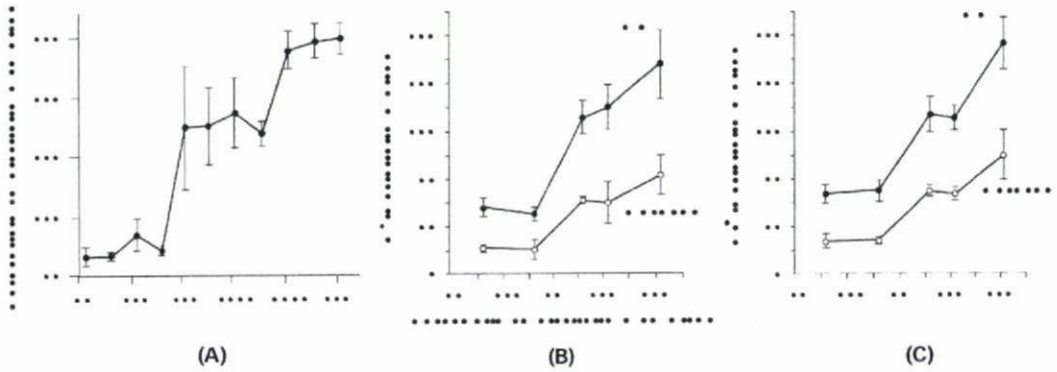

FIGURE 3.2 (A) The cytosolic osmolarity of Paramecium multimicronucleatum increases in steps rather than linearly as the external osmolarity increases. Plateaus exist at approximately 75, 160, and 245 mOsmol/L. (From Stock, C. et al., J. Exp. Biol., 204, 291, 2001. With permission.) (B) The K^+ activities of both the cytosol (open circles) and CV (closed circles) also increase in steps as the external osmolarity rises. (C) The cytosolic (open circles) and CV (closed circles) Cl^- activities also increase in steps with increasing external osmolarity. (B and C from Stock, C. et al., J Cell Sci., 115, 2339, 2002. With permission.)

increasing external osmolarities, at least in an external osmolarity range from 40 to 170 mOsmol/kg of cells.[31,112] Amoeba proteus was found to have a cytosolic osmolality of ~240 mOsmol/L when cultured in a medium containing 0.7 mg KCl, 0.8 mg $CaCl_2$, and 0.8 mg $MgSO_4$–$7H_2O$ per liter.[86]

Paramecium multimicronucleatum cells living in an external environment having an osmolarity from 4 to 64 mOsmol/L exhibit a relatively constant cytosolic osmolarity of ~75 mOsmol/L.[109] Thus, the cytosol of this cell, like other freshwater protozoa, is also hyperosmotic to its environment; however, as the environmental osmolarity increased beyond 75 mOsmol/L, it was observed that the osmolarity of the cytosol dramatically increased stepwise rather than linearly when three critical cytosolic osmolarities were exceeded.[109] These three key osmolarities were at approximately 75, 160, and 245 mOsmol/L (see Figure 3.2A). When these barriers were approached or crossed, water segregation by the CVC was temporarily disrupted. Once disruption had occurred, time was necessary for water segregation to restart. During this time, the cytosolic osmolarity rapidly increased to the next plateau level of either 160 or 245 mOsmol/L.

B. Structure of CVCs

The contractile vacuole of the CVC appears in wall-less, single-celled organisms to be a single-membrane-lined compartment.[6,92] Based on electron microscopy, the membrane of the CV itself is decorated with neither ribosomes nor an extensive cytosolic coat such as clathrin or the COPI or COPII coats of some membranes of the endocytic and biosynthetic pathways,[5,6,46,47] and they lack, for the most part, the luminal polysaccharide lining such as occurs in lysosomes and some stages of food vacuoles.[5,6] Molecules usually associated with clathrin have been reported to be associated with the CV membrane of Dictyostelium in developmental stages[66,88] or under certain experimental treatments.[46] The major specialization noted so far is its tendency to be continuous with a meshwork of tubules or with much smaller vesicles that have a peg-like decoration and, in some cells, an uncharacterized luminal lining. In several cases, the cytosolic decorations are known to be V-type proton-translocating ATPase complexes (V-H^+-ATPases).[32,47,76] Genes encoding some of the subunits of the V-ATPase complex have been cloned and sequenced from a few protozoa.[34,124,125]

In the smallest CVs, which appear in the small green algae such as Chlamydomonas[69] and in the zoospores of Oomycetes such as Phytophthora,[76] the CV is composed of vesicles that are either undecorated or decorated on their cytosolic sides with these pegs. Some of these vesicles will fuse together to form a vacuole as water crosses their membrane. In Chlamydomonas reinhartii, this

vacuole eventually passes its content to the exterior of the cell through the plasma-membrane-lined flagellar pocket. In this case, the CV may not fuse with the plasma membrane in the conventional way but one or a number of minipores may open between the closely opposed CV membrane and the plasma membrane to allow for exocytosis of the fluid content of the contractile vacuole.[69] Such an exocytic mechanism was first proposed for the CVC of a trypanosomatid protozoan, Leptomonas collosoma.[67]

Some algal cells such as Poterioochromonas show a particularly clear difference between the CV membrane that is undecorated and the decorated flattened or tubular membranes that bind to and extend from the smooth CV membrane.[126] These tubules do not appear to expand to become part of the CV membrane, as neither their cytosolic pegs nor their luminal fibrous contents are found to be part of the CV membrane proper. In other algae, the decorated tubules are more spherical and, after fusion with the CV membrane, remain as decorated patches protruding from the CV surface into the cytosol.[44,45]

In ciliates, the number of CVs per cell or some of the parameters of the CV cycle (e.g., CV volume and rate of expulsion) vary positively or negatively depending on the size of the cell.[70] Flagellated protozoa usually have one or two small CVs located in a pocket at the base of the flagellum. Amoebae, such as Amoeba proteus, have one CV, which is not fixed in place but can lie close to the nucleus and then migrate to the uroid (posterior) region, where it will dock at and fuse with an apparently unspecialized region of the plasma membrane.[1,23] Giant amoebae, such as Chaos carolinensis,[98] have many CVs, as does the ciliate Ichthyophthirius, the causative agent of white spot disease of freshwater fish.[18] Dictyostelium discoideum has an extensively studied CVC system that consists of one or two main bladders, which originally were reported to contain the marker enzyme alkaline phosphatase,[97] but the reliability of this marker has been questioned recently.[21] Extending from these bladders is a network of tubules and smaller expanded compartments that remain close to the cell surface.[47] All of these membranes, when viewed in quick-frozen, deep-etched replicas and in replicas of freeze-dried fragments of disrupted cells, bear V-ATPase pegs of 15 nm; even the largest bladders that fuse with the plasma membrane have pegs.[46,47] Using an antibody specific for the B-subunit of the V_1 subcomplex of the V-ATPase holoenzyme, Heuser et al.[47] showed that these 15-nm pegs clump in the presence of this B-subunit antibody which confirms that they contain components of the V-ATPase complexes. A monoclonal antibody (mAb) specific for the 100-kDa accessory protein of the V-ATPase of Dictyostelium and other antibodies such as that to calmodulin were used to fluorescently label the tubules and saccules[33,130] of this CVC in interphase as well as in dividing cells.[131]

The CVC seems to have reached its largest size and complexity in the ciliated protozoa. For electron micrographs of such CVCs in the ciliates, see appropriate chapters in Allen's website (www.pbrc.hawaii.edu/allen): Tetrahymena, Chapter 18, Figures 50 and 55; Paramecium, Chapter 9; Nassula, Chapter 16, Figure 15; Vorticella, Chapter 19, Figures 1i and 24 to 27; Coleps, Chapter 11, Figure 7a; and Didinium, Chapter 13, Figure 9. Most ciliated protozoa have one or two CVs (but occasionally many more) that are composed of a smooth undecorated membrane that is in contact with a three-dimensional spongiome of membranes that encircles or extends from the CV. This peripheral mass of membranes includes both smooth and decorated tubules that are not easily distinguished from each other in transmission electron micrographs of many ciliates. Presumably, the smooth membranes can become part of the CV membrane to provide for CV enlargement while the decorated tubules do not become part of the expanding CV membrane. The decorated or peg-bearing membranes, for most of the time, remain as tubules even when they are continuous with the smooth, undecorated membrane meshwork.[73,74] Under hyperosmotic stress, however, exposure to cold or during cell division all or some of these tubules in Paramecium round into vesicles and separate from the smooth membranes of the radial arms.[34]

The CVs of ciliates are not free to move about in the cell but are attached to the surface of the cell, each at an indentation of the plasma membrane called a CV pore.[72] This pore (or often several closely spaced pores) is located at a specific location on the surface of the cell. Where the pore is

located seems to be unique to different groups of ciliates; for example, in Tetrahymena two pores lie near the posterior end of the cell and both are attached to the same CV.[7] In Vorticella, several pores are attached to one CV that opens into a pellicle-lined chamber that in turn opens into the peristome region of the cell (see Allen's website, Chapter 19, Figures 1i and 24 to 27). In Paramecium, which typically has two CVs per cell, pores are found on the dorsal somatic surface of the cell, one on the anterior-dorsal half and one on the posterior-dorsal half of the cell. Only one pore is associated with each CV. In most ciliates, if not all, the pores are short cylindrical or funnel-shaped indentations of the plasma membrane whose cytosolic surfaces are each supported by one or more helically wrapped microtubules. Five to ten bands of microtubules originate from or against these helically wound pore microtubules and radiate out over the CV membrane where they are bound to and hold the CV against the bottom of the pore. In most ciliates, these bands of microtubules are relatively short and do not extend much beyond the surface of the expanded CV. In Paramecium, however, these radiating microtubules extend far from the CV membrane where they pass into the cytosol, remaining near the cell surface, for many micrometers.[8,34] The number of microtubular ribbons determines the number of radial arms (Figure 3.1), as well as the number of collecting canals and thus the overall radial shape of the CVC.

