These results are also supported by the recent identification of chromosome 7 in man as the preferential hybridization site of ^{125}I-labeled human H4 mRNA (*18*). Moreover, these data imply that the genes for more than one of the five major histones are clustered in one chromosomal region. This is not surprising since in both sea urchin and *Drosophila* the five histone genes are arranged in a tandemly arrayed repeat unit (*11*, *12*, *19*); in *D. melanogaster* histone genes are located in region 39D-E on chromosome 2 (*19*, *20*); and hybridization of histone mRNA with restriction endonuclease-cleaved DNA human placenta suggested clustering of the genes in a unit of at least 10×10^6 daltons (*21*). Since there are approximately 40 copies of each histone gene per human haploid genome (*22*) and chromosome segment 7q32-36 contains many times the amount of DNA needed to encode these sequences (*23*), nonhistone DNA may be interspersed among histone-coding regions in man.

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23. If each human histone linear repeat unit is 10×10^6 daltons, and the unit is repeated 40 times per haploid genome (*22*), approximately 4×10^8 daltons of DNA would be needed for these sequences. Since the human haploid genome contains 1.8×10^{12} daltons of DNA [B. Lewin, *Gene Expression* (Wiley, New York, 1974), vol. 2, p. 7] and segment 7q32-36 comprises 0.97 percent of the total chromosome complement length, 1.75×10^{10} daltons of DNA may be contained in 7q32-36, of which up to 97 percent may be nonhistone sequences.
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Temperature Dependence of ADH-Induced Water Flow and Intramembranous Particle Aggregates in Toad Bladder

Abstract. *Antidiuretic hormone (ADH)-induced luminal intramembranous particle aggregates and hormonally stimulated water flow in toad urinary bladder are reduced simultaneously with a reduction in temperature. When water movement is factored by the aggregation response, the apparent activation energy for this process decreases from 12.1 ± 1.6 to 3.0 ± 2.3 kilocalories per mole. The data are consistent with the view that the particle aggregates contain sites for transmembrane water movement and that these sites behave as pores.*

It is well known that water permeability of toad urinary bladder is enhanced in response to antidiuretic hormone (ADH). This response is mediated by intracellular adenosine 3′,5′-monophosphate (cyclic AMP) (*1*) and appears principally to involve an alteration of the luminal membrane of granular-type epithelial cells (*2*). Freeze-fracture electron microscopy reveals that stimulation of amphibian urinary bladder with ADH also induces a structural alteration in the luminal membrane of granular cells (*3*). This consists of aggregated intramembranous particles that are organized in linear arrays at multiple sites on the inner (protoplasmic) fracture face and complementary grooves on the apposed (exoplasmic) face. It occurs in both the absence and presence of a transbladder osmotic gradient and it can be induced by stimulation with cyclic AMP (*4*). In quantitative terms the number of aggregates per area of granular cell luminal membrane and the cumulative area of membrane occupied by aggregates are related linearly to ADH-induced levels of bladder water permeability (*3*, *4*) and both are either inhibited or enhanced with either selective inhibition (*5*) or accentuation (*6*) of the hydro-osmotic effect of ADH. Although it has not yet been shown that these sites of particle aggregation are or contain the actual sites for ADH-induced transmembrane water movement, this appears to be a reasonable hypothesis.

We studied this hypothesis by assessing the dependence of the aggregation and water permeability responses to ADH on temperature. In addition, by normalizing ADH-stimulated water movement for the effect of temperature on membrane morphology, which until now had not been fully realized, and by calculating the activation energy (E_A) for this process from the Arrhenius equation, we considered the question of whether ADH-stimulated water permeation of the luminal membrane of the granular cell involves aqueous channels ("pores") or a solubility-diffusion process.

