Immunofluorescence of Vasopressin and Oxytocin in the Rat Hypothalamo-Neurohypophyseal System*

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With 6 Figures

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Summary

The present paper deals with the development of an immunofluorescence procedure that allows specific localization of vasopressin and oxytocin in the hypothalamo-neurohypophyseal system (HNS) of the rat.

Antibodies against arginine-vasopressin (AVP), lysine-vasopressin (LVP) and oxytocin were raised by injecting these hormones that were covalently bound to thyroglobulin into rabbits. The vasopressin-immunized rabbits showed periods of diabetes insipidus, while histology of the HNS revealed an intact neurosecretory system with signs of increased endogenous hormone synthesis in the supraoptic nucleus and increased release in the neurohypophysis of some rabbits. The daily water intake of the oxytocin-immunized rabbits was similar to that of control rabbits. The development of antibodies against vasopressin as measured in the immunofluorescence procedure showed a course that was quite different from the curve of the titer as determined by radioimmunoassay (RIA). Also the specificity of the antibodies used in the immunofluorescence procedure was found to be quite different from their specificity in a RIA system. Potency and specificity of the antibodies have to be studied therefore within the immunofluorescence procedure itself.

Using freshly frozen acetone-postfixed hypothalami or pituitaries, no sharp localization of immunofluorescence could be obtained in the HNS. Therefore prefixation was performed. Both, the type and the duration of prefixation revealed quite different results regarding the immunofluorescence in the neurosecretory cell bodies in the hypothalamus and of their endings

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in the neurohypophysis. The best immunofluorescence results were obtained using 6 hours glyoxal-prefixation for the hypothalamus and 24 hours formalin-prefixation for the pituitary.

The cross-reaction of the antibodies for oxytocin or vasopressin was tested on synthetic hormones that were bound to CNBr-activated agarose beads and mounted on glass slides. All anti-plasmas showed cross-reaction on beads containing the heterologous antigen. The plasmas were purified by incubation with beads containing the heterologous hormone until the cross-reacting component had been removed.

Using purified antibodies, the distribution of oxytocin and vasopressin cells within the HNS was investigated. More oxytocin containing cells were localized in the rostral part and more vasopressin in the caudal part of both, the supraoptic (SON) and paraventricular nucleus (PVN). Comparable percentages of oxytocin and vasopressin containing cells were found in the SON and PVN. The absolute amount of oxytocin containing cells was 2.5 times more in the SON than in the PVN, which seems to contradict the "classical" view that the PVN predominantly or entirely synthesizes oxytocin.

In addition, fluorescence was found using antibodies against vasopressin in the suprachiasmatic nucleus in Wistar rats and heterozygous Brattleboro rats, but not in this nucleus of homozygous Brattleboros.

Introduction

By 1894 Ramon y Cajal had described fibers that originate in hypothalamic cells and innervate the neurohypophysis. It was, however, not until 1948 that Bargmann concluded from Gomori-stained sections that axons issuing from the supraoptic (SON) and paraventricular (PVN) nuclei transported neurosecretory material into the neurohypophysis that was synthesized in their cell bodies (for an historical review see *Anderson and Haymaker, 1974*). Since the work of *Olivecrona (1957)* and *Nibbelink (1961)*, a functional differentiation between the SON and PVN has been assumed, specifically that the SON synthesizes mainly vasopressin, and that the PVN is predominantly or entirely responsible for oxytocin production. This functional division between the SON and PVN has recently become into dispute again (*Burford et al., 1974; Dyer et al., 1973; Lincoln and Wakerley, 1974*). Also the possible sites of neurohypophyseal hormone release are not yet settled. Literature is accumulating about possible sites of neurohypophyseal hormone release beyond the neurohypophysis, *i.e.* into the portal capillaries (*Stutinsky, 1970; Zimmerman et al., 1973 a*), into the third ventricle and the pars intermedia of the pituitary (*Stutinsky, 1970*), and into different parts of the central nervous system (*Sterba, 1974*).

Since the sites of production, transport, release and action of oxytocin and vasopressin are of great interest in view of possible functional roles of these hormones, a technique was developed for *specific in situ* localization of vasopressin and oxytocin by means of immunofluorescence (Swaab and Pool, 1975). Brattleboro rats homozygous for diabetes insipidus were used as control for specificity of vasopressin immunofluorescence. These animals, which have a hereditary form of hypothalamic diabetes insipidus, do not have any measurable amount of vasopressin in their hypothalamo-neurohypophyseal system (HNS) (Valtin *et al.*, 1965; Van Wimersma Greidanus *et al.*, 1974).

