Preparation of a Luteinizing Hormone Releasing Factor from Sheep Hypothalamus

The evidence that has accumulated over the past several years from a variety of sources, firmly establishes that the hypothalamus plays an important role in the release, and perhaps also the production, of hypophyseal hormones[1]. While analysis of the humoral factor causing release of ACTH (the so-called CRF) has received the most attention, isolation of specific factors controlling other hypophyseal hormones, including various gonadotropins is currently under investigation in many laboratories. Release of luteinizing hormone (LH) from the hypophyses of rats has been accomplished using acid extracts of rat, sheep and beef hypothalamic tissue[2–5]. COURRIER et al.[2] first started purification of these extracts by acetone precipitation. We[5] have previously taken advantage of this method and used double acetone precipitations to obtain active fractions, not only from sheep hypothalamus, but also from brain tissue, exclusive of hypothalamus. Release of endogenous LH using the brain extracts could be ascertained by observing ovulation in mature follicles of androgenized female rats, but not by significant depletion of ovarian ascorbic acid.

The present report outlines a simple procedure for obtaining an LH-releasing factor from sheep hypothalamic tissue. The extracts obtained are not pure, and probably are of little value for chemical studies. However, they can be given intravenously in very large doses without toxic effects and are offered as a useful tool in acute and chronic functional studies of hypothalamic control.

Pieces of sheep hypothalamus, 25 mm square and approximately 7 mm thick, weighing 4.6 ± 0.9 g, were purchased from Cornish Laboratories, Berkeley (Calif.). This tissue, obtained from slaughtered females and castrate males, was frozen as quickly as possible after removal from the animal and maintained at $-20°C$ until extraction. The pieces were examined for remnants of hypophyseal tissue which would obviously contaminate extracts with gonadotropins.

The frozen tissue was thawed, finely minced in a blendor with acetate buffer at pH 4.6, 0.01 μ ionic strength, and centrifuged at 12,000 r.p.m. for 30 min in a refrigerated centrifuge. One half of this extract was immediately lyophilized (extract No. 3-1, Figure 1), while to the remainder was added 4 vol of acetone. The acetone-insoluble material was collected by centrifugation, resuspended in buffer and again precipitated with acetone (extract No. 3-2). The precipitate was washed twice with acetone and dried *in vacuo*. From the lyophilized and acetone fractions, a solution, containing an equivalent of 10 pieces of hypothalami per ml, was prepared, and assayed by the ovarian ascorbic acid depletion (OAAD) method of PARLOW[6].

The Table compares the percent depletion obtained with extracts and ovine LH(NIH-LH)[7]. Immature Holtzman strain female rats were used, with the material being injected *via* a tail vein in a period of 15 to 30 sec, and ascorbic acid determined in one ovary 4 h later. Brain tissue, exclusive of hypothalamus, extracted in the same manner as that outlined above for the acetone fraction, was also tested. The dosage of this material consistently used was an amount of extract equal to 30 g of fresh brain tissue, which is also equivalent, in weight, to about 6.5 pieces of hypothalami. The value shown for brain extract is the average depletion obtained in 5 assays, using 3 different extracts. In each of these assays, some depletion was obtained, but in only one was the amount significantly greater than that obtained with saline.

Since the acetone precipitates appear to contain essentially all the activity extractable with buffered acetate, modification in this procedure was attempted. In subsequent extractions, time was saved by first mincing the tissue with acetone, followed by acetate extraction, all other aspects of the procedure being the same as those outlined in Figure 1. With this procedure centrifugation

