nificance. In apomixis, sexual recombination is bypassed and the genotype of the progeny is identical to that of the parent. Apomictic plants are often unable to complete normal meiosis and may be highly heterozygous. Their breeding system is an escape from sterility and retains heterozygosity. Cleistogamy is an extreme form of inbreeding, with normal meiosis and recombination, and as in all autogamous plants the genetic consequence of the breeding system is a rapid shift toward homozygosity. I eventually rejected apomixis as unlikely for these reasons: (i) The anther is always present and always contains pollen that stains well with aniline blue. Development proceeds normally from pollen mother cells to tetrads to small but otherwise typical pollen, and in one case normal meiosis was observed. (ii) Development of the embryo sac is likewise perfectly normal, from spore mother cell to egg. (iii) Embryogenesis is also just what one would expect in a normally sexual plant.

A better method for discovering the path of the pollen tubes was clearly needed. Since pollen tubes often contain callose, a substance which can be made to fluoresce in the presence of aniline blue, cleistogamous flowers were embedded and sectioned longitudinally at 17 μm, then stained with a dilute buffered solution of aniline blue (*2*). This temporary preparation was examined with a Nikon Apophot microscope equipped with a 200-watt mercury burner for fluorescence, a BG 12 exciter filter, and a yellow (OG 530) barrier filter. Under these conditions, callose in the pollen grains and pollen tubes fluoresces bright yellow or yellow-green. Photographs were taken with a Nikon AFM camera using Kodak Tri-X pan film for black and white and high-speed Ektachrome, exposed and push-developed at ASA 400, for color slides (Figs. 4 to 6).

The pollen grains germinate inside the indehiscent anther and fill it with pollen tubes. Many pollen tubes grow down through the short filament and into the receptacle of the flower (Fig. 4). From there they turn upward (Fig. 5) and grow into the locule of each carpel, through the space between the ovule and the wall of the carpel, and into the nucellar beak of the ovule (Fig. 6). After the basic pathway was thus discovered, whole mounts of stamens (Figs. 7 and 8) and ovules (Fig. 9) were cleared with NaOH and then studied with fluorescence. In Fig. 9 several pollen tubes may be seen in the nucellar beak of the ovule, with one (fluorescing only faintly) penetrating deep into the ovule and presumably effecting fertilization. Since there are only two ovules in the flower, one per carpel, the relatively few pollen grains are more than ample. Since the whole flower is so small, the seemingly tortuous path of the pollen tubes is actually quite short, much shorter than that of a pollen tube that grows through the style of a chasmogamous flower, which is often 3 mm long.

Given the topography of these flowers, it is very difficult to get a single section that shows the complete path of pollen tubes from the anther locule to the nucellar beak; it is usually not all in one plane. However, Figs. 4 to 6 are photographs of sections of a single flower and together they show the path quite clearly. Many preparations have shown identical results in several species of *Janusia*, *Gaudichaudia*, and *Camarea*; *Aspicarpa*, which has similar cleistogamous flowers in some species, is expected to have the same method of fertilization.

The unusual and cryptic self-fertilization found in the cleistogamous flowers of the Malpighiaceae is quite unlike anything previously reported for angiosperms and suggests some interesting evolutionary questions. These include the way in which such a radical reorientation of the pollen tubes arose; the nature of the intermediates that bridged the evolutionary gap between chasmogamous flowers with styles and dehiscent anthers and cleistogamous flowers with no functional styles and indehiscent anthers; and how independent the genetic systems are that control the development of the two types of flowers, which are often produced simultaneously. It is worth mentioning that the single stamen in the cleistogamous flowers is one of the six present in the chasmogamous flowers in the more primitive members of this group (tribe Gaudichaudieae), but in the more advanced species that stamen has been lost from the chasmogamous flowers while remaining in the cleistogamous flowers. This suggests some evolutionary divergence in the parts of the genome controlling production of these flowers.

