AN EVALUATION OF THE ROLE OF HYPOTHALAMIC ENZYMES IN THE METABOLISM OF OXYTOCIN AND VASOPRESSIN

by

I.J. Lloyd, B.Sc.

In order to evaluate fully the physiological significance of a hormone it is essential to know the secretory rates and the circulating levels of that hormone over a wide range of physiological situations. In the case of the neurohypophysial hormones these can only be measured in a very limited number of situations.

The work reported below was undertaken to evaluate the suggestion that two hypothalamic enzymes, one in the supernatant and the other in the mitochondrial fraction, reflect the synthesis and/or the secretion of oxytocin (Hooper, 1966 a, b).

Two methods of approach were used; (a) the enzyme activity in additional physiological and experimental states was measured, and (b) preparatory to testing the hypothesis directly a method of extracting and

purifying the hormones from fresh glands was attempted.

The enzyme-containing fractions were prepared as described by Hooper (1966a).

When the enzyme activity was measured using oxytocin and vasopressin separately it was found that the supernatant enzyme inactivated both hormones to approximately the same extent, whereas the particulate enzyme inactivated vasopressin much more effectively than oxytocin. Following mating, the particulate enzyme showed an increase in activity at 9 and 12 hrs. The supernatant enzyme showed an apparent slight increase at 12 hrs followed by a decrease in activity at 36 and 48 hrs. During dehydration induced by administration of 3% NaCl for 3 days the supernatant enzyme activity was doubled. The particulate enzyme was also approximately doubled when tissue concentrations up to 70 µg/ml were used. Above this concentration there was a progressive decrease in activity. In animals that had been overhydrated by i.p. administration of 8% glucose corresponding to 10% bodyweight every 8 hrs for 3 days the supernatant enzyme was doubled while the particulate was halved. In animals that were overhydrated but stressed due to gut damage both the en-

zymes were increased, the former five-fold the latter only slightly. Enzyme activity was tested for in one dog diagnosed as suffering from diabetes insipidus. Activity was only found in the supernatant fraction.

The pattern of enzyme activity throughout pregnancy is known (Hooper, 1966 a, b). The body water/kg body weight and body water turnover were measured to see whether there was any correlation with enzyme activity. A correlation was found between body water turnover and enzyme activity. With the exception of days 10-20 of pregnancy the body water turnover was increased when the ratio of supernatant/particulate enzyme was increased above the control value, and decreased when the ratio was decreased.

A method suitable for the partial purification of neurohypophysial hormones from small numbers of fresh glands has been devised. Homogenization in 0.5 ml of 0.1 N HCl/gland followed by salt precipitation and chromatography on a long Sephadex G-25 column results in separation of the hormones from the high and much of the low molecular weight material with a 70% recovery of hormone.

The finding that the enzymes respond to the transient physiological stimuli of mating suggests a physiological role for them. The time course of the change in the supernatant enzyme shows that this enzyme does not, as has been suggested (Hooper, 1958), reflect luteinizing hormone secretion. The fact that particulate enzyme inactivates vasopressin more effectively than oxytocin, that its activity reflects vasopressin secretion during dehydration and overhydration and that no activity was found in the diabetic dog suggests that this enzyme is involved in vasopressin and not oxytocin metabolism.

Since the supernatant enzyme inactivates both hormones to the same extent it seems probable that it is not involved in the metabolism of oxytocin itself, but rather a substance similar to it.

A correlation has emerged between the ratio of the enzyme activities and water metabolism. A significant finding is that in overhydrated animals the ratio supernatant/particulate enzyme is increased, a result of an increase in the former and a decrease in the latter; in overhydrated stressed animals in diuresis the enzyme ratio was similar. In this case the

particulate enzyme appeared to be increased, the ratio being maintained by a very large increase in the supernatant enzyme. Vasopressin secretion in the latter case would be expected to be increased due to the stress. This suggests that diuresis is achieved by a balance of actions of vasopressin and a diuretic agent, the latter being an oxytocic-like substance or even oxytocin itself. This possibility is at present being investigated further.

References

Hooper, K.C. (1966a). Biochem. J., 99, 128.

Hooper, K.C. (1966b). Biochem. J., 100, 823.

Hooper, K.C. (1968). Biochem. J., 110, 151.

AN EVALUATION OF THE ROLE OF HYPOTHALAMIC ENZYMES IN THE METABOLISM OF OXYTOCIN AND VASOPRESSIN

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine.

by

Iouan John Lloyd, B.Sc.

Department of Veterinary Physiology
University of Glasgow

April 1970

ACKNOWLEDGEMENTS

The author would like to thank Professor W. Mulligan of the University of Glasgow for his encouragement and also for providing the facilities for much of the work. The author also wishes to thank Dr. K.C. Hooper of the University of Sheffield under whose supervision the post-mating experiments were performed. Thanks are also due to Professor W.I.M. McIntyre and Dr. G.W. Crighton of the University of Glasgow for making available the dog examined, Dr. W.R. Tindall of Organon Laboratories Ltd. for supplying much of the concentrated hormone preparation and the acetone dried powders, Mr. E. Glancy, Mr. L. Arhens and Mrs. J. Glazier for technical assistance and finally Mrs. A.R. Lloyd for much constructive criticism of the manuscript.

CONTENTS

SECTION A

MEASUREMENTS OF ENZYME ACTIVITY IN ADDITIONAL PHYSIOLOGICAL AND EXPERIMENTAL STATES: CORRELATION OF ENZYME ACTIVITY WITH BODY WATER METABOLISM

SECTION B

EXTRACTION AND PURIFICATION OF OXYTOCIN AND VASOPRESSIN FROM POSTERIOR PITUITARY MATERIAL

GENERAL INTRODUCTION

Extracts of the neurohypophysis have been shown to possess four main biological effects. Howell (1898) demonstrated a vasopressor effect. A myogenic effect on uterine muscle was shown by Dale (1906, 1909), a year later Ott and Scott (1910) showed a milk ejection effect and finally Frank (1912), Fanni (1913) and Von den Velden (1913) demonstrated an anti-diuretic effect.

The Functional Unit

The neurohypophysis arises totally from neural tissue and the functional unit in mammals comprises: (a) Specialized hypothalamic nuclei, namely the supra-optic and paraventricular nuclei although the possibility of other nuclei being involved cannot be excluded (for references see Christ, 1966); (b) The median eminence; (c) The infundibular stem (neurohypophysial tract or the pituitary stalk) which comprises nerve fibres of the hypothalamic neurones in the supraoptic, paraventricular nuclei and other nuclei that may be involved; (d) The infundibular process (posterior pituitary or lobe, neural lobe, or the pars nervosa) which consists of the distal portion of the nerve fibres that made up the infundibular stem, glial cells which were called pituicytes by Bucy (1932)

and an abundance of blood capillaries. The terminals of the nerve fibres are expanded and contain membranous electron dense vesicles. These neurosecretory vesicles, containing the active principles, are seen to end in close proximity to the capillaries.

After forty years of controversy it is now established that the infundibular process itself does not elaborate the biologically active principles, but that they are elaborated in the hypothalamic nuclei and pass in membranous vesicles, the neurosecretory vesicles, intra-axonally to the infundibular process where they are stored, and subsequently released into the circulation. This interpretation resulted from the work of Bargmann (1949) who applied Gomori's acid-permanganate-chrom-alum-haematoxylin technique to the neurohypophysial system and found that the stain stained selective regions throughout the system and from the accurate conceptualization of Scharrer and Scharrer (1954). Ortmann (1951) and Hild and Zettler (1953 a and b) found a correlation between the amount of biologically active material and the stainable material, suggesting that the stain stained the active principles. It was also found that stain-

able material tended to accumulate above a cut in the infundibular stem (Hild and Zettler, 1953a). Hild (1954) observed and filmed in tissue culture the transport of granules in a distal direction down the axons of living supraoptic cells in dog tissue. That the above is the functional behaviour of the system has been confirmed many times subsequently.

The Hormones and Neurophysin

For years following the discovery of the biological activities of the neurohypophysial extract, the question was posed whether one single substance was responsible for these biological properties or whether each was related to a separate substance. Abel, Rouiller and Geiling (1932) and Abel (1930) suggested that one principle alone was responsible for the biological activities and suggested that the presence of two substances sharing the biological activity, as suggested by Dudley (1919), was a result of the splitting of the hormone by the excessively violent extraction. The work of Abel and his colleagues was supported by MacArther (1931) and Rosenfeld (1940), the latter describing a fraction of molecular weight, 20,000 -

30,000 possessing all the biological activities. On treating with boiling acetic acid followed by ultracentrifugation, it was possible to observe the presence of biologically active substances of much lower molecular weight, suggesting that these were active fragments resulting from cleavage of the natural hormone. Van Dyke, Chow Greep and Rothen (1942) came to the same conclusion. They described what they considered to be a pure protein with a molecular weight of 30,000 and possessing all of the biological activities.

Dudley (1919) and Kamm, Aldrich, Grote, Rowe and Bugbee (1928) showed that it was possible to obtain from the neurohypophysis two preparations, one containing oxytocic activity and the other vasopressor activity. Several workers confirmed these findings, and ultimately Du Vigneaud (1954--1955) succeeded in obtaining in a pure state two principles, one possessing oxytocic and the other vasopressor properties. These became known as oxytocin and vasopressin or alternatively antidiuretic hormone (A.D.H.). Both principles were small peptides, each with a molecular weight of about 1,000.

Using the protein Van Dyke considered to be homogeneous and to possess all the biological properties of the neurohypophysis, Acher and Fromageot (1957) showed that the two preparations described by Du Vigneaud as oxytocin and vasopressin could be separated from it by electrophoresis, electrodialysis and 5% trichloroacetic acid. Ginsburg and Ireland (1964) showed that dilution alone is sufficient to dissociate oxytocin and vasopressin from the main protein, and suggested an electrostatic bond between the two. Frankland, Hollenberg, Hope and Schater (1965) found that gel filtration in long Sephadex columns using 0.1 N formic acid elutant was sufficient to dissociate the hormones from the main protein. Acher and Fromageot (1957) also showed that the V/O (vasopressin/oxytocin) ratio in the neurohypophysis as assessed biologically varied in different physiological states, for example with age and during lactation. These findings have been amply confirmed subsequently and also extended to other physiological states, for example dehydration (Lederis, 1962) and pregnancy (Heller, 1958).

The finding that the hormones can be dissociated from the main protein by exceedingly mild procedures suggests strongly that the concept of the unitary hormone is incorrect. This is further supported by the variation in the V/O ratio. Some elements of caution must be applied to this interpretation since the levels of the hormones were inferred from their biological effect and not from chemical analysis. It is just conceivable that present in these tissues is some agent or agents which may alter the sensitivity of one or both of the biological effects.

The concensus of opinion is that the biological effects of the neurohypophysis are the sum of two hormones, oxytocin and vasopressin (A.D.H.), and that these hormones are loosely bound, probably electrostatically, to an inert protein or proteins of molecular weight 30,000. The name neurophysin has been suggested for this group of proteins.

The existence of a neurophysin-hormone complex raises the question whether such an association has any biological significance or whether it is brought about fortuitously during the course of the extraction.

It is worthy of note that the complex contains oxytocic and vasopressor activities in the ratio 1:1, a proportion which is also found in the gland extract. It is also present in all the species so far examined (Acher, 1963).

As mentioned above, the Gomori-Bargmann-Chromic haematoxylin technique is thought to stain that part of the cell which contains the biological activities. The hormones themselves are not responsible for the stain; on the other hand, neurophysin when treated with the stain yields a colour similar to that obtained in the cell (Acher, 1958), a finding that is supported by Sloper (1954). It would thus appear that neurophysin could well play some role in the hypothalamic metabolism or transport of the hormones, presumably as a carrier protein. Acher (1966) suggested at that time that it was premature to make a positive statement concerning the existence of any biological significance in the neurophysin-hormone complex.

The Structure of the Hormones

The structure of the two hormones oxytocin and vasopressin is known. Du Vigneaud, Ressler and Trippett (1953), and Tuppy (1953) simultaneously proposed the structure for oxytocin, which at the same time was synthesised by Du Vigneaud and co-workers. It is an octapeptide with a molecular weight of 1,007, and with the structure shown below.

Cy.S-Tyr-Ileu-Glu(NH_4)-Asp(NH_4)-Cy.S-Pro-Leu-Gly(NH_4)

Amino acid sequence of oxytocin

In all the mammalian species so far examined the oxytocic principle has been identical.

The structure of hog and beef vasopressin was determined and its synthesis achieved by Du Vigneaud and co-workers (1954-5). It too was found to be an octapeptide, and its molecular weight was found to be 1,084; the structure is shown below.

Cy.S-Tyr-Phe-Gly(NH_4)-Asp(NH_4)-Cy.S-Pro-Arg-Gly(NH_4)

Amino acid sequence of arginine vasopressin

As can be seen, the hormones differ in only two amino acids, one at position 3 and the other at position 8. In contrast to oxytocin there are two types of vasopressin. The more common is shown above. It contains arginine in position 8 and is designated arginine vasopressin. This form has been found in all the species so far examined except the pig family which has lysine in place of arginine at position 8. This form is predictably designated lysine-vasopressin.

Synthesis

Little is known concerning the biosynthesis of the neurohypophysial hormones; the parakaryon in the hypothalamic supraoptic or paraventricular nucleus would appear to be required for synthesis of the neurosecretory material. This has been suggested on morphological grounds and has been supported by electron-microscopic studies (Bern and Knowles, 1966) and cell fractionation with ultracentrifugation (Sachs, 1963).

Oxytocin and vasopressin have been recovered from the infundibular process, median eminence and the supraoptic and paraventricular nuclei (Lederis, 1962; Barrer, Heller and Lederis, 1963); thus the hormones could well be synthesised in any or all of these sites.

Following transection of the infundibular stem in dog, the neurones continue to synthesise vasopressin (Hild and Zettler, 1953). In such neurones, synthesis must be a property of the neuronal cell body, the proximal nerve fibre, or both. The synthesis in the neurone is associated with the ribosomes and at the onset may be inhibited by puromycin (Sachs, 1966).

It is not known whether complete synthesis occurs in the cell body or whether it is merely started there and continues during the course of transport to the infundibular process. It has been suggested that the ribosomal R.N.A. is necessary only for the formation of a precursor of the active hormone and that the formation of vasopressin from the precursor occurs later, during migration down the axon (Sachs, 1966; Porlanova, Bissel and Sachs, 1966). The conversion of the precursor to A.D.H. can occur <u>in vitro</u> in a cell-free system (Porlanova, Bissel and Sachs, 1966).

<u>Secretion</u>

There are many indications that stimuli for synthesis of the hormones are closely linked with stimuli for release of the hormones and for the speeding up of intraneuronal transport. Adult rats when subjected to

dehydration, thus causing a release of vasopressin, increase the neurohypophysial content of the hormone for the first 48 hours (Amis and Van Dyke, 1950; Dicker and Munn, 1957).

A similar situation has been observed following haemorrhage (Ginsburg and Brown, 1957), which again causes release of vasopressin (Ginsburg and Heller, 1953). Edstrom and Eichner (1958) have shown that the amounts of R.N.A. in the cytoplasm and nucleolus of cells in the supraoptic nucleus increase significantly with administration of sodium chloride, which is known to release vasopressin.

Takabataka and Sachs (1964) suggest that the stored pool of vasopressin in the infundibular process is heterogeneous and contains a "quickly releasable component" of approximately 10-20% of the total hormone together with a slowly releasable component.

It is not known whether the free hormones are released into the capillaries of the infundibular process, or whether they are released together with the carrier protein. Sachs, Fawcett and Haller (1967) believe the latter to be the case. Finally the entire

neurosecretory vesicle may be extruded. Occasionally they have been located within the capillaries both with the light microscope (Hanström, 1952; Scharrer and Frandson, 1954; Paulson, 1956) and the electron microscope (Heller and Lederis, 1962). They have never been detected in the interstitial spaces on the other hand; this and the infrequence of their occurrence in the capillaries suggest that these findings are in fact artifacts.

Originally it was suggested that there was a simultaneous liberation of the oxytocin and vasopressin on electrical stimulation (Harris, 1947), osmotic (Abrahams and Pickford, 1954) and chemical stimulation (Chamorro and Minz, 1955). Cross (1961), Heller (1961) and Farrel, Fabre and Rauschkold (1968) concluded that at the very least the release of the two hormones is to some degree independent. The preferential release of one hormone appears to be determined by the type of stimulus applied. In suckling, the release of oxytocin to vasopressin is in the ratio of 100:1 (Peeters and Coussens, 1950; Cross, 1951), while haemorrhage in rats raised the vasopressin activity in blood without much effect on

oxytocin (Ginsburg and Smith, 1959). In rabbits on the other hand no alteration in oxytocin levels was observed (Chadbury and Walker, 1958).

Physiological Effects and Secretory Stimuli

Controlled vasopressin release is undoubtedly important in regulating water excretion. The absence of vasopressin leads to rapid depletion of body fluids and to cardiovascular collapse if water is not freely ingested. Relatively minute amounts of vasopressin can prevent this water loss. The body maintains a constant water content despite a fluctuating intake. In normal subjects this is achieved by graded degrees of antidiuresis which are controlled by regulated vasopressin release.

This effect is achieved because in the absence of vasopressin the distal convoluting tubules and collecting duct are impermeable to water. It has been suggested that the effect of vasopressin on the distal convolution and collecting duct is similar to that which it exerts in amphibian skin and bladder, namely that it alters pore size and so permits the outward movement of water from the tubules (Koefoed-Johnson and Ussingm, 1953; Andersson and Ussingm, 1957; Maffy,

Mays, Landin and Leaf, 1960; Sawyer, 1957, 1960). Stimuli causing the release of vasopressin are many. Verney (1947) showed that the injection of a hypertonic solution of sodium chloride into a common carotid of conscious dogs produced an inhibition of water diuresis by release of vasopressin. These results are in favour of a physiological importance of the osmotic release of vasopressin, since increases of serum osmotic pressure even on the first day of water withdrawal in man (Rubini, Wolf and Moroney, 1939) and the dog (Wolf and Eddy, 1957) are of a similar order to those in Verney's experiments. An increased osmotic pressure of the extracellular fluids may not be the only factor involved in the release of vasopressin in dehydration since contraction of body fluid volumes can also evoke a release (see Sawyer and Mills, 1966). It is generally agreed that a decrease in blood volume or extracellular fluid evokes an antidiuresis. In haemorrhage an elevated titre of vasopressin has been reported many times. The increase appears to be related to a fall in pressure (see Heller and Ginsburg, 1966; Sawyer and Mills, 1966). The receptors for this release have been the subject of much debate. It appears

certain that "volume" receptors in the atria and possibly elsewhere in the thoracic circulation are involved, together with the baroreceptors and possibly the chemoreceptors (Sawyer and Mills, 1966; Farrel, Faber and Rauschild, 1968). However, owing to the often incomplete and sometimes contradictory results, the exact physiological meaning of vasopressin release in response to fluid loss or hypotension is at present obscure.

The above are the most generally accepted physiological roles for vasopressin. Two physiological roles for oxytocin have been clearly established. Suckling initiates a reflex release of oxytocin which effects contraction of the myoepithelial cells surrounding the alveoli of the mammary glands. This reaction results in the expression of milk stores and aids the efficient transfer of milk from mother to young (see Benson and Fitzpatrick, 1966). The receptors for this neurohumoral reflex are located within the nipple; temperature and tactile receptors are presumably involved. Visual stimuli may also play some role (Peeters, Stormorken and Vanschoubroek, 1960). There may exist

supplementary mechanisms which assist milk letdown, possibly myoepithelial contraction evoked by direct mechanical stimulation and intrinsic reflexes within the lactating gland (Zachs, 1962). Folley and Knaggs (1966) found that milk ejection in goats could occur in the absence of oxytocin. This adds support to the previous statement.

Most investigators agree that oxytocin has some role in parturition. That oxytocin is released during some stage of labour is in little doubt (see Fitzpatrick, 1966). The role of oxytocin in the physiological process of birth, however, is not well defined. Fuchs (1964) concluded that oxytocin initiates labour in the rabbit. In other studies where the plasma levels have been measured during parturition in various species (Van Dongen and Hays, 1966; Knaggs, 1963; Fitzpatrick and Walamsley, 1962; Fitzpatrick, 1961; Cech, Brovetto, Cabot, Frelity and Caldeyro-Barcia, 1965; Folley and Knaggs, 1965), oxytocin levels have been found to be low prior to and during the first stage of labour and to rise rapidly to high levels in the second stage of labour. Following expulsion of the foetus, the oxytocin levels fall rapidly to the levels present prior to the

onset of parturition. These reports suggest that labour is initiated by another mechanism, and dilation of the birth canal results in reflex release of oxytocin that assists in delivery. Folley and Knaggs (1965) suggest that dilation of the cervex by the head of the foetus causes release of oxytocin which greatly augments uterine contractions which hasten expulsion at the time when the foetus is in potential danger of cord constrictions. Ferguson (1941) and Fitzpatrick and Walamsley (1962) have both presented evidence that this release is reflex in nature; the latter group of workers demonstrated that spinal block in the cow results in arrest of the progress of labour and a fall in plasma oxytocin.

On the other hand it has been reported that cervical dilatation in women does not result in oxytocin release (Sals, Fisch, Schwartz, 1965) and that normal birth may occur after destruction of the neurohypophysis.

The above are the generally accepted roles of vasopressin and oxytocin. Many other biological effects have been described. It remains in doubt whether these effects are physiological or pharmacological.

Increased uterine activity, milk ejection and antidiuresis have been reported following coitus in many species. (Van Denmark and Hays, 1952, 1953; Evans, 1933; Florey and Walton, 1932; Walton, 1960; Pickles, 1953; Harris and Pickles, 1953; Campbell and Peterson, 1953; Debacherе and Peteers, 1960; Debacherе, Peteers and Tuyttens, 1961; Franko, Frigberg and Karvonen, 1953).

The responses in the three target organs provide satisfactory evidence that the stimulus of coitus elicits the release of the neurohypophysial hormones. The presence of increased plasma levels however has not been convincingly established. This may merely represent the difficulty in measuring a transient surge of unknown duration and exact time of release. It has been tempting to interpret this release as being a reflex release resulting from local stimulation of the genitalia with the possible function of aiding sperm ascent. This may well be an oversimplification, for genital afferent impulses are by no means the only source of impulses impinging on the hypothalamus. Courtship phenomena without any physical contact between male and female apparently release oxytocin

(Harris, 1955; Van Donmark and Hays, 1952); in the powerful C.N.S. stimulation during mating, physical stimulation of the vagina and cervex is only one component.

In many species the concept of rapid sperm ascent has to be re-evaluated in the light of Du Buisson and Dauzier's (1955) work, which showed that sperm ascent in sheep is, contrary to what was believed, slow. Their work was based on the estimation of the distribution pattern throughout the tract at given periods, and not as previously on the location of literally one or two spermatozoa in the fallopian tubes as an index of sperm ascent. Even in those animals where rapid sperm ascent is thought to take place there is no direct proof that this ascent has been brought about by the release of the neurohypophysial hormones resulting from physical or psychological stimuli of coitus.

On renal tubules, oxytocin and vasopressin seem to have essentially the same types of effect, differing only in the relative doses required. Both hormones lead to an increase in the production of cyclic 3'5'-A.M.P. by the epithelial cells which induces permeability changes in the distal nephron (Orloff and Handler, 1965).

Antidiuresis is not a necessary accompaniment of the administration of neurohypophysial hormones, at least in some species. Kramer, Grinell and Duff (1966) have shown in rat that vasopressin at a dose of 1 mμ unit by single injection or 0.1 mμ unit/min by infusion causes a modest but definite diuresis. At higher concentrations, 5 mμ unit, the anticipated water retention occurs. During infusion, sodium excretion was enhanced during the diuresis; the results after a single injection were less consistent. Oxytocin in moderate doses often causes a prolonged diuresis (Chang, 1965). This diuresis is sometimes preceded by a brief antidiuresis. In some species at least, e.g. the rat, the diuresis is in part a consequence of a saluresis (see Pickford, 1966).

Both vasopressin and oxytocin in dogs and rats increase excretion of sodium, oxytocin being the more effective (Kramer, Grinnell and Duff, 1966). Lees and Lockett (1944) found that oxytocin sometimes caused a diuresis, natriuresis and kaluresis in the absence of a change in inulin clearance which indicates that electrolyte loss is not necessarily a matter of increased filtration rate.

Vasopressin, though it increases overall peripheral resistance, has been reported to increase renal blood flow at dose levels insufficient to cause a rise in systemic pressure (Barrer, 1963).

It is clear that the neurohypophysial hormones can affect the cardiovascular system, although the physiological significance, if any, is in doubt. The pressor effect of vasopressin is in no doubt. Nakane and Fisher (1963) found that oxytocin decreased the mean arterial pressure, the left atrial pressure and the total peripheral resistance, reactions causing a modest increase in heart rate and myocardial contractility. It was suggested that the action of oxytocin was a direct effect on the vasculature, causing dilatation.

Similar findings have been reported by Pickford and her group (see Pickford, 1966). They found that vasodilatation, including renal vasodilatation is lost and often converted to a constriction following ganglionic blockage, or surgical sympathectomy. It was suggested that the fraction of epinephrine released by sympathetic nerves is of functional significance and is essential for the dilator effect of oxytocin.

Steroids appear to modify the vascular response of oxytocin (see Pickford, 1966). Of interest here is the suggestion that oestrogens appear to interfere with the synthesis of epinephrine or its release from the sympathetic nerves (Haig, Lloyd and Pickford, 1965).

The vascular effects of vasopressin are also influenced by the presence of other hormones. Hyperresponsiveness to vasopressin is seen in D.C.A. hypertensive rats (Hinke, 1965). Phelan (1966) reports that renal hypertensive rats are also hyperreactive to vasopressin. Pressor responses to catecholamines are potentiated by the prior administration of non-pressor doses of vasopressin (Bartlestone and Nasmith, 1965).

Oxytocin has been shown to have antiarrythmic properties. It prevents E.C.G. changes due to hypoxia and terminates ventricular fibrillation produced by picrotoxin (Melville and Varma, 1961). It is also effective in preventing and arresting ventricular fibrillation caused by other means (see Farrel, Fabre and Rauschild, 1968).

It has been demonstrated that there is an increased tendency to atheroselerosis in animals given long term injections of vasopressin. (Cooper and Gutstein, 1966).

Researchers in dairy husbandry have been intriguod by the observation that a cow can be brought into heat quickly by large injections of oxytocin (Labhsetwar, Collins, Tyler and Casida, 1964; Armstrong and Hansel, 1959). For this effect to be seen, the uterus must be present (Armstrong, Hansel, 1958).

Vasopressin is claimed to have a direct effect on the submaxillary gland (Planel, Soleilhavoup and Tixador, 1965). It causes a prompt increase in glycogen content in the gland, followed by a depletion after an hour, returning in three hours to normal. Lawson and Dragstodt (1964) found that vasopressin decreased gastric secretion in dogs.

Oxytocin in large doses consistently induces hyperglycemia (Baisset, Dang-Trang and Montastruc, 1965). Insulin is also liberated (Balasse, 1964; Balasse, Rasio and Conrad, 1965). Balasse and his coworkers discounted the idea that the insulin release is secondary to hyperglycemia.

Vaughan (1964) found that vasopressin increases fat hydrolysis with the release of free fatty acids and glycerol *in vitro* in the intact animal. Goldman (1964) found that the opposite occurs in man. De Wied and Bohus (1966) suggest that vasopressin given to rats can aid the formation and retention of memory. Hilton, Scian, Westerman and Kruesi (1959) found that dogs' adrenal perfused by an isolated organ technique with vasopressin, resulted in a significant increase in hydrocortisone output. Hume, 1958; Royce and Sawyer, 1959; Girout, Stachenko and Pillota, 1958; and Bohus and Endroczo, 1961, have reported similar findings. Telegdy and Fendler (1964) claim that both oxytocin and vasopressin increase adrenal progesterone and corticosteroid secretion. Hilton (1960) injected oxytocin into the circulation of an adrenal pouch preparation in hypophysectomized dogs and observed no increase in hydrocortisone output. Kovach, Monos and Koltay (1965) gave oxytocin intravenously and observed no effect on steroidogenesis even in large doses. In the rat, vasopressin appears to have no effect on steroidogenesis either *in vivo* or *in vitro* (Mailhe-Voloss, Koch, Ducommun and Fortier, 1964).

Difficulties in Assessing the Physiological Effects

From what has been said above it can be seen that these hormones are capable of causing a great many effects in the body. These results have in the main been obtained by injecting exogenous hormone into the animal and locating a response. The interpretation in physiological terms of data from this type of experiment is very much open to question, as it is always uncertain whether the response is a physiological one. All that we can be certain of is that the hormones are potentially capable of that particular response. This information is useful but it cannot be directly extrapolated into an explanation of a physiological mechanism. To do this two additional pieces of information are required. Firstly we need to know the concentration of endogenous free hormone at the active site, or to the first approximation the concentration of hormone in the blood plus, ideally, information about the binding to carrier proteins, if any. This would inform us whether sufficient hormone is present to elicit the potential response. Caution must also be applied here because we cannot assume that the threshold for a response is the same in different

physiological states. We must be increasingly aware of the synergistic and antagonistic effects of hormones on each other.

The second piece of information that we require is whether the appropriate stimuli in a postulated physiological mechanism are capable of causing the release of the hormones. This is best studied by measuring the concentration of hormone passing from the gland. Information about the secretory rate and pattern yields information about the control of an endocrine gland, and the blood concentration about the possible physiological effect of the hormones.

In the case of the neurohypophysial hormones we are painfully ignorant of these criteria for almost all physiological conditions. Because of the inaccessability of the gland it is as yet impossible to measure secretory rate from the venous outflow from the gland. The nearest approach we can get is to measure jugular blood concentrations. Here again we are faced with difficulties because to date methods are not available to measure normal physiological levels, let alone variations. These measurements are also difficult to

perform and suffer from lack of precision and the labile nature of the hormones. To date no chemical or physical methods of measurement have been devised, consequently quantitation has been dependent on bioassay with all its inherent difficulties. As a result few determinations have been made.

The assay of vasopressin requires extraction from the plasma, removal of other prossor substances and interfering proteins, concentration, and bioassay in suitable animals. Most methods require the removal of the red cells, precipitation of proteins with trichloroacetic acid, extraction of the precipitate and chromatography on an ion exchange column. The bioassay is usually carried out in a rat, normally using the antidiuretic response, in ethanol-anaesthetized fasted and hydrated animals. With these procedures a minimum sensitivity of 2-5 μu can be expected. Since circulating levels of vasopressin in normal states have been estimated to be below 5 μu/ml of plasma (Share, 1965) the method is of limited value at physiological levels of hormone.

Extreme modifications (Tata and Gauer, 1966; Czaczjes, Kleeman and Koenig, 1964) have produced assays sensitive to less than a micro-unit of vasopressin. However, results are variable even in experienced hands (Tata and Gauer, 1966; James and Lee, 1965).

Immunological methods for the assay of vasopressin are being devised. Permutt, Parker and Utiger (1966) couple vasopressin to bovine albumen for an antigen. This method is as yet only sensitive to 6 μu of vasopressin.

Procedures for the preparation and bioassay of oxytocin are generally inferior to those for vasopressin. Numerous and varied bioassays have been employed; in vivo contractile response of the uterus (Saameli, 1963), the in vitro contractile response of the uterus (Coutinko and Csapo, 1959), degrees of milk ejection from time of exposure to milk ejection (Van Dongen and Hays, 1966), in vitro contractility of mammary gland tissue, and depression of blood pressure in fowls (Coon, 1939; Chan and Du Vigneaud, 1966).

From what has been said above it is not at all surprising that our knowledge of the above two factors is very sparse. It is not without significance that those cases where there exists general agreement as to the physiological roles of the hormones e.g. lactation, parturition and dehydration are where there exist measurable blood levels and a great many determinations of those have been made. This has resulted not only in the concentrations being known but more importantly, probably, the pattern of secretion is also known. For almost all other physiological conditions we are painfully ignorant of what is happening with regard to the neurohypophysial hormones. Because of the inability to determine the above factors directly, workers in the field have been forced to try other methods of determining them. Both (a) changes in the amount of hormone in the gland, and (b) the light or electronmicroscopic appearance of the gland have been taken as indices of secretion rates and of circulating hormone levels.

Much of the work evaluating the physiological role of these hormones has been based on the assump-

tion that a disappearance of stainable material as seen with the light microscope can be correlated with secretion of hormones (Rothballer, 1953; Leveque and Scharrer, 1953). Moses, Leveque, Giambatista and Lloyd (1963) have reported that reduction in stainable material can occur without concomitant depletion of the hormones. The disappearance of electron-dense material in the elementary granules of the neurohypophysis has also been taken as an index of secretion (Palay, 1955; Hartman, 1958; Bodian, 1963). Daniel and Lederis (1966) have evidence that this phenomenon bears no necessary relationship to the hormone content of the gland.

The content of hormone in the gland as measured by bioassay procedures has been used as a measure of secretion (Daniel and Lederis, 1966; Moses, 1963; Fendler, 1961; Fendler, Endrocai and Lissak, 1965). The interpretation of these results is complicated by the fact that the hormone content is a momentary balance between the rate of synthesis and the rate of secretion (Sawyer and Mills, 1966; Takabatka and Sachs, 1964).

It should also be mentioned that even the measurement of systemic blood levels is not without its dif-

ficulties when used as an index of the secretory rate, although there can be little doubt that it is the best method available. The blood concentration is a balance between secretion and metabolism, and the rate of metabolism has been shown to alter at different plasma levels and also states of hydration.

It can be seen that at present for the majority of physiological conditions there are no available methods for determining the plasma concentrations or for determining the rate of synthesis and secretion. The lack of information that would be available if such methods existed may mean that additional functions that the hormones may have are at present unsuspected or not established. This might well be particularly true in the case of oxytocin, for it seems very suspicious that this hormone should be present in females in very substantial amounts throughout their lives when, according to present reasoning, it is only required at infrequent intervals. Even more surprising is that it should be present in the male in equivalent amounts where it has no known functions. It also seems rather coincidental that these two hormones should have in general opposite effects on the

vascular system, oxytocin in most situations acting as a vasodilator and vasopressin as a vasoconstrictor. Another point is the suspicion that, although there certainly is preferential secretion, there is a tendency for both hormones to be secreted together. A knowledge of the above factors would certainly help to throw light on additional functions, if any.

A Possible Index of Hormone Synthesis and Secretion

A method of determining the secretory pattern of oxytocin which would be far superior to any of the available methods has been suggested by K.C. Hooper. If established, it could well be invaluable in furthering our knowledge of the physiology of oxytocin and in theorising about additional functions that the posterior pituitary gland may have.

During a study of hypothalamic peptidases he located two enzyme systems that were capable of inactivating the neurohypophysial hormones. He considered that these enzymes were distinct from the enzymes previously described, and from the study of their activity during pregnancy, parturition and lactation he concluded that they were involved in the hypothalamic metabolism

of oxytocin, and that they reflected the rate of synthesis of the hormone. As mentioned above, there appear good grounds for thinking that synthesis follows secretion quite closely. Hooper's experiments are described below.

Hooper's Experiments on Hypothalamic Enzymes that Inactivate the Neurohypophysial Hormones

In 1962 he examined the catabolism of four physiological polypeptides, namely bradykinin, oxytocin, vasopressin and substance P, by homogenates of dog hypothalamus. Dogs of either sex were anaesthetised and killed by bleeding from the neck vessels. The hypothalamus was dissected out and homogenized in 10 vol of distilled water and extensively dialysed at $4^{\circ}C$. The homogenate was incubated at $37^{\circ}C$ for various times and at different pH values with the four substrates. Enzyme action was stopped by immersion in boiling water for 15 minutes. A control incubation containing protein that had been denatured by heat prior to incubation was included. The residual hormone was estimated by standard bioassay procedures, oxytocin was measured by the avian-depressor method of Coon (1939) or with the isolated rat uterus. Vasopressin was measured by

the presor method of Dekanski (1952). Bradykinin and substance P were estimated with isolated guinea-pig ileum.

The system was shown to be incapable of synthesising the hormones at pH 7.0 and 37°C. Incubation of the homogenate with the hormones led to the destruction of the hormones; this did not occur if the homogenate had been boiled prior to incubation.

The pH optima curves for oxytocin, vasopressin and bradykinin destruction were similar, namely pH 7-8. The peak of optimum inactivation of substance P was somewhat lower, pH 5-6. The progress curve of inactivation of the hormones with time at pH 7.3 and 37°C did not follow precisely that predicted by first-order kinetics, since the rate of inactivation decreased with time. The relation between time, concentration and inactivation of the hormones showed an approximate linear relationship between tissue nitrogen and the logarithm of the residual concentrations of the hormones. Substance P in all the experiments showed the greater deviation. This was accounted for by the contamination of the specimen with small amounts of other

physiologically active material (Clough, Gaddum and Hooper, unpublished results).

The homogenate inactivated oxytocin approximately 2.8 times as rapidly as vasopressin when the substrates were incubated separately. When both substrates were incubated together the rates of destruction of the two peptides were approximately the same, the rate of both being about the same as that for vasopressin when used alone. No detectable interference from the mixed substrates was encountered during the bioassay of hormones.

When vasopressin and substance P were incubated together, the presence of vasopressin caused only a slight decrease in the rate of inactivation of substance P. The effect of substance P on the inactivation of vasopressin could not be assessed owing to limitations of the bioassay method: substance P in the mixed substrate caused a depressor effect on the rat blood pressure. The enzymes appeared to have no requirement for metal ions since cyanide, fluoride and EDTA did not inhibit their action. Neither iodoacetamide nor di-isopropyl phosphorofluoridate had any significant inhibitory action. Copper ions inhibited

the enzyme action, whereas neither iron nor zinc ions had any effect. The above substances have been shown to have very little effect on the stability of vasopressin or on the biological assays (Hooper, 1959).

Hooper concluded on the basis of pH optima, co-factor and metal requirements that the enzymes were distinct from hypothalamic enzymes previously described. Although the number of enzymes involved in the inactivation of the peptides was not known, he suggested that it was probable from the results of the mixed oxytocin and vasopressin substrates that these two hormones were inactivated by the same enzyme. He suggested three possible explanations. First, that the hormones are destroyed by the same enzyme which has a higher affinity and lower maximum velocity of destruction for vasopressin. Secondly, the hormones are inactivated by different enzymes, the observed decrease in the rate of inactivation of oxytocin being due to the presence of competitive substrates present in the impure sample of vasopressin. Thirdly, if two enzymes are involved, each may be inhibited by the substrate for the other, or by substances present in the impure samples of the hormones.

The fact that vasopressin did not interfere with the destruction of substance P, and also the difference between the optima pH of inactivation of the polypeptides, suggested to him that different enzymes are responsible for the destruction of the two substances.

Three possible physiological functions for the enzymes were suggested. They may prevent diffusion of the peptides into the surrounding tissue and serve to limit them to the particles in which they occur. The enzyme destroying substance P may possibly serve to terminate its action if substance P does act as a transmitting agent (Lembeck, 1953). Finally, the enzyme may assist in the maintenance of the amino acid pool in the hypothalamus, and the destruction of the four peptides may be incidental.

A study of the distribution of the enzymes, using as substrates vasopressin and bradykinin, in dog brain was undertaken (Hooper, 1963). The methods used were the same as those used previously (Hooper, 1962). Six regions of the brain examined were: (1) caudate nucleus (2) cerebellar cortex (3) cortex (4) hypothalamus (excluding corpora mamillaria and median eminence) (5) thalamus and (6) white matter. Dorsal roots, ventral roots and dorsal root ganglia were also examined for enzyme activity against the four peptides. He assumed

from previous work that oxytocin and vasopressin were inactivated by the same enzyme. It was also found that the intracellular distribution of enzymes destroying oxytocin was paralleled within the limits of the experimental methods employed, by the distribution of enzymes inactivating vasopressin (K.C. Hooper, unpublished work). This supported the hypothesis. Thus the distribution pattern obtained for the inactivation of vasopressin should also apply to oxytocin. The relative concentrations of vasopressin inactivating enzymes in the six regions of brain referred to a hypothalamic content of 10 for two dogs are given in table 1. From this it can be seen that the level is somewhat higher in the hypothalamus than in the other regions. White matter can be seen to contain considerably less than regions containing grey matter. The inactivation of bradykinin by brain tissue is shown in table 2. Again white matter contains less enzyme than grey, although in the case of bradykinin there is no difference between the enzyme activity in the six regions examined.

Neither the dorsal nor ventral roots contained detectable amounts of enzyme inactivating oxytocin at

Table 1

Inactivation of vasopressin by brain tissue (Hooper, 1963)

Tissue	Enzyme distribution (Hypothalamic content = 10)	
	Dog 1	Dog 2
Caudate nucleus	5.8	9.1*
Cerebellum	8.1	12.3*
Cortex	5.8	5.8
Hypothalamus	10.0	10.0
Thalamus	8.1	4.2
White matter	3.3	2.7

* Values probably artificially high owing to the presence of vasodepressor substances in media.

Table 2

Inactivation of bradykinin by brain tissue (Hooper, 1963)

Tissue	Enzyme distribution (Hypothalamic content = 10)
Caudate nucleus	9.8
Cerebellum	10.2
Cortex	10.0
Hypothalamus	10.0
Thalamus	9.8
White matter	6.1

a tissue concentration of 100 μg nondiffusible nitrogen/ml. At this level hypothalamic homogenate contained easily detectable amounts of enzyme. There was an indication that the dorsal root ganglia preparations that contained large amounts of tissue caused a slight loss of hormone. A similar situation existed when vasopressin was used as substrate, although in this case two out of four animals showed significant loss of hormone when the ventral roots were examined.

Bradykinin, in contrast to oxytocin and vasopressin, was inactivated by all three regions, although a difference in enzyme content in the three regions was noted. The ratio of activity was 108 for ganglia, 60 for dorsal roots and 20 for ventral roots.

Tissue from the three sites contained similar quantities of activity for the inactivation of substance P. Hooper concluded that the unequal distribution of enzymes inactivating vasopressin and presumably oxytocin, bradykinin and substance P suggested that different enzymes are involved. The inability of spinal roots and ganglia to inactivate oxytocin and vasopressin showed that certain hypothalamic enzymes

are not normal constituents of all nerve tissue. The restricted distribution also suggested that those enzymes have a special function.

The intracellular distribution of the enzymes in the non-pregnant and pregnant dog was investigated by Hooper (1964). The methods he employed were similar to those described in the Methods Section of this thesis. Enzyme activity was detected in the mitochondrial fraction isolated following centrifugation at 7,600 g and in the supernatant fraction isolated following centrifugation at 25,000 g. Subfractionation of the mitochondrial fraction was performed as described in the Methods Section. This procedure split the mitochondrial fraction obtained following centrifugation at 7,600 g into three distinct bands. Enzyme activity was found among the particles of heavy and intermediate density.

Electron micrographs of the three groups of particles suggested that the light fraction consisted largely of myelin threads. The intermediate fraction contained numerous structures, including nerve endings and fragments of axons. The heavy layer contained what appeared to be swollen mitochondria along with other unidentified material.

The stages of pregnancy were broken down by Hooper into three broad divisions depending on hair development. 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 changes in the enzyme activity and distribution are shown in table 3. These show that in early pregnancy the particulate enzyme becomes distributed throughout the three layers and by mid-pregnancy it is confined to the light layer where the majority of it appears to remain through to late pregnancy.

It was concluded that the above-mentioned enzymes have a more restricted intracellular distribution than the other peptidases from brain tissue. It was also stated that the variations in the distribution of enzymes inactivating oxytocin suggest that pregnancy is probably accompanied by a change in the metabolism of oxytocin in the hypothalamus. One function of the enzymes could be of a protective nature analagous to the plasma enzymes seen in human beings and anthropoidal apes during pregnancy, and that it is also possible that the enzymes may exert some control over

Table 3

Enzyme activity in non-pregnant and pregnant dog hypothalamic tissue. The activity is expressed in arbitrary units.

State of pregnancy	Supernatant	Light mitochondrial	Intermediate mitochondrial	Heavy mitochondrial
Non-pregnant	5	0	1	1
Early-pregnancy	-	4	3	3
Mid-pregnancy	1	3	0	0
Late-pregnancy	7	10	2	0

oxytocin production by varying the rate of hormone turnover during pregnancy.

Similar oxytocin inactivating enzymes were found in rabbit hypothalamus. The intracellular distribution differed slightly from that encountered in dogs in that the particulate bound enzyme was confined to the intermediate layer following the density gradient centrifugation (Hooper, 1964, 1966a). The enzyme activity and distribution throughout pregnancy was also determined. 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 and this then remained fairly constant although an increase in scatter was noted towards the end of pregnancy. The particulate enzyme was elevated by about the eighth day, and like the supernatant enzyme the concentration increased until a maximum was reached by about the tenth day, after which it remained fairly constant during the remainder of pregnancy. The scatter in this enzyme fraction was less than in the supernatant. Although the increases were detectable by the sixth and eighth day, the initial increases must have occurred before

this since the tissue concentrations used were below the concentration required to detect the enzymes in the non-pregnant animal. After the tenth day of pregnancy the specific activities of both enzymes were similar. Initially the specific activity of the supernatant enzyme was approximately three times that of the particulate. By comparison with the slopes obtained by plotting enzyme activity against tissue concentration, it was found that the particulate fraction had increased three and a half times. The supernatant had not increased to anything like the same extent. Hooper suggested that the enzymes reflect the rate of hormone production; his main basis for this was the results obtained during parturition and lactation which were at that time unpublished. The increase in enzyme concentration during pregnancy coincides approximately with the period of blastocyst spacing in the uterus. This suggested to Hooper that an increase in production of oxytocin occurs during the time of blastocyst implantation and that there may be some relation between the two effects.

Oxytocin is known to be released during parturition and lactation. Hooper (1966b) investigated enzyme

activity during these times. Enzyme activities were measured in animals from six hours to twenty-seven days after the birth of the young. The size of litters was not controlled. In a second series of animals the litters were removed shortly after parturition and the does were kept in isolation until required for estimation of hypothalamic enzymes. In the period following parturition the soluble enzyme showed peak activity (three times the pregnancy level), declining to the pregnancy level by day four post partum, at which level it was maintained as long as suckling continued. The particulate enzyme showed no peak activity following parturition and declined to the non-pregnant level by day seven. When the litters were removed both enzymes reverted to the control levels more quickly, by days four and ten to eleven for the particulate and supernatant respectively.

In two papers published in late 1968 from Hooper's laboratory the effects of steroids on the hypothalamic enzymes were described. Firth and Hooper (1968) described the effect of 17α-ethinyloestradiol-17β on the enzyme activity of hypothalamic homogenate. One group of animals was injected i.m. with 0.5 μg of the steroid daily for three days, a second group was in-

jected once with 0.5 µg and killed seven hours after injection, and a third group was killed eighteen hours after a single injection of 0.5 µg of steroid. A fourth group was used as a control. Oxytocin was again used as the substrate. In the animal injected for three days there was a considerable increase in enzyme activity. Animals killed eighteen hours after injection also showed a considerable elevation but this was significantly less than that obtained after three days of injection. After seven hours the enzyme level was slightly elevated.

