The Distribution of Hypothalamic Peptidases in Pregnant and Non-Pregnant Dogs

By K. C. HOOPER

Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

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It appears to be generally accepted that oxytocin and vasopressin are synthesized in the hypothalamus and stored in the neurohypophysis. A variation in the hormone content of the neurohypophysis in physiological conditions involving the release of large amounts of oxytocin or vasopressin might be expected. For example, it has been established that dehydration and lactation deplete the neurohypophysis of hormones or neurosecretory material (for references see Ortmann, 1960). Some change in the metabolism of oxytocin might be expected during pregnancy. However, the concentration of hormones in pituitaries of pregnant rats did not differ greatly from that found in non-pregnant animals (Acher, Chauvet & Olivry, 1956). In view of this observation, it was decided to find out whether or not the hypothalamic enzymes inactivating oxytocin (Hooper, 1962) varied during pregnancy, for, if this were the case, it would appear probable that the metabolism of oxytocin in the hypothalamus changed during pregnancy.

The present paper describes the intracellular distribution of hypothalamic enzymes inactivating oxytocin in a series of pregnant and non-pregnant dogs, and discusses certain points arising from the observed distribution patterns.

METHODS

Preparation of fractions from dog hypothalamus. Mongrel dogs of both sexes were anaesthetized with ether or with chloroform and killed by bleeding from the common carotid artery. The brain was rapidly removed and the hypothalamus dissected out and weighed. Hypothalami were homogenized in 9 vol. of 0.25M-sucrose in a hand-operated homogenizer, and approximately 0.2 vol. of homogenate was kept for enzyme-activity and nitrogen determinations. The remaining homogenate was centrifuged successively for 30 min. at 600g, 60 min. at 7600g and 60 min. at 25000g at 2°, to give nuclear, mitochondrial, microsomal and supernatant fractions respectively. In some experiments the first centrifuging was for 15 min. at 600g. The particulate fractions were washed once with 0.25M-sucrose by using the same volumes and conditions of sedimentation originally employed to prepare the fractions; the washings were discarded. The unwashed mitochondrial fractions obtained from eight hypothalami were centrifuged in a gradient of 0.32M-, 0.8M- and 1.2M-sucrose solutions. The density-gradient system was prepared in a manner similar to that

described by Hebb & Whittaker (1958) and Whittaker (1959), its application differing only in that it was used immediately after layering and not after equilibration. After centrifuging in a Spinco model L preparative ultracentrifuge at approx. 7.5×10^6 g/min. (60 min. at 39000 rev./min.) in a SW39 centrifuge head, three well-defined layers of particles were obtained. The layers were separated one from another by the test-tube-slicing technique, and the concentration of the sucrose in each fraction was made approx. 0.25M by the addition of water. The particles were then sedimented by centrifuging for 60 min. at 25000g at 2°. The pellets were suspended in water (approx. 1 ml.), and all samples, including the homogenate, were dialysed against running tap-water for 16 hr. The non-diffusible nitrogen 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. Oxytocin (1 i.u./ml.) was incubated at 37° for 3 hr. with tissue fractions at pH 7.3 (0.2 vol. of 0.4M- Na_2HPO_4 - NaH_2PO_4 buffer). Enzyme action was stopped by heating in a boiling-water bath for 15 min. The residual concentration of oxytocin was measured on the isolated rat uterus by using a bracket-type assay (Hooper & Jessup, 1959). The recovery of hormone from the incubation medium was expressed as a percentage of hormone recovered from a control medium; the control medium contained an equal amount of tissue that had been heated for 15 min. in a boiling-water bath before incubation with hormone. The residual concentrations of oxytocin have been converted into units of enzyme activity by reference to a curve relating enzyme activity and residual concentrations of oxytocin (Hooper, 1962).

Materials. The materials were as described by Hooper (1963).