Whether this mechanism is limited to the Malpighiaceae remains to be seen. Perhaps other plants in which apomixis has been inferred but not rigorously demonstrated should be reexamined for the possible presence of some form of cryptic self-fertilization.

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Ala-Gly- and Val-Asp-[Arg^8]-Vasopressin: Bovine Storage Forms of Arginine Vasopressin with Natriuretic Activity

Abstract. *Extracts of fresh-frozen bovine neurohypophysis were purified by chromatographic techniques to isolate and characterize the components that produce natriuresis in nondiuretic dogs. Two compounds with natriuretic properties similar to those of synthetic arginine vasopressin accounted for most of the natriuretic activity and appeared to be the prevalent vasopressin-like molecules in the extract. These peptides were Ala-Gly-[Arg^8]-vasopressin and Val-Asp-[Arg^8]-vasopressin; the natriuretic potency of each appeared to be similar to synthetic arginine vasopressin and could be observed with doses in the range of 50 picomoles. In the dog the most conspicuous difference between synthetic arginine vasopressin and the new vasopressin peptides was the smaller pressor responses to natriuretic doses of the new compounds.*

Evidence suggests that a humoral factor may contribute to the natriuresis (urinary excretion of sodium) produced by the expansion of extracellular volume (*1*, *2*). We have attempted to determine the sites of origin of such natriuretic substances, assaying their activity in nondiuretic male mongrel dogs (*3*). Selective natriuretic activity was observed in extracts prepared from bovine posterior pituitary lobes (*4*). These results provided the impetus for our further evaluation of the substances that produced this response. The absence of pressor activity in some of our early partially purified fractions made it unlikely that the natriuretic activity was produced entirely by arginine vasopressin, a known natriuretic substance (*5*).

We used extraction procedures similar

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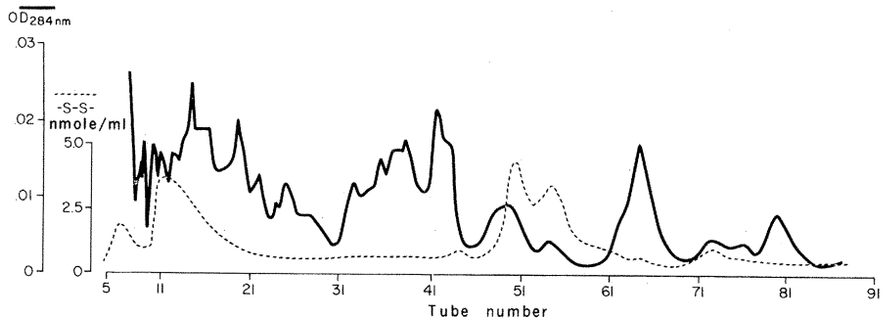

Fig. 1. Chromatographic separation of partially purified posterior pituitary extract on Partisil-10 SCX (step 6). The sample, derived from the purification of 10 g of dried bovine posterior pituitary lobes, was dissolved in 85 m*M* acetic acid in 10 percent *n*-propanol and applied at tube 3. At tube 10 a linear gradient was begun with 24 m*M* pyridine acetate, *p*H 5.0, in 10 percent *n*-propanol which increased at a rate of 0.6 percent per minute. The flow rate was 160 ml per hour and tube collections were for 2.5 minutes each. The absorbance of the eluate was monitored at 284 nm. The concentration of disulfide-reacting material was assayed in portions from each tube by a modification of an automated disulfide technique (*18*). The tubes were pooled for repeated chromatography on the basis of their disulfide content. The Ala-Gly-[Arg^8]-vasopressin was obtained from rechromatography of the disulfide peak that was maximal at tube 50. Approximately 95 nmole of disulfide was present in this peak. The Val-Asp-[Arg^8]-vasopressin was obtained from rechromatography of the disulfide material that peaked at tube 54. Approximately 80 nmole of disulfide was contained in this peak. With the chromatographic conditions used in this run, [Arg^8]-vasopressin would emerge around tube 56. The disulfide elution pattern, amino acid composition, and absence of any contaminating amino acid sequence in the natriuretic peaks, all suggest that the [Arg^8]-vasopressin content of the material applied to the column was less than 10 percent of the amount present as Val-Asp-[Arg^8]-vasopressin.

