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Proton-Translocating Adenosinetriphosphatase in Rough and Smooth Microsomes from Rat Liver†

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ABSTRACT: Rat liver smooth and rough microsomal membranes exhibit an ATP-dependent H^+ transport which can be inhibited by sulfhydryl reagents and dicyclohexylcarbodiimide but is resistant to oligomycin. On the basis of inhibitor sensitivities and substrate specificities, this H^+ pump was found to be different from that of mitochondria, lysosomes, gastric H^+–K^+-ATPase, and yeast plasma membrane H^+-ATPase but to resemble that of endocytic vesicles and the H^+ pump responsible for urinary acidification. The transport process is accelerated by valinomycin in the presence of potassium, suggesting that it is an electrogenic pump. The same fractions were enriched in an ATPase with inhibitor sensitivities similar to those of the transport activity. It is possible that the proton electrochemical gradients generated by this pump may play a role in the translocation of proteins and sugars, two of the major functions of these structures.

Intracellular organelles exist frequently as membrane-bound compartments allowing the development of microclimates where the concentration of various substances could be maintained at levels different from those in the cytoplasm. It is now clear that the organellar membranes possess a variety of transport activities that generate gradients of concentration and potential that can be used not only to create these microclimates but also to act as driving forces for the accumulation or extrusion of solutes in these compartments. Recent studies on the function of microsomal vesicles have uncovered the important role that the endoplasmic reticulum plays in the synthesis and packaging of secretory and membrane proteins and in their initial glycosylation. We were stimulated by the observation that monensin, a protonophore which exchanges H^+ for Na^+ or K^+, had dramatic effects on the function and structure of these organelles (Tartakoff & Vassalli, 1978; Ledger et al., 1983). Since one possible mechanism by which monensin could exert its effect is by collapsing a pH gradient in these organelles, we tested microsomes for the ability to transport protons. We found that these membranes contain a proton-translocating ATPase which appears to be different from the mitochondrial F_0–F_1-ATPase and the gastric H^+–K^+-ATPase.

Materials and Methods

Rat liver microsomes were prepared according to the procedure of Adelman et al. (1973). All low-speed centrifugations were performed in a Sorvall Model RC-5B centrifuge and the high-speed spins in a Beckman Model L5-50 ultracentrifuge. All steps were performed at ~4 °C. Briefly, fresh livers were excised, placed in ice-cold 0.25 M sucrose, cut into five pieces each, blotted on filter paper, and forced through a stainless-steel screen with a mesh size of 1 × 1 mm into 1 M sucrose (2 mL/g of tissue). This was homogenized with eight strokes of a motor-driven Teflon pestle and glass homogenizer at 1500 rpm (Caframo type RZR50, Warton, Ontario), filtered

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Table I: Enzyme Activities in Various Cellular Fractions[a]

	protein (mg)	activity (μmol of P_i min^{-1}) oligomycin-sensitive ATPase	DCCD-sensitive ATPase	glucose-6-phosphatase
homogenate	1247 ± 365	142 ± 29	1 ± 1	94 ± 27
postnuclear supernate	1019 ± 331	146 ± 29	8 ± 11	83 ± 14
postmitochondrial supernate	786 ± 195	64 ± 6	5 ± 5	71 ± 15
high-speed supernate	423 ± 79	7 ± 4	2 ± 2	7 ± 2
smooth membranes	68 ± 23	9 ± 4	2 ± 0.4	21 ± 7
rough membranes	38 ± 14	3 ± 2	0.5 ± 0.1	15 ± 6

[a] Fractionation was performed as described in the text. Results are means ± standard deviations of at least three preparations.

through gauze, mixed with an equal volume of 2.5 M sucrose, and centrifuged for 45 min at 100000g to pellet nuclei. The postnuclear supernatant was mixed with half its volume of water and centrifuged for 15 min at 22000g to bring down the mitochondria. The postmitochondrial supernatant was saved, and the mitochondrial pellet was washed twice by resuspending in 12 mL of a 9:1 0.5 M sucrose:inhibitory supernate[1] mixture and centrifuging for 15 min at 17000g_{max}. The combined postmitochondrial supernatant was centrifuged for 15 min at 17000g_{max} again for the final postmitochondrial supernate. The mitochondrial pellets were combined and saved.