RESULTS

The intracellular distribution of enzymes destroying oxytocin was measured in 12 hypothalami. In the first two experiments, nuclear, mitochondrial and microsomal fractions were obtained as described above and the results listed in Table 1 show that a sedimentable enzyme was distributed in a variable manner between nuclear and mitochondrial fractions. When nuclear material was sedimented at 9000 g/min. (dogs 3-6), no detectable activity was present in this fraction and the sedimentable enzyme was associated almost completely with the mitochondrial fraction. In all instances, mitochondrial and supernatant fractions

contained enzymes inactivating oxytocin. Of the five microsomal samples tested, four did not contain detectable amounts of activity, whereas the fifth contained approx. 15% of the total activity found in this hypothalamus.

Evidence suggesting that particulate and soluble enzymes are not identical. The activity of the supernatant fraction could be due to an enzyme released from particulate matter during homogenization of the tissue. Enzymes appearing in the supernatant of a 0.25 M-sucrose homogenate are, however, usually regarded as true cytoplasmic enzymes, and it is unlikely that the soluble enzyme is of lysosomal origin in view of the report that 0.25 M-sucrose does not disrupt these particles (de Duve, 1959). In all but one experiment, the sum of the enzyme activities after fractionation was greater than that in the original homogenate, the average value obtained in six experiments being approx. 160%. This value would doubtlessly be higher still if the recovery of nitrogen in Expts. 3 and 6 had been better. A recovery in excess of 100% would be expected if the homogenate contained more than one enzyme inactivating oxytocin. The graph from which the activity of the individual fractions was calculated represents the relation between residual hormone and homogenate concentrations (Hooper, 1962). Should the homogenate contain several enzymes acting on oxytocin, the observed rate of substrate disappearance would be less than the sum of the individual rates of the various enzymes in the homogenate. The overall rate would be largely controlled by the enzyme having the greatest affinity for oxytocin, for incubation conditions are such that the kinetic system approaches that of a first-order reaction (Hooper, 1962).

Subfractionation of the mitochondrial fraction. The mitochondrial fractions obtained from one male and three female animals were separated into three components by centrifuging in a sucrose gradient. The heavy fraction which sedimented in 1.2 M-sucrose consisted of a well-packed layer of sticky material. The particles of intermediate density occurred immediately below the interface of the 0.8 M- and 1.2 M-sucrose solutions, and the layer of light particles was found below the interface of the 0.8 M- and 0.32 M-sucrose solutions. Layers of particles were separated by optically clear regions provided that the system was not overloaded; satisfactory separations were obtained by distributing the mitochondrial fraction from one hypothalamus among three centrifuge tubes.

It is apparent from Table 2 that the activity is distributed in a variable manner among the particles of heavy and intermediate density, and it is probable that a slight variation in the composition of the density-gradient system would result in a more definite localization of the activity. Particles of light density contained little or no enzyme.

The three subfractions obtained from the mitochondrial fraction were examined in the electron microscope by using a negative-staining technique (Brenner & Horne, 1959). A detailed interpretation of the micrographs was not possible owing to lack of information about the dog hypothalamus. However, the light fraction appeared to consist largely of myelin threads. The intermediate fraction contained material similar in appearance to the nerve endings found in guinea-pig brain (Gray & Whitaker, 1962); of the numerous other structures present in this fraction, some appeared to be fragments of axons. Large particles resembling swollen

Table 1. *Inactivation of oxytocin by fractions obtained from homogenate of dog hypothalamus*

Homogenate was separated into nuclear, mitochondrial, microsomal and supernatant fractions as described in the Methods section. Fractions were incubated with oxytocin (2.5 i.u.) and 0.4 M-phosphate buffer, pH 7.3 (0.5 ml.), for 3 hr. at 37° in a total volume of 2.5 ml., and the residual hormone was measured with isolated rat uterus. The residual hormone concentration was converted into enzyme units (Hooper, 1962). A/N is enzyme specific activity in units/mg. of non-diffusible nitrogen. The mitochondrial fractions obtained from dogs 5 and 6 were fractionated further (see Table 2).

Dog	Homogenate		Nuclear fraction		Mitochondrial fraction		Microsomal fraction		Supernatant fraction		Recovery of nitrogen (%)	Recovery of enzyme (%)
	Enzyme units	A/N	Enzyme units	A/N	Enzyme units	A/N	Enzyme units	A/N	Enzyme units	A/N		
1 (♀)	12.4	2.4	4.6	2.9*	4.5	2.1	0	0	5.3	4.4	95	116
2 (♂)	10.6	3.0	13.8	6.6*	7.1	3.9	0	0	3.4	3.6	106	229
3 (♂)	18.8	3.1	0	0	3.9	3.0	0	0	8.0	7.9	72	63
4 (♂)	14.6	2.0	0	0	16.6	4.4	4.3	6.2	7.6	4.1	107	195
5 (♂)	5.8	1.0	0	0	See Table 2		Not tested	Not tested	4.7	2.9	84	183
6 (♀)	2.6	0.5	0	0	See Table 2		0	0	2.9	2.4	68	188

* Nuclear fraction sedimented at 600g/30 min.; in experiments 3-6 the nuclear fractions was sedimented at 600g/15 min.

Table 2. *Inactivation of oxytocin by mitochondrial subfractions obtained from dog hypothalamus*

The mitochondrial fractions were subfractionated by means of a sucrose gradient as described in the Methods section. The remainder of method was as described in Table 1.

Dog	Heavy fraction		Intermediate fraction		Light fraction	
	Enzyme units	A/N	Enzyme units	A/N	Enzyme units	A/N
5 (♂)	4.7	7.3	1.2	2.2	0	0
6 (♀)	0.8	1.9	1.2	2.7	0	0
7 (♀)	1.4	1.6	1.6	1.3	0	0
8 (♀)	1.3	2.7	0	0	0	0

Table 3. *Inactivation of oxytocin by fractions obtained from pregnant-dog hypothalamus*

The methods were as described in Tables 1 and 2.

Dog	Heavy fraction		Intermediate fraction		Light fraction		Supernatant fraction	
	Enzyme units	A/N	Enzyme units	A/N	Enzyme units	A/N	Enzyme units	A/N
1 (early pregnancy)	2.6	3.7	3.0	3.8	4.1	4.6	Not tested	Not tested
2 (mid-pregnancy)	0	0	0	0	3.1	4.5	1.4	0.9
3 (late pregnancy)	0	0	0	0	4.9	6.8	5.6	4.7
4 (late pregnancy)	0	0	3.9	6.9	17.9	44.8	9.0	6.9

mitochondria, with other unidentified material, were contained in the heavy layer.

Distribution of enzymes inactivating oxytocin in hypothalami obtained from pregnant animals. The precise duration of pregnancy of these animals was not known, for they were obtained from outside sources. For the purposes of the present investigation, the duration of pregnancy was classified into one of three rather broad stages, based on hair development of the foetus. In the category defined as early pregnancy the foetus was entirely devoid of hair, in mid-pregnancy it was partially covered with hair, and in late pregnancy hair development was complete.

The distribution of enzymes in the first two available animals (dogs 2 and 4; see Table 3) showed that nuclear and microsomal fractions did not contain detectable activity; consequently, the corresponding fractions obtained from dogs 1 and 3 were not examined.

The results listed in Tables 2 and 3 show that a change in enzyme distribution occurs during pregnancy. During late pregnancy the heavy fraction did not contain detectable amounts of activity, whereas, in contrast, the corresponding fraction from the four non-pregnant animals possessed activity. A greater difference between the enzyme distribution of the two groups is shown in the light fraction. Non-pregnant animals did not possess detectable enzyme in the light fraction; however, the four pregnant animals possessed appreciable activity, and during late pregnancy the major part of the particulate bound activity of the hypothalamus

appears to be contained in this fraction. The activity of the supernatant fraction appeared to increase during late pregnancy; however, it is doubtful whether the values are significantly different from those found for non-pregnant animals (see Table 1).