The authors implied that there may be some connection between the increase in enzyme activity and the availability of releasing factors.

Hooper (1968) examined the effects of ovariectomy and injected oestradiol monobenzoate on the enzyme activity in the supernatant and intermediate mitochondrial fraction. Animals were ovariectomized and killed at two, six and thirty-two weeks after the operation and the enzyme activity measured. In animals killed at two weeks no enzyme activity was detected in the particulate group, and only very low activity in the supernatant fraction. In the group killed at six weeks the

particulate bound enzyme had returned to the control level. The supernatant fraction had also increased but was considerably below the control level. By thirty-two weeks the particulate bound enzyme was again the same as the controls, and the supernatant enzyme was intermediate between that found in animals sacrificed at six weeks and the intact animals.

The results obtained from intact animals injected with doses of oestradiol monobenzoate ranging from 15 µg to 120 µg showed that the supernatant enzyme was elevated, whereas the activity in the particulate fraction was not altered. The activity also seemed to be independent of the dose used, for points obtained with 15 µg doses lay virtually on the same curve as those obtained with 120 µg doses.

Hooper concluded that the ovary has some influence on the enzyme activity and that the influence is probably hormonal in nature, since afferent neural pathways between the ovary and the hypothalamus have not been established. The gradual return of enzyme activity following ovariectomy may be due to compensatory mechanisms of adrenal origin.

Hooper suggested a correlation between the enzyme activity and hormonal stimuli that are considered to inhibit the release of certain gonadotrophic hormones. Physiological conditions under which release of luteinising hormone is suppressed e.g. during pregnancy and lactation are accompanied by increased peptidase activity; conversely, conditions where gonadotrophic release would be expected to increase e.g. after ovariectomy, are accompanied by decreased peptidase activity.

In late 1965 when the work reported below was started, all that could be said with certainty was that enzymes that were capable of inactivating oxytocin and vasopressin <u>in vitro</u> were located in sub-cellular fractions of certain regions of the brain. The enzymes had a higher concentration in the hypothalamus than in other regions of the brain and they were different from enzymes that inactivated bradykinin and substance P. Their optimum pH and their behaviour with a number of enzyme inhibitors was known. The changes that occurred during pregnancy in the dog and rabbit and during lactation in the rabbit were known.

It was thought by Hooper that the enzymes reflected oxytocin production. This was based largely on three pieces of information; firstly that these enzymes interacted with oxytocin; secondly that the supernatant enzyme showed peak activity at the time of parturition in the rabbit; and finally that the supernatant enzyme level was maintained above control levels by lactation. The latter again was demonstrated in the rabbit.

This circumstantial information was by no means conclusive. Apart from describing how the enzymes change in additional physiological states, little more can be said with certainty now than could in October 1965.

It seemed to the author, then as now, that if it could be established that these enzymes reflect qualitatively the rate of synthesis (synthesis and secretion are thought to go hand in hand) of one or ideally both hormones, each enzyme being an index of one of the hormones, then this would be a potentially significant advance in our knowledge of the physiology of the hormones.

Possible Approaches for Further Examination of the Enzymes

To try to establish that the enzymes do reflect the rate of synthesis and secretion of the hormones, two lines of research could be taken. The first would be to investigate changes in enzyme activity in additional physiological states in order to accumulate more data, thus providing additional circumstantial evidence for or against the hypothesis. The second and by far the more scientifically valid would be to test the hypothesis directly.

As mentioned previously, the hormones stored in the infundibular process represent the balance between synthesis and secretion. The former is a function of the rate of synthesis and the speed of transport of the neurosecretory vesicles from the hypothalamus to the infundibular process. The gland contains a considerable store of the hormones, and from the structure of the gland it would not appear that newly synthesised hormone is immediately secreted on reaching the gland. It would seem more likely that the hormone on reaching the gland resides there for some time before secretion. If such a situation does exist and if

a radio-active labelled amino acid which is a constituent of the hormone is injected into the third ventricle, then a graph of labelled hormone extracted from the pituitary against time from the injection would be expected to be plateau shaped.

The time lag before the onset of the rise would be determined by the speed of transport of the neurosecretory vesicles. The rate of rise of the graph would be determined by both the rate of synthesis and the speed of transport. The height of the plateau would be determined by the rate of synthesis, and the duration of the plateau and rate of fall would be a function of the rate of secretion.

This is based on a number of assumptions, the first being that the amount of isotope incorporated into the hormone depends on the rate of synthesis. This seems a reasonable assumption since the labelled amino acid will, following injection, diffuse throughout the C.S.F. and hence be removed from the vicinity of hormone synthesis. It is only available for incorporation for a limited time which should not alter much. Thus one would expect incorporation to depend

on rate of synthesis. There may be interference from changes in rate of other synthetic mechanisms utilizing the same labelled amino-acids.

The second assumption is that all the labelled hormone reaches the pituitary and resides there for some time before secretion of it starts. Thirdly, that all hormone synthesised at the same time is homogeneous when it comes to that batch's turn for secretion.

If however such a clear-cut situation does not exist, it should still be possible to tell whether the rate of synthesis has changed in different situations, although interpretation will be more difficult and equivocal. Interpretation would be helped if the urine was collected, the hormone extracted from it and the radioactivity monitored, and similarly with the blood levels. Unfortunately, it is almost certain that the concentrations of hormones in these last two fluids are too small for detection. If they were not, we would probably have a deeper understanding of the hormones.

If it is possible to derive an index of hormone synthesis as described above, and the amount of hor-

mone in the pituitary is measured as well as monotoring the radioactivity, then it may be possible to ascertain the trend of the secretory pattern in the different physiological states tested. If it is possible to obtain these two parameters, they could be compared with the enzyme changes to see whether any correlation exists between one or other or both of them, and so test the hypothesis directly.

35-S-cystine would be a suitable amino acid to use. Sachs (1963) and Sloper, Arnolt and King (1960) have both found that it is incorporated into the hormones in vivo. It could be introduced into the third ventricle via a previously implanted cannula. These can be left in situ almost indefinately (Professor W. Feldberg, personal communication). A second practical route of administration which does lead to incorporation is into the sub-arachnoid space (Professor H. Heller, personal communication). The former route, although more difficult, is favoured since it causes less disturbance to the animal. The latter route necessitates slight anaesthesia and this may well interfere with the rate of synthesis.

Following the introduction of the isotope, the animals could be killed by decapitation and the pituitaries rapidly dissected out and extracted. This method means that apart from the trauma of death which is unavoidable, we would be dealing essentially with normal animals. The major disadvantage of the method is that it is hoped to build up a dynamic picture from a series of "stills", and each "still" will be of a different animal or a planned group of animals. This result will obviously be affected by individual variation. This can be overcome to some extent if sufficient animals are included in each determination. The variation should then tend to average out. Because of this variation and the complete absence of information about the effects of age, weight etc. in the function of the hypothalamo-neurohypophysial system, care would have to be taken to select animals as comparable in terms of age, size etc. as possible. Further, it would be useful in the case of vasopressin to keep potential test animals in metabolism cages prior to selection. Thus only animals whose water intake, urine output and density are of the same order would be selected. Other screening tests for neurohypophysial function may also be applicable prior to selection.

In the work reported below both these experimental approaches were used. Section A contains the work undertaken to test the hypothesis indirectly by measuring enzyme activity in additional physiological and experimental states. In order to test the hypothesis directly by the approach described above, it is essential to derive a method of extracting the hormones in a pure state from the pituitary. Section B contains a description of the work undertaken to derive a method of extraction and purification.

SECTION A

MEASUREMENTS OF ENZYME ACTIVITY IN ADDITIONAL PHYSIOLOGICAL AND EXPERIMENTAL STATES: CORRELATION OF ENZYME ACTIVITY WITH BODY WATER METABOLISM

CHAPTER I

INTRODUCTION

The object of the work reported below was to examine further the hypothesis that the enzymes have some functional correlation with the neurohypophysial hormones.

Parturition and lactation are dramatic conditions involving changes in many systems in the body. It was thought that as the particulate enzyme was not so effective at inactivating oxytocin and because it did not show peak activity at the time of parturition, that the enzyme may not be primarily concerned in the metabolism of oxytocin but rather with vasopressin or some other substance. An investigation of the enzyme activity during the postcoital period was undertaken because this is a transient physiological stimulus, and certainly a more limited one than that of pregnancy or lactation. Also there is a considerable body of evidence which suggests that both the neurohypophysial hormones are secreted at this time in many species, and further there is a well-defined increase in luteinizing hormone secretion during this period

in the rabbit, a reflex ovulator. This situation is thus ideal for evaluating the possibility of the enzymes being involved with luteinising hormone.

Subsequently the possibility of the particulate enzyme being concerned with vasopressin metabolism was examined. This was done firstly by comparing the inactivation of the hormones by the enzymes and subsequently by assaying the enzyme activities during dehydration and overhydration, two conditions where there are well-defined changes in secretion and synthesis of vasopressin. One dog diagnosed as suffering from diabetes insipidus became available and the hypothalamus from this animal was also examined.

During the course of the overhydration experiments three animals became obviously stressed. This was subsequently found to be the result of perforation of the gut. These animals were sacrificed and the enzyme activity measured.

The results obtained during dehydration and overhydration experiments suggested the possibility of the particulate enzyme being involved in the metabolism of vasopressin. During pregnancy well-defined changes in

the body water content, plasma volume and in the osmolarity of the blood have been described. It seemed reasonable to suppose that some of these changes at least, and in particular the reported haemodilution, might be accompanied by changes in the vasopressin status of the animals. It was thus decided to measure these factors as well as body water turnover to see whether any correlations between them and the enzyme activities existed.

CHAPTER II

METHODS AND MATERIALS USED IN THE ENZYME ASSAYS

Experimental Animals

The animals used were female rabbits of the New Zealand White strain and were of reproductive age. They were kept in isolation for about a month prior to use to ensure that none were pregnant or pseudo-pregnant. The animals were obtained in all cases from outside sources. Unless otherwise stated, they were allowed food and water without restriction.

a) Preparation of animals in the experiments to study the enzyme changes in the post-coital period

The control group were treated as stated above, and were removed from the animal house immediately prior to killing. The experimental group were individually put into the male's cage and left for thirty minutes, after which they were kept in isolation until killed. That successful mating had taken place was assessed visually, it being assumed that the mating had been successful when the buck recoiled off the doe. In animals killed twelve hours or more after mating, the fallopian tubes were dissected out and the contents

washed out with saline. The effluent was then examined for the presence of ova.

b) Preparation of normal, dehydrated and overhydrated animals

The normal animals were treated as described for the control group in the previous section. Dehydrated rabbits were produced by the administration of 3% NaCl as drinking water for three days. The animals were weighed before and after administration of the 3% NaCl. Where stated, animals were kept in metabolism cages for four days prior to and during the course of the treatment, and the urine volume and density were recorded daily. The other animals were kept in the animal house and removed immediately prior to death.

The overhydrated animals were kept in metabolism cages for four days before overhydration and their urine volume and density measured before and during overhydration. They were overhydrated by i.p. injection every eight hours for three days of 0.8% glucose corresponding to 10% of body weight. The infusion of the glucose solution, containing 1 ml Veterinary Mylipen (Procaine penicillin G Aqueous suspension 300,000

units/ml, Glaxo Laboratories Ltd., Greenford, England) per litre, was performed under sterile conditions, the solution having previously been boiled and subsequently brought to 37°C before infusion. The fur on the abdomen was shaved and the skin sterilized using alcohol. To ensure that the needle, a 26 G$\frac{3}{8}$ Yale disposable needle, did not puncture the gut, the needle was attached to a 2 ml syringe for piercing the abdominal wall. The plunger was retracted, and if no gut contents were aspirated the syringe was disconnected and the needle attached to the infusion apparatus. The latter consisted of a length of Silicone rubber tubing 6 mm in diameter (Esko Rubber Co. Ltd.) leading from the glucose reservoir to the animal via a Watson-Marlow H.R. Flow Inducer, type NHRE. Both the tubing and glucose reservoir had previously been sterilized by boiling. The glucose was infused at a rate of 10 ml/min. With the animals cradled in the experimenter's lap no sedation was found necessary for this procedure. The animals were also treated with 1 ml Mylipen i.m. twice daily.

c) Preparation of the dog suffering from diabetes insipidus

The dog (Case Number 37363, Dept. of Veterinary Medicine, University of Glasgow) was a West Highland terrier, about six years old. It was obtained through the courtesy of Professor W.I.M. McIntyre and Dr. G.W. Crighton of the Dept. of Veterinary Medicine of the University of Glasgow. The dog was kept in the medical ward of the Veterinary Hospital and was allowed food and water without restriction. More details are given in the results section.

Fractionation of the Hypothalamus (Hooper, 1966)

Rabbits were killed by a blow on the neck and in the case of the animals used in the pre- and post-coital period, by bleeding out from severed neck vessels. In subsequent experiments the animals were decapitated following the blow on the neck. The second procedure was adopted because at this time the pituitary glands were required. The dog was killed by bleeding out following an i.v. injection of Veterinary Nembutal (Abbot Laboratories Ltd., Agro-Vet Division, Kent).

In all cases the brain was rapidly removed and the hypothalamus dissected out after removal of the pial vessels, corpora mammillaria and the optic nerve. In all subsequent manoevers all glassware, solutions and centrifuges used were pre-cooled to 4°C. Hypothalami were homogenized in 9 vol of 0.25 M sucrose in a hand-operated homogeniser and the homogenate centrifuged at 4°C successively for 15 min at 600 g, 60 min at 7,600 g, and 60 min at 25,000 g to give nuclear mitochondrial, microsomal and supernatant fractions respectively. The mitochondrial fractions were then centrifuged through a discontinuous density gradient system prepared from 0.32, 0.8 and 1.2 M sucrose solutions. This system was prepared by layering the sucrose solutions, and was used immediately after preparation. Three well-defined layers of particles were obtained after centrifugation in a Spinco model L preparative ultracentrifuge for 60 min at 39,000 r.p.m. in a S.W.39 rotor. The layers were separated from one another by the test-tube-slicing technique. Following separation, the concentration of sucrose in each fraction was made approximately 0.25 M by the addition of water. The particles were then sedimented

by centrifugation for 60 min at 25,000 *g* at 4°C. The pellets were suspended in a little water (about 0.5 ml) and all the fractions unless otherwise stated were dialysed at 4°C against water overnight. The volumes of the dialysed fractions were recorded, and the nitrogen content was measured by a micro-Kjeldahl method.

Micro-Kjeldahl Determination of Non-diffusible Nitrogen

With the exception of the experiments in the post-coital period where the samples were done in duplicate, all the other determinations were done in triplicate. In each batch blank titrations, containing water and the other reagents, and "standards" containing urea (1 mg/ml solution) and the other reagents were included. The samples were added to 15 ml Kjeldahl digestion flasks, 0.1-0.2 mg of Cu-K_2SO_4 catalyst (1 *g* of cupric sulphate ground to a fine powder with 10 *g* of potassium sulphate) was added, followed by 1 ml of nitrogen free concentrated H_2SO_4. Finally 0.5 ml of selenium catalyst solution (2.34 *g* of sodium selenate in 150 ml of nitrogen free concentrated H_2SO_4) was added. The contents of the flasks were then digested for $7\frac{1}{2}$ hours on a Gallenkamp digestion rack.

During digestion a length of clean paper was placed in the vicinity of the mouths of the flasks. In the case of any sign of "spitting" that flask was discarded. Following digestion the contents of each flask were thoroughly washed into an upright Markham still, about 10 ml of 40% NaOH was added, and the distillation started. 10 ml of distillate was collected into containers containing 1 ml Tashiro's indicator (40 mg methyl red and 20 mg methylene blue dissolved in 100 ml of 50% ethanol. 1 ml of this solution was then diluted with 32 ml of 1% boric acid to give the working indicator). The distillate was then titrated against N/140 HCl prepared by diluting N/10 HCl Volumetric solutions (B.D.H. Ltd., Poole, Dorset).

Incubation and Measurement of Enzyme Activity

Enzyme activity was expressed as a function of the destruction of substrate, oxytocin or vasopressin (Pitocin and Pitressin respectively, Park, Davis and Co., Hounslow, Middlesex) resulting from incubation with hypothalamic fractions. Tissue fractions ranging from 20-200 µg of non-diffusible nitrogen were incubated with 0.5 i.u. of the substrate at 37°C for 3 hrs

at pH 7.3 (0.2 ml of 0.4 M Na_2HPO_4-NaH_2PO_4 buffer), the total volume of the incubation medium being made up to 1 ml with water. The incubation flasks (7.5 x 0.6 cm rimless test-tubes) were inclined at 20° to the horizontal in a circular rack and totally submerged in a water-bath and rotated at 10-15 r.p.m. The temperature of the water-bath was maintained at 37°C. Enzyme activity was stopped by heating in a boiling water-bath for 15 min and the residual hormone concentration was measured on the isolated rat uterus in the case of oxytocin, and the rat pressor assay (Dekanski, 1952) for vasopressin. A bracket type of assay (Hooper and Jessup, 1959) was used.

The amount of hormone detected after incubation was expressed in terms of the amount of hormone recovered from a control medium. The control medium differed from that used in the definitive experiment in that it contained tissue extract which had been denatured by heating for 15 min in a boiling water-bath before the addition of hormone and subsequent incubation. Residual hormone concentration was expressed as $\log A_o/A$ where A_o and A are the amounts of hormone recovered from the control and definitive incubations respectively.

Assay Procedures

a) Oxytocin

In all cases oxytocin was assayed on the isolated rat uterus. Female albino rats weighing 200-300 gm were brought from the animal house the evening before use, and were injected sub-cutaneously with 50 µgm stilboestrol diluted with arachis oil. This ensured that they were in oestrous when used the following morning. They were killed by a blow on the head and the uterus was rapidly removed, the adhering fat was trimmed away and one horn suspended in a 3 ml organ bath containing de Jalons solution (9 gm NaCl, 0.4 gm KCl, 0.0855 gm $CaCl_2$, 0.5 gm $NaHCO_3$, 0.5 gm glucose/litre) at 31°C and oxygenated by a stream of fine air bubbles from the bottom of the bath. The uterus was attached by a fine cotton thread to the stainless steel hook at the bottom of the organ bath, the other end was attached again with a cotton thread to an isotonic writing lever and the record was recorded on a smoked drum. A tension of 1.5 g was applied to the uterus. The other horn of the uterus was stored in de Jalons solution at 4°C and used subsequently on the same day if required.

Doses were injected regularly at 5 min intervals and the dose washed out immediately the maximum response was over. A bracket type of assay (Hooper and Jessup, 1959) was employed. The sensitivity of the uterus was determined by adding a range of standard doses. The sensitivity was found to vary in a sigmoid fashion. Doses lying on the linear region of the graph were chosen for the assay. Two or three dose levels of "standard" were given before and after the unknown, the unknown being diluted until responses were approximately equal to the standards.

b) Vasopressin

Vasopressin was measured by the pressor method of Dokanski (1952) on rats. Male albino rats weighing 200-300 g were anaesthetized with urethane (175 mg/100 g body weight) injected sub-cutaneously. The anaesthetic was supplemented with ether for the operative procedures. The trachea was cannulated with a short piece of polythene tubing about 2.5 mm in diameter and one carotid artery was dissected out ready for cannulation. The femoral vein close to the inguinal ligament was cannulated with a short length of Portex nylon tubing (pp 25) into which a 26 G disposable

hyperdermic needle fitted. The abdominal muscles were retracted to expose the inguinal ligament. The superficial pudencal vein was retracted and the femoral vein was separated from the adhering tissue. By slightly elevating the femoral vein, the deep branch reaching the femoral vein became visible. This was found and tied off to prevent bleeding that might occur during cannulation. The cannula was tied into the femoral vein by two ligatures and was made more secure by suturing it to the rat's thigh and also by strapping it to the operating table. Heparin (200 u per 100 gm body weight) was injected through the venous cannula and was washed in with saline. The carotid cannula (Portex tubing tapered to a tip 1.2 mm external diameter, filled with saline and connected to a Candon rat mercury manometer) was tied in. Dibenamine, 100 µg per 100 gm body weight, was injected twice at an interval of 10 min. Following the injection of dibenamine, there is a progressive fall in blood pressure for about 20 min. If after the pressure had stabilised there was a significant rise in blood pressure following an injection of about 0.4 ml saline, a third dose of dibenamine was injected. All

solutions, warmed to body temperature, were injected through the venous cannula by means of a 1 cc tuberculin syringe and 26 g 3/8 disposable needle, and were washed in with saline using a second tuberculin syringe. The total volume of sample plus saline in every case was adjusted to 0.4 ml and successive injections were given as soon as the pressure returned to the base line.

A bracket type of assay was used. Two dose levels of "standard", usually 6 and 10 m.u. or 10 and 15 m.u., were given before and after two dose levels of the unknown diluted to approximately the same potency as the standards.

CHAPTER III

METHODS EMPLOYED IN THE EXPERIMENTS ON BODY WATER METABOLISM

Preparation of Animals

The animals were kept in standard metabolism cages for 7 days prior to the start of the experiment and for the duration of the experiment. They were removed to breeding cages immediately before the expected time of delivery. They were allowed food and water without restriction.

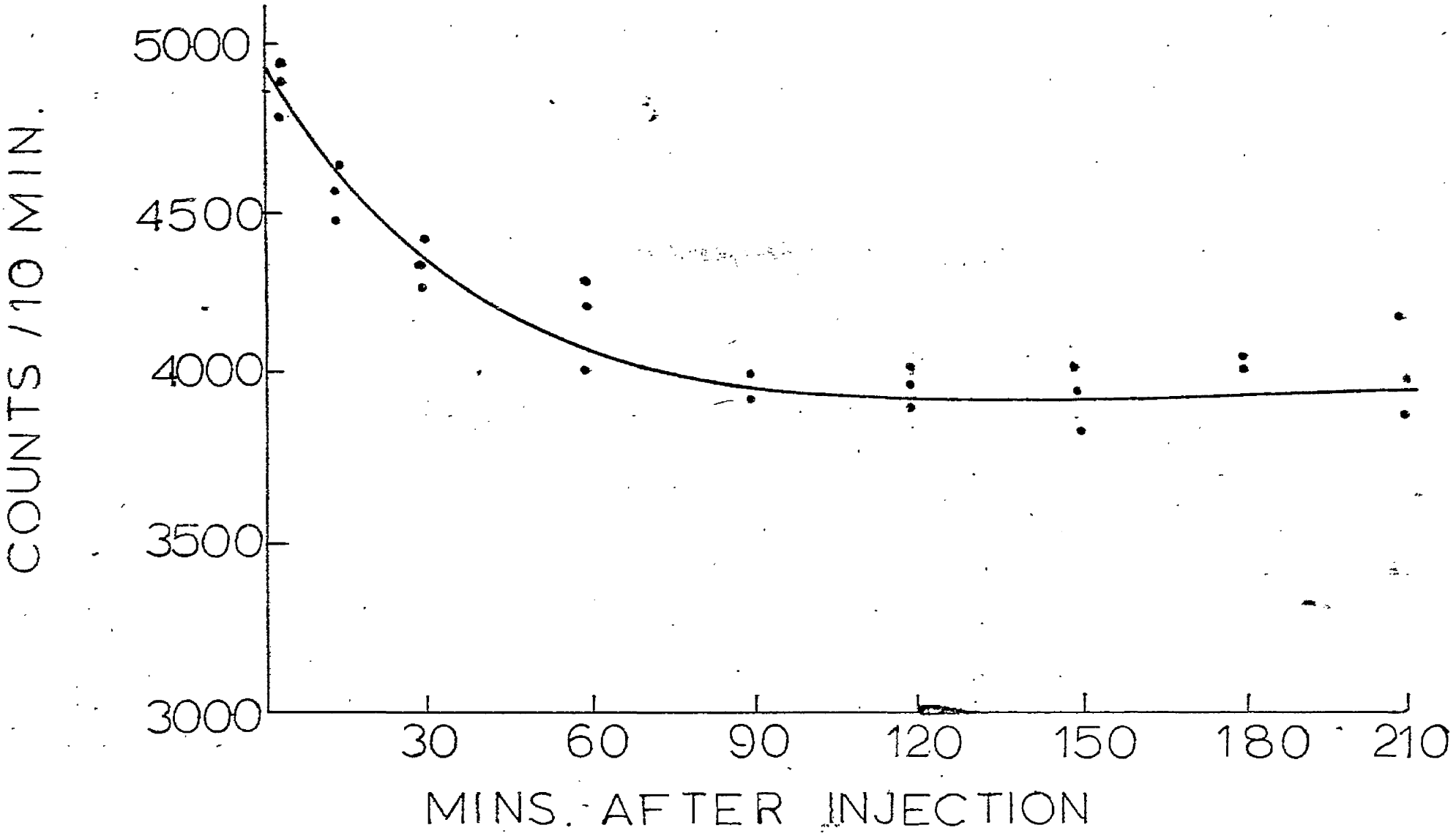
Total Body Water Determination

The index of total body water was taken as the tritiated water space and was measured using the dilution principle.

The equilibration characteristics of tritiated saline in six animals were determined. Figure 1 shows a typical disappearance curve of tritiated saline from the plasma following injection of the saline into the blood. This shows that equilibration occurs between 60 and 90 min. Thereafter it remains relatively constant at least for the 2 hrs in which it was determined.

Figure 1

DECAY OF PLASMA ACTIVITY FOLLOWING I.V. INJECTION OF TRITIATED SALINE



The determination of body water was based on these characteristics. Blood samples were taken 90 min following injection of a weighed amount of tritiated saline and every 30 min thereafter for 2 hrs. The body water was then determined by comparing the average activity in these samples with the activity of a weighed amount of the tritiated saline diluted in a known volume of saline, usually 1 or 2 l.

In the experiments reported below, serial determinations were made. The increment in activity following an injection was determined by taking blood samples prior to the injection and subtracting the activity in the pre-injection samples from the activity in the post-injection samples.

The tritiated saline was prepared by diluting Stock tritiated water (Radiochemical Centre, Amersham, England) containing about 5 m.c./ml with saline until the activity was in the region of 200-300 μc/ml.

The region around the marginal ear veins of both ears was shaved and the vein which was to be subjected to venopuncture was dilated by warming, then pierced

with the point of a scalpel blade. The blood, if flowing freely, was collected into glass centrifuge tubes containing a small amount of Heparin (Pularin, Evans Medical Ltd., Liverpool, England). Usually two samples were collected. A weighed amount of tritiated saline was then injected into a dilated marginal ear vein. Following a delay of 90 min the first post-injection blood sample was collected, as described above, from the ear opposite to the one injected. Thereafter blood samples were collected at 30 min intervals for a maximum time of 2 hrs.

The blood samples were centrifuged at 3,000 r.p.m. for 15 min and the plasma transferred with a Pasteur pipette to a small test tube. 0.5 ml of the plasma was pipetted in duplicate into centrifuge tubes, an equal volume of 20% T.C.A. was added and the contents were thoroughly mixed using a Whirlimixer (Scientific Industries Inc. (U.K.) Ltd., England). The denatured protein was then precipitated by centrifugation at 3,000 r.p.m. for 15 min. The protein-free supernatants were then transferred to small bottles which were sealed and stored until required.

The radio-activity in each blood sample was counted in triplicate. 0.25 ml of the sample was transferred to a counting vial using an Autozero precision pipette and 6 ml of liquid scintillator was added, followed by 5 ml of absolute ethanol. The contents were mixed and cooled to 4°C, the activity being counted at this temperature in a Tricarb Liquid Scintillator Counter for 10 min.

The liquid scintillator was prepared by dissolving 4 g of P.P.O. (Packard Instruments Co., Illinois, U.S.A.) in 1 litre of scintillation grade toluene (Nuclear Enterprises, Edinburgh, Scotland).

Body Water Turnover Determinations

Tritiated saline containing approximately 800-1500 μc/ml was injected into the animals and blood samples were collected 90 min post-injection. The time of sampling was noted and thereafter daily samples were collected, the time of collection of each sample being recorded. The blood samples were prepared for storage as described above.

Subsequently all the samples for each animal were prepared and counted at the same session. Again each

sample was prepared for counting as described in the previous section and as before the samples were counted in triplicate. The disappearance characteristics were represented graphically by plotting the log of the counts against the time of collection of the samples.

A visual examination of the disappearance characteristics of all the animals showed what appeared to be distinct variations in the gradients of the lines at different periods during pregnancy. As a result of this visual examination, pregnancy was divided into four periods.

The comparison of the lines in each period was achieved by calculating the regression characteristics as described by Snedecor (1956) and the significance of the differences in regression characteristics was calculated by co-variance analysis (Snedecor, 1956).

Plasma Volume Determinations

This was measured by the dilution principle using radio-active iodine ^{131}I or ^{125}I (Radiochemical Centre, Amersham, England) labelled albumen. The plasma volume was determined by injecting a weighed amount of label-

led albumin into the marginal ear vein and comparing the average activity of a 5 min and 10 min post-injection blood sample with the activity of a weighed amount of the labelled albumin diluted in a known volume of saline, usually 100 ml.

The experiments reported below were serial studies involving repeated determinations. For convenience the albumin was labelled in bulk. Crystallized rabbit albumin (Mann Research Laboratories, Division of Becton, Dickinson & Co. B - D, New York) was labelled by the method described by McFarlane (1958).

Initially the entire sample was kept at -15°C in one container. This necessitated thawing the sample and subsequently re-freezing what remained for later use. The repeated thawing and freezing was suspected of altering a proportion of the protein in such a way that the rabbits could differentiate it and rapidly remove it from the circulation. Because of this the albumin labelled subsequently was stored in small aliquots so that each determination of plasma volume would be carried out using protein that had been frozen and thawed only once. Following thawing, the al-

bumin solution was centrifuged to remove any particulate matter and a weighed amount was injected into the marginal ear vein. Blood samples were collected from the marginal ear vein of the other ear at 5 and 10 min. The blood samples were centrifuged at 3,000 r.p.m. for 15 min and the plasma collected. Samples in duplicate were prepared for counting by taking 0.5 ml of the plasma and adding to it 2 ml of 0.02 N NaOH. The activity was then counted for 100 sec.

Statistics

The standard deviations, t - test, regression coefficients, correlation coefficients and covariance analysis were all calculated by the methods described by Snedecor (1956).

CHAPTER IV

RESULTS OF ENZYME ASSAY STUDIES

For measuring the enzyme activity during the post-coital period, Pitocin (synthetic oxytocin) was used as the substrate for the enzymes. The possibility of one of the enzymes (the particulate enzyme) being involved in the metabolism of vasopressin was then tested for. The first experiment undertaken was a comparison of the inactivation of the two hormones by the enzymes from control animals. An examination of the inactivation of oxytocin by control animals was performed again because the work was being undertaken in a different laboratory. The results nevertheless were of the same order as those obtained previously. During this experiment it was found that the pressor assay was the more successful; the oxytocin assays more often than not failed to give a satisfactory and sustained dose response curve. The reason for this remains unknown. Consequently the enzyme activity in the subsequent experiments was determined using vasopressin as substrate and the pressor assay for determination of the activity.

For convenience in presenting the results, the experiment determining the enzyme activity during the post-coital period using oxytocin as substrate has been reported separately from the others.

The Activity of Hypothalamic Enzymes Inactivating the Neurohypophysial Hormones During the Post-coital Period Using Oxytocin as Substrate

In these experiments the animals were used in groups of threes. The tissue of each animal was extracted and assayed individually for enzyme activity. A control group of 23 animals was used, the test series being mated once and killed at a recorded time after mating. The animals were killed and the tissue extracted as described in the Methods Section. The tissue of each animal was extracted and split into two aliquots, each of 100 µg. One of these was used to measure the enzyme activity and the other was used as a control following boiling to denature the protein.

In the control group three experiments were performed where the tissue from the three animals was combined and an activity/tissue concentration curve obtained. This was found to be linear and activity could not be detected below 50 µg of tissue extract.

In the experiments where it was not possible to use 100 µg of tissue extract, the amount used was recorded and the activity adjusted to a 100 µg amount. This adjustment was achieved by making use of the fact that 50 µg amounts are just below threshold and the activity varies in a linear fashion with increasing concentrations. A calculation was then made based on a linear progression from 50 µg through the point obtained to 100 µg. Although this is valid for the control group, in the test series where the activity is altered, this is not strictly true; where an increase in activity has occurred, the adjustment would tend to overestimate the amount since the threshold of activity would be a little below 50 µg. The converse would hold true for a situation in which the enzyme activity has decreased. The majority (13 out of 16) of the adjustments were only from 80-100 µg, which should not result in a large error. Since only 16 out of 100 estimates required adjustment, there should not be a significant alteration in the basic picture. Where the adjustments have been made is shown in appendix 1.

The animals

Table 4 shows the details of the weights of the animals and of the hypothalamic tissue dissected out.

Precision of the techniques

a) Nitrogen determinations

Estimates were performed in duplicate. The average coefficient of variance ($\frac{S.D.}{mean} \times 100$) for all the duplicate titrations was 4.8 ± 4.1% with a range of 0-24%. The average coefficient of variance in each tissue fraction and the distribution of the coefficients of variance are shown in table 5. There was no significant difference between the discrepancies in the supernatant and particulate fractions.

b) Assays of oxytocin

The average coefficient of variance of all the assays was 9 ± 5.6% with a range of 0-43%. The average values in the particulate and supernatant fractions both pre- and post-coitus and the distribution are shown in table 6. The differences between the groups are not statistically significant.

Table 4

Weights of animals and hypothalami in the control and post-coital groups

Hours after mating	No. of experiments	No. of animals	Weight of animals (kg)	Range	Weight of hypothalami (mg)	Range
Pre-coitus	8	23	2.25±0.75	1.4-4.0	133±26	84-183
Post-coitus						
1	1	3	3.03±0.29	2.7-3.2	86±11	76-98
2	1	3	2.33±0.36	1.9-2.7	112±16	100-130
4	1	3	2.70±0.44	2.4-3.2	82±13	70-96
5	1	2	2.80±0.80	2.0-3.6	99±17	87-111
9	1	3	3.20±0.23	3.0-3.4	109±11	97-118
12	1	3	2.30±0.37	2.0-2.5	130±6	123-136
16	1	2	2.85±0.23	2.7-3.1	74±8	68-80
20	1	3	3.21±0.45	2.8-3.7	90±9	84-101
24	1	3	3.27±0.54	2.7-3.7	89±3	85-91
36	1	3	4.08±0.71	3.7-4.9	111±20	95-133
48	1	2	3.95±0.50	3.6-4.3	120±4	117-123
72	1	2	3.77±0.31	3.6-4.0	102±9	96-108
96	1	2	2.60±0.14	2.5-2.7	111±37	85-137

Table 5

The average coefficient of variance of the nitrogen titrations and the distribution in the tissue fractions examined

Fraction	Coef. of variance	Range	No. of titrations in each range			
			0%	1-5%	5-10%	10%
Blank	6.9±6.7	0-24	6	2	7	4
Soluble	4.4±3.5	0-11	7	28	9	3
Particulate	4.2±3.4	0-14.7	8	19	8	3

Table 6

The average coefficient of variance of the assay determinations and the distribution in the tissue fractions examined pre- and post-coitus

Group	Coef. of variance	Range	Distribution			
			0%	0-5%	5-10%	10%
Supernatant (control)	7.9±3.7	1.6-23	0	8	24	11
Supernatant (post-coital)	10±7.9	0.3-43	0	10	25	19
Particulate (control)	8.9±4.4	0-16	1	8	16	16
Particulate (post-coital)	9.0±5.1	0-25	1	13	17	22
TOTAL	9.0±5.6	0-43	2	39	82	68

Non-diffusible nitrogen content of the tissue fractions in the control and post-coital periods

Figures 2 and 3 and tables 7 and 8 show the non-diffusible nitrogen content/mg wet weight of tissue in the particulate and supernatant fractions respectively. The horizontal lines represent the control levels and the shaded area the standard deviations. From these it can be seen that the content of the non-diffusible nitrogen in the particulate fraction is elevated at 12 and 16 hrs. At 20 hrs the content was at the control level and between 36 and 48 hrs it began increasing, the increase becoming significant at 72 and 96 hrs. The supernatant fraction showed an apparent gradual rise which increased throughout the whole time. At 96 hrs post-mating the increase was statistically significant.

Enzyme activity in the control and post-coital periods

The recoveries of the control incubations of the supernatant and particulate enzymes were 359 ± 48 (range 252-442) and 390 ± 59 (range 248-467) respectively. These do not differ significantly.

Figure 2

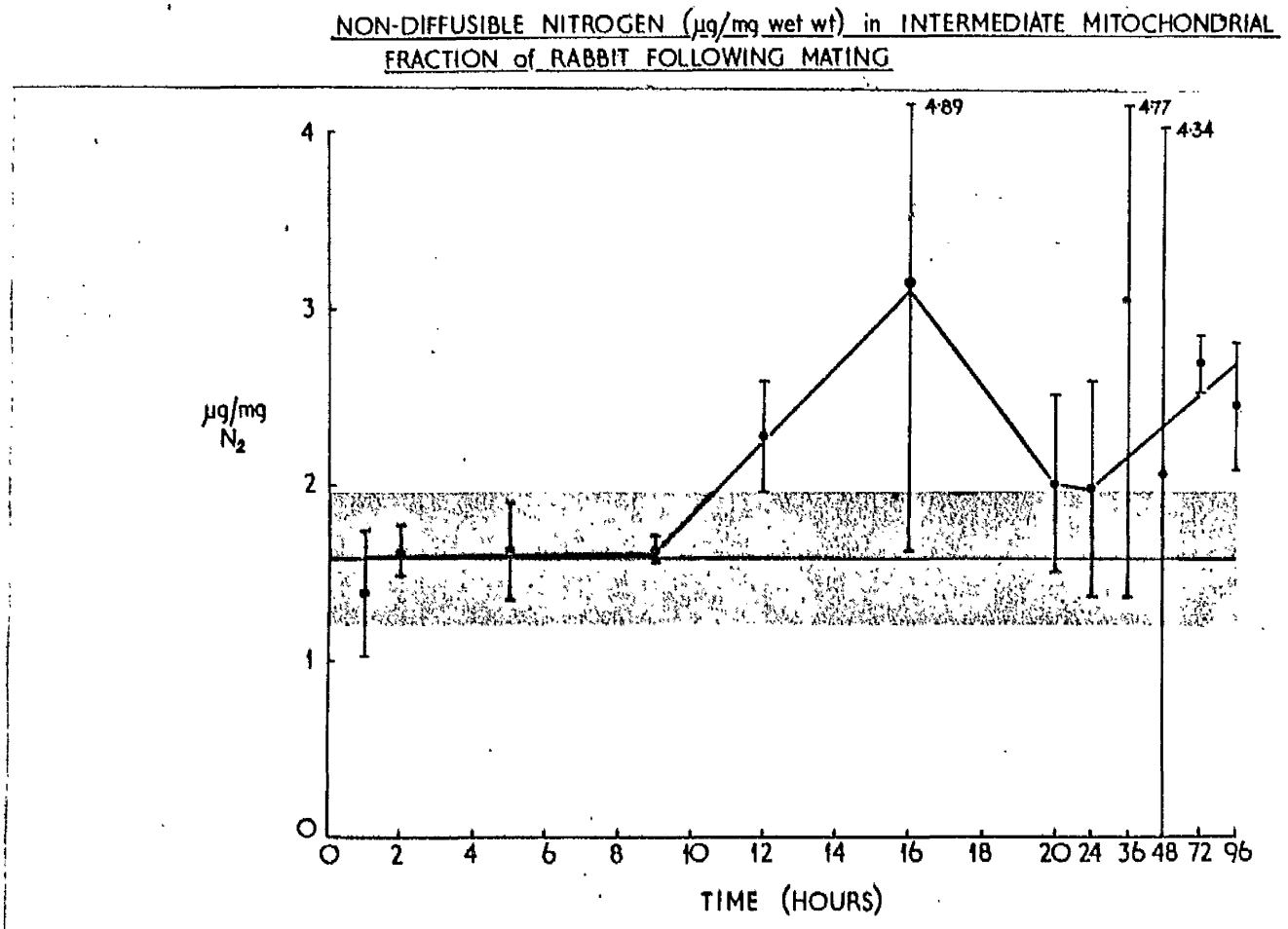

NON-DIFFUSIBLE NITROGEN (µg/mg wet wt) in INTERMEDIATE MITOCHONDRIAL FRACTION of RABBIT FOLLOWING MATING
µg/mg N2
4
3
2
1
0
4·89
4·77
4·34
0 2 4 6 8 10 12 14 16 18 20 24 36 48 72 96
TIME (HOURS)

Figure 3

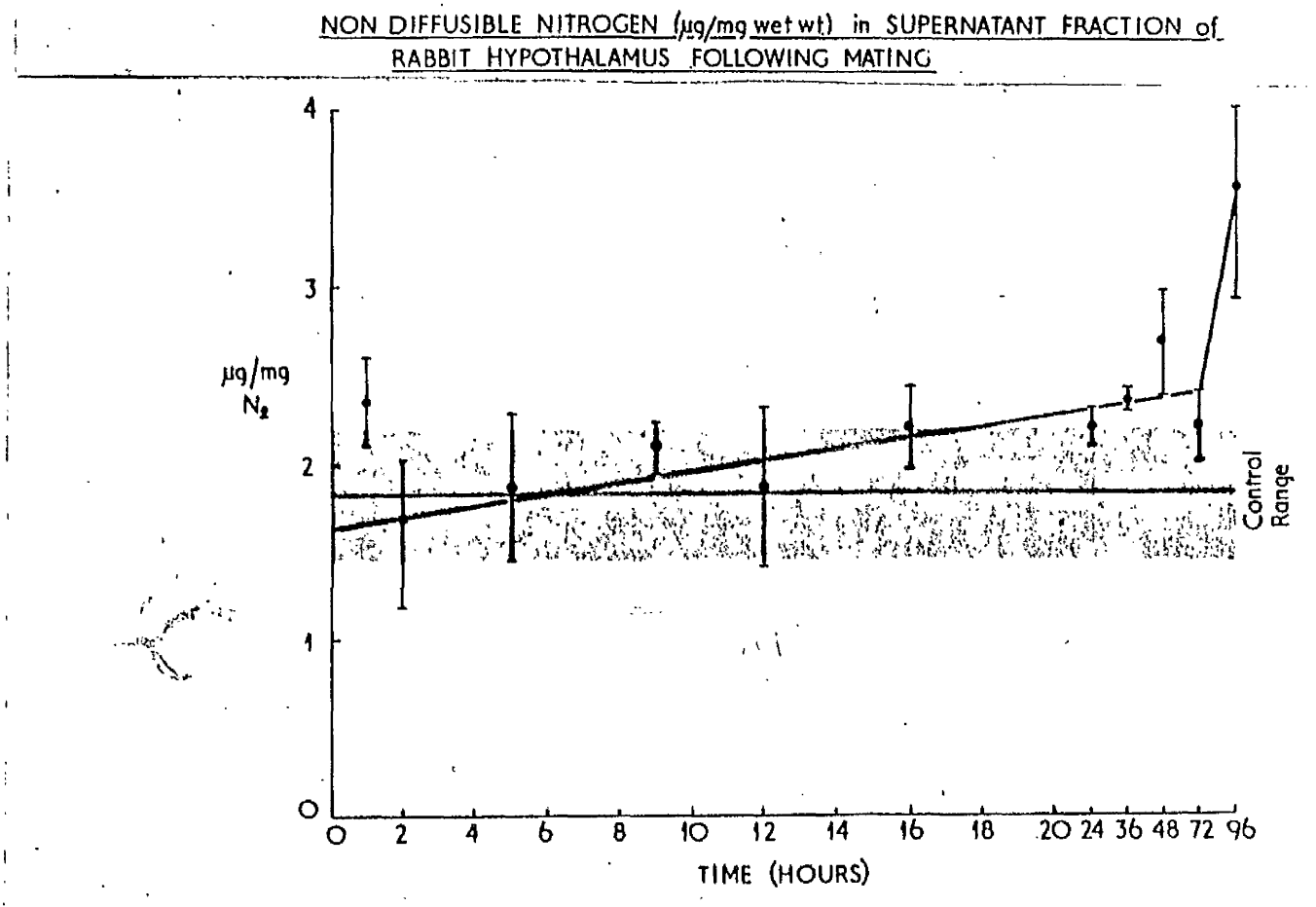

NON DIFFUSIBLE NITROGEN (µg/mg wet wt) in SUPERNATANT FRACTION of RABBIT HYPOTHALAMUS FOLLOWING MATING
µg/mg N2
4
3
2
1
0
Control Range
0 2 4 6 8 10 12 14 16 18 20 24 36 48 72 96
TIME (HOURS)

Table 7

Nitrogen recovery in the particulate fraction in the control and post-coital animals

Hours after mating	No. of experiments	No. of animals	$\mu g\ N_2$ / mg wet wt	Range	Significance P
Control	8	23	1.58±0.39	1.13-2.40	-
1	1	3	1.38±0.36	1.13-1.79	N.S.
2	1	3	1.62±0.15	1.50-1.79	N.S.
4	1	3	-	-	-
5	1	2	1.57±0.32	1.34-1.80	N.S.
9	1	3	1.63±0.07	1.60-1.70	N.S.
12	1	3	2.28±0.31	2.07-2.50	N.S.
16	1	2	2.90±1.56	1.79-4.00	N.S.
20	1	3	2.10±0.61	1.66-2.38	N.S.
24	1	3	1.98±0.62	1.32-2.58	N.S.
46	1	3	3.06±1.04	2.80-4.21	N.S.
48	1	2	2.07±2.27	1.09-3.31	N.S.
72	1	2	2.70±0.16	2.59-2.81	<0.05
96	1	2	2.46±0.50	2.10-2.82	<0.05

Table 8

Nitrogen recovery in the supernatant fraction in the control and post-coital animals

Hours after mating	No. of experiments	No. of animals	μg N_2 mg wet wt	Range	Significance P
Control	8	23	1.82±0.38	0.94-2.85	-
1	1	3	2.36±0.25	2.12-2.62	N.S.
2	1	3	1.66±0.49	1.30-2.23	N.S.
4	1	3	-	-	-
5	1	2	1.87±0.43	1.44-2.30	N.S.
9	1	3	2.07±0.06	2.01-2.13	N.S.
12	1	3	1.52±0.52	1.02-2.06	N.S.
16	1	2	2.26±0.36	2.00-2.53	N.S.
20	1	3	-	-	-
24	1	3	2.20±0.16	2.09-2.39	N.S.
36	1	3	2.32±0.05	2.24-2.40	N.S.
48	1	2	2.68±0.45	2.36-2.99	N.S.
72	1	2	2.21±0.21	2.06-2.36	N.S.
96	1	2	3.49±1.52	2.41-4.57	<0.05

The changes in enzyme activity in the particulate fraction following mating are shown in table 9 and figure 4. The changes in the activity in the supernatant fraction are shown in table 10 and figure 5. The horizontal lines represent the enzyme level in the control animals and the shaded areas the standard deviations. These results show that in the particulate fraction there is peak activity. The increase began between 5 and 9 hrs, reached a peak at 12 hrs, returning to the normal range by 16 hrs and remained within the control range for the remainder of the time. The supernatant fraction showed a slight apparent rise of similar time course to that obtained above, followed by a transient decrease starting after 24 hrs and before 36 hrs, returning to the control level by 72 hrs.

