encoding *N*-sulfotransferase.
14. Tumors were excised after cervical dislocation of the animal. Cell suspensions were generated by teasing tumors through cloth (bridal veil). The suspension was treated overnight with gentamycin sulfate (50 μg/ml), streptomycin sulfate (100 μg/ml), and penicillin G (100 U/ml), and then the medium was changed to remove tissue debris. One day later, the cells were used for the indicated experiments.
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16. The possibility that the failure of mutants 745 and 761 to form tumors is due to a secondary mutation unrelated to the defect in proteoglycan synthesis seems unlikely, because the same secondary mutation would have had to occur in the different strains. We also found that mutant 605 formed tumors efficiently (4 of 4). Mutant 605 is defective in the transport of inorganic sulfate from the medium (Fig. 1), but it synthesizes fully sulfated proteoglycans using inorganic sulfate generated from the catabolism of sulfur-containing amino acids (*10*).
17. D. M. Kingsley, K. F. Kozarsky, L. Hobbie, M. Krieger, *Cell* **44**, 749 (1986). Strain ldlD (from M. Krieger, Massachusetts Institute of Technology) lacks the 4-epimerase involved in the interconversion of UDP-esters of galactose and glucose or *N*-acetylgalactosamine and *N*-acetylglucosamine. It fails to synthesize both Asn-linked and Ser- or Thr-linked oligosaccharides on glycoproteins when the growth medium lacks galactose and *N*-acetylgalactosamine.
18. One millicurie of carrier-free $Na^{35}SO_4$ (Amersham, Arlington, IL) was diluted to 0.5 ml with phosphate-buffered saline, filter-sterilized, and injected intraperitoneally. Eighteen hours later, the tumors were excised, weighed, and extracted with 4*M* guanidine hydrochloride containing leupeptin (0.5 μg/ml), pepstatin A (1 μg/ml), 2 m*M* phenylmethylsulfonylfluoride, 20 m*M* *N*-ethylmaleimide, 10 m*M* EDTA, and 50 m*M* sodium acetate, *p*H 6.0 [V. C. Hascall and J. H. Kimura, *Methods Enzymol.* **82**, 769 (1982)]. The extracts were filtered through gauze, and the filtrate was dialyzed against 0.3*M* NaCl, 4*M* urea, and 50 m*M* sodium acetate buffer, *p*H 6, containing protease inhibitors. The sample was next passed over a small column (0.5 ml) of DEAE-Sephacel (Pharmacia), and non-proteoglycan contaminants were washed from the column with 15 ml of solution. We recovered the proteoglycans by increasing the salt concentration to 1*M*. A portion of this material was counted by liquid scintillation spectrometry.
19. The material in the tumor that migrated in the region of chondroitin sulfate has not been identified (Fig. 2B), but more highly sulfated forms of heparan sulfate proteoglycan elute in this position.
20. For animal cell replica plating, see J. D. Esko, *Methods Enzymol.* **129**, 237 (1986); *Methods Cell Biol.*, in press.
21. To test how many wild-type cells might be needed to satisfy the proteoglycan requirement of the mutant, we examined the cell composition of tumors derived from mixtures of 10^7 mutant 745 cells and 10, 10^2, 10^3, 10^4, 10^5, or 10^6 OUA-1 cells, a CHO mutant resistant to 1 m*M* ouabain. OUA-1 synthesizes the same proteoglycans as those produced by CHO-K1 and forms tumors efficiently (14 of 15). Tumors arose from mixtures containing $\geq 10^3$ OUA-1 cells, and incubation of cell suspensions from each mixed tumor in growth medium with and without ouabain showed that the ratio of the two cell types in each tumor was within a factor of 2 to 8 of their ratio in the original mixture injected into the animals.
22. BALB/c mice were γ-irradiated with 650 rads from a ^{60}Co source. One day later, the animals were injected with cells. Nude/beige mice contain about 1/7 as many NK cells as homozygous nude mice (*24*). SCID mice, provided by J. McGhee (University of Alabama at Birmingham), were maintained under sterile conditions in a Trexler isolator. CHO cells formed tumors rapidly in SCID mice and metastasized to other sites.
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A Novel Target for Antidiuretic Hormone in Insects

