ผลของการใส่ห่วงคุมกำเนิดชนิดไหมต่อโปรเจสเตอโรนรีเซปเตอร์ในมดลูกหนู

นางสาวฉวีวรรณ อภิสิทธิ์ไพศาล

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THE EFFECT OF SILK - SUTURE INTRAUTERINE DEVICE ON PROGESTERONE RECEPTORS IN RAT UTERUS

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หัวข้อวิทยานิพนธ์ ผลของการใส่ห่วงคุมกำเนิดชนิดไหมต่อโปรเจสเตอโรนรีเซปเตอร์ในมดลูกหนู

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บทคัดย่อ

การศึกษาผลของห่วงคุมกำเนิดชนิดไหม (silk-suture IUD) ต่อปริมาณและการจับกับฮอร์โมนของโปรเจสเตอโรนรีเซปเตอร์ในมดลูกหนู โดยใช้โปรเจสเตอโรนที่ติดฉลากด้วยไฮโดรเจน-3 (^{3}H-progesterone) เป็นตัวจับจำเพาะ พบว่า ห่วงคุมกำเนิดไม่มีผลต่อความสามารถในการจับกับฮอร์โมนของโปรเจสเตอโรนรีเซปเตอร์ เนื่องจากค่า K_d (dissociation constant) ของโปรเจสเตอโรนรีเซปเตอร์ในไซโทปลาสมของมดลูกข้างที่ใส่ห่วงและไม่ใส่ห่วงมีค่าใกล้เคียงกัน ไม่ว่าจะเปรียบเทียบในระยะเดียวกันหรือต่างระยะกันของวงจรสืบพันธุ์ (estrous cycle) กล่าวคือ ค่า K_d เฉลี่ยของโปรเจสเตอโรนรีเซปเตอร์ในมดลูกข้างที่ไม่ใส่ห่วงมีค่าเท่ากับ 0.59 ± 0.03 นาโนโมล/ลิตร และข้างที่ใส่ห่วงมีค่าเท่ากับ 0.56 ± 0.02 นาโนโมล/ลิตร ในทำนองเดียวกันค่า K_d ของโปรเจสเตอโรนรีเซปเตอร์ในนิวเคลียสของมดลูกข้างที่ใส่ห่วงและไม่ใส่ห่วงก็ไม่แตกต่างกัน คือมีค่าเท่ากับ 0.89 ± 0.10 นาโนโมล/ลิตร ในมดลูกข้างที่ใส่ห่วงและเท่ากับ 0.89 ± 0.06 นาโนโมล/ลิตร ในมดลูกข้างที่ไม่ใส่ห่วง

เมื่อศึกษาผลของห่วงคุมกำเนิดต่อปริมาณโปรเจสเตอโรนรีเซปเตอร์ในไซโทปลาสมที่ระยะต่างๆของวงจรสืบพันธุ์ พบว่า ทุกระยะของวงจรสืบพันธุ์ โปรเจสเตอโรนรีเซปเตอร์ในไซโทปลาสมของมดลูกข้างที่ใส่ห่วงจะมีปริมาณต่ำกว่าข้างที่ไม่ใส่ห่วงอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) แต่รูปแบบของการเปลี่ยนแปลงปริมาณโปรเจสเตอโรนรีเซปเตอร์ในไซโทปลาสมที่ระยะต่างๆของวงจรสืบพันธุ์ของมดลูกทั้งสองข้างยังคงเหมือนกัน กล่าวคือ พบโปรเจสเตอโรนรีเซปเตอร์ในไซโทปลาสมระดับต่ำสุดในระยะเมทอีสตรัส และระดับสูงสุดในระยะอีสตรัส จากการศึกษาผลของห่วง-

คุมกำเนิดในหนูซึ่งถูกตัดรังไข่ต่อการสังเคราะห์โปรเจสเตอโรนรีเซปเตอร์เมื่อใช้อีสโตรเจนเป็นสารเหนี่ยวนำ และต่อการเคลื่อนที่ของรีเซปเตอร์จากไซโตปลาสมเข้าไปในนิวเคลียส พบว่า หนูซึ่งถูกตัดรังไข่และไม่ได้รับการฉีดฮอร์โมนมีปริมาณโปรเจสเตอโรนรีเซปเตอร์ในไซโตปลาสมของมดลูกข้างที่ไม่ใส่ห่วงมากกว่าข้างที่ใส่ห่วงอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) การฉีดอีสโตรเจน (17β-estradiol) ในหนูซึ่งถูกตัดรังไข่จะทำให้โปรเจสเตอโรนรีเซปเตอร์ในไซโตปลาสมของมดลูกทั้งสองข้างเพิ่มขึ้นเท่ากัน คือเพิ่มจากเมื่อไม่ได้ฉีดอีสโตรเจน 1-2 เท่า แต่ปริมาณโปรเจสเตอโรนรีเซปเตอร์ในไซโตปลาสมของมดลูกข้างที่ใส่ห่วงยังคงมีค่าเป็น 60 - 70% ของข้างที่ไม่ใส่ห่วง นอกจากนี้การเคลื่อนย้ายของโปรเจสเตอโรนรีเซปเตอร์จากไซโตปลาสมไปยังนิวเคลียสในมดลูกข้างที่ใส่ห่วงต่ำกว่าข้างที่ไม่ใส่ห่วง 10% ซึ่งอยู่ในขีดจำกัดของความแปรปรวนในการหาปริมาณรีเซปเตอร์ ดังนั้น ข้อมูลนี้จึงไม่อาจสรุปลงไปแน่นอนได้ว่าห่วงคุมกำเนิดมีผลทำให้การเคลื่อนย้ายของโปรเจสเตอโรนรีเซปเตอร์จากไซโตปลาสมไปยังนิวเคลียสของมดลูกข้างที่ใส่ห่วงลดลง

เมื่อศึกษาคุณสมบัติการเซดิเมนท์ของโปรเจสเตอโรนรีเซปเตอร์ในไซโตปลาสมโดยการปั่นในสารละลายซูโครส (sucrose gradient centrifugation) ปรากฏว่า ไม่ว่าจะใช้ศึกษาจากระบบของโปรเจสเตอโรนที่ติดฉลากด้วยไฮโดรเจน-3หรือ ^{3}H-ORG 2058 เป็นตัวจับจำเพาะ สัมประสิทธิ์การเซดิเมนท์ของโปรเจสเตอโรนรีเซปเตอร์ในไซโตปลาสมของมดลูกทั้งสองข้างมีค่าเท่ากันเป็น 4S เมื่อเปรียบเทียบกับ BSA (4.6S)

จากผลการทดลองทั้งหมดนี้ชี้แนะว่า ห่วงคุมกำเนิดชนิดไหมไม่มีผลต่อความสามารถในการจับกับฮอร์โมน และคุณสมบัติเซดิเมนเตชั่นของโปรเจสเตอโรนรีเซปเตอร์ แต่มีผลทำให้ปริมาณโปรเจสเตอโรนรีเซปเตอร์ลดลง ซึ่งการศึกษาวิจัยนี้ยังไม่มีข้อสรุปถึงสาเหตุที่แท้จริงของการลดลงดังกล่าว อย่างไรก็ตามการลดปริมาณของโปรเจสเตอโรนรีเซปเตอร์ในมดลูกเมื่อใส่ห่วงคุมกำเนิดอาจทำให้ความไว (sensitivity) ของมดลูกต่อโปรเจสเตอโรนเปลี่ยนไป อันน่าจะเป็นกลไกอย่างหนึ่งของการคุมกำเนิดโดยไปทำให้การฝังตัวของบลาสโตซีสต์ในวันที่ 5 ล้มเหลว

Thesis Title The Effect of Silk-suture Intrauterine Device on Progesterone Receptors in Rat Uterus

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ABSTRACT

The effect of silk thread suture intrauterine device (IUD) on the binding characteristics and concentration of progesterone receptor in rat uterus was investigated by using ^{3}H-progesterone as specific binding ligand. The IUD showed no effect on the binding affinity of progesterone receptor. The dissociation constant (K_d) of cytosolic progesterone receptor (PR_c) in the control and IUD horns were similar whether they were compared in the same stage or between stages of estrous cycle. The mean K_d of PR_c in the control horn was 0.59 $\pm$ 0.03 nmol/l and was 0.56 $\pm$ 0.02 nmol/l in the IUD horn. Similarly , there was no significant difference in the K_d of nuclear progesterone receptor (PR_n) between the control and IUD horns. The K_d value of PR_n was 0.89 $\pm$ 0.10 nmol/l in the control horn and was 0.89 $\pm$ 0.06 nmol/l in the IUD horn.

The effect of IUD on the PR_c concentration during estrous cycle was investigated. The concentration of PR_c in IUD horn was significantly lower ($P<0.05$) than that of the control horn at all stages of estrous cycle. The pattern of variation of the receptor level during estrous cycle were similar in both the control and IUD horns. Minimum PR_c was observed at metestrus and the maximum level was at estrus. The effect of IUD on progesterone receptor induced systhesis by estrogen

and on its translocation was observed in ovariectomized rats. It was shown that, in unprimed ovariectomized rat uterus, the PR_c in control horn was significantly ($P<0.05$) higher than that of the IUD horn. In estrogen-primed ovariectomized rats, the PR_c in both the control and IUD horns was similarly increased about 1 to 2 - fold. The amount of PR_c in the IUD horn was still about 60-70% to that of the control horn. Translocation of progesterone receptor from the cytoplasm into nucleus in the IUD horn was 10% lower than that of the control horn which is still in the variation limit of progesterone receptor measurement. It is therefore not yet conclusive whether IUD really lowers translocation of PR_c into the uterine nuclei.

A study with sucrose gradient centrifugation demonstrated that the sedimentation coefficient of PR_c in both the control and the IUD horns was the same at 4S when compared to that of the BSA (4.6S) either ^{3}H-progesterone or ^{3}H-ORG 2058 was used as the specific binding ligand.

All these results indicated that IUD had no effect on binding affinity and sedimentation property of progesterone receptor. It significantly caused a decrease in progesterone receptor concentration of which the underlying mechanism is not yet available from present data. The reduction of progesterone receptor in the presence of an IUD however may alter the uterine sensitivity to progesterone which probably contribute to the contraceptive effect by causing failure of blastocyst implantation on Day 5.

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ABBREVIATIONS

BSA	Bovine serum albumin
CBG	Cortisol binding globulin
DNA	Deoxyribonucleic acid
E_2	17β- estradiol
FSH	Follicle stimulating hormone
G_nRH	Gonadotropin releasing hormone
IUD	Intrauterine device
K_d	Dissociation constant
LH	Luteinizing hormone
P	Progesterone
PR_c	Cytosolic progesterone receptor
PR_n	Nuclear progesterone receptor
S.E.M.	Standard error of mean

CHAPTER I

INTRODUCTION

The intrauterine device (IUD) provides an effective mean of contraception. It has the advantage of low cost, ease of use, and reversibility to normal fertility after removal of the device. The most frequent side effects associated with the presence of an IUD are bleeding and pain. Pelvic inflammatory disease is relatively rare and perforation of the uterus is even rarer (1). The IUD used by women are made of plain plastic, copper wire or IUD coated with certain chemicals (2). Many investigators have tried to modify the IUD by adding a pharmacologically active agent to the inert device in an effort to reduce its side effects and to increase its efficacy. There are only two types of medicated IUD which have been more extensively tested in women, namely the copper releasing IUD and the progestagen releasing IUD (3).

Effect of IUD on reproductive process

Previous observations from experimental animals indicated that an IUD had different mechanism of action on different spicies. However, a general conclusion might be drawn that the physical presence of IUD blocks certain stages of fertilization, or it may induce some drastic biochemical changes which interfere with blastocyst implantation and embryonic development. In the sheep, for example, IUD prevented fertilization by affecting sperm transport mechanism (4), and it also seemed to stimulate phagocytosis and / or cytolysis of the sperm (5). In the rabbit, the presence of an IUD was associated with the increase of prostaglandin $F_{1\alpha}$ and $F_{2\alpha}$ in the uterus (6) which

were believed to be harmful to the survival of blastocyst and embryo (7, 8) *in utero*. The IUD also affected ovarian function, such as inhibiting ovulation in Indian water buffaloes (9). There were indications that an IUD may interfere with the development of copora lutea in sheep , guinea pig , cattle , goat and pig (10, 11, 12). In addition, the IUD caused the leucocytic infiltration in the endometrium of several species including human (11). It is widely believed that the infiltrating macrophages from inflammation or their lysate eventually destroyed the sperm or blastocyst (13, 14, 15). Prevention of blastocyst implantation by IUD was observed in many species such as mouse (16), rat (17, 18, 19, 20), and human (21, 22). Batta and Chaudhury (18) proposed that there was liberation of some active antifertility substances which prevented blastocyst implantation in rats. They observed the effect of uterine anastomosis on the action of an IUD in the rat (19). Normally, the rat has a bicornuate uterus and the contraceptive action of an IUD is strictly localized at the IUD containing horn (19). When the uterine lumens were connected , however, bilateral contraceptive effect was observed. There were many more evidences showing that IUD caused alterations in the morphology and physiology of the endometrium in rat and human (15, 23, 24, 25, 26). The abnormality of the endometrium might be the reason which made implantation impossible (24, 25). Webb (27) investigated the effect of copper IUD in the rat and concluded that copper from the copper IUD prevented the development from the morula stage to the blastocyst.