Smooth and rough microsomes were then obtained from a sucrose density gradient where 11 mL of postmitochondrial supernate was underlayed with 2 mL of 3:1 2 M sucrose:inhibitory supernatant (final molarity ~1.5 M) and 0.5 mL of a solution containing 2 M sucrose, 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[2] 25 mM KCl, and 5 mM $MgCl_2$, pH 7.5, and centrifuged for 18–20 h at 200000g_{max}. Rough membranes banded at the interface between steps of 1.265 and 1.202 g/mL densities while the smooth membranes band between densities of 1.202 and 1.085 g/mL. The membranes were aspirated separately by using a syringe and steel cannula diluted with twice their volume in transport medium (0.25 M sucrose, 150 mM KCl, 6 mM $MgCl_2$, 2 mM Tris base, and 2 mM MES titrated to pH 7.0 with HCl) and pelleted by centrifuging for 1 h at 46800g_{max} in a Sorvall centrifuge using the SM 24 rotor. These pellets were resuspended in 10 mL of transport medium per fraction (smooth and rough), and portions were either used immediately in transport experiments or frozen and stored at −20 °C for subsequent enzyme assays. In one preparation, dithiothreitol was added to all solutions to a final concentration of 1 mM.

Transport Assays. Smooth and rough microsomes were separated into two aliquots and incubated on ice for 1 h with oligomycin in the presence or absence of the inhibitors in the same final concentrations used in the ATPase assays (see below). This concentration of oligomycin completely abolished H^+ transport in the mitochondrial fraction of rat liver (see below).

Membranes and transport media (at room temperature) were placed in a cuvette to a total volume of 1.5 mL, and the difference in absorbance at 492–540nm was measured in a dual-wavelength spectrophotometer (University of Pennsylvania Instrument Shop). This signal, an index of the concentration of the monomeric form of acridine orange in free solution, was displayed continuously on a chart recorder. Unless otherwise stated, 6 μM acridine orange, 1 μM valinomycin, and 10 μg/mL oligomycin were present in all assay media. After the absorbance signal reached a stable base line, transport was initiated by adding ATP to a final concentration of 0.4 mM. (The ATP stock solution of 30 mM was titrated to pH 7.0 with Tris). After steady-state uptake of acridine orange occurred, nigericin was added to a final concentration of 1.3 μM to collapse the pH gradient across the vesicles.

Enzyme Assays. Oligomycin and DCCD-sensitive ATPase activities were measured as described before (Gluck et al., 1982), glucose-6-phosphatase activity was measured by the method of Baginski et al. (1974), phosphate was measured by the method of Baginski & Zak (1960), and protein was measured with Bio-Rad (Bio-Rad Corp., Richmond, CA) reagent using bovine serum albumin as a standard. All fractions were diluted in 0.25 M sucrose. Assays were performed in duplicate for protein and acid phosphatase, and in triplicate for the others.

In the ATPase and transport assays, the effect of inhibitors was tested following preincubation of the sample in the inhibitor on ice for at least 1 h.

Results

Using the fractionation procedure of Adelman et al. (1973), we obtained smooth and rough endoplasmic reticulum fractions which were enriched in glucose-6-phosphatase, a marker enzyme of these structures (Table I). These two fractions contained 43% of the glucose-6-phosphatase activity of the postnuclear supernate but only 8% of the mitochondrial marker, the oligomycin-sensitive ATPase. Hence, they formed a reasonable starting point to search for the presence of a proton-translocating ATPase. To define the characteristics of this H^+ pump in these fractions, we measured proton transport by using the uptake of the weak base acridine orange into the microsomal vesicles in response to the addition of ATP (Figure 1). When the inside of a microsomal vesicle becomes acidic, it will accumulate acridine orange since the protonated form of that dye is much less permeable than the free base. As the concentration of acridine orange increases, it tends to form dimers and higher order multimers with a resultant change in its spectral properties. Using a dual-wavelength spectrophotometer tuned to the absorption characteristics of the monomer, one can measure its uptake as an index of the rate of H^+ transport into the vesicles. Addition of ATP to smooth or rough membranes resulted in a rapid uptake of acridine orange (Figure 1). To demonstrate that this uptake is due to the development of a pH gradient, we added the proton ionophore nigericin and found that this agent caused

[1] To prepare the ribonuclease inhibitory supernatant, we centrifuged the 0.25 M sucrose homogenate for 20 min at 40000g in the Beckman ultracentrifuge using the SW-40 rotor. The supernatant from that spin was recentrifuged for 2 h at 100000g. The supernatant, termed inhibitory supernatant, was divided into 10-mL aliquots and stored at −20 °C until used.

