STUDIES OF THE SHEEP NEUROHYPOPHYSIS DURING
PREGNANCY AND FOETAL DEVELOPMENT

by

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We accept this thesis as conforming to the
required standard

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Date Sept. 24, 1968
ABSTRACT

The biological activities of the neurohypophyses of pregnant and embryonic sheep, Ovis aries were studied. The levels of hormonal activities of the neurohypophyses varied with the stage of pregnancy, and were always lower than levels in the control (non-pregnant) glands. Chemical studies of the purified oxytocic and vasopressor moieties of the pregnant sheep have indicated that these agents are oxytocin and arginine vasopressin.

An increase of biologically active agents of the posterior pituitary was detected concomitant with embryonic development. The amount of vasopressor activity greatly surpassed the oxytocic activity in the neurohypophyses of foetal sheep all through gestation, but the difference became smaller with the advancement of intrauterine life. Pharmacological and chemical studies indicated oxytocin and arginine vasopressin as the active peptides of the foetal neurohypophysis. In addition to these two neurohypophysial peptides characteristic of the mammals, the presence of a third peptide was indicated. Pharmacological studies indicate that this third neurohypophysial peptide of the foetuses may be arginine vasotocin, the antidiuretic principle of lower vertebrates.
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ABBREVIATIONS USED

ala - alanine
arg - arginine
asp - aspartic acid
cyS - cystine
cyt - cysteic acid
glu - glutamic
gly - glycine
his - histidine
ileu - isoleucine
leu - leucine
lys - lysine
met - methionine
pro - proline
phe - phenylalanine
ser - serine
tyr - tyrosine
thre - threonine
val - valine
PLATE I

Diagram of the pituitary of the sheep

Ovis aries.

Mid sagittal plane.

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INTRODUCTION

The Hypothalamo-Hypophyseal System of the Adult Mammal

Scharrer (1944, 1954) and Bargman (1951) using Gomori's chrom-alum haematoxylin phloxin stain, demonstrated the presence of neurosecretory granules in the supraoptic and para-ventricular nuclei of the hypothalamus. These same granules were distributed along the axons of nerve fibers, leading from the nuclei to the pars nervosa, and within the pars nervosa itself. Further histological studies following sections of the tract from the hypothalamus to the posterior lobe, showed accumulation of secretory material rostral to the cut, indicating the direction of movement of the neurosecretory material. These workers formulated the generally accepted hypothalamo-hypophyseal concept of the posterior pituitary, which pointed to the hypothalamus as the place of synthesis, and the pars nervosa as the storage organ for the neurohypophyseal hormones.

The physiological actions of posterior lobe extracts were discovered just before the turn of the century. Injections of the extracts caused elevation of blood pressure (Oliver and Shafer, 1895), contractions of the uterus (Dale, 1906) and ejection of milk from the mammary gland (Ott and Scott, 1910). The active agents were separated in 1928 by Kamm, Aldrich, Grote, Rowe and Bugbee, identified and synthetised by
The active principles were identified as two cyclic octapeptides, oxytocin and vasopressin; they shared a molecular weight of approximately 1,000, but differed from one another in two amino acids.

The primary action of oxytocin is on the mammary gland during lactation, where it is responsible for the ejection of milk, as a response to suckling by the young. Its ability to contract the uterus is also well demonstrated, and it appears to cause rhythmic contractions during labour. Various secondary actions can be elicited by the administration of oxytocin, and among them are the following: dilation of blood vessels in the extremities, increased glomerular filtration rate in the kidney, and a possible capacity to enhance electrolyte excretion by the kidneys (Pickford, 1960, 1964). These secondary responses were elicited by pharmacological dose levels of the hormone; consequently, their importance to the organism under physiological conditions is far from proven.

The first action of vasopressin to be discovered was its pharmacological action in causing constriction of blood vessels (Oliver and Shafer, 1895); this property of vasopressin is still commonly used to detect and assay the peptide in extracts of the posterior lobe. The physiological significance of vasopressin, however, lies in its very potent anti-diuretic effect. It promotes water reabsorption by acting on the distal tubules and collecting ducts of the
kidney. Besides its action on membranes in the kidney, it is attributed with the capacity to act similarly on membranes of other organs. Wakim (1966) has summarized the available evidence for some of the extrarenal actions elicited by the hormone: after injections of vasopressin there is a marked fall in the excretion of fluid from the pancreas, from the liver, and from the sweat glands. Water absorption through the isolated frog skin is also increased in the presence of this hormone (Ussing and Anderson, 1957). The accumulated data shows that vasopressin acts on the membranes of widely varied organs, with the same end result - waterconservation.

The ratio of vasopressin/oxytocin (V/O) is approximately one in the posterior lobe of most mammals. The hormones are stored attached to, and probably released accompanied by, a large protein molecule (M.W. 25000) named neurophysine (Heller and Ginsburg, 1966). On each neurophysine molecule there is one binding site for oxytocin and another for vasopressin. The sites are specific for the terminal amino acids of the respective octapeptide (Ginsburg, 1964). Binding of the terminal amino acids does not inactivate the hormones, the complex exhibits both vasopressor and oxytocic activities (van Dyke, et al., 1954).

The existance of a common carrier for the two octapeptides, raises one of the most interesting, and still unresolved questions, concerning the mechanism of release of the hormones. The release of one neurohypophyseal peptide is usually accompanied by the release of the other, in accord with the existance of a common carrier, however,
under many circumstances one of the hormones is released in much greater quantities than the other. Following haemorrhage in anaesthetised rats (Heller, 1961), or the severe dehydration of dogs and rats (Pickford, 1964), a preferential release of antidiuretic hormone occurs. On the other hand, lactation appears to preferentially deplete the gland of its stored oxytocin. The mechanism by which this differential release is accomplished is under extensive investigation (Ginsburg, 1964; Dicker, 1966), but remains unresolved at the present.

The Neurohypophysis during Pregnancy and Lactation

During pregnancy, an additional burden is placed on the water regulatory system, and it is rather crucial to keep the uterus quiescent, to prevent expulsion of the foetus. Considering that the main actions of the neurohypophysial hormones are on water regulation and on the uterus, a change in the level of stored neurohypophysial hormones would not be unexpected. However, direct studies on rat neurohypophyses by Acher, Chauvet and Olivry (1956) and by Heller and Lederis (1959), failed to show any changes in either the mean hormonal content of the gland, or in the vasopressor/oxytocic (V/O) ratios at any stage of pregnancy. Their studies did not include animals shortly before, or at parturition.

Although no changes in the neurohypophysial hormone levels were detected during pregnancy in the rat, other evidence (Heller, 1957) indicates an influence of the reproductive cycle on the level of the posterior pituitary
hormones. In the rat the amounts of vasopressin and oxytocin were found to rise during the follicular phase and to drop at metestrous. In the same species, the diuretic response to water was also impaired immediately after cessation of estrous (Ginsburg, 1950 as quoted by Heller, 1961).

Lactation depletes the posterior pituitaries of dogs and rats (van Dyke, Adamson and Engel, 1955; Heller and Lederis, 1959) of its oxytocic and vasopressor activities. Some authors have reported that the V/O ratio rises due to a preferential depletion of oxytocin (Acher, Chauvet, Olivry, 1956; van Dyke et al., 1955) but others have found it unaltered, (Maculay, Landgrebe and Waring 1950). Heller (1961) have pointed out that those workers who utilized acetone dried glands observed a rise in V/O ratio, but those who worked on fresh glands found the ratio unaltered. He has suggested that the apparent contradiction in these results can be explained on the basis of a preferential solubility of oxytocin in acetone.

The Hypothalamo-Hypophysial System of the Mammalian Foetus

Histological studies of the embryonic development of the hypothalamo-hypophysial system are far more numerous than studies of its hormonal content. Bernischke and McKay (1953) and Meitner (1961), working in the human, found the first neurosecretory material in the hypothalamic nuclei in the fourth month of gestation. Only after a further three weeks of development, could secretory material be detected in the
posterior lobe of the pituitary. These observations are confirmed by studies in the cow (Kivalo and Talanti, 1957), mouse, rat and dog embryos (Yakovleva, 1966). In all the mammalian foetuses so far studied, some of the workers also noted an earlier appearance and greater abundance of secretory material in the supra-optic than in the paraventricular nucleus (Yakovleva, 1966). These findings point to an earlier embryonic development of the hypothalamic nuclei than the posterior lobe of the pituitary, and to an earlier appearance of the supra-optic than the paraventricular nucleus.

There is little evidence available concerning the biological activities present in the foetal neurohypophysis. Oxytocic and vasopressor activities have been detected by Dicker and Tyler (1953) in the posterior pituitaries of dog, cat and human foetuses. They found a ratio of vasopressor to oxytocic activity as high as 28 to 1 in the human foetus at 112 days of gestation. At 190 days of intrauterine life the ratio falls to 5 and reaches unity at birth. In the cat where the V/O ratio in the adult is 1.2, the ratio at 54 days of gestational age is 4.7, and at 21 days of post partum is still as high as 3.3. These results illustrate the predominance of vasopressor activity over the oxytocic activity during intrauterine life. The vasopressor activity remains higher until birth in human and even long after birth in the cat. It is suggested that the relative maturity of the animal at birth determines how near to unity the ratio between
these two active principles is at this time.

Although in the human the studies of the neurohypophysis during embryonic development, performed by Dicker and Tyler, are relatively complete, human tissue usually is available only a considerable time after death. In view of the very rapid depletion of the posterior pituitary of its active agents following death, a general breakdown of tissues post mortem could have contributed to the low potencies and high ratios reported by these authors in the human foetuses. Further doubt is cast on these results by Heller (1961), who again suggests, that these high ratios, at least in part, could be attributed to the effect of acetone, used in collecting the glands: "The proportion of vasopressor to oxytocic activity in immature posterior pituitary glands have been reported to be very high. But Heller and Lederis could recently show, that in infant rats such high ratios were obtained only when glands were treated with acetone before the usual extraction in dilute acid."

No attempts have been made previously, to identify the active agents in the foetal neurohypophysis, by either pharmacological or chemical means. The only indication to the possible nature of these active peptides are the studies of Heller (1959), performed in immature rats. By paper-chromatographic studies he identified the active agents in the immature rat neurohypophysis as oxytocin and vasopressin.

**Statement of the Problem**

Previous work on the pharmacology of the neuro-
hypophysis during pregnancy, is limited to one species only, the rat. The neurohypophyses of very few species have been studied during their foetal development. In the foetus, only the results obtained in the human are at all extensive, but they are of uncertain value, since the tissues may well have been obtained a considerable time after death. Only the studies in the cat are clearly reliable, and these are limited to one gestational age only.

In the work performed here, investigations were extended to a further species, the sheep, Ovis aries. All tissues were dissected immediately after death and at known stages of pregnancy. Healthy foetuses were delivered by caesarian section, and their neurohypophyses were compared directly with those of their mothers. Due to the considerable doubt cast on results obtained in acetone dried glands, parallel studies were carried out on acetone dried and lyophilised tissues.

Since there is no previous data on the chemical nature of the active agents in the foetus, preliminary efforts were also directed towards the identification of these agents.
MATERIALS AND METHODS

Collection and Storage of Materials

Posterior pituitaries from sheep *Ovis aries* of the Clunrose breed were collected in the 1964 and 1965 breeding seasons. The samples were taken from 3 year old pregnant females and from their foetuses, at known gestational ages, together with controls from non-breeding 3 year old females.

The animals were anaesthetised by intravenous injection of Chloralose (30 mg/Kg as a 40 mg/ml solution), an anaesthetic reported to be without any effect on the hormone content of the posterior pituitary (Ginsburg and Brown, 1956).

The foetuses were delivered by Caesarian section and the placenta tied off and cut. The posterior lobe of the pituitary was removed from the pregnant female and the foetus in the following manner:

The superficial skin was removed with a scalpel, the cranium cut with a Desouter Saw, to expose the entire brain. The frontal end of the brain was lifted up, and proceeding caudally the optic nerves, the infundibular stem, the cranial nerves, and all other connections to the cranial floor were severed, exposing the pituitary. Then the entire pituitary was removed from the sella turcica, and separated into the anterior and posterior lobes. The posterior lobes
were dried either in acetone (1964 season) or lyophilised (1965 season).

The acetone dried tissues were dissected into acetone which had been dried over CaCl₂. The acetone was decanted and exchanged at least three times during the first 24 hours. The glands were then subjected to prolonged acetone extraction by being stored for 12-14 months in dry acetone at room temperature. After removal from the fluid, the glands were exposed to air to remove residual acetone, and then sealed into glass tubes and stored at 4°C until the time of extraction.

Lyophilised tissues were dissected into glass tubes submerged in a dry-ice methanol mixture. The frozen samples were placed on a vacuum pump, evacuated at 0.1 mm Hg pressure for 24 hours, and stored at room temperature over P₂O₅ until the end of the season. The samples were sealed at the end of the season and stored at 4°C until extraction.