Extensive research on the biochemical changes due to the presence of an IUD in the uterus were done. In women fitted with Lippes loop, there was a marked increase in total protein and non-

protein nitrogen levels of the uterine fluid (28). Similar observation was found in the rat fitted with a silk-suture IUD by Yaovapolkul (29) and Jantaraniyom (30). In addition, the presence of a silk-suture IUD in the rat uterus was associated with the increase of DNA , RNA contents (30) ; inorganic phosphate , Ca^{2+} (29) ; several enzymes (31, 32) and amino acids(33). Yaovapolkul (29) demonstrated that when injected uterine fluid obtained from the IUD fitted horn of the rat into the right uterine horn, and the uterine fluid from the control horn was injected into the left horn of a Day 4 pregnant rat , implantation was inhibited in the right horn while normal implantation was allowed to occure in the left horn. She further studied the active substances in the uterine fluid which may be responsible for this action and concluded that the contraceptive effect of the IUD fluid might reside on a high molecular weight protein. In order to be functionally active , this protein seemed to require inorganic phosphate as well. Phlummanus (34) further supported this result and indicated that specific non-covalent binding between inorganic phosphate and some proteins could mediate the antifertility effect of the IUD by interacting with the smooth muscle of the uterus to make it unsuitable for implantation and normal development of the fetuses. Jantaraniyom (30) demonstrated that the proteins from the IUD and control uterine fluids were different both qualitatively and quantitatively. Her studies suggested that a newly synthesized protein and possibly the lack of some pre-existing proteins may be responsible for the anti-implantation activity of IUD and the contraceptive action. Ghosh , Roy and Kar (35) reported that, in ovariectomized rat, copper IUD could influence the sensitivity of uterus to ovarian hormones as revealed by the uptake of labelled steroids. They observed that estradiol uptake was significantly higher

in the contralateral control horn when compared to that of the IUD horn. On the contary , uterus sensitivity to progesterone was more in the IUD horn than the control horn. The effect of progesterone on the morphology of endometrium was observed from women using either a de - vice which release different amount of progesterone or an inactive placebo device (26). They found that progesterone released from the IUD affected the uterus similarly to that reported in women using a combined oral contraceptive pill. The IUD with a high progesterone releasing rate gave a high frequency of depressed endometria with atrophy of the glands and a diffuse decidual reaction of the stroma (26). Seshadri , et al (36) also found that when progesterone capsule was implanted into one uterine horn of the mature rabbit, while the other horn recieved placebo , progesterone released from the capsule completely inhibited implantation at the horn that progesterone capsule was implanted. Janne and Ylostalo (21) indicated that progesterone released from the IUD has a depressive action on the estrogen and progesterone receptor levels which may contribute to the contraceptive effectiveness of the IUD.

Although many observations on the action of an IUD were carried out leading to various hypothesis, sometimes even contradictory , the precise mechanism whereby the IUD produces it contraceptive effect is still not clearly understood. It seems very likely that complex mechanisms resulted from many biological events might ultimately lead to contraception.

Ovarian hormones and maintenance of ovarian cycle and pregnancy

As we generally know, estrogen and progesterone have an important role in regulating ovarian cycle and pregnancy. In the rat, the

estrous cycle is similar to the menstrual cycle in women. It is four to five days in lenght and is devided into four stages. namely: proestrus, estrus, metestrus and diestrus (37). The estrous cycle, as well as the menstrual cycle, is regulated by hormones from the hypothalamus, the pituitary and the ovary. Gonadotropin releasing hormone (G_nRH) from the hypothalamus stimulates the anterior pituitary to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates the growth and maturation of immature follicles and prepares them for ovulation. LH acts synergistically with FSH to cause ovulation of mature follicles as well as secretion of estrogen (38) . After ovulation, progesterone is secreted from the corpus luteum. If pregnancy does not occure, the corpus luteum regresses and serum progesterone level falls while serum estrogen increases. This will initiate another round of estrous cycle. When pregnency occures, the corpus luteum does not regress but continues to secrete progesterone, and it is believed that prolactin is the major luteotropic stimulus which transforms an estrous or menstrual cycle into a pregnancy by prolonging progesterone secretion from the corpus luteum(37). Progesterone plays an essential part in all stages of pregnancy. Its functions are to prepare uterus for blastocyst implantation, to maintain pregnancy and to stimulate mammary development. In the rat, progesterone influences placental growth since treatment of castrated pregnant rats with progesterone induces placental hypertrophy (39, 40). In addition, pregnant mice which were ovariectomized 3 days after mating could maintain pregnancy by progesterone treatment (41). Bennett, et al (42) observed that progesterone is necessary during a prolonged delay in nidation to maintain the viability of unimplanted ova and the uterine receptivity for implantation. Although how progesterone prepares the uterus

for implantation is unclear, there are evidences that, in the rat, uterine preparation for stromal mitosis just 24 hours before implantation and decidualization on Day 5 of pregnancy requires a precise temporal exposure to progesterone and estrogen (42, 43). The uterus must first be exposed to progesterone for at least 48 hours, followed by 17β- estradiol released between 20.00 hours of Day 3 and 04.00 hours of Day 4 of pregnancy (44). Only after this sequence will a blastocyst induce the decidual response (44). In animals which implantation is delayed by continuous injection of progesterone, implantation of the blastocyst can occure 24 hours after an injection of estradiol (44). Davies and Ryan (45) suggested that the modulation of progesterone concentration in target cells may be influenced not only by plasma concentration but by changes in receptor protein activity. Studies with the rat and rabbit have established that a relationship exists between the uterine progesterone concentration and the continuation of gestation. In the pregnant rat, myometrial progesterone binding sites increased from Day 3-4, and then the concentration of the binding sites decreased to a low value on Day 15 until term (46).

Mechanism of action of progesterone

The mechanism of progesterone action in target cell is illustrated in Figure 1 (47). The first step of hormone action is the binding of the hormone molecule to its specific receptor. When progesterone enters the target cell, probably by simple diffusion, it binds to the progesterone specific receptor in the cytoplasm. This is followed by a change in the conformation of the hormone-receptor complex which is then translocated into the nucleus and binds with specific acceptor sites in the nucleus and induces transcription of specific proteins.

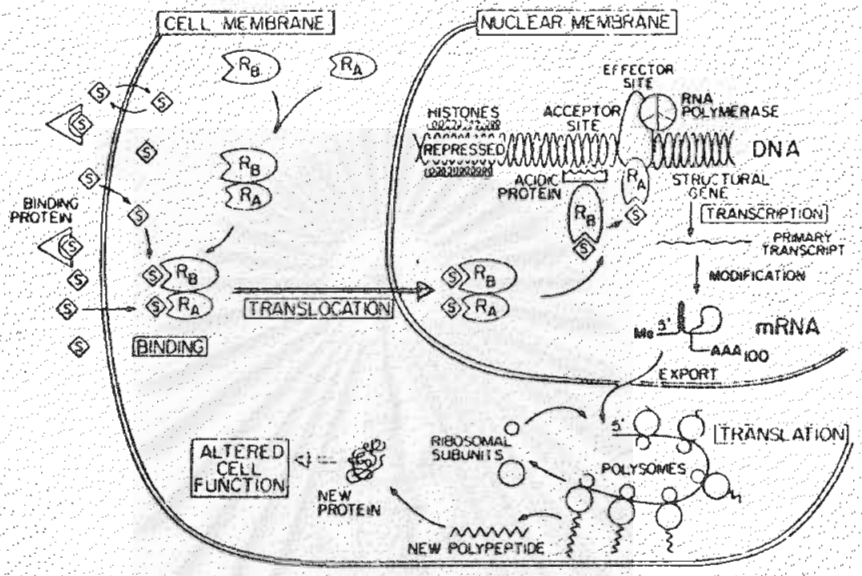

Figure 1 Proposed model of the mechanism of action of progesterone in target cell ; based upon chick oviduct. In a target cell , the intact 6S dimer receptor molecule binds two molecule of hormone , forming a complex then enters the nucleus and become attached to the chromatin. The complex stimulates the transcription of particular genes , so that RNA encoding the information in those genes is synthesized. On the ribosome , the RNA is translated into proteins.

R_A = receptor A subunit , R_B = receptor B subunit , S = steroid

(O ' Malley , B.W. , et al , 1977 ; (47))

Thus the presence of the hormone receptors will reflect the responsiveness of the target cell to the hormone. Non-target cell will contain none or only a few molecules of its specific receptor.

The properties of purified progesterone receptor have been investigated in many species and in general they are quite similar. The properties of chick oviduct progesterone receptor was extensively reviewed by O'Malley and his coworkers (47,48). Progesterone receptor is a protein with a molecular weight of about 200,000 daltons. The intact receptor molecule is a dimer in which each subunit is a different protein of quite similar size. Each subunit has a single specific binding site which binds tightly to progesterone. The dissociation constant (K_d) of the hormone-receptor complex is between 10^{-8}- 10^{-9} mol/l (48). The subunit are roughly cigar-shaped ; when they associate together as a dimer they probably lie side by side. Progesterone receptor is a heat-labile molecule. The sedimentation coefficient of each subunit is 4S. In a dimeric form , it sediments at 6S. It may undergo salt-dependent aggregation into a 8S form (48). During the estrous cycle , the variation in uterine progesterone receptor levels were correlated with the pattern of estrogen and progesterone secretion (49). The progesterone receptor synthesis was induced by estrogen and a negative control may be exerted by progesterone itself (50). In maximal sensitized mammalian cell the progesterone receptor is about 40,000 molecules per cell (51). In serum and uterine cytosol of rats , there was another non-specific , low affinity and high capacity progesterone binder. This progesterone binder is a cortisol binding globulin (CBG)-like protein. It sediments at 4-5S and is heat stable , and can bind to other steroids such as cortisol (49). Thus , to determine the progesterone receptor it is necessary to eliminate the interference of

this CBG-like protein from binding to progesterone. In this study, excess hydrocortisone was added to all assay tubes to eliminate such effect.

Research aim

This thesis is aimed at investigating any possible correlation between the contraceptive effect of IUD and progesterone action in the uterus. In this investigation, I postulate a hypothesis that in rat uterus, IUD may alter the uterine sensitivity to progesterone and thus leads to failure of blastocyst implantation. Such change may resulted from 3 possibilities.

1. The IUD may affect the concentration of progesterone receptor in rat uterus.

2. The IUD may affect some physicochemical properties of progesterone receptor and causes change in the behavior of the receptor.

3. The IUD may affect both the concentration and physicochemical properties of progesterone receptor.

In an attempt to test this hypothesis, the binding capacity to progesterone receptor and the dissociation constant (K_d) of the progesterone receptor were determined by labelling the receptor with radioactive progesterone and analysed from scatchard plot (52). The sedimentation property of the progesterone receptor was determined by sucrose density gradient centrifugation.

During the course of my study , Myatt, et al (53) had published their results on the alteration of uterine cytosolic and nuclear progesterone receptors during estrous cycle and early pregnancy of rat bearing a silk-suture IUD. They found that the dissociation constant of PR_c and PR_n for IUD and control uterine horns were similar. The PR_c concentration in the IUD horn was always lower than that of the control

horn but varied in a similar pattern during estrous cycle. The pattern of PR_n in IUD horn was in reversal with the result observed in the control horn. The PR_n concentrations were not significantly different between both horns during early pregnancy.