The Behaviour of Hypothalamic Enzymes Inactivating the Neurohypophysial Hormones in: (a) Control Animals Using Oxytocin and Vasopressin Separately as Substrates; (b) Dehydrated and Overhydrated Animals Using Vasopressin as Substrate; (c) Stressed Animals Using Vasopressin as Substrate

In these experiments the animals were treated in groups of threes or fours. The animals were killed as

INACTIVATION of OXYTOCIN by INTERMEDIATE MITOCHONDRIA FRACTION of RABBIT HYPOTHALAMUS FOLLOWING MATING

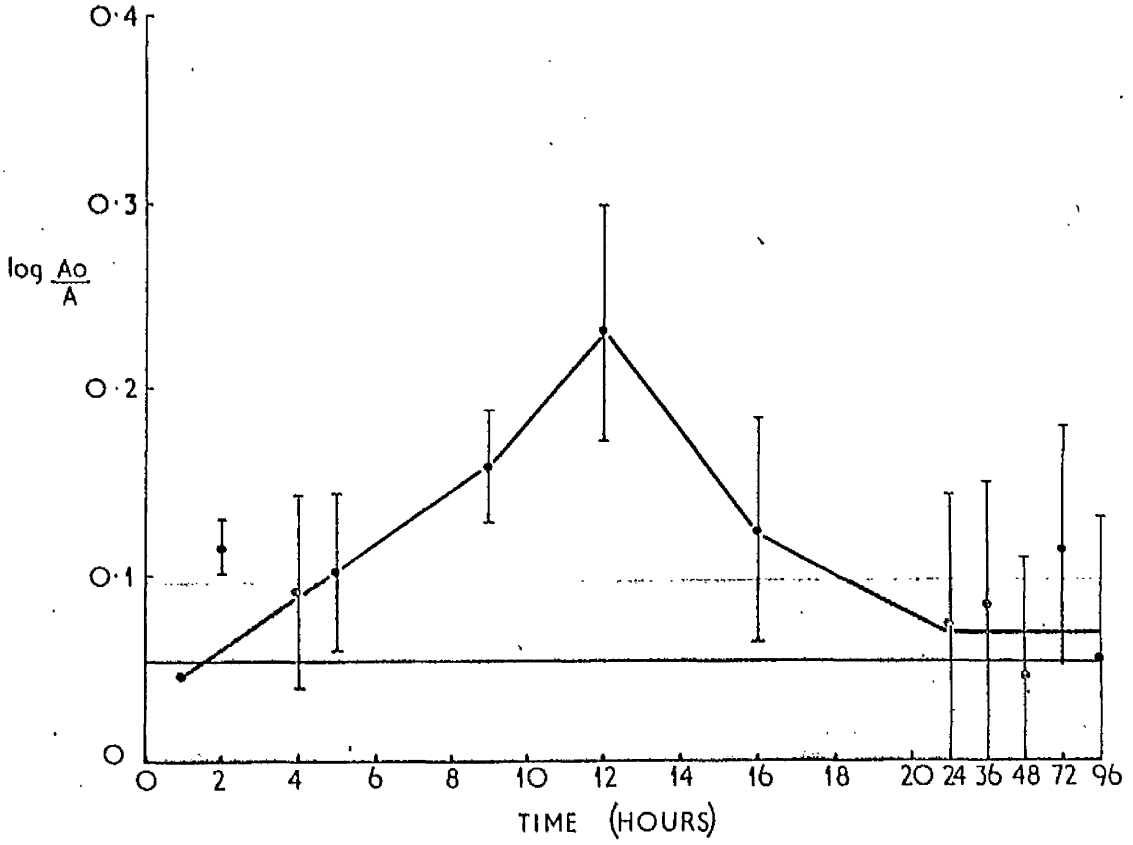


Figure 5

INACTIVATION of OXYTOCIN by SUPERNATANT FRACTION of RABBIT HYPOTHALAMUS FOLLOWING MATING

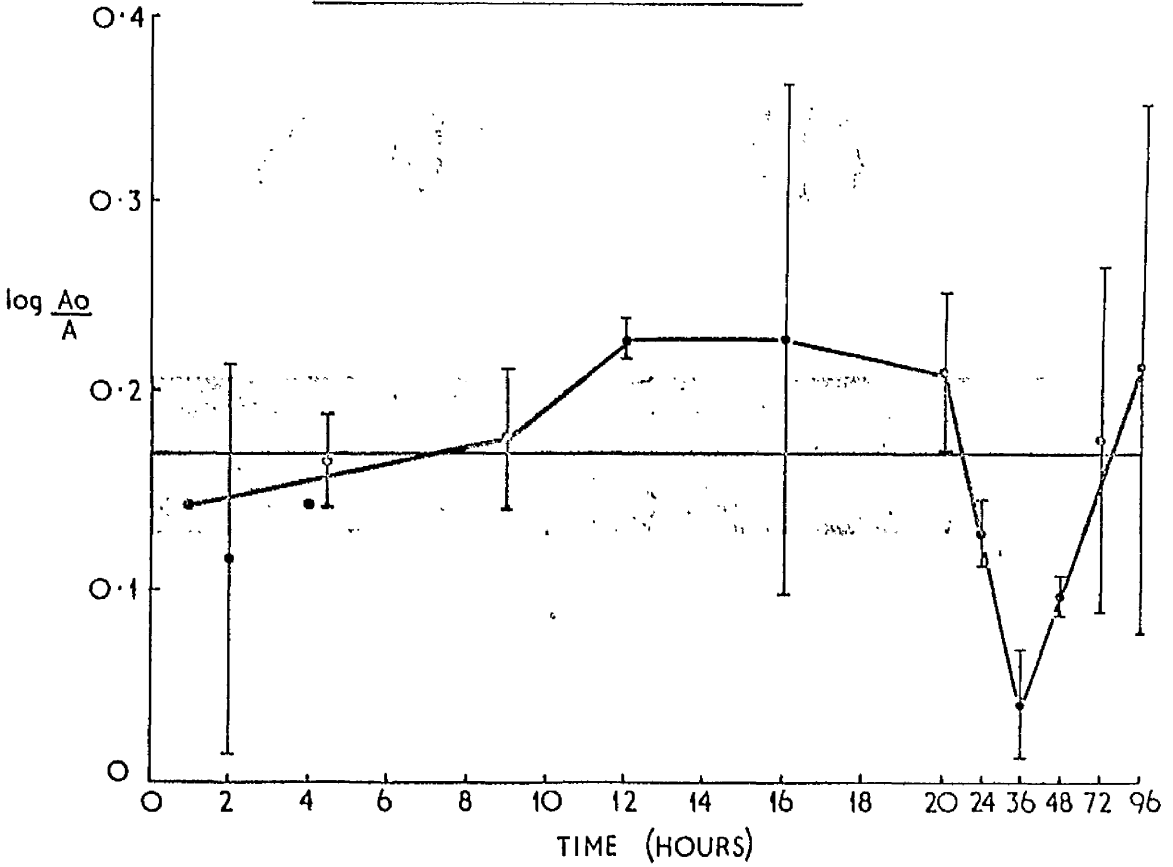


Table 9

Enzyme activity in the particulate fraction during the control and post-coital periods

Hours after mating	No. of experiments	No. of animals	$\log \frac{A}{A}o$	% change	Range	Significance P
Control	5	14	0.05±0.05	-	0.00-0.16	-
1	1	3	0.04±0.01	-14.4	0.04-0.05	N.S.
2	1	3	0.11±0.02	+11.7	0.10-0.13	< 0.05
4	1	3	0.09±0.05	+73.1	0.06-0.15	N.S.
5	1	2	0.10±0.04	+92.0	0.07-0.13	N.S.
9	1	3	0.16±0.03	+200	0.13-0.19	< 0.01
12	1	3	0.23±0.07	+344	0.16-0.27	< 0.01
16	1	2	0.12±0.06	+136	0.08-0.17	N.S.
20	1	-	-	-	-	-
24	1	3	0.07±0.07	+34	0.00-0.14	N.S.
36	1	3	0.08±0.07	+59.6	0.04-0.16	N.S.
48	1	2	0.05±0.06	-13.5	0.00-0.09	N.S.
72	1	2	0.11±0.07	+117	0.07-0.16	N.S.
96	1	2	0.06±0.08	+ 5.8	0.00-0.11	N.S.

Table 10

Enzyme activity in the supernatant fraction in the control and post-coital animals

Hours after mating	No. of experiments	No. of animals	$\log \frac{A}{A_o}$	% change	Range	Significance P
Control	5	15	0.17±0.04	-	0.11-0.22	-
1	1	2	0.14±0.01	-15.5	0.14-0.15	N.S.
2	1	3	0.12±0.10	-30	0.00-0.19	N.S.
4	1	3	0.14±0.01	-15	0.13-0.16	N.S.
5	1	2	0.17±0.03	- 1.8	0.15-0.19	N.S.
9	1	3	0.18±0.04	+ 5.4	0.13-0.20	N.S.
12	1	2	0.23±0.02	+35.7	0.22-0.24	N.S.
16	1	2	0.23±0.13	+35.7	0.14-0.32	N.S.
20	1	3	0.21±0.40	+26.2	0.18-0.24	N.S.
24	1	2	0.13±0.02	-23.8	0.12-0.14	N.S.
36	1	3	0.04±0.03	-75.6	0.01-0.07	$<$0.01
48	1	2	0.09±0.01	-42.3	0.09-0.10	$<$0.02
72	1	2	0.21±1.30	+ 5.4	0.12-0.24	N.S.
96	1	2	0.21±0.14	+27.4	0.12-0.31	N.S.

described in the Methods Section. The dissected hypothalami were combined and extracted as described. Aliquots of the cellular extracts ranging from 20-200 µg of non-diffusible nitrogen were incubated. In each incubation at least two control incubations were included, one containing denatured tissue extract and the other containing only buffer and hormone. Only the supernatant and intermediate mitochondrial fractions were tested in the control and stressed groups. Hooper (1966a) showed that enzyme activity was confined to these fractions in control animals. In the stressed group the nuclear fractions etc. were unfortunately lost. In the overhydrated and dehydrated groups all the fractions were tested for enzyme activity.

The animals

Table 11 shows the details of weights of the animals and the hypothalamic tissue dissected out. Table 12 shows the average change in urine density and volume pre and post the administration of the 3% NaCl in the one experiment where this was determined in the dehydrated group. This shows that there was a statistically significant increase in the density of the urine. There was also an apparent decrease in the

Table 11

Weights of animals and hypothalamic tissue dissected out in experiments testing enzyme activity in control, dehydrated, overhydrated and stressed animals

	No. of experiments	No. of animals	Wt of animals kg	Range kg	Wt of hypothalamus mg	Range mg
Control oxytocin as substrate	3	10	3.2±0.2	2.9-3.7	89±8	80-100
Control A.D.H. as substrate	5	20	3.2±0.5	2.6-4.4	104±16	81-130
Dehydrated	5	16	3.4±0.4	2.7-4.2	112±17	74-136
Overhydrated	2	5	3.0±0.7	2.3-4.5	106±17	84-134
Stressed	1	3	3.6±0.5	3.1-4.2	99±17	85-118

Table 12

Average urine density and volume pre- and during dehydration

Expt.	Animal	Urine density		% Change	Significance P	Urine volume		% Change	Significance P
		Pre-hydration	Post-hydration			Pre-hydration	Post-hydration		
1	1	1.016±0.005	1.044±0.002	+24	<0.01	221±105	68±61	-68	<0.1
1	2	1.027±0.007	1.041±0.005	+14	<0.05	235±126	141±43	-66	<0.2
1	3	1.023±0.004	1.053±0.012	+31	<0.02	283±42	110±56	-61	<0.02
TOTAL		1.023±0.004	1.046±0.006	+23±9	<0.01	246±32	106±37	-65±3.6	<0.01

urine volume passed each day. The decrease was not statistically significant. Unfortunately it was not possible to determine these parameters routinely. The animals in this group also lost weight, presumably due to loss of water. The average loss of weight was -10 ± 6% (range 0-23%). The distribution of the percentage weights lost is shown in table 13.

Table 14 shows the changes in urine density and volume pre and post overhydration. There is a statistically significant decrease in density. The average decrease was -19 ± 10% (range -8 - -31%). There was also a statistically significant increase in the urine volume which averaged 184 ± 14% (range 85-427%). During the time of the overhydration the animals showed no gain in weight, indicating that they were not accumulating water.

Post-mortem examination of the gut revealed no visible abnormalities except in the first attempt. In this case the infusions were undertaken using a 100 ml syringe and a 16-G disposable needle to infuse the glucose. The animals very quickly began showing signs of stress and were sacrificed after 36 hrs by which time they had received 20% of their body weights of

Table 13

Percentage change in weights of animals subjected to dehydration for 3 days

% change in weight	0 - -5%	-5 - -10%	-10 - -15%	-15 - -20%	-20%
No. of animals	2	7	5	1	1

Table 14

Average urine density and volume pre- and during overhydration

Expt.	Animal	Urine density		% Change	Significance P	Urine volume		% Change	Significance P
		Pre-hydration	Post-hydration			Pre-hydration	Post-hydration		
1	1	1.012±0.002	1.004±0.002	- 8	<0.05	276±142	557±93	+109	<0.05
2	1	1.019±0.009	1.002±0.001	-17	<0.05	221±85	460±45	+108	<0.02
2	2	1.033±0.006	1.002±0.006	-31	<0.01	120±30	633±64	+427	<0.01
2	3	1.028±0.013	1.001±0	-27	<0.05	220±156	653±101	+186	<0.01
2	4	1.016±0.004	1.003±0	-13	<0.01	353±46	630±260	+ 85	<0.01
TOTAL		1.022±0.008	1.002±0.001	-19±10	<0.01	236±85	587±80	+183±142	<0.01

glucose solution. Post-mortem examination revealed that animals 1 and 2 had severe inflamation of the gut and massive fibrinogen deposits. Animal 3, in addition to the above, had a perforation of the caecum.

The urine volumes prior to and during the overhydration procedures are shown in table 15, together with the density during the overhydration procedures. These show that there was an increase in the urine output in all animals. The average increase was 154 ± 59% (range 92-209%). This average increase is statistically significant, and of the same order as that obtained in animals that were successfully overhydrated. The average density was found to be somewhat higher than that found in animals that had been successfully overhydrated, but was considerably lower than that routinely encountered in normal animals.

Precision and validity of the techniques

a) Nitrogen determinations

These were determined as described in the Methods Section. Samples were determined in triplicate. The average coefficient of variance for all the determinations was 3.6 ± 4.3% (range 0-22%). The average co-

Table 15

Urine volume before and dyring overhydration and urine density during overhydration in stressed animals

Animal	Urine density		% change	Urine volume		% change
	Pre-hydration	Post-hydration		Pre-hydration	Post-hydration	
1	-	1.008±0.003	-	208	545±7	+162
2	-	1.006±0.001	-	176	545±21	+209
3	-	1.009±0.001	-	156	300±113	+ 92
AVERAGE	-	1.008±0.002	-	180±26	473±124	154±59

efficient of variance and the distribution within each tissue fraction are shown in table 16. These values are of the same order as those found in the previous experiment. The difference between the fractions is not significant, neither was a significant difference found between the same tissue fractions in the different experimental groups. Table 17 shows the values for the two enzyme-containing fractions, the supernatant and the intermediate mitochondrial (particulate) in the control, overhydrated and dehydrated groups.

b) Assays for oxytocin and vasopressin

Preliminary experiments were carried out to test the influence of the following factors on the pressor assay for vasopressin: (i) the time interval of injection on the response, (ii) the dose response relationship, (iii) the presence of possible interfering agents in the tissue of normal, dehydrated and overhydrated animals, and (iv) the precision of the assays.

i) Influence of frequency of injection on the response: 10 mU doses were injected in pairs at time intervals of 7 and 14 min and the responses compared. The total volume injected, as in all

Table 16

Average coefficient of variance of the titration in the nitrogen determinations and their distribution in each tissue fraction

Fraction	Coef. of variance	Range	Distribution				Total
			0%	1-5%	5-10%	10%	
Blank	4.7±6.0	0-22	5	6	1	3	15
Soluble	2.4±1.7	0-6.3	2	13	1	0	16
Particulate	3.8±4.4	0-15.6	5	7	3	2	17
Nuclear	6.3±6.5	2-10.7	0	1	0	1	2
Light mit.	6.3±6.2	4.3-10	0	2	1	1	4
Heavy mit.	0.6±1.2	0-2.4	3	1	0	1	4
Microsomes	1.7±2.4	0-3.4	1	1	0	0	2

Table 17

Average coefficient of variance of the nitrogen determinations in each enzyme containing tissue fraction in each experimental group

Expt. state	Blank		Supernatant		Particulate	
	Coef. of variance	Range	Coef. of variance	Range	Coef. of variance	Range
Control	4.7±6.0	0-22	2.14±1.37	0-4.6	4.71±5.08	0-15.6
Dehydrated	4.7±6.0	0-22	1.40±1.8	0-4.4	3.5 ± 2.4	0-6
Overhydrated	4.7±6.0	0-22	4.0 ± 2.4	1.4-6.5	3.7 ± 6.5	0-11
Stressed	-	-	-	-	-	-

assays, was 0.4 ml. Table 18 expresses the results obtained numerically, together with the percentage difference of the 14 min response compared to the 7 min response. These results show no systematic difference, thus indicating that the time of injection is not critical and that subsequent injections are valid when the pressure returns to approximately the pre-injection level.

ii) Dose response relationship: Doses ranging from 1-25 mU were injected into a pressor preparation prepared as described in the Methods Section. Table 19 shows the regression characteristics of the dose response and the log dose response curves. The log dose results are also shown graphically in figure 6. These results show that there is a linear relationship between the dose and response, at least between the limits 1-25 mU of vasopressin.

iii) Test for possible interfering agents: The finding that there was no significant difference between the two control incubations, one containing dona-

Table 18

Effect of time interval between injections on the pressor response to injections of 10 mU of pitressin

Time between injections	Response in mm Hg						
14 mins	30	25.5	22	22	20	19	15
7 mins	27.5	25	24	21	20	19	19
% difference in response	+8	+2	-9	+4.5	0	0	-21

Table 19

Regression characteristics of the dose and log dose response curve of pitressin on the rat blood pressure

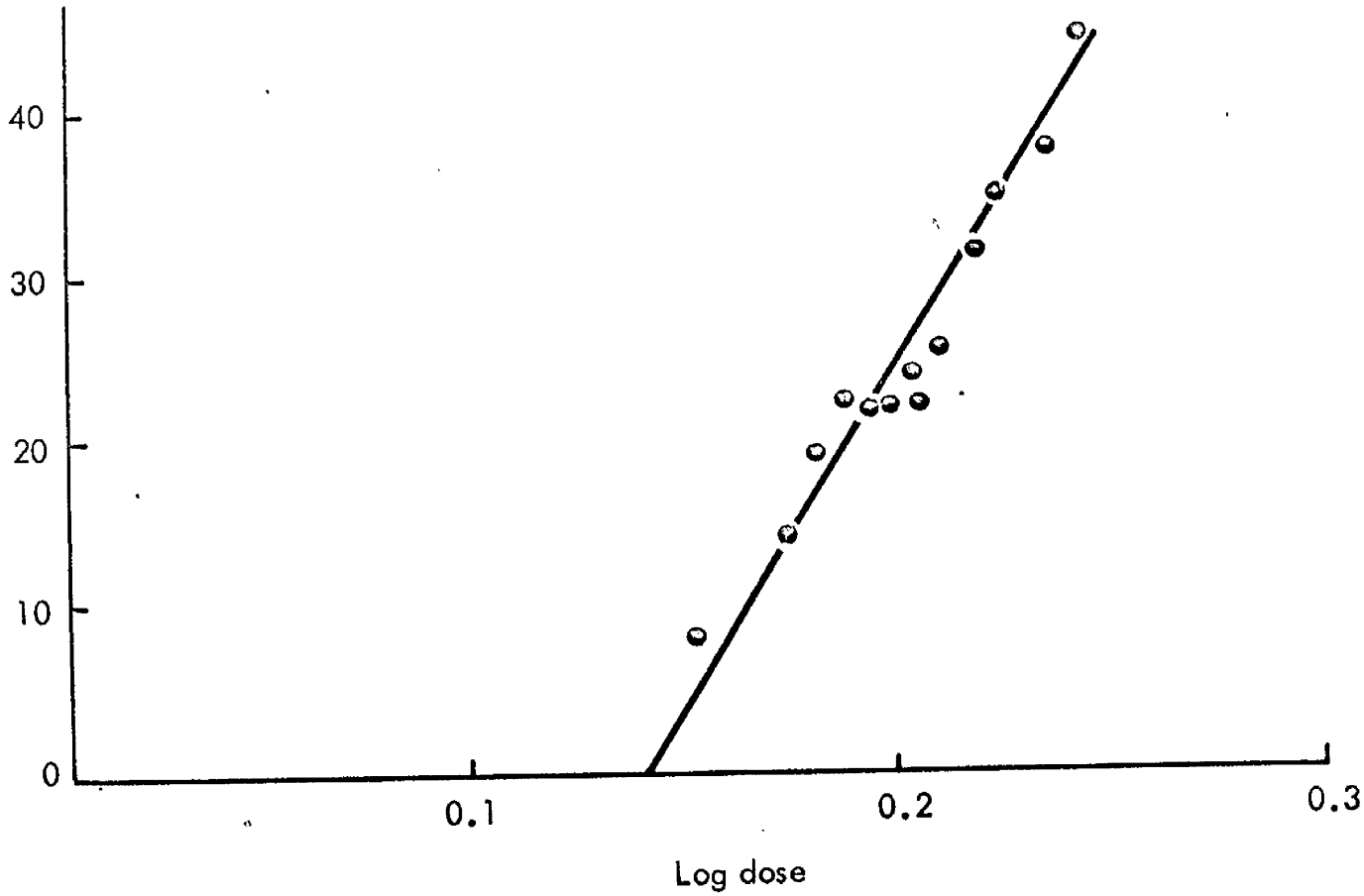
	Refression coefficient	Correlation coefficient	S.D. of regression	S.D. of regression coefficient	Significance of regression
Log dose response	3.998	0.961***	0.335	0.261	11.506***
Dose response	1.629	0.968***	0.3055	0.128	12.730***

** Significant at $P < 0.05$

*** Significant at $P < 0.01$

Figure 6

Log dose response of pitressin on the blood pressure of the rat



tured tissue extract and the other only buffer and hormone (see table 28), suggests that there is no heat stable agent present initially that interferes with the bioassay. This statement is true for the three experimental states: control, dehydrated and overhydrated. It is however possible that during the course of the incubations some endogenous material may have been modified, resulting in a heat stable agent possessing either pressor or depressor properties, or capable of blocking the action of vasopressin.

These possibilities were tested for by extracting the tissue as described in the Methods Section and incubating it as described again in the Methods Section, but with the exception that vasopressin was not added to the incubation media. The incubates, containing in the region of 100 µg/ml, were then denatured as described and tested for pressor or depressor effects. 0.25 ml of the sample was injected into the pressor preparation in each case and was washed in with 0.15 ml of saline, thus bringing the total volume to the standard 0.4 ml. The volume of the sample injected,

0.25 ml, was greater than that normally required for assay purposes.

The second possibility was tested for by adding a known amount of hormone to a known volume of sample and re-incubating for 3 hrs. The hormone was then estimated without further heating. Only the supernatant and particulate fractions were examined in the control group. Hooper (1966a) showed that the enzyme activity was confined to these fractions. In the dehydrated and overhydrated groups all the fractions were tested.

No indication of either a pressor or depressor agent was present in any of the fractions in any of the experimental states. Neither was there any indication of a heat stable agent interfering with the pressor effect of vasopressin being formed during the course of the incubation. The recoveries of the added hormone compared with the theoretical value were the same within the experimental error of the method. The results are shown in table 20 for the experiments using tissue from the control, dehydrated and overhydrated groups.

Table 20

Values of recovered hormone compared to the theoretical values in the test for interfering agents in the three groups

Tissue fraction	Control Group			Dehydrated Group			Overhydrated Group		
	Conc. N_2 µg/ml	Theor. value	Assayed value	Conc. N_2 µg/ml	Theor. value	Assayed value	Conc. µg/ml	Theor. value	Assayed value
Intermediate mitochondria	100	10 15	9.8±0.5 15.4±0.8	94	10 15	10.1±0.1 14.7±2.8	96	10 15	11.4±2.6 14.6±0.4
Supernatant	130	10 15	10.2±1.0 15.0±0.6	128	10 15	9.8±0.4 15.0±0.0	114	6 12	6.3±0.4 10.6±1.1
Light mitochondria	not tested			112	10 15	8.5±1.1 15.2±2.0	Lost		
Heavy mitochondria	not tested			-	-	-	Lost		
Nuclear	not tested			168	10 15	10.4±0.6 15.3±0.8	Lost		
Microsome	not tested			80	10 15	10.0±1.3 15.3±0.4	Lost		

iv) Precision of the assays: The average coefficient of variance in the supernatant and particulate fractions in each experimental condition are shown in tables 21-23. None of these differ significantly from each other.

Non-diffusible nitrogen content of the tissue fractions in the experimental states

The non-diffusible nitrogen/mg wet weight of tissue in the particulate and supernatant fractions in each of the experimental states is shown in tables 24 and 25. There is no significant difference between any of these. Tables 26 and 27 show the non-diffusible nitrogen in the remaining fractions in the dehydrated and overhydrated groups respectively. Unfortunately the values in the control group were not determined. Thus it is not possible to say whether they differ from the control group or not.

Enzyme activity in the experimental states

The average recoveries from the control incubations of the supernatant and particulate fractions in all the experimental conditions tested are shown in table 28. The recoveries in the oxytocin series are somewhat higher than in the vasopressin series. Vasopressin is known to be more unstable in alkali than oxytocin.

Table 21

Average coefficient of variance of the assays in the supernatant and particulate fractions from normal animals using oxytocin as substrate

Experimental group	Control		Definitive	
	Coefficient of variance	Range	Coefficient of variance	Range
Supernatant	10.5±1.5	9-12	8.1±4.5	3.7-15.8
Particulate	7.8±4.6	1-11.2	8.1±3.5	2.9-13

Table 22

Average coefficient of variance of the assays using the particulate fraction and vasopressin as substrate in all the experimental groups

Experimental group	Control		Definitive	
	Coefficient of variance	Range	Coefficient of variance	Range
Control	7.6±2.3	5.2-11	8.5±7.2	3.9±27.3
Dehydrated	10.1±3.3	5.9-14.5	6.6±5.2	1.2-22.6
Overhydrated	9.8±3.8	7.1-12.5	7.3±1.8	5.7-10.6
Stressed	-	-	-	-

Table 23

Average coefficient of variance of the assays using the soluble fraction and vasopressin as substrate in all the experimental states

Experimental group	Control		Definitive	
	Coefficient of variance	Range	Coefficient of variance	Range
Control	8.0±2.7	6.1- 9.9	8.8±2.8	6.2-13.7
Dehydrated	6.1±3.2	4.8- 8.4	11.2±6.7	5.1- 4.3
Overhydrated	10.7±2.3	9.1-12.3	6.6±1.9	4.5- 8.8
Stressed	-	-	-	-

Table 24

Non-diffusible nitrogen/mg wet wt in the particulate fraction in the control, dehydrated and overhydrated groups

State	No. of expts.	No. of animals	$\mu g\ N_2$/ mg wet wt	Range	Significance P
Control	8	30	1.29±0.78	0.52-2.74	-
Dehydrated	5	16	1.15±0.49	0.67-1.84	N.S.
Overhydrated	2	5	1.56±0.21	1.41-1.71	N.S.

Table 25

Non-diffusible nitrogen/mg wet wt in the soluble fraction in the control, dehydrated and overhydrated groups

State	No. of expts.	No. of animals	$\mu g\ N_2$ mg wet wt	Range	Significance P
Control	8	30	2.22±0.5	1.31–2.79	–
Dehydrated	5	16	2.35±0.54	1.76–3.15	N.S.
Overhydrated	2	5	1.86±0.76	1.32–2.39	N.S.

Table 26

Non-diffusible nitrogen/mg wet wt in the nuclear, light mitochondrial, heavy mitochondrial and the microsomal fractions in the dehydrated group

Tissue fraction	No. of expts.	No. of animals	$\mu g\ N_2$/ mg wet wt	Range
Nuclear	1	4	3.67	-
Light mitochondria	3	10	0.88±0.49	0.47-0.75
Heavy mitochondria	3	10	0.86±0.35	0.53-1.22
Microsomes	1	4	1.19	-

Table 27

Non-diffusible nitrogen/mg wet wt in the nuclear, light mitochondrial, heavy mitochondrial and the microsomal fractions in the overhydrated group

Tissue fraction	No. of expts.	No. of animals	/ug N_2/ mg wet wt	Range
Nuclear	-	-	-	-
Light mitochondria	1	4	1.29	-
Heavy mitochondria	1	4	1.23	-
Microsomes	1	4	0.95	-

Table 28

Recoveries of hormones in the two control incubations

Experimental group	Group	Recovery with tissue	Range	Recovery no tissue	Range	% Recovery with tissue	% Recovery no tissue extract
OXYTOCIN Control	Particulate	413±10	388-430	-	-	83%	-
	Supernatant	431±28	407-469	-	-	86%	-
VASOPRESSIN Control	Particulate	303±28	267-338	292±34	274-334	61%	54.4%
	Supernatant	351±38	319-384	356±26	330-370	70%	71%
Dehydrated	Particulate	265±47	227-322	250±25	214-283	53%	50%
	Supernatant	363±142	316-520	372±148	299-525	73%	74%
Overhydrated	Particulate	305±22	248-313	286±35	255-304	61%	57%
	Supernatant	277±34	260-313	273±25	245-308	55%	55%

a) Control animals using oxytocin and vasopressin as substrates

Table 29 and figure 7 show the results obtained using oxytocin as substrate. Table 30 and figure 8 show the results obtained using vasopressin as substrate. A comparison of these results indicates that the supernatant enzyme inactivates both hormones to approximately the same extent, whereas the particulate enzyme is much more effective at inactivating vasopressin than oxytocin. Vasopressin was inactivated to approximately the same extent as that achieved when using the supernatant enzyme. Table 31 shows the percentage change in regression coefficients and the adjusted means for 100 μg N_2 when vasopressin was used as a substrate in place of oxytocin. The increase in both in the particulate fraction is highly significant. The adjusted mean for 100 μg of non-diffusible nitrogen was calculated by first calculating the adjusted mean for the regression line as described by Snedecor (1956). This value was then adjusted to the corresponding value at 100 μg using the regression coefficient that had previously been determined.

Figure 7

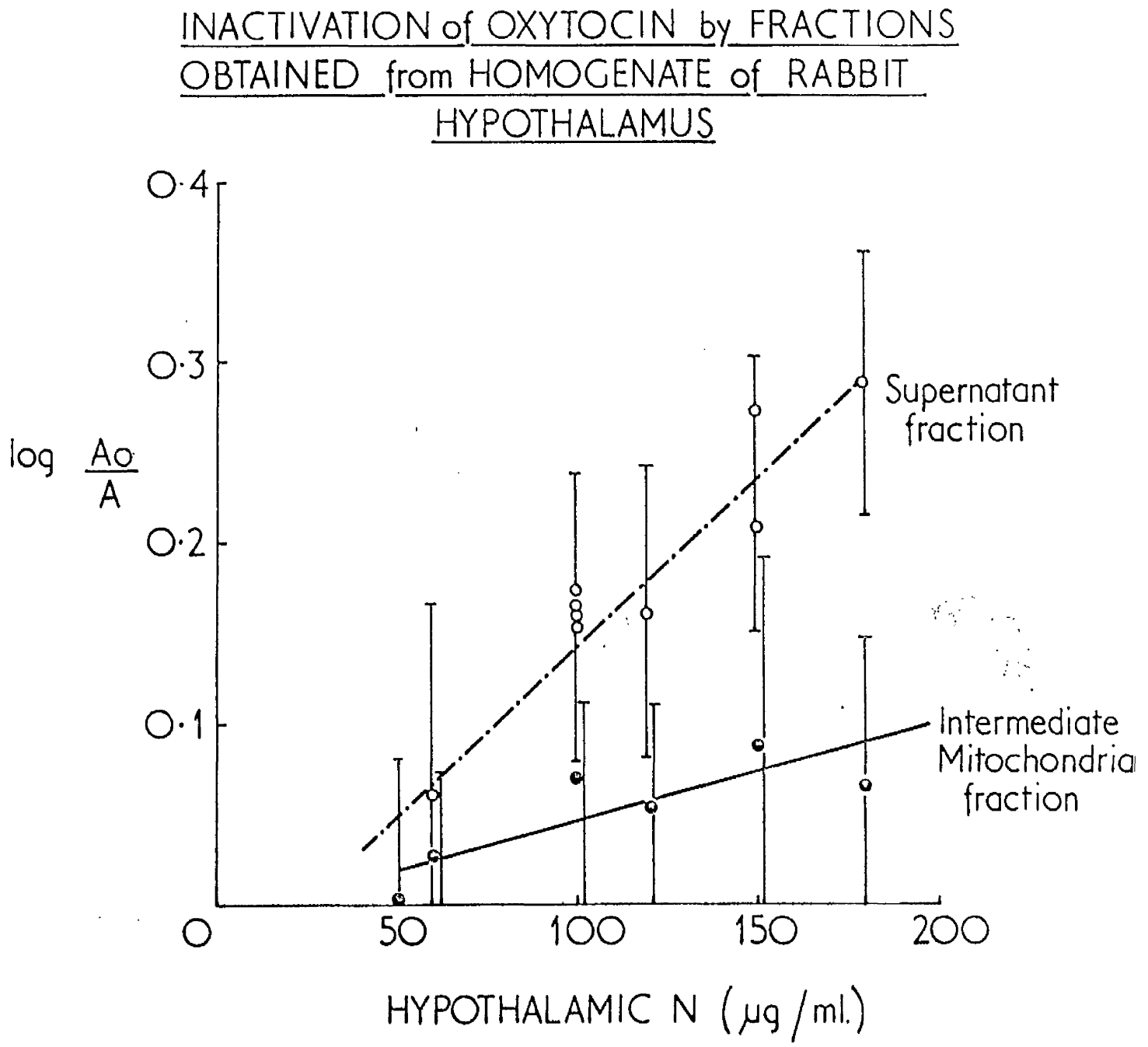

INACTIVATION of OXYTOCIN by FRACTIONS OBTAINED from HOMOGENATE of RABBIT HYPOTHALAMUS
0·4
0·3
0·2
0·1
log Ao/A
Supernatant fraction
Intermediate Mitochondria fraction
0
50
100
150
200
HYPOTHALAMIC N (µg/ml.)

Figure 8

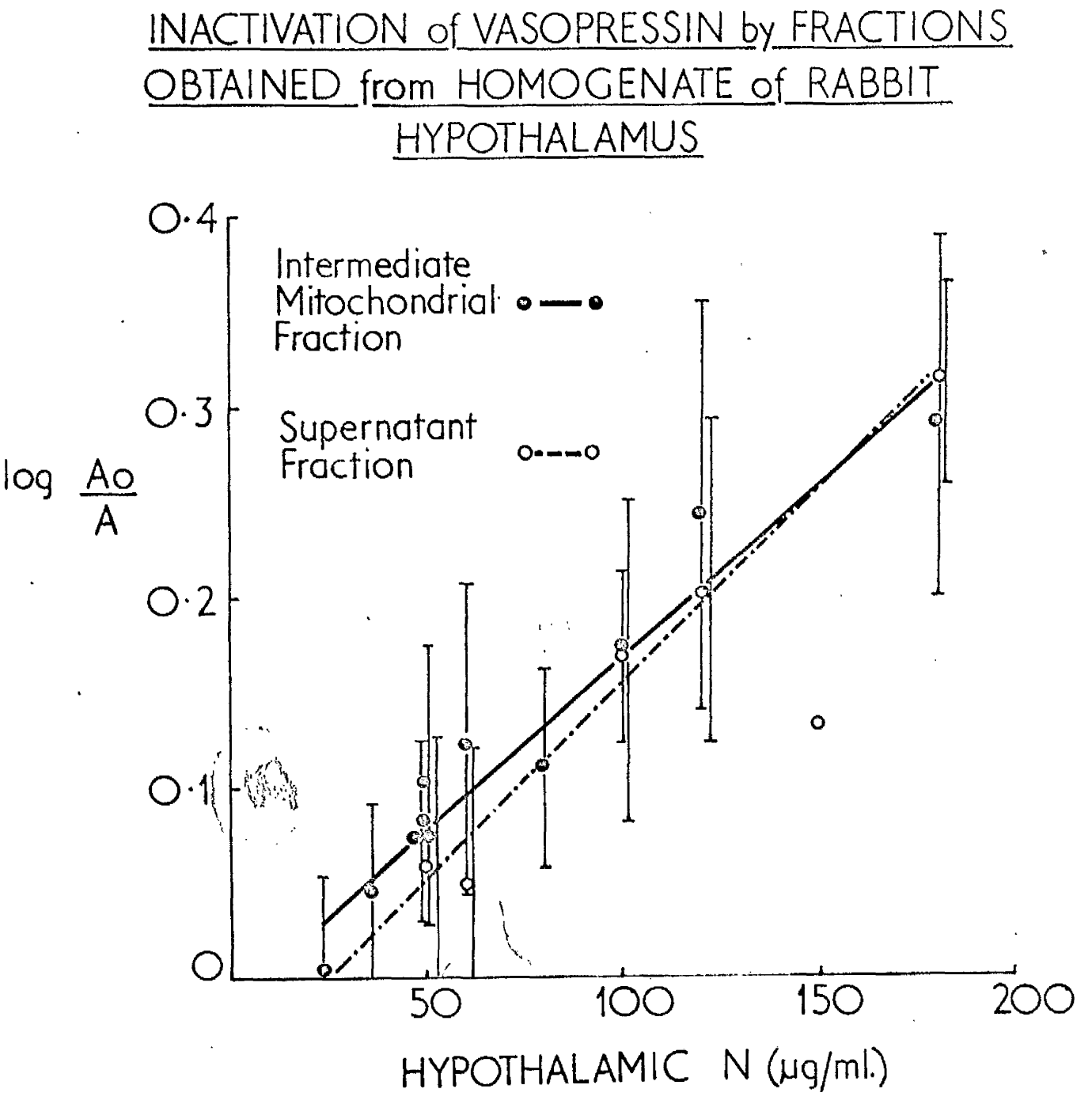

INACTIVATION of VASOPRESSIN by FRACTIONS OBTAINED from HOMOGENATE of RABBIT HYPOTHALAMUS
0·4
0·3
0·2
0·1
0
log Ao/A
Intermediate Mitochondrial Fraction
Supernatant Fraction
50
100
150
200
HYPOTHALAMIC N (µg/ml.)

Table 29

Regression characteristics of enzyme activity in control animals using oxytocin as substrate

Enzyme	Regression coefficient	Correlation coefficient	S.D of regression	S.D of regression coefficient	Adjusted mean 100μg
Supernatant	0.00183	0.991***	0.001	0.0001	0.139
Particulate	0.0004	0.735	0.024	0.0002	0.049

** Significant at $P < 0.05$

*** Significant at $P < 0.01$

Table 30

Regression characteristics of enzyme activity in control animals using vasopressin as substrate

Enzyme	Regression coefficient	Correlation coefficient	S.D of regression	S.D of regression coefficient	Adjusted mean 100/ g
Supernatant	0.00169	0.875***	0.00529	0.00046	0.166
Particulate	0.00183	0.971***	0.00223	0.00016	0.167

** Significant at $P < 0.05$

*** Significant at $P < 0.01$

Table 31

Percentage change in enzyme activity when vasopressin is used instead of oxytocin

Enzyme	% change of regression coefficient using ADH	Significance P	% change in adjusted mean using ADH	Significance P
Supernatant	-7.65	N.S.	+19.4	N.S.
Particulate	+4475	< 0.01	+244.2	< 0.01

b) Dehydrated and overhydrated animals using vasopressin as substrate

Table 32 and figure 9 show the supernatant enzyme activity in all three experimental states: control, dehydrated and overhydrated. In both the overhydrated and dehydrated groups the activity was approximately doubled. Table 33 records the percentage change in the regression coefficients and the adjusted means at 100 μg. In each case the increase was significant.

