5. A. E. Szulman, *N. Engl. J. Med.* **286**, 1028 (1973).
6. W. P. Faulk, A. Temple, R. E. Lovins, N. Smith, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1974 (1978).
7. J. A. McIntyre and W. P. Faulk, *J. Exp. Med.* **149**, 824 (1979).
8. W. P. Faulk, J. A. McIntyre, B. L. Hsi, in *Transplantation and Clinical Immunology*, J. P. Revillard *et al.*, Eds. (Exerpta Medica, Amsterdam, 1980), pp. 143–150.
9. J. A. McIntyre and W. P. Faulk, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4029 (1979).
10. ———, *Hum. Immunol.* **4**, 27 (1982).
11. J. O. Kim and C. W. Mueller, *Introduction to Factor Analysis* (Sage, Beverly Hills, Calif., 1978).
12. H. H. Harmon, *Modern Factor Analysis* (Univ. of Chicago Press, Chicago, ed. 3, 1976).
13. Statistical Analysis System Institute, Inc., *SAS User's Guide* (SAS Institute, Cary, N.C., 1979).
14. W. P. Faulk and J. A. McIntyre, *Transplantation* **32**, 1 (1981).
15. J. A. McIntyre and W. P. Faulk, *Am. J. Reprod. Immunol.*, in press.
16. L. Komlos, R. Zamir, H. Joshua, I. Halbrecht, *Clin. Immunol. Immunopathol.* **7**, 330 (1977).
17. A. E. Beer *et al.*, *Am. J. Obstet. Gynecol.* **141**, 987 (1981).
18. B. Schacter *et al.*, *Lancet* **1979-I**, 796 (1979).
19. J. A. McIntyre and W. P. Faulk, in *Immunological Aspects of Placenta Proteins*, A. Klopper, Ed. (Holt-Sanders, Eastbourne, United Kingdom, 1982), pp. 1–11.
20. C. Taylor and W. P. Faulk, *Lancet* **1981-II**, 68 (1981).
21. J. A. McIntyre and W. P. Faulk, *Fertil. Steril.*, in press.
22. N. C. Smith, M. G. Brush, S. Luckett, *Nature (London)* **252**, 302 (1974).
23. Supported in part by the March of Dimes Birth Defects Foundation, the Human Embryology and Pregnancy Foundation, and the Luzern Medical Research Institute.

15 June 1983; accepted 30 August 1983

Dynorphin-A-(1–8) Is Contained Within Vasopressin Neurosecretory Vesicles in Rat Pituitary

Abstract. *Dynorphin-A-(1–8), an opioid peptide widely distributed in the rat central nervous system, is present in vasopressin-containing neurosecretory cells terminating in the neural lobe of the pituitary. Electron microscopic immunocytochemistry reveals that dynorphin-A-(1–8) is contained within the same neurosecretory vesicles as vasopressin and vasopressin-associated neurophysin in the neural lobe of the rat. The results indicate that dynorphin may be released in the pituitary concomitantly with vasopressin during the antidiuretic response.*

Dynorphin A was originally isolated from porcine pituitary and gut as an extremely potent 17-amino-acid opioid peptide with leucine-enkephalin ([Leu]-enkephalin) at its amino terminus (*1*). It is widely distributed in rat brain (*2*), and has been shown to be present within vasopressin-containing magnocellular neurons (*3*). The amino-terminal octapeptide dynorphin-A-(1–8) is the predominant form of dynorphin in rat brain (*4*). The messenger RNA sequence for the prodynorphin precursor has been determined by cloning and sequence analysis of complementary DNA (*5*). The precursor contains the sequence for a previously isolated 32-amino-acid peptide consisting of dynorphin A at the amino terminus and a related tridecapeptide (dynorphin B) at the carboxyl terminus (*6*). The precursor also contains the sequence for α-neo-endorphin, which explains the identical distributions by immunocytochemistry of dynorphin and α-neo-endorphin (*2*), and the approximately equimolar concentrations of dynorphin-A-(1–8) with α-neo-endorphin in all regions of rat brain (*4*). Immunoreactive dynorphin A and α-neo-endorphin are released in approximately equal amounts in a Ca^{2+}-dependent manner from isolated neurointermediate lobes of the pituitaries of developing postnatal rats (*7*). In view of the possible influence of opioid peptides on the release of vasopressin and oxytocin in the neurohypophysis (*8*), it is important to characterize the synthesis, processing, packaging, and release of dynorphin and related peptides in magnocellular neurosecretory cells. Dynorphin-like immunoreactivity in rat neurointermediate lobe homogenates migrates in sucrose density gradients at the same rate as vasopressin (*9*), which suggests that dynorphin may be located in vasopressin neurosecretory vesicles. [Met]Enkephalin or [Leu]enkephalin immunoreactivity (or both) has been located in neurosecretory vesicles in oxytocin or vasopressin axons (or both) in rat (*10*) and cat (*11*) neurohypophysis by electron microscopic (EM) immunocytochemistry. However, [Leu]enkephalin immunoreactivity has also been located by EM immunocytochemistry in fibers making synaptoid contacts with neurohypophyseal glial cells (pituicytes), but no evidence was found for the coexistence of the enkephalins with vasopressin or oxytocin in the same terminals (*12*).

