HORMONAL REGULATION OF MYOMETRIAL GAP JUNCTIONS: CONTROL BY ESTROGEN, PROGESTERONE AND PROSTAGLANDINS

BY

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HORMONAL REGULATION OF MYOMETRIAL GAP JUNCTIONS

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ABSTRACT

The muscle of the pregnant uterus transforms into an active and reactive tissue at term which is necessary for expulsion of the fetus and termination of pregnancy. Gap junctions develop between the smooth muscle cells of the uterus immediately prior to term and disappear after delivery. Gap junctions may be providing the structural basis for improved communication between myometrial cells during labor thereby leading to the synchronized coordinated muscle activity required for effective labor. Although gap junctions appear to have significant implications in the termination of pregnancy, the regulation of their presence in the myometrium is not completely understood but may depend upon hormonal changes that occur prior to parturition.

In the studies reported in this thesis, quantitative thin section electron microscopy showed that high doses of estradiol stimulated, whereas progesterone when administered with estradiol, suppressed the presence of myometrial gap junctions in non-pregnant animals. The steroid hormones regulate the presence of gap junctions by controlling the synthesis of the junction proteins probably through their receptor mechanism. In addition, high doses of estradiol may selectively stimulate the synthesis of a prostaglandin which may be required for the development of gap junctions. Prostaglandins appear to be involved in junction regulation since inhibitors of cyclo-oxygenase potentiated estradiol stimulation of gap junctions. Moreover, treatment of pregnant animals with estradiol resulted in the presence of numerous myometrial gap junctions and abortion. These results demonstrate that

the steroid hormones and prostaglandins regulate the presence of myometrial gap junctions and that their presence in the myometrium may be a requirement for the occurrence of term as well as preterm labor.

Cell-to-cell communication via gap junctions is also believed to be involved in the control of cellular and tissue growth. The myometrium grows dramatically throughout pregnancy to accommodate the growing fetus. Moreover, the steroid hormones, in particular estradiol, stimulates growth of the uterus and prostaglandins also appear to be involved in the process. Since the hormones and prostaglandins regulate uterine growth and the presence of myometrial gap junctions (see above), the relationship between the two events was further evaluated in this thesis. Studies on non-pregnant animals after various hormonal treatments and in untreated pregnant animals demonstrated that regulation of uterine growth (of which the myometrium is a major component) and the presence of gap junctions in the myometrium are dependent upon the hormonal environment. The results suggest a complex interaction between the steroid hormones, a product of the cyclo-oxygenase and/or lipoxygenase pathway for control of gap junctions and myometrial growth. Myometrial growth may occur because of the lack of gap junctions but the cessation of growth of this tissue does not depend on the presence of the junctions. The development and presence of gap junctions, however, may be partially dependent upon myometrial growth.

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TABLE OF CONTENTS

	page
DESCRIPTIVE NOTE	ii
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
LIST OF FIGURES	xvi
LIST OF TABLES	xxii
Chapter 1	
General introduction and review of the literature	1
1A. Gap junctions	1
1A.1 Structure	2
1A.2 Function	13
1A.3 Regulation of gap junction permeability	23
1A.4 Formation of gap junctions	25
1A.5 Regulation of gap junction formation	29
1A.6 Degradation	31
1A.7 Methods for quantitation of gap junctions	36
1A.7.1 Thin section morphometry	36
1A.7.1.1 Applications and limitations	37
1A.7.2 Freeze-fracture morphometry	38
1A.7.2.1 Applications and limitations	39
1B. Presence of gap junctions in smooth muscle	42

1B.1 Introduction 42

1B.2 Myometrial gap junctions 47

1B.2.1 Presence in the myometrium 47

1B.2.2 Function of myometrial gap junctions 49

1B.2.3 Functional control of myometrial gap junctions 53

1B.2.4 Regulation of myometrial gap junction formation 54

1B.2.4.1 Steroid hormones 54

1B.2.4.2 Prostaglandins 57

1B.2.5 Regulation of gap junctions in non-pegnant animals 58

1B.2.6 Degradation of myometrial gap junctions 60

1C. Control of labor 63

1C.1 Introduction 63

1C.2 Endocrine changes at parturition 64

1C.2.1 Progesterone 65

1C.2.2 Estrogen 68

1C.2.3 Prostaglandins 72

1C.3 Hormonal profile of the reproductive cycle 75

1C.4 Hormonal control of myometrial activity 79

1C.4.1 Steroid hormones 79

1.C.4.1.1 Mechanism of steroid hormone action 83

1C.4.1.1a Classical theory of steroid receptor interactions 83
Estrogen 83
Progesterone 87
1C.4.1.1b Other possible mechanisms of steroid hormone action 92
1C.4.2 Prostaglandins 96
1C.4.2.1 Mechanism of action for prostaglandins 97
1C.5 Neural control of myometrial activity 98
1C.6 Gap junctions in control of labor 101

1D. Control of tissue growth 103
1D.1 Introduction 103
1D.2 Gap junctions between cells and growth control 104
1D.3 Communication incompetence of cells and growth control 106
1D.4 Gap junctions in cancerous tissue 110
1D.5 Steroid hormones and tissue growth 112
1D.5.1 Control of uterine growth 113
1D.6 Steroid hormones, gap junctions and uterine growth .. 116
1D.7 Steroid hormone dependent cancer 117
1D.8 Relationship between steroid hormones, gap junctions and tissue growth 121

1E. Purpose of the present study 122

Chapter 2

Objectives of this study 123

2.1 Introduction 124

2.2 Objectives 128

2.2.1 Primary objectives 128

2.2.1.1 Regulation of myometrial gap junctions by steroid hormones 128

2.2.1.1a Estrogen 128

2.2.1.1b Progesterone 130

2.2.1.2 Regulation of myometrial gap junctions by prostaglandins 130

2.2.1.3 Regulation of uterine growth by steroid hormones 132

2.2.1.3a Estradiol 132

2.2.1.3b Progesterone 133

2.2.1.4 Regulation of uterine growth by prostaglandins 134

2.2.1.5 Relationship between uterine growth and presence of myometrial gap junctions 134

2.2.1.5a Immature rats 135

2.2.1.5b Ovariectomized mature rats 135

2.2.1.5c Pregnant animals 136

2.3 Significance 136

Chapter 3

Materials and methods 141

3.1 Animals 142

3.1.1 Ovariectomy 142

3.1.2 Treatment schedule 143

3.1.2.1 Immature rats 143

3.1.2.2 Ovariectomized mature rats 144

3.1.2.3 Pregnant rats 145

3.2 Tissue collection and processing for microscopy 145

3.2.1 Thin section electron microscopy 145

3.2.2 Thin section microscopy: Quantitative measurements of gap junctions 146

3.2.3 Freeze-fracture electron microscopy 148

3.2.4 Freeze-fracture morphometry 149

3.2.5 Light microscopy 150

3.2.6 Light microscopy morphometry 151

3.2.7 Light microscopy autoradiography 152

3.3 Incorporation of tritiated thymidine 153

3.4 Determination of uterine wet and dry weights 154

3.5 Estimation of 17B-estradiol and prostaglandin F levels ... 154

3.6 Uptake of tritiated estradiol-17B 155

3.7 Statistical comparisons 156

Chapter 4

Control of myometrial gap junctions by steroid hormones 157

4.1 Introduction 158

4.2 Results 161

4.2.1 Effects of estrogen and progesterone 161

4.2.2 Time course of gap junction development: Tissue from immature rats 169

4.2.2.1 Effects of estradiol 169

4.2.2.2 Effects of diethylstilbestrol 184

4.2.2.3 Effects of progesterone 187

4.2.2.4 Effects of tamoxifen 192

4.2.2.5 Effects of cycloheximide 195

4.2.3 Tissues from ovariectomized mature rats 198

4.2.3.1 Effects of estradiol 198

4.2.3.2 Effects of progesterone 202

4.2.4 Tissues from pregnant rats 202

4.3 Discussion 224

4.3.1 Regulation of gap junctions by steroid hormones 224

4.3.1.1 Regulation in non-pregnant animals 225

4.3.1.2 Regulation in pregnant tissues 237

4.3.2 Summary 241

Chapter 5

Regulation of myometrial gap junctions by prostaglandins 243

5.1 Introduction 244

5.2 Results 246

5.2.1 Tissues from immature rats 246

5.2.1.1 Effects of indomethacin 246

5.2.1.2 Effects of sodium meclofenamate 252

5.2.2 Tissues from ovariectomized mature rats 254

5.2.2.1 Effects of indomethacin 254

5.2.3 Effects of estradiol and/or indomethacin on plasma estradiol levels 264

5.2.4 Effects of indomethacin on tritiated estradiol uptake by uterine tissue 267

5.2.5 Effects of estradiol and/or indomethacin on uterine tissue prostaglandin F levels 267

5.3 Discussion 273

5.4 Summary 281

Chapter 6

Regulation of uterine growth and presence of gap junctions ... 283

6.1 Introduction 284

6.2 Results 290

6.2.1 Immature rats 290

6.2.1.1 Effects of estradiol 290

6.2.1.2 Effects of progesterone 325
6.2.1.3 Effects of tamoxifen 340
6.2.1.4 Effects of indomethacin 346
6.2.1.5 Relationship between uterine weight increase and the presence of myometrial gap junctions 348
6.2.2 Ovariectomized mature rats 361
6.2.2.1 Effects of estradiol 361
6.2.2.2 Effects of indomethacin 369
6.2.2.3 Relationship between uterine weight increase and presence of myometrial gap junctions ... 375
6.2.3 Pregnant rats 380
6.2.3.1 Relationship between uterine weight increase and presence of myometrial gap junctions ... 383
6.3 Discussion 388
6.3.1 Regulation of uterine growth 389
6.3.1.1 Hypertrophy versus hyperplasia 389
6.3.1.1a Immature rats 389
6.3.1.1b Ovariectomized mature rats 397
6.3.1.1c Pregnant animals 398
6.3.1.2 Summary 400
6.3.2 Control by steroid hormones 401
6.3.2.1 Steroid receptor mechanism 401
6.3.2.1a Estrogen 401
6.3.2.1b Progesterone 402
6.3.2.1c Tamoxifen 406

6.3.2.2 Adenylate cyclase mechanism 409
6.3.3 Control by prostaglandins 410
6.3,4 Control by distention 417
6.3.5 Summary 418
6.3.6 Relationship between myometrial growth and presence of gap junctions 419
6.3.6a Independence of myometrial growth and gap junctions 421
6.3.6b Absence of gap junctions and myometrial growth 422
6.3.6c Cessation of myometrial growth and development of gap junctions 426
6.3.6d Myometrial growth as a component for the development of gap junctions 430
6.3.6e Summary 436

Chapter 7

Summary and conclusions 437

7.1 Regulation of myometrial gap junctions by steroid hormones 438
7.1a Summary of estrogen studies 438
7.1ab Conclusions 441
7.1b Summary of progesterone studies 441
7.1bc Conclusions 442
7.2 Regulation of myometrial gap junctions by prostaglandins . 443

7.2a Summary of studies 443

7.2b Conclusions 444

7.3 Regulation of uterine growth by steroid hormones 445

7.3a Summary of estradiol studies 445

7.3ab Conclusions 447

7.3b Summary of progesterone studies 448

7.3bc Conclusions 448

7.4 Regulation of uterine growth by prostaglandins 448

7.4a Summary of studies 448

7.4b Conclusions 449

7.5 Relationship between uterine growth and presence of myometrial gap junctions 450

7.5a Summary of immature rat studies 450

7.5b Summary of studies on ovariectomized mature rats 451

7.5c Summary of studies on pregnant animals 452

7.5d Conclusions 453

7.6 Future studies 453

Chapter 8

References 458

LIST OF FIGURES

page

1. High magnification electron micrograph of a gap junction as seen in a thin section 4
2. High magnification electron micrograph of a gap junction as seen in a membrane replicate 8
3. Electron micrographs showing both fracture faces of a gap junction 9
4. Diagrammatic illustrations of freeze-fracturing showing how the fracture faces of a gap junction arise 11
5. High magnification electron micrograph showing an annular gap junction 33
6. Schematic representation of the steroid receptor mechanism 91
7. Size distribution histogram of gap junctions found in estradiol treated immature rats and in pregnant delivering animals 166
8. High magnification electron micrographs of thin sections showing gap junctions in myometrium of (A) immature rats treated with estradiol, and (B) delivering animals 168
9. Time course for gap junction development in immature rat myometrium 171
10. Dose response curve for estradiol on the myometrial membrane area of gap junctions in immature rats 175

11. Electron micrograph of a myometrial membrane replica from immature rat showing at high magnification a gap junction 178
12. Low magnification replica from myometrium of immature rat treated with estradiol showing two gap junctions 180
13. Temporal pattern of gap junction development following a single injection of estradiol 183
14. Time course for the development of gap junctions in immature rat myometrium after daily treatments with diethylstilbestrol 186
15. Effects of progesterone on estradiol stimulation of gap junctions over 1-6 days 189
16. Effects of tamoxifen on estradiol stimulation of gap junctions over 1-6 days 194
17. Time course of gap junction development in myometrium of ovariectomized mature rats treated daily with estradiol 200
18. Low magnification electron micrograph from a thin section of myometrium from a day 17 pregnant rat 209
19. Electron micrographs of thin sections of myometrium from delivering rats showing gap junctions between the muscle cells 211
20. Electron micrograph of a replicate from delivering rat myometrium showing several gap junctions in the fracture plane 213
21. Electron micrographs of thin sections of myometrium from pregnant rats undergoing premature delivery and

showing several gap junctions between the muscle cells after treatment with estradiol 220
22. Electron micrograph of a replicate from premature delivering rat myometrium after treatment with estradiol showing several gap junctions 222
23. Effects of indomethacin on the development of myometrial gap junctions in ovariectomized mature animals treated with estradiol 256
24. Electron micrographs of thin sections of myometrium from ovariectomized mature rats treated with estradiol plus indomethacin showing several gap junctions between myometrial cells 260
25. Plasma estradiol levels of ovariectomized mature rats treated with estradiol with and without indomethacin 266
26. Tritiated estradiol content in serum and uterine tissue of ovariectomized mature rats treated with indomethacin . 269
27. Prostaglandin F content in uterine tissue of ovariectomized mature rats treated with estradiol with and without indomethacin 272
28. Photos of intact uteri from immature rats treated with (A) sesame oil, and (B) estradiol 292
29. Wet weight profile of uteri from immature rats treated with estradiol over 1-8 days 294
30. Uterine wet weight changes normalized to rat body weight over 1-8 days after treatment with estradiol 297

31. Changes in uterine dry weight over 1-6 days after treatment with estradiol 299
32. Changes in uterine dry weight over 1-6 days normalized to rat body weight 302
33. Effects of estradiol treatment on normalized cross-sectional area of (A) the whole uterus, (B) myometrium, and (C) endometrium 305
34. Light micrographs of transverse sections of uteri from (A) control, and (B) estradiol treated rats 309
35. Effects of estradiol on the normalized cross-sectional area of the circular (A) and longitudinal (B) muscle layers of the immature rat myometrium 312
36. Time course of tritiated thymidine incorporation into uterine DNA of immature rats after treatment with estradiol over 1-6 days 316
37. Effects of estradiol treatment on total uterine DNA content of immature rats 318
38. Nucleus labeling indices of the myometrium (A) and endometrium (B) after daily treatment with estradiol over 1-6 days 321
39. Immature rat uterine wet weight profile over 1-6 days after treatment with a single injection of estradiol at zero time (day 0) 323
40. Effects of estradiol on normalized uterine wet weight changes over 1-6 days from immature rats treated with a single injection of estradiol at zero time 327

41. Uterine wet weight profiles from immature rats treated with progesterone and progesterone plus estradiol 330
42. Normalized uterine wet weight profiles from immature rats treated with progesterone and progesterone plus estradiol 332
43. Effects of estradiol, progesterone and estradiol plus progesterone treatments on immature rat uterine wet weight in animals primed with estradiol 336
44. Normalized wet weight profiles from estradiol primed immature rats treated with estradiol, progesterone and estradiol plus progesterone 339
45. Effects of tamoxifen on uterine wet weight of immature rats over 1-6 days 342
46. Normalized uterine wet weight profiles from estradiol, tamoxifen and estradiol plus tamoxifen treated immature rats 345
47. Changes in normalized uterine dry weight and area of myometrial gap junctions in immature rats after treatment with 50 (A) and 500 (B) ug/day estradiol 350
48. Normalized uterine wet weight changes and the time course for the development of gap junctions in immature rat myometrium over 1-6 days after treatment with a single injection of estradiol 353
49. Changes in normalized uterine wet weight and area of myometrial gap junctions after treatment with estradiol with and without progesterone over 1-6 days 356

50. Normalized uterine wet weight changes and changes in the area of myometrial gap junctions in immature rats treated with estradiol with and without tamoxifen 359
51. Wet and dry weight profiles of ovariectomized mature rat uteri treated with estradiol 363
52. Effects of estradiol treatment on normalized uterine wet weight changes of ovariectomized mature rats 365
53. Profiles of normalized dry weight of ovariectomized mature rats treated with estradiol 368
54. Changes in normalized uterine dry weight and area of myometrial gap junctions in ovariectomized mature rats treated with estradiol at 50 (A) and 500 (B) ug/day for 1-4 days 377
55. Uterine wet and dry weight profiles of untreated pregnant animals throughout gestation, during delivery and one day post-partum 382
56. Normalized uterine wet and dry weight profiles of pregnant animals throughout gestation, during delivery and one day post-partum 385
57. Changes in normalized uterine dry weight and area of gap junctions in myometrium of pregnant animals throughout gestation, during delivery and one day post-partum 387

LIST OF TABLES

page

1. Effect of hormone treatment on gap junctions in rat myometrium 163
2. Effects of estradiol and/or progesterone on myometrial gap junctions in immature rats treated for 4 days 191
3. Effects of estradiol and/or cycloheximide on myometrial gap junctions in immature rats 197
4. Effects of progesterone on myometrial gap junctions in ovariectomized mature rats treated for 3 days 203
5. Effects of estradiol on myometrial gap junctions in pregnant animals 206
6. Effects of various doses of estradiol on sustaining the presence of myometrial gap junctions of pregnancy 223
7. Effects of estradiol and/or indomethacin on myometrial gap junctions in immature rats 248
8. Effects of estradiol plus indomethacin with and without progesterone on myometrial gap junctions in immature rats 251
9. Effects of estradiol and/or sodium meclofenamate on myometrial gap junctions in immature rats 253
10. Effects of progesterone on myometrial gap junctions in ovariectomized mature rats treated with estradiol plus indomethacin 261

11. Effects of estradiol and/or indomethacin on myometrial gap junctions in ovariectomized mature rats .. 263
12. Effects of indomethacin and estradiol on uterine wet weight of immature rats 347
13. Effects of indomethacin and estradiol on normalized uterine wet weight and myometrial gap junctions in immature rats 360
14. Effects of estradiol on uterine weight of ovariectomized mature rats 370
15. Effects of indomethacin on uterine wet weight of ovariectomized mature rats 371
16. Effects of indomethacin on uterine wet and dry weights of ovariectomized mature rats 373
17. Effects of indomethacin on estradiol induced uterine weight of ovariectomized mature rats 374
18. Effects of indomethacin and estradiol on normalized uterine wet weight and myometrial gap junctions in ovariectomized mature rats 379

This thesis is dedicated to my late son Aaron,
and late father, Malcolm MacKenzie

CHAPTER 1

GENERAL INTRODUCTION AND REVIEW OF THE LITERATURE

1A. GAP JUNCTIONS

1A.1 Structure:

Gap junctions have been described in a variety of cell types (Dewey and Barr, 1962; Gabella, 1973; Henderson, 1975; Bennett and Goodenough, 1978; Peracchia, 1980; Larsen, 1983). A gap junction is a structure that appears as an area of close opposition between two adjacent cell plasma membranes. When examined by thin section electron microscopy after fixation with glutaraldehyde followed by osmium tetroxide and en bloc staining with uranyl acetate, gap junctions cut in cross-section consist of regions where the extracellular space between the plasma membranes of the two cells is reduced to a very uniform 2-3 nm electron-lucent gap (Figure 1). This structure of a gap junction representing the plasma membranes and gap between the two cells is visualized as four electron-opaque and three electron-lucent lines comprising a septilaminar structure 15-20 nm thick. The thin extracellular space at gap junctions has been confirmed by its permeability to extracellular stains, such as colloidal lanthanum. Gap junctions fixed in the presence of this colloidal suspension show in cross-section a dense line where the lanthanum has penetrated the gap (Henderson, 1975; Peracchia, 1980).

It is now clear that the apparent gap of this junction as viewed in thin sections is only a limitation of this microscopic technique. The junctional membranes are in contact with each other in discrete spots where intramembrane particles, protruding from opposite membrane

Figure 1. High magnification electron micrograph from a thin section of the myometrium showing a gap junction between two smooth muscle cells. At this high magnification the 7-lined structure of the junction is apparent. Marker is 0.1 um, X 101,000.

surfaces, align with each other and bridge the gap between membranes (Peracchia, 1980; Loewenstein, 1981). The usual observation of an uninterrupted gap in thin sections (Figure 1) is due to the low electron opacity of the particles bridging the gap (Peracchia, 1980). These protruding particles, however, are occasionally seen in thin sections (Peracchia, 1973).

Visualization of the particles protruding into the extracellular space of a gap junction was first accomplished by using extracellular stains. Colloidal lanthanum applied to tissues with gap junctions infiltrates the extracellular space and surrounds the intramembrane particles within the gap (Henderson, 1975; Peracchia, 1980; Larsen, 1983). When a gap junction exposed to lanthanum has been sectioned tangentially, the lanthanum outlines an array of hexagonal structures delineating the intramembrane particles (Henderson, 1975; Peracchia, 1980). The array of structures are viewed as 7.0-7.5 nm electron-lucent spots (the protruding particles) surrounded by an electron-opaque network (lanthanum filled extracellular spaces of 1-2 nm). The center-to-center spacing of the intramembrane particles was estimated at 9-10 nm (McNutt and Weinstein, 1970). Revel and Karnovsky (1967) observed that the gap junction particles outlined by lanthanum exhibited electron dense cores approximately 1 nm wide. These cores are thought to represent the channels between the cytoplasm of the two cells connected by the gap junction.

Thin section electron microscopy cannot clearly resolve the substructure of membranes of gap junctions and it cannot ascertain whether the hexagonal array of particles revealed by lanthanum staining

are an integral part of the membrane structure or simply fixtures attached to the external membrane surface. With the introduction of freeze-fracture electron microscopy for the study of membrane junctions, the latter question and many others concerning the structure of gap junctions have been answered. The fracture plane of a membrane occurs along the interface running between the two monolayers of lipids, thus , freeze-fracturing splits the membrane down the middle. As a result, the internal aspects of either the protoplasmic (P face) or the extracellular (E face) membrane surfaces are exposed. A fracture plane through one membrane of a gap junction reveals on the P face an array of particles 7-8 nm in diameter with an average center-to-center spacing of approximately 10 nm (Figure 2) (McNutt and Weinstein, 1970; Henderson, 1975; Bennett and Goodenough, 1978; Peracchia, 1980). The fracture plane, however, often steps from one cell membrane to the other showing both faces of the gap junction. When these are present in the replicate, a gap junction appears as an aggregate of particles on the P face in mammalian tissues as described, as well as an array of complementary pits displayed on the E face (Figure 3). This image of a gap junction arises from arrays of particles embedded in each membrane and protruding from it to make contact with similar particles of the opposite membrane; the bumps represent partially exposed particles, the pits represent the impressions left by the particles cleaved away with the protoplasmic leaflet (Figure 4) (Peracchia, 1980). In high resolution replicas of gap junctions the particles exhibit central pits suggesting the presence of a pore or channel (McNutt and Weinstein, 1970; Peracchia, 1980) supporting earlier observations implicated from

Figure 2. Electron micrograph of a plasma membrane fracture surface from a replicate of the myometrium showing at high magnification an aggregation of membrane particles on the protoplasmic fracture (PF) face representing a gap junction (arrow). Open arrow shows shadow angle. Marker is 0.1 um, X 460,000.

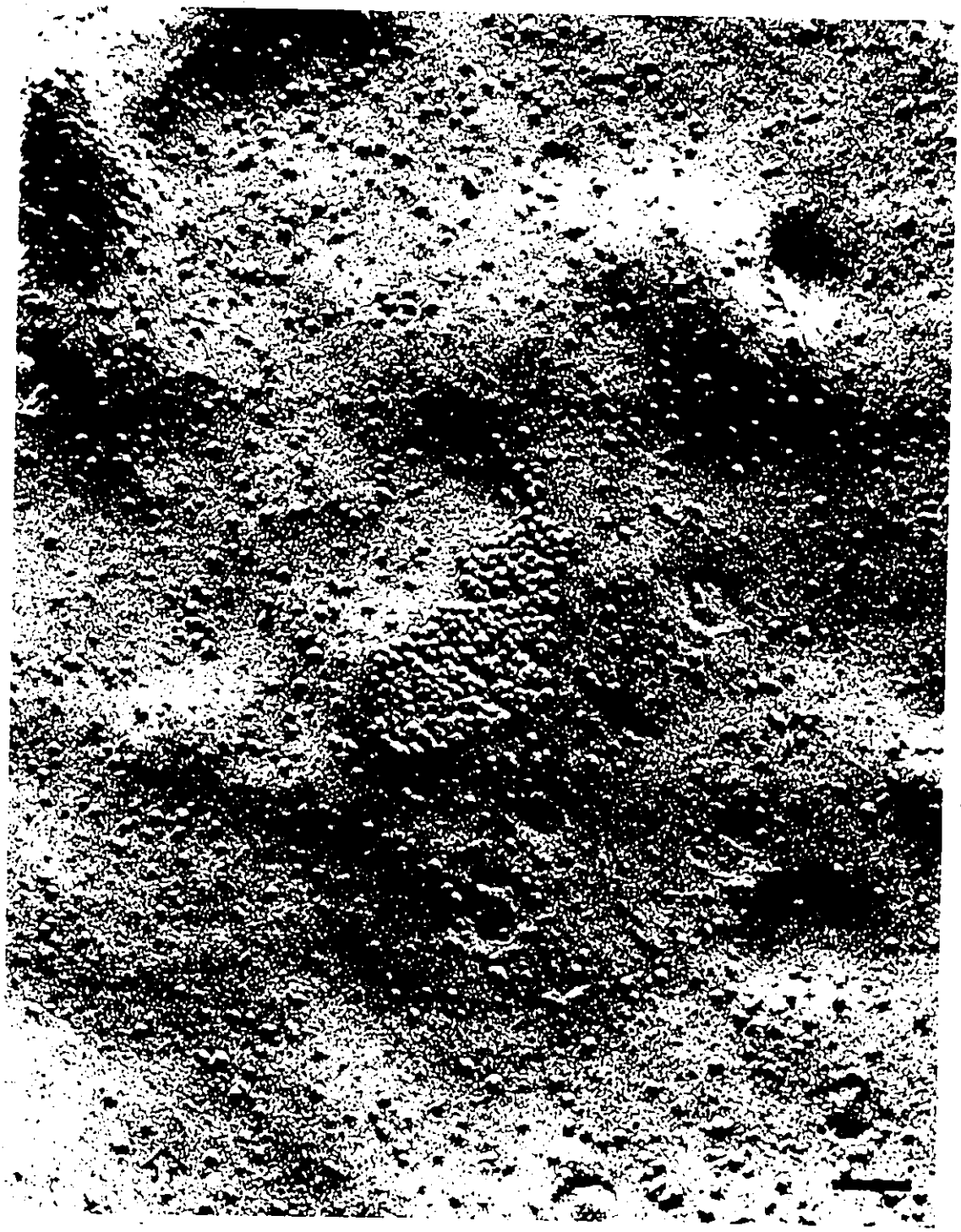


Figure 3. High magnification electron micrographs from freeze-fracture replicas of the myometrium showing the fracture faces of a gap junction. A) aggregation of junction particles on the protoplasmic fracture (PF) face and complementary pits on the extracellular (EF) face, X 425,000. B) a gap junction seen on the EF fracture face of the cell membrane showing intramembrane pits; particles on the PF face are evident at the periphery of the junction (solid arrow), X 562,500. C) a small gap junction on the EF face of a muscle cell membrane revealing intramembrane pits, X 425,000. Open arrows on each panel show shadow angle. Markers are 0.1 um.

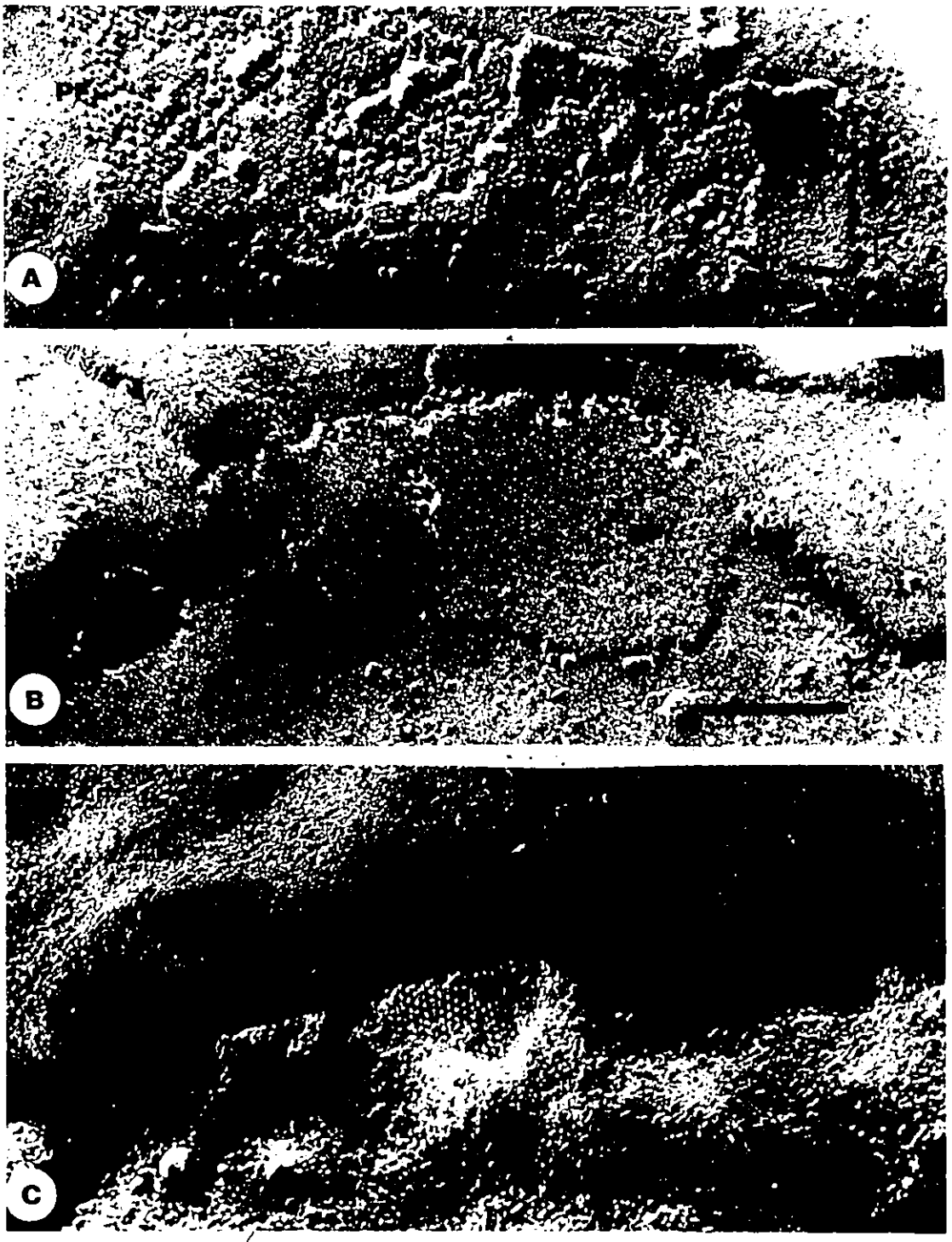
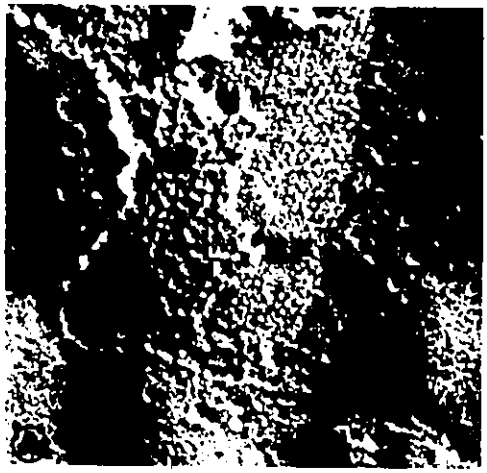
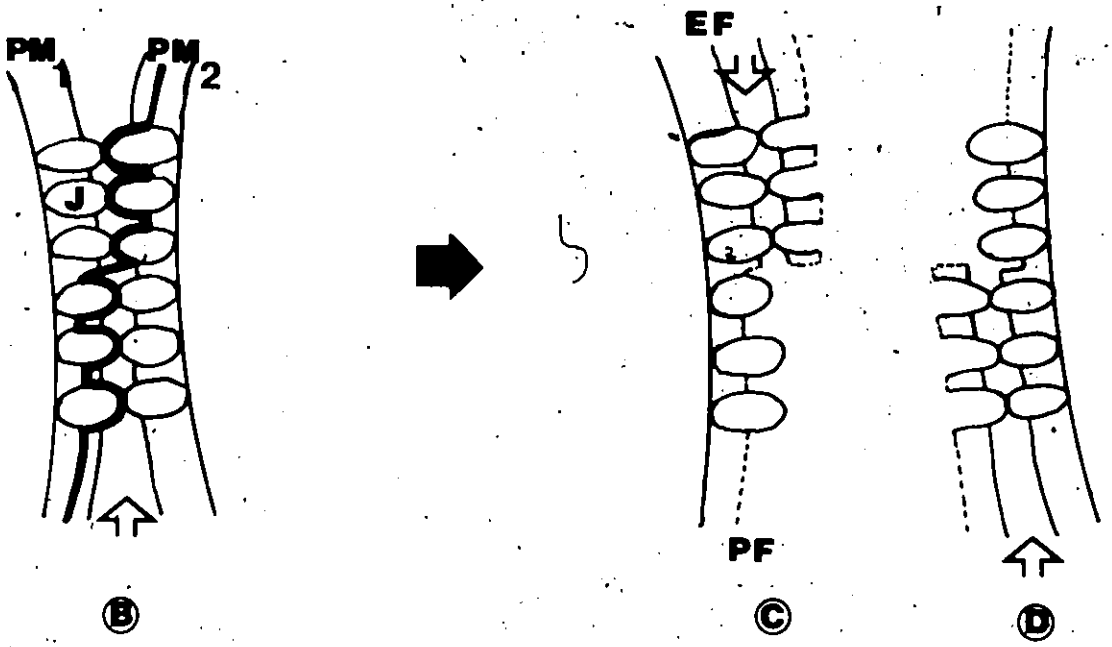

A
B
C

Figure 4. Electron micrograph from a replica of myometrial smooth muscle showing the PF and EF faces of a gap junction (A). Schematic diagrams (B,C,D) of gap junction structure in profile illustrating how the fracture faces of (A) arise. The gap junction membrane contains an array of intramembrane particles represented by J in plasma membrane of one cell (PM_1) and their complements in an apposed membrane, PM_2 (B). In freeze-fracture microscopy, the plane of fracture (indicated by the heavy line in B) splits the membrane of one cell down the middle and often jumps to the other membrane comprising the junction. When this occurs, the fracture plane exposes a portion of the intramembrane particles on the protoplasmic (PF) fracture surface and their complementary pits on the extracellular (EF) surface (C). Figure D represents parts of membranes split off and discarded during fracturing. Open arrows in B and C indicate extracellular space between the junction membranes and dotted lines in C and D illustrate the fracture plane.




PM 1
PM 2
J
EF
PF
Ⓑ
Ⓒ
Ⓓ

lanthanum stained gap junctions. In mammalian tissues the particles more frequently adhere to the P face than on the E face of the membrane. There are tissues (i.e. arthropod tissues) in which gap junctions have reversed fracture faces with particles on the E face and complementary pits on the P face (Gilula, 1972). Freeze-fracture studies of arthropod tissues containing gap junctions have allowed the morphology of both the cytoplasmic and extracellular ends of the intramembrane particles to be visualized. Estimates of the heights of the particles confirm the hypothesis that the particles span the membrane thickness and protrude from both membrane surfaces (Peracchia, 1980).

Models of gap junctions based upon observations from sections through lanthanum stained junctions and freeze-fracture replication have depicted the intramembrane particles (connexons) to be composed of six main subunits arranged around a center channel. X-ray diffraction and electron optic studies on isolated gap junctions have also revealed that the connexon units of a gap junction are composed of six protein subunits with a central hydrophilic channel (Makowski et al., 1977; Unwin and Zampighi, 1980). Three dimensional Fourier analysis of electron microscopic views of isolated gap junctions confirmed the hexameric structure of the gap junction connexons and have suggested that the protein subunits of each connexon are roughly rod shaped and on a slight tilt with respect to the axis of the channel (Unwin and Zampighi, 1980). The existence of proteins in the extracellular space at gap junctions is believed to be the protruding connexons bridging the gap between the two cells (Makowski et al., 1977). The bonding of connexon pairs forms the membrane channel connecting the cytoplasm of

the two cells.

Many attempts have been made to analyze the biochemical composition of gap junctions. Unfortunately, because of the differences in techniques between laboratories and the difficulties isolating purified fractions of gap junctions, the results have been difficult to interpret since a wide variety of proteins with diverse molecular weights have been demonstrated (Peracchia, 1980; Larsen, 1983). Recently, it has been accepted that the major gap junction protein is a component with a molecular weight of 25,000-28,000. Peracchia (1980) has predicted that each gap junction connexon could be composed of approximately 25 polypeptide chains, with each of the six subunits being composed of at least 4 polypeptide chains oriented perpendicular to the membrane surface. More recently, antibody cross-reactivity studies, peptide mapping and amino acid sequencing techniques have indicated that the major polypeptide of gap junctions vary between certain tissues (Larsen, 1983). Therefore, although gap junctions from various tissues appear structurally similar, the proteins which make up the connexons may be significantly different.

1A.2 Function:

Gap junction connexons within the opposed cell membranes are thought to align themselves and create channels from the cytoplasm of one cell to the cytoplasm of the other cell. The channels formed by the gap junction connexons are believed to be sites for electrical and metabolic coupling between cells (Henderson, 1975; Loewenstein and Rose, 1978; Peracchia, 1980; Hooper and Subak-Sharpe, 1980). There is

convincing evidence that gap junctions form a pathway between coupled cells to provide for the passage of current (electrical coupling or ionic coupling) ; gap junctions are low resistance or low impedance contacts between cells (Henderson, 1975; Griepp and Revel, 1977; Bennett and Goodenough, 1978; Peracchia, 1980, Loewenstein, 1981). Low resistance junctions were first detected by their ability to provide channels for the exchange of small ions between cell interiors thereby coupling cells electrically allowing for the propagation of action potentials in excitable tissues. This type of coupled cell system is demonstrated with electrophysiological methods to measure the electrical resistance across two cells. The basic experiment involves passing an electric current pulse with the aid of an intracellular microelectrode into the interior of one cell and measuring the corresponding electrical potential in a neighbouring cell. If the two cells are connected by low resistance junction channels, current injected into the cell will enter into the other cell and produce a corresponding change in electrical potential.

Electrical coupling between cells was first demonstrated in the early 1950's. Weidmann (1952) first showed that cardiac cells were electrically coupled which was followed shortly after by similar observations between neurons of the crayfish (Furshpan and Potter, 1959). In the ensuing years the observation of electrical communication between various excitable cells became widespread involving a variety of species (Peracchia, 1980). In the early 1960's it also became evident that non-excitable cells were electrically coupled. With the demonstration of electrical coupling by Loewenstein and Kanno (1964) and

Kuffler and Potter (1964) between salivary gland cells of insect larvae and glial cells of the leech respectively, it was apparent that electrical communication was inherent to cells capable of generating and transmitting electrical impulses. Similar observations to date have been observed in most cell types except cells which are not part of an organized tissue (i.e. blood cells) (see Peracchia, 1980 and Larsen, 1983 for review).

That gap junctions are low resistance pathways which provide sites of coupling between cells has been supported by several lines of evidence. Cardiac muscle treated with hypertonic solutions results in cell shrinkage and selective disruption of gap junctions. It has been shown that intercellular resistance increases concomitantly with this structural event implying that gap junctions are involved in electrical coupling of those cells (Barr et al., 1965). Loss of cell coupling in correlation with the disruption of gap junctions has also been demonstrated in smooth muscle (Barr et al., 1968) and in crayfish axons (Asada and Bennett, 1971; Pappas et al., 1971). Similar observations have also been reported in studies which do not involve disruption of gap junctional membrane. Conformational rearrangements in gap junction channel structure between crayfish giant axons have been detected in parallel with changes in electrical coupling resistance (Peracchia and Dulhunty, 1974; 1976). Furthermore, gap junctions are the only discernable junctional specializations present in some communicating cell types (Gilula et al., 1972; Johnson et al., 1974; Bennett, 1977; Sheridan et al., 1978). Moreover, fusion of communication- incompetent cells, which are not electrically coupled and do not possess gap

junctions, with communicating cells yields communication- competent hybrids (Loewenstein, 1979; 1981). In addition, the size of gap junctions increase progressively as electrical coupling improves (Johnson et al., 1974). Therefore, experimental treatment that modulates gap junction frequency or size results in corresponding changes in the degree of electrical coupling between cells (Flagg-Newton et al., 1981; Meyer et al., 1981). Physiological and morphological data have also been correlated resulting in junctional characteristics specific for electrical resistivity. Estimates of specific junctional resistance are several orders of magnitude lower than that of a non-junctional membrane (Peracchia, 1980). For example, a junctional membrane resistance of approximately 0.01 ohms/cm^2 in cultured Novikoff hepatoma cells (Sheridan et al., 1978) confirms ultrastructural studies suggesting that the gap junction is composed of channels, approximately 2 nm in diameter and 15-20 nm long, having a core resistivity comparable with that of the cytoplasm.

Whether gap junctions are necessary for electrical coupling or whether they are the only type of cell-to-cell contact which can accomplish electrical communication between cells is a matter considered by all investigators. Numerous studies have shown that when cells structurally connected by gap junctions are injected with current pulses , a voltage change occurs in an adjacent cell (Bennett and Goodenough, 1978; Peracchia, 1980). Contrary to these findings, electrical coupling between cells in some tissues and among cell aggregates has been observed in the absence of ultrastructurally defined gap junctions (Daniel et al., 1976; Kuriyama and Suzuki, 1976; Sperelakis, 1979;

Clapham et al., 1980). For this reason these investigators have proposed that organized gap junctions are not necessary for electrotonic coupling of cells. There has been speculation that other low resistance pathways exist between cells for current flow (Daniel et al., 1976) or that cells may be functionally coupled by other mechanisms (Sperelakis, 1979). Recently, however, intracellular electrical studies have directly correlated coupling between myometrial cells with the presence of gap junctions thereby supporting the hypothesis that electrical communication between cells is mediated by the junctions (Sims et al., 1982).

It should be noted that in many instances the correlation between electrical coupling and the absence of gap junctions between cells (as above) has not been determined from the same sample preparations. Electrical and structural analysis must complement each other in an investigation to resolve whether electrical coupling occurs via either the gap junctions or rather another undefined low resistance pathway by some other coupling mechanism. Alternatively, coupling could occur in the absence of visible gap junctions. Connexons, the individual components of a gap junction, are recognized as being the functional units of the junctions. A single channel created by one connexon may represent a functional communicating link between two cells. Coupling may also be mediated by small aggregates of connexons scattered throughout the plasma membrane. Freeze-fracture microscopy can detect individual and small aggregates of membrane proteins and gap junctions composed of only two or three particles may exist but cannot be recognized as such with this technique. Moreover, it must be stressed

that not all membrane proteins seen by freeze- fracture microscopy are necessarily connexons. The term gap junction refers to an aggregate of membrane particles at the interface between two cells when viewed by freeze-fracture microscopy. Therefore, can a single connexon warrant the term gap junction when it is not by definition an aggregate? Furthermore, what is the minimum number of connexons that constitute an aggregate, thereby being referred to as a gap junction?. If single connexons or even small aggregates are termed gap junctions then it is imperative that the putative junction exists in regions where adjacent cells come into close proximity. In addition, an aggregate of membrane particles may not necessarily have corresponding partners on an adjacent cell. Therefore, demonstration of membrane particles would be equivocal evidence for the presence of connexons. Membrane particle aggregates should demonstrate corresponding membrane pits from the adjoining cells as evidence for a gap junction (Peracchia, 1980). Thus, only when the above concepts are considered and explicit criteria are proposed for defining a gap junction ultrastructurally, can the underlying uncertainty of the structural basis of cell-to-cell coupling be resolved. However, if small aggregates of intramembrane particles represent functional gap junctions then why are gap junctions often composed of hundreds of connexon units? Although there is no definite answer to this question we may speculate that the increased frequency of connexons may further reduce the junctional resistance between cells acting in the manner of a safety device or complying to the needs for functional stability of the particular cells involved. However, whereas the necessity of gap junctions is questioned by all, cumulative evidence

strongly suggests such a role for the gap junction and it is generally believed that these junctions are sufficient for electrical coupling of cells.

The demonstration that cells of non-excitable tissues were coupled electrically lead to the assumption that molecules other than small inorganic ions may also be transferred from cell-to-cell because transmission of electrical impulses is not generally considered a necessary physiological property of these tissues. The finding that fluorescein, a larger hydrophilic molecule (MW 332) , could traverse a junctional pathway between non-excitable cells of the insect salivary gland supported this assumption (Loewenstein and Kanno, 1964). Shortly after this observation fluorescein was shown to cross gap junctions from a wide variety of vertebrate and invertebrate cells (Peracchia, 1980; Loewenstein, 1981). The cell-to-cell transfer of fluorescein was then extended to include a variety of other molecules of similar and somewhat larger size. Light absorbing hydrophilic molecules and radioactively labeled molecules (ranging from 300 to approximately 1500 MW) have been shown to diffuse between coupled cells and most, if not all of these molecules, are much more permeant from cell-to-cell than outward through non-junctional membrane (Peracchia, 1980; Loewenstein, 1981). Proof that the pathways for dye transfer are not continuous with the extracellular space was demonstrated by showing that the dyes do not enter the cells when present in the extracellular space or conversely, do not leak out of cells. Since the dyes (i.e. Procion and Lucifer yellow) do not enter or leave the cells across the plasma membrane, it is reasonable to conclude that the passage of these molecules between

cells occurs via a route insulated from the extracellular space. These studies and others using fluorescent dyes and radioactive tracers have concluded therefore, that charged and uncharged molecules diffuse between cells probably by way of the gap junctions. The permeability limits of gap junctions are consistent with other evidence suggesting the existence of a 1-2 nm channel created by the gap junction connexons (Peracchia, 1980).

It is quite clear from the electrical and tracer studies that cells are linked by membrane channels that provide structural continuity between cells. It is logical to assume, therefore, based on the evidence that tracer molecules up to 1500 MW can diffuse from cell-to-cell, that many non-excitable cells exhibit gap junction communication-competence and that a variety of cytoplasmic molecules in situ (i.e. metabolic intermediates) could be traversing the junctional membrane between cells. Evidence for direct intercellular exchange of metabolic intermediates between cells was first obtained from in vitro culture studies on hamster fibroblasts (Subak-Sharpe et al., 1969). These authors found that certain mutant cells deficient in a purine or pyrimidine synthetic pathway were unable to incorporate exogenous base or nucleotide into their nucleic acids unless they were grown in mixed culture with wild type cells. Autoradiography showed that only when mutant cells were in contact with the wild type did they possess products of the enzyme in which they were deficient. This indicates that the missing metabolite was passed from the wild type cell to the mutant by passing the enzyme deficiency thereby supporting the assumption that metabolic cooperation requires direct cell-to-cell

interaction.

A metabolic cooperation method providing convincing evidence for the intercellular exchange of nucleotides and other small metabolites which does not employ mutant cells was introduced by Pitts and Simms (1977). Donor cells in their technique are loaded with a radioactive precursor which is converted to an intermediate metabolite and retained in the cell. The donor cells are then cocultured with unloaded cells and transfer of the metabolite from loaded cells to unloaded cells is assayed by autoradiography. Employing this technique a larger variety of metabolic intermediates (i.e. uridine, thymidine, glucose, various amino acids and cyclic adenosine monophosphate [cAMP]) were found to pass readily from cell-to-cell (Peracchia, 1980; Hertzberg et al., 1981). The cell-to-cell transfer of metabolic intermediates seems to be via gap junctions because correlated electrophysiological and metabolic cooperation studies have shown that cell types incapable of junctional communication are also incapable of metabolic cooperation (Loewenstein, 1979). Thus, there is much evidence to support the hypothesis that gap junctions provide a pathway for the direct exchange of metabolites (metabolic coupling) between cells via permeable intercellular channels (see Bennett and Goodenough, 1978; Peracchia, 1980; Hooper and Subak-Sharpe, 1980; Loewenstein, 1981).

Cell-to-cell communication via gap junctions has also been proposed as a possible mechanism for the transmission of information between cells necessary for regulation of growth, differentiation and development (Pitts, 1978; Loewenstein, 1979; 1981). Cyclic AMP which plays an important role in the regulation of cell function including

growth (Loewenstein, 1979) is transferred between cells coupled by gap junctions (Peracchia, 1980; Hertzberg et al., 1981). The proposed role for gap junctions in regulation of growth is outlined in detail in section 1D.

The existence of gap junctions between cells of embryonic tissues implies that gap junctions may in some way participate in development and differentiation, perhaps by allowing the cell-to-cell transfer of morphogens (Furshpan and Potter, 1968; Crick, 1970; Wolpert, 1971). If gap junctions were to play a role in development then there should be spatial or temporal changes in communication- competence between cells reflecting different developmental pathways. Evidence from the squid and amphibian embryo and insect epidermis, however, have shown no restriction of communication across regions where there was a discontinuity in the developmental field (Furshpan and Porter, 1968; Warner, 1973; Caveney, 1974) thereby arguing against a critical role of gap junctions in development. Another limitation of theories relating gap junctions to development and differentiation is the undefined nature of the morphogenic molecules that might be transferred between cells.

Recently direct evidence supporting a role for gap junctions in development has been established through the use of antibodies to the gap junction protein (Warner et al., 1984). Antibodies to the liver gap junction protein were microinjected into one identified cell of the 8-cell stage Xenopus embryo. This treatment selectively disrupted both dye transfer and electrical coupling between the progeny cells. Moreover, injection of the antibody into the embryo resulted in developmental defects which were attributable to the injected cell of

the embryo. These results indicate and provide the first direct evidence of a role for junctional communication in development and differentiation.

1A.3 Regulation of gap junction permeability:

Recently it has become apparent that cells connected by gap junctions may not necessarily show a free exchange of current, dyes or isotopes (Flagg-Newton and Loewenstein, 1979; Peracchia, 1980; Loewenstein, 1981). Electrophysiological evidence has shown that junctions can rapidly switch from a low to a high resistance pathway (Peracchia, 1980). It is now recognized that the channels created by the gap junction connexons may not always be in the open state (Bennett and Goodenough, 1978; Flagg-Newton and Loewenstein, 1979; Peracchia, 1980; Unwin and Zampighi, 1980). There may be times when the channels are open and times when they are closed. This uncoupling mechanism may act as a safety device to uncouple injured cells or to uncouple cells when metabolically or electrically desirable (Loewenstein and Rose, 1978; Loewenstein, 1981).

Several mechanisms for the control of gap junction permeability have been considered. Functional uncoupling has been achieved by changes in cytoplasmic levels of calcium and hydrogen ions (Gilula and Epstein, 1976; Peracchia, 1980; Loewenstein, 1981; Hertzberg et al., 1981; Spray et al., 1981; Peracchia and Bernardini, 1984). Increased intracellular levels of free calcium and decreased intracellular pH cause a rapid and reversible decrease in gap junction conductance, independent of each other. Exactly how calcium or hydrogen ions regulate junctional

conductance remains incompletely understood. Calcium may directly occlude the channel (Loewenstein, 1981) or calcium and hydrogen may cause a change in junction channel conformation resulting in blockage (Peracchia, 1980; Loewenstein, 1981). Peracchia (1980) speculates that uncoupling agents such as calcium or pairs of hydrogen ions act directly on gap junction connexons by binding to negative sites on the cytoplasmic ends of each connexon neutralizing the charge and causing a narrowing and closure of the intercellular channels. Calcium and pH could also cause realignment of connexon subunits and thereby regulate junctional permeability (Unwin and Zampighi, 1980).

Calmodulin is also thought to be involved in regulating gap junction permeability. Calmodulin inhibitors, trifluoperazine and calmidazolium, reversibly inhibit electrical uncoupling of cells (Lees-Miller and Caveney, 1982; Peracchia and Bernardini, 1984). Furthermore, calmodulin binds to gap junction proteins in either a calcium dependent or independent manner depending on the source of tissue (Hertzberg and Gilula, 1981; Welsh et al., 1981; 1982). Peracchia and Bernardini (1984) suggests that activated calmodulin may directly induce a conformational change in the gap junction channel protein resulting in channel occlusion. It has been demonstrated that calcium induces a conformational change in the lens gap junction protein through calmodulin activation (Girsch and Peracchia, 1982) supporting this hypothesis in addition to the calcium mechanism of uncoupling as described previously. However, that gap junction proteins vary between different species or different tissues from the same animal in chemical composition and structure, a calmodulin independent mode of uncoupling

cannot be excluded (Peracchia and Bernardini 1984).

Changes in gap junction structure accompanies the transition in junctional permeability from low to high resistance states. Freeze-fracture microscopy has revealed connexon packing to shift from loosely packed to tightly and hexagonally packed arrays in response to uncoupling stimuli (Peracchia, 1980). Peracchia proposes that this structural change is part of the uncoupling mechanism, possibly through conformational rearrangement in the connexon protein resulting in closing of the channel.

It appears therefore, that gap junctions when present in the open state are capable of providing a communication system between cells. The most basic physiological role of the gap junction is probably homeostatic (Loewenstein, 1981), buffering variations in channel permeant molecules between cells. In this way gap junctions could provide the physical basis of molecular cell-to-cell communication via transmission of regulatory signals; electrical signals, regulatory molecules such as cyclic nucleotides and nutrients (metabolic coupling) and possibly, but speculatively, growth regulating factor(s) (Loewenstein, 1981)

1A.4 Formation of gap junctions:

Gap junctions are present between most cell types during some phase of life of the cell (Griepp and Revel, 1977; Bennett and Goodenough, 1978; Peracchia, 1980; Larsen, 1983). The only cell types known to be incapable of intercellular communication are ones that either do not undergo cell division, such as skeletal muscle (gap

junctions are present in developing muscle, i.e. between myoblasts) or cells which are not part of an organized tissue (i.e. blood cells). Gap junctions have also been shown to form between a variety of cultured cells from different species and different cell types (Peracchia, 1980; Larsen, 1983).

Gap junctions appear to be dynamic structures in most cell systems. The formation of gap junctions has been studied primarily in vitro where cells are separated and allowed to reaggregate and form communicating junctions (Pitts, 1977; Larsen, 1977). Gap junction formation has been evaluated structurally and functionally as previously described.

Several successive steps in the formation of gap junctions have been demonstrated with the electron microscope (Johnson et al., 1974). The appearance of specialized areas of the membrane called formation plaques or recruitment centers scarce of protein particles except for large intramembrane gap junction precursor particles, measuring 10-11 nm in diameter, have been observed (Johnson et al., 1974; Bennett and Goodenough, 1978; Larsen, 1983). When formation plaques are present the distance between membranes of the cells is greater than the spacing between membranes when gap junctions have formed. Thereafter, small aggregates of 8-9 nm gap junction particles appear in the plaque region followed by an enlargement of the 8-9 nm particle aggregates and the disappearance of the precursor particles.

Gap junctions form relatively fast after cells come into contact with each other (i.e. a few minutes up to one hour depending on the cell type, Johnson et al., 1974). This suggests that the machinery and

precursors for the formation of gap junctions are readily available in cells or at cell surfaces (Revel et al., 1978). According to Revel it is unlikely that with the shorter times there is the induction of synthesis of new proteins after contact between interacting cells but the longer times do allow for the synthesis and secretion of new protein. The formation of gap junctions in most tissues appears to depend on the synthesis of protein (Griepp and Revel, 1977). However, Epstein et al., (1977) reported the formation of gap junctions between Novikoff hepatoma cells in vitro in the absence of protein synthesis indicating either a recycling of preexisting dissociated junction proteins at the cell surface through several cycles of disaggregation- reaggregation or the presence of large amounts of the precursor of the gap junction protein in another intracellular pool (Bennett and Goodenough, 1978). Another possibility is that the formation of the junctions may require a proteolytic step which initiates the assembly of precursors in the cell membrane (Revel et al., 1978). Freeze-fracture electron microscopy of protease inhibited cultures has revealed unusual intramembrane particle aggregation when compared to normal junctions in untreated samples (Griepp and Revel, 1977). This data may support the above hypothesis.

The formation of gap junctions according to the available literature is summarized as follows: synthesis of gap junction proteins (monomers) are secreted to the cell surface where they associate with each other at the level of the formation plaque to form the 10-11 nm proconnexons. The proconnexons undergo proteolytic cleavage changing the conformation of the junction protein which results in the end-to-end association of connexons and formation of channels between the

neighbouring membrane (Revel et al., 1978).

The assembly of connexons into gap junctions and junction growth within the plasma membrane is not completely understood. Gap junctions may form and grow through the coalescence of small aggregates of connexons to form larger plaques (Bennett and Goodenough, 1978; Larsen, 1983) or alternatively, gap junctions could form by the recruitment of individual connexons. This step may be controlled by the fluidity of the membrane because agents which cause an increase in clustering of gap junction particles are also thought to alter the membrane fluidity. However, it should be noted that not all systems use the formation plaque as a recruiting center for the initial aggregation of gap junction precursors (Larsen, 1983). Moreover, extremely large gap junctions observed between some cells seem to result from the recruitment of small plaques within the cell membrane because small junction plaques are infrequent or completely absent from the membrane possessing the large gap junctions. Another possibility is that the formation and growth of gap junctions may be controlled by cytoplasmic filaments or other cytoskeleton structures. It has been reported that colchicine disrupts the formation of gap junctions between embryonic cells at the time when their assembly through accretion of intramembrane particles takes place (Meller, 1981). Colchicine administered after this stage of junction formation had no effect on the structure of gap junctions. This relationship between the effect of colchicine and inhibition of gap junction formation suggests that cytoskeletal structures may play a role in the assembly of junctional contacts since this drug works through disruption of microtubules. Alternatively, the

possibility that no in situ factor(s) directly governs the translateral migration of free junctional precursors resulting in the aggregation of gap junction particles cannot be excluded because of the vast array of variations in junction shape, size and packing patterns of junctional subunits between species and cell types within the same animal.

1A.5 Regulation of gap junction formation:

Gap junction size and total membrane area vary considerably in different tissues and organisms. This may reflect variations in the control of gap junction formation between tissues and species, and thus has attracted much attention.

In most species it appears that protein synthesis in response to various agents is necessary for the formation of gap junctions (see above). The physiological agents known to influence gap junction formation have been outlined in a number of reviews (Griepp and Revel, 1977; Sheridan, 1978). Of the agents listed, much investigation has been directed towards the role of cyclic nucleotides in regulation of the junctions. Cyclic AMP has been demonstrated to increase the size of gap junctions between many mammalian and invertebrate cell lines (Hax et al., 1974; Johnson et al., 1974; Sheridan, 1978; Flagg-Newton, 1980; Garfield et al., 1980b; Flagg-Newton and Loewenstein, 1981; Flagg-Newton et al., 1981). Moreover, agents which stimulate adenylate cyclase in mammalian cells (i.e. beta adrenergic catecholamines) thereby elevating cAMP levels also increase the size of the junctions (Garfield et al., 1980b; Radu et al., 1982). On the other hand, cGMP may have an inhibitory effect on the formation of gap junctions (Sheridan, 1978).

However, intercellular resistivity between invertebrate epidermal cells increases after exposure to cAMP possible through an effect of the cyclic nucleotide on the formation, structure and/or function of gap junctions (Caveney, 1978).

Decker (1976) has also reported that the peptide hormone, thyroxine, influences the formation of gap junctions between ependymoglial cells of the amphibian larvae. Similarly, vitamin A and ecdyosone induce the development of gap junctions between epidermal cells in the chick embryo and metamorphosing insect, respectively (Elias and Friend, 1976; Caveney, 1978). Stimulation of gap junction formation between granulosa cells of the ovary has also been demonstrated after administration of gonadotropins and estrogen (Merk et al., 1982). Estrogen also stimulates an increase in the frequency of gap junctions between the smooth muscle cells of the myometrium from non-pregnant (Dahl and Berger, 1978; Garfield et al., 1980a; Merk et al., 1980; Burghardt et al., 1984a) and pregnant animals in vitro (Garfield et al., 1978; 1980a). Garfield and coworkers (1980a) have also demonstrated that prostaglandins may be involved in the regulation of myometrial gap junctions. Prostaglandins have also been implicated in the regulation of gap junction formation between cells of other tissues (Radu et al., 1982). Since prostaglandins have been reported to modulate cAMP levels (Vesin et al., 1979; Kuehl and Egan, 1980), the regulation of gap junction formation in the myometrium and other tissues by this agent may result through a cAMP effect, however, this possibility has not been ascertained. It is apparent, nevertheless, that inhibitors of protein synthesis prevent the effects of these agents on gap junction formation.

1A.6 Degradation:

The mode of gap junction disappearance or removal from cell membranes in vivo has been reported to occur via internalization through an invagination and endocytotic process by one of the cells connected by the junction. In thin sections internalized gap junctions appear annular (Figure 5) and have been demonstrated in a variety of tissues including cancers (Merk et al., 1973; Albertini and Anderson, 1975; Griepp and Revel, 1977; Larsen, 1977; Pfeifer, 1980; Agus, 1981; Larsen, 1983). These annular gap junctions in most instances show no evidence of contact with the plasma membrane representing true intracellular structures since extracellular tracers are excluded from the annular gap junction complex (Garant, 1972; Merk et al., 1973; Albertini et al., 1975). Moreover, the individuality of the annular form of gap junctions has been verified by serial sectioning (Merk et al ., 1973). It has been suggested that annular gap junctions likely represent an intermediate step in a process which eventually leads to junction degradation following internalization by endocytosis (Garant, 1972; Merk et al., 1973; Albertini and Anderson, 1974; Albertini et al., 1975; Gilula, 1977; Griepp and Revel, 1977; Larsen, 1977; 1983).

Once internalized, gap junctions are thought to be degraded through a lysosomal mechanism (Griepp and Revel, 1977; Larsen, 1977; 1983; Larsen et al., 1979; Pfeifer, 1980). The sequence of events involved in this process, however, have not been described in detail. It is possible that the annular gap junction represents an heterophagosome and directly fuses with lysosomes as the starting event for lysosomal

Figure 5. High magnification electron micrograph of an annular gap junction in a smooth muscle cell. The micrograph shows the membrane structure of the annular junction and lack of association of material with inner aspect of annular membranes. Marker is 0.1 um, X 120,000.

digestion (Pfeifer, 1980). There is, however, no morphological evidence to support this mechanism. Alternatively, the internalized gap junction may be handled by the cell like other organelles that are to be degraded by autophagy, rather than like heterophagic vacuoles (Pfeifer, 1980). Following autophagic sequestration of an internalized gap junction, the newly formed autophagosome would fuse with the lysosome and be digested. Ultrastructural evidence has supported this mechanism of internalized gap junction degradation (Gilula, 1977; Pfeifer, 1978; 1979). However, since gap junctions make up only a small fraction of the total cell membrane area of a tissue and that extensive enlargement of gap junctions through recruitment of smaller junctional plaques may be required for endocytosis thereby resulting in a small number of internalized gap junctions, it would be difficult to analyze this process of junction degradation (Pfeifer, 1980; Larsen, 1983).

There has also been reports from vertebrate tissues that when coupled cells separate there was no evidence for gap junction internalization (Revel, 1978; Yancy et al., 1978; Yee and Revel, 1978). According to these investigators the way in which gap junctions may be removed from those tissues is by dispersion of their component particles over the membrane, although this process has never been reported in vertebrates. Freeze-fracture analysis of gap junction breakdown in vivo in metamorphosing insects, however, has supported such dispersal of gap junction particles (Lane and Swales, 1980). They have shown that mature larval gap junctions breakdown by a streaming out of junctional particles in linear arrays away from the plaque periphery. As metamorphism proceeds, the gap junctional particles are dispersed over

the intramembranous surface rather then being disposed of by internalization. Preceding the dispersion of junctional particles, particle free aisles of the membrane with linear strands of particles straddling them are also present (Lane and Swales, 1980). These authors have suggested that this change in form of the membrane surface , which seems to be the primary event in junctional transformation in the insect, may itself modify or initiate particle dispersion by inducing an increase in membrane fluidity. The reason why this form of gap junction degradation has not been detected in vertebrates may be the result of fracturing differences between the two groups. Arthropod systems have inverted gap junctions which fracture to reveal particles on the E face rather than on the P face. The E face possesses many fewer particles compared to the P face of eucaryotic cells, thus making it easier to detect such alterations in distribution of junction particles.

In vertebrate tissue, gap junctions which are taken up into the cytoplasm have been reported to lose their crisp, flattened topography and develop irregular blebs indicating that particle dispersion may precede junctional endocytosis (Larsen, 1983). Furthermore, it has also been shown that gap junctions between liver cells disappear and than reappear within short time intervals (i.e. between 22 and 48 hours) after partial hepatectomy (Yee and Revel, 1978). It seems reasonable, therefore, to assume that a redistribution rather than true degradation of gap junction particles exist enabling subsequent reutilization for junctional reformation process (Lane, 1978; Revel, 1978).

1A.7 Methods for quantitation of gap junctions:

Two basic ultrastructural techniques are available for the quantitation of gap junctions between cells in any tissue; thin section and freeze-fracture electron microscopy. In thin section microscopy the occurrence of membrane regions occupied by gap junctions identified by five or seven lined structures are determined, whereas in freeze-fracture microscopy the presence of groups or aggregates of particles or pits in replicates of the membrane are counted.

1A.7.1 Thin section morphometry:

Several techniques for analysis and quantitation of gap junctions in thin sections have been developed. Although gap junction length measurements can be made directly on high magnification electron micrographs, accurate measurements depend on the use of quantitative morphometric analysis employing precise sterological methods. Utilizing these methods one can determine the number and area of gap junctions. The most suitable method, however, is to determine junction area which represents the product of gap junction size and frequency and then compare the area of gap junctions to the area of plasma membrane. To accomplish this, the length of the cell plasma membrane is determined by either tracing with a planimeter or by sterological methods. The area of plasma membrane is then calculated from this measurement. The sterological method for the estimation of plasma membrane length, which is based upon principles described by others (Weibel et al., 1969; Weibel, 1973) is generally adopted because it is convenient, less tedious, very rapid and has been demonstrated to yield values for

membrane lengths comparable with those obtained from tracing techniques (Garfield et al., 1980a).

Quantitative techniques in which gap junction frequency is related to other parameters (i.e. such as number of gap junctions per grid square) has also been used in several studies (Merk et al., 1980; Burghardt et al., 1984a; 1984b). It should be noted, however, that this technique provides only semi- quantitative data on gap junction frequency because it is based on the assumption that approximately the same length of plasma membrane is present in every grid square. In many instances this assumption will not be justified, for example following natural cellular hypertrophy, hypotrophy or hyperplasia and hypoplasia and when these events are initiated experimentally. Moreover, this method of quantitation does not take into account gap junction size or area.

1A.7.1.1 Applications and limitations:

Thin section quantitative morphometry on gap junctions has been used to analyze changes in junction size, frequency and area of plasma membrane occupied by these structures in tissues under control and experimental conditions. This technique has been employed successfully by several authors studying the mechanisms responsible for the regulation of gap junctions in the myometrium (Garfield et al., 1980a; Wathes and Porter, 1984). Quantitative thin section morphometry can also be used in evaluating the effectiveness of gap junction isolation procedures by determining the relative number of gap junctions in various cell fractions (Kachura et al., 1983).

One major limitation of quantitative morphometric analysis of thin sections is that the frequency of gap junctions may be underestimated. This results from the inability to identify all the junctions in sections cut at angles which do not reveal the lined structure at least at some portion of the junction. Thin section microscopy does not detect small gap junctions (approximately 30 nm or from 1 to 3 connexons) and this would also lead to an underestimation of the frequency and membrane area of gap junctions.

1A.7.2 Freeze-fracture morphometry:

Quantitative morphometry of gap junctions in freeze-fracture replicas allows the analysis of parameters similar to those outlined for thin sections. It has been suggested, however, that for gap junction demonstration and analysis the freeze-fracture method may be more dependable or accurate than thin section morphometry for various cell types (Griepp and Revel, 1977; Gabella and Blundell, 1981). These claims have been made even though there are only a small number of quantitative studies of gap junctions using freeze- fracture morphometry and no studies comparing the two methods.

Measurements of gap junction area in freeze-fracture replicas can be made by tracing the outline of the junctional and non-junctional plasma membrane fracture faces with either a planimeter or some similar technique and calculating the area sustained by this border. The sterological method described for thin section morphometry can also be applied in estimating plasma membrane and junctional membrane areas in fracture replicas. As well, the frequency of gap junctions can be

of the fetuses (Garfield et al., 1982a). In the same study treatment of ovariectomized rats with exogenous progesterone was sufficient to maintain circulating progesterone levels, prevent the development of myometrial gap junctions and prolong pregnancy. Treatment of similar ovariectomized rats with estradiol resulted in the development of myometrial gap junctions and premature labor but both responses occurred much earlier than in untreated ovariectomized controls (Garfield et al., 1982a). Ovariectomized rats treated exogenously with both estrogen and progesterone maintained circulating hormone levels similar to the levels of intact pregnant vehicle controls and prevented the premature labor.

The appearance of myometrial gap junctions and preterm labor in ovariectomized midterm pregnant rats was associated with hormonal changes similar to that observed prior to normal labor. Exogenous administration of steroid hormones to ovariectomized rats resulted in the maintenance of pregnancy and prevented the development of gap junctions in the myometrium, while administration of estrogen stimulated the presence of gap junctions and initiated premature labor. These findings suggest that progesterone withdrawal or increased estrogen levels may be involved in regulating gap junction development in vivo. In support of these assumptions there is a good correlation between the increased levels of estrogen, decreased levels of progesterone, increased ratios of estrogen to progesterone in maternal and fetal blood and uterine tissues and increased myometrial gap junction area during spontaneous parturition in sheep and rats (Garfield et al., 1979b; Puri and Garfield, 1982).

Withdrawal of progesterone, however, may not be involved in

regulating myometrial gap junctions in all animal species. In the guinea pig large numbers of gap junctions are present at the time of parturition (Garfield et al., 1982b) in the presence of high levels of plasma progesterone (Challis et al., 1971). Furthermore exogenous treatment of progesterone to the guinea pig does not prolong pregnancy (Zarrow et al., 1963; Schofield , 1964). Whether progesterone withdrawal occurs in myometrial tissue of this species or plays any role in myometrial gap junction development remains uncertain.

Investigation into the hormonal regulation of myometrial gap junctions has also been carried out in an in vitro culture system. The advantage of this system is that it allows a variety of agents, thought to be involved in the regulation of gap junctions, to be tested in the absence of other possible complicating humoral factors present in situ. Studies on myometrial tissues from midterm pregnant rats incubated in vitro have revealed that the presence of gap junctions does not require either exogenous estrogen or progesterone, but estrogen stimulates and progesterone, when administered together with estrogen, inhibits the development of gap junctions (Garfield et al., 1978; 1980a). These results are consistent with the hypothesis that withdrawal of an inhibitory factor (i.e. progesterone) and increases in a stimulatory factor (i.e. estrogen), that precede and accompany the development of myometrial gap junctions in parturient animals are responsible for the regulation of their presence.

Protein synthesis appears to be an essential step in myometrial gap junction formation. The changes in levels of steroid hormones may stimulate the synthesis of gap junction proteins. It has been well

present within small aggregations of particles (i.e. 2-4 particles) is very unlikely. Thus, it would be impossible to interpret small aggregates in steps between cell membranes as gap junctions according to these criteria. If the majority of gap junctions in a tissue are small gap junctions, then considerable error will be introduced into any quantitative analysis.

Another disadvantage of freeze-fracture morphometry is its application to a tissue that contracts isotonically, for example, in smooth muscle. Some of the gap junctions in smooth muscle of the myometrium are located on cytoplasmic extensions between smooth muscle cells (Garfield et al., 1978). Conceivably, the junctions may not be included in the fracture plane because the membrane of the cytoplasmic extensions would not frequently occur within the same plane of fracture as the flat surface of cells on large areas of the membrane replicate. The fracture plane may selectively pass through a site on the cellular extension other than the junction. Also, it is extremely difficult to identify gap junctions in small areas of membrane surrounded by extracellular material. Thus, quantitating gap junctions under these circumstances may result in an underestimation of the proportion of gap junction membrane.

1B. PRESENCE OF GAP JUNCTIONS IN SMOOTH MUSCLE

1B.1 Introduction:

During the 1960's, gap junctions between smooth muscle cells were first described by Dewey and Barr (1962). In subsequent years the occurrence of gap junctions in smooth muscles has been widely investigated by electron microscopy and it was found that these junctions are present in most types of smooth muscle (i.e. smooth muscle from the gastrointestinal track, uterus, vas deferens, urinary track, various pupillae and blood vessels, refer to the review by Henderson, 1975). There are, however, certain layers of smooth muscle that may not contain significant numbers of gap junctions. For example, the longitudinal muscle layer of the small intestine of the dog and guinea pig lack gap junctions (Henderson et al., 1971; Gabella, 1972) whereas the circular muscle layer contains a high frequency of them. Furthermore, in the vas deferens gap junctions have been observed by some (Cobb and Bennett, 1969), however, there are many more studies in which gap junctions were not detected in this tissue (Henderson, 1975). According to Henderson (1975), it is possible that in the vas deferens gap junctions may be present only in the circular muscle layer similar to that observed in the small intestine. This assumption was based on the fact that it has been shown that gap junctions are absent from the longitudinal muscle layer of this tissue (Merrillees, 1968) and that in other studies the muscle layer examined is quite frequently not identified.

Thin section electron microscopy has been used in all the early studies on gap junctions. It is possible that the type of fixation may influence the occurrence of gap junctions in smooth muscle. In the uterus myometrial gap junctions were observed in tissues after fixation in permanganate (Bergman, 1968). Fixation with permanganate tends to reduce the separation between cells (decrease extracellular space) and cause changes in cell shape (Bergman, 1968; Henderson et al., 1971). This form of tissue fixation therefore, may produce gap junction like structures between cells and result in an overestimation in the frequency of the junctions. In addition, in taenia coli gap junctions have only been identified in permanganate fixed tissue (Dewey and Barr, 1964; Bennett and Rogers, 1967; Cobb and Bennett, 1969; Nishihara, 1970). Furthermore, when glutaraldehyde is used as the fixative, which allows gap junction demonstration in many types of smooth muscle, it can induce cell shrinkage and therefore a certain amount of separation between cells. It has been suggested that this may open up gap junctions and render them undetectable which could also account for their absence in certain tissues (Gabella and Blundell, 1981). This is not likely since gap junctions have some mechanical strength in linking cells together (Henderson et al., 1971).

These technical problems associated with thin section electron microscopy are partly overcome by freeze-fracture microscopy. The possibility that junctions may be absent in thin sections due to cell shrinkage should not impair the detection of gap junctions as seen in replicas since the connexons remain at the site of junctions even when the cells have been separated (Goodenough and Gilula, 1974). The absence

of gap junctions from the longitudinal muscle layer of the small intestine has been confirmed by freeze-fracture analysis suggesting genuine structural differences between the longitudinal and circular muscularis. In addition, only a few and very small gap junctions have been identified in taenia coli using freeze-fracture microscopy (Gabella, 1976; Fry et al., 1977; Gabella and Blundell, 1981) contrary to the numerous junctions observed in thin sections from permanganate fixed tissues. Freeze fracture microscopy is now frequently used in studying gap junctions and for confirming their presence in a tissue when they have been identified in thin sections.

The presence of gap junctions in vascular smooth muscles was also controversial during early studies on these tissues. Many close oppositions in vascular smooth muscle were originally classified as gap junctions based on the assumption that junction appearance was altered by fixation conditions (Rhodin, 1967). Gap junctions, as defined ultrastructurally, have been demonstrated in smooth muscle of only larger vessels such as the aorta, pulmonary artery and portal mesenteric vein (Henderson, 1975).

Gap junctions are also found in smooth muscle of the uterus. Their presence in this tissue is of great concern to many because the myometrium only intermittently possesses gap junctions. This has been clearly demonstrated in a variety of species by Garfield and collaborators (see section 1B.2). In the uterus towards the end of gestation immediately prior to parturition, the myometrial cells rapidly develop gap junctions only to lose them twenty-four hours after labor. Furthermore, myometrial gap junctions are present in non-pregnant

animals only when the animals have been treated with estrogen (see section 1B.2). Thus, because of the intriguing nature of their presence in the myometrium, their mechanism of regulation is a concern to investigators in reproduction and as well, is the basis for the experimental results present in this dissertation.

Most smooth muscles behave as a functional syncytium. Stimulation of one region leads to a rapid spread of excitation to all regions. From the preceding section it appears that low resistance coupling of cells via gap junctions provides pathways for ion flow through interior of cells implying that these junctions are sites of electrical coupling between smooth muscle cells. Several smooth muscles have been demonstrated to exhibit electrical cell-to-cell coupling and cable like behavior (Abe and Tomita, 1968; Barr et al., 1968; Daniel and Lodge, 1973; Daniel et al., 1976; Kuriyama and Suzuki, 1976a). However, ultrastructural studies failed to detect gap junctions between smooth muscle cells (Garfield and Daniel, 1974; Daniel et al., 1976; Gabella, 1976; Fry et al., 1977). The failure to find gap junctions in coupled systems lead to the proposal that gap junctions may not be necessary for electrical coupling in smooth muscle (Daniel et al., 1976). Those negative results, however, are inconclusive in implying that gap junctions do not mediate the transmission of electrical signals between smooth muscle cells for the following reasons. Ultrastructural studies were not carried out on the same tissues samples that were used for electrophysiological analysis. Gap junctions develop during in vitro incubation of myometrial tissue (Garfield et al., 1978; 1980a) and during electrical recording (Sims et al., 1982). Therefore, gap

junctions may develop in other smooth muscles during electrophysiological studies accounting for coupling of cells. It is also possible that tissues which have been reported to lack gap junctions completely may develop them temporarily at critical moments. Another possibility is that small gap junctions, not detectable by electron microscopy (see above, section 1A.2), may be present between smooth muscle cells thereby justifying coupling between the cells. In addition, small gap junctions may be present in smooth muscle and under physiological conditions may not be coupling cells functionally. When the tissue is removed from the animal the physiological regulation of the junctions may be removed and coupling between cells becomes evident. Recently, ultrastructural analysis of myometrial samples studied electrophysiologically have shown a distinct correlation between electrical coupling and the presence of gap junctions between the smooth muscle cells (Sims et al., 1982).

Henderson (1975) has suggested that gap junctions may also serve as points of mechanical attachment between smooth muscle cells. In the circular muscle of the small intestine intact gap junctions have been observed after hypertonic shrinkage of the cells (Henderson et al., 1971). However, it has also been reported that gap junctions in taenia coli smooth muscle are destroyed following similar treatment (Barr et al., 1968). These results must be interpreted with caution because numerous gap junctions in taenia coli are only observed when the tissue is fixed with permanganate. Therefore, although the assumption that gap junctions provide mechanical strength at points of attachment between smooth muscle cells seems logical, especially in such a tissue that is

active and reactive, further investigation into this possible function for gap junctions is required.

Gap junctions are present between cells of various smooth muscles and appear to provide the physical basis for electrical communication between the muscle cells. The junctions, however, are present between smooth muscle cells of the uterus only intermittently and it seems that these membrane structures may rapidly develop and disappear from the cell surface depending on the physiological condition. This intriguing aspect of gap junctions in this tissue has lead to detailed investigations into gap junction modulation and function in the myometrium.

1B.2 MYOMETRIAL GAP JUNCTIONS

1B.2.1 Presence in the myometrium:

The presence of gap junctions in the myometrium of pregnant animals has been demonstrated by thin section and freeze-fracture electron microscopy in a number of recent studies (Garfield et al., 1977; 1978; 1979a; 1979b; 1980a; 1980b; 1982a; 1982b; Garfield and Hayashi, 1981; Wathes and Porter, 1982; Demianczuk et al., 1984). These authors have shown that gap junctions are present between smooth muscle cells of the myometrium immediately prior to, during and for a short time after parturition.

