent bonds. The B-subunits bind specifically to a membrane receptor, part of which has been identified as ganglioside GM_1. The damaging action of fragment A_1 is to activate adenylate cyclase and this provokes secretion of electrolytes and water. The precise site of interaction between A_1 and adenylate cyclase, a membrane-associated enzyme, has been disputed (Fig. 2). Kinetic arguments and effects of antisera on intact and ruptured cells have by now brought forward sufficient arguments for an intracellular site of action of the A_1 fragment, although the precise reactions have not yet been identified.

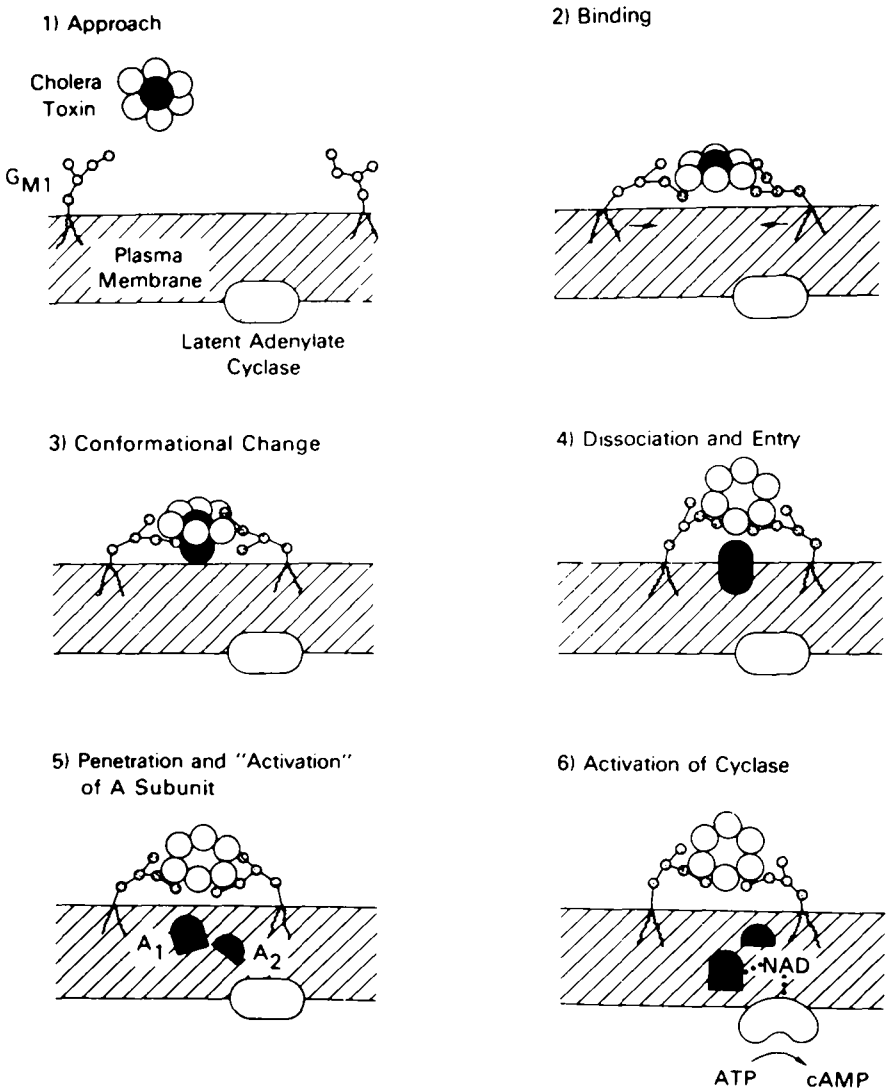


Fig. 2. Hypothetical mechanism for the binding of cholera toxin to the cell surface and subsequent activation of adenylate cyclase. From Fishman and Brady [42], copyright March 13th, 1980, by the American Association for Advancement of Science. cAMP, cyclic AMP.

Ps. aeruginosa exotoxin has the same intracellular action on elongation factor EF-2 as diphtheria toxin. It differs from diphtheria toxin mainly in that its conversion in vitro into the active form does not require any proteolytic step but is accomplished by simultaneous treatment with disulfide bond-reducing agents like mercaptoethanol, dithiothreitol or cysteine and a protein denaturant like urea or SDS. In this case, activation seems to result only from a conformational change that exposes the previously buried active site. It has not yet been shown that this mode of activation is sufficient to provoke the entry of the active part of the toxin into sensitive cells. Proteolytic fragmentation of this toxin has been demonstrated to yield a fragment that is enzymatically active (A fragment?) [44]. The plant toxins, abrin and ricin, inhibit protein synthesis in animal cells by inactivation

of 60 S ribosomal subunits. They are made of two subunits A and B, connected by a disulfide bridge. The B chain binds to the cell membrane via a glycoprotein receptor and facilitates the transfer of the active A subunits into the cytoplasm.

The bacteriotoxin, colicin E_3, is produced by strains of *E. coli* carrying the colicin E_3 plasmid. It is secreted as a complex with a small peptide immunity factor that is able to prevent its enzymatic action which is the cleavage of 16 S RNA. It is not yet clear whether this immunity factor is involved in the binding of toxin to the cell membrane or if any proteolytic cleavage is necessary for the entry of an active part of the toxin.

The significant characteristics of these toxins therefore can be summarized. Intracellularly acting toxins consist of two separate or separable active and binding components (A and B). These A fragments have enzymatic properties responsible for the toxic effects on sensitive cells. The A components cross the cell membrane and reach either the cytoplasm or the cytoplasmic side of the membrane. The structures of A components are compatible with the idea that they enter by unfolding-diffusion through some narrow channel and reform once inside the cell. The B components bind to the cell membrane and do not enter cells.

These well established characteristics of some toxins that have an intracytoplasmic site of action have led Gill [40] to examine the possible modes of transmembrane passage of these proteins and to propose a generalization of the model of Boquet et al. [43] for the diphtheria toxin. Some features of this model need to be further confirmed but it is clear it is the most satisfactory to date.

V. Translocation via vectorial processing: transport of proteins into mitochondria and chloroplasts

Biogenesis of mitochondria and chloroplasts is controlled through the cooperation between their own genetic system and the nuclear genome. The latter encodes about 90% of the proteins of the mitochondria. This feature has made the mitochondrion a much studied system for the elucidation of the molecular events underlying protein segregation from the cytosol to the mitochondria [14].

Since excellent reviews on this topic have been recently published [46–48], we will briefly summarize the present state of the art. The scheme proposed by Kellems et al. [49] in which proteins are incorporated through ribosomes bound to mitochondrial membrane in a cotranslational manner has, to date, proved to be successful only with yeast mitochondria, since no ribosomes have been found to be bound to such organelles in other species. In disagreement with this proposal, evidence is accumulating that cytoplasmically produced proteins are incorporated into mitochondria and chloroplasts in a post-translational manner. First evidence resulted from kinetic studies of radioactively labelled proteins, for instance, rat liver ATPase subunits [50] or proteins of *N. crassa* for which it was shown that incorporation presents a lag period [51] and is insensitive to inhibitors of protein synthesis [52] which clearly suggested the existence of an extramitochondrial pool.

More recent experiments demonstrated the existence of precursor forms of enzymes which can be segregated into mitochondria and chloroplasts. These include spinach and pea ribulosebisphosphate carboxylase, a chloroplastic enzyme [53,56], and yeast mitochondrial enzymes: subunits V of the cytochrome bc_1 complex [55] and three subunits of F_1-ATPase (Fig. 3) [54]. These precursor forms resemble those described for secretory proteins in the 'signal-sequence' hypothesis (see below) in that they are of higher molecu-

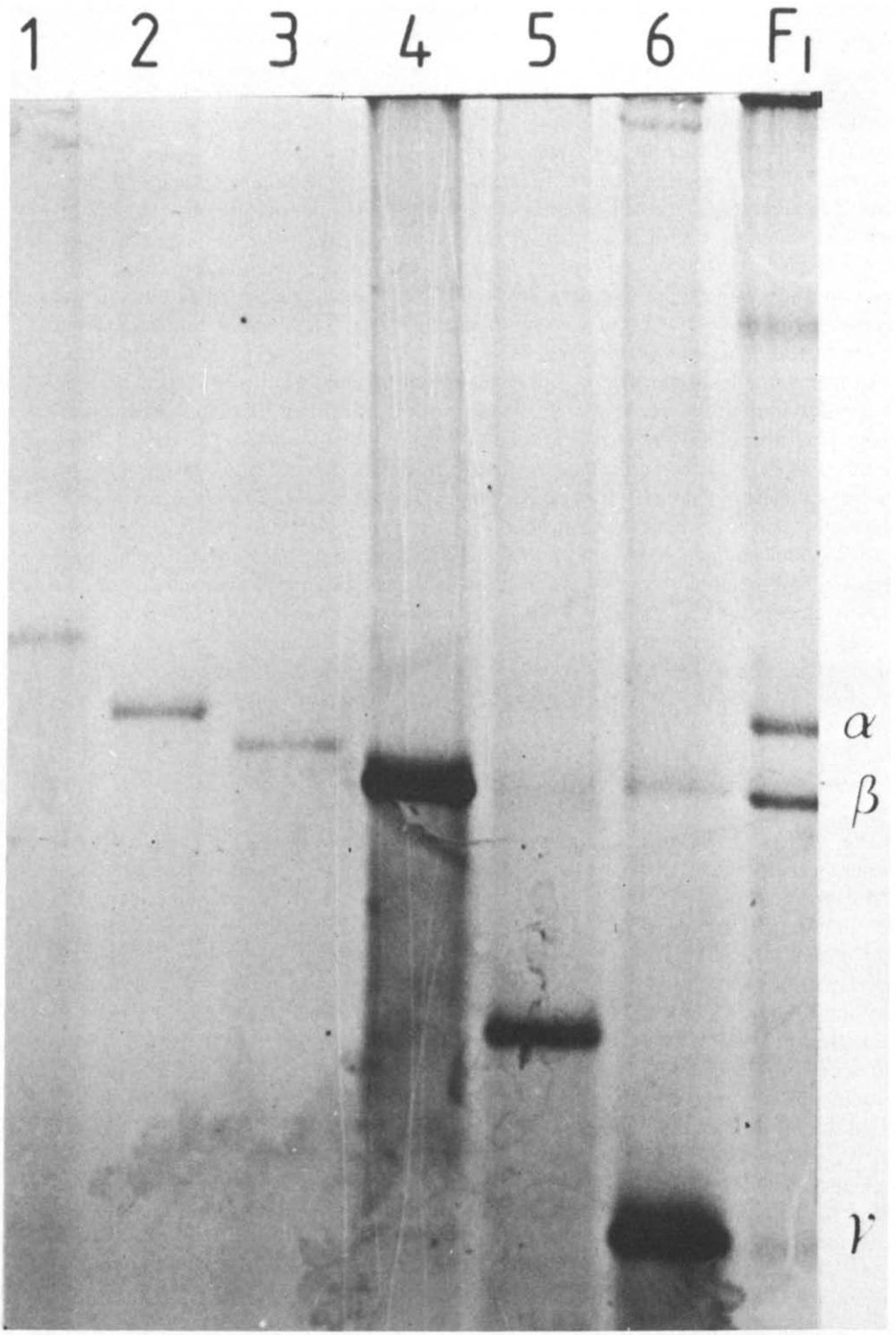


Fig. 3. Comparison between 'mature' F_1-ATPase subunits and subunits synthesized in vitro. Subunits synthesized in vitro were immunoprecipitated with subunit-specific antisera from a reticulocyte lysate programmed with yeast RNA. Mature subunits were isolated in a similar fashion from isolated mitochondria of yeast cells that had been grown for more than 10 generations in the presence of $^{35}SO_4^{2-}$. Track F_1 contains a mature F_1-ATPase standard that had been immunoprecipitated with antiserum against F_1 from mitochondria labelled with $^{35}SO_4^{2-}$ in vivo. An autoradiogram of the dried SDS-polyacrylamide gel slab is shown. Track 1, precursor to α; track 2, mature α; track 3, precursor to β; track 4, mature β; track 5, precursor to γ; track 6, mature γ. Ikram Mangroobhai et al. 1541

lar weight than the mature forms and seem to yield their mature counterpart after a proteolytic cleavage of the chain. However, unlike precursors of secretory proteins, these mitochondrial precursors do not need a hydrophobic N-terminal signal sequence. Highfield and Ellis [56] showed that the precursor for the small subunits of ribulosebisphosphate carboxylase has a much lower isoelectric point than the mature one, which indicates that the extra sequences are rich in acidic amino acids. Furthermore, a striking difference exists in that incorporation of the cytoplasmically derived mitochondrial precursors into their target organelles is a post-translational event, occurring in vitro in the absence of any element of the protein-synthesis machinery. This departs from the favored scheme of the signal-sequence hypothesis of Blobel and Dobberstein (see Section VI) in which membrane incorporation of proteins is driven by elongation of the chain through ribosomes bound to the membrane. By analogy with this vectorial translation mechanism, Schatz [47] introduced the term 'vectorial processing' which is intended to describe how a modification or 'processing' of the protein (proteolysis, for instance), taking place on one side of a membrane, can drive the vectorial event of transmembrane translocation. It appears that the processing step is needed to create the required asymmetry and irreversibility of the phenomenon. Somehow different is the case of proteins inserted in the inner mitochondrial membrane which have to cross only the outer membrane, and do not possess any cleavable signal sequence. This is the case for cytochrome *c*, of which insertion is ensured by the addition of the heme group [26], and of the adenine nucleotide transporter [47]. The existence of a soluble polyprotein precursor to subunits IV–VII of mitochondrial cytochrome oxidase was established [57]; the subunits are inserted by several cleavages of the precursor.

