THE EFFECTS OF LIVER PREPARATIONS ON PROLACTIN RELEASE FROM PITUITARY TISSUE IN VITRO

By

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LIVER EFFECTS ON PROLACTIN RELEASE *IN VITRO*

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ABSTRACT

The effects of liver on pituitary prolactin (PRL) secretion were investigated using an *in vitro* organ culture system. It was determined that various preparations of liver when coincubated with pituitary tissue resulted in a diminished PRL content in the bathing culture medium. Female Wistar rats (>200gm) provided the source for liver chunks (∿150mg) that were coincubated with pituitary tissue obtained from female Wistar rats (>200gm) primed with estradiol-17-β and progesterone. The PRL content of the culture medium obtained from the liver - pituitary coincubate was reduced significantly ($p<.001$) compared to control. This effect could not be attributed to PRL degradation and/or binding to liver and was therefore interpreted as inhibition of secretion. The effect was partly but not completely antagonized by the addition of Haldol at concentrations ranging up to 1000nM.

It was also noted that the diminished PRL content was observed when methanol extracts of male and female livers were coincubated with male and female (primed and unprimed) pituitary tissue. It is concluded that factors associated with liver and extracts of liver inhibit the secretion of prolactin. These factors are thought not to mediate all of their actions through the dopamine receptor as maximally effective concentrations of Haldol only partially eliminate this inhibition.

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PREFACE

PREFACE

The impetus for this thesis was provided by preliminary *in vitro* studies (28) which suggested that livers and extracts of liver possess factors that stimulate the secretion of both prolactin (PRL) and growth hormone (GH) from rat pituitary tissue. In the event that factors are identified which are capable of stimulating the release of these hormones, their physiological role and relationship to the hypothalamic factors regulating PRL and GH release remain to be determined.

Because of the greater potential for clinical application (i.e. treatment of dwarfism), my primary interest was the pursuit of the liver factor that would act directly on the pituitary to release GH. The ensuing work (not included in this thesis) suggested that no GH stimulating entity was present in liver, nor was their evidence for a PRL stimulating factor. My efforts revealed the presence, however, of a factor that may, *in vitro*, inhibit the release of prolactin. It was decided that the liver activity should be systematically screened indirectly (by antagonists) for known PRL inhibitory factors (PIF's). The final chapter of the Results section documents an initial effort directed at defining the involvement of liver dopamine as the source of inhibition. Although this study showed a significant ($<.025$) interaction between various Haldol (a liquid preparation of a dopamine antagonist) concentrations and the control and liver groups, findings

suggested that the liver inhibition was not mediated in any large part by dopamine. The introduction of the thesis discusses the wide variety of known PIF's and of PRL releasing factors (PRF's) that could have accounted for any activity altering prolactin secretion.

SECTION I

REVIEW OF LITERATURE

Chapter 1

Hypothalamic Inhibitory Control of Prolactin Secretion

A body of evidence implicates the central nervous system (CNS) as the major locus tonically inhibiting the secretion of prolactin from the pituitary latotrope. Removing the anterior pituitary from the hypothalamic vascular link (either by pituitary stalk sectioning or adenohypophysial transplantation to the renal capsule) results in a marked and prolonged elevation in serum prolactin (45, 26, 20). Furthermore hypothalamic extracts possess the ability to inhibit prolactin secretion both *in vitro* and *in vivo* (83, 63, 78). Several substances have been scrutinized for their involvement in pituitary secretion as the physiologic Prolactin Inhibiting Factor (PIF), but to date only dopamine has received the critical recognition as such.

1-1 Dopamine, Evidence for Its Role as the PIF

The presence of distinct dopamine (DA) containing neurons in the hypothalamus was initially established by Fuxe and Hokfelt using the Falck-Hillarp histochemical fluorescence technique (29). The cell bodies of these tuberoinfundibular dopamine (TIDA) neurons reside primarily in the arcuate nucleus with axons terminating in the external layer of the median eminence. Ultrastructurally, it has been suggested that TIDA nerve terminals appose the fenestrated capillaries forming the primary capillary plexus of hypophyseal portal vessels (37). These anatomical studies provided a major impetus for the investigation of dopamine as a

neurohumor regulating directly pituitary function.

The pharmacological evidence for dopamine inhibition of prolactin release is extensive. A high dose, 5μg/mℓ, of dopamine administered *in vitro* to a pituitary incubation system significantly inhibited prolactin secretion yet was without effect on growth hormone (4). This direct effect of dopamine was confirmed by MacLeod and Lehmeyer who were, in addition, able to demonstrate a similar response with the DA agonists apomorphine and ergocryptine (56). These *in vitro* effects of DA and it's agonists were reversed in a dose dependent fashion by the addition of DA blocking agents, specifically haloperidol and perphenazine.

Data first demonstrating dopamine interaction with a specific receptor in pituitary tissue was obtained by Brown, Seeman, and Lee (7). Subsequent to this, numerous groups have identified presumptive dopamine receptors using varied radioligands and donor species. This included ^{3}H dopamine binding to bovine, rat, and ovine pituitary (10, 16); ^{3}H spiroperidol to ovine and bovine pituitaries (17). Haloperidol labelling of dopamine receptors was used to illustrate that the majority of binding sites appear localized on lactotrope membranes (32).

Administration *in vivo*, of the dopmaine precursor "L-Dopa" to animals with hypothalamic lesions associated with hyperprolactin states, diminishes the exaggerated prolactin levels (20). Furthermore, pretreatment with a dopa decarboxylase inhibitor which prevents the synthesis of dopamine, resulted in a failure of L-Dopa to diminish serum prolactin levels in the ectopic adenohypophyseal preparation (19).

A recent review tabulated findings of dopamine related drugs on *in vivo* prolactin secretion (see Table 1) (93).

TABLE 1

In vivo Effects of Dopamine-Related drugs on Prolactin (PRL) Secretion

Drug	Action	Change	Model
dihydroxyphenylalanine (l-Dopa)	DA precursor	↑PRL	- resting levels - sleep related increase - ectopic pituitaries - hypothalamic lesion
dopamine	DA agonist	↓PRL	- resting levels - hyperprolactin
ergots	DA agonist	↓PRL	- hyperprolactinemia
apomorphine	DA agonist	↓PRL	- hyperprolactinemia
phenothiazines	DA antagonist	↑PRL	- resting levels
butyrophenones	DA antagonist	↑PRL	- resting levels
substituted benzamides	DA antagonist	↑PRL	- resting levels - l-Dopa suppression

NOTE: this table is adapted in an abbreviated form from "Weiner, R.I., and Bethea, C.L." chapter entitled "Hypothalamic Control of Prolactin Section" in "Prolactin", pp. 19-55, edited by Robert B. Jaffe, Elsevier North-Holland/New York 1981. (ref. 93).

Chemical determinations aimed at correlating portal blood dopamine levels with prolactin have been accomplished using radio-enzymatic techniques (2) or liquid chromatographic-electrochemical assays (67, 31). All reports to date have claimed that dopamine concentrations in hypophyseal stalk blood are higher than in the peripheral circulation (2, 67). The DA concentrations present in the portal vasculature are of a range (i.e. 0.7-9.0 ng/ml) capable of suppressing prolactin secretion (31, 79). After pretreatment with α-methyl-p-tyrosine, an inhibitor of the enzyme tyrosine hydroxylase (required for DA synthesis) (82), the group of Gibbs and Neill infused dopamine to attain arterial plasma concentrations approximating values seen in hypophyseal portal vessels (i.e. ∿9ng/ml). Such loading culminated in the diminution of plasma prolactin to 70% of pretreatment levels. The same approach directed against animals with lesions of the median eminence resulted in a suppression of PRL to 42% of prelesioned values. However, the administration of dopamine failed to diminish the prolactin elevation associated with proestrus.

The group of Plotsky, Gibbs, and Neill were unable to demonstrate a significant correlation between hypophyseal portal serum dopamine levels and peripheral serum prolactin values (67). Additionally, they could not demonstrate a significant difference in stalk levels of dopamine during proestrus and diestrus -2, associated with varied prolactin levels in peripheral circulation. Ben-Jonathan et al have not substaniated these findings (2). This dispute may be resolved with further studies monitoring not only stalk concentrations of dopamine, and peripheral plasma prolactin levels but also measuring estradiol levels (which have been neglected in

previous efforts). From the data cited above, it appears as if prolactin serum values derive from more than just a simple inverse relation to portal dopamine concentrations. A variety of uncontrolled parameters including estradiol modulation of lactotrope sensitivity could account for the lack of such a direct relationship (61).

Most recently, there is a new theory regarding the source of the dopamine prolactin inhibitory activity (66). The contention is that dopamine fibres descending the pituitary stalk impinge onto short posterior-anterior interhypophyseal vessels. This is substantiated by studies involving the removal of the posterior pituitary and the monitoring of plasma PRL (which increases subsequent to this). Posterior pituitary extracts were measured for PIF activity and it was found that such activity could be antagonized by the addition of "+ butaclamol".

1-2 Other Catecholamines as Hypothalamic PIF's

Much of the initial work on catecholamine regulation of prolactin secretion focused on norepinephrine and epinephrine. The findings support an inhibitory action, in vitro, of these substances, however, the literature does reveal disparity. MacLeod demonstrated that norepinephrine and epinephrine, not their metabolites, significantly inhibited in vitro prolactin secretion at high doses ($\sim 10^{-6}$M) (55). Talwalker and co-workers failed to demonstrate such an in vitro effect (83), while Gala and Reese reported that microgram concentrations of epinephrine promoted in vitro prolactin secretion (30). However, the group of Jacobs et al, using concentrations of epinephrine identical to those used by Gala and Reese showed an inhibition in vitro (43).

It should be noted that no known neurons with cell bodies in the

hypothalamus are thought to synthesize norepinephrine under normal conditions (for review see ref.# 62). Analysis of portal blood revealed that neither norepinephrine nor epinephrine levels were found to be elevated when contrasted to peripheral blood levels (2). If norepinephrine and epinephrine exert any direct inhibition on prolactin secretion it would be of a tonic nature.