to those used previously for extracting oxytocin and vasopressin from the posterior lobe of the pituitary (*6*), except that we lyophilized the fresh-frozen glands prior to fat extraction with acetone to minimize any autolytic process that might occur. To extract the peptides we used 175 ml of 0.2*M* acetic acid per 10 g of posterior pituitary powder. The stirred mixture was heated to 75°C and then cooled to room temperature over a 4-hour period and stored at 4°C overnight. After centrifugation, the supernatant was partially deproteinized by adding ethanol to achieve a 70 percent (by volume) final concentration (230 ml of ethanol and 100 ml of supernatant). The mixture was filtered through a glass fiber filter to remove precipitate, lyophilized, reconstituted in 20 ml of 1*M* acetic acid, and stored frozen. At this stage of purification, the extract from 10 g of lyophilized posterior pituitary glands contained approximately 1.1 g of protein. We found the assay of this material for natriuretic activity to be less reliable

Table 1. Chromatographic procedures for purification of posterior pituitary extracts.

Column packing	Initial conditions	Eluting conditions	Materials collected	Natriuretic activity (*3*)
		Step 1		
LH 20 (Pharmacia) (5 by 50 cm)	0.33*M* acetic acid in 67 percent *n*-propanol. The sample is dissolved in a solution of similar composition, then adjusted to *p*H 3 with hydrochloric acid	0.33*M* acetic acid in 67 percent *n*-propanol	All material absorbing at 280 nm emerging before the salt peak	510,000 units per 0.8 g of protein
		Step 2		
Carboxymethylcellulose (Whatman) (2.5 by 25 cm)	12 m*M* ammonium acetate buffer, *p*H 4.5, in 70 percent methyl Cellosolve.	12 m*M* ammonium acetate, *p*H 4.5, in 70 percent methyl Cellosolve	All eluates until the 254-nm absorbance stabilizes	460,000 units per 0.3 g of protein
		Step 3		
Carboxymethylcellulose (Whatman) (1.2 by 25 cm)	35 m*M* ammonium acetate, *p*H 4.5, in 10 percent methyl Cellosolve	12 m*M* ammonium acetate, *p*H 4.5, in 70 percent methyl Cellosolve	All 254-nm absorbance eluted with initial and eluting buffers	340,000 units per < 0.01 g of protein; 6000 nmole of disulfide
		Step 4		
SP-glycophase CPG250 (Corning) (1.5 by 18 cm)	0.17*M* acetic acid in 50 percent *n*-propanol	23 m*M* pyridine acetate, *p*H 5.0, in 50 percent *n*-propanol	All 280-nm absorbing material eluted by the initial and eluting buffers	270,000 units per 4000 nmole of disulfide
		Step 5		
SP-glycophase CPG250 (Corning) (0.9 by 12 cm)	0.17*M* acetic acid in 50 percent *n*-propanol	1.7*M* acetic acid in 50 percent *n*-propanol	All 280-nm absorbing material eluted by the initial and eluting buffers	210,000 units per 1500 nmole of disulfide
		Step 6		
Partisil-10 SCX (Whatman) (0.9 by 25 cm)	17 m*M* pyridine acetate, *p*H 5.0, in 10 percent *n*-propanol	Created by application of a linear gradient with 24 m*M* pyridine acetate, *p*H 5.0, in 10 percent *n*-propanol started approximately 25 minutes after sample application	Disulfide containing peaks that emerge approximately 100 minutes after sample application	160,000 units eluted; 120,000 units per 300 nmole of disulfide in peaks isolated for rechromatography
Partisil-10 SCX (Whatman) (0.9 by 25 cm)	Rechromatography*	Rechromatography*	Ala-Gly-[Arg^8]-vasopressin; Val-Asp-[Arg^8]-vasopressin	320 ± 50/nmole (N = 4)†; 580 ± 108/nmole (N = 5)†

*The isolated disulfide peaks were rechromatographed according to the conditions specified in step 6. †Mean (± standard error) values shown with sample number. The differences in natriuretic activity of each peptide were not significant.

than assays of purer fractions and attributed this variability to the presence of impurities.

