

## Estrogen-Directed Synthesis of Specific Prostaglandins in Uterus

(prostaglandin  $F_{2\alpha}$ /guanosine 3':5'-cyclic monophosphate/adenosine 3':5'-cyclic monophosphate/  
indomethacin)

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**ABSTRACT** Endogenous uterine prostaglandin levels were monitored in the cycling rat and prostaglandins of the F-type were found to rise at proestrus when estrogen levels have been shown to be maximal. Evidence that this is an estrogen-induced event is furnished by the finding that estradiol-17 $\beta$  caused a similar rise in prostaglandin F levels in uteri of ovariectomized rats, an action blocked by coadministration of progesterone. Examination of *in vitro* prostaglandin synthetase by uterine microsomal fractions from cycling rats and estrogen-treated ovariectomized rats revealed that the action of estrogen to control prostaglandin synthesis in a directional manner is accomplished, at least in part, by regulation of the prostaglandin synthetase complex, resulting in a patterned alteration in the ratio of prostaglandin F to prostaglandin E. These results demonstrate that prostaglandins are involved in the expression of estrogen action in the rat uterus.

Estrogen administration has been shown to effect the release of prostaglandin  $F_{2\alpha}$  (PGF $_{2\alpha}$ ) into uterine fluids of the guinea pig (1) and monkey (2). This prostaglandin, which has a potent contractile effect on the isolated uterus, has been identified in sheep as the luteolytic agent produced by the uterus which subsequently acts on the ovary to bring about luteal regression (3). Estrogen administration has also been shown to be associated with uterine hyperemia (4). Such hyperemia has been related to elevations in prostaglandin E (PGE) levels (5, 6). These findings, plus new data demonstrating the existence of receptors unique to PGE $_1$  and PGF $_{2\alpha}$  in the uterus (7) and corpus luteum (8, 9), pose the possibility that PGEs and PGF $_{2\alpha}$ , produced in the uterus as a consequence of estrogen action, play distinctly different roles in the reproductive processes.

Recently, the action of estrogen on the uterus of the ovariectomized rat has been shown to be associated with a rise in guanosine 3':5'-cyclic monophosphate (cyclic GMP) (10), an observation strengthened by the finding that cyclic GMP is maximal in the normal cycling rat at proestrus (10), a time when estrogen levels reach their peak value (11). Although there is evidence that the contractile effect of PGF $_{2\alpha}$  on the uterus (N. D. Goldberg *et al.*, personal communication and ref. 21) as well as on the canine saphenous and lung veins is associated with a rise in cyclic GMP levels (12, 13), estrogen itself exhibits no such acute contractile action on the isolated uterus. Thus, in view of the demonstrated ability of estrogen to trigger the production of both cyclic GMP and PGF by this organ, we were prompted to examine these events in more detail.

Abbreviations: PG, prostaglandin; PGF $_{2\alpha}$ , prostaglandin  $F_{2\alpha}$ ; PGE, prostaglandin E; cyclic GMP, guanosine 3':5'-cyclic monophosphate; cyclic AMP, adenosine 3':5'-cyclic monophosphate.

Emphasis was placed on the estrogen-regulated biosynthesis of prostaglandins E and F, as well as the interrelationships between these prostaglandins and adenosine 3':5'-cyclic monophosphate (cyclic AMP) and cyclic GMP. The results of these studies are the substance of this report.