The present paper deals with technical details on the optimal conditions of the procedure: the choice of the best antibody samples for immunofluorescence, the purification of these antibodies and the importance of the type and duration of tissue fixation. Data are presented on the distribution of vasopressin and oxytocin containing cells within the SON and PVN. In addition, the possibility of vasopressin production beyond the HNS, *i.e.* in the suprachiasmatic nucleus will be discussed.

Materials and Methods

Production of Antibodies

Antibodies were raised in 15 rabbits [New Zealand rabbits, TNO, Zeist, or bastards, Centr. Lab. of the Neth. Red Cross Blood Transfusion Service (CLB), Amsterdam], weighing about 3.5–4.5 kg. The animals were immunized (see Table 1) against 8-arginine-vasopressin (AVP, Ferring lot UA2818, $n = 6$), 8-lysine-vasopressin (LVP, Sandoz lot 1710, $n = 6$), or oxytocin (Sigma, lot 103C-2910, $n = 3$). The antigens were covalently bound to thyroglobulin (bovine, Sigma; porcine, Koch-Light or Sigma) using carbodiimide (Sigma) (Skowsky and Fisher, 1972). The conjugates were emulsified in Freund's adjuvant (Difco) prior to injection. Repeated injections were given *s.c.* (in the back) and *i.m.* (in the hind legs). The rabbits were bled from the ear artery once before immunization and at regular intervals during the immunization procedure. Blood was collected in EDTA-, citrate- or heparin-containing tubes. The final collection was by cardiac puncture under Nembutal anaesthesia into citrate-containing bottles. Blood was centrifuged at 3000 rpm. for 15–25 min and plasma was collected and stored at -30°C . The anti-vasopressin plasmas were raised by Drs. H. J. G. Hollemans, A. P. M. Schellekens and J. L. Toubert (University Clinic for Internal Medicine, Amsterdam), and the anti-oxytocin plasmas by ourselves. During the course of the immunization procedure the potency of the plasmas in the immunofluorescence procedure was determined quantitatively on neurohypophyseal sections.

The hypothalamo-neurohypophyseal system of 9 vasopressin-immunized rabbits and 4 controls were studied histologically and histochemically (hypothalamus and neurohypophysis of # 119, 125, 123, 120, 124, 118, 194, and only the neurohypophysis of # 757 and 126). The rest of the anti-vasopressin rabbits and all the anti-oxytocin rabbits are still alive. The tissues were fixed in 4 % glyoxal and washed for 24 hours (*Sabatini et al.*, 1963). After freezing the tissue blocks in liquid nitrogen, they were stored at -80°C until use. Cryostat sections ($16\text{ }\mu\text{m}$) were prepared and placed on albuminized slides. Alternating hypothalamic sections were stained with thionin (*Windle et al.*, 1943), and for the Golgi-apparatus enzyme thiamine diphosphate phosphohydrolase (TPP-ase) (*Swaab and Jongkind*, 1970). The last procedure was modified such that incubation was performed using thiamine pyrophosphate chloride (TPP-Cl, Sigma) as a substrate and incubating at 37°C for 10 min (cf. *Iijima*, 1969). The pituitary sections were stained with thionin.

Tissues, Fixatives and Immunofluorescence Procedure

Male Wistar rats and male Brattleboro rats (either homozygous or heterozygous for *diabetes insipidus*) were used for the present study. They weighed about 200 g and were obtained from TNO, Zeist. The Brattleboros were checked for homo- or heterozygosis in metabolism cages (see *Swaab et al.*, 1973). The animals received tap water and standard chow *ad libitum*. The different experimental groups were chosen at random and the rats were killed by decapitation. The hypothalamus and pituitary gland were dissected out within 2 min and either prefixed or frozen immediately in liquid nitrogen. Prefixation was performed in 4 % glyoxal or 4 % formalin at 4°C , after which the tissues were washed in buffered sucrose (*Sabatini et al.*, 1963). The tissues were subsequently frozen in liquid nitrogen and stored at -80°C until use.

Cryostat sections ($6\text{ }\mu\text{m}$) were made between -15°C and -30°C and collected on albuminized slides. Sections of freshly frozen tissues were post-fixed in acetone (Merck, pro analysi) at room temperature for 10 min, after which they were dried. All sections were stored overnight at -30°C in a closed slide box.

