Some Observations on the Behaviour of Hypothalamic Enzymes during the Time of Blastocyst Implantation in the Rabbit

By K. C. HOOPER*

*Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge
and Department of Physiology, University of Birmingham*

(Received 11 October 1965)

1. Hypothalami from pregnant and non-pregnant rabbits were homogenized in sucrose and separated into fractions by differential and density-gradient centrifugation. 2. The fractions were examined for enzymes capable of inactivating oxytocin. 3. Enzyme activity was detected in the supernatant and one particulate fraction of both pregnant and non-pregnant animals. 4. The specific activity of the enzymes increased during early pregnancy, and coincided with the time of blastocyst spacing and implantation in the uterus.

The precise function of oxytocin during parturition is not yet established; however, it is very probable that the hormone plays an important role during pregnancy and parturition (for references see Cross, 1959). No change in hormone content of rat pituitaries has been noticed during various stages of pregnancy (Acher, Chauvet & Olivry, 1956); even so, the rate of oxytocin metabolism could be expected to alter during pregnancy. A change in metabolism would be detected more readily in the hypothalamus than in the pituitary, for it is generally accepted that the posterior-pituitary hormones are synthesized in the hypothalamus and stored in the pituitary.

The dog hypothalamus contains enzymes capable of inactivating oxytocin (Hooper, 1962, 1963, 1964). The enzymes also occur in other parts of the central nervous system, and the distribution appears to correspond to regions containing a large number of nerve cells with the hypothalamus containing the greatest specific activity (Hooper, 1963). The enzymes possess a somewhat restricted substrate specificity (Hooper, 1962) and occur in two groups, one being associated with particulate matter and the other with a 25000g supernatant fraction. In a preliminary investigation with a small series of pregnant animals, it was found that the concentration of particulate-bound enzyme increased during pregnancy (Hooper, 1964). Certain of these characteristics, taken together, suggest that the enzymes may be concerned with some aspect of oxytocin metabolism in the hypothalamus.

The hypothalamic-pituitary region of the dog is somewhat different from that of most other animals

in that the ratio of vasopressin to oxytocin in the hypothalamus is markedly different from that in the pituitary. It seems possible therefore that the metabolism of oxytocin and vasopressin in the dog is different from that in most mammals. The present work shows how the concentrations of hypothalamic enzymes change during pregnancy. The rabbit was chosen as experimental animal, first to determine whether or not the changes observed in the dog were specific to that species, and secondly to correlate changes in enzyme concentration with other effects known to occur during pregnancy. A preliminary account of this work has appeared (Hooper, 1965a).

METHODS

Animals. Female rabbits of New Zealand White strain were employed. Animals were kept isolated for about 1 month before use to ensure that none was pregnant or pseudo-pregnant. Rabbits used for the pregnant animal series were mated once and kept in isolation until killed. Animals were bled out by severing neck vessels and excised uteri were examined for foetuses or blastocysts. Only those animals possessing foetuses or blastocysts were considered to be pregnant, and mated animals not having detectable signs of pregnancy were discarded.

Fractionation of hypothalamus. The brain was rapidly removed and the hypothalamus dissected out after removal of optic nerves and corpora mamillaria. Hypothalami were 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 600g, 60 min. at 7600g and 60 min. at 25000g to give nuclear, mitochondrial, microsomal and supernatant fractions respectively. The nuclear fraction was washed once with 0.25 M-sucrose by using the same volume and conditions of sedimentation originally employed to prepare the fraction. The supernatant was

* Present address: Department of Physiology, University of Birmingham.

drained as free as possible from the microsomal fraction. The unwashed mitochondrial fraction was centrifuged at approx. $7.5 \times 10^6 g$ -min. (Spinco model L preparative ultracentrifuge, SW39 centrifuge head) through a discontinuous gradient made from 0.32M-, 0.8M- and 1.2M-sucrose solutions. The three layers of particles were separated one from another, and the concentration of sucrose in each fraction was adjusted to approx. 0.25M by the addition of water. The particles were then sedimented by centrifuging for 60 min. at 25000g. The pellets were suspended in a little water (0.5 ml.) and all samples were dialysed overnight at a water temperature not greater than 8°. The non-diffusible N content of each fraction was measured by a micro-Kjeldahl method.

