Transmembrane proteins and soluble proteins destined for secretion are synthesized in the endoplasmic reticulum (ER) and traverse multiple compartments en route to their ultimate destination. Nascent polypeptides are folded and core glycosylated in the ER and are shuttled to the Golgi complex via a specialized vesicular compartment, the ER-to-Golgi intermediate compartment (ERGIC). As they traverse the Golgi complex, these immature proteins are sequentially modified by an array of glycosylating enzymes and can undergo other posttranslational modifications, including sulfation. The modified (glyco)proteins exit the complex via the trans-Golgi network (TGN) and are either directly delivered to their target destination or are packaged into secretory granules, where they remain poised for secretion upon stimulation (FIGURE 1). Lipids also undergo glycosylation and sulfation in the Golgi complex.

Accurate delivery of biosynthetic cargo to its intended destination is critical, and cells have therefore evolved sophisticated strategies to identify and properly route individual molecules along the secretory pathway. One such strategy is to control the interaction between cargo molecules and their receptors by modulating their state of protonation. Hence strict regulation of the pH prevailing in the secretory pathway is fundamental for proper protein sorting and processing. The measurement, determinants, and regulation of the pH of the different subcompartments of the secretory pathway are the focus of this review.

The Importance of pH

The central role of pH in secretory function is best illustrated by a variety of experiments in which protein processing and traffic were found to be disrupted when the luminal H⁺ concentration of one or more compartments was forcibly altered, generally using weak bases or ionophores. When the pH gradient across Golgi membranes was collapsed, the posttranslational modifications and processing of secreted proteins were impaired (5), cargo was misdirected (6), and the integrity of the organelle itself was compromised, as judged by the redistribution of resident marker enzymes (25, 33). Proper targeting of resident TGN proteins, such as TGN38 and furin, was found to be similarly pH dependent (8). Lastly, sorting and proteolytic maturation of prohormones in secretory granules are also strictly pH dependent, requiring an acidic environment (38).

Alterations of the pH of the secretory pathway are not only a convenient experimental paradigm to study the role of protonation but also occur in nature, often with dire consequences. The M2 proton channel of influenza viruses has been shown to alkalinize the lumen of the Golgi apparatus, thereby delaying protein delivery to the plasma membrane, a step that is critical in enabling virus assembly (15, 36). Abnormalities noted in tumors and papillomas have also been attributed to modification of the pH of the secretory compartment (20, 44).

The mechanism(s) underlying the alterations summarized above are rarely understood. The catalytic activity of multiple processing enzymes is compromised when the native pH is experimentally altered. In addition, the ability of receptors to deliver cargo or retrieve essential components is known to be, at least in some instances, critically dependent on pH (9). Moreover, vesicular traffic itself is exquisitely pH dependent, an effect that has been variously attributed to changes in membrane fusion (42) or fission (2, 14). Considered collectively, these effects emphasize the crucial importance of pH regulation in the secretory pathway.

Measuring pH in the Secretory Pathway

The simplest and most convenient method to measure organellar pH is through the targeted delivery of pH-sensitive spectroscopic probes. Indeed, this approach has proven fruitful in
REVIEWS

measuring the pH of various endocytic compartments, which are easily accessible to external probes that become internalized by fluid-phase pinocytosis. By comparison, measurement of the pH along the secretory pathway has been considerably more difficult, due primarily to the restricted accessibility of this compartment. Because the secretory system cannot be readily reached by impermeant probes, early approaches to the measurement of its pH relied on partition probes that traverse membranes. One such probe is 3-(2,4-dinitroanilino)-3′-amino-N-methylidipropylamine (DAMP), a weak base that accumulates in the lumen of acidic organelles, where it can be fixed. Immunostaining of DAMP in conjunction with electron microscopy was used to make the first measurements of pH in the secretory pathway (1). Although this was unquestionably a landmark study, the need for more precise, dynamic, and convenient methods became painfully apparent. Alternative, indirect approaches were developed in parallel to estimate the pH of secretory compartments. These were based on the assessment of the in situ efficiency of endogenous enzymes of the pathway, selected for having pronounced pH sensitivity. The prevailing pH was deduced by comparing their activity in vivo with profiles of the pH dependence of their activity in vitro. The pH of secretory granules was initially estimated in this manner (43). As in the case of DAMP, however, these measurements were approximate and lacked temporal resolution.

