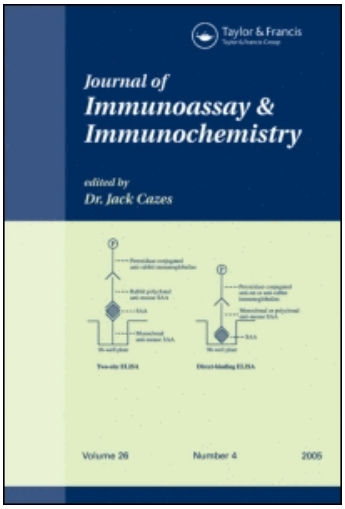

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Enzyme Linked Immunosorbent Assay for the Neurohypophyseal Hormones Arginine Vasotocin and Isotocin

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ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE NEUROHYPOPHYSEAL HORMONES ARGININE VASOTOCIN AND ISOTOCIN

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ABSTRACT

A competitive enzyme linked immunosorbent assay (ELISA) using new polyclonal antibodies was developed for the first time for each of two neurohypophyseal hormones: arginine vasotocin (AVT, ubiquitous in vertebrates) and isotocin (IT, restricted to teleost fish). The antibodies obtained were highly specific, showed no cross-reaction between the two peptides and were able to suppress the activity of the peptides in *in vitro* bioassays. An original feature of the assays was the covalent binding of the peptidic antigen to Covalink microplates. Competition was thereafter made between this bound antigen and the antigen in samples, for a fixed amount of the relevant antibody. Revelation used peroxidase-labeled antibodies. These ELISAs were sensitive enough to detect 1 ng/ml and 0.1 ng/ml for AVT and IT respectively. Moreover, for the first time, the two hormones were measured separately, each in the presence of the other, and shown to exist as circulating hormones in fish.

(**KEY WORDS:** ELISA - polyclonal antibodies - neurohypophyseal peptides - vasotocin - isotocin.)

INTRODUCTION

Neurohypophyseal peptides have represented for decades a fascinating chapter of vertebrate neuroendocrinology. In mammals, the two main hormones,

arginine vasopressin (AVP) and oxytocin (OT), while very close by their molecular structure (Table 1), exert much dissimilar functions. They are attracting renewed attention owing to the recent discovery of their central effects, which appear numerous and of utmost importance, and of their local intervention in newly discovered production/target sites like the reproductive system or the adrenal cortex. Among vertebrate classes, fish in total undoubtedly possess the largest number of these hormones since no less than 7 peptides have been identified in that group. The neurohypophysis of teleost fish produces arginine vasotocin (AVT), in common with the other sub-mammalian vertebrates, and isotocin (IT) which is restricted to teleosts. Interestingly however, AVT or AVT-like material has been found by bioassay or radioimmunoassay in fetal mammalian pituitary gland, newborn human cerebrospinal fluid and some mammalian, including human, pineal gland (see 1, 2).

Despite many investigations, the physiological functions of these hormones are still poorly defined in fish. The largest number of *in vivo* studies were directed towards the role of these peptides in osmoregulatory processes (3). However, their actions were often obscured by a systemic vasopressor effect induced simultaneously. In addition, the doses used varied considerably, so that it was difficult to predict whether they were physiological or not. For example, AVT provoked diuresis or antidiuresis depending on the amount administered (4, 5). More interestingly in this respect, AVT or IT were shown to inhibit cyclic AMP production in several fish tissues (6, 7, 8, 9) and to act by means of a new membrane receptor type (10, 11, 12).

The physiological relevance of these results cannot be established without proving the presence of circulating levels of these peptides. In mammals, plasma levels of neurohypophyseal peptides are currently measured by radioimmunoassay, RIA (13, 14, 15) and rarely by enzyme linked immunosorbent assay, ELISA (16, 17). Attempts to measure AVT (or related peptides) in hypophysis or plasma samples were described in different groups of lower vertebrates and invertebrates using bioassays (18, 19), high performance liquid chromatography, HPLC (20) or RIA. Sensitive radioimmunoassays have been made available to measure AVT in humans, birds, reptiles, amphibians, fish and gastropods. However, in most cases, the antibodies used in these assays were

TABLE 1

Amino Acid Sequence of Main Neurohypophyseal Peptides and Synthetic Analogues.

	AMINO ACID POSITION								
	1	2	3	4	5	6	7	8	9
NATURAL ANALOGUES:	⌜					⌝			
arginine vasotocin (AVT)	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Arg	Gly
isotocin (IT)	-	-	Ile	Ser	-	-	-	Ile	-
arginine vasopressin (AVP)	-	-	Phe	Gln	-	-	-	Arg	-
oxytocin (OT)	-	-	Ile	Gln	-	-	-	Leu	-
mesotocin (MT)	-	-	Ile	Gln	-	-	-	Ile	-
lysine vasopressin (LVP)	-	-	Phe	Gln	-	-	-	Lys	-
SYNTHETIC ANALOGUES:									
lysine vasotocin (LVT)	-	-	Ile	Gln	-	-	-	Lys	-
tocinoic acid (TOC)	-	-	Ile	Gln	-	-OH			
pressinoic acid (PRES)	-	-	Phe	Gln	-	-OH			

raised against AVP (21, 22, 23) or against lysine vasopressin, LVP (24), instead of against AVT itself. Authors have used a property of these antibodies to crossreact highly with AVT (where AVT and AVP are not simultaneously present). Therefore, very few RIAs have been set up using anti-AVT antibodies (1, 2, 25, 26, 27). To our knowledge, no RIA measurement was reported concerning isotocin. Futhermore, until now, no non-isotopic immunoassay method concerning AVT or IT has been published. Important advantages of the method compared to RIA are that no radioactivity is needed and problems linked to antigen damage as a result of its iodination are circumvented.