Extraction

Single glands of adults or foetuses were homogenized in 0.25% acetic acid at 2 mg/ml with a Thomas Extractor. The homogenate was heated for 3 minutes in a boiling water-bath, cooled, and filtered on Whatman #1 filterpaper. The extracts were immediately assayed and the remaining solution stored in the refrigerator at 4°C.

Estimation of Biological Activities

The biological activity of crude extracts, and
purified products, were estimated by means of four assay methods.

a. Isolated rat uterus assay for oxytocic activity.
b. Rat Vasopressor assay
c. Rat antidiuretic assay
d. Frog waterbalance assay

In all types of bioassay Holton's (1948) "4 point" method was used for more accurate determination of potency (and statistical estimation of possible errors).

a. Isolated rat uterus oxytocic assay

Method, as described by Holton (1948) and modified by Munsick, 1960: Virgin albino females of the Wistar strain were selected in full or pro-oestrous, by microscopic examination of their vaginal smears. The rats were stunned with a blow on the head and killed by decapitation. The uterus, exposed by a midventral and two sagittal incisions, was carefully dissected, with the ovaries intact and part of the vagina attached. The dissected uterus was transferred into a petri-dish containing van Dyke-Hastings solution (composition follows), all remaining fat and connective tissue removed, and the uterus divided into its two horns. The vaginal end of one horn was tied with silk thread to a glass hook, pulled from an air supply tube. A longer piece of thread was sewn through the ovary. The glass hook with the uterus was then immersed in a 5 ml muscle bath containing van Dyke-Hastings solution, and
connected to a supply of 5% CO₂-95% O₂. The free end of the thread sewn to the ovary was secured to a carefully balanced writing lever, which in turn rested against the smoked drum of a Palmer Kymograph.

The muscle bath was connected to a reservoir system for van Dyke-Hastings solution, and the whole complex was kept at constant temperature (32°C) in a water-bath by a Bromwell Thermomix pump.

At 5 minute intervals, doses of standard (Syntocinon, 10 IU/ml, Sandoz) alternated with doses of unknown extract were pipetted into the muscle bath. After the response reached its maximum, the van Dyke-Hastings solution in the muscle bath was exchanged with fresh solution from the reservoir, by means of a rubber bulb attached to the reservoir.

Two types of van Dyke-Hastings solution were used to support the uterus, differing from one another only in the presence or absence of Mg²⁺ (Munsick, 1960).

The van Dyke-Hastings solution was prepared fresh daily by the use of two stock solutions.

(1) **van-Dyke Hastings solution without Mg²⁺**

<table>
<thead>
<tr>
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<tr>
<td>NaCl</td>
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<tr>
<td>NaHCO₃</td>
<td>51.170g</td>
</tr>
<tr>
<td>KCl</td>
<td>9.082g</td>
</tr>
<tr>
<td>Phenol red Na salt</td>
<td>0.054g</td>
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</table>

Combined, made up to 2 liters in dist H₂O
Stock Solution B

Na$_2$HPO$_4$ 22.714g up to 1 liter with dist H$_2$O
NaH$_2$PO$_4$ 5.520g up to 1 liter with dist H$_2$O

Approximately equal volumes of NaH$_2$PO$_4$ and Na$_2$HPO$_4$ were mixed, and the pH adjusted to 7.4

The final mixture in the reservoir contained:

200 ml of stock solution A
20 ml of stock solution B
2 ml of 0.5 M CaCl$_2$
1 g dextrose

Combined, and made up to 2 liters with dist H$_2$O

(2) van-Dyke-Hastings solution with Mg$^{++}$

Preparation of the van Dyke-Hastings solution with Mg$^{++}$ is identical to the one described for van Dyke-Hastings solution without Mg$^{++}$, with the exception that the final mixture in the reservoir contains

200 ml of stock A
20 ml of stock B
2 ml of 0.5 M CaCl$_2$
1 g dextrose

And an additional:

2 ml of 0.5 M MgCl$_2$

Combined, made up to 2 liters in dist H$_2$O

b. Rat vasopressor assay

The pressor activity of posterior lobe extracts was
determined as described by van Dyke, Adamson and Engel, 1955.

Wistar rats were anesthetised by subcutaneous injections of urethane (175 mg/100g body wt) and Dibenzylene (0.5 mg/100g). Dibenzylene, an adrenergic blocking agent was used to lower and stabilize the blood pressure, preventing interference with the pressor assay by various adrenergic pressor and depressor agents.

The rats were ready for surgery in 30-45 minutes, following the administration of the anaesthetic.

The skin on the throat was cut, the jagular vein located and freed from connective tissue. Two ligatures were placed around the vein, and the one distal to the heart tied. The vein was punctured with a 22 guage needle, a cannula (PE 10 Intermedic) inserted, and secured with the two ligatures. The carotid artery and trachea were cannulated in the same manner, using PE 50 Intermedic tubing for the artery and PE 250 for the trachea.

To prevent formation of blood clots during the assay 0.5 mg Heparin solution (in 0.9% NaCl) was injected per 100 g body wt. intravenously.

Injections of standard and unknown solutions were administered through the cannula in the jagular vein. The blood pressure was recorded from the carotid cannula by means of a Stratham 23AA transducer, coupled to a Beckman dynograph recorder.

The assay was carried out by injections of two doses
of standard (Pitreesin 20 IU/ml, Parke Davies Co.) matched for magnitude of responses by two doses unknown solution, at 10 minute intervals. For estimation of potency Holton's "4 point" statistical method was applied.

c. Rat antidiuretic assay

The antidiuretic potency of posterior pituitary extracts was estimated in anesthetised rats by the method of Sawyer, (1961).

Male albino rats of Wistar strain were located to 5% of their body weight with 12% ethanol, by means of a stomach tube. Anesthesia occurred between 25-40 minutes. To ensure complete insensitivity to the surgical procedure, Xylocaine Hydrochloride (2% solution, without epinephrine, Astra Pharmaceuticals) was applied locally to the areas of incision.

The jugular vein and trachea were cannulated as described for the pressor assay. The bladder was cannulated in the following manner: ½ inch midventral incision was made through the skin and abdominal muscle approximately ½ inch anterior to the penis. The bladder was eased through the incision and cut in a relatively avascular area. The end of a PE 200 Intermedic cannula, which had been flared by heat, was inserted through the incision and tied into place with a ligature close to the urethral exit. To ensure no loss of urine during the assay, the penis was tied with another ligature. The incision in the abdominal wall was sutured.

The prepared rat was hydrated up to 8% of its body weight, and the lost urine replaced throughout the assay with
a solution of 1.5% ethanol in 0.5% NaCl, by way of a stomach tube.

The cannula from the bladder was connected to a Palmer drop counter and recorder. Responses to standard (Pitressin 20 IU/ml Parke Davies Co.) and unknown solutions were recorded on the smoked drum of a Palmer Kymograph, as a decrease in the number of drops per unit time.

Injections were administered through the cannula leading to the jugular vein at times when the urine flow had returned to the baseline level. For estimation of potency, the 4 point statistical analysis of Holton was employed.

d. Frog waterbalance assay

Frog waterbalance assay was carried out according to Sawyer 1960. The apparatus for the assay consisted of an open glass tube, flared at one end. Near the other end a glass rod was sealed to the tube to form a handle. This glass tube was suspended in a larger outer container into which a glass tube was pulled near the base, to be connected to an air supply.

To carry out the assay, frogs of the species _Rana catesbiana_ were pithed, the bladder exposed and removed. The dissected bladder was divided into its two lobes, the cut end of a half bladder was pulled over the flared end of the glass tube, and secured onto it with a silk thread. The glass tube was filled with 5 ml of dist $H_2O$ and suspended in 25 ml frog Ringers solution contained in the outer bath (composition follows).

The bladders were weighed every 15 minutes and were
considered adequate for assay when they showed a very slow steady loss of weight.

Doses of standard (Syntocinon 10 IU/ml, Sandoz) or unknown solution were administered into the outer bath. The rate of water-loss from the tube through the bladder was measured by weighing the glass tubes with the attached bladders every 15 minutes throughout the assay. The weight loss during the period of 15-45 minutes following the addition of the standard or the pituitary extract was considered the response. After 45 minutes the frog Ringer's solution in the outer bath was exchanged with fresh solution and the bladder was allowed 45 minutes to recover in which time the rate of water-loss returned to the baseline level.

The Ringer's solution used in the frog waterbalance assay was prepared from two stock solutions.

**Stock solution A**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>94.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>3.7 g</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>5.3 g</td>
</tr>
</tbody>
</table>

**Stock solution B**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>2.0 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>40.0 g</td>
</tr>
<tr>
<td>glucose</td>
<td>4.0 g</td>
</tr>
<tr>
<td>phenol red</td>
<td>12.0 mg</td>
</tr>
</tbody>
</table>

50 ml of stock solution A and stock solution B were mixed and made up to 1 liter in distilled H₂O
Standards

The synthetic oxytocin preparation, Syntocinon (Sandoz) used as standard in the rat uterus and frog water-balance assays, was assayed against the U.S. International Standard. The potency was in good agreement with that stated by the company and no adjustment of the results was necessary.

The commercial vasopressin preparation, Pitressin, used as standard in the rat vasopressor and antidiuretic assays is a partially purified mixture of arginine and lysine vasopressin. The two peptides differ in their respective vasopressor and antidiuretic potencies, and no information is available from the company as to the ratio of the two peptides present in a particular preparation of Pitressin. Pitressin, assayed against the U.S. International Standard was found to have a lower antidiuretic than vasopressor activity. Correction in the results were made to compensate for this discrepancy.

Methods of Purification

For the separation of biologically active principles from the crude extracts two methods were applied:

a. Gel filtration

b. Paper chromatography

a. Gel Filtration

Purifications of the active principles was achieved by passing the posterior lobe extracts through a (Dextran) column of Sephadex G 15 (Pharmacia, Uppsala).
The procedure used was as follows:

**i. Preparation of the Sephadex**

A 40-120 μ particle size Sephadex G-15, with water regain value of 1.5 ± 0.2 g/g and bed volume of 3 ml/g dry gel, was used. The dry gel was added to a large volume of 0.2 M acetic acid. The slurry thus formed was mixed for one hour on a magnetic stirrer, allowed to settle for 30 minutes, and the acetic acid decanted and discarded along with the fine non-settling fraction. The process was repeated four times. After the fourth washing, the remaining Sephadex was taken up in enough 0.2 M acetic acid to form a thick slurry, and used to build the column.

**ii. Preparation of the column**

A Kontes chromaflex (100 cm x 25 mm volume 450 ml) column was used either by itself, or extended with a Kontes chromaflex (100 cm x 25 mm volume 450 ml) extender to double the height and volume. The column was fixed vertically onto a stand, with the help of a plumb line, and filled with 0.2 M acetic acid. A ½ inch thick layer of glass wool was placed at the bottom, followed by a similar layer of small glass beads. A large funnel was fitted to the top of the column by means of a rubber stopper, and filled with 0.2 M acetic acid. The Sephadex G-15 slurry was added to the acetic acid in the funnel, and was stirred continuously with an electric mixer. Additional slurry was added into the funnel at intervals. The Sephadex gel was packed into a single column over a period of 18 hours, and into a double column over a period of 36 hours.
iii. Purification procedure

Before using the column, 0.2 M acetic acid was allowed to run through it for a period of 6 hours. At the completion of the acetic acid washing, the liquid above the gel surface was allowed to run down until the meniscus reached the surface. The posterior lobe extracts (see extraction) were carefully applied to the top of the column, and permitted to run into the gel. The column was filled with acetic acid (0.2 M) and connected to a reservoir containing the same.

The solution which had passed through the column was collected in 2.8 ml fractions, by the use of an LKB Ultrorac Type 7000 fraction collector.

b. Paper chromatography

The paper chromatography techniques used, were as described by Heller and Pickering (1961) and Perks (1966). Butanol, acetic acid, water in a 4:1:5 volume were shaken in a separatory funnel for 15 minutes immediately after mixing, and every 15 minutes thereafter for a period of 1½ hours. The solvent system then was allowed to separate into two layers. The aqueous layer was placed into four Petri dishes in the bottom of a 12" x 12" x 24" glass chromatography tank. The lid of the tank was sealed into place with "Lubrisseal", and the system was equilibrated at 20°C for one hour.

Standards and unknown solution were applied to a 24 x 54 cm sheet of Whatman 3MM chromatography paper, with the use of 1 ml tuberculin syringes. Cold air from a portable
hairdryer was allowed to pass through the origin, to speed up the drying of the spots.

The chromatogram was then placed in the equilibrated chromatographic tank, and the system was allowed to equilibrate for a further hour. At the end of the equilibration period the butanol layer of the solvent mixture was added to the solvent bath through openings in the glass lid. The opening in the lid was closed with the rubber stopper it held previously. The system was allowed to run for 12 hours. At the completion of the run, the chromatogram was removed, the solvent front marked, and the remaining solvent driven off by a cool flow of air.