Only in the peniculine ciliates, to which Paramecium belongs, are such elaborate CVCs found. Thus the complexity of the CVC seems to have reached its apex in Paramecium and its close relatives. In these ciliates, the CVC has a strict spatial separation of smooth membranes from decorated membranes so these two populations of membranes are easily distinguished in transmission electron micrographs as well as in immunologically labeled fluorescence micrographs (Figure 3.1). Only the smooth spongiome is in contact with the microtubular ribbons, and these ribbons permit the formation of the long collecting canals in Paramecium by allowing the tubular smooth spongiome to form linear rows of 40-nm circular connections that lie between the subdivisions of the ribbons where the tubules then expand into the canals.[43]

In summary, many CVCs are composed of at least two pools of membrane: (1) a smooth membrane that can expand from a tubular form into fluid reservoirs including the CV, ampulli, and collecting canals, and (2) a decorated form that maintains a tubular or small-vesicule shape with a greatly reduced volume-to-surface area ratio. This latter decorated form can fuse with the smooth form but does not appear to fuse with the plasma membrane and does not contact microtubules. The smooth form can fuse both with the decorated form and with the plasma membrane and, in Paramecium, it also binds to microtubules.[120] In Dictyostelium, both tubules and bladders have only one type of membrane based on their decoration with pegs,[46,47] although biochemically two types of membranes were reported.[87] Chlamydomonas has also been reported to have only one type of membrane.[69] A third pool of membrane, consisting of vesicles called acidocalcisomes, has been observed in the smaller miroorganisms, including Trypanosoma,[28,75] Chlamydomonas,[102] and Dictyostelium,[71] that can apparently fuse with some part of the CVC. The spongiome membrane pools, once they are formed in the cell, appear to remain separate from other endomembranes in the cytoplasm, including the endoplasmic reticulum, the Golgi cisternae, the endosomal membrane system, and the digestive vacuole membrane system.[36] CVC membranes must originate from the endoplasmic reticulum and pass through the Golgi stacks as their membranes form and become differentiated, but the process of new CVC membrane formation has not been studied in detail. CVC membranes appear to be slow to break down, as no trace of these membranes has been observed inside autophagosomes, which are most often seen in Paramecium to contain the breakdown products of mitochondria.

C. Effects of External Osmolarity on CVC Morphology

The rate of fluid segregation and expulsion by the CVC (R_{CVC})[109] varies as the external osmolarity of the bathing solution varies. When the cell is placed in solutions hypoosmotic to the cytosol, the rate of fluid accumulation increases. This may be detected as an increase in the maximum CV

diameter before fluid expulsion, as a decrease in time between successive CV expulsions, or by a combination of both parameters. No morphological change in the fine structure of the CVC was noted following relatively small hypoosmotic changes. The usual maximum size of a CV adapted to standard saline in Paramecium multimicronucleatum is 13 • m in diameter,[50] while CVs of Amoeba proteus can be 27 to 45 • m in diameter.[23,86] The filling and expulsion cycle of P. multimicronucleatum growing in 80 mOsmol/L is completed in about 10 sec.[50] In the epimastigotes of Trypanosoma cruzi, the CVC cycle lasts 60 to 75 sec;[15] in the zoospores of Phythophthora, the cycle is completed in 6 sec;[76] and, in Dictyostelium, it is completed in 3 to 4 sec.[37]

Morphological changes in the water segregation system of Paramecium multimicronucleatum occur when cell cultures are subjected to pronounced decreases in external osmolarities or when cell cultures have been subjected to a high external Ca^{2+} concentration.[53] These changes involve the production of: (1) longer and bifurcated radial arms, (2) more radial arms per CVC, and (3) the development of additional CVCs per cell. The maximum number of CVCs observed in one P. multimicronucleatum cell was seven.[53] Additional CVCs over the usual interphase number had been reported before,[8,57] but this phenomenon is now known to be triggered either by a significant decrease in the external osmolarity or by a significant rise in the external calcium concentration.[53] On the other hand, an equally large increase in external K^+ concentration was not found to lead to extra CVCs.[53]

The response of the CVC to hyperosmotic conditions can also be complex. When the cell is placed in hyperosmotic solutions, the rate of water accumulation will soon fall to zero and the CVC will become inactive. In high hyperosmotic conditions, the decorated tubules around the radial arms, at least in Paramecium, will disappear and can no longer be detected immunologically when V-ATPase-specific mAb labeling is used.[39,52] Instead, in these cases, the mAb becomes dispersed throughout the cytosol. Observed by electron microscopy, the decorated tubules will have lost their tubular shape and will have expanded into vesicles of various sizes that are no longer connected to the smooth spongiome.[34,52] If the cell remains in the high hyperosmotic solution long enough, around 8 hours, the decorated tubules will gradually reappear around the radial arms, and CVC activity will be partially restored.[52] If the cell is returned to a hypoosmotic medium, the decorated tubules begin to reappear in 20 min and the CV will return to full activity within 1 hr.[39]

D. Ions and Osmolytes of the Cytosol and CVC

The older literature on inorganic ion concentrations (K^+, Na^+, and Cl^-) in the cytosol of protozoa was summarized by Prusch.[94] K^+ is present in most freshwater protozoa at a concentration of ~25 to 35 mmol/kg wet weight of cells, with amoebae generally containing concentrations near the low end of the range. The marine ciliate Miamiensis avidus has a cytosolic K^+ concentration of 74 mmol/kg. The concentration of Na^+ in the cytosol was more variable and generally lower, ranging from 0.5 to 20 mmol/kg, with the exception of the marine ciliate, which had a Na^+ concentration of 88 mmol/kg. Cl^- was low in both freshwater and marine cells, measuring 0.36 to 16 mmol/kg, much lower than the Cl^- concentration in the external medium, which measured up to 550 mmol/L in saltwater. Only in the amoebae Chaos carolinensis and Amoeba proteus were the Cl^- concentrations in the cytosol considerably higher than in the external medium. In these earlier studies, in no case was enough Cl^- reported in the cytosol to counterbalance all inorganic cation concentrations present.

Studies performed to determine if active transport of ions is occurring across the plasma membrane and if this active transport is coupled reported that Na^+ and K^+ are apparently both transported actively in Acanthamoeba but they were not coupled.[63] In Amoeba proteus, both Na^+ and K^+ are actively transported across the plasma membrane, K^+ is actively accumulated, and Na^+ is actively eliminated.[96] The ability of the cell to regulate Na^+ was dependent on Ca^{2+} in the external medium. Both Na^+ and K^+ can be actively transported in Tetrahymena, whereas Cl^- is apparently distributed passively.[10,30]

Marine and brackish water ciliates, as expected, had much higher cytosolic osmolarities which resulted from a summation of their inorganic ions and free amino acid concentrations, as well as other unspecified intracellular participants that might include organic osmolytes. The marine ciliate Miamiensis avidus, a facultative parasite living on the seahorse, has lower concentrations of Na^+ and Cl^- in its cytosol than are present in the external environment.[55] These ions vary with changes in salinity, increasing with increased salinity but always remaining lower than their concentrations in the external medium. The cytosolic concentration of K^+ was much higher than the exterior K^+ concentration, but this was affected less by changes in salinity than were the Na^+ and Cl^- concentrations. Osmolarities changed rapidly, within 10 min. A 30-min exposure to a changed external osmolarity caused swelling or shrinkage of the cell under hypoosmotic or hyperosmotic stress, respectively. The cells then returned to their approximate original volume in about 90 min. The total cytosolic osmolarity of M. avidus that resulted from both inorganic ions and free amino acids was about 540 mmol/kg cells in seawater. The free amino acid concentration was 317 mmol/kg cells, and alanine, glycine, and proline made up 73% of the free amino acids.[56] The concentration of free amino acids increased and decreased with salinity changes; 25% seawater resulted in a 76% reduction in free amino acids, and 200% seawater resulted in an increase of 22% free amino acids over that in 100% seawater. These changes were completed in 20 min and were apparently the result of the metabolic release of bound amino acids during hyperosmotic stress rather than amino acid uptake from the medium.[56]

The brackish water ciliate Paramecium calkinsi can be adapted (over a month) to osmolarities from 10 to 2000 mOsmol/L but it does not divide above 1000 mOsmol/L. It uses both organic and inorganic osmolytes for osmoregulation. Cells exposed to hyperosmotic changes will increase their free amino acid concentrations, particularly alanine and proline, but this requires several hours in the case of alanine and much longer in the case of proline. Upon exposure to hypoosmotic stress, however, much of the free alanine and proline of the cell are released from the cell (within 5 min), and these can be recovered in the external medium. Thus, free amino acids, particularly alanine and proline, play an important role in osmoregulation when the protozoan is subjected to hypoosmotic stress, but the increase of free amino acids in the cytosol would be too slow to quickly reestablish the required hyperosmotic cytosol when the cell is under hyperosmotic stress.[24] Dictyostelium also secretes half or more of its load of amino acids, particularly glycine, alanine, and proline, when it encounters hypoosmotic stress.[107]

The in situ ionic contents of the CVC have only recently been determined in a living protozoan cell. Earlier attempts to determine CVC osmolarity relied on micropuncture and freezing point depression techniques and, for ionic content, on helium-glow photometry to collect and assay cellular fluids.[98,104] This led to the conclusion that the CV fluid of an amoeba was hypoosmotic to the cytosol. This finding has been difficult to reconcile with generally accepted ideas of water permeability of cellular membranes, based on the obvious accumulation of fluid within the CV, and has required some innovative speculation as to how water could accumulate against a hypoosmotic gradient in the CV, a question still unresolved in cells such as Dictyostelium.[46,107] Recently, with the use of ion-selective microelectrodes, it has been possible to actually measure the ionic activities of several major inorganic ions present in living Paramecium multimicronucleatum, both in the cytosol of the cell and at the same time in the CV of the cell.[110] In cells adapted to a 24-mOsmol/L standard saline solution that did not contain Na^+ (as used by Paramecium electrophysiologists[83]), the cytosol had a 22.6 K^+ activity (all ionic activities are in mmol/L) compared to 56.0 in the CV, 3.9 of Na^+ in the cytosol (presumably carried over in the cell from the previous culture conditions) compared to 4.7 in the CV, and 27.3 of Cl^- in the cytosol compared to 66.5 in the CV. Ca^{2+} activity in the cytosol was too low to measure by this ion-selective microelectrode technique but was measurable at 0.23 in the CV. Thus, the major inorganic ions in the CV of Paramecium are K^+ and Cl^-. These results show that the cytosolic Cl^- activity in freshwater protozoa is actually much higher than the older determinations had reported (see Table 1 in Prusch).[94] Thus, Cl^- can act as the counterbalancing anion for most, if not all, of the free inorganic cations present in the cytosol and

TABLE 3.1
Ratios of K^+ and Cl^- Activities in the CV Fluid Compared to Those in the Cytosol

	Adaptation Solution/Experimental Condition									
External Osmolarity	K^+-Containing		Choline-Containing		Ca^{2+}-Containing		Furosemide		DMSO	
(mOsmol/L)	K^+	Cl^-	K^+	Cl^-	K^+	Cl^-	K^+	Cl^-	K^+	Cl^-
24	2.5	2.4	2.4	2.1	2.3		2.4	2.4	2.5	2.5
64	2.4	2.5	—	—	—	—	—	—	—	—
104	2.1	1.9	—	—	—	—	—	—	—	—
124	2.3	2.0	2.5	2.0	2.4	2.0	—	—	—	—
164	2.1	2.3	—	—	—	—	—	—	—	—

Source: Stock, C. et al., J. Cell Sci., 115, 2339, 2002. With permission.

in the CV. It is not yet known, however, if K^+ and Cl^- are the major osmolytes in the CVCs of other cells that have this organelle.

