When the miracidium approaches an unsuitable candidate snail host, or an unsuitable portion such as the shell of a natural intermediate host, it appears to probe the surface with its apical papilla before backing off and swimming away. In laboratory exposures, the snail is sometimes able to dislodge attached miracidia by contraction of its head or foot against the shell. When this happens, the detached miracidia will swim away.

Figure 2 illustrates a *G. huronensis* miracidium on the foot of a young *P. gyrina* snail 1 minute after attachment. While attached, it continues to be very active, extending and contracting in an effort to gain entrance into the snail. Once penetration begins, it can be completely enclosed in the host's tegument within a few minutes. The tegument of the miracidium has many dermal plates covered by cilia (Figs. 2–4). The cilia curl during fixation, appearing as white spheres on the dermal plates. The areas lying between the dermal plates lack cilia but contain small tissue evaginations.

Advanced stages of penetration by the miracidia are shown in Figs. 3 and 4. In Fig. 3 the miracidium appears to be halfway into the host tissue, with only the posterior portion of the middle row of dermal plates and the posterior row still visible. In Fig. 4 penetration is nearly complete, and in less than a minute the miracidium will have disappeared. The tegument of the snail surrounding the miracidium then appears to reseal, with the tissue completely filling the opening left by the penetrating miracidium. Although we examined nearly a hundred snails after penetration, the absence of such openings strongly suggests that penetration leaves no scars.

Scanning electron microscopy not only allows investigators to examine the snail's tegument and the attachment apparatus in topographical detail not seen with light or transmission electron microscopes, but it also may provide information essential to biochemists, malacologists, physiologists, and behaviorists. These specialists need to combine their efforts to assist in finding methods of controlling schistosomiasis, a disease that now is of worldwide significance.

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8. Material was fixed in 3 to 5 percent buffered formalin, gradually dehydrated in ethanol, transferred into increasing concentrations of amyl acetate, and then dried at the critical point with carbon dioxide. Specimens were mounted on metal stubs and then coated with approximately 200 to 300 Å of gold. Micrographs were taken with a Jeol-JSM-U3 scanning electron microscope.
9. This work was supported by Rockefeller Foundation grant RF 710 48 and by H. H. Rackham Faculty Research grant 135028 from the University of Michigan. We thank W. C. Bigelow, Engineering Department at the University of Michigan, for the use of the SEM as well as his staff for their assistance in the preparation of the SEM materials.

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What Retains Water in Living Cells?

Abstract. *Three types of evidence are presented showing that the retention of cell water does not necessarily depend on the possession of an intact cell membrane. The data agree with the concept that water retention in cells is due to multilayer adsorption on proteins and that the maintenance of the normal state of water relies on the presence of adenosine triphosphate as a cardinal adsorbent, controlling the protein conformations.*

There are two opposing views on the mechanism controlling the retention of water in the cell. In one view the major instruments for cell water retention are the cell membrane and a battery of postulated pumps in the membrane (*1*), which regulate the total amount of free solutes and hence osmotic activity in the cell; osmotic activity in turn determines intracellular water content. With the exception of a small percentage in the form of water of hydration on proteins and other macromolecules, cell water itself is considered to be in the free state. Metabolism provides adenosine triphosphate (ATP) as a fuel to operate the pumps. In this view, the integrity of the cell membrane is essential for the retention of cell water.

According to an alternative view, the association-induction hypothesis, the bulk of cell water is retained in the cell in a physical state different from that in a dilute aqueous solution; the major instrument for the regulation of the cell water is the cell proteins (*2*, *3*). The maintenance of cell proteins in a certain "extended" conformation, where the backbone peptide groups polarize, orient, and retain deep layers of water, depends on the adsorption of ATP on certain controlling "cardinal sites." According to this hypothesis, the function of metabolism is to provide ATP,