Longitudinal strips of the dry chromatograms were cut at the width of the origins plus an extra 0.5 cm to allow for diffusion on each side. The lengthwise strips were divided into ten equal parts between the origin and the solvent front, each resulting square corresponding to 0.1 R_f unit. The squares were numbered, folded and eluted individually in 5 ml beakers, which contained 1 ml of 0.25% acetic acid. The elution was carried out for a period of 6 hours at 4°C. The eluate was then recovered from the paper, by pressure, and stored at 4°C until analysed for biological activities.

Chemical Methods for the Analysis of Peptides

a. Estimation of total peptide concentration

The estimation of total peptide concentration was carried out by the colorimetric method of Lowry, Rosebrough,
Farr and Randall (1951) as follows:

i. Reagents

A - 2% Na₂CO₃ in 0.1 N NaOH

B - 0.5% CuSO₄ 5H₂O in 1% K tartarate

C - alkaline copper solution

50:1 mixture of reagent A + B

D - 1 N Folin-Ciocalteau Phenol reagent

ii. Standards

A series of standards 10, 25, 50, 100, 150, 200 and 300 ug were prepared by dilution from a 50 mg % Bovine Serum Albumin Stock (fraction v Armour Pharmaceuticals).

iii. Measurement of peptide content

0.2 ml of the unknown and the standards were pipetted into a series of 2 ml testubes. To each sample 1 ml of reagent C was added, shaken and allowed to stand for 15-30 minutes. 0.1 ml of Folin Ciocalteau reagent was then added very rapidly and mixed immediately. After a period of 40-60 minutes the colour was fully developed. The samples were read against a blank on a Unicam SP 600 spectrophotometer at a wavelength of 750 millimicrons, using a red filter photocell.

b. Hydrolysis of the peptides

Fractions with identical biological activities, obtained from the Sephadex column, were pooled and lyophilised. The residue was dissolved in dist H₂O and lyophilised again. The procedure was repeated at least three times in order to get rid of all acetic acid remaining in the residue. With the last washing, the samples were transferred into a glass
bulb. 4 ml of triple distilled HCl was added to the dry residue, and N$_2$ gas bubbled through the liquid for 15 minutes in order to remove trapped air. The samples were then frozen in liquid nitrogen and evacuated for 30 minutes at 10 µ Hg pressure on a vacuum pump. At the end of the 30 minutes, the glass bulb was sealed with a blowtorch, under vacuum, and transferred into an oven at 108°C (Fisher Isotemp) for 22 hours.

At the end of the 22 hours, the samples were removed from the oven, allowed to cool and transferred into 50 ml flash evaporator flasks. Further cooling, for 15 minutes, followed in a deep-freeze.

The flasks, whilst still cold, were attached to a flash evaporator, and allowed to warm slowly under vacuum and with continuous turning of the rotor. When the samples were at approximately room temperature, they were gradually lowered into a 37°C waterbath, and dried. The dry powder was dissolved in iced, dist H$_2$O and evaporated again, to wash out the remaining HCl. The washing was repeated at least three times until no traces of HCl remained.

c. **Amino acid analysis**

The amino acid analysis was performed by thin layer chromatography of the dansyl derivatives of the amino acids, as described by Levison, 1967.

i. **Preparation of the plates**

One part of the silica gel was stirred into a slurry in 2 parts (w/v) of glass distilled water, for approximately three minutes. The slurry was spread in 250 micron thickness onto
20" x 20" glass plates with the aid of a Warner-Chilcott spreader.

The plates were allowed to dry at room temperature, until the silica became opaque (10-20 minutes). They were then warmed in a stream of hot air for 10 minutes, until the silica became dry, and placed for 30 minutes in a drying oven at 110°C.

**ii. Dansylation of the hydrolysates**

Five microliters of 0.2N NaHCO₃, and five microliters of standard dansyl chloride* were added to one micromole hydrolysate. The reaction was allowed to proceed for three hours. Amino acids were dansylated in the same manner to provide standards for chromatographic comparisons.

**iii. Chromatography of the hydrolysates**

One microlitre of the hydrolysates, containing 1 muM of peptide was applied to the plates, beside one milimicromole samples of the standard amino acids. The plates were placed in thin layer chromatographic tanks which contained a small amount of the appropriate solvent system, and the system was allowed to equilibrate for 30 minutes. At the end of the 30 minutes equilibration period, the pan at the bottom of the tank was filled with the solvent to be in contact with the edge of the plate. The solvent was allowed to run up the plates until it reached within an inch from the top. The plates were then removed from the tanks, and in a few minutes, when they appeared dry, the spots for the standards and the unknowns were located under ultraviolet light and

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* 1 di-methylaminonaphtaline 5 sulphanylichloride
marked. The Rf values were read, and direct comparisons with the standards permitted identification of the amino acids in the hydrolysates.

Four solvent systems were selected for their ability to separate various groups of amino acids.

Solvent system 1; chloroform: benzyl alcohol: acetic acid, 70:30:3
Solvent system 2; benzyl alcohol: pyridine: acetic acid, 16:4:1
Solvent system 3; butanol: acetic acid: water, 1:5:4
Solvent system 4; chloroform: butanol: acetic acid, 6:3:1

Statistical Methods

Mean hormonal potencies are calculated by the use of weighted means according to K.A. Brownlee (1965) using the formula:

\[ \bar{X} = \frac{\sum_{j}^{k} w_j x_j}{\sum_{j}^{k} w_j} \]

where \( w_i = 1/6^2 \)

and confidence limits at the 5% probability level were assigned using the following formula:

\[ \sqrt{\frac{V-T}{x}} \]

where \( V_x = \frac{1}{\sum_{i}^{k} w_i} \)

The ratios of vasopressor or antidiuretic activity to oxytocic activity were calculated by the formula (Bliss, 1956):
Confidence limits were assigned at the 5% probability level according to the formula:

\[ X^* = \bar{M} \pm \sqrt{\left( M^2 - \frac{a^2}{b^2} - \frac{6^2}{\delta^2 b^2 t^2} \right) \frac{a^2}{b^2} + \frac{6^2}{\delta^2 b^2 t^2} \frac{a^2}{b^2}} \]
SECTION I

STUDIES ON THE PREGNANT SHEEP

Posterior pituitary glands from pregnant sheep at two stages of pregnancy, and from control non-pregnant sheep were collected throughout two breeding seasons. The biological activities of the crude extracts were determined. Crude extracts were purified, and the purified product was analysed for amino acid composition.

The two stages of pregnancy, represented in the samples collected, were 89-91 days and 138-141 days. Since the duration of pregnancy in the sheep is 145-147 days, these stages correspond to about mid-pregnancy, and to just a few days prior to parturition, respectively.

1. Biological activities of the crude extracts

The biological activities of three to five individual glands, at 89-91 days, and 138-141 days of gestation were estimated from both acetone dried and lyophilised material. To complete each series, two controls from non-pregnant animals were included. In the acetone dried series, one gland, the only one available during the process of natural delivery, is also included. Representative results from these bio-assays are shown in Fig. 1.

All glands exhibited oxytocic, vasopressor and anti-diuretic activities. The results are presented in Table I for acetone dried, and in Table II for lyophilised tissue.
Figure 1

Sample records from rat uterus, vasopressor and antidiuretic assays.

Rat uterus assay

A = 0.08 ml of 1:250, 2 mg/ml posterior pituitary extract of pregnant sheep.
B = 0.08 ml of 1:1000 synthetic oxytocin (Syntocinon, 10 I.U./ml).
C = 0.12 ml of 1:1000 synthetic oxytocin.
D = 0.12 ml of 1:250 of 2 mg/ml extract

Rat vasopressor assay

A = 0.1 ml of 1:60, 2 mg/ml posterior pituitary extract of pregnant sheep.
B = 0.06 ml of 1:400 standard vasopressin (Pitressin, 20 I.U./mg).
C = 0.12 ml of 1:400 standard vasopressin.
D = 0.2 ml of 1:60 posterior pituitary extract

Rat antidiuretic assay

A = 0.05 ml of 1:40,000 standard vasopressin.
B = 0.05 ml of 1:2,000, 2 mg/ml posterior pituitary extract.
C = 0.1 ml of 1:40,000 standard vasopressin
D = 0.1 ml of 1:2,000 posterior pituitary extract.
Rat Uterus Assay

Vasopressor Assay

Antidiuretic Assay
Weighted means were calculated for corresponding stages of gestation by the statistical method described on p. 25 in Methods.

The mean oxytocic, vasopressor and antidiuretic activities in the acetone dried glands are presented in Fig. 2. At 90 days of pregnancy the oxytocic activity was 60% below that in the non-pregnant adult sheep; this fall is followed by a relative rise at 140 days, but the potency remained below the control level throughout gestation.

The changes in vasopressor and antidiuretic activities paralleled those of the oxytocic activity. The one gland taken at delivery revealed a marked increase in oxytocic potency, and in terms of oxytocic activity, it was by far the most active pituitary tissue encountered in the course of this investigation. The apparent increase in oxytocic activity was accompanied by a much lesser increase in the vasopressor and antidiuretic activities.

The estimation of the biological activities of the lyophilised glands of pregnant females showed changes in hormonal content, similar to those found in acetone dried tissues. The mean potencies are shown in Fig. 3. Again, a decrease in the stored level of the oxytocic, vasopressor and antidiuretic activities was found at 90 days of pregnancy, followed by a relative rise in all three activities at 140 days of gestation.

In Fig. 4, the hormonal levels during gestation are compared directly in acetone dried and lyophilised glands.
<table>
<thead>
<tr>
<th>No.</th>
<th>Gest. Days</th>
<th>RU mU/mg</th>
<th>Pressor mU/mg</th>
<th>ADH mU/mg</th>
<th>V/O Ratio</th>
<th>ADH/O Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>90</td>
<td>505.0 ±32.2</td>
<td>467.5 ±44.0</td>
<td>402.0 ±28.6</td>
<td>0.92 ±0.10</td>
<td>0.80 ±0.55</td>
</tr>
<tr>
<td>4</td>
<td>89</td>
<td>677.5 ±96.0</td>
<td>341.3 ±37.5</td>
<td>479.8 ±101.6</td>
<td>0.50 ±0.11</td>
<td>0.70 ±0.12</td>
</tr>
<tr>
<td>7</td>
<td>91</td>
<td>1191.6 ±150.0</td>
<td>373.6 ±33.0</td>
<td>568.2 ±82.5</td>
<td>0.31 ±0.05</td>
<td>0.47 ±0.08</td>
</tr>
<tr>
<td>8</td>
<td>138</td>
<td>748.8 ±120.0</td>
<td>478.9 ±74.1</td>
<td>542.0 ±321.0</td>
<td>0.64 ±0.14</td>
<td>0.74 ±0.12</td>
</tr>
<tr>
<td>9</td>
<td>138</td>
<td>1536.2 ±252.8</td>
<td>1086.1 ±148.8</td>
<td>1275.7 ±384.3</td>
<td>0.71 ±0.14</td>
<td>0.83 ±0.11</td>
</tr>
<tr>
<td>13</td>
<td>138</td>
<td>1080.1 ±82.5</td>
<td>664.5 ±49.1</td>
<td>567.0 ±133.0</td>
<td>0.62 ±0.06</td>
<td>0.55 ±0.36</td>
</tr>
<tr>
<td>27</td>
<td>Term</td>
<td>2454.4 ±802.0</td>
<td>1015.9 ±188.4</td>
<td>910.8 ±159.0</td>
<td>0.41 ±0.14</td>
<td>0.37 ±0.13</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>1954.4 ±134.9</td>
<td>1220.0 ±140.5</td>
<td>1480.6 ±461.1</td>
<td>0.62 ±0.04</td>
<td>0.76 ±0.17</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>1350.5 ±205.6</td>
<td>1416.8 ±430.0</td>
<td></td>
<td>1.05 ±0.34</td>
<td></td>
</tr>
</tbody>
</table>
# TABLE II