A potentially important finding was that the ratios of both K^+ and Cl^- activities in the CV compared to the K^+ and Cl^- activities in the cytosol were maintained at between a 2.0- and 2.4-fold higher level in the CV over that of the cytosol, and these ratios stayed within this narrow range even as the ion concentrations in the cytosol and CV increased as the external osmolarity was increased from 24 to 164 mOsmol/L (see Table 3.1).[110] This suggests the presence of a mechanism in the cell, probably in the CV membrane, that maintains the K^+ and Cl^- activities of the CV at 2.0 to 2.4 times that of the cytosol. The activities of these two ions within the CV and presumably the overall osmolarity of the CV are therefore regulated by the cytosolic osmolarity and by the individual ionic activities in the cytosol, rather than the CVC determining or regulating the cytosolic ionic composition or the overall cytosolic osmolarity. Thus, the CVC does not control the osmolarity of the cytosol; rather, the cytosol, with a possible contributing role by the CVC membrane, determines the osmolarity of the CV.

As was the case for cytosolic osmolarity (Figure 3.2A), the individual ionic activities of K^+ and Cl^- increased in both the cytosol and CV in steps instead of linearly (Figure 3.2B and C). These steps occurred at the same external osmolarities as the increases in cytosolic osmolarity, at ~75 and ~160 mOsmol/L. It seems likely that either K^+ is actively transported into the cytosol from the external medium together with Cl^- or active K^+ transport is followed by Cl^- moving through Cl^- channels. These two ion species may then be moved together or separately into the CV, and water will enter both the cytosol and the CVC passively by osmosis.

As already mentioned, the presence of different single ion species or a mixture of ion species outside the cell combined with changes in external osmolarity will ultimately affect the cytosolic osmolarity and cytosolic ionic composition as well as the osmolarity and ionic composition of the CVC fluid (Figure 3.3) in a complex way. Single-ion species or a mixture of ion species outside the cell will also affect the rate of fluid expulsion by the CVC (R_{CVC}).[111] When the bathing solution to which Paramecium was adapted for 18 hr contained: (1) 2-mmol/L K^+ in MOP-KOH buffer, (2) 2-mmol/L Na^+ in MOP–NaOH buffer, (3) a mixture of 1-mmol/L K^+ and 1-mmol/L Na^+ in MOP–KOH buffer, or (4) 2 mmol/L of the organic cation choline without buffer, it was observed that the fluid segregation (or expulsion) rate (R_{CVC}) was the same in cells adapted to either K^+ or Na^+ alone; however, when K^+ and Na^+ were both present at 1 mmol/L each, thus making a total of 2 mmol/L, R_{CVC} was almost twofold higher (Table 3.2). In the solution enriched in the organic cation choline, R_{CVC} was about half the rate of that in Na^+ or K^+ alone. The sum total of K^+, Na^+, Ca^{2+}, and Cl^- activities in the CV fluid remained about equal in cells adapted to K^+ or Na^+ alone for a particular external osmolarity (e.g., 24 mOsmol/L), but when both K^+ and Na^+ were present

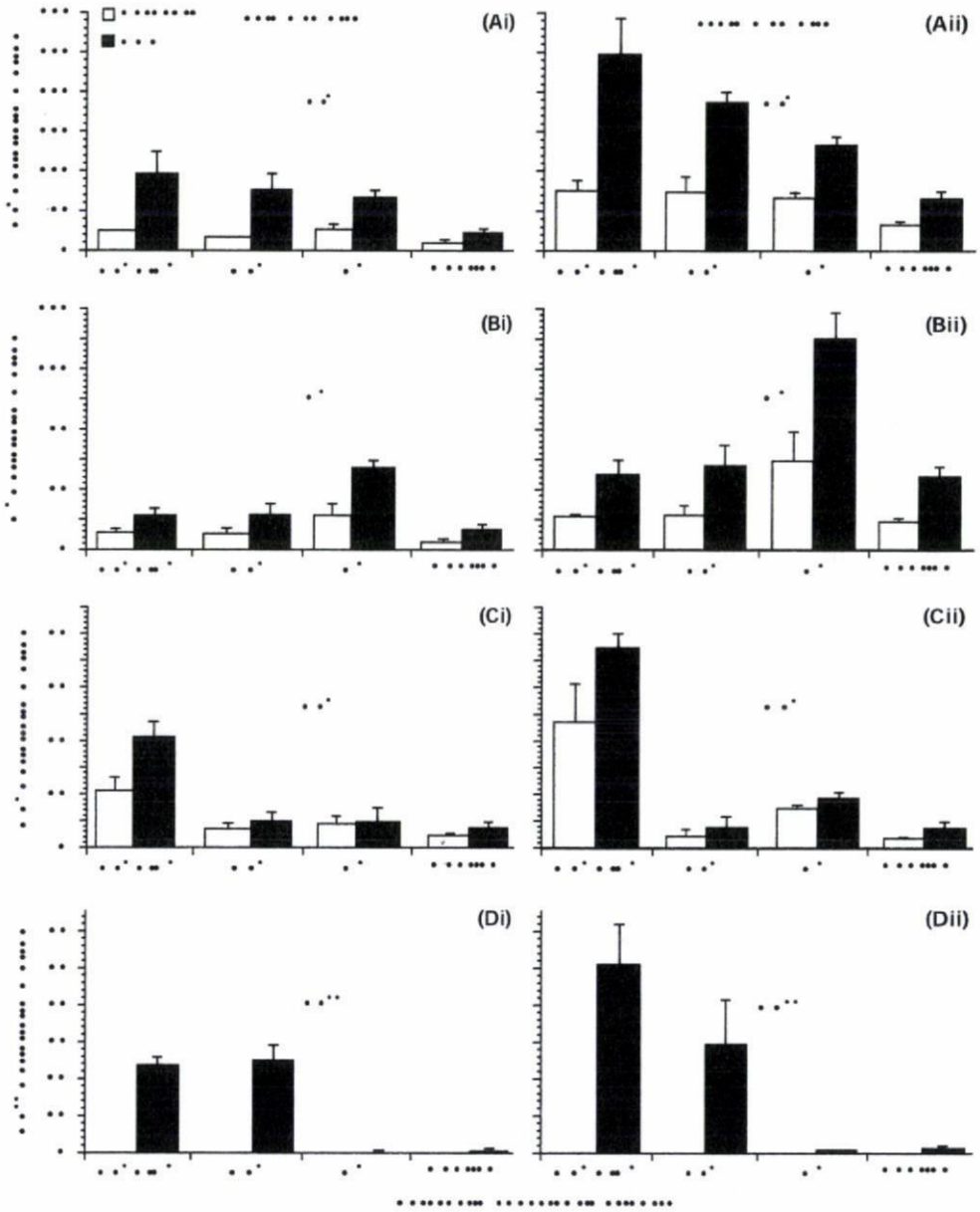

FIGURE 3.3 Ionic activities of (A) Cl^-, (B) K^+, (C) Na^+, and (D) Ca^{2+} in the cytosol (white bars) and CV fluid (black bars) of Paramecium multimicronucletum as a function of the external osmolality. The left column is of cells adapted to 24 mOsmol/L (Ai to Di), and the right column to 124 mOsmol/L (Aii to Dii). The two adaptation osmolalities also contained four ionic conditions: (1) 1-mM K^+ plus 1-mM Na^+; (2) 2-mM Na^+ alone; (3) 2-mM K^+ alone; and (4) 2-mM choline alone as the monovalent cations. (Numerical values for the CV fluid are reported in Table 3.2). Vertical lines represent SD. (From Stock, C. et al., Eur. J. Cell Biol., 81, 505, 2002. With permission.)

together the sum total of their ionic activities within the CV was significantly higher. The presence of both K^+ and Na^+ in the adaptation solution did not lead to an increase in the cytosolic osmolarity but did increase the estimated osmolarity of the CV fluid (due mostly to the rise in Na^+ activity in the CV); consequently, the osmotic gradient between the cytosol and the CV fluid was significantly

TABLE 3.2
Overview of Ionic Activities in Contractile Vacuole, Overall Cytosolic Osmolarity, Estimated Osmotic Gradient across the Contractile Vacuole Membrane, Rate of Fluid Segregation, and Membrane Potential of Contractile Vacuole Complex in **Paramecium multimicronucleatum** Cells Adapted to 24- or 124-mOsmol/L Solutions Containing $K^+ + Na^+$, Na^+, K^+, or Choline as the Monovalent Cation

	Adaptation Solution							
Monovalent	24 mOsmol/L				124 mOsmol/L			
Cation Species	$K^+ + Na^+$	Na^+	K^+	Choline	$K^+ + Na^+$	Na^+	K^+	Choline
$a_{Cl^-_{CV}}$ (mmol/L)	96	77	67	33	244	187	131	66
$a_{Na^+_{CV}}$ (mmol/L)	20	5	5	4	38	4	10	4
$a_{K^+_{CV}}$ (mmol/L)	23	24	56	14	51	57	141	50
$a_{Ca^{2+}_{CV}}$ (mmol/L)	23	25	0.2	0.7	51	29	0.7	1.2
$\bullet a_{ion_{CV}}$ (mmol/L)	162	131	128.2	51.7	384	277	282.7	122.2
Osm_c (mOsmol/L)	60	56	66	32	168	165	178	132
Osmotic gradient$_{CV}$[a]	102	75	62.2	19.7	216	112	104.7	−9.8
R_{CVC} (fL/sec)	131	67	69	33	34	20	18	3
V_{eVC} (mV)	81	95	84	93	79	87	84	87

[a] Estimated by subtracting Osm_c from $\bullet a_{ions_{CV}}$.