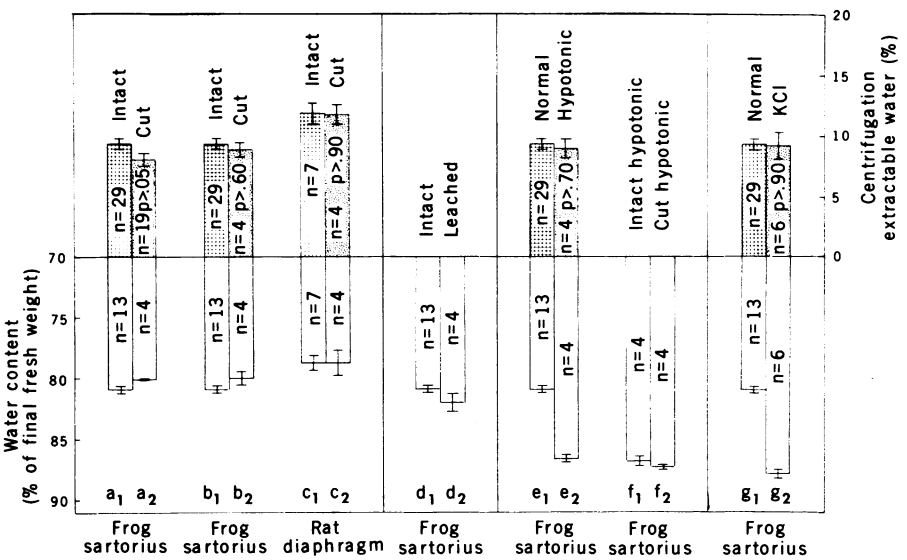

Fig. 1. Data show no significant change in CEF in response to cutting into 2- to 4-mm segments and to swelling brought about by exposure to (e) hypotonic solution (osmolarity 20 percent that of normal Ringer solution, 4 hours at 0°C) and (g) a high KCl concentration (93 m*M*, 72 hours at 4°C). Muscles of b_2 were incubated in a Ca-free Ringer solution containing 1 m*M* ethylenediaminetetraacetic acid (EDTA) for 30 minutes at 4°C before cutting. (d) Removal of intracellular solutes produced no significant change in the amount of water retained in muscles after centrifugation at 1000*g* for 4 minutes. (f) Similar water contents of intact and cut muscles after exposure to hypotonic solution (osmolarity 40 percent that of normal Ringer solution, 5 hours at 0°C) but in this case the muscles were blotted but not centrifuged. The letters a_1, a_2, b_1, b_2, and so on are experiment designations for easier reference.

not as a fuel, but as a "cardinal adsorbent," whose adsorption and electronic interaction elevate the key protoplasmic protein-water-ion system to a higher-energy cooperative state (*4*). [For evidence that ATP, without undergoing hydrolysis, can control both the conformation of a protein on which it adsorbs and the affinity for solute adsorption on distant sites, see (*5*).] The integrity of the cell membrane, in this view, is not indispensable for the retention of water.

This report describes new experimental findings, obtained by a recently described simple centrifugation technique (*6*): frog sartorius muscles (*Rana pipiens pipiens* Schreber) were placed on a deck of wetted filter paper in a moisture-proof packet; centrifugation for 4 minutes at a relative centrifugal force varying between 400 and 1500*g* extracted a constant volume of tissue water, equal to the volume of the extracellular fluid in the muscles.

To test the alternative theories of water retention we asked, Would the same amount of water be extracted by this centrifugation procedure after the intactness of the cell membrane has been destroyed? To answer this question we studied the muscle cells.

A frog sartorius muscle consists of many parallel single cells elongated in shape, each approximately 3 cm in length, extending from one end of the muscle cell to the other (*7*). To destroy the intactness of the cell membrane, we made about 15 cuts at 2-mm intervals on alternate sides of each muscle. These cuts extended from one edge of the muscle almost to the other edge. The great majority of cell segments in a cut muscle were 2 mm long; the remainder were 4 mm long.

Figure 1, a and b, shows that the amount of fluid removable by centrifugation at 1000*g* for 4 minutes (centrifugation extractable fluid or CEF) remained the same in intact frog sartorius muscles and in muscles cut into open-ended segments.