1-3 γ-Aminobutyric Acid (GABA)

A divergence of prolactin changes is observed following GABA administration. Intracerebral ventricular (ICVT) infusion of GABA or it's agonist muscimol reportedly results in an elevation of serum prolactin (60). Intravenous infusions of GABA or Muscimol inhibited prolactin secretion (78, 36). Such discrepent actions may manifest in part through preferential action on varied neural paths. The possibility that GABA inhibition of PRL secretion is mediated directly at the pituitary locus was examined with most reports claiming that a direct inhibitory action of prolactin is possible *in vitro* (36, 24, 78, 14). In experiments using muscimol, the concentrations required to drop prolactin values to 75 and 56% of baseline values were 10^{-7} and 10^{-5}M, respectively. However, Mioduszewski *et al* could not demonstrate an inhibitory action of GABA (doses ranging from 5 to 50 mg/100gm) in animals with ectopic pituitaries (60).

Studies using ^{3}H GABA or ^{3}H muscimol reveal that specific binding is present in pituitary tissue with a Kd value of 33nM (34, 35). Studies also reveal that the *in vitro* inhibitory effects of GABA and Muscimol are antagonized by bicuculline and picrotoxin, both putative GABA antagonists, and not by the DA blockers α-flupentixol and sulpiride

(35, 24).

Anatomic evidence for a tuberoinfundibular GABAergic pathway is accumulating. Tappaz et al have reviewed the work they and others have put forth in support of such a pathway (85, 84). This includes the localization of GABA, glutamic acid decarbosylase (GAD activity localization, GAD immunoreactivity localization) and ^{3}H GABA autoradiography demonstrating uptake.

Recently, Racagni et al, have shown that GABA is present in anterior pituitary tissues and that no GAD activity is noted (69). When ectopic anterior pituitaries are examined for GABA a significant diminution is observed when contrasted with controls. Furthermore, injections of ethonolamine-O-sulphate (EOS), a blocker of the enzyme GABA transaminase (which degrades GABA), are associated with elevations in GABA content of pituitary gland. The conclusion derived from such findings is that pituitary GABA content originates from a tuberoinfundibular neuronal pathway that secretes GABA into the portal system. I have been unable to obtain any reports from the literature documenting direct portal blood determinations of GABA.

1-4 Acetylcholine (ACh)

There is some pharmacologic evidence suggesting that acetylcholine may be a PIF. High doses of ACh and related agonists diminish prolactin secretion in vitro, an effect which is attenuated with a muscarinic antagonist (81). When several brain regions were examined, the levels of the ACh synthesizing enzyme "choline acetyl transferase" were lowest in the hypothalamus where most activity was found in the median eminence (9). I am not aware of any reports documenting direct measurement of ACh in hypophyseal portal blood.

Chapter 2

Hypothalamic Prolactin Releasing Factors (PRF)

As stated previously the predominant effect the hypothalamus exerts over prolactin secretion is inhibitory. There are instances when prolactin surges and basal levels increase such as during proestrus or as in periods of stress of a varied nature (61). These increments in prolactin secretion may result from diminutions in the secretion of PIF's; there is however, evidence to suggest that some of the elevated prolactin responses are due to secretion of PRF(s). These include;

(1) isolated hypothalamic extracts may promote prolactin secretion both *in vivo* and *in vitro* (vide infra).

(2) during periods of infusion using maximally effective doses of dopamine, prolactin can be elevated under certain conditions (13).

(3) the fluctuations in portal dopamine levels are not always of a sufficient magnitude to account for elevations in prolactin (61).

The ensuing account will provide a brief review of the major substances scrutinized for their potential involvement as PRF's.

2-1 Thyrotropin Releasing Hormone (TRH)

TRH is a tripeptide first isolated from porcine stalk median eminence and identified by Schally, Folkers, and Bowers (77, 6). The initial suggestion that TRH may, in addition to its action on TSH secretion, promote prolactin secretion stemmed from the work of Tashjian

and co-workers using an *in vitro* monitor of secretion from a pituitary tumor culture (86). These findings were eventually corroborated both *in vitro* and *in vivo* (54). It has been established, in addition, that concentrations of TRH that promote prolactin release *in vitro*, are well within the range found in hypophyseal portal blood (96, 25).

In rats, physiological monitoring reveals that there are parallel elevations in serum PRL and TSH following suckling (5). However, the TSH peak is seen to occur after the rise in PRL. Administration of a TRH antisera is associated with a decline in the surge of prolactin at proestrus (50). Paradoxically, no TSH elevation was seen to occur during the normal proestrus PRL rise. This may in fact be accounted for by concurrent elevations in other PRF's or diminutions in PIF's which would not alter TSH secretory profiles. One other curious finding is noted following ICVT administration of TRH; the prolactin elevation associated with suckling is attenuated following such treatment (15).

There may exist numerous other PRF's, as hypothalamic extracts devoid of TRH are capable of promoting PRL secretion (76, 5). A clear and concise role ascertained for TRH, must await the elucidation of other PRF's and PIF's.

2-2 Vasoactive Intestinal Polypeptide (VIP)

Vasoactive intestinal polypeptide is a 28 amino acid polypeptide first isolated from porcine duodenum (73). Immunoreactivity studies have suggested that it is present throughout the central nervous system, including the hypothalamus (3). Deafferentiation of the medial basal hypothalamus (MBH) resulted in a 40% drop in the caudal portion of the MBH as detected by RIA with no alteration of rostral MBH levels.

Furthermore, it was subsequently determined that VIP immunoreactivity is located in vesicles and that release from the hypothalamus is evoked by potassium (21). Said and Porter have determined that immunoreactive VIP-like activity is present in portal blood (mean ∿ 995pg/ml) almost 20 times as great as peripheral blood (mean ∿ 52pg/ml) (74). This evidence is consistent with and suggestive of a neurohumoral substance capable of regulating anterior pituitary function.

Endocrine studies using a variety of routes of administration produce similar findings. Both ICVT and iv application of VIP result in dose-related elevations of serum prolactin (48). However, *in vitro* studies yield conflicting results; Kato *et al* suggest that VIP's *in vitro* promotion of prolactin secretion is due not to a direct stimulatory action of its own, rather an interferance at the level of the pituitary with DA inhibition (48). Vijayan *et al*, claim that VIP is exerting stimulatory action at the hypothalamic rather than pituitary locus (90); however, members of this team later went onto reveal an action directly on the pituitary (75). This latter finding is in general agreement with other reports (80, 22, 27).

The mechanism of VIP stimulation awaits further clarification. It has been revealed that there are VIP receptors present on plasma membranes of human prolactin secreting tumors (1), and that VIP administration to such tumors and GH 3 tumors stimulate cAMP production (1, 33).

Unfortunately, at the present time, there is a paucity of information correlating varied states of prolactin secretion with VIP levels in hypophyseal portal blood.

2-3 Other Neuropeptides as PRF's

The recent exponential growth in the neurosciences on the investigation of neuropeptides has witnessed a flurry of work aimed at elucidating the potential endocrine involvement of such peptides. A few of the peptides being considered as potential modulators of prolactin secretion will be discussed here.

-A- Substance P

The 11 amino acid peptide was first described by von Euler in 1931, but it was not until 1971 that Chang and Leaman isolated and identified the structure of this peptide from the hypothalamus (see for further details #12). Substance P immunoreactivity is present in the hypothalamus with a rich network of terminals in the external median eminence of primates - this however, is not the case with rats (38, 39).

Kato and co-workers reported that following i.v. administration of substance P to rats with large hypothalamic lesions, there is an elevation in serum prolactin (46). A direct dose related effect stimulating in vitro prolactin release has been shown by Vijayan and McCann with doses ranging from 5 to 500 ng/ml (88). This group also found that 2 µg but not 0.5 µg of substance P when injected ICVT produced a significant elevation in serum prolactin. The increment in prolactin secretion following 1µg injection i.v. of substance P was of a greater magnitude that the response seen with 2 µg of substance P ICVT.

-B- Neurotensin

Neurotensin is a tridecapeptide which when administered i.v. in a dose of 1 µg prompted an elevation in prolactin secretion (88). This work has been substantiated by Rivier et al, (70). Injection into the ventricles of either 0.5 or of 2.0 µg elicited an inhibitory response in prolactin secretion (88). In the same paper using an in vitro preparation of pituitary, they documented that doses of 50 µg/ml or higher stimulated prolactin secretion. The finding was not substantiated by Frohman's lab, however, no data were presented (57). However, Enjalbert's group replicated their original findings (57) and revealed that the stimulatory action appears to be independent of dopmaine or GABA receptors (23).

-C- Cholecystokinin (CCK)

Immunoreactive CCK is found to be present throughout the CNS including the lateral hypothalamus (18). Administration by way of cerebral ventricle of 40 µg of CCK elevated serum prolactin whereas doses of 4 and 500 µg were without effect (89). Intravenous administration of doses of CCK ranging from 4 to 1000 µg yielded a dose related rise in prolactin. This group then claimed that no direct effect on pituitary could be demonstrated using a hemipituitary organ culture.

-D- Endorphins

Although ICVT and i.v. administration of the endogenous opioids or synthetic long acting analogs prompted a prolactin elevation, it has been reported that no direct stimulation on the pituitary for prolactin secretion occurs (47, 8, 71) although one report is not in agreement (53).

In conclusion, although much of the work seems supportive of a

role for neuropeptides in prolactin secretin, much of the evidence is scant using apparent pharmacologic doses. In vivo monitoring of hypophyseal portal neuropeptide levels and correlation with prolactin secretory profiles are indicated. Table 2 contains a summary of the findings for neuropeptides and for most of the monoamines.