### MATERIALS AND METHODS

Estradiol-17 $\beta$  benzoate was obtained from Steraloids, progesterone from Nutritional Biochemicals Co., indomethacin from Merck & Co. and Metofane<sup>®</sup> (2,2-dichloro-1,1-difluoroethyl methyl ether) from Pitman-Moore, Inc. [ $5,6\text{-}^3\text{H}$ ]PGE $_1$  (S.A. = 86 Ci/mmol) and [ $5,6\text{-}^3\text{H}$ ]PGF $_{1\alpha}$  (S.A. = 75 Ci/mmol) were purchased from New England Nuclear, [ $8\text{-}^3\text{H}$ ]adenosine 3':5'-cyclic monophosphate (S.A. = 28 Ci/mmol) from Schwarz/Mann, an [ $8\text{-}^3\text{H}$ ]guanosine 3':5'-cyclic monophosphate (S.A. = 15 Ci/mmol) from Amersham/Searle. Cyclic GMP antigen (2'-O-succinyl  $^{125}\text{I}$ -labeled tyrosine methyl ester) and the corresponding antiserum were obtained from Collaborative Research, Inc. Antisera to PGE and PGF were kindly provided by Dr. H. R. Behrman of the Merck Institute.

Female Charles River rats (150-250 g) were used throughout these studies. The stages of their estrus cycle were determined by microscopic examination of vaginal smears. Castrated animals were ovariectomized 1-4 months prior to experimentation. Steroids were administered as solutions in sesame oil; indomethacin was administered as a uniform suspension in sesame oil. All animals were injected and subsequently decapitated while anesthetized with Metofane<sup>®</sup>. Immediately following decapitation and exsanguination, the uteri were denuded of fat, excised and quickly frozen in liquid Freon 12 at dry ice temperature.

*Separation and Determination of Prostaglandins.* Frozen uterine tissue was added to 3 ml of isotonic saline to which was added 5000 cpm each of tritiated PGE $_1$  and PGF $_{1\alpha}$ , and sonicated for 30-60 sec in a Polytron (PT 10 ST) homogenizer at a pulse frequency of 7300 Hz. The homogenate was then brought to pH 3.5 with 0.4 ml of 0.12 M citric acid and extracted with 1  $\times$  4 ml and 2  $\times$  2.5 ml of ethyl acetate. The organic phases were combined and backwashed with 2  $\times$  1 ml of water. The ethyl acetate was reduced to dryness under nitrogen, and the residue taken up in 1 ml of aqueous methanol (90% MeOH 10% H $_2$ O). The aqueous methanol was then extracted with hexane (2  $\times$  2 ml). The combined organic phases were backwashed with 1 ml of aqueous methanol. The aqueous methanolic phases were combined and reduced to dryness under nitrogen. The residue was dissolved in ethyl acetate/methanol (50:50), applied to a 1  $\times$  20 cm lane of silica gel G on an Anal-

tech prescored thin-layer chromatography plate and developed in Andersen's FVI solvent system (14) composed of ethyl acetate/acetone/acetic acid (90:10:1). After exposure of markers to iodine vapor to locate the prostaglandins, the unexposed PGE ( $R_F = 0.40$ ) and PGF ( $R_F = 0.20$ ) zones from the samples were scraped from the plate. The silica gel was wet with water, extracted with 2 ml of ethyl acetate, and the ethyl acetate dried under nitrogen. The residues were dissolved in methanol for radioimmune assay, as described (15, 16).

**Separation and Determination of Cyclic Nucleotides.** A trace amount (3000 cpm) of either tritiated cyclic AMP or cyclic GMP, both purified on Dowex-1, was added to 3 ml aliquots of cold 10% trichloroacetic acid for recovery purposes. The frozen tissue was transferred to the acid solution and sonicated in a Polytron (PT 10ST) homogenizer at a pulse frequency of 7300 Hz for 30–60 sec. The insoluble material was removed by centrifugation and analyzed for protein content. The supernatant fraction was extracted with three 5 ml portions of diethyl ether to remove the trichloroacetic acid and then chromatographed in the following manner. The solution was applied to a 0.5 cm  $\times$  0.5 cm column of dry Baker neutral aluminum oxide (17, 18). The eluate was allowed to flow directly onto a second column (0.5 cm diameter  $\times$  2.5 cm) of Bio-Rad AG 1-x8 (100–200 mesh) in the formate form and this eluate discarded. The cyclic AMP was eluted by passing 4 ml of 1 N HCOOH over the aluminum oxide column and onto the AG 1-x8 column. Elution was continued with 4 ml of 1 N HCOOH over the AG 1-x8 column only, and the two 4 ml eluates containing cyclic AMP were pooled. Cyclic GMP was next eluted with 12 ml of 4 N HCOOH over the AG 1-x8 column only. All eluates were taken to dryness on a Buchler Evapo-Mix at 50°, then placed under pump vacuum for 10 min at room temperature to remove residual formic acid. Cyclic AMP was determined by the Gilman procedure (19).

