The Effects of Ovariectomy and Injected Oestradiol Monobenzoate on Polypeptide Metabolism in the Hypothalamus

By K. C. HOOPER

Department of Physiology, University of Sheffield

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1. The activities of two groups of peptidases separated from a homogenate of rabbit hypothalamus were determined (*a*) in adult female animals, (*b*) in ovariectomized animals and (*c*) in intact female animals after injection of oestradiol monobenzoate (15–120 μ g.). 2. Ovariectomy decreased the enzyme activity initially; the activity in the particulate group of enzymes subsequently returned to normal whereas the activity of the supernatant fraction was less than normal 8 months after operation. 3. Injection of oestradiol monobenzoate increased the enzyme activity in the supernatant fraction to that observed in pregnant animals and in suckled lactating animals. 4. There is a correlation between changes in enzyme activity of the supernatant fraction and conditions that are known to influence gonadotrophin secretion.

Previous work showed that the activities of certain peptidases in the hypothalamus varied according to the physiological state of the animal. An increase in activity was noted after parturition and during lactation (Hooper, 1966*b*), and also during pregnancy (Hooper, 1964, 1966*a*). The substrate used to measure peptidase activity was the hormone oxytocin, and it was considered that some of the enzymes measured under the experimental conditions were implicated in oxytocin metabolism, for oxytocin is known to be released during parturition and by the stimulus of suckling. The observation that pregnancy was accompanied by an increase in peptidase activity suggested that there might be some correlation between peptidase activity in the hypothalamus and the control of gonadotrophin secretion from the anterior pituitary, for gonadotrophin secretion is known to be inhibited by suckling and during pregnancy (for references see Harris & Campbell, 1966). Many factors are known to inhibit gonadotrophin secretion, among them being elevated concentrations of circulating oestrogenic hormones.

The present work was undertaken to evaluate the influence of the ovary and of ovarian hormones on the metabolism of polypeptides by the hypothalamus. This paper describes the effect of ovariectomy and of the administration of oestradiol monobenzoate to intact female rabbits, and discusses some of the implications that arise from the results. A preliminary account of part of this work has been given (Hooper, 1966*c*).

METHODS

Animals. Adult female rabbits of the New Zealand white strain were kept separately and isolated for about 3 weeks to ensure that none was pregnant or pseudo-pregnant. Bilateral ovariectomy was performed by the mid-line incision approach, with Nembutal (0.5 ml./kg. body wt.) used as main anaesthetic, the required state of anaesthesia being obtained with ether. Intact female animals were injected intramuscularly for 3 consecutive days with a fixed dose of oestradiol monobenzoate diluted with arachis oil, and were killed on the fourth day. The doses used were 15 μ g., 30 μ g., 60 μ g. and 120 μ g. of oestrogen.

Fractionation of the hypothalamus. Animals were killed by a blow on the neck and bled out; the brain was rapidly excised and the hypothalamus dissected out after removal of the optic chiasma and corpora mamillaria. The uterus and ovaries were also removed for histological examination. The hypothalamus was homogenized in 9 vol. of 0.25 M-sucrose in a hand-operated homogenizer, and the homogenate was centrifuged at 2° successively for 15 min. at 600*g*, 60 min. at 7600*g* and 60 min. at 25000*g* to give nuclear, mitochondrial, microsomal and supernatant fractions respectively. The unwashed mitochondrial fraction was fractionated further by centrifugation at approx. 7.5×10^6 *g*-min. (Spinco model L preparative ultracentrifuge with a SW39 rotor head) through a discontinuous gradient made from 0.32 M-, 0.8 M- and 1.2 M-sucrose solutions. Three layers of particles were separated and, of these, only the middle fraction was kept. The concentration of sucrose in it was adjusted to approx. 0.25 M by the addition of water, and it and the supernatant fraction were dialysed against tap water overnight at 4°. The non-diffusible nitrogen content of the two fractions was measured by a micro-Kjeldahl method.

Measurement of enzyme activity. The enzyme activities of the two fractions were determined in terms of their ability to cause loss of oxytocic activity under specified conditions. Oxytocin (0.5 i.u./ml.) was incubated with the specified fractions at 37° for 3 hr. at pH 7.2–7.3 (0.2 vol. of 0.4 M- Na_2HPO_4 – NaH_2PO_4 buffer). Enzyme action was stopped by heating the mixture in a boiling-water bath for 15 min. Residual oxytocin was measured on the isolated rat uterus by using a bracketing type of assay (Hooper & Jessup, 1959), and the fiducial limits at a probability of 0.95 were calculated by the method of Burn (1952). The recovery of hormone from the medium after incubation is expressed in terms of hormone recovered from a control experiment, i.e. as $\log(A_0/A)$, where A_0 and A are the concentrations of oxytocin (milliunits/ml.) in the control and test experiments respectively.

The control experiment was used to indicate loss of substrate due to factors other than enzyme action, and also to check that the fractions contained no detectable amounts of oxytocic substances that might interfere with bioassay procedures. The media of the control and test experiments contained equal quantities of the same tissue fraction, but they differed in that the control contained protein that had been denatured by heating for 15 min. in a boiling-water bath before incubation with the hormone.

Materials. Di-Menformon (oestradiol monobenzoate B.P.; 5 mg./ml.) was obtained from Organon Laboratories Ltd., London, W.C. 2; Nembutal Veterinary (pentobarbitone sodium B.P.; 60 mg./ml.) was obtained from Abbott Laboratories Ltd., Queenborough, Kent. Pitocin (Parke, Davis and Co. Ltd., Hounslow, Middx.), a synthetic preparation of oxytocin, was used as enzyme substrate.

RESULTS

Enzyme activity in intact female rabbits. Previous work on the intracellular distribution of hypothalamic enzymes active against oxytocin (Hooper, 1966a) showed that detectable activity was present only in the supernatant and in the particulate fraction prepared as described in the Methods section. The relation between the inactivation of oxytocin and the concentration of hypothalamic nitrogen for both the supernatant and the particulate fraction is shown in Fig. 1. This relation is essentially the same as that reported by Hooper (1966a), but additional points and fiducial limits are shown.

Enzyme activity in ovariectomized animals. The first group of rabbits were killed 6 weeks after the removal of ovaries. The relation between enzyme activity and tissue concentration is shown in Fig. 2. A comparison of the curves in Figs. 1 and 2 shows that there was no detectable difference at a probability of 0.95 between the activities of the particulate fractions from ovariectomized and from intact animals. However, the supernatant fraction obtained from ovariectomized animals possessed a lower activity than did the corresponding fraction from intact animals. Two other groups of ovariectomized animals were examined, one killed 8

Fig. 1. Relation between enzyme activity and concentration of tissue. Rabbit hypothalamus was homogenized and separated into fractions. The supernatant fraction (○) and one particulate fraction (●) were incubated with oxytocin for 3 hr. at 37°, and the residual biological activity was measured. For experimental details, see the text. A_0 and A , control and test polypeptide concentrations respectively.

Fig. 2. Hypothalamic enzyme activity after ovariectomy. Adult female rabbits were ovariectomized and the activities of the supernatant enzymes (□, ○ and △) and the particulate enzymes (■, ● and ▲) were measured as described in Fig. 1. Post-ovariectomy period: □ and ■, 2 weeks; ○ and ●, 6 weeks; △ and ▲, 8 months.

months after removal of ovaries, and the other killed 2 weeks after ovariectomy. The results of these two series are also plotted in Fig. 2. The 8-month post-operative series showed that the activity of the particulate fraction was similar to that found in intact animals. However, the activity of the supernatant fraction was intermediate between that of intact animals and that of animals that had been ovariectomized for 6 weeks. Animals killed 2 weeks after ovariectomy did not possess detectable amounts of activity in the particulate fraction, and possessed only very low activity in the supernatant fraction.

