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Ann. Rev. Biochem. 1986. 55:663–700
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ACIDIFICATION OF THE ENDOCYTIC AND EXOCYTIC PATHWAYS

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0066-4154/86/0701-0663$02.00

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SUMMARY AND PERSPECTIVES

Most of the membrane organelles in a typical eukaryotic cell belong to the elements of the exo- and endocytic pathways, referred to collectively as the vacuolar system. They include the endoplasmic reticulum, the Golgi complex, the secretory vacuoles, the endosomes, and the lysosomes and other organelles involved in biosynthesis, processing, transport, storage, release, and degradation of soluble and membrane-bound macromolecules. Both the inward- and the outward-directed pathways consist of a series of organelles through which material passes in an orderly and sequential fashion by means of specific transport vesicles and membrane fission and fusion reactions. Both pathways effect molecular sorting of soluble and membrane-bound components, and both pathways have in some cells the task of storage of molecules for subsequent mobilization. Mechanisms for membrane retrieval between the compartments are in place to ensure the balance in membrane flow. The pathways are, also, functionally interconnected at one or more levels.

An important similarity among most of the organelles of the vacuolar system is the presence of H^+-ATPases responsible for generating an internal acidic environment. The acidity of lysosomes and certain secretory vesicles such as chromaffin granules has long been known, but only recently has it become clear that many other vacuolar organelles such as endosomes, the Golgi complex, and coated vesicles are also acidic. In the endocytic pathway incoming material encounters progressively decreasing pH as it moves through the prelysosomal compartments towards the lysosomal compartment. A similar trend of progressively decreasing pH is seen in the exocytic pathways followed by many secretory products (i.e. from the endoplasmic reticulum to secretory vesicles). The properties of the proton pumps responsible for generating the proton gradients are similar in all these organelles; they are electrogenic, they show clear-cut substrate specificity for ATP, they are inhibited by low concentrations of *N*-ethylmaleimide, and judging by their insensitivity to vanadate they may not have a phosphorylated intermediate. The composite data indicates that the H^+-ATPases encountered in the vacuolar system are distinct from the mitochondrial F_1F_0-type ATPases as well as the plasma membrane-type H^+-ATPases found in the gastric mucosa, yeast, and other fungi. We call these pumps "vacuolar H^+-ATPases," and describe, in this review, their properties in some detail.

The pH in the various organelles differs, and the functions of acidity are complex and variable. In lysosomes, the low pH provides favorable conditions for enzymatic hydrolyses. In chromaffin granules, the proton gradient is used as an energy source for the coupled transport of biogenic amines. In receptor-mediated endocytosis the difference in pH between the endosome and the extracellular environment is used by the cell to provide asymmetry to the

recycling circuit between the two compartments. The pH difference allows, in this case, incoming receptors, ligands, and fluid-phase components to display different properties in intra- and extracellular compartments. Exposure to the endosomal low pH induces conformational changes which in turn can lead to the dissociation of receptor-ligand complexes, changes in ligand solubility, activation of latent activities, etc. In many cases these alterations determine the subsequent sorting and fate of the incoming molecules. The principle of acid activation in the endocytic pathway is exploited by viruses and bacterial toxins whose penetration into the host cell cytoplasm is triggered by acid-induced conformational changes.

The major challenges are now to identify and characterize the vacuolar H^+-ATPases, to determine how they are targeted to the various organelles of residence, and to elucidate how the pH in these organelles is regulated. It will also be important to determine how receptors and other migratory molecules respond to acidity at a molecular level, and to determine what role the decreasing pH and possibly other differences in ionic composition have when considered from the point of view of entire pathways.

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THE ORGANELLES OF THE EXO- AND ENDOCYTIC PATHWAYS

The Endocytic Pathway

Endocytosis is the general term used for the internalization of extracellular fluid or particles by invagination and pinching off of the plasma membrane (for reviews see 1–7). Endocytosis serves an important role in uptake of nutrients, scavenging of extracellular material, and in the internalization of receptor-bound ligands such as hormones, growth factors, lipoproteins, and antibodies. A distinction is usually made between internalization of large particles *(phagocytosis)* and small particles, solutes, and fluid *(pinocytosis)*. The molecular processes underlying phagocytosis and pinocytosis are different and can be operationally distinguished. Phagocytosis is particle-activated and cytochalasin-sensitive, and it is usually a property of professional phagocytic cells (amoebae, macrophages, polymorphonuclear leukocytes, etc). Pinocytic uptake, on the other hand, is continuous even in the absence of added ligands. It is cytochalasin-resistant, and it is observed in variable extent in nearly all cell types.

The organelles involved in phagocytosis are the plasma membrane, the prelysosomal phagocytic vacuoles—*the phagosomes*—, and the fusion product between a phagosome and one or more primary or secondary lysosomes—*the phagolysosomes* (Figure 1). Of these the phagolysosomes are definitely acidic as first demonstrated by Metchnikoff in 1893 (8), who fed litmus paper to protozoa and observed it changing color from blue to red. It is likely, however,

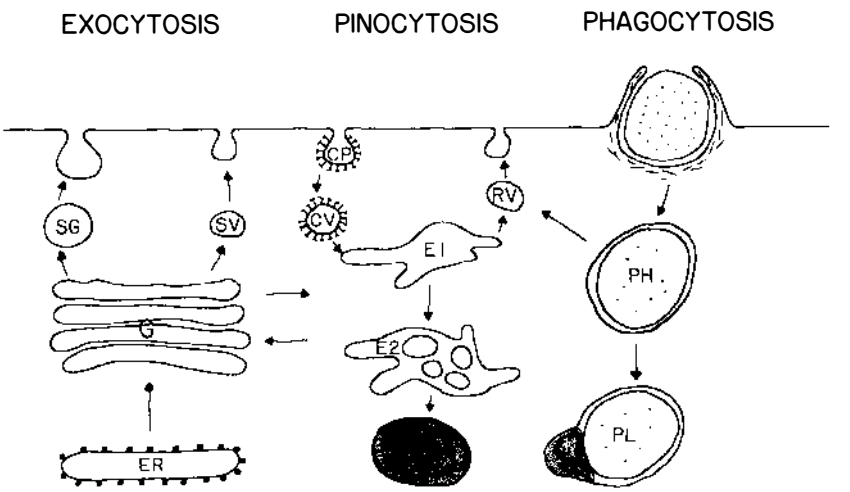

Figure 1 A schematic view of membrane traffic in the vacuolar system. Abbreviations used: ER, endoplasmic reticulum; SG, secretory granule; SV, secretory vesicle; L, lysosome; E1, peripheral endosome; E2, perinuclear endosome; CV, coated vesicle; RV, recycling vesicle; CP, coated pit; PL, phagolysosome; PH, phagosome. The exocytic pathway branches within the Golgi complex into a regulated route (SG) and a constitutive route (SV).

that phagosomes begin to become acidic even before they fuse with a lysosome (9–11).

The general pathways of pinocytosis are also schematically depicted in Figure 1. *Coated pits* and *coated vesicles* constitute major vehicles for pinocytic uptake of both fluid, membrane-bound ligands, and receptors (see 4, 5, 7, 12). Pathways involving uncoated vesicles may also exist (13–18), but information about their relative importance is lacking. Whereas isolated coated vesicle preparations have been found to contain proton ATPase of the vacuolar type (19, 20), it remains unclear whether the ATPase is present specifically in the endocytic coated vesicles (21–23).

The *endosomes* constitute the next station in the pathway (24–34). These relatively recently discovered prelysosomal organelles (26) represent a heterogeneous, complex set of vacuoles located in the peripheral and perinuclear cytoplasm. The endosomes (or subpopulations thereof) have also been called receptosomes (27), CURL (28), endocytic vesicles (30), etc. They consist of complex vacuolar and tubular elements, and many have the appearence of multivesicular bodies (30a). They constitute the main site for sorting of endocytosed material and membrane recycling in the cell. For recent reviews the reader is referred to Ref. 25, 31–34. Endosomes are acidic (35–42), and the acid pH is crucial for many of their functions.

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The secondary *lysosomes*, the main intracellular digestive compartment, constitute the terminal compartment of the pathway. With a pH of 4.5–5.0, the lysosomes are the most acidic organelles in animal cell (43–45).

The Exocytic Pathways

The exocytic pathways are responsible for secretion, posttranslational modification, and transport of lipid and protein to the plasma membrane, the lysosomes, and the organelles of the vacuolar system. The main organelles are the *endoplasmic reticulum*, the *Golgi apparatus*, and *secretory granules* (Figure 1). The secretory granules are either part of a constitutive exocytic pathway, or they belong to regulated pathways where exocytosis is triggered by specific stimuli (45a). The pH of the endoplasmic reticulum is not known, but it is usually assumed to be close to neutrality. It is becoming increasingly clear that elements of the Golgi complex contain proton pumps, and are mildly acidic (22, 23, 46). Many exocytic granules of the triggered type contain vacuolar ATPases, and acidity is important for their proper function (47–50). Figure 1 does not depict the various coated and noncoated transport vesicles thought to function as carriers between organelles, some of which may also be acidic (19, 20, 23).

CHARACTERISTICS OF PROTON ATPases

There are three general categories of proton ATPases in eukaryotic cells: ATPases of the mitochondrial-bacterial F_1F_0 class, the plasma membrane proton ATPases, and the vacuolar ATPases. They are easily distinguished on the basis of their sensitivities to a variety of inhibitory agents as well as on the basis of their general functional characteristics. This information is summarized in Table 1.