Cyclic GMP was measured by a modification of the radioimmune assay of Steiner *et al.* (20). The residue was dissolved in 1.0 ml of 40 mM Tris-HCl, pH 7.5, containing 2 mM MgSO<sub>4</sub>. An aliquot was counted in a Packard liquid scintillation counter to calculate recovery. Of four 100  $\mu$ l aliquots incubated at 30° for 1 hr, two were incubated with phosphodiesterase and then all were heated at 95° for 3 min. <sup>125</sup>I-labeled antigen, antiserum and 50 mM sodium acetate buffer, pH 6.2 (final volume 500  $\mu$ l) were added to the cooled tubes and allowed to equilibrate overnight (15–20 hr) at 0°. Each sample was diluted with 3 ml of cold 50 mM sodium acetate buffer and immediately poured over a HAWP 02500 (0.45  $\mu$ m) Millipore filter. The sample tube was washed twice with 3 ml of the same buffer and the washings passed through the filter. The filter was finally washed with an additional 3 ml of buffer. The radioactivity of the antigen-antibody complex retained on the filter was determined directly in a Packard gamma counter. The cyclic GMP measurements include corrections for phosphodiesterase hydrolysis, and the mass of the added tritiated cyclic GMP.

**Preparation of Microsomes.** Uteri were homogenized (1 part tissue/3 parts buffer) in 0.125 M Na-EDTA buffer (pH 8.3) in a Kontes® all-glass homogenizer at 4°. The homogenates were centrifuged for 10 min at 10,000  $\times g$  in an International (model PR-2) refrigerated centrifuge. The supernatant phases were then centrifuged for 2 hr at 105,000  $\times g$  in a Beckman (model L2-65B) ultracentrifuge. The pellets were taken up in

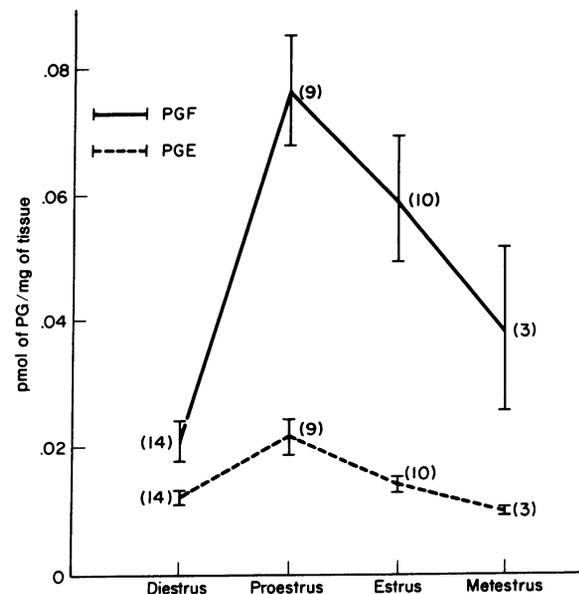


FIG. 1. Changes in uterine prostaglandin levels during the estrus cycle of the rat. Animals were sacrificed at the stages indicated and prostaglandins were extracted and measured as described. PGF levels rose significantly at proestrus as compared to metestrus ( $P < 0.05$ ) and diestrus ( $P < 0.001$ ). The difference between estrus and proestrus was not significant although that between estrus and diestrus was significant ( $P < 0.001$ ). The graph shows mean values  $\pm$  SEM, with the number of observations in parentheses.