[2] Abbreviations: ER, endoplasmic reticulum; DCCD, dicyclohexylcarbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; PCMBS, *p*-(chloromercuri)-benzenesulfonate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NEM, *N*-ethylmaleimide; DTT, dithiothreitol.

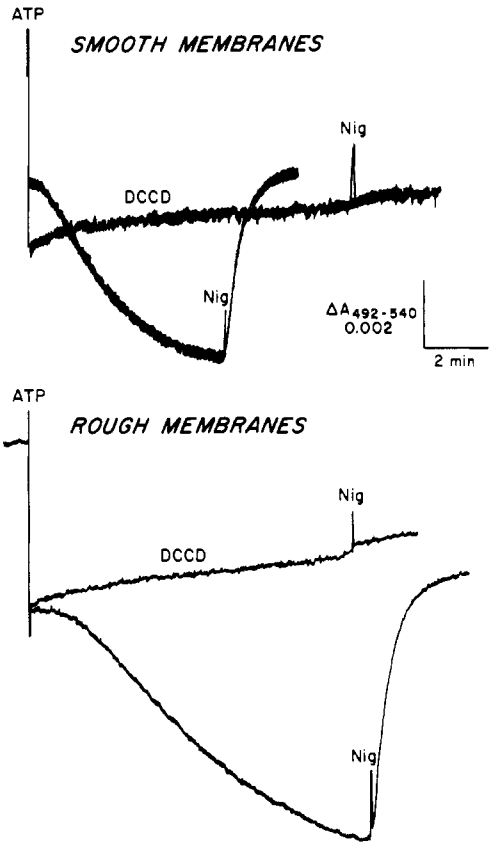

FIGURE 1: H^+ transport in smooth and rough microsomal vesicles. Fractions were isolated as described in the text. ATP-dependent H^+ transport into the vesicles is measured as the uptake of acridine orange determined in a dual-wavelength spectrophotometer by using the two wavelengths shown. Each assay contained in 1.5 mL of assay medium 800 μg of protein, 10 μg/mL oligomycin, and 1 μM valinomycin. Final ATP concentration was 0.4 mM. Nigericin was added to a final concentration of 1 μM. In the DCCD experiments, the vesicles were preincubated with 200 μM DCCD for 1 h on ice.

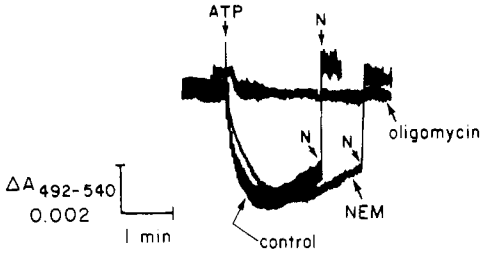

FIGURE 2: H^+ transport in submitochondrial particles of rat liver. Assay conditions were as in the legend to Figure 1. No oligomycin was added except as indicated, where it was present at a concentration of 33 ng/mL. In the trace labeled NEM, 50 μM *N*-ethylmaleimide was present. Forty-four micrograms of protein was used in each assay. At the arrows marked N, 2 μM nigericin was added.

a rapid discharge of the accumulated dye (Figure 1). Preincubation of the microsomal vesicles with DCCD, an inhibitor of many proton-translocating ATPases, prevented the uptake of the dye. These results demonstrate that microsomal vesicles contain a DCCD-sensitive ATP-driven H^+ pump. Addition of ATP always resulted in a decrease in the signal regardless of the presence of vesicles. This is likely due to interaction of the nucleotide with the acridine. Further, there was frequently some delay of about 30 s before acidification started for which we have no explanation at the present time.