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Diuresis in insects is controlled by two antagonistic hormone groups: diuretic hormones, which promote water loss, and antidiuretic hormones, which inhibit it. All known antidiuretic factors act solely to promote fluid reabsorption by the hindgut and do not affect secretion by the Malpighian tubules. In the house cricket, *Acheta domesticus*, an antidiuretic hormone was found that inhibits fluid secretion by the Malpighian tubules but has no effect on the hindgut. Correlations were found between the density of neurosecretory granules and the presence of antidiuretic hormone in the corpora cardiaca, suggesting that the hormone is released from specific axons. Its release is triggered by dehydration; the hormone is detectable in the hemolymph of water-deprived crickets. These results imply that an unusual mechanism regulates water balance in these insects.

THE CURRENT MODEL FOR THE CONTROL of diuresis in non–blood-feeding insects is based on the presence of two antagonistic hormone groups. Diuretic hormones (DHs) stimulate fluid secretion by the Malpighian tubules and inhibit reabsorption by the rectum (*1*), whereas an antidiuretic hormone (ADH) enhances fluid resorption by the hindgut (*2*, *3*). It was reported that ADH could also inhibit fluid secretion (*4*); however, those results subsequently could not be duplicated (*3*, *5*, *6*). On the basis of in vitro measurements of DH degradation rates, and in the absence of data confirming the action of ADH on tubules, it was proposed that tubular secretion is reduced solely by enzymatic destruction of DH and by a reduction in DH release (*2*, *7*).

We have reported that diuresis by the Malpighian tubules of the house cricket, *Acheta domesticus*, was stimulated by homogenates of the primary neurohemal organs [corpora cardiaca (CC)] as well as homogenates of several other ganglia (*8*). To distinguish between the physiological and pharmacological effects of such treatments, we also performed bioassays on extracts of the hemolymph to determine which factors were present under various conditions of water stress. We determined that hemolymph could either stimulate or inhibit fluid secretion by the tubules in vitro, depending on the hydration state of the donor animal.

To produce different hydration states, crickets were subjected to one of three preliminary treatments. One group of insects was freely provided with food and water for 48 hours (control). A second group had access to food and water for 24 hours, after

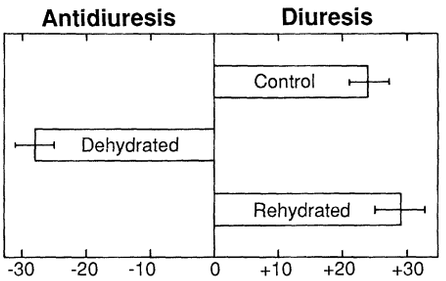

Fig. 1. Histogram of the maximum change in secretion rate by the Malpighian tubules in vitro (mean ± SE) in response to methanolic extracts of hemolymph. Hemolymph from crickets allowed free access to food and water (control, $n = 9$) acts as a diuretic, promoting fluid secretion by the tubules. When control crickets were held at 0% RH for 24 hours (dehydrated, $n = 12$) the hemolymph acted as an antidiuretic, inhibiting fluid secretion. Dehydrated crickets allowed access to water for 1 to 2 hours (rehydrated, $n = 8$) produced hemolymph which also acted as a diuretic, with a slightly greater effect than observed in the controls (not significant at $P = 0.05$, Student t test).

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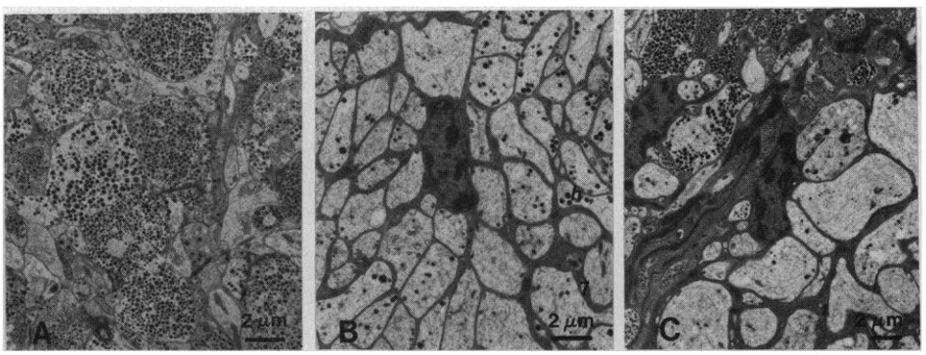