The next morning the closed slide boxes were brought at room temperature, and the sections were treated to produce immunofluorescence. The procedure consisted of:

- a) a 30 min incubation with the rabbit plasma diluted 1:80 (non-purified plasmas) or 1:40 (purified plasmas) with buffered NaCl, pH 7.4 (140.4 mM NaCl, 9 mM Na_2HPO_4 and 1.3 mM NaH_2PO_4 , pH 7.4);
- b) washing three times with buffered NaCl (once for 30 sec, twice for 15 min);
- c) a 30 min incubation with horse anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (PK-17-2-F³, CLB) (FITC-conjugate), diluted 1:70 with buffered NaCl;
- d) three washings with buffered NaCl (see b);

e) if counterstaining was needed the sections were incubated for 1 min in ethidium bromide 7.9×10^{-4} mM (Boots Pure Drug Co., Ltd.) and washed in buffered NaCl for 1 min;

f) the sections were embedded in a mixture of equal volumes of glycerin (Brocades) and buffered NaCl, and mounted with nail polish.

All washings and incubations were performed at room temperature (18–23 °C). The washings were performed with gentle shaking. Control incubations were done similarly using pre-immune (control) rabbit plasma in step a.

Purification of Plasmas

All anti-oxytocin and anti-vasopressin plasmas cross-reacted with their respective heterologous hormone vasopressin or oxytocin (Swaab and Pool, 1975) and purification of the antibodies was necessary. Anti-vasopressin and anti-oxytocin plasmas were purified by incubation with agarose beads (CNBr-activated Sepharose-4B, Pharmacia) coupled to their heterologous hormones. Purification was performed until the immunofluorescence on heterologous hormone-covered beads equalled or was lower than the fluorescence found on the same beads with control plasma at the same dilution. In addition, for purified anti-vasopressin plasmas no fluorescence had to be found on neurohypophyses of homozygous Brattleboro rats (for an extensive description of the procedure see Swaab and Pool, 1975).

Fluorescence Microscopy

Fluorescence of tissue sections was performed with a Leitz orthoplan fluorescence microscope using epi-illumination. An oil-immersion objective (22×) was used. The light source was a xenon arc (XBO 75 W, Osram) which was operated on a stabilized current (Leitz, power supply type 500-175). For excitation of FITC (470–490 nm) a KP 500 and a GG 475 filter (2 mm) were used in combination with a BG 38 filter. An extra BG 38 filter was placed in front of the xenon arc. Barrier filter K 530 was placed in the emission pathway. All filters were obtained from Leitz. The photometer attachment (Knott, type MFLK, BN 5006 T) was equipped with a stabilized high voltage supply (Knott, NSHM, type BN 600 S/N 716), and connected to a digital voltmeter (Schneider electronique mn 124). Measurements were performed within 2 sec of the light reaching the section. A peripheral part of the neurohypophysis was measured (see results) and a central part of the SON. The quantitative fluorescence data are given as the difference between the fluorescence in the neurohypophysis or SON and that in the anterior lobe of the pituitary (= pars distalis) or anterior hypothalamus, respectively, in the same section. This background correction was similar to the values obtained from SON or neurohypophysis using control plasma. Each experiment consisted of measurements from 25 sections (5 sections per rat) using a 5 mm diaphragm which covered a diameter of 120 μ m on the section. Photographs were taken, using an Ilford HP4 800 ASA film and exposure times of 15–45 sec. Statistical differences were tested using Student's t-test. A probability of < 0.05 was considered significant.

Results

Production of Antibodies

All vasopressin-immunized rabbits showed periods of diabetes insipidus within 4 months after the first injection. They developed a hypotonic polyuria and a polydipsia sometimes exceeding the normal water intake more than tenfold. No glucose was found in the urine. Diabetes insipidus was not observed in the oxytocin-immunized rabbits, that drank similar amounts per day (23—66 ml/kg b.w) as the controls (31—64 ml/kg b.w.).

As judged in thionin-stained sections, the histology of the HNS of these anti-vasopressin rabbits that were studied always revealed an intact HNS, being fully comparable to that of the controls. An infiltrate was never observed. In some rabbits (# 125, # 194) an

Fig. 1. Activity of the Golgi apparatus enzyme thiamine diphosphate phosphohydrolase (TPP-ase) in the supraoptic nucleus (SON) of a control rabbit (C) and in the SON of anti-vasopressin rabbit # 125.

