BINDING OF OXYTOCIN TO RECEPTORS IN LACTATING MAMMARY GLAND

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ABSTRACT

Oxytocin is a nonapeptide hormone which is synthesized in the hypothalamus and stored in the posterior pituitary gland (neurohypophysis). It has two known biological activities in mammals: 1) Oxytocin stimulates uterine contraction. Its precise role in the initiation of labour and function during parturition is not as yet well defined; 2) Oxytocin causes milk ejection from the mammary gland during lactation. This research has been involved with the latter aspect.

In mammary gland, the target cells for oxytocin are myoepithelial cells. Interaction of oxytocin with specific receptor molecules, thought to be present in the outer, or plasma membrane of these cells causes their contraction and results in ejection of milk from the gland. The goal of this research has been to identify specific receptors for oxytocin in mammary gland from lactating rabbits, to examine some properties of the binding of oxytocin to its receptor and to solubilize the receptor with detergents to permit future examination of its properties in isolation from other plasma membrane components.

Receptors for oxytocin can be identified in mammary gland from lactating rabbits by using a radioactive hormone, tritiated oxytocin ([^{3}H]-oxytocin) to bind to the receptors. This receptor meets several criteria of specificity for oxytocin. The active oxytocin analog [1-deamino]-oxytocin competes with [^{3}H]-oxytocin for binding; the almost inactive analog [4-proline]-oxytocin did not. The receptor was detectable in the target tissue, mammary gland, but was not detectable in a non-target tissue, rabbit liver. The receptor had a high affinity for oxytocin (K_d = 3.2 x 10^{-9} M) and had a maximal binding capacity in the preparation used for these studies of

385×10^{-15} moles per mg protein. A high affinity for the ligand is characteristic of hormone-receptor interactions. In view of the amount of oxytocin required for a biological response, the binding capacity found in this study indicates the presence of "spare" receptors for oxytocin.

The binding of [^{3}H]-oxytocin required the presence of divalent cations and was inhibited in the presence of EDTA. Re-addition of Mg^{2+} restored binding activity. Binding of [^{3}H]-oxytocin was reversible. At 37°C, dissociation of approximately 90% of the bound oxytocin required 30 minutes.

Partial purification of the particulate receptors was done by sucrose density gradient centrifugation. In one fraction, oxytocin binding activity was enriched 5-6 fold. 5'-Nucleotidase, a plasma membrane marker enzyme, was also enriched in this fraction to a similar extent. This provides evidence that the oxytocin receptor is present on the plasma membranes of its target cells.

The particulate oxytocin receptor was treated with the detergents deoxycholic acid, Triton X-100 and Lubrol-PX in an attempt to obtain a soluble receptor. Such treatment prevented the binding of [^{3}H]-oxytocin. The amount of binding ability destroyed was dependent upon the concentration of detergent employed. Removal of the detergent did not restore the ability to bind oxytocin. Incubation of the particulate receptor with [^{3}H]-oxytocin prior to detergent treatment permitted the recovery of a hormone-receptor complex. Most of the complex remained in the particulate fraction. Approximately 25% was still present in the supernatant following centrifugation at 210,000 x $\bar{g}_{av.}$ for 30 minutes. This portion of the hormone-receptor complex may be considered "solubilized", as judged by this single criterion.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

av.	=	average
c̄	=	containing
cAMP	=	3', 5'-cyclic adenosine monophosphate
Ci	=	Curie = 2.22×10^{12} disintegrations per minute
cm	=	centimetre = 10^{-2} metres
CMC	=	critical micellar concentration
DOC	=	deoxycholic acid
DPM	=	disintegrations per minute
ED_{50}	=	one-half the maximally effective concentration
EDTA	=	ethylenediamine tetraacetic acid
f	=	femto = 10^{-15}
$\bar{g}$	=	acceleration due to gravity
g	=	gram
h	=	hour
hCG	=	human chorionic gonadotropin
K	=	kilo = 10^{3}
K_d	=	dissociation constant
μ	=	micro = 10^{-6}
m	=	milli = 10^{-3}
M	=	molar
max.	=	maximum
mg	=	milligram = 10^{-3} gram
min	=	minute

ml	=	millilitre = 10^{-3} litres
N	=	Normal
nm	=	nanometre = 10^{-9} metres
p	=	pico = 10^{-12}
PAGE	=	polyacrylamide gel electrophoresis
PEG	=	polyethylene glycol
P_i	=	inorganic phosphate
R_f	=	Void volume/Elution volume (Partition chromatography), or Component migration/Solvent front (Thin-layer chromatography)
S	=	Svedberg unit = 10^{-13} seconds
S.D.	=	standard deviation
SDS	=	sodium dodecyl sulfate
TM	=	Tris-Magnesium buffer
TMG	=	Tris-Magnesium-Gelatin buffer
Tris	=	Tris (hydroxymethyl) aminomethane
U	=	International unit
V	=	Volts

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INTRODUCTION

Membrane biochemistry and endocrinology have met at a challenging interface in the study of membrane bound hormone receptors. Although in the past receptors were spoken of as a concept more than a molecular entity, now they are defined as, "Molecules uniquely capable of recognizing and interacting with a ligand with a high degree of selectivity and affinity and that in addition possess the capability to convey the occurrence of the interaction to biochemical processes that find expression in metabolically significant events" (Cuatrecasas, 1972b). One such class of receptors is that for the peptide hormones. Because these receptors are membrane bound, they require solubilization and purification from the other membrane components if they are to be studied. This approach provides an alternative to the studies on the relationship between chemical structure and biological activity of the neurohypophysial hormones. Although a large amount of data has been collected as a result of these studies, it has not provided enough information to evaluate the mechanism of action of neurohypophysial hormones. This problem has been recently reviewed by Jard and Bockaert (1975). Once a purified receptor molecule has been obtained, answers to several questions can be sought, eg: what constitutes the receptor "active sites"; what structural changes occur upon binding of the hormone; what other membrane components interact structurally or functionally with a receptor; how similar are receptors from the hormones different target tissues; how are receptors altered in relation to different hormonal or physiological states

of the tissue; can a physiologically responsive system be reconstituted from the isolated receptor and membrane components. Such studies are, of course, in their early stages and many difficulties have yet to be overcome. The studies reported in this thesis are concerned with the purification, solubilization and examination of some properties of a component present in rabbit mammary gland which binds the neurohypophysial hormone, oxytocin. This component is thought to be a specific receptor for oxytocin.

I. The Neurohypophysial Hormone, Oxytocin

a. Discovery and Chemical Structure

Oxytocin (Figure 1) belongs to a group of chemically related neurohypophysial hormones found in mammals, birds, amphibians, reptiles and fish. These compounds contain nine amino acid residues. Two hemicystine residues, at positions one and six form a disulfide bond to give a twenty-atom ring. In addition to oxytocin, two related hormones are found in mammals, 8-arginine-vasopressin and 8-lysine-vasopressin. 8-Lysine-vasopressin is present in some ungulates.

Oliver and Shafer (1895) were the first to discover the vasopressor activity of mammalian pituitary extracts. The presence of two active fractions in pituitary extract was later demonstrated by Dudly (1919). Kamm and co-workers (1928) achieved some separation of the two active peptides which they called alpha- and beta-hypophamine. These names were later changed to oxytocin and vasopressin, respectively (Bugbee and Kamm, 1928). It was not until 1949 that Livermore and du Vigneaud purified a preparation of oxytocin which was highly potent.

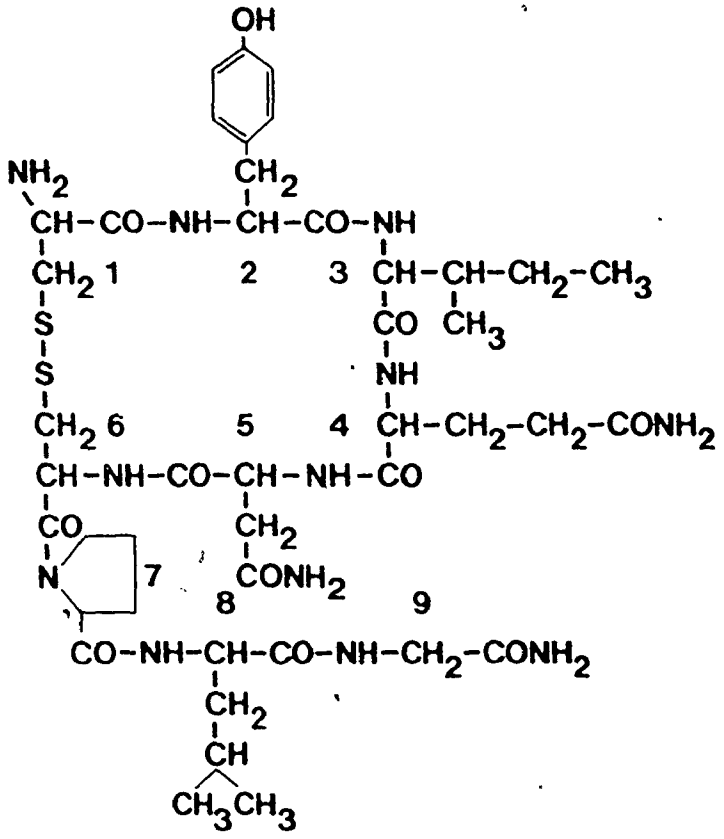


FIGURE 1. Structure of Oxytocin. The numbers indicate the following amino acid residues.

1 = Cysteine
2 = Tyrosine
3 = Isoleucine
4 = Glutamine
5 = Asparagine
6 = Cysteine
7 = Proline
8 = Leucine
9 = Glycinamide

The chemical structure of oxytocin was arrived at and proposed independently by Tuppy and coworkers (Tuppy 1953, Tuppy & Michl, 1953) and du Vigneaud and coworkers (du Vigneaud et al., 1953). The proposed structure was confirmed by the chemical synthesis (du Vigneaud et al., 1953, 1954).

b. Milk ejection activity of oxytocin

Ott and Scott (1910) demonstrated that a posterior pituitary extract given intravenously, increased milk flow from the cannulated teat of a lactating goat. Similar work with other species including man rapidly followed. Schafer (1915) suggested that posterior pituitary extracts caused expulsion of milk from mammary gland by causing contraction of tissue in the walls of the mammary alveoli rather than by direct action on the secretory epithelium. He demonstrated this by photographing cat alveoli following injection of a posterior pituitary extract. Alveoli taken from the region of an intact nipple were distended whereas those from the region of a nipple excised to allow free flow of milk were empty. In addition, repeated administration of the extract did not lead to milk ejection unless time was allowed for prior accumulation of milk. Gaines (1915) extended the work by showing that a central nervous system reflex, initiated by suckling, was involved. Furthermore suckling at one teat led to an increased intramammary pressure in the opposite gland as well as the one stimulated by suckling.

Using fractionated posterior pituitary extracts, Ely and Peterson (1939, 1940, 1941) were able to show that the hormone involved in milk-ejection was oxytocin. Using partially fractionated posterior pituitary extract they showed that the fraction containing more oxytocin was more

potent in causing milk-ejection in cows. The ability of oxytocin to cause milk-ejection, mimicking the effect of suckling, has supported the belief that the hormone responsible for milk-ejection is oxytocin. For a thorough review of the role of oxytocin in milk ejection, see Bisset (1968).

The contractile cell responsible for the ejection of milk from the alveoli in response to oxytocin is the myoepithelial cell, also called basket cells or myothelia. Similar cells are found in a variety of glandular tissues such as salivary and lacrimal glands. Benda described the presence of myoepithelial cells in mammary gland in 1894. However, it was not until the work of Richardson (1947, 1949, 1950a, 1950b) that this cell was convincingly shown to be the target cell for oxytocin. Using a technique of silver impregnation to selectively stain myoepithelial cells, he described the morphological changes of the cells during contractions of the mammary gland alveoli. He observed that the thin processes of these cells were shorter and thicker when the alveoli contracted and that folds in the alveoli were apparently caused by the myoepithelial cells. These findings in goat mammary gland were confirmed and extended to other species (man, dog, cat, rabbit, rat) by Linzell (1952, 1959).

The effect of myoepithelial cells in facilitating milk ejection is two-fold. As described above, one effect is the squeezing of the milk-distended alveoli. In addition, the myoepithelium which extends longitudinally onto the small collecting ducts contract to shorten and widen the ducts, thus reducing resistance to the flow of milk from the contracting alveoli. Linzell (1954, 1955) demonstrated this process <u>in situ</u> in

lactating mice, rats, guinea pigs and rabbits. Contraction and widening of the ducts was observed even when the alveoli were empty, demonstrating that the dilatation of the duct was not a passive expansion due to milk being forced into the ducts. (For reviews, see Folley and Knaggs, 1970; Hamperl, 1970). In addition to the direct observation of the myoepithelial contractile ability, the presence of actomyosin has been shown in these cells. Archer and Kao (1968), using a fluorescein labeled antibody to actomyosin, observed fluorescent staining of human mammary tissue in the regions corresponding to the myoepithelium. Other tissue such as salivary gland also reacted positively in regions containing myoepithelial cells.

c. Other biological activities of the neurohypophysial hormones.

In addition to the milk-ejection activity already discussed, there are several other recognized biological activities of the neurohypophysial hormones. Dale (1906, 1909) demonstrated their uterotonic activity. The mammalian antidiuretic activity was discovered shortly thereafter (Farini, 1913; von der Velden, 1913) followed by the discovery of their water retention activity in amphibia (Brunn, 1921). In birds, the hormones lower the blood pressure whereas in mammals, they raise the blood pressure (Paton and Watson, 1912). Five of the biological activities are used for bioassay and characterization of neurohypophysial hormones and their analogs (Table 1).

Neurohypophysial hormones, particularly oxytocin, also have metabolic effects apparently unrelated to the activities mentioned above (Mirsky, 1968). The most thoroughly investigated is the insulin-like activity of oxytocin. Like insulin, oxytocin acts on adipose tissue in

TABLE 1

Biological Activities of Naturally Occurring Neurohypophysial Hormones*

HORMONE	BIOLOGICAL ACTIVITY (Potencies are given in International Units** per mg)				
	Milk-ejecting (Rabbit)	Vasodepressor (Chicken)	Uterotonic (Rat Uterus)	Pressor (Rat)	Antidiuretic (Rat)
Oxytocin	450 ± 30	450 ± 30	450 ± 30	5 ± 1	5 ± 1
Arginine-Vasopressin (2-Phe- 8-Arg-Oxytocin)	69 ± 9	62 ± 6	17 ± 4	412 ± 41	465 ± 45
Lysine-Vasopressin (2-Phe-8-Lys-Oxytocin)	63 ± 10	42 ± 5	5 ± 0.5	285 ± 21	260
Arginine-Vasotocin (8-Arg-Oxytocin)	220	300 ± 42	120 ± 16	255 ± 16	260 ± 37
Mesotocin (8-Ile-Oxytocin)	330 ± 21	502 ± 37	291 ± 21	6.3 ± 0.8	1.1 ± 0.1
Isotocin (4-Ser- 8-Ile-Oxytocin)	290 ± 15	310 ± 15	145 ± 12	0.06 ± 0.01	0.18 ± 0.03
+Glumitocin (4-Ser- 8-Gln-Oxytocin)	53	---	10	0.4	0.5

* Modified from Berde, B. and Boissonnas, R.A. in, "Handbook of Experimental Pharmacology", Vol. 23, Ed. by B. Berde, pp. 806-807. Springer-Verlag. 1968.

** International Units are based on United States Pharmacopeia reference standards.

\+ From: Sawyer *et al*. 1969.

vitro to increase glucose uptake, glucose oxidation, lipogenesis from glucose and acetate, and protein synthesis. Glucose oxidation is stimulated both *in vivo* and *in vitro* and a positive correlation exists between the uterotonic activities of oxytocin and its analogs and the glucose oxidation activities of these compounds. The significance of the metabolic effects of these hormones is not known.

II. Binding Studies with the Neurohypophysial Hormones

a. Binding of Oxytocin to Mammalian Tissues

The possibility of studying binding of a hormone to its target tissues has come about recently as a result of the availability of tritiated or radioactive iodinated hormones. All studies thus far reported on oxytocin binding, with one exception, have utilized oxytocin tritiated in the tyrosine ring. The study which was done with monoiodo-oxytocin (^{125}I-oxytocin) showed that this compound has altered biological activities, exhibiting only 10-40% of the activity of oxytocin in stimulating adenylate cyclase in toad bladder epithelium and 75-80% of its ability to stimulate glucose oxidation in isolated fat cells (Thompson *et al.*, 1972). Monoiodo-oxytocin was bound by isolated fat cells but a variety of related peptides (lysine-vasopressin and arginine-vasopressin) were almost equally potent in competing with the iodinated hormone for the binding sites. No binding to rat uterus or toad bladder was observed. Other biological activities which are characteristic of oxytocin were not examined. The altered properties of the iodinated hormone indicate that iodine is not a satisfactory choice of isotope for binding studies with this hormone.

Several previous studies concerned with binding of [^{3}H]-oxytocin to mammary gland have been reported. Egan and Livingston (1971, 1973)

demonstrated uptake of oxytocin by pieces of mammary gland from lactating rats. This uptake appeared to be specific since non-radioactive oxytocin competed effectively for the binding sites. Uptake by the non-target tissues, heart and skeletal muscle, was much lower than uptake by mammary gland, and competition with cold oxytocin was either very slight or nil.

Similar work has been done by Soloff and co-workers (1972), who used pieces of mammary tissue from lactating rats. They observed greater uptake of the hormone in mammary gland than in the non-target tissue, abdominal muscle. They extended the work by showing that non-radioactive oxytocin and active analogs competed for the uptake of [^{3}H]-oxytocin. The degree to which the analogs competed was related to their milk-ejection potentcy. This finding strengthened the possibility that specific hormone receptors were responsible for the uptake of [^{3}H]-oxytocin in lactating mammary gland. In a continuation of this work, Soloff and Swartz (1973) examined sub-cellular fractions of mammary gland from lactating rats. Particulate material sedimenting at 1,000, 20,000 and 105,000 x $\bar{g}$ as well as the 105,000 x $\bar{g}$ supernatant was examined for binding of [^{3}H]-oxytocin. Binding was observed in all of these fractions, with the greatest amount of specific binding occurring in the 105,000 x $\bar{g}$ pellet. The 20,000 x $\bar{g}$ pellet was, however, used for their subsequent studies. Attempts to correlate the amount of binding in a fraction to the presence of marker enzymes for specific organelles such as 5'-nucleotidase for plasma membranes and succinic dehydrogenase for mitochondria were not successful. Characterization of binding to the 20,000 x $\bar{g}$ pellet showed that divalent cations were required for binding. The most effective were Co^{2+} and Mn^{2+}, followed by Mg^{2+} and Zn^{2+}. Calcium ions

were not required for optimal binding. It has long been recognized that divalent cations enhance the potency of oxytocin, both in rat uterus (Fraser, 1939; Stewart, 1949) and rat mammary gland (Somlyo et al., 1966). In the studies of Soloff and Swartz (1973), binding was optimal at pH 7.6, and the time course of binding at 20°C showed maximal binding after approximately 40 minutes. As was observed with pieces of mammary gland, non-radioactive analogs competed for binding in relation to their biological potency. Scatchard analysis (Scatchard, 1949) indicated a K_d of 9.5×10^{-10} M and a binding capacity of 0.28 pmoles per milligram protein for oxytocin.

The uterus, another target tissue for oxytocin, has also been shown to bind [^{3}H]-oxytocin. Sjöholm and Rydén (1969) observed a rapid uptake of [^{3}H]-oxytocin by the uterine horns of non-pregnant rats which reached a maximum in less than 60 seconds after the intravenous injection of the radioactive peptide. This high initial uptake was interpreted as indicating preferential uptake of oxytocin by uterus. Egan and Livingston (1973) demonstrated specific uptake of [^{3}H]-oxytocin by pieces of rat uterus. Estrogen treatment, which is known to increase uterine sensitivity to oxytocin (Csapo, 1960, 1961), increased the uptake of oxytocin. Conversely, progesterone treatment lowered the uptake of oxytocin to levels observed in non-target tissues. Soloff and Swartz (1974) have studied uterine oxytocin receptors in rat and sow using the techniques already described for mammary gland. The properties of this receptor were similar to those of the mammary gland receptor with respect to divalent cation requirement, pH optimum, and time required for binding. Scatchard analysis indicated

a K_d of 1.8×10^{-9}M with a capacity of 0.18 pmoles/mg protein. For the sow receptor, these values were 1.5×10^{-9}M and 0.15 pmoles/mg protein. Differences were observed in the ability of some analogs to compete with [^{3}H]-oxytocin binding to the rat and the sow uterine receptors, but these differences were attributed to the possibility of the analogs having different potencies in the sow and in the rat.

Human uteri (Soloff et al., 1974) have also been examined for specific oxytocin binding. Receptors were found in pregnant uteri (14-16 weeks) with properties similar to those in the rat and sow. Scatchard plots (Scatchard, 1949) indicated a K_d of approximately 2×10^{-9}M and a capacity of 0.185 pmoles per mg protein. Oxytocin analogs competed with [^{3}H]-oxytocin binding in relation to their biological potency.

The effect of estrogens on rat uterine oxytocin receptors has been recently studied (Soloff, 1975a). Soloff found that subcutaneous injections of diethylstilbestrol led to an apparent increase in both affinity and number of binding sites present per uterus. The increase in affinity was apparent 6 hours after treatment, whereas the increase in binding sites was seen 12 hours after treatment. After 24 hours, the longest time studied, the affinity increased 4.2 times and the number of binding sites per uterus increased 2.1 times. Whether these alterations were due to synthesis of new receptors or modification and unmasking of existing receptors was not determined. However, the fact that an increase in affinity is observed prior to an increase in the number of binding sites suggests that, at least in part, existing sites may be modified by estrogen treatment.

Although mammary gland and uterus are the two accepted targets for oxytocin, two other tissues have been studied with respect to oxytocin binding. Rorie and Newton (1965) demonstrated that both puerperal and non-puerperal fallopian tubes respond to oxytocin (8μU/ml) with increased amplitude of contraction. Puerperal tubes increased frequency of contraction as well, but non-puerperal tubes did not. Soloff (1975b) studied binding of [^{3}H]-oxytocin to unpurified subcellular fractions of rat oviduct. High affinity sites (K_d=5.4 x 10^{-10}M) were found. Analogs competed for binding in relation to their biological potency with the exception of lysine-vasopressin which showed more binding than would be expected on the basis of its uterotonic activity. Uptake by oviduct has also been shown autoradiographically (Soloff, 1975a). Although these studies indicate interaction of oxytocin with some component of oviductal tissue, they do not establish oviduct as a physiologically significant target tissue since the role of oxytocin in movement of ova through this organ in mammals has not been proven.

As previously discussed, oxytocin stimulates glucose oxidation in adipose tissue. Binding of [^{3}H]-oxytocin has been reported for rat periepididimal fat cells (Bonne and Cohen, 1975). A K_d of 5 x 10^{-9}M and a capacity of 3 x 10^4 sites per cell was found. This binding did not appear to involve insulin receptors. The physiological significance of oxytocin receptors on fat cells is not known.

b. Binding of Oxytocin to Non-Mammalian Tissues

Neurohypophysial hormones stimulate water retention in amphibia. Therefore, frog skin and bladder have been studied to investigate the presence of specific receptors for these hormones. Oxytocin induces two

responses in both of these tissue, a hydroosmotic and a natriferic response. The receptors for these responses appear to be separate structures since oxytocin and analog peptides have different ratios of natriferic to hydroosmotic activity. Bockaert and coworkers (1970) observed specific binding of [^{3}H]- oxytocin to frog skin epithelium with a time course which approximated the time course of the natriferic response. Criteria of specificity were, as in the mammalian studies, competition by oxytocin and analogs to a degree similar to their biological activities. They could not, however, detect specific binding to the frog bladder. Although they asserted that this finding confirmed an earlier study (Gulyassy and Edelman, 1965) in which specific binding by toad bladder could not be detected, this conclusion is doubtful since the very low specific activity (140 mCi/mMole) used in this early study would make detection of specific binding at low hormone concentrations difficult. A continuation of the study with frog skin epithelium (Bockaert et al., 1972) established the presence of a population of binding sites with the characteristics expected of receptors responsible for the natriferic response. These characteristics included a somewhat faster time course for binding than for the biological response and an apparent K_d value of 2.5×10^{-9}M. This value was very similar to that determined from the dose response curve. In addition, competition for binding by analogs occurred in approximate relation to their biological potency. In addition to the binding sites responsible for the biological response, another set of sites was observed which had a high capacity but low affinity for oxytocin. These sites could not be related to a biological activity of oxytocin. Another phenomenon observed in this system was an apparently irreversible

component of binding. Some of the oxytocin bound was not removed by a one hour wash in Ringer's solution. However, the addition of 10mM dithiothreitol to the media resulted in release of this material. A similar observation made in the study of vasopressin binding to renal medulla (Schwartz et al., 1960), led to the suggestion that covalent bonding of the hormone to a receptor site through a disulfide bond might be required for hormonal activity. However, analogs which lack the disulfide bond retain some biological activity (Jošt and Rudinger, 1967). Therefore, it is unlikely that a process of disulfide exchange is required for biological activity. The irreversible component of binding observed in frog skin is probably not related to the natriferic response.

III. Binding Studies with Other Hormones

A large number of binding studies have been carried out with all classes of hormones. This discussion will be confined to receptors for peptide hormones, and in particular to those receptors which have been solubilized with the use of detergents or other membrane disrupting treatments. The hormones used in these studies can be divided into those which are iodinated and those which are tritiated to obtain a radioactive compound. In general, the larger peptide hormones are iodinated since apparently the large size of the iodine atom does not lead to severe biological alterations of these compounds. The smaller hormones such as oxytocin, vasopressin and angiotensin are tritiated because, as mentioned previously, the introduction of a large iodine atom leads to alteration of their biological properties. There are several technical and theoretical disadvantages to both isotopes. These are outlined in Table 2. The hormones which will be discussed are: ACTH, gonadotropins, glucagon,

TABLE 2

Advantages and Disadvantages of the Use of Tritiated or Iodinated Hormones for Receptor Binding Studies

^{3}H	^{125}I or ^{131}I
Advantages:	Advantages:
1. Does not seem to alter biological properties of the hormone.	1. Iodination can be done quickly in the laboratory. 2. Prolonged storage of iodinated peptide not required. 3. Very high specific activities (1000-3000 Ci/mmole) can be obtained which makes receptor detection more sensitive.
Disadvantages:	Disadvantages:
1. Usually obtained commercially due to difficult preparation. Cost is high. 2. Must be stored for prolonged periods since it is obtained commercially. 3. Requires frequent checks of purity. Stability of such compounds is not well characterized. 4. Relatively low specific activity of (29.4 Ci/mmole) compounds makes detection much less sensitive.	1. Alters the biological properties of small peptide hormones such as angiotensin and the neurohypophysial hormones. 2. May cause alterations in larger peptide hormones if mild conditions are not used for iodination.

insulin, prolactin, angiotensin and vasopressin. A comprehensive discussion would be outside the scope of this thesis but what follows should give an idea of the findings obtained from a variety of peptide hormone-receptor systems.

a. Adrenocorticotropic Hormone (ACTH)

ACTH, a pituitary hormone responsible for stimulating steroidogenesis in the adrenal cortex was the first hormone for which a receptor was extracted from a subcellular fraction. The group of Pastan (Lefkowitz et al., 1970; Pastan et al., 1970) using a murine adrenal tumour responsive to ACTH, solubilized the hormone receptor by passing a low speed subcellular fraction suspended in an emulsion of phosphatidylethanolamine containing NaF through a French press. A clear extract containing the receptor was obtained by centrifugation at 105,000 x $\bar{g}$ for 1 h. The size of the adrenal ACTH receptor in this extract was estimated to be 3-7 x 10^6 daltons, suggested that the soluble material was not a single monomolecular protein species. A major point of interest in this study was the observation that not only ACTH receptor, but also the adenylate cyclase activity was solubilized and remained sensitive to stimulation by ACTH.

Attempts to solubilize with Triton X-100 were unsuccessful, as were attempts to use acetone, sodium dodecylsulfate, sodium deoxycholate or sonication in the absence of a phospholipid emulsion.

b. Gonadotropins

Gonadotropin receptors from both ovary and testis have been examined using ^{125}I-hCG and a membrane fraction sedimented at 1500 x $\bar{g}$ for 15 minutes (Catt, et al., 1972). Binding was higher at 24°C than at 4°C or 37°C. The rate of binding was found to be quite slow,

taking up to 24 hours at 24°C to plateau. As with ACTH, these hormones act through cAMP to stimulate steroidogenesis in their target tissues (Dufau, et al., 1973a). An increase in cAMP production could be observed as early as one minute following addition of hCG to rat testis and became maximal after 3 hours. It would, therefore, appear that the time required for maximal binding cannot be correlated with the time required for this maximal biological response. The comparatively long time required for maximal binding to be reached suggested that only a small number of binding sites need be occupied in order to elicit a physiological response, and that the testis contains a large number of spare receptors. Scatchard analysis gave a K_d of 0.77×10^{-10} M and a capacity of 0.93×10^{-12} moles of receptor per testis.