Fig. 2. (**A**) Cross-section through the ventral portion, near the midline, of CC from a control cricket. All of the axons are full of neurosecretory material. (**B**) Section through the same region of the CC of a dehydrated cricket, that shows these axons are now almost completely void of neurosecretory granules (NSG). (**C**) Section through the CC of a dehydrated cricket, taken near the boundary of the area we have identified as containing the ADH. The axons in the lower right of the plate have released all of their NSG, whereas axons farther from the aorta have released only part of their NSG. The full axons in the upper portion of this plate have not yet been stimulated to release their NSG.

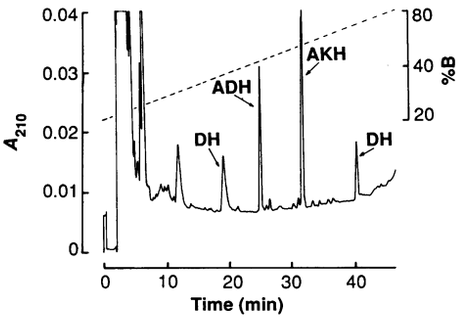

Fig. 3. Chromatogram obtained from 18 pairs of CC from control crickets, showing the distribution of neurosecretory material. Identification of peaks was based on hyperlipemic activity in *Locusta* (AKH) (*11*), or stimulation (DH) or inhibition (ADH) of fluid secretion by *Acheta* Malpighian tubules in vitro. The functions of the remaining peaks are unknown. Dashed line indicates the slope of the solvent gradient, expressed as a percentage of solvent B.

which they were transferred to a desiccator over concentrated sulfuric acid [0% relative humidity (RH)] for another 24 hours (dehydrated). The third group was treated identically to the dehydrates, but at the end of the dehydration period the insects were allowed free access to food and water for 1 to 2 hours (rehydrated). Samples of hemolymph were obtained from crickets by established methods (*5*, *9*) and added to 4.0 volumes of absolute methanol. The mixture was centrifuged (10,000*g* for 10 min) to remove insoluble proteins, and the supernatant was evaporated to dryness. Hemolymph extracts were resuspended in either distilled water or insect saline.

Malpighian-tubule secretion rates are determined by collecting the urine from all the tubules by means of a catheter immersed in oil and by measuring the diameter of the collected droplets (*8*). Control preparations initially secrete approximately 4 μl of urine per hour. The effects of hemolymph on the secretion rate of Malpighian tubules in vitro are shown in Fig. 1. Hemolymph prepared from control insects consistently increased tubule secretion rates by 13 to 36% (24 ± 3%, mean ± SE). This increase corresponded to a stimulus of 0.01 to 0.02 CC equivalents (*10*) and indicated that crickets with free access to water generally maintain low levels of circulating DH. These results are consistent with our observation that freshly isolated tubules initially produced the slightly hypo-osmotic urine characteristic of CC-stimulated diuresis (*8*). When crickets were deprived of water for 24 hours, the amount of hemolymph that could be collected dropped from 20 to 30 μl (control) to 4 or 5 μl. Even when diluted in 1.5 ml of bathing saline, this small volume of hemolymph produced a strongly antidiuretic effect, decreasing fluid secretion by nearly 30% [$t(19)=11.56$, two-tailed; $P < 0.001$]. Although rehydrated crickets had more DH in the hemolymph than did controls, the rate increase was statistically nonsignificant. Higher DH levels in rehydrated insects would be consistent with an overshoot release following rehydration.