TABLE 2

Prolactin Response to Varied Chemical Stimuli

Agent/Route	ICVT	IV	in vitro	in vitro culture system
TRH	↑provoked	↑e	↑e	hemipit., tissue cult
VIP	↑	↑	↑,→	monolayer, hemipit.
Substance P	↑	↑,↑a	↑	hemipit.
Neurotensin	↑	↑,↑a	↑	hemipit.
CCK	↑,→	↑	→	hemipit.
Gastrin	↑	→	→	hemipit.
SRIF		→	→,↓(hi dose)	monolayer
Endorphins	↑A	↑	→,↑	monolayer
GABA	↑	↑	↓,→	(hemipit?), ectopic pit.
DA		↓	↓,↑(low dose)	hemipit., monolayer
NE		↓,→	↓,→	hemipit.
E			↓,→,↑	hemipit.
ACh	↓	↓	↓(hi dose)	cell culture

(NOTE: this table is in part modified from ref.#42)

e = estrogen primed

a = anaesthetized animals

Chapter 3

Liver As A Source for Factor(s) Altering Prolactin Secretion

The literature would appear to indicate that the liver is a most unlikely tissue source for the isolation of factor(s) capable of affecting prolactin secretion *in vitro*. The liver derives embryologically from the endoderm, and is not known to produce any neuropeptide(s) which, according to the APUD theory (64, 65) derive only from cells of neuroectodermal origin. Monoamines, which, as stated previously, affect PRL secretion, are found to be present in the liver as a result of the organ's autonomic innervation (see review by Waynehautt) (92). The total catecholamine content of the gland is low ($\sim$ .06 - 0.10 µg NE+E+DA/gm) (40) and as such it would be surprising that this gland's autonomic compartment could alter the *in vitro* secretion of prolactin.

To contrast these reports, there are several lines of evidence to warrant examination for a liver factor influencing PRL secretion. The claim that only cells of neuroectodermal origin produce neuropeptides has received criticism, most notably from LeDouarin (52). Using the chick-quail chimera, she demonstrated that some peptide secreting cells are of endodermal origin. In addition, there is a curious clinical picture associated with alcohol induced liver cirrhosis - at times patients with this liver dysfunction present with gynecomastia and hyperprolactinemia (87). It has been suggested by Vanthiel *et al*, that these findings stem in part from the liver's inability in metabolizing estrone, however, the possibility remains that part of the prolactin elevation stems from a

diminution in the elaboration by the liver of a prolactin inhibitory factor. Digressing somewhat, liver in now regarded as an endocrine organ integral in the mediation of some of growth hormone's actions. Pituitary growth hormone secretion stimulates, via specific liver membrane receptors, the release into the circulation of a family of peptides collectively termed the somatomedins (see review by 91). It has been implied from varied disease states (e.g. Laron dwarfism) that the somatomedins feedback negatively onto the growth hormone axis.

Based upon reports of specific prolactin receptors in liver membrane fractions from female rat (49), one may draw parallels with the growth hormone axis, and therein propose an hypothesis amenable to testing. It would appear logical that, as specific receptors are found in the liver, there occurs a physiological response in liver to prolactin and that the liver would possess a means of providing feedback modulation of the incoming prolactin signal. Initial unpublished observations in our lab suggested the presence of just such a modulator (W.C. Friend personal communications). Using the hemipituitary organ culture as the *in vitro* model, preliminary findings claimed that liver and liver extract from female rats promoted prolactin secretion. The current thesis attempts to reveal the presence of a liver factor altering prolactin secretion.

SECTION II

OBJECTIVES OF INVESTIGATION

OBJECTIVES OF INVESTIGATION

As stated previously, a series of preliminary experiments in our laboratory suggested the presence of a prolactin releasing activity (PRA) in female Wistar rat (∿ 250 gm) livers. The initial intentions of the current thesis were to:

(1) replicate the findings obtained from the preliminary studies using the same detailed protocol for the extraction of the PRA from liver.

(2) attempt to characterize the nature of the PRA as particular to prolactin secretion or as a nonspecific stimulus for the release of pituitary content.

(3) partially isolate the PRA and examine the possibility that this substance exhibits identity with another PRF or PRF(s) already known.

However, due to my inability in replicating the initial unpublished observations, a new set of goals were established:

(1) demonstrate by replication my own finding that liver "chunks" (∿ 100 mg) possess a prolactin inhibitory activity (PIA) in vitro.

(2) determine whether or not the inferred PIA is an artifact of measurement. Specifically, determine whether apparent inhibition is due to prolactin binding and/or degradation by liver or whether prolactin secretion is inhibited.

(3) determine whether the PIA is in part or in totallity accounted for by an already discovered PIF.

SECTION III

MATERIALS AND METHODS

Chapter 1

The Radioimmunoassay of Rat Prolactin

1-1 The Iodination of Rat Prolactin

In a modification of the Hunter and Greenwood method (41), rat prolactin standard for radioiodination (NIAMDD Rat Prolactin: RP-I-3, RP-I-5) is reacted with $Na^{125}I$ through the aid of the oxidizing agent Chloramine T.

The following solutions are required and prepared prior to iodination:

(1) 0.5M Phosphate Buffer pH = 7.5

- solution "A" is a 0.5M solution of NaH_2PO_4 (anhydrous) in distilled water.
- solution "B' is a 0.5M solution of NaH_2PO_4-H_2O made in distilled water.
- solutions "A" and "B" are added together in a ratio of 6.7:1 (volume "A": volume "B") and stirred with a magnetic stirring bar. After mixing, the solution is brought to a pH of 7.5 and refrigerated at 4^oC until the time of iodination. For iodination purposes, the solution must be redissolved thoroughly at $\sim 37^oC$ as crystallization occurs.

(2) 0.05M Phosphate Buffer

- a volume of the 0.5M Phosphate buffer (pH=7.5) prepared as above, is diluted 1:10 with distilled water.

(3) 5% Bovine Serum Albumin (BSA) Buffer

- 5gm of Bovine Serum Albumin powder (Sigma, Cohn Fraction V) are added to 100 ml of distilled water. Aliquots of 1 ml are stored in glass tubes (10X50mm) and frozen at -20°C until time of iodination. On the day of iodination, the following solutions were prepared;

(4) Chloramine T solution

- chloramine T reagent (Eastman) is prepared as a solution of 1.75 mg/ml of 0.05M Phosphate buffer (pH=7.5) (see I-#2), and is stored in a 12X75mm glass test tube enclosed with tin foil (as the chemical is light sensitive).

(5) Na_2SO_5 (Sodium Metabisulphite) solution.

- a solution containing 2.4 mg Na_2SO_5 (BDH chemicals) per ml of 0.05M Phosphate buffer (see I-#2) is prepared in a 12X75mm glass test tube.

(6) NaI (Sodium Iodide) solution

- 10mg of NaI (BDH chemicals) is added for every ml of 0.05M Phosphate buffer (see I-#2) and is stored in a 12X75mm glass test tube.

Procedure

- approximately 5µg of rat prolactin iodination standard is weighed on a CAHN 21 automatic electrobalance and then added to a 12X75mm borosilicate glass test tube containing 50µl of 0.5 M phosphate buffer (see I-#1). To this reaction tube, is added 5µl of Na ^{125}I (100 mCi/ml.) (Amersham) with the aid of a "1-20λ" variable Gilson Pipetman. The reation is initiated by the addition of 25µl of the Chloramine T solution (see I-#4). After 45 seconds of intermittent gentle vortex mixing, the oxidation reaction is terminated by the addition of 50µl of the sodium metabisulphate solution (see I-#5). To this reaction tube is added 50µl of the NaI solution (seeI-#6) and 50µl of the 5% B.S.A. solution (see I-#3). This entire volume is applied to a Sephadex G-50 column (Pharmacia) eluted with 0.05M phosphate buffer (see I-#2) while fractions are collected as 32 drops per 12X75mm glass test tube. The ^{125}I peaks are determined with 0.1 minute counting in the Micromedic Systems MS 588 gamma-counter of 20µl samples from the collection tubes. The radioactive peaks are then correlated with "Dextran Blue" (Pharmacia) and free ^{125}I peaks determined previously so as to verify the labelled hormone peak. The fractions containing labelled hormone are divided into 50µl aliquots, "snap frozen" in an acetone/dry ice bath, covered with "Parafilm", and stored at -20°C in 12X75mm borosilicate glass test tubes until time of assay.

NOTE: ^{125}I-rat prolactin (NEN) has been employed in some instances.

1-2 The Immunoassay of Rat Prolactin

The following solutions are required for double antibody radioimmunoassay;

(1) 1% BSA Buffer

- in a 2000 ml Pyrex beaker, the following reagents are added to 1800 ml of distilled water; 2.84gm of Na_2HPO_4, 17.52gm of NaCl, 18.6gm of EDTA, 20gm of BSA powder (Sigma, Cohn Fraction V) and 0.2gm of Thimerosol. This solution is mixed for approximately one hour, at room temperature, using a magnetic stirring bar. Following this, the pH of the solution is adjusted to 7.6 with concentrated sodium hydroxide. The volume of this solution, then is brought up by the addition of 200ml of distilled water; this being further mixed for some ten minutes. The entire solution is filtered through a Millipore Sc. 8.0µm filter into a Florence flask. The buffer solution is poured into a 1 liter plastic flask for storage, air-tight at $4^{o}C$.

(2) Rat Prolactin Assay Standard

- rat prolactin reference preparation (NIAMDD rat prolactin: RP-2) is obtained as a 10µg lyophilate (plus powdered BSA) and is dissolved by adding 1ml of distilled water to the vial. This 10µg/ml solution of reference preparation is diluted to 1µg/ml by adding it to 9mls of 1% BSA buffer (see 2-#1). After thorough mixing, 1ml aliquots are prepared in glass vials, labelled as "rat prolactin standard 'A'-1µg/ml" and frozen at $-20^{o}C$. For assay purposes, "rat prolactin standard 'A'" must be further diluted yielding preparations of 100ng/ml. One ml. of "standard 'A'" is added to 9mls of 1% BSA buffer. This solution, after complete mixing, is divided into 0.6ml aliquots in glass

vials, capped with plastic screw-on tops, and labelled as 'rat prolactin standard 'B' - 100ng/ml". These vials are individually stored frozen at -20°C until time of assay, wherein each vial should provide enough material for a single assay's standard curve.

Rat prolactin reference preparation, NIAMDD rat prolactin RP-1, was prepared as in the instruction sheet accompanying the kit. Vials containing 0.6ml aliquots of 100ng/ml concentration of preparation in 1% BSA were frozen at -20°C until time of assay.

