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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16. Frog voluntary muscles, which are known to be highly resistant to anoxia and cyanide (*2*), also showed the least swelling and gain in CEF in response to NaCN [G. N. Ling and R. W. Gerard, *J. Cell. Comp. Physiol.* **34**, 383 (1949)].
17. Supported in part by NIH grants I-ROI-CA163OI-OI and 2-ROI-GM11422-IIA1 and ONR contract NR 105-326. The John A. Hartford Foundation provided many of the basic facilities. We thank M. DeFeo for her invaluable help.

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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-

cer-associated breasts (containing cancer or contralateral to breasts that contained cancer) were studied by a subgross sampling technique with histological confirmation by using a modification of methods previously described (*5, 6*). All focal subgross lesions that were observed with the dissecting microscope at ×4 to ×10 were photographed, and the three-dimensional subgross appearance correlated with conventional histopathology of the identical lesions. The method permits the quantitation of the entire dysplastic, hyperplastic, anaplastic, and neoplastic content of whole breasts.

A group of lesions, called atypical lobules (AL), were observed more frequently in the cancer-associated breasts than in the routine autopsy breasts (Table 1 and Fig. 1). Other kinds of lesions showed no or lesser positive correlation with coincident cancer. Like the HAN of the mouse (Fig. 2), AL are lobular in character (Fig. 3) and show variable degrees of epithelial anaplasia, forming a continuum between normal epithelium and carcinoma in situ, usually of the common "ductal carcinoma in situ" type (Figs. 4 and 5). The *t*-test was used to examine the hypothesis that the means of the populations of the two samples (routine autopsy and cancer-associated) are equal when the sigmas are equal but unknown. If the two samples are drawn from the same population they must necessarily have the same sigma and mean. If the *t*-test rejects the hypothesis that the means are equal while the sigmas are equal but unknown, then the populations are different within the confidence interval that is drawn from the *t*-test. In this instance the *t*-test indicates that the two samples are not drawn from the same population, with less than 1 percent chance for error. All *P* values were less than .01.

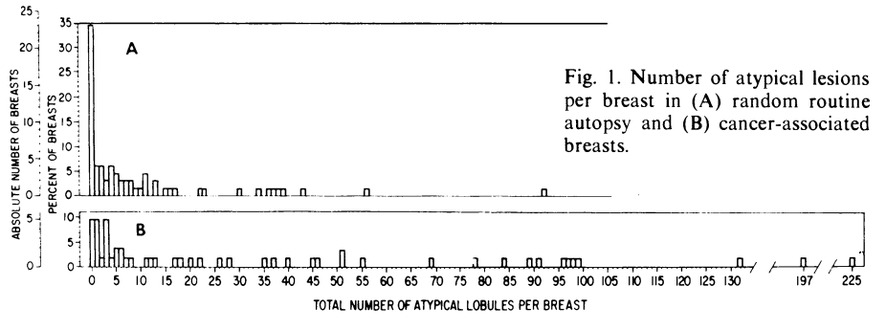

Fig. 1. Number of atypical lesions per breast in (A) random routine autopsy and (B) cancer-associated breasts.

