Possible causes of apoptotic volume decrease: an attempt at quantitative review

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Model MA. Possible causes of apoptotic volume decrease: an attempt at quantitative review. Am J Physiol Cell Physiol 306: C417–C424, 2014. First published November 6, 2013; doi:10.1152/ajpcell.00328.2013.—Cell shrinkage and dehydration are essential characteristics of apoptosis, and loss of as much as half of the initial cell volume is not uncommon. This phenomenon is usually explained by efflux of K⁺ and Cl⁻. We reexamine this hypothesis on the basis of the available data for ion concentrations and the requirements for osmotic equilibrium and electroneutrality. In addition to ion loss, we discuss the possible impacts of several other processes: efflux of low-molecular-weight osmolytes, acidification of the cytosol, effects of water channels and pumps, heterogeneity of intracellular water, and dissociation of apoptotic bodies. We conclude that the most mammalian cells are theoretically capable of reducing their volume by 15–20% through ion loss or a decrease in cytosolic pH, although, in reality, the contribution of these mechanisms to apoptotic shrinkage may be smaller. Transitions between osmotically active and inactive water pools might influence cell volume as well; these mechanisms are poorly understood but are amenable to experimental study. Dissociation of apoptotic bodies is a separate mechanism of volume reduction and should be monitored closely; this can be best achieved by measurement of intracellular water, rather than cell volume.

We start with the most trivial mechanism. As mentioned above, apoptotic cells can reduce their volume by loss of water (which is the essence of AVD) or by separation of apoptotic bodies. It is frequently stated that formation of apoptotic bodies occurs only during the final stages of apoptosis, but this is not always the case; for example, the protein kinase inhibitor staurosporine, which is frequently used to study AVD, tends to cause rapid cell fragmentation in diverse cell types (86). However, reliable detection of dissociated apoptotic bodies is far from simple, and there have been few detailed investigations of the methodology of such an assay (133). Thus it is possible that, in some cases, populations of cells with greatly reduced sizes found on flow cytometric scattergrams or on particle analyzers represented cell fragments, rather than severely dehydrated whole cells. This would have caused an overestimation of the AVD effect. Microscopic observation is more likely to detect dissociated apoptotic bodies, but only if they remain attached to the substrate close to the parent cells.

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More reliable assessment of the AVD would be possible by direct measurement of intracellular water. Water measurement methods based on population analysis include gradient density centrifugation (5, 127), NMR (108), quantification of intracellular markers (61, 63), or drying and weighing (98). Microscopic approaches would require determination of the refractive index, which can be accomplished by quantitative phase microscopy (86); indeed, Model and Schonbrun (86) presented evidence that staurosporine and actinomycin D induce dehydration (AVD) as well as loss of dry mass.

**Loss of K⁺ and Cl⁻**

The prevailing view is that K⁺ permeability increases early in the apoptotic process; some of the K⁺ exits the cell, thus creating an imbalance in osmotic pressure and driving water out of the cell. Quite often, prevention of K⁺ efflux, by blockade of K⁺ channels or elevation of extracellular K⁺, will suppress AVD and other apoptotic changes (14, 119, 125). To ensure overall electroneutrality, efflux of K⁺ must be balanced by accumulation of Na⁺ or loss of Cl⁻, and there is abundant evidence that both processes occur in apoptosis (14, 93). Activation of Na⁺ influx has been implicated in apoptotic shrinkage (13); however, since equimolar replacement of K⁺ for Na⁺ is not expected to produce a net loss of water, the effect was probably indirect. The majority of studies of AVD have focused on characterization of K⁺ channels involved in apoptosis and Cl⁻ channels as their counterparts (14, 71, 74, 94, 119, 125, 126). Whether K⁺ or Cl⁻ channels act as initiators of shrinkage is of secondary importance, since, within this model, both processes should be tightly coupled.