(3) Antiserum To Rat Prolactin- "1st Antibody"

- the antiserum directed against rat prolactin (prepared in rabbits) was obtained from the NIH (NIAMDD anti rat prolactin: S-7, S-8).

Anti rat prolactin S-8 was obtained in liquid form as 1ml and at time of reception was diluted in 9mls of 1% BSA buffer yielding a 1:10 dilution (volume: volume). Subsequently, 200µl aliquots were placed into Eppendorf plastic vials and frozen at -20°C until time of assay. At this point, the 200µl aliquots are added to 59.8 mls of 1% BSA buffer and after thorough mixing yield a final dilution of 1:3000 (volume: volume).

Anti rat prolactin S-7 was obtained as 400µl in liquid form and diluted immediately to a 1:10 solution by the addition of 3.6mls of 1% BSA buffer. Aliquote of size 100µl were prepared and frozen at -20°C until time of assay, at which point they were added to 39.9mls of 1% BSA buffer yeilding a final dilution of 1:4000 (volume:volume).

with the aid of Lang-Levy type micropipettes, 0,2,5,10,25, and 50µl of "rat prolactin standard 'B'" prepared as above. These volumes possess 0 (0µl), 0.2 (2µl), 0.5 (5µl), 1 (10µl), 2.5 (25µl), and 5 (50µl) namograms of rat prolactin reference. As such the standard curves contain 0,4,10,20,50, and 100ng of rat prolactin reference preparation per ml when projected onto 50µl sample sizes. The standard curve is completed by the addition of a pair of "Totals" and a pair of "NSB's" (non-specific binding). The amount of 1% BSA buffer in the "Totals" is 950µl/tube and the "NSB's" receive 550µl/tube. These tubes do not receive "rat prolactin standard 'B'" reference preparation.

At the same time, incubation experiment samples frozen at -20°C, are thawed then vortex mixed to ensure homogeneity of the sample. A volume of sample; sized 50µl, is added along with 400µl of 1% BSA buffer to 12X75mm borosilicate glass test tubes. Duplicates are made for each sample. The sample and buffer are added simultaneously with the aid of a Micromedic Systems automated pipetting station (Model 25000). The design of an assay requires that duplicates containing 50µl of known serum concentrations (of low, medium, and high range) and 400µl of 1% BSA buffer are interposed at the beginning, middle, and end of the sample series, for the assessment of inter - and intraassay variabilities. In addition, a pair of "0's" and a pair of "4's" are situated after 100 tubes while another pair of "0's" along with a pair of "10's" are located after the next 100 tubes. All tubes in the assay, with the exception of the "Totals" and the "NSB's" receive 100µl of the first antibody (i.e. anti

rat prolactin antisera; as above) delivered through a Hamilton gas-tight syringe attached to a Hamilton repeating dispenser. All tubes are vortex mixed, covered with a cellophane wrap and kept at 4°C for a minimum of 18 hours. Following this, 50µl of ^{125}I-rat prolactin, diluted to N5000 cpm in 1% BSA buffer, are added to all tubes with the aid of a 50µl Hamilton repeating dispenser syringe. The tubes are vortex mixed and left at room temperature with a cellophane wrap cover, for a period of 24 hours. When this time has elapsed, 100µl of the anti-"anti rat prolactin" antiserum preparation (2nd antibody; as above) is added to all tubes but the "Totals". Then 100µl of the carrier NRS solution (as above) and 200µl of the PEG 6000 solution are added to all tubes excluding 'Totals". All additions are performed with Hamilton repeating dispenser syringes of either the 100µl (2nd antibody) or 200µl (PEG) variety. Samples are vortex mixed and after a one and a half hour incubation at room temperature, are spun at 3200 r.p.m. for 20 minutes (4-8°C) on a Beckman J-6 centrifuge. One decants the "supernatant" off of all tubes but the "Totals". All tubes have the "pellet" fraction counted in a gamma counter (either a Micromedic Systems Model MS 588 or a Searle Analytic Model 1285). Samples are counted for 5 minutes with the number of counts in that time frame coded onto teletype tape. The teletype tape is brought to computation services wherein the data is obtained from computations performed on a Hewlit Packard "HP 3000" computer. The program developed by Mr. C. Davis is termed "RUN RIA" (personal communications).

TABLE 1

Sample Standard Curve

	Day 1			Day 2		Day 3	
Tube	BSA Buffer	Hormone Standard	Antibody #1	Labelled Hormone	Antibody #2	Carrier	Polyeth. Glycol
T	950µl	0µl	0µl	50µl	0µl	0µl	0µl
T	950µ	0µl	0µl	50µl	0µl	0µl	0µl
NSB	550	0	0	50	100	100	200
	550	0	0	50	100	100	200
0	450	0	100	50	100	100	200
0	450	0	100	50	100	100	200
4	448	2	100	50	100	100	200
4	448	2	100	50	100	100	200
10	445	5	100	50	100	100	200
10	445	5	100	50	100	100	200
20	440	10	100	50	100	100	200
20	440	10	100	50	100	100	200
50	425	25	100	50	100	100	200
50	425	25	100	50	100	100	200
100	400	50	100	50	100	100	200
100	400	50	100	50	100	100	200
Sample	400	50µl	100	50	100	100	200
Sample	400	Sample	100	50	100	100	200

Control "1" is present in tubes #1,2; 91, 92; 191, 192; 291, 292

Control "2" is present in tubes #3,4; 93, 94; 193, 194, 293, 294

Control "3" is present in tubes #5,6; 95, 96; 195, 196; 295, 296

Chapter 2

The Dissection And Incubation of Rat Anterior Pituitary Gland

Female Wistar rats weighing between 200 and 300gm are obtained from Woodlyn breeding farms. The animals are housed 3 per cage, maintained under 12/12 lighting conditions, and are fed ad libitum with Purina Rat Chow. After a minimum of one week duration for stabilization under such conditions, the animals are used in experiments. On the day of the pituitary incubation, the animals are sacrificed by rapid decapitation with a Wahmann guilotine. The brain is revealed by cutting right and left parasagittal lines with scissors, commencing from the foramen magnum and proceeding through to the coronal suture. The above flap is reflected in a caudal to rostral fashion allowing removal of the brain from the fossa in a reverse direction with the aid of blunt forceps. Meninges at the base of the fossa are removed with the aid of blunt forceps, exposing the pituitary. After swabbing excess blood away with a Kleenex tissue, one then extirpates the posterior lobe with fine forceps. The anterior pituitary is bisected longitudinally in the mid sagittal plane with a fine scalpel. The two paired hemipituitaries are "preincubated" together in a glass scintillation vial containing 3mls of Medium 199 (GIBCO) pregassed with 95%O_2,5%CO_2. The vial is placed in a Dubnoff metabolic shaker with bath temperature at 37^{o}C and aerated constantly with a 95%O_2-5%CO_2 gas mixture. After a period of one hour, the hemipituitaries are

separated from the same vial and placed into "incubation" vials individually, containing 3mls of medium 199. (Further detail used in individual studies is provided in the "materials & methods" section of the appropriate experiment).

SECTION IV

RESULTS

Chapter 1

Effects Of Liver Extract On Prolactin Release in vitro

1-1 Introduction

Unpublished observations in our laboratory suggested that preparations of female rat livers when co-incubated with female pituitary tissue promoted the release of prolactin (PRL) (28). This finding was obtained from studies designed to compare the influence various experimental tissues (e.g. hypothalamus) exerted over pituitary tissue when compared with control tissues (of which liver was included). Following this finding, additional studies were pursued with the resultant formulation of a detailed protocol for the partial isolation of this PRL releasing activity (PRA).

The objectives of the current experiment were to re-examine the influence that the liver preparation, as formulated previously, had on pituitary tissue. In addition, a subsequent purification step (i.e. dichloro-methane extraction) was to be assessed for any recovery of PRA. Finally, as all previous efforts used female tissues, it was decided that male tissues would also be examined in an identical fashion for any PRA.

1-2 Materials and Methods

(f) Animals

Female and male Wistar rats (200gm) were obtained through Woodlyn breeding farms. The animals were housed 3 per cage by sex,