Two series of experiments were performed. In the first, paired hemibladders ($N = 6$) from large female Dominican toads (*Bufo marinus*) were prepared as sacs on the ends of glass tubes. All bladders were washed inside (mucosal) and out (serosal) with Ringer solution (111 m*M* NaCl, 3.5 m*M* KCl, 2.5 m*M* $NaHCO_3$, 1.0 m*M* $CaCl_2$, *p*H 7.6 to 8.2, 220 mOsm per kilogram of H_2O), then filled with Ringer solution diluted 1:5 with distilled water and suspended in an aerated Ringer bath. Bladders were permitted to stabilize for 30 minutes while transbladder electrical potential (PD) was monitored with calomel electrodes and a Keithley electrometer (610 C). If the PD was less than 20 mV at the end of this period for either hemibladder of a pair, the experiment was terminated. Otherwise, the mucosal contents of both

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bladders were exchanged with fresh one-fifth strength Ringer solution at either 23.6° ± 0.6° or 10.5° ± 0.4°C and the bladders were resuspended in fresh aerated baths, which were at comparable temperatures. After a 30-minute baseline period both bladders of a pair were treated with 20 mU of ADH (arginine vasopressin; Sigma) per milliliter of serosal bathing medium for 20 minutes, then fixed with 2.5 percent glutaraldehyde in 0.1*M* cacodylate buffer (*p*H 7.4) for 15 minutes. Throughout the pre-ADH and ADH stimulation periods, the temperature of the serosal baths for cold-treated bladders was regulated at about 10°C with a Coleman cold plate and the bath temperature for these bladders, as well as for bladders at room temperature (~ 24°C), was recorded every 10 minutes during the course of an experiment. Osmotic water flow in all bladders was assessed gravimetrically (*7*) 15 minutes before and at 10-minute intervals after the addition of ADH, and was expressed per unit bladder area (which was calculated from measurements of mucosal volume, assuming bladder sacs to be spheres).

The glutaraldehyde-fixed tissues of these experiments were stored in cacodylate buffer at 4°C until they were processed for freeze fracture in a Balzers freeze-etch unit (BAF 301), as described previously (*3*, *4*). Quantitative electron microscopy was performed with a Zeiss electron microscope (10 A) without knowledge of tissue status (cold or room temperature). This approach consisted of taking a random micrograph of the protoplasmic fracture face from each of at least ten randomly selected granular cells for every bladder studied. The area of membrane observed in each micrograph was about 23.5 μm^2. For quantitative purposes the number of aggregates was counted at a magnification of ×45,000 and the area of membrane occupied by each was evaluated by planimetry with an Elographics digitizer (E 241) on-line to a Wang programmable calculator (720 C).

In the second series of experiments, the fixed-sac technique of Eggena (*8*) was used to study paired bladders from six additional toads. Both bladders of a pair were stimulated with ADH for 20 minutes at room temperature (24.3° ± 0.4°C) and then fixed from the mucosal surface for 5 minutes with 1 percent glutaraldehyde in 0.05*M* cacodylate buffer. Thereafter, they were thoroughly rinsed, refilled with one-fifth strength Ringer solution, resuspended in fresh Ringer baths either at room temperature (24.5° ± 0.3°C) or cold (11.7° ± 0.3°C), and assessed gravimetrically for osmotic water movement over two sequential 10-minute intervals.

Statistical comparisons of both physiologic and morphologic data for significant differences ($P < .05$) were made with Student's *t*-test for paired or unpaired observations, as required (*9*).

In the first experimental series, baseline osmotic water flow at 10.5° ± 0.4°C (0.15 ± 0.04 mg/min-cm²) was not changed from that at 23.6° ± 0.6°C (0.15 ± 0.05 mg/min-cm²). In separately conducted, parallel experiments ($N = 3$), intramembranous particle aggregates were not observed at either of these temperatures in the absence of ADH. It was therefore unnecessary to anticipate differences in the aggregation response to ADH for cold alone.