We have been able to locate dynorphin-A-(1–8) on the EM level by using a recently developed postembedding immunostaining method that stains intensely without the use of etching or enzymatic treatment of the sections. In addition, the use of serial ultrathin sections has allowed us to unequivocally compare the intraaxonal distributions of dynorphin-A-(1–8), vasopressin, and oxytocin.

Adult heterozygous Brattleboro rats were perfused through the heart with 4 percent paraformaldehyde, 2 percent glutaraldehyde, 0.2 percent picric acid, and 0.1*M* sodium cacodylate (*p*H 6). The pituitaries were embedded in LR White

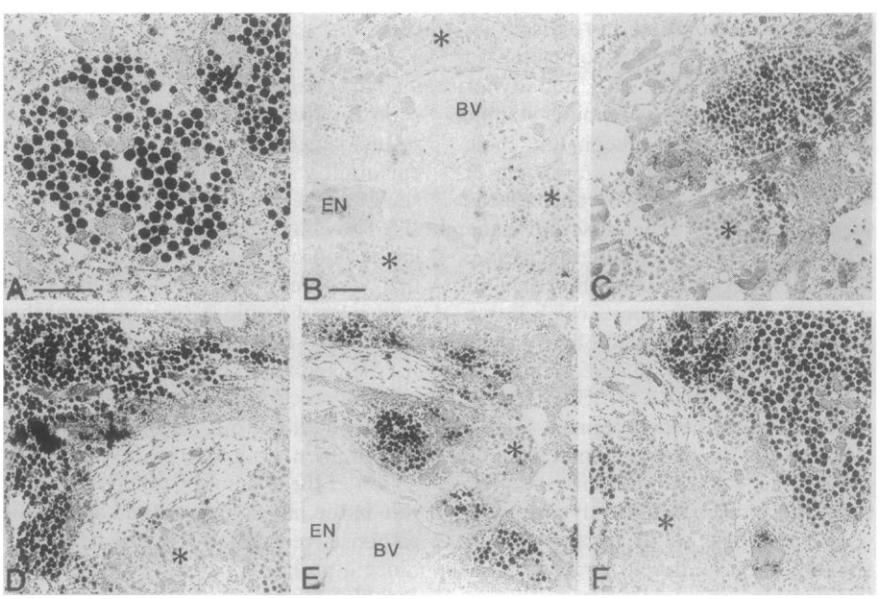

Fig. 1. Immunostaining of neurosecretory vesicles for dynorphin-A-(1–8) in the rat posterior pituitary. (A) Section stained with dynorphin-A-(1–8) antiserum. (B) Stained with dynorphin-A-(1–8) antiserum absorbed with dynorphin-A-(1–8). No staining is present. (C) Dynorphin-A-(1–8) antiserum absorbed with dynorphin-A-(1–13). Staining is partially inhibited. No inhibition of staining results from absorbing the dynorphin-A-(1–8) antiserum with [Leu]enkephalin (D), arginine vasopressin (E), or cholecystokinin (F). Asterisk, unlabeled axon. Abbreviations: *BV*, blood vessel; *PN*, pituicyte (glial) nucleus; *EN*, endothelial cell nucleus. Bars represent 1.0 μm; (C) to (F) are printed at the same magnification as (B).