The major concern whenever proton transport is found in a subcellular fraction is that it is due to inside-out vesicles from broken mitochondria (submitochondrial particles) which have

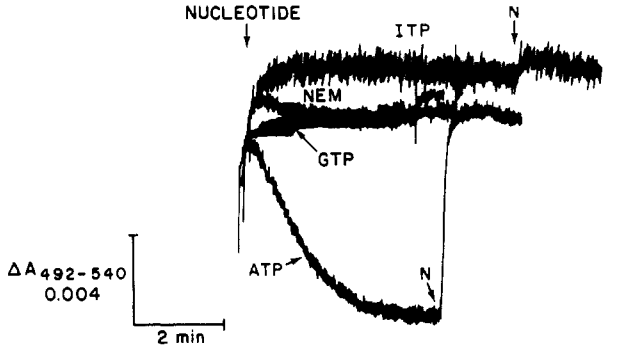

FIGURE 3: H^+ transport in smooth microsomal vesicles. Assay conditions are as in the legend to Figure 1. Transport was started with additions of 0.4 mM ATP in the traces labeled ATP and NEM. In the latter, 50 μM *N*-ethylmaleimide was present. In the other two traces, 0.4 mM GTP or ITP was added. Nigericin was added at the arrows marked N to a final concentration of 1 μM.

Table II: Initial Rate of Uptake of Acridine Orange in Smooth ER Membranes Measured as the Steepest Tangent to the Decline of the Absorbance Signal[a]

assay		transport rate [absorbance units s^{-1} (mg of protein)$^{-1}$ × 10^{-5}]
A (no DTT)	smooth membranes	2.54 ± 0.32
	+200 μM DCCD	0
	+100 μM PCMBS	0
	+1 mM azide	0
	+100 μM NBD-Cl	0
	+20 μM Cu^{2+}	2.14
	+50 μM NEM	0
B (+1 mM DTT)	smooth membranes + val	6.98 ± 0.55
	smooth membrane − val	5.73 ± 0.17
	+200 μM DCCD	0
	+50 μM vanadate	6.56
	+5 mM azide	1.23
	+1 mM azide	4.05
	+100 μM NBD-Cl	0

[a] Each assay contained (unless otherwise stated) 800 μg of protein, 3 μM acridine orange, 10 μg/mL oligomycin, and 1 μM valinomycin (val). Transport was initiated by adding ATP to a final concentration of 0.4 mM. For the inhibitor studies, all membranes were preincubated in the stated concentration of the inhibitor for 1 h at 4 °C. Results are means ± standard deviations of at least three preparations.

a potent H^+-translocating ATPase that is also inhibited by DCCD. The mitochondrial H^+ pump, however, is exquisitely sensitive to oligomycin. We prepared submitochondrial particles from rat liver by using the method of Beyer (1967). The ATP-induced H^+ transport in these vesicles was completely abolished by 33 ng/mL oligomycin (Figure 2). Furthermore, 50 μM *N*-ethylmaleimide (NEM) had no effect on the mitochondrial pump (Figure 2) but completely inhibited the microsomal H^+ pump (Figure 3). These studies show that these two ATPases can be readily separated from each other. All the transport experiments shown here were performed in the presence of 10 μg/mL oligomycin. This oligomycin resistance and NEM sensitivity were recently found to be a characteristic of a number of proton pumps including those from lysosomes (Ohkuma et al., 1982), chromaffin granules (Njus et al., 1981), and urinary epithelia (Gluck et al., 1982).

To provide a semiquantitative measure of the H^+ uptake in these vesicles, we estimated the maximal rate of uptake by plotting the steepest tangent to the initial decline in the absorbance signal.[3] This was used to test the effect of various

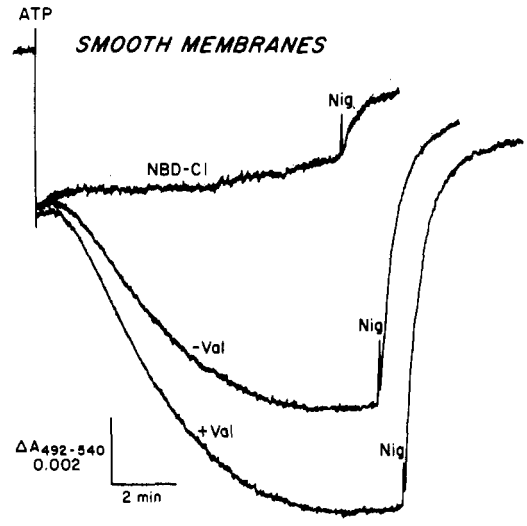

FIGURE 4: H^+ transport in smooth membranes measured as described in the legend to Figure 1 (±valinomycin). In one experiment here, micromolar concentrations of valinomycin were added (+val tracing). In the NBD-Cl tracing, the vesicles were preincubated with the inhibitor for 1 h on ice. All had 800 μg of protein per assay.