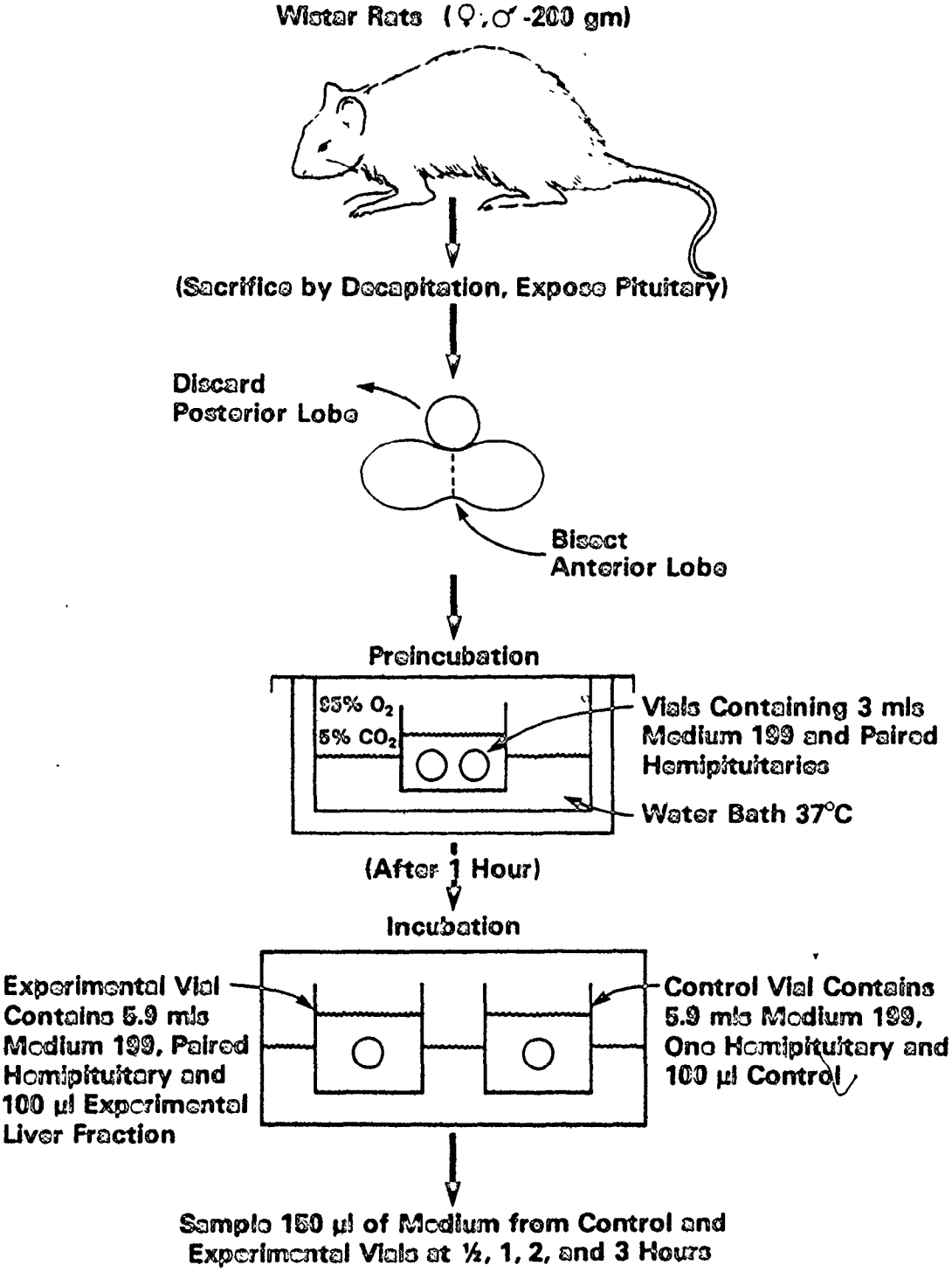
provided with food and water ad libitum, and maintained at 20-23°C under 12/12 artificial fluorescent lighting. After a minimum of one week acclimatization, the animals were sacrificed for purposes of either liver extraction or of pituitary incubation (vide infra).

(ii) Pituitary Incubations (refer to figure 1)

Prolactin releasing activity was to be measured using an *in vitro* hemipituitary organ culture as described by Talwalker (83). The experiments, of which there were two major kinds (examination of female tissues for PRA, and of male tissues for PRA), were performed on separate days. On the day of the incubation experiments, the animals were sacrificed by rapid decapitation using a guillotine. The pituitary glands were exposed, the posterior lobe discarded, and the adenohypophysis bisected in a longitudinal plane. The experiment required that one hemipituitary serve as control for its paired half. The hemipituitaries were for 1 hour first "pre-incubated" in vials containing 3mls of medium 199 (GIBCO). These vials were placed in a Dubnoff metabolic shaker with bath temperature of 37°C, all of which was enclosed and aerated with a 95%O_2, 5%CO_2 gas mixture. The hemipituitaries were then transferred to vials containing 5.9mls of fresh medium 199 for an "incubation" of 3 hours under the same experimental conditions listed above. Vials for the control condition received one hemipituitary and 100µl of the control solution. Three fractions were tested; crude, aqueous, and organic (see below). At 1/2, 1, 2, and 3 hours, 150µl of the bathing medium were taken, and diluted for purposes of hormone analysis. These samples were then frozen and stored at -20°C until time of assay.

Figure 1. System used for the coincubation of liver extracts (crude, aqueous, organic) with pituitary tissue.

INCUBATION SYSTEM



(iii) Preparation of the Crude, Aqueous, and Organic Liver Fractions (refer to figure 2)

The liver extracts were prepared from Wistar rats (housed as above) several days prior to the incubation studies. The animals were rapidly decapitated, and livers immediately removed, cut into small chunks, and weighed. The liver chunks were homogenized in ice cold absolute methanol (J.T. Baker Chemical Co.) (8.1ml MeOH/gm wet liver) using a Sorvall Omnimix. When a consistent homogenate was obtained, it was centrifuged at $0^{o}C$, 16000g, for 30 minutes in a Sorvall RC2-13 centrifuge. The supernatant was pooled, the methanol was then evaporated using a rotary evaporator (bath temperature of $\sim 30^{o}C$). The remaining sample was then frozen in an acetone/dry ice bath and lyophilized for storage. On the day of the incubation experiment, the lyophilate was suspended in physiological saline (Harleco) (50µl physiol. sal./70mg wet liver). As previous findings reported that this fraction possessed PRA, a portion of this fraction termed "crude" was allocated for testing. The remainder of this fraction was further processed by adding it to an equal volume of dichloro-methane (Fisher Scientific). The resultant aqueous and organic phases were partitioned in a separatory funnel. The aqueous phase was collected and again added to an equal volume of dichloro-methane for shaking and subsequent separation. This process was performed a total of three times. This procedure yielded an "aqueous fraction" for testing and an organic phase for further handling. The dichloro-methane component of the organic phase was evaporated off with nitrogen gas. This phase was reconstituted to its original volume by

adding the appropriate amount of physiological saline. This yielded the preparation termed "organic fraction", which was tested for PRA along with the other two fractions.

The control for the crude fraction was an equivalent volume of physiologic saline. The controls for the aqueous and organic fractions were obtained in a similar fashion to the preparation of the fractions - the only difference being the use of physiologic saline in lieu of the crude fraction to be applied to dichloro-methane.

(iv) Hormone Assay

Rat prolactin was determined using a double antibody radioimmunoassay with 1st antibody and rat prolactin standard (RP-1) supplied by the National Pituitary Agency under a program supported by the NIAMDD. The intraassay coefficients of variability were calculated as described previously (72) and found to be 6.6%, 8.2%, and 10.2% for low, mid, and high range values respectively. The between assay variations were 28%, 15.2% and 8.3% respectively for low, mid, and high range controls. The assay sensitivity was 4ng/ml.

(v) Statistical Analysis

Analysis of the data was performed using a two-way analysis of variance corrected for repeated measures (95).

1-3 Results

The results of the incubation study using female tissues (i.e. varied extracts from female liver, coincubated with female hemipituitaries) are shown in Table 1. The values represent the mean (± s.e.m.) levels of rat prolactin in the culture medium of the controls (n=5) and liver (n=5) groups and are expressed as ng (RP-1)/ml culture medium. Statistical analysis revealed that for all studies (control vs crude, control vs aqueous, and control vs organic fractions) there were no significant interactions between Time (1/2, 1, 2, and 3 hours) and Group (Control vs Liver Fraction). There was no evidence supporting the presence of a PRA in any of the liver extract preparations. Examination of the findings for the study of the aqueous extract revealed significantly lower levels of PRL ($p<.025$, $F=5.99$) in vials with the extract than in appropriately paired controls (see Table 3B). Mean values for vials containing liver extract were 518 ± 57 ng/ml culture medium while for the control 646 ± 58 ng/ml. Table 3A also reveals that in all instances there was a significant Time effect. Mean Time values are expressed for each fraction study performed.

Examination of data obtained from the coincubation studies using male tissue revealed no significant interaction between Time and Grouping (see Table 2). There was a significant Time effect on prolactin content in all but the study examining the organic fraction (see Table 4A). There was no evidence demonstrating any PRA present in male liver extracts. It was observed that there were significantly diminished PRL values ($p<.001$, $F=17.1$) in the vials containing crude male liver extract when contrasted to the vials containing the crude control (see Table 4B).

TABLE 1
Liver Extract Effect on Pituitary Incubation Using Female Tissues
(i.e. female livers, pituitaries)
- values expressed are ng RP-1 (rat Prolactin)/ml Med 199 ± s.e.m.

	1/2 Hour		1 Hour		2 Hours		3 Hours	
	Control	Liver Extract	Control	Liver Extract	Control	Liver Extract	Control	Liver Extract
Crude (n=5)	672 ±132	638 ±41	865 ±81	878 ±40	1217 ±126	1157 ±76	910 ±245	1104 ±100
Aqueous (n=5)	372 ±67	321 ±59	525 ±47	474 ±115	909 ±113	551 ±78	780 ±31	729 ±136
Organic (n=5)	561 ±111	471 ±96	591 ±65	662 ±89	906 ±107	1041 ±171	944 ±58	896 ±62

TABLE 2
Liver Extract Effect on Pituitary Incubation Using Male Tissues
(i.e. male livers, pituitaries)
- values expressed are ng RP-1 (rat Prolactin)/ml Medium 199 ± s.e.m.

	1/2 Hour		1 Hour		2 Hours		3 Hours	
	Control	Liver Extract	Control	Liver Extract	Control	Liver Extract	Control	Liver Extract
Crude (n=5)	129 ±53	106 ±24	170 ±42	139 ±54	267 ±72	214 ±67	332 ±65	262 ±65
Aqueous (n=5)	153 ±60	209 ±102	243 ±71	265 ±116	258 ±68	263 ±69	455 ±120	389 ±87
Organic (n=5)	270 ±290	228 ±186	184 ±14	235 ±106	244 ±33	264 ±80	291 ±55	279 ±76

TABLE 3

Significant Effects in Studies Using Female Tissues
- values expressed are mean prolactin values in ng/ml of culture medium ± s.e.m.

- A - Time Effects

Study Significance	Crude vs Control $p < .001$	Aqueous vs Control $p < .001$	Organic vs Control $p < .001$
1/2 hour	655 ± 65	346 ± 43	516 ± 71
1 hour	872 ± 43	499 ± 59	626 ± 53
2 hours	1187 ± 70	730 ± 88	974 ± 98
3 hours	1006 ± 129	754 ± 66	920 ± 41

- B - Grouping Effects

Study Significance	Crude vs Control not significant	Aqueous vs Control $p < .025$	Organic vs Control not significant
Control	916 ± 85	646 ± 58	751 ± 57
Liver Extract	944 ± 57	518 ± 57	767 ± 72

TABLE 4

Significant Effects in Studies Using Male Tissues
- values expressed are mean prolactin values in ng/ml of culture medium ± s.e.m.

