It has been suggested that the external shell growth lines of *Nautilus* are laid down daily (and these lines have been used as a basis for arguments about lunar orbital evolution) (*5*). However, the growth lines are difficult to count because of the wide variation in relief and spacing. The number of growth lines on the dated increments of *N. belauensis* ranges from 1 to 2.7 per millimeter of shell across the venter. In time, these represent from 4 to 25.4 days per growth line (mean, 10.6 days); the four youngest animals (at release) show less variation—4 to 8.2 days per growth line (mean, 6.8 days). These values clearly contradict summary claims of daily growth lines in *Nautilus* (*5*).

These records show that in its natural habitat *Nautilus* grows more slowly than had been thought. Estimates derived from *Nautilus* growth rates in aquariums are higher than those reported here; daily rates of 0.15 mm (*2*), 0.25 mm (*2*), and 0.15 to 0.25 mm (*9*) were reported for *N. macromphalus* and *N. pompilius*, whereas the highest growth rate observed for a marked specimen of *N. belauensis* was 0.12 mm per day. The data from captive animals may be high because the animals had regular and often unlimited food and were all younger than the animals that were marked and released. Thus, the highest rate (0.12 mm per day or 43.8 mm per year) from a submature animal is used as an average approximation and gives age estimates ranging from 14.5 to 17.2 years for the seven specimens of *N. belauensis* in Table 1.

Nautilus exhibits determinate growth, reaching a mature stage after which there is no more growth. There is evidence of considerable longevity beyond maturity: one animal, caught 4 years after it had been logged as mature and released, had not grown during the intervening period. It is likely that *Nautilus* lives 5 to 10 years after reaching maturity, and its life-span may be greater than 20 years. This is in striking contrast to the relatively short life-span of most squids and octopods and provides another line of evidence of the great contrast in life strategies between the once successful and often dominant ectocochliate cephalopods and the modern dibranchiates (*10*).

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References and Notes

1. O. W. Holmes, *The Autocrat of the Breakfast Table* (Houghton Mifflin, Boston, 1858).
2. A. W. Martin *et al.*, *Neues Jahrb. Geol. Palaeontol. Abh.* **156**, 207 (1978); P. Ward *et al.*, *Paleobiology* **7**, 481 (1981).
3. E. J. Denton and J. B. Gilpin-Brown, *J. Mar. Biol. Assoc. U.K.* **47**, 723 (1966).
4. J. K. Cochran, D. M. Rye, N. H. Landman, *Paleobiology* **7**, 469 (1981).
5. P. G. K. Kahn and S. M. Pompea, *Nature (London)* **275**, 606 (1978).
6. W. B. Saunders and C. Spinosa, *Science* **204**, 1199 (1979).
7. In Palau, *N. belauensis* occurs on forereef slopes, at depths of 90 to 500 m. Of the 2387 animals trapped, processed, and released between 1977 and 1982, 300 were released in 1977, 424 in 1978, 233 in 1979, 452 in 1981, and 978 in 1982. All had first been weighed, measured, and sexed, and their relative maturity recorded. Each shell was numbered with an adhesive label or engraved number near the umbilicus. After processing, they were released at depths of 15 to 50 m on the reef face above the trap site [W. B. Saunders, *Veliger* **24**, 1 (1981); _____ and C. Spinosa, *Paleobiology* **4**, 349 (1978)].
8. W. H. Everhart and W. D. Youngs, *Principles of Fishery Science* (Cornell Univ. Press, Ithaca, N.Y., 1981); G. G. Simpson, A. Roe, R. C. Lewontin, *Quantitative Zoology* (Harcourt, Brace & World, New York, 1960).
9. P. D. Ward and J. Chamberlain, *Nature (London)* **304**, 57 (1983).
10. M. J. Wells, *New Sci.* **100**, 332 (1983).
11. This research was based at the Micronesian Mariculture Demonstration Center, Koror, Palau, West Caroline Islands, and was under the auspices of the Department of Marine Resources, with support of T. Paulis, N. Idechong, and F. Perron. I thank C. Spinosa, L. Davis, G. Heslinga, G. Monaco, J. Chamberlain, A. Swan, and particularly M. Weekley for technical and field assistance. Supported by NSF grant EAR 81-00629 and by the National Geographic Society.