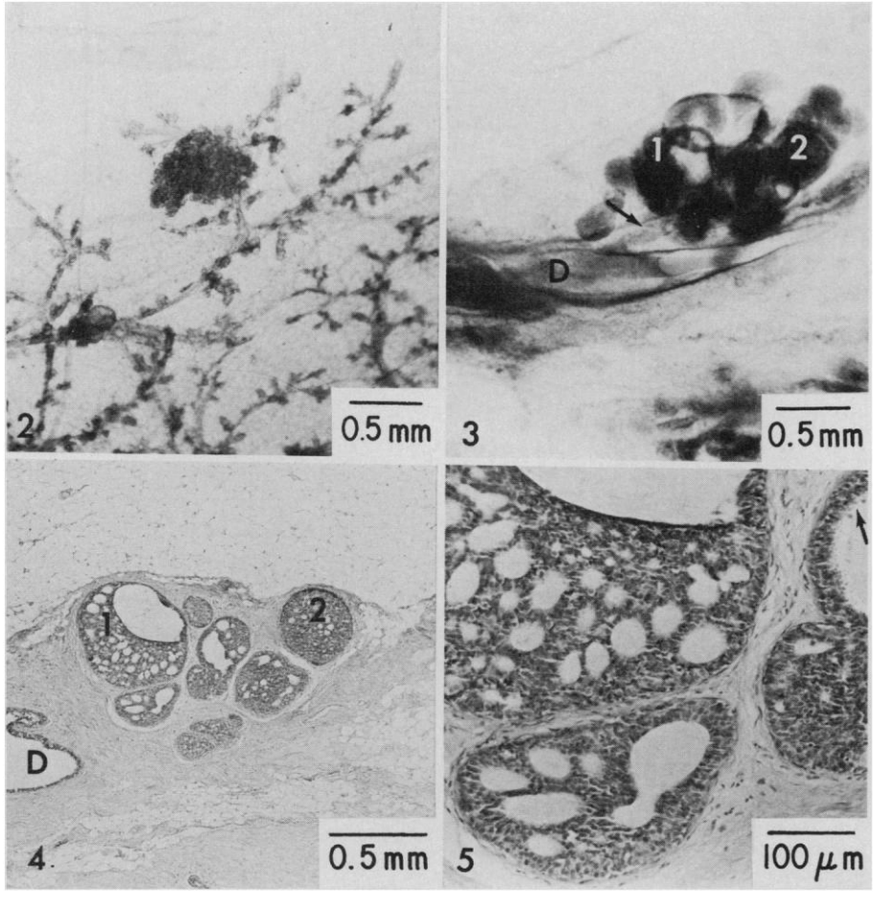

Fig. 2. Hyperplastic alveolar nodule (HAN) of retired breeder C3H/Crgl mouse is shown in the top center. Individual alveoli are visible, especially at the edges of the HAN. Hematoxylin stain (× 22). Fig. 3. An atypical lobule (AL) in a cancerous breast from an 80-year-old human female. She had the other breast removed for cancer at age 75. In the 2-mm-thick slice of breast, the AL is seen as a cluster of ovoid structures. Some of them are partially filled with cells (1), others are completely filled with cells (2). The arrow points to the small duct that drains the AL into a larger duct (D); hematoxylin stain (×18.9). Fig. 4. Histologic section of the AL depicted in Fig. 3. The two structures labeled 1 and 2 correspond to those labeled similarly in Fig. 3. Some unfolding parts of the lobule are of similar diameter to the larger duct (D) (×18.9). Fig. 5. Part of structure labeled 1 in Fig. 4 is seen in upper left corner. A cartwheel pattern is prevalent and it is of grade IV atypia (we call carcinoma in situ grade V atypia). The arrow points to an area of grade 1 atypia where the luminal cells display cytoplasmic blebs (×120).

The morphology indicates that AL are lobular in character and resemble the well-known precancerous HAN of mice of high mammary cancer strains. This agrees with our previous results (*6*), which suggest that ductal carcinoma in situ of the human breast is of lobular origin. Progressive distension of ductules with dysplastic and anaplastic cellular elements leads to unfolding and coalescence of the ductules within the lobule to form larger ovoid structures (Fig. 3). Such lesions falsely appear to be small ducts in conventional histology slides (Figs. 4 and 5). However, the microarchitecture shows a cluster of larger structures to be drained by a small duct, as is the pattern of normal lobules (Fig. 3).

Our data (Table 1 and Fig. 1) appear to indicate that the presence of AL in breast biopsies should alert clinicians and pathologists that such women may be prone to develop breast cancer, especially if AL are abundant.

Although cystic distension of ducts and lobules was more frequent in cancer-associated breasts than in autopsy specimens, the degree of cystic disease did not appear to be associated either with abundance of AL or their degree of atypia.

This indirect evidence therefore supports

the hypothesis that AL are the common kind of precancerous lesions in the human breast. In our opinion, every effort should be made to prove or disprove this hypothesis by direct experimental means.

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Messenger RNA Induction of Fast Sodium Ion Channels in Cultured Cardiac Myoblasts

Abstract. *Incubation with adult heart messenger RNA caused the appearance of fast sodium ion channels in young myocardial cells whose development had been arrested in vitro. The induction was blocked by cycloheximide, indicating dependence on protein synthesis. Thus, cardiac myoblasts can be made to differentiate in vitro, and membrane properties can be altered by exogenous RNA.*