0.125 M Na-EDTA (pH 8.3) buffer to a final protein concentration of about 3 mg/ml, and rehomogenized to disperse the microsomes.

**Prostaglandin Synthetase Incubation.** The reaction mixture consisted of 500  $\mu$ g of bovine serum albumin (Pentex, fraction V),  $5 \times 10^{-4}$  M hydroquinone,  $2 \times 10^{-3}$  M reduced glutathione,  $3 \times 10^{-5}$  M arachidonic acid, and 0.5 ml (about 1.5 mg of protein) of the microsomes in a final volume of 1.0 ml of Na-EDTA buffer (pH 8.3). After incubation for 30 min at 37° in a Dubnoff shaker, the reaction was terminated by the addition of 1.0 ml of 0.12 M citric acid. Tritiated PGE<sub>1</sub> and PGF<sub>1 $\alpha$</sub>  were added for recovery. The PG extractions and radioimmune assays were performed as described in a section above.

## RESULTS

### Changes in uterine prostaglandin levels during the estrus cycle

Endogenous levels of PGs were monitored in rats during stages of the estrus cycle (Fig. 1). PGF levels at metestrus and diestrus were similar and low, but increased rapidly at proestrus to a value 4-fold greater. The concentration of PGF at estrus continued to exceed that at metestrus and diestrus but proved to be more variable during this stage of the cycle. In contrast, uterine levels of PGE remained relatively constant throughout the cycle with only a small increment occurring at proestrus.

### Effects of estradiol and progesterone on uterine prostaglandin levels

Ovariectomized rats were treated with estradiol-17 $\beta$  benzoate (1  $\mu$ g in oil administered subcutaneously) for three consecutive

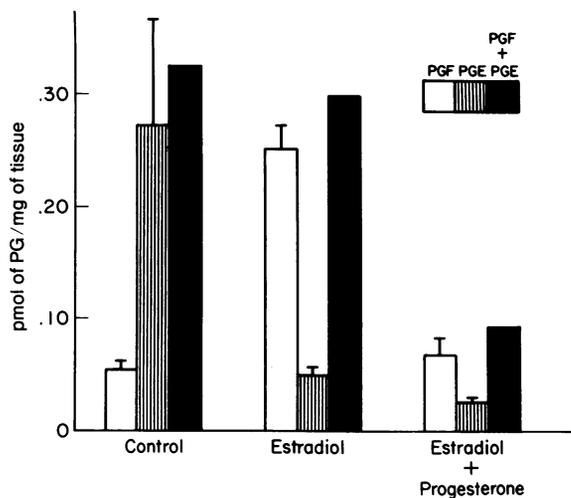


FIG. 2. Effects of estradiol-17 $\beta$  benzoate and progesterone on uterine prostaglandin levels in the rat. Castrated animals were treated subcutaneously with estradiol-17 $\beta$  benzoate (1  $\mu$ g daily) or estradiol-17 $\beta$  benzoate (1  $\mu$ g) plus progesterone (4 mg daily) for 3 days prior to sacrifice. The rise in PGF levels induced by estrogen is highly significant ( $P < 0.001$ ), as is the depression of PGE ( $P < 0.05$ ). The blockade of the rise in PGF by progesterone is evident ( $P < 0.05$ ). The graph shows mean values  $\pm$  SEM.

days. Uteri were removed 3–4 hr after the final injection. It is noteworthy that endogenous PGE levels rose at least 10-fold following ovariectomy; subsequent estrogen treatment resulted in a decrease in PGE with a concomitant elevation in PGF to levels at least 3-fold higher than those routinely observed at proestrus (Fig. 2). In some instances an increase in total prostaglandins occurred as a consequence of estrogen action, but the rise in PGF and depression in PGE were consistent observations. Administration of progesterone (4 mg in oil administered subcutaneously) for 3 consecutive days had no effect on PGF levels, although it appeared to depress PGE levels (data not shown). Progesterone, coadministered with estradiol, completely blunted the estrogen-induced stimulation of PGF.