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Inhibition of Aldosterone Production by an Atrial Extract

Abstract. *Crude extracts of rat atria reduced the basal amount of aldosterone released from rat zona glomerulosa cells and partially inhibited aldosterone stimulation by adrenocorticotropic hormone and angiotensin II. The destruction of this activity by trypsin suggests that the active factor is a peptide, possibly atrial natriuretic factor. These data suggest that atrial natriuretic factor affects sodium excretion by the kidneys both directly and through the inhibition of aldosterone production.*

Peptides contained in mammalian atria are reported to affect kidney function (*1–6*) and smooth muscle relaxation (*7, 8*). Atrial extracts injected into rats induce a prompt natriuresis and diuresis (*1–6*). The site of action of the atrial extracts in the kidney is primarily the medullary collecting duct (*2*). Mammalian atrial cardiocytes contain specific granules resembling those found in peptide-secreting cells of endocrine organs (*9*). These granules are probably storage sites for the atrial natriuretic factor (*10, 11*). In the rat, the number of granules changes when water and electrolyte balance are altered (*12, 13*). Adrenalectomy causes a slight increase in atrial granularity, and administration of 1 percent sodium chloride to adrenalectomized rats decreases atrial granularity (*12*). The atrial natriuretic factor is heat-stable and trypsin-sensitive (*3, 7, 13*). Recent characterization of this factor indicates that there are two low-molecular-weight peptides, atriopeptin I and atriopeptin II, containing 21 and 23 amino acids residues, respectively. Both of these peptides have potent natriuretic, diuretic, and vasorelaxant activities, and both appear to derive from a common precursor of molecular weight 20,000 to 30,000 (*8*).

Earlier studies on the control of aldosterone secretion suggested that an inhibiting system regulates secretion. In 1958, Mills *et al.* (*14*) reported increased aldosterone secretion in the dog during constriction of the inferior vena cava and decreased secretion when the constriction was released; sectioning the cervical vagi did not affect the increase in secretion but prevented the decrease. In 1959, Anderson *et al.* (*15*) showed that stretching of the right atria inhibited aldoster-

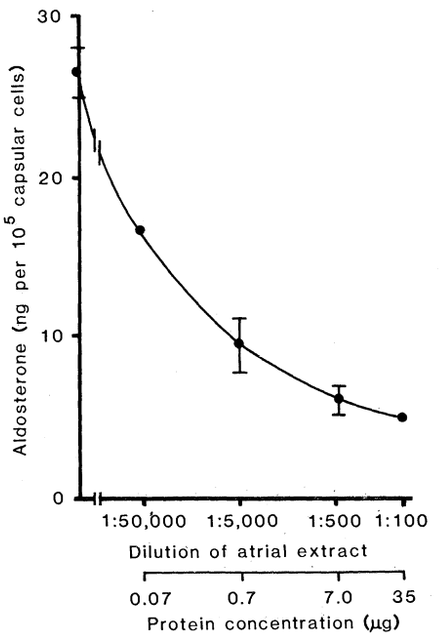

Fig. 1. Inhibition of secretion of aldosterone from unstimulated capsular cells by an atrial extract. The point without atrial extract and the points of 1:5000 and 1:500 dilution of the atrial extract each represent the mean ± standard error of five experiments. The other two points show the mean of duplicate incubations in one experiment. One milliliter of incubation Medium 199 contained a final dilution of atrial extract as indicated on the abscissa. Immediately beneath the dilution is shown the amount of atrial extract protein that was added to 1 ml of incubation media for a given dilution.

one secretion in the dog, and in 1964, Gann and Travis (*16*) presented evidence for an inhibitor of aldosterone secretion. We therefore investigated whether atrial extracts have an effect on aldosterone and corticosterone production in rat adrenal collagenase–dispersed capsular (zona glomerulosa) and decapsular cells (fasciculata medullary cells). We also examined the effects of atrial extracts on the dose-response curves for aldosterone in the presence of adrenocorticotropic hormone (ACTH) and angiotensin II. We found that the atrial extract inhibited basal aldosterone production in a dose-dependent fashion and reduced the sensitivity of the glomerulosa cells to ACTH and angiotensin II.

