Position of the Methionine Residue in Bovine Neurophysin II[1]

In 1942 VAN DYKE et al.[2] described a protein fraction from bovine posterior pituitary lobes which exhibited oxytocic, pressor and antidiuretic activities. Subsequently, studies[3] have shown that this 'VAN DYKE protein' consists of neurohypophyseal hormones associated in non-covalent linkage with several closely related proteins, which CHAUVET et al.[4] designated as neurophysin. Evidence has accumulated which suggests that under a variety of conditions neurophysins reversibly form aggregates. For bovine neurophysins a monomeric molecular weight of approximately 10,000 has been reported[5]. One of the major components of the neurophysin fraction, neurophysin II, has been described by HOLLENBERG and HOPE[6]; a conspicuous feature of this protein is its single methionine residue. The successful scission of the amide chain at the carboxyl site of a methionine residue by the non-enzymatic cleaving reagent cyanogen bromide[7] has been demonstrated with many peptidyl substrates[8]. It was hoped that the cleavage of neurophysin II at the methionine position would result in 2 fragments of suitable size for a variety of further studies.

With this as background neurophysin II was purified by a modification of the procedure of HOLLENBERG and HOPE[6], and checked for the absence of neurohypophyseal hormones by avian vasodepressor[9] and rat pressor assay[10], and by the lack of precipitation with specific antibodies to oxytocin[11]; neurophysin II was tested for homogeneity by disc electrophoresis[12], isoelectric focusing[13] and amino acid analysis[14]. This material (10 mg), dissolved in 6 ml of 0.1 *N* HCl, was allowed to react with 60 mg of cyanogen bromide. The mixture, stirred until a homogenous solution resulted and allowed to stand at room temperature for 16 h, was diluted with 12 ml of glass distilled water and lyophilized. This powder was then taken up in a minimal amount of *N* formic acid and subjected to gel filtration on a Sephadex G-75 column. Elution with *N* formic acid resulted in two UV-absorbing peaks (254 nm); the first (CNBr-1) emerged about midway between the void- and bed-volume and the second (CNBr-2) at the bed-volume. The N-terminal of CNBr-1 was found to be serine. Amino acid analysis after acid hydrolysis with 6*N* HCl at 110 °C for 22 h showed that CNBr-2 contained 2 ninhydrin-active components, alanine and homoserine lactone, the latter resulting from methionine during the cyanogen bromide reaction. These results confirm that alanine is the N-terminal residue of bovine neurophysin II[5a] and show that methionine occupies the penultimate N-terminal position, and serine the third position, in this protein.

Zusammenfassung. Durch Spaltung mit CNBr wurde gezeigt, dass Neurophysin II aus Rinderhypophyse nur eine Methionin-Einheit enthält und dass die N-terminale Sequenz Ala-Met ist.

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Identification of Teleost Gonadotrope Cells Using Methallibure (I.C.I. 33, 828) and Thiourea

In recent years increasing attention has been paid to the experimental identification of functional cell types in the teleost pituitary (see review by VAN OORDT[1]). Associated with this has been the realization that empirical staining techniques have, by themselves, only a morphological value, a fact emphasized in teleosts by the general failure of tinctorial methods to distinguish between thyrotrope and gonadotrope cells in the proximal pars distalis (PPD) of the pituitary gland[2].

In attempts to identify these cell types the most common approach has been to look for parallel changes in the pituitary following experimentally-induced changes in its target organs. In this way the identification of thyrotrope cells has been successfully accomplished in

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many species, both by radiothyroidectomy[3] and by treatment with goitrogens[4]. Only a few attempts have been made, however, to identify gonadotrope cells experimentally. SOKOL[4] observed changes in the adenohypophysis following gonadectomy in *Poecilia* (Lebistes) *reticulata*, and GESKE[5] used sex steroid administration to identify gonadotropes in the same species.

The recent development of non-steroidal chemical inhibitors of gonadotropic function allows a new approach to this problem. The compound 1-*o*C-methylallyl-6-methyldithiobiurea (Methallibure, I.C.I. 33, 828) was used in this study. Methallibure has been shown to inhibit gametogenesis in mammals[6,7], frogs[8] and teleost fish[9,10]. There is strong evidence from work on mammals[6,11,12] that this compound inhibits the production or secretion of pituitary gonadotropins, rather than inhibiting gametogenesis by a direct effect on the gonads. The results reported here indicate that Methallibure may be a useful tool in the identification of functional cell types in the teleost hypophysis.