VI. Translocation via vectorial translation: the signal-sequence hypothesis

The synthesis and segregation of secretory proteins across the endoplasmic reticulum membrane is the most studied example of transmembrane movement of proteins. Work by Palade and his coworkers [8,58–60] established the role of the ribosomes bound to the endoplasmic reticulum in the synthesis of secretory proteins. Blobel and Sabatini [61] proposed that nascent polypeptide chains of secretory proteins shared some common feature which allows them to interact with the membrane during their synthesis on membrane-bound ribosomes. Later, Milstein et al. [62] demonstrated that the cell-free translation product of the mRNA for immunoglobulin light chain was larger than the authentic isolated protein. These authors proposed, independently of Blobel and Dobberstein [63,64], that an N-terminal supplementary sequence could account for the observed discrepancy in size between the cell-free product and the mature protein and could play a signaling role for its segregation (Fig. 4).

Similar experiments in several laboratories confirmed the existence of the in vitro precursor for immunoglobulin light chain, and its partial sequencing demonstrated that the extension was indeed at the amino terminus of the chain and was rich in hydrophobic amino acids. These facts led Blobel and Dobberstein [64] to propose a completed scheme, the 'signal hypothesis', to account for the segregation of secretory proteins across the reticulum membrane.

In brief, the essential feature of the signal hypothesis is the occurrence of a sequence of codons, located to the right of the initiation codon, which is characteristic of the mRNA, of which the translation products are to be transferred across a membrane. Translation of this sequence yields a unique sequence of amino acids, 16–30 residues in length,

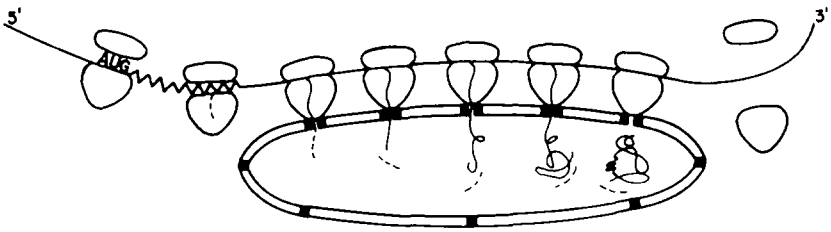


Fig. 4. Illustration of the essential features of the signal hypothesis for the transfer of proteins across membranes. Signal codons after the initiation codon, AUG, are indicated by a zig-zag region in the mRNA. The signal-sequence region of the nascent chain is indicated by a dashed line. Endoproteolytic removal of the signal sequence before chain completion is indicated by the presence of signal peptides (indicated by short dashed lines) within the intracisternal space. From Blobel and Dobberstein [63].

on the amino terminal end of the nascent chain, which is called the signal peptide or signal sequence. This signal peptide has the function of promoting the attachment of the free ribosome to the membrane. Upon its emergence from the large subunit of ribosomes, it binds to specific receptors on the membrane and penetrates into it. This causes association of ribosomes' receptor proteins with the membrane, thereby creating a tunnel and permitting the passage of the signal peptide and the following chain. The signal peptide is proteolytically removed by a signal peptidase before the polypeptide chain is completed. After release of the nascent chain into the transmembrane space, the ribosome is detached from the membrane and the tunnel is eliminated.

The two main characteristics of this signal-sequence hypothesis are that the information for the segregation of a protein is encoded in the mRNA itself and is not in the protein-synthesizing machinery, and that the incorporation of a protein into a membrane is a strictly cotranslational process (vectorial translation).

The validity of the signal hypothesis has been broadly demonstrated for several eukaryotic secretory proteins as well as for a number of bacterial periplasmic proteins (see Refs. 48 and 65–67). It has also been shown that some of the integral membrane proteins located on the extracytoplasmic side of the membrane are inserted according to the scheme of the signal hypothesis [68–70]. In addition, a thermodynamic treatment of this hypothesis was performed recently [71].

Due to the rapid expansion of the literature in this precise area, we shall not present an extensive coverage but rather focus on the more uncertain or contradictory elements published to date. We will discuss the indispensability and hydrophobicity of the signal sequences, the mode of action of the signal peptidase, the ribosome-membrane interaction, some more recent results obtained by genetic techniques and some modifications of the hypothesis introduced to account for recent results contradicting its statements.

VI.A. Is an N-terminal peptide extension obligatory?

A much discussed exception to the signal-hypothesis mechanism is ovalbumin synthesized by chicken oviduct cells. Ovalbumin is synthesized in its final and mature form and cotranslationally segregated into reticulum membrane [72]. However, experiments on cell-free translocation in the presence of microsomal membranes showed that ovalbumin can effectively compete for segregation with other presecretory proteins [73]. The conclusion drawn for these experiments was that ovalbumin posesses a functional equivalent of a signal sequence, although its N-terminal sequence has no particular hydropho-

bicity. This apparent contradiction was recently resolved when it was shown that a signal-equivalent peptide can be proteolytically released from ovalbumin and that this sequence, which contains all the competing activity, is located more than 200 residues into the polypeptide chain [74,75]. More recently, other translocated proteins have been shown to be synthesized without the occurrence of a larger precursor form. These are the envelope glycoprotein PE_2 of Sindbis virus [76], *E. coli* outer and inner membrane proteins encoded by F sex factor [77], and rat liver cytochrome *P*-450 [78]. These exceptions clearly point out that proteolytic processing is not essential for incorporation of some proteins into membranes or their secretion.

VIB. Hydrophobicity of the signal sequence

The only common feature of several N-terminal signal peptides that have been sequenced is a generally marked hydrophobicity. Exclusively hydrophobic regions of 10–12 amino acids are to be found within the central portion of the sequences, but no homology in their primary structure is discernible between the sequenced signal-peptides [48,79]. Therefore, the mode of action of the signal peptidase, which cleaves off the signal peptide during segregation, is difficult to imagine. Furthermore, a number of these precursors have been shown to be cleaved accurately during in vitro experiments in media supplemented with microsomal membranes of various origins from several species [80, 81]. It was also concluded from these experiments that the signal peptidase activity is latent in the rough endoplasmic reticulum. Recently, it was demonstrated that this activity is a neutral endopeptidase and may be a metalloprotein [74]. Its mode of action is still unclear but it is apparent that the cleavage mechanism, as well as the membrane insertion process, does not recognize the primary structure alone. It has been suggested that a common three-dimensional folding of the sequences is responsible for the recognition by the membrane apparatus [79,81].

VIC. Ribosome-membrane interactions

A recent survey of this question is to be found in the review of Shore and Tata [82]. Recent developments have characterized integral glycoproteins of rat liver microsomal membranes which have ribosomal binding ability. The participation of these ribophorins in the interaction between the nascent polypeptide chain with its signal sequence, ribosome and membrane was proposed [83]. Another approach was an in vitro reconstitution of the translocation activity from tryptic extract of canine pancreas microsomes [84]. In this case, the translocation activity was found to be represented by transmembrane proteins possessing two distinct domains: a cytosol-exposed domain which may be the recognition site and a membrane integrated area, which may interact with the nascent chain during its passage across the lipid bilayer. In contradiction with these data, smooth microsomes of various origins, which are devoid of ribophorins, have been shown in vitro to be able to perform the proteolytic cleavage of the signal peptide of placental lactogen hormone as well as the glycosylation of a subunit of chorionic gonadotropin [85]. These results indicate that the necessity for the completion of transmembrane segregation of secretory proteins of ribophorins strictly sequestered in one membrane compartment is questionable.

VII). Genetic aspects of the signal hypothesis

An impressive result on the secretion of ovalbumin has been obtained by recombinant DNA methods [86]. Chicken ovalbumin structural gene has been fused to *E. coli* lac control regions in a plasmid. *E. coli* cells containing the plasmid synthesized and excreted a protein identified as ovalbumin. These results confirm that common features exist in the secretion recognition apparatus found in widely differing species. A confirmation of the principal tenet of the signal hypothesis, i.e., that the information for the segregation of a protein is encoded in the gene itself, has been the isolation and partial characterization of *E. coli* mutants accumulating the precursor of secreted proteins in the cytoplasm. Such mutations affected the secretion of the maltose binding protein [87] and the lambda phage receptor [88]. In the latter case, mutations were shown to be located very early in the gene, in a region corresponding to the NH_2-terminus of the protein. One of the mutations is a deletion, yielding a cytosolic protein of lower molecular weight than the mature receptor, another is a point mutation which yields to the accumulation in the cytosol of a protein of higher molecular weight than the mature protein. Although these results are consistent with the signal hypothesis for transport of bacterial outer membrane proteins, the precise localisation of the mutations into a transient N-terminal signal sequence has not yet been established. On the other hand, a point mutation in the signal sequence of the lipoprotein precursor of *E. coli* is able to prevent its cleavage, but not its insertion. The protein is incorporated in the outer membrane but with some differences: other post-translational modifications are affected and part of the secreted protein remains associated with the inner membrane [89]. These data tend to confirm that the proteolytic cleavage of a larger precursor is not essential for the translocation of some proteins (modified prolipoprotein, ovalbumin, cytochrome *P*-450).

More contradictory results have been obtained using gene fusion techniques to assemble an NH_2-terminal sequence of the product of lam β gene (an outer membrane protein) and a major portion of the COOH-terminal sequence of a cytosolic protein, β-galactosidase [90]. The fused strains produce a hybrid protein showing β-galactosidase activity. The hybrid protein is excreted towards the outer membrane only when 2/3 of the lam β gene protein is present. These results confirm that the information necessary to direct the lam β protein to an outer membrane location is localized within the lam β gene itself. However, it appears that more information than a simple N-terminal sequence is needed to ensure excretion of such hybrid protein.