#### Effects of indomethacin upon uterine PG and cyclic nucleotide levels

Administration of indomethacin (1 mg daily) subcutaneously with estradiol antagonized the estrogen-induced rise in uterine PGF (Fig. 3A) and actually lowered both PGE and PGF to below control values. This prostaglandin synthetase inhibitor did not block the estrogen-stimulated rise in cyclic GMP levels (Fig. 3B). The apparent stimulatory action of indomethacin upon cyclic GMP levels may be due to its action as a cyclic nucleotide phosphodiesterase inhibitor. In any event, the rise in cyclic GMP was not statistically significant.

#### Changes in uterine prostaglandin synthetase activity during the estrus cycle

It is evident from the data in Table 1 that rat uterine microsomes contain endogenous PGF and PGE, as expressed by the 0-time levels. The content of PGF increased progressively from diestrus to proestrus and reached a maximum value at estrus. Changes in PGE content, although small, follow the reverse order. Incubation of these microsomal preparations in the presence of added arachidonic acid and cofactors gave *de novo* PGF synthesis. This *de novo* PGF synthesis is seen to

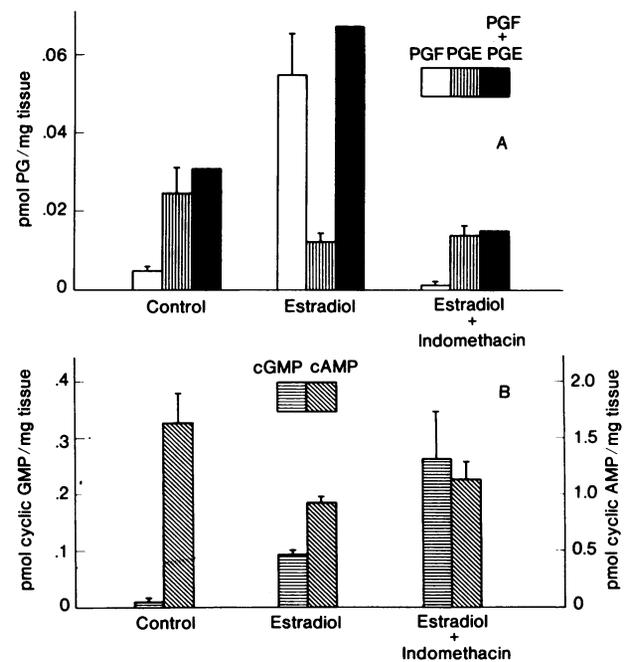


FIG. 3. Effects of indomethacin on uterine PG and cyclic nucleotide levels in the rat. Ovariectomized animals were treated subcutaneously with estradiol-17 $\beta$  benzoate (1  $\mu$ g daily) or estradiol-17 $\beta$  benzoate (1  $\mu$ g) plus indomethacin (1 mg daily) for 4 days prior to sacrifice. A complete inhibition of the estrogen-induced rise in PGF is seen to occur. The apparent rise in cyclic GMP caused by indomethacin is not statistically significant ( $P > 0.3$ ). The graph shows mean values  $\pm$  SEM.

increase from diestrus to proestrus and attain a 7-fold increment at estrus as compared to diestrus. Although the changes are less dramatic, it is evident that the ability to synthesize PGE follows the reverse course, so that the *de novo* PGF/PGE at diestrus (0.38) progresses to a maximum value of 7.61 at estrus.

#### The effect of *in vivo* administration of estradiol-17 $\beta$ benzoate to ovariectomized rats on *in vitro* uterine microsomal prostaglandin synthetase activity

Estrogen treatment produced two dramatic effects in the rat uterus (Table 2). Estradiol increased the endogenous prostaglandin content of the microsomes from a basal level at ovariectomy, which was similar to that noted at diestrus, to an elevated level similar to that routinely found at estrus. Estradiol also increased the ability of the isolated microsomes to biosynthesize PGF in preference to PGE in the presence of arachidonic acid and cofactors. This directional effect of estrogen is most evident when the data are viewed as PGF/PGE ratios. Additional evidence for an association of PGF with estrogen can be found by a comparison of microsomal data from diestrus and ovariectomized rats. Removal of the source of estrogen by ovariectomy resulted in a lower PGF/PGE ratio.

