On the Purifiication of Reptilian (Turtle) Pituitary Gonadotropin[1] (36146)

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(Introduced by Choh Hao Li)

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With the exception of a recent report on the preparation of avian gonadotropins (1) and a few reports on the fractionation of piscine pituitaries (2, 3) there is a paucity of biochemical information on the pituitary hormones of nonmammalian species. In particular, there is no information, whatsoever, on reptilian adenohypophysial hormones. In this preliminary study, we have attempted by means of very simple fractionation procedures, to obtain data which may be useful in the future, for the preparation of reptilian gonadotropin(s). It is firmly established that mammalian pituitaries contain two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH, interstitial cell-stimulating hormone, ICSH). Mammalian FSH and LH can be clearly separated in a variety of species by ammonium sulfate fractionation (4–7). Thus, LH (ICSH) is known to be almost quantitatively precipitated by half saturation with $(NH_4)_2SO_4$, leaving FSH in solution. We show here that $(NH_4)_2SO_4$ fractionation can be effectively employed for the partial purification of the gonadotropin(s) from turtle pituitaries (*Pseudemys scripta*). In addition, information is obtained on the solubility characteristics of turtle gonadotropin(s) compared to mammalian LH and FSH.

Materials and Methods. Turtles were purchased from a supplier in Wisconsin. All were large adults with carapace lengths between 16 and 21 cm. Two separate batches of pituitary were employed. The first consisted of 50 glands (191 mg wet weight) from male turtles received in mid-July; testes showed early stages of spermatogenic recrudescence. The second batch of 110 glands (290 mg wet weight) were obtained from mixed sexes (80 males, 30 females) in mid-October. Ovaries were regressed but testes were enlarged and active spermatogenically. In both cases, turtles were killed by decapitation and the glands (only the pars distalis was used) were placed on Dry Ice immediately upon removal and stored at $-20°$ until extracted. Pituitary tissue was homogenized with 0.9% saline in an all glass hand homogenizer and stirred for two hours thereafter. All procedures were performed in an ice bath or at 4°. Various degrees of saturation of ammonium sulfate (referred to as SAS) were achieved by addition of the solid salt as calculated from the nomogram published by Dixon (8). The fractions obtained were dissolved or suspended in a minimal volume of water, dialyzed against water, and lyophilized. Bioassays were performed in the male lizards, *Anolis carolinensis*, as described previously (9–11). Briefly, hormones were injected subcutaneously every other day for 10 days into either "physiologically" or surgically hypophysectomized adult males at 32°. Doses refer to the amount of hormone given per injection. Testis weights were used to quantify responses and routine histology was employed to assess spermatogenic activity. Hypertrophy of the epididymis and renal sex segment were taken as indices of testicular

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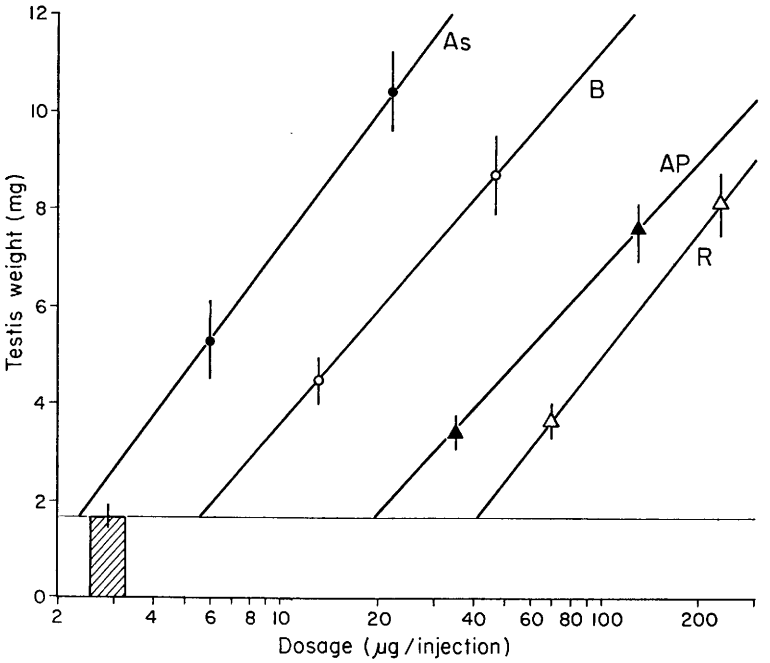

FIG. 1. Assay of gonadotropic activity of 4 fractions prepared from the adenohypophysis of adult male turtles, *Pseudemys scripta* (Exp. 1). Materials were injected subcutaneously in 5 equal injections on alternate days into adult male lizards at 32° with a short photoperiod to effect physiological hypophysectomy. Testes were fully regressed and quiescent at the start of treatment and remained so in saline treated controls (mean and SE for testis weights in controls shown by the cross-hatched bar and vertical line in lower left). Circles or triangles and vertical lines show the mean and SE for groups of 7 animals used for assay of each of 4 turtles pituitary fractions at 2 doses each. All responses were significantly greater than controls and all curves parallel. With a 10 μg dose of NIH-FSH-S8, testes weighed 13.0 ± 0.5 mg.

androgen production (steroidogenesis). Specific activities were obtained from multiple dose assays in which the preparations were compared with the NIH-FSH-S8 standard (a gift of the Endocrinology Study Section, NIH). In addition to the basic assay in the lizard, several fractions were tested for LH activity using the ovarian ascorbic acid depletion (OAAD) bioassay in the rat (12).