Note: $a_{ion_{CV}}$, Cl^-, Na^+, K^+, and Ca^{2+} activities in the contractile vacuole; $\bullet a_{ion_{CV}}$, the sum of all ion activities in the CV; Osm_c, the overall cystosolic osmolarity; R_{CVC}, the rate of fluid segregation; V_{CVC}, the CVC membrane potential. The values shown are rounded numbers.

Source: Stock, C. et al., Eur. J. Cell Biol., 81, 505, 2002. With permission.

higher when both K^+ and Na^+ were present together. All of the above values determined for 124-mOsmol/L-exposed cells were higher by equivalent amounts (the external osmolarity had been increased by adding sorbitol). Thus, cells adapted to a mixture of K^+ and Na^+ exhibited a higher osmotic gradient across the CVC membrane than if either K^+ or Na^+ had been present alone. Water also flowed into the CVC faster which was reflected by the higher R_{CVC} in the mixture of K^+ and Na^+.[111]

When adapted to the organic cation choline, most corresponding values were significantly lower—50 to 80% or more lower, except for the cytosolic osmolarity, which was lower by only ~25%. The CVC was still active in cells in choline but its R_{CVC} in an external osmolarity of 124 mOsmol/L was only 3 fL/sec (one femtoliter = 10^{-15} L) compared to 33 fL/sec when in a 24-mOsmol/L adaptation solution—a 10-fold decrease.[111]

Na^+ was accumulated significantly in the CVC and the cytosol only when external Na^+ was present together with K^+. This probably indicates that a significant part of the Na^+ in Paramecium is cotransported with K^+ across both membranes. Some Na^+ cotransport was previously proposed for Tetrahymena pyriformis.[10,31] At the higher 124-mOsmol/L osmolarity a higher amount of Na^+ was present in both the cytosol and the CV when the cells had been adapted to K^+ solution alone rather than to Na^+ solution alone. On the other hand, K^+ was taken up much more rapidly when the cells were adapted to K^+ alone, in both the cytosol and CV, as opposed to when a mixture of Na^+ and K^+ was present (Figure 3.3).

Exposing cells to a medium with high levels of calcium, as would be expected, did not produce a significant rise of calcium in the cytosol but did result in a profound accumulation of calcium activity in the CVC fluid.[110] The calcium activity in the CVC of calcium-exposed cells in an external

osmolarity of 24 mOsmol/L was between 20 and 25 mmol/L, whereas in 124-mOsmol/L-exposed cells it was 50 mmol/L. This huge increase over the activity in the cytosol was true only in cells adapted to Na^+ plus K^+ or in Na^+ alone; very little calcium was found in the CVs of K^+-adapted or choline-adapted cells. This indicated that calcium entered the cell in association with Na^+ rather than with K^+, but once in the cytosol Ca^{2+} is quickly transferred to calcium storage sites, such as the alveolar sacs of ciliated protozoa[42,93,108] or it is transferred to the CVC compartments for excretion from the cell.[110,111]

Although each CVC in Paramecium is estimated to have millions of V-H^+-ATPase enzymes, the fluid of the CV does not become acid as it does in phagosomes that have far fewer V-H^+-ATPases per unit of membrane area.[32,51] By using ion-selective microelectrodes filled with a cocktail sensitive to H^+ activity, the in vivo pH of the CV fluid was found in Paramecium to be only mildly acid (pH of 6.4), whereas that of the cytosol of the same cell was neutral (pH of 7.0). Altering the external osmolarity from 24 or 124 mOsmol/L had no effect on the pH of either the cytosol or the CV. Thus, most of the protons inside the CVC lumen are either not present in ionic form or they are quickly exchanged for cations (K^+, Na^+, and Ca^{2+}) during the import of these cations into the CVC that help to give rise to some of the +80-mV luminal electrochemical charge across the CV membrane.[118] The primary role of the V-ATPases in the CVC membrane is clearly to energize the CVC membrane rather than to produce a strongly acidic compartment.

E. Electrophysiology of the CVC of Paramecium

Electrophysiological techniques were used to determine if the CVC of Paramecium had an electrical potential across its membrane, as well as to estimate the membrane fusion and fission events that occur and the resistance/conductance of its membranes to ion flow. A fine-tipped microelectrode was inserted into the living CV and an electrical potential of +80 mV was recorded relative to the cytosol.[118] A continuous recording of several filling and expulsion cycles of the same CV showed that, just before expulsion, the electrical potential dropped precipitously to a level near +10 mV (Figure 3.4). In addition, input capacitance measurements made at the same time indicated that the radial arms had become disconnected from the CV shortly before expulsion of the CV concomitant with the CV undergoing a rounding or contraction process. Thus, at the time of expulsion, the CV was no longer in continuity, with its electrogenic source providing most of the +80-mV electrochemical potential of the CVC. The electrogenic source was therefore found to apparently reside in the V-type proton pumps arrayed on the decorated tubules that, in turn, are found only along the radial arms. At this point, the CV membrane fused with the plasma membrane at the bottom of the CV pore, and the CV was emptied by cytosolic pressure.[84] The pore then closed by fission, and the CV membrane was resealed against the exterior of the cell. After resealing, the CV registered a brief period of negative potential before the electrical potential quickly returned again to +80 mV as the several radial arms quickly reassociated with the CV. Input capacitance, which was low during the expulsion phase, rapidly returned to its earlier higher plateau value, indicating that the radial arms had once again fused with the membrane of the collapsed CV. These measurements also showed that the conductance (the reciprocal of input resistance) was high when the radial arms were attached to the CV but fell rapidly when the CV was no longer attached to the decorated tubules via the smooth spongiome located along the radial arms. Thus, the CV membrane itself seems to have little electrical conductance compared to the total of all the membranes of the CVC.

By determining the diameter of the CV and the membrane area of the CV, it was evident that the visible CV itself did not suddenly return to its maximum diameter (Figure 3.4). As expected, the microscopically visible CV grew more or less linearly during the filling phase following the initial emptying of the engorged ampulli into the CV;[118] however, the input capacitance measurements showed that the total membrane area of the CVC system had rapidly reconnected with the CV membrane long before the microscopically visible CV had returned to its maximum diameter. This confirmed that the CV during expulsion did not vesiculate into a myriad of individual vesicles but

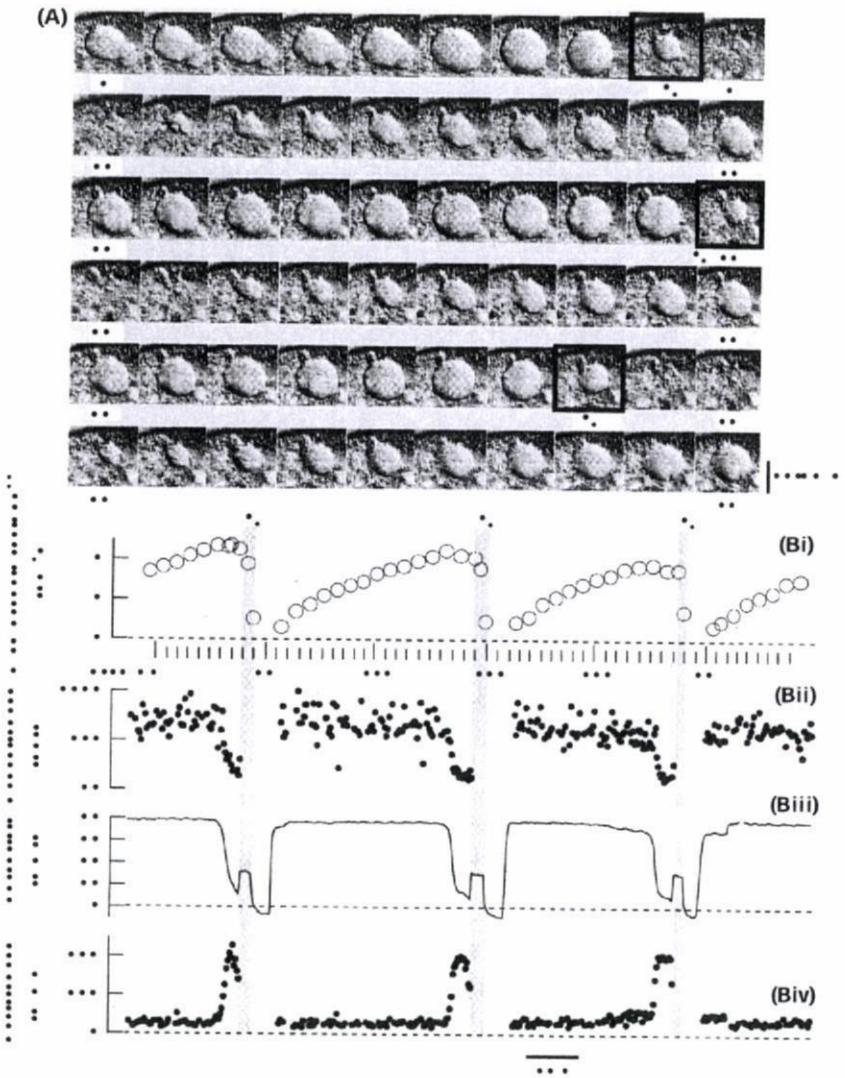

FIGURE 3.4 Electrophysiological parameters of a microelectrode-impaled contractile vacuole of a Paramecium multimicronucleatum cell during three successive exocytotic cycles. (A) A series of 60 consecutive images of the contractile vacuole profile taken at 2-sec intervals. Some frames (0–59) are numbered. Black-bordered images correspond to the fluid expulsion phases (e_1–e_3). (Bi) The contractile vacuole membrane area in each frame. (Bii) Input capacitance of the organelle. (Biii) Membrane potential of the organelle with reference to the cytosolic potential. (Biv) Input resistance of the organelle. (From Tominaga, T. et al., J. Exp. Biol. 201, 451, 1998. With permission.)

underwent a process of total membrane tubulation where the membrane of the CV reverted into a three-dimensional array of contiguous 40-nm tubules that maintained continuity with a single CV system or with its radial arms (Figure 3.5). Apparently, the only fission occurring along the radial arms was when the membrane of each arm separated as a unit from the CV membrane prior to rounding. During CV expulsion, the collapsed CV could not be detected by light microscopy, so it superficially appeared that the CV had vanished; however, as fluid flowed back into the CV compartment from the reconnected radial arms, the tubules from the collapsed CV reexpanded to form the membrane of the once again microscopically visible CV (see Allen's website, Chapter 9, Video 1).