The effect of temperature on ADH-

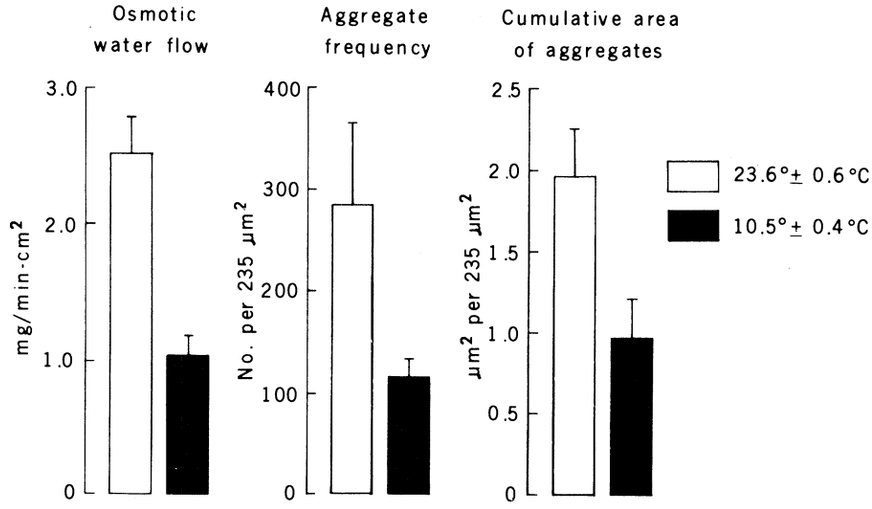

Fig. 1. Effect of temperature on ADH-stimulated osmotic water flow and intramembranous particle aggregation in paired toad urinary bladders ($N = 6$). Means and standard errors are shown.

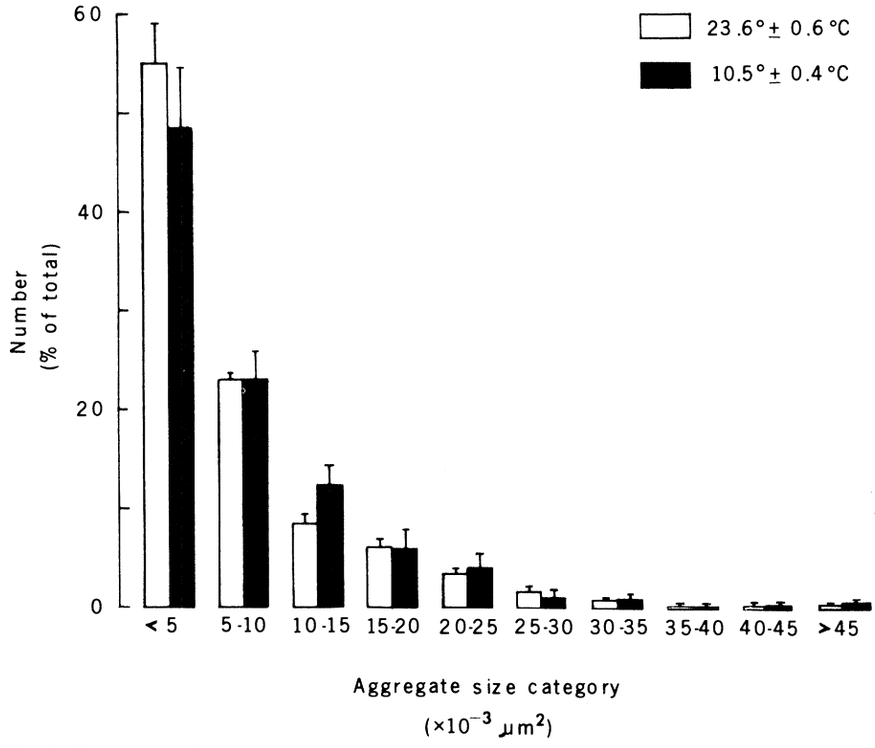

Fig. 2. Frequency histogram of aggregate size (surface area) for paired toad urinary bladders ($N = 6$), which were ADH-stimulated at either 23.6° ± 0.6° or 10.5° ± 0.4°C. Means and standard errors are shown. Mean aggregate size for bladders at room temperature (6.9 ± 0.8 × 10^{-3} μm^2) was not significantly different ($.3 > P > .4$) from that for bladders in the cold (8.0 ± 1.2 × 10^{-3} μm^2).

