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FINAL REPORT

Research to Determine the Role of Gravity in Neurosecretory Physiology

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Prepared by: L. E. Clemens
J. S. Life, Ph.D.

Submitted to: Office of Life Sciences
(Code MMR)
Attn: Dr. Joseph Saunders
NASA, Washington, DC

COMAR SPACE/DEFENSE

A Division of

COMAR CORPORATION

2265 Cole Street
Birmingham, Michigan 48008
Telephone: (313) 647-1304

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I. BIOLOGICAL REVIEW AND RATIONALE*

The supraoptic and paraventricular nuclei of the hypothalamus are connected by a bundle of non-myelinated nerve fibers to the neural lobe of the hypophysis. The function of this hypothalamo-hypophyseal system was discovered by Fisher and Ingram and confirmed by more recent studies showing that the posterior lobe hormones are synthesized in the supraoptic and paraventricular nuclei, or in the preoptic nuclei of lower vertebrates. The hormones are separately "packaged" in small secretory granules in association with a larger carrier protein molecule and migrate down nerve fibers, ultimately accumulating in the posterior lobe of the pituitary. This accumulation of granules in neurohypophyseal nerve endings is taken as evidence that neuro-secretory material is transported distally by axoplasmic flow.

The mechanism(s) by which granule migration or axoplasmic flow occurs is not known and constitutes an important biologic question. Because of the anatomic relationship of the hypothalamus and hypophysis, it appears reasonable that gravity could influence the migration of these neurosecretory granules. If not, however, other flow control mechanisms must be responsible. There are arguments suggesting that the combined carrier protein

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*For a more complete discussion and bibliographic citation see S/D Technical Proposals P69-171, dated 19 March 1969, and P70-129A, dated 18 September 1970, submitted earlier. See also Appendix A of this report.

and hormone package may be just large enough to be affected by Newtonian forces rather than the Brownian dynamics, differential concentration or active transport mechanisms usually implicated in molecular transportation. Nevertheless, there is sufficient evidence to warrant an empiric test of the role of gravity in regulating neurosecretory activity of the posterior pituitary. The pragmatic significance of such a study is evident when one considers space flight. It has long been known that weightlessness causes water loss. Several mechanisms have been implicated, but, as categorically stated by Academician V. Parin in a recent article on life in orbital stations, "...still we do not know why the organism is dehydrated in weightlessness." Parin implicates gravity and suggests that the hypothalamus may be involved. Clearly, a deeper understanding of gravity's role in hypothalamic secretion would produce important benefits for aerospace and terrestrial medicine.

II. EXPERIMENTATION

To determine the role gravity may play in neurosecretory physiology we proposed to study the hypothalamo-hypophyseal system under a variety of modes in the one-G environment. The test animal we chose to investigate was a fresh water teleost, the goldfish. Fishes have a distinct advantage over other vertebrates since their orientation to gravity can be altered with little difficulty, thereby allowing gravity to act on the hypothalamo-hypophyseal system in a variety of axes.

The organ of equilibrium in fishes is located in the pars superior and consists of the semicircular canals and their ampullae and a sac-like vesicle, the utriculus. In bony fishes the utriculus contains an otolith called the lapillus. The lapillus rests horizontally on the hairs of the sensory cells of the crista utriculi and responds to the force of gravity, thereby stimulating the sensory cells of the cristae. This stimulation of hair cells works in conjunction with sensors in the lower portion of the retina to maintain balance. Thus, with both eyes and utriculi intact, light from above and gravitational force from below keep the fish oriented in an upright position.

If one utriculus is removed, however, or a strong beam of light is directed toward the fish at a right angle instead of from above, the subject can be made to lean to one side or the other. When a fish has its utriculi removed from both inner ears, it can be made to swim at a 90^{o} angle to the normal gravitational field if illuminated from the side, or even upside down if lighted from below.

Utilizing these techniques, we have conducted a study to vary precisely the direction of gravitational force relative to the anatomic axis of the hypothalamo-hypophyseal system. This experimental technique was employed to assess the influence of altered gravitational orientations on neurosecretory granule regulation.

During early work we designed an environmental control system, developed surgical procedures for bilaterally eliminating

the utriculi in fish, and induced voluntary re-orientation relative to the Earth's gravitational field with altered light cues. (See Figure 1.)

An electronic method (based upon variable impedance) was designed and used to continuously assess and record the position of the fish relative to gravity. This sensor was incorporated into the walls of a Plexiglas environmental chamber. Water flowed continuously through this chamber at a controlled rate producing slow swimming movements of the fish and consequent horizontal ($+G_x$) orientation in the field. Light entered the chamber through a narrow translucent stripe along the long axis on the side of the otherwise opaque chamber. In our laboratory, fishes, surgically deprived of their inner ear mechanisms, oriented to this external light stimulus. By gradual rotation of the chamber and its translucent stripe, the position of the fish relative to the G field was altered.