The usual quantitative statement of the above-described model is as follows. The balance of osmotic pressure requires that

\[
[\text{Na}] + [\text{K}] + [\text{Cl}] + [A] = P \tag{1}
\]

where \(P\) is the external osmolarity and the square brackets represent intracellular concentrations. In some cases, intracellular bicarbonate can be abundant enough to be entered in Eq. 1, but, following other authors, we omit it here. The quantity \([A]\) represents the effective average osmolarity of intracellular organic matter; the nature of the most osmotically active components and the magnitude of \([A]\) depend on the species and tissue (21). Proteins only add several millimolar to intracellular osmolarity (84), and most of the contribution comes from free amino acids, peptides, and diverse compounds such as ATP, phosphorylcreatine, taurine, betaine, carnitine, sarcosine, myoinositol, and ascorbate. Perhaps there is no better way to evaluate \([A]\) than as the difference between \(P\) and the sum of concentrations of monovalent ions (79), which yields the typical values of \([A]\) \(\sim 110–140\) mM (1, 12, 45, 98, 111, 114, 129). Notwithstanding its diverse nature, the analysis below does not require that \(A\) be further divided into subcategories as long as the total amount of organic matter is assumed constant. One important simplification inherent in Eq. 1 is the substitution of ion activities with concentrations; its possible significance is discussed in *Osmotically Inactive Volume*. The other assumption is that the system is at equilibrium with respect to water. The rate of equilibration of osmotic pressure depends on membrane permeability for water and is usually much faster than the development of apoptosis.

The second condition used to obtain the formula for cell volume comes from the requirement for electroneutrality. The slight excess of negative over positive charges, which is responsible for the generation of the negative cell potential, is much smaller than the individual concentrations and can be ignored for our purposes. Because the total amount of intracellular K⁺ and Na⁺ is always greater than that of Cl⁻, \(A\) has to carry an overall negative charge whose average magnitude (per osmole) we denote \(z\):

\[
[\text{Na}] + [\text{K}] = [\text{Cl}] + z[A] \tag{2}
\]

We assume for now that the total cellular content of impermeable osmolytes \(A_T\) remains constant, and

\[
[A] = A_T/V \tag{3}
\]

With use of Eqs. 1–3, volume \((V)\) can be expressed through the other parameters, namely

\[
V = \frac{(1 + z)A_T}{P - 2[\text{Cl}]} \tag{4}
\]

(39, 59) or

\[
V = \frac{(1 - z)A_T}{P - 2([\text{Na}] + [\text{K}])} \tag{5}
\]

From a practical standpoint, Eq. 5 is less useful than Eq. 4, because the difference between two large quantities in the denominator would be more difficult to determine with sufficient precision, especially when \(z \sim 1\) and \(P \sim 2([\text{Na}] + [\text{K}])\), as is the case, for example, with Ehrlich ascites cells (98). It is important to emphasize that we are only assuming that electrical and osmotic forces are in equilibrium and that intracellular ion concentrations are known from the experiment. We are not considering how a given state has been achieved and why the ion concentrations are at a particular level. Detailed theoretical treatment of cell volume dynamics that relates ion fluxes with specific molecular mechanisms, such as the channel conductance and the activity of the Na⁺/K⁺ pump (3, 39, 59), including specific applications to AVD (100, 116), can be found elsewhere.

As the cell transitions from the healthy state to apoptosis, the relative change in the volume will be

\[
\frac{V_2}{V_1} = \frac{A_T^{1+2z}}{A_T^{1+z_1}} \cdot \frac{1 + z_2}{1 + z_1} \cdot \frac{P - 2[\text{Cl}]}{P - 2[\text{Cl}]} \tag{6}
\]

For constant \(A_T\) and \(z\), Eq. 6 is reduced to

\[
\frac{V_2}{V_1} = \frac{P - 2[\text{Cl}]}{P - 2[\text{Cl}]} \tag{7}
\]

It follows that if \(P = 300\) mM and Cl⁻ concentration drops from the initial 30 mM to zero, the resulting volume decrease would be 20%. In fact, water does not occupy the entire cell, and not all water is osmotically active (OA; see below); for use in Eq. 1, it would be more correct to associate the quantity \(V\) with the volume occupied by OA intracellular water. In this case, the extent of shrinkage would be smaller. The OA fraction of the total volume varies between cell types and has been measured as 81% in human oocytes (89), 77% in neutrophils (112), 67% in Ehrlich ascites cells (51), 52% in human erythrocytes (23) and spermatozoa (44), and 43% in Madin-
Darby canine kidney cells (130). If we assume conservatively that OA water occupies 75% of the cell volume in a healthy cell, the extent of shrinkage accompanying complete loss of 30 mM Cl\(^-\) would be close to 15%, while the occasionally observed 50% shrinkage would be only possible through a complete loss of 100 mM Cl\(^-\).