Results. Experiment 1. In this experiment, 191 mg of turtle pituitaries were homogenized and extracted with saline. The saline extract (pH 5.5) was adjusted to 0.5 saturation with $(NH_4)_2SO_4$ and the resultant precipitate collected by centrifugation, suspended in H_2O, and dialyzed. Following dialysis, insoluble material was separated and two fractions were obtained and lyophilized: AP, the insoluble material; AS, the soluble fraction. The 0.5 saturated supernatant was adjusted to 0.8 saturation of $(NH_4)_2SO_4$, and the resultant precipitate was dissolved, dialyzed, and lyophilized (fraction B).

Each fraction was tested in "physiologically" hypophysectomized *Anolis* at 2 doses. All four fractions contained gonadotropic activity as judged by the stimulation of testis growth and spermatogenesis. Response curves for testis weight for all fractions were parallel (in test for nonparallelism, $F = 0.80$ where $F\ .05 = 2.78$) but marked differences were evident in their relative potencies (Fig. 1 and Table I). Spermatogenesis had progressed to the formation of spermatozoa in the group treated with the high dose (22 μg) of fraction B, but only to the stage of spermatocytes or early spermatids in the others. Also, stimulation of steroidogenesis was evident only in the group receiving the high dose of fraction B (all 7 animals had enlarged and

TABLE I. Yields and Potencies of Turtle Pituitary Fractions.

	Fraction	Yield (mg)	Potency[a] (units/mg)	Total Activity units[a]	Total Activity %
Exp. 1 (50 glands; 191 mg)	Residue, R	16.5	0.016	0.26	21
	0–0.5 SAS, AS (soluble)	1.5	0.3	0.45	36
	0–0.5 SAS, AP (insoluble)	9.0	0.026	0.23	18
	0.5–0.8 SAS, B	3.0	0.1	0.30	24
				1.24	100
Exp. 2 (100 glands; 290 mg)	Residue, R	15	—	—	—
	0–0.3 SAS, A	13.5	0.07	0.95	35
	0.3–0.6 SAS, B′	15.0	0.12[b]	1.80	66
	0.6–0.8 SAS, C	3.4	0.0007	0.002	nil
				2.75	100

[a] Expressed in terms of NIH-FSH-S1 (1 unit/mg).

[b] Average of two separate assays in which the potency was estimated to be 0.10 and 0.14 units/mg.

secretory epididymides).

Table I summarizes the yield and activities of the four fractions based on the lizard assay. The index of precision for the assay was 0.17. It can be seen that although a majority of the activity was precipitated at 0.5 SAS, at least 24% was found in the fraction B, precipitating between 0.5 and 0.8 SAS. The residue, R, as well as the precipitate that formed upon dialysis, AP, were each of very low specific activity.

Experiment 2. In view of the division of activity into two fractions obtained above, the conditions in the experiment were modified in an effort to concentrate the gonadotropic activity maximally into one fraction. Thus, 290 mg of turtle pituitaries were handled as above, but fractions were obtained at 0–0.3 SAS (fraction A), 0.3–0.6 SAS (fraction B′) and 0.6–0.8 SAS (fraction C). These fractions were assayed using surgically hypophysectomized *Anolis*.

The yields and activities are summarized in Table I and show that fractions A and B′ contain virtually all the biological activity. Of these two fractions, B′ was almost twice as active as A. Fraction C (0.6–0.8 SAS) was virtually devoid of activity: both the yield and specific activity were very low. The dose response to fraction B′ was parallel to that obtained with ovine FSH (Fig. 2). The low dose (20 μg) of fraction B′ maintained all stages of spermatogenesis but did not stimulate androgenesis. The high dose (100 μg) of fraction B′ was highly effective in stimulating both spermatogenesis (including testis growth), and androgenesis since both the epididymides and renal sex segments were hypertrophied. Fraction C only barely maintained spermatogenesis, and the initial low weight of the testes. Fraction A (tested in a separate assay together with B′) at 30 μg maintained spermatogenesis with slight androgen stimulation.