The removal of labyrinthine structures was performed by making a small incision (3mm) behind each eye in fish, anesthetized with MS222, and carefully removing the vestibular structures with dissecting forceps. The entire procedure was done with the aid of a dissecting microscope. Within minutes the fish would start swimming in a tumbling manner, completely disoriented. This behavior continued until the second day when the fish began orienting toward the single bright light source illuminating the otherwise darkened aquarium. It was at this time that the operated fish also began eating and interacting with

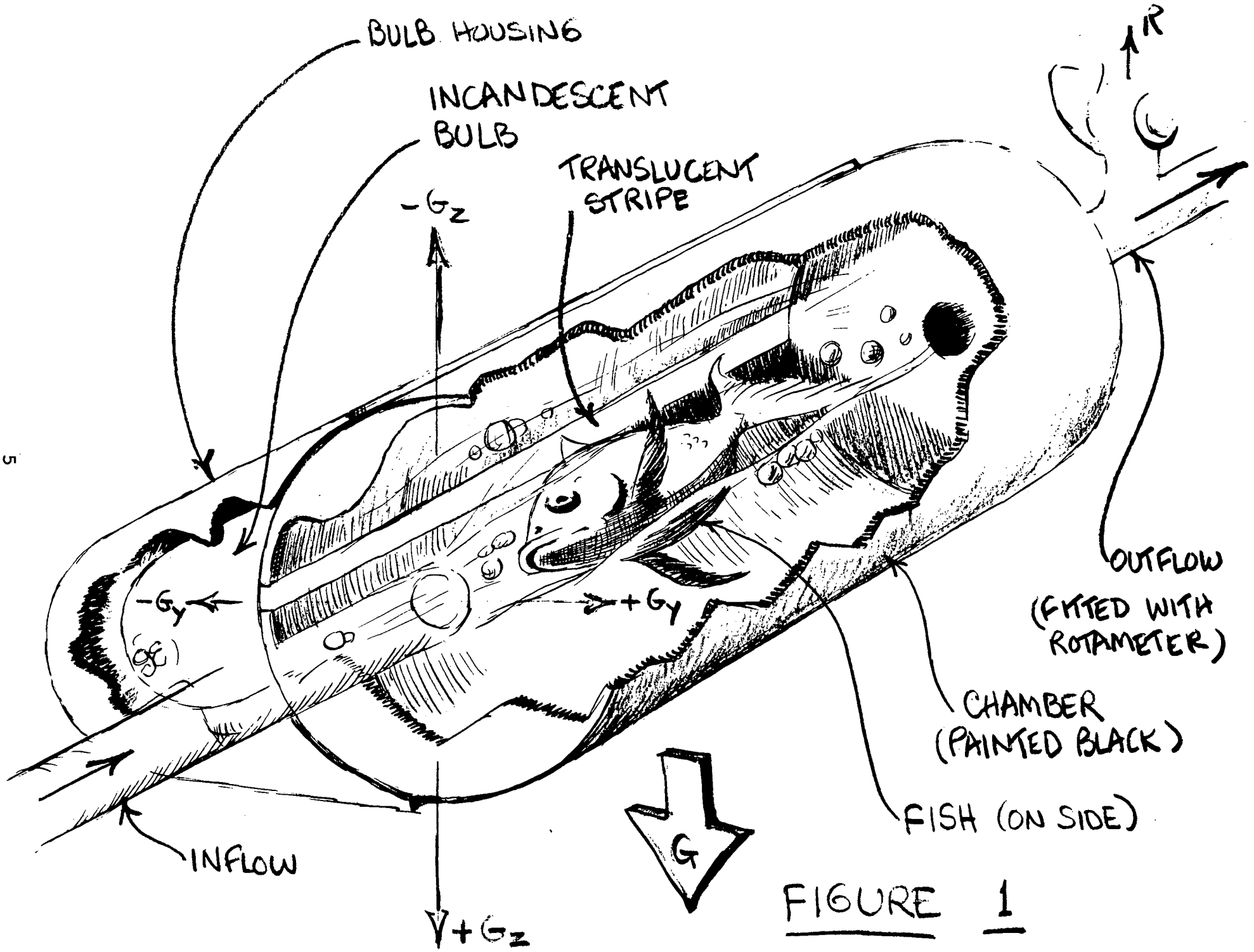
BULB HOUSING
INCANDESCENT BULB
TRANSLUCENT STRIPE
-Gz
IR
-Gy
+Gy
OUTFLOW
(FITTED WITH ROTAMETER)
CHAMBER
(PAINTED BLACK)
FISH (ON SIDE)
INFLOW
G
+Gz

FIGURE 1

other fish in the aquarium. All behavior appeared normal except for the orientation of the operated fish. As the light source was incrementally moved around to the bottom of the aquarium, the operated fish again demonstrated disoriented swimming behavior, but soon "locked on" the light and swam inverted.