Twelve mature female specimens of the large freshwater Serranid, *Plectroplites ambiguus* Richardson, were divided into 4 groups of 3 fish. Each group was housed in a 300 l aquarium with aerated filtered water at 22 °C and under long (14 h) daylength conditions. The fish were fed regularly and treated as follows:

Group 1 – Methallibure. A suspension containing 1.0 g Methallibure in 100 ml of distilled water was prepared. The suspension was maintained with Tween 80 (2 drops/10 ml) and was added to the aquarium water 4 times per week in 5.0 ml doses.

Group 2 – Thiourea. These fish received 4×5.0 ml doses per week of a solution containing 2.0 g thiourea in 100 ml distilled water.

Group 3 – Methallibure + Thiourea. This group received both compounds in the same dosages as above.

Group 4 – Control. Both the control group and the thiourea-treated fish received the same amounts of the wetting agent Tween 80 as the other groups (i.e. 4 drops/week).

Half of the water in each tank (150 l) was replaced each week with fresh, dechlorinated water. After 5 weeks the fish were sacrificed and their pituitary glands dissected free and fixed in 10% formalin. Serial sections were cut at 5 µm and stained with aldehyde-thionine-PAS-yellow naphtol[13].

Results. Pituitary glands from the control fish showed deeply-staining, granular basophil (i.e. PAS-positive) cells throughout the PPD (Figure). The dorsal edge of the PPD is occupied by acidophil cells surrounding the branches of the neurohypophysis. Below these small, rounded, tightly-packed basophils (Type 1 in Figure) occupy the bulk of the central region of the PPD, with scattered groups of acidophils amongst them. In the rostro-ventral region of the PPD, and extending posteriorly as a ventral rim to the pars intermedia, are larger basophils of more angular appearance (Type II). With the aldehydethionine-PAS-yellow napthol technique the cytoplasmic granula of these peripheral cells stained a liver-red colour while those of the Type I basophils stained deep purple, presumably due to a greater affinity for aldehyde thionine (cf. *Mugil cephalis*[14]).

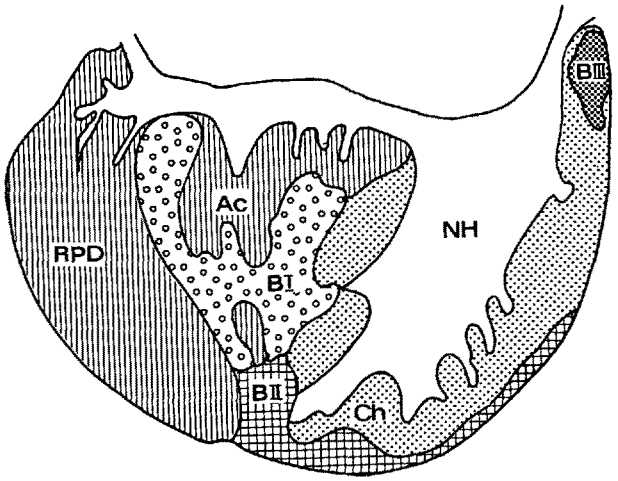
Thiourea treatment caused degranulation and vacuolization of the Type I basophils. The Type II cells were unaffected, remaining large and granular. The fish in this group showed thyroid hyperplasia (increase in follicle cell height and loss of colloid). At the dosage level used here thiourea did not adversely affect the gonads; all fish in this group remained in breeding condition.

Pituitaries from Methallibure-treated fish showed marked degranulation of the Type II basophils. They appeared small and flattened, but no vacuoles were observed in these cells. The ovaries from these fish showed advanced atresia of yolky eggs and inhibition of vitellogenesis in developing oocytes.

The Type I basophils, although staining much more deeply than those on the periphery, were none the less not unaffected by Methallibure. These cells appeared to be slightly degranulated as compared to the controls, and some vacuolization was apparent.

Although no histological abnormalities could be detected in the thyroid follicles of these fish, the situation may be complex. In rats and mice, TULLOCH et al.[15] found that Methallibure affects thyroid function both directly, by inhibiting the protein-binding of iodine, and indirectly by influencing pituitary thyrotropic activity. The expected goitrogenic effect of Methallibure was masked by its inhibitory effect on TSH secretion.

It thus seems not unlikely that the partial degranulation of Type I basophils following Methallibure treatment represents an inhibition of TSH production which is not reflected by thyroid cytology.