However, before these results could be interpreted to determine whether an intact cell membrane is essential for the retention of the normal amount of cell water, it was necessary to find out if new cell membranes were regenerated at the cut ends (*8*). We tested for membrane regeneration by four different methods, studying (i) the permeability of the cut ends of the muscle to labeled sucrose, D-arabinose, and sodium (*7*); (ii) the outward diffusion of K^+ through the cut end (*9*); (iii) the extractability of water in the presence and absence of Ca^{2+}; and (iv) the electrical potential at the cut surface. All four methods showed that no regeneration of the destroyed cell membrane occurred [for details, see (*10*)].

As in the case of frog sartorius muscles, cutting into 2- and 4-mm segments produced no significant change in the CEF of Sprague-Dawley rat diaphragm muscles (*11*) (Fig. 1c).

The secondary role of the intactness of the cell membrane in regulating the water contents of the cell is also shown in Fig. 1f, but in a different way. Here intact frog sartorius muscles and their pairs, which were cut into 2- and 4-mm segments, were exposed for 4 hours at 0°C to a hypotonic solution (total osmolarity 20 percent that of a normal Ringer solution). Swelling occurred in both. Within the limits of statistical error, cut and intact muscles swelled to the same degree (Fig. 1f).

In maintaining the normal amount of water in the cells, the greater significance of cell proteins than of either the intactness of the cell membrane (and pumps) or the presence of the ions and other solutes in the cell is further shown in Fig. 1d. By leaching frog sartorius muscles in several changes of a large volume of cold, distilled, ion-free water, the bulk of intracellular ions were removed (*12*). The only nonaqueous major component left in the cells was the proteins. After centrifugation for 4 minutes at 1000*g* the leached muscles retained an amount of water equal to that in the intact muscles (*13*). Such extensively leached muscles have lost the intactness of

