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The metabolic clearance of arginine vasopressin in the amniotic sac of the fetal guinea pig

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THE METABOLIC CLEARANCE OF ARGININE VASOPRESSIN IN THE

AMNIOTIC SAC OF THE FETAL GUINEA PIG

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
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ABSTRACT

A route for arginine vasopressin (AVP) clearance in the fetus has yet to be established. Ervin et al. (*Am. J. Physiol.* 250:E253-8, 1986) reported that AVP may be absorbed in the ovine fetal gut and remain biologically active. Thus, an AVP clearance problem could exist in the fetus since fetal urinary AVP may be recirculated. The results of the present study indicate that in the fetal guinea pig AVP is metabolized in the amniotic sac. When fetal urine and amniotic fluid (AF) were fractionated on HPLC, AVP was identified only in fetal urine. Moreover, when AVP and inulin were injected into the AF *in vivo* during the last week of gestation, the AVP/inulin ratio decreased (regression, $p=0.01$) over a 2 hour period, indicating a swallowing-independent disappearance of AVP. Also, the rate of AVP disappearance increased (regression, $p<0.01$) with increasing initial AF AVP concentration. When tritiated AVP was similarly injected, the radioactivity/inulin ratio remained constant, suggesting that AVP was not diffusing out of the amniotic sac. Two hours after the injection the AF was collected and fractionated by HPLC, and only 42% of the total radioactivity was intact AVP. The remainder was identified in 2 other products (M1 and M2). Tritiated AVP incubated with AF and amnionic membrane *in vitro* produced both M1 and M2. Tritiated AVP incubated with amnionic membrane in artificial amniotic solution produced M1 with no M2. Tritiated AVP incubated in only AF produced

M2 with no M1. Thus, neither metabolite was necessary for the production of the other, M1 was amnionic membrane dependent and M2 was AF dependent. The amnionic membrane metabolic system was functioning as early as 34 days of gestation (term = 68 days) whereas the AF enzyme system was not effective until 54 days of gestation, further support that the 2 metabolic systems are independent of each other. M2 comigrates with des-glycinamide AVP in three different HPLC buffers and crossreacts only with an AVP antiserum that detects des-glycinamide AVP. M2 production in the amniotic fluid can be blocked with the trypsin inhibitor aprotinin, suggesting that the amniotic fluid enzyme responsible for M2 production is trypsin-like.

In establishing the guinea pig as an animal model, amniotic fluid osmolality (OSM), and sodium (Na), potassium (K) and cortisol concentrations were measured in the second and third trimesters of gestation. Amniotic fluid OSM did not change and K concentration increased (regression, $p<0.01$) with gestational age in contrast to reported human values in which OSM decreased and K remained constant with age. Amniotic fluid Na levels decreased (regression, $p<0.01$) and cortisol levels increased (regression, $p<0.01$) with age in the guinea pig, similar to results reported in humans. Fetal plasma and urine OSM, Na and K were also measured in the late third trimester. Fetal urine was found to be slightly hypertonic compared to fetal plasma (328 ± 8 mOsm/kg H_2O vs. 308 ± 3 mOsm/kg H_2O, $p<0.05$), and urinary Na concentration was lower than fetal plasma (127.2 ± 1.0 mEq/l vs. 139.9 ± 0.4 mEq/l, $p<0.05$), which indicated that the guinea pig fetal kidney or bladder may be involved with the reabsorption of water and Na. Some other undetermined osmotically active substances besides Na and K are responsible for most of the fetal urine osmotic activity since Na, K, and attendant anions account for less than 15% of the urine osmolality. A difference was discovered between Na and K levels in the amniotic fluid and fetal urine: although fetal urine was hypertonic to amniotic fluid (328 $\pm$ 8 mOsm/kg H_2O vs. 287 $\pm$ 2 mOsm/kg H_2O, $p<0.05$), urinary Na and K concentrations were lower than amniotic fluid concentrations (by 115.8 mEq/l and 6.3 mEq/l, respectively). Therefore, fetal urine is modified in the amniotic sac. Amniotic fluid OSM measured in fetuses in the late third trimester was constant (287 $\pm$ 2 mOsm/kg H_2O) despite a large variation in urine osmolalities (288 to 393 mOsm/kg H_2O). The regulation of amniotic fluid OSM is not well understood but a hormonal regulation of water and electrolyte movement across the amnion is believed to be involved. The present study was unable to demonstrate an influence of amniotic fluid AVP on amniotic fluid OSM.

In conclusion, the amniotic sac may play a role in the clearance of AVP, as well as the clearance of other proteins, from the fetal system. Amniotic fluid OSM appears to be tightly regulated and the mechanisms behind this regulation remain unresolved.

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LIST OF ABBREVIATIONS

AVP .... Arginine Vasopressin (2.5 pg = 1 μU)

dgAVP .... Des-Glycinamide Vasopressin

OT .... Oxytocin

PA .... Pressinoic Acid

VT .... Vasotocin

PREFACE

When Oliver and Schafer (1895) discovered that extracts of the posterior pituitary caused a marked and rapid increase in blood pressure, endocrinology was not yet a science and "hormone" was not yet a word. Endocrinology began with the discovery of secretin by Bayliss and Starling (1902); it was not appreciated until then that chemical influences on the body could occur independent of the nervous system. Starling coined the word "hormone" from the greek "hormon" which means "setting in motion". Oliver and Schafer's discovery of the hormone later named "vasopressin" (due to its pressor action via arteriolar constriction and increased heart rate) "set into motion" the science of water and electrolyte endocrinology.

Since then the understanding of the functional significance of this hormone has greatly expanded. In 1901, Magnus and Schafer reported that posterior pituitary extracts, in addition to having pressor actions, could also be diuretic. In retrospect, it is clear that the diuretic action was due to its pressor effects at higher doses, leading to a so-called "pressure diuresis". However, Farini in 1913 showed that posterior pituitary extracts were also antidiuretic since such extracts could correct diabetes insipidus (for reference see Heller, 1974). Starling and Verney in 1924 demonstrated the antidiuretic action on the isolated kidney. The principle of "free water clearance" was introduced by Smith in 1937. In 1947 Verney developed the concept of the osmoreceptor and demonstrated the osmotic control of vasopressin release. During this time the field of biochemistry was beginning a new era with the Nobel prize winning work of du Vigneaud (1953) that characterized the structure of both oxytocin and vasopressin. These represented the first peptide molecules to be fully characterized and synthesized. Over the next two decades, exciting discoveries on all fronts of vasopressin research were made: the understanding of the control of vasopressin secretion, its metabolism, its actions, and the mechanisms underlying these actions.

The discovery of new methodology is often followed by great advances in science. Accordingly, the invention of the radioimmunoassay (RIA) by Berson and Yalow in 1956 opened the doors to a more sophisticated world of research. Until this time, hormonal measurements were obtained by a bioassay in which body fluids were injected into an animal and assessed for a particular physiological effect. The first RIA for vasopressin validated against an antidiuretic bioassay was developed by Miller and Moses in 1971. With the refinement of the vasopressin RIA in the 1970's came the ability to detect low concentrations and small changes in hormone levels under various physiological conditions. This increased sensitivity of hormone measurement enabled the study of vasopressin action at endogenous physiological hormone concentrations. Also, smaller sample sizes were now sufficient for analyses.

As more was known about the actions of vasopressin in the adult animal, interest in the ontogeny of this hormone grew. Of importance to the study of vasopressin in the fetus, was the advancement of fetal research methodologies during this time. Physical restrictions such as fetal size and accessibility of the *in utero* environment were no longer barriers to the study of fetal development. Thus, with both the ability to measure vasopressin at low concentrations and the improved accessibility of the fetus, a spectacular burst of research on the development of the neurohypophysial hormones in the fetus has been seen in the last decade.

CHAPTER I

INTRODUCTION

DEVELOPMENT OF THE FETAL VASOPRESSIN SYSTEM

THE APPEARANCE OF VASOPRESSIN IN THE FETUS

The fetal arginine vasopressin (AVP) system develops early in gestation. AVP is detectable in the neurohypophysis as early as 11 weeks of gestation in the human fetus (Burford and Robinson, 1982). Pituitary AVP content in the human increases with gestational age from about 0.07 mU/mg pituitary gland wet weight at 10 to 14 weeks to 1.5 mU/mg at 25 to 29 weeks to 4.65 mU/mg at 35 to 40 weeks (Fisher, 1979). In the fetal lamb, AVP is found in the pituitary from 120 days of gestation (term = 148 days; Skowsky and Fisher, 1977). AVP has also been detected in the fetal guinea pig neurohypophysis from 20 days of gestation (term = 68 days; Burton and Forsling, 1972).

Vasotocin (VT), an AVP analogue and the antidiuretic hormone of amphibians, is also found in the mammalian fetal pituitary very early in development, but progressively decreases towards term. Pavel first suggested that VT was secreted by the ependyndymal cells of the bovine fetal pineal gland (Pavel, 1971) and later demonstrated that rat pineal VT content decreased from the fetus to the newborn to the adult (Pavel et al., 1975). VT has since been identified in the pituitary by several investigators. Smith and McIntosh (1983) have demonstrated, by RIA combined with high pressure liquid chromatography (HPLC), the presence of VT in human fetal pituitary extracts as early as 70 days of gestation (0.32 ng/gland). In the last two weeks of gestation in the ovine fetus, the AVP pituitary content has been measured at 5.7 $\pm$ 2.9 mU/mg gland wet weight and the VT pituitary content at 0.8 $\pm$ 0.2 mU/mg gland wet weight (Skowsky and Fisher, 1977). Vizsolyi and Perks (1969) showed by bioassay that the neurohypophysis of fetal fur seals at 19% of term (the earliest measured in gestation) demonstrated 115.7 mU/mg dry gland weight vasopressor activity. In addition, they demonstrated frog bladder activity, postulated to represent VT activity, in extracts of the pituitary glands of both fetal sheep and fetal fur seals. It is possible that these measurements by bioassay and RIA with AVP antisera reflect a substance that is similar to but not identical to VT, since at least one group of investigators (Dogterom, et al., 1980) was not able to detect any VT immunoreactivity by RIA with a VT-specific antiserum. However, the verification of RIA activity by HPLC of human pituitary extracts by Smith and McIntosh (1983), and a recent study which isolated VT by HPLC in ovine fetal fluids (Ervin et al., 1985) seem to indicate that this hormone actually does exist in the fetus. The role of VT in the developing fetus is uncertain. It may be involved with water regulation in the fetus or could simply be an example of how "ontogeny recapitulates phylogeny" and is present only as a primitive form of AVP.

Vasopressin, identified by RIA is present not only in the pituitary gland and pineal gland but also in the subcommissural organ (Dogterom et al., 1980). In addition, AVP was detected by immunohistochemistry in the supraoptic nucleus (SON), median eminence, and neurohypophysis of the fetal guinea pig on day 45 of gestation (term = 68 days; Silverman, 1975). Buijs and associates (Buijs et al.,1980) have shown with immunocytochemistry that cells containing AVP can be detected in the SON from day 16 of gestation and in the paraventricular nuclei (PVN) from day 18 of gestation in the fetal rat (term = 22 days). They were also able to trace exohypothalamic AVP containing fibers to various other brain structures including the olfactory bulb, ependyma, amygdala, and corpus callosum.

Vasopressin levels of 0.08 and 0.6 pg/ml were measured by RIA in the cerebrospinal fluid (CSF) of two human hydrocephalic fetuses at 33 and 34 weeks of gestation (Punnonen et al., 1984). Vasopressin levels in the CSF of normal fetuses have not been reported. The CSF of healthy neonates at 2 to 48 days of age are about 6 pg/ml (Artman et al., 1984).

Plasma AVP levels can be detected throughout the third trimester in fetal sheep (e.g. Alexander et al., 1971a; Rurak, 1976; Stark et al., 1981). At 101 to 120 days of gestation fetal sheep plasma levels are 1.9 $\pm$ 0.4 μU/ml and from 121 days to term levels are 0.7 $\pm$ 0.3 μU/ml (Fisher, 1979). Ervin and colleagues (1985) measured by RIA and verified by HPLC the presence of AVP and VT in ovine fetal plasma at 3.83 ± 1.02 pg/ml and 2.42 ± 0.15 pg/ml, respectively. Plasma AVP levels in human fetuses have never been measured before parturition. Vasopressin levels of arterial and venous umbilical cord blood of "non-stressed" human fetuses sampled immediately after caesarean section delivery were measured by RIA to be 10.2 pg/ml and 4.1 pg/ml, respectively (median values; Johannesen et al., 1985). Several studies have reported high AVP levels in cord blood of human neonates after vaginal delivery but these levels may be influenced by fetal stress (e.g. Leung et al., 1980; Rees et al., 1980; DeVane and Porter, 1980) and probably do not reflect normal fetal plasma levels. Still, plasma AVP levels measured in fetal sheep and goats 15 minutes after delivery by cesarean section without labor are 5 times higher than levels before delivery, demonstrating an increase in plasma AVP which is independent of arterial pressure and blood gasses (Leffler et al., 1985).

The fetal AVP system appears to be independent of the maternal AVP system. Previous studies suggest, for instance, that AVP does not cross the placental barrier in the maternal to fetal direction (Czernichow, 1979). After in vivo maternal intravenous infusion of ^{125}I-AVP in the sheep, maternal plasma ^{125}I was measured at 2,500 counts/ml/5 min whereas no radioactivity could be measured in the fetus (Stegner et al., 1984). Also, several studies have demonstrated that there is no correlation between fetal and maternal plasma AVP levels (e.g. Daniel et al., 1982; DeVane et al., 1982; Leake et al, 1979).

Fetal urinary AVP levels have been well documented in sheep. Fetal urine AVP levels measured by RIA were 5.36 $\pm$ 0.55 pg/ml and VT levels were 1.44 $\pm$ 0.18 pg/ml (Ervin et al., 1985); the presence of these hormones was verified by HPLC.

When the present study was initiated the presence of AVP in amniotic fluid had not yet been verified. Early attempts to measure AVP in the amniotic fluid were unsuccessful with mostly undetectable, or very low levels reported (Alexander et al., 1971a). Swaab and Oosterbaan (1983) attempted to assess the contribution of the fetal brain to oxytocin (OT) and AVP levels in amniotic fluid. While they were able to demonstrate that there were no differences in amniotic fluid OT levels between normal and anencephalic rat or human fetuses, their AVP results were inconclusive since most of their amniotic fluid AVP levels were below the detectable limits of their radioimmunoassay. Since then several studies, including the amniotic fluid baseline study of this thesis, have measured amniotic fluid AVP levels by RIA. Human amniotic fluid levels at the end of gestation have been reported at 2.0 pg/ml (Wiriyathian et al., 1983), 3.0 pg/ml (Artman et al., 1984), 8.6 pg/ml (Johannesen, et al., 1985) and 39.4 pg/ml (Merialdi and Fatone, 1985). Human amniotic fluid levels at mid gestation have been reported at 3.0 pg/ml (Artman et al., 1984) and 9.0 pg/ml (Johannesen et al., 1985). The presence of ovine amniotic fluid AVP and VT have been verified by HPLC (Ervin et al., 1985). However, attempts to verify the presence of AVP in guinea pig or human amniotic fluid by HPLC methods successfully used to verify AVP presence in plasma and urine were not successful in our laboratory (unpublished results).

CONTROL OF FETAL VASOPRESSIN SECRETION

Fetal vasopressin secretion and hypoxia.

The increase in cord blood AVP concentration in response to stress at parturition of fetuses delivered vaginally is perhaps due to asphyxia. In chronically catheterized sheep, fetal arterial blood was sampled before labor induced with adrenocorticotrophic hormone, and during delivery. Fetal plasma AVP concentration increased from 2.1 $\pm$ 1.4 pg/ml before labor to 584.2 $\pm$ 433.0 pg/ml at delivery. Fetal plasma levels were inversely correlated with fetal arterial pH and pO_2 (Stark et al., 1981). Fetal sheep at 100 to 140 days of gestation were also shown to have elevated AVP levels in response to maternal hypoxia induced by the administration of 10% O_2 in nitrogen (Alexander et al., 1972; Stark et al., 1984). Fetal asphyxia induced by the administration of sodium pentobarbital in the maternal sheep also caused fetal plasma concentration to rise (DeVane et al., 1982). Fetal hypoxia induced by clamping the umbilical cord or administration of 10% O_2 to the pregnant ewe was shown to elevate both fetal plasma (by approximately 200 pg/ml) and amniotic fluid (by approximately 14 pg/ml) AVP concentrations in the fetal sheep after 30 minutes of exposure to hypoxia (Stark et al., 1984).

Fetal vasopressin secretion and osmoregulation.

Fetal AVP concentration increases in response to osmolar stimulation. Hyperosmolality was induced directly by infusion of hypertonic saline into fetal sheep or indirectly by infusion of hypertonic saline into maternal sheep which caused a fetal to maternal flow of water. Both stimuli resulted in increased fetal secretion of AVP (Leake et al., 1979). Fetal plasma AVP concentration also increased in response to infusion of furosemide in the fetal sheep, possibly via stimulation of the renin-angiotensin system (Siegel et al., 1980). A hyperosmotic challenge to the fetus independent of changes in fetal blood volume results in an increase in fetal plasma AVP and an increase in urine osmolality and urine sodium and chloride excretion, indicating that AVP can cause the concentration of urine (Woods et al., 1986).

Fetal vasopressin secretion and hypotension.

Volume control of AVP secretion is also operating in fetal sheep by the end of gestation. Hemorrhage in fetal sheep has been shown to cause an increase in fetal sheep AVP plasma levels in the third trimester (Alexander et al., 1971b) and in fetuses as young as 59 days of gestation (Drummond et al., 1980). A threshold for AVP secretion in response to slow hemorrhage in fetal sheep in the third trimester was defined at about a 13% blood volume loss (Ross et al., 1986b). Isovolemic hypotension induced by intravenous infusion of nitroprusside in fetal sheep also caused an increase in fetal plasma AVP levels which in turn caused a decrease in both free water and osmolar clearance (Ross et al., 1986a). There apparently is an age-related response to hypotension as the peak AVP levels seen in nitroprusside-induced hypotension in sheep fetuses less than 68% of gestation was 7.8 $\pm$ 2.7 pg/ml, and the peak AVP levels in fetuses greater than 89% of gestation was 63.5 $\pm$ 20 pg/ml (Rose et al., 1981).

VASOPRESSIN ACTIONS IN THE FETUS

Vasopressin and the fetal cardiovascular system.

Intravenous infusion of AVP in the sheep fetus causes an increase in fetal mean arterial blood pressure, a decrease in heart rate, and the redistribution of blood flow to the heart, brain and placenta, with a reduction of flow to the gastrointestinal organs and periphery (Iwamoto et al., 1979). High AVP levels found in cord blood at parturition were found to be correlated with high blood pressures and peripheral vasoconstriction indicated by low skin temperatures in human neonates delivered vaginally (Pohjavuori and Fyhrquist, 1980). The AVP secreted in response to hypoxia may shunt blood flow from the periphery towards the heart and brain (Iwamoto et al., 1979).
When an AVP antagonist was injected into the blood of fetal sheep, there was a greater fall in blood pressure in response to a 2% hemorrhage, indicating that AVP is necessary for the cardiovascular responses necessary for blood pressure regulation (Kelly et al., 1983).

Vasopressin and the fetal kidney..

The fetal kidney is able to respond to AVP during the third trimester and the relationship between plasma osmolality, urine osmolality and plasma AVP levels that is found in the fetus is similar to that found in the adult (Daniel et al., 1982). Vasopressin infusion into fetal sheep of more than 112 days of gestation results in an increase in urine osmolality and a decrease in free water clearance (Robillard and Weitzman, 1980). When an AVP antidiuretic antagonist is infused intravenously into the sheep fetus, no change in urine osmolality is observed indicating that changes in urine osmolality are mediated by fetal AVP (Woods et al., 1986).

Vasopressin is believed to be the stimulus for the renal response to hemorrhage where urine volume, and urinary osmolar, sodium, and potassium excretion have all been shown to decrease, along with the increase in fetal AVP secretion (Ross et al., 1986b). It is possible, however, that these responses could be independent of AVP and totally explained by a decrease in GFR.

Vasopressin does not affect glomerular filtration rate (Robillard and Weitzman, 1980) or renal blood flow (Iwamoto et al., 1979) in the fetus. A natriuresis is caused by AVP intravenous infusion which may partially account for the increase in urine osmolality. It has been hypothesized that the natriuresis obtained with high levels of AVP infusion is due to a decrease in plasma renin activity resulting from an increase in blood pressure (Robillard and Weitzman, 1980).

Maximum ovine fetal urine osmolality (336 $\pm$ 22 mOsm/kg H_2O) was achieved with plasma AVP concentrations of about 5 µU/ml which is similar to the plasma AVP concentrations required for maximal urine concentration in adults. However, it appears that the responsiveness of the fetal kidney to AVP may be less than the responsiveness in the adult kidney since this same urine osmolality can be achieved with only 0.7 µU/ml in the adult and the slope of the regression line relating plasma AVP to urine osmolality is less than the slope seen in adults (Robillard and Weitzman, 1980).

Urine flow rate was significantly reduced in sheep fetuses infused at low AVP rates (1.35 $pg \cdot ml^{-1} \cdot hr^{-1}$; Lingwood et al., 1978) but was not affected at higher AVP rates (plasma AVP concentration = 140 pg/ml, infusion rate = 600 $\mu U \cdot min^{-1} \cdot kg^{-1}$ (Robillard and Weitzman, 1980); and plasma AVP = 15-25 µU/ml, 1.2 ng/min (Woods et al., 1986)). It has been suggested that at high AVP doses, the pressor effects of AVP (as evidenced by an increase in mean arterial blood pressure) results in an increase in glomerular filtration rate and a pressure diuresis which counteracts the AVP antidiuretic action (Woods et al., 1986).

Fetal urine in humans and sheep is hypotonic relative to adult urine. This could be due to insufficient AVP levels, the unresponsiveness of the fetal kidney to AVP, or the incomplete elaboration of the AVP effect on an immature kidney. The above results indicate that AVP is present in sufficient concentrations in the fetus and that the fetal kidney is able to respond to this hormone. It is now believed that the fetus' hypotonic urine is the result of the lack of a fully developed concentration gradient in the fetal kidney and not low fetal renal responsiveness to AVP (Ervin et al., 1986a).

Recently, it has been shown that increased plasma VT can also increase urine osmolality to a maximum of 292 $\pm$ 19 mOsm/kg H_2O in fetal sheep via an increase in osmotic clearance and a decrease in free water clearance with no change in urine flow rate (Ervin et al., 1986). The increase in urine osmolality was attributed to an increase in sodium excretion. These investigators postulated that VT in fetuses may be more important in sodium regulation than water regulation.

Vasopressin and the fetal lung.

During *in utero* life, the fetus produces lung fluid which contributes to amniotic fluid volume. At birth, however, it is necessary for this lung fluid production to cease. It has been demonstrated that AVP can promote lung liquid reabsorption in fetal goats (Perks and Cassin, 1982). Also, intravenous AVP infusion causes a decrease in the production of fetal lung liquid in fetal sheep with no change in lung fluid osmolality (Ross et al., 1984; Perks and Cassin, 1985).

Since both the decrease in lung fluid and the decrease in urine volume caused by AVP act to decrease amniotic fluid production, AVP is believed to be involved in the regulation of amniotic fluid volume. In addition to its actions on the fetal kidney and lung, AVP has been demonstrated to affect fluid movement across the fetal membranes which is another way it may be involved in amniotic fluid volume regulation.

Vasopressin effects on water and ion movement across fetal membranes.

THE EFFECT OF VASOPRESSIN ON THE AMNION. Under the influence of osmotic and hydrostatic gradients from the amniotic fluid to the maternal fluid, Vizsolyi and Perks (1974) showed that water flowed from the fetal to maternal side of the amnionic membrane. When AVP or AVT was added to the fetal surface of the amnion, the flow of water slowed or reversed to a maternal to fetal direction. Manku and colleagues confirmed these findings and also described an effect of cortisol which reversed the AVP effect (Manku et al., 1975). In another series of *in vitro* experiments Holt and Perks demonstrated that AVP applied to the fetal surface of the amnion resulted in an increase in the flux of tritiated water from the maternal to fetal side with a corresponding increase in the flux of sodium in the same direction (Holt and Perks, 1977).

THE EFFECT OF VASOPRESSIN ON FETAL SKIN. Prior to keratinization before 45 days in the guinea pig fetus, the fetal skin is believed to be involved with the exchange of water between the amniotic fluid and fetal interstitium (Stiles and Power, 1983). *In vitro* experiments on fetal guinea pig skin at midterm showed that AVP added to the serosal side of the skin in pharmacological doses of 500 to 1000 mU/ml caused an increase in tritiated water movement across the skin towards the fetus (Perks, 1977). In fetal sheep at 47 to 97 days of gestation, AVP was demonstrated to cause an increase in the sodium uptake by the fetus via the skin (France, 1974). Thus, at least in early gestation, AVP can also regulate water and sodium movement across this fetal membrane.

THE EFFECT OF VASOPRESSIN ON THE FETAL BLADDER. Fetal guinea pig bladder urine was found to be hypertonic to fetal kidney urine (Boylan, 1958) which suggests that water can be absorbed from the fetal bladder. This hypothesis was verified when Perks (1977) demonstrated with in vitro experiments that 100 mU/ml of AVP added to the serosal surface of the bladder caused an increase in water movement towards the fetus.

In vitro studies with late gestation fetal sheep bladders showed that AVP added to the serosal side of the membrane caused an increased efflux of sodium from the mucosal to the serosal surface whereas, in early gestation, the reverse was found where there was a net influx of sodium from the mucosal to serosal side (France et al., 1972). The results seen with the late gestation fetal sheep may explain the hypotonic, low sodium urine found in fetal pig bladders (Stanier, 1971). The discrepancy in the results between early and late gestation fetal sheep indicates that physiological regulatory systems may change during gestation and generalizing results to all gestational ages and all species may not be warranted. In any case, these studies demonstrate that AVP may be involved in fetal water and sodium regulation via actions on the bladder.

VASOPRESSIN CLEARANCE

Because of the potential effects of vasopressin on the developing fetus, it is important to understand how the fetus is able to maintain homeostatic control of this hormone.

The fetus is presented with a special problem in clearing this hormone from its system due to its enclosed *in utero* environment. AVP excreted with urine is cleared from the adult system but can remain in the extracorporeal amniotic fluid circulation of the fetus. In fact, Ervin and his colleagues (1986b) have provided evidence that AVP can be recirculated via swallowing of amniotic fluid AVP. After instilling 25 ug of AVP into the amniotic sac, amniotic fluid AVP concentrations increased to about 6 ng/ml. Assuming the amniotic fluid volume in the fetal sheep to be about 1000 ml, the amnitoic fluid concentration should have been approximately 25 ng/ml and the reason for the recovery of less than 25% of the AVP injection into the sac was unclear. Nevertheless, plasma AVP levels increased in the fetus from about 2.5 pg/ml at baseline to about 25 pg/ml 120 minutes after injection. In addition, the AVP thus absorbed remained biologically active, resulting in an increase in urine osmolality from about 175 mOsm/kg H_2O before injection to greater than 300 mOsm/kg H_2O by 90 minutes after injection.

The fetus must have some way of removing this hormone from its system to prevent adverse effects on fetal cardiovascular function and water and electrolyte balance. However, the mechanism of this clearance is still poorly understood.

VASOPRESSIN CLEARANCE IN THE ADULT

In examining the special problems the fetus may face in AVP clearance, it is helpful to first briefly review the basic processes which are involved with the clearance of this hormone in the adult.

Several studies have documented the average half life of AVP in the plasma of a variety of species to be about 3.5 to 4.5 minutes which is relatively rapid when compared to the half life of other hormones in the blood. There appears to be at least 2 components involved with the clearance of AVP from the plasma; a fast component responsible for an AVP plasma half life of about 1.4 min and a slower component responsible for an AVP plasma half life of about 4.1 min (Weitzman and Fisher, 1978). Total plasma clearance rates in dogs, estimated by the steady state constant infusion technique, with low AVP infusion rates, was reported at 37.4 $\pm$ 7.4 $ml \cdot kg^{-1} \cdot min^{-1}$ at an infusion rate of 34 $\mu U \cdot kg^{-1} \cdot min^{-1}$. The rate of plasma clearance was increased at low plasma AVP concentrations and constant at plasma concentrations higher than 5 $\mu U/ml$ (Weitzman and Fisher, 1978), indicating that AVP clearance may be saturable. In contrast, plasma clearance rates in humans estimated by the same technique but with higher infusion rates (10 to 350 $\mu U \cdot kg^{-1} \cdot min^{-1}$) showed that AVP clearance rate increased linearly (6.8 $\pm$ 2.3 to 17.9 $\pm$ 2.0 $\mu U \cdot kg^{-1} \cdot min^{-1}$) with increasing infusion rates. These investigators attribute this increase in clearance rate to a possible decrease in plasma binding of AVP at high concentrations.

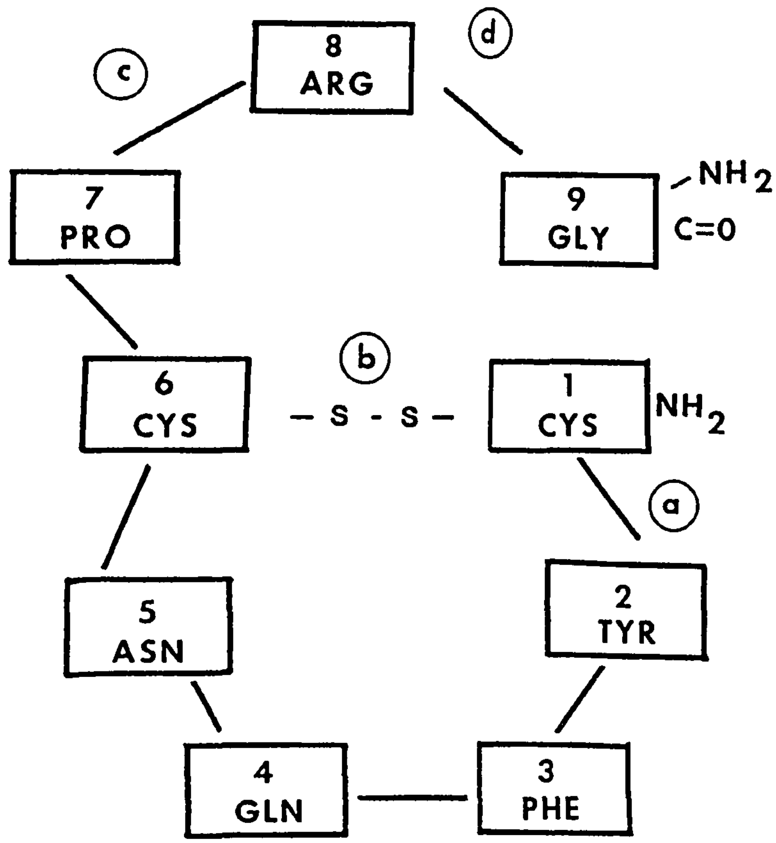
Plasma clearance is a composite of all the ways AVP can be cleared in the body. Plasma inactivation of AVP is believed to contribute very little to the overall clearance of AVP _in vivo_. Renal and splanchnic bed (including the liver and intestines) clearance account for 80 to 90% of the clearance of AVP in the body (Lauson, 1974; Share et al., 1985). Urinary clearance via urinary excretion (Lauson, 1974) or renal metabolism (Benmansour et al., 1982; Claybaugh and Sato, 1985) probably accounts for the AVP not cleared by these organ systems.

Degradation of AVP in the body may be accomplished by a variety of proteolytic enzymes. So far, 4 major sites of cleavage of the AVP molecule and the enzymes involved with these cleavages (fig. 1) have been identified (Walter and Simmons, 1977):

1. Post-proline cleavage. Cleavage between proline and arginine (amino acids 7 and 8) has been demonstrated in mammalian and bird kidney, and rat and rabbit brain extracts, and in the toad urinary bladder. The post-proline endopeptidase has been chemically purified and identified as having a molecular weight of about 115,000 (Koida and Walter, 1976).

2. Cleavage of the terminal glycinamide (Gly-NH_2) residue. Trypsin-like activity has been described in the liberation of glycinamide from AVP. Chymotrypsin-like activity is responsible for this cleavage between amino acids 8 & 9 in OT, but not for AVP.

figure 1. Enzymatic cleavage sites for AVP. The four enzymes thought to act on AVP are: vasopressinase of pregnancy (a), thioglycolate (b), post-proline cleaving enzyme (c), and trypsin (d). The post-proline and cysteine-tyrosine cleavage sites are thought to be relatively more important for the inactivation of AVP.


8
ARG
c
d
7
PRO
9
GLY
NH2
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CYS
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CYS
NH2
a
5
ASN
2
TYR
4
GLN
3
PHE

Carboxamidopeptidase, so far only found in the toad bladder, is able to liberate glycinamide in several AVP analogues. Although glycinamide has been detected after incubation with kidney extracts and membrane-bound enzyme preparations, des-glycinamide AVP (the AVP molecule minus the last amino acid) has never been isolated (Nardacci et al., 1975; Walter and Bowman, 1973; Walter and Shlank, 1975). Thus, it is believed that glycinamide may be released only after post-proline or some other cleavage of the AVP molecule.

3. Disulfide bond cleavage. The disulfide bond between the two cysteine amino acids in the "ring" of the AVP molecule can be cleaved by thiol:protein-disulfide oxidoreductase. This enzyme has been isolated in a variety of tissues including rat and bovine liver, bovine pancreas, and human placenta (Walter and Simmons, 1977).

4. Cleavage between amino acids 1 and 2. The "vasopressinase" (also called "oxytocinase") found in the blood of pregnant women is actually a cystine amino peptidase (CAP). This enzyme is believed to be synthesized in the placenta (Rosenbloom et al., 1975). It cleaves the peptide bond between amino acids 1 and 2 in the ring portion of the molecule and proceeds to cleave the rest of the ring in sequence. CAP inactivates both OT and AVP equally well. The importance of this enzyme in the clearance of AVP and OT during pregnancy is unclear.

Radiolabelled hormones are useful in the study of hormone degradation, as metabolic products undetectable by radioimmunoassay can be traced. Caution must be exercised in the use of analogues such that changes in the physicochemical properties of a hormone, such as changes in half life (Stegner et al., 1984a) due to the blockage or exposure of cleavage sites and migration of the hormone on HPLC, are prevented. The recent commercial availablilty of AVP tritiated at the third amino acid phenylalanine has enhanced the study of AVP metabolism _in vivo_ and _in vitro_. Clearance of ^{125}I-AVP or ^{131}I- AVP is relatively inhibited as compared to tritiated AVP (^{3}H-AVP; Weitzman and Fisher 1978) or unlabelled AVP (Shade and Share, 1976). Because the tritium label is small, the migration patterns on HPLC of ^{3}H-AVP and unlabelled AVP are identical (unpublished results of this laboratory).

VASOPRESSIN CLEARANCE IN THE FETUS

If there is no metabolism of fetal plasma AVP by the fetus, it is added intact via the urine into the amniotic sac, and if there is no loss or breakdown of this amniotic fluid AVP such as swallowing and reabsorption by the gut, then with normal pituitary secretion this hormone would accumulate in the fetus to harmful levels. That is not the case is indicated by the fact that measurements of fetal plasma AVP levels are reasonably low (see above). Therefore AVP must be cleared by some means. It is necessary to establish whether AVP is cleared by the fetus or in the amniotic sac, or whether the fetus is simply dependent upon maternal clearance of this hormone.

AVP is not cleared from the fetal system by simple fetal-to-maternal transfer across the placenta to the maternal circulation (Wiriyathian et al., 1983). When as much as 10 U of AVP was injected into the fetal jugular vein, maternal plasma AVP levels did not increase above baseline values (Leake et al., 1979).

