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 originally 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 (28-30°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 10 x 10 magnification during 8:00-9:00 AM. Only those animals with at least two successive normal estrous cycle were used in the experiments.

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2. Chemicals

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

[5,6,8,9,11,12,14,15-^{3}H] arachidonic acid in toluene (specific activity 4.85 TBq/mmol) was purchased from Amersham International Limited. Stocks of the radioisotopes were kept in absolute ethanol at 4°C in small aliquots. They were prepared by passing stream of

nitrogen gas until the commercially obtained radioisotope in toluene were dried and then redissolved in absolute ethanol.

2,5-Diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyl-oxazolyl) benzene (POPOP) were products of Sigma Chemical Company. Naphthalene (scintillation grade) and Silica Gel G (type 60; for thin-layer chromatography) were obtained from Merck Company. Ethylene glycol (analytical grade) was bought from J.T. Baker Chemical Company; p-dioxane from May and Baker Limited. L-epinephrine and polyoxyethylen-sorbitan-monopalmitate (Tween 40) were from Fluka AG, Chemische Febrik; reduced glutathione from BDH Chemical Limited. Arachidonic acid (purity 90%), prostaglandin E_1, prostaglandin E_2, prostaglandin $F_{1\alpha}$, prostaglandin $F_{2\alpha}$ (tris salt), indomethacin, 3-hydroxy-1-methyl-5,6-indolinedione (adrenochrome) and bovine serum albumin (fraction V) were also from Sigma Chemical Company.

Phenol Reagent (Folin-Ciocalteu Reagent) was prepared in our laboratory by the method of Folin and Ciocalteu (139).

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

3. Instruments

Analytical balance, model H10Tw	(Mettler Instrument, Inc.)
Motor-driven homogenizer	(Arthur H. Thomas Company)
Beckman refrigerated centrifuge, model J-21C	(Beckman Instruments, Inc.)
Beckman ultracentrifuge, model L8-70	(Beckman Instruments, Inc.)
Beckman spectrophotometer, model 25	(Beckman Instruments, Inc.)
Spectronic 20	(Bauch and Lomb, Inc.)

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Spectronic 2000	(Bauch and Lomb, Inc.)
Gilson pipetman	(Rainin Instrument Company, Inc.)
Thin-layer chromatography equipment	(Desaga Associate Companies)
Packard PRIAS Tris-Carb Liquid Scintillation Spectrometer, model PL	(Packard Instrument Company, Inc.)
Autocal pH meter, model PHM 83	(Radiometer)

B. METHODS

1. Insertion of silk-suture intrauterine device (IUD)

All surgical procedures were carried out through a mid-ventral laparotomy under ether (anesthetic grade, May and Baker Limited) anesthesia, and were 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 (140) and is illustrated in Figure 7. This uterine horn would be called "IUD horn" in the text. The left horn was sham operated by passing a needle and suture thread through the uterine lumen. This uterine horn will serve as the control horn. Animals were rested for at least 2 weeks before further used in the investigation. Subsequent vaginal smears showed that these rats still retained normal estrous cycle.

2. Preparation of crude prostaglandin synthetase

The method for preparation of crude prostaglandin synthetase complex from rat uterine horn follows essentially the method described by Flower et al (141). The animals were killed by decapitation, then quickly removed the control and IUD uterine horns which were separately