In the present study, we describe a specific competitive ELISA to measure AVT or IT. The first part of this work was to design appropriate antigens in order to produce specific anti-AVT or IT antibodies in rabbits and to set up the assay. The second was to characterize the antibodies regarding their titer and specificity. Furthermore, the efficiency of these antibodies in inhibiting some hormonal (AVT or IT) effects was studied on classical bioassays (on frog skin and urinary bladder). The third part of this work was to apply the method to measure blood levels of AVT and IT in the rainbow trout.

MATERIALS AND METHODS

A-Chemicals

Arginine vasotocin (AVT), isotocin (IT), arginine vasopressin (AVP), oxytocin (OT), mesotocin (MT), lysine vasopressin (LVP), lysine vasotocin (LVT), tocinoic acid (TOC), pressinoic acid (PRES) were from Bachem, California. Bovine neurophysins I (NP I) and II (NP II), angiotensin II (A II), urotensin II (U II), human atrial natriuretic factor (ANF) and neurotensin (NT) were from Sigma. Porcine insulin (INS), human insulin-like growth factors I (IGFI) and II (IGFII) were generously given by Dr Y. Lemarchand-Brustel (Nice).

B-Anti-AVT or IT Antisera Production

Hormone-carrier conjugation

AVT and IT have a molecular weight around 1000, too low to induce a consistent immunological response in rabbits. Their immunological potency was increased by conjugating them as haptens with a heavier carrier. For this purpose we used the keyhole limpet hemocyanin (KLH, from Sigma) coupled to the peptides with glutaraldehyde. KLH was preferred to bovine serum albumin (BSA, Boehringer) because the latter was used routinely to decrease the non specific binding in the ELISA. Briefly, 10 mg of KLH were mixed in 2ml phosphate buffer saline (PBS), pH 7.4, containing 5 mg of pure AVT (or IT, prepared in 10% N,N dimethylformamide, DMF from Sigma). Then, 20 µl of glutaraldehyde 25% (Serva) were added to the mixture and gently agitated at room temperature for 15 min. After this time, 10 more µl of glutaraldehyde 25% were added and the resulting solution was agitated for 15 min. The conjugation process was stopped by adding 400 µl of glycine 1M, pH 6.3. Five minutes later, the mixture was introduced in a dialysis bag (exclusion size ≤10 kD, Polylabo) which was placed in 2 liters of PBS and left overnight at room temperature. The dialysed mixture

was centrifuged 5 min at 1600g, the supernatant containing the KLH-conjugated peptide was separated from the pellet and aliquoted as 400 µl fractions which were kept frozen (-20°C), to be used as the source of antigen for the immunization procedure.

Immunization

Six rabbits (obtained from a local supplier), 3 for each peptide, were used for immunization. An emulsion was prepared by adding 400 µl of sterile distilled water and 800 µl of Freund's complete adjuvant (Sigma) to one conjugate fraction (400 µl). Each rabbit received 500 µl of the emulsion in 3 subcutaneous injections between the shoulders. This step was repeated twice (3 weeks delay in between). The first bleeding was made 10 days after the last injection, before booster injections. The antisera recovered were divided into 50 to 500 µl fractions which were kept frozen at -20°C. A code number was given to each antiserum retained, using the name of the immunizing peptide, followed by rabbit and batch numbers (e.g. anti-AVT-1 b2).

C-Bioassays

They were performed on isolated tissues of the frog Rana esculenta. Urinary bladders dissected out and filled with distilled water were placed in 40 ml of air-gassed amphibian Ringer solution (NaCl 112 mM, KCl 5 mM, $CaCl_2$ 1 mM, $NaHCO_3$ 2.5 mM, glucose 5.55 mM, pH 7.4). The osmotic water loss was monitored by weight variation every 5 min and the response to neurohypophyseal peptide stimulation followed for 20 min. Considering that the bladder volume decreased all along the experiment, the results were expressed by the relative weight variation during 5 min versus the preceeding bladder weight ($-\Delta P/P$). The consequence of the antibody binding to AVT or IT was estimated by comparing the water loss induced by 10^{-9}M AVT or 10^{-7}M IT in the absence or in the presence of a dilution range of the respective antibody (from 1:80,000 to 1:10,000 for anti-AVT and from 1:50,000 to 1:5,000 for anti-IT).

Frog skins were placed in conventional Ussing chambers modified from Ussing and Zerahn (28) with automatic recording of the short-circuit current

which, in this tissue, is equal to the active transport of sodium. The tissue area between chambers was 0.6 cm^2 and each chamber contained 1 ml of amphibian Ringer solution gassed with air. As for the bladder, the anti-neurohypophyseal peptide antibodies were used to inhibit the stimulatory effect of AVT (or IT) on the transport of sodium.

Each experiment was repeated 6 times. For the sake of simplicity, representative figures are given in Results.

D-ELISA Procedure

The method is based on competition between the neurohypophyseal peptide in samples and the same peptide immobilized in microplate wells, and this for a limiting amount of specific antibody. Because of their small molecular size, the peptides do not bind easily to classical plates. We therefore made use of specific microplates (Covalink, Nunc, Denmark) designed to increase the quantity of coated peptide and to produce homogeneous orientation of the peptide in each well. These plates are known to possess NH radicals reacting with disuccinimidyl suberate (DSS, Pierce). DSS is a specific linker which may bind covalently to 2 NH radicals, one belonging to the plate and the other to the peptide. Preliminary experiments carried out to determine the best conditions to run the assay led us to choose the following parameters: coating concentration of each peptide, concentration of BSA used to reduce the non specific binding, optimal dilution of each antiserum, duration and temperature of each step of the assay. The final optimized conditions were as follows.