In addition to the re-orientation studies described above, a major effort was directed to the biochemical investigations needed to determine the presence of vasopressin in the neurohypophysis and peripheral target organs. It was necessary to perform an accurate bio-assay of arginine-vasotocin (fish vasopressin) in tissue sections taken from the hypothalamus, infundibulum and neurohypophysis of fishes exposed to different gravitational orientations to assess the gravitational effects on neurosecretory regulation. There is no good procedure for the detection and assay of endogenous arginine-vasotocin prior to or after its release from the pituitary. Gormori's chrome hematoxylin and other histologic stains lack specificity and cannot be used with confidence to selectively identify vasotocin neurosecretory granules. Therefore, we pursued a new approach, utilizing fluorescent antibody techniques, to assay arginine-vasotocin at its sites of synthesis, transport and storage. To achieve this objective we synthesized arginine-vasotocin, developed antibodies to the synthesized hormone, tagged the antibody with a fluorescing molecule, and determined cross-reactivity of the antibody with the endogenous hormone.

The antigenicity of vasopressin, oxytocin and arginine-vasotocin has been questionable due to their low molecular

weight. Recently, however, Spragg, et al, successfully produced antibodies to bradykinin (nine amino acids) through both coupling and polymerization techniques. We utilized Freund's adjuvant and employed similar methods to determine the best procedure for making antibodies to arginine-vasotocin. These antibodies were, in turn, used to detect endogenous vasopressin by labeling the molecules with a fluorescent tag and selectively staining sections of hypothalamus, infundibulum and pituitary. Rabbits were innoculated with synthetic arginine-vasotocin and antibody activity was determined periodically using the ring test. Titers were pooled and globulin fraction purification was performed using the Ethodin (Rivanol) procedure described by Horejsi and Semtana.

Anti-arginine-vasotocin was coupled with Rhodamine B-200 (a fluorescent tag) and the conjugated globulin was chromatographed on a Sephadex G-25 column with phosphate buffered saline (pH 7.2) for purification. The conjugated globulin was subsequently stored in a frozen state until it was used to identify endogenous arginine-vasotocin in the hypothalamo-neurohypophyseal system. Because of low yields of our synthetic arginine-vasotocin, we also made antibodies in goats to commercially available oxytocin in the belief that these antibodies would cross-react with endogenous fish arginine-vasotocin thereby enhancing our fluorescent antibody staining capability. We tagged these antibodies and also determined their cross reactivity.

In spite of all these efforts, we were unable to employ the fluorescent antibody procedure successfully as an accurate assay for arginine-vasotocin. Clearly, another assay was needed.

Since it had been previously demonstrated that one of the first responses of toad bladder to vasopressin (the anti-diuretic principle of the mammalian posterior pituitary) was an increase in the level of intracellular cyclic AMP (adenosine-3', 5' -monophosphate), (Orloff, et al, 1965), we decided to develop an assay for this nucleotide in the kidneys of experimental and control fish as a measure of the arginine-vasotocin concentration. The rationale for this indirect approach is well founded since cyclic AMP is known to act as a second messenger whose function is to mediate a variety of hormonal responses (Robison, et al, 1971), including those associated with the neurohypophysis. Formation of cAMP in response to a hormone is dependent upon an enzyme, adenyl cyclase, that catalyzes the reaction ATP adenyl cyclase cAMP + PPi. Once released from the posterior pituitary into the blood, the octapeptides do not indiscriminately activate the adenyl cyclase enzyme in all tissues, but they are restrictive in their action to only the adenyl cyclase of target organs. The specificity of the response seems to be with the enzyme system itself, for other hormones that increase cAMP in their target tissue do not alter the level of cAMP in other, non-target tissue. Dousa, et al, 1970, and Bently, 1970, have suggested that the specific

hormone receptors necessary for the activation of adenyl cyclase are closely associated with, if not an actual part of, the enzyme molecule itself.