Solubilization of these receptors has been accomplished with non-ionic detergents (Charreau et al., 1974) and they have been found to exist in multiple forms. The receptors were stable to exposure to 1% Triton X-100 at 4°C for 30 min. The soluble receptors had a sedimentation coefficient of 6.5S as compared to 7.5S for the hormone-receptor complex. Removal of detergent caused conversion of the 7.5S form to an 8.8S form, which could also be obtained if hormone was incubated with receptor prior to detergent treatment. It was also observed that prior incubation of the receptor with hormone conferred on it a greater stability than the unoccupied receptor when stored at 0-4°C. Thus, pre-incubation with hormone altered the stability of the receptor.

In addition to the gonadotropin receptor from rat testis, the receptor from rat ovary has also been solubilized (Dufau et al., 1974). Again, the receptors were stable to 1% Triton X-100. Density gradient

centrifugation was used to derive an estimated molecular weight of 228,000. As with the testicular receptor, a different sedimentation coefficient, 6.0-6.7S, was observed when no hormone was bound to the receptor as compared to the 7.5S hormone-receptor complex. Furthermore, an 8.8S complex was obtained if incubation with hormone was carried out prior to detergent treatment. In general, it appears that gonadotropin receptors solubilized from rat testis and ovary are quite similar.

c. Prolactin

Prolactin is of interest to these studies with oxytocin because rabbit mammary gland is a target tissue for both of them. Birkinshaw and Falconer (1972) observed autoradiographically a localization of iodinated prolactin in the region of the alveolar secretory membrane on the side adjacent to the vascular supply. The ductal side of the membranes showed no hormone bound even when it was administered into the ducts. The mean half-life of the hormone in the circulation was 16 minutes. When bound to its target tissue the half-life was prolonged to approximately 50 hours. These studies, done primarily in vivo, prompted Shiu and coworkers (1973) to develop an assay for prolactin based on binding of [^{125}I]-prolactin to receptors present in a membrane fraction from mid-pregnant rabbit mammary gland. This assay was found useful for prolactin and other hormones with lactogenic activity such as human growth hormone. In a later study (Shiu and Friesen, 1974a) the characteristics of prolactin binding to mammary membranes were investigated. Binding was affected by temperature, being higher at 37°C than at 23°C or 0°C. Approximately 3 h were required for binding to plateau at 37°C. Dissociation of the hormone from the receptor was also dependent on temperature. At 0°C only

about 5% of the bound hormone dissociated after 25 hrs, compared to 80-85% at 37°C. Binding showed a pH optimum between 7.5-8.5. The dissociation constant found both from Scatchard plots and Lineweaver-Burk plots was 3.4×10^{-10}M.

The prolactin receptor has been solubilized using Triton X-100 (Shiu and Friesen, 1974b). A membrane fraction prepared from rabbit mammary gland was treated with 1% Triton X-100 for 30 min at 23°C, and the supernatant remaining following centrifugation for 2 hrs at 200,000 x $\bar{g}$ contained the prolactin receptor. One difficulty which arose in this work was the alteration of the physical properties of [^{125}I]-prolactin by concentrations of Triton X-100 higher than 0.01%. For this reason, [^{125}I]-growth hormone which is unaffected by Triton and, in rabbits, has the same lactogenic potency as prolactin was used for this study. This soluble receptor had an apparent molecular weight of 220,000. Unlike the situation with other soluble receptors, the affinity of the hormone for the solubilized receptor was five times higher than for the particulate receptor. A partial purification (1500-fold) of the receptor was done by affinity chromatography using growth hormone coupled to agarose. Previous work (Turkington, 1970) had shown a complex of prolactin coupled to Sepharose to be fully biologically active.

d. Glucagon

The hepatic receptor for this hormone has been extensively studied, and much of the information has been discussed in a recent review (Blecher, et al., 1974). This is a convenient system for study since glucagon stimulates adenylate cyclase activity (Pohl et al., 1971), and therefore, allows a correlation of binding with biological activity.

In the particulate system, a single dissociation constant of approximately 10^{-10}M has been observed. Binding at 30°C is maximal after approximately 10 minutes. Dissociation of bound glucagon at 30°C is somewhat slower (Rodbell et al., 1971), reaching only 50% after 1 hour. The range of concentration for both glucagon binding and the glucagon stimulated adenylate cyclase activity were quite similar. Treatment with phospholipase-A led to loss of sensitivity of adenylate cyclase to glucagon and also abolished glucagon binding, implying a role for phospholipids as part of the receptor molecule. This observation is of interest since Schneider and Edelhoch (1972) have demonstrated binding of glucagon to the phospholipid, lysolecithin.

Early attempts to solubilize the adenylate cyclase as well as the binding activity were not successful. Triton X-100 and sodium deoxycholate both caused a complete loss of glucagon and NaF stimulated adenylate cyclase activity (Birnbaumer, et al., 1971). More recent work (Giorgio et al., 1974) has demonstrated that detergents inhibit hormone binding. It was essential to first remove the non-ionic detergent (Lubrol-PX) by ultrafiltration and then carry out hormone binding or to pre-incubate the plasma membrane material with the hormone prior to detergent treatment. The solubilized receptor had a dissociation constant of 1.14×10^{-10}M, very similar to that also observed for the particulate receptor. However, another low affinity site was observed. Due to its very low affinity it was not possible to accurately calculate its dissociation constant. An approximate molecular weight of 190,000 was calculated by exclusion chromatography on Agarose. In relation to the glucagon-stimulated adenylate cyclase activities the receptor was

apparently dissociated from the adenylate cyclase and the solubilized enzyme was not sensitive to the hormone. Therefore, it is not possible to state unequivocally that the hormone receptor studied was also responsible for adenylate cyclase stimulation.

e. Insulin

Receptors from several tissues for this hormone have been studied extensively, and probably constitute the most studied hormone-receptor system. A number of recent reviews discuss this area in detail (Katzen & Soderman, 1972; De Meyts, 1976; Kahn, 1976a). Progress has been rapid for several reasons: the studies employ [^{125}I]-insulin which can be easily prepared as needed, the receptor molecule itself appears to be quite stable, remaining capable of binding insulin after treatment with phospholipases or organic solvent or detergents (Cuatrecasas et al., 1971) and also the hormone remains active when coupled to Sepharose (Cuatrecasas, 1969), which has permitted purification of the receptor using affinity chromatography (Katzen and Soderman, 1972; Cuatrecasas, 1972a). Extensive work in this area has been done by Cuatrecasas, who has studied particulate receptors from both fat and liver cell membranes and found their characteristics to be very similar or identical (Cuatrecasas et al., 1971). Using these two tissues, he achieved solubilization of the insulin receptor using the non-ionic detergents Triton X-100 and Lubrol PX (Cuatrecasas, 1972b, 1972c). Unlike other systems, the formation of the hormone-receptor complex was not prevented until concentrations of Triton X-100 greater than 0.15% were reached. Another advantageous finding was the apparent unmasking of receptor sites by detergent, so that the amount of binding in the

supernatant was almost double that observed in the original particulate material. The dissociation constant was altered by solubilization, going from 5 - 9 x 10^{-13}M in intact cell or particulate fraction to 13 x 10^{-13}M with the solubilized material. Other characteristics of the liver and fat cell receptors were, as with the particulate system, similar or identical. In addition, the soluble receptor was quite stable to treatment by organic solvents, phospholipases, and high salt concentration, but was destroyed by trypsin. The sedimentation coefficient of the solubilized receptor was approximately 11S, and its molecular weight estimated to be 300,000. Attempts to obtain smaller components which would bind insulin were not successful.

Another source of insulin receptors which can be solubilized without the use of detergents is human lymphocytes (Gavin et al., 1972). Cultured lymphocytes released insulin receptors when shaken in buffer at 30°C for 70 min. The receptors then present in the supernatant bound [^{125}I]-insulin with the same characteristics as intact lymphocytes.

It is interesting to note that progress with this hormone has reached the point that Pullen and co-workers (1976) were able to study the receptor binding region of the insulin molecule in detail and suggest that synthesis of a simplified analog containing this portion of the molecule could make fully active "insulin" easily available.

f. Angiotensin-II

Angiotensin-II is a small peptide (8 amino acid residues) which causes contraction of smooth muscle. Early studies with [^{3}H]-angiotensin of low specific activity (300 μCi/mg) demonstrated that following infusion of the labeled peptide into rats, the kidneys, adrenals

and uterus were the tissues which retained the highest amounts of radioactive angiotensin (Bumpus et al., 1964). [^{125}I]-angiotensin has been used to study binding to several tissues, and uterus and kidney were again found to bind greater quantities than other tissues such as liver, spleen, muscle and lungs (Goodfriend and Lin, 1970; Lin and Goodfriend, 1970). However, as discussed previously, iodination of small peptide hormones can lead to significant alterations in biological activity. Mono-iodinated angiotensin had only 25-80% of the biological activity of native angiotensin, depending upon the assay system used (Lin et al., 1970). Di-iodoangiotensin was even less active. Recent work on the angiotensin receptor has utilized [^{3}H]-angiotensin. Glossman and coworkers (1974), studying the angiotensin-II receptors of bovine and rat adrenal cortex, found that the uptake of [^{3}H]-angiotensin by an impure membrane fraction was rapid, being maximal at 30 minutes. Dissociation was also rapid, taking approximately 30 min to reach 85% dissociation. Analogs were found to compete with angiotensin in approximate relation to their biological potencies. Brecher and coworkers (1974), in a less detailed study with adrenal glands also demonstrated binding in an extract of the tissue prepared in 0.4 M KCl, and obtained very similar results. Treatment with KCl might not have resulted in a truly solubilized receptor, since a very low centrifugal force was used to prepare the extract. Two other tissues widely used to study angiotensin binding are rabbit aorta and rat uterus. Although neither of these organs are thought to be physiological target organs, both contract in response to pharmacological doses of this compound. Since the K_d for binding closely approximates the ED_{50} of the biological response, they are considered valid model systems

(Devynck and Meyer, 1975). Rabbit aorta has been carefully studied, and receptors have been demonstrated in intact aorta (Baudouin et al., 1971), in the microsomal membranes (Baudouin et al., 1972), in a fraction derived from plasma membranes (Devynck et al., 1973) and in a 200,000 x $\bar{g}$ supernatant of a microsomal fraction treated with 0.2-1% deoxycholic acid (Devynck et al., 1974). Thus, in this case an ionic detergent was used successfully. Solubilization led to a decrease in the K_d from 5.5×10^{-8}M to 2.0×10^{-8}M in the microsomal membranes.

In addition to binding of the hormone, Ca^{2+} release has also been observed (Baudouin, et al., 1972; Limas and Cohn, 1973). This supports the view that the binding observed is related to the stimulus which leads to contraction in this tissue. In addition, a conformational change in membranes in response to angiotensin has been observed using spin label probes (Schreier-Muccillo, et al., 1974). The relationship of this conformational change to the contraction or Ca^{2+} release has not been established.

g. Vasopressin

Vasopressin, a nona-peptide neurohypophysial hormone structurally similar to oxytocin, has as its principal target tissue renal medulla where it stimulates production of cAMP and, physiologically, causes water retention. Extensive studies using [^{3}H]-lysine-vasopressin and plasma membranes derived from pig renal medulla established that stimulation of adenylate cyclase activation in this system was closely correlated with the amount of vasopressin bound (Bockaert et al., 1973). Analogs competed for binding with lysine-vasopressin in approximately the same relation-

ship as their ability to cause activation of adenylate cyclase. This, in addition to the fact that the time course of binding was essentially identical with stimulation of cAMP production, led the authors to conclude that the receptors studied were involved in the increased synthesis of cAMP and were the physiological receptors for lysine-vasopressin. These findings were confirmed and extended by examining the characteristics of a large number of analogs of vasopressin (Roy et al., 1975a) and of oxytocin (Roy et al., 1975b) in this system. This same approach has proven useful in studies on the solubilization of the vasopressin receptor. Neer (1973) reported the solubilization of adenylate cyclase from rat renal medulla using the detergent Lubrol-PX at a concentration of 1%. The solubilized adenylate cyclase preparation was sensitive to the addition of lysine-vasopressin at a concentration of 2-4 x 10^{-6}M which led to a 1.5-fold stimulation over the basal activity. Although this was not a large response, it does indicate that some of the receptor-adenylate cyclase complexes were intact and at least partially functional. Stimulation was not observed in the presence of detergent, nor were attempts at solubilization successful when purified membrane material was used instead of whole tissue. Although this latter phenomenon was not explained, it has been observed in other systems (Pohl et al., 1971). Although the main emphasis was on adenylate cyclase, this work may be the first successful attempt to solubilize receptors for neurohypophysial hormones. A more recent and detailed study of lysine-vasopressin receptors solubilized from pig kidney plasma membranes (Roy et al., 1975c) found that both adenylate cyclase and the vasopressin receptor could be solubilized with 0.5% Triton X-100. However, the adeny-

late cyclase was no longer sensitive to the hormone. Yields of only approximately 30% of the receptor were obtained unless the membrane bound receptor was first occupied with [^{3}H]-vasopressin. When incubation with [^{3}H]-vasopressin was carried out prior to detergent addition, the yield increased to 65-100%. The authors interpret the differences in yields as indication that the receptors exist in two forms, an accessible and an inaccessible form. The inaccessible form occurs in the absence of hormone. When pre-incubation is done, the accessible form is favoured and the presence of hormone-receptor complexes can be shown. Although this interpretation is consistent with the observations there are other possible explanations. For example, detergent could bind to the hormone receptor site and prevent the hormone from having access to it, or the presence of bound hormone could stabilize the receptor and prevent its partial denaturation.

IV. Detergents and the Solubilization of Membrane Proteins

The use of detergents for solubilization of biological membranes has recently been reviewed by Helenius and Simons (1975), and Coleman (1974). In this thesis the discussion will concentrate on detergents which have been used for the solubilization of peptide hormone receptors in general and particularly for oxytocin receptors.

Membrane proteins have been assigned to two classes, those which are loosely bound to the membrane, extrinsic proteins, and those which are firmly bound, intrinsic proteins (Capaldi and Green, 1972). The latter are presumably the more lipophilic proteins and are tightly held within the membrane lipid matrix. While extrinsic proteins can often be removed by fairly gentle methods such as the use of chelating agents, removal of the intrinsic proteins requires more vigorous treatment. Detergents are the

most frequently used agents for the removal of these components from the membrane. Detergents, like some other lipids, contain both hydrophilic and hydrophobic regions and are therefore amphiphiles. Two major classes of detergents exist: ionic and non-ionic. Of the ionic detergents, the most commonly used is deoxycholic acid (DOC) (Figure 2), a component of bile salts. Of the non-ionic detergents, the most commonly used is Triton X-100 (Figure 3) with the Lubrols being used with success in some systems.

Physical properties which are characteristic of each detergent are the critical micellar concentration (CMC) and aggregation number. The CMC is the concentration above which detergent molecules begin to associate to form aggregates or micelles. Ionic detergents have higher CMC values than non-ionic detergents. The CMC for DOC is 4-6 mM and for Triton X-100 approximately 0.24 mM. The aggregation number indicates how many molecules associate to form a micelle, which in turn determines micellar weight. DOC, with an aggregation number of 4-10 has a micellar weight of 1700-4200 daltons. Non-ionic detergents, however, form quite large micelles. Triton X-100 has an aggregation number of 140 with a micellar weight of 90,000 daltons.

It has been claimed that these detergents can extract many membrane proteins without disturbing their native, active conformation (Tzagoloff and Penefsky, 1971). The interaction of proteins with detergents has been extensively studied. Helenius and Simons (1972) examined this interaction using both hydrophilic and delipidated lipophilic proteins. Hydrophilic proteins such as albumin, aldolase and transferrin showed very little or no binding of the various detergents used in the study.

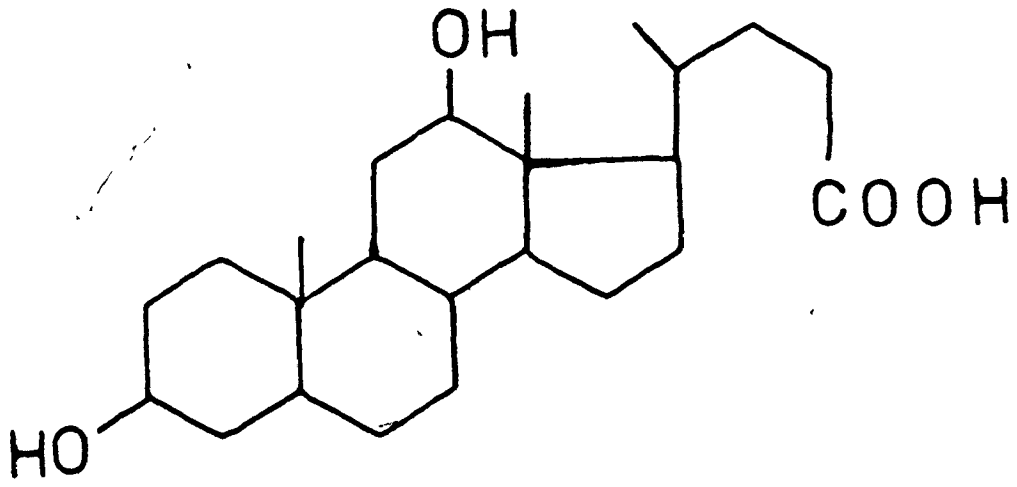


FIGURE 2. Structure of the Ionic Detergent Deoxycholic Acid (DOC)

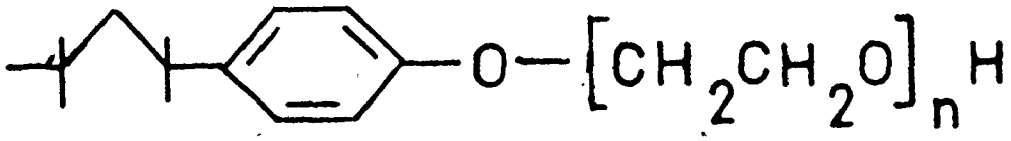


FIGURE 3. Structure of the Non-ionic Detergent Triton X-100. The characteristic structure of the Triton series of detergents is polyoxyethylene p-t-octylphenol. Triton X-100 is a mixture of molecules containing 9 and 10 oxyethylene units.

Lipophilic proteins such as Semliki Forest virus envelope and human erythrocyte stroma bound large amounts of DOC and Triton X-100, approximating 70% of their weight. On the basis of these studies they postulated that hydrophobic interactions are primarily responsible for the detergent-lipophilic protein interaction. Makino and co-workers (1973), using bovine serum albumin as a model protein confirmed the work of Helenius and Simons. DOC and Triton X-100 were shown to bind exclusively to high affinity sites on the protein. Binding to a limited number of such sites did not lead to co-operativity of binding. Since co-operative binding of detergents is a major cause for denaturation of proteins (as in the case of sodium dodecylsulfate), when DOC and Triton X-100 are used the risk of denaturation is markedly reduced. Co-operative binding of detergents requires higher concentrations of free (as opposed to micellar) detergent. Since the CMCs of Triton X-100 and other non-ionic detergents are quite low and the CMC for DOC is still not high enough for co-operative binding to occur, the high concentrations required for co-operative binding cannot be seen. Thus, these detergents are restricted from reaching free concentrations which could cause denaturation. It can be concluded that, "if a membrane protein can be extracted with detergents at all, the chances of obtaining it in its native conformation or something close to it are greatest if this class of reagent (DOC or Triton X-100) is used" (Makino et al., 1973).

Some studies have been carried out to clarify the parameters necessary for efficient solubilization of membranes by detergents. Miller (1970) found that erythrocyte membranes could be almost totally solubilized by Triton X-100 with a 2.7 mM Tris buffer of pH of 7.0.

Divalent cations (Ca^{2+} or Mg^{2+}) inhibited solubilization. However, addition of EDTA did not reverse this inhibition. It was concluded that the critical parameter was the solute concentration. Addition of EDTA raised the solute concentration which also inhibited solubilization. In a report conflicting with Miller (1970), Tzagoloff and Penefsky (1971) stated that Triton X-100 solubilized proteins more effectively in the presence of salts such as KCl at a concentration of 0.2-1.0 M. Salts were also found to increase DOC solubilization of mitochondrial membrane proteins (Burkhard and Kropf, 1964). Keeping the pH slightly alkaline seemed to increase the efficiency of membrane protein solubilization.

The lipid composition of membranes as well as the vescicle size has been shown to alter susceptibility to solubilization by Triton X-100. Onoue and Kitagawa (1976) demonstrated that liposomes of rat liver phosphatidylcholine and egg lecithin were resistant to detergent action. Furthermore, introduction of cholesterol into the liposomes inhibited lysis by Triton X-100. If these results, derived from an artificial system, are applicable to biological systems, a wide variation in susceptibility to detergent action can be expected depending upon the lipid composition of the organelle and tissue from which the membranes are derived.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

During the course of this study, the following tissues were used: rat kidney medulla, rat uterus, rat lactating mammary gland and rabbit lactating mammary gland. In addition, rabbit liver was used as a control tissue. Sprague-Dawley rats (200-250 g) and New Zealand rabbits (4-5 Kg) were used. Rats were sacrificed by decapitation and rabbits by injection of 5 ml of pentobarbital (65 mg/ml) into a marginal ear vein. The animals were obtained from local suppliers through the Health Sciences Centre.

Kidneys were removed from rats of either sex, the capsule and peri-renal fat removed, and the cortex dissected from the medulla. The medullary tissue was palced immediately in ice-cold buffer used for the preparation of plasma membranes.

Uteri were obtained from rats which were treated for three days with diethylstilbestrol (100 ug per day in a single subcutaneous injection) in sesame oil. Following decapitation, the uteri were removed and adhering membrane and fat removed. Each horn was cut longitudinally and the endometrium scraped from the underlying myometrium.

Mammary gland was obtained from rats approximately 12-15 days post-partum. Following removal, the tissue was frozen in liquid N_2 until used. Approximately 40-50 g of tissue was obtained from each animal.

Rabbit mammary gland, the principal tissue used in this study, was obtained 10-12 days post-partum from a female having a litter of at least three. In the later experiments, the litter was removed 24 hours prior to sacrifice of the animal. Mammary tissue was excised, and

as much membranous material removed as possible. The tissue was then frozen immediately in liquid N_2. Frozen tissue was stored at -60°C, with portions being removed and thawed as required. No loss of oxytocin binding ability was noticed after storage for several weeks.

Rabbit mammary gland was chosen for the following reasons: (1) a large amount of tissue can be obtained from a single animal; (2) rabbit mammary gland contracts specifically to oxytocin. Other compounds such as bradykinin and angiotensin elicit a milk ejection response in both rats and guinea pigs, but not in rabbits (Bisset, 1968).

Subcellular Fractionation and Centrifugation

In the subcellular fractionation procedures described subsequently, all steps were carried out at 0-4°C unless otherwise stated. All centrifugations were done either in an International Equipment Company (I.E.C.) centrifuge Model PR-J which was equipped, when necessary, with a high-speed attachment (I.E.C. 2768), or in an I.E.C. ultra-centrifuge Model B-60. Gravitational forces are reported as the force at the middle of the centrifuge tube.

Sucrose gradients (10 ml; 10-40%) were prepared either in thin-walled polyallomer or cellulose nitrate tubes using a Buchler double conical chamber gradient former attached to a Buchler Polystaltic pump. Gradients were left at 4°C overnight prior to use. Gradient fractions were collected from the bottom of the tube. Periodically, gradients were checked for consistency and linearity by examination of refractive index of 0.5 ml fractions with a Bausch and Lomb refractometer.

Preparation of Plasma Membranes from Rat Kidney Medulla

Several methods for the preparation of plasma membranes from rat kidney have been reported (Manitius, et al., 1968; Fitzpatrick, et al., 1969; Price, et al., 1972). The procedures of Manitius and co-workers (1968) and Fitzpatrick and co-workers (1969) were used and compared. The procedure of Manitius was used as modified by Ray (1970) by the addition of 0.5 mM $CaCl_2$ to the medium. This modification improved both the yield and the purity of the plasma membrane fraction which was obtained, as compared to the unmodified procedure. Figure 4 outlines this procedure.

Preparation of Plasma Membranes from Uteri and Mammary Gland

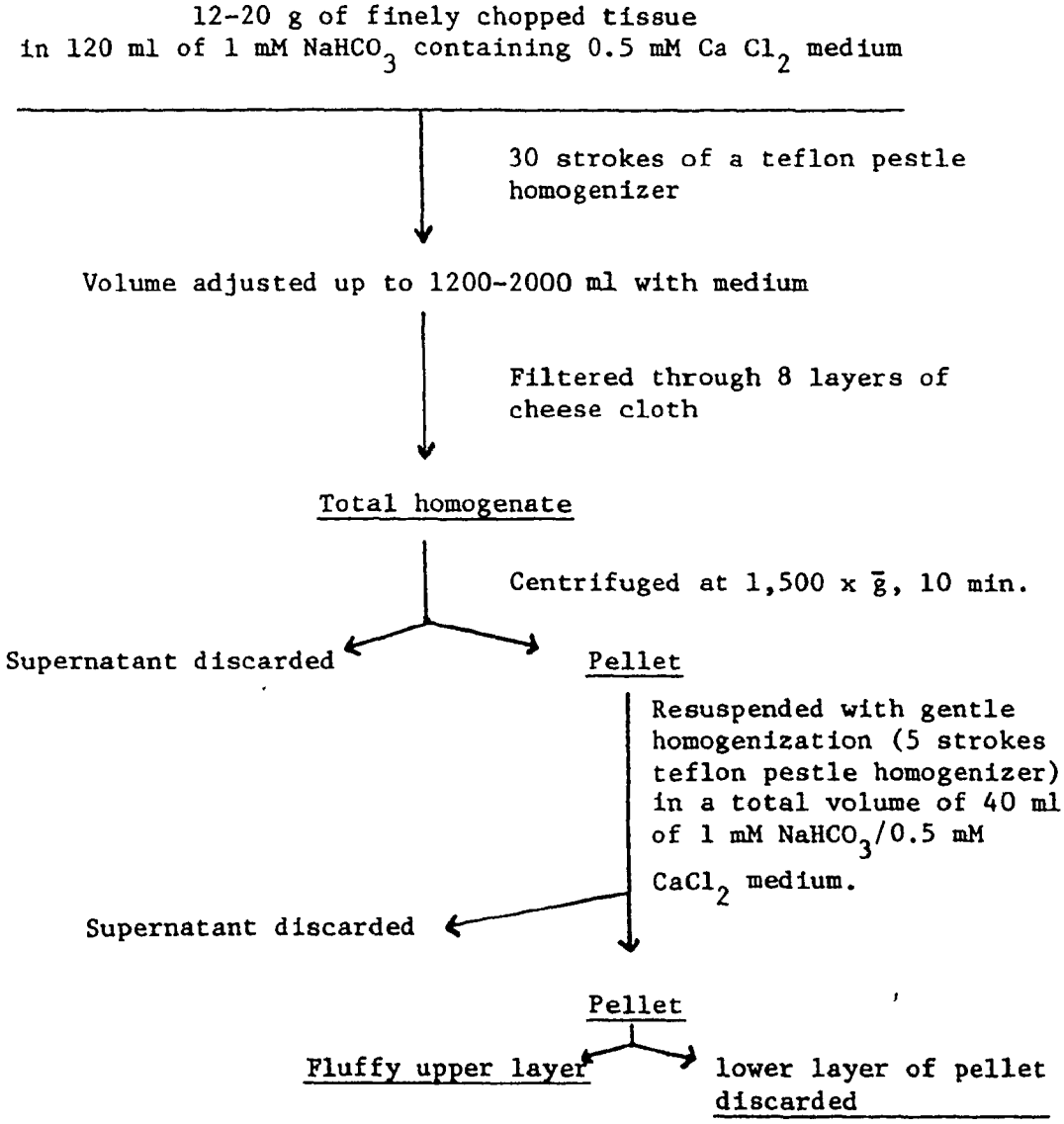
Membranes were prepared from estrogenized rat uteri using the procedure of Kidwai and co-workers (1971). This procedure was also used successfully for the preparation of plasma membrane fractions from non-estrogenized rhesus monkey uteri and from lactating rat and rabbit mammary gland. Figure 5 outlines this procedure.