inhibitors. As seen in Table II, assay A, DCCD (a carboxyl-reactive agent), NEM (a sulfhydryl-alkylating agent), PCMBS (an organomercurial), and NBD-Cl (a tyrosine-reactive agent) completely inhibited the transport. (The latter reagent can also react with sulfhydryl groups). Since the effects of NEM and PCMBS suggested that the pump contained a critical sulfhydryl group, we prepared the membranes in the presence of 1 mM dithiothreitol and found the transport rate to be higher (Table II, assay B, and Figure 3 as compared to Figure 1, smooth membranes). It is noteworthy that preincubation of the vesicles with NBD-Cl for 1 h inhibited transport, an effect that was not reversed by subsequent treatment with dithiothreitol. In this respect, this ATPase is different from the chloroplast and mitochondrial F_0–F_1–ATPases where dithiothreitol readily reversed the inhibition by NBD-Cl. Further, we found that azide inhibited the transport rate in the microsomes but 50 μM vanadate did not. At this concentration, vanadate completely inhibits the proton-translocating ATPases of yeast plasma membrane (Addison & Scarborough, 1981) and stomach (Sachs et al., 1983) as well as other ATPases such as (Na,K)-ATPase and sarcoplasmic reticulum Ca-ATPase (O'Neal et al., 1979).

Recent studies have identified a proton-translocating ATPase in lysosomes. Since microsomal fractions, especially rough membranes, are frequently heavily contaminated with lysosomes, it is imperative to rule out the possibility that what we term the microsomal proton pump is not simply an artifact of lysosomal contamination. Ohkuma et al. (1982) recently found that rat liver lysosomes transport protons in response to addition of GTP and ITP almost as well as in response to addition of ATP. However, microsomes responded only to ATP; GTP and ITP (Figure 3) did not induce H^+ transport. These studies suggest that the microsomal transport activity is not due to mitochondrial or lysosomal contamination and that these membranes possess a H^+-ATPase that is distinct from that of the F_0–F_1-ATPase, the lysosomal ATPase, and the gastric H^+–K^+-ATPase.

The above results demonstrate that the microsomal membranes contain an ATP-dependent proton pump that can generate a pH gradient. To test for the capacity of this pump to generate a membrane potential, we added valinomycin in the presence of K^+. Figure 4 shows that valinomycin accelerated the rate of development of a pH gradient as well as increased its magnitude. Since valinomycin shunts any potential developed by the pump, the increase in the pH gradient implies that the H^+ pump initially induced a positive potential in the vesicles. This potential acts as an adverse gradient which would slow down the transport rate of an electrogenic pump. This result is only suggestive of electrogenicity and needs to be confirmed by more direct tests.

We compared the characteristics of H^+ transport in rough and smooth vesicles and found them to be similar. Both were stimulated by valinomycin and were inhibited by *N*-ethylmaleimide, DCCD, and NBD-Cl. However, the transport activity in the rough membranes was much more labile than that in smooth membranes. It was routinely possible to store smooth membranes (in media containing ATP and dithiothreitol) at −70 °C. H^+ transport in rough membranes was only detected in freshly prepared membranes.

Using the characteristics identified above, we measured ATP hydrolysis in an attempt to find the enzymatic equivalent of the proton pump. Previous studies on urinary epithelia suggested that DCCD might be a useful label (Gluck et al., 1982). However, DCCD inhibits many ATPases including the mitochondrial ATPase, the (Na,K)-ATPase, and the Ca-ATPase. To detect ATPase activity due to the microsomal H^+ pump without having the results confounded by the presence of the other ATPases, we measured the effect of DCCD on ATP hydrolysis by microsomal membranes that have been preincubated in the presence of 10 μg/mL oligomycin, 1 mM ouabain, and 1 mM EGTA. We shall term this activity the DCCD-sensitive ATPase. We found that there was little activity in the liver homogenate (Table I) [specific activity 1.06 ± 1.84 (±standard deviation) nmol min^{-1} (mg of protein)$^{-1}$]. This activity increased in the postnuclear supernatant to 6.2 ± 6.9 nmol min^{-1} (mg of protein)$^{-1}$. (In both of these fractions, this activity was frequently absent). In the smooth membranes, the specific activity was 27 ± 4.6 nmol min^{-1} (mg of protein)$^{-1}$ while in the rough membrane fraction it was 12 ± 4.4 nmol min^{-1} mg of protein^{-1}. There was no activity in the mitochondrial fraction. The smooth and rough membranes contained 43% of the total activity of the postmitochondrial supernatant. We also tested the effect of other inhibitors on the ATPase activity, and although we found that PCMBS, azide, NBD-Cl, and NEM inhibited the ATPase, the effects were small and sufficiently variable to prevent us from making any strong conclusions.