Female Sprague-Dawley rats (200 to 250 g) maintained on a regular sodium diet were used in each experiment. Collagenase-dispersed capsular and decapsular cells were harvested as described earlier (*17*). After the capsular and decapsular cells were harvested, the cell suspensions (average cell count, 60,000 per tube) were incubated in 1 ml of Medium 199 (Gibco) (K^+, 5.4 meq/liter) containing bovine serum albumin (Sigma) (2 mg/ml) with various amounts of ACTH (*1–24*) (Sigma) (10^{-11} to $10^{-8}M$), angiotensin II (Sigma) (10^{-11} to $10^{-8}M$), and atrial or ventricular extract (diluted 1:50,000 to 1:100) for 2 hours at 37°C in an atmosphere of 95 percent O_2 and 5 percent CO_2. Atrial and ventricular tissues of rat heart were homogenized separately with a Polytron (Brinkmann) for 10 to 15 seconds in 10 m*M* phosphate-buffered saline (PBS, *p*H 7.4) (1:5, weight to volume), placed in a boiling water bath for 10 minutes, and centrifuged at 25,000*g* at 4°C for 30 minutes. The supernatants were removed and stored at −20°C (*3*). The protein concentration of these extracts was 3.5 mg/ml measured by the Lowry method (*18*). Injection of 1 mg of the atrial extract into control rats increased sodium excretion by a factor of 14, and one-half of this dose increased sodium excretion by a factor of 7. The ventricular extract had little or no natriuretic activity. The atrial extract also relaxed K^+-contracted aortic smooth muscle in vitro.

Aldosterone and corticosterone were measured directly by radioimmunoassay (*17, 19*). To rule out the possibility of interference in the radioimmunoassay of aldosterone, we showed that there was no alteration of the standard curve for aldosterone when the atrial extracts were added. To investigate a possible toxic effect on the glomerulosa cells, we performed a trypan blue exclusion experiment before and after incubation with the atrial extract; no significant difference in cell viability was found. The ventricular extract had no effect on aldosterone release from the capsular cells, whereas the atrial extract decreased it in a dose-dependent manner (Fig. 1). A 1:500 dilution of the atrial extract, containing 7 μg of protein, lowered basal aldosterone release by 76 percent (Fig. 1; basal value, 25.6 ± 1.6 ng per 10^5 capsular cells; at 1:500 dilution of the atrial extract the value was 6.1 ± 0.9; $P < 0.001$). A 1:50,000 dilution caused a 37 percent inhibition of basal aldosterone release (Fig. 1). In addition, the atrial extract (1:500) reduced the sensitivity of the glomerulosa cells to ACTH and shifted the dose-response curve to the right (Fig. 2A). However, the maximum response ($10^{-8}M$) was nearly the same with or without atrial extract. The inhibition of the action of angiotensin II was even more striking (Fig. 2B). The maximum response in the presence of the atrial extract was still considerably less than the control response, even at $10^{-8}M$ of angiotensin II. The atrial and ventricular extracts had little or no effect on corticosterone release from the decapsular cells. To examine the effect of trypsin on the aldosterone-inhibiting effect of the atrial extract, we incubated 1 ml of the atrial extract with 1 ml of trypsin solution (Sigma) (12 μg of trypsin per milliliter of PBS) at 37°C for 2 hours. The treatment was stopped by incubating the mixture for 20 minutes with 20 μg of lima bean trypsin inhibitor (Sigma). Trypsin treatment of the atrial extract almost totally abolished the effect on aldosterone release both in the basal state and in response to ACTH.

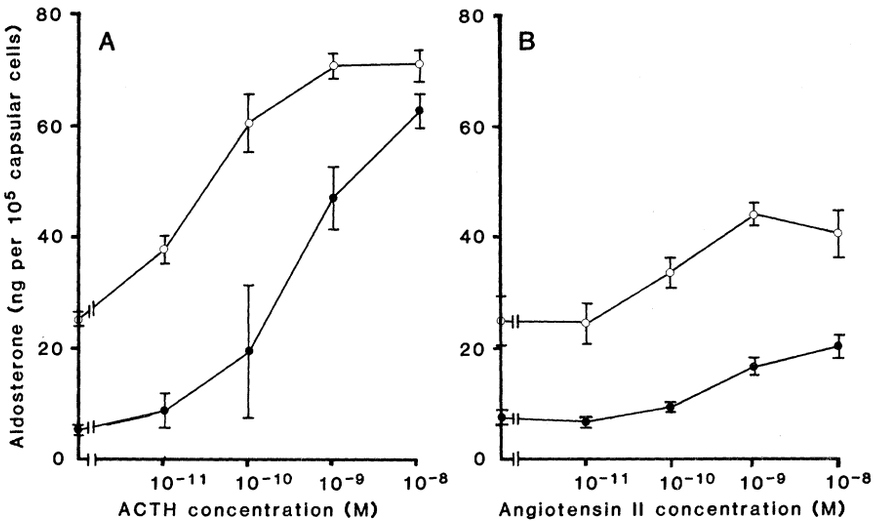

Fig. 2. (A) Aldosterone production by ACTH (●) with atrial extract (1:500 dilution) and (○) without atrial extract. Each point represents the mean ± standard error of five separate experiments. In each experiment ACTH concentrations are expressed as moles per liter of incubation medium. (B) Aldosterone production by angiotensin II (AII) (●) with atrial extract (1:500 dilution) and (○) without atrial extract. Each point represents the mean ± standard error of three experiments. Angiotensin II concentrations are expressed as moles per liter of incubation medium.