Preparation of an Oxytocin Binding Particulate Fraction from Rabbit Mammary Gland

The desired weight of frozen mammary gland, usually 40 g, was weighed, thawed in 4 volumes of ice-cold mannitol medium (Kidwai, et al., 1971) and thoroughly minced with scissors. Portions of approximately 50 ml were transferred to a polycarbonate tube and homogenized with a Polytron homogenizer equipped with a PT-10 probe (Brinkmann Instruments). Homogenization was done at the maximum speed of the Polytron for four seconds. Between bursts, the tissue was left on ice for approximately one minute and the probe was cleared of tissue if necessary. As each portion of tissue was homogenized, it was filtered through two layers of cheese-cloth into a cooled graduated cylinder. After adjusting the final volume to five tissue volumes, the homogenate

FIGURE 4

Flow diagram for Rat Kidney Plasma Membrane Preparation
(modified from Manitius et al., 1968)



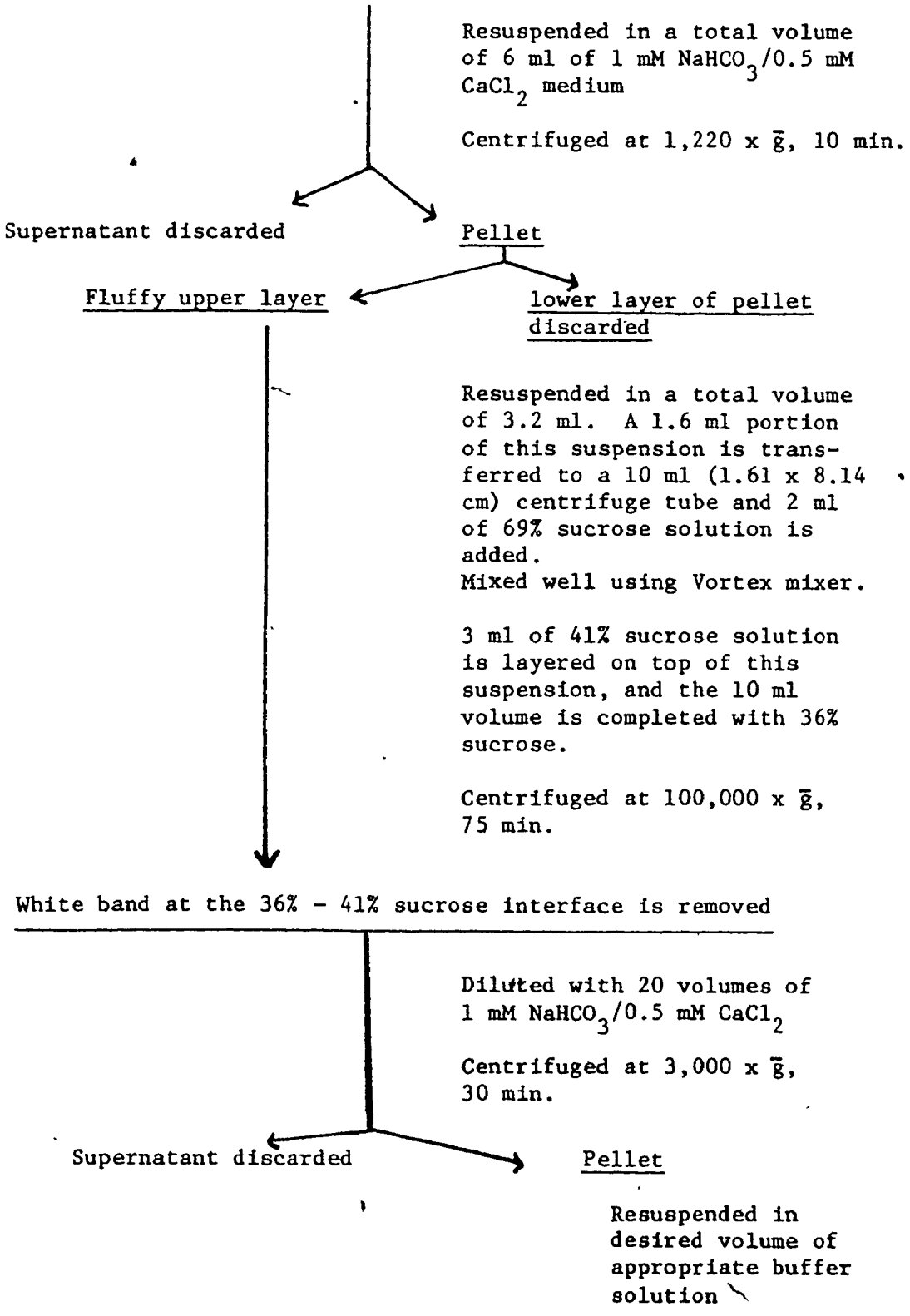
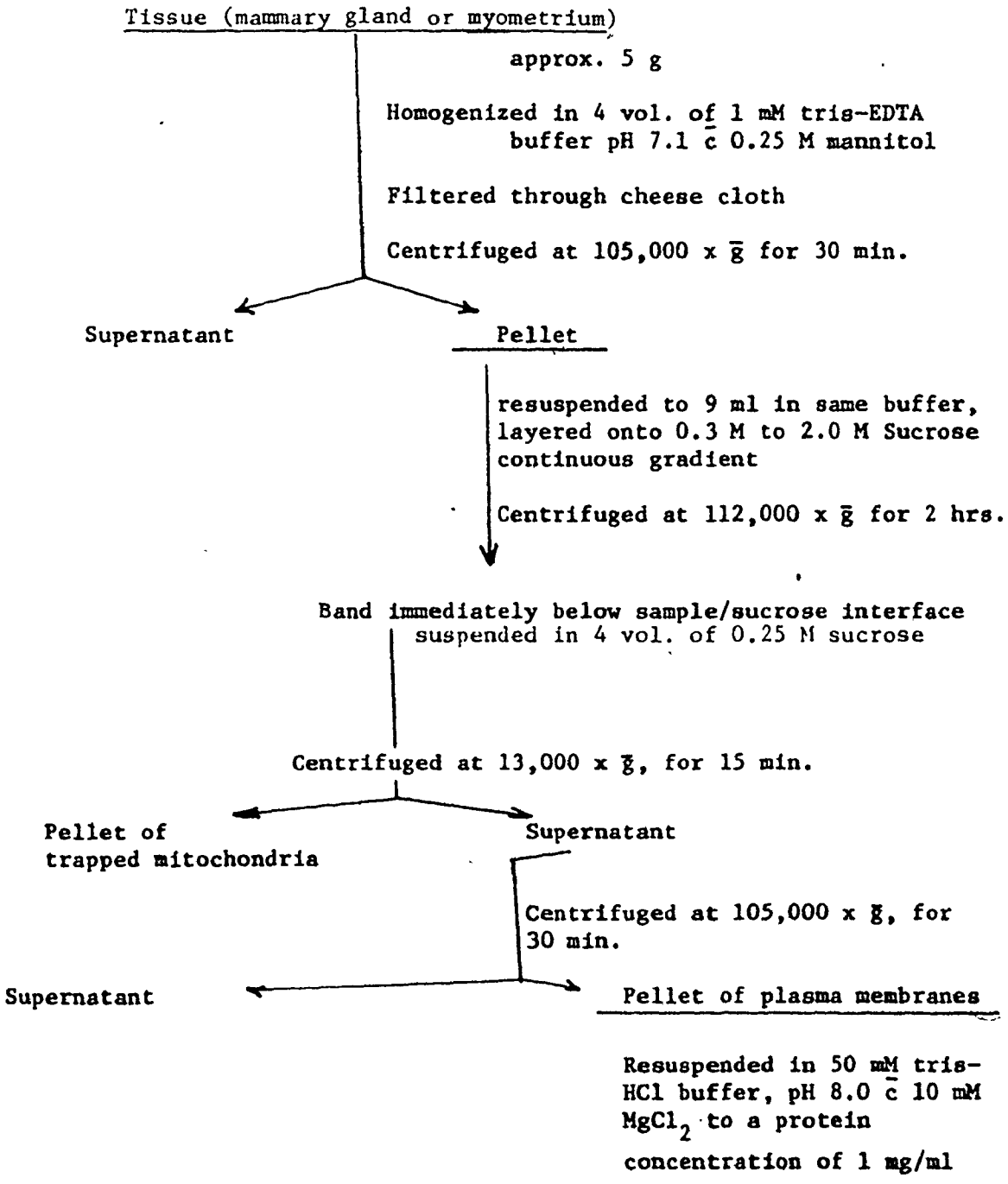

Resuspended in a total volume of 6 ml of 1 mM $NaHCO_3$/0.5 mM $CaCl_2$ medium
Centrifuged at 1,220 x $\bar{g}$, 10 min.
Supernatant discarded
Pellet
Fluffy upper layer
lower layer of pellet discarded
Resuspended in a total volume of 3.2 ml. A 1.6 ml portion of this suspension is transferred to a 10 ml (1.61 x 8.14 cm) centrifuge tube and 2 ml of 69% sucrose solution is added.
Mixed well using Vortex mixer.
3 ml of 41% sucrose solution is layered on top of this suspension, and the 10 ml volume is completed with 36% sucrose.
Centrifuged at 100,000 x $\bar{g}$, 75 min.
White band at the 36% - 41% sucrose interface is removed
Diluted with 20 volumes of 1 mM $NaHCO_3$/0.5 mM $CaCl_2$
Centrifuged at 3,000 x $\bar{g}$, 30 min.
Supernatant discarded
Pellet
Resuspended in desired volume of appropriate buffer solution

FIGURE 5

Flow Diagram for Uterus and Mammary Gland Plasma Membrane Preparation



was transferred to 30 ml polycarbonate centrifuge tubes and centrifuged 15 min at 150 x $\bar{g}$.

Three distinct layers were then visible; a top fatty layer which was discarded, an opaque beige supernatant which was retained, and a lightly packed white pellet which was discarded. The volume of the supernatant was again adjusted to five tissue volumes and transferred to clean 30 ml centrifuge tubes and centrifuged 1 h at 140,000 x $\bar{g}$.

The resulting pellets were resuspended in 5 ml of STKM medium (Sucrose, 0.25 M; Tris base, 20 mM; KCl, 10 mM; $MgCl_2$, 4 mM; pH adjusted to 8.0 with 1N HCl) and recentrifuged at 140,000 x $\bar{g}$. The resulting pellets were stored frozen at -60°C. When detergent treatment was done, the detergent was added to the STKM to obtain the desired concentration. Details of the detergent treatment vary in different series of experiments and are given in the legend of the appropriate figure.

The final pellets of the non-detergent treated pellet had two distinct layers, a lower white layer and an upper brownish layer. The lower layer did not bind [^{3}H]-oxytocin whereas the upper layer did. Unless specified otherwise, "140,000 x $\bar{g}$ pellet" refers to this upper portion which was scraped free with a spatula and resuspended. The lower layer was discarded.

Preparation of this fraction had the following advantages: (1) time of preparation was short (approximately 5 h); (2) a large quantity of material was obtained which could be used immediately, stored for future use, or carried on for further purification. This fraction, was used for much of the work reported in this thesis.

Further Purification of the Impure Oxytocin Binding Fraction or 140,000 x $\bar{g}$ Pellet

Fractionation of the 140,000 x $\bar{g}$ pellet on sucrose gradients (see "Subcellular Fractionation and Centrifugation") yielded a fraction enriched in oxytocin binding activity. The 140,000 x $\bar{g}$ pellet was resuspended in 50 mM Tris-HCl, buffer (TM buffer) pH 8 containing 10 mM $MgCl_2$, to a protein concentration of 10-15 mg per ml. Two ml were then layered onto the 10 ml sucrose gradients. Gradients were centrifuged for 20 hours at 140,000 x $\bar{g}$ using an I.E.C. SB-283 swinging bucket rotor. Following centrifugation, a distinct white band was present in the lower third of the gradient (30-40% sucrose). The lower thirds of the gradients were collected and pooled. An equal volume of TM buffer was added and the dilute material was centrifuged at 140,000 x $\bar{g}$ in 30 ml polycarbonate tubes in an I.E.C. A-237 rotor. The pelleted material was kept frozen at -60°C.

Tritiated Oxytocin

Oxytocin tritiated in the ring of the tyrosine residue was obtained from Schwartz-Mann Radiochemicals (Orangeville, New York). In previously reported work by other groups, on the binding of oxytocin to uterine and mammary gland subcellular fractions, [^{3}H]-oxytocin (16 Ci/mmole) was obtained from the same source. Their control for purity was reported to be that 90% of the radioactivity was found to migrate on thin layer chromatogram to the same place as did authentic oxytocin (Soloff and Swartz , 1973). Using the same solvent system and an additional one, we obtained the same result. However, the biological activity of this material was lower than expected (184 Units per mg of oxytocic activity

instead of 450-500 Units per mg). Contamination with peptide fragments arising from synthesis could lead to misinterpretation of binding results due to competition of these fragments with [^{3}H]-oxytocin. Therefore, because of the low biological activity, purification was essential. Thin layer chromatography was run on this material using the solvent systems: 1-butanol-acetic acid-water (4:1:5), and 1-butanol-pyridine-acetic acid-water (3:2:0.6:2.4). At the position corresponding to the R_f for oxytocin in these solvent systems (0.26 ± 0.09 and 0.60 ± 0.08, respectively) 90-94% of the radioactivity was measured. When this material was purified by partition chromatography on Sephadex G-25 (fine) (Yamashiro, 1964) in a column of 80 cm x 1.5 cm using the solvent system 1-butanol-water-acetic acid-pyridine (1000:950:35:15), four major regions of radioactivity were detected in the eluates (Figure 6). The material eluted at the R_f corresponding to oxytocin (0.30) was further purified by gel filtration in the same Sephadex G-25 column. Elution with 0.2N acetic acid gave a single peak of radioactive material at the elution volume corresponding to oxytocin with an R_f= 0.41 (Figure 7). The material was assayed for milk-ejection activity (Fielitz, et al, 1970). The specific activity found for this purified [^{3}H]-oxytocin was 25 Ci/mmole, and the milk ejection-like activity was 376 U/mg.

Incubation Conditions for Binding of Tritiated Oxytocin

Binding of oxytocin was studied under conditions similar to those reported by Soloff and Swartz (1973). Incubations were done at room temperature (20-22°C) for 1 hour in 1.5 ml Eppendorf Microfuge plastic tubes. The total volume of the incubation mixture was 250 µl and contained the following:

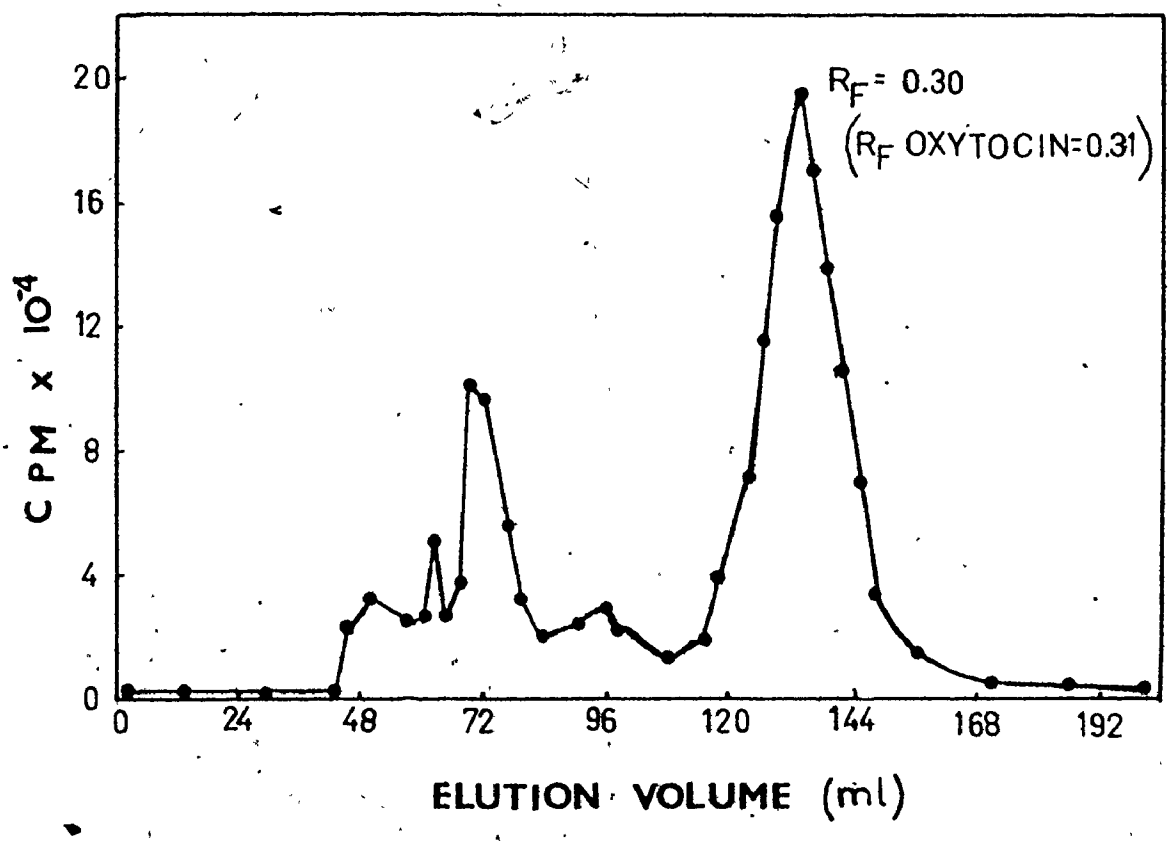


FIGURE 6. Purification of [^{3}H]-oxytocin.

Partition chromatography on Sephadex G-25 (fine). Detailed description of the procedures is in the text.

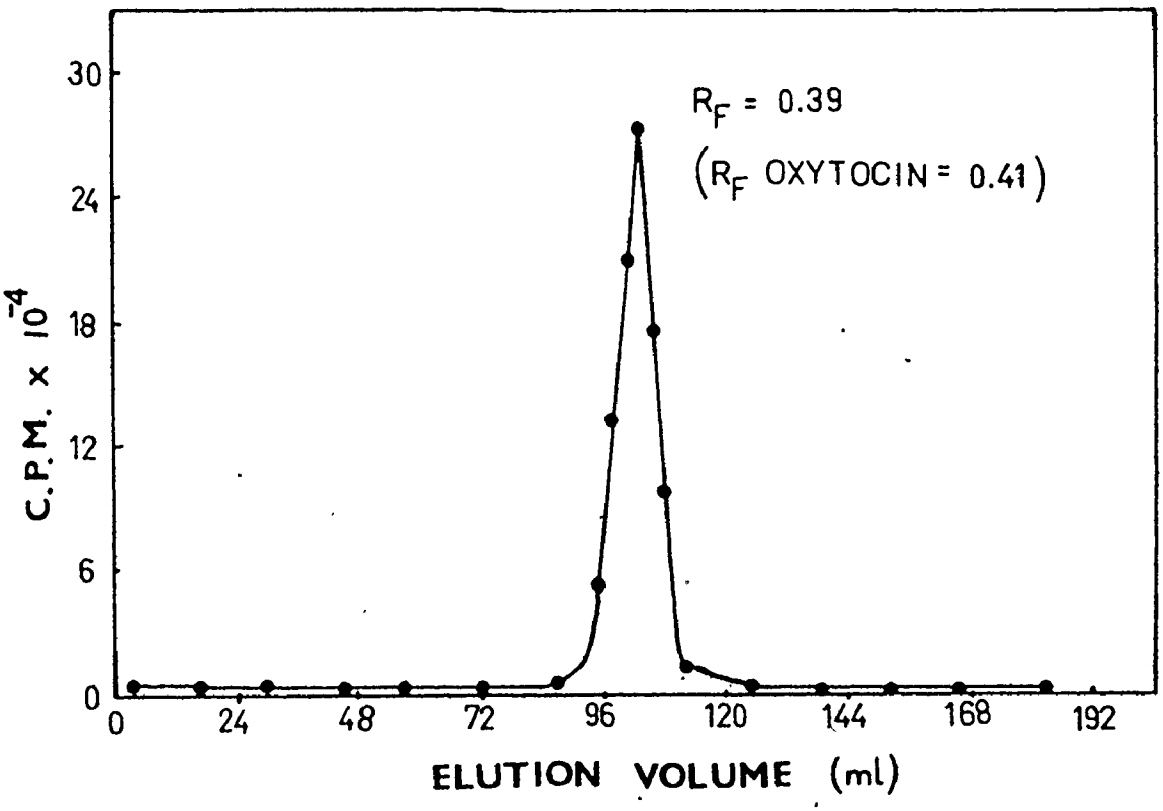


FIGURE 7. Purification of [^{3}H]-oxytocin.

Gel filtration on Sephadex G-25 (fine). Details of the procedure are given in the text.

(1.) Oxytocin binding fraction, 100 or 200 µl. The protein concentration was usually 5 mg/ml in a buffer containing 50 mM Tris-base, 10 mM $MgCl_2$ and 0.1% gelatin adjusted to pH 8.0 with HCl (TMG buffer). (2.) [^{3}H]-oxytocin containing 11,500 DPM in 0.2 ng (approximately 100 µU milk-ejecting activity) to give a final concentration of the hormone of 8×10^{-10} M. (3.) Non-radioactive oxytocin when required in a 100-fold excess of [^{3}H]-oxytocin to give a final concentration of 8×10^{-10} M. (4.) TMG buffer in the requird volume to bring the final volume to 250 µl.

The buffer and the protein mixture were pre-incubated for 10 minutes at 20°C and the incubations were started by the addition of tritiated or tritiated plus non-radioactive oxytocin. The incubation was ended either by centrifugation or by the addition of polyethylene glycol-6000 and subsequent centrifugation as described below.

Centrifugation Procedure

Following incubations, the tubes were centrifuged at 4°C for 10 minutes at 20,000 x $\bar{g}$ in an I.E.C. PR-J centrifuge equipped with a high speed attachment. In contrast to the fresh membrane material (see: Figure 5), the frozen and thawed membrane material (140,000 x $\bar{g}$ pellet) which was routinely used for oxytocin binding assays would sediment under these conditions. Supernatants were removed and 1 ml of TMG added to each pellet. The pellets were resuspended with the help of a Vortex mixer and centrifuged again under the same conditions. This wash was then removed and the sides of the tube wiped with tissue paper to remove any liquid. The bottoms of the tubes containing the pellets were cut of into 1 ml of Nuclear Chicago Solubilizer (NCS, Amersham-Searle) in a 13 x 100 mm glass tube and left

overnight. When the pellets were solubilized (sometimes requiring slight warming the next day), 50 µl of glacial acetic acid was added to each tube to acidify the solution and prevent chemiluminescence, followed by 5 ml of scintillation fluid. The tubes were decanted into scintillation vials and rinsed with another 5 ml of scintillation fluid and the samples counted.

Polyethlene Glycol-6000 (PEG) Procedure

The procedure used was modified from that described by Cuatrecasas (1972b). After the incubation with [^{3}H]-oxytocin, 0.5 ml of cold 0.1% bovine gamma-globulin (fraction II) dissolved in TMG was added, followed immediately by 0.5 ml of cold 25% PEG dissolved in 50 mM Tris buffer, pH 8, containing 10 mM $MgCl_2$. After thorough mixing, the tubes were left on ice for 15 minutes and centrifuged as described in "Centrifugation Procedure". The pellets were washed with 1 ml of 10% PEG in TM buffer and re-centrifuged. Pellets were then prepared and counted as described in "Centrifugation Procedure". The purpose of the PEG treatment was to precipitate material which would not sediment using the centrifugation assay in experiments in which a solubilized receptor was studied.

The value shown for specific oxytocin binding is the difference between the mean of duplicate determinations containing [^{3}H]-oxytocin only (total binding), and the mean of duplicate determinations containing [^{3}H]-oxytocin and non-radioactive oxytocin (non-specific binding). Blanks containing no protein but containing [^{3}H]-oxytocin only or [^{3}H]-oxytocin plus non-radioactive oxytocin were done. The value of the blank was subtracted from the corresponding samples containing protein. These corrected values were used to calculate the means from which specific binding was determined. Experimental error is expressed as the sum of the ranges from the means of

the total and non-specific binding.

Counting of Radioactivity

All counting was done in a Beckman LSC-233 liquid scintillation counter. Efficiency (30-40%) was determined using the external standard ratios method, and disintegrations per minute calculated. The scintillation fluid routinely used contained 5.0 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis (2-(5-phenyloxazolyl)) benzene per litre toluene. When the material was derived from sucrose gradients, the cocktail of Anderson and McClure (1973) was used.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

PAGE in the presence of sodium dodecyl sulfate (SDS) was carried out following the method of Fairbanks and co-workers (1971). A sample of the plasma membrane preparation containing approximately 250 µg of protein was centrifuged at 100,000 x $\bar{g}$ for 30 minutes. The pellet so obtained was resuspended in 250 µl of a 10 mM Tris-HCl buffer solution, pH 8.0, containing 1 mM EDTA, 1% SDS, 10% sucrose, 40 mM dithiotheitol, and 10 µg per ml pyronin-Y as a tracking dye. The suspension was then heated at 37°C for 30 minutes. Twenty-five to 50 µl aliquots of this solution were used for running the gels.

A solution of 5.6% acrylamide monomer was allowed to set in glass tubes to a length of 10 cm. Polymerization was allowed to proceed overnight. The plasma membrane solution was layered on top of the gel and overlaid with 0.3 ml of a 40 mM Tris, 20 mM sodium-acetate buffer at pH 7.4, containing 2 mM EDTA and 1% SDS. Electrophoresis was carried out in a Hoefer unit with the voltage gradient at 6 V/cm until the tracking dye

had reached 1 cm from the bottom of the gel (approximately 3 hours). The gels were then removed from the tubes and the position of the tracking dye marked. The gels were stained with Coomassie blue and were treated in a diffusion destainer in several stages:

1. 25% isopropyl alcohol/10% acetic acid/0.025% Coomassie blue, overnight;
2. 10% isopropyl alcohol/10% acetic acid/0.0025% Coomassie blue, 6-9 hours;
3. 10% acetic acid/0.0025% or less Coomassie blue, overnight;
4. 10% acetic acid; several hours until the background of the gel is clear.

The gels were then scanned at 550 nm. A calibration curve was obtained by running 6 proteins of known molecular weight in the range 135,000-12,000 (Figure 8). The following proteins were used: Escherichia coli β-galactosidase, 135,000; bovine serum albumin, 68,000; ovalbumin, 45,000; pepsin, 35,000; cytochrome c dimer, 24,000; myoglobin, 17,000; and cytochrome c monomer, 12,000. All were obtained from Sigma Chemical Company, except bovine serum albumin which was obtained as the crystallized protein from Pentex Chemicals.

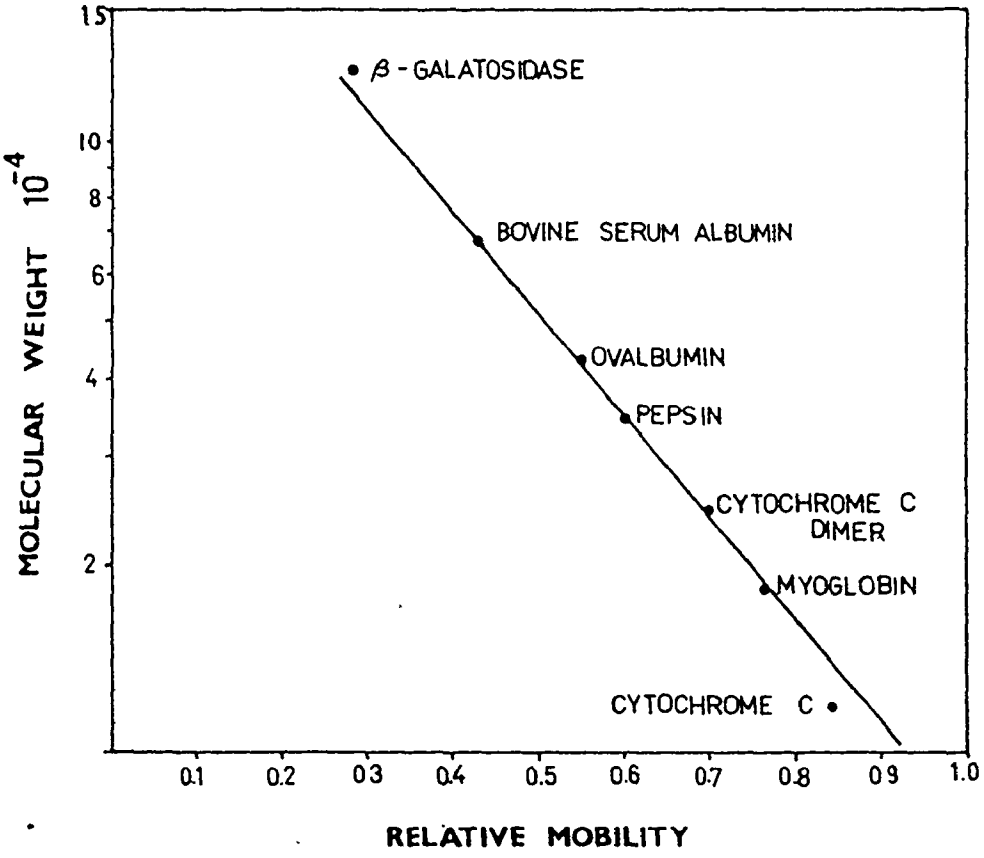


FIGURE 8. SDS-PAGE Standard Curve for Molecular Weights.