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Animals

Virgin female albino rats of the Charles Foster strain, aged between 3-4 months and weigh between 250-300 grams were used. They were obtained from The Department of Physiology, Faculty of Medicine, Chulalongkorn University ; and propagated at The Department of Biochemistry, Faculty of Science, Chulalongkorn University. The animals were kept at room temperature (28-35 ^{o}C) and were exposed to natural light and darkness. Food (rat chow ; product of Kasetsart University) and water were available ad libitum. Cytology of the vaginal smears were daily examined under light microscope at 10x10 magnification during 8:00 - 9:00 AM. Only those animals with at least two successive normal estrous cycle were used in the experiments.

The rats were divided into two groups :

Group I : Normal female rats fitted with a silk-suture intrauterine device at the right uterine horn. The contralateral horn served as control horn.

Group II : Ovariectomized rats fitted with a silk-suture intrauterine device at the right uterine horn.

2. Chemicals

All chemicals were obtained commercially and were of reagent grade or better.

[1,2,6,7-^{3}H] Progesterone (specific activity 3.22 TBq/mmol) and ^{3}H-ORG 2058 (16α-ethyl-21-hydroxy-19-nor 6,7-^{3}H pregn-4-ene-3, 20-dione ; specific activity 1.55 TBq/mmol) were perchased from Amersham International Limited. Stocks of the radioisotopes were kept in absolute ethanol at 4 ^{o}C. They were prepared by passing stream of nitrogen gas until the commercially obtained radioisotopes in toluene were dried and then redissolved in absolute ethanol.

Progesterone ; hydrocortisone and 17β-estradiol were products of BDH Chemical Limited. Monothioglyceral ; activated chacoal (Norit A) ; 2,5-Diphenyloxazole (PPO ; reagent grade) and 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP ; reagent geade) were obtained from Sigma Chemical Company. Dextran T-70 was from Pharmacia Fine Chemicals ; Toluene (analytical grade) from Mallinckrodt, Inc. ; crystalline sucrose (density gradient grade) from Schwarz / Mann Division of Becton, Dickinson and Company ; crystalline bovine serum albumin and calf thymus DNA (Type I grade) were also from Sigma Chemical Company.

Phenol Reagent (Folin - Ciocalteu Reagent) was prepared in our laboratory by the method of Folin and Ciocalteu (52).

The sources of other special chemicals and reagents will be specified in the text.

B.METHODS

1. Insertion of silk - suture IUD and ovariectomy

Under ether (anesthetic grade ; May and Baker Limited) anesthesia, a mid - ventral laparotomy was performed. The surgical procedure was carried out under full aseptic precautions. Silk-suture (number 5/0) was inserted into the anterior third of the right horn uterus at estrus stage by the method described by Doyle and Margolis

(17) and is illustrated in Figure 2. The left horn was sham operated by passing a needle and the suture thread through the uterine lumen. This uterine horn will serve as control horn. Animals were rested for at least 15 days before further used in the investigation. Subsequent vaginal smears showed that these rats still retained normal estrous cycle.

Ovariectomy of both horns was performed just before the animals were inserted with an IUD. To ensure complete removal of the ovaries, vaginal smear was examined. Those showing entirely leucocytes in vaginal cytology were allowed 2-3 weeks rest and were used for studies of the effect of IUD on nuclear progesterone receptor and the effect of IUD on progesterone receptor responsiveness to estrogen administration.

2. Preparation of cytosolic receptors

Preparation of the cytosolic receptor is essentially the method described by Feil , et al (49). The animals were killed by heavy ether anesthesis and the IUD and control uteri were removed immediately and separately immerged into ice-cold 0.9% ($^{W}/_{V}$) NaCl solution. Further steps would be carried out at 4 ^{o}C unless otherwise specified. Fat and mesenteries were trimmed off. The uteri were then blotted, weighed, finely chopped with scissors and homogenized in a motor-driven homogenizer with three 10 seconds-bursts in 2 ml per uterus of Tris-EDTA buffer (50 mM Tris-HCl , 10 mM EDTA-disodium , 12 mM monothioglyceral containing 30% $^{V}/_{V}$ glycerol, pH 7.4) The homogenate was centrifuged in L8-70 Beckman Ultracentrifuge (Beckman Instruments, Inc.) at 150,000xg for 60 minutes in a SW 50.1 rotor. The supernatant was collected and used for the determination of the number of receptor sites and dissociation constant of cytosolic progesterone receptors as inter-

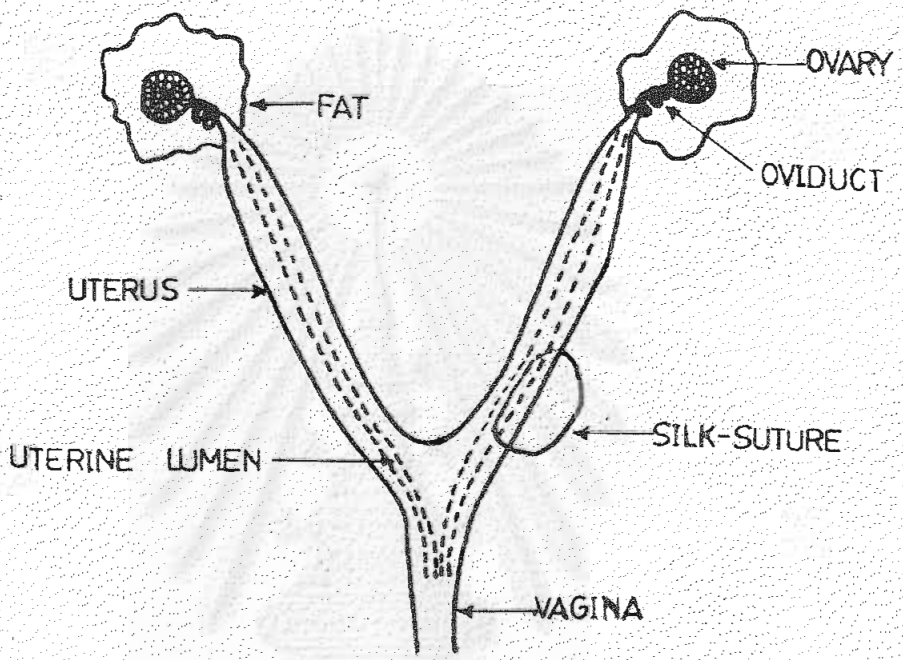

FIGURE 2 DIAGRAMATIC REPRESENTATION OF THE RAT UTERI WITH AN INTRAUTERINE DEVICE

preted from Scatchard analysis (52).

3. Preparation of nuclear receptors

Ovariectomized rats were divided into 3 groups and were subjected to different treatment.

Group A : Animals were not treated with any hormones.

Group B : Animals were treated with 17β-estradiol for 2 days.

Group C : Animals were treated with 17β-estradiol for 2 days following by a single dose of progesterone on the third day, and were killed one hour later.

Hormone treatment prior to analysis of receptors was described by Vu Hai and Milgrom (55). They were firstly primed for 2 days with 17β-estradiol (5 μg in 0.25 ml sesame oil per day) by subcutaneous injection. On the third day, the rats recieved another subcutaneuos injection of progesterone (1 mg in 0.25 ml sesame oil) and were quickly killed one hour later by heavy ether anesthesia. The IUD and control uterine horns were removed immediately and homogenized as described previously in the preparation of cytosolic receptor by the method of Milgrom , et al (50). The uterine homogenate in Tris-EDTA buffer was centrifuged at 1000xg for 15 minutes at 4 °C with Beckmam J-21 Centrifuge (Beckman Instruments , Inc.) to yield a crude nuclear pellet. The crude pellet was further washed twice with 3 ml of ice-cold Tris-EDTA buffer and then was devided into two parts : one part was used for DNA determination ; the other part was used for the studies of nuclear progesterone receptor. The latter part of the pellet was extracted for 30 minutes at 4 ml of Tris-EDTA buffer containing 0.4 M KCl. The extract was recentrifuged at 150,000xg for 60 minutes at 4 °C in L8-70 Beckman Ultracentrifuge using SW 50.1 rotor. The supernatant was collected and

would be used for nuclear receptor assay. The supernatant obtained from previous centrifugation at 1,000xg was recentrifuged at 150,000xg for 60 minutes at 4 °C (50). The clear supernatant was used for cytosolic receptor assay.

Both the nuclear and cytosolic progesterone receptors were analysed for number of binding sites and dissociation constant by Scatchard analysis (52).

4. Assay of progesterone receptor

The measurement of PR_c was done by the modified method of Kurl and Borthwick (56). The PR_n measurement was done by the modified methods of Vu Hai and Milgrom (55) , and Walters and Clark (57). Since progesterone receptor was unstable and a heat labile molecule , so in this study the assay of nuclear progesterone receptor was carried out at 4 °C. Many investigator had demonstrated that the exchange of endogeneous progesterone with ^{3}H-progesterone could occur even at low temperature (0-4 °C) (55, 56, 57, 58).

Duplicate samples of 100 µl (containing approximately 4-8 mg protein per milliliter) cytosol or nuclear fractions were incubated at 4 °C for 16-18 hours with 50 µl of ^{3}H-progesterone in Tris-EDTA buffer containing ethanol not more than 10% of final volume (concentration ranging between 0.2 - 2.0 nmol/l) , with or without a 200-fold excess of unlabelled progesterone (5 µl). The sample with excess unlabelled progesterone was for the assay of non-specific hormone binding proteins. To all incubation tubes , 5 µl of 100-fold excess of hydrocortisone was added for 10 minutes prior to incubation with ^{3}H-progesterone. After incubation , 200 µl ice-cold dextran-coated chacoal suspension (0.5% Norit A , 0.05% dextran T-70 in Tris-EDTA buffer pH 7.4

with 30% $^{v}/_{v}$ glycerol) were added to remove the unbound steroid. The suspension was briefly agitated and was incubated at 4 °C for an additional 10 minutes before centrifugation at 1000xg for 10 minutes at 4 °C in a Beckman J-21 Centrifuge (Beckman Instruments, Inc.). The clear supernatant was decanted into scintillation vials containing 5 ml toluene scintillation fluid (0.3% PPO , 0.01% POPOP and 33.3% Triton X-100 in Toluene ; (59)) and the amount of radioactivity was measured in a Packard PRIAS Model PL Liquid Scintillation Counter (Packard Instrument Company , Inc.). Counting efficiency of the system is 35-40 % as calibrated by using commercially available external ^{3}H standard (Packard Instrument Company , Inc.).

Protein was determined by the method of Lowry, et al (60) with crystalline bovine serum albumin serving as standard. DNA concentration was determined by the method of Giles and Myers (61) using calf thymus DNA stored in 10 % perchloric acid as standard.

5. Sedimentation analysis

Sucrose gradient preparation

Four milliliters of 5-20% $^{w}/_{w}$ sucrose gradient in Tris-EDTA buffer containing 10 % $^{v}/_{v}$ glycerol were performed by hand-layering four sucrose solution with increasing concentration (5, 10, 15, 20%) into a 5 ml polyallomer tube. These four sucrose concentrations were prepared according to the dilution chart (see Appendix I). The layers were allowed to diffuse at room temperature for 1 hour before being used. The gradient prepared by this method was a linear gradient (see Appendix II).

Sample preparation

The sample for sedimentation analysis were prepared from uteri

of rats at estrus stage. The 150,000xg cytosol receptor was prepared according to the process described in the preparation of cytosolic receptor. The samples were diluted to approximately 7-10 mg/ml protein with Tris-EDTA buffer which contained only 10% $^{v}/_{v}$ glycerol (62). The samples must be pretreated before layering onto the gradient following the method of Toft and Sherman (63). Two hundred of the cytosol solution was preincubated with a 100-fold excess (1 μmol/l at final concentration) of hydrocortisone for 10 minutes at 4 °C ; and then incubated with either 2.0 pmol ^{3}H-progesterone or ^{3}H-ORG 2058 for 2 hours at 4 °C. The unbound steroid was removed by incubation with 200 μl of dextran-coated charcoal suspension (0.5% Norit A , 0.05% dextran T-70 in Tris-EDTA buffer , pH 7.4 , containing 10% $^{v}/_{v}$ glycerol) for 10 minutes at 4 °C. The suspension was centrifuged at 1000xg for 10 minutes at 4 °C in a Beckman J-21 Centrifuge and the clear supernatant was then ready for sedimentation studies.