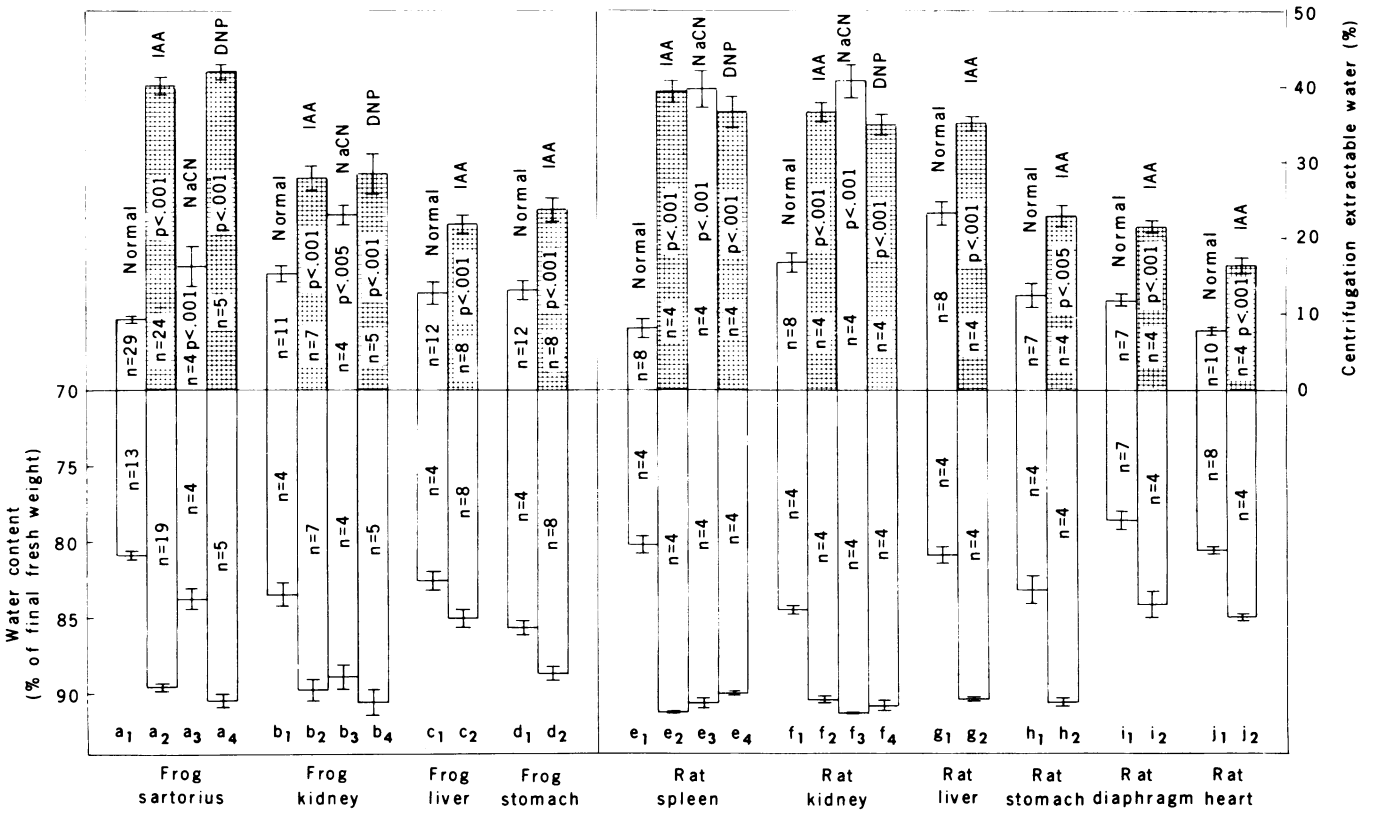

Fig. 2. Effects of metabolic poisons on the CEF and total water contents of frog and rat tissues. Before centrifugation poisoned tissued were exposed for 7 days at 4°C to a Ringer solution containing 1 m*M* IAA, 1 m*M* NaCN, or 1 m*M* 2,4-dinitrophenol (DNP).

the cell membrane as well as the bulk of intracellular solutes. In the conventional membrane-pump model, only a few percent of the observed water retention would be expected.

Summarizing the three types of studies shown in Fig. 1, we conclude that the intactness of the cell membrane and even the presence of the intracellular solutes are not indispensable for the retention of an amount of cell water equal to that found in normal living muscle cells. All three sets of data are in harmony with the view that the seats of water retention in living cells are the cellular proteins.

We next investigated whether metabolism plays a role in the retention of water, as suggested by the association-induction hypothesis. To this end, we studied the effects on a variety of frog and rat tissues of three metabolic poisons: NaCN (1 m*M*), which blocks respiration; 2,4-dinitrophenol (1 m*M*), which uncouples oxidative phosphorylation; and sodium iodoacetate (IAA), which inhibits glycolysis by blocking glyceraldehyde-3-phosphate dehydrogenase. In response to these poisons, there was as a rule an increase in the total water content of the tissues (downward bars in Fig. 2). Extensive ultrastructural changes within the cell in response to metabolic inhibitors have been described (*14, 15*). These changes include a massive increase in cytoplasmic volume and swelling and shape distortion of mitochondria and endoplasmic reticulum. Thus, swelling in response to metabolic interference reflects primarily an increase in intracellular water content in both amphibian and mammalian tissues. Qualitatively similar intracellular changes were also reported for tissues that had been exposed to hypotonic solution and to KCl at high concentration (*15*).

Figure 2 shows that in response to the metabolic inhibitors, there were also highly significant gains in CEF in each of the four frog and six rat tissues (*16*). No change in CEF was observed in swollen frog muscles exposed to hypotonic solution and to KCl (Fig. 1, e and g). Thus, increase of CEF is not a necessary result of swelling, but it is characteristic of swelling induced by metabolic poisons.

These findings are in harmony with (but do not prove) the concept that effective interference with metabolism leads to a fall of ATP in the cells and that without ATP the proteins cooperatively shift to a different conformation in which the backbone NHCO groups, which polarize deep layers of water in the normal resting state, form hydrogen bonds with other NHCO groups of water in the α-helix or with other proteins (for example, a β-pleated sheet). A substantial amount of the cell water now reverts to a normal liquid state and is removable by the centrifugation procedure.