It has been shown that whole body metabolic clearance rates of AVP in fetal sheep average about 15.4 $\pm$ 0.6 $ml \cdot kg^{-1} \cdot min^{-1}$ and do not change over a wide range of low AVP infusion rates (10 to 500 $\mu U \cdot kg^{-1} \cdot min^{-1}$ (Stegner et al., 1984b). Vasopressin plasma clearance rates have been reported at 60.5 $\pm$ 8.72 $ml \cdot kg^{-1} \cdot min^{-1}$ at high infusion rate (up to 8.73 mU/min) in the fetal lamb (Wiriyathian et al., 1983). Also, vasopressin plasma clearance rates did not correlate with fetal age or fetal body weight. These authors also reported that the half life of AVP in fetal plasma was between 2.8 and 10.95 minutes. Vasotocin plasma clearance rate was reported at 45.8 $ml \cdot kg^{-1} \cdot min^{-1}$ at a VT infusion rate of 1 or 2 $ng \cdot kg^{-1} \cdot min^{-1}$ (Ervin et al., 1986a). These studies suggest that a clearance mechanism within the fetal system does exist.

Hepatic and renal clearance. A site for fetal AVP metabolism has not yet been identified. Neither the liver nor the kidney seem to play major roles in AVP metabolism in the fetus as they do in the adult. Although AVP clearance by the fetal liver has not yet been examined, the fetal liver is thought to have little effect on the metabolism of other substrates since much of the blood flow from the placenta bypasses the liver via the ductus venous (Jones and Rolph, 1985). Also, swallowed AVP appears in the blood. Very little is known about protein metabolism in the fetal liver, but of the enzymes known to be present in the fetal liver early in gestation, none have been shown to be involved with AVP metabolism in the adult.

The renal clearance of AVP in the fetus has never been measured directly by calculation of the difference between renal arterial and venous AVP values. In fetal sheep, renal AVP clearance (C_{AVP}) was reported at 1 $ml \cdot kg^{-1} \cdot min^{-1}$ which accounted for less than 7% of the total AVP cleared from the body (Daniel et al., 1980).

Placental clearance. It is generally believed, that the transplacental clearance of AVP is unlikely since indirect evidence supports the idea that the placenta is impermeable to AVP. Although Jones and Rurak (1976) estimated the placental clearance to equal 90% they used a technique of comparing umbilical artery and uterine artery and since placental blood flow is high, their calculations could be erroneous. Forsling and Fenton (1977) have shown that perfusion of guinea pig placentas with low concentrations of AVP does not result in a significant decrease in AVP concentration across the placent, and found no fetal to maternal transfer of AVP. Despite infusion of 10 U of AVP into fetal sheep, maternal plasma AVP concentrations do not change (Leake et al., 1979). Furthermore, Stegner and his colleagues have suggested that placental clearance is insignificant in fetal sheep since calculated clearance rates for fetuses and neonatal lambs are similar (Stegner et al., 1984b). This observation would imply also that the recirculated AVP, that is, the AVP swallowed with amniotic fluid that reappears in the circulation, is also negligible.

Thus, the amniotic sac into which vasopressin is excreted along with fetal urine is a possible site for metabolism.

Amniotic fluid clearance. In examining the amniotic compartment as a clearance organ, its unique characteristics must first be considered. The concentration of a hormone in the amniotic fluid is affected by changes in amniotic fluid volume, the rate of addition of the hormone into the amniotic sac, and its rate of removal.

AMNIOTIC FLUID VOLUME. Amniotic fluid volume affects the concentrations of amniotic fluid constituents. If, for example, the addition of a hormone is kept constant but the volume to which it is added increases, the concentration of hormone will decrease. Despite much research and speculation, little is known about the control of amniotic volume regulation, and the effect of volume on amniotic fluid hormone concentrations is difficult to evaluate. If AVP is able to influence amniotic water and electrolyte movement across the amnionic membrane, it would also affect amniotic fluid volume and thus its concentration in the amniotic sac. It would be interesting to examine what role AVP plays in the *in vivo* regulation of amniotic fluid volume. However, regardless of the amniotic fluid volume effect on amniotic fluid AVP concentration, this is not of major importance when considering the fetal AVP recirculation problem. If there is no clearance or metabolism of amniotic fluid AVP, the hormone will accumulate in the sac no matter what the amniotic fluid volume and initial concentration.

ADDITION OF HORMONE INTO THE AMNIOTIC SAC. There are several possible sources of amniotic fluid hormones: the fetus, the mother, the placenta, the amnion and the decidua. It is possible for the sources of a hormone to change over gestation, making the rate of hormone entry into the amniotic sac more difficult to assess. For example, the major contributors of estriol early in gestation are the fetal skin, umbilical cord, and placenta, whereas near term, the major sources are the fetal intestinal and urinary tracts (Belisle and Tulchinsky, 1980). This, however, does not appear to be the case for AVP. The only known source of amniotic fluid AVP, to date, is the fetus. This is supported by the finding that in heterozygous Brattleboro rat fetuses, amniotic fluid AVP could be detected even if in very low levels but AVP was not detected in homozygous fetuses (Oosterbaan et al., 1985). AVP is believed to enter the amniotic sac via fetal urination. AVP does not cross the placental barrier, so maternal AVP does not contribute to amniotic fluid AVP levels. The placenta, amnion, and the decidua have not been shown to synthesize AVP.

REMOVAL OF HORMONE FROM THE AMNIOTIC SAC. Although the entry of AVP into the amniotic sac is rather simple, its removal is more complex. The rate of removal of a hormone from the fetal system depends upon metabolism in the fetus itself (e.g. by the fetal lungs, fetal liver, fetal skin, fetal kidney), or direct transfer of the hormone across the placenta or amnionic membrane, or the presence of enzymatic systems in the amniotic sac which can degrade the hormone to an inactive form. No fetal organ has been shown to effectively metabolize AVP so it is probably added to the amniotic sac intact as is evidenced by the measurements of fetal urine AVP levels by RIA with HPLC verification (Ervin et al., 1985). Also, there is no evidence demonstrating AVP transfer across the placenta or leakage out across the amnionic membrane. However, several pieces of evidence suggest that AVP may be metabolized in the amniotic sac.

The clearance of protein (albumin, gamma G and gamma A immunoglobulins, and the protein hormones human chorionic gonadotropin and growth hormone) from the amniotic fluid has been demonstrated in the human fetus near term (Gitlin et al., 1972). The amount of amniotic fluid protein cleared, determined by the disappearance of radioactive label, was about 0.24 gm/kg fetal weight. Because the proteins studied differed in physicochemical properties and were still cleared at the same rate, it was concluded that the clearance of protein from the amniotic fluid was not due to diffusion or to active transport, but no mechanism of clearance was identified. It is probable that the amniotic fluid is capable of degrading proteins including AVP via proteolytic enzyme action.

Proteolytic activity has been demonstrated in the amniotic fluid by several laboratories. Proteolytic activity in amniotic fluid samples collected from humans at 36 to 42 weeks of gestation was demonstrated using hemoglobin as a substrate and measuring liberated amino acids (Sanyal et al., 1977). No proteolytic activity could be detected in amniotic fluid samples between 28 and 35 weeks of gestation. Although previous reports speculated that the source of the proteolytic enzyme may be fetal meconium, no difference in proteolytic activity in samples with and without meconium was found. These authors suggested that the proteolytic enzyme in the amniotic fluid may be pepsinogen since the pH optimum and Km values were similar to those found in gastric juice. Gastrointestinal hormones have recently been measured in amniotic fluid samples from human fetuses at 16 to 20 weeks of gestation. Gastrin, gastric inhibitory polypeptide, pancreatic polypeptide, glucagon and enteroglucagon have all been detected, but the significance of their presence in the amniotic fluid is unknown (Aynsley-Green et al., 1985).

Trypsin has been measured in amniotic fluid by a RIA specific for trypsin with no crossreactivity with carboxypeptidase, elastase, or chymotrypsin. Trypsin was detectable in human amniotic fluid as early as 12 weeks of gestation and levels at mid pregnancy were 350 $\pm$ 260 ng/ml (Pocknee and Abramovich, 1982). At term, trypsin levels were undetectable in most amniotic fluid samples but present in all samples with meconium. These authors suggest that amniotic fluid trypsin originates in the fetal pancreas since pancreatic levels at midterm are about 900 ng/mg tissue. Supporting this is the demonstration of a trypsinogen activator in the fetal pancreas and proteolytic activity in meconium (Lieberman, 1966).

Clinically, it has been proposed that the measurement of amniotic fluid trypsin (Brock et al., 1984) and trypsin-alpha-1 antitrypsin complex (Borgstrom et al., 1984) be used for diagnosis of cystic fibrosis since their concentrations in fetuses with cystic fibrosis are very low. These studies of cystic fibrosis fetuses suggest that the low amniotic fluid trypsin levels may be associated with an abnormality in fetal defecation which supports the notion of meconium being the main source of amniotic fluid trypsin. No trypsin could be detected in fetal urine, kidney and lung, and there was no correlation between amniotic fluid trypsin levels and fetal or maternal plasma levels. Therefore, these are unlikely sources for amniotic fluid trypsin.

The actions of trypsin in the amniotic fluid have not been defined. One possible adverse action of this amniotic fluid enzyme could be its involvement with the premature rupture of the amnionic membrane at term. Trypsin activity in human amniotic fluid collected before delivery was measured at 14 $\pm$ 20 U/l with corresponding alpha-1 antitrypsin concentration at 25.2 $\pm$ 20.2 mg/dl. Alpha-1 antitrypsin appears to control the trypsin activity in amniotic fluid and defends against proteolytic attacks by this enzyme which can degrade the type III collagen of the amniochorion. In cases of premature rupture of the membranes, trypsin activity is high and alpha-1 antitrypsin levels are low (Kanayama et al., 1986). Another possible action of trypsin is the conversion of renin in amniotic fluid and fetal plasma from an inactive to active form, as has been idicated by <u>in vitro</u> studies (Morris and Lumbers, 1972; Takada et al., 1986).

These reports of amniotic fluid proteolytic enzymes have all focused on the use of their presence in the amniotic fluid as an indication of fetal maturity. No connection between the presence of these enzymes and a possible role of these enzymes in fetal peptide clearance in the amniotic sac have been made. True, the effectiveness of these proteolytic enzymes in the presence of amniotic fluid protease inhibitors such as alpha-1 antitrypsin and alpha-1 antichymotrypsin (Bhat and Pattabiraman, 1980) is questionable, but the rate of enzyme proteolysis may be faster than the rate of inhibition. Nevertheless, it is very likely that amniotic fluid enzymes are actively involved in the clearance of proteins, including AVP, from the fetal system.

Rosenbloom et al. (1975) reported vasopressinase (cystine amino peptidase) activity in not only human maternal serum but in term umbilical cord serum and amniotic fluid as well. Pitressin (1000 μU) was incubated in 0.25% normal rabbit serum in phosphate buffered saline at $37^{o}C$ for up to 2 hours with 50 μl of either human maternal serum, cord serum, or amniotic fluid obtained at delivery. Remaining AVP content in the samples was measured by RIA. Maternal serum degraded AVP _in vitro_ at 53 $mU \cdot ml^{-1} \cdot min^{-1}$ umbilical cord serum and amniotic fluid were shown to degrade AVP at much slower rates of 9.2 and 16.6 $\mu U \cdot ml^{-1} \cdot min^{-1}$, respectively. Vasopressinase activity in the blood of the neonate disappears within 72 hours after birth. Vasopressinase activity was not detected in maternal sera collected in rats, rabbits and sheep nor in sheep neonatal sera and sheep amniotic fluid. Thus, a species difference in vasopressinase activity may exist.

The vasopressinase activity in amniotic fluid collected from humans at 16 and 39 weeks of gestation was further studied by incubating unlabelled AVP with amniotic fluid at room temperature for one hour (Artman et al., 1984). Vasopressin was degraded at about 12% per hour in late term amniotic fluid and no degradation of AVP was seen in early gestation amniotic fluid. The recovery of AVP added to amniotic fluid (5 or 7.5 μU/ml) was 84%. The recovery of VT added to amniotic fluid (10 or 25 pg/ml) was 61%. Again, the significance of an amniotic fluid vasopressinase enzyme system as a route for AVP clearance in the fetus was not addressed. These investigators point out that amniotic fluid vasopressinase activity is low, and only 0.4% of the activity found in maternal serum, and allude to the presence of cystine aminopeptidase in lower concentration in amniotic fluid than in maternal serum. Instead, the difference in activity found between maternal serum and amniotic fluid could indicate the presence of a totally different enzyme in the amniotic fluid. In light of the documented presence of other amniotic fluid enzymes such as trypsin which have the ability to degrade AVP, this hypothesis is not unreasonable.

STATEMENT OF PROBLEM

A route for AVP clearance in the fetus has yet to be established. None of the known AVP clearance sites in the adult seem to play a significant role in the clearance of AVP in the fetus. Simple fetal to maternal transfer of AVP across the placenta does not occur and the placenta does not appear to be involved with fetal AVP clearance. Several facts suggest that the amniotic compartment is a likely site for AVP clearance.

Various proteolytic enzymes have been identified in the amniotic fluid, and protein clearance in the amniotic fluid has been demonstrated.

The objective of this study is to determine the significance of the amniotic compartment as a site for fetal AVP metabolism.

The study of fetal physiology requires consideration of the complexities due to the special situation of the developing fetus. Care must be taken when interpreting experimental results. Maternal effects must not be confused for independent fetal responses and functions. Also, since the fetus is constantly changing, physiological mechanisms in the fetus may be different at different stages of gestation and in different degrees.

It will first be necessary to examine amniotic fluid dynamics in the animal model chosen because of possible unique species characteristics in amniotic fluid composition and control of amniotic fluid volume. Next, whether metabolism of AVP can occur in the amniotic sac *in vivo* will be established. *In vitro* experiments will then be conducted studying the metabolism of AVP in isolated preparations of amniotic fluid and amnionic membrane to isolate these systems from maternal and fetal factors that may influence *in vivo* results. If metabolism is discovered, the *in vitro* experiments will also enable the isolation of amniotic fluid dependent metabolism from amnionic membrane dependent metabolism.

The preliminary characterization of any metabolic products and enzymes involved in their production will be attempted.

Once metabolism by the amniotic fluid and/or amnionic membrane has been established, the onset of the ability to metabolize AVP and the changes in the relative importance of these two systems over gestation will be investigated.

CHAPTER II

MATERIALS AND METHODS

ANIMALS

The guinea pig was chosen as the animal model for this study because its relatively short gestational period (approximately 68 days) is long enough to differentiate between the different stages in development. Also, the guinea pig has on the average three to five fetuses per litter which allows several experiments to be done in the same animal. These two qualities of short gestation and multiparous pregnancy allowed the performance of more experiments with fewer animals in a shorter period of time. In addition, the guinea pig's hemomonochorial placenta is morphologically similar to the human placenta. Further, the guinea pig fetus is fairly large, and more mature at birth in comparison to other small laboratory animals, which allows for adequate sample size for analyses, and the ability to trace to some extent the development of the fetus in utero.

Adult female Hartley albino guinea pigs (Charles River Laboratories, Portage, Michigan) were bred by daily monitoring of vaginal membrane openings for accurate timed pregnancies. Mating of females occured within 24 hours of vaginal membrane opening. Females were behaviorally receptive with vaginal membranes open for about 12 hours approximately every sixteen days (average length of estrus cycle) (Wagner and Manning, 1976).

In vivo experiments were conducted with guinea pigs in the last week of gestation. Guinea pigs in the second (22-44 days) and third (45 days-term) trimesters of gestation were used in studies establishing baseline amniotic fluid electrolyte concentrations and for the *in vitro* experiments.

The animals were housed and experiments performed in AAALAC certified facilities. Guinea pigs were exposed to a 12 hour light/ 12 hour dark cycle at a controlled room temperature of 78^{0}C, and given food (Purina Guinea Pig Chow) and water ad lib.

All procedures were approved by the Animal Use Committee, Clinical Investigation Committee, and Radioisotope Committee of Tripler Army Medical Center.

ANALYSES

OSMOLALITY AND ELECTROLYTES

Amniotic fluid, fetal urine, fetal plasma, and maternal plasma samples were analyzed for osmolality, sodium, and potassium within 24 hours after collection. Samples were stored either on ice or in the refrigerator at 4^{0}C until analyses could be performed. Osmolality was measured using either a freezing point depression osmometer (Advanced Instruments Micro-Osmometer Model 3MO, Needham Heights, MA) or a vapor pressure (dew point depression) osmometer (Wescor, Inc. Model 5100B, Logan, Utah). The accuracy of both instruments in the range of osmolalities measured, was within 290 $\pm$ 3 mOsm/kg H_2O.

Sodium and potassium were measured using a flame photometer (Instrumentation Laboratory AutoCal Flame Photometer 643, Lexington, MA). The accuracy of plasma measurements was sodium $\pm$ 1.0 mEq/l and potassium $\pm$ 0.05 mEq/l. The precision for urine and amniotic fluid samples was $\pm$ 1.0 mEq/l for both sodium and potassium.

INULIN

Inulin was measured using a spectrophotometric assay adapted from the methods of Davidson and Sackner (1963). Plasma samples were first diluted (1 part to 5 parts water) and then deproteinized by adding 0.1 ml 6.1 N TCA per 1 ml of diluted plasma. After shaking for 30 minutes, plasma samples were centrifuged and the supernatant saved for the assay. Urine samples were diluted 50 µl up to 1.5 ml so that the estimated inulin concentration would fall within the range of the standard curve. All samples were stored at $4^{o}C$ until assayed 24 to 48 hours later.

Inulin (Sigma, I-3754) standards at concentrations of 0.005, 0.01, 0.02, 0.03 and 0.04 mg/ml were prepared for the standard curve. Two aliquots of 0.5 ml from each sample was dispensed into 12x75 borosilicate tubes. Anthrone reagent (3.0 ml, 0.08% anthrone in 79% H_2SO_4) was added to each tube. After tightly covering tubes with parafilm, all samples were carefully inverted several times to assure proper mixing, and chilled for 1 minute in ice water. The samples were then incubated at $37^{o}C$ for 50 minutes, and chilled in ice water for another minute. The absorbance of each sample was then measured at 620 nm on a Coleman Junior II spectrophotometer Model 6/20. The intraassay coefficient of variablity (c.v.) was 1.0%. The interassay c.v. was 2.4%.

MEMBRANE WEIGHTS AND PROTEIN ANALYSIS

Wet and dry weights (procedure adapted from Dr. Walter Morishige's methods) were obtained from amnionic membrane sections used in the *in vitro* studies of amnionic membrane-dependent metabolism development over gestation. Glass coverslips were dried in an oven at $55^{o}C$ for 2 to 4 hours and then brought to room temperature in a dessicator before obtaining tare weights using a microbalance (Mettler Analytical Electronic Balance Model ME30). Two or three 1 cm^2 pieces of amnionic membrane were cut from different parts of the membrane used in the experiment. Each piece was carefully blotted and placed on its own coverslip. Wet weights were immediately obtained. The membranes were then dried in the oven at $55^{o}C$ for two to three days. After this time, samples were allowed to reach room temperature in a desicator, and then weighed for a first dry weight. This dry weight procedure was repeated with the same samples to verify a true dry weight.

Protein was measured using the procedure of Lowry (Lowry et al., 1951). Two or three 1 cm^2 pieces of tissue from different parts of the amnionic membrane were each dissolved in 0.5 ml 1N NaOH in a borosilicate tube by gentle warming (covering tube with parafilm and heating in oven at 55^0 C for five minutes) and vortexing. Standards of 10, 20, 40, 60 and 100 μg/ml were prepared in duplicate from bovine serum albumin (Sigma, A-7888, St. Louis, MO) for the standard curve. Samples were assayed at doses of 50, 100 and 200 μl Absorbances were measured with a Coleman Junior II Spectrophotometer Model 6/20 at 640 nm. The intraassay c.v. was 2.4% and the interassay c.v. was 5.3%.

HORMONAL ASSAYS

Cortisol Assay. Cortisol concentrations were measured by radioimmunoassay (RIA) (Clinical Assays Gammacoat) of unextracted samples. The intraassay c.v. was 3.4%; the interassay c.v. was 5.0%.

Vasopressin Assay. AVP concentration was measured by RIA (developed by Dr. John Claybaugh) of both unextracted and extracted amniotic fluid, urine and plasma.

SAMPLE PREPARATION. Unextracted samples were kept frozen at $-20^{O}C$ until the day of the assay. The plasma samples to be extracted were first prepared by acidifying plasma with 0.1 ml 1 N HCl per ml plasma before freezing. Urine and amniotic fluid were stored at $-20^{O}C$ and later acidified to a pH of 2.0 with HCl on the day of the assay. Since at least 2.0 ml of sample was used for extraction, those samples of less volume were diluted up to 2.0 ml with 0.9% NaCl before acidification.

Acidified samples were extracted using octadecyl silane cartridges (Sep-Pak C-18, Water Associates, Milford, Massachusetts) by a modification of the method of LaRochelle et al. (1980). Cartridges were primed with first methanol (5 ml), then 8 M urea (5 ml), and then distilled water (10 ml) before applying the acidified samples (2 to 4 ml). The samples were then washed with 10 ml of distilled water, followed by 10 ml of 4% acetic acid. AVP was eluted from the cartridge with 4% acetic acid in 40% ethanol (5 ml) into 12 x 75 borosilicate tubes coated with assay buffer (0.33% NaCl, 0.1% bovine serum albumin (Sigma, A-7888, St. Louis, MO) in 0.1 M $NaPO_4$ pH 7.2). Samples were dried down using a Savant Speed Vac Concentrator.

PREPARATION OF ANTISERA. Antisera were prepared according to the method of Skowsky and Fisher (1972), using either 8-lysine vasopressin (LVP) or 8-arginine vasopressin (AVP)(Sigma, V-0377) conjugated to bovine thyroglobulin (Sigma) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (The Ott Company, Muskegon, MI) with a conjugation molar ratio of 118 : 1 : 200, respectively.

Four different antisera -- AS 96, AS 95, AS 09, and AS 101 (the first two were developed against LVP and the second two were developed against AVP) at dilutions of 1:30,000, 1:9,000, 1:30,000, and 1:3,000, respectively -- were used in radioimmunoassays depending on their crossreactivities with various vasopressin analogues. These dilutions allowed for 40 to 65% binding of I^{125} labelled AVP with no unlabelledAVP present (BO/T). The calculated crossreactivities of these antisera can be seen in Table 1.

Table 1

Crossreactivities (%) of Vasopressin Antisera

Antisera Number	AS 96	AS 95	AS 09	AS 101
Original Hapten	LVP	LVP	AVP	AVP
vasotocin	< 0.05	< 0.05	68.8	< 0.05
oxytocin	< 0.05	< 0.05	< 0.05	< 0.05
des glycinamide AVP	< 0.05	59.8	< 0.05	52.4
pressinoic acid	0	0	0	7.2

RADIOIMMUNOASSAY. A 72 hour/48 hour disequilibrium assay as used to measure AVP, vasotocin (VT), and des glycinamide vasopressin (dgAVP) in the samples collected. AVP was used to generate the standard curves used in all assays. Standards were set up in duplicate at doses of 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 pg/400 μl assay buffer (0.33% NaCl 0.1% bovine serum albumin (BSA) 0.1M sodium phosphate pH 7.2). Samples were assayed at a variety of doses and brought up to a volume of 400 μl with assay buffer. The antiserum at the dilution listed above was added in a 50 μl volume. The assay was then kept at $4^{o}C$ for 72 hours to allow the AVP in the standards and samples to bind to the antiserum before competing with ^{125}I-AVP labelled AVP for antiserum binding sites. ^{125}I-AVP (New England Nuclear, Boston, MA) was diluted to obtain approximately 3000 counts per minute (CPM) in 50 μl. A 50 μl volume of ^{125}I-AVP was added to each assay tube and samples allowed to incubate at $4^{o}C$ for another 48 hours. The final volume of the assay tube was 0.5 ml. Antiserum-bound ^{125}I-AVP was separated from unbound ^{125}I-AVP by adding 300 μl of BSA coated charcoal (0.8% Norit A charcoal 0.16% BSA in assay buffer) to each tube and centrifuging at 2000 rpm (1,100 x G) for 20 minutes. Calculations of AVP concentrations used both bound and unbound counts.

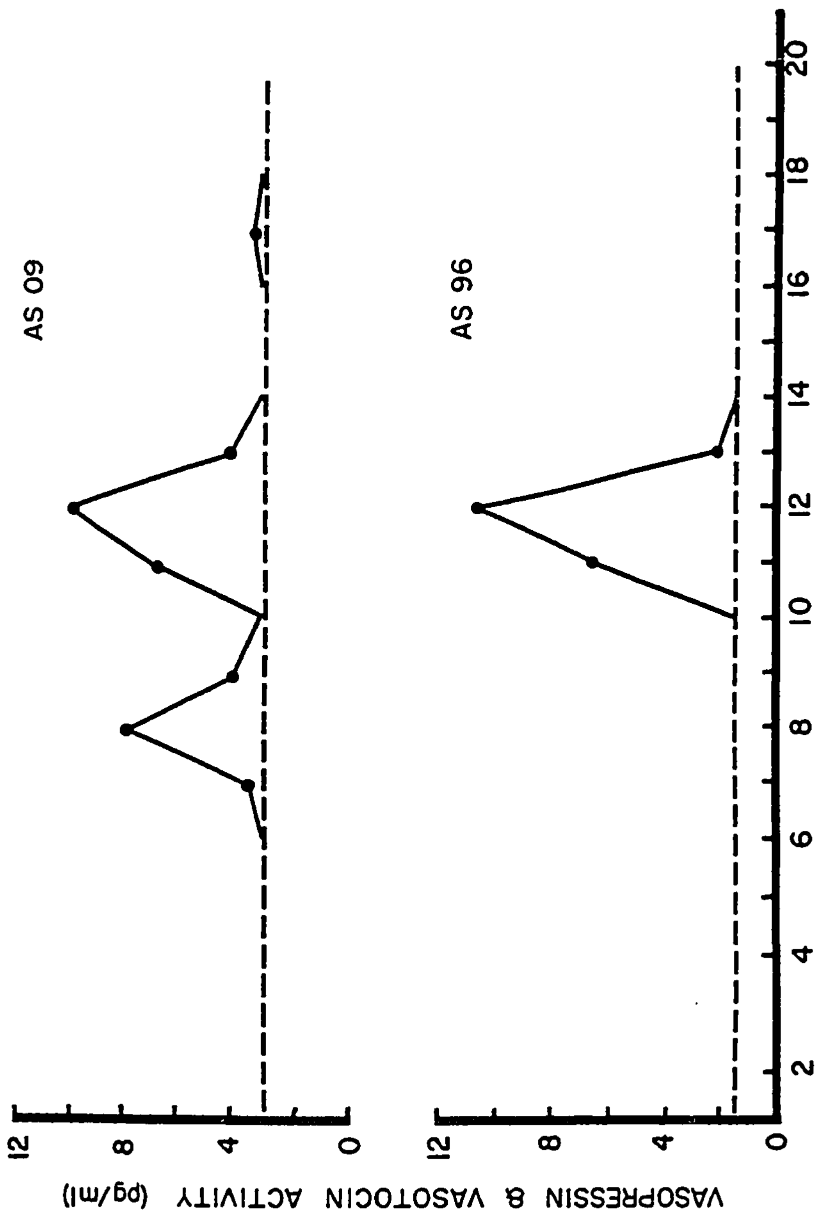
A 100 μU/ml AVP standard solution was used as a control in all assays to determine the c.v. between and within assays. The between assays c.v.'s were: AS 96, 8.0%; AS 09, 11.7%; AS95, 6.1%; AS 101, 1.6%. The within assay c.v.'s were: AS 96, 6.2%; AS 09, 4.9%; AS 95, 8.5%; AS 101, 12.0%. The lower limit of the assay sensitivity was determined by dividing the lowest dose used in the standard curve by the highest sample volume. The lowest dose was determined by a logit value of 3.0 or less, and linear with the higher doses of AVP in the curve. If not linear, for instance, it would improve the linear correlation coefficient if the points were removed.

ASSAYS FOR BASELINE STUDIES. All amniotic fluid samples analyzed for AVP and VT concentration in the baseline studies were measured using two different AVP antisera: AS 09 which crossreacts 68.8% with VT, and AS 96 which crossreacts less than 0.05% with VT (fig. 2). VT concentration was thus determined by subtraction of the levels measured with the "AVP only" antiserum (AS 96) from levels measured with the antiserum which measures both AVP and VT (AS 09).

ASSAYS FOR ALL OTHER STUDIES. All samples not processed by HPLC were extracted and assayed with AS 96 since this antiserum showed the least crossreactivity with AVP analogues. All samples from the AVP metabolism studies which were processed by HPLC were assayed with AS 95 since this antiserum could detect dgAVP (a suspected metabolite).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC).

The HPLC system consisted of: a solvent programmer (Beckman, Berkeley, CA, Controller 420), 2 pumps (Beckman Models 100A and 110A), micro injector, and an ultrasphere octadecyl silane 0.5 µm 4.6mm I.D. 25 cm column (Beckman 235329). Three different buffer systems were used for analysis. The first system (method of Ervin et al., 1985) employed a 15 to 30% linear acetonitrile (Solvent A; 80% CH_3CN; American Burdick and Jackson, Muskegon, MI) in 0.1% trifluoroacetic acid (TFA; Solvent B; Sigma,T-6508) gradient over 20 minutes at a flow rate of 1.5 ml/min. Forty 750 µl fractions were collected with a figure 2. HPLC demonstration of crossreactivity of AVP antisera 09 and 96 with VT. A mixture of 60% AVP and 40% VT standards was separated on a C_{18} column. (Eluent: 39% CH_3OH in 0.05 M CH_3COONH_4 (pH 6.5). Flow rate: 1.0 ml/min. Fractions: 1.0 ml/fraction.) Each fraction was assayed by both antisera. AS 09 crossreacts with VT. AS 96 detects only AVP activity.

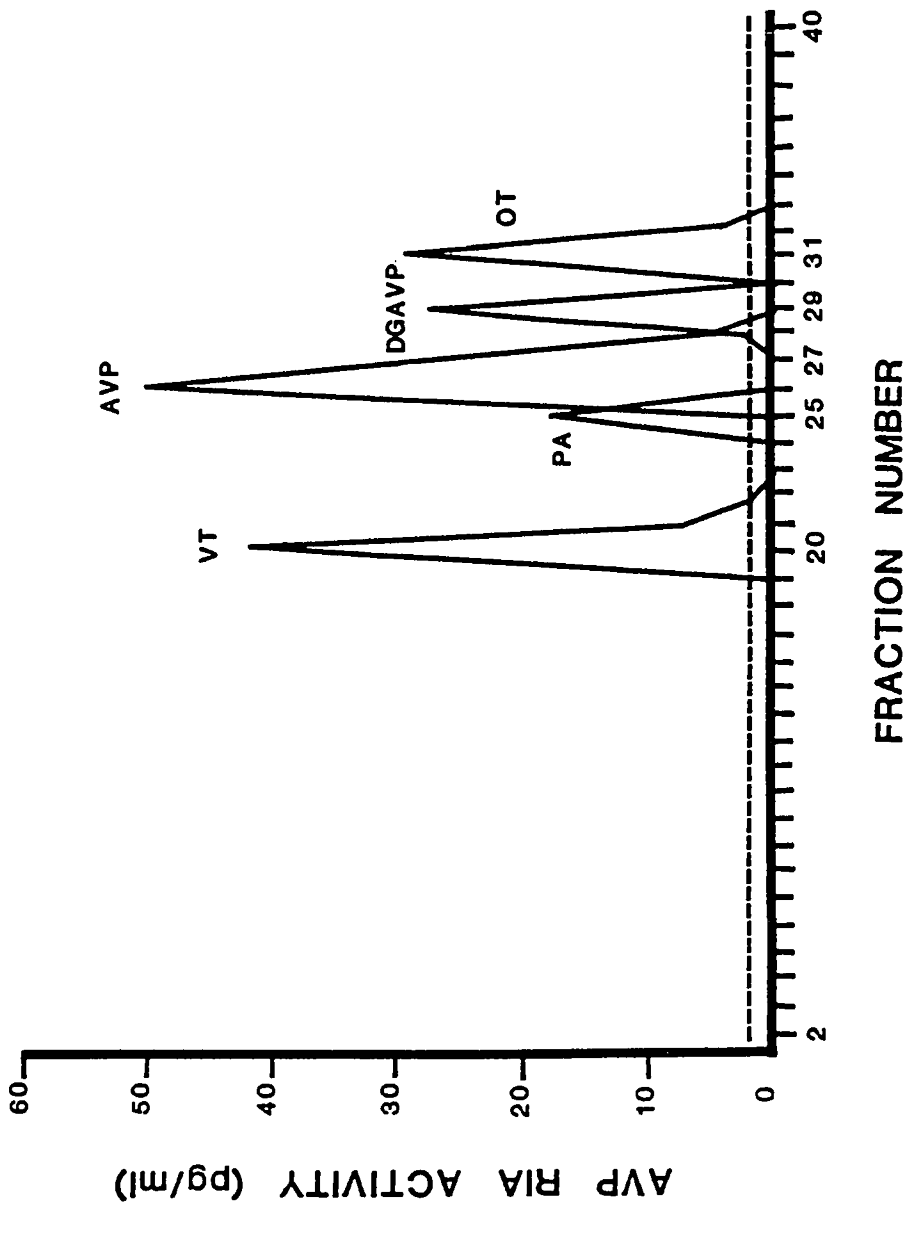

AS 09
AS 96
FRACTION (ml)
VASOPRESSIN & VASOTOCIN ACTIVITY (pg/ml)
0
4
8
12
2
4
6
8
10
12
14
16
18
20

fraction collector (Gilson, Madison, Wisconsin, Model FC-80K). Two other buffer systems, a 18% CH_3CN 82% 0.01 M ammonium acetate (CH_3COONH_4) ph 4.0 buffer (Larsen et al., 1979) and a 39% methanol (CH_3OH) 61% 0.05 M CH_3COONH_4 pH 6.5 buffer (Lindberg, 1980) were used to confirm the separation of AVP from metabolic products and some AVP analogues. A flow rate of 1.0 ml/min was used and 25 1.0 ml fractions were collected for both of these buffers. Sample size for all three buffer systems was 250 µl.

Which buffer system was chosen for analysis was dependent upon the sample analyzed. The 39% CH_3OH buffer was used for separation of AVP from dgAVP and VT. The gradient CH_3CN buffer system was used to better isolate smaller metabolic products that tend to migrate with the HPLC front with the non-gradient buffer systems. The 18% CH_3CN buffer was used to verify the isolation of the metabolic products with the other two buffer systems.

HPLC fractions were assayed for AVP RIA activity by drying down 500 ul of the fraction and bringing the dried down sample back into solution with 400 µl assay buffer. Antiserum (50 µl) was then added and the entire tube incorporated into the assay. The elution profiles for AVP and its various analogues for the acetonitrile gradient buffer system) can be seen in figure 3. AVP and VT were assayed with AS 09, OT and dgAVP were assayed with AS 95, and pressinoic acid was assayed with AS 101. AVP, VT, OT, dgAVP, and pressinoic acid, elute at fractions 27, 20, 31, 29, and 25, respectively.

figure 3. HPLC migration patterns of AVP and various AVP analogues. (Eluent: 15-30% CH_3CN in 0.1 % TFA over 20 minutes. Flow rate: 1.5 ml/min. Fractions: 750 µl/fraction.) AVP elutes at fraction 27. Vasotocin (VT), pressinoic acid (PA), des glycinamide AVP (dgAVP), and oxytocin (OT) elute at fractions 20, 25, 29, and 31, respectively.