Sedimentation of receptors in sucrose gradient

Two hundred microliters of the pretreated cytosol solution were applied onto the sucrose gradient and centrifuged in a L8-70 Beckman Ultracentrifuge using a SW 50.1 rotor at 115,000xg , 4 °C for 16 hours. At the end of the centrifugation , fractions were collected dropwise from the bottom of the tube (approximately 200 μl per fraction). About 26-28 fractions were obtained. All fractions were assayed for radioactivity in 5 ml of toluene scintillation fluid (0.3% PPO , 0.01% POPOP , 33.3% Triton X-100 in Toluene,(59)) with a Packard PRIAS Model PL liquid scintillation counter with 35-38% counting efficiency of the system.

The sedimentation of the migrated steroid-binding components was compared with that of crystalline BSA solution (10 mg/ml) cen-

trifuged in a parallel gradient. The migration of protein in the gradient was determined by measuring absorbancy at 260 and 280 nm in a Beckman Model 25 Spectrophotometer (Beckman Instruments , Inc.).

6. Statistical analysis

The procedure for evaluating the significant of difference is the Student 's t-test. To judge whether the observed difference is significant , 95% is used as level of significance. In this study , the hypothesis was tested both by comparision between two population means and between pair (see Appendix III).

CHAPTER III

RESULTS

1 Effect of IUD on cytosolic progesterone receptor concentration during estrous cycle

The concentration of cytosolic progesterone receptor (PR_c) was analysed from Scatchard plot. An example of the Scatchard plot of PR_c was shown in Figure 3.

The concentration of PR_c during estrous cycle in the control and IUD horns is shown in Table 1. The concentration is given as the concentration of binding sites per mg protein , per mg DNA and per uterine horn. The result clearly demonstrated that at all stages of estrous cycle , there was a significant decrease in the PR_c level in the IUD horn. It must be noted here , however , that at estrus and diestrus , the amount of PR_c when expressed as femtomol per uterine horn in the IUD horn was significantly ($P<0.05$) less than that of the control horn only when tested by the paired comparison test. They were not different from the control when tested by the two population mean. The differences in the PR_c levels between the control and IUD horns during the four stages of estrous cycle are more clearly demonstrated in Figure 4 and 5. The variation of the receptor level during estrous cycle follows the same pattern in IUD and control horns. Minimum PR_c was observed at metestrus. The receptor concentration then gradually increased during diestrus and proestrus to reach maximum at estrus , followed by a sharp decline in metestrus. It also shows here that the amount of progesterone receptor in the IUD horn were about 57-65% , 50-67% and 64-82% that of the control horn when the concen-

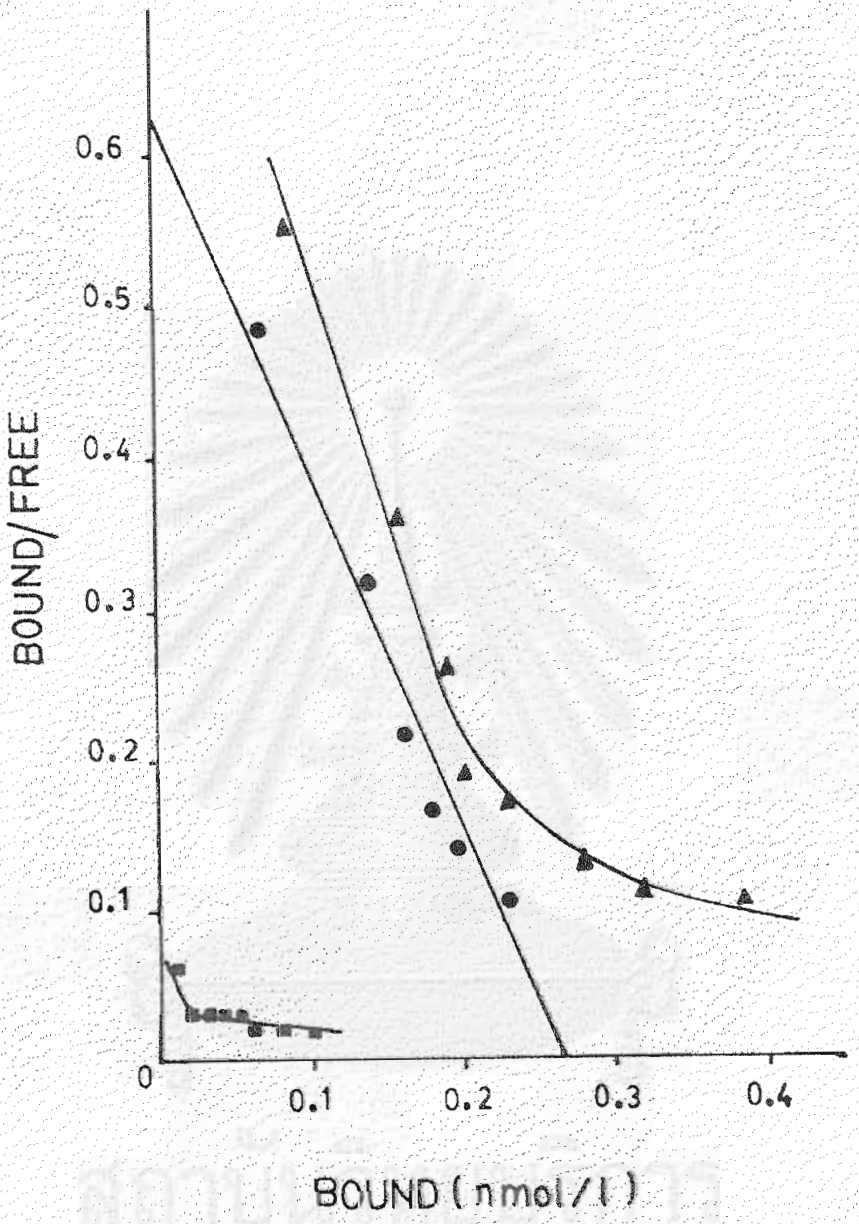

Figure 3 Scatchard plot of cytosolic progesterone receptor. The detailed procedure was as described in the assay of progesterone receptor. The number of progesterone binding site was obtained from X-intercept and K_d was estimated from the slope.

▲—▲ Total binding

●—● Specific binding

■—■ Non-specific binding

Table 1 The concentrations of cytosolic progesterone receptor in control and IUD horns of the rat during the four stages of estrous cycle.

Stages of estrous cycle	Number of experiment	Concentration of cytosolic progesterone receptor								
		fmol / mg protein			fmol / mg DNA			fmol / uterine horn		
		CONTROL	IUD	P	CONTROL	IUD	P	CONTROL	IUD	P
Proestrus	6	29.4±2.3	17.3±1.9	P<0.05	733.4±116.7	420.1± 70.8	P<0.05	536.0± 36.1	408.6±52.9	P<0.05 *
Estrus	6	37.0±3.1	24.2±2.4	P<0.05	1254.7±205.4	708.4±110.6	P<0.05	636.5± 63.1	522.3±62.2	P<0.05
Metestrus	6	14.2±0.8	8.1±0.3	P<0.05	296.4± 42.6	153.1± 15.8	P<0.05	230.4± 34.8	146.7±18.4	P<0.05 *
Diestrus	6	18.0±2.5	11.4±1.7	P<0.05	353.2± 42.7	236.3± 35.1	P<0.05	258.2± 33.9	208.8±34.1	P<0.05

Results are given as the mean ± standard error of mean (S.E.M.) . Two rats were used per experiment. P value was calculated from the Student 's t test. Significant difference was tested by two population means and paired comparison test.

P^{*} = P value is less than 0.05 only when tested by paired comparison test.

P = P value is less than 0.05 when tested by both methods.

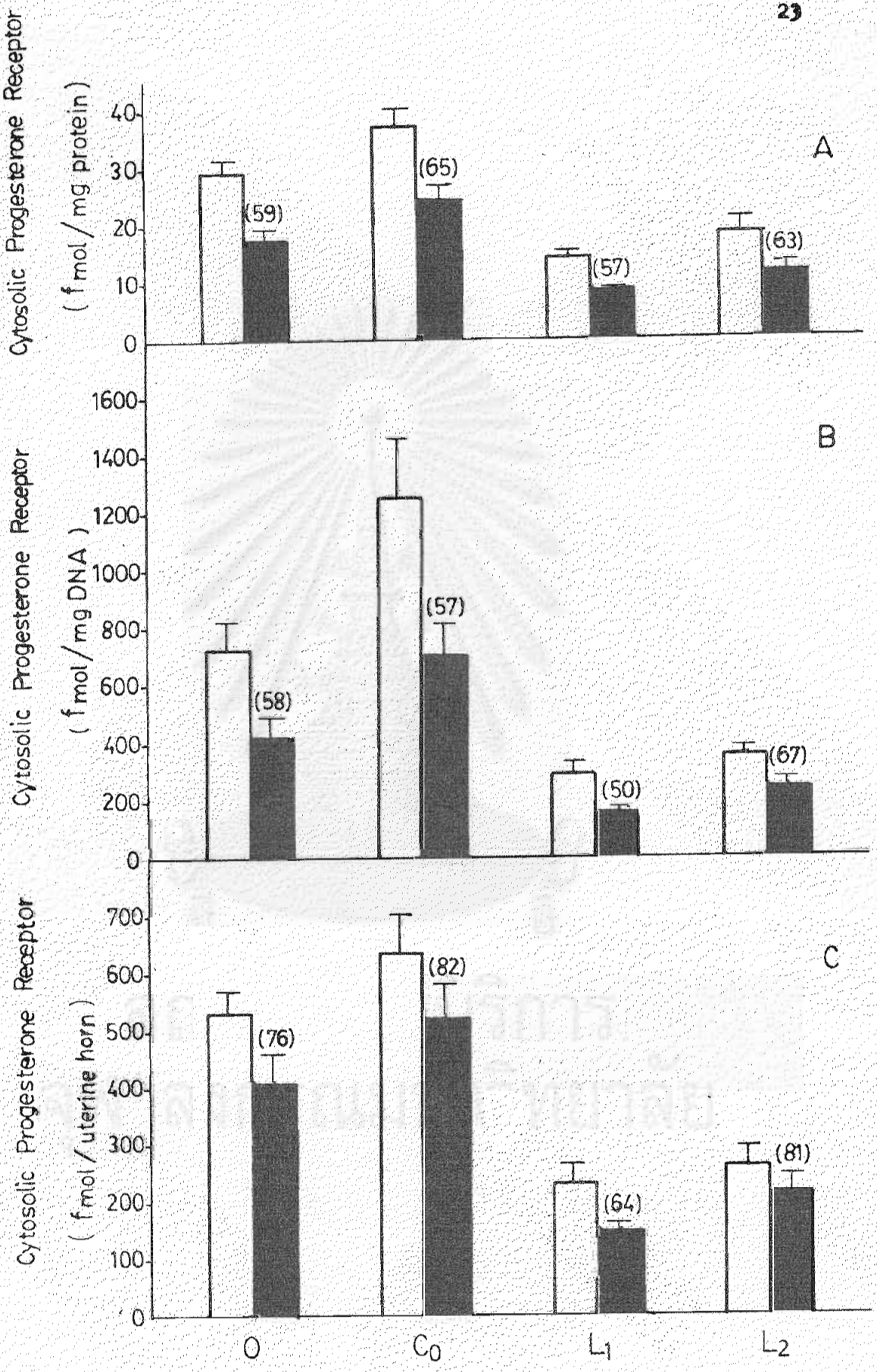
Cytosolic Progesterone Receptor
(f mol / mg protein)
A
(59)
(65)
(57)
(63)
Cytosolic Progesterone Receptor
(f mol / mg DNA)
B
(58)
(57)
(50)
(67)
Cytosolic Progesterone Receptor
(f mol / uterine horn)
C
(76)
(82)
(64)
(81)
O
C0
L1
L2
Stages of Estrous Cycle