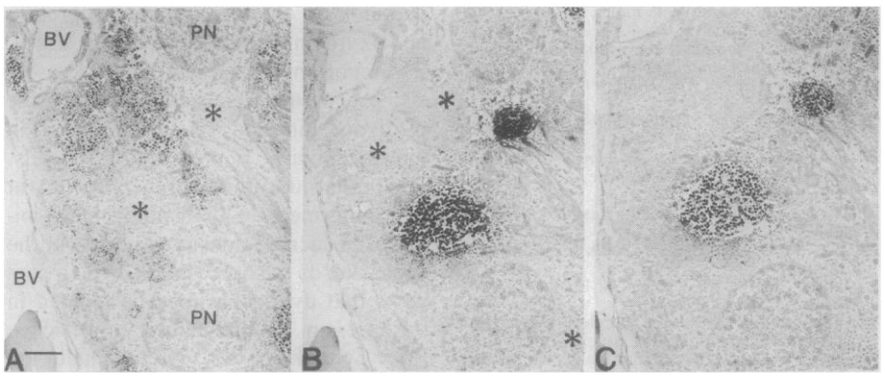

Fig. 2. Serial sections of rat posterior pituitary stained with (A) a monoclonal antibody to oxytocin-associated neurophysin, (B) dynorphin-A-(1–8) antiserum, and (C) a monoclonal antibody to vasopressin-associated neurophysin. Symbols are as in Fig. 1. Bar, 2.0 μm.

resin (Fullam) (*13*), and silver sections were cut and picked up on nickel grids. For dynorphin staining, the grids were placed sequentially on drops of antiserum specific for dynorphin-A-(1–8) (*4*) diluted 1:400 in 10 percent normal goat serum for 20 minutes, biotinylated goat antiserum to rabbit immunoglobulin (Vector, 1:300, 10 minutes), avidin-biotinylated peroxidase complex (Vector, 10 μl/ml, 10 minutes), 0.5 percent diaminobenzidine (DAB) containing 0.01 percent H_2O_2 (2 minutes), and 0.5 percent osmium tetroxide (OsO_4, 30 seconds). All 160-nm neurosecretory vesicles in a subpopulation of axons and axonal endings were intensely stained for dynorphin-A-(1–8) (Figs. 1, A and D to F, and 2B). To ascertain the staining specificity of the dynorphin-A-(1–8) antiserum, solid-phase absorption controls were performed with peptides bound to Affigel-10 beads (Bio-Rad). A 100-μl sample of antiserum (1:400) was incubated overnight at 4°C with 20 μl of beads coupled to 0.05 mg of dynorphin-A-(1–8), dynorphin-A-(1–13), [Leu]enkephalin, arginine vasopressin, or cholecystokinin. Dynorphin-A-(1–8) completely inhibited staining (Fig. 1B), and dynorphin-A-(1–13) partially inhibited staining (Fig. 1C), but [Leu]enkephalin, cholecystokinin, and vasopressin had no effect (Fig. 1, D to F). We conclude that the specificity of the antiserum under these conditions is restricted to parts of the dynorphin molecule other than the [Leu]enkephalin segment, which is consistent with the liquid-phase radioimmunoassay (RIA) characterization reported previously (*4*, *14*). Since dynorphin-A-(1–8) is the predominant form of dynorphin in the pituitary and elsewhere (*4*), the staining seen with this antiserum is probably due mostly to dynorphin-A-(1–8), although some fraction of the staining could be due to larger forms of this peptide.

To ensure that the subpopulation of axons staining for dynorphin consisted only of the vasopressin axons (as a further test of the specificity of staining under these conditions), serial thin sections were cut and sequential sections were stained with a monoclonal antibody to oxytocin-associated neurophysin (NP-OT), dynorphin-A-(1–8) antiserum, and a monoclonal antibody to vasopressin-associated neurophysin (NP-VP). The immunocytochemical specificities of the monoclonal antibodies will be described in detail elsewhere (*15*). For neurophysin staining, grids were placed on drops of hybridoma-conditioned medium diluted in 10 percent normal goat serum (1:1000 for NP-OT, 1:200 for NP-VP, 10 minutes), goat antiserum to mouse immunoglobulin coupled to peroxidase (1:40, Boehringer Mannheim, 10 minutes), and DAB/H_2O_2 and OsO_4 as for dynorphin staining. The two monoclonal antibodies stained 160-nm neurosecretory vesicles in mutually exclusive populations of axons (Fig. 2). Dynorphin-A-(1–8) staining was confined to the neurosecretory vesicles in the axons staining for NP-VP (Fig. 2).