stimulated osmotic water movement and particle aggregation is illustrated in Fig. 1. During the 10-minute interval before tissue fixation, ADH-stimulated osmotic water flow was inhibited ($P < .001$) by 59.1 ± 5.5 percent in the cold as compared to the level observed at room temperature. In these same tissues, the number of aggregates per area of membrane and the total area of membrane which they occupied were reduced by 51.5 ± 7.2 percent ($P < .05$) and 47.9 ± 7.7 percent ($P < .025$), respectively. As shown in Fig. 2, cold did not appear to influence the relative distribution of aggregate sizes, and mean aggregate areas for different conditions of temperature were comparable ($8.0 \pm 1.2 \times 10^{-3}$ μm^2 in the cold and $6.9 \pm 0.8 \times 10^{-3}$ μm^2 at room temperature).

If the hypothesis that sites of particle aggregation are or contain sites for ADH-stimulated transmembrane water flow is considered, it would seem reasonable (because of the influence of temperature on the viscosity of water) that if the aggregation response to ADH is reduced by the cold, then bulk water movement across toad bladder (under constant conditions of osmotic gradient) would in relative terms be reduced to some small additional extent. The data given above support this concept. The purpose of the experiments with the fixed-sac technique of Eggena (*8*) was to quantitate this hypothetical effect. Because in these experiments both bladders of a pair were fixed with glutaraldehyde after 20 minutes of ADH stimulation at room temperature, they presumably had the same number of aggregates. Any difference in water flow would therefore be attributable solely to the effect of temperature on water. For these fixed-bladder preparations osmotic water flow was indeed slightly, but significantly ($P < .025$), lower in the cold than at room temperature (1.12 ± 0.09 as opposed to 1.40 ± 0.12 mg/min-cm^2). The relative inhibition with cold was 18.8 ± 6.5 percent. This would quantitatively appear to explain the slight discrepancy between the 59.1 ± 5.5 percent inhibition of the water flow response to ADH and, for example, the 47.9 ± 7.7 percent inhibition of the aggregation response, as measured by the cumulative area of membrane occupied by aggregates, which was suggested ($.1 < P < .15$) in our primary experiments. That is (with no change in aggregate size), if aggregates are or contain sites for water movement, the predicted total inhibition of ADH-stimulated water flow with cold should approximate the level of inhibition of the aggregation response in the cold (~ 48 percent) plus the product of the uninhibited portion of the aggregation response in the cold (~ 52 percent) and the inhibition of water movement due to the physical effect of cold (~ 19 percent). This calculated value from average relative data is about 58 percent—that is, $100 \times [0.48 + (0.52 \times 0.19)]$, which is close to the overall observed mean value of about 59 percent.

The effect of temperature on membrane morphology which we found appears fundamentally important for the assessment of the apparent E_A for ADH-stimulated water movement across toad bladder. In one of the most well-accepted investigations to define E_A for this process in toad bladder, which took place before the aggregation response to ADH was discovered, the possibility that the number of sites for water movement might be reduced with cooler temperatures was not considered (*10*). However, because the aggregation response to ADH is related to membrane water permeability (*2–5*), if the effect of cold on this response is not considered, E_A for water movement is necessarily overestimated. The previous estimate of E_A for water movement across toad bladder, in which the effect of cold on membrane structure and function was not taken into account, was ~ 10 kcal/mole (*10*). With our data, if the aggregation response in relation to water movement is disregarded, E_A for the process is 12.1 ± 1.6 kcal/mole, which closely approximates this previous estimate. On the other hand, when ADH-stimulated water movement is factored by the cumulative area of membrane occupied by aggregates, E_A is reduced significantly ($P < .01$) to 3.0 ± 2.3 kcal/mole.