Figure 4

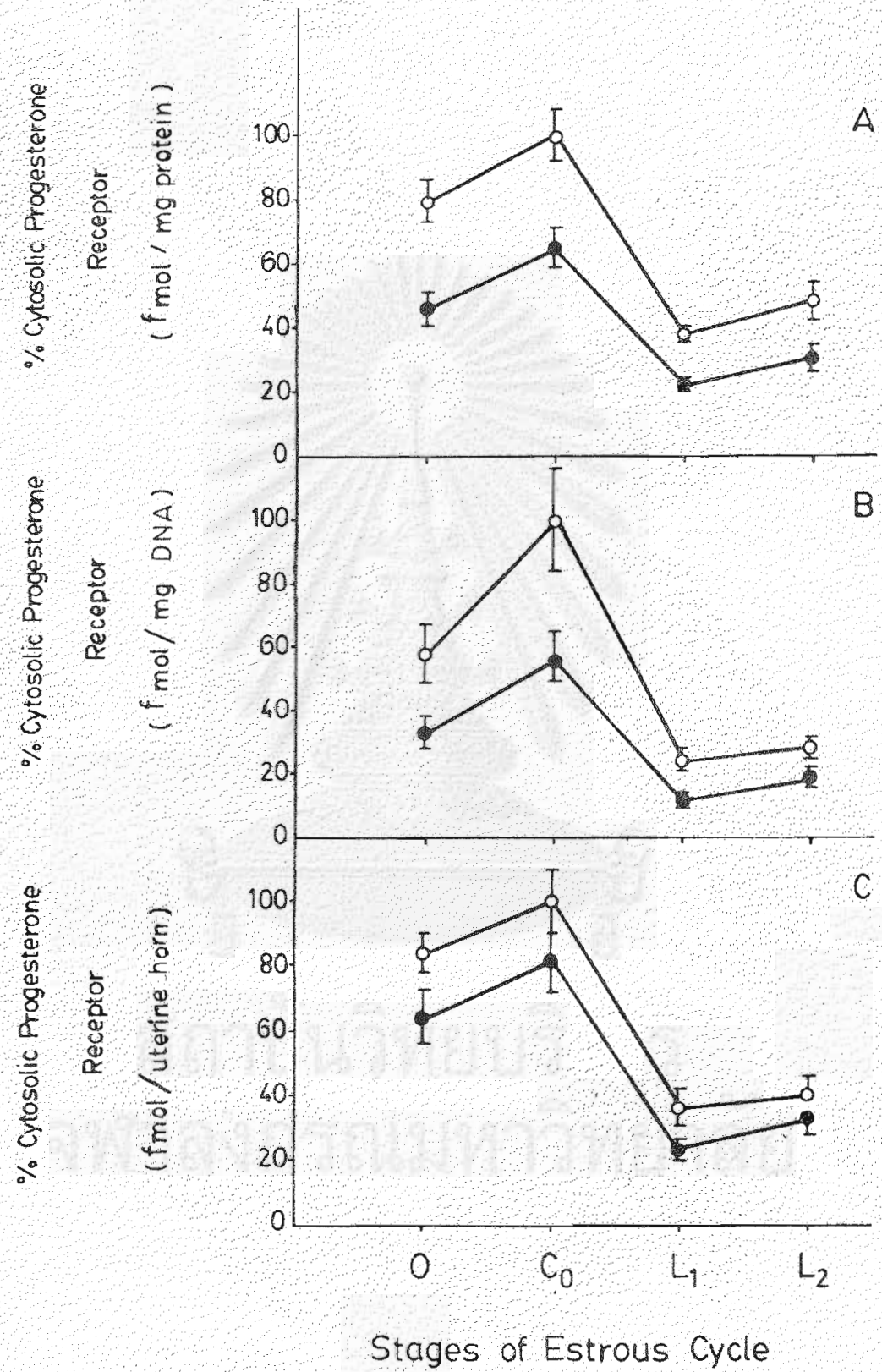
% Cytosolic Progesterone Receptor (f mol / mg protein)
% Cytosolic Progesterone Receptor (f mol / mg DNA)
% Cytosolic Progesterone Receptor (f mol / uterine horn)
A
B
C
100
80
60
40
20
0
O
C0
L1
L2
Stages of Estrous Cycle

Figure 5

tration of the receptor was presented as per mg protein , per mg DNA and per uterine horn , respectively (Figure 4A , B , C). The highest difference was observed at the metestrus (Figure 4A , B , C) , and the least difference was during the diestrus and estrus in Figure 4A and 4C. In Figure 4B the least difference was during diestrus. Figure 5 shows the percentage of the PR_c at various stages of the estrous cycle of both horns as compared to the maximum stage at estrus of the control horn. Larger differences in variation between the control and the IUD horns were observed if the concentration was calculated on per milligram DNA basis (Figure 5B).

2 Effect of IUD on progesterone receptor binding to hormone during estrous cycle

In order to study whether IUD had any effect on the binding affinity of progesterone receptor , the dissociation constant (K_d) of both cytosolic and nuclear progesterone receptor (PR_c and PR_n) were determined by incubating the receptor containing solution with varying amount of ^3H-progesterone (0.2 - 2.0 nmol/l) and then analysed the result from the Scatchard plot (52).

2.1 Dissociation constant of PR_c

The K_d of PR_c of the control and IUD horns were determined at all four stages of estrous cycle. Table 2 shows that the K_d values of the receptor in the control and the IUD horns were similar whether they were compared in the same stage or between stages of estrous cycle. The mean K_d of cytosolic progesterone receptor was 0.59 ± 0.03 nmol/l in the control horn and was 0.56 ± 0.02 nmol/l in the IUD horn (Table 2).

Table 2 The dissociation constant (K_d) of cytosolic progesterone receptor in control and IUD horns of the rats during the four stages of estrous cycle.

Stages of estrous cycle	Number of experiment	Dissociation constant (K_d) of cytosolic progesterone receptor (nmol/l)		
		CONTROL	IUD	P
Proestrus	6	0.60 ± 0.15	0.53 ± 0.15	NS
Estrus	6	0.59 ± 0.19	0.59 ± 0.19	NS
Metestrus	6	0.58 ± 0.12	0.61 ± 0.08	NS
Diestrus	6	0.60 ± 0.15	0.59 ± 0.11	NS
$\bar{X}$ ± S.E.M.	-	0.59 ± 0.03	0.56 ± 0.02	NS

Results are given as the menas ± S.E.M. (2 rats were used per experiment) P value was calculated from Student ' s t test and the significant difference was tested by both two population means and paired comparison test.

NS = not significant

2.2 Dissociation constant of PR_n

The K_d of PR_n in normal rat was determined by the method similar to that of the cytosolic receptor. Since the receptor concentration in this experiment was very low (5.28 fmol/mg protein for control horn and 3.72 fmol/mg protein for IUD horn) , that the K_d could not be suitably determined from the Scatchard plot. In order to prevent endogeneous progesterone from binding to the PR_n and therefore masked the observation , the K_d of PR_n was determined from ovariectomized rats. The rats were firstly primed with 17β-estradiol for 2 days. On the third day , the animals were killed after 1 hour of progesterone injection and determined for the K_d values of PR_n in the control and the IUD horns. The K_d of PR_n in the control horn was 0.89 ± 0.10 nmol/l and 0.89 ± 0.06 nmol/l in the IUD horn (Table 3). The affinity of PR_n was less than that of PR_c , however.

3 Effect of IUD on induction of progesterone receptor synthesis by estrogen

Since the synthesis of progesterone receptor was stimulated by estrogen (49, 50) , so in this study I attempted to observe whether IUD had any effect on this process.

To eliminate any effect of endogenous ovarian hormones and to reduce the concentration of progesterone receptors to its basal level so that clearly changes in the level of the receptor , if any , may be detected , ovariectomized rats were used in the following studies.

3.1 Response of cytosolic progesterone receptor

After ovariectomy (Group A rats) , the concentration of PR_c in both the control and IUD horns dropped markedly to about the

Table 3 The dissociation constant (K_d) of nuclear progesterone receptor in control and IUD horns in ovariectomized rats.

Experimental number	Dissociation constant (K_d) of nuclear progesterone receptor (nmol/l)		
	CONTROL	IUD	P
1	0.94	0.90	-
2	1.00	0.94	-
3	0.83	0.94	-
4	1.03	0.91	-
5	0.88	0.80	-
6	0.79	0.78	-
7	0.76	0.93	-
$\bar{X} \pm$ S.E.M.	0.89 $\pm$ 0.01	0.89 $\pm$ 0.06	NS

The rats were primed for 2 days with 17β-estradiol and the K_d of the nuclear progesterone receptor was determined on the third day , 1 hour after progesterone injection Experimental condition was described in method . The results are expressed as the mean $\pm$ S.E.M. and P value was calculated from Student ' s t-test. Significant difference was tested by two population menas and the paired comparison test.

NS = not significant

level observed in metestrus of normal rat. The result is shown in Table 4. The concentration of PR_c in the IUD horn was significantly ($P<0.05$) less than that of the control horn. Figure 6 shows that the progesterone receptor concentration in the IUD horn is about 58% , 61% and 63% that of the control horn when the concentration of the receptor was expressed as per mg protein , per mg DNA and per uterine horn , respectively. Ovariectomized rats (Group B rats) treated with 17β-estradiol alone for 2 days prior to killing showed about 1-fold increase in the PR_c in both the control and the IUD horns when the concentration was calculated as per mg protein and per mg DNA. A greater increased of about 2-fold was observed when the receptor concentration was per uterine horn (Table 4). In this condition , the amount of receptor in the IUD horn was still significantly less than that of the control horn. The amount of PR_c expressed as per mg protein , per mg DNA and per uterine horn in the IUD horn was respectively about 61% , 70% and 65% that of the control horn (Figure 6). In ovariectomized rat , estrogen primed plus progesterone-treated rats (Group C) , injection of progesterone 1 hour prior to killing on the third day caused a decrease in the PR_c in both the control and the IUD horns , when compared to rats of Group B , which was only treated with estrogen (Table 4). The result also indicated that receptor concentration of this group fell to about the same level in both the control and the IUD horns. However , the progesterone mediated decrease in the receptor level was always greater in the control horn than the IUD horn whether observed on the basis of per mg protein (59%) , per mg DNA (52%) , or per uterine horn (40%) (Figure 6). In addition , it must be noted here that the reduction in the receptor was more in both horns when the concentration was given as per uterine horn. Moreover , the

Table 4 The concentration of cytosolic progesterone receptor in the control and IUD horns of ovariectomized rats.

Group of rats	Hormonal treatment	Number of experiments	Concentration of cytosolic progesterone receptor								
			f mol / mg protein			f mol / mg DNA			f mol / uterine horn		
			CONTROL	IUD	P	CONTROL	IUD	P	CONTROL	IUD	P
A	None	3	12.0±2.3	7.0±0.4	P<0.05	111.0±24.5	67.5± 0.7	P<0.05	87.1± 5.3	55.3±14.8	P<0.05
B	E_2	3	23.2±1.2	14.2±2.7	P<0.05	225.9±34.9	157.7±24.1	P<0.05	235.8±30.3	153.2±23.8	P<0.05
C	E_2 + P	7	10.1±0.4	8.9±0.5	NS	110.8± 7.6	103.1±11.3	NS	67.7± 3.6	62.9± 5.0	NS

The cytosolic progesterone receptor concentration was measured on the 15 th day after ovariectomy. The condition of hormone treatment was previously described in Methods. Results are given as the means ± S.E.M. (3 rats were used per experiment). P value was calculated from the Student 's t test. Significant difference was tested by two population means and the paired comparison test.

NS = not significant , E_2 = 17β - estradiol , P = progesterone

Figure 6 Effect of estrogen and progesterone administration on the concentration of cytosolic progesterone receptor in control (□) and IUD (■) horns of the ovariectomized rats. The receptor concentration was expressed as fmol/mg protein (A) , fmol/mg DNA (B) and fmol/uterine horn (C). The untreated and E_2 bars represent the mean ± S.E.M. from 3 experiments. The number in the bracket is the percent amount of cytosolic progesterone receptor in the IUD horn when compared to that of the control horn at the same condition.