DISCUSSION

Distribution of hypothalamic enzymes in non-pregnant animals. The results indicate that the intracellular distribution of enzymes inactivating oxytocin in the dog hypothalamus is well-defined. The sedimentable enzyme is associated predominantly with a fraction largely composed of mitochondria and which also would be expected to contain neurosecretory material. The enzyme may therefore originate in either of these particles: in addition, the possibility of its being of lysosomal origin cannot be excluded, for it is not possible as yet to distinguish brain lysosomes from brain mitochondria (Beaufay, Berleur & Doyen, 1957). The intracellular distribution of other proteolytic enzymes in the hypothalamus does not appear to be known. However, a comparison with the distributions of enzymes found in homogenate of whole brain can be made. The cathepsins of rat and rabbit brain occur predominantly in the mitochondrial fraction, and decrease progressively in the nuclear, microsomal and soluble fractions (Lajtha, 1961): however, it was not possible to associate the sedimentable cathepsins of cat and rat brain with mitochondria (de Duve, 1959). Of the neutral proteinases, one occurs largely in the mitochondrial

fraction of sheep brain, with smaller amounts of the enzyme being found in the nuclear, microsomal and soluble fractions (Lajtha, 1961). The di- and tri-peptidases of rabbit brain occur mainly in the 'cytoplasmic' fraction, with some activity being present in the nuclear and mitochondrial fractions (Hanson & Tendis, 1954). It is apparent that the above-mentioned enzymes possess a more general intracellular distribution than do those which destroy oxytocin.

Distribution of hypothalamic enzymes inactivating oxytocin in pregnant animals. A variation in the distribution of enzymes inactivating oxytocin shows that, in the dog at least, pregnancy is probably accompanied by a change in the metabolism of oxytocin in the hypothalamus. Further experimental results are required to indicate what function the enzymes serve during pregnancy. However, one function may be of a protective nature. In human beings and anthropoid apes, an enzyme(s) capable of inactivating oxytocin appears in the plasma during early pregnancy. The concentration of the plasma enzyme(s) increases as pregnancy progresses, and it has been suggested that the enzyme(s) decreases the amount of oxytocin in the blood during gestation (for references see Semm, 1960). Similar enzymes have not been found in the plasma of other mammals (Werle, Semm & Enzenbach, 1950). Since dog plasma does not contain an enzyme inactivating the hormone, the hypothalamus may exert some control over oxytocin production by varying the rate of hormone turnover during pregnancy.

SUMMARY

1. The intracellular distribution of enzymes inactivating oxytocin in hypothalami obtained from pregnant and non-pregnant dogs has been measured.

2. Hypothalami were homogenized in a 0.25M-sucrose solution and the homogenate separated into nuclear, mitochondrial, microsomal and supernatant fractions by differential centrifuging.

3. The nuclear and microsomal fractions generally did not contain detectable amounts of enzyme destroying the hormone whereas the mitochondrial and supernatant fractions did.

4. The mitochondrial fraction was further fractionated into three components by centrifuging in

a gradient comprising 0.32M-, 0.8M- and 1.2M-sucrose solutions.

5. For non-pregnant animals, the fraction containing particles of least density did not contain detectable amounts of enzyme. The remaining fractions contained enzymes inactivating oxytocin. For pregnant animals, a change in the distribution of enzymes was observed. The activity was very low in the heavy fraction whereas activity in the least-dense fraction accounted for the major part of the particulate bound enzyme in the hypothalamus.

6. The variation in enzyme distribution suggests that there is a change in the pattern of oxytocin metabolism in the hypothalamus during pregnancy.

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