Solutions

DSS solution: 100 µg/ml sodium phosphate buffer 0.1M, pH 7.4;

Coating buffer: Na_2CO_3 0.05M , $NaHCO_3$ 0.05M pH 9.6;

Sodium phosphate buffer 0.01M pH 7.4 (PB);

Blocking buffer: PB + 1% BSA;

Washing buffer: PB + 0.05% Tween 20 (Polyoxyethylen-sorbitan monolaurate, Sigma);

Diluting buffer: PB + 1% BSA + 0.05% Tween 20;

Substrate solution: citrate phosphate buffer (citric acid 0.1M, Na_2HPO_4 0.2M pH 5) + 0.3% orthophenylenediamine dihydrochloride (OPD, Sigma) + 0.02% H_2O_2 (Prolabo);

H_2SO_4, 2M.

Coating

Plates were pretreated with DSS solution (100 µl/well) for 2h at 4°C. After this period, plates were rapidly emptied and 100 µl of peptide (AVT or IT) or BSA (to determine the non specific binding) diluted to 0.5 µg/ml in coating buffer were added to the wells. Plates were left to incubate for 1 hour at 37°C, then overnight at 4°C.

Blocking

Plates were emptied and saturated with blocking buffer (200 µl/well) for 2 hours at 37°C.

Washing

Plates were carefully emptied and filled with washing buffer (300 µl/well) and left for 2 min before they were emptied again. This step was repeated 3 times.

Competition

Plates were filled with standards or samples (100 µl/well), then with specific anti-AVT or anti-IT antibody (100 µl/well) and left overnight at 4°C. Standards, samples and antibodies were prepared in diluting buffer. Plates were washed as above.

Developing

This procedure was carried out in several steps.

Swine immunoglobulins to rabbit immunoglobulins (Dakopatts) were used as second antibodies. They were diluted 5,000 times in diluting buffer, thereafter added to each well (200 µl/well) and incubated for 1h at room temperature. Plates were washed as above.

Soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase (PAP, Dakopatts) was diluted 5,000 times in diluting buffer, added to each well (200 µl/well) and incubated for 45 min at room temperature. Plates were washed as above.

Plates were filled with substrate solution (200 µl/well). After 30 min in the dark at room temperature, the reaction was stopped by adding 2M H_2SO_4 (50 µl/well).

Absorbance was read at 492 nm in an automatic plate reader (Titertek, Multiskan MCC/340 from Flow Laboratory, France).

Calculations

The optical density (OD) measured corresponded to the total binding (specific + non specific) of the anti-peptide antibody in each well. BSA-coated wells yielded the non specific signal. Specific binding (B) was expressed by the difference: Total minus Non specific OD.

In competition tests, Bo represented the maximum binding of anti-peptide antibody to the coated peptide and corresponded to the specific OD calculated in the absence of soluble peptide. The ratio B/Bo was calculated for each dose of soluble peptide. The standard curve expressed by B/Bo as a function of soluble peptide concentrations was converted into a "logit" representation to yield a linear relationship by using the least square method. The "logit" curve was used to estimate peptide concentrations in unknown samples.

The ELISA standard curve also provided two main parameters: The D_{10} and D_{50} expressing the concentrations of AVT or IT which decreased the B/B0 by 10 and 50 % respectively from its maximum value.

The specificity of antisera was analyzed in the presence of various molecules (related peptides and others) susceptible of recognition by the antibodies. These molecules, in increasing concentration, were allowed to compete with a fixed amount of AVT or IT coated in the wells. Cross-reactivity was expressed (in %) by the ratio between the respective D_{50} of the relevant peptide and the tested molecule.

Representative curves of antibody titre and specificity and hormone assay are provided in Results.

E-Trout Plasma Extracts

Rainbow trout, Salmo gairdneri (average weight 220g), were purchased from a local fish farm and maintained at 12-15°C in the laboratory, in running tap water (freshwater, FW-adapted trout), in constant photoperiod (12 h light per day).

Blood was collected from the caudal blood vein with a heparinized syringe, centrifuged for 10 min at 1600g and plasma was stored at -20°C. AVT and IT extraction was made by reverse-phase chromatography using disposable Sep-Pak C_{18} cartridges (Millipore UK Ltd). First, a 2 ml plasma sample was mixed with 2 ml sodium phosphate buffer, 0.05 M, pH 7.4. The cartridge was activated with 5 ml of methanol followed by 20 ml of distilled water. Secondly, the diluted plasma was pushed slowly through the column over 4 min. The column was subsequently rinsed with 20 ml of 4% acetic acid. Then, 3 ml of methanol were brought into the column, left 3 min and flushed afterwards. The eluate, which contained the neurohypophyseal peptides, was dried at 37°C under an air stream and stored at -20°C. For measurement by ELISA, the sample was reconstituted in the appropriate volume of diluting buffer. The extraction efficiency, determined by recovery of either ^{125}I-labelled AVT or ^{125}I-labelled IT added to a plasma before extraction, was estimated around 80%.

RESULTS

A-Properties of the Antibodies

Antisera titers

After each rabbit bleeding, the titer of the anti-AVT or IT antibody was estimated by measuring its binding to AVT or IT coated into the wells. For this purpose, various antibody dilutions were used. As usual, the titers obtained were different between rabbits and, for a given rabbit, between the successive blood collections. For the following experiments, we selected the anti-AVT-1 b2 antibody in a final dilution of 1:50,000 and the anti-IT-3 b2 antibody in a final dilution of 1:20,000 (Figure 1), these values corresponding to the antibody titers.

