of approximately 5×10^{-12} to 5×10^{-16} mole. Therefore, droplet diameter need not be considered in calculating the specific activity of β-glucuronidase in a single cell since the number of product molecules formed depends only on the amount of enzyme in the cell. However, if impurities are present in the assay mixture and contribute to background fluorescence the correction for this contribution does depend on droplet size since the amount of background fluorescence is proportional to droplet volume.

In the micro droplet assay the amount of product formed is directly proportional to the amount of enzyme present in the droplet (Fig. 1B). If the enzyme is first assayed by conventional means and then distributed in micro droplets, the same specific activity is obtained in both assays. This being so, we have calculated the sensitivity of our instrumentation. The minimum detectable amount of reaction product is 5×10^{-16} mole. The turnover number (that is, the number of product molecules formed per enzyme molecule per minute) for purified murine β-glucuronidase is approximately 6×10^3 at 37°C with the nitrophenyl substrate (*5*). We found the 4-methylumbelliferone substrate approximately as active. From these data we calculate that approximately 800 enzyme molecules are detected in a 1-hour incubation.

The range and distribution of β-glucuronidase activity in single liver cells is shown in Fig. 2. If the cells are separated into categories of small, medium, and large cells, we find that enzyme activity generally increased with cell size. This observation and the fact that cell size increases with the ploidy of the cell in liver (*6*) suggests that β-glucuronidase activity is a function of the number of gene copies for that enzyme.

The micro droplet assay is versatile. Almost any enzyme that can form a fluorescent product directly or through a coupled reaction can be assayed by this procedure. The sensitivity depends largely on the instrumentation used to measure fluorescence. Our method combines sensitivity with simplicity, making it possible to assay β-glucuronidase in as many as 100 individual cells in a few hours easily and accurately.

LINDA WUDL*
KENNETH PAIGEN
Department of Molecular Biology, Roswell Park Memorial Institute, Buffalo, New York 14203

References and Notes

1. P. C. Maloney and B. Rotman, *J. Mol. Biol.* **73**, 77 (1973); B. Rotman, *Proc. Natl. Acad. Sci. U.S.A.* **41**, 1981 (1961).
2. We thank Janice Peterson for her assistance in preparing the cells according to the method of R. B. Howard, J. C. Lee, and L. A. Pesch [*J. Cell Biol.* **57**, 642 (1973)].
3. If bovine serum albumin is used it should be exhaustively dialyzed to remove fluorescent impurities.
4. It is important that the primary filter does not fluoresce itself; otherwise the measured background will be too high.
5. S. Tomino, personal communication.
6. C. J. Epstein, *Proc. Natl. Acad. Sci. U.S.A.* **57**, 327 (1967)
7. Supported in part by NSF research grant GB 3049 and PHS grant GM 19521.
* Present address: Merck Institute, Rahway, New Jersey.

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Angiotensin II– and Angiotensin III–Induced Aldosterone Release in vivo in the Rat

Abstract. *The potential role of angiotensin II and its heptapeptide metabolite, des-aspartyl-angiotensin II, was studied in the conscious unanesthetized rat. Aldosterone release was induced by both peptides at physiologic doses (0.72 nanogram per minute). [1-Sarcosyl-8-alanyl]angiotensin II (P-113 inhibited angiotensin II more effectively than des-aspartyl-angiotensin II (101 percent as compared to 82 percent). These results indicate that angiotensin controls aldosterone release in the rat and that des-aspartyl-angiotensin II (that is, angiotensin III) may be important in this sequence.*

There are proponents (*1–4*) and opponents (*5*) to angiotensin's role in mediating aldosterone release in the rat. This controversy centers around studies with rats stressed by surgery and anesthesia. Since anesthesia inevitably induces renin release in the intact rat (*6*), we questioned the state of adrenal cortical sensitivity of the previous reports. We have found a remarkable responsiveness of aldosterone release to angiotensin infusion in the unanesthetized rat, suggesting that anesthesia introduced an important artifact in the previous studies. We now report our comparison of the effects of the heptapeptide des-aspartyl-angiotensin II (angiotensin III), and of the octapeptide angiotensin II on the adrenal receptor, using the new selective angiotensin antagonist, P-113 ([1-sarcosyl-8-alanyl]angiotensin II).