Note the increased distribution and activity of TPP-ase in the SON cells of # 125 (Objective 22 $\times$)

increased TPP-ase activity was found in sections of the SON (Fig. 1 a, b) while an increased concentration of pituicytes was observed in sections of the neurohypophyses of rabbits # 125, # 194, # 126 (for codes of rabbits see Table 1).

Table 1. *Codes of the Rabbits in which Antibodies Were Raised*

	Antigen		
	Oxytocin	AVP	LVP
Code of rabbit	0—1	123	118
	0—2	124	119
	0—3	125	120
		126	121
		127	194
		758	757

AVP = arginine-vasopressin

LVP = lysine-vasopressin

Vasopressin and oxytocin antibodies as measured with the immunofluorescence procedure developed after 1 to 2 months of immunization. Maximal fluorescence values were measured after 2 to 5 months after which the fluorescence decreased. The plasmas with high fluorescence capacity were used for the studies described below. Two examples of vasopressin antibody development are shown in figure 2. The titer, in the same samples, revealed by radioimmunoassay followed a quite different course from that revealed by immunofluorescence.

With all tested plasmas of the different rabbits immunolocalization of hormones in the HNS was possible, although great differences in the intensity of fluorescence were found.

Influence of Fixation (Fig. 3; Table 2, 3)

The optimal fixation conditions for immunofluorescence of neurohypophyseal hormones were determined using anti-vasopressin plasmas that cross-reacted strongly with oxytocin (see below). Glyoxal and formalin fixations were performed for 24 hours in these experiments, followed by 24 hours of washing.

Using freshly frozen *acetone-postfixed hypothalami* no sharp cellular localization of immunofluorescence could be obtained, and tissue morphology was poor (Fig. 3 a). In these tissues, the SON, PVN, "islands" of ectopic magnocellular elements, supraoptico-para-

Fig. 2a, b. Development of immunofluorescence capacity of anti-arginine-vasopressin rabbit # 126 (a) and anti-lysine-vasopressin rabbit # 121 (b). Each arrow indicates one immunization. For immunofluorescence determinations (●—●) plasmas were diluted 1:80 and fluorescence was measured on hypophyseal sections of Wistar rats. The vertical bars indicate the SEM. For background corrections see Table 2.

Radioimmunoassay titer determinations (●---●) were performed in duplicate by Dr. A. P. M. Schellekens (University Clinic for Internal Medicine, Wilhelmina Gasthuis, Amsterdam) in the same plasma samples.

Series of plasma dilutions, stepwise diluted with a factor two, were made in a 0.02 M phosphate buffer (pH 7.6, containing 0.25 % BSA). Dilutions (50 μ l) were incubated for 48 hours at 4 °C with 10 μ l phosphate buffer containing approximately 5000 cpm 125 I-AVP (approx. 2.5 pg). The volume was brought to 1 ml with phosphate buffer. Bound and free hormone were separated by adding 1 ml of norite-dextran and centrifuging for 10 min at 3000 rpm. The pellet was counted in a L.K.B. γ -spectrometer. The titer is expressed as the antiserum dilution necessary for 50 % binding of 2.5 pg 125 I-AVP in 1ml buffer.

Note the difference in course of intensity of fluorescence and titer

Fig. 3. Immunofluorescence using unpurified vasopressin antiplasmas # 121 of the supraoptic nucleus (SON) (a, c, e) and neurohypophysis (b, d, f) of the rat using different fixation procedures (Objective 22 $\times$).

a, b: Acetone-postfixed tissues; note the diffuse staining of the SON and of the neurohypophysis.

c, d: Formalin-prefixed tissues; note that in the SON only fibers show fluorescence and the bright fluorescence of the neurohypophysis.

e, f: Glyoxal-prefixed tissues; note the bright fluorescence in SON cells and fibers, the diffuse staining of the neurohypophysis and high background of the intermediate and anterior lobe of the pituitary. O = optic chiasm, N = neurohypophysis, PI = pars intermedia of the pituitary, PD = pars distalis of the pituitary

ventriculo-neurohypophyseal tractus (SO-PV-NH tractus) in the median eminence and the neurohypophysis showed immunofluorescence. In addition, the suprachiasmatic nucleus and fibers in the anterior hypothalamus (AH) between the PVN and the SON appeared to be stained. The fluorescence was found to be "specific", *i.e.* it did not show up in alternate sections incubated with pre-immune (control) rabbit plasma. This is in contrast to the "non-specific" autofluorescence of the ependymal lining of the third ventricle and the intima of the large blood vessels basal to the SON, both of which gave fluorescence when control plasma was used.