Figure 10 shows the enzyme activity in the particulate fraction in the control and dehydrated groups. It can be seen that the particulate bound enzyme activity is approximately doubled when small amounts, up to 70 μg of non-diffusible nitrogen, of tissue fraction were used. Above this concentration there is what appears to be a progressive decrease in enzyme activity. One possible explanation of this strange result is that present in this tissue is a metabolite with a higher affinity for the enzyme than vasopressin. If the graph is considered only up to the 70 μg point then both the regression coefficients and the adjusted means are significantly increased (see tables 34 and 35). Table 34 and figure 11 show the enzyme activity

Figure 9

INACTIVATION of VASOPRESSIN by SUPERNATANT FRACTION of RABBIT HYPOTHALAMUS from CONTROL, DEHYDRATED and OVERHYDRATED RABBITS

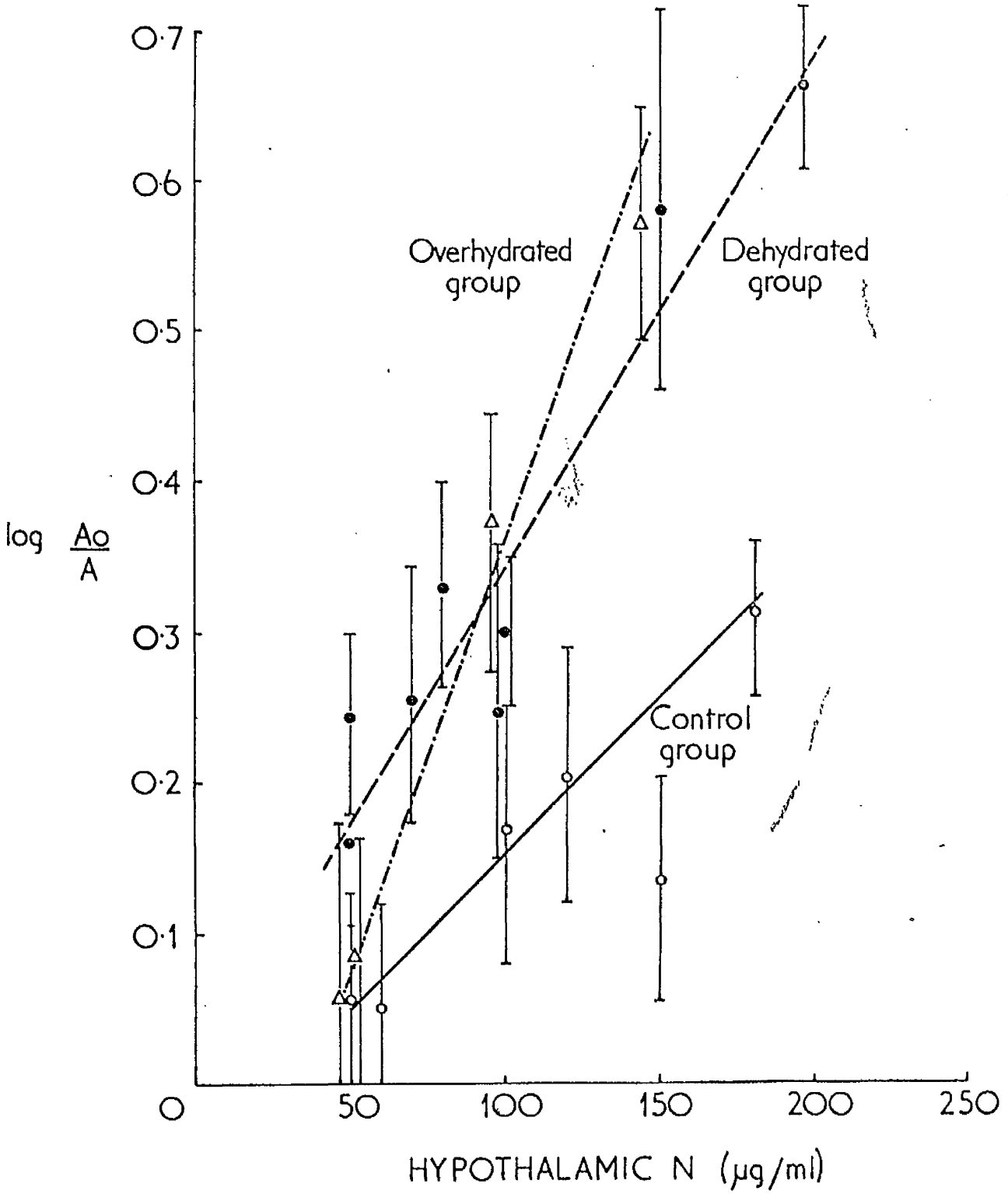


Table 32

Regression characteristics of the supernatant fraction in the control, dehydrated and overhydrated groups

Experimental groups	Regression coefficient	Correlation coefficient	S.D of regression	S.D of regression coefficient	Adjusted mean 100/g
Control	0.00169	0.8746**	0.00529	0.00046	0.166
Dehydrated	0.00342	0.9527***	0.00600	0.00046	0.35
Overhydrated	0.00534	0.9957***	0.00264	0.00033	0.352

** Significant at $P < 0.05$

*** Significant at $P < 0.01$

Table 33

Change in the regression coefficient and adjusted means in the supernatant enzyme in dehydrated and overhydrated groups from the control group

Experimental group	% change of regression coefficient	Significance P	% change of adjusted mean (100 μg N_2)	Significance P
Dehydrated	+102.4	< 0.01	+110.8	< 0.05
Overhydrated	+216.0	< 0.05	+112.0	< 0.01

Figure 10

INACTIVATION of VASOPRESSIN by INTERMEDIATE MITOCHONDRIA FRACTION of RABBIT HYPOTHALAMUS from DEHYDRATED RABBITS

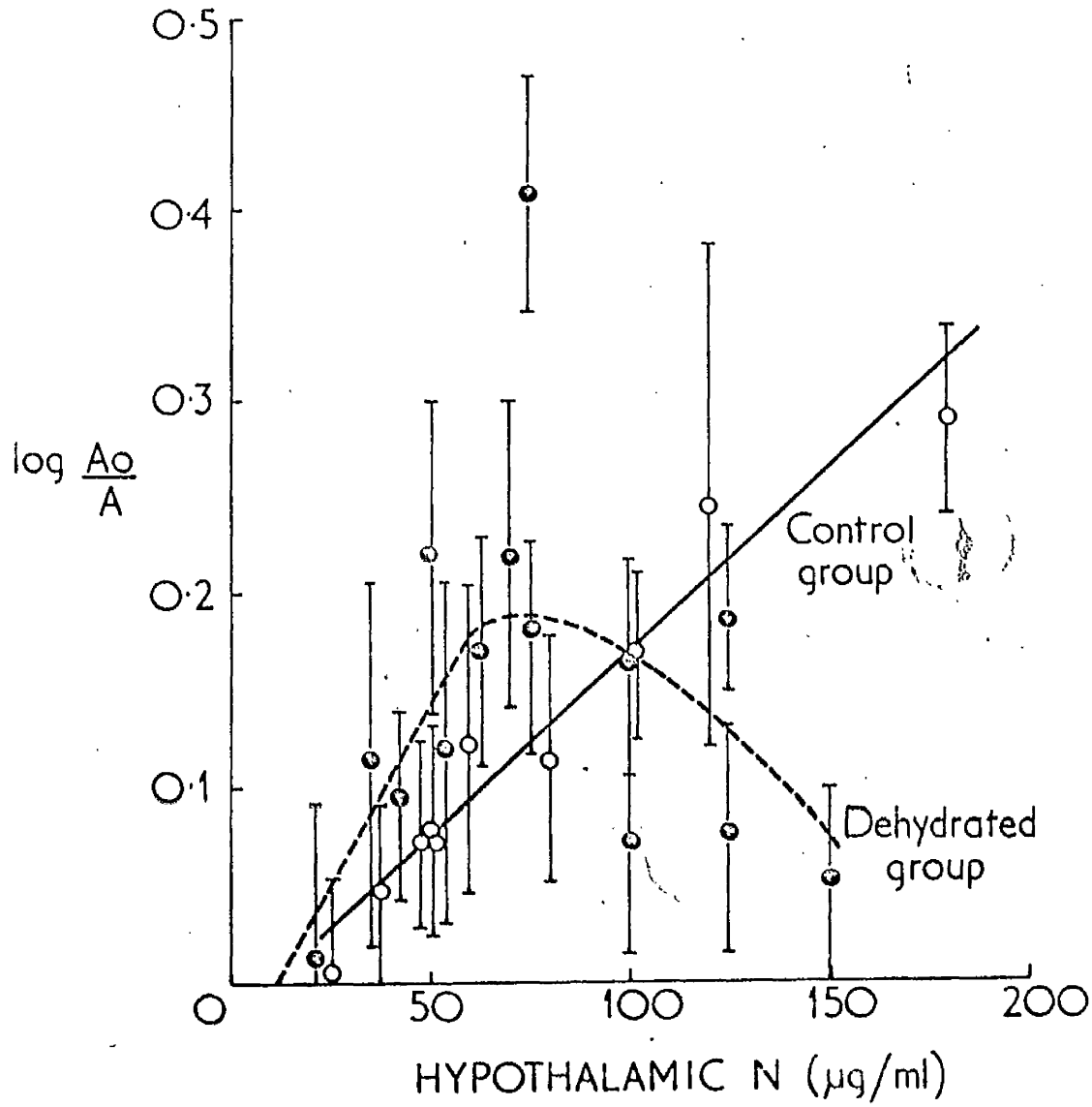


Figure 11

INACTIVATION of VASOPRESSIN by INTERMEDIATE MITOCHONDRIA FRACTION of RABBIT HYPOTHALAMUS from OVERHYDRATED RABBITS

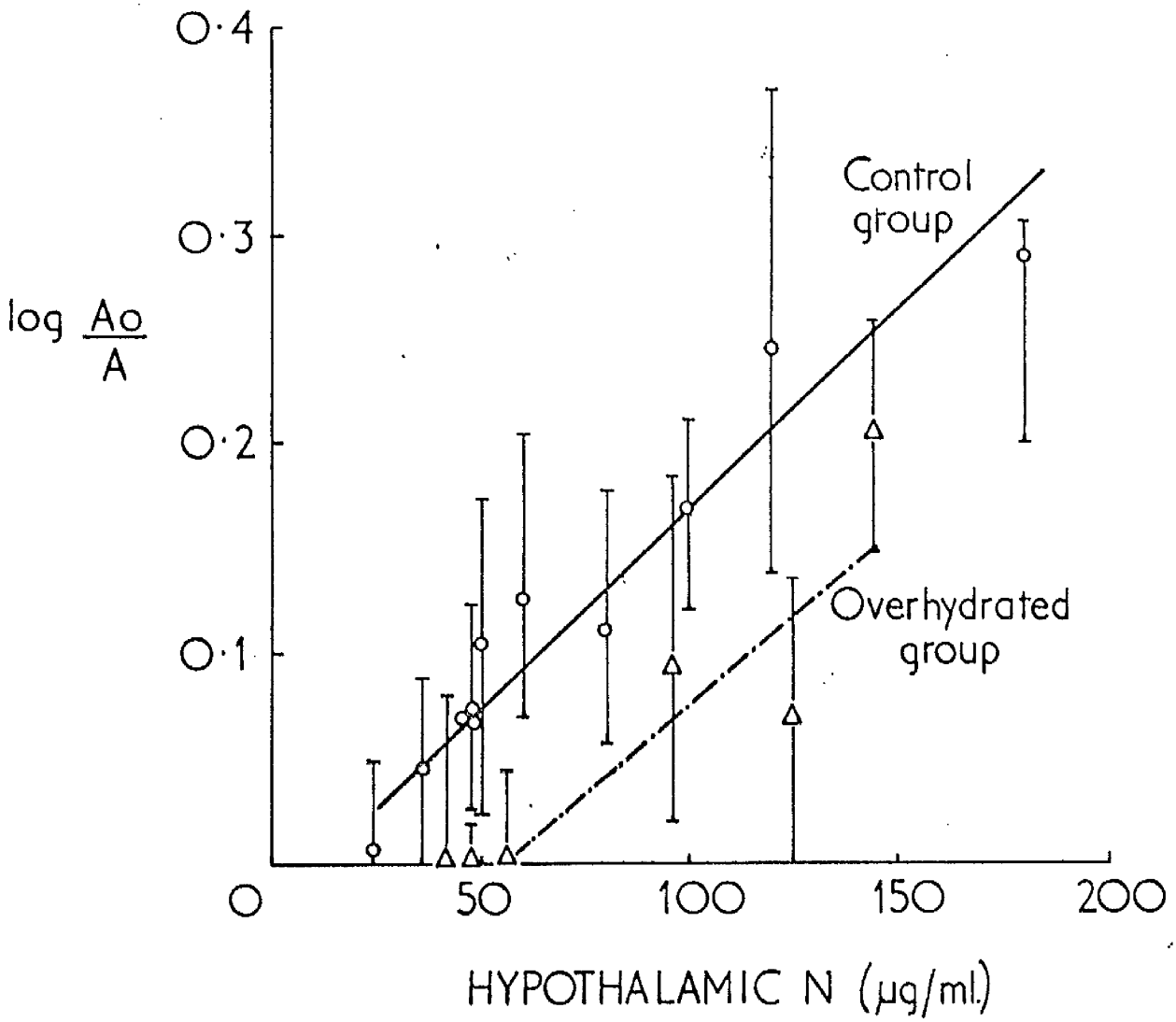


Table 34

Regression characteristics of the particulate fraction in the control, dehydrated and overhydrated groups

Experimental groups	Regression coefficient	Correlation coefficient	S.D of regression	S.D of regression coefficient	Adjusted mean 100µg
Control	0.00183	0.9711***	0.00223	0.00016	0.167
Dehydrated*	0.00374	0.842**	0.00435	0.00106	0.332
Overhydrated	0.00169	0.830	0.0050	0.00075	0.082

* Characteristics up to 70µg non-diffusible nitrogen

** Significant at $P < 0.05$

*** Significant at $P < 0.01$

Table 35

Change in the regression coefficients and adjusted means in the particulate enzyme in dehydrated and overhydrated groups from the control group

Experimental group	% change of regression coefficient	Significance P	% change of adjusted mean (100 μg N_2)	Significance P
Dehydrated	+104	<0.01	+99	<0.01
Overhydrated	- 8	N.S.	-50	<0.01

in the particulate fraction in the overhydrated and control groups. The regression coefficient does not differ significantly. The adjusted mean on the other hand is significantly reduced. It is approximately half that of the control (see table 35).

The nuclear, heavy and light mitochondrial and microsomal fractions were tested for enzyme activity. The enzyme activity in these fractions in the dehydrated group is shown in table 36. No regression characteristics are shown for the microsome fraction. It was possible to make only one estimation, at 80 µg non-diffusible nitrogen. Log A_o/A at this concentration was 0.03. This is in the same range as the results obtained in the other fractions. Effectively then there is no activity in these fractions.

In the overhydrated group the activity was determined only at one concentration. These findings are shown in table 37. From these it can be seen that there is virtually no activity in the light mitochondrial, heavy mitochondrial or the nuclear fractions. In the microsomal fraction the activity was 0.07 at a tissue concentration of 80 µg.

Table 36

Regression characteristics of enzyme activity of the heavy and light mitochondrial fraction etc. in dehydrated animals

Tissue fraction	Regression coefficient	Correlation coefficient	S.D of regression	S.D of regression coefficient	Adjusted mean at 100 μg N_2
Nuclear	0	0	0	0	0
Light mitochondria	0.00025	-0.946	0.00140	0.00086	0.03
Heavy mitochondria	0.00017	0.7679	0.00251	0.0001	0.031
Microsomes	-	-	-	-	-

** Significant at $P < 0.05$

*** Significant at $P < 0.01$

Table 37

Enzyme activity of the heavy and light mitochondrial fraction etc. in the overhydrated animals

Tissue fraction	Tissue concentration µg N_2/ml	Log $\frac{A}{A}o$
Nuclear	126	0.04
Light mitochondria	70	0.02
Heavy mitochondria	76	0.02
Microsomes	80	0.07

c) Stressed animals using vasopressin as substrate

Table 38 shows the enzyme activity in the stressed animals, while table 39 shows the percentage change from the control levels. These show that the particulate enzyme appears to be slightly elevated above the control level while the supernatant enzyme on the other hand is very considerably increased. The apparent increase in the particulate enzyme is not statistically significant. That of the supernatant enzyme, on the other hand, is.

Enzyme Activity in a Dog Diagnosed as Suffering from Diabetes Insipidus

Clinical history

Prior to an onset of excessive thirst, polyurea and occasional vomiting, the dog suffered from diarrhoea. This cleared soon after the onset of the symptoms of diabetes insipidus. The appetite was good and there was no vomiting, but some diarrhoea recurred later when the dog developed distemper pneumonia. The water intake was in the region of 7-8 l/day and the dog was incontinent. Administration of Pitressin 0.5 U three times a day reduced the water intake to 1 l/day by the fourth day of treatment and the water intake

Table 38

Regression characteristics of enzyme activity in over-hydrated and stressed animals

Enzyme	Rgeression coefficient	Correlation coefficient	S.D.of regression	S.D. of regression coefficient	Adjusted mean 100/g
Supernatant	0.0092	0.9950**	0.0396	0.0009	0.690
Particulate	0.0032	0.9942**	0.0245	0.0003	0.170

** Significant at P<0.05

** Significant at P<0.01

Table 39

Percentage changes in enzyme activity in stressed animals

Enzyme	% change of regression coefficient	Significance P	% change of adjusted means	Significance P
Supernatant	+ 400	< 0.05	+ 422	< 0.05
Particulate	+ 47	N.S.	+ 31	N.S.

fluctuated around this point (range 1-1.7 l/day). No other clinical or biochemical abnormalities were discovered and the dog did not show any deterioration in body condition until it developed distemper pneumonia.

Post-mortem examination

Distemper pneumonia and diabetes insipidus were diagnosed. There were no abnormalities of the brain, but there was a kidney lesion with paleness of colour and a few irregular white foci in the cortices. On histological examination some necrosis of the proximal convoluted tubules of the kidney was found.

Enzyme activity

Table 40 shows the results obtained. These show that some enzyme activity was present in the supernatant fraction. None was detected in any of the other fractions in the concentration used. Enzyme activity was normally found at these concentrations in the rabbit.

Table 40

Enzyme activity in the tissue fractions of the dog diagnosed as suffering from diabetes insipidus

Supernatant		Intermediate mitochondria		Light mitochondria		Heavy mitochondria		Nuclear		Microsomes	
µg N_2/ml	log $\frac{A}{A}o$	µg N_2/ml	log $\frac{A}{A}o$	µg N_2/ml	log $\frac{A}{A}o$	µg N_2/ml	log $\frac{A}{A}o$	µg N_2/ml	log $\frac{A}{A}o$	µg N_2/ml	log $\frac{A}{A}o$
50	0	50	0.03	80	0	60	0	48	0.02	-	-
100	0.09	100	0								

CHAPTER V

RESULTS OF BODY WATER METABOLISM DURING PREGNANCY

The results obtained in the experiments on enzyme activity in the experimental situations described in the last section suggested the possibility that the enzyme might be concerned in vasopressin metabolism and further that the ratio of the amounts of enzyme might be important in water metabolism. To test this hypothesis further, experiments were undertaken to measure changes in total body water and body water turnover, both pre- and during pregnancy. Two unsuccessful experiments were undertaken, one to measure plasma volume and one to measure plasma osmolarity.

Total Body Water/Kg Body Weight

This parameter was determined as described in the Methods Section. In each case at least three estimations were undertaken before mating, thus each animal acted as its own control. The results suggest an apparent increase occurring early in pregnancy, before five days, and remaining fairly constant throughout pregnancy. The average increase was 5.2 $\pm$ 5% with a range of -1.5 - +20.8. Five out of six animals showed

the same order and pattern of increase. These results are shown in table 41 and figures 12 and 13. During the post-partum period, in one animal (animal 1) suckling its young, the body water/kg body weight was below the pre-partum period at four days post-partum and further decreased nine days post-partum. It was still above the pre-pregnancy level at this time. In another animal (animal 3) that killed its young, the body water was -13% below the pre-pregnancy level at day four post-partum.

Body Water Turnover

In this case also determinations were made prior to pregnancy; thus each animal again acted as its own control. From a visual examination of the tritiated water plasma clearance curves the gestation period appeared to be divided into four parts: days 0-4, days 4-11, days 11-20 and day 20 to the end of pregnancy. The results indicated an apparent decrease during the first period, during the second period the turnover rate appeared to increase, it increased further during the third period, and finally in the fourth period it decreased. The regression coefficients, correlation coefficients and the statistical significances are shown in table 42. Table 43 shows the percentage changes

Table 41

Changes in body water/kg body weight during pregnancy

Time after mating in days	Identity of animals (No. 1-6)	% change in body water/kg	Significance P
+ 5	1	+ 2.9	<0.4
+ 6	4	+ 3.7	<0.05
+ 7	2	+ 7.3	<0.2
+ 8	5	+ 2.1	<0.2
+10	3	- 1.5	<0.5
+11	6	+ 4.5	<0.1
+16	4	+ 3.7	<0.2
+18	6	+ 4.2	<0.2
+19	5	+ 1.8	<0.3
+20	2	+ 7.4	<0.1
+21	1	+ 3.9	<0.4
+25	3	+20.8	<0.2
+27	4	+ 2.3	<0.2
+28	1	+ 7.9	<0.2
	2	+ 7.0	<0.4
	Postpartum		
+ 4	1	+ 6.0	<0.3
	3	-13.8	<0.1
+ 9	1	+ 4.0	<0.3

Figure 12

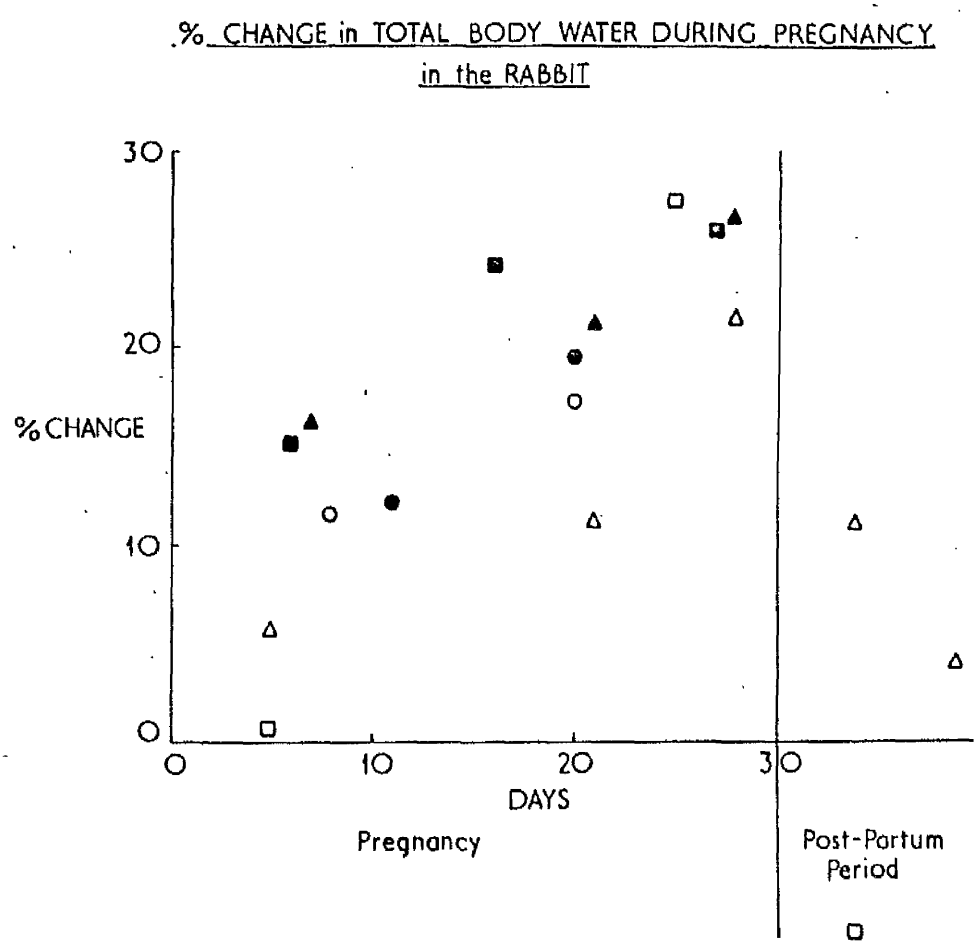
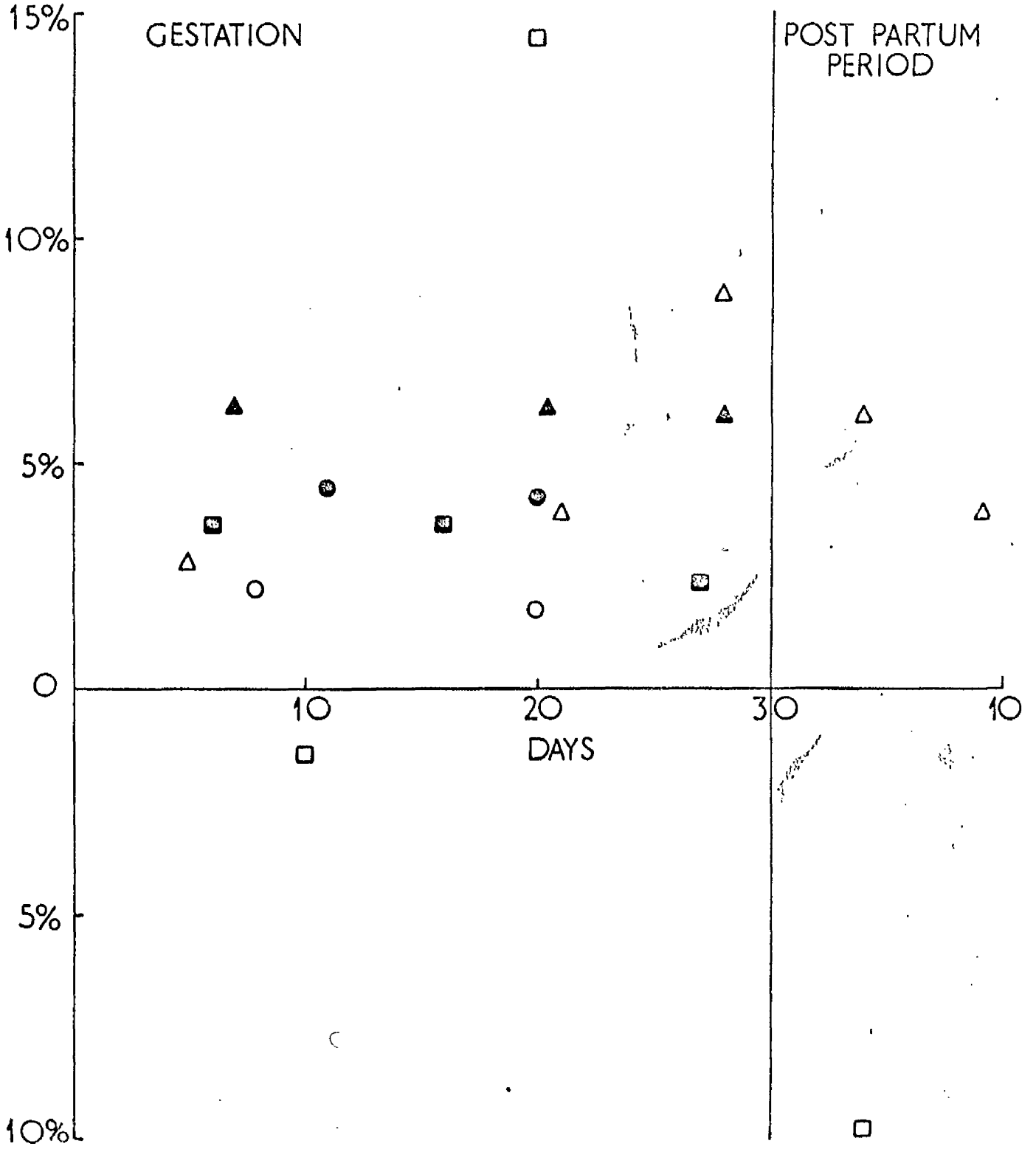

% CHANGE in TOTAL BODY WATER DURING PREGNANCY
in the RABBIT
30
20
10
0
% CHANGE
0
10
20
30
DAYS
Pregnancy
Post-Partum
Period

Figure 13

PERCENTAGE CHANGE of BODY WATER/Kg in RABBITS DURING PREGNANCY



from control value of the regression coefficients. Figures 14-19 show the individual regression lines obtained.

Plasma Volume

The above concludes the useful biological data accumulated. The remaining experiments were unsuccessful as far as this was concerned. However, one extremely useful piece of information concerning technique emerged during the first determination of plasma volume. The albumin was labelled as described and stored in bulk at -15°C. While determining the plasma volume in the pre-pregnancy period, it was found that each determination was greater than the former one. An aliquot of the albumin solution was dialysed and the dialysate monitored for radio-activity. None was found to have dialysed from the dialysis bag. Following precipitation of the protein with 20% T.C.A. only insignificant activity was present in the supernatant (<2%), the remainder being found in the precipitate. It is certain then that the label was not becoming detached from the protein. What would appear to be the case is that the freezing and thawing caused denaturation of some of the protein and that the animal was differentiating this protein and removing it from the circulation. A

Table 42

Regression characteristics of plasma tritium oxide clearance curves pre- and during pregnancy

Expt.	Animal	Control		Days 0-4		Days 4-11		Days 11-20		Days 20-28	
		Regr. coeff.	Corr. coeff.	Regr. coeff.	Corr. coeff.	Regr. coeff.	Corr. coeff.	Regr. coeff.	Corr. coeff.	Regr. coeff.	Corr. coeff.
1	1	-0.064*** ±0.003	-0.99***	-0.038 ±0.018	-0.73	-0.048*** ±0.008	-0.92***	-0.049*** ±0.012	-0.83***	-0.057	-0.96
1	2	-0.059*** ±0.002	-0.99***	-0.052 ±0.024	-0.76	-0.059 ±0.006	-0.88	-0.073*** ±0.007	-0.96***	-0.044 ±0.018	-0.91
1	3	-0.057*** ±0.002	-0.99***	-0.049 ±0.031	-0.67	-0.071*** ±0.022	-0.83**	-0.067*** ±0.008	-0.95***	-0.062 -	-0.92
2	2	-0.082*** ±0.002	-0.99***	-0.022 ±0.03	-0.59	-0.093 ±0.015	-0.89	-0.148 ±0.078	-0.85	-0.058** ±0.004	-0.99**
2	3	-0.066*** ±0.006	-0.97***	-0.023*** ±0.004	-0.99***	-0.069*** ±0.005	-0.99***	-0.087*** ±0.004	-0.99***	-0.049*** ±0.002	-0.99***
2	4	-0.087*** ±0.012	-0.98***	-0.046** ±0.011	-0.95**	-0.128*** ±0.004	-0.99***	-0.175*** ±0.007	-0.99***	-0.118 ±0.148	-0.62
Average		-0.069 ±0.012		-0.038 ±0.013		-0.078 ±0.029		-0.100 ±0.05		-0.065 ±0.027	

cont.../42

Table 42 cont...

** Significant at $P < 0.05$

*** Significant at $P < 0.01$

Experiment 1, A 1

Second period average of days 4- 7 (regr. coeff. -0.059 ± 0.066; corr. coeff. -0.66)
and days 7-12 (regr. coeff. $-0.060^{**} \pm 0.018$; corr. coeff. -0.88)

Experiment 2, A 2

Second period average of days 3- 6 (regr. coeff. $-0.093^{***} \pm 0.022$; corr. coeff. -0.99^{***})
and days 6-11 (regr. coeff. -0.093 ± 0.027; corr. coeff. -0.89^{**})

Experiment 2, A 4

Turnover during days 0-3 (regr. coeff. -0.029 ± 0.005; corr. coeff. -0.984)

Table 43

Percentage changes in regression coefficients of the plasma tritium oxide clearance curves during pregnancy

Expt.	Animal	% change days 0-4	Significance P	% change days 4-11	Significance P	% change days 11-20	Significance P	% change days 20-28	Significance P
1	1	-41	<0.05	-25	<0.05	-23	N.S.	-11	N.S.
1	2	-12	N.S.	0	N.S.	+24	<0.05	-25	N.S.
1	3	-16	N.S.	+23	N.S.	+18	N.S.	+ 9	N.S.
2	2	-30	<0.01	+12	3-6 N.S. 6-11<0.01	+65	11-15 N.S. 16-20<0.01	-29	<0.05
2	3	-65	<0.05	+ 6	N.S.	+33	N.S.	-25	N.S.
2	4	-67	N.S.	+49	<0.01	+100	<0.01	+35	N.S.
Average		-38±23	< 0.05	+11±42	N.S.	+36±42	N.S.	-8±25	N.S.

Figure 14

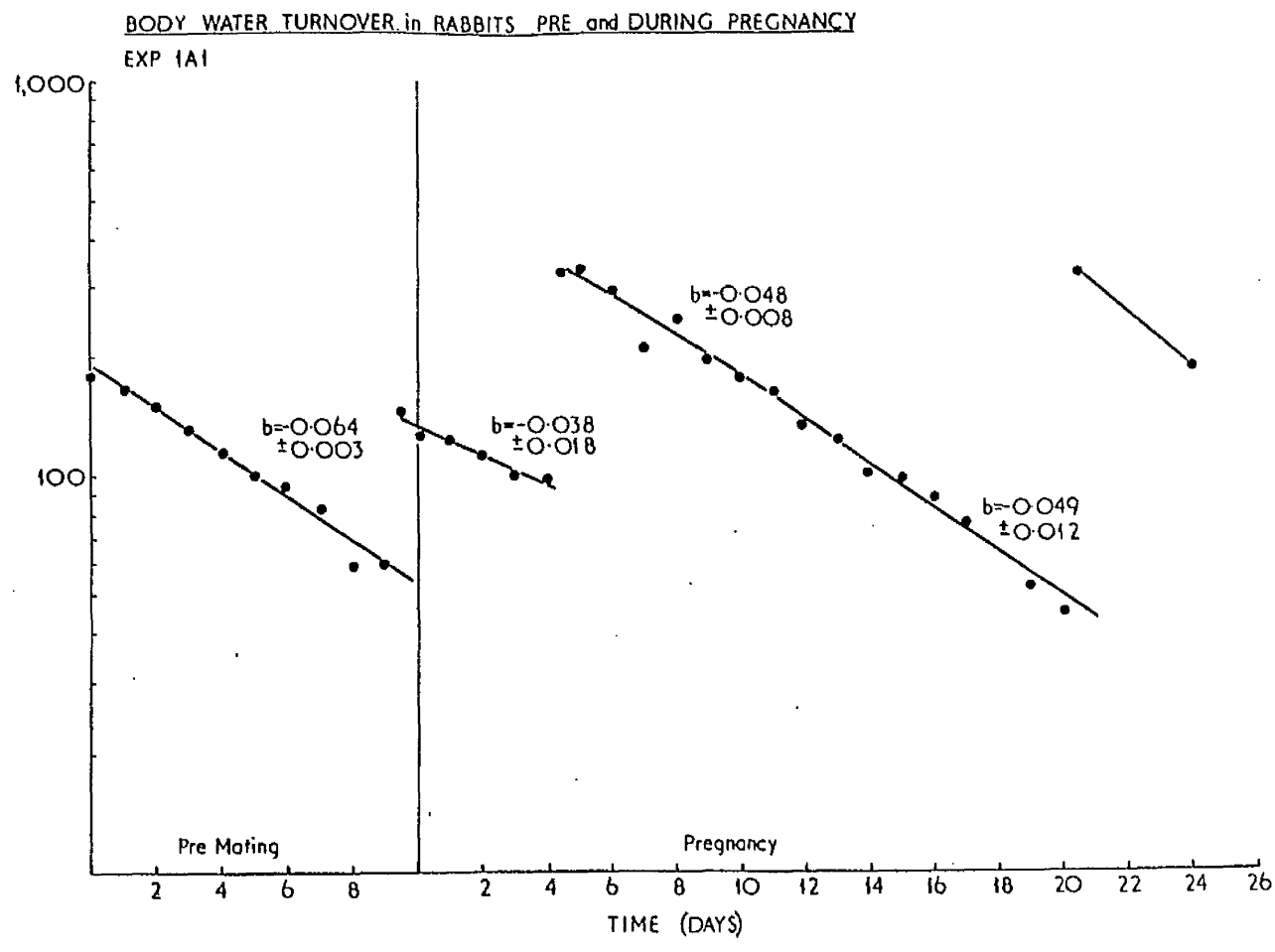


Figure 15

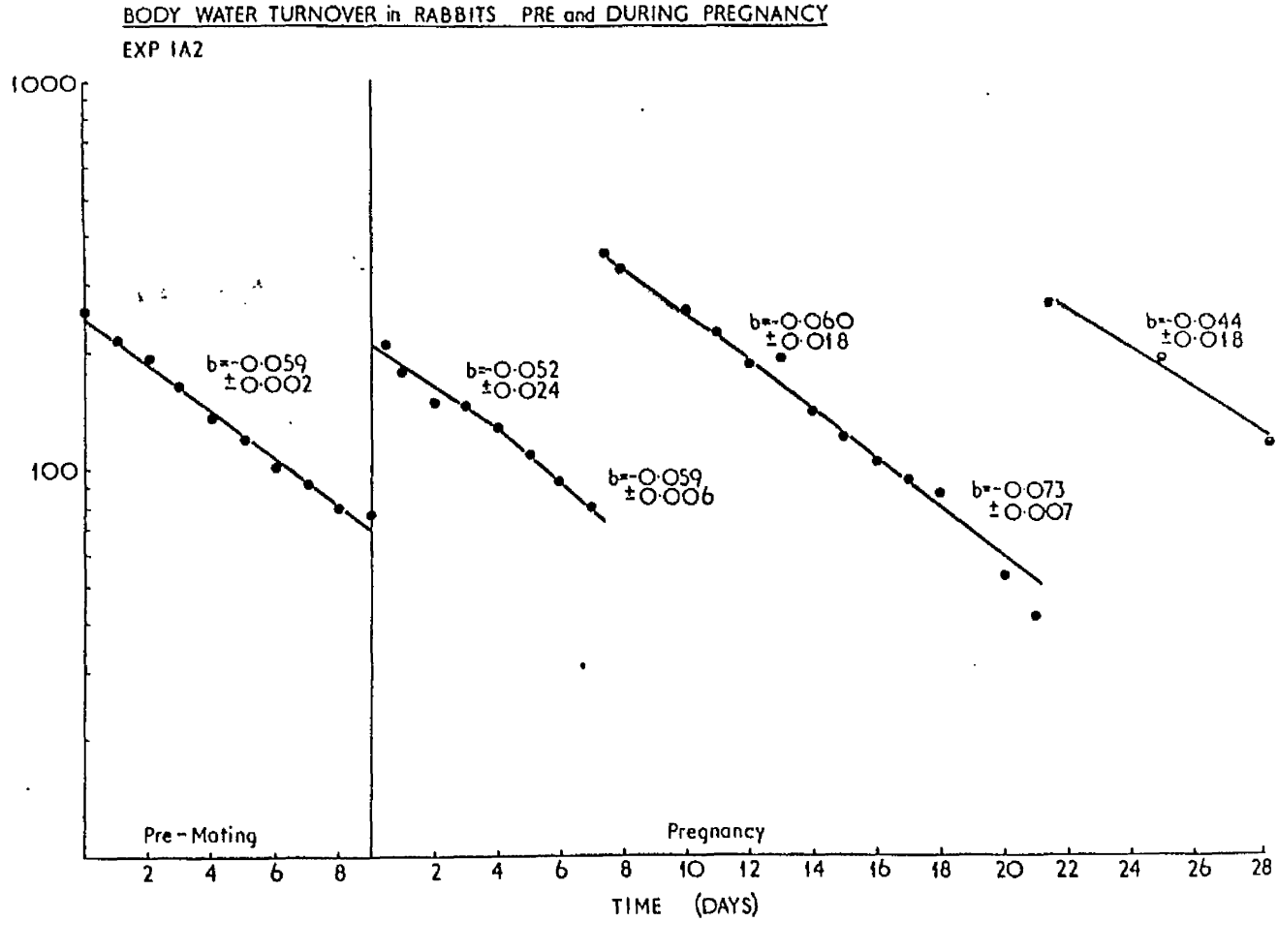


Figure 16

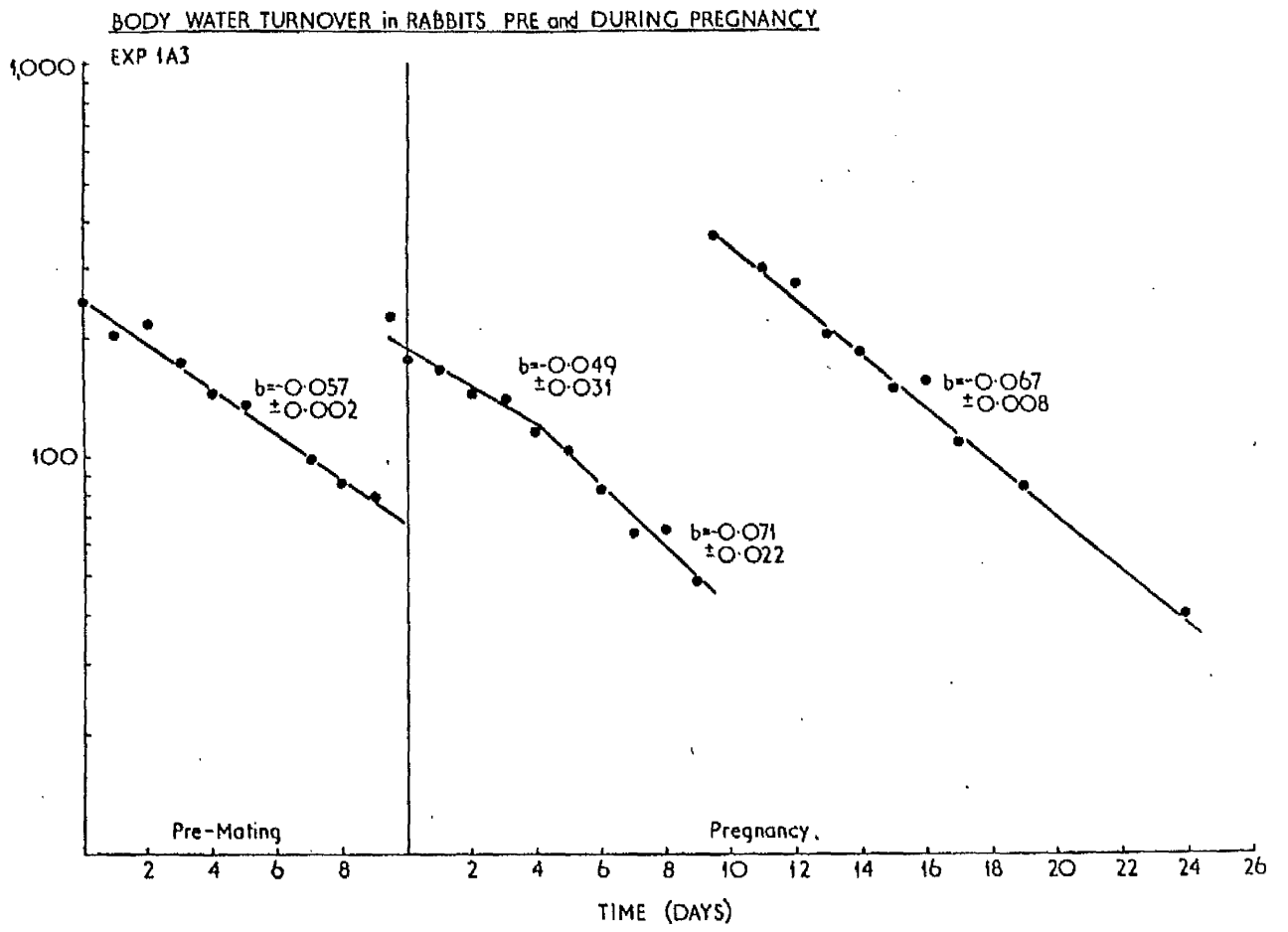


Figure 17

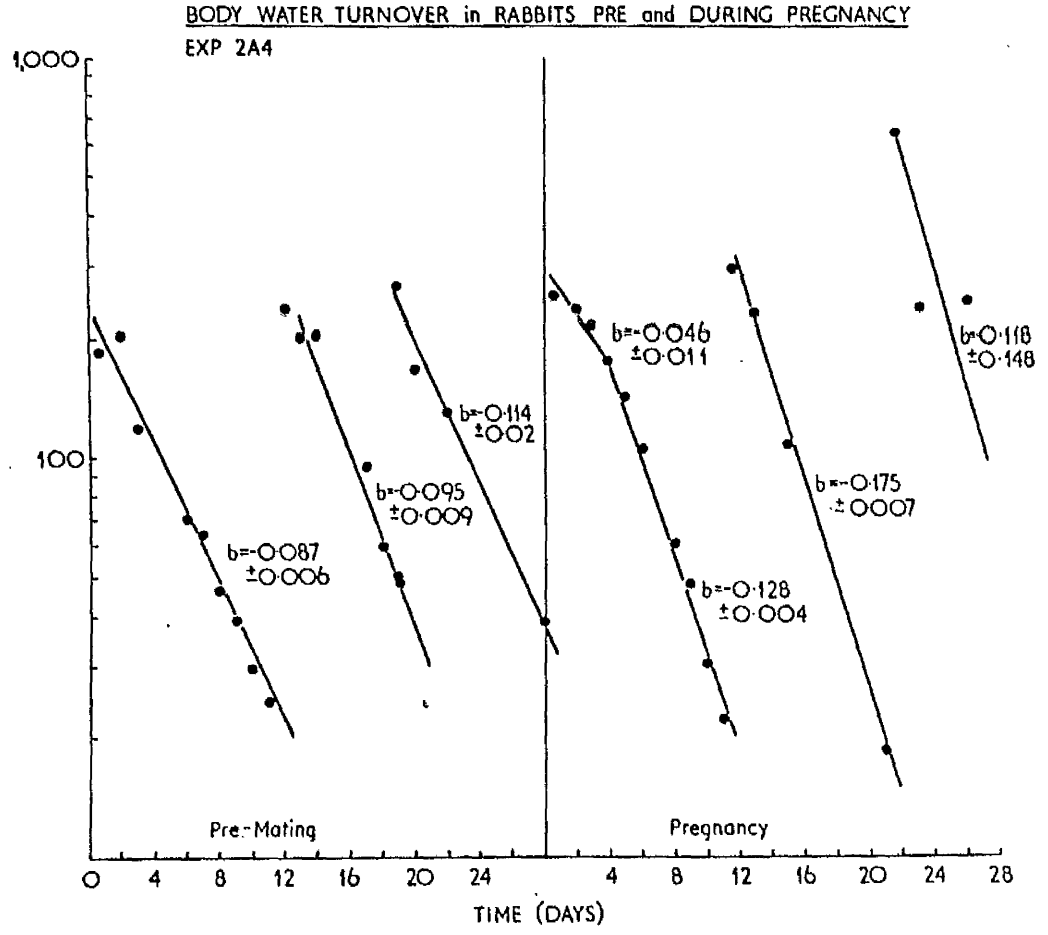


Figure 18

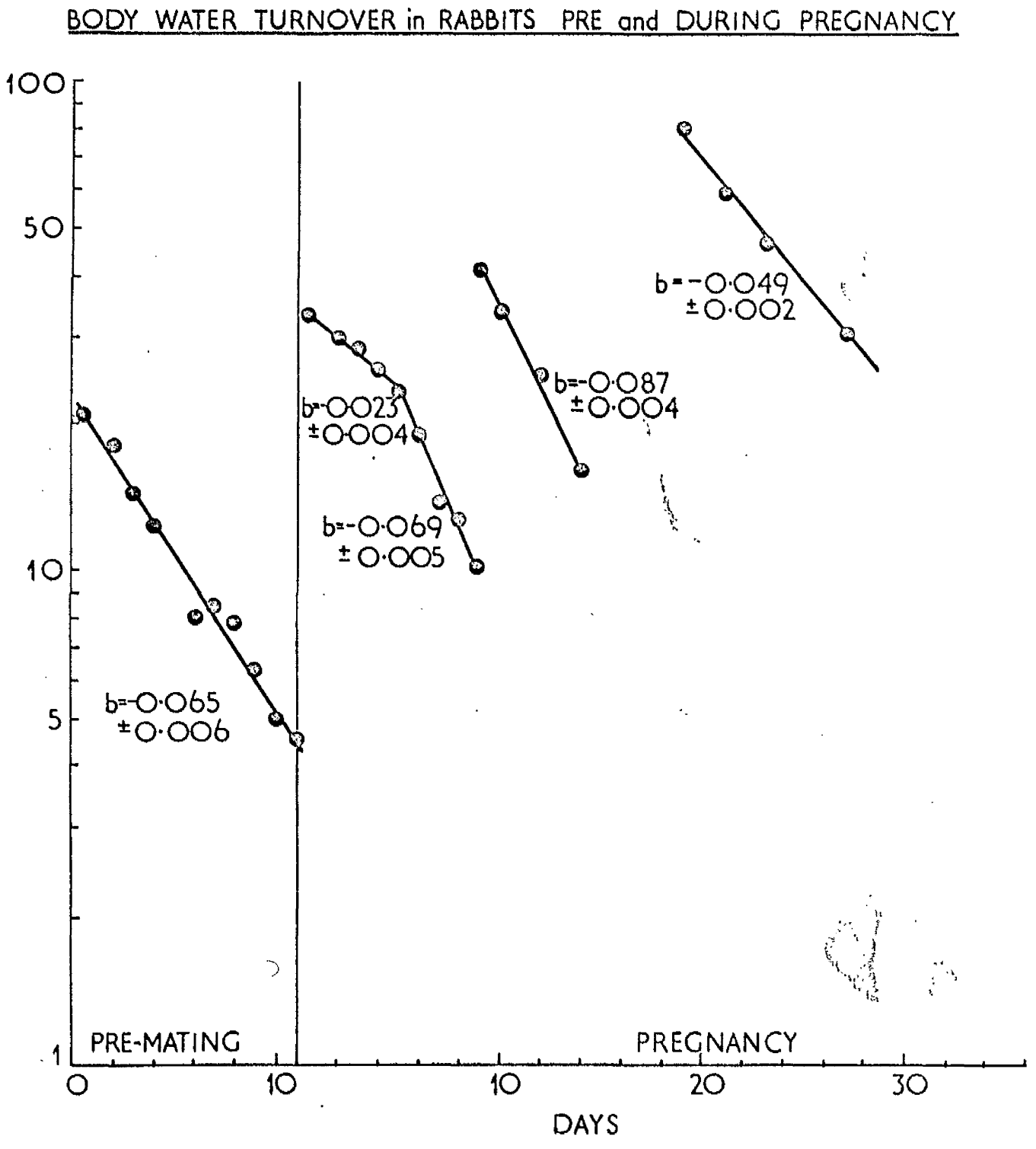
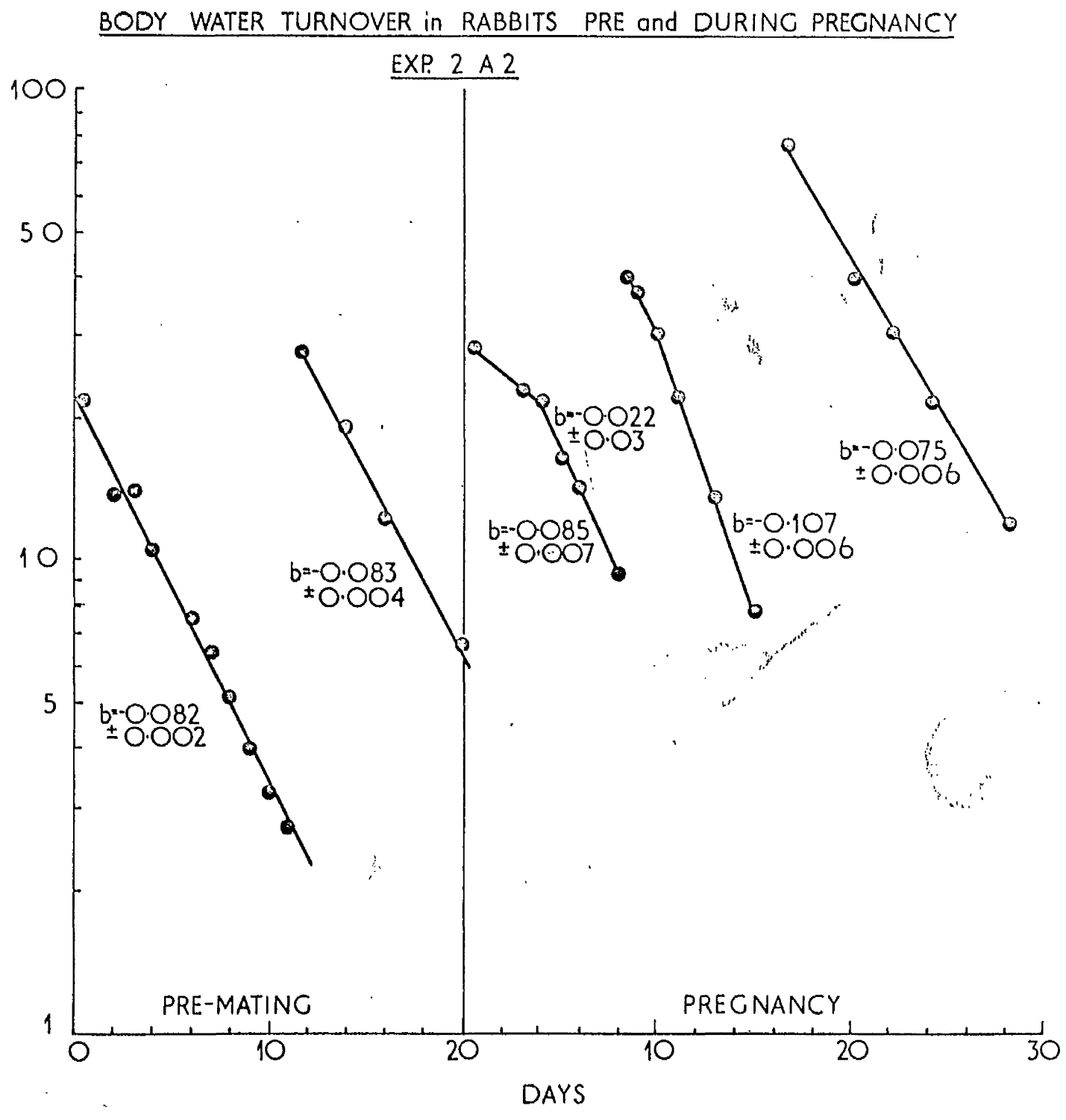

BODY WATER TURNOVER in RABBITS PRE and DURING PREGNANCY
100
50
10
5
1
b=-0·065
±0·006
b=-0·023
±0·004
b=-0·069
±0·005
b=-0·087
±0·004
b=-0·049
±0·002
PRE-MATING
PREGNANCY
0
10
10
20
30
DAYS

Figure 19


BODY WATER TURNOVER in RABBITS PRE and DURING PREGNANCY
EXP. 2 A 2
100
50
10
5
1
b=-0·082 ±0·002
b=-0·083 ±0·004
b=-0·022 ±0·03
b=-0085 ±0·007
b=-0·107 ±0·006
b=-0·075 ±0·006
PRE-MATING
PREGNANCY
0
10
20
10
20
30
DAYS

new batch of protein was then labelled and stored at the same temperature, but this time in small aliquots. Each sample was thus only frozen and thawed once before being used to determine the plasma volume. The results of repeated determinations on animals showed good reproducoability, the determinations invariably being within ± 4%.

The conclusion that freezing and thawing causes some change in the protein which the rabbit can detect and remove from the circulation seems inescapable. Thus in using labelled albumin it is essential to use the technique of storing it in small aliquots so that the material will only be frozen and thawed once, unless a fresh batch is made for each day's determination.

It is unfortunate that having resolved the problem described above, the animals in the second experiment were not fertilized on mating. The results are thus of no use in this context since there appears to be no information concerning the enzyme activity during pseudo-pregnancy. The results are therefore not included. This is also the case with the plasma osmolarities.

Table 44 shows the results obtained in repeated determinations of plasma volume on the same animals using labelled albumin which was stored in bulk, thus involving repeated freezing and thawing, and albumin that was stored in small aliquots which entailed only one freezing and thawing before the determination was made.

Table 44

Comparison of repeated estimations of plasma volume/kg body weight of normal animals using (1) labelled albumin that had been stored in bulk, thus involving repeated freezings and thawings and (2) labelled albumin that had been stored in small aloquots. Both preparations were used in the same animals but at a time interval of one month. Figures in brackets are the percentage discrepancy between successive estimations

Estimates	ANIMALS							
	A Preparations		B Preparations		C Preparations		D Preparations	
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
1	28.1	29.6	23.4	26.6	29.6	29.6	27.3	31.0
2	35.9(27)	29.6(0)	25.3(8)	27.8(4)	36.8(25)	30.5(3)	35.9(31)	33.1(6)
3	38.7(10)	27.8(6)	33.4(23)	28.5(1)	40.4(12)	31.2(3)	46.0(28)	30.7(7)
4	-	-	38.9(16)	28.5(0)	-	-	-	-

CHAPTER VI

DISCUSSION

General Considerations

It can be seen from what has been stated in the general introduction that basically the situation which presents itself is that the homogenate of dog hypothalamus possesses the ability to inactivate enzymically oxytocin and vasopressin, oxytocin being inactivated to a greater extent than vasopressin. Following cellular fractionation, both the supernatant and the mitochondrial fractions of brain tissue were able to inactivate oxytocin enzymically. Oxytocin only was used as substrate in those experiments and it was found that the supernatant fraction was 3 times as effective as the particulate. An analogous situation was found in the rabbit.

In order to evaluate the significance and possible functions of these enzymes, it is necessary to discuss three basic and crucial questions. The first is whether the enzyme action measured in the *in vitro* system used in the experiments is a genuine reflection of an important *in vivo* system. This question must be asked since

enzymes are notoriously delicate substances, the nature of their action being very easily alterable by bio-chemical manoeuvers. The procedures used in the experiments described above were exceedingly mild, involving only homogenization, centrifugation and dialysis, all of which were performed at $4^{o}C$. Because of this it would seem reasonable to assume that the enzyme activities found in these systems reflect biological activities and not artifacts created by altered enzyme characteristics brought about by the technique.

One change is possible, namely that the enzyme action may be proceeding in the reverse direction to that present <u>in vivo</u>. This could be due to the obviously altered dynamic equilibrium in the <u>in vitro</u> system.

The second question is whether the enzyme activity measured is an index of a specific physiological phenomenon or whether it is unspecific. It is unfortunate that enzymes are not absolutely specific. It is a common occurrence for an enzyme to interact less effectively with a substrate that is similar to but not identical with its natural substrate. The question that concerns us here is whether the enzyme activity measured is the measure of a specific enzyme, in which

case the measured activity would give an index of some specific physiological phenomenon and changes in measured enzyme activity would signify changes in that phenomenon; or whether the activity is a result of many enzymes involved in different processes interacting with the neurohypophysial hormones because of a superficial similarity between them and their natural substrates. If the latter is the case then the measured enzyme activity would be of dubious significance.

To attempt to evaluate this question, the early work of Hooper (1962) must be referred to. He found from an examination of the enzyme kinetics using dog hypothalamus homogenate that the enzyme characteristics did not follow precisely the behaviour predicted by first-order kinetics. The characteristics, however, did not differ greatly from those predicted. This suggests that even when both enzymes are used together, the enzyme system inactivating the neurohypophysial hormones is relatively simple, in all probability involving few enzymes and it is likely that only the two enzymes described above are involved.

Other findings infer that we are dealing with a specific enzyme system. These are the restricted intracellular distribution of the enzymes and that bradykinin and substance P, two peptides normally found in brain tissue, are inactivated by different enzymes or enzyme systems. Moreover the enzymes are not universally present in all brain tissue and lastly the distribution of the enzymes inactivating the neurohypophysial hormones differs from that of those inactivating bradykinin and substance P. These observations tend to suggest that the enzymes have specific functions, that the activities measured are not a consequence of unspecific activity, and that it is possible that the activity offers an index of some physiological phenomenon.

It is unfortunate that with the present state of knowledge no conclusive answer can be given to this question.

If the enzyme activity in each fraction is not a consequence of a specific enzyme system, then the work reported here is of doubtful biological value. It is the uncertainty surrounding this question that is the

weak point in the work and any conclusions drawn from the results.

If it is the case that the enzyme activity measures the activity of a specific enzyme, then the third question that must be asked is: what is the natural substrate for the enzyme? Until this question is answered, data concerning changes in enzyme activity, will be, if the assumption made in the second question is correct, a valid index of some changing physiological phenomenon; but what that phenomenon might be would be doubtful as would the nature of a mediating agent, if any. It is with this question that the work reported here is mainly concerned. It is probably true to say that the more certain the answer to this question becomes, the greater becomes the credulity that can be given to suggestions that the enzyme activities are a result of specific enzymes.

This question would best be resolved by the difficult and complex process of isolating the enzymes in a pure state and then characterizing the nature of the enzymic action. This has not been done so far. The approach used was more indirect. As stated in the general introduction, there is evidence that suggests

a possible involvement of the enzyme and oxytocin metabolism. The approach adopted to examine this possibility further was to test the enzyme activity in physiological and experimental conditions where there are known or probable changes in neurohypophysial hormone metabolism, and to correlate the enzyme changes with the hormone alterations. The limitation of this approach and a criticism that can be validly made is the certainty that every situation, physiological or experimental to which an animal is subjected causes changes, not in one physiological system alone, but in many. This must apply to the experimental conditions here; changes that are known and no doubt some that are unknown occur, together with changes in the metabolism of the neurohypophysial hormones. This complicates considerably the interpretation of the data. Consequently any conclusions must be offered tentatively.

It is in the light of these limitations that this work must be considered, most importantly the doubt concerning the specificity of the enzymes, but also the equivocal nature of the probable natural substrates of the enzymes. The doubt concerning the nature of the

natural substrate of the enzymes will be reduced the more instances of correlation that are found.

If a correlation not previously suspected should be found between the enzyme activities and a physiological phenomenon, and if administration of the agent with which the enzymes are suspected to be involved should alter that phenomenon in the direction implied by the correlation, then this would provide convincing evidence in favour of the involvement of the enzymes with that agent. This situation has in part been encountered with regard to enzyme activity and water diuresis. Unfortunately attempts to induce the suspected changes with the suspected agent are as yet incomplete. This will be discussed in more detail later.

Before the experimental results are discussed, it is appropriate here to deal with some general considerations concerning a possible correlation between the enzymes and a hormone metabolism. If the hypothesis that the enzymes are genuinely involved in the metabolism of oxytocin or any other hormone is correct, then changes in enzyme activity must either initiate changes in the synthesis and/or secretion of the hormones concerned, or alternatively changes in synthesis

or secretion must initiate changes in enzyme activity. If the former situation exists, then it would be expected that the enzymes would be located at the site of synthesis and release. In the case of the neurohypophysial hormone this is so; the hormones are synthesised in two hypothalamic nuclei, the paraventricular nucleus and the supraoptic nucleus, the enzymes being present in highest concentration in the hypothalamus. However, the enzymes are not confined to the hypothalamus, but are to be found, in lesser concentration, in other regions of the brain in dog (Hooper, 1963). This does not prove but nevertheless suggests strongly that if there is a direct functional relationship between hormone and enzyme, the secretion of the hormone alters enzyme activity rather than vice versa; for it does seem unlikely that the secretion or synthesis should be initiated by alterations in enzymes distributed throughout the brain.

It is significant that a close examination of the results reported here as well as Hooper's reveals that the enzymes in the two fractions do not behave identically when undergoing changes. Both enzymes were elevated during pregnancy, the particulate 3 times and

the supernatant only slightly (Hooper, 1966a). During the post-partum period it was the supernatant enzyme only which showed peak activity, and similarly during lactation it was again this fraction that was maintained at elevated levels as long as lactation continued, the particulate enzyme activity declining to control levels (Hooper, 1966b). Similarly it was found that the enzymes behaved differently in the experiments involving the injection of steroids, ovariectomy (Hooper, 1968; Firth and Hooper, 1968) and in particular during overhydration. In the latter case the behaviour of the enzymes was diametrically opposed.

These findings suggest that the two enzymes are not involved with the same phenomenon, namely oxytocin secretion, but that the enzymes are involved with different phenomena and should be treated as separate and distinct entities.

This contradicts the interpretation put forward by Hooper (1964, 1966 a, b). He suggests that both enzymes are involved in the metabolism of oxytocin. Again in 1968, Hooper and Firth implied that both enzymes are involved in the same process, this time the metabolism of gonadotrophic releasing factors and in

particular the factor involved in the release of luteinizing hormone.

If it is accepted that the enzymes are separate entities and the first hypothesis is considered, it can be seen from Hooper's results (1966b) that the supernatant enzyme follows the established secretory pattern of oxytocin more closely than the particulate bound enzyme. Consequently it would appear that it is the supernatant enzyme that is most probably involved in oxytocin metabolism.

Following from this, the function of the particulate enzyme must now be considered. A number of observations suggest that it may be concerned in vasopressin metabolism; it does not, for example, inactivate oxytocin as effectively as the supernatant enzyme, which suggests that the enzyme may be primarily concerned in vasopressin metabolism rather than with oxytocin, and the noticeable inactivation of oxytocin by the enzyme is a consequence of the very similar structure of the two hormones.

The changes in this enzyme during pregnancy and its return to control levels following parturition

could well be due to changes in water metabolism present during pregnancy, and which also return towards normal after parturition (see Hytten and Liech, 1963). A suggestive observation which indicates that the particulate enzyme is concerned with vasopressin metabolism and the supernatant enzyme with oxytocin, is the finding that oxytocinases in many tissues are found in the supernatant fractions of tissue homogenates; and vasopressinases are found in the particulate fractions or vice versa (see Heller and Ginsburg, 1966).

The experiments reported in Chapter IV of this thesis were designed to test this hypothesis. It was felt that the work of Hooper (1966 a, b) upon which this hypothesis draws heavily for its inception and support, suffers from the disadvantage that pregnancy and lactation are two somewhat dramatic conditions involving changes in almost every system in the body. His further work involved the effects of ovariectomy and injected steroids in intact and ovariectomized animals. It is never certain, when drugs or hormones are injected into preparations, whether a physiological or pharmacological response is obtained; moreover

in the latter case it is not known what effect the injected material has on other systems.

Enzyme Activity in the Post-coital Period

A study of changes in enzyme activity in the immediate post-coital period minimizes these disadvantages discussed above. The stimulus of coitus is physiological and transient and although it initiates a chain of reactions, pregnancy or pseudopregnancy, the changes in the metabolism and endocrine pattern immediately following coitus should be minimal, although it is certain that changes do occur.

A very great advantage of examining enzyme activity at this time is that the exact time and duration of release of luteinizing hormone in rabbit, a reflex ovulator, is known (Hillard, Spies, Lucas and Sawyer, 1968). This is useful for evaluating the second hypothesis, namely the involvement of the enzymes with the releasing factors for luteinizing hormone proposed by Hooper (1968), and Firth and Hooper (1968). Further, there is a large body of evidence which suggests that the neurohypophysial hormones are released at about the time of coitus in many species.

Unfortunately, it has not been clearly established that this occurs in the rabbit. Elevated blood levels have not been established in any species. The release of these hormones has been infered from the effect on the three main target organs, namely the kidney, mammary gland and uterus. It appears that little work has been undertaken on this in the rabbit. Ferguson (1941) reported that stimulation of the cervix and vagina, the former being the more potent stimulus, caused an oxytocic response in the uterus. This was certainly caused by a humoral agent coming from the head. Ferguson suggested that such stimulation caused a reflex release of oxytocin. Cross (1958) found no oxytocic response in spayed oestrogen-treated animals which were subjected to similar stimulation. There was also a difference in anaesthetic used. Ferguson used 1% chlorolase 15% urethane, and Cross nembutal. Fuchs, Olsen and Petersen (1965) repeated the work with essentially negative results. They suggested that the difference between their results and Ferguson's might have been quantitative rather than qualitative, and that Ferguson's preparation may have suppressed spontaneous activity of the uterus, thus rendering discernible the response to small amounts of oxytocin.

The rationale that has been applied to the release of oxytocin is that it aids the rapid ascent of sperm in the reproductive tract. The rabbit, unlike many species, has a slow rate of sperm ascent. Superficially this would suggest against the release of oxytocin following coitus in the rabbit. However, there is no evidence which suggests that oxytocin is required for rapid sperm ascent, nor does exogenous oxytocin invariably increase the rate of ascent. Moreover fertility is not impaired in the absence of endogenous oxytocin (see Fitzpatrick, 1966).

The entire concept of rapid sperm ascent ought to be re-examined, as stated on p. 19, where reference is made to the meticulous work of du Buissons and Dauzier (1955) which shows that the sperm ascent in sheep, contrary to what had previously been accepted, is relatively slow. Again with reference to the sheep, Debachere, and Peters and Tyttens (1961) have shown by means of elegant cross-circulation experiments that oxytocin is released on vaginal and cervical stimulation. It would thus appear that oxytocin secretion at this time does not necessarily result in rapid sperm ascent. Most investigators (see Fitzpatrick, 1966)

agree that the reflex is more adapted to the expulsion of the foetus than sperm ascent. Consequently it would appear that since a post-coital release of the neurohypophysial hormones is evident in a great many species, from the work of Ferguson and Fuchs team, from the fact that there is a release of oxytocin in the sheep, an animal with a relatively slow sperm ascent, and finally that the reflex is probably concerned with the expulsion of the foetus, a reflex required in all species, it would be reasonable to expect that a similar release occurs in the rabbit.

The observations of Firth and Hooper (1968) are of considerable use in evaluating the results discussed here on the enzyme changes following mating. They found that following i.m. injection of 0.5 µg 17α-ethinyloestradiol-17β the enzyme activity in hypothalamic homogenate was elevated 7 hrs after the injection. At 18 hrs after injection the response was further enhanced. Since homogenate was used, the time course of the change of the enzymes individually is unknown. However, injection of oestradiol monobenzoate resulted in elevation of the supernatant enzyme and caused no change in the particulate enzyme. It would thus seem likely that 17α-ethinyloestradiol-17β would have a si-

milar effect. What can be said with certainty is that one at least and possibly both of the enzymes respond to a stimulus within 7 hrs. It would seem likely from what has been said above concerning the response to oestradiol monobenzoate, that it is the supernatant enzyme alone which responds.

Duration of a response to a transient stimulus is unknown since i.m. injections of steroids in oil are absorbed into the circulation slowly. Thus the duration of the stimulus in these experiments is unknown, since the steroids would diffuse into the blood over an unknown length of time.

If a functional relationship does exist between the enzymes and the hormones in question, then the effect of the injected steroids and ovariectomy would either alter the hormone secretion, and this in turn the activities of the enzymes; or the steroids would alter the activities of the enzymes and this would initiate changes in hormone secretion. As has already been stated, it is to be suspected that the former is more probably the case.

That the steroid status of the animals can alter the metabolism of the hormones has been suggested. Starup and Ostergaard (1966) suggest that ovulation inhibitors which are generally steroids act on the hypothalamus, altering gonadotrophic release. Dingman and Despointes (1956) and Gaunt, Lloyd and Chart (1957) suggest that steroids may influence the neurohypophysial hormone metabolism by acting on the hypothalamus. More recently the findings of the latter two groups of workers has been supported by work performed in the Department of Pharmacology of the University of Bristol (Professor H. Heller, personal communication). They have found that marked changes in the pituitary $^{V}/_{O}$ ratio are present in the castrated animal. Administration of gonadal steroids reverses those changes. Full details of this work are eagerly awaited. If the situation described above is the case, then a response to changes in secretion of the neurohypophysial or lutoinizing hormone should result in an enzyme change within 7 hrs.

The correlation suggested by Hooper (1968) and Firth and Hooper (1968) between enzymes and gonadotrophic release is an inverse correlation. It would

appear that the correlation was primarily between the supernatant enzyme and luteinizing hormone. Hilliard, Spies, Lucas and Sawyer (1968) have demonstrated that the preovulatory surge of luteinizing hormone occurs at the time of, or immediately following coitus and lasts 5-6 hrs. If there is a direct functional relationship between the two, then the supernatant enzyme activity should be suppressed within 7 hrs of coitus. The suppression of enzyme activity reported in Chapter IV, however does not appear until after 24 and before 36 hrs. There is thus an unexplained delay of 17 hrs from the expected enzyme index of the preovulatory surge of luteinizing hormone until the depression of enzyme activity was recorded. At the time or soon after, one would expect a decrease in activity there is in fact an apparent slight increase in activity. It would thus appear that a direct functional relationship between the supernatant enzyme and luteinizing hormone does not exist.

It would seem unlikely that the enzymes, because of their widespread distribution, are involved with the releasing factors for the anterior pituitary hormones since they are thought to be synthesised in the

hypothalamus alone; and also since they have a direct blood supply into the anterior pituitary, their concentration in the peripheral blood would be exceedingly small, much smaller than the oxytocin concentration. Consequently it would seem unlikely that an enzyme system distributed throughout the brain would be involved. If on the other hand the enzyme is involved with luteinizing hormone itself, or with the releasing factors, we are faced with a disturbing problem in analysing the results since the substrate used to measure the enzyme activity was not luteinizing hormone releasing factor, the enzyme's natural substrate; yet the enzyme acted on oxytocin as substrate. This is quite a common phenomenon in enzymology for an enzyme to act, less effectively, on compounds similar to but not identical with its natural substrate. What is disturbing is that if an enzyme involved in the metabolism of luteinizing hormone releasing factor acts on oxytocin, then it is possible that enzymes involved with other peptide hormones etc. do likewise. This conclusion is not suggested by the results discussed above, concerning the kinetic studies carried out by Hooper (1962). However, in the absence of strong evidence in favour of the specificity of the enzymes,

this disturbing possibility must be kept in mind. It is just possible that what is being measured is the sum total of several enzymes acting on oxytocin because of a superficial similarity between it and their natural substrate.

As has already been stated, the work of Hooper (1966 a, b) showed that a closer correlation exists between the supernatant enzyme and the known secretory pattern of oxytocin. If as reasoned above there is a post-coital release of oxytocin, then the apparent slight increase in the supernatant enzyme may be a consequence of this release. The time course does not fit exactly with what would be expected, there being a delay of 5 hrs before the appearance of the enzyme response, which as reasoned above would be expected at, or before 7 hrs. If, however, the response to a transient stimulus is of long duration, reaching a peak in about 12 hrs, then the enzyme results obtained here would be compatible with a slight post-coital release of oxytocin. The secretory pattern following coitus of oxytocin other than a post-coital surge, is not known. Thus the transient decrease in activity that occurred at 36-48 hrs cannot be satisfactorily accounted for.

It has been pointed out above that there is no correlation between the particulate enzyme and oxytocin, the enzyme showed no peak activity at the time of parturition, nor was an elevated enzyme level maintained by lactation. As reasoned above, it is possible that the particulate enzyme is involved with vasopressin metabolism. If as in many species, rat (Franko, Friberg and Karvonen, 1953) and man (Friberg, 1953; Harris and Pickles, 1953) for example, there is also a post-coital release of vasopressin, then the increase in particulate enzyme may be a consequence of this. Figure 4 shows that the response appears to start well within 7 hrs after coitus and reaches a peak at +12 hrs, declining to control levels by +24 hrs. If the response is merely a consequence of the post-coital release, then the response to a transient stimulus would be prolonged with a maximum at +12 hrs. This would confirm the suggestion offered above correlating the supernatant enzyme with a post-coital surge of oxytocin.

References to work suggesting that steroid hormones influence the metabolism of neurohypophysial hormones have already been cited. It appears to be un-

known what the exact effects of the various steroids are, nor is it known what they influence in altering the $^{V}/_{o}$ ratio in the posterior pituitary. They could alter either synthesis or secretion, or both. The suggestion that they act on the hypothalamus would in all possibility mean that they alter the rate of synthesis. The changes in enzymes noted in these experiments may be a consequence not only of a post-coital surge of the neurohypophysial hormones but also of an altered secretory pattern of the hormones induced by the changing steroid status of the animals during this time. 20α-hydroxypregn-4-en-3one(20 -OH) blood titres are high for the first 10-12 hrs after coitus, for example (Hillard, Spies, and Sawyer, 1968). It is quite possible that the significant increase in particulate enzyme at +2 hrs (table 9) may be the enzyme index of a post-coital release of vasopressin, while the remainder of the response may be due to the altering steroid status. Unfortunately the pattern of secretion of the neurohypophysial hormones during this period is not known. It does seem a little coincidental that the enzyme activity should follow the steroid secretion so closely.

Without the knowledge of how the various steroids influence the neurohypophysial hormones, or a detailed knowledge of the steroid status of the animal during this time, it seems unprofitable to try to analyse further the enzyme responses in terms of them.

What does emerge from this series of experiments is that no correlation exists between the enzymes and luteinizing hormone. The finding that enzyme activity alters quite quickly after a transient physiological stimulus and during the early stages of the ensuing chain of events, pregnancy or pseudopregnancy, is indicative of the fact that the activity of the enzymes is specific rather than non-specific and that it responds to relatively mild physiological stimuli.

Finally the results obtained are compatible with the suggestion that the supernatant enzyme is concerned in the metabolism of oxytocin and that this enzyme offers an index of the secretion of the hormone, while the particulate bound enzyme does the same for vasopressin. This deduction does, however, depend on a number of assumptions.

Enzyme Activity in Control Animals Using Oxytocin and Vasopressin as Substrates

To examine the hypothesis further, the effects of each enzyme on the hormones was tested separately, and also changes in enzyme activity in overhydrated and dehydrated animals were examined.

The progress curves of the enzymic inactivation of oxytocin by each enzyme are shown in figure 7 and the regression characteristics are shown in table 29. The results are in close agreement with those obtained by Hooper (1962) using dog tissue, and Hooper (1966a) using rabbit tissue fractions. They show that the supernatant fraction inactivates oxytocin approximately 3 times as effectively as the mitochondrial fraction.

When vasopressin was used as substrate, the results (figure 8 and table 30) showed that the supernatant enzyme inactivated the hormone to the same extent as it did oxytocin. The particulate fraction on the other hand was much more effective at inactivating vasopressin (figure 8). It inactivated vasopressin as effectively as the supernatant enzyme. This represents an increase in the adjusted mean of approximately 244%, while the increase in regression coefficient was 4475%.

The very marked increase in regression coefficient and correlation coefficient when vasopressin was used supports the hypothesis that this enzyme is more likely to be concerned in vasopressin metabolism than oxytocin. Because of the very low regression coefficient obtained when oxytocin was used as substrate for this enzyme and the low correlation coefficient, it would seem quite unlikely that the enzyme has any function in oxytocin metabolism.

The finding that the supernatant enzyme inactivates both hormones to approximately the same extent suggests very strongly that this enzyme is not primarily involved with oxytocin metabolism. One would expect it to inactivate vasopressin to a limited extent, comparable to the situation existing when oxytocin was used as substrate and the particulate fraction tested for enzyme activity, but certainly not to an equal extent. It would seem much more likely that the enzyme is concerned with the metabolism of some material that is unrelated to either of the hormones and that it inactivates the hormone because of structural similarities.

Enzyme Activity in Dehydrated Animals

Administration of 3% NaCl as drinking water is a generally accepted way of producing dehydration. The mechanism is twofold. The first component is restriction of water intake, the animals tending not to drink the solution, and the second mechanism is the action of the ingested solution as an osmotic diuretic. During the course of the three days of treatment the animals lost weight. It seems highly likely that the loss of weight, or at least a large part of it, is due to water depletion. In the three animals where it was possible to determine the urine output and density pre- and post-treatment, there was in all a statistically significant increase in density. Both these observations, loss of weight and increased urine density, show that dehydration was in all probability achieved by this treatment. The results showed that, as in the control animals, the enzyme activity is confined to the supernatant and the mitochondrial fractions. The supernatant fraction showed a statistically significant increase of 120% in regression coefficient and an increase of 110% in adjusted mean. The results obtained with the particulate group are interesting (figure 10; table 34). There is a stati-

stically significant increase in regression coefficient and adjusted mean of 140% and 99% respectively when tissue concentrations up to 70 µg non-diffusible nitrogen were used. Above this concentration there was a progressive decrease in enzyme activity, with increasing tissue concentration. The explanation offered for this strange result is that present in this tissue is a metabolite with a higher affinity for the enzyme than vasopressin, which blocks the action of the enzyme on vasopressin. This metabolite must be a macromolecule since the tissue was extensively dialysed. Mitochondria are membraneous structures and contain enzymes in the structure spacially oriented to one another as well as containing enzymes in solution. Possible changes in membrane characteristics causing altered spacial orientation or loss of soluble enzymes resulting in decreased inactivation seem unlikely. If this is the case there would be a reduction in inactivation of hormone at all concentrations. This is not borne out by the results.

No certain suggestion as to the nature of the interfering agent or agents can be offered. Neurophysin has been suggested as one possibility (Dr. D.B. Hope,

personal communication). This would tend to bind vasopressin, thus protecting it during the course of the incubation. Later, when the enzyme activity was stopped by boiling, the neurophysin would be denatured and the hormone released. Boiling the hormone - neurophysin complex is an established method of dissociating the complex. Moreover, as the tissue concentration increases so would the neurophysin content, thus adding a greater degree of hormone protection. This agrees with the experimental findings just described.

Against this possibility is the fact that the hypothalamic content of the neurohypophysial hormones is known to be exceedingly small and it is customarily supposed that this is also the case with neurophysin. If in the course of future work it is shown that this agent is in fact neurophysin, then it must be asked why the stimulus of dehydration stimulates the production of neurophysin and possibly the hormones to such an extent, whereas other stimuli which increase the rate of synthesis of the hormones do not do so to nearly the same extent.

However, the relevant question with regard to the problem being examined in this thesis is: does this possible inhibitor act *in vivo* as well as in the *in vitro* system used here? The answer can only be guessed at. It does, however, seem unlikely that the body should increase an enzyme activity and then block its action. This would suggest that the blocking is an artifact and that it does not operate *in vivo*. If this in fact is the case, then the actual activity *in vivo* must be more than doubled. This is compatible with the hypothesis that the particulate enzyme is concerned with the vasopressin metabolism, for there is no doubt whatsoever that dehydration is the most potent stimulus for increased secretion and presumably synthesis of vasopressin.

The increase in the supernatant enzyme, according to the hypothesis under test, suggests an increase in oxytocin secretion during dehydration. Lederis (1962) found in rabbits that the neurohypophysial content of both oxytocin and vasopressin was depleted in animals deprived of water for considerable periods. It would seem very probable that the depletion is a consequence of a prolonged demand not being met by adequate syn-

thesis. If this is so, then oxytocin is also secreted in the dehydrated rabbit. This would be compatible with the hypothesis that the supernatant enzyme is concerned in oxytocin metabolism.

Enzyme Activity in Overhydrated Animals

During the overhydration procedure the average urine flow increased by 183%. A significant increase occurred in all animals. The density in all animals was significantly reduced in all cases, resulting in an average density of 1.002. This shows clearly that the animals were all in a state of anti-diuresis, a time when circulating vasopressin is minimal. There was no increase in weight on the morning the animals were sacrificed, eight hours after the last infusion. They were thus not accumulating fluid so the effects of water intoxication etc. can be excluded. Unfortunately, the degree of stress the animals endured with its adrenocortical consequences is not known. A fluid intake of 10% body weight every eight hours is a considerable water load. In retrospect the author feels that he was over-zealous in being sure that a prolonged anti-diuresis was achieved. Another point of concern is that a major danger of i.p. infusion of fluid is

the possibility of gut damage or irritation. Post-mortem examination of the gut showed no visible abnormalities. This observation gives no grounds for assuming irritation; on the other hand neither does it prove the absence of irritation and stress. We thus have a system where there is a considerable antidiuresis and a possible but unknown degree of trauma.

As in the dehydrated animals, the enzyme activity was confined to the supernatant and intermediate mitochondrial fractions. There was an indication that some activity might be present in the microsomal fraction. If this is the case, then its significance is not understood. In all the other states tested, enzyme activity is confined to the supernatant and intermediate mitochondrial fractions. One feels that before this result can be accepted as genuine it has to be confirmed. This has not been done so far.

However, in the two enzyme-containing fractions the changes recorded were a 216% increase in regression coefficient and a 112% increase in the adjusted mean in the supernatant enzyme. Both of these differed significantly from the control. The particulate enzyme showed a decrease in activity, the regression coeffi-

cient was reduced by 18% and the adjusted mean by 48%. The former result, owing to a scatter in the results, was not significant whereas the latter result was significant.

The decrease in particulate enzyme is what would be expected if the enzyme is concerned with vasopressin metabolism, for vasopressin secretion and blood levels are minimal during anti-diuresis.

The author is not aware of any information concerning oxytocin blood levels or secretion during overhydration. The increase in enzyme activity would, if the hypothesis is correct, suggest an elevated blood level of oxytocin. Of interest here is the finding that the diuresis following atrial distension is thought to be of humoral origin, and that this humor comes from the head (Carswell, Hainsworth and Ledsome, 1968). One wonders whether this factor might not be oxytocin. Atrial distension simulates extra cellular fluid expansion, a condition causing salauresis. Oxytocin has been reported by many workers to cause a salauresis when infused in some preparations (see Pickford, 1966). This finding suggests that oxytocin, or an oxytocin-like substrance, may be released at this time and play

a part in the diuretic response resulting from actual or simulated fluid expansion.

Enzyme Activity in a Dog Diagnosed as Suffering from Diabetes Insipidus

The final experimental condition examined, which could add data which might be helpful in evaluating the hypothesis under test, was the examination of one dog diagnosed as suffering from diabetes insipidus. Unfortunately one cannot be entirely sure that the genesis of the classic symptoms of diabetes insipidus was in fact a consequence of the lack of vasopressin. Certainly the dog exhibited polyurea and excessive thirst (7-8 l/day intake on admission to hospital). Again it certainly responded to therapeutic doses of pitressin (0.5 U t.t.d.) and this was effective in reducing urine volume and water consumption after the typical latency period. Subjective observation of the urine suggested to the house physician that the urine was extremely dilute. These observations indicate that the kidney was able to respond to vasopressin and that the symptoms were the result of vasopressin deficiency.

It is unfortunate but understandable that the veterinary officers in charge of the case did not use

any tests to examine the possibility of the thirst centres being damaged, resulting in excessive intake. This could have been examined by restricting the fluid intake and examining the concentrating ability of the kidney. Neither were any tests of the nicotine type performed to see if an anti-diuretic effect could be elicited by pharmacological agents known to elicit the release of the hormone in normal animals, nor was the response of the kidney to graded doses of vasopressin after the latency period during which the concentrating ability is restored during the first few days of treatment, examined. This would have given an idea as to whether the kidney responded to physiological levels or only to pharmacological levels. If a response was only achieved in the latter case, then the possibility of the response being due to the vasoconstrictor properties of the hormone on the kidney could not be excluded. This might be expected to result in some degree of toxaemia. None was noticed, which suggests against this. The finding at post-mortem examination that there was a kidney lesion with necrosis of some of the proximal tubules, shows that the suspected lack of vasopressin was certainly not the only factor involved.

However, there would appear to be grounds for assuming that vasopressin deficiency was a contributory factor. The situation was further complicated before the dog was sacrificed due to its developing distemper pneumonia.

The results of enzyme activity measurements showed that enzyme activity was present in significant amounts only in the supernatant fraction. The activity in a 100 μg fraction was $(0.09\frac{\log A}{A}o)$. This is approximately half that present in equivalent fractions of rabbit tissue. It has been shown in work reported above that the enzyme activities in both fractions in stressed animals are increased. Provided then that all types of stress have the same effect on enzymes, an elevated enzyme level would be expected. We can thus be certain that the particulate bound enzyme is markedly reduced if present and it is very probably absent. This finding is certainly compatible with the hypothesis that this enzyme is involved with the metabolism of vasopressin. It seems too coincidental that the dog should have the symptoms of diabetes insipidus and a total lack of particulate enzyme without there being some connection between them.

The finding that there is some activity in the supernatant fraction is interesting. Sawyer and Valtin (1965) found that extracts of the posterior pituitaries of rats with congenital diabetes insipidus contained oxytocin. When the extracts were injected into normal rats they induced a water diuresis. From pharmacological studies they concluded that this agent was oxytocin and they suggested that oxytocin inhibits an A.D.H. anti-diuresis. If a similar situation existed in this dog, then the finding is compatible with the involvement of the supernatant enzyme with oxytocin metabolism.

Enzyme Activity in Stressed and Overhydrated Animals

The first attempt to overhydrate animals was unsuccessful. The animals became obviously stressed, were sacrificed and the enzyme activity measured. Post-mortem examination revealed perforation of the caecum in one animal, and extensive inflamation accompanied by massive fibrinogen deposits in the others.

At the time of sacrifice the animals had received a total of 20% of their body weight of glucose solution in 36 hrs. The volume given was half that planned, since the volume infused was reduced after the

first signs of stress following the initial infusion.

During the infusion period the animals showed a statistically significant increase in urine output. The increase, although less than that in the animals that were successfully overhydrated, does not differ significantly. The density of the urine was somewhat higher than that found in animals which were successfully overhydrated, 1.009 as compared to 1.002. Unfortunately the density prior to treatment was not recorded. The post-treatment density, however, is less than that routinely encountered in normal animals, 1.009 compared to 1.023.

Consequently it seems that the animals were in a state of anti-diuresis. The presence of enzyme activity in fractions other than the supernatant and intermediate mitochondrial was not tested for. In the two fractions tested, both showed an increase in enzyme activity. The particulate increased only slightly while the supernatant increased by 400-500%.

All that can be said with certainty concerning oxytocin secretion during stress is that there is an increased content of the hormone in the posterior pi-

tuitary. Some investigators have taken this to mean that there is an increased secretion of the hormone. This conclusion is disputable on this evidence alone. Talcisnik and Deis (1964) suggest that the opposite occurs. They used weight gain of the litters of stressed mothers as an index of oxytocin secretion. This index alone is insufficient unless the quantity and detailed content of the milk is considered. It seems highly probable that under stressed conditions the composition undergoes significant alterations. Thus it is difficult to offer any opinion concerning any correlation between the enzyme and oxytocin secretion in this situation.

Nevertheless, this is an extremely interesting result, and together with the finding that the supernatant fraction is elevated in apparently unstressed overhydrated animals undergoing anti-diuresis, may well turn out to be the most significant and potentially important finding reported here.

In this preparation, the stimulus of overhydration would tend to reduce the circulating level of vasopressin, and the stress factor would tend to increase it. Pain is an established and well-defined

stimulus for the release of vasopressin (see Pickford, 1966). The finding that the animals were in a state of diuresis would suggest that the former stimulus was dominant over the second. This, according to the hypothesis under test, would imply that the particulate enzyme would be suppressed. In contrast, the observed results show that the enzyme is increased slightly above the control levels, and yet the animals were in diuresis. This might suggest that the enzyme is not involved in the metabolism of vasopressin, although it could be compatible with the hypothesis in view of possible changes in renal blood flow and G.F.R. These were not measured.

A finding that must be strongly emphasised is that the supernatant enzyme is very markedly elevated, considerably more so than the elevation found in animals that were successfully overhydrated where no obvious stress was present, and the particulate enzyme was suppressed. This could suggest that the supernatant bound enzyme is involved in the metabolism and reflects the level of an agent that has a diuretic effect. If the particulate enzyme monitors vasopressin or some other anti-diuretic agent, the result obtained here implies that the level of the diuretic

agent in this situation needs to be greatly increased to counter the effects of increased anti-diuretic agent. This means that water excretion may be the consequence of a balance of effects of two agents, an anti-diuretic and a diuretic one.

Could these agents be in fact oxytocin and vasopressin? With respect to the possibility discussed above, it should be remembered that during dehydration the particulate enzyme was, if the explanation concerning the possible inhibitor is accepted, approximately doubled up to concentrations of 70 μg even in the presence of the powerful inhibiting agent. Presumably the actual increase of enzyme is greater than this. The supernatant enzyme was also doubled. This means that in all probability the ratio of particulate/supernatant enzyme activity is increased above the control level. During overhydration the particulate enzyme was halved and the supernatant enzyme doubled, thus markedly decreasing the ratio. Again this was the case in the stressed animals but here, although the ratio was of the same order, the absolute amounts were increased. This suggests that the ratios of the agents are of considerable importance.

Body Water Turnover During Pregnancy

During the time that these experiments were being performed, it was felt that the well-documented increase in percentage body water, the increase in plasma volume and the decrease in the blood osmolarity during pregnancy might reasonably be expected to be brought about in part, or alternatively that they may initiate, changes in the metabolism of vasopressin. There appeared to be no information available about the time course of these events in the rabbit. Consequently it was decided to measure these parameters to see whether the changes corresponded to the changes in enzyme activities. As the total body water was being measured at the time, it was decided to measure body water turnover also. This would be an extremely easy matter. Changes in body water turnover would very largely be a consequence of altered excretion of water by the kidneys since the animals were maintained under the same conditions as far as temperature and exercise were concerned. There would be some increase in the metabolic rate of the animal due to pregnancy and the change in thyroid status, which would cause increased loss of water via the lungs and skin.

The results obtained in these experiments showed that during the first three or four days of pregnancy the rate of body water turnover is decreased. From days 4-11 it increased to about or just above the control level. It increased further between days 11-20. Finally between days 20-30 it decreased to or just below the control level.

The initial decrease is accompanied by an increase in particulate enzyme/supernatant enzyme ratio. This is the consequence of an initial increase in the particulate enzyme and subsequently, as the activity returns to the control level, the supernatant enzyme activity decreases. From day 4-11 the body water turnover is increased slightly above control levels (11 ± 24%). Hooper (1966a) found that the supernatant enzyme is increased above control levels by day 6, and continues to increase until day 10 post-mating, after which time it remains constant. No increase in the activity of the particulate bound enzyme was detected until day 8, on the other hand. The activity then increased rapidly, reaching a maximum again by day 10 post-mating and again remaining constant throughout pregnancy. This means that during the days preceding day

10 the supernatant enzyme activity is dominant over the particulate. In Hooper's experiments the concentration of the tissue fractions used for incubation was below detectable levels when control animals were tested. Thus as the increase in supernatant enzyme activity was detected at day 6, the increase must have started before this, which would mean that the supernatant enzyme would be proportionately greater than the particulate from before day 6. The body water turnover during days 11-20 increased further. The average increase was 38 ± 42%. During this period both enzymes had reached their plateau of activity, the particulate having increased to a greater extent than the supernatant, the enzyme ratio being such that a decreased turnover would be expected. During the final period the ratio of enzyme activity was the same as that in the previous period, but in this case the body water turnover had decreased to control level or slightly below it.

The changes were not significant in all the animals tested. 3 out of 6 in the first and third period, 2 out of 6 in the second and 1 in the third period showed significant changes. One suspects, however,

that the changes are genuine from the fact that 5 out of 6 animals showed the same pattern of change.

These results show that there is a measure of correlation between the enzyme ratio and turnover, and presumably water excretion by the kidney. The period containing days 11-20 was the only one that did not fit into the predicted pattern. If there is a direct functional relationship between the ratio of the enzymes and water excretion, then this finding would suggest that the ratio of the enzymes plays some role in altering excretion, rather than the other way about. If the reverse were true, then the correlation should hold good in all circumstances; whereas if the former is the case, discrepancies could reasonably be expected since more than one factor is concerned in modifying water excretion, aldesteron, vasopressin and adrenalin, to name but a few.

One difficulty in interpreting these results is the great many changes that occur during pregnancy which make it probable that many correlations could be made to account for the changes in body water turnover and also the changes in enzyme activity. However, it does seem rather coincidental considering the fin-

dings in the dehydrated, overhydrated and stressed animals that a similar situation should exist during the first 4 days of pregnancy, unless it is accepted that there is some connection; similarly during days 4-11, although here the situation is complicated by the fact that progesterone appears at this time and increases until day 20, after which it decreases (Hilliard, Spies and Sawyer, 1968). Progesterone is a well-known aldosterone antagonist which would tend, unless counteracted, to cause salt and water loss with the resulting increase in body water turnover. There is no reason why there should not be more than one factor contributing to this, and it is quite possible that both progesterone and the consequence of the enzyme ratio contribute to the increase during days 4-11. After this the enzyme ratio would tend to favour water retention, but its effects are surpassed by the progesterone effect. One might expect that the water loss caused by progesterone might initiate the water-conserving mechanisms during the entire course of its secretion. If it is accepted that an increased particulate enzyme/supernatant enzyme ratio signifies this, this does in fact happen from day 10 onwards. It is difficult to understand why the water loss should

be accentuated by the consequences of the enzyme changes during days 4-10.

Correlation Between Enzyme Activity and Water Excretion

If there is a genuine functional correlation between enzyme activity and water excretion, then it certainly would appear that there is some mediating and probably humoral agent involved. If the enzymes are involved in the synthesis or release of that agent, then because of the distribution of the enzyme they would have to be synthesised throughout these regions of the brain. This seems highly unlikely. It is much more probable that they act as protective enzymes, as suggested above, in which case the agents might originate anywhere in the body.

In view of what has been discussed above concerning an involvement of the enzymes with the metabolism of the neurohypophysial hormones, it would seem reasonable to suspect at first that these might be the mediating agent. The involvement of vasopressin and the particulate bound enzyme certainly has an appeal because of the former's well-known role in controlling water excretion. The direction of changes in the particulate enzyme and possibly vasopressin secretion is

what would be expected, bearing in mind the recorded alterations in water loss and turnover; for example, during dehydration when turnover would be reduced, the circulating levels of vasopressin would be high and the enzyme is high also. The reverse is true of overhydration.

It would seem reasonable to state that vasopressin is possibly or even probably one of the agents and is represented by the particulate enzyme. It is more doubtful what the mediating agent of the supernatant enzyme is. There are only two circumstances in which there is a reasonable correlation between it and oxytocin secretion, and in addition the observation that both of the neurohypophysial hormones are inactivated to the same extent by the enzyme is damaging. It is difficult to see what alternative could apply; the postulated saluretic hormone (Cort, 1968) is a possibility. Because of the similarity of the optimum pH etc. of the enzymes and the analogous situation found in other tissues for oxytocinases and vasopressinases, it appears unwise to disregard the possibility completely. The fact that vasopressin is involved to such an extent with water excretion; that oxytocin

has no established functions other than parturition and milk ejection; that the hormones are often secreted together; that they come from the same place and that so many of nature's phenomena occur in pairs, leads one to think that oxytocin might be the diuretic agent involved although there is no sound evidence to support this.

It must be emphasised that it is not established that the correlation between the enzymes and water excretion is a functional and not a fortuitous one.

Previously a similar doubt was encountered concerning the correlation of vasopressin with the particulate enzyme. The situation that presents itself is an ideal test for the validity of those two suggestions concerning functional correlations. What can be predicted from the second hypothesis is that changes in water excretion are in part initiated by two humoral agents, monitored by the enzyme activity. The first hypothesis implies a functional correlation between these two same enzymes and vasopressin and possibly oxytocin. If then the two suspected agents elicit the predicted responses in water excretion, this would indeed provide extremely persuasive evidence

that the two theories are substantially true.

It is on these lines that work is now proceeding. It seemed sensible to start the search for the diuretic agent with oxytocin. To date, the experiments are still in the initial stages, but it has very recently been shown by the author that oxytocin may have a diuretic action when small doses (200-400 m.u. S.C. twice a day) are given.

In addition to suspecting this possibility, there are many reports which show that this hormone can influence kidney function (see Pickford, 1966). The results discussed above indicate that it is the balance between the two agents that might be important. There would seem to be a number of areas in which this balance may act. The first would be on the renal haemodynamics, an essential component of renal functions. Sellwood and Verney (1955) suggested that there are changes in the calibre of the renal vasculature with changes in renal activity. A great many workers have demonstrated that the neurohypophysial hormones can affect the renal vasculature, vasopressin in general acting as a vasoconstrictor agent, and oxytocin as a vasodilator agent. These effects are greatly modified

by the presence of other agents, particularly the steroid hormones. This aspect of the action of the neurohypophysial hormones has been admirably reviewed by Pickford (1966). The observation that vasopressin in very small doses can induce a diuresis suggests that vasopressin plays some role in kidney function apart from its effect on the permeability of the tubules (Grinnell, Kramer, Duff and Lydon, 1968). Pickford (1966) made the suggestion that Sellwood and Verney's observation may be accounted for by certain blood vessels being more sensitive to the withdrawal of vasopressin than the renal tubules.