#### DISCUSSION

The findings that uterine PGF levels are elevated at proestrus in normal cycling rats, and are dramatically increased by estrogen treatment of ovariectomized rats reveal a striking parallel with uterine cyclic GMP concentrations (10). They also suggest that the uterine PGF increases, like those of cy-

TABLE 1. Prostaglandin synthetase activity in uterine microsomes from normal cycling rats

Stage of cycle	Time (min)	pg of PGF	$\Delta$	pg of PGE	$\Delta$	$\frac{\Delta \text{PGF}}{\Delta \text{PGE}}$
		mg of protein		mg of protein		
Diestrus	0	490		995		
	30	741	251	1650	655	0.38
Proestrus	0	849		921		
	30	1813	964	1312	391	2.47
Estrus	0	1137		867		
	30	2933	1796	1103	236	7.61

The microsomal fractions from uteri from normal cycling rats were evaluated for the ability to convert arachidonic acid to prostaglandins. Cofactor concentrations, buffer, and incubation conditions were as described in the *text*. Prostaglandins were isolated and analyzed by radioimmune assay.

elic GMP (10), are an estrogen-related event, since it is well established that estrogen levels peak at proestrus in the cycling rat (11). The parallel between PGF and cyclic GMP changes was also observed when progesterone was coadministered with estradiol-17 $\beta$  benzoate to ovariectomized rats. Progesterone completely blunted the stimulatory effect of estrogen on uterine PGF content, and this action is similar to its previously observed prevention of the estrogen-induced increase in cyclic GMP (10).

It has been suggested that in situations where PGE and PGF have opposing actions, these effects are mediated through cyclic AMP and cyclic GMP, respectively (21). This concept appears to be true with respect to PG action in certain veins wherein the contractile effect of PGF<sub>2 $\alpha$</sub>  is associated with a rise in cyclic GMP, in contrast to the relaxing action of PGE which is associated with a rise in cyclic AMP (12, 13). Despite the fact that estrogen is capable of effecting an increase in cyclic GMP levels in the rat uterus *in vitro*, no acute contractile response is associated with this phenomenon. Furthermore, the alterations of cyclic GMP levels that occur as a consequence of estrogen action are confined chiefly to the endometrium (27), and prostaglandin synthetase activity is also restricted to the endometrial portion of the rat uterus (22). The contractile action of the uterus is confined to the myometrial portion of this organ. Thus, the interrelationship between PFG and cyclic GMP would appear to be of an entirely different nature in the action of estrogen upon the uterus as compared to the PGF-induced contractile response. That this is in fact the case is evident by the findings reported here that indomethacin is capable of completely blocking the estrogen-induced rise in uterine PFG levels with no concurrent effect upon the rise in cyclic GMP. It is necessary to conclude, therefore, that the rise in PGF that occurs as a consequence of estrogen action is either independent of or secondary to the rise in cyclic GMP.

That estrogen can alter prostaglandin synthetase *per se*, resulting in a significant increase in the PGF/PGE ratio, casts doubt upon a current hypothesis (23) that tissue levels of prostaglandins are regulated solely by the availability of substrate precursor acids. Although precursor acids are important

TABLE 2. Effect of estradiol-17 $\beta$  benzoate on prostaglandin synthetase activity in uterine microsomes from ovariectomized rats

Treatment	Time (min)	pg of PGF	$\Delta$	pg of PGE	$\Delta$	$\frac{\Delta \text{PGF}}{\Delta \text{PGE}}$
		mg of protein		mg of protein		
None	0	612		878		
	30	681	69	1373	495	0.14
Estradiol	0	1285		777		
	30	2195	910	860	83	10.96