Electrical activity can be recorded in the precardiac areas (anterolateral blastoderm) of the 20-hour embryo (*1*) and a sequence of changes in electrophysiological properties occurs during normal development of chick embryonic myocardial cells in situ (*2–4*). Up to 4 days in ovo, a time when the heart tube is undergoing complex morphogenetic movements, the young myoblasts lack fast Na^+ channels (*2–4*). This is indicated by the fact that the young cells retain slow rates of rise when the membrane is hyperpolarized to a take-off potential of –80 mv. Their action potentials are generated by kinetically slow Na^+ channels, which confer slow maximal rates of rise ($+\dot{V}_{max}$) (usually less than 25 volt/sec) and which are insensitive to tetrodotoxin (TTX), a blocker of fast Na^+ channels (Fig. 1, A and B). Sensitivity to TTX first appears on about day 5, marking the initial appearance of fast Na^+ channels (*2–4*). From day 5 to day 8, the action potentials have intermediate rates of rise (30 to 80 volt/sec) and partial sensitivity to TTX; that is, TTX reduces $+\dot{V}_{max}$ to a low value (5 to 10 volt/sec), about the same as that found in young cells. The inward current during these residual action potentials must be carried by slow Na^+ channels, which are not blocked by TTX. Thus, the membrane contains both TTX-sensitive fast Na^+ channels and TTX-insensitive slow Na^+ channels at this stage of development. After day 8, TTX completely abolishes all excitability despite intense stimulation (Fig. 1, C and D), indicating that the density of functional slow Na^+ channels has diminished. The $+\dot{V}_{max}$ values increase progressively to about 150 volt/sec by day 18, suggesting the acquisition of an increased density of fast Na^+ channels. The resting potential also increases gradually during development from about –35 mv on day 2 to about –80 mv by day 12 (*2–4*). The increase in resting potential coincides with a decrease in the incidence of pacemaker activity. Both of these changes can be accounted for by an increase in K^+ permeability (P_K) during development (*2, 5*).

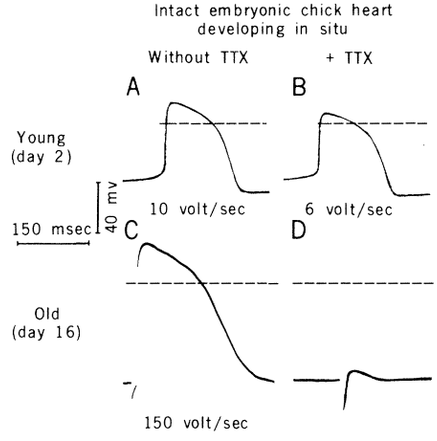

Fig. 1. Electrophysiological properties of young (day 2) (A and B) and old (day 16) (C and D) chick embryonic hearts developing in situ. (A) Control action potential with slow (< 20 volt/sec) $+\dot{V}_{max}$ and with pacemaker potential. (B) After addition of TTX (1 μg/ml), the action potential rate of rise and overshoot are unchanged. (C) Rapidly rising recording ($+\dot{V}_{max}$ of 150 volt/sec) from ventricular cell with a high resting potential (–80 mv). Note absence of pacemaker potential. (D) Tetrodotoxin (0.1 μg/ml) abolished all excitability despite intense field stimulation. Calibrations apply throughout. The horizontal broken line gives zero potential. Extracellular electric field stimulation was given in (C) and (D) (note artifacts).

The normal sequence of membrane electrical differentiation can be interrupted in vitro. Sperelakis and co-workers (*6*) have shown that development is arrested at the stage reached at the time of explantation when hearts or fragments of hearts are placed in organ culture. For example, young hearts retain their high density of TTX-insensitive slow Na^+ channels and fail to gain fast Na^+ channels in vitro.

When young embryonic cells are cultured as monolayers or as reaggregates, they do not further differentiate; that is, they retain characteristically young properties, including TTX-insensitive slowly rising action potentials and pacemaker activity (*7*) [although limited development may be achieved in some cells (*8*)], as found in the case of organ-cultured young hearts. When cells are dissociated from old embryonic hearts (14 to 20 days in ovo) with trypsin and subsequently surface-cultured as monolayers, the electrical properties revert to resemble those of the young heart; that is, the cells lose their TTX-sensitive fast Na^+ channels, regain a high density of slow Na^+ channels, and resume pacemaker activity (*3, 9*). This reversion can be partially prevented by culture in elevated K^+ (*10*), and complete retention of highly differentiated electrophysiological properties in vitro can be achieved in spherical reaggregates (*11*).

Niu and co-workers (*12*) have reported the induction by adult chicken heart RNA of spontaneously beating tubes and sheets of cardiac cells in cultured postnodal pieces of chick blastoderm; the effect became apparent about 5 days after RNA treatment. We have recently corroborated these results and have recorded typical cardiac action potentials from these cells (unpublished observations). The induction of cardiac tissue in undifferentiated postnodal pieces indicates that determination, as well as differentiation, can be produced in vitro. This led us to test whether cultured young embryonic myocardial cells in organ-cultured hearts and in spherical reaggregate cultures could be induced to develop fast Na^+ channels by treatment with adult heart messenger RNA (mRNA) in vitro. As indicated by rapidly rising action potentials and TTX sensitivity, we conclude that such induction does occur.

For organ culture, spontaneously con-