E_2 = 17β-estradiol , P = Progesterone

(Data derived from Table 4)

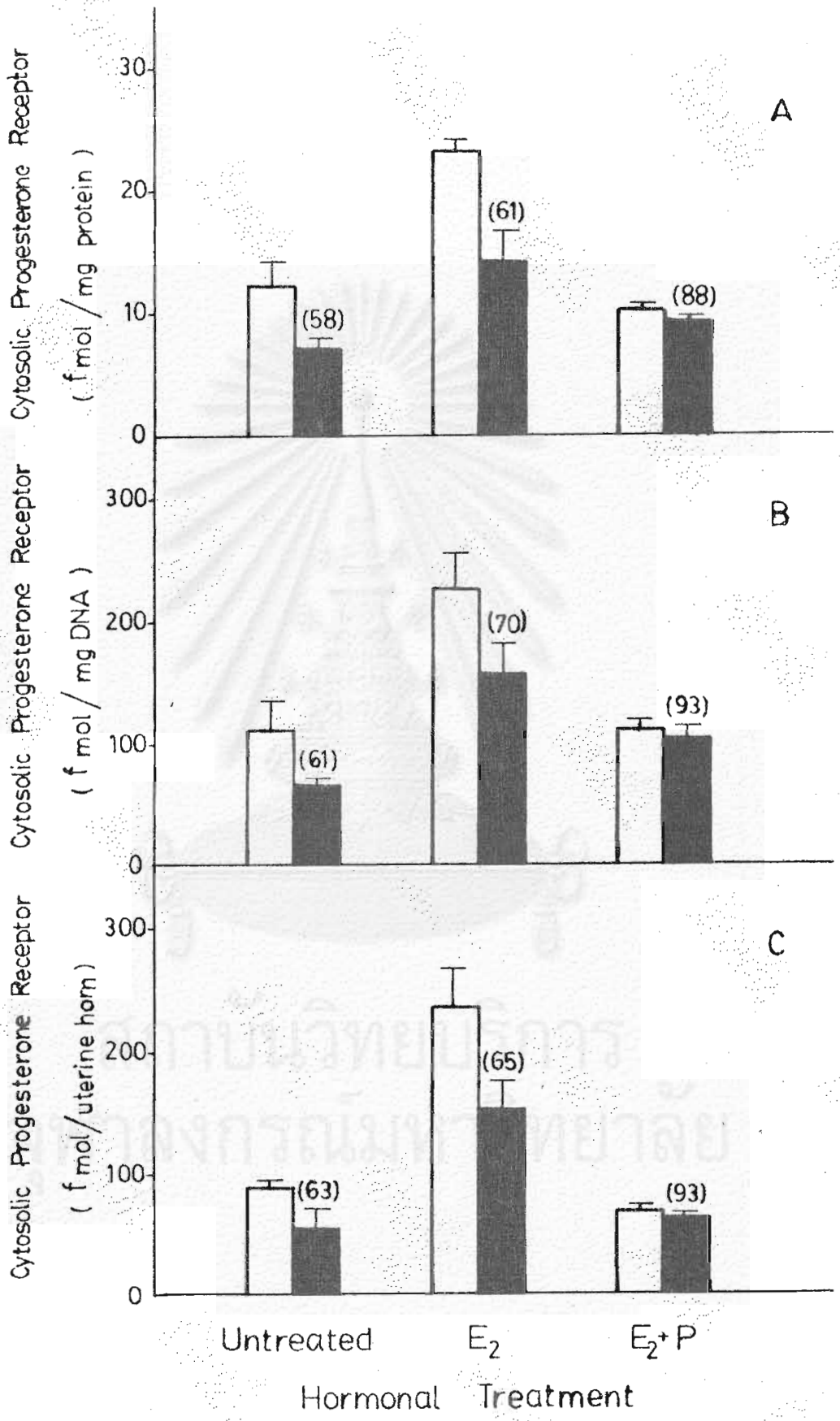
Cytosolic Progesterone Receptor
(f mol / mg protein)
30
20
10
0
A
(58)
(61)
(88)
Cytosolic Progesterone Receptor
(f mol / mg DNA)
300
200
100
0
B
(61)
(70)
(93)
Cytosolic Progesterone Receptor
(f mol/uterine horn)
300
200
100
0
C
(63)
(65)
(93)
Untreated
E2
E2+P
Hormonal Treatment

Figure 6

level of receptor in the IUD horns were still significantly higher than the basal level observed in Group A rats except when the concentration was per uterine horn. In the control horn , the treatment of progesterone brought the receptor down to the same level as that of non-hormonal treated-ovariectomized rats again with exception when the concentration was per uterine horn.

3.2 Nuclear progesterone receptor

The concentration of nuclear progesterone receptor was analysed from Scatchard plot. An example of the Scatchard plot of PR_n was shown in Figure 7.

Since the control and the IUD horns of Group C rats showed a considerable decreased in the cytosolic progesterone receptor concentration when compared with that of the Group B rats , it was interesting to test whether the decreased level was due to transport of cytosolic progesterone receptor into the nucleus. The concentration of the PR_n was thus measured in Group C rats. The result is shown in Table 5. The PR_n in the control horn was significantly ($P<0.05$) higher than that of the IUD horn. The amount of PR_n in the IUD horn was about 60% that of the control horn whether the concentration of receptor was expressed as per mg protein , per mg DNA or per uterine horn. In addition , it must be noted here that the PR_c in the IUD horn was 56% , 35% and 36% mobilized into the nucleus when the concentration of receptor was expressed as per mg protein , per mg DNA and per uterine horn , respectively. In the control horn , the mobilization of the PR_c into the nucleus was 64% , 45% and 45% when the concentration of receptor was expressed as per mg protein , per mg DNA and per uterine horn , respectively. Figure 8 summarized the effect of progesterone administration on cytosolic and nuclear receptors in the control and

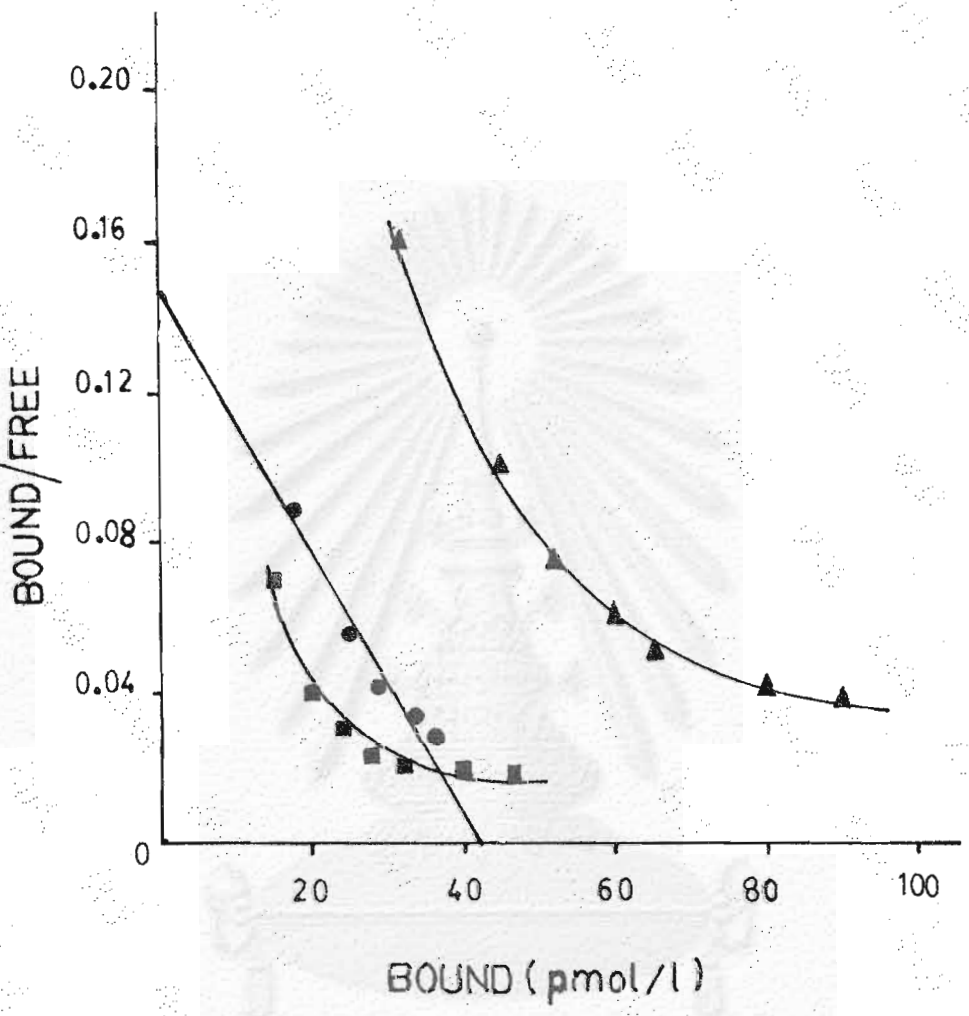

Figure 7 Scatchard plot of nuclear progesterone receptor. The detailed procedured was as described in the assay of progesterone receptor. The number of progesterone binding site was obtained from X-intercept and K_d was estimated from the slope.

▲—▲ Total binding

○—○ Specific binding

■—■ Non-specific binding

Table 5 The concentration of nuclear progesterone receptor in control and IUD horns of ovariectomized rats.

Experimental number	Nuclear progesterone receptor concentration								
	fmol / mg protein			fmol / mg DNA			fmol / uterine horn		
	CONTROL	IUD	P	CONTROL	IUD	P	CONTROL	IUD	P
1	21.18	10.00	-	146.43	69.15	-	73.80	34.85	-
2	26.82	15.91	-	109.26	66.67	-	94.40	56.00	-
3	17.27	13.08	-	87.00	56.33	-	43.07	38.53	-
4	11.43	6.45	-	65.73	43.01	-	35.20	21.00	-
5	17.69	10.00	-	69.17	45.71	-	55.20	33.60	-
6	19.33	15.00	-	89.64	68.52	-	53.65	38.85	-
7	14.61	8.75	-	60.32	46.85	-	36.10	27.30	-
$\bar{X}$ ± S.E.M.	18.3±1.7	11.3±1.2	P<0.05	89.6±10.6	56.6±4.0	P<0.05	55.9±7.5	35.7±3.8	P<0.05

The animals were primed for two days with 17β-estradiol (5 μg in 0.25 ml sesame oil). On day 3 , after 1 hour of progesterone (1 mg in 0.25 ml sesame oil) injection, the rats were killed and assayed for the nuclear progesterone receptors as described in Methods. (3 rats were used per experiment) P value was calculated from the Student's t test. Significant difference was tested by two population means and the paired comparison test.

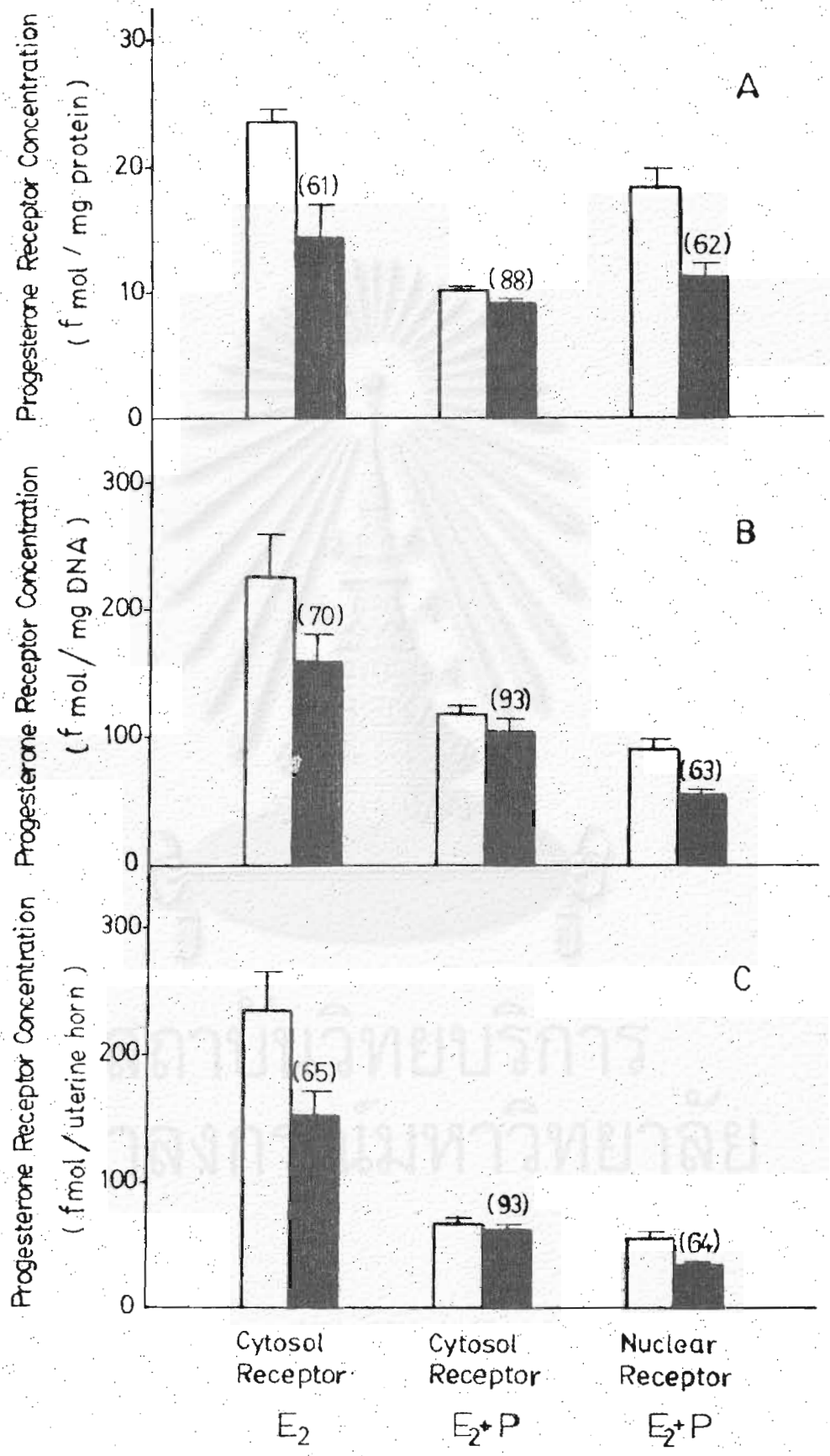
Progesterone Receptor Concentration
(f mol / mg protein)
30
20
10
0
A
(61)
(88)
(62)
Progesterone Receptor Concentration
(f mol / mg DNA)
300
200
100
0
B
(70)
(93)
(63)
Progesterone Receptor Concentration
(f mol / uterine horn)
300
200
100
0
C
(65)
(93)
(64)
Cytosol Receptor
E2
Cytosol Receptor
E2+P
Nuclear Receptor
E2+P

Figure 8

IUD horns. The data was derived from Table 4 and 5.