The first direct and dynamic measurements of pH in organelles of the secretory pathway were initiated in the mid-1990s by delivery of pH-sensitive fluorophores to the Golgi complex. Seksek et al. (39) exploited the mysterious ability of small (70-nm) liposomes to selectively fuse with the Golgi complex to deliver to this organelle soluble probes that were retained transiently. Shortly thereafter, Kim et al. (22) took advantage of the retrograde pathway that directs certain surface receptors to the Golgi stacks. These authors managed to probe the Golgi with fluorescein, a pH-sensitive fluorophore, by conjugating it to verotoxin, a bacterial product that undergoes retrograde transport following binding to surface glycolipids. In both studies, fluorescence microsco-

FIGURE 1. Comparison of the steady-state pH of the compartments of secretory and endocytic pathways
Numbers indicate the approximate luminal pH of the specified compartment. TGN, trans-Golgi network.
Py was the method chosen for continuous detection of organelar pH, and in situ calibrations using ionophores made accurate quantitation possible.

The identification of organelle-specific targeting sequences, combined with the advent of molecular engineering, made it possible to design chimeric proteins bearing exogenous moieties that could be selectively directed to the secretory pathway. The chimeras could be engineered to contain epitopes recognized by readily available antibodies, proteins like avidin that have well-defined ligands, or, more recently, fluorescent proteins. In one instance (10), the TGN-resident proteins furin and TGN38 were tagged with an epitope, which could be ligated by a fluorescent antibody during their brief transit through the plasmalemma, where they become exposed to exogenously added probes (FIGURE 2A). In another elegant study (46), chimeric avidin was targeted to secretory compartments and a pH-sensitive fluorescent derivative of biotin was then added to label the resulting complex (FIGURE 2B). In yet other studies (26), pH-sensitive variants of the green fluorescent protein (GFP) of Aequora victoria have been directed to specific organelles by fusion with the appropriate targeting sequences (FIGURE 2C). Sensitive and reproducible measurements can be performed by using two such GFP variants: pHluorin (29) and dual-emission GFP (deGFP). Both proteins undergo spectral shifts with changing pH and display a distinct isobestic point. Measurement of the ratio of fluorescence intensity at two suitably chosen wavelengths makes the determinations independent of photobleaching and of alteration in the focal plane, a common occurrence during the course of organelar fluorescence determination, allowing for increased precision. Note that although pHluorin undergoes an excitation shift, it is the emission spectrum that shifts in the case of deGFP (FIGURE 3).

The pH values estimated for the individual sub-compartments of the secretory pathway are summarized in FIGURE 1. As with most biological phenomena, some variability has been reported, which is most likely attributable to differences in the cell type used. Yet, considering the wide range of cells and approaches used, it is remarkable that a reasonable consensus has been reached regarding the absolute pH of the individual organelles. The endoplasmic reticulum pH is generally thought to be near neutral, similar to the cytoplasmic pH (21, 45). However, the compartments of the secretory pathway become progressively more acidic thereafter, as the products of secretion approach their final destination. The cis-Golgi is measurably more acidic (pH = 6.7) than the

**FIGURE 2.** Methods used for measurement of the pH of secretory organelles

A: cells are transfected with a TGN-targeted chimera that bears a luminal (extracellular) epitope tag. TGN targeting is accomplished by using constructs based on TGN38 or furin. When expressed, the chimera cycles between the TGN, where most of the proteins reside at steady state, and the plasma membranes, where they sojourn transiently. Addition of FITC-conjugated antibodies to the epitope tag enables the investigator to label the surface-exposed chimeric protein with the pH-sensitive fluorescein moiety of FITC. The labeled protein then cycles back to the TGN, where it accumulates. B: cells are transfected with a Golgi-targeted chimera that bears a luminal avidin moiety. Golgi targeting is accomplished by using a sialyltransferase construct. Addition of membrane-permeant biotin derivatives labeled with a pH-sensitive probe, followed by washing of unbound probe, enables the investigator to measure Golgi pH. C: cells are transfected with a Golgi-targeted chimera that bears a luminal fluorescent protein that is pH sensitive in the physiological range.
Determinants of pH along the secretory pathway

The progressive acidification encountered along the secretory pathway has critical implications for the processing and sorting of cargo. How is it determined and maintained? There are several factors that cooperatively dictate the pH of the individual organelles. Vacuolar H+-ATPases are the primary and perhaps the sole source of proton delivery to the organelar lumen. Because they are electrogenic, the activity of the ATPases is affected by the prevailing transmembrane potential, which is in turn a function of the permeability to other, bystander ions. The steady-state pH attained by an organelle will also be affected by the rate at which H+ or its equivalents (e.g., OH−, HCO3−) “leak” back into the cytosol. A brief description of the nature and contribution of each of these factors is presented in the following sections.

V-ATPases

Proton pumps of the vacuolar or V-ATPase type are found in a wide array of intracellular com-partments and in virtually all cell types. Their primary function is to transfer protons from the cytoplasmic compartment to the lumen of membrane-bound organelles in an ATP-dependent manner. They are multisubunit complexes (FIGURE 4) composed of two large domains: the V₀ domain is an integral membrane complex made up of five different types of subunits, some in multiple copies, and mediates proton translocation across the bilayer; the V₁ domain is a peripheral complex composed of eight different subunit types involved in converting the energy derived from ATP hydrolysis into the mechanical force required for proton translocation. By analogy with the mitochondrial ATPase, the V-ATPase is believed to function as a rotating motor to propel protons across the membrane. The most compelling evidence of the central role of V-ATPases in organelar acidification is provided by the use of macrolide antibiotics like bafilomycin A₁, which promptly and completely dissipate the pH gradient across all compartments of the secretory and endocytic pathways.

If similar, perhaps even identical, V-ATPases establish the pH gradient across all secretory organelles, what accounts for the differences in pH of the individual compartments? One obvious possibility is that the density of pumps varies among the organelles. Remarkably, to our knowledge, no systematic studies exist comparing the pump density of purified subcompartments of the secretory pathway. In contrast, there is a growing body of evidence supporting the notion that the intrinsic activity of individual V-ATPases is subject to regulation. Since both domains of the pump are required for proton translocation, disassembly of the complex is predicted to curtail its activity. Accordingly, in yeast, the degree of assembly of the V₀ and V₁ domains appears to be variable and acutely controlled by the availability of glucose, with up to 75% of the V-ATPase complexes becoming disassembled within minutes of hexose deprivation (19). Reassembly and reinitiation of proton transport occur when glucose is restored, validating the concept that V-ATPase activity can be reversibly controlled by assembly/disassembly. Further support of this notion was recently provided by the identification of the glycolytic enzyme aldolase as a regulator of the expression, assembly, and activity of V-ATPases (27). The interaction between this enzyme and the V-ATPase increases markedly in the presence of glucose, suggesting that aldolase may act as a glucose sensor for proton pump regulation. The RAVE complex (regulator of the H+-ATPase of the vacuolar and endosomal membranes), which consists of Skp1, Rav1, and Rav2, was also found to be a key regulator of V-ATPase activity by binding to the V₁ domain (40).

V-ATPase activity can seemingly be regulated
also when the holoenzyme undergoes redox changes. Disulfide bond formation between cysteines of the catalytic subunits of the V₁ domain has been reported to inhibit the ATPase (11). This control mechanism appears to operate in vivo, since fully assembled V-ATPases displaying varying levels of activity are found in different compartments. In agreement with this notion, a significant fraction of V-ATPases isolated from native clathrin-coated vesicles is present in the inactivated, oxidized state, whereas in synaptic vesicles the pumps are largely in the active, fully reduced state (35). Once formed, the inhibitory disulfide bond is not readily cleaved by normal cytoplasmic levels of reduced glutathione, indicating the possible involvement of an internal thiodisulfide rearrangement (13).

Modulation of the catalytic activity of the pumps by ancillary factors is yet another potential regulatory mechanism. Both inhibitory and stimulatory factors have been described but not thoroughly characterized. Finally, considering the complex subunit composition of the V-ATPases and the existence of multiple isoforms of some of these subunits, it is entirely conceivable that structurally and therefore functionally distinct types of pumps may exist in different organelles. The contribution of each of the variables described above has not been ascertained, but it seems increasingly likely that varying pump activity is a key contributor to establishing the gradient of pH along the secretory pathway.

Membrane potential and counterion permeability

V-ATPases translocate protons, generating an electrical potential (inside positive) across the organelar membrane (FIGURE 5, left). Although the magnitude of the potential has not been reliably measured, it is nevertheless expected that the buildup of positive charges will antagonize further inward transport of protons while promoting their outward "leak" (FIGURE 5, center). In principle, the maximum voltage attained as a result of pump activity can be calculated from thermodynamic considerations, knowing the transport stoichiometry and the energy of hydrolysis of ATP. However, thermodynamic equilibrium is not likely to be achieved, since the organelar membranes exhibit passive permeability to protons and to counterions. Their fluxes tend to collapse the electrogenic potential of the pump, reaching a steady state at an intermediate voltage that is dictated by the relative magnitudes of the rate of pumping and of the passive permeabilities. Using a null-point titration approach, Shapiro et al. (37) estimated the K⁺ concentration inside the Golgi to be 107 mM, somewhat lower than that of the cytosol. If the K⁺ conductance of the Golgi is confirmed to be sizable, as suggested by earlier determinations (12), this finding implies that the membrane potential across the Golgi membrane is modest, far from approaching the theoretical maximum.

In view of their abundance and comparatively high conductive permeability across other cellular membranes, K⁺ and Cl⁻ are favored candidates as counterions able to compensate the electrogenic displacement of protons by the V-ATPases of the secretory pathway (FIGURE 5, right). Indeed, available measurements suggest that these ions are rather permeable across normal secretory membranes (10, 37, 45, 46). Accordingly, increasing cation permeability further by addition of a conductive ionophore had little effect on Golgi pH, suggesting that the endogenous counterion permeability does not limit the rate of proton pumping (45).

Although in normal cells counterion permeability is seemingly not limiting, it was proposed that the secretory pathway of cells from cystic fibrosis patients is abnormally alkaline, due to the lack of functional CFTR (3, 4). Because CFTR is a Cl⁻-selective channel, these observations would imply that this anion constitutes the main source of counterion conductance. However, the findings of Barasch et al. have been challenged by other groups, who failed to see pH abnormalities in cystic fibrosis cells (7, 28).

In comparison with the plasma membrane, where extensive knowledge has been gathered, the identification of ion transporters in the secretory pathway lags sorely behind. Several classes of Cl⁻ channels have been shown to be expressed in intra-
underlying molecular entities, it is obvious that the counterion conductance of the membranes of the secretory pathway is substantial. Therefore, the prevailing membrane potential is likely to be low and, as such, a minor contributor to the electrochemical gradient generated by the proton pump. Instead, the chemical H+ concentration gradient is in all likelihood the predominant component of the proton-motive force. As for the membrane potential, the theoretical maximal H+ concentration gradient generated by the V-ATPases can be calculated on a thermodynamic basis, yielding luminal pH levels. These are clearly much more acidic than the pH values determined experimentally, suggesting that other factors prevent the system from approaching thermodynamic equilibrium at the steady state. The existence of a proton (equivalent) back-flux or "leak" is the most likely explanation for this discrepancy (FIGURE 5, center). Indeed, the presence of a sizable leak is most easily demonstrated by the addition of macrolide pump inhibitors such as concanamycin. The steady-state organellar acidification begins to dissipate almost immediately, at a rate that is indicative of the leak permeability and proportional to the luminal buffering power.

Proton "leak"

Despite our incomplete understanding of the underlying molecular entities, it is obvious that the counterion conductance of the membranes of the secretory pathway is substantial. Therefore, the prevailing membrane potential is likely to be low and, as such, a minor contributor to the electrochemical gradient generated by the proton pump. Instead, the chemical H+ concentration gradient is in all likelihood the predominant component of the proton-motive force. As for the membrane potential, the theoretical maximal H+ concentration gradient generated by the V-ATPases can be calculated on a thermodynamic basis, yielding luminal pH levels. These are clearly much more acidic than the pH values determined experimentally, suggesting that other factors prevent the system from approaching thermodynamic equilibrium at the steady state. The existence of a proton (equivalent) back-flux or "leak" is the most likely explanation for this discrepancy (FIGURE 5, center). Indeed, the presence of a sizable leak is most easily demonstrated by the addition of macrolide pump inhibitors such as concanamycin. The steady-state organellar acidification begins to dissipate almost immediately, at a rate that is indicative of the leak permeability and proportional to the luminal buffering power.

The exact mechanism(s) responsible for the proton (equivalent) leak are not at all well defined. NHE7, a recently identified isoform of the Na+/H+ exchanger family, has been localized to the TGN (32), but its role in pH regulation has not yet been established. Indeed, attempts to demonstrate Na+/H+ exchange activity in the secretory pathway have been unsuccessful (10). Ca2+ accumulates in the lumen of at least some secretory organelles, which could conceivably result from Ca2+/H+ exchange, but this mechanism remains to be...
demonstrated experimentally. Similarly, exchange of Cl\(^–\) for OH\(^–\) or HCO\(_3^–\) may contribute to the leak. Anion exchangers have been detected in the secretary (Golgi) compartment (16, 17), but direct evidence of any contribution to organellar pH homeostasis is lacking. In fact, omission of HCO\(_3^–\) appears to have little effect on steady-state pH.

Although no firm evidence exists for a role of electroneutral exchangers in secretory pH homeostasis, there are indications that a proton (equivalent) conductive pathway, possibly a channel, contributes to the leak. Demaurex and colleagues (10) found that the leakage of protons from the Golgi could be reduced by addition of micromolar concentrations of Zn\(^{2+}\). By analogy with Zn\(^{2+}\)-sensitive pathways described at the plasmalemma, these authors suggested that proton-conductive channels may exist in the membranes of secretory organelles. The proton-selective channel found in the plasma membrane of several cell types has very low unitary conductance and is exquisitely voltage and pH sensitive. These properties have not been defined in the case of the Zn\(^{2+}\)-sensitive pathway of the secretory pathway, but the high temperature coefficient of the plasmalemmal channel is shared by the Golgi proton leak (37).

Establishment and regulation of pH in the secretory pathway

An analysis of the contribution of the rate of pumping and of the leak to the establishment of pH can be performed even if the responsible molecular entities have not been fully defined. This systematic task was undertaken by Machen and his team (45). These authors analyzed in detail not only the absolute magnitude of the luminal pH of each subcompartment but also the rates of pumping and leakage and the contribution of counterion permeability to these processes. Machen and colleagues concluded that two main factors contribute to the development of a progressively more acidic luminal pH along the secretory pathway. First, they estimated that pump activity (possibly, but not necessarily, a reflection of the density of active pumps) increases from the ER to the Golgi and thereafter. Secondly, and perhaps more importantly, they determined that the leak decreased progressively, facilitating the retention of the protons pumped by the V-ATPase (FIGURE 6). These two effects synergize to ensure that the late stages of the secretory pathway (e.g., secretory granules) are much more acidic than the early ones (e.g., the ER). Variations in counterion permeability seem not to influence greatly the development of the pH gradient.