**POTENCIES AND THE RATIOS OF THE POTENCIES IN THE LYOPHILISED TISSUE OF PREGNANT SHEEP**

<table>
<thead>
<tr>
<th>No.</th>
<th>Gest. Days</th>
<th>RU mU/mg</th>
<th>Pressor mU/mg</th>
<th>ADH mU/mg</th>
<th>V/O Ratio</th>
<th>A/O Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>87</td>
<td>1706.0 +296.0</td>
<td>1273.0 +243.5</td>
<td>1115.4 +543.9</td>
<td>0.75 +0.18</td>
<td>0.65 +0.3</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>1405.8 +106.5</td>
<td>963.5 +83.6</td>
<td>1565.1 +937.3</td>
<td>0.70 +0.07</td>
<td>1.1 +0.64</td>
</tr>
<tr>
<td>16</td>
<td>90</td>
<td>1231.0 +117.0</td>
<td>1005.5 +216.4</td>
<td>999.0 +682.6</td>
<td>0.82 +0.18</td>
<td>0.81 +0.50</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>1220.6 +101.0</td>
<td>893.0 +74.9</td>
<td>963.4 +208.8</td>
<td>0.73 +0.141</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>89</td>
<td>1213.4 +109.0</td>
<td>850.0 +265.0</td>
<td>963.4 +208.8</td>
<td>0.70 +0.23</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>141</td>
<td>1261.2 +171.0</td>
<td>926.4 +242.8</td>
<td>963.4 +208.8</td>
<td>0.73 +0.31</td>
<td>0.76 +0.79</td>
</tr>
<tr>
<td>22</td>
<td>143</td>
<td>1689.6 +101.9</td>
<td>1069.2 +344.1</td>
<td>1526.1 +643.0</td>
<td>0.63 +0.15</td>
<td>0.90 +0.38</td>
</tr>
<tr>
<td>23</td>
<td>141</td>
<td>1662.2 +117.5</td>
<td>1041.5 +88.0</td>
<td>915.9 +248.8</td>
<td>0.63 +0.06</td>
<td>0.54 +0.31</td>
</tr>
<tr>
<td>10</td>
<td>140</td>
<td>1520.1 +135.5</td>
<td>1388.6 +139.6</td>
<td>1050.4 +205.1</td>
<td>1.05 +0.28</td>
<td>0.86 +0.35</td>
</tr>
<tr>
<td>17</td>
<td>137</td>
<td>1483.5 +180.5</td>
<td>750.1 +49.6</td>
<td>1083.9 +366.9</td>
<td>0.73 +0.21</td>
<td>0.59 +0.35</td>
</tr>
</tbody>
</table>

1 Control | 1224.6 +231.0 | 1297.4 +272.4 | 1050.4 +205.1 | 1.05 +0.28 | 0.86 +0.35 |

2 Control | 1568.9 +127.2 | 1464.1 +215.7 | 1267.6 +458.1 | 0.93 +0.25 | 0.81 +0.29 |

3 Control | 2094.2 +306.9 | 1502.0 +299.8 | 1083.9 +366.9 | 0.73 +0.21 | 0.59 +0.35 |
The single value obtained in the acetone dried series is omitted here, since there was no lyophilised sample for comparison. The graph illustrates the similarity in the pattern of change seen during pregnancy; tissues taken in two different breeding seasons, and subjected to two different drying procedures, gave essentially similar results. The fact that the overall changes are very similar in the two different groups of sheep suggests that the alterations in the level of stored hormones are meaningful, and that they are not due to random variability. It appears probable that acetone treatment does not obscure the changes which occur in the neurohypophysis during gestation, but only magnifies them.

The ratios of vasopressor to oxytocic activity (V/O) and of antidiuretic to oxytocic activity (A/O), were calculated for individual glands, and confidence limits at $P < 0.05$ assigned according to the method of Bliss (1956). The results are included in Table I and Table II. Ratios of the mean potencies were calculated by the same statistical method (see p.25 Methods) and the results for both acetone dried and lyophilised tissue are given in Fig. 5. Comparison between the ratios of glands from control non-pregnant sheep, and those from pregnant animals, following either lyophilisation or acetone treatment, reveals no statistically significant change in ratios.

2. **Purification and amino acid analysis of the active principles of pregnant sheep**

Samples of the neurohypophysial extracts of pregnant
Figure 2

Weighted means of oxytocic, vasopressor and antidiuretic activities in the acetone dried neurohypophyses of control and pregnant sheep.

$mU/mg = \text{milliunit biological activity/mg dry tissue}$
PREGNANT SHEEP (ACETONE DRIED)

Oxytocic

Vasopressor

Antidiuretic

Days Gestation

0 50 100 147

Days Gestation

0 50 100 147

Days Gestation

0 50 100 147

mU/mg

control term control term control term
Figure 3

Weighted means of oxytocic, vasopressor and antidiuretic activities in the lyophilised neurohypophyses of control and pregnant sheep.

$mU/mg = \text{milliunit biological activity/mg dry tissue}$
PREGNANT SHEEP (LYOPHILISED)

Oxytocic

Vasopressor

Antidiuretic

Days Gestation

Days Gestation

Days Gestation

mU/mg
sheep were pooled for chemical studies. Because of the insensitivity of chemical methods as compared to pharmacological analysis, it was necessary to mix the extracts without regard to the stage of pregnancy at which they were taken, or to the method of drying. The mixed samples were regarded merely as examples of posterior pituitary extracts from sheep in a pregnant state, and they later served as controls for the studies of the foetal neurohypophysial agents. The pooled samples were purified by a new modification of the gel filtration method, and the eluates from the column were analysed for protein content (Lowry peptide), and for biological activities (oxytocic and vasopressor assay). Details of these estimations are given in the section on methods. The purified agents were partially analysed for their chemical structure by the use of thin layer chromatography.

a. Purification by gel filtration

15 ml of neurohypophysial extract at a concentration of 2 mg/ml, and which contained a total of 27.0 ± 0.9 I.U. (International Unit) of rat uterus and 24.0 ± 3.0 I.U. of vasopressor activity, was applied to a 100 cm column of Sephadex G-15 dextran gel which was suspended in 0.2 M acetic acid (see Methods). A reservoir filled with 0.2 M acetic acid was connected to the column, and the acetic acid was allowed to flow through at a rate of 16 ml/hr. The first 100 ml of eluate represented the void volume of the column and was discarded. The next 150 fractions of 2.8 ml each were then collected into test tubes in a fraction collector.
Figure 4

Comparison between the biological activities of the acetone dried and lyophilised neurohypophyses of control and pregnant sheep.

$mU/mg = \text{milliunit/mg dry tissue}$
PREGNANT SHEEP

- lyophilised
- acetone dried

Oxytocic

Days Gestation

Vasopressor

Antidiuretic

Days Gestation

0 50 100 147

term

0 50 100 147

term

0 50 100 147

term

Days Gestation
Figure 5

Ratios of biological activities in acetone dried and lyophilised neurohypophyses of control and pregnant sheep.

Ratio of V/O = milliunit of vasopressor activity per mg dry tissue/milliunit of oxytocic activity per mg dry tissue

Ratio of A/O = milliunit of antidiuretic activity per mg dry tissue/milliunit oxytocic activity per mg dry tissue
RATIO OF ACTIVITIES, PREGNANT SHEEP

V/O

A/O

Ratio of V/O

Ratio of A/O

Days Gestation

Days Gestation

0 50 100 147 term

0 50 100 147 term

○ lyophilised
○ acetone dried
The fractions were analysed for protein content, vasopressor and oxytocic activity, and the results are given in Fig. 6. The mass of protein, as determined by the Lowry peptide determination, came off the column in the first 50 fractions and showed a remarkable separation of the active principles. The analysis for biological activities revealed that the vasopressor and oxytocic activities were also partially separated in the eluate. The recovery for oxytocic activity was just above 100%; the apparent increase in the total oxytocic moiety is undoubtedly an artifact, due to the inherent error of the estimation. The recovery for vasopressor activity was approximately 80%.

The partial resolution of the active principles by the G-15 column, suggested the possibility that an increase in the length of the column, might result in a complete resolution of the two biologically active agents. Therefore, an extender was used to build a 200 cm column. 15 ml of extract at 2 mg/ml, representing 27.0 ± 0.9 I.U. total oxytocic and 24.0 ± 3.0 I.U. total vasopressor activity, was subjected to the same procedure as used previously for the short column. The fractions were analysed as before, and the results are presented in Fig. 7. There was a complete separation of the two active principles. Large proteins were eluted first, with oxytocic and vasopressor activities following, in that order. The increased length of the column did not alter the recovery for either oxytocin or vasopressin; the recovery of oxytocin was again approximately 100% and that of vasopressin.
Figure 6

Purification of the active neurohypophysial agents of pregnant sheep by the use of 100 cm Sephadex G-15 column.
Purification of Pregnant Sheep Extract
on 100 cm. Sephadex G-15 column

Tube Number (1 tube = 2.8 ml)
approximately 80%. No increase in conductivity, above that of the supporting 0.2 M acetic acid, was detected in any of the fractions, so that no electrolyte peak could be resolved; this was probably because of the low salt content of the crude extract.

The peaks of biological activities showed chemical evidence for their peptide content. In the oxytocic peak (Fig. 7), 5 μg/ml Lowry peptide content, corresponded to 600 mU/ml oxytocic activity, or 120 I.U. oxytocic activity/mg Lowry peptide. This value compares favourably with the 133 I.U./mg peptide obtained for pure synthetic oxytocin by Perks and Sawyer (1965). On the basis of this measurement, the oxytocic peak in Fig. 7 appears to be almost, or perhaps completely, pure.

The Lowry peptide content of the vasopressor peak is even smaller than that of the oxytocic peak. Again, this suggests a pure or almost pure peptide. The oxytocic activity in the vasopressor peak can be completely accounted for by the 3-5% intrinsic oxytocic activity of vasopressin (Adamson, Engel, van Dyke, 1954).

b. Amino acid analysis

After various estimations of the different fractions were completed, those eluates which contained identical biological activities were pooled, and the pooled samples were hydrolysed in 6 N HCl. The hydrolysates were then mixed with dansyl chloride, to form dansyl derivatives of their constituent amino acids. The reacted hydrolysates
Figure 7

Purification of the biologically active principles from the crude extracts of pregnant sheep neurohypophyses, by the use of 200 cm Sephadex G-15 column.
Purification of Pregnant Sheep Extract
on 200 cm. Sephadex G-15 column

Tube Number (1 tube = 2.8 ml)
were applied to thin layer (silica) chromatographic plates, alongside standard dansylated amino acids (see Methods).

The plates were developed in four solvent systems which were selected to give optimal separations of the 16 amino acids used as standards. The solvent systems were:

1. chloroform: benzyl alcohol: acetic acid, (70:30:3)
2. benzyl alcohol: pyridine: acetic acid, (16:4:1)
3. butanol: acetic acid: water (1:5:4)
4. chloroform: butanol: acetic acid, (6:3:1)

As yet, no solvent system has been found to separate mono-dansylated lysine from dansyl-arginine. After chromatography was complete, the spots were made visible under ultraviolet light and their position marked. A representative plate is shown in Fig. 8.

Because of the known variability of the $R_f$ values of dansyl derivatives between different experiments (Perks, personal communication), an effort was made to employ direct comparison of the constituent amino acids of the hydrolysates with the standards on the same plates. However, the $R_f$ values of the standards were also calculated on each plate and compared directly to the $R_f$-s of the amino acids of the unknown (Fig. 9). In the eluates which contained the oxytocic
Figure 8

Sample plate of the thin layer chromatography of dansyl derivatives of standard amino acids and the hydrolysates of the oxytocic and vasopressor moieties of the adult pregnant sheep, and of the hydrolysate of the foetal vasopressor moiety

DNS = dansyl
moiety, the eight constituent amino acids of oxytocin were identified; these were - tyrosine, isoleucine, glutamic acid, aspartic acid, proline, leucine, glycine and cysteic acid. Acid hydrolysis would have caused the loss of ammonia from glutamine and asparagine to form the (identified) glutamic and aspartic acids. In addition to the eight amino acids listed, the hydrolysates contained lysine, alanine, and a further amino acid which could have been either monodansylated lysine or arginine. Lysine is a known contaminant of hydrolysates of neurohypophysial peptides (Perks and Sawyer, 1965), and alanine may be generated by the breakdown of cystein (Perks, personal communication). Due to the similar mobility of mono-dansylated lysine and arginine, it cannot be determined which of the two amino acids was present in the hydrolysate. The presence of mono-dansylated lysine, however, is the most likely possibility, since this amino acid was seen to be present in its di-dansylated form, and both dansyl derivatives are frequently formed simultaneously.

Analysis of the vasopressor peak showed the eight constituent amino acids of arginine vasopressin, that is to say - tyrosine, phenylalanine, glutamic acid, aspartic acid, proline, arginine, and cysteic acid in place of cystein. Again, lysine and alanine were detected in addition to the above amino acids.

It may be concluded that the amino acids known to be present in oxytocin and vasopressin were identified in
Graphed $R_f$ values of dansylated standard amino acids, and the dansylated hydrolysates of the oxytocic and vasopressor moieties from the purified extracts of the neurohypophyses of adult pregnant sheep.