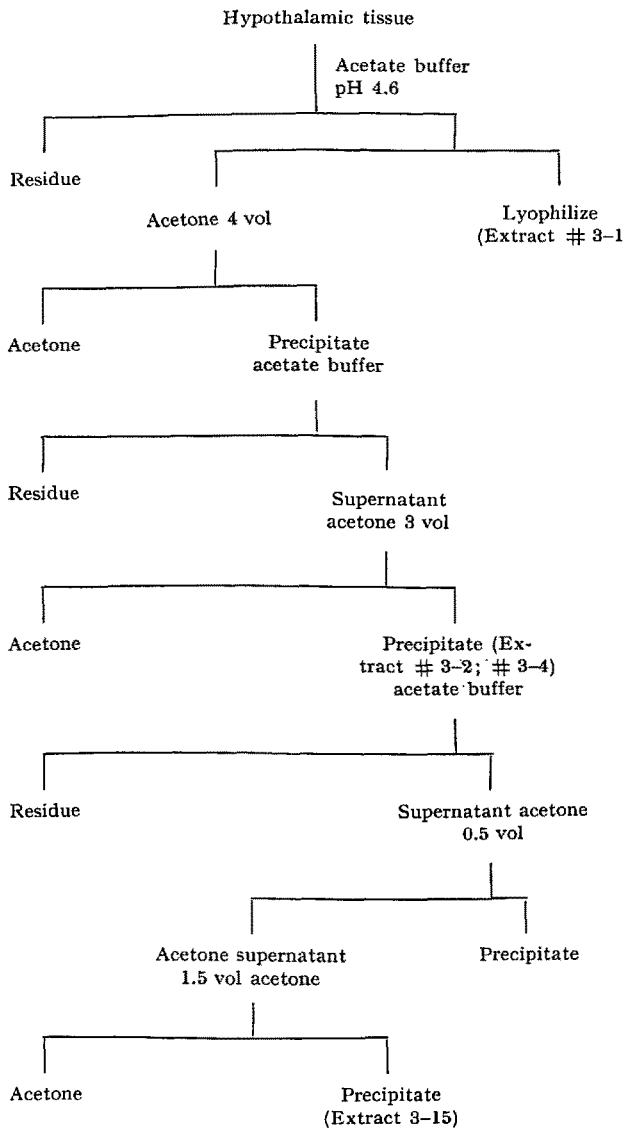

Fig. 1. Flow sheet for obtaining hypothalamic extracts by acetone precipitation.

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[2] R. COURRIER, R. GUILLEMIN, M. JUSTISZ, E. SAKIZ, and P. ASCHEIM, C.R. Acad. Sci. (Paris) *253*, 922 (1961).
[3] S. M. MCCANN, Am. J. Physiol. *202*, 395 (1962).
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[5] D. C. JOHNSON, Endocrinology *72*, 832 (1963).
[6] A. F. PARLOW, in *Human Pituitary Gonadotropins* (Ed. A. ALBERT, Charles C. Thomas, Springfield, Ill. 1961), p. 300.
[7] Standard luteinizing hormone was a gift of the Endocrinology Study Section, National Institutes of Health.

Results in the OAAD assay using extracts or ovine LH

Material	Dose	Ascorbic acid depletion (% ± S.E.)
LH (ovine) NIH-LH	5.0 μg	37.5 ± 1.8n $P < 0.001$
LH + anti-LH	5.0 μg + 0.25 ml	14.1 ± 2.1n
Hypothalamus extract 3–1	10.0 pieces	56.6 ± 1.9
Hypothalamus extract 3–2	10.0 pieces	54.0 ± 2.1
Brain extract (5 assays)	6.5 pieces equivalent	7.7 ± 3.2
Hypothalamus extract 3–4	5.0 pieces	27.6 ± 1.3n $P < 0.001$
Hypothalamus extract 3–4 + heat	5.0 pieces	7.7 ± 2.1n
Hypothalamus extract 3–15	2.5 pieces	24.1 ± 1.7
Hypothalamus 3–15 + anti-LH	2.5 pieces	22.9 ± 2.4

of the extract required only 10 min at 3000 r.p.m. No loss in activity was found using this early acetone treatment (Table, extract No. 3-4).

Heating extract No. 3-4 to 100°C in a water bath for 1 min, brought about the formation of a precipitate, and a concomitant loss (72% $P < 0.01$) in activity. This result, which confirms our earlier report[5], is in contrast to the findings of McCANN[3] and NIKITOVITCH-WINER[4], who reported that heat did not affect the LH-releasing activity of their extracts. McCANN[3] used the fact that heat destroyed the activity of hypophyseal LH, but not LH-releasing factor, as evidence that the latter was not merely a gonadotropin contaminated extract. Whether the discrepancy in heat lability between extracts is due to a species difference or to the degree of purity is as yet undetermined.