AVP RIA ACTIVITY (pg/ml)
0
10
20
30
40
50
60
FRACTION NUMBER
2
20
25
27
29
31
40
VT
PA
AVP
DGAVP
OT

Fractions were analyzed for tritium by adding 5.0 ml of scintillation cocktail (Opti-Fluor, Packard, Downer's Grove, Illinois) to 500 μl aliquots in plastic scintillation vials and counting for 2 minutes on a liquid scintillation counter (Tm Analytic 6881, Elk Grove Village, Illinois). Tritium (^3H) was expressed as counts per minute (CPM). Since the counting efficiency of the scintillation counter was about 60%, the CPMs obtained may be converted to DPMs with the following equation:

$$DPM = \frac{CPM}{0.60}$$

EXPERIMENTAL DESIGN

SURGICAL PREPARATION

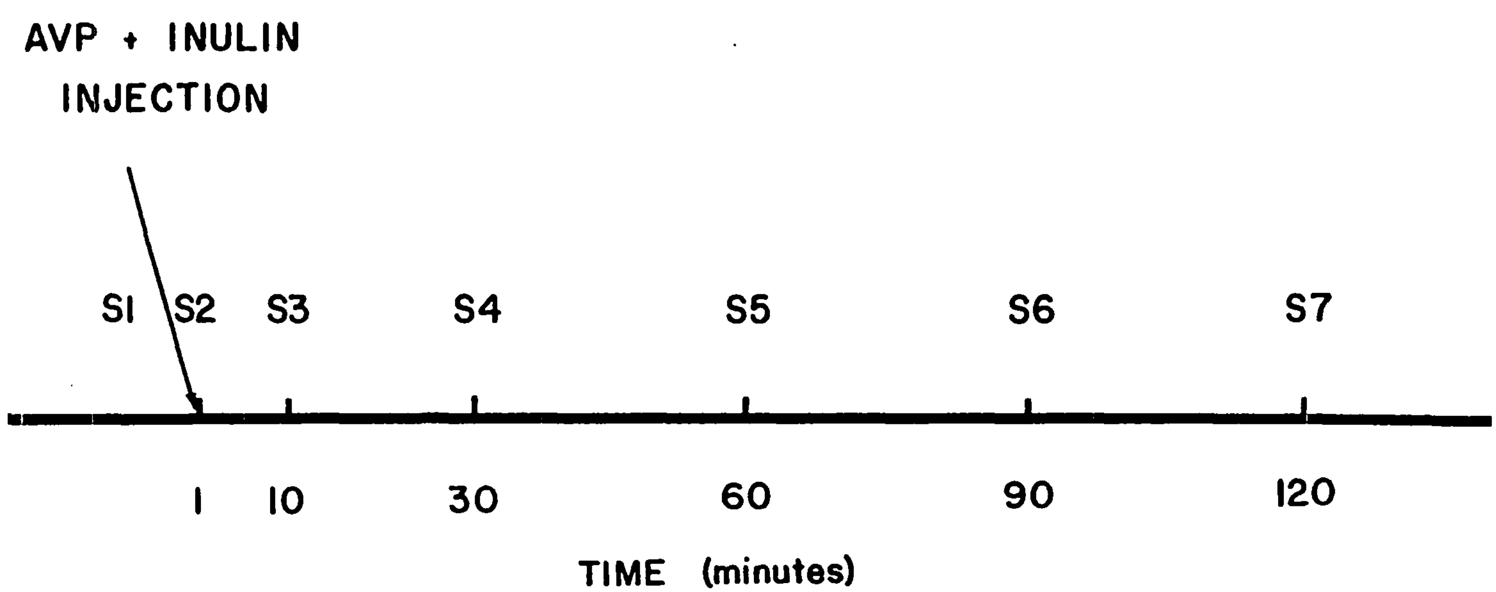
Maternal guinea pigs were anesthetized with either ketamine HCl (IM), 44 mg/kg body weight and xylazine (IM), 5-10 mg/kg body weight, or Halothane (1.5%-3.0%, 2-4 L O_2/min) for the initial baseline studies. Only Halothane anesthesia was used for all other experiments.

After sterile preparation of the abdomen, the uterine horns were exposed via a midline incision in the lower abdomen.

SAMPLING PROCEDURES

Baseline Studies. Amniotic fluid samples were collected by inserting a 26 gauge 1/2" needle through both the uterine wall and amnion. Fluid was withrdrawn with a 1 ml or 3 ml syringe. The entire amniotic fluid volume was collected from 36 fetuses at days 37 to 67 of gestation. The abdominal incision was sutured closed and the mother was allowed to recover. Samples were immediately put on ice and aliquots were separated for the different analyses.

In Vivo Studies. The maternal guinea pig was kept on a heating pad and its body temperature was carefully maintained throughout the experiment. Maintenance of maternal rectal body temperature above $36^{o}C$ was vital for the survival of the fetuses until the end of the experiment. Amniotic fluid samples were collected via multiple needle punctures, according to the experimental protocol seen in figure 4. After an initial baseline sample of amniotic fluid (500 µl) was obtained, either 5mg inulin, or 12.5 ng AVP + 5 mg inulin, or 12.5 ng ^{3}H-AVP (vasopressin, 8-L-arginine, [phenylalanyl-3,4,5-^{3}H(N)]; New England Nuclear, MA) + 5 mg inulin was injected in 500 µl of artificial amniotic solution into the amniotic sac. Artificial amniotic solution was made according to Manku et al. (1975): sodium, potassium, calcium, and magnesium (125, 6.2, 4.5, and 2.3 mEq/l, respectively); glucose (1.0 g/l); 10 ml/l 0.2 M phosphate buffer at pH 7.8; osm = 280 mOsm/kg H_2O. One ml of amniotic fluid was withdrawn and flushed back into the amniotic sac three times to assure proper mixing of the injection fluid. Amniotic fluid samples (300 µl) were figure 4. Sampling times for *in vivo* experiments. After an initial baseline sample (500 μl), either inulin (5 mg) or AVP (12.5 pg) + inulin (5 mg) was injected into the amniotic sac. Amniotic fluid samples (300 μl) were then taken at 1, 10, 30, 60, 90, and 120 minutes after injection.

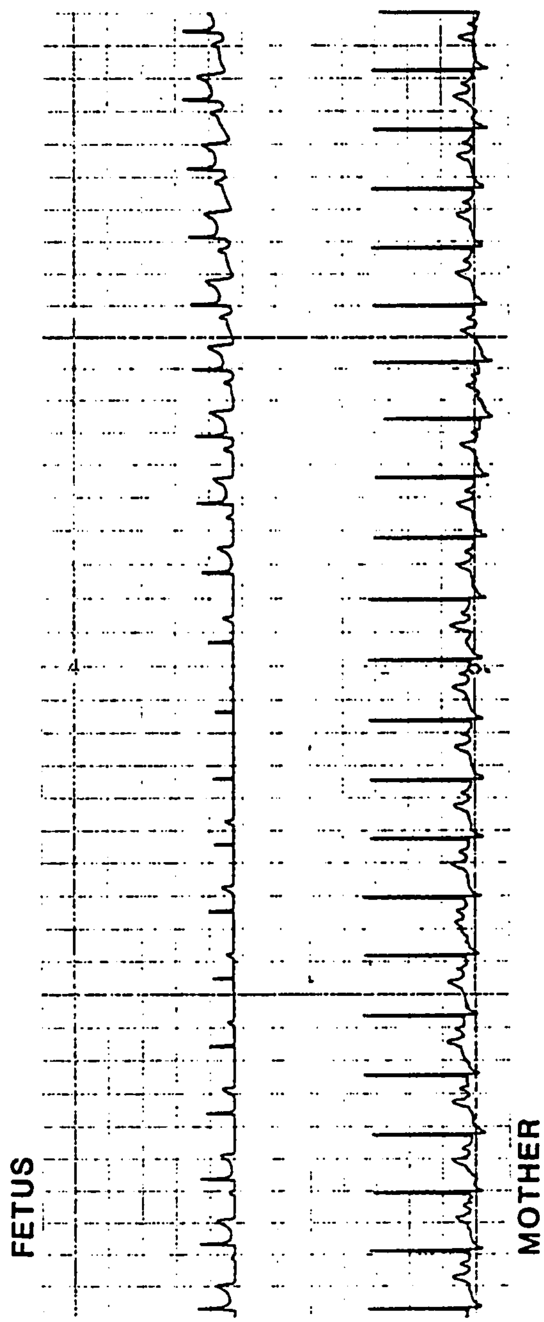

AVP + INULIN
INJECTION
SI
S2
S3
S4
S5
S6
S7
1
10
30
60
90
120
TIME (minutes)

then taken 1, 10, 30, 60, 90, and 120 minutes after injection. The abdominal incision was fastened shut with hemostats between sampling to minimize loss of body heat.

VERIFICATION OF THE VIABILITY OF THE FETUSES. The viability of the fetuses during the 120 minute sampling period was tested by obtaining heart rate before the first sample and after the last sample was taken. Fetal EKG probes were made by soldering 26 gauge needles to the ends of disposable EKG electrodes (Deseret, EKG stripper, No. 413). The needles were placed on the fetus with probe 1 in the left shoulder, probe 2 in the right flank, and probe 3 in the left hind quarter. Heart rate was recorded using an Electronics for Medicine Recorder (Patient Monitor Model OM, Honeywell, Pleasantville, NY). Fetal and maternal heart rates were obtained simultaneously so as not to confuse fetal tracings with maternal tracings (fig. 5). This test of viability was done in 6 of the in vivo experiments.

After the 120 minute sample, all remaining amniotic fluid was withdrawn from the sac. Fetal and maternal urine was collected by exposing the bladder and directly inserting a 26 gauge needle through the bladder wall. Fetal blood was obtained via an 18 gauge needle inserted into the vena cava. Maternal plasma was obtained via cardiac puncture. Animals were euthanized with 1 ml T-61 euthanasia solution (IC; Hoechst, Somerville, N.J.).

In Vitro Studies. Amniotic fluid samples were obtained for the in vitro studies as they were for the baseline studies. Amnionic membranes were collected by making a small incision in the uterus and figure 5. Heart rate tracings of maternal and fetal guinea pigs. Example of recordings made at the end of the *in vivo* experiments to verify fetal viability. These tracings were taken simultaneously so as not to confuse impulses from the mother with that of the fetus. Maternal and fetal heart rates were 165 and 150 beats/min, respectively (chart speed: 25 mm/sec).


FETUS
MOTHER

gently extracting the fetus such that the amnion clearly separated from the chorion. The amnion was cut around the umbilical cord and peeled away from the fetus in one piece. The membrane was immediately put into 37^{o}C artificial amniotic solution bubbled with air. After the amniotic fluid and amnionic membranes were obtained, fetal and maternal urine and plasma were collected, and the animal euthanized, according to the procedures described above.

Either amniotic fluid (after centrifuging at 2000 rpm for 5 minutes to remove meconium) or artificial amniotic solution was measured into 13 x 100 polystyrene tubes (see "Experiments" below). Fluids were bubbled with air via spinal needles (20 to 25 gauge) connected by extension tubing to an aquarium pump. The temperature of the fluids were kept constant at 37^{o}C with a Temp Blok Module Heater (Scientific Products H2025-1).

The amnionic membrane was cut into 1 inch by 1 inch squares (6.45 cm^{2}) using a self healing artist's cutting board and a #10 scalpel blade and put into the bubbling artificial amniotic solution as quickly as possible.

After an initial baseline sample was collected (100 µl/ml total volume of incubated fluid), ^{3}H-AVP (2.5 ng/ml) was added to the bubbled amniotic fluid or amnionic membrane in artificial amniotic solution (100 µl/ml of fluid). Samples (200 µl) were collected 1 minute and 120 minutes after addition of the ^{3}H-AVP.

EXPERIMENTS

In Vivo Studies.

ASSESSMENT OF AMNIOTIC FLUID DYNAMICS. Inulin (Sigma, I-3754) was used as a reference marker to determine amniotic fluid volume since it was used successfully by other investigators (nelsen et al., 1954; Brans et al., 1984) in assessing amniotic fluid dynamics. Also, no inulin was detected in the plasma collected at the end of the experiment. This indicated that the inulin after being swallowed was not passed through the fetal gut to be recirculated in the amniotic fluid during the 120 minute sampling period. Thus, recirculated inulin did not interfere with the amniotic fluid volume estimations.

Amniotic fluid volume was estimated by the following equation:

$$\text{Volume (ml)} = \frac{\text{Amount of inulin injected into sac (mg)}}{\text{Amniotic fluid inulin concentration (mg/ml)}}$$

The inulin concentration at 60 minutes after injection was used in the volume calculations since this allowed enough time for the inulin marker to be completely mixed within the entire amniotic cavity. Swallowing rate was determined based on the assumption that there is no net fluid removal across the amnionic membrane and that amniotic fluid volume is not changing:

$$\text{Swallowing rate (ml/hr)} = \text{rate of inulin removal (ml/hr)}$$

$$= \text{IDR (\%/hr)} \times \text{Amniotic fluid volume (ml)}$$

where $\text{IDR} = \text{inulin disappearance rate (\%/hr)}$

Turnover time for the entire amniotic fluid volume was determined by the rate of inulin disappearance and was based on the assumption that inulin was not leaking out across the amnionic membrane:

$$\text{Turnover time} = \frac{\text{amniotic fluid volume (ml)}}{\text{rate of inulin removal (ml/hr)}}$$

STUDIES EXAMINING THE EFFECTS OF VASOPRESSIN ON AMNIOTIC FLUID VOLUME AND OSMOLALITY. Either inulin alone (n=4) or AVP and inulin (n=6) injections into the amniotic sac were made to determine whether AVP may affect amniotic fluid volume or osmolality. Samples were collected according to the protocol described above, and analyzed for osmolality, sodium, potassium, and inulin. Amniotic fluid volume estimations were made using inulin concentrations as described above.

DEMONSTRATION OF VASOPRESSIN DISAPPEARANCE. AVP and inulin were simultaneously injected into the amniotic sac (n=6) and the disappearance of intact AVP (measured by RIA with AS 96 which does not crossreact with metabolic products) was compared with the disappearance rate of inulin. A decrease in the AVP to inulin ratio over time would indicate a swallowing-independent disappearance of AVP from the amniotic sac. This would suggest that AVP was either leaking out of the compartment faster than inulin or that it was being metabolized.

DEMONSTRATION OF VASOPRESSIN METABOLISM. ^{3}H-AVP and inulin were simultaneously injected into the amniotic sac (n=5). The tritium concentration per inulin concentration was measured to determine whether any tritium was leaking out of the amniotic sac.

Samples collected 10, 30, 60 and 120 minutes after injection were analyzed by HPLC. Metabolites were identified as the tritiated fractions which did not co-migrate with standard ^{3}H-AVP. The peaks isolated by HPLC were expressed as percent of total radioactivity detected in the HPLC analysis.

<u>In Vitro Studies</u>.

DETERMINATION OF THE SITE OF VASOPRESSIN METABOLISM IN THE AMNIOTIC SAC. ^{3}H-AVP (1000 pg) was incubated at 37^{o}C and bubbled with air for 120 minutes in one of the 4 preparations listed below:

1. artificial amniotic solution (4 ml)(n=4)
2. amnionic membrane (38.7 cm^2 = 6 in^2) +
   amniotic fluid (4 ml)(n=4)
3. amnionic membrane (38.7 cm^2 = 6 in^2) +
   artificial amniotic solution (4 ml)(n=4)
4. amniotic fluid alone (4 ml)(n=4)

Thus, by examining all possible combinations, these studies would indicate whether metabolism was taking place in the amniotic fluid, on the amnionic membrane, neither site, or a combination of both.

PARTIAL CHARACTERIZATION OF METABOLITES AND METABOLIC ENZYMES. The 120 minute samples from 3 in vitro amnionic membrane + amniotic fluid experiments were analyzed with the 15-30% CH_3CN gradient, the 39% CH_3OH, and the 18% CH_3CN buffer systems to verify the isolation of just two peaks of radioactivity besides the ^{3}H-AVP peak. Also, the co-migration with analogues of AVP in HPLC of the metabolites isolated was checked in the three different HPLC buffers. Tritium peaks detected on the HPLC other than intact ^{3}H-AVP were metabolic products which contained the amino acid phenylalanine, since it was here that the molecule was labeled.

Since the metabolite produced in the amniotic fluid appeared to be dgAVP, it was likely that the enzyme involved with its production was trypsin-like. To characterize this enzyme, aprotinin, a trypsin inhibitor (Sigma, A 4529), was simultaneously incubated 4.5 TIU/ml) along with ^{3}H-AVP (1000 pg) in the amniotic fluid (4 ml) only preparation (n=4). The HPLC analysis of the 120 minute sample of the aprotinin and amniotic fluid preparation was compared with the analyses of the amniotic fluid only and artificial amniotic saline preparations. If the enzyme involved with the production of this metabolite was trypsin, or trypsin-like, the aprotinin would be able to block, at least partially, the production of the metabolite in the amniotic fluid.

EXAMINATION OF THE PHYSIOLOGICAL DEVELOPMENT OF THE METABOLIC SYSTEM. The development of the ability to metabolize AVP in the amniotic sac in the second half of gestation was examined. Amniotic fluid and membranes were collected from fetus from 34 to 62 days of gestation. ^{3}H-AVP (250 pg) was incubated with amniotic fluid-only (1 ml)(n= 26) and amnionic membrane (1 in^2 = 6.45 cm^2)(n=22) in artificial amniotic solution (1 ml) preparations. The 120 minute samples were analyzed by HPLC and the amnionic membrane results were compared with the amniotic fluid results.

STATISTICAL ANALYSIS

Simple regression analysis was used to relate the changes in amniotic fluid osmolality, sodium, potassium, and cortisol to gestational age. Comparison between baseline AVP and VT levels measured by RIA at three gestational stages (late 2nd trimester (34-45 days), early 3rd trimester (46-56 days), and late 3rd trimester (57-68 days)) was analyzed by one-way ANOVA and a Duncan's multiple range post hoc test. Amniotic fluid and fetal urine, fetal plasma, and maternal plasma osmolality, sodium, and potassium concentration in the last week of gestation were compared by one-way ANOVA with a Duncan's multiple range post hoc test and a multiple regression analysis with amniotic fluid parameters as the dependent variable and all other parameters as the independent variables. The relationship between amniotic fluid osmolality and fetal urine osmolality was analyzed by simple regression analysis. Simple regression analyses were also used to determine the relationships between amniotic fluid concentrations of AVP, tritium, osmolality, sodium, and potassium with time after injection in the *in vivo* experiments, and the relationship between the rate of AVP disappearance and initial amniotic fluid AVP concentration. The AVP concentration expressed per fraction of initial *in vivo* amniotic fluid concentration over time was analyzed by exponential regression. A t-test of slopes was used to test any difference between control and AVP injections in the *in vivo* experiments. The relationship between M1 and M2 production and gestational age was analyzed by simple linear regression. All results are expressed as mean $\pm$ standard error of the mean. A $p<0.05$ significance level was used for all analyses.

CHAPTER III

RESULTS

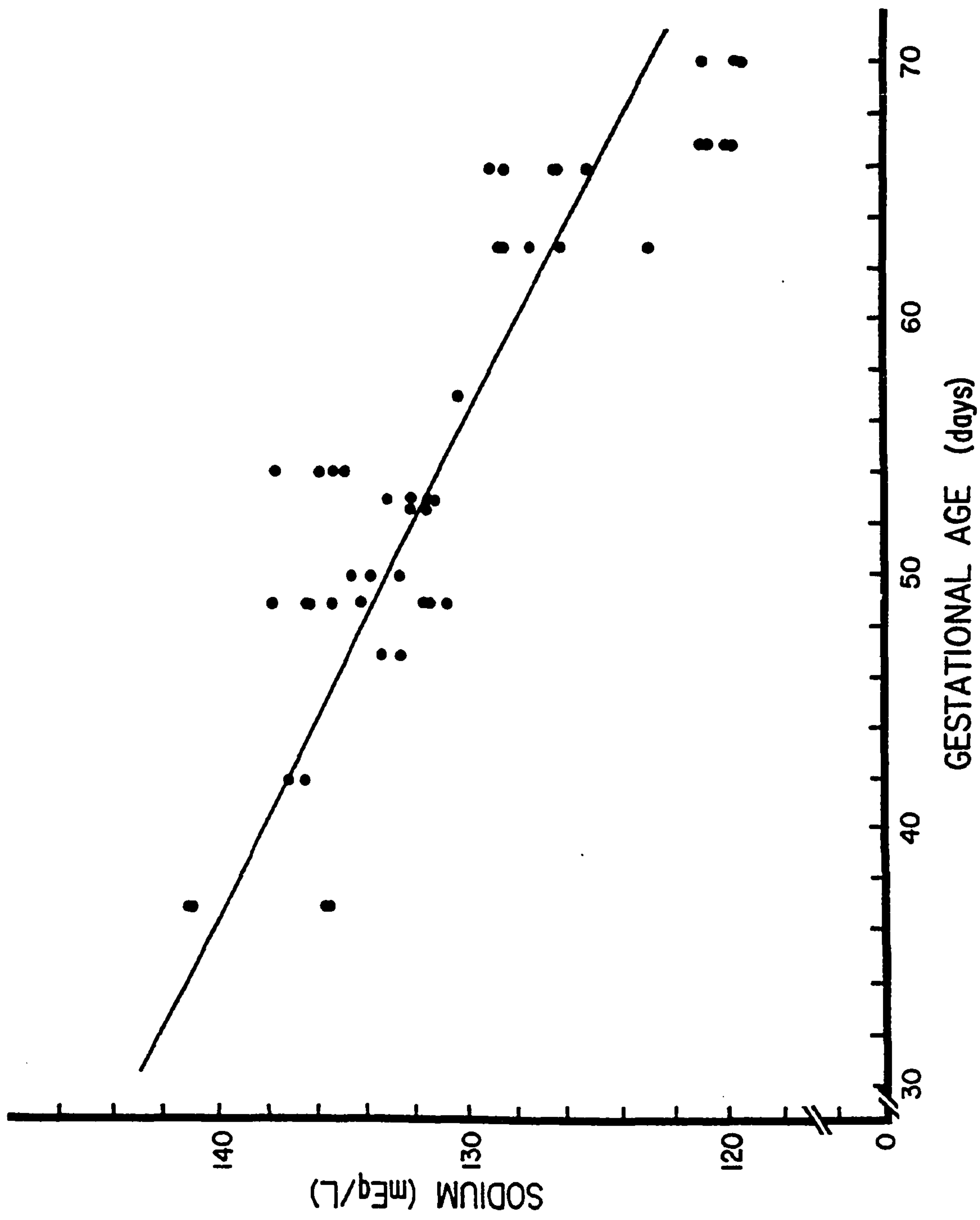
PART I. INITIAL STUDIES OF OSMOLALITY, SODIUM, POTASSIUM, AND HORMONE BASELINE VALUES IN THE AMNIOTIC FLUID

AMNIOTIC FLUID OSMOLALITY, SODIUM AND POTASSIUM CONCENTRATION: INFLUENCE OF FETAL AGE AND FETAL PLASMA, FETAL URINE, AND MATERNAL PLASMA

Age Related Changes. A baseline study was conducted to establish osmolality and sodium and potassium concentration in the amniotic fluid of guinea pigs between 37 and 67 days of gestation (term = 68 days).

Sodium concentration decreased linearly with gestational age ($r=-0.88$, $p<0.001$) (fig. 6) as has been previously observed in humans (Lind et al., 1969). Potassium concentration increased over gestation ($r=0.94$, $p<0.001$) (fig. 7) in contrast to reports in humans (Bonses, 1966). Osmolality (275 $\pm$ 1 mOsm/kg H_2O) did not vary with gestational age (fig. 8) possibly because the decrease in sodium concentration (about 20 mEq/l) is completely balanced by an increase in potassium concentration.

Comparison of Amniotic Fluid and Fetal Urine, Fetal Plasma, and Maternal Plasma Osmolality and Sodium and Potassium Concentration in the Last Week of Gestation. Figure 9 summarizes the maternal plasma, figure 6. Amniotic fluid sodium concentration over gestation. Sodium decreased linearly with gestational age ($r = -0.88$, $p < 0.001$).



SODIUM (mEq/L)
140
130
120
0
30
40
50
60
70
GESTATIONAL AGE (days)

figure 7. Amniotic fluid potassium concentration over gestation. Potassium increased with gestational age ($r=0.94$, $p<0.001$).

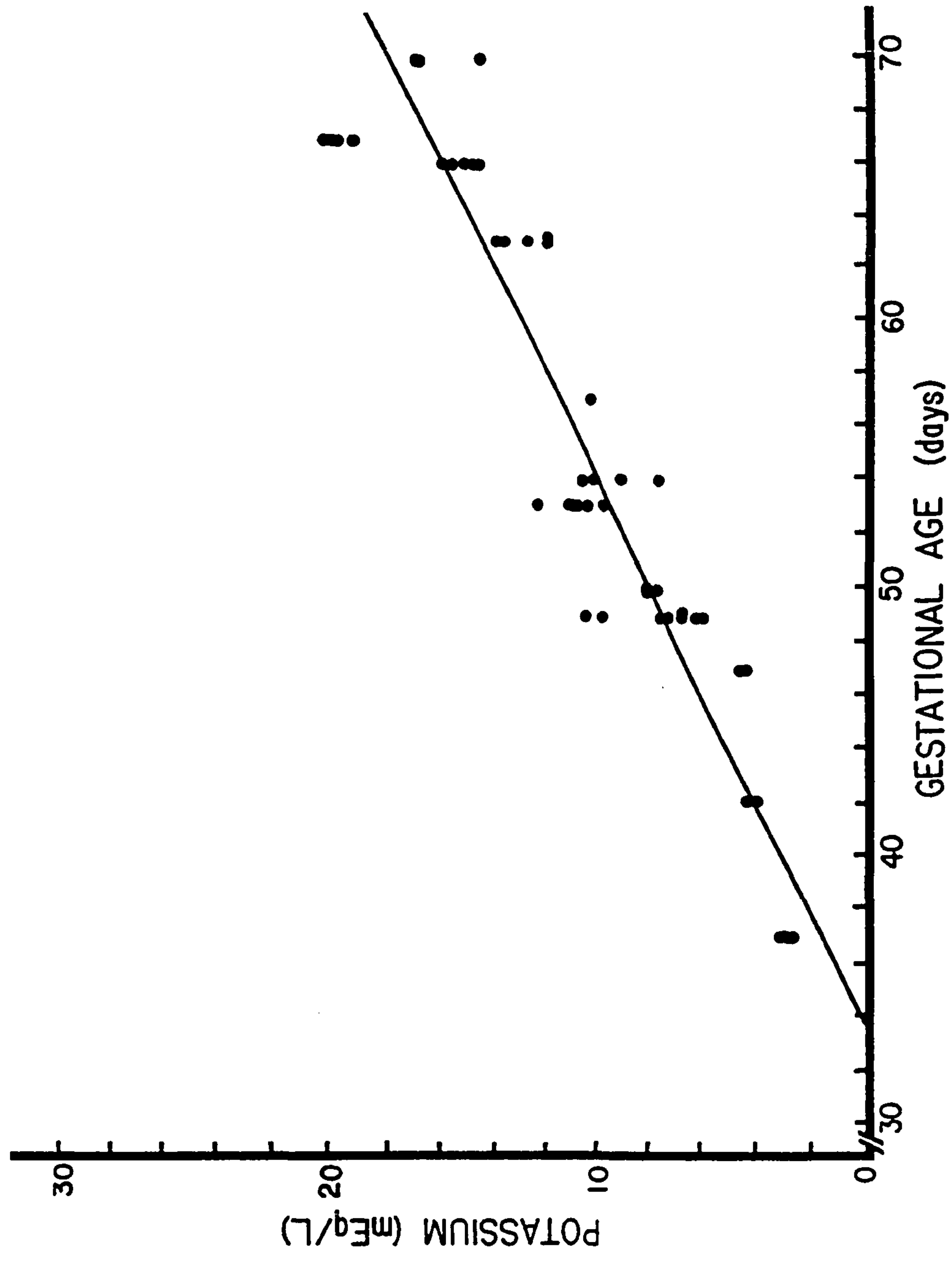

POTASSIUM (mEq/L)
0
10
20
30
GESTATIONAL AGE (days)
30
40
50
60
70

figure 8. Amniotic fluid osmolality over gestation. Osmolality (275 ± 1 mOsm/kg H_2O) did not vary with gestational age.

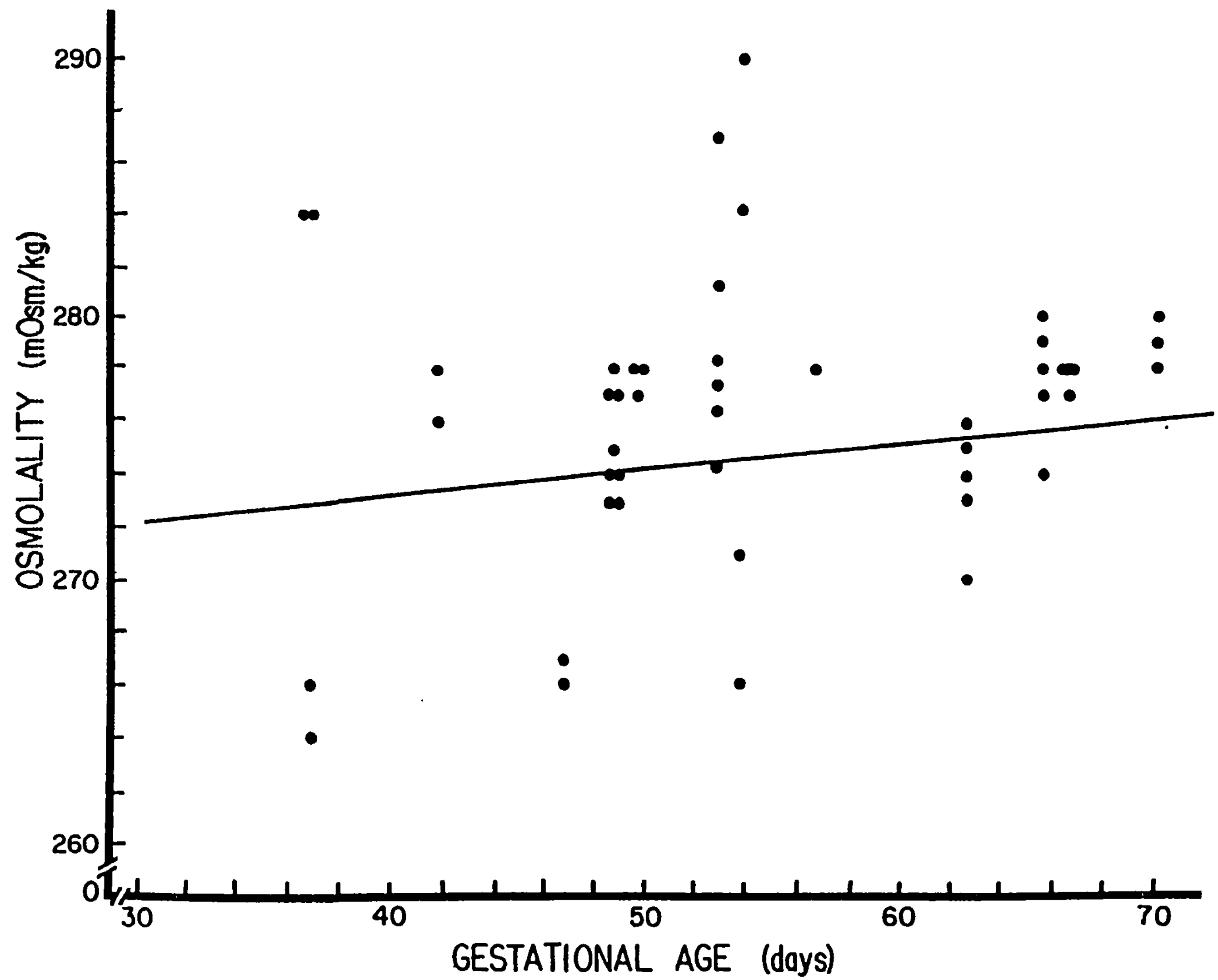
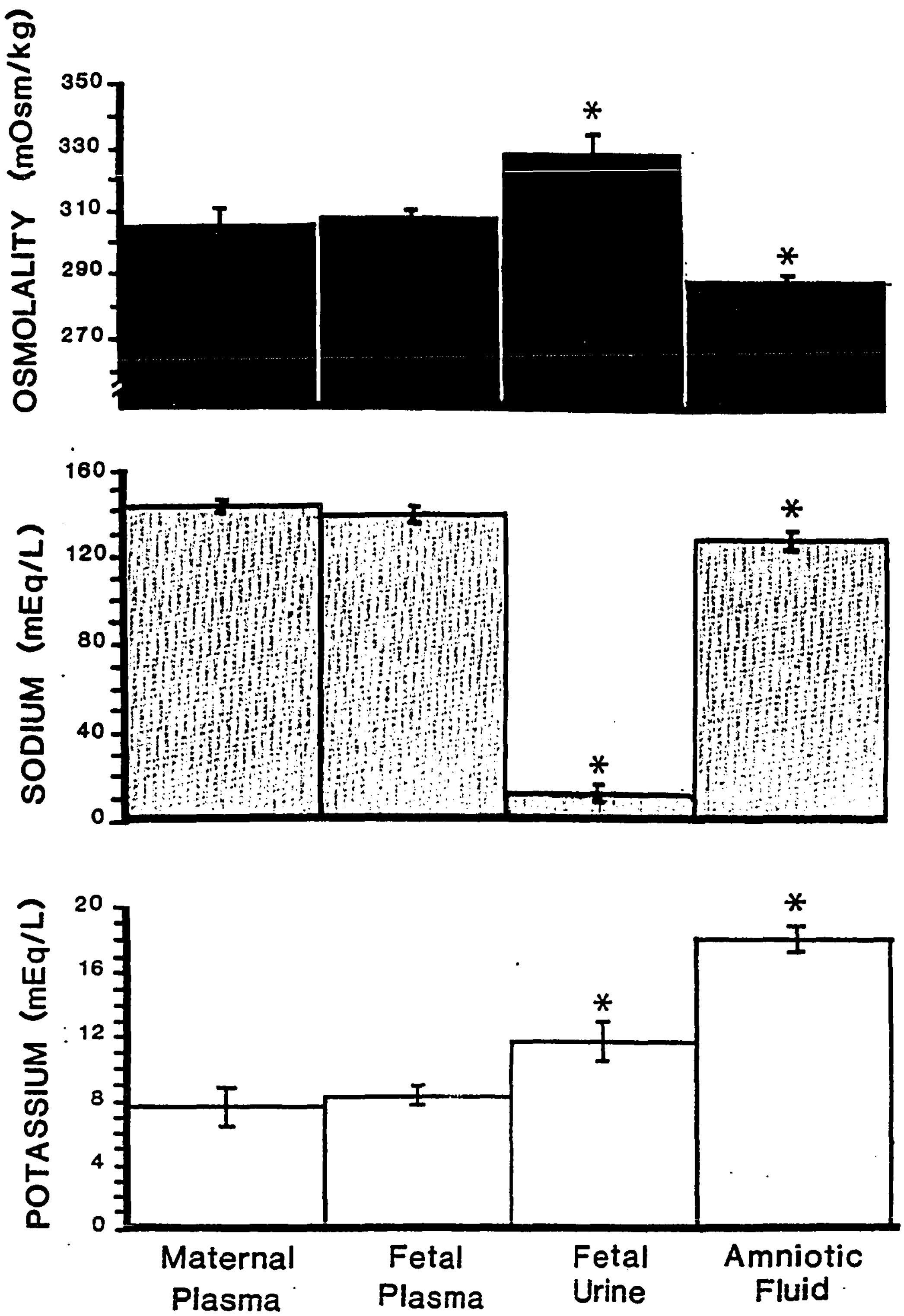

290
280
270
260
0
OSMOLALITY (mOsm/kg)
30
40
50
60
70
GESTATIONAL AGE (days)

figure 9. Average osmolalities, and sodium and potassium concentrations in maternal plasma, fetal plasma, fetal urine, and amniotic fluid in the last week of gestation (mean $\pm$ S.E., * = $p<0.05$). Fetal plasma and maternal plasma values were not different from each other. Fetal urine values were significantly different from fetal plasma and amniotic fluid values. Amniotic fluid values were significantly different from and were not dependent upon fetal urine, fetal plasma or maternal plasma values (MREG).