Details are given in the text.

Scanning of the stained gels at 550 nm was done on a Gilford 2400 spectrophotometer equipped with a Gilford linear transport device. A scan rate of 1 cm/minute was used at a chart speed of 1 inch/minute.

5' - Nucleotidase Assay

5'-Nucleotidase was assayed using the incubation conditions of Heppel and Hilmoe (1955). Inorganic phosphate released during the incubation of subcellular fractions with 5'-AMP was measured using the procedure of Bonting and co-workers (1961).

Protease Assay

Protease activity was measured by the method of Hatton and Regoeczi (1973) using [^{125}I]-casein supplied by Dr. E. Regoeczi, as substrate. Incubations were done at 25°C for 18 hours in the same buffer used for incubation of subcellular fractions with oxytocin. The same final concentration of subcellular fraction protein (4 mg/ml) was used as in the incubations with oxytocin.

Cytochrome c Oxidase Assay

Cytochrome c oxidase activity was determined using the method of Cooperstein and Lazarow (1951).

Glucose 6-Phosphatase Assay

Glucose 6-phosphatase was determined using the method of Harper (1965).

β-Glucuronidase Assay

β-glucuronidase was determined using the method of Fishman and Bernfeld (1955).

Glutathione-Insulin Transhydrogenase Assay

Glutathione-insulin transhydrogenase activity was determined using the method of Varandani (Varandani and Tomizawa, 1966; Varandani, 1973).

Protein Determination

Protein concentrations were estimated by the method of Lowry and co-workers (1951), using bovine serum albumin (Pentex) as standard. When EDTA was present, samples were dialyzed against distilled water overnight prior to protein determination (Ward and Fastiggi, 1972).

Electron Microscopy

The samples were fixed by exposing them for 24 hours to 2% glutaraldehyde in Krebs-Ringer buffer, followed by 3 hours in a secondary fixative, 1% osmium tetraoxide. Staining was accomplished by an overnight exposure to 5% uranyl acetate in distilled water. The samples were imbedded in Spurr's soft epoxy resin and segments with a thickness of 50-100 nm were cut for examination on a Phillips 300 transmission electron microscope.

Reagents

Unless indicated otherwise, all reagents and solvents were of analytical grade and were obtained from Fischer Chemical Company or British Drug House Ltd. Tris-base, Triton X-100, Lubrol-PX, dithio-

threitol and bovine gamma-globulin fraction II were obtained from Sigma Chemical Co. (St. Louis, Missouri). Polyacrylamide gel reagents including the SDS used in the gels, were obtained fron the Bio-Rad Laboratories (Mississauga, Ontario). SDS used in the electrophoresis buffer and polyethylene glycol-6000 were obtained from Matheson, Coleman and Bell (Norwood, Ohio) and Baker (Phillipsburg, New Jersey), respectively. Gelatin was obtained from Difco (Detroit, Michigan). The non-radioactive peptides oxytocin, [1-deamino]-oxytocin and [4-proline]-oxytocin were supplied by Dr. B. M. Ferrier.

RESULTS

I. Purification of Plasma Membrane Fractions from Neurohypophysial Hormone Target Organs

Initially, I examined the binding of [^{3}H]-arginine vasopressin to purified plasma membranes derived from rat kidney cortex. Considerable time was spent in exploring methods suitable for the preparation of reasonably pure plasma membranes. To examine the purity of the membrane preparation, several enzyme activities characteristic of various subcellular organelles were measured. These were 5'-nucleotidase for plasma membranes, cytochrome c oxidase for mitochondria, β-glucuronidase for lysosomes, and glucose 6-phosphatase for endoplasmic reticulum. Table 3 shows the various activities found in a typical kidney plasma membrane preparation. Electron microscopy of this preparation also showed enrichment of plasma membranes.

The specific activity of the available tritiated arginine-vasopressin was not high enough (3-4 Ci/mole) to enable the study of high-affinity binding sites for the hormone. However, the preparation of plasma membranes and the use of techniques to examine the purity of the fraction (e.g. enzyme markers, electron microscopy) was part of the experience which was of great help in the further studies which were carried out. As part of the studies on the binding of oxytocin to target tissues, uterine and mammary gland membranes were prepared from rat, rabbit and rhesus monkey. 5'-Nucleotidase activities in the homogenates and the membrane fractions are shown in Table 4. Membrane preparations were significantly enriched in this plasma membrane marker enzyme. Two preparations were done with rhesus monkey uterus. Although this would have been a desirable tissue to use since only one study of

TABLE 3

Marker Enzyme Activities* in a Fraction Enriched in Plasma Membranes Prepared from Rat Kidney Medulla

Enzyme	Whole Homogenate	Plasma Membrane	Plasma Membrane / Whole Homogenate	(n)
5'-Nucleotidase μmoles Pi/hr./mg. protein	6.07 ± 0.23	43.0 ± 12.1	7.1	(4)
Glucose-6-Phosphatase μmoles Pi/hr./mg. protein	5.0 ± 0.2	0.9 ± 0.1	0.2	(2)
β-Glucuronidase μg Phenolphthalein/hr./mg. protein	26.1 ± 0.7	<1	<0.04	(2)
Cytochrome-c-Oxidase Units of Cooperstein and Lazarow (1951)	107.8 ± 10.0	54.3 ± 6.5	0.5	(2)

* Values shown are mean ± range.

TABLE 4

5'-Nucleotidase Activities in Homogenates and Plasma Membrane Fractions from Neurohypophysial Hormone Target Tissues

Tissue	Whole Homogenate*	Plasma Membranes*	Enrichment	(n)
Rat Uterus	13.8 ± 1.7	51.9 ± 6.1	3.8	3
Rhesus Monkey Uterus	4.6 ± 0.5	13.2 ± 1.0	2.9	2
Rat Mammary Gland	3.1 ± 0.1	8.4 ± 0.9	2.7	3
Rabbit Mammary Gland	0.6 ± 0.1	2.7 ± 0.6	4.5	3

*(μmoles Pi/hr/mg of protein; mean values ± range)

oxytocin binding has been carried out with primate uterine tissue (Soloff et al., 1974), the irregular supply of small quantities of tissue from animals on which there were no reliable records of age or hormonal status made the continuation of this work impractical.

The use of marker enzymes as a means of characterizing the membrane fractions required the use of large proportions of the material obtained in each preparation. Since yields of plasma membrane enriched material were already small, other procedures were used to monitor the preparation of plasma membranes. For this reason sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was tried as a procedure which could be used to characterize each preparation and would require little material (50 µg protein or less). Gel scans for the tissues used are shown in Figures 9 a-d. Although major features of the patterns obtained for each tissue were generally similar, variations in peak positions and intensities were observed. Therefore, while potentially useful information was obtained with this technique, it did not prove satisfactory for consistent characterization of the membrane fractions obtained.

The purified plasma membrane fractions obtained were used for initial binding studies described in the following section.

Once a satisfactory oxytocin binding fraction had been obtained from rabbit mammary gland, attempts were made to prepare a fraction from it which would show an enriched oxytocin binding ability. Specific oxytocin binding activity was shown by first preincubating the 140,000 x $\bar{g}$ pellet, prepared as described above, with [^{3}H]-oxytocin and fractionating the material on a sucrose density gradient (Figure 10). These

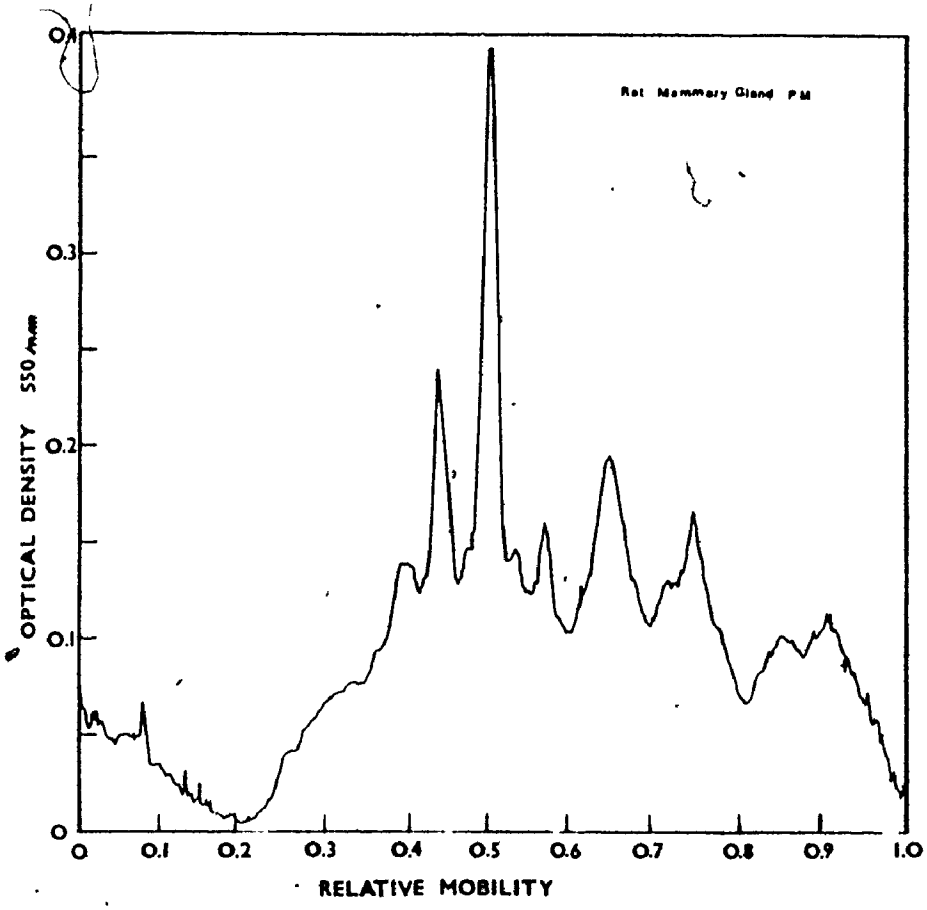


FIGURE 9.a. SDS-PAGE Pattern of Rat Mammary Gland Plasma Membranes. After staining, the gels were scanned at 550 nm. Details of the procedure are given in the section on Materials and Methods.

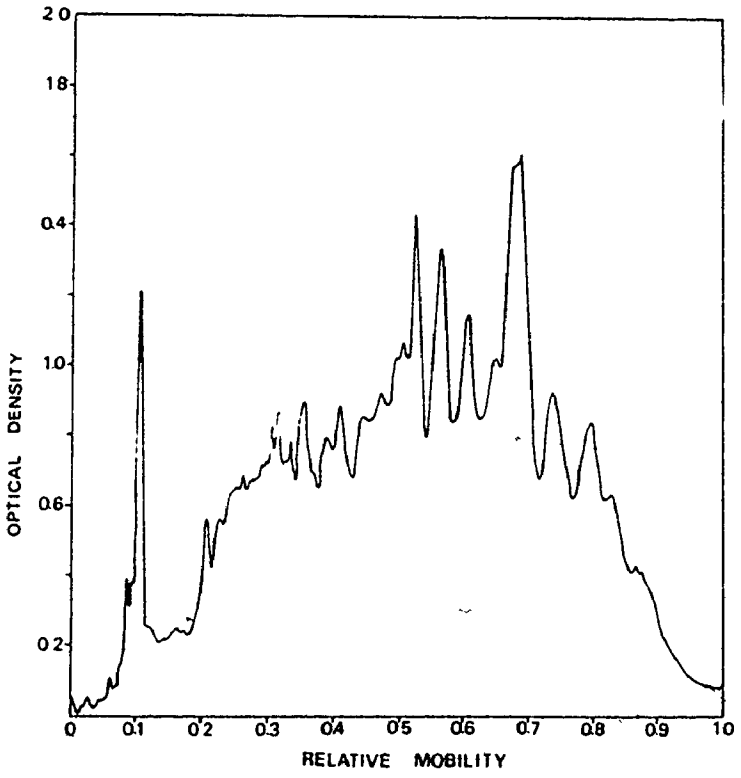


FIGURE 9.b. SDS-PAGE Pattern of Rabbit Mammary Gland Plasma Membranes. After staining the gels were scanned at 550 nm. Details of the procedure are given in the section on Material and Methods.

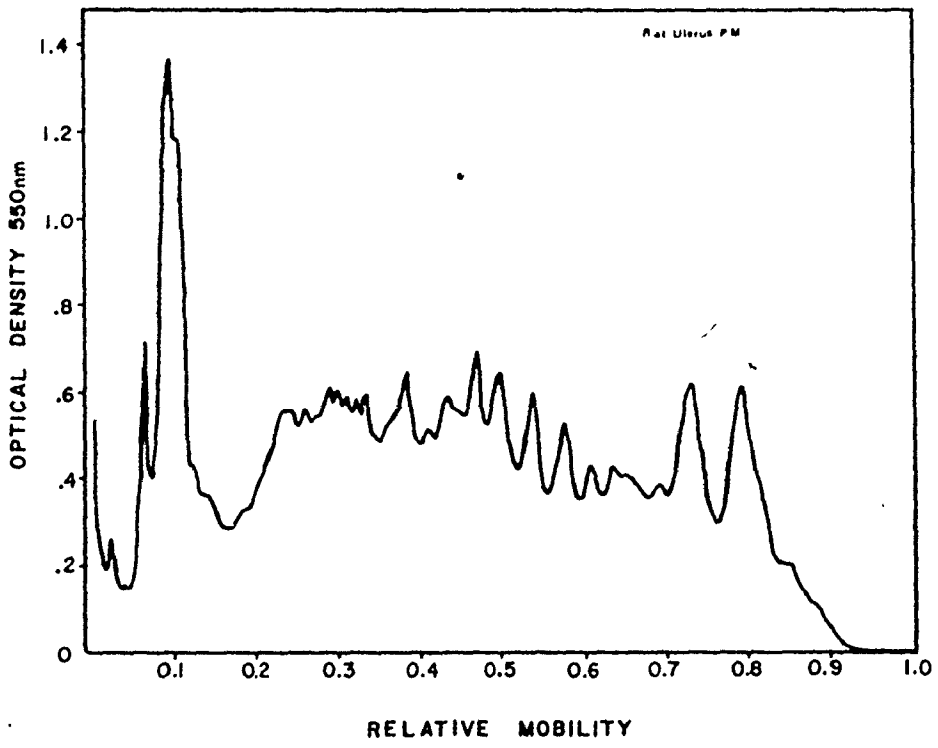


FIGURE 9.c. SDS-PAGE Pattern of Rat Uterus Plasma Membranes. After staining the gels were scanned at 550 nm. Details of the procedure are given in the section on Material and Methods.

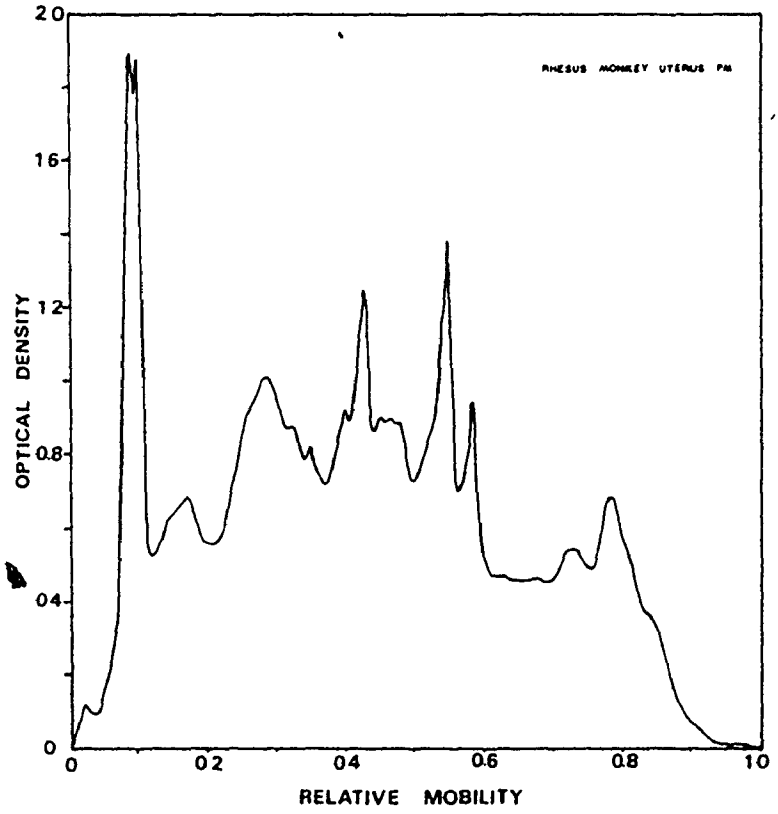


FIGURE 9.d. SDS-PAGE Pattern of Rhesus Monkey Uterus Plasma Membranes. After staining, the gels were scanned at 550 nm. Details of the procedure are given in the section on Materials and Methods.

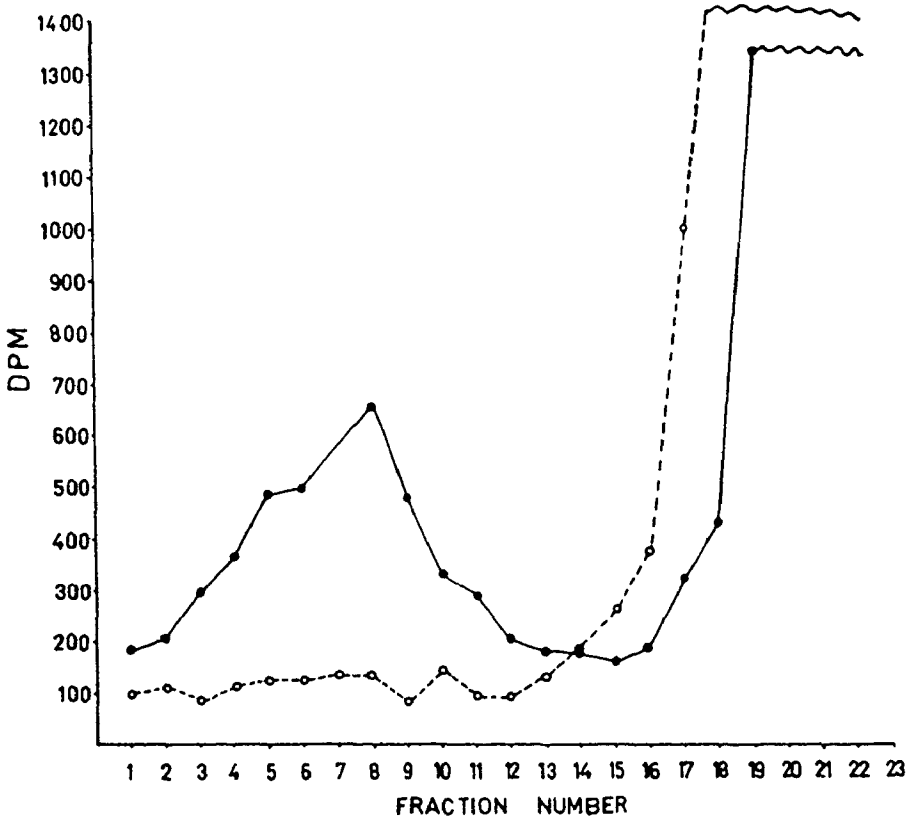


FIGURE 10. Sucrose Density Gradient of Oxytocin Binding Fractions from Rabbit Mammary Gland.

Incubations of the upper layer of a 140,000 x $\bar{g}$ pellet with [^{3}H]-oxytocin (volume 1.25 ml) were layered onto 10 ml gradients of 10%-40% sucrose in TM buffer. After centrifugation as described in the text, the tubes were pierced and 0.5 ml fraction collected and counted for radioactivity. Duplicate gradients were done which gave similar results as the one shown. The large radioactivity at the top of the gradient is due to unbound [^{3}H]-oxytocin.

(●) [^{3}H]-Oxytocin only

(o) [^{3}H]-Oxytocin + 100-fold excess of cold oxytocin.

results led to the use of sucrose gradients for a preparative method to obtain an enriched oxytocin binding fraction. Table 5a shows the results of two representative experiments in which 140,000 x $\bar{g}$ pellets from a rabbit mammary gland were purified using a sucrose density gradient. The material sedimenting into the lower 1/3 of these gradients showed the greatest enrichment in oxytocin binding, and was also enriched in 5'-nucleotidase activity (Table 5b). An electron micrograph of the oxytocin binding material obtained by collecting the lower 2/3 of the gradients (20-40% sucrose) is shown in Figure 11.

II. Initial Binding Studies with Oxytocin

Tritiated Oxytocin

One of the major problems encountered in working with oxytocin is the difficulty in obtaining radioactively labelled hormone which is chemically stable and retains full biological activity. As was already pointed out (pp. 38-39), the studies described in this thesis were carried out using [^{3}H]-oxytocin supplied by Schwarz-Mann Radiochemicals. This [^{3}H]-oxytocin was purified by partition chromatography, followed by gel filtration in Sephadex G-25. Some of the initial studies were carried out with the [^{3}H]-oxytocin as it came from the supplier. Dr. M. Soloff of Toledo, Ohio has used [^{3}H]-oxytocin from the same supplier in his studies on the binding of oxytocin to various mammalian target tissues. He found that storage at -70°C for one year did not significantly alter the binding activity of the [^{3}H]-oxytocin. Partition chromatography on Sephadex G-25 of previously purified [^{3}H]-oxytocin stored for approximately six months in this laboratory showed the presence of a considerable amount of a "fast moving material" (Figure 12). This material was run in gel filtration on Sephadex G-25 mixed with 1.5 mg of highly purified cold oxytocin.

TABLE 5a

Sucrose gradient purification of high speed (140,000 x $\bar{g}$) pellet material from rabbit mammary gland

	Expt. 1		Expt. 2	
High Speed Pellet				
Protein/gradient	20	mg	20	mg
Binding/mg protein	1540	DPM	1400	DPM
Total binding per gradient	30800	DPM	28000	DPM
Pellet				
Protein/gradient/fraction	12.9	mg	14.5	mg
Binding/mg protein	600	DPM	212	DPM
Binding/gradient/fraction	7740	DPM	3074	DPM
Lower Third				
Protein/gradient/fraction	1.7	mg	0.92	mg
Binding/mg protein	8340	DPM	9155	DPM
Binding/gradient/fraction	14178	DPM	8423	DPM
Middle Third				
Protein/gradient/fraction	0.34	mg	0.1	mg
Binding/mg protein	4260	DPM	7966	DPM
Binding/gradient/fraction	1448	DPM	797	DPM
Upper Third				
Protein/gradient/fraction	0.14	mg	0.27	mg
Binding/mg protein	0		151.2	DPM
Binding/gradient/fraction	0		40.8	DPM

Incubations were carried out under the conditions described in Materials and Methods, pp. 41-42.

Table 5b

5'-Nucleotidase Activities in the 140,000 x $\bar{g}$ Pellet Prior to Sucrose Density Gradient Purification and in the Fraction from the Lower 1/3 of the Gradient.

Experiment	5'-Nucleotidase Activities (μmoles P released/hour/mg protein)		
	140,000 x g Pellet	Lower 1/3 of Gradient	Enrichment
1	2.26±0.45	10.57±1.46	4.7
2	2.20±0.20	17.17±1.58	7.8

Values are expressed as the mean±S.D. of four determinations.

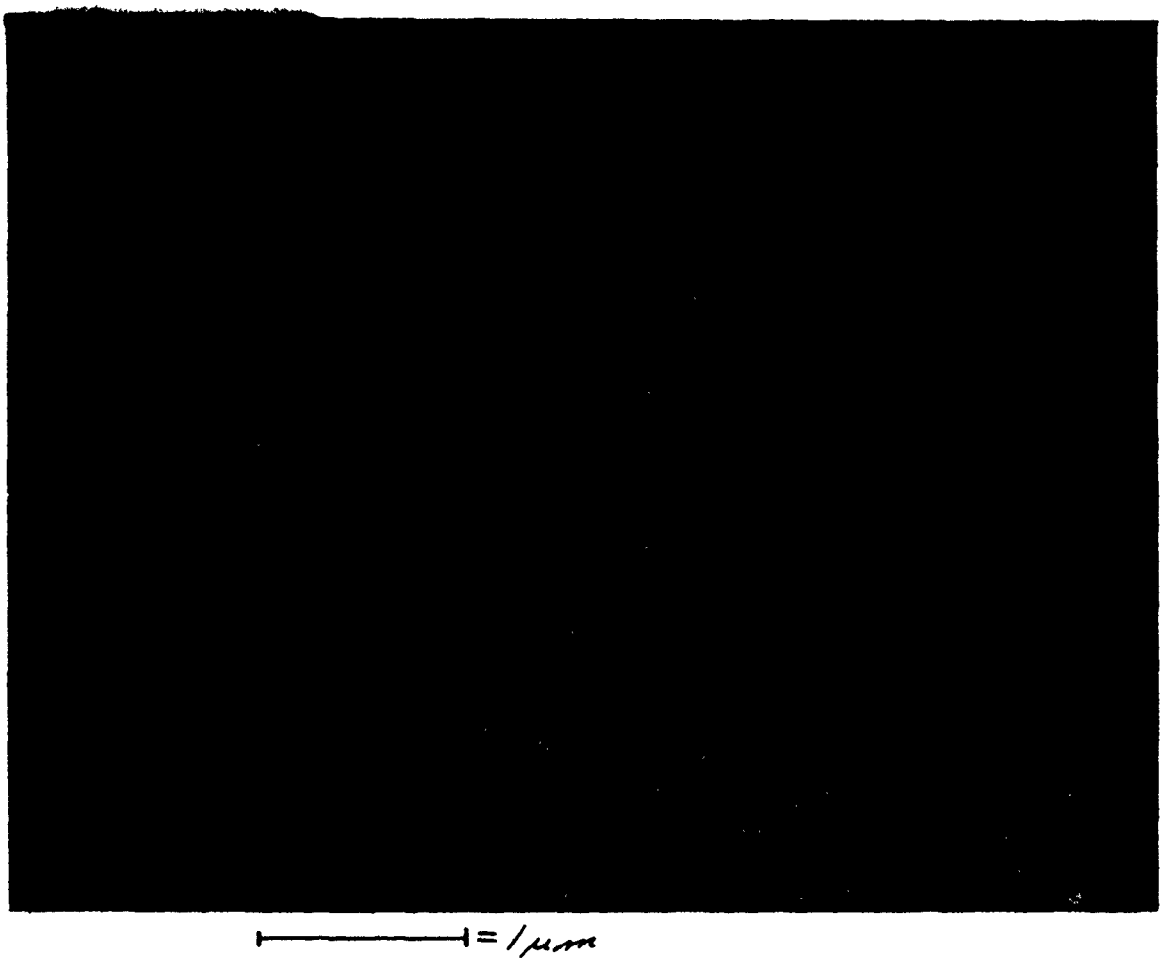


Figure 11. Oxytocin Binding Fraction from Rabbit Mammary Gland. Electron micrograph (magnification = 26,850) of a sucrose density gradient purified 140,000 x g pellet from lactating rabbit mammary gland.

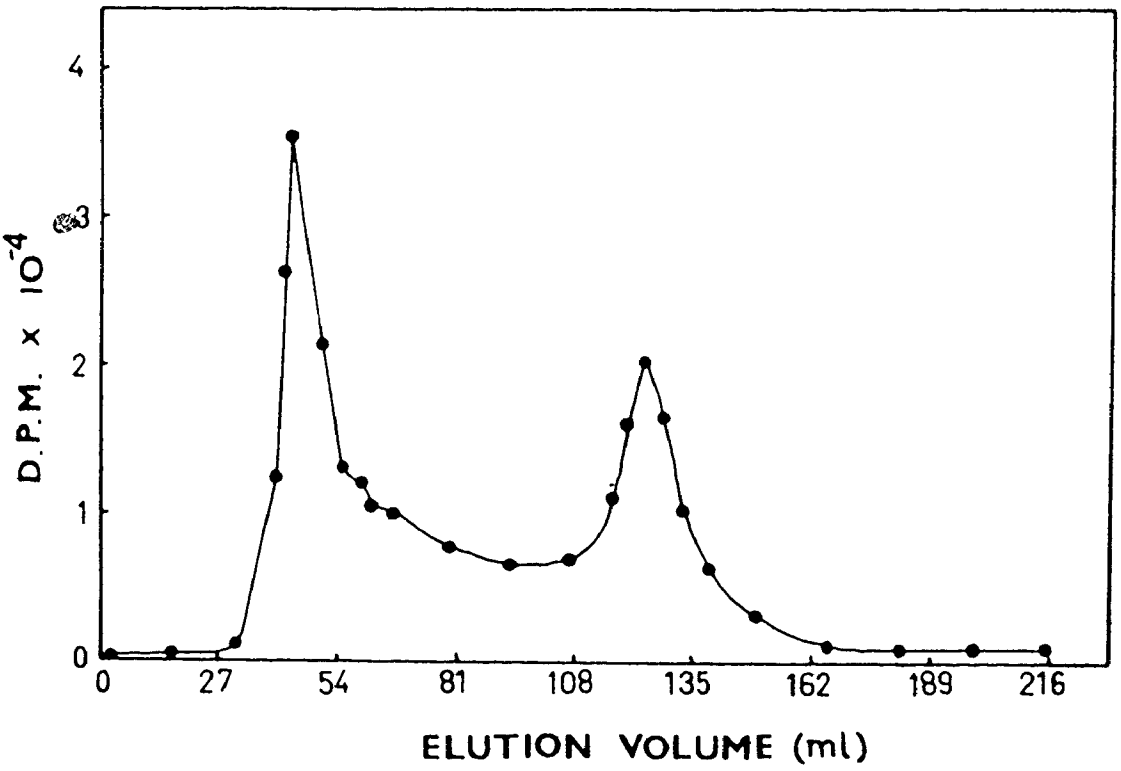


FIGURE 12. Purification of [^{3}H]-oxytocin after 6 months storage at -60°C. Partition chromatography on Sephadex G-25 (fine) was carried out as described under Material and Methods. The fractions corresponding to the "fast moving material" (32-72) were collected and used for the run illustrated in Figure 13.