Antisera specificity

The two hormones were compared to other neurohypophyseal peptides or synthetic analogues (see amino acid sequences in Table 1). Specificity of the anti-AVT antibody appears in Figure 2a. Only four peptides were recognized by

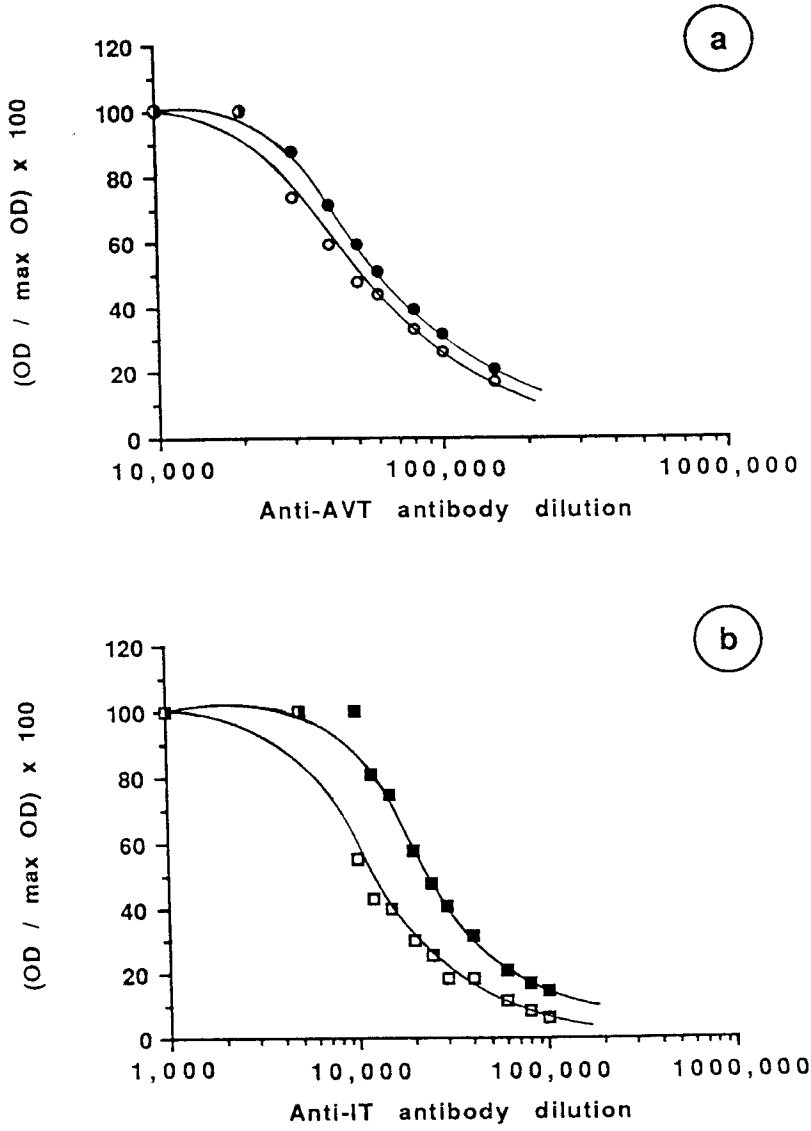


FIGURE 1 Titer determination of the anti-AVT (a) or anti-IT (b) antisera obtained from batch 1 (open symbols) and batch 2 (closed symbols).

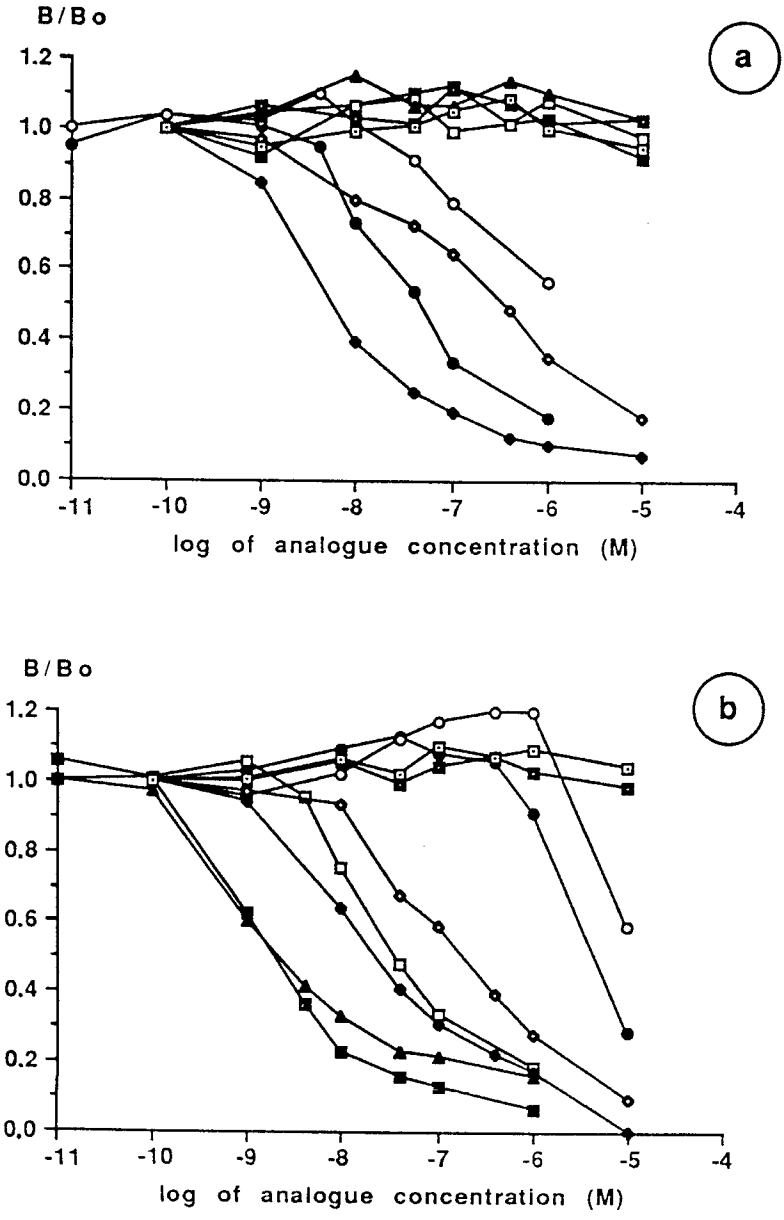


FIGURE 2 Specificity of anti-AVT (a) or IT (b) antibodies. Neurohypophyseal peptides or analogues allowed to compete with coated AVT (a) or IT (b) are: ● AVT, ■ IT, ○ AVP, □ OT, ▲ MT, ◇ LVP, ◆ LVT, ▫ TOC, ⊡ PRES.