Fraction B′ (from Exp. 2) was tested in the mammalian OAAD assay at a total dose of 100 μg (equivalent to 1.7 mg wet weight of whole pituitary). The response did not differ significantly from control values, indicating LH activity to be *less than* 0.6 μg NIH-LH-S1 or 0.06 units LH/mg. To determine whether some LH activity might have

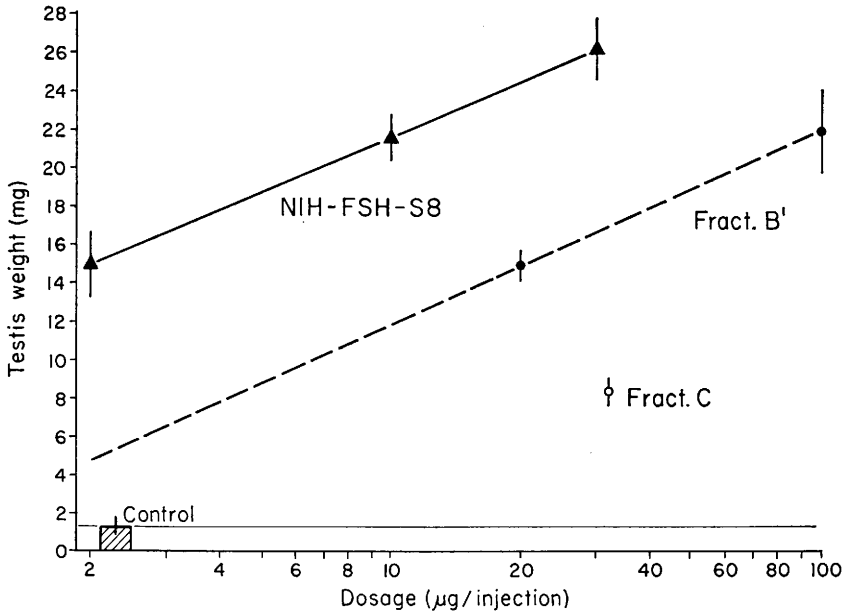

FIG. 2. Gonadotropin assay for fractions B′ and C from turtle pituitaries (Exp. 2) and NIH-FSH standard using surgically hypophysectomized *Anolis* lizards. Data presented as in Fig. 1. Testes showed early stages of spermatogenic recrudescence and average 7 mg at the start of treatment.

been lost in fractionation, homogenates of several separate batches of whole turtle pituitaries were also tested in the OAAD system. In the first test, a total dose of 1 mg wet weight was ineffective; in a second test, 8 mg fresh weight of turtle pituitary was found to be without activity. Thus, these tests indicate LH activity to be *less than* 0.08 μg NIH-LH-S1/mg.

All mammalian species of FSH which have been tested contain sialic acid, which when removed enzymically with neuraminidase results in loss of biological activity (6). A small quantity of fraction B′ (Exp. 2) was treated with neuraminidase and found to be unaffected when tested in the lizard: a 100 μg dose of hormone treated with neuraminidase was indistinguishable from the same dose of untreated material (Fig. 2). In other experiments (Licht and Papkoff, unpublished data), neuraminidase treatment was found to inactivate pituitary extracts or purified gonadotropins from a variety of vertebrates, when assayed in the lizard, including several species of squamate reptiles (snakes and lizards), but whole pituitaries from three species of turtles were unaffected. Thus, with respect to sialic acid, the turtle gonadotropin behaves like mammalian (ovine, bovine, human) LH (13).

Discussion. To summarize, we have found that small quantities of turtle pituitary tissue can be fractionated with ammonium sulfate, yielding a more concentrated gonadotropin fraction that can be employed for biological studies. The behavior of the material in ammonium sulfate solutions suggests a closer resemblance to mammalian LH than FSH. The lack of effect of neuraminidase on turtle gonadotropin also supports this view. Biological studies with purified mammalian hormones in lizards, however, suggest that reptilian gonadotropin has more of an "FSH-like" character (9–11, 14). With regard to the biological activity of this turtle gonadotropin preparation, tests in the lizard indicate that it has both FSH and LH-like activities if gametogenesis and steroidogenesis are taken as separate indices of these two types of hormonal activities. However, when compared with the effects of purified mammalian hormones in the lizard, the behavior and potency

of turtle gonadotropin is more like FSH and LH. As far as its behavior in mammalian bioassay systems are concerned, we can state that the turtle gonadotropin lacks LH activity, but tests for possible FSH activity have not yet been performed.

It remains to be determined whether the reptile possesses two gonadotropins as is the case in mammals and some birds. On the basis of the present study, there was no evidence for two separate gonadotropic principles in the turtle. The various active fractions from the pituitary differed only quantitatively. Cases in which only spermatogenesis was stimulated are best explained in terms of a low dose of gonadotropin (*e.g.*, FSH) rather than as evidence for a hormone that differentially regulated spermatogenesis. There was no evidence of an LH-like fraction that differentially affected steroidogenesis.

Summary. Turtle (*Pseudemys scripta*) pituitaries have been homogenized, extracted with saline, and the gonadotropin partially purified by ammonium sulfate fractionation. The bulk of the gonadotropin was found to precipitate between 0.3 and 0.6 saturated ammonium sulfate which is similar to the solubility behavior of ovine LH. No separation of gonadotropic activities (*i.e.*, LH and FSH) was evidenced on the basis of biological activity as tested in the lizard. The fraction most active in the lizard was inactive in mammalian bioassays. Neuraminidase did not affect the activity of the reptilian preparation. The reptilian gonadotropin resembles mammalian LH from a chemical point of view, but biological studies in the lizard suggest it has more of an "FSH-like" character.

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