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Accessory Reproductive Organs in Mammals: Control of Cell and Tissue Transport by Sex Hormones

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I. Introduction

VERTEBRATE reproductive organs are wholly dependent for structural integrity, function, and growth upon steroid hormones secreted by the ovary and testis. The precise means by which the hormones affect underlying metabolic events in soluble and formed portions of the cell are not known. That the hormones involved (estrogen, progesterone, and testosterone) regulate transport of ions, water, and nutrients in respective target organs is documented in this review. Data are evaluated from the standpoint of biological significance of the effect, and whether the control of transport is causally related to the capacity of the hormones to regulate metabolism and specific organ function. The data are discussed in the context of theories of hormonal modes and mechanisms of action. The subject is treated comprehensively. That is, transport is perceived in its broadest sense physiologically to mean the "carrying" of molecules across vascular and interstitial barriers as well as across cell membranes. All organ components are included that might govern permeability between blood and target cell cytoplasm.

The scope of the project required placement of certain limitations on coverage. Reports published during the past 15 years are emphasized, and only mammalian systems are discussed although there exists a rich literature concerning non-mammalian systems of potential interest to students of hormone mechanism of action. The hormonal targets covered are the sex accessory organs. By definition, these are the gamete-carrying tubes of the reproductive tracts proper and glands connected with them through ducts. In males, discussion is virtually limited to the glandular prostate and seminal vesicles due to a scarcity of pertinent information on the remainder of the system. Data of interest concerning preputial glands are included although these glands are not directly connected with the tract. The female accessory organs are the oviducts, uterus, vagina, and vestibular (Bartholin's) glands. Information on the uterus predominates because of intensive use of that organ as a model system in the study of hormone action. The secondary sex characters (external genitalia, mammary glands, perineal sex skin, scrotum) are other targets sensitive to steroid hormones but are omitted from coverage, as are the primary sex organs, the gonads. Also due to space limitations, discussion of hormone-stimulated changes in accessory organs during pregnancy is severely restricted.

II. Hormonal Control of Connective Tissue Formation, Structure, and Function

The regulation of accessory organ connective tissue cells and their secretions (ground substance and collagen) is reviewed in this section from the standpoint that these secretions are the immediate environment of target cells that undergo growth cycles in response to steroid hormones.

As an environment, connective tissue is the medium of gas and nutrient exchange between blood and target cells, but is at the same time a principal locus of organ enlargement, reorganization, and regression that occur sequentially in estrous cycles and pregnant states. These changes, also controlled by the steroid hormones, importantly affect movements of water ions, and organic metabolites to and from growth-responsive cells.

Hormones regulate development and composition of connective tissues generally (cf. 14, 138, 449) and actions of ovarian steroids on the female reproductive tract are among the most striking examples of such control. Edematous swelling, principally in the subepithelial stroma, occurs in the uterine endometrium during estrous cycle and pregnancy and was recognized by early workers to involve profound alterations in the character of the intercellular matrix (8, 28, 295). These changes are fully reversed after endometrial subsidence late in the cycle and in postpartum regression. Qualitatively similar changes occur in highly responsive secondary sex tissues, *e.g.*, primate sex skin (8, 37, 256, 566) and cock's comb (139, 211) as affected by estrogen and testosterone, respectively; testosterone also increases hydration and extracellular volume of scrotal skin (555) but a possible relationship with connective tissue metabolism has not been assessed. In the accessory organs, two types of edematous conditions may be distinguished: One is typically the acute response observed in uteri of ovariectomized animals beginning 1 to 3 hours after an injection of estrogen (17, 419, 486), an effect traceable to changes in local blood flow and capillary permeability (cf. sections III and IV). The other hydrated state is longer in duration and associated with fundamental changes in the character of the extracellular "compartment." The physiology of edema *per se* in sex accessory tissues is fully discussed in sections III and IV. The focus here is primarily upon regulation of components of the connective tissue itself.

Connective tissue of tubular organs has been described as largely a hydrophilic colloidal solution, the ground substance, existing in an amorphous sol-gel equilibrium. The equilibrium is subject to shift depending on local conditions (*cf.* 172) and physiological properties change accordingly. Thus, in the more aggregated state, movement of ions relative to one another is restricted; K for example is selectively immobilized. In normal loose connective tissue or in that rendered more disaggregated and water soluble through hormone action, the mobility of K relative to Na is increased (256). The matrix is a mixture of mucopolysaccharides, polymers of two or more kinds of sugars (heteropolysaccharides) covalently bound to a protein core; the polymers are arranged bristle-like in the protein, exposing a large surface area capable of holding substantial amounts of tissue fluid. A principal heteropolysaccharide is hyaluronic acid (long polymers of glucosamine and n-acetylglucosamine) but the matrix includes chondroitin and chondroitin sulfate (polymers of glucuronate and n-acetylgalactosamine) in varying amounts depending on location. Tensile strength in the ground substance is provided by relatively insoluble fibrous strands of collagen, elastin, and reticular fiber networks usually close to basement membranes. Other constituents more or less in solution include tropocollagen (subunits that aggregate covalently to form collagen), depolymerization products of mucopolysaccharides, glycoproteins, all components of a plasma dialysate, and significant amounts of plasma protein (*cf.* 395). Cells embedded in the matrix include fibroblasts (which produce collagen and matrix), undifferentiated mesenchymal cells, mast cells, fat cells, and macrophages. Mast cells are the main repository of the body for histamine, serotonin, and heparin (a sulfated mucopolysaccharide); disucssion of the occurrence and hormone sensitivity of mast cells in reproductive tracts is deferred to the section on circulation (III C). Quantitation of collagen in

reproductive organs has involved hydrolysis, then measurement usually of hydroxyproline, an amino acid peculiar to collagen, or incorporation and hydroxylation *in situ* of either lysine or proline-^{14}C. Biochemical studies of ground substance exploit incorporation of ^{35}S and glucose-^{14}C or measurement of constituent hexosamines. The amino acid composition of uterine collagen has been reported (212).

A. Collagen

The uterus seems to have a greater capacity for synthesizing and breaking down collagen than any tissue studied (*cf.* 275). In adult rats the uterus is well supplied with discrete bundles of thick collagen strands that penetrate the endometrial stroma throughout (152, 307, 318). These fray, break up, and ultimately disappear after ovariectomy. The same occurs in pregnancy after placentation such that at parturition the endometrium is free of collagen bundles (152, 153). The process in pregnancy, however, is accompained by substantial edema (153, 295) and thus occurs under different physiological conditions than in the castrate state. Fainstat (153) observed histologically a spectrum of collagen fiber degeneration and edematous states in layers peripheral to artificially induced decidua. In the postpartum uterus, collagen fibers reappeared after the nursing period and three weeks after resumption of normal cycles (152). Uteri of rats ovariectomized at parturition remained permanently devoid of fibers but could be stimulated to produce full complements in both endometrium and myometrium by 10 daily injections of estrogen beginning at parturition (153).

Quantitative measurements of changes in uterine collagen (hydroxyproline) agree with histological findings when it is appreciated that hydrolysates of whole organs contain hydroxyproline from collagen precursors, from collagen previously depolymerized *in vivo* and from intact collagen bundles of both stroma and muscle. Proline is hydroxylated only as collagen is being synthesized (see section II D); an increase in hydroxyproline therefore reflects collagen synthesis or turnover but not necessarily collagen organized as discrete connective tissue bundles. Thus, in apparent contrast with histological findings (*v.s.*), collagen content of uterine horns with fetuses was observed to rise continuously through pregnancy and to drop sharply after parturition (215–217, 366, 557), but organized collagen appeared to decrease while a net increase occurred in the composite of collagen precursors and breakdown products.

Collagen content of horns without fetuses in pregnant rats (from unilateral ovariectomy) remained constant at levels lower than pregnant horns but higher than horns in normal cycling animals. In the horns with fetuses, collagen content at placental sites was significantly lower than in the remainder of the horn that was distended with fluid. These and other observations led Harkness and Harkness (216) to conclude that mechanical distention is a principal, direct stimulus of collagen synthesis. The conclusion is supported by later findings that artificial distention of uteri in estrogen-progesterone-relaxin-primed rats induced collagen synthesis as well as cell division in both epithelium and connective tissue (107, 214, 505). Any distension-induced cell division in epithelium and stroma is an addition to mitosis already stimulated by estrogen plus progesterone (*e.g.*, 339).

Uterine collagen content decreases markedly after parturition to levels lower than in normal non-pregnant uteri; 85% of the total is degraded within 4 days, a rate of loss much more rapid than after castration (215, 217, 557). Daily administration of estradiol over the same period prevents most of this loss, an effect apparently due to inhibition of collagen breakdown by estrogen rather than stimulation of new synthesis (437, 557). Thus, under treatment, free hydroxyproline content decreased (557). Also, after collagen had first been labeled *in vivo* during pregnancy with

proline-^{14}C, more of the prelabeled collagen was present in treated uteri than in controls 3 days postpartum with no decrease in specific activity of labeled hydroxyproline residues. Incorporation of proline-^{14}C given postpartum was not affected by estradiol and less hydroxyproline derivative appeared in the urine of treated rats than of controls (437). Both progesterone (178) and estradiol (557) inhibit the sharp decrease in uterine weight that normally occurs postpartum but the inhibiting effect of estradiol on collagen breakdown is greater than on weight (557).

Activities of hydroxyproline epimerase and proline oxidase were shown histochemically to be markedly increased in fibroblasts of myometrium, basal mucosa, and around blood vessels (387) late in pregnancy and in early postpartum uteri of rats and women, but amounts of a variety of acid hydrolases were found by Ryan and Woessner (437) to be unaffected by estradiol in the postpartum uterus. Discussion of hydrolases will be resumed below in connection with activity of lysosomes.

During the estrous cycle, uterine collagen content generally increases from diestrus to estrus in rats (218, 365, 474) and opossums (363) but analysis of specific stages in the proliferative phase by Smith and Kaltreider (474) showed the increase actually occurs between early and late estrus after having dropped to a cycle minimum at proestrus. The content continues to climb after ovulation and decreases again with regression of the corpus luteum in diestrus (365, 474). The size of the uterine collagenous framework is maximum at estrus with a second increase at diestrus and is depressed below normal by ovariectomy (106). The collagen content of the uterus falls after ovariectomy, but injections of estradiol over 2 or more days reverses the decline (218, 243, 275, 474) and increases the size of the collagenous framework (102, 106). The vagina is relatively insensitive to estrogen with respect to collagen (215, 364) but responds when treated by injection over long intervals (318). Vaginal collagen was found not to vary with the cycle (365). After prolonged periods under castrate condition the uterus apparently becomes resistant to hormone stimulation; Harkness et al. (218) could observe only slight changes in collagen in rats treated by injection 2 to 3 months after operation. Results with progesterone are equivocal; given with estrogen, both enhancement (106, 474) and some inhibition (243) of estrogen effects on collagen have been reported. Progesterone alone was weakly positive at best (102, 106, 152, 243, 273, 275, 474) and prolonged treatment (13 days) caused losses in total collagen to below castrate levels (474).

Some metabolic turnover of uterine collagen may occur early both in treated (229) and cycling animals, whereas a minimum of 2 days of treatment with estrogen is required for increased net synthesis of total collagenous material (243) in accord with an apparent lag observed during the cycle prior to ovulation (*v.s.*). Incorporation of lysine-^{14}C into hydroxylsyl residues of collagen *in vitro* was maximal in slices of uteri from preovulatory animals and less from those in the midst of estrus (274). Uteri taken from castrates given a single, microgram dose of estradiol incorporated significant amounts of lysine-^{14}C 16 hours after injection with hormone but not after 3 hours (275); other intervals were not studied and the exact time course of the effect is not known. Estradiol was ineffective when added directly to media in incubations of uteri (275). Rat uterine protocollagen hydroxylase activity decreased to 15% of normal after ovariectomy but was restored to normal levels after 3 days of treatment with estradiol; progesterone alone increased activity to 30% of normal in 3 days but the combination of hormones was no more effective than estrogen alone (273).

B. Ground Substance

Ground substance in the female tract follows a somewhat different cyclic pattern than collagen. Hexosamine content (365),

uptake of ^{35}S (496), and metachromatic staining due to acid mucopolysaccharides (144, 564) progressively increase in the uterus from diestrus to ovulation. Hexosamines then fall (365) and staining reactions are sharply diminished in the stroma by the third postovulatory day, being retained (strongly) only in epithelial cells and glandular lumina (144, 564). The latter staining was resistant to hyaluronidase, indicating absence of chondroitin sulfates. In castrate rats and rabbits, incorporation of ^{35}S into the uterine stroma (564) and into sulfated mucopolysaccharide fractions of whole uteri (465) was very low and incorporation of glucose-^{14}C into hyaluronic acid did not occur (465). Injections of estradiol markedly increased total organ hexosamine (364), uronic acid (290), and incorporation of ^{35}S into the luminal two-thirds of the stroma (564) cohabitant with areas of increased metachromasia (475, 564). With continued exposure to estrogen, labeled material decreased in the stroma while it increased in the lumen and lining epithelium (564). Heavier mucopolysaccharide staining also was associated with stromal small-vessel walls and collagen fibers, and with luminal surface membranes, microvilli, and endoplasmic reticulum (but not Golgi bodies) of epithelial cells (475). Measurements with ^{35}S and hexose-^{14}C in estrogen-stimulated uteri indicated rapid turnover of macromolecules with half-lives for hyaluronic acid (465) and sulfated compounds (465, 564) of about 24 and 32 hours, respectively. The time course of net mucopolysaccharide synthesis in the uterus after a single dose of estrogen showed a rapid rise beginning 6 hours after hormone injection, with incorporated label from glucose-^{14}C first appearing between 3 to 6 hours (465). Progesterone or progestins given with estrogen inhibited estrogen-induced staining for mucopolysaccharides (475, 564) and ^{35}S uptake (564) in uterine stroma but simultaneously enhanced staining and labeling in epithelia and in luminal secretion. Progesterone appears to inhibit estrogen action on mucopolysaccharides by depressing synthesis rather than increasing breakdown (564). Total hexosamine content rose in the vagina after estrogen injection (364) but did not vary during the cycle (365). Incorporation of ^{35}S in the uterine cervix (in contrast with the uterine body), vagina, and oviducts after estrogen was much more intense in epithelia than stroma and with time appeared in luminal secretions (564); total ground substance in the cervical stroma was increased, however (307), as in the uterine body. Mucopolysaccharides of skin and intestine were not affected by estrogen treatment under conditions of these experiments (364, 465).