An intense and sharply localized fluorescence was observed in *hypothalami prefixed in glyoxal* (Fig. 3 e, 4). The over-all localization was fully comparable to acetone-postfixed hypothalami, except for the absence of the "non-specific" ependymal fluorescence. Immunofluorescence was, however, clearly localized in cytoplasm and fibers of magnocellular elements. In the suprachiasmatic nucleus the immunofluorescence was also localized in cells and fibers (Fig. 4).

In *formalin-prefixed hypothalami*, the SON or PVN cell bodies were not stained but their fibers stained brightly (Fig. 3 c). No staining was found in the suprachiasmatic nucleus. The intima of the large vessels showed "non-specific" autofluorescence.

In *acetone-postfixed pituitaries*, the neurohypophyseal (Fig. 3 b) immunofluorescence was distributed diffusely, and varied strongly in intensity from section to section. Very weak and diffuse staining was found in the intermediate and anterior lobe of the pituitary.

In *glyoxal-prefixed pituitaries* the intermediate and anterior lobe showed more fluorescence than after the other procedures, while the relatively weak fluorescence in the neurohypophysis was diffusely distributed (Fig. 3 f).

After *formalin fixation* (Fig. 3 d) a very bright immunofluorescence was sharply localized in the neurohypophysis mainly in small grains. Immunofluorescence had its highest intensity in the peripheral part of the neurohypophysis. No immunofluorescence was found in the intermediate or anterior lobe of the pituitary using this fixative.

Fig. 4. Immunofluorescence in glyoxal-fixed hypothalamic sections of Wistar rats using unpurified anti-vasopressin # 126 (Objective 22×).

- a: Fluorescent cells in the paraventricular nucleus (PVN). Note pearl necklace shape of the fibers.
- b: Immunofluorescence in accessory neurosecretory cell groups surrounded by PVN fibers.
- c: Immunofluorescence in cells of the supraoptico-paraventriculo-neurohypophyseal tract in the intermediate zone of the median eminence.
- d: Immunofluorescence in cells of the suprachiasmatic nucleus. III = third ventricle.

Immunofluorescence was not found in the pituitaries after any of the three fixation procedures, incubated with control serum.

Quantitative measurements of immunofluorescence confirmed the findings discussed above (Table 2). Formalin-fixed neurohypophyses showed the highest fluorescence with relatively low background values, as measured in the anterior lobe of the hypophysis. In the SON the same can be said of glyoxal fixation.

Table 2. *Influence of Some Fixatives on Immunofluorescence in the Supra-optic Nucleus (SON) and Neurohypophysis*

	Post-fixation	Pre-fixation	
	Acetone (10 minutes)	Formalin (24 hours)	Glyoxal (24 hours)
SON	320 (24.9)	196 (17.1)	377 (13.2)
Neurohypophysis	240 (26.3)	877 (58.3)	539 (30.4)

Mean quantitative fluorescence data (and SEM) were obtained by measuring 5 sections of neurohypophysis or supraoptic nucleus (SON) per rat in 5 rats using anti-vasopressin plasma # 121. For calculation of the data presented, background fluorescence as measured in the anterior lobe of the pituitary was subtracted from that obtained in the neurohypophysis of the same section. For the SON the value of the hypothalamus anterior was subtracted from that obtained in the SON. In the neurohypophysis formalin-prefixation gave the best results and in the hypothalamus glyoxal-prefixation (both differed significantly $p < 0.001$ from the next highest value).

The effect of fixation was also studied quantitatively and qualitatively using 3 groups of 5 Wistar rats each with plasma # 126 (not reported in the present study). This experiment revealed essentially the same results.

Shorter fixation (6 hours fixation and 18 hours washing) using plasma # 125 gave 21 % less fluorescence in the neurohypophysis but 70 % more fluorescence in the SON (Table 3) together with a good morphology. Shortening of the fixation time in glyoxal to 3 hours, however produced a picture comparable to that of acetone-postfixed hypothalamus. The fluorescence was not restricted to the cell bodies anymore. Other 24 hours fixation procedures followed by 24 hours washings were also tried. Bouin's fixative and 5 % TCA in physiological saline gave unsatisfactory results. Therefore, 6 hours glyoxal-prefixation for the hypothalamus and 24 hours formalin-prefixation for the pituitary were used in the rest of the study.