VIII. Other models for translocation of secretory and membrane proteins

A somewhat different model has been proposed by DiRienzo and coworkers [66], based on the precursor structure of the prolipoprotein of *E. coli*. This model proposes that the hydrophobic signal sequence of prolipoprotein (and other outer membrane proteins) assumes an inverted transmembrane orientation and that the entering nascent polypeptide chain forms a peptide loop across the membrane rather than penetrating through a membrane pore (Fig. 5). Such a loop mechanism could depend more on secondary or tertiary structure and could explain the observed lack of specificity of both binding and processing of proteins of various origins. Recent results (see Ref. 74), showing that some eukaryotic signal sequences remain outside microsomal membranes in in vitro segregation experiments, tend to support the validity of such a loop mechanism. This mechanism could also explain the insertion of ovalbumin by an internal signal-sequence equivalent

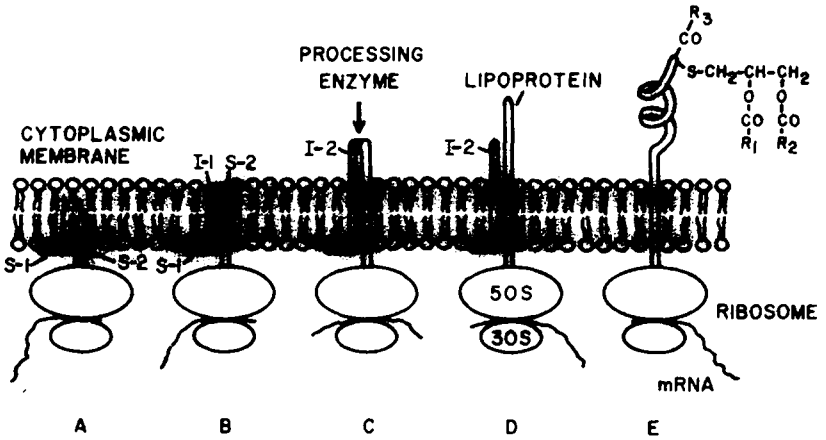


Fig. 5. Proposed mechanism of translocation of the prolipoprotein across the cytoplasmic membrane. Sections of the extended signal peptide region of the prolipoprotein that have a specific function during translocaton are designated S-1, I-1, S-2 and I-2. From DiRienzo et al. [66].

and the incorporation of polytopic integral membrane proteins such as bacteriorhodopsin [74]. Blobel and collaborators [48] have presented a revised version of the signal hypothesis to account for some of the recent experimental data in the field. This broader conceptual framework integrates both cotranslational (vectorial translation) and post-translational (vectorial processing) translocation of proteins. It is proposed that the term, signal sequence, defines a segment of the polypeptide chain, cleaved or not, which induces the translocation of the protein across a membrane via its interaction with a specific translocator. These signal sequences are specific for receptors in different types of membrane such as the endoplasmic reticulum, the inner mitochondrial membrane or the plasma membrane of prokaryotic cells. The signal sequences could be viewed as addresses indicating into which membrane a protein is due to insert. By analogy, the existence of stop-transfer sequences for integral membrane proteins is postulated, these serving to interrupt the translocation, thus embedding the protein in the lipid bilayer. The addition of hypothetical sorting sequences permitted Blobel [91] to propose a general scheme for membrane topogenesis, where the necessary topological information which determines the final localization of many proteins is contained in a few specific sequences which are part of the protein.

A somewhat different model has been presented by Wickner [92]. This model, termed the membrane trigger hypothesis, emphasizes the importance of induced folding of a polypeptide into a conformation highly favorable for its insertion into a membrane, rather than calling for a peptide transport system and specific ribosome-membrane interactions mediated by a signal sequence. In this model, the role of the signal sequence is to alter the folding pathway, whether during or after protein synthesis, the driving force for the insertion of the protein being of a self-assembly type. This model is the most satisfactory for such membrane proteins as erythrocyte band 3 glycoprotein, which is inserted with its N-terminus exposed to the cytoplasm and its C-terminus on the exterior of the cell or bacteriorhodopsin, a polytopic integral membrane protein, which spans the membrane seven times. This stimulating hypothesis stresses the importance of membrane-triggered conformational changes in the migrating protein and is quite close to the view

expressed by ourselves from a different conceptual and experimental approach [15–18].

Finally, it should be mentioned that very little has been published on the influence of the physical state of the membrane on the segregation or insertion of proteins. However, a recent paper dealing with the dependence of the assembly of outer membrane proteins of *E. coli* upon lipid fluidity has demonstrated variable behavior of different proteins [83]. The three major outer membrane proteins were found to be synthesized and assembled by different mechanisms on which the fluidity state of the membrane had different effects.

As a conclusion to the first chapters, it should be stressed that no unique mechanism can, to date, describe all the transmembrane movements of proteins. Furthermore, many different mechanisms seem to coexist in the same cell: cotranslational with or without cleavage of a signal sequence and post-translational (for example, in a hepatocyte prepro-albumin, cytochrome *P*-450 and mitochondrial proteins).

Nevertheless, the conformational and organizational modifications of both the translocating protein and the membrane components are still to be elucidated.

VII. Shedding of immunoglobulins

Protein translocation through biological membranes occurs, inter alia, during protein turnover of the outer surface of membranes and in shedding phenomena. In a more general way, shedding of surface protein is a phenomenon integrated with intracellular exchanges modulating different metabolic steps. The mere fact that shed protein exogenously added to cell cultures stimulates cell growth illustrates this integration [94]. In this chapter, we will only refer to the shedding of immunoglobulins (IgM) in B-lymphocytes as an example of protein transmembrane translocation in a complex integrated system.

Immunoglobulins are known to exist in different molecular forms, variously localized in the diverse compartments of B lymphocytes, thus:

(a) an intracellular pool of IgM, of which the molecular size is 8 S;

(b) a pool of membrane-bound 8 S IgM which corresponds to the shed molecules;

(c) a pool of 19 S IgM, which is the pentameric form of the 8 S molecule and corresponds to the massively secreted form after differentiation of the B lymphocyte into the 'plasma cell'. Whereas very little detectable Golgi structure is to be found in resting lymphocytes, it develops in these cells after induction by mutagenic agents and by differentiation [95,96].

This has led to the proposal for the secretion of the 19 S immunoglobulins by a pathway analogous to that of digestive enzymes in pancreatic cells (see next chapter) [97].

The situation is, however, quite different for the shedding of 8 S IgM, and raises the following questions: (a) how are the surface immunoglobulins synthesized and transported? (b) how are they bound and released from the membrane?

VIIA. Synthesis and transport of 8 S IgM

Different possible schemes for the synthesis and transport of surface immunoglobulins have been proposed.

The 8 S immunoglobulins, with their carbohydrate moiety already attached, are found first in the cytoplasmic pool. The latency time between the beginning of their synthesis and their appearance on the outer surface of the membrane is about 1 h. To account for

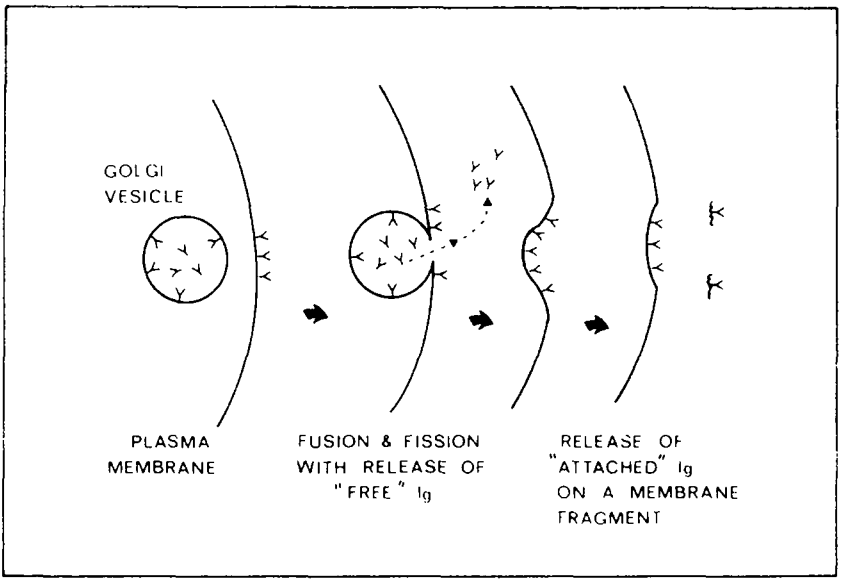


Fig. 6. Model for intracellular transport of 'free' and cell surface Ig. From Vitetta and Uhr [98].

these transport steps a number of hypotheses have been put forward.

(1) Synthesis and transport would follow a parallel route to that of the 19 S IgM and it would thus be secreted by exocytosis. The essential difference being that the monomer (8 S) would remain bound to the post-Golgian vesicles, whereas this is not the case for the 19 S pentameric form (Fig. 6) [98]. The fact that almost no Golgi structure is to be found in resting B lymphocytes would be explained by the presence of relatively few surface 8 S immunoglobulins compared to the massive 19 S IgM secretion; thus, a presence supported by a very reduced reticular structure. The existence of two different pathways for 8 S and 19 S IgM synthesis and transport is, however, suggested by different turnover of these immunoglobulins after induction and differentiation of B lymphocytes. Following induction, surface immunoglobulins disappear and then reappear at higher concentrations. In parallel, an increase in their turnover is observed.

(2) Synthesis would take place on free ribosomes and the translocation would occur directly starting with a possible precursor molecule, thus following a different pathway from that of exocytosis.

(3) A third hypothesis proposes direct secretion through the plasma membrane via attached ribosomes, following the vectorial secretion pathway described above. However, this hypothesis is in contradiction with the observation that 8 S immunoglobulins exist in a free form in the cytosol [99].

VIIB. Fixation of 8 S IgM on the membrane surface

Whereas the 19 S IgM is a very soluble molecular form, bound 8 S IgM appears to be difficult to remove from the membrane. And this despite the fact that the composition of these two forms is very comparable, if not identical, with respect to the very close composition of their carbohydrate moieties and their immunological behavior [100]. Criteria of accessibility to antibodies for the two immunoglobulins show that the fixation of the

8 S IgM to the membrane occurs at the Fcμ region which also contains the carbohydrate [101,102]. According to the usual classification of membrane proteins, bound 8 S IgM appears as an intrinsic protein [103,104].

VIIC. Mechanisms of 8 S IgM shedding

The amphiphilic nature of the 8 S IgM is clearly described by the fact that on the one hand this molecule is firmly bound to the membrane, but that on the other hand it possesses hydrophilic characteristics due to the presence of its carbohydrate moiety. The release of 8 S IgM thus raises the question of what happens to the hydrophobic part, which must disappear from its surface and sink into the more hydrophobic parts of the 8 S IgM entity, leading to a profound reorganization of the whole molecular structure. This supposes that 8 S immunoglobulins are shed intact in the extracellular medium either by pinching off microvilli or by another mechanism involving the interaction between membranes and IgM. Another hypothesis is that immunoglobulins are shed by the action of a protease. This is favored by the fact that in tumor cells, shedding is increased and so is protease activity. This is also illustrated by the increased shedding through slight trypsinization of normal cells. The above hypothesis, however, does not account for the existence on the membrane surface of specific interaction sites for IgM and more particularly for 8 S IgM [105,106].

VIID. Shedding of antigen-antibody complexes

When antigen-antibody complexes occur at the membrane surface, two pathways account for their elimination, endocytosis (about 90%) and shedding (about 10%). Both of these pathways will provoke and enhance the immune response, the first by direct stimulation of B lymphocytes, the second via the fixation of the released complex on the macrophages, thereby facilitating further interaction between B and T cells [107].

Which is thus the shedding mechanism? It is possible that the fixation of the antibody on the IgM induces an allosteric modification leading to a lesser affinity of the complex for the membrane and its subsequent shedding [108]. A similar mechanism might be implied in the shedding of surface 8 S IgM alone, but here the slow release could find its origin in both the fluctuations of the IgM structure and the modifications inherent to the association between IgM and the surface. In any case, this phenomenon requires both protein transmembrane movement and the oscillation between an intrinsic form (thus hydrophobic) to a soluble form (thus hydrophilic) of this protein.

