A Specific and Sensitive Bioassay for Arginine–Vasotocin: Description, Validation, and Some Applications in Lower and Higher Vertebrates

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A new bioassay for arginine-vasotocin (AVT) is described. The technique, based on the record in vitro isometric contractions employs eel ventral aortic strips mounted in a small Perspex microchamber (0.5 ml) in relation with a Statham strain gauge. AVT induces, in the most favorable cases, isometric contractions at a concentration down to 10⁻¹⁶ M., and a good dose–response relationship is obtained. In order to test the validity of this method, tissue extracts of neurohypophysis and pineal glands as well as biological fluids (plasma and urine samples) from different vertebrates were tested simultaneously with one and/or two radioimmunoassays (RIA). Except in the lamprey and the mammalian pineal gland, in most cases, a good correlation exists between bioassay and radioimmunoassay. Results are discussed on structure–activity relationship basis. It is concluded that the eel ventral aorta strip bioassay appears as a suitable and a very specific technique for AVT determination in lower as well as higher vertebrates.

Arginine–vasotocin (AVT), a neurohypophysial peptide present in all nonmammalian vertebrates and possibly some mammals (Viszloyi and Perks, 1969; Pavel, 1978), has been determined by radioimmunoassay (RIA) using antibodies against lysine- or arginine–vasopressin (Rosenbloom and Fisher, 1974; Holder et al., 1979) or oxytocin (Legros et al., 1971). To date, only three specific AVT antisera have been described (Dogterom et al., 1979, 1980; Fernstrom et al., 1980; Negro-Villar et al., 1980). These assays have, however, given controversial results, especially for the mammal. Further, in nonmammalian vertebrates, AVT in the body fluids appears to be present at concentrations at or about the limit of the sensitivities of the RIAs, and Bioassays such as the urinary bladder hydroosmotic test (Bentley, 1969) have failed to demonstrate AVT activity in fish plasma even under stress conditions (Bentley, 1971). More sensitive and specific assays for AVT are clearly desirable.

Vasoactivity is a characteristic of AVT both in vivo (Chan and Chester Jones, 1969; Maetz and Rankin, 1969; Sawyer et al., 1976) and in vitro (Somlyo and Somlyo, 1968). Previous investigations showed AVT to contract aortic and bulbous strips (Holder, 1976; Holder and Guerné, 1976); bulbous preparation gave variable sensitivities so that ventral aortic strips of the eel became the preparation of choice.

The present paper describes this new bioassay for AVT and validates its use in vertebrates.

MATERIALS AND METHODS

Animals

Eight to fifteen animals from the following species were used for radioimmuno- and bioassays.

Cyclostomes. Lampetra planeri (lamprey) from rivers of the region of Clermont–Ferrand (France).
lampreys were dissected at the latest 24 hr after being caught.

Fishes. Anguilla anguilla (eel) obtained from the affluents of the Rhine in Alsace (France). They were kept in aerated running fresh water at a temperature of 11 to 14°. Salmo gairdneri (trout) obtained from a fish breeder in Alsace. They were kept in aerated running fresh water at the same temperature as before.

Amphibians, Reptiles, Birds, and Mammals. Rana esculenta (frog), Testudo hermanni (tortoise), Lacerta agilis (lizard), Natrix tessellata (snake), Gallus domesticus (chicken), Coturnix japonica (quail), rat, hamster, and rabbits were used respectively.

Surgical Techniques

Eel blood and urine sampling. After anesthesia in a 30%/oo ether bath for 15 min the eel was wrapped in dampened paper and placed on an operating table. Anesthesia was maintained during the operation by injecting a few milliliters of freshly prepared anesthetic solution into the bucco-pharyngeal cavity.

Int.aortica cannula: This cannula (thir polyvinyl tube) was introduced about 2 cm into the ventral aorta, in a posterior direction, according to the method described by Kirsch (1972a,b). The cannula passed through a rubber jacket which covered the aorta and made the area of the surgical wound impermeable.