OSMOLALITY (mOsm/kg)
350
330
310
290
270
SODIUM (mEq/L)
160
120
80
40
0
POTASSIUM (mEq/L)
20
16
12
8
4
0
Maternal Plasma
Fetal Plasma
Fetal Urine
Amniotic Fluid

fetal plasma, fetal urine, and amniotic fluid osmolalities and sodium and potassium concentrations. There were no differences in plasma osmolality or electrolyte concentration between the fetus and mother. Osmolality was greater in the fetal urine (328 $\pm$ 8 mOsm/kg H_2O) than the fetal plasma (308 $\pm$ 3 mOsm/kg H_2O, $p<0.05$), indicating that the fetus is capable of modifying the filtered plasma by the reabsorption of free water in the kidney or in the bladder. Furthermore, sodium evidently was reabsorbed by the fetal renal tubular system or in the fetal bladder since sodium concentration decreased in the urine despite an increased osmolality. The increase in potassium concentration between fetal plasma and fetal urine was about 4 mEq/l ($p<0.05$), which, with attendant anions would constitute about 8 mOsm. This increase in potassium concentration could be accounted for by the renal tubular removal of water in the formation of the urine and does not necessarily represent any specific renal tubular actions on this ion.

As the urine passes into the amniotic sac further modifications occur. Osmolality decreased from 328 $\pm$ 8 mOsm/kg H_2O in the urine to 287 $\pm$ 2 mOsm/kg H_2O in the amniotic fluid, which was also significantly lower than fetal plasma ($p<0.05$). Despite this decrease in osmolality, both sodium and potassium concentrations were increased in the amniotic fluid compared to the fetal urine (by 115.8 mEq/l and 6.3 mEq/l, respectively). The amniotic fluid sodium concentration, was lower than that of maternal and fetal plasma (127.2 $\pm$ 1.0 mEq/l vs. 142.0 $\pm$ 1.4 and 139.9 $\pm$ 0.4 mEq/l, $p<0.05$), and the amniotic fluid potassium concentration was higher than fetal plasma concentration (18.1 ± 0.5 mEq/l vs. 8.2 ± 0.3 mEq/l, $p<0.05$). Multiple regression analysis showed that amniotic fluid osmolality and sodium and potassium concentrations are not dependent upon fetal urine, fetal plasma or maternal plasma values.

AF osmolalities measured in baseline samples were constant (287 ± 2 mOsm/kg H_2O) among fetuses in the third trimester, and were independent of urine osmolality (fig. 10). The amniotic fluid osmolality of 11 fetuses ranged between 277 and 297 mOsm/kg H_2O despite a large variation in urine osmolalities (288 to 393 mOsm/kg H_2O).

These results indicate that there are mechanisms which provide a relatively constant external osmotic environment for the developing fetus. The possible nature of these mechanisms will be discussed.

BASELINE HORMONE DETERMINATIONS AND IDENTIFICATION OF AVP IN THE AMNIOTIC FLUID AND FETAL PLASMA AND URINE

Baseline Amniotic Fluid Cortisol, Vasopressin and Vasotocin RIA Measurements. Since *in vitro* studies by others (Manku et al., 1975; Vizsolyi and Perks, 1974) showed that AVP, VT and CORT influence the movement of water across the amnionic membrane, these hormones were also measured in the baseline samples in the present studies. Cortisol levels increased sharply near term ($r=0.721$ $p<0.001$) (fig. 11), as has been found in humans (e.g. Aderjan et al., 1977; Blankstein et al., 1980; Murphy et al., 1975; Sippell et al., 1981) and rabbits (Barr et al., 1980).

figure 10. Relationship between amniotic fluid osmolality and fetal urine osmolality. Despite a wide variation of urine osmolality (288 to 393 mOsm/kg H_2O), amniotic fluid osmolality remained between 277 and 297 mOsm/kg H_2O.

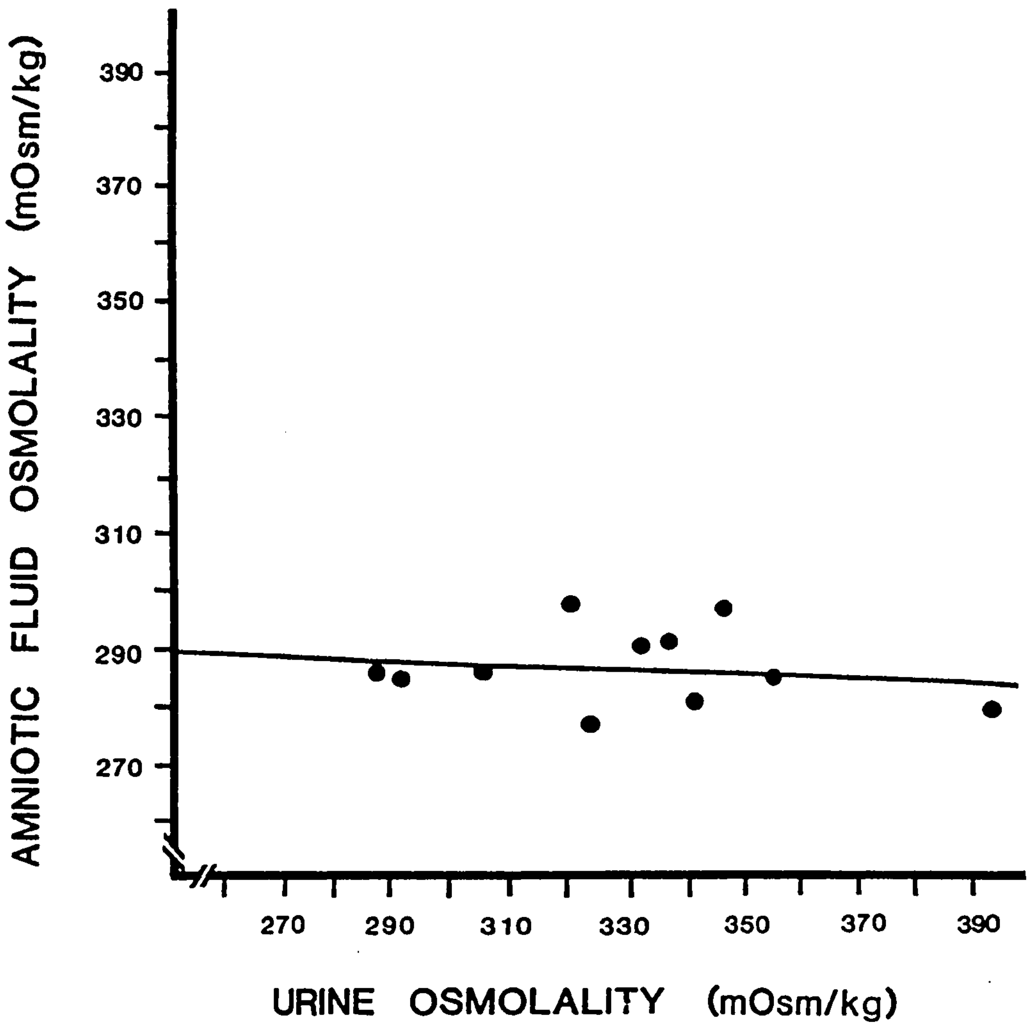
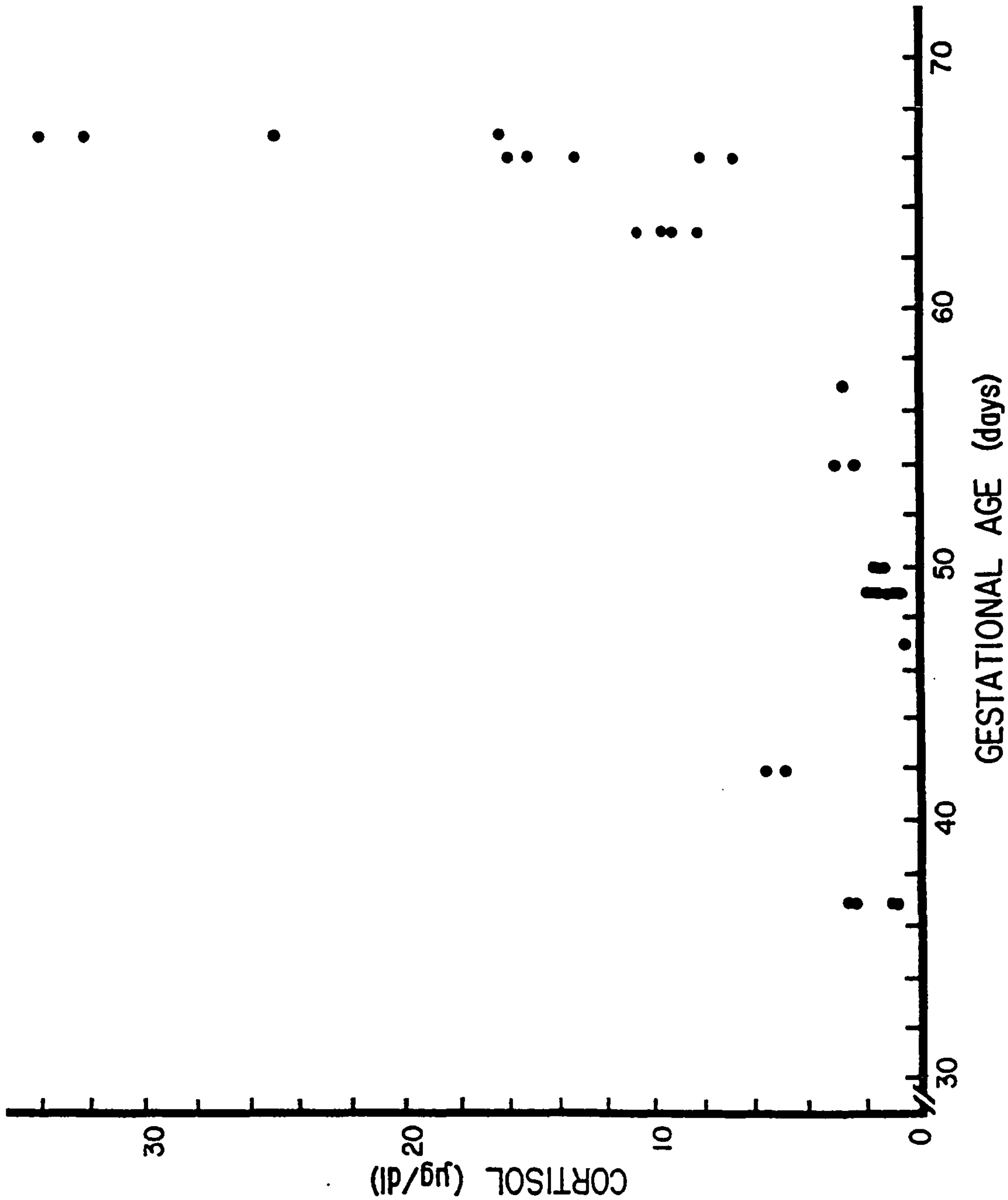

AMNIOTIC FLUID OSMOLALITY (mOsm/kg)
390
370
350
330
310
290
270
270
290
310
330
350
370
390
URINE OSMOLALITY (mOsm/kg)

figure 11. Amniotic fluid cortisol levels over gestation. Cortisol levels increased sharply near term ($r=0.72$, $p<0.001$).

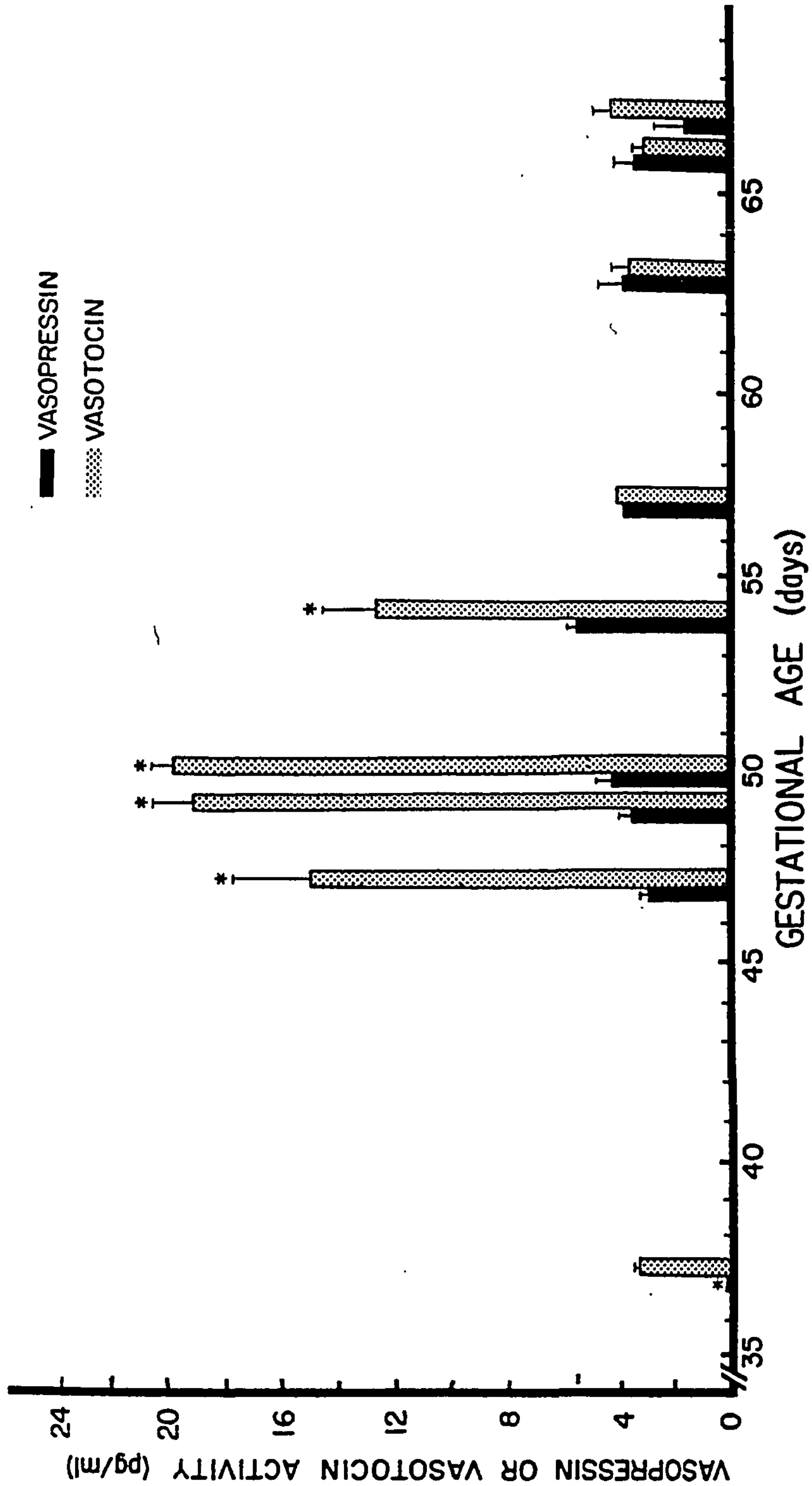

CORTISOL (µg/dl)
0
10
20
30
GESTATIONAL AGE (days)
30
40
50
60
70

When these baseline studies were initiated, AVP levels had not yet been measured in amniotic fluid. Since then, Artman et al. (1984) reported that VT concentration in human amniotic fluid decreased from the second to third trimester while AVP levels remain unchanged. The results of the present study employing similar RIA techniques showed that guinea pig amniotic fluid AVP and VT concentrations in the third trimester (days 46 to 69 of gestation) exhibit the same pattern as that seen in humans in the second and third trimesters (fig. 12). Apparent AVP concentration significantly increased (ANOVA, $p<0.01$) from undetectable levels in the second trimester (days 23 to 45 of gestation) to 4.03 ± 0.30 pg/ml in the early third trimester but then remained relatively constant until the end of gestation. The average AVP level in the third trimester was 3.67 ± 1.61 pg/ml. Also, similar to previous studies, the apparent VT levels, measured by the RIA subtraction technique, increased between the second and third trimesters and then decreased (ANOVA, $p<0.001$) from 17.99 ± 1.05 pg/ml in the early third trimester to 3.87 ± 0.18 pg/ml in the late third trimester. The VT levels at the end of gestation were not different from those measured in the second trimester.

There were problems, however with this assessment of AVP and VT which will become evident below.

<u>Identification of Endogenous Vasopressin in Fetal Plasma, Fetal Urine, and Amniotic Fluid</u>. It is believed that the source of amniotic fluid AVP is fetal urine since this hormone does not cross the

figure 12. Amniotic fluid AVP and VT concentrations over gestation (measured by RIA). AVP concentration significantly increased (* ANOVA, $p<0.01$) from undetectable levels in the second trimester to 4.026 ± 0.30 pg/ml in the early third trimester but then remained relatively constant until the end of gestation. VT levels significantly decreased (* ANOVA, $p<0.001$) from 17.99 ± 1.05 pg/ml in the early third trimester to 3.87 ± 0.18 pg/ml in the late third trimester.

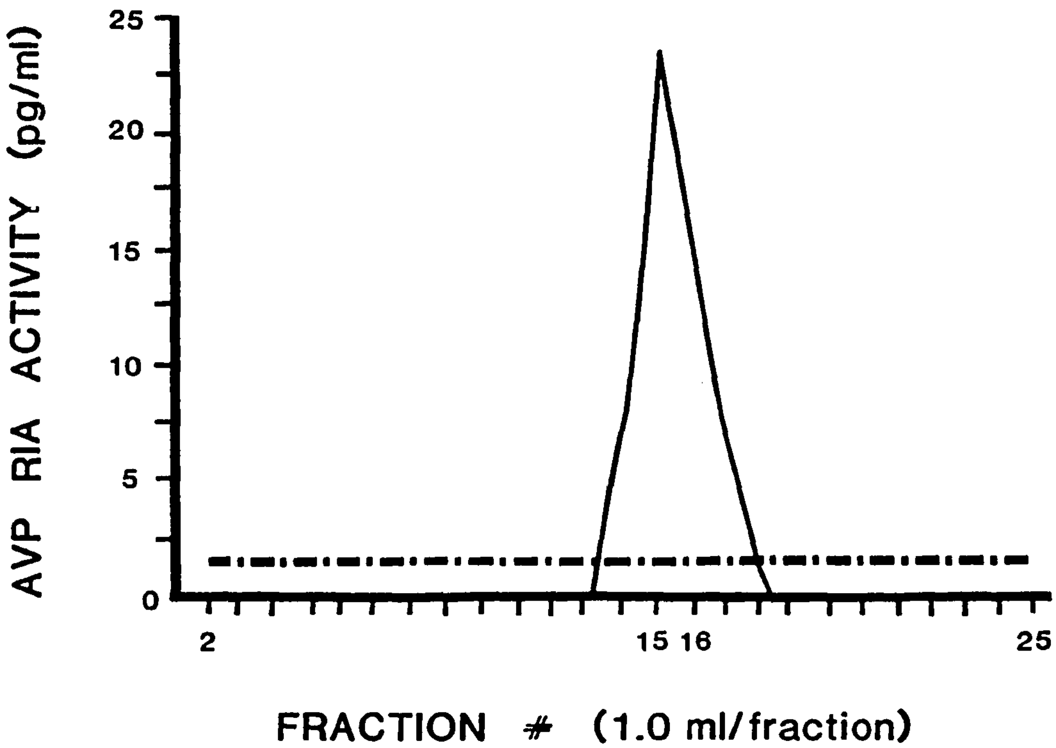

VASOPRESSIN
VASOTOCIN
VASOPRESSIN OR VASOTOCIN ACTIVITY (pg/ml)
0
4
8
12
16
20
24
GESTATIONAL AGE (days)
35
40
45
50
55
60
65

placental barrier (Alexander et al., 1971a; Forsling and Fenton, 1977). In order to verify the endogenous presence of AVP in amniotic fluid, it was first necessary to demonstrate AVP presence in both fetal plasma and fetal urine.

VASOPRESSIN IN FETAL PLASMA. AVP was present in the guinea pig fetal plasma. In the last week of gestation, fetal plasma AVP levels measured by RIA of extracted samples averaged 16.52 $\pm$ 2.45 pg/ml (plasma osmolality of 304 $\pm$ 1 mOsm/kg H_2O). These measurements were verified as representing true AVP by demonstrating that the radioimmunoactive peak measured in fetal plasma co-migrates with standard AVP on the HPLC (fig. 13). Although an AVP antiserum (AS 95) which crossreacts with dgAVP was used to measure radioimmunoactivity, no peak which corresponds to dgAVP was detected in these samples. In 39% CH_3OH buffer AVP elutes at fractions 15 and 16, and dgAVP elutes at fractions 11 and 12.

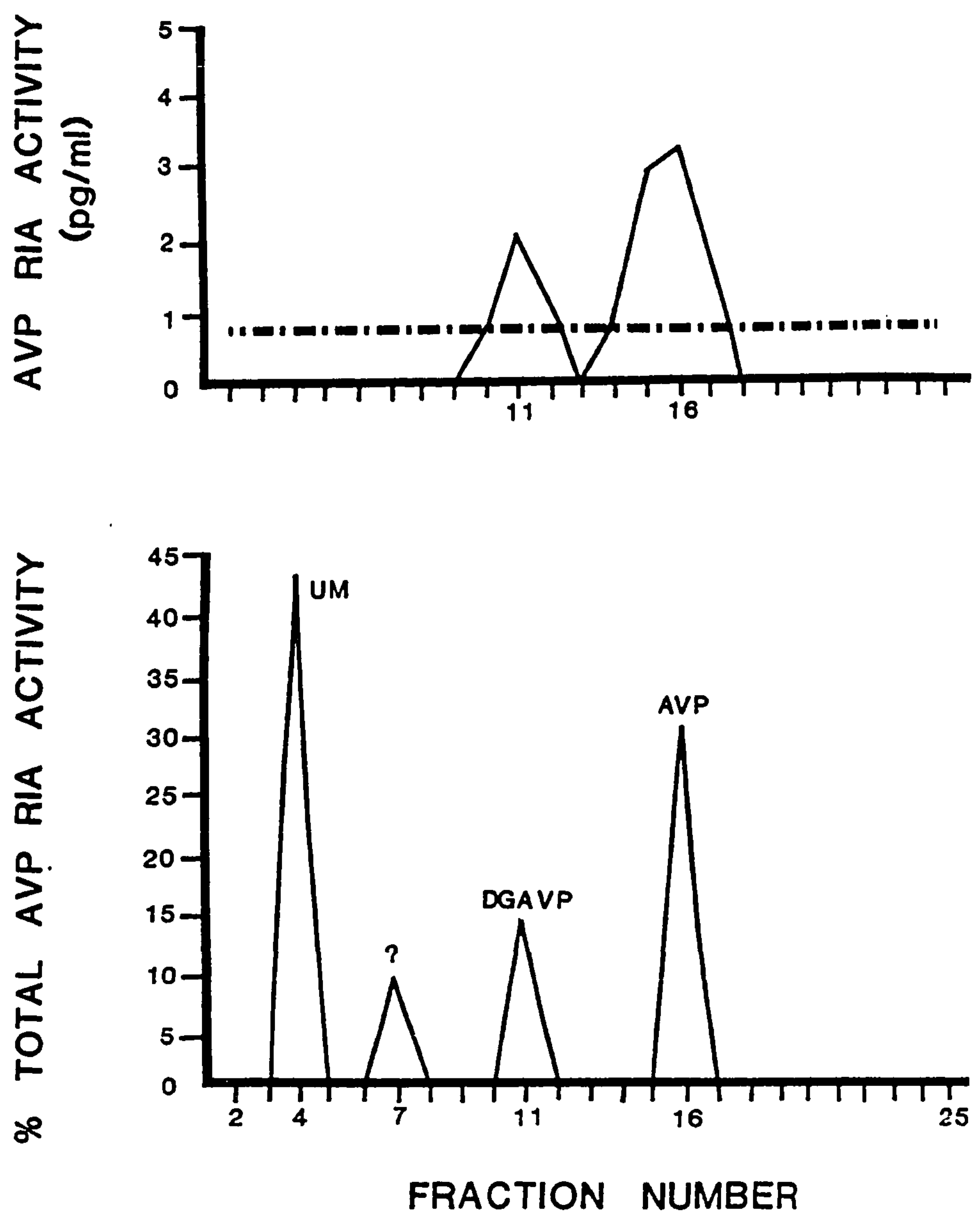
VASOPRESSIN IN FETAL URINE. Intact AVP was also present in guinea pig fetal urine. The average fetal urine AVP level in the last week of gestation measured by RIA of extracted samples was 58.42 $\pm$ 7.20 pg/ml (urine osmolality of 340 $\pm$ 9 mosm/kg H_2O). A radioimmunoactive peak which co-migrates with standard AVP on the HPLC was isolated in both extracted and unextracted fetal urine (fig. 14). In addition to AVP, other radioimmunoactive peaks were also detected in unextracted urine. One product which elutes at fraction 4 in the 15-30% CH_3CN gradient buffer and the 39% CH_3OH buffer may be a possible urinary metabolite -- which also elutes at fraction 4 --

figure 13. HPLC elution profile of fetal plasma extracted with octadecyl silane (Waters Sep-Pak C_{18} cartridge). Eluent: 39% CH_3OH in 0.05 M CH_3COONH_4 (pH 6.5). Although HPLC fractions were analyzed by radioimmunoassay with AS 95 which crossreacts 60% with dgAVP no other peak other than one which co-migrated with standard AVP seen at fraction 16 could be detected. Radioimmunoactivity is represented in pg/ml. (The dashed line represents the lowest detectable limit of the assay.)



AVP RIA ACTIVITY (pg/ml)
25
20
15
10
5
0
2
15 16
25
FRACTION # (1.0 ml/fraction)

figure 14. HPLC elution profiles of (A) extracted (octadecyl silane, Waters Sep-Pak C_{18} cartridge) and (B) unextracted fetal urine. Eluent: 39% CH_3OH in 0.05 M CH_3COONH_4 (pH 6.5). In extracted urine a peak which co-migrated with standard dgAVP (fraction 11) and a peak which co-migrated with standard AVP (fraction 16) were detected by radioimmunoassay with AS 95. Radioimmunoactivity is represented in pg/ml. (The dashed line represents the lowest detectable limit of the assay.) In unextracted urine two other peaks which did not co-migrate with either dgAVP or AVP were also detected. Peaks are represented as % total radioimmunoactivity in the samples.

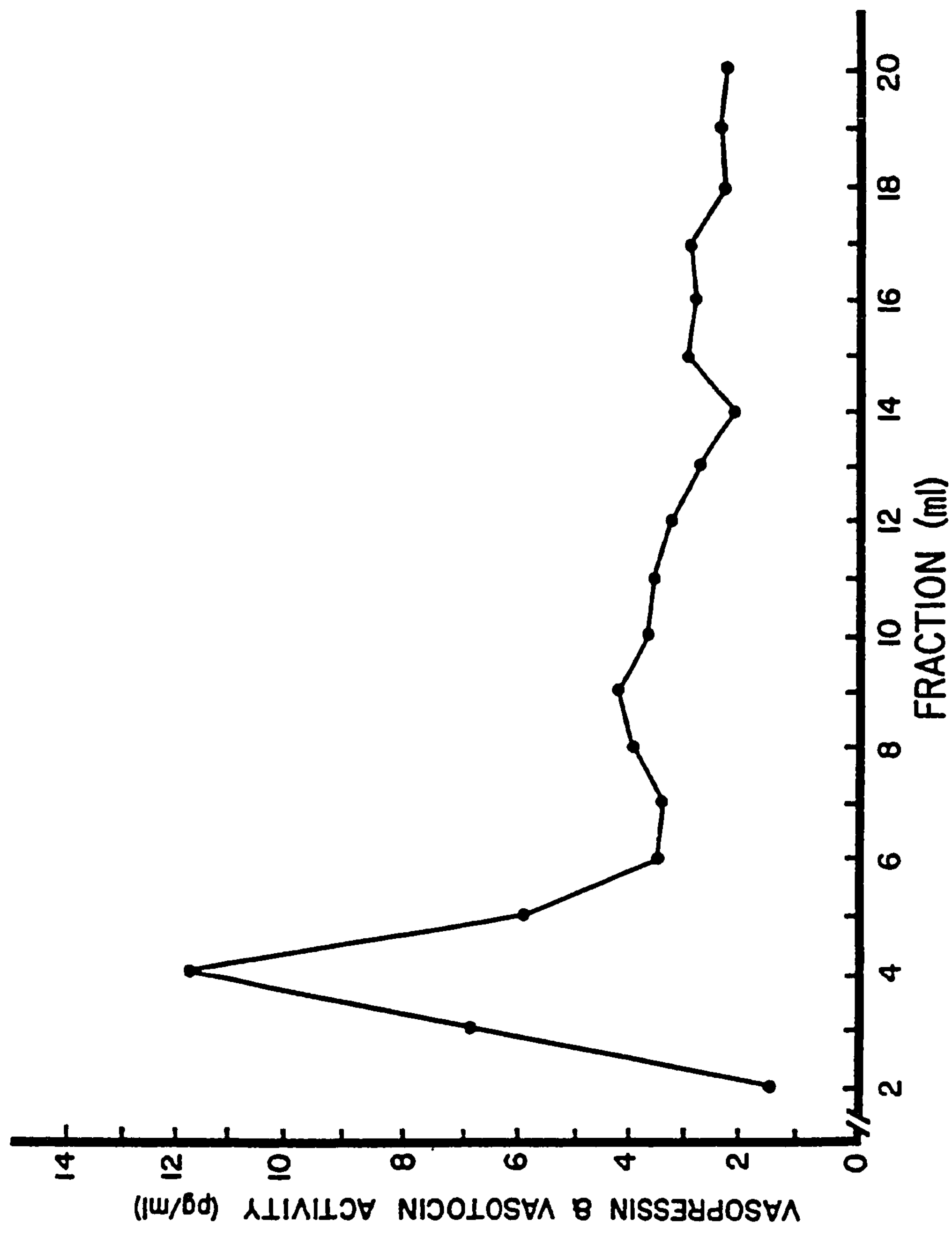

AVP RIA ACTIVITY (pg/ml)
5
4
3
2
1
0
11
16
% TOTAL AVP RIA ACTIVITY
45
40
35
30
25
20
15
10
5
0
UM
?
DGAVP
AVP
2
4
7
11
16
25
FRACTION NUMBER

found in several other species (Claybaugh and Sato, 1985). Two additional peaks have been separated using the 39% CH_3OH buffer system; one which migrates at fraction 7 and one which migrates at fraction 12. The product at fraction 7 has not been identified. The product at fraction 12 co-migrates with standard dgAVP.

When fetal urine was extracted and spiked with standard AVP and standard dgAVP, the corresponding fractions increased accordingly with 72% spike recovery.

VASOPRESSIN IN AMNIOTIC FLUID. Although AVP levels measured by RIA in the baseline studies were similar to those levels reported by Artman et al. (1984), neither intact AVP nor VT could be verified by HPLC analysis. Twenty ml of amniotic fluid pooled from 14 fetuses of 49 to 51 days gestation -- the period when VT concentrations were highest -- were extracted with CG-50 ion exchange and concentrated to 1 ml. The concentrate was then analyzed by HPLC with the 39% CH_3OH buffer system. Twenty 1 ml fractions were collected and assayed for AVP and VT with an AVP antiserum which crossreacts with VT (AS 09). Only a peak which eluted at the HPLC front was recovered (fig. 15). No peaks which coincided with AVP and VT standards (fractions 12 and 7, respectively) were recovered. Therefore, the RIA activity measured in the baseline amniotic fluid samples was apparently a result of interference with the assay and not actually due to either AVP or VT.

The amniotic fluid AVP levels measured in the last week of gestation by RIA of extracted samples averaged 1.70 $\pm$ 0.15 pg/ml (osmolality of 284 $\pm$ 1 mosm/kg H_2O), but again, a radioimmunoactive figure 15. HPLC elution profile of CG-50 extracted pooled amniotic fluid, gestation 49-51 days. Eluent: 39% CH_3OH in 0.05 M CH_3COONH_4 (pH 6.5). AVP RIA activity was measured with AS 09 which crossreacts with VT. No peaks which coincided with the fractions where AVP and VT elute (fractions 12 and 7, respectively) were isolated.


VASOPRESSIN & VASOTOCIN ACTIVITY (pg/ml)
0
2
4
6
8
10
12
14
FRACTION (ml)
2
4
6
8
10
12
14
16
18
20

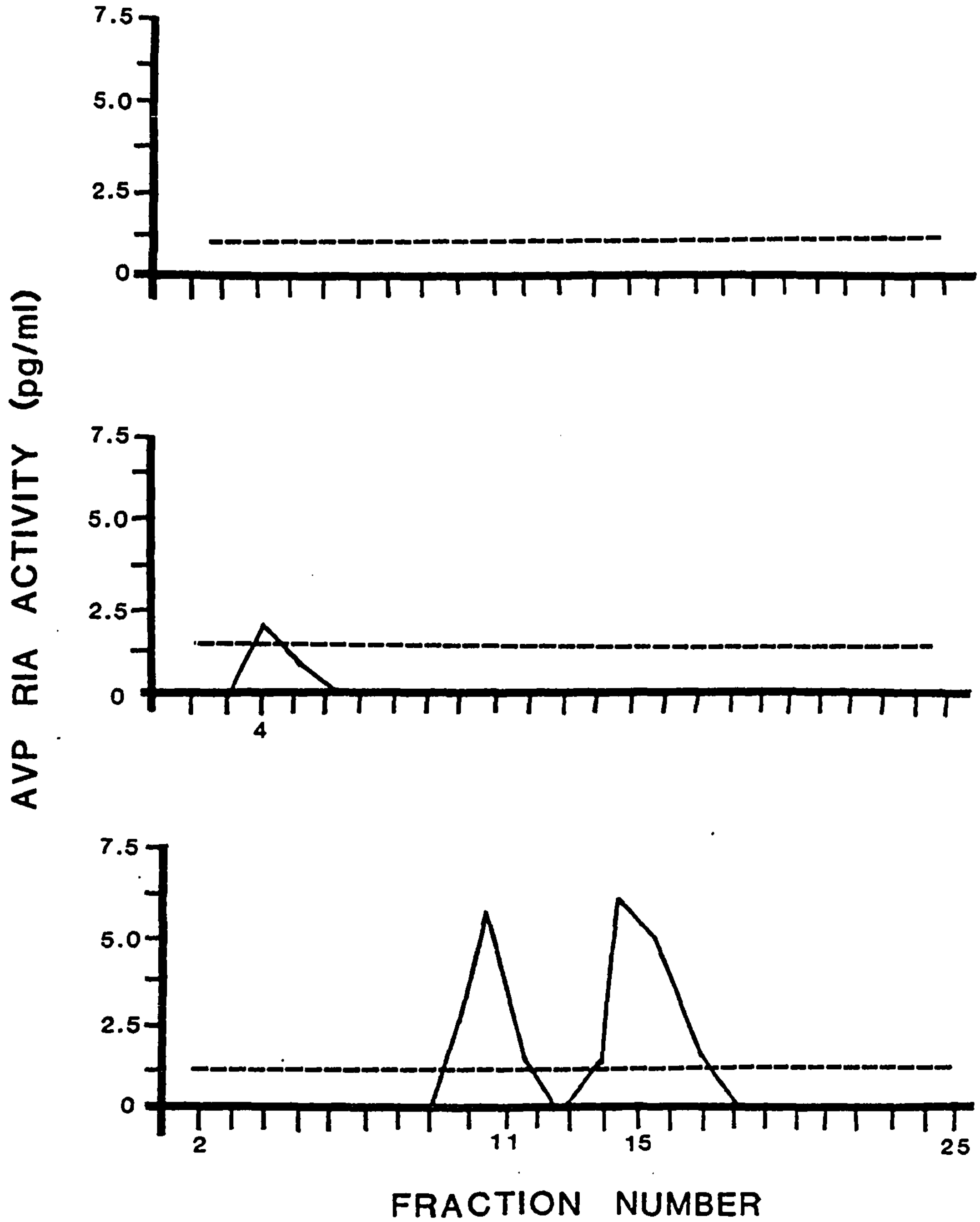
peak which co-migrated with standard vasopressin could not be isolated. In six HPLC analyses of both unextracted and extracted (by sep-pak C_{18} octadecyl silane) amniotic fluid samples concentrated 8 to 22 times, no recovery of a radioimmunoactive peak corresponding to standard AVP could be isolated (fig. 16). However, when amniotic fluid samples were spiked with AVP and dgAVP, the standards were isolated with 86% spike recovery.