- A - Time Effects

Study Significance	Crude vs Control $p < .001$	Aqueous vs Control $p < .001$	Organic vs Control not significant
1/2 hour	118 ± 13	181 ± 27	249 ± 73
1 hour	154 ± 15	254 ± 29	209 ± 24
2 hours	241 ± 23	261 ± 21	254 ± 19
3 hours	297 ± 23	422 ± 33	285 ± 20

- B - Grouping Effects

Study Significance	Crude vs Control $p < .001$	Aqueous vs Control not significant	Organic vs Control not significant
Control	225 ± 22	277 ± 30	247 ± 32
Liver Extract	180 ± 18	282 ± 25	252 ± 25

1-4 Discussion

Previous unpublished reports have claimed that liver and methanol extracts of liver from female Wistar rats promote the *in vitro* secretion of prolactin. Using identical methodology, this study has been unable to demonstrate the presence of such a liver PRA obtained from female rats. Furthermore, no evidence for any PRA derived from male livers could be shown. Moreover, this report has demonstrated that, under certain conditions, liver extract co-incubations with hemipituitaries are associated with a diminished content of prolactin in the bathing culture medium when contrasted with the appropriately paired control condition. Specifically, "aqueous fraction" extracts obtained from female livers and "crude fraction" extracts obtained from male livers are the conditions responsible for the above mentioned finding.

The interpretation of these findings by this author is that these preparations of liver may possess a factor which inhibits prolactin secretion *in vitro* - i.e. a prolactin inhibitory factor (PIF). It should be cautioned that this inferrence is based upon repetitive sampling from a vial with a fixed volume. As such, one is measuring prolactin content in the medium and not necessarily secretory profiles. Alternative explanations for the diminished prolactin in the vials with liver extracts may be entertained. The findings may be due to enzymatic degradation of PRL by liver extracts and/or binding of PRL to receptors present in the liver extract. This would require that these liver enzymes and/or receptors would be soluble in absolute methanol.

That liver receptors for PRL account for the reduced content in liver extract co-incubations, seems an unlikely explanation. Receptors for prolactin are not normally found in male livers (68). It remains possible that methanol soluble liver enzymes present in both male and female tissues could account for the diminished content. Further studies eliminating this possibility will be required before a definitive statement regarding the presence of a liver PIF can be made.

The findings of this study appear to contradict earlier efforts within our laboratory. The source of the contradiction may be in the nature and handling of the liver extracts. Despite all efforts to minimize variation in technique, the possibility remains that some minor difference in preparation (e.g. time) of the extract occurs. It is not unlikely that as estrous cycles were not controlled for, the tissues used in this study may respond differently. The influence that steroidal priming plays must further be characterized before it can be stated that no liver PRA exists.

Chapter 2

Influences of Liver Extract on the Secretion of Prolactin From Steroid Primed Pituitaries in vitro

2-1 Introduction

The previous chapter fails to confirm the presence of a prolactin releasing activity (PRA) in liver extracts. On the contrary, it suggests that certain liver extract fractions may possess a prolactin inhibitory activity (PIA). It was concluded that the discrepancies may arise from at least two potential sources - a minor difference in the handling of the liver extracts, and/or unresponsive tissues (e.g. pituitary) due to varied hormonal milieus of the animals prior to sacrifice.

With regard to the latter case, it has long been recognized that prolactin secretion varies during the estrous cycle (see review by 61). Specifically, markedly elevated prolactin levels are seen during rises of both estradiol - 17-β and progesterone. In vitro studies reveal that pituitary secretion of prolactin is augmented when the tissue culture medium is enriched with estradiol - 17-β (59). The estrogen can interact with TRH binding to potentiate the TRH stimulated release of PRL (11). Furthermore, estradiol when previously incubated with monolayer cell cultures of pituitary gland, can partially reverse the inhibitory effect on PRL secretion exerted by dopamine agonists (51). As a result of the above mentioned findings,

it was decided that a steroidal preparation (consisting of estradiol-17-β and progesterone) would be employed in an effort to potentiate the pituitary responsiveness to stimulation and possibly to diminish any inhibitory influence.

2-2 Materials and Methods

(i) Animals

Female Wistar rats (∿ 200 gm) obtained through Woodlyn breeding farms were group housed, provided with food and water ad libitum, and maintained under 12/12 lighting. The animals were acclimatized for a minimum of one week under the above conditions before use in experiments. The animals donating livers were sacrificed several days prior to the incubation study.

(ii) Steroidal Priming

In a protocol established previously (58) animals donating pituitaries were sensitized to stimulation using a steroidal preparation. The animals received for 3 days prior to sacrifice, subcutaneous injections of suspensions containing 50μg of estradiol - 17-β and 25 mg progesterone (Sigma Chemicals) in 0.5 ml propylene glycol.

(iii) Pituitary Incubations

As described previously, liver extracts (crude, organic, and aqueous) were assessed for any activity altering PRL secretion using an organ culture system. The incubation procedure has been documented in detail elsewhere (see Section III - Chapter 2, or Section IV - Chapter 1).

(iv) Preparation of the Crude, Aqueous, and Organic Liver Fractions

The preparation of the liver extracts for incubation studies has been detailed elsewhere (see Section IV - Chapter 1).

(v) Hormone Assay

A double antibody radioimmunoassay was empolyed using NIAMDD standards and reagents for the detection of rat prolactin. A detailed description has been provided in Section III - Chapter 1.

(vi) Statistical Analysis

The data obtained from the study was analyzed using a two-way analysis of variance corrected for repeated measures (95).

2-3 Results

The results of the study using liver extracts (crude, aqueous, and organic fractions) coincubated with steroidal primed hemipituitaries, are listed in Table 1. Values expressed represent the mean (± S.E.M.) levels of rat prolactin in the culture medium of the controls (n=5) and liver groups (n=5) and are expressed as ng (RP-1)/ml culture medium. Statistical analysis revealed that for all three studies (crude vs control, aqueous vs control, and organic vs control), there was no significant interaction between Group and Time. In all three studies there was a significant time effect ("crude" study - $p<.001$, $F=49.1$; "organic" study - $p<.001$, $F=14.4$; "aqueous" study - $p<.001$, $F= 45.5$). The combined (i.e. collapsed between control and liver extract) mean value for the individual times are listed in Table 2A. This study did not reveal the presence of a PRA from any liver extract fraction. There was, however, a

TABLE 1

Liver Extract Effect on Steroidal Primed Pituitaries in vitro
- values expressed are ng(RP-1)/ml Medium 199 ± s.e.m.

	1/2 Hour		1 Hour		2 Hours		3 Hours	
	Control	Liver Extract	Control	Liver Extract	Control	Liver Extract	Control	Liver Extract
Crude (n=5)	318 ±50	237 ±39	632 ±48	424 ±66	801 ±68	636 ±88	851 ±46	786 ±126
Aqueous (n=5)	392 ±43	410 ±82	556 ±36	494 ±35	941 ±103	901 ±74	971 ±69	870 ±44
Organic (n=5)	350 ±63	351 ±57	485 ±95	366 ±43	762 ±165	749 ±118	849 ±162	701 ±86

TABLE 2

Significant Effects
- values expressed are mean prolactin values in ng/ml of culture medium ± s.e.m.

- A - Time Effects

Study Significance	Crude vs Control p < .001	Aqueous vs Control p < .001	Organic vs Control p < .001
1/2 hour	277 ± 33	400 ± 44	350 ± 40
1 hour	528 ± 52	525 ± 26	426 ± 53
2 hours	718 ± 59	921 ± 60	755 ± 96
3 hours	818 ± 64	921 ± 42	775 ± 90

- B - Grouping Effects

Study Significance	Crude vs Control p < .001	Aqueous vs Control not significant	Organic vs Control not significant
Control	651 ± 54	715 ± 65	611 ± 75
Liver Extract	521 ± 62	669 ± 58	542 ± 56

significant group effect (i.e. control vs liver) for the study using the crude liver extract ($p<.001$, $F = 14.9$) so that levels in the liver group were reduced. The mean values for the individual group effects in each study are listed in Table 2-B.

2-4 Discussion

We have examined pituitary response to liver extract under a sensitized pituitary state. Specifically, in this study we have examined the response to liver extracts of pituitaries sensitized to high levels of estradiol and progesterone. In the previous study pituitaries obtained from animals expected to have low non-cyclical estrogen and progesterone levels (i.e. males), and pituitaries from normal cycling females were examined for their response to liver extracts. In no instance was there evidence supporting a stimulated PRL response to liver extract. Moreover, there was what may be interpreted as an inhibitory response to several extracts. The previous chapter documented that male crude liver extracts apparently inhibited male pituitary PRL secretion, and that aqueous fractions from female liver extracts apparently inhibited PRL secretion from female pituitaries. The present study suggested that female liver crude extract also inhibits PRL secretion from steroid primed pituitaries. It appears unlikely that the source of variation between the original studies showing PRA and these studies is the liver itself as the liver extracts were obtained from males (with low estrodial and progesterone levels) and from normally cycling females on several instances. The most acceptable explanation,

assuming that the original findings were accurate, is that variations occur in the handling of the liver while the various extracts were prepared. Since the original observations conclude that liver chunks possessed PRA in vitro, then if liver handling is crucial, studies using chunks of liver should reproduce the original effect.

As for the finding that diminished levels of PRL are found in vials co-incubated with liver extract, it remains uncertain that this is a PRL inhibiting activity or related to other factors. Further studies examining liver breakdown and/or binding of PRL must be conducted to eliminate these possibilities. If inhibition is still suggested, then a careful elimination of known PIF's (using antagonists) should be conducted.