the antibody. With reference to AVT recognition taken as 100%, lysine vasopressin, LVP (11%) and arginine vasopressin, AVP (3%) were poor competitors. However, lysine vasotocin, or LVT, a non-natural analogue, was 7 times better competitor than AVT itself. Cross-reactivity with other peptides was not significantly different from 0 (Table 2).

Results concerning the anti-IT antibody are presented in Figure 2b. A small number of peptides bound to the antibody but with much lower affinity in comparison to IT (100 %): namely LVT (9%), OT (5%) and LVP (1%). Interestingly mesotocin, MT, was recognized as well as IT. Cross-reaction with other peptides was not significantly different from 0 (Table 2).

Cross-reaction was also measured for a number of natural peptides. Neurophysins I and II (generally co-secreted with neurohypophyseal hormones), Angiotensin II and Atrial Natriuretic Factor competed less than 4.4% (Table 2). Other peptides (neurotensin, urotensin II, insulin and insulin-like growth factors I and II), not all homologous however, led values not significantly different from 0.

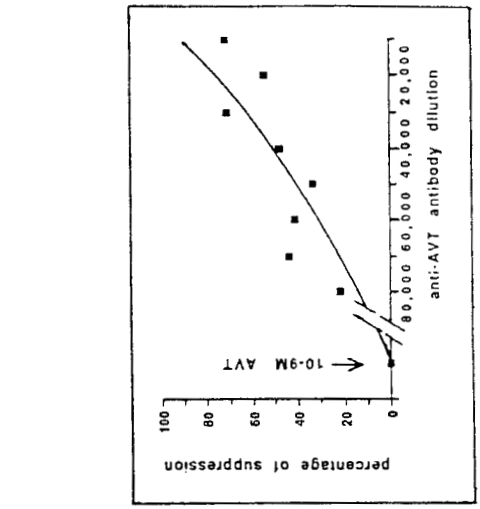
Biological tests in the presence of antisera

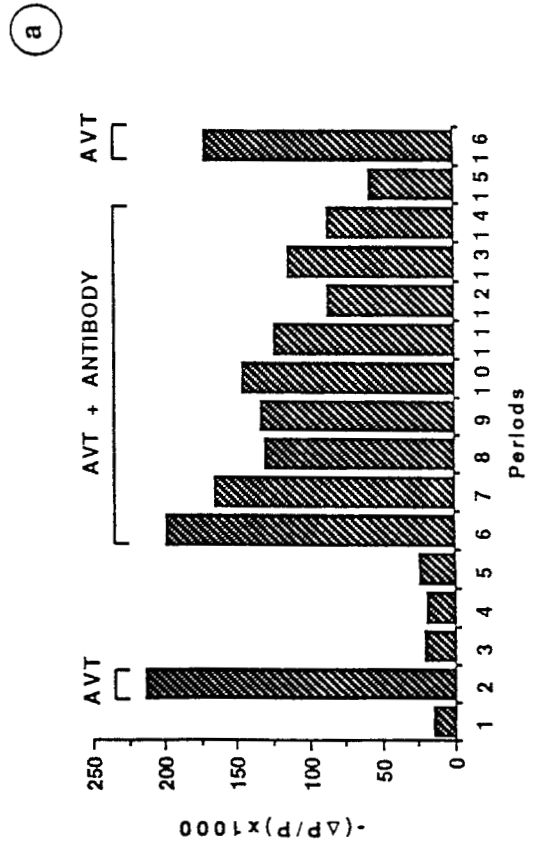
Control experiments on frog bladders (not shown) indicated that in the absence of AVT or IT, the basal osmotic water movement remained low and constant for 5 hours. In the following experiments, AVT and IT were added each at a dose giving a sub-maximal effect. Figure 3 (a and b) illustrates two typical experiments showing that a prime dose of 10^{-9}M AVT and 10^{-7}M IT induced an immediate and large increase in water loss (which was multiplied 11.5 fold by AVT and 5.6 fold by IT, on average). The stimulation induced by IT was lower by about 50% than that produced by AVT. These stimulatory effects were totally reversed after a 20 min period in Ringer. Controls indicated that antisera given alone had no effect on water transport. When the bladder was incubated in the simultaneous presence of the hormone (at the above fixed dose) and the corresponding antiserum (in increasing amounts) there was a gradual decrease in water movement. The efficiency of our antibodies to offset the AVT- or IT-induced biological effect was expressed as the "D_{50} dilution", i.e. the dilution of antibody producing 50% inhibition. The D_{50} so-defined was 1:40,000 and 1:10,000 for

TABLE 2

Cross-reaction of anti-AVT and anti-IT Antibodies with Neurohypophyseal Peptide Analogues and other Peptide Molecules.
% cross-reactivity was expressed as:
(D_{50} for AVT or IT / D_{50} for the substance tested) x 100.