Male Wistar rats (300 to 350 g) were housed individually and exposed to light by an automated system from 6 a.m. to 6 p.m. The animals were given free access to tap water and Purina rat chow containing 152 meq of sodium per kilogram of food.

Experiments were done 24 hours after placement of a polyethylene catheter in the jugular vein, which was exteriorized posteriorly. Control rats were infused with 0.1*M* tris buffer, *p*H 7.5, in 0.2 percent lysozyme, and the experimental rats were infused with the same buffer containing des-aspartyl-angiotensin II (purchased from Schwarz-Mann; labeled heptapeptide) or angiotensin II amide (Ciba). All infusions were administered at a rate of 25 μl/min for 30 minutes. At the end of the infusion, the rats were decapitated, and blood was collected from the aorta in siliconized glass tubes kept on ice. The samples, after being allowed to clot, were centrifuged at 12,000*g* at 4°C for 20 minutes, and the serum was frozen (− 20°C) in capped plastic tubes until they were assayed.

Another group of identically prepared rats were given preliminary treatment with P-113 in the following manner. The P-113 (0.6 mg/kg) was injected subcutaneously (time zero). Ten minutes later, P-113 (10 mg/kg) was administered subcutaneously in 15 percent Pharmagel A in saline to ensure prolonged action (*7*). At 20 minutes, the 30-minute infusion was initiated as before with tris (control), angiotensin II, or the heptapeptide. The peptides were infused at a dose of 100 ng/min. At 50 minutes, the animals were decapitated and blood was collected for radioimmunoassay of aldosterone.

All samples were assayed for aldosterone according to the immunopurification method of Gomez-Sanchez *et al.* (*8*) with the modification of a 1 : 1,000,000 dilution of antibody to aldosterone (obtained from the National Institutes of Health) being used for the radioimmunoassay.

The results of the peptide infusions are shown in Fig. 1. Both peptides elicited dose-related aldosterone release over a wide range. The response of aldosterone release to the infusion at 0.72 ng/min was significant in both

cases ($P < .05$ for angiotensin II; $P < .005$ for angiotensin III). There was no significant difference at any given dose between the response elicited by angiotensin II and that elicited by des-aspartyl-angiotensin II. Another experiment showed that there was no significant difference between tris-infused rats and noninfused control rats (2.05 ± 0.16 ng/100 ml as compared to 2.30 ± 0.24 ng/100 ml).

The effects on aldosterone of both peptides (100 ng/min) were inhibited by preliminary treatment with P-113, although the inhibition was more complete with angiotensin II (101 percent) than with des-aspartyl-angiotensin II (82 percent) (Fig. 2).

Several investigators have suggested that the rat is unique among mammalian species commonly used for experimental purposes in that the renin-angiotensin axis does not control release of aldosterone from the rat adrenal cortex (*5*). Others (*2*, *4*) disagree, asserting that the significance of angiotensin as a physiological factor in the control of aldosterone may have been masked experimentally by introduced artifacts [for example, nonphysiological doses of angiotensin or anesthesia (or both) and surgical stress]. This controversy should be resolved because an inexpensive, readily available model is needed for reproducible studies of the renin-angiotensin-aldosterone axis.

The validity of previous experiments with anesthetized animals is open to question, since anesthetics tend to elicit the release of aldosterone via activation of the renin-angiotensin system (*7*). Thus, the high baseline rates of renin and aldosterone secretion in anesthetized animals could alter the response to infused angiotensin or obscure the statistical significance of the angiotensin-induced increment in aldosterone release. Coleman *et al.* (*9*) have further substantiated this contention by showing attenuation of angiotensin-induced aldosterone release in pentobarbital anesthetized rats, as compared to conscious rats.