A further purification is effected by removal of an inactive fraction which precipitates with 33% acetone. Addition of another 1.5 volumes of acetone, to bring the concentration to 66%, precipitates the active material. While fractions are obtained at 75 and 80% acetone, they are inactive in causing LH release. The washed and dried final product is a reddish-brown powder, weighing 2.3 mg per piece of hypothalamus, or 0.5 mg/g of original tissue (extract No. 3-15). The dose response curve for this material in the OAAD assay is linear (Figure 2) with a λ-value of 0.303.

Hypophysectomized animals have generally served as controls to demonstrate that hypothalamic extracts are active only in the presence of the hypophysis. However, this test has the difficulty, which has been emphasized by GUILLIMEN and SAKIZ[8] that ovarian ascorbic acid levels fall rapidly following hypophysectomy and LH does not deplete this further. Therefore a simplified control procedure was attempted using an immune reaction. Antiserum to sheep hypophysis (acetone dried anterior lobes) was prepared in rabbits. 15 min prior to administration of test material in the OAAD assay, 0.25 ml of this antiserum was given intravenously. The effectiveness of this amount in reducing (by 62%) the activity of 5 μg of ovine LH is shown in the Table (LH + anti-LH). In contrast, there is no significant difference between the ascorbic acid depletion obtained with hypothalamic extract and extract plus antiserum. Had any of the activity in the extract, which caused less depletion than 5 μg of LH, been due to gonadotropin contamination, the antiserum would certainly have reduced the activity.

A criticism of the OAAD assay is that vasopressin, which is likely to be a contaminant of hypophyseal stalk and median eminence extracts, causes ascorbic acid depletion. Ovulation of anovulatory androgenized female rats is not obtained with either vasopressin or oxytocin[6] and this test was customarily employed in examining the various fractions in the extractions outlined above. However, a disadvantage in using these females is that they must be prepared well in advance of their use (about 60 days). The Weaver Finch assay of WITSCHI[9], which is a sensitive, specific, and simple test for LH, and not affected by posterior lobe hormones was therefore tried.

The birds used were *Steganura paradisea*, commonly called Paradise Wydahs. The subtail feathers of mature females were plucked and the feather follicles allowed to reorganize and grow for 5 to 7 days. The material to be assayed was then administered intramuscularly in a total volume of 1 ml distributed in four injections within a period of 48 h. Standard LH[7] was tested simultaneously and always gave a positive response with 5 μg. With hypothalamic extract No. 3-15 all 6 birds tested gave a strong positive reaction after receiving a total dose of extract equivalent to 4 hypothalami, as did the 5 birds receiving the 1 piece dose. Only 1 of 3 birds showed a melanin deposition during the time of injection with a total dose of 0.75 pieces. No positive responses were obtained with brain extracts using an equivalent of 5 pieces of hypothalami per bird (extract equal to 25 g of brain tissue). Also, vasopressin (Parke, Davis & Co., Pitressin) at 4 units and oxytocin (Parke, Davis & Co., Pitocin) at 2 units failed to cause a positive reaction in any of the 5 birds tested with each compound.

The degree of contamination with other hypophyseal controlling factors was not determined. However, chronic

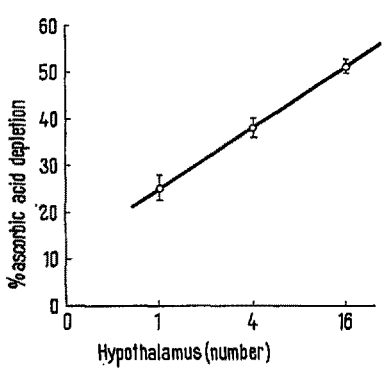

Fig. 2. The log dose-response curve for purified sheep hypothalamic extract (Extract No. 3–15). Vertical lines indicate standard error in groups of 6 animals.

[8] R. GUILLIMEN and E. SAKIZ, Endocrinology *72*, 813 (1963).
[9] E. WITSCHI, Mem. Soc. Endocr. *4*, 149 (1955).

treatment of male and female rats with 2.5 pieces intravenously twice per day for 7 days does not cause a change in adrenal weight[10].

Résumé. Un matériel provoquant la libération d'hormone lutéinisante (LH) d'hypophyse de rats femelles (impubères ou mûres), aussi bien que d'hypophyse d'oiseaux femelles (*Steganura paradisea*), a été obtenu par la précipitation à l'acétone d'extraits acides de l'hypothalamus de mouton. L'évidence que l'activité de ces extraits n'est pas due a la contamination LH a été obtenue grâce à une méthode sérologique. L'antisérum préparé contre l'extrait d'hypophyse de mouton inhibe la capacité de l'LH de mouton d'épuiser l'acide ascorbique de l'ovaire, mais pas celle de l'extrait hypothalamique.