Median saggital section of pituitary of *Plectroplites ambiguus*. Diagram showing distribution of main cell types. Ac, acidophils of proximal pars distalis (PPD); B I, Type I basophils (thyrotropes) in PPD; BII, Type II basophils (gonadotropes) in PPD; B III, basophils of pars intermedia; Ch, chromophobes of pars intermedia; NH, neurohypophysis; RPD, rostral pars distalis.

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Fish that received both Methallibure and thiourea showed degranulation of both types of basophil in the PPD. The ovaries from these fish showed atresia and blockage of vitellogenesis. Thyroid follicle cells appeared to show a somewhat less marked loss of colloid than in fish treated with thiourea alone.

On the basis of these results it seems reasonable to suggest that the small central Type I basophils are thyrotropes and the larger ventral Type II cells are gonadotropes. Methallibure appears to act by inhibiting the production of pituitary gonadotropin(s) and reducing the production of TSH.

Preliminary results for another teleost, *Hypseleotris galii*[16], suggest a similar distribution of these cell types. The work reported here is part of a study of the reproductive physiology of several teleost species using Methallibure treatment to replace the classical technique of surgical hypophysectomy[17].

Zusammenfassung. Die versuchsweise Identifizierung gonadotroper Zellen der Teleostier-Hypophyse (*Plectoplites ambiguus*) gelang nach Behandlung mit Methallibure (I.C.I. 33, 828) und Thiourea.

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[16] N. J. MACKAY, in preparation.

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Pineal Weight Response to a Dietary Variable in *Microtus montanus*

Several investigators have demonstrated a correlation between changes in the pineal gland and the reproductive system relative to photoperiod. The laboratory rat maintained under constant dark has a decreased incidence of estrus associated with an increase in pineal weight and hydroxy-indole-*O*-methyl (HIOMT) transferase activity[1]. In constant light, estrus is prolonged, ovarian hypertrophy is observed and pineal weight and HIOMT activity are decreased[2]. HOFFMAN and REITER[3] demonstrated in hamsters that gonadal atrophy which occurs as a result of blinding or artificial short days (1 h of light) is prevented in pinealectomized animals.

It has been established for a number of different species that long photoperiod is highly stimulatory to reproduction while short photoperiod has no effect or is a negative stimulus. The montane meadow vole, *Microtus montanus*, a strict herbivore, shows a positive reproductive response to long photoperiod[4]. Furthermore, NEGUS and PINTER[5] have demonstrated a positive reproductive response to supplements of fresh green plants in the diet of this species.

The present study was undertaken to determine whether or not changes in photoperiod or addition of fresh green plants to the diet could affect weight changes in the pineal gland of *M. montanus*. Most of the animals used in this study were young subadults of uniform age and size, obtained from an outbred colony of *M. montanus* maintained in our laboratory. One group of experimental animals was obtained directly from wild populations at the Jackson Hole Biological Research Station, Wyoming. This group was taken into captivity during late October, 1968, when the entire population was in a non-breeding condition. From time of capture and throughout the experimental period, these animals were maintained under short photoperiod (8L, 16D).

Animals were caged either in pairs or singly and were given Purina rabbit chow and water ad libitum. The outer leaves of head lettuce were fed as a dietary supplement to experimental animals. Daily artificial illumination provided by cool white fluorescent lights (250 ft candles) was controlled by a 24-h clock-switch. Temperature was maintained at 70–72 °F.

Generally, the laboratory animals were maintained under a particular light regime for 21 days prior to a succeeding 21 days of experimental diet administration. At the termination of each experiment, animals were sacrificed in the middle of the dark period (Table I). The wild caught animals were maintained under 8 h

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Table I. Effect of daily dietary supplement of fresh plant substances on pineal weight in *Microtus montanus*

Group	No. of animals	Sex	Light regime	Treatment	$\bar{x}$ Pineal weight (mg)	Level of significance (P)
1	9	♀	8L 16D	Greens	0.096 ± 0.047 [a]	0.025
	9	♀	8L 16D	No greens	0.152 ± 0.057	
2	18	♂	8L 16D	Greens	0.096 ± 0.028	0.025
	16	♂	8L 16D	No greens	0.123 ± 0.043	
3	16	♂	12L 12D	Greens	0.091 ± 0.040	0.025
	19	♂	12L 12D	No greens	0.130 ± 0.068	
4	27	♂	16L 8D	Greens	0.086 ± 0.040	0.01
	24	♂	16L 8D	No greens	0.116 ± 0.047	

[a] Standard Deviation.