All NP-VP–containing axons examined in serial sections were also found to stain for dynorphin-A-(1–8). Thus, our results suggest that this peptide is present in all vasopressin-containing neurosecretory vesicles in rat neurohypophysis. The colocalization of these peptides in the neurosecretory vesicles indicates that vasopressin and dynorphin-A-(1–8) (and related peptides such as dynorphin A, α-neo-endorphin and possibly [Leu]enkephalin, formed from the dynorphin precursor) are probably released concomitantly from vasopressin terminals in the neurohypophysis. The site of action of these opioid peptides and their role in the physiology of the antidiuretic response remain to be characterized. Since a prohormone-converting enzyme capable of processing another opiate peptide precursor, pro-opiomelanocortin, has been detected in neurosecretory granules from the neural lobe (*16*), it will be important to determine whether the same enzyme is used intragranularly to process both the vasopressin and dynorphin precursor proteins.

MARK H. WHITNALL
HAROLD GAINER
Laboratory of Neurochemistry and Neuroimmunology, National Institute of Child Health and Human Development, Bethesda, Maryland 20205
BRIAN M. COX
CHRISTOPHER J. MOLINEAUX
Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

References and Notes

1. B. M. Cox, K. E. Opheim, H. Teschemacher, A. Goldstein, *Life Sci.* **16**, 1777 (1975); A. Goldstein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6666 (1979); A. Goldstein *et al.*, *ibid.* **78**, 7219 (1981); S. Tachibana, K. Araki, S. Ohya, S. Yoshida, *Nature (London)* **295**, 339 (1982).
2. C. J. Molineaux, G. Feuerstein, A. L. Faden, B. M. Cox, *Neurosci. Lett.* **33**, 179 (1982); S. J. Watson, H. Khachaturian, D. Coy, L. Taylor, H. Akil, *Life Sci.* **31**, 1773 (1982); S. J. Watson, H. Khachaturian, H. Akil, D. H. Coy, A. Goldstein, *Science* **218**, 1134 (1982); E. Weber, K. A. Roth, J. D. Barchas, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3062 (1982); S. J. Watson *et al.*, *ibid.* **80**, 891 (1983); E. Weber and J. D. Barchas, *ibid.*, p. 1125.
3. S. J. Watson *et al.*, *Science* **216**, 85 (1982).
4. E. Weber, C. J. Evans, J. D. Barchas, *Nature (London)* **299**, 77 (1982); R. I. Cone, E. Weber, J. Barchas, A. Goldstein, *J. Neurosci.*, in press.
5. H. Kakidani *et al.*, *Nature (London)* **298**, 245 (1982).
6. W. Fischli, A. Goldstein, M. W. Hunkapillar, L. E. Hood, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5435 (1982).
7. B. R. Seizinger *et al.*, *Life Sci.* **31**, 1757 (1982).
8. J. Rossier *et al.*, *Nature (London)* **277**, 653 (1979); G. Clarke, P. Wood, L. Merrick, D. W. Lincoln, *ibid.* **282**, 746 (1979); L. L. Iversen, S. D. Iversen, F. E. Bloom, *ibid.* **284**, 350 (1980); S. L. Lightman, M. Ninkovic, S. P. Hunt, L. L. Iversen, *ibid.* **305**, 235 (1983); P. Micevych and R. Elde, *J. Comp. Neurol.* **190**, 135 (1980); S. L. Lightman, L. L. Iversen, M. L. Forsling, *J. Neurosci.* **2**, 78 (1982); W. Knepel and D. K. Meyer, *J. Physiol. (London)* **341**, 507 (1983).
9. C. J. Molineaux and B. M. Cox, *Life Sci.* **31**, 1765 (1982).
10. R. Martin and K. H. Voigt, *ibid.*, p. 1727; R. Martin *et al.*, *Neuroscience* **8**, 213 (1983).
11. H. D. Coulter, R. P. Elde, S. L. Unverzagt, *Peptides* **2** (Suppl. 1), 51 (1981).
12. F. W. van Leeuwen, C. W. Pool, A. A. Sluiter, *Neuroscience* **8**, 229 (1983).
13. G. R. Newman, B. Jasani, E. D. Williams, *Histochem. J.* **15**, 543 (1983). LR White is a recently formulated hydrophilic mixture of acrylic monomers that tolerates partial dehydration of tissue (up to only 70 percent ethanol) before embedding. This obviates the need for postfixation in osmium tetroxide, which can impair immunoreactivity. Unlike methacrylates, LR White is not subject to polymerization artifact and is stable in the electron beam without support films. Also LR White sections can be immunostained without the etching procedures that obscure ultrastructure in immunostaining with the traditional hydrophobic epoxides and polyesters.
14. The previously reported cross-reactivity of dynorphin-A-(1–13) for the dynorphin-A-(1–8) antiserum in RIA is only 0.001 percent (*4*). The clear partial inhibition of staining by solid-phase absorption with dynorphin-A-(1–13) demonstrates the inadequacy of using liquid-phase absorption controls to characterize antibody specificities in solid-phase assays such as immunocytochemistry.
15. These monoclonal antibodies to NP-VP and NP-OT specifically stain neurosecretory vesicles in vasopressin and oxytocin axonal endings, respectively, and the endings of the neural lobe of

the Brattleboro rat, which does not contain vasopressin or NP-VP, stains only with the NP-OT antibody [M. H. Whitnall, J. Ben-Barak, K. Ozato, S. Key, H. Gainer, in preparation].
16. T. L. Chang, H. Gainer, J. T. Russell, Y. P. Loh, *Endocrinology* **11**, 1607 (1982); Y. P. Loh and H. Gainer, in *Brain Peptides*, D. Krieger, J. B. Martin, M. J. Brownstein, Eds. (Wiley, New York, 1983), in press.
17. We thank E. Weber for donating the dynorphin-A-(1–8) antiserum and for providing a copy of a manuscript in press.

7 October 1983; accepted 17 October 1983

Dietary Chloride as a Determinant of "Sodium-Dependent" Hypertension

Abstract. *The uninephrectomized rat given desoxycorticosterone (DOC) provides a classic model of "sodium-dependent" hypertension. In such rats, the extent to which a given dietary intake of sodium induced an increase in blood pressure depended on whether or not the anionic component of the sdoium salt was chloride. With normal and high dietary intakes of sodium, sodium chloride induced increases in blood pressure much greater than that induced by approximately equimolar amounts of sodium bicarbonate, sodium ascorbate, or a combination of sodium bicarbonate and sodium ascorbate. A normal amount of dietary sodium chloride induced hypertension, whereas an equimolar amount of sodium bicarbonate did not increase blood pressure. This difference could not be attributed to differences in sodium or potassium balances, weight gain, or caloric intake. The DOC model of "sodium-dependent" hypertension might better be considered sodium chloride–dependent.*

In many clinical settings, restriction of dietary sodium chloride (NaCl) can mitigate, and its supplementation can exacerbate, hypertension, an abnormally increased blood pressure (*1*). Although Ambard and Beaujard suggested in 1904 that increased retention of chloride might be important in the pathogenesis of hypertension (*2*), Dahl, in 1954, stated the now prevalent view that "the sodium ion alone is important" [in the pathogenesis of hypertension] (*3*). This view, however, is based almost entirely on studies in which dietary sodium has been varied mainly by varying the dietary intake of NaCl (*4*). It is now recognized that the chloride ion (*5*), like the bicarbonate ion (*6, 7*), can be specifically transported by a variety of tissues and exert its own biological effects. The pathogenesis of NaCl-dependent hypertension might therefore depend on the dietary intake of chloride. We now report a positive test of this hypothesis in a well-characterized model of "sodium-dependent" hypertension, the uninephrectomized rat given desoxycorticosterone and dietary sodium (*8*). This mineralocorticoid hormone stimulates the kidney to reclaim both sodium and chloride and can induce continued retention of both ions when administered over time in pharmacological amounts (*9*).