Urinary bladder cannulation: It was made according to the method described by Kirsch (1972), which inserts a flexible polyvinyl cannula in the bladder via the urethra and passing through a small orifice made in the body wall.

Frog blood sampling. Blood sampling in anesthetized and cannulated animals was made according to the technique described by Schroeder (1981).

Anesthetized animals: Frogs are anesthetized by means of a 6% Tricaine methane sulfonate solution (MS 222) injected into the dorsal lymphatic sac (0.1 ml/30 g body wt). After anesthesia a skin and muscle incision is made at the thoracic level, and the pericardium and frenulum are cut. Blood is then slowly collected by means of a 1-ml heparinized (500 U/ml-1) syringe.

Cannulated animals: Frogs are anesthetized by immersion in a 1.5% Urathan solution. After thoracic skin and muscle incision, a catheter (Silastic medical grade tubing, 0.5-mm i.d., 0.9 mm o.d.) pierced in the middle part is introduced into the frog ventricle. A small polyvinyl round is applied against the external ventricular wall in order to keep the catheter in position. On the external body side it is kept on the dorsal skin with a small hook. Blood sampling can be made 24 hr after cannulation.

Bioassay Apparatus

A Perspex microchamber, immersed in a temperature-regulated water bath (+18°), contains aerated (sterilized air) Ringer solution to which test substances are added. The isolated vessel is connected to a strain gauge (Statham Type G10 B) and activity monitored (Fig. 1). The microchamber and strain gauge are mounted on a steel column that allows vertical adjustment.

A second system above the strain gauge permits independent movement of the microchamber. This arrangement allows ready regulation of initial stretch applied. In addition, a blocking device mounted on the strain gauge is used during the mounting procedure. The steel column, the aeration system, and the water bath are mounted on an antivibratory stand to minimize extraneous disturbance to the highly sensitive recording system. The Perspex microchamber is 30 mm long, 25 mm wide, and 15 mm deep, and contains a volume of about 0.5 ml (Fig. 2). Inside there are two vertical channels containing, respectively, the blood vessel (4-mm diameter) and providing aeration (3-mm diameter).

Preparation of the Eel Ventral Aorta and Practical Realization of the Bioassay

The ventral aorta is dissected from the decapitated freshwater silver eel (300–400 g), cut in helical strips, and suspended vertically in the Perspex microchamber containing freshwater eel Ringer solution. An initial tension of between 300 and 700 mg is applied. This isolated preparation is perfused overnight (3 ml/hr) to stabilize it. After equilibration the Ringer perfusion is stopped and the standard hormone, drugs, tissue, and plasma extracts or urine samples are injected into the

1 Composition of the Ringer solution (in mM): NaCl (114.4), NaHCO3 (26.2), Na2HPO4 (1.6), KCl (2.0), KH2PO4 (0.3), CaCl2 (1.3), MgCl2 (10), (NH4)2SO4 (0.4), glucose (10); pH 7.4–7.5.
Fig. 2. Bioassay microchamber. Detailed view of the experimental bioassay apparatus: 1, microchamber; 2, ventral aorta strip; 3, stainless-steel stalk; 4, strain gauge with blocking device; 5, Teflon plugs with and without catheters; 6, Ringer solution catheter; 7, compressed air catheter; 8, Ringer aspiration catheter; 9, microsyringe used for sample injection; and 10, outside thermoregulated water bath.

Fig. 3. Verification of the bioassay procedure. Example of eel ventral aorta contractions recording: $S_1$ (standard 1), 6.6 pg AVT/ml$^{-1}$; $S_2$ (standard 2), 4.1 pg AVT/ml$^{-1}$; $U$ (unknown), 5.3 pg AVT/ml$^{-1}$; (a) first half aorta, (b) second half aorta from the same eel, (R) Ringer perfusion.

is recorded. When the maximal response is reached, the hormone is removed by washing the strip with Ringer solution at the same rate as before until the baseline is reached. Return to baseline, however, required 30–90 min, and only six (or at most seven) contractions can be recorded with one aorta strip in the same day, i.e., two doses of standard AVT (twice) and one dose of sample (twice). In our three-point assay the two doses of standard AVT and the dose of sample are each tested four times. This requires two aorta strips, which are dissected from the same eel by cutting the aorta in two equal parts 8–10 mm long and 2 mm wide, mounted in two separate microchambers.