Proton ATPases of the F_1F_0 class occur in the inner mitochondrial membrane, in chloroplasts, and (in prokaryotes) in the plasma membrane (for review, see Refs. 61, 69). While the precise subunit compositions of F_1F_0-type enzymes can vary, their general structural features are highly conserved. They consist of a water-soluble domain referred to as F_1, and a hydrophobic transmembrane domain, F_0. F_1 is composed of at least five polypeptide chains ranging in molecular mass from <10 kd to 50–60 kd (the total molecular mass is approximately 380 kd) (61, 70). In contrast, the F_0 domain consists of at least three small subunits (each <20 kd) and presumably represents the transmembrane proton channel (70). The F_1 complex is responsible for the ATPase and ATP-synthase activities. The reaction cycle of F_1F_0 ATPases does not appear to involve the formation of a phosphoenzyme intermediate.

Plasma membrane proton ATPases are typified by the proton pumps found in the plasma membranes of *Neurospora* and yeast, where their function is

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Table 1 Characteristics of the proton ATPases

Inhibitor[c]	Mitochondrial F_1F_0	ATPase CLASS Plasma membrane Gastric	Plasma membrane Fungal	Vacuolar
Oligomycin	+	-		–
Efrapeptin	+	–		–
Azide	+	–	–	
NBD-Cl^-	+	–	–	+[a]
DCCD	+			+[b]
Vanadate	–	+	+	–
Ouabain				
NEM	–	–	–	+
Tributyl tin	+	–	–	+
DIDS	?	–	–	+
Zn^{2+}	?	?	?	+
Nitrate	?	?	?	+
Properties				
Molecular mass (kd)	320–390	94–115	100	80–116?
No. of subunits	8–10	1	1	?
Electrogenic	+	–	+	+
Anion stimulation	Cl^-	–	Cl^-	Cl^-
Cation stimulation	–	K^-	–	
Mg^{2+} dependence	+	+	+	+
Phosphorylated intermediate	–	+	+	?
References	(54, 58, 61, 62, 70)	(54, 64, 66, 71–73)	(51, 52, 63)	(51, 53, 55, 57, 59, 60, 65, 68)

[a] Concentrations required for inhibition are at least 10-fold lower than those required to inhibit ATPases of the F_1F_0 class.

[b] Generally, DCCD concentrations needed for inhibition are at least 10-fold higher than those required to inhibit ATPases of the F_1F_0 class.

[c] Abbreviations used: NBD-Cl^-, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; DCCD, dicyclohexylcarbodiimide; NEM, N-ethylmaleimide; DIDS, 4,4'-diisothiocyanostilbene-2,2-disulfonic acid.

analogous to that of the Na^+,K^+-ATPase of the mammalian plasma membrane: i.e. generation of a transmembrane electrical potential (52, 57, 63). In mammals, the only known proton ATPase of this type is the K^+,H^+-ATPase of the gastric parietal cell whose major function is the secretion of acid into the stomach (64, 66). Plasma membrane proton pumps form stable phosphorylated intermediates and are sensitive to inhibition by the transition-state analogue sodium orthovanadate (71, 72). Thus, these proton pumps are similar in

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reaction mechanism and inhibitor sensitivity to other well-studied ion transport ATPases, such as the Na^+,K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase. While these enzymes can all be considered to belong to the same general class of ATPases, it should be pointed out that the proton ATPases (as well as the Ca^{2+}-ATPase) are insensitive to ouabain, a potent inhibitor of the Na^+,K^+-ATPase.

Both the fungal proton ATPase and the gastric mucosa K^+,H^+-ATPase have been isolated and found to consist of a major 95–115-kd polypeptide chain which contains the catalytic portion of these molecules and is phosphorylated during the reaction cycle (64, 68). A tryptic peptide containing the fluorescein isothiocyanate (FITC)–binding site—presumably the ATP-binding site—of the H^+,K^+-ATPase is closely related to the corresponding regions of the Na^+, K^+-, and Ca^{2+}-ATPases, whose complete amino acid sequences have recently been deduced from cloned cDNAs (73–75).

While the plasma membrane proton ATPases may have similar structures and reaction mechanisms, their ion transport characteristics differ. Proton transport by the H^+,K^+-ATPase across the parietal cell plasma membrane into the stomach lumen is molecularly coupled to the transport of K^+ from the stomach lumen into the parietal cell: i.e. ATP drives an obligatory electroneutral exchange of K^+ for H^+ (64, 66). Na^+ cannot substitute for K^+. In contrast, proton translocation via the fungal plasma membrane ATPase does not require direct coupling to another cation (or anion) and is thus definitely electrogenic (52, 63).

The vacuolar proton ATPases are distinguished from the other two classes by virtue of their inhibitor specificities, lack of coupling to counter-ion transport (Table 1), and intracellular distribution. They are not inhibited by oligomycin, efrapeptin, azide, aurovertin (mitochondrial inhibitors), nor by classical inhibitors of the plasma membrane—type ATPases, such as vanadate and ouabain. The only known "specific" inhibitors are the alkylating reagents such as *N*-ethylmaleimide (NEM) and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (19, 20, 36, 47, 60). While these agents will inhibit ATPases of other classes, they are effective against vacuolar ATPases at relatively low concentrations (e.g. complete inhibition by NEM can be obtained at 10 μM) (50). Conversely, dicyclohexylcarbodiimide (DCCD), which is a potent inhibitor of F_1F_0 ATPases at micromolar concentrations, is only effective against vacuolar ATPases in the millimolar range (19, 36, 76). Dauromycin, an antibiotic with some detergentlike properties, has also been reported to be a selective inhibitor of the vacuolar enzymes (77).

Vacuolar ATPases appear to be electrogenic, proton translocation proceeding without direct molecular coupling to other cations or anions (unlike the K^+,H^+-ATPase of the gastric mucosa, see above). As a result, acidification is accompanied by the generation of an interior positive membrane potential.

Since this potential difference prevents the continued translocation of protons into the vesicle lumen, it must be dissipated by the influx of external anions (negative charges) or the efflux of internal alkali cations (positive charges). A diagram illustrating the ion fluxes that may accompany vacuole acidification is given in Figure 2. A more detailed description of the proton ATPases found in endocytic and secretory organelles will be given below.

IDENTIFICATION OF ACIDIC VACUOLAR ORGANELLES

A variety of biochemical, functional, and morphological approaches have been used to identify acidic compartments and to measure vacuole acidification in intact cells and cell-free systems. Clearly, organelles for which all three types of data exist constitute the most convincing demonstrations. At present, these include lysosomes, endosomes, and certain secretory granules. In this section we summarize the general methods used to study organelle acidification in order to establish the criteria that have been used to identify vesicles with a low pH.

Measurement of Vacuole Acidification

LIPOPHILIC WEAK BASES The most common and convenient method to study vacuolar acidification relies on the use of lipophilic weak bases. These agents are membrane-permeant when uncharged at neutral pH and relatively membrane-impermeable once protonated. Thus, if allowed to equilibrate with either intact cells or isolated organelles, weak bases will accumulate within membrane vesicles that have acidic internal pH. In quantitative terms, the degree of accumulation will depend on the magnitude of the transmembrane pH gradient and on the total internal volume of the acidic vesicles.

Biochemical determinations of acidification using lipophilic weak bases are

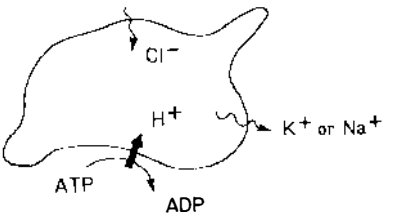

Figure 2 Mechanism of ATP-dependent acidification of endocytic and secretory organelles. A vacuolar ATPase catalyzes the electrogenic translocation of protons into the vacuole creating both a positive internal membrane potential and a low internal pH. The membrane potential is dissipated by passive influx of permeant cytoplasmic anions (Cl^-) or efflux of internal cations (K^+).

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generally made in one of two ways. First, optical measurements are possible using dyes such as acridine orange or 9-amino acridine (19, 47, 67, 78). These dyes exhibit characteristic alterations in their absorbance spectra and fluorescence intensity as a function of concentration. Consequently, acidification can be followed by the quenching of acridine fluorescence which occurs as the dye becomes concentrated within acidic vesicles. The fluorescence quenching of these dyes results from the concentration-dependent increase in resonance energy transfer among individual acridine molecules. While neither fluorescence nor absorbance measurements can be converted into actual pH values, radiolabeled weak bases such as ^{14}C-methylamine can be used to obtain quantitative data (20, 76, 79, 80). Following equilibration with the labeled base, the vesicles are collected by centrifugation (or filtration) and the radioactivity determined. Since it is also possible to measure the internal volume of the vesicle population, one can obtain the intravesicular concentration of the pH probe and estimate the internal pH.

While rapid and convenient, these methods are limited by the fact that they are nonselective and thus only as reliable as the homogeneity of the organelle fraction used. Even a slight contamination could yield aberrant results if the contaminating membranes contained a proton pump and accounted for a disproportionately large fraction of the total intravesicular volume.

The ability of lipophilic weak bases to partition into any vesicle with a low internal pH has been exploited to allow the morphological identification of acidic organelles in intact cells. At the level of the fluorescence microscope, acridine orange has long been used for this purpose. This approach has been particularly useful in tissue culture cells where it is relatively specific for lysosomes and phagolysosomes, the most acidic intracellular compartments (81, 82). More recently weak bases such as the probes dinitro-amino-phenyl (DAMP) and primaquine (21–23) have been developed for use in conjunction with electron microscopy. High-affinity antibodies to dinitrophenol or primaquine (respectively) allow the localization of these probes by immunocytochemistry using the immunoperoxidase technique or using protein A–gold conjugates on ultrathin cryosections or Lowacryl sections.

SELECTIVE LABELING WITH ENDOCYTIC TRACERS Specific methods have been developed to study the acidification of endocytic organelles by selectively introducing pH probes into endosomes or lysosomes by endocytosis. This approach was first introduced by Ohkuma & Poole (44), who found that fluorescein isothiocyanate–labeled dextran (FITC-dextran), a macromolecular marker of fluid-phase pinocytosis, could also be used as an intracellular pH probe. The intensity and excitation spectrum of fluorescein fluorescence are titratable functions of pH. Therefore, standard curves can be constructed relating fluorescence intensity (or excitation maxima) and pH. The relationship

between fluorescence and pH is also independent of the concentration or ionic environment of the fluorochrome. By incubating cultured macrophages for several hr at 37°C in medium containing FITC-dextran, the marker was selectively localized in lysosomes. Since the dextran polymer is not a substrate for lysosomal hydrolases, the marker remained intralysosomal almost indefinitely. Extracellular FITC-dextran was removed, the cells transferred to a spectrofluorometer, and the intralysosomal pH estimated to be approximately 4.8 (44). Addition of weak bases (e.g. ammonium chloride, chloroquine) or the carboxylic ionophores nigericin or monensin rapidly (in <30 sec) increased the amount of intracellular fluorescence to a level close to the pH of the external medium, reflecting the dissipation by these agents of the pH gradient across the lysosomal membrane (see below). Removal of the inhibitors from the medium resulted in the restoration of the acidic pH, indicating that the lysosomes were capable of re-acidification, in a fashion that appeared to depend on cytoplasmic ATP concentration (83). Recently, this approach has been used to study acidification of endocytic vesicles in cell-free systems (see below).

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FUNCTIONAL MEASUREMENTS OF VESICLE ACIDIFICATION A third general approach to identify acidic compartments makes use of acid-sensitive properties of incoming ligands or the intrinsic enzyme activites present in the organelles of interest. For example, the known effect of low pH on the fusogenic activity of enveloped animal viruses has been exploited to identify endosomes as the first acidic compartment encountered by newly internalized virus particles (38) (see below). The pH in lysosomes has been assessed using amino acid methyl esters, which are hydrolyzed by the acid esterases and specifically accumulate in the lysosomes (84).

Functional assays for acidification have also been developed for a variety of acidic secretory granules, such as chromaffin granules and platelet dense granules (50, 85, 86). Each of these organelles has a transporter to facilitate the uptake and concentration of biogenic amines in response to a pH gradient (see below). By monitoring the accumulation of labeled serotonin or 5-hydroxytryptamine, one can obtain an indirect but accurate measure of granule acidification. It is important to note that even if the granule fraction is contaminated by other acidic organelles, the coupled assay ensures that only granule acidification will be measured, since only the granule membrane possesses the amine transporter.

ACIDIFICATION OF ENDOCYTIC AND SECRETORY VACUOLES

Lysosomes

A number of laboratories have shown that in intact cells, the intralysosomal environment is maintained at a pH of 4.6–5.0 (9, 35, 43, 44). It is now

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well-established that the low pH is not caused by a Donnan potential for protons, as originally thought (for detailed review see 45), but is due to the activity of a proton ATPase (56, 60, 76). Among the first and most convincing demonstrations was provided by Ohkuma and coworkers (60). Rats were given parenteral injections of FITC-dextran which accumulated in lysosomes following endocytosis. Enriched lysosome fractions from the liver were prepared and acidification determined using the intralysosomal FITC-dextran as a pH probe. The lysosomes retained an internal pH of 5.0 after isolation and were shown to have a finite but low intrinsic permeability to H^+, K^+, Na^+. Addition of Mg^{2+}-ATP led to a rapid decrease in internal pH which was insensitive to oligomycin, NaN_3, DCCD, and vanadate but could be completely blocked by 1 mM NEM. Substitution of Mg^{2+} by Cu^{2+} or Zn^{2+} also completely prevented acidification. GTP was found to support acidification activity nearly as well as ATP, although the possible role of nucleoside diphosphokinase activity which could interconvert GTP and ATP was not assessed. The external cation composition (Na^+, K^+, or choline) had no significant effect while permeant external anions potentiated ATP-dependent proton translocation. The anion conductance exhibited selectivity for $Br^- \geq Cl^- >$ phosphate $> F^-$, sulfate. Acidification was reduced in the absence of external anions.

While these results were compatible with an electrogenic mechanism for the proton ATPase (Figure 2), they did not exclude the possibility of electroneutral transport of protons and anions. Although initial reports suggested that protons may be cotransported with external phosphate anions (76), recent evidence has demonstrated that lysosomal acidification is electrogenic (56, 59, 87). Using the potential sensitive fluorescent dye Di-S-C_3 (5), Harikumar & Reeves (56) showed, for example, that rat kidney lysosomes developed an interior positive membrane potential after addition of ATP. The presence of a permeant external anion (Cl^-) abolished the effect. By preventing the development of an electrical gradient for protons, external anions facilitate the development of a greater chemical gradient for protons, i.e. a more acidic intralysosomal pH.

The lysosomal proton pump has not yet been isolated. However, Reggio et al (81) identified an antibody to lysosomal membranes that recognizes a 100-kd polypeptide cross-reactive on Western blots with a preparation of the 100-kd H^+/K^+-ATPase isolated from hog gastric mucosa. Whether this antigen is in fact the lysosomal proton ATPase remains to be determined. These results do suggest, however, the interesting possibility that although on mechanistic grounds the H^+/K^+-ATPase and the lysosomal proton ATPase fall into distinct classes of enzyme, at the molecular level they may be related.

Endosomes

The first direct evidence indicating that endosomes are acidic was provided by Tycko & Maxfield (35). FITC was coupled to alpha-2 macroglobulin and incubated with cultured fibroblasts at 37°C to permit its receptor-mediated

endocytosis. Incubation times were brief in an attempt to ensure that most of the internalized ligand was selectively localized in endosomes. Individual cells were analyzed by fluorescence microscopy at two excitation wavelengths and the FITC-labeled alpha-2 macroglobulin found in acidic compartments within 15–20 min after internalization. Since little of the ligand was expected to have entered lysosomes at this time point, it appeared likely that acidification occurred in a prelysosomal compartment, i.e. endosomes.

Similar findings have been made using a variety of other FITC-conjugated receptor-bound ligands and markers of fluid phase endocytosis. In addition to single-cell measurements made by fluorescence microscopy (39–41, 88), endosome acidification has also been monitored in cell suspensions by fluorescence spectrophotometry (37, 41a) and flow cytofluorometry (42). Experiments using FITC-labeled transferrin have been particularly conclusive because this ligand enters the endosomal compartment but escapes transport to lysosomes (37) (see below).

While it is difficult to convert optical measurements of FITC fluorescence in intact cells to actual pH values (10), a consensus appears to have been reached that endosomes maintain an internal pH of between 5 and 6, i.e. somewhat less acidic than lysosomes. Part of the difficulty in establishing a more precise figure reflects the likelihood that endosomal pH may vary over a considerable range, even within the same cell. For example, internalized pH-sensitive markers often appear to be transferred from less acidic to progressivley more acidic endosomes as a function of time after endocytosis (10, 41a, 42, 88). The method and cell type used for pH measurements may also influence the results. For example, using spectrofluorometric measurements, FITC-transferrin was found to reach endosomes of pH 5–6 in K562 cells (37). However, in Chinese hamster ovary (CHO) cells, single-cell measurements made using fluorescence microscopy do not detect FITC-transferrin in vesicles of pH less than 6.5 (39).

Like lysosomes, endosomes lower their internal pH by an ATP-dependent mechanism. This was first demonstrated using crude fractions of endosomes which had been selectively labeled with FITC-dextran (36, 41a). Galloway et al (36) established that the endosomal proton pump was distinct from the F_1F_0- and plasma membrane-type proton ATPases, but very similar to the lysosomal ATPase. Its inhibitor sensitivity is typical for vacuolar ATPases (Table 1). Similar results have been obtained using fluorescent pH probes coupled to ligands for receptor-mediated endocytosis, such as alpha-2 macroglobulin (40), asialoorosomucoid, and transferrin, except that the endosomal membrane has a high permeability for protons (R. Fuchs, I. Mellman, in preparation). Thus, endosomes labeled by markers of fluid phase endocytosis and by receptor-mediated endocytosis are functionally equivalent with respect to their capacity for ATP-dependent acidification.

In recent work, we have further characterized the mechanism of endosome

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acidification using highly purified fractions of rat liver endosomes obtained by free flow electrophoresis and sucrose density gradient centrifugation (R. Fuchs, I. Mellman, in preparation; M. Marsh, S. Schmid, H. Kern, E. Harms, I. Mellman, A. Helenius, in preparation). The bioenergetics of ATP-dependent proton transport in endosomes were found to be very similar to those of lysosomes, except that the endosomal membrane seemed somewhat more permeable to protons and K^+, and less permeable to Cl^-. Unlike lysosomes, however, GTP did not substitute for ATP. Endosome acidification was also shown to be electrogenic.

Analogous results have been obtained for two other classes of nonlysosomal endocytic vesicles. ATP-dependent acidification of FITC-dextran-labeled vesicles isolated from rat kidney proximal tubule also appears to be electrogenic and largely inhibitable by NEM (65). In contrast to endosomes from rat liver, fibroblasts, and macrophages, the proton ATPase of these kidney vesicles may be directly stimulated by Cl^-. Similarly, "multivesicular bodies," endocytic organelles isolated from the livers of estradiol-treated rats, have been shown using nonselective pH probes to possess an electrogenic proton ATPase similar to the proton pumps of endosomes and lysosomes (67).

Taken together, these studies show that the ability of endosomes to establish and maintain a proton gradient of one-to-two orders of magnitude requires not only the activity of an NEM-sensitive, ATP-driven electrogenic proton pump, but also a regulated permeability of the endosomal membrane to certain anions and/or cations.

Coated Vesicles

Considerable interest has been generated by reports that, like endosomes and lysosomes, coated vesicles contain an ATP-driven proton pump. Coated vesicle fractions prepared by standard techniques from bovine brain (19, 20, 90) and from rat liver (89) have been examined. ATP-dependent acidification occurs via a typical vacuolar proton ATPase (Table 1). As described above for endosomes and lysosomes, proton translocation in coated vesicle fractions also appears to be electrogenic, with acidification being favored by the presence of external permeant anions (Cl^-, Br^-, but not gluconate) or by voltage clamp conditions (internal and external K^+ in the presence of valinomycin).

Racker and colleagues have recently been able to solubilize the active ATPase from bovine brain coated vesicle membranes using deoxycholate (91). Functional reconstitution into liposomes was also accomplished from the crude solubilizate; interestingly, the addition of phosphatidyl serine (PS) was found to stabilize the enzyme during solubilization. Partial purification of the deoxycholate-solubilized ATPase has indicated that the ATPase activity copurifies with a polypeptide of 116 kd (91). Unfortunately, it has not yet been possible to reconstitute the purified ATPase into liposomes. Therefore its final

identification and the identification of other polypeptides that may be part of the proton pump must await further investigation.

While considerable information has been obtained describing coated vesicle acidification, some basic problems remain. First, the intracellular origin of the vesicles used is unknown. For example, it is not clear what fraction of the vesicles are endocytic or Golgi-derived. Coated vesicle fractions have recently been shown to consist of biochemically and immunologically distinct subpopulations (92, 93). Accordingly, the implication that proton pumps in the plasma membrane are internalized in coated vesicles and subsequently delivered to endosomes, although possible, is without experimental support. Indeed, evidence obtained using the cytochemical probes of intravesicular pH (DAMP, primaquine; see above) suggests that peripheral or endocytic coated vesicles do not have an acidic internal pH (21–23). In contrast, Golgi-region coated vesicles often label with these reagents. A second major problem concerns the purity of coated vesicle preparations. When purified by established techniques, they may be contaminated by proton pump–containing uncoated membranes such as submitochondrial particles (94) or endosomes.

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Golgi Complex and Endoplasmic Reticulum

Since certain carboxylic ionophores (e.g. monensin) and in some instances lipophilic weak bases can disrupt normal Golgi function (see below), it has been suspected that Golgi vesicles and cisternae might maintain an acidic internal pH. Direct evidence that Golgi membranes are capable of ATP-dependent acidification first came from in vitro studies of rat liver Golgi fractions (47, 95). Glickman et al (47) showed that the Golgi-associated proton pump was a typical vacuolar ATPase and that Golgi membranes contained a discrete Cl^- conductance. Interestingly, similar data were obtained for fractions of rat liver rough and smooth endoplasmic reticulum (96).

Independent confirmation that elements of the Golgi may contain a proton pump was provided by electron microscopic immunocytochemistry. Using the pH-sensitive cytochemical probes DAMP and primaquine, the acidic internal pH of Golgi-associated vesicles and, occasionally, the *trans*-most Golgi cisterna, has been obtained in a number of cell types (21–23). Localization of these probes within elements of the endoplasmic reticulum has not been observed, suggesting that this organelle does not possess a proton pump or that the endoplasmic reticulum ATPase is used to establish electrical as opposed to pH gradients.

Secretory Granules

Most of the secretory granules studied thus far have been found to have an acidic pH and an ATP-driven proton pump. These include: chromaffin granules from adrenal medulla (97, 98), platelet dense granules (53) neurosecretory granules from the pituitary (78), and cholinergic synaptic vesicles (100, 101).

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In contrast, the storage granules of the rat parotid gland are thought not to be acidic or to contain a proton ATPase (102). While the functions of low intragranular pH may vary (see below), it is clear that the proton ATPase of each of these organelles closely reflects the properties of the ATPases already described for lysosomes, endosomes, coated vesicles, and Golgi membranes (Table 1).

Acidification has been most extensively studied in the bovine adrenal chromaffin granule. While the ATPase of these granules was originally thought to be of the F_1F_0 class, the work of Nelson and colleagues (98, 103) has demonstrated that chromaffin granule ATPase is a typical vacuolar proton pump. Chromaffin granule acidification is NEM-sensitive, electrogenic, and attains an internal pH of ~5.7, as measured by ^{14}C-methylamine partitioning and ^{31}P-ATP nuclear magnetic resonance (104).

Chromaffin granules are a source for purification of the ATPase since inhibition of ATP-dependent proton translocation by NEM correlates well with the inhibition of up to 70% of the ATP hydrolytic activity associated with the purified membranes (103; G. Dean, P. Nelson, G. Rudnick, submitted for publication). These results suggest (but do not prove) that the major ATPase activity associated with chromaffin granules is due to the proton pump. The ATPase has been solubilized in cholate and $C_{12}E_9$ and functionally reconstituted into liposomes (103). As is the case with the coated vesicle ATPase, addition of phosphatidyl serine stabilizes the detergent-solubilized enzyme. Partial purification of the granule proton pump has been accomplished, and NEM-sensitive ATPase activity copurifies with four polypeptides ranging in molecular mass from 115 kd to 20 kd (105, 105a; G. Dean, C. Galloway, I. Mellman, G. Rudnick, unpublished results). A photoactivatable ATP analogue, 3'-*O*-(4-benzoyl)benzoyl-ATP, specifically labels only the 98-kd polypeptide (C. Galloway, G. Dean, G. Rudnick, I. Mellman, unpublished results). In contrast, NEM labels the 115-kd and 57-kd polypeptides. Final identification of the polypeptides that comprise the ATPase must await its complete purification and functional reconstitution.

One other acidic secretory vesicle that deserves note is found in various urinary epithelia, such as the amphibian bladder and the mammalian collecting duct (82, 106). These vesicles contain a vacuolar-type proton ATPase which can be inserted into the apical plasma membrane by exocytic fusion, resulting in urinary acidification. A potent stimulus for the secretory event appears to be increased carbon dioxide concentrations (107). Once inserted into the cell surface, these proton pumps can be retrieved by endocytosis and apparently stored in the intracellular vesicles. This system provides the only example of regulated translocation of proton ATPases from one compartment to another. It also constitutes the only direct evidence, by electrophysiological measurement, that vacuolar ATPases can be present on the plasma membrane.

In a preliminary report, the kidney ATPase has been partially purified from

CHAPS-solubilized membranes (107a). Functional reconstitution into liposomes was reported and proton pumping activity found to copurify with several polypeptides, the largest of which was 80 K.

Yeast and Plant Vacuoles

Many fungal and plant cells have intracellular vacuoles that maintain an acidic internal pH (for review, see Ref. 50). Best studied are the vacuoles of yeast and *Neurospora*, which have been isolated and found to contain an ATP-dependent proton pump (51, 57). It is now clear that the yeast vacuole is analogous to the mammalian cell's lysosome, since it contains an array of hydrolytic enzymes and continuously receives extracellular macromolecules internalized by endocytosis (108). Like mammalian lysosomes, ATP-driven acidification of isolated yeast vacuoles is sensitive to NEM, high concentrations of DCCD, Cu^{2+}, and Zn^{2+}.

Uchida et al (68) recently reported the isolation of the yeast vacuole ATPase. The purified enzyme consisted of three polypeptides of 89 kd, 64 kd, and 19.5 kd. While these polypeptides differ somewhat from those reported for the putative coated vesicle and chromaffin granule ATPases, the possibility of proteolysis by vacuole hydrolases has not yet been eliminated. Moreover, the purified yeast enzyme has not yet been reconstituted, so its identification remains tentative.

Partial purification of the vacuole ATPase from corn coleoptyles has also been accomplished (108a,b). In this case, however, functional proton pumping activity is associated with an 80 K polypeptide.

It is clear that there is no consensus regarding the structure of the proton ATPase from any single source. Thus, determining the structural relationships of proton pumps from different acidic organelles must await considerable additional information.

Regulation of Intravesicular pH

If the same or similar proton pumps are present in all endocytic and exocytic membranes, how is their internal pH regulated? As discussed above, there is ample evidence that different endosomal compartments may have different pH's, and that endosomes and secretory granules have a less acidic pH than lysosomes. Regulation could occur by controlling the number of ATPase molecules present per organelle, or by modulating the activity (e.g. by phosphorylation or some other covalent modification) of a constant number of ATPases. Alternatively, the observations that many organelles have defined permeabilities to anions, protons, and other cations suggests that net proton flux and accumulation can be controlled by regulating the ion permeability characteristics of each membrane. For example, rat liver endosomes appear more permeable to K^+ and less permeable to Cl^- than rat liver lysosomes. Therefore,

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the endosomes may be less acidic because they may be less able to accumulate protons (limited Cl^- permeability will favor the development of a membrane potential, limiting proton accumulation) and less able to retain protons (cytoplasmic K^+ can more easily exchange with internal H^+ if the conductance to both ions is significant).

INHIBITORS OF VACUOLAR ACIDIFICATION IN INTACT CELLS

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In addition to their use as nonselective pH-probes in vitro, lysosomotropic, or more accurately "acidotropic" (109), weak bases are widely used to elevate the pH within acidic vacuoles of living cells. Agents such as ammonium chloride, chloroquine, and methylamine are relatively lipophilic in their unprotonated form and they penetrate the membranes of cells and vacuoles. Upon entering an acidic environment they become protonated and too polar to escape rapidly through the membrane. The equilibrium thus favors accumulation within the organelles (109, 110). The increase in vacuolar pH, which can amount to as much as 1–2 pH units and which occurs within a minute or two after addition of the agents to the medium, is due to the neutralization of protons by the weak base and possibly to the loss of the buffering capacity (45, 110). The effect of the acidotropic weak bases depends on the concentration of the drugs, acidity of the organelles, and on the pH of the medium. When more than one base is present the effects are additive.

The main cellular effects of acidotropic weak bases are related to the increase in vacuolar pH. Lysosomal degradation by acidic hydrolases is inhibited (109–113), molecular sorting and recycling in endosomes is affected (114–116), virus and toxin entry is blocked (117–119), and exocytosis in the regulated pathway is disturbed (49), just to mention a few examples. The acidotropic weak bases are unfortunately not free of undesirable side effects. The most important is the swelling of the acidic vacuoles leading to a dramatic vacuolization of the cell's cytoplasm (120–123). This effect may severely affect the pathways under study. Some of the amines also directly inhibit certain enzymes such as transglutaminase (124) and cathepsin D (121). It has, moreover, been suggested that intracellular fusion events may be inhibited (125, 126). For a recent review on the effects and side effects of these agents the reader is referred to Dean et al (126).

The second group of agents used to elevate vacuolar pH are the carboxylic ionophores such as monensin, nigericin, and X537A. They intercalate into membranes and mediate exchange of monovalent cations through the membrane (127). Given the high concentration of K^+ in the cytoplasm their major effect on the acidic organelles is to exchange protons for potassium ions, thereby effecting a rise in vacuolar pH. The overall effects on vacuolar function

are similar to those of lysosomotropic agents (41, 128–133). But some differences are also evident: e.g. they do not affect pH as rapidly and their effects on Golgi function tend to be more marked at low concentrations than those on other acidic organelles (133). The reason for these differences is not known.

ROLE OF ACIDIFICATION IN THE ENDOCYTIC PATHWAYS

Material internalized by the phagocytosis and pinocytosis is usually exposed to decreasing pH very soon after internalization. It only takes five minutes or less before the pH of the compartment in which the material is located drops well below the extracellular level (9–11, 35, 37–42, 134–136).

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Phagocytosis

It is likely that phagosomes become acidic prior to the onset of fusion with lysosomes, although acidification may be preceded by a transient alkalinization of the phagosome's interior (9–11). The nature of this acidification reaction has not been studied in detail. If it is caused by a vacuolar ATPase similar to that found in endosomes, which seems likely, the enzyme must either be derived from the plasma membrane as a component of the phagosomal membrane, or be delivered to the newly formed phagosome by fusion of ATPase-carrying vesicles. Although not usually considered intrinsic to the plasma membrane, vacuolar ATPases have been observed in the plasma membrane under some conditions. Gluck et al (137) found that urinary acidification can occur by insertion of a vacuolar type ATPase into the luminal membrane of the epithelial cells (see above); while in *Paramecium,* which depends on phagocytosis for uptake of nutrients, evidence has been obtained for the fusion of newly formed digestive vacuoles with smaller, nonlysosomal vacuoles ("acidosomes") around the time when the pH begins to decrease (138). This suggests that the ATPase might be delivered to the phagosome from an intracellular source. Both of these examples are from highly specialized cell types and may not reflect the situation in most mammalian cells.

It is not clear why phagosomes are acidic. It has been proposed that acid pH might facilitate the fusion of phagosomes with lysosomes (139), although it does not seem to be a prerequisite (82). Intracellular parasites such *Legionella pneumophilia* and *Toxoplasma gondii,* which multiply in phagosomes (140), may be able to inhibit phagosomal acidification and in this way avoid acid-inactivation or phagosome-lysosome fusion followed by digestion in phagolysosomes (139, 141).

Receptor-Mediated Endocytosis

RECEPTOR RECYCLING Whereas the same uncertainty applies to the origin of the proton pumps in endosomes, the functions of the acidic pH are much better understood than that of the phagosome. Low pH has emerged as an important

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factor regulating receptor traffic during receptor-mediated endocytosis and receptor recycling. In a general sense, the acidity of endosomes provides the feature of asymmetry which is required in any cyclic pathway involved in net transport. The receptors, ligands, or receptor-ligand complexes are differentially affected by the pH in the various compartments of the recycling circuit and their properties are changed accordingly. The elegance of using pH in this way is the ease by which receptors in the pathway can be individually modulated. Receptor systems vary greatly in their responses to acid pH. In this section, we focus on some of the general aspects that have emerged from the study of a variety of such systems. For recent reviews on the topic the reader is referred to (3, 17, 25, 31, 34, 142).

Receptors usually recycle between the plasma membrane and the endosome compartment. The intracellular portion of the recycling circuit takes as a rule 15 min or less, and the plasma membrane phase can be as short as a few minutes, depending on the properties of the receptor, and whether ligand is present (143–145). Receptor recycling provides a rapid and economical way to utilize receptors for multiple rounds of uptake. In many cases the receptor has been found to recycle at a finite basal rate even in the absence of added ligand. This has been suggested for at least three receptors, the Fc receptor (146, 153, 154), the LDL receptor (147), and the transferrin receptor (148–150). Indirect evidence for a large number of receptors is provided by the observation that they are depleted from the cell surface in the presence of acidotropic agents and monensin, again irrespective of whether ligands are added or not (114–116, 129, 151, 152). The basal recycling probably reflects the continuous nonspecific pinocytic uptake of membrane and fluid which is generally thought to involve the internalization of 50–200% of the plasma membrane surface area per hour (155, 156). The frequency at which a receptor molecule can be predicted to be internalized is thus once every 0.5–2 hours. The actual rates of basal recycling are, however, variable depending on whether the particular receptors are efficiently integrated into coated pits in their unoccupied state (7, 147, 150, 157–159). LDL receptors in fibroblasts are, for example, localized preferentially in coated pits even in the absence of ligand (7, 147). Many other receptors move rapidly into the coated microdomains of the cell surface only when associated with ligand, the addition of which apparently enhances the uptake rate of the receptor above the basal rate (157, 158).

Upon entering the endosomal compartment, the unoccupied receptors and receptor-ligand complexes encounter a pH of 5.0–6.5, a condition that alters properties of many complexes and profoundly affects their fate. The responses that the incoming receptor-ligand complexes and free receptors display fall roughly into three categories: those where the receptor (or receptor with bound ligand) is reversibly altered by acid pH, those where only the ligand is modified, and those where both ligand and receptor are unaffected by low pH. These receptor types will be discussed below.

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RECEPTORS ALTERED BY LOW PH Receptors are often found to bind their ligands with high or intermediate affinity at neutral pH (the pH of the extracellular medium) and only weakly in mildly acidic pH (the pH of the endosome). The receptor-ligand complexes formed on the plasma membrane therefore dissociate once they reach the endosome. The receptor is free to recycle to the plasma membrane, bind new ligand molecules, and continue through repeated cycles of uptake (for reviews see 25, 31, 33, 109, 159). The dissociated ligands become part of the fluid volume of the endosome, and usually get delivered to lysosomes. Only a fraction (one third or less) of ligand is typically secreted as a consequence of the fusion of recycling vesicles with the plasma membrane (160). The low pH in endosomes thus regulates the sorting of ligand from the receptor.

The molecular basis for the change in binding affinity is not clear. The limited data available suggests that it is primarily the receptor that reacts to low pH and not the ligand. Several of the receptors that have lectin activity (the asialoglycoprotein receptor, the mannose receptor, and the mannose-6-phosphate receptors) mediate uptake of a large variety of glycosylated ligands including "neoglycoproteins" with artificially attached carbohydrate side chains (161, 162). As it seems unlikely that all such ligands behave similarly when exposed to acid pH, the receptor is probably the main target for pH regulation. The recycling of unoccupied receptors is, moreover, often found to be inhibited by agents that elevate endosomal pH (see below). More direct evidence for acid-induced conformational changes in the asialoglycoprotein receptor and the epidermal growth factor (EGF) receptor have been obtained by DiPaola & Maxfield (163), who found that the receptors are differentially cleaved by trypsin, radioiodinated by lactoperoxidase, and labeled by hydrophilic photolabels at acidic and neutral pH. Much additional work is needed to clarify at the molecular level the changes that bring about the altered receptor properties.

When endosomal acidification is inhibited by the addition of acidotropic agents or carboxylic ionophores, intracellular dissociation of the receptor-ligand complex is fully or partially blocked (30, 144, 164). This prevents further binding of ligand to the receptor, and internalization of ligand may come to a halt after one round of uptake (114–116, 129, 151, 152, 165–169a). The actual fate of the nondissociated complexes is variable. Usually, the majority of receptor-ligand complexes accumulate in the highly swollen intracellular vacuoles, and the cell surface is correspondingly depleted of most of its receptor molecules. It is significant that acidotropic agents and carboxylic ionophores block not only the recycling of occupied receptors but also the constitutive recycling of unoccupied receptors. Treatment of cells with the drugs at 37°C in the absence of ligand thus usually results in a dramatic decrease of receptor number on the cell surface which may or may not be further accentuated by the

presence of ligand. The vacuoles in which receptors and receptor-ligand complexes accumulate are probably endosomal, having a low buoyant density and a low content of lysosomal marker enzymes (170, 171). A definite identification of them as bona fide endosomes is, however, somewhat arbitrary owing to the drastic changes in morphology, size, and properties of acidic organelles in the drug-treated cells mentioned above.

Why are the receptors and receptor-ligand complexes in the drug-treated cells not recycled, and why are they not delivered to lysosomes? Studies with receptors and fluid-phase markers suggest that the recycling pathway and the pathway to lysosomes may only be partially blocked by the presence of inhibitors; receptors apparently continue to be recycled and delivered to the lysosomes albeit at a reduced rates (172–173a). The reasons for the block in the traffic of most acid-sensitive receptors are probably quite complex. A major contributing factor may be a trivial one: receptor accumulation in vacuoles may simply reflect the massive redistribution of membrane into the cell and the change in vacuolar structure induced by the drugs. As already mentioned above it is known that more than half of the cell surface is internalized after addition of acidotropic bases, and recent studies on primaquine effects on hepatocytes suggest even more dramatic increases in vacuolar surface area at the expense of the plasma membrane (123). This redistribution alone could explain most of the internalization effect. The size of individual vacuoles, moreover, increases drastically, which must have other functional consequences. The swelling of the vacuoles may, for instance, prevent their movement in the cytoplasm, making productive contacts with other organelles such as lysosomes more difficult (173). It is interesting, in this context, that (hydroxymethyl)-aminomethane (Tris) has been shown to inhibit insulin receptor recycling but not its transport to lysosomes (159). Tris belongs to a group of acidotropic weak bases whose vacuolating effects are less drastic than most of the more commonly used aciditropic bases (120).

Another possible explanation for the inhibition is that receptors in their neutral pH conformation are somehow prevented from entering the recycling or the lysosome-directed pathways in the endosome. According to this view, endosomes would possess a mechanism for sorting receptors on the basis of their conformation, aggregation state, charge, or other pH-dependent property. It is quite possible that such a mechanism exists but the molecular principles remain to be elucidated.

THE LIGAND IS SENSITIVE TO ACID PH The prime example of this class is the uptake system for transferrin, the major iron-transporting protein in serum. It binds two ferric iron atoms with very high affinity and delivers them to cells that carry functional transferrin receptors (see 174). The receptor is a disulfide-linked dimer of two 90K transmembrane glycoproteins (see 175, 176). The

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ligand-receptor complex is rapidly internalized through coated pits and recycled via endosomes to the cell surface (177–181). In the process, transferrin donates one or both of its iron atoms to ferritin at an intracellular site. After returning to the cell surface, the apotransferrin-receptor complex dissociates and both its components participate in subsequent rounds of iron transport.

Studies by Karin & Mintz (179), Klausner et al (182), Octave et al (183), and Dautry-Varsat et al (184) have shown that this elegant and economical pathway depends on carefully adjusted pH dependencies between the carrier protein and the receptor on the one hand, and between the iron atoms and the transferrin molecule on the other. While transferrin has quite a high affinity for its receptor at neutral pH, apotransferrin has high affinity at acidic pH but relatively low affinity at neutral pH. The iron, which is extremely tightly bound to transferrin at neutral pH, on the other hand, dissociates easily at mildly acidic pH (185, 186). These facts, in conjunction with the low endosomal pH, presumably suffice to explain why the transferrin is stripped of its iron, why the apotransferrin reappears in circulation, and why the majority of the receptor and transferrin escape degradation in lysosomes. Addition of lysosomotropic agents has a clear-cut inhibitory effect on iron accumulation by the cell, but recycling continues albeit at a reduced rate (187–190).

Other systems where the primary action of pH is on the ligand include viruses and toxins, which are described below in detail. The receptor-dependent recycling of high-density lipoprotein (HDL) in macrophages (191) may also belong in this category, although it is unclear what role the acid pH may have. The internalized lipoprotein is thought to bind cholesterol in the endosomes and then transport it out of the cell. This is the first case in which receptor-mediated endocytosis may remove material from cells.

LIGAND-RECEPTOR COMPLEXES ARE INSENSITIVE TO ACID PH The best known examples of this class are the receptors for choriogonadotropin (172) and immunoglobulins (154, 192–195). Here, the receptor-ligand complexes are either destroyed in lysosomes or transported across epithelial cells. In most of these cases the receptors are "sacrificial," i.e. they are only used once before being either degraded in the lysosomes or proteolytically cleaved so that ligand-binding portion is removed.

The IgG Fc receptor (a 55-kd transmembrane glycoprotein) of macrophages is a well-studied example of pH-insensitive receptors (153, 153a, 154, 173a). The receptor binds to the Fc portion of antibodies present on soluble immune complexes and antibody-coated particles. Internalization occurs by coated vesicles or phagosomes, depending on the size of the particle, and the complexes become sequestered with the receptors into endosomes or phagosomes. The ligands do not dissociate at acid pH, and the receptor is therefore not liberated for recycling. Instead the entire complex is destroyed in the lysosomes

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or phagolysosome, and the receptor is thus effectively "down-regulated." Continued uptake requires new receptor synthesis.

Studies on the Fc receptor have provided a first clue as to the principles governing the sorting events in the endosomal compartment (153, 153a, 173a). It has been shown that the valency of the ligand that is bound alters the intracellular pathway. In unoccupied form, or when bound to a monovalent ligand, the receptor displays normal basal recycling into and out from endosomes. It is only when associated with a polyvalent ligand that the receptor-ligand complex is directed to the lysosomal route. Receptor traffic and "down regulation" is thus dependent on the valency of the ligand and presumably the aggregation state of the receptor-ligand complex. It is conceivable that pH-dependent aggregation/disaggregation determines the fate of other receptors in a similar way, and that clustered receptors are handled differently in endosomes than monomeric.

A different situation is encountered during transepithelial transport of IgG in the intestine of newborn rats via the IgG-receptor; this receptor has high affinity for IgG at neutral and acidic pH which allows the complex to form in the intestinal lumen and pass through endosomes undissociated on their way from the apical to the basal surface of the cells (192, 193). The relatively high pH in the interstitial fluid at the basolateral side is thought to induce dissociation of the transepithelially transported IgG. In this particular case, it is not yet known whether the receptor is reutilized.

GENERAL CONSIDERATIONS The properties of receptor-mediated uptake and its dependence on endosomal pH may in specific receptor systems be much more complex than suggested by our simple division into three receptor categories. It is possible, and probably quite common, that receptor systems fit the criteria outlined above only partially. Only a fraction of the ligand may, for instance, dissociate during the time the complexes spend in endosomes. It is also important to recognize that the properties of a receptor uptake system may vary depending on cell type or physiological state, a point clearly illustrated by insulin receptor studies (see 159).

Another complication is that the pH is not uniform throughout the various elements of the endosomal compartment. Ligands encounter decreasing pH with time in the endosomal compartment (41a, 42). In most cells the average, initial endosomal pH is probably no lower than about pH 5.5–6.0. Within the next 30 minutes it drops to around 5, and finally reaches its minimum within lysosomes where values as low as 4.5–5.0 have been recorded. Judging by the kinetics of recycling (about 10–15 min for the intracellular phase), most of the acid-sensitive receptors do not remain in the endosomal compartment long enough to encounter the more acidic pH values. This is in agreement with the observation that acid-releasable ligands usually dissociate at mildly acidic pH

values, i.e. at values close to pH 6.0. Given the kinetics of the recycling pathway, ligands that dissociate at pH values lower than this may not be able to dissociate effectively in time to be recycled. Such considerations also affect virus entry. We have shown that wild-type Semliki Forest viruses which penetrate at pH 6.0 do so only 4 min after internalization in BHK21 cells, whereas a mutant virus that is activated at pH 5.3 penetrates 40 minutes later (196). Such a delay, caused by the relatively low activation pH, may be important in the infection of cells such as nerve cells where the peripheral endosomes can be distant from the cell body and located in a region where penetration would not result in efficient infection (196a). Morphological localization studies have shown that endosomes with mildly acidic pH tend to be located in the peripheral cytoplasm and those with more acidic pH are perinuclear (88). The peripheral endosomes are the ones into which incoming material is initially channeled.

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Virus Entry

The entry of enveloped animal virus provides one of the most dramatic and well-studied examples of the role of vacuolar acidity. It also provides the clearest examples of what changes membrane proteins can undergo when exposed to mildly acidic pH in endosomes.

A large number of viruses have been identified that depend on adsorbtive endocytosis and acid pH for their penetration (for reviews see 197–201). In the best-studied cases, Influenza virus and Semliki Forest virus, acid pH in endosomes induces a conformational change in the spike glycoproteins of the virus, which are thus activated to mediate the fusion of the viral membrane with the vacuolar membrane (202–207). As a result, the viral genome and other components inside the viral membrane are released into the cytoplasmic compartment. The group of viruses suspected to follow a similar pathway is rapidly expanding. It already includes enveloped viruses from the following families: toga viruses, rhabdoviruses, orthomyxoviruses, baculoviruses, and retroviruses. Evidence has also been obtained for endocytic, acid-triggered penetration of some non-enveloped viruses such as poliovirus, reovirus, and adenovirus (208–211). In general, viruses that follow this pathway of entry are distinguished by the fact that their entry is inhibited by acidotropic weak bases and carboxylic ionophores (212–214), and the enveloped ones frequently display membrane fusion activity when present in media of low pH in vitro (204, 207, 215, 216).

Semliki Forest virus and Influenza virus particles are internalized by adsorptive endocytosis in coated pits, and, in the case of Influenza, in small noncoated vesicles as well (202, 217, 218). The virus particles are next delivered to endosomes from which penetration usually occurs (203, 205). The fusion activity of Semliki Forest virus requires a pH of 6.1 or below, while the pH

needed for Influenza is between pH 5.1 and 6.0 depending on the strain. The Semliki Forest virus spikes responsible for the fusion are heterotrimers containing two integral membrane glycoproteins (E1 and E2, 49K and 51K respectively) and a peripheral one (E3, 10K) (219). When exposed to acid, both E1 and E2 undergo irreversible conformational changes (220). The change in E1 depends in addition on the presence of cholesterol. The steroid requirement is particularly interesting because Semliki Forest virus can only fuse with membranes that contain cholesterol or other steroids with a 3-β-hydroxyl group (220, 221).

The Influenza hemagglutinin is a homotrimer where each unit is composed of two disulfide-bonded subunits, HA1 (56K) and HA2 (24K). The structure of the external domain of the hemagglutinin has been determined by X-ray crystallography (222), the major antigenic epitopes have been located on the molecule (223), and large amounts of structural and functional information is available. It is known that the HA1 subunits form the top domain of the spike and carry a binding site for sialic acid responsible for virus attachment to the cell surface. The stem of the 13.5-nm-long spike is mainly composed of the three HA2 subunits. The N-terminal of HA2 located in the stem region is hydrophobic and highly conserved, and several lines of evidence indicate that it plays a central role in the fusion reaction.

Studies using biochemical, immunochemical, genetic, and morphological techniques (224–232) have shown that an irreversible conformational change accompanies the acid-induced activation of HA. The change involves partial or total dissociation of the three subunits of HA leading to the opening of the molecule along its long axis. As the three subunits become separated from each other at the top of the molecule apolar moities previously hidden within the stem become accessible. The most favored view is that the exposure of the hydrophobic moiety, which includes the N-terminal peptides of the three HA2 subunits, allows the protein to become hydrophobically attached to the target membrane. The dual attachment in the viral and the target membrane may bring the membranes into close enough proximity to allow fusion.

While much remains to be learned about the mechanism of the fusion reaction it is already clear that it is exposure to low pH that induces the dramatic changes in the spike glycoproteins. Recent genetic studies show that single mutations in HA that affect intersubunit salt bridges, the cavity in which the hydrophobic N-terminal of HA2 is located, or the N-terminal of HA2 sequence itself, can change the efficiency of fusion and its pH dependence (224–226, 232). Mutants of Semliki Forest virus with altered pH dependence have been also isolated and partially characterized (196).

Protein Toxins

Many bacterial and plant toxins are thought to enter the vacuolar apparatus of animal cells by adsorbtive endocytosis, and some have been shown to depend

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on acidic pH for their penetration (234–236). The best characterized of these are diphtheria toxin, *Pseudomonas aeruginosa* exotoxin, and the plant toxin modeccin (234–240). Each consists of two subunits, A and B, of which the smaller (A) carries the toxic activity and the larger (B) is needed for translocation of the A chain into the cytoplasm. The A-chains inhibit protein synthesis either by ADP-ribosylating elongation factor 2 (diphtheria toxin and *Pseudomonas* endotoxin) or by inactivating the 60S ribosomal subunit (modeccin). Diphtheria toxin is best characterized in terms of structure and biological effect. After binding to yet unidentified receptors the toxin is internalized. Penetration occurs from endosomes and requires an acidic environment (pH < 5.5) (241, 242). Intoxication is blocked by agents that elevate vacuolar pH, and the block can be bypassed by lowering medium pH to 5.0 and below, which induces penetration directly through the plasma membrane (119, 241–245).

Several approaches have shown that diphtheria toxin undergoes an irreversible change in conformation and solubility properties when exposed to acidic pH. It binds nonionic detergent and it attaches firmly to artificial membranes (246–248). There is also evidence from model membrane studies suggesting that it can form transmembrane, voltage-dependent anion channels (249–252), and it has been assumed that these channels allow the passage of the A-chain through the membrane. The exact nature of the conformational change remains unclear, but it is assumed that some of the relatively long hydrophobic stretches in the sequence of the B subunit may become exposed, and that these insert into the endosomal membrane. Fluorescence spectroscopy suggests exposure of buried tryptophan-containing sites in the molecule (253). Only relatively small changes in secondary structure are, however, detected by circular dichroism measurements (254). Whereas the critical role of low pH seems relatively well-established the details involved in the actual penetration reaction remain unclear.

The Functions of Lysosomes

The lysosomes are the most acidic compartments of most cells. The low pH facilitates the activity of lysosomal hydrolases, most of which have pH optima in the 4.5–5.5 range. The low pH may be generally advantageous for hydrolytic reactions, and many substrates become acid-denatured and thus more susceptible to enzymatic degradation. A further advantage is the safety aspect; by confining most of their hydrolytic enzymes into lysosomes and adjusting their pH optima to extreme values, the cell diminishes the risks involved if there is "accidental" release of the enzymes into the cytoplasm and extracellular fluid.

Whether the pH of lysosomes has other functions is not clear. It is conceivable that the proton gradient could be used for coupled transport of degradation products out of the lysosomes. This has recently been proposed for the transport of cysteine (255). The low pH could also be useful in the uptake of weak bases

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of physiological importance, and facilitate the escape of weak acids into the cytosolic compartment.

ROLE OF ACIDITY IN THE EXOCYTIC PATHWAY

It is becoming increasingly apparent that low intravesicular pH plays important roles in various stages of the exocytic pathway. Thus far, the transport of certain small molecules into secretion granules, the efficient targeting and storage of secretory proteins, and the orderly flow of traffic along the secretory pathway have all been found to directly or indirectly depend on pH gradients established by vacuolar proton ATPases.

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Coupled Transport of Biogenic Amines into Secretory Granules

Many acidic secretory granules use an electrochemical proton gradient to mediate the coupled transport of biogenic amines (for review, see Ref. 48, 50). The best-known examples include the adrenal chromaffin granule (epinephrine), the platelet dense granule (serotonin), and the synaptic vesicle (catecholamines, acetylcholine) (53, 86, 97, 98, 103, 104). In each case, generation of the electrochemical proton potential is accomplished by vacuolar-type proton ATPases described in detail above. While the precise details of amine transport remain to be determined, it is well-established that the granule membrane contains a reserpine-sensitive transporter that can mediate the exchange of cytoplasmic amines for internal protons. The transporter appears to be relatively nonspecific, and can accept a variety of substrates: e.g. epinephrine, norepinephrine, serotonin, and dopamine. Although both neutral and protonated amines can be transported into the granule, both cases result in the efflux of net positive charge (e.g. one H^+ per unprotonated amine, two H^+ per protonated amine). In this way, the granule can efficiently accumulate amines to internal concentrations as high as 0.1 M. In vitro, amine transport can be stimulated by ATP-driven proton pumping or by artificially imposed diffusion potentials (48, 50). Either an electrical or chemical proton gradient can be chemiosmotically coupled to amine accumulation in isolated granules.

Not all secretory granules utilize transmembrane proton gradients to drive the accumulation of biogenic amines. For example, secretory granules found in the neurohypophysis have a proton ATPase and acidic interiors, but serve to package and store polypeptide hormones (78–80). While the function of low pH in these organelles is not yet clear, it has been suggested that the acidic internal environment provides the optimal conditions for the proteolytic processing enzymes that cleave prohormones into mature species (46). In addition, low pH may play a role in the biogenesis of these organelles, by influencing the targeting of newly synthesized hormones into the nascent granule (see below).

Other secretory granules, such as those found in the rat parotid gland, seem to contain neither a proton pump nor an acidic internal pH (102). Therefore, an obligatory role for pH gradients in events such as the fusion of the granule and plasma membranes during exocytosis appears unlikely.

Targeting of Secretory Proteins

Exocytosis of secretory proteins by mammalian cells can occur along regulated or unregulated (constitutive) pathways (1, 2, 128) (Figure 1). The "regulated pathway" is characterized by the storage of a protein in secretory granules and released only after a cell receives the appropriate stimulus. In general, these granules form at the *trans* face of the Golgi apparatus and contain high concentrations of the secreted protein. This type of exocytosis is generally associated with specialized secretory cells, such as the pancreatic acinar cell or the neurosecretory cells of the pituitary gland. In contrast, the "constitutive pathway" is characterized by the continuous, unregulated release of secretory products directly following their synthesis. There is no significant storage or concentration of the secreted proteins in intracellular vesicles prior to release. The constitutive pathway is characteristic of most cell types, such as fibroblasts or plasma cells, which continuously secrete proteins such as fibronectin and immunoglobulin, respectively. The constitutive pathway also operates in many cells that are capable of regulated secretion (49, 128, 256). What determines whether a particular protein is secreted in a regulated or a constitutive manner is not yet known. However, there is evidence to suggest that acidic pH in organelles of the exocytic pathway plays an important regulatory role.

The AtT-20 cell line is derived from a mouse pituitary tumor and is capable of the regulated secretion of mature ACTH as well as the constitutive exocytosis of other secreted and membrane proteins (49, 256). A fraction of immature ACTH (i.e. proopiomelanocortin, POMC) is also released constitutively, suggesting that packaging in secretory granules is a prerequisite for maturation of the hormone precursor. Kelly and colleagues have been able to transfect AtT-20 cells with plasmids containing the genes for other regulated secretory proteins, such as insulin and trypsinogen, which are not normally expressed by pituitary cells (257, 258). The fact that these proteins are efficiently packaged and stored in cytoplasmic secretory granules and are subject to regulated exocytosis by the addition of cyclic AMP suggests the existence of a highly conserved and generalized mechanism which facilites the proper targeting of certain secretory proteins to storage granules. Conceivably this mechanism relies on some type of secretory protein receptor; e.g. a receptor that might mediate the selective transport of newly synthesized proteins from the Golgi to the forming granule.