There have been few direct measurements in absolute units of chloride loss in apoptotic cells that can be correlated with cell volume decrease; some of the available results are listed in Table 1. It is evident that the shrinkage is always greater than the value calculated from Eq. 7; in at least one case (98; see also 102), intracellular Cl\(^-\) did not decrease at all and, at later times, started to rise. Furthermore, neurons typically have low Cl\(^-\) concentrations (19), and thus there is no possibility that Cl\(^-\) concentration will decrease substantially. Nevertheless, neurons, like other cells, undergo marked shrinkage during apoptosis (14, 18, 121). Large experimental errors in Cl\(^-\) measurements are unlikely, because ion concentrations have been measured by four different techniques: radioactive (129) or fluorescent ratiometric (50) probes, coulometric titration (98), and X-ray microanalysis (4, 6).

Another possible experimental approach to test the role of Cl\(^-\) in apoptotic shrinkage is stimulation of apoptosis in a low-Cl\(^-\) medium. However, exposure to such a medium alone may cause apoptosis (80); the 15–20% shrinkage observed by Maeno et al. (80) was probably a direct consequence of Cl\(^-\) loss, rather than AVD. Treatment of Jurkat cells by Fas ligand produced shrinkage that did not depend on Cl\(^-\) in the medium (50). Thus we have to conclude that while it is likely that Cl\(^-\) efflux may sometimes contribute to apoptotic shrinkage, it is not significant enough to fully account for all cases of AVD.

This result must be balanced against numerous documented reports of protective action of K\(^+\) and Cl\(^-\) channel inhibitors. It is significant that, in almost all cases when ion channel blockers prevented shrinkage, they also prevented cell death. With few exceptions, such as that reported by Beauvais et al. (11), there are hardly any reports of selective inhibition of AVD while allowing caspase activation, DNA fragmentation, and other manifestations of apoptosis. One possible and frequently invoked explanation for this is that AVD or associated ion changes represent a critical step in the apoptotic cascade that is upstream of other observable changes (92); therefore, prevention of shrinkage abrogates the entire process of cell death. However, in some apoptotic models, shrinkage is definitely preceded by other apoptotic reactions, such as chromatin condensation, mitochondrial depolarization, caspase activation, and membrane blebbing (60, 85, 90). Experiments in which ion channel blockers or high-K\(^+\) media would be applied after the first signs of apoptosis but before the onset of shrinkage might help clarify the matter. Additionally, the assumption of constant $z$ and $A_T$ may have to be revised (see below).

### Decrease in the Fixed Negative Charge

As we adhere to our assumption that all the parameters in Eqs. 1 and 2 are independent, we find from Eq. 6 that a decrease in $z$ to allow additional efflux of cations could provide an alternative mechanism of shrinkage. A possible mechanism of neutralization of negative charges, namely, the acidification of the cytosol, has been proposed by Fraser et al. (40). Although the physiological concentrations of protons are much less than $[A]$, the large buffering capacity of the cell material ensures that a 1-unit decrease in pH would require neutralization of 40–80 mM of charges (20), i.e., roughly half of the initial amount. Indeed, apoptosis is frequently accompanied by an early acidification of the cytosol (71, 83), and a decrease in cytosolic pH can reach 0.5–1 pH units (47, 77). In particular, staurosporine, Fas, cycloheximide, etoposide, and UV light can cause a 0.5- to 0.7-unit drop of intracellular pH (9, 47, 83). In agreement with the hypothesis of volume regulation by pH, the mitochondrial matrix becomes more alkaline during apoptosis (66, 83), and, at the same time, mitochondria exhibit pronounced swelling (17, 27, 104, 118). Interestingly, cytosolic alkalization during apoptosis is also sometimes observed, usually as a transient early event (66), and, conversely, a temporary cell volume increase at the beginning of apoptotic transformation has been described as well (60, 97, 98; unpublished observations).