Thus, although intact AVP may be added to the amniotic compartment along with fetal urine, these results indicate that it is either leaking out of the compartment or being broken down here, such that intact AVP can no longer be separated by HPLC and measured by RIA.

In addition, AVP levels measured in the amniotic fluid after injection of exogenous AVP into the amniotic sac (see below) were much less than what would be expected based on calculations of the amount of AVP injected and amniotic fluid volume. These two findings of unsuccessful characterization by HPLC of endogenous AVP RIA activity and incomplete recovery of injected AVP indicated that AVP was somehow disappearing from the amniotic sac.

Since AVP could not be identified in the amniotic fluid, it may not be a likely candidate for influencing amniotic fluid dynamics as hypothesized (see introduction). The next series of experiments, designed to determine whether AVP injected into the amniotic sac might affect amniotic fluid osmolality, supports the suspicion that AVP is not involved in osmotic regulation of amniotic fluid.

figure 16. HPLC elution profiles of sep-pak extracted (A), unextracted (B), and extracted + VP-dgAVP spiked (C) amniotic fluid in the last week of gestation. Eluent: 39% CH_3OH in 0.05 M CH_3COONH_4 (pH 6.5). A radioimmunoactive peak which co-migrated with either standard AVP or dgAVP could not be detected in either extracted or unextracted samples. However, when urine was spiked with AVP and dgAVP before extraction, the standards were recovered.


AVP RIA ACTIVITY (pg/ml)
7.5
5.0
2.5
0
4
2
11
15
25
FRACTION NUMBER

EFFECTS OF EXOGENOUS AVP ON AMNIOTIC FLUID VOLUME, OSMOLALITY AND ELECTROLYTE CONCENTRATIONS, AND ASSESSMENTS OF VOLUME AND TURNOVER RATE

Volume. Inulin was initially used as a reference marker to assess amniotic fluid volume and was injected with a control injection of artificial amniotic solution or an injection of 12.5 ng of AVP. Amniotic fluid volume estimations were calculated from the inulin concentration at 60 minutes after injection since it was not until then that complete mixing of the inulin marker in the amniotic compartment was achieved. Average amniotic fluid volume of 9 fetuses in the last week of gestation was 9.2 $\pm$ 0.7 ml). Turnover time for the entire amniotic fluid volume calculated from inulin disappearance, averaged 14.1 $\pm$ 3.0 hours. If the removal of amniotic fluid is due only to swallowing and that there is no movement of fluid across the amnion, the swallowing rate is estimated at approximately 0.85 $\pm$ 0.15 ml/hr (see methods for calculations). Regression analysis showed that there was no correlation between amniotic fluid osmolality and amniotic fluid volume. However, both osmolality and volume were within limited ranges which would tend to statistically work against achieving an accurate assessment of the relationship between these two parameters.

Because of the insensitivity of this method of volume estimation to very small changes in volume and the required mixing time for the inulin marker, an accurate measurement of amniotic fluid volume change in the 120 minute period after AVP injection could not be obtained.

Osmolality, Sodium and Potassium. The same osmolality and electrolyte changes were seen after AVP injection into the amniotic compartment as those seen after control inulin injection into the amniotic compartment. Amniotic fluid sodium and potassium concentrations did not change over time (fig. 17 and 18). Osmolality significantly increased over the 120 minutes following both control and AVP injection ($r=0.63$ and 0.56, respectively; $p<0.02$) (fig. 19). There was no difference between the rates of the osmolality increase seen in the AVP injection and control injection experiments (t-test of slopes).

Thus, no effect of AVP on either amniotic fluid osmolality nor amniotic fluid electrolytes could be established.

figure 17. Amniotic fluid sodium concentration 30 to 120 minutes after control (———) or 12.5 ng AVP (------) *in vivo* injection into the amniotic sac. Sodium levels did not change over time.

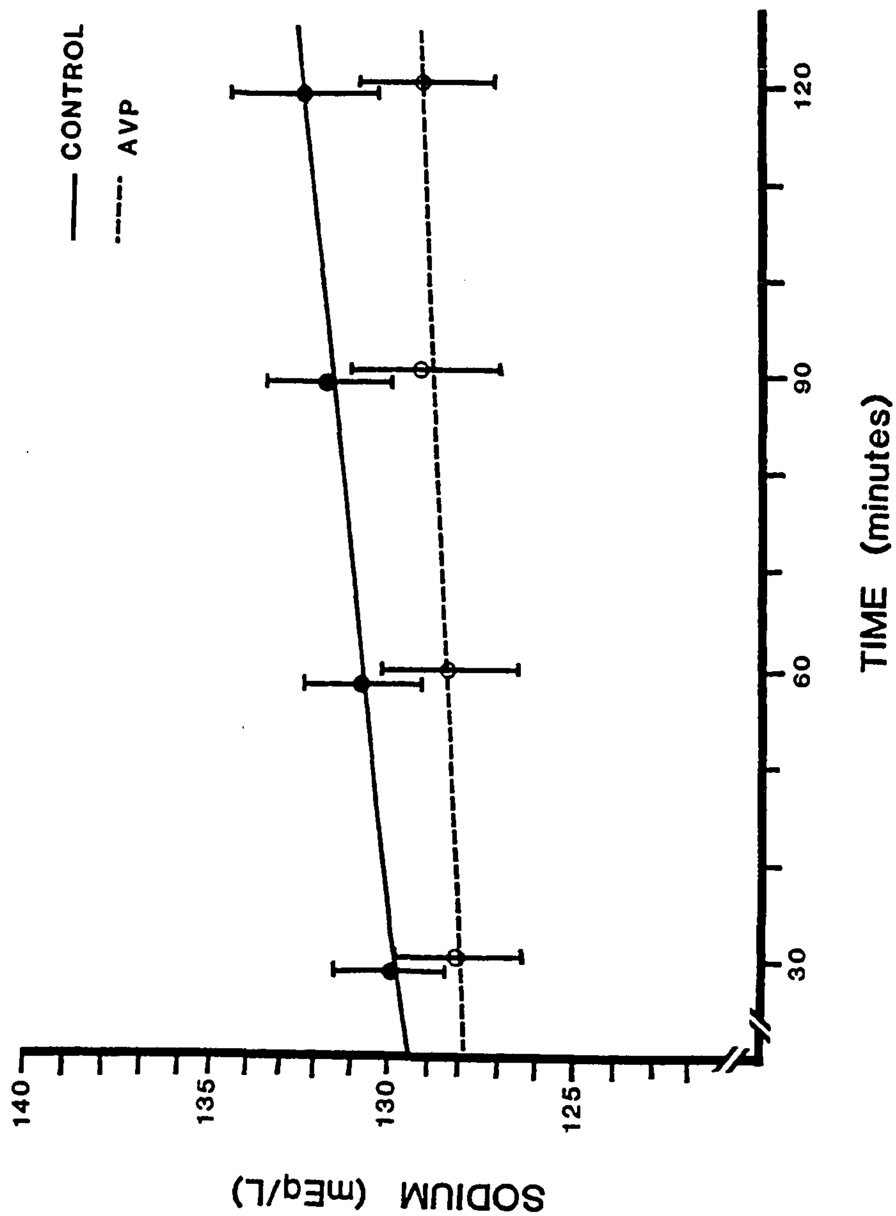

CONTROL
AVP
140
135
130
125
SODIUM (mEq/L)
30
60
90
120
TIME (minutes)

figure 18. Amniotic fluid potassium concentration 30 to 120 minutes after control (————) or 12.5 ng AVP (– – – – –) *in vivo* injection into the amniotic sac. Potassium levels did not change over time.

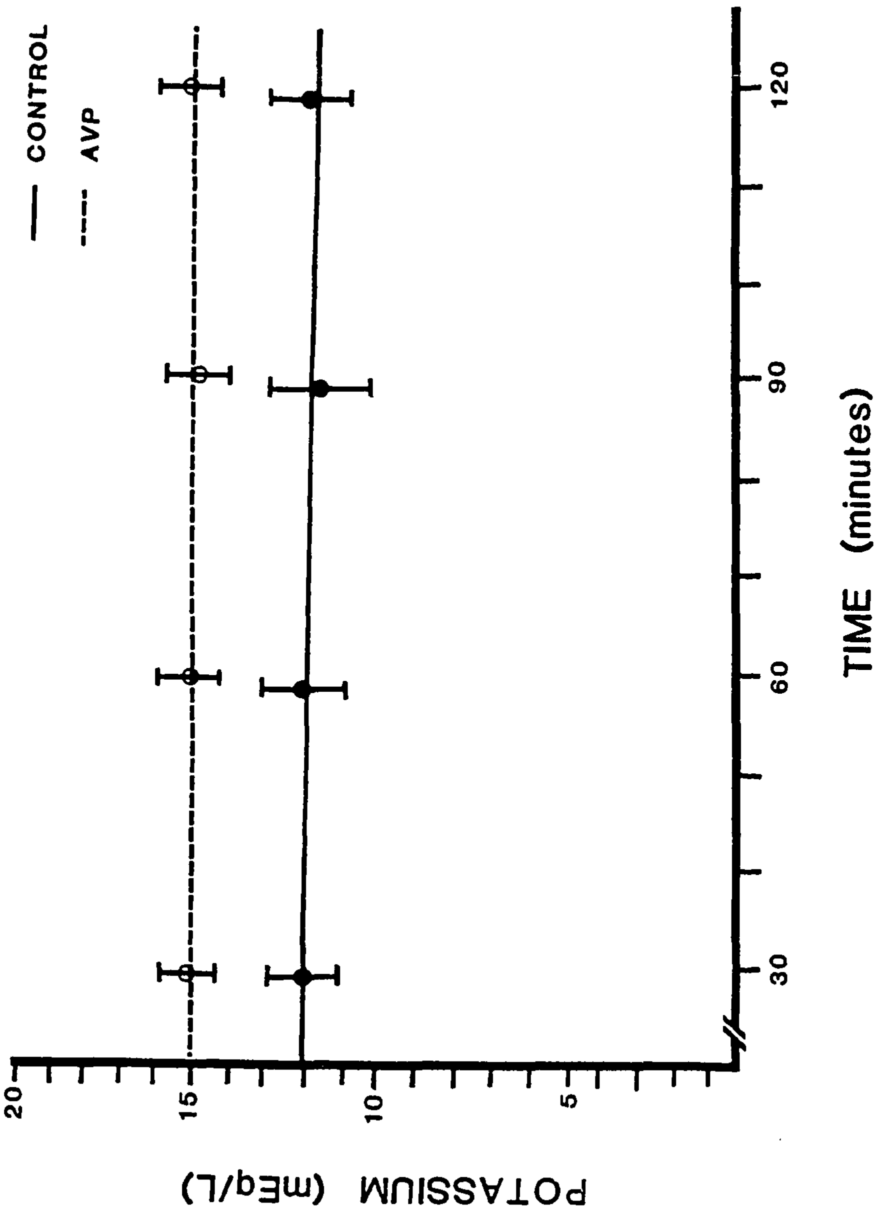
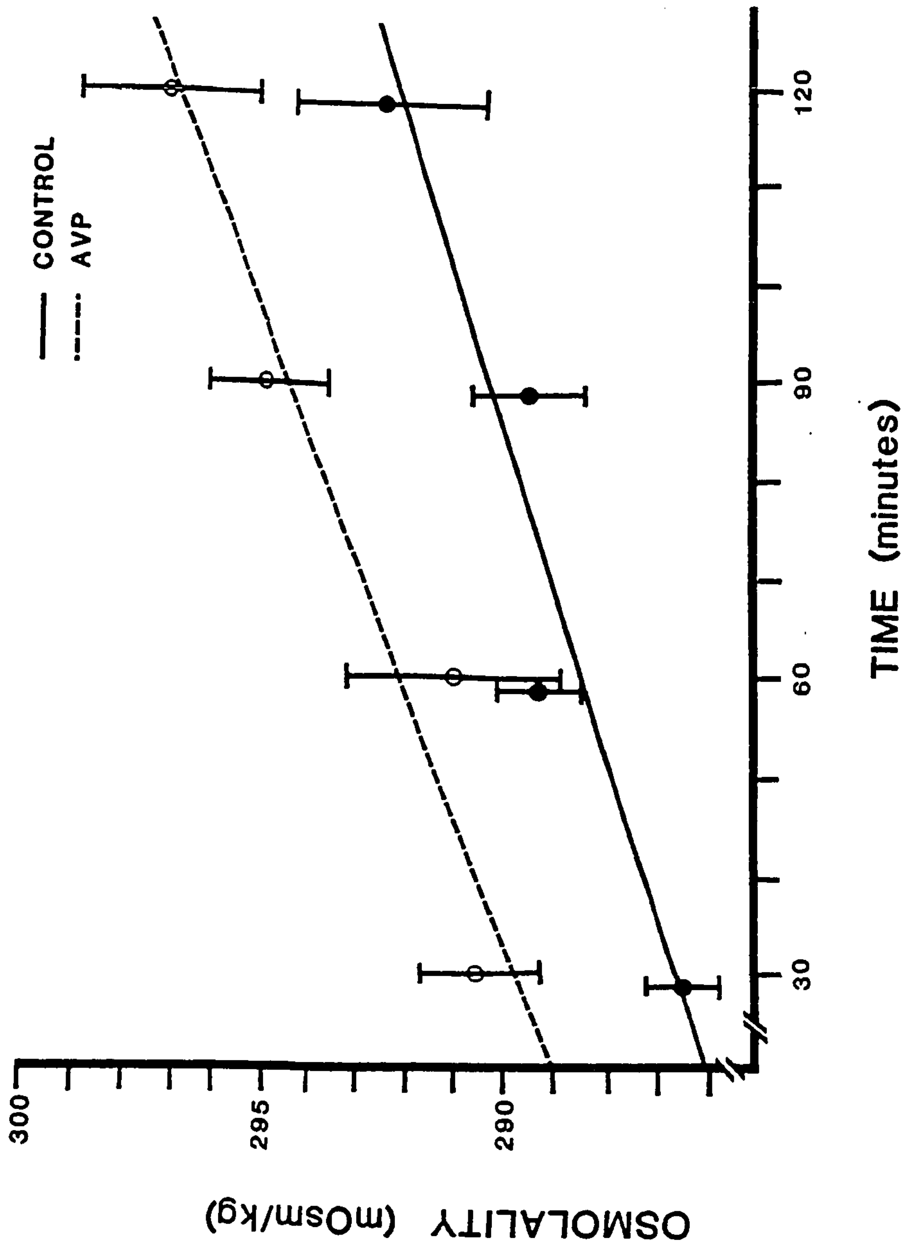

CONTROL
AVP
POTASSIUM (mEq/L)
20
15
10
5
TIME (minutes)
30
60
90
120

figure 19. Amniotic fluid osmolality 30 to 120 minutes after control (———) or 12.5 ng AVP (------) *in vivo* injection into the amniotic sac. Osmolality increased significantly following both control and AVP injections ($r=0.63$ and 0.56, respectively, $p<0.02$). There was no difference between the slopes of the two lines.



CONTROL
AVP
OSMOLALITY (mOsm/kg)
300
295
290
TIME (minutes)
30
60
90
120

CONCLUSIONS FOR PART I

The following is a summary of the findings of these initial studies:

1. Amniotic fluid osmolality did not change over gestation possibly because the decrease in sodium concentration over gestation is completely balanced by an increase in potassium concentration.

2. The guinea pig fetal kidney and/or bladder in the last week of gestation is functioning to alter urine composition.

3. Amniotic fluid osmolality and electrolyte levels are different from fetal urine and fetal and maternal plasma levels in a manner which indicates that the fetal urine presented to the amniotic sac is modified such that amniotic fluid is not simply a result of fetal urine equilibrating with fetal or maternal plasma.

4. Intact AVP is removed from the amniotic compartment.

5. AVP was not shown by *in vivo* studies to have an affect on amniotic fluid osmolality or electrolyte composition.

Hence, these initial studies suggested two possible directions for further work:

1. a study of the mechanisms underlying the regulation of amniotic fluid osmolality by:
    a. examining the movement of sodium, potassium and water across the amnionic membrane, and
    b. determining the relationship between amniotic fluid and fetal urine,

or

2. an examination of the role of the amniotic sac in the metabolism of AVP.

Since previous reports have been unsuccessful in identifying a metabolic site for fetal AVP clearance (see introduction), the major emphasis of this project was directed towards a study of AVP metabolism in the amniotic sac.

PART II. METABOLISM OF VASOPRESSIN IN THE AMNIOTIC SAC.

AVP-INULIN EXPERIMENTS

When AVP and inulin are injected into the amniotic sac *in vivo*, intact AVP concentration (measured by RIA with AS 96 which does not crossreact with metabolic products) divided by inulin concentration significantly decreased over time ($n = 6$; $p = 0.01$) (fig. 20). The average correlation coefficient for all six experiments was 0.97 (range: $r = 0.92$ to $r = 0.99$). This decrease in the AVP to inulin ratio indicates a swallowing-independent clearance of AVP from the amniotic sac.

The rate of AVP disappearance was dependent upon the initial concentration of the hormone (fig. 21). Regression analysis showed that the rate of AVP disappearance significantly increased with increasing initial concentration ($n = 6$; $p = 0.01$; $r = 0.92$). The same dose of 12.5 ng of AVP was injected into the amniotic compartment in all six experiments but the concentration of AVP per ml of amniotic fluid ranged between 697.8 pg/ml and 1900.2 pg/ml because of differences in fetal size and thus amniotic fluid volume. The rate of AVP disappearance for the six experiments varied between -2.13 and -8.77 $pg \cdot (mg\ inulin)^{-1} \cdot min^{-1}$.

Because of the dependence of rate of AVP disappearance upon hormone concentration, AVP disappearance over time should be a curvilinear relationship and not a linear one as it appears in figure 20. In fact, the relationship between AVP concentration, figure 20. AVP concentration in the amniotic fluid over two hours following *in vivo* injection of 12.5 ng of AVP into the amniotic sac. AVP concentration significantly decreased (n=6, mean $\pm$ S.E., r=0.92, p=0.01) over time. AVP concentration was measured by RIA with an antiserum (AS 96) which does not crossreact with any known AVP metabolites. AVP is represented as pg/mg inulin to correct for any disappearance due to swallowing.

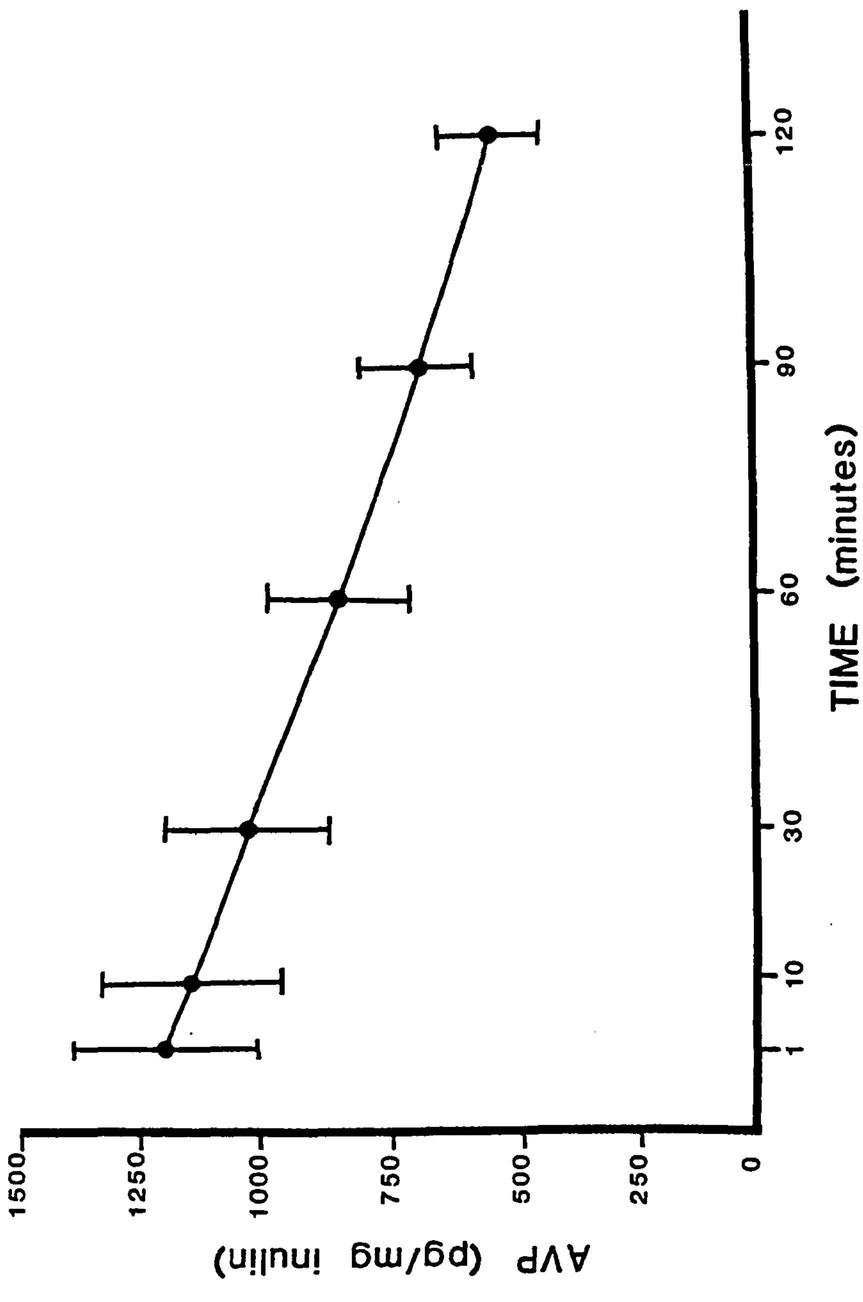
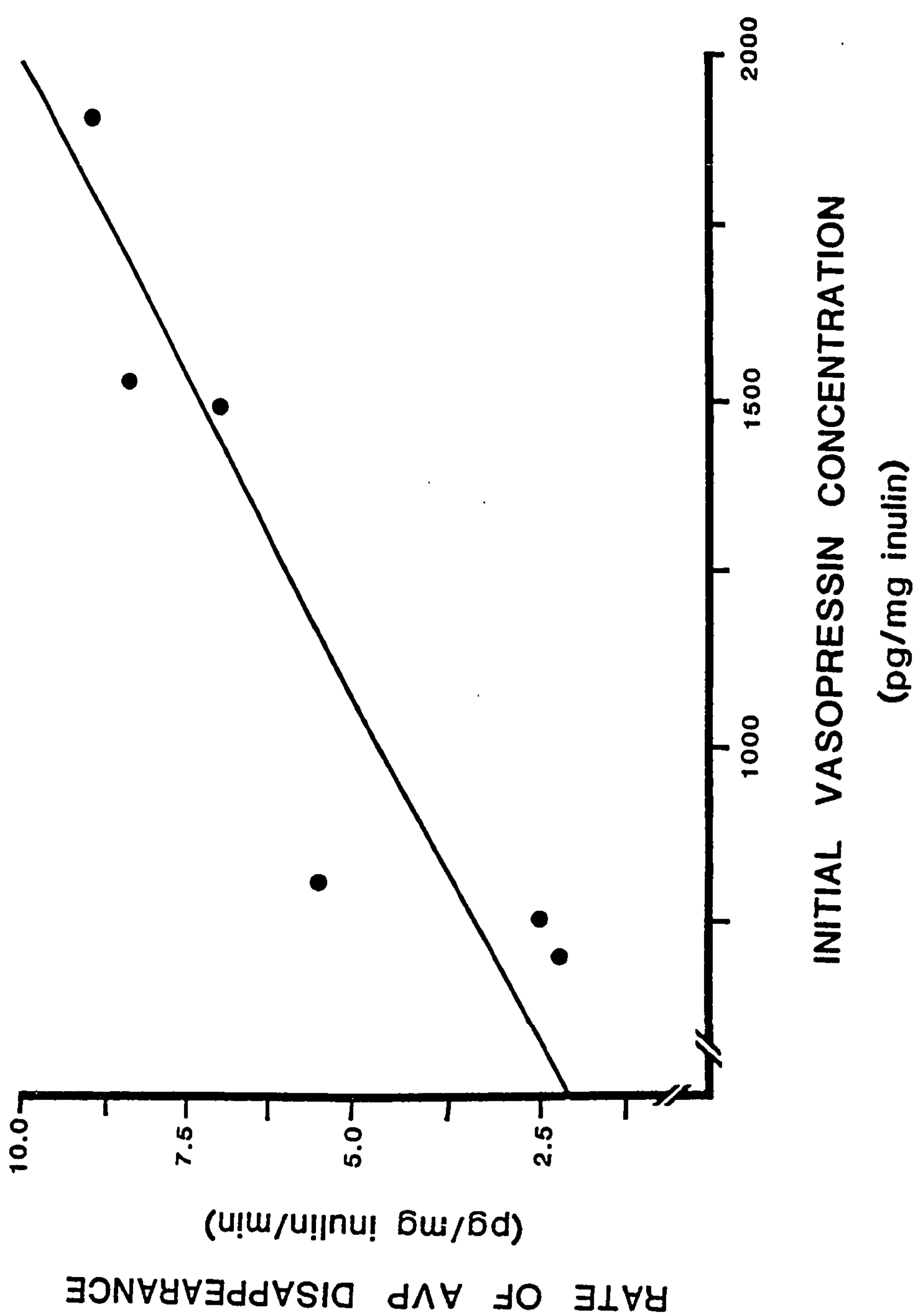

AVP (pg/mg inulin)
1500
1250
1000
750
500
250
0
1
10
30
60
90
120
TIME (minutes)

figure 21. The rate of AVP disappearance in the amniotic sac was dependent upon initial AVP concentration. The rate of AVP disappearance from the amniotic sac independent of swallowing significantly increased (n=6, r=0.92, p=0.01) with initial vasopressin concentration.


RATE OF AVP DISAPPEARANCE
(pg/mg inulin/min)
10.0
7.5
5.0
2.5
INITIAL VASOPRESSIN CONCENTRATION
(pg/mg inulin)
1000
1500
2000

expressed as the fraction of initial concentration (to correct for the variation in initial concentrations), and time (fig. 22) can be described by the equation

$$Y = 1.022 \cdot e^{(-0.000065)X}$$

(n=6, F=873.62, r=-0.998).

TRITIATED VASOPRESSIN EXPERIMENTS

The results of the ^{3}H-AVP experiments (n=5) indicate that AVP was not simply diffusing out of the amniotic compartment, but rather, was being metabolized since there was no change in radioactivity expressed per mg inulin over time (fig. 23).

The HPLC analysis of the samples collected over a 120 minute period after the ^{3}H-AVP injection demonstrated that AVP was broken down into at least two products (fig. 24). In addition to AVP which eluted at fraction 26 with a 15-30% CH_3CN gradient, two other products were isolated: one that eluted at fraction 11, and one that eluted at fraction 28. The product which eluted at fraction 11 was designated as M1, and the fraction at 28 as M2. All of the radioactivity injected into the sac was accounted for by these 3 peaks.

HPLC analysis of samples obtained at 10, 30, 60 and 120 minutes after addition of ^{3}H-AVP to the amniotic fluid in five experiments are shown in figure 25. Amounts of AVP, M1, and M2 are represented as the percent of total radioactivity detected in the HPLC fractions.

Vasopressin Metabolism. ^{3}H-AVP significantly decreased over time ($p = 0.01$), confirming the radioimmunoassay results (fig. 25). There figure 22. Amniotic fluid concentration of AVP _in vivo_, expressed as the fraction of initial concentration, vs. time. The dependence of the rate of AVP disappearance on concentration results in a curvilinear relationship between amniotic fluid concentration and time. The relationship between AVP concentration and time can be described by the equation $Y = 1.02 \cdot e^{(-0.000065)X}$ ($n=6$, $F=873.62$, $r=-0.998$).

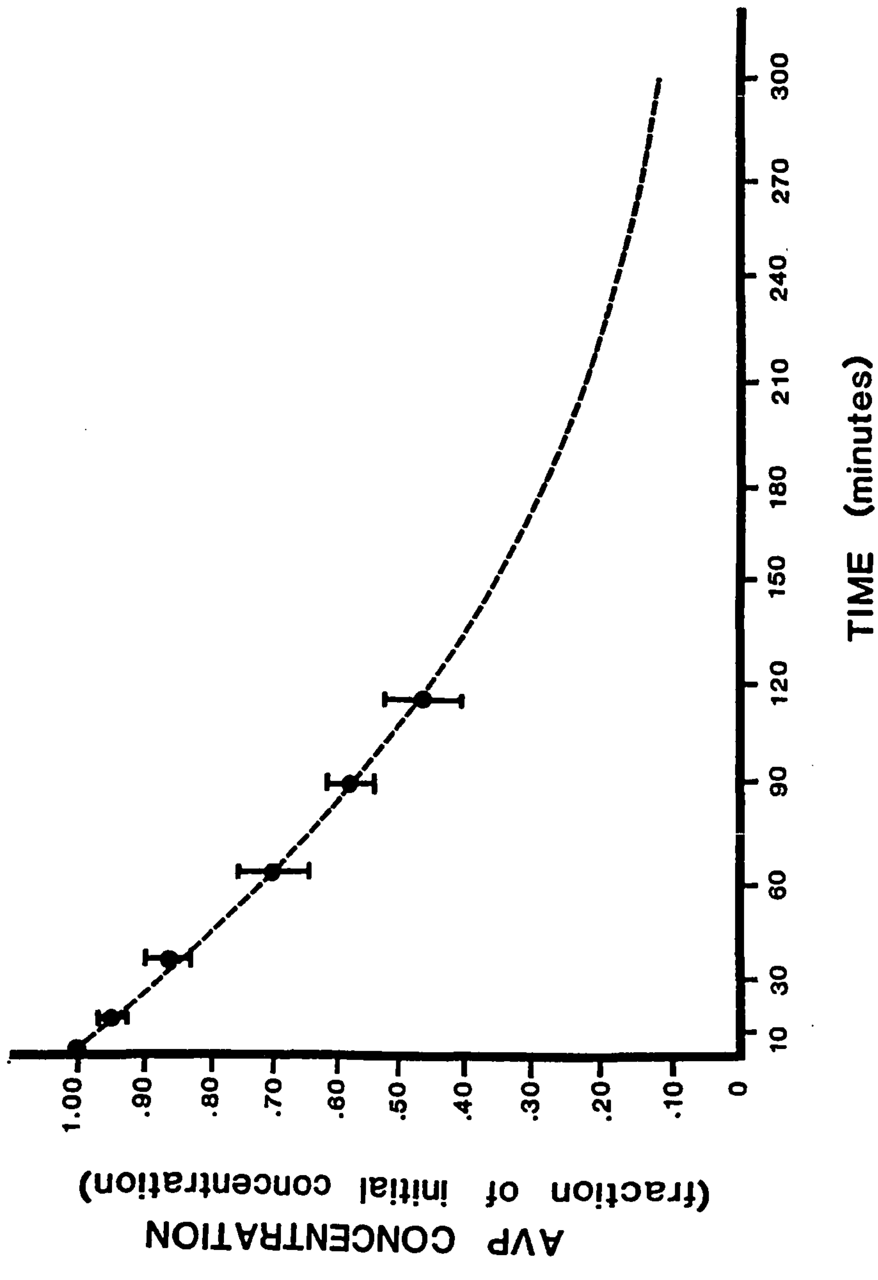
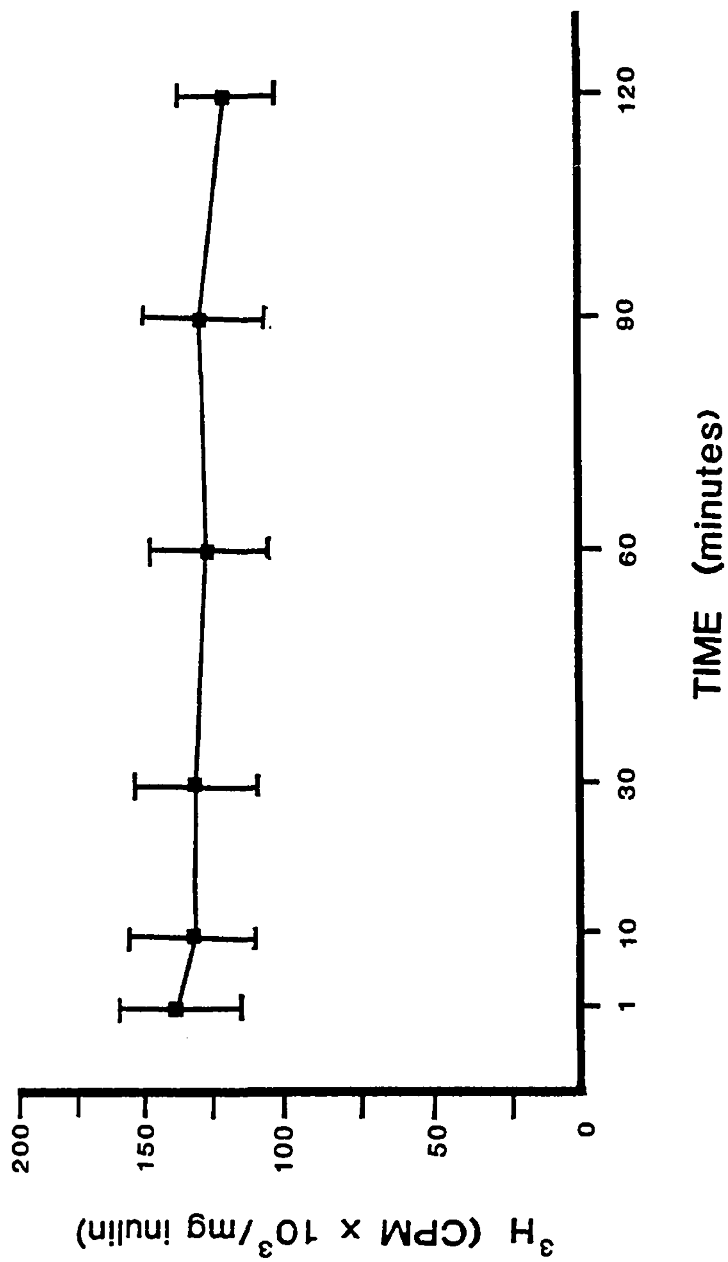
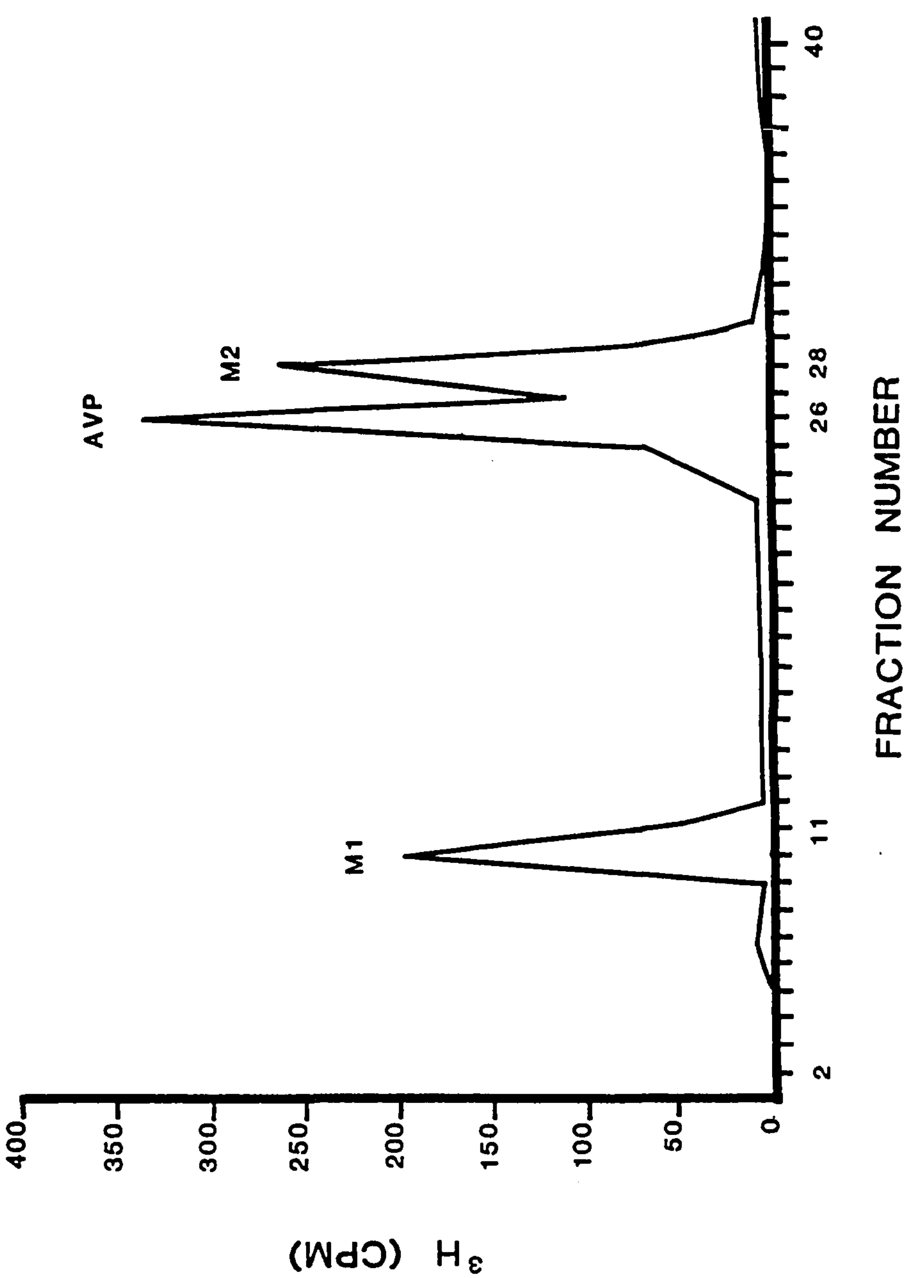

AVP CONCENTRATION
(fraction of initial concentration)
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
0
10
30
60
90
120
150
180
210
240
270
300
TIME (minutes)

figure 23. Tritium vs. time (n=5, mean $\pm$ S.E.). ^{3}H counts per minute per mg inulin did not change over the two hours after injection of ^{3}H-AVP into the amniotic sac *in vivo*, indicating that ^{3}H was not leaking out of the sac across the amnionic membrane.