C. Relation of Hydrolases and Lysosomal Function

1. Female. Catabolism of collagen and ground substance in female reproductive tracts has been studied in relation to normal breakdown and remodeling during the estrous cycle and after pregnancy. Interest in the problem continues and has been approached more recently from the standpoint of the function of lysosomes, membrane-bound organelles that contain an array of acid hydrolases including ribo- and deoxyribonucleases, peptidases, phosphatases, esterases, β-glucuronidase, and collagenase (123). Uteri of the several species tested are relatively high in hydrolase activities, particularly in proproteolytic enzymes (7, 472); see Woessner (558) for details of work prior to 1967. Under acute conditions, within 4 hours of an estrogen injection, activities of lysosomal acid hydrolases increase in epithelium and stroma of uterus and vagina (536) and in preputial glands (503).

Most hydrolase measurements have been made in organs actively growing under hormonal stimulation. Observed enzyme changes have not been distinguished from the stimulated general increase in protein synthesis and it is not clear therefore whether enzyme change represents (a) activation or *de novo* synthesis or (b) primary or secondary action of the hor-

mone. However, evidence exists to support an hypothesis that primary estrogen action may be multifaceted and compartmentalized in a given tissue (see section III). From this standpoint, special significance may be attached to hydrolases discretely packaged in lysosomes, the increasing presence of which seem timed for the normal involution of diestrus and postpregnancy. Thus, enzyme-laden lysosomes are more abundant in luminal and glandular epithelia of rat (19), mouse (473), and human (448) uteri from proestrus onward, with the appearance of the largest lysosomes coinciding with reduction in cell height at diestrus (19). In the ultrastructural study of rabbit endometrium by Smith and Henzl (475), marked increases were seen in size and numbers of epithelial lysosomes during chronic estrogen administration to castrates. Golgi cysternae were more numerous and cysternae and lysosomes more densely reactive for marker acid phosphatase. Stromal lysosomes were infrequent after estrogen alone but injection of progestin with estradiol increased the number and staining in both stroma and epithelium. Myometrium also was affected; 3 days of treatment with estradiol alone or with progesterone increased the proteolytic enzymes, phosphatases, and ribonuclease in the muscle. The enzymes were concentrated in "light and heavy mitochondrial" fractions which included lysosomes of differing sizes; activities increased to a maximum by the 10th daily injection and then declined with further treatment (177).

Experimental withdrawal of estrogen plus progesterone after 9 days of treatment produced endometrial changes of a different character (475). Shortly after withdrawal, macrophages appeared between epithelial cells, and lysosomes in stromal cells stained intensely for phosphatase. By 48 hours, the lining epithelium contained large enzyme-reactive lysosomes and still larger reactive autophagic bodies enclosing cell parts in stages of digestion (475). With time, lysosomal and autophagic structures appeared in subepithelial areas and finally throughout the stroma (316, 475). Continued estrogen treatment after withdrawal of progesterone prevented formation of these regressive structures (475), thus indicating a specific requirement for estrogen deprivation in normal diestral involution.

In postpartum uterine involution, loss of collagen and ground substance accompanied enlargement of stromal cells and loss of cytoplasm in epithelial and smooth muscle cells previously enlarged during pregnancy (316, 322). Collagenase, other proteolytic enzymes (366, 437, 472, 557), and phosphatase (316) either remained at the high levels seen in pregnancy, or increased and then declined. Marked enzyme activity was observed in granules of epithelial cells particularly at placental detachment sites, in stromal macrophages, in other cells throughout the stroma (316), and in lysosomal fractions from centrifuged homogenates (557). As in diestrus, postpartum involution seems to be triggered by hormone withdrawal, but the known regressive changes postpartum have not been linked uniformly to loss of hormone influence. For example, injections of estradiol or progesterone beginning at term retard involution; estrogen exerts this effect in part at least by inhibiting collagen breakdown (*v.s.*), yet no effects of estrogen on levels or activities of hydrolases (including collagenase) could be detected (437, 557). Progesterone on the other hand, added to cultures of postpartum uterine tissue, was observed to depress both collagenase activity of the cells and release of collagen peptides into the medium (249).

In general, observations are consistent with a theory that normal remodeling of existing uterine cells and breakup of cellular and extracellular material during the cycle and postpartum are mediated by lysosomal hydrolases previously formed or activated by ovarian hormones (19, 178, 316, 475, 557). Smith and Henzl (475) put it concisely: "while the endometrium proliferates, it accumulates destructive potential." Further, the sequential occurrence of lysosomes and other autolytic bodies in

epithelium and then stroma is consistent with other observations that suggest the two tissue layers are functionally interdependent (see section D, below). Hormonal control of hydrolase liberation within cells or to the exterior is subtle and seems to differ with site and conditions. Thus, lysosomes and related bodies with encapsulated enzymes, their formation stimulated by hormones in growing tissues, are permitted by those hormones to remodel intracellularly but are otherwise held in check. Regressive enzyme activity on a more massive scale, including disaggregation and hydrolysis of extracellular material as occurs in diestrus and postpartum, seems to be triggered by normal withdrawal of hormones; experimental maintenance of high estrogen (475) or progesterone (178) levels prevented normal regressive changes of the diestrus and postpartum types, respectively. On the other hand, intracellular remodeling (particularly in the luminal epithelium), which seems to be more or less a continuous process, may be regulated by positive hormone action. For example, progesterone seems to increase lability of lysosomal membranes (*cf.* 475). Also, Szego *et al.* (503) recently showed that isolated rat uterine lysosomes were more labile after estrogen treatment than in controls, as measured by release of hydrolases into the suspending medium after lytic stress; the effect was detectable 15 minutes after injection of estrogen. Similar results were obtained with preputial gland lysosomes of either sex in response to estradiol or testosterone. Based on these and other data, Szego (500) has proposed a general theory involving lysosomal activity as the mediating mechanism in a variety of cytoplasmic and nuclear events stimulated by gonadal hormones [see also remarks on steroid-lysosome interaction by Weissman (538)].

2. *Male.* Acid hydrolases occur in all male sex accessory organs but are especially prominent in tissue and secretion of the prostatic lobes (*cf.* 67, 188, 227, 281, 325, 401). Hydrolase activities, particularly that of acid phosphatase, have been associated in light- and electronmicroscope studies with prostatic lysosomes (67, 213, 226, 302, 506); the association has been discussed in reviews by Brandes (67), Dott (137), and Williams-Ashman and Reddi (550). Lysosomes and lysosome-like bodies occur abundantly in prostatic epithelial cells of both normal and castrated animals but their intracellular distributions differ under the two conditions, implying that the hydrolases they contain have different functions. The prostatic enzymes "concentrate" in large numbers of lysosomes when loss of cytoplasm and reduction of organelles takes place after castration (67, 228, 303), and the concentration seems to be causally linked to this regression. For example, aminopeptidase activity is greatly increased in prostatic lobes after castration or estrogen treatment (285). Association of lysosome activity with degenerative changes also has been seen in cultured prostates in the absence of testosterone or in the presence of estrogen (175, 302), and in prostates (67) and seminal vesicles (321) of animals chronically treated with estradiol. Lysosomes (321) and total hydrolase activity (67, 188, 227, 422) eventually decrease in castrates but losses are reversible with testosterone treatment. Under testosterone influence, however, enzyme-rich particles of a different specialization appear; in normal prostatic epithelium Helminen and Ericsson (226) observed secretory granules, considered to be primary lysosomes, migrating from Golgi bodies to the apical cytoplasm, apparently without engaging in digestive events *en route*. These eventually discharged acid phosphatase and presumably other hydrolases to the alveolar lumen through fusion with the apical plasma membrane. The extracellular function of hydrolases in prostatic *secretion* is unknown.

D. Summary and Comment on Hormone Action Mechanisms in Connective Tissue

Measurements in normal animals and in castrates after hormone replacement permit the following conclusions: Increased rates of turnover and net synthesis of uterine stromal collagen and ground substance coincide with maximum estrogen secretion; experiments confirm that estrogen is the primary stimulant Timing of events in the two connective tissue components differ in the rest of the cycle apparently because of differential sensitivity to progesterone. Progesterone tends to enhance estrogen action on collagen but inhibits stimulation of matrix formation by estrogen. Formation of collagen and matrix mucopolysaccharides seem to be separately controlled by estrogen and these syntheses in turn are separable from the stimulation of general protein synthesis. For example, several works cited include measurements of non-collagenous protein; rates of change in content or labeling of non-collagenous protein and in organ dry weight consistently differed from rates for connective tissue in the same animals. Withdrawal of hormones, by gonadectomy in either sex or that normal to diestrus or postpregnancy in females, triggers breakdown of connective tissue and "degenerative" changes elsewhere in accessory organs. Details vary with organ and circumstance but general patterns may be discerned. Gonadal steroid hormones maintain basal numbers of lysosomes and associated hydrolases, or with time stimulate increases in their number and activity. Under positive hormonal control lysosomal activity is confined largely to intracellular remodeling or to contributing hydrolases to an exocrine secretory product. Meanwhile, properties of lysosomal membranes seem to alter such that on hormone withdrawal hydrolase and autophagic activities are diverted to reducing cytoplasm and disaggregating organized connective tissue.

The foregoing indicates connective tissue in uterine stroma and that directly supporting epithelia, muscle, and vessels undergo continuous physical change during the estrous cycle. The physiological behavior of connective tissue, as a diffusion "barrier" between capillaries and target cells, may be expected to shift accordingly. Through much of the cycle, collagen and ground substance are increasingly deposited; in rodent uteri at least, collagen is organized and maintained as well-formed, coarse bundles of fibers. The uterine wall progressively thickens through the follicular and luteal phases of the cycle because of muscle hypertrophy and mucosal hyperplasia. Assuming other factors to be constant, the general denseness of fibrillar networks, their compression between expanding muscle cells, the lengthening of diffusion paths, and differential binding in both collagen and matrix may be expected to impede interstitial flow of ions, water, and organic metabolites. However, in midcycle (proestrus, estrus) when estrogen secretion is maximal, the collagenous framework and encompassing matrix is greatly stretched and hydrated by pressure of accumulated luminal fluid and tissue edema, respectively. The wall is otherwise morphologically thick but has temporarily been thinned by distention. These changes coincide with increased motility in myometrium and sharply increased uptake of water, ions (particularly Na), and organic substrates in cellular and extracellular compartments; qualitatively similar changes occur in castrates after estrogen treatment (*cf.* section IV). Causal relationships between the connective tissue changes and physiological events in midcycle have not been studied but are probable. For example, uterine edema may be traced to increases in blood flow, capillary permeability, and tissue osmotic pressure stimulated by estrogen (*cf.* section III) but binding of the extra water by large amounts of connective tissue almost certainly contributes to the effect. In early diestrus by contrast, the uterine wall is structurally thin from reductions in tissue

water, in stromal width, and in cell dimensions throughout; hydrolases reduce cytoplasm and connective tissue but remaining collagen bundles are closely compacted (153). Intracellular distances are shortened but the denseness of extracellular space, coupled with low blood flow rate, may account in part for cycle minima in tissue uptake of plasma constituents and in cell metabolism. Subtler effects of hormones on metabolism, cellular transport, and smooth muscle behavior (sections III and IV) are mediated in part by changes in the cellular microenvironment that may in turn reflect alterations in the physical state of connective tissue components.

The general literature on connective tissues establishes fibroblasts and fibrocytes as principal origins of matrix and tropocollagen (*cf.* 429, 537) but there is little direct evidence that these cells in reproductive organs are principal sites of hormone-regulated synthesis. Glandular epithelia and possible smooth muscle (62, 423, 430, 435) apparently contribute oligosaccharidic mucins (glycoproteins) as well as some mucopolysaccharides and collagen to the uterine connective tissue. Still, fibroblast hypertrophy with greatly enlarged rough endoplasmic reticulum and Golgi structures have been observed in the rat uterus in estrus and after estrogen treatment (430) and in the stimulated mouse cervix (307). Ozzello (391) reported direct estrogenic stimulation of mucopolysaccharide secretion by human embryonal subcutaneous fibroblasts in culture, although estrogen depressed fibroblast growth rate. Inhibition of cell division by both estrogen and testosterone is a general finding in cultured fibroblasts and other cells from non-target tissues (*cf.* 301). Studies of stroma explanted from reproductive tracts are few and results difficult to interpret. Cultured juvenile prostates exhibited epithelial regression but increased growth of stromal cells and fibers; adding testosterone restored the epithelium but retarded the stroma, whereas addition of estradiol stimulated the stroma without apparent effect on epithelial regression (301). Study of estrogen effects on female reproductive stroma *in vitro* has been neglected and deserves intensive study. A rationale for study is based on well-known observations that explanted epithelial layers of male and female sex accessory organs proliferate when exposed to testosterone and estrogen, respectively; it is not generally appreciated, however, that such growth occurs consistently only if underlying stroma is attached (*cf.* 301). A functional relationship between stroma and epithelium is indicated and its potential importance in understanding gonadal hormone regulation of epithelial growth *in vivo* cannot be exaggerated.

Mechanisms of estrogen action in matrix and collagen formation are unknown, but promising leads have emerged that bear on this and the broader problem of hormonal control of specialized cellular function. Collagen biosynthesis with its special characteristics is particularly intriguing as a possible model system for study of hormone mechanisms. In general, protocollagen peptides formed on fibroblast polyribosomes are routed through the Golgi complex where they are assembled into triple-stranded tropocollagen units (537); these are secreted and then polymerized into collagen fibers extracellularly (429). Amino acids of collagen include approximately 30% glycine, 20% proline and hydroxyproline mixture, and 1% hydroxylysine, of which hydroxyproline and hydroxylysine are unique to collagen. Codons for these hydroxylated amino acids do not exist and it is clear from other evidence that they derive from lysine and specifically positioned proline residues, both of which undergo hydroxylation only after incorporation into the lengthening peptide chain (*cf.* 187, 353). Hydroxylation of either amino acid requires a microsome-associated hydroxylase, ferrous ions, α-ketoglutarate (as specific cofactor), ascorbic acid, and molecular oxygen (187, 244, 326, 403, 521). Collagen formation is impaired by ascorbic acid deficiency

(scurvy), the impairment traceable to a requirement for ascorbate in peptide hydroxylation. This and other evidence suggest hydroxylation of proline is essential for extracellular aggregation of subunits into collagen and perhaps for secretion earlier of the subunits (*cf.* 187). Hydroxylysine residues in collagen are important as points of attachment of polysaccharide chains.