Demumbrum, Keller, Lerhoff and Purser (1954) showed that renal function including blood flow was strikingly reduced following neurohypophysectomy. Renal blood flow was restored by administering oxytocin and vasopressin together, and oxytocin alone. Goldman (1968) also found that oxytocin in physiological doses significantly increased blood flow to the kidney. That oxytocin and vasopressin may be acting antagonistically is suggested by Sawyer and Valtin (1965) who showed that oxytocin inhibits a vasopressin antidiuresis. Of interest here are the findings that the diuresis following atrial distention is possibly of

humoral origin, and that this humor comes from the head (Carswell, Hainsworth and Ledsome, 1968). There is thus evidence from other work to support the suggestion put forward above.

Another possible site of action of oxytocin and vasopressin is on the active transport of sodium out of the ascending limb of the loop of Henle. The intrarenal gradient of osmotic pressure is increased in the medulla during anti-diuresis induced by vasopressin, and decreased when the animal undergoes diuresis. It would seem that this may be a consequence of changes in renal haemodynamics, including changed vasa recta flow, or of an altered active transport of sodium out of the ascending limb, or both.

In the epithelial cells of lower vertebrates, those of the amphibian skin and bladder, it is well known that the neurohypophysial hormones not only increase the permeability but also stimulate the active transport of sodium (Bentley and Heller, 1963; Bourguet and Maetz, 1961). The results of the two groups mentioned above lead them to suggest that there are structures through which the hormones are able to stimulate the active transport of sodium without affecting

the water permeability at the same time.

Jard and Morel (1963) studied the effects of oxytocin and lysine vasopressin on the renal handling of sodium in the rat, using clearance techniques. With oxytocin they found a variable effect; either no change occurred or a saluresis was present. The results with lysine vasopressin were much the same except that where a saluresis did occur it was of a considerably lesser magnitude than that which occurred when oxytocin was used. These changes occurred in the absence of changes in the glomerular filtration rate.

Using doses of oxytocin sufficient to cause an anti-diuresis, they found in the animals showing saluresis that the increased secretion of sodium per unit volume of filtrate showed a linear relationship with decrease in urine flow. A similar correlation was found when lysine vasopressin was used, although the regression coefficient was considerably less than that obtained when oxytocin was used.

This could mean that oxytocin may have an inhibiting effect on the active transport in the ascending limb of the loop of Henle. Thus more sodium would be presented to the distal convoluted tubules and conse-

quently lost. Prevention of this loss would occur by increased aldosterone secretion. As altered aldosterone secretion is a somewhat slow response, in short term experiments the loss would not be countered.

If vasopressin on the other hand increases the active transport from the ascending limb of the loop of Henle, this could be a factor involved in the increase in interstitial osmolarity that occurs during anti-diuresis. The loss occurring in the urine during these experiments could be a consequence of slight diffusion into the collecting ducts, the amount increasing as the osmolarity increases.

This would indicate that the control of the concentrating ability of the kidney is achieved by coordinated alterations of the osmolarity and permeability of the distal convoluted tubules and collecting ducts.

It has been suggested above that it is possible that the renal haemodynamics and active transport of sodium out of the ascending limb of the loop of Henle may be controlled by a balance of action of vasopressin and oxytocin. It is also possible that the same

applies to the permeability of the distal convoluted tubules and collecting ducts. That this might be the case is indicated by the numerous reports that oxytocin in large doses causes an anti-diuresis. If both oxytocin and vasopressin compete for the same receptor sites and if oxytocin is less effective in causing the permeability changes then this possibility would certainly be true.

In view of all these possibilities it is conceivable that a given degree of water excretion or retention could be brought about in different circumstances by altering these factors to various degrees. This would give the kidney great versatility of function.

It must, however, be emphasised that these suggestions are purely speculative, and that details of the modes of function are not clearly formulated.

The suggestion that some biological effects may be the result of a balance of these hormones has the appeal that it accounts for the presence of the considerable store of oxytocin in females throughout their lives, despite the fact that it is apparently only required at coitus, during parturition and lactation.

It further accounts for the possession in the male of equivalent amounts to those found in the female, although it has no known function in the male. Finally it would account for the fact that when there is secretion of one of the hormones there is quite often secretion of the other. Further details of this latter factor will become available when better assay techniques are available.

Total Body Water and Plasma Volume Changes During Pregnancy

As mentioned above, an attempt to correlate enzyme activity with water metabolism was made by seeing if the enzyme changes corresponded with the well-documented increases in the body water content/kg body weight, plasma volume and the decrease in plasma during osmolarity pregnancy. During these changes it would seem reasonable to expect that vasopressin metabolism would be altered. The osmolar changes could suggest that a new setting for the control of vasopressin secretion would occur. One might expect the circulating levels to be elevated.

Unfortunately both the plasma volume and osmolarity determinations were unsuccessful. The increase

in body water however was successfully measured. This parameter showed an apparent increase which occurred before day 5 post-partum and remained fairly steady throughout pregnancy. The changes measured were not statistically significant by the t-test, but the fact that 5 out of 6 animals showed the same pattern of increase suggests that the response, although not statistically significant by the t-test and the number of determinations made may well be genuine.

The increase, it will be noted, occurred during the initial decrease in body water turnover, a time when the ratio of particulate enzyme activity/supernatant enzyme activity is elevated. The increase in body water however remains constant in the face of a decreased enzyme ratio during days 4-11. Consequently it is difficult to see how the increase can be a result of altered vasopressin and oxytocin metabolism. The result appears to throw no light on the role of the enzymes.

A possible correlation may be present in the post-partum period. In one animal nursing its litter, the increase in body water was intermediate between the pre-partum period and the control level on day 5 and

only slightly above the control level on day 10. Hooper (1966b) has shown that the particulate enzyme returns to control levels from pre-partum levels by about day 11 post-partum.

In contrast, in one animal which killed her litter and consequently was not suckling, the body water/kg body weight was below control levels by day 5 post-partum. Hooper (1966b) has shown that the particulate enzyme in such animals declines to control level. This observation again adds little to an understanding of the functions of the enzymes.

Conclusion

As stated at the beginning of this discussion, the question of whether the enzymes are in fact functionally involved in the metabolism of the neurohypophysial hormones, and if so what aspect they monitor, cannot be answered conclusively by these experiments. What can be concluded is that the enzymes respond rapidly and significantly with much less extensive physiological stimuli than pregnancy or lactation. This suggests that they may be involved in some specific process, and that they are quite sensitive to changes in that process. The results during the post-coital

period render the suggestion of an inverse correlation between enzyme activity and luteinizing hormone secretion untenable. In addition more data has been accumulated to test the correlation of enzymes with the neurohypophysial hormone metabolism.

With regard to the supernatant enzyme, the correlation between the enzyme and oxytocin requirements during lactation and possibly during pregnancy has been supplemented by the finding that the enzyme level is increased during dehydration where there is evidence to suggest that oxytocin is secreted. The postulated correlation is also to some extent supported by the finding that the enzyme is present in the hypothalamic tissue of the dog diagnosed as suffering from diabetes insipidus. Sawyer and Valen (1965) found oxytocin present in rats with diabetes insipidus.

A very significant finding is that the enzyme inactivates both oxytocin and vasopressin to the same extent. This would suggest that oxytocin itself is not the natural substrate of the enzyme.

Although no conclusive statement can be made until additional biochemical characterisation, particularly of the pH optima of the individual enzymes using

both hormones as substrate is undertaken, this last finding suggests strongly against the postulated correlation between the supernatant enzyme and oxytocin.

In the case of the particulate enzyme, the finding that it inactivates vasopressin much more effectively than oxytocin; that the activity is increased in dehydration and decreased in overhydration; and finally that the enzyme was absent from the dog suffering from diabetes insipidus, all support the hypothesis that there is a correlation between this enzyme and vasopressin requirements.

It is suggested that if there is an involvement of the enzymes with these hormones, then their function would appear to be one of a protective nature, that is the inactivation of the hormones reaching the brain tissue either via the circulation, by way of diffusion from their sites of synthesis or by secretion into the C.S.F. (Vorherr, Bradbury, Hoghoughi and Kleeman, 1968).

It must be emphasised that no definitive statement can be made as to whether the correlations are functional or fortuitous.

Since all the conditions where there are established neurohypophysial involvements have been examined and still the picture is equivocal, it is unlikely that further experiments of this nature will significantly clarify the picture.

A correlation has also emerged between water excretion and the ratios of the enzyme activities. Again it must be emphasised that there is no direct proof that this correlation is a functional one.

This situation where two possible functional correlations have been proposed gives high hopes that the question still existing may soon be resolved, as described in the foregoing discussion.

SECTION B

EXTRACTION AND PURIFICATION OF OXYTOCIN AND VASOPRESSIN FROM POSTERIOR PITUITARY MATERIAL

CHAPTER I

INTRODUCTION

In the General Introduction possible approaches for evaluating the physiological significance of the enzymes were discussed. It was stated that the most scientifically valid method of evaluating the suggestion that the enzymes reflect oxytocin secretion and synthesis was to test the hypothesis directly, and a possible way of doing this was discussed. It was also stated that an essential requirement of this approach was a method of isolating quantitatively from fresh glands the neurohypophysial hormones in a high state of purity. This section of the thesis describes the work undertaken to obtain a suitable method of purification.

The first attempts to purify the active principles were made at the beginning of this century, but it was not until over 50 years later that the purification was achieved by du Vigneaud and his colleagues (1954-55). They extracted the gland with hot 0.25% acetic acid. Then, following precipitation of the active principles with ammonium sulphate, they re-dis-

solved the precipitate with glacial acetic acid and re-precipitated oxytocin and vasopressin with acetone, ether and petroleum ether. Finally they applied the techniques of counter-current distribution to isolate the hormones in a pure state.

More recent methods consciously utilise the electrostatic binding of the hormones to neurophysin. These methods entail the following steps: (1) extraction of the acetone dried powder of the glands, (2) precipitation of the high from the low molecular weight material (the hormone-neurophysin complex is in the high molecular weight group), (3) re-dissolving the precipitate from (2) and removing any free peptides by dialysis, (4) splitting the hormone-neurophysin complex by one of the many methods described and separating the hormones from the high molecular weight material, and (5) finally separating and purifying the hormones.

With very few exceptions the methods that have been described in the literature have entailed the use of large amounts of material. Usually the material used has been the acetone dried powder of the posterior pituitary glands. The yields from these

methods have varied between 30-60%. Sachs (1960) on the other hand has described a method of extracting vasopressin from fresh hypothalamic tissue in a high state of purity, using ion exchange chromatography. Unfortunately his losses were large and variable.

When the work reported here was started, Sephadex gel-filtration techniques were in common use. These techniques are exceedingly gentle and recoveries in the region of 100% are frequently recorded. Furthermore, a search of the literature revealed that a method of separating the high and low molecular weight material of posterior pituitary homogenate using a gel-filtration technique had been described by Lindner, Elmquist and Porath (1959). In addition Frankland, Hollenburg, Hope and Schater (1966) had described a method of dissociating the hormone-neurophysin complex and also separating the high and low molecular weight materials from each other in one step, using gel-filtration. It was also claimed by these workers that oxytocin and vasopressin were separated from each other in the same step. Using only gel-filtration it thus seemed that it might be possible to extract the hormone in a pure state with a recovery of not very much less than 100%.

It was first decided to see whether this was in fact possible. If it was, it would remove the slight uncertainty present in the alternative method of detecting physiological change in the rate of synthesis of the hormones in the face of considerable and variable losses during the course of the purification. This alternative method would entail assaying the hormone content of the gland before purifying the hormones, and then re-assaying the hormone content following completion of the purification. In this way the percentage losses in each experiment would be determined and the amount of radioactive incorporation could be adjusted accordingly. The main uncertainty here is whether all the initial hormone activity is a consequence of the actual hormones and not in part the consequence of some other agent or agents. However, because of the high concentration of the hormones in the gland involving considerable dilution of the extract before bioassay, it is probable that oxytocic and vasopressor agents other than oxytocin and vasopressin would be diluted to such an extent that their concentrations would be insignificant. To the author's knowledge this, however, has not been shown experimentally to be the case.

CHAPTER II

METHODS AND MATERIALS

Although the ultimate aim was to obtain a method of purifying the neurohypophysial hormones from fresh glands, preliminary work was undertaken using acetone dried pig posterior pituitary powder (supplied through the courtesy of Dr. W.J. Tindall, Organon Laboratories Ltd., Mordon, Surrey, England). The reason for this was to gain some experience in the techniques, using large amounts of material in order to facilitate chromatographic examination. To obtain comparable concentrations using fresh glands would have entailed mass slaughter of rabbits and great expense.

Extraction of Material

a) Acetone dried powder

In these experiments the powder was extracted by placing a weighed amount in a conical flask and adding to it the required volume of buffer which had been precooled to $4^{\circ}C$. The conical flask was then placed in a large beaker containing ice cubes. In this way the temperature of the extraction mixture was kept low during the course of the extraction. Extraction was effected

by continual stirring for 5 hrs, using a magnetic stirrer. The solid material was removed by centrifugation at 3,000 r.p.m. for 60 min at 4°C.

b) Fresh glands

The rabbits were killed by a blow on the neck followed by decapitation. The pituitary glands were dissected out after removal of the brain and cutting through the sphenoid bone posteriorly and laterally. A period of 10-15 min usually elapsed from the time of death until the gland was removed. Most of this time was occupied with removing the hypothalamus and homogenizing it in preparation for the enzyme assays described above. As soon as the glands were removed they were stored at 4°C until homogenized. They were homogenized at 0°C for 30 min in 0.5 ml of 0.1 N HCl/gland in an all-glass homogenizer. The particulate material was removed by centrifugation at 3,000 r.p.m. for 60 min at 4°C.

Preparation of Columns

a) Sephadex columns

The Sephadex G-25 (fine) (Pharmacia, Uppsala, Sweden) was allowed to swell in 10 vols of buffer at room temperature for 24 hrs with occasional swirling.

In the case of the 150 x 1 or 150 x 2 cm columns used for dissociating the hormone-neurophysin complex and separating the hormones from the neurophysin, they were prepared as described by Frankland, Hollenburg, Hope and Schater (1966). The Sephadex G-25 powder was suspended in 50% acetic acid and de-aerated for 4 hrs under reduced pressure with occasional swirling. Water was then added to the suspension until a suspension of one part of sedimented Sephadex to two parts of N acetic acid was obtained. This was then used to pour the columns.

Before pouring, the outlets of all the glass columns were plugged with glass wool, care being taken to avoid the trapping of air bubbles. Filling the column with buffer prior to inserting the moist glass wool plug was found to be a satisfactory method. A large filter funnel was then sealed onto the top of the column and partly filled with buffer. The tube was adjusted to the vertical position using a spirit level. An electrically driven stirrer was placed in the funnel just above the outlet and rotated slowly. The swollen Sephadex was brought into suspension by swirling and was poured in its entirety into the funnel. When about 5 cm of Sephadex had settled, the out-

let was opened allowing a very slow flow. When the required height plus 5 cm had settled, the funnel was removed and replaced by the tube leading from the buffer reservoir. An extra 5 cm of Sephadex was allowed to settle to allow for subsequent settling. The flow rate was adjusted by adjusting the height of the reservoir. All columns were washed for 24 hrs before use.

The columns were originally loaded by layering the sample below the surface of the buffer using a L-shaped Pasteur pipette. This method, however, proved unsatisfactory and subsequently the sample was layered onto the head of the column just as the buffer was disappearing into the column. When the sample was likewise disappearing it was washed in 3 times with 2-3 ml of buffer. As the final washing was entering the column, a head of buffer was layered onto the head of the column, and the reservoir connected to the head of the column. During all the operations described above, great care was taken not to disturb the surface of the column. Fractions of effluent were collected in a LKB fraction collector.

b) Carboxymethylcellulose columns

Whatman's (fine grade) carboxymethylcellulose

powder was routinely washed before use. The dry material was allowed to sink into 0.5 M NaCl-0.5 M NaOH solution. Following swirling, the "fines" remaining in suspension after the bulk of the adsorbent had settled were decanted off. This was repeated twice. Following this the flask was swirled to bring the adsorbent into suspension which was then filtered and washed thoroughly with additional 0.5 M NaCl-0.5 M NaOH. This was followed by the addition of sufficient 1 N HCl to make a strongly acid suspension, which was quickly washed free of acid with a large volume of distilled water. The washing was again undertaken using filtration. The filter cake was then suspended again in 0.5 M NaCl-0.5 M NaOH and washed free of alkali with water. Finally the adsorbent was equilibrated with the buffer by suspending it in a large volume followed by several washings on the filter. The pH of the suspension was then checked and if required, adjusted to the required pH with addition of the acid component of the buffer. The adsorbent was finally washed 3 times on the filter with the starting buffer and then transferred to a beaker where it was re-suspended in sufficient buffer to make about 50 ml of suspension for each gram of dry adsorbent. While

in this state the suspension was checked for the presence of "fines". If present they were removed by decanting.

The glass columns, usually 1 ml tuberculine syringes into which a small glass wool plug had been inserted, were connected to a filter funnel by tubing filled with buffer, care being taken to eliminate all bubbles. The suspension of adsorbent was then poured in its entirety into the funnel and the adsorbent allowed to settle. At intervals the tubing was clamped and disconnected from the column and slight pressure was applied to the settled adsorbent. After all the adsorbent had settled, the column was connected to the reservoir and washed with approximately 200 ml of the starting buffer.

The gradient elution system used was a linear one. Two identical flasks capable of holding 200 ml were used. These were connected by a syphon. The mixing of the two buffers in the mixing flask was achieved using a magnetic stirrer.

The samples, which had been dissolved in the starting buffer, were loaded in the same way as samples loaded onto the Sephadex columns. The columns were

then washed through with 10 ml of the starting buffer before the gradient elution was started.

Analytical Procedures

The course of the purification was followed by assay of the biological activity of oxytocin, and by paper and thin layer chromatography.

a) Bioassay of oxytocin

This was determined on the isolated rat uterus by the technique described in Section A of this thesis.

b) Paper chromatography

The paper chromatography technique employed was that described by Heller and Lederis (1958). Whatman's Number 1 paper was washed with 1 N HCl followed by thorough washing with distilled water, employing at least 10 changes. The paper was thoroughly dried and samples applied using a Hamilton 100 µl syringe (Hamilton Co. Inc., California), the aim being to keep the spot as compact as possible. The chromatogram was run using descending chromatography for 24-48 hrs, using n-butanol:acetic acid:water (5:1:4) as solvent. At completion of the run the strips were removed from the tank, heated at 60°C for 2 hrs and hung overnight, following which they were further heated at 60°C for

30 min. The strips were stained using the method of Reindel and Hoppe (1954). The strips were submerged in watery alcohol:acetone (1:1) solution. The excess solution was removed with filter paper and the strips chlorinated. The latter was achieved by placing the strips, loosely rolled, in a desiccator containing 10 ml N/10 $KMnO_4$ in the lower compartment. 10 ml of 10% HCl was added, the cover was put on and the desiccator gently rocked for 5 min. The strips were then removed and hung in a stream of air for 5-10 min after which they were immersed in a solution of 1 part M/20 KI:1 part of saturated o-toludine in 2% acetic acid. The o-toludine was stirred for 3 hrs into the 2% acetic acid using a magnetic stirrer. The solution was then filtered prior to use. The colour developed rapidly, reaching a maximum intensity in about 2 min following which it faded. The spots were ringed and a map made of each strip.

c) Thin layer chromatography

The method used was that described by Ferguson (1965). The adsorbent was Whatman's Thin Layer Chromedia CC 41 (W. and R. Balston, Ltd., England) and calcium free cellulose powder MN 300 (Macherey, Nagal and Co., Germany, supplied by Camlab ((Glass)) Ltd.,

Cambridge). The latter was used in cases where the hormones were eluted off the adsorbent and assayed. The plates were prepared by mixing, using a magnetic stirrer, 1.5 g of dry cellulose powder with 9 ml of water until a smooth slurry resulted. The slurry was spread evenly on completely grease free glass plates using a S.T.L. manually operated thin-layer coater type CC/5065 (Baird and Tatlock ((London)) Ltd.). The plates were air dried in a dust free environment, care being taken to store them in a horizontal plane. The plates were activated by heating at 105°C for 10 min. When the plates had cooled the sample was applied using a Hamilton 100 l syringe, and the plates run using a single-phase solvent system, namely n-butanol: acetic acid:water (6:2:2). The solvent was prepared fresh before each experiment and was placed in a chromatography tank which had already been saturated with solvent vapour. An ascending chromatogram was run until the front had moved 13-15 cm from the origin; 3-4 hrs was usually sufficient for this. The plates were removed from the tank and dried. They were then stained as described previously using the method of Reindel and Hoppe (1954), the difference being that the solutions were applied by spraying instead of immersion. Where

samples were eluted for assay of biological activity, the plates were again dried and strips of cellulose corresponding to 0.5 or 1 xf unit were scraped off and packed into small column sticks, thus forming small columns. The adsorbed material was then eluted using 1 ml of 0.25% acetic acid. To increase the flow rate, the elutant was collected into a test-tube contained in a pressure flask and the pressure inside the flask was reduced using a water suction pump. Before assay, all samples were brought to a pH of 7 with Na_2CO_3, the pH being assessed using narrow range indicator paper.

d) Analysis of the effluent off the columns

The ultra-violet absorption of the effluent was measured at 280 mμ using a Unicam Spectrophotometer and silica glass cuvettes. The protein concentration was measured by the method of Lowry, Rosebrough, Farr and Randall (1951), using the double strength technique. Determinations were performed in duplicate and the colour read at 750 mμ using a Unicam Spectrophotometer.

CHAPTER III

RESULTS AND DISCUSSION OF THE ATTEMPTED PURIFICATION OF THE NEUROHYPOPHYSIAL HORMONES FROM ACETONE DRIED POWDER OF THE POSTERIOR LOBE USING GEL-FILTRATION FOR THE SEPARATION OF THE HIGH FROM LOW MOLECULAR WEIGHT MATERIAL

This method entailed extracting the tissue with 0.2 M pyridine - 0.5 M acetic acid buffer pH 5.9, followed by chromatography of the supernatant fraction on a 60 x 4 cm Sephadex G-25 column equilibrated with the same buffer. The high molecular weight peak was then reduced by rotary evaporation at 37°C and the hormone-neurophysin complex split by chromatography on a 150 x 2 cm Sephadex G-25 column equilibrated with 0.1 N formic acid and eluted with the same buffer.

The course of the purification was followed by paper and thin layer chromatography and by assaying the oxytocic activity both before and after each stage. The samples to be assayed were all neutralized to pH 7 with Na_2CO_3 using narrow range indicator paper. Preliminary tests were performed to test the validity of the assays in the different solvents.

Validity of the Assays

(1) The pH of the sample was not found to be critical providing it was greater than pH 6 and lower than pH 9. This was found to apply even when volumes as great as 0.5 ml were injected into the organ bath.

(2) The amount of NaCl present in the sample was not critical. When large amounts were present, far in excess of that ever present in the samples tested, an effect was seen. The oxytocic response which usually started 30-45 seconds after administering the dose was blocked, the uterus relaxed and after 3 min it went into a series of near maximum contractions. The test was not quantitated. Increasing amounts of NaCl were added to aliquots of pitocin solution, and the test and standard solutions were injected alternatively and the responses compared.

(3) A similar test to (2) was performed to see if the presence of acetate ions interfered in any way with the assay. 0.1 ml of pitocin was diluted with 100 ml of 0.25% acetic acid, aliquots were neutralized with Na_2CO_3 and equal doses were administered alternatively. No difference was found between the test and standard solutions (pitocin diluted with de Jalon's solution).

(4) A similar test to (3) was performed using 0.1 N

HCl instead of acetic acid. In this case the result was quantitated. No difference was again found between the test and standard solutions; 1 U of standard solution was equivalent to 1.08 ± 0.2 U of the test solution. This result was to be anticipated from the results of (2).

(5) The effect of pyridine acetic acid buffer on the assay was also tested for. The buffer itself caused no contraction of the uterus. Pitocin added to the buffer did cause the uterus to contract; it was, however, impossible to obtain a reliable dose response curve. When samples containing pyridine required quantifying, the pyridine was removed by rotary evaporation at 37°C or by freeze drying.

Extraction

Two experiments were performed. 300 mg of the acetone dried powder (containing approximately 2 U oxytocic activity/mg) was extracted as described in the Methods Section. The insoluble residue was centrifuged at 3,000 r.p.m. for 60 min. The supernatant in each case was decanted off and a small (0.5 ml) aliquot taken for thin layer chromatography, using the method described by Ferguson (1965).

The results of the chromatographic examination are shown in table 45. The rf of the spots of the individual chromatograms have been adjusted to a rf for the pitocin reference marker of 0.7. This makes the rfs directly comparable. From these results it can be seen that 4 spots were obtained in experiment 1 compared with 5 in experiment 2, and of these only 3 show similar rfs. No explanation was found for this discrepancy.

Separation of High and Low Molecular Weight Material

A measured volume of the extraction mixture was then loaded onto the 60 x 4 Sephadex G-25 column. The effluent was examined for ultra-violet absorption and protein concentration. In experiment 2 the protein concentration was not determined. The results are shown in figures 20 and 21. In experiment 1 it can be seen that there is close agreement between the U-V absorption and the protein concentration. The variation in the U-V with the volume of effluent shows close agreement in the two experiments.

In both cases peak A (volume 175-250 ml in experiment 1, and volume 190-290 ml in experiment 2) was reduced to 4 ml. A 1 ml aliquot from each of these

Table 45

Thin layer chromatography of extracted acetone dried powder of the posterior pituitary using the method described by Ferguson (1965)

Experiment	1	2
Rfs of spots	0	0
	0.37	0.23
	0.70	0.39
	0.90	0.52
	-	0.70
Rf of pitocin	0.70	0.70
Rf of pitressin	-	0.29

Figure 20

Gel-filtration of extracted acetone dried powder of the posterior pituitary on a 60 x4 cm Sephadex G-25 column (experiment 1).

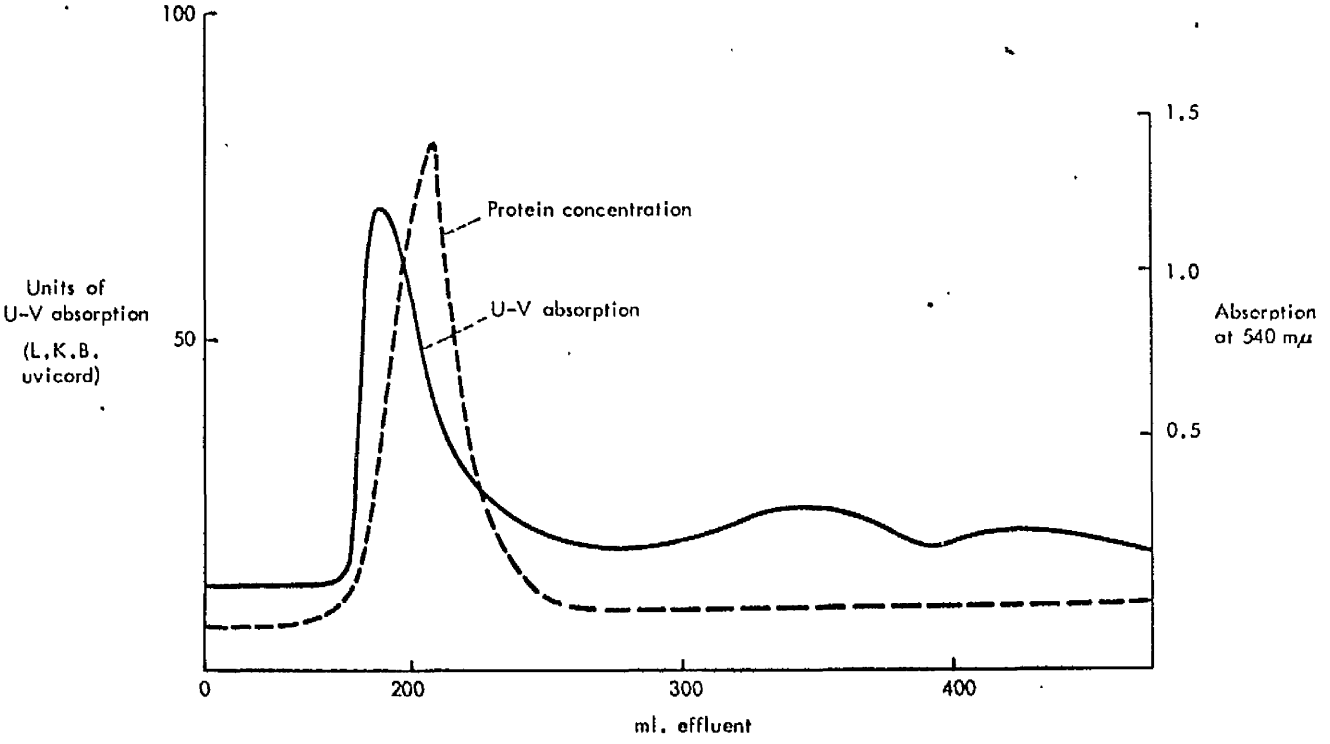
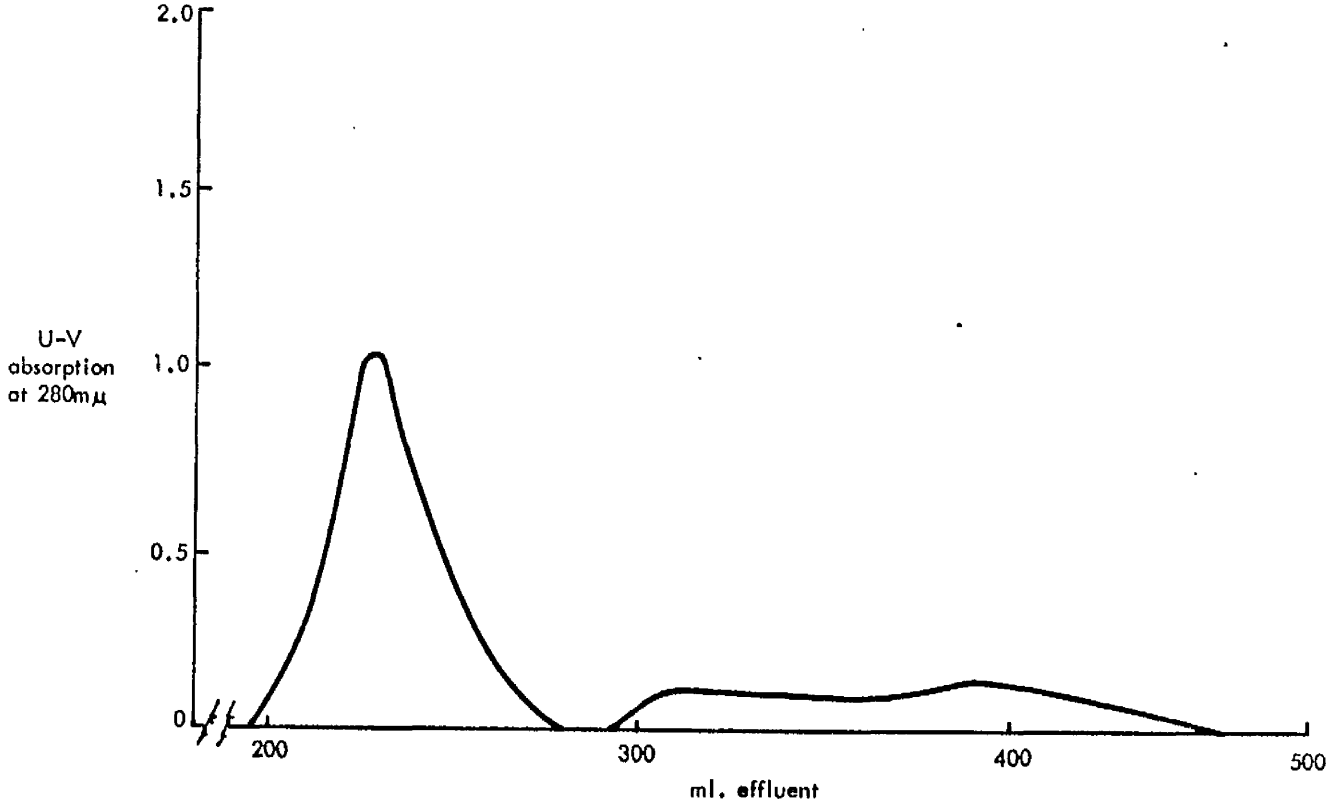


Figure 21

Gel-filtration of extracted acetone dried powder of the posterior pituitary on a 60 x 4 cm Sephadex G-25 column (experiment2)



reduced fractions was taken for chromatographic examination. In experiment 1, peak B (volume 300-400 ml) was similarly reduced. In experiment 2 the following volumes: 290-440 ml; 440-560 ml; and 560-630 ml were also reduced. All these fractions were examined chromatographically using the method described by Ferguson (1965). In experiment 2 this method was supplemented by paper chromatography using two solvent systems: butanol, acetic acid and water (5:1:4); and pyridine, butanol, acetic acid and water (20:30:6:24). The results of the chromatographic examination are shown in table 46.

The Dissociation of the Hormone-neurophysin Complex on a 150 x 2 cm Sephadex G-25 Column

A measured volume of the remainder of peak A in both experiments was then loaded onto the 150 x 2 cm Sephadex G-25 column. The effluent of both these columns was examined for U-V absorption and in the case of experiment 1 the protein concentration was also determined. The results are shown in figures 22 and 23. These show a close agreement between U-V absorption and volume of effluent in each case. In experiment 1 there is also close agreement between the U-V absorption and the protein concentration.

Table 46

Chromatographic examination of the effluent from the 60 x 4 cm Sephadex G-25 columns. The rfs followed by (A.D.H.) are the rfs of pitressin markers. Pitocin was always included as a marker, and all rfs are adjusted to a rf of 0.7 for pitocin

Experiment	1	2		
Method	T.L.C.	T.L.C.	Paper	Paper
System	But:AcAc:H_2O (6:2:2)	But:AcAc:H_2O (6:2:2)	But:AcAc:H_2O (5:1:4)	But:AcAc:Pyr:H_2O (20:30:6:24)
Samples Peak A	0 0.26 0.50 0.81	0 0.80 0.30(A.D.H.)	0 0.34 0.64 0.70 0.85 1.00	lost
Vol 300 -500 (exp 1). 290-440(exp 2)	0.29 0.67 0.89	0.27 0.50 0.77 0.29(A.D.H.)	0.36 0.78 0.37(A.D.H.)	0.38 0.50 0.60
Vol 440-560 exp 2		0.33 0.53 0.71 0.28(A.D.H.)	0.32 0.37(A.D.H.)	0.20 0.42 0.60 0.39(A.D.H.)

Figure 22

Gel-filtration of the high molecular weight material of acetone dried posterior pituitary powder on a 150 x 2 cm Sephadex G-25 column (experiment 1)

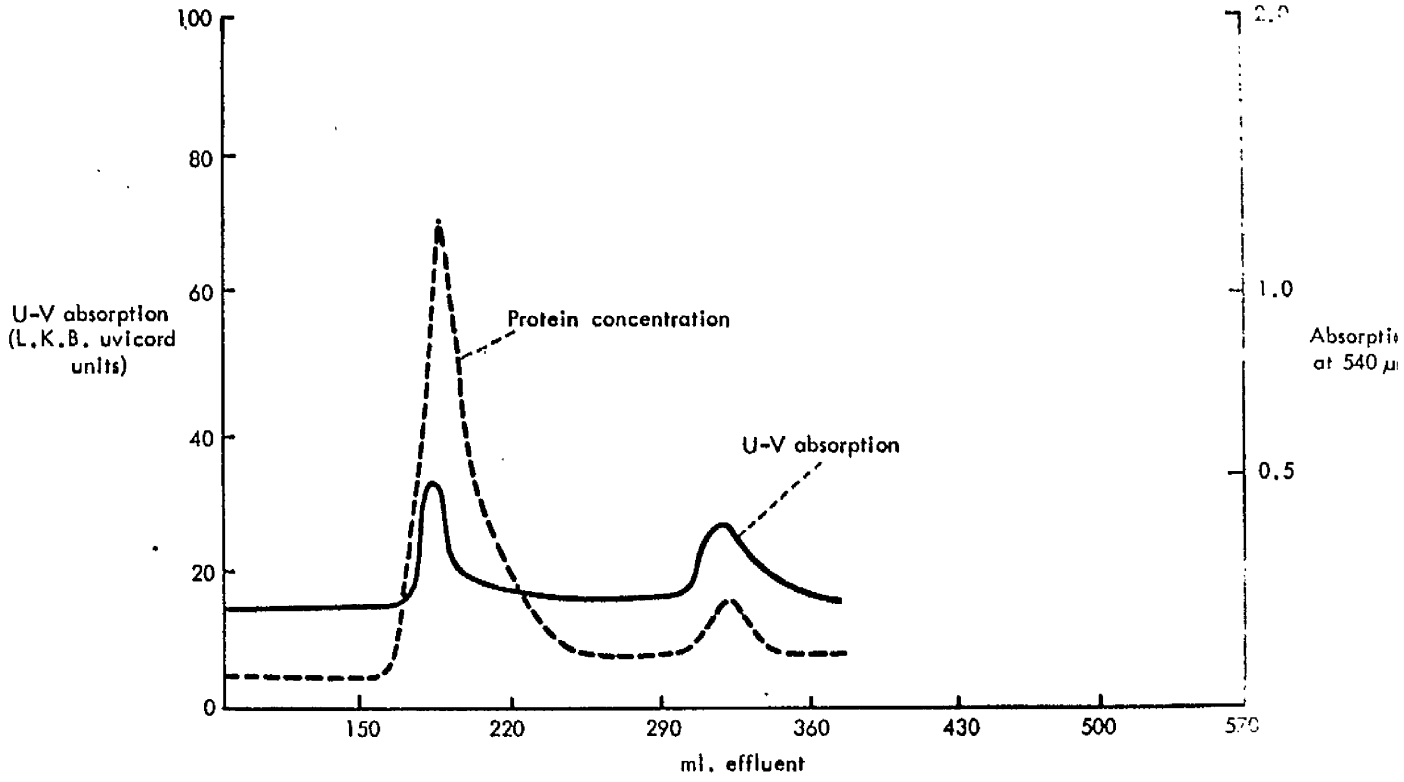
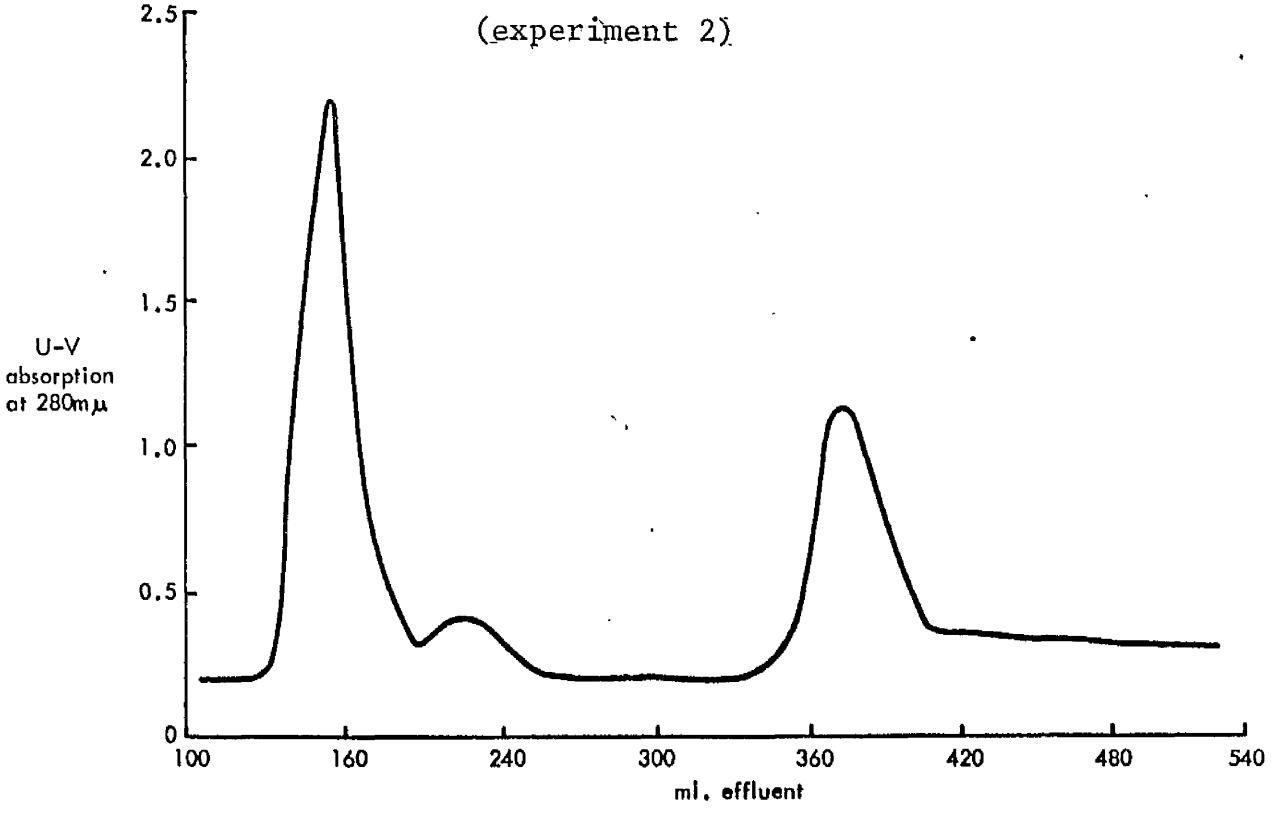


Figure 23

Gel-filtration of the high molecular weight material of acetone dried posterior pituitary powder on a 150 x 2 cm Sephadex G-25 column (experiment 2)



In experiment 1 the peaks A and B (volumes 140-196 ml and 310-332 ml respectively) were both reduced to a small volume and an aliquot taken for chromatographic examination. The results are shown in table 47. Similarly in experiment 2 peak A (volume 132-196 ml) and peak B (volume 330-410 ml) were reduced by rotary evaporation. In addition volume 196-246 ml and volume 438-660 ml were also reduced and examined. The results are shown in table 47.

Ion Exchange Separation of the Hormones

In experiment 2, a measured volume of the reduced effluent of peak B (volume 330-410 ml) from the 150 x 2 cm column was desalted on a 30 x 1 cm Sephadex G-25 column that had been equilibrated with 0.2 M-pyridine-acetic acid buffer pH 7.0. The sample was eluted using the same buffer. The U-V absorption of the effluent is shown in figure 24. One peak was present. A sample of the peak was taken, reduced by rotary evaporation and examined chromatographically using paper chromatography and butanol:pyridine: acetic acid:water (30:20:6:24) as solvent. The results are shown in table 48. Four spots were present.

Table 47

Chromatographic examination of the effluent from the 150 x 2 cm Sephadex G-25 columns

Experiment	1	2		
Method	T.L.C.	T.L.C.	Paper	Paper
System	But:AcAc:H_2O (6:2:2)	But:AcAc:H_2O (6:2:2)	But:AcAc:H_2O (5:1:4)	But:AcAc:Pyr:H_2O (20:30:6:24)
Samples				
Peak A	0.31 0.24(A.D.H.)	0.28 0.76 0.30(A.D.H.)	lost	0 0.62
Peak B	0.24 0.63 0.24(A.D.H.)	0.49 0.22(A.D.H.)	lost	0.48 0.68 0.45(A.D.H.)
Volume (196-246ml)	-	0.55 0.19(A.D.H.)	lost	0.55 0.86
Volume (438-660ml)	-	0.55	lost	nothing

Figure 24

Gel-filtration of the hormone containing peak from the 150 x 2 cm Sephadex G-25 column on a 30 x 1 cm Sephadex G-25 column

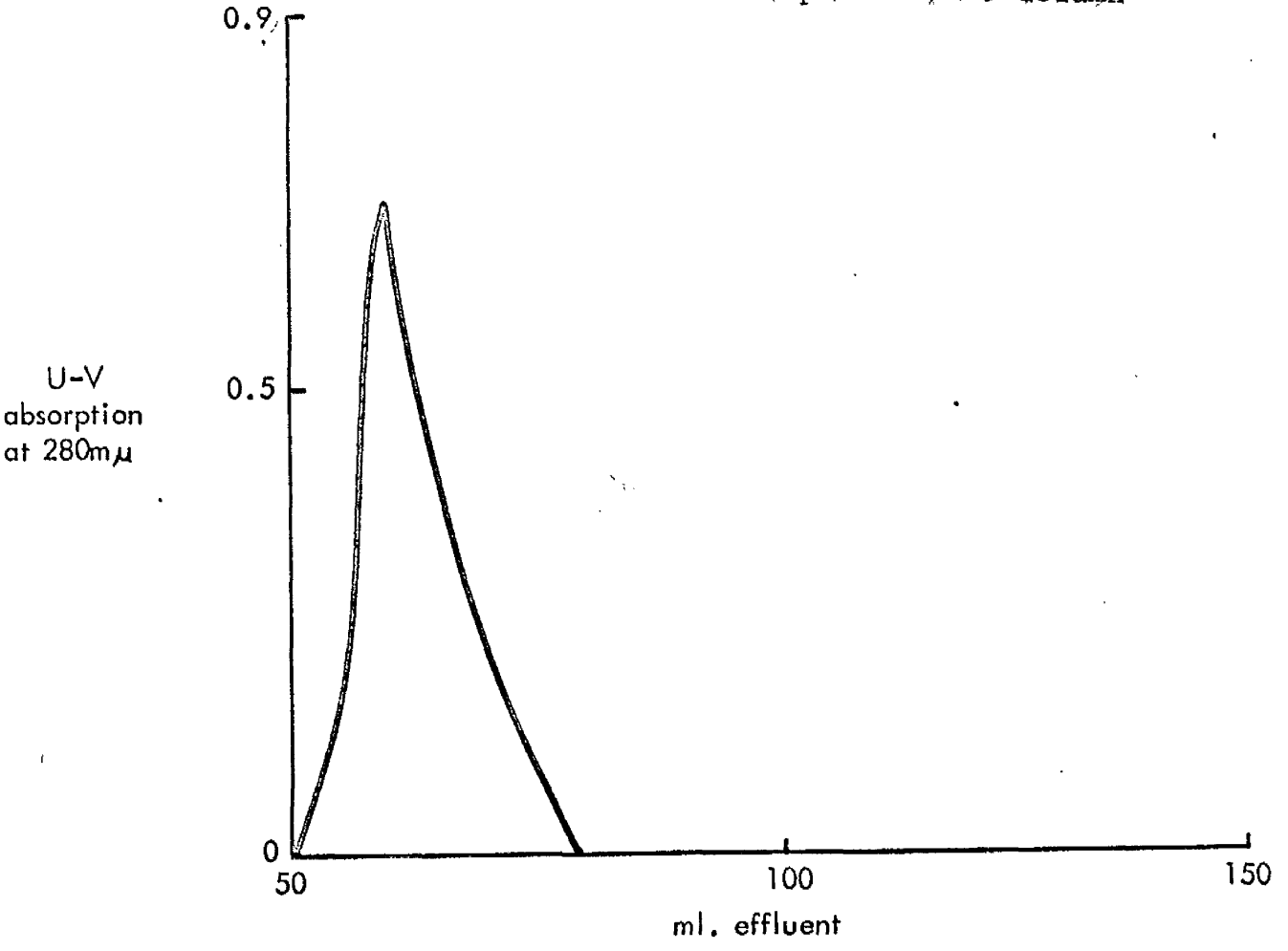


Table 48

Paper chromatography examination using butanol : acetic acid: pyridine: water as solvent of the peak off a desalting column and of the effluent off the C.M.C. column

Fraction	Peak of 20 x 1 cm G-25 column	0.2 M pyridine acetic acid buffer	2 M pyridine acetic acid buffer	Oxytocin	ADH
Rfs	0.07	0.03	0.18		0
	0.31	0.32	0.31	0.24	0.12
	0.43	0.41	0.41	0.42	0.18
	0.53	0.48	0.52		
			0.62		

The rest of the sample was loaded without reduction in volume onto a 5 x 1 cm C.M.C. column equilibrated with 0.2 M-pyridine-acetic acid buffer pH 7.0, and eluted with the same buffer. Following this, the elutant was changed to 2 M-pyridine-acetic acid buffer pH 7.3. Using this system, oxytocin should pass straight through the column, and vasopressin should be retained and subsequently eluted with the second buffer (Dr. D.R. Ferguson, personal communication). The two groups of elutant were reduced in volume and examined using paper chromatography and butanol:pyridine:acetic acid:water (30:20:6:24) as solvent. The results are shown in table 48. Four spots were present in the reduced sample of the 0.2 M buffer, and five were present in the 2 M buffer.