### Table 1. Experimental and theoretical parameters related to apoptotic shrinkage

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Treatment</th>
<th>[Cl] (mM), apo/con</th>
<th>V(_{\text{meas}}), ap/con</th>
<th>V(_{\text{calc}}), ap/con</th>
<th>[zA], ap/con</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937</td>
<td>Stauroporine, 5 h</td>
<td>31/41</td>
<td>0.86</td>
<td>0.92</td>
<td>0.99</td>
<td>129</td>
</tr>
<tr>
<td>U937</td>
<td>Stauroporine, 1 h</td>
<td>22/25</td>
<td>0.68</td>
<td>0.98</td>
<td>0.76</td>
<td>6*</td>
</tr>
<tr>
<td>Ehrlich ascites</td>
<td>Cisplatin, 10 h</td>
<td>22/25</td>
<td>0.97</td>
<td>&gt;1</td>
<td>0.89</td>
<td>98</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Fas ligand, 4 h</td>
<td>48/58</td>
<td>0.7-0.8</td>
<td>0.91</td>
<td>0.91</td>
<td>110</td>
</tr>
<tr>
<td>Monocytes</td>
<td>OxLDL, 3 h</td>
<td>91</td>
<td>0.67</td>
<td>0.80</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>Etoposide</td>
<td>0.81</td>
<td>0.81</td>
<td>0.97</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>LNCaP</td>
<td>Etoposide</td>
<td>0.45</td>
<td>0.97</td>
<td>0.97</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>U937</td>
<td>UV</td>
<td>0.97</td>
<td>0.93</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[Cl], intracellular Cl\(^-\) concentration; V\(_{\text{meas}}\), measured cell volume; V\(_{\text{calc}}\), volume calculated from Eq. 7; [zA], fixed negative charges calculated from Eq. 2; apo/con, ratio of apoptosis to control; OxLDL, oxidized LDL; ND, not determined. *Results were reported only in mmol/kg dry wt; to convert these numbers into concentrations, we used the water content measured by Yurinskaya et al. (129) under similar conditions. †Volume changes were roughly estimated from Ref. 16, where the experiments were performed under similar conditions.

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Some quantitative estimates of this effect can be made. If, for the sake of simplicity, \( A_T \) and \([\text{Cl}]\) are assumed constant, then

\[
\frac{V_2}{V_1} = \frac{1 + z_2}{1 + z_1} = 1 + \frac{(z_1 - \Delta z)}{1 + z_1} \tag{8}
\]

While it appears difficult to measure \( z \) directly, \(^1\) the product \( z[A] \) and its change in the course of apoptosis can be calculated from \( \text{Eq. 2} \) if the \( K^+ \), \( Na^+ \), and \( \text{Cl}^- \) data are available. Importantly, since all the parameters in \( \text{Eq. 2} \) relate to the same volume, it is not necessary to know the concentrations: elemental analysis of dry samples would suffice. Such measurements have been done by Fernández-Segura and collaborators and in the laboratory of Vereninov. With the proviso that estimation of \( z[A] \) depends on three sets of experimental data, with the correspondingly larger error, the results are summarized in Table 1. There is, indeed, an indication of a net charge change only slightly during apoptosis (the volume in those experiments also underwent only minute changes during the same time period, but, surprisingly, cell water was significantly reduced). The results of Yurinskaya et al. (129) point to the same time period, but, surprisingly, cell water was significantly reduced. The results of Yurinskaya et al. (129) point to \( z = 0.7–1.3 \) for U937 cells. On the basis of the data not just for fixed osmoles (taurine is no longer considered fixed) and \( \text{Eq. 2} \) remains unchanged, because taurine does not carry an electrical charge at neutral pH (48, 68). Thus, for cell volume, we now have

\[
V = \frac{(1 + z)A_T}{P - 2[\text{Cl}] - [T]} \tag{11}
\]

To exert a measurable effect on \( V \), the loss of taurine must be comparable with \( P - 2[\text{Cl}] \). Given that taurine concentration in most mammalian tissues is in the low millimolar range (21, 54, 96, 109, 124), it is doubtful that taurine alone can be directly responsible for significant shrinkage in these cell types. There is not enough information about apoptosis-induced efflux of amino acids and other low-molecular-weight osmoles.

