THE EFFECT OF OVARIECTOMY ON THE ACTIVITY OF CERTAIN ENZYMES IN THE FEMALE RAT HYPOTHALAMUS

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ABSTRACT

Previous work in the rabbit has shown that the activity of certain peptidases in the hypothalamus which inactivate oxytocin, changes with stimuli known to release gonadotrophins, and may be used as an index of gonadotrophin hormone release (Hooper 1966a, b, 1968; Frith & Hooper 1971a, b). Using this approach, a study was made of the activities of similar peptidases in the rat hypothalamus following ovariectomy, a condition known to cause gonadotrophin release. Enzyme activity in the supernatant fraction was found to decrease progressively with time after ovariectomy, until 42 days after operation, thereafter maintaining a level not significantly different from that at 42 days; there was no detectable difference in particulate enzyme activity after ovariectomy. An inverse relationship between supernatant enzyme activity and luteinizing hormone levels is suggested. It is concluded that a similar relationship to that in the rabbit exists between enzyme activity in the rat hypothalamus and the release of luteinizing hormone-releasing factor from the tissue.

The hypothalamus of the rabbit contains certain enzymes capable of inactivating oxytocin (Hooper 1966a, b, 1968). The activity of these enzymes has been shown to vary after stimuli known to alter gonadotrophin release from the anterior pituitary: for example, enzyme activity is decreased by ovariectomy and increased by oestradiol monobenzoate injection (Hooper 1968). Further, it has been suggested that the enzyme system inactivating oxytocin in the rabbit can be used as an index of gonadotrophin release (Frith & Hooper 1971a) and it would seem probable that changes in enzyme activity can be related to luteini-
zing hormone-releasing factor (LH-RF) release (Frith & Hooper 1971b). Following these findings, it was decided to make a change of experimental animal from the rabbit to the rat, an animal exhibiting an oestrous cycle, so that enzyme activity could be investigated during the different hormonal regimes associated with this cycle. However, it was first found necessary to determine if conclusions drawn from work in the rabbit were applicable to the rat, before further work on the oestrous cycle could be attempted.

In the rat, there is considerable documentation of both gonadotrophin and LH-RF levels in various physiological conditions (for references, see McCann & Porter 1969 and Everett 1969). Bioassay and radioimmunoassay determinations have clearly established that luteinizing hormone (LH) secretion can be altered in response to changes in gonadal steroid levels; there are conflicting views on hypothalamic LH-RF levels during such changes (Chowers & McCann 1965; Piacsek & Meites 1966). However, there appears to be a lack of evidence of enzymes capable of inactivating oxytocin in the rat hypothalamus, and of any correlation between these and LH and LH-RF levels.

The present work was undertaken to demonstrate the presence of such enzymes in the rat hypothalamus, the influence of ovariectomy upon them and to determine if conclusions drawn from work with the rabbit are applicable to the rat. It also provides some discussion of the implications arising from the results obtained.

**MATERIALS AND METHODS**

*Animals.* – Adult, virgin, albino female rats of the Sheffield strain, weighing 250–250 g were used. These animals were kept under standard conditions, and were divided into two groups: – a control group of female rats, randomly selected with respect to the oestrous cycle, and a second group of female rats, bilaterally ovariectomised under ether anaesthetic and killed at various intervals after operation.

*Preparation of the hypothalamus.* – Animals were killed at approximately the same time of day (08.00 GMT) under ether anaesthetic, by bleeding out. The brain was quickly removed and the hypothalamus dissected out after removal of the optic chiasma. Each hypothalamus was weighed and those from six animals bulked together to provide sufficient tissue for enzyme determinations. Homogenization was performed in 8 vol. 0.25 M sucrose in a hand-operated homogenizer (all previously to 4°C). The homogenate was centrifuged at 2°C for 15 minutes at 600 g to give a nuclear/cell debris fraction and a supernatant fraction; the former was discarded as it was found to contain no significant enzyme activity, and the latter was centrifuged again, for 1 h at 25 000 g, giving a particulate fraction (mitochondria, microsomes) and a supernatant fraction. The particulate fraction was resuspended in about 0.8 ml double distilled water, and both this and the supernatant fraction were dialysed overnight against distilled water at 4°C. The non-diffusible nitrogen content of the two fractions was determined by a micro-Kjeldahl technique.
**Measurement of enzyme activity.** – The enzyme activity in both particulate and supernatant fractions was measured by estimating the loss of oxytocin activity incurred when the fractions were incubated with oxytocin under controlled conditions. Oxytocin (500 mU) was incubated with varying amounts of the specified fractions at 37°C for 8 hours at pH 7.8 (0.2 vol. of 0.4 m Na₂HPO₄ – NaH₂PO₄ buffer). Enzyme action was terminated by heating the mixture in a boiling water bath for 15 minutes. A control was prepared by boiling a standard amount of each fraction before incubation; solutions were stored at -20°C until required for assay. Residual oxytocin after incubation was measured on the isolated rat uterus using a bracketing type of assay (Hooper & Jessup 1959). The recovery of oxytocin from the medium after incubation is expressed in terms of substrate recovered from the control experiment i.e. as log Ao/A, where Ao and A are the concentrations of oxytocin (mU/ml) in the control and test experiments respectively. Log Ao/A was plotted against the non-diffusible nitrogen content of hypothalamus homogenate used. Fiducial limits at a probability of 0.95 were calculated (Burn 1952).

**Materials.** – Pitocin® (Parke Davis and Co., Hounslow, Middlesex), a synthetic preparation of oxytocin, was used as enzyme substrate.

**RESULTS**

*Enzyme activity in the hypothalamus of randomly-picked normal female rats*

Three groups of six rats each were used here. The activities of the two fractions capable of inactivating oxytocin are shown in Fig. 1, which clearly demonstrates enzyme activity to be linearly related to the amount of tissue incubated (in µg non-diffusible nitrogen). The supernatant fraction has a specific activity approximately three times that of the particulate fraction. These findings agree closely with those obtained in the rabbit (Hooper 1968; Frith & Hooper 1971a).

*Enzyme activity in the hypothalamus of female rats after ovariectomy*

Two groups of six rats each were used in the determination of enzyme activity 7, 14, 42 and 83 days after ovariectomy; the relation between enzyme activity and tissue concentration at these intervals after ovariectomy is shown in Figs. 2 and 3. A comparison of the curves in Figs. 1 and 2 shows there is no detectable difference in particulate fraction activity at a probability of 0.95 between intact and ovariectomised animals. However, the supernatant fractions obtained from ovariectomised animals had a progressively lower activity with time than the same fraction from intact animals (Fig. 3). Activity was significantly lower 7 days after ovariectomy and continued to decrease to its level 42 days after operation (Fig. 4), thereafter maintaining a level not significantly different from that at 42 days.
Levels of enzyme activity in the hypothalami of 3 groups of 6 rats (randomly-selected with respect to oestrous cycle); three estimations made from each group; ± fiducial limits; • supernatant enzyme; ○ particulate bound enzyme.

DISCUSSION

The depression of supernatant activity can be correlated with and would seem to be related to luteinizing hormone (LH) levels following ovariectomy, although there was no significant change in particulate activity. Pituitary LH content has been shown to increase significantly with time after ovariectomy, though this only represents the balance between synthesis and release in the pituitary (Chowers & McCann 1965; Piacsek & Meites 1966; Labhsetwar 1969). A more appropriate approach would be to measure serum LH; using a radioimmunoassay technique, this has been demonstrated to increase following ovariectomy, rising rapidly over the first 14 days and reaching a plateau level after 35 days (Gay & Midgley 1969). Comparison of these findings with supernatant enzyme activity over this period and beyond indicates that there is an extremely close inverse relationship between the two. Taken with previously published work in the rabbit (Hooper 1966a,b, 1968; Frith & Hooper 1971a,b), it is quite clear that, in the rat, enzyme activity and gonadotrophin release from the anterior pituitary (and hence LH-RF from the hypothalamus) can be correlated in a similar way.

To provide the increased pituitary and serum LH levels after ovariectomy,
Fig. 2.
Levels of particulate bound enzyme activity at various time intervals after ovariectomy. 2 groups of 6 rats were used for each experiment; three estimations made from each group; ± fiducial limits; □ 7 days after operation, ◇ 14 days after operation, ○ 42 days after operation, △ 83 days after operation.

Fig. 3.
Levels of supernatant enzyme activity at various time intervals after ovariectomy. 2 groups of 6 rats were used for each experiment (three estimations made from each group); ± fiducial limits; ■ 7 days after operation, ● 14 days after operation, ◆ 42 days after operation, ▲ 83 days after operation.
Fig. 4.
Levels of activity of a constant amount of supernatant enzyme (189 µg.) at various time intervals after ovariectomy; ± fiducial limits.