LIST OF SUBSTANCES TESTED	PERCENTAGE OF CROSS-REACTIVITY	
	anti-AVT antibody	anti-IT antibody
AVT	100	0.04
IT	< 0.45	100
AVP	2.81	0.02
OT	< 0.45	5.25
MT	< 0.45	100
LVP	11.25	1.05
LVT	726	8.75
TOC	< 0.45	< 0.02
PRES	< 0.45	< 0.02
NP I	2.67	0.41
NP II	0.61	0.04
A II	0.07	0.00
ANF	4.38	2.40





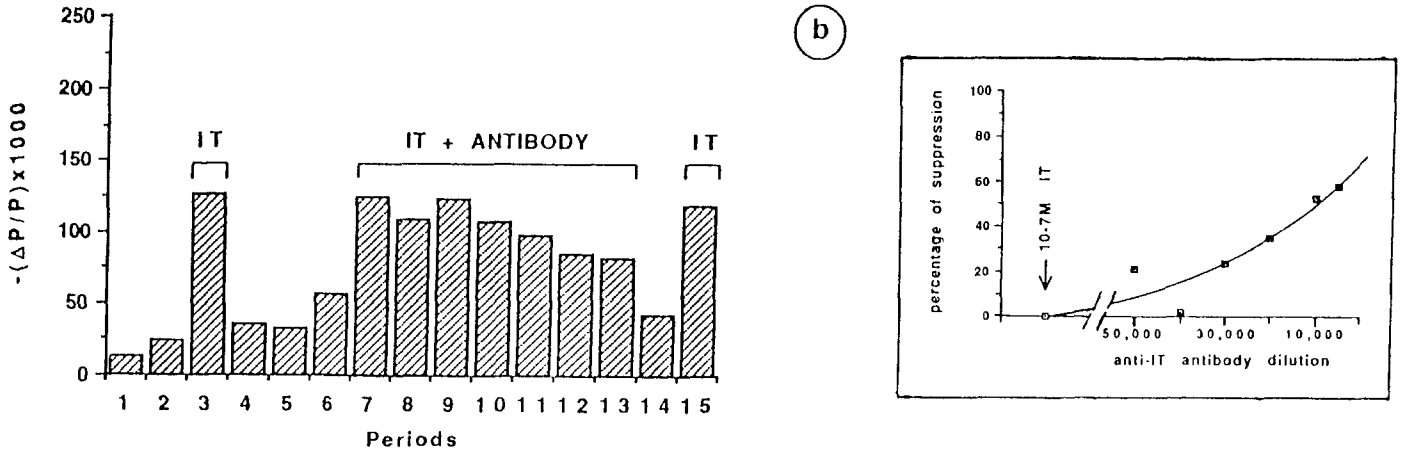


FIGURE 3 Inhibition by the antibodies of water transport (expressed by relative weight loss) in frog urinary bladder stimulated by the neurohypophyseal peptides. Numbers below solid bars refer to successive 20 mn periods.
a) Effect of the anti-AVT antibody. The excised and filled bladder was exposed to Ringer, 10^{-9}M AVT, Ringer, anti-AVT antibody diluted 1:10,000, Ringer (periods 1, 2, 3, 4, 5 respectively), then to 10^{-9}M AVT alone (6) or in presence of increasing quantities (dilutions 1:80,000 to 1:10,000) of the antibody (7 to 14). The urinary bladder was then washed with Ringer and weighed again after exposure to 10^{-9}M AVT (15 and 16).
b) Effect of the anti-IT antibody. Conditions were successively Ringer, 10^{-8}M IT, 10^{-7}M IT, Ringer, anti-IT antibody diluted 1:5,000, Ringer (periods 1, 2, 3, 4, 5, 6 respectively), then 10^{-7}M IT alone (7) or in presence of various dilutions of the antibody (1:50,000 to 1:10,000, 8 to 12; 1:5,000, 13). The urinary bladder was then washed with Ringer and weighed again after exposure to 10^{-7}M IT (14 and 15).
Inserts: Results expressed as percentage of suppression of the AVT (a) or IT (b) induced stimulatory effect, by dilutions (same as above) of the corresponding antibody.

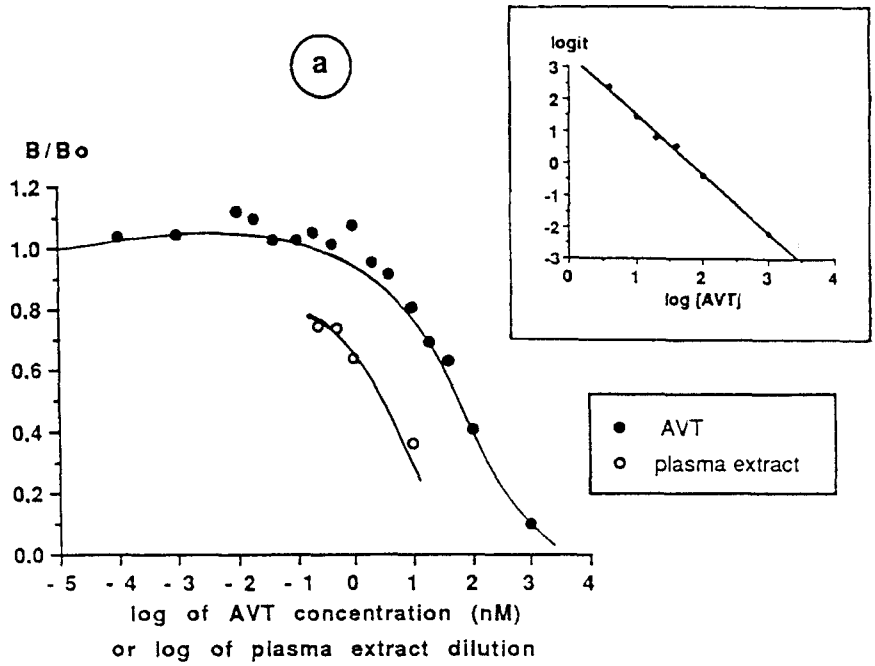


FIGURE 4 ELISA calibration curves for AVT (a) and IT (b).
Closed symbols: curve obtained with the peptide.
Opened symbols: curve drawn with different dilutions of a plasma extract.
Inserts: "logit" representation of each standard curve.

anti-AVT and IT antibodies respectively. Noteworthy, removal of the antibody at the end of the experiment restored full responsiveness to the hormone.