Table 3. *Influence of the Duration of Fixation on Immunofluorescence in the Supraoptic Nucleus (SON) and Neurohypophysis*

		Duration of fixation	
	Pre-fixation	24 hours	6 hours
SON	Glyoxal	205 (10.8)	348 (22.3)
Neurohypophysis	Formalin	374 (19.6)	297 (12.9)

Mean quantitative fluorescence data (and SEM) were obtained by measuring 30 sections of neurohypophysis or supraoptic nucleus (SON) of 6 rats using anti-vasopressin plasma # 125. The values were corrected for background as given in Table 2. In both, the neurohypophysis and the SON the differences between 6 and 24 hours of fixation were highly significant ($p < 0.002$).

Purification of Antibodies

Anti-vasopressin plasmas that revealed bright fluorescence in Wistar neurohypophyses showed comparable intensities of fluorescence in neurohypophyses of homozygous Brattleboro rats. In both groups the fluorescence increased linearly with decreasing plasma dilutions in the range of 1:160 to 1:40 (Fig. 5 a, b). Anti-vasopressin plasmas produced moreover a fluorescence in the hypothalamus of these homozygous Brattleboro rats that was similar to that obtained with anti-oxytocin in homo- and heterozygous Brattleboro rats.

As a specificity test, LVP, AVP or oxytocin were covalently bound to CNBr-activated agarose beads. These hormone-coupled beads were mounted on glass slides and treated in the same way for immunofluorescence as tissue sections. Anti-oxytocin plasmas produced intense fluorescence on beads containing oxytocin and only slight fluorescence (8—32 % of the value found on oxytocin containing beads) on beads containing LVP or AVP. Anti-vasopressin plasmas produced intense fluorescence on beads containing either vasopressin or oxytocin (52—129 % of the value found on vasopressin containing beads).

These data made it clear that purification of the plasmas was necessary. The purification procedure consisted of incubation of diluted plasmas with beads containing their heterologous antigen. Preincubation of the plasmas with beads containing the homologous hormones completely removed the capacity for immunofluorescence. Preincubation of anti-vasopressin with oxytocin coupled to beads completely removed cross-reacting components from the plasmas as tested on beads coupled to the heterologous hormone and placed on glass slides. Moreover, purified anti-vasopressin plasmas did not stain

Fig. 5 a, b. Examples of immunofluorescence in the neurohypophysis of Wistar and of Brattleboro rats homozygous for diabetes insipidus (HOM. D.I.). Different dilutions (1:160, 1:80 and 1:40) in buffered NaCl of unpurified anti-vasopressin # 125 (a) and # 126 (b) were used. The values were corrected for background fluorescence (*cf.* Table 2). The vertical bars represent the SEM, each point is a mean of 5 immunofluorescence readings. Note the linear increase in the range of the antibody dilutions used

the HNS of homozygous Brattleboro rats anymore. For further details on the cross-reactivity of the plasmas and their purification see *Swaab and Pool (1975)*.

Immunolocalization of Oxytocin and Vasopressin Containing Cells

The distribution of oxytocin and vasopressin containing cells was performed in serial sections of the SON and PVN of 5 male Wistar rats. The first section of each group of 6 sections (6 μ m) was incubated with purified anti-vasopressin, and the second with purified anti-oxytocin. The other 4 sections were skipped. The sections were counterstained with ethidium bromide in order to determine the amount of cells that did not show fluorescence. The countings revealed that oxytocin was localized more in the rostral part and vasopressin more in the caudal part, both in the SON and PVN. An example of these data is given in figure 6. In the SON of these 5 Wistar rats 52.8 % (SEM 2.5) of the cells appeared to contain vasopressin, 31.4 % (SEM 1.1) oxytocin, and 15.8 % (SEM 1.8) did not stain. In the PVN 50.6 % (SEM 2.0) of the cells contained vasopressin, 40.1 % (SEM 2.3) oxytocin, while 9.3 % (SEM 1.1) did not stain.