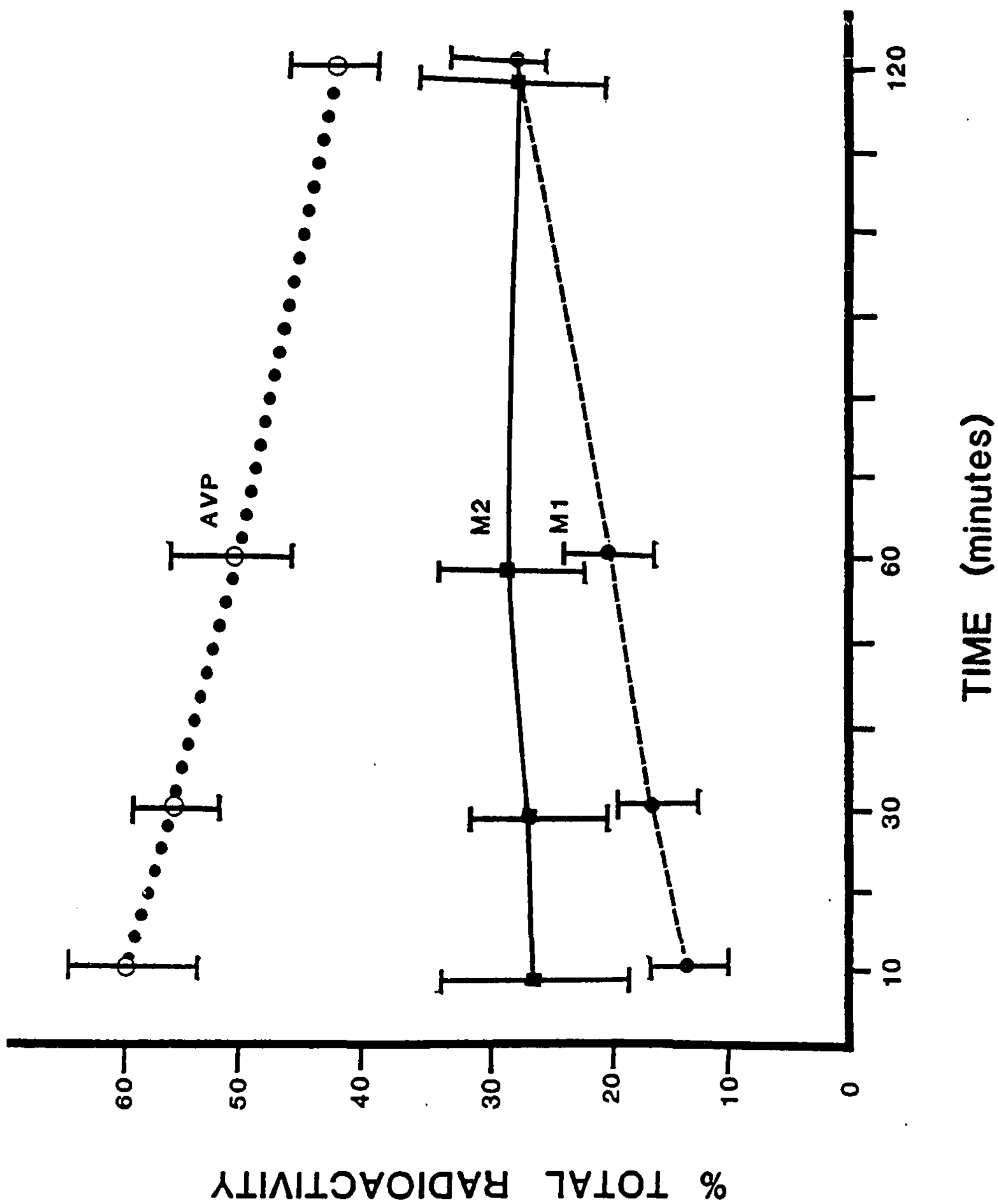
³H (CPM x 10³/mg inulin)
200
150
100
50
0
1
10
30
60
90
120
TIME (minutes)

figure 24. Representative HPLC elution profile of an amniotic fluid sample collected two hours after *in vivo* injection of ^{3}H-AVP. (Eluent: 15-30% CH_3CN in 0.1 % TFA over 20 minutes. Flow rate: 1.5 ml/min. Fractions: 750 µl/fraction.) Peaks are measured as ^{3}H counts per minute. A peak which co-migrated with standard AVP was isolated at fraction 26. Two other products were separated: one which eluted at fractions 10 & 11 (M1), and one which eluted at fraction 28 (M2).



³H (CPM)
400
350
300
250
200
150
100
50
0
2
11
26
28
40
FRACTION NUMBER
M1
AVP
M2

figure 25. AVP (●●●●●), M1(------), and M2(———), represented as % total radioactivity in amniotic fluid samples collected at 10, 30, 60, and 120 minutes after injection of ^{3}H-AVP into the amniotic sac in vivo (n=5, mean ± S.E.). AVP significantly decreased from 10 minutes to 120 minutes after injection (p<0.01). M1 produced significantly increased between 10 minutes and 120 minutes (p<0.001).



AVP
M2
M1
% TOTAL RADIOACTIVITY
0
10
20
30
40
50
60
10
30
60
120
TIME (minutes)

was a rapid disappearance of ^{3}H-AVP during the first 10 minutes after injection during which time about 40% was metabolized. This rapid phase was followed by a slower rate of metabolism where ^{3}H-AVP decreased from 59.82 $\pm$ 5.13 percent to 42.84 $\pm$ 3.5 percent of the total radioactivity.

M1 Production. M1 product significantly increased from 13.46 $\pm$ 2.03 % total radioactivity at 10 minutes to 28.78 $\pm$ 2.68 % total radioactivity at 120 minutes ($p<0.001$)(fig. 25). These results indicate that M1 was being produced faster than it was being removed from the compartment via swallowing.

M2 Production. M2 was produced rapidly following injection of ^{3}H-AVP since at 10 minutes 26.61 $\pm$ 7.07 percent of the total radioactivity was accounted for by M2 (fig. 25). In fact, the major portion of the rapid drop in ^{3}H-AVP in the first 10 minutes was attributable to M2 production. From 10 to 120 minutes the percent of total radioactivity accounted for by M2 did not change and averaged 27.64 $\pm$ 0.55 percent. M2, therefore, was being removed, by either swallowing or further metabolism to M1, at about the same rate as it was being produced.

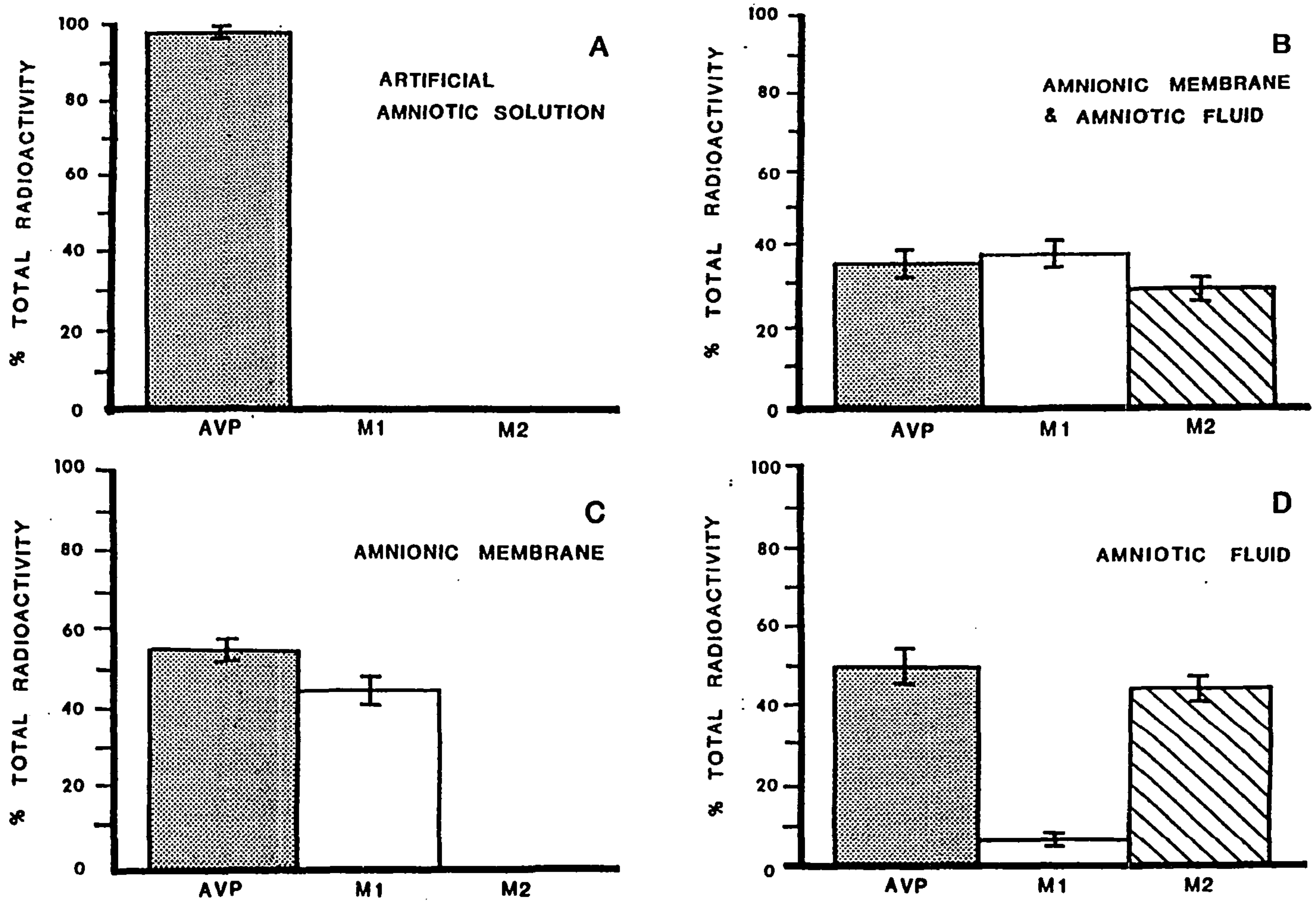
DETERMINATION OF THE SITE OF VASOPRESSIN METABOLISM

^{3}H-AVP incubated *in vitro* in artificial amniotic solution remained intact after 120 minutes (fig. 26a). However, when ^{3}H-AVP figure 26a. % total radioactivity represented by AVP, M1 and M2 after incubation of 1000 pg ^{3}H-AVP in 4 ml artificial amniotic solution for 2 hours at 37^{o}C. AVP remained intact and accounts for all of the radioactivity. There was no production of either M1 or M2. (n=6, mean $\pm$ S.E.)

figure 26b. % total radioactivity represented by AVP, M1 and M2 after incubation of 1000 pg ^{3}H-AVP with 38.7 cm^2 of amnionic membrane in 4 ml amniotic fluid for 2 hours at 37^o C. About 65% of AVP was metabolized into both M1 and M2 with M1 accounting for 37% and M2 accounting for 28% of the total radioactivity. (n=5, mean $\pm$ S.E.)

figure 26c. % total radioactivity represented by AVP, M1 and M2 after incubation of 1000 pg ^{3}H-AVP with 38.7 cm^2 of amnionic membrane in 4 ml artificial amniotic solution for 2 hours at 37^{o}C. M1 represented 45% of the total radioactivity. There was no M2 production. (n=6, mean $\pm$ S.E.)

figure 26d. % total radioactivity represented by AVP, M1 and M2 after incubation of 1000 pg ^{3}H-AVP with 4 ml amniotic fluid for 2 hours at 37^{o}C. M2 represented 44% of the total radioactivity. M1 production (6%) was slight. (n=4, mean $\pm$ S.E.)



A
ARTIFICIAL
AMNIOTIC SOLUTION
B
AMNIONIC MEMBRANE
& AMNIOTIC FLUID
C
AMNIONIC MEMBRANE
D
AMNIOTIC FLUID
% TOTAL RADIOACTIVITY
0
20
40
60
80
100
AVP
M1
M2

was incubated with both amnionic membrane and amniotic fluid, it was broken down into the two products M1 and M2 as was seen in the *in vivo* studies (fig. 26b).

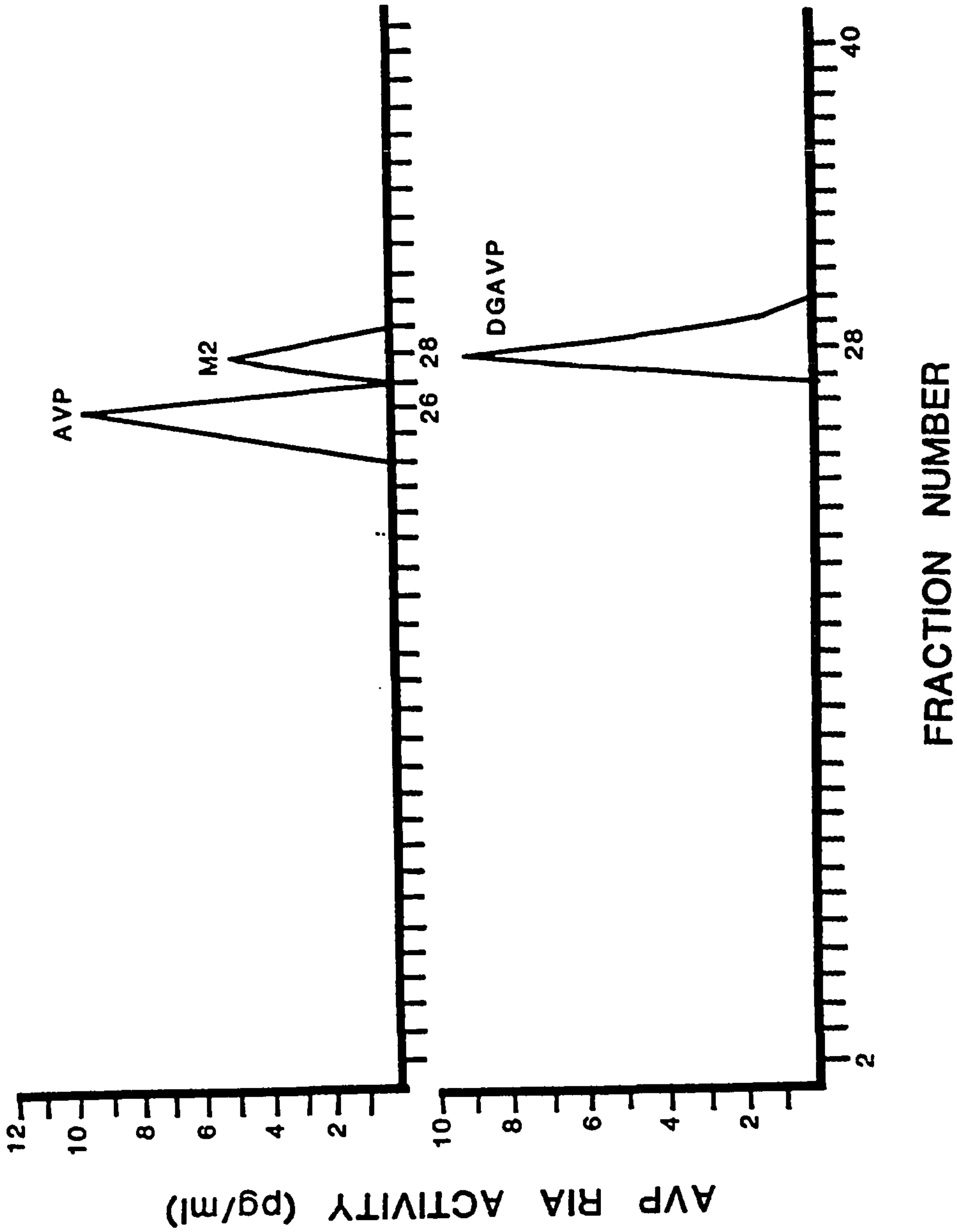
When ^{3}H-AVP was incubated with amnionic membrane alone in artificial solution, only M1 was produced (fig. 26c). ^{3}H-AVP incubated in amniotic fluid alone, produced M2 with slight production of M1 (fig. 26d).

These results indicate that M1 production was primarily membrane dependent and M2 production was fluid dependent. Also, the presence of either metabolite does not seem to be necessary for the production of the other, as M1 can be produced with no M2 present, and M2 can be produced with little or no M1 present.

PARTIAL CHARACTERIZATION OF M2 AND A TRYPSIN-LIKE ENZYME

Several experiments suggest that the M2 metabolite is dgAVP. First, M2 produced *in vivo* can be detected by radioimmunoassay with an AVP antiserum which cross-reacts about 60% with dgAVP (fig. 27). Also, M2 co-migrated on HPLC with dgAVP in two other buffer systems in addition to the 15-30% CH_3CN gradient system seen in figure 27. In 18% CH_3CN, M2 co-migrated with dgAVP at fraction 18 (fig. 28). In 39% CH_3OH, both M2 and dgAVP eluted at fractions 10 and 11 (fig. 29).

Furthermore, if the M2 product is indeed dgAVP, the enzyme responsible for its production is most likely trypsin or a trypsin-like enzyme (see introduction). Studies utilizing aprotinin demonstrated that M2 production can be completely blocked when this figure 27. HPLC elution profiles of amniotic fluid 10 minutes after ^{3}H-AVP injection *in vivo* and standard dgAVP. (Eluent: 15 - 30% CH_3CN in 0.1 % TFA over 20 minutes. Flow rate: 1.5 ml/min. Fractions: 750 μl/fraction.) Peaks were measured by RIA with AVP AS 95 (60% crossreactive with dgAVP) and represented in pg/ml. Both M2 and standard dgAVP co-migrated at fraction 28 and were immunoreactive with AS 95.



AVP
M2
DGAVP
26
28
2
40
FRACTION NUMBER
AVP RIA ACTIVITY (pg/ml)
12
10
8
6
4
2

figure 28. HPLC elution profiles of standard dgAVP and AVP (bottom) and of a sample collected after incubation of 1000 pg ^{3}H-AVP with 25.8 cm^2 of amnionic membrane and 4 ml amniotic fluid _in vitro_ (top) in 18% CH_3CN (Eluent: 18% CH_3CN in 0.01 M CH_3COONH_4 pH 4.0). AVP eluted at fractions 18 & 19. M2 and dgAVP eluted at fraction 13. M1 eluted at fraction 5. No other peaks besides AVP, M1 and M2 were detected in the amnionic membrane-amniotic fluid sample.

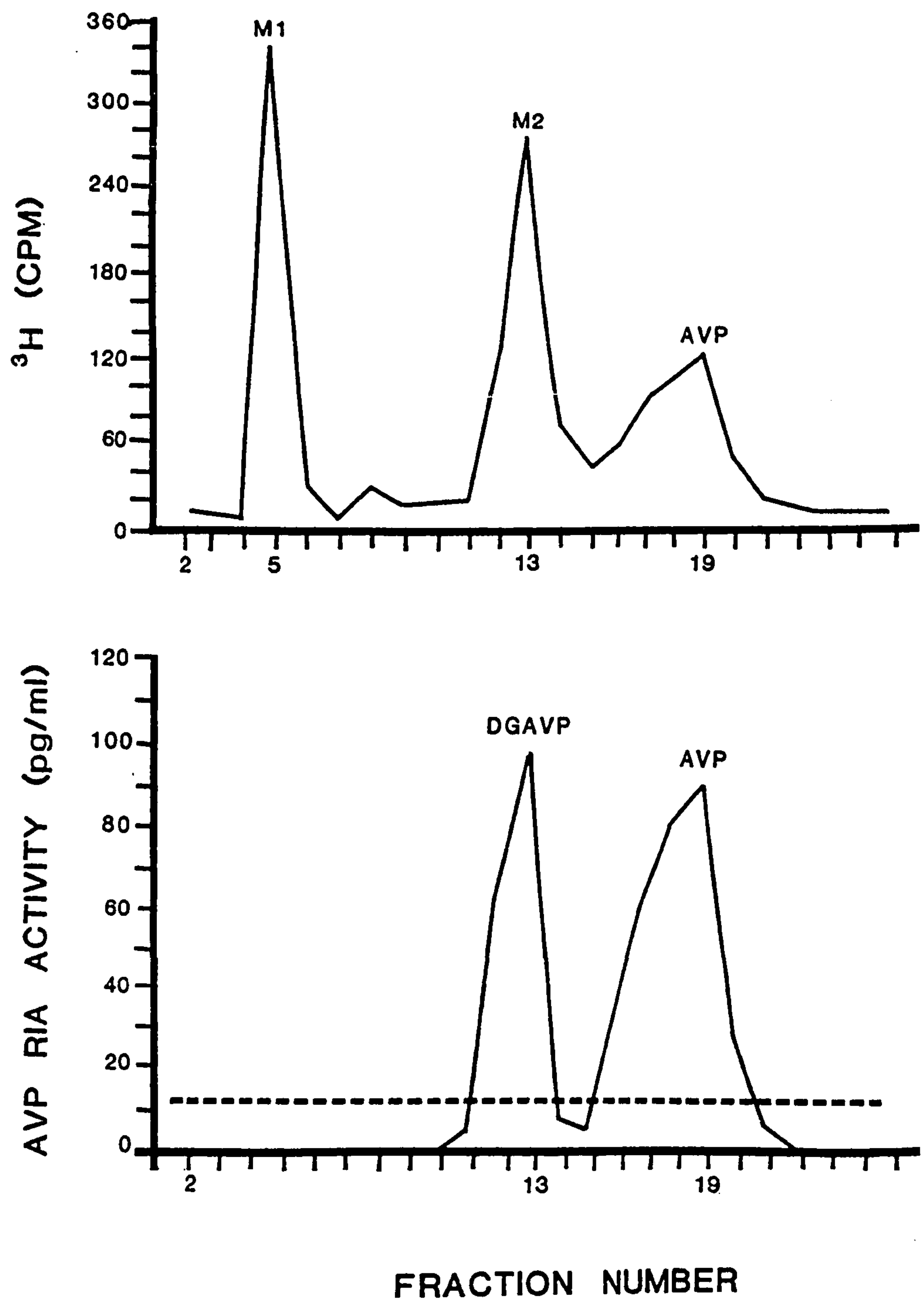
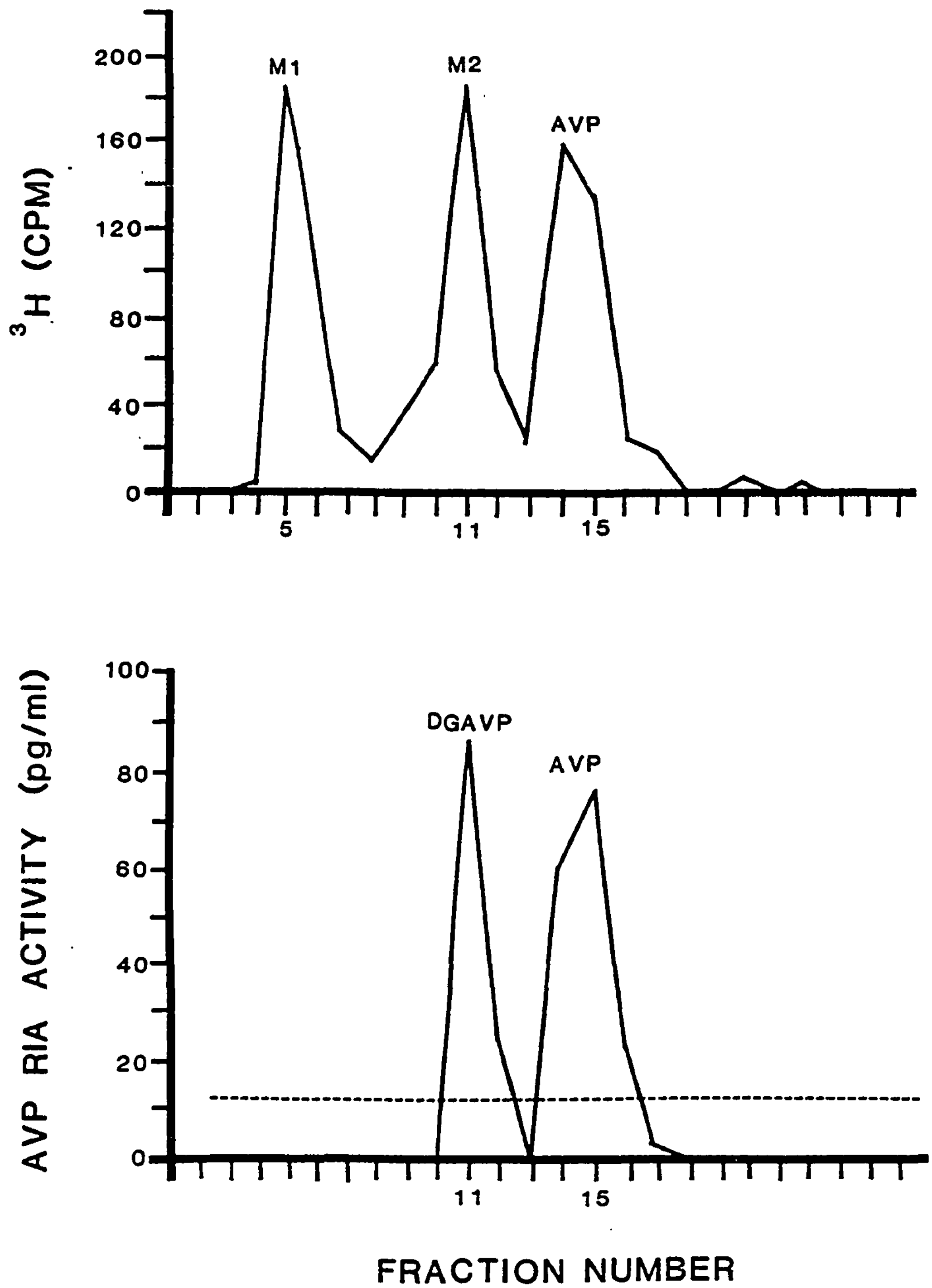

M1
M2
AVP
^3H (CPM)
360
300
240
180
120
60
0
2
5
13
19
DGAVP
AVP
AVP RIA ACTIVITY (pg/ml)
120
100
80
60
40
20
0
2
13
19
FRACTION NUMBER

figure 29. HPLC elution profiles of standard dgAVP and AVP (bottom) and of sample collected after incubation of 1000 pg ^{3}H-AVP with 25.8 cm^2 of amnionic membrane and 4 ml amniotic fluid in vitro (top) in 39% CH_3OH (Eluent: 39% CH_3OH in 0.05 M CH_3COONH_4 pH 6.5). AVP eluted at fractions 14 & 15. M2 and dgAVP eluted at fraction 11. M1 eluted at fraction 6. No other peaks besides AVP, M1 and M2 were detected in the amnionic membrane-amniotic fluid sample.



M1
M2
AVP
200
160
120
80
40
0
³H (CPM)
5
11
15
DGAVP
AVP
100
80
60
40
20
0
AVP RIA ACTIVITY (pg/ml)
11
15
FRACTION NUMBER

trypsin inhibitor is incubated along with AVP in amniotic fluid *in vitro* (fig. 30).

DEVELOPMENT OF VASOPRESSIN METABOLISM IN THE AMNIOTIC COMPARTMENT OVER GESTATION

Data from the initial baseline studies (Part I) suggested that the amniotic sac metabolic systems may be functioning early in gestation since as early as 37 days of gestation amniotic fluid AVP levels were low (under 4.0 pg/ml). Therefore, a series of *in vitro* experiments were conducted to determine: 1. if the metabolic systems were functioning early in gestation, 2. whether the time of development of the membrane and fluid systems differed, and 3. if the capacity of the membrane and fluid systems increased over gestation.

Amnionic Membrane-Dependnent AVP Metabolism. The average amnionic membrane weights and protein content per square centimeter of membrane at various gestational ages can be seen in Table 2. M1 production (fig. 31) is represented as fmoles produced per minute per square inch of membrane. The molar conversion is made with the assumption that the tritium label on the phenylalanine is not altered such that the CPM per mole does not change as ^{3}H-AVP is metabolized into M1 or M2. M1 production is also represented per mg protein content or per mg dry weight in Table 2 for comparison. M1 production expressed per membrane area was chosen for analysis since it seemed logical that M1 production would be more dependent upon surface area, and thus the number of AVP receptors available, rather than the weight figure 30. HPLC profiles of ^3H-AVP incubated in artificial amniotic solution (A), amniotic fluid (B), and amniotic fluid + aprotinin (C). (Eluent: 39% CH_3OH in 0.05 M CH_3COONH_4 pH 6.5). Peaks were measured in ^3H counts per minute. AVP eluted at fraction 16 and M2 eluted at fraction 11. AVP remained intact in artificial amniotic solution. AVP was metabolized to M2 with slight production of M1 (fraction 6) in amniotic fluid. Aprotinin prevented the formation of M2 (but not M1) in the amniotic fluid and AVP remained intact.