The results of this experiment (Figure 13) showed that a major component of the stored, previously purified radioactive hormone was not [^{3}H]-oxytocin. Since this material had some of the chromatographic behaviour of oxytocin dimer, it was reduced in liquid ammonia and metallic sodium, and then oxidized with hydrogen peroxide. Subsequent partition chromatography and gel filtration failed to show the presence of [^{3}H]-oxytocin. This indicates that the "fast running material" which was isolated from the Sephadex purification of [^{3}H]-oxytocin was not dimer. Storage for a month at -60°C has also produced some "fast moving material" from previously purified oxytocin.

In every case, after the repurification steps, the milk-ejection potency of the [^{3}H]-oxytocin was what it was expected for a specific activity of 25 Ci/mmole.

Initial binding studies with [^{3}H]-oxytocin were carried out with total homogenate and a plasma membrane fraction from both lactating rat mammary gland (Figure 14.a. and b.) and lactating rabbit mammary gland (Figure 15.a. and b.). As expected, the plasma membrane fraction in both cases bound more [^{3}H]-oxytocin per mg protein than did the whole homogenate. The binding represented in these figures is total rather than specific binding. When a 100-fold excess of unlabeled oxytocin was added to a set of incubations with [^{3}H]-oxytocin, a significant component of the total binding was eliminated (Figure 16). The difference between the total binding of [^{3}H]-oxytocin and the binding in the presence of the unlabeled hormone was considered to be the specific binding.

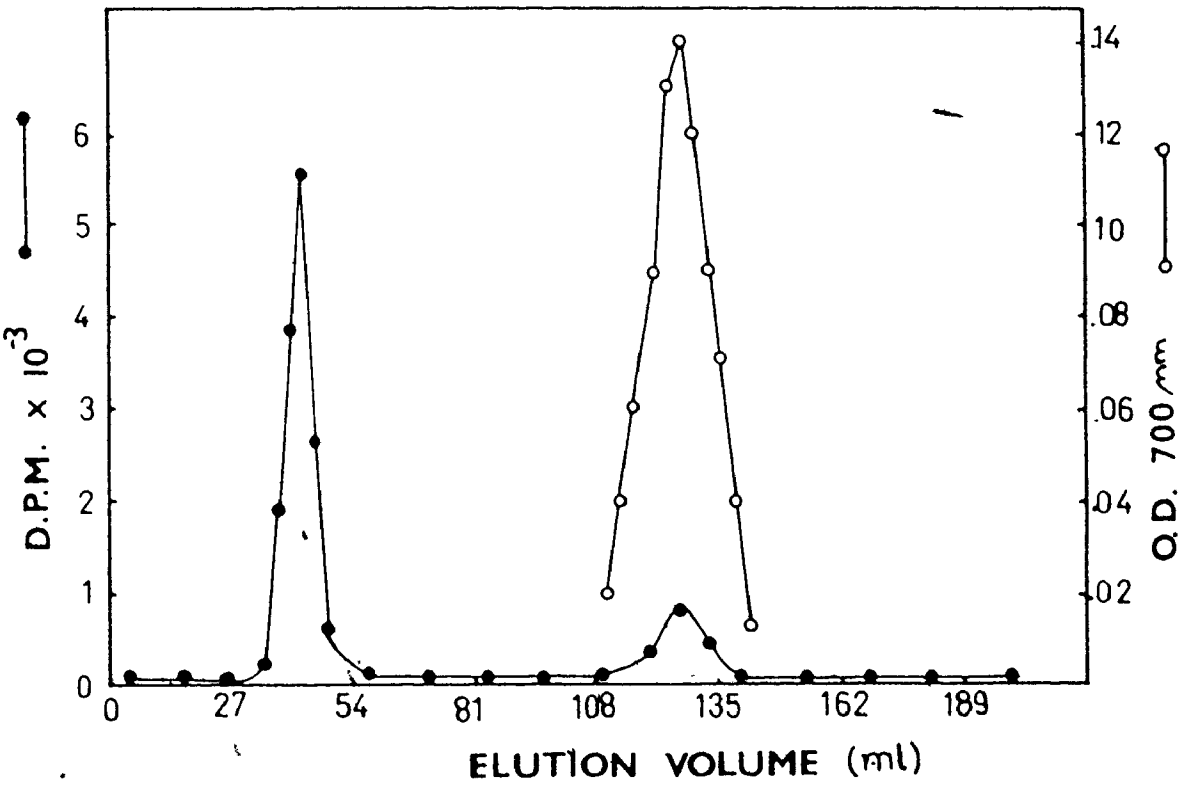


FIGURE 13. Co-chromatography of the "Fast Moving Material" from the purification of [^{3}H]-oxytocin. The material from fractions 32-72 illustrated in Figure 12 together with authentic oxytocin was run in gel filtration in Sephadex G-25 (fine) using 0.2N acetic acid as eluant. At the elution volume corresponding to oxytocin, a small amount of radioactivity can be seen.

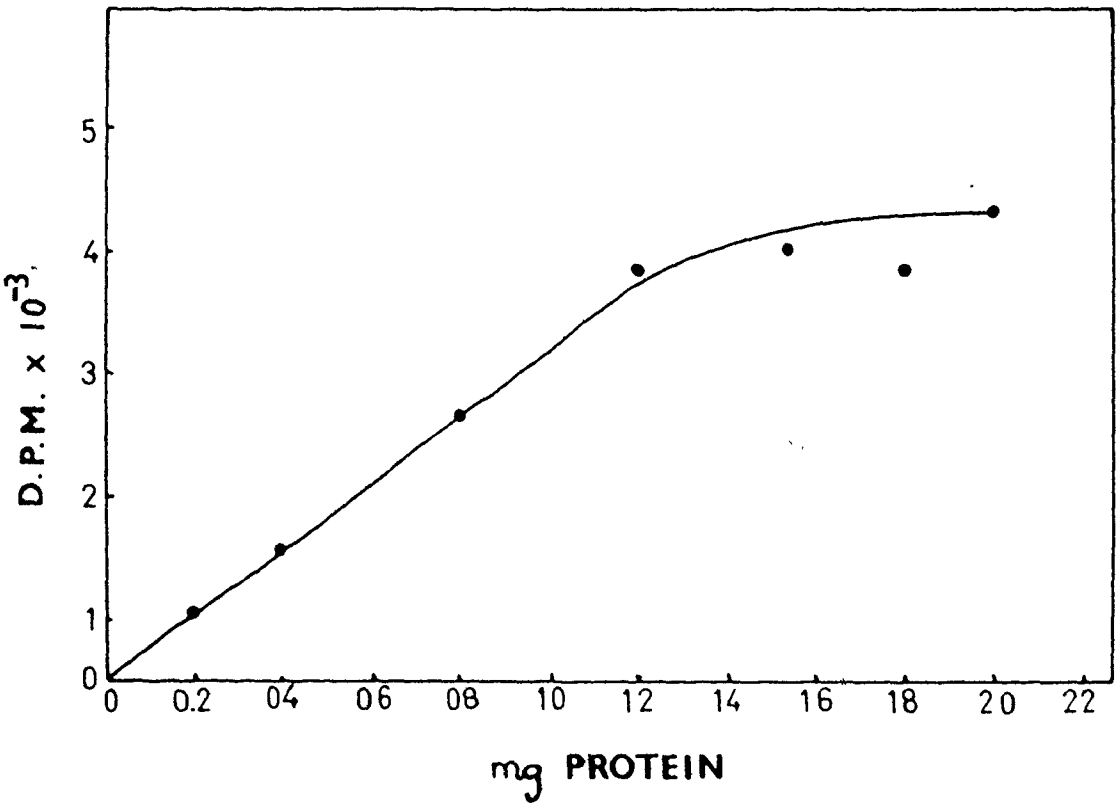


FIGURE 14.a. Binding of [^{3}H]-Oxytocin to Lactating Rat Mammary Gland Homogenate. The tissue homogenate was prepared according to the procedure shown in Figure 5. The binding was done by the centrifugation method. The results of a representative experiment are shown.

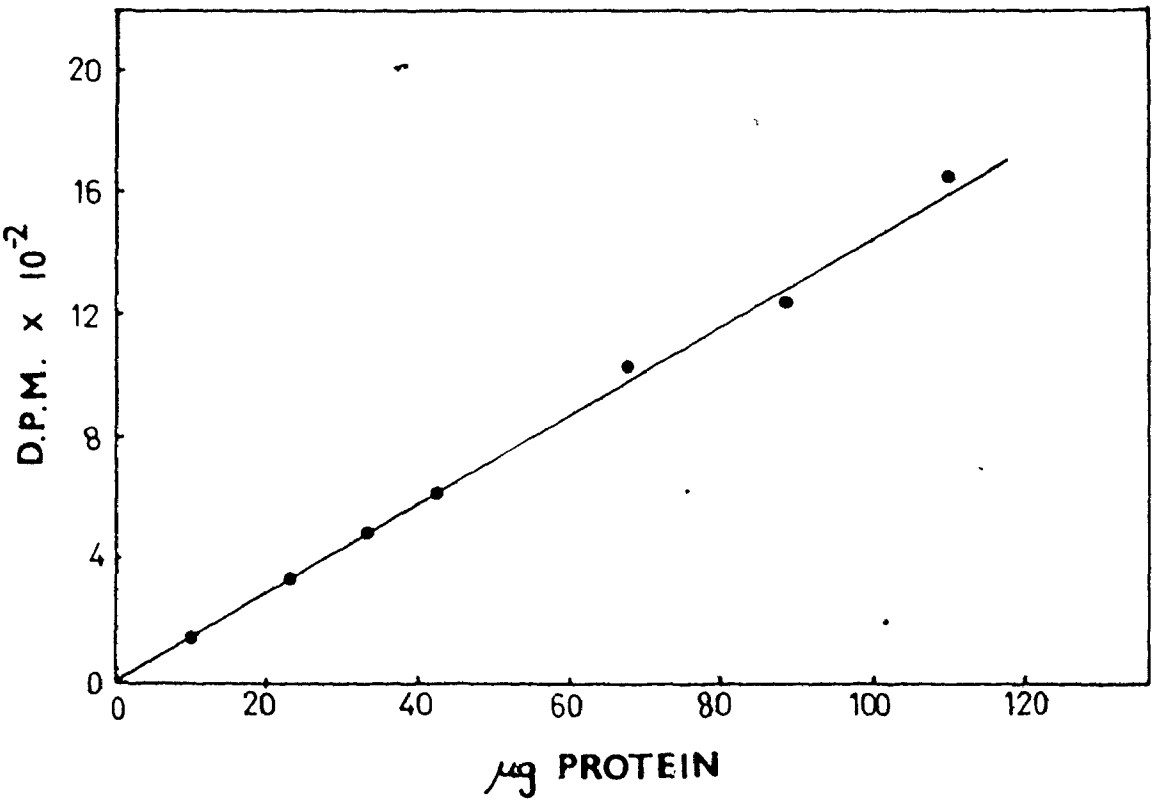


FIGURE 14.b. Binding of [^{3}H]-Oxytocin to Lactating Rat Mammary Gland Plasma Membranes. The plasma membrane fraction was prepared according to the procedure outlined in Figure 5. The binding was done by the centrifugation method. The results of a representative experiment are shown.

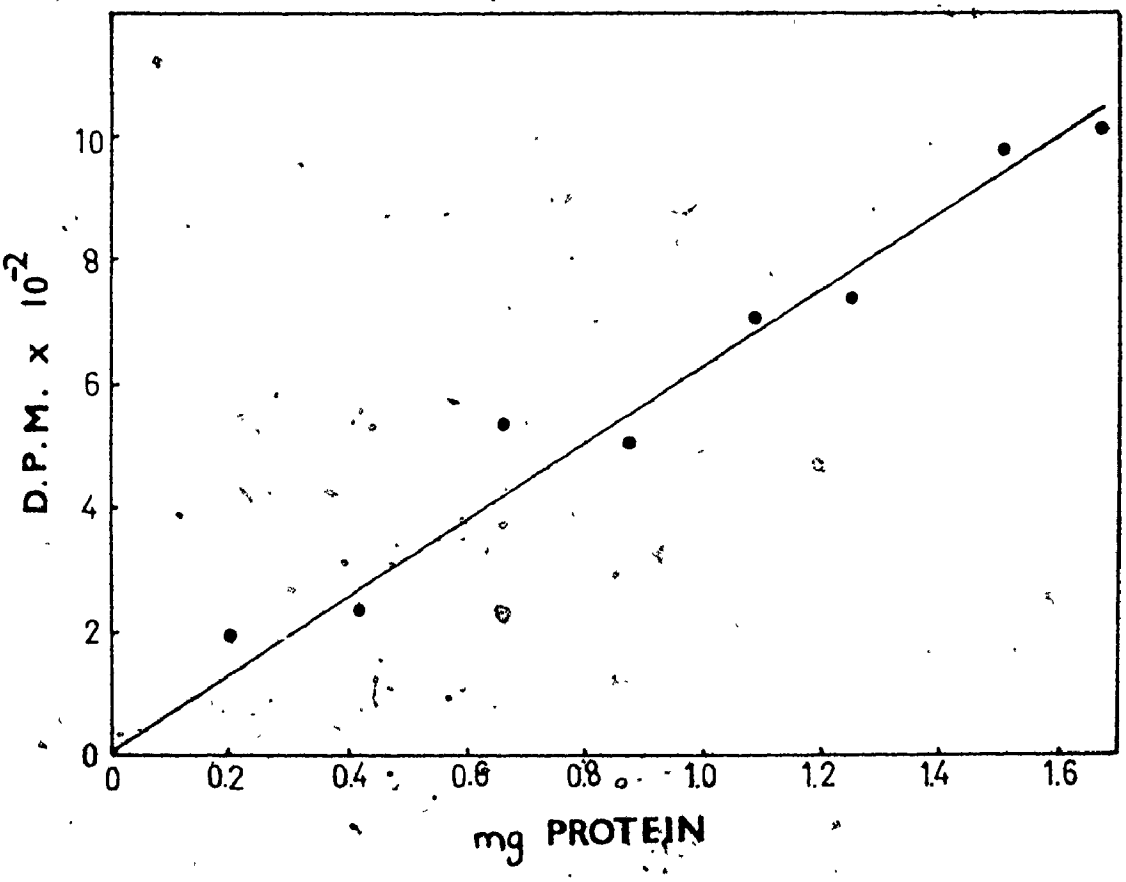


FIGURE 15.a. Binding of [^{3}H]-Oxytocin to Lactating Rabbit Mammary Gland Homogenate. The tissue homogenate was prepared according to the procedure shown in Figure 5. The binding was done by the centrifugation method. The results of a representative experiment are shown.

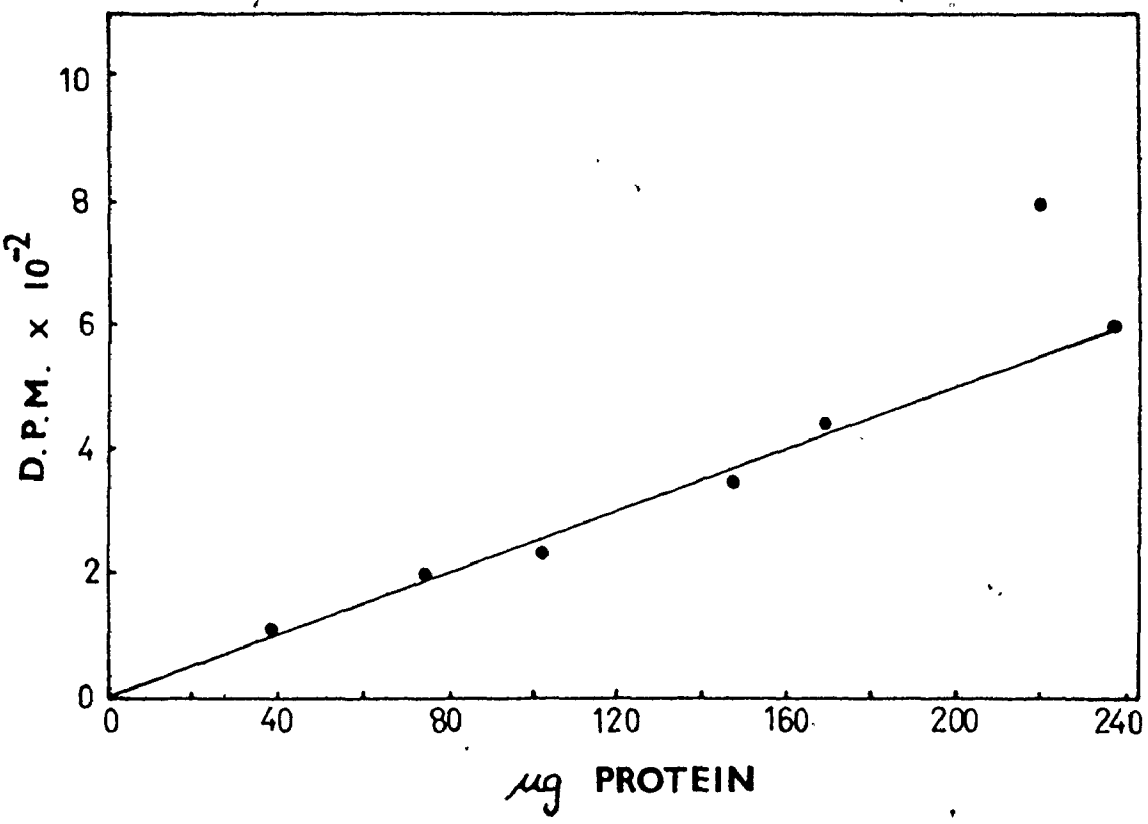


FIGURE 15.b. Binding of [^{3}H]-Oxytocin to Lactating Rabbit Mammary Gland Plasma Membranes. The plasma membrane fraction was prepared according to the procedure outlined in Figure 5. The binding was done by the centrifugation method. The results of a representative experiment are shown.

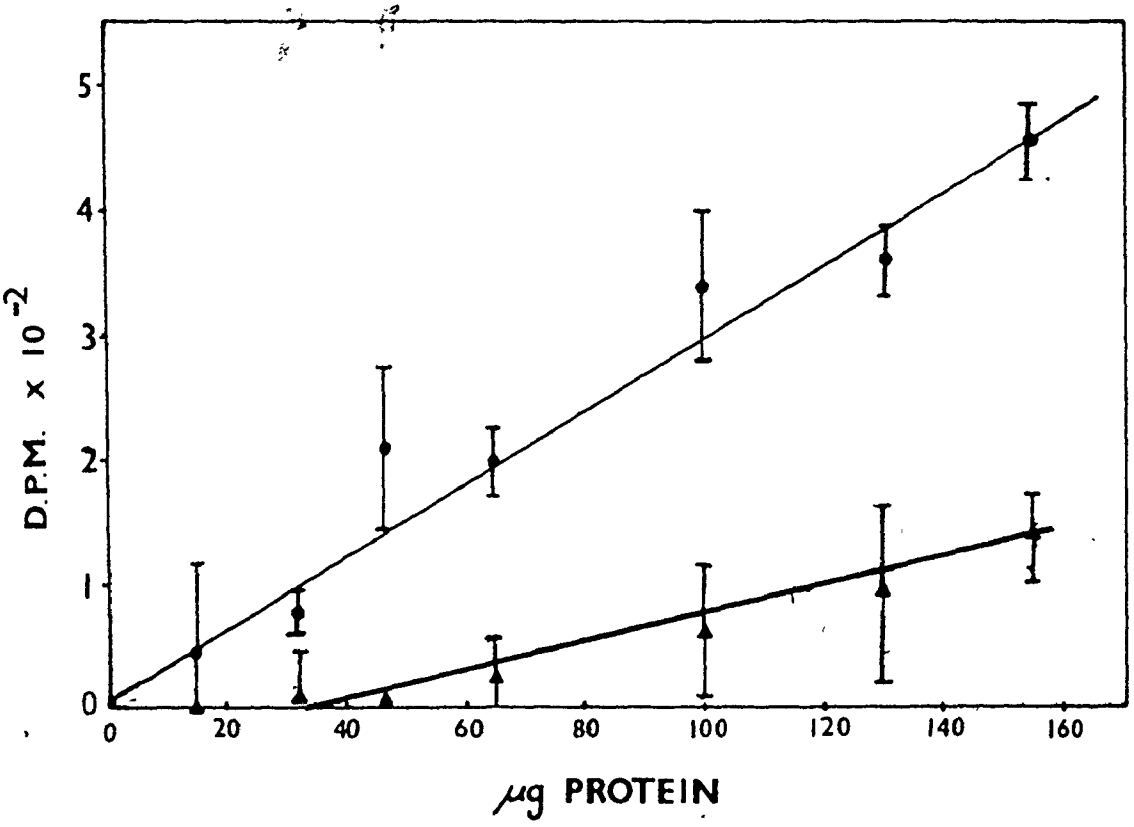


FIGURE 16. Binding of [^{3}H]-Oxytocin to Lactating Rabbit Mammary Gland Plasma Membranes. Total binding (●); non-specific binding (▲) shown when a 100-fold excess of oxytocin is added to the incubation mixture. Binding conditions as in Figure 14.b.
The mean ± the range of duplicate determinations is shown.

Subsequent experiments were carried out with the high speed (140,000 x $\bar{g}$) pellet of lactating rabbit mammary gland described in Materials and Methods. The two distinct layers of this pellet, the upper beige layer and the lower whitish layer, were examined for their ability to bind [^{3}H]-oxytocin. Table 6 shows the specific binding observed in these fractions. The upper layer showed a significant binding of [^{3}H]-oxytocin. The amount of [^{3}H]-oxytocin bound by the lower layer was approximately 10-20% of that bound by the upper layer.

In order to further examine the specificity of the binding of [^{3}H]-oxytocin to this fraction, rabbit liver was fractionated in a similar manner. Table 7 shows that no significant binding occurred with fractions from this non-target organ.

III. Properties of the Oxytocin Binding Fraction from Lactating Rabbit Mammary Gland

Several properties of the binding process were examined. In most cases, these parameters have been previously examined by others in other tissues. Since the primary objective of this project was the solubilization of the receptor rather than the quantitative characterization of the membrane bound receptor, a detailed examination of these parameters was not undertaken.

Binding of oxytocin to its receptor was, as expected, affected by pH. Although Soloff and co-workers (1973) reported maximal binding with a subcellular fraction from rat mammary gland at pH 7.6, we did not find a statistically significant difference in binding in rabbit mammary gland between pH 7.6 and 8.0 (Figure 17). Since the latter pH resulted in a higher mean binding value, it was routinely used for binding assays.

TABLE 6

[^{3}H]-Oxytocin Binding in the Upper and Lower Layers of the High Speed (140,000 x $\bar{g}$) Pellet Prepared from Rabbit Mammary Gland

Fraction	Specific binding (DPM/mg protein)	
	Preparation #1	Preparation #2
Upper portion	666 ± 30	912 ± 39
Lower portion	156 ± 50	106 ± 27

Values shown are mean ± range of 3 determinations.

TABLE 7

Binding of Tritiated Oxytocin to Rabbit Liver Fractions (DPM per mg of protein)

Tissue Fraction	Total	Non-specific	Specific
Whole Homogenate	159 ± 10	180 ± 10	0
Low Speed Supernatant (150 x $\bar{g}$)	68 ± 1	50 ± 11	18 ± 12
Low Speed Pellet (150 x $\bar{g}$)	303 ± 13	250 ± 10	53 ± 23
High Speed Pellet (140,000 x $\bar{g}$)	204 ± 6	182 ± 2	22 ± 8

Values shown are mean ± range of 3 determinations.

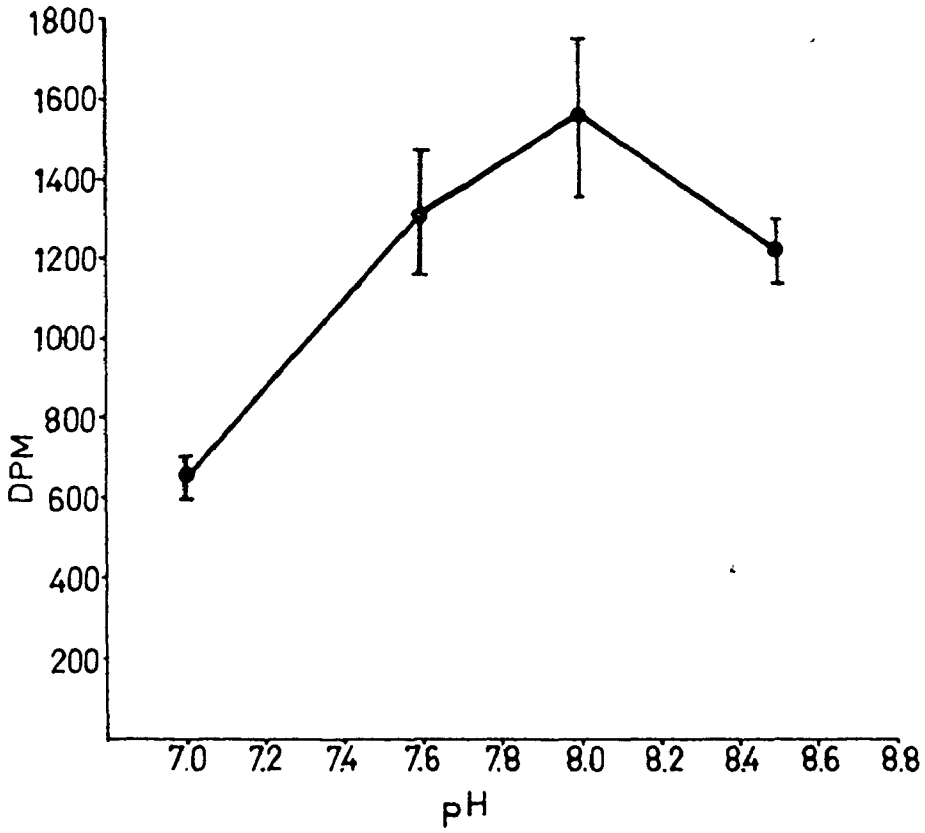


FIGURE 17. Effect of pH on the Specific Binding of [^{3}H]-Oxytocin to High Speed (140,000 x g) Pellet from Lactating Rabbit Mammary Gland. Mean ± S.D. values of three determinations are shown.

The conditions of pH 8.0 and 20°C have been also selected because they fall within the range of optimal binding conditions of neurohypophysial hormones to target tissues (Bockaert et al, 1970; Bockaert et al, 1973; Soloff and Swartz, 1973). Binding was found by Soloff and co-workers (1973) to increase until incubation had been carried out for approximately 60 minutes. For the lactating rabbit mammary gland system, the time found optimal by Soloff and co-workers was also used for the experiments presented in this thesis. Using the 140,000 x $\bar{g}$ pellet, the time required for maximal binding was found to be 100 min (Figure 18).

Competition of hormone analogs of varying biological potency has been used to establish whether or not observed binding is specific or non-specific. The highly potent analog [1-deamino]-oxytocin (Ferrier et al, 1965) and the almost inactive analog [4-proline]-oxytocin (Sawyer et al, 1969) (Table 8) were used to examine the ability of these peptides to compete with [^{3}H]-oxytocin for binding to the receptor. Insulin, which is a peptide hormone having structural analogies with oxytocin such as a disulfide bridge in a 20-membered intra-chain ring, was also tried. Figure 19 shows that whereas oxytocin and [1-deamino]-oxytocin were effective in competing for binding, [4-proline]-oxytocin and insulin were not.