The use of crude tissue homogenates was not informative in the identification of the source of the antidiuretic factor inasmuch as all of the tissue homogenates tested (CC, pars intercerebralis, subesophageal ganglion, and terminal abdominal ganglion) produced only a diuretic effect (*8*). An ultrastructural examination of the CC, however, revealed major differences between control and dehydrated crickets. Electron micrographs of the CC (Fig. 2) showed that a specific group of neurosecretory axons, located in the posterior mid-ventral region, had released their neurosecretory material in water-stressed, but not control, crickets. In a separate experiment using control and dehydrated crickets, the CC were fixed for transmission electron microscopy (TEM), and the hemolymph was collected from the same insects. In all cases ($n = 4$, control; $n = 5$, dehydrated) ADH was present in the hemolymph only if these particular CC axons were empty of neurosecretory granules. The effects of homogenates and extracts of CC were always stimulatory (that is, diuretic) (*8*) even though these organs contained the ADH. By implication, either much less ADH than DH is present in the CC, or the DHs are considerably more potent than the ADH.

To confirm that the CC were indeed the source of the ADH, methanolic extracts of CC were fractionated by high-performance liquid chromatography (HPLC), and the fractions were tested for ADH activity on Malpighian tubules. Separations were performed by established techniques for the isolation of adipokinetic hormones (AKHs)

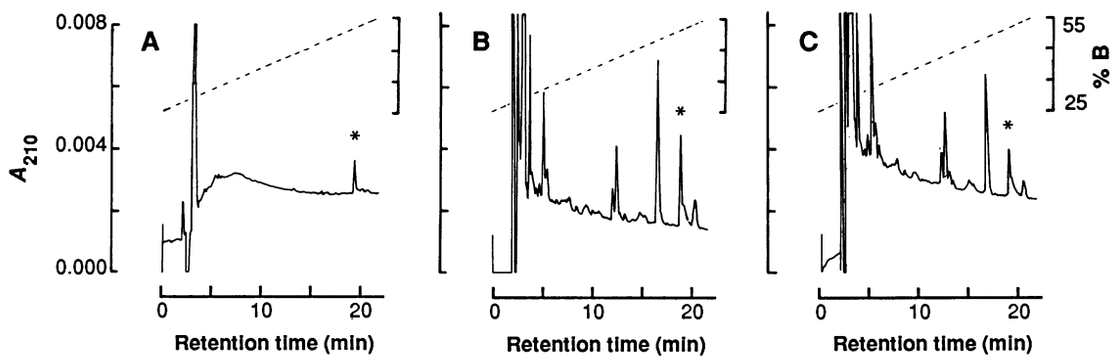

Fig. 4. Chromatograms of CC extracts from individual crickets. ADH peaks are indicated by asterisks. (**A**) One CC equivalent of ADH (Fig. 3) rechromatographed on the new gradient (*13*). (**B**) The CC extract from a control cricket. (**C**) The CC extract from a 48-hour dehydrated cricket. Dashed lines represent solvent gradient, as in Fig. 3.

(*11*). Typical chromatograms showed seven major peaks (Fig. 3). On the basis of a limited number of bioassays ($n = 3$), we have tentatively assigned DH activity to two different peaks, whereas the ADH activity is associated exclusively with one peak ($n = 8$). The purified ADH is highly potent, with 1.0 CC equivalent inhibiting secretion by 70 ± 8% (mean ± SE). With the standard gradient (*11*), the ADH peak elutes 6.4 min before the AKH peak (*12*). To obtain sufficient material to bioassay required that pooled samples of ten or more CC be chromatographed. In general, however, the use of pooled samples masks individual variability to the extent that differences between control and experimental treatments are often concealed (*9*). This difficulty was addressed by modifying the HPLC method to optimize ADH separation and by increasing the sensitivity to the point where the CC of individual crickets could be successfully chromatographed (*13*). Crickets were first treated as described for the preparation of hemolymph extracts with the exception that the desiccation time was increased to 48 hours to maximize the effect. The use of individual CC precluded accurate bioassays; therefore a sample of the ADH peak recovered by the standard HPLC method was reinjected into the column to serve as a marker (Fig. 4A). Individual CC from control (Fig. 4B) and dehydrated (Fig. 4C) crickets were then chromatographed over the same gradient. The size of the ADH peak in the CC of dehydrated crickets was significantly smaller than in controls, whereas, overall, the other peaks were not changed (Fig. 4, B and C). Integration of each ADH peak showed that the area (*14*) decreased from 68.7 ± 9.4 (control, $n = 6$, mean ± SE) to 39.5 ± 5.3 (dehydrated, $n = 6$), a 42% decrease [$t(10) = 2.693$, one-tailed; $P < 0.05$].