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10. The question of membrane regeneration is important since biologists have often described the formation of a "membrane" when naked protoplasm of protozoa, marine eggs, and muscle cells comes into contact with seawater. Our evidence of the lack of membrane regeneration is fourfold. (i) The increased permeability of the cut ends of the muscle cells toward sucrose, D-arabinose, and Na^+ remains unchanged for 24 hours after the cut at 25°C under sterile conditions (*7*). (ii) Under conditions where only the cut end is directly exposed to an external solute, the cells lose K^+ continually in the same way as an open-ended capillary (*9*). (iii) As shown in Fig. 1, the behavior of the cut muscle is the same whether the bathing Ringer solution contains its normal quantity of free Ca^{2+} or contains no Ca^{2+} (Ca^{2+}-free and contains EDTA). It is well known that the "membrane" formation does not take place without external Ca^{2+} (*8*). (iv) Using a capillary microelectrode, the resting potential of the cut end of the muscle cells was found to drop from 89 ± 0.4 to 13 ± 0.9 mv immediately after cutting. Sterile incubation at 25°C for 24 hours in a complete Ringer GIB medium [G. N. Ling and G. Bohr, *Physiol. Chem. Phys.* **1**, 591 (1969)] led not to a rise but to a further drop of the potential to 5 ± 0.8 mv while the intact end retained a potential of 84 ± 2.1 mv.
11. In this case, the control diaphragm muscle was also cut, but only once, at the insertion on the rib cage.
12. The residual ionic concentrations, from four sets of analyses, were (in micromoles per gram, fresh weight) K, 0.242 ± 0.242; Na, 1.34 ± 0.68; Mg, 1.90 ± 0.30; and Ca, 0.225 ± 0.093.
13. Although the same amount of centrifugation-resistant water (1000*g*, 4 minutes) per gram of dry matter was found in the leached muscles as in normal muscle, this does not necessarily mean that the water was in the same physical state. Evidence for differences based on solute distribution pattern and nuclear magnetic resonance relaxation time studies will be presented elsewhere (G. N. Ling, in preparation).
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Preneoplastic Lesions in the Human Breast

Abstract. *A subgross sampling technique with histological confirmation was used to study the pathology of 119 whole human breasts, either cancer-associated (that is, containing cancer or contralateral to a cancer) or taken from random routine autopsies. Atypical lobules were observed much more frequently in the cancer-associated group than in the group of routine autopsy breasts. Atypical lobules showed varying degrees of anaplasia that formed a continuum between normal epithelium and carcinoma in situ, usually of the common ductal type. As apparent markers for increased cancer risk, atypical lobules in the human breast may be homologous to hyperplastic alveolar nodules that are abundant in high mammary cancer strains of mice. This indirect evidence supports the hypothesis that atypical lesions are common preneoplastic lesions in the human mammary gland.*

Hyperplastic alveolar nodules (HAN) were first observed in the mammary glands of mice by Apolant (*1*) and by Haaland (*2*).

Table 1. Comparison of 119 autopsy and cancer-associated breasts.

Item	Autopsy	Cancer-associated
Number of breasts	67	52
Average age (years)	63.47	60.80
Age range (years)	25–96	28–89
Average number of AL per breast	9.96	37.40
Range in number of AL per breast	0–92	0–225

These HAN are lobulo-alveolar, they are more frequent in the mammary glands of strains of mice that have a high frequency of mammary adenocarcinoma than in strains with a low incidence, they increase in frequency with age, and they have been shown by direct experimental means to be precancerous to the common mammary adenocarcinomas of mice (*3*). We report findings that suggest that a similar lobular lesion in humans is commonly precancerous to human mammary carcinoma. Some of these data have been previously reported in abstract form (*4*).

The pathology of 67 whole human breasts from routine autopsies and 52 can-