LH-RF release and hypophysial portal blood levels would also be expected to be elevated. Chowers & McCann (1965) found that 21 days after ovariectomy, hypothalamic LH-releasing activity was not influenced, even though pituitary LH was elevated. However, the quantity of LH-RF in the hypothalamus in this case must again represent the balance between synthesis and release. Studies by Piacsek & Meites (1966), on the contrary, showed that hypothalamic LH-RF decreased as a result of ovariectomy, a similar result to that found independently by Moszkowska & Kordon (1965). This decrease does not parallel the changes in pituitary LH but could represent an increased LH-RF release rather than synthesis. Only direct measurement in the hypophysial portal system can provide a true picture of hypothalamic LH-RF response to ovariectomy. Initial work (Fink & Harris 1970) suggests portal levels do increase after ovariectomy; however, until more detailed studies are made, no real comparison with supernatant activity can be made. If LH-RF portal levels do parallel the change in serum LH, then a similar inverse relationship would be expected.

The use of oxytocin as substrate for measuring enzyme activity could suggest that oxytocin or a similar chemical substance may be involved in gonadotrophin release. At one time, it was thought that oxytocin and LH-RF might be identical (Martini et al. 1959); in revealing the structure of LH-RF, Schally et al. (1971),
indisputably show this to be untrue. Some structural similarities do exist, but it is without doubt that LH-RF is a separate entity. This does not rule out the possibility that oxytocin or a similar compound may be involved in gonadotrophin release, as there are numerous examples indicating that oxytocin may well be involved in such a mechanism in the rabbit and rat (Brinkley & Nalbandov 1963; Endröczi & Hilliard 1965; Martini 1966; Berthelay et al. 1967; Melin 1971). Also of particular interest is the report by Barnafi & Croxatto (1966) that ovariectomy in the rat decreases the oxytocin content of the posterior pituitary 30 days after operation. This could indicate an increased release of oxytocin from the posterior pituitary in response to ovariectomy, and, taken with the previous evidence, adds further credability to the suggestion that oxytocin is involved with LH release in some way.

Hopkinson & Hooper (1970) have shown that other enzyme system in the rabbit such as cystine aminopeptidase, leucine aminopeptidase, carboxypeptidase and chymotrypsin fail to show any response to stimuli known to alter gonadotrophin secretion patterns. Cystine aminopeptidase activity in the female rat hypothalamus does increase with oestradiol monobenzoate injection (Heil et al. 1971), a condition affecting gonadotrophin secretion and also increasing supernatant activity (Hooper 1968; Griffiths & Hooper, unpublished observations), but it is unlikely that the two enzyme systems are comparable. It is more than likely that these enzymes in the hypothalamus inactivate oxytocin at a site other than the cystine, in a similar way to the enzymes inactivating oxytocin in rat kidney (Koida et al. 1971), rat uterus (Glass et al. 1970), and toad bladder (Glass et al. 1969). So, it would appear that these enzymes which respond to stimuli influencing gonadotrophin secretion are quite specific.

Since oxytocin is used as substrate to evaluate enzyme activity, it would seem reasonable to assume that the two groups of enzymes investigated are involved in the metabolism of oxytocin or similar polypeptides in the hypothalamus, as suggested by Hooper (1968). One such polypeptide could be LH-RF. From this evidence presented here and previous work suggesting the inverse relationship between LH (and presumably LH-RF) and enzyme activity, it seems likely that these enzymes could be involved with LH-RF metabolism. The other alternative is that they are implicated with oxytocin metabolism and that oxytocin plays some role in the availability, release, or action of LH-RF. Oxytocin itself has a function in reproduction and considering that vasopressin (closely related to oxytocin, secreted similarly by the posterior pituitary and with a role in controlling water retention and blood pressure) plays a part in corticotrophin-releasing factor (CRF) release (Hedge & Smelik 1969; Yates et al. 1971), this could be the case.
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REFERENCES

Schally A. V., Arimura A., Baba Y., Nair R. M. G., Matsuo H., Redding T. W.,
San Francisco Hilton (1971).
Yates F. E., Russell S. M., Dallman M. F., Hedge G. A., McCann S. M. & Dhariwal

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