Example of application: Verification of the bioassay procedure. Verification is made by use of synthetic AVT. Three solutions were prepared in eel Ringer solution: $S_1$, $S_2$, and $U$. Solution $U$ was considered the "unknown," and the following concentrations (pg/ml$^{-1}$) were chosen: $S_1$ (standard 1), 6.6; $S_2$ (standard 2), 4.1; and $U$ (unknown), 5.3. When the eel aorta strips are equilibrated and the Ringer perfusion stopped, $S_1$ and $S_2$ were first injected in the microchambers for initial check of "dose–response" validity. Then, $U$, $S_1$, and $S_2$, were injected and four contractions recorded for each hormone solution (Fig. 3). In all cases only 10 μl of each solution was used and after contraction recording the corresponding developed isometric force was determined (Table 1). Then constants $K_1$ and $K_2$ are calculated:

"unknown" developed isometric force

$$K_1 = \frac{\text{"unknown" developed isometric force}}{S_1 \text{ developed isometric force}}.$$ 

"unknown" developed isometric force

$$K_2 = \frac{\text{"unknown" developed isometric force}}{S_2 \text{ developed isometric force}}.$$
### TABLE I
VALUES OF THE DEVELOPED ISOMETRIC FORCES FOR STANDARD AND "UNKNOWN" AVT SOLUTIONS

<table>
<thead>
<tr>
<th>AVT concentration in the injected samples (pg/10 μl)</th>
<th>Standards</th>
<th>&quot;Unknown&quot;</th>
<th>Constants</th>
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<tbody>
<tr>
<td></td>
<td>$S_1$</td>
<td>$S_2$</td>
<td>$U$</td>
</tr>
<tr>
<td>Developed isometric force (mg)</td>
<td>82</td>
<td>88</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>78</td>
<td>52</td>
</tr>
<tr>
<td>Mean</td>
<td>80.5</td>
<td>43.7</td>
<td>57.5</td>
</tr>
<tr>
<td>Standard error (σ)</td>
<td></td>
<td></td>
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<tr>
<td>Error (%)</td>
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</table>

The "unknown" concentration is determined according to

$$U = K_1 \text{(mean)}$$

$\times$ (standard $S_1$ concentration),

$$U = K_2 \text{(mean)}$$

$\times$ (standard $S_2$ concentration).

The "unknown" AVT solution was initially chosen as 0.053 pg/10 μl injected in the microchamber. Using our eel aorta bioassay the following values are obtained. With $S_1$ standard: $U = 0.047 \pm 0.003$ pg AVT/10 μl; with $S_2$ standard: $U = 0.054 \pm 0.001$ pg AVT/10 μl.

Our method applied to many other assays gives errors from 2% (min) to 23% (max). This represents a valuable result in respect to the very small hormone concentrations used.

### Extraction of Tissue and Plasma

For bioassay and radioimmunoassay, neurohypophyses and pineal glands were extracted in 0.1 N HCl (or in hot 0.25% acetic acid for 5 min) and centrifuged. The pellet was discarded and the supernatant (diluted in Ringer solution) assayed. To compare bioassay with radioimmunoassays each extract was divided into three parts and run simultaneously in the eel ventral aorta test, the RIA-I and RIA-II systems (see below).

An adaptation of the acetone method (Ginsburg and Smith, 1959) for rat plasma oxytocin was used for eel plasma. This method gives recoveries of 64 ± 1% and produces clear extracts.

Frog (R. esculenta) plasma was extracted using an adaptation of the Skowsky et al. (1974) bentonite technique. This gives recoveries of 66.8 ± 2%. In both cases the extracts were evaporated to dryness under a gentle stream of compressed air and redissolved in the bioassay or RIA buffer.