Fig. 6. Percentage of oxytocin and vasopressin containing magnocellular elements from rostral to caudal in the supraoptic (SON) and paraventricular nucleus (PVN) of one Wistar rat in groups of frontal sections (see material and methods). Note the decreasing percentage of oxytocin containing cells and increasing percentage of vasopressin containing cells into the caudal direction

Fluorescence in the Suprachiasmatic Nucleus (Fig. 4)

Fluorescence was found in the cell bodies and fibers of the supra-chiasmatic nucleus in Wistar rats using non-purified as well as purified anti-vasopressin plasmas. No fluorescence was found in this area using anti-oxytocin plasma. In heterozygous Brattleboro rats the findings were similar to those in Wistar rats, while in homozygous Brattleboros no fluorescence was observed in the supra-chiasmatic nucleus using anti-vasopressin plasma.

Discussion

All vasopressin-immunized rabbits developed a diabetes insipidus, despite the absence of any sign of HNS destruction. On the contrary, the HNS of these animals showed signs of activation of neuro-secretory activity. Increased activity of the Golgi-apparatus enzyme TPP-ase (Fig. 1) was found in the SON of some of these animals, indicating increased hormone synthesis (cf. *Swaab*, 1970). Quantification of the changes in enzyme distribution, as was performed in SON and PVN in rat was, however, not possible in the rabbits due to the narrow and protracted shape of the SON in this animal. The enhanced concentration of pituicytes in the neurohypophysis of some rabbits indicates increased hormone release (*Boer et al.*, in prep.). These signs of increased HNS activity and the fact that diabetes insipidus disappeared with passage of time, shows that the antibodies do not destroy the vasopressin producing neurons. The diabetes insipidus is therefore probably caused by *in vivo* inactivation of endogenous vasopressin by the produced antibodies (cf. *Miller and Moses*, 1968), which induces increased hormone production. A hyper-activity of the organ that synthesizes the hormone against which antibodies are being raised is also observed in other endocrine systems, e.g. in the gonads (*Cameron et al.*, 1974; *Ferin et al.*, 1974).

Although a high specificity of the antibodies was suggested by the absence of diabetes insipidus in oxytocin-injected rabbits, the antibodies clearly did cross-react in the immunofluorescence procedure, causing the fluorescence observed in homozygous Brattleboro rats using anti-vasopressin plasmas (*Swaab and Pool*, 1975).

Great differences were found between the specificity in relation to the heterologous hormone in a radioimmunoassay (RIA) system and in immunofluorescence. Various anti-vasopressin plasmas that showed negligible cross-reaction to oxytocin in a RIA system revealed bright fluorescence on oxytocin-covered beads and in the HNS of homozygous Brattleboro rats. This paradox might be explained by

the very different relative concentrations of antigen and antibodies used in the two techniques. In addition, however, specificity in a RIA system depends only on the specificity of the competitive reaction between unlabeled hormone and tracer while specificity in an immunofluorescence (IF) system depends on the lack of antibodies against related compounds. The ideal plasma for IF has thus to be monospecific, while this is not necessary the case for RIA. As well, RIA titer determinations did not give data comparable to the potency of the plasma for immunofluorescence in sections. During the period that the antibodies were raised, quite different curves were obtained for the intensity of immunofluorescence and the value of the RIA determined titer (Fig. 2). The possibility exists therefore, that for the two techniques by preference a different part of the antibody population is used. Consequently, it is not sufficient to rely upon specificity data or titer values obtained in a RIA for antibodies that are to be used in immunofluorescence as is generally done in literature (e.g. *Burlet et al.*, 1973). Specificity and potency have to be studied using the immunofluorescence procedure itself.

Using acetone-postfixation, no sharp localization of immunofluorescence could be obtained within the neurosecretory cells. Therefore prefixation of the tissues was tried. Because they produced intense and sharply localized fluorescence in the rat, formalin was chosen for the neurohypophysis and glyoxal for the hypothalamus. Clear-cut differences in sensitivity for each kind of fixation were observed such that glyoxal made hormone localization in the cell bodies possible and formalin had a similar effect in fibers. In the SO-PV-NH tractus, both procedures revealed good fluorescence. This difference in sensitivity for fixation between the cell bodies and fibers may explain why *Leclerc and Pelletier* (1974) could not demonstrate vasopressin in the perikarya of the neurosecretory neurons. Fixation is thus not only important for a good morphology of the tissues and the intensity of the immunostaining, but may also determine the ultimate site on which a compound is located. In addition, these findings may point to a change in configuration of neurohypophyseal hormones during their transport (cf. *Zambrano and De Robertis*, 1966).