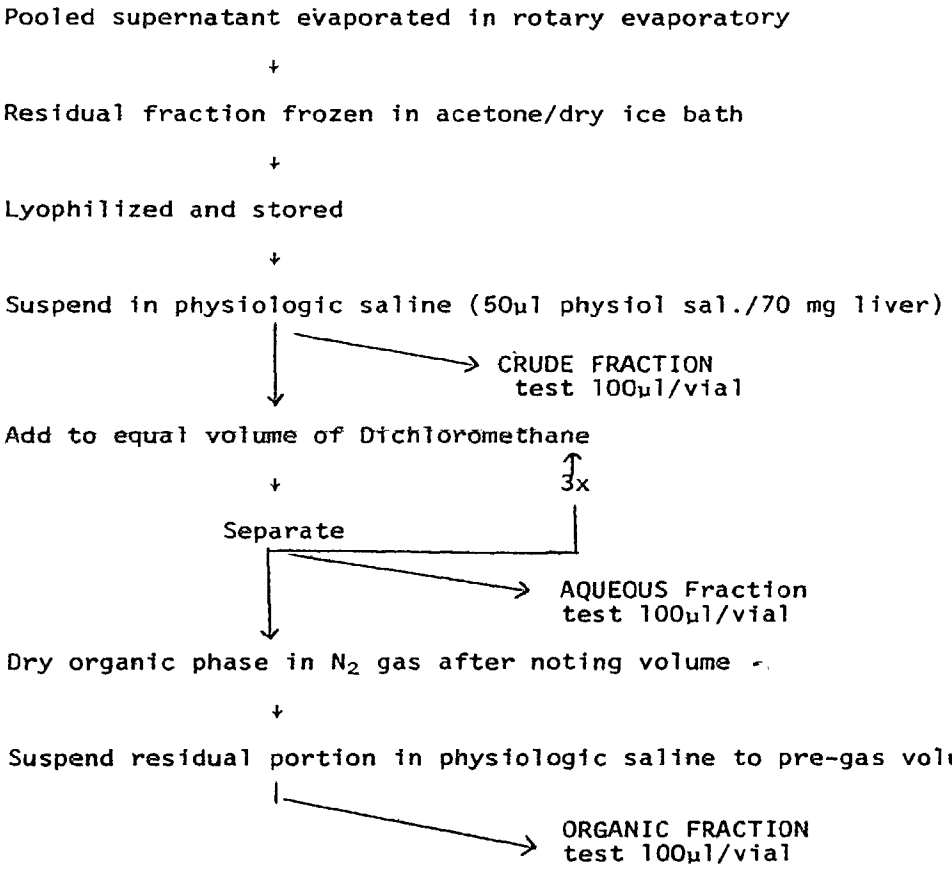
Chapter 3

An Examination of The Possible Inhibitory Role Exerted by Liver on Prolactin Release in vitro

3-1 Introduction

In light of the findings mentioned in previous chapters, a series of preliminary studies were conducted in an attempt to reconcile differences in findings between my own and early efforts in our laboratory. The results of these studies revealed that no PRA was discerned in liver chunks (in additon to the liver extracts) under a variety of steroid primed milieus. Furthermore, there was a consistent, apparent "inhibitory" influence on PRL secretion exerted by the liver chunks. It was noted that this influence was most accentuated when the animals donating pituitaries received steroidol priming (as described in Chapter 2) and the liver-donating animals received the vehicle (propylene glycol) injections.

There were two specific objectives of the present study. It would be determined whether PRL is subject to liver degradation and/or binding at an appreciable extent accounting for apparent inhibitory activity. In addition, using the butyrophenone neuroleptic "Haloperidol", the role that liver dopamine has upon this apparent inhibition would be determined.



3-2 Materials and Methods

(i) Animals

Thirty female Wistar rats weighing ~ 200 gms were obtained from Woodlyn breeding farms. The animals were kept in groups of 3 per cage, provided with food and water ad libitum, and maintained at 20-23°C under 12/12 lighting. All animals were acclimatized for a minimum of one week prior to sacrifice. Six animals would yield liver tissues for analysis on the pituitaries obtained from the remaining 24 animals. All animals were sacrificed by decapitation on the same days.

(ii) Steroidal Priming

Animals donating pituitary tissue were injected s.c. with a solution of 0.5 ml propylene glycol containing 50µg of estradiol - 17-β (Sigma) and 25 mg of progesterone (Sigma). The animals donating liver tissues received for 3 days prior to sacrifice, 0.5 ml of propylene glycol.

(iii) PRL Degradation/Binding by Liver Study

Preincubation medium was collected and pooled following the

The data obtained from the uptake/degradation study are listed in Table 1. The values expressed are the mean (n=6) content of rat PRL in ng RP-2/ml of culture medium. Statistical analysis revealed no significant interaction between group and time. Furthermore, there was no significant effect of either Time or Group on the vial content of prolactin.

Table 2 contains the mean data obtained from the Haldol study with values expressed in ng RP-2/ml of culture medium ± S.E.M. Statistical analysis revealed there to be a highly significant Time X Group interaction ($p<.001$, $F=8.5$). The mean values for this interaction are tabulated in Table 3A and are graphed in figure 1. There was a signficiant interaction ($p<.025$) between drug concentration and cell group (i.e. liver or blank). The mean values for this interaction are listed in Table 3B and are plotted in graph form in figure 2. Analysis of the data revealed that both the group effect and the time effect were highly significant ($p<.001$), whereas there was no significant drug effect. The mean values for these effects are provided in Table 3c and 3D.

TABLE 1

Prolactin Uptake/Degradation by Liver Study
- values expressed are ng RP-2 (rat Prolactin)/ml Med. 199 ± s.e.m.
(n=6)

1/2 Hour		1 Hour		2 Hours		3 Hours	
Control	Liver	Control	Liver	Control	Liver	Control	Liver
2438 ±228	2799 ±373	2607 ±421	3174 ±673	2618 ±522	2132 ±177	3347 ±470	2562 ±300

TABLE 2

Haloperidol Influence on Liver Inhibition of Prolactin Secretion in vitro
- values expressed are ng RP-2 (rat prolactin)/ml Medium 199 ± s.em.

	1/2 Hour		1 Hour		2 Hours		3 Hours	
	Control	Liver	Control	Liver	Control	Liver	Control	Liver
OnM Haldol	607 ±127	284 ±39	1168 ±331	471 ±57	1797 ±399	693 ±78	2307 ±498	872 ±128
10nM Haldol	477 ±68	372 ±49	788 ±112	780 ±247	1290 ±161	901 ±138	2249 ±482	1066 ±129
100nM Haldol	559 ±59	483 ±97	1066 ±166	690 ±130	1763 ±221	954 ±147	2151 ±488	1357 ±209
1000nM Haldol	457 ±65	356 ±18	857 ±80	584 ±52	1536 ±208	921 ±150	1635 ±153	1249 ±251

TABLE 3

Significant Effects in Haldol Study
- values expressed are mean levels in ng RP-2/ml Med. 199 ± s.e.m.

- A - Time X Group Interaction (p<.001)

	1/2 Hour	1 Hour	2 Hours	3 Hours
Control	525 ±41	970 ±97	1596 ±130	2085 ±208
Liver	374 ±31	633 ±71	867 ±65	1136 ±95

- B - Drug x Group (p<.025)

	0nM Haldol	10nM Haldol	100nM Haldol	1000nM Haldol
Control	1469 ±215	1200 ±186	1384 ±184	1121 ±120
Liver	581 ±60	779 ±90	871 ±98	777 ±99

- C - Time Effect (p<.001)

1/2 Hour	1 Hour	2 Hours	3 Hours
449±4	801±9	1231±13	1610±19

- D - Group Effect (p<.001)

Control	1294 ± 9
Liver Chunk	752 ± 5

Figure 1. Effect of liver on prolactin secretion from pituitary over Time. Values expressed are ng/ml of RP-2 standard ± s.e.m. (o control, o liver).