Studies on a variety of pregnant animals (rats: Garfield et al., 1977; 1978; Wathes and Porter, 1982; sheep: Garfield et al., 1979a;

1979b; guinea pig: Garfield et al., 1979a; 1982; rabbits: Demianczuk et al., 1984; baboons: personal communication from Dr. R.E. Garfield) and humans (Garfield et al., 1979a; Garfield and Hayashi, 1981), have shown a) gap junctions are absent or present at an extremely low frequency throughout gestation, b) at the end of term, prior to parturition, the frequency and size of gap junctions begin to increase, c) during parturition the frequency and size of gap junctions are at a maximum, the junctions are occupying a larger proportion of myometrial membrane area, and d) within twenty-four hours after delivery of the fetus gap junctions begin to disappear from the myometrium. Similarly, in pregnant animals ovariectomized on day 16 of gestation and in others treated with an abortifacient agent, myometrial gap junctions were present in increased numbers and all animals delivered prematurely (Garfield et al., 1977; 1982a; 1982b). Gap junctions are also present in human myometrium from women in preterm labor (Garfield and Hayashi, 1981).

The ultrastructure of the myometrium from non-pregnant animals has also been studied to determine if gap junctions are present between the smooth muscle cells. In studies reported gap junctions were not visible in the myometrium of immature (intact and hypophysectomized) and mature ovariectomized rats and mature virgin mice (Bergman, 1968; Dahl and Berger, 1978; Merk et al., 1980; Burghardt et al., 1984a; 1984b). Gap junctions are present, however, between myometrial cells of these non-pregnant animals after in vivo treatment with very high doses of estrogen. Large numbers of gap junctions are also present in myometrial tissues from non-pregnant animals incubated in vitro and treated with

estrogen (Garfield et al., 1978).

1B.2.2 Function of myometrial gap junctions:

Gap junctions are present between uterine smooth muscle cells of pregnant animals exclusively during term and preterm labor when the myometrium is extremely active and reactive and in the myometrium of non-pregnant animals treated with estrogen which results in spontaneous and rhythmic electrical and mechanical activity (Marshall, 1959; Csapo, 1962; Kao, 1977). These studies suggest that gap junctions play an important role in the function of the uterus.

Garfield and associates have postulated that the presence of gap junctions in the myometrium exclusively at term has significant implications in the maintenance and termination of pregnancy. The increased frequency of gap junctions between myometrial cells during labor may be necessary for the coordinated muscle contractility required for the expulsion of the fetus and termination of pregnancy. It is well known that parturition in all species is accompanied by the development of increased muscular activity of the uterine wall. This activity is thought to be synchronous and coordinated and arise because of a rapid propagation of action potentials throughout the myometrium (Csapo, 1981). The rapid formation of gap junctions prior to and during labor may allow parturition to proceed by providing large areas of low resistance between cells, thereby promoting the spread of current throughout the myometrium resulting in synchronized uterine activity. On the contrary, the absence of gap junctions from the myometrium throughout gestation may maintain pregnancy by limiting electrical

and/or metabolic communication between cells preventing coordinated activity within the uterus.

The relationship between the presence of myometrial gap junctions and electrical coupling between cells during parturition has recently been evaluated. Intracellular microelectrodes have been used to determine space constants and electrical resistance of the myometrium from rats prior to and during labor and delivery (Sims et al., 1982). This study demonstrated larger space constants and lower electrical impedance in the myometrium in tissues from delivering animals. These results provide electrophysiological evidence for improved electrical coupling of myometrial cells during parturition when gap junctions are present.

Electromyographic and intrauterine pressure devices have recorded in vivo electrical and mechanical activity of the myometrium in a variety of animals at various stages of pregnancy and parturition (Fuchs, 1969; Csapo, 1981; Demianczuk et al., 1984). These studies have indicated that electrical activity at different regions of the myometrium throughout pregnancy was infrequent and of low amplitude while that of parturition was rhythmic bursts of short duration at an extremely high frequency. Myometrial membrane area of gap junctions was low throughout gestation followed by a significant increase at the time of delivery. These results also provide evidence indicating increased cell-to-cell coupling and propagation of electrical events from region to region of the myometrium during parturition. Thus, electrical and mechanical activity required for effective labor occurs only when large numbers of gap junctions are present between myometrial cells. Taken

together with the results from the former study (i.e. Sims et al., 1982), it is clearly evident that one role for gap junctions in the myometrium is to provide low resistance electrical coupling between cells.

Another possible role for gap junctions in the myometrium may be to couple cells for metabolic cooperation. Studies on the structure and function of gap junctions (see section 1A.2) have shown that substances other than small ions are exchanged by cells connected by gap junctions. In addition, many non-excitable cell types exhibit gap junction communication competence supporting the belief that these cell-to-cell contacts provide the basis for a mode of coupling independent of the transmission of electrical signals. Loewenstein (1981) has postulated that the most basic function of a gap junction is to maintain homeostasis and to buffer variations in channel-permeant molecules. These molecules including nutrients, regulatory molecules such as cyclic nucleotides and perhaps growth regulating factors would be rapidly distributed between all cells coupled by the junctions. That gap junctions provide the basis for metabolic coupling between myometrial or other smooth muscle cells, however, has not been demonstrated. Preliminary experiments directed towards this question have indicated that in vitro diffusion of 2-deoxyglucose in myometrial strips is greater in tissue from delivering animals (Cole et al., 1983). This suggests that myometrial cells are better coupled metabolically during parturition when gap junctions are present in high frequency. The significance of metabolic coupling during parturition, however, is not completely understood. It is possible that metabolic cooperation of

myometrial cells is essential for the energy expenditure required for the expulsive force of the myometrium during labor.

Another role for gap junctions may be to control responses of the myometrium to drugs and receptor mechanisms. When gap junctions are present in the myometrium the tissue would be expected to be more sensitive to a given agonist than a tissue without gap junctions. The presence of gap junctions between cells may mean that only some of the receptors for the agonist need to be occupied as the reaction which is generated when the receptors are occupied is propagated from cell-to-cell via the junctions. On the other hand, when gap junctions are absent from the tissue a higher concentration of the agonist may be required to produce a response because the receptors on most of the cells would have to be occupied. Perhaps this mechanism is responsible for increased reactivity and sensitivity of the myometrium to oxytocin and other agonists at term (Soloff et al., 1979).

Gap junctions in a tissue may in a similar way play a role in controlling responses to the release of transmitters from nerves. It has been proposed that gap junctions are necessary for coupling of smooth muscle cells in sparsely innervated tissue where only a few smooth muscle cells in a muscle bundle are innervated (Burnstock, 1980). The electrical response generated in these cells by the release of transmitter and interaction with membrane receptors is believed to be transmitted from cell-to-cell via gap junctions. This response mechanism may be important in the myometrium of parturient animals. In the myometrium adrenergic nerves are thought to degenerate during late stages of gestation (Sporrong et al., 1978; Thorbert et al., 1979;

Marshall, 1981) when gap junctions are present between muscle cells. Therefore, the sparsely innervated myometrium of late gestation and parturition may require the presence of gap junctions for neural responses of the uterus.

1B.2.3 Functional control of myometrial gap junctions:

It is likely that the channels created by gap junction connexons in the myometrium can exist in either an open or closed state as they do in other cell systems (see section 1A.3). The presence of gap junctions in the myometrium during parturition when the muscle is active and reactive, implies that the smooth muscle cells are functionally coupled and the channels created by the connexons are open. Garfield et al., (1980b) have demonstrated electron opaque deposits associated with myometrial gap junctions in tissue from midterm pregnant rats incubated in vitro with β-adrenergic agonists. These agonists activate adenylate cyclase causing elevations in cAMP levels which results in relaxation of the smooth muscle. The presence of electron dense deposits associated with the junctions may indicate that myometrial gap junctions are associated with a cAMP-dependent calcium transport or binding process (Garfield et al., 1980b). Similar deposits have been observed in other tissues where they are believed to represent calcium phosphate crystals formed during hydrolysis of adenosine triphosphate by an enzyme associated with gap junctions (Larsen, 1975; 1977b).

Gap junctions may also represent aggregates of receptors or adenylate cyclase because there is good correlation between binding of some hormones, the activation of adenylate cyclase and the presence of

gap junctions in a variety of tissues (Larsen, 1977b). Receptor sites for oxytocin increase prior to and during parturition (Soloff et al., 1979), coinciding with the presence of gap junctions between myometrial cells. If exteriorized gap junction connexons are oxytocin binding sites, the binding could result in a conformational change in the connexons and alter the permeability of the junction channels. Further studies are needed to clarify this relationship, if it exists in the myometrium.

1B.2:4 Regulation of myometrial gap junction formation:

Garfield and associates have carried out several studies investigating the mechanisms which control the presence of myometrial gap junctions. The presence of gap junctions in the myometrium of parturient animals appears to be regulated by the changes in levels of steroid hormones and prostaglandins which immediately precede labor.

1B.2.4.1 Steroid hormones:

It has been proposed that the steroid hormones regulate gap junction development in the myometrium (Garfield et al., 1977; 1978; 1979a; 1979b; 1980a; 1980b; 1981; 1982a; 1982b; Puri and Garfield, 1982). Progesterone treatment of term pregnant rats prevented the increase in myometrial gap junctions and normal labor (Garfield et al., 1980a). Other studies in which pregnant animals were ovariectomized on day 16 of gestation to deprive the animals of their primary source of steroid hormones resulted in a withdrawal of circulating progesterone, inhibition of myometrial gap junction development and premature delivery.

of the fetuses (Garfield et al., 1982a). In the same study treatment of ovariectomized rats with exogenous progesterone was sufficient to maintain circulating progesterone levels, prevent the development of myometrial gap junctions and prolong pregnancy. Treatment of similar ovariectomized rats with estradiol resulted in the development of myometrial gap junctions and premature labor but both responses occurred much earlier than in untreated ovariectomized controls (Garfield et al., 1982a). Ovariectomized rats treated exogenously with both estrogen and progesterone maintained circulating hormone levels similar to the levels of intact pregnant vehicle controls and prevented the premature labor.

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regulating myometrial gap junctions in all animal species. In the guinea pig large numbers of gap junctions are present at the time of parturition (Garfield et al., 1982b) in the presence of high levels of plasma progesterone (Challis et al., 1971). Furthermore exogenous treatment of progesterone to the guinea pig does not prolong pregnancy (Zarrow et al., 1963; Schofield , 1964). Whether progesterone withdrawal occurs in myometrial tissue of this species or plays any role in myometrial gap junction development remains uncertain.

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Protein synthesis appears to be an essential step in myometrial gap junction formation. The changes in levels of steroid hormones may stimulate the synthesis of gap junction proteins. It has been well

established that steroid hormones regulate protein synthesis in the uterus (see section 1C.4.1.1). Furthermore, in the myometrium estrogen stimulation is required to achieve the full development of gap junctions as well as to permit the inhibition exerted by progesterone (Garfield et al., 1980a). An inhibitor of protein synthesis, cycloheximide, prevents the expression of gap junctions in myometrial tissues incubated in vitro (Garfield et al., 1980b). It has also been suggested that estrogen stimulation results in the transcription of a specific mRNA coding for the protein associated with gap junctions (Dahl et al., 1980). These studies confirm that protein synthesis is essential for the expression of gap junctions in the myometrium.

1B.2.4.2 Prostaglandins:

The work of Garfield and associates have also implicated a direct influence of prostaglandins in the development of myometrial gap junctions. Indomethacin, (a prostaglandin synthesis inhibitor), an inhibitor of thromboxane A_2 synthesis and a prostacyclin analogue (carbacyclin) prevent the appearance of myometrial gap junctions in vitro (Garfield et al., 1980a; 1980b). These studies indicate that there may be prostaglandins that stimulate gap junction development (i.e. thromboxane and other possible endoperoxides) and other prostaglandins that inhibit the process (i.e. prostacyclin). It has been proposed that prostaglandins may control the aggregation of gap junction connexons by effecting cross-linking reactions or by altering the fluidity of the membrane (Garfield et al., 1980b). Alternatively, the prostaglandins may influence the presence of myometrial gap junctions by regulating the

steroid receptors. Prostaglandins are synthesized by the uterus (Phillips and Poyser, 1981) and are thought to play a role in regulating uterine contractions (Thorburn and Challis, 1979). Consequently, the increase in prostaglandin synthesis immediately before and during labor (Thorburn and Challis, 1979; Puri and Garfield, 1982) may be involved in the in vivo regulation of myometrial gap junctions.

Prostaglandins may also indirectly increase the synthesis of gap junction connexons rather than, or in addition to acting directly on gap junction development. The administration of prostaglandins to terminate pregnancy may alter steroid hormone synthesis and effect changes in the hormonal profile similar to that observed prior to and during labor. Termination of pregnancy in rats with $PGF_{2\alpha}$ has been related to changes in progesterone levels (Fuchs, 1978).

1B.2.5 Regulation of gap junctions in non-pregnant animals:

Hormonal regulation of myometrial gap junctions has also been investigated in non-pregnant animals. Bergman (1968) showed gap junctions between myometrial cells when he injected virgin mature rats with a 500 ug/day dose of diethylstilbestrol over a period of five days. Bergman's results and conclusions, however, were criticized because they were based on myometrial tissues fixed in permanganate which is thought to destroy and obliterate detail in uterine fine structure and produce "gap junction" like contacts between cell (Garfield and Daniel, 1974; Daniel et al., 1976). Thin section electron micrographs presented in Bergman's report are questionable with respect to demonstrating gap junctions between the muscle cells. In addition, he did not provide any

quantitative estimates supporting a significant increase in the frequency, size or membrane area of the junctions. Later studies carried out on glutaraldehyde and osmium fixed tissues have supported the relationship between estrogenic stimulation and the presence of myometrial gap junctions in mature virgin mice (Dahl and Berger, 1978) and in intact and hypophysectomized immature rats (Merk et al., 1980; Burghardt et al., 1984a; 1984b). These studies demonstrate that in the absence of ovarian and adrenal steroid hormone production, gap junctions are present in the myometrium of non-pregnant animals after treatment with high doses of estrogen. However, Garfield et al., (1978) failed to find myometrial gap junctions when lower doses of estradiol were injected into rats. In the study by Dahl and Berger (1978), they reported that formation plaques and preformed junctions were not detected by freeze-fracture microscopy in the myometrium of virgin mice treated with estrogen, indicating that totally new gap junction formation occurred in response to hormone treatment. These studies indicate that estrogen is involved in regulating the development of gap junctions in vivo. On the other hand, Wathes and Porter (1982) have shown that estrogen (7.5 ug) administered to ovariectomized post-partum animals stimulates large numbers of myometrial gap junctions only when the uterus is distended suggesting an indirect effect of estrogen on junctional membrane. Therefore, studies in the regulation of myometrial gap junctions in tissue from non-pregnant animals are limited. Further investigation is required for clarification of the role of steroid hormones in the control of gap junction development in these animals.

1B.2.6 Degradation of myometrial gap junctions:

Gap junctions once formed between uterine smooth muscle cells during labor significantly decrease in frequency to preterm levels following delivery of the fetus (Garfield et al., 1979; Wathes and Porter, 1982; Demianczuk et al., 1984). The mechanism by which gap junctions are removed from between myometrial cells is not clear. It is likely that gap junctions become interiorized within one of the cells connected by the junction and are digested by an endocytotic-lysosomal mechanism (see section 1A:5). The observation of annular gap junctions in thin sections of myometrial tissue supports this mechanism of destruction (Garfield et al., 1980b). The interiorized gap junctions are independent of the plasma membrane and adjoining cell as verified with extracellular tracers (personal communication from Dr. R.E. Garfield). Garfield et al., (1980b) observed extremely large gap junctions on invaginations between cells suggesting an initial step of interiorization possibly through the aggregation of smaller junction plaques and similar to that observed in other cell systems (Larsen, 1983). Based upon the size and area of annular gap junctions in the myometrium and size and area of intercellular junctions, Garfield et al., (1980b) have suggested that approximately ten of the smaller gap junctions could aggregate together prior to interiorization. They have also postulated that approximately twenty annular gap junctions would have to form between any two myometrial cells to degrade all of the intercellular junctions.

Another possibility is that the junctional connexons may detach

any connections between cells and individual connexons become dispersed within the cell membrane (see section 1A.5). Dahl and Berger (1978) have shown that following parturition in the mouse gap junctions seem to disaggregate and that strands or patches of particles were observed implying dispersal of gap junction connexons as the mechanism for disappearance from the myometrium. According to their study, gap junctions were maximally developed on the day of delivery and one day after parturition. The frequency and size of gap junctions began to decrease forty-eight hours after parturition. The post-partum disappearance of gap junctions from the myometrium as they report is not consistent with many other studies in a variety of animal species (Garfield et al., 1978; Puri and Garfield, 1982; Wathes and Porter, 1982; Demianczuk et al., 1984). These studies have shown that the frequency, size and myometrial membrane area of gap junctions decreases to near preterm levels one day post-partum and are almost undetectable by forty-eight hours after parturition. It is possible that that the small junctions observed by Dahl and Berger (1978) two days after parturition may represent gap junctions that have not coalesced with other junctions as the initial step of junction interiorization.

The studies outlined above show that gap junctions are present, only intermittently, between the smooth muscle cells of the uterus immediately prior to labor, during parturition and for a short time after delivery and in non-pregnant animals treated with estrogen. In addition, it has also been demonstrated how hormonal changes can be translated into structural changes in the myometrium. The presence of gap junctions in the myometrium of pregnant animals at term appears to

be requisite for labor and necessary for coordinated electrical and mechanical activity required for the termination of pregnancy. Immediately following parturition, gap junctions disappear from the myometrium. Moreover, the rapid development and regression of the junctions from the myometrium may imply that smooth muscle tissues that have been characterized as possessing gap junctions may loose them temporarily whereas tissues which are devoid of the junctions may possess them transiently at certain times under appropriate conditions.

1C. CONTROL OF LABOR

1C.1 Introduction:

The uterus is a relatively quiescent organ throughout most of pregnancy allowing for an environment essential for implantation and development of the fetus. Normally at term when the fetus has developed sufficiently to survive outside the mother, the muscle of the uterus undergoes a transition from the calm gestational state to an active and reactive tissue contracting rhythmically and forcefully resulting in the expulsion of the fetus and termination of pregnancy.

The underlying basis for this conversion in uterine activity at the end of gestation that is essential for successful labor has been the focus of debate. It is thought that the sequence of hormonal changes that precede labor (term or preterm labor) is responsible for the transition of myometrial activity required for parturition. Whether the maintenance of pregnancy and the conversion from relative quiescence to the activity of labor is a consequence of a release from an inhibition or the result of a lack of stimulation is still a matter of controversy. It is known, however, that myometrial tissue from term and preterm delivering animals are capable of generating similar tension when stimulated (Csapo, 1977). Therefore, the onset of labor cannot be a consequence of a change in the working capacity of the muscle. Numerous studies in animals have indicated that myometrial activity is under control by hormonal mechanisms (Liggins, 1979; Anderson et al., 1981; Challis and Mitchell, 1981; Csapo, 1981) and that the maintenance of

pregnancy and initiation of labor is accomplished through the action of the hormones on the myometrium.

1C.2 Endocrine changes at parturition:

A cascade of endocrine events occurs late in pregnancy and these changes have been elucidated in a variety of experimental animals, as well as in humans (Heap et al., 1973; Nathanielsz, 1978; Liggins, 1979; Thorburn and Challis, 1979; Challis and Mitchell, 1981; Csapo, 1981; Liggins, 1981; Puri and Garfield, 1982). These experimental animals have been grouped into three categories with respect to investigations into the hormonal control of labor. The categories are: 1) species in which the corpus luteum is the sole source of progesterone (i.e. rabbit), 2) species in which the corpus luteum is the main source of progesterone although the placenta secretes sufficient progesterone late in gestation to maintain pregnancy but requires luteolysis at term for the withdrawal of progesterone (i.e. rat), and 3) species at the other extreme in which the corpus luteum contributes little progesterone throughout much of pregnancy and the placenta is the major source of the hormone (i.e. primates and sheep). The species within the third group, however, are different in that the placental progesterone declines sharply at term in the sheep whereas the levels in primates remain constant. Thus it appears that the mechanisms of parturition are identifiable as luteolysis (regression of the corpus luteum); inhibition of placental progesterone production; and a mechanism apparently independent of progesterone.

1C.2.1 Progesterone:

In species such as the rat in which luteolysis is essential for labor to occur, the hormonal changes of parturition have been clearly described (Thorburn and Challis, 1979; Puri and Garfield, 1982). Progesterone production is predominately ovarian but a placental contribution late in gestation is supplementary. The principle cells of the ovary responsible for the production of progesterone are those of the corpus luteum. The corpus luteum synthesizes cholesterol from acetate and then converts it to 5-pregnenolone. 5-pregnenolone is then hydroxylated by 3B-hydroxysteroid dehydrogenase to form progesterone.

When mating occurs cervical stimulation causes the release of luteotropic hormone from the pituitary which maintains the corpus luteum for continued production of progesterone. Luteotropic hormone suppresses the activity of 20α-steroid dehydrogenase which is responsible for the catabolism of progesterone by converting the hormone to a less active 20^{α}-hydroxyl derivative. If nidation occurs luteotropins from the placenta relieve the pituitary of this function. Functional persistence of the corpus luteum throughout pregnancy allowing for the continued secretion of progesterone is the result of the production and secretion of chorionic gonadotropin by the chorion cells of the placenta.

The high circulating plasma levels of progesterone throughout gestation falls sharply a few days prior to term or preterm labor. This decrease in secretion of progesterone into the blood coincides with a decline in the secretion of luteotropic hormone from the placenta. This leads to a decrease in the synthesis of progesterone through the removal

of the inhibition of 20α-steroid dehydrogenase activity. In addition, the high levels of progesterone during gestation inhibits luteinizing hormone release from the pituitary which stimulates the activity of 20α-steroid dehydrogenase. Therefore, decreased synthesis of progesterone prior to term results from the withdrawal of luteotropic hormone together with a subsequent removal of the inhibition to luteinizing hormone release.

Luteolysis may result from either a withdrawal of luteotropic support, from a luteolytic signal, or from both. Rat placental lactogen, a luteotropic factor (Thorburn and Challis, 1979) progressively falls in concentration prior to term and may initiate labor (Shiu et al., 1973). On the other hand, prostaglandin $F_{2\alpha}$ has been implicated in luteal regression. On day 22 of gestation in the rat when the progesterone levels have fallen, there is a concomitant rise in prostaglandin $F_{2\alpha}$ levels in the uterus. That progesterone levels fall prior to the increase in prostaglandin $F_{2\alpha}$, however, would indicate that this prostaglandin is not required for luteolysis. Williams et al., (1974) suggests that the increase in prostaglandin $F_{2\alpha}$ levels at term brings luteolysis to completion.

In species dependent on the placenta for the production and secretion of progesterone (i.e. sheep and primates), the circulating levels of progesterone are relatively high throughout gestation. Placental progesterone secretion in the sheep falls profoundly near term or preterm labor (Nathanielsz, 1978; Thorburn and Challis, 1979) and is believed to be a prerequisite for parturition. Progesterone is synthesized de novo by the placenta. The placenta has enzymes for the

synthesis of cholesterol from acetate and for the production of 5-pregnenplone from cholesterol. These enzymatic conversions together with those involved in the formation of progesterone in the placenta are similar to, or the same as the enzymatic mechanisms found in other endocrine tissues such as described in the corpus luteum of the ovary.

The lytic agent responsible for placentolysis (Liggins, 1981) in the sheep and decrease in placental production of progesterone prior to labor appears to be of fetal origin. In the sheep, the fetal pituitary-adrenal axis has been shown to play a central role in the mechanisms that initiate parturition (Nathanielsz, 1978; Thorburn and Challis, 1979; Challis and Mitchell, 1981). Labor can be induced by infusing adrenocorticotropic hormone or glucocorticoids to the fetal lamb *in utero*, or prevented by surgically removing the fetal but not the maternal pituitary or adrenal glands. The secretion of fetal cortisol triggers the activation of placental steroid 17α-hydroxylase (Flint *et al*., 1975). The activity of this enzyme is low throughout pregnancy in the sheep, but as cortisol concentrations rise in the fetal circulation as term approaches, 17α-hydroxylase is activated. As a consequence, progesterone is metabolized leading to a decrease in maternal plasma and tissue concentrations.

In contrast, primates are dependent on the placenta for progesterone production and secretion but the circulating levels of the hormone remain constant throughout gestation and labor. There is no experimental evidence, as in the sheep, to support a decrease in plasma progesterone concentration prior to parturition. Moreover, there is no persuasive evidence to show that fetal cortisol triggers parturition in

primates as described in the sheep because in both the monkey and human exogenous glucocorticoids fail to induce parturition (Nathanielsz, 1978; Challis and Mitchell, 1981). Similarly, both fetal hypophysectomy and decapitation cause disordered pregnancy lengths in the rhesus monkey and human pregnancies complicated by anencephaly are approximately normal (Liggins, 1981). Primate placentas lack the enzyme 17α-hydroxylase thereby explaining the ineffectiveness of fetal cortisol and the high levels of progesterone at term. Thus, the role of progesterone in the initiation of primate parturition is less clear and has been the subject of much discussion.

1C.2.2 Estrogen:

Circulating plasma and tissue levels of estrogen also change as term approaches during pregnancy. Estrogen production in the rat (species dependent on the corpus luteum for progesterone) is mainly ovarian (Thorburn and Challis, 1979). The theca interna cells are the primary source of estrogen which is synthesized through the common steroid biosynthetic pathway from acetate via cholesterol, pregnenolone and progesterone. In the theca cells estrogen production for the most part is through pregnenolone. 5-pregnenolone is hydroxylated forming the corresponding hydroxysteroid. A desmolase enzyme then cleaves the 17-β side chain of pregnenolone to form dehydroepiandrosterone which is converted to its 3-keto derivative (5-androstene-3, 17-dione) by the enzyme Δ5,3βhydroxysteroid dehydrogenase before being aromatized to estrogen. Estrogen is also synthesized and secreted to a much lesser extent by the granulosa cells and corpus luteum. Progesterone

synthesized by these cells can be converted into 4-androstenedione similar to dehydroepiandrosterone from pregnenolone in the theca cells. 4-androstenedione is aromatized to estrone and then a 17-dehydrogenase enzyme converts estrone into estrogen. Similarly, androstenedione can be converted to testosterone by a 17-dehydrogenase enzyme and then aromatized to estrogen.

Estradiol levels are low throughout most of pregnancy in the rat. The secretion of estradiol, however, begins to increase a few days before and reaches peak levels the day of parturition. It is believed that the pituitary controls the increased secretion of estradiol seen in pregnant rats before expected parturition because hypophysectomized animals show no similar increase (Waynforth and Robertson, 1972). The principle site of origin of the circulating estradiol in pregnant rats appears to be the ovary. The corpus luteum secretes estradiol but the major source is the non-luteal tissue, the follicle containing part of the ovary. The secretory activity of the follicular tissue correlates with the high content of estradiol found in the non-luteal tissue on day 21 of pregnancy (Waynforth and Robertson, 1972). An increase in pituitary FSH prior to parturition reaching maximum levels on day 21 (Greenwald, 1966) supports the follicular source of estradiol. In addition to the ovarian source of estradiol in the rat, the fetus and placenta also contribute to the circulating estradiol levels at term (Gibori and Sridaran, 1981). Since the fetal ovaries have no steroidgenic activity and the fetal liver is rich in aromatase enzyme (Quattropani and Weisz, 1973; Schindler, 1975), the probable site of estradiol production in the rat fetus is the hepatic tissue (Gibori and

Sridaran, 1981). The placental contribution of estradiol arises from the production of androgen precursors which are aromatized in peripheral tissue. Whether the fetal liver can aromatize placental androgens to estradiol remains to be shown.

The changes in maternal plasma estrogen levels in the sheep (dependent on the placenta for hormone production) has been well documented (Challis, 1971; Thorburn and Challis, 1979; Anderson et al., 1981). Twelve to twenty four hours before delivery the plasma estrogen levels dramatically rise and it appears that the sequence of enzymatic events that control the production of placental progesterone is responsible for this increase. Immediately prior to term fetal cortisol triggers placental steroid 17α- hydroxylase which metabolizes progesterone into estrogen. There are three major steps in the conversion of progesterone to estrogen by the sheep placenta. First, 17-hydroxylation of progesterone to 17α-hydroxyprogesterone followed by cleavage of the C-17,20 bond by C-17,20 lyase to produce androstenedione and finally the aromatization of this androgen to estrogen.

The primate placenta also produces estrogen throughout pregnancy. There is, however, no dramatic increase in maternal estrogen levels within twenty-four hours of parturition similar to that observed in the sheep. Estrogen levels do increase over the last course of gestation in both women and rhesus monkey (Anderson et al., 1981; Challis and Mitchell, 1981). Unlike the sheep placenta, the primate placenta is not capable of synthesizing estrogen de novo from acetate or cholesterol, possessing no 17α-hydroxylase or C-17,20 lyase activity. Instead, the primate placenta has a great capacity to carry out

aromatization of C-19 androgenic steroid to estrogen. The androgen precursors for estrogen biosynthesis in the placenta come from both the fetal and maternal adrenal cortex (Nathanielsz, 1978; Thorburn and Challis, 1979; Anderson et al., 1981; Challis and Mitchell, 1981; Liggins, 1981). The fetal primate adrenal lacks both Δ5,Δ4- isomerase and 3β-hydroxysteroid dehydrogenase. As a result, the fetal adrenal secretes large quantities of the Δ5-androgen, dehydroepiandrosterone sulphate. The biosynthesis of this is as described in the theca cells of the ovary. Following 16α-hydroxylation of dehydroepiandrosterone sulphate in the fetal liver to form 16α-hydroxydehydroepiandrosterone sulphate, hydrolysis of the conjugate by placental sulphatase enzyme liberates the unconjugated 16α-hydroxydehydroepiandrosterone which is then aromatized to estriol by the placenta. Estriol synthesis, therefore, is a reflection of the capacity of the fetal adrenal cortex to provide the precursor as 16α-hydroxydehydroepiandrosterone and is independent of the pathway for placental estrone and estradiol-17β synthesis. Siiteri and MacDonald (1966) have shown that the human placenta utilizes approximately equal amounts of dehydroepiandrosterone from the maternal and fetal adrenals for the production of estradiol whereas, the contribution of maternal dehydroepiandrosterone for estriol production is less than 10%. The steroid sulphatase activity in the placenta hydrolyzes the sulphated dehydroepiandrosterone to yield the unconjugated form which is transformed in the placenta to form androstenedione, then aromatized to estrone and estradiol-17β.

In pregnant women with anencephalic fetuses which have an atrophic adrenal cortex, there is greatly diminished estriol secretion.

In this condition, androstenedione and dehydroepiansterone are apparently supplied by the maternal adrenal cortex in quantities sufficient to provide precursors for aromatization to estrone and estradiol.

1C.2.3 Prostaglandins:

Prostaglandins are now widely accepted as an important link between the various mechanisms involved in the initiation of parturition. This is true for all species studied regardless of whether the maintenance of pregnancy is corpus luteum or placenta dependent. Similar to the changes in steroid hormones around parturition, prostaglandin levels also change. In this section I will describe the synthesis and degradation of prostaglandins as a general occurrence in all species investigated.

The availability of arachidonic acid to prostaglandin synthetase, the precursor of $PGF_{2\alpha}$, PGE_1, PGE_2, PGI_2 and TXA_2 (thromboxane A_2), is usually considered to determine the rate of synthesis of the prostaglandins. In the cell arachidonic acid exists as various esters, predominately glycerolphospholipids. Release of arachidonic acid into the free form is controlled by the activity of an acyl hydrolase, phospholipase A_2, which is present in an inactive membrane bound form localized mainly in lysosomes of decidua and fetal membranes (Liggins, 1981). Physiological stimulation or inhibition of prostaglandin synthesis is thought to occur by altering the stability of membranes binding phospholipase A_2. Once phospholipase A_2 liberates arachidonic acid it must then reach the sites of prostaglandin synthesis

which are all uterine tissues including myometrium, endometrium, chorion, amnion and fetal membranes (Liggins, 1981). Towards term in the human, decidua and fetal membranes incorporate large quantities of arachidonic acid into the glycerolphospholipid stores and the amnion has appreciable phospholipase A_2 activity. These events parallel the rise in prostaglandin levels at term suggesting that phospholipase A_2 is the major regulatory step in prostaglandin synthesis (MacDonald et al., 1978).

Tissues capable of synthesizing prostaglandins also contain a catabolic enzyme, 15-hydroxy-prostaglandin dehydrogenase. The activity of this enzyme is very high throughout pregnancy in all species and is almost undetectable at the time of parturition. For example, in the pregnant rat uterus, the activity of this enzyme is high during pregnancy and then the activity begins to decrease on day 19 of gestation and becomes progressively less active until days 21-23 when it is almost undetectable (Nathanielsz, 1978: Thorburn and Challis, 1979; Liggins, 1981). It appears that the steroid hormones control the activity of 15-hydroxy-prostaglandin dehydrogenase. The rise in prostaglandin levels prior to parturition in the rat coincides with the fall in progesterone and increase in estrogen production. In addition, estrogen treatment depresses and progesterone treatment stimulates the activity of 15-hydroxy- prostaglandin dehydrogenase (Liggins, 1981). These observations support the hypothesis that during pregnancy high levels of enzyme activity prevent the synthesis of uterine prostaglandins essential for the maintenance of pregnancy. In species such as the rat, the decline or withdrawal of progesterone and increase

in estrogen leads to an increase in tissue concentration of prostaglandins. In the rat prostaglandins are also considered to be involved in luteolysis thereby helping to bring about the decrease in progesterone production from the corpus luteum. In the human, however, 15-hydroxy-prostaglandin-dehydrogenase activity in the placenta does not vary with gestational age or with the stage of labor (Nathanielsz, 1978) suggesting that uterine degradation of prostaglandins is unlikely to have an important function in the initiation of human labor (Liggins, 1981).

It is quite apparent that the metabolism and synthesis of prostaglandins during gestation and prior to parturition results from the change in the ratio of estrogen to progesterone. Subsequently, factors regulating steroid synthesis would also be an important link in the control of prostaglandins (i.e. fetus). Administration of estrogen stimulates release of prostaglandins from the uterus and progesterone coadministered with estrogen inhibits estrogen stimulated prostaglandin synthesis (Liggins, 1981). The steroid hormones are believed to regulate the synthesis of prostaglandins by uterine tissue through a direct action on the lysosomes of decidua and fetal membranes (Challis and Mitchell, 1981; Liggins, 1981). Progesterone exerts a stabilizing effect on the lysosomal membrane preventing prostaglandin synthesis and release. Estrogen, on the other hand, has a labilizing effect on the lysosomal membrane to initiate the synthesis and release of prostaglandins. The steroid hormones regulating the stability of the lysosomal membrane would control the levels of tissue phospholipase A_2 activity thereby monitoring the synthesis of prostaglandins.

Alternatively, progesterone may be causing the storage of prostaglandin precursors in decidual and other tissues while the action of estrogen may be mediated by the activation of cyclooxygenase (Liggins, 1981).

Species in which the ratio of estrogen to progesterone remains static at the time of parturition (i.e. human), the above mechanisms regulating the release of prostaglandins at parturition are less satisfactory. In these species a variety of alternative hypotheses have been proposed. A progesterone binding protein in the cytosol of near term fetal membranes may compete with progesterone binding sites on lysosomes causing labilization of the lysosomes and activation of phospholipase A_2 (MacDonald et al., 1978). It is also possible that estrogens in the amniotic fluid gain access to fetal membranes interacting with lysosomes and exerting a labilizing effect on their membranes (Challis and Mitchell, 1981). The near term human fetal adrenals may also secrete increasing quantities of non-glucocorticoids which could be excreted into the amniotic sac in fetal urine and act on lysosomes in fetal membranes to initiate prostaglandin synthesis and release (Liggins, 1981).

1C.3 Hormonal profile of the reproductive cycle:

Details of the mammalian reproductive cycle are best known in the laboratory animals and in the human. The endocrine changes and control of the cycle are similar between these species, however, the duration of the cycle varies considerably. For example, in the rat the cycle is over a four day period, whereas in the human the cycle ranges from twenty-one to thirty-six days (Fink, 1976).

The first stage of the cycle is the follicular phase. This stage is characterized by follicular growth and secretion of estrogens (i.e. estrone, estriol and estradiol) (Owens, 1975; Fink, 1976). Follicular growth and subsequent estrogen production is stimulated by high plasma levels of follicle stimulating hormone (FSH) during the luteal phase and menstrual period of the cycle (see below). The elevated estrogen levels during the follicular phase sensitizes follicular tissue for a greater response to FSH.

Increased estrogen production by the maturing follicle is a positive stimulus for the secretion of luteinizing hormone (LH). Fink (1976) has reported that this is initiated by the steroid increasing the responsiveness of the pituitary gland to gonadotropin releasing hormone, which stimulates the pituitary to discharge gonadotropins, and possibly through a direct stimulation of the gland for the release of LH. LH secretion rises gradually and at the time of ovulation (i.e. rupture of the mature follicle) dramatically shoots up to peak plasma levels. Coincident with this event is a smaller increase in plasma levels of FSH, a dramatic fall in estrogen secretion and the beginning of the synthesis and secretion of progesterone. The surge of LH prior to and at the time of ovulation leads to the increase in ovarian progesterone secretion (Fink, 1976).

The corpus luteum is formed following ovulation and defines the second stage of the cycle as the luteal phase. It has been reported that luteinization is stimulated by the LH surge of ovulation (Owen, 1975), but others have suggested that the release from an inhibitory effect by the ovum of the mature follicle may be more important in initiating this

stage of the cycle (Nalbandov, 1970). The life cycle of the corpus is characterized by a sudden fall in LH and FSH secretion, a gradual increase in the secretion of progesterone and a second but smaller rise in estrogen levels. This change in estrogen would be expected to stimulate LH (as above), but it does not as revealed by the low plasma LH levels. A possible explanation for this paradox is that progesterone in high concentration may be suppressing LH release from the pituitary (Owen, 1975). Towards the end of the luteal phase, new follicular growth begins (controlled by FSH as indicated above) in preparation for another cycle. At this time of the cycle, which is accompanied by a fall in both estrogen and progesterone levels, menstruation begins during which the inner portion of the endometrium is exfoliated. The steroid hormones throughout the cycle prepare the uterus to receive the fertilized egg in preparation for pregnancy. If conception does not occur then the former event, menstruation, proceeds.

The reproductive cycle is also characterized by changes in the levels of uterine prostaglandins. During the follicular and luteal phase when estrogen levels are elevated there is a marked increase in the concentration of the prostaglandins $F_{2\alpha}$ and E_2 (Downie et al., 1974; Maathus and Kelly, 1978). This correlation between endometrial prostaglandins and estrogen concentration reflects an effect of estrogen on the synthesis of prostaglandins (see section 1C.2.3). The levels of prostaglandins are also high at menstruation (Downie et al., 1974; Maathus and Kelly, 1978). Although the levels of prostaglandins throughout the cycle have been well documented, their physiological function(s) are not exactly understood. It is known that the myometrium

is active throughout the cycle, in particular at the time of menstruation (Csapo and Pinto-Duntas, 1966). Prostaglandins have been implicated in the regulation of myometrial contractility (Thorburn and Challis, 1979) and painful contractions of the myometrium (dysmenorrhea) frequently accompanies menstruation. Therefore, the high levels of prostaglandins may contract the myometrium during menses and give rise to dysmenorrhea as based on observations that the contractions can be alleviated by inhibitors of prostaglandin synthesis (Pulkinen and Csapo, 1978; Fuchs et al., 1979).

Ovulation and menstruation are preceded and controlled by changes in levels of the steroid hormones and prostaglandins. Similarly, estrogen, progesterone and prostaglandins levels change prior to term or preterm labor which precede and control myometrial contractility of labor (Thorburn and Challis, 1979; Garfield et al., 1978; 1982). Moreover, it appears that the development of gap junctions between myometrial cells at these times of pregnancy are necessary for synchronized coordinated muscle activity and that their presence may be controlled by the change in levels of hormones and prostaglandins (Garfield et al., 1978). Therefore, it is tempting to speculate that the changes in the synthesis and secretion of the steroid hormones and prostaglandins that accompany ovulation and menstruation may stimulate the development of myometrial gap junctions leading to activation of the muscle and dysmenorrhea. In addition, the inhibitors of prostaglandin synthesis may relieve the pain associated with dysmenorrhea (Pulkinen and Csapo, 1978; Fuchs et al., 1979) by interfering with development of the junctions in the myometrium. Garfield and Hayashi (1980) carried out

an ultrastructural study on the myometrium from women at various stages of the cycle and found gap junctions between the smooth muscle cells in some of the tissues from women with dysmenorrhea, during menstruation and at other times during the cycle. Those studies, however, were performed on tissue removed from women who underwent hysterectomy for secondary dysmenorrhea at various stages of the menstrual cycle. Therefore, the presence or absence of the junctions at any particular time of the cycle studied may not reflect physiological changes but may rather be related to various pathological conditions or gynecological problems from which the women suffer. Ideally, this type of analysis should be performed on tissue samples from women with normal menses or primary dysmenorrhea with no underlying pathological problems, however, it is not possible to obtain the tissues.

1C.4 Hormonal control of myometrial activity:

1C.4.1 Steroid hormones:

In 1956 Csapo proposed the progesterone block theory to explain how the hormone is responsible for the maintenance of pregnancy. According to this theory the myometrium is intrinsically active but suppressed throughout gestation. Release of the suppression results in the initiation of labor, whether normal or premature. He has shown that when progesterone levels decline at either term or preterm, the myometrium is converted from an inactive to an active and reactive muscle. The theory stated that the maintenance of pregnancy was dependent upon the ability of progesterone to suppress myometrial

contractions and when the hormone levels decline the suppression is removed and the myometrium undergoes synchronized contractions. Progesterone was believed to interfere with excitation and impair electrical coupling of myometrial cells (Csapo, 1962; 1977) but the mechanism in which progesterone might block activity was not elucidated. Thus, the effects of high levels of progesterone throughout pregnancy may reduce the synchrony of muscle contractions so that activity is localized thereby maintaining the contents of the uterus.

Since the proposal of the progesterone block theory, experimental evidence has also suggested that the uterus is inert throughout gestation but is reactive to various stimulants (i.e. estrogen, prostaglandins, oxytocin and uterine stretch). Sufficient concentrations of these agonists are not present until the end of gestation at which time they stimulate myometrial activity and initiate labor.

A compromise that accommodates both the effects of suppressors and stimulates for the regulation of uterine activity was the "see-saw" theory proposed later by Csapo (1977). This theory appeared to provide the most suitable explanation for the onset of labor. The "see-saw" theory predicted that the maintenance of pregnancy involved a balance of two opposing forces in the myometrium, that of inhibition by progesterone (suppressor) and that of uterine activation by estrogen and prostaglandins (stimulants). The onset of labor, according to this theory, depends upon a regulatory imbalance in favor of the stimulants (decrease in progesterone versus increases in estrogen and prostaglandins). The endocrine changes at parturition as described in

the previous section appear to be consistent with Csapo's theories. Progesterone appears to be essential for maintenance of pregnancy in many species (i.e. rat) and ovariectomy before day 18 of gestation causes preterm labor (Garfield et al., 1982a). Exogenous progesterone will maintain pregnancy in ovariectomized animals and when administered to intact pregnant rats at term, labor is prevented and pregnancy is prolonged (Heap et al., 1973). As well, estrogens in vivo have a stimulatory effect on the uterus (Heap et al., 1973; Anderson et al., 1981) and it has been recognized that estrogen treatment during pregnancy causes abortion or premature labor (Evans et al., 1981; Dawe et al., 1982). The increase estrogen levels at term is therefore, believed to be an important step in the sequence of events leading to parturition (Thorburn and Challis, 1979).

The mechanisms of parturition differ considerably among species making it extremely difficult to present a single sequence of events leading to the initiation of labor that is applicable to all animals. For example, in some animals and humans in which the placenta is the source of progesterone, there is no significant decline in progesterone levels prior to labor thereby refuting the theories of Csapo. It appears, however, that in humans progesterone is required for the maintenance of pregnancy because when progesterone levels decline from lutectomy before the luteal-placental shift in the synthesis of the steroids abortion ensues (Csapo and Pulkinenen, 1978). Furthermore, antiprogesterone agents, which produce the same effect as progesterone withdrawal, are effective abortifacients at any time during human pregnancy. In addition, the progesterone binding proteins in fetal

membranes as described by MacDonald et al., (1978) may be of great significance in the initiation of human labor. These studies suggest that a functional withdrawal of progesterone could occur without a decrease in the level of the hormone which would be consistent with Csapo's theories.

The basis for the effects of the steroid hormones on myometrial function have recently been described in detail (Garfield, 1984). The steroid hormones regulate myogenic mechanisms in muscle cells: estrogen stimulates and progesterone inhibits myogenic properties. The action of the steroid hormones can occur in two separate phases; that of delayed and acute effects. Both hormones can elicit immediate effects either directly on calcium binding and transport or indirectly on prostaglandin production (Thorburn and Challis, 1979). The acute effects of the steroids may be independent of the classical hormone receptor and synthetic mechanisms (see below) and could possible result through an effect on adenylate cyclase and cAMP. The delayed effects, on the other hand, are probably mediated through the steroid receptor mechanism and involve the stimulation or inhibition of protein synthesis. According to Garfield (1984) the synthesis of protein is likely to be important because the evolution of contractility and labor occurs gradually and may involve the synthesis of proteins necessary for effective labor, including those involved in excitation-contraction coupling, receptors, contractile filaments, adenosine triphosphate and gap junctions.

1C.4.1.1 Mechanism of steroid hormone action:

1C.4.1.1a Classical theory of steroid-receptor interaction:

Estrogen:

Upon entry into the target cell estrogen combines with a receptor protein (estrophilin) in the cytoplasm. The cytosol binding component exists as two binding sites, Type I and Type II (Clark et al., 1979). Type I has a high affinity for estrogen and is depleted from the cytosol after an injection of the hormone. Type II has a lower affinity for estrogen and it is not depleted from the cytosol after hormone treatment. It has been postulated that Type II binding sites may be extracellular binding proteins that play a role in accumulating estrogen within the cell or that they may represent a precursor form of Type I sites (Clark et al., 1979). Uterine estrogen cytosol receptor sediments out as an 8S band on sucrose gradients but in the presence of high salt concentrations the 8S complex is reversibly dissociated into subunits that sediment at 4S with a molecular weight of approximately 60-70K (Toft and Gorski, 1966).

Once the estrogen-receptor complex is formed in the cytoplasm an activation or transformation of the receptor occurs. This is an estrogen and temperature dependent conformational change modifying the receptors intrinsic activity and its sedimentation characteristics. According to Notides (1979) the estrogen induced transformation of the receptor may be attributed to a change in one or more of the following properties of the 4S binding protein: a) shape, producing a form with hydrodynamic properties that permit faster sedimentation to the activated 5S complex;

b) partial specific volume or density, or c) mass by association with another macromolecule. Notides and Nielson (1974) suggested that the 5S activated receptor is formed by association of the 4S protein with another macromolecule, independent of any enzyme action. A hypothetical model of the regulatory interaction of the estrogen-receeptor in vivo indicates three separate molecular interactions each with an equilibrium that influences the final product, the formation of the 5S estrogen binding protein dimer (Notides, 1979). According to this model the 4S receptor complex is associated with a macromolecular inhibitor in an inactive conformation confined to the cytoplasmic compartment. The binding of estrogen to the 4S complex serves to shift the 4S form from its inhibited complex to an unobstructive 4S estrogen-receptor complex available for the formation of the 5S form. The final equilibrium is between the 4S complex and its complementary monomer (E-?) forming the 5S dimer, the biochemically active macromolecule essential for estrogen action.

The activation of the cytosol estrogen-receptor results in an accumulation of the complex in the nucleus of the cell, representing the well known cytoplasmic to nucleus translocation phenomenon (O'Malley and Means, 1974). Two specific estrogen binding sites are also present in the nucleus. One site confirms to the Type I site that was depleted from the cytosol whereas the nuclear Type II site is not the same as the Type II site present in the cytoplasm (Clark et al., 1979). The nuclear Type II sites are hormone and tissue specific and occur in the nucleus after treatment with estrogen. Although the function of this binding site has not been ascertained, they may represent: a) a component of the

machinery involved in processing the Type I nuclear complex and bring about recycling or replenishment of the receptor; b) an integral component of the mechanism that controls RNA and/or DNA synthesis; c) a component of the off-reaction involved either in turning off receptor stimulated events or in removing the hormone and/or the receptor from the nucleus, or d) an amplification mechanism to amplify the nuclear events initiated by binding of the receptor to acceptor sites (Clark et al., 1979; Eriksson et al., 1980). We must also consider the possibility that the nuclear Type II site has no function at all in the nucleus and may represent an inactive form of the receptor processed in the nucleus. This is not likely because it has been suggested in a couple of recent studies that the nuclear Type II sites are specific for estrogenic responses and are highly correlated with uterine growth (Eriksson et al., 1980; Markaverich et al., 1981).

When the hormone-receptor complex is in the nucleus it binds to the chromatin material (King and Inman, 1966). The binding to nuclear acceptor sites is thought to initiate the events leading to the expression of estrogen action. Although the complete interaction of hormone-receptor complexes in target cell nuclei may involve various aspects in the stimulation of RNA synthesis and processing, a particular striking effect is observed on the RNA polymerase system. Administration of estrogen to immature rats initiates an increase in uterine RNA polymerase II activity within 15-30 minutes after treatment which is followed 2-4 hours later by an increase in the activity of RNA polymerase I and a second rise in polymerase II activity (Brothwick and Smellie, 1975; Hardin et al., 1976). Only the long acting estrogens

(i.e. estradiol) cause the second elevation in RNA polymerase II activity together with prolonged stimulation of polymerase I.

The stimulation of RNA synthesis appears to be a complicated process. There are numerous machinery components of the transcriptional events that might be modified by estrogen-receptor complexes. Hardin et al., (1979), however, suggested that the increase in RNA synthesis by estrogen treatment results from an increase in the number of specific RNA chain initiation sites. Kastern et al., (1981) have also implied that this increase is caused by an estrogen stimulated increase in RNA polymerase molecules. The structure of chromatin, therefore, plays an important role in the regulation of RNA synthesis in response to estrogen. Following RNA synthesis, proteins are synthesized (Means and Hamilton, 1966) including the receptor (Jensen, 1979).

Protein synthesis results from the ability of estrogen to regulate the synthesis of newly formed ribosomes (Moore and Hamilton, 1964), together with its regulation of the functional capacity of the ribosomes to synthesize protein. Estrogen treatment for several days also causes the aggregation of polysomes (Means et al., 1971). Withdrawal of the hormone results in polysome disaggregation and cessation of protein synthesis. It was therefore concluded in a review by Wyss (1974), that estrogen stimulates protein synthesis by exerting a control at both the transcriptional and translational level.

Estrogen also induces DNA synthesis, an event leading to cellular proliferation (Stormshak et al., 1978). Estrogen acts to reduce the duration of the cell cycle at the expense of the G1 and S periods (G1 is the period during which the cell prepares for DNA synthesis

through increased synthesis of RNA and protein; S period is DNA synthesis). The early responses of the target cell to estrogen are prerequisites for preparing the cell for subsequent replication, however, they cannot by themselves promote DNA synthesis. The retention of estrogen-receptor complex in the nucleus is required to elicit DNA synthesis and this process appears to be dependent on specific estrogen regulated events during the G1 period.

Progesterone:

There is considerable evidence to support a receptor mediated mechanism for progesterone similar to that described for estrogen. Proteins binding progesterone have been identified in several progesterone target tissues. The cytosol progesterone receptor sediments out as a 4S band when analyzed in a sucrose gradient containing a salt. When the salt is omitted, the sedimentation results in two distinct bands. The cytoplasmic receptor therefore, is recognized as being composed of an A and B subunit (molecular weights 72 and 117K respectively) each capable of binding one molecule of progesterone (Schrader and O'Malley, 1978). The next step in the action of this hormone involves the transfer of the steroid-receptor complex to the nucleus. Labeled progesterone injected into an animal is localized in the nucleus of target cells supporting a nuclear translocation (O'Malley et al., 1971). Results of many investigations indicate that when the progesterone- receptor complex is in the nucleus, it exerts its effect by binding to the chromatin and causing an increase in the number of RNA polymerase initiation sites (Schrader and O'Malley, 1978). Therefore,

similar to that of estrogen, the progesterone-receptor complex may be acting directly on chromatin to enhance the number of initiation sites for RNA synthesis leading to the synthesis of specific mRNA's.

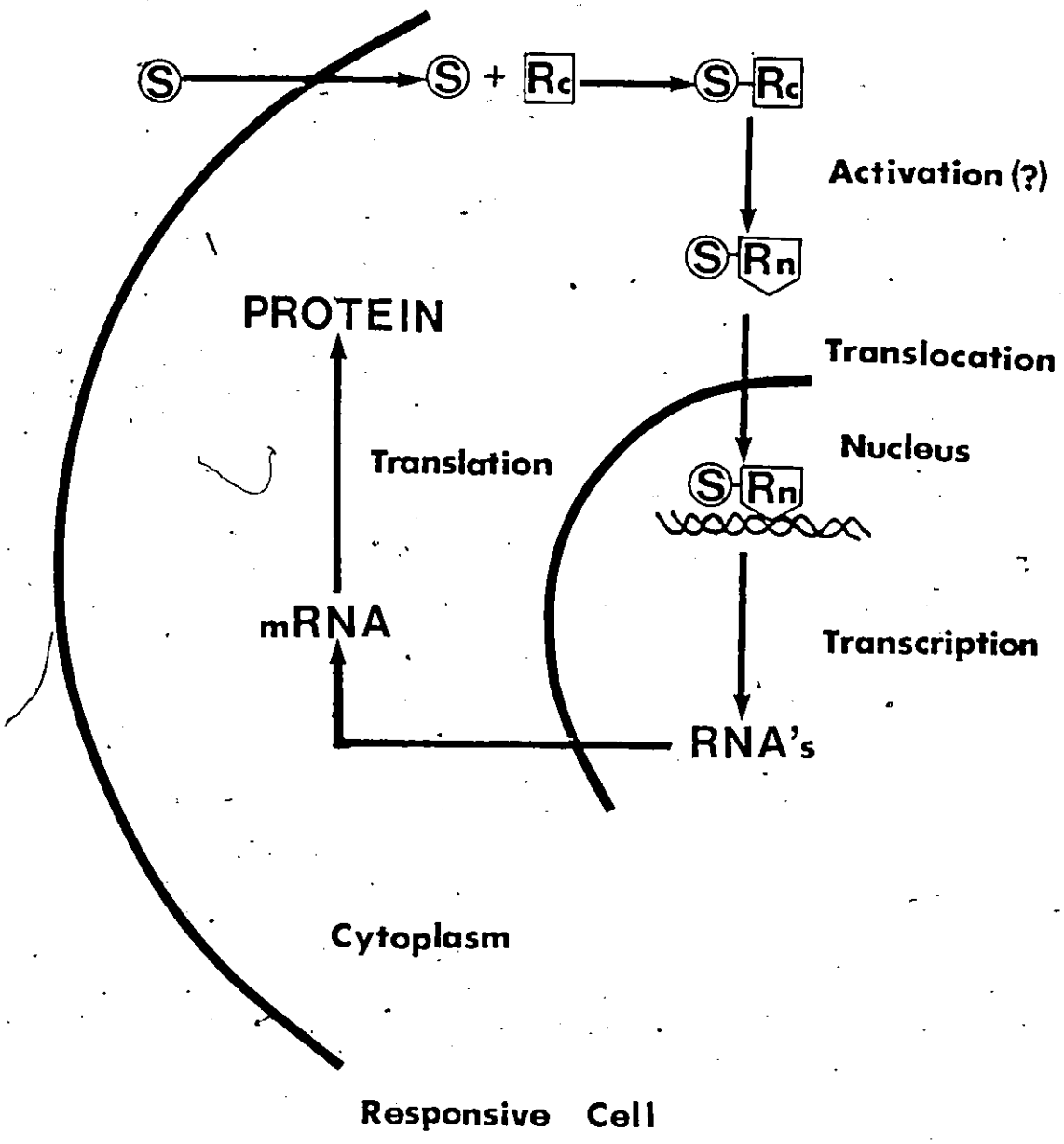
Spelberg et al., (1972) have shown that acidic (non-histone) proteins of chromatin (designated AP_3) are involved in binding the progesterone-receptor complex. Furthermore, AP_3 binds subunit B and the intact form of the receptor complex whereas subunit A only binds to pure DNA (Schrader and O'Malley, 1972; Schrader et al., 1972; 1975), suggesting that the A subunit could be the actual gene regulatory protein and the B subunit could specify where the A subunit is to localize. According to O'Malley and Schrader (1976), progesterone exerts its ultimate effects (RNA, protein and DNA synthesis) by influencing the transcription of DNA as follows: The hormone on the B subunit causes expression of a chromatin acceptor-protein binding site. This chromatin affinity results in a net translocation of the receptor to the nucleus where the receptor binds to the chromatin through the B subunit on the AP_3 component of the chromatin protein. The receptor complex then dissociates to liberate the DNA binding subunit A still bearing its hormone molecule. Subunit A attaches to and interacts with DNA causing the helix to open allowing a molecule of RNA polymerase to occupy an initiation site resulting in the transcription of a new mRNA. This newly synthesized mRNA is then transferred to the cytoplasm where it serves as a template for the synthesis of protein specific for the target cell.

It should be noted that estrogen regulates the synthesis of the cytoplasmic receptor for progesterone (Horwitz and McGuire, 1978). Thus, estrogen may be necessary for some of the effects of progesterone.

Furthermore, progesterone has been shown to inhibit estrogen dependent protein synthesis (Hseuh et al., 1975).

The classical "two-step" model of steroid hormone intercellular action (Figure 6), as described above, suggests that cytoplasmic receptors can exist either steroid-bound or unbound but nuclear receptors can only exist in the steroid-bound form. Recently, however, it has been reported that unbound receptors are present in the cell nucleus of estrogen target tissues (Carlson and Gorski, 1980; Clark et al., 1982) thereby refuting the interpretation of the two-step model of steroid action. These unbound nuclear receptors possess properties characteristic of the nuclear Type I receptor and are present in purified preparations of nuclei (Zava and McGuire, 1977). This data suggests that in intact cells unbound receptors may exist in a state of equilibrium between the cytoplasmic and nuclear compartments with the equilibrium in favor of the cytoplasm after tissue homogenization (Sheridan et al., 1979). Furthermore, Welshons et al., (1984) have shown that unbound receptors are present in the nucleus of cells from rat pituitary tumors and they have proposed that in the intact cell there may be no nuclear translocation of the receptor as part of the steroid response but rather an increase in receptor affinity for nuclear elements. This conclusion, however, must be interpreted with caution because the cells were from a cancerous tissue implying that alterations in the steroid mechanism may have been inherent to those cells. In addition, a recent study using immuno- cytochemical staining of a monoclonal antibody generated against the estrogen receptor protein has revealed that specific staining was confined to the nucleus of the cells

Figure 6. Schematic representation of the receptor mechanism of steroid hormone action in target cells. The hormone (S) enters the cell and binds to an extranuclear receptor protein (R_c), inducing its conversion to an activated form (R_n) that is translocated to the nucleus where the hormone–receptor complex interacts with chromatin material altering genetic transcription enhancing the production of RNA's for the synthesis of proteins.


S
S + Rc
S Rc
Activation (?)
S Rn
PROTEIN
Translocation
Translation
Nucleus
S Rn
mRNA
Transcription
RNA's
Cytoplasm
Responsive Cell

studied (King and Greene, 1984). Although these studies indicate that the estrogen receptor may reside exclusively in the nucleus of the cells of steroid target tissues they nevertheless are still in agreement with intracellular steroid receptors as originally outlined by the two-step model of steroid action.

1C.4.1.1b Other possible mechanisms for steroid hormone action:

The current concept for the mechanism of steroid hormone action is based on the receptor system within target cells (see above). Although this system is well supported, it has been suggested that the steroid hormones act by an additional mechanism or mechanisms. Of great interest and amongst many unanswered questions is the involvement of the plasma membrane in the interaction of steroid hormones with target cells. It is possible that the hormone entering the cell triggers a response prior to and independent of the hormone-receptor mediated effects on gene transcription. In addition, the localization of the steroid receptor in the absence of the hormone is not known. It has been proposed that the receptor may be attached to or be a component of the plasma membrane from which it is easily detached during homogenization and hence obtained in the cytoplasmic fraction (Jackson and Chalkley, 1974).

Based on these assumptions investigations attempting to define the "other" mechanism for steroid hormone action have been carried out. The amphibian oocyte system has been the primary focus of several studies. These investigations have revealed significant differences in

the mechanism through which progesterone exerts its effects. In a review by Baulieu (1978), he has summarized the effects of progesterone on Xenopus laevis oocytes which indicates that progesterone triggers processes (i.e. protein synthesis) independent of nuclear functioning. Moreover, it is thought that no intracellular receptor exists in oocytes because endogenous but not exogenous progesterone initiates responses. He has implied that calcium may be the second messenger of progesterone action in the oocyte, a concept which envisages that progesterone action includes the release of membrane bound calcium ions into the cytoplasm. More recent studies supporting this theory also indicate that progesterone exerts its effect by down regulation of adenylate cyclase, possibly via calcium release from specific membrane sites (Kostellow et al., 1980; Finidori-Lepicard et al., 1981). This membrane bound receptor for progesterone appears to be different from the "classical" steroid receptor, however, no direct studies of the interaction between the oocyte membrane and progesterone have been carried out (Baulieu, 1978). In smooth muscle if progesterone had this effect it would lead to contraction. Csapo, however, thought progesterone decreased calcium levels in smooth muscle cells.

Although the Xenopus oocyte system suggests a membrane- receptor -hormone interaction as the mechanism through which progesterone exerts its effects, this does not seem to be the mechanism through which progesterone initiates its effect in other cells. It has been shown that progesterone action in the uterus is not mediated at the membrane level by influencing adenylate cyclase but rather triggers biochemical processes after binding to intracellular receptors (Rubanyi, 1980).

Moreover, there is unfortunately no evidence of a similar system for the mechanism of estrogen action. Furthermore, there is little supportive evidence for a steroid hormone-membrane interaction mechanism in somatic target cells. There is no reason, however, to exclude this possibility, either alone in cells or associated with the classical receptor system. For example, many target tissues and organs for steroid hormones do not exhibit the dramatic growth that occurs in the uterus in response to estrogen. In addition, some of the growth related effects of estrogen on the uterus can be mimicked by other treatments. Cholera toxin injected into ovariectomized hamsters and rats causes an estrogen-like increase in uterine wet weight and mitotic index (Alleva and Lamanna, 1979; Stewart and Webster, 1983). Stewart and Webster (1983) have also shown that the estrogen-like effect of cholera toxin was neither the result of nuclear accumulation of the estrogen receptor nor the synthesis of uterine induced proteins, which is known to be correlated with the translocation of the receptor to the nucleus (Katzenellenbogen et al., 1980). It also has been reported that 4-mercuri-17β- estradiol induces hypertrophy of the uterus in the absence of the cytoplasmic translocation and that sequential estrogen treatment inhibits uterine DNA synthesis but not the translocation of the receptor (Muldoon, 1971; Stormshak et al., 1976).

If estrogen's mode of action in the uterus is by another or an additional mechanism this may involve an estrogen receptor which is apparently not translocated from the cytoplasm to the nucleus. This could be the plasma membrane receptor for estrogen reported by Pietras and Szego (1979). Estrogen has been shown to stimulate adenylate cyclase

(a membrane associated enzyme) resulting in increased cAMP levels in uterine tissue (Szego and Davis, 1967). Therefore, it is tempting to speculate the interaction of estrogen with a membrane receptor as whole or part of its effects in the uterus. The only drawback in accepting such an hypothesis is the lack of detailed investigations on this proposed membrane receptor and the reproducibility of the data reported by Szego and Davis (1967).

A completely different receptor system, the "eosinophil receptor" for estrogen, independent of the classical hormone receptor system has been demonstrated in the uterus (Tchernitchin et al., 1976a; 1976b; Tchernitchin and Tchernitchin, 1976; Tchernitchin and Galand, 1982). Although both receptor systems are functional in the uterus, the eosinophil receptor system is believed to mediate early estrogenic responses such as uterine water imbibition and increased vascular permeability. Tchernitchin et al., (1976b) have demonstrated that eosinophils are directly attracted to the uterus after estrogen treatment before several changes in the early parameters of estrogen stimulation. They propose that this occurs because estrogen bound to the cytoplasmic membrane of eosinophils (and/or to the uterine capillary wall) could also have an affinity for the receptors in the capillary wall (and/or the receptors in the cytoplasmic membrane of the eosinophils from the uterine blood vessels) which would allow attachment of the eosinophils to the capillary wall in the uterus representing the initial step towards eosinophil penetration into the uterus (Tchernitchin et al., 1976b). They have further suggested that the hydrolytic enzymes released from the uterine eosinophils produce a

depolymerization of uterine mucopolysaccharides which cause an increase in vascular permeability and water imbibition. Early increase in uterine wet weight parallels estrogen-induced uterine eosinophilia (Tchernitchin et al., 1976a). More recently, in ovariectomized adrenalectomized rats treated with estradiol, Tchernitchin and Galand (1982) have separated estrogenic effects using actinomycin D into genomic and non-genomic responses. RNA and protein synthesis (genomic responses to estrogen) were inhibited by actinomycin D whereas increases in early uterine wet weight, uterine eosinophilia and glycogen content were not. Similarly, cortisol which decreases the number of eosinophils in the blood and therefore limits their availability for migration to the uterus, drastically reduced estrogen-induced uterine eosinophilia and early uterine wet weight response but did not interfere with estrogen-induced uterine RNA and protein synthesis (Tchernitchin et al., 1976a). It is possible to speculate therefore, that the latter estrogenic responses are of a non-genomic origin, implicating multiple mechanisms of action of this hormone. The simultaneous presence of estrogen receptors in the cytoplasmic membrane of eosinophils and in the wall of uterine capillaries and small blood vessels but not in other organs as demonstrated by autoradiographic studies (Tchernitchin et al., 1976b) may explain the organ specificity of the estrogen-induced uterine eosinophilia.

1C.4.2 Prostaglandins:

Several lines of evidence implicate a role for prostaglandins in the sequence of events resulting in term or preterm labor. The rate of

prostaglandin production increases during term and preterm labor, exogenous prostaglandins stimulate the myometrium to contract and induces labor at any stage of pregnancy, and inhibitors of prostaglandin synthesis suppresses myometrial contractions and labor (Thorburn and Challis, 1979; Garfield, 1984). These observations are applicable to all species investigated and although they do not enable us to define their precise role we can conclude that endogenous prostaglandins are involved in parturition.

That the primary effect of prostaglandins at term is exerted by increased synthesis or by increased myometrial sensitivity, or both, is still controversial. Some products of the cyclo-oxygenase pathway have been shown to stimulate myometrial contractility ($PGF_{2\alpha}$, PGE_1, PGE_2), while others inhibit the process (PGI_2). Prostaglandin $F_{2\alpha}$ is also thought to function as a luteolytic agent and may play an important role in corpus luteum regression in species dependent on the corpus luteum for maintenance of pregnancy. The role of steroid hormones in the regulation of prostaglandin synthesis is well established (see section 1C.2.3). Furthermore, estrogen has been shown to inhibit uterine prostacyclin production (PGI_2) which is known to suppress myometrial activity. Therefore, the maintenance of pregnancy may be determined by a balance of stimulatory and inhibitory prostaglandins (Thorburn and Challis, 1979).

1C.4.2.1 Mechanism of action for prostaglandins:

Prostaglandins appear to have direct and indirect effects on myometrial contractility. Stimulatory prostaglandins (i.e. $PGF_{2\alpha}$) are

thought to act mainly by promoting the influx of calcium from the extracellular fluid or by liberating calcium from intracellular binding sites (Liggins, 1979). These prostaglandins cause membrane depolarization which increases the frequency of action potential bursts (Kuriyama and Suzuki, 1976b). However, $PGF_{2\alpha}$ is not effective as a stimulant in animals under progesterone dominance (Csapo, 1981). Therefore, termination of pregnancy by this prostaglandin in animals such as the rat has been attributed to indirect effects of $PGF_{2\alpha}$ on luteolysis resulting in a withdrawal of progesterone (Fuchs, 1978).

Inhibitory prostaglandins (i.e. PGI_2) are thought to exert their action through effects on adenylate cyclase. This prostanoid has been shown to cause an elevation of intracellular cAMP which may in turn activate transport mechanisms to reduce cytoplasmic calcium levels and promote muscle relaxation (Vesin et al., 1979).

More recently, studies have indicated that prostaglandins may control the steroid receptors or binding of the steroid to its receptor (Sanfilippo et al., 1983; Clerc-Hofmann et al., 1983). Although further investigation into this phenomenon is required, it may nevertheless have important implications in the hormonal changes around parturition. For example, prostaglandins may be responsible for a functional progesterone withdrawal through a decrease in the steroid receptor number or binding ability. This may explain why a decrease in progesterone level is not necessary for initiation of labor in some species (i.e. humans).

1C.5 Neural control of myometrial activity:

Smooth muscle of the uterus may also be under a neurogenic

control. That is the muscle activity is influenced through a direct effect of nerve transmitters. However, according to the literature there may not be a neural control of myometrial activity during pregnancy. These conclusions were based on the observations that transection of the spinal cord or peripheral nerves has no influence on pregnancy or labor. According to Garfield (1984), the exclusion of neurogenic input as above, may be a misconception since transection or cutting of nerves may not completely eliminate all nerve connections to the uterus and may not exclude local control by nerves.

Marshall (1981) has provided evidence that adrenergic nerves disappear from the uterus in most species a few days prior to term. She has hypothesized that uterine nerves degenerate at the end of gestation and that the initiation of labor may be related to this phenomenon. Therefore, throughout pregnancy myometrial activity may be suppressed by adrenergic nerves and with their disappearance at term the muscle activity may increase (Garfield, 1984). In addition, it has also been reported that the steroid hormones regulate the adrenergic receptors (Carsten, 1968; Cornett et al., 1981; Hoffman et al., 1981). Estrogen stimulates the alpha receptors thus increasing contraction whereas progesterone sensitizes the beta receptor and enhances relaxation. There are, however, species differences and differences between the circular and longitudinal muscle layers. If estrogen stimulates the alpha adrenergic receptor which may be involved in myometrial activity then it is difficult to relate this relationship with adrenergic degeneration as term approaches when the myometrium becomes increasingly active and reactive. However, the nervous input to the uterus and nerve-

muscle interactions appear to be complex and require further investigation to clarify if neural control is important in regulating contractility of the myometrium.

Following denervation of smooth muscle cells of the vas deferens in the guinea-pig there is a marked increase in the number of gap junctions between the muscle cells (Westfall et al., 1977). This structural change in the muscle after denervation is thought to be responsible for increased electrophysiological activity and spontaneous contractions of this muscle (Marshall, 1981). Those observations may have significant implications if extrapolated to the uterus since the work of Garfield (see section 1B.2.2) has shown that gap junctions develop in the myometrium only at term in pregnancy and appear to be necessary for the occurrence of parturition. In the myometrium, a factor which may also be involved in regulating the presence of gap junctions, by analogy with other smooth muscles (as above), is functional denervation. Whether junction development is related to denervation in the myometrium, however, remains to be established. Burden et al., (1979) have reported that bilateral section of the pelvic parasympathetic nerves blocks parturition in the rat but did not alter the development of gap junctions between myometrial smooth muscle cells. This suggests that denervation may mediate the appearance of myometrial gap junctions and that the junctions may not be involved in parturition. However, in the group of animals surgically denervated, fetuses were usually trapped in the cervix after day 22 of pregnancy. This indicates that some form of labor occurred when gap junctions were present in the myometrium. Furthermore, these observations suggest that normal

delivery may have been prevented because the cervix was not dilated sufficiently to allow the passage of the fetus. This would indicate that innervation of the cervix may be important for normal labor.

1C.6 Gap junctions in control of labor:

I have previously described the function and regulation of myometrial gap junctions. In a recent review, Garfield (1984) has summarized the control of myometrial gap junctions and their significance in the initiation of term or preterm labor. In brief his studies demonstrate, regardless of the species,: 1) myometrial gap junctions are absent or present in low frequency throughout pregnancy, 2) at term, myometrial gap junction frequency increases reaching maximum frequency and size during delivery of the fetus, 3) gap junctions revert back to the predelivery state, low frequency and small size, immediately following parturition, and 4) myometrial gap junctions are present in tissues from pregnant animals induced to deliver prematurely and in human myometrium during preterm labor. Gap junctions are present in the myometrium under all conditions studied that lead to parturition (oxytocin and prostaglandin treatment) and are absent in tissues from control animals or animals treated similarly but hormonally prevented from delivering (progesterone treatment). Therefore, myometrial gap junctions appear to be necessary for term or preterm parturition.

Garfield (1984) suggests that the presence of myometrial gap junctions during term or preterm labor is controlled by the changes in the steroid hormones and prostaglandins which precede and accompany these events. Evidence from many studies involving a variety of species

support this hypothesis. Moreover, gap junctions improve electrical and metabolic coupling and their presence in the myometrium during term or preterm labor may be the structural basis for improved propagation or conduction of signals thereby allowing for the transformation of the inactive myometrium of gestation to an electrical and metabolic syncytium essential for the active and reactive muscle of labor to expel the contents of the uterus. Thus, myometrial gap junctions may be essential for normal term labor and if precipitated early results in premature labor.

1D. CONTROL OF TISSUE GROWTH

1D.1 Introduction:

Communication between cells within an organ and between organs may be necessary for the control of some types of tissue growth and there is a number of ways in which cells can communicate with each other. For example, communication among a population of cells can be accomplished by the release of a chemical signal into the circulatory system, transmission of messages through the extracellular space, across close contacts between cells or by direct cell-to-cell communication via structural connections between the cells. Alternatively, communication between cells may not be necessary for the control of growth of all tissues.

Since it has been well established that cells make permeable junctions that allow the passage of molecules between cell interiors, it is logical to assume that cell-to-cell communication may be a primary event in controlling growth of cells in tissues. Growth can occur as a result of cellular hypertrophy (increase in cell size) or hyperplasia (cell division). Therefore, gap junctions which provide channels connecting the cytoplasm of two cells may be pathways for the transfer of growth controlling molecules between populations of cells. An hypothesis proposed by Loewenstein (1979) states that junctional membrane channels are instrumental in the cell-to-cell transmission of molecules necessary for the control of cell division (i.e. an inhibitor). Alternatively, a defect in the step which involves the transmission of a signal between cells, whether completely absent or

just obstructed, may result in a disturbance of cellular growth control. This form of abnormal growth (i.e. cancer) may be envisioned as gap junction communication incompetent, however, Loewenstein (1979) emphasizes that not all growth control incompetent cell types will be junctional communication- incompetent nor all noncommunicating cells to be abnormal since transmission of a growth regulatory factor represents just one possible control site. According to Loewenstein (1979) growth control consists of five stages : (1) generation of control signal, (2) transmission of the signal, (3) reception of the signal, (4) comparison of signals, and (5) the effector processes of cell division. Uncontrolled growth can be the result of a genetic defect at any or all of the five general stages of control. Therefore, junctional communication-incompetence should be found only among the categories in which uncontrolled growth results from alterations at control stage 2, signal transmission.

1D.2 Gap junctions between cells and growth control:

In an attempt to support the growth control hypothesis proposed by Loewenstein (1979) a broad variety of growing cell types and tissues have been examined for junctional communication-competence. Those reports, reviewed by Azarnia and Larsen (1977) and Loewenstein (1979) have indicated that all experimental investigation into growing cells have yielded uniform results. All cell types of organized tissue capable of dividing but not in the active process of division were found to be communication-competent as indicated by the cell-to-cell transfer of ions, fluorescent labeled molecules and the presence of gap junctions

between the cells. Moreover, experimentation employing cell culture techniques has also related junctional communication in the control of growth. Following experimental wounding of cells in confluent culture, the cells at the wound border respond by dividing until the injury heals over. This process is initiated as follows: (1) the cytoplasmic calcium levels in injured cells rise resulting in closure of their junctional channels, thereby uncoupling communication from all neighbouring cells, (2) the junctional channels of intact cells at the wound border close by the calcium mechanism, producing a new diffusion boundary for the coupled cell system, and (3) newly joining cells form communication channels after the healing process is over (Loewenstein, 1979).

The examples sited above imply that cell growth or proliferation occurs in the absence of gap junctions between the cells and that the presence of the junctions was correlated with the cessation of growth. There are, however, many cell types that grow and divide in the presence of gap junctions, in particular those from developing tissues of embryonic origin (Furshpan and Potter, 1968; Crick, 1970; Wolpert, 1971; Loewenstein, 1979). In embryonic tissue, growth and proliferation are finely regulated and gap junctions are present between the cells. Moreover, the cessation of growth of these tissues has been related to the disappearance of the junctions (Fujisawa et al., 1976). Similarly, the presence of gap junctions has also been correlated with proliferation of cells in adult regenerating tissue (Loewenstein, 1979). However, the cessation of growth in these tissues does not correlate with the regression of gap junctions from the tissue. Therefore, it appears that in some tissues growth or proliferation occurs in the

absence of gap junctions and intercellular communication whereas in others the junctions are present during growth.

1D.3 Communication-incompetence of cells and growth control:

According to Loewenstein's hypothesis some communication-incompetent cells may not be under any growth control regulation. This has important implications in cancerous tissue and thus, several tumor cell lines have been analyzed in order to establish the relationship between the above parameters in these tissues.

Electrical measurements and dye transfer studies, performed on cells in situ from tumors isolated from experimental animals and patients undergoing surgery, failed to detect cell-to-cell coupling under conditions in which coupling was found in normal tissue counterparts (Azarnia and Larsen, 1977; Loewenstein, 1979). On the basis of these findings it was suggested that growth control of cells in those malignant tumors was lost because of a predominance of communication-incompetent cells. Contrary to these findings, some solid tumors are well coupled and gap junctions have been revealed between the cells by electron microscopic techniques (Jamakosmanovic and Loewenstein, 1968; Sheridan, 1970; Johnson and Sheridan, 1971). The analysis of the presence or absence of cell coupling in solid tumors, however, must be treated with caution because of the heterogeneity of cell populations in the tumors.

The study of tumor cells under in vitro culture conditions has allowed a more precise analysis of the relationship between cell communication and growth regulation, especially in tumors composed of

both cancerous and normal cell types. Two classes of cell types derived from tissue explants of a Morris hepatoma (two cell types of epithelial morphology and one of fibroblastic morphology) have been studied in detail. The epithelial cell types are communication-incompetent by both electrophysiological and metabolic cooperation criteria; they are not electrically coupled and they fail to transfer fluorescein or endogenous hypoxanthine nucleotides from cell-to-cell (Azarnia and Larsen, 1977; Loewenstein, 1979). These communication-incompetent cell types are also growth control incompetent since they are density-independent in culture (i.e. they grow to densities of $> 10^6$ cells/cm^2) and they are tumorigenic (Azarnia and Loewenstein, 1971). The cell type of fibroblastic morphology from this hepatoma, however, is communication-competent. Interestingly, a Morris hepatoma when grown in cell culture forms gap junctions only between a few cells in the centers of the colonies it grows in and fluorescein diffusion is restricted in a fashion consistent with the junctional distribution (Azarnia and Larsen, 1977). Although their studies did not indicate which cell type possessed the gap junctions, it may be assumed that they are of the fibroblast population as based on the previous discussion. Numerous other in vitro investigations on cancer cell lines and strains from a variety of sources (i.e. human mammary carcinoma, X-irradiated hamster embryo cells, derivatives of mouse L cell line) have all revealed that the cells are communication-incompetent and under no growth control (Loewenstein, 1979).

A number of tumor cell lines have been shown to be partly communication-incompetent (Loewenstein, 1979). Cell communication in

hepatoma cells (strain SA-21, Azarnia and Loewenstein, 1976) is evident but the development of their communication is abnormally slow. Electrical coupling of those cells is approximately 15 times slower then in normal liver cells and fluorescein transfer occurs in only a small fraction of the tumor cells compared with 100% in the normal counterparts (Azarnia and Loewenstein, 1976). This marked reduction in cell-to-cell communication between these hepatoma cells may reflect a retardation in the growth and developmental process of gap junctions or alterations in cell-to-cell channel structure reducing the channel pore and thereby restricting passage of growth regulatory factors between the cells. Loewenstein (1979) probed the permeability of those junctions with molecules of various sizes and found that the junctional defect severely limited the exchange of molecules between cells. Thus, he concluded that alterations in the structure of gap junction channels may conceivably be as important in the etiology of uncontrolled growth of this cell line as the more radical defect (i.e. absence of gap junctions) as previously described in other cancer cells.