### Radioimmunoassays

Radioimmunoassay for AVT using an AVP antibody (RIA-I). An antibody raised against AVP (L 35) was used. Titer, radiodination, specificity, and sensitivity of this RIA have been described previously (Holder et al., 1979). The greatest sensitivity is obtained with AVP and AVT. Since, so far as is known, AVP is not present in nonmammalian vertebrates, this RIA can be applied to measure AVT in such vertebrates. There is no cross-reactivity with oxytocin, isotocin, and mesotocin, even when present at concentrations at more than 1 ng/0.1 ml.

Radioimmunoassay for AVT using an AVT antibody (RIA-II). Procedures for raising antibodies, preparation, and purification of labeled hormone, standard curves, and extraction procedures of the hormone with activated Vycor glass powder have been described (Dogterom et al., 1979, 1980).

### Hormones and Drugs

The following were used for bioassay and radioimmunoassays determinations: arginine–vasotocin, 78 U/mg oxytocic activity (Hoffmann La Roche, Switzerland); isotocin, 390 U/mg oxytocic activity (UCB, Bioproducts Peptide Department, Belgium); mesotocin, 200 U/mg oxytocic activity (Dr. Vandesande, The Netherlands) and 390 U/mg oxytocic activity (Dr. Sawyer, USA); oxytocin, 430 IU/mg (Ferring, Germany); arginine–vasopressin, 400 IU/mg (Ferring, Germany); melatonin, serotonin creatinine sulfate, and noradrenaline (Fluka, Switzerland); adrenaline (Sigma, USA); and antiserum L 35 (Dr. Lutz-Bucher, France).
Statistical Analysis

The Student Fisher $t$ test has been applied to compare the mean values in the different assays.

RESULTS

Properties and validation of the bioassay

Response to synthetic AVT. The responses of the eel ventral aortic strip to synthetic AVT were examined at concentrations between $10^{-6}$ and $10^{-16} M$. For each dose the recorded response was expressed as percentage of the maximal contractile force (Fig. 4). There was a good correlation between hormone concentration and isometric contraction, and eel aortic strip responds to AVT 1–6 min after hormone addition. It is possible to work at two sensitivity levels: one corresponding to AVT at $10^{-16} M$ occurs under the most favorable conditions, while the other is more usual employs 5–10 µl of a $6.6 \times 10^{-12} M$ AVT solution (final concentration $6.6 \times 10^{-14}$ and $1.32 \times 10^{-13} M$, respectively). The aortic strips give reproducible responses to AVT, except as noted during January and February when there is a significant reduction in sensitivity.

Comparison of AVT with other substances. To assess the sensitivity and specificity of the bioassay, the smallest effective concentrations of AVT and related hormones were compared. In addition, substances known to be present in the pineal gland were tested. Table 2 shows

<table>
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<th>TABLE 2</th>
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<tr>
<td><strong>EEL AORTA BIOASSAY: COMPARISON OF THE SMALLEST EFFICIENTLY CONCENTRATIONS OF AVT AND RELATED SUBSTANCES</strong></td>
</tr>
<tr>
<td>Substances</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Arginine–vasotocin (AVT)</td>
</tr>
<tr>
<td>Isotocin (ICT)</td>
</tr>
<tr>
<td>Oxytocin</td>
</tr>
<tr>
<td>Arginine–vasopressin (AVP)</td>
</tr>
<tr>
<td>Mesotocin (MT)</td>
</tr>
<tr>
<td>Adrenaline</td>
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<td>Noradrenaline</td>
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<td>Melatonin</td>
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* In the most favorable cases.
that AVT induces, in the most favorable cases, isometric contractions down to concentrations of $10^{-10} \text{ M}$. All the other substances tested also induce contractions (except serotonin at $6 \times 10^{-5} \text{ M}$), but much greater concentrations are required.