Is the pH of each one of the secretary organelles always the same, or is it modulated under physiological or pathological conditions? A recent report indicates that inositol 3,4,5,6-tetrakisphosphate can inhibit acidification of insulin-secretory granules (34). The polyphosphoinositol did not seem to affect V-ATPase activity in vitro, nor did it increase the rate of proton leakage, leading to speculation that it may exert its effects by inhibiting charge neutralization by blocking ClC channels. This has yet to be proven conclusively. In addition, activation of protein kinase C has also been noted to depress Golgi acidification (39). These observa-

![Figure 6](http://physiologyonline.physiology.org/)

**FIGURE 6. The balance of proton pumps and leaks dictates pH**

A: determination of the rate of proton (equivalent) leakage from various secretory compartments. Proton pumping was inhibited by a supramaximal dose of bafilomycin, and the lumen of each individual compartment was artificially acidified to pH 6.0 by using an ammonium prepulse. The rate of dissipation of the imposed acidification was used to estimate the passive permeability. B: model of the mechanism responsible for the establishment of differential pH levels along the regulated secretory pathway. The progressive acidification that develops from endoplasmic reticulum (ER) to Golgi to the secretory granules is generated by a gradual increase in the density of active H\(^+\) pumps, in parallel with a gradual decrease in proton (equivalent) permeability. Figure adapted from Ref. 45, with permission.
tions suggest that the pH of the secretory pathway may be under regulation by second messengers. Because of the multiplicity of steps controlled by the luminal concentration of protons (see above), biosynthesis, processing, and export of secretory products could conceivably be regulated acutely by changing the luminal pH. This tantalizing notion is, in our view, not entirely farfetched and certainly deserving of experimental testing.

In summary, despite its obvious importance, we know remarkably little about the physiology of pH regulation in the secretory pathway. More detailed molecular studies are required to establish the density of the pumps in the individual compartments and their subunit and isomorph composition. The conductive properties of the secretory membranes and the underlying molecular entities also need to be better understood. Last, but not least, we will need to find out whether the luminal pH is modulated under various physiological conditions, including stimulation by agonists or when cells enter the mitotic cycle. As in the past, breakthroughs in our understanding of these areas will likely come in the wake of the development of improved methods to monitor the properties of intact organelles in situ in a continuous and noninvasive manner.

Original work in our laboratory is supported by the Canadian Cystic Fibrosis Foundation and the Canadian Institutes of Health Research. P. Paroutis is supported by a fellowship from the Research Training Competition (RESTRACOMP) at the Hospital for Sick Children. S. Grinstein is the current holder of the Pitblado Chair in Cell Biology. S. Grinstein is the current holder of the Pitblado Chair in Cell Biology.

References


In Forthcoming Issue

**Emerging Topics:** First Encounter: How Pathogens Compromise Epithelial Transport

**Karl Kunzelmann**

Deciphering the Renal Code: Advances in Conditional Gene Targeting

**Susan Quaggin**

A Hypothesis About the Role of Adult Neurogenesis in Hippocampal Function

**Fred Gage**

The Architecture of the Active Zone in the Presynaptic Nerve Terminal

**Hugo Bellen**

Why Calcium-Stimulated Adenylyl Cyclases

**Daniel Storm**

Connexins: Gaps in our Knowledge of Vascular Function

**Brian Duling**

Potassium Channels in Cell Proliferation

**Luis Pardo**

A Two-Holed Story: Structural Secrets About Chloride Channels Become Unraveled

**Michael Pusch**

Sensing Chemoattractant Gradients

**Peter Devreotes**

The Nuclear Envelope and Human Disease

**Howard Worman**