Measurement of enzymic inactivation of oxytocin. Enzyme activity was determined by measuring the loss of oxytocin occasioned on incubation with tissue fractions under specified conditions. Oxytocin (0.5 i.u./ml.) was incubated with tissue at 37° for 3 hr. at pH 7.3 (0.2 vol. of 0.4M- Na_2HPO_4 - NaH_2PO_4 buffer). Enzyme action was stopped by heating the medium in a boiling-water bath for 15 min., and residual oxytocin was measured on the isolated rat uterus by using a bracket type of assay (Hooper & Jessup, 1959). The recovery of hormone from the medium after incubation is expressed as a percentage of the hormone recovered from a control experiment. The control experiment was employed to test for any loss of hormone due to factors other than enzyme action, e.g. absorption, and to check that the fractions contained no detectable amounts of oxytocic substances that might interfere with the bioassay procedure. The media of the control and definitive experiments contained equal quantities of the same tissue fraction, but the control contained protein that had been denatured by heating for 15 min. in a boiling-water bath before incubation with hormone.

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

RESULTS

Previous work on the intracellular distribution of hypothalamic enzymes in the pregnant and non-pregnant bitch (Hooper, 1964) showed that each

fraction obtained from one hypothalamus contained sufficient material for 100–150 μg . of N to be used/ml. of incubation medium. Slightly different incubation conditions were used with rabbit hypothalamic fractions since smaller quantities of material were available. The mean weights of hypothalami obtained from 18 non-pregnant female rabbits and from 16 pregnant rabbits, together with the mean diffusible nitrogen content of each fraction, are given in Table 1.

The results listed in Table 1 show that there is a greater scatter in the measured nitrogen content of particulate fractions than there is in the supernatant fractions; this scatter probably reflects the difficulty in obtaining reproducible samples of suspensions of particles. The ratio of the nitrogen content of the fractions, based on a supernatant value of 1.00, is also listed in Table 1. With the exception of the ratios of the nuclear fractions, the two series show close agreement.

Since the nitrogen contents of mitochondrial, microsomal and supernatant fractions from pregnant animals are greater than those noted in similar fractions from non-pregnant animals, it seems probable that homogenate of pregnant-rabbit hypothalami contained a larger number of undisrupted cells.

Preliminary work indicated that each fraction contained approx. 130 μg . of non-diffusible N for incubation purposes, which was sufficient for control and test incubations at a concentration of 60 μg . of N/ml. (The difference between the total amount of non-diffusible nitrogen listed in Table 1 and the amount available for incubation purposes was that required for nitrogen determination.) Further work showed that the 'microsomal' and 'heavy-mitochondrial' fractions did not always contain sufficient material for two incubations at a concentration of 60 μg . of N/ml., and on such occasions the mean control value obtained for the

Table 1. *Non-diffusible nitrogen content of fractions obtained from rabbit hypothalamus*

Hypothalami obtained from pregnant and non-pregnant rabbits were homogenized in 0.25M-sucrose and fractionated. The mean ($\pm$ s.e.m.) nitrogen contents of the dialysed fractions are listed. The mean weight of hypothalami from non-pregnant animals was 149 mg. wet wt. of tissue (s.e.m. 20 mg.), and from pregnant animals 147 mg. (s.e.m. 18 mg.).