- **d. NH$_2$** = dansylated ammonia
- **di-lys** = di- dansyl derivative of lysine
- **ε-Lys** = mono- dansyl derivative of lysine

Amino acids of the unknowns are labelled only where identification of their $R_f$ values correspond closely to the $R_f$ of a standard amino acid.
<table>
<thead>
<tr>
<th>SOLVENT SYSTEM 1</th>
<th>SOLVENT SYSTEM 2</th>
<th>SOLVENT SYSTEM 3</th>
<th>SOLVENT SYSTEM 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>vasopressor</td>
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<td>vasopressor</td>
<td>vasopressor</td>
</tr>
<tr>
<td>standard</td>
<td>standard</td>
<td>standard</td>
<td>standard</td>
</tr>
<tr>
<td>oxytocin</td>
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<td>oxytocin</td>
<td>oxytocin</td>
</tr>
<tr>
<td>( R_f )</td>
<td>( R_f )</td>
<td>( R_f )</td>
<td>( R_f )</td>
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</tr>
<tr>
<td>0.9 -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8 -</td>
<td>( \text{leu} )</td>
<td>( \text{leu} )</td>
<td>( \text{Val} )</td>
</tr>
<tr>
<td></td>
<td>( \text{d.NH}_2 )</td>
<td>( \text{Val} )</td>
<td>( \text{d.NH}_2 )</td>
</tr>
<tr>
<td>0.7 -</td>
<td>( \text{Pro} )</td>
<td>( \text{Pro} )</td>
<td>( \text{Pro} )</td>
</tr>
<tr>
<td>0.6 -</td>
<td>( \text{Ala} )</td>
<td>( \text{Ala} )</td>
<td>( \text{Ala} )</td>
</tr>
<tr>
<td>0.5 -</td>
<td>( \text{Gly} )</td>
<td>( \text{d.Lys} )</td>
<td>( \text{d.Lys} )</td>
</tr>
<tr>
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<td>( \text{Tyr} )</td>
<td>( \text{Tyr} )</td>
<td>( \text{Tyr} )</td>
</tr>
<tr>
<td>0.3 -</td>
<td>( \text{His} )</td>
<td>( \text{d.Lys} )</td>
<td>( \text{d.Lys} )</td>
</tr>
<tr>
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<td>( \text{Glu} )</td>
<td>( \text{Tyr} )</td>
<td>( \text{Tyr} )</td>
</tr>
<tr>
<td>0.1 -</td>
<td>( \text{Glu} )</td>
<td>( \text{Tyr} )</td>
<td>( \text{Tyr} )</td>
</tr>
</tbody>
</table>

The table lists amino acids and peptides for different solvent systems. Each solvent system (1-4) has a set of compounds with varying \( R_f \) values. The compounds include vasopressin, oxytocin, and various amino acids such as leucine, valine, glycine, and tryptophan.
the fractions exhibiting the appropriate biological activities. The presence of additional amino acids in both hydrolysates is probably due to the breakdown of constituent amino acids, together with contamination from glassware. The extent of purification (i.e. the proportion of the additional amino acids present) could not be determined, since the thin layer separation of dansyl amino acids is a difficult method to make accurately quantitative. However, the limited amount of material available made it impossible to use any other quantitative method of amino acid analysis. Nevertheless, as far as it could be determined, the purification procedure appeared to be very good for such a simple system, since contaminant amino acids were few, and their dansyl derivatives neither trailed nor overloaded the chromatograms. On the basis of this mainly qualitative system, the purified product from the G-15 dextran columns appeared to be almost pure.
DISCUSSION
(Section I)

The results reported here have shown a reduction in the level of the posterior pituitary hormones during the course of pregnancy. The level of both oxytocic and vasopressor activities were lowest at 90 days of gestation. Although the amount of the stored hormones remained below that of the control non-pregnant animal throughout gestation, a trend towards increased storage could be seen at 140 days. The single gland taken during natural labour appeared to show a remarkable increase in the oxytocic activity. The same pattern in the changing hormonal content of the pituitary during pregnancy was observed in two different breeding seasons. Therefore, the fall in the peptide content of the neurohypophysis during mid-pregnancy would appear to be significant since it is difficult to attribute it to a chance variability which might occur within one breeding season. Further, since it occurred in both lyophilised and acetone dried tissues, it is unlikely to be an artifact caused by the method of drying. No significant changes in the ratio of vasopressor to oxytocic activity were detected at any stage of pregnancy.

The close link between oxytocin and reproduction, and the importance of antidiuretic hormone in water metabolism, which must change during pregnancy, would suggest that
changes in the hormonal content of the neurohypophysis during the course of pregnancy might be anticipated, and the results presented here demonstrated that such changes do, in fact, occur. Previous attempts, limited to the rat, failed to find any such changes in either the level or the ratio of the neurohypophysial peptides during pregnancy (Heller and Lederis, 1956; Acher et al., 1956). However, variations have been seen to occur in connection with other aspects of reproduction, such as in the estrous cycle, or in lactation. Heller (1958) detected phasic changes in the amount of stored peptides in the posterior pituitary of rats, and these changes corresponded with particular stages in the estrous cycle. Levels of vasopressor and oxytocic activities were found to be higher in the follicular phase than in metestrous. A loss of the active agents from the posterior pituitary of dogs and rats during lactation was also observed (Dicker and Tyler, 1953; Heller and Lederis, 1957; Acher et al., 1956). A fall in the content of both hormones was found by all investigators, but there was a disagreement concerning the nature of the changes in the V/O ratios. Heller (1961), in reviewing these results, noted that changes in ratios were found by workers using acetone dried glands, but not by those working with fresh glands. He attributed the rise in ratio to a preferential solubility of oxytocin in acetone.

The possible preferential solubility of oxytocin in acetone from glands taken from animals under certain physiological conditions, raises doubt concerning those
results obtained from acetone dried tissues. The studies in pregnant sheep presented here do not confirm such preferential solubility of oxytocin, since the ratios of the hormones were similar in both lyophilised and acetone dried tissues, even when acetone treatment was prolonged. However, estimation of the absolute hormonal potencies, following acetone treatment, showed a loss of both oxytocic and vasopressor activities from glands taken during pregnancy, but not from the controls.

The experiments reported here were designed primarily to evaluate changes in biological activities during the course of pregnancy, but the greater loss of hormonal activity following acetone treatment is an interesting problem in itself. Possibly, the answer to this problem lies in an altered binding of the active principles during pregnancy. Sachs (1967) in his studies of neurohypophysial response to haemorrhage, noted an initial increase in the amount of vasopressin released into the bloodstream, until approximately 10-20% of the total antidiuretic hormone content of the posterior pituitary was depleted. However, further haemorrhage could not elicit any additional release of the antidiuretic hormone from the neurohypophysis. Sachs suggests that the stored vasopressin is heterogenous, consisting of a quickly and a slowly releasable component, and with the two components differing from one another in their binding within the gland. An increase in the quickly releasable, or loosely bound component of the neurohypophysial hormones,
during pregnancy, might account for the greater loss of activity which occurred during acetone treatment of the glands of pregnant animals.

In previous work, two basic methods were utilised for the purification of neurohypophysial peptides. The first, used by Acher (1955) and Chauvet (1960), made use of the specific affinity of the peptides for the carrier protein, neurophysine. In this method, the complex formed between the neurohypophysial octapeptides and neurophysine was precipitated by NaCl, and dialysed to eliminate contaminating peptides. The neurophysine was then precipitated with trichloracetic acid, and the active agents were left in solution. The active agents were separated from one another on ion exchange columns. The relative simplicity of this method makes it attractive; however, it came under criticism because of the danger of contaminating the purified products with bovine neurohypophysial peptides which remained unremoved during the original preparation of the bovine neurophysine.

The other more generally used purification method involves simple gel filtration of crude extracts, followed by the use of ion-exchange columns for the resolution of the two neurohypophysial peptides (Porath and Flodin, 1959; Yamashiro, 1964).

In 1966, Frankland and his co-workers combined the two methods, and subjected neurophysine-peptide complexes, obtained by Acher's method, to gel filtration on G-25 Sephadex.
supported in 1 N acetic acid and performed the chromato-
graphic separation with 0.1 M formic acid as the suspension
fluid. They report that this two step method resulted in a
complete separation of the two active peptides from the
carrier protein.

The purification procedure used in the present
investigation was designed to simplify and improve currently
accepted methods of gel filtration, as applied to
neurohypophysial peptides. The use of formic acid was
avoided, since it is a potent reducing agent. It is possible
that it could effect biological activity by causing chemical
changes in the neurohypophysial peptides, and, further, it
might have detrimental effects on the preparations used for
biological assays. In contrast, the acetic acid which was
used in the purification procedure reported here, is a weak
acid, which has no reducing potency, and is a normal
component of metabolising systems. The procedure also
eliminated such severe steps as treatment with trichloracetic
acid, and steps which might add artifacts such as the
possible addition of foreign peptides with neurophysine. It
was a simple and relatively mild system, yet it resolved
highly purified neurohypophysial peptides in a single step.

The results of the amino acid analysis were in good
agreement with those of Acher and Fromageot (1957), who
identified oxytocin and arginine vasopressin as the active
peptides of sheep neurohypophysis of unknown reproductive
condition.
SECTION II


In this part of the investigation, posterior pituitaries of sheep embryos at different gestational ages, and of newborn lambs, were assayed for their biological activities. Samples at corresponding stages of intrauterine development were pooled and subjected to purification procedures; the neurohypophyses of early foetuses were purified by paper chromatography, and of the late (140 days) foetuses by gel filtration.

The duration of gestation is 145-147 days in the sheep. Foetuses at 89-91 days of gestational age weighed only 500-600 grams, lacked fur, and showed the disproportionate head measurement of the early foetus. By 140 days of gestational age, which is only a few days prior to parturition, the foetuses weighed 4-5 kgs, were covered with fur, and generally appeared to be fully developed.

1. Biological activities of the foetal neurohypophysis

Single glands of foetuses at 89-91 days, and at 138-141 days of gestational age were collected immediately after death. The glands were individually extracted and assayed for oxytocic, vasopressor and antidiuretic activities. Similar
studies were made on glands taken from lambs at birth and at 13-15 days of post-natal age, in order to determine whether trends seen during foetal development might continue into early post-natal life. Collections were made in two different breeding seasons. The glands in the first breeding season were acetone dried and allowed to stand in acetone up to an additional 14 months. Posterior pituitaries in the second breeding season were immediately lyophilised and stored over P$_2$O$_5$ for a similar period (see Methods).

Vasopressor, antidiuretic and oxytocic activities were detected in all glands. The results of the biological assays are presented in Table III for acetone dried, and in Table IV for lyophilised tissues. Weighted means were calculated for groups of tissues at the same developmental stage, and confidence limits assigned at the 5% probability level as described in the Methods.

a. **Acetone dried tissue**

The weighted means of oxytocic, vasopressor and antidiuretic activities for corresponding developmental stages are plotted in Fig. 10. The average oxytocic activity at 90 days of gestation was low, and amounted to only 10 mU/mg dry powder. Since the average weight of the glands at the early stage of gestational age is only 0.8 mg, the total oxytocic activity in the early foetal sheep pituitary did not exceed 8 mU/gland in the acetone dried tissue. In the same glands the vasopressor and antidiuretic activities were notably higher and averaged 120 mU/mg, or a total of 96 mU/
**TABLE III**

POTENCIES AND THE RATIOS OF THE POTENCIES IN THE ACETONE-DRIED TISSUES OF SHEEP FOETUSES AND LAMBS

<table>
<thead>
<tr>
<th>No.</th>
<th>Gest. Days</th>
<th>RU mU/mg</th>
<th>Pressor mU/mg</th>
<th>ADH mU/mg</th>
<th>V/O Ratio</th>
<th>A/O Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b</td>
<td>89</td>
<td>14.0</td>
<td>113.0 ±37.5</td>
<td>149.6 ±21.3</td>
<td>10.0</td>
<td>13.6</td>
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<tr>
<td>2</td>
<td>90</td>
<td>8.3</td>
<td>170.6 ±32.5</td>
<td>144.9 ±19.4</td>
<td>20.0</td>
<td>17.0</td>
</tr>
<tr>
<td>6a</td>
<td>91</td>
<td>10.2</td>
<td>122.8 ±59.0</td>
<td>109.0 ±10.0</td>
<td>12.0</td>
<td>10.0</td>
</tr>
<tr>
<td>29</td>
<td>87</td>
<td>31.8 ±2.0</td>
<td>557.0 ±102.0</td>
<td>769.1 ±135.0</td>
<td>18.0 ±2.0</td>
<td>24.2 ±6.8</td>
</tr>
<tr>
<td>8</td>
<td>138</td>
<td>157.0 ±12.2</td>
<td>1237.0 ±191.4</td>
<td>2004.0 ±1022.0</td>
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<td>12.7 ±6.0</td>
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<tr>
<td>9b</td>
<td>138</td>
<td>93.3 ±16.9</td>
<td>713.4 ±124.2</td>
<td>879.6 ±284.0</td>
<td>7.6 ±1.83</td>
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<tr>
<td>13</td>
<td>138</td>
<td>59.9 ±7.6</td>
<td>653.6 ±116.4</td>
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<td>11.0 ±1.8</td>
<td>10.0 ±5.0</td>
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<tr>
<td>27c</td>
<td>Term</td>
<td>196.0 ±35.1</td>
<td>901.2 ±199.9</td>
<td>921.0 ±212.5</td>
<td>4.6 ±1.24</td>
<td>4.7 ±1.24</td>
</tr>
<tr>
<td>1</td>
<td>15 p.n.</td>
<td>63.0 ±7.8</td>
<td>263.0 ±53.6</td>
<td>201.6 ±47.0</td>
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<td>3.2 ±0.08</td>
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<tr>
<td>2</td>
<td>13 p.n.</td>
<td>101.7 ±7.6</td>
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<td>164.3 ±55.2</td>
<td>3.3 ±0.76</td>
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</tr>
</tbody>
</table>
gland. At 140 days, a 10 fold increase in the oxytocic and an approximately 8 fold increase in the vasopressor and antidiuretic potencies were detected. At the same time, the weight of the glands increased from 0.8 mg to 3.5 mg, making the total increase in the amount of stored peptides 45 fold. A further rise in potency was detected at parturition in the oxytocic and vasopressor, but not the antidiuretic activities. This discrepancy between the vasopressor and antidiuretic activities is probably not significant in view of the overlapping confidence limits. After birth, all three biological activities appeared to be significantly lower than at late gestation.