Partial communication-incompetence between tumor cells have also been demonstrated by a sensitive metabolic cooperation technique that assays the degree of communication by the number of cell survivors in coculture with donor cells containing a toxic metabolite (i.è. 6 thioguanylic acid). Cell types from four transformed human cell lines, two fibrosarcoma lines and a HeLa cell derivative D9-AH-2 (Loewenstein, 1979) communicate but they transfer the toxic metabolite to a lesser degree than normal cells under the same conditions.

The various communication defective cell types discussed above

indicate that this abnormality is common among malignant tumor tissues. However, whether or not the communication defect is the actual cause of the cell malignancy is a concern to all investigators. One approach towards answering this question is through genetic analysis of this cancerous condition, to determine whether the uncontrolled growth behavior is genetically related to the communication defect. Through utilization of somatic hybrids between communication-incompetent cancer cells and communication- competent cells, the communication defect and the growth defect should be traceable to the level of a single chromosome (Loewenstein, 1979). Unfortunately this has not been accomplished but it has been possible to establish a genetic correlation between the two defects. Results from experiments using hybrids of communicating liver cells and cancerous communication-incompetent hepatoma cells showed that normal growth properties, density- dependent growth and non-tumorigenicity, were dominate in the hybrids suggesting that the junctional defect was causally related to the malignancy (Azarnia and Loewenstein, 1973). Furthermore, similar results were obtained when human skin fibroblast cells, which are coupled and have gap junctions, were fused with mouse Cl-1D cells, which are not coupled and lack gap junctions (Azarnia et al., 1974). In the early generation of hybrids, cells resembled the human parent cells in both coupling and the presence of junctions while they contained nearly the complete complement of parent chromosomes. When gap junction communication was present between the hybrid cells they were not cancerous. The hybrid system between human and mouse cell lines is genetically less stable and as a result the hybrid generations progressively lost human chromosomes

in vitro producing segregates. These segregates had reverted to the noncoupling and junction deficient trait of the mouse parent cell indicating that as communication competence was lost, so was growth control. In this example the human cells appear to contribute a genetic factor to the hybrids that corrects the communication-incompetency of the mouse cells. Further analysis of the human/mouse segregates have indicated that the communication and growth phenotypes do not segregate (Loewenstein, 1979). Thus, Loewenstein concluded that the genetic determinant supplied by the communication-competent parent cell to the incompetent parent cell during segregation behaves as if it were located on the same chromosome as the determinant correcting the growth defect; the genetic determinants for junctional communication and growth control are insegregable.

1D.4 Gap junctions in cancerous tissue:

Several excellent and comprehensive reviews of the possible relationship between intercellular communication (i.e. gap junctions) and control of growth or proliferation of cells have been published (Weinstein et al., 1976; Loewenstein, 1979; Hooper and Subak-Sharpe, 1981; Loewenstein, 1981). Only a few of the studies, however, have supported conclusions with ultrastructural evidence. A number of other studies have examined solid tumors for the presence or absence of gap junctions using transmission and freeze-fracture electron microscopy. It has been reported that gap junctions are scarce but present between cells of some tumors (i.e. human cervical squamous cell carcinoma, McNutt et al., 1971; mouse skin squamous cell carcinoma,

Martinez-Palomo, 1970) while completely absent from others (i.e. human cervical squamous cell carcinoma undifferentiated cell, McNutt et al., 1971; human ovary granulosa-theca tumor cell, Brausilver et al., 1974). Solid tumors in many cases are not homogeneous but consist of structurally and functionally different cell populations. Therefore, we cannot exclude the possibility that the junctions observed in tumors are associated with nonmalignant cells or abnormal cells at a particular stage of development. As an example, in the human cervical squamous cell carcinoma, gap junction occurrence was four per cell in the differentiated cell population and zero per cell in the undifferentiated cells compared to a frequency of approximately two hundred per cell in normal cervical epithelium (Weinstein et al., 1976). These structural studies on solid tumors, however, were not correlated with studies on junctional communication and therefore a direct relationship between communication-competency with the absence or presence of gap junctions can not be made.

Moreover, the absence of gap junctions from tumors must also be interpreted with caution. Many studies examining the occurrence of gap junctions have employed thin section electron microscopy. Gap junctions are not easily identified in thin sections of tumor tissue and are often confused with tight junctions in epithelial cells. It is possible that the difficulty in detecting gap junctions by this method may explain their absence or extremely low frequency in many tumors. Freeze-fracture electron microscopy carried out on all tumor tissue may resolve this matter.

Studies on cancer cells in vitro have directly correlated

ultrastructural evidence with functional coupling between cells. Homogenous cell populations from a communication- incompetent mouse cell line showed no detectable gap junctions (Gilula et al.,1972). Similarly, communication-incompetent cancerous parent cells and communication-incompetent segregate hybrid cells had no gap junctions whereas, the fully competent parent cells and early generation hybrids possessed numerous junctions. Observations similar to these have also been reported in normal and malignant human fibroblast cell lines in tissue culture (Azarnia and Loewenstein, 1977). Therefore, in many tissues the absence of gap junctions from between cells is correlated with abnormal growth of the tissue.

I.D.5 Steroid hormones and tissue growth:

The steroid hormones, estrogen and progesterone, have important regulatory roles in the growth, development and maintenance of tissues and organs of and associated with the female reproductive tract. When the ovaries mature at puberty increasing amounts of the steroid hormones are secreted in response to increased output of gonadotropins from the pituitary. The steroid hormones then accelerate the growth of the uterus, vagina, accessory sex glands, external gentilia, pelvis, breasts and pubic and auxiliary hair.

In the uterus estrogen stimulates growth of the glandular epithelium in the endometrium as well as myometrial cells. During pregnancy the uterus enlarges, due in part to the action of the steroid hormones and also to the stimulation (i.e. distention) from the growing fetus. The uterus in the prepubertal human female weighs about 10-15

grams; its weight increases five to six fold with the onset of sexual development and in pregnancy it may reach weights of 800-1000 grams (Heap et al., 1973). The growth is due to both cellular hypertrophy and hyperplasia.

The action of progesterone is greatly influenced by the extent to which the uterus has been exposed to estrogen, due to the fact that estrogen regulates the synthesis of the progesterone receptor. Progesterone is responsible for the growth of the epithelium and glands of the endometrium for the reception of the fertilized ovum and for changes in the estrogen-primed endometrium to the secretory type.

Both steroid hormones also influence the growth of the mammary glands, which is observed best during pregnancy. The glandular elements of the mammary gland are under a combined influence of estrogen and progesterone, with estrogen stimulating the growth of the ductile components while that of the glands themselves is stimulated by progesterone. Growth of the mammary glands and duct development are also seen in nonpregnant animals treated with the steroid hormones.

1D.5.1 Control of uterine growth:

With the onset of sexual maturity the uterus exhibits two types of growth: 1) that which occurs periodically with the reproductive cycle and is mainly under the control of the ovarian steroid hormones, and 2) that which takes place during pregnancy under the stimulus of the steroid hormones together with the physical effects of the presence of the growing conceptus.

The influences of the steroid hormones on uterine growth are

usually investigated in the immature and ovariectomized mature animal. Estrogen influences uterine growth by stimulating a number of uterine events: hyperemia, fluid accumulation, hyperplasia of glands and epithelium and hypertrophy and hyperplasia of myometrial cells (Carsten, 1968). Hypertrophy and hyperplasia in response to estrogen is associated with increased synthesis of RNA, protein and DNA, the amount of sarcoplasm and various estrogen specific enzymes associated with the synthesis of protein and metabolism (see Carsten, 1968). Changes in uterine connective tissue cells also occur after estrogen treatment. In the non-primed uterus, the connective tissue cells appear as fibroblasts. After estrogen stimulation there is an enlargement and increase in the number of golgi complexes and rough endoplasmic reticulum in the fibroblasts. A marked increase in total content of uterine collagen and an increase in the number of collagen fiber bundles occurs in response to estrogen stimulation thereby adding to the increase in weight of the uterus (Ross and Klebanoff, 1967).

The growth stimulating effect of progesterone is dependent on stimulation by estrogen and seems to be primarily in the endometrium (Carsten, 1968; Boomsma et al., 1982). Progesterone exerts a general effect of hyperplasia on these cells. In addition, progesterone also causes further hypertrophy of the myometrium and synthesis of collagen thereby adding to the growth of the uterus.

Stretch or distention of the uterus also acts as a local factor controlling uterine growth. Distention induced growth involves all components of the uterus with the myometrium affected to the greatest degree. This form of growth is solely of myometrial hypertrophy

(deMattos et al., 1967; Carsten, 1968) resulting in an increase in muscle wall thickness. Myometrial hypertrophy induced by distention is controlled by a physical factor, the length of the myometrial cell under tension and results in an increase in RNA and protein synthesis by the muscle cell (deMattos et al., 1967).

The growth of the uterus during pregnancy results from a combination of the effects of the steroid hormones and distention. Hyperplasia occurs early in pregnancy and precedes the implantation of the blastocyst. This growth process is under steroid control being mainly influenced by progesterone. Shortly after implantation hyperplasia of all uterine cells ceases and subsequent growth of the uterus is accomplished by hypertrophy of the connective tissue and new and old smooth muscle cells. Hypertrophy of the myometrium throughout the latter part of pregnancy results from systematic and local estrogen concentrations and distention induced by the growing fetus (Carsten, 1968).

Uterine growth is under dominant control by the steroid hormones, in particular estrogen. Although several mechanisms of action for estrogen on the uterus have been suggested (see section 1C.4.1.1), it is generally accepted that estrogen stimulates various uterine parameters including uterine growth through the cytoplasmic-nuclear receptor system. The effect is a complex phenomenon involving numerous specific biochemical events in a temporally ordered sequence. In brief, after a single injection of estradiol to the immature rat, there is a rapid nuclear translocation (within 1-2 hours) of the cytoplasmic receptor. This is followed by increases in water imbibition, hyperemia,

amino acid and nucleotide uptake, activation of RNA polymerase I and II, lipid, RNA and protein synthesis (Clark et al., 1979) representing early estradiol-induced uterine responses. These events lead to the late responses which are sustained RNA polymerase I and II activity, uterine cellular hypertrophy and hyperplasia, with a peak of DNA synthesis and mitosis occurring approximately twenty-four hours after hormone administration (Kaye et al., 1972; Stormshak et al., 1976; Kirkland et al., 1979). The overall response can therefore be viewed as a set of early tissue responses, thought to represent a preparation for growth, and a set of later uterine responses involving DNA synthesis, cell division and uterine growth (Kirkland et al., 1981). Uterine growth, however, irrespective of early estrogenic responses, requires the long term nuclear retention of the hormone-receptor complex. This is thought to involve the nuclear Type II binding sites stimulated by estrogen treatment (Markaverich et al., 1984). They have demonstrated that estradiol stimulation of uterine growth is antagonized by compounds (i.e. progesterone) which block the stimulation of nuclear Type II sites.

1D.6 Steroid hormones, gap junctions and uterine growth:

In a previous section (see section 1C) I have discussed in detail the relationship between the steroid hormones and the presence of gap junctions in the uterus. Since the steroid hormones, in particular estrogen, regulate uterine growth and gap junctions it is important to ascertain the relationship, if any, between the hormonal stimulation of uterine growth and presence of gap junctions. It is interesting and

perhaps significant that in the uterus, which grows tremendously during pregnancy, gap junctions are absent or present at an extremely low frequency. The cessation of uterine growth either at term or before is accompanied by hormonal changes and the development of myometrial gap junctions (Garfield, 1984). It is apparent then that the uterus grows throughout pregnancy in the absence of gap junctions and that at the end of pregnancy growth ceases and gap junctions develop. These observations are consistent with and support Loewenstein's (1979) hypothesis that an alteration of gap junctions may lead to the normal or abnormal growth or proliferation of cells.

1D.7 Steroid hormone dependent cancer:

During the late 1800's, Beatson reported that bilateral oophorectomy caused a striking regression of advanced metastatic breast cancer in premenopausal women. Since that time it has been recognized that some human breast cancers depend on the steroid sex hormones for their proliferation. As a result of this conclusion, hormone deprivation through endocrine ablation became a common therapy for advanced breast cancer in postmenopausal women (Huggins and Bergenstal, 1952; Pearson et al., 1956). In a recent article Kaufman (1981) reported that radiotherapists in the 1930's also concluded that estrogen could stimulate breast cancer. At that time it was believed that the larger, or more rapidly growing, tumors were more sensitive to irradiation and estrogen was therefore administered to primary inoperable breast cancer patients weeks before radiotherapy. When the patient returned for further therapy, however, their tumors had regressed. This regression of

breast tumors by large doses of estrogen was further substantiated by the work of Haddow et al., (1944).

The evidence that estrogen is able to initiate and stimulate growth of breast cancer is considerable. Noble and Collip (1941) had shown that mammary tumors induced by estrogen treatment regressed when the exogenous hormone source was removed. Cutts and Noble (1964) provided further support for this phenomenon and concluded that estrogens do contribute to mammary tumor production in animal models.

The use of exogenous estrogen has also led to an increased risk of developing endometrial cancer (Smith et al., 1975). These authors showed that the risk of endometrial cancer was 4.5 times greater among women exposed to estrogen therapy. This increased risk, however, only occurs when estrogen is used for more than 3.5 years (Hulka et al., 1980). A study by Elwood et al., (1977) indicated that incidence of endometrial cancer begins to rise sharply at about age 40 and proceeds through to the late 50's; thereafter there is a gradual decline. This type of cancer appears from about age 20 to 50, suggesting that endometrial cancer may be caused by some hormonal aberration during the reproductive years.

Endometrial cancer has also been associated with an estrogen producing ovarian tumor (McDonald et al., 1976). Because of this association it has been suggested that estrogens secreted by the tumor may be the causative agent for the malignancy (Gusberg and Kardon, 1971). The results of another study have shown that estradiol was the major unconjugated hormone secreted by the ovarian thecoma suggesting that this was the major factor which caused the endometrial carcinoma

(Reed et al., 1979). Additional support for an association of estrogen with certain cancers comes from the study of Sutherland et al., (1980). They reported that leiomyomatosis peritonealis dissemination, associated with pregnancy or with oral contraceptives, was controlled hormonally. The etiology of endometrial cancer therefore, may be similar to that of breast cancer (Gambrell, 1979). The biological basis for this is that both are neoplasms of estrogen target organs.

Steroid hormone action in target cells is mediated through specific intracellular receptors. Tissues devoid of receptors appear not to respond to hormonal influence. Folca et al., (1961) demonstrated that women whose metastatic breast tumors concentrated tritiated estrogen responded well when the hormone source was removed. Experimental mammary tumors induced in the rat were also found to resemble the uterus in their incorporation of estradiol in vivo (King et al., 1965). It was further shown that most of the estrogen, both in these adenocarcinomas and in other tissues, was associated with an acid-labile nonhistone component of the nuclear chromatin (King et al., 1966). This suggested that if breast cancer depends on estrogenic stimulation for its growth it might be expected to contain the cytoplasmic receptor. Alternatively, mammary cancers which have escaped from hormone dependency might not produce the receptor since they no longer need them (Jensen, 1981). This seems to be the case since a number of investigations indicate that cancers lacking the ability to bind estrogen rarely respond to endocrine therapy, whereas most (but not all) patients whose tumor contains estrogen receptors benefit from such treatment (Engelsman et al., 1973; Maass et al., 1973; Saulov et al., 1974).

The discovery of estrogen receptors in hormone responsive cancers has provided a useful method for predicting response to endocrine therapy (Leclercq and Heuson, 1979). The failure of a certain percentage of estrogen positive tumors to respond to endocrine therapy could reflect the fact that, although present, the receptors are nonfunctional (Jensen et al., 1976). In an attempt to identify cancers with nonfunctional estrogen receptors, Horwitz et al., (1975) have proposed that measurements of the progesterone receptor, the synthesis of which is known to be controlled by estrogen, might serve as a marker for estrogen dependency in hormone related cancers; the presence of the progesterone receptor in a tumor would indicate that the intracellular mechanism of action for estrogen is functional. More recent work has substantiated this hypothesis by indicating that progesterone receptor analysis further enhances the selectivity of patients with advanced breast cancer for hormonal therapy (Horwitz and McGuire, 1978; Bloom et al., 1980).

It is evident that estrogen can play a role in the induction and promote growth of tumors; there is virtually no evidence, however, that they are themselves carcinogens (King, 1971). The mode of action of estrogen in promoting cancer remains unclear; it is not even certain whether estrogen follows the current hypothesis for the hormones mode of action. However, the fact that estrogen binds to receptors in some tumors leads one to suspect that there may be some common pathways.

1D.8 Relationship between steroid hormones, gap junctions and tissue growth:

It is reasonable to suggest from the foregoing discussion that initiation and progression of growth (including abnormal growth) in tissues that contain steroid receptors may be regulated in part by gap junctions. Steroid hormones (particularly estrogen) stimulate growth of hormone dependent tissues and estrogen is also associated with the regulation of gap junction development between cells of such tissues. This implies a possible relationship between these parameters and growth of the myometrium. However, the relationship between the steroid hormones, gap junctions and growth is not clear. It is possible that the two events occur simultaneously but independently in response to hormonal influence. Alternatively, growth in response to hormones may be dependent upon the absence or presence of gap junctions.

Human mammary carcinoma has been shown to be neither electrically or metabolically coupled, that is communication-incompetent (Loewenstein, 1979). This implies that gap junctions are not present between the cells of this tumor or that the junctions are not functional. This suggests that a relationship between steroid hormones, gap junctions and abnormal growth of this tissue appears to be complex and requires further investigation for clarification. However, this poses a number of questions which must be be considered before any definitive correlation can be inferred between gap junctions and growth. For example, (A) the interpretation of the term "growth"; proliferative growth may be associated with cell coupling in a totally different way to hypertrophy; (B) at different stages of tumor development alterations

or reversals of the correlation between growth and cell coupling are possible; (C) the degree of differentiation of the malignancy may well effect the relationship; it is possible that the proliferating cells of a well differentiated tumor may differ in their dependence on coupling from those of an anaplastic tumor derived from the same tissue or cell type, and (D) the tissue of origin may clearly be, of itself, a source of variability.

1E. Purpose of the present study:

From the foregoing discussion it is obvious (1) that regulation of myometrial gap junctions by steroid hormones and prostaglandins is not completely understood in non-pregnant or pregnant animals, and (2) that the relationship between the control and presence of gap junctions and regulation of myometrial growth is not known. The purposes of the investigations reported in this thesis were: A) to determine the role of the steroid hormones and prostaglandins in regulation of myometrial gap junctions, and B) to define the relationship between regulation and presence of gap junctions and growth of the myometrium.

CHAPTER 2

OBJECTIVES OF THIS STUDY

2.1 Introduction:

Over the last three decades several significant advances have been made in understanding the events which culminate in normal labor. Many steps in the cascade of events leading to parturition have been elucidated. The complete series of events, however, has not been established. Specifically, the factors directly governing the activity of the myometrium are incompletely understood. It is of paramount importance then to investigate the regulation of myometrial activity in order to enhance our understanding of the initiation of labor.

The myometrium undergoes a transition from a relatively quiescent state during gestation to an active and reactive tissue at parturition. The muscle cells during parturition are synchronized in electrical and mechanical activity which results in the coordinated contractile activity of the myometrium necessary for the expulsion of the fetus and termination of pregnancy.

The conversion of uterine muscle at the end of gestation is rapid and the factors controlling the transition have not been ascertained. Our understanding of the conversion in myometrial activity, however, has been advanced by the finding that gap junctions exist between myometrial cells in the rat uterus during labor (Garfield et al., 1977; 1978; 1980; 1982). These muscle cell-to-muscle cell contacts were not present at any other time throughout gestation. These findings lead to the hypothesis that the development of myometrial gap junctions, specifically at the end of gestation, may be the structural basis for improved synchronized activity within the uterus allowing for the rapid transition of the myometrium

required for an effective labor.

In the ensuing years several studies on other animal species, including humans, have also shown results similar to that in the rat uterus; gap junctions are present between myometrial cells only immediately prior to, during and for a short period following parturition (Garfield et al., 1979a; 1979b; 1981; 1982a). Since labor is preceded and accompanied by hormonal changes, it is therefore reasonable to speculate that hormones regulate the presence of the junctions. In vitro investigations have demonstrated that hormones involved in the regulation of parturition influence the presence of gap junctions in the myometrium (Garfield et al., 1978; 1980a; 1980b). It is apparent that modulation of gap junction formation by agents involved in the initiation of parturition is a common mode of action, but whether the effect of these agents on labor results solely through regulation of gap junctions remains uncertain. Nevertheless, these findings suggest that gap junction formation in the myometrium may be a critical step, possibly one of the final requirements in the cascade of events leading to the initiation of parturition.

Regulation of myometrial gap junctions has also been studied in non-pregnant animals. Gap junctions appear between myometrial cells of the immature and ovariectomized mature rat and mature virgin mouse after several days treatment with large doses of estrogen (Dahl and Berger, 1978; Merk et al., 1980; Burghardt et al., 1984a). Moreover, myometrial tissues from immature rats incubated in vitro and treated with estrogen have a greater area of gap junctions. When progesterone is administered to the incubation medium together with estrogen, there is a suppression

of the stimulation of gap junction development (Garfield et al., 1978). Although studies on myometrial gap junction regulation in non-pregnant animals are limited and not detailed, they are consistent with the hypothesis that steroid hormones may regulate the presence of gap junctions in the myometrium.

It is also well known that ovarian hormones influence the spontaneous contractility of the non-pregnant myometrium (Marshall, 1962). Several in vitro and in vivo investigations on the contractile activity of the myometrium from various species have demonstrated that during the reproductive cycle the electrical and mechanical activities of the muscle undergo characteristic changes that can be correlated with circulating blood steroid levels (Fuchs and Fuchs, 1963; Talo and Karki, 1976; Ishikawa and Fuchs, 1978). Those reports suggest that estrogen stimulates the potential for uterine contractile activity, whereas progesterone on the other hand, appears to act as an antiestrogen with regards to spontaneous generation of electrical activity and conduction of activity within the tissue. Moreover, other studies have also shown that treatment of non-pregnant animals with estrogen promotes regularity and improves coupling and contractile activity of uterine muscle, while progesterone treatment resulted in irregularity of contractile activity (Marshall, 1959; 1962; Fuchs, 1976; Kao, 1977 Bortoff and Gilloteaux, 1980). In addition, prostaglandins stimulate the non-pregnant myometrium to contract both in vitro and in vivo (Maathus and Kelley, 1978).

The steroid hormones and prostaglandins are believed to regulate the presence of gap junctions in the myometrium (see above) and available evidence suggests that gap junctions may be present between myometrial

cells during selective periods of the reproductive cycle (Garfield and Hayashi, 1980). It is tempting to speculate therefore, that gap junctions may play a role in uterine contractility and functioning in general and not only during parturition. If so, then this may have further importance if extended to other tissues because gap junctions are present between most cell types (see Chapter 1) ,however, regulation of their presence between other cells is not fully understood.

In addition, the uterus dramatically increases in size throughout gestation to accommodate the growing fetus. It is well known that steroid hormones and other factors control uterine growth (Carsten, 1968). Recently, there have been many advances in our understanding of receptor mechanisms for the effects of steroid hormones in cellular growth. Unfortunately, however, the studies do not explain how the hormones control this phenomenon.

Gap junctions have been implicated as sites for the control of growth or proliferation of cells in normal and abnormal tissues (Loewenstein, 1979). Studies carried out in Garfield's laboratory implicate the steroid hormones in the control of junctional development and regulation in the myometrium. Since the steroid hormones control uterine growth and the presence of myometrial gap junctions it is possible therefore, that the mechanism by which steroid hormones control uterine growth may be through regulation of gap junctions.

2.2 Objectives:

2.2.1 Primary Objectives:

The primary objectives of this study were (1) to determine the role of the steroid hormones and prostaglandins in regulation of myometrial gap junctions, and (2) to define the relationship between the control of the junctions and uterine growth?

2.2.1.1 Regulation of myometrial gap junctions by steroid hormones:

The following questions were posed to determine the role of the steroid hormones in regulation of gap junctions.

2.2.1.1a Estrogen:

1) Do immature rats treated with estradiol develop myometrial gap junctions?
2) If gap junctions develop in response to estradiol will treatment with a synthetic estrogen, diethystilbestrol, stimulate the formation of gap junctions?
3) If gap junctions develop in response to estrogen treatment how do the junctions compare with those that form spontaneously in the parturient myometrium with respect to frequency, size and area of membrane they occupy?
4) If repeated injections of estrogens stimulate the presence of myometrial gap junctions in the immature rat, what is the time course for the development of the junctions?

5) Are repeated estradiol injections required for the development of gap junctions? Will gap junctions form in the myometrium after a single injection of the hormone and if so, when are they present?

6) Is the development of gap junctions a dose related estradiol effect?

7) Are the steroid receptors involved in regulation of gap junctions? What effect will an antiestrogen have on estradiol stimulation of gap junctions?

8) Is protein synthesis required for the expression of of gap junctions after estradiol treatment?

9) If gap junction development can be induced by treatment with estradiol in the immature rat, can similar treatments stimulate the formation of gap junctions in the myometrium of ovariectomized mature animals?

10) If estradiol is effective in ovariectomized mature rats, what is the time course for the development of the junctions?

11) Do gap junctions develop in ovariectomized mature animals treated with the same doses of estradiol used with the immature rats?

12) If estradiol treatment of non-pregnant animals induces gap junction development, will similar treatments stimulate the formation of gap junctions in the myometrium of pregnant rats? If so, what is the time

course for gap junction development in the pregnant animals?

14) If estradiol is effective (as above), what is the outcome of pregnancy in the animals treated with the hormone?

2.2.1.1b Progesterone:

1) Do immature rats treated with progesterone develop myometrial gap junctions?
2) Is progesterone capable of counteracting the effects of estradiol and/or diethystilbestrol on myometrial gap junctions?
3) If progesterone suppresses the estrogen induction of gap junctions when is the effect evident?
4) Is the inhibitory effect of progesterone influenced by the dose of the hormone used?
5) If progesterone is inhibitory in the immature rat will it also suppress the estradiol effect on the junctions in the myometrium of ovariectomized mature animals?

2.2.1.2 Regulation of myometrial gap junctions by prostaglandins:

The following questions were posed to determine the role of prostaglandins in the regulation of gap junctions.

1) Do immature rats treated with indomethacin develop myometrial gap junctions?

2) What effect does indomethacin have on estradiol stimulation of gap junctions in the immature animals?

3) If indomethacin influences the estradiol induction of myometrial gap junctions, what is the effect of progesterone in the immature rat treated with both estradiol plus indomethacin?

4) Does sodium meclofenamate, another inhibitor of prostaglandin synthesis, influence the estradiol stimulation of myometrial gap junctions?
If so, is the effect of sodium meclofenamate similar to that of indomethacin?

5) Does indomethacin influence the estradiol stimulation of myometrial gap junctions in ovariectomized mature rats?

6) If indomethacin is effective (as above) when is the effect evident?

7) If prostaglandins are involved in regulation of myometrial gap junctions, what is their mechanism of action?

8) What effect does indomethacin have on the plasma levels of estradiol in ovariectomized mature animals?

9) Does indomethacin alter the uptake of estradiol by uterine tissue from ovariectomized mature rats?

10) Are prostaglandins synthesized by uterine tissue after treatment with estradiol?

11) Is uterine prostaglandin synthesis inhibited by

indomethacin?

2.2.1.3 Regulation of uterine growth by steroid hormones:

The following questions were asked in determining the role of the steroid hormones in control of uterine growth.

2.2.1.3a Estradiol:

1) Does estradiol stimulate growth of the immature rat uterus? If so, what is the time course for growth in response to repeated injections of estradiol?
2) Does estradiol treatment induce an increase in both the wet and dry mass of the uterus?
3) How is uterine growth influenced by the dose of estradiol injected?
4) Do the endometrial and myometrial tissues of the uterus grow in response to estradiol treatment?
5) What effect does estradiol have on the cross-sectional areas of uterine tissues? Are both tissue layers equally affected by estradiol treatment?
6) Is there a similar growth response of the circular and longitudinal muscle layers of the myometrium after estradiol treatment?
7) How does the uterus grow in response to estradiol stimulation? Does uterine growth occur via cellular hypertrophy or hyperplasia?
8) How does repeated injections of estradiol influence

the incorporation of thymidine into DNA of uterine cells?

9) If thymidine is incorporated into uterine cells, does it occur in all uterine tissues?

10) What is the temporal pattern of total uterine DNA content after repeated injections of estradiol?

11) Are repeated injections of estradiol necessary for uterine growth? What is the time course for uterine growth following a single injection of estradiol?

12) Is the steroid receptor mechanism involved in regulation of uterine growth? What effect will an antiestrogen have on estradiol induced uterine growth?

13) If estradiol stimulates uterine growth in immature rats does the hormone have a similar effect in ovariectomized mature animals? If so, what is the time course of uterine growth (wet and dry mass) in those animals?

14) What is the time course of uterine growth (wet and dry mass) in untreated pregnant rats throughout gestation, during parturition and post-partum?

2.2.1.3.b Progesterone:

1) What effect does progesterone have on uterine growth in the immature rat?

2) Can progesterone suppress estradiol induced uterine growth? If so, then what is the time course for suppression

exerted by progesterone?

3) Does estradiol priming of uterine tissue influence the time course of progesterones inhibitory effect on estradiol induced growth?

2.2.1.4 Regulation of uterine growth by prostaglandins:

The following questions were posed to determine the role of prostaglandins in the regulation of uterine growth.

1) Does indomethacin treatment stimulate uterine growth in the immature rat?
2) What effect does indomethacin have on estradiol stimulation of uterine growth in those animals?
3) Does indomethacin treatment of ovariectomized mature rats induce uterine growth? If so, what effect does indomethacin have on uterine wet and dry mass?
4) If indomethacin influences uterine growth, what effect does this prostaglandin synthesis inhibitor have on estradiol induced uterine growth in ovariectomized mature rats?
5) What effect does indomethacin have on uterine weight of intact mature rats?

2.2.1.5 Relationship between uterine growth and presence of myometrial gap junctions:

To analyze the relationship between gap junctions and growth the

following questions were asked.

2.2.1.5a Immature rats:

1) What is the relationship between uterine growth and the quantity of myometrial gap junctions: A) after repeated injections of estradiol, and B) after a single injection of estradiol?
2) Does uterine growth (hypertrophy or hyperplasia) occur simultaneously or independently of the presence of gap junctions?
3) Do agents which alter the development of myometrial gap junctions in response to estradiol interfere with uterine growth? A) tamoxifen, B) progesterone, and C) indomethacin.

2.2.1.5b Ovariectomized mature rats:

1) What is the relationship between uterine growth and the presence of myometrial gap junctions induced by estradiol? Is the relationship between growth and gap junctions similar to that seen in immature animals (see above)?
2) Does uterine growth occur simultaneously or independently of the presence of gap junctions?
3) Is the effect of indomethacin, alone or together with estradiol on myometrial gap junctions, correlated with any alteration in uterine growth?

2.2.1.5c Pregnant animals:

1) What is the relationship between uterine growth and the presence of myometrial gap junctions of untreated pregnant rats throughout gestation, during labor and post-partum?

   Is the relationship similar to that found in non-pregnant immature and ovariectomized animals after treatment with steroid hormones (see above)?

2) Does uterine growth occur when gap junctions are present or absent from the myometrium?

2.3 Significance:

Mechanisms underlying labor, whether term or preterm, are not completely understood. However, it is well known that preceding the initiation of term or preterm labor changes occur in the ratio of circulating estrogen-to-progesterone levels and uterine tissue concentrations of prostaglandins (Thorburn and Challis, 1979; Puri and Garfield, 1982). Furthermore, morphological changes occur within the myometrial cells at term (i.e. gap junction formation) presumably secondarily to the changes in steroid hormones and prostaglandins (Garfield et al., 1980a). Since both the endocrine and structural changes appear to be necessary for the initiation of labor, it is reasonable to think that a causal relationship may exist between the two events. If the steroid hormones and prostaglandins regulate the presence of gap junctions in the myometrium than understanding this regulation may have significant implications in our comprehension and management of labor.

Moreover, under abnormal circumstances the life of the newborn or fetus may be compromised if labor begins early and results in premature birth or if labor is delayed at term. Preterm labor is the leading cause of perinatal morbidity and mortality. Preterm birth is responsible for approximately 85% of early neonatal deaths not due to lethal congenital deformations (Rush et al., 1979). Furthermore, early neonatal mortality is high when preterm labor is spontaneous but low when preterm labor is elective. Therefore, preventing spontaneous preterm labor would considerably reduce neonatal mortality. Since the presence of gap junctions between myometrial cells immediately prior to and during term or preterm delivery may be a critical event, possibly one of the final requirements necessary for effective labor, then understanding the mechanisms that control their development may allow us to stimulate the process when necessary or inhibit it when appropriate.

Uterine contractile activity is also evident in the non-pregnant uterus, in particular during the menstrual period of the reproductive cycle. Frequently, painful contractions of the myometrium (primary dysmenorrhea) accompany menstruation. These contractions result in severe cramps in the lower abdominal cavity and lumbar region and are accompanied by sweating, nausea, severe headaches, vomiting, diarrhea, and occasional dizziness and fainting. It has been reported that 25 to 50% of fertile women experience these contractions; 1 out of 10 women have these pains which force them to avoid normal activities for 1 to 3 days each month (Dandenell et al., 1979). Strong myometrial contractions of menstruation are correlated with changes in levels of steroid hormones and prostaglandins, similar to that observed in the pregnant uterus at

term. Moreover, there is evidence suggesting that gap junctions may be present between myometrial cells during menstruation and in patients with primary dysmenorrhea. The presence of gap junctions may be the underlying basis of this gynecological problem. Understanding regulation of the presence of gap junctions in the myometrium therefore, would have significant implications in the control of uterine activity and may benefit in alleviating the painful contractions of menstruation which handicap a large percentage of the female population.

Non-pregnant animals may serve as good working models for investigating the regulation of myometrial gap junctions. In animals, such as the immature and ovariectomized mature rat, the ovarian production of the steroid hormones is negligible. This allows for manipulation of the hormonal milieu through exogenous administration of the steroid hormones and provides answers to questions as those outlined above. In addition, factors other than the steroid hormones which may be involved in regulating gap junctions can be studied using these animal models. Furthermore, an understanding of gap junction regulation in non-pregnant animals may further our knowledge of their regulation in the pregnant myometrium and of myometrial activity in general.

An understanding of regulation of gap junctions in the myometrium may shed light on the control of the junctions in tissues other than smooth muscle. Gap junction occurrence is widespread throughout mammalian tissues, however, factors regulating their presence are not well understood. It may be possible to extrapolate information from the myometrium to cells in other tissues, in particular, tissues responsive to the steroid hormones. Furthermore, it is believed that gap junctions

are involved in the regulation of cellular growth. However, the exact role of the junctions in growth is confusing since it appears to vary between types of tissue. Since uterine growth can be manipulated in non-pregnant animals, then it should be possible to determine if factors which regulate gap junctions in the uterus have any influence on uterine growth. If so, then one could determine if any consistent relationship between the two events exists after various treatments which affect both growth and junctions. Therefore, this study may also enhance our understanding of tissue growth and mechanisms for growth regulation. Moreover, the mechanisms responsible for the unregulated growth or proliferation of cancer cells are not known. Some cancer tissues in women, including tumors of the mammary gland and endometrium, appear to be regulated by the steroid hormones secreted from the ovary, the placenta in pregnant women, and the adrenal gland. It has long been recognized that ovariectomy results in regression or remission of advanced cancer in premenopausal women. Other observations and studies have lead to the conclusion that these cancers depend upon the steroid sex hormones. Indeed estrogen has been identified as the hormone responsible for growth in some conditions. Recently there have been many advances in the understanding of receptor mechanisms for the effects of estrogen on cell proliferation. There have been attempts to identify therapeutic schedules dependent upon receptor analysis from cancerous tissues taken from women. Unfortunately these studies do not explain how estrogen, reacting through its receptor mechanism, controls cell proliferation.

It has been suggested that altered cell-to-cell communication may

be in part responsible for abnormal growth of cancerous tissues. Cell-to-cell communication is thought to occur via gap junctions. It has been postulated that A) the junctions or channels created by the junction may be necessary for cell-to-cell transmission of molecules which control growth or proliferation, and B) absence of junctional communication results in disturbance of growth (Loewenstein, 1979). This study may contribute important information about the regulation of gap junctions in the myometrium and other tissues including cancers where receptors are present for the steroid hormones. Furthermore, it is hoped that this study will broaden our knowledge in understanding the relationship between gap junctions and growth which may lead to a better comprehension of abnormal growth.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals:

Immature (21-30 days old weighing 30-60 gm), mature non-pregnant and pregnant female Wistar rats (250-350 gm) with a gestational age of 22-23 days were used to study the hormonal regulation of myometrial gap junctions. Most non-pregnant mature animals were bilaterally ovariectomized and allowed to recover for three weeks before treatments began. Pregnant rats were grouped as ante-partum (days 16-19 of gestation) and parturient (day 22-23 of gestation after commencement of vaginal bleeding or expulsion of at least one fetus) animals. Non-pregnant rats were housed 4 to 5 per cage whereas pregnant animals were caged individually. All animals were maintained at a constant temperature (22 C), with a photoperiod of 12 hours light: 12 hours dark and were fed Purina rat chow and water ad libitum.

3.1.1 Ovariectomy:

In order to investigate steroid hormone regulation of myometrial gap junctions in adult rats, mature non-pregnant animals were bilaterally ovariectomized to deprive them of their primary source of the steroid hormones. The surgical procedure was carried out under aseptic conditions with the rats under ether anesthesia. Following one midline incision through the skin on the back, the skin was pulled over to each side and blunt dissection was performed through the abdominal wall over the ovaries. Once each ovary was externalized, a single suture was placed around the oviduct. After excision of the ovaries

the uterine horns were allowed to retract into the abdominal cavity and then the abdominal wall and dorsal skin incisions were sutured closed. The animals were allowed to recover for 21 days before experimental procedures began.

3.1.2 Treatment Schedule:

3.1.2.1 Immature rats:

Animals were injected subcutaneously with estradiol-17B (50, 150, 300, 500, 750 and 1000 ug/day) and diethylstilbestrol (500 ug/day) alone or together with progesterone (2 mg/day or 2.5 mg twice daily). The steroid hormones were administered in 0.1 ml sesame oil. Tamoxifen suspended (5 mg/0.1 ml) in sesame oil and cycloheximide dissolved (100 ug/0.1 ml) in physiological saline were injected subcutaneously with and without estradiol (500 ug/day). Indomethacin (0.2 mg/0.1 ml twice daily) in sesame oil was also administered alone or concomitantly with estradiol (50 and 500 ug/day) and estradiol (500 ug/day) plus progesterone (2.5 mg twice daily). Sodium meclofenamate dissolved in saline was administered at 0.7 mg/0.1 ml/day by itself or together with estradiol (500 ug/day). Control animals received 0.1 ml sesame oil or physiological saline.

The injections were started on the twenty-second day of age (unless otherwise stated) and were repeated every twenty-four hours for 1-8 days as indicated for the particular experiment. Twenty-four hours after the required number of injections the rats were weighed and then killed by cervical dislocation following a blow to the head. The uterus

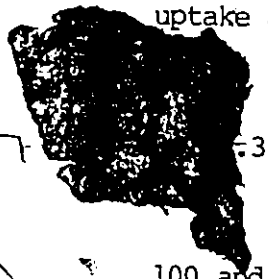
from each animal was quickly removed, cleaned of adjoining fatty tissue and mesentery and tissue samples were either fixed for thin section and freeze-fracture electron microscopic studies or used for wet and dry weight estimations, tritiated thymidine uptake studies, deoxyribonucleic acid (DNA) assays or light microscopy histology and autoradiography.

3.1.2.2 Ovariectomized mature rats:

Ovariectomized rats were injected subcutaneously with 0.1 ml sesame oil alone or containing estradiol (0.025, 0.10, 50 and 500 ug/day) and/or indomethacin (2 mg twice daily) for 1-6 days, as indicated. Progesterone (2.5 mg twice daily) was administered alone or simultaneously with estradiol (500 ug/day) for three days. Control animals received sesame oil alone. Intact mature rats were also treated with sesame oil with and without indomethacin for 3 or 6 days.

Twenty-four hours after the required number of injections, the rats were weighed and killed as described for the immature animals. The uterus from each animal was rapidly removed, cleaned and tissue samples were fixed for thin section electron microscopic studies or wet and dry weight estimations. In other animals blood and uterine tissue samples were collected after ether anesthesia. Samples of blood collected by cardiac puncture (8 ml) using heparinized syringes were immediately centrifuged in graduated centrifuge tubes at 2500 rpm for 10 minutes at 4 C. Plasma was separated and immediately frozen in liquid nitrogen and kept frozen at -20 C until assayed for estradiol levels. After removing and cleaning the uterus, some tissue samples were also immediately

frozen and stored at -20 C until processed for prostaglandin assays. Uterine tissues from ovariectomized rats were also used for estradiol uptake and retention studies.



3.3 Pregnant rats:

Animals were injected subcutaneously with estradiol (5, 25, 50, 100 and 500 ug/day) in 0.1 ml sesame oil for 1-3 days starting either on day 16 or day 21 of gestation. Control rats received the same volume of sesame oil. Twenty-four hours after the required number of treatments or once labor commenced (vaginal bleeding observed or delivery of at least on pup) the rats were weighed and killed as previously described. Uterine tissues were immediately removed and prepared for ultrastructural analysis by thin section and freeze-fracture electron microscopy. Pregnant animals were also killed on various days of gestation (days 6, 11, 16, 18 and 20), during parturition and one day post-partum.The uteri were removed, cleaned and used for weight and dry weight estimations.

3.2 Tissue collection and processing for microscopy:

3.2.1 Thin section electron microscopy:

Small longitudinal strips (ca. 3 mm by 1 cm) of uterine tissue were removed from the central portion of each uterine horn, stretched 1.5 times the excised length to maintain their in situ length and pinned out on dental wax with the serosal surface up. This procedure is routinely performed to minimize the degree of contraction of the

myometrial samples when immersed into the fixative solution. For preservation of cellular detail and membrane ultrastructure, the tissue samples were fixed for 2 hours at room temperature in 75 mM sodium cacodylate buffer (pH 7.4) containing 2% glutaraldehyde, 4.5% sucrose and 1 mM calcium chloride. After fixation in glutaraldehyde the tissues were washed for 1 hour in 100 mM cacodylate buffer containing 6% sucrose and 1.25 mM calcium chloride followed by postfixation in 2% osmium tetroxide in 50 mM cacodylate buffer (pH 7.4) for 90 minutes. All tissues were stained en bloc with saturated aqueous uranyl acetate for 60 minutes, then dehydrated through a graded ethanol series and embedded in Spurr resin.

The tissues were oriented so that the outer longitudinal muscle layer of the uterine strips was cut in cross-section. Thin sections of the longitudinal muscle were cut with either a Porter-Blum (MT2-B) or Reichert Ultracut ultramicrotome equipped with a diamond knife. Sections were mounted on 200 mesh copper grids, stained with lead citrate for 2 minutes and then examined using either a Philips 300 or 301 electron microscope. In all cases the myometrium was examined in transverse orientation.

3.2.2 Thin section morphometry: Quantitative measurements of gap junctions:

The multi-purpose test grid was used to estimate the length of myometrial smooth muscle cell plasma membrane. From one thin section overlying a grid square 20-22 non-overlapping and randomly selected photographs (ca. X4,200 magnification) were taken of the longitudinal.

muscle cells cut in cross-section. The negatives were enlarged 8 times to a final print magnification of X33,600 on 20 x 25 cm photographic paper. A transparent multi-purpose coherent test grid was superimposed over each photograph and the number of intersections of the grid lines with the plasma membrane were counted. The length of membrane present is mathematically related to the number of intersections between the grid lines and the smooth muscle cell plasma membrane. Since the smooth muscle cells are often oriented in an anisotropic manner on the photographs, it was necessary to make two intersection counts at right angles to each other and to use the average of those counts. The total length of non-junctional smooth muscle membrane in each photograph was then calculated according to the following formula:

$$Ln = \pi/2 \times I/Lp \times A \times CF$$

where Ln is the length of non-junctional membrane; I is the average number of intersects determined by two estimates taken at right angles to each other; Lp is the total length of probe lines on the test grid (138.6 cm); A is the area of the photograph examined in the grid system (384 cm^2) and CF is the correction factor for the magnification. The total length of membrane scanned was the sum of the membrane lengths in each photograph.

Gap junctions were identified and measured in photographs or negatives enlarged to X 100,000 magnification according to the following criteria: A) a membrane opposition demonstrating a 5 or 7 lined multi-laminar morphology at some point along its length. If seven lines were evident, the gap between the two unit membranes must be approximately 2 nm wide, B) the gap junction must be connecting two

smooth muscle cells together, and C) the total thickness of the junction must be about 15-20 nm. The total length of gap junction membrane was calculated as the sum of all individual gap junctions in each photograph. The final values obtained were the number and size of gap junctions and the length of non-junctional membrane. From these values the data were expressed as the frequency of gap junctions (number of gap junctions per 1000 um plasma membrane), size or length of gap junctions (determined with a mm scale on photographs at X 100,000 magnification) and the proportional area of gap junctional membrane per area of plasma membrane, expressed as a percentage using the formula:

$$Aj/An = (Lj \times 2/Ln + Lj \times 2) \times 100$$

where Aj and An are the area of gap junctional and non-junctional membrane respectively; Lj is the total cross-sectional length of gap junctional membrane and Ln is the length of non-junctional membrane measured. Gap junctions comprise membrane components of two cells and in estimates of gap junction area both components must be considered. Therefore, the cross-sectional length of gap junctions (Lj) is corrected (X2) to obtain the area of gap junctional membrane (Aj).

3.2.3 Freeze-fracture electron microscopy:

Tissues samples for freeze-fracture analysis were fixed in 2% glutaraldehyde as described for thin section microscopy. The tissues were rinsed in 100 mM cacodylate buffer (pH 7.4) containing 6% sucrose and 1.25 mM calcium chloride for 1 hour and then infiltrated for 1.5 hours through a graded series of glycerol (10, 20 and 30%) in

cacodylate buffer. The endometrium was teased away from the muscle layers while in glycerol and the muscle was cut into small samples 1-1.5 mm^3. The tissue samples were mounted on gold alloy specimen discs (3-4 tissue samples per disc) such that the longitudinal muscle layer was upper most and the samples formed a small pyramid suspended in a drop of 30% glycerol. Discs of tissue were rapidly frozen in the liquid phase of Freon-22 cooled in liquid nitrogen for 15 seconds and subsequently stored in liquid nitrogen until fractured. The tissues were freeze-fractured at -105 C at 10^{-7} Torr in a Balzers 301 freeze-etch unit equipped with platinum and carbon electron guns. The fractured tissues were coated with a thin layer of platinum (2200 V, 70 mA for 7-10 seconds) at a 45 degree angle to the tissue followed immediately by carbon (2400, 110 mA for 10-20 seconds) at a 90 degree angle to the tissue. After shadowing with platinum and carbon, the tissues were immersed in methanol and transferred through a gradually diluted methanol-water solution and finally into distilled water. Adherent tissue was removed from the replicas by digestion in commercial bleach for 2 hours. The replicas were rinsed in distilled water 3 times and then retrieved on 200 mesh collodian coated grids. The replicas were examined using either a Philips 300 or 301 electron microscope.

3.2.4 Freeze-fracture morphometry:

The area of plasma membrane in freeze-fractured replicas was determined by tracing the perimeter of the membrane regions with a microplanimeter on a series of photographs printed at X 33,600

magnification on 20 x 25 cm photographic paper. The total area of membrane is the sum of the membrane areas in each photograph.

Gap junction frequency was determined by a visual count on each of the photographs from the series used to estimate plasma membrane area. Each gap junction was further enlarged and printed at X 100,000 magnification for diameter measurements. The higher magnification increased the precision of this measurement. The final values obtained were the number and diameter of gap junctions and the area of plasma membrane. From these values the data were expressed as the frequency (number of gap junctions per 1000 um^2 plasma membrane) and size or mean diameter of gap junctions.

3.2.5 Light microscopy:

A small portion (ca. 5 mm) of the entire cross-section of the uterine wall from the mid region of the uterine horn was removed and fixed by immersion into 10% phosphate buffered formalin. After fixation the tissues were dehydrated in methyl alcohol, cleaned using xylene and embedded in paraffin wax. Five-micron thick sections were cut and then stained with Harris hematoxylin and 2% aqueous eosin. The sections were then dried, cleaned, mounted in permount on microscope slides and examined and photographed with a Zeiss photomicroscope 3 equipped with an automatic 35 mm camera. Tissue processing and sectioning were done in collaboration with Ms. S. Putns, chief technologist in the Department of Pathology, McMaster University Health Sciences Center.

3.2.6 Light microscopy morphometry:

The multi-purpose test grid system was used to estimate surface areas of uterine tissue layers from immature rats. Thick sections of the uterus were photographed at X 31.25 magnification, enlarged and printed on 20 x 25 cm photographic paper at a final magnification of X 300. The transparent multi-purpose test grid was superimposed over each photograph and the number of times each test point of the grid lines fell within a tissue layer were counted. The number of test points falling within the tissue in relation to the total number of test points is an estimate of the area of the tissue in the section. The area measurements were carried out on cross-sections of the whole uterus, longitudinal and circular muscle layer (separately and together) and the endometrium. The endometrium measured consisted of the luminal epithelial cells and stroma to the circular muscle layer. The myometrium was measured from the stromal/ circular muscle boundary to the serosal surface excluding the connective tissue layer separating the circular and longitudinal muscle layers. The tissue cross-sections were not always oriented in an isotropic fashion on the photographs, therefore two test point counts were made at right angles to each other and the average of the counts was used in the measurements. The total area of tissue in each photograph was calculated according to the formula:

$$Au = Pi/Pt \times A \times CF$$

Au is the area of uterine tissue; Pi is the average number of test points determined by estimates taken at right angles to each other; Pt is the total number of test points on the test grid; A is the area

of the grid system and CF is the correction factor for the magnification.

3.2.7 Light microscopy autoradiography:

Immature rats were injected with estradiol (50 and 500 ug/day) for 1-6 days. Twenty-four hours after the required number of injection the uterus from each animal was removed, cleaned and incubated in vitro for 1 hour in the presence of ^{3}H-thymidine. Following the incubation period a small sample of uterine tissue was removed , fixed in glutaraldehyde and osmium tetroxide, dehydrated in alcohol and embedded in Spurr resin as outlined previously for thin section electron microscopy excluding the step of en bloc staining with uranyl acetate. The remaining tissue was used for thymidine incorporation measurements and DNA assays (see section 3.3 below)

For autoradiography, 1 um thick sections were cut with a Reichert Ultracut ultramicrotome equipped with a glass knife. The sections were mounted on glass slides and coated with Kodak NTB II nuclear track emulsion by dipping the slides into the emulsion for approximately 1 second. The emulsion was allowed to dry and the slides were placed into light-tight boxes containing drierite and stored at 4 C for 2 weeks. After the exposure period, the sections were developed in Kodak developer D-19 for 4 minutes, cleared and fixed in Kodak rapid fixer for 5 minutes, washed and allowed to dry. Sections were stained with 1% toluidine blue in borax, examined and photographed using a Zeiss photomicroscope 3 equipped with an automatic 35 mm camera.

3.3 Incorporation of tritiated thymidine:

To investigate the effects of estradiol treatment on DNA synthesis, uterine incorporation of ^{3}H-thymidine was measured according to the procedure of Harris and Gorski (1978). After *in vivo* hormone treatment uteri were removed from immature animals, cleaned, cut open longitudinally along the mesometrial attachment, rinsed in Krebs-Ringer solution (composition mmole/liter:KCl 4.6; $CaCl_2$ 2.5; $MgSO_4$ 1.2; NaH_2PO_4 1.2; $NaHCO_3$ 21.9; glucose 11.1; pH 7.4) at 4 C and then incubated *in vitro* for 1 hour in Krebs-Ringer solution (two uteri/2 ml) containing ^{3}H-thymidine (2 uCi/ml; 16.2 Ci/mole). Throughout the incubation period the medium was aerated with a mixture of 95% O_2-5% CO_2 and maintained at 37 C. At the end of the incubation period the uteri were rinsed in ice-cold Krebs-Ringer solution and then homogenized in 3 ml cold 0.5M $HClO_4$. The homogenate was centrifuged at 800 x g for 10 minutes. The resulting pellet was resuspended and washed three times in 4 ml cold 0.3M $HClO_4$. The final pellet was resuspended in 2 ml cold 0.5M $HClO_4$ and hydrolyzed by heating in a boiling water bath for 30 minutes. Samples were cooled at 4 C for 30 minutes followed by a centrifugation at 800 x g for an additional 10 minutes. Portions of the supernatant (0.25 ml aliquots) were added to scintillation vials containing 10 ml Aquasol. The solutions were counted for ^{3}H-thymidine in a Beckman scintillation counter with internal standards for correction of quenching. Counting for radioactive incorporation was done in triplicate. From the remaining supernatant, a 1.0 ml aliquot (in duplicate) was also taken for the colourimetric estimation of DNA

(Burton, 1956) with calf thymus DNA as the standard.

3.4 Determination of uterine wet and dry weights:

Uteri removed from animals were cleaned of adjoining fatty tissue and mesentery, cut open longitudinally along the mesometrial attachment and blotted twice on filter paper. Each uterus was immediately weighed on a Mettler AE 160 balance. For determination of uterine dry weight the uterine tissues were placed in preweighed dry tin weighing dishes and dried at 100 C to a constant weight. The uterine weights were normalized to animal body weight by dividing the uterine wet and dry weights by the total body weight for each animal.

3.5 Estimation of 17B-estradiol and prostaglandin F levels:

Serum 17B-estradiol and tissue prostaglandin F levels were measured using specific radioimmunoassay. Serum samples were diluted with physiological saline and following administration of labeled steroid the samples were extracted twice with diethyl ether. The quantitative determination of estradiol was performed with an antiserum raised against estradiol-6-(O-carboxy-methyl)- oxime-bovin serum albumin at a final dilution of 1:3000. 2,4,6,7-[^{3}H]-estradiol-17B (110 Ci/mmole) was used as the tracer. The separation of free and bound steroid was achieved using dextran coated charcoal. The sensitivity of the assay was 5 pg and the coefficient of variation between and within assays was < 10%.

To measure prostaglandin F levels, the tissue samples were acidified to pH 4.5 using citric acid and then homogenized and

extracted with ethyl acetate. Prostaglandin F was separated from other prostaglandins on silicic acid columns using benzene:ethyl acetate: methanol as developing solvents. Antiserum raised against prostaglandin F-bovine serum albumin and 5,6,8,9,11,12,14,15-[^{3}H] prostaglandin F (135 Ci/mmole) were used to measure prostaglandin F levels. Free and bound hormone were separated by dextran coated charcoal. The high reproducibility of the assay was indicated by a < 10% coefficient of inter and intraassay variations.

3.6 Uptake of tritiated estradiol-17B:

To investigate the influence of prostaglandins on uterine tissue levels of estradiol the effect of indomethacin (an inhibitor of prostaglandin synthesis) on the uptake of ^{3}H-estradiol by the rat uterus was studied. Ovariectomized mature rats were divided into two groups; to one group of animals indomethacin (5 mg/kg body weight) was injected twice daily for three days, whereas the other group of animals received the vehicle alone. On the fourth day 2,4,6,7-[^{3}H]-estradiol (110 Ci/mmole) was injected (6 uCi in 0.25 ml sterile saline) through the jugular vein to all rats under ether anesthesia. One hour later the animals were killed and uterine tissue was immediately collected. The tissue was homogenized in Tris-HCl buffer (0.01M, pH 7.4) containing 0.25M sucrose and 0.003M calcium chloride. The homogenate was extracted with hexane and the dried extract was chromatographed through Sephadex LH-20 columns to separate estradiol from any metabolites. The [^{3}H]-estradiol fraction was counted in a liquid scintillation counter at an efficiency of 52%.

The serum and tissue levels of estradiol and prostaglandin F and tissue uptake of [^{3}H]-estradiol were carried out in collaboration with Dr. C. P. Puri at the Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, Missouri.

3.7 Statistical comparisons:

The Mann Whitney U test was used for statistical comparisons on quantitative measurements of gap junctions between groups on different days of treatment. The unpaired Student's two tailed t-test was used to test differences between groups for all other experimental data. An analysis a variance was used to indicate differences for multiple comparisons of all data between samples from one treatment group on different days of treatment. A significance level of 5% was used for the tests.

CHAPTER 4

CONTROL OF MYOMETRIAL GAP JUNCTIONS BY STEROID HORMONES

4.1 Introduction:

The relatively quiescent myometrium of gestation transforms into an active and reactive tissue during labor eliciting strong synchronized contractions resulting in the expulsion of the fetus and termination of pregnancy (Csapo, 1981). Although the factors controlling the myometrium during parturition appear complex, Garfield et al., (1977; 1978; 1979) have postulated that the development of gap junctions between these smooth muscle cells is necessary for the occurrence of term or preterm labor.

Gap junctions are believed to regulate tissue activity by providing the basis for electrical and metabolic coupling between cells of various tissues (Bennett, 1973; Griepp and Revel, 1977; Peracchia, 1980; Hooper and Subak-Sharpe, 1981; Loewenstein, 1981). The area of gap junction contact increases rapidly between the smooth muscle cells of the uterus in a variety of species during labor and decreases following parturition (Garfield et al., 1977; 1978; 1979). The appearance of gap junctions between myometrial cells at term may furnish a large membrane area of cellular communication that is sufficient for the synchronized muscle contractility of labor leading to the effective termination of pregnancy (Garfield et al., 1977; 1978). Evidence that improvement in electrical coupling accompanies the increase in myometrial gap junctional area during parturition has been provided from microelectrode and impedance studies (Sims et al., 1982). Specifically, the appearance of gap junctions in the myometrium at term was correlated with a decrease in junctional resistance and an

increase in the length constant for the spatial decay of electrotonic potentials. These data suggest that gap junctions function to improve electrical coupling between smooth muscle cells in the uterus.

The mechanism responsible for controlling the presence of myometrial gap junctions during parturition has not been clearly established. It is likely that the changes in the steroid hormones and prostaglandins which precede or accompany the presence of gap junctions are responsible for their appearance (Garfield et al., 1980a). Prior to parturition in the rat, rabbit and sheep there is a pronounced progesterone withdrawal followed by an increase in the concentration of estrogen (Bedford et al., 1972; Csapo, 1975). It has been shown that there is a dramatic increase in myometrial gap junction area coinciding with the change in the hormone levels at term in the rat and sheep (Garfield et al., 1978; 1979; 1980a; 1982a). Ovariectomized, preterm pregnant rats treated with estradiol have myometrial gap junctions and abort their fetuses but progesterone treatment prevented both the appearance of gap junctions and premature labor (Garfield et al., 1982a). Gap junctions were also scarce in the myometrium of term pregnant rats treated with progesterone which prevented labor and extended the gestation period (Garfield et al., 1978). These studies indicate that gap junctions may be necessary for labor, whether normal or premature, and that their presence in the myometrium may be regulated by steroid hormones.

In other studies of non-pregnant animals, it has been reported that myometrial gap junction size and frequency increased with estrogen treatment. Early studies by Bergman (1968) suggested that the number

of junctions in the myometrium of non-pregnant mature rats increased following diethylstilbestrol treatment. Similarly, Dahl and Berger, (1978) and Merk et al., (1980) have also shown that myometrial gap junctions are present in mature virgin mice and immature rats after treatment over several days with very high doses of estrogen (Progynon) and diethystilbestrol, respectively. However, Garfield et al., (1978), failed to find myometrial gap junctions when lower doses of estradiol were injected into rats. Other studies showed that myometrial tissues from immature rats incubated in vitro and treated with estradiol have a greater area of gap junctions (Garfield et al., 1980a). Recently, a messenger RNA preparation extracted from the myometrium of rats treated with an unspecified estrogen has been suggested to increase cellular coupling between cultured cells of a mouse cell line (CL-ID) which normally are neither electrically coupled nor contain gap junctions (Dahl et al., 1980). The above studies demonstrate that gap junctions are present in non-pregnant uterine tissue after treatment with high doses of estrogen. Gap junctions are also present between granulosa cells (Merk et al., 1972; Burghardt and Anderson, 1981) and theca interna cells (Burghardt and Anderson, 1981) of the rat ovary following estradiol administration.

The present study was designed to determine the effects of steroid hormones on the presence of myometrial gap junctions in non-pregnant (immature and ovariectomized mature) and pregnant rats. The results indicate that estrogen stimulates whereas progesterone inhibits the presence of myometrial gap junctions and that their presence between uterine smooth muscle cells is required for the

occurrence of term or preterm labor.

4.2 Results:

4.2.1 Effects of estrogen and progesterone:

Table 1 shows the effects of estradiol-17B and diethylstilbestrol with and without progesterone on myometrial gap junctions in immature rats injected over a 6-day period. There were no gap junctions in tissues taken from control rats injected with the vehicle alone or in animals treated with progesterone (2 mg/day for 6 days). In tissues from rats treated with 50 ug/day estradiol only one small gap junction was observed. However, after treatment with 500 ug/day estradiol, the frequency (number of gap junctions/1000 um membrane) and area of gap junctions increased significantly ($P<0.005$ and $P<0.01$, respectively). Diethylstilbestrol administered at the same concentration (500 ug/day) caused increases similar to that observed with estradiol. Treatment of animals with estradiol or diethylstilbestrol (500 ug/day) plus progesterone (2 mg/day) significantly reduced ($P<0.01$, $P<0.05$) the frequency of gap junctions from 3.44 ± 0.58 and 1.53 ± 0.58 to 0.93 ± 0.58 and 0.24 ± 0.17 per 1000 um membrane, respectively (Table 1). The mean size of the junctions in tissues from rats injected concomitantly with either estrogen plus progesterone were larger, but the differences were insignificant compared to treatment with the estrogens alone. Simultaneous progesterone administration also decreased the percentage of membrane area occupied by gap junctions, however, the values were

Table 1. Number, size and area of gap junctions (GJs) between longitudinal myometrial smooth muscle cells are taken from immature rats treated with sesame oil (control) or estradiol (E_2) and diethylstilbestrol (Des) with and without progesterone (P_4) for six days and from pregnant rats during delivery. No. of tissues, total number of tissues examined. Number in parentheses indicate the number of animals from which tissues were taken; No. of GJs, total number of gap junctions found within 20-22 non-overlapping photomicrographs of each tissue; No. of GJs/ 1,000 um membrane, frequency of gap junctions per 1,000 um measured membrane length; Size of GJs, lengths of each gap junction; Area GJs/Area Plasma Membrane, area of gap junctions expressed as percentage of gap junction area per area plasma membrane. Frequency, size and area of gap junctions are given as mean $\pm$ S.E. Mean values with same superscripts are not significantly different ($P>0.05$).

TABLE 1. EFFECT OF HORMONE TREATMENT ON GAP JUNCTIONS (GJs) IN RAT MYOMETRIUM

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane,% (Mean ± S.E.)
Immature Rats						
Control	8(4)	0	11,047	0	0	0
P_4 (2 mg/day X 6 days)	4(4)	0	5,946	0	0	0
E_2 (50 ug/day X 6 days)	8(4)	1	9,710	0.11 ± 0.11^{1}	36	0.001 ± 0.001^{a}
E_2 plus P_4	8(4)	0	11,521	0	0	0
E_2 (500 ug/day X 6 days)	8(4)	30	8,902	3.44 ± 0.52^{2}	89 ± 8^{A}	$0.061 \pm 0.010^{b,f}$
E_2 plus P_4	8(4)	8	8,490	$0.93 \pm 0.58^{1,3}$	$142 \pm 28^{A,B,E}$	$0.025 \pm 0.011^{b,d}$
Des (500 ug/day X 6 days)	8(4)	13	8,931	$1.53 \pm 0.58^{2,3}$	$127 \pm 31^{A,E}$	$0.036 \pm 0.014^{b,e}$
Des plus P_4	8(4)	3	10,640	0.24 ± 0.17^{1}	$153 \pm 30^{C,E,F}$	$0.007 \pm 0.005^{c,d,e}$
Pregnant Rats						
Delivery	15(10)	38	13,599	3.19 ± 0.046^{2}	$224 \pm 23^{D,F}$	0.112 ± 0.012^{f}

not significantly different compared to those observed after estradiol or diethystilbestrol treatment.

Quantitative values of gap junctions in the myometrium of delivering rats is also expressed in Table 1 for comparison. The mean frequency and size of the junctions present in tissues from parturient rats were 3.19 ± 0.46/1000 um membrane and 224 ± 23 nm, respectively. Gap junctions also occupied 0.112 ± 0.012 percent of the plasma membrane area of the myometrial cells during labor. It is interesting to note that the frequency of gap junctions in delivering tissue was not significantly different ($P>0.05$) compared with that in immature rats treated with estradiol (500 ug/day) for six days. The mean size of the junctions present in the myometrium of the delivering rats, however, was significantly larger ($P<0.001$).

Figure 7 shows histograms of the distribution of gap junction sizes in myometria of immature rats treated with estradiol (500 ug/day) for six days and those of delivering animals. It is apparent that the distribution of junction sizes was different between the groups. The range of sizes and the size with highest frequency of the gap junctions were greater in tissues from delivering animals as compared to myometrial tissues from estradiol treated immature rats.

Figure 8 shows high magnification electron micrographs of a gap junction from the myometrium of estradiol treated immature rats (Figure 8A) and a larger gap junction as observed in the myometrium from delivering animals (Figure 8B). At this high magnification the seven lined structure of the gap junction is apparent.

Figure 7. Size distribution histograms of lengths of gap junctions found in thin sections of immature rat myometrium treated for 6 days with estradiol and in myometrium from delivering rats.

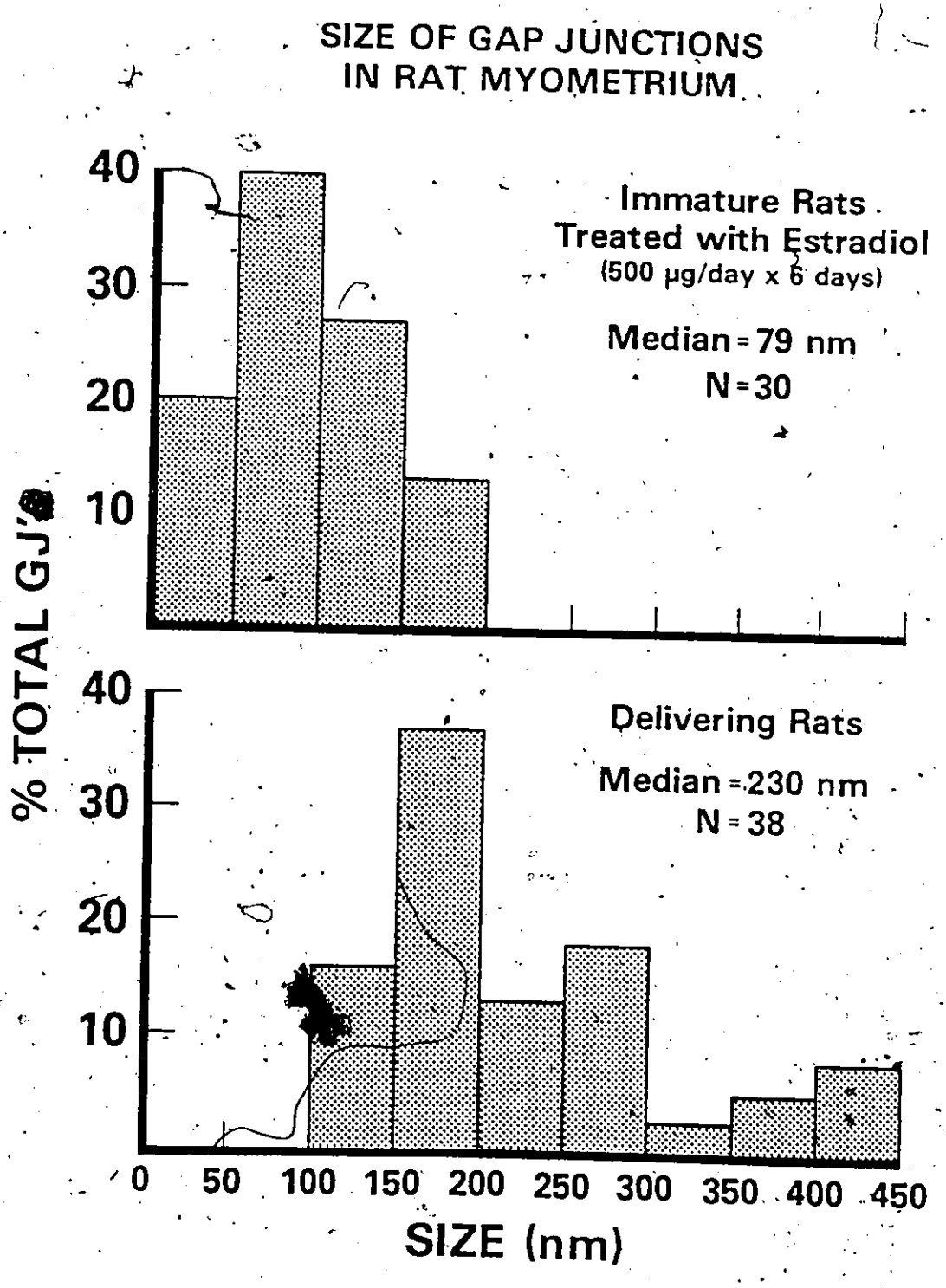
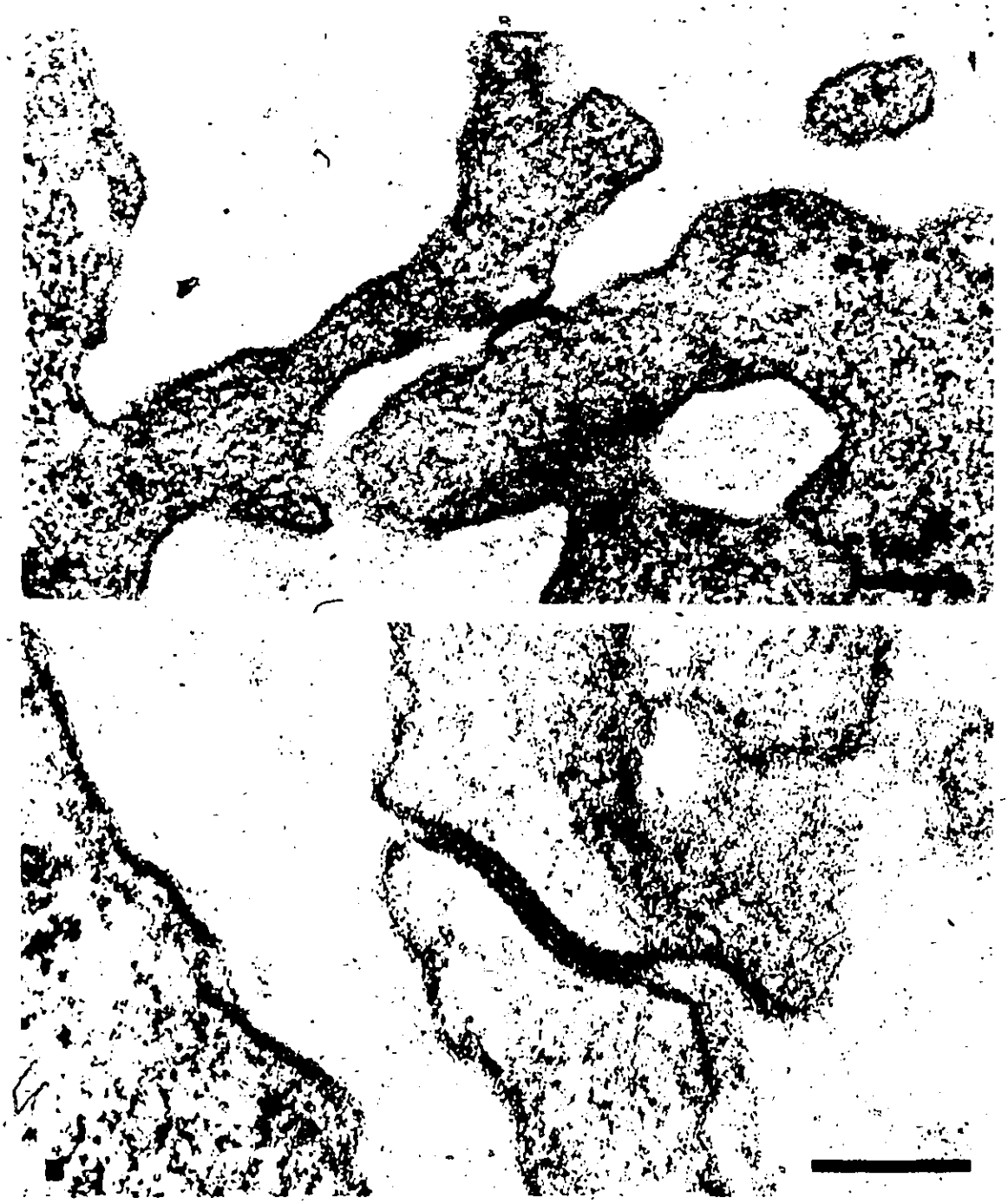

SIZE OF GAP JUNCTIONS
IN RAT MYOMETRIUM
Immature Rats
Treated with Estradiol
(500 µg/day x 6 days)
Median = 79 nm
N = 30
Delivering Rats
Median = 230 nm
N = 38
% TOTAL GJ's
40
30
20
10
0
50
100
150
200
250
300
350
400
450
SIZE (nm)

Figure 8. High magnification electron micrographs of thin sections showing profiles of a small gap junction from the myometrium of immature estradiol treated rats; Marker is 0.1 um, X 290,000 (A) and a large gap junction as observed in the myometrium from delivering animals; Marker is 0.1 um, X 458,000 (B).



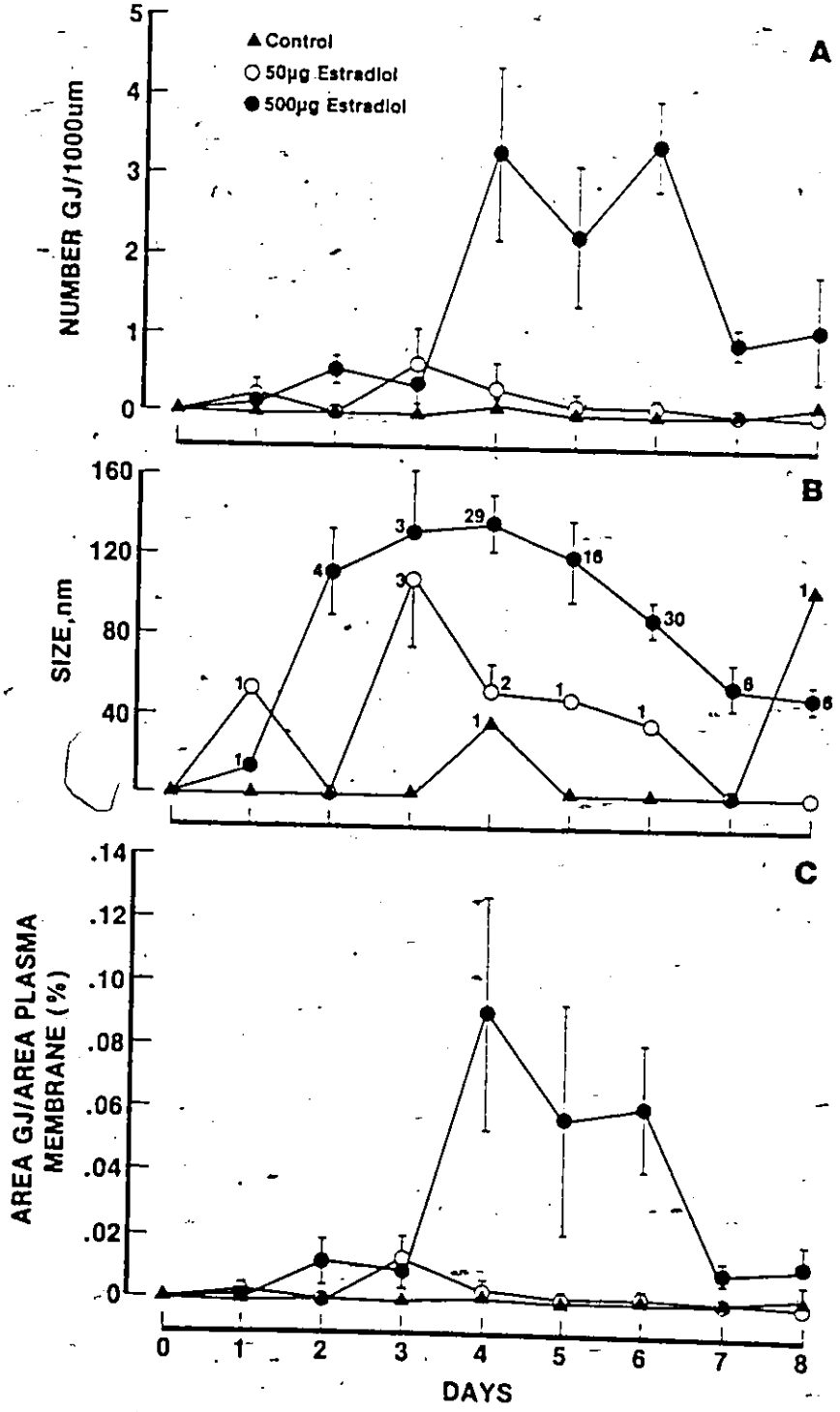
4.2.2 Time course of gap junction development: Tissues from immature rats:

4.2.2.1 Effects of estradiol:

To determine gap junction changes with low and high doses of estradiol throughout an 8 day period, daily determinations of myometrial gap junctions in immature rats were carried out. Immature rats were injected subcutaneously with sesame oil with and without estradiol (50 and 500 ug/day) for 1-8 days. Gap junctions were infrequent in myometrial tissues from control animals treated with only sesame oil throughout the injection period (Figure 9A). Similarly, gap junctions were present at a very low frequency between uterine smooth muscle cells from rats treated with 50 ug/day estradiol. The frequency of gap junctions in tissues from rats treated with the higher dose of estradiol (500 ug/day) was also low after the first three days of treatment. Thereafter, however, the frequency increased to 3.300 $\pm$ 1.260 after the fourth injection ($P<0.05$, Figure 9A). The increased frequency was maintained with the fifth and sixth injections of estradiol but thereafter decreased significantly with further injections ($P<0.05$). The frequency of gap junctions in myometrial tissues from immature rats treated with 500 ug/day estradiol was significantly larger on days four ($P<0.05$) five ($P<0.01$) and six ($P<0.01$) compared with tissues from rats treated with 50 ug/day estradiol.

The mean size of gap junctions stimulated by the low dose of estradiol over the 1-8 day period were small, on average less than 50

Figure 9. Effects of daily estradiol treatments on the presence of gap junctions in immature rat myometrium. (A) Number GJ/1000 um, frequency of gap junctions per 1000 um measured membrane length; (B) Size, length (nm) of each gap junction; (C) Area of GJ/area of plasma membrane, area of gap junction is expressed as percentage of gap junctions per area plasma membrane. Frequency, size and area of gap junctions are given as mean values ± S.E. of 4-6 tissues for each treatment on each day. Numbers for the size panel represent the total number of gap junctions found on that specified day.


▲Control
○ 50µg Estradiol
● 500µg Estradiol
A
NUMBER GJ/1000um
5
4
3
2
1
0
B
SIZE,nm
160
120
80
40
C
AREA GJ/AREA PLASMA MEMBRANE (%)
.14
.12
.10
.08
.06
.04
.02
0
0 1 2 3 4 5 6 7 8
DAYS

nm (Figure 9B). Similarly, the one gap junction observed between myometrial cells in the control tissue after three days of treatment with sesame oil was approximately 40 nm in length. The mean sizes of gap junctions in tissues from animals treated with the high dose of estradiol over the 1-8 day period were significantly larger from two ($P<0.05$) through five ($P<0.005$) days of treatment compared with those in rats treated for eight days (Figure 9B). Gap junction size was only significantly larger ($P<0.05$) in tissues from animals treated four days with 500 ug/day estradiol compared to those in tissues from rats treated with 50 ug/day estradiol for the same period.

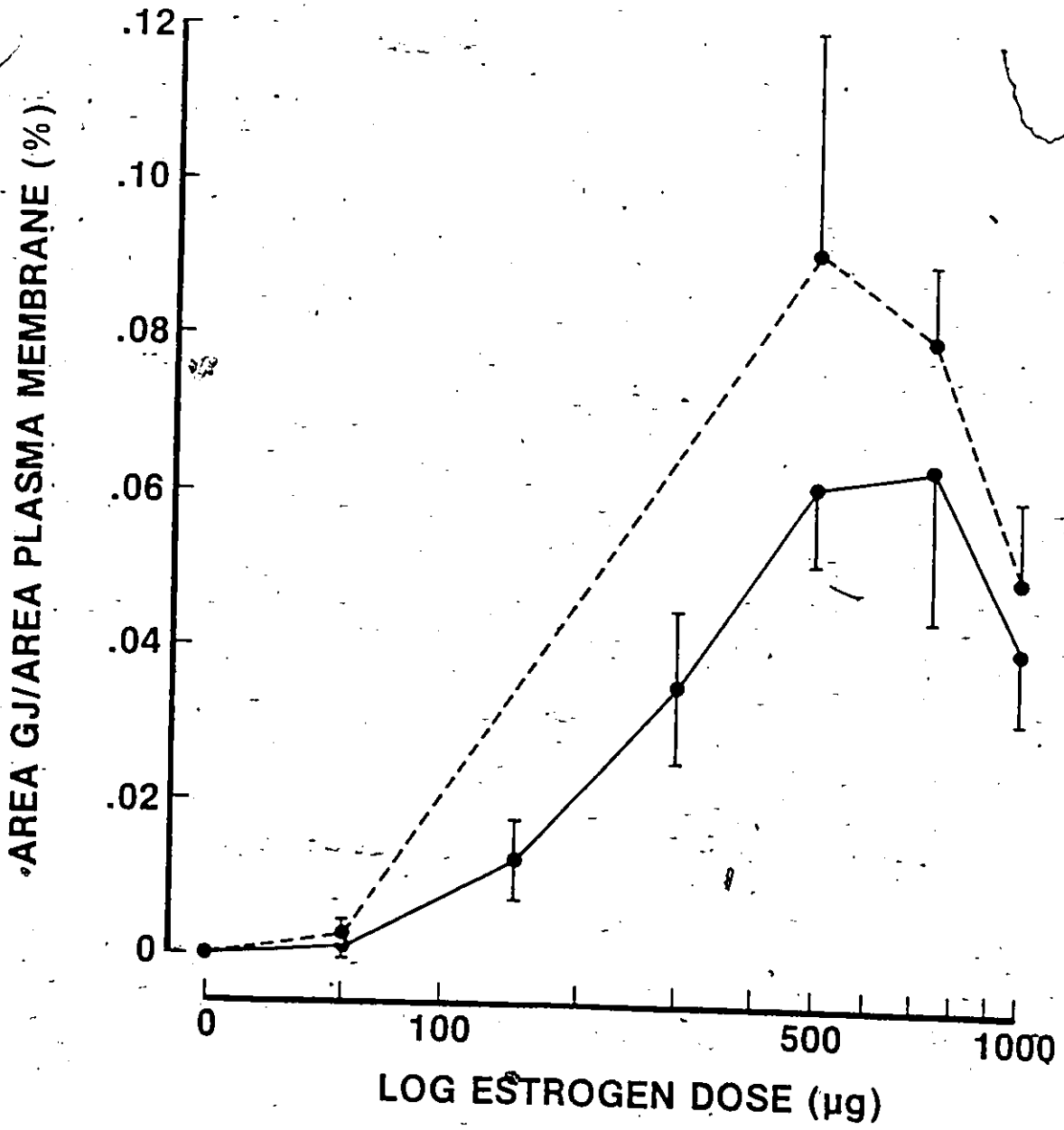
The changes in the frequency (Figure 9A) and sizes (Figure 9B) of gap junctions resulted in changes in gap junctional membrane area (Figure 9C). The percentage of myometrial membrane area occupied by gap junctions was extremely low throughout the injection period in tissues from control animals treated with sesame oil and in rats treated with 50 ug/day estradiol. Gap junctions present in tissues from rats treated with this dose of estradiol occupied less than 0.01 % of the myometrial membrane area over the course of the experiment (Figure 9C). The percentage of membrane area occupied by gap junctions was significantly increased by 500 ug/day estradiol after the fourth ($P<0.05$), fifth and sixth ($P<0.01$) injections (Figure 9C) compared with control or 50 ug/day estradiol treated animals. The membrane area occupied by gap junctions decreased to a significantly lower percentage value after seven and eight days of treatment ($P<0.05$).

To determine the effects of different doses of estradiol on the presence of myometrial gap junctions, immature rats were injected over

four and six days with 50, 150, 300, 500, 750 and 1000 ug/day estradiol. A dose response curve of estradiol on the membrane area of gap junctions is shown in Figure 10. The percentage of myometrial membrane area occupied by gap junctions was extremely low after treatment with 50 ug/day estradiol for four and six days. However, the percentage of membrane area occupied by gap junctions increased in response to treatment with all other doses of estradiol over the four and six day injection period. After treatment with 500 ug/day estradiol for four days the percentage of membrane area occupied by gap junctions was significantly higher ($P<0.05$) compared with that in animals treated with 50 ug/day estradiol over the same period. The membrane area of gap junctions decreased after treatment with 750 and 1000 ug/day estradiol compared to the treatment with 500 ug/day, but were significantly greater ($P<0.05$) than the membrane area after treatment with 50 ug/day estradiol. In animals treated for six days a significant increase ($P<0.01$) in gap junctional membrane area occurred after treatment with 300 ug/day compared with the low percentage value observed after treatment with 50 ug/day estradiol over the same period. The percentage of membrane area occupied by gap junctions increased further to a near maximum level after treatment with 500 ug/day and was similar to that in tissues from animals treated with 750 ug/day estradiol. Following treatment with 1000 ug/day estradiol the membrane area of gap junctions decreased insignificantly compared to 500 or 750 ug/day but was significantly larger ($P<0.01$) then after 50 ug/day estradiol treatment.

Analysis on gap junctions by freeze-fracture electron microscopy of uterine smooth muscle has also been carried out. Many

Figure 10. Dose response curve of estradiol on the myometrial membrane area of gap junctions (GJ) in immature animals. Rats were treated with estradiol for 4 (---) and 6 (——) days with each dose of estradiol; 24 hours after the last injection animals were killed, and myometrial tissue was removed and processed for electron microscopy. Data presented as mean values ± S.E. of 4-6 tissues.

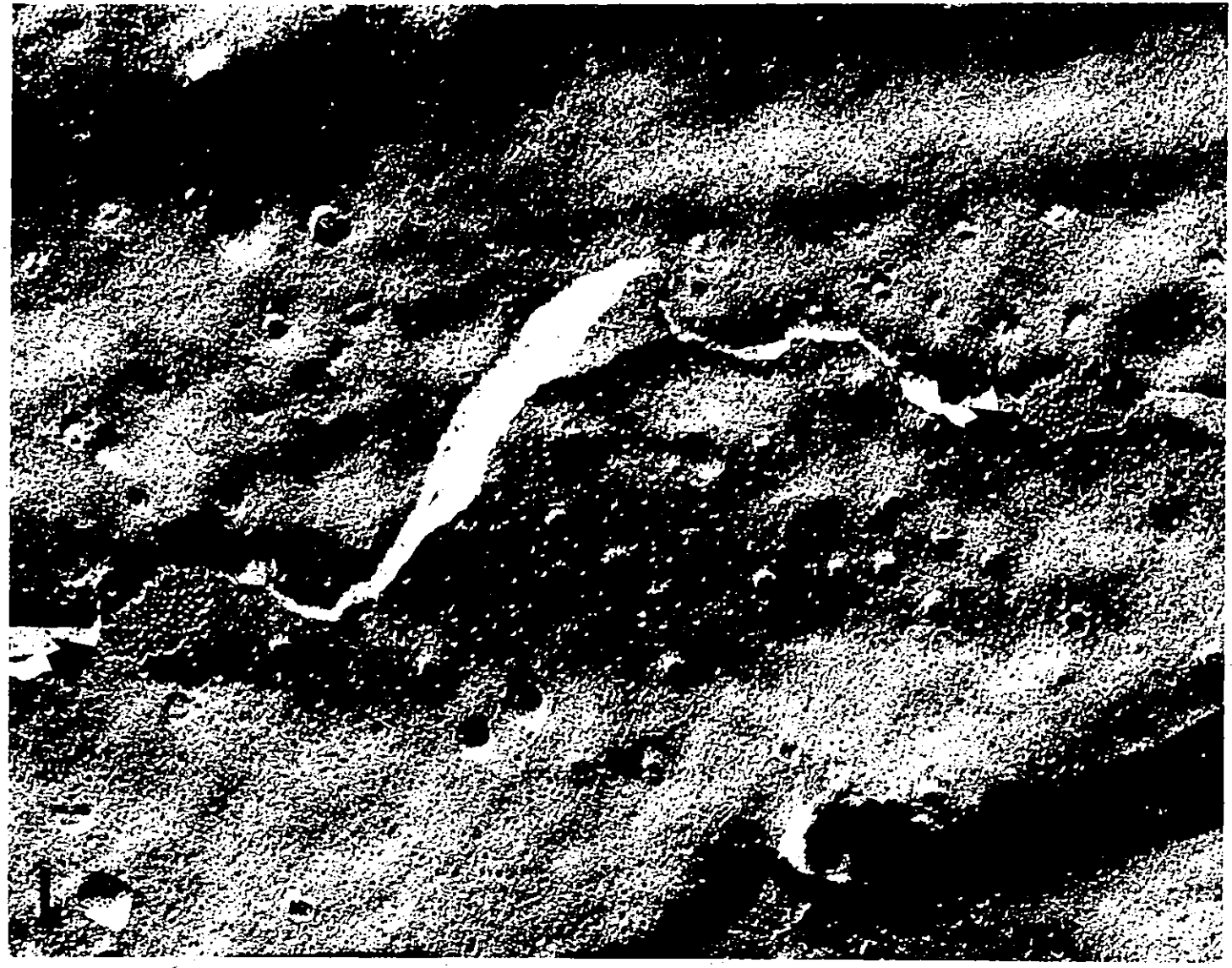

.12
.10
.08
.06
.04
.02
0
AREA GJ/AREA PLASMA MEMBRANE (%)
0
100
500
1000
LOG ESTROGEN DOSE (µg)

well prepared replicas of longitudinal myometrial smooth muscle from immature rats treated with sesame oil with and without estradiol (50 and 500 ug/day) for four days, representing several hundred squared micrometers of membrane area, were examined for the presence of gap junctions. In replicas of tissue from control and 50 ug/day estradiol treated rats gap junctions were undetectable or present at a very low frequency similar to that observed in thin sections (Figure 9A). Replicas of myometrial tissues from immature rats treated with 500 ug/day estradiol for four days contained gap junctions at a frequency of approximately 10 gap junctions/1000 um^2. The mean diameter of gap junctions in freeze-fracture replicates of 500 ug/day estradiol treated tissues was 154 ± 25 nm, similar to values obtained from thin sections after the same treatment (Figure 9B). Gap junctions as seen in freeze-fractured replicas of longitudinal smooth muscle are shown in Figures 11 and 12. At high magnification (Figure 11) gap junctions are seen as a tight clustering of intramembrane protein particles on the protoplasmic fracture face (PF). Figure 12 shows two gap junctions in the same field after treatment with estradiol for four days.

The preceding data illustrates the effects of consecutive daily treatments of estradiol on the presence of myometrial gap junctions. To determine if repeated hormone injections were necessary for the presence of myometrial gap junctions, immature rats were treated with a single injection of sesame oil with and without estradiol (50 and 500 ug) at zero time (day 0) and daily microscopic analysis for the presence of gap junctions in the myometrium were carried out over 1-6 days. Figure 13A shows that gap junctions were not present in tissues

Figure 11. Electron micrograph of a freeze-fracture replica from the myometrium of an immature rat treated with estradiol daily for 4 days showing at high magnification gap junctions (solid arrows) on the protoplasmic fracture (PF) face; gap junction in the center of the micrograph shows clusters of particles on the PF face with corresponding pits on the EF (extracellular fracture face). Open arrow shows shadow angle. Marker is 0.1 um, X 170,000.

Figure 12. Electron micrograph of a fracture replica from myometrium of immature rats treated for 4 days with estradiol showing at low magnification two gap junctions (solid arrows) in the same field. Both fracture faces of the junctions are evident. Open arrow shows shadow angle. Marker is 0.1 um, X 95,000.



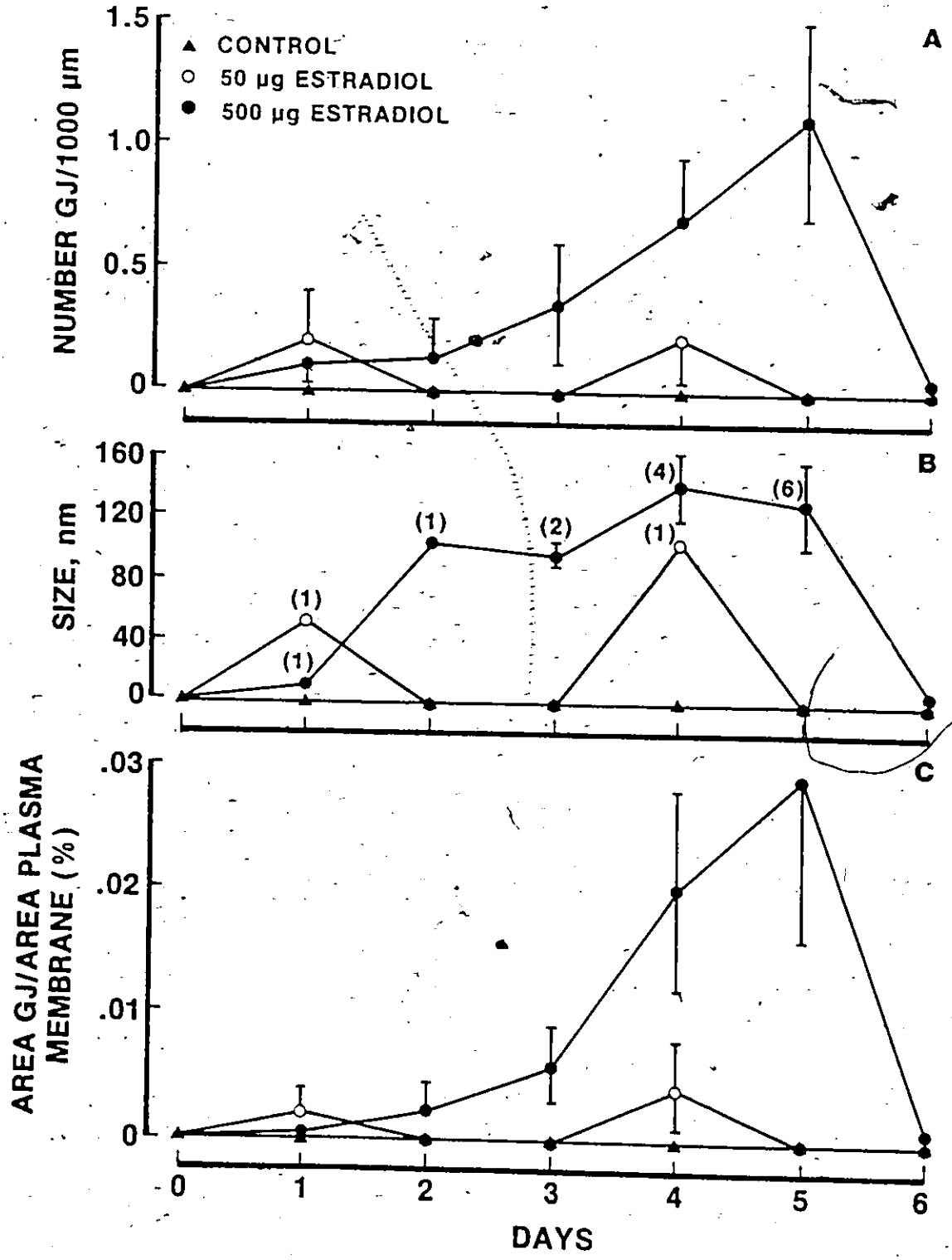
from control animals and when present in tissues from rats treated with 50 ug estradiol, they were detectable at an extremely low frequency one and four days after the treatment. In animals treated with 500 ug estradiol the frequency of myometrial gap junctions gradually increased from day 0 to a maximum value five days after the injection of the hormone (1.140 ± 0.497 gap junctions/1000 um membrane). This increase was not significantly greater than control or 50 ug estradiol treated tissues on the same day. Gap junctions were not detected in the myometrium six days after treatment with 500 ug estradiol.

The mean sizes of gap junctions on days one and four after the treatment with 50 ug estradiol were 53 and 104 nm, respectively (Figure 13B). In animals treated with a single injection of 500 ug estradiol the size of the gap junction observed in the myometrium on day one was very small followed by an increase to a near maximum size (104 nm) two days after the hormone treatment. The size was maintained on day three followed by an non-significant increase to a maximum value (142 ± 25 nm) four days after the treatment (Figure 13B).

The percentage of membrane area occupied by gap junctions in tissues from animals treated with 50 ug estradiol was extremely low over the 1-6 day period (Figure 13C). The membrane area occupied by gap junctions following treatment with 500 ug estradiol increased gradually from day 0 (zero time) to a maximum percentage (0.029 ± 0.016) value five days after the injection of estradiol but was not significant compared with the control or 50 ug estradiol treated tissues on the same day.

The maximum frequency and membrane area occupied by gap

Figure 13. Effects of a single injection of estradiol (on day 0) on the frequency (A), size (B) and membrane area (C) of gap junctions in the myometrium of immature rats determined daily over 1-6 days. Data as described in Figure 9.

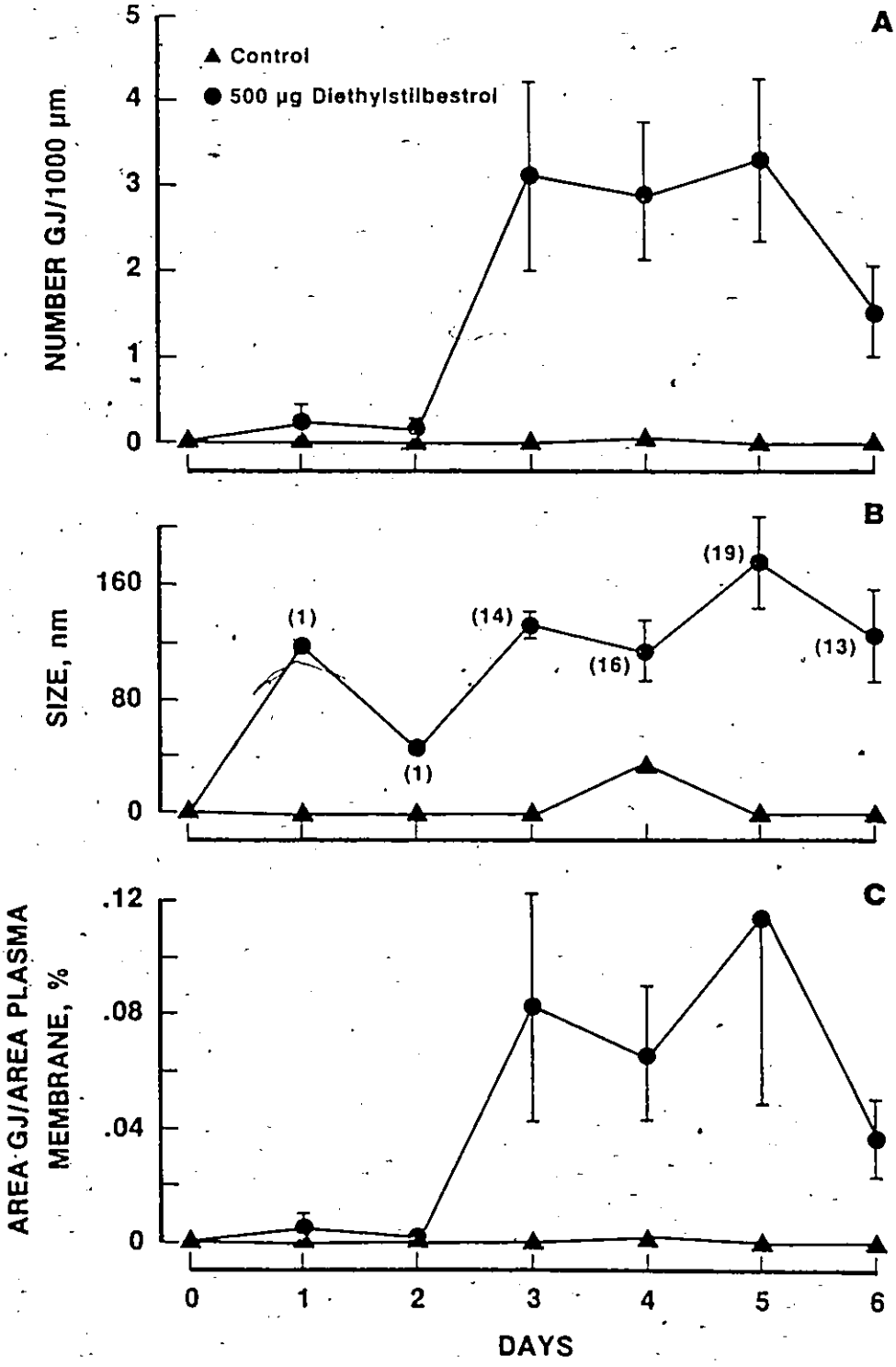

CONTROL
50 µg ESTRADIOL
500 µg ESTRADIOL
A
NUMBER GJ/1000 µm
1.5
1.0
0.5
0
B
SIZE, nm
160
120
80
40
0
(1)
(1)
(1)
(2)
(1)
(4)
(6)
C
AREA GJ/AREA PLASMA MEMBRANE (%)
.03
.02
.01
0
0
1
2
3
4
5
6
DAYS

junctions five days after the single injection of 500 ug estradiol were considerably smaller compared to the values from animals treated daily with estradiol (500 ug/day) over the 1-6 day period (mean frequency: 1.140 ± 0.497 compared to 3.440 ± 0.520 junctions/1000 um membrane, $P<0.05$; mean percent membrane area: 0.029 ± 0.016 compared to 0.091 ± 0.038 percent, $P<0.05$). The maximum mean size of gap junctions observed in myometrial tissues from animals in both treatment groups was similar (142 ± 25 nm after a single injection of estradiol compared to 136 ± 14 nm after repeated injections).

4.2.2.2 Effects of diethylstilbestrol:

Quantitative measurements on gap junctions in immature rats treated with 500 ug/day diethylstilbestrol for 1-6 days are represented in Figure 14. Gap junctions were not present in control animals treated with only sesame oil. Gap junctions were present at an extremely low frequency throughout the first two days of diethylstilbestrol treatment followed by a significant increase ($P<0.05$) to 3.250 ± 1.500 junctions/1000 um membrane after the third injection (Figure 14A). The increased frequency was maintained with the fourth and fifth injections. Following the sixth injection there was a non- significant decrease in the mean frequency ($P>0.05$). The mean size of the gap junctions was similar throughout three to six days of treatment (Figure 14B), whereas the gap junction membrane area (Figure 14C) was significantly greater ($P<0.05$) after the third injection. Following the sixth injection there was an insignificant ($P<0.05$) decline in the gap junction area.

Figure 14. Effects of daily diethylstilbestrol treatments on the frequency (A), size (B) and membrane area (C) of gap junctions in immature rat myometrium. Data as described in Figure 9.


A
▲ Control
● 500 µg Diethylstilbestrol
NUMBER GJ/1000 µm
5
4
3
2
1
0
B
SIZE, nm
160
80
0
(1)
(1)
(14)
(16)
(19)
(13)
C
AREA GJ/AREA PLASMA MEMBRANE, %
.12
.08
.04
0
0
1
2
3
4
5
6
DAYS

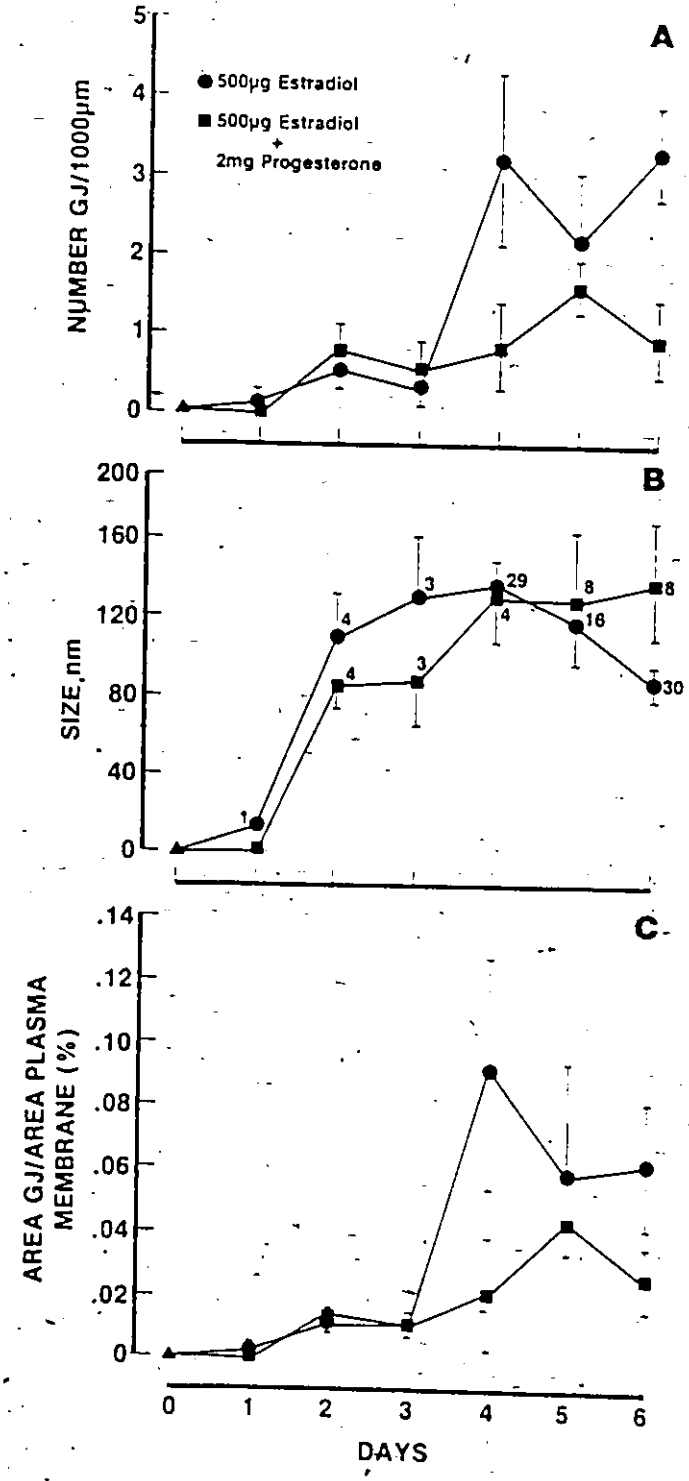
In myometrial tissues from immature rats treated with 500 ug/day diethylstilbestrol (Figure 14), the frequencies and gap junction membrane areas were similar compared to those observed in animals treated with 500 ug/day estradiol (Figure 9). However, the increase in gap junction frequency and area occurred 24 hours earlier when diethylstilbestrol was administered. Following the third diethylstilbestrol injection gap junctions were significantly increased ($P<0.05$) compared to the peak following the fourth injection in the estradiol treated rats.

4.2.2.3 Effects of progesterone:

To determine the effects of progesterone on the presence of gap junctions after estradiol treatment, progesterone (2 mg/day) was administered alone or simultaneously with estradiol (500 ug/day) for a period of 1-6 days. Figure 15A shows that progesterone treatment had no effect on the low frequency of gap junctions throughout the first three days. Thereafter, the increased frequencies stimulated by estradiol alone on days four, five and six were reduced by concomitant progesterone treatment reaching significance after the sixth injection (3.44 ± 0.52 compared to 0.93 ± 0.58 gap junctions/ 1000 um membrane, $P<0.01$).

The size of gap junctions that formed in response to estradiol plus progesterone treatments remained constant throughout the six day injection period (Figure 15B). No significant difference in the sizes were observed when compared with the size of the junctions in the estradiol treated tissues.

Figure 15. Effects of progesterone on the frequency (A), size (B) and membrane area (C) of gap junctions in immature rat myometrium. Progesterone was administered concomitantly with estradiol daily over 1-6 days to determine its effect on estradiol stimulation of gap junctions. Data as described in Figure 9.


A
● 500µg Estradiol
■ 500µg Estradiol + 2mg Progesterone
NUMBER GJ/1000µm
5
4
3
2
1
0
B
SIZE, nm
200
160
120
80
40
0
1
4
3
4
3
4
29
8
16
8
30
C
AREA GJ/AREA PLASMA MEMBRANE (%)
.14
.12
.10
.08
.06
.04
.02
0
0 1 2 3 4 5 6
DAYS

Concomitant injections of estradiol plus progesterone also reduced the percentage of membrane area occupied by gap junctions (Figure 15C). The percentage of membrane area was similar in tissues from animals treated with estradiol or estradiol plus progesterone over the first three days of treatment. After the fourth, fifth and sixth injections simultaneous administration of estradiol and progesterone resulted in a suppression of the membrane area occupied by gap junctions compared with the estradiol treated tissues. The decreases, however, were not significantly different between the two treatment groups. The mean values of all frequencies and gap junctional membrane areas combined from days four, five and six from the estradiol treated animals were significantly larger ($P<0.05$) compared with the combined results over the same period from the estradiol plus progesterone treated animals. No gap junctions were found in myometrial tissues from immature rats treated with progesterone alone (data not shown).

Table 2 shows the effect of a higher dose of progesterone on gap junctions after treatment with estradiol. Progesterone (2.5 mg twice daily) administered concomitantly with estradiol (500 ug/ day) for four days significantly suppressed the frequency (3.300 $\pm$ 1.260 vs 0.330 $\pm$ 0.220 junctions/1000 um membrane, $P<0.05$), size (130 $\pm$ 6 vs 69 $\pm$ 5 nm, $p<0.001$) and membrane area occupied by gap junctions (0.091 $\pm$ 0.038 vs 0.004 $\pm$ 0.003 percent of the membrane, $P<0.05$) compared to the treatment with estradiol alone. Gap junctions were present at an extremely low frequency in control tissues and were undetectable in tissues from animals treated with progesterone alone.

TABLE 2. EFFECTS OF ESTRADIOL AND/OR PROGESTERONE ON MYOMETRIAL GAP JUNCTIONS (GJs) IN IMMATURE RATS TREATED FOR FOUR DAYS

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane,% (Mean ± S.E.)
Control	6(6)	1	9,299	0.103 ± 0.103	36	0.001 ± 0.001
Progesterone (2.5 mg twice daily)	4(4)	0	5,895	0	0	0
Estradiol (500 ug/day)	6(6)	29	8,963	3.300 ± 1.260 *	136 ± 14 **	0.091 ± 0.038 *
Estradiol plus Progesterone	6(6)	3	9,020	0.336 ± 0.221	69 ± 5	0.004 ± 0.003

Data are as described in Table 1. Immature rats were injected daily for 4 days (time period required for maximum frequency of gap junctions following estradiol treatment, Figure 9) to determine the effect of a higher dose of progesterone on estradiol stimulation of gap junctions. Levels of significance: * $P<0.05$; ** $P<0.01$.

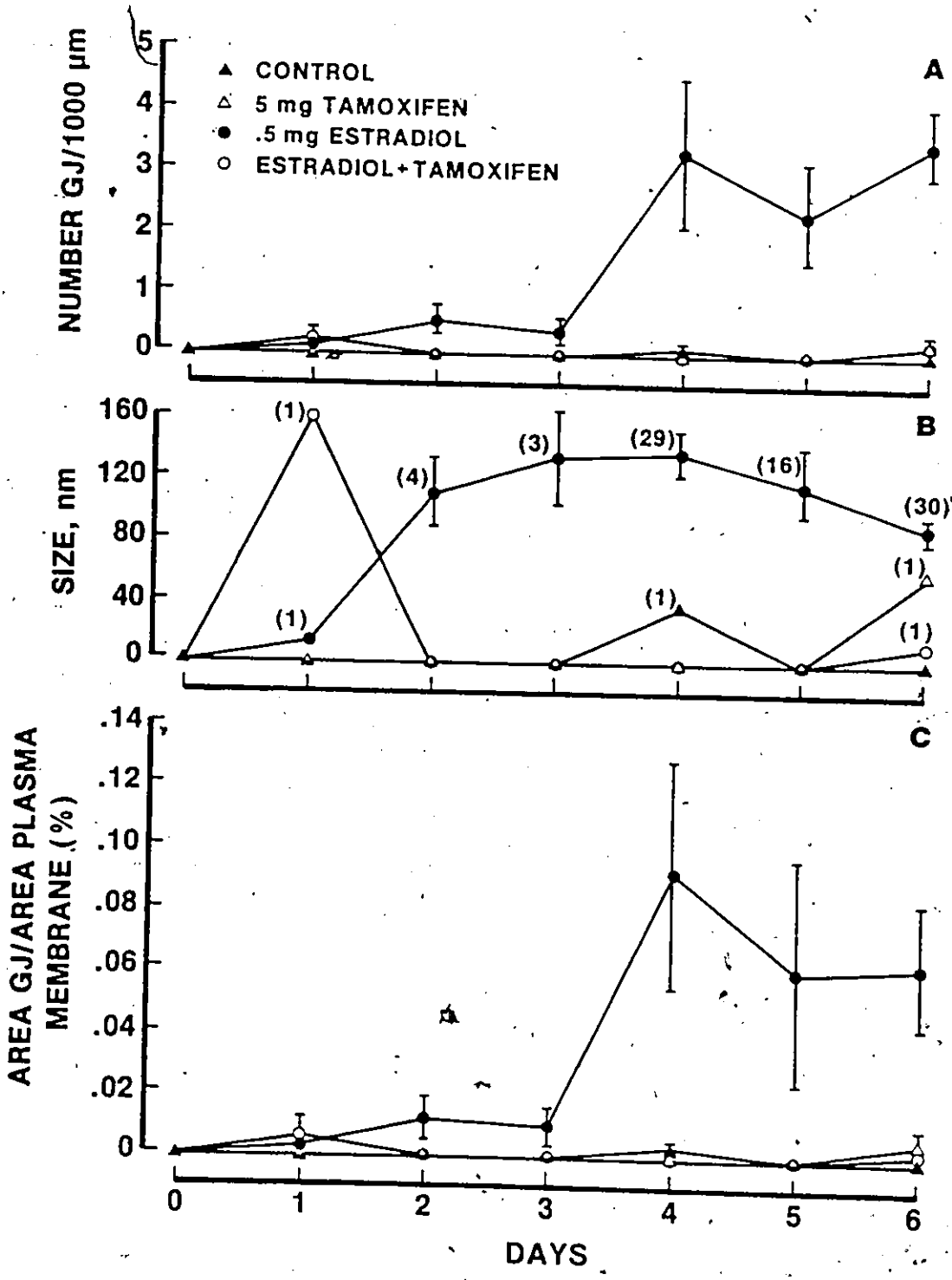
4.2.2.4 Effects of tamoxifen:

To analyse the effects of an antiestrogen on the presence of myometrial gap junctions in the immature rats, tamoxifen (5 mg/day) was administered alone or together with 500 ug/day estradiol for 1-6 days. Gap junctions were absent or present at an extremely low frequency in tissues from control animals and rats treated with tamoxifen alone over the 1-6 day period (Figure 16A). Similarly, the frequency of myometrial gap junctions was low over the first three days of treatment with 500 ug/day estradiol. Thereafter, however, the frequency was significantly elevated ($P<0.05$) after the four, five and six days of treatment. When tamoxifen was injected simultaneously with estradiol over the 1-6 day period gap junctions were present at a very low frequency compared with estradiol treatment alone and similar to that of control and tamoxifen treated animals.

The size of gap junctions in tissues from control and tamoxifen treated animals after four and six injections were very small (40 and 60 nm respectively, Figure 16B). The mean size of gap junctions stimulated by estradiol increased to a near maximum size after two days of treatment which was maintained with repeated injections over the remainder of the six day period. In animals treated with estradiol plus tamoxifen the two gap junctions measured over the treatment period varied in size from approximately 160 to 30 nms after one and six injections, respectively.

The percentage of myometrial membrane area occupied by gap junctions in tissues from control, tamoxifen and estradiol plus

Figure 16. Effects of tamoxifen on myometrial gap junction frequency (A), size (B) and membrane area (C) in the immature rat. Tamoxifen was administered alone or together with estradiol daily over 1-6 days to determine its effects on estradiol induced gap junction formation. Data as described in Figure 9.


NUMBER GJ/1000 µm
CONTROL
5 mg TAMOXIFEN
.5 mg ESTRADIOL
ESTRADIOL+TAMOXIFEN
A
SIZE, nm
B
AREA GJ/AREA PLASMA MEMBRANE (%)
C
DAYS

tamoxifen treated animals over the 1-6 day period and in animals treated with estradiol for 1-3 days was extremely low (Figure 16C). The gap junctional membrane area after four days of estradiol treatment was significantly larger ($P<0.05$) compared to the controls treated with sesame oil alone. The increase in membrane area of gap junctions after estradiol treatment was maintained with repeated injections and was significantly greater ($P<0.01$) after six days of treatment compared with the membrane area in control, tamoxifen or estradiol and tamoxifen treated tissues.

4.2.2.5 Effects of cycloheximide:

To evaluate the effects of cycloheximide on the presence of myometrial gap junctions in immature rats, cycloheximide (100 ug) was administered alone or together with estradiol (500 ug/day) at zero time (day 0) or on day one and ultrastructural analysis for myometrial gap junctions were carried out after four and five days of treatment. Table 3 shows that gap junctions were present at an extremely low frequency and small size occupying a small percentage of the myometrial membrane area in tissues from control animals treated with sesame oil over the four days. Gap junctions were also not detectable in the myometrium from rats after four or five days following treatment with cycloheximide alone on day 0 or day 1 and in control tissues after five days of oil injections. The concomitant administration of cycloheximide (100 ug) with estradiol (500 ug/day) to immature rats on day 0 had no influence on the frequency or membrane area of gap junctions in the myometrium after a further four or five days of treatment with

Table 3. Data are as described in Table 1. Immature rats were injected daily for 4 and 5 days with estradiol with and without an injection of cycloheximide (100 ug) on day 0 (zero time) or day 1 to determine the effect of protein synthesis on myometrial gap junctions.

TABLE 3. EFFECT OF ESTRADIOL (E_2) AND/OR CYCLOHEXIMIDE (Cyclo.) ON MYOMETRIAL GAP JUNCTIONS IN IMMATURE RATS

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
4 Days						
Control	6(6)	1	9,299	0.103 ± 0.103^{1}	36	0.001 ± 0.001^{a}
Cyclo. (day 0)	2(2)	0	2,863	0	0	0
Cyclo. (day 1)	2(2)	0	3,091	0	0	0
E_2 (500 ug/day)	6(6)	29	8,963	3.300 ± 1.260^{2}	136 ± 14^{A}	0.091 ± 0.038^{b}
E_2 plus Cyclo. (day 0)	4(4)	27	6,157	4.250 ± 0.569^{2}	96 ± 6^{B}	0.082 ± 0.013^{b}
E_2 plus Cyclo. (day 1)	4(4)	7	5,563	0.962 ± 0.339^{1}	100 ± 12^{AB}	0.023 ± 0.006^{c}
5 Days						
Control	7(7)	0	10,394	0	0	0
Cyclo. (day 0)	2(2)	0	2,956	0	0	0
Cyclo. (day 1)	2(2)	0	2,793	0	0	0
E_2 (500 ug/day)	6(6)	16	7,600	2.260 ± 0.830^{1}	119 ± 21^{A}	0.057 ± 0.036^{a}
E_2 plus Cyclo. (day 0)	3(3)	13	3,943	3.300 ± 0.190^{1}	78 ± 6^{B}	0.051 ± 0.003^{a}
E_2 plus Cyclo. (day 1)	4(4)	2	5,466	0.367 ± 0.212^{2}	101 ± 18^{AB}	0.007 ± 0.004^{b}

estradiol compared with values from animals treated with estradiol alone over the same periods (Table 3). The mean size of the junctions in tissues from animals treated with cycloheximide plus estradiol on day 0, however, were smaller than those in tissues from rats treated with estradiol alone after both treatment periods.

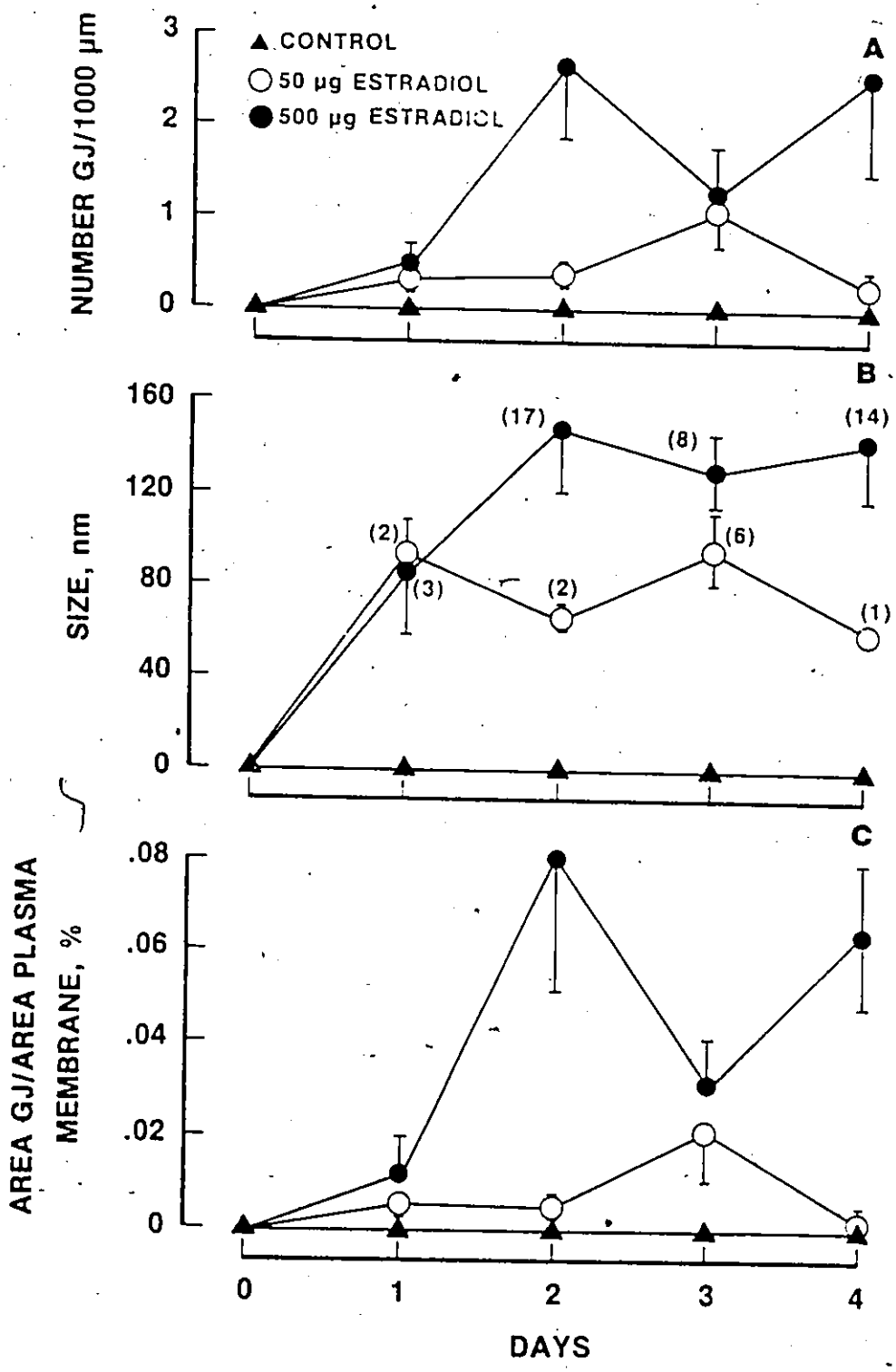
When animals injected with estradiol were treated with a simultaneous dose of cycloheximide on day one, twenty-four hours after the first day of treatment with estradiol, there was a significant suppression in the frequency ($P<0.01$ and $P<0.05$) and percentage ($P<0.01$) of membrane area occupied by gap junctions after continued treatment with estradiol over four and five days compared with the estradiol treatment alone (Table 3). After four and five days of treatment the mean gap junction size was smaller in the group of animals treated with cycloheximide on day one with a significant difference ($P<0.05$) occurring after four days.

4.2.3 Tissues from ovariectomized mature rats:

4.2.3.1 Effects of estradiol:

Figure 17 shows the effect of estradiol (50 and 500 ug/day) on the presence of myometrial gap junctions in ovariectomized mature rats treated for 1-4 days (i.e., up to the time at which the estradiol effect was maximum in the immature rat). Gap junctions were not detectable in control tissues over the 1-4 day period (Figure 17A). In animals treated with 50 ug/day estradiol the frequency of gap junctions was very low over the same four day period. Treatment with

Figure 17. Effects of estradiol on the frequency (A), size (B) and membrane area (C) of myometrial gap junctions in ovariectomized mature rats. Estradiol was administered daily over a four day period. Data as described in Figure 9.


NUMBER GJ/1000 µm
3
2
1
0
▲ CONTROL
○ 50 µg ESTRADIOL
● 500 µg ESTRADIOL
A
B
SIZE, nm
160
120
80
40
0
(17)
(8)
(14)
(2)
(6)
(3)
(2)
(1)
C
AREA GJ/AREA PLASMA MEMBRANE, %
.08
.06
.04
.02
0
0
1
2
3
4
DAYS

500 ug/day estradiol resulted in a small but insignificant increase in the frequency of myometrial gap junctions after the first day of treatment. Twenty-four hours after the second 500 ug/day estradiol injection the frequency of gap junctions increased significantly ($P<0.05$) to 2.700 ± 0.720 gap junctions/1000 um membrane compared with control or 50 ug/day estradiol treated animals. This frequency was maintained with further estradiol injections and after four days of treatment was significantly greater ($P<0.05$) compared with the frequency of gap junctions in tissues from animals treated with the lower dose of estradiol over the same period.

The mean size of the gap junctions in tissues from animals treated with either dose of estradiol were similar after the first day of treatment (Figure 17B). Thereafter there was no further increase in the mean gap junction size with repeated injections of 50 ug/day estradiol. The mean junction size in tissues from animals treated with 500 ug/day estradiol increased to a maximum value after two days of treatment ($P<0.05$ compared with the mean size after treatment with 50 ug/day estradiol for the same period). The maximum mean size remained constant throughout the remainder of the treatment period.

Figure 17C shows the membrane area of gap junctions over the 1-4 day treatment period. The percentage of membrane area occupied by gap junctions after treatment with 50 ug/day estradiol was extremely low on all days over the four day period. Gap junctions occupied less than 0.02 % of the membrane area over the course of the experiment. The increase in frequency and size of the gap junctions after the second injection of 500 ug/day estradiol resulted in a significant increase

($P<0.05$) in the membrane area of gap junctions. This increase was significant compared with control and 50 ug/day estradiol treated animals. The membrane area decreased non-significantly after three and four days of treatment but after four days was still significantly larger ($P<0.05$) compared to the treatment with 50 ug/day estradiol. The quantitative values for gap junctions stimulated by the high dose of estradiol in ovariectomized mature rats were similar to those observed in immature myometria (Figure 9). However, gap junction peak values appear earlier in the mature animals.

4.2.3.2 Effects of progesterone:

To determine the effects of progesterone on myometrial gap junctions in ovariectomized mature animals following estradiol treatment, progesterone (2.5 mg twice daily) was administered alone or together with 500 ug/day estradiol for three days. Table 4 shows that gap junctions were not detected in tissues from control or progesterone treated animals. The mean frequency ($P<0.05$) and size of gap junctions present after estradiol treatment were suppressed by simultaneous progesterone injections. This inhibitory effect by progesterone resulted in a significant ($P<0.05$) reduction in the percentage of membrane area occupied by junctions compared with that observed after estradiol treatment alone (0.040 ± 0.006 vs 0.002 ± 0.002 percent).

4.2.4 Tissues from pregnant rats:

To determine if estradiol (50 or 500 ug/day) treatment would stimulate the development of myometrial gap junctions in pregnant

TABLE 4. EFFECTS OF PROGESTERONE ON MYOMETRIAL GAP JUNCTIONS (GJs) IN OVARIECTOMIZED MATURE RATS TREATED FOR THREE DAYS

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
Control	4(4)	0	6,589	0	0	0
Progesterone (2.5 mg twice daily)	4(4)	0	5,991	0	0	0
Estradiol (500 ug/day)	4(4)	8	5,937	1.270 ± 0.063 *	132 ± 16	0.040 ± 0.016 *
Estradiol plus Progesterone	4(4)	1	5,614	0.166 ± 0.166	59	0.002 ± 0.002

Data are as described in Table 1. Results from ovariectomized mature rats treated daily for 3 days with estradiol and/or progesterone. Progesterone was administered at 2.5 mg twice each day because of the greater suppression on the presence of gap junctions as observed in the immature rats (Table 2). Level of significance: * $P<0.05$.

animals and to determine the outcome of the hormonal treatment on pregnancy, estradiol was administered to pregnant rats starting on day 16 of pregnancy. Pregnant rats treated with sesame oil alone represented the controls. Table 5 shows the results of the quantitative analysis of the frequency, size and membrane area of gap junctions in the longitudinal muscle layer of the pregnant rat myometrium from day 17 to day 21 of gestation and during delivery. In the control animals gap junctions were present between the smooth muscle cells at a very low frequency from day 17 through to day 20 of pregnancy (ranging from 0.212 to 0.393 gap junctions/ 1000 um nonjunctional membrane). However, the frequency of myometrial gap junctions was significantly ($P<0.001$) higher (2.010 ± 0.359 junctions/1000 um membrane) in rats killed on day 21 of pregnancy compared with the mean frequency on day 20. The number of gap junctions in the myometrium was also significantly higher in tissues from animals during delivery (day 22) compared with tissues at ($P<0.01$) or prior to day 21 ($P<0.001$). Gap junctions were present in all tissues from animals during delivery, with a mean frequency of 5.710 ± 1.227 gap junctions/1000 um membrane.

The mean size of gap junctions was also significantly larger in myometrial tissues from animals on day 21 ($P<0.01$) and during delivery (day 22) compared with the sizes in animals between days 17 and 20 of pregnancy (Table 5). The average measured length of gap junctions in tissues from animals on days 20, 21 and 22 were 128 ± 34, 248 ± 21 and 280 ± 48 nm, respectively.

The increase in frequency and size of myometrial gap junctions on day 21 of pregnancy and during delivery resulted in corresponding

Table 5. Data are as described in Table 1. Results show the time course for the development of gap junctions in the myometrium of pregnant rats from day 17 of pregnancy through delivery on day 22 (controls) and from pregnant animals treated with estradiol starting on day 16 of gestation. Statistical comparisons were made between mean values on different days for each group of animals. Animals treated with estradiol underwent premature delivery on day 20 (50 ug/day estradiol) and day 19 (500 ug/day estradiol). Frequencies and membrane areas of gap junctions during the preterm labor were similar to control values from spontaneous delivery on day 22. The mean size of the junctions was greater in the control tissues. On day 19 approximately 24 hours after the third day of treatment, pregnant animals received a fourth injection of 500 ug/day estradiol (day *19). All those animals delivered within 3 hours after treatment. Mean values with the same superscripts are not significantly different ($P>0.05$).

TABLE 5. EFFECTS OF ESTRADIOL (E_2) ON MYOMETRIAL GAP JUNCTIONS IN PREGNANT RATS

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
Control						
day: 17	14(10)	4	14,702	$0.272 \pm 0.118^{1}_{1}$	$97 \pm 4^{A}_{A}$	$0.006 \pm 0.002^{a}_{a}$
18	16(13)	4	17,584	$0.212 \pm 0.112^{1}_{1}$	$90 \pm 18^{A}_{A}$	$0.005 \pm 0.003^{a}_{a}$
19	19(13)	8	20,688	$0.393 \pm 0.109^{1}_{1}$	$97 \pm 19^{A}_{A}$	$0.007 \pm 0.002^{a}_{a}$
20	14(12)	7	18,264	$0.327 \pm 0.126^{1}_{2}$	$128 \pm 34^{A}_{B}$	$0.009 \pm 0.003^{a}_{b}$
21	24(24)	55	28,050	$2.010 \pm 0.359^{2}_{3,*}$	$248 \pm 21^{B}_{B,\Psi}$	$0.089 \pm 0.019^{b}_{c,\Phi}$
22 (delivery)	10(10)	60	11,050	$5.710 \pm 1.227^{3,*}$	$280 \pm 48^{B,\Psi}$	$0.264 \pm 0.071^{c,\Phi}$
E_2 (50 ug/day)						
day: 17	8(4)	11	9,474	$1.182 \pm 0.495^{4}_{4,5}$	$175 \pm 28^{C,E}_{C}$	$0.041 \pm 0.019^{d}_{d,e}$
18	8(4)	32	8,660	$3.621 \pm 1.220^{4}_{4}$	$134 \pm 11^{C}_{D}$	$0.100 \pm 0.046^{d}_{d}$
19	8(4)	11	8,871	$1.401 \pm 0.546^{4}_{5,*}$	$101 \pm 13^{D}_{E,\Psi\Psi}$	$0.028 \pm 0.011^{d}_{e,\Phi}$
20	6(3)	32	6,856	$4.622 \pm 1.240^{5,*}$	$186 \pm 17^{E,\Psi\Psi}$	$0.230 \pm 0.068^{e,\Phi}$
E_2 (500 ug/day)						
day: 17	8(4)	45	8,609	$5.401 \pm 1.340^{6}_{6}$	$138 \pm 8^{F}_{F}$	$0.154 \pm 0.045^{f}_{f}$
18	7(4)	29	8,516	$3.802 \pm 0.486^{6,*}_{6,*}$	$152 \pm 19^{F}_{F,\Psi\Psi}$	$0.117 \pm 0.098^{f}_{f,\Phi}$
19	11(6)	60	10,887	$5.521 \pm 1.701^{6,*}_{7,**}$	$178 \pm 14^{F,\Psi\Psi}_{F,\Psi\Psi}$	$0.190 \pm 0.061^{f,\Phi}_{g,\Phi\Phi}$
*19	8(4)	127	8,482	$14.10 \pm 4.410^{7,**}$	$156 \pm 7^{F,\Psi\Psi}$	$0.426 \pm 0.148^{g,\Phi\Phi}$

increase in the myometrial membrane area of gap junctions. The percentage of membrane area occupied by gap junctions on day 21 of pregnancy (0.089 $\pm$ 0.019 percent) was significantly greater ($P<0.005$) compared to tissues taken prior to that time. During delivery the membrane area of gap junctions (0.264 $\pm$ 0.071 percent) was also significantly larger than in tissues on day 21 ($P<0.05$) or days 17 through 20 of pregnancy ($P<0.001$).

Figures 18-20 show electron micrographs of longitudinal smooth muscle cells from control pregnant animals. Gap junctions were infrequent between the muscle cells from animals on day 17 of pregnancy (Figure 18) compared with the high frequency observed in tissues from delivering animals (Figure 19A). At higher magnification, the seven lined appearance of the gap junction with a gap (ca. 2 nm) between outer leaflets of the membranes was usually apparent (Figure 19B). Freeze-fracture microscopy of the longitudinal smooth muscle cells from the myometrium of delivering rats also revealed the presence of gap junctions. Figure 20 shows a low magnification micrograph of a replica illustrating several gap junctions in the fracture plane.

Daily determinations of gap junction frequency, size and membrane area in the myometrium from pregnant rats treated with estradiol (50 and 500 ug/day) for three or four days beginning on day 16 of gestation are also shown in Table 5. The mean frequency of gap junctions twenty-four hours after one (day 17), two (day 18) or three (day 19) days of treatment with 50 ug/day estradiol were larger than corresponding controls with a significant difference ($P<0.01$) after two days of treatment. On day 20 of pregnancy after treatment with

Figure 18. Low magnification electron micrograph from a thin section of myometrium from day 17 pregnant rat showing profiles of several smooth muscle cells. Gap junctions are not evident between the muscle cells, however, note the many close oppositions. Marker is 1 um, X 16,000.

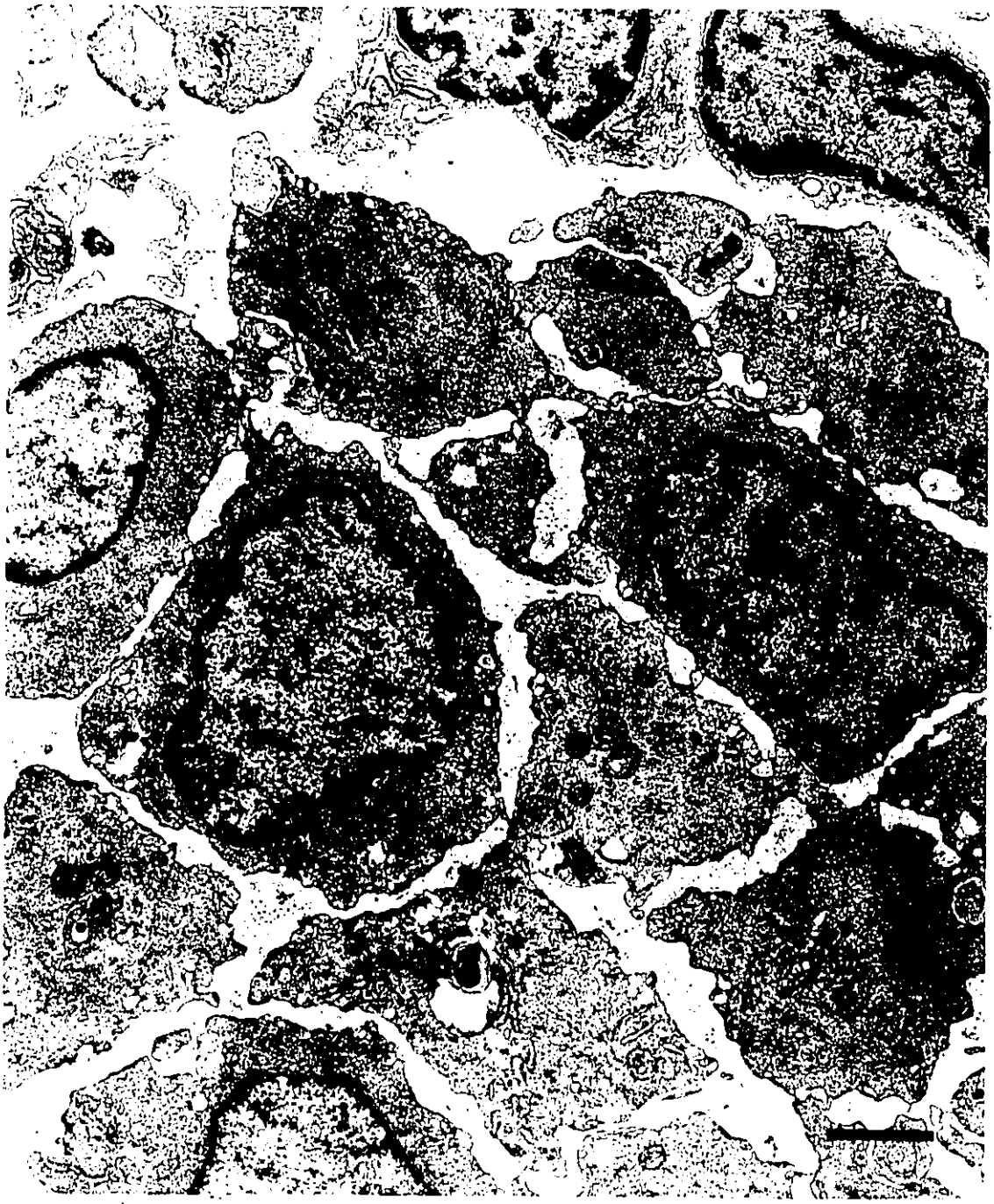


Figure 19. Electron micrographs of thin sections of myometrium from delivering rats showing gap junctions between the muscle cells. A) low magnification micrograph showing profiles of several smooth muscle cells and abundance of gap junctions (arrows) in this tissue. Marker is 1 um, X 91,000. B) high magnification of a gap junction between two smooth muscle cells illustrating the multilaminar structure of the junction. Marker is 0.1 um, X 290,000.

Figure 20. Electron micrograph of plasma membrane fracture surface from a replicate of delivering rat myometrium. At this magnification several gap junctions (solid arrows) are observed on the protoplasmic fracture (PF) face. Open arrow shows shadow angle. Marker is 1 um, X 34,000.

estradiol (50 ug/day) for four days the frequency was significantly larger ($P<0.01$) compared with control animals on the same day of gestation. The frequency of myometrial gap junctions after four days treatment on day 20 of pregnancy was also significantly larger ($P<0.05$) than in treated animals on days 17 and 19 of gestation.

The mean size of gap junctions in tissues from animals treated with 50 ug/day estradiol were significantly greater ($P<0.05$) on days 17 and 20 of pregnancy compared with the control values. Twenty-four hours after four days of treatment on day 20 of pregnancy the average measured length of gap junctions was 186 $\pm$ 17 nm, a significant increase ($P<0.05$) compared with the mean sizes on day 18 and day 19 after estradiol treatment. The increased frequencies and sizes of gap junctions in estradiol treated rats on days 17 through 19 of pregnancy resulted in a increase in the membrane area of gap junctions. The myometrial membrane area occupied by gap junctions after two (0.100 $\pm$ 0.046 percent) and four (0.230 $\pm$ 0.068 percent) days of estradiol treatment were significantly greater ($P<0.01$) compared with the control animals on the same days of pregnancy.

In the group of three pregnant rats treated with 50 ug/day estradiol for four days, two of the animals delivered prematurely on day 20 of pregnancy and in the other rat the fetuses were dislodged and compacted into the cervix region of the uterus. Myometrial tissues from all the rats contained gap junctions at a frequency and membrane area similar to that observed in tissues from control delivering animals (day 22). The sizes of the gap junctions in control tissues on day 22, however, were significantly larger ($P<0.05$) than in animals

treated with estradiol (50 ug/day) for four days.

Pregnant animals treated with 500 ug/day estradiol for 1-3 days (days 17 to 19 of pregnancy) contained gap junctions in the myometrium at a frequency significantly greater ($P<0.01$) compared with control animals over the same period (Table 5). Similarly, the frequency of myometrial gap junctions in tissues from pregnant animals treated with 500 ug/day estradiol was significantly larger than in animals treated with 50 ug/day estradiol for one (day 17, $P<0.01$) and three (day 19, $P<0.05$) days of treatment. No significant differences were observed between the frequency of gap junctions on day 17, day 18 and day 19 of gestation after treatment with 500 ug/day estradiol.

Gap junction sizes in tissues from animals treated with 500 ug/day estradiol were significantly larger ($P<0.05$) than the junctions in control pregnant animals on days 17, 18 and 19 of pregnancy. The mean measured length of junctions in tissues from rats treated for two days with estradiol (500 ug/day) were similar with the values after treatment with 50 ug/day estradiol over the same period. There was, however, a significant increase ($P<0.05$) in gap junction sizes on day 19 of gestation three days after 500 ug/day estradiol treatment compared to the treatment with the lower dose of the hormone. Gap junction size in tissues on days 17 through 19 after treatment with 500 ug/day estradiol were not significantly different compared to each other.

The percentage of membrane area occupied by gap junctions was significantly increased after treatment with 500 ug/day estradiol on day 17 (0.154 ± 0.045), day 18 (0.117 ± 0.298) and day 19 ($0.190 \pm$

0.061) of pregnancy compared with control ($P<0.01$) and 50 ug/day estradiol treatment for one ($P<0.05$) and three days ($P<0.01$). The membrane area of gap junctions was not significantly different on any of the days after treatment with 500 ug/day estradiol.

In the group of animals treated with 500 ug/day estradiol for three days (days 17 to 19 of pregnancy), two rats delivered prematurely and four rats showed vaginal bleeding on day 19 of gestation, approximately twenty-four hours after the third estradiol injection. It is interesting to note that the frequency of myometrial gap junctions in tissues from all the rats at that time were similar to that in control delivering animals (5.521 ± 1.710 in the estradiol treated group compared to 5.710 ± 1.227 junctions/1000 um membrane in control animals). The membrane area occupied by gap junctions in the treated animals was lower, but not significantly different compared with that in the control delivering rats. The mean size of the gap junctions, however, was significantly larger ($P<0.05$) in control delivering animals than in animals on day 19 after treatment with 500 ug/day estradiol. On day 19 of pregnancy (day *19), four pregnant animals were also treated with a fourth injection of estradiol (500 ug). Within three hours after the injection on day *19 all four rats underwent a premature labor. The frequency (14.10 ± 4.410 junctions/1000 um membrane) and membrane area (0.426 ± 0.148 percent) of gap junctions in myometrial tissues from the premature laboring animals treated with estradiol were significantly larger ($P<0.05$) compared with control delivering rats on day 22 of gestation. The mean size of the gap junctions in the estradiol treated tissues was smaller ($P<0.05$) than in

the control animals.

Figure 21A shows an electron micrograph from a thin section of the myometrium from a pregnant rat that delivered prematurely after treatment with estradiol (500 ug/day). Gap junctions were frequently seen between the smooth muscle cells of the myometrium similar to that in control delivering animals, however, the size of the junctions in animals treated with estradiol were smaller (compare Figures 18 and 21). At high magnification (Figure 21B) the multilaminar appearance of the gap junction was visible. Figure 22 shows a low magnification micrograph of a replica from the longitudinal muscle of the myometrium of pregnant rats treated with estradiol illustrating several gap junctions in the fracture plane.

To determine the effects of estradiol on sustaining the presence of gap junctions in the myometrium during delivery into the post-partum period, estradiol (5, 25, 100 and 500 ug/day) was injected into pregnant animals starting on day 21 of pregnancy. The injections were repeated daily for three days totalling three injections per rat (i.e. injected on day 21, day 22 (during delivery) and one day post-partum). All the rats were killed 48 hours after delivery, twenty-four hours after three days of treatment. The control and treated animals delivered on the morning of day 22 of pregnancy (i.e. approximately 20-24 hours after the first injection of estradiol). Table 6 shows that by 48 hours after delivery gap junctions were not detectable in myometrial tissues from control animals treated with sesame oil alone. Similarly, treatment with estradiol at any dose examined was not able to sustain the presence of gap junctions in the

Figure 21. Electron micrographs of uterine longitudinal smooth muscle cells from pregnant rats undergoing premature delivery after treatments with estradiol (500 ug/day). A) at low magnification a large number of gap junctions (arrows) are observed between the cells of this tissue. Marker is 1 um, X 25,000. B) at higher magnification the 7-lined structure of a gap junctions is apparent. Marker is 0.1 um, X 306,000.

Figure 22. Low magnification micrograph of a replica from the myometrium of a pregnant animal treated with estradiol (500 ug/day) and undergoing premature delivery. Several gap junctions (solid arrows) are present in the fracture plane. Open arrow shows shadow angle. Marker is 1 um, X 50,000.

TABLE 6. EFFECTS OF VARIOUS DOSES OF ESTRADIOL (E_2) ON SUSTAINING THE PRESENCE OF MYOMETRIAL GAP JUNCTIONS (GJs) OF PREGNANCY

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
Control	7(7)	0	5,492	0[1]	0	0[a]
E_2 (ug/day)						
5	5(5)	2	4,263	0.544 ± 0.335[1]	98 ± 38[A]	0.010 ± 0.006[a]
25	5(5)	0	3,602	0	0	0
100	5(5)	2	3,845	0.744 ± 0.744[1]	210 ± 60[B]	0.031 ± 0.031[a]
500	5(5)	3	3,680	0.792 ± 0.792[1]	144 ± 32[A,B]	0.023 ± 0.023[a]

Data are as described in Table 1. Estradiol was injected starting on day 21 of pregnancy. Injections were repeated daily; total number of injections per rat was 3. The control and treated animals delivered on the morning of day 22 of pregnancy, approximately 24 hours after the first injection of estradiol. All rats were killed 48 hours after delivery.

myometrium since the junctions were absent or present at an extremely low frequency and reduced sizes occupying a very small percentage of the myometrial membrane area. The frequency and area of gap junctions in tissues from rats treated with 5, 100 and 500 ug/day estradiol were not significantly greater than zero in the control tissues.

4.3 Discussion:

4.3.1 Regulation of gap junctions by steroid hormones:

This study demonstrates that administration of high doses of estradiol and diethylstilbestrol to immature rats stimulate the presence of myometrial gap junctions. Similarly, previous studies from this laboratory have demonstrated that gap junctions are present in myometrial tissues *in vitro* after treatment with estradiol (Garfield *et al.*, 1980a). Furthermore, it has been suggested that the appearance of myometrial gap junctions immediately prior to and during parturition may occur as a result of the change in steroid hormone levels (Garfield *et al.*, 1978; Puri and Garfield, 1982). Studies from other laboratories have also provided support for the hormonal regulation of myometrial gap junctions since treatment of mature mice or immature rats with high doses of estrogen (Progynon) and diethylstilbestrol stimulates their presence (Dahl and Berger, 1978; Merk *et al.*, 1980). The results of the present investigation support these studies and show that high doses of estradiol and diethylstilbestrol stimulate the presence of myometrial gap junctions in immature rats. In addition, the present study also demonstrates the presence of gap junctions in

the myometrium of ovariectomized mature rats and pregnant animals treated with the same high doses of estradiol. Furthermore, pregnant animals treated with the high dose of estradiol developed numerous myometrial gap junctions and aborted their fetuses.

4.3.1.1 Regulation in non-pregnant animals:

In a recent study on the hormonal regulation of myometrial gap junctions, it was reported that 5 and 50 ug/day estradiol benzoate and diethylstilbestrol treatment for five days to immature rats stimulated the development of a large number of gap junctions in the myometrium (Burghardt et al., 1984a). In addition, that study also demonstrated the presence of gap junctions in the myometrium twenty-four hours after one day of treatment with 500 ug/ day estradiol benzoate. Their observations are similar with the results of this study, however, the quantitative determinations they have reported are much different than those presented here. It should be noted that the method employed by Burghardt et al., (1984a) in quantitating gap junctions consisted of counting the number of gap junctions per grid square, assuming an approximately equal length of membrane in each square. However, this method of quantitation is not without difficulties, for example, when there is hypertrophy or hyperplasia of the smooth muscle cells. Moreover, their method does not take into consideration gap junction size or membrane area and therefore, would provide only semiquantitative data compared to our methods.

In this study gap junctions were identified and quantitated by thin-section and freeze-fracture electron microscopy morphometric

methods. The calculated frequency and percentage of membrane area occupied by gap junctions in thin sections of tissues from immature rats after four days of estradiol treatment were much larger than that determined by freeze-fracture electron microscopy. Considering the gap junctional membrane area (0.091 percent) and mean junction size (130 nm) found in thin sections after four days treatment with estradiol, approximately 58 gap junctions per 1000 um^2 membrane area should be found in freeze-fracture replicas. In many (n=5) well prepared replicas of longitudinal myometrial smooth muscle, however, only a small number of junction plaques with dimensions similar to the gap junctions observed in thin sections were found. This discrepancy between thin section and freeze-fracture techniques may occur because gap junctions are usually, but not always, located on cytoplasmic extensions between myometrial smooth muscle cells (Garfield et al., 1978). It is likely that this would result in an underestimation of the proportion of gap junction membrane by freeze-fracturing because the membrane of the cytoplasmic extensions would not occur within the same plane of fracture as the flat surfaces of cells on large areas of the membrane replicate. Also, it is extremely difficult to identify gap junctions in small areas of membrane surrounded by extracellular material. Considerable stretching of uterine smooth muscle prior to fixation for freeze-fracture electron microscopy may provide membrane surfaces without numerous projections allowing for a more accurate estimation of the frequency and area of gap junctions.

In an earlier study by Dahl and Berger (1978) myometrial gap junctions were present after an injection of a high dose (1 mg) of

estrogen (Progynon). These authors examined freeze-fractured replicas of the myometrium and demonstrated small aggregates of intramembranous particles which were interpreted as gap junctions 24 hours after estrogen treatment. They have shown aggregates of intramembranous particles with dimensions similar to gap junctions that I have observed in thin sections only after repeated injections of the estrogen. Small aggregations of intramembranous particles, suggesting the presence of small gap junctions, have also been described in various other types of smooth muscle by freeze-fracture microscopy (Fry et al., 1977; Peracchia, 1977). In the present study, aggregation of small numbers of particles (less than five) were frequently observed by themselves or together with larger gap junction-like plaques on the plane of fracture of the flat surface of myometrial cells. There is no reason, however, to believe that these small aggregations are located on areas of the cell membrane in contact with another cell. Arrays of membrane particles should not be considered junctions unless membrane opposition occurs as revealed by the fracture plane passing through both membranes comprising the gap junction (see Chapter 1, section 1A.2).

Estradiol elicits a response through a specific protein receptor within the cell (Gorski and Gannon, 1976; Jensen, 1979). Although low doses of the hormone are sufficient to induce a physiological response, such as growth of the uterus (Anderson et al., 1975), it is not exactly known why the higher dose of estradiol is required for stimulating the presence of gap junctions the myometrium. It is apparent, however, that the high dose (500 ug/day) is optimal for maximum stimulation of the junctions because higher doses of

estradiol do not further increase junction number, size or membrane area. Moreover, it is not exactly known if the presence of gap junctions in the myometrium results from estradiol stimulation of their development, inhibition of junction degradation or an effect on both processes. It is well known that estradiol regulates protein synthesis in the uterus (Gorski et al., 1968) and the present study shows that de novo protein synthesis may be necessary for the presence of gap junctions in the immature rat myometrium in response to estradiol. Inhibition of protein synthesis by cycloheximide prevents the increase in frequency, size and membrane area of gap junctions in myometrial tissues from animals treated with estradiol. Although this study does not provide evidence showing that cycloheximide reduced protein synthesis in the immature uterus, it has previously been reported that cycloheximide at a concentration similar to that employed in this study blocks the incorporation of [^{14}C]-leucine into trichloroacetic acid-precipitable protein in the immature (Sariff and Gorski, 1971), intact and ovariectomized mature rat (Cidlowski and Muldoon, 1978). Furthermore, cycloheximide has also been shown to prevent uterine responses to estrogen, such as estrogen induced myometrial contractions and steroid hormone receptor synthesis (Rexroad, 1978; Dix and Jordan, 1980). In addition, it has been reported that cycloheximide inhibits increased numbers and sizes of gap junctions in myometrial tissues incubated in vitro and formation of the junctions between other cells (Decker et al., 1978; Garfield et al., 1980b). Other studies, however, have indicated that de novo protein synthesis may not be required for gap junction formation in

all cell types (Epstein et al., 1977; Kannan and Daniel, 1978;).

The results of this study suggest that estradiol stimulation may result in the transcription of a specific mRNA for the proteins which form the molecular structure of the gap junctions. Dahl et al., (1980) have reported that a mRNA preparation extracted from the myometrium of estrogen treated rats induces gap junction formation in a cell line deficient in the junctions. Those results, however, must be interpreted with caution because the authors did not specify the dose or estrogen used, the number of treatments the animals received, or how long after the treatments began did they extract the mRNA. The data of my investigation indicate that these questions are of paramount importance for carrying out such an experiment. Furthermore, the structural and functional evidence they report are not very convincing. However, although that study is subject to criticism it nevertheless suggests that RNA and protein synthesis may be necessary for the expression of gap junctions. It is possible therefore, that estradiol stimulates the synthesis of the gap junction protein but that another pathway activated by estradiol in a dose responsive manner (Figure 10) is required for the appearance of gap junctions in the myometrium. It is tempting to speculate that a selective stimulation of a prostanoid by high doses of estradiol may be necessary for myometrial gap junction formation because prostaglandins have been implicated in the regulation of myometrial gap junctions (Garfield et al., 1980a).

In this study, cycloheximide prevented the increase in myometrial gap junctions only when administered concomitantly with the second injection of estradiol, twenty-four hours after the first day of

treatment. Cycloheximide at a concentration similar to that used in this investigation prevents estradiol induced uterine protein synthesis by more than 95% for approximately six hours after administration (Sariff and Gorski, 1971). Thereafter, however, the ability of cycloheximide to reduce the estrogenic response gradually diminishes to less than 50% after 10-15 hours. Cidlowski and Muldoon (1978) have also demonstrated that cycloheximide blocked estradiol induced uterine protein synthesis only when administered concomitantly with the hormone. When cycloheximide was administered 30 minutes after estradiol there was a partial suppression of protein synthesis and the inhibitory effect was completely absent when given one hour after estradiol. That concomitant administration of cycloheximide with estradiol at zero time in this study failed to prevent the appearance of gap junctions in the myometrium after continued hormone treatment, may reflect the inability of the single injection of cycloheximide to completely block total protein synthesis. Another possibility is that the synthesis of gap junction proteins may occur at a different time than other proteins in general and this may not be blocked by cycloheximide based on the time scale of this inhibitors effect. Assuming that within twenty-four hours after an injection of estradiol, uterine proteins (including the gap junction protein) are synthesized. Administration of cycloheximide simultaneously with a second injection of estradiol on day two prevents the appearance of gap junctions in the myometrium on days four and five after continued hormone treatment. This may indicate that a high concentration of connexons may be required for gap junction formation and this level may not be attained when protein synthesis is prevented

on the second day of treatment. Alternatively, a sufficient quantity of gap junction proteins may be synthesized after one treatment with estradiol but the "other factor" which appears to be required for estradiol stimulation of gap junctions may be suppressed by administration of cycloheximide on the second day of treatment. These results further suggest that protein synthesis appears to be required for at least 48 hours in response to repeated estradiol injections for the expression of gap junctions in the myometrium. This is supported by the observation that a single injection of estradiol at zero time was not sufficient to induce the presence of numerous myometrial gap junctions over a 1-6 day period. Furthermore, these data indicate that continued occupation of the steroid receptors may be required for estradiol stimulation of gap junctions.

There are delays in the onset of gap junctions following treatment with estradiol and diethystilbestrol in the immature and/or ovariectomized mature rat (Figures 9, 14 and 17). A similar delay is also apparent after the hormonal change prior to parturition and it has been attributed to the period of protein synthesis and assembly into gap junction plaques (Garfield et al., 1980a; 1980b). The time difference in this delay between the immature and ovariectomized mature rats treated with estradiol may indicate that the gap junction aggregation mechanism, steroid receptors or some other mechanism is differentially stimulated or not exactly the same in each group of animals. Similarly, the earlier appearance of gap junctions in immature myometrium after diethylstilbestrol treatment may indicate that the non-steroidal estrogen is more efficient for stimulating the

presence of gap junctions. It is not likely that diethylstilbestrol has a greater potency than estradiol for stimulating the synthesis of the gap junction proteins since both hormones at the same dose induce similar increases in frequency, size and area of the junctions over the treatment period. It is possible that the two estrogens do not equally interact with the immature myometrial estrogen- receptor. There are conflicting reports in the literature concerning the affinities of diethylstilbestrol and estradiol for the estrogen-receptor in a variety of tissues, however, it has been shown that diethylstilbestrol has a higher affinity than estradiol for the estrogen-receptor in human (Robel et al., 1981) and rat uteri (Gorski et al., 1968). Another possible explanation for the earlier effect of diethylstilbestrol on the presence of gap junctions which cannot be ruled out is that the two estrogens are delivered from the administration site to the uterus at different times or that the uterus takes up the estrogens at different rates.

The frequency of gap junctions present in immature and ovariectomized mature rat myometrium following estradiol treatment is similar to that observed in myometrial tissues from delivering animals (compare Figures 9 and 17 with Table 1). However, the sizes of the junctions in delivering myometrium were larger than those in the non-pregnant tissues. In a previous study it was suggested that in delivering tissue prostaglandins may facilitate the aggregation of gap junction proteins (Garfield et al., 1980a). The results of the present study may indicate that the aggregation mechanism is not exactly the same in non-pregnant myometrial tissues. The frequency of gap

junctions also decreases in immature rats after continued estradiol and diethylstilbestrol treatment (Figures 9 and 14). Why gap junctions disappear or the mechanism responsible for gap junction degradation in the myometrium is not completely understood. Gap junctions also disappear following parturition and it has been suggested to occur via junction internalization followed by digestion through an endocytotic-lysosomal mechanism (Garfield et al., 1980b). Junction degradation following parturition also coincides with the post-partum decline in levels of steroid hormones which may [illegible]tiate the internalization process. Similarly, it has been shown that estrogen-receptors are significantly suppressed in uteri from ovariectomized rats treated daily for five days with 50 ug/day estradiol (Manni et al., 1981). Thus, it is tempting to speculate that continued estradiol treatment results in receptor suppression accompanied by the removal of gap junctions from the myometrium.

Progesterone administered concomitantly with high doses of estradiol to immature and ovariectomized mature animals suppresses the presence of myometrial gap junctions but progesterone alone has no effect on the junctions. These results can be explained on the basis of the mechanism of action of steroid hormones. The immature and ovariectomized mature rat is exposed to low levels of sex steroids (Dohler and Wuttke, 1975), and estradiol is required for the synthesis of the progesterone receptor (Vu Hai et al., 1977; Manni et al., 1981; Keeping and Lyttle, 1982), explaining the ineffectiveness of progesterone when administered alone. Estradiol treatment, however, stimulates the synthesis of progesterone receptors thus allowing

progesterone to exert its action. Progesterone suppression probably occurs as a result of inhibiting estradiol stimulation of protein synthesis through the steroid receptor mechanism (Bhakoo and Katzenellenbogen, 1977; Okulicz et al., 1981). The effects of progesterone on estradiol induced uterine responses and possible mechanism of progesterone action are discussed in detail in Chapter 6. This antagonistic effect of progesterone on the action of estradiol has been previously reported in sex hormone target tissues (West et al., 1976; Sumida et al., 1981; Sakamoto et al., 1983), including inhibition of myometrial gap junctions in mature animals (Garfield et al., 1980a). Although it is evident that progesterone suppresses the estradiol stimulated presence of gap junctions, the size of the junctions is not influenced by the low dose progesterone treatment (Figure 15). This may indicate that a mechanism, independent of the steroid receptor mechanism, may be regulating the aggregation of gap junction proteins into plaques prior to degradation.

The incompleteness of progesterone's inhibitory effect in vivo may be related to the dose of the hormone administered. When progesterone is given at a higher dose a further significant suppression on the presence of gap junctions becomes apparent (Tables 2 and 5). This may indicate that a particular progesterone-to- estradiol ratio is critical and must be reached for maximal inhibition of gap junctions. In this study a progesterone-to-estradiol ratio of 10:1 effectively diminished the response but did not abolish it. Recently it was reported that a progesterone-to-estrogen ratio of 50:1 but not 1:1 or 10:1 resulted in similar effects to that reported here (Burghardt et

al., 1984a). The greater ratio between the steroid hormones in their study may reflect the different estrogen used, however, those results are subject to criticism because of the methods they used to quantitate gap junctions in the myometrium. Similarly, the high plasma levels of progesterone throughout pregnancy may prevent the development of myometrial gap junctions (Garfield et al., 1978) which may be required to maintain the uterus in a quiescent state throughout gestation and be partially responsible for the progesterone block theory of uterine contractions as proposed by Csapo in 1956.

The synthetic non-steroidal antiestrogen tamoxifen did not promote the induction of myometrial gap junctions in the immature rat over a 1-6 day period. Moreover, tamoxifen was found to completely suppress the ability of estradiol in stimulating the presence of gap junctions in the myometrium. It is generally thought that tamoxifen acts as a partial agonist-antagonist to estrogenic uterine responses (Jordan and Dix, 1979). However, with respect to the parameter of myometrial gap junction stimulation by estradiol, tamoxifen acts as a pure estrogen antagonist.

Tamoxifen exerts its antiestrogenic effect by binding to the cytoplasmic estradiol receptor followed by the translocation of the antiestrogen-receptor complex into the nucleus of the cell (Katzenellenbogen et al., 1978). The mechanism by which the tamoxifen-receptor complex inhibits estradiol induced uterine responses, however, has not been resolved but appears to result from altering the steroid receptor mechanism (the effects of tamoxifen on estradiol induced uterine responses and possible mechanisms of action are discussed in

detail in Chapter 6). Therefore, the antiestrogenic effects of tamoxifen implies that the steroid receptor mechanism for estradiol is involved in the regulation of myometrial gap junctions.

Other studies have suggested an indirect effect of estradiol on the presence of myometrial gap junctions. Wathes and Porter (1982) reported that an injection of estradiol (7.5 ug) was not sufficient to stimulate myometrial gap junctions in ovariectomized rats to the level found during parturition. However, when the uterine horns of the animals were distended with intrauterine balloons gap junctions were detectable following estradiol treatment, and the frequency of gap junctions approximated that found in delivering tissue. They suggest that estradiol and uterine distention together are required for the presence of gap junctions. The results of this study, however, clearly show that a large dose of estradiol is sufficient to stimulate the presence of large numbers of myometrial gap junctions in the absence of distention. Uterine distention on the other hand, may initiate an event necessary for the assembly of proteins into gap junction structures. It is well established that uterine distention causes an increase in the synthesis of uterine prostaglandins (Csapo, 1977) and that prostaglandins may be involved in regulation of gap junctions (Garfield et al., 1980a). The increase in gap junctions with uterine distention plus estradiol treatment as reported by Wathes and Porter (1982) may result from the stimulation of the synthesis of a specific prostaglandin which complements the low dose of estradiol for the appearance of gap junctions. My studies clearly indicate that prostaglandins are involved in regulation of myometrial gap junctions

(see Chapter 5). In addition, it is also evident from this study that the mechanism(s) regulating the presence of gap junctions does not require the presence of the fetus and/or placenta.

4.3.1.2 Regulation in pregnant tissues:

The results of this study show that gap junctions are present between uterine smooth muscle cells in increased numbers and size immediately prior to and during spontaneous parturition as shown previously (see Chapter 1). These results are consistent with the hypothesis that the presence of gap junctions in the myometrium exclusively at the end of gestation is significant for the termination of pregnancy (Garfield et al., 1978). High levels of progesterone throughout gestation may inhibit the development of myometrial gap junctions thereby suppressing coordinated myometrial activity, but when progesterone levels decline and estradiol levels increase prior to labor, lead to the appearance of gap junctions in the myometrium.

The evidence presented in this study also clearly shows that pregnant rats treated with the high dose (500 ug/day) of estradiol developed numerous myometrial gap junctions and aborted their fetuses. These results support the hypothesis of a hormonal regulation of myometrial gap junctions and that the development of the junctions between uterine smooth muscle cells is necessary for the occurrence of term as well as preterm labor (Garfield et al., 1977; 1978; 1979). Gap junctions also developed in the myometrium of pregnant rats treated with the lower dose of estradiol (50 ug/day), however, only some of the animals appeared to undergo premature labor as observed after treatment

with the higher (500 ug/day) dose of the hormone. Furthermore, more injections of the lower dose of estradiol were required compared with that of the higher dose of the hormone. Vaginal bleeding was evident in most of the animals that developed numerous myometrial gap junctions and in the animals that did not deliver their pups prematurely, the fetuses were dislodged and compacted in the lower uterus. These results indicate that the animals had undergone some form of labor but that the cervix may not have dilated sufficiently to allow the passage of the fetuses.

The appearance of gap junctions at term in the rat is accompanied by an increase in plasma estradiol levels (Fuchs, 1978) and a 3 to 4 fold increase in the tissue levels of the hormone (Puri and Garfield, 1982). In addition, estrogens seem to be essential for normal and preterm labor (Csapo et al., 1973b; Garfield et al., 1982a). The data of this study indicates that an elevation in estradiol levels above that normally found before progesterone withdrawal may not be sufficient for gap junction formation and/or labor since three days of treatment with estradiol was required to induce the development of myometrial gap junctions and preterm labor. In the rat, progesterone plasma and tissue levels begin to decline on day 19 of pregnancy and are significantly lower than in samples taken prior to this day of gestation (Puri and Garfield, 1982). However, it has been reported that estradiol treatment to pregnant animals results in a reduction in the progesterone levels and termination of pregnancy (Evans et al., 1981). Therefore, the results of this study may indicate that by elevating the estradiol levels prior to the withdrawal of progesterone observed at

term, progesterone withdrawal may be achieved allowing the estradiol induced expression of myometrial gap junctions. Another possibility is that the ratio between steroids rather than the absolute values of the steroid hormones in pregnant and non- pregnant animals is the critical factor controlling the regulation of gap junction development. It is tempting to speculate that the estrogen induced termination of pregnancy in the guinea pig and ewe reported elsewhere (Restall et al., 1976; Evans et al., 1981; Dawe et al., 1982) resulted from the stimulation of myometrial gap junctions in response to changes in ratio's of estradiol-to-progesterone.

Besides the mechanisms regulating the presence of gap junctions in the myometrium at term, there is a process that controls the disappearance of gap junctions from the myometrium during the post-partum period. It is possible that the decrease in gap junctions post-partum is related to the decline in steroid hormones in this period and the absence of sufficient gap junction stimulus regulating their synthesis. However, results presented in this study show that estradiol, which can induce myometrial gap junction formation prior to term in the rat, is unable to sustain the presence of the junctions in the myometrium when administered on day 21 of pregnancy, during delivery and one day post-partum. Similarly, Berezin et al., (1982) have shown that the presence or function of the ovaries is not necessary for resolving myometrial gap junctions after parturition. However, Saito et al., (1984) have suggested that increased myometrial cellular levels of progesterone and progesterone receptors due to post-partum ovulation in the rat may serve as the factor responsible

for the disappearance of gap junctions. This seems unlikely since gap junctions disappear from the myometrium following parturition when the ovaries have been removed (Berezin et al., 1982). Moreover, as reported in this study gap junctions develop and disappear from the immature rat myometrium in response to continued treatment with estradiol over a 1-8 day period, in the absence of progesterone. Furthermore, the post-partum receptor observation reported by Saito et al., (1984) are difficult to conceive for the following reason. Post-partum ovulation results in increased progesterone levels, however, they reported a corresponding increase in the number of progesterone receptors. Although progesterone receptor synthesis is regulated by estradiol (Keeping and Lyttle, 1982) they report the increase in progesterone-receptors during the post-partum period when estradiol levels and receptor number are at their lowest levels.

That an ovarian hormone is not required for the disappearance of gap junctions from the myometrium indicates that some other control system is responsible for resolving myometrial gap junctions. Berezin et al., (1982) have suggested that the progression of events leading to gap junction formation, labor and disappearance of gap junctions may be programmed by some control system prior to day 21 before gap junctions appear in most animals. In tissue culture of myometrium from pregnant and non-pregnant animals gap junctions persist for long periods of time (Garfield et al., 1980a) suggesting that preprogramming of their disappearance seems unlikely. Another possibility is that events associated with parturition (i.e. loss of the placenta and uterine distention) may be involved in controlling the regression of gap

junctions from the myometrium. If such factors are involved in the process this may explain why gap junctions persist at plateau levels under in vitro conditions. Wathes and Porter (1982) have demonstrated that mechanical distention of the post-partum uterus, supplemented with estradiol treatment, maintains the frequency of myometrial gap junctions after labor. Distention of the uterus causes the synthesis and release of prostaglandins (Csapo, 1977) and prostaglandins appear to be involved in the regulation of myometrial gap junctions (see Chapter 5). High estrogenic conditions maintained by exogenous estradiol administration to pregnant animals in this study may have been insufficient in sustaining the presence of gap junctions in the myometrium due to the absence of uterine distention and prostaglandin production. During the post-partum period prostacyclin synthesis may increase and this prostanoid may inhibit gap junction formation (Garfield et al., 1980a). It is possible that uterine distention may decrease the synthesis of prostacyclin while selectively stimulates other prostaglandins that may be required for gap junction formation and the prevention of uterine involution. Subsequently, after delivery of the fetuses the uterus undergoes involution resulting from removal of the stimulation exerted by the steroid hormones, prostaglandins and conceptuses involving a decrease in myometrial cell size and number which could result in the disappearance of gap junctions from the myometrium.

4.3.2 Summary:

Myometrial tissues from immature, ovariectomized mature and

pregnant rats were examined by thin section and/or freeze-fracture electron microscopy for the presence of gap junctions following treatment with the steroid hormones. The results indicate that the steroid hormones regulate the presence of gap junctions in the myometrium. Estrogens stimulate whereas progesterone, when administered simultaneously with estrogen, suppresses the presence of myometrial gap junctions. The steroid hormones may influence the presence of gap junctions by regulating the synthesis of the gap junction protein through the steroid receptor mechanism. Also, pregnant animals treated with estradiol develop numerous myometrial gap junctions and deliver prematurely. These results indicate that the development of gap junctions in the myometrium is a requirement for the occurrence of term, as well as preterm labor.

Furthermore, my results show that gap junctions develop only after treatment with high doses of estrogen and that there is a delay after treatment before the junctions appear. These observations suggest that high doses of estrogen may stimulate another mechanism necessary for the development of gap junctions (i.e. prostaglandins) see Chapter 5. In addition, I have also evaluated the regulation of myometrial growth by the steroid hormones and the relationship between growth and the development of gap junctions (Chapter 6). The results suggest that myometrial growth may be necessary for the appearance of the junctions which could account for the delay in the onset of gap junctions following hormone treatment.

CHAPTER 5

REGULATION OF MYOMETRIAL GAP JUNCTIONS BY PROSTAGLANDINS

5.1 Introduction:

Parturition in a variety of animal species (Garfield et al., 1979; Puri and Garfield, 1982; see Chapter 4), including humans (Garfield et al., 1979; Garfield and Hayashi, 1981) is accompanied by the development of gap junctions between the muscle cells of the uterus. The presence of these specialized cell-to-cell contacts, exclusively at term and during labor, results in increased electrical (Sims et al., 1982) and metabolic coupling (Cole et al., 1983) of the muscle cells, possibly prerequisites for increased uterine contractility necessary for expulsion of the fetuses.

Although the stimulation or inhibition of myometrial gap junctions have significant implications in the management of labor, the regulation of their formation is not completely understood. The results presented in Chapter 4 support the hypothesis that the steroid hormones regulate the presence of gap junctions in the myometrium and that their presence in the myometrium of pregnant animals appears to be a requisite for the occurrence of labor (see Chapter 1). However, in addition to the role of the steroid hormones in regulating the presence of gap junctions in the myometrium there appears to be another factor directly involved in the process, possibly the result of selective stimulation by estradiol in a dose responsive fashion (see Chapter 4).

The presence of myometrial gap junctions in parturient animals is accompanied by increased plasma and tissue levels of prostaglandins in addition to the changes in the steroid hormones (Puri and Garfield, 1982). The increased production of prostaglandins at term is believed

to be stimulated by the change in levels of the steroid hormones (Nathanielsz, 1978). Prostaglandins may be required for the regulation of myometrial gap junctions because indomethacin, an inhibitor of prostaglandin synthesis, prevents the appearance of gap junctions in tissue from pregnant animals in vitro (Garfield et al., 1980b). Prostaglandins stimulate the myometrium to contract (Csapo, 1981) and alter steroid hormone production (Horton and Poyser, 1976). Administration of prostaglandin $F_{2\alpha}$ to pregnant animals induces abortion or premature labor and treatment with inhibitors of prostaglandin synthesis prolongs pregnancy (Csapo et al., 1973a; 1973b; Challis et al., 1975; Fuchs, 1977). Therefore, termination of pregnancy by prostaglandins may result from a direct influence on the regulation of myometrial gap junctions and/or an indirect effect on the steroid hormone levels.

In this study the effects of indomethacin and sodium meclofenamate (inhibitors of prostaglandin synthesis) on the in vivo steroid hormone regulation of myometrial gap junctions was examined in the immature and/or ovariectomized mature rat. In addition, plasma estradiol levels, uterine tissue uptake and retention of estradiol and prostaglandin F content were measured in ovariectomized mature rats treated with estradiol and indomethacin. The results show that inhibitors of prostaglandin synthesis potentiate the estradiol stimulation of myometrial gap junctions and that progesterone is still capable of suppressing this effect. Moreover, uterine tissue from animals treated with indomethacin take up and retain ^{3}H-estradiol to a greater extent than control tissues. These data clearly indicate that

prostaglandins are involved in the regulation of myometrial gap junctions possibly through an effect on the steroid receptors.

5.2 Results:

5.2.1 Tissues from immature rats:

5.2.1.1 Effects of indomethacin:

To evaluate the effects of a prostaglandin synthesis inhibitor on the presence of myometrial gap junctions, indomethacin (0.2 mg twice daily) was administered with and without estradiol (50 and 500 ug/day) to immature rats for three and four days. Table 7 shows that concomitant injections of estradiol plus indomethacin enhanced the action of both doses of estradiol in stimulating the presence of gap junctions in the myometrium. The very low mean frequencies of gap junctions in tissues from rats treated for three days with 50 and 500 ug/day estradiol were significantly increased by indomethacin to 3.425 $\pm$ 0.912 ($P<0.05$) and 5.425 $\pm$ 1.220 ($P<0.01$) junctions/1000 um membrane, respectively.

The mean size of the gap junctions that formed in response to either dose of estradiol plus indomethacin were not significantly ($P>0.05$) different to the mean sizes observed after estradiol treatment alone. Concomitant administration of indomethacin with both doses of estradiol also resulted in an increase in the percentage of myometrial membrane area occupied by gap junctions, however, a significant difference ($P<0.05$) occurred only with the 500 ug/day dose of

Table 7. Data are as described in Table 1. Immature rats were injected daily for 3 and 4 days with estradiol (E_2) with and without indomethacin (IDM) to determine the effects of prostaglandin synthesis on myometrial gap junctions. Statistical comparisons were made between estradiol and estradiol plus indomethacin values. Levels of significance: * $P<0.05$; ** $P<0.01$.

TABLE 7. EFFECTS OF ESTRADIOL (E_2) AND/OR INDOMETHACIN (IDM) ON MYOMETRIAL GAP JUNCTIONS(GJs) IN IMMATURE RATS

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
3 days						
Control	6(6)	0	8,380	0	0	0
IDM (0.2 mg twice daily)	5(5)	0	5,281	0	0	0
E_2 (50 ug/day)	4(4)	3	4,926	0.650 ± 0.450*	107 ± 38	0.013 ± 0.007
E_2 plus IDM	4(4)	19	5,550	3.425 ± 0.912	121 ± 13	0.081 ± 0.036
E_2 (500 ug/day)	6(6)	3	7,704	0.363 ± 0.238**	132 ± 30	0.009 ± 0.006**
E_2 plus IDM	4(4)	30	5,405	5.425 ± 1.200	106 ± 53	0.121 ± 0.030
4 days						
Control	6(6)	1	9,299	0.103 ± 0.103	36	0.001 ± 0.001
IDM (0.2 mg twice daily)	4(4)	0	5,935	0	0	0
E_2 (50 ug/day)	4(4)	2	5,323	0.325 ± 0.325*	52 ± 14	0.003 ± 0.003
E_2 plus IDM	4(4)	18	6,054	3.170 ± 0.767	93 ± 11	0.061 ± 0.024
E_2 (500 ug/day)	6(6)	29	8,963	3.300 ± 1.260	135 ± 14	0.091 ± 0.038
E_2 plus IDM	4(4)	43	5,237	8.185 ± 3.707	114 ± 8	0.186 ± 0.080

estradiol. Gap junctions were not observed in tissues from control rats treated with sesame oil or in animals treated with indomethacin alone for the three day period.

Similarly, when gap junctions were infrequent in myometrial tissues from immature rats treated four days with 50 ug/day estradiol or present at a high frequency after treatment with 500 ug/day estradiol alone, concomitant injections of indomethacin resulted in a further increase in the frequencies of gap junctions reaching values of 3.170 ± 0.767 ($P<0.05$) junctions/1000 um membrane when administered with 50 ug/day estradiol and 8.185 ± 3.707 junctions/1000 um membrane with 500 ug/day estradiol (Table 7). No effect on gap junction size was observed when indomethacin was administered with either dose of estradiol. The percentage of myometrial membrane area occupied by gap junctions, however, increased but insignificantly to 0.061 ± 0.024 and 0.186 ± 0.080 percent after 50 and 500 ug/day estradiol plus indomethacin, respectively.

The effect of progesterone on the indomethacin enhancement of estradiol stimulation of myometrial gap junctions is shown in Table 8. Gap junctions were not detected in myometrial tissues from control animals and rats treated with indomethacin or progesterone alone for three days. Progesterone (2.5 mg twice daily) administered simultaneously with estradiol (50 and 500 ug/day) plus indomethacin (0.2 mg twice daily) for three days significantly ($P<0.05$) suppressed the frequencies of myometrial gap junctions compared with that observed in tissues from rats treated with estradiol plus indomethacin. The decreased frequencies together with an insignificant decrease in the

Table 8. Data are as described in Table 1. Progesterone was administered simultaneously to immature rats with estradiol plus indomethacin daily for 3 days to determine whether the steroid would suppress the effect of estradiol plus indomethacin on the presence of gap junctions in the myometrium. Level of significance: * $P<0.05$.

TABLE 8. EFFECTS OF ESTRADIOL (E_2) PLUS INDOMETHACIN (IDM) WITH AND WITHOUT PROGESTERONE (P_4) ON MYOMETRIAL GAP JUNCTIONS (GJs) IN IMMATURE RATS

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
3 days						
Control	6(6)	0	8,380	0	0	0
IDM (0.2 mg twice daily)	5(5)	0	5,281	0	0	0
P_4 (2.5 mg twice daily)	4(4)	0	4,850	0	0	0
E_2 (50 ug/day) plus IDM	4(4)	19	5,550	3.425 ± 0.912	121 ± 13	0.081 ± 0.036
E_2 plus IDM plus P_4	4(4)	4	5,557	0.725 ± 0.500 *	107 ± 35	0.015 ± 0.012
E_2 (500 ug/day) plus IDM	4(4)	30	5,405	5.425 ± 1.220 *	106 ± 53	0.121 ± 0.030 *
E_2 plus IDM plus P_4	5(4)	2	8,002	0.240 ± 0.147	104 ± 15	0.005 ± 0.003

mean size of the gap junctions following concomitant progesterone treatment also resulted in suppression of the membrane area occupied by the junctions. Administration of progesterone together with 50 and 500 ug/day estradiol plus indomethacin prevented the increase in the gap junctional membrane area compared to treatments with estradiol plus indomethacin alone (from 0.081 ± 0.036 and 0.121 ± 0.030 percent after treatment with 50 and 500 ug/day estradiol plus indomethacin respectively to 0.015 ± 0.012 and 0.005 ± 0.003 percent ($P<0.05$) after treatment with 50 and 500 ug/day estradiol plus indomethacin together with progesterone). Note that the quantitative measurements after suppression by simultaneous administration of progesterone with estradiol (50 and 500 ug/day) plus indomethacin were similar to those after treatment with either dose of estradiol alone (see Table 6).

5.2.1.2 Effects of sodium meclofenamate:

Table 9 shows the effects of sodium meclofenamate (an inhibitor of prostaglandin synthesis) on the presence of myometrial gap junctions in the immature rat after estradiol treatment for three days. Gap junctions were undetectable in myometrial tissues from control rats and animals treated with sodium meclofenamate (0.7 mg/day) alone. However, when administered concomitantly with 500 ug/day estradiol, sodium meclofenamate caused a significant increase ($P<0.01$) in the frequency (0.363 ± 0.238 to 3.620 ± 0.596 junctions/1000 um membrane) and the myometrial membrane area occupied by gap junctions (0.009 ± 0.006 to 0.067 ± 0.018 percent) compared with the values determined in tissues from animals treated with estradiol alone. The mean gap junction size

TABLE 9. EFFECTS OF ESTRADIOL (E_2) AND/OR SODIUM MECLOFENAMATE (MFA) ON MYOMETRIAL GAP JUNCTIONS (GJs) IN IMMATURE RATS

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
3 days						
Control	6(6)	0	8,380	0	0	0
MFA (0.7 mg/day)	2(2)	0	3,628	0	0	0
E_2 (500 ug/day)	6(6)	3	7,704	0.363 ± 0.238 **	132 ± 30	0.009 ± 0.006 **
E_2 plus MFA	4(4)	21	5,905	3.620 ± 0.596	91 ± 10	0.067 ± 0.018

Data are as described in Table 1. Results from immature rats treated daily for 3 days (when a very low frequency of gap junctions is present after estradiol treatment) with estradiol (E_2) and/or sodium meclofenamate (MFA) on myometrial gap junctions. Level of significance: * $P<0.01$.

in myometrial tissues after treatment with sodium meclofenamate together with estradol was smaller, but not significantly different to the mean size in the estradiol treated tissues.

5.2.2 Tissues from ovariectomized mature rats:

5.2.2.1 Effects of indomethacin:

Figure 23 shows the effects of indomethacin on the presence of gap junctions in ovariectomized mature rats treated with 500 ug/day estradiol. Myometrial gap junctions were not present in tissues from control animals treated daily with sesame oil and present at an extremely low frequency and small sizes in tissues from rats treated with indomethacin over the 1-4 day period (Figure 23A). In animals treated with 500 ug/day estradiol the frequency of gap junctions was low after one day of treatment but thereafter, was significantly elevated ($P<0.05$) compared with that in control tissues. The size of the gap junctions in myometrial tissues increased to a near maximum value after two days of treatment with estradiol and was maintained over the remainder of the treatment period (Figure 23B). The increase in the frequency and size of gap junctions after two days of estradiol treatment resulted in a corresponding increase in the percentage of membrane area occupied by the junctions (Figure 23C). The percentage of gap junction membrane area decreased but insignificantly after three and four days of treatment.

Similar to that observed in the immature rat (see Table 7), concomitant indomethacin treatment (2 mg twice daily) enhanced the

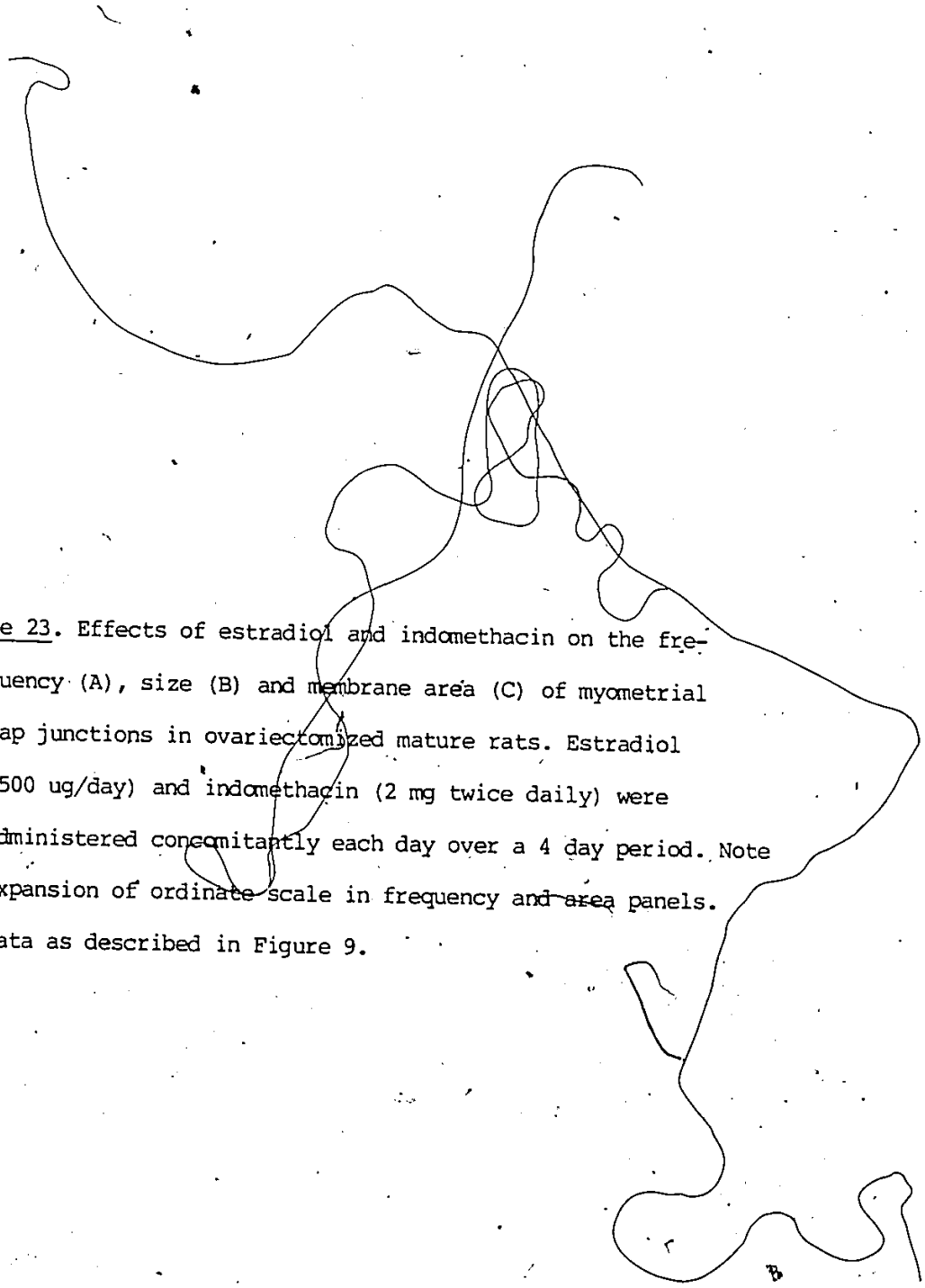
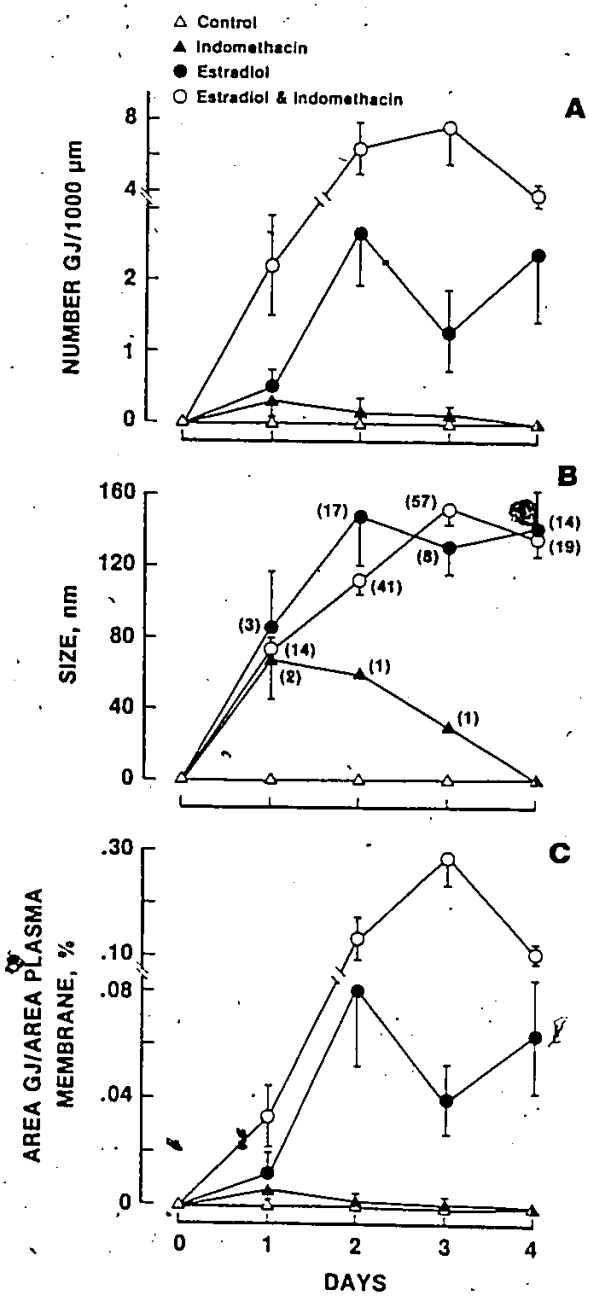


Figure 23. Effects of estradiol and indomethacin on the frequency (A), size (B) and membrane area (C) of myometrial gap junctions in ovariectomized mature rats. Estradiol (500 ug/day) and indomethacin (2 mg twice daily) were administered concomitantly each day over a 4 day period. Note expansion of ordinate scale in frequency and area panels. Data as described in Figure 9.


Control
Indomethacin
Estradiol
Estradiol & Indomethacin
A
NUMBER GJ/1000 µm
8
4
2
1
0
B
SIZE, nm
160
120
80
40
0
(17)
(57)
(14)
(19)
(8)
(41)
(3)
(14)
(2)
(1)
(1)
C
AREA GJ/AREA PLASMA MEMBRANE, %
.30
.10
.08
.04
0
0
1
2
3
4
DAYS

effect of estradiol (500 ug/day) in stimulating the presence of myometrial gap junctions (Figure 23). The mean frequency of gap junctions in tissues from rats treated with estradiol and indomethacin increased from 2.200 ± 0.730 junctions/1000 um membrane after the first injection to 6.500 ± 1.700 after two days of treatment, a significant increase ($P<0.05$) compared with control animals and rats treated with indomethacin alone (Figure 23A). The frequency increased further with continued treatment to a maximum value of 7.700 ± 2.300 junctions/1000 um membrane after three days of treatment. Following the fourth injection the frequency significantly ($P<0.05$) decreased to 3.900 ± 0.590 junctions/1000 um membrane. All mean frequencies observed after treatment with estradiol plus indomethacin were greater than those in response to estradiol alone, however, a significant difference ($P<0.05$) occurred only after the third day of treatment.

The mean sizes of gap junctions following treatment with estradiol plus indomethacin increased over the injection period reaching a maximum size after three days (Figure 23B). The simultaneous administration of indomethacin together with estradiol did not significantly effect the sizes of the gap junctions compared to the treatment with estradiol alone.

Indomethacin, when administered with estradiol, also enhanced the gap junction membrane area (Figure 23C). The membrane area was increased after the first day of treatment to 0.033 ± 0.014 percent from 0.013 ± 0.007 percent after treatment with estradiol alone. The indomethacin effect continued until a maximum of 0.284 ± 0.056 percent of the membrane was occupied by gap junctions after the third

injection, a significant increase ($P<0.05$) when compared with the estradiol treated animals. Figure 24A shows a low magnification electron micrograph from a thin section of the myometrium from ovariectomized mature animals treated with estradiol plus indomethacin. Gap junctions were frequently observed between the muscle cells. At high magnification the structure of the junction is apparent (Figure 24B).

To determine the effects of progesterone on the indomethacin potentiation of estradiol stimulation of myometrial gap junctions, progesterone was administered simultaneously with estradiol plus indomethacin for three days. Gap junctions were not observed in tissues from control rats and animals treated with progesterone (2.5 mg twice daily) alone and present at an extremely low frequency and small size after treatment with indomethacin (2 mg twice daily) after the three day period (Table 10). Progesterone administered concomitantly with estradiol (500 ug/day) plus indomethacin for three days significantly suppressed the frequency ($P<0.05$) and membrane area ($P<0.05$) of gap junctions compared with the values in tissues from animals treated with estradiol plus indomethacin for the same period. There was no significant difference in the mean size of the junctions in the myometrium from animals in both treatment groups.

The effect on indomethacin together with lower doses of estradiol on gap junction formation was studied in ovariectomized rats treated simultaneously with 0.025, 0.10 or 50 ug/day estradiol plus indomethacin (2 mg twice a day) for 6 days. Table 11 shows that gap junctions were present in low numbers in myometrial tissues from

Figure 24. Electron micrographs from thin sections of myometrium from ovariectomized mature rats treated with estradiol plus indomethacin. A) At low magnification several profiles of longitudinal muscle cells are seen with many gap junctions (arrows) between the cells. Marker is 1 um, X 27,500. B) The septilaminar structure of a gap junction is apparent at higher magnification. Marker is 0.1 um, X 374,000.

A
B

TABLE 10. EFFECTS OF PROGESTERONE (P_4) ON MYOMETRIAL GAP JUNCTIONS (GJs) IN OVARIECTOMIZED MATURE RATS TREATED WITH E_2 PLUS IDM

Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
3 days						
Control	4(4)	0	6,589	0	0	0
IDM (2 mg twice daily)	4(4)	1	6,600	0.160 ± 0.160	30	0.001 ± 0.001
P_4 (2.5 mg twice daily)	4(3)	0	5,890	0	0	0
E_2 (500 ug/day)	4(4)	8	5,937	1.300 ± 0.620	132 ± 16	0.040 ± 0.016
E_2 plus IDM	4(4)	57	6,278	7.730 ± 2.300*	152 ± 9	0.284 ± 0.056*
E_2 plus IDM plus P_4	4(4)	6	5,482	1.077 ± 0.448	149 ± 15	0.032 ± 0.013

Data are as described in Table 1. Results from ovariectomized mature rats treated daily for 3 days with estradiol (E_2), indomethacin (IDM), progesterone (P_4) and estradiol plus indomethacin. Progesterone (at 2.5 mg twice daily because of the greater suppression on the presence of gap junctions as observed in the immature rats) was administered together with E_2 plus IDM to determine if it would suppress the effect of both drugs on the presence of gap junctions. Level of significance: *P<0.05.

Table 11. Data are as described in Table 1. Ovariectomized mature rats were treated daily over 6 days with estradiol (E_2, 0.025, 0.10 and 50 ug/day) to determine the effect of lower doses of the hormone on the presence of gap junctions in the myometrium. Indomethacin (IDM, 2 mg twice daily) was administered alone or together with each dose of estradiol to determine if it would enhance the effect of estradiol on the junctions as previously demonstrated in ovariectomized animals.

TABLE 11. EFFECTS OF ESTRADIOL (E_2) AND/OR INDOMETHACIN (IDM) ON MYOMETRIAL GAP JUNCTIONS (GJs) IN OVARIECTOMIZED RATS

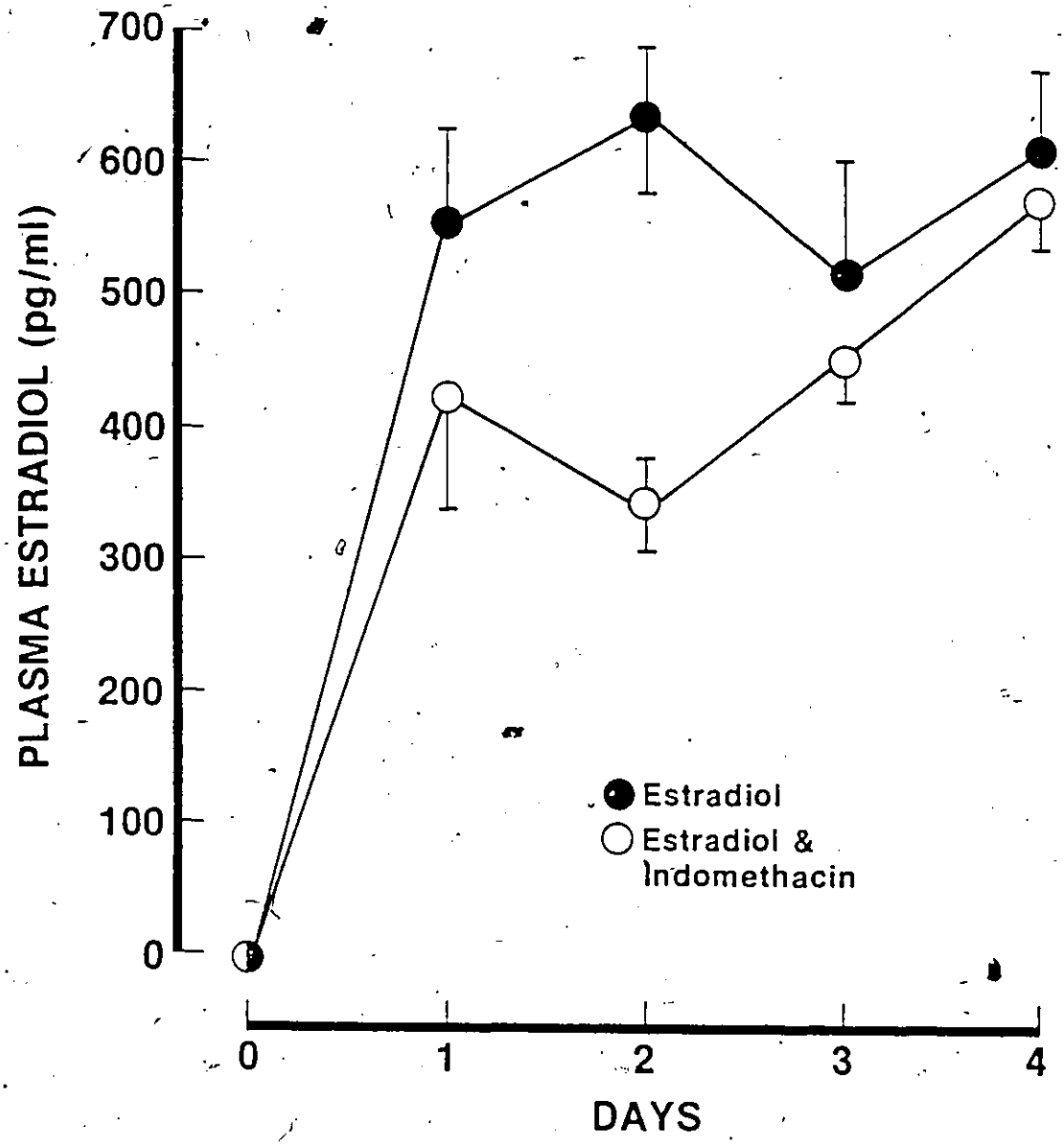
Treatment	No. of Tissues	No. of GJs	Length of Membrane Surveyed, um	No. of GJs/1000 um Membrane (Mean ± S.E.)	Mean Size GJs (nm ± S.E.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
6 days						
Control	7(5)	1	9,731	0.133 ± 0.133^{1}	67	0.002 ± 0.002^{a}
IDM (2 mg twice daily)	6(6)	2	8,510	0.262 ± 0.169^{1}	$99 \pm 9^{A,B}$	0.005 ± 0.005^{a}
E_2 (0.025 ug/day)	3(3)	0	4,778	0	0	0
E_2 (0.10 ug/day)	4(4)	1	5,857	0.163 ± 0.163^{1}	59	0.002 ± 0.002^{a}
E_2 (50 ug/day)	4(4)	3	3,420	0.690 ± 0.230^{1}	$151 \pm 47^{B,C}$	0.021 ± 0.010^{a}
E_2 (0.025 ug/day) plus IDM	3(3)	0	4,752	0	0	0
E_2 (0.10 ug/day) plus IDM	2(3)	2	2,863	0.560 ± 0.560^{1}	66 ± 7^{A}	0.007 ± 0.007^{a}
E_2 (50 ug/day) plus IDM	4(4)	31	3,912	8.200 ± 1.600^{2}	200 ± 26^{C}	0.336 ± 0.133^{b}

control and indomethacin treated rats. Gap junctions were also infrequent in myometrium of rats treated with 0.025 ug/day estradiol, however, with increasing estradiol doses up to 50 ug/day, the frequency and area of membrane occupied by junctions increased but not significantly above control values. Concomitant treatment with indomethacin produced significant results only when administered with 50 ug/day estradiol and increased the frequency and gap junction membrane area to 0.336 ± 0.133 percent of the plasma membrane area ($P<0.05$ when compared with estradiol treatment alone). Indomethacin administered together with 50 ug/day estradiol over the six day period resulted in a non-significant increase in the sizes of the junctions compared with the average measured length in tissues from animals treated with only estradiol.

5.2.3 Effects of estradiol and/or indomethacin on plasma estradiol levels:

The blood plasma estradiol concentration determined in ovariectomized rats after 1-4 injections of estradiol (500 ug/day) or concomitant injections of estradiol and indomethacin are shown in Figure 25. There were only trace amounts of estradiol in control ovariectomized rats daily over the 4 day injection period. The plasma estradiol content of animals injected with 500 ug/day estradiol increased from trace quantities at zero time (day 0) to a near maximum mean level of 552.75 ± 72.20 pg/ml twenty-four hours after the first injection. The level of plasma estradiol fluctuated with repeated injections of the hormone but showed no significant differences between

Figure 25. Effects of estradiol (500 ug/day) with and without indomethacin (2 mg twice daily) on plasma estradiol levels of ovariectomized mature rats over a 4 day period. Data presented as mean values ± S.E. of 4-6 animals on each day.


700
600
500
400
300
200
100
0
PLASMA ESTRADIOL (pg/ml)
Estradiol
Estradiol &
Indomethacin
0
1
2
3
4
DAYS

days one through four of the treatment period. In animals treated with estradiol plus indomethacin the plasma estradiol levels increased after the first day of treatment to a mean of 424.5 ± 92.20 pg/ml. No further increase was observed until after four days of treatment when the plasma level of estradiol was measured at 570.0 ± 34.00 pg/ml. In animals treated with estradiol plus indomethacin the plasma estradiol levels were lower than in animals given only estradiol throughout 1-4 days. However, significant differences in estradiol levels between the two groups occurred only after the second injection (638 ± 57.2 pg/ml vs 448 ± 26 pg/ml, $P<0.05$).

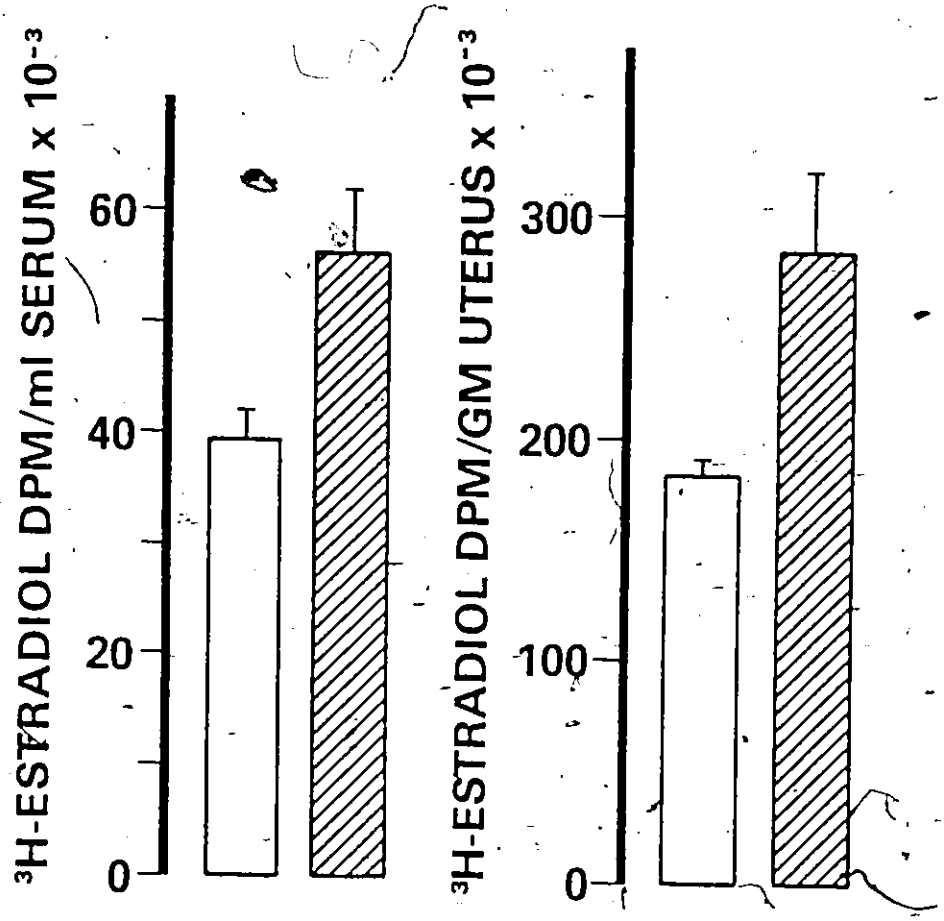
5.2.4 Effects of indomethacin on tritiated estradiol uptake:

Figure 26 shows the ^{3}H-estradiol content of peripheral serum and uterine tissue from ovariectomized rats treated with and without indomethacin for 3 days. In animals treated with indomethacin (5 mg/kg body weight twice daily for three days) the uterine tissue uptake of ^{3}H-estradiol was significantly higher ($P<0.01$) than in the controls (282 dpm/mg compared to 182 dpm/mg). The serum concentration of ^{3}H-estradiol was also significantly higher ($P<0.01$) in the indomethacin treated rats (56 ± 6 compared to 39 ± 3 dpm/ml in the plasma from the control animals).

5.2.5 Effects of estradiol and/or indomethacin on uterine tissue prostaglandin levels:

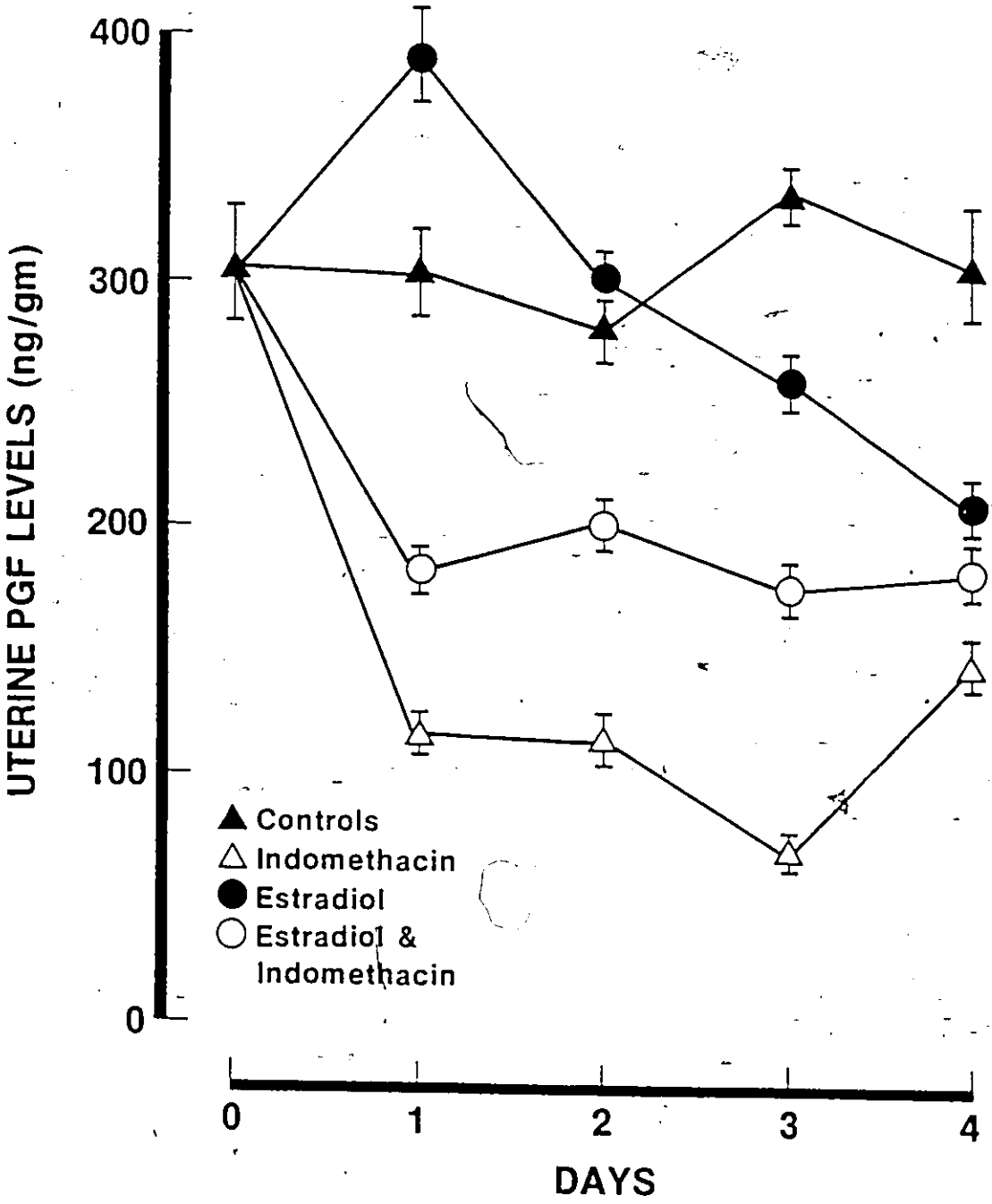
To examine the effect of estradiol with and without indomethacin on the levels of prostaglandin F in uterine tissue,

Figure 26. ^{3}H-estradiol content of serum and uterine tissues one hour after injection of the isotope into mature ovariectomized rats pretreated with indomethacin. Data presented as mean values ± S.E. of 6 animals in each group.


^{3}H-ESTRADIOL DPM/ml SERUM x 10^{-3}
60
40
20
0
^{3}H-ESTRADIOL DPM/GM UTERUS x 10^{-3}
300
200
100
0

prostaglandin F content was measured in the different groups of ovariectomized rats (Figure 27). At zero time (day 0) in control tissues the mean uterine prostaglandin F content was 309 ± 16.3 ng/gm tissue. The daily prostaglandin F content remained fairly constant in tissues from control animals over the 1-4 day period. Administration of estradiol (500 ug/day) for 1-4 days resulted in a significant increase in uterine prostaglandin F content after the first injection to 395.8 ± 14.5 ng/gm compared with control values of 308.6 ± 11.6 ng/gm ($P<0.001$). However, the prostaglandin F content in uterine tissues from rats treated with estradiol for two days was significantly lower ($P<0.001$) compared to the content on day one. Thereafter, the prostaglandin F content in the estradiol treated group progressively declined to values lower than that observed in control tissues after three ($P<0.01$) and four ($P<0.001$) days of treatment. Indomethacin significantly reduced the uterine prostaglandin F content after the first injection to 115.1 ± 3.8 ng/gm tissue ($P<0.0005$) compared to the zero time control value. This level was maintained with the second indomethacin injection followed by a further significant ($P<0.01$) decrease after three days of treatment. After four days of treatment the uterine content of prostaglandin F was significantly ($P<0.001$) elevated compared to that after three days treatment with indomethacin. The uterine content of prostaglandin F in animals treated with indomethacin was significantly lower ($P<0.0005$) compared with the control values on each day over the four day treatment period. Simultaneous administration of estradiol and indomethacin also resulted in a significant decrease in uterine prostaglandin F content after the

Figure 27. Prostaglandin F content in uterine tissue of ovariectomized mature rats over a 4 day period after treatment with estradiol (500 ug/day) and/or indomethacin (2 mg twice daily). Data presented as mean values ± S.E. of 4-6 tissues per group on each day.


400
300
200
100
0
UTERINE PGF LEVELS (ng/gm)
Controls
Indomethacin
Estradiol
Estradiol &
Indomethacin
0
1
2
3
4
DAYS

first injection (186.9 ± 5.2 ng/gm compared with 308.6 ± 16.3 ng/gm tissue in control animals at zero time, $P<0.0005$). No further reduction occurred with repeated injections over the remainder of the treatment period. However, uterine prostaglandin F content was significantly higher in the estradiol plus indomethacin treated animals as compared with those treated with indomethacin alone for the 1-4 day injection period ($P<0.001$).

5.3 Discussion:

The results of this study clearly show that indomethacin and sodium meclofenamate, cyclo-oxygenase inhibitors, potentiate the estradiol stimulation of myometrial gap junctions in vivo in the immature and/or ovariectomized mature rat. This may mean that products of the cyclo-oxygenase pathway play an inhibitory role in directly effecting gap junctions such as their assembly, or that products of the lipoxygenase pathway stimulate gap junctions and when the cyclo-oxygenase pathway is inhibited greater amounts of lipoxygenase products are produced to influence the junctions. Another possibility is that products of cyclo-oxygenase inhibit some step in the steroid-receptor complex leading to gap junction expression. Also, a direct effect of indomethacin or sodium meclofenamate at any point cannot be ruled out. However, both inhibitors of prostaglandin synthesis had no effect on the junctions. Whatever the mechanism for potentiation by these compounds, it is obvious that the mechanism for regulation of myometrial gap junctions is complex relevant to uterine physiology and requires further study.

Prostaglandin synthesis is known to be stimulated by estradiol (Nathanielsz, 1978) and this study shows that myometrial gap junctions increase after inhibition of prostaglandin synthesis. If products of cyclo-oxygenase suppress the presence of gap junctions, then it is not evident how gap junctions appear when prostaglandins are elevated after estradiol treatment. It has been shown that prostaglandin synthesis in response to estrogen may be independent of steroid receptor mediated events and this study shows that the uterine content of prostaglandins decline with repeated injections of estradiol. Therefore, it would not be impossible for gap junctions to develop in response to estradiol provided increased prostaglandin levels are not maintained (see below).

Similarly, if products of cyclo-oxygenase inhibit gap junctions in pregnant myometrium as they may do in nonpregnant animals as shown in this study, then it is not evident how gap junctions develop during parturition when cyclo-oxygenase products are produced profusely by uterine tissues (Nathanielsz, 1978; Puri and Garfield, 1982). However, there is evidence that prostacyclin, a cyclo-oxygenase product, may inhibit development of myometrial gap junctions *in vitro* (Garfield *et al.*, 1980b) and there are other studies which show that estrogen inhibits prostacyclin production (Gimeno *et al.*, 1980; Thaler-Dao *et al.*, 1982). Prostacyclin, produced by the myometrium of pregnant human and rat uterus, has been shown to inhibit *in vitro* activity of pregnant human myometrial strips and to relax vascular smooth muscle, respectively (Williams *et al.*, 1978; Omini *et al.*, 1979; Thorburn and Challis, 1979). Clark *et al.*, (1981) and Lye and Challis (1982) have also reported that in the non-pregnant ewe prostacyclin decreases

uterine contractile activity *in vivo*. The inhibition by prostacyclin has been correlated with increased uterine cAMP content (Omini *et al.*, 1979) and cAMP has been suggested to be involved in regulating gap junction development in the myometrium (Garfield *et al.*, 1980b) and between other mammalian cells (Flagg-Newton, 1980; Flagg-Newton and Loewenstein, 1981; Flagg-Newton *et al.*, 1981). Throughout gestation when gap junctions are absent from the myometrium (Garfield *et al.*, 1978; 1980a; Puri and Garfield, 1982) and estrogen levels are low, prostacyclin levels may be relatively high (Thorburn and Challis, 1979). At term estrogen levels increase (Puri and Garfield, 1982), prostacyclin levels may decrease (Thorburn and Challis, 1979) and myometrial gap junctions develop (Puri and Garfield, 1982). Therefore, prostacyclin may normally prevent gap junction development but after estrogen treatment or during periods when estrogen levels are elevated and prostacyclin levels depressed, gap junctions may form.

It is also possible that the mechanisms for regulation of the junctions by prostaglandins may be different in tissues from pregnant animals. Treatment of myometrial tissues from pregnant animals *in vitro* with indomethacin depresses the frequency of gap junctions (Garfield *et al.*, 1980b) indicating that either there are differences between pregnant and non-pregnant animals or there are differences accounted for by the *in vitro* treatment. It should be noted that gap junctions form spontaneously between myometrial cells in tissues from pregnant animals incubated *in vitro* but that a greater number of the junctions form after addition of estrogen to the incubation media (Garfield *et al.*, 1978). The effect of indomethacin on estradiol

stimulation of gap junctions in vitro therefore, remains to be resolved. Gap junctions may develop between myometrial cells from mid-term pregnant rats incubated in vitro as a direct result of removing the progesterone block which is dominant in vivo during this stage of pregnancy. Moreover, the inhibitory action of prostacyclin in regulating gap junction development (as above) may be lost in vitro allowing for the expression of the junctions. Alternatively, there also appears to be prostanoids which may stimulate gap junction development. Garfield et al., (1980b) has shown that indomethacin, an inhibitor of thromboxane A_2 synthesis and a thromboxane A_2 antagonist suppress the spontaneous formation of gap junctions in vitro. Furthermore, endoperoxides over come the gap junctional inhibitory effect of indomethacin (Garfield et al., 1980a). In addition, uterine distention causes the synthesis and release of prostaglandins (Csapo, 1977) and mechanical distention of the uterus results in a slight increase in the frequency of myometrial gap junctions (Wathes and Porter, 1982). This effect of uterine distention, however, was further increased by low doses of estradiol. Throughout pregnancy endoperoxides and thromboxane A_2 may not be synthesized, possibly through inhibition by high levels of progesterone (see Chapter 1). Upon removal of uterine tissue from pregnant animals and stretching the tissue in vitro, endoperoxides and thromboxane A_2 may be synthesized resulting in the appearance of gap junctions. Consequently, at the end of term the synthesis of prostaglandins may be changed in favor of the endoperoxides and thromboxane A_2 which stimulate the formation of gap junctions. Therefore, the influence of the endoperoxide, thromboxane and

prostacyclin system in regulating gap junctions could be a subsequent mechanism in addition to the steroid hormones.

Indomethacin has also been reported to prevent estrogen stimulation of myometrial gap junctions in immature hypophysectomized rats in vivo (Burghardt et al., 1984a). In that study the animals were primed with indomethacin prior to concomitant treatment with estradiol benzoate together with indomethacin for five days. This protocol was not followed in the investigation reported here, which may explain the inhibition of gap junction development. I have carried out a preliminary study to examine the effects of indomethacin priming on estradiol stimulation of myometrial gap junctions in the immature rat. Although the results are preliminary and require further investigation, they show no inhibitory effect on estradiol stimulation of myometrial gap junctions. At present, interpretation of the results from the study of Burghardt et al., (1984a) is difficult but may reflect the method used in quantitating gap junctions (see Chapter 4) or the surgical treatment to the animals.

Recently, Garfield has shown that indomethacin enhanced the development of myometrial gap junctions in some human tissues incubated in vitro, similar to the in vivo results of this study, whereas in other human tissues the cyclo-oxygenase inhibitor prevented gap junction formation (private communication from Dr.R.E. Garfield). Indomethacin treatment in vitro stimulated gap junction development only in human tissues that did not develop the junctions spontaneously. On the other hand, in tissues that developed numerous gap junctions in vitro, indomethacin had an inhibitory effect. It is interesting to note

that the original observation that indomethacin prevented the appearance of gap junctions in vitro (Garfield et al., 1980b) occurred in tissues from which control pieces of the same tissues developed numerous gap junctions spontaneously. Taken together with the results of this study, it seems likely that more than one prostaglandin is involved in the regulation of gap junctions in vitro or in vivo and possibly that a balance between products of the cyclo-oxygenase and lipoxygenase pathways is required for controlling the presence of gap junctions in the myometrium.

If products of the lipoxygenase pathway stimulate gap junctions and if these substances are produced in increased amounts during estradiol treatment or parturition, then this may account for the observations of this study. Evidence is limited but one study has shown that term uterine and intrauterine tissues synthesize several lipoxygenase products (Mitchell et al., 1983). Furthermore, in other tissues it has been shown that lipoxygenase products inhibit prostacyclin biosynthesis (Moncada et al., 1976; Turk et al., 1980) and enhances the synthesis of thromboxane A_2 (Sirois et al., 1980; Omini et al., 1981). It is well known that estrogens stimulate prostaglandin synthesis by the uterus and the stimulatory effect of the steriod on the myometrium has been attributed to this phenomenon (Nathanielsz, 1978; Gawienowski and Chatterjee, 1980; Soto-Feine et al., 1981). Recently, it has also been shown that fairly high doses of estrogen selectively stimulates the lipoxygenase pathway in other tissues and that lipoxygenase products increase after inhibition of the cyclo-oxygenase pathway (Guivernau et al., 1980; 1982). Perhaps the

difference between the low and high doses of estradiol on gap junctions may be that the latter is stimulating this pathway. When an inhibitor of cyclo-oxygenase is administered to ovariectomized mature rats together with low doses of estrogen (Table 11), more lipoxygenase products would be produced and then myometrial gap junctions may form. The results of this study do not rule out the existence of the lipoxygenase pathway in the uterus of non-pregnant animals. It is tempting to speculate the existence of this system of regulation in the uterus and that lipoxygenase products may be involved in gap junction formation in the myometrium. If so, then these results may be of greater significance as lipoxygenase products are thought to be involved in increased reactivity of other smooth muscles such as those in airways (Anderson et al., 1982) and gap junctions may possibly be involved.

Plasma estradiol levels are lower in animals injected concomitantly with estradiol and indomethacin than those administered estradiol alone (Figure 25). It is possible that indomethacin treatment influences the metabolism of estradiol resulting in lower plasma levels of the hormone. This is not likely because in the animals treated with indomethacin plus estradiol there is a greater estrogenic response. The serum levels and uterine uptake and retention of ^{3}H-estradiol are also higher in ovariectomized rats pretreated with indomethacin (Figure 26). However, the plasma levels of estradiol from the two groups (Figures 25 and 26) cannot be compared as values represented in Figure 25 are from animals treated daily with high doses of estradiol. These results indicate that uterine tissues may contain

greater numbers of cytoplasmic estradiol receptors when exposed to indomethacin and this could explain the lower circulating hormone levels in animals treated with both compounds. Support for this hypothesis is provided by recent studies showing that prostaglandin synthetase inhibitors elevate the binding capacity of uterine cytoplasmic estrogen receptors (Sanfilippo et al., 1983) and that polyunsaturated fatty acids, such as prostaglandins, prevent binding of estradiol to its receptor in myometria of immature rats (Clerc-Hofman et al., 1983). This may mean that products of the cyclo-oxygenase pathway regulate gap junctions by controlling the steriod-receptor complex. These results are compatible with the hypothesis that prostaglandins down regulate the steroid-receptor mechanism. I know of no other evidence indicating that prostaglandins control the expression of the steroid hormone-receptor complex. This phenomenon may have significant implications in the initiation of parturition in species such as the guinea-pig and human where labor procedes in the absence of plasma and tissue progesterone withdrawal. If a prostanoid is regulating the progesterone receptors, it is possible that a functional progesterone withdrawal could occur independent of the levels of the hormone.

If the above mechanism is correct, then the changes in the uterine prostaglandin levels following injections with estradiol may explain the slow response to the development of gap junctions. Uterine prostaglandin levels increase following the first injection and then decline to lower than control levels with repeated injections of estradiol (Figure 27). These results are similar to that observed by

Castracane and Jordan (1975) and Barcikowski et al., (1974) in the rat and ewe, respectively. Together, these results suggest that uterine prostaglandin synthesis become refractory when continuously exposed to the hormone. Perhaps the subsequent suppression of prostaglandins by estradiol is required for initiation of event(s) necessary for gap junction development. However, the prostaglandin measured in this study may not be the one involved in regulation of gap junctions or the steroid receptors. Other endoperoxides and thromboxane may also be synthesized therefore, prostanoids may be a more accurate term and should be used when talking about the synthesis of prostaglandins. Estradiol-stimulated prostaglandin synthesis in the rat uterus appears to be independent of receptor mediated events and occurs prior to hormone-receptor stimulation of protein synthesis (Castracane and Jordan, 1976). It is likely that the initial increased prostaglandin production after estradiol treatment is suppressing the hormonal effect through a down regulation of the hormone receptor in the cytoplasm of myometrial cells. Similarly, indomethacin administered with estradiol results in significantly lower uterine prostaglandin levels after the first injection concomitant with the formation of gap junctions (Figures 23 and 27). Prostaglandins are not considered to be stored in tissues to any great extent and since their synthesis is depressed with indomethacin, hormone-receptor complexes may not be inhibited and allow estradiol to express its action on gap junction protein synthesis.

5.4 Summary:

The results of this study indicate that the steroid hormones

and prostaglandins regulate myometrial gap junction formation. The steroid hormones influence the presence of gap junctions by regulating the synthesis of the gap junction protein (see Chapter 4) together with a selective stimulation of a prostaglandin. It appears that the mechanism of action of estradiol and prostaglandins in regulating myometrial gap junction formation is complex and may involve the cyclo-oxygenase pathway, lipoxygenase pathway and/or an effect on the steroid receptors or stimulation of myometrial growth (see Chapter 6). Further investigations are necessary to clarify the mechanism for control of the presence of gap junctions in the myometrium.

CHAPTER 6

REGULATION OF UTERINE GROWTH AND PRESENCE OF GAP JUNCTIONS

6.1 Introduction

Gap junctions have been observed to be present in increased numbers and size between myometrial cells of the pregnant uterus of various species immediately prior to and during term or preterm labor and their development appears to be regulated by the steroid hormones and prostaglandins (see Chapters 4 and 5). Furthermore, the results presented in Chapter 4 indicate that the myometrium of intact pregnant animals develop a high frequency of gap junctions in response to estradiol treatment and the animals labor prematurely. These results provide support for the hypothesis that the steroid hormones regulate the presence of gap junctions in the myometrium.

It has also been reported that treatment of non-pregnant animals with high doses of estrogen for several days stimulates the presence of gap junctions in the myometrium (see Chapter 1). The results in Chapter 4 demonstrate the regulation of myometrial gap junctions in the immature and ovariectomized mature rat by the steroid hormones. Gap junctions were observed between the smooth muscle cells of the myometrium only after continuous treatment with large doses of estradiol or diethylstilbestrol. The appearance of gap junctions in the immature rat myometrium occurred after four consecutive days of treatment and appeared to be a dose related effect with 500 ug/day estradiol being optimal for maximum stimulation. Ovariectomized mature rats treated with the same high dose of estradiol also developed numerous myometrial gap junctions, however, the appearance of the junctions between myometrial cells occurred much earlier than observed

in the immature animal. The expression of gap junctions in the myometrium in response to estrogen treatment was suppressed when progesterone was administered with either estradiol or diethylstilbestrol. Similarly, myometrial tissues from immature rats incubated *in vitro* develop a greater membrane area of gap junctions when treated with estradiol whereas progesterone, when added with estradiol to the incubation media, prevented the increase in the frequency of gap junctions (Garfield *et al.*, 1980a). In addition, prostaglandins also appear to be involved in the steroid hormone regulation of myometrial gap junctions (see Chapter 5). Concomitant administration of indomethacin or sodium meclofenamate with estradiol to immature or ovariectomized mature rats enhanced the estradiol stimulation of gap junctions. Moreover, progesterone was still capable of suppressing the increase in frequency of gap junctions in response to estradiol and indomethacin. These results indicate that the presence of myometrial gap junctions is controlled by the steroid hormones and prostaglandins.

Estradiol stimulates the normal growth of the uterus (Clark *et al.*, 1978). Steroid hormones are thought to exert their biological action through specific receptors, which, when in the nucleus of the target cell alters the pattern of genetic transcription (Gorski and Gannon, 1976). The ultimate expression of estradiol is stimulation of RNA, protein and DNA synthesis resulting in cell growth and proliferation (Anderson *et al.*, 1975; Clark *et al.*, 1978; Stormshak *et al.*, 1978). Moreover, progesterone has been shown to inhibit estradiol induced uterine responses (Bhakoo and Katzenellenbogen, 1977;

Markaverich et al., 1981; Sumida et al., 1981).

In addition, estradiol stimulation has been reported to increase uterine cAMP levels (Szego and Davis, 1967) and cAMP is believed to be involved in the control of cellular proliferation (Millis et al., 1974). Whether cAMP is involved in the stimulation of uterine growth by estradiol, however, remains to be resolved. Estradiol also stimulates the production of prostaglandins by the uterus (Castracane and Jordan, 1976). It is well known that prostaglandins participate in various reproductive processes and that inhibitors of cyclo-oxygenase prevent their action through inhibition of prostaglandin synthesis (Csapo et al., 1973; Jordan and Castracane, 1976; Fuchs, 1978; Chan et al., 1983). Prostaglandins have also been shown to promote or inhibit cellular proliferation in a variety of tissues (Egg et al., 1978; Jubiz et al., 1979; Goodwin, 1981; Wiley et al., 1983). Therefore, if prostaglandins or their inhibitors influence uterine growth, these compounds may have profound effects on uterine function.

Intercellular communication is believed to be involved in the normal controlled growth of a tissue. Pathways between cells via gap junctions are thought to be required for direct cell-to-cell communication (Peracchia, 1980; Hooper and Subak-Sharpe, 1981; Loewenstein, 1981). Loewenstein (1979) has proposed that gap junctions play a critical role in the control of cell growth by allowing nutrients, metabolites and specific growth regulating molecules to move directly from cell-to-cell. This implies therefore, that alterations in the structure and/or function of gap junctions between cells may be

responsible for abnormal growth of tissues (Loewenstein, 1981). In a number of reviews it has been reported that cell types of organized adult tissues capable of dividing, but not in the process of division, are communication-competent as revealed by the cell-to-cell transfer of ions, fluorescent labeled molecules and the presence of gap junctions between the cells (Azarnia and Larsen, 1977; Loewenstein, 1979). Moreover, cells of many cancerous tissues are not electrically coupled and gap junctions are absent from between the cells, suggesting a predominance of communication-incompetent cells in those malignant tumors (Loewenstein, 1979). Gap junctions have been observed to be present between the cells of some malignant tumors, however, Loewenstein has suggested that they may exist in a non-functional state thereby not coupling the cells together.

Cell culture techniques have also related gap junctional communication to the control of growth. When cells in confluent cultures, which are coupled by gap junctions, are experimentally wounded the division of cells at the injured site is accompanied by a decrease in communication with neighbouring uninjured cells (Loewenstein, 1979). Similarly, results from experiments using hybrids of communicating cells and cancerous non-communicating cells have shown that a junctional defect was related to abnormal proliferation of cells (Azarnia et al., 1974). In addition, cancer cells in culture are communication-incompetent and are under no growth control (Loewenstein, 1979).

Cell coupling via gap junctions is present during the phase of rapid cell division in adult regenerating tissue and embryonic tissue

where cellular growth and differentiation is extensive and finely regulated. However, whether the presence of gap junctions in these tissues are necessary for cellular proliferation or represent a structural feature required for cellular differentiation remains uncertain. Recently, the use of antibodies to the gap junction protein have supported the latter role for gap junctions (Warner et al., 1984). Furthermore, in intestinal smooth muscle where growth in response to experimental stenosis is due to hypertrophy and hyperplasia, there is a greater number and size of gap junctions between the cells (Gabella, 1975; 1979a; 1979b). Although Gabella has indicated the presence of gap junctions during hypertrophy of the cells he does not distinguish which growth process was responsible for the increase in the gap junction frequency. Brent and Stephens (1975) have reported that hypertrophy of the smooth muscle in the urinary bladder of adult rabbits precedes hyperplasia. The occurrence of hyperplasia has also been observed in a variety of differentiated smooth muscle cells (Cobb and Bennett, 1970; Chamely and Campbell, 1974; Kamio et al., 1977), in normal mature smooth muscle and during regeneration , repair and wound healing in adult smooth muscle (McGeachie, 1971). Unfortunately, however, these studies have not reported on the incidence of gap junctions between smooth muscle cells. Therefore, it appears that in some tissues including smooth muscle gap junctions are present during growth of the cells and that in other tissues gap junctions are absent.

The pregnant uterus grows dramatically throughout gestation to accommodate the growing fetus and the growth of the uterus ceases prior to parturition (Carsten, 1968; Afting and Elce, 1978) when gap

junctions are present in the myometrium (Garfield et al., 1978). Uterine growth, of which a major component is due to the myometrium, is controlled by the steroid hormones and prostaglandins appear to be involved in the process (Carsten, 1968; Puri et al., 1984). The steroid hormones and prostaglandins also regulate the presence of myometrial gap junctions (see Chapters 4 and 5). It is possible therefore, that some relationship exists between growth of the uterus and the presence of gap junctions between the cells. Myometrial growth (either hypertrophy or hyperplasia) may occur in the absence of gap junctions. Alternatively, myometrial growth in response to the hormones may be required for the presence of the junctions at the end of pregnancy.

In this study the relationship between the presence of gap junctions and growth of the myometrium has been evaluated in non-pregnant rats after various hormonal treatments and in pregnant animals on various days throughout gestation, during parturition and one day post-partum. The results show that myometrial growth occurs primarily in the absence of gap junctions. Regulation of myometrial growth and the presence of gap junctions in non-pregnant and pregnant animals appears to be dependent upon the hormonal environment. The results also suggest a complex interaction between estradiol, a product of the cyclo-oxygenase or lipoxygenase pathway, gap junctions and myometrial growth. Growth may occur in part because of the lack of gap junctions and the presence of gap junctions may be partially dependent upon myometrial growth.

6.2 Results:

6.2.1 Immature rats:

6.2.1.1 Effects of estradiol:

Administration of estradiol to immature animals results in a dramatic growth of the uterus. Figure 28 shows photomicrographs of intact uteri from immature rats treated with sesame oil (Figure 28A) and estradiol (Figure 28B). Growth in response to estradiol treatment resulted in an increase in the length and diameter of the bicornate uterus compared with the organs from control animals treated with oil alone.

To determine the time course of uterine growth with two different doses of estradiol, daily determinations of uterine weight were carried out over an 8 day period. Immature rats were injected subcutaneously with sesame oil (0.1 ml) with and without estradiol (50 and 500 ug/day) daily for 1-8 days. Figure 29 shows that 50 and 500 ug/day doses of estradiol induced similar increases in uterine wet weight. The wet weight of the tissues from animals treated with either 50 ug or 500 ug/day estradiol increased rapidly and significantly ($P<0.001$) after the first day of treatment compared with the uterine wet weights from control rats injected with sesame oil. The uterine wet weights in animals treated with either dose of estradiol increased progressively until the eighth and last day of treatment. No significant differences were observed between uterine wet weights in animals treated with 50 and 500 ug/day estradiol on any day of the eight day treatment period. There was a small, but significant increase

Figure 28. Photos of intact uteri from immature rats treated with sesame oil (A) and estradiol (B) for 6 days illustrating the effect of the steroid hormone on size of the bicornate uterus.

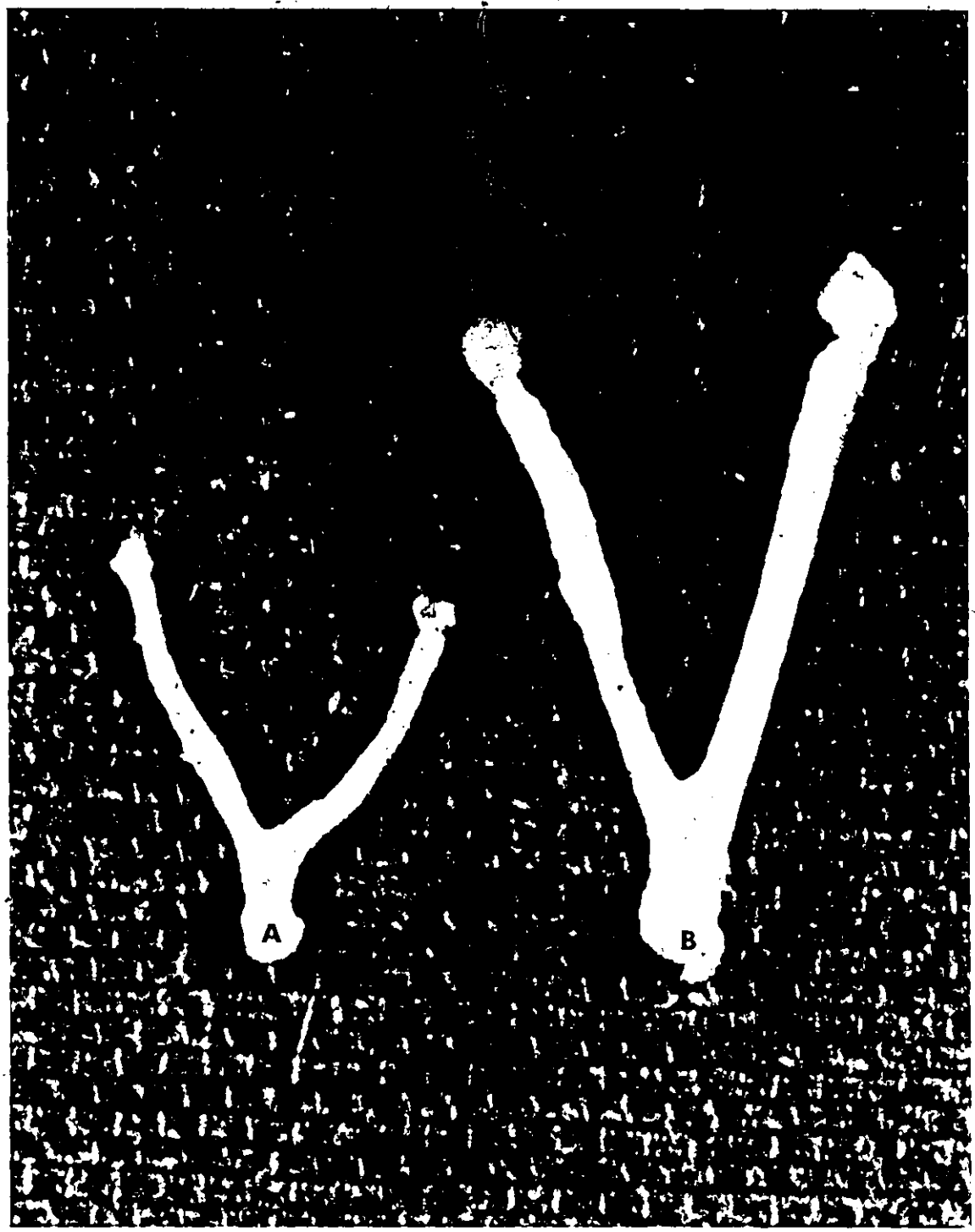
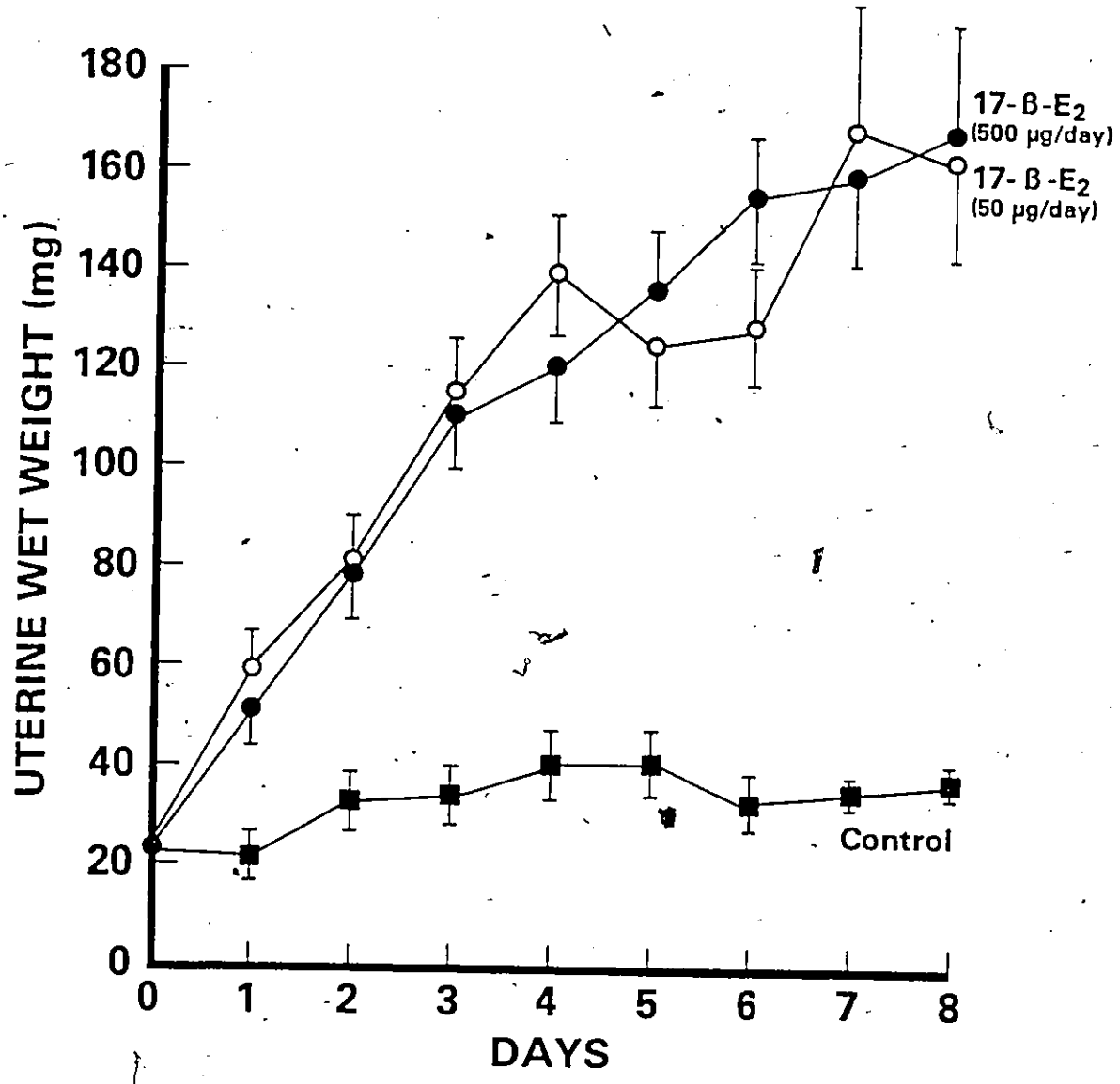

A
B

Figure 29. Effects of estradiol (50 and 500 ug/day for 1-8 days) on the wet weight of uteri from immature rats. There were no significant differences in the uterine weights between the two doses of estradiol over the 8 day period. Data are presented as mean values ± S.E. of 6 uteri on each day.


180
160
140
120
100
80
60
40
20
0
UTERINE WET WEIGHT (mg)
0
1
2
3
4
5
6
7
8
DAYS
17-β-E2
(500 µg/day)
17-β-E2
(50 µg/day)
Control

($P<0.01$), in the uterine wet weight of control animals from zero time over the same eight day period.

The estradiol induced uterine wet weight gain over the 1-8 days period normalized to animal body weight is illustrated in Figure 30. Normalized uterine wet weight increased from 0.325 ± 0.012 mg/gm body weight at zero time in control animals to 1.020 ± 0.035 and 1.110 ± 0.060 after the first day of treatment with 50 and 500 ug/day estradiol respectively, a significant increase ($P<0.001$) compared with the control normalized uterine weight on the same day. A near maximum response in uterine weight gain was observed after three days of treatment with both doses of estradiol (0.650 ± 0.038 mg/gm body weight in the control group to 1.930 ± 0.073 and 2.120 ± 0.054 in the estradiol treated animals, $P<0.001$) followed by non-significant ($P>0.05$) increases with repeated injections over the remainder of the eight day treatment period. No daily differences were observed between animals treated with 50 and 500 ug/day estradiol. Tissues from control animals showed a significant increase ($P<0.01$) in normalized uterine wet weight after the second day of treatment, however, mean normalized uterine weights of control animals were significantly lower ($P<0.001$) than corresponding weights from estradiol treated animals at all times over the 1-8 days of treatment.

Figure 31 shows uterine dry weights determined from immature rats treated for 1-6 days with 50 and 500 ug/day estradiol. Dry weights were similarly increased by both doses of estradiol over the treatment period. Treatment with 50 ug/day estradiol resulted in a significantly larger ($P<0.01$) mean dry uterine weight after two days of

Figure 30. Effects of estradiol on uterine wet weight normalized to immature rat body weight over 1-8 days. Data are presented as mean values ± S.E. of 6 uteri on each day.

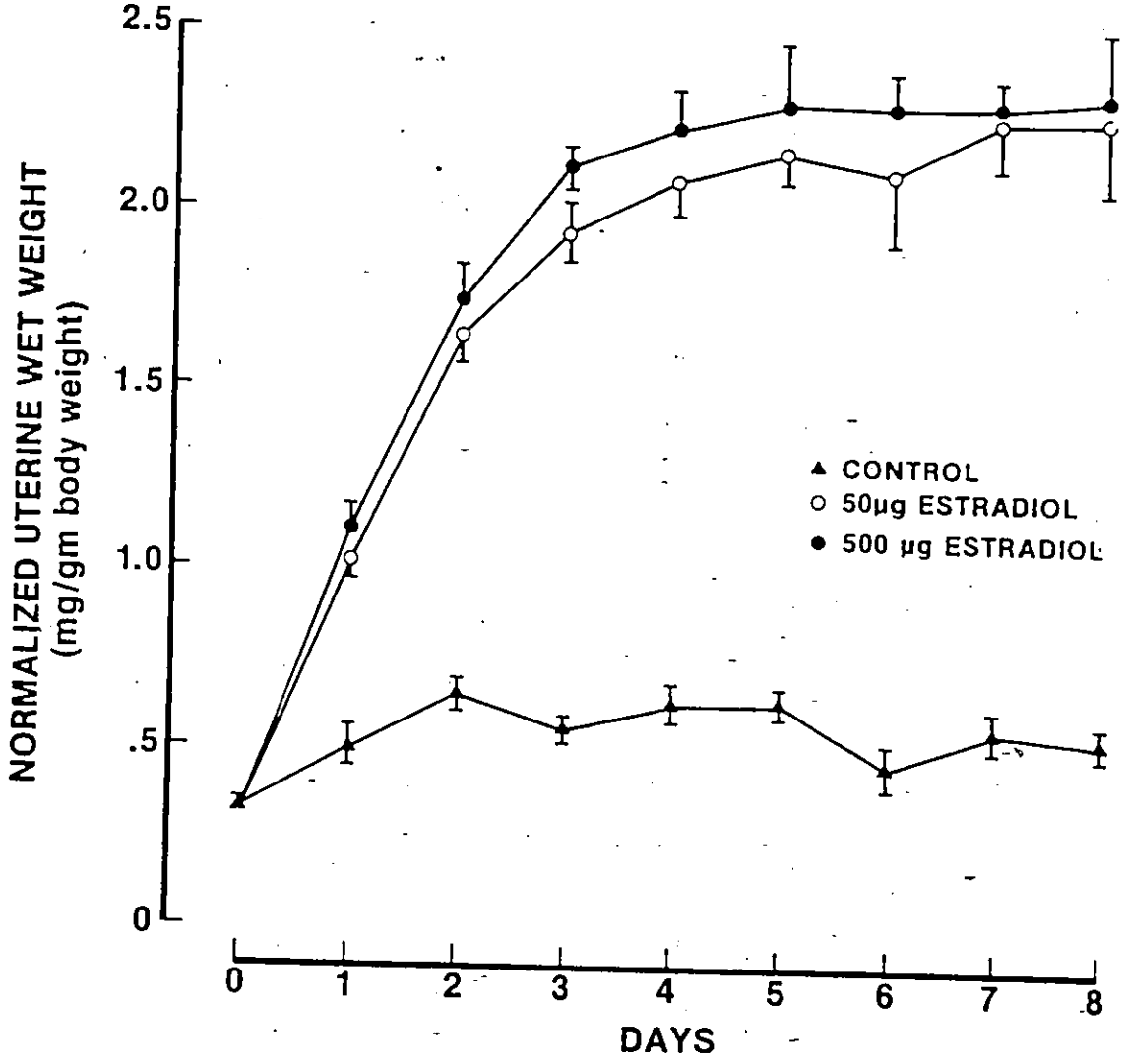
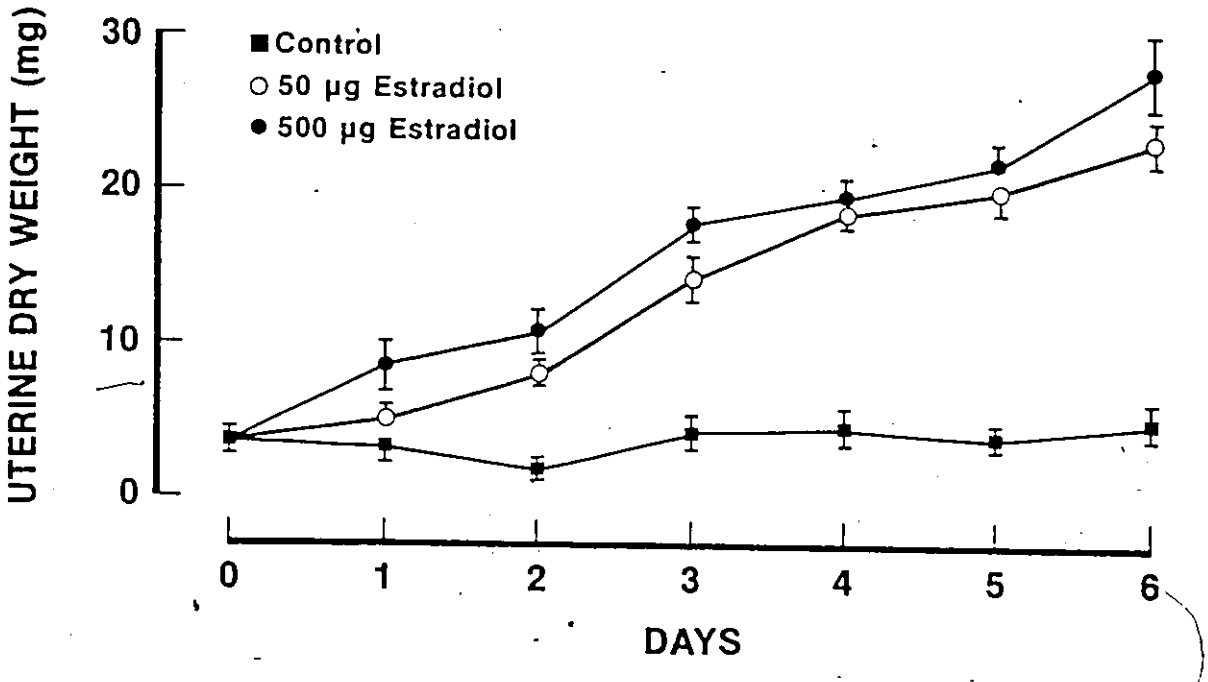

NORMALIZED UTERINE WET WEIGHT
(mg/gm body weight)
2.5
2.0
1.5
1.0
.5
0
0
1
2
3
4
5
6
7
8
DAYS
▲ CONTROL
○ 50µg ESTRADIOL
● 500 µg ESTRADIOL

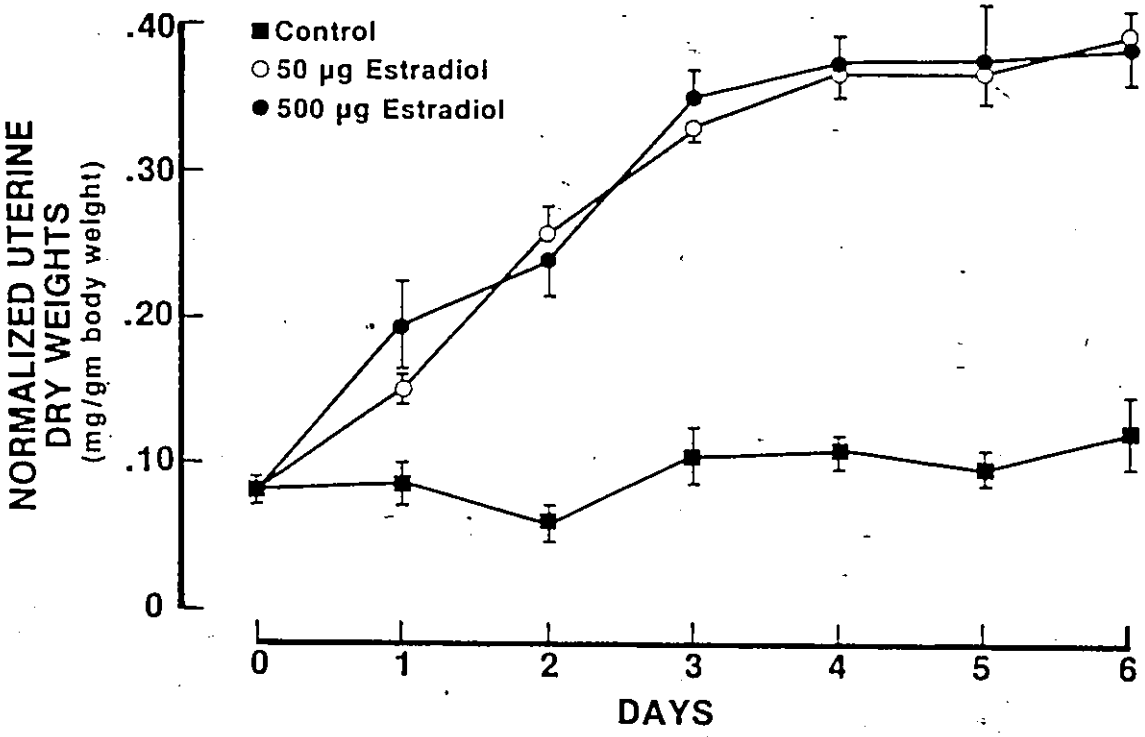
Figure 31. Effects of estradiol treatment on uterine dry weight determined daily over 1-6 days. Data are presented as mean values ± S.E. of 6 uteri on each day.


UTERINE DRY WEIGHT (mg)
30
20
10
0
■ Control
○ 50 µg Estradiol
● 500 µg Estradiol
0
1
2
3
4
5
6
DAYS

treatment compared with tissues from control animals. Thereafter, the uterine dry weight progressively increased to a maximum value after six days of treatment (24.3 $\pm$ 1.60 mg compared with 5.60 $\pm$ 1.40 mg dry weight of control tissue after six days, $P<0.001$). In animals treated with 500 ug/day estradiol, the uterine dry weight was significantly elevated ($P<0.01$) after the first day of treatment followed by a further increase with repeated injections to a significantly larger ($P<0.001$) mean dry weight after six days compared with that measured from the control animals. No significant differences between the dry weights from animals treated with 50 ug or 500 ug/day estradiol over the 1-6 day period were observed. There was a slight, but non-significant increase in uterine dry weights of control animals from zero time over the same six day period.

Uterine dry weights normalized to body weight (Figure 32) showed similar increases in response to 50 and 500 ug/day estradiol over 1-6 days. Normalized dry weights were significantly increased ($P<0.01$) after the first day of treatment with both doses of estradiol compared with the normalized dry weight of uterine tissue from control animals. The normalized dry weights continued to increase with repeated estradiol (50 and 500 ug/day) treatment reaching near maximum values after three days of treatment. This increase in normalized dry weight was significant ($P<0.001$) compared with the three day control normalized dry weight. With further injections of 50 ug/day estradiol, a non-significant increase was observed on day four and five followed by a significant increase ($P<0.005$ compared with the mean normalized dry weight after three days treatment with 50 ug/day estradiol) to a

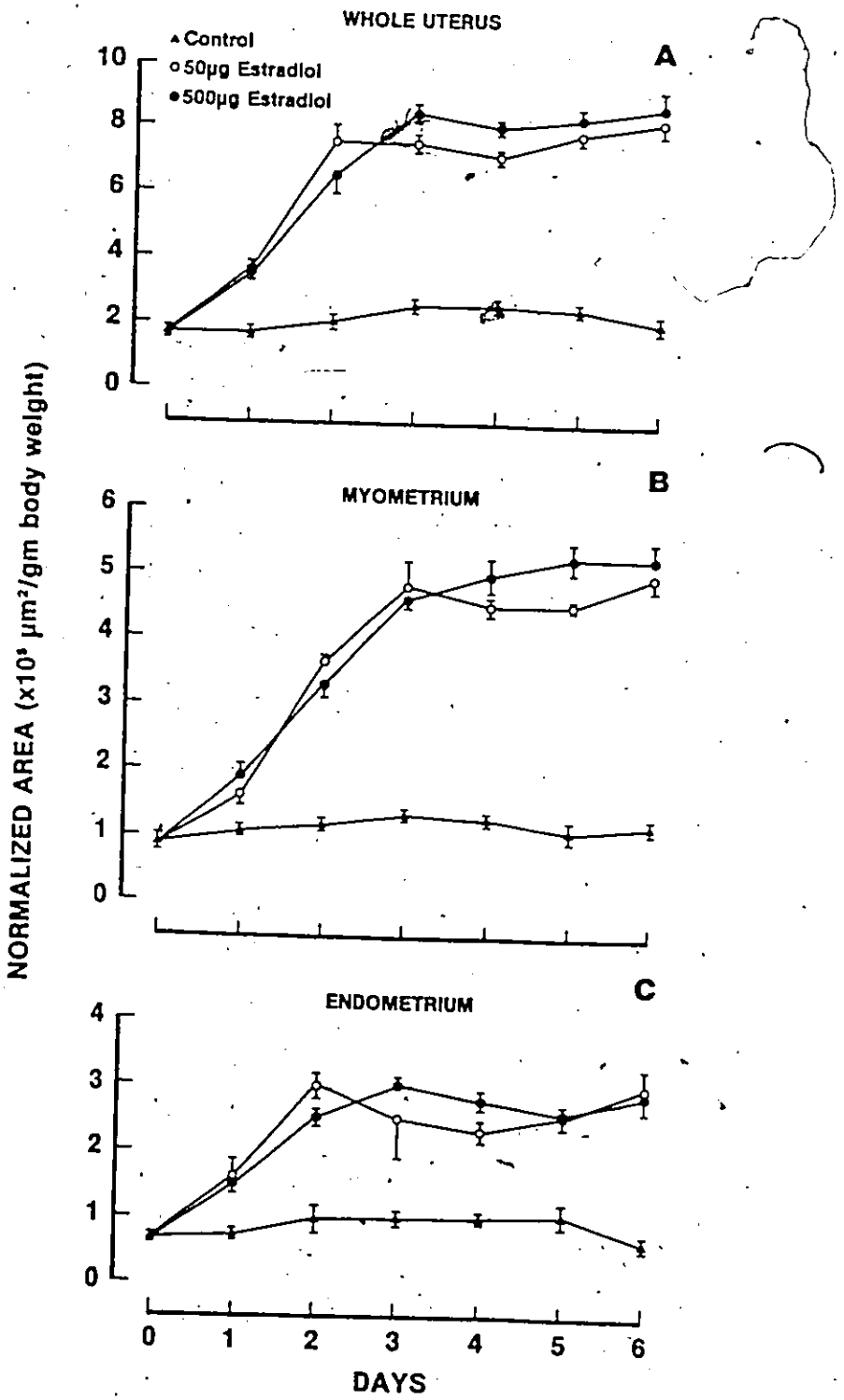
Figure 32. Uterine dry weight increases normalized to immature rat body weight determined daily over 1-6 days after treatment with estradiol. Data are presented as mean values ± S.E. of 6 uteri on each day.


NORMALIZED UTERINE DRY WEIGHTS
(mg/gm body weight)
.40
.30
.20
.10
0
Control
50 µg Estradiol
500 µg Estradiol
0
1
2
3
4
5
6
DAYS

maximum normalized uterine dry weight after six days of treatment . The normalized uterine dry weight after six days treatment was significantly greater ($P<0.001$) than the mean value from control tissues on the same day. Continued treatment with 500 ug/day estradiol resulted in no further significant increases in normalized dry weight beyond that observed after three days of treatment. No significant daily differences were observed between the effects of either dose of estradiol on the normalized uterine dry weight. Normalized uterine dry weights from control animals increased slightly, but not significantly over the same treatment period.

The effects of daily injections of estradiol on uterine cross-sectional area measured from light micrographs is shown in Figure 33. Analysis of cross-sectional area of uteri from immature control and rats treated with estradiol (50 and 500 ug/day) for 1-6 days are shown. Treatment with 50 and 500 ug/day estradiol resulted in similar and significant increases ($P<0.005$) in total normalized uterine cross-sectional area (um^2/gm body weight) after the first day of treatment compared with the cross-sectional area of control uterine tissue (Figure 33A). Thereafter, the normalized uterine area increased to near maximum values after two and three days of treatment with 50 and 500 ug/day estradiol respectively. No further significant increase in the cross-sectional area occurred beyond this time. Treatment with 50 and 500 ug estradiol/day increased the normalized cross-sectional area of the immature uterus from $1.70 \pm 0.01 \times 10^5$ um^2 at zero time in control animals to 8.20 ± 0.32 and $8.80 \pm 0.55 \times 10^5$ um^2 respectively after the sixth day of treatment, a significant increase ($P<0.001$)

Figure 33. Effects of estradiol treatment over 1-6 days on normalized cross-sectional area of (A) the whole uterus, (B) myometrium and (C) endometrium. Cross-sectional area measurements were made from light micrographs of transverse sections through the uterus (see Figure 34). Data are presented as mean values ± S.E. of 3-4 transverse sections on each day.


WHOLE UTERUS
▲Control
○50µg Estradiol
●500µg Estradiol
A
B
MYOMETRIUM
C
ENDOMETRIUM
NORMALIZED AREA (x10³ µm²/gm body weight)
DAYS
0
1
2
3
4
5
6

compared to control values on the same day. The results show no significant variations between the two doses of estradiol on the total normalized uterine cross-sectional area on any day over the six day period. A small and gradual increase in the normalized cross-sectional area of uterine tissue from control animals treated with sesame oil was observed from zero time over the first three days of the treatment period. However, the mean values from control animals were significantly lower ($P<0.001$) compared to those from 50 and 500 ug/day estradiol treated animals throughout the six day period.

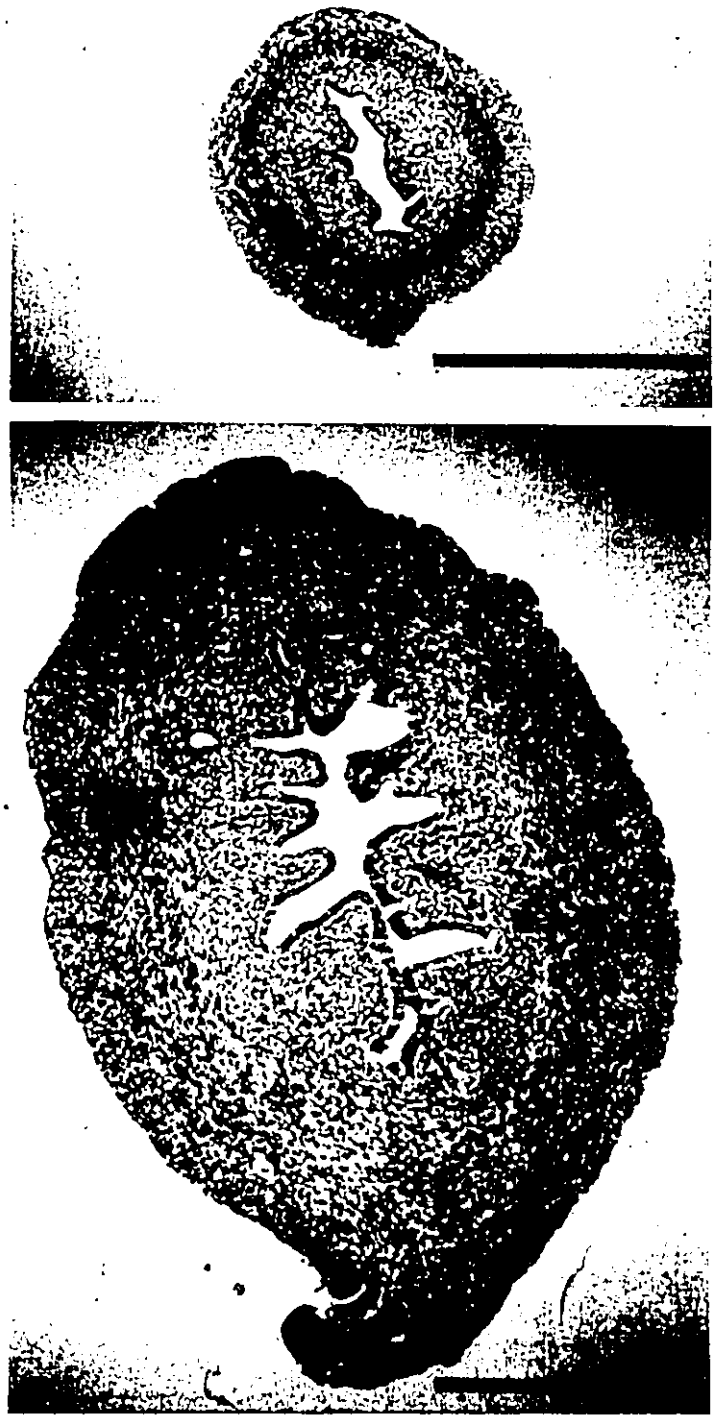
The normalized cross-sectional area of the myometrium determined daily for 1-6 days is shown in Figure 33B. Treatment with both 50 and 500 ug/day doses of estradiol resulted in a similar increase in the normalized area over the six day period. A significant increase ($P<0.05$) in the normalized myometrial cross-sectional area occurred after the first day with both doses of estradiol compared to the control tissues, followed by a continuous increase with repeated injections to a near maximum normalized area after three days of treatment ($1.40 \pm 0.01 \times 10^5$ um^2 in the control group to 4.80 ± 0.42 and $4.60 \pm 0.06 \times 10^5$ um^2 in the 50 and 500 ug/day estradiol treated groups respectively, $P<0.001$). Thereafter, the normalized cross-sectional area increased, but not significantly, to maximum values on day six. A significant difference ($P<0.05$) between the effects of 50 and 500 ug/day estradiol on the normalized cross-sectional area was observed after five days of treatment. A slight and non-significant increase in normalized myometrial cross-sectional area occurred in control uterine tissue from zero time over the first three days of the

treatment period.

The effects of daily injections of estradiol on the normalized cross-sectional area of the endometrium is also shown in Figure 33. Treatment with 50 and 500 ug/day estradiol resulted in similar increases in the cross-sectional area to significantly higher values ($P<0.01$) after the first day of treatment compared to the control normalized endometrial cross-sectional area on the same day (Figure 33C). Thereafter, there was a progressive increase in the normalized cross-sectional area of the endometrium to maximum values after two and three days of treatment with 50 and 500 ug/day estradiol, respectively ($P<0.01$ compared to the controls on the same day). There were no significant differences between the effects of either dose of estradiol on the normalized cross- sectional area on any day over the six days of treatment. Normalized endometrial cross-sectional area in control uterine tissue also showed a slight increase from zero time over the 1-6 day period, but on all days was significantly lower ($P<0.01$) than the corresponding estradiol treatments. Light micrographs of uterine cross-sections from immature rats treated with sesame oil with and without estradiol are shown in Figure 34.

The total increase in normalized uterine cross-sectional area (Figure 33A) over 1-6 days of estradiol (50 and 500 ug/day) treatment was due to increases in both the myometrial and endometrial area's because significant increases occurred in both tissue layers during the treatment period compared to tissue from control animals (Figure 33B and 33C respectively). It should be noted, however, that in zero time control animals the myometrial area was approximately 57% and the

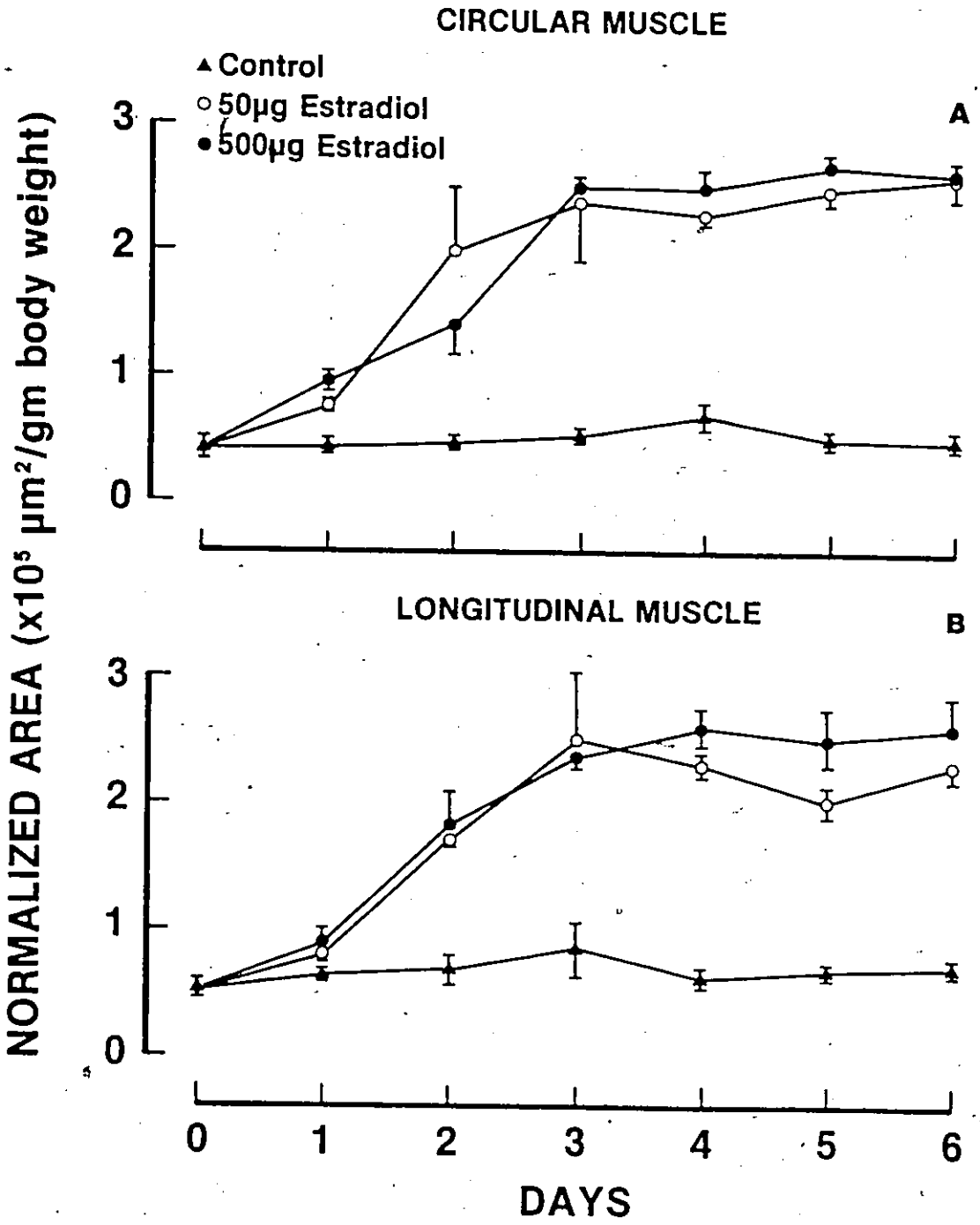
Figure 34. Light micrographs of transverse sections of uteri from control animals (A) and those treated with estradiol for 6 days (B). Histology of the uterus is illustrated in panel B showing the endometrium (E) and myometrium composed of circular (C) and longitudinal (L) muscle layers. Marker is 3 mm.



endometrial area approximately 43% of the total uterine cross-sectional area. Furthermore, after six days of treatment with either dose of estradiol, the myometrial and endometrial area's accounted for approximately 64% and 36% of the total uterine area respectively. Therefore, the myometrial tissue layer accounted for approximately 60% of the total uterine area before and after treatment at various times.

Figure 35 shows the effects of daily injections of estradiol (50 and 500 ug/day) for 1-6 days on the normalized cross-sectional area of the circular and longitudinal muscle layers of the immature rat myometrium. Following treatment with 50 and 500 ug/day estradiol there was a significant increase ($P<0.05$) in the normalized cross-sectional area of the circular muscle after the first day of treatment compared to the control measurements (Figure 35A). Thereafter, the circular muscle normalized cross-sectional area increased with repeated injections of both doses of estradiol until a near maximum value was observed after three days of treatment ($P<0.001$ compared to the three day control area measurement). Maximum normalized area of the circular muscle induced by 50 and 500 ug/day doses of estradiol occurred after the fifth and sixth days of treatment, but they were not significantly larger than the measurements after three days. The normalized cross-sectional areas measured on days five and six of treatment were significantly greater ($P<0.001$) than the corresponding control values. No significant differences between the effects of either dose of estradiol on the normalized cross-sectional area of the circular muscle was observed over the 1-6 day period. Uterine tissues from control animals showed no significant increase in the circular muscle

Figure 35. Effects of daily treatments of estradiol over 1-6 days on normalized cross-sectional areas of the circular (A) and longitudinal (B) muscle layers of the immature rat myometrium. Data are presented as mean values ± S.E. of 3-4 transverse sections on each day.


CIRCULAR MUSCLE
▲Control
○50µg Estradiol
●500µg Estradiol
A
B
LONGITUDINAL MUSCLE
NORMALIZED AREA (x10³ µm²/gm body weight)
0
1
2
3
0
1
2
3
4
5
6
DAYS

cross-sectional area over the course of the experiment.

Similarly, 50 and 500 ug/day estradiol for 1-6 days increased the normalized cross-sectional area of the longitudinal muscle layer (Figure 35B). Treatment with both doses of estradiol increased the normalized area of the longitudinal muscle area to a significantly higher value ($P<0.01$) after two days of treatment compared to the controls. Following further hormone treatment, the normalized cross-sectional area increased to maximum and near maximum values after three days of treatment with 50 and 500 ug/day of estradiol, respectively (2.40 ± 0.57 and $2.50 \pm 0.01 \times 10^5$ um^2). No further significant changes occurred in the normalized cross-sectional area of the longitudinal muscle with continued hormone treatment. There were no daily significant differences between the effects of either dose of estradiol over the six day period. Administration of sesame oil to the control animals had no significant influence on daily normalized cross-sectional areas compared to that at zero time. At zero time in control tissue, the circular and longitudinal muscle area were each approximately 50% of the total myometrial area. Similarly, after six days of treatment with 50 and 500 ug/day doses of estradiol, the area's of each muscle layer was approximately 50% of the myometrial area after the same treatment period. However, the circular and longitudinal muscle increased by approximately 550% and 515% respectively, after six days of estradiol treatment.

To determine whether the uterine weight increase induced by estradiol resulted from hypertrophy, hyperplasia, or a combination of both tissue growth processes, uterine DNA synthesis (measured by the

incorporation of ^{3}H-thymidine) and total DNA content were measured in immature rats treated with estradiol (50 and 500 ug/day) for 1-6 days. Figure 36 illustrates the time courses of uterine incorporation of ^{3}H-thymidine in immature rats treated with both doses of estradiol. Estradiol at both doses showed almost identical patterns; the time of maximal ^{3}H-thymidine incorporation measured in vitro and expressed as a percentage of the control values occurred twenty-four hours after the first injection. Twenty-four hours after the second day of treatment with either 50 or 500 ug/day doses of estradiol, the incorporation of labeled thymidine into DNA was less than that which occurred after the first injection. The in vitro incorporation of thymidine into DNA after the second injection and over the remaining four days of treatment was below that observed for tissues from control animals (less than 40% of the control value after six days of treatment). However, after three days of treatment with estradiol at both doses the incorporation of ^{3}H-thymidine into DNA increased to a second smaller peak of approximately 70% of the control value.

The total content of uterine DNA in response to estradiol treatment over 1-6 days is shown in Figure 37. Total DNA content (expressed as a percentage of the control values) in uterine tissue from animals treated with 50 and 500 ug/day estradiol was elevated twenty-four hours after the first injection and continued to increase with repeated injections of estradiol to maximum levels after four days of treatment. Uterine DNA content thereafter, decreased slightly over the last two days of the six day period.

Thymidine labeling indices seen in estradiol stimulated

Figure 36. Time course of tritiated thymidine incorporation into DNA of immature rat uterine tissue *in vitro* after *in vivo* treatment with estradiol for 1-6 days. Data from treated animals are expressed as a percentage of the control values.

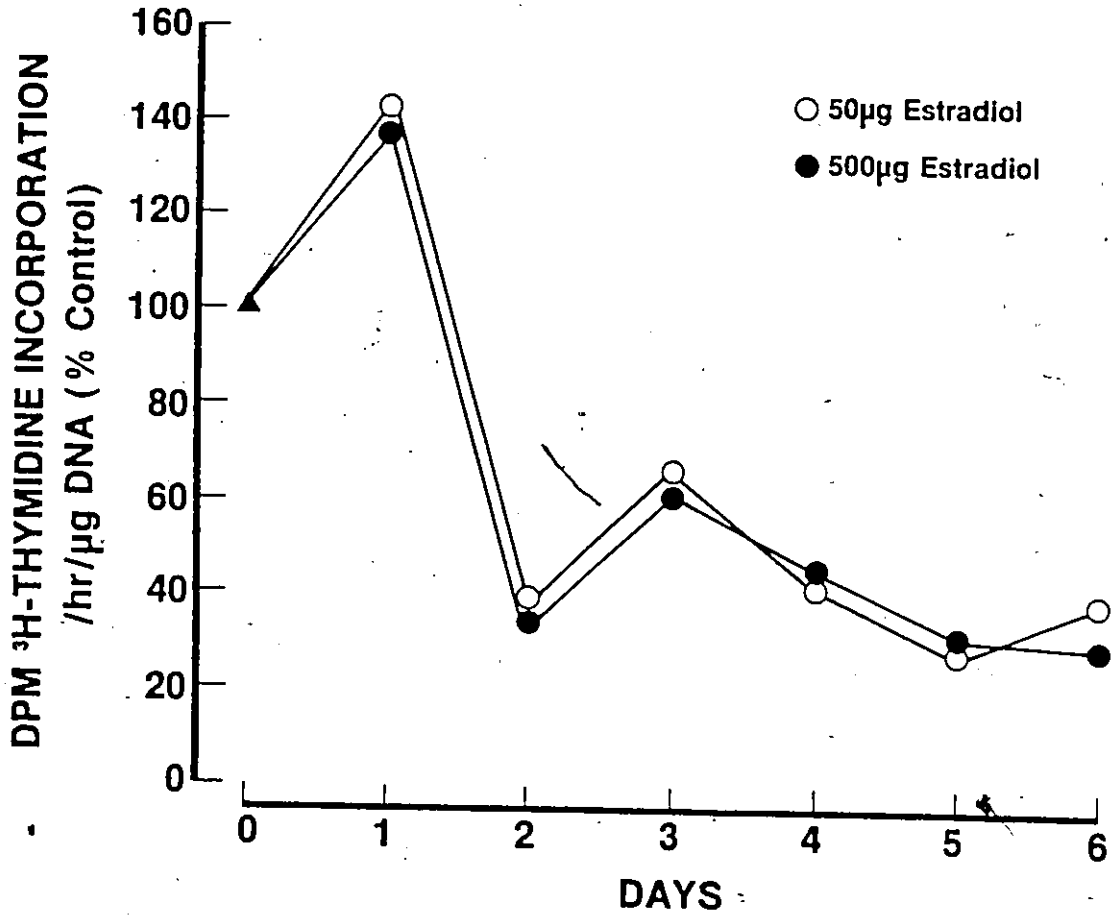
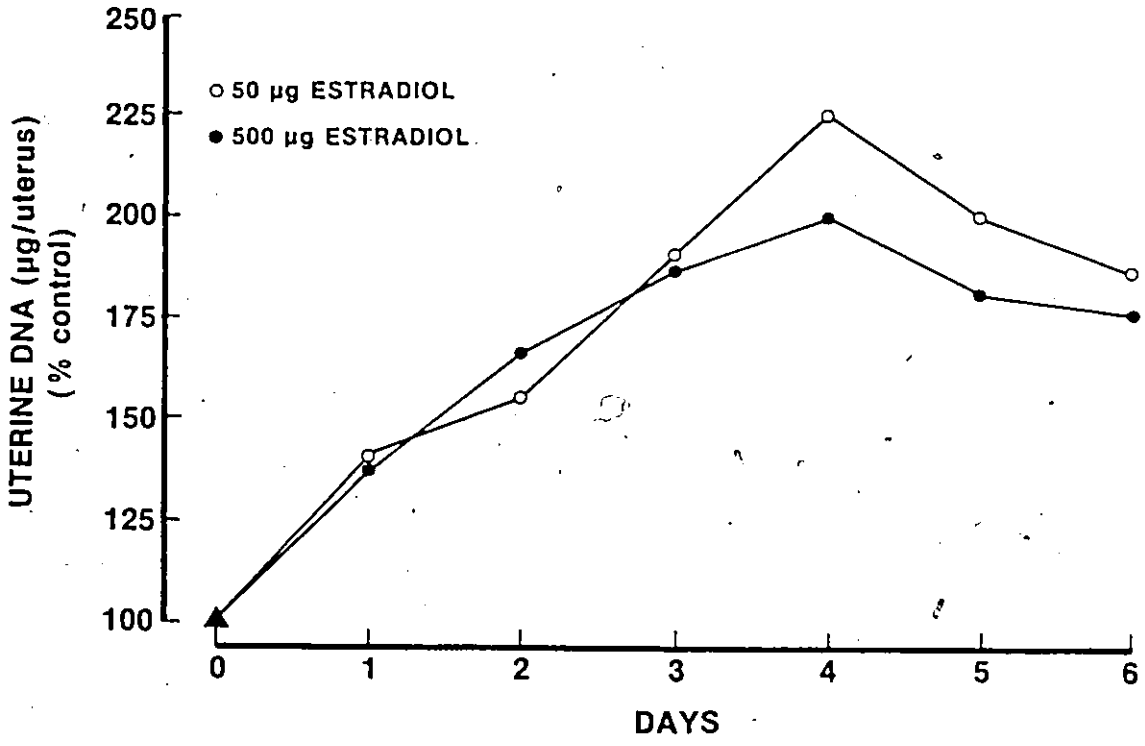

DPM ³H-THYMIDINE INCORPORATION
/hr/µg DNA (% Control)
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
DAYS
○ 50µg Estradiol
● 500µg Estradiol

Figure 37. Effects of daily estradiol treatment over 1-6 days on total immature rat uterine DNA content. Data are expressed as a percentage of the control values.


250
225
200
175
150
125
100
UTERINE DNA (µg/uterus)
(% control)
○ 50 µg ESTRADIOL
● 500 µg ESTRADIOL
0
1
2
3
4
5
6
DAYS

immature rat uterine tissue expressed as a percentage of the control values are shown in Figure 38. In the immature rat uterus estradiol at 50 and 500 ug/day similarly stimulated a maximum increase in the labeling index in the myometrium (Figure 38A) and endometrium (Figure 38B) twenty-four hours after treatment. The myometrial labeling index showed a decrease after the second day of estradiol treatment to values lower than in control tissues. Thereafter, there was a further gradual and progressive decline in the labeling index of the myometrium with repeated hormone treatments to approximately 25% of the control value at the end of the six day period. Similarly, the labeling index of the endometrium decreased to below control levels after two days of estradiol treatment (50 and 500 ug/day). The endometrial labeling index, however, rose slightly to a second smaller peak after three days, then fell progressively to approximately 25% of the control value after the sixth day of treatment.

The preceding data illustrated uterine weight increases in response to chronic administration of estradiol. To determine if repeated injections of estradiol were necessary to initiate increases in uterine weight, immature rats were injected with a single injection of estradiol (50 and 500 ug) at zero time (day 0) and daily determinations of uterine wet weight were carried out for 1-6 days. Figure 39 shows the wet weight changes over a six day period after a single injection of both doses of estradiol. Uterine wet weights were increased significantly ($P<0.01$) one day after treatment with either dose of estradiol compared to the control value. In animals treated with 50 and 500 ug estradiol the uterine wet weight increased

Figure 38. Effects of estradiol on nucleus labeling index of the myometrium (A) and endometrium (B). Labeling indices were determined by light microscopy autoradiography after incorporation of labeled thymidine into uterine DNA of immature rats treated with the steroid hormone. Data for the treated animals are expressed as a percentage of control values.

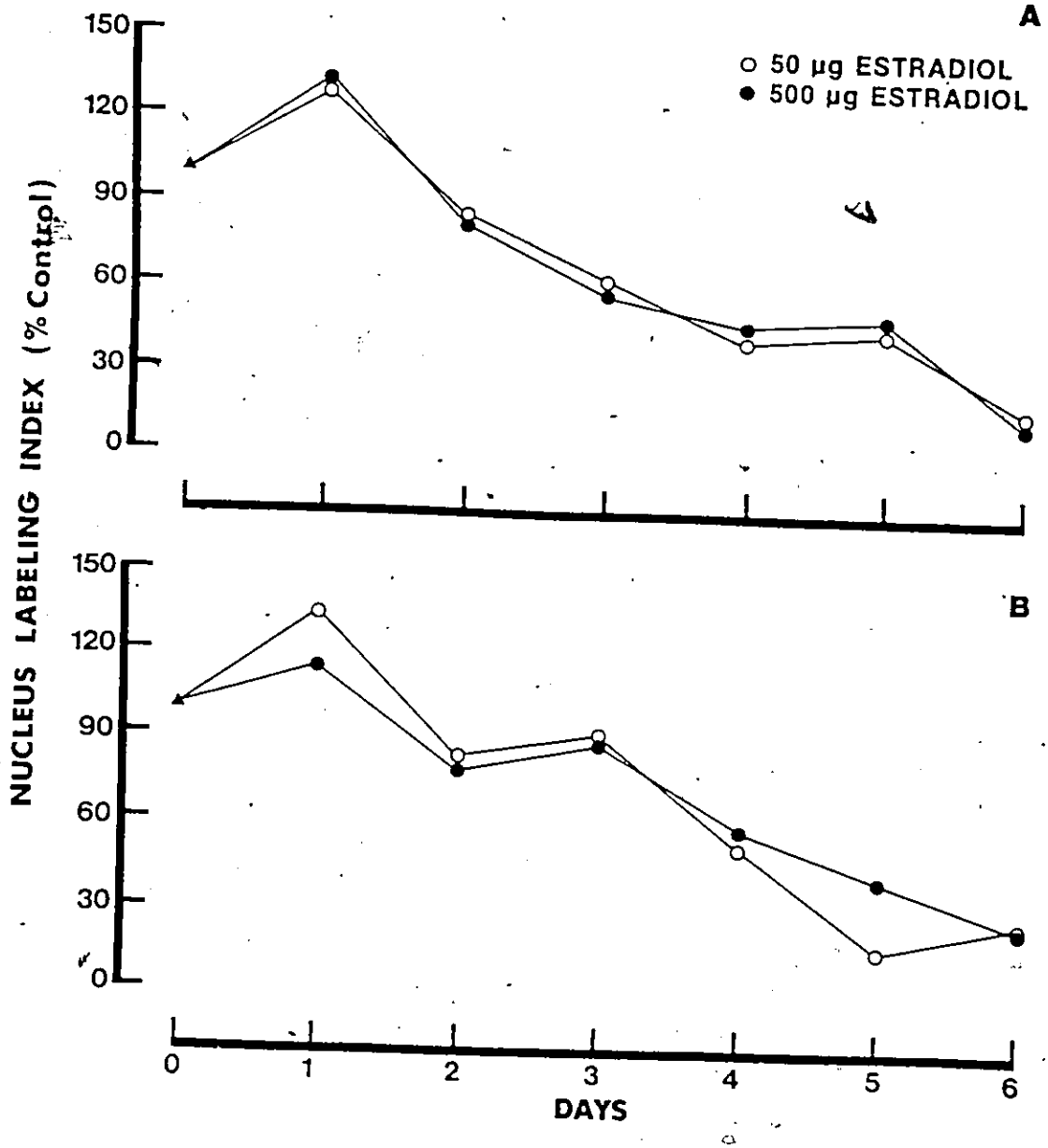
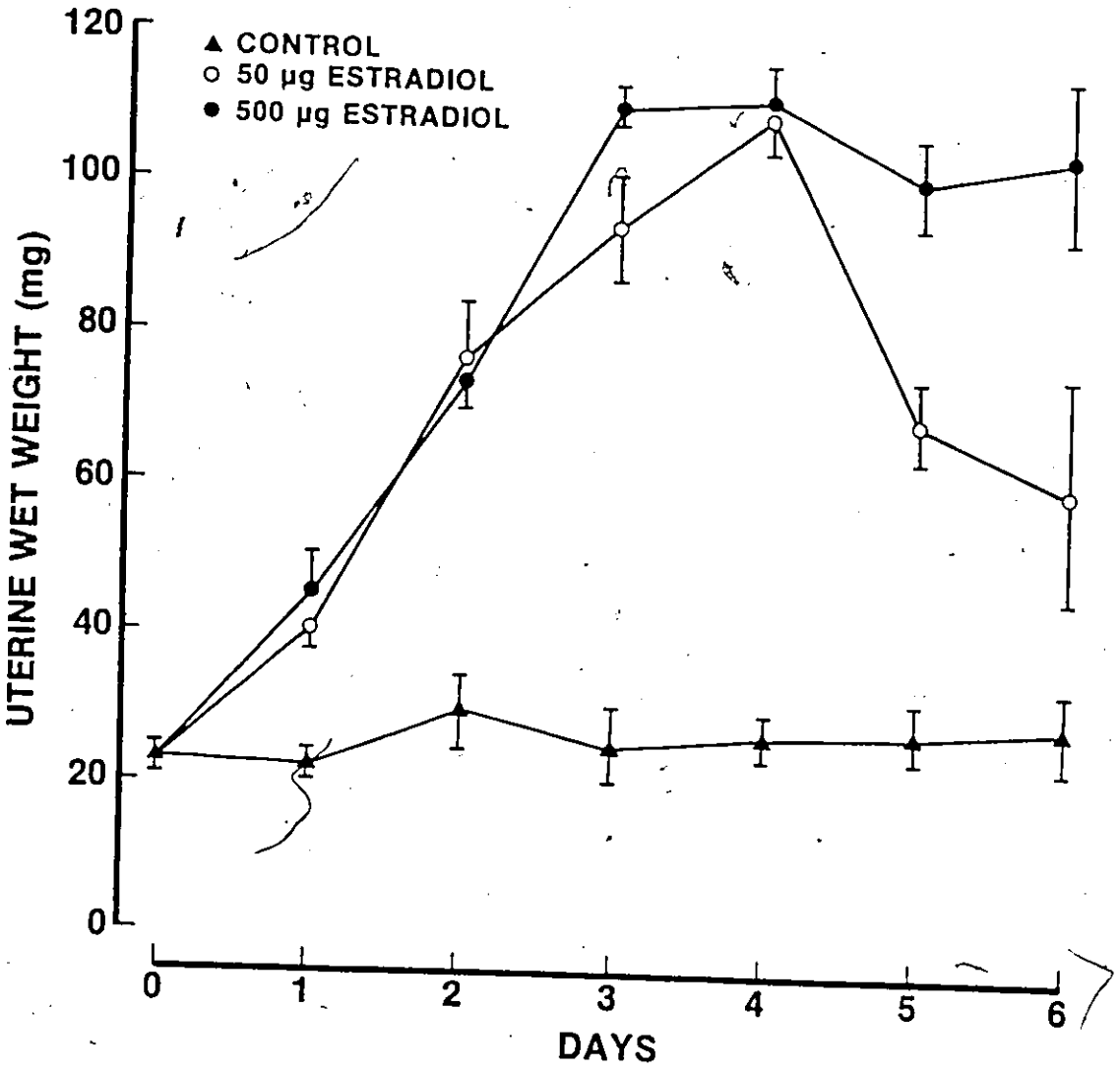

A
○ 50 µg ESTRADIOL
● 500 µg ESTRADIOL
150
120
90
60
30
0
B
150
120
90
60
30
0
NUCLEUS LABELING INDEX (% Control)
0
1
2
3
4
5
6
DAYS

Figure 39. Immature rat uterine wet weight profiles over 1-6 days after treatment with a single injection of estradiol at zero time (day 0). Data are presented as mean values ± S.E. of 6 uteri on each day.


▲ CONTROL
○ 50 µg ESTRADIOL
● 500 µg ESTRADIOL
UTERINE WET WEIGHT (mg)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
DAYS

continually to a peak weight three and four days after treatment, respectively. Thereafter, however, the uterine weight in animals treated with 50 ug estradiol decreased significantly ($P<0.01$) by five and six days after hormone treatment at zero time. The uterine weight measured six days after treatment with 50 ug estradiol was not significantly larger ($P>0.05$) than the control value. In animals treated at zero time with 500 ug estradiol the uterine wet weight decreased slightly, but not significantly, five and six days after hormone treatment. The wet weight measured six days after estradiol (500 ug) treatment was significantly larger ($P<0.001$) than the corresponding control value. A significant variation between uterine weights in animals treated with 50 and 500 ug estradiol occurred five days after hormone treatment at zero time. Uterine wet weights from control animals showed a slight increase from zero time over the same six day period.

The maximum wet weight on day four in response to a single injection of 50 ug estradiol at zero time was smaller than the wet weight measured on the same day after four repeated injections of the hormone. However, the maximum weight observed three days after a single injection of 500 ug estradiol was similar to the weight gain after three consecutive days of treatment with 500 ug/day estradiol (compare Figures 29 and 39). Maximum wet weight gains with a single injection of 50 and 500 ug estradiol, however, were considerably smaller than those measured after repeated hormone injections.

Uterine weight normalized to body weight over the 1-6 day period after a single injection of estradiol (50 and 500 ug) at zero

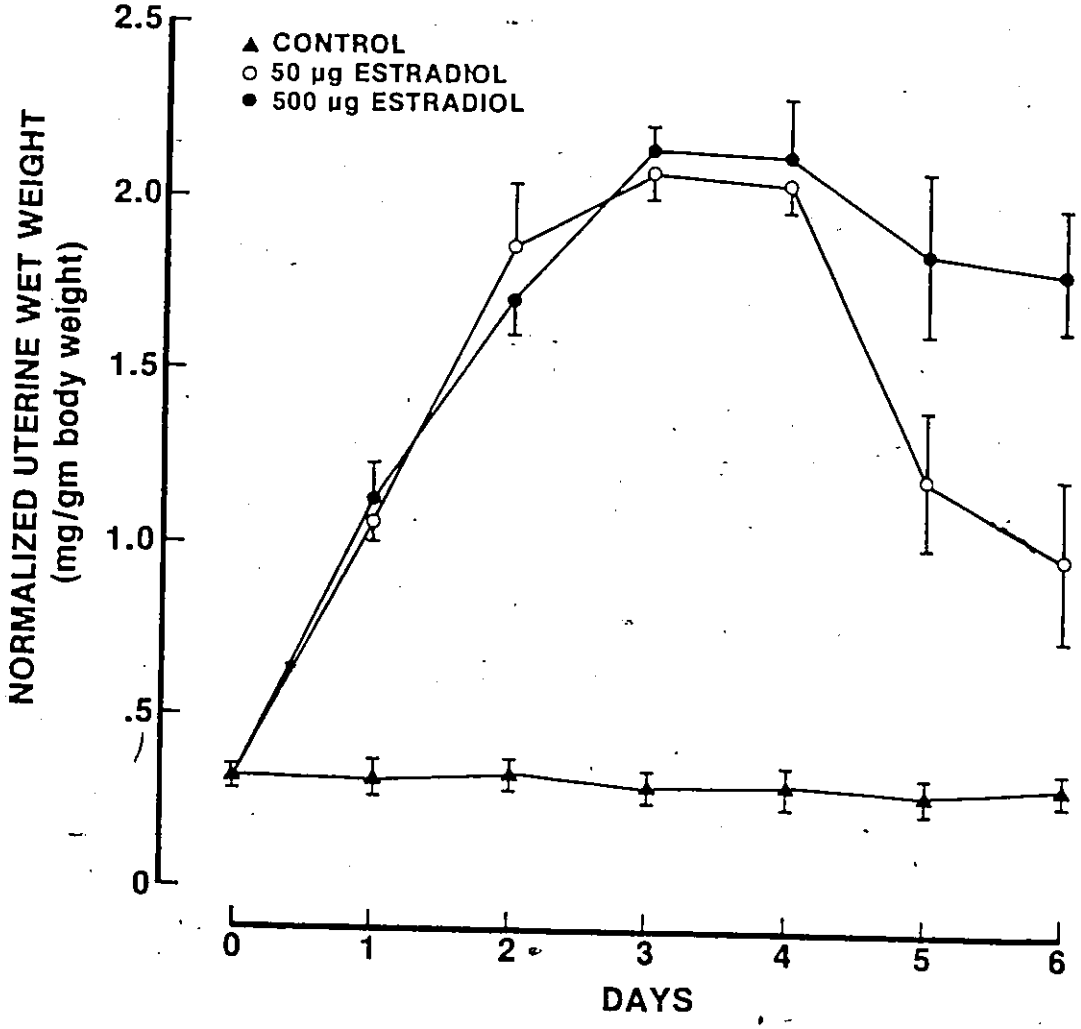
time, show a similar weight profile as the absolute wet weights presented in Figure 39. Normalized uterine wet weights increased from 0.325 ± 0.012 mg/gm body weight at zero time in control animals to 2.080 ± 0.080 and 2.150 ± 0.060 mg/gm body weight twenty-four hours after treatment with 50 and 500 ug estradiol respectively (Figure 40), representing a significant increase ($P<0.005$) compared to the normalized control uterine weight. Normalized weights continued to increase to maximum values three days after the injection of both doses of estradiol. The maximum weights were maintained on the fourth day after treatment but thereafter, decreased to 0.980 ± 0.240 ($P<0.01$) and 1.810 ± 0.230 mg/gm body weight six days after treatment with 50 and 500 ug estradiol, respectively. No significant differences between the effects of 50 and 500 ug doses of estradiol on normalized wet weight were observed on any day over the six day period. A slight but non-significant increase in normalized wet weight was observed in control animals from zero time over the course of the experiment.

The maximum normalized weight gains in response to a single injection of both doses of estradiol on day three were similar to normalized weights measured after repeated injections of the hormone over the same period (compare Figures 30 and 40). A single injection of estradiol, however, was not sufficient to maintain the wet weight gain beyond that observed three days after hormone treatment.

6.2.1.2 Effects of progesterone:

To determine the effects of progesterone on uterine wet weight, progesterone (2 mg/day) was administered alone or simultaneously with

Figure 40. Effects of estradiol on normalized uterine wet weight changes over 1-6 days from immature rats treated with single injection of estradiol at zero time (day 0). Data are presented as mean values ± S.E. of 6 uteri on each day.


▲ CONTROL
○ 50 µg ESTRADIOL
● 500 µg ESTRADIOL
NORMALIZED UTERINE WET WEIGHT
(mg/gm body weight)
2.5
2.0
1.5
1.0
.5
0
0
1
2
3
4
5
6
DAYS

estradiol (500 ug/day) for a period of 1-6 days. Figure 41 shows that progesterone administered alone for two, four and six days resulted in a small, but significant increase ($P<0.01$) in uterine wet weight from zero time controls. However, the wet weight increase was not significantly larger ($P>0.05$) compared to the wet weights of control uterine tissue over the course of the experiment. Uterine weights in the control animals increased slightly over the same six day period. Administration of progesterone together with estradiol had no influence on uterine wet weight increases compared to that induced by estradiol alone over the first three days of treatment. Twenty-four hours after the first injection of estradiol plus progesterone the uterine wet weight increased significantly ($P<0.05$) compared to the control values. Thereafter, the wet weight increased to maximum values after three days of treatment ($P<0.001$ compared to the three day control weight) and similar to the wet weight from estradiol treated animals over the same period. No further increase in wet weight occurred in animals treated with estradiol plus progesterone over the remainder of the six day period. Twenty-four hours after the fourth day of treatment, concomitant aadministration of progesterone together with estradiol resulted in a suppression of the estradiol induced uterine weight reaching significantly lower levels after five and six days of treatment compared to the estradiol treated animals.

Uterine wet weight changes (normalized to rat body weight) in response to progesterone or progesterone plus estradiol treatment are illustrated in Figure 42. Progesterone (2 mg/day) administered alone caused a small but significant increase ($P<0.01$) in the normalized

Figure 41. Uterine wet weight profiles from immature rats treated with progesterone and progesterone plus estradiol over 1-6 days. Data are presented as mean values ± S.E. of 4-6 uteri per group on each day.

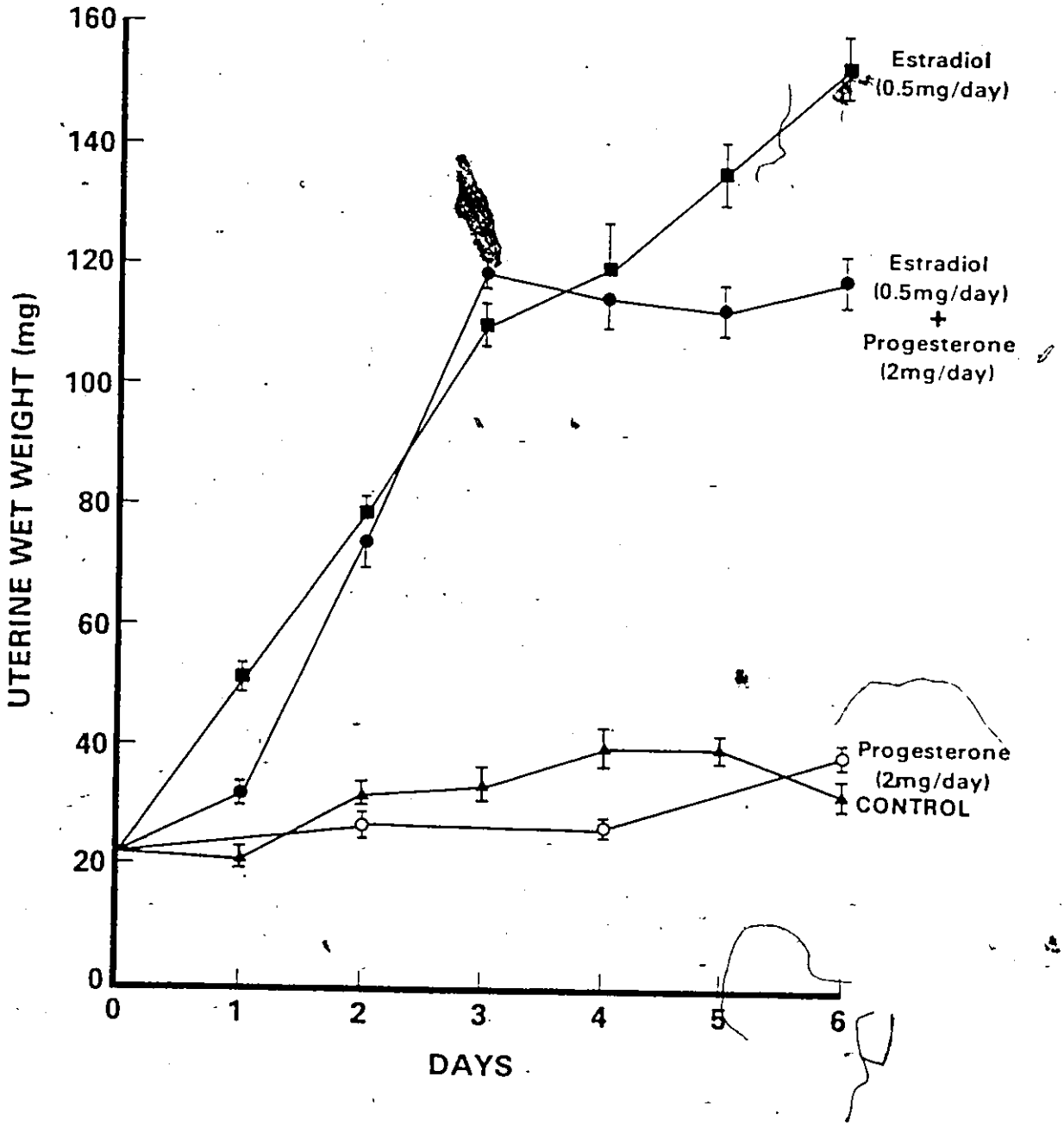
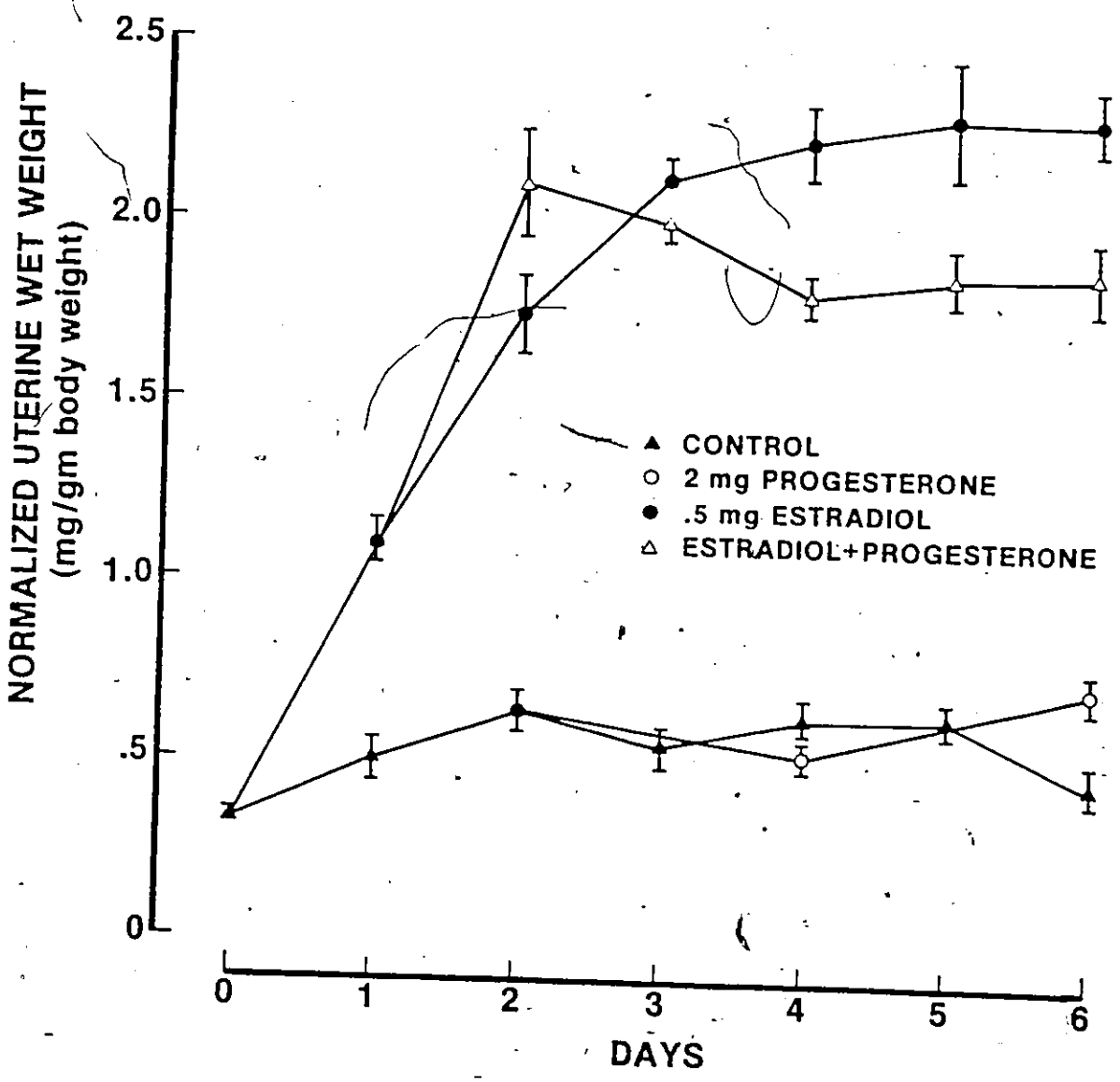

160
140
120
100
80
60
40
20
0
UTERINE WET WEIGHT (mg)
0
1
2
3
4
5
6
DAYS
Estradiol
(0.5mg/day)
Estradiol
(0.5mg/day)
+
Progesterone
(2mg/day)
Progesterone
(2mg/day)
CONTROL

Figure 42. Normalized uterine wet weight profiles from immature rats after treatment with progesterone and progesterone plus estradiol for 1-6 days. Data are presented as mean values ± S.E. of 4-6 uteri per group on each day.

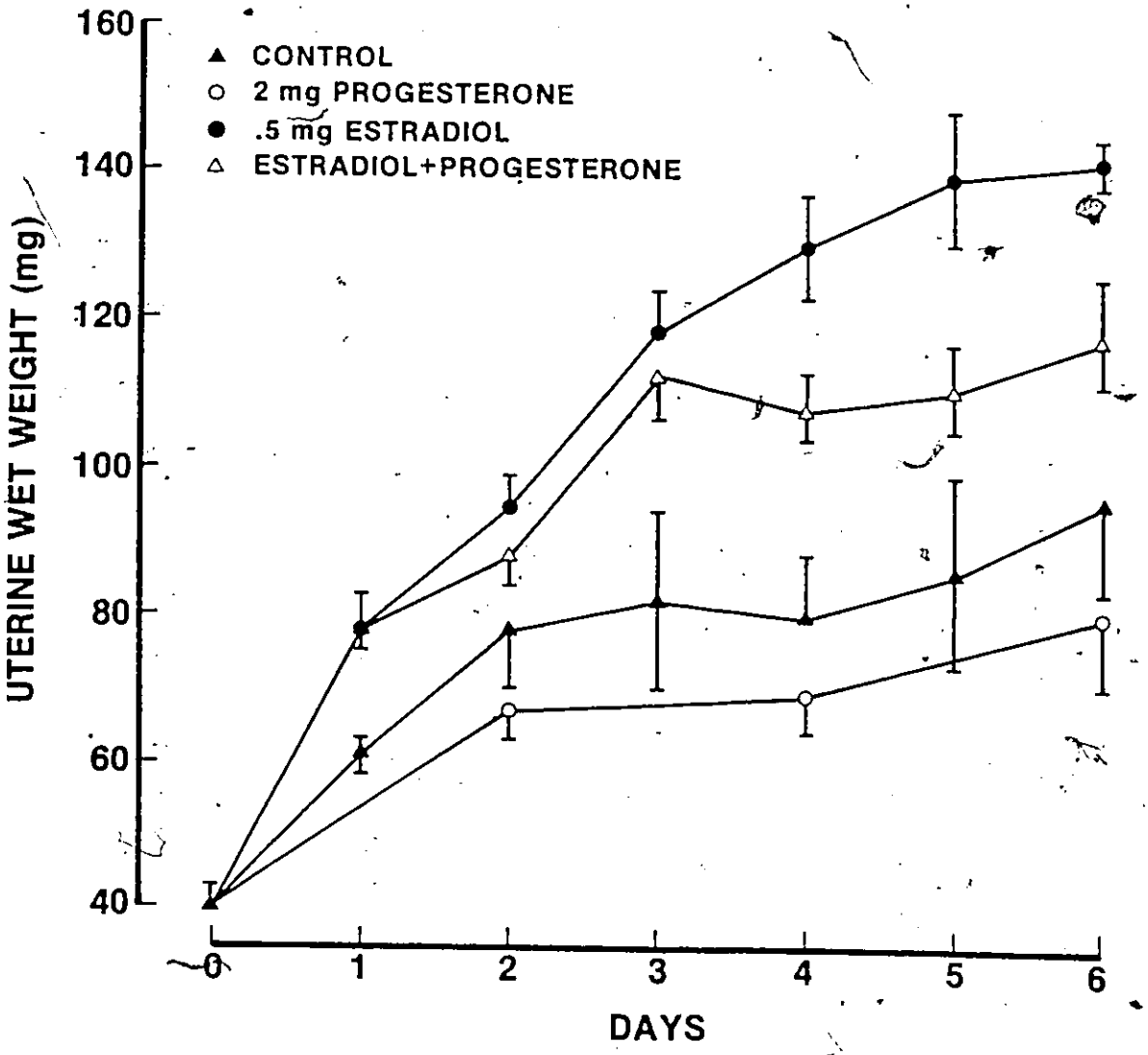

NORMALIZED UTERINE WET WEIGHT
(mg/gm body weight)
2.5
2.0
1.5
1.0
.5
0
CONTROL
2 mg PROGESTERONE
.5 mg ESTRADIOL
ESTRADIOL+PROGESTERONE
0
1
2
3
4
5
6
DAYS

uterine wet weight after two days compared to the normalized uterine weight at zero time in control animals and was elevated over four and six days of treatment. Normalized weight of control uterine tissue also increased significantly ($P<0.01$) over the six day period. A difference between the normalized weights of uterine tissue from control and progesterone treated animals occurred after six days of treatment ($P<0.05$). Progesterone administered simultaneously with estradiol (500 ug/day) for 1-6 days had no significant effect either above or below that observed with estradiol alone over the first two days of treatment, similar to the absolute wet weights expressed in Figure 41. The normalized uterine weight was significantly increased ($P<0.001$) after the first day of treatment with estradiol plus progesterone compared to the control values. A further increase to a maximum normalized weight occurred after two days of treatment (0.650 ± 0.040 mg/gm body weight in control animals compared to 2.100 ± 0.150 mg/gm body weight in the estradiol plus progesterone treated group, $P<0.001$). Normalized wet weight did not increase as much as with estradiol alone from three through six days with repeated injections of estradiol plus progesterone, but were significantly larger ($P<0.001$) compared to the control values. The normalized weights induced by estradiol treatment on days four, five and six were significantly suppressed (day four $P<0.01$, day five and six $P<0.05$) by concomitant progesterone treatment (i.e. 2.300 ± 0.090 mg/gm body weight in the estradiol treated animals after six days compared to 1.860 ± 0.110 mg/gm body weight after six days treatment with estradiol plus progesterone).

To evaluate the effect of estradiol priming on the influence of

progesterone on uterine wet weight, immature rats were injected with 50 ug estradiol approximately sixteen hours prior to treatment with progesterone (2 mg/day) or progesterone plus estradiol (500 ug/day). Figure 43 shows a significant increase ($P<0.01$) in the control uterine wet weight twenty-four hours after the first injection of sesame oil compared to the zero time control value. The control uterine weight increased thereafter over the six day period to a significantly larger ($P<0.01$) and maximum weight after six days of treatment (95.40 $\pm$ 12.40 mg compared to 40.50 $\pm$ 2.100 at zero time). Similarly, uterine wet weight was significantly increased ($P<0.001$) after two days of treatment with progesterone compared to the zero time control followed by a gradual insignificant increase after six days of treatment. Uterine wet weights on day two, four and six in response to progesterone were lower but not significantly different than control weights on the same days. Estradiol (500 ug/day) administration resulted in a significant increase ($P<0.001$) in the uterine weight after the first day of treatment compared to the control value. With repeated injections of estradiol (i.e. 2-6 days) the uterine weight increased gradually and progressively to a maximum weight gain after six days of treatment (141.0 $\pm$ 2.800 mg, $P<0.05$ compared to the six day control weight). Simultaneous treatment with estradiol plus progesterone did not alter the uterine weight increase compared to that induced by estradiol alone after the first day of treatment. The uterine weights on days two and three after estradiol plus progesterone treatment were lower, but did not differ significantly compared to the weights after treatment with estradiol alone over the same days.

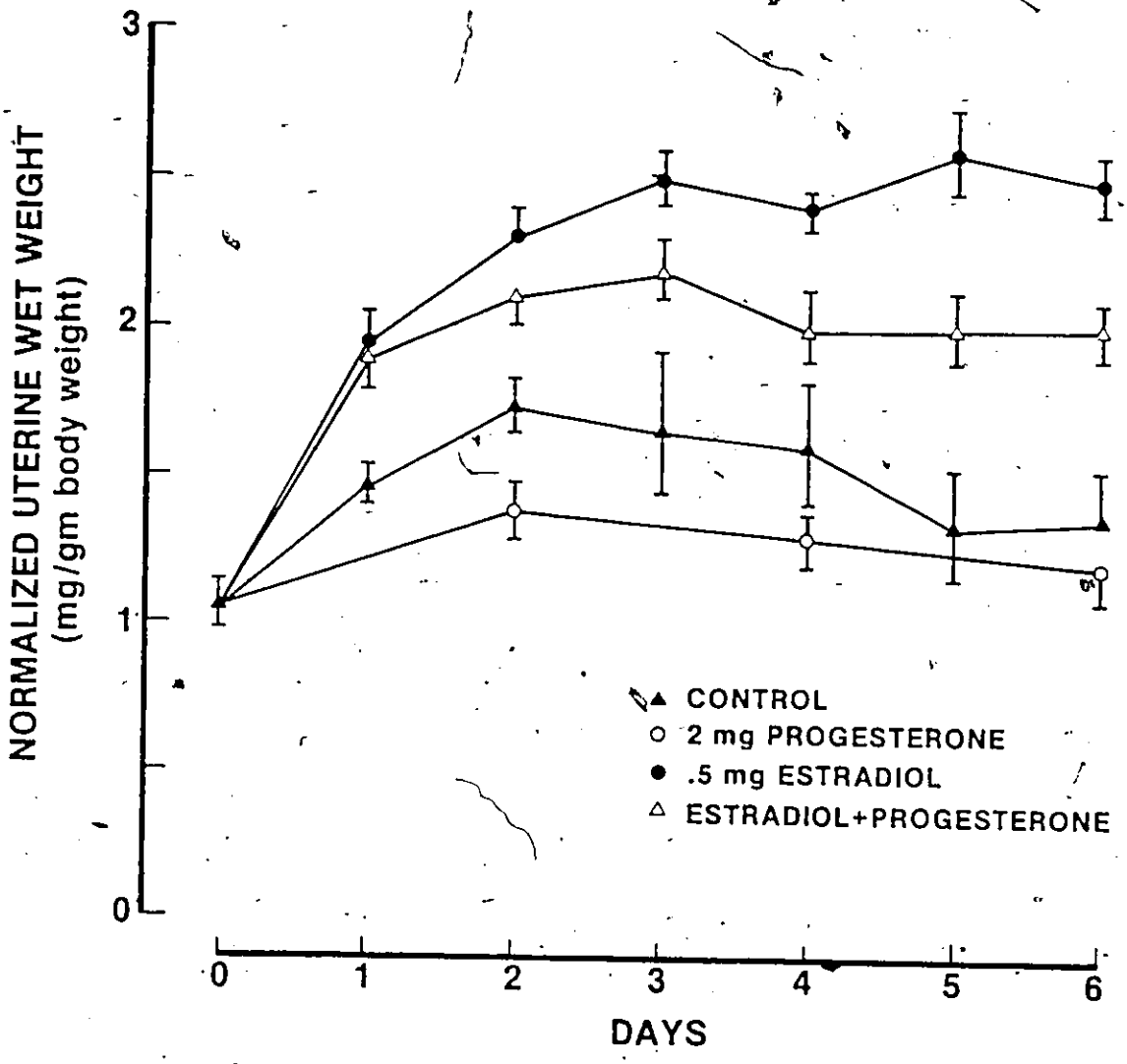
Figure 43. Effects of estradiol, progesterone and estradiol plus progesterone treatments on immature rat uterine wet weight in animals primed with estradiol. Approximately 16 hours prior to the first day of the treatment period, all animals were injected with 50 ug of estradiol. Data are presented as mean values ± S.E. of 4-6 uteri per group on each day.


CONTROL
2 mg PROGESTERONE
.5 mg ESTRADIOL
ESTRADIOL+PROGESTERONE
UTERINE WET WEIGHT (mg)
160
140
120
100
80
60
40
0
1
2
3
4
5
6
DAYS

However, from four through six days of treatment with the combination of the two hormones, there were significant suppressions ($P<0.05$) in uterine wet weights compared to the weights from the estradiol treated animals. Mean wet weights measured from 2-6 days of treatment with estradiol plus progesterone were larger but not significantly different than corresponding control weights.

Normalized uterine wet weights following 50 ug estradiol priming are shown in Figure 44 and are similar to the absolute wet weight changes in Figure 43. The normalized wet weight of control uterine tissue was significantly elevated ($P<0.01$) after the first and second days (1.490 ± 0.040 and 1.760 ± 0.080 mg/gm body weight respectively) compared to the zero time control normalized weight (1.060 ± 0.060 mg/gm body weight). The control normalized weight decreased insignificantly (compared to the two day control normalized weight) over the remaining four days of the six day period. Treatment with progesterone (2 mg/day) alone caused a small non-significant increase in the normalized weight after two, four and six days of treatment. The mean normalized weights in animals treated with progesterone were lower than the control normalized weights, however, the differences between the two groups were not significant at any time. A significant normalized weight gain occurred after one day of treatment with 500 ug/day estradiol ($P<0.01$ compared to the control value). After the second day of treatment with estradiol a near maximum gain in the normalized weight occurred which was significantly greater ($P<0.01$) than the control value on the same day (from 1.760 ± 0.080 mg/gm body weight in the control group to 2.300 ± 0.080 mg/gm body

Figure 44. Normalized uterine wet weight profiles from estradiol primed immature rats treated with estradiol, progesterone and estradiol plus progesterone. Data are presented as mean values ± S.E. of 4-6 uteri on each day.

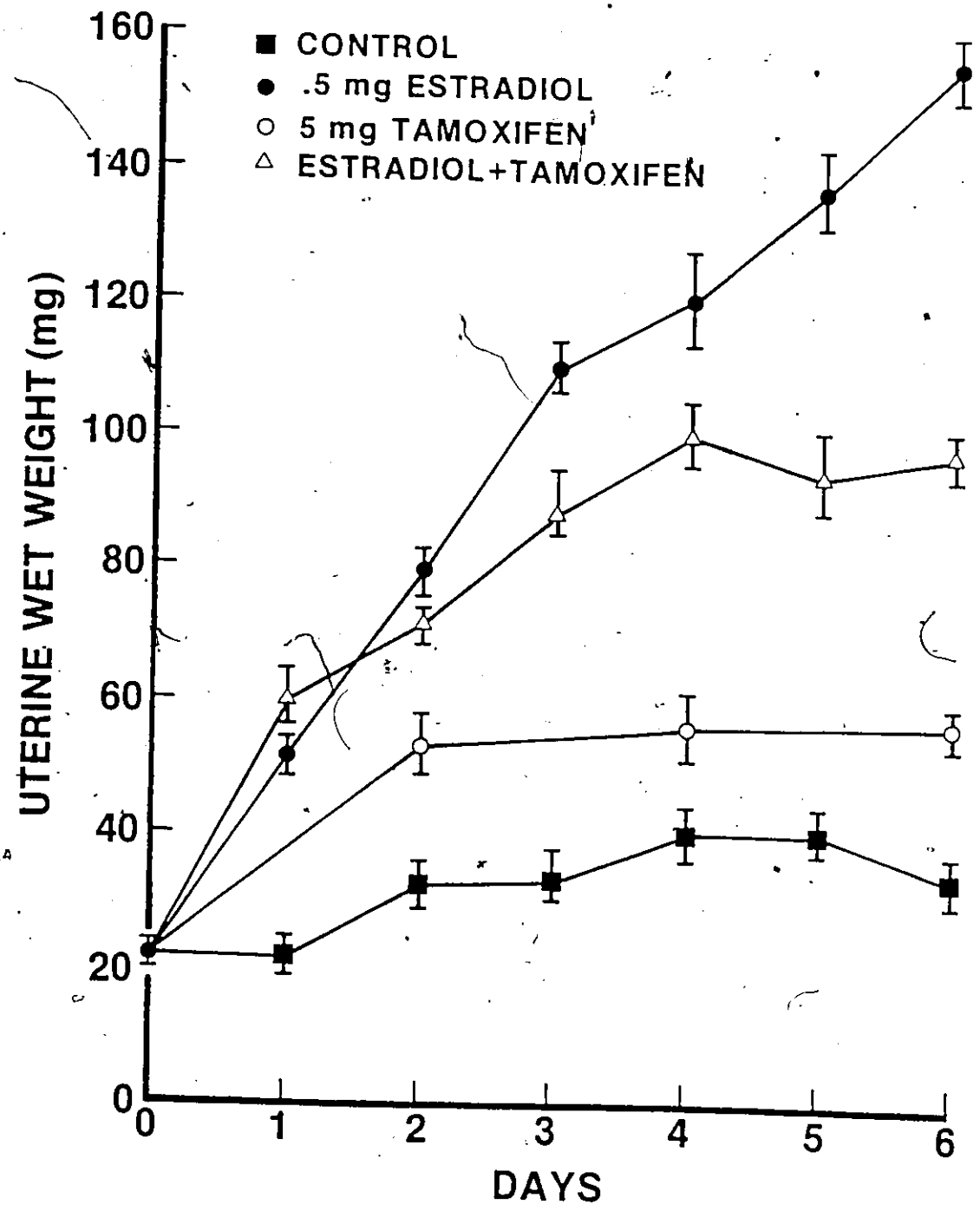

NORMALIZED UTERINE WET WEIGHT
(mg/gm body weight)
3
2
1
0
0
1
2
3
4
5
6
DAYS
CONTROL
2 mg PROGESTERONE
.5 mg ESTRADIOL
ESTRADIOL+PROGESTERONE

weight in the estradiol treated animals). No further significant increase in normalized wet weight occurred over the remainder of the six day period. Progesterone (2 mg/day) administered simultaneously with estradiol resulted in a significant ($P<0.01$ compared to the control) and almost similar increase in the normalized weight after one day of treatment as compared to the estradiol treated group. With continued treatment of the two hormones, however, normalized weight increases, compared to estradiol treatment alone, were suppressed and reached levels of significance ($P<0.05$) twenty-four hours after the third injection. Normalized wet weights induced by treatment with estradiol plus progesterone were significantly larger ($P<0.05$) than controls after five and six days of treatment.

6.2.1.3 Effects of tamoxifen:

To analyze the effects of the antiestrogen tamoxifen on uterine wet weight, immature animals were treated with tamoxifen (5 mg/day) alone and together with estradiol (500 ug/day) for a period of 1-6 days. Figure 45 shows that the uterine wet weight increased significantly to a peak value after two days treatment with tamoxifen (22.20 $\pm$ 2.000 mg at zero time in control animals compared to 53.80 $\pm$ 3.400 mg in the treated group, $P<0.001$). The increase was significantly larger ($P<0.05$) than the control uterine weight and smaller than the uterine weight from animals treated with estradiol for the same period ($P<0.05$). No further increase in weight occurred over six days of treatment with tamoxifen. Tamoxifen administered concomitantly with estradiol did not effect ($P>0.05$) the wet weight

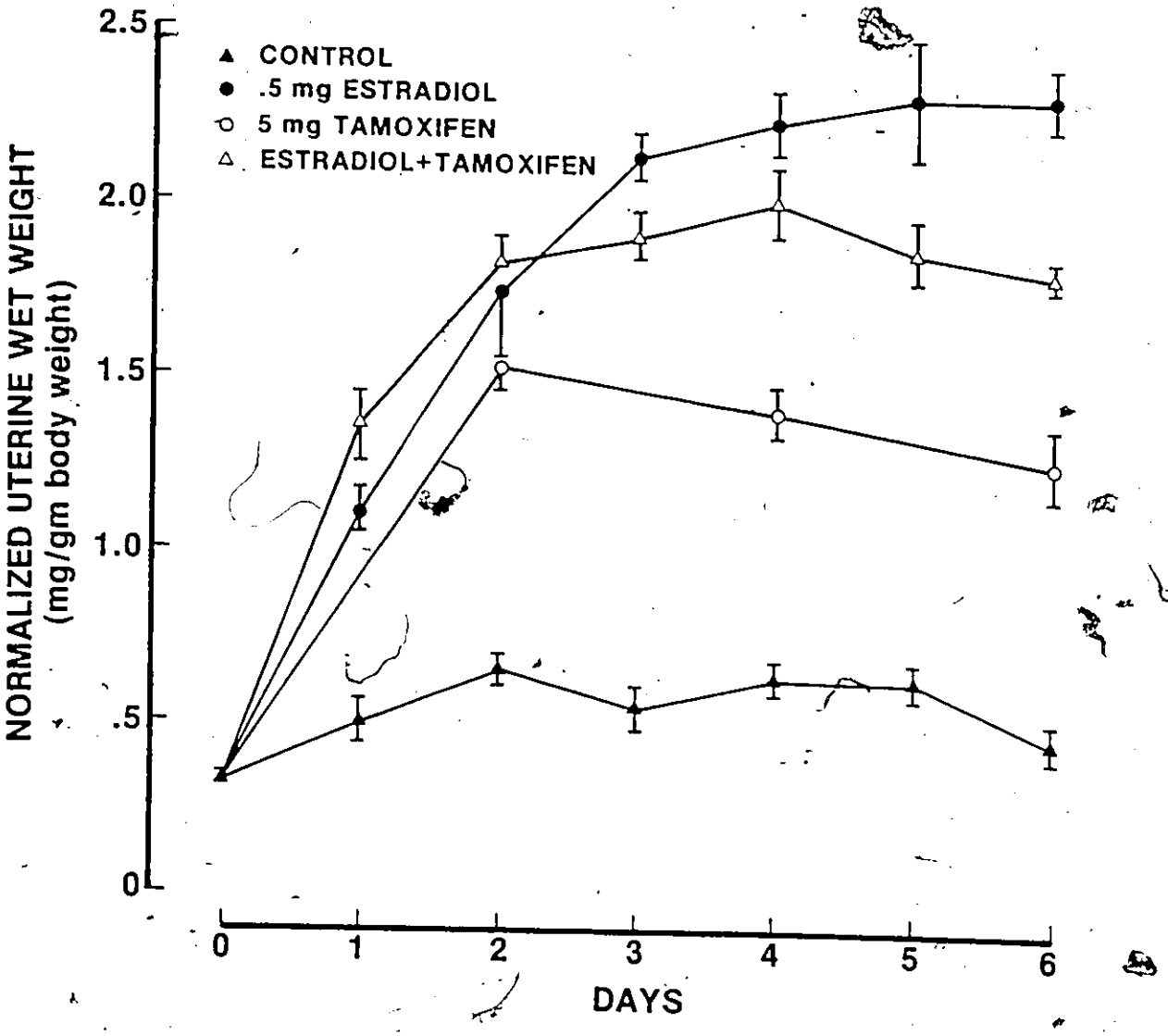
Figure 45. Effects of tamoxifen on uterine wet weight of immature animals. Immature rats were treated with tamoxifen, estradiol and tamoxifen plus estradiol for 1-6 days to determine the effect of tamoxifen on estradiol induced weight increases. Data are presented as mean values ± S.E. of 6 uteri per group on each day.


CONTROL
.5 mg ESTRADIOL
5 mg TAMOXIFEN
ESTRADIOL+TAMOXIFEN
UTERINE WET WEIGHT (mg)
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
DAYS

increases induced by estradiol after one and two days of treatment, but thereafter suppressed the estradiol stimulated increases in wet weight over the remainder of the six day treatment period. The uterine wet weights measured from days three through six after estradiol plus tamoxifen injections were significantly lower ($P<0.05$ and .01) than corresponding weights from the estradiol treated animals and significantly larger ($P<0.01$) than wet weights from control animals and rats treated with tamoxifen alone.

Similar effects of tamoxifen, alone or together with estradiol, on absolute uterine wet weights were also demonstrated if the weights are normalized to animal body weight. Figure 46 illustrates changes in normalized wet weights over a 6 day period in immature rats treated with tamoxifen (5 mg/day) with and without estradiol (500 ug/day). Treatment with tamoxifen resulted in a significant increase ($P<0.001$) in the normalized wet weight after two days compared to the control values, followed by a non-significant decrease after the sixth injection. Normalized weights determined after two, four and six days of treatment with tamoxifen were significantly greater ($P<0.01$) than control normalized weights on the same days. Simultaneous administration of tamoxifen together with estradiol showed no influence on the normalized weight increases as induced by estradiol over the first two days of treatment. Concomitant treatment with both compounds resulted in a significant increase ($P<0.001$) in the normalized weight after one and two days of treatment compared to the control. With continued treatment of estradiol plus tamoxifen however, the normalized wet weight gains induced by estradiol over three through six days of

Figure 46. Normalized uterine wet weight profiles from estradiol, tamoxifen and estradiol plus tamoxifen treated immature rats over 1-6 days. Data are presented as mean values ± S.E. of 6 uteri per group on each day.


CONTROL
.5 mg ESTRADIOL
5 mg TAMOXIFEN
ESTRADIOL+TAMOXIFEN
NORMALIZED UTERINE WET WEIGHT
(mg/gm body weight)
2.5
2.0
1.5
1.0
.5
0
0
1
2
3
4
5
6
DAYS

treatment were suppressed to significantly lower ($P<0.01$) values after six days, from 2.300 ± 0.090 mg/gm body weight in the estradiol treated group to 1.800 ± 0.040 mg/gm body weight in animals treated with estradiol plus tamoxifen. Normalized uterine weights from animals treated with estradiol and tamoxifen were significantly larger ($P<0.001$) than control values over the six day period and the normalized weights after four and six days of treatment were also larger compared to that observed in animals treated with tamoxifen alone for the same period.

6.2.1.4 Effects of indomethacin:

To evaluate the influence of cyclo-oxygenase inhibition on estradiol stimulated increases in uterine wet weight, indomethacin (0.2 mg twice daily) was administered with and without estradiol (50 and 500 ug/day) for three days to immature rats. Administration of indomethacin resulted in a significant increase ($P<0.05$) in uterine wet and normalized wet weight after three days of treatment from 34.25 ± 2.744 mg and 0.549 ± 0.041 mg/gm body weight in the control group to 63.12 ± 6.645 mg and 1.347 ± 0.160 mg/gm body weight in the treated animals (Table 12). Simultaneous administration of indomethacin together with estradiol (50 and 500 ug/day) for three days resulted in no further wet weight increases beyond that attributable to either estradiol dose alone. Indomethacin caused a slight but insignificant decrease in the mean body weight when administered by itself or together with estradiol for three days.

TABLE 12. EFFECTS OF INDOMETHACIN (IDM) AND ESTRADIOL (E_2) ON UTERINE WET WEIGHT OF IMMATURE RATS

Treatment	Number of Tissues	Body Weight (gm)	Wet Weight (mg)	Normalized Wet Weight (mg/gm body wt.)
3 days				
Control	6	62.3 ± 2.55	34.2 ± 2.77	0.55 ± 0.04
IDM (0.2 mg twice daily)	4	49.5 ± 8.43	63.1 ± 6.64*	1.35 ± 0.16*
E_2 (50 ug/day)	6	59.8 ± 2.98	114 ± 4.66	1.93 ± 0.07
E_2 plus IDM	4	53.0 ± 1.08	110 ± 4.59	2.07 ± 0.06
E_2 (500 ug/day)	6	52.2 ± 1.30	110 ± 3.67	2.11 ± 0.05
E_2 plus IDM	4	47.7 ± 0.63	106 ± 2.05	2.22 ± 0.021

Data presented as mean values ± S.E.

Level of significance: * $P<0.05$.

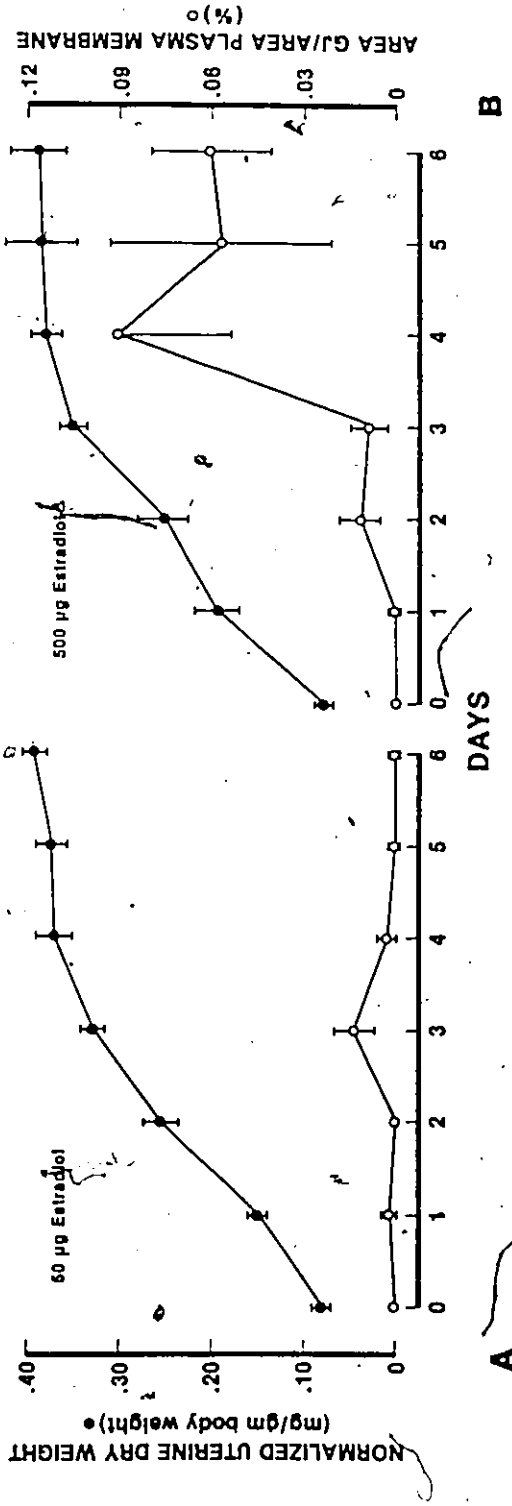
6.2.1.5 Relationship between uterine weight increase and the presence of myometrial gap junctions in immature rat:

Figure 47 illustrates the relationship between estradiol (50 and 500 ug/day) induced increases in normalized uterine dry weight and myometrial gap junctions in immature rats. The effects of both doses of estradiol on the normalized dry weight profile over the 1-6 day period have been described previously (see Figure 32). Similarly, the effects of 50 and 500 ug/day estradiol on the presence of myometrial gap junctions in the immature rat have been described in Chapter 4.

The normalized dry weight of the immature uterus increased rapidly and progressively over the first three days of treatment with 50 ug/day estradiol (Figure 47A). Thereafter, there was no further significant increase in the normalized weight over the remainder of the treatment period. Gap junctions were absent or present at a low frequency and small size, and occupied an extremely small percentage of the myometrial membrane area (less than 0.010%) throughout the six day treatment period in tissues from immature rats treated with 50 ug/day estradiol (Figure 47A). These results show that the rapid increase in weight of the uterus (of which a major contribution is the myometrium, see Figure 33) following estradiol treatment (zero time through day three) occurred when the myometrial membrane area of gap junctions was extremely low. Furthermore, cessation of the uterine dry weight gain after three days of treatment with estradiol (50 ug/day) also occurred when gap junctions were essentially not detected in the myometrium.

Normalized uterine dry weight from immature rats treated with 500 ug/day estradiol increased rapidly to a maximum weight after three

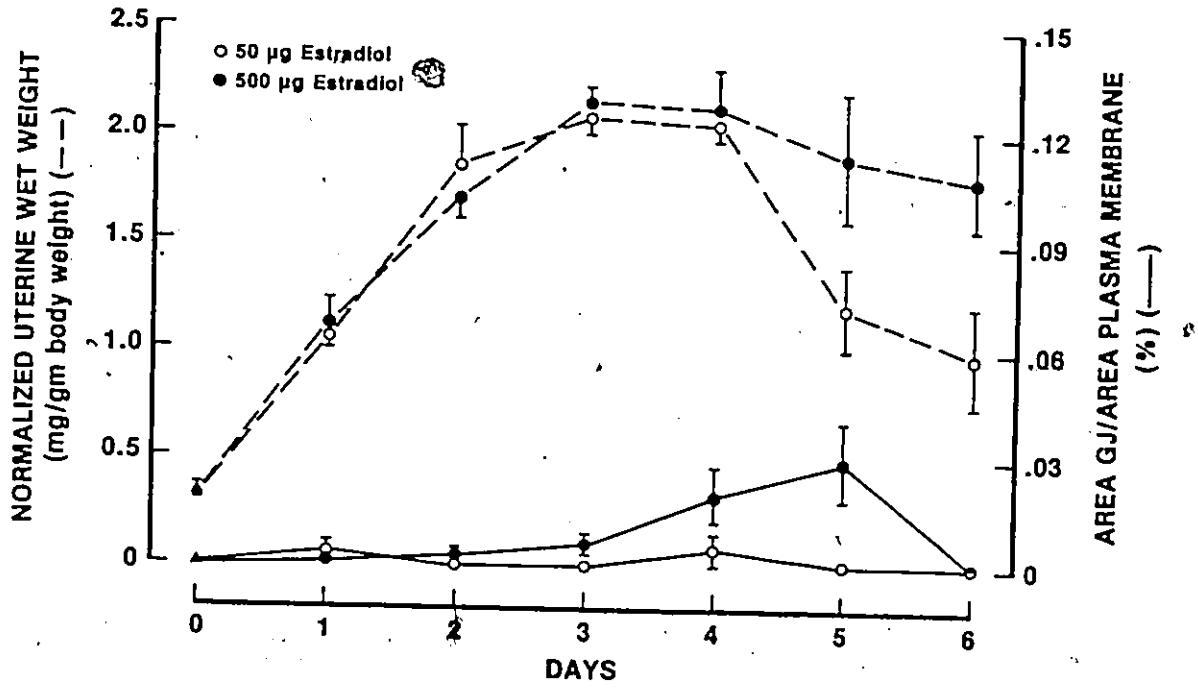
Figure 47. Changes in normalized uterine dry weight and area of myometrial junctions in immature rats after treatment with 50 (A) and 500 (B) ug/day estradiol for 1-6 days showing the relationship between the two events. Data are presented as mean values ± S.E. from 6 animals per treatment on each day.


NORMALIZED UTERINE DRY WEIGHT
(mg/gm body weight)•
.40
.30
.20
.10
0
60 µg Estradiol
500 µg Estradiol
A
B
DAYS
0
1
2
3
4
5
6
AREA GJ/AREA PLASMA MEMBRANE
(%)•
.12
.09
.06
.03
0

days of treatment (Figure 47B), similar to that observed after treatment with the lower dose of estradiol (Figure 47A). No further significant increase in the normalized dry weight occurred with continued hormone treatment. The percentage of myometrial membrane area occupied by gap junctions was extremely low throughout the first three days of treatment, but thereafter was significantly elevated after four ($P<0.05$), five and six ($P<0.01$) days of treatment (Figure 47B). It is apparent from these results that the rapid increase in dry weight of the uterus occurred when the myometrial membrane area of gap junctions was very low and similar to the response in rats treated with 50 ug/day estradiol. However, after the uterine dry weight reached a plateau there was a significant increase in the membrane area of myometrial gap junctions.

The relationship between uterine wet weight increases and myometrial gap junctions over the six days after a single injection of estradiol (50 and 500 ug) at zero time (day 0) is shown in Figure 48. Normalized uterine wet weight increased rapidly over the first three days after treatment with both doses of estradiol followed by a decline over the remainder of the six day period. Gap junctions were virtually undetectable in the myometrium from immature animals over the six days after treatment with 50 ug estradiol at zero time (Figure 48). The percentage of myometrial membrane area occupied by junctions after treatment with 500 ug estradiol was also negligible over the first three days and extremely low thereafter, occupying less than 0.030% of the membrane area. The increase in uterine weight over the first three days following a single injection of estradiol (50 and 500 ug) occurred

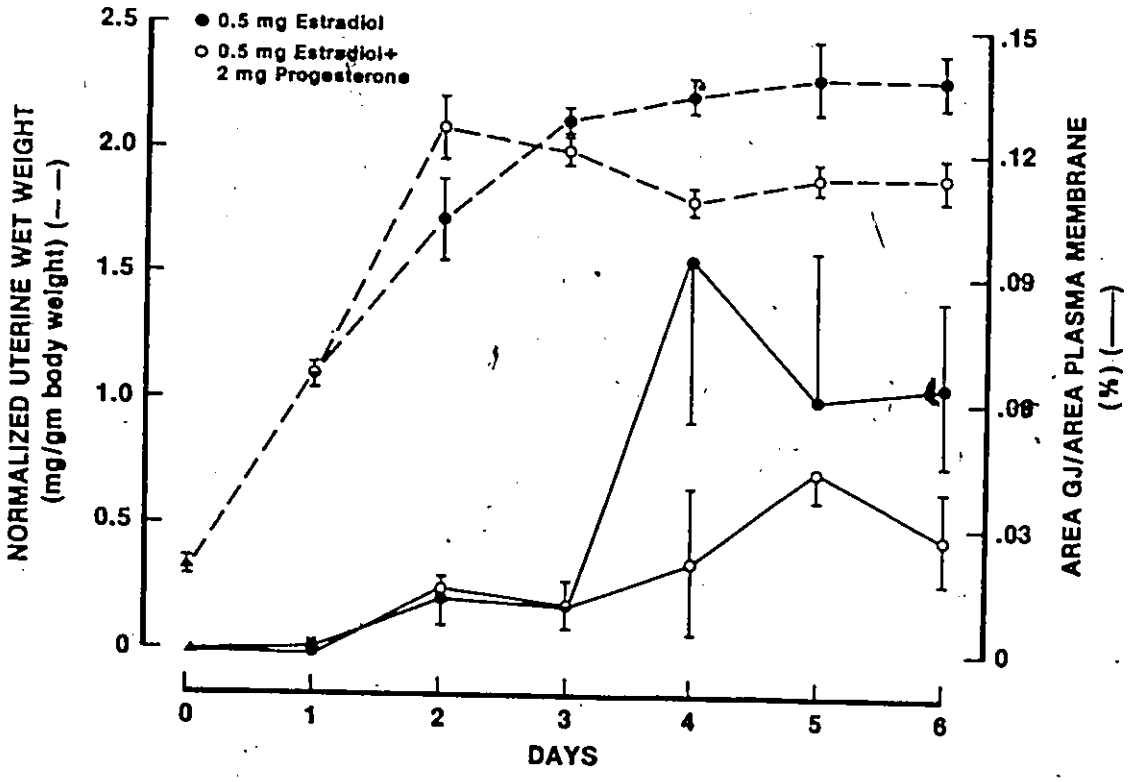
Figure 48. Normalized uterine wet weight changes and the time course for the development of gap junctions in the myometrium of immature rats over 1-6 days after treatment with a single injection of estradiol (50 and 500 ug) at zero time (day 0). Data are presented as mean values ± S.E. of 4-6 animals per group on each day.


○ 50 µg Estradiol
● 500 µg Estradiol
NORMALIZED UTERINE WET WEIGHT (mg/gm body weight) (——)
AREA GJ/AREA PLASMA MEMBRANE (%) (——)
2.5
2.0
1.5
1.0
0.5
0
.15
.12
.09
.06
.03
0
0
1
2
3
4
5
6
DAYS

when the myometrial membrane area of gap junctions was extremely low (i.e. less than 0.006%). The decrease in uterine weight after this time also occurred when an extremely small percentage of the myometrial membrane area was occupied by gap junctions.

Figure 49 shows the effects of progesterone on estradiol induced increases in uterine weight and presence of myometrial gap junctions over 1-6 days in the immature rat. Progesterone, when administered together with estradiol, suppressed the maximum uterine wet weight gain as induced by estradiol alone. Progesterone under these conditions also inhibited the development of myometrial gap junctions in response to estradiol treatment. The normalized uterine wet weight increases induced by estradiol over three to six days of treatment were significantly suppressed ($P<0.05$) beginning after the fourth concomitant injection of estradiol plus progesterone (Figure 49). The significant increase in percentage of myometrial membrane area of gap junctions after four, five and six days of estradiol treatment were also suppressed by simultaneous injections of progesterone (Figure 49). The rapid increase in normalized wet weight over the first three days of estradiol treatment occurred when the membrane area occupied by gap junctions was extremely low. A significant increase in myometrial gap junction area was observed only after the uterine weight gain ceased. Similarly, concomitant administration of progesterone together with estradiol did not effect the estradiol induced weight gain over the first three days when the gap junctional membrane area was low. Progesterone suppression of estradiol induced wet weight on days four, five and six was accompanied

Figure 49. Changes in normalized uterine wet weight and area of myometrial gap junctions of immature animals after treatment with estradiol with and without progesterone over 1-6 days showing the relationship between the two events. Data are presented as mean values ± S.E. of 4-6 uteri per group on each day.


● 0.5 mg Estradiol
○ 0.5 mg Estradiol+
2 mg Progesterone
NORMALIZED UTERINE WET WEIGHT
(mg/gm body weight) (— —)
AREA GJ/AREA PLASMA MEMBRANE
(%) (——)
DAYS
2.5
2.0
1.5
1.0
0.5
0
.15
.12
.09
.06
.03
0
0
1
2
3
4
5
6

by inhibition of the percentage of myometrial membrane area of the junctions.

The effects of tamoxifen on estradiol stimulated normalized wet weight and myometrial gap junction area in immature rats are shown in Figure 50. Increased normalized uterine wet weight after two days of treatment with estradiol plus tamoxifen was followed by a suppression of the wet weight gain as induced by estradiol alone over the remaining four days of the treatment period. Tamoxifen also completely prevented the estradiol stimulation of myometrial gap junctions. The stimulation of uterine weight by estradiol and estradiol plus tamoxifen and the suppression of estradiol induced weight by tamoxifen occurred when the gap junction myometrial membrane area was extremely low.

Table 13 shows the effects of indomethacin on the estradiol stimulated normalized wet weight and myometrial gap junction area in immature rats after three days of treatment. Indomethacin treatment caused a significant increase ($P<0.05$) in the normalized wet weight compared to the control value. Administration of indomethacin together with either dose of estradiol, however, did not have any influence on the normalized wet weight compared to the weight measured after estradiol treatment alone. Concomitant injections of indomethacin together with estradiol (50 and 500 ug/day) resulted in a further increase in the membrane area of gap junctions. These results indicate that uterine weight did not increase when gap junctions occupied a larger percentage of the myometrial membrane area. Note, as well, that treatment with indomethacin alone significantly increased the normalized wet weight compared to the control weight and that no gap

Figure 50. Normalized uterine wet weight changes and changes in the area of myometrial gap junctions in immature rats after treatment with estradiol with and without tamoxifen for 1-6 days illustrating the temporal patterns of the two events. Data are presented as mean values ± S.E. of 4-6 animals per group on each day.

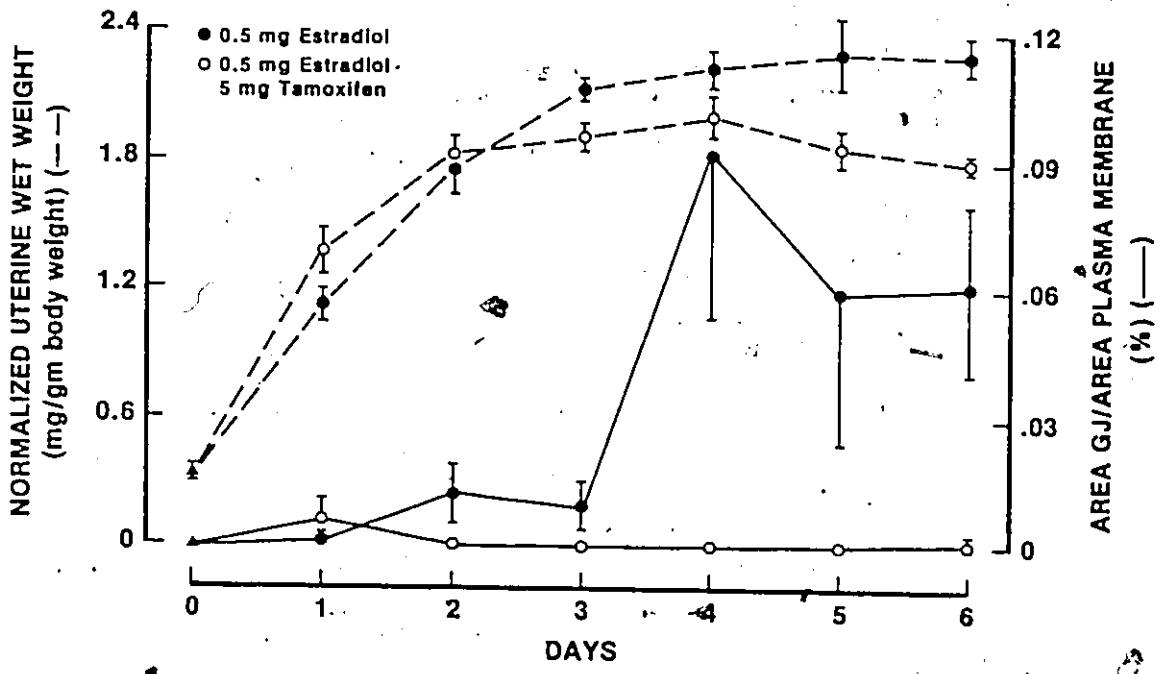

● 0.5 mg Estradiol
○ 0.5 mg Estradiol - 5 mg Tamoxifen
NORMALIZED UTERINE WET WEIGHT (mg/gm body weight) (— —)
AREA GJ/AREA PLASMA MEMBRANE (%) (——)
2.4
1.8
1.2
0.6
0
.12
.09
.06
.03
0
0
1
2
3
4
5
6
DAYS

TABLE 13. EFFECTS OF INDOMETHACIN (IDM) AND ESTRADIOL (E_2) ON NORMALIZED UTERINE WET WEIGHT AND MYOMETRIAL GAP JUNCTIONS (GJs) IN IMMATURE RATS

Treatment	Normalized Wet Weight (mg/gm body wt.)	Area GJs/Area Plasma Membrane, % (Mean ± S.E.)
3 days		
Control	0.549 ± 0.041	0
IDM (0.2 mg twice daily)	1.347 ± 0.160*	0
E_2 (50 ug/day)	1.930 ± 0.073	0.013 ± 0.007
E_2 plus IDM	2.075 ± 0.062	0.081 ± 0.036
E_2 (500 ug/day)	2.110 ± 0.054	0.009 ± 0.006
E_2 plus IDM	2.220 ± 0.021	0.121 ± 0.030**

Data presented as mean values ± S.E.

Levels of significance: * $P<0.05$; ** $P<0.01$.

junctions were detected between myometrial cells.

6.2.2 Ovariectomized mature rats:

6.2.2.1 Effects of estradiol:

Uterine wet and dry weights were determined from ovariectomized mature rats treated with estradiol (50 and 500 ug/day) for 1-4 days (i.e. up to the time at which the estradiol induced weight increases were maximum in the immature rat). Treatment with 50 ug/day estradiol resulted in a significant increase in the wet and dry weights of tissues after one ($P<0.01$ and $P<0.05$) and two ($P<0.001$ and $P<0.05$) days of treatment respectively, compared to the control weights (Figure 51). Uterine wet and dry weights continued to increase with repeated injections of 50 ug/day estradiol to peak values after the fourth day of treatment. Administration of estradiol at 500 ug/day also caused significant increases ($P<0.01$ and $P<0.05$) in uterine wet and dry weights twenty-four hours after the first injection compared to the controls. No further increase in the wet or dry weight occurred with continued treatment of 500 ug/day estradiol over the four day period. Significant variations ($P<0.05$) between the effects of 50 and 500 ug/day estradiol doses on uterine wet and dry weights were observed after four days of treatment. There were no changes in wet and dry weights of control uterine tissue from zero time over the same four day period.

Figure 52 shows the changes in normalized uterine wet weight in ovariectomized mature rats treated with estradiol (50 and 500 ug/day)

Figure 51. Wet and dry weight profiles of ovariectomized mature rat uteri after treatment with estradiol for 1-4 days. Note the expansion of the ordinate scale at .07 gms. Data are presented as mean values ± S.E. of 5-6 uteri on each day.

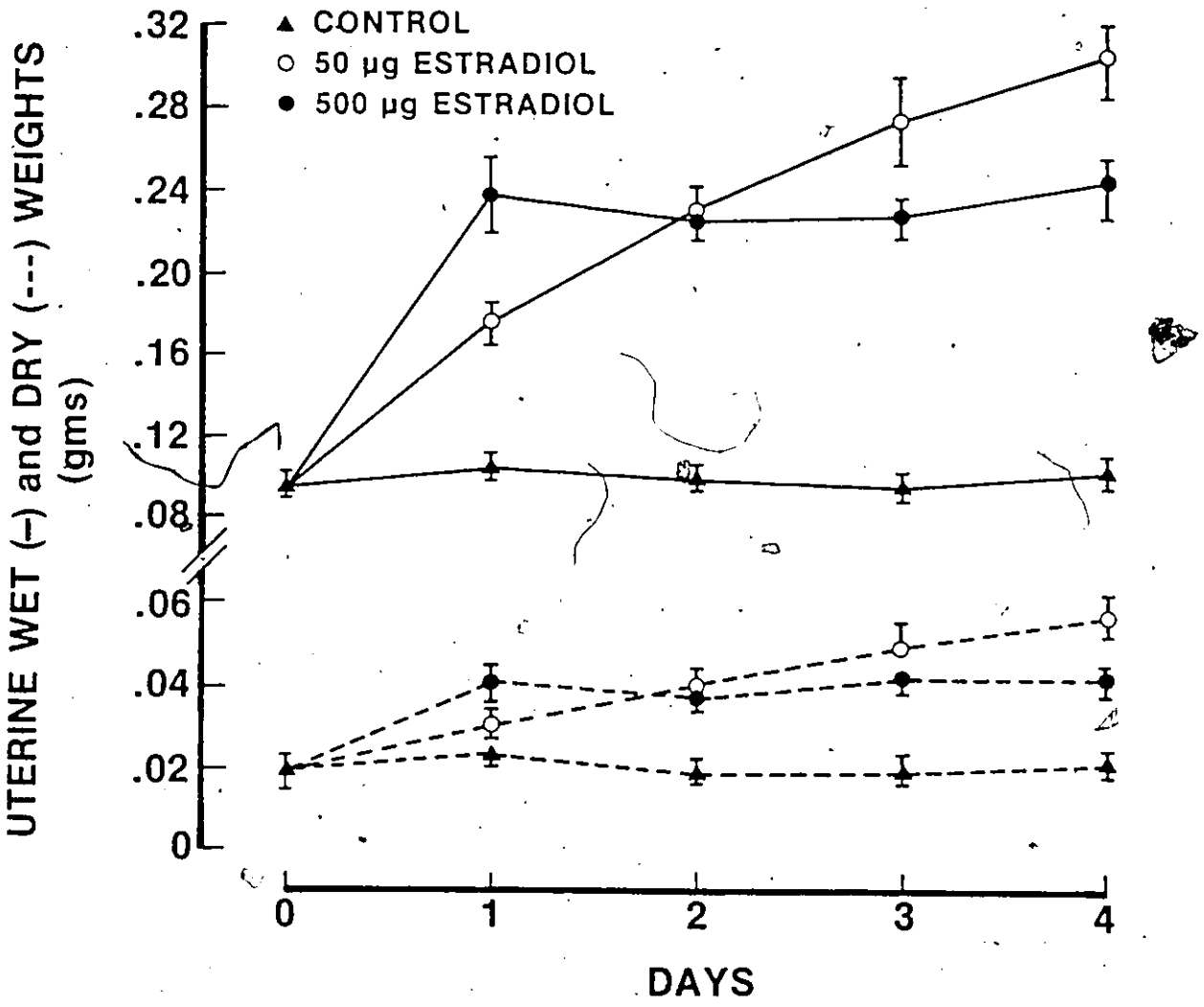
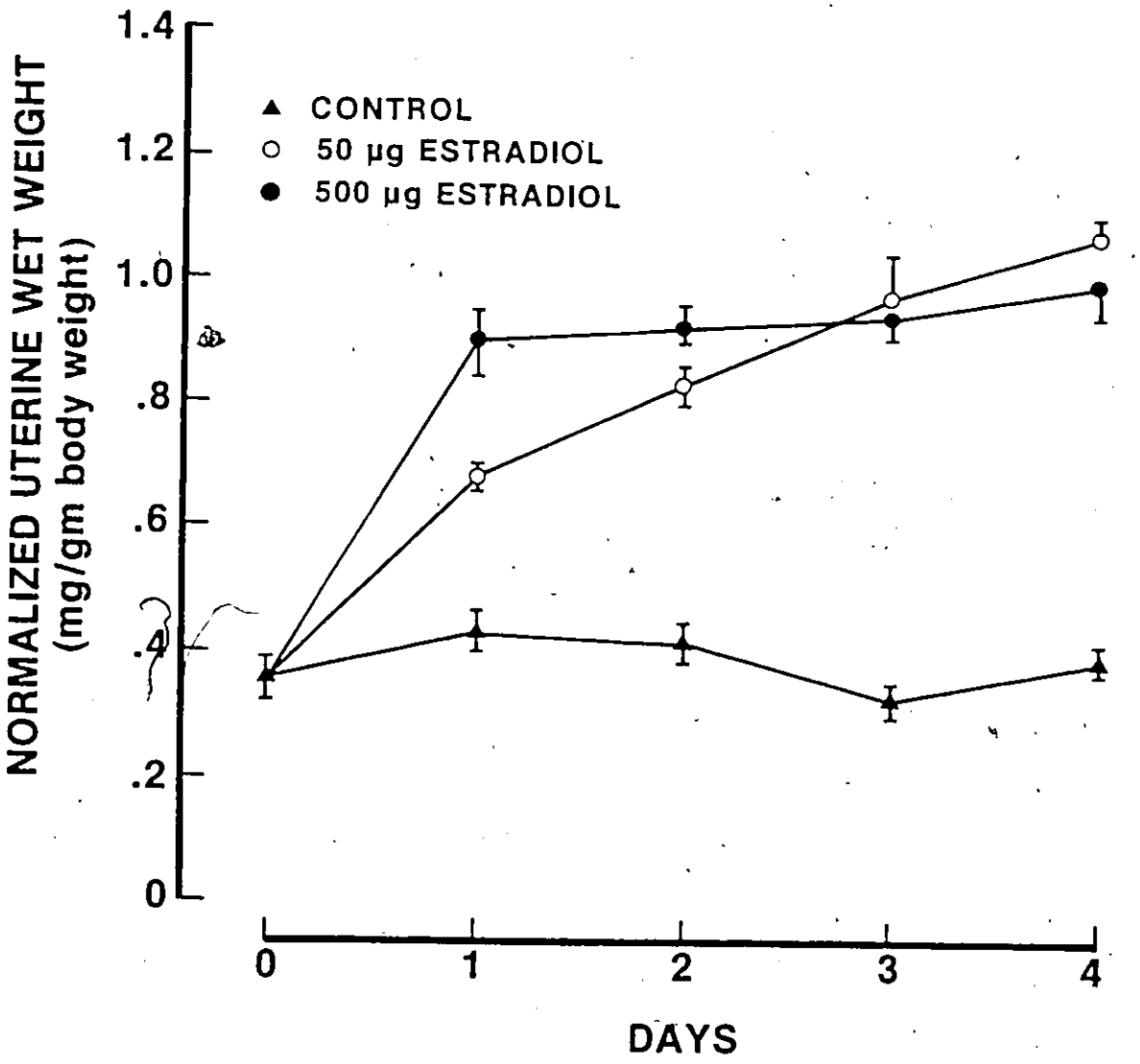

▲ CONTROL
○ 50 µg ESTRADIOL
● 500 µg ESTRADIOL
UTERINE WET (–) and DRY (---) WEIGHTS (gms)
.32
.28
.24
.20
.16
.12
.08
.06
.04
.02
0
0
1
2
3
4
DAYS

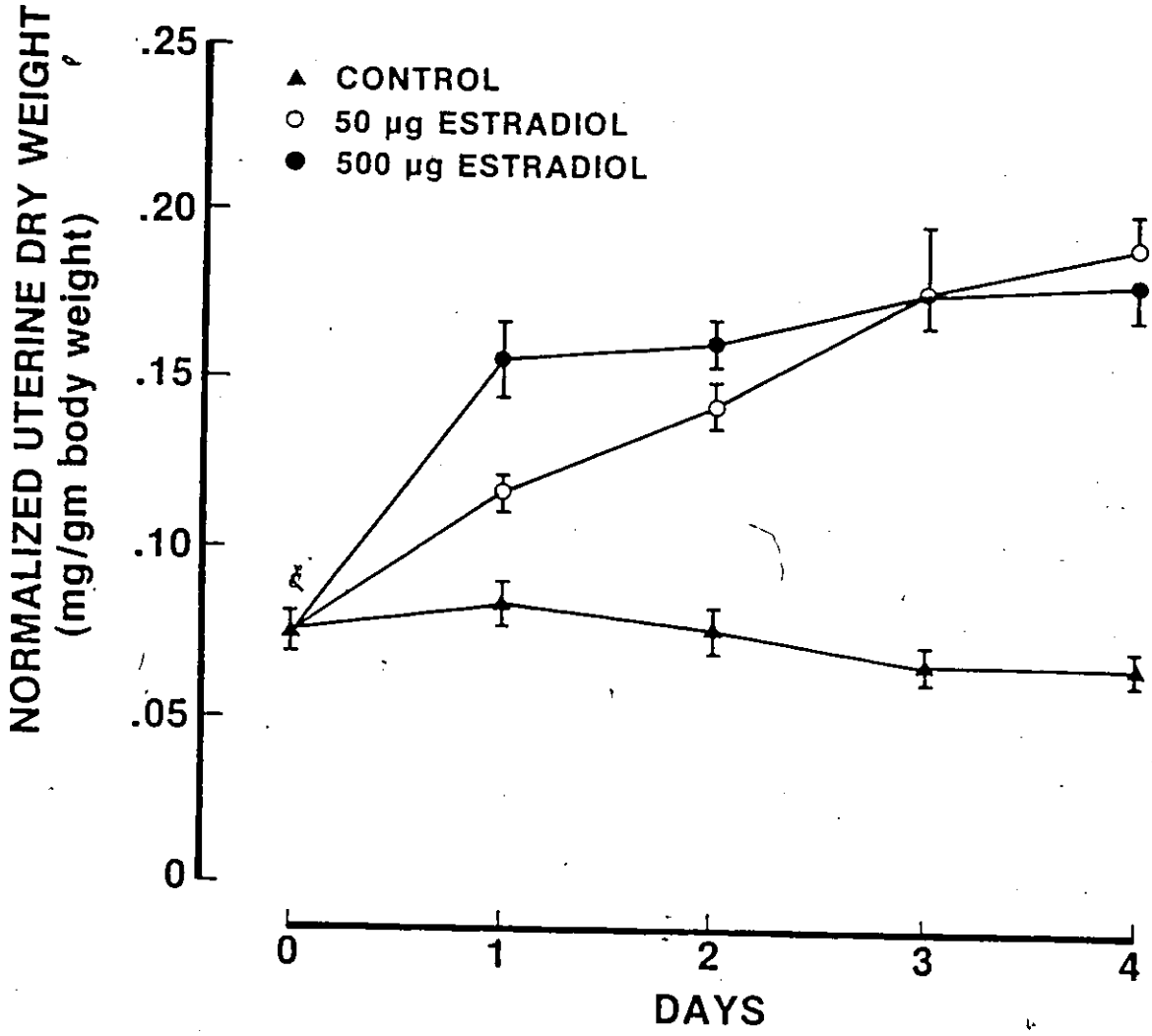
Figure 52. Effects of estradiol treatment on normalized uterine wet weight changes from ovariectomized mature rats over 1-4 days. Data are presented as mean values ± S.E. of 5-6 uteri on each day.


NORMALIZED UTERINE WET WEIGHT
(mg/gm body weight)
1.4
1.2
1.0
.8
.6
.4
.2
0
CONTROL
50 µg ESTRADIOL
500 µg ESTRADIOL
0
1
2
3
4
DAYS

over a 1-4 day period. The daily changes in normalized wet weight are similar to the absolute wet weight changes presented in Figure 51. Treatment with 50 ug/day estradiol resulted in a significant increase ($P<0.01$) in the normalized weight after one day of treatment compared to the control normalized uterine weight. The normalized uterine weight increased thereafter with further estradiol treatment to a maximum value at the end of the four day period. Similarly, in animals treated with 500 ug/day estradiol, the normalized weight was significantly elevated ($P<0.01$) after the first day of treatment compared to the control value, however, no further significant increase occurred with repeated injections of the hormone. No significant differences between the effects of 50 and 500 ug/day doses of estradiol were observed on any day of the four day period. Normalized wet weights from control animals were similar from zero time over the course of the experiment.

Normalized uterine dry weights from ovariectomized mature rats treated for 1-4 days with 50 and 500 ug/day estradiol are shown in Figure 53. Administration of 50 ug/day estradiol caused a significant increase ($P<0.05$) in the normalized dry weight after the first day of treatment compared to the mean normalized dry weight from control animals on the same day. Normalized dry weight increased further with repeated hormone injections to a maximum value after four days of treatment (0.190 ± 0.010 mg/gm body weight, $P<0.001$ when compared to the control value). Estradiol at 500 ug/day also increased the normalized dry weight to a near maximum value (0.156 ± 0.014 mg/gm body weight) twenty-four hours after the first injection, representing a significant increase ($P<0.05$) when compared to the control value. With

Figure 53. Profiles of normalized dry weight from ovariectomized mature rats treated with estradiol for 1-4 days. Data are presented as mean values ± S.E. of 4-6 uteri on each day.


NORMALIZED UTERINE DRY WEIGHT
(mg/gm body weight)
.25
.20
.15
.10
.05
0
▲ CONTROL
○ 50 µg ESTRADIOL
● 500 µg ESTRADIOL
0
1
2
3
4
DAYS

continued treatment with 500 ug/day estradiol, the normalized dry weight increased but not significantly over the last three days of the treatment period. All mean normalized dry weights from 50 and 500 ug/day estradiol treated animals were significantly larger ($P<0.05$ to $P<0.001$) than normalized control weights from zero time over the four day period. There were no significant differences between the effects of either dose of estradiol over the 1-4 days of treatment. Normalized uterine dry weights from control animals showed a slight insignificant decrease for zero time over the same four day period.

Effects of various doses of estradiol (up to and including 50 ug/day) on uterine wet and normalized wet weight in ovariectomized mature rats was also determined after six days of treatment. Administration of estradiol at 0.025, 0.10 and 50 ug/day for six days increased uterine weight in a dose-responsive manner (Table 14). Mean wet weights measured after treatment with 0.10 and 50 ug/day estradiol were significantly larger ($P<0.05$ and $P<0.001$) compared to the control uterine weights and the mean values after treatment with 0.025 ug/day estradiol.

6.2.2.2 Effects of indomethacin:

To determine the effects of indomethacin on estradiol stimulated increases in uterine wet weight in ovariectomized mature rats, indomethacin (5 or 10 mg/kg body weight twice daily) was injected alone or together with estradiol (0.025, 0.10 and 50 ug/day) for three or six days. Table 15 shows the uterine wet weight from ovariectomized mature rats treated with and without indomethacin. In animals treated

Table 14. EFFECTS OF ESTRADIOL ON UTERINE WEIGHT IN OVARIECTOMIZED MATURE RATS

Treatment	Number of Tissues	Body Weight (gm)	Wet Weight (mg)	Normalized Wet Weight (mg/gm body weight)
6 days				
Control	7	265 ± 3	104 ± 9.70[a]	0.39 ± 0.03[A]
Estradiol				
0.025 ug/day	7	271 ± 8	117 ± 12.0[a]	0.43 ± 0.04[A]
0.10 ug/day	7	278 ± 6	168 ± 15.0[b]	0.61 ± 0.05[B]
50 ug/day	7	258 ± 11	370 ± 14.0[c]	1.43 ± 0.09[C]

Data presented as mean values ± S.E.

Mean values with same superscript letters within the same column are not significantly different (P>0.05).

TABLE 15. EFFECTS OF INDOMETHACIN (IDM) ON UTERINE WET WEIGHT OF OVARIECTOMIZED MATURE RATS

Treatment	Number of Tissues	Uterine Wet Weight (mg)
3 days		
Control	7	93 ± 12
IDM (5 mg/kg body weight)	7	109 ± 4.0 *
6 days		
Intact Control	7	252 ± 22
Intact plus IDM (10mg/kg body weight)	7	234 ± 26
Ovariectomized Control	7	104 ± 10
Ovariectomized plus IDM	7	157 ± 13 **

Data presented as mean values ± S.E.

Levels of significance: * $P<0.05$; ** $P<0.01$.

with indomethacin (5 mg/kg body weight twice daily for three days) the uterine wet weight showed a slight, but significant ($P<0.05$), increase (21%) after three days of treatment compared to the control values. Administration of indomethacin (10 mg/kg body weight twice daily) for six days to ovariectomized mature rats also resulted in a significant increase ($P<0.01$) in uterine weight from 104 $\pm$ 9.70 mg in the control group to 157 $\pm$ 12.9 mg in the treated group (Table 15). Injections of indomethacin into intact cycling rats for the same period, however, produced no similar effect on uterine wet weight. Wet weights from intact animals were larger than those of the ovariectomized rats.

Table 16 shows uterine wet and dry weights from intact and ovariectomized mature rats treated for six days with indomethacin (10 mg/kg body weight twice daily). The intact control uterine weight was larger than that observed in Table 15, possibly because the two groups of animals may not have been in the same stage of the estrous cycle. However, the results again show that uterine wet weight, as well as dry weight, from the ovariectomized rats were significantly lower ($P<0.05$) than the intact controls. Furthermore, uterine wet and dry weights of ovariectomized animals treated with indomethacin were significantly greater ($P<0.005$) than the ovariectomized control group.

The effects of indomethacin on estradiol induced uterine wet and normalized wet weight in ovariectomized mature rats treated for six days is shown in Table 17. Treatment with indomethacin (2 mg twice daily) caused a significant increase ($P<0.01$) in uterine weight compared to the control animals treated with sesame oil alone. Simultaneous administration of indomethacin with 0.025 or 0.10 ug/day

TABLE 16. EFFECTS OF INDOMETHACIN (IDM) ON UTERINE WET AND DRY WEIGHTS OF OVARIECTOMIZED MATURE RATS

Treatment	Number of Tissues	Wet Weight (mg)	Dry Weight (mg)
6 days			
Intact Control	10	504 ± 36 *	94 ± 6.0 *
Ovariectomized Control	8	104 ± 8.2 *	25 ± 1.6 *
Ovariectomized plus IDM (10 mg/ kg body weight)	10	232 ± 34	46 ± 5.6

Data are presented as mean values ± S.E.

Level of significance: * $P<0.05$.

TABLE 17. EFFECTS OF INDOMETHACIN (IDM) ON ESTRADIOL (E_2) INDUCED UTERINE WEIGHT OF OVARIECTOMIZED MATURE RATS

Treatment	Number of Tissues	Body Weight (gm)	Wet Weight (mg)	Normalized Wet Weight (mg/gm body weight)
6 days				
Control	7	265 ± 3	104 ± 10	0.39 ± 0.03
IDM (2 mg twice daily)	7	239 ± 5	157 ± 13**	0.66 ± 0.05**
E_2 (0.025 ug/day)	7	271 ± 8	117 ± 12	0.43 ± 0.04
E_2 plus IDM	7	241 ± 6	167 ± 12*	0.69 ± 0.05*
E_2 (0.10 ug/day)	7	278 ± 6	168 ± 15	0.61 ± 0.05
E_2 plus IDM	7	238 ± 9	243 ± 37*	1.02 ± 0.15*
E_2 (50 ug/day)	7	258 ± 11	370 ± 14	1.43 ± 0.09
E_2 plus IDM	7	230 ± 7	360 ± 6	1.56 ± 0.07

Data presented as mean values ± S.E.

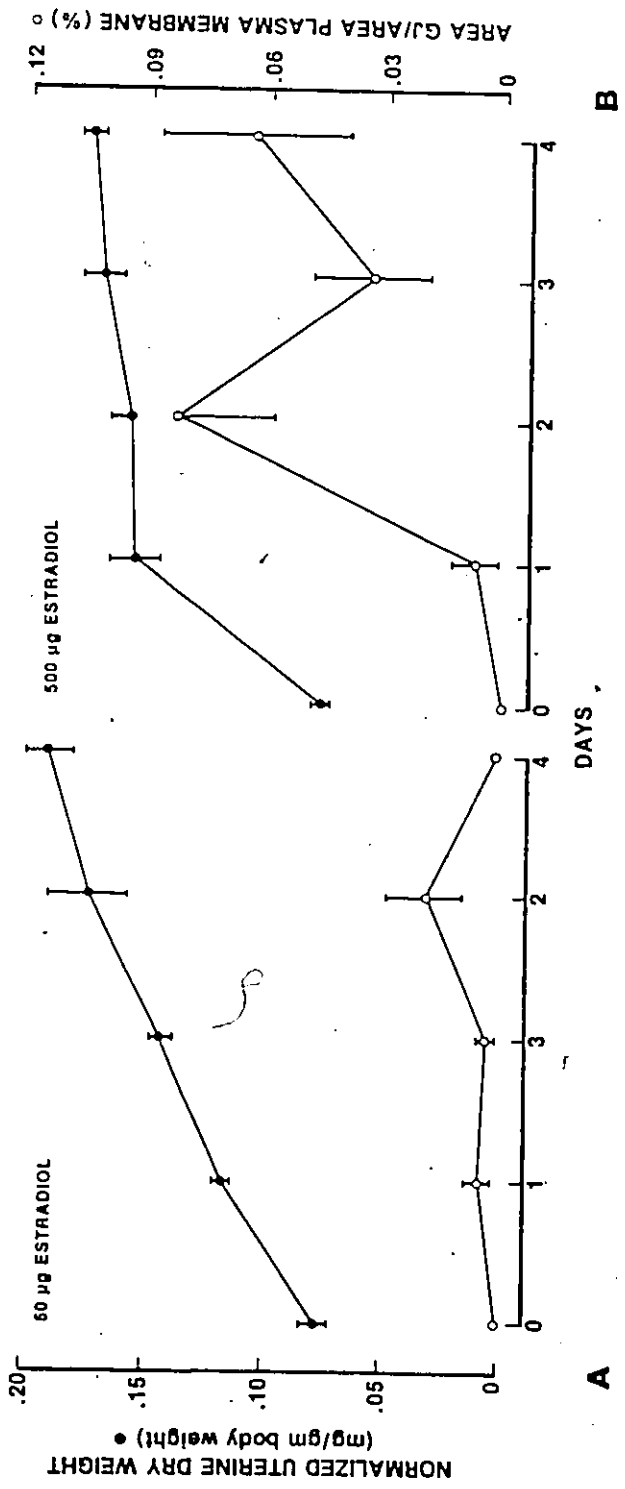
Levels of significance: * $P<0.05$; ** $P<0.01$.

estradiol resulted in uterine wet and normalized wet weight increases (167 ± 12.0 and 243 ± 37 mg, and 0.693 ± 0.049 and 1.021 ± 0.155 mg/gm body weight respectively) which were significantly greater ($P<0.05$) than weight gains due to the estradiol doses alone (117 ± 12.0 mg and 168 ± 15.0 mg, and 0.432 ± 0.044 and 0.604 ± 0.053 mg/gm body weight, respectively). However, when the dose of estradiol was increased to 50 ug/day, concomitant administration of indomethacin caused no further increase in uterine wet weight beyond that attributable to estradiol. Indomethacin treatment caused a non-significant decrease in the mean body weight of the ovariectomized rats (Table 17).

6.2.2.3 Relationship between uterine weight increase and presence of myometrial gap junctions in ovariectomized mature rat:

The relationship between normalized uterine dry weights and the percentage of myometrial membrane area of gap junctions in ovariectomized mature rats treated with 50 and 500 ug/day estradiol for 1-4 days is shown in Figure 54. The effects of estradiol on normalized dry weights and myometrial gap junctions over the four day period have previously been described (see Figure 53 and Chapter 4). Normalized uterine dry weight increased progressively in response to treatment with 50 ug/day estradiol over the four day period (Figure 54A). In myometrial tissues from animals treated with this dose of estradiol, the percentage of membrane area occupied by gap junctions was very low over the same four day treatment period (Figure 54A). The increase in the normalized dry weight of the ovariectomized mature rat uterus over 1-4 days of estradiol treatment occurred when gap junctions occupied a

Figure 54. Changes in normalized uterine dry weight and area of myometrial gap junctions in ovariectomized mature animals after treatment with estradiol at 50 (A) and 500 (B) ug/day for 1-4 days showing the relationship between time courses of the two events. Data are presented as mean values ± S.E. of 5-6 animals per group on each day.


NORMALIZED UTERINE DRY WEIGHT
(mg/gm body weight) •
.20
.15
.10
.05
0
60 µg ESTRADIOL
500 µg ESTRADIOL
A
B
0
1
2
3
4
DAYS
AREA GJ/AREA PLASMA MEMBRANE (%) ○
.12
.09
.06
.03
0

very small percentage of the myometrial membrane area.

The normalized dry weight of the ovariectomized mature rat uterus increased to a peak value after one day of treatment with 500 ug/day estradiol (Figure 54B). Continued treatment with this dose of estradiol for three more days had no significant influence on the normalized dry weight beyond that measured on day one. The percentage of myometrial membrane area occupied by gap junctions was extremely low (0.013 ± 0.007 percent of the membrane) after the first injection of estradiol at 500 ug/day (Figure 54B). Twenty-four hours after the second injection of estradiol (500 ug/day), the percentage of membrane area occupied by the junctions was significantly increased ($P<0.05$) to 0.082 ± 0.030 percent. Throughout the remainder of the four day period the junctional membrane area decreased, however, the decrease was not significant compared to the maximum percentage value after two days of treatment. Uterine weight increases in response to 500 ug/day estradiol treatment occurred only when the membrane area of gap junctions in the myometrium was extremely low. The presence of gap junctions between the myometrial cells following treatment with this dose of estradiol occurred only after no further increase in weight of the uterus was observed.

Table 18 shows the relationship between the effects of various doses of estradiol plus indomethacin on the normalized uterine wet weight and myometrial gap junction area in ovariectomized mature rats treated for six days. The effect of estradiol (0.025, 0.10 and 50 ug/day) on the normalized uterine wet weight after six days of treatment was accompanied by a low frequency of gap junctions in the

TABLE 18. EFFECTS OF INDOMETHACIN (IDM) AND ESTRADIOL (E_2) ON NORMALIZED UTERINE WET WEIGHT AND MYOMETRIAL GAP JUNCTIONS IN OVARIECTOMIZED MATURE RATS

Treatment	Normalized Wet Weight (mg/gm body weight)	Area GJs/Area Plasma Membrane,% (Mean ± S.E.)
Control	0.392 ± 0.036	0.002 ± 0.002
IDM (2 mg twice daily)	0.656 ± 0.053**	0.005 ± 0.005
E_2 (0.025 ug/day)	0.432 ± 0.044	0
E_2 plus IDM	0.693 ± 0.049*	0
E_2 (0.10 ug/day)	0.604 ± 0.053	0.002 ± 0.002
E_2 plus IDM	1.021 ± 0.155*	0.007 ± 0.007
E_2 (50 ug/day)	1.430 ± 0.092	0.021 ± 0.010
E_2 plus IDM	1.565 ± 0.073	0.336 ± 0.133*

Data presented as mean values ± S.E.

Levels of significance: * $P<0.05$; ** $P<0.01$.

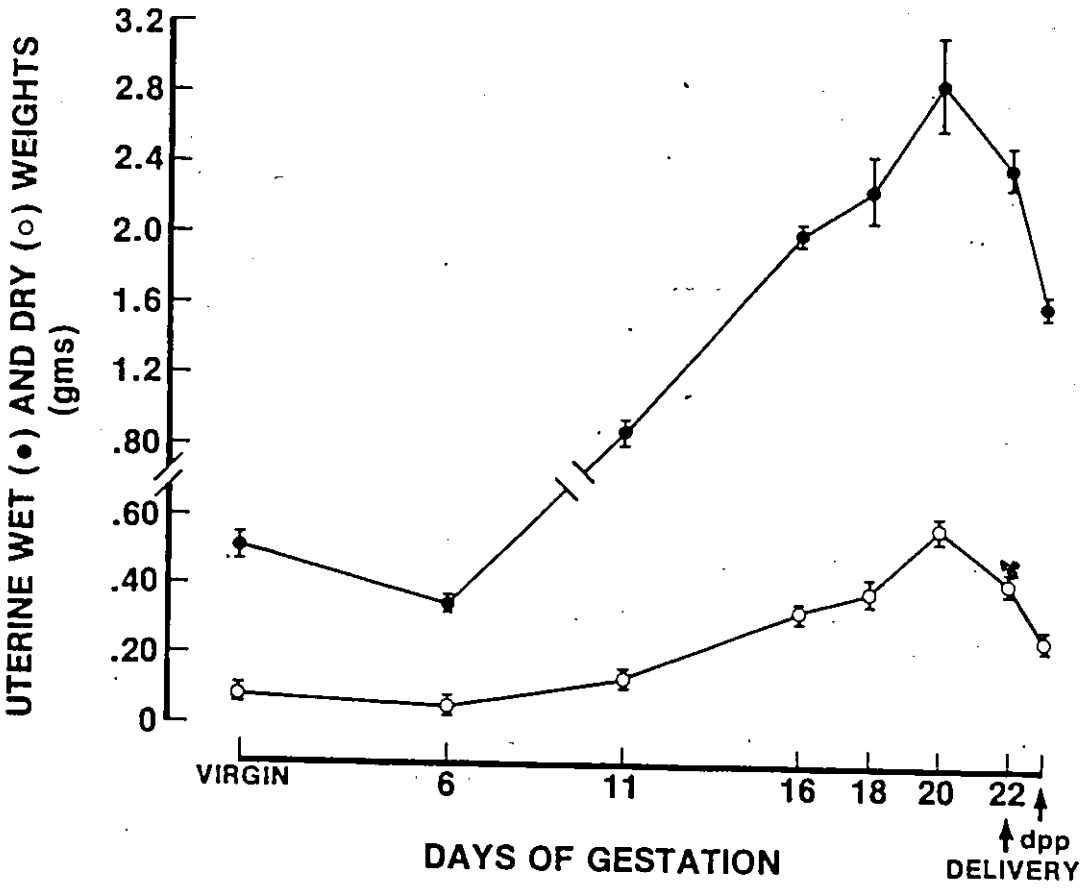
myometrium, occupying an extremely small percentage of the myometrial membrane area. Furthermore, concomitant injections of indomethacin together with estradiol (0.025 and 0.10 ug/day) enhanced the estradiol stimulated weight gains but did not influence the percentage of membrane area occupied by gap junctions. Indomethacin alone also stimulated an increase in the normalized uterine wet weight and had no effect on the junctional membrane area. Moreover, indomethacin administered together with 50 ug/day estradiol did not potentiate the wet weight gain induced by estradiol alone, but significantly enhanced the myometrial membrane area of gap junctions. Thus uterine weight increases occurred when the myometrial membrane area of gap junctions was extremely low. When no further increase in weight occurred the gap junctional membrane area was significantly elevated.

6.2.3 Pregnant rats:

Uterine wet and dry weights of pregnant rats were determined on various days of gestation, during parturition and one day post-partum. Figure 55 shows that uterine wet and dry weights decreased slightly from the non-pregnant virgin state until day 6 of gestation. Thereafter, uterine wet and dry weights increased progressively with gestational age to maximum weights on day 20 of pregnancy ($P<0.001$ when compared to the wet and dry weights determined on day 6 of pregnancy). Uterine weights decreased thereafter and the weights were significantly lower compared to day 20 ($P<0.05$ and $P<0.01$) twenty-four hours after parturition.

Uterine wet and dry weights, determined over the 22 day

Figure 55. Uterine wet and dry weight profiles from untreated pregnant animals throughout gestation, during delivery and one day post-partum (dpp). Note the expansion of the ordinate scale at .70 gms and on the wet weight curve. Data are presented as mean values ± S.E. of 4-5 uteri on each day.


UTERINE WET (•) AND DRY (○) WEIGHTS
(gms)
3.2
2.8
2.4
2.0
1.6
1.2
.80
.60
.40
.20
0
VIRGIN
6
11
16
18
20
22
dpp
DELIVERY
DAYS OF GESTATION

gestation period, during parturition and one day post-partum, normalized to rat body weight are illustrated in Figure 56. The normalized weight profiles are similar to that for the absolute wet and dry weight changes shown in Figure 56. Normalized wet and dry weights decreased slightly over the first week of pregnancy. The uterine weights were increased by day 11 of gestation, but were not significantly larger compared to the weight measurements on day 6. Thereafter, the normalized wet and dry weight increased significantly ($P<0.01$ and $P<0.05$) to near maximum values on day 16 of gestation. Normalized wet and dry weights increased insignificantly to maximum weights on day 20 of pregnancy which were maintained during parturition and then the weights decreased significantly ($P<0.05$) twenty-four hours post-partum.

6.2.3.1 Relationship between uterine weight increase and the presence of myometrial gap junctions in pregnant rats:

Figure 57 shows the normalized uterine dry weights in relationship to the myometrial membrane area occupied by gap junctions in untreated pregnant animals on various days of gestation, during parturition and twenty-four hours after delivery of the fetuses. The normalized uterine dry weight and myometrial gap junction profile have been described above (see Figure 56) and in Chapter 4. Normalized dry weight of uterine tissue from pregnant animals increased progressively from day 6 through day 20 of gestation at which time a peak of normalized dry weight was reached. This weight was maintained during parturition followed by a significant decline one day after labor.

Figure 56. Normalized uterine wet and dry weight profiles from pregnant animals throughout gestation, during delivery and one day post-partum (dpp). Data are presented as mean values ± S.E. of 4-5 uteri on each day.

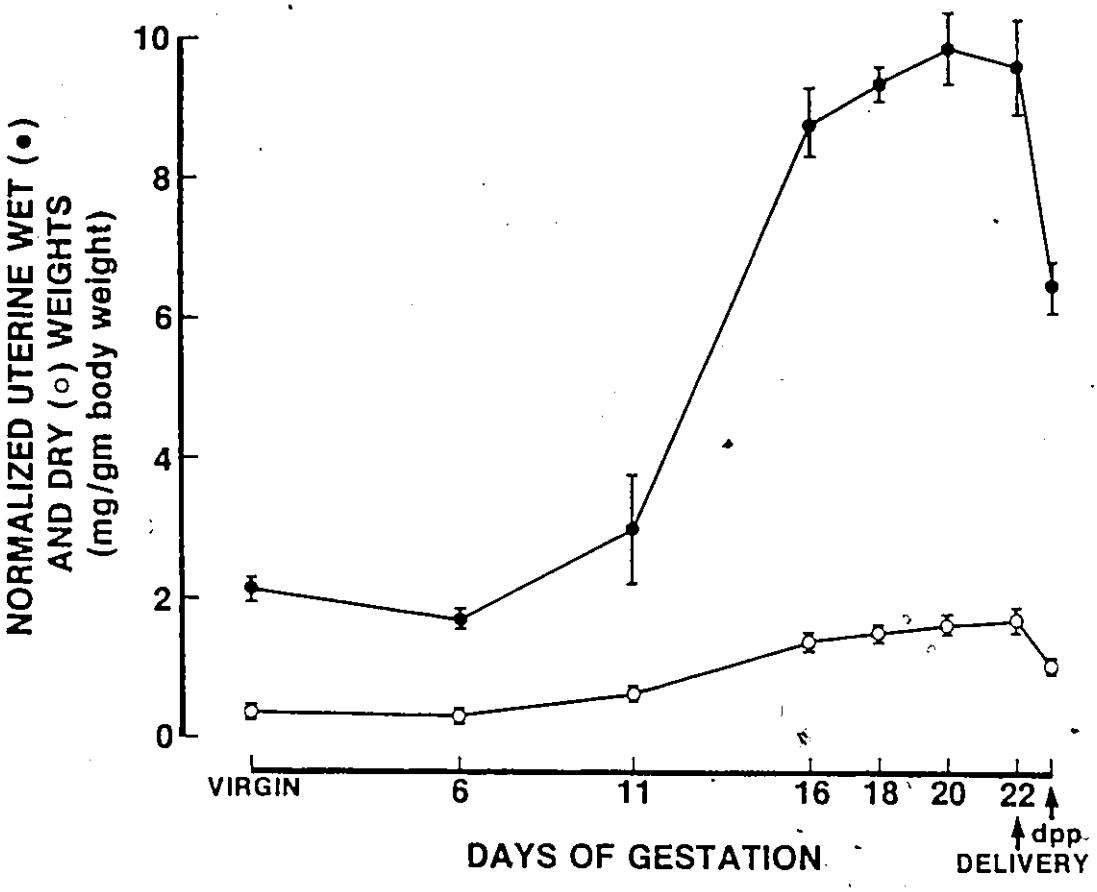
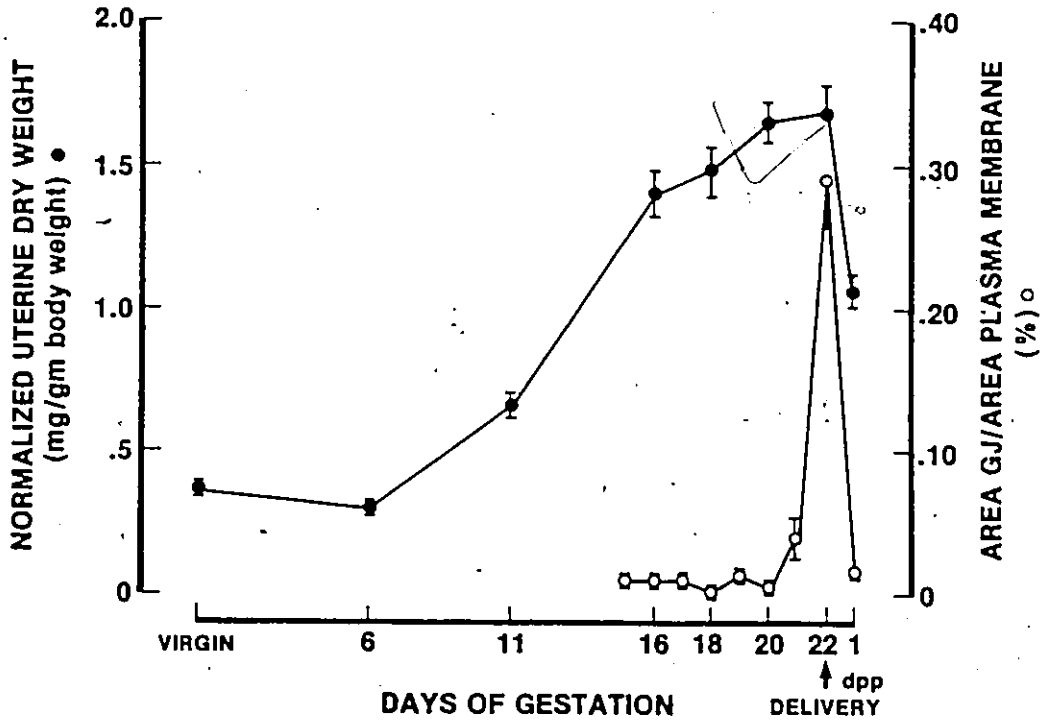

NORMALIZED UTERINE WET (•)
AND DRY (○) WEIGHTS
(mg/gm body weight)
10
8
6
4
2
0
VIRGIN
6
11
16
18
20
22
dpp
DELIVERY
DAYS OF GESTATION

Figure 57. Changes in normalized uterine dry weight and area of gap junctions in the myometrium of pregnant animals throughout gestation, during delivery and one day post-partum (dpp) illustrating the relationship between the temporal patterns of the two events. Data are presented as mean values ± S.E. from 4-6 animals per group on each day.


NORMALIZED UTERINE DRY WEIGHT
(mg/gm body weight) ●
2.0
1.5
1.0
.5
0
.40
.30
.20
.10
0
AREA GJ/AREA PLASMA MEMBRANE
(%) ○
VIRGIN
6
11
16
18
20
22
1
dpp
DELIVERY
DAYS OF GESTATION

Myometrial tissues examined for the presence of gap junctions revealed that the junctions were undetectable or present at an extremely low frequency occupying a very small percentage of the muscle membrane area throughout gestation (Figure 57). The membrane area of gap junctions began to increase on day 21 of gestation, immediately prior to labor, reaching maximum levels during delivery. Twenty-four hours after delivery, the junctional membrane area significantly decreased ($P<0.001$) to preterm levels (Figure 57). The rapid and progressive increase in normalized dry weight of the uterus from pregnant rats occurred when gap junctions were infrequent in the myometrium. When no further increase in uterine weight was evident, the myometrial membrane area of gap junctions increased. Gap junction area rapidly declined twenty-four hours post-partum when the uterine weight also decreased.

6.3 Discussion

The observations reported in this study show that low and high doses of estradiol stimulate an increase in the wet and dry weight of the immature rat uterus. Moreover, the antiestrogens progesterone and tamoxifen partially suppress the estradiol induced increase in uterine wet weight. Cross-sectional area analysis of uterine tissue layers revealed that the myometrium was a major portion of the total area of the uterus prior to and after treatment with estradiol. The increase in weight of the uterus in response to estradiol results from both hyperplasia and hypertrophy with the former growth process preceding the latter. Light microscopy autoradiography showed that hyperplasia of uterine cells occurred in both the myometrial and endometrial tissue

layer. Furthermore, indomethacin appears to have a uterotropic action in the uterus. In addition, the present study also demonstrates a similar effect of estradiol and indomethacin on uterine weight in ovariectomized mature rats and shows the uterine weight profile from untreated pregnant rats throughout gestation, during parturition and one day post-partum.

In this study I have also evaluated the possible association between uterine and myometrial growth and the presence of gap junctions in the myometrium in response to a variety of treatments. The results show that gap junction area is low after treatments which stimulate growth, and that gap junctions appear after some treatments when growth ceases. These results raise the following questions: 1) Is the absence of gap junctions necessary for growth? 2) When gap junctions form does growth cease? 3) Is growth necessary for the presence of gap junctions?

6.3.1 Regulation of uterine growth

6.3.1.1 Hypertrophy versus hyperplasia:

6.3.1.1a Immature rats:

The present study shows that repeated injections of estradiol (50 and 500 ug/day) similarly stimulate uterine weight (absolute and normalized weight values) in the immature rats. The weight increase can result from either hypertrophy (increase in cell size) or hyperplasia (cell multiplication) of uterine cells. This growth effect of estradiol on the uterus is illustrated in the myometrium and

endometrium, however, the increase in the myometrial area accounts for the major portion (approximately 60%, Figure 33) of the total increase in the area of the uterus after estradiol treatment. This study demonstrates that estradiol induced an increase in the amount of uterine tissue dry mass of approximately 600% ([final weight-initial weight/initial weight] X 100). This suggests the involvement of synthetic mechanisms in estradiol induced growth, but it does not differentiate hypertrophy from hyperplasia as the cause. It is evident, however, that estradiol administration stimulates DNA synthesis, as measured by the incorporation of tritiated-thymidine into DNA (Figure 36). Treatment with estradiol resulted in a net synthesis of DNA and, as compared with the increase in dry mass, an equal increase in the total amount of uterine DNA (Figure 37). These results indicate cellular multiplication (hyperplasia) in the growth promoting effect of estradiol in the immature rat uterus. However, total DNA does not indicate which layer (i.e. myometrium or endometrium) of the uterus involved in the hyperplastic process.

Total uterine DNA content increases in response to estradiol reaching maximum levels after four days of treatment (Figure 37), whereas, tritiated-thymidine incorporation into DNA peaks after one injection (Figure 36). Thymidine incorporation is a measure of cells preparing to divide but at that time have not undergone the process of division. As a result, the DNA synthesized after one injection of estradiol would not be measured until the assay on day two of the treatment period. The increase in total DNA content determined on day one therefore, represents the DNA present in the tissue before

estradiol stimulation and from cells that have divided between zero time and twenty-four hours after the first injection of the hormone. Likewise, the DNA measured after two days of treatment reflects the DNA synthesized between days one and the time of the assay on day two. A second, but smaller peak, of tritiated-thymidine incorporation also occurred after three days of estradiol treatment indicating that a population of uterine cells were preparing to undergo a second round of DNA synthesis. Although the second peak of thymidine incorporation was below that observed in control animals, the DNA synthesized would not be detectable until the assay twenty-four hours later. This data would explain the increase and peak total uterine DNA content on day four of the treatment period.

The results (Figure 36) show a peak of tritiated-thymidine incorporation in the immature uterus twenty-four hours after the first injection of estradiol indicating that cells were preparing to undergo division. Twenty-four hours after the second injection of estradiol, however, incorporation of labeled thymidine into DNA was less than that which occurred after the first injection and below that in control rats. The decrease in DNA synthesis was sustained with repeated injections of estradiol over six days. These results suggest that DNA synthesis and cell division become refractory to multiple injections of estradiol. The underlying basis for this suppression is not known but it has been demonstrated that the sustained presence of estradiol is necessary to evoke the observed state of uterine unresponsiveness and that the refractoriness is dose dependent (Stormshak et al., 1976). Uterine refractoriness in DNA synthesis may be due to the lack of

available cytoplasmic receptors since it has been reported that consecutive daily treatments of estradiol resulted in a reduction in estradiol receptors (Manni et al., 1981). However, since depletion of uterine cytoplasmic receptors in their study occurred much later than DNA refractoriness observed in this study (Figure 36) after repeated estradiol treatment, this is not the likely mechanism responsible for the reduction in stimulation of DNA synthesis. Alternatively, the ability of receptors to bind estradiol or lack of translocation of the estradiol receptor complex to the nucleus could account for these observations. Stormshak et al., (1976) have reported that specifically bound uterine nuclear estradiol after successive daily injections of the hormone was similar to that of control animals. These observations indicate that cytoplasmic receptors were available for translocation of estradiol to the nucleus after repeated injections. Furthermore, the measurable quantities of nuclear bound estradiol demonstrated in that study were not due to retention of estradiol-receptor complex after hormone treatment because levels of specifically bound estradiol in the nucleus twenty-four hours after one, two or three days of treatment were low and comparable to that present in uterine nuclei of control rats. Thus it appears that the uterine refractoriness phenomenon, once induced, is independent of the ability of receptors to bind estradiol or of the hormone-receptor complex to be translocated into the nucleus. Metabolic activities which occur prior to DNA synthesis and cell division, but are essential for the latter event, are also inhibited by continuous estradiol administration (Stormshak et al., 1976). These observations imply that cells may be blocked in the resting or

presynthetic stage of the cell cycle. This could account for the suppression in uterine cell proliferation because the effect of estradiol on uterine growth is believed to be at the expense of the presynthetic period: estradiol treatment decreases the duration of this stage of the cycle (Stormshak et al., 1978).

Although the mechanism through which chronic estradiol administration inhibits uterine DNA synthesis remains to be ascertained, it is evident nevertheless, that estradiol under these conditions alters a critical step(s) required for DNA synthesis and cell division through limiting the ability of cells to respond in this manner to the steroid hormone. Since, in the presence of large doses of estradiol the refractoriness is irreversible as compared to lower dose of the hormone, estradiol in a dose responsive manner may cause the accumulation or synthesis of a specific product which inhibits the estrogenic response of DNA synthesis (Stormshak et al., 1976). A further understanding of this phenomenon therefore may enhance our comprehension of the regulation of cellular hyperplasia in steroid target tissues. Moreover, the effect of continuous estradiol exposure on cellular proliferation may explain why chronic estrogen therapy is effective as treatment in certain steroid-dependent tumors (Kaufman, 1981).

The increase in weight of the uterus is due to hyperplasia and hypertrophy of uterine cells. The dry weight of the immature rat uterus in response to either low or high doses of estradiol over the 1-6 day period increased by a factor of approximately 7 from 3.75 mg to 26.4 mg (Figure 31) during which time the DNA, and presumably the

number of cells, increased by a factor of about 3 (from 43.14 ug to 134 ug/uterus, Figure 37). The average cell size must therefore have increased by a factor of about 2.35. This indicates that hyperplasia of uterine cells occurred to a slightly greater extent than did hypertrophy of the cells over the treatment period. Moreover, the relative rapidness in the onset of DNA synthesis (tritiated-thymidine incorporation) would suggest that hyperplasia occurred prior to hypertrophy of uterine cells in response to estradiol treatment. As far as I'm aware there are no other similar detailed analysis of immature animals. In addition, the myometrial cross-sectional area accounts for approximately 60% of the total uterine area before and after estradiol treatment (Figure 33). Moreover, the cross-sectional area of the myometrium increased to near a plateau level after three days of treatment. Since cellular hyperplasia occurred over the first twenty-four hours after estradiol treatment (see above), the further increase in area of the myometrium must have resulted from hypertrophic growth of the cells. Although a detailed study on cell diameter was not carried out, the myometrial cells appeared larger in thin sections after estradiol treatment for six days indicating hypertrophic growth. Similarly, cross-sections of the uterine wall as seen under the light microscope show larger cells after the treatment period compared to control tissues (Figure 34).

Estradiol induced cell division in both myometrial and endometrial tissues. Autoradiography of the rat uterus labeled with tritiated thymidine twenty-four hours after each daily treatment with estradiol over the 1-6 day period revealed a nuclear labeling index in

both the myometrium and endometrium (Figure 38). The number of labeled nuclei in these tissues was greater than control uteri one day after the first injection. Thereafter, the nuclear labeling index fell and was below control values over the remainder of the six day period. Similar results have been reported by other groups suggesting that larger doses of estradiol stimulates myometrial and endometrial cell division (Kaye et al., 1972; Kirkland et al., 1979; 1981; Mukka et al., 1982). These results also show that the changes in nuclei labeling index are closely correlated with increased thymidine incorporation into DNA assayed during in vitro incubation of the rat uterus. It is interesting to note that when comparing DNA synthesis (thymidine incorporation, Figure 36) and nuclear labeling indices (Figure 38), the second smaller peak of thymidine uptake on day three was correlated with a corresponding increase in the number of labeled cells in the endometrium. These results suggest that a population of cells in the endometrium entered a second round of DNA synthesis. Luminal and glandular epithelial cells account for approximately 10% of the total uterine cell population (Kirkland et al., 1979) and could account for the increased thymidine incorporation and nuclear labeling if they were to undergo DNA synthesis two days after the initial peak on day one. Alternatively, DNA synthesis may occur in the luminal epithelium and endometrium after one day of treatment and the second increase on day three may occur in the glandular cells (Tachi et al ., 1972; Kirkland et al., 1981). The uterus is a complex target organ for estradiol and the epithelial, endometrial and myometrial cell types may differ in their ability to respond to estrogenic stimulation by entering into DNA

synthesis and cell division . The possibility that a population of cells undergoes a second round of DNA synthesis or responds to estradiol on a different time scale is likely (Quarmby and Korach, 1984).

A single injection of either low or high doses of estradiol induced increases in uterine wet and normalized wet weight to peak values three days after the injection. Thereafter there was a decrease in the wet weight of the uterine tissues in all estradiol treated animals (Figures 39 and 40). Although a detailed investigation into the effects of a single injection of estradiol on uterine growth parameters was not carried out, it is evident that a single injection of estradiol stimulated an increase in uterine mass. Other studies show that DNA synthesis after a single injection of estradiol returns to pretreatment values 36 to 48 hours after hormone treatment (Kirkland et al., 1981; Mukka et al., 1982). This suggests that uterine growth under the conditions of this study may also result from hyperplasia and hypertrophy of uterine cells as described previously. It appears therefore, that a single injection of estradiol stimulates uterine growth but it is not capable of maintaining the growth over a six day period. The decrease in uterine growth three days after a single injection of estradiol may reflect a withdrawal of the hormone resulting in uterine atrophy. These results have further significant implications in explaining the effect of consecutive daily injections of estradiol on DNA synthesis. Twenty-four hours after estradiol treatment thymidine incorporation into DNA was maximal (Figure 36) followed by a decrease to levels similar to or below that in zero time

controls after a single or two days of hormone treatment. The peak tritiated thymidine incorporation 24 hours after one injection of estradiol indicates cells preparing to undergo DNA synthesis and cell division. Once triggered into the DNA synthetic stage of the cell cycle, the sequence of replication and division goes to completion (Martin et al., 1976). Therefore, administration of the second injection of estradiol on day one may not influence DNA synthesis because the cells have not completed the replication and division process. Only after the process is over would a response be observed as demonstrated by the smaller peak of thymidine incorporation on day three of the treatment period. This may mean that estradiol treatment does not suppress or inhibit DNA synthesis but rather cells cannot respond to the hormone.

6.3.1.1b Ovariectomized mature rats:

Estradiol induced increases in absolute and normalized wet and dry weights of uterine tissue of ovariectomized mature rats over a four day period. These results are somewhat similar to the results seen with immature rats (see above). There are, however, differences in uterine weight increases between ovariectomized mature and immature rats in response to estradiol (compare Figures 26, 28 and 55, 56). The time required to attain a near maximum growth response in the ovariectomized mature animals treated with estradiol (1-2 days) is much shorter than the time in the immature rats (3 days). This time difference indicates that the steroid receptors or some other step in

the mechanism responsible for uterine growth is not exactly the same in the two groups of animals. The mature rats have been exposed to estradiol previously so they may have some steroid receptors available for a hormonal response following ovariectomy. However, this seems unlikely since receptor assays have reported very low numbers of the steroid receptor in mature rats 3 weeks after the surgery (Feherty et al., 1970). It is possible that the time required to generate new receptors after hormonal treatment may be more rapid in ovariectomized mature animals.

The results of this study demonstrate uterine growth in ovariectomized mature rats treated with estradiol, however, the growth process(es) responsible for the increase has not been ascertained but presumably they are the same as in the immature rat. The observations from the immature animals would suggest that uterine growth in the mature rats may result from both hyperplasia and hypertrophy (see above). That the cross-sectional size of myometrial cells are larger after estradiol treatment compared to control cells supports hypertrophic growth similar to that observed in the immature animals.

6.3.1.1c Pregnant animals:

The uterus of untreated pregnant animals increased dramatically during gestation, beginning after the first week of pregnancy to reach a maximum weight prior to parturition (Figures 55 and 56). I did not carefully try to characterize the contribution of hyperplasia and hypertrophy to the growth process during pregnancy but from earlier

observations in the immature and ovariectomized mature rats it is likely that both forms of growth would play a role in the pregnant uterus. The growth of the uterus during pregnancy is regulated systematically by the steroid hormones and locally by the presence of the fetus(es) and, in some species, by local secretion of hormones by the placenta (Carsten, 1968). There is, however, some concern in the relative roles of hypertrophic and hyperplastic growth processes in the uterus throughout pregnancy as induced by these stimulations. It has been reported that hyperplasia and hypertrophy contribute equally (Afting and Elce, 1978) to the uterine growth during pregnancy, while others suggest that hypertrophic growth occurs to a greater extent (Carsten, 1968). Moreover, Carsten (1968) suggests that growth promoting effects of the steroid hormones and growing fetus consist of hyperplasia and hypertrophy; hyperplasia is restricted to the early part of pregnancy; hypertrophy is associated with the slow growth throughout pregnancy. The period of cell division declines upon implantation and ceases by mid-pregnancy. Thereafter, the rapid growth of the uterus occurs simultaneously with the growth of the fetus. Thus, distention induced by the growing fetus(es) together with hormonal influences appear to regulate this growth period. The growth consists mainly of hypertrophy of all cells, however, the effect on the myometrium is more pronounced. This is similar to the observations in the non-pregnant animals in which the weight gain of the uterus after hormonal treatment was mainly due to an increase in the area of the myometrium (see above). As well, there is an increase in the collagen content adding to the growth and weight of the pregnant uterus

(Harkness and Morake, 1956; Ross and Klebanoff, 1967; Carsten, 1968). The uterus is believed to stop growing prior to parturition concomitant with the change in fetal shape from a spheroid to a cylinder or elongated shape, thereby altering the influence of distention on the uterine wall (Carsten, 1968).

My study also shows that uterine weight of pregnant animals was dramatically reduced one day after parturition (Figures 55 and 56). This decrease probably reflects uterine involution and atrophy in the absence of stimulation by the steroid hormones and fetus. Afting and Elce (1978) have reported that the rapid post-partum involution involves hypoplasia, accounting for approximately one third of the reduction in the size of the uterus, and hypotrophy of cells for the remaining two thirds in size reduction. Recently it has been shown that autophagocytosis in smooth muscle cells occurs as a means to reduce both cell number and size, and that macrophages and fibroblasts endocytose the collagen to further reduce the size of the uterus (Henell et al., 1983).

6.3.1.2 Summary:

Low and high doses of estradiol stimulate an increase in weight of the immature rat uterus. The weight increase in response to estradiol treatment is evident in both the myometrium and endometrium, however, the increase in size of the myometrium compared to the endometrium accounted for a greater proportion of the uterus. The increase in weight of the uterus resulted from hyperplastic and hypertrophic growth of myometrial and endometrial cells. The rapid

onset of hyperplastic growth and appearance of thymidine nuclear labeled cells compared with the time of maximal myometrial growth implies that cellular hyperplasia preceded hypertrophic growth after treatment with estradiol. Although the studies on ovariectomized mature rats treated with estradiol and on untreated pregnant animals were not as detailed as the study of the immature rats, the data suggests, together with published information from other investigators, that myometrial growth in both groups of animals results from both hyperplasia and hypertrophy.

6.3.2 Control by steroid hormones

6.3.2.1 Steroid receptor mechanism:

6.3.2.1a Estrogen:

The results discussed above show that estradiol stimulates uterine hyperplasia and hypertrophy in the rat. These results, however, do not indicate the mechanism through which the steroid hormones work in inducing the effects. It is possible that the effects of estradiol are mediated through adenylate cyclase and cAMP or prostaglandins as described below, however, it is generally believed that the mechanism of action of estradiol is through the steroid receptors and the classical receptor mechanism is described in detail in Chapter 1. Briefly this theory is based on the assumptions that estradiol enters cells, target tissues contain receptors, the receptors in the absence of estradiol are located in the cytoplasm, the estradiol receptor

complex is translocated to the nucleus, and a consequence of the presence of the estradiol receptors in the nucleus is an alteration in the pattern of gene transcription. The ultimate effects of estradiol stimulation are increased RNA and protein synthesis leading to cell growth, and DNA synthesis necessary for cellular proliferation. The following discussion supports this mechanism of steroid action in the rat uterus and suggests that an estradiol-receptor mechanism is responsible for effects on uterine growth.

6.3.2.1b Progesterone:

Simultaneous treatment with progesterone plus estradiol results in a restrictive effect of the former hormone on the estradiol induced increase in uterine growth (Figures 41 and 42). This suppression exerted by progesterone, however, occurred only after three days of treatment. These results can be explained on the basis of the mechanism of steroid hormone action. The ovarian production of steroids in the immature animals is negligible and exposure to significant levels of estradiol is required for the synthesis of the progesterone receptor in hormone target tissues (Dohler and Wuttke, 1975; Vu Hai et al., 1977; Manni et al., 1981; Boomsma et al., 1982; Keeping and Lyttle, 1982). Upon exposure to estradiol, progesterone receptors are synthesized allowing progesterone to exert its inhibitory effect. That progesterone suppression occurred only after three days of treatment may reflect the time required for the synthesis of a sufficient quantity of receptors allowing for progesterone to inhibit the effects of the doses of estradiol employed in this study. Therefore, the interactions between

estradiol and progesterone should be significantly influenced by the time relationship of the administration of the hormones. This is demonstrated in the study in which animals were primed with estradiol prior to concomitant estradiol plus progesterone injections. Under these conditions progesterone suppression of the estradiol induced growth of the immature rat uterus was evident twenty-four hours earlier than in the unprimed animals (Figures 43 and 44). Moreover, progesterone administered alone to primed or unprimed animals induced a slight but significant increase in uterine wet weight. This suggests that the uterus of these animals is exposed to some endogenously produced estrogen. Since the adrenals of the prepubertal animal may produce some steroids (Ramaley, 1979), the adrenal output may explain this observation. Alternatively, it may be possible that progesterone can initiate progestational responses independent of a progesterone receptor.

The magnitude of the response to progesterone alone was lower than that observed with either estradiol or estradiol plus progesterone treatments (Figures 41-44). Progesterone stimulates hypertrophy and hyperplasia of uterine luminal and glandular epithelial cells (West et al., 1976; Bhakoo and Katzenellenbogen, 1977; Boomsma et al., 1982). Since the epithelial cells comprise approximately 10% of the total uterine cell population (Kirkland et al., 1979), a direct growth effect of progesterone on these cells in the rat uterus could account for the small weight increase observed in this study.

Progesterone suppression of estradiol induced uterine growth and other estrogenic responses (i.e. glucose metabolism, fluid

imbibition, protein, RNA and DNA synthesis, cytoplasmic and nuclear estradiol receptor synthesis) have been reported (Bhakoo and Katzenellenbogen, 1977; Markaverich et al., 1981; Okulicz et al., 1981a; 1981b; Sumida et al., 1981; Sakamoto et al., 1983), but the basis of their antagonistic interactions remain unclear. It is generally believed that the antagonistic action of progesterone results through down regulation of the estradiol receptor system. Whether this effect occurs on the cytoplasmic or nuclear receptor remains controversial. It has been reported that progesterone selectively interferes with the de novo synthesis of the cytoplasmic estradiol receptor in the immature uterus (Bhakoo and Katzenellenbogen, 1977). This assumption has been supported by studies in other tissues. During the luteal phase (progesterone dominated) of the menstrual cycle the cytoplasmic fraction of the human uterus and oviduct binds considerably less estrogen than during the follicular phase (Evans and Hahnel, 1971; Brenner et al., 1974). In addition, the rabbit and rat uterus is less able to take up and retain estrogen following pseudopregnancy and during ovoimplantation, respectively (Jacobson et al., 1972; Mester et al., 1974).

On the other hand, it has been suggested from observations in a variety of species that the capacity of progesterone to antagonize estradiol action results from an influence on the nuclear estradiol receptor (Markaverich et al., 1981; Okulicz et al., 1981a; 1981b; Sumida et al., 1981). This effect of progesterone may occur through a control over the nuclear estradiol receptor retention because progesterone treatment has been shown to induce a rapid decrease in

total concentration of the occupied form of the nuclear receptor in the hamster and guinea-pig (Okulicz et al., 1981a; Sunida et al., 1981). Okulicz et al., (1981b) have provided a hypothetical model for progesterone action on the nuclear receptor. They have demonstrated a unique progesterone induced activity which acts on the occupied form of the estradiol receptor and proposed that nuclear retention of the estradiol-receptor complex, which is responsible for estrogen dependent gene expression, is controlled by a phosphorylation- dephosphorylation mechanism. Progesterone may induce an enzyme activator which rapidly and selectively dephosphorylates nuclear receptors. This would inactivate the nuclear estradiol receptor mechanism and attenuate the estrogenic response. That the estradiol receptor may be a phosphoprotein supports this model (Auricchio et al., 1981; 1982).

Progesterone suppression of estradiol induced uterine growth in the rat is also accompanied by a reduction in nuclear Type II estradiol binding sites (Markaverich et al., 1981). These binding sites are stimulated by estradiol treatment and it has been speculated that they may be an intermediate step in the mechanism by which estradiol causes uterine growth (Eriksson et al., 1980). The suppression of uterine growth by progesterone (Markaverich et al., 1981) occurs even though the estradiol receptor undergoes translocation to the nucleus, nuclear retention and cytoplasmic replenishment. Whether the receptor mechanism characteristics reported in above study in response to progesterone are different from that previously described is the result of species differences or whether uterine growth and other responses induced by estradiol are initiated

through independent nuclear events is not understood.

The incompleteness of the expression exerted by progesterone on estradiol induced uterine growth as shown in this study may be related to the dose of progesterone administered. Results in Chapter 4 show that a estrogenic response (i.e. gap junction formation) is further suppressed by progesterone when the dose was increased above that employed in the uterine weight measurements. This may indicate, as suggested for hormonal regulation of myometrial gap junctions (see Chapter 4), that a particular progesterone:estradiol ratio is critical and must be reached for maximal suppression of uterine growth.

6.3.2.1c Tamoxifen:

The effect of a synthetic non-steroidal antiestrogen on estradiol induced uterine growth was also investigated in this study. Tamoxifen administered concomitantly with estradiol suppressed uterine wet increases induced by estradiol beginning after two days of treatment (Figures 45 and 46). Moreover, tamoxifen alone increased the uterine wet weight. In the rat uterus, tamoxifen binds to the cytoplasmic estradiol receptor causing the translocation of the receptor into the nucleus (Jordan et al., 1977; 1978; Jordan and Naylor, 1979; Katzenellenbogen et al., 1978; Clark et al., 1973). Therefore, it appears that both estradiol and tamoxifen follow the same pathway in the early stages of their action which probably explains the partial uterotrophic response of tamoxifen when administered alone and why there was no initial suppression when administered together with

estradiol. In addition, it has also been demonstrated that tamoxifen stimulates progesterone receptor, RNA and protein synthesis (Koseki et al., 1977; Jordan and Dix, 1979; Spona and Leibl, 1981; Kurl and Borthwich, 1980; Waters and Knowler, 1981). Tamoxifen does not stimulate a consistent increase in immature rat DNA content (Jordan and Dix, 1979) implying that increase in uterine weight by tamoxifen is due to hypertrophy of the cells. It appears therefore, that tamoxifen is able to initiate but not maintain stimulated uterine hypertrophy in response to repeated injections.

Tamoxifen is not as efficient as estradiol in stimulating increases in uterine weight. Whether this results from a slower uptake of tamoxifen into cells or perhaps some intrinsic property of the antiestrogen remains uncertain. It is also possible that the delayed response could reflect a weak agonist activity of the antiestrogen or that tamoxifen must be metabolized and converted to a more active compound prior to its action. That tamoxifen metabolites (i.e. monohydroxytamoxifen) have enhanced affinities for the estradiol receptor would support this theory (Sutherland and Murphy, 1982). Another possibility is that tamoxifen-induced uterine effects on gene transcription may have no relationship to estradiol induced effects because tamoxifen does not translocate the estradiol receptor into the nucleus or stimulate RNA synthesis to the same magnitude as estradiol (Koseki et al., 1977; Waters and Knowler, 1981).

The reason why tamoxifen cannot maintain uterine growth or stimulate a late estrogenic effect but can suppress estradiol induced uterine responses is not clear. It has been reported that tamoxifen

stimulation results in replenishment of cytoplasmic estradiol receptors which are capable of being translocated to the nucleus (Koseki et al., 1977). These observations may indicate that the nucleus is the likely site of the inhibitory action of tamoxifen. Nuclear retention of the estradiol-receptor complex is a requisite for expression of estrogenic responses. Koseki et al., (1977) have shown that the tamoxifen-receptor complex is retained in the nucleus for a much longer period of time than estradiol, even though tamoxifen was not able to maintain an estrogenic response. This observation lead to the suggestion that the inability of the cell to process or remove the tamoxifen-receptor complex rapidly from the nucleus, as it does with estradiol, might somehow interfere with continued uterine stimulation. Tamoxifen stimulates RNA and protein synthesis but when administered with estradiol, tamoxifen suppresses the estradiol induced stimulation of these events. This implies that the antiestrogen may be working beyond the level of receptor-chromatin interaction. Recently it has been shown that tamoxifen binds to two components in rat uterine cytosol, a highly specific high-affinity saturable binding site for the antiestrogen and the estradiol receptor (Sutherland et al., 1980). However, no evidence is available indicating that this binding site mediates the effects of the antiestrogen. Baudendistel and Ruh (1978) have also shown that the estradiol- receptor complex and the antiestrogen-receptor complex bind to different chromatin loci in the immature rat uterus. Similar observations have been reportedin the chick oviduct (Sutherland and Murphy, 1982). Prolonged nuclear retention of the antiestrogen-receptor complex and the biological ineffectiveness characteristic of

the antiestrogen may therefore be the result of the binding, at least in part, of the antiestrogen-receptor complex to different nuclear loci or sites which do not initiate long term estrogenic responses. Subsequently, with estradiol-receptor bound to non-estrogenic nuclear sites by the antiestrogen, events inherent to the action of estradiol would effectively be suppressed. Whether the non-estrogenic loci binding by antiestrogen-receptor complexes is a consequence of the cytosolic antiestrogen binding site described above or perhaps a conformational change in the estradiol-receptor induced by antiestrogen binding (Spona and Leibl, 1981) remains to be resolved.

6.3.2.2 Adenylate cyclase mechanism:

It is generally thought that estradiol stimulates uterine growth through its receptor system inducing protein and DNA synthesis (Sheehan et al., 1981; Mukku et al., 1982). It has been suggested, however, that stimulation of uterine growth by estradiol involves an additional mechanism of estrogen action (Stewart and Webster, 1983). A second mechanism of estrogen action in the uterus may involve a plasma membrane receptor (Pietras and Szego, 1979) which when bound to by estradiol could stimulate adenylate cyclase thereby initiating the steroid hormone induced response. Estrogen has been shown to stimulate adenylate cyclase in the uterus causing an elevation in cAMP levels (Szego and Davis, 1967). Furthermore, cAMP has been implicated as a chemical modulator of cell proliferation. However, the possibility that cAMP can regulate DNA synthesis and growth is still a matter of controversy. It has been reported that cAMP acts as a growth promoting

agent and brings about the general proliferation of the uterus (Jain, 1978). In addition, adenylate cyclase activity and increased cAMP levels precede, but return to basal level, prior to DNA synthesis in cultured vascular smooth muscle and in several other kinds of eukaryotic cells (MacManus et al., 1973; Millis et al., 1974; Boyton and Whitfield, 1983; Franks et al., 1984). This suggests that an increase in adenylate cyclase activity may be a prerequisite for DNA synthesis, possible for the progression of the cell cycle from the presynthetic period to that of DNA synthesis. However, if adenylate cyclase activity remains elevated or cells in culture are treated with analogues of cAMP, cell division is prevented (Millis et al ., 1974). Cyclic AMP may play a regulatory role in the cell cycle with the G2 period being the most likely site of action (Willingham et al., 1972; Millis et al., 1974). Therefore, it appears from these studies that cAMP may inhibit growth by inhibiting cell division and having no direct influence on DNA synthesis.

I have not examined the role of adenylate cyclase or cAMP in regulation of uterine growth. Generally my results support the steroid receptor mechanism. However, my studies do not rule out this possibility that adenylate cyclase or cAMP is involved in the mechanism of action of estradiol on regulating uterine growth.

6.3.3 Control by prostaglandins:

The observations reported in this study show that indomethacin has a possible uterotropic effect on the immature and ovariectomized mature rat uterus. Indomethacin administered over a three day period to

immature rats or a six day period to ovariectomized mature animals caused a marked increase in uterine wet and/or dry weights (Tables 12, 15 and 16). Moreover, when indomethacin was given with increasing doses of estradiol (up to 50 ug/day), the prostaglandin synthesis inhibitor caused uterine weight increases which were greater than that observed with estradiol alone. Furthermore, I have shown that the uptake and retention of tritiated-estradiol were greater in the uterus of rats treated with indomethacin and that prostaglandin levels (PGF) were lower after treatment with indomethacin (see Chapter 5). These results indicate that prostaglandins may suppress uterine growth, that prostaglandin inhibition may result in increased uterine growth by steps independent of but additive to those produced by estradiol or that indomethacin may directly effect growth by a mechanism not attributed to its inhibitory effect on prostaglandin synthesis. At present the underlying basis for the uterotropic response to indomethacin is not understood, but it is clear that indomethacin may have either direct or indirect effects through inhibition of prostaglandin synthesis.

It should be noted that these experiments were done after three and six days of treatment in immature and ovariectomized mature rats, respectively. A detailed temporal study was not carried out to define when the effects of indomethacin first appear and when they peak, or if they are dose-dependent. However, the results do indicate that the uterus is larger in immature and ovariectomized rats even after three days of treatment with lower doses of indomethacin (Tables 12 and 15).

Estradiol stimulates uterine growth by a mechanism which involves interaction of hormone with receptors and the increase of synthetic processes within the cells (see above). It is most likely that the increase in uterine weight observed in response to indomethacin is due to an effect of this agent or the decrease in prostaglandin synthesis on the steroid-receptor mechanism. The results indicate that the increase in uterine growth with indomethacin is due to an increase in dry weight of the tissues (Table 16), suggesting that synthetic processes are involved and that the weight gain is not due to water uptake alone.

Other studies have also demonstrated an effect of indomethacin on the uterine growth. In immature rats, primed with pregnant mare's serum, administration of indomethacin caused a ballooning of the uterus and increased uterine weight (Armstrong and Grinwich, 1972). Simultaneous administration of lutenizing hormone to those rats caused ovulation and prevented indomethacin- induced uterine growth. These observations parallel the results of this study and indicate that the increase in uterine weight caused by indomethacin occurs only when estradiol levels are low. Similarly, indomethacin treatment of pseudopregnant ovariectomized mice and rats resulted in increased uterine weight and decreased prostaglandin synthesis (Rankin et al., 1979; Kennedy and Lukash, 1982). However, uterine weights are usually much larger in intact and estradiol treated animals than in ovariectomized rats, so a net effect of indomethacin in intact rats may not be readily apparent. The action of indomethacin may be mediated through changes in uterine blood flow. Estradiol is thought to increase

uterine blood flow in the rat by altering prostaglandin levels (Ryan et al., 1974), and estradiol might synergize with indomethacin to increase blood flow, thereby increasing the wet weight. However, this study shows an increase in dry weight of the uterus in response to indomethacin and others have shown that indomethacin prevents the increase in blood flow produced by estradiol (Ryan et al., 1974).

The uterotropic actions of indomethacin in ovariectomized rats, as well as its synergism with estradiol may result from a direct, intrinsic estrogenic activity of indomethacin. However, in a recent study it was shown that administration of antiestrogens, progesterone and testosterone, failed to prevent indomethacin induced uterine weight increase (Puri et al., 1984). Furthermore, there is no other evidence that would support a direct effect of indomethacin.

Alternatively, the uterotropic action of indomethacin may be indirect, through altering prostaglandin levels. Since the results show that indomethacin, a cyclo-oxygenase inhibitor, stimulates uterine growth, this may mean that either products of cyclo-oxygenase inhibit growth or that lipoxygenase products stimulate uterine growth.

Products of the cyclo-oxygenase pathway may regulate uterine growth by effecting the estradiol receptor complex. The uptake and retention of tritiated-estradiol were higher in ovariectomized rats treated with indomethacin (see Chapter 5). These results indicate that uterine tissues from rats treated with indomethacin may contain greater numbers of receptors or that the receptor binding capacity for estradiol is increased. Support for this hypothesis is provided by recent studies showing that prostaglandin synthetase inhibitors elevate

uterine cytoplasmic estradiol receptor binding (San-Filippo et al., 1983) and that polyunsaturated fatty acids, such as the prostaglandins, prevent binding of estradiol to its cytoplasmic receptor in the myometria of immature rats (Clerc- Hoffmann et al., 1983). Therefore, the results of this study could be explained if products of cyclo-oxygenase control growth by down regulation of the steroid receptors. However, this suggested mechanism does not account for uterine growth by indomethacin in the absence of estradiol (Tables 12, 15, 16 and 17) unless the very low levels of estradiol found in control ovariectomized animals (Puri et al., 1984) are sufficient to initiate the above events.

It is known that estradiol stimulates prostaglandin synthesis (Castracane and Jordan, 1976; Nathanielsz, 1978) and prostaglandins have been implicated in the control of cell proliferation. If products of cyclo-oxygenase inhibit growth (as above) it is difficult to conceive how uterine growth occurs when prostaglandins are elevated after estradiol treatment. However, it has been shown in the rat uterus that estradiol stimulated prostaglandin synthesis may be independent of receptor mediated events, that it occurs prior to hormone -regulated protein synthesis, and it declines following repeated estradiol injections (Castarcane and Jordan, 1975; 1976). Therefore, it would not be impossible for uterine growth to occur in response to estradiol, provided increased prostaglandin levels are not maintained. Results presented in Chapter 5 indicate that maximum prostaglandin synthesis occurs in ovariectomized mature rats after one day of treatment with estradiol, similar to the time period of uterine growth reported in

this study. The prostanoid measured in that study, however, does not necessarily mean that it is the prostaglandin involved in growth regulation.

Another possibility is that estradiol may selectively stimulate the cyclo-oxygenase pathway to produce prostanoids which do not effect the steroid receptor or uterine growth. In this respect, estradiol has been shown to inhibit prostacyclin production in the rat uterus (Thaler-Dao et al., 1982). If prostaglandin F inhibits uterine growth, then concomitant administration of indomethacin plus prostaglandin F should prevent the increase in growth. This study, however, was not carried out in this investigation.

Inhibition of the cyclo-oxygenase pathway may also increase the production of lipoxygenase products which could influence the growth of the uterus. Recent studies have shown increased synthesis of lipoxygenase products in response to indomethacin and estrogen (Guivernau et al., 1980; 1982). Therefore, it is tempting to speculate that products of lipoxygenase pathway stimulate uterine growth. That no further increase in uterine weight with estradiol (50 and 500 ug/day) plus indomethacin was observed, however, may rule out this possibility.

Several studies have also shown that prostaglandins regulate growth in other tissues. Prostaglandin E_1 inhibits proliferation of lymphocytes, aortic smooth muscle cells, bone marrow cells, baby hamster kidney cells, and a variety of tumor cell lines such as B-16 melanoma cells, L-929 cells and Lewis lung carcinoma (Johnson and Pastan, 1971; Thomas et al., 1974; Santoro et al., 1976; Huttner et al., 1977; Aglitetta et al., 1980; Horn et al., 1981; Wiley et al.,

1983). Prostaglandin $F_{2\alpha}$ has also been shown to inhibit growth of a hormone-dependent mammary tumor in the mouse (Jubiz et al., 1979). In addition, indomethacin stimulates the growth of various cell line in vitro (Thomas et al., 1974; Santoro et al., 1976; Aglitetta et al., 1980), similar to the in vivo effect reported in this study.

The mechanism through which prostaglandins inhibit cell proliferation is not completely understood. Several prostaglandins are known to stimulate adenylate cyclase activity thereby raising intracellular cAMP levels. Since it has been demonstrated that cAMP inhibits cellular proliferation (see above) the majority of the studies have suggested that prostaglandin induced suppression of cell proliferation is mediated through cAMP. Recently, however, Wiley et al., (1983) have shown that prostaglandin E_1 affects DNA synthesis independent of prostaglandin increases in cAMP in culture baby hamster kidney cells. Furthermore, they have shown that prostaglandins block cell proliferation by inhibiting S-period DNA synthesis, a different site of the cell cycle compared to that suggested for the action of cAMP. Prostaglandin $F_{2\alpha}$ suppression of the hormone-dependent mammary tumor was also associated with a reduction in serum progesterone concentration and was abolished by concomitant progesterone administration (Jubiz et al., I979). This suggests that prostaglandin $F_{2\alpha}$ suppressed growth by inhibiting endogenous progesterone synthesis. Thus, although prostaglandins can inhibit cellular proliferation, the mechanism through which they work remains to be resolved but may vary with cell types.

It has also been reported that prostaglandins can induce cell

division. Prostaglandin $F_{2\alpha}$ added to mouse fibroblast cultures initiates DNA synthesis and cell proliferation (Jimenez de Asua et al., 1975). The rapid phase of cell proliferation and increase in DNA content in human uterine smooth muscle cells in culture is also accompanied by an increase in prostacyclin levels (Cassey et al., 1984). Furthermore, increased amounts of prostaglandins have been detected in a variety of tumors and inhibitors of prostaglandin synthesis have been shown to suppress tumor growth (Goodwin, 1981). These findings in tumor tissue have been correlated with changes in intracellular levels of cyclic nucleotides, implying a possible mechanism of action for prostaglandins in these tissues.

6.3.4 Control by distention:

The effect of uterine distention has also been suggested as a factor of uterine growth (Csapo et al., 1965; deMattos et al., 1968; Carsten, 1968). Uterine distention by intrauterine devices results in an increase in uterine weight. Distention induced growth involves all uterine tissue with the myometrium affected to the greatest degree. Growth induced by distention is due to hypertrophy of the cells (see Carsten, 1968). The mechanism by which distention induces cellular hypertrophy is not clear, but it is evident that the steroid hormones mediate that effect (Csapo et al., 1965). Moreover, distention of uterine tissue stimulates prostaglandin synthesis and release which may be responsible for the growth response (see above). It has also been suggested that throughout pregnancy the pressure exerted on the uterine wall by the growing fetus enhances uterine growth in addition to

hormonal influences (Carsten, 1968). My studies on non-pregnant animals suggest that distention may not be required for uterine growth. It is possible, however, that the increase in uterine luminal fluid after treatment with estradiol may contribute to the hormonal effect on growth but after indomethacin treatment the uterus grows in the absence of fluid accumulation. Furthermore, rat fetal growth occurs primarily after days 17 and 18 of pregnancy (Puri and Garfield, 1982) after the uterus has grown to approximately 86% of its final weight (see Figures 55 and 56).

6.3.5 Summary:

These results demonstrate that progesterone and tamoxifen, when administered concomitantly with estradiol, suppressed the estradiol stimulation of uterine growth. Since it is generally believed that these antiestrogens inhibit or alter nuclear function of the estradiol-receptor complex thereby attenuating estrogenic responses, it can be concluded that estradiol induced increases in uterine growth is mediated through the estradiol-receptor system. In addition, it is also evident from this investigation that indomethacin has a uterotropic influence on the rat uterus. It appears that the mechanism of indomethacin stimulated uterine growth is complex and may involve either a direct or indirect effect on the cytoplasmic estradiol-receptor by influencing the production of prostanoids, either from the cyclo-oxygenase or lipoxygenase pathways. Further studies are needed to clarify the mechanism.

It is possible that uterine growth can be initiated by mechanisms independent or in addition to the estradiol-receptor system.

However, whether cAMP or prostaglandins play a role in regulation of uterine growth independently or act as intermediary or second messengers in the action of estradiol has not been ascertained. Although the involvement of another or an additional mechanism of estradiol action in the uterus cannot be excluded, the results of this study support the "classical" receptor theory for estradiol action in the stimulation of uterine growth.

6.3.6 Relationship between myometrial growth and the presence of gap junctions:

The gap junction is an ubiquitous structure of various tissues and organs of both adults and embryos, and it is believed that they are involved in the control of cellular growth (Loewenstein, 1979). There are, however, variances in the relationship between growth and gap junctions depending on the source of the tissue. Many adult cells grow primarily in the absence of gap junctions, whereas in other tissues in particular those of embryonic origin, growth occurs only when the junctions are present between cells. In addition there may also be, at least theoretically, another group of tissues in which no relationship exists between cellular growth and gap junctions. Growth of the myometrium is regulated by steroid hormones and prostaglandins (Carsten, 1968; Puri et al., 1984). Moreover, the presence of gap junctions in the myometrium is also regulated by the same factors (see Chapters 4 and 5). Furthermore, during pregnancy or after hormonal treatments of non-pregnant animals the uterus grows and gap junctions

eventually form. It is tempting to speculate, therefore, that myometrial growth is somehow related to the presence or absence of gap junctions.

I have evaluated the relationship between the presence of gap junctions in the myometrium and myometrial growth in non- pregnant animals after various hormonal treatments and in untreated pregnant rats on different days of pregnancy, during partutition and one day post-partum. Myometrial growth (hyperplasia and hypertrophy, see above) in immature animals treated with estradiol (50 and 500 ug/day) occurs rapidly reaching near maximum normalized values twenty-four hours after three consecutive days of hormone treatment. Thereafter no further significant increase in growth occurs over a six day period. Gap junctions were not present in the myometrium during the period of growth but developed between the myometrial cells of the immature rat uterus only in response to high doses of estradiol (500 ug/day) and only after growth of the myometrium had reached maximum weight. Similarly, in ovariectomized mature rats treated with the same doses of estradiol, myometrial growth was rapid occurring over the first twenty-four hours after animals were treated with 500 ug/ day estradiol. Gap junctions were present between myometrial cells of the mature animals only after two days of hormone (500 ug/day) treatment when no further growth was apparent. Moreover, in untreated pregnant rats growth of the myometrium occurred beginning after the first week of pregnancy and proceeded to reach near maximum levels on day 20 of gestation. Gap junctions were undetectable throughout gestation but were present at a very low frequency on day 21 and at maximum frequency, size and membrane area on day 22 during delivery. Both the

growth of the pregnant myometrium and the presence of gap junctions decreased dramatically immediately following parturition. The results of this study therefore, indicate that there may be either a dependent relationship between myometrial growth and its cessation and respectively the absence or presence of gap junctions, or that they are independent events. It is interesting to note that in all three animal models studied the relationship between the pattern of growth and development of gap junctions when present were similar, however, the time sequences were different for the three models. Growth occurred in all cases in the absence of the junctions and gap junctions when present between myometrial cells developed when growth ceased. Tentatively, one could argue from these results that (1) the absence of gap junctions is necessary for growth, (2) the presence and development of the junctions results in cessation of growth, (3) growth is necessary for the presence of gap junctions, or (4) that gap junctions and growth are unrelated or independent events which are controlled by similar hormonal conditions.

6.3.6a Independence of myometrial growth and gap junctions:

That myometrial growth and gap junctions are unrelated or independent events is not likely for the following reasons. In all animal models studied the rapid period of myometrial growth occurred in the absence of myometrial gap junctions, and cessation of growth and the appearance of gap junctions when present occurred at approximately the same time frame regardless of the treatment regimens (Figures 47, 54, 57). Furthermore, in animals treated with agents (i.e.

progesterone and tamoxifen) which interfere with the development of myometrial gap junctions, maximum growth was not achieved (Figures 49 and 50). In addition, indomethacin treatment induced myometrial growth in immature and ovariectomized mature rats and potentiated estradiol stimulation of uterine growth only when gap junctions were not detected in the myometrium. When gap junctions were present between myometrial cells, indomethacin had no effect on growth (Tables 13 and 18). Similarly, indomethacin treatment enhanced estradiol stimulation of myometrial gap junctions when no further growth occurred. Therefore, it appears that myometrial growth and gap junctions are interrelated events and that they may be controlled by similar hormonal conditions.

6.3.6b Absence of gap junctions and myometrial growth:

The absence of gap junctions from the myometrium may be necessary for growth of this tissue. The observations reported in this study clearly show that myometrial growth in the immature and ovariectomized mature rat treated with estradiol and in untreated pregnant animals occurred when the myometrial membrane area of gap junctions was extremely low (Figures 47, 54, 57). Moreover, uterine growth stimulated by indomethacin and its potentiation of estradiol induced growth occurred when gap junctions were not observed in the myometrium (Tables 13 and 18). These results support the above hypothesis for the absence of gap junctions and intercellular communication in the control of myometrial growth.

The correlation between the lack of intercellular communication via gap junctions and myometrial growth is similar to that in other

tissues. It has been reported that between many adult cells in culture there is an absence of junctions and junctional communication between cells during periods of cellular growth based on evidence from electrical, dye transfer and ultrastructural studies (Loewenstein, 1979). Furthermore, electron microscopy has demonstrated an absence or paucity of gap junctions in several abnormal growing tissues (i.e. human cervical squamous cell carcinoma, mouse breast adenocarcinoma, rat hepatoma, mouse skin squamous cell carcinoma, rat mesothelioma and human ovary granulosa-theca tumor, see Loewenstein, 1979; 1981 for review). Note that some of these abnormal growths are in steroid hormone target tissues similar to the myometrium. Other in vitro studies, in which communication and structural evidence has been directly correlated in homogenous cancer cell populations, have shown that communication incompetent tumors cells possessed no gap junctions and were under no growth control (see Loewenstein, 1979 and Larsen, 1983 for review). Similarly, hybrids of communicating cells under growth control and cancerous communication incompetent cells have revealed that a junctional defect was causally related to the cell malignancy (Azarnia et al., 1974). These results are similar to those in the myometrium and support the theory that lack of junctional communication may be necessary for cells to grow.

On the other hand, some tumor cells are apparently well endowed with gap junctions as determined ultrastructurally (Larsen, 1983). It has been suggested that other defects in the intercellular communication mechanism (i.e. perhaps closure of junction channels) could account for the lack of growth control in these tissues

(Loewenstein, 1979). The presence of gap junctions between cells does not mean they are functionally coupling cells together. These studies have led to the hypothesis that a defect or alteration in the structure and/or function of the junctions may be an underlying basis for abnormal growth of cells and tissues including cancers (Loewenstein, 1979). However, no study has clearly distinguished between growing and nongrowing cells of a tumor cell population.

The relationship between the absence of gap junctions and myometrial growth demonstrated in this study and in other tissues may not be common to all cell and tissue types. There is a widespread occurrence of gap junctions between cells of embryonic tissues in which cellular growth is extensive and finely regulated (Ito and Hori, 1966; Furshpan and Potter, 1968; Loewenstein, 1979). Although the junctions are present in developing tissues their role, whether for cellular and tissue growth or for differentiation and morphogenesis, is not well understood. It has been suggested that diffusion of morphogenic substances through populations of embryonic cells via gap junctions may be a selective way of determining developmental patterns (Crick, 1970; Wolpert, 1971). Evidence demonstrating that no change in junctional communication existed across regions where there was a major discontinuity in the developmental field argues against a role for gap junctions in development (Furshpan and Potter, 1968; Caveney, 1974). Recently the use of antibodies to the gap junction protein has implied a role for the junctions in early neural embryonic development (Warner et al., 1984), however, others have reported that early steps in neural induction can occur in the absence of any cell-to-cell contacts

indicating that morphogenic signals are transmitted between cells via another mechanism (Toivonen et al., 1975). According to Slack (1984), regional specificity of neural induction (i.e. regions inducing corresponding regions) indicates that the process is probably more complex than the transmission of a single substance. These studies imply that understanding the role for gap junctions in development awaits clarification of the inducing factors. Moreover, Warner et al., (1984) have reported that embryonic cells injected with the gap junction protein antibody continued to grow and proliferate at the same rate as other cells in the embryo. This may suggest that gap junctions do not have any control over cellular growth at that level. If the junctions are not involved in growth of embryonic cells then this may be similar to growth in the myometrium and other tissues during which period gap junctions are absent from between the cells. However, there appears to be some role for the presence of gap junctions in control of proliferation during growth in some embryonic tissues because it has been reported that a correspondence exists between cessation of cellular growth in the developing chick retina and the disappearance of the junctions from between the cells (Fujisawa et al., 1976; Hayes, 1977).

Studies on adult regenerating tissues also indicate the presence of gap junctions during the period of cellular growth. In the rat liver regenerating after surgical removal of a piece of tissue, electrophysiological studies have revealed cell coupling during the phase of rapid cell division and throughout the phase when cell growth levels off (Loewenstein, 1979). In other studies structural evidence

has demonstrated paucity of gap junctions in liver regenerating tissue prior to the rapid phase of cell division (Yee, 1972). The few junctions observed by Yee were much smaller than in unoperated controls suggesting that alterations in gap junction structure and perhaps function may have been responsible for growth in those tissues, If so, then these adult cells may be similar to others including the myometrium and grow essentially in the absence of gap junctions.

Although there is evidence in some tissues, including the myometrium (this study), that gap junctions are absent during growth, this relationship may be required only if the lack of cell-to-cell communication is needed for the growth processes. Exactly how intercellular communication may control growth is not completely understood. However, Loewenstein (1979) has proposed that in the absence of gap junction channels between cell interiors growth regulating molecules (i.e. inhibitors) are not transmitted between cells and growth is not impeded. The lack of transmission of growth regulating molecules between myometrial cells may be the underlying basis for growth of this tissue and others.

6.3.6c Cessation of myometrial growth and development of gap junctions

The results of this investigation on the myometrium are consistent with the report that some types of cellular growth and proliferation occur in the absence of gap junctions. As a corollary to this, development of gap junctions between the smooth muscle cells would result in the cessation of myometrial growth. In the myometrium

of immature and ovariectomized mature rats treated with estradiol and in untreated pregnant animals, gap junctions when present between the smooth muscle cells (i.e. after large doses of the hormone) occupied a significant area of myometrial membrane only after growth of the tissue ceased (Figures 47B, 54B, 57). Furthermore, when indomethacin was administered with high doses of estradiol, no further increase in myometrial growth occurred and there was a very large percentage of the myometrial membrane area occupied by gap junctions (Tables 13 and 18). These observations would indicate that cessation of myometrial growth may be directly correlated with the development of gap junctions. However, the temporal pattern of myometrial growth compared with that for the development of gap junctions in non-pregnant animals after estradiol treatment and in untreated pregnant rats does not support a role for gap junctions in cessation of growth. If the presence of gap junctions is necessary for regulation of myometrial growth then the development of the junctions should accompany or precede the cessation of growth. This time course relationship between the two events, however, does not appear to exist in the myometrium. In the animals groups studied, myometrial growth reached a plateau approximately twenty-four hours prior to the appearance of gap junctions between the smooth muscle cells. Furthermore, the myometrium of the immature and ovariectomized mature rat, under certain experimental conditions (i.e. low dose estradiol treatment) stopped growing in the absence of gap junctions. It may be possible that gap junctions develop in response to this low dose of estradiol but are too small to be detected by thin section electron microscopy. However, in freeze-fracture studies of

myometrial tissue from immature animals treated with 50 ug/day estradiol for four days, gap junctions were not observed (see Chapter 4) indicating that the latter possibility is not likely. Similarly, myometrial growth ceased in the immature rat after treatment with antiestrogens, tamoxifen and progesterone, and after a single injection of estradiol in the absence of significant levels of myometrial gap junctions. Therefore, these findings suggest that no direct or consistent correlation exists between the cessation of myometrial growth and the development of gap junctions. If gap junctions are not necessary for the cessation of growth, the absence of gap junctions may not be needed for the initiation or maintenance of growth (see above).

In addition, the results described in the preceding section of this chapter demonstrate myometrial hyperplasia in the immature rat over the first twenty-four hours after estradiol treatment (Figure 36). The further increase in uterine mass and myometrial area therefore, must have resulted from an increase in the size (hypertrophy) of the muscle cells. Moreover, the presence of gap junctions in the myometrium occurred after four consecutive days of estradiol treatment (Figure 47B). It is generally believed that gap junctions are involved in the control of cellular proliferation (Loewenstein, 1979). It is obvious from these results that the presence of gap junctions does not regulate myometrial cell proliferation (hyperplasia). This finding adds further support against a role for gap junctions in the control of myometrial growth.

Direct evidence in support of a role for gap junctions in regulation of myometrial growth could be obtained from an experiment in

which gap junctions are induced to develop prior to any growth. If the presence of the junctions is necessary for cessation of growth or conversely, their absence required for growth (see above), then growth should not occur in the above experiment. At present, however, I know of no procedure that would enable this investigation to be carried out in the myometrium.

These results suggest that the absence or presence of gap junctions between myometrial cells is not necessary for control of myometrial growth. If transmission of a regulatory molecule is essential for control of growth of the myometrium as proposed by Loewenstein, then it may be possible that this occurs via a mechanism independent of the gap junctional communication system. Much evidence has accumulated involving the cell surface through contact inhibition as an important component of cellular growth control. Density dependent inhibition through cell-to-cell contact has been suggested to act as a suppressor of cell growth possibly through the generation of intracellular negative signals for growth (see Lieberman and Glaser, 1982). This form of growth control is not evident in abnormal growing cell populations and thus, may be the cause of the cell malignancy (Holley, 1975). Cell-to-cell contact inhibition could account for the control of myometrial growth observed in this study but is not likely since contacts between cells are evident during the period of myometrial growth. Alternatively, regulatory signals could be transmitted between cells independent of any cell-to-cell contacts through the circulatory system, the extracellular space or from cell-to-cell across close membrane oppositions.

Another possibility is that communication between myometrial cells may not be required for the control of growth. A number of other possible mechanisms may be involved in growth regulation. In the myometrium and other tissues negative feed back mechanisms within the tissue representing a self inhibitory control, between tissues or within individual cells of a tissue may exist (Goss, 1972). This type of growth control could account for growth regulation in the myometrium. That continuous stimulation through repeated estradiol treatment does not result in continuous myometrial growth would support this form of growth control. In addition, myometrial growth in non-pregnant animals treated with estradiol does not compare with that in untreated pregnant rats suggesting that some form of intrinsic regulation is dominant and monitoring the size of the uterus and growth of the myometrium. Alternatively, growth of the myometrium throughout pregnancy may also reflect the functional demand theory in which the activity of the organ and tissue regulates its growth (Goss, 1972).

6.3.6d Myometrial growth as a component for the development of gap junctions:

Myometrial growth (hyperplasia and hypertrophy) in response to estradiol or the stimulation during pregnancy may in part be necessary for the development of gap junctions. In all models (immature, ovariectomized mature and pregnant) studied gap junctions when present developed after growth of the myometrium (Figures 47B, 54B, 57). Studies on the immature rat demonstrated that both myometrial hyperplasia and hypertrophy account for the growth of this tissue in

response to hormonal treatments (see above). Myometrial hyperplasia occurred rapidly over the first twenty-four hours of the six day treatment period. This indicates therefore, that the further increase in the area of the myometrium to a plateau level after three days of treatment results from hypertrophic growth of the muscle cells. The development of myometrial gap junctions, however, occurred after four days of treatment and after growth occurred. These observations indicate that growth of the myometrial cells may be necessary for the appearance of the gap junctions. Although a detailed investigation into uterine growth of the ovariectomized mature and pregnant rat was not carried out in this study, the evidence presented in this and the preceding two chapters would suggest that the concepts and mechanisms regulating myometrial growth and gap junctions may be similar between the non-pregnant and pregnant animals. Therefore, conclusions drawn from the immature rat model studies on growth may also conceivably apply to the ovariectomized and pregnant rats . Moreover, in the ovariectomized mature and pregnant animals the development of gap junctions was always preceded by growth. It is obvious, however, that myometrial growth by itself is not sufficient stimulus for the development of gap junctions as various treatments, which result in growth to an equivalent extent, did not result in the appearance of the junctions (i.e. low doses of estradiol and tamoxifen, Figures 47A, 50, 54A).

Support for the hypothesis that myometrial growth is in part a necessary component for development of the junctions comes from studies with antiestrogens. Progesterone and tamoxifen prevented that maximum

growth attained when administered together with estradiol, and the time course of their effect is compatible to that for the antiestrogenic suppression of gap junctions (Figures 49 and 50). The suppression of myometrial growth in response to these agents occurred prior to their effect on gap junctions which would be expected if the suppression of growth is responsible for the lower frequency of junctions. This suggests that if optimal growth is not achieved neither is the full expression of gap junctions and that alteration of gap junction development through interference with myometrial growth may be consequence of the latter event. Similarly, myometrial hypertrophy is not maintained with a single injection of estradiol and gap junctions are not induced (Figure 48).

Additional evidence to support the above hypothesis comes from studies on post-partum animals. The atrophy of the pregnant uterus within twenty-four hours after delivery is accompanied by a dramatic decrease in the frequency of gap junctions (Figure 57). This observation is similar to those above with the antiestrogens and support the hypothesis that growth may be necessary for the presence of gap junctions. Further support for this concept has been provided by studies carried out by Wathes and Porter (1982). Recently they have demonstrated that distention of the uterus immediately after parturition with an intrauterine balloon prevented the decrease in size of the uterus and the complete disappearance of myometrial gap junctions. If the decrease in the size of the myometrium post- partum is responsible for the regression of gap junctions we would expect atrophication to precede the disappearance of the junctions. I have

not carried out a detailed time analysis of the regression of the junctions or decrease in uterine weight post-partum but the results suggest that the disappearance of gap junctions accompanies atrophy so the results would support the concept that the reduction in size of the uterus may be partially responsible for the disappearance of gap junctions. Therefore, the decrease in gap junctions post-partum is probably controlled by several factors including changes in steroid hormones and their receptors, decreased prostaglandin synthesis, and atrophy with the accompanied destruction of cells and connective tissue.

It is not readily apparent why growth may be required for the development of gap junctions. An increase in cell size may result in a larger proportion of cell membranes in close proximity to each other which may provide a better opportunity for the formation of gap junctions. It is possible that there may be a greater area of close contact between cells after estradiol treatment. This seems unlikely because cells appear to be in close contact even in untreated control tissues and tissues from treated animals prior to junction development. However, there have been no quantitative studies to describe this phenomenon in more detail.

The evidence presented in this study showing the dependence of gap junctions on growth is mainly circumstantial. Some insight may be gained if complete or incomplete gap junctions could be induced to form in the absence of any growth, but at present I don't know how this could be achieved. There have been two studies which may contribute to our understanding of the growth-gap junction relationship. One study

shows that myometrial gap junctions are present during preterm labor on day 18 of gestation suggesting that maximum growth may not be necessary for the development of gap junctions (Garfield et a)., 1982). Although there were no measurements of uterine growth in that study the results shown in Figure 57 reveal that 86% of the uterine growth has occurred at day 18 of pregnancy. This growth may have been sufficient for the development of the junctions. In addition, uterine tissue from immature rats incubated in vitro for 48 hours develop small numbers of gap junctions in the myometrium (Garfield et al., 1980a). One would expect that these tissues don't grow during the in vitro incubation. This would suggest that junctions developed in the absence of myometrial growth. However, tissue samples were not weighed in the study so direct correlation between the two events can not be made. Furthermore, the in vitro development of gap junctions is known to be regulated differently than in vivo (see Chapter 5).

Other studies have also reported observations similar to these in the myometrium. Intestinal circular smooth muscle undergoes hypertrophic growth in response to experimental stenosis followed by a corresponding increase in the number and size of gap junctions between the cells (Gabella, 1979; 1979b). However, a detailed study of the time sequence of growth and gap junctions was not done.

The results of the preceding section of this chapter and those presented in Chapters 4 and 5 suggest that myometrial growth and gap junctions are regulated by the steroid hormones probably through the steroid receptor mechanism. However, the temporal pattern of growth and development of gap junctions are not on the same time frame. Growth

occurs rapidly after hormone treatment whereas gap junctions develop later in the non-pregnant animals. Similarly, throughout gestation the pregnant myometrium grows followed by the development of the junctions. If the same mechanism regulates both events and growth regulates the appearance of gap junctions then it is evident why the two occur on different time scales. What is not apparent is if proteins synthesized for growth are provided at the same time as gap junction proteins. During growth proteins are synthesized and include, at least presumably as indicated by the cycloheximide results presented in Chapter 4, the gap junction proteins. However, the time delay between start of growth and development of gap junctions in the pregnant myometrium (approximately 16 days) is much longer than that in non-pregnant animals (approximately 2-4 days). The changes in levels of steroid hormones and prostaglandins thought to be necessary for junction development in the pregnant myometrium (Puri and Garfield, 1982) occurs on day 19 of pregnancy, 2-3 days prior to labor and the appearance of gap junctions. This delay is similar to that seen in non-pregnant animals after hormonal treatment suggesting that during pregnancy gap junction proteins are synthesized and aggregated into junctions after growth occurred. Therefore, later development of gap junctions in the myometrium of treated non-pregnant and untreated pregnant animals after growth may reflect the period required for protein aggregation into junctional plaques necessary for identification by electron microscopy. This process may require another mechanism, perhaps prostaglandins, as discussed previously (see Chapters 4 and 5).

6.3.6e Summary:

The results of this study show that myometrial growth in non-pregnant animals after various hormonal treatments and in untreated pregnant rats occurred when the myometrial membrane area of gap junctions was extremely low. Gap junctions when present between the myometrial smooth muscle cells of non-pregnant and pregnant animals, developed after growth of the myometrium. The junctions, however, do not develop under all conditions when growth ceases. These results imply: 1) That growth of the myometrium occurs in the absence of myometrial gap junctions and intercellular communication. 2) Cessation of myometrial growth does not depend on the presence of gap junctions. It is possible that cell-to-cell transmission of regulatory molecules may occur via a mechanism independent of the junctional communication system thereby accounting for the cessation of growth. Alternatively, however, self inhibition of myometrial growth in the treated non-pregnant animals or untreated pregnant animals may be the underlying basis for growth control in this tissue. 3) Growth of myometrial cells may in part be a necessary component for the development of gap junctions.

CHAPTER 7

SUMMARY AND CONCLUSIONS

In this final chapter concerning the experimental aspects of my work, I will briefly summarize my data and indicate the conclusions I have drawn from the studies that were presented in this thesis. In addition, I will outline possible future directions of research along the lines demonstrated by the investigations I have carried out.

7.1 Regulation of myometrial gap junctions by steroid hormones:

7.1a Summary of estrogen studies:

1) Immature rats treated with estradiol developed myometrial gap junctions (Table 1).
2) Treatment with the synthetic estrogen diethylstilbestrol induced the development of myometrial gap junctions in the immature rat (Table 1).
3) The frequency of gap junctions in the myometrium of immature rats treated for 6 days with estradiol was similar to that observed during spontaneous parturition. The size of the junctions induced by estradiol treatment, however, were significantly smaller compared with the sizes during labor. Similar frequency and reduced size in the immature rat myometrium resulted in a smaller percentage of the membrane area occupied by gap junctions in the non-pregnant animals (Table 1).
4) Gap junctions developed in the myometrium of immature rats treated with estradiol and diethylstilbestrol daily

over a 1-8 day period. Estradiol induced the presence of gap junctions in the myometrium only after four consecutive days of treatment. The junctions were maintained between the smooth muscle cells after five and six days of treatment but thereafter significantly declined with further injections of estradiol. In animals treated with diethylstilbestrol, gap junctions developed and disappeared from the myometrium twenty-four hours earlier than after estradiol treatment. The frequency, size and membrane area of gap junctions induced by either estradiol or diethylstilbestrol were similar.

5) Repeated injections of estradiol were required for the development of gap junctions. Gap junctions were present at an extremely low frequency and reduced sizes occupying a very small percentage of myometrial membrane area five days after a single injection of estradiol (Figure 13).

6) The development of gap junctions in the immature rat myometrium was a dose related estradiol effect. Estradiol at 500 ug/day was the optimal dose for stimulation of gap junctions (Figure 10).

7) The antiestrogen tamoxifen did not induce the presence of gap junctions in the myometrium of immature rats and it completely suppressed estradiol stimulation of the junctions when administered concomitantly with the steroid hormone (Figure 16).

8) Cycloheximide administered simultaneously with estradiol

suppressed the estradiol stimulation of myometrial gap junctions in the immature rat (Table 3).

9) Similar to that in the immature rat, gap junctions developed between myometrial cells of ovariectomized mature animals treated with estradiol (Figure 17).

10) Gap junctions developed in the myometrium of ovariectomized mature rats in response to repeated injections of estradiol (Figure 17). Gap junctions were present between the smooth muscle cells after two days of treatment and were maintained in the myometrium by continued hormone treatment over a four day period. The frequency, size and membrane area occupied by junctions in the ovariectomized mature rat myometrium were similar to those induced by estradiol in the immature animals, however, the effect of the hormone treatment occurred earlier in the mature animals.

11) High (500 ug/day) but not low (50 ug/day) doses of estradiol stimulated gap junction development in ovariectomized mature rat myometrium (Figure 17).

12) Estradiol treatment stimulated the development of gap junctions in the myometrium of pregnant animals (Table 5). Daily injections of estradiol (50 and 500 ug/day) starting on day 16 of gestation resulted in a gradual increase in the frequency of gap junctions reaching maximum levels on day 19 of pregnancy.

13) Pregnant animals treated with the high dose of estradiol

delivered prematurely on day 19 of pregnancy approximately twenty-four hours after the third day of treatment (Table 5). Treatment with 50 ug/day estradiol resulted in some form of premature labor on day 20 of gestation (Table 5).

14) Administration of estradiol on day 21, during delivery and one day post-partum was not able to sustain the presence of gap junctions in the myometrium that developed during labor (Table 6).

7.1ab Conclusions:

Estrogens stimulate the presence of gap junctions between myometrial cells of the immature, ovariectomized mature and pregnant rat uterus. Estrogen must be administered at a high dose and repeated injections of the hormone are necessary for this estrogenic effect. The steroid hormone influences the presence of gap junctions by regulating the synthesis of the gap junction protein probably through the classical steroid receptor mechanism. It is also possible that estrogen treatment influences the production of prostaglandins which are important in gap junction regulation (see below). The induction of myometrial gap junctions and premature delivery of pregnant animals by estradiol indicates that the development of gap junctions in the myometrium is a requirement for the occurrence of term as well as preterm labor.

7.1b Summary of progesterone studies:

1) Progesterone treatment did not stimulate the development

of gap junctions in the immature rat myometrium (Table 1).

2) Progesterone when administered simultaneously with either estradiol or diethylstilbestrol suppressed the estrogen stimulation of myometrial gap junctions in the immature animals (Table 1).

3) Concomitant treatment of immature rats with progesterone plus estradiol daily for 1-6 days had no effect on the low frequency of gap junctions induced by estradiol alone over the first three days of treatment. The significant increase in gap junctions with further injections of estradiol was suppressed by continued treatment with progesterone plus estradiol (Figure 15).

4) The inhibitory effect of progesterone was influenced by the dose of the hormone used. When the dose of progesterone was increased (i.e. from 2 mg/day to 2.5 mg twice daily) there was a further suppression on estradiol stimulation of gap junctions (Table 2).

5) Progesterone when administered together with estradiol to ovariectomized mature rats, also suppressed the estradiol stimulation of myometrial gap junctions (Table 4).

7.1bc Conclusions:

The steroid hormones regulate the presence of gap junctions in the myometrium. Progesterone, when administered simultaneously with estradiol, suppresses the estrogen stimulation of myometrial gap junctions. Progesterone, working through the steroid receptor mechanism,

inhibits the effect of estrogen probably by interfering with estrogen stimulation of protein synthesis.

7.2 Regulation of myometrial gap junctions by prostaglandins:

7.2a Summary of studies:

1) Immature rats treated with indomethacin did not develop myometrial gap junctions (Table 7).
2) Indomethacin administered concomitantly with estradiol potentiated the effect of estradiol on gap junctions in the immature rat myometrium (Table 7).
3) Progesterone, when administered to immature animals treated with estradiol plus indomethacin, was still capable of suppressing the stimulation of gap junctions (Table 8).
4) Treatment of immature rats with sodium meclofenamate plus estradiol enhanced the stimulation of myometrial gap junctions similar to that observed with indomethacin (Table 9).
5) Indomethacin was also capable of potentiating the estradiol induction of gap junctions in ovariectomized mature rats (Table 11, Figure 23).
6) The effect of indomethacin on estradiol stimulation of gap junctions in the myometrium of mature ovariectomized animals was rapid and occurred on each day over 1-4 days of treatment (Figure 23).

7) Progesterone was capable of suppressing the effects of estradiol plus indomethacin on myometrial gap junctions in ovariectomized mature rats (Table 10).

8) Ovariectomized mature rats treated with estradiol plus indomethacin had lower plasma levels of estradiol compared with animals treated with estradiol alone (Figure 25).

9) Indomethacin treatment resulted in an increase in uptake and retention of estradiol in uterine tissue of ovariectomized mature rats (Figure 26).

10) Estradiol stimulated uterine prostaglandin synthesis. Uterine tissue content of prostaglandin F increased after one injection of estradiol but with further hormone treatment the level of prostaglandin decreased to below control levels after four days of treatment (Figure 27).

11) Indomethacin significantly suppressed the synthesis of prostaglandin F by uterine tissue. The inhibition was evident after the first day of treatment and was maintained over the remainder of a four day treatment period (Figure 27).

7.2b Conclusions:

Indomethacin and sodium meclofenamate, inhibitors of cyclo-oxygenase, potentiate the effect of estradiol in stimulating gap junction development in the myometrium. This may mean that a prostanoid or prostanoids of the cyclo-oxygenase pathway are involved in the steroid hormone regulation of myometrial gap junctions. The product(s) of the

cyclo-oxygenase pathway (i.e possibly prostacyclin) may be regulating gap junctions by controlling the level of steroid receptors. In addition, inhibition of cyclo-oxygenase may result in an increase in products of the lipoxygenase pathway which may be involved in junction regulation at the level of steroid receptors or other steps in the mechanism of steroid hormone action.

7.3 Regulation of uterine growth by steroid hormones:

7.3a Summary of estradiol studies:

1) Estradiol stimulated growth of the immature rat uterus (Figure 28). Growth of the uterus was rapid and progressive reaching a plateau after three days of a 1-8 day treatment period (Figures 30 and 32).
2) Treatment with estradiol induced similar increases in both wet and dry mass of the immature rat uterus over the 8 day period (Figures 30 and 32).
3) 50 and 500 ug/day estradiol induced similar increases in uterine growth over the treatment period. Lower doses of estradiol (0.025 and 0.10 ug/day) stimulated uterine growth in a dose responsive fashion (Table 14).
4) Both myometrial and endometrial tissues grew in response to estradiol treatment (Figure 33).
5) The cross-sectional area of the myometrium and endometrium increased after estradiol treatment. The growth of the myometrium was a major component in the

response of the uterus after treatment with estradiol (Figure 33).

6) Estradiol treatment stimulated a similar increase in the circular and longitudinal muscle layers of the myometrium (Figure 34).

7) Uterine growth in response to estradiol treatment resulted from cellular hyperplasia and hypertrophy.

8) Incorporation of tritiated thymidine into uterine tissue increased to a maximum twenty-four hours after the first injection of estradiol but thereafter decreased with further hormone treatment. On day three of the treatment period a second, but smaller peak, of thymidine uptake occurred (Figure 36).

9) Tritiated thymidine was incorporated into cells of the myometrial and endometrial tissues. The smaller peak of thymidine uptake on day 3 appeared to occur only in the endometrial tissue (Figure 38).

10) Total uterine DNA content increased progressively from zero time to a maximum level on day four of the six day period. With further injections of estradiol the DNA content decreased slightly when expressed as a percentage of control values (Figure 37).

11) Repeated injections of estradiol were not necessary for stimulation of uterine growth. Uterine growth in response to a single injection of estradiol progressed rapidly reaching a maximum value on day three of a six day period.

Thereafter, however, uterine growth decreased (Figure 40).

12) Tamoxifen administered together with estradiol prevented the maximum increase in uterine growth compared with estradiol treatment alone (Figures 45 and 46).

13) Estradiol also stimulated uterine growth in ovariectomized mature rats. Uterine wet and dry weight increased to near maximum values after the first day of treatment. No further significant increase occurred with continued treatment over the four-day period (Figures 51-53).

14) In untreated pregnant rats uterine growth was not evident over the first 6 days of gestation. Thereafter, the wet and dry weights increased progressively to maximum weights on day 20 of pregnancy. The weights decreased slightly by the time of parturition followed by a significant decline one day post-partum (Figures 55 and 56).

7.3ab Conclusions:

Estradiol at low and high doses stimulates uterine growth in immature and ovariectomized mature rats and the action of the steroid on growth appears to be mediated through the steroid receptor mechanism. Repeated injections of estradiol are necessary for maintaining growth of the uterus when maximum mass is attained. Growth occurs in all uterine tissues, however, the increase in weight of the uterus is due primarily to the increase in myometrial area. Myometrial and endometrial hyperplasia accounts for the initial growth response after treatment with estradiol followed by an increase in cell size (hypertrophy).

7.3b Summary of progesterone studies:

1) Progesterone had no effect on uterine growth when administered by itself (Figures 41 and 42).
2) Progesterone administered concomitantly with estradiol over a 1-6 day period partially suppressed estradiol induced uterine growth. The suppression of growth was evident only after three days of treatment (Figure 42).
3) Estradiol priming of uterine tissues resulted in an earlier suppression of growth by progesterone. In addition, progesterone induced a slight but significant increase in uterine growth after estradiol priming (Figures 43 and 44).

7.3bc Conclusions:

The steroid hormones regulate uterine growth. Estradiol stimulates whereas progesterone, when administered with estradiol, suppresses uterine growth. Progesterone's inhibitory action is probably mediated through the steroid receptor mechanism.

7.4 Regulation of uterine growth by prostaglandins:

7.4a Summary of studies:

1) Indomethacin treatment stimulated uterine growth in the immature rat (Table 12).
2) Indomethacin administered simultaneously with estradiol

(50 and 500 ug/day) to immature rats had no effect on uterine growth compared with estradiol treatment alone (Table 12).

3) Treatment of ovariectomized mature rats with indomethacin resulted in an increase in uterine weight (wet and dry mass) (Tables 15-17).

4) Indomethacin administered together with estradiol (0.025 and 0.10 ug/day) for six days to ovariectomized animals caused a further increase in uterine growth beyond that attributable to estradiol alone. However, when indomethacin was administered simultaneously with 50 ug/day estradiol no further additive increase in uterine growth was evident (Table 17).

5) Indomethacin treatment had no significant effect on uterine growth of intact mature animals (Table 15).

7.4b Conclusions:

Indomethacin has a uterotropic action in the uterus. This may mean that product(s) of the cyclo-oxygenase pathway are involved in regulation of uterine growth. Prostaglandins may suppress uterine growth and inhibitors of cyclo-oxygenase may result in growth by steps independent of but additive to those produced by estradiol. Inhibitors of the cyclo-oxygenase pathway may also increase the production of lipoxygenase products which could influence growth of the uterus.

7.5 Relationship between uterine growth and presence of myometrial gap junctions:

7.5a Summary of immature rat studies:

1) Uterine growth (mainly myometrial) occurred rapidly and progressively over the first three days of treatment with estradiol (50 and 500 ug/day). After day 3 of a six day period no further significant increase in uterine weight and thus growth was apparent. Myometrial gap junctions were absent or present at an extremely low frequency and membrane area during the period of myometrial growth in response to both doses of estradiol. The junctions when present between the smooth muscle cells (i.e. only after treatment with 500 ug/day estradiol) occupied a large percentage of myometrial membrane area approximately twenty-four hours after growth reached a plateau (Figure 47). Similarly, uterine growth progressively increased to a plateau three days after a single injection of either 50 or 500 ug estradiol. This growth period was followed by a significant decline 5 and 6 days after hormone treatment. Significant levels of gap junctions were not induced over the 6 day period by a single injection of estradiol (Figure 48).

2) Uterine growth resulted from cellular proliferation (hyperplasia) of myometrial cells over the first twenty-four hours after starting treatment with estradiol (50

and 500 ug/day) for 1-6 days (Figure 36). Further growth of the myometrium resulted from hypertrophy of the muscle cells. Hyperplasia and hypertrophy of myometrial cells occurred in the absence of gap junctions.

3) Treatment with tamoxifen or progesterone together with estradiol (500 ug/day) prevented maximum uterine growth and gap junction development compared with that induced by estradiol alone. The time courses for their effects on growth and gap junctions were similar. However, the suppression of growth preceded the agents effects on gap junctions (Figures 49 and 50). Immature animals treated with indomethacin plus estradiol (50 and 500 ug/day) developed numerous myometrial gap junctions but had no additive effect on uterine growth. Indomethacin by itself, however, stimulated uterine growth but not gap junction development (Table 13).

7.5b Summary of studies on ovariectomized mature rats:

1) Uterine growth in response to estradiol treatment (50 and 500 ug/day) over 1-4 days occurred in the absence of myometrial gap junctions. When the junctions developed (i.e. only after treatment with 500 ug/day estradiol) they were present in the myometrium after growth occurred and had reached a plateau level (Figure 54). The results were essentially the same as those from the immature rats (see above).

2) Uterine growth of ovariectomized mature animals treated

with estradiol occurred independently of the presence of gap junctions in the myometrium because gap junctions did not develop in all tissues even though growth stopped and growth ceased before gap junctions appeared.

3) Indomethacin treatment stimulated uterine growth but not gap junction development in ovariectomized animals (Table 18). When administered together with low doses of estradiol (0.025 and 0.10 ug/day) for 6 days, indomethacin caused a further increase in uterine growth compared with that induced by estradiol alone. No effect on gap junctions occurred in response to the above treatments (Table 18). However, when indomethacin was administered simultaneously with 50 ug/day estradiol for the 6 days, no effect on uterine growth was observed but there was a significant enhancement of the frequency and membrane area of the junctions in the myometrium (Table 18).

7.5c Summary of studies on pregnant animals:

1) Uterine growth throughout pregnancy occurred in the absence of myometrial gap junctions (Figure 57). Growth of the pregnant uterus reached a near maximum level on day 20 followed by a slight non-significant increase by day 22 during delivery. Within twenty-four hours after parturition the size of the uterus dramatically decreased. Gap junctions were present in the myometrium at an extremely low frequency occupying a small percentage of the membrane

area throughout gestation. The membrane area of junctions began to increase on day 21 and was maximum on day 22 during delivery. One day post-partum the membrane area occupied by gap junctions returned to prepartum levels. The relationship between uterine growth and gap junctions was similar to those observed in non-pregnant treated animals described above.

2) Growth of the untreated pregnant uterus occurred in the absence of myometrial gap junctions.

7.5d Conclusions:

Myometrial growth, whether in non-pregnant animals treated with hormones or in untreated pregnant rats, occurs in the absence of myometrial gap junctions, but the cessation of growth does not depend on the development and presence of the junctions in this tissue. Growth of myometrial cells, however, may in part be a necessary component for the development of gap junctions.

7.6 Future studies:

The work described in this thesis has established a role of steroid hormones and prostaglandins in regulation of myometrial gap junctions and it has also defined the possible relationship between uterine growth and gap junctions. The experiments reported here have generated new questions for further investigation. The following studies appear to be pertinent and would corroborate the experimental observations reported in this thesis.

1. I have quantitated the regulation of myometrial gap junctions by thin section morphometry. This technique is tedious and requires an enormous amount of time to accomplish (i.e. I have measured smooth muscle membrane length, quantitated and identified gap junctions in over 15,000 photomicrographs). This method is also, obviously, very expensive. It would therefore, be advantageous to develop a more practical method to quantitate gap junctions. Recently, the use of antibodies to the gap junction protein have been developed in other studies. The use of antibodies to gap junctions may enhance the technique and precision of quantitating myometrial gap junctions. However, this form of investigation is still in experimental stages and awaits time before it could be applied to studies as outline here. Alternatively, a detailed and carefully executed freeze-fracture microscopic study may prove beneficial in gap junction quantitation. However, I've outlined some of the difficulties with this technique in my thesis.
2. My results show that the steroid hormones regulate the presence of myometrial gap junctions by influencing protein synthesis probably through their receptor mechanism. Analysis of steroid receptors in relationship to the development of gap junctions in the immature, ovariectomized mature and pregnant rats treated with steroid hormones would substantiate my conclusions. In addition, junctions disappear from the myometrium, however, the underlying basis for this event is not known but may be related to a decrease in receptors for the steroid hormones.

Furthermore, receptor analyses may shed light on an explanation for the time delays in the appearance of the junctions in the animal models that I have studied.

3. I have suggested that junctional protein synthesis via the steroid receptor mechanism accounts for the observations reported in my thesis. Uterine protein synthesis may be stimulated by other mechanisms, however, I have no data to accept or refute such a possibility. Studies with cAMP and agents which act on adenylate cyclase would answer this question. Moreover, studies with estradiol and progesterone together with an assay for adenylate cyclase activity would indicate if the latter is involved in the mechanism of action of the steroids on regulating the presence of gap junctions.
4. It is evident from this report that the ratio rather than the absolute levels of the steroid hormones may be a critical factor in controlling the expression of gap junctions. To determine the ratio between estradiol and progesterone one would have to measure plasma and/or tissue levels of the steroids.
5. Estradiol stimulates gap junction development in a dose responsive fashion probably by alteration of prostaglandin synthesis as indicated by the effects of inhibitors of cyclo-oxygenase, in addition to protein synthesis. Alternatively, lipoxygenase products may also be involved. To determine their role, if any, specific inhibitors of this pathway would have to be used. Furthermore, assays for various prostaglandins of both arachidonic acid pathways may provide a more complete overview of the

prostanoids involved in regulating gap junctions.

6. I have suggested, based upon indirect evidence, that prostanoids may regulate gap junctions through an effect on the steroid receptors. If this is true then prostanoids should either control the number and/or binding ability of the receptors. Direct evidence in support of this hypothesis would be provided through receptor studies in conjunction with those outlined above (see 5).

7. Inhibitors of cyclo-oxygenase enhance the development of myometrial gap junctions after treatment with estradiol. Furthermore, estradiol treatment stimulates junction development in the myometrium of pregnant animals and results in premature delivery. It would be interesting to determine what effect indomethacin might have on gap junctions and pregnancy since prostaglandins are involved in both events. In addition, treatment of pregnant animals with agents such as tamoxifen may also support the role of estradiol and gap junctions in pregnancy and labor.

8. Studies on treated pregnant animals imply that junctions form and that they are functionally coupling cells together based on the observation that the animals delivered prematurely. Functional studies would provide direct support for this assumption. Also it would be interesting to know if the junctions that form in tissue from non-pregnant animals after hormonal treatment are coupling cells functionally. Functional coupling of myometrial cells can be measured by electrophysiological (Sims et al., 1982) techniques (intra and extracellular recordings) and by the

cell-to-cell diffusion of metabolites such as 2-deoxyglucose (Cole et al., 1984).

9. Regulation of gap junction development in myometrial tissues incubated in vitro appears to be different than in vivo. Repeating the studies outlined in this thesis in vitro may allow us to determine mechanism(s) of in vitro regulation and how they differ from those determined in vivo. Also, it would be interesting to determine the mechanisms for control of gap junctions in cultured myometrial cells.

10. Steroid hormones and prostaglandins regulate growth of the myometrium and development of gap junctions in this tissue. Cessation of myometrial growth, however, does not require the presence of gap junctions as in other tissues. The steroids and prostaglandins may regulate growth possibly through alterations in the steroid receptors. Receptor analysis would therefore, allow us to determine the role of the steroids and prostaglandins in cessation of growth.

11. Growth of myometrial cells may in part be a necessary component for the development of gap junctions. However, we don't know why growth is to be required. It is possible that growth results in a larger proportion of membranes in close proximity to each other which provides a better opportunity for the junctions to develop. A detailed quantitative study on close contacts between myometrial cells would have to be carried out to support this theory.

CHAPTER 8

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