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THE IMPORTANCE OF BEING ACID: THE ROLE OF ACIDIFICATION IN INTRACELLULAR MEMBRANE TRAFFIC

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Introduction

Ever since the early work of Elie Metchnikoff at the turn of the century, it has been clear that the ability of vacuolar organelles to generate and maintain pH plays a crucial role in a variety of critical cellular functions. Using a variety of crude pH indicators such as neutral red or litmus particles, Metchnikoff observed that bacteria ingested by phagocytic cells of the immune system were transferred to acidic structures in the cytoplasm, killed and then digested. Without any understanding or knowledge of membranes, organelles or proton pumps, Metchnikoff drew accurate conclusions concerning the necessity of an acidic milieu for intracellular digestion, the role of cellular energy in maintaining the acidic pH, and the likelihood that the acidity was due to diffusible protons as opposed to the infusion of acidic substances such as DNA (which had, in fact, been offered as an explanation by some of Metchnikoff's contemporaries).

The past 80 years have illustrated just how remarkable Metchnikoff's insights were. However, as is amply demonstrated in the chapters contained within this volume, the molecular mechanisms underlying the formation and regulation of transmembrane pH gradients are now coming to be described in impressive and exciting detail. Much of this work has resulted from efforts to understand the function of acid- and alkali-secreting epithelia. However, a considerable amount has been learned about the function of V-ATPase-dependent acidification of intracellular organelles. Here, one finds carefully regulated patterns of pH gradients amongst organelles which are both dynamic and functionally interconnected. As described below, pH in intracellular organelles is intimately associated with the function of the biosynthetic and endocytic pathways as well as with issues pertaining to the entry and immune response to pathogens. Accordingly, it will also be important to elucidate the mechanisms responsible for controlling acidification and V-ATPase activity in endocytic and secretory organelles.

Functions of acidification in the endocytic and secretory pathways

In the secretory pathway, the best described role for acidic pH is in the packaging and/or processing of secretory granule contents (Mellman and Helenius, 1986). For example, acidic pH provides the proper conditions for the activation of highly specific endoproteases that are responsible for the post-translational cleavage of prohormone precursors, an event which typically occurs in the lumen of mature endocrine or

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neuroendocrine secretory granules. Interestingly, granules in exocrine cells – which contain zymogens that are activated only after secretion – generally lack detectable V-ATPase activity and have a neutral internal pH. In cholinergic synaptic vesicles as well as certain types of endocrine granules (e.g. chromaffin granules), V-ATPase activity is also responsible for the concentration of many biogenic amines, whose transport from the cytosol into the granule lumen requires transembrane proton gradients. Although it is also clear that elements of the *trans*-face of the Golgi complex are slightly acidic, the function of low pH in this compartment is less certain. Conceivably, the acidic pH assists in the condensation of nascent granule content, in proteolytic cleavage of membrane proteins, or in providing the proper environment for the activity of terminal glycosyltransferases.

On the endocytic pathway, acidification plays a wider variety of roles that are important not only for normal cellular function but also for infection by a wide variety of viruses as well as many protozoan and bacterial pathogens. Understanding the relationship between these events and the regulation of acidification of endocytic organelles has been a major focus of our group over the past several years. The remainder of this review will be concerned with our collective understanding of this problem.

The pathway of receptor-mediated endocytosis

Although many details remain to be resolved, the basic features of the pathway of receptor-mediated endocytosis are well known (Kornfeld and Mellman, 1989). As shown in Fig. 1, receptor–ligand complexes that form at the plasma membrane are typically internalized in clathrin-coated pits and coated vesicles. Within seconds after their formation, these vesicles uncoat and fuse with early endosomes, a population of tubules and vesicles found generally in the peripheral cytoplasm. In early endosomes, the slightly acidic internal pH (<6.3–5.5) is sufficient to facilitate the dissociation of ligands from their receptors, allowing the newly vacated receptors to return (or recycle) back to the cell surface for re-use. In contrast, the dissociated ligands now free in the early endosome's lumen are transferred to a distinct population of structures referred to as late endosomes and then finally to lysosomes for degradation. The low-pH-mediated discharge of ligands, and the subsequent 'sorting' of the discharged ligands from their receptors, is one of the hallmarks of receptor-mediated endocytosis and is to a large extent responsible for the ability of cells to take up and concentrate extracellular macromolecules.

As is evident from Fig. 1, incoming ligands and receptors encounter progressively more hostile environments the farther into the endocytic pathway they penetrate. Thus, whereas early endosomes are generally given as having pH values ranging from 5.5 to 6.3, the pH of late endosomes is typically less than 5.5, and that of lysosomes in most cells can be as low as 4.6 (Kornfeld and Mellman, 1989). Moreover, as the pH decreases, the content of acid hydrolases increases: early endosomes generally contain very little in the way of lysosomal-type hydrolases, but late endosomes and lysosomes both have high concentrations of these enzymes. Thus, early endosomes provide an optimal environment for most plasma membrane receptors to deposit their ligands and return to the cell surface while minimizing their risk of degradation. In a sense, pH provides the asymmetry that

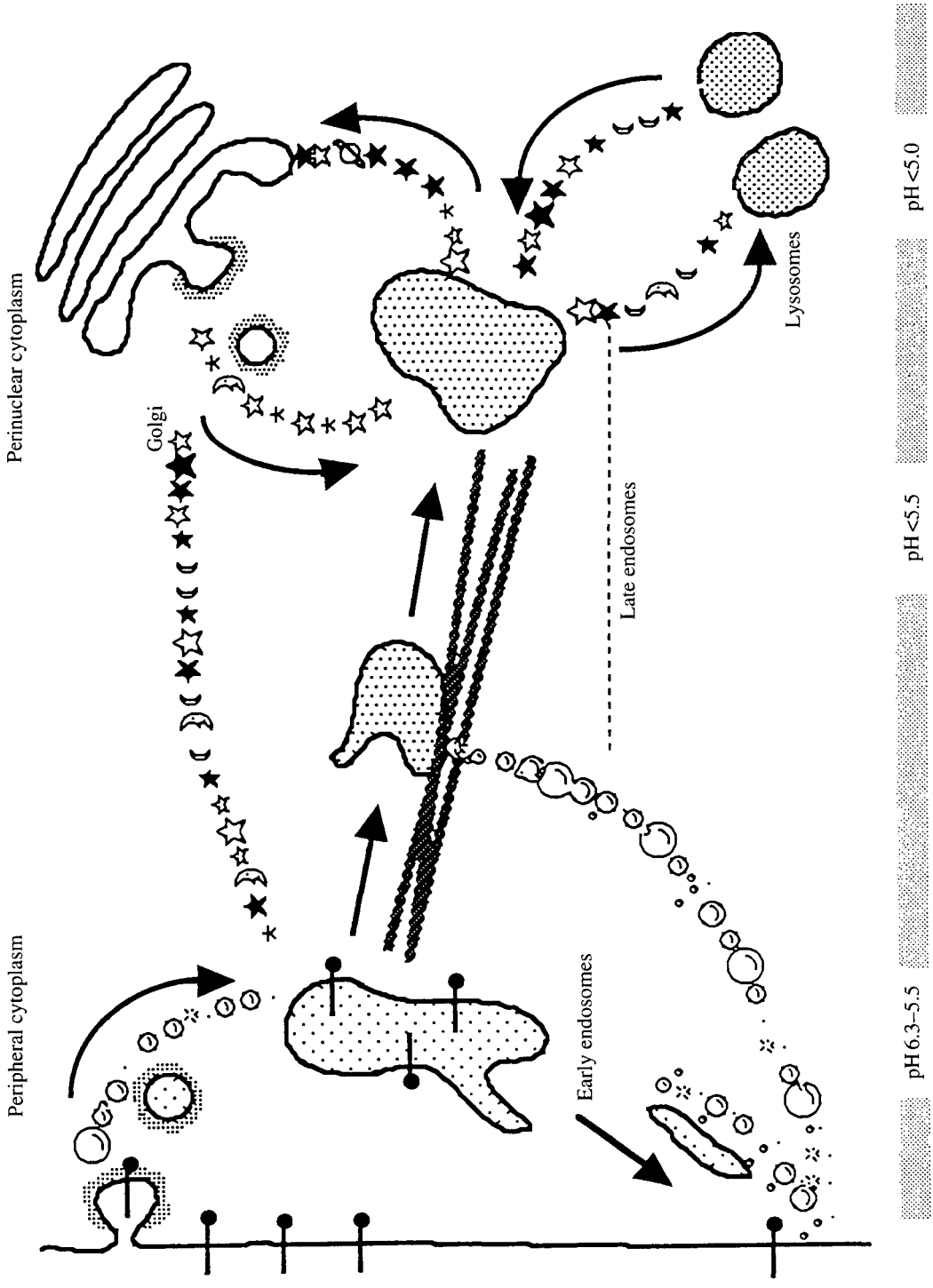

Fig. 1. Membrane transport during endocytosis (modified from Kornfeld and Mellman, 1989)

allows receptors to distinguish between the intracellular and environments, thus enabling accumulation of the ligands they internalize.

Several lines of evidence have contributed to the appreciation that later endocytic compartments have generally lower internal pH values than do early compartments. Many experiments have relied on the use of ligands coupled to pH-sensitive fluorochromes, such as fluorescein, in conjunction with fluorescence microscopy or flow cytofluorometry to monitor endosomal pH in living cells (Murphy and Cantor, 1984; Yamashiro *et al.* 1984). Although such experiments have yielded important insights, quantitative fluorescence measurements in intact cells have a number of limitations. Accordingly, we developed an independent approach that makes use of the pH-sensitive conformational changes exhibited by viral spike glycoproteins as 'biological pH probes' (Kielian *et al.* 1984; Kielian and Helenius, 1986; Schmid *et al.* 1989). It is well known that a wide variety of enveloped animal viruses infect their target cells by opportunistically making use of the endocytic pathway. In general, such viruses bind to cell surface receptors and are rapidly internalized. After delivery to acidic endosomes, the spike glycoproteins typically assume an 'acid conformation' which is thought to expose a domain that facilitates the fusion of the viral envelope with the limiting membrane of the endosome. This event exposes the viral nucleocapsid to the cytosol, thus initiating replication.

Since the fusion pH of different viruses can vary, the intracellular site at which the appropriate pH is reached – and thus from which viral penetration occurs – can vary depending on the virus. We have made use of this feature to monitor the rate and intracellular site at which incoming viruses are exposed to their threshold pH for fusion. This approach was facilitated by the availability of wild-type and mutant forms of a well-characterized alphavirus (Semliki Forest virus, SFV) whose spike glycoproteins reach their acid conformations at pH 6.3 and pH 5.3, respectively (Schmid *et al.* 1989). Using biochemical and immunological assays for acquisition of the acid conformation in conjunction with cell fractionation and immunofluorescence microscopy, we were able to establish that incoming virus first entered a compartment of 'neutral' pH (>6.3) before arriving in early endosomes at a pH between 6.3 and 5.3. pH values less than 5.3 were not reached until delivery to late endosomes.

Although the internal pH of endosomes decreases the closer one gets to lysosomes, it is apparent that the vesicles or tubules involved in the recycling of receptors from endosomes back to the cell surface actually have a more neutral pH than the endosomes from which they are formed. This difference was first suggested by experiments using fluorescein isothiocyanate (FITC)-labeled transferrin (Tfn), a ligand that remains bound to its receptor after delivery to early endosomes and during its subsequent transport back to the plasma membrane (Yamashiro *et al.* 1984).

Regulation of acidification on the endocytic pathway

It is clear from the above considerations that each of the major organelles involved in the endocytosis and recycling of receptors and ligands has a unique pH in intact cells. These features are also reflected by the acidification properties of endocytic organelles *in vitro*.

Clathrin-coated vesicles

We have recently found that endocytic coated vesicles from rat liver are completely incapable of ATP-dependent acidification (R. Fuchs and I. Mellman, in preparation). Unlike previous experiments, we selectively monitored acidification in endocytic coated vesicles by labeling them with FITC-conjugated endocytic tracers prior to isolation. The labelling was accomplished by perfusing rat livers with either FITC-asialo-orosomucoid or FITC-dextran for 1–2 min at 37 °C. Enriched coated vesicle and endosome fractions were prepared by density gradient centrifugation and ATP-dependent acidification was monitored by FITC fluorescence quenching. We found that the most highly purified coated vesicle fractions exhibited no decrease in FITC fluorescence upon ATP addition, although the corresponding endosome fractions were acidification-competent. It was unlikely that the coated vesicle fractions contained an acidification inhibitor or were prepared in a manner that destroyed V-ATPase activity, since using acridine orange as an extrinsic pH probe demonstrated significant acidification in the coated-vesicle fractions. This residual activity was due either to contaminating smooth membranes (endosomes) or to Golgi-derived coated vesicles. Moreover, we were able to show that the FITC-containing coated vesicles were capable of maintaining proton gradients since creation of a diffusion potential for protons (dilution of KCl-loaded vesicles into acidic buffer in the presence of nigericin) resulted in the expected decrease in FITC fluorescence. These results suggest either that one or more V-ATPase subunits are missing from endocytic coated vesicles or that the V-ATPase is regulated in some other fashion.

Early endosomes, late endosomes and lysosomes

Early and late endosomes, again selectively labeled with FITC-conjugated endocytic tracers, isolated from both cultured cells and from rat liver, have also been found to exhibit markedly distinct acidification properties. In general, larger signals are obtained from early than from late endosomes; lysosomes exhibit even larger decreases in pH (Schmid *et al.* 1988; Fuchs *et al.* 1989; Fuchs and Mellman, 1989; Schmid *et al.* 1989). Since these fractions are likely to contain mixed populations of acidification-competent and -incompetent vesicles, it is impossible to assign precise values for the extent of acidification in each case. On average, however, ΔpH values vary from 0.3 pH units to 1.0 pH unit. At present, it is not clear why these different organelle populations exhibit quantitatively different capacities for acidification. Since the other ion permeability characteristics of these membranes do not vary in the same way, it is likely that the differing ΔpH values reflect (in part) either the concentration or activity of the V-ATPases found in each. It will now be important to use these cell fractions of different acidification activities to establish the spectrum of V-ATPase subunits present.

One contributing factor to maintaining a relatively mild pH in early endosomes, however, is the presence of active Na^+/K^+-ATPase in these fractions (Fuchs *et al.* 1989). Both *in vitro* and in intact cells, there is good evidence that conditions that favor Na^+/K^+-ATPase activity oppose V-ATPase-dependent acidification, apparently as a result of the increased interior-positive membrane potential in the presence of an active Na^+/K^+-ATPase.

Recycling vesicles

Based on fluorescence measurements in intact cells, Maxfield and co-workers first suggested that recycling receptors, such as Tfn, exit from acidic early endosomes and enter a compartment of neutral pH recycling vesicles prior to returning to the plasma membrane (Yamashiro *et al.* 1984). Although the results are still preliminary, it appears that, when these vesicles are selectively labeled and isolated, they (like endocytic coated vesicles) exhibit negligible V-ATPase-dependent acidification activity. We have found that Chinese hamster ovary cells induced to express high levels of the ras-like GTP-binding protein rab4A accumulate Tfn in a compartment with the characteristics of post-endosomal recycling vesicles (van der Sluijs *et al.* 1992). In accord with their apparent lack of acidification in intact cells, these vesicle do not acidify in response to ATP *in vitro*.

Thus, structures that mediate both the entry into (coated vesicles) and exit from (recycling vesicles) early endosomes appear to be incapable of ATP-dependent acidification. If this is indeed the case, then either the distribution or the activity of the V-ATPase must be very tightly controlled during constitutive membrane traffic between endosomes and the plasma membrane. Clearly, this observation has a number of important implications for our understanding of the biogenesis, regulation and intracellular transport of the V-ATPase itself.

Functions of pH control on the endocytic pathway

The fact that different stations on the endocytic pathway can exhibit such decidedly different capacities to generate and maintain pH gradients suggests that the pH differences must have some underlying functional significance. A clue is provided by a consideration of the relative pH sensitivities of various receptor–ligand complexes. Ligands that bind to receptors that recycle rapidly between early endosomes and the plasma membrane all dissociate at relatively mild pH values. Examples include low-density lipoprotein and α_2-macroglobulin, both of which dissociate from their receptors at pH values below 6.8. In contrast, receptors that do not rapidly return to the plasma membrane, but are involved in continuous recycling between endosomes and the Golgi complex, discharge their ligands at the much more acidic pH values (<5.5) more typically found in late endosomes. Perhaps the best examples of such receptors are the cation-dependent and cation-independent mannose 6-phosphate receptors that are responsible for the targeting of newly synthesized and/or internalized lysosomal enzymes to lysosomes. Finally, receptors that fail to discharge their ligands at all (or at pH values below those normally reached in endocytic organelles) typically fail to recycle and are degraded in lysosomes along with their bound ligands. This failure of ligand dissociation is the basis for most forms of down-regulation where receptors are irreversibly removed from the plasma membrane following ligand internalization. Perhaps one of the best studied examples of such a receptor is the Fc receptor for IgG (Mellman and Plutner, 1984; Mellman and Ukkonen, 1984; Ukkonen *et al.* 1986).

If ligand dissociation is ordinarily a necessary prerequisite for receptor recycling, then

controlling the endosome population in which dissociation occurs will, in turn, determine the fate of any given receptor. As illustrated in Fig. 1, discharge at the mild pH found in early endosomes results in rapid recycling, discharge at the pH found in late endosomes results in recycling to the Golgi, discharge at lysosomal pH (or no discharge at all) results in receptor degradation. Clearly, this possibility will require additional work before it can be regarded as certain, but it does provide an attractive and biologically consistent explanation for the degree of acidification control observed within a pathway that is remarkably interconnected and dynamic.

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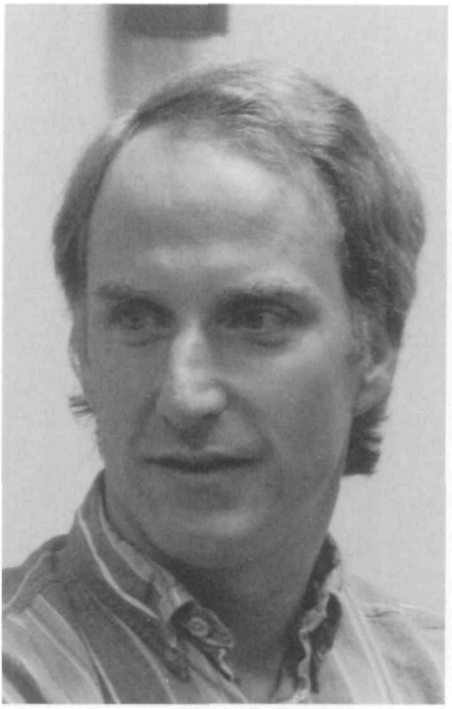
CHAPTER 2. Biochemistry and molecular genetics of fungal vacuoles



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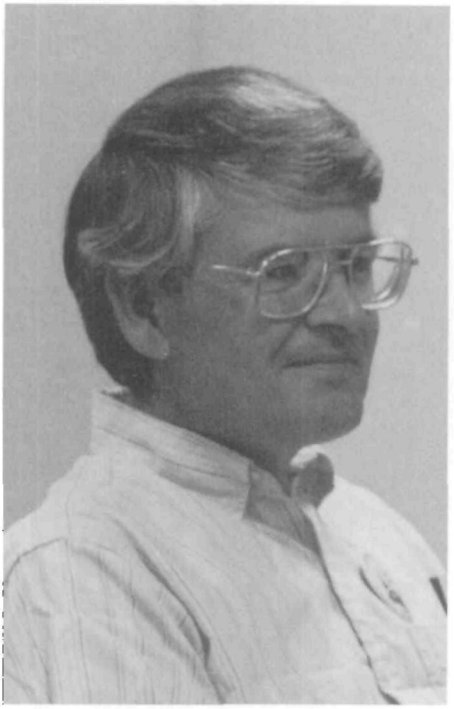

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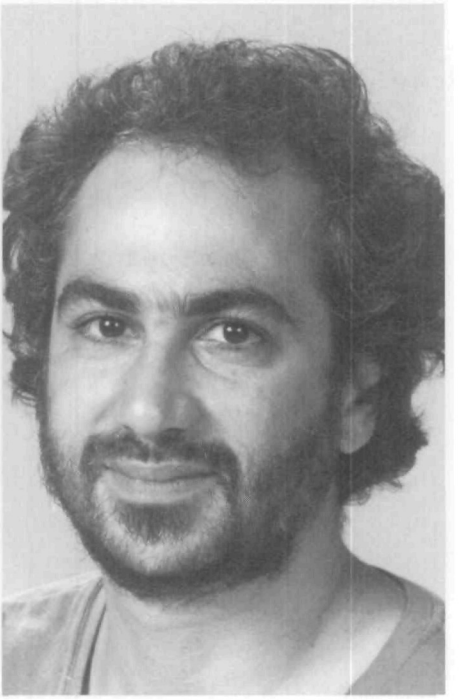

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