The V/O and A/O ratios, at a high value in the early gestational age, exhibited a very steady change throughout gestation, and are presented in Fig. 11. At 90 days of gestation the ratios approximated 14:1, which reflected the preponderance of the vasopressor and antidiuretic activities at this early stage of gestation. The proportionally greater accumulation of oxytocic activity in the neurohypophysis with the advancement of gestation and early post-natal development, resulted in the steady fall of the ratios throughout gestation. At 13-15 days of post-natal age, the ratio of vasopressor to oxytocic activity was still far from the adult ratio of approximately 1, and was found to be 3:1 in favour of the vasopressor moiety.

b. Lyophilised tissue

The weighted means for oxytocic, vasopressor and antidiuretic activities at corresponding gestational stages
### TABLE IV

POTENCIES AND THE RATIOS OF THE POTENCIES IN THE LYOPHILISED TISSUES OF SHEEP FOETUSES AND LAMBS

<table>
<thead>
<tr>
<th>No.</th>
<th>Gest Days</th>
<th>RU mU/mg</th>
<th>Pressor mU/mg</th>
<th>ADH mU/mg</th>
<th>V/O Ratio</th>
<th>ADH/O Ratio</th>
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<td>2a</td>
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<td>+156.0</td>
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<tr>
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</tr>
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were plotted in Fig. 12. The average oxytocic activity at 90 days of gestational age was low, and amounted to only 60 mU/mg dry powder. The average weight of a lyophilised gland at this stage was approximately 0.6 mg and, therefore, the total oxytocic activity in the early stage of gestation did not exceed a total of 40 mU/mg. In the same glands, the vasopressor activity was approximately 900 mU/mg, and the antidiuretic activity 1200 mU/mg; this corresponds to a total of 550 mU/gland vasopressor and 720 mU/gland antidiuretic activity. At 140 days of gestation, an increase in all biological activities was detected, but the increase did not appear to be as great as was detected in the acetone dried tissues. A fall in the posterior pituitary content of the active agents was again demonstrated at 13-15 days of post-natal age, but in contrast to the acetone dried tissues, a fall in potency also occurred at the time of natural delivery.

The V/O and A/O ratios, presented in Fig. 12, exhibited a similar pattern of steadily decreasing values as was already noted in the acetone dried tissues.

c. **Comparison of biological activities in acetone dried and lyophilised glands**

The two series of tissues were similar in showing an increased accumulation of the active agents with the advancement of gestation, a predominance of vasopressor and antidiuretic activities over the oxytocic activity during embryonic development, and an apparent reduction in the
Figure 10

Weighted means of biological activities in acetone dried neurohypophyses of sheep foetuses and lambs.

mU/mg = milliunit per mg dry gland
SHEEP FOETUS (ACETONE DRIED)

Days Gestation

ADH

pressor

oxytocin

2000

1500

1000

500

mU/mg

10 30 50 70 90 110

130 147

25

term Post Natal
level of active peptides after birth. The changes in ratios during intrauterine life and early post-natal development have shown essentially the same pattern in both series, as illustrated in Fig. 13.

However, the two series differed from one another in several other respects. The difference between the acetone dried and lyophilised glands was very pronounced in the absolute hormonal potencies. At 90 days of gestation oxytocic and vasopressor activities were 80-90% lower in the acetone dried than in the lyophilised glands. At 140 days, the loss of activity for both biological activities amounted to approximately 30%, and after birth again a greater loss of activity was detected, approximately 50-60%. From these values it appeared that the extent of acetone extraction was closely similar for the oxytocic and vasopressor activities, but varied from one stage of development to the other. Acetone extracted a high proportion of both active agents at the early stage of embryonic and at post-natal development, but exhibited relatively little influence on the hormonal content of the pituitary in late gestation.

In the lyophilised series, a consistently higher antidiuretic than vasopressor activity was detected, but in the acetone dried series a good correspondance between the two activities was found. In view of the fact that vasopressor and antidiuretic assays supposedly measure the
Ratios of biological activities in the acetone dried neurohypophyses of sheep foetuses and lambs.

Ratio of $V/O = \frac{\text{weighted mean of vasopressor activity per mg dry gland}}{\text{weighted mean of oxytocic activity per mg dry gland}}$

Ratio of $A/O = \frac{\text{weighted mean of antidiuretic activity per mg dry gland}}{\text{weighted mean of oxytocic activity per mg dry gland}}$
Ratio of Activities: Sheep Fetus vs. Adult Dried
biological activities of the same peptide, this finding was unexpected.

Finally, in the acetone dried series a fall in potency of the neural lobe was detected after delivery, while in the lyophilised glands the oxytocic, vasopressor and antidiuretic activities were found to be already lower at the time of delivery. Since these estimations are based on single glands in both cases, the discrepancy could be due to individual variations. However, it could also represent a depletion of the posterior pituitary of the lamb during delivery. Although both glands were taken in the late phase of the first stage of delivery, it is possible that labour had been more arduous in the case of the lamb in which the posterior pituitary was consequently lyophilised; extra depletion could have occurred as a result of extra stress.

2. Purification and amino acid analysis of the active agents in the neurohypophyses of sheep foetuses

In view of the high V/O and A/O ratios detected in the neurohypophyses of sheep foetuses, a possible separation and identification of the active principles appeared to be especially important. Arginine vasopressin has an intrinsic rat uterus activity as well as vasopressor activity, and these are in an approximate V/O ratio of 20:1 (Berde, Boissonnas, 1966). Since the ratios recorded in the individual glands of sheep foetuses often approached this figure, it seemed possible that the early sheep foetus possessed arginine
Figure 12

Weighted means of biological activities in the lyophilised neurohypophyses of sheep foetuses and lambs.

mU/mg = milliunit biological activity/mg dry gland
SHEEP FOETUS (LYOPHILISED)

Days Gestation

mU/mg

ADH

pressor

oxytocic

0 10 30 50 70 90 110 130 147 term Post Natal
vasopressin alone, and was free of oxytocin.

However, the low biological activities and limited amount of material in the early foetuses made purification by gel filtration impossible. Therefore, the pooled sample of posterior pituitaries of early foetuses, which contained both acetone dried and lyophilised glands, was subjected to paper chromatography in order to determine whether an activity peak, typical of oxytocin itself could be resolved from the mixture.

Neurohypophysial extracts from the later, 140 days old foetuses were subjected to gel filtration, and the purified principles were analysed for their amino acid composition.

a. **Partial purification of foetal neurohypophysial principles by paper chromatography**

The posterior pituitaries of 22 foetuses at 89-91 days of gestational age, with a total dry weight of 14 mg, were extracted in 1 ml of 0.25% acetic acid (see Methods). 0.84 ml of extract, which represented a total of 350 mU of oxytocic and 7250 mU of vasopressor activity, was applied to Whatmann #3MM chromatography paper. 500 mU synthetic oxytocin ("Syntocinon") and 12500 mU commercial vasopressin preparation ("Pitressin") were run alongside the extracts as standards.

The chromatogram was developed in butanol/acetic acid/water (4:1:5) for a period of 13 hours at room temperature. After completion of the development, the chromatogram was dried in a cool stream of air, and when dry, cut into squares,
Figure 13

Ratios of biological activities in the lyophilised neurohypophyses of sheep foetuses and lambs.

Ratio of V/O = weighted mean of vasopressor activity/mg dry tissue/weighted mean of oxytocic activity per mg dry tissue.

Ratio of A/O = weighted mean of antidiuretic activity per mg dry tissue/weighted mean of oxytocic activity per mg dry tissue.
of which each one represented 0.1 of an $R_f$ unit (see Methods).

Each square was eluted in 0.25% acetic acid and analysed for biological activities. The results are presented in Fig. 15, and show a successful separation of oxytocin from vasopressin. Synthetic oxytocin was located at $R_f 0.5-0.7$, and arginine/lysine vasopressin (Pitressin) at $R_f 0.3-0.4$, with a trace (15 mU) overlapping into $R_f 0.5$. From the 500 mU synthetic oxytocin applied, a total of 163 mU was recovered, representing a 32% recovery. The 4000 mU Pitressin recovered was again a 32% recovery.

Both oxytocic and vasopressor activities were detected in the chromatogram of the foetal posterior pituitary extracts. Oxytocic activity was found from $R_f 0.2$ to 0.7, and vasopressor activity in $R_f 0.2$ to 0.4, with traces of activity in $R_f 0.5$ and 0.6. The rat uterus activity in $R_f 0.5-0.7$ corresponds to the location of synthetic oxytocin, and the rat uterus activity in $R_f$ units corresponding to those of the vasopressor activity can be partially explained by the intrinsic oxytocic activity of vasopressin. In view of the rat uterus activity in $R_f 0.5-0.7$, which runs ahead of vasopressin, and corresponds well to the location of the synthetic oxytocin, it is reasonably certain that the neurohypophyses of early foetuses contain oxytocin itself. The oxytocic activity recovered in $R_f 0.5-0.7$ is approximately 32% of the rat uterus activity detected in the crude extracts,
Figure 14

Comparison of ratios of biological activities between the acetone dried and lyophilised tissue of sheep foetuses and lambs.

Ratio of V/O = weighted means of vasopressor activity per mg dry tissue/weighted means of oxytocic activity per mg dry tissue.

Ratio of A/O = weighted means of antidiuretic activity per mg dry tissue/weighted means of oxytocic activity per mg dry tissue.
RATIO OF ACTIVITIES, FOETAL SHEEP

- Lyophilised
- Acetone dried

Days Gestation

Ratio of V/O

Ratio of A/O

Term Post Natal
which gives a recovery, similar to the recovery found for the standards.

Vasopressor activity, from the foetal extracts was located at $R_f$ 0.2-0.4 with traces in $R_f$ 0.5 and 0.6 as was seen also in the case of standard vasopressin. This is in good agreement with the $R_f$ values of arginine/lysine vasopressin (Pitressin) and indicates the probable presence of arginine vasopressin in the early foetus.

In the chromatogram of early foetuses, rat uterus activity was detected in the $R_f$ units corresponding to the vasopressor activity as well as in those which corresponded to the $R_f$ values of oxytocin itself. As pointed out earlier, the presence of rat uterus activity in the vasopressor peak was not entirely surprising, in view of the intrinsic oxytocic activity possessed by vasopressin. However, the magnitude of this activity was found to be 5 times too great to be accounted for by the amount of vasopressin present. This discrepancy suggested the possible presence of a slow-moving principle, with chromatographic behaviour similar to that of arginine vasopressin, but which contrasted with it in possessing a relatively higher intrinsic oxytocic activity. The only known naturally occurring peptide with the properties approximating to these, is arginine vasotocin. Arginine vasotocin is distinguished from the other neurohypophysial peptides, by its very high activity in promoting water transport through the isolated frog bladder. Therefore, in order to determine the possible presence of arginine vasotocin
Figure 15

Paper chromatogram of crude extract of the neurohypophyses of sheep foetuses at 89-91 days of gestational age.

Black = rat uterus activity
Striped = vasopressor activity
White = frog waterbalance activity
Synthetic Oxytocin
Rat Uterus
Standard Vasopressin
Vasopressor
Sheep Foetus
within the vasopressor peak, frog bladder assay was carried out. As shown in Fig. 16, a very high frog bladder activity was detected at the peak of the vasopressor activity, and a lesser quantity was found in the two \( R_f \) units on either side. The frog bladder activity, which amounted to 38,000 mU in the region between \( R_f 0.2-0.3 \), is 450 times too great to be attributable to the amount of vasopressin present. It is 100 times too great to be elicited by the amount of rat uterus activity in this region, if this rat uterus activity was the result of a possible trailing of oxytocin into the vasopressor peak. In any case, such trailing is most unlikely in view of the small amount of oxytocin present. Therefore, there is a strong suggestion that the neurohypophyses of early foetuses contain arginine vasotocin in addition to arginine vasopressin and oxytocin.

b. Gel filtration of crude neurohypophysial extracts from late foetuses

Since separation and a high degree of purification was achieved with maternal extracts by the use of 200 cm G-15 Sephadex columns, the same method was applied to the purification of neurohypophysial extracts of late foetuses. Acetone dried and lyophilised neurohypophysial extracts of sheep foetuses at 138-141 days of gestation age were pooled, and extracted in 0.25% acetic acid. A total of 15 ml extract, representing 4.4 \( \pm \) 0.48 I.U. oxytocic and 34.0 \( \pm \) 8.0 I.U. vasopressor activity, were applied to a 200 cm G-15 Sephadex column. The eluate from the column was collected in
Figure 16

Purification of crude extract of sheep foetuses at 138-141 days of gestational age, on 200 cm Sephadex G-15 column.
Purification of Foetal Sheep Extract on 200 cm. Sephadex G-15 column

Tube Number (1 tube = 2.8 ml)

- Lowry
- Vasopressor
- Oxytocic
2.8 ml fractions as previously described for the maternal extracts and the fractions were analysed for biological activities, protein content and conductivity. The results are shown in Fig. 17.