For these reasons, the dose-response relation of angiotensin-induced aldosterone release was determined in the unanesthetized rat. The angiotensin infusion rate of 0.72 ng/min approximates a serum renin activity of 3.2 ng/ml per hour on the basis of the following calculations. A 300-g rat with a plasma volume of 13.5 ml (*10*) should produce 43 ng of angiotensin per hour, which is equivalent to an

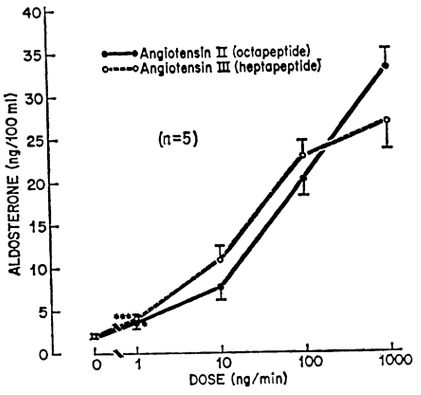

Fig. 1. Dose-response of angiotensin II (octapeptide)– and angiotensin III (heptapeptide)–mediated aldosterone release in conscious, ambulatory rats. Dose in nanograms per minute includes 28 percent moisture. Each value represents the mean $\pm$ the standard error of five rats; * indicates $P < .05$; *** indicates $P < .005$. Higher doses are each significantly greater than control values with $P < .001$.

infusion rate of 0.72 ng/min. Rats ingesting a diet containing 54 meq of sodium per kilogram of food had an even higher serum renin activity (5.4 ng/ml) per hour when the plasma was incubated at *p*H 6.5, which produces 26 percent more angiotensin I as compared to incubation at *p*H 7.4 (*11*). Thus, a physiological rate of angiotensin II infusion resulted in significant release of aldosterone in the rat. This response may have been even greater had the rats been fed a low sodium diet since the aldosterone response to angiotensin II is greater in sodium-depleted rats (*2*).

The aldosterone-releasing potency of

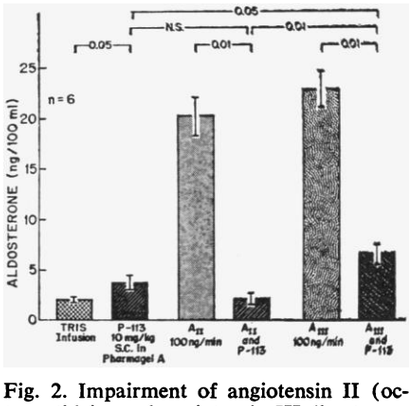

Fig. 2. Impairment of angiotensin II (octapeptide)– and angiotensin III (heptapeptide)–induced aldosterone release by P-113. Significance of difference between respective means is indicated by *P* values and connecting brackets. Each value represents the mean $\pm$ the standard error for six rats.

des-aspartyl-angiotensin II was at least as great as that of angiotensin II in conscious, ambulatory rats (Fig. 1). Since this peptide is formed from angiotensin II in vivo, it is not unreasonable to suggest that it contributes to aldosterone release. The contribution of both emphasizes the importance of the renin-angiotensin cascade in the control of aldosterone release in the unanesthetized rat as well as in other species.

P-113 is a competitive inhibitor of angiotensin II on vascular smooth muscle in rats (*12*). The decrease in aldosterone release in response to infusions of the angiotension II and des-aspartyl-angiotensin II in doses of 100 ng/min after preliminary treatment with P-113 demonstrates that P-113 inhibits the effects of both angiotensin II and the des-aspartyl-angiotensin II on the adrenal cortex. It should be emphasized that the inhibition by P-113 of the effect of the des-aspartyl-angiotensin II (82 percent) was less complete than with angiotensin II (101 percent). This corroborates the observations in vitro of Chiu and Peach (*13*) and Brecher *et al.* (*14*), who used isolated adrenal cells in which the heptapeptide has a greater affinity for the receptor site than does the octapeptide.

The enzymatic hydrolysis of the naturally occurring angiotensin II by angiotensinase A (an aminopeptidase) occurs by removal of the NH_2-terminal aspartyl residue producing des-aspartyl-angiotensin II as a metabolite (*15*). This compound has been reported to have only 22 percent of the pressor activity of angiotensin II and only 18 percent of its activity on isolated rat uterus (*16*). This heptapeptide has been shown only qualitatively to increase aldosterone secretion in sheep in vivo (*17*) and to release aldosterone in vitro (*13*). After removal of the NH_2-terminal aspartate, the NH_2-terminal arginine was removed leaving the des-aspartyl-arginyl-angiotensin II (hexapeptide) metabolite (*18*), which has practically no activity on vascular smooth muscle (*16*) or the adrenal cortex (*17*). On the other hand, synthetic angiotensin II amide, used in these experiments, has been shown to be more rapidly metabolized to the inactive hexapeptide (*18*), possibly by the rapid removal of the two NH_2-terminal amino acids (that is, asparaginyl-arginine) by a different aminopeptidase (*15*). Thus, angiotensin II amide may stimulate aldosterone release without

involving the heptapeptide metabolite and remain almost as active as the heptapeptide in this respect.

We conclude the following: (i) The renin-angiotensin system functions in the physiological control of aldosterone secretion from the rat adrenal cortex, and other investigators were dealing with artifacts of anesthesia and surgical stress. (ii) (The rat adrenal cortex in vivo is at least as responsive to the naturally occurring des-aspartyl-angiotensin II metabolite of angiotensin II as it is to angiotensin II itself. (iii) The angiotensin antagonist P-113 acts in vivo to inhibit the effects of these peptides on the rat adrenal cortex. (iv) In view of these observations in vivo and the studies in vitro (*17*), we suggest that the naturally occurring heptapeptide, des-aspartyl-angiotensin II, be called angiotensin III.

WILLIAM B. CAMPBELL
STEPHEN N. BROOKS
WILLIAM A. PETTINGER
Departments of Pharmacology and *Internal Medicine, University of Texas Southwestern Medical School, Dallas*

References and Notes

1. B. Singer, C. Losito, S. Salmon, *Endocrinology* **74**, 325 (1964).
2. G. A. Kinson and B. Singer, *ibid.* **83**, 1108 (1968).
3. M. L. Dufau and B. Kliman, *ibid.*, p. 180.
4. ———, *ibid.* **82**, 29 (1968).
5. N. J. Marieb and P. J. Mulrow, *ibid.* **76**, 657 (1965); E A. Eilers and R. E. Peterson, *Aldosterone* (Davis, Philadelphia, 1964), pp. 251–264; R. Cade and T. Perenich, *Am. J. Physiol.* **208**, 1026 (1965).
6. W. A. Pettinger, K. Tanaka, K. Keeton, W. B. Campbell, S. N. Brooks, in preparation.
7. K. Tanaka and W. A. Pettinger, *J. Pharmacol. Exp. Ther.* **188**, 229 (1974).
8. C. Gomez-Sanchez, D. C. Kem, N. M. Kaplan, *J. Clin. Endocrinol. Metab.* **36**, 795 (1973).
9. T. G. Coleman, R. E. McCaa, C. A. McCaa *Clin. Res.* **22**, 17A (1974).
10. R. F. Pitts, *Physiology of the Kidney and Body Fluids* (Year Book, Chicago, 1968), p. 22.
11. W. A. Pettinger, M. Marchelle, L. Augusto, *Am. J. Physiol.* **221**, 1071 (1971).
12. P. T. Pals, F. D. Masucci, G. S. Denning, F. Sipos, D. C. Fessler, *Circ. Res.* **29**, 673 (1971); H. Gavras, H. R. Brunner, E. D. Vaughan, Jr., J. H. Laragh, *Science* **180**, 1369 (1971).
13. A. Chiu and M. Peach, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
14. P. Brecher, M. Tabacchi, H. Y. Pyun, A. V. Chobanian, *Biochem. Biophys. Res. Commun.* **54**, 1511 (1973).
15. I. Nagatsu, L. Gillespie, J. E. Folk, G. G. Glenner, *Biochem. Pharmacol.* **14**, 721 (1965).
16. R Schwizer, *J. Pure Appl. Chem.* **6**, 265 (1963).
17. J. R. Blair-West, J. P. Coghlan, D. A. Denton, J. W. Funder, B. A. Scoggins, R. D. Wright, *J. Clin. Endocrinol. Metab.* **32**, 575 (1971).
18. A. V. Notargiacomo and J. N. Cohn, *Proc. Soc. Exp. Biol. Med.* **134**, 149 (1970).
19. Supported by AHA grant No. 85090 (1918-51-85090), the Norwich Pharmacal Co., the Merck and Hoffmann–La Roche foundations, and by the Pharmaceutical Manufacturing Association. The aldosterone antibody was provided by the National Institute of Arthritis and Metabolic Diseases, and P-113 was supplied by Dr. Castellion of the Norwich Pharmacal Company.