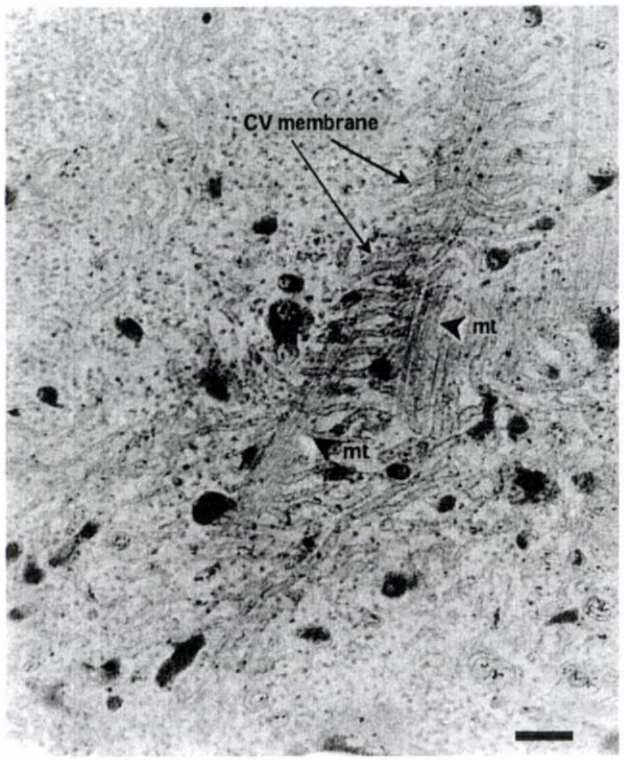

FIGURE 3.5 During systole the CV membrane collapses into 40-nm tubules (arrows) that tend to lie crossways to the microtubular ribbons (arrowheads) that extend from the CV pore. Transmission electron micrograph of chemically fixed, broken cell; electron opaque material entered the disrupted CV prior to or during tubulation. Scale bar, 0.2 • m.

To confirm that the radial arms are the sites of the electrogenic engines of the CVC of Paramecium, compressed cells were studied.[39] In these compressed cells, CV potential was found to increase in steps after the expulsion phase (systole), with each step representing the reattachment of one or a few radial arms with the CV. Compressing the cell seemed to interfere with and slow reattachment of the arms. In noncompressed cells, arms seem to reattach simultaneously. A stepwise reduction in CV potential was also observed in these compressed cells at the start of rounding prior to opening of the CV pore.

The number of functional V-ATPases did not directly determine the rate of fluid segregation in the CVC nor did different osmolarities significantly change the CV membrane potential.[39] The CV membrane potential rose to +80 mV or slightly higher and stayed there in cells adapted to external osmolarities from 4 to 124 mOsmol/L. The rate of fluid segregation by the CVC (R_{CVC}) was reduced from 98 to 20 fL/sec as cells were adapted upward through this range of osmolarities. No changes in the final immunologically fluorescent images of the radial arms were observed in cells that had changed from a R_{CVC} of 98 fL/sec to 20 fL/sec, which supported the conclusion that the number of functional V-ATPases did not change significantly. When hypoosmotically adapted cells were changed to a much higher hyperosmotic condition (from 4 mOsmol/L to 124 mOsmol/L) for 30 min and then returned to the original hypoosmotic conditions, it was observed that the fluorescently labeled decorated tubules of the radial arms had disappeared and the potential of the tubulated CV membranes had fallen drastically. This was based on the fact that 20 min after the return of the cell to a hypoosmotic standard saline solution, as radial arm fluorescence began to reappear, the CV potential was now only +44 mV. The potential reached +80 mV by 60 min.

R_{CVC} that had fallen to 0 increased over time from 58 fL/sec at 20 min to ~100 fL/sec at 60 min. Hypoosmotic environments (going directly from 124 mOsmol/L to 4 mOsmol/L) had no effect on the CV membrane potential but did result in an increase of R_{CVC} from 20 fL/sec at 124 mOsmol/L to 103 fL/sec at 4 mOsmol/L. No change was observed in the fluorescent images of the radial arms during this drastic hypoosmotic change.

Exposure of cells to 30 nmol/L of concanamycin B, an inhibitor of V-type ATPases, for 30 min resulted in a 50% decrease from +80 to +40 mV of CV membrane potential in 4-mOsmol/L-adapted cells, and the R_{CVC} decreased 43%.[39] These experiments help to confirm that the membrane potential is generated by voltage-producing processes occurring in the CVC membranes. These processes involve the V-ATPase enzymes that pump protons into the lumen of the decorated tubules. When the cell is placed in a strongly hyperosmotic environment, the V-ATPase holoenzymes fall apart, the CV membrane loses most of its electrochemical potential, and the CVC is no longer functional in eliminating water and electrolyte ions from the cell. It then takes 60 to 120 min for the total population of V-ATPases to reassemble, for the +80-mV CV membrane potential to be restored, and for the fluid segregation rate to return to its normal activity level.[39]

Because the CV potential remains the same at widely different external osmolarities but R_{CVC} changes significantly under this same range (i.e., decreasing with increasing osmolarity), it appears that the CV potential is kept at a maximum to provide for the exchange of K^+ and other cations for protons. Such an exchange may than be followed by the attraction of Cl^- into the CV lumen by the positive electrical gradient of protons, or Cl^- may enter by cotransport of K^+ and Cl^- into the CV lumen. A CV pH of 6.4 argues for proton exchange as the CV only becomes mildly acid.[111] The resultant accumulation of ions in the CVC (K^+ and Cl^- are 2.0 to 2.4 times higher) provides the osmotic gradient that will support the flow of water into the CVC by osmosis. The sum of the activities of the common inorganic ions in the CV is much higher than the activities of these same ions in the cytosol that contribute to the lower osmolarity of the cytosol (see Table 3.2).[111]

F. Membrane Dynamics of the CVC

Because of its apparent periodic contractility, the contractile vacuole has fascinated observers of protozoa from the very early days of microscopy. At the end of a cycle of filling, the CV was observed to fuse with the cell membrane and to disappear from view as its contents were released. It was generally assumed that this contractility was caused by an actin–myosin cytoskeletal system that surrounded the CV; however, no such system has ever been observed either by electron microscopy or by immunological labeling techniques. Only recently has it been possible to begin to understand what precedes fusion of the CV with the plasma membrane and what happens to the CV membrane once fusion occurs. A combination of electron microscopy, electrophysiology, and biophysical techniques has led to a partial understanding of the events that occur at the end of the filling phase (diastole) and during the expulsion phase (systole).

Electron microscopy of Paramecium demonstrated that the CV membrane during systole collapses not into a flattened sac but into a meshwork of 40-nm tubules (Figure 3.5), which branch from each other to form a meshwork that remains bound to the noncontractile microtubular ribbons radiating from the cytosolic funnel-shaped surface of the CV pore.[4,85,120] The particular combination of molecular components that make up the bilayer of the CV membrane probably ensures that the membrane returns to a tubular form when the internal hydrostatic pressure of the CV is released. Bending energy is stored in the membrane when the tubules are forced into a more planar shape, and this energy is released when the membrane is allowed again to become tubular.[84] Although this bending energy is not sufficient to account for the rapid expulsion of the fluid from the CV in a living cell, it does seem to be sufficient to expel the fluid at the lower rate observed when a CV was still able to fuse with the plasma membrane of a ruptured cell where the cytosolic pressure had been eliminated.[84]

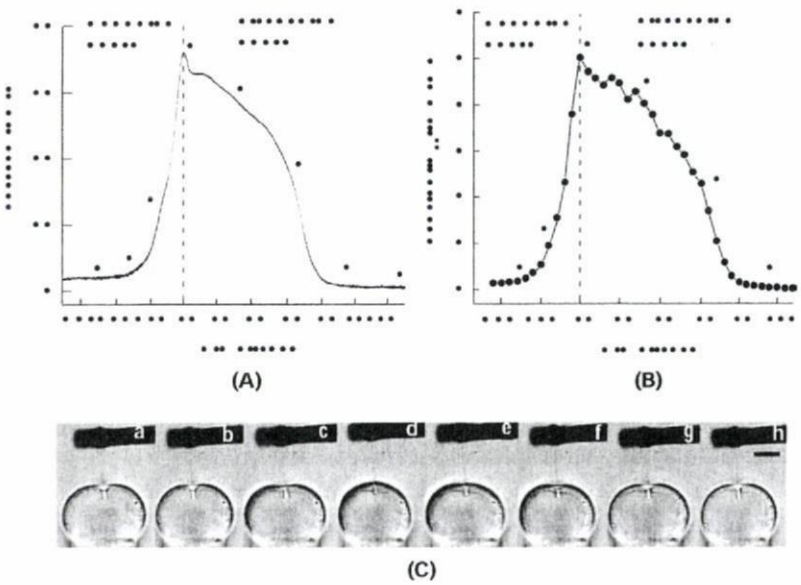

FIGURE 3.6 (A) A trace of the output voltage of a position sensor that follows the tip of a cantilever pressed against an in vitro CV. The output voltage varies with the force (F) generated by the rounding/relaxing cycle of the in intro CV. (B) A single tension (T_{cv})-developing cycle of an in vitro CV. (C) Each image labeled by a letter was taken at the time corresponding to each letter beside the trace of (A) F and (B) T_{cv}. (Adapted from Tani, T. et al., J. Cell Sci., 114, 785, 2001. With permission.)