Discussion

The present results demonstrate the existence of a proton-translocating ATPase in rough and smooth microsomal membranes. We suggest that these membranes are largely derived from endoplasmic reticulum. The fractionation method used has been extensively characterized by Adelman et al. (1973), and on the basis of morphological and enzymatic

[3] The steady-state uptake of acridine orange is proportional to the pH gradient developed. The size of the pH gradient depends on the H^+ influx minus the leak of protons. The latter is given by the proton permeability times the electrochemical driving force. The initial uptake of acridine orange should then approximate the ATP-driven H^+ influx since the electrochemical gradient is small. This is valid provided the permeability of the free base is not limiting. As seen on addition of nigericin in Figures 1 and 2, there is "instantaneous" discharge of acridine orange, indicating that this dye is freely permeable. To compare the effect of various inhibitors, two assumptions are made; one is that the inhibitor has no effect on the buffering power of the vesicles, and the other is that it is not a proton conductor. Because these two assumptions as well as others need to be rigorously tested, this method should not be considered in any way quantitative. However, it is a useful qualitative tool for comparing the effect of various inhibitors.

criteria, they have shown these membranes to be the endoplasmic reticulum (Adelman et al., 1973). We have repeated the enzymatic evaluation and obtained results quantitatively similar to theirs. Despite this, it must be emphasized that cell fractionation methods are an imperfect approach to localize a specific enzyme to an organelle. Until we obtain an immunoelectron-microscopic localization using a specific antibody to this enzyme, our conclusions must be considered tentative. However, we feel that the finding of a proton pump in these membranes is sufficiently provocative to warrant further work on this subject.

There has been much recent interest in the role that membrane potential and pH gradients could play in the regulation of a variety of cellular events. A proton-translocating ATPase has been identified in a number of intracellular organelles including lysosomes (Schneider, 1981; Ohkuma et al., 1982; Reeves & Reames, 1981), chromaffin granules (Njus et al., 1981), and clathrin-coated Golgi and endocytic vesicles (Forgac et al., 1983; Stone et al., 1983; Galloway et al., 1983; Glickman et al., 1983). Where tested, these ATPases have all been inhibited by sulfhydryl reagents but not by oligomycin or vanadate. These important characteristics distinguish these enzymes from the mitochondrial F_0–F_1-ATPase and the gastric H^+–K^+-ATPase, the former being inhibited by oligomycin and the latter by vanadate. This ATPase had nucleotide specificity different from that of the lysosmal H^+ pump. Whether this constitutes a fundamental difference between the microsomal and the lysosomal enzymes will have to await purification of the two pumps.

It is possible that this new ATPase represents a single H^+ pump that is widely distributed in intracellular organelles. Should that prove to be the case, it would not be surprising that the endoplasmic reticulum also contains it since that structure is the site of synthesis of the membrane proteins of lysosomes and other organelles. Its presence in the ER may simply be a consequence of the fact that it is synthesizd there. Further, it may serve some of the vital functions that are performed by this structure such as the transport of proteins or sugars across its membrane. Recent studies on protein transport across artificial and natural membranes have suggested that at least for some proteins, e.g., diphtheria toxin (Draper & Simon, 1980; Sandvig & Olsnes, 1980), some viruses (Helenius et al., 1980), and possibly epidermal growth factor (King & Cuatraecassas, 1982), transport across membranes requires the presence of a pH gradient or a membrane potential. A low pH in the ER could also facilitate folding of the amino terminal of the peptide chain to allow pulling the protein into the vesicle. Sugar transport in a variety of prokaryotic and eukaryotic cells is frequently coupled to a transmembrane electrochemical gradient. It is possible that the proton electrochemical gradient produced by the microsomal proton pump discussed above could generate a driving force for it. Needless to say, these are only speculations at present, and experiments need to be performed to test these hypotheses.

Registry No. ATPase, 9000-83-3; H^+, 12408-02-5.

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