Fractions obtained from hypothalamus	Non-pregnant animal		Pregnant animal	
	μg . of N/fraction	Ratio of N content (soluble=1.00)	μg . of N/fraction	Ratio of N content (soluble=1.00)
Nuclear	322 ($\pm$ 100)	0.89	433 ($\pm$ 159)	1.43
Heavy*	158 ($\pm$ 53)	0.44	143 ($\pm$ 39)	0.47
Intermediate*	327 ($\pm$ 124)	0.91	286 ($\pm$ 103)	0.95
Light*	256 ($\pm$ 88)	0.71	212 ($\pm$ 50)	0.70
Microsomal	102 ($\pm$ 40)	0.28	78 ($\pm$ 27)	0.26
Supernatant	360 ($\pm$ 58)	1.00	303 ($\pm$ 62)	1.00

* Obtained by a sucrose-density-gradient fractionation of the mitochondrial fraction.

remaining fractions was used. The mean recovery of substrate from control experiments of both series does not differ significantly from 100% at $P=0.05$.

Intracellular distribution of enzymes in non-pregnant animals. With the occasional exception of the supernatant, hypothalamic fractions did not contain detectable amounts of activity. Supernatant fractions from seven animals were incubated at $60\mu\text{g.}$ of N/ml.; two of the seven contained detectable amounts of enzyme, whereas none was detected in the remaining five. Apparently, $60\mu\text{g.}$ of N/ml. is just below the concentration required to detect enzyme in most normal animals. Detectable amounts of enzyme were present in the supernatant and 'intermediate-mitochondrial' fractions when concentrations in excess of $60\mu\text{g.}$ of N/ml. were used. Activity was not detected in the nuclear, microsomal, 'heavy-mitochondrial' and 'light-mitochondrial' fractions when incubated at a concentration of $240\mu\text{g.}$ of N/ml. Fig. 1 shows the relation between the logarithm of residual hormone concentration and tissue concentration (expressed as non-diffusible nitrogen). Each point on the graph was obtained by using a different hypothalamus or groups of hypothalami. Fig. 1 therefore includes variations between individual animals. A comparison of the slopes suggests that the specific activity of the supernatant fraction is approximately 3 times that of the particulate fraction.

Intracellular distribution of enzymes in pregnant animals. Rabbits were mated once and killed at specific times after mating. Incubation of hypo-

thalamic fractions at $60\mu\text{g.}$ of N/ml. showed that only the supernatant and 'intermediate-mitochondrial' fractions possessed detectable enzyme activity after a certain stage of pregnancy. The residual hormone concentrations obtained after incubation with the two fractions were plotted against duration of pregnancy (Fig. 2). A detectable increase in the activity of the supernatant fraction occurred at about the sixth day after mating. By about the tenth day a mean maximum value had been reached that then remained fairly constant although an increase in scatter was noticeable towards the end of pregnancy. The particulate-bound enzyme was detectable by about the eighth day after mating, and like the supernatant enzyme the concentration increased until a mean maximum value was reached at about the tenth day, after which it remained fairly constant for the remainder of pregnancy with somewhat less scatter than that noticed for the soluble enzyme. Although the increase in particulate-fraction activity was detected on the eighth day, the initial increase must have occurred before the eighth day, for activities of fractions from non-pregnant animals are below the limit of detection. After the tenth day of pregnancy the specific activities of both fractions were similar.

The increase in enzyme activity occasioned by pregnancy was measured by comparing the slopes of enzyme activity curves for the two series. Fig. 3 shows the relation between residual substrate and enzyme concentrations for two animals in the seventeenth day of pregnancy. A comparison of the slopes in Figs. 1 and 3 shows that the activity of the particulate fraction has increased about $3\frac{1}{2}$ -fold. The activity of the supernatant fraction has not increased to the same extent; however, the

Fig. 1. Inactivation of oxytocin by fractions obtained from homogenate of rabbit hypothalamus. Oxytocin was incubated with fractions for 3 hr. at 37° and residual biological activity measured: O, soluble fraction; ●, particulate fraction. A_0 and A are control and residual polypeptide concentrations respectively.

Fig. 2. Progress curve for destruction of oxytocin by homogenate of pregnant-rabbit hypothalamus: O, soluble fraction; ●, particulate fraction. A_0 and A are control and residual polypeptide concentrations respectively.

Fig. 3. Inactivation of oxytocin by fractions obtained from homogenate of pregnant-rabbit hypothalamus: ○, soluble fraction; ●, particulate fraction. A_0 and A are control and residual polypeptide concentrations respectively.

difference between the slopes of the curves must be significant for the results listed in Fig. 2 show that an increase in activity of the supernatant does occur during pregnancy.