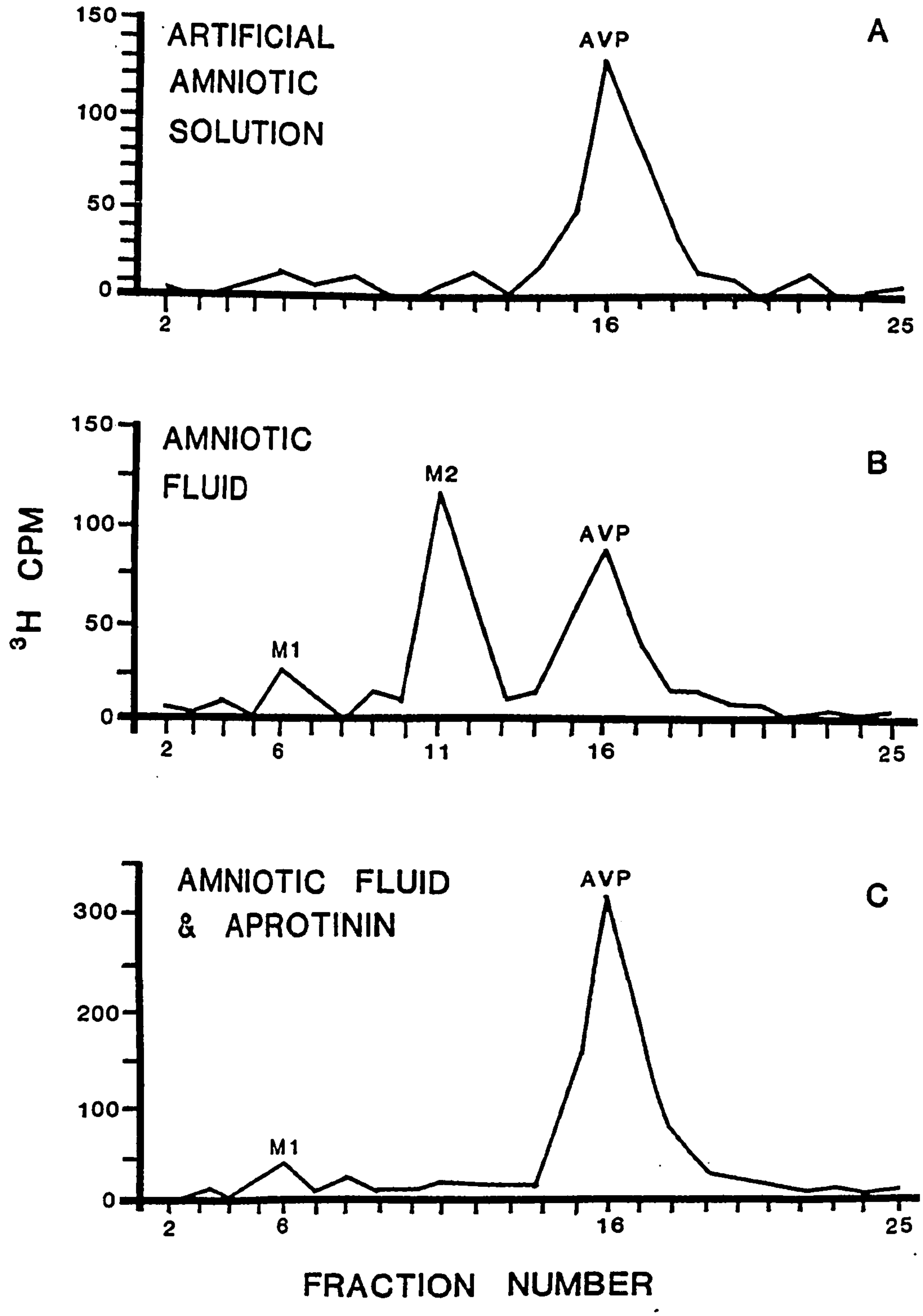
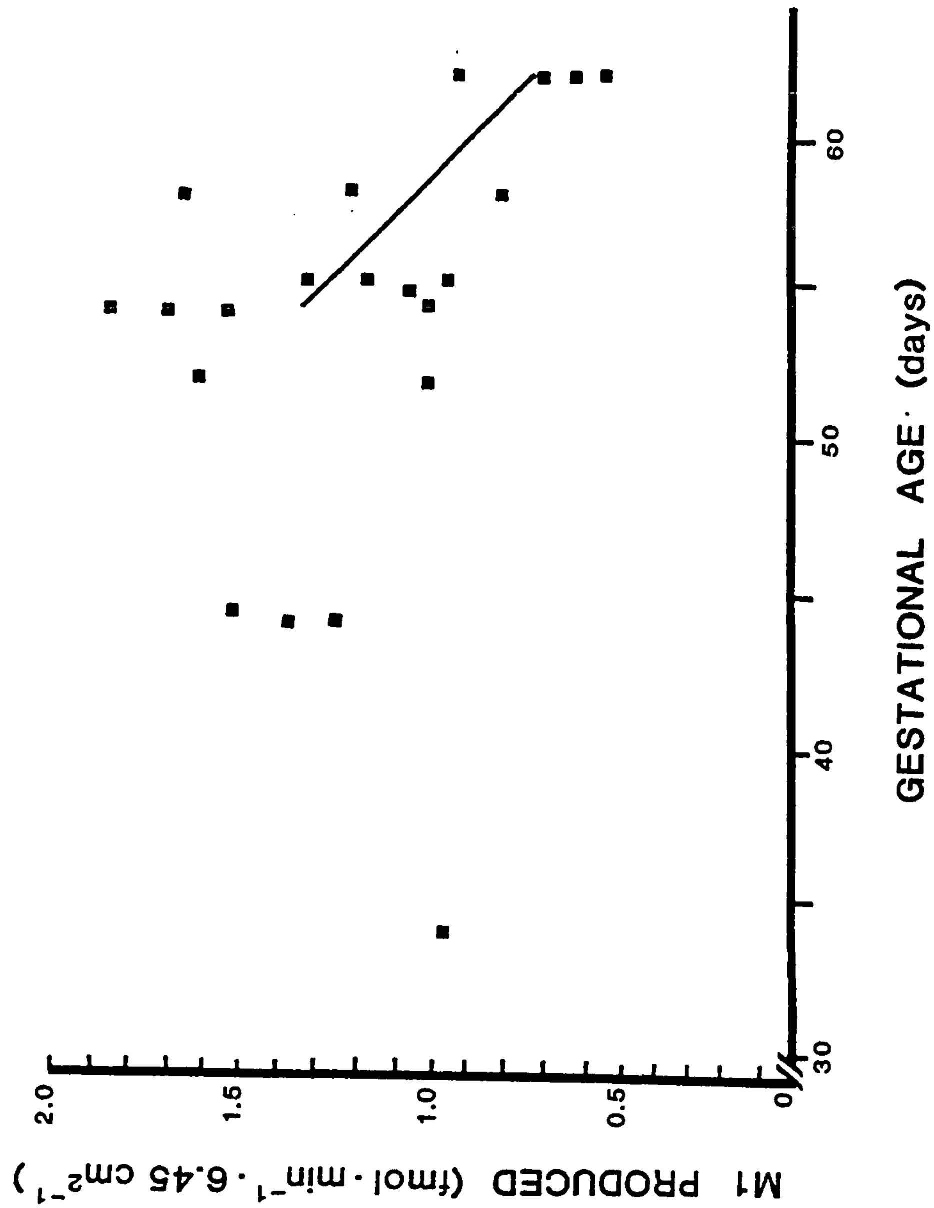

ARTIFICIAL AMNIOTIC SOLUTION
AVP
A
150
100
50
0
2
16
25
AMNIOTIC FLUID
M2
AVP
M1
B
^{3}H CPM
150
100
50
0
2
6
11
16
25
AMNIOTIC FLUID & APROTININ
AVP
M1
C
300
200
100
0
2
6
16
25
FRACTION NUMBER

Table 2

Amnionic Membrane Wet and Dry Weights and Protein Content

GESTATIONAL AGE (days)	WET WEIGHT (mg/cm^2)	DRY WEIGHT (mg/cm^2)	PROTEIN ($\mu g/cm^2$)	M1 PRODUCED (fmol/min) (per 6.45 cm^2)	(per mg dry wt)	(per mg protein)
39	1.869	0.264	813	0.958	0.562	0.183
44	3.240	0.288	1006	1.259	0.678	0.194
44	1.680	0.195	616	1.543	1.227	0.388
44	2.730	0.254	537	1.381	0.843	0.399
52	3.330	0.328	692	1.612	0.762	0.361
52	1.400	0.179	339	1.040	0.901	0.476
54	6.468	0.434	1153	1.856	0.663	0.250
54	5.974	0.396	969	1.021	0.400	0.163
54	6.550	0.492	1150	1.696	0.534	0.229
54	6.892	0.596	1022	1.549	0.403	0.235
55	9.400	0.522	1270	0.525	0.156	0.064
55	9.402	0.475	1211	1.314	0.429	0.168
55	8.678	0.456	1309	0.958	0.326	0.113
55	6.626	0.470	1242	1.169	0.386	0.146
58	8.366	0.670	1360	1.219	0.282	0.139
58	6.864	0.460	988	1.656	0.558	0.260
58	5.714	0.414	900	0.828	0.310	0.143
58	7.287	0.462	1065	1.234	0.414	0.180
62	10.729	0.594	917	0.944	0.246	0.160
62	10.415	0.584	764	0.572	0.152	0.116
62	13.506	0.526	902	0.618	0.182	0.106
62	8.904	0.360	663	0.715	0.308	0.167
MEAN	6.637	0.428	949	1.167	0.487	0.211
S.D.	3.163	0.131	263	0.377	0.265	0.104
N	22	22	22	22	22	22
S.E.	0.674	0.028	56	0.080	0.056	0.022

figure 31. Development of amnionic membrane-dependent metabolism over gestation. AVP was metabolized by the amnionic membrane to M1 as early as 34 days of gestation. M1 production was represented as fmol$\cdot$min$^{-1}\cdot(6.45\ cm^2)^{-1}$ membrane. From 54 to 62 days of gestation, M1 production slightly decreased ($r=-0.57$, $b=-0.07$, $p<0.02$).


2.0
1.5
1.0
0.5
0
M1 PRODUCED (fmol · min^{-1} · 6.45 cm$^{2^{-1}}$)
30
40
50
60
GESTATIONAL AGE (days)

or thickness of the membrane. M1 production appeared as early as 34 days and was present for the rest of gestation (fig. 31). The average rate of ^{3}H-AVP metabolized to M1 per 6.45 cm^2 (1 square inch) of membrane was 1.17 $\pm$ 0.08 fmol/min.

The amount of M1 produced significantly decreased when M2 production appeared after 52 days of gestation (see below) ($r=-0.57$, $p=0.02$). The importance of this decrease is difficult to determine since even if the production per area of membrane is less, there is more membrane area as the fetus gets older, and so the total capacity of the membrane metabolic system would not decrease. However, it is interesting to note that the M1 production rate does not decrease until after the fluid system is active.

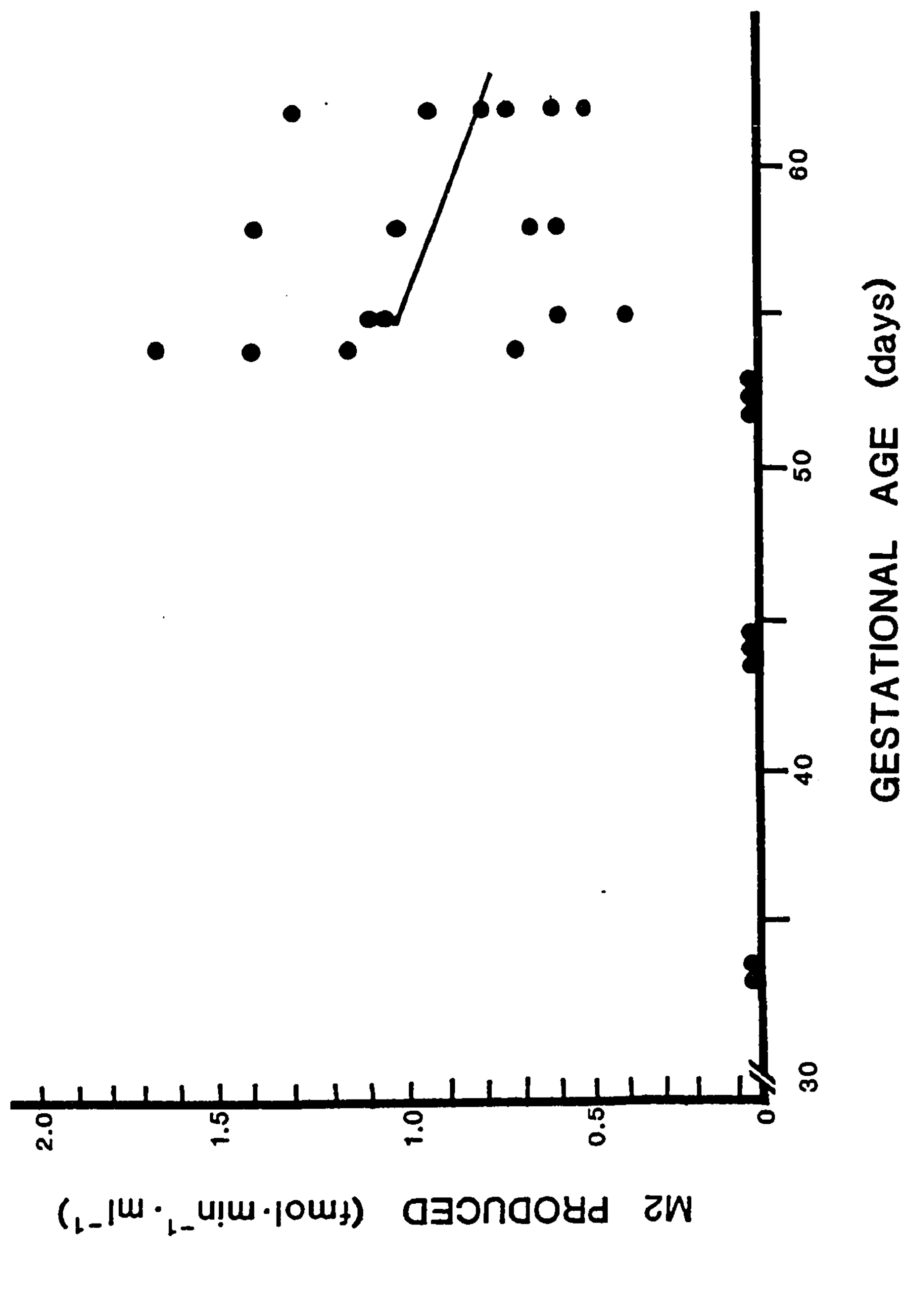
There is no M2 produced by the amnionic membrane at any gestational age.

Amniotic Fluid-Dependent AVP Metabolism. No M2 is produced in the amniotic fluid until after day 52 of gestation (fig. 32). From 54 to 62 days of gestation the average rate of ^{3}H-AVP metabolized to M2 per ml of amniotic fluid was 0.90 $\pm$ 0.08 fmol/min.

The rate of metabolite production by the amniotic fluid system, like the membrane system, also decreased after 54 days of gestation ($r=-0.51$, $p<0.05$). Again, the importance of this is questionable since the total volume of amniotic fluid increases until the end of gestation, so the capacity of the amniotic fluid metabolic system may not decrease.

M1 production in the amniotic fluid at any age was negligible.

figure 32. Development of amniotic fluid-dependent metabolism over gestation. AVP was not metabolized in the amniotic fluid until after 52 days of gestation. M2 production is represented as $fmol{\cdot}min^{-1}{\cdot}ml^{-1}$ fluid. From 54 to 65 days of gestation M2 production decreased ($r=-0.51$, $b=-4.36$, $p<0.05$). M1 production was negligible.


M2 PRODUCED (fmol·min⁻¹·ml⁻¹)
2.0
1.5
1.0
0.5
0
30
40
50
60
GESTATIONAL AGE (days)

CONCLUSIONS FOR PART II

The following is a summary of the findings of the studies on the metabolism of AVP in the guinea pig amniotic sac:

1. AVP is cleared from the amniotic sac.

2. This clearance is due to the metabolism of AVP into two products, M1 and M2.

3. M1 is membrane-dependent. M2 is fluid-dependent. Neither metabolite is necessary for the production of the other.

4. M2 crossreacts with an AVP antiserum that detects dgAVP. M2 co-migrates with dgAVP on HPLC in three different buffer systems.

5. The fluid component responsible for the production of M2 is a trypsin-like enzyme.

6. The membrane-dependent metabolic system develops as early as 34 days of gestation (term = 68 days). The fluid-dependent system does not appear until the last two weeks of gestation.

CHAPTER IV

DISCUSSION

OSMOTIC REGULATION IN THE FETUS

The present amniotic fluid baseline study showed that the fetus in the third trimester is able to modify filtered plasma by the reabsorption of water and sodium in either the fetal kidney or bladder. From these studies, it was also evident that the fetal urine was modified within the amniotic sac resulting in the formation of amniotic fluid which was hypotonic to fetal and maternal plasma. Previous studies with fetal sheep have demonstrated that fetal osmoregulation is independent of maternal osmoregulation (Leake et al., 1979; Leake et al., 1983). It is possible that the fetus is able to independently regulate its plasma osmolality via these fluid modifications. Although the regulation of fetal plasma osmolality was not the focus of this study, the initial baseline analyses unveiled some interesting observations that may contribute to a better understanding of osmotic regulation in the fetal guinea pig.

URINE OSMOLALITY IN THE GUINEA PIG FETUS

The fetal guinea pig develops to a fairly advanced state of maturation *in utero*; it is possible for the newborn guinea pig to survive on dry food and be weaned on the day of birth (Wagner and

Manning, 1976). Unlike most other fetal animals, the guinea pig is able to form a hypertonic urine in the last few weeks of gestation (Kleinman, 1970). The fetal guinea pig urine measured in this study was found to be hypertonic to fetal plasma, confirming findings by others (Boylan et al., 1958; Merlet-Benichou and deRouffignac, 1977). Few studies have investigated the ionic constituents of guinea pig fetal urine. In the single study found for comparison of guinea pig fetal urine osmolality, sodium and potassium concentrations, similar patterns were observed (Merlet-Benichou and deRouffignac, 1977). The fetal urine was hyperosmotic to fetal plasma, indicating a renal tubular or bladder reabsorption of water. Urinary sodium concentration was lower and potassium concentration was higher than the plasma concentrations, which indicate that sodium is reabsorbed in the kidney or bladder, and potassium is concentrated as a result of water reabsorption. Although the fetal urine ionic patterns in the present study were qualitatively similar to the patterns of this other study, sodium and potassium concentrations were lower than those reported in that study. This is probably due to the different procedures used in obtaining the fetal urine samples. Vasopressin may have been elevated in their study due to a longer period of anesthesia and stress during fetal arterial and venous cannulation before the collection of urine during cannulation of the bladder. The sodium and potassium levels were perhaps elevated due to an increase in AVP causing a pressure diuresis which inturn would tend to increase the renal excretion of sodium and bring urine osmolality closer to plasma osmolality. In the present study, fetal urine was collected under conditions of less stress within 30 minutes of anesthesia.

The present study and that of Merlet-Benichou and de Rouffignac (1977) are generally in agreement also in that there is a large variation of both sodium and potassium concentrations despite a comparatively narrow range of osmolalities. In fact, in the present study, neither sodium nor potassium concentration correlated with urine osmolality. Similarly, Boylan et al. (1958) found that chloride concentrations of guinea pig fetal bladder urine were highly variable and no relationship between the urine osmolality and the concentration of this anion was evident. If sodium and potassium are present in the urine mainly as chloride salts, both the low sodium and potassium values found in this study and the levels measured by Merlet-Benichou and de Rouffignac are in agreement with the variable chloride concentrations, and are not unrealistic.

These ionic concentrations and osmolality determinations are different than those observed in the chronically cannulated sheep fetus. In such preparations, fetal urine osmolality, and sodium and potassium concentrations were about 188 $\pm$ 31 mOsm/kg, 56.9 $\pm$ 8.6 mEq/l and 16.7 $\pm$7.9 mEq/l, respectively. The differences in the guinea pig and sheep values may be due to species differences, but at least part of the difference is due to the collection of fetal sheep urine after catheterization of the bladder. If urine is collected via catheterization and the bladder is not allowed to fill, then the urine formed in the kidney will not be influenced by the actions of the fetal bladder on water and solute reabsorption. It has been demonstrated that the guinea pig fetal bladder may be involved with the reabsorption of water since the guinea pig fetal bladder urine is hypertonic to newly formed urine in the kidney (Boylan et al., 1958) and it is possible that the urine of the fetal sheep may be modified in the bladder also. Also, sodium movement across the bladder wall seems to be influenced by AVP (France et al., 1972). Furthermore, Boylan et al. (1958) found that urine in the bladder has a low urea/creatinine concentration ratio compared to kidney urine which they attributed to slow bladder emptying of urine and a possible bladder permeability to urea with low permeability to creatinine.

In common with all the reported fetal guinea pig urine measurements is a discrepancy between urine sodium and potassium concentrations and osmolality. These results indicate that some other undetermined osmotically active substance(s) other than sodium and potassium are primarily responsible for fetal guinea pig urine osmolality. Subsequent to this study, fetal urine urea, creatinine and glucose levels were measured in our laboratory, but none of these solutes could explain the unaccounted for osmotic activity in the urine. A similar pattern of high osmolality with low sodium concentration has been reported (Tomoda et al., 1987) in the amniotic fluid of dead sheep fetuses. The authors were also unable to identify a substance which could account for much of the amniotic fluid osmolality. Whether or not the mechanisms and solutes responsible for the majority of the osmolality in these two situations are the same is uncertain.

CHANGES IN AMNIOTIC FLUID COMPOSITION OVER GESTATION

Amniotic fluid sodium concentration was found to decrease, potassium concentration increased, and osmolality did not change over gestation. These patterns over gestation are somewhat different than those seen in humans where potassium concentration did not change and osmolality decreased (Bonses, 1972; Lind et al., 1969). The amniotic fluid osmolality in this study did not change over gestation due to the increase in potassium concentration matching the decrease in sodium concentration. Since the major source of amniotic fluid is fetal urine, this difference may be a reflection of the fact that the guinea pig fetal kidney is more mature, and with the reabsorption of water from the fetal bladder, the fetal guinea pig is able to form a concentrated urine with a corresponding increase in potassium concentration. It would follow, therefore, that since the human fetus does not form a concentrated urine, potassium concentration would remain constant throughout gestation. The decrease in sodium concentration over gestation in both human and guineq pig amniotic fluid may reflect the increased ability of the fetus to reabsorb sodium as it develops.

THE MAINTENANCE OF AMNIOTIC FLUID OSMOLALITY

The initial baseline results also indicate that amniotic fluid osmolality is maintained within a narrow range. The necessity of a stable osmotic environment for the fetus is illustrated by the use of intra-amniotic injection of hypertonic solution in the clinical interruption of pregnancy (Wagner, 1966). When amniotic fluid osmolality is drastically changed, the fetus is aborted. Studies investigating amniotic fluid dynamics have demonstrated that amniotic fluid solute concentration is maintained in the face of experimental manipulations. When 25 ml of amniotic fluid in the rhesus monkey is replaced with distilled water, the resulting decreased amniotic fluid osmolality returns to normal in about 18 hours (Schruefer et al., 1972). There is also evidence of specific ionic regulation. For instance, when 20 to 30% of amniotic fluid in fetal sheep is replaced with isotonic solutions of saline mannitol or dextrose, sodium potassium and chloride concentrations return to normal in 3 to 6 hours (Lingwood et al., 1980) This represents a shorter interval than the amniotic fluid turnover time which is about 24 hours in the sheep (Tomoda et al., 1985) and suggests a membrane transport function. These studies support the concept that, in addition to the replacement of swallowed fluid with the addition of urine, water or solute movement across the amnion also plays a role in the maintenance of amniotic fluid osmolality.

Perhaps one of the most interesting observations from the initial baseline study in the present experiments, was that not only is amniotic fluid osmolality maintained in a narrow range, but also the amniotic fluid was kept slightly hypotonic to maternal and fetal plasma, confirming findings by others (Ross et al., 1985; Tomoda et al., 1987). The mechanism by which this hypotonicity is maintained has not been identified. Amniotic fluid was always hypotonic to fetal urine despite what seemed to be an influx of sodium into the amniotic sac since sodium increased between the urine and amniotic fluid by about 115 mEq/l. The efflux of some other substance must have accompanied this influx of sodium in order to not only keep amniotic fluid osmolality from increasing, but resulting in a net decrease. Thus, it appears that another osmotically active substance is leaving or a large amount of free water is entering the amniotic sac at an even faster rate than sodium and potassium are entering the sac. It is unlikely that water enters the sac against a hydrostatic gradient. Therefore, the hypotonicity is most likely a result of relative impermeability of the amnion to water compared to some other, as yet unidentified, osmotic solute that is leaving the sac. Urea is at least partially accountable for this, but the sodium influx more than balances the urea efflux. Thus, other substances in addition to urea are leaving the sac.

VASOPRESSIN AND AMNIOTIC FLUID OSMOLALITY

Since AVP is excreted along with fetal urine into the amniotic cavity, it has been postulated that AVP may be involved in the control of amniotic fluid osmolality. Ross et al. (1985) have demonstrated that intravenous infusion of AVP into the fetus will result in increased amniotic fluid osmolality. These investigators suggest that the influence of AVP on amniotic fluid osmolality is due to its actions on the kidney to conserve fluid and form a concentrated urine. In support of this hypothesis, Woods (1986) demonstrated that the increase in amniotic fluid osmolality following fetal and maternal intravenous injection of hypertonic saline is due to an increase in fetal urine osmolality. When an AVP antagonist is adminstered to the fetus, the increase in fetal urine osmolality is blocked and the increase in amniotic fluid osmolality is blunted.

It is also possible that AVP may be involved with amniotic fluid osmotic regulation by influencing the movement of water across the amnion. This hypothesis was supported by _in vitro_ studies with isolated guinea pig amnionic membranes which indicated that AVP may be influencing amniotic fluid volume and composition by causing water movement across the membrane into the sac (Manku et al., 1975; Vizsolyi and Perks, 1974). It is unlikely that both of these mechanisms are involved with amniotic fluid osmoregulation since they have opposite effects on osmolality: AVP actions on the kidney result in the concentration of amniotic fluid, and AVP actions on the amnion would result in the dilution of amniotic fluid.

The results of AVP injections into the amniotic fluid of late gestation guinea pig fetuses in the present study were unable to demonstrate an effect of amniotic fluid AVP on either amniotic fluid osmolality or electrolytes within 2 hours after injection. These findings do not support the hypothesis that AVP influences amnionic membrane water permeability. AVP injected directly into the amniotic sac may not have influenced water and solute movement across the amnion because amniotic fluid AVP was metabolized as was demonstrated by the fetal AVP metabolism experiments of the second part of this study.

In summary, the present initial baseline study confirms previous findings that fetal guinea pig urine is hypertonic to plasma, evidence that the fetus is capable of water reabsorption via the kidney or bladder. The guinea pig fetal kidney is probably more mature than that of the human fetus as is indicated by this ability to form a hypertonic urine whereas the human fetus produces a hypotonic urine. Further, whereas the human amniotic fluid potassium concentration does not change with gestational age, the guinea pig amniotic fluid potassium concentration increases, reflecting the formation of a more concentrated urine via water reabsorption with a corresponding increase in potassium concentration. The mechanism by which the guinea pig fetus is able to produce a hypertonic urine despite very low sodium and potassium concentrations remains unresolved because a large portion of the osmotically active particles have not been identified. Regardless of whether the fetal urine presented to the amniotic sac is hypertonic or hypotonic, the amniotic fluid in sheep, human and guinea pigs, is usually slightly hypotonic to fetal plasma. This hypotonic fluid that the fetus swallows perhaps provides the fetus a means by which it can control its osmolality independent of the mother via excretion of a hypertonic or hypotonic urine in the face of various states of maternal hydration. The factors responsible for the maintenance of a hypotonic amniotic fluid also have yet to be elucidated. Finally, AVP injected in vivo into the amniotic sac had no effect on amniotic fluid osmolality perhaps because it was degraded in the amniotic sac, and thus began part 2 of this study.

AVP METABOLISM IN THE AMNIOTIC SAC

The mechanism by which the fetus is able to maintain homeostatic control of AVP in its circulation is not understood since a route for fetal AVP clearance has yet to be established. The results of this study indicate that the amniotic sac is a site for fetal AVP metabolism.

EVIDENCE OF VASOPRESSIN CLEARANCE IN THE AMNIOTIC SAC

Vasopressin is added to the amniotic sac intact as was verified by HPLC analysis of fetal plasma and urine. However, no intact AVP in the amniotic fluid could be isolated by HPLC. A swallowing-independent disappearance of AVP from the amniotic sac was demonstrated by the more rapid decrease in AVP concentration relative to inulin concentration.

VASOPRESSIN DISAPPEARANCE RATES IN THE AMNIOTIC SAC

The rate of AVP disappearance in the amniotic compartment increased as the initial amniotic fluid AVP concentration increased. Although different enzyme kinetics may exist in amniotic fluid versus plasma, the relationship between the rate of AVP disappearance in the amniotic sac and AVP concentration observed in this study is similar to the relationship between AVP plasma clearance rates and AVP plasma concentrations reported in humans (Moses and Steciak, 1986) and in dogs (Sondeen and Claybaugh, 1987). These studies found that plasma

AVP clearance rates also increased with increased AVP concentrations. This is in contrast to the kinetics of plasma clearance at low plasma AVP levels in the adult dog described by Weitzman and Fisher (1978); they found that plasma clearance rate increased with decreased plasma AVP concentrations. The difference between the results of these studies could reflect differences in the range of AVP concentrations studied. The concentrations studied by Weitzman and Fisher were lower than the more pharmacological concentrations used in the study by Moses and Steciak (1986) and the study by Sondeen and Claybaugh (in review). Moses and Steciak described their findings of increased plasma clearance rates at higher AVP concentrations to be attributed to decreased plasma binding of AVP at high levels, and thus greater AVP filtration and excretion by the kidney. The increase in amniotic fluid AVP clearance rate seen in this study may be a result of decreased binding to amniotic fluid proteins which make the molecule more susceptible to enzymatic cleavage, or to a possible mass action effect of large amounts of substrate in the presence of an unlimited supply of enzyme.

The disappearance of AVP from the amniotic sac appears to follow classical first order kinetics. The half-life of AVP in the amniotic sac _in vivo_ was about 110 minutes. It should be noted that this _in vivo_ half-life is a reflection of a combination of both fluid and membrane metabolic influences. In order to establish true enzyme kinetics, an AVP dose response study needs to be done _in vitro_ with purified fluid enzymes and membrane enzymes. Also, the concentrations of AVP in these _in vitro_ preparations need to be measured over a longer time course of at least three half-lives (i.e. 330 minutes) to establish a better curve.

EVIDENCE OF THE METABOLISM OF VASOPRESSIN

The results of _in vivo_ and _in vitro_ experiments demonstrate that AVP is degraded in the amniotic compartment into at least 2 products. It should be noted that other metabolic products may have been present, but only those products which contained the tritium labelled phenylalanine amino acid could be detected. _In vitro_ studies have demonstrated that there are two separate enzymatic systems that metabolize AVP in the amniotic sac: a fluid metabolic component, and a membrane metabolic component. The two components are independent of each other. The fluid metabolic product was designated as M1 and the membrane metabolic product was designated as M2. The rate of M2 formation _in vivo_ is rapid and could represent a high affinity "fast component" of clearance. M1 production is slower but steady and may be equivalent to what is referred to by Weitzman and Fisher (1978) in the plasma clearance of AVP as the "elimination component". The differences in rates of M1 and M2 production within the first 10 minutes after AVP injection could indicate that the amniotic fluid enzyme responsible for M2 production is readily available, whereas AVP must first come into contact with the amnionic membrane bound enzyme before M1 production can occur. In addition, M2 production does not change between 10 and 120 minutes after injection, indicating that either M2 production has stopped due to the depletion of fluid enzymes, or that M2 is being removed at the same rate as it is being produced. The removal of M2 in vivo may involve further conversion to M1 by the membrane metabolic system. Although the in vitro studies of the isolated fluid and membrane systems show that neither the production of M1 nor M2 is dependent upon one another, it is still possible that in vivo, the M2 produced by the fluid enzyme system is further acted upon by the membrane metabolic system. This needs to be verified by incubating purified M2 with amnionic membrane in vitro.

Vasopressin degradation in amniotic fluid has been previously documented but never recognized for its possible role in fetal AVP clearance. There have been several reports of undetectable or at best, very low levels of amniotic fluid AVP (e.g. Alexander et al., 1971a; Swaab and Oosterbaan, 1983). Perks and colleagues conducted experiments to define a role for AVP action on the guinea pig amnion even though they "failed to find vasopressin in normal amniotic fluid from either the guinea pig or human" (Perks, 1977). They suggested that AVP may gain access to the amnion via passage from blood vessels of the outer membrane between the amnion and yolk sac. They did not consider the possibility that AVP may have been present in the amniotic fluid but was then metabolized by either fluid enzymes or upon binding to amnionic membrane receptors.

Wiriyathian et al. (1983) dismissed the amniotic sac as a possible site for fetal AVP clearance since they were able to see an increase in amniotic fluid AVP concentration from 1.6 $\pm$ 0.14 μU/ml at baseline to 5.2 ± 1.03 μU/ml 60 minutes after a 75 minute infusion of AVP at rates between 1.94 and 8.73 mU/min was stopped. Considering the very large amount of AVP that was infused into the fetus (654.75 mU at the highest rate) it is surprising that a higher amniotic fluid AVP concentration was not observed. These investigators also noted that the clearance rate of AVP from the fetal plasma was very rapid (60.5 ± 8.72 $ml \cdot kg^{-1} \cdot min^{-1}$) and despite such high infusion rates, the highest plasma AVP concentration achieved was only 30 μU/ml. Amniotic sac metabolism may have contributed to this clearance of AVP. Still, the increase in amniotic fluid AVP concentration from 1.6 to 5.2 μU/ml indicates that the capacity of the amniotic sac clearance system was exceeded.

The study done by Ervin et al. (1986b), which described a recirculation of amniotic fluid AVP in the fetus, also overlooked the significance of their low recovery of exogenous AVP injected into the amniotic compartment. They attributed the recovery of only 25% of the 25 μg of AVP injected into the amniotic sac to "procedural losses resulting from poor mixing...adsorption to test tubes, syringes, or catheters and nonspecific adsorption of the injected AVP to membranes and proteins". It is highly likely, in light of the findings of the present study, that much of their injected AVP was metabolized.

The discovery of AVP breakdown in human amniotic fluid by Rosenbloom et al. (1975) was not given much importance by these authors due to the low rate of degradation relative to that found in maternal serum. The amniotic fluid metabolism rate of 2500 pg/ml of

AVP was calculated by them to be about 40 $pg \cdot ml^{-1} \cdot min^{-1}$. This is equal to about 40 $fmol \cdot ml^{-1} \cdot min^{-1}$ (molecular weight of AVP = 1084 g). This rate is higher than the 1 $fmol \cdot ml^{-1} \cdot min^{-1}$ metabolism of 250 pg/ml found in the _in vitro_ experiments of this present study. Artman et al. (1984) demonstrated a 12% per hour decrease in 22.5 pg/ml of AVP incubated with amniotic fluid, which is equal to a rate of 0.038 fmol/min. The differences in amniotic fluid metabolism rates may be due to differences in the concentrations of AVP studied as seen in the plasma clearance studies discussed above. The differences in rates may also reflect species differences. For instance, as presented later, with the same _in vitro_ experimental methods used for the present studies, human amniotic fluid clearly degrades AVP faster and more completely than guinea pig amniotic fluid. These amniotic fluid AVP metabolism rates are not insignificant, however, when the relatively large volume of the amniotic fluid is taken into account. The metabolic capacity of the amniotic sac is further enhanced with the ability of the amnionic membrane, in addition to the amniotic fluid, to metabolize AVP as demonstrated in the present work .

CAPACITY OF THE AMNIOTIC SAC METABOLIC SYSTEMS

The metabolic capacity of the amniotic sac needs to be further studied with dose response tests. The results of this study show that the amniotic compartment is able to handle the metabolism of large amounts of AVP since approximately 65% of 1000 pg ^{3}H-AVP incubated _in vitro_ with amnionic membrane and amniotic fluid was metabolized in

2 hours. It should be recalled that the turnover time of the amniotic fluid is about 14 hours. Other investigators have found that amniotic fluid levels of AVP remain elevated up to 24 hours after experimental manipulation (Stark et al., 1984) and for at least 2 hours after large pharmacological infusions of AVP into the fetus (Wiriyathian et al., 1983), suggesting that the amniotic sac clearance system is saturable. Daniel et al. (1980) estimated urinary AVP excretion rates in unstressed sheep fetuses to be about 2.07 $\pm$ 0.20 $pg \cdot kg^{-1} \cdot min^{-1}$. This can easily be matched by the calculated amniotic fluid clearance rates and thus, the amniotic sac, especially when one considers the contribution of the amnionic membrane, appears to be able to metabolize the AVP with which it is normally presented. Nevertheless, the importance of the amniotic sac in the overall clearance of AVP from the fetal plasma needs to be considered. Wiriyathian et al. (1983) reported an overall AVP plasma clearance rate in the fetal sheep of about 60 $ml \cdot kg^{-1} \cdot min^{-1}$ which is equal to about 120 ml/min if fetal weight is estimated at about 2 kg. Woods et al. (1986) reported glomerular filtration rate (GFR) in the fetal sheep to be about 4 ml/min. Thus, even if all of the AVP filtered and excreted was metabolized in the amniotic sac such that the amniotic sac clearance rate was equal to GFR, amniotic sac metabolism would account for only one thirtieth of the total plasma clearance of AVP. Therefore, although the capacity of the amniotic sac metabolic system appears to be high, the importance of this system in overall fetal plasma AVP regulation and osmotic homeostasis is uncertain.

PARTIAL CHARACTERIZATION OF THE METABOLIC PRODUCTS

The *in vitro* results strongly suggest that M2 is des-glycinamide AVP. This metabolic product has not yet been found in any other biological system. It had seemed, thus far, that cleavage at this site of the AVP molecule is only possible after a post-proline cleavage, since no intact des-glycinamide AVP product had been found in previous studies of AVP metabolism, and only the glycinamide residue has been identified. The evidence presented that suggests that M2 is des-glycinamide AVP is based on two findings. First, an antiserum that crossreacts with des-glycinamide AVP also crossreacts with M2 whereas other antisera do not. Second, M2 comigrates with des-glycinamide AVP on three different buffer systems utilized in HPLC. The M1 product has yet to be characterized. So far it is known that M1 contains the tritiated phenylalanine in the ring portion of the AVP molecule. The amino acid composition of both M1 and M2 should be determined after isolation by HPLC of a large enough quantity of these products.