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[10] This research was done in the Departments of Urology and Zoology, State University of Iowa, Iowa City (Iowa) and supported by a grant (C-3993) from the U.S. Public Health Service.

Physiological Role of Coronary Constriction in the Tortoise (*Emys orbicularis* L.)

Unlike warm-blooded animals, the coronaries of the tortoise are unable to adapt themselves to any changes of cardiac metabolism[1]. It is precisely for this reason that the direct constrictor effect of the catecholamines is revealed in the coronaries of the tortoise. In the mammalian heart the constrictor effect of catecholamines shows itself only when these are released at the nerve endings of coronary vasomotor fibres[2,3]. It may be added besides that, as shown conclusively by Drury and Sumbal[4], sympathetic vasoconstrictor fibres run to the coronaries of the tortoise, just as they do to the mammalian coronaries[2,3].

However, the physiological role played by the coronary constrictor system of the tortoise has not yet been ascertained. We assumed this role to consist in the regulation of the cardiac metabolic level. In order to verify this hypothesis, a set-up isolated from the surrounding airspace and consisting of rubber tubes and a reservoir, has been substituted by us for the whole circulatory system of the tortoise, under urethane narcosis. The cardiac oxygen consumption was measured by a special low-capacity (0.5 ml) densitometer cuvette. The experimental set-up did not influence coronary circulation.

Coronary constriction was elicited by local application of diluted adrenaline – or noradrenaline – solution to the surface of the heart. Our findings, in full accordance with those of Drury and Smith[5], have shown that this procedure resulted in large and extensive coronary circulation. The filled coronaries were reduced to hardly visible bundles. Simultaneously, coronary constriction greatly decreased cardiac oxygen consumption and, in many cases, completely stopped it. Figure 1 shows that local administration of 1 µg noradrenaline resulted in total anaerobiosis over a certain period. It is a notable fact that neither in this experiment, nor in others made by us, could a reduction of the ventricular contractile force or of the pressure in the aorta be observed during the anoxic periods. For these reasons temporary anaerobiotic cardiac activity should be considered a physiological state in the tortoise.

Further experiments showed that, when necessary, the tortoise in fact makes use of coronary constriction conducive to anaerobiosis. In these latter experiments coronary flow in waking tortoises of unimpaired circulation was measured by means of small, heated thermistor-beads introduced into the heart. The lungs were artificially ventilated with the aid of a pump. As can be seen in Figure 2, interruption of breathing elicited considerable coronary constriction, the flow being restored after breathing had been started again. Constriction set in so fast that it could only be attributed to a reflex mechanism. This explanation is supported by the fact that constriction cannot be elicited by interrupting the breathing of deeply narcotized animals.

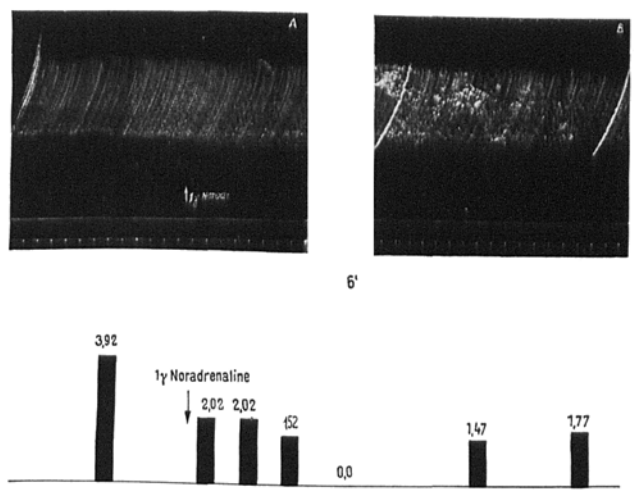

Fig. 1. Application of 1 µg noradrenaline to the coronaries of the tortoise decreases cardiac O_2 consumption. Between A and B there was a period of total anaerobiosis exceeding 6 min. From above downwards: ventricular activity, time signal (30 sec), diagram of O_2 consumption. The numbers above the columns refer to the calculated O_2 consumption in µl/30 sec.

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