Bioassay Examination

In experiment 2 the course of the purification was also followed by bioassay, see table 49. From this it can be seen that only 11% of the oxytocic material extracted was recovered in the final stage. During the various stages of the extraction, measured volumes were taken at each stage for chromatographic and bioassay examination. This has been taken into account in deriving the recoveries. The effluent from the

Table 49

Oxytocin activity recovered during the course of the purification

Sample	Recovery U	Percentage recovery
Extraction mixture	250±32	42
Peak A col 1	129±16	32
Peak B col 2	120±20	91
0.2M pyridine acetic acid buffer	28±5	25
	Overall recovery	11

60 x 4 cm and the 150 x 2 cm columns was also examined qualitatively for the presence of oxytocic activity. In column 1 activity was found not only in the high molecular weight peak but also in volumes 300-500 ml. In column 2 activity was confined to the low molecular weight peak.

Discussion

Unfortunately because of losses the course of purification can only be followed by the thin layer chromatography method, and here one is made very suspicious of the significance of these results because of the lack of agreement between the two experiments. The course of the purification is almost impossible to follow from examination of the chromatograms. One would expect to find the spots present in the extraction mixture being orientated in the various fractions during the course of the purification. This however is not the case. For example in experiment 1, 4 spots are present in the extraction mixture which following chromatography on a 60 x 4 cm Sephadex G-25 column, gave a total of 7 spots. Of the 4 spots in the high molecular weight peak, these yielded only 3 in the effluent of the 150 x 2 cm column.

This discrepancy could be explained in one of two ways, or probably a mixture of both. Firstly by faulty technique; this is suggested by the lack of agreement in the chromatograms from the two experiments. The second explanation is that the initial mixture is extremely complex and interference between some of the components occurs. Heller and Lederis (1958) chromatographed simpler mixtures. They chromatographed samples in which the proteins had been precipitated with 5% T.C.A. Ferguson did not stain his chromatograms but located the hormones using bioassay following elution of strips of C.M.C. corresponding to rf intervals of 0.1.

It is known that the rf values of materials are affected by a number of conditions. An investigation of the possible factors involved here was not pursued. Because of the number of variables involved, it does not seem profitable to analyse in detail the course of the purification in terms of the chromatographic data, or the anomalous behaviour of the chromatographs.

The chromatography of the extraction mixture on the 60 x 4 cm Sephadex G-25 columns shows close agreement in the two experiments. These differ however from

the results of Linder, Elmquist and Porath (1959) who obtained two distinct peaks, with the entire oxytocin activity confined to the first. In these experiments only one distinct peak was obtained together with a long low peak indicating material with a large range of molecular weights. This second peak was also found to contain oxytocic activity. The presence of oxytocin in this second peak may be explained by the fact that in the present experiments the concentration of the extract was less than in Porath's experiments. Ginsburg and Ireland (1964) have shown that dilution alone is sufficient to dissociate the hormone-neurophysin complex. This would then account for the low recovery, 32% of the oxytocic substance in the first peak, and the presence of oxytocin in the low molecular weight fractions.

The discrepancy in the second peak cannot be satisfactorily explained. Enzymic degradation of neurophysin is known to occur in this system (Dr. D.B. Hope, personal communication) and this might be expected to result in fragments of varying molecular weight, which is suggested by the long low peak. The question that arises is why this phenomenon was not seen in Porath's

results. It is difficult to see how concentration could make any great difference.

Stage 3, the dissociation and separation of the neurophysin-hormone complex, was by far the most successful stage. The recovery in experiment 2 was good and the U-V absorption pattern in the two experiments showed close agreement.

The thin layer chromatography of the hormone peak in experiment 1 showed only two spots with rf corresponding to vasopressin and oxytocin. This was not the case in experiment 2 which showed only one spot with an rf (49) intermediate between vasopressin and oxytocin. Using paper chromatography and butanol, pyridine, acetic acid and water, two spots with rfs corresponding to the two hormones were obtained. Following the reduction in volume of an aliquot of this peak after the supposed buffer exchange (subsequently it was discovered that a Sephadex G-25 column is insufficient to effect complete separation of the hormones from small ions) 4 spots were obtained. This discrepancy could be the result of the alteration of some of the hormones by the rotary evaporation. The composition of the solvent used in each case was not identical.

This too may be a factor involved in this discrepancy.

The recovery following ion exchange chromatography was very low (25%) and the chromatographic examination did not, even remotely, suggest a separation. There were 4 spots in the 0.2 M buffer and 5 in the 2 M buffer. Separation may have taken place and alteration of the hormones occurred during rotary evaporation, not the most gentle of techniques.

In conclusion it must be stated that these extraction attempts, with the exception of stage 3, were unsuccessful. The chromatographic examination of the fractions added considerably to the confusion. Here the experimental technique was probably partly at fault.

No preliminary experiments were undertaken to examine the effects of differences in the composition of the solvent of the material to be chromatographed. This is a serious omission and a knowledge of this might have helped considerably in analysing the results.

It seemed obvious that a great deal of work would be required before the paper and thin layer chromato-

graphy techniques could be used as a reliable guide to the course of purification. It was felt that it would be more profitable in future experiments to follow the course of the purification using only bioassay techniques and then subject the final products to a rigorous test for purity, using amino acid analysis, chromatography and melting point.

Stages 1 and 2 showed a poor recovery of oxytocin. The explanation for this, given above, seems very probable. If this is the case the method would be completely useless when only 3 or 4 fresh glands were extracted, for in this case the amount of hormones present would be only approximately 6 U (6-12 μg). To achieve a sufficiently high concentration to prevent the dissociation of the neurophysin-hormone complex, minute volumes of buffer would have to be used, and this would greatly decrease the accuracy of the method and the recoveries. It would also be impossible to maintain the concentration during column chromatography. Dilution must take place. Due to these reasons it was decided to abandon the method. Subsequently the initial separation of the high and low molecular material was achieved by salt precipitation.

CHAPTER IV

RESULTS AND DISCUSSION OF THE ATTEMPTED PURIFICATION OF THE NEUROHYPOPHYSIAL HORMONES FROM ACETONE DRIED POWDER OF THE POSTERIOR PITUITARY LOBE USING SALT PRECIPITATION FOR THE SEPARATION OF THE HIGH FROM LOW MOLECULAR WEIGHT MATERIAL

In this series of experiments the acetone powder was extracted as described above, the only difference being that the solvent used was 0.1 N HCl (pH 1.5). Following extraction, the insoluble material was again removed by centrifugation at 3,000 r.p.m. The volume of the supernatant was recorded accurately and Analar NaCl added to give a concentration of NaCl equivalent to 15 g/100 ml. The solution was well shaken to ensure that it all dissolved, and was stored at $4^{\circ}C$ for 24 hrs with occasional shaking to allow the precipitate to form. The precipitate was then centrifuged at 3,000 r.p.m. at $4^{\circ}C$ for 60 min. The supernatant was separated from the precipitate and the latter dissolved in water; if difficulty was encountered, a few drops of concentrated acetic acid were added.

The next step in the method described by Hope and co-workers (1966) was dialysis. They were using

higher concentrations and were not concerned with hormone recoveries. Because of the dilution effect on splitting the hormone-neurophysin complex, it was decided first to see if dialysis tubing offered an effective barrier to oxytocin. A trial dialysis of pitocin and the hormone-neurophysin complex against acetic acid-pyridine buffer (pH 5.9) showed that conventional dialysis tubing does not offer an effective barrier to oxytocin. In view of the results from the trial dialysis, it was decided to omit the dialysis step. The presence of NaCl did not affect the dissociation of the complex on the 150 x 1 cm Sephadex G-25 column.

A preliminary experiment showed that no oxytocin was inactivated when stored in 0.1 N HCl at 4°C for 4 days.

As stated above, the rotary evaporation could possibly result in the alteration of the hormones. Because of this it was decided in future to reduce the volumes by freeze-drying. Frankland, Hollenburg, Hope and Schafer (1966) found that by using a 150 x 2 cm column, as well as splitting the complex they also achieved in the same step the separation of oxytocin from vasopressin.

This latter step did not occur when using a 150 x 1 cm column. In the experiments reported above, the expected separation of oxytocin from vasopressin did not occur. It was thus decided that in future experiments a 150 x 1 cm column would be used. This would reduce the time required for separation and also minimize the possibility of the loss of the sample or part of it on the column.

Extraction and Salt Precipitation

Five experiments in all were undertaken. In experiments 1 and 2, 200 mg and 100 mg of acetone dried powder were extracted respectively with acid (5 ml/100 mg). The remaining 3 experiments involved aliquots of the same extraction. 400 mg of powder was extracted with 10 ml of acid and 3 ml aliquots taken. The results of the precipitation are shown in table 50. These results show that there is a reasonable degree of repetition in the percentage of activity precipitated and in the total activity recovered (80%). They also show that the percentage of activity precipitated increases as the concentration of extract increases. In experiments 1 and 2 where the concentration was 20 mg/ml, the percentage of activity precipitated was in the region of 60%, whereas in the remaining experiments where

Table 50

Results of salt precipitation of extracted acetone dried powder

Experiment	Units of oxytocic activity in extraction mixture	Units of oxtocic activity in salt precipitate	Units of oxtocic activity in supernatant	Percentage activity precipitated	Percentage activity recovered precipitated + supernatant
1	373±17	201±30	50±6	60	83
2	166±8	109±18	37±1	66	85
3	188±24	136±18	15±3	90	76
4	188±24	150±22	34±8	82	98
5	188±24	134±16	26±4	84	89

the concentration was 40 mg/ml, the precipitated activity was about 80%. To the supernatant of experiment 2 an additional precipitation was performed. The additional oxytocin activity precipitated was 12 ± 1 U. This gives an overall recovery in the salt precipitate of 72%.

Dialysis

Three trial dialyses were performed, one containing pitocin, and two containing redissolved salt precipitates containing approximately 30 u/ml. These were dialysed against 1000 ml pyridine-acetic acid buffer (pH 5.9) overnight, the bags were washed out with 0.25% acetic acid and the pyridine removed by freeze-drying. Following this, the samples were diluted with 0.25% acetic acid and neutralized prior to assay with Na_2CO_3. Acetic acid was used because the hormones are more stable in acid solutions. Table 51 shows the results obtained and shows moreover that the dialysis bag offers no effective barrier to oxytocin.

The Dissociation of the Hormone-neurophysin Complex on a 150 x 1 cm Sephadex G-25 Column

In both experiments 1 and 2 an aliquot of the dissolved salt precipitate was loaded onto the columns.

Table 51

Dialysis of pitocin and redissolved salt precipitate against pyridine-acetic acid buffer (pH 5.9) for 20 hours

Experiment	Material dialysed	Activity U/ml	Initial activity U	Activity following dialysis	Activity recovered
-	Pitocin	10	5	negligable	0
1	Salt precipitate	26	13±2	2.9±0.6	12
2	Salt precipitate	28	23±4	3.2±0.7	14

The U-V absorption is shown in figures 25 and 26. Peaks A (volume 19 - 40 ml in experiment 1; volume 20 - 40 ml in experiment 2), peaks B (volume 40 - 75 ml in experiment 1; volume 40 - 75 ml in experiment 2), volume 75 - 144 ml in experiment 1 and volume 75 - 125 ml in experiment 2 were freeze-dried. In experiment 1 the dried powders were redissolved in 0.25% acetic acid and assayed for oxytocin after neutralizing with Na_2CO_3.

In experiment 2 the dried powder from peak A and volume 75 - 125 ml was dissolved in 0.25% acetic acid and assayed for oxytocin. The hormone (peak B) was dissolved in 0.001 M pyridine-acetic buffer pH 3.7, a small aliquot was taken and the pyridine removed by freeze-drying, after which it was dissolved and assayed for oxytocin. The remainder was chromatographed on a 5 x 1 cm C.M.C. ion exchange column.

Table 52 shows the results of the bioassay of the effluent. Approximately 90% of the activity was recovered and all of this was confined to peak B. No oxytocic activity was found in the other two fractions examined.

Figure 25

Gel-filtration of the salt precipitate from acetone dried posterior pituitary powder on a 150 x 1 cm Sephadex G-25 column (experiment 1)

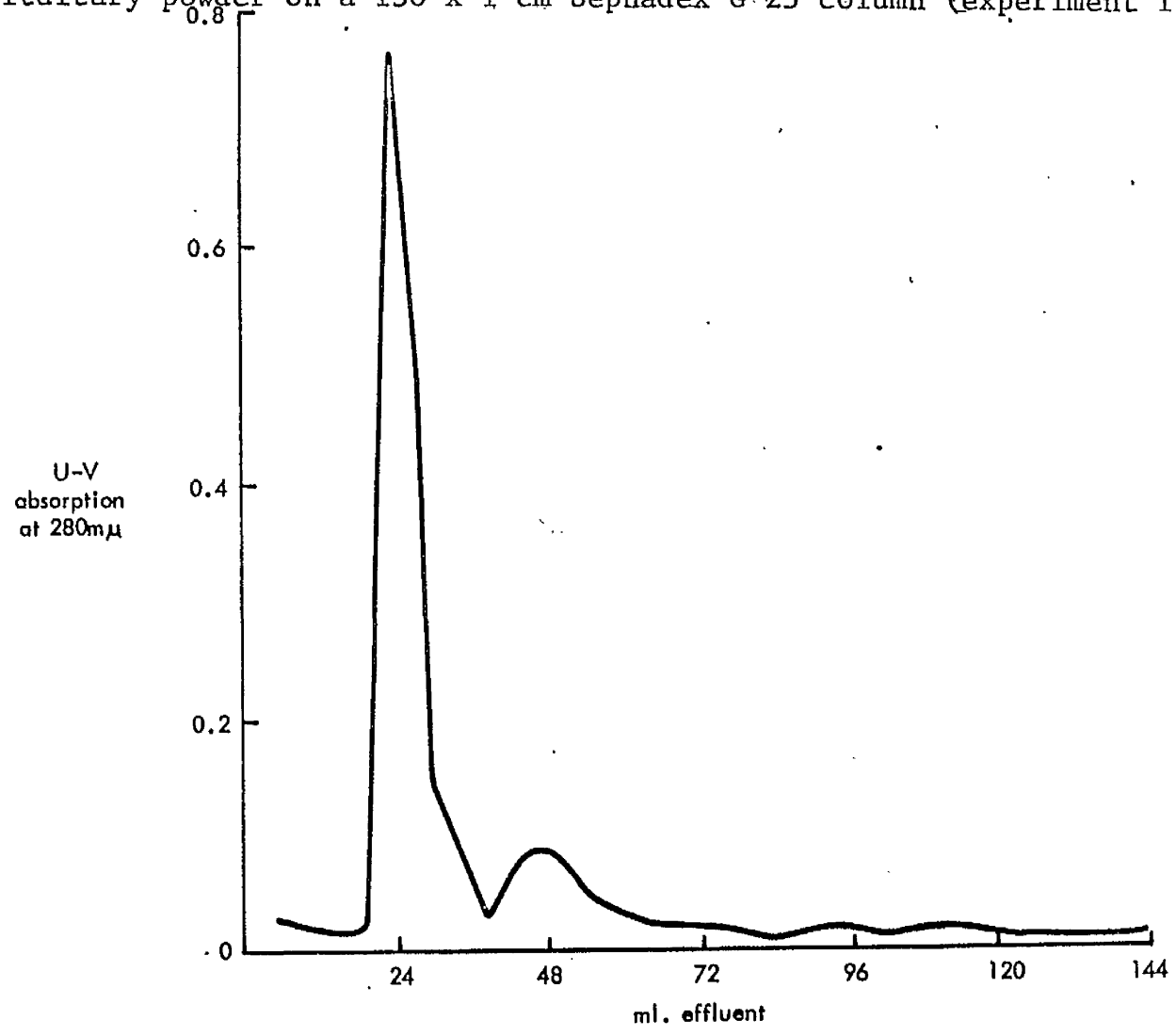


Table 52

Recovery of oxytocin in the elutant from the 150 x 1 cm Sephadex G-25 columns

Experiment	Activity loaded onto columns	Activity recovered in peak B	Percentage recovered	Activity in peak A	Activity in volume 75-150 ml
1	58±7	53±9	92	None	None
2	54±9	50±6	91	None	None

Ion Exchange Separation of the Hormones

An aliquot of the hormone peak containing 10 U oxytocic activity dissolved in 0.001 M pyridine-acetic acid buffer (pH 3.7) was loaded onto a 5 x 1 cm C.M.C. ion exchange column that had been equilibrated with the same buffer. Following loading the buffer was changed twice, once with 0.2 M pyridine-acetic acid buffer pH 7.0 and secondly with 2 M pyridine-acetic acid buffer pH 7.3. Using this system, both hormones should be tightly held during the loading with the 0.001 M buffer. On changing to the 0.2 M buffer the oxytocin should be eluted, and finally A.D.H. should be eluted with the 2 M buffer. In the case of the latter two buffers, acetic acid was added to the effluent because the hormones are more stable in an acid medium. The three eluates (0.001 M, 0.2 M and 2 M buffers) were freeze-dried and then dissolved in 0.25% acetic acid. These were then assayed for oxytocic activity. Activity was located only in the 0.001 M fraction; 4 ± 1 U of the 10 U which had been loaded were recovered.

In an attempt to discover the possible causes of this failure an aliquot of the hormone peak from the

Sephadex column, which had not been used, was tested for the presence of sodium using the simple flame test, and the presence of chloride ions was tested for using the silver nitrate test. Both these tests were positive. This was confirmed by running a sodium chloride solution on a 150 x 1 cm Sephadex G-25 column and testing the eluate for sodium chloride. Sodium and chloride ions were found in the eluate in volume 54 - 66 ml; the hormones were eluted in volume 40 - 75 ml. Thus both the hormones and small ions are eluted together.

Formic acid should have been removed by freeze-drying. Unfortunately no test was used for this substance. It is assumed that the failure of the ion exchange separation was either fully or in part a consequence of the presence of the strong sodium and chlorine ions.

The recoveries in experiments 2 and 3 at the end of stage 3 were 56% and 63% respectively.

Discussion

The overall recovery of the hormone following chromatography on the 150 x 1 cm Sephadex G-25 column

using the present system was approximately twice that obtained previously, where the first stage was attempted using gel-filtration. This alone makes the present system infinately more preferable. The recovery could also be increased slightly by additional salt precipitations.

The presence of NaCl in the hormone peak presented a problem at the time this work was done. The salt could not be removed by gel-filtration or by dialysis. The passage of time has however resolved this problem, for in the meantime Sephadex G-10 and G-15 have become available. These are tighter meshed gels, and would certainly separate the salt from the hormones.

Although the recoveries in this series were markedly improved and of the same order, differing in the two experiments by 7%, the original aim of detecting physiological differences in hormone content from the purified hormones seemed remote because of high losses and differences in recovery even when only half the extraction had been completed. Further, in extracting fresh glands one would expect these losses to be even higher and probably even more variable.

However, the object of these experiments was achieved. They were undertaken to see whether it was possible to purify the hormones with sufficient reproducibility in the recoveries to detect physiological changes. This is not possible.

CHAPTER V

RESULTS AND DISCUSSION OF THE ATTEMPTED PURIFICATION OF THE NEUROHYPOPHYSIAL HORMONES FROM FRESH GLANDS

As stated in the previous section, it does not appear possible to purify the hormones with a sufficiently reproducible recovery to fulfil the requirements demanded for the experiments that were originally contemplated. These were described in the General Introduction. An alternative method would be to extract the fresh glands, assay the hormone activity, purify the hormones and then re-assay the purified hormone and monitor the radio-active incorporation. The amount and the radio-active incorporation could then be scaled up by a quotient corresponding to the quotient original activity/final activity, and these values compared in the different physiological states. The validity of this approach depends upon all the oxytocic and vasopressor activities in the extracted glands being caused by the hormones and not in part by any other agent. It was to avoid this contigency that the experiment described above was undertaken.

An attempt was consequently made to test whether

all the oxytocic activity in the homogenate of a fresh gland was caused by oxytocin alone.

Examination of Factors Contributing to Oxytocic Activity in the Homogenate of Fresh Glands

In this and in most of the subsequent experiments in which fresh glands were extracted, the animals were killed by a blow on the neck and decapitated. The pituitary glands were dissected out after removal of the brain and cutting through the spenoid bone posteriorly and laterally. A time lapse of approximately 10-15 min elapsed from the time of death and the final removal of the glands. Most of this time was occupied with removing the hypothalamus and homogenizing it in preparation for the enzyme assays described above. As soon as the glands were removed they were stored at $4^{\circ}C$ until homogenization in, usually, 0.5 ml 0.1 N HCl/gland.

Following precipitation of the insoluble material and separation of the supernatant, 0.05 ml of the supernatant was spotted onto a thin layer chromatography plate. A second spot containing the extract plus pitocin (200 mU), and finally a third spot which was a pitocin marker were spotted onto the same 20 x 20 cm

plate. Following the run, the plate was dried and the band of cellulose corresponding to the path of each spot was divided into segments corresponding to rfs of 0.1. These were individually scraped off and packed into small column sticks which were then eluted under pressure with 1 ml 0.25% acetic acid. This was then neutralized immediately prior to assay, at which time the oxytocin activity was qualitatively examined on the isolated rat uterus. Two separate experiments were performed. The results obtained are shown in table 53. These show that the oxytocic activity of the extraction mixture, extraction mixture plus pitocin and pitocin all occur at the same rf values.

Bioassay of the extraction mixture showed that fresh glands contained between 500-1000 mU of oxytocic activity. In assaying the extraction mixture, taking the lower estimate of oxytocic activity in the gland, the maximum equivalent of gland injected into the organ bath is 1/25. Usually the equivalent of 1/85 - 1/50 of a gland is injected. In the present experiment the equivalent of 1/10 of a gland was spotted onto the plates; following elution, $\frac{1}{2}$ of the neutralized effluent was injected into the organ bath, thus giving an equivalent of 1/20 of a gland. Fergu-

Table 53

Oxytocic activity in fractions eluted from thin layer chromatography plates using the method described by Ferguson (1965). Fractions correspond to rf intervals of 0.1

Samples	Activity at different rf values									
	0-0.1	0.1-0.2	0.2-0.3	0.3-0.4	0.4-0.5	0.5-0.6	0.6-0.7	0.7-0.8	0.8-0.9	0.9-1.0
Extraction mixture	-	-	-	-	-	-	+	+	-	-
Extraction mixture +pitocin	-	-	-	-	-	-	+	+	-	-
Pitocin	-	-	-	-	-	-	+	+	-	-
Extraction mixture	-	-	-	-	-	-	-	+	+	-
Extraction mixture +pitocin	-	-	-	-	-	-	+	+	+	-
Pitocin	-	-	-	-	-	-	+	+	+	-

son found a 40% recovery of oxytocin. Assuming this to be general for all substances, then the equivalent of 1/50 of a gland was injected. During the course of the scanning, 5 mU of pitocin were injected at regular intervals into the bath. This dose consistently gave a substantial response throughout both experiments.

From this it can be seen that even at the low estimate of the content of the gland (500 mU), in most cases of bioassay (using 1/85 - 1/50 of a gland) no agents having a different rf to oxytocin interfere with the assay. This does not necessarily mean that oxytocin alone is responsible for the activity.

To state this two assumptions must be made. The first is that only oxytocin is present at the relevant rf. It is possible that some other substance is also present, and if this possesses oxytocic activity, then this interfering agent would have remained undetected. Secondly it must be assumed that the loss of activity of all substances during the chromatographic separation is no greater than 40%. If an oxytocic agent suffered a greater loss than this, then again it may not be true to say that no interference occurs when

1/50 of a gland is injected. It is however generally assumed that no interference occurs when assaying extracts of the infundibular process.

Although only oxytocin and vasopressin were to be extracted, whole glands were used and not merely the infundibular process. In rabbit the infundibular process is not well-delineated from the rest of the gland, and even if as much of the anterior and intermediate part of the gland as possible is trimmed away, inevitably in an attempt not to discard any of the infundibular process some of the anterior pituitary will be included. These experiments were designed to detect the interfering agents. The inclusion of the entire gland would improve the chance of detecting the presence of such agents in the fragments of the anterior pituitary adhering to a dissected infundibular process.

Extraction of Fresh Glands

Four experiments were attempted. The glands were dissected out and homogenized as described in the Methods Section in the case of experiments 3 and 4. In experiments 1 and 2 the glands were homogenized in 5 ml of buffer. Again oxytocin alone was assayed. The activity from the four experiments is shown in table 54. Experiments 1 and 2 show a much lower activity than the remaining two experiments. One probable explana-

Table 54

Oxytocic activity in homogenate of fresh glands

Experiment	No. of glands	Oxytocic activity	Oxytocic activity gland	Activity in residue	Percentage activity in residue
1*	1	655±35	655±35	None	0
2*	1	533±56	533±56	None	0
3	3	2774±109	925	295±25	19
4	4	6669±980	1667	Lost	-

* Attempted to dissect free the infundibular process

tion for this is that in these two experiments an attempt was made to dissect out the infundibular process. It is possible that in doing so some of the infundibular process was dissected out in error. The experimenter did not have a great deal of confidence in this operation.

In the remaining two experiments whole glands were used. As the results show, the extracted activity in these glands is very much higher than in the previous two. There is however a considerable difference between the two, much greater than one would expect from biological variation. Apparently no published work gives the range of activity per gland in rabbit glands. The V/O ratio and activity per mg wet weight is quoted instead. As the weight of the infundibular process is not known for these experiments, it is not possible to compare these recoveries with those found in other laboratories.

The residue of the glands was extracted further with 2-4 ml of 0,1 N HCl for a number of hours and assayed. The results are shown in table 54. Activity was only found in one of the 4 experiments.

Salt Precipitation

Table 55 shows the results obtained by precipitating the activity from the supernatant of the homogenate. It must be remembered that in experiments 1 and 2 the volume of HCl used was 10 times greater than that used in experiments 3 and 4. In experiment 1 no activity was precipitated. The recovery was also very slow (33%). No explanation can be offered for this. In all probability the absence of precipitated activity is a consequence of the low concentration and pH. In experiment 2 the concentration was the same, but the pH was raised by the addition of NaOH to pH 5.9. Here precipitation, although low (7.5%), is higher than in the previous experiment, showing that in all probability the binding of the hormone to neurophysin is greater at this pH. This agrees with the findings of Ginsberg and Ireland (1964). In the remaining two experiments where the concentration was increased 10 times, the percentage activity precipitated was increased to 70-90%. This again confirms Ginsberg and Ireland's (1964) findings of the effects of dilution on the dissociation of the complex.

It can be stated in conclusion that the salting

Table 55

Results of salt precipitation of homogenate of fresh glands

Experiment	pH	Activity in extract	Activity in first precipitate	Activity in second precipitate	Activity in supernatant	Percentage activity in precipitate	Percentage activity recovered
1*	1.5	655±35	None	-	215±62	0	33
2*	5.5	533±56	39±5	-	489±33	7.5	99
3	5.9	2774±37	917±10	150±37	288±34	70	90
4	5.9	6669±980	5625±358	-	569±48	89	97

* Attempted to dissect the infundibular process free

out procedure for small numbers of fresh glands is a practical proposition.

The Dissociation of the Hormone-neurophysin Complex on a 150 x 1 cm Sephadex G-25 Column

Of the dissolved salt precipitate (0.7 ml), a 0.5 ml aliquot from experiment 4 was loaded onto a 150 x 1 cm Sephadex G-25 column, prepared as described previously. The U-V absorption of the effluent is shown in figure 27. Peak A (volume 17 - 39 ml), peak B (volume 39 - 70 ml) and volume 70 - 84 ml were freeze-dried, the dried powder of peak A and volume 70 - 84 ml being dissolved in 0.25% acetic acid and neutralized immediately prior to assay. The hormone-containing peak, peak B, was dissolved in 1 ml distilled water, a 0.1 ml aliquot was taken for assay and the remainder immediately desalted on a 60 x 1 cm Sephadex G-10 column equilibrated with 0.001 M-pyridine-acetic acid buffer (pH 3.7) and eluted with the same buffer. The U-V absorption of the effluent is shown in figure 28, there being only 1 peak present. A sample of the peak was tested for the presence of NaCl. This, unlike the hormone-containing peak (peak B) in the previous experimental method, was negative. The assay results for the effluent off the 150 x 1 cm column are shown in

Figure 27

Gel-filtration of the salt precipitate from fresh glands on a 150 x 1 cm Sephadex G-25 column

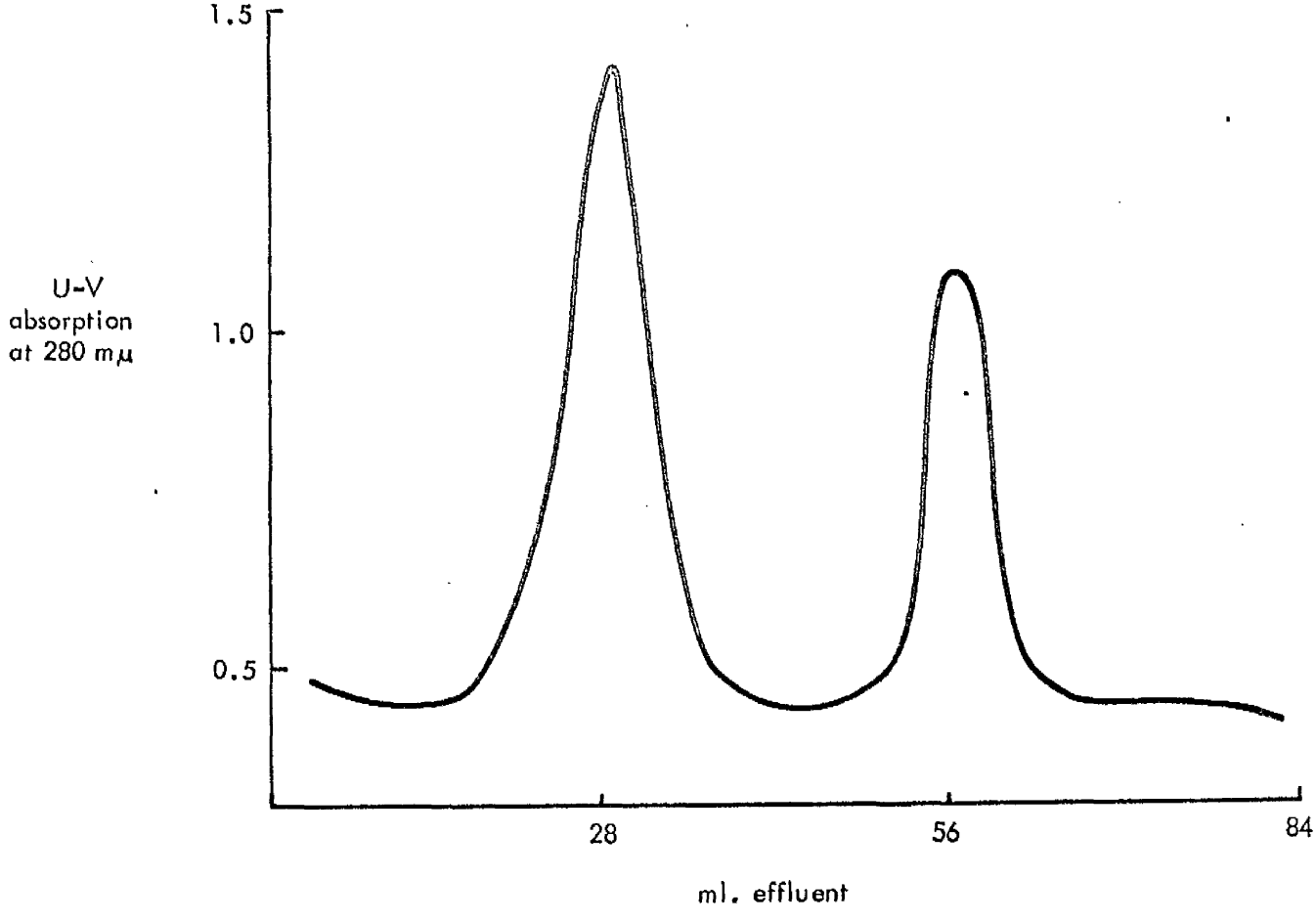


Figure 28

Gel-filtration of the hormone containing peak from the 150 x 1 cm Sephadex G-25 column on a 60 x 1 cm Sephadex G-10 column

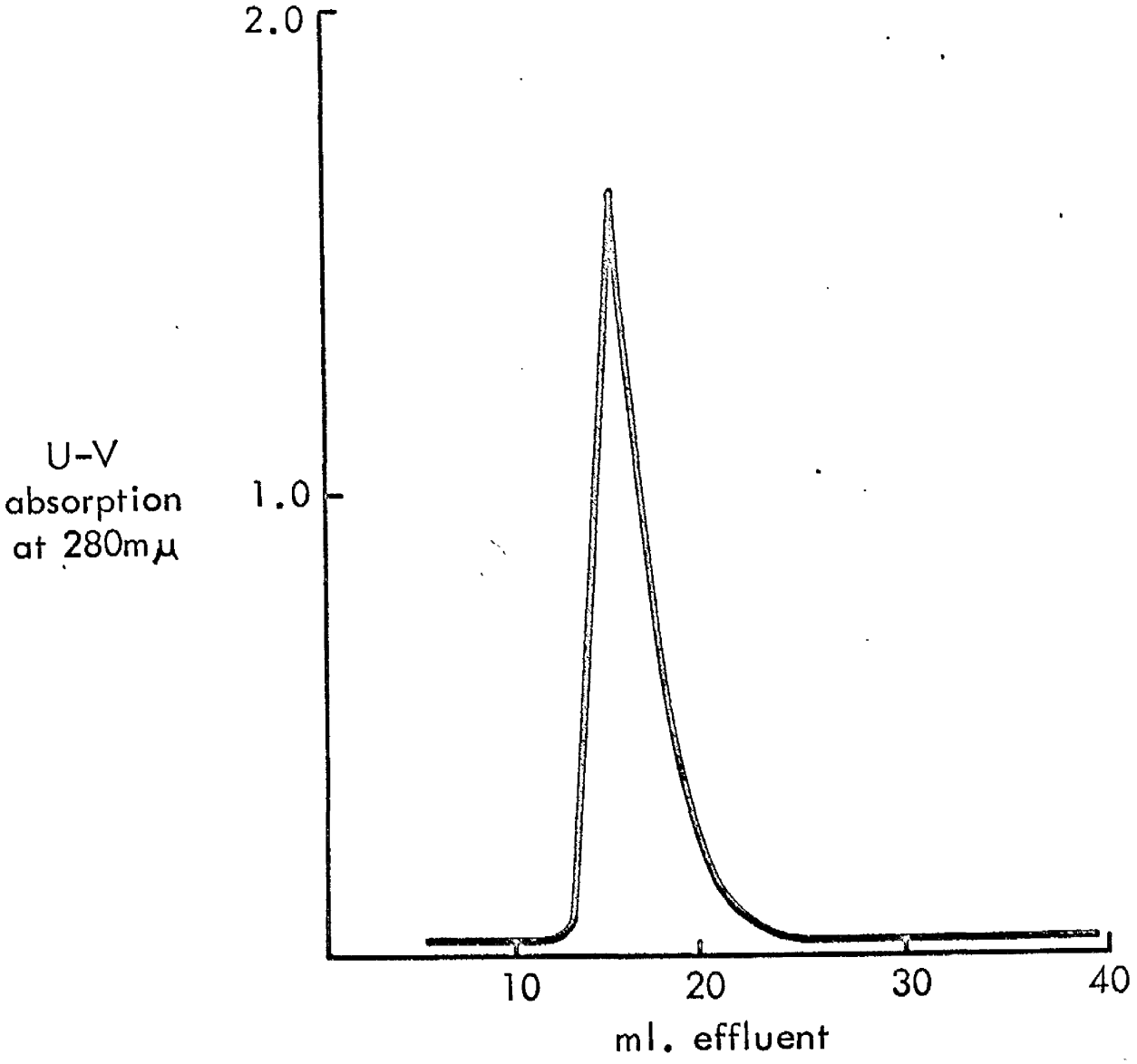


table 56. These show that the activity is not so well localized as in the previous experiments; 4% was present in the first peak and 18% present in the effluent after the hormone-containing peak. However, 75% of the activity was recorded in the hormone peak, giving an overall recovery of 68% activity in the hormone peak. This is comparable with the recoveries obtained when using the acetone powder. The method is thus reasonably effective for fresh glands.

Ion Exchange Separation of the Hormones

It is unfortunate that to date no extract of fresh gland has been subjected to ion exchange chromatography. All that has so far been achieved is the characterization of a gradient elution of a small (5 x 0.3 cm) C.M.C. column using small volumes of buffers. A small column was decided upon because of the minute (µg range) amount of the material to be purified. The main difficulty encountered with these columns is the resistance to flow that they offer. This problem has not yet been overcome; there still exists a tendency for buffer in the higher molar ranges to cease completely to flow.

A linear gradient elution system was devised,

Table 56

Oxytocic activity from fresh glands in the effluent from a 150 x 1 cm Sephadex G-25 column

Fraction	Activity recovered (mU)	Percentage recovery
Peak A (volume 17-39 ml)	150±8	4
Peak B (volume 39-70 ml)	3020±217	75
Volume 70-84 ml	756±29	18
	Total recovery	97

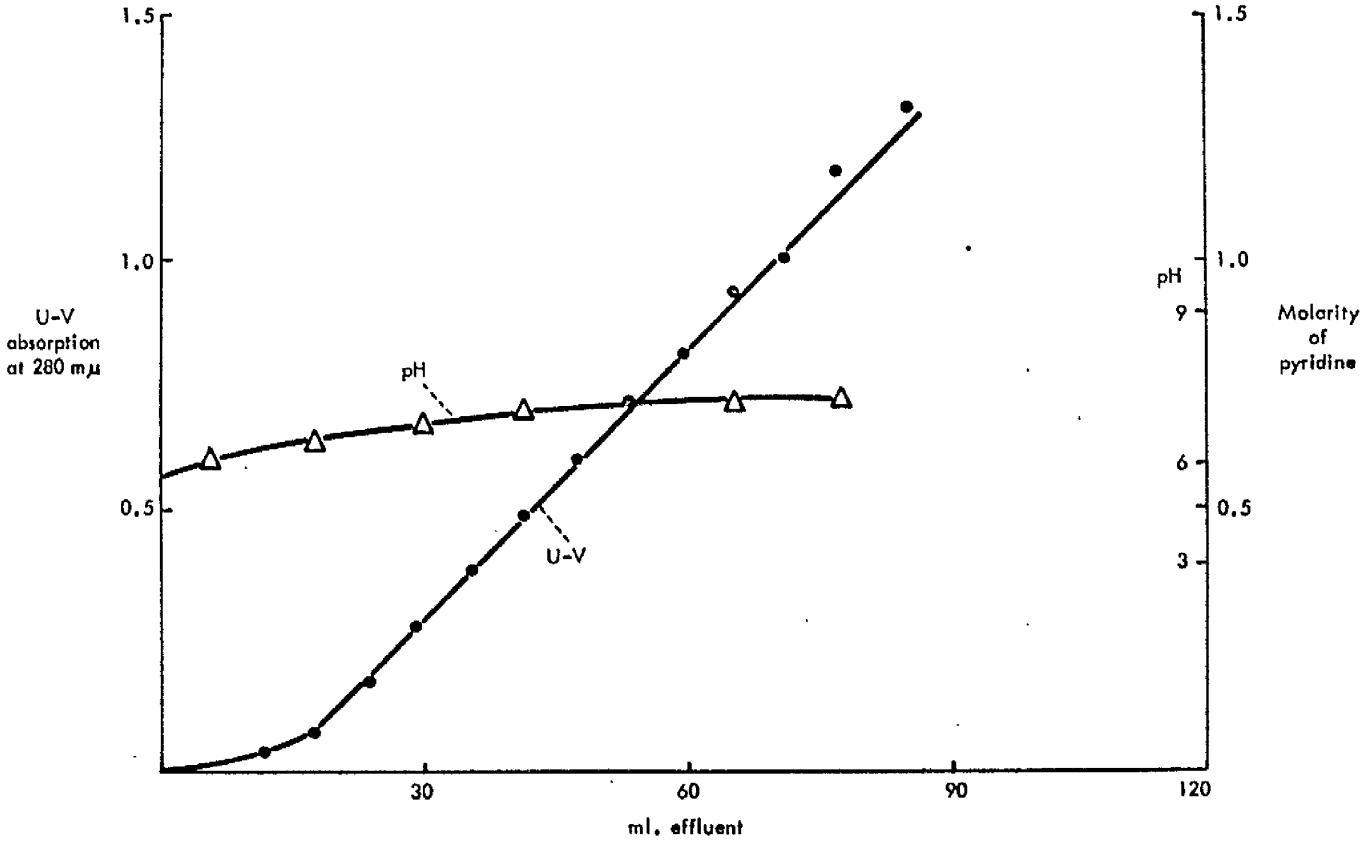
based upon the work of Schally, Lipscomb and Guillemin (1959) who found that oxytocin is eluted from these columns using 0.2 M pyridine-actic acid buffer pH 7.0 and vasopressin with a 2 M pyridine-actic acid buffer pH 7.3. The system was designed for the elution to be completed in 100 ml. In this system it is probable that the molarity plays a more important role than pH (Dr. D.R. Ferguson, personal communication).

Two experiments were undertaken, the first simply to show the elution characteristics. No material was loaded onto the column for this experiment. The pH of the effluent was measured and the progressive increase in molarity from the starting buffer, 0.001 M pyridine-acetic acid buffer pH 3.7, was measured by virtue of the U-V absorptive capacity of pyridine. It was found that by using a compromise between the optimum U-V absorption wavelength of both pyridine and protein, both could be measured. The most effective wavelength was found to be 284 mμ. Figure 29 shows the change in pH and molarity with effluent.

One similar experiment was performed where approximately 50 U each of pitressin and pitocin were loaded onto the column. The hormone had been chromatographed

Figure 29

Characteristics of the pyridine-acetic acid buffer gradient elution



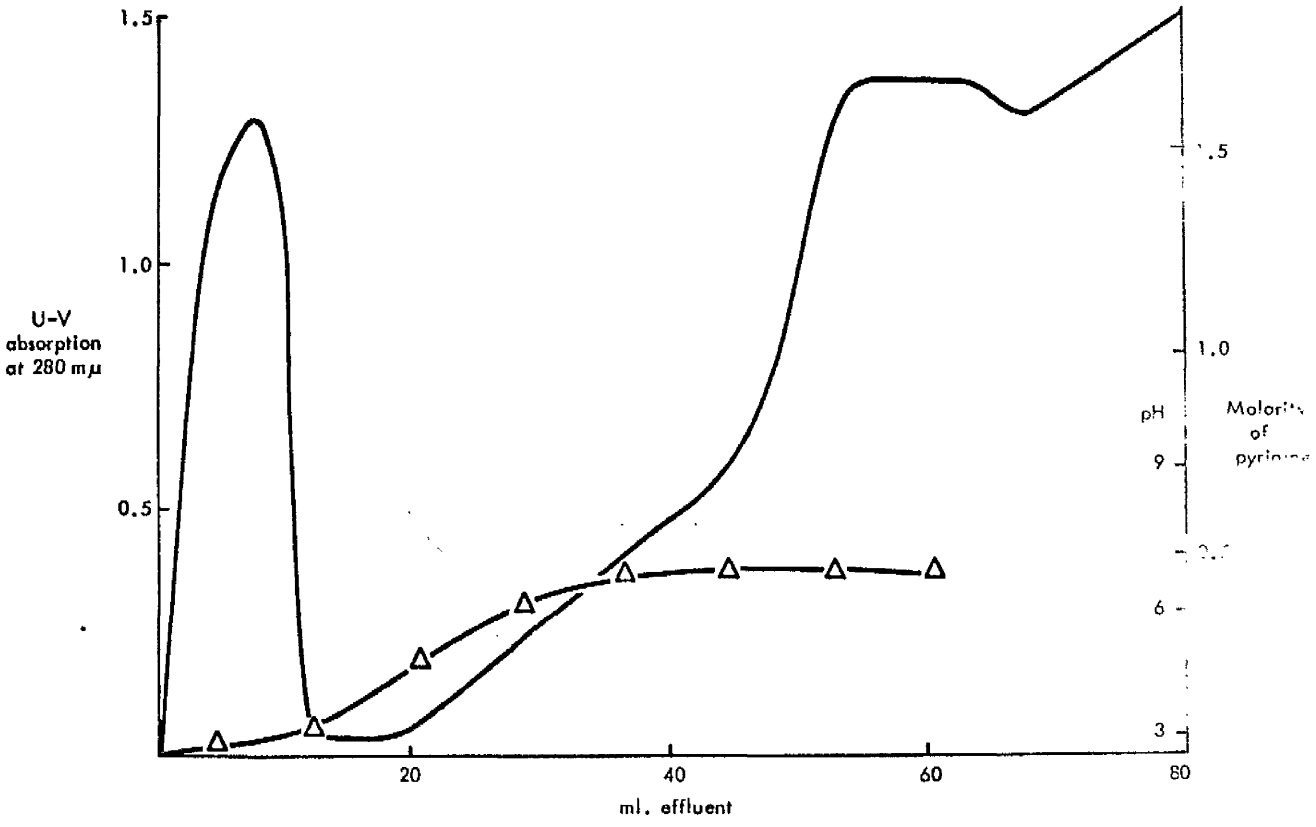
on a 50 x 1 cm G-10 Sephadex column to remove the chlorobutanol preservative. The column had been equilibrated with 0.001 M pyridine-acetic acid buffer, and the same buffer was used as elutant, thus effecting a buffer exchange. The U-V absorption of the effluent off the C.M.C. column is shown in figure 30. The fractions were freeze-dried to remove pyridine, re-dissolved, neutralized and assayed. On scanning the effluent for oxytocic activity, this was found in the first peak (volume 0 - 12 ml) and in volumes 32 - 44 ml. Prossor activity was similarly located. When an aliquot of each of these fractions was taken and diluted 10^3 times, it was found on injecting 0.1 ml of these into the assay preparations, oxytocic activity only was found to be present in volume 0 - 16 ml and vasopressor activity only in volume 32 - 44 ml, suggesting that oxytocin and vasopressin had been separated.