PARTIAL CHARACTERIZATION OF THE AMNIOTIC FLUID ENZYME

Results of the aprotinin experiments suggest that the AF metabolism of AVP is accomplished by a trypsin-like enzyme which is capable of cleaving the terminal glycinamide. This also suggests that its proteolytic action may not be specific for AVP since this is a general proteolytic enzyme. If this is so, it is probable that the amniotic compartment plays a significant role in the clearance of AVP, and may be a general metabolic site for other proteins as well. On the other hand, the enzyme is somewhat specific for certain peptides such as AVP since OT, which lacks the necessary peptide bonds vulnerable to trypsin, escapes degradation and is easily measured in amniotic fluid (Swaab and Oosterbaan, 1983). Enzyme studies show that OT is vulnerable to chymotrypsin which cleaves the terminal glycinamide from that hormone (Walter and Simmons, 1977). Further studies on the specificity of the amniotic fluid and amnionic membrane enzymes can be done if the enzymes can be further purified.

DEVELOPMENT OF THE AMNIOTIC SAC METABOLIC SYSTEMS OVER GESTATION

The amnionic membrane enzyme system functions as early as 34 days of gestation (term = 68 days) whereas the amniotic fluid enzyme system is not effective until 54 days of gestation (see figs. 31 and 32), further support that the two metabolic systems are independent of each other. It is interesting that both the findings of this study and that of Artman et al. (1984) demonstrate AVP degradation in the amniotic fluid only in late term. If the amniotic fluid enzyme responsible for AVP metabolism is trypsin, rather than "trypsin-like" as discussed below, it may originate in the gastrointestinal (G.I.) tract and enter the amniotic sac via fetal meconium. Others have postulated this source of amniotic fluid trypsin (Pocknee and Abramovich, 1982). Thus, the appearance of this enzyme is dependent upon the maturation of the fetal G.I. system. The amnionic membrane which is responsible for the formation of M1, on the other hand, is present from the beginning of gestation. The amnion could therefore serve as an early means of AVP clearance which is later augmented by the fluid system.

The trypsin found in amniotic fluid by some investigators is reported to be present in human amniotic fluid in early gestation but not in late gestation unless meconium is present, which is not in agreement with this study's finding that AVP degradation occurred via the trypsin-like enzyme in the amniotic fluid only in late gestation. The difference in the apparent appearance of the amniotic fluid trypsin enzyme may reflect a species difference between humans and guinea pigs. On the other hand, it is also possible that the enzyme responsible for amniotic fluid AVP degradation is just trypsin-like and not true trypsin since trypsin has not been measured directly in this present study. The appearance of amniotic fluid AVP degradation only in late gestation is similar to findings that proteolytic activity in human amniotic fluid is absent before 36 weeks of gestation which suggested the use of the presence of amniotic fluid proteolytic activity as an index of fetal maturity (Sanyal et al., 1977).

POSSIBLE SOURCES OF THE AMNIOTIC FLUID ENZYMES

Another likely source of the amniotic fluid enzyme besides fetal meconium is the fetal urine. Some apparent production of M2 was detected in the fetal urine (see fig. 14). The metabolism of AVP in the fetal urine should be tested by incubating ^{3}H-AVP with fetal urine. It does appear, however, that even if AVP may be degraded in the fetal urine, the metabolism by urinary enzymes is not as efficient as amniotic fluid metabolism since intact AVP can be detected in fetal urine whereas no AVP can be isolated in amniotic fluid. Perhaps anti-proteolytic enzyme activity , for example alpha-1 antitrypsin activity, in the urine is more effective than such activity in the amniotic fluid. It may be that degradation in the urine is less because the urinary enzymes are passed into the amniotic sac and the time of incubation of these enzymes with AVP in the bladder is short. Other possible sources of the amniotic fluid enzyme are the placenta and decidua.

POSSIBLE BIOACTIVITY OF THE METABOLITES

It is possible that the M1 and M2 metabolic products may have some bioactivity. Central actions of AVP metabolites have been described by Burbach and Lebouille (1983) which support the concept that AVP or OT are precursors for peptide fragments with specific functions. Perks (1977) described a substance in human AF similar to that found by Carretero et al. (1971) that produced an antidiuretic response which "was closely similar to that of AVP". This substance eluted close to but not with AVP on Sephadex and may be the M1 product of this study. It is unlikely that this substance would be M2 since des-glycinamide has no antidiuretic activity (Manning and Sawyer, 1977; unpublished results from this laboratory). This would not rule out a possible membrane effect of this molecule on the amnionic membrane, however. Perhaps the active molecule which influences amniotic fluid dynamics and movement of water and solutes across the amnion is not the entire AVP molecule but just a portion of it. It may be that in order for AVP to have an effect on the amnion, it has to first be converted to this active form much like the way angiotensin II and not the parent molecules angiotensinogen and angiotensin I is the active molecule. The bioactivity of the M1 and M2 metabolites can be studied in an Ussing chamber set up of the amnionic membrane once these metabolites are isolated in large enough quantities .

FATE OF THE METABOLIC PRODUCTS

Regardless of the bioactivity of the M1 and M2 products, the fate of these products in the amniotic sac should be considered. It is unlikely that these products accumulate in the amniotic sac throughout gestation, especially if they do possess some bioactivity. Furthermore, M2 which is detectable directly by RIA in our laboratory, is not detectable in the amniotic fluid unless exogenous AVP is added. It is more likely that these metabolites are eventually further degraded to smaller peptide fragments or even to single amino acids once the first enzymatic cleavage of the intact AVP molecule has occurred. Verhoef et al. (1986) have shown that carboxypeptidase may be invoved in further transformation of des-glycinamide AVP to AVP 1-7 (des-gly, des-arg). Carboxypeptidase may be present in amniotic fluid, but this degradation may not have been detected in this study since its rate is very slow (e.g. the half life of dgAVP in rat plasma is 5.8 hours). It is possible that M2 is further converted to M1 _in vivo_ (again, this can be tested by incubating purified M2 with amnionic membrane). It is believed that amino acids swallowed from the amniotic fluid may be incorporated by the fetus into protein (Pitkin and Reynolds, 1975). If not used for protein synthesis, once peptide fragments are swallowed, they can be further degraded in the fetal G.I. tract and passed on to the maternal circulation. This is evidenced by the demonstration of ^{125}I label appearing in maternal urine within 24 hours after intraamniotic injection of radiolabelled protein (Gitlin et al., 1972).

STUDIES OF VASOPRESSIN METABOLISM BY HUMAN AMNIOTIC FLUID AND AMNIONIC MEMBRANES

Because of species differences noted with the study of vasopressinase (Rosenbloom et al., 1975), preliminary results of _in vitro_ experiments have been conducted on human amniotic fluid and amnionic membranes. It appears that the same type of enzymatic processes found in the guinea pig are present in the human. Both the M1 and M2 metabolites were produced in term (greater that 35 weeks of gestation) amniotic fluid and amnionic membranes. The human amnionic membrane like the guinea pig amnionic membrane only produced M1 with no M2 production. However, whereas in the guinea pig M2 is produced in the amniotic fluid with only very little M1 production, the human amniotic fluid is able to produce M2 and considerable amounts of M1 as well. Early gestation (16-20 weeks) amniotic fluid also has the ability to metabolize AVP to both M1 and M2 in contrast to guinea pig results which showed that the amniotic fluid enzyme system was not operating until late gestation. No studies have yet been done with early gestation human amnionic membranes.

These initial results also suggest that the human enzyme systems metabolize AVP at faster rates and have much greater capacities than the guinea pig enzyme systems. In 120 minutes, both the human amniotic membrane and amniotic fluid at term were able to metabolize about 95% of the 250 pg of AVP injected, which is equal to a metabolism rate of about 2 $fmol \cdot ml^{-1} \cdot min^{-1}$.

In conclusion, the amniotic compartment may play a role in the clearance of AVP in the guinea pig fetus. The rate of AVP metabolism is dependent upon the initial amniotic fluid AVP concentration. The amniotic sac metabolism involves two separate metabolic components which are independent of each other: an amniotic fluid component, and an amnionic membrane component. The fluid component is responsible for the production of M2 and the membrane component is responsible for the production of M1. The M2 metabolite appears to be des glycinamide AVP. If this is true, this is the first time this metabolic product has been isolated in a biological system. The amnionic membrane metabolic system functions early in gestation but the amniotic fluid metabolic system does not contribute to AVP degradation until after 54 days of gestation. Finally, the amniotic fluid enzyme responsible for the production of M2 appears to be trypsin. If this is so, the amniotic compartment may be important for not only the clearance of AVP from the fetal system, but other proteins as well.

BIBLIOGRAPHY

Aderjan, R., W. Rauh, P. Vecsei, U. Lorenz, and H. Ruttgers. 1977. Determination of cortisol, tetrahydrocortisol, tetrahydrocortisone, corticosterone, and aldosterone in human amniotic fluid. J. Steroid Biochem. 8: 525-528.

Alexander, D.P., H.G. Britton, M.L. Forsling, D.A. Nixon, and J.G. Ratcliffe. 1971a. The concentrations of adrenocorticotrophin, vasopressin and oxytocin in the foetal and maternal plasma of the sheep in the latter half of gestation. J. Endocrinology 49: 179-180.

Alexander, D.P., H.G. Britton, M.L. Forsling, D.A. Nixon, and J.G. Ratcliffe. 1971b. The release of corticotrophin and vasopressin in the foetal sheep in response to haemorrhage. J. Physiol. 213: 31P-32P.

Alexander, D.P., M.L. Forsling, M.J. Martin, D.A. Nixon, J.G. Ratcliffe, D. Redstone, and D. Tunbridge. 1972. The effect of maternal hypoxia on fetal pituitary hormone release in the sheep. Biol. Neonate 21: 219-228.

Artman, H.G., R.D. Leake, R.E. Weitzman, W.H. Sawyer, and D.A. Fisher. 1984. Radioimmunoassay of vasotocin, vasopressin, and oxytocin in human neonatal cerebrospinal and amniotic fluid. Dev. Pharmacol. Ther. 7: 39-49.

Aynsley-Green, A., I.Z. Mackenzie, P.A. Jenkins, G. Soltesz, and S.R. Bloom. 1985. The metabolic and endocrine milieu of the human fetus at 16-20 weeks of gestation. In The Physiological Development of the Fetus and the Newborn. Eds., C.T. Jones and P.W. Nathanielsz. Academic Press. London.

Barr, H.A., M.A. Lugg, and T.E. Nicholas. 1980. Cortisone and cortisol in maternal and fetal blood and in amniotic fluid during the final ten days of gestation in the rabbit. Biol. Neonate 38(3-4): 214-220.

Bayliss, W.M. and E.H. Starling. 1902. The mechanism of pancreatic secretion. J. Physiol. 28: 325-353.

Belisle, S. and D. Tulchinsky. 1980. Amniotic fluid hormones. In Maternal-Fetal Endocrinology. Eds., D. Tulchinsky and K.J. Ryan. W.B. Saunders Company. Philadelphia. p. 169-195.

Benmansour, M., M. Rainfray, F. Paillard, and R. Ardaillou. 1982. Metabolic clearance rate of immunoreactive vasopressin in man. European J. of Clin. Invest. 12: 475-480.

Berson, S.A. and R.S. Yalow. 1968. Radioimmunoassay: a status report. Hospital Practice 3(11): 73-83.

Berson, S.A., R.S. Yalow, A. Bauman, M.A. Rothschild, and K. Newerly. 1956. Insulin-I^{131} metabolism in human subjects: Demonstration of insulin binding globulin in the circulation of insulin treated subjects. J. Clin. Invest. 35(2): 170-190.

Bhat, A.R. and T.N. Pattabiraman. 1980. Comparative study of proteinase inhibitors in human amniotic fluid and maternal serum. Br. J. Obstet. Gynaecology. 87: 1109-1114.

Blankstein, J., K. Fujieda, F.I. Reyes, C. Faiman, and J.S. Winter. 1980. Cortisol, 11-desoxycortisol, and 21-desoxycortisol concentrations in amniotic fluid during normal pregnancy. Am. J. Obstet. Gynecol. 137: 781-784.

Bonses, R.W. 1966. Composition of amniotic fluid. Clin. Obstet. Gynec. 9(2): 441-448.

Bonses, R.W. 1972. The chemistry and physiology of amniotic fluid. In Antenatal Diagnosis. Ed., A. Dorfman. The University of Chicago Press. Chicago. p. 3-16.

Borgstrom, A., T. Sveger, S. Kullander, L. Svanberg, and P. Hosli. 1984. Determination and characterization of immunoreactive trypsin in amniotic fluid from normal and cystic fibrosis fetuses. Pediatric Research 18(10): 957-959.

Boylan, J.W., E.P. Colbourn, and R.A. McCance. 1958. Renal function in the foetal and new-born guinea-pig. J. Physiol. 141: 323-331.

Brans, Y.W., T.J. Kuehl, R.H. Hayashi, D.L. Shannon, and P. Reyes. 1984. Chemical dilution and clearance studies to estimate amniotic fluid volume and amniotic fluid ingestion in normal baboon pregnancies. Am. J. Primatology 6: 31-40.

Brock, D.J.H., C. Hayward, C. Gosden, and C. Rodeck. 1984. Immunoreactive trypsin and the prenatal diagnosis of cystic fibrosis. Br. J. Obstet. Gynaecol. 91: 449-452.

Buijs, R.M., D.N. Velis, and D.F. Swaab. 1980. Ontogeny of vasopressin and oxytocin in the fetal rat: early vasopressinergic innervation of the fetal brain. Peptides 1(4): 315-324.

Burbach, J.P.H. and J.L.M. Lebouille. 1983. Proteolytic conversion of arginine-vasopressin and oxytocin by brain synaptic membranes. J. Biological Chemistry 258(3): 1487-1494.

Burford, G.D. and I.C.A.F. Robinson. 1982. Oxytocin, vasopressin and neurophysins in the hypothalamo-neurohypophysial system of the human fetus. J. Endocr. 95:403-408.

Burton, A.M. and M.L. Forsling. 1972. Hormone content of the neurohypophysis in foetal, new-born and adult guinea-pigs. J. Physiol. 221: 6P-7P.

Carretero, O.A., B. Bujak, A. Hodari, C. Hodgkinson and F. Bumpus. 1971. Identificaiton of a pressor polypeptide in human amniotic fluid. Am. J. Obstet. Gynecol. 111(8): 1075-1082.

Claybaugh, J.R. and A.K. Sato. 1985. Factors influencing urinary vasopressin concentration. Fed. Proc. 44(1): 62-65.

Czernichow, P. 1979. Vasopressin in fetal sheep: a review. J. Physiol. 75(1): 33-36.

Daniel, S.S., R.I. Stark, M.K. Husain, U.M. Sanocka, and L.S. James. 1980. Excretion of vasopressin (VP) in the hypoxic lamb. Pediatric Research 14: 618.

Daniel, S.S., R.I. Stark, M.K. Husain, L.V. Baxi, and L.S. James. 1982. Role of vasopressin in fetal homeostasis. Am. J. Physiol. 242(6): F740-F744.

Davidson, W.D. and M.A. Sackner. 1963. Simplification of the anthrone method for the determination of inulin in clearance studies. J. Lab. Clin. Med. 62(2): 351-356.

DeVane, G.W. and J.C Porter. 1980. An apparent stress-induced release of arginine vasopressin by human neonates. J. Clin. Endocrinol. Metab. 51(6): 1412-1416.

DeVane, G.W., R.P. Naden, J.C. Porter and C.R. Rosenfeld. 1982. Mechanism of arginine vasopressin release in the sheep fetus. Pediatric Res. 16(6): 504-507.

Dogterom, J., F.G.M. Snijdewint, P. Pevet, and D.F. Swaab. 1980. Studies of the presence of vasopressin, oxytocin and vasotocin in the pineal gland, subcommissural organ and fetal pituitary gland: failure to demonstrate vasotocin in mammals. J. Endocr. 84: 115-123.

Drummond, W.H., A.M. Rudolph, L.C. Keil, P.D. Gluckman, A.A. MacDonald, and M.A. Heymann. 1980. Arginine vasopressin and prolactin after hemorrhage in the fetal lamb. Am. J. Physiol. 238: E214-E219.

DuVigneaud, V., C. Lawler, and E. Popenoe. 1953. Enzymatic cleavage of glycinamide from vasopressin and a proposed structure for this pressor-antidiuretic hormone of the posterior pituitary. J. Amer. Chem. Soc. 75: 4880-4881.

DuVigneaud, V., C. Ressler, and S. Trippett. 1953. The sequence of amino-acids in oxytocin, with a proprosal for the structure of oxytocin. J. Biol. Chem. 205(2): 949-957.

Ervin, M.G., R.D. Leake, M.G. Ross, G.C. Calvario, and D.A. Fisher. 1985. Arginine vasotocin in ovine fetal blood, urine, and amniotic fluid. J. Clin. Invest. 75:1696-1701.

Ervin, M.G., M.G. Ross, R.D. Leake, and D.A. Fisher. 1986a. Changes in steady state plasma arginine vasotocin levels affect ovine fetal renal and cardiovascular function. Endocrinology 118(2): 759-765.

Ervin, M.G., M.G. Ross, R.D. Leake, and D.A. Fisher. 1986b. Fetal recirculation of amniotic fluid arginine vasopressin. Am. J. Physiol. 250: E253-E258.

Fisher, D.A. 1979. Fetal endocrinology: endocrine disease and pregnancy. In Endocrinology Vol. 3. Eds., DeGroot, L.J., G.F. Cahill, Jr., W.D. Odell, L. Martini, J.T. Potts, Jr., D.H. Nelson, E. Steinberger and A.I. Winegrad. Grune and Stratton. New York p. 1649-1663.

Forsling, M.L. and E. Fenton. 1977. Permeability of the foetal guinea-pig placenta to arginine vasopressin. J. Endocr. 72:409-410.

France, V.M. 1974. Sodium uptake by the skin of foetal sheep. J. Physiol. 240(2): 27P.

France, V.M., N.R. Saunders, and M.W. Stanier. 1972. Sodium transport in foetal sheep urinary bladder. J. Physiol. 224: 23P.

Gitlin, D., J. Kumate, C. Morales, L. Noriega, and N. Arevalo. 1972. The turnover time of amniotic fluid protein in the human conceptus. Am. J. Obstet. Gynecol. 113(5): 632-645.

Heller, H. 1974. History of neurohypophysial research. In Handbook of Physiology Sec. 7: Endocrinology Vol. IV The Pituitary Gland and Its Neuroendocrine Control, Part 1. Eds., E. Knobil and W.H. Sawyer. Am. Physiol. Soc. Washington, D.C. p. 103-117.

Holt, W.F. and A.M. Perks. 1977. The influence of vasopressin on the passage of tritated water through the isolated amniotic membrane and other tissues from the fetal guinea pig. Can. J. Zool. 55(9): 1393-1403.

Iwamoto, H.S., A.M. Rudolph, L.C. Keil, and M.A. Heymann. 1979. Hemodynamic responses of the sheep fetus to vasopressin infusion. Circ. Res. 44(3): 430-436.

Johannesen, P., E.B. Pedersen, and A.B. Rasmussen. 1985. Arginine vasopressin in amniotic fluid, arterial and venous cord plasma and maternal venous plasma. Gynecol. Obstet. Invest. 19:192-195.

Jones, C.T. and T.P. Rolph. 1985. Metabolism during fetal life: a functional assessment of metabolic development. Physiological Reviews 65(2): 357-430.

Jones, C.T. and D. Rurak. 1976. The distribution and clearances of hormones and metabolites in the circulation of the foetal sheep. Quarterly J. Experimental Physiology 61: 287-295.

Kanayama, N., H. Kamijo, T. Terao, K. Horiuchi, and D. Fujimoto. 1986. The relationship between trypsin activity in amniotic fluid and premature rupture of membranes. Am. J. Obstet. Gynecol. 155: 1043-1048.

Kelly, R.T., J.C. Rose, P.J. Meis, B. Hargrave, and M. Morris. 1983. Vasopressin is important for restoring cardiovascular homeostasis in fetal lambs subjected to hemorrhage. Am. J. Obstet. Gynecol. 146: 807-812.

Kleinman, L.I. 1970. Physiology of the perinatal kidney. In Physiology of the Perinatal Period Functional and Biochemical Development in Mammals Vol. 2. Ed., Stave, Uwe. Meredith Corporation. New York. p. 679-702.

Koida, M. and R. Walter. 1976. Post-proline cleaving enzyme: purification of this endopeptidase by affinity chromatography. J. Biol. Chem. 251: 7593-7599.

LaRochelle, Jr., F.T., W.G. North and P. Stern. 1980. A new extraction of arginine vasopressin from blood: the use of octadecasilyl-silica. Pflugers Arch. 387: 79-81.

Larsen, B., B.L. Fox, M.F. Burke and V.J. Hruby. 1979. The separation of peptide hormone diastereoisomers by reverse phase high pressure liquid chromatography. Int. J. Peptide Protein Res. 13: 12-21.

Lauson, H.D. 1974. Metabolism of the neurohypophysial hormones. In Handbook of Physiology, Section 7, Endocrinology, Vol IV, The Pituitary and Its Neuroendocrine Control, Part 1. Eds., E. Knobil and W.H. Sawyer. Am. Physiol. Soc. Washington, D.C. p. 287-393.

Leake, R.D., H. Stegner, S.M. Palmer, G.K. Oakes, and D.A. Fisher. 1983. Arginine vasopressin and arginine vasotocin inhibit ovine fetal/maternal water transfer. Pediatr. Res. 17:583-586.

Leake, R.D. and R.E. Weitzman. 1979. Developmental pharmacokinetics of the posterior pituitary hormones. Clinics in Pharmacology 6(1): 65-85.

Leake, R.D., R.E. Weitzman, R.M. Effros, S.R. Siegel, and D.A. Fisher. 1979. Maternal fetal osmolar homeostasis: fetal posterior pituitary autonomy. Pediat. Res. 13: 841-844.

Leffler, C.W., J. Crofton, D.P. Brooks, L. Share, J.R. Hessler, and R.S. Green. 1985. Changes in plasma arginine vasopressin during transition from fetus to newborn following minimal trauma delivery of lambs and goats. Biol. Neonate 48: 43-48.

Leung, A.K., R.G. McArthur, D.D. McMillan, D. Ko, J.S. Deacon, J.T. Parboosingh, and K.P. Lederis. 1980. Circulating antidiuretic hormone during labour and in the newborn. Acta Paediatr. Scand. 69(4): 505-510.

Lieberman, J. 1966. Proteolytic enzyme activity in fetal pancreas and meconium. Gastroenterology 50(2): 183-190.

Lind, T., F.M. Parkin, and G.A. Cheyne. 1969. Biochemical and cytological changes in liquor amnii with advancing gestation. J. Obstet. Gynaecol. Br. Commonwealth 76(8): 673-683.

Lindberg, G. 1980. Separation of vasopressin analogues by reversed-phase high-performance liquid chromatography. J. Chromatography 193: 427-431.

Lingwood, B.E., K.J. Hardy, J.G. Long, M. McPhee, and E.M. Wintour. 1980. Amniotic fluid volume and composition following experimental manipulations in sheep. Obstetrics and Gynecology 56(4): 451-458.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Magnus, R. and E.A. Schafer. 1901. The action of pituitary extracts upon the kidney. J. Physiol. 27: ix-x.

Manku, M.S., J.P. Mtabaji, and D.F. Horrobin. 1975. Effect of cortisol, prolactin and ADH on the amniotic membrane. Nature 258: 78-80.

Manning, M. and W.H. Sawyer. 1977. Structure-activity studies on oxytocin and vasopressin 1954-1976. From empiricism to design. In Neurohypophysis. Eds., A.M. Moses and L. Share. Karger. Basel. p. 9-21.

Merialdi, A. and M. Fatone. 1985. The probable role of antidiuretic hormone in the pathophysiology of fetal secondary growth defect. In The Physiological Development of the Fetus and the Newborn. Eds., C.T. Jones and P.W. Nathanielsz. Academic Press. London. p. 561-564.

Merlet-Benichou, C. and C. de Rouffignac. 1977. Renal clearance studies in fetal and young guinea pigs: effect of salt loading. Am. J. Physiol. 232(2): F178-F185.

Miller, M. and A.M. Moses. 1972. Radioimmunoassay of urinary antidiuretic hormone in man: response to water load and dehydration in normal subjects. J. Clin. Endocrinol. Metab. 34: 537-545.

Morris, B.J. and E.R. Lumbers. 1972. The activation of renin in human amniotic fluid by proteolytic enzymes. Biochim. Biophys. Acta 289: 385-391.

Moses, A.M. and E. Steciak. 1986. Urinary and metabolic clearances of arginine vasopressin in normal subjects. Am. J. Physiol. 251: R365-R370.

Murphy, B.E.P., J. Patrick, and R.L. Denton. 1975. Cortisol in amniotic fluid during human gestation. J. Clin. Endocrinol. Metab. 40: 164-167.

Nardacci, N.J., S. Mukhopadhyay, and B.J. Campbell. 1975. Partial purification and characterization of the antidiuretic hormone inactivating enzyme from renal plasma membranes. Biochim. Biophys. Acta 377: 146-157.

Nelsen, E.D., D.L. Hutchinson, R.L. Hallet and A.A. Plentl. 1954. Dilution methods for determination of amniotic fluid volume. Obstetrics and Gynecology 3(6): 598-607.

Oliver, G. and E.A. Schafer. 1895. On the physiological action of extracts of pituitary body and certain other glandular organs. J. Physiol. 18: 277-279.

Oosterbaan, H.P., D.F. Swaab and G.J. Boer. 1985. The origin of amniotic oxytocin and vasopressin and their relationship to intra-uterine growth retardation. In The Physiological Development of the Fetus and the Newborn. Eds., C.T. Jones and P.W. Nathanielsz. Academic Press. London.

Pavel, S. 1971. Evidence for the ependymal origin of arginine vasotocin in the bovine pineal gland. Endocrinology 89: 613-614.

Pavel, S., R. Goldstein, and M. Calb. 1975. Vasotocin content in the pineal gland of foetal, newborn and adult male rats. J. Endocr. 66: 283-284.

Perks, A.M. 1977. Developmental and evolutionary aspects of the neurohypophysis. Am. Zool. 17: 833-849.

Perks, A.M. and S. Cassin. 1982. The effects of arginine vasopressin and other factors on the production of lung fluid in fetal goats. Chest 81S: 63S-65S.

Perks, A.M. and S. Cassin. 1985. The effects of arginine vasopressin on lung liquid secretion in chronic fetal sheep. In The Physiological Development of the Fetus and Newborn. Eds., Jones, C.T. and P.W. Nathanielsz. Academic Press. London. p. 253-257.

Pitkin, R.M. and W.A Reynolds. 1975. Fetal ingestion and metabolism of amniotic fluid protein. Am. J. Obstet. Gynecol. 123(4): 356-363.

Pocknee, R.C. and D.R. Abramovich. 1982. Origin and levels of trypsin in amniotic fluid throughout pregnancy. Br. J. Obstet. Gynaecol. 89: 142-144.

Pohjavuori, M. and F. Fyhrquist. 1980. Hemodynamic significance of vasopressin in the newborn infant. J. Pediatr. 97(3): 462-465.

Punnonen, R., R. Tuimala, and O. Viinamaki. 1984. Vasopressin in fetal cerebrospinal fluid. Early Human Development 9: 309-311.

Rees, L., M.L. Forsling, and C.G.D. Brook. 1980. Vasopressin concentratins in the neonatal period. Clin. Endocrinology 12(4): 357-362.

Robillard, J.E. and R.E. Weitzman. 1980. Developmental aspects of the fetal renal response to exogenous arginine vasopressin. Am. J. Physiol. 238: F407-F414.

Rose, J.C., P.J. Meis, and M. Morris. 1981. Ontogeny of endocrine (ACTH, vasopressin, cortisol) responses to hypotension in lamb fetuses. Am. J. Physiol. 240(6): E656-661.

Rosenbloom, A.A., J. Sack, and D.A. Fisher. 1975. The circulating vasopressinase of pregnancy: species comparison with radioimmunoassay. Am. J. Obstet. Gynecol. 121(3): 316-320.

Ross, M.G., M.G. Ervin, R.D. Leake, and D.A. Fisher. 1985. Amniotic fluid ionic concentration in response to chronic fetal vasopressin infusion. Am. J. Physiol. 249: E287-E291.

Ross, M.G., G. Ervin, R.D. Leake, P. Fu, and D.A. Fisher. 1984. Fetal lung liquid regulation by neuropeptides. Am. J. Obstet. Gynecol. 150: 421-425.

Ross, M.G., M.G. Ervin, R.D. Leake, O. Habeeb, and D.A. Fisher. 1986a. Isovolemic hypotension in ovine fetus: plasma arginine vasopressin response and urinary effects. Am. J. Physiol. 250: E564-E569.

Ross, M.G., M.G. Ervin, R.D. Leake, J.A. Humme, and D.A. Fisher. 1986b. Continuous ovine fetal hemorrhage: sensitivity of plasma and urine arginine vasopressin response. Am. J. Physiol. 251: E464-E469.

Rurak, D.W. 1976. Plasma vasopressin in foetal lambs. J. Physiol. 256(1): 36P-37P.

Sanyal, A.K., C. Dey, P.K. Debnath, D. Sharma, and R. Chatterjee. 1977. Presence of proteolytic activity in the amniotic fluid. Indian J. Med. Res. 65(6): 822-828.

Schruefer, J.J., A.E. Seeds, R.E. Behrman, A.E. Hellegers, and P.D. Bruns. 1972. Changes in amniotic fluid volume and total solute concnentration in the rhesus monkey following replacement with distilled water. Am. J. Obstet. Gynecol. 112(6): 807-815.

Shade, R.E. and L. Share. 1976. Metabolic clearance of immunoreactive vasopressin and immunoreactive [^{131}I]iodo vasopressin in the hypophysectomized dog. Endocrinology 99(5): 1199-1206.

Share, L., T. Kimura, K. Matsui, R.E. Shade, and J.T. Crofton. 1985. Metabolism of vasopressin. Fed. Proc. 44:59-61.

Siegel, S.R., R.D. Leake, R.E. Weitzman, and D.A. Fisher. 1980. Effects of furosemide and acute salt loading on vasopressin and renin secretion in the fetal lamb. Pediatric Res. 14(7): 869-871.

Silverman, A.J. 1975. The hypothalamic magnocellular neurosecretory system of the guinea pig II. Immunohistochemical localization of neurophysin and vasopressin in the fetus. Am. J. Anat. 144(4): 445-459.

Sippell, W.G. 1981. Unconjugated cortisol and corticosterone levels in amniotic fluid. Am. J. Obstet. Gynecol. 140(3): 353-354.

Skowsky, W.R. and D.A. Fisher. 1972. The use of thyroglobulin to induce antigencity to small molecules. J. Lab. Clin. Med. 80(1): 134-144.

Skowsky, W.R. and D.A. Fisher. 1977. Fetal neurohypophyseal arginine vasopressin and arginine vasotocin in man and sheep. Pediatric. Res. 11: 627-630.

Smith, A. and N. McIntosh. 1983. Neurohypophyseal peptides in the human fetus: presence in pituitary extracts of immunoreactive arginine-vasotocin. J. Endocrinology 99: 441-445.

Smith, H.W. 1937. The physiology of the kidney. Oxford University Press. New York.

Sondeen, J.L. and J.R. Claybaugh. 1987. Clearance, plasma binding, and urinary excretion of vasopressin in conscious dogs. In review.

Stanier, M.W. 1971. Osmolarity of urine from renal pelvis and bladder of foetal and post-natal pigs. J. Physiol. 218: 30P-31P.

Stark, R.I., S.S. Daniel, M.K. Husain, J. Milliez, H.O. Morishima, L.S. James, and R. Van De Wiele. 1981. Release of vasopressin by the fetal lamb during premature parturition induced with corticotropin. Pediatric Res. 15(9): 1261-1265.

Stark, R.I., S.S. Daniel, M.K. Husain, U.M Sanocka, A.B. Zubrow, and L.S. James. 1984. Vasopressin concentration in amniotic fluid as an index of fetal hypoxia: mechanism of release in sheep. Pediatric Res. 18(6): 552-558.

Starling, E.H. and E.B. Verney. 1924. The secretion of urine as studied on the isolated kidney. Proc. Roy. Soc., London, Ser. B 97:321-363.

Stegner, H., H.G. Artman, R.D. Leake, and D.A. Fisher. 1984a. DDAVP (1-desamino-8-D-arginine vasopressin) clearance rate. Acta Endocrinologica 107(2): 204-206.

Stegner, H., R.D. Leake, S.M. Palmer, A.M. Morris, and D.A. Fisher. 1984b. Arginine vasopressin metabolic clearance and production rates in fetal sheep, pregnant ewes, and lambs. Dev. Pharmacol. Ther. 7: 87-93.

Stiles, B. and G.G. Power. 1983. Changes in permeability of fetal guinea pig skin during gestation. J. Dev. Physiol. 5(6): 405-411.

Swaab, D.F. and H.P. Oosterbaan. 1983. Exclusion of the fetal brain as the main source of rat and human amniotic fluid oxytocin. Br. J. Obstet. Gynaecol. 90(12): 1160-1167.

Takada, Y. S.W. Brown, and E.G. Erdos. 1986. Activation of plasma and amniotic prorenin by metalloprotease and trypsin. J. Clin. Endocrinol. Metab. 62: 569-576.

Tomoda, S., R.A. Brace, and L.D. Longo. 1985. Amniotic fluid volume and fetal swallowing rate in sheep. Am. J. Physiol. 249: R133-R138.

Tomoda, S., R.A. Brace, and L.D. Longo. 1987. Amniotic fluid volume regulation: basal volumes and responses to fluid infusion or withdrawal in sheep. Am. J. Physiol. 252: R380-R387.

Verhoef, J., H.M. van den Wildenberg, and J.W. van Nispen. 1986. [^{3}H]9-desglycinamide,8-arginine vasopressin: metabolism and in-vivo fate. J. Endocrinology 110: 557-562.

Verney, E.B. 1947. The antidiuretic hormone and the factors which determine its release. Proc. Roy. Soc. London, Ser. B. 135: 25-106.

Vizsolyi, E. and A.M. Perks. 1969. New neurohypophysial principle in foetal mammals. Nature 223: 1169-1171.

Vizsolyi, E. and A.M. Perks. 1974. The effect of arginine vasotocin on the isolated amniotic membrane of the guinea pig. Can. J. Zool. 52: 371-386.

Wagner, G. 1966. Induction of abortion or labor by intra-amniotic injection of hypertonic solutions. Clin. Obstet. Gynec. 9(2): 520-534.

Wagner, J.E. and P.J. Manning (eds.). 1976. The Biology of the Guinea Pig. Academic Press. New York.

Walter, R. And R.H. Bowman. 1973. Mechanism of inactivation of vasopressin and oxytocin by the isolated perfused rat kidney. Endocrinology 92: 189-193.

Walter, R. and H. Shlank. 1975. Differences in the enzymatic inactivation of arginine vasopressin and oxytocin by rat kidney homogenate. Endocrinology 96: 811-814.

Walter, R. and W.H. Simmons. 1977. Metabolism of neurohypophyseal hormones: considerations from a molecular viewpoint. In Neurohypophysis. Eds., Moses, A.M. and L. Share. Karger. Basel. p. 167-188.

Weitzman, R. E. and D.A. Fisher. 1978. Arginine vasopressin metabolism in dogs. I. Evidence for a receptor-mediated mechanism. Am. J. Physiol. 235(6): E591-E597.

Weitzman, R.E., T.H. Glatz, and D.A. Fisher. 1978. The effect of hemorrhage and hypertonic saline upon plasma oxytocin and arginine vasopressin in conscious dogs. Endocrinology 103(6): 2154-2160.

Wiriyathian, S., J.C. Porter, R.P. Naden, and C.R. Rosenfeld. 1983. Cardiovascular effects and clearance of arginine vasopressin in the fetal lamb. Am. J. Physiol. 245(1): E24-E31.

Woods, L.L. 1986. Fetal renal contribution to amniotic fluid osmolality durin maternal hypertonicity. Am J. Physiology 250: R235-R239.

Woods, L., C.Y. Cheung, G.G. Power, and R.A. Brace. 1986. Role of arginine vasopressin in fetal renal response to hypertonicity. Am. J. Physiol. 251: F156-F163.