The first 150 ml of eluate represented the void volume and was discarded. The mass of proteins, as determined by the Lowry peptide method, were concentrated in the first 40-70 fractions. The oxytocic activity was well separated from the Lowry peptide section by 70 fractions. A further 20 fractions divided the vasopressin from the oxytocic peak. The marked separation of the active agents from the protein fractions, and the complete resolution of the oxytocic and vasopressor activities indicated that a good purification of the hormones was achieved. The 2.0 I.U. oxytocic activity detected in the oxytocic peak represented a 100% recovery, if the contribution of the large amount of vasopressin to the oxytocic activity of the crude extract is taken into account. The recovery for vasopressin was again approximately 80%.

The pooled fractions of the oxytocic peak were assayed for their oxytocic activity both in the presence and absence of 0.5 mM MgCl₂. The ratio of oxytocic activity with Mg²⁺/without Mg²⁺ was found to be 0.84, which is in good agreement with the value of 0.86 reported by Munsick (1960) for the potentiation of synthetic oxytocin. Since oxytocin is the only known neurohypophysial principle in which this ratio is less than 1, this result strongly suggests that the rat uterus
Figure 17

Sample plate of thin layer chromatography of the dansyl derivatives of standard amino acids, and of the hydrolysate of the oxytocic moiety of the adult pregnant sheep and the foetus.

DNS = dansyl
solvent front

DNS hydrolysates
Oxytocic
Adult Foetal

DNS Tyr
DNS Pro
DNS Leu
DNS Ileu

DNS Gly
DNS Asp
DNS Cyt

origin

di DNS Lys
DNS Gfu
contracting principle of the late foetus is oxytocin itself.

The estimation of the oxytocic activity throughout the vasopressor peak, showed that one fraction (187 in Fig. 17) contained an unusually high oxytocic activity, which could not be accounted for by the intrinsic oxytocic activity of vasopressin. Frog waterbalance assay was carried out on this fraction to determine whether it might contain arginine vasotocin, as the work presented above had suggested that this peptide might be present in the young foetuses. The total frog waterbalance activity in the 2.8 ml of eluate present in this tube was found to be 5,600 mU. This value is 80 times greater than could be expected from arginine vasopressin alone, if it were present in the quantity indicated by the pressor assay. Again, this suggests the presence of a peptide other than vasopressin, a peptide possessing unusually high frog bladder activity. The same arguments given previously for the similar activity found in the early foetus suggest that this peptide might be arginine vasotocin.

c. Amino acid analysis of the purified peptides of the late foetuses

The oxytocic and vasopressor fractions of 140 days old foetuses, obtained from the G-15 Sephadex column were pooled, lyophilised and hydrolysed in 6N HCl (see p. 22 Methods). The hydrolysates were reacted with the fluorescent
Figure 18

Graphed $R_f$ values of dansylated standard amino acids, and the dansylated hydrolysate of the foetal vasopressor moiety.

d. $\text{NH}_2$ = dansyl ammonia
di- Lys = di- dansyl derivative of lysine
$\xi$- lysine = mono-dansylated derivative of lysine

Amino acids of the unknowns are labelled only where identification of their $R_f$ values correspond closely to the $R_f$ of a standard amino acid.
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marker, dansyl chloride, as described in detail on p. 23

The resulting amino acid complexes were applied to thin layer silica plates, beside dansylated amino acid standards. The hydrolysates from the foetus were developed on the plates parallel to hydrolysates from the adult, to provide a direct comparison between the amino acid composition of the two. The plates were run in four different solvent systems for optimal resolution of the standard amino acids.

The constituent amino acids in the hydrolysates of the maternal and foetal oxytocic moieties had shown an identical chromatographic behaviour, as illustrated by the sample plate given in Fig. 17. The more complete analysis of the maternal material had shown the eight constituent amino acids of oxytocin, as already discussed in Section I, and since the foetal oxytocic principle exhibited the same chromatographic pattern, this indicates that the amino acid composition of the foetal oxytocic peptide is the same as that of the adult. When taken in conjunction with the pharmacological data, this strongly suggests that oxytocin itself is the oxytocic agent of the late foetuses.

The much greater amount of vasopressor agent in the late foetuses allowed a more complete analysis of the constituent amino acids of this peptide. The $R_f$ values of the standard amino acids and the constituent amino acids of the hydrolysate are shown in Fig. 18. The constituent
amino acids identified were: phenylalanine, arginine, aspartic acid, proline, glycine, glutamin acid, tyrosine, and cysteic acid. In addition to the eight constituent amino acids of arginine vasopressin, lysine and alanine were also detected. It is assumed that these two amino acids were introduced into the hydrolysates during processing either by the breakdown of constituent amino acids (as in the case of alanine), or as contamination from the glassware.
DISCUSSION

(Section II)

The estimation of biological activities in sheep foetuses and lambs have shown a steadily increasing accumulation of the biologically active principles throughout gestation. Increasing amounts of hormonal activities with the advancement of embryonic development were characteristic of both the acetone dried and lyophilised glands. Around the time of birth a secondary fall in both oxytocic and vasopressor activities were found. This fall in potency appeared to occur at the time of natural delivery in the lyophilised series, but occurred only after birth in the acetone dried series. The ratio of vasopressor to oxytocic and of antidiuretic to oxytocic activities were high at the early gestational age (90 days), and in some cases approached the value of 20. During the course of embryonic development and early post-natal life, these very high ratios decreased steadily. The results in the sheep foetuses and newborn lambs agree generally with those reported by Dicker and Tyler (1953 a, b) for human and dog foetuses, with respect to the originally high, and steadily declining V/O ratios during gestation.

The high ratio between the vasopressor and oxytocic activities in the foetal neurohypophysis is the result of the
very low oxytocic activities during embryonic development. As before, in connection with various other aspects of the work reported here, the question arises as to whether the low oxytocic content of the immature posterior pituitary is a product of depressed hypothalamic secretion, or to an increased depletion of the gland of its oxytocic moiety. The latter possibility appears to be an unlikely one. On the basis of the primary actions of oxytocin in the adult organism, where it is connected with reproduction and the nursing of the young, it is hard to envisage a possible function for this agent in the foetal organism.

The more likely possibility appears to be a depressed hypothalamic production of the oxytocic agent. Histological evidence supports this hypothesis. Staining with Gomori's chrome haematoxylin phloxin stain shows an earlier embryonic appearance of stainable material in the supra-optic nucleus, than in the bodies of the paraventricular cells of human foetuses (Meitner, 1959). Since the paraventricular nucleus is mainly responsible for the production of vasopressin (Heller, 1966), this observation points to a later embryonic development of the oxytocin producing center. A later embryonic development of the paraventricular nucleus has been demonstrated in other species as well, such as the mouse, rat and calf (Yakovleva, 1966).

It is necessary to point out, that histological evidence, for the presence of neurohypophysial hormones is often unreliable, and biological activities can often be
detected before the appearance of secretory material (Heller, 1961). Further, a disappearance of these granules can occur without a loss in potency (Farrell, 1967). However, the large number of similar reports, which give histological evidence for a time difference between the appearance of secretory material in the two hypothalamic nuclei, seem to make a strong argument in favour of a later embryonic appearance of the oxytocin synthetising structures.

In the results concerning the neurohypophysial hormone content of the sheep foetuses reported here, a consistent loss of biological activity was found following prolonged exposure of the glands to acetone. This problem has already been discussed in Section I, in connection with the same phenomena in pregnant females. The studies on foetal neurohypophyses only emphasised further the fact that acetone leaches out both active agents to approximately the same degree. However, the extent of this removal varies with different physiological conditions. In the foetal glands the greatest loss of activity was observed at 90 days of gestation, and a lower loss occurred during the early postnatal age. On the other hand, there was only a slight loss of activity in late gestation, when the foetuses had been in utero for 140 days.

The comparison of potencies in the lyophilised and acetone dried glands of sheep foetuses have also shown a consistently and significantly higher anti-diuretic than vasopressor activity in the lyophilised but not in the
acetone dried tissues. Again, only suggestions can be made as to the possible factors contributing to the greater antidiuretic, than vasopressor activities recorded in the lyophilised glands of foetuses. Since these two bio-assays supposedly measure the potency of the same hormone; the higher antidiuretic potency could be due to either an additional antidiuretic agent in the hypophysis of the foetuses or possibly to the presence of a vasodepressor agent. Due to lack of material, no tests could be carried out to determine the possible presence of either agent. In view of the fact, however, that other workers have come across the same phenomena (Hild and Zettler, 1953), and that various reports claim the separation of the vasopressor from the antidiuretic principle (Kammen and De Wield, 1960) the problem warrants further investigation.

The purification and analysis of the active principles of the foetal sheep neurohypophysis have shown the presence of an oxytocic and vasopressor principle. The $R_f$ values of the biologically active fractions in the posterior pituitary extracts of the foetuses at 90 days of gestational age suggest that these active agents are: oxytocin and arginine vasopressin. Again, in the older, 140 days old embryos, both the pharmacological evidence and the amino acid analysis point to oxytocin and arginine vasopressin being the active agents.

In addition to the two neurohypophysial peptides typical of the mammal, the presence of a third peptide was
detected. The chemical behaviour and pharmacological properties of this third peptide suggest that it might be arginine vasotocin, the antidiuretic principle of lower vertebrates. The amount of this peptide appears to decline with the advancement of gestation. Calculations, which converted the biological activities to mg peptide present, indicated that in early gestation (90 days) arginine vasopressin and arginine vasotocin are present in an approximately 1:1 ratio. By 140 days of intrauterine life the ratio of the two peptides was approximately 120 in favour of arginine vasopressin.
GENERAL DISCUSSION

The data obtained in the sheep during pregnancy and embryonic development, have shown that changes occur in the neurohypophysis of both the foetus and the mother during the course of pregnancy. About mid-pregnancy, the neurohypophysis of the mother contains a reduced level of its active agents, and although there is an increase in potencies towards late gestation, the hormonal levels do not return to the control, non-pregnant level throughout the duration of pregnancy.

During embryonic development, an increasing accumulation of biologically active agents were detected. The presence of both peptides, characteristic of the adult mammal, oxytocin and vasopressin have been shown to be present in the foetus. In addition to these active principles, the presence of a third neurohypophysial peptide, probably arginine vasotocin, has been indicated. The neurohypophysis of the embryo is characterised by a predominance of vasopressor over oxytocic activity.

The results in both the pregnant adult and in the foetus have been discussed briefly in the light of the limited literature, directly concerned with these topics. However, the most important questions have not yet been touched on; these concern the relationship of the changes in
the neurohypophysis to the more general picture of pregnancy and foetal development, and to the possible physiological role of the active agents in the metabolism of the embryo. This general discussion will attempt to develop some of these problems. However, because of the scarcity of information, many of the following considerations are purely speculative.

1. Changes in hormonal level of the neurohypophysis during pregnancy

The lowered activity of the neurohypophysis during pregnancy raises the question of whether this depletion is due to a depressed hypothalamic production of hormones, or to a greater release into the bloodstream. Either change might be related to changes in other endocrine glands, and therefore, a brief description of the primary endocrine modifications which take place must be given.

The main endocrine changes during pregnancy are the elevated estrogen and progesterone levels, which may originate to a large extent from the corpus luteum, or from the placenta. In the sheep, where the placenta takes over the production of these steroids at 50-60 days of gestation (Marshall, 1959), the progesterone level rises to 6 ug/ml in the peripheral blood of the pregnant female at 40 days of gestation and remains at this level until 100 days. At about 100 days the blood progesterone level rises and reaches a second plateau at 12 ug/ml blood, and this is maintained up to 36 hrs post parturition (Neher and Zarrow, 1958). Although
there is no information concerning the estrogen levels in the sheep during pregnancy, in the goat and the cow, which are similar to the sheep in their hormonal control of gestation, estrogen level was reported to rise steadily in the second half of pregnancy and reached a maximum value at term (Barrie, Patterson and Underhill, 1935; Bromskov, 1939).

Evidence concerning the possible action of the steroid hormones and the posterior pituitary peptides is suggestive, but sketchy.

At the level of the target organs, progesterone has been shown to decrease the sensitivity of the uterus to oxytocin, whilst estrogen enhances the action of this peptide (Fitzpatrick, 1966). The target organs of vasopressin react in a similar manner to progesterone and estrogen; sensitivity is lessened in the presence of the former and increased by the latter steroid. Cobo (1967) reported a high threshold for antidiuretic responses to vasopressin in pregnant women, and Pickford (1966) found a similar effect of progesterone on the salt excretion and vascular response to the same principle.