Data from the experiments with fixed-bladder preparations validate the latter procedure, in which the aggregation response to ADH is used as a scale factor for water movement. For these experiments it was not necessary to normalize water movement for the effect of temperature on membrane morphology because the bladders were treated identically until they were fixed. The apparent E_A for water movement in these experiments was 2.9 ± 1.0 kcal/mole, which is in general agreement with Eggena's observations for a comparable temperature range (*8*) and not significantly different ($P > .9$) from the E_A value derived by factoring for the effect of cold on the aggregation response to ADH (3.0 ± 2.3 kcal/mole). That channels within the luminal membrane of toad bladder granular cells for water movement must be postulated (as opposed to general membrane sites at which transmembrane water movement would take place by a solubility-diffusion process) is evident because E_A for volume water flow across native lipid bilayer membranes approximates 12.7 ± 0.3 kcal/mole (*11*), which is about four times greater than our refined estimate for this process. This argument has already been made, but on different theoretical grounds (*12*). Moreover, to extend the basis of our argument, our estimate of E_A for ADH-stimulated osmotic water movement is close to the value of 4.6 kcal/mole for viscous water movement (*13*).

Considering that sites of particle aggregation may contain sites for water permeation under conditions of ADH stimulation, it remains to be established whether water permeation might occur through the particles themselves or between them. Because the radius of an average site of particle aggregation approximates 450 to 600 Å, as an entire structure, it almost certainly could not be rationalized as being uniformly water permeable. Others have reported depressions within intramembranous particles (*14*), and these can be seen in particles comprising aggregates as well. Whether these depressions could be the openings of patent water channels within ADH-induced aggregated intramembranous particles is not known. In fact, it has never been established that particles comprising ADH-induced sites of aggregation actually span the entire membrane from the extracellular to the cytoplasmic surface. At this point, it seems equally reasonable to postulate that membrane lipids between particles comprising aggregates might be sufficiently disorganized to permit porelike water movement. Finer details of water permeation of the luminal membrane of toad bladder with ADH-stimulation must therefore await further study.

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Müllerian Duct Regression in the Embryo Correlated with Cytotoxic Activity Against Human Ovarian Cancer

Abstract. *A significant cytotoxicity index was obtained when human ovarian cancer cells in a microcytotoxicity assay were exposed during the S (DNA-synthesizing) phase of the cell cycle to purified fractions of testis exhibiting high Müllerian inhibiting substance bioactivity. The same effect was not observed when these fractions were tested against human glioblastoma or fibroblast lines. Most human ovarian cancers are said to resemble Müllerian tissues histologically. Müllerian inhibiting substance may thus deserve further study as a potential chemotherapeutic agent.*

Embryonic development is dependent on a series of intricate systemic and local signals that stimulate organ differentiation, or, conversely, cause dissolution or even self-destruction leading to disappearance of parts or all of an organ system. Müllerian inhibiting substance (MIS), a fetal testicular product (*1*), initiates regression of the Müllerian duct in the male embryo of many mammalian species. The Müllerian ducts in females develop into the uterus, fallopian tube, and upper vagina (*2*). The common epithelial carcinomas of the ovary resemble histologically the tissues derived from the Müllerian duct (*3*), that is, fallopian tube, endometrium, and endocervix; MIS may have an inhibitory effect that is specific for fallopian, endometrial, and cervical tumors as well as ovarian tumors. In this study, we tested the cytotoxic effects of partially purified (*4*) MIS-active fractions (*5*) of testes from newborn calves (*6*) against a human ovarian cancer in tissue culture (*7*).