**Response to endogenous and exogenous AVT.** Experiments were carried on endogenous (eel plasma) and exogenous (synthetic) AVT. Figure 5 shows that with a percentage error of 9 to 20% the response of the isolated aorta is summative for the two hormones. Exogenous AVT does not disappear.

**Response to synthetic AVT incubated with the AVP antiserum.** Synthetic AVT was incubated with the L 35 antibody at +37°C. Four experiments were performed: two with a $0.5 \times 10^{-3}$ final antibody dilution, and two with a $0.5 \times 10^{-5}$ final antibody dilution. In the first experiment the AVT activity disappears after an incubation of 1 to 3 hr. In the second experiment, the biological AVT disappears after an incubation of 4 to 6 hr.

**Applications: Comparison of Radioimmunoassays and Bioassay for the Same Extracts**

Neurohypophysial extracts. Table 3 summarizes the results obtained from the neurohypophysial extracts of the various vertebrates. The AVT content varies with species, but there is good correlation between the three systems for any given extract, except for the trout and the tortoise where significant differences are apparent.

Pineal extracts. Pineal extracts of the...
same species of nonmammalian and mammalian (Table 4) vertebrates were tested in the three assays. It appears that AVT activity is detectable in all the nonmammalian vertebrates (except in the lamprey, RIA-II) and there is a good agreement between the three assays in trout, tortoise, lizard, and snake. In frog, quail, and chicken the RIA-II system gave significantly lower values than either the RIA-I system or the bioassay ($P < 0.01$). Among the mammals biological and RIA-I immunoreactivity was detectable with the rabbit displaying highest values. RIA-I gave no detectable AVT.

**AVT in plasma.** Preliminary extraction of the hormone from eel and frog plasma is necessary, since, in general, unextracted plasma displays only weak AVT activity, and there is a clear discrepancy between bioassay and RIA-I values (Pollatz et al., 1979; Schroeder et al., 1979, 1980). Extracted plasma AVT values are similar in bioassay or RIA-I (Table 5). In the frog the lowest plasma hormone concentration appears in cannulated animals.

**AVT in urine.** Preliminary experiments showed that it was not necessary to use previously extracted urine for AVT bioassay and RIA determinations (Guerné et al., 1977). We report the measurement of urine AVT activity (Table 6) in an unanesthetized freshwater eel with cannulated urinary bladder. The bioassay and RIA-I correlate well.

**DISCUSSION**

In vertebrates general AVT responsive tissues include the smooth muscle of the female genital tract (Lederis, 1970; La-
pointe, 1969, 1977), isolated blood vessels (Somlyo and Somlyo, 1968), and isolated gills (Maetz and Rankin, 1969; Rankin and Maetz, 1971). Indeed, Heller et al. (1970) found the oviduct of Necturus maculosus to respond to $10^{-16} \text{M}$ AVT and concentration similar to that observed in the present studies using the eel ventral aortic strip. Apart from the present studies none of these preparations have been suggested as practical quantitative bioassays.

The isolated urinary bladder of the frog (Sawyer, 1960) or toad (Bentley, 1969) are currently used for AVT bioassay, but at best concentrations of about $10^{-12} \text{M}$ are detectable. In these hydroosmotic tests, 10 ml of Ringer solution is required, so that about 10 pg AVT must be present. In the eel bioassay described the equivalent amount of AVT is about 0.05 pg (contained in 0.5 ml). This sensitivity is much greater than other bioassays and available immunoassays, and makes the preparation of obvious application in studies of animals from which only tiny volumes of blood can be obtained.

The data on the properties and validity of the eel ventral aortic strip bioassay may be summarized as follows. Isoleucine at position 3 donates activity, since AVP reduces activity; polar residues at position 4 are important, since isotocin is the next most active substance after AVT. These are all structure–activity characteristics typical of neurohypophysial peptides in hydroosmotic tests.

The eel bioassay reported also appears specific for AVT since after incubation with antisera the AVT biological activity disappears in a few hours, demonstrating that the biological activity corresponds to the immunoreactivity of the antibody and that the aorta response is completely due to the hormone.

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**REFERENCES**