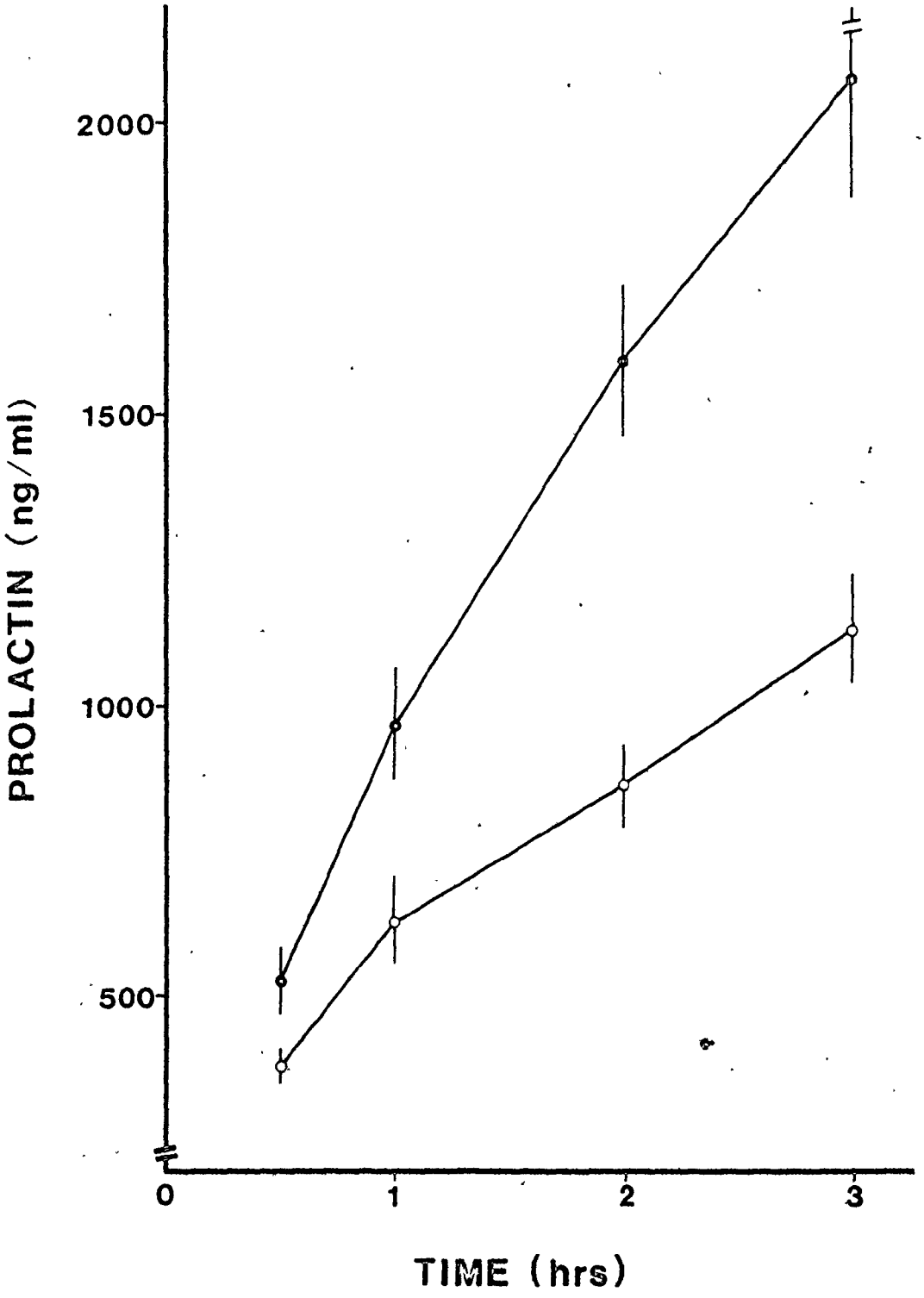
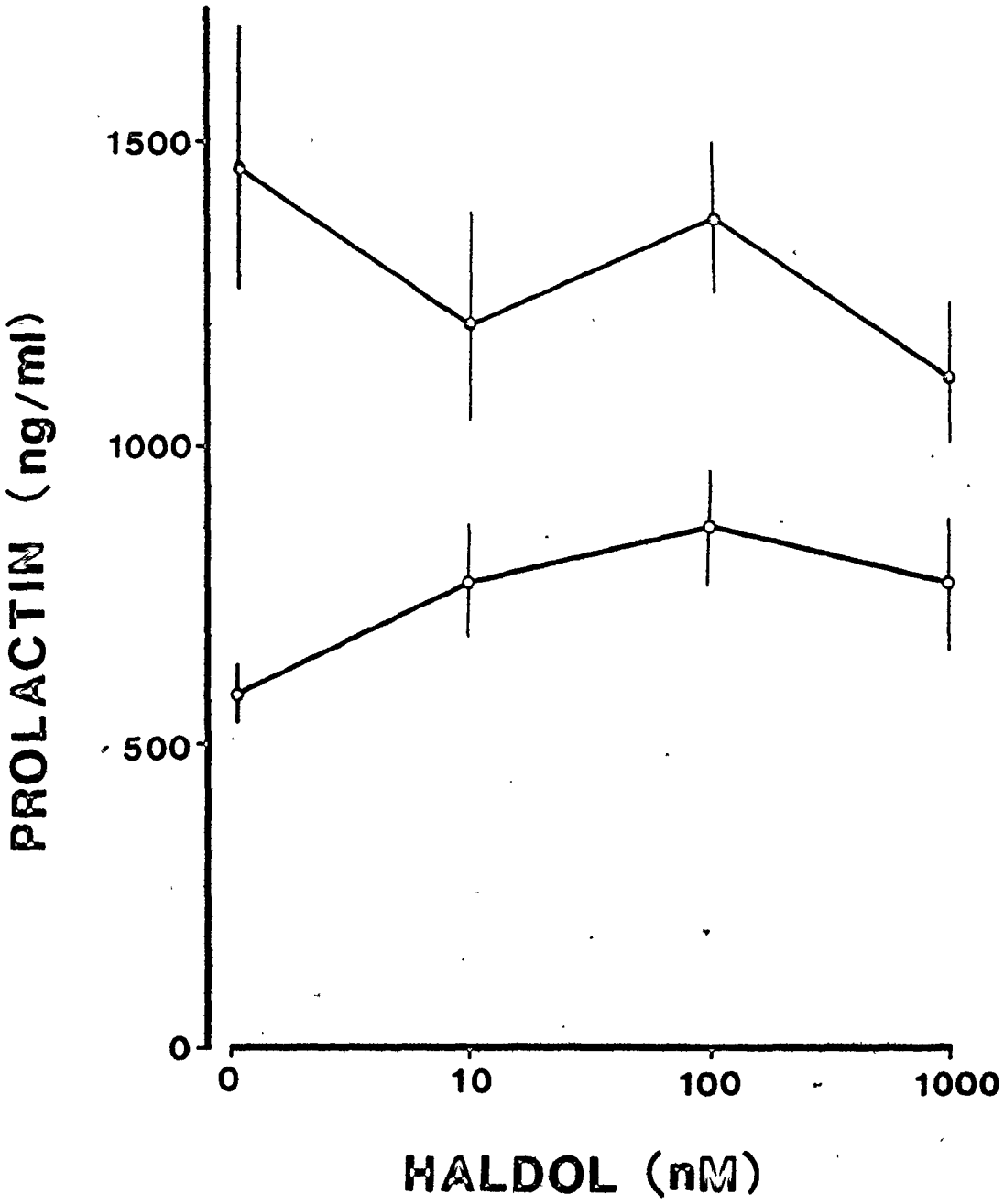

2000
1500
1000
500
PROLACTIN (ng/ml)
0
1
2
3
TIME (hrs)

Figure 2. Effect of Haldol on liver inhibition of Prolactin secretion Values expressed are ng/ml of RP-2 standard ± s.e.m. (● control, o liver).


1500
1000
500
0
PROLACTIN (ng/ml)
0
10
100
1000
HALDOL (nM)

Discussion

The results of the experiments presented in this chapter provide further information on the nature of the liver activity associated with diminished prolactin levels. In the first part of the study, it was determined that the liver was not degrading nor was it binding prolactin to any appreciable extent. That is to say, there was no significant effect of Time during the period studied over the content of PRL in the culture medium. Furthermore, liver chunk effects on the vial content of PRL were not different from the control condition over the period of time studied. In light of this evidence, it was concluded that the phenomena of diminished PRL in the culture medium of vials containing liver chunks is, in all probability, a true inhibition of secretion. In an effort to obtain some information on the nature of the liver inhibitory factor, a dopamine blocking agent (Haldol) was assessed for any antagonism of liver inhibitory activity. The concentrations of Haldol used were based upon data obtained from our laboratory. Using a similar *in vitro* preparation for the incubation of pituitary tissue, concentrations of dopamine in the order of 10^{-6}M, inhibit by 50% the secretion of PRL into the culture medium (44). 100nM of Haldol prevented this 50% inhibition by dopamine. We have estimated that the inhibition of PRL by 150 mg liver chunks ranges from 30-60%, as such, it was decided that 1000 nM of Haldol would be the maximum concentration studied. In the hope of obtaining useful information, it was decided

that additionally, concentrations less than 1000nM of Haldol (I.e. 100, 10, and 0nM) were to be tested.

The study confirms the presence in liver chunks, of a factor that inhibits PRL secretion, in vitro. Although, the study revealed a significant ($p<.025$) interaction between Haldol concentrations and groupings (see Table 3B and figure 2), the 1000nM of Haldol failed to abolish the liver inhibition. Post hoc statistical anaylsis using T-tests, suggests that the interaction is due in part to an inhibition by Haldol of the PRL secretion in the controls. There is a significant difference ($p<.02$) between the maximum value of the the control at 0nM and it's minimum value at 1000nM. The most dramatic difference for the liver group is only slightly signficiant ($p<.05$) and occurs with a minimum value at 0nM and a maximum value at 100nM Haldol. As the most dramatic change in secretion for the liver groups occurs between 0 and 100nM and as there is no significant difference for this group between 10 and 1000nM, it appears unlikely that the concentrations of Haldol examined were not of a sufficient magnitude. Confirmation of this would require measurement of haloperidol in the culture medium of haloperidol in the culture medium to rule out degradation of this antagonist by liver. This technique was not available to us at the time of experimentation.

In conclusion, the results of the studies indicate that a liver factor inhibits PRL secretion and that this factor is partially antagonized by the neuroleptic Haloperidol.

SECTION V

GENERAL DISCUSSION OF FINDINGS

General Discussion

The thesis documents studies directed at examining the liver for the presence of a substance(s) reported to alter prolactin secretion, in vitro. Contrary to preliminary unpublished efforts, I have been unable to demonstrate the presence of a liver factor that stimulates prolactin release. I have demonstrated an association between liver and diminished prolactin content in culture medium. The liver preparations revealing such an association have been aqueous fractions of female livers, crude fractions of female livers, liver chunks of female animals, and crude fractions of male livers.

The data suggest that the liver association is not due to degradation and/or binding of PRL to liver, rather more likely due to a factor that inhibits PRL secretion. This factor is not completely antagonized by 1000nM Haldol. Because the factor is soluble in absolute methanol it is suggested that this factor is not protein. Similarly, because activity has been delineated in aqueous fractions following dichloro methane extraction, it is more than likely not a lipid (as most lipids are insoluble in aqueous solutions).

One known PIF was examined indirectly - namely dopamine. In pursuing the project further there would be at least two possible routes to take. One could continue, as for the Haldol study, eliminating known PIF's such as GABA, Ach, etc., by employing known antagonists of these agents and assessing alterations in liver PRL inhibitory activity.

The alternative approach for obtaining insight as to the nature of this liver PIA would be the examination of the physico chemical propterties of the factor. That is to say, one could determine the size of the substance with dialysis membranes or further elaborate upon solubility characteristics of the factor.

There are, of course, both limitations and benefits inherent in these two approaches. A systematic elimination of known PIF's could leave the investigator with very little information were this agent to be novel. On the other hand, detailed attempts at characterizing the properties of this substance might provide the investigator with information previously worked out for a particular known PIF.

There is, in addition, some essential groundwork to be performed before continuing the isolation of the substance. One should examine for the possibility that this phenomena is dose dependant. One should assess any cytotoxic activity of this factor. One should also determine the effect this agent has on other anterior pituitary cells and on their secretions. Finally, studies should be directed at determining the nature of the disparities in findings by this author and another.

SECTION VI

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