The frog skin bioassay yielded essentially similar results with AVT and its antibody (not shown). However no response was obtained with IT at any concentration up to 10^{-7}M (probably due to a lack of tissue sensitivity), nor with the anti-IT antibody tested alone or in presence of IT. Anti-IT antibody used in presence of AVT did not offset the AVT-induced biological effect, again suggesting the high degree of specificity of our anti-IT antibody.

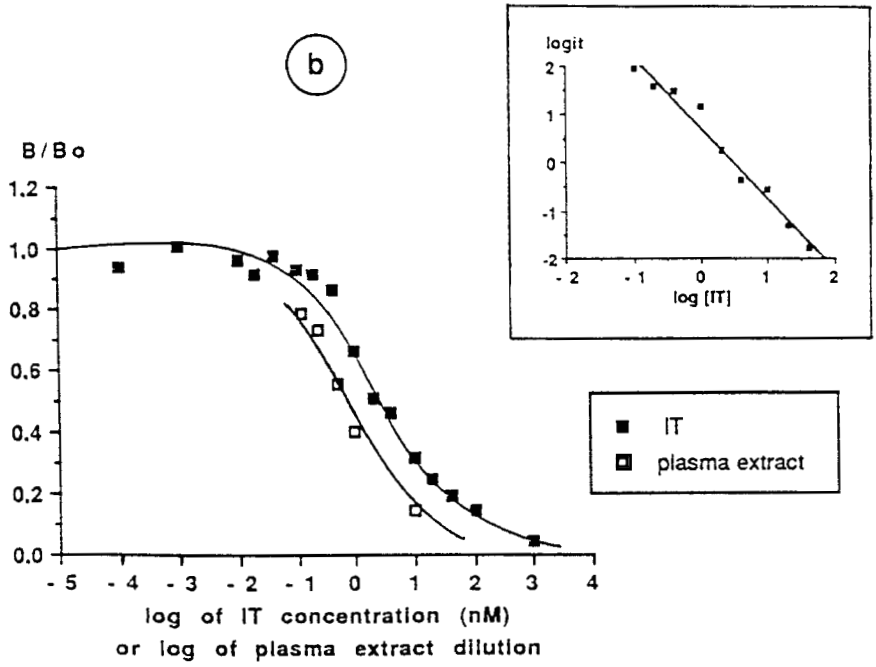


FIGURE 4 Continued

B-Characteristics of the ELISAs

Standard curves

Figure 4 represents typical calibration curves for AVT (a) and IT (b) assays. The "logit" linear representations were drawn from classical standard curves (inserts) for routine determination of peptide concentrations. The calculated regression coefficients were > 0.99.

The D_{10} and D_{50} defined above were 1 and 60 ng/ml respectively in AVT assay (Figure 4a) and 0.1 and 2.3 ng/ml in IT assay (Figure 4b).

Precision

In order to estimate the precision of each assay, extracts were prepared from pooled plasmas and hormone contents were measured in different conditions.

Samples were assayed 8 times in the same set of experiments and in 5 different series. The corresponding intra- and inter-assay variation coefficients were respectively 11% and 15% for AVT, 10% and 11% for IT. In addition, clear parallelism was noted between curves drawn with AVT or IT and those built with dilutions of plasma extracts (Figure 4, a and b).

C-Application to Hormone Levels in Blood

Setting up the assays allowed us to quantify peptide levels in FW-adapted trout plasma in resting conditions. AVT and IT circulating amounts were estimated to be 26.2 +/- 3.8 (n=19) and 5.5 +/- 1.3 (n=16) nM respectively.

DISCUSSION

The ultimate purpose of our investigation was, by estimating the circulating levels of neurohypophyseal peptides in fish, to provide the awaited firm evidence that the effects of these hormones recorded by many authors during the last thirty years are of physiological relevance (3). This significance was questioned on the ground that neurohypophysis, owing to its anatomical position and vascularization in fish pituitary, may simply provide local stimulation of the anterior lobe and on the contention that the peptide doses used in *in vivo* experiments were too high.

In addition, AVT is of general interest in the evolution of neurohypophyseal hormones since it has long been known to be present in all submammalian vertebrates and is also believed to occur in mammalian fetus and epiphysis (1, 2).

The present ELISA method is the first assay proposed for the two teleost fish hormones, arginine vasotocin and isotocin, by means of specific antibodies raised in the rabbit.

The method we have designed possesses all the advantages of reliable ELISA tests (sensitivity, specificity and reproducibility). Its main feature is the use of covalent binding of the peptidic antigen onto Covalink microplates. Then, competition was made between this bound antigen and the antigen in samples, for a fixed amount of the relevant antibody, and the result was visualized by using peroxidase-labeled antibodies.

The antibody titers are in a convenient range for immunoassays: 1:50,000 and 1:20,000 for the anti-AVT and IT antibodies respectively. Under these conditions, the background represents 15% of the maximum.