VIII. Externalization-internalization of proteins via the exocytotic-endocytotic cycle

The functional analysis of the pancreatic exocrine cell induced Palade [8] to propose a multi-step scheme for the secretory process (Fig. 7). Anatomical and biochemical approaches have been instrumental in the formulation of similar processes for a large number of secretory systems [9,10]. The general pathway is that secretory proteins are sequestered in various intracellular compartments through which they travel from the rough endoplasmic reticulum to the plasma membranes. In these compartments, the secretory proteins are discharged and the membranes of the secretory vesicles become incorporated in the plasma membrane. To account for the constant dimensions of a secretory cell, a coupled mechanism exocytosis-endocytosis has been proposed (for recent

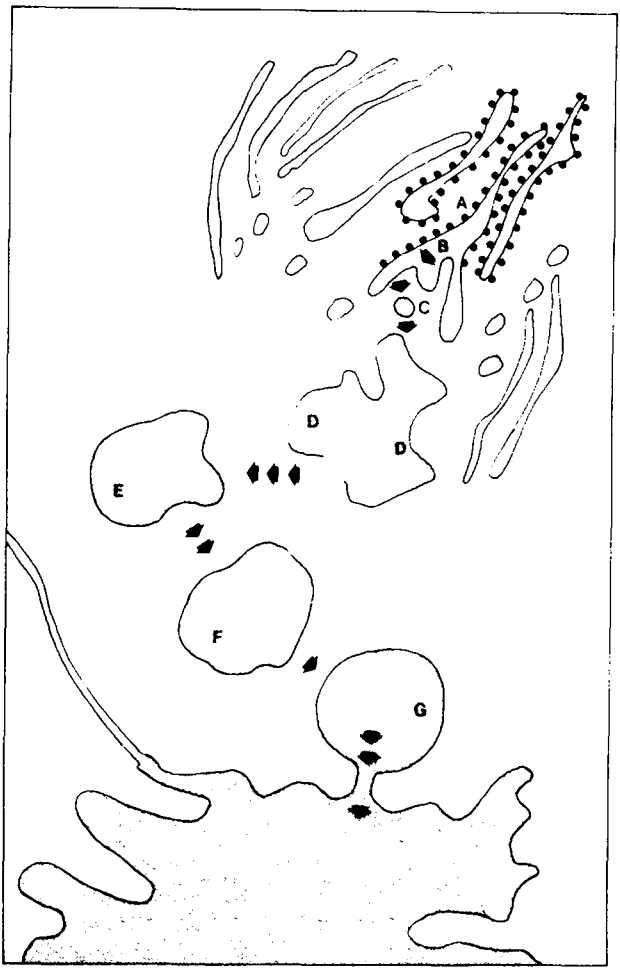


Fig. 7. Schematic representation of the pancreatic acinar cell. The postulated sequence of events from synthesis to discharge of secretory proteins is indicated by the numbers and arrows. (A) Synthesis on the bound ribosomes of the rough endoplasmic reticulum and discharge into the cisternal space. (B) Intermediary aggregation as 'intercisternal granules' which is observed only under particular conditions of starvation and refeeding. (C) Transport via small smooth-surface 'Golgi vesicles' which bud from the transitional elements of the rough endoplasmic reticulum. (D) Accumulation within condensing vacuoles. (E) Condensation within condensing vacuoles. (F) Storage as mature zymogen granules. (G) Discharge to the acinar lumen. Inspired by Tartakoff et al. [60].

reviews see Refs. 109 and 110). However, endocytosis also refers to the process by which cells take up, under given conditions, macromolecules from extracellular fluid. The fate of endocytotic vacuoles follows different pathways, as most incoming vesicles can fuse with pre-existing membrane-bound granules (lysosomes). In some instances, vacuoles may traverse the cell and discharge their content by exocytosis or finally the vacuoles could remain intracellular without fusing with lysosomes. In brief, endocytosis is thought to bring about internalization of considerable amounts of proteins and/or selected portions of the cell surface.

VIIIA. Protein externalization by exocytosis

Views on the secretory processes have been strongly conditioned by information on gland cells. The various steps leading to secretion stem principally from the functional analysis of the pancreatic exocrine cell [8]. In general, the secretory proteins are sequestered within discontinuous membrane structures, passing successively from the rough endoplasmic reticulum, to the Golgi region, to condensing vacuoles and to zymogen granules where they are stored before discharge into the intracellular space. Six successive functional steps were recognized and associated with protein transfer: synthesis, segregation and intracellular transport, concentration, intracellular storage and discharge. In contrast to this orthodox view of intracellular transport, it has also been proposed that some or all the secretory proteins could be temporally and functionally located in the cytosol [111].

VIIIB. Synthesis, segregation and intracellular transport

There is much evidence suggesting that secretory proteins are synthesized on the ribosomes of the rough endoplasmic reticulum and enter the membrane-delimited cavities of the cisternae of the endoplasmic reticulum [112,113]. In the case of pancreatic secretory proteins, their segregation was shown to occur via a metabolically short-lived signal sequence in the nascent polypeptide chain [114] (see Section VI). In the internal space of endoplasmic reticulum, a series of co- or post-translational modifications (removal of the signal sequence, glycosylation, hydroxylation of amino acid residues [62,115,116]) will take place before the proteins move into the region of the Golgi apparatus. It is generally admitted that the segregated protein molecules which enter the lumen or are still membrane bound (specially glycoproteins) are incapable of diffusion back across the membrane. This distribution of the secretory proteins within the endoplasmic reticulum system is, with few exceptions [117–119], probably homogeneous.

From the endoplasmic reticulum, the proteins will be transported into the region of the Golgi complex. Two general hypotheses have been formulated to account for this transport. In the guinea-pig pancreas [8,120], the results have been interpreted in terms of a physical discontinuity of the two apparatuses. The functional connection is then thought to be assured by endoplasmic reticulum-derived vesicles that unload by 'shuttling' their content (secretory proteins) into the cavities of the Golgi complex. It has been supposed that fusion-fission of the membrane of the shuttling vesicles is the energy-dependent process in the transport of secretory proteins. An alternative possibility has emerged from the hepatocyte system. It favors a more direct transport route involving direct lumenal continuity between the endoplasmic reticulum internal and the Golgi sacs [121].

The two transport hypotheses are similar in that both envisage the continuous maintenance of the secretory macromolecules within membrane-delineated compartments. On the other hand, kinetic observations reported by Rothman [122], McGregor et al. [123], Slaby and Bryan [124] are inconsistent with the general lines of this more classical view, in that it has been found that small amounts of labelled proteins are secreted a few minutes after the addition of radioactive amino acids. This transport time is incompatible with segregation and storage within granules. The results can best be explained by postulating the existence of an independent transport pathway by-passing the vesicular storage route.

In spite of the large number of reports concerning the mechanism regulating intracellular transport and the possible driving forces involved, the knowledge of these processes is still limited and contradictory [58,59,125]. In this respect, the role played by microtubules and microfilament systems is obscure. Much of the information is derived from the use of drugs that have a wide spectrum of effects. Thus, the consequence of transport of secretory proteins is not a specific consequence of the structural depolymerization of the cytoskeleton, but perhaps a more general disturbance of cytoplasmic membrane interactions [126–128].

VIIIC. Concentration, intracellular storage, discharge

It is generally accepted that in the region of the Golgi apparatus, secretory molecules are concentrated, packed and redistributed into discrete organelles. In the process of concentration, the segregated proteins will undergo molecular modification. Among these modifications, attachment of sugar residues to glycoproteins, the synthesis and sulfatation of carbohydrate chains of protidoglycans [129–131] and the processing of some proteins, of which the size must be reduced by controlled cleavage before release from the cell, have all been shown to occur [65]. A further metabolic event that occurs in the Golgi complex is ion accumulation. It is known that Zn^{2+} or Ca^{2+} can accumulate in much higher amounts in Golgi-enriched fractions than in mitochondria or in microsomes. These observations are important because they show that the ionic content of the Golgi complex is different from the ionic content of the endoplasmic reticulum [132,133]. There is evidence that protein and polysaccharides could form complexes through ionic association (see Refs. 10 and 134). In some cases, a primary role might be played by organic polyanions or by Ca^{2+}, either alone or in conjunction with nucleotides and acidic proteins [135,136].

In parallel with these biochemical events, the Golgi complex is thought to be involved in the redistribution of the segregated proteins derived from the endoplasmic reticulum. Although it has been suggested that redistribution of secretory proteins by the Golgi complex is a general phenomenon in secretory systems, some intriguing questions remain still unsolved. For instance, in the exocrine pancreas, using immunochemical techniques, a homogeneous distribution of zymogens has been shown without qualitative regional differences within the gland, in each acinar cell or granule of the Golgi elements [134]. On the contrary, cytochemical results indicate that lysosomal enzymes appear in immature secretion granules, but in mature organelles they are no longer visible [137]. These two types of data might suggest that secretory proteins in the ionic environment of the Golgi complex interact with each other to form supramolecular complexes that would then be segregated in different organelles through fusion-fission of the limiting membrane. In contrast, in many other cases like the parotid gland, it has been suggested that exportable proteins leave the endoplasmic reticulum already enveloped by the secretory granule membrane [138,139].

In addition, Rothman and Isenman [140] reported numerous examples of non-parallel or selective zymogen secretion, that cannot be explained solely on the basis of exocytotic protein secretion especially if one takes into account that zymogen granules are of mixed enzyme composition. In this respect, experiments have been reported that describe the ability of single cells to carry out 'multiple vesicular secretions' concomitantly, suggesting that this could be a frequently occurring phenomenon [141–144]. Thus, the processes of redistribution, intracellular storage and granule formation of secretion products show a

large variability according to the different cytological systems.

The final event is the discharge of secretory proteins towards the extracellular environment. This secretory material is prepared in the cytoplasm, within membrane-limited organelles. In terms of the classical theory, discharge will occur by exocytosis, following fusion-fission of the limiting membrane of granules or vesicles with the plasmalemma. The final results of fusion-fission will be the establishment of continuity between two lipid bilayers. The original evidence for exocytosis arose from a combination of biochemical and microscopic studies of the pancreatic secretory cells. More recently, these studies have been refined by a detailed analysis of the various membranes participating in the discharge of the secretion products including their chemical composition, structure, biogenesis and turnover (for recent reviews, see Refs. 110 and 145).

In considering the localization of exocytosis at the cell surface, a wide spectrum of situations can be recognized ranging from the occurrence of exocytosis all over the cell surface to the limited discharge of specific portions of the plasmalemma. In some cells, however, different phenomena existing either in the discharge of the granule content within lysosomes or within granules previously discharged have also been described (see Refs. 110 and 145).

In contrast, kinetic studies of product discharge and intracellular distribution of secretory products have led to the proposal of alternative models of secretion. In the exocrine pancreas, for instance, secretion has been reported to continue after the complete disappearance of granules. This observation together with cell fractionation studies led Rothman [111] to propose the selective permeability of the granule and plasma membrane to zymogens ranging in molecular weight between 13 000 and 70 000.

VIII.D. Origin of cellular membranes in the secretion processes

A simple and attractive concept to explain the biogenesis of cellular membranes is that they accompany secretory products from the endoplasmic reticulum through the plasmalemma [146]. However, this simple scheme is inconsistent with the fact that the four membrane systems involved in transport and discharge of secretion products possess unique biochemical structural features and composition, and that the rates of membrane biogenesis and intracellular transport processes are of a different order of magnitude [147].

An alternative explanation of this hypothesis is that the Golgi complex and various other internal cellular membranes should be regarded as examples of transitory structures. During transit, the membrane will be modified by addition and elimination including molecular modification. This mechanism is termed as flow hypothesis (for review, see report by Morré et al. [148]).

A final mechanism for the biogenesis of membrane could involve direct insertion of proteins synthesized on free or particulate ribosomes into the appropriate membrane (for a detailed discussion, see Sections V and VI).

IX. Protein internalization by endocytosis

Exocytosis and endocytosis are two complementary mechanisms that have to be examined as an entity if one is going to approach and integrate protein externalization and internalization phenomena. We shall examine here pinocytosis, the intention being to follow the diverse mechanisms involved in protein internalization and subsequent externalization.

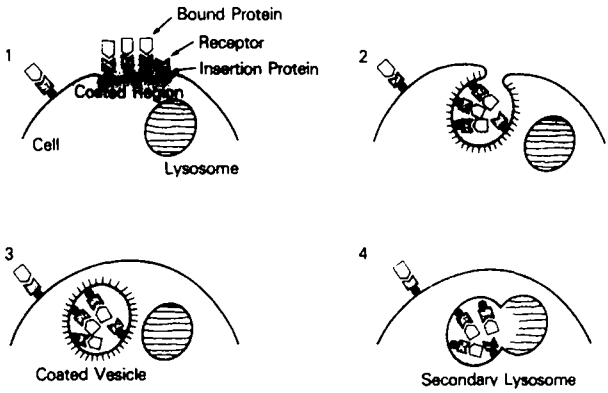


Fig. 8. In this model, receptors are clustered at specialized small areas of the surface membrane known as coated regions which are predestined to become sites for pinocytosis. Clustering allows for a high ratio of receptor-mediated uptake to membrane internalized uptake. As shown here, clustering is promoted by a second insertion protein or a second specialized region on a single polypeptide chain receptor (solid circles) which has a high affinity for the coated regions. From Neville and Chang [45].

Four steps stand out in this process (Fig. 8):
(1) the recognition and fixation of the molecule to the membrane,
(2) the formation of a vesicle,
(3) the fate of that vesicle,
(4) the recycling of the membrane proteins.

IXA. Recognition and fixation of the molecule to the membrane

Fluid phase pinocytosis does not seem to require preliminary recognition of the molecule prior to its endocytosis by the membrane. Thus, any molecule present in the close neighborhood of the membrane will possibly be internalized. This seems to be the case for horseradish peroxidase which is endocytosed without any previous fixation to the membrane (see Ref. 109). The situation is different in the case of adsorptive pinocytosis. The existence of fixation sites whether specific or non-specific seems to be a prerequisite. But whereas the internalization of a bound molecule can be fortuitous, in cells endocytosing continuously and at a constant rate, the binding of a molecule can also strongly stimulate endocytosis. The valency of the bound molecule also seems to play a role in the rate of its endocytosis. Bivalent or multivalent concanavalin A increases pinocytosis by macrophages by a factor of 3 [149], whereas monovalent succinyl-concanavalin A does not stimulate it [109]. But in some cases where a receptor seems required, as for the internalization of the low density lipoprotein (LDL), the endocytosis of the receptor occurs even in absence of LDL [150]. There appears to be a broad specificity for endocytosed molecules, although some of them are able to stimulate pinocytosis.