Not only does this smooth part of the CV spongiome membrane exist as a highly curved membrane when it is at its lowest energy state, but it also has other unique properties that are revealed during the rounding phase of the CV that occurs just before systole. At this point, an innate timing mechanism is triggered that leads to an increase in tension within the CV membrane of 35 times its resting level (Figure 3.6).[115,116] This timing mechanism is a unique property of the smooth spongiome membrane itself and may involve an enzyme system that is dependent on adenosine triphosphate (ATP) as its energy source. These enzymes may bring about a reversible modification of the membrane structure. Any isolated part of the fragmented smooth spongiome membrane can undergo its own cycle of rounding and relaxing as long as sufficiently undiluted cytosol is still present that contains ATP and other undetermined cofactors.[114,119] No master pacemaker exists to regulate the rounding and relaxing cycles of all vesicles derived from the same CVC to keep their cycles in phase. Even the cycles of the two parts of the same isolated CV, when it is pinched in two by a microneedle, will soon go out of phase with each other (see Allen's website, Chapter 9, Video 3).[114]

The isolated CV of Amoeba proteus has also been shown to contract when in the presence of ATP and appropriate ions.[86,95] No fibrous system has been reported to be associated with this CV, not even microtubules. Nishihara et al.[86] showed that isolated CVs from Amoeba proteus would suddenly shrink or burst after 2 to 3 min of exposure to 1-mmol/L ATP. Only the ATP nucleotide had this effect. Though these results remain unexplained, the studies suggest that a mechanism such as that observed in Paramecium may cause apparent contraction of the CV of Amoeba proteus when this organelle is freed from the cell and supplied with ATP.

Thus, what was perceived as contraction in the CV actually is a periodic rapid buildup of membrane tension which has the effect, in Paramecium, of causing the CV to round up and to proceed to separate from its attached ampulli and their collecting canal extensions. Just before the CV membrane fuses with the plasma membrane, the CV seems to begin to relax, as its diameter increases slightly, indicating that relaxation of the membrane tension has begun prior to the actual

fusion of the CV with the pore membrane.[113] Once the CV and plasma membrane bilayers have completely fused, the natural fluid properties of the bilayer will cause the initial pore opening to expand to the edge of the pore indentation. The contents of the CV will be pushed out of the cell by the cytosolic pressure, which is a product of ongoing osmosis into a cytosol that is hyperosmotic to the environment.[84] This is also presumed to be true in other cells such as Dictyostelium.[12] It is at this time that the CV membrane collapses into the meshwork of 40-nm tubules. The meshwork of tubules effectively closes the pore, and this allows the pore membrane to separate from the CV membrane by the fission of the last single, small 40-nm membrane neck that links the CV membrane to the pore membrane.

Fluid does not reenter the CV lumen until the CV has reattached to the ampulli. Fusion probably requires the same complement of fusion proteins as those known to be present in membrane fusion sites universally. The gene coding for the N-ethylmaleimide-sensitve factor (NSF)[80] protein in Paramecium has been cloned in P. tetraurelia and localized by anti-NSF antibody to the junctions between the ampulli and the CV, among other cellular sites.[60] This protein regulates interactions of the soluble NSF attachment protein (SNAP) receptors (SNAREs). Genes for both synaptobrevin-like and syntaxin-like SNAREs that are specific for the CVC of P. tetraurelia have also been identified.[61,103] SNAREs from both the vesicle membrane and target membrane must be complexed in trans configuration before fusion can occur.[106]

Fluid continues to flow into each ampullus during systole, showing that the entire mechanism required for fluid accumulation is present in each radial arm but not in the CV itself. The one component known to be present in the radial arms that is not in the CV in Paramecium is the array of V-ATPase holoenzymes present only in the decorated tubules. The membrane of the decorated tubules differs from the smooth spongiome. Although this membrane forms 50-nm tubules when the V-ATPases are complete and organized into helices, it loses its tubular shape and vesiculates when the cell is placed under hyperosmotic stress or is subjected to cold.[32,52] Under these conditions, the V-ATPases disassemble, at least in part. Membranes of the decorated tubules are thus fundamentally different from the 40-nm tubule-forming, smooth spongiome, because these decorated membranes revert to a spherical shape, not a tubular shape, at their lowest energy state. Tubulation of the membrane of the decorated tubules may depend on the helical associations of the complete V-ATPase holoenzymes, which promote reshaping of the spherical, more planar membrane into bundles of 50-nm tubules, probably one bundle per each large vesicle.[2]

G. Comparing CVCs

A close comparison of the CVC of Paramecium with that of the CVC of Dictyostelium is useful and informative, as these two cells, although vastly different in size and structural complexity, have CVCs that share important features and taken together help us understand what is unique about the CVC that sets it apart from other organelles in cells. The CVC of Dictyostelium is composed of one or two relatively large 1- to 3-• m-diameter, membrane-limited bladders or CVs that are connected to a meshwork of secondary saccules by membrane tubules that in motile cells lie close to the substrate-facing surface of the cell.[47] All of these structures are studded with V-ATPase pegs, although the observation of a peg in a deep-etch replica does not tell us if the enzyme is, in fact, complete and capable of pumping protons.[46,47] The bladders lie close to the plasma membrane and are associated with this membrane by a layer of palisade-like connections, which, in all likelihood, include the protein drainin,[12] Rab-like GTPase proteins,[17,41] and membrane fusion complexes consisting of, at a minimum, SNAREs, SNAPs, and NSF proteins. Surrounding the cytosolic side of this docking site is an annulus of actin filaments that is in contact with the plasma membrane but does not extend over the CV membrane itself.[46] Fusion of these two membranes is followed by the contraction and emptying of the CV but not, under normal conditions, the mixing of the components of the plasma membrane with the CV membrane.[36,46] The CV first rounds before fusion and then collapses after fusion is completed; its membrane flattens and tubulates during systole but does not

vanish into the cytosol. The tubules of the CV can be refilled to restore the bladder.[36,47] The V-ATPase pegs remain with the CV membrane and actin remains only on the luminal surface of the plasma membrane. Only when experimental conditions were used was intermixing of these two membranes seen, and under these conditions clathrin-coated regions arose, and coated vesicles would form that could potentially collect the intramembrane CV components into coated pits to reorganize the CV.[46]

The contraction of the CV during systole, although thought in the past to be actomyosin driven,[27,35] is probably not[46] because cytosolic pressure and the release of bending energy in the CV membrane are sufficient for fluid release, as has been calculated for Paramecium.[84,85] No F-actin has ever been seen bound to membranes of the CVs in Dictyostelium[46] or Paramecium.[5] Heuser and his coworkers[20,22,38,47] concluded that an asymmetrical distribution of phospholipids in the CV membranes may account for the contraction of the CV as its membrane rapidly tubulates during systole. The transient presence of a protein known as LvsA (for large-volume sphere A) in Dictyostelium which is related to a protein in mammalian cells that has a sphingomyelinase activating activity can be used in support of this conclusion.[26,37] This protein is associated with the CV only in late stages of the CV cycle and during the return of the more planar CV membrane to a tubular form. LvsA is required for the localization of calmodulin to the CV membrane in Dictyostelium.[37]

Thus, contraction of the CV during systole may be, in part, a return of the more planar membrane of the CV to a tubular form; however, as mentioned above, experiments with isolated CVs from Paramecium have shown that, during the rounding phase of the CV prior to systole, the tension of the CV membrane increases 35-fold, and this increase in tension is apparently ATP driven.[115] Thus, we know that the CVs of both Paramecium and Dictyostelium will automatically return to a tubular form during systole (possibly with the aid of phospholipid-altering enzymes) so their underlying CV membrane bilayers seem to be similar in this respect. The V-ATPase pegs on the CVCs of Dictyostelium are not organized into helical patterns as they are on the decorated tubules of Paramecium,[2] so this pattern was not required for membrane tubulation of the spongiome in Dictyostelium, nor is it required for tubulation of the smooth spongiome of Paramecium.

Unfortunately, little is known about the tension in the CV prior to systole in Dictyostelium except that the CV has been reported to round up as it does in ciliates.[36,38] The lumen of the bladder then separates from the tubules by membrane fission, or the tubules may simply constrict so their lumens become disconnected from the bladder's lumen.[38] As in Paramecium, cytosolic pressure is also thought to be sufficient to produce fluid discharge from the open CV in this cell.[12]

We conclude that contraction of the CV probably has two parts: (1) a rapid buildup of tension immediately prior to systole that results in the rounding of the CV and its detachment from the tubules and the bulk of the CVC, and (2) the rapid return of the CV membrane to a tubular form after CV membrane fusion with the plasma membrane. Both aspects are visible and measurable in Paramecium,[114,115] but in Dictyostelium a buildup of tension has not yet been documented, only tubulation of the CV. In Paramecium, any part of the smooth spongiome when isolated from the rest of the CVC can cycle between a membrane with increased tension (rounding) and one with relaxed tension (tubulation) (see Allen's Web site, Chapter 9, Videos 2 and 5). The CVC membrane of Dictyostelium has not yet been studied in vitro, but the fact that any part of its fragmented CVC membrane during mitosis or in multinuclear cells can undergo rounding[38] prior to systole which then leads to tubulation during systole strongly suggests that this membrane may undergo the same tension increases followed by relaxation and tubulation that occur in Paramecium. Do isolated CV membranes from Dictyostelium continue to round and relax in vitro, as such membranes do in Paramecium, independent of the ability of the vesicle to accumulate additional fluid? A positive answer to this question would establish that the membranes of CV organelles are indeed unique and are strikingly different biophysically from most other membranes of living organisms. Such membranes may fall under the category of the little-studied cubic membranes.[9]

Molecular biological studies of the CVC are more advanced in Dictyostelium than in Paramecium or other protozoa. Several early studies were focused on the proteins of the individual subunits of the V-ATPase that is enriched in CVC membranes.[68,117,128] Similar studies have now been performed on the V-ATPase subunits of Paramecium tetraurelia,[124,125] and one report on P. multimicronucleatum has been published.[34] Other studies have dealt with the small GTPases of the Rho family of proteins and its Rac subfamily, as well as on the Rab family of regulatory proteins and the proteins that, in turn, regulate these small GTPases by promoting the release of guanosine diphosphate (GDP) from the GTPase to allow a guanine exchange factor (GEF) to insert a guanosine triphosphate (GTP) in its place. Two Rabs, Rab D and Rab 11, have been identified in the CVC of Dictyostelium that presumably act as molecular switches for regulating some aspect of the CV activity.[17,41] Another protein, DRG, which has a GTPase-activating domain (i.e., a GAP), has been identified in Dictyostelium; it functions in both the Rac (actin-modifying) and Rab (membrane-trafficking) pathways and appears to be important in CVC regulation.[64] Also, a Rho GDP-dissociation inhibitor (RhoGDI-1) that might also act on both the Rho and Rab pathways has been localized to the CV in Dictyostelium.[99]

Copine A (CpnA), another protein associated with the CV of Dictyostelium, is a soluble calcium-dependent, membrane-binding protein that may be involved in membrane trafficking pathways or signaling pathways. CpnA has been localized to the CVC as well as other organelles and may only localize to the CVC membrane during a rise in cytosolic calcium concentration.[25] Another protein recently found to associate with the CV of Dictyostelium is a protein known as VwkA for its von Willebrand Factor A-like motif that contains a conserved • -kinase catalytic domain that is reported to be present in myosin heavy-chain kinases (MHCKs). This factor may influence myosin II abundance and assembly at the CV membrane of Dictyostelium.[14]

These studies have revealed proteins that are for the most part peripherally or transiently associated with the CV of Dictyostelium. Currently, their roles seem to be mostly regulatory and are not yet precisely understood. They will likely be shown to be important in CV function or development in the future as more becomes known about the complete pathways involved. Such studies are obviously just beginning, and it is necessary to expand these studies to other cells that have CVCs and to complete the entire pathways in Dictyostelium.

Studies of another CVC-possessing organism that is of significant medical interest is that of the parasite Trypanosoma cruzi, the causative agent of Chagas disease. Chagas disease is a major problem in Latin America, where it has infected more than 11 million people and 40 million more are at risk.[121] This parasitic flagellate has a CVC adjacent to its flagellar pocket that consists of a vacuole and spongiome. The CVC has a pulsation cycle that lasts 60 to 75 sec.[19] Recent work shows that the membranes of the CVC contain an aquaporin water channel. The gene for this protein was cloned and a polypeptide with a molecular mass of 24.7 kDa (23 residues) was produced.[79] The polypeptide had similarities to other known aquaporins, including the signature Asn–Pro–Ala motif that forms an aqueous channel through the membrane bilayer.[90,127]

Not only was the aquaporin found to be part of the CVC in this cell but it was also present in the membranes of acidocalcisome vesicles.[28,29] These vesicles have a high content of pyrophosphate (PPi), polyphosphate (poly P), calcium, and magnesium, as well as other elements.[105] In addition, membranes of acidocalcisomes also contain two types of proton pumps, a V-H^+-ATPase as well as a pyrophosphatase (V-H^+-PPase) proton pump, and they also have a vacuolar Ca^{2+}-ATPase. As mentioned above, these vesicles, first described in Trypanosoma cruzi, have also been found in Chlamydomonas reinhardtii[102] and Dictyostelium discoideum[71] and seem to be linked to the functioning of the CVC.[28,100] Placing epimastigotes of T. cruzi under hypoosmotic stress caused the acidocalcisomes to migrate to and apparently fuse with the CVC, as fluorescently labeled acidocalcisomes accumulate at and cause the CV to fluoresce more brightly. Acidocalcisomes themselves can swell by 50% when they are exposed to hypoosmotic conditions.[100]

Thus, fusion of acidocalcisomes with a CVC would add free amino acids (mainly arginine and lysine), pyrophosphates, and polyphosphates (that may be reduced to inorganic phosphate), as well as the inorganic ions present in their lumens, along with the integral membrane complexes

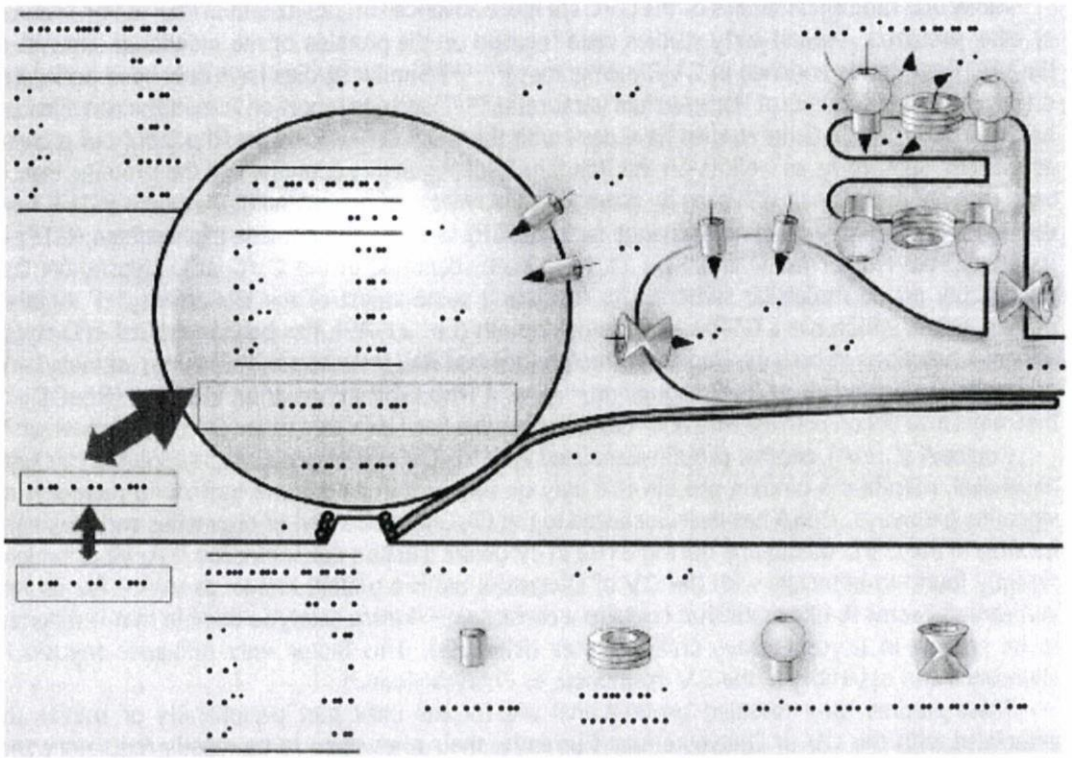

FIGURE 3.7 Summary of the inorganic ions in the standard saline solution, the cytosol, and the rounded CV of Paramecium multimicronuleatum just before fluid expulsion (systole). In situ cytosolic and CV ionic activities in mmol/L and pH were determined with ion-selective microelectrodes inserted into living cells.[109,110] The osmolarities of the saline solution and cytosol were determined as reported.[108,109] The presence and location of aquaporin, cation/H^+ exchangers, and anion channels are only speculative. The location of V-H^+-ATPase was determined by immunofluorescence and electrophysiology.[32,39,117] Abbreviations: CV, contractile vacuole; AP, ampullus; CO, collecting canal; DS, decorated spongiome; P, CV pore; MT, microtubular ribbon; CM, cell membrane.

V-ATPases, V-H^+-PPases, vacuolar Ca^{2+} pumps, and aquaporins to the CVC. This contribution would favor movement of water osmotically into the CVC; however, how widespread acidocalcisomes are in cells containing CVCs has not been determined. We have never observed electron-opaque vesicles around a CVC nor such bodies fusing with the CVC in electron micrographs of intact Paramecium cells.

H. The Role of CVC in Osmoregulation

Cellular osmoregulation involves at least two interacting processes: (1) the acquisition or production of osmolytes that are dissolved in the cytoplasmic fluid phase of the cell, and (2) the balancing of cellular water, which constantly flows into the cell by osmosis to maintain the cytosol at its required hyperosmotic level. Thus, the primary role of the CVC is to facilitate the second process, to sequester excess water from the cell and to expel this water to the exterior of the cell. Paramecium does this principally by transferring the inorganic ions K^+ and Cl^- to the CVC at a level 2.0 to 2.4 times higher than that in the cytosol (Figure 3.7). A secondary function is to sequester and excrete cations such as Ca^{2+} and Na^+ from the cell.

To accomplish this, the CVC membranes must contain mechanisms such as cation/H^+ exchangers and cotransporters for concentrating K^+ and Na^+, Ca^{2+} pumps such as those identified in

Dictyostelium,[77,78] and/or anion channels for the entry of Cl^- into their lumens. Although such mechanisms remain to be studied in detail, it is now clear that the membranes of the CVC are highly enriched in V-ATPases. These V-ATPases are not used to form a highly acid compartment but are important for energizing the membrane to establish the +80-mV luminal electrochemical charge and for providing protons that are then available to be exchanged for the cations that increase the osmolarity of the CV and so promote osmosis across the CVC membrane.

Water then enters the CV by osmosis, probably through aquaporin water channels, as we first postulated for Paramecium[118] and which is now confirmed by molecular techniques for the CVCs of trypanosomes.[13,29] Periodically, an innate timing mechanism, which in Paramecium is not tied to the volume of the CV, triggers the CV to round up and at the same time to separate from its radial arms and proton pumps. This precedes the fusion of the CV membrane with the plasma membrane and the opening of the CV pore so expulsion of the contents of the CV, both osmolytes (such as K^+ and Cl^-) and water, occurs. After CV emptying, both the plasma membrane and CV membrane separate and reseal, and the collapsed and tubulated CV membrane will fuse again with each ampullus. The ampulli will empty their accumulated fluid content into the tubules of the collapsed CV which will cause these tubules to swell into a vacuole.

This scheme implies that only those osmolytes that are trapped in the CV during rounding will be expelled from the cell during the expulsion process. Osmolytes remaining in the radial arms will be retained as they are no longer in continuity with the CV during systole. During each cycle the cell will maintain its K^+ and Cl^- levels in the CVC using the energy of the proton gradient produced by the huge number of V-ATPases in the CVC membrane for the import of additional cations. Currently, no experimental evidence has demonstrated a mechanism for retrieving and returning osmolytes from the rounded CV back to the cytosol during the very short period of time that the CV is separated from the CVC and is in the rounded phase. An osmolyte retrieval mechanism in the CV membrane would probably defeat much of the purpose of water segregation and expulsion by the CVC, as water would rapidly flow out of the CV back into the cytosol as the osmolytes are retrieved from the CV, particularly as the CV membrane now seems to contain aquaporins.

In contrast to what was reported for amoebae,[98,104] the osmolarity of the CV of Paramecium is not hypoosmotic (hypotonic) to the cytosol.[109,110] Earlier techniques used to measure the osmolarity and ionic contents of the CV in amoebae may have been inadequate to provide reliable results, so these earlier studies should be repeated with improved techniques, preferably on living cells. In Paramecium not only do we find K^+ activity 2.0 to 2.4 times higher in the CV than in the cytosol but we also find Cl^- activity equally as high or higher (when the Ca^{2+} concentration is high externally) which can account for most if not all of the counterbalancing anions.

III. CELL VOLUME CONTROL IN **PARAMECIUM** AND PARASITIC PROTOZOA

A. Volume Adaptation to the External Osmolarity

1. Adapted Cells Remain Osmotically Swollen

We previously found that the cytosolic osmolarity (C_{cyt}) of a Paramecium multimicronucleatum cell changed stepwise at ~75 or 160 mOsmol/L when the adaptation osmolarity (C_{adp}) is continuously changed. That is, C_{cyt} is ~75, ~160, and ~245 mOsmol/L when C_{adp} is (1) less than 75, (2) more than 75 but less than 160, and (3) more than 160, respectively (see Figure 3.2A and Figure 3.8B).[109] This finding implies that an active change in C_{cyt} takes place when the external osmolarity is changed beyond these osmolarities. Hereafter, these two osmolarities (~75 and ~160 mOsmol/L) and also ~245 mOsmol/L (see the legend for Figure 3.8) will each be termed a critical osmolarity (C_N), as these cause an active change in C_{cyt} (i.e., an activation of a hypothetical osmolyte-transport mechanism).[54] This finding also implies that C_{cyt} will normally be higher than C_{adp}, and, therefore, an adapted cell will remain osmotically swollen.

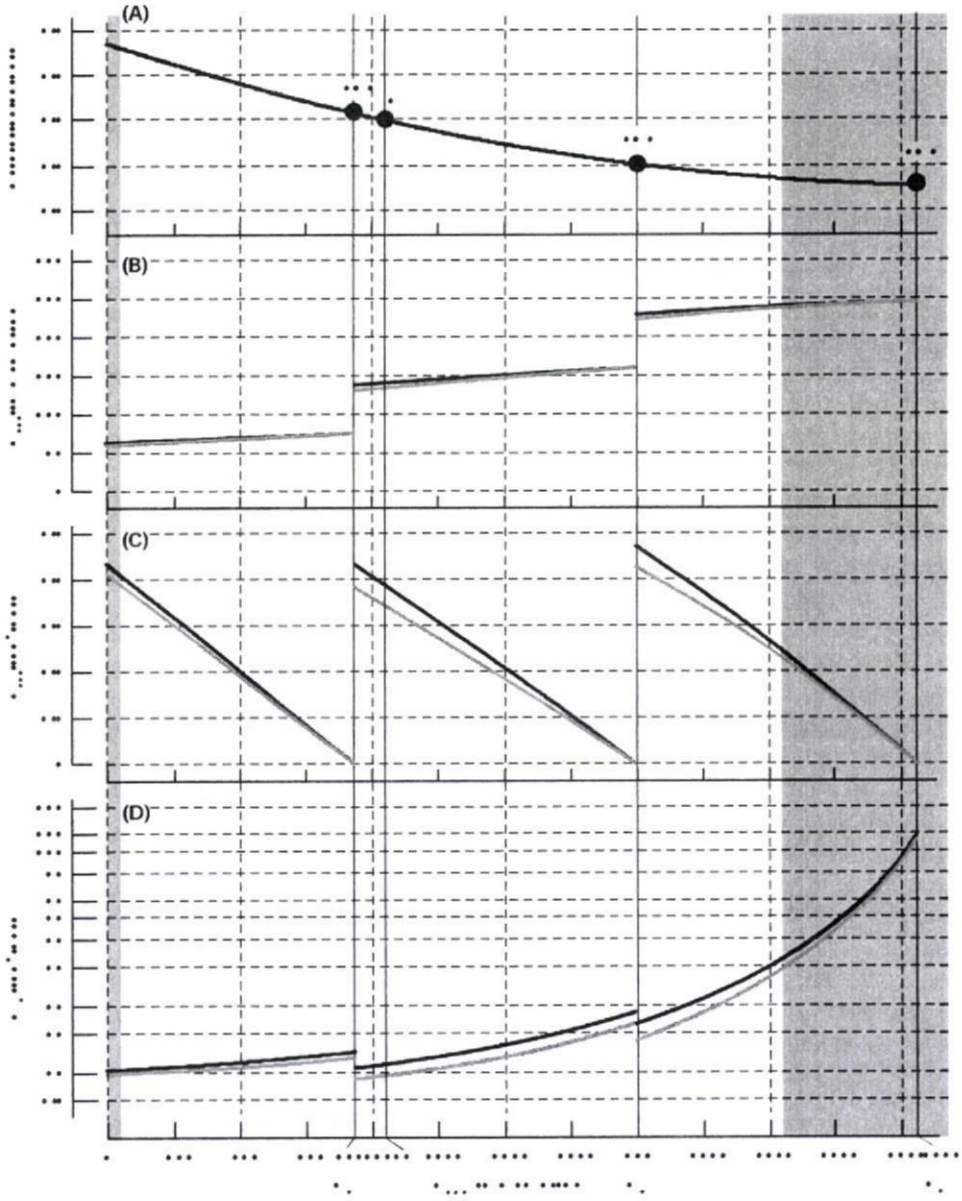

FIGURE 3.8 (A) Cell volume (v); (B) cytosolic osmolarity (C_{cyt}); (C) hydrostatic pressure of the cytosol with reference to the external (adaptation) solution (P_{cyt}); and (D) bulk modulus of the cell (M_B) in Paramecium multimicronucleatum cells plotted against the adaptation osmolarity (C_{adp}). A filled circle labeled v corresponds to the reference cell volume (1.0) (i.e., the volume of the cell adapted to 84 mOsmol/L, which is the osmolarity of the axenic culture medium). All cell volumes are presented as the values relative to the reference volume. Three filled circles labeled vN correspond to the natural volumes of the cells for three C_{adp} ranges ($C_{adp} < 75$, $75 < C_{adp} < 160$, and $C_{adp} > 160$ mOsmol/L). C_N represents the critical osmolarities for three stepwise changes in C_{cyt} (75, 160, and 245 mOsmol/L). In B, C, and D, the values for respective parameters were estimated based on the assumption that the amount of the osmotically nonactive portion of the cell (v_{na}) is 20% (black lines) and 40% (gray lines) of the reference cell volume. The left gray column is for C_{adp} ranging from 0 to 4 mOsmol/L; the right gray column is for C_{adp} ranging from 204 to 245 mOsmol/L. These C_{adp} ranges in gray are nonexperimental, and all the values in these regions were obtained by extrapolation. (Modified from Iwamoto, M. et al., J. Exp. Biol., 208, 523, 2005. With permission.)

The extent of swelling depends on the osmotic pressure of the cytosol with reference to the external solution (π_{cyt}), which is proportional to the difference between C_{cyt} and C_{adp}. π_{cyt} is written as:

$$\pi_{cyt} = \phi C_{cyt} - C_{adp} \phi R T \tag{3.1}$$

where R and T are the gas constant and the absolute temperature, respectively.

2. Osmotic Pressure Balances Hydrostatic Pressure in Cytosol in Adapted Cells

In an adapted cell, π_{cyt} equals or balances the hydrostatic pressure in the cytosol with reference to the external solution (P_{cyt}). P_{cyt} is generated when an elastic membrane and its associated cytoskeletal structures, which surround the cell, are expanded as the cell is osmotically swollen. The balance can be written as:

$$\pi_{cyt} = P_{cyt} \tag{3.2}$$

If either one or both of these pressures become modified, the cell volume will change until a new balance between these pressures is established (Figure 3.9). The cell elasticity, or physical resistance to swelling or shrinking, can be represented as the modulus of volume elasticity—that is, the bulk modulus (M_B). The M_B of a Paramecium cell adapted to an osmolarity (C_{adp_n}) is defined as:

$$M_{B_n} = \frac{\dfrac{\pi_{cyt_{n+1}} \pi_{cyt_n}}{V_{n+1} - V_n}}{V_n} \cdot \frac{\dfrac{\pi_{cyt_n}}{V_n}}{V_n} \cdot \frac{V_n \pi_{cyt_n}}{V_n} \tag{3.3}$$

where n stands for the nth experiment among a series of experiments with varied C_{adp}, and n+1 stands for the (n + 1)th experiment employing a C_{adp} that is slightly different from that in the preceding nth experiment; v is the volume of the cell in either experiment n or n + 1, and π_{cyt_n} can be written as:

$$\pi_{cyt_n} = \phi C_{cyt_n} - C_{adp_n} \phi R T \tag{3.4}$$

3. Volume of the Cell Adapted to a New Osmolarity Will Always Change as Adaptation Osmolarity Changes

The volume of a cell (v) that has been adapted for several hours to a given C_{adp} will then continuously change as the C_{adp} is continuously raised in an osmolarity range from 4 to 204 mOsmol/L. The value for v relative to that in the original culture medium employed (84 mOsmol/L) changes by a ratio of ~1.16 to ~0.87 as C_{adp} changes from 4 to 204 mOsmol/L (Figure 3.8A).[54]

4. Estimation of C_{cyt}, π_{cyt}, and M_B of Cells Adapted to Varied C_{adp}

The hypothetical osmolyte-transport mechanism responsible for the stepwise change in C_{cyt} is not activated by a change in C_{adp} within a range where no critical osmolarity (C_N) is crossed, so the number of osmolytes in the cytosol (N) remains unchanged regardless of a change in v due to a change in C_{adp}.[54] Within such a C_{adp} range, C_{cyt_n} can be written as:

$$C_{cyt_n} = \frac{N}{V_n - V_{na}} \tag{3.4}$$