Other investigators have shown that a natriuretic factor is contained in atrial extracts but not in the ventricular extracts (*1–4*). Both diuresis and natriuresis induced by atrial extracts are heat-stable and trypsin-sensitive (*3, 7, 13*). In our study, the ventricular extract had no effect on aldosterone release, but the atrial extract decreased it in a dose-dependent manner. The aldosterone-inhibiting effect of atrial extract was heat-stable and sensitive to proteolytic digestion. Furthermore, our atrial extract contained diuretic and natriuretic activity and relaxed vascular smooth muscle. Therefore, our data suggest that the active substance inhibiting aldosterone production is a peptide, as is atrial natriuretic factor. The aldosterone-inhibiting effect of atrial natriuretic factor is similar to that observed when the calcium concentration of the medium is reduced or when calcium channel blockers are used (*20*). Such an action could also be explored for the atrial extract. In conclusion, extracts of rat atria have an inhibiting action on aldosterone production in vitro. Whether this factor is a physiological inhibitor of aldosterone secretion remains to be determined.

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References and Notes

1. A. J. deBold, H. B. Borenstein, A. T. Veress, H. Sonnenberg, *Life Sci.* **28**, 89 (1981).
2. H. Sonnenberg, W. A. Cupples, A. J. deBold, A. T. Veress, *Can. J. Physiol. Pharmacol.* **60**, 1149 (1982).
3. N. C. Trippodo, A. A. MacPhee, F. E. Cole, H. L. Blakesley, *Proc. Soc. Exp. Biol. Med.* **170**, 502 (1982).
4. R. Keeler, *Can. J. Physiol. Pharmacol.* **60**, 1078 (1982).
5. A. J. deBold, *Proc. Soc. Exp. Biol. Med.* **170**, 133 (1982).
6. N. C. Trippodo, A. A. MacPhee, F. E. Cole, *Hypertension* **5** (Suppl. I), I-88 (1983).
7. M. G. Currie *et al.*, *Science* **221**, 71 (1983).
8. _____, *ibid.* **223**, 67 (1984).
9. A. J. deBold, *J. Mol. Cell Cardiol.* **10**, 717 (1978).
10. _____, *Can. J. Physiol. Pharmacol.* **60**, 324 (1982).
11. R. Garcia *et al.*, *Experientia* **38**, 1071 (1982).
12. A. J. deBold, *Proc. Soc. Exp. Biol. Med.* **161**, 508 (1979).
13. G. Thibault, R. Garcia, M. Cantin, J. Genest, *Hypertension* **5** (Suppl. I), I-75 (1983).
14. I. H. Mills, A. Casper, F. C. Bartter, *Science* **128**, 1140 (1958).
15. C. H. Anderson, M. McCally, G. L. Farrel, *Endocrinology* **64**, 202 (1959).
16. D. S. Gann and R. H. Travis, *Am. J. Physiol.* **207**, 1095 (1964).
17. H. Matsuoka, S. Y. Tan, P. J. Mulrow, *Prostaglandins* **19**, 291 (1980).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
19. D. M. Mayes, *Endocrine Sciences Laboratory Procedure Manual* (Tarzana, Calif., 1983).
20. J. L. Fakunding and K. Catt, *Endocrinology* **107**, 1345 (1980).

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Reversal of Adriamycin Resistance by Verapamil in Human Ovarian Cancer

Abstract. *The effectiveness of adriamycin in the treatment of ovarian cancer and other human tumors has been limited by the development of drug resistance. Verapamil, a calcium channel blocking agent, completely reversed adriamycin resistance in human ovarian cancer cells with moderate (three- to sixfold) degrees of resistance and partially reversed resistance in highly (150-fold) resistant cells. The potentiating effect of verapamil was due to inhibition of adriamycin efflux in the resistant cells. These results have led to a clinical trial of adriamycin and verapamil in refractory ovarian cancer patients.*