The effect of divalent cations on the oxytocic response of uterus (Fraser, 1939; Stewart, 1949) and mammary gland (Somlyo, et al, 1966) is well established. In addition, Soloff and co-workers (1973) found divalent cations to be essential for oxytocin binding to a rat mammary gland. The order of effectiveness of the different cations they examined was approximately the same as reported by Schild (1969) on their effectiveness in potentiating the uterotonic effect of oxytocin on the isolated

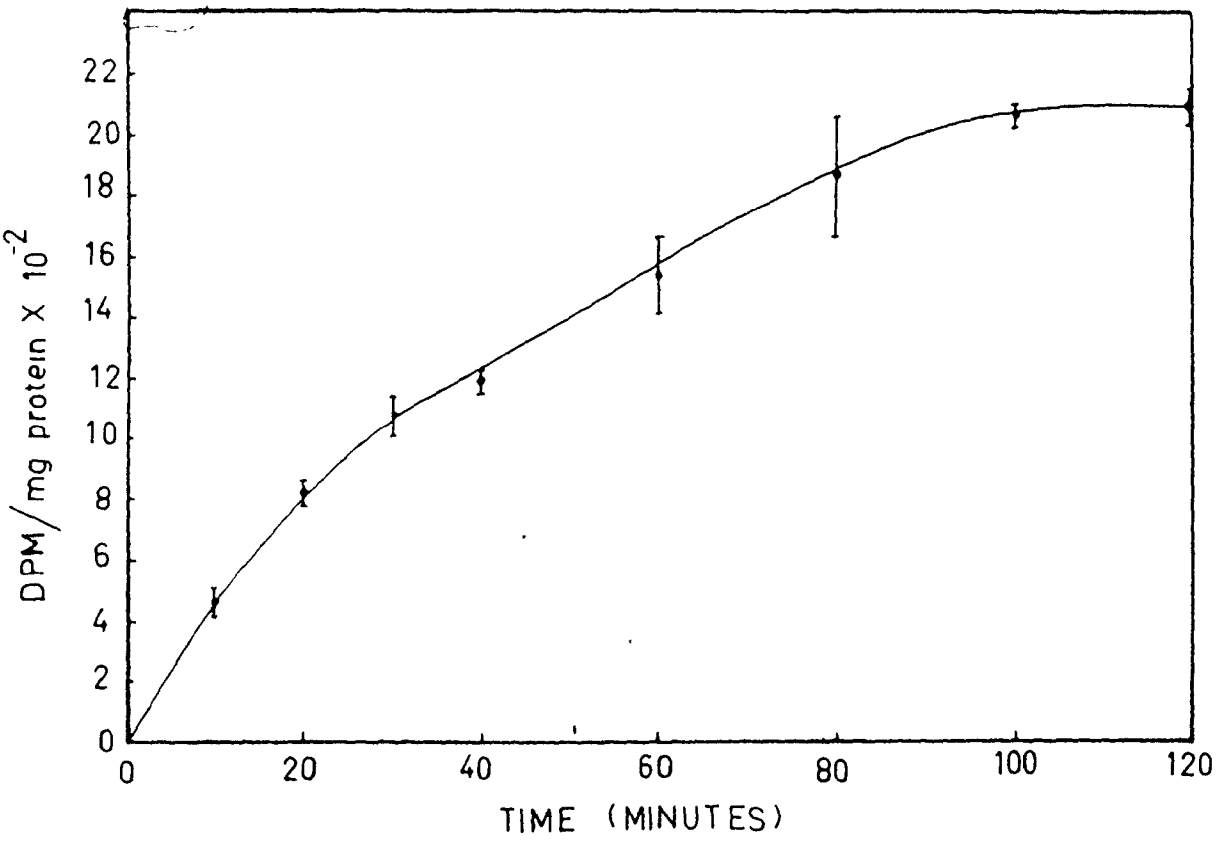


FIGURE 18. Specific Binding of [^{3}H]-Oxytocin to High Speed (140,000 x $\bar{g}$) Pellet from Lactating Rabbit Mammary Gland. Binding was studied using the centrifugation method. The only variable was the time of incubation. Values reported are mean ± range of 3 experiments.

TABLE 8

Biological Activities of [1-Deamino]-Oxytocin and [4-Proline]-Oxytocin

PEPTIDE	BIOLOGICAL ACTIVITY (Potencies are given in International Units per mg)				
	Milk-ejecting (Rabbit)	Vasodepressor (Chicken)	Uterotonic (Rat Uterus)	Pressor (Rat)	Antidiuretic (Rat)
*Oxytocin	450	450	450	5	5
+[1-Deamino]-Oxytocin	540	975	803	1.5	20
ǂ[4-Proline]-Oxytocin	0.04	---	0.007	---	$< 6 \times 10^{-5}$

* Modified from Berde, B. and Boissonnas, R.A. in, "Handbook of Experimental Pharmacology" Vol. 23, Ed. by B. Berde, pp. 806-807. Springer-Verlag. 1968.

\+ Modified from Ferrier, B. M., Jarvis, D. and du Vigneaud, V. (1965). J. Biol. Chem. 240, 4264-4266.

ǂ Modified from Sawyer, W. H., Wuu, T. C., Baxter, J. W. M. and Manning, M. (1969). Endocrinology 85, 385-388.

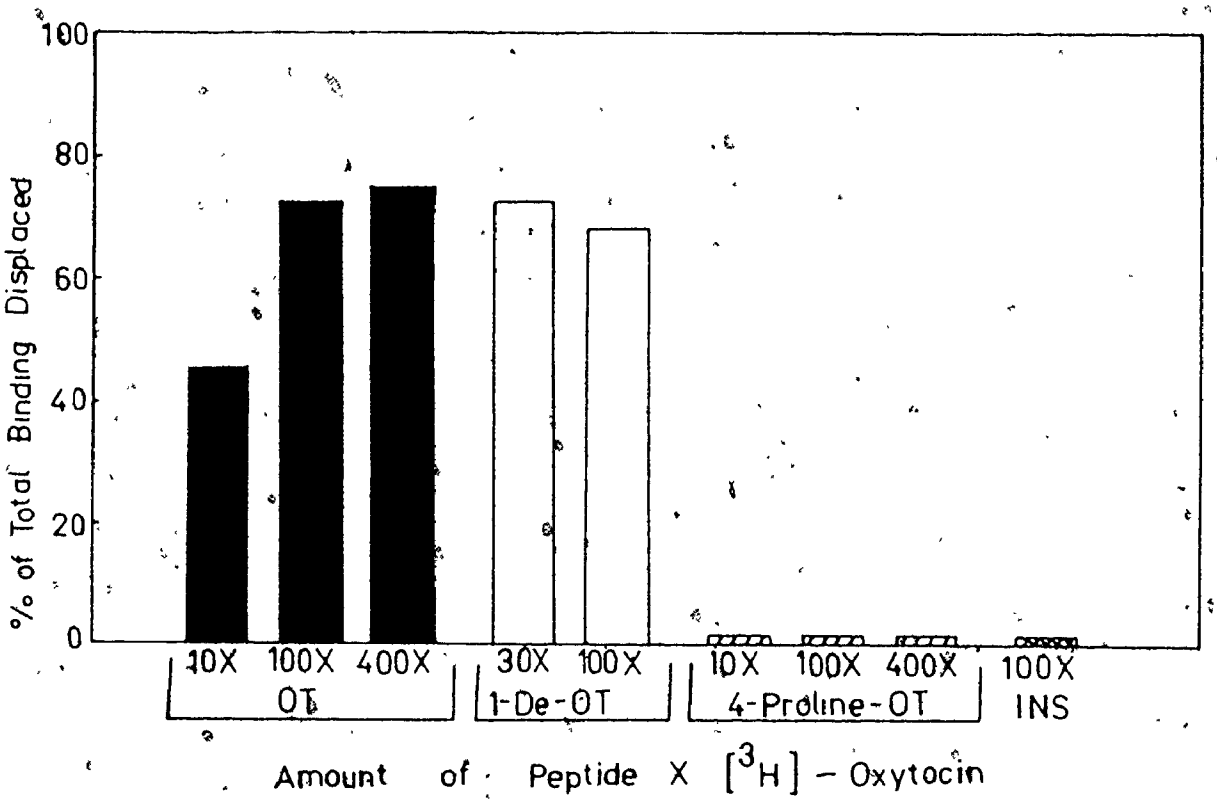


FIGURE 19. Displacement of [^{3}H]-Oxytocin from the High Speed (140,000 x g) Pellet by Different Peptides.

Excess peptide is expressed as a ratio of the molar concentrations of the non-radioactive peptides and [^{3}H]-oxytocin.

rat uterus. When incubations were done without Mg^{2+} present, but contain- EDTA (5 mM and 10 mM), binding was eliminated (Table 9a). This inhibitory effect was reversible as shown when an EDTA treated pellet was resuspended in buffer containing 10 mM Mg^{2+} (Table 9b).

Temperature-dependent oxytocin binding reversibility had not previously been examined in any tissue or subcellular fraction. The addition to the mammary gland fraction of a 500-fold excess of unlabelled oxytocin following 1 hour incubation with [^{3}H]-oxytocin, and subsequent incubation for 30 minutes showed that at 0°C binding was essentially irreversible. However, at 20°C and at 37°C, the binding was reversible (Figure 20).

In an attempt to obtain information concerning the saturability of the binding sites, the number of different binding sites and an estimate of the dissociation constant of each of the kinds of sites, a Scatchard plot (Scatchard, 1949) was done at 20°C. The data obtained indicate saturable binding sites with a K_d of approximately 3.2×10^{-9} M and a binding capacity of 385 fmoles per mg protein. A second binding component was not saturable in the range of concentrations examined, and presumable represents non-specific binding (Figure 21).

TABLE 9a

Effects of [EDTA] on [^{3}H]-Oxytocin Binding to High Speed (140,000 x $\bar{g}$) pellet Material from Rabbit Mammary Gland

[EDTA] (mM)	DPM per mg of protein*
0	2500 ± 30
1	2250 ± 139
5	0
10	0

*mean ± range of 3 determinations

TABLE 9b

Reversibility of the EDTA Effect on [^{3}H]-Oxytocin Binding to High Speed (140,000 x $\bar{g}$) Pellet Material from Rabbit Mammary Gland

Treatment	DPM per mg of protein*
TMG buffer	2800 ± 55
5 mM EDTA	90 ± 50
5 mM EDTA then resuspended in TMG buffer	3200 ± 370

*mean ± range of 3 determinations

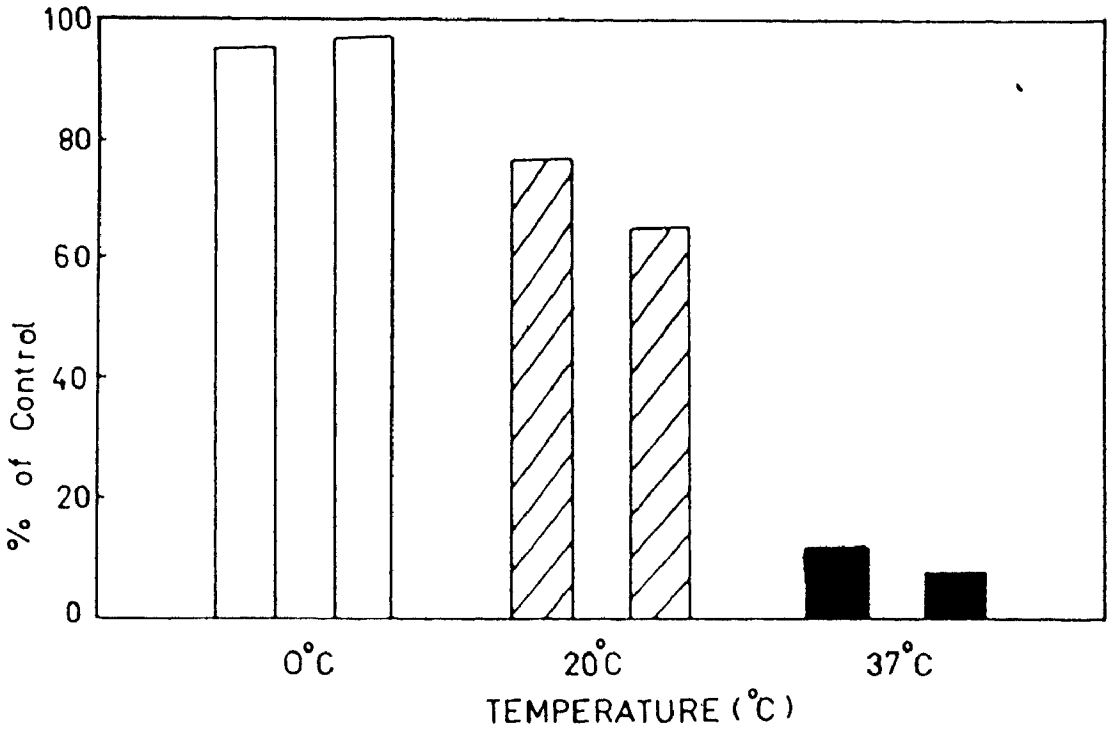


FIGURE 20. Temperature-Dependent Reversibility of Oxytocin Binding. The high speed (140,000 x $\bar{g}$) pellet from lactating rabbit mammary gland was incubated with [^{3}H]-oxytocin at 0°C, 20°C and 37°C, during 1 hr. Then a 500-fold excess of cold oxytocin was added in 50 µl and the mixture incubated for an additional 30 minutes. The results are expressed as a percentage of the control determinations in which no cold oxytocin was added. Mean ± range of 2 experiments are shown.

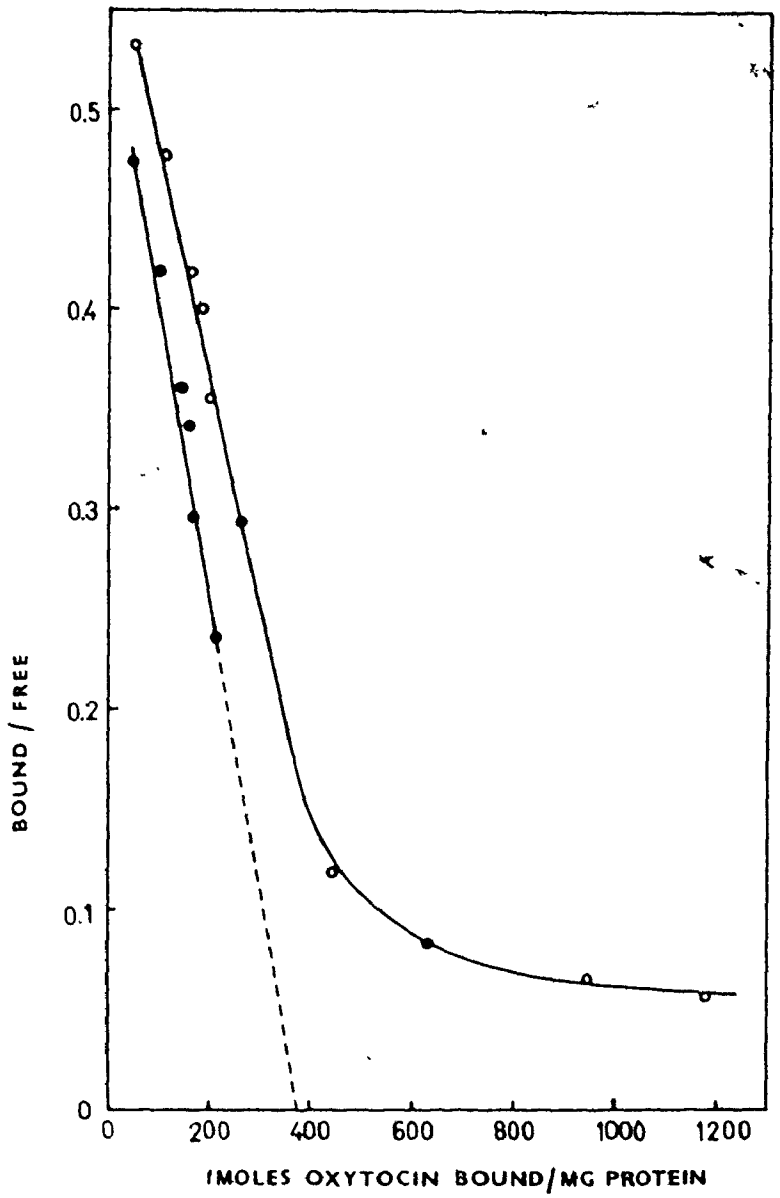


FIGURE 21. Scatchard Plot for Oxytocin Binding to High Speed (140,000 x g) Pellet from Lactating Rabbit Mammary Gland.
1 mg of protein (EDTA washed) was incubated for 2 hours with 100,000 DPM of [^{3}H]-oxytocin, and increasing amounts of non-radioactive oxytocin ranging from 0 to 200 ug. The PEG assay was used to determine the amount of bound oxytocin. The concentration centration of free oxytocin was calculated from the difference between total concentration and the amount bound. Values are expressed as the mean of triplicate determinations.

O—O Uncorrected data.

●—● Data corrected for non-specific binding (Chamness and McGuire, 1975).

IV. Solubilization Studies on the Oxytocin Binding Component of Lactating Rabbit Mammary Gland

A variety of treatments and procedures were carried out in attempts to solubilize the oxytocin receptor from lactating rabbit mammary gland. Initial experiments were done with deoxycholate (DOC) added to the final resuspension during preparation of the high speed (140,000 x $\bar{g}$) pellet (Table 10). This treatment abolished oxytocin binding ability in the pellet. However, the supernatant did not contain any detectable binding activity when examined using a variety of procedures described subsequently. An experiment was carried out in which the 140,000 x $\bar{g}$ pellet was treated for 15 minutes on ice with 0.25% DOC in TM buffer. Oxytocin binding ability was abolished in this material. The whole mixture (particulate and soluble material) was then dialyzed during 24 hours against TM buffer to remove the DOC. The removal of the detergent did not restore binding ability to the DOC treated material. Thus DOC treatment failed to produce a solubilized fraction which retained binding activity even when the detergent was removed.

The effect of three concentrations of the non-ionic detergent, Triton X-100 on the binding of oxytocin to the 140,000 x $\bar{g}$ pellet was studied (Figure 22). The amount of oxytocin binding ability remaining in the pellet was lowered with increasing detergent concentration. On the basis of these experiments, a concentration of 0.1% Triton was chosen for future experiments.

Since Triton X-100 treatment had removed oxytocin binding ability, which could have been solubilized into the supernatant, the detergent treated supernatants were examined by SDS-PAGE. Figures 23.a, b show the result of the SDS-PAGE analysis of 0.1% Triton X-100 treated supernatants. The patterns consistently showed an increase in a component with

TABLE 10

The Effect of 1% DOC on the Binding of [^{3}H]-Oxytocin to Lactating Rabbit Mammary Gland High Speed (140,000 x $\bar{g}$) Pellets

Treatment	Specific Binding			
	Experiment 1		Experiment 2	
	0.5 mg Protein	1.0 mg Protein	0.5 mg Protein	1.0 mg Protein
Untreated Pellets	767 ± 43	1311 ± 66	909 ± 83	1500 ± 99
1% DOC Treated Pellets	31 ± 22	26 ± 25	16 ± 9	10 ± 19

D.P.M. mean values ± range corresponding to 3 determinations are given for each protein concentration.

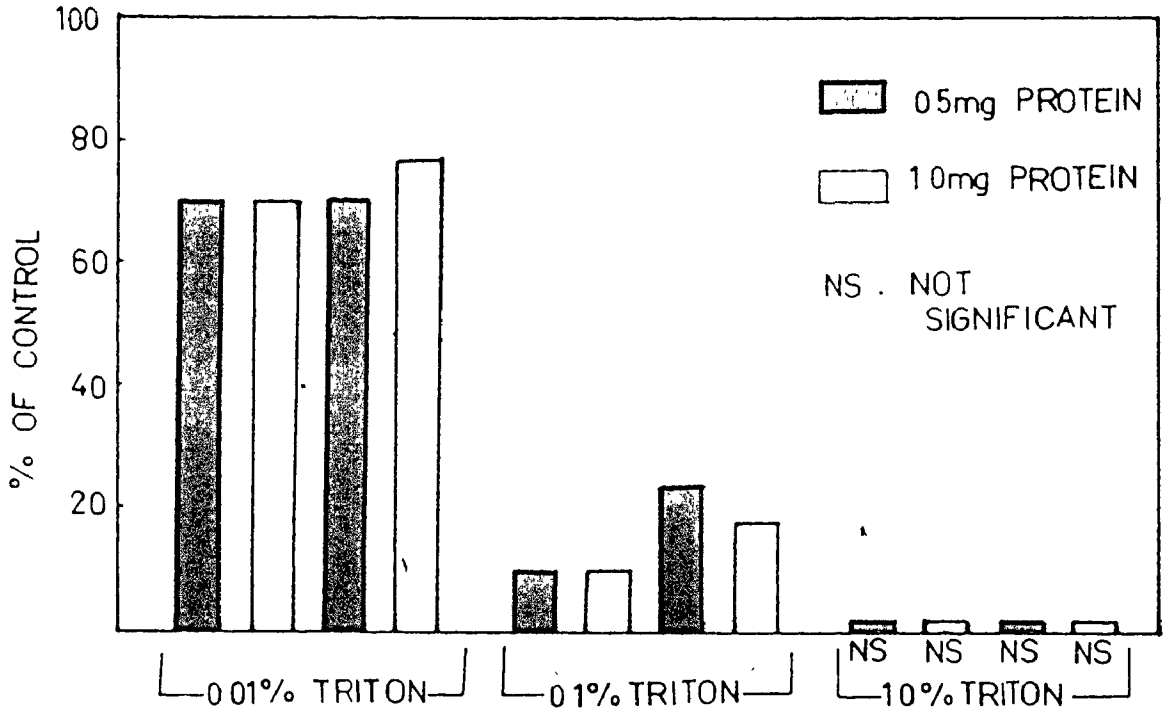


Figure 22. Effect of Triton X-100 on binding of [^{3}H]-oxytocin to high-speed pellets from lactating rabbit mammary gland. The detergent treatment was done at the concentrations indicated at 0°C during 15 minutes.

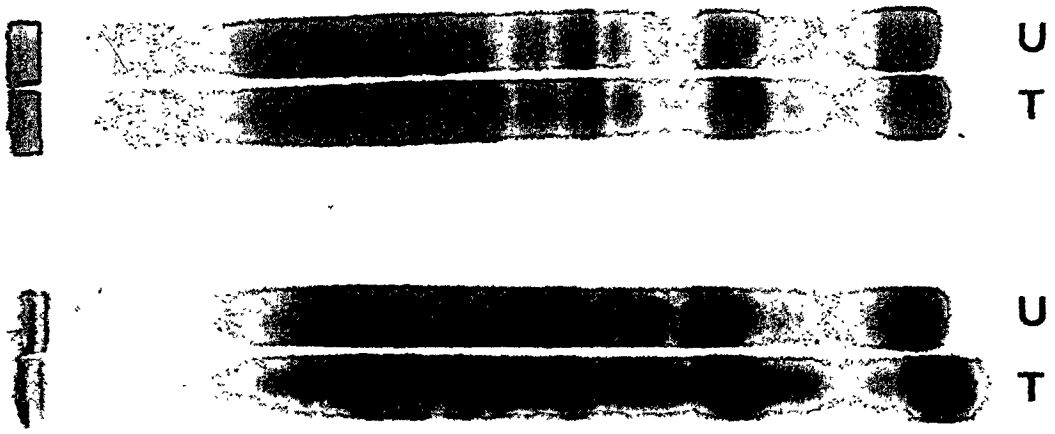


FIGURE 23.a. SDS-PAGE Analysis of Supernatants from Lactating Rabbit Mammary Gland Oxytocin Binding Fraction. The high speed (140,000 x g) pellet was treated with 0.1% Triton X-100 and the supernatant analysed by SDS-PAGE. The stained gels from two representative experiments are shown, corresponding to the supernatant from detergent treated (T) and untreated material (U).

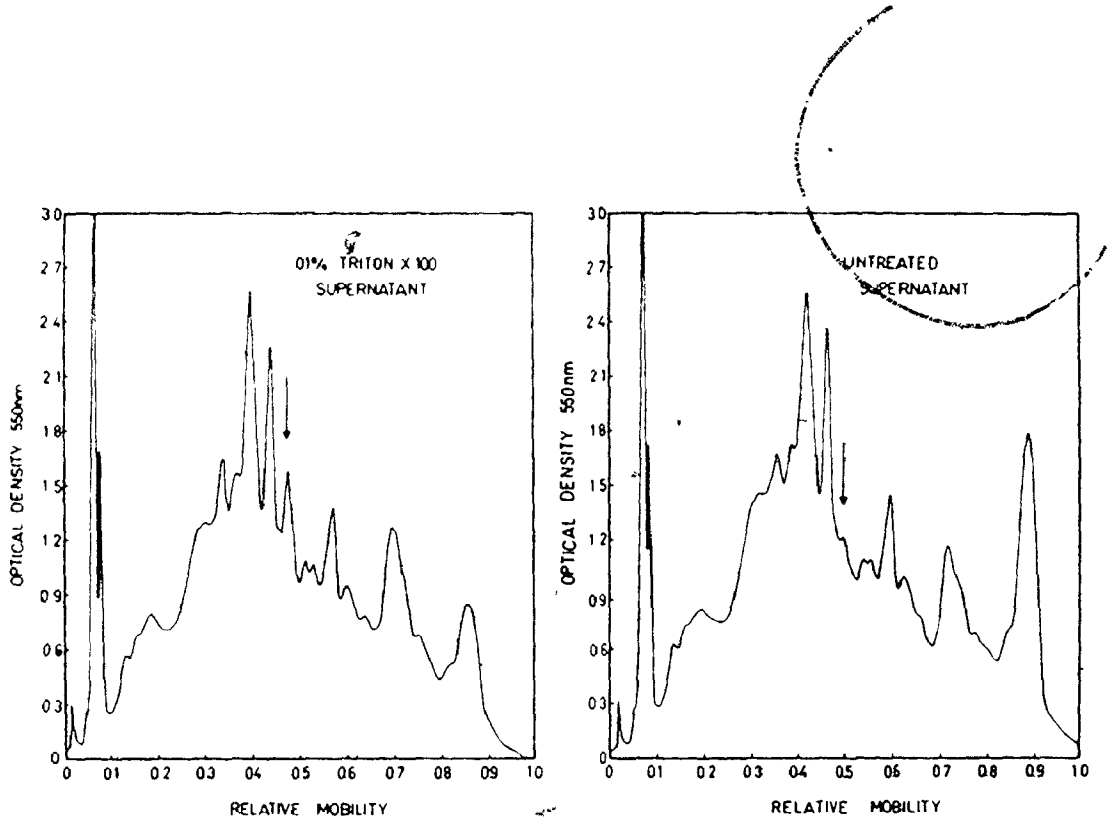


FIGURE 23.b. SDS-PAGE Analysis of Supernatants from Lactating Rabbit Mammary Gland Oxytocin Binding Fraction. Scans at 550 nm of two of the gels in Figure 23.a. are shown.

an approximate molecular weight of 55,000 present in the detergent treated supernatants. Subsequent SDS-PAGE analysis of supernatants obtained by EDTA treatment (described subsequently) of the impure binding fraction showed an apparently identical component. However, in the case of EDTA treatment, the pellets retained their full oxytocin binding activity.

Conditions which affect the ability of Triton X-100 to solubilize membrane proteins have also been examined. Triton X-100 had, thus far, been used in the presence of Mg^{2+} ions. Metal ions have been shown to stabilize membrane structure and their removal results in membrane solubilization (Reynolds and Trayer, 1971). In order to investigate the effectiveness of Triton in the absence of divalent cations, and also the use of EDTA itself as a potential solubilizing agent, the following experiments were done. The high speed pellet was treated with 5 mM Tris-buffer pH 8.0 containing 5 mM Mg^{2+} , 5 mM Mg^{2+} plus 0.1% Triton X-100, 5 mM EDTA or 5 mM EDTA plus 0.1% Triton X-100 (Table 11). EDTA treatment left only 34% of the initial protein insoluble, but the oxytocin binding activity in this protein increased to 158% of the control. Mg^{2+} alone left 61% of the initial protein, and oxytocin binding ability increased to 110% of the control. No binding was detected in the supernatants. Thus, these treatments were not suitable for solubilization of the oxytocin receptor. A control experiment done with equimolar concentrations of EDTA and Mg^{2+} did not result in an increase on protein solubilization over that observed with 5 mM Mg^{2+} alone, showing that the cause of EDTA-induced solubilization may be related to the chelation of divalent cations.

TABLE 11

Protein Removal and Remaining Oxytocin Binding Ability in Rabbit Mammary Gland Pellets Treated with Mg or EDTA in the Presence or Absence of 0.1% Triton X-100

Treatment	Protein Present	% of Control	(DPM per mg protein) Oxytocin Binding	% of Control
Control	30.4 mg	100	1030 ± 85	100
Mg^{2+} only	18.7 mg	61	1140 ± 60	110
Mg^{2+} - TX-100	14.0 mg	46	0	0
EDTA only	10.3 mg	34	1630 ± 100	158
EDTA + TX-100	7.5 mg	25	0	0

Values shown are mean ± range of three determinations.