Discussion and Conclusion

As stated previously, it is generally assumed that no interference occurs when assaying extracts of the infundibular process. The concentration of hormone present necessitates a dilution sufficient to reduce

Figure 30

Chromatography of Pitocin and Pitressin on aC.M.C. column using a pyridine-acetic acid gradient elution system



the concentration of other probable substances to below threshold. To the author's knowledge this has not been shown experimentally to be the case. The experiments reported above showed no evidence of interfering agents; however, before embarking on a long and time-consuming series of experiments based on this assumption it seems sensible to investigate it further. The experiments described above should be repeated and extended also to vasopressin using larger amounts of material and additional chromatographic systems. In addition the oxytocin and vasopressin spots should be eluted and subjected to exhaustive analytical examination.

The experiment also showed that the neurophysin-hormone complex is split in this chromatographic system, or alternatively that oxytocin and the complex have the same rf value.

The results of the actual purification are gratifying. They show that the method as far as the chromatography on the 150 x 1 cm Sephadex G-25 column is suitable for small numbers of fresh glands. The ion exchange chromatography on the other hand requires considerable work before a reasonable evaluation of

the step can be made. It seems likely that a larger diametered column will have to be used.

REFERENCES

Abel, J.J. (1930). J. Pharmacol. 40, 139-169.

Abel, J.J., Rouiller, C.A. and Geiling, E.M.K. (1923). J. Pharmacol. 22, 289-316.

Abrahams, V.C. and Pickford, M. (1954). J. Physiol. 126, 320-346.

Acher, R. (1958). In Zweites Intern. Sympos. ueber Neurosekretion, pp. 71-78. Springer, Berlin.

Acher, R. (1963). Biochim. Biophys. Acta, 42, 379-380.

Acher, R. (1966). In the Pituitary Gland, vol. 3, pp. 269-287. Eds. H. Heller and B.T. Donovan, London. Butterworths & Co. Ltd.

Acher, R. and Fromageot, Cl. (1957). In Neurohypophysis. Ed. H. Heller. Butterworths, London.

Ames, R.G. and van Dyke, H.B. (1950). Proc. Soc. Exp. Biol. N.Y., 75, 417-420.

Anderson, B. and Ussing, H.H. (1957). Acta Physiol. Scand., 39, 228-239.

Armstrong, D.T. and Hansel, W. (1959). J. Dairy Sci., 42, 333-342.

Baisset, A., Dang-Trang, L. and Montastruc, P. (1965). Compt. Rend. Soc. Biol., 158, 1602-4.

Balasse, E. (1964). Compt. Rend. Soc. Biol., 158, 1165-68

Balasse, E., Rasio, E. and Conrad, V. (1965). Arch. Intern. Physiol. Biochem., 73, 27-32.

Bargmann, W. (1949). Z. Zellforsch., 37, 583-596.

Barrer, G.R. (1963). J. Physiol. (Lond.), 169, 62-72.

Bartlestone, H.J. and Nasmith, P.A. (1965). Am. J. Physiol., 208, 754-762.

Benson, G.K. and Fitzpatric, R.J. (1966). In the Pituitary Gland, vol. 3, pp. 414-452. Eds. Harris, G.W. and Donovan, B.T., Butterworths, London, Ltd.

Bentley, J.P. and Heller, H. (1962). Gen. Comp. Endocrin., 2, 6.

Bern, H.A. and Knowles, F.G.W. (1966). In Neuroendocrinology, I, pp. 139-186. Eds. L. Martini and W.F. Ganong, Acad. Press. N.Y.

du Biusson, F. du M. and Dauzier, L. (1955). C.R. Soc. Biol. Paris, 149, 76-79.

Bodian, D. (1963). Bull. Johns Hopkins Hosp. 113, 57-93.

Bohus, B. and Endröczi, E. (1961). Acta. Physiol. Acad. Sci. Hung., 20, 285-297.

Bourguet, J. and Maetz, J. (1961). Biochim. Biophys. Acta, 52, 552.

Brarrer, R. Heller, H. and Lederis, K. (1963). Proc. Roy. Soc. B., 158, 388-416.

Bucy, P.C. (1932). In Cytology and Cellular Pathology of the Nervous System. Ed. W. Penfield, vol. 2, pp. 705-738. N.Y. Hoeber.

Campbell, B. and Petersen, W.E. (1953). Hum. Biol., 25, 165-168.

Carswell, F., Hainsworth, R. and Ledsome, J. (1968). J. Physiol. (Lond.)

Chadbury, R.R. and Walker, J.M. (1958). J. Physiol., 143, 16p

Chang, W.Y. (1965). Endocrin., 77, 1097-1104.

Chang, W.Y. and du Vigneaud, V. (1962). Endo., 71, 977-982.

Christ, J.F. (1966). In the Pituitary Gland, vol. 3, pp. 62-130. Ed. G.W. Harris and B.T. Donovan, London: Butterworth & Co. Ltd.

Cech, J.A., Brovetto, J., Cabot, H.M., Frehty, C.A. and Caldegro-Barcia, R. (1965). Am. J. Obzet. and Gynecol., 91, 10-17.

Coon, J.M. (1939). Arch. int. Pharmacodyn., 62, 79-99.

Cooper, J. and Gutstein, W.H. (1966). Circ. Res., 10, 925-932.

Cort, (1968). Nephron, 5(6).

Coutinho, E.M. and Csaho, A. (1959). J. Gen. Physiol., 43, 13-27.

Cross, B.A. (1958). J. Endocrin., 16, 237-260.

Cross, B.A. (1951). J. Physiol., 114, 447-453.

Cross, B.A. (1961). In Oxytocin, pp. 24-46. Eds. Caldeyro-Barcia and H. Heller. Pergamon Press, London.

Czaczhes, J.W. and Kleeman, C.R. (1964). J. Clin. Invest., 43, 1625-40.

Dale, H.H. (1906). J. Physiol., 34, 163-206.

Dale, H.H. (1909). Biochem. J., 4, 427-447.

Daniel, A.R. and Lederis, K. (1966). J. Endocrimol., 34, 91-104.

Debackere, M. and Peeters, G. (1960). Archir. int. Pharmacodyn., 126, 486-488.

Debackere, M. and Peeters, G. and Tuyttens, N. (1961). J. Endocrin., 22, 321-334.

Debackere, M., Peeters, G. and Tuyttens, N. (1961). J. Endocrin., 22, 321-334.

Dekanski, J. (1952). Brit. J. Pharmacol., 7, 567.

Demumbrum, T.W., Keller, A.D., Lerhoff, A.L. and Purser, R.M. (1954). Am. J. Physiol., 179, 429-434.

Dicker, S.E. and Nunn, J. (1957). J. Physiol., 141, 332-336.

Dingman, J.F. and Despointes, R.H. (1956). J. Clin. Endocrin. and Metabol., 16, 936.

Dudley, H.W. (1919). J. Pharmacol., 14, 295-312.

Edstrom, J.E. and Eichner, D. (1958). Nature, Lond., 181, 619.

Eranko, O., Frigberg, O. and Karvonen, M.J. (1953). Acta Endocrin. Kbh., 12, 197-200.

Evans, E.I. (1933). Am. J. Physiol., 105, 287-293.

Farme, F. (1913). Wein klin. Wschr., 26, 1867.

Farrel, G., Fabre, C.F. and Rauschkold, E.W. (1968). An. Rev. Physiol., 30, 557-588.

Fendler, K. (1961). Acta Physiol., Acad. Sci. Hung., 20, 89-92.

Fendler, K., Endroczi, E. and Lissak, K. (1965). Acta. Physiol. Acad. Sci. Hung., 27, 275-278.

Ferguson, J.K.W. (1941). Surg. Gynec. Obstet., 73, 359-366.

Ferguson, D.R. (1965). J. Endocrin., 32, 119-120.

Firth, D.A., Hooper, K.C. (1968). Biochem. J., 108, 510.

Fitzpatrick, R.J. (1961). J. Endocrin., 22, 19-20.

Fitzpatrick, R.J. (1966). In the Pituitary Gland, vol. 3, pp. 453-504. Eds. G.W. Harris and B.T. Donovan. Butterworths, London, Ltd.

Fitzpatrick, R.J. and Walamsby, C.F. (1962). J. Physiol. (Lond.), 163, 13.-14p.

Florey, H. and Walton, A. (1932). J. Physiol., 74, 5-6p.

Folley, S.J. and Knaggs, G.S. (1965). J. Endocrin., 33, 301-315.

Folley, S.J. and Knaggs, G.S. (1966). J. Endocrin., 34, 197-214.

Frank, E. (1912). Berliner klin. Wschr., 49, 293-328.

Frankland, B.T.B., Hollenburg, M.D., Hohe, D.B. and Schater, B.A. (1966). Brit. J. Pharmacol., 26, 502-510.

Fuchs, A.R. (1964). J. Endocrin., 30, 217-224.

Fuchs, A.R., Olsen, Petersen, (1965). Acta Endocrin. Cph., 50, 239-248.

Gaunt, R., Lloyd, C.W. and Chart, J.J. (1957). In Neurohypophysis, pp. 233-251. Ed. H. Heller, N.Y. Acad. Press.

Ginsburg, M. and Brown, L.M. (1957). Brit. J. Pharm., 14, 327-333.

Ginsburg, M. and Heller, H. (1953). J. Endo., 9, 274-282.

Ginsburg, M. and Ireland, M. (1964). J. Endocrin, 30, 131-145.

Ginsburg, M. and Smith, M.W. (1959). Int. Cong. Physiol. Sci., 169, 15-16.

Giroud, C.J.P., Stachenko, J. and Piletta, P. (1958). Ciba Symposium on Aldosterone, pp. 56-72. Eds. A.F. Muller and M. O'Connor, London.

Goldman, H. (1968). Am. J. Physiol., 214, 860.

Goldman, J.K. (1964). Proc. Soc. Exptl. Biol. Med., 117, 164-166.

Grinnell, E.H., Kramer, Duff and Lyon (1968). Endocrin., 83, 199-206.

Haig, A.L., Lloyd, S. Pickford, M. (1965). J. Physiol. (Lond.), 148, 625-632.

Hanström, B. (1952). K. Fysiogr. Sallsk. Lund. Förk., 22, 1-8.

Harris, G.W. (1947). Phil. Trans. B., 232, 385-441.

Harris, G.W. (1955). Neural Control of the Pituitary Gland. Edward Arnold, London.

Harris, G.W. and Pickles, V.R. (1953). Nature, Lond., 172, 1049.

Hartman, J.F. (1958). Z. Zellforsk. Mikroskop. Anat., 48, 291-198.

Heller, H. (1958). In Endo. of Reprod. Ed. C.W. Lloyd, p. 365.

Heller, H. (1961). In Oxytocin. Eds. R. Caldeyro-Barcia and H. Heller, Perganon Press, London.

Heller, H. and Ginsburg, M. (1966). In Pituitary Gland, vol. 3, pp. 330-373. Eds. G.W. Harris and B.T. Donovan. Butterworths, London Ltd.,

Heller, H. and Lederis, K. (1958). Nature (Lond.), 182, 1231.

Heller, H. and Lederis, K. (1969). In Neurosecretion, pp. 35-46. Eds. H. Heller and R.B. Clark. Mem Soc. Endocrin. No. 12.

Hild, W. (1954). Z. Zellforsch., 40, 257-312.

Hild, W. and Zettler, G. (1953a). Pflg. Arch. Ges. Physiol., 257, 169-201.

Hild, W. and Zettler, G. (1953b). Z. ges. exp. Med., 120, 236-243.

Hilliard, J., Spiers, H.G. and Sawyer, C.H. (1968). Endocrin., 82, 157.

Hilton, J.G. (1960). Circulation, 21, 1038-46.

Hilton, J.G., Scian, L.F., Westerman, C.D. and Kruesi, O.R. (1959). Proc. Soc. Exptl. Biol. Med., 100, 523-524.

Hinke, J.A.M. (1965). Circ. Res., 17, 359-371.

Hooper, K.C. (1959). J. Physiol. (Lond.), 148, 283.

Hooper, K.C. (1962). Biochem. J., 83, 511.

Hooper, K.C. (1963). Biochem. J., 88, 398.

Hooper, K.C. (1964). Biochem. J., 90, 584.

Hooper, K.C. (1966a). Biochem. J., 99, 128.

Hooper, K.C. (1966b). Biochem. J., 100, 823.

Hooper, K.C. (1968). Biochem. J., 110, 151.

Hooper, K.C. and Jessuh, D.C. (1959). J. Physiol. (Lond.), 146, 539.

Howell, W.H. (1898). J. Exp. Med., 3, 245-258.

Hume, D.M. (1958). In Pathphysiologia Dioncephalica, pp. 217-228. Ed. S.B. Curri, Springer, Vienna.

James, J.J. and Lee, J. (1965). J. Endocrin, 33, 329-330.

Jard, S. and Morel, F. (1963). Am. J. Physiol., 204, 227.

Kamm, O, Aldrich, T.B., Grote, I.W., Rowe, L.W. and Bugbee, E.P. (1928). J. Amer. Chem. Soc., 50, 573-601.

Knaggs, G.S. (1963). J. Endocrin., 26, 24-25.

Koefoed-Johnson, V. and Ussing, H.H. (1953). Acta Physiol. Scand., 28, 60-76.

Kovack, A.G.G., Monas, E. and Koltay, E. (1965). Acta Physiol. Acad. Sci. Hung., 28, 155-161.

Kramer, J., Grinnell, E.H. and Duff, W.M. (1966). Am. J. Med. Sci., 252, 59-61.

Labhsetwar, A.P., Collins, W.E., Tyler, W.J. and Casida, L.E. (1964). J. Reprod. Fert., 8, 77-83.

Lawson, L.J. and Dragstedt, L.R. (1964). Surg. Forum, 15, 118-120.

Lederis, K. (1962). In Neurosecretion, pp. 227-244. Eds. H. Heller and R.B. Clark. Mem. Soc. Endocrin. No. 12.

Lees, P. and Locke, H.M.F. (1964). J. Physiol. (Lond.), 171, 403-410.

Lembeck, F. (1953). Arch. exp. Path. Pharmak., 219, 197.

Leveque, T.F. and Scharrer, E. (1953). Endocrin., 65, 909-919.

Lindner, E.B., Elmquist, A. and Porath, J. (1959). Nature (Lond.), 184, 1565.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem., 193, 265.

MacArthur, C.G. (1931). Science, 73, 448.

McFarlane, A.S. (1958). Nature (Lond.), 182, 53.

Maffy, R.H., Hays, R.M., Lamdin, E. and Leaf, A. (1960). J. Clin. Invest., 30, 630-641.

Melville, K.I. and Varma, D.R. (1961). Brit. J. Pharmacol., 17, 218-223.

Mialhe-Volosse, C., Koch, B., Ducommun, P. and Fortier, C. (1964). Rev. Can. Biol., 23(4), 469-472.

Moses, A.M. (1963). Endocrin., 73, 230-236.

Moses, A.M., Leveque, T.F., Giambatista, M. and Lloyd, C.W. (1963). J. Endocrin., 26, 273-278.

Nakano, J. and Fisher, R.D. (1963). J. Pharmacol. Exptl. Therap., 142, 206-214.

Orloff, J. and Handler, J.S. (1965). J. Clin. Path., 18, 533-542.

Ortmann, R. (1951). Z. Zellforsch., 36, 92-140.

Ott, I. and Scott, J.C. (1910). Proc. Soc. exp. Biol. N.Y. 8, 48-49.

Palay, S.L. (1955). Anat. Rec., 121, 348.

Paulson, K. (1956). Acta neurovag. Wein, 13, 209-220.

Permutt, M.A., Parker, C.W. and Utiger, R.D. (1966). Endo., 78, 809-819.

Peeters, G. and Coussens, R. (1950). Arch. int. Pharmacodyn., 84, 209-220.

Peeters, G., Stormorken, H. and Vanschoubroek, F. (1966). J. Endocrin., 20, 163-172.

Phelan, E.L. (1966). Am. Heart. J., 71, 50-57.

Pickford, M. (1966). In the Pituitary Gland, vol. 3, pp. 374-398. Eds. G.W. Harris and B.T. Donovan. Butterworths, London.

Pickles, V.R. (1953). J. Obset. Gynaec. Brit. Cwlth., 60, 301-311.

Planel, H., Soleilhavoup, J.P. and Tiscado, R. (1964). Compt. Rend. Soc. Biol., 19, 2177-78.

Porlanova, R., Bissel, E.C. and Sachs, H. (1966). Fed. Proc., 25, 795.

Reindel, F. and Hoppe, W. (1954). Ber. deutsch. Chem. Ges., 87, 1103.

Rosenfeld, M. (1940). Bull. Johns Hopk. Hosp., 66, 398-403.

Rothballer, A.B. (1953). Anat. Rec., 115, 21-41.

Royce, P.C. and Sayers, G. (1958). Proc. Soc. Exptl. Biol. Med., 98, 70-74.

Rubini, M.E., Wolf, A.V. and Moroney, W.H. (1956). Res. Rep. WRAIR. Cited from Wolf (1958). Thirst. Charles C. Thomas, Springfield Ill.

Saameli, K. (1963). Am. J. Obset. Gynecol., 85, 186-192.

Sachs, H. (1960). J. Neurochem., 5, 297-303.

Sachs, H. (1963). Neurochem., 10, 289-97.

Sachs, H. (1963). Neurochem., 10, 299.

Sachs, H. (1966). In Protides of Biological Fluids, pp. 181. Ed. H. Peeters, Elsevier, N.Y.

Sachs, H., Fawcett, C.P. and Haller, E.W. (1967). Proc. 49th Meeting Endo. Soc., 1967, Bal Harbour, Florida

Sala, N.L., Fisch, L. and Swarez, R.L. (1965). Am. J. Obset. Gynecol., 91, 1090-94.

Sawyer, W.H. (1957). In the Neurohypophysis, pp. 171-179. Ed. H. Heller, Butterworths, London.

Sawyer, W.H. (1960). Endocri., 66, 112-120.

Sawyer, W.H. and Mills, E. (1966). In Neuroendocrinology, I, pp. 187-211. Ed. L. Martini and W.F. Ganong. Academic Press, N.Y.

Sawyer, W.H. and Valtin (1965). Endocrin., 76, 999-1001.

Schally, A.V., Lipscomb, H.S. and Guillman, R. (1959). Biochim. Biophys. Acta., 31, 252-254.

Scharrer, E. and Frandson, R.D. (1954). Anat. Rec., 118, 350-351.

Scharrer, E. and Scharrer, B. (1954). Recent Progo. Hormone Res., 10, 183-240.

Sellwood, R.V. and Verney, E.B. (1955). Phil. Trans., 238, 361-396.

Share, L. (1967). Am. J. Med., 42, 701-712.

Sloper, J.C. (1954). J. Anat., 88, 576.

Sloper, J.C., Arnott, D.J. and King, B.C. (1960). J. Endocrin., 20, 9.

Snedecor, G.W. (1956). Statistical Methods. 5th Ed. Ames, Iowa. Iowa State College Press.

Starup, J. and Ostergaard, E. (1966). Acta Endocrin., Kbh., 52, 292.

Tababatake, Y. and Sachs, H. (1964). Endocrin., 75, 934-942.

Taleisnik, S. and Deis, R.P. (1964). Am. J. Physiol., 207, 1394-98.

Tata, P.S. and Gauor, O.H. (1966). Arch. Ges. Physiol., 290, 279-285.

Telegdy, G. and Fendler, K. (1964). Acta Physiol. Acad. Sci. Hung., 25, 359-364.

Tuhhy, H. (1953). Monatsch. Chem., 84, 1011-1020.

van Denmark, N.L. and Hays, R.L. (1952). Am. J. Physiol., 170, 518-521.

van Denmark, N.L. and Hays, R.L. (1953). Endocrin., 52, 634-637.

van den Velden, R. (1913). Berliner Klin. Wschr., 50, 2083-86.

van Dongen, C.F. and Hays R.L. (1966). Reprod. and Fert., 11, 317-323.

van Dongen, C.F. and Hays, R.L. (1966). Endocrin., 78, 1-6.

van Dyke, H.B., Chow, B.F. Greek, H.O. and Rothen, A. (1942). J. Pharmacol., 74, 190-209.

Vaughan, M. (1964). Am. J. Physiol., 207, 1166-68.

Verney, E.B. (1947). Proc. Roy. Soc. B., 135, 25-106.

du Vigneaud, V. (1954-55). The Harvey Lectures series L, 1-26.

du Vigneaud, V., Ressler, C. and Trippet, S.J. (1953). J. Biol. Chem., 205, 949.

Vorherr, H., Bradburg, M.W.B., Hoghough, M. and Kleeman, C.R. (1968). Endocrin., 83, 246.

Walton, A. (1960). In Marshall's Physiol. of Reprod., II, pp. 130-160. Ed. A.S. Parks, Longmans Green, London.

de Weid, D. and Bohus, B. (1966). Nature, 217, 1484-86.

Wolf, A.V. and Eddy, H.A. (1957). Res. Rep. WRAIR, 57-90. Cited from A.V. Wolf (1958). Thirst. Charles, C. Thomas, Springfield, Ill.

Zaks, M.G. (1962). In the Motor Apparatus of the Mammary Gland. Ed. A.T. Cowie. Oliver & Boyd, London.

APPENDICES

APPENDIX 1*

Results of individual experiments determining enzyme activity in the intermediate mitochondrial and supernatant fractions in normal and post-coital animals

* All values for log A_o/A are adjusted to a value corresponding to a tissue concentration of 100 μg

Particulate enzyme results

Experiment	Weight of animals (Kg)	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) blank	Definitive	Nitrogen conc (μg N_2/0.1 ml)	Amount of tissue used (μg)	Ao	A	logAo/A
C	1.5	113	5.7±0.4	-	-	-	-	-	-
	1.9	100	5.7±0.4	18.6±1.1	12.9±1.2	100	434±19	166±18	0.416±0.07
	2.0	109	5.7±0.4	11.7±0	6.0±0.4	60	434±19	266±9	0.28±0.06
C	1.9	137	6.0±0	18.7±0.8	12.7±0.8	100	406±40	245±5	0.22±0.10
	2.0	141	6.0±0	28.0±0	11.0±0	100	406±40	291±45	0.14±0.18
	1.8	130	6.0±0	16.0±0	10.0±0	60	406±40	300±50	0.19±0.20
C	1.6	156	7.0±0	23±0	8.0±0	100	353±25	240±25	0.16±0.13
	1.4	137	7.0±0	21.7±0.4	7.3±0.4	100	413±33	435±0	0±0.08
	1.8	130	7.0± 0	29.5±0.7	11.3±0.7	100	248±34	247±20	0±0.16
C*	2.3	133	4.5±0.7	19.3±0.8	14.8±1.0	50	402±49	406±38	0±0.15
	2.3	162	4.5±0.7	19.3±0.8	14.8±1.0	100	402±49	277±44	0.16±0.2
	2.2	122	4.5±0.7	19.3±0.8	14.8±1.0	150	402±49	328±40	0.03±0.17
	-	-	-	-	-	200	402±49	254±15	0.06±0.14
C	2.0	163	6.0 -	14.7±0.4	8.7±0.4	100	365±56	333±34	0.04±0.18
	2.0	151	6.0 -	16.0 -	10.5 -	100	370±40	341±18	0.04±0.12
	2.0	147	6.0 -	16.5± 0	10.5±0	100	385±34	313±36	0.09±0.14
C*	2.0	160	6.7±0.3	28.5±0.7	10.8±0.8	50	467±25	451±50	0.02±0.12
	2.0	161	6.7±0.3	28.5±0.7	10.8±0.8	50	467±25	474±30	0±0.08
	2.0	183	6.7±0.3	28.5±0.7	10.8±0.8	100	467±25	396±25	0.07±0.08
C	2.8	84	4.5±0.7	17.0±0	12.5±0.7	100	368±50	411±34	0.05±0.15

*Tissue extracts lumped together

Particulate enzyme results (cont.)

Experiment	Weight of animals (Kg)	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) blank	Nitrogen titrations (μg N_2) definitive	Nitrogen conc. (g/ N_2/0.1 ml)	Amount of tissue used (μg)	Ao	A	logAo/A
	2.8	118	4.5±0.7	17.0±0	12.5±0.7	100	411±50	368±34	0.05±0.15
	2.8	118	4.5±0.7	16.5±0.7	12.0±1	100	421±40	454±10	0±0.10
	2.7	115	4.5±0.7	13.0±0	8.5±0.7	100	408±36	325±38	0.09±0.15
C	3.6	117	7.0 -	14.0±0	7.0±0	100	411±68	274±12	0.18±0.17
	4.0	106	7.0 -	21.5±0.7	14.5±0.7	100	404±9	339±30	0.09±0.09
+1 hr	3.2	84	7.0±0	17.0±1.4	10.0±1.4	100	500±20	449±34	0.04±0.09
	2.7	76	7.0±0	14.0±1.3	7.0±1.3	100	416±8	366±53	0.05±0.15
	3.7	98	7.0±0	16.5±0.7	9.5±0.7	100	410±0	391±19	0.04±0.05
+2 hr*	2.65	107	7.5±0	14.5±0.7	7.0±0.7	50	478±43	451±51	0.01±0.14
	2.0	100	7.5±0	14.5±0.7	7.0±0.7	100	478±43	387±31	0.11±0.12
	2.4	130	7.5±0.	14.5±0.7	7.0±0.7	100	478±43	352±60	0.13±0.19
+4 hr*	2.2	81	7.5±0.7	14.2±0.5	6.7±0.9	50	461±50	399±48	0.15±0.16
	2.5	96	7.5±0.7	14.2±0.5	6.7±0.9	100	461±50	403±13	0.06±0.11
	3.2	70	7.5±0.7	14.2±0.5	6.7±0.9	100	461±50	421±18	0.06±0.12
+5 hr	2.8	111	4.5±0.7	13.0±0	8.5±0.7	80	385±28	351±22	0.07±0.09
	2.0	87	4.5±0.7	14.5 -	10.0 -	80	413±17	339±13	0.13±0.06
+9 hr	3.5	97	6.3±0.4	14.7±0.3	8.3±0.6	100	470±50	335±25	0.15±0.13
	3.0	111	6.3±0.4	15.7±1.0	9.4±1	100	467±13	302±9	0.19±0.04
	3.15	118	6.3±0.4	13.0±0	6.7±0.4	100	445±39	326±36	0.13±0.14
+12 hr	2.5	136	8.0±0.7	17.0±0	9.0±0.7	100	439±60	239±21	0.27±0.16
	2.4	132	8.0±0.7	17.7±0.4	9.6±0.8	100	459±20	319±10	0.15±0.05
	2.0	123	8.0±0.7	13.0±0.7	5.0±1	60	400±3	271±27	0.17±0.11

Particulate enzyme results (cont.)

Experiments	Weight of animals (Kg)	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) balank	Definitive	Nitrogen cont. (μg N_2/0.1 ml)	Amount of tissue used (μg)	Ao	A	log Ao/A
	3.1	68	7.0±1.4	12.5±0	5.5±1.4	100	449±29	372±19	0.08±0.08
+24 hr	3.7	91	9.0±1.4	17.5±1.4	8.5±2	100	368±27	392±46	0± 0.14
	3.5	85	9.0±1.4	18.5±2.0	9.5±2.4	100	367±24	265±29	0.14±0.13
	2.7	91	9.0±1.4	16.7±2.5	7.7±2.8	100	375±21	333±19	0.08±0.08
+36 hr	3.7	104	9.5±0.7	22.0±0	12.5±0.7	100	403±55	398±10	0.040.14
	4.9	95	9.5±0.7	17.0±0	7.5±0.7	100	345±31	308±28	0.05±0.13
	3.65	133	9.5±0.7	23.5±0.7	13.5±1.0	100	380±45	281±40	0.16±0.19
+48 hr	3.6	123	4.0±0	18.5±0.7	14.5±0.7	100	426±60	350±56	0.09±0.21
	43	117	4.0±0	21.5±0.7	17.5±0.7	100	423±58	447±12	0±0.13
+72 hr	4.0	96	10.5±0.4	22.5±0.7	12.0±1.0	100	353±32	243±48	0.16±0.20
	3.55	108	10.5±0.4	16.5±0.7	6.0±1	100	437±21	344±90	0.11±0.27
+96 hr	2.7	137	10.5±0	21.5±5.7	11.0±5.7	100	248±39	251±35	0±0.21
	2.5	85	10.5±0	23.7±0.4	13.2±0.4	100	273±40	213±31	0.11±0.20

Supernatant enzyme results

Experiment	Weight of animals (Kg)	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) blank	definitive	Nitrogen conc. (μg N_2/0.1ml)	Amount of tissue used (μg)	Ao	A	log Ao/A
C	1.5	113	5.5±0.4	18.3±0.6	12.8±0.6	100	428±14	174±11	0.34±0.70
	1.9	100	5.5±0.4	22.5±0.4	17.0±0.57	100	313±5	262±25	0.08±0.10
C	1.9	180	6.0±0	12.5 -	6.5 -	100	330±22	171±32	0.31±0.19
	1.8	143	6.0±0	12.7±1	6.7±1	100	442±48	175±23	0.38±0.17
	2.1	130	6.0±0	13.2±0.4	7.2±0.4	100	400±21	190±29	0.31±0.16
C	1.9	137	6.0±0	18.6±0.8	6.3±0.8	100	331±6	187±20	0.22±0.11
	2.0	141	6.0±0	19.2±0.7	6.6±0.7	100	330±44	255±20	0.11±0.15
	1.8	130	6.0±0	19.2±0	6.6±0	100	404±28	266±22	0.15±0.11
C*	2.3	133	4.5±0.7	15±0.4	10.5±0.4	50	427±48	430±40	0±0.15
	2.3	162	4.5±0.7	15±0.4	10.5±0.4	100	427±48	354±33	0.08±0.15
	2.2	122	4.5±0.7	15±0.4	10.5±0.4	150	427±48	240±7	0.13±0.12
C	2.0	163	6.0 -	18.6±0.8	12.6±0.8	100//	295±35	231±27	0.11±0.12
	2.0	151	6.0 -	23.0±0	17.0±0	100	318±13	205±89	0.19±0.43
	2.0	147	6.0 -	24.7±0.4	18.7±0.4	100	308±	223±96	0.14±0.43
C*	2.0	160	6.7±0.3	20.7±0.4	14.0±0.5	50	423±59	389±59	0.13±0.20
	2.0	161	6.7±0.3	20.6±0.4	14.0±0.5	100	423±59	288±32	0.16±0.16
	2.0	133	6.7±0.3	20.7±0.4	14.0±0.5	150	423±59	220±13	0.17±0.15
C	2.8	84	4.5±0.7	15.0±1.4	10.5±1.5	100	370±35	209±34	0.24±0.18
	2.8	115	4.5±0.7	9.5±1.0	5.0±1.0	80	303±34	234±29	0.14±0.15
	2.7	118	4.5±0.7	11.0±0.7	6.5±0.7	100	336±30	236±13	0.15±0.10
C	3.6	106	7.0 -	19.5±2.0	12.5±2.0	100	359±31	237±23	0.18±0.12
	4.0	106	7.0 -	12.0±1.4	5.0±1.4	80	362±27	225±25	0.25±0.14
	2.7	90	7.0 -	12.5±0.7	5.5±0.7	80	348±27	251±11	0.13±0.20

Supernatant enzyme results

Experiment	Weight of animals (Kg)	Weight of hypothalamus (Mg)	Nitrogen titrations (µg N_2) blank	definitive	Nitrogen cont. (µg N_2/0.1 ml)	Amount of tissue used µg	Ao	A	logAo/A
+1 hr	2.7	76	7.0±0	15,5±2.1	8.5±2.1	80	452±28	324±18	0.19±0.09
	3.2	98	7.0±0	14.5±0.7	7.5±0.7	80	395±32	284±15	0.18±0.10
+2 hr*	2.6	107	7.5±0	15.7±1.1	8.0±1.1	50	358±40	365±30	0±0.14
	2.4	130	7.5±0	15.7±1.1	8.0±1.1	100	358±40	249±14	0.16±0.13
	2.0	100	7.5±0	15.7±1.1	8.0±1.1	130	258±40	191±29	0.19±0.19
+4 hr	2.2	81	7.5±0.7	14.0±0.5	6.5±0.9	80	472±43	387±10	0.15±0.09
	3.2	96	7.5±0.7	14.5±0.5	7.0±0.9	100	475±20	353±16	0.13±0.06
	2.5	76	7.5±0.7	15.0±0.5	7.5±0.9	100	460±26	330±21	0.14±0.09
+5 hr	2.8	111	4.5±0.7	11.3±0.4	6.8±0.8	80	301±32	270±25	0.19±0.14
	2.0	87	4.5±0.7	12.4±0.4	7.9±0.8	80	416±21	339±1	0.15±0.05
	3.6	108	4.5±0.7	10.8±0.4	6.3±0.4	80	385±28	319±14	0.15±0.08
+9 hr	3.5	97	6.3±0.4	13.5±0	7.2±0.4	100	365±21	278±41	0.13±0.16
	3.0	111	6.3±0.4	11.7±0.4	5.4±0.6	100	449±30	287±30	0.19±0.11
	3.2	118	6.3±0.4	14.0 -	7.7±0.4	100	444±17	279±27	0.20±0.10
+12 hr	2.5	136	8.0±0.7	18.0±0	10.0±0.7	100	452±34	265±35	0.24±0.15
	2.4	132	8.0±0.7	11.7±0.4	2.7±0.8	100	-	-	-
	2.0	123	8.0±0.7	16.5±0	8.5±0.7	100	384±41	245±31	0.22±0.17
+16 hr	3.1	80	7.0±1.4	15.8±0.4	8.8±1.5	100	-	-	-
	2.7	95	7.0±1.4	13.3±0.4	6.3±1.5	100	398±41	196±13	0.32±0.12
	3.1	68	7.0±1.4	16.8±0.4	9.8±1.5	100	410±41	301±32	0.14±0.14
+20 hr	3.7	86	5.3±0.4	11.7±0.4	6.4±0.6	80	411±35	283±12	0.24±0.03
	3.1	101	5.3±0.4	14.0 -	8.7±0.4	-	-	-	-

Supernatant enzyme results

Experiment	Weight of animals (Kg)	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) blank	Nitrogen titrations (μg N_2) definitive	Nitrogen cont (μg N_2/0.1 ml)	Amount of tissue used (μg)	Ao	A	log Ao/A
[illegible]	2.8	84	5.3±0.4	14.0±0.7	8.7±0.8	80	420±12	332±11	0.18±0.04
+24 hr	3.7	91	9.0±1.4	13.7±0.4	4.7±1.5	100	-	-	-
	3.5	85	9.0±1.4	15.2±0.4	6.2±1.5	100	432±35	312±18	0.14±0.10
	2.7	91	9.0±1.4	18.5±0.7	9.5±1.6	100	413±37	319±9	0.12±0.09
+36 hr	3.7	104	9.5±0.7	17.5±0.7	8.0±1.0	100	361±25	350±10	0.01±0.07
	4.9	95	9.5±0.7	17.5±0.7	8.0±1.0	100	306±61	277±57	0.04±0.29
	3.7	133	9.5±0.7	18.5±0.7	9.0±1.0	100	261±22	221±21	0.07±0.12
+48 hr	3.6	123	4.0±0	10.5±0.7	5.5±0.7	100	423±33	343±44	0.09±0.16
	4.3	117	4.0±0	19.0±2.8	15.0±2.8	100	430±40	339±19	0.10±0.11
+72 hr	4.0	96	10.5±0.4	15.0 -	4.5 -	100	374±23	213±16	0.24±0.09
	3.5	108	10.5±0.4	17.0±1.4	5.5±1.5	100	327±32	254±14	0.11±0.09
+96 hr	2.7	137	10.5±0	18.7±0.4	8.2±0.4	100	475±17	363±52	0.12±0.14
	2.5	85	10.5±0	17.8±1.8	7.3±1.0	100	428±22	210±19	0.31±0.10

APPENDIX 11

Results of individual experiments measuring enzyme activity in:

(1) Control animals using oxtocin as substrate in (a) the particulate fraction and (b) in the supernatant fraction

(2) Control animals using vasopressin as substrate in (a) the particulate fraction and (b) the supernatant fraction

(3) Dehydrated animals using vasopressin as substrate in (a) the particulate fraction and (b) the supernatant fraction

(4) Overhydrated animals using vasopressin as substrate in (a) the particulate fraction and (b) the supernatant fraction

(5) Stressed and overhydrated animals using vasopressin assubstrate

(1) Enzyme activity in control animals using oxytocin as substrate (a) particulate enzyme

Experiment	Weight of animals (Kg)	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) blank	Nitrogen titrations (μg N_2) definitive	Nitrogen conc (μg N_2/0.1ml)	Amount of tissue used (μg)	Ao	A	logAo/A
1	3.1	83	15.5±0	27.3±1.1	11.1±1	100	469±4	366±14	0.07
2	3.3	89	11±0	31.2±0.3	20.2±0.3	60	424±40	404±15	0.03±0.10
	3.6	80	"	"	"	120	"	373±17	0.05±0.10
	3.0	96	"	"	"	180	"	363±23	0.06±0.11
	3.7	106	-	-	-	-	-	-	-
	2.9	82	-	-	-	-	-	-	-
3	3.1	85	14.5±0.5	24.8±0.8	20.3±0.1	50	402±49	406±38	0±0.15
	3.4	83	"	"	"	150	328±40	329±40	0.09±0.17
	3.3	85	"	"	"	-	-	-	-

(1) Enzyme activity in control animals using oxytocin as substrate (b) supernatant enzyme

Experiment	Weight of animals (Kg)	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) blank	definitive	Nitrogen conc (μg N_2/0.1 ml)	Amount of tissue used (μg)	Ao (a) tissue (b) no tissue	A	A	log
1	3.1	83	15.5±0	27.5±0.7	12.0±0.7	100	469±4	329	0.15	
	3.3	89	"	"	"	150	"	290±12	0.21±0.08	
2	3.0	98	11.0±0	34.5±0	23.5±0	60	404±52	358±45	0.06±0.14	
	3.6	80	"	"	"	120	436±42	290±28	0.16±0.11	
	3.0	97	"	"	"	180	-	217±16	0.29±0.09	
	3.7	107	-	-	-	-	-	-	-	
	2.9	82	-	-	-	-	-	-	-	
3	3.1	85	14.5±0.5	34.7±1.6	20.2±1.7	100	428±48	294±15	0.16±0.12	
	3.4	83	"	"	"	150	-	240±7	0.27±0.11	

(2) Enzyme activity in control animals using vasopressin as substrate (a) particulate enzyme

Experiment	Weight of animals (kg)	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) blank	Nitrogen titrations (μg N_2) definitive	Nitogen conc. (μg N_2/0.1 ml)	Amount of tissue used (μg)	Ao (1)tissue (2)no tissue	A	logAo/A
4	4.4	124	9.0±0.2	17.0±0	6±0.2	50	310±22	235±15	0.10±0.19
	3.8	127	"	"	"	100	312±23	210±8	0.17±0.08
	3.5	130	"	"	"	-	-	-	-
	3.5	100	"	"	"	-	-	-	-
5	2.9	104	12.5±0	35.5±0.7	23.5±0.7	60	292±58	209±15	0.123±0.08
	2.6	84	"	"	"	120	278±31	158±27	0.25±0.17
	2.8	96	"	"	"	180	-	143±39	0.29±0.28
	2.6	127	"	"	"	-	-	-	-
6	2.9	107	9.7±1.1	19.0±1.1	9.3±1.1	50	338±30	280±11	0.08±0.05
	3.1	89	"	"	"	50	324±20	283±10	0.08±0.05
	3.3	95	"	"	"	80	-	261±23	0.11±0.08
	3.8	97	"	"	"	80	-	252±15	0.11±0.07
	2.8	81	"	"	"	-	-	-	-
7	3.1	111	13.3±0	25.0±0	21.7±0	24	267±31	266±17	0±0.07
	3.2	109	"	"	"	36	274±14	243±31	0.05±0.09
	2.9	100	"	"	"	48	-	228±16	0.10±0.07

(2) Enzyme activity in control animals using vasopressin as substrate (b) supernatant enzyme

Experiment	Weight of animals (Kg)	Weight of hypothal-amus (mg)	Nitrogen titrations (μg N_2) blank	definitive	Nitrogen conc (μg N_2/0.1 ml)	Amount of tissue used (μg)	Ao (1) tissue (2) no tissue	A	logAo/A
4	4.4	125	9.0±0.2	37.5±0.8	28.5±0.7	50	423±42	371±25	0.06±0.12
	3.8	127	"	"	"	100	–	278±27	0.17±0.13
	3.5	130	"	"	"	150	–	310±22	0.14±0.12
	3.5	102	"	"	"	–	–	–	–
5	2.9	104	12.5±0	61.7±0.6	49.2±0.6	60	319±30	290±29	0.05±0.10
	2.6	85	"	"	"	120	330±21	204±28	0.20±0.13
	2.8	96	"	"	"	180	–	157±11	0.31±0.07
	2.6	127	"	"	"	–	–	–	–

(3) Enzyme activity in dehydrated animals using vasopressin as substrate (a) particulate enzyme

per-ent	Weight of animals (Kg)	Loss of weight %	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) blank	definitive	Nitrogen conc (μg N_2/0.1 ml)	Amount of tissue used (μg)	Ao (1) tissue (2) no tissue	A	logAo/A
8	3.9	-14	115	12.5±0.7	39.0±0	26.5±0.7	21	286±31	278±21	0.01±0.13
	4.0	-12	109	"	"	"	42	-	231±3	0.10±0.11
	4.2	-9	129	"	"	"	63	-	195±44	0.17±0.25
9	3.2	-15	102	13.0±1.4	20.0±0	7±1.4	35	256±34	180±12	0.12±0.14
	3.3	-25	102	"	"	"	50	214±18	131±8	0.22±0.14
	3.0	-9	96	"	"	"	70	-	140±10	0.22±0.13
10	3.2	0	121	17.5±0.1	41.0±1.0	23.5±1.0	75	227±7	92±6	0.41±0.08
	3.4	-10	123	"	"	"	100	245±19	201±9	0.07±0.07
	4.1	-8	125	"	"	"	125	-	199±18	0.07±0.11
	-	-	-	-	-	-	150	-	209±7	0.05±0.06
11	3.3	-2	107	15.5±0.7	26.0±1.0	11.5±1.2	75	306±18	207±13	0.18±0.09
	3.2	-13	116	"	"	"	100	218	211±15	0.16±0.09
	3.72	-20	137	"	"	"	-	-	-	-
12	3.0	-9	95	14.0±0	27.0±0	14±0	54	289±33	217±19	0.12±0.09
	2.8	-9	116	"	"	"	-	283±41	-	-
	2.7	-7	94	"	"	"	-	-	-	-

(3) Enzyme activity in dehydrated animals using vasopressin as substrate (b) supernatant enzyme

Experiment	Weight of animals (Kg)	Loss of weight %	Weight of hypothalamus (mg)	Nitrogen titrations (μg N_2) blank	Nitrogen titrations (μg N_2) definitive	Nitrogen conc. (μg N_2/0.1ml)	Amount of tissue used (μg)	Ao (1) tissue (2) no tissue	A	logAo/A
8	3.9	-14	115	12.5±0.7	27.1±0.3	14.6±0.3	98	316±18	175±25	0.15±0.15
	4.0	-12	109	"	"	"	196	299±20	67±4	0.66±0.07
	4.2	-9	129	"	"	"	-	-	-	-
9	3.2	-15	102	13.0±1.4	34±1.5	21.3±2	50	515±33	297±15	0.24±0.05
	3.3	-25	102	"	"	"	100	525±62	261±14	0.30±0.06
	3.0	-9	96	"	"	"	150	-	151±33	0.58±0.24
12	3.0	-9	85	14.0±0	19.5±0	5.5±0	50	279±26	212±24	0.16±0.12
	2.8	-9	116	"	"	"	70	293±18	159±20	0.26±0.13
	2.7	-6	94	"	"	"	80	-	98±12	0.33±0.09

(4) Enzyme activity in overhydrated animals using vasopressin as substrate (a) particulate enzyme

Experiment	Weight of animals before treatment (Kg)	Weight of animals post-treatment (Kg)	Weight of hypothalam (mg)	Nitrogen titrations (μg N_2) Blank	definitive	Nitrogen conc. (μg N_2/0.1 ml)	Amountof tissue used (μg)	Ao	A	log Ao/A
13	2.5	2.5	120	0.13±0	37.0±0	24.0±0	48	248±17	277±18	0±0.07
2.3	2.3	2.3	124	"	"	"	96	261±12	206±22	0.0[illegible]±0.11
	2.7	2.8	93	"	"	"	125	-	220±15	0.06±0.0[illegible]
	2.8	2.7	94	"	"	"	144	-	159±[illegible]	0.21±0.07
14	-	3.1	110	0.14±0.1	21.0±2	7±2	42	258±32	256±18	0±0.07
	-	-	-	-	-	-	-	255±38	274±16	0±0.06

(4) Enzyme activity in overhydrated animals using vasopressin as substrate (b) supernatant enzyme

13	2.5=	2.5	120	0.13±0	25.0±0	12.0±0	48	280±65	215±19	0.06±0.13
	2.3	2.3	124	"	"	"	96	245±35	106±6	0.34±0.11
	2.7	2.8	93	"	"	"	144	_	66±3	0.57±0.11
	2.8	2.7	94	"	"	"	196	_	0	+
14	1-	3.1	110	0.14±0.1	25.0±0.4	11±0.6	50	318±37	253±19	0.09±0.08
	-	-	-	-	-	-	-	305±34	-	-

(5) Enzyme activity in stressed and overhydrated animals using vasopressin as substrate

Animal	Weight of animals prior to treatment	Weight of animals post treatment	Weight of hypothalami
1	4.1	4.5	118
2	3.5	3.2	95
3	3.1	2.9	85

Nitrogen titrations

blank 4.5±0.4±0

particulate 15.2±0

supernatant 16.2±0.7

Amount of tissue used (g)	Blank Ao (1) tissue (2) no tissue	A	log Ao/A	Amount of tissue used (g)	Ao (1) tissue (2)no tissue	A	log Ao/A
30	313±11	320±9	0	25	326±37	337±36	0
100	304±14	203±10	0.19±0.05	70	308±19	99±10	0.49
150	-	150±14	0.32±0.10	80	[illegible]	101±12	0.48