The total activity of one hypothalamus cannot be readily estimated by the assay method; however, an approximation of the relative activities in the two fractions can be made. Table 1 lists the mean nitrogen content of supernatant and 'intermediate-particulate' fractions from one hypothalamus as 360 μg. and 327 μg. respectively. Incubation media containing 360 μg. of N/ml. of supernatant would decrease the oxytocin to non-detectable concentrations. The kinetics of this type of reaction approximate to that of a first-order reaction (Hooper, 1962), and Fig. 1 indicates that the supernatant fraction from one hypothalamus contains sufficient enzyme to destroy 99% of substrate in 3 hr. The particulate-bound enzyme of one hypothalamus would inactivate only 80% of the substrate under similar conditions, i.e. it is at least 20-fold less effective than the supernatant fraction.

The particulate-bound enzyme of the dog hypothalamus was associated with the 'heavy-mitochondrial' fraction (Hooper, 1964). The distribution of the particulate-bound enzyme in the rabbit hypothalamus was somewhat different in that it was located in the 'intermediate-mitochondrial' fraction. The difference in distribution does not necessarily mean that the enzymes are associated with morphologically distinct particles in the two species, for it is possible that morphologically identical particles from rabbit and dog hypothalamus are of different density.

DISCUSSION

Oxytocin is known to be inactivated enzymically by blood of pregnant women and anthropoid apes; similar enzymes do not appear to be present in other pregnant mammals (Werle, Semm & Enzenbach, 1950). The placenta is thought to produce the enzyme that appears in humans, and it has been detected as early as the sixteenth day of pregnancy (Werle & Semm, 1951). The activity rises continuously during pregnancy, reaches maximum values at delivery and falls rapidly after parturition. The significance of blood oxytocinase is not understood, and it has been suggested that an equilibrium is reached between oxytocin and enzyme in the blood (Semm, 1961). With the exception of some enzymes found in the dog hypothalamus, nothing is known of tissue oxytocinases during pregnancy.

The mechanism of oxytocin synthesis in the hypothalamus is unknown. However, it is probable that some provision exists for hormone catabolism in the hypothalamus in order to complete the metabolic turnover of the peptide. Several stimuli known to release oxytocin act via hypothalamic centres, e.g. the hypothalamic milk-ejection centre (Andersson & McCann, 1955). These stimuli would in all probability have an effect on oxytocin metabolism in the hypothalamus. An increase in the rate of hormone synthesis may be followed, after a certain time lag, by an increase in the rate of hormone catabolism. It is suggested that the increase in the concentration of peptidases inactivating oxytocin reflects an increase in hormone production. Evidence supporting the hypothesis was obtained from lactating rabbits where suckled animals possessed higher peptidase activities than did animals from which litters had been removed shortly after parturition (Hooper, 1965b). The measurement of hypothalamic peptidases as an index of hormone production may be applicable to hormones other than oxytocin.

The increase in enzyme concentration during pregnancy coincides, approximately, with the period of blastocyst spacing in the uterus. According to Böving (1959), rabbit blastocysts are scattered randomly along the uterine horn 3–5 days after ovulation; between the fifth and seventh day they expand and are spaced equal distances one from another, whereas after the seventh day they are so large that they cannot be propelled. After ovulation, blastocyst implantation normally occurs on the seventh day in the rabbit, and on the eighth or ninth day in the human being.

The present work suggests that an increase in oxytocin production probably occurs during the time of blastocyst implantation and that there may be some relation between the two effects: (a) the

hypothalamus plays a part in blastocyst spacing and implantation that is manifested by an increase in the concentration of hypothalamic enzymes; (b) the spacing and implantation of blastocysts stimulates the production of hypothalamic enzymes via a neural pathway; or (c) the increase in concentration of hypothalamic enzymes is under the control of the corpus luteum.

I thank Miss Priscilla Higginbotham for technical assistance.

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