4 Effect of IUD on sedimentation property of cytosolic progesterone receptor

Earlier experiments suggested that IUD reduces the concentration of PR_c , but does not cause any change in the affinity of the receptor to its specific hormone. It is interesting to observe further whether IUD induces any changes in the sedimentation coefficient of the PR_c.

I firstly attempted to use ^{3}H-progesterone as the specific binding ligand of the PR_c from rat uterus at estrus. The sedimentation profiles of both the control and IUD uterine preparations through 5-20% sucrose density gradient were illustrated in Figure 9A. Both the control and the IUD preparations showed only a small 4S peak of the hormone-protein complex. As the sedimentation peaks obtained in this experiment were very small. I further performed another experiment using ^{3}H-ORG 2058 as the binding ligand since this synthetic progesterone binds with the receptor better than ^{3}H-progesterone and it does not bind with corticosteroid binding protein (53). The result was shown in Figure 9B. It still showed that the hormone-bound protein from the control and IUD cytosols had the same sedimentation coefficient at 4S. The peak of the receptor detected by using ^{3}H-ORG 2058 , however , was more prominent and sharper.

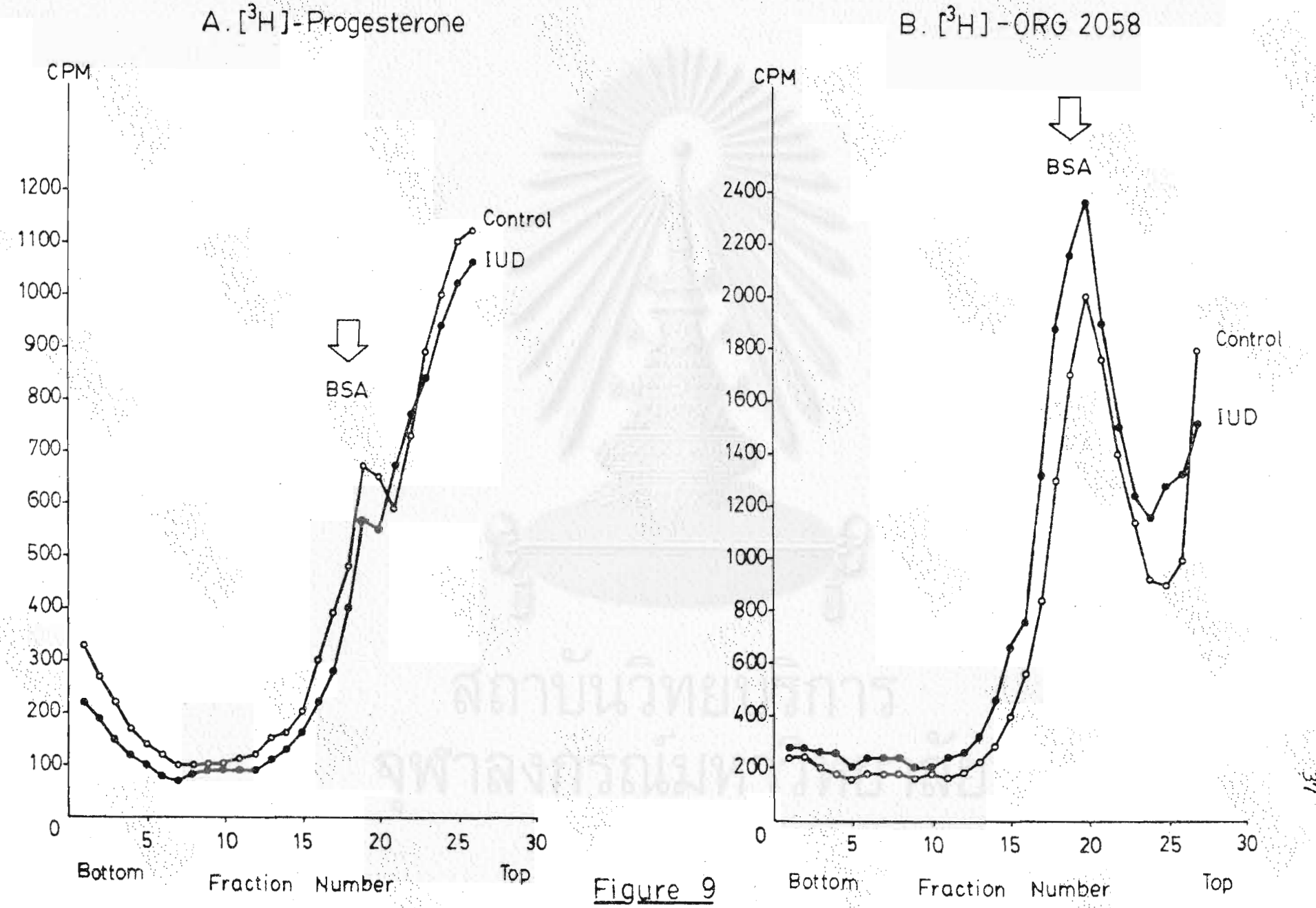
A. [3H]-Progesterone
CPM
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Control
IUD
BSA
5
10
15
20
25
30
Bottom
Fraction Number
Top
B. [3H]-ORG 2058
CPM
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
BSA
Control
IUD
5
10
15
20
25
30
Bottom
Fraction Number
Top

Figure 9

DISCUSSION

The important of hormonal influences on embryonic development and implantation has long been recognized. It has been shown that the implantation of blastocysts in rat requires preparing of the uterus with estrogen and progesterone (42, 43, 44). Since it was found that IUD prevents blastocyst implantation in rat (17, 18, 19, 20) , I proposed here that one of the mechanisms by which an IUD may act is due to its influence on the sensitivity of uterus to ovarian hormones and consequently impairs the implantation processes. It is now well established that the actions of progesterone and estrogen on target tissue are mediated by the binding of these hormones to their specific receptors (57, 64) and the concentration of hormone receptor in the target tissue will reflect the responsiveness of that tissue to the hormone. Hence , in this study I measured and compared the progesterone receptor between the control and IUD horns.

The reduction of PR_c level in the uterine endometrium caused by the presence of an IUD was observed in women by Janne and Ylostalo (21). They reported that the amounts of both estrogen and progesterone receptors in the uterine endometrium were significantly reduced in women having a progesterone releasing IUD. The levels of cytosolic estrogen and progesterone receptors measured from these women with an IUD represented only about 20% of those of normal women. It should be noted that since they did not measure the nuclear receptor levels , the reported reduction of steroid receptors might not present the true picture of the influence of an IUD and therefore the mobilization of progesterone receptor from cytoplasm into nucleus was not included in their

consideration. My study also confirm the markedly reduction of PR_c in the IUD-bearing uterine horn of the rat. The PR_c in the IUD horn of the rat was significantly ($P<0.05$) lower than that of the control horn at all four stages of estrous cycle (Table 1). This may be resulted from a) reduced synthesis of progesterone receptor in the IUD horn or b) increased mobilization of PR_c into nucleus in the IUD horn without any alteration in synthetic events. As there are indications that progesterone receptor synthesis is induced by estrogen in the rat uterus (49, 55) and in this organ of many other organisms (50, 65, 66) it is resonable to test the first criterion by comparing the estrogenic induction of progesterone receptor between the control and IUD horns. In untreated ovariectomized rats , (Group A , Table 4) , the concentration of PR_c in IUD horn was approximately 38% lower than that of the control horn. In the estrogen-primed ovariectomized rats (Group B , Table 4) the concentration of PR_c in both the control and the IUD horns increased about 1-2 fold of the Group A rats (Table 4 and Figure 6). This clearly demonstrated that estrogen does induce synthesis of progesterone receptor and the degree of induction was similar in both the control and the IUD horns. However , the PR_c level in the IUD horn was 30-40% less than that of the control horn (Group B, Table 4).

The second criterion of IUD action was tested by observing the concentrations of cytosolic and nuclear progesterone receptors in the control and IUD horns of estrogen and progesterone-treated ovariectomized rats (Group C rats). It was shown that the concentration of PR_c in both control and IUD horns of the ovariectomized rats (Group C, Table 4) was similar when treated with both estrogen and progesterone. When the PR_c of Group B (E_2 primed) and Group C (E_2+P primed) was

compared (Figure 6) , it was shown that the translocation of PR_c into the nucleus in IUD horn was about 10% lower than that of the control horn. This 10% difference observed was within the higher variation limit of analysis (4-10%) , therefore my result does not strongly indicate that the IUD caused a slight decrease in the translocation of progesterone receptor from cytoplasm into the nucleus. Since my study suggested that both the IUD and control horns responed to estrogen at the same extent and IUD might not stimulate translocation of progesterone receptor from cytoplasm into nucleus , the reason for observing lower progesterone receptor in the IUD horn still remains unanswered. There were some reports in mice (67) and women (15, 24) that the presence of an IUD caused cell proliferation and changes in the morphology of uterine endometrium. I also observed that the IUD horn contained about 1.3 fold more of both protein and DNA comparing to the control horn. Taking into account of these evidences , the lower amount of progesterone receptor observed in the IUD horn might be explained by the assumption that the proliferated cells in the IUD horn were not the target cells for estrogen and progesterone. Base on this assumption the increased cell population would thus increase the value of the denominator and thus lower the calculated amount of progesterone receptor either expressed as femtomol per mg protein or femtomol per mg DNA in the IUD horn. However , there is no direct evidence that the proliferated cells in the IUD horn are non-target cells for estrogen and progesterone , thus the above explanation is only a suggestive possibility which needs further investigation. I must also point out that the turnover rate of progesterone receptor in both uterine horns are not drtermined. It is interesting , therefore , to study also the turnover rate of progesterone receptor in the IUD horn which may help

to provide more understanding on the effect of IUD on progesterone receptor.

Sometimes in my study , I observed that at estrus the IUD horn released uterine fluid slightly later than that of the control horn although the rat 's estrous cycle appeared normal by vaginal smear. Reduction of progesterone receptor in IUD horn may be responsible for the observed delay by causing low steroid response and therefore altered the physiology of uterus during estrous cycle in that horn. Although the PR_c level was always lower in the IUD horn , the pattern in which the PR_c level fluctuated during the estrous cycle was similar in both horns (Figure 5). The result was supported by the result of Myatt , et al (53) , and it agreed with the observation that the rat 's estrous cycle was still normal with the presence of an IUD although the IUD horn may reach each stage a little later. Smith, et al (68) reported that plasma estrogen gradually increased in the evening of diestrus to reach maximum on the morning of proestrus and then declined on the evening of proestrus to reach baseline at estrus. There was another small peak of estrogen during the evening of metestrus to the early morning of diestrus. Circulating progesterone had one prominent peak on the afternoon of proestrus and declined quite sharply on the evening to reach baseline at the morning of estrus. There was also another minor peak of progesterone in the evening of metestrus which overlaped with the small peak of estrogen (68). Theoretically , in accordance with the patterns of estrogen and progesterone in the plasma , the maximum progesterone receptor should be reached in the late morning of proestrus and become minimum at estrus or metestrus. In the present study , maximum progesterone receptor was observed at estrus which was the time when plasma estrogen and proges-

terone was low (68). This result was in contrast with that reported by Myatt , et al (53) and Vu Hai , et al (69). They observed that the maximum value of PR_c was at proestrus which corresponded with the plasma estrogen and progesterone levels at that time. My result showed minimum PR_c level was at metestrus which was the same as that reported by Myatt , et al (53) and Vu Hai , et al (69). The receptor level was corresponded with the observed decrease in plasma estrogen and progesterone levels at that time (68, 70). It must be noted here that the amount of progesterone receptor measured in this investigation were lower than that reported in rat uterus of other strains such as Wistar rats (53) and Spraque-Dawley rats (69).