IXB. Formation of vesicles

Formation of vesicles is often preceded by capping of surface proteins, although this appears to be independent and without any correlation with vesicle formation which can occur in its absence (see Ref. 151). In fact, the capping of surface immunoglobulins in murine lymphocytes induced by anti-immunoglobulin antibodies is rapidly followed by

the endocytosis of the ligand-receptor complex [152], whereas a monovalent antibody, such as Fab-anti-immunoglobulin, which does not induce capping, is internalized as well in pinocytotic vesicles [153,154]. When proteins are internalized by receptor-mediated endocytosis [155], clustering occurs before internalization. Firstly, coated pits appear and then endocytosis occurs, apparently via coated vesicles. Clustering of LDL receptors occurs before their binding with LDL [156] whereas in the case of the epidermal growth factor (EGF, another molecule internalized via coated vesicles, clustering occurs after its binding with receptor sites [157]. Haigler et al. [158] observed, however, internalization of ferritin-conjugated EGF via both coated vesicles and via a process involving neither coated pits nor coated vesicles in human A431 carcinoma cells. Very little is known of the mechanisms of membrane fusion involved in the closing process of the vesicle.

The presence of divalent cations seems to be implied in this event. The possibility of lysophosphatides playing some role in this fusion was proposed (see Ref. 109). The phenomenon seems to require energy and appears to be temperature dependent. However, some of the processes involved in the formation of the vesicle are unclear.

IXC. Fate of endocytosed vesicles

The fate of the endocytosed vesicles seems to be extremely variable and depends both on the nature of the internalized molecule and on the cellular membrane carrying out this endocytosis. Many of the steps involved in vesicular segregation are still not well understood. The most usual evolution of the vesicle is the lysosomal degradation of its components. A relatively simple case, which illustrates the fate of molecules internalized by endocytosis, is that of the maternal IgG transferred into the fetal bloodstream, or that of the yolk protein which accumulates in the yolk granules (see, for example, Ref. 159). In both these cases, the proteins are redistributed in another compartment which is extracellular in the first case, and intracellular in the second. Some authors also report the presence of endocytosed molecules in the Golgi region or even in the nucleus. The degradation, via the lysosomal pathway, of these internalized proteins is illustrated by the progressive disappearance of endocytosed enzymatic activities such as horseradish peroxidase or by the appearance of iodinated tyrosine when the proteins were previously iodinated on this amino acid, and finally by the localization of pinocytosed markers by electron microscopy.

Do the endocytosed vesicles fuse directly with the lysosomes, or does a first fusion occur, as a intermediary step, in the Golgi region? Both cases have been observed. Thus, uptake of horseradish peroxidase by macrophages is rapidly followed by degradation of the molecule and seems to follow the first pathway [160]. Lysosomal delivery has been demonstrated directly by electron microscopy after internalization of LDL or epidermal growth factor [150]. On the other hand, the pinocytosis of lysosomal enzymes seems on some occasions to follow the second scheme. After endocytosis, the enzymes appear first in what could be Golgi-endoplasmic reticulum-lysosome-derived vesicles and could then be transferred progressively in the lysosomes until an equilibrium of about 50% is reached in both these compartments [161].

The endocytosis of insulin seems to follow a more complex pathway with most of the internalized hormone being degraded via the lysosomal route [162] and only a fraction of the receptor-bound hormone being delivered to the Golgi apparatus [163] and to the nucleus [164]. Another interesting case is that of the endocytosis of transferrin, where transferrin-loaded vesicles fuse with the lysosomes with transferrin being slowly released

into the cytosol without degradation [165].

It was also possible to show, by following segregation of labelled precursors, that the retrieved organelles of exocrine and endocrine secretory cells gather and accumulate in the Golgi area. Their distribution seems to vary in different organs [110].

After intense chemical stimulation of the adrenal medulla, accumulation of marker molecules is mainly observed in the lysosomes, few being found in the Golgi area. However, after sustained stimulation of endocrine pancreas, the markers appear in both lysosomes and the Golgi area [10]. In the cholinergic system, the vesicles seem to be re-utilized without returning towards the perikaryon, but the route followed is still unclear. According to Heuser and Reese [166], they fuse first with the large cisternae present in the nerve terminals from which new synaptic vesicles could originate by budding. According to Cecarelli et al. [167], the uncoated vesicles would be derived directly from the presynaptic membrane and might be available for re-use in transmitter discharge. Some authors observe that some of the retrieved vesicles fuse into multivesicular bodies which are then transported to the perikaryon [168,169]. There is thus a great diversity in the fate of endocytotic vesicles, since it varies with the nature of the molecule to be internalized and with the cellular membrane involved in the process of endocytosis.

IXD. Recycling of the membrane proteins

There is little doubt as to the re-utilization of the endocytosed membrane by the cell. However, the refilling mechanism and its localization in exocrine and endocrine glands vary with the cell type and are still hypothetical. The re-utilization and refilling seem to occur by fusion, either with the Golgi membrane or with membranes of the granule type present in the Golgi area [10]. In adrenergic neurones, a part at least of the internalized granule membranes is transported towards the perikaryon by the rapid retrograde system, as suggested by the parallel transport of dopamine β-hydroxylase [170]. On the contrary, re-utilization of the cholinergic membranes does not seem to require a complex intracellular processing and seems to refill at the nerve-ending level, as proteins do not seem to be associated with the products of cholinergic secretion. A parallel case which illustrates well the ambiguity existing in this field is given by the cholinergic discharge of acetylcholine, where it is not yet clear if the vesicular pathway is a prerequisite. Thus, Dunant and Israël [171] have shown the existence in nerve terminal of two compartments for acetylcholine, a bound one associated with vesicles and a free one. During stimulation, it seems that the second population is utilized, the first one being only utilized in case of prolonged non-physiological stimulation.

In the receptor-mediated endocytosis of LDL, this receptor does not seem to be hydrolysed while in transit in the lysosomes and could thus be re-utilized at the plasma membrane level [172]. Because protein synthesis could not account for this major internalization of LDL, the situation is analogous to the internalization of transcobalamin II [173] and asialoglycoprotein receptor [174]. Nevertheless, in the case of the internalization of EGF, a decrease of 80% in the involved plasma membrane receptors is observed, the cell being only able to internalize EGF at a rate of 20% of the initial rate. This could be explained by a corresponding degradation of the initial receptor [175].

The above results clearly suggest the necessity of vesicle recycling. In receptor-mediated endocytosis, results are still too contradictory to allow for any generalizations. In concluding this chapter, it is of interest to stress some of the problems raised by endocytosis:

(1) do the marker molecules used to follow vesicular displacement remain associated with the vesicular structure in the course of this displacement? For instance, in the case of fibroblasts the vesicle content, which includes the extracellular tracers, is released within lysosomes, while the vesicle membrane is able to separate from the lysosomes and is probably recycled back to the plasmalemma [110];

(2) in most of the marker-tracer experiments, results were obtained by overstimulation of the cellular material. The physiological relevance of such observations should be queried [110];

(3) in the case of receptor-mediated endocytosis, are proteins with different destinations endocytosed in the same vesicles? If affirmative, then subsequent resegregation mechanisms are still in doubt [155].

X. Discussion

XA. The membrane model

The question of protein externalization from and internalization into membrane systems suffers from a fundamental dichotomy of principles. On the one hand, there exists a set of biological events dependent on protein transmembrane movements such as renewal of proteins in membranes and organelles and shedding of IgM. On the other hand, a conceptual barrier is against such protein translocation. Is it possible to reconcile these apparently opposing aspects?

Here, it is important to stress that most of the available information on biological membrane dynamics stems from the study of intrinsic dynamics of the membrane usually in a steady state. When compared to the wealth of information derived from the statistical analysis of membranes, there exist little data on dynamic behavior induced by changing environment. Yet it is obvious that by 'freezing' the parameters bound to the environment, one can only gather dynamic information expressed by the constituents of the membrane in a steady-state equilibrium. This, of course, does not add to our understanding of membrane dynamics with respect to those aspects bound to the continuous interaction between the membrane and its surroundings.

XA-1. Lipids

The dynamic element introduced by the lipids alone could first be described by a predominant, but not unique, contribution to an asymmetrical bilayer structure wherein the lipids are endowed with lateral diffusion in both layers. Such diffusion is characterized by a high speed of the order of 10^{-8} $cm^2 \cdot s^{-1}$ [176–178]. On this must be superimposed a *trans-gauche* conversion by a 120° rapid rotation of the carbon-carbon bond. The average life-time of these conformations is of the order of 10^{-6} s [179,180]. On the other hand, the transmembrane exchange of lipids (flip-flop) in a resting membrane seems generally to be a very slow phenomenon with a life-time of hours at 37°C [181–184].

In apparent contradiction to these results is the finding of a rapid asymmetrical translocation of newly synthesized phospholipids from the plasma membrane inner face towards the outer face during membrane assembly [68,185,186]. In the case of the transbilayer movement of lysophosphatidylcholine, in glycophorin-containing vesicles, this movement seems to be protein-mediated [187].

Many constraining elements modify the motional freedom of lipids in biological membranes. A typical example is cholesterol, which inserts itself with its β-OH function in the

phospholipid layer, interacting in the region of the polar heads of the phospholipids. The remainder of the molecule intercalates in a parallel way to the hydrophobic tails of the phospholipids. The effect of cholesterol usually restricts the motional freedom of the lipids, since it has a tendency to condense the average surface area per molecule and it reduces the movement of the hydrocarbon chains in the fluid-lipid bilayer. However, it also has an opposing effect by dissociating the all-*trans* structures in the crystalline areas. Thus, cholesterol has a complex influence on the global membrane fluidity [188,189]. With the exception of the recent observations on the 'movement' of newly synthesized lipids in the membrane, the above description of lipids' behavior in the resting membrane leads to a two-dimensional dynamic model structure.

The influence of cations is ambiguous on this structure [190,191]. From the change in the bulk charge of the polar heads following the interaction with cations, a parallel increase in membrane fluidity due to repulsion of neighboring molecules would be expected. Nevertheless, cations have a complex pattern of interaction with the membrane constituents [192,193].

XA-2. Proteins

The usual working model of a fluid mosaic should not be conceptually modified by the presence of proteins in the membrane. The topographical relationship of proteins to lipids was first defined according to accessibility criteria in the presence or absence of detergents under defined assay conditions. Two extreme types of topographical interaction for proteins have been defined in connection with the hydrophilic, more peripheral, or with the hydrophobic, more integral, contacts with the membrane. These are (a) the peripheral or extrinsic proteins showing only superficial interactions of the electrostatic type, with either the polar heads of the lipids or with the apparent charges of the proteins of the membrane and (b) the integral or intrinsic proteins, embedded in the lipid bilayer of the membrane and showing an amphiphatic type of interaction both with the hydrophobic and the hydrophilic areas of this membrane. Some of these proteins or protein complexes span the whole thickness of the membrane [194–202].

This distinction is, however, very ambiguous and many intermediate situations can be distinguished in relation to the many possible interactions of protein with both hydrophilic and hydrophobic components and as a function of changing exogenous conditions. In general, the presence of protein modifies the fluidity of the membrane. Plasma membrane carbohydrates, whether bound to proteins or to lipids, are external.

In the present two-dimensional view of the membrane, proteins appear somewhat as icebergs floating on an ocean of lipids. However, the protein content of biological membranes can vary from 20% (myelin membrane) to 50% (plasma-type membrane) up to 75% (inner mitochondrial membrane). The following approximate calculation is possible. Taking an average molecular weight of 50 000 for the protein and 600 for the lipids, with respective cross sections of 1256 $Å^2$ (40 Å diameter) and 49 $Å^2$ (7 Å diameter plus diameter occupied by kinks) [203], considering that the lipids are present in a bilayered structure and that the proteins are scattered at random, then 21 'bilipids' (42 lipid molecules) are necessary to encircle each protein molecule. In the case of the mitochondrion, the actual figure is 28 lipid molecules per protein molecule. If one imagines two extreme situations: (a) completely embedded proteins, with a minimal hydrophobic area of contact of 10 Å in diameter (see Fig. 9) and the rest of the protein molecule extruding on both sides of the membrane, the distance between proteins due to the available lipids would be 28 Å, which leads to a tight contact between the extruding parts; and (b) globular

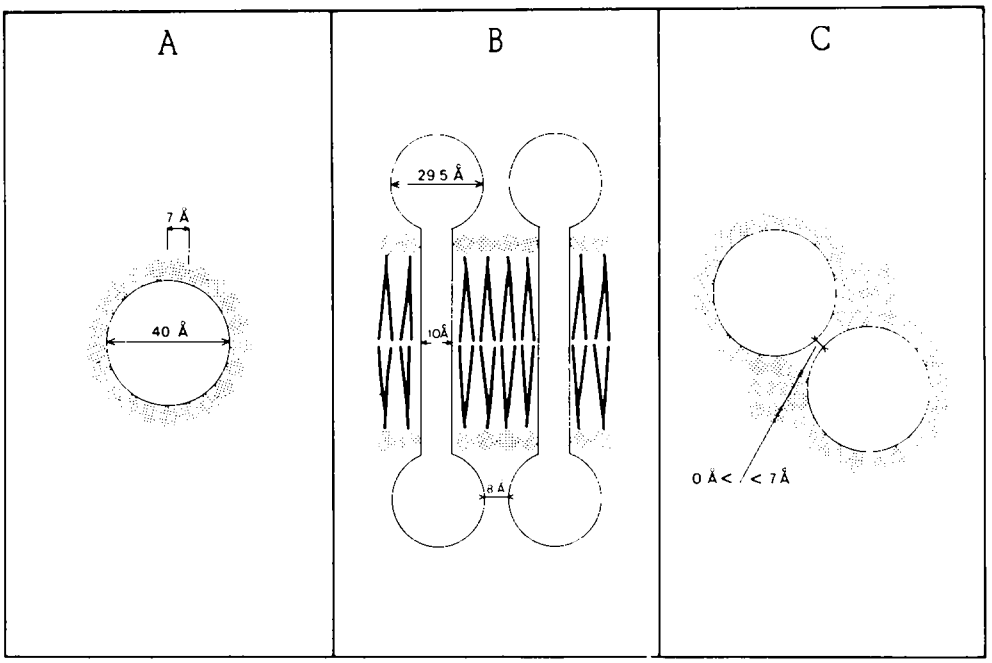


Fig. 9. Schematic representation of protein-protein proximity in mitochondrial membrane as deduced from protein-lipid ratio. A, top view of a protein molecule, 40 Å in diameter, surrounded by 21 lipid heads, 7 Å in diameter. B, lateral view of two protein molecules totally embedded in mitochondrial membrane and having minimal contact area of 10 Å in diameter. Their structure was extrapolated from an initial sphere 40 Å in diameter. Distance between the extruding heads of the protein molecules is about 8 Å, taking into account a ratio of protein to lipids of 1 : 28. C, top view of two protein molecules totally embedded into mitochondrial membrane having maximal contact area. Distance between the two molecules is less than 7 Å, taking into account the same ratio of protein to lipid as an B.

embedded proteins, 40 Å in diameter, the distance between them would be between 0 and 7 Å. Thus, a tight packing of proteins will exist in both cases. For plasma membranes, the ratio of bilipids to proteins is 42, which would lead in the case of the last hypothesis to a distance of 28 Å between proteins 40 Å in diameter and for the myelin membrane that distance becomes 108 Å. Of course, all the proteins belonging to the membrane complex are not intrinsic, since they are not all equally embedded. They are often in aggregate form, for example. Thus, the above figures will fall short of the theoretical values and suggest that for the average biological membrane, the contemporary view of protein icebergs floating on an ocean of lipids is at least inadequate and should be modified, taking into account a high surface concentration of protein relative to the lipids; without, however, excluding the possibility for more specific lateral diffusion for those proteins embedded in lipid-rich areas. Although physiologically and functionally important, lipids do not represent major quantitative constituents in the inner mitochondria membrane. This was demonstrated over a decade ago by Fleischer et al. [205], who showed that lipid-depleted mitochondria still present an electron-microscopically indentifiable structure. Nevertheless, the functional role of specific lipids has been demonstrated in experiments on reconstitution of membranes such as mitochondrial membranes, the cytochrome oxidase system, the insertion of the Ca^{2+} pump in erythrocytes, etc. (see Ref. 204).

XB. Multiplicity of the translocating mechanisms

XB-1. Recognition-translocation step

All the above concepts support the idea of a biological membrane, of which the surface hydrophobic characteristics might be modified, at least locally, by high protein concentrations. Such a membrane will be endowed with a system of reading and recognition, the complexity of which will be related to the multiplicity of the membrane proteins and the many possible arrangements and combinations between its diverse constituents. Owing to the electrostatic and electromagnetic type (van der Waals', London) of interactions that govern these rearrangements, this leads to a considerable flexibility of the system, guided both by the large number of constituents and by their cognitive and self-organizing patterns.

Consequently, one can imagine that a protein or any other molecule or complex structure that approaches the membrane will induce a set of continuously localized modifications. This initiates a recognition or receptor site in the membrane at the available elements. One of the consequences of this 'best-fit-like' interaction might be the translocation of the inducing molecule or complex system. Such a conceptual approach applied to the translocation mechanisms described in this review can be paraphrased as:

(a) all four systems have in common a recognition step, followed by internalization or externalization of proteins;

(b) they differ in the nature of the inducing signal. Firstly, in the vectorial secretion, the inducing recognition process is at the one time a synthesizing ribosome plus the N-terminal hydrophobic sequence of the protein to be translocated. Secondly, in the precursor-induced internalization, it is some precursor form of the protein itself that carries the information for recognition and translocation. Thirdly, in the tetanus toxin type of internalization, it is an associated protein which induces membrane recognition and modification prior to internalization, and finally, in the large-amplitude protein movement and in the equilibration of digestive enzymes, it is some defined change in the environment such as succinate or pH, which is responsible for both membrane reorganization and change in protein configuration necessary for translocation (Fig. 10).

Thus, all four mechanisms prior to translocation require the modification of both the structure of the protein to be translocated and some area of the membrane to recognize or to be recognized. Whether the signal inducing the necessary membrane modification lies within the N-terminal sequence or in some triggered refolding of the protein, a primary sequence of events seems to be required to put both membrane and protein into proper conjunction (ribosomes, N-terminal hydrophobic sequence, associated protein or other environmental factors). However, from our present understanding of these phenomena, none of the proposed mechanisms lead to a clear molecular view of the protein translocation step itself. None of the proposed mechanisms can justify the passage through the currently used membrane model. The most documented observation of the hydrophobic signal sequence, where proteins are translocated with or without the help of ribosomes, can at most account for a specific site of interaction between protein and membrane prior to translocation. The presence of such a hydrophobic segment could be a possible vector to orientate this interaction. However, two questions arise: (a) as assumed by the current membrane model, biological membranes turn their hydrophilic areas towards the water-rich exogenous and endogenous environment. Therefore, how does such an orientation favor a privileged interaction between these hydrophilic-rich areas with a hydrophobic segment of the protein? (b) If such an interaction, nevertheless, does occur (and the evi-

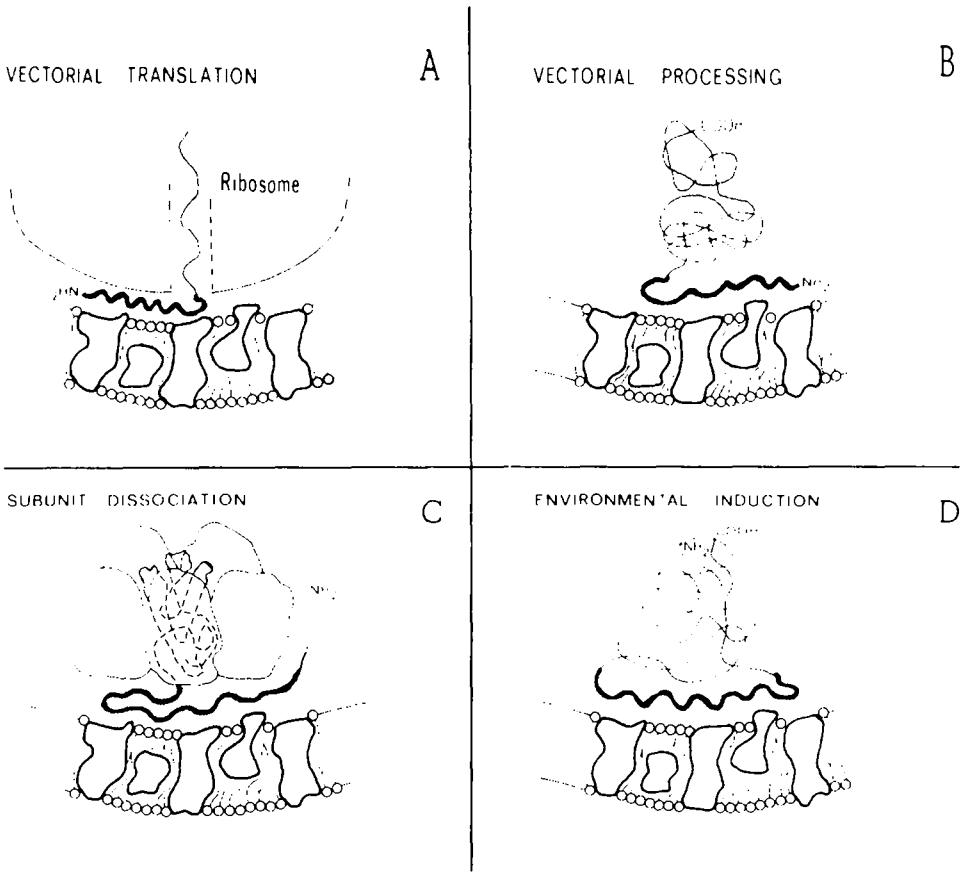


Fig. 10. Interacting area between protein to be internalized and membrane. A and B, N-terminal sequence involved in primary interaction. C and D, N-terminal sequence not necessarily involved in primary interaction.

dence shows that it does indeed occur), how do the hydrophilic segments present in that very same protein find their way through the hydrophobic areas of the lipid bilayer?

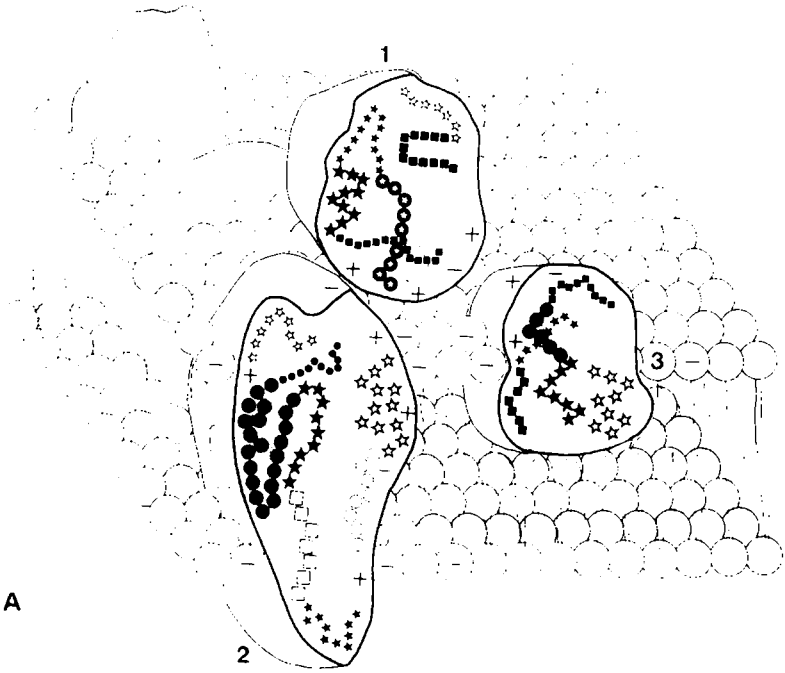
This again raises the earlier part of this discussion where a minor role was proposed for the lipids in connection with their relatively low concentration, thus making it possible for the protein to be translocated to seek privileged, less hydrophobic areas of contact and hence penetrate within the membrane. But is a specialized N-terminal segment a prerequisite for contact and translocation or, more generally, does a protein need to fulfil some structural and configurational requirements to meet the demands for translocation?

Three examples serve to illustrate the second possibility and suggest that the presence of an N-terminal hydrophobic segment on the protein is not a prerequisite condition for translocation.

(a) In the case of ovalbumin, glycoprotein PE_2, cytochrome *P*-450 translocated via the vectorial translation process, no such sequence is known to exist [72,73,76,78].

(b) In genetic grafting, it is necessary to add to a non-secretory protein (β-galactosidase) the equivalent of 2/3 of an excreted protein (lam β gene product), with a view to transform the first protein into a secretory structure. The addition of the N-terminal peptide is not sufficient [90].

(c) In translocation of mitochondrial aspartate aminotransferase and malate dehydro-



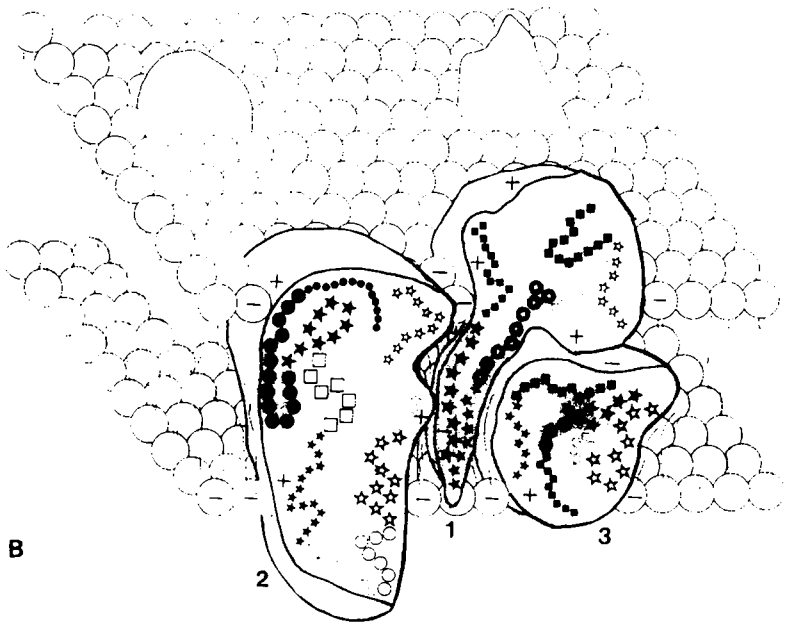


Fig. 11. A and B.

Fig. 11. Schematic sequence of protein internalization via environmental induction. Large symbols, hydrophobic peptide segments; small symbols, hydrophilic peptide segments. A, protein 1 is external to membrane surface. B, protein 1 internalizes starting with a non-terminal segment (see also Ref. 63). Proteins 2 and 3 change configuration while protein 3 sinks partially into the membrane. C, protein 1 completes internalization with concomitant change of configuration of 2 and 3 while protein 2 follows partially protein 1 in its movement.

genase through the mitochondrial inner membrane, reversible modification of the environment alone provokes reversible translocation of these enzymes [15- 20].

In all three of the above examples, something other than the N-terminal peptide seems to be required for the proper translocation configuration of the protein to materialize.

XB-2. Translocation and asymmetry

Clipping the signal sequence of a translocating protein constitutes a natural tool to create the asymmetry necessary for further compartmentation of the neosynthesized proteins.

However, for proteins which do not possess such a signal it is most probable that asymmetry of the membrane itself suffices as a signal for further compartmentation. The recognition between protein and membrane is not the same on both sides of the membrane. Self-organizing processes and membrane asymmetry have been implied to account for the vectorial incorporation of the major capsid protein of M13 bacteriophage and for the selective incorporation of membrane proteins into proteoliposomes of different composition. This is also illustrated by the reversible translocation of mitochondrial aspartate aminotransferase.

The reorganization of the membrane prior to the externalization of the enzyme seems to occur in two steps, the first of which requires the integrity of the surface charges, the second a closer and specific interaction between the membrane and the signal. If one of these recognition parameters is partially immobilized by cross-linking the membrane with bifunctional agents of the imidate type, to preserve the surface charge, then an inducer of aspartate aminotransferase release (succinate) still provokes a change in membranal conformation, which is detectable by the change in fluoresence of the 8-anilino-1-naphthalenesulfonic acid marker. However, no concomitant release of the enzyme is detectable. This might suggest that in the absence of a more specific and closer type of interaction between the signal and the membrane, the release event could occur. On the contrary, such partially cross-linked membranes are still able to internalize (at least partially) the previously released enzyme, upon removal of the exogenous succinate. This would suggest that the asymmetry of the membrane, superimposed on structuring factors of the environment, is a part of the conditions required for the segregation-localization process of mitochondrial aspartate aminotransferase to occur [227].

XB-3. Site and channel

The essential question remaining is how a protein, such as aspartate aminotransferase, which is endowed with apparent hydrophilic properties, can acquire transitorily partial hydrophobic characteristics making it possible for it to cross the membrane 'barrier'. The arguments making this step conceptually acceptable are as follow:

(1) Considering the high concentration of proteins and their possible segregation in most biological membranes, it is conceivable that proteins could cross membranes through protein-modulable structures, rather than through a hydrophobic barrier alone.

(2) If one accepts that the protein-membrane interaction is of a type analogous to that occurring in the best-fit association between ligand and enzyme [206], then the possibility could be envisaged that the protein to be translocated adapts its structure by covering or exposing hydrophilic or hydrophobic groups in response to the changing demands of the membrane. This, in conjunction with the change in environment, might eventually lead to the passage of the involved proteins through the membrane (Fig. 11).

(3) Finally, highly hydrophobic proteins exist which are soluble in chloroform/methanol mixtures and which appear to be reversibly transformable into hydrophilic forms [207]. Interesting examples of this hydrophilic-hydrophobic relationship are: (a) the CF1 factor, which is a hydrophilic peripheral component of the mitochondrial inner membrane and is composed of five highly hydrophobic subunits [208]; (b) a soluble succinate-binding protein which is released into the intermembranal mitochondrial space upon addition of succinate and precipitates upon dialysis of succinate if detergent is omitted from the dialysis [226]; (c) malate dehydrogenase is a water-soluble dimeric enzyme, of which the monomeric form interacts actively with phospholipid vesicles [36].

As proteins do cross biological membranes it can be hypothesized that it is for all the above reasons. The hydrophobic signal sequence might account for a selective first fixation event, which may be a prerequisite in some step of membrane building. Nevertheless, the protein coil erected from the polysome structure has an approximate cross-section of 10 Å. If this coil crosses a 70 Å thick membrane, its average interacting surface will be of the order of 2200 $Å^2$. This is far from being negligible in terms of hydrophobic-hydrophilic interactions and is only half of the interaction surface of a globular protein 40 Å in diameter completely embedded in the membrane.

Thus, the signal sequence by itself cannot explain how proteins cross membranes, but neither do other postulated mechanisms. However, in the case of large-amplitude protein movement, the triggering signal is exterior to the protein to be translocated, it can be singularized and its effects measured. On the other hand, the cohesion of the biological membrane being ensured by non-covalent bonds, the insertion of a protein in this structure might imply a sequence of interactions in a succession of environments. Thus, it might be favored by the presence of a signal sequence on the protein to be inserted and rendered more likely by a 'structured' interaction of the type polysomes-membrane-signal sequence.

The universality of this mechanism seems, however, contradicted by the diversity of the mechanisms already known.

XC. Thermodynamics

Thermodynamic data justifying transmembranal protein movement are certainly very scarce. However, such movement could perhaps be accounted for by the exchange of protein configurational energy, the order-disorder relationship of the system being dependent on the self-organizing properties of the membrane and on the qualitative and quantitative reorganization of the water molecules present either inside or in direct proximity to the membrane.

XC-1. Parameters

The difficulty with a thermodynamic approach to protein translocation, independently of the mechanism involved, is that it should take into account the global thermodynamic state of the membrane. The question is then to define the components that participate in the membrane reorganization or influence its structure in the course of protein translocation. Moreover, in vivo, it has to integrate the retarded diffusion in the neighborhood of the membrane caused by the cytosol gel.

The influence of environment on membrane structure is directly suggested by our own results showing a modification of the protein composition in perimembranal compartments induced by changes in environment, and also appears elsewhere in the literature where a relationship between membrane protein extraction and conditions of membrane preparation was described [207,209].

Some authors already attempted to compare membrane behavior by analogy either with the properties of isolated protein or with lipid vesicle behavior. It is advantageous to examine the thermodynamic approaches to these systems.

(a) Analogy with the protein structure. Many approaches have been used for the thermodynamic analysis of protein behavior [210,211]. We will limit ourselves to an enumeration of the interactions considered by Ikegami [212]: (1) the structure of a protein is characterized by the state of secondary binding between unique pairs of specific sites on the peptide chain; (2) each secondary bond fluctuates by thermal agitation, between the bound and unbound state; (3) each secondary bond is endowed with the same properties, the same energy and same number of close neighbors; (4) the polypeptide chain favors only binding with unique specific sites.

(b) Analogy with lipid structure in vesicles. Another approach could be made by analogy with that of Pink and Chapman [213] on a lipid membrane model structure. A number of similarities exist, moreover, with the protein model. In this approach: (1) the interaction energies between *trans* and *gauche* lipid chains are considered, (2) the surface

pressures due to the polar heads of the lipids are accounted for, (3) repercussions of the introduction of intrinsic proteins are sought, the protein showing a unique structure and being in a frozen position.

Both the above models could be used to some advantage for a thermodynamic understanding of biological membranes. However, parameters introducing the relative mobility of the different interacting components would have to be considered. This would increase the degree of freedom of the system. These parameters could explain the existence of many structural states of the membrane and induced modifications of these states by molecular signals in the proximity of the membrane.

Traüble et al. [214] have developed an approach in this direction, studying the influence of local pH changes on membrane structure, or the influence of various ions on the thermotropic transition point of the membrane. Another approach performed on artificial membranes, based on the thermodynamics of irreversible systems, has been used to study ion fluxes through artificial membranes to which ATPases were bound. These systems show the existence of different types of flux, the passage from one type of flux to another being spontaneous [215].

XC-2. For a thermodynamic approach to protein translocation through biological membranes

To justify membrane cohesion, Singer [216] favored hydrophobic forces and argued for the necessity of the maximation of these forces so as to minimize the free energy of the system. Such a view is the result of the membrane model chosen a priori in which the major role is attributed to the lipid structures and the hydrophobic interactions, at the expense of the other types of interaction.

These assumptions are bound to lead to a conceptual prevention of protein translocation through the membrane barrier. The extension of the model and the introduction of protein-protein and protein-lipid type interactions, as well as the fluctuation of the existing structures and interactions between these structures may open new perspectives to allow for protein translocation. This seems even more obvious if one takes into account the preponderant quantitative role played by protein in most biological membranes. As proteins do cross membranes in many instances, a thermodynamic model is required to account for it. What are the interacting parameters involved?

(1) In the case of protein translocation via exocytosis, the thermodynamic scheme would have to take into account the following complex interacting multiple steps [8].

(a) formation of a vesicle and inclusion of a protein therein;

(b) approach by the vesicle to the membrane and fusion with it;

(c) formation of an inverted micelle and its crossing the membrane;

(d) release of the intravesicular proteins;

(e) destruction or recycling of the vesicular non-released constituents.

(2) In the case of translocation via environmental induction, the following molecular steps have to be considered. In fact, this only amounts to an analysis of the molecular events required for a protein to cross the membrane barrier whether vesicular or otherwise. These steps would thus be: (a) the structural modification of the protein to expose temporarily the functional peptide segments on the exterior surface in order to maximize the following hydrophobic interaction with the membrane. Since for a given protein, hydrophobic residues always exist in contact with the hydrophilic environment, so reciprocally, this is conceivable [217]; (b) in parallel, 'unveiling' of the more hydrophobic areas of the membrane, so as to facilitate protein translocation (Fig. 11).

If the energy required for such a reorganization is only of the order of 100 kcal per residue, then it is feasible to envisage such a process as being thermodynamically favorable, or at least, not unfavorable [218]. Moreover, if one accepts the more general assumption that for a surface structure to cross a water/solvent interface the energy required is proportional to this surface, then it becomes much easier to conceive crossing this interface by a protein than by a vesicle.

In agreement with this view is the membrane-trigger hypothesis proposed by Wickner [92] where the interaction of a protein with the lipid bilayer triggers spontaneous folding of the protein without additional catalysis. The insertion or translocation of a protein within or through a membrane being then dependent upon the folding of the protein and the availability of an appropriate lipid bilayer.

XC-3. Transmembrane translocation and the membrane

The previous examples only take into account the thermodynamic balance of the proteins or vesicles directly involved in membrane crossing. However, a total view would include the thermodynamic state of the membrane itself. Indeed, the new environment associated with the translocation of the protein leads to a new organization of the membrane. This is observed in the case of aspartate aminotransferase, where its translocation through the inner mitochondrial membrane is associated with a large-amplitude protein movement between the different membranal and perimembranal compartments. Also, in the case of membrane protein turnover or biogenesis, the incorporation of a new protein must necessarily modify the structure of the membrane at least locally, and create a new state of symmetry.

More generally, this may be compared with the current thermodynamic analysis of transport phenomena, in which the membrane is considered as inert and the thermodynamic properties of substrates and carriers are not taken into consideration. In the case of the sodium pump, for instance, this has led to an imbalance between transport and coupled energy [219–222].

XC-4. Energy dependence and self-organization

Recent data imply that energy, in the form of ATP, is required for the internalization of the cytoplasmic precursor of some mitochondrial enzymes [47]. Interpretation of this type of result must allow for the fact that the inhibitors used to deplete mitochondria of their ATP or to uncouple the organelles strongly interact with and modify diverse membrane components, making it unclear as to the possible participation of ATP in the translocation mechanism. The fact that mitochondrial aspartate aminotransferase can be translocated inside the mitochondria against its own gradient could be explained on the basis of both the existence of receptor sites on the inner face and the self-organizing properties of the membrane. The receptor site's affinity would vary in parallel with the presence or absence of the movement effector (succinate). The loss of affinity would be accompanied, followed or preceded by a reorganization of the membrane leading to a cascade of events, one of which would be the release of the enzyme. The removal of the movement effector would reverse this sequence of events. This is partially illustrated by the reversible sequence of protein release into the mitochondrial intermembranal space as a function of effector concentration.

XD. View on an integrated system

In more integrated systems such as exocytosis-endocytosis, many unknown steps are involved. The mechanism of endocytosis and exocytosis was essentially built on the

necessity of segregation of hydrophilic enzymes and proteins and on the assumption that proteins are unable to cross membranes other than by vectorial secretion. Perhaps, in accepting the other more recently described mechanisms, a few of the existing discrepancies could be discarded.

Some criticisms of exocytosis have been brought forward by Rothman [111,122] and bear essentially on two points: the sequestration step and the fusion-fission step. Some obscure points of the exocytotic-endocytotic system reside (a) in the difference in the kinetics of turnover between vesicular membranes and secretory proteins and (b) in the discontinuum existing between the different membrane structures: rough endoplasmic reticulum-Golgi associated with vesicular secretion. None of the present evidence can fully account quantitatively for such discrepancies. There are indications that compensatory mechanisms such as the inter rough endoplasmic reticulum-Golgi shuttle by involving fusion and budding might account for the recovery of membrane material. Such a mechanism could account for partial recovery of some of the membrane material of which the turnover time is much slower than that of the proteins secreted in parallel. It presents an enormous degree of complexity as compared to direct protein translocation [223] which, except for Rothman's work [111,122], has never been examined in exocytosis. It also poses the very complex thermodynamic problem of vesicular fusion, which involves opposite membranes' behavior. On the one hand, lipid lateral diffusion is required for fusion whereas on the other hand, restriction of the very same behavior is a prerequisite for endocytotic recovery. Restriction could be supported by the cytoskeleton but this has so far not been demonstrated [9]. In the present context, endocytosis has to be the 'enantiomorphic' partner of exocytosis. In contradistinction to the restrictive hypothesis is the secretory system of the lymphocyte where exocytosis was associated with active secretion of IgM and where capping and patching phenomena accompanying this secretion were accounted for by lipid lateral diffusion [7,224]. This does not favor the restrictive step necessary for parallel endocytosis. In the present state of integration of secretion by exocytosis, all intermediate steps that could not be interpreted in terms of vectorial secretion or fusion-fission were considered as artefacts. This is the case, for instance, for the presence of secretory proteins in cytosol extract which was considered as stemming from the destruction either of prevesicular vacuoles or microsomal vesicles during the separation of the diverse fractions [60,225]. This is a real possibility; however, it cannot be excluded that some of these proteins occur as normal constituents of the cytosol. In fact, Rothman suggests the possibility of an equilibrium occurring between secretory proteins in the cytosolic compartment and in the zymogen granules of the pancreas. Also, the fact that 35% of the cell sap proteins labelled with ^{3}H and reabsorbed by the various membrane fractions of the cell homogenate was interpreted as a non-specific fixation of proteins issuing from lysed organelles [60,225]. It would have been of interest to test whether such refixation occurs only on the outer face of zymogen granules or on the inside of these structures as well. An asymmetrical rebinding or internalization could have had many specific functional implications. Globally, the fact that after stimulation of some secretory tissues the total number of vesicles does not seem to decrease and that the empty vesicles coexist with full ones raises [9] many intriguing questions. These examples stress the difficulties of quantitative and conceptual integration of the exocytosis-endocytosis shuttle mechanism.

Can these apparent contradictions be dismissed by taking into account mechanisms other than vectorial secretion? If one simply admits the possibility of empty-vesicle internalizing specific proteins (as mitochondria do), then the presence of secretory proteins in

cytosol, their 'absorption' on to the membrane structure and the coexistence of full and empty vesicles could be justified.

If this mechanism is further extended to the rough endoplasmic reticulum-Golgi system, then, in parallel to the shuttle mechanism, one could allow for secretory proteins to pass from one structure to the other, thus bridging the morphological and conceptual gap between these structures. This would justify and supplement the poorly documented shuttle mechanism with regard to the presence of vacuolar proteins in the cell sap. This approach could thus initiate an interesting working hypothesis to solve the problem of secretion by exocytosis.

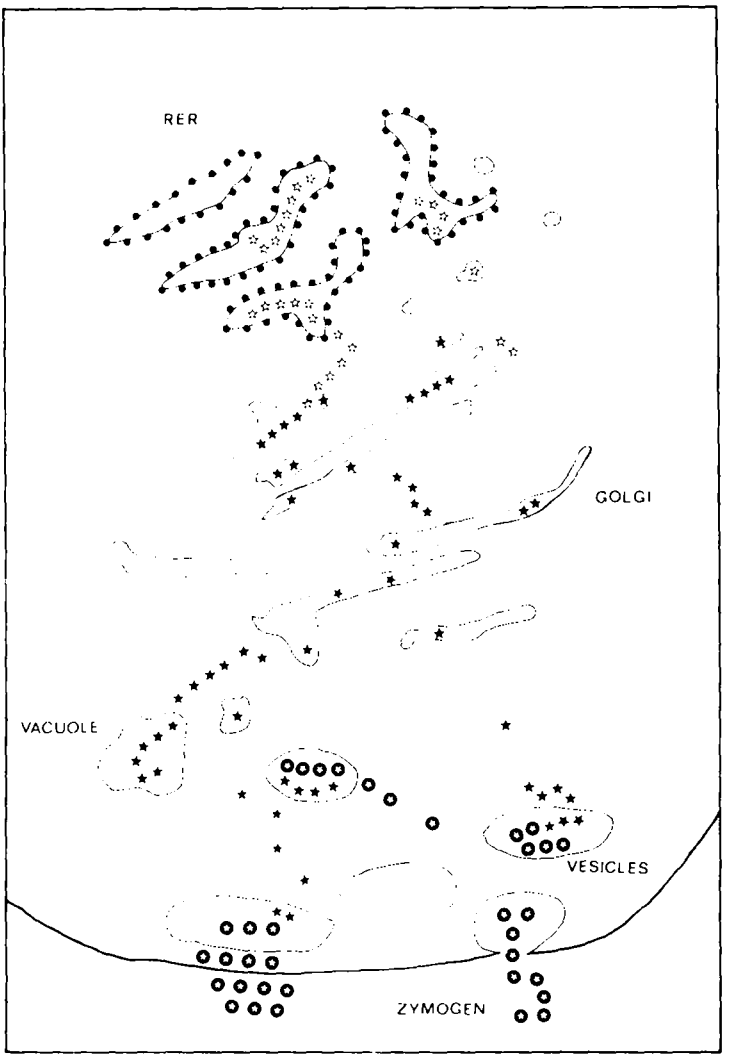


Fig. 12. Hypothetical parallel pathway to exocytosis taking into account a protein translocation mechanism other than that driven by vectorial translocation. Different stars represent proteins in different states of maturation. Emptying and refilling of vesicles into and from cytoplasmic pool of secretory proteins is visualized. RER, rough endoplasmic reticulum.

In the case of intramitochondrial large-amplitude protein movements, it is clear that some intravesicular proteins are able to reappear in different subvesicular compartments in response to specific demands of the environment. In the specific case of the externalization of mitochondrial aspartate aminotransferase and malate dehydrogenase, this follows the 'quantal' law, in that the amplitude of the response is, up to an optimum, a function of both the concentration and the nature of the exogenous releasing signal [17,18].

If this behavior is extrapolated to the exocytotic system, then in parallel to the vesicular release by fusion, one could imagine a speculative step whereby some of the secretory proteins mature and are stored in the vesicles, and could be released through the vesicular membrane near an activated site of the inner side of the plasma membrane. The released or equilibrated products would be eventually released through the plasma membrane with the activated inner side serving as the signal for this two-step release. The empty vesicle could then refill from the cytosolic pool of precursor secretory proteins (Fig. 12). The signal mediating the internalization would be either carried by the precursor secretory protein, and/or by environmental factors such as pH, Ca^{2+}, ATP, glucose gradient or by the new asymmetry of the empty vesicle, of which the inner-face configuration would be different from that of the full vesicle. This speculation could explain the existence of apparently intact empty vesicles upon discharge, account for the cytosolic pool of secretory proteins and allow for multiple recycling of the vesicle. Such a scheme reconciles exocytosis-endocytosis and the other known mechanisms for protein translocation.

In summary, a hypothetical sequence of events has been postulated in parallel to the exocytosis, which would account for some of the missing steps and discrepancies of the system.

XI. Conclusions

Towards a unified concept of protein translocation

The molecular mechanism that is the working membrane code involved in protein translocation is unknown. Nevertheless, due to the nature of the interactions between membrane components, the general rules governing both membrane communication with the environment and within itself would be expected to be of the bimodal type. Within such a context, protein translocation could be described by the following mechanisms given with appropriate examples:

(1) Translocation via vectorial translocation: in which the initiating signal is a macromolecular structure (ribosome or protein complex) in close association with the protein to be translocated which binds and modifies the membrane prior to translocation. This is the case for secretion of protein into the rough endoplasmic reticulum at the start of polysome attachment;

(2) Translocation via subunit dissociation: in which the initiating signal is a binding macromolecular structure, for instance, the B protein of tetanus toxin, which binds to GM_1 gangliosides of the membrane prior to injection of the A fragment in the membrane. In this case, it is the binding macromolecular structure associated to the protein to be translocated which defines the area for further protein penetration;

(3) Translocation via vectorial processing: in which it is some precursor form of the protein alone that bears the information signal (probably on its N-terminal) which induces the membrane conformation prior to the translocation step. This has been shown for the translocation of the cytoplasmic components of mitochondrial ATPase and cytochrome bc_1 complex;

(4) Translocation via environment induction: in which the environmental signal, the protein to be translocated and the membrane itself cooperate in a dynamic way to induce the proper membrane configuration required for translocation. This is the case for the internalization into mitochondria of cytoplasmically synthesized aspartate aminotransferase and malate dehydrogenase. It is both the movement-effector signal and the protein which would define the area for further protein penetration.

All these mechanisms have in common as a first step the inducing signal, which always precedes a multi-step translocation. Thus, they could be described as a signal-induced multi-step protein translocation. New definitions must then be introduced: signal sequence(s) is any domain(s) of the polypeptide chain involved in the recognition step of the interactions with the membrane. Precursor describes the state of the protein before its insertion into the membrane. Processing depicts any structural modification, possibly reversible, of the precursor and participation in the segregation process, sometimes ensuring its irreversibility by some type of covalent modification. Such processing would include proteolysis (vectorial translation and vectorial processing), disulfide bond reduction (peptide toxins) and conformation changes or subunit association/dissociation.

In conclusion, the externalization and internalization of proteins from and into cells and organelles may be described by a variety of mechanisms. The analysis of their common features has led to broader yet more unifying concepts of membrane dynamics, which should permit more meaningful experimental design and interpretation in the future.

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