In both cases where detergent was used, additional protein was solubilized over the corresponding control without detergent, and oxytocin binding ability in the remaining insoluble protein was abolished. No binding activity could be detected in the supernatants obtained subsequent to detergent treatment, either before or after detergent removal.

The use of EDTA wash of the impure binding fraction has consistently been observed to enhance binding by what seems to be removal of contaminating protein. This has provided an additional procedure to achieve some purification of the receptor. This step is easy to perform and has been used later to enhance oxytocin binding in impure binding fractions.

The results of the attempted solubilization of the oxytocin receptor presented thus far have stressed the loss of binding ability in the particulate fractions. The supernatant fractions have also been examined for oxytocin binding activity. Techniques tried were: exclusion chromatography, sucrose density gradient centrifugation, the use of dextran coated charcoal to remove unbound ligand, and finally the PEG-6000 assay procedure described previously. With these procedures, no binding has been detected in detergent treated supernatants. Removal of the detergent by gel filtration or by repeated washing of the solubilized protein using an Amicon Centriflo filter, did not result in binding. The technique for removal of Triton X-100 described by Holloway (1973) was tried unsuccessfully. Therefore, even though a large effort was placed on this aspect of the work and a variety of techniques employed, the supernatant fractions prepared by prior treatment with detergent followed by incubation with [^{3}H]-oxytocin did not show any detectable oxytocin binding activity.

Another procedure tried was to solubilize the hormone-receptor complex. The 140,000 x $\bar{g}$ pellet was incubated with [^{3}H]-oxytocin prior to the addition of detergent. The supernatant fraction was then examined for an increase in specifically bound oxytocin over the controls to which no detergent was added.

An initial experiment was done in which the effect of detergent addition following incubation with [^{3}H]-oxytocin was examined (Table 12). No separation into supernatant and particulate fraction was done in this experiment. Detergent treatment at 0-4°C following incubation with the hormone permitted recovery of specific oxytocin binding, whereas detergent treatment prior to incubation with [^{3}H]-oxytocin abolished binding. In subsequent experiments with hormone pretreatment, separation of the binding into the supernatant fraction and the particulate fraction was examined. Table 13 shows the effect of 0.1% Triton X-100 on these two fractions. Detergent treatment after pre-incubation with the hormone resulted in an increase of the specific binding in the supernatant to approximately twice that observed in the controls which were not detergent treated. The total recovery of binding was almost quantitative. When the concentration of Triton X-100 was increased to 1.0% in the same kind of experiment, an increase in the specific binding was again observed in the supernatant, when compared to the controls (Table 14). However, the increase was less marked and, more significantly, the recovery of specific binding in the detergent treated samples was only about 50-75% of that observed in the control samples. Thus, a high concentration of detergent, even after hormone pre-incubation seems to be detrimental to the stability of the hormone-receptor complex. When sucrose density gradient purified material

TABLE 12

Comparison of Specific Oxytocin Binding in High Speed (140,000 x $\bar{g}$) Pellets from Rabbit Mammary Gland Prior to and Following Incubation with Tritiated Oxytocin. Effect of Preincubation with Oxytocin on Detergent-Treatment. The mean ± range values of three determinations are shown.

Treatment	Specific Binding (DPM/mg of protein) 1% Lubrol-PX	1% Triton X-100
#1: No Detergent Added	664 ± 110 (100%)	772 ± 58 (100%)
#2: Detergent treatment; no preincubation with [^{3}H]-oxytocin	35 ± 24 (5.3% ± 4.6)	* (Not significantly different from background)
#3: Detergent treatment; preincubation with [^{3}H]-oxytocin	502 ± 25 (75.6% ± 16.9)	635 ± 51 (82.3% ± 10.3)

TABLE 13

Effect of 0.1% Triton X-100 on the Appearance of Specific Oxytocin Binding in a 20,000 x g Supernatant following Preincubation with [^{3}H]-Oxytocin*

	Control (DPM)			0.1% TX-100 (DPM)			
Time ǂ	Pellet	Supernatant	Total	Pellet	Supernatant	Total	% of Control
15 min.	10860 ± 355	3600 ± 185	14460 ± 540	5780 ± 220	7540 ± 270	13320 ± 490	92 ± 7 %
60 min.	10580 ± 120	3530 ± 510	14110 ± 630	6140 ± 160	6740 ± 300	12880 ± 460	91 ± 8 %

Mean values ± range of two determinations are shown.

* Incubations were scaled up 5-fold from the conditions described in Materials and Methods (pp. 41-42).

ǂ Refers to the time of detergent treatment.

TABLE 14

Effect of 1.0% Triton X-100 on the Appearance of Specific Oxytocin Binding in a 20,000 x g Supernatant Following Preincubation with [^{3}H]-Oxytocin*

	CONTROL (DPM)			1.0% TX-100 (DPM)			
	Pellet	Supernatant	Total	Pellet	Supernatant	Total	% of Control
Time‡							
15 min.	11050 ± 360	3750 ± 430	14800 ± 790	2410 ± 75	4540 ± 270	6950 ± 345	47 ± 10%
60 min.	9755 ± 330	2310 ± 140	12065 ± 470	2215 ± 145	3340 ± 190	5555 ± 335	46 ± 10%

Mean values ± range of three determinations are shown.

* Incubations were scaled up 5-fold from the conditions described in Materials and Methods (pp. 41-42).

‡ Refers to the time of detergent treatment.

was examined in the same way, the same result was found (Table 15). In this experiment, 15 minutes was used for detergent treatment since from the previous experiments it was known that the length of detergent treatment had no effect on our results.

In order to examine the effect of increased centrifugal force on the 20,000 x $\bar{g}$ supernatant binding fraction, experiments were done in which identical samples were centrifuged at 140,000 x $\bar{g}$ for 30 minutes rather than 20,000 x $\bar{g}$ for 30 minutes. This treatment lowered, but did not eliminate the binding from the supernatant (Table 16). When no detergent was present, centrifugation at 140,000 x $\bar{g}$ removed all specific binding from the supernatant.

Further examination of the solubilized binding material prepared by treatment of the oxytocin pre-incubated material with 0.1% Triton X-100 was done to see whether this material could be removed from the supernatant by centrifugation at a higher speed. After oxytocin pre-incubation and treatment with detergent, centrifugation at 210,000 x $\bar{g}_{av.}$ (280,000 x $\bar{g}_{max.}$) for 30 minutes was done. As can be seen from the results shown in Table 17, no loss of oxytocin binding material was observed,

As shown by the time course of binding (Figure 18), binding was maximal at approximately 100 minutes. Therefore, more soluble hormone-receptor complex should be obtained by extending the pre-incubation time to 2 hours. This was observed, as shown in Table 18. The amount of labelled hormone-receptor complex solubilized after a 2 hour pre-incubation increased, while the percentage of total binding solubilized remained unchanged.

To study the specificity of the binding of this solubilized material, the effects of oxytocin analogs were examined. A similar approach as

the one previously described for the 140,000 x $\bar{g}$ pellet (Figure 19) was used. As can be seen from the results shown in Figure 24, the competition of [1-deamino]-oxytocin with [^{3}H]-oxytocin for the solubilized receptor was of similar magnitude as that observed between unlabelled oxytocin and [^{3}H]-oxytocin, while [4-proline]-oxytocin did not compete significantly.

TABLE 15

Effect of Preincubation with [^{3}H]-oxytocin and Subsequent 0.1% Triton X-100 Treatment of a Sucrose Gradient Purified Binding Fraction on the Appearance of Oxytocin Binding in a 20,000 x $\bar{g}$ Supernatant

	Pellet (DPM)	Supernatant (DPM)	Total (DPM)	% of Control
Control	17840 ± 535	5780 ± 525	23620 ± 1060	100
0.1% TX-100	13900 ± 1250	8490 ± 1010	22390 ± 2260	95

Mean values ± range of three determinations are shown.

TABLE 16

Effect of High Speed (140,000 x $\bar{g}$) Centrifugation on Oxytocin Binding Remaining in Detergent Treated and Untreated 20,000 x $\bar{g}$ Supernatants Which Were Prepared Following Preincubation with [^{3}H]-Oxytocin

Treatment	No Detergent (DPM)	0.1% TX-100 (DPM)
No Centrifugation	2090 ± 120	2510 ± 50
140,000 x g 30 min.	0	780 ± 35

Mean values ± range of three determinations are shown.

Table 17

Effect of Increased Centrifugal Force on Specific Oxytocin Binding in Detergent-Treated Supernatants

(DPM average values ± range of two determinations are shown)

Centrifugation Force	Experiment 1	Experiment 2
140,000 x $\bar{g}$ (av.)	1521 ± 201	1172 ± 67
210,000 x $\bar{g}$ (av.)	1654 ± 440	1230 ± 117

The 140,000 x $\bar{g}$ rabbit mammary gland pellet was pre-incubated with [^{3}H]-oxytocin and then treated with 0.1% Triton X-100 for 15 minutes at 0-4°C. After centrifugation during 30 minutes the specific binding of oxytocin remaining in the supernatants was measured using the PEG-6000 assay (see: Materials and Methods).

TABLE 18

Effect of Pre-incubation Time on the Amount of Hormone-Receptor Complex in a Detergent-treated 210,000 x $\bar{g}$ Pellet and Supernatant

DPM average values ± range of two determinations are shown

Fraction	1 hour pre-incubation		2 hour pre-incubation		% Increase	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
210,000 x $\bar{g}$ Pellet	6307 ± 65	5798 ± 187	10,021 ± 30	8102 ± 373	159	140
210,000 x $\bar{g}$ Supernatant	1784 ± 396 (22.0%)	1592 ± 201 (21.5%)	2684 ± 275 (21.1%)	2802 ± 161 (25.7%)	150	176

The 140,000 x $\bar{g}$ rabbit mammary gland pellet was pre-incubated with [^{3}H]-oxytocin and [^{3}H]-oxytocin plus non-radioactive oxytocin as described in Materials and Methods. Treatment with 0.1% Triton X-100 was followed by centrifugation at 210,000 x $\bar{g}_{av}$ for 30 minutes. Binding in the supernatant was determined using the PEG-6000 assay. Values in parentheses indicate the proportion of the total binding which was solubilized.

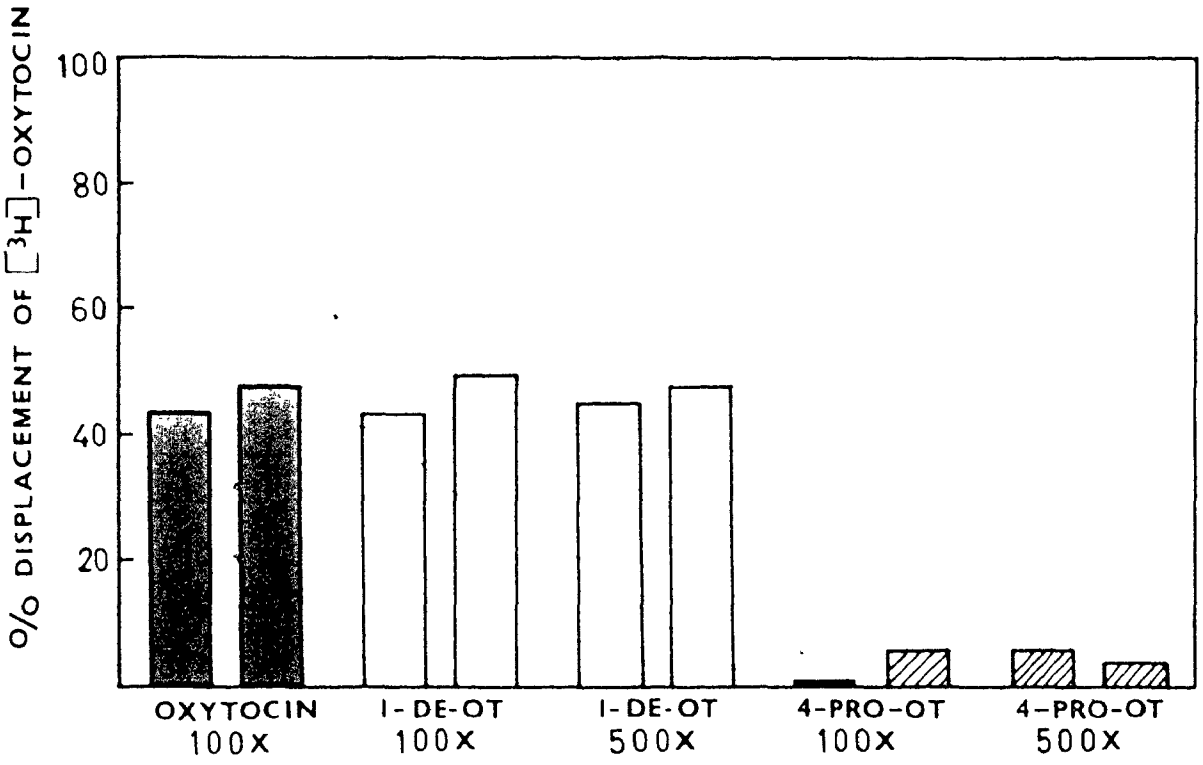


FIGURE 24. Displacement of Oxytocin from the Preincubated, Detergent Treated Soluble Binding Fraction by Analogs of Oxytocin. 140,000 x $\bar{g}$ pellet protein was incubated with [^{3}H]-oxytocin and the peptides indicated prior to the addition of detergent. Triton X-100 was added to a final concentration of 0.1% and the incubations were placed on ice for 15 min. They were then centrifuged at 210,000 x $\bar{g}_{av.}$ for 30 min and the specific oxytocin binding present in the supernatants was determined using the PEG assay. Results of two experiments are shown.

DISCUSSION

I. The Choice of Experimental Systems

a. The Choice of Oxytocin Target Tissue: Lactating Mammary Gland

In the initial stages of the research reported in this thesis, several oxytocin target tissues were examined (rat and rhesus monkey uterine smooth muscle, rat and rabbit lactating mammary gland). The lactating rabbit mammary gland was chosen for the following reasons:
1) Large amounts of tissue can be obtained at a reasonable cost.
2) After lactation is established, the physiological and hormonal status of the tissue does not change significantly for a period of three weeks.
3) It is a highly specific tissue for oxytocin.

Both uterus and mammary gland, the two target organs for oxytocin which are believed to be related to the hormones physiological role, are composed of a variety of cell types (for reviews see: Hamperl, 1970; O'Rahilly, 1973). The oxytocin target cells in the uterus are present in the myometrium, which can be dissected out from the rest of the tissue. Since the wet weight of the estrogen-dominated rabbit uterus is approximately 5 grams, the yield of myometrial tissue is very small. In lactating mammary gland, the cells which are known to be a target for oxytocin, the myoepithelial cells, are a minority. However, the wet weight of a lactating rabbit mammary gland is 100-200 grams. This was sufficient to yield a reasonable amount of oxytocin binding material which was required for the receptor solubilization studies reported here.

To obtain uteri which have been exposed to a uniform hormonal status, the animals must be pretreated with pharmacological doses of

estrogens. With mammary tissue, however, after lactation is established at 7-10 days post-partum, the hormonal status of this tissue is reasonably constant, and the sensitivity of the tissue of rabbit mammary gland to oxytocin remains maximal until 40 days post-partum (Bisset, 1968). For this study, lactating mammary tissue was obtained from rabbits between the 10th and 12th days of lactation.

The myometrium contracts in response to several compounds. In addition to oxytocin, the peptides angiotensin II and bradykinin have been shown to stimulate this tissue. The lactating rabbit mammary gland, however, is very specifically responsive to oxytocin (Bisset, 1968).

In order to examine the suitability of lactating mammary gland for these studies, preliminary studies were carried out with both lactating rat and rabbit mammary gland. These were designed to examine: 1) the dependence of oxytocin binding on protein concentration; 2) the possibility that, as with other peptide hormones, plasma membranes contain the receptor for oxytocin; 3) the competition of non-radioactive oxytocin with [^{3}H]-oxytocin for specific receptor sites.

The total binding of [^{3}H]-oxytocin to a homogenate prepared from lactating rat mammary gland showed that at high protein concentrations, the linearity of the graph of oxytocin bound versus protein concentration was lost (Figure 14a). This phenomenon could be due to enzymatic degration of the [^{3}H]-oxytocin at higher protein concentrations. Although linearity was not lost with an analagous fraction from lactating rabbit mammary gland (Figure 15a), the maximum amount of protein used (1.7 mg) was less than that used in experiments with the rat mammary gland system (2.0 mg). The experiments are, therefore, not strictly comparable.

When a fraction enriched in plasma membranes from lactating rat mammary gland was used, an approximate increase of 4-fold in the binding of oxytocin was observed (Figure 14b). A similar increase was observed with a plasma membrane preparation from lactating rabbit mammary gland (Figure 15b). These experiments (Figures 14a-15b) indicated that binding of oxytocin to fractions from lactating rat and rabbit mammary gland could be measured and that the binding component appeared to be located in a fraction enriched in plasma membranes. The fact that non-radioactive oxytocin competed with [^{3}H]-oxytocin for binding supported the premise that the binding was due to a binding component specific for oxytocin, as judged by this single criterion (Figure 16).

Another reason for choosing lactating mammary gland was the fact that other studies being carried out in this laboratory have provided additional information about other properties of this tissue.

b. The Choice of an Oxytocin Binding Fraction

Once the decision had been made to use lactating rabbit mammary gland for this study, it was necessary to choose between a crude membrane fraction or a purified plasma membrane fraction prepared from this tissue A crude membrane fraction (140,000 x $\bar{g}$ pellet) was chosen because a large amount of oxytocin binding material could be prepared in a relatively short length of time. Material sufficient for 16 experiments could be prepared in 8-10 hours. This permitted storage of material which could be used as needed, and facilitated this study. In contrast to this, the preparation of a fraction enriched in plasma membranes (Figure 5) required 8-10 hours to prepare a small amount of material (less than 5 mg of protein) sufficient for one experiment. Preparation time would have been a

limiting factor in this case. Since oxytocin binding to the impure fractions could be detected with sufficient sensitivity to permit study of the effects of detergents on the oxytocin receptor, the expenditure of the additional time required for preparation of a purer fraction was not judged to be warranted.

II. Determination of Specific Binding of [^{3}H]-oxytocin to Rabbit Mammary Gland Subcellular Fractions.

This section discusses in more detail some aspects of the conditions and techniques used to determine the specific binding of [^{3}H]-oxytocin to subcellular fractions prepared from lactating rabbit mammary gland. Two other factors, the non-specific binding of [^{3}H]-oxytocin, and the possible degradation of the labelled oxytocin during the incubations carried out in this study will also be discussed.

a. The Concentration of Oxytocin and Incubation Time Used for These Studies

The concentration of [^{3}H]-oxytocin routinely used in the binding assays was approximately 0.8 nM. The preliminary binding studies discussed in the previous section (Figures 14a-16) indicated that this concentration resulted in sufficient radioactive labelling of the various subcellular fractions to permit study of the formation of the oxytocin-receptor complex. However, on the basis of the K_d of 9.5×10^{-10} M found by Soloff and Swartz (1973) for the rat mammary gland oxytocin receptor, it was not expected that 0.8 nM oxytocin would be sufficient to saturate all the available receptor sites. Scatchard analysis (Scatchard, 1949), discussed in the next section, has confirmed that an oxytocin concentration of 0.8 nM was not sufficient to saturate the receptor sites since a

K_d of 3.2×10^{-9} M was found for the rabbit mammary gland receptor. This was not considered to be of concern in relation to the studies reported in this thesis since much of the work carried out was comparative, i.e. comparison of detergent treated fractions with untreated fractions or comparison of various concentrations of detergent on a subcellular fraction. Study of a labelled fraction of the receptors was considered adequate for establishing conditions suitable for solubilization of the receptor. Then, once these conditions were found, higher concentrations could be used to study the solubilized receptor. The uncertainty and difficulty in obtaining [^{3}H]-oxytocin were influential on the above decisions.

A one hour incubation was routinely used for these studies. Soloff and Swartz (1973) found that a one hour incubation was required for maximal oxytocin binding to lactating rat mammary gland. In view of the rapid response (<1 minute) to oxytocin which is observed in vivo or with isolated organ preparations, this time seemed adequate. However, later examination of this problem has shown that in this system, 1 hour was not sufficient for maximal binding (Figure 18). Approximately 100 minutes were required for maximal binding to occur. The longer time required for [^{3}H]-oxytocin binding to lactating rabbit mammary gland may have been due to the fact that Soloff and Swartz (1973) used approximately 6 nM [^{3}H]-oxytocin for determination of the time course of binding. However, lower concentrations, also in the range of 0.8 nM, were used by them for some studies. Regardless of the cause for this difference in the two systems, interpretations of the data and conclusions reached on the basis of the data obtained in this study are not dependent upon maximal or equilibrium binding, with the exception of the Scatchard plot which

was done under equilibrium conditions.

b. Assay Procedures for the Determination of [^{3}H]-oxytocin binding

Two procedures, the centrifugation assay and the PEG-6000 assay have been used to determine oxytocin binding. Initial studies with the particulate receptor were done using the centrifugation assay. This technique is not, however, suitable for the assay of soluble receptors. PEG-6000 has been used successfully as a precipitant to facilitate assay of several other hormone-receptor complexes (Cuatrecasas, 1972b; Shiu and Friesen, 1974b). When oxytocin binding to a particulate fraction was determined using both the centrifugation assay and the PEG-6000 assay, the two techniques gave the same results. This indicated that PEG-6000 did not alter binding to the particulate fraction. It was also found in this study that the detergent solubilized hormone-receptor complex was precipitated by 10% (w/v) PEG-6000. Recovery of detergent solubilized specific binding by this procedure was quantitative when compared to a control not treated with detergent (Table 13). In view of these observations, the PEG-6000 assay was then used routinely for determining oxytocin binding to both particulate and soluble fractions.

c. Determination of Total, Specific and Non-specific Binding

Total binding of a ligand to a cellular or subcellular fraction does not necessarily give a valid estimate of the amount of ligand bound to specific ligand receptors. There is, usually, a component of non-specific binding which must be estimated and subtracted from the total binding in order to estimate specific binding more accurately. Determination of the specific and non-specific components of binding is done by simultaneously carrying out two sets of incubations, one set containing

only radioactive ligand, the other containing both radioactive ligand and an excess of non-radioactive ligand. The excess non-radioactive ligand competes with the radioactive ligand for the high affinity receptors, but not for the low affinity receptors responsible for the non-specific binding. Specific binding is calculated by subtracting the value obtained for the amount of radioactivity bound in the presence of the non-radioactive ligand from that obtained in the absence of the non-radioactive ligand.

The amount of non-radioactive ligand employed for this purpose varies widely. Soloff and Swartz (1973) used approximately a 10-fold excess of non-radioactive oxytocin for much of their work, although up to a 160-fold excess was used for some experiments. In this study, a 100-fold excess was routinely used. A 10-fold excess resulted in a 45% reduction in bound [^{3}H]-oxytocin, whereas a 100-fold excess increased this to approximately 70%. A 400-fold excess did not significantly increase the amount of [^{3}H]-oxytocin displaced (Figure 19). Data from Soloff and Swartz (1973) indicate that they observed a displacement of approximately 60% of the bound [^{3}H]-oxytocin when using a 10-fold excess of non-radioactive oxytocin. Although direct comparisons are not possible, these results indicate that under the conditions used both systems show non-specific binding to the particulate fractions in the range of 30-40% of the total binding.

d. Degradation of Oxytocin

Two enzyme activities capable of degrading oxytocin during incubation with the 140,000 x $\bar{g}$ pellet have been examined. These are: 1) glutathione-insulin transhydrogenase (GIT), and 2) protease. The activity of GIT increases during lactation and this enzyme has been shown

to degrade oxytocin *in vitro* (Ferrier, *et al.*, 1973). It opens the 20-atom ring present in oxytocin by reducing the disulfide bond between residues 1 and 6 (Figure 1). GIT activity was not found in the 140,000 x $\bar{g}$ rabbit mammary gland pellet when measured by the method of Varandani (see: Materials and Methods). The assay was carried out in thr presence of glutathione which is required for enzyme activity. Since the tissue fractions used for the oxytocin binding assays were washed and sedimented several times prior to incubation with oxytocin, the presence of glutathione, and therefore, of GIT activity was unlikely.

Protease activity of the 140,000 x $\bar{g}$ pellet was also examined using [^{125}I]-casein as substrate (Hatton and Regoeczi, 1975). During an 18-hour incubation using the same protein concentration employed for the oxytocin binding assay (4 mg/ml), only 1% of the radioactivity became soluble in trichloroacetic acid. The relatively short incubation time used for the oxytocin binding assay (1 hour) makes significant oxytocin degradation by protease activity unlikely. However, as a precaution, 0.1% gelatin was always present in the binding assays in order to provide an alternative substrate for proteases which might otherwise degrade oxytocin.

III. The Nature of the Rabbit Mammary Gland Oxytocin Binding Component

Several lines of evidence support the conclusion that the component of rabbit mammary gland which was examined during this study is a physiological receptor for oxytocin in this tissue. The evidence was as follows:

1) The receptor was not uniformly distributed in the material prepared from mammary tissue and was not present in a non-target organ. It is

well established that peptide hormones bind to receptors present in the plasma membranes of their target cells (Kahn, 1976b). Specific binding was primarily found in the membranous material which constituted the brownish upper layer of the 140,000 x $\bar{g}$ pellet (Table 6). The white lower portion of the pellet, possibly derived from connective tissue, did not show significant amounts of oxytocin binding. This supports the view that a specific component of the membranous fraction was responsible for the binding. When a non-target organ was fractionated following the same method used for the mammary gland, no significant binding of oxytocin was observed with any of the fractions (Table 7). The binding component appeared to be specific to the target tissue and absent in a non-target tissue.

2) The active analog [1-deamino]-oxytocin competed with [^{3}H]-oxytocin for binding, but the almost inactive analog, [4-proline]-oxytocin, did not. [1-Deamino]-oxytocin is approximately 20% more active than oxytocin in causing milk ejection (Table 8). On this basis, it would be expected to compete with [^{3}H]-oxytocin for specific binding sites. Conversely, [4-proline]-oxytocin has less than 0.01% of the milk ejection activity of oxytocin (Table 8) and would not be expected to compete significantly for the oxytocin receptors. This is, in fact, what was observed when these two analogs were used to compete with [^{3}H]-oxytocin for binding to the specific sites (Figure 19). This behavior of analogs is consistent with the conclusion that the binding component examined was a specific oxytocin receptor. Soloff and Swartz (1973) have also shown that [4-proline]-oxytocin did not compete with [^{3}H]-oxytocin for binding

to rat mammary gland receptors. Insulin did not compete with [^{3}H]-oxytocin for binding. Insulin, like oxytocin, has a disulfide containing 20-atom ring.

3) Magnesium ions were essential for specific binding (Table 9). Soloff and Swartz (1973) also found the presence of divalent cations essential for specific oxytocin binding to rat mammary gland. The potentiation of oxytocic activity by Mg^{2+} was mentioned previously (page 10). Somlyo and co-workers (1966) demonstrated that addition of 1.2 mM Mg^{2+} to a rabbit mammary gland strip, previously contracted by oxytocin or vasopressin, resulted in a secondary contraction with a magnitude of from 0.2 to 14 times the original contraction. In view of these findings, the Mg^{2+} requirement for binding observed in this study supports the other evidence that the binding was occurring to a physiological receptor.

Binding did not occur in the absence of Mg^{2+}, but was restored when Mg^{2+} was added. Thus, the loss of binding observed in the absence of Mg^{2+} was reversible (Table 9b). It is possible that divalent cations such as Mg^{2+} form a dissociable part of the oxytocin receptor and that loss of this component inactivated the receptor.

4) Oxytocin binding was a reversible process (Figure 20). Although the reversibility was low at 0°C, 20-30% of the hormone dissociated from the receptor during 30 minutes at 20°C and 90-95% dissociated during 30 minutes at 37°C. Although the reversibility of the response to oxytocin is much faster, it is probable that other factors such as hormone degradation also control the reversibility <u>in vivo</u> or in the isolated target organ. At 37°C, 25 hours are required for the dissociation of 90-95% of prolactin from membranes prepared from rabbit mammary gland (Shiu and

Friesen, 1974a), with slower dissociation rates observed at lower temperatures. Cuatrecasas and Hollenberg (1976) have pointed out that, "... reversibility of binding may prove to be characteristic of the majority of hormone-receptor interactions."

5) Scatchard analysis (Scatchard, 1949) has demonstrated high affinity sites with characteristics similar to those observed in rat mammary gland by Soloff and Swartz (1973). Using the 140,000 x $\bar{g}$ pellet from lactating rabbit mammary gland, a binding capacity of approximately 385 fmoles per milligram protein and a K_d of 3.2 x 10^{-9} M was obtained after correction for non-specific binding had been made using the procedure of Chamness and McGuire (1975) (Figure 21). Soloff and Swartz (1973) obtained a binding capacity of 275 fmoles per miligram protein and a K_d of 0.95 x 10^{-9}M. No corrections were made by these workers for non-specific binding.