Aquaporins and Water Pumps

Aquaporins enhance the rate of water exchange between the cell and the environment. Inhibition of aquaporins with \( \text{HgCl}_2 \) prevented AVD as well as other apoptotic changes in several in vitro systems (58); similar results have been reported by others (37, 57, 67, 134). Since aquaporins do not actively pump water into or out of the cell, they appear to be the necessary components, rather than the driving force, behind AVD.

The initially controversial hypothesis of active water transport seems to be gaining acceptance (10, 131, 132). It has been found that ion-transporting activity of several symporters [e.g., \( K^+\text{Cl}^- \) cotransporter isofrom 4 (KCC4) and \( \text{Na}^+\text{K}^+\text{Cl}^- \) cotransporter isofrom 1 (NKCC1)] is accompanied by transfer of as much as several hundred water molecules per cycle. This water transport can take place against a large osmotic gradient (131) and, in principle, should be capable of producing cell dehydration when directed outward. Incidentally, the assumption of this mechanism may invalidate \( \text{Eq. 1} \) and the subsequent analysis, because water no longer has to be at equilibrium.

The direction of electroneutral transport follows the chemical gradient; therefore, NKCC activation should, rather, favor water intake (2). Indeed, experimental evidence suggests that NKCC activity offers some protection against apoptosis (71), although theoretical modeling shows that this effect may depend on other parameters (115). On the other hand, \( K^+\text{Cl}^- \) cotransport is directed outside the cell and is apparently responsible for much of \( K^+ \) efflux in Ehrlich ascites cells treated with hydrogen peroxide (69). Cells in those experiments shrank by 20%, but data are not sufficient to verify whether water pumping may have contributed to the volume loss. Apparently, only transporters of the cotransport type have the water-pumping ability (T. Zeuthen, personal communication).

\[^1\] Although theoretical modeling shows that this effect may depend on other parameters (115).
and when cotransporters do not become activated in apoptosis, the involvement of this mechanism appears less likely.

**Osmotically Inactive Volume**

There is no question that scientific progress is largely driven by a “desire to simplify things” (122). Treatment of the cellular aqueous phase as a diluted solution of salts and metabolites has been one such successful simplification. However, it may become necessary at some point to adopt a more realistic, even if logically less streamlined, view.

Cytoplasm contains up to 30–40% protein and, thus, is an extremely crowded space. The effects of macromolecular crowding on reaction rates and on the conformation of proteins have been extensively discussed (135). The other consequence of crowding is that a substantial fraction of intracellular water is associated with proteins. In their native state, proteins are surrounded by a shell of tightly bound water molecules that make up as much as 60% of the protein mass (7, 25, 78, 95). Conformational changes of proteins are often accompanied by a release or uptake of large amounts of water (7, 24, 95, 131). For example, in experiments in which BSA was placed in a dialysis bag, every treatment that reduced protein solubility (elevated temperature, urea, or decrease in pH) caused expulsion of some water from the BSA solution (22).

These chemical effects may partly underlie volume regulation at the cellular level. Reduced water in sickle red blood cells containing aggregated hemoglobin (42, 75, 99) may be analogous to the loss of water from partially denatured BSA (22). Comparison of the OA volumes derived from Boyle-Van’t Hoff plots with total water measured by drying showed that part of intracellular water does not participate in the osmotic balance (30, 42, 43). Importantly, osmotically inactive (OI) water appears to be a dynamic quantity that varies with the physiological state of the cell. In the old study on sea urchin eggs, the OI fraction increased from 8% to 28% following fertilization (105); another study on Ehrlich ascites cells showed large variations in OI (although not in total) water during the cell cycle (31). Accumulation of OI water has also been observed following incubation of erythroblast-like leukemic cells at 4°C (52). The interesting observation that up to half of all water in fibroblasts is generated by metabolism, rather than supplied by the environment, stands somewhat apart, but one may hypothesize that metabolic water is also osmotically inactive, judging by the slow rate of its exchange (64).