Enzyme activity of intact animals treated with oestradiol monobenzoate. The results obtained from these animals are shown in Fig. 3. The activity

Fig. 3. Hypothalamic enzyme activity in intact female rabbits after injection of oestradiol monobenzoate. Rabbits were injected for 3 consecutive days and killed on the fourth day. The activities of the supernatant enzymes (Δ , ∇ , $\square$ and $\circ$) and the particulate enzymes ($\blacktriangle$, $\blacktriangledown$, $\blacksquare$ and $\bullet$) were measured as described for Fig. 1. Doses: Δ and $\blacktriangle$, 15 $\mu\text{g.}$; ∇ and $\blacktriangledown$, 30 $\mu\text{g.}$; $\square$ and $\blacksquare$, 60 $\mu\text{g.}$; $\circ$ and $\bullet$, 120 $\mu\text{g.}$

seemed to be independent of the amount of oestradiol injected, for points obtained with 15 $\mu\text{g.}$ doses lay virtually on the same curve as those obtained with 120 $\mu\text{g.}$ doses. The activity of the particulate fraction was not significantly different from that found in intact animals, whereas the activity of the supernatant fraction was greater than that found in normal animals.

DISCUSSION

The results show that the ovary has some influence on polypeptide metabolism in the hypothalamus. The effect of withdrawal of the main source of ovarian hormones is apparently at its greatest shortly after ovariectomy, for enzyme activity was very low 2 weeks after removal of the glands. After 6 weeks the activity of the particulate group of enzymes had reached normal values whereas the activity of the enzymes in the supernatant was still depressed. After 8 months the activity in the supernatant had risen but was still below that present in intact animals. Ovarian influence on the hypothalamic enzymes is probably hormonal in nature, for afferent neural pathways between ovary and hypothalamus have not yet been established. The gradual return of peptidase activity in the hypothalamus after ovariectomy

may be due to a compensatory mechanism of adrenal origin.

Attempts to mimic ovarian hypersecretory effects were made by injecting oestradiol monobenzoate into intact animals. The doses of hormone employed (15–120 $\mu\text{g.}$) are generally considered to be within the normal physiological range for the pregnant rabbit (see, e.g., Schofield, 1962). The minimum dose was as effective as the higher ones, indicating that the response was probably maximal. The effect produced is noteworthy in that it reveals a difference in response between the particulate and the supernatant groups of peptidases; a difference in the behaviour of the two groups in response to suckling was noted by Hooper (1966b). The activity of the particulate enzymes was not altered by administration of oestradiol, but that of the supernatant enzymes was increased. This effect is similar to that observed after the time of blastocyst implantation in pregnant rabbits and also in suckled lactating animals.

The results show that there is correlation between the activity of certain hypothalamic peptidases and hormonal stimuli that are considered to inhibit the release of certain gonadotrophic hormones. Physiological conditions under which release of luteinizing hormone is suppressed, e.g. during pregnancy and lactation, are accompanied by increased peptidase activity; conversely, conditions where gonadotrophin release would be expected to increase, e.g. after ovariectomy, are accompanied by decreased peptidase activity. Since oxytocin was employed as substrate to measure peptidase activity, it seems reasonable to assume that the two groups of enzymes investigated are involved in the metabolism of oxytocin or similar polypeptides present in the hypothalamus.

Measurement of enzyme activities may offer a biochemical approach to the study of hormonal feedback mechanisms in the hypothalamus.

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REFERENCES

- Burn, J. H. (1952). *Biological Standardization*, 2nd ed., p. 37. London: Oxford University Press.
- Harris, G. W. & Campbell, H. J. (1966). In *The Pituitary Gland*, vol. 2, pp. 99–165. Ed. by Harris, G. W. & Donovan, B. T. London: Butterworth and Co. Ltd.
- Hooper, K. C. (1964). *Biochem. J.* **90**, 584.
- Hooper, K. C. (1966a). *Biochem. J.* **99**, 129.
- Hooper, K. C. (1966b). *Biochem. J.* **100**, 823.
- Hooper, K. C. (1966c). *Biochem. J.* **100**, 37 f.
- Hooper, K. C. & Jessup, D. C. (1959). *J. Physiol.* **146**, 539.
- Schofield, B. M. (1962). *J. Endocrin.* **25**, 95.