We have developed an in vitro preparation of the perfused rectum of *Acheta* that is identical in all important respects to that used for the Malpighian tubules (*15*). Using this preparation as a bioassay, we showed that crude CC homogenate increased fluid absorption by the rectum sixfold, that is, it acted as an ADH (*16*). The HPLC-purified ADH, which inhibits Malpighian tubule secretion, has no effect on reabsorption in this preparation ($n = 8$) and therefore is not the same as the CC factor that increases fluid absorption by the rectum (*16*).

These experiments show the presence in insects of an ADH that directly inhibits Malpighian tubule secretion in vitro without altering rectal reabsorption. In the cricket *Acheta domesticus*, ADH is released from specific neurosecretory axon endings in the CC in response to water stress and is transported from the CC to the tubules by way of the hemolymph. The existing model for the control of Malpighian tubule function (*2*) may not be universal among phytophagous insects, since we have demonstrated that in addition to the controls described by the model, *Acheta* possesses a neurohormone capable of directly inhibiting tubule secretion.

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10. CC equivalent dosages are based on the data from the dose-response curve to crude CC homogenates in (*8*).
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12. The AKH peak was identified by the hyperlipemic response this fraction produced in *Locusta migratoria*, as described in (*11*).
13. The extracts and column were identical to those described in (*11*). Solvent A (water) and solvent B (60% acetonitrile in water) were optically balanced against each other with TFA. Approximate concentrations of TFA were 0.427% (solvent A) and 0.350% (solvent B). The solvents were applied as a linear gradient (25 to 55% B in 20 min; 1.0 ml/min of flow rate) and the eluant monitored at 210 nm.
14. Peak area is expressed in arbitrary units.
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Coding of Two Sphingolipid Activator Proteins (SAP-1 and SAP-2) by Same Genetic Locus

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Several complementary DNAs (cDNAs) coding for sphingolipid activator protein–2 (SAP-2) were isolated from a λgt-11 human hepatoma library by means of polyclonal antibodies. The nucleotide sequence of the largest cDNA was colinear with the derived amino acid sequence of SAP-2 and with the nucleotide sequence of the cDNA coding for the 70-kilodalton precursor of SAP-1 (SAP precursor cDNA). The coding sequence for mature SAP-2 was located 3′ to that coding for SAP-1 in the SAP precursor cDNA. Both SAP-1 and SAP-2 appeared to be derived by proteolytic processing from a common precursor that is coded by a genetic locus on human chromosome 10. Two other domains similar to SAP-1 and SAP-2 were also identified in SAP precursor protein. Each of the four domains was approximately 80 amino acid residues long, had nearly identical placement of cysteine residues, potential glycosylation sites, and proline residues. Each domain also contained internal amino acid sequences capable of forming amphipathic helices separated by helix breakers to give a cylindrical hydrophobic domain that is probably stabilized by disulfide bridges. Protein immunoblotting experiments indicated that SAP precursor protein (70 kilodaltons) as well as immunoreactive SAP-like proteins of intermediate sizes (65, 50, and 31 kilodaltons) are present in most human tissues.

SPHINGOLIPID ACTIVATOR PROTEINS (SAPs) are small (8 to 13 kD), heat-stable proteins required for the hydrolysis of sphingolipids by specific lysosomal hydrolases (*1*). To date, three different SAPs have been identified. SAP-1 activates the hydrolysis of cerebroside sulfate, GM1 ganglioside, and globotriaosylceramide by arylsulfatase A (E.C. 3.1.6.1), acid β-galactosidase (E.C. 3.2.1.23), and α-galactosidase (E.C. 3.2.1.22), respectively (*2*). Human deficiency of SAP-1 results in tissue