The ovarian tumor was surgically removed from a 56-year-old woman in 1971 and has been serially subcultured since then. Histologically, the tumor is described as a papillary serous cystadenocarcinoma that is moderately well differentiated. The epithelioid line doubles every 28 hours, has a near diploid karyotype, and can produce tumors with a histology similar to the original tumor when 10^4 or more cells are heterotransplanted into the hamster cheek pouch (*7*, *8*). This line has been serially subcultured in our laboratory in stationary monolayers at 37°C in Eagle's minimum essential medium (MEM) containing 15 percent fetal calf serum, 1 percent penicillin (10,000 unit/ml), and streptomycin (10,000 μg/ml). As control lines we used a human fibroblast strain derived from the foreskin of a 1-year-old undergoing circumcision, and a glioblastoma (non-Müllerian duct tumor) line (*9*). The control lines are grown in stationary monolayers at 37°C in F10 nutrient medium containing 10 percent fetal calf serum and 1 percent penicillin and streptomycin. All lines are free of mycoplasma (*10*).

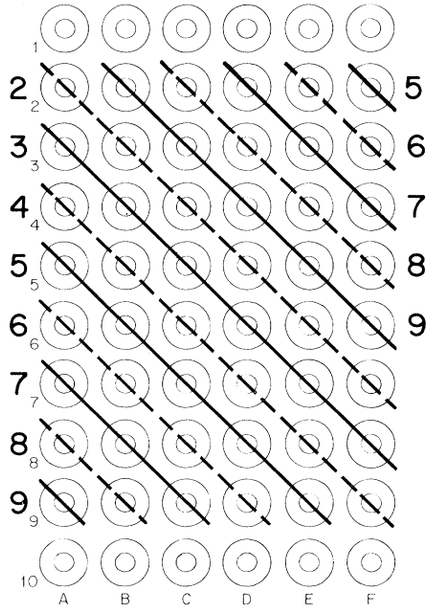

Fig. 1. Evaporation was minimized by applying experimental and controlled fractions on the microtest plates in the template illustrated and by surrounding the plates with moist gauze in a small plastic food box with the lid slightly ajar at 37°C in a 5 percent CO_2 humidified incubator. Rows 1 and 10 were eliminated.

By methods adapted from those of Wood and Morton (*11*), monolayers of the cell lines, after they approached confluency (3 days after a 1:2 subculture), were washed with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution, dispersed with 0.25 percent trypsin-EDTA (Gibco), and counted in a hemocytometer. Appropriate dilutions for each cell line were made with either MEM or F10 containing 20 percent fetal calf serum, and 300 to 400 cells were delivered to each well of a Falcon 3034 microtest plate in a 0.01-ml volume with a six-barrel Teresaki syringe. Plates were surrounded by moistened gauze, placed in a plastic box with the lid ajar (*12*), and incubated at 37°C in a humidified incubator with an atmosphere containing 5 percent CO_2 and 95 percent air. The following morning medium was blotted from the wells and replaced with new medium (0.01 ml per well). Testis fractions and controls were then added in 0.01-ml portions (total volume, 0.02 ml per well) according to the pattern shown in Fig. 1 (*12*). After a 24-hour incubation period, the plates were washed and stained with Giemsa, and the adherent cells were counted on a projection screen. Six replicate wells were used for each test fraction. The microtest plates contained phosphate-buffered saline (PBS) as a negative control, fractions from newborn calf heart (no Müllerian duct regression) as a tissue negative control, and fractions from newborn calf testes that were either inactive (biochemical negative controls) or active (test substance) in the organ culture assay. The counts of the 18 PBS control replicate wells were averaged and compared with the average of the six replicates of each fraction tested, and a cytotoxicity index [CI = (control well counts − test well counts)/control well counts] was calculated separately for each plate. A CI greater than 0.25 differed significantly from the controls ($P < .01$, Student's t-test). Müllerian duct regression activity was simultaneously determined for each test fraction, and the activity in an organ culture assay was correlated with the cytotoxicity assay on the human ovarian cancer cells.

Newborn calf testes were diced rapidly in an automatic tissue chopper, suspended in a 1*M* guanidine hydrochloride

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