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Defensive Use by an Insect of a Plant Resin

Abstract. *Larvae of the sawfly* Neodiprion sertifer *(Hymenoptera: Diprionidae), when disturbed, discharge an oily oral effluent essentially identical chemically to the terpenoid resin of its host plant* (Pinus sylvestris). *The resin is sequestered by the larva upon feeding, and stored in two compressible diverticular pouches of the foregut. The fluid is effectively deterrent to predators. The defensive use by an insect of a plant resin provides an instance of secondary utilization by a herbivore of the protective chemical weaponry of its host.*

Many animals, when attacked, traumatized, or otherwise "stressed," discharge enteric contents. Insects in particular are prone to regurgitate or defecate when handled, as anyone knows who has collected grasshoppers, caterpillars, or beetles in the field. What seems generally unrecognized is that in some species these discharge mechanisms serve primarily for protection. We here report on the defensive function, chemical nature, and dietary origin of the oral effluent of the larva of the sawfly *Neodiprion sertifer*.

Like some of its relatives, *N. sertifer* is a common pest. Gregarious as a larva (*1, 2*) it feeds on such conifers as Scotch pine (*Pinus sylvestris*), denuding branches of their needles (Fig. 1A). When disturbed, the larva shows a diversity of startle responses (*2*), involving most commonly the rearing of the front end, and the emission from the mouth of a droplet of fluid (Fig. 1B). The droplet is viscous, immiscible with water, and of the consistency and unmistakable odor of *Pinus* resin. When poked or pinched, the larva revolves its body and, in an obvious defensive maneuver, dabs the droplet directly on the offending object (Fig. 1C). The response is quick and accurate, and the larva can reach any part of its back and sides with the mouth, except the region just behind the head.

To test the proposition (*2*) that the oral effluent represents resin from the host plant regurgitated by the larva after ingestion, chemical analyses were made of effluent and of resin obtained from needles and branches of *P. sylvestris*. Effluent was collected by pinching larvae with forceps and placing capillary tubes over their mouths so as to trap the disgorged fluid (90 mg of material was "milked" from 500 medium to full-sized larvae). Branch resin was obtained by scraping the oozings from bark incisions made on needle-bearing twigs comparable to those ordinarily beset by larvae. Needle resin was obtained by extracting whole needles with methylene chloride, or by squeezing freshly transected needles and taking up in microcapillary tubes the droplets that emerged from the resin ducts at the severed surface. The compounds identified, and their distribution in the three samples, are given in Fig. 2 and Table 1. It is clear, in accord with previous findings (*3, 4*), that branch resin differs chemically from needle resin. Both resins contain the two volatile monoterpenes, α-pinene and β-pinene, responsible for the characteristic odor of *Pinus* resin, but only branch resin contains a complex mixture of resin acids. Needle resin has only one major acidic component, pinifolic acid. The oral effluent contains all the identified components.

The analytical procedures were as follows. Components 1 and 2, detected by gas chromatography–mass spectroscopy (GC-MS) of the larval effluent, were identified as α-pinene and β-pinene by GC-MS comparison with authentic samples. The presence of car-

Fig. 1. (A) Cluster of larvae of *Neodiprion sertifer* on twig of *Pinus sylvestris*. (B) "Altered" larva, raising its front end and regurgitating. (C) Larva, pinched in forceps, responding by dabbing regurgitated fluid onto the instrument. (D) Larva dabbing fluid on an attacking ant (*Formica exsectoides*). (E to G) Larva within cocoon responding to prodding of its rear (with pin shown at lower left) by revolving and rotating its body so as to bring its fluid-laden mouthparts to bear upon the pin. (The cocoon has been cut open and covered over with glass, through which the photographs were taken.) (H) Foregut of larva (mouth at upper center) showing the two muscled diverticular pouches in which the resin is stored (muscles appear as bright bands in partially polarized transmitted light). (I) Comparable to preceding, but consisting of the cuticular lining only (muscles and other cellular components have been removed by treatment with hot potassium hydroxide). (J) Comparable to preceding, but of a prepupa. The arrow points to the sealed junction where the foregut formerly opened to the midgut. (K) Enlargement of the sealed foregut-midgut junction [region shown by arrow in (J)] showing the plug that effects the seal. (L) Pupa in cocoon, shedding the larval skin with the attached resin sac (photograph of a specimen that died during the molt). (M) Resin sac such as is characteristically left behind in the cocoon after emergence of the adult.