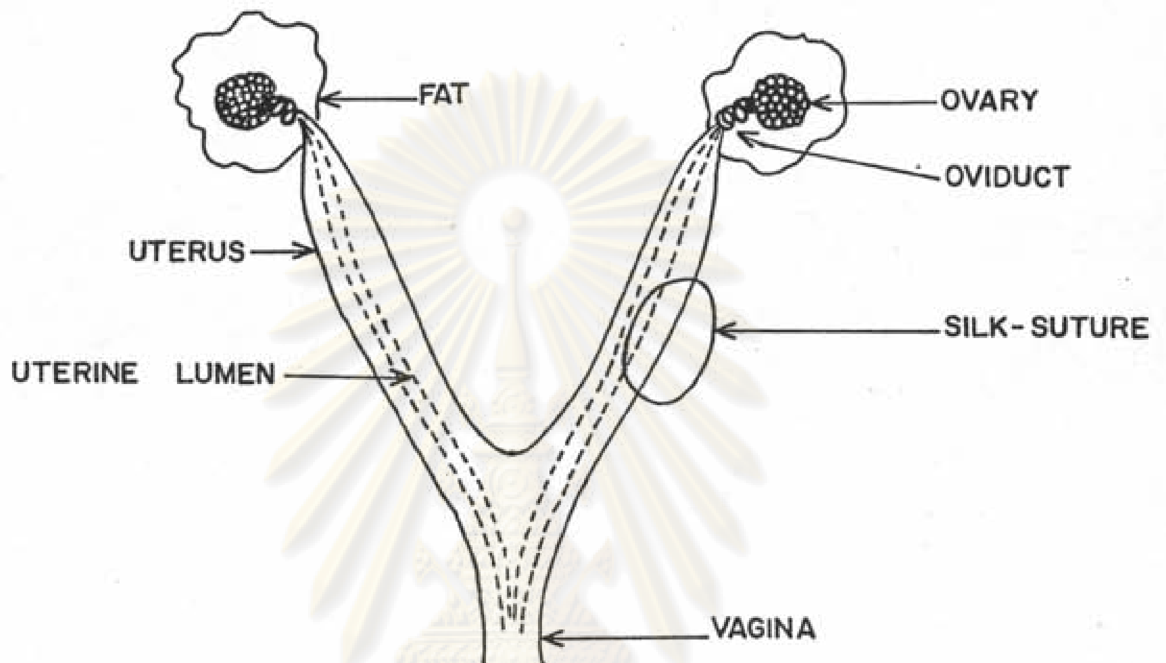

Figure 7 Diagramatic representation of the rat uteri with a silk-suture intrauterine device.

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immerged into ice-cold 0.9% (w/v) NaCl solution. All further steps would be carried out at 4°C unless otherwise specified. Fat and mesenteries were trimmed off. The uterine horns were then blotted, weighed, finely chopped with scissors and homogenized in a motor-driven homogenizer with three 10 second-bursts in ten volumes (w/v) per uterine horn of 50 mM Tris·HCl buffer, pH 8.2. The homogenate was centrifuged in a J-21C Beckman Centrifuge at 12,000 x g for 15 min in a JA-20 rotor. The supernatant was collected and then passed through a double-layer cheesecloth. The filtered supernatant was further recentrifuged in a L8-70 Beckman Ultracentrifuge at 100,000 x g for 2.5 h in a SW 50.1 rotor, to yield a crude microsomal pellet. The crude pellet was then washed twice with 50 mM Tris·HCl buffer, pH 8.2, suspended in the same buffer, and stored at -70°C until used in the next day. This is the crude prostaglandin synthetase mentioned in the text.

3. The cytosol fraction (CF)

The modulation of prostaglandin synthetase activity by cytosolic factors was also tested in this study. For preparation of cytosol fraction , the uterine homogenate was centrifuged at 100,000 x g for 2.5 h. The supernatant collected is "the cytosol fraction" in the text.

To obtain boiled cytosol, the cytosol fraction was boiled for 5 min and centrifuged at 3,000 x g for 5 min to remove denatured protein. The supernatant was used for further assay on its effect on prostaglandin synthetase.

4. Radiochromatographic assay of prostaglandin synthesis

This method based on the use of ^{3}H-arachidonic acid as the

precursor for prostaglandin synthesis. The radioactive products can be extracted, separated by thin-layer chromatography (TLC) and quantitative for their respective radioactivities.

4.1 The synthesis of prostaglandins from arachidonic acid

The assay system is based on the method described by Flower *et al*. (141) which for the bovine seminal vesicle gland. Some modifications for studies in rat uterus is therefore essential.

The reaction was carried out at $37^{o}C$ for 5 min in a 500 µl reaction volume. The crude enzyme complex (600 µg protein) was incubated in 50 mM Tris·HCl buffer, pH 8.2, containing 1 mM ^{3}H-arachidonic acid (final specific activity 2.43 TBq/mmol, 10^{6} dpm/ml); 5 mM L-epinephrine and 5 mM reduced glutathione. The reaction was initiated by the addition of enzyme and terminated by the addition of 250 µl 1 N HCl.

4.2 Extraction of arachidonic acid metabolites

Prostaglandins and other metabolites were extracted by adding 1.5 ml of ethyl acetate. Samples were mixed on the Vortex Mixer for 15 to 20 sec, and centrifuged at 2,000 x g for 5 min in a clinical centrifuge. A 1.0 ml aliquot of each ethyl acetate layer was withdrawn and placed in a separate tube. The added 10 µl (1 mg/ml each) of a mixture of unlabelled PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$ (tris salt) in ethanol will serve as carriers and internal markers. The ethyl acetate solution was dried under nitrogen and the residue was dissolved in 50 µl absolute ethanol. This would be immediately used for subsequent separation by TLC.

4.3 Thin-Layer Chromatography

The clean glass plates (0.40x20x20 cm) were coated by spreading a mixture of 30 g Silica Gel G and 60 ml water using the applicator designed by Stahl. The coating thickness was approximately 0.25 mm. The plates were activated by heating for 30 min at 110-115°C and kept in a closed box until used. The plates were developed for 40-60 min in one of the two solvent systems.