Because of the degree of specificity present in this system, plus the fact that adenyl cyclase can be activated by nanogram quantities of hormone, it seemed possible that the biological activity of endogenous arginine-vasotocin in goldfish renal tissues could be determined by measuring adenyl cyclase activity. Accordingly, preliminary experiments were performed and we were, indeed, able to demonstrate the presence of an adenyl cyclase - cyclic AMP generating system, presumably dependent on arginine-vasotocin. Adenyl cyclase activity was measured by determining its ability to convert H^3ATP to H^3-cAMP. This product was separated from intermediate substances in the homogenate by paper chromatography, and then quantified by liquid scintillation counting. Tissues were collected from experimental and control fishes and assayed for endogenous levels of adenyl cyclase (Menon, et al, 1971). Also see Table I. Because the endogenous adenyl cyclase activity in kidney tissues is regulated by the circulating levels of arginine-vasopressin released from the neurohypophysis, we measured this enzyme's activity in the kidneys of three different experimental groups in order to determine indirectly the effect of gravity on the release of this peptide hormone. Group I was a control, consisting of unoperated fish, orienting to an overhead light source. Groups II and III were labyrinthectomized, with Group II orienting to an overhead light, and Group III to an inverted light source. We found that

TABLE I

EXPERIMENTAL PROCEDURE FLOW SHEET

1. Remove Kidney

2. Homogenize in .01/M Tris Buffer pH 7.5
   5% w/v

3. Incubate 50ul homogenate with reaction mixture, 130ul, containing ATP-H^3 for 15 minutes at 37° C.

4. Reaction ATP-H^3 $\xrightarrow{\text{Adenyl Cyclase}}$ cAMP-H^3 + P_iP_i

5. Chromatograph 100ul of reaction mixture to separate cAMP-H^3 from other reaction products.

6. Identify cAMP with standards and determine cAMP-H^3 formed by liquid scintillation counting techniques

after four days of swimming in an inverted position, the fish in Group II were no longer capable of maintaining this position and simply rested on the bottom of the aquarium. The adenyl cyclase activity in the kidneys of Groups II and III were therefore assayed three days after the removal of the otoliths. The results from these studies are summarized in Tables II and III.

III. DISCUSSION

The preceding sections describe the work performed under Contract No. NASw-2196. This study represents an initial attempt to understand the role of gravity in regulating the synthesis, transport, storage and release of octapeptides from the hypothalamo-neurohypophyseal system. Labyrinthectomized goldfish employed in this study were forced to live in an altered gravitational orientation ($-G_z$). After three days of exposure to this orientation these animals demonstrated reduced levels of adenyl cyclase activity in their kidneys when compared to labyrinthectomized and unoperated animals exposed to normal gravitational orientations ($+G_z$) for the same period of time.

If fish renal adenyl cyclase activity is related to blood neurohypophyseal hormone levels, as is the case with mammals (Robison, et al), this study suggests that gravity may well play a role in the regulation of the posterior pituitary functions. The differences observed in this study between control and experimental fish may, however, have resulted from

TABLE II

ADENYL CYCLASE ACTIVITY
AS MEASURED BY cpm of cAMP-H^3
PRODUCED FROM ATP-H^3

Group I Unoperated Controls Normal light ($+G_z$)	Group II Operated Controls Normal Light ($+G_z$)	Group III Operated Animals Inverted ($-G_z$)
n = 10*	n = 10*	n = 10*
$\overline{X}$ = 10,779 $\pm$ 1175** cpm	$\overline{X}$ = 4,632 $\pm$ 739** cpm	$\overline{X}$ - 3,661 $\pm$ 817**
Group I vs Group II t = 9.344 p = .005	Group II vs Group III t + 1.86 p = .05	

* n = the number of assays performed. Each assay was done with the pooled kidney tissue from four fish.

** $\overline{X}$ = mean $\pm$ standard error.

TABLE III

Kidney Weight/Body Weight

Group I Unoperated Control Normal Light ($+G_z$)	Group II Operated Controls Normal Light ($+G_z$)	Group III Operated Fish Inverted ($-G_z$)
n = 40*	n = 40*	n = 40*
$\overline{X}$ = 0.67 $\pm$ 0.23**	$\overline{X}$ = 0.61 $\pm$ 0.20**	$\overline{X}$ = 0.76 $\pm$ 0.18
Group I vs Group II	Group II vs Group III	
t = 0.828	t = 0.99	
p .0	p .1	

* n = number of fish/group

** $\overline{X}$ = mean $\pm$ standard error

altered biochemical and physiological processes or other stresses associated with the experimental conditions required to produce altered gravitational orientation on the neurohypophyseal-hypothalamo system of animals in an earth-based study. Future studies in the weightless environment of space are clearly needed, therefore, to validate this study. Blood levels of vasopressin and urinary outputs should be investigated in astronauts before and during space flight to provide more direct evidence of the effect of gravity (or reduced gravity) on vasopressin regulation and water balance.

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