Similar percentages of vasopressin and oxytocin containing cells were found in the SON and PVN using purified antibodies. Based on the first formula of *Abercrombie* (1946), the total number of cells can be estimated to be 6430 in the SON and 2100 in the magnocellular part of the PVN. The total number of oxytocin containing cells in the SON (2019) was therefore more than two times higher than that in the PVN (842). Even if all unstained cells of the PVN contained oxytocin, the SON would still have about two times more oxytocin

containing cells than the PVN. These findings seem to contradict the "classical" view that the SON predominantly or entirely synthesizes vasopressin and the PVN oxytocin (*Olivecrona*, 1957; *Lederis*, 1962). Our results on oxytocin and vasopressin distribution agree however with more recent indirect data like the distribution of neurophysins (*Zimmerman et al.*, 1973 b), hormone assays (*Dyer et al.*, 1973) and labeling experiments (*Burford et al.*, 1974). The rough isolation techniques of *Dyer et al.* (1973) do not allow for discrimination between the presence of a hormone in cell bodies of a particular area and that in fibers coming from elsewhere. Electrophysiological observations (*Lincoln and Wakerley*, 1974) also support the idea that, in the rat, the SON is at least as important for oxytocin production as the PVN.

Whether vasopressin and oxytocin are synthesized in separate cells (*LaBella*, 1968) cannot be proved definitively by the present technique. Our data are, however, consistent with this possibility. The sum of the percentages of cells stained by either of the two antibodies ranged from 84—91 %. So we suggest that no consistent double staining of cells occurred. In addition, sometimes parts of the same cells were detectable in the two adjacent sections and all cases noted it stained only with one, not both antibodies. Moreover, oxytocin or vasopressin containing cells showed a characteristic distribution, not only in the serial sections from rostral to caudal (Fig. 6), but also within one section. In the SON, oxytocin containing cells were observed mainly in the dorsal part while vasopressin containing cells were located mainly in its ventral part. These observations all point to separate cells for oxytocin and vasopressin synthesis. *Leclerc and Pelletier* (1974) reported that all nerve endings in the neurohypophysis showed a positive immunolocalization with anti-vasopressin, but this finding might be explained by cross-reaction with oxytocin. The question whether a given neurosecretory cell is involved in the production of only one of the hormones is elaborated at present by staining both hormones in a different way and in the same section using immunoperoxidase techniques.

Neurohypophyseal hormones are found to be located also outside the HNS *i.e.* in the suprachiasmatic nucleus (*Swaab and Pool*, 1975; *Zimmerman*, 1974). The question arises whether the immunofluorescence observed in the suprachiasmatic nucleus really represents vasopressin or only a closely related compound. The fluorescence in these cells was not found using anti-oxytocin plasmas, and remained visible after purification of anti-vasopressin (*Swaab and Pool*, 1975). In addition, homozygous Brattleboro rats do not show immunofluorescence in the suprachiasmatic nucleus while in heterozygous

animals the picture is similar to that of normal Wistar rats. These data strongly suggest that these cells really contain vasopressin rather than a related compound. Confirmation of this point, *e.g.* by RIA using isolated tissue from this area, seems however worthwhile. Neither the function nor the efferent pathways of the suprachiasmatic cells are known. Moreover it is not yet settled whether these cells only *contain* vasopressin or do also *synthetize* this hormone. The ultra-microscopic pictures of suprachiasmatic cells are highly suggestive of secretory activity. The cells contain granulated vesicles (*Suburo and De Iraldi*, 1969) of similar diameter (1700 Å) as HNS neurons. In addition to vasopressin, neurophysins are also found in these cells (*Zimmerman*, 1974; *Vandesande et al.*, 1974). It seems probable therefore that the suprachiasmatic cells do not only contain vasopressin but are also able to synthetize this hormone.

In conclusions we can state that, if appropriate purification of antibodies is performed and the right fixation procedure is used, specific localization of vasopressin and oxytocin in nervous tissue is possible. Further, since ultramicroscopic localization of the hormones using peroxidase is also possible with these antibodies (*Van Leeuwen and Swaab*, 1975), immunolocalization procedures are a promising tool for the elucidation of the process and sites of synthesis and release of neurohypophyseal hormones and may thus contribute to the knowledge about the functions of these hormones.

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