The solvent systems used were :

S_1 : ethyl acetate : acetone : acetic acid = 90:10:1

S_2 : organic phase of ethyl acetate : 2,4,4-trimethylpentane : acetic acid : water = 11:5:2:10

The developing solvents were equilibrated in close glass tank for 5 min before used. All solvents were redistilled before used.

Twenty microlitres of the extract was applied onto glass-coated silica gel thin-layer plate by calibrated microcapillary. The plate was developed to a distance of 16 cm in either solvent system S_1 or S_2. If solvent system S_2 was employed, the plate would be developed twice in the same dimension and same solvent system. At the same time, standards PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$ and arachidonic acid were run parallelly on the same TLC plate. The spots were detected by exposure to iodine vapor and the R_f values were determined.

4.4 Quantitation of prostaglandin synthesis

Small zones (about 0.5 cm each) were scraped off the plates, placed directly into vials containing 5 ml of Bray's solution

(6% naphthalene, 0.4% PPO, 0.02% POPOP, 10% methanol, 2% ethylene glycol in dioxane), mixed vigorously, and counted for the radioactivity on a Packard PRIAS Model PL Liquid Scintillation Spectrometer. Counting efficiency of the system is 35-40% as calibrated by using commercially available isotope (Packard Instrument Company, Inc.) and determined by the channel ratio. Care were taken so that each scraped zone did not contain overlapping bands of the separated products.

5. Spectrophotometric assay of prostaglandin synthesis

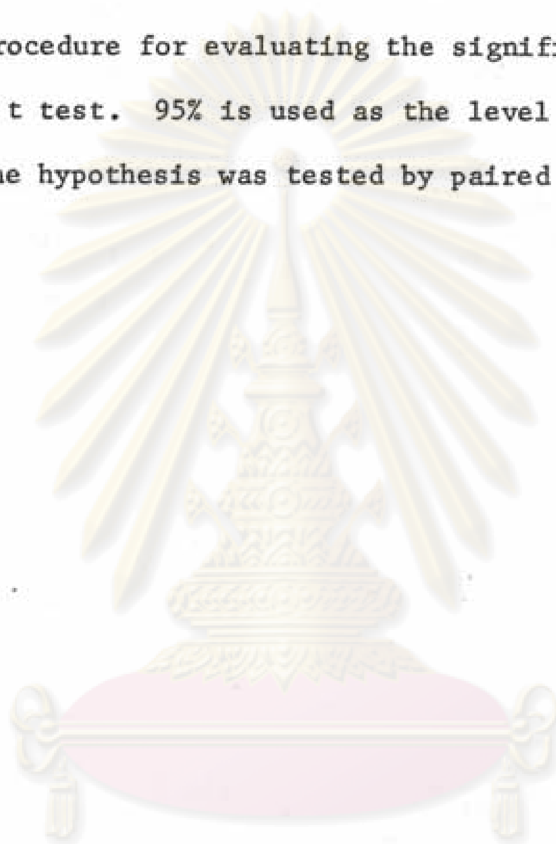
The spectrophotometric measurement of prostaglandin synthetase activity was done by the modified method from the method described by Takeguchi and Sih (138). L-epinephrine can be converted into adrenochrome by the enzyme complex in the presence of arachidonic acid. The adrenochrome formed can be detected spectrophotometrically at 480 nm if glutathione is omitted from the system.

The reaction mixture contains: crude enzyme (varying amount of protein), L-epinephrine (6.0 mM) and arachidonic acid (0.5 mM) in 50 mM Tris·HCl-2% Tween 40 buffer, pH 8.2. The crude enzyme was preincubated with L-epinephrine at 25°C for 5 min. The reaction was initiated by the addition of arachidonic acid. The total volume of the reaction mixture is 1.5 ml. The absorbancy was recorded on a Spectronic 2000. The change in absorbancy at 480 nm (ΔA_{480}) after the addition of arachidonic acid was taken as a measure of the adrenochrome formation. A blank was always run parallelly. The blank cuvette contains no L-epinephrine but is identical otherwise. The reaction was usually run for 12 min. The activity was expressed as velocity of enzyme reaction (ΔA_{480} per min) or µg of adrenochrome formed per min and the specific

activity was μg of adrenochrome formed per min per mg protein. Protein concentration was estimated by Lowry's method (142), using bovine serum albumin as standard.

6. Statistical analysis

The procedure for evaluating the significant of difference is the Student's t test. 95% is used as the level of significance. In this study, the hypothesis was tested by paired comparison (see Appendix I).



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