The chromatographic procedures that we developed for further purification of the posterior pituitary extracts are shown in Table 1. At each stage of purification we monitored the natriuretic activity of the fractions to ensure that the major portion of natriuretic activity was being retained for further purification (Table 1). Throughout the purification process we moved solvent by lyophilization or rotary evaporation at room temperature and used 0.1*M* to 0.5*M* acetic acid in 10 percent *n*-propanol as a solvent to redissolve the dried material.

Initially we observed a high degree of interaction of the active material with contaminants, as judged by the marked effect of sample size on chromatographic behavior. We fractionated the crude extract obtained from 10 g of dried posterior pituitary according to the procedures shown in Table 1. The fractions obtained by the early procedures maintained natriuretic activity in a heterogeneous pool, only trivial amounts of natriuretic activity being removed with the contaminants. After the second run on the sulfonated glass column (step 5), the results obtained with subsequent chromatography were reproducible even at different sample concentrations. Figure 1 shows the isolation of two natriuretic disulfide-containing peaks. Both peaks typically contained approximately 70 percent of the natriuretic activity present in the column eluate. Rechromatography of the individual peaks yielded symmetrical peaks that were chemically analyzed. This was accomplished by extensive reduction of the material with 0.01*M* dithioerythritol and radioactive alkylation with 0.02*M* ^{3}H-labeled iodoacetic acid in 7*M* guanidine hydrochloride. The material was freed of excess reagent by molecular sieve chromatography and again lyophilized to dryness. The material was subjected to acid hydrolysis for 24 hours and then examined with a modified Technicon amino acid analyzer. Amino acid sequence analysis was performed in the Beckman 890C Sequenator utilizing Polybrene (*7*) and with a dimethylallylamine program. Identification of phenylthiohydantoin amino acids was achieved by high-pressure liquid chromatography, thin-layer chromatography, liquid scintillation counting and, if necessary, back hydrolysis to the parent amino acid (*7, 8*). Both natriuretic peaks were judged to be at least 90 percent pure by amino acid compositional analysis and amino acid sequence analysis. Peak 1 was found to be Ala-Gly-[Arg^8]-vasopressin, and peak 2, Val-Asp-[Arg^8]-vasopressin.

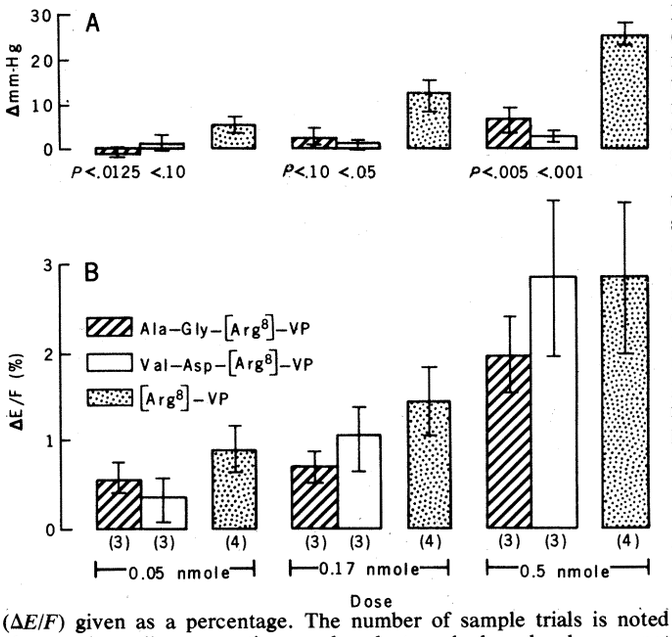

Fig. 2. Summary of (A) pressor and (B) natriuretic responses to 0.5-, 0.17-, and 0.5-nmole doses of Ala-Gly-[Arg^8]-vasopressin, Val-Asp-[Arg^8]-vasopressin, and synthetic [Arg^8]-vasopressin. Mean (± standard error) values are shown. Pressor responses were the peak changes in arterial pressure observed after the injection of material. The changes in sodium excretion produced by an injection of test material are the absolute increments (Δ) in fractional sodium excretion expressed as the ratio of sodium excreted to that filtered (Δ*E*/*F*) given as a percentage. The number of sample trials is noted below each bar. The changes in sodium excretion produced at each dose level were not significantly different for any of the peptides; the *P* values ranged from < .2 to < .98. The *P* values comparing the pressor responses of Ala-Gly-[Arg^8]-vasopressin and Val-Asp-[Arg^8]-vasopressin to [Arg^8]-vasopressin are displayed below the respective columns and were calculated with Student's *t*-test.

These vasopressin compounds and synthetic arginine vasopressin were assayed for pressor and natriuretic activities in anesthetized mongrel male dogs with our usual testing procedures (*3*). In most of these studies we alternated increasing doses of two of these compounds, waiting 40 to 60 minutes after each injection to permit the effects of a given compound to dissipate before injecting another dose of the other compound. In all of our studies we observed a dose-related natriuretic effect with each peptide; this effect was qualitatively similar in terms of onset of action and duration for each and was not accompanied by a significant alteration in the rate of creatinine clearance. In Fig. 2 the natriuretic responses are tabulated as the absolute increment in fractional sodium excretion for each dose of material. A dose-related increase in sodium excretion was observed that was similar for each peptide. Synthetic [Arg^8]-vasopressin produced significantly greater pressor responses in comparison to equivalent doses of Ala-Gly-[Arg^8]-vasopressin and Val-Asp-[Arg^8]-vasopressin.

These newly described peptides displayed biological activities in species other than the dog. Both compounds exhibited antidiuretic and vasopressor activity in rats, with a potency in the range of 25 percent of synthetic arginine vasopressin (*9*). Both compounds also displayed natriferic and hydroosmotic activity in the toad urinary bladder (*10*) with a relative potency similar to that of synthetic arginine vasopressin. It is not known whether these biological activities represent the activity of the intact molecules or whether they are the result of hydrolysis to arginine vasopressin.

That these compounds have never been isolated previously provides a strong argument for the presence of peptidases that can hydrolyze both compounds to [Arg^8]-vasopressin. In retrospect, we recognize that our use of lyophilized, fresh-frozen glands protected our starting material against potential loss by proteolytic activity. In addition, we prevented exposure of these compounds to bacterial peptidases by using redistilled water that was free of bacterial contamination and remained sterile with the addition of *n*-propanol.

There is now evidence that several peptide hormones, including bovine pituitary growth hormone (*11, 12*), prolactin (*12, 13*), parathyroid hormone (*14*), and insulin (*15*) are synthesized as portions of larger precursor peptides which undergo sequential hydrolytic cleavage to yield the principal form of the hormone that is secreted from the gland. Thus, both Ala-Gly-[Arg^8]-vasopressin and Val-Asp-[Arg^8]-vasopressin may well be intermediates in the synthesis of arginine vasopressin which would qualify them for consideration as prohormones. Since our current chemical techniques are not sufficiently sensitive to evaluate the circulatory forms of arginine vasopressin,

we do not know whether the new peptides are strictly storage forms or may be the form of the secreted hormone. In this regard, it is important to note that a current radioimmunoassay technique (*16*) is unable to distinguish between synthetic arginine vasopressin and either of these compounds (*17*).

We were surprised to find two different molecules in the bovine neurohypophysis that may serve a similar function. Whether these are allotypic variants between members of the species or isotypes present in all individuals can best be answered by isolating the products from single rather than pooled pituitaries. If these two molecules are isotypes, a more interesting question remains. Are the two peptides a result of biosynthetic heterogeneity or a consequence of different anatomical sites of synthesis within the hypothalamus?