Adriamycin is a widely used anticancer agent whose effectiveness has been limited, in part, by the frequent development of drug resistance (*1*). The impact of drug resistance on the clinical usefulness of adriamycin is particularly evident in ovarian cancer, where adriamycin has a 40 percent response rate in previously untreated patients (*2*). The median duration of response is 3 months, and when the drug is administered in combination with alkylating agents the response duration is increased to only 7 to 9 months (*3*). Neither the nature of the intrinsic resistance of the patients who do not respond to treatment with adriamycin nor the acquired resistance that develops rapidly in patients who initially respond to the drug has been well characterized. Studies performed with P388 leukemia cells in vitro have shown that adriamycin-resistant cells have a decreased intracellular accumulation of adriamycin primarily because of an enhanced active efflux (*4, 5*). Verapamil, a calcium channel blocker, produces a seven- to tenfold enhancement of adriamycin cytotoxicity in such cells (*6*) and a twofold enhancement in human hematopoietic tumor cell lines (*7*). In studies of mice bearing daunomycin-resistant Ehrlich ascites tumor, the combination of verapamil plus daunomycin produced a doubling of survival rate compared to treatment with daunomycin alone (*8*). These observations, together with the availability of human tumor cell lines with varying degrees of resistance to adriamycin (*9, 10*), led us to examine the effect of verapamil on adriamycin resistance in ovarian cancer.

The ovarian cancer cell lines used in this study included (i) two cell lines sensitive in vitro to adriamycin [A1847 and A2780 (*11*)] and their adriamycin-resistant variants (1847^{AD} and 2780^{AD}) produced in vitro; (ii) cell lines NIH:OVCAR-2, -3, and -4 derived from ovarian cancer patients resistant to combination chemotherapy with and without adriamycin; and (iii) a fibroblast cell line established from the ovarian cancer patient from whom OVCAR-4 was established.

The concentration of verapamil used in cytotoxicity studies in the ovarian cancer cell lines encompassed the clinically achievable peak concentrations (250 to 1000 ng/ml) seen in patients who receive verapamil (Table 1) (*12, 13*). Verapamil completely reversed adriamycin resistance (Fig. 1A) and cross-resistance to vinblastine in 1847^{AD}, a line six times more resistant to adriamycin than its parent line. A similar dose-modifying factor (DMF) of verapamil on adriamycin cytotoxicity was observed with 2780^{AD}, a cell line 150 times more resistant to adriamycin than A2780. The DMF ranged from 3 to 12 as the verapamil dose was raised from 300 to 1000 ng/ml. However, in contrast to its effect on 1847^{AD}, verapamil did not restore sensitivity to adriamycin in 2780^{AD} to the corresponding sensitivity of the parent cell line.

Potentiation of adriamycin cytotoxicity by verapamil in the human ovarian cancer cell lines A2780 and A1847 established from previously untreated patients was also observed (Table 1). However, the degree of potentiation in these cell lines (DMF's of 1.5 and 3) was less than that observed in cell lines with resistance induced in vitro. Potentiation was greater in A1847 compared to A2780, even though A1847 is endogenously more resistant to adriamycin than is A2780. The DMF of verapamil was also smaller in the OVCAR cell lines (1.5 to 4) compared to that in the lines with resistance acquired in vitro. The optimal DMF was achieved in the clonogenic assay when the cells were exposed to verapamil for 24 hours.

In the absence of verapamil, the net

Table 1. Cytotoxicity of adriamycin alone and in combination with verapamil in human ovarian cancer cell lines.

Cell line	Resistance produced		ICR_{50} adriamycin‡ (uM)	ICR_{50} adriamycin + verapamil (DMF)§
	In vivo*	In vitro†		
A1847			0.044	0.013 (3.3)
A2780			0.015	0.01 (1.5)
1847^{AD}		AD	0.18	0.03 (6)
2780^{AD}		AD	2.5	0.8 (3 to 6)
NIH:OVCAR-2	CP, AA		0.088	0.037 (2.4)‖
NIH:OVCAR-3	AA, AD, CP		0.03	0.02 (1.5)‖
NIH:OVCAR-4	AA, AD, CP		0.13	0.03 (4.3)‖

*Indicates cell line established from patient clinically refractory to indicated drugs: AA, alkylating agent; AD, adriamycin; CP, cisplatin. †Resistance was induced in vitro by stepwise incubation with adriamycin. ‡ICR_{50} refers to the drug concentration that results in a 50 percent reduction in colony formation (cell lines 2780, 2780^{AD}, 1847, 1847^{AD}, OVCAR −3 and −4) or a 50 percent reduction in cell count (OVCAR-2) after a 24-hour exposure to adriamycin plus verapamil. Colony formation was assessed 14 days after plating in agar, and cell count was assessed 7 days after exposure to drugs. §DMF (dose-modifying factor) represents the ratio of ICR_{50} (adriamycin) to ICR_{50} (adriamycin plus verapamil). Verapamil concentration was 250 to 500 ng/ml. ‖Maximum DMF achieved with verapamil at 3 μg/ml concentration.