Upon the finding that IUD reduces the amount of progesterone receptor during estrous cycle , we may relate such finding to the contraceptive action of an IUD during pregnancy. In pregnancy , uterine preparation for stromal mitosis before implantation and decidualization requires a precise temporal exposure to progesterone and estrogen (43, 71, 72). In the rats , the uterus must first be exposed to progesterone for at least 48 hours follow by estradiol released during the evening of Day 3 to the early morning of Day 4 (43). The timing of implantation is precise and if blastocyst did not implant during 12.00 hour of Day 5 in the rat , it was no longer able to implant later (73). In the present study , I have demonstrated that the PR_c (Table 1) and the PR_n (Table 5) contents were less in the IUD horn. These may cause some delay in responsiveness to progesterone which make the uteus refractory to blastocyst implantation on Day 5. Chikusu , et al (74) reported that the PR_n level in rat uterine myometrium increased from Day 2-3 to reach maximum at Day 5 which was the day of blastocyst implantation. A relationship between desidual regression and decline in

progesterone receptor concentration was reported by Peleg , et al (75). Myatt , et al (53) found that PR_c in IUD horn of the rat fell on the evening of Day 5 and the morning of Day 6. They suggested that this may be a reason of failure to decidualization in this horn : delay responsiveness of the uterus to hormone influences results in the delay of receptivity of the uterus for blastocyst implantation.

Similar K_d of PR_c in the IUD and control horns throughout the four stages of estrous cycle was demonstrated (Table 2). The K_d of PR_n in the IUD horn was also the same as that of the control horn (Table 3). The result was similar with that reported by Myatt , et al (53). It indicated that the silk-suture IUD had no effect on the hormone binding affinity of PR_c and PR_n. The possibility that IUD may induce physical changes in the receptor molecule was also investigated by comparing the sedimentation property of PR_c between control and IUD horns. My result suggested that IUD had not induced any change in the molecular size , and probably also the conformation of the receptor molecule , since the PR_c in the control and IUD horns both sedimented at 4S (Figure 9) when using BSA (4.6S) as the molecular weight marker. In this study , I cannot obtain the 6S peak in the sedimentation profile. The receptors observed were the monomeric 4S subunits. So any aggregation , if any , of the progesterone receptor which associated with the IUD cannot be observed here. It is interesting to observe further whether the IUD has any effect on the aggregation of progesterone receptor which may lead to alter the receptor molecule.

In conclusion , my study showed that the IUD had no effect on the binding affinity and the sedimentation property of the progesterone receptor. It probably has an effect on the concentration of progesterone receptor by reducing its concentration in the uterus. The de-

crease of progesterone receptor in the presence of an IUD may alter the needed physiology of the uterine endometrium. This factor rendered the uterus to become less sensitive to hormone and consequently to blastocyst implantation.

This investigation bring us to understand more in the mechanism of action of an IUD which is important for improving and increasing the efficacy of an IUD. It also provides us some understanding about the role of progesterone in reproduction.

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APPENDIX

Appendix I Sucrose Dilution Chart for % ($^{W}/_{W}$) Concentration

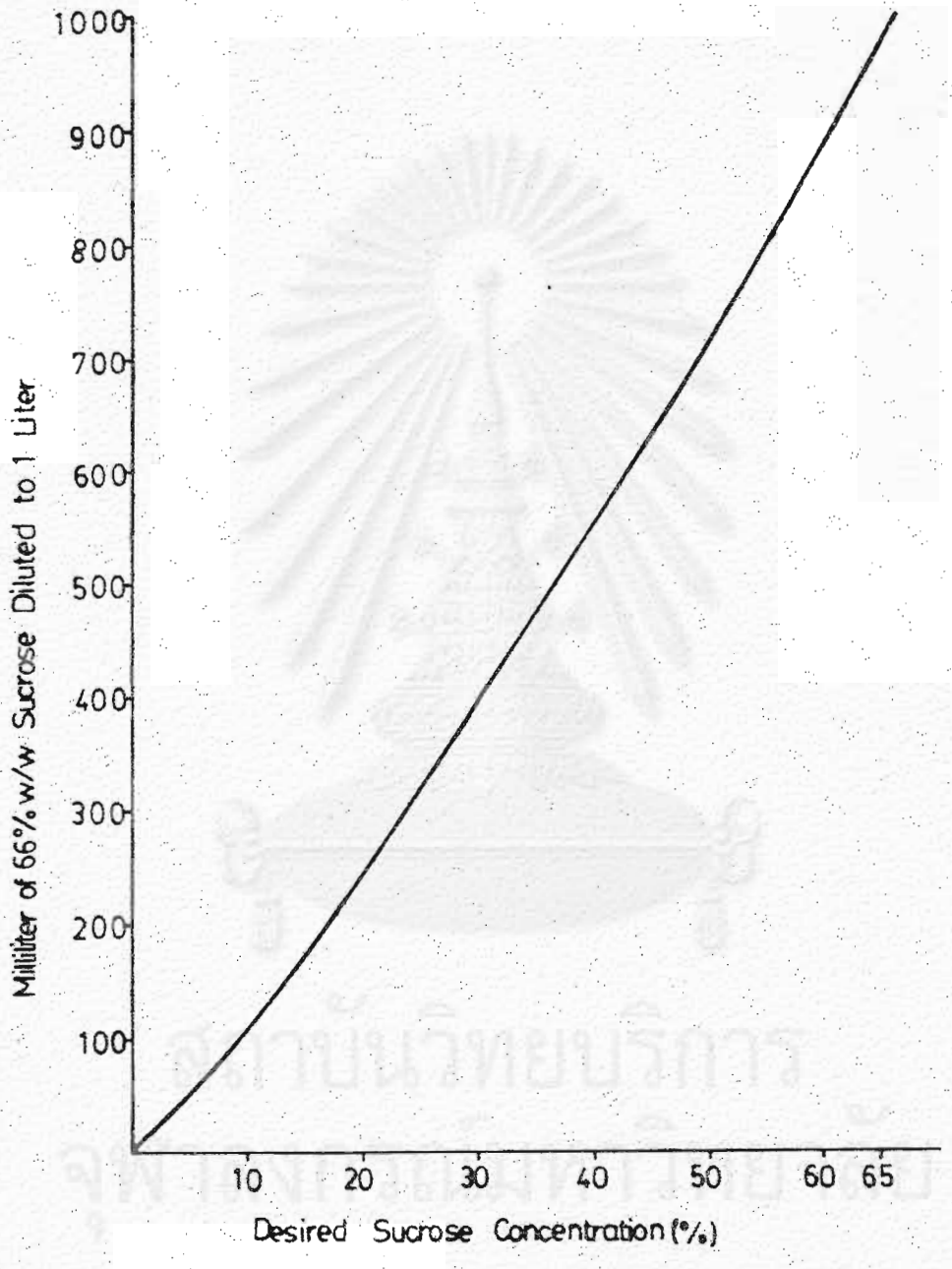

Figure 10 Sucrose dilution chart for % ($^{W}/_{W}$) concentrations. This chart is used for making the desired sucrose concentration (in % $^{W}/_{W}$) from the 66% ($^{W}/_{W}$) stock sucrose solution.

(Griffin , C.M. ; (62))

Appendix II Linear Gradient of 5-20 % ($^{W}/_{W}$) Sucrose

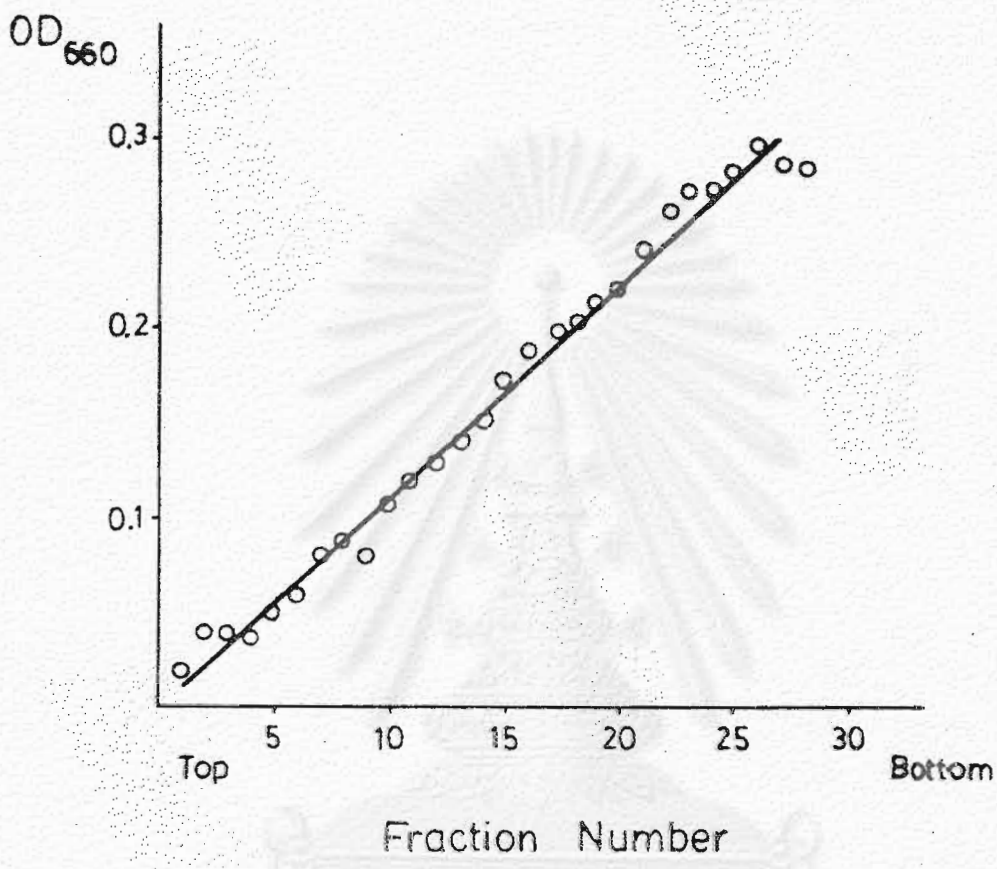

Figure 11 A manually layer linear gradient of 5-20% $^{W}/_{W}$ sucrose. The gradient was performed by hand-layering four sucrose solution with increasing concentrations (5, 10, 15, 20) containing 20 mg% methylene blue into a 5 ml polyallomer tube. The tube was allow to stand for 1 hour at room temperature. It was then punctured at the bottom and fractions were collected dropwise at approximately 200 µl per fraction. All fractions were diluted to 1 ml with distilled water and absorbancy was measured at 660 nm in a Beckman Model 25 spectrophotometer (Beckman Instrument , Inc.).

Appendix III Formulae of Student 's t-Test by Two Population Means Comparison and Paired Comparison

a) The formula for Student 's t-test between two population means (76) is :

$$t = \frac{(\bar{X}_1 - \bar{X}_2) - (\mu_1 - \mu_2)}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

$\bar{X}$ = The sample mean

S = Standard deviation

μ = The population variance

n = The number of samples

b) The formula for paired comparison test (76) is :

$$t = \frac{\bar{d} - \mu_d}{S_{\bar{d}}}$$

$$\bar{d} = \frac{d_i}{n}$$

$$d_i = X_2 - X_1$$

$$\mu_d = \mu_2 - \mu_1$$

$$S_{\bar{d}} = \frac{S_d}{\sqrt{n}}$$

$$S_d = \frac{n\Sigma d_1^2 - (\Sigma d_i)^2}{n(n-1)}$$

BIOGRAPHY

Miss Chawiwan Apisitpaisarn was born on April 5 , 1955 in Pattani , Thailand. She graduated with the Bachelor degree of Science in General Science from the Faculty of Science , Chulalongkorn University in 1979.