The main interest of our antisera resides in their high degree of specificity for the corresponding peptide. Thus the anti-AVT antibody recognized AVT, used as reference (100%), and LVP and AVP to much lower extents (11 and 3% respectively). Surprisingly, LVT was 7 times better competitor than AVT but this peptide is not present in any species. Cross-reaction with other analogues was not significantly different from 0 (Table 2). With respect to the molecular structure (Table 1), it is possible to speculate about the amino acids playing a major role in recognition. Tocinoic acid, which represents the cycle of AVT (and also of MT, OT and LVT), and pressinoic acid that of AVP (and LVP), are not bound by the anti-AVT antibody. This would indicate the importance of the linear tripeptide. The analogues recognized by this antibody (LVT>AVT>LVP>AVP) are structurally closely related. If we consider changes in the tripeptide, only the amino acid in position 8 varies (Arg or Lys). These two amino acids are polar and possess a lateral chain positively charged at pH7. They are structurally close enough but there is better specificity in favour of Lys. In addition, this difference is the only one existing between LVT and AVT on the one hand, and between LVP and AVP on the other hand, which may explain the respective affinities obtained. A second difference exists between the four analogues: Ile in position 3 in AVT and LVT cycle is replaced by Phe in AVP and LVP. Both amino acids are apolar, but the aliphatic Ile is better recognized than the aromatic Phe, hence the above displacement sequence. The other analogues (IT, MT, OT), which belong to the "oxytocin family", do not cross-react with this antibody. By comparison to AVT, the peptide cycle is the same except that in IT the Gln in position 4 is replaced by Ser. These two amino acids are polar and possess a lateral chain *not charged at pH*

7 which may not affect the relative affinity of the antibody. By contrast, substitution of the polar Arg in position 8 (AVT) by the apolar Ile (in IT and MT) or Leu (in OT) leads to the loss of recognition. Thus, the presence of a polar amino acid in position 8 seems essential.

Results concerning the anti-IT antibody showed that with respect to IT, LVT (9%), OT (5%) and LVP (1%) were little bound. MT, which is not present in teleosts, was recognized as much as IT, while cross-reactivities with the other peptides were not significantly different from 0 (Table 2). Again, the use of tocinoic and pressinoic acids led to the conclusion that the cycle alone was not sufficient. The binding of analogues was in the following order: IT=MT>LVT>OT>LVP>AVT>AVP. IT and MT differ only by the amino acid in position 4: Ser in IT, Gln in MT. These two polar amino acids possess a lateral chain not charged at pH 7 and may be substituted for each other without consequence. In the tripeptide sequence, the amino acid in position 8 plays a key role as shown by the order: Ile (non polar, in IT) > Lys (polar, with lateral chain positively charged at pH7, in LVT or LVP) > Leu (non polar, in OT) > Arg (polar, with lateral chain positively charged at pH7, in AVT or AVP). These observations cannot presumably be explained only by a difference in polarity, but may result also from the spatial arrangement of the amino acid molecules (linear for Ile and Lys, or branched for Leu and Arg) and/or the attached radicals (-CH_3 for Ile and Leu, or -NH_2 for Lys and Arg). In the cycle, the amino acid in position 3 also seems to play a major role. Replacement of Ile (non polar, in LVT, AVT and IT) by Phe (non polar, in LVP and AVP), the only change between LVT and LVP and between AVT and AVP, decreased the degree of recognition but did not suppress it.

Our anti-AVT or IT antibody had weak or no affinity at all for molecules which do not belong to the neurohypophyseal peptide family (Table 2). No cross-reaction exceeded about 4%. Although some of these other substances were not fish hormones, they are active in fish by physiological tests.

In conclusion: 1) positions 3 and 8 in neurohypophyseal or related peptides play a key role as shown by antibody specificity. Most of the antisera previously raised against neurohypophyseal peptides react with the cycle moiety of the molecules, only a few with the linear part (17, 29, 30, 31), rarely with both (32)

as in the present study, which may explain the high degree of specificity of our antibodies; 2) an essential feature is that each of our antibodies is very specific for either AVT or IT and did not cross-react at all with the other. This provides for the first time a way to measure each hormone safely in the presence of the other, as occurs in teleost blood and pituitary gland.

An additional proof for the reliability of these antibodies is that they proved able to suppress biological effects induced by the corresponding peptide. This means that each antibody bound sufficiently to the hormone as to diminish (and presumably suppress at higher antibody dose) its biological activity. Furthermore, a simple wash was sufficient to remove the antibody since full biological effect was recovered when the peptide alone was added again.

To our knowledge, no immunoassay has been reported for IT previously. Therefore the present test is the only one available to measure plasma levels of this hormone in fish. Concerning AVT, while a number of RIAs have been set up in humans, birds, reptiles, amphibians and gastropods (1, 2, 18, 22, 24, 33), only three have been presented in teleost fish (21, 27, 34). Recently, Balment and coworkers have provided the first radioimmunological estimates of AVT in teleost fish (flounder, trout and eel) by means of anti-AVP antibodies cross-reacting sufficiently with AVT (34, 35).

As an immediate application of the ELISA method, we have measured AVT and IT in plasmas of FW-adapted trout. The AVT levels were higher than IT (see above). Perrott et al. (34) have estimated AVT blood concentrations as $(1.83 \pm 0.30)\times10^{-10}$M and $(8.4 \pm 3.0)\times10^{-11}$M in FW-adapted trout and flounder, respectively. These values are lower than ours. The difference between flounder and trout may be related to species. For trout, the difference between the two estimates may be due to various factors such as animal strain or physiological condition, antibodies and techniques used. This difference did not arise from the specificity of our antibody, since no other peptide was shown to interfere in our assay (see Figure 2, Table 2). The parallelism obtained between curves built up with dilutions of plasma extracts and AVT or IT standard curves was also in line with the absence of interfering substances (Figure 4). Similarly, this difference cannot be explained by handling stress because we obtained same values as in unstressed catheterized animals. Finally, bioassays on frog skin and urinary

bladder showed plasma peptide levels in accordance with those obtained by ELISA.

In conclusion, the ELISA method we propose appears adequate to estimate AVT and IT separately in the same samples of plasma or pituitary extract in teleost fish. In addition, these measurements will be possible for fish adapted to various salinities or other environmental conditions or physiological states. Future studies should allow us to clarify the role that neurohypophyseal hormones may play in teleost osmoregulation. An unexpected outcome is the observation that the anti-IT antibody may be used to determine plasma levels of mesotocin, where isotocin is not present. The latter condition prevails in Amphibians.

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