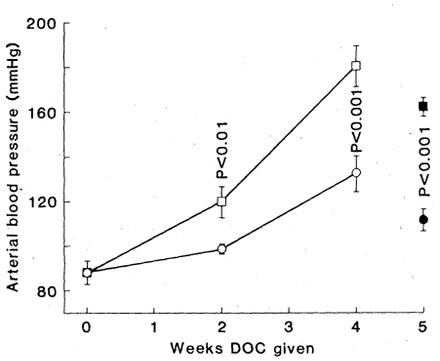

Fig. 1. Squares and circles represent the measured blood pressure of rats that consumed an average of 40.4 mmole of sodium chloride per week (N = 8) or 40.9 mmole of a combination of sodium bicarbonate and sodium ascorbate per week (N = 8), respectively. Open symbols denote systolic pressures measured with the tail plethysmography method. Closed symbols denote mean arterial pressures measured directly through femoral artery catheters placed 3 hours beforehand. Symbols and their brackets indicate means ± standard errors. P values indicate statistical differences (one-tailed Student's t-test) between the group receiving sodium chloride and the group receiving sodium bicarbonate and sodium ascorbate at the times indicated. In a similar experiment (results not depicted), the blood pressures in six rats given DOC and NaCl (38.5 mmole per week) were significantly greater than those in six rats given DOC and $NaHCO_3$ (38.8 mmole per week).

Male Sprague-Dawley rats (100 to 150 g) were uninephrectomized and individually housed in metabolic cages (Nalge Company, No. 650-0100) so that food and fluid intake could be controlled and sodium and potassium balances cumulatively measured. Desoxycorticosterone (DOC) was injected intramuscularly as Percorten pivalate (Ciba-Geigy), 50 mg/kg initially and 25 mg/kg each week thereafter. All animals were fed a NaCl-deficient, natural ingredient diet (Teklad) (sodium content less than 0.03 mmole/g). Dietary sodium was thus provided mainly by adding sodium to the drinking water as near equimolar amounts of NaCl, sodium bicarbonate ($NaHCO_3$), sodium ascorbate (Na-ascorbate), or a combination of sodium bicarbonate and sodium ascorbate ($NaHCO_3$/Na-ascorbate). The sodium concentrations of the solutions ranged from 70 to 172 mmole/liter depending on the weekly dietary sodium intake being studied. The daily amount of food and fluid provided to each animal drinking the solution of NaCl was equal to that amount consumed by a partner drinking the solution of $NaHCO_3$ or Na-ascorbate or both. The animals drinking the solution of NaCl usually consumed all of the food and fluid provided to them.

Systolic blood pressures and body weights were similar for all rats before DOC was administered. Afterward, at dietary intakes of sodium ranging from approximately 17 to 40 mmole per week, the systolic or mean blood pressures of rats given NaCl were significantly greater than those of rats given $NaHCO_3$ or Na-ascorbate or both (Figs. 1 and 2). At the lowest dietary intake of sodium employed, approximately 17 mmole per week, the blood pressure of rats given $NaHCO_3$ with DOC was not significantly different from that of rats given NaCl without DOC (Fig. 2). With DOC, however, this normal amount of NaCl (*10*,

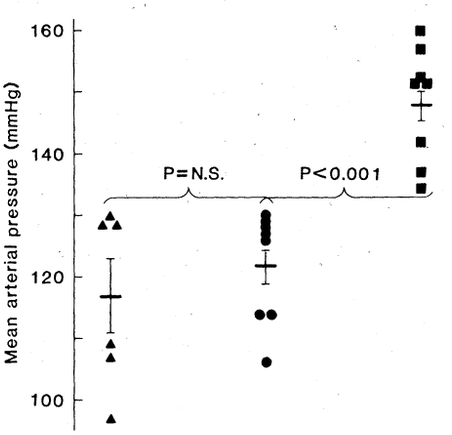

Fig. 2. Mean arterial pressures (measured through femoral artery catheters implanted 24 hours beforehand) in conscious, unrestrained, uninephrectomized rats given sodium chloride without desoxycorticosterone pivalate (DOC) (▲, N = 6), sodium bicarbonate with DOC (●, N = 8), or sodium chloride with DOC (■, N = 8), in amounts of 16.1, 17.9, and 16.6 mmole of sodium per week, respectively, for 5 weeks. Horizontal bars and their brackets indicate group means ± standard errors. P values indicate statistical differences between group means by Student's one-tailed t-test with the Bonferonni correction for multiple comparisons. Metabolic data from these studies are presented in Table 1. In a similar experiment (results not depicted), the mean arterial pressures of eight rats given NaCl, 22.8 mmole per week for 5 weeks, with DOC were significantly greater than those of five rats given sodium ascorbate, 24.7 mmole per week for 5 weeks, with DOC (mean = 169 ± 7 versus 134 ± 3 mmHg, respectively; $P < 0.01$).