The K_d represents the concentration of ligand required for half-maximal occupancy of the available receptors. If the value of 3.2 x 10^{-9} M is converted to International Units of milk ejecting activity per millilitre, a value of 1.6 mU/ml is obtained. This is a high and perhaps a non-physiological concentration and would elicit more than a half-maximal response in the mammary gland <u>in vivo</u>. Two possibilities arise:

1) Isolation of the receptors alters the K_d which, <u>in vivo</u> is much lower;

2) Only a small percentage of the total oxytocin receptors need be occupied in order for a physiological response to be elicited. The first possibility seems improbable since Soloff (1976) has reported a K_d of 1.8 x 10^{-9}M for intact myoepithelial cells from rat mammary gland. This closely approximates the values for the systems under discussion. The second possibility appears more likely. This conclusion is supported by

estimating the amount of oxytocin which could be bound per gram wet weight of mammary tissue based on the value of 385 fmoles/mg protein in the 140,000 x $\bar{g}$ pellet. The value obtained is approximately 1.5 mU/g wet weight. This is a very high capacity, and is additional evidence for a large reserve of "spare receptors". It must be remembered, however, that this high binding capacity was observed after a 2 hour incubation required to reach equilibrium (Figure 18). Only a small proportion of these receptors would be occupied in the short time (less than 1 minute) required for a biological response to be elicited. The function of this apparent receptor reserve is not known.

6) In addition to the five major points discussed above, the effect of pH upon oxytocin binding was also examined. Maximum binding was observed in the region of pH 7.6-8.0 (Figure 17). Soloff and Swartz (1973) observed a pH maximum for binding with rat mammary gland around pH 7.6. The maximum observed in this study was in good agreement with that found for rat mammary gland.

The data discussed above support the premise that the oxytocin binding component which was observed in rabbit mammary gland is a physiological receptor for this hormone. However, a more satisfactory indication that the hormone binding observed in these subcellular systems involved a physiological receptor would require more direct correlation of hormone binding with a biological response. This has not yet been done for any mammalian oxytocin-receptor system.

IV. Partial Purification of the Plasma Membrane Fraction Containing Oxytocin Receptors

The impure 140,000 x $\bar{g}$ pellet prepared from lactating rabbit

mammary gland provided, for this study, a satisfactory alternative to the preparation of a purified plasma membrane fraction from this tissue. However, the preparation of a purified plasma membrane fraction was desirable for some purposes such as investigation of the premise that the oxytocin receptor was located on the plasma membranes from this tissue. Soloff and Swartz (1973) examined 5'-nucleotidase activity, an enzyme shown by Keenan and co-workers (1970) to be a valid marker for mammary gland plasma membranes, in sub-cellular fractions from lactating rat mammary gland. No correlation of 5'-nucleotidase activity with oxytocin binding activity was found in any of the sub-cellular fractions.

Chang and co-workers (1975) found that prelabelling the plasma membranes of adipocytes or hepatocytes with [^{125}I]-ligands such as insulin, cholera toxin, lectins, wheat germ agglutinin or concanavalin A prior to homogenization and sub-cellular fractionation facilitated the isolation of fractions enriched in plasma membranes. As observed with the oxytocin-receptor complex (Figure 20), the receptor-ligand complexes did not dissociate appreciably if kept at 4^{o}C. Prelabelling of plasma membranes prior to their isolation has also been used successfully by Rittenhouse-Simmons and Deykin (1976) who used [^{125}I]-lectin as a marker for platelet membranes, and by Luzio and co-workers (1976) who used an [^{125}I]-immunoglobulin prepared against rat fat cell membranes as a marker for those membranes. The advantages of the procedure are that it permits more precise identification of a particular plasma membrane fraction, e.g. that containing insulin receptors, since ligands can be used which interact with a specific receptor. Furthermore, a higher yield of plasma membranes is obtained since identification of the plasma membrane fractions can

be done with more certainty. In a heterogeneous tissue such as mammary gland, these are important considerations since plasma membranes from various cell types are present, and most of the plasma membranes are not derived from myoepithelial cells.

Prelabelling of receptors has not been widely used in hormone binding studies. Brecher and co-workers (1974) found that an angiotensin-II-receptor complex prepared from rat adrenal gland could be separated on a 10-40% sucrose density gradient. They did not, however, examine their hormone-receptor preparation for plasma membrane marker enzyme activities. Soloff and Swartz (1973) were able to detect specific binding of oxytocin in subcellular fractions of lactating rat mammary gland by incubating small pieces of gland with [^{3}H]-oxytocin prior to homogenization and fractionation of the tissue. Examination of 1,000 x $\bar{g}$, 20,000 x $\bar{g}$, and 105,000 x $\bar{g}$ pellets showed that both 5'-nucleotidase and oxytocin binding activities were distributed in the three fractions, and no correlation existed between them. This wide distribution and lack of correlation of the two activities could be due to heterogeneity in the size of the plasma membrane fragments.

When the oxytocin receptors present in the 140,000 x $\bar{g}$ pellet from lactating rabbit mammary gland were pre-incubated with [^{3}H]-oxytocin and the material subsequently fractionated on a 10-40% sucrose density gradient, a broad peak of radioactivity was found in the gradient. The addition of unlabelled oxytocin during pre-incubation of the receptors suppressed this peak which, therefore, appears to have been due primarily to specific oxytocin binding (Figure 10). These experiments indicated that an oxytocin binding fraction could be obtained by this procedure. In subsequent preparative experiments, fractions isolated from the upper,

middle and lower thirds of the sucrose density gradients were incubated with oxytocin following centrifugation (Table 5a). The observation that the lower and middle thirds of the gradient contained material which showed the greatest enrichment (approximately 5-6 fold and 3-5 fold respectively) of oxytocin binding was in agreement with the previous data (Figure 10). Electron microscopy of these combined fractions showed that they were not homogeneous (Figure 11). A variety of membrane fragments can be seen which appear to be mainly plasma membranes and rough endoplasmic reticulum. The significant increase in 5'-nucleotidase activity (4.7-7.8 fold) found in the material located in the lower third of the gradient (Table 5b) strengthened the premise that this fraction was enriched in plasma membranes and is additional evidence that the oxytocin receptors are present in the plasma membranes of its target cells in mammary gland.

Although this gradient purified material has been used for some work in this study (Table 15) and appears to behave towards detergents similarly to the impure 140,000 x $\bar{g}$ pellet, the problems of small amounts of material and long preparation times were not overcome by this procedure. This precluded routine use of this fraction. However, the use of this material or its further purification may facilitate future aspects of this study.

V. Attempted Solubilization of the Oxytocin Receptor without Preincubation with [^{3}H]-oxytocin.

a. The effect of Detergents upon the Oxytocin Receptor of Rabbit Mammary Gland

A number of hormone receptors have been solubilized by treatment with detergents (see: Introduction). Although DOC is considered to be

a fairly mild detergent for the solubilization of membrane components (Tzagoloff and Penefsky, 1971; Makino, et al., 1973), treatment of the 140,000 x $\bar{g}$ pellet from lactating rabbit mammary gland with 1% DOC resulted in a quantitative loss of oxytocin binding (Table 10). More extensive work was done with Triton X-100, a non-ionic detergent (Figure 3). Treatment of the 140,000 x $\bar{g}$ lactating mammary gland pellet with three concentrations of Triton X-100 showed that the loss of binding activity of the particulate material was dependent on the concentration of Triton X-100 employed (Figure 22). Lubrol-PX, also a non-ionic detergent, gave essentially the same results. Regardless of the detergent used, extensive washing of the pellet to remove free detergent did not restore binding to its original level.

The supernatants, both from the DOC and Triton X-100 treated pellets, did not show any oxytocin binding activity when incubated with [^{3}H]-oxytocin and assayed using the PEG-6000 procedure. Lowering the concentration of DOC present in these supernatants by dialysis against detergent free buffer did not restore binding activity, nor did lowering the concentration of free Triton X-100 in the supernatants using an Amicon Centriflo-filter.

Several interpretations of these results are possible: 1) The receptor was solubilized but inactivated by the detergent treatment; 2) The receptor was not solubilized but remained, in an inactive form, in the particulate fraction; 3) The detergent caused inactivation of the receptor by dissociating it into two or more components and separating at least one into the supernatant. Several experiments were done to examine the latter possibility. A 140,000 x $\bar{g}$ pellet was treated with

0.25% DOC, which was just sufficient to abolish oxytocin binding. Pellet and supernatant were not separated as in other experiments. Instead, the entire treated material was dialyzed against detergent free TM buffer to lower the DOC concentration. Restoration of oxytocin binding ability would have indicated that more than one component required for binding had recombined to reconstitute an active receptor. However, no reactivation of oxytocin binding ability was observed. A control pellet, dialyzed but not detergent treated, maintained its ability to bind oxytocin. This negative result does not eliminate the possibility of a multicomponent receptor, but only shows that the components, should they exist, cannot recombine underthe conditions used. A similar experiment was not carried out with Triton X-100 because of the long times required for dialysis of this detergent (Egan, et al., 1976). However, another approach was taken with this detergent. Most of the protein present in the 140,000 x $\bar{g}$ pellet was not solubilized by Triton X-100. If the receptor were composed of several components, and one was being solubilized while others remained in the particulate fraction, increasing the total proportion of the protein solubilized would increase the probability of having the entire receptor solubilized, perhaps in an active form. Since EDTA had been previously shown not to be irreversibly damaging to hormone binding (Tables 9a,b), and since EDTA has also been used for the solubilization of membrane proteins (Reynolds and Trayer, 1971), treatment of the 140,000 x $\bar{g}$ pellet from lactating rabbit mammary gland simultaneously with Triton X-100 and EDTA was done (Table 11). It can be seen that EDTA alone resulted in a 27% increase in protein removal over that removed by washing with Mg^{2+} containing buffer. The addition of 0.1% Triton X-100 along

with EDTA increased the protein removal so that 75% of the protein was solubilized and 25% remained particulate. The detergent treated particulate fractions did not retain oxytocin binding ability, and despite the large increase in protein solubilization caused by EDTA, the supernatants also did not show oxytocin binding either prior to or following treatment to lower the concentration of Triton X-100. Thus, this experiment also did not make it possible to locate an active receptor in the soluble fraction, or to determine whether the receptor consisted of a single molecule or several components.

Since it was not possible to detect oxytocin binding in supernatants prepared from detergent treated 140,000 x $\bar{g}$ pellets, SDS-gel electrophoresis was done on the supernatants to examine the proteins that had been solubilized. The appearance of a single additional protein band in the detergent treated fraction seemed promising, since this band could have been related to the oxytocin receptor (Figures 23 a, b). Subsequent work, however, showed that a protein with the same relative mobility could be extracted from the 140,000 x $\bar{g}$ pellet by EDTA treatment without impairing the oxytocin binding ability of the EDTA extracted particulate fraction. It therefore appeared likely that the protein extracted by detergent treatment was not related to the oxytocin receptor. Scatchard plot results (Figure 21), obtained after the SDS-gel electrophoresis of the detergent treated supernatants was done, indicated that this electrophoresis procedure might not have been sufficiently sensitive to detect a solubilized receptor. It can be seem that the 140,000 x $\bar{g}$ pellet had a binding capacity of 385 fmoles oxytocin per mg protein. If a molecular weight similar to those found for other receptors of 200,000 is assumed for the

oxytocin receptor (Kahn, 1976b), quantitative extraction of the receptor from approximately 10 mg of 140,000 x $\bar{g}$ pellet protein would yield approximately 0.75 µg of oxytocin receptor protein. This is close to the limit of detectibility of the SDS-electrophoresis procedure used (Fairbanks, et al., 1971).

b. Possible Causes for the Deleterious Effects of Detergents on the Oxytocin Receptor of Lactating Mammary Gland

As discussed in the preceding section, detergents have a deleterious effect upon the oxytocin receptor from rabbit mammary gland. Although it has not been possible from the work carried out in this study to determine the cause of this effect, several explanations have been mentioned and will be expanded upon in this section

1. The receptor is very labile. This seems to be unlikely since the detergents, at the concentrations and conditions used, have a mild effect on proteins. That the receptor was not labile toall manipulation was shown by its stability to freeze-drying. The resulting lyophilized material could be resuspended in TMG buffer and retained full oxytocin binding activity. The term "labile" is quite general however. It is difficult to eliminate this possibility since a minor structural alteration in the receptor could have extensive effects upon its ability to bind oxytocin.

2. Detergents bind to the receptor, perhaps at or near the site of hormone recognition, and prevent hormone binding. In this study it has been observed that the receptor is not solubilized or inactivated by compounds other than detergents. Extraction of the 140,000 x $\bar{g}$ pellet with 0.5 M KCl did not affect oxytocin binding. EDTA also did not sol-

ubilize or inactivate the oxytocin receptor, although it did remove a large proportion of the other proteins present in the 140,000 x $\bar{g}$ pellet (Table 11). Although the oxytocin receptor cannot be functionally considered an intrinsic membrane protein on the basis of these observations, they do indicate that the receptor is more firmly held within the membrane lipid matrix than other proteins which are removed by the treatments. In contrast, it was observed that even a 15 minute exposure at 0°C to 0.1% Triton X-100 resulted in a loss of approximately 90% of the oxytocin binding activity (Figure 22). A similar lability was also observed with DOC and Lubrol-PX. Thus, the hydrophilic agents were without effect, whereas detergents exerted a rapid and deleterious effect on the oxytocin receptor. These observations indicate that the receptor has some lipophilic character, as would be expected of a membrane bound protein (Strittmatter, et al., 1972). Several studies have shown that lipophilic proteins bind greater amounts of DOC and Triton X-100 than do hydrophilic proteins (Clarke, 1975; Makino, et al., 1973; Helenius and Simons, 1972), and that membrane proteins can bind Triton X-100 micelles as well as detergent monomers. The micellar weight of Triton X-100 is approximately 90,000 (Helenius and Simons, 1975). The binding of a single molecule or micelle of the detergent per receptor could prevent hormone binding by making the oxytocin binding site inaccessible to the hormone. Evidence for lipophilicity at the hormone recognition site has been obtained from pharmacological studies. When a methylene group was omitted from [3-valine]-oxytocin or [3-norvaline]-oxytocin a loss in potency resulted (Nesvadba, et al., 1963). Furthermore, substitution of an oxygen atom for a methylene group as in [3-O-methyl-

threonine]-oxytocin again reduced the potency of the analog. Since [3-O-methyl-threonine]-oxytocin is nearly isosteric with oxytocin (Chimiak and Rudinger, 1965), the effect of this substitution cannot be entirely attributed to a change in the conformation of the compound. In a review by Rudinger and co-workers (1972) it was stated, "...that the oxytocin receptor of the uterus, and by analogy, of the rat mammary gland myoepithelium, has a lipophilic sterically rather strictly defined region whose interaction with the side chains in sequence position three makes an important contribution to hormone binding." The results presented in this thesis are consistent with this statement.

Other membrane bound receptors appear to be lipophilic. This has been determined by estimating the partial specific volume of the receptor-Triton X-100 complex. Triton X-100 has a partial specific volume of 0.99 cm^3/g. Binding of the detergent to proteins results in perturbation of their partial specific volume from the value of 0.72-0.74 normally observed for proteins. Meunier and co-workers (1972), working with the acetylcholine receptor of Electrophorus electricus, and Cuatrecasas (1972c) working with the hepatic insulin receptor both obtained results indicative of significant binding of Triton X-100 to these receptors. Since membrane bound receptors transmit signals from the extracellular side to the intracellular side of the target cell membrane, it is possible that the receptors are deeply imbedded within the lipid matrix on the membrane. Based on observations made during this study, pharmacological studies with hormone analogs, and properties of other membrane bound receptors, it is reasonable to propose that detergents exert their deleterious effect on the oxytocin receptor by interacting with lipophilic sites which may include the hor-

mone binding site.

3. Some essential components of the receptor (e.g. phospholipids) are removed by detergent treatment. This possibility was discussed in the preceding section (see: section Va) and cannot be eliminated on the basis of the experiments described in that section. This discussion will focus on the possible role of lipids as a component of the oxytocin receptor. Evidence for a lipid component requirement for hormone binding has been reported for the following receptors: glucagon (Rubaclava and Rodbell, 1973); gonadotropins (Dufau et al., 1973b; Haour and Saxena, 1974; Azhar, et al., 1976a, 1976b); prolactin (Shiu and Friesen, 1974b); prostaglandin $F_{2\alpha}$ (Rao, 1976). The evidence has been obtained primarily by treatment of hormone binding fractions with phospholipases. In one case, separation of the receptor into protein and lipid components was achieved and, subsequently, reconstitution of an active gonadotropin receptor from the components was accomplished (Haour and Saxena, 1974). Although thorough examination of the involvement of lipids in neurohypophysial hormone receptors has not yet been carried out, there are some studies in non-physiological systems which indicate that this requires further study. Kafka and Pak (1969, 1972) found that oxytocin and vasopressin alter water permeability and Ca^{2+} binding of lipid monolayers composed of monooctadecyl phosphate, stearic acid or stearyl alcohol. Graziani and Livne (1973) extended this work and obtained results of more physiological significance by showing that lysine-vasopressin increased water permeability through a phosphatidylcholine bilayer. Both Ca^{2+} and prostaglandin E_2, which inhibit the antidiuretic activity of vasopressin in vivo, also inhibited the increased permeability of the lipid bilayer

if added prior to the lysine-vasopressin.

Soloff and Swartz (1973) examined the effect of phospholipases on oxytocin binding to rat mammary gland membranes. Phospholipase C treatment resulted in a 10% reduction of oxytocin binding under the single set of conditions used for phospholipase treatment. Phospholipase D had no effect on oxytocin binding. Phospholipase A reduced binding by approximately 75%, but non-enzymatic impurities were suspected of being responsible for this effect. In a recent review, Soloff (1976) states, "No definite conclusions can be drawn as to the phospholipid nature of the receptor site, because the inhibition of oxytocin binding by a crude preparation of phospholipase A could be accounted for by non-catalytic activity." Preliminary studies by J. Warr in this laboratory indicate that treatment of the 140,000 x $\bar{g}$ pellet from lactating rabbit mammary gland with phospholipase C which has been purified from protease activity causes a reduction in oxytocin binding which is dependent upon the concentration of phospholipase employed.

Detergents are known to displace lipids from membrane proteins, and the displacement of lipids is dependent upon the concentration of detergent used (Helenius and Soderland, 1973). In this study, the extent of oxytocin receptor inactivation was found to be dependent upon detergent concentration (Figure 22). The possibility that lipids are an essential part of the native oxytocin receptor of rabbit mammary gland, and that receptor inactivation is due to their removal by detergent treatment is consistent with the data obtained in this study.

VI. The Effect of Preincubation of [^{3}H]-oxytocin on the Oxytocin Receptor and Receptor Solubilization

The data discussed in the preceding section were collected in studies of detergent treatment of material containing oxytocin receptors prior to incubation with [^{3}H]-oxytocin. This section will deal with the results obtained when lactating rabbit mammary gland pellets were incubated with [^{3}H]-oxytocin before detergent treatment. The results indicate that this procedure resulted in: 1) preservation of the oxytocin binding ability of the receptor and 2) partial solubilization of the hormone-receptor complex.

a. Protection of the Oxytocin Receptor from Detergent Inactivation by Preincubation with [^{3}H]-oxytocin.

As previously shown, treatment of the 140,000 x $\bar{g}$ pellet from lactating rabbit mammary gland with 1% Triton X-100 prior to incubation with [^{3}H]-oxytocin resulted in loss of the ability of this fraction to bind [^{3}H]-oxytocin. An identical result was obtained with 1% Lubrol-PX. However, if the 140,000 x $\bar{g}$ pellet was first incubated with [^{3}H]-oxytocin, and subsequently treated with detergent, a significant proportion of the [^{3}H]-oxytocin-receptor complex was recovered from the supernatant by precipitation with PEG-6000 (Tables 12, 14). Lowering the concentration of Triton X-100 to 0.1% resulted in a quantitative recovery of binding (Table 13). The occupation of the receptor site by oxytocin prior to the addition of detergent seems to have a protective effect on the oxytocin receptor against inactivation by the detergents.

This protective effect has been observed with other hormones. Azhar and co-workers (1976a) found that treatment of bovine corpus luteum plasma membranes with phospholipases A or C resulted in reduced binding of [^{125}I]-hCG. However, if the membranes were first incubated with the

hormone, the phospholipase treatment had no apparent effect on the preformed hormone-receptor complex. Thus, the formation of the hormone-receptor complex either protected a phospholipid component of the receptor from hydrolysis, or once the hormone was bound, the phospholipid was no longer required. In either event, the phenomenon of protection of a receptor by preincubation with hormone was indicative of the necessity for the interaction of a lipid for the initial hormone-receptor binding. With pig renal receptors for [8-lysine]-vasopressin, another neurohypophysial hormone, Roy and co-workers (1975c) using 0.5% Triton X-100, obtained a 30% yield of solubilized [8-lysine]-vasopressin receptors if detergent treatment preceded incubation with the hormone. If preincubation with [8-lysine]-vasopressin was done prior to the detergent treatment, the yield of the solubilized hormone-receptor complex was 65-100%.

In section V of this Discussion, three possible explanations for the detergent inactivation of the oxytocin receptor were discussed. None of these can be eliminated on the basis of the results obtained with the preincubation experiments. The formation of a hormone-receptor complex prior to solubilization may: 1) prevent a conformational change in the receptor molecule; 2) prevent displacement by detergents of a phospholipid or other component of the receptor; 3) permit the formation of a stable hormone-receptor complex which might not further require the phospholipid or other receptor component which can be subsequently removed by detergent treatment; 4) allow occupation of the receptor site with [^{3}H]-oxytocin which would then prevent the binding of detergents to the hormone recognition site. Since detergents such as Triton X-100 bind primarily to high affinity sites (see: Introduction), their displacement from the

oxytocin receptors once bound could be difficult.

b. Partial Solubilization of the Hormone-Receptor Complex

Various criteria have been used to define the solubilization of membrane components. Coleman (1974) stated, "Solubilization as applied to membranes rarely means the same as it does in tradional terms, i.e. making an homogeneous solution of individual molecules or ions. It often means only size decrease and is usually defined in terms of experimental convenience. Since ultracentrifugation is often the discriminating step, "solubilized" often describes that material which will not sediment at about 1 x 10^7 $\bar{g}$-minutes." He also points out that even with this criterion, the supernatants could contain small membrane fragments, lipoprotein particles or micelles. Razin (1972) suggests that a valid centrifugal criterion of solubilization is no sedimentation after 1 hour at 100,000 x g, which is equivalent to 0.6 x 10^7 $\bar{g}$-minutes. In the initial experiments, the solubilization of the oxytocin receptors was measured after centrifugation at 20,000 x $\bar{g}$ for 30 minutes (Tables 13, 14, 15). A higher centrifugal force, 140,000 x $\bar{g}_{av.}$ for 30 minutes was subsequently used (Table 16). A fraction of the material which was present in the 20,000 x $\bar{g}$ supernatants also remained in the 140,000 x $\bar{g}$ supernatant. Further increase of the centrifugal force to 210,000 x $\bar{g}_{av.}$ (280,000 x $\bar{g}_{max.}$) did not result in any reduction in the amount of solubilized receptors from that observed in a 140,000 x $\bar{g}_{av.}$ supernatant (Table 17). Since centrifugation at 210,000 x $\bar{g}_{av.}$ (280,000 x $\bar{g}_{max.}$) for 30 minutes is equivalent to 0.63 x 10^7 $\bar{g}$-minutes (0.84 x 10^7 $\bar{g}$-minutes), the conditions used in this study meet the criterion of Coleman (1974) of "about 1 x 10^7 $\bar{g}$ -minutes". The centrifugation criterion of Razin (1972) has been exceeded.

This is, of course, a single criterion based on the fact that sedimentation rate is directly proportional to gravitational force. Other parameters such as the shape and density of the hormone-receptor complex and the density and viscosity of the medium used for centrifugation have not been defined, either in this study or by Coleman (1974) or Razin (1972). Since in this and similar studies (Roy, et al., 1975c), a buffered aqueous solution without sucrose, glycerol, etc. was used, these parameters may be considered to be constant. More precise criteria for considering a membrane protein as soluble should undoubtedly evolve as more physical characteristics of these proteins become known. From the data obtained in this study using centrifugation as a criterion, the oxytocin-receptor complex prepared from rabbit mammary gland with Triton X-100 can be considered partially soluble.

c. Characteristics of the Soluble Complex

The same criterion for the determination of specific binding was applied to the soluble complex as had previously been used for the particulate receptor (see: Section II). In addition, the oxytocin analogs, [1-deamino]-oxytocin and [4-proline]-oxytocin were used to examine their ability to bind to the solubilized receptors. Since incubation with the analogs was not possible following detergent treatment, it was necessary to incubate the analogs with the particulate fraction in the presence of [^{3}H]-oxytocin and to treat with detergent subsequently. As with the particulate fraction, the results showed that [1-deamino]-oxytocin did compete with [^{3}H]-oxytocin for binding to the receptors which were subsequently solubilized, and that [4-proline]-oxytocin did not compete (Figure 24).

In the experiments described above (Figure 24), the percentage of [^{3}H]-oxytocin displaced by oxytocin or [1-deamino]-oxytocin was lower with the soluble receptor(40-45%) than it had been with the particulate receptor (65-70%; Figure 19). Calculation of a ratio of non-specific/specific binding for the particulate and soluble systems gave a value of approximately 0.54 for the particulate systems and 1.2 for the soluble systems. Similar data were reported for the particulate and soluble [8-lysine]-vasopressin receptor of pig kidney (Roy, et al., 1975c). They observed a non-specific/specific binding ratio of 0.71 in the absence of detergent and 2.7 for the receptor in the presence of 0.05% Triton X-100. Increasing the concentration of Triton X-100 to 0.5% decreased this latter ratio to approximately 0.4. Although the cause of this phenomenon is not known, it is possible that at lower detergent concentrations mixed micelles or vesicles of protein, lipid and detergent were formed which could entrap the hormone, whereas at higher concentrations of detergent removal of lipids from the receptor would be more complete and such micelles might not exist. The stepwise removal of lipids from proteins with the intermediate formation of micelles has been observed in another system (Helenius and Soderland, 1973).

The next point to be considered is the extent of solubilization of the hormone-receptor complex. Treatment with 0.1% Triton X-100 resulted in solubilization of 21.1 to 25.7% of the total binding (Table 18). This was independent of the amount of hormone-receptor complex formed. Increasing the amount of hormone-receptor complex was done by lengthening the incubation time from 1 hour to 2 hours. This resulted in a 140-176% increase in specific binding. This increase was expected in view of the

time course of binding discussed previously (Figure 21). Previous experiments (Table 11) made it possible to compare this removal of hormone-receptor complex with the removal of total protein from the 140,000 x $\bar{g}$ pellet. In these experiments 0.1% Triton X-100 removed approximately 54% of the protein present in the 140,000 x $\bar{g}$ pellet. If this value is compared to the 21-25% removal of the oxytocin-receptor complex observed in these latter experiments (Table 18), it can be seen that removal of the hormone-receptor complex is low in relation to total protein removal. These data are indicative that the receptor is more tightly held than other proteins in the membrane lipid matrix. This observation is additional evidence for the previously discussed possibility that the receptor is an "intrinsic" membrane protein.

SUMMARY

The results obtained in this study show that lactating rabbit mammary gland contains a component which binds the neurohypophysial hormone, oxytocin. This component meets criteria which indicate that it is specific for oxytocin such as:

1. Displacement of [^{3}H]-oxytocin from the binding component by an active analog of oxytocin.
2. No displacement of [^{3}H]-oxytocin from the binding component by an inactive analog of oxytocin.
3. A high binding affinity (3.2×10^{-9} M) of the receptor for oxytocin, a characteristic property of hormone-receptor interactions.

In addition, partial purification of the oxytocin binding component indicates that it is present in the plasma membrane fraction, as are other peptide hormone receptors.

The receptor was labile in the presence of the detergents deoxycholic acid, Triton X-100, and Lubrol-PX. The extent of inactivation of the oxytocin receptor was dependent on the concentration of detergent used. Preincubation of the receptor with oxytocin prior to detergent treatment made it possible to detect the preformed oxytocin-receptor complex both in a particulate and soluble fraction. The solubilized oxytocin-receptor complex did not sediment when centrifuged at 0.63×10^7 $\bar{g}$-minutes which meets one of the criteria for solubilization used by other workers.

The "protective effect" of preincubation with hormone has been observed with other hormone-receptor systems, including that for [8-lysine]-

vasopressin. The "protective effect" which oxytocin exerts on the receptor is discussed in relation to lipophilicity at the hormone recognition site of the receptor and also with respect to a possible lipid component required for hormone-receptor interaction.

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