Ovariectomized Charles River female rats were injected subcutaneously with vehicle or estradiol-17 $\beta$  benzoate (1  $\mu$ g daily) for 3 days prior to sacrifice. The uteri were removed, and microsomes prepared as described in the *text*. The microsomal fractions were then studied for their ability to convert arachidonic acid to prostaglandins. Cofactors, buffer, and incubation conditions were as described in the *text*. Prostaglandins were isolated and analyzed by radioimmune assay.

in regard to total prostaglandin levels in tissues, it is unlikely that our observed hormonally-induced changes in prostaglandin ratios can be explained on such grounds alone. The data presented here (Table 1) demonstrating the acute alterations in the ability of the microsomal fraction of the rat uterus to convert exogenous arachidonic acid to PGF<sub>2 $\alpha$</sub>  clearly show that this enzyme complex, prostaglandin synthetase, or associated cofactors, are subject to orderly fluctuations during the estrus cycle. It is unlikely that these changes are due to cofactors, since the microsomal preparation was freed from its cytosol, the source of the cofactors, and the arachidonic acid incubations were carried out in a synthetic medium with a defined cofactor content. We believe, therefore, that the alterations in the PGF/PGE ratio are attributable to fluctuations in the composition of prostaglandin synthetase *per se*. That this is an estrogen-induced event is suggested by the initial rise in the ability of the enzyme complex to effect increased conversion of arachidonic acid at proestrus, a time when estrogen levels reach their peak value. The observation that estrogen is capable of causing a preferential increase in PGF levels as well as in the ability to synthesize PGF in microsomes from the uteri of ovariectomized rats (Table 2) solidifies this estrogen-PGF interrelationship with respect to PG synthetase activity.

The E- and F-type prostaglandins differ qualitatively and quantitatively in their actions on many organs. It has been proposed that these prostaglandins interact with receptors unique to each, thus, resulting in a different series of intracellular events (21, 24). In such a scheme, the nature of the events triggered would be a function of the amounts of individual prostaglandins present, the number of individual receptors available, i.e., for PGE, PGF, or both. The ability of the estrogen (and progesterone) produced by the ovary to regulate the synthesis of prostaglandins by the uterus in a directional and cyclic manner is consistent with the unique roles played by PGEs and PGF<sub>2 $\alpha$</sub>  in the reproductive processes. One of the initial effects of estrogen upon the uterus is to induce a rapid hyperemia (25). There is much evidence to show that this action of estrogen is prostaglandin-mediated

(5), and studies in the gravid uterus implicate PGEs as those involved in this phenomenon (6). On the other hand, PGF<sub>2α</sub> has been identified as the substance produced by the uterus that acts on neighboring ovarian tissue to effect luteal regression and an accompanying depression of progesterone levels (3). The contractile action of PGF<sub>2α</sub> on the myometrium is a classic phenomenon. Whether the precise levels of the individual prostaglandins determine the nature of the events triggered or whether this is simply a function of the PGF/PGE ratio, which varies widely in the cycle, remains to be determined. However, it is clear that estrogen and the counter action of progesterone combine to play a central role in regulating uterine prostaglandin as well as regulating cyclic nucleotide levels (10). Although the role of cyclic GMP in uterine function remains to be established, the involvement of prostaglandins has been implicated in several parameters including parturition, estrus regulation, and uterine blood circulation.

It is evident from the present studies that estrogen is capable of regulating the synthesis of prostaglandins both quantitatively and qualitatively in the uterus of the rat. Contrary to the current concept that availability of prostaglandin precursor acids is the sole factor in controlling tissue levels of PGs, the data reported here offer evidence to show that the enzyme complex, prostaglandin synthetase, is subject to hormonal control in a finely regulated manner. It is evident from these actions of estrogen and the counter action of progesterone, that prostaglandins are involved in the expression of the action of these steroidal sex hormones.

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