At the level of the hypothalamic nuclei, Cross and Silver (1965) reported depressed activity of both the supra-optic and paraventricular nuclei, following administration of progesterone.

Besides evidence for the influence of steroid hormones on the production and effectiveness of the neurohypophysial
peptides, these peptides may in turn effect the steroid levels themselves directly or indirectly, through the mediation of the gonadotrophic hormones of the adenohypophysis. The role of the neurohypophysial hormones as releasing factors for the gonadotrophic hormones has been reviewed by Martini (1966), and only one example will be given here. It has been shown that large doses of oxytocin will bring cattle into premature estrous, and it is presumed that the peptide inhibits progesterone production, so that the consequent fall in blood level unleashes the release of gonadotrophic hormones from the adenohypophysis (Labsathwar et al., 1964).

These fragmentary pieces of evidence encourage the speculation that the elevated progesterone level renders the target organs less sensitive to the neurohypophysial hormones at 90 days of pregnancy. This in turn might result in an increased release from the posterior pituitary of its active agents, through secondary mechanisms, only partly understood at the present. At the same time, the elevated progesterone level might also partially inhibit hypothalamic production of the peptides. The combined effect of an increased release and partially inhibited production could result in the lowered activity of the neurohypophysis as found in the studies of pregnant sheep at 90 days of gestation.

In the latter part of pregnancy, the rising estrogen level, which reaches its maximum at term, could possibly
counteract the action of progesterone, both at the level of the target organ and in the hypothalamus. The rising estrogen level would reestablish a balance nearer to that of the control, where the release of the neurohypophysial peptides will be decreased and the production by the hypothalamus increased. This would result in a greater accumulation of neurosecretory material in the neurohypophysis and would explain the secondary rise in activities found at 140 days or pregnancy in the sheep.

2. The possible role of the antidiuretic principle, during embryonic development

The relative abundance of the vasopressor or antidiuretic principle in the neurohypophysis of the foetus, represents one of the most interesting and still unresolved problems of posterior pituitary function during embryonic development. It is not certain whether this predominance of the antidiuretic activity during intrauterine life represents an earlier appearance of the synthetic mechanism for the production of the antidiuretic agent or whether it reflects the greater use of this agent by the organism. However, this brief discussion will attempt to show a possible use for the antidiuretic principle by the foetal organism.

General evidence indicates a role for the antidiuretic activity in the volume regulation of the amniotic fluid. Berniscke and McKay (1953) correlated histological
studies of the posterior pituitary of human foetuses with the volume of amniotic fluid present, and found that foetuses with very little stainable material in the pituitary have a great excess of amniotic fluid, and conversely, an abundance of secretory material was associated with smaller amniotic fluid volume. On the basis of these observations it might appear that the foetal neurohypophysis plays some part in the regulation of the amniotic fluid volume, but very little is known about the possible target organs which might be concerned. The primary action of the antidiuretic hormone in the adult would suggest that the foetal kidney could be a possible target organ for the action of the embryonic antidiuretic principle. However, Heller (1949) working with both newborn humans and rats, failed to elicit either a diuretic response to waterload, or an antidiuretic response to dehydration. On the other hand, Pauline (1962) recorded that hydration of the foetal sheep at 130 days of gestation resulted in an increased glomerular filtration rate and elevated urine flow. Injections of vasopressin brought both the elevated filtration rate and urine flow back to a control level. However, vasopressin was found to be without effect by both the above workers in animals where filtration rate and urine flow have not been previously elevated. Vasopressin at no time promoted the formation of hypertonic urine in immature animals. On the basis of these and several other experiments Pauline concluded that the immature kidney is unable to concentrate its urine, and attributes this failure
to an electrolyte reabsorption by the immature kidney, which exceeds the reabsorption of water. However, in passing, it should be pointed out that the failure to produce hypertonic urine by the foetus is not surprising, since it is practically impossible to produce a shortage of water in the foetus, which can draw all it needs from the maternal organism. Furthermore, the discharge of such a hypertonic urine into the embryonic fluid compartments would appear to be questionable benefit for both the mother and the foetus.

However, the production of hypertonic urine is not the only form of water regulation available to an animal. For example, the amphibian may utilise water transport through the skin and the bladder (Sawyer, 1967). Nor are the membranes of the kidney the only structures in an adult mammal where vasopressin can promote water reabsorption; this is shown by the action of the peptide on the membranes of the pancreas, liver and sweat glands, where it reduces the volume of secretion (Wakim, 1967).

In the foetal organism too, there are many plausible alternate target organs, for a possible effect of the antidiuretic principle, besides the kidneys. A brief description of the production of the amniotic fluid, and the compartments in which it is contained suggest several possible points of action for the antidiuretic agent.

In foetal sheep of less than 90 days of gestational age, urine from the bladder passes into the allantoic sac. Beyond this age, the urine passes into the amniotic sac in
progressively greater amounts (Pauline, Nixon, 1962). The rate of foetal urine production is more than adequate to account for the volume of embryonic fluid, but it is by no means totally responsible for the production and maintenance of the fluid environment of the foetus. In the sheep, the buccal cavity and the foetal lungs can secrete up to 15 ml of fluid per hour (Reynolds, 1953). The swallowing of amniotic fluid is also known to occur, and absorption from the gastrointestinal tract also contributes to the exchange of water and electrolytes between the foetus and the embryonic fluids.

Therefore, the possible target organs of the embryonic neurohypophysial peptides could include the allantoic and/or the amniotic membranes outside the foetus, or the lungs and buccal cavity within the foetal organism. There is no evidence in the literature to indicate whether any of these membranes could respond to vasopressin or to foetal neurohypophysial extract. More research will have to be done before the great abundance of antidiuretic principle reported here can be fitted into the general picture of the embryonic development, and the physiology of the foetus. However, the great number of potential target organs and the possible need of the foetus to regulate its aqueous environment suggests that this hormone may well be important to the organism during its intrauterine life.

3. The importance of the presence of arginine vasotocin in the foetus
The high frog waterbalance activity detected in the early sheep foetuses, with its possible implication that the early foetal neurohypophysis contains a third active peptide, arginine vasotocin, is important for more than one reason. Firstly, it is the first time that this antidiuretic agent, typical of lower vertebrates, has been found in a mammalian organism. Secondly, it raises the possibility that the target organs of the foetus at the earlier stages of embryonic development could be responsive to arginine vasotocin, rather than to arginine vasopressin.

Arginine vasotocin promotes water reabsorption in the amphibian kidney, and also regulates water uptake through the skin and bladder of certain amphibians (Sawyer, 1967). It might be speculated that the action of arginine vasotocin on extrarenal membranes could be of special relevance to the foetus, where it might promote water movement through the embryonic membranes, through the lungs or through the buccal cavity. Further, it may be of some significance, that arginine vasotocin increases the electrolyte excretion and influences the glomerular filtration rate of lungfish and certain other species. These two latter actions of arginine vasotocin might be important to the foetal organism, considering that electrolyte reabsorption in the foetus exceeds water reabsorption, and that regulation of the volume of the produced urine could be demonstrated on the level of changes in the glomerular filtration rate only. Arginine vasotocin that could possibly aid in the regulatory processes involved
in the amniotic fluid formation, and the electrolyte balance of the early foetus.

However, the demonstration of the presence of arginine vasotocin in mammalian foetuses, has another significance. It is important in considerations of the phylogenetic distribution of the neurohypophysial antidiuretic principle.

The neurohypophysis of most vertebrate classes contain an active rat uterus contracting principle, and an antidiuretic principle. In the oxytocin like rat uterus contracting principles, a great many mutations appear to have occurred during evolution. The structure of the active oxytocic principle can vary not only from one class of vertebrates to another, but also between species within the same class (Sawyer, 1967).

On the other hand arginine vasotocin exhibits a remarkable stability throughout the phylogenetic tree of vertebrates. There is evidence for its existance in all vertebrates except for the Mammals (Vliegenthart and Versteeg, 1967; Sawyer, 1967).

Vliegenthart and Versteeg (1967) have reviewed the evolutionary aspects of the neurohypophysial peptides and they have pointed out that the Cyclostomes possess only one peptide, arginine vasotocin, but all other vertebrate classes synthetise two active peptides. They suggest that the two active peptides evolved by a doubling of the vasotocin gene. Vasotocin remained unchanged, but further mutations took place
in the neutral peptide, and produced a series of oxytocin analogs. A point mutation in the vasotocin gene produced arginine vasopressin, the antidiuretic hormone of most mammals. In the Suina, a further mutation replaced arginine with lysine to produce lysine vasopressin.

The probable presence of arginine vasotocin in mammalian foetuses, as found here, does not fit the picture of neurohypophysial evolution which is accepted at present. The presence of three active agents in the foetus, contradicts the theory that a point mutation in the vasotocin gene produced arginine vasopressin. Such a point mutation would suggest the complete disappearance of the parent gene. A parallel presence of vasopressin and vasotocin would call for a further doubling of the vasotocin gene, and if such doubling of the gene indeed occurred, some neurons of the mammalian neurohypophysis might retain the capacity to produce arginine vasotocin in later life also.

The decrease in the total amount of arginine vasotocin and in the ratio of arginine vasopressin/arginine vasotocin, toward the end of intrauterine life, as was found in the sheep and discussed earlier, might be the result of the following possibilities. Firstly, in early embryonic life, there may be no call for the release of either antidiuretic principle, and the product of the more primitive gene arginine vasotocin dominates. With the advancement of gestation, possibly the gene producing the antidiuretic agent of the adult mammalian neurohypophysis could become dominant. Secondly, the foetal
membranes could be more sensitive to arginine vasotocin in early gestation, and therefore physiological stimuli might be in favour of the production of this agent. In later embryonic life, and after birth, the mammalian target organs may become sensitive to vasopressin, and the physiological stimuli could promote the production of arginine vasopressin. The vasotocin producing neurons could consequently either degenerate, or they could produce such small quantities that the presence of arginine vasotocin might not be detectable by conventional studies.

In passing it is worthwhile to point out that the presence of three neurohypophysial peptides was also demonstrated in some of the Suina, where some species produce both arginine and lysine vasopressin, in addition to oxytocin (Heller, 1966). However, this situation is not analogous to the simultaneous presence of arginine vasopressin and arginine vasotocin in the foetus, since the presence of the two vasopressins in the pig family can be explained by cross-breeding between species elaborating one or the other of the vasopressins, and producing offsprings where both the arginine and lysine vasopressin producing genes survive. In the case of the foetus the presence of the two antidiuretic principles could not be accounted for in this manner, since there is no known adult mammal that has arginine vasotocin as the active antidiuretic agent. Although only suggestions can be made at the present as to the possible genetic implications of the presence of arginine vasotocin in the pituitary of a
mammalian foetus, it could become an important aid to the understanding of the evolution of the neurohypophysial peptides.
SUMMARY

The posterior pituitaries of pregnant sheep were collected throughout two breeding seasons, and were either dried in acetone, or lyophilised. Extracts of the posterior pituitaries were analysed for biological activities, purified, and the purified agents examined for their amino acid composition. The results of these studies have shown:

(1) A fall in potency for the oxytocic, vasopressor and antidiuretic activities occurred in the neurohypophyses during pregnancy. The loss of activity was found to be more marked in the earlier (90 days) stage of pregnancy, than in the later (140 days) stage. At the time of natural delivery, a very marked increase in the oxytocic activity of the posterior pituitary of the mother was detected.

(2) The pattern of changes in the biological activities throughout pregnancy were similar in the acetone dried and lyophilised tissues, but acetone treatment resulted in a greater loss of the biological activities from the neurohypophyses of pregnant sheep.

(3) In the foetus, with the advancement of gestation, an accumulation of all active principles of the neurohypophyses was found. At around the time of birth a secondary fall in potency was detected.

(4) The vasopressor and antidiuretic activities
greatly surpassed the oxytocic activity all through gestation as was shown by the large V/O and A/O ratios. The relatively greater accumulation of the oxytocic moiety, during the latter half of embryonic development, resulted in the declining V/O and A/O ratios at the later stages of intrauterine life, and early post natal age.

(6) In the acetone treated glands, biological activities were found to be lower for oxytocic vasopressor and antidiuret activities, than in the lyophilised glands. The loss of activity from the acetone treated tissue was of the same magnitude for both oxytocic and vasopressor activities, but varied from one stage of development to the other.

(7) A new modification of the gel filtration method was employed, to achieve a one step purification of the biologically active principles from the crude neurohypophyseal extracts of the pregnant sheep, and sheep foetuses.

(8) The amino acid analysis of the purified neurohypophyseal agents have indicated that these are oxytocin and arginine vasopressin.

(9) Paper chromatographic studies of the neurohypophyseal extracts of early (90 days) foetuses again indicated oxytocin and arginine vasopressin as the active agents of these glands.

(10) An unusually high frog waterbalance activity in the purified neurohypophyseal extracts of sheep foetuses, indicated the presence of a third peptide in these extracts.
It is suggested that this third peptide is arginine vasotocin, the antidiuretic principle of lower vertebrates.
LITERATURE CITED


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