While the existence of intracellular receptors for the transport of secreted proteins remains to be demonstrated, it is of interest that acidophilic agents such as chloroquine dramatically change the pattern of secretion in AtT-20 cells

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(49). Chloroquine-treated cells can no longer efficiently package POMC in secretory granules. Instead, the hormone is diverted from the regulated pathway to the constitutive pathway and exocytosed continuously, without modulation by added cyclic AMP. While the mode of chloroquine action is not known, one can draw analogies with its effects on receptor-mediated endocytosis. Since chloroquine would be expected to elevate the internal pH of the Golgi, Golgi-associated vesicles, and the secretory granules, it is conceivable that the low-pH-facilitated discharge of POMC from its receptor is inhibited, tying up all available receptors and leading to the constitutive release of newly synthesized hormone. A similar series of events is believed to occur in the transport of newly synthesized lysosomal enzymes in chloroquine-treated cells (161). Enzyme release from the mannose-6-phosphate receptor is inhibited by the elevated pH, the pool of free receptors decreases, and the enzymes are released constitutively into the medium. Whatever the mechanism leading to the packaging of ACTH in secretory granules, it seems clear that acidic pH in one or more organelles plays a critical role in the sorting events that are known to occur.

pH and Membrane Traffic on the Exocytic Pathway

EFFECTS OF MONENSIN Treatment of cells with carboxylic ionophores can lead to dramatic alterations in the processing and transport of membrane and secretory proteins on the exocytic pathway (for review, see Ref. 128). As first shown by Tartakoff & Vassalli (259), monensin arrests the secretion of immunoglobulin by plasma cells within the Golgi. Since the intracellular immunoglobulin fails to receive terminal galactose or sialic acid residues, it has been suggested that the block occurs at medial Golgi cisternae, prior to the compartment containing terminal transferase activities (260, 261). However, the effects of monensin on transport are not always predictable. For instance, in hepatoma cells, neither transport transferrin nor vesicular stomatitis virus G protein reaches the plasma membrane following monensin treatment but terminal glycosylation is blocked only for transferrin (262, 263). Interestingly, both proteins can be localized by immunocytochemistry to the same Golgi vesicles. Yet another variation is the mouse macrophage Fc receptor, which reaches the plasma membrane in monensin-treated cells but fails to be galactosylated (264). Some of these differences may be due to differences in the cell types used and to variations in the rate that individual proteins are transported through the Golgi. Nevertheless, it seems likely that monensin does not exert a single, well-defined block in the exocytic pathway.

Monensin facilitates the electroneutral exchange of Na^+, K^+, and H^+. As such, it would be expected to dissipate transmembrane pH gradients in Golgi compartments, an effect often invoked as its mechanism of inhibition. However, any possible K^+ and/or Na^+ gradients would also be disrupted. In addition, monensin may exert other effects on the cell that could indirectly affect Golgi

function; one such effect is the hyperpolarization of the electrical potential across the plasma membrane (128).

Nevertheless, since monensin causes a rapid (within 30 min) and dramatic dilation of Golgi cisternae and Golgi-associated vesicles (128), it seem clear that the drug does directly affect the internal ionic environment of the Golgi apparatus. Why should the disruption of Golgi pH or cation gradients lead to dilation, inhibition of transport, and aberrant glycoprotein processing? One possibility is that monensin treatment inhibits membrane vesiculation, thereby slowing the rate at which membrane leaves the *trans* Golgi. Alternatively, the dissipation of transmembrane ion gradients may disrupt the transport of sugar nucleotide precursors or other metabolites into or out from the Golgi, conceivably leading to less efficient glycosyltransferase activity and possibly osmotic imbalance. However, the available evidence suggests that the transport of at least sugar nucleotide precursors is not coupled to transmembrane ion gradients (265).

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EFFECTS OF ACIDOTROPIC AGENTS Although the influences of acidophilic agents on the exocytic pathway have been less extensively studied, several examples are known in which the transport of membrane and secretory proteins is altered by amines. Agents such as chloroquine can cause dilation of Golgi cisternae and Golgi-associated vesicles in a manner at least superficially similar to monensin (121). In hepatoma cells, primaquine will greatly slow the secretion of albumin and transferrin, as well as prevent the budding of vesicular stomatitis virus at the plasma membrane (266). In contrast to the effects of monensin in the same cells, terminal glycosylation of G protein is not inhibited by primaquine treatment. This is consistent with the observation that the primaquine block seems to occur at the *trans*-most cisterna of the Golgi stack. In cultured kidney epithelial cells, transport of the Influenza virus spike glycoprotein, hemagglutinin, is also slowed in the presence of NH_4Cl (267).

Unlike monensin, acidotropic drugs would be expected only to dissipate transmembrane pH gradients. Therefore, all of these effects should be a direct or indirect reflection of an elevation of intravesicular pH. As discussed earlier, it is conceivable that this pH change affects the interaction of proteins on the exocytic pathway with a possible class of receptors responsible for transporting newly synthesized membrane and secretory proteins from the Golgi to the plasma membrane. In the AtT-20 cell experiments (see above), it was not determined whether chloroquine slowed the overall rate of constitutive secretion in addition to diverting ACTH from the regulated pathway.

GENETIC APPROACHES

The importance of intravesicular acidification in the endocytic and exocytic pathways is illustrated by mutant cell lines that exhibit acidification defects

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both in intact cells and in vitro (41a, 268–271). In general, these cell lines were isolated by selection for the ability to grow in the presence of agents (e.g. bacterial toxins, enveloped viruses) which require low intravesicular pH to penetrate the cytosol and kill their host cell. Both nonlethal and temperature-sensitive mutations have been isolated.

Among the best-characterized mutants is the series of CHO cell lines isolated by Robbins and colleagues (260, 271, 268). These cells exhibited defects in the ATP-dependent acidification of endosomes in vitro. In contrast, lysosome acidification—both in vitro and in vivo—appears normal. Genetic complementation analysis revealed that the mutants fall into two distinct complementation groups, designated *end-1* and *end-2;* thus, at least two genes may control endosome acidification in CHO cells.

Cells of both complementation groups are phenotypically similar and exhibit pleiotropic defects in the endocytic pathway consistent with defective endosome acidification: resistance to a variety of low-pH-requiring viruses and toxins; reduced uptake of exogenously added lysosomal enzymes via the mannose-6-phosphate receptor; failure to dicharge iron from internalized transferrin [also postulated as being the reason for the lethality of the temperature-sensitive phenotype (269)]; inefficient retention of newly synthesized lysosomal enzymes. Interestingly, these cells also exhibit aberrant Golgi-related functions, and, for example, defects in the terminal glycosylation of some viral spike glycoproteins and several endogenous secretory glycoproteins (e.g. fibronectin) (268; A. Robbins, in preparation). The wild-type phenotype is restored for each of these defects in complementing *end-1* × *end-2* hybrids, correlating with the restoration of endosome acidification activity. That the effects of these mutations is a reflection of altered acidification or ion transport activity is further supported by the fact that low concentrations of monensin closely mimic the mutant phenotype (268).

The nature of the molecular defect in either *end-1* or *end-2* cells is unknown. Nevertheless, these observations do suggest the possibility that endosomes and Golgi membranes share at least one gene product, related to ion transport, which is required for the normal function of both organelles. For example, the mutations could affect a subunit common to the endosomal and Golgi proton pumps. Alternatively, they may affect a shared anion or cation transporter whose activity indirectly controls intravesicular acidification. In any event, localization of the defects in these cells may permit the demonstration that the acidification mechanisms and ion permeabilities defined in cell-free systems also function in intact cells. They may also permit a definition of the biochemical and ontogenetic relationship between ion transport mechanisms in the endocytic and exocytic pathways.

Since the mutant gene products have not yet been identified, it is difficult to speculate on the significance of the observation that both the *end-1* and *end-2* mutations affect endosome acidification without affecting the acidification of

lysosomes. This observation may indicate that there are differences between the acidification mechanisms of endosomes and lysosomes. On the other hand, they may simply reflect a quantitative difference in the concentration of a mutant protein in the two organelles. For example, if a mutation partially affects the V_{max} of a proton pump shared by endosomes and lysosomes, and if lysosomes contain many more copies of the pump than do endosomes, the observed phenotype might be expected to result. Therefore, it is not yet possible to conclude on the basis of the genetic findings that there exist fundamental differences in the acidification of endosomes and lysosomes.

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CONCLUSIONS

It is clear that the acidification of intracellular organelles plays numerous and critical roles in maintaining the normal function of the endocytic and exocytic pathways. In every case studied thus far, acidification is mediated by a unique class of ATP-driven electrogenic proton pumps, which we have referred to as the "vacuolar H^+-ATPases." The characteristics of the ATPases found in all endocytic and exocytic organelles are strikingly similar, suggesting the possibility that the same proton pump is distributed throughout the vacuolar apparatus. Although the definitive identification and isolation of the ATPase of any organelle has yet to be reported, this suggestion is further supported by the genetic evidence, which shows that two distinct mutations affecting endosome acidification also affect Golgi functions in a manner at least superficially similar to the effects of monensin and acidophilic agents on the exocytic pathway. However, crucial questions remain as to the biogenesis, origin, and inter-organelle transit of the ATPase. While it is appealing to suggest that the proton pump exists on the plasma membrane, is internalized in coated vesicles, and is subsequently distributed throughout the vacuolar system, no information exists to support this scheme.

If the same (or greatly similar) proton pumps are present in all endocytic and exocytic membranes, how is their internal pH regulated? There is ample evidence that different endosomal compartments may have different pH's, and that endosomes and secretory granules have a less acidic pH than lysosomes. Regulation could occur by controlling the number of ATPase molecules present per organelle, or by modulating the activity (e.g. by phosphorylation or some other covalent modification) of a constant number of ATPases. Alternatively, the recent finding that many organelles have defined permeabilities to anions, protons, and other cations suggests that net proton flux and accumulation can be controlled by regulating the ion permeability characteristics of each membrane. For example, rat liver endosomes appear more permeable to K^+ and less permeable to Cl^- than rat liver lysosomes. Therefore, the endosomes may be less acidic because they may be less able to accumulate protons (limited Cl^-

permeability will favor the development of a membrane potential, limiting proton accumulation) and less able to retain protons (cytoplasmic K^+ can more easily exchange with internal H^+ if the conductances for both ions is significant).

Now that the basic role and mechanism of organelle acidification has been defined, future work must concentrate on the crucial questions of the regulation, biogenesis, and functions of acidification in the endocytic and exocytic pathways. It is already clear that such work will require the combined application of biochemical, morphological, and genetic approaches.

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