Not only water, but also intracellular ions, have rather special properties. By comparing the readings of ion-selective electrodes with the values measured spectrophotometrically or by electron probe microanalysis, the activity coefficients were found to be 0.08/0.48 for Na⁺ and 1.2/0.67 for K⁺ in mature/immature frog oocytes (113). Large deviations of ion activities from those in solution are not uncommon in cells (55, 79) and have been demonstrated even in cell-free systems (123). Activity coefficients larger than unity likely indicate a decrease in the amount of solvent water (24).

The question pertinent to the subject of this review is whether cell water content can be affected by apoptotic restructuring of the cell. Unfortunately, the answer to this question is unknown until careful measurements are done. Without necessarily subscribing to any of the particular models of intracellular water [which can be found, for example, in a review by Shepherd (106)] and limiting ourselves to the empirical quantities of OA and OI water, the hypothetical scenarios could be as follows. First, ions may redistribute to OI water, which, as far as the osmotic pressure is concerned, would be identical to ions exiting the cell with the ensuing loss of water; this could manifest itself by their reduced activity. Of the data listed in Table 1, only Jurkat cells treated by Fas ligand were measured by a method capable of detecting a change in activity, and the decrease in Cl⁻ in that experiment was only 10 mM. The second, and perhaps more promising, hypothesis is that the initially OI water may shift to the OA fraction, from where it will be induced to vacate the cell. In other words, healthy cells would have large reserves of OI water, and apoptotic cells would have less. It is difficult to speculate what might be causing such shifts during apoptosis, as it is difficult to know what caused them in the above-mentioned experiments. In looking for processes capable of causing large-scale changes in physicochemical properties of the cytosol, activation of proteolytic enzymes is the natural candidate. Indeed, 5–10% of all proteins become cleaved during apoptosis (29, 107), which may result in an increased exposure of hydrophobic residues. Major changes in the cytoskeleton that develop in apoptotic cells (17, 88) may also affect water structure. Our understanding of water organization in the cell is too rudimentary to allow us to decide whether these processes are likely to produce a major efflux of water. The role of OI compartments in apoptosis awaits its experimental verification.

**Conclusions**

Several conceivable mechanisms of apoptotic shrinkage have been considered (Fig. 1). Of these, the efflux of monovalent ions has been by far the most favored in the literature, followed by the efflux of low-molecular-weight osmolytes. Although dissociation
of apoptotic bodies is a well-recognized process, it can easily go unnoticed. The other possibilities have received relatively little attention, especially with regard to apoptosis. Our main conclusions can be summarized as follows.

Typical intracellular concentrations of ions should be sufficient to cause a moderate AVD, in agreement with the accepted views. As a test for this mechanism, a decrease in intracellular Cl− concentration may be more revealing than a decrease in K+, because the latter is frequently balanced, at least partially, by accumulation of Na+.

Intracellular acidification is the other mechanism capable of causing some shrinkage through facilitation of K+ efflux.

Quantitative data are not sufficient to implicate organic osmolytes or active water transport in apoptotic volume regulation.

The above-described mechanisms can hardly explain shrinkage beyond 15–20% of the initial volume. Such shrinkage can be caused by separation of apoptotic bodies, which should be monitored by measurement of intracellular water, rather than cell volume.

When apoptotic body formation can be ruled out as a reason for a major volume shrinkage, we suggest measurement of the OI water as the next step.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author.

AUTHOR CONTRIBUTIONS

M.A.M. drafted the manuscript; M.A.M. edited and revised the manuscript; M.A.M. approved the final version of the manuscript.

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