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4. Acetic acid extracts of bovine pituitary glands were fractionated on P-2 Biogel polyacrylamide beads equilibrated with 0.1*M* acetic acid. Portions of the column eluate were pooled, lyophilized, and dissolved in normal saline to test for natriuretic activity. Significant natriuresis occurred in fractions that emerged after the salt peak. This response with the pituitary extract was at least an order of magnitude greater than the response to similar fractions prepared from acetic acid extracts of cerebral cortex, kidney, liver, lung, and skeletal muscle. Subsequently, we observed that the posterior lobe of the pituitary, which accounts for only 20 percent of the weight of the whole gland, contains approximately 90 percent of the natriuretic activity [H. J. Gitelman and W. B. Blythe, *Clin. Res.* **20**, 594 (Abstr.) (1972).
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Circadian Rhythms in *Neurospora crassa*: Oligomycin-Resistant Mutations Affect Periodicity

Abstract. *Nuclear mutations conferring resistance to oligomycin, a mitochondrial inhibitor, shorten the period of the circadian conidiation rhythm of* Neurospora crassa *from the normal 21.5 hours to 18 to 19 hours and slow the linear growth rate by 30 percent. These* oli^r *mutations map very close to* frq, *a locus at which mutations affecting periodicity have been previously obtained. The possibilities are discussed that mitochondria are involved in circadian rhythm generation and that certain period-length mutations affect mitochondrial functions.*

The band (*bd*) strain of *Neurospora crassa* exhibits a spore-forming rhythm when grown on solid media in the dark (*1*). This conidiation rhythm is deemed circadian by three criteria: (i) it has a free-running period length close to 24 hours, (ii) the phase of the rhythm is sensitive to exposures to light, and (iii) the period length is relatively insensitive to growth temperature (*2*). In order to further the understanding of the biochemical basis of circadian timekeeping in *Neurospora*, the effects on circadian rhythmicity of a variety of auxotrophic and morphological mutations were previously investigated (*3*).

We now report that, upon crossing oligomycin-resistance mutations (*oli*^{r}) into *bd*, the normal circadian period of 21.5 hours for *Neurospora* is shortened to 18 to 19 hours. This result is of interest for two reasons. The first is that these *oli*^{r} mutations map very close to another set of circadian period mutants: those at the *frq* (frequency) locus (*4*). The second is that oligomycin resistance appears to be due to changes in the primary structure of a particular mitochondrial protein. Sebald has reported that similar *oli*^{r} mutations lead to changes in the primary structure of a small polypeptide component, subunit 9, of the F_0 membrane portion of the mitochondrial adenosine triphosphate (ATP) synthetase (*5*).

The *bd csp-1* strain of *N. crassa* was used for all genetic crosses. The *bd* mutation allowed the clear expression of a conidiation pattern in closed petri dishes (*1*). The *csp-1* mutation (conidial separation) allowed easy handling of the dishes without self-reinoculation (*6*). The period length was determined as reported (*7*). All petri dish cultures were grown at 22°C on Vogel's minimal medium with 0.5 percent maltose as the carbon source, and inositol at 0.05 mg/ml for those strains carrying the *inl* marker. Crosses were performed with SC media (*8*).

Four oligomycin-resistant strains, *oli*^{r} (16-1), *oli*^{r} (16-3), *oli*^{r} (16-14), and *oli*^{r} (16-16) and one revertant to oligomycin sensitivity, *oli*^{s} (16-16R45), were donated by Edwards (*9*). It is not known whether these strains represent different alleles of the *oli*^{r} locus or are independent isolates of the same mutation. These *oli*^{r} mutations were isolated in a background containing the Has, Azs, and *inl* mutations. The Has and Azs mutations block alternate respiratory pathways in *N. crassa* (*10*). When the *oli*^{r} strains were crossed to *bd csp-1*, all four *oli*^{r} mutants gave the same result. The *bd csp-1 oli*^{r} progeny all showed a shortening of the circadian pe-

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