Estrogen might control collagen synthesis through production of fibroblast RNAs[1] appropriate for the coding or assembly of tropocollagen peptides or attendant enzymes. In view of a lack of information on that point, it is equally probable that the hormone affects cytoplasmic events, thereby altering the rate of extracellular collagen deposition by prior action on hydroxylation. Consistent with the latter mechanism, rat uterine protocollagen hydroxylase activity rises after treatment with estrogen (273). Of related significance, uterine slices from estrogen-treated rats, incubated with lysine-^{14}C, contained 16 times more label (specific activity) in collagen hydroxylysine than control subjects, whereas incorporation into collagen lysine increased only 4 to 5 times in the same groups (275). Other mechanisms could involve availability of proline or cofactors known to be rate-limiting. Estrogenic control may be highly indirect. Kao *et al.* (274) found incorporation of lysine-^{14}C into uterine collagen *in vitro* to be virtually suppressed by omission of NaCl from the medium. This suggests mediation by ions, possibly related to the net movement of Na into the uterus that occurs *in vivo* within 3 hours after injection with estrogen (114, 267, 486).

Hormonal control mechanisms for synthesis of ground substance mucopolysaccharides are even less clear. The matter is complicated by the fact that both the sugar and protein moieties of these macromolecules are synthesized in the same cell and packaged together before secretion, involving both endoplasmic reticular and Golgi systems. Protein synthesis, sulfation, and hexose transport are among limiting steps possibly subject to hormonal control. Pool sizes of UTP, UDP-glucose and UDP-n-acetylglucosamine enlarge in uteri of castrates 1 to 2 hours after estradiol injection. Whether the increases in uridine nucleotides arise from *de novo* synthesis or from hormone-induced breakdown of nucleic acids is not known (181), but metabolism of the nucleotide-hexoses involved in synthesis of sugar macromolecules is another possible control point. The effect on uridine nucleotides coincides with accelerated intracellular uptake of 5- and 6-C sugars stimulated by estrogen (426, 482) but apparently precedes both incorporation of hexose into mucopolysaccharides (465) and net synthesis of general uterine protein (379).

III. Hormonal Control of Local Circulation

Organ metabolism is subject to modulation through change in blood supply. Change is effected by the net of any alterations in systemic blood pressure, local arteriovenous flow rates, functional capillary volume, and capillary permeability. These factors influence metabolism by controlling temperature, hydration, pH, and rates of by-product removal, in addition to affecting nutrient and oxygen supply. Local vascular beds are subject in turn to feedback control by cells they supply but positive control by endocrine and/or autonomic nervous systems is far more significant. Responses of target organs to their

[1] The abbreviations used are (in alphabetical order): ADP, adenosine diphosphate; AIB, α-amino isobutyric acid; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CA, cytosine arabinoside; CMP, cytidine monophosphate; CTP, cytidine triphosphate; DHT, dihydrotestosterone; DNA, deoxyribonuclieic acid; EDTA, diaminoethanetetraacetate; GTP, guanosine triphosphate; NAD, NADH, nicotinamide adenine dinucleotide and its reduced form; NADP, NADPH, nicotinamide adenine dinucleotide phosphate and its reduced form; PG, prostaglandins; RNA, ribonucleic acid; UDP, uridine diphosphate; UDGP, uridine diphosphoglucose; UDPAG, uridine diphospho-N-acetylglucosamine; UTP, uridine triphosphate.

respective hormones are known to include a vascular effect, usually reactive hyperemia, and accessory reproductive organs are among the most sensitive in this regard. Hormone-induced vascular responses in the accessory organs are surveyed below. Relationships of the induced vascular changes with hormone-stimulated growth are discussed, and information on modes of action of gonadal hormones on these vascular beds is evaluated. The functional neuroanatomy of mammalian female tracts is described in several articles (4, 35, 70, 78, 95, 294, 390, 394, 466, 561) and a review (334a). For vascular anatomy and for circulatory physiology of pregnant states, see reviews (15, 245, 311, 328, 366, 404, 407), the comprehensive work on rat uterine vasculature by Williams (548), and other articles (50, 133, 135, 158, 159, 200, 241, 293, 457). Pertinent aspects of structure in male accessory organs have been described (*cf.* 127, 146, 164, 257, 298, 415, 534).

A. Blood Flow

1. Female. Observations aided by Lucite rod translumination and dyes injected intravenously established that uterine vascularity increases during the follicular phase of the estrous cycle. By late diestrus in rodents, change from the resting state of mid-diestrus is already evident; vasodilation of extrauterine, then myometrial, plexes has taken place and hyperemia of the uterine antimesometrial quadrant has been established. Thereafter to proestrus, dilation and hyperemia proceed throughout the endometrium progressively inward toward the lumen, affecting in latter stages the subepithelial capillary plexus (548). Distention of the uterus is characteristic of the follicular phase, first from tissue edema then intensified by luminal accumulation of fluid, and is clearly preceded by the vascular effects. During estrus after maximum distention, the uterus begins a "collapse" initiated by expulsion of luminal fluid. In metestrus, vessels are somewhat reduced though venous channels appear more dilated [also characteristic of human oviducal veins (292)], and the subepithelial capillaries together with basket-like networks around the shorter endometrial glands are prominent. Through metestrus to mid-diestrus vascular activity progressively decreases (548).

Quantitative and semiquantitative measurements of uterine blood flow rates in the cycles of several species (see 366 for methods) generally conform with the foregoing description of structural changes in the rat. Expressed as organ total flow/min, rates in ewes (195) and women (402) increase in the presence of growing follicles to a maximum during estrus and gradually decline after ovulation. This same pattern qualitatively was reported by Markee (330, 332) in classical experiments on monkey and guinea pig endometria transplanted homologously to the iris and observed through the anterior chamber; largest apparent flows judged by color occurred in the follicular half of the cycles of the hosts and appeared maximal at estrus. Total flow/min in sows, however, has been reported maximal during the luteal phase (130). Expressed as rates per unit of organ weight, results seem to differ with species, but differences may be more a function of variations in timing of maximum uterine weight increases through water uptake and growth than true species differences in flow pattern. Thus, rates per unit of weight in both sows (130) and ewes (239) were maximal in the presence of well-developed follicles and lowest in luteal phases which, in the sow at least, coincide with the largest weight increase; in rats (291, 357) and mice (50) rates were lowest at estrus (maximum weight) and highest in diestrus.

Despite some possible species variation in timing of maximum flow, it is clear from detailed observations of normal cycles that increase in flow over the resting state originates in late diestrus coincident with initial growth of follicles (50, 120, 291, 330, 357, 392, 548). Estrogen is the hormone responsible for the vascular change. A single dose of estradiol to immature or

castrate animals causes marked increases in flow rate (196, 240, 264, 483, 487) and in uterine blood volume as measured by amount and distribution of inert intravascular particles (236, 392, 502, 548), albumin-^{131}I, or labeled red cells (67a, 120, 395). Vascular pool size enlarges in proportion to the increase in uterine wet weight and does not seem to change when expressed on a wet weight basis (98, 267, 395), but the increase in blood volume becomes strikingly apparent when expressed as an organ total (98) or per unit organ dry weight (120, 395). Under more or less acute conditions of treatment (a few hours), progesterone or other steroids alone have no effect (120, 196) and given with estradiol usually inhibit (196, 502). Acute estrogen treatment increases vaginal blood flow (3) although to less extent than in the uterus. Flow in tissues outside the reproductive tract do not seem affected (67a, 483; *v.i.*). The influence of the hormone is not secondary to general systemic effects since blood pressure or cardiac output under these acute conditions are not changed (240, 522, 526); after several hours to days exposure, however, estrogens tend to increase cardiac output and to lower general blood pressure both in mammals (132, 233, 522, 526) and birds (492). With chronic treatment, estrogen in the rodent uterus stimulates occurrence of dense networks of uniform-sized capillaries throughout (548) and greatly increases the size of veins (165). Chronic treatment also causes cell division in uterine vascular endothelia of monkeys (234), and vastly increases uterine blood flow rates in sows (131); progesterone generally potentiates these estrogen effects (except blood flow) but is inactive alone.

The effect of estrogen on uterine blood flow and volume is rapid and its timing is significant in considering mechanism as well as possible cause-effect relationships between blood flow and early metabolic responses to the hormone. The rapid onset of the blood flow effect was first observed by Markee (330). Injection of estrogen into guinea pigs with intraocular transplants of endometrium produced within 20 minutes a sustained "blush" in the transplant similar to that seen at estrus. With ink-injected preparations, Williams (548) could distinguish dilation within an hour of hormone administration. In quantitative time-course studies, Spaziani and Suddick (483) showed that uterine blood flow rate in the rat increases significantly within 30 minutes after an intravenous injection of estradiol, and rises linearly thereafter to 4 hours. The 30-minute response time has since also been shown in ovine uterus by Huckabee *et al.* (240), Resnik *et al.* (406), and Spellacy *et al.* (487); ovine vagina responds within an hour (3). The time of onset, magnitude and duration of uterine blood flow was not affected by doses of actinomycin D that blocked up to 75% of new RNA synthesis (405). Uterine blood volume in mice was increased 100% over controls 30 minutes after an intravenous dose of estradiol (67a).

2. *Male.* Accessory organs have been less studied in the male. Testosterone increases blood flow rates in primate prostates (452) and dilates small and large vessels in rat seminal vesicles, simultaneously altering the pattern of flow (201, 286) and total vascular space (42). Direct *in vivo* visualization with quartz rod illumination by Knisely *et al.* (286) showed dilation of smallest arterioles and venules of seminal vesicles beginning 1 to 38 minutes after a single intravenous injection of testosterone; the effect was relatively transient, lasting 11 to 119 minutes (286). In contrast, uterine dilation after a single estrogen dose is sustained for several hours.

B. Capillary Permeability

Capillary beds of uterus and other portions of the female tract dilated by estrogen become increasingly permeable. Normal tracts of ewes (100, 222), ferrets (209), and rabbits (222, 392) showed increased extravasation of intravenous dyes or colloidal particles throughout the follicular phase,

beginning in late diestrus (392), reaching a maximum at estrus, and diminishing in luteal phases (100, 222, 392). Passage of marker substances from the circulation into tissue was greater in all phases than in ovariectomized preparations. Increasing leakiness of the capillary system was earlier suggested by the finding (18, 529) that uterine water content increased progressively from late diestrus to proestrus. Astwood (18) discovered the edema effect could be reproduced in immature rats with an injection of estrogen. Water uptake was significantly elevated 2 hours after injection, near maximal at the 3rd hour (486) and sustained with some diminution through the 12th hour, with secondary rises from 20 to 30 hours coincident with organ growth (myometrial hypertrophy, stromal-epithelial cell division) (17, 419, 502).

In male systems by contrast, the sharp but transient rise in vascularity after injection of testosterone (*v.s.*) is not accompanied or followed by edema formation. Time course studies by Mills and Spaziani (355) showed no change in water content of prostate, seminal vesicles, and levator ani muscle from 3 to 18 hours after hormone administration. A 12-hour increase reported earlier by Rudolph and Samuels (432) was not confirmed by subsequent work of Rudolph and Starnes (433).

Experiments with systemically injected trypan blue in female rats (89, 167, 223, 224) and rabbits (223, 224) permit visualization of the extent and specificity of the increase in capillary permeability. The early time course of dye uptake by the uterus exactly parallels that for water (89, 167, 223, 224), and tissue staining is particularly heavy in the stroma (399). Several other body tissues take up dye, but edema (82, 486) and increases in dye uptake (224) after acute hormone treatment are confined to the uterus and vagina (however, see 565). Given in physiological amounts, only estrogens were found effective among the gonadal and adrenal steroid hormones tested (223, 224), although deoxycorticosterone and progesterone in larger amounts have some stimulatory effect in 6 hours (224, 565). Also, ion uptake (Na, I) by the uterus rose in proportion to the amounts of water in the earlier stages of edema formation and was largely confined to the extracellular space (267, 486). From 3 hours onward, the kinetics of uptake of ions, water, and other plasma constituents are complicated by differential penetration into cells of both myometrium and endometrium (see section IV A).

The occurrence of edema after estrogen treatment, accompanied by extravasation of vital dyes and plasma constituents of small molecular weight, could result alone from estrogen's stimulation of local blood flow, hyperemia of capillary beds, and dilation of individual capillaries which exposes a larger total surface area of microvascular wall to diffusion and hydrostatic forces. Some venule constriction may contribute to the edema effect, but local stasis of the rich uterine-vaginal lymphatic systems does not occur (223, 556) and no evidence exists for acute changes in general systemic blood pressure, blood volume, or cardiac output after estrogen administration (*v.s.*).

The observed local changes in hemodynamics undoubtedly contribute to edema production but two lines of evidence indicate strongly that estrogen also directly or indirectly increases actual capillary porosity, resulting in bulk flows across capillary walls: Large amounts of plasma protein accompany edema fluid into uterine tissue (205, 262, 267, 395, 396), sufficient to increase significantly uterine dry weight within 4 hours after estrogen injection. The resulting rise in interstitial colloid osmotic pressure is sufficient to account for edema that is sustained over several hours. Extravasated fluid with dissolved constituents in the estrogen-stimulated uterus, therefore, more resembles plasma than a plasma ultrafiltrate. Secondly, direct evidence of increased porosity is provided by electron microscopical studies. Inert tracer particles or albumin-^{131}I were observed passing between endothelial cells, and in membrane-bound vesicles near apposed

cell membranes of adjoining cells, during proestrus (392) and after a single estrogen injection (167); specialized junctional complexes between apposed membranes, earlier a continuous barrier, became discontinuous, creating open pathways between endothelial cells (392). There is an overall impression of formation, in capillaries and small venules, of transient, reversible interendothelial gaps without a widening of the intercellular spaces (167, 205, 392). The conditions suggest those of acute inflammation but structural differences have been pointed out between effects of inflammation and of estrogen injection (167, 392).

C. Modes of Hormonal Regulation

Studies of the mechanism of estrogen action in altering vascular beds and blood flow in female accessory organs may be grouped in one or the other of two operating hypotheses that are at least implicit in these investigations: 1) Estrogen controls vascular smooth muscle neurogenically by modifying the activity of autonomic innervation or concentrations of neurotransmitter substances. 2) Control by estrogen is mediated humorally by affecting concentrations, activation, or release of vasodilating substances (*e.g.*, histamine, serotonin, kinins, prostaglandins) from local repositories.

A third possibility, namely a direct estrogen action on vascular smooth muscle has received little attention but should be explored. The presence of modifiable myogenic activity in uterine vessels is suggested by a common experience of workers in this field (*e.g.*, 239): the lightest touch of the *in situ* organ with a probe causes intense and shifting redness in and near the site, thus illustrating the extreme sensitivity of the organ to virtually any stimulus. Also pertinent is the discovery by Markee (330, 331) of slow-wave (15–80 seconds depending on species), rhythmic pulsations of blood flow in endometrium transplanted to the iris (*v.s.*); the frequency of blush-pale cycles varied with the time of day and estrous cycle stage of hosts and were specific for endometrium since the rhythmic phenomenon was absent in transplants of several other tissues (330). Pulsations persisted in transplants to ovariectomized hosts and could be induced to sustained blush (resembling estrus) by estrogen injection; they persisted, moreover, with no changes in frequency, after cervical sympathectomy or stimulation, or after artificial lowering of systemic blood pressure. Pulsations began as early as 24 hours after transplantation, which was judged sufficient time to re-establish circulation but insufficient to re-establish innervation (330). These observations suggest intrinsic myogenic activity of endometrial vascular muscle that might be modified directly by estrogen. However, hormone experiments by Markee were done with well-established transplants in which autonomic innervation from the iris or mediation by diffusing neurotransmitter had not actually been ruled out.

1. Neurogenic Mediation. A neurogenic mechanism mediating estrogen-induced vasodilation would require the presence either of dilator nerve connections, presumably cholinergic in character, or tonic adrenergic constricter activity which would permit vasodilation when blocked. Studies with modern fluorescence microscopical and histological techniques in several species confirm older physiological work (*cf.* 450, 460) that the uterus, vagina, and oviduct receive both sympathetic and parasympathetic axons. Acetylcholine (*cf.* 4), acetylcholinesterase (*cf.* 35), and adrenergic transmitters particularly norephinephrine (4, 155, 346, 389, 439, 466) have been identified in these nerves and their terminals. Adham and Schenk (4) observed exclusively cholinergic fibers in and near the surface epithelia of the uterus and vagina while both adrenergic (4, 155, 466) and cholinergic materials were evident in myometrial and basal endometrial layers. Histochemical study of uterine arterial walls in guinea pigs (35) showed dense

plexes of fine fibers with high contents of acetycholinesterase and catecholamines. McKercher *et al.* (346) report adrenergic nerve terminals in the rat uterus to be almost exclusively periarterial.

A. CHOLINERGIC. Though cholinergic fibers exist in the reproductive tract (*v.s.*), their function is obscure. Stimulation-inhibition studies by Bell (35) did not show cholinergic dilator nerve activity in isolated uterine arteries of guinea pigs. Greis *et al.* (198) conclude that parasympathetic uterine vascular innervation is absent in the ewe and work by Schofield (450) indicates an absence of functional cholinergic fibers in hypogastric nerves of rabbits. However, Sherif (460) in an early study reported an acetylcholine-like substance in perfusates of isolated canine uterus after stimulation of uterine nerves and inhibition of cholinesterase with eserine. Also in the dog uterus, Ryan *et al.* (439) demonstrated a vasodilation response that could be "unmasked" by nerve stimulation after blockade of adrenergic activity with guanethidine or reserpine; vasodilation did not consistently occur after guanethidine but vasodilation, when obtained, was enhanced by cholinesterase inhibitors and abolished with atropine. Estrogen, however, given by perfusion or injection, did not produce vasodilation in this dog preparation (438).

Other studies, directly designed to explore possible cholinergic mechanisms in estrogen-induced vasodilation, used uterine infusion of acetylcholine, application of cholinergic blocking drugs to animals treated with injections of estrogen, or direct estimation of acetylcholine content after hormone treatment. Atropine failed to block estrogen-stimulated vasodilation in rats (223, 236), rabbits (223), and intraocular transplants of rabbit endometrium (260), but did inhibit the response in mice (225). Local infusion of acetylcholine produced vasodilation in ewes (199) and dogs (439), but in one study of the dog vasodilation resulted from applying atropine (5). Results in rats have been mixed: acetylcholine alone produced no response (11, 236) but enhanced the estrogen effect when given with the hormone (11). In larger doses (236), or topically applied (356), acetylcholine caused vasoconstriction. The neuronal content of acetylcholine in the rat uterus was found lowest in diestrus and highest during estrus (4). Chronic (10 days) estrogen treatment caused histochemically demonstrable increases in the acetylcholinesterase content of uterine cholinergic nerves (145). Uterine levels of acetylcholine are not apparently affected by estrogen under *acute* conditions of treatment in rats and cats (*cf.* 407), but an old disagreement about the rabbit remains unresolved; Reynolds and colleagues (*cf.* 407) reported a rise in rabbit uterus an hour after estradiol injection (but not after diethylstilbestrol), whereas Emmens *et al.* (148) could find no change in the content or synthesis of acetylcholine, or in cholinesterase activity.

From the foregoing, it is evident the uterus is supplied with cholinergic fibers. Also, estrogens cause vasodilation and hyperemia in the uterus, but experimental evidence does not support the hypothesis that acetylcholine mediates this response. Experiments suggest the presence of neurally-based vasodilation in the canine uterus, but evidence for such control generally in non-pregnant females of other species is negative or inconclusive, and in no case has estrogen-induced dilation been linked to nerve action. The functions of uterine cholinergic nerves remain unknown.

B. ADRENERGIC. Electrical stimulation of the uterine sympathetic nerve supply uniformly leads to vasoconstriction (35, 197, 421, 439). The effect can be distinguished from contraction of the whole uterus that also usually occurs (439). The vasoconstriction is effected adrenergically since it is mimicked by arterial infusion of epinephrine or norepinephrine (35, 236, 304, 331, 356, 421) and is blocked by adrenergic inhibitors (35, 197, 439).

Estrogens influence the amounts of cate-

cholamines in uterine neurons and possibly in extraneuronal sites. The catecholamine content in rat uterine neurons was found by Adham and Schenk (4) to be lowest in diestrus and to increase through proestrus to a maximum at estrus, coincident with rising titers of secreted estrogen. A similar pattern was reported by Rudzik and Miller (434) for catecholamines in rat uterine extracts, but Oskarsson (389) could detect no significant cyclic change in the norepinephrine component. Ovariectomy (389) or partial denervation of uteri in rats (26, 389) lowered the catecholamine content. The epinephrine content in rat uterine extracts was elevated after 1 to 4 days of treatment with estrogen, whereas norepinephrine showed only a transient rise in the first 2 days (434, 490, 491). Rudzik and Miller (434) observed that longer treatment depressed the epinephrine content toward castrate levels, which probably accounts for the failure of Oskarsson (389) to find any change in epinephrine after 3 weeks exposure to estrogen.

Chronic estrogen administration to ovariectomized rabbits (155, 231, 466) and guinea pigs (154) raised norepinephrine to intact control levels in the characteristic short adrenergic neurons that innervate smooth muscle of the uterus, vagina, and oviduct in these species, but not in the longer axons to the ovary and heart (155, 231, 466); the estrogen response was inhibited by progesterone. The rat uterus in contrast receives mainly, if not exclusively, "long" adrenergic nerves in which the norepinephrine content could not be seen to change after ovariectomy or 1 week of treatment with estrogen (154). Other experiments in rats suggest an *acute* response to estradiol, in which norepinephrine is *depleted*; lowered concentrations were observed in uterine periarterial nerves by 1 hour and in uterine extracts as early as 15 minutes (346).

Relevant to the catecholamine content, the uterus can accumulate or "bind" catecholamines as shown with the tritiated compounds injected systemically (189, 560). Uterine uptake of epinephrine-^{3}H in the rat was highest at estrus and lowest at diestrus while norepinephrine uptake showed the reverse cyclic pattern (560). Estradiol injection doubled epinephrine uptake in ovariectomized animals (599). Results of uptake studies are consistent with data that indicate the uterine catecholamines originate outside the uterus; for example, the rat uterus does not appear to contain a methyl transferase system required for epinephrine synthesis (491). The adrenals are not the source of the catecholamines since estradiol increases uterine epinephrine content in adrenalectomized or adrenal-demedullated rats (491).

The biological significance of the effects of estrogen on uterine catecholamine content or accumulation is unknown. Indeed, there is no convincing evidence that the uterus requires catecholamines or even a nervous system for normal function; denervation of the rat uterus (resulting in depletion of 80% of its norepinephrine content) does not interfere with the estrous cycle changes, conception, or pregnancy (26). Also, total depletion of uterine norepinephrine content with 6-hydroxydopamine does not interfere with the full ability of estrogen to increase blood volume (67b). In male organs by contrast, nervous elements as well as cholinergic and adrenergic contents have been implicated in the delivery (*cf.* 146, 298, 415, 534) and possibly the synthesis (*cf.* 317) of secretory products. Subtle influences of adrenergic agents on uterine metabolism (24) and myometrial contraction (*cf.* 270, 296 and section IV A, 2, c) exist but in relation to estrogen action are usually inhibitory (26, 304).

Summary of adrenergic aspects: electrical stimulation of the uterine sympathetic nerve supply, infusions of catecholamines and application of inhibitors indicate the system is adrenergic-vasoconstrictive. Chronic exposure to estrogen raises the uterine uptake and content of catecholamines, whereas acute treatment decreases the content. Estrogen vasodilates the

uterus and conditions are not known in which the hormone is vasoconstrictive. Denervation or depletion of catecholamines does not interfere with the estrogen responses. The dilation effect of the hormone does not seem to involve adrenergic nerves and has not been linked to changes in the organ content of catecholamines.

2. *Humoral Mediation.* A. HISTAMINE. In the *female*, a purely humoral mechanism mediating the vasodilation response to estrogen, involving release of endogenous uterine histamine, has been proposed by Spaziani and Szego (484, 485). Work by Shelesnyak and colleagues (*cf.* 328) implicating histamine in the mechanism of nidation, and scattered reports in older literature (*cf.* 484, 485 for complete summary) suggested histamine mediation of localized hyperemia. Holden (236) observed in 1939 that histamine, alone among several bioactive agents applied to the rat uterus, produced vasodilation and hyperemia. Upon exploring drugs effects, others noted histamine injections caused estrus-like vaginal smears in rats (388) and guinea pigs (91) and uterine growth in rats (11) and rabbits (90). The antihistamine, pyranisamine maleate (Neo-Antergan), produced persistent diestrus-metestrus vaginal smears (388) and tripelennamine (Pyribenzamine) reduced the effect of exogenous histamine in the uterus (11). Formalization of the hypothesis by Spaziani and Szego (484) was based on results of direct experiments. Uteri of ovariectomized adult rats contain 7 μg of histamine per g of fresh tissue (2 μg/g in intact rats), similar to values in the guinea pig (362) and in human beings (143, 202). A single dose of estradiol lowered the uterine histamine concentration 17% within 4 hours (484). The decrease was due to depletion of local tissue stores and not a result of greater enzymatic destruction (484). Further, depletion was not a secondary consequence of estrogen-induced tissue edema since blockade of water uptake with cortisol (502) did not interfere with histamine reduction by the estrogen (484); cortisol alone had no effect. The capacity of estrogens to mobilize uterine histamine was confirmed and extended by Shelesnyak (458) with the bioassay procedure for histamine and subsequently by Szego and coworkers (*cf.* 499) and McKercher *et al.* (346) with fluorometric and isotope dilution methods. Depletions of 30 to 40% of organ content have been detected (346, 458, 499) and shown to begin within an hour of hormone injection (346, 499). Histamine content is restored to normal by 14 hours (484) and the precursor, histidine, by 4 hours (346). In the estrous cycle (rats) the lowest histamine content was during diestrus II (329), coincident with earliest estrogen secretion and hyperemia (*v.s.*).

Local or parenteral injection of histamine has been shown repeatedly to produce uterine vasodilation and hyperemia, indistinguishable from the normal response to estrogen, in rats (122, 236, 305, 356, 484, 504) and dogs (5) although Bell (35) found histamine (and serotonin) to be vasoconstrictive in isolated uterine arteries of guinea pigs. Measured 4 hours after injection in rats, vasodilation was elicited by 5 μg of histamine · HCl given intraluminally, the lowest dosage tested (485). In the same interval, histamine caused an estrogen-like increase in capillary permeability to trypan blue (86). Also consistent with the histamine mediation hypothesis, direct application to uteri of the histamine releasing agents, compound 48/80 (485), methyl salicylate, and nicotinic acid (499) produced hyperemia and edema; Mongar and Schild (362) had shown that uterine histamine is released by 48/80. Further, antihistamines given by intraluminal injection suppressed the usual edema response to estradiol (485); diphenhydramine (Benadryl) and chlorprophenpyridamine (Clor-Trimeton) effectively inhibited while tripelennamine (Pyribenzamine) and pyranisamine maleate (Neo-Antergan) did not (485). Tripelennamine also did not prevent cyclic hyperemia of intraocular endome-

trial transplants (261). Diphenhydramine and chlorprophenpyridamine do not contain the intercyclic N atom common to the ineffective compounds, indicating structural specificity for the action in the rat uterus (485).

Uterine intraluminal injections were used in the latter experiments since giving the drugs systemically in amounts sufficient to affect the uterus is toxic to the whole animal. The procedure is without doubt unphysiological both in terms of injection route and the large doses necessary for effective absorption and action. These methods were justified nevertheless in the original experiments (485) from the standpoint that negative results from application of histamine or antihistamines would seriously weaken the histamine hypothesis, whereas positive findings would provide a degree of support. Birchall and Halkerston (52) showed diphenhydramine and other antihistamines were effective in equal degree in blocking uterine RNA synthesis despite differences in antihistaminic potency. These results, and their data that suggest antihistamines form insoluble complexes with RNA, indicate that these compounds affect metabolic processes nonspecifically and independent of their antihistaminic properties; the authors (52) properly caution against over-reliance on use of antihistamines to support a histamine hypothesis. Cecil *et al.* (86) questioned results with intraluminally-administered antihistamines on grounds of their finding damaged or missing epithelium. That these results may be due to technique and not to drug effects is indicated by their unique findings of tissue damage also after local injection of saline, estradiol, or histamine, and of edema production with antihistamines (86). Others cannot confirm the presence of edema after antihistamine injection (52, 485) and no tissue damage was observed, nor does damage appear in published photomicrographs, in studies (481, 504) with luminal injections that compare effects of estradiol, saline, and histamine on epithelial cell division. Indeed, uteri treated by injections of histamine are capable of normal implantation and maintenance of embryos (162).

In the *male*, histamine concentrations found in rat prostatic lobes, seminal vesicles, and ductus deferens (367) are comparable to those in the uterus. Castration did not significantly alter the histamine content of these organs in mice and rabbits (16), and an increase reported in rat prostate glands after castration and a lowering by testosterone (16) could not be confirmed in studies with both acute and chronic testosterone therapy (367).

B. MAST CELLS. In the *female*, the mechanism of uterine histamine release by estrogen is not known but may be similar to that of anaphylaxis observed in the sensitized uterus upon exposure to antigens (12, 64, 362). The sites of action both for antigens (64) and estrogens appear to be mast cells, which in the body as a whole are principal repositories of histamine and, in the rat and mouse at least, of heparin and 5-hydroxytryptamine (serotonin) (*cf.* 414, 455). Among organs, the uterus is relatively sensitive to release of its high-moderate histamine supply (64, 362) and to dissolution of its mast cells (64). Mast cells occur throughout the female reproductive tract but are more numerous in stromal parts of vagina and cervix (497) and in uterine myometrium than in endometrium (220, 309, 340, 471, 497). They are evenly distributed along the length of the uterine horns in rodents (220, 309, 340, 539) but across the uterine body are more concentrated in the antimesometrial quadrant, in the richly vascular region between muscle layers (220, 309). The antimesometrial quadrant of the rat is also the usual site of blastocyst implantation. In or near the uterine body, the largest numbers of mast cells occur in the mesometrial root (309) where they are strikingly arranged along blood vessels entering the uterus.

Uterine mast cells are fewer in number during the follicular phase of the estrous

cycle than the luteal phase in rats (173, 309, 327), guinea pigs (220), and cows (247); one study reports no change in rats (340) and cycle patterns in human uterus are not clear (345, 444). Mast cells in endometrium disappear 24 to 36 hours before implantation in the rat, coinciding with an estrogen surge associated with the decidual response (459). Effects of estrogen administration on mast cell numbers appear to depend on length of treatment, tissue, and species. Thus, injections over 12 hours to several days decreased the numbers of mast cells in the uterus (41, 173, 220, 246, 253, 327, 471, 497, 539; however see 39) and vagina (253, 541), whereas longer treatment increased the number in the mouse vagina (540) and guinea pig uterus (246); progesterone had no effect alone and slightly inhibited the estrogen action (173, 327, 471). The estrogen effect appears to be specific for the reproductive tract since mast cell numbers are not affected elsewhere (253, 309).

Estrogen presumably lowers mast cell numbers by causing their degranulation and breakup in the manner seen after application of histamine releasers such as 48/80. With acute estrogen treatment however (4 hours), LeVier and Spaziani (309) saw no change in cell numbers in the uterus or mesometrium, and conclude that early histamine release induced by estrogen (*v.s.*) occurs without disruption of mast cells, or that estrogen activates storage sites other than mast cells. Recent direct studies by McKercher *et al.* (346) indicate the former interpretation is correct although the latter is not ruled out; uterine mast cell fluorescence due to histamine was depleted an hour after estrogen injection and remained depleted over 4 hours without apparent degranulation (346). Similarly, histamine is released from the thyroid gland in response to thyrotropin without degranulation of its mast cells (150). The ovaries release histamine and become hyperemic in response to luteinizing hormone (*cf.* 499) but ovarian mast cells have not been studied. Non-mast cell stores of histamine apparently exist in the uterus and might be mobilized by estrogen; Brody *et al.* (67b) report the uterus capable of vasodilating to estrogen after the mast cells of the organ have been depleted with 48/80; extraction of mast cell-depleted uteri revealed 75% of the histamine of the organ still present and presumably available for further mobilization. In relation to the mechanism of action of estrogen an interesting observation by Di Carli *et al.* (122) should be followed up; histamine was given by subcutaneous injection to one of two groups of rats given estradiol-^{3}H. Uteri (but not other organs) of the histamine-treated group took up 375% more labeled hormone than the control group.

Mast cells occur in all layers of *male* sex accessory organs, usually in association with blood vessels, and strikingly so in the outer adventitial layer of seminal vesicles (McDonnell and Spaziani, unpublished observations). Generally, they are more numerous in ventral and dorsal prostatic lobes than in the lateral prostate (507) or seminal vesicles, but their numbers are not affected by castration or testosterone replacement (McDonnell and Spaziani, unpublished). The rapid but transient increase of blood flow observed in male accessory reproductive organs after testosterone administration (*v.s.*) is not apparently mediated by histamine release.

C. SEROTONIN, RENIN, AND KININS. Other bioactive substances potentially capable of mediating estrogen-induced vasodilation have received some study in female reproductive tracts. Small amounts of serotonin (50-fold less than histamine) occur in uterine mast cells and about 30% of the store is released by estrogen at the same time histamine is liberated (346). Snyder *et al.* (477) could not detect endogenous serotonin in extracts of pooled uteri, although the organs took up and bound circulating serotonin-^{14}C in a pattern similar to that of norepinephrine (*v.s.*; 477). Serotonin could be raised to detectable levels by infusing its precursor, 5-hydroxytryptophan; appropriate decarboxylase activity

occurs in the organ and varies with the estrous cycle, but is markedly depressed by estrogen administration (*cf.* 477). The serotonin antagonist, lysergic acid diethylamide, blocked estrogen-induced hyperemia in rat uteri (504). The cumulative evidence, however, does not support a significant role for serotonin in mediating the uterine vascular response to estrogen.

Uteri of rabbits (51, 151, 163, 436) and dogs (235) contain renin concentrations normally 10-fold less than in kidney but which may increase 100-fold during pregnancy (51, 163, 235). Castration decreased, and estrogen treatment elevated, concentrations both in the uterus *in situ* and in endometrial transplants (151). It should be noted that renin-angiotensin in extra-uterine sites is usually vasoconstrictive. The function of a renin-angiotensin system in the uterus is not known.

Local injection of bradykinin was without effect on uterine blood flow in the rat (499); this powerful vasopeptide and related kinins have not otherwise been studied in reproductive tracts.

D. PROSTAGLANDINS. Prostaglandins PGE_1 and PGA_1 (but not $PGF_{2\alpha}$) lowered uterine vascular resistance when perfused, without affecting myometrial contraction; PGE_1 redistributed blood flow to the endometrium (93, 438). Paradoxically, these prostaglandins and PGF enhanced the vasoconstrictor response to infused norepinephrine (93, 438). $PGF_{2\alpha}$ and lesser amounts of PGE have been detected in ovine uterus (554), and its venous blood (59, 514) during the 3 days of the cycle just prior to ovulation. The same was observed in guinea pigs, in which venous $PGF_{2\alpha}$ also was elevated after estrogen treatment (60). In the rat, Saksena and Harper (441) reported no significant change in uterine PGF content through the estrous cycle, and decreases in content 3 to 12 hours after injection of estrogen or progesterone. However, Ryan and colleagues (440) found that PGF content was raised 80 to 90% 2 hours after estrogen treatment. The increase due to estrogen was abolished by 2 days of pretreatment with indomethacin, an inhibitor of prostaglandin synthesis. However, the same dosage of indomethacin only partially reduced estrogen-stimulated vasodilation. Similar results were obtained with higher doses of meclofemanic acid, another inhibitor of prostaglandin synthesis (440).

These findings are the basis of an hypothesis that prostaglandins mediate estrogen-induced vasodilation (440). However, the data cited are not entirely consistent with the hypothesis. The sharp rise in prostaglandins observed in uterine vein blood near ovulation does correlate with increased estrogen secretion from the mature ovarian follicles. Uterine venous blood at that point in the cycle contained both prostaglandin series PGE and PGF (*v.s.*). However, after estrogen injection only PGF could be detected (60). As noted above, PGE—not PGF—was found effective in dilating uterine vessels. If prostaglandins mediate estrogen-induced vasodilation, then PGE should be found consistently in the venous outflow after hormone injection. Also in relation to the mediation hypothesis, the functional significance of an estrogen-induced rise in uterine prostaglandin content is not clear. A rise in content would be consistent with the hypothesis if net prostaglandin synthesis increased and was coupled with product release from cells. In this light, the data of Saksena and Harper (441) and of Ryan *et al.* (440) are not necessarily in conflict, and together may mean that an initial burst of induced synthesis is followed shortly by depletion (release) to below basal levels. Additional study is required, especially to resolve discordancies apparently due to species differences, but present information is sufficient to suggest the likelihood of a role for prostaglandins in mediating estrogen-regulated vascular changes. Relative to histamine, for which the same role has been proposed and supported (*v.s.*), it may be noted the rat uterus contains 100 times more histamine than prostaglandins (compare 484 and 440). However, infused

prostaglandins PGE effectively dilate uterine vessels in nanogram quantities (93), whereas microgram amounts of histamine appear to be required for the same effect (481, 485).

D. Relation of Increased Blood Flow to Growth

It has occurred to several workers that the biological significance of hormone-stimulated vasodilation may lie in enrichment of the cellular environment. Enhancement of substrate availability through vasodilation and increased capillary permeability may be the immediate stimulus for hypertrophy and cell division that occurs in the female reproductive tract during the cycle or after estrogen administration. Several studies support this view. Incubation of uterine segments for 18 hours in an enriched medium was sufficient alone to stimulate incorporation of glycine into nucleic acids and to stimulate the activity of amino acid activating enzymes. The extent of stimulation was equivalent to that obtained by 18 hours of treatment with estradiol *in vivo* (369). Similarly, vaginal tissue cultured in biological or complete synthetic media grew and epithelia cornified in the absence of estrogens (259, 337), although estrogen added to the medium augmented these effects (45, 259). Hechter *et al.* (225) measured *in vivo* effects on uterine growth (change in dry weight) after producing hyperemia with yohimbine, and after attempts to inhibit estrogen-stimulated vasodilation with atropine. Although growth generally could be correlated with occurrence of hyperemia, their equivocal results might have been due to inconsistent effects of the dilating and inhibiting agents used (*cf*, 236 and discussion above of atropine and acetylcholine studies). Exogenous histamine dilates vessels of the reproductive tract (*v.s.*) and under a variety of conditions stimulates growth in uteri of rats (11, 305, 481, 504), rabbits (90), and guinea pigs (420). Growth and cornification of vaginal epithelia (91, 338, 388) also occurs. Single injections of histamine or serotonin into the uterine lumen trigger epithelial cell division in 18 to 24 hours, after first producing vasodilation and edema (481, 504). In colchicinized rats increases in mitotic rate resulting from histamine or serotonin were statistically the same as the rate from estradiol given by injection (481). The histamine analogue, 3-(β-aminoethyl) pyrazole, has virtually no vasodilating effect in the uterus and did not affect the water content or mitotic rate over saline controls (481). It should be noted that any substance given intraluminally, including isotonic saline (481) or inert pellets (408), that distends the uterus even slightly, stimulates a basal level of cell division. However, injection of a vasodilator doubles the basal rate of mitosis (481).

The adrenal glucocortical steroid hormone, cortisol, blocks edema resulting from estrogen (486, 502, 530), histamine (481, 485), or histamine releasers (485). Cortisol also blocks increased ion (21, 486) and protein (395) permeability that accompanies the edema. These and other findings suggest cortisol antagonizes estrogen in the uterus by an independent action at the capillary level, consistent with its general action of decreasing capillary permeability, rather than by direct molecular competition or interaction with estrogens (485). Cortisol decreases the mitotic effect of estradiol or histamine in direct proportion to which it diminishes vasodilation and edema (481). Of related interest, delay of cortisol injection for 1 hour after injection of estrogen produces a greater suppressive effect on edema and mitosis than when the hormones are introduced together; delay in administration of cortisol for 2 hours or longer permits estrogen to escape inhibition (480). These observations point to the first 30 to 90 minutes of estrogen interaction with target tissue as critical for development of cellular events leading to growth (480). That an anti-inflammatory agent (cortisol) can suppress the growth effect of estrogen suggests that early estrogen effects are critical

to growth and are dependent on hyperemia.

Together, these studies establish a positive correlation between stimulation of local vasculature and occurrence of growth. Virtually any substance that produced uterine vasodilation and edema also triggered a growth response; with estrogen present, agents that depressed hyperemia similarly depressed growth. The correlation suggests that vascular effects triggered by estrogen are causal in the chain of responses ending in hypertrophy and cell division. Since uterine substrate supplies increase with hyperemia, the mechanism in growth stimulation may be assumed to depend upon substrate concentration as rate-limiting. A functional relationship between hyperemia and growth would demand that metabolic pathways linked to the growth process and stimulated by estrogen should also be stimulated by non-specific vasodilating agents. Results of test experiments are generally consistent with that expectation. Thus, histamine, like estrogen, increased glycine incorporation into uterine proteins, lipids, and nucleic acid purines (499). Similarly, glycogen synthesis and phosphorylase activity were elevated by histamine, estradiol (88, 305, 361), or the histamine releaser, 48/80 (361), and were depressed along with uterine weight when blood flow was restricted by epinephrine-induced vasoconstriction (305). However, these experiments do not distinguish between effects that may result from vasodilation and direct non-specific effects the dilating agents themselves may have on target cell metabolism. Also, serotonin, given by any route, did not affect glycogen or phosphorylase; histamine, effective when administered intraperitoneally, had no effect on these parameters when injected into the uterine lumen (305). Finally, workers in this field with few exceptions have noted that effects on metabolism and growth produced by vasodilating agents rarely measure on an absolute scale to full-blown estrogen responses, although they favorably compare statistically. It can be argued that these inconsistencies arise from use of agents which differ from estrogen in absorption through the unnatural routes employed; non-specific, semi-toxic side effects of the dosages used may also interfere with the responses. A full-blown response might be expected to occur when the vasodilator substance is endogenous ("intrinsic") and is released from cellular loci in close apposition to blood vessels. In short, most evidence points to a causal relationship in the stimulated uterus between growth and prior occurrence of vasodilation-hyperemia, but that evidence is circumstantial. Gonadal hormones also effect substrate transport in target cells (*cf.* section IV) but functional links between (a) enrichment of the cellular environment through increased vascular permeability, (b) transport, and (c) growth have not been established conclusively.

E. Summary and Conclusions

Studies in normal and hormone-treated castrates of both sexes have established that increased blood volume, flow rate, hyperemia, and enlarged microvascular surface area are characteristic early responses of accessory reproductive organs to gonadal hormones. The effects are measurable within several minutes of hormone administration and, in the female tract, are sustained for several hours. In the uterus, initial estrogenic stimulation of blood flow is followed by tissue uptake of water and other plasma constituents, a result in part of physiological and structural changes permitting bulk flows through increasingly porous capillary walls. Assessment of data bearing on the mechanism of estrogen-stimulated hyperemia leads to the following conclusions: (a) Initiation of hyperemia is not dependent upon genome activation in target cells; the response occurs at least simultaneously with earliest detected increases in protein and RNA synthesis (*cf.* section IV C, D and refs. 25, 184, 207), precedes changes in RNA polymerase activity (*cf.* section IV D

and refs. 179, 207), and is not sensitive to actinomycin D. (b) Direct hormone interaction with vascular smooth muscle appears unlikely but has not been ruled out. (c) Studies of the uterine autonomic nerve supply, including both adrenergic and cholinergic elements, and results of denervation, indicate that the nervous system does not mediate vasodilation and hyperemia. (d) Estrogen controls uterine uptake and content of adrenergic substances, but these are functionally vasoconstrictive in the uterus. Results from a variety of experiments with acetylcholine are largely negative. (e) Estrogens mobilize histamine from mast cells and possibly from other uterine stores of the amine. Additional direct and indirect evidence supports a purely humoral mechanism, involving mediation by histamine, as the currently plausible explanation of control of uterine blood flow by estrogen. Information is emerging that suggests uterine prostaglandins also may be involved. The function of hyperemic responses in reproductive organs is not fully clear but circumstantial evidence indicates that the stimulation of growth by gonadal hormones depends in part upon prior stimulation of hyperemia.

IV. Control of Cellular Transport and Tissue Distribution of Molecules; Relations to Cellular Structure, Physiology, and Metabolism

A. Ions and Water

Hormonal control of electrolyte distributions and transport in vertebrate accessory reproductive organs has received intensive study only in the mammalian uterus and that emphasis is adopted here. For this survey, uterine studies are divided into two groups, those involving measurements in the whole organ and those primarily concerned with the myometrium. The division is not entirely arbitrary since it reflects somewhat different experimental objectives. Most of the former are oriented to an understanding of early estrogen action on metabolism and cell division mainly in the endometrium. The technical problem of isolating viable endometrial tissue has been a determining factor in experimental design, so that the bulk of work has been performed on the whole organ with all the attendant difficulties in interpretation that approach entails; analysis of endometrium by difference from whole organ has been attempted. Myometrium on the other hand has been studied extensively as an isolated smooth muscle preparation, primarily by muscle physiologists; information on ion compartmentation and shift has been sought as a basis for understanding excitation, membrane potentials and contraction, and changes in these properties as these have been influenced by estrogen or progesterone-dominated states of the cycle or pregnancy have not always been of prime consideration.

1. Whole Organs. A. EXTRACELLULAR VOLUME AND WATER DISTRIBUTION. Accurate determination of ion and organic metabolite distribution rests on reliable means of measuring tissue extracellular volume. Estimations in the uterus derive (a) from measurements *in vitro* of wash-out rates of Na and Cl, ions assumed from findings of skeletal muscles to be largely or exclusively extracellular, and (b) from uptake *in vivo* or *in vitro* or organic molecules (*e.g.*, sucrose, inulin) assumed to be inert under the conditions employed and sufficiently large so as not to penetrate cells, rapidly and uniformly distributed in extracellular compartments and not bound to tissue components. Evidence to be described suggests that intracellular concentrations of Na and Cl are sufficiently high in the uterus that these ions are of questionable use for measuring the extracellular space. Accordingly, inulin and sucrose spaces are accepted here as the more reliable of the indicators usually employed (*cf.* 63) although there are indications that short-term exposure of tissue to sorbitol (66a) or to a relatively new marker, diaminoethanetetraacetate (EDTA) saturated with ^{60}Co (66a, 472a), may be the methods of choice. With whatever substance, results with the uterus may be accepted with

confidence only in immature, spayed, or anestrous preparations since other evidence (*v.i.*) suggests sucrose, inulin, and even plasma proteins enter cells in advanced states of estrogen-induced edema.

The extracellular compartment of whole uterus is larger than most tissues, including intestine. Under conditions of apparent steady-state exchange, insulin-^{14}C (411, 482) and sucrose-^{14}C (266, 411, 482) spaces of spayed-adult or immature rat uterus indicate an extracellular compartment of 40 to 46% of tissue volume (55 to 59% of total tissue water). Calculated as volume fraction of uterine weight or of total uterine water, similar values were obtained with sucrose in immature rabbit uteri after spans of 3 to 90 minutes of sucrose circulation time (378). Inulin spaces, obtained from outward diffusion or from direct extraction of uteri preloaded *in vitro*, were 41% for the immature cat (114) but only 27% (114) to 33% (269) for immature rabbits. Estimates of extracellular volume based on Na or Cl concentrations in tissue and serum assume the ions are extracellular and that uterine interstitial fluid resembles a plasma ultrafiltrate with ion concentrations relative to serum modified according to Donnan distribution ratios (*cf.* 565). Sodium spaces so derived for rat uteri range from 35 to 64% (98, 204, 267, 508); values reported for immature and spayed rabbits are 61% (114) and 45% (55), respectively, and 75% for the immature cat (114). Chloride spaces of 50 to 54% have been calculated for the rat (98, 508) and 65% in rabbits (378), and shown to be considerably higher than sodium spaces when determined together in the same studies. The reverse pattern appears in immature rabbit and cat uteri in which sodium spaces exceed the chloride space (114). Thiocyanate when used as a marker ion in the spayed rat gave an extracellular volume of only 29% (98). Studies which compare endometrium separate from myometrium show extracellular volumes (per cent of total water, calculated from rapidly diffusing Na), respectively, to be 71 and 38% in spayed rabbits (55) and 49 and 46% in spayed cows (221).

A single estrogen injection to immature or spayed adult rats causes two cycles of uterine weight change over 72 hours: a rapid rise within the first 4 to 6 hours, a decline by hour 12 and a second peak at 30 hours followed by gradual decline toward control levels (17, 82, 98, 508, 565). The first cycle of weight change through 12 to 15 hours is due almost entirely to shifts of water into and out of the organ as a whole (17, 98, 262, 486, 502, 508). These initial changes have generated considerable interest since they precede growth, *i.e.*, the stromal cell division and general hypertrophic increase in protoplasm with proportional increase in water that account for the second weight increase (*cf.* 17, 419, 481, 504, 508). Similar effects and time course have been observed in oviducts of immature hens after a single dose of estrogen (87). The cycles of weight change reflect cellular events that, once set into motion by the hormone, appear to proceed to completion as a programmed sequence since these are not significantly altered by additional injections of estrogen (82) or by delayed administration of an estrogen inhibitor (480). Progesterone increases weight but the magnitude and timing differs from that caused by estrogen (565).

The earliest increase in uterine weight in response to estrogen results from tissue uptake of water that is significantly higher by the second hour and near maximum by hour 3 (486). The water is taken up into expanded extracellular compartments as indicated by increased tissue distribution of sucrose (266, 482), inulin (482), and albumin (267, 395), and by calculations from absolute increases in "extracellular" ions (sodium, chloride, and thiocyanate) (98, 114, 508). Tissue *concentrations* of markers such as sucrose, expressed as amount per unit of fresh tissue weight, do not change in the first 3 hours after estrogen injection, indicating that the marker is taken up in proportion to the rise in tissue weight while increasing in absolute

amounts per uterus (482). Albumin-^{131}I is taken up more slowly through the increasingly permeable capillary bed (*cf.* section III) and apparently accumulates without free exchange between interstitial fluid and plasma (267, 395). Subsequently, 3 to 8 hours after injection, estrogen directly or indirectly increases permeability of uterine cells to water and to molecules as large as sucrose, inulin, and possibly plasma protein (395, 396). The extra water, initially confined to extracellular compartments, is taken up by cells to an estimated extent of 20% of the excess, as determined from ion concentrations (98, 508) and by direct observation of stromal cell enlargement (17). After a 3-hour period of equilibration in the edema fluid, sucrose and inulin concentrations sharply increase in disproportion to tissue weight, thus indicating some cellular penetration.

In normal male rats, extracellular volumes (Na space) of ventral prostate and seminal vesicles average 9% and 14% of the tissues, respectively (433). The volumes increase after castration to 30 to 40% [measured by Na (433) or inulin (355, 513) distribution], in part at the expense of cellular space. A single injection of testosterone does not alter extracellular volume or percentage of water content in 18 hours (355), although by hour 24 both parameters and total wet weight (433), cell height, and nuclear volume (*cf.* 401) are increased. Thereafter to 96 hours, extracellular volumes progressively decrease toward normal as cellular hydration rises (433) in concert with restoration of cell fine structure and mass (*cf.* 401).

B. ION DISTRIBUTION. Ion penetration also is affected by estrogen. The uterus in any physiological state has long been known to contain greater amounts of Na, Cl, and Ca, and less K, Mg, and P than skeletal or cardiac muscle (*cf.* 114, 324, 535, 546). The influence of the hormone on distribution of these ions in the uterus as a whole is seen most clearly in the acutely-treated ovariectomized animal. In the first 3 hours of stimulation, estradiol increased amounts of ^{24}Na taken up by the uterus from the circulation in the same time course as, and proportional to, water uptake (486), while not altering Na uptake in liver (262, 486), kidney, spleen, small intestine, skeletal muscle, or skin (486). A movement of ^{42}K into the uterine extracellular space similarly occurs but is masked by an initial amount of ^{42}K that rapidly enters and equilibrates with intracellular K pools (486). Uterine absolute blood volume meanwhile increases (120, 395), but whole organ ion uptake is too large to be accounted for by amounts trapped in the expanded vascular space (98, 267), and concentrations in the general circulation do not change under estrogen treatment (98, 114, 166, 508).

In the subsequent period of stimulation, 3 to 8 hours after estrogen injection (immature or spayed adult rats), absolute amounts of Na, K, Cl, P, Ca, and Mg per uterus, and concentrations of all but K, P, and Mg, are elevated, with particularly striking changes in Na and Cl (98, 508). In adult rats, the increases in Na relative to Cl (262, 263) and in Cl itself (98) were found to be in greater concentrations than would be expected in a plasma ultrafiltrate; increased cellular penetration of both ions is indicated. The immature rat uterus contains unusually high Cl relative to Na, thus suggesting some Cl is normally intracellular (508); 6 hours after estrogen injection, the Na:Cl ratio in now edematous uteri revert to a value expected for an ultrafiltrate, but some cellular uptake has not been ruled out (508). The increased tissue uptake of such cations as K and Mg apparently results in enrichment of the intracellular pool of these ions (98, 508). During the growth phase response to estrogen, beyond 18 hours after injection, the uterus doubles in weight through increase in solids as well as total water. Solids in addition to new structural protein and nucleoprotein (98, 419, 502) include at least twice-control amounts per uterus of all ions measured (508). The increases are proportional to the increment in solid tissue mass, and redistributions occur such that relative extracellular volume and ion *concentra-*

tions revert to near control values (508). Typical of growing tissue, however, relative cell volume is increased (98, 508) and K content (508) and concentration (98) are disproportionately higher.

Qualitatively comparable events were observed by Daniel and Daniel (114) in immature cats and rabbits treated chronically (estrogen injections over several days) in which the growth state of the uterus more approximates that of normal estrus. Amounts of both Na and K per unit of dry weight of uterus were considerably higher than in control subjects, except for Na in the cat. Concentrations of K per unit of wet weight were also greater than control subjects in every case, but Na concentrations were decreased. Progesterone given with estrogen for 3 days after 6 days of estrogen priming generally depressed ion levels and extracellular volume below levels seen with estrogen alone. Cellular volume relative to extracellular volume was expanded by estrogen and accompanied by a decrease in total intracellular cation concentration (114). As in the rat (*v.s.*), rabbit and cat uterine Na and Cl spaces exceed the inulin space, indicating that substantial portions of these ion contents are normally intracellular (109), a conclusion supported by studies of diffusion from uterine segments into ion-free media. Diffusion rate curves for Na showed two slopes, suggesting a portion less freely diffusible, hence intracellular or possibly bound. That virtually all tissue Cl diffused freely does not rule out the probability of intracellular Cl. Hormone treatment generally had little effect on the content of the slowly-diffusing (cellular) Na, although progesterone increased its diffusion significantly in the rabbit (114).

Bitman *et al.* (55) analyzed separated endometrium and myometrium in one of the few such studies extant. Results in estrous and pseudopregnant rabbits were the same as after chronic treatment of castrates with estrogen and estrogen-progesterone, respectively. Combined electrolyte concentrations (Na plus K) were higher in endometrium than myometrium in each physiological state. Estrogen (but not progesterone) increased the percentage of water content of endometrium. However, the distribution of water in the endometrium was not changed by estrogen but was markedly shifted to the intracellular space by progesterone. Concentrations of Na were equally high in control and estrogenized (edematous) endometria, and low after progesterone, reflecting proportionally larger extracellular spaces in the former. Concentrations of K showed the reverse pattern, being much higher after progesterone, presumably reflecting expansion of the cellular compartment. In the myometrium, the water content did not change significantly with treatment; changes in Na and K concentrations and in extracellular space were qualitatively similar to those in the endometrium, although ion shifts in the muscle were generally far less marked (55, 255). Unlike endometrium, myometrial K concentrations were higher than Na under all conditions. Total electrolyte concentration was lowered by hormone treatment in the endometrium (55) but raised in the myometrium, both in immature (269) and spayed adult (55) rabbits. In bovine endometrium by contrast, totals were slightly raised under hormonal influence (221). Differences in totals between endometrium and myometrium, compared in spayed, estrous, and luteal cows, were small. Extracellular space and Na-water uptakes were increased in bovine endometrium during estrus but the large increases in K concentration and intracellular water seen in adult rabbit endometrium under progesterone domination (55) did not occur in luteal cows (221). Bovine myometrium was less affected by estrogen than was rabbit muscle but more affected by progesterone in decreasing extracellular water and Na concentrations (221). Similarly, progesterone reduced Na in guinea pig myometrium without significantly affecting total K (242).

2. *Uterine Smooth Muscle.* Most analyses of ion concentrations and shifts in uterine muscle have been conducted as a

background for, or in conjunction with, studies on the electrochemical basis of myometrial contraction. Studies on ion distribution in differing hormonal states are few and characteristically feature chronic hormone treatment to simulate preovulatory, postovulatory, or pregnancy conditions; findings prior to 1966 have been tabulated by Kao (270) in a critical review of myometrial physiology.

A. MONOVALENT IONS. Information to date from analyses directed exclusively to myometrium *in vivo* conforms in basic features with findings in the earlier studies described above in which rabbit myometrium was compared with endometrium (55). In general, water content and extracellular space of rabbit muscle are slightly elevated after estrogen treatment (238, 254) but not further changed with progesterone (*cf.* 238, 270). Also, total K concentrations generally are higher than Na under all conditions (109, 110, 238, 255, 272), but highest relative to Na after exposure to hormones if treated myometrium can be compared with the untreated whole organ as control (269). Rat (84) and cat (109) myometria, however, are richer in Na than K under most conditions. Estrogen generally does not change or slightly depresses total Na concentrations (109, 151), while concurrent progesterone treatment tends to lower Na concentrations below estrogen-dominated levels (109, 242). Total Cl concentration was not affected by these hormones. Cation totals (Na plus K) are hyperosmotic to plasma under all conditions (109) and hormone treatment increases the sum of ion concentrations (55, 221, 238). Compared with skeletal muscle, myometrium contains higher total Na and Cl concentrations. In a study of total ions in fresh rat myometrium as a function of the estrous cycle, Smith and Bianchi (472a) found K and Mg concentrations to be lowest in proestrus (55 and 4.6 mEq/l, resp.), to rise at estrus (to 69 and 5.6 mEq/l) and to remain relatively constant thereafter through diestrus; Na and Ca concentrations did not change. The extracellular space of "fresh," estrogen-dominated rabbit muscle, determined in short-term incubations with ^{14}C-sorbitol or ^{60}CoEDTA, was 52 to 54% of the tissue volume (66a). This agrees with the 53% (^{60}CoEDTA) found in myometrium from proestrous rats; values for estrus, metestrus, and diestrus were 45%, 58%, and 53%, respectively (472a).

In terms of distribution, calculations indicate that intracellular K concentrations are vastly greater than Na as expected, but also cell Cl is unusually high (*cf.* 270). In rabbits at least, the calculated intracellular concentration of each ion, particularly of Na, tends to be higher in progesterone- than in estrogen-dominated cells (*cf.* 238, 270).

Expressed in relative terms, *i.e.*, the concentration of one ion compared with another, these data on fresh muscle have qualitative validity, but values reported for absolute concentrations are open to question as reliable bases for calculations of equilibrium potentials or for consideration of diffusion gradients and active transport. Concentrations reported for given animal species and hormonal status vary from laboratory to laboratory according to whether ion extractions were direct, or followed periods of soaking in balanced media for dissection or for equilibration prior to measuring membrane potentials. Kao and colleagues (*cf.* 270) claim instantaneous changes occur on excising the uterus, principally a loss of intracellular K which then diffuses to bathing media on immersion; net amounts of Na meanwhile are taken up from the media. These shifts are such that a K:Na total concentration ratio greater than unity in fresh, unbathed myometrial strips can be lowered rapidly toward 0.5 by a few minutes of incubation (272). Accordingly, differences in technique alone may account for the range of "fresh" tissue intracellular K concentrations from 139 (115), 158 (238), 169 (66a), 188 (110) to 194 (269) mEq/l of cell water, reported for estrogen-dominated myometrium. Disparities also arise from differing

methods of estimating extracellular space. Still another factor is the estrous cycle stage of donor animals at the time of sacrifice; myometrium taken from estrous, metestrous, or diestrous rats lost 32% of K and 21% of Mg during incubation but no changes were seen in proestrous muscle; Na and Ca concentrations did not change during incubation regardless of cycle stage of donors (472a).

Without consistent data on basal distribution of ions in myometrium *in vivo*, it has not been possible to conclude precisely what effect hormones have on these distributions. Another hindrance is that muscles from estrogen- and progesterone-treated animals have been compared frequently against one another but rarely against diestrous or spayed control animals. Still, some trends are apparent. Findings on myometrium "*in situ*" rather consistently show that changes in ion concentration under differing hormonal states, when changes can be observed at all, are not large (*e.g.*, 55, 115, 270). Indeed, Kao (269, 270, 272) and Jones (255) compared estrogen- with progesterone-dominated fresh rabbit myometrium and could not find significant differences in total tissue concentrations of Na, K, or Cl. Casteels and Kuriyama (84) could detect no change in totals or cellular distribution between anestrous, estrous, and pregnancy myometria of rats. Negative findings also were reported by Bulbring *et al.* (72), who compared myometrial ion concentrations in several hormonal states and pregnancy in cats and guinea pigs, except that intracellular Cl rose markedly in cats during pregnancy. Similarly, Smith and Bianchi (472a) saw no change in total Na concentrations through the estrous cycle; K also stayed relatively constant except for a low point during proestrus.

Nevertheless, real differences in physiological properties exist between estrogen- and progesterone-dominated myometria, as revealed by measurements of ion shifts *in vitro* under a variety of conditions and changes in ion-based electrical properties of single cells (*v.i.*). Operating hypotheses have emerged to reconcile these observations with instances of failure to find changes in ion concentrations *in vivo*. These state in effect that the hormones must bring about qualitative changes that (a) cause differing permeabilities to individual ions, and (b) permit differing capacities for creating and maintaining subtle but functionally significant differences in cellular-extracellular distributions. Thus, rabbit muscle on immersion in standard media rapidly gained Na and then slowly removed it as incubation progressed (272); meanwhile K, rapidly lost to the medium initially, was regained with time, although levels after 4 hours did not reach starting concentrations (110, 270). Under these conditions, muscle from estrogen-primed, progesterone-treated rabbits regained K (255, 272) and expelled excess Na (272) at faster rates than muscle exposed to estrogen alone. In a study by Jones (255), kinetics of K movements showed complex dependence on K concentration of the medium but progesterone generally caused greater K accumulation and permeation in both directions, with increased selectivity toward K over Na. Other workers (84, 186, 258, 333) reach essentially the same conclusion regarding progesterone action with less direct approaches. For example, Casteels and Kuriyama (84) compared observed membrane potentials with calculated K equilibrium potentials as a function of increasing external K concentration in pregnant (*i.e.*, progesterone-dominated), and in non-pregnant (anestrous, estrous) rat myometria; with a 10-fold increase in external K, membrane potentials changed to a greater extent in pregnant than in non-pregnant muscle (84).

Earlier work by Daniel and colleagues (*cf.* 117) suggested the extrusion of Na *in vitro*, and possibly the reaccumalation of K, were active processes. Rabbit and cat uterine segments subjected to metabolic inhibition by cold in K-free medium lost K and gained an equivalent amount of Na and smaller amounts of Cl (115). Returned

to 37°C and aerated medium containing K, these segments regained half the K lost, extruded equivalent Na, and exhibited normal contractility and action potentials; separate analysis of myometrium alone indicated these ion shifts had occurred throughout the uterus, although largely in the muscle. *In vivo* pretreatment with progesterone caused less leakage in the cold, both K loss and Na gain, than with estrogen and, accordingly, less of a shift during recovery and warming. Apparent active outward transport of Na during warm recovery was prevented by the absence of K in the external medium, while normal reaccumulation of K occurred independently of external Na (115). Extrusion of Na and uptake of K during recovery also was diminished or prevented by inhibitors of glycolysis, by dinitrophenol, and by ouabain (which interferes with Na-K-ATPase carrier systems), but not by anoxia or most inhibitors of cellular oxidation at the level of terminal electron transfers (116).

These data for cat and rabbit myometria suggest Na expulsion is an active process dependent on energy from aerobic glycolysis and linked to inward K transport. The findings were not considered conclusive in either regard, however, since (a) little is known about actual effects of metabolic inhibitors on smooth muscle metabolism, (b) observed actions of cold in reversing normal gradients, and of inhibitors in preventing their recovery (particularly of dinitrophenol), could as well have resulted from depolarization of the muscle and attendant leakage of Na and K down their electrochemical gradients rather than from inhibition of an energy supply, and (c) evidence suggests that Na extrusion is uncoupled from K uptake by dinitrophenol (116). The basic questions of active transport for Na and K, their mechanisms and their possible interdependence were further explored in rat myometrium (110, 111, 118, 118a–c, 404a, 509a,b). As in cat and rabbit muscle, rat myometrium in a cold, K-free medium takes up NaCl and loses K; on rewarming in a normal medium the process is reversed, the tissue meanwhile also losing weight through loss of excess water as Na is extruded (110, 404a). The cold-induced net loss of K showed more than one rate constant and was accompanied by contraction (110). Effects of cold could be mimicked at 37°C by application of metabolic inhibitors and were not prevented by an absence of external K (111). There remains some uncertainty whether K loss due to cold or inhibitors arises through direct inhibition of metabolism or through depolarization by inhibition of the electrogenic Na pump of the muscle (110, 111, 118b,c, 509a). At 37°C and in normal medium, K exchange is virtually in steady state, influx approximating efflux, but a K-free medium causes a 50% decrease in K efflux. These observations suggest at least a portion of the K movement is exchange diffusion (110, 111). Ouabain only minimally affected K movements normally or on warm recovery from cold (111, 118a) and K fluxes did not always relate to muscle ATP content (118b). Na fluxes also exhibit complex kinetics; uterine segments preloaded with Na by cold exposure showed, on warming, three rates of efflux and an inexchangeable fraction (118). Ouabain depressed extrusion of an intracellular Na fraction but the effect was regarded as incomplete. Further, a K-free solution, also known to inhibit transport ATPases, did not clearly affect Na extrusion and the inhibiting effects of ouabain were not relieved by a high K media; both means of inhibiting ATPase increased Na influx (118a). Experimental depletion of the ATP supply in the tissue was more effective in inhibiting Na extrusion, increasing Na influx, and accelerating K efflux than was application of ATPase inhibitors (118b). Also, ATP depletion caused water uptake (mimicking the effect of cold) but ATPase inhibitors did not, while in both instances Na influx was increased (118a,b). Other observations on tissue volume changes due to water uptake or loss are pertinent. Changes in myome-

trial volume are dependent on metabolism but not on Na or K pumping since the volume changes occurred in the absence of external Na or K. Water loss during warming recovery is dependent on Ca and the presence of a permeable anion and is prevented by isopropylnorepinephrine (404a).

The combined data on fluxes of Na, K, and water and their inhibition indicate that compartmentation, movements, and driving mechanisms in myometrium are exceedingly complex. For rat myometrium, it seems that, under appropriate conditions, fluxes of Na, K, and water can at least partially be dissociated from one another. Extrusion of excess intracellular Na is active but there is no convincing indication of active K influx linked to Na efflux and mediated by active transport (118c). It is noted, however, that the ouabain-sensitive electrogenic pump of late-pregnancy uterus depends on extracellular K (509a,b). These and other data from Daniel's group suggest the presence of two Na pumps, both ATP-dependent. One, ouabain-sensitive, controls movements of Na and K but not water. The other, insensitive to ouabain and to K levels, controls Na and water (118a,b). Also, it seems that the transport ATPase system in the rat uterus is unusual (111, 118a,b). Other studies of rat uterine ATPases, discussed below in section A, 3, generally support this last impression. A mechanochemical model has been proposed to explain the ouabain-insensitive component controlling cell volume and Na extrusion. These effects are viewed as resulting from changes in ion binding in, and/or contraction of, micropinocytotic vesicles of the myometrial membrane that are open to the extracellular compartment (118c, 404a).

Kao and coworkers (*cf.* 270, 272) have summarized evidence from their own and Daniel's laboratories that points strongly, though not conclusively, to the existence of linked, active transport systems for Na and K in rabbit myometrium. On cooling, ion concentrations appear to redistribute passively as they approach concentrations of the bathing medium, whereas restoration toward normal tissue distribution on warming features net uphill fluxes that can only reasonably be explained by active pumping both for Na and K. The latter requires the presence specifically of carbohydrate for energy and is sensitive to at least one group of metabolic inhibitors. Also, the net accumulation of K is in excess of that required for simple Donnan distribution. That the Na pump is linked to K uptake is indicated by fluxes that are roughly equal and reciprocal, by the inability of myometrium to produce net Na extrusion in a medium lacking K, and by the marked reduction of ^{22}Na efflux from Na-preloaded muscle into K-free medium. However, dissociation of K uptake from Na extrusion can occur under certain experimental conditions (116, 270).

Beyond the work cited, which indicates progesterone-treated muscle is more "efficient" than estrogen-treated in restoring normal ion balance *in vitro*, little more can be said at present regarding gonadal hormone control of K and Na distribution and movement in myometrium. Possible modulating roles for insulin and adrenal mineralocorticosteroids have not been explored seriously. Further understanding of endocrine control will likely advance only so far as additional basic information is gathered on myometrial cells as metabolic units and as components of excitable tissue. It would be of interest to learn, for example, the physiological meaning of the extraordinarily high Na:K ratio found in rat myometrial cell membranes (81); concentrations in mmoles/Kg of dry weight membranes for Na, K, Ca, and Mg were, respectively, 75, 2, 4, and 3. In the latter study, as in several others cited, work was done entirely on myometrium from intact animals chronically treated with estrogen, a treatment applied for no other apparent reason than to produce enlarged cells.

B. DIVALENT CATIONS. The distribution and movements of divalent cations have been studied in particular relation to myometrial contractile mechanisms. Spontan-

eous contractions of the normally polarized muscle *in vitro* are accompanied by depolarization, propagation of action potentials, and Na entry, all of which favor contraction and speed the maximum contractions induced by natural effectors such as oxytocin and acetylcholine. Contractions (spontaneous and induced) are abolished, and action potentials disappear, if Ca is first depleted by prolonged immersion of the muscle in Ca-free solution (99, 117, 141, 268, 334). Spontaneous activity, after initial reactive depolarization and contraction, is similarly abolished by immersion in solutions (Na-free) that maintain a depolarized state; oxytocin and acetylcholine nevertheless can trigger contraction under these conditions if some Ca is present (*cf.* 113, 117). Normal contractions did not change significantly after substituting Sr or Ba for external Ca (113, 268, 334), findings of theoretical interest that do not detract from the basic function apparent for Ca *in vivo*. Increasing Mg up to 10-fold in the bathing medium did not alter the resting potential level but depressed the action potential frequency and contraction amplitude, whereas elevations of Ca increased the resting potential without affecting the action potential frequency or contraction; eliminating external Mg did not affect myometrial behavior. It was concluded that any normal action of Mg was unlikely to correspond to that of Ca (334), and in high concentration may interfere with Ca function (113). These and other data demonstrate a fundamental requirement for Ca, more basic for contraction in this system than depolarization and propagation of action potentials (*cf.* 63, 117).

Models developed to explain functions of Ca in myometrium, both in spontaneous contractions and in those triggered by drugs in the absence of action potentials, presuppose direct interaction of Ca with intracellular contractile elements (as in other muscle) and postulate the compartmentalization of the ion in at least two functional sites (*cf.* 113). Reducing external Ca concentration 10-fold below normal depolarizes and eventually depresses spontaneous activity of single myometrial cells; reintroduction of the normal Ca medium restores activity and does so at a faster rate than that at which the activity had decayed (334). Similarly, reducing Ca by acute application of moderate amounts of EDTA causes depolarization and a submaximal contraction (but not if all Ca had previously been removed) (113). These results are representative of those interpreted to indicate that Ca resident in "superficial" binding sites, in or on the surface membrane and immediately accessible to EDTA, serves to regularize membrane function in spontaneous (depolarization-induced) activity. That is, Ca is believed to stabilize membranes of excitable cells by controlling the permeability of the resting membrane. Application of large amounts of EDTA for prolonged periods chelates sufficient tissue Ca to suppress all responsiveness. However, for a time after immersion in the presence of high concentrations of EDTA (or Ca-free solution), but before complete suppression, the depolarized muscle continues to respond to drugs although responsiveness diminishes. After depletion, restoration of full responsiveness to drugs requires several minutes of reexposure to a normal Ca solution, whereas normal contractility due to Ca alone is restored in seconds (112, 117). Similarly, contraction induced by depolarization decays more rapidly in a Ca-free medium than that induced by acetylcholine, and finally a stage is reached at which depolarization is ineffective but the muscle still responds to acetylcholine (141). The same behavior has been noted, incidentally, for seminal vesicle contraction (142). These results, together with those on drug action described in greater detail above and on other smooth muscles, suggest a secondary repository of Ca, "sequestering sites" which are less readily accessible to EDTA. Mobilization of Ca from these sites might explain contractions to drugs in muscle that is depolarized and devoid of action potentials (*cf.* 113 for full arguments and description of models).

Sequestering sites presumably exist in

one or a combination of the inner surface of the vesiculated plasma membrane (*cf.* 128), the smooth endoplasmic ("sarcoplasmic") reticulum (*cf.* 75, 409, 430, 478), or other myoplasmic organelles (75, 430). Surface membranes of myometrial cells become much ruffled and pinocytotic-like vesicles (caveolae) increase in number during estrus and after estrogen treatment (430). But consideration of sequestering sites in estrogen-stimulated uterine muscle is complicated by the presence of rough endoplasmic reticulum and Golgi systems that enlarge markedly, become closely apposed to the surface, ahd have been observed actually to fill outpocketings of the surface membrane (430).

Uteri from estrogen-treated rats were found to contain 2.25 mmoles of Ca/Kg of fresh weight, of which 20% was not exchangeable; distribution of the exchangeable Ca (80%) was estimated to be 35% extracellular and 45% intracellular (528). The cellular concentration was below that of ionic Ca in serum and well below the theoretical electrochemical equilibrium value expected for intracellular Ca. Metabolic inhibitors caused net ^{45}Ca accumulation, a result apparently of increased Ca influx without change in exit rate. It was concluded that Ca transport is active and involves a mechanism that tends to prevent Ca entry (294a, 528). Ouabain did not affect Ca transport (528) in the rat, but one cannot conclude from this observation that Ca transport is necessarily independent of Na-K-activated ATPase activity; transport ATPase systems in rat myometrium have been shown to be atypical (*cf.* section on monovalent cations, above, and on ATPase activity, below).

Solutions which contain high concentrations of K, or oxytocin, which depolarize myometrium and induce or enhance contractions, have been used in attempts to determine whether the contractile response might be initiated by a net cellular gain of Ca (*e.g.*, 294a, b, 446, 527, 528). Flux analyses and estimation of Ca spaces under a variety of experimental conditions have yet to provide a clear answer to the question. One obstacle to obtaining unambiguous results has been contraction *per se* which slows Ca movements in and out of the muscle through constriction or other alteration in the extracellular compartment (294a, 528). These effects too are not subject to simple interpretation since Ca efflux also was slowed during contracture in muscle not permitted to shorten (294a). Uptake of ^{45}Ca was not slowed in tissue allowed to equilibrate with ^{45}Ca in a normal medium and to remain exposed to tracer during and after contraction. In this and similar preparations no significant net increase in Ca could be seen to be associated with contraction (249a), although some exchange of cellular Ca for labeled extracellular Ca apparently occurs (294a, b, 527, 528); the exchange seems to be one-for-one (294a). Experiments of this kind do not rule out the possibility of net Ca entry into cells, since the net gain presumably required to initiate contraction could be smaller than present methods can detect. However, in the absence of such a demonstration, a model featuring activation by unbinding or other mobilization of existing cellular Ca (*v.s.* and 113, 478a) would seem to be favored.

Lanthanum ion, which displaces Ca from superficial sites and does not enter cells (*cf.* 178a), inhibits contraction due to high K or to acetylcholine and has been used to study the different mechanisms (*v.s.*) underlying responses to the two agents in rat myometrium. Inhibition of K-induced contractions required 100-fold less La than was required to inhibit contractions to acetylcholine, and recovery of responsiveness to acetylcholine after removing La was much more rapid than recovery of the response to K (178a). Other data suggested that removal of superficial Ca in the uterus is not linked to release of less superficial Ca, or not linked in the same way as in ileal smooth muscle. This may explain why an acetylcholine-induced contraction is not readily inhibited by attachment of La to superficial Ca sites in the uterus but is readily inhibited in the ileum (178a). Differential effects of the

kind described support a model, namely that contractions due to a drug such as acetylcholine are stimulated by Ca release from sequestered sites, whereas contraction by depolarization and propagated action potentials results from decreased membrane affinity for Ca leading to Ca release from superficial sites. It seems that contractions from either kind of stimulus have in common the basic requirement of a direct action of Ca ions on the contractile apparatus. These ions appear to be supplied by release from cellular sources and not immediately by net influx from the outside.

Spontaneous relaxation presumably arises from rebinding or sequestration of free Ca ions in both surface and interior sites (*cf.* 113). Studies on subcellular fractions of myometrium further support these concepts. Calcium was observed to be bound by microsomes (31, 32) and mitochondria (32) of rat (31, 32) and bovine (83) muscle; vesicles were not present in the rat microsomal preparation. Binding in all fractions was ATP-dependent; ATPase activity was present in each fraction of bovine tissue but was highest in the microsomes (83). Low temperatures inhibited Ca binding generally, suggesting a metabolic requirement, but a possible link between ATP hydrolysis and active Ca uptake by the membrane systems has not been established. Binding was inhibited by K or Na in mitochondria but not in microsomes, and other qualitative differences between the two fractions were noted (32). Cyclic AMP occurs in the microsomal fraction but the addition of cyclic AMP or caffeine had no effect on Ca transport (*cf.* 31). However, Ca transport may be involved in myometrial relaxation induced by *beta*-adrenergic agents. Epinephrine and isoproterenol hyperpolarize membranes, relax the uterus (129a,b), and increase tissue cyclic AMP (cAMP) levels, effects that are mimicked by dibutyryl cAMP and enhanced by phosphodiesterase inhibitors (*cf.* 295a; section c, next). These responses are apparently accompanied by passive increases in K permeability and/or decreases in Na permeability; a dependence on metabolism is indicated but Na pumping is not involved (*cf.* 335). The effects are abolished by La, are dependent on the external Ca concentration and are accompanied by a net Ca efflux (295a,b). In general, relaxants that increase cAMP (isoproterenol, papaverine, dibutyryl cAMP) decrease tissue Ca. Non-adrenergic relaxants ("D-600," a derivative of verapamil) do not affect cAMP levels and appear to inhibit Ca influx. Thus, actions of both classes of relaxants may be reconciled through a common effect of decreasing cell Ca, although different mechanisms are involved. Outward extrusion of Ca, as stimulated by *beta*-adrenergic drugs, may provide some of the current for hyperpolarization (295a, b). A composite model for the mechanism of relaxation might include the cause-effect sequence, increase in cAMP levels, stimulation of energy-producing reactions, active extrusion of Ca, relaxation (295a). The relative contributions to relaxation of net decreases in cell free Ca through efflux *vs.* rebinding or sequestration are not known.

All studies cited on divalent cations employed uteri exclusively from estrogen-treated animals or from intact untreated adults without regard for stage of estrous cycle. Little meaningful information therefore can be extracted on the degree to which ion transport, binding, *etc.* may be dependent upon, or modified by, steroid hormones. Some information is available on endocrine control of overall content. Myometrium contains more Ca, less Mg and a lower Mg:Ca ratio than skeletal muscle (535). Estrogen given to spayed (535) or immature (360) rats increased the Mg content and the Mg:Ca ratio over controls. Supplementing treatment with progesterone did not further change the content (360, 535). No difference in the Ca content could be detected during the estrous cycle of rats (84, 472a) but the content decreased significantly during pregnancy (84). Mg was 18% lower during

proestrus than in the rest of the cycle (472a).

C. RELATION TO ELECTRICAL AND MECHANICAL ACTIVITY. Myometrium subjected *in vivo* to hormone influence or deprivation exhibits differences in electrophysiological properties and in spontaneous and induced contractility. The following summary of major findings is intended principally to supplement reviews of the subject (2, 270, 296) and to integrate, to the limited extent currently possible, muscle behavior with ion and hormone data of the preceding sections.

Isolated whole uterine horns or myometrium from immatures or spayed adults are minimally active. Contraction cycles, when these can be observed at all, are short, relatively weak and uncoordinated along the length of the organ (66, 104, 258, 333); action potentials that may accompany contraction are few with a very low amplitude (258, 268). As first shown by Bozler (66), electrical excitability to stimulation is relatively low or absent. Single muscle cells of castrate rats (333) and immature rabbits (186) show resting membrane potentials of 30 to 35 mv, which correlates well with the minimal excitability of these preparations.

Original studies of the estrous cycle by Katzenstein and Soskin (279) showed diestrous rat uteri to be less electrically excitable to stimulation than estrous organs. With surface recording and stimulation, Melton and Saldiver (349) have reported details for the full rat cycle. Proestrous and estrous uteri exhibited large spontaneous contractions accompanied by bursts of action potentials, interspersed by periods of electrical and mechanical silence. Stimulated action potentials were conducted the full length of uterine horns. Metestrous uteri showed lower conduction velocities and groups of spikes separated by continuous discharge of low activity. Diestrus was characterized by a continuous, spontaneous electrical discharge with no grouping of spikes; contractions were small and uncoordinated and relaxation was incomplete. Similar changes in motility occur during the guinea pig cycle (160). Gonadotropin combinations given to spayed rats produced electrical responses of the proestrus-estrus type (349).

Responses to *estrogen* given by injection resemble those in proestrus-estrus just described and contrast markedly with activity in spayed controls. Estrogen has been observed to increase the resting membrane potential of myometrial cells by 10 to 20 mv (186, 258, 333, 515). However, Casteels and Kuriyama (84) and Bulbring *et al.* (72) could not detect significant change in resting potential between anestrous and estrous stages in rats or guinea pigs. "Anestrous" is not defined in these publications and it is not clear whether the animals were spayed or in diestrus; if the latter, precise staging (criteria not mentioned) becomes important since animals in late diestrus are already under estrogen influence (see section III) and the authors may have been comparing two estrogen-dominated stages. It was noted (72) that 2 days of treatment of immature guinea pigs with estrogen raised the average resting potential from 38 to 42 mv (range shift from 25–45 to 35–45 mv). Under the influence of estrogen, excitability to induced stimulation is high (66, 442). Spontaneous contractions are rhythmic, of greater amplitude (*cf.* 104) and duration (451), and are accompanied by synchronous bursts of action potentials (66, 268, 333, 442, 515) that appear to initiate contractions and to govern contraction frequency, magnitude, and duration (333). The electrical activity is myogenic since it continues in the presence of nerve block (333). Increased electrical activity can be observed within 24 hours after a single injection of estrogen in the rabbit (268) and within 2 hours in the rat (349); with longer treatment, grouped spikes with a higher amplitude, appear (268). Estrogen also appears to increase Fallopian tubal motility (79, 125) and conduction of electrical activity (71); contractions were characterized as high in frequency but of low amplitude, whereas under pro-

gesterone frequency decreased while amplitude rose (125).

In estrogen-dominated uteri, oxytocin, acetylcholine and stretching slightly depolarize the muscle, increase the frequency of action potential discharge (333), and generally cause or enhance contractility (219, 247, 282, 333, 358, 516). Norepinephrine, in the estrogenized uterus (129, 493), and prostaglandins of series E and Fα (219) also stimulate contraction although less is known of accompanying electrical effects. Epinephrine by contrast suppresses action potentials, hyperpolarizes membranes (247, 333), and generally relaxes the uterus (219, 247, 333, 398, 516); the latter effect is also characteristic of norepinephrine (282, 310, 516) in all but estrogen-dominated stages (129, 517). The suppressive effects described for epinephrine are blocked by inhibitors of *beta-* (but not of *alpha-*) adrenergic membrane receptors (*cf.* 335). Some exceptions to these general patterns due to species difference or special conditions are summarized by Miller (354).

Cyclic AMP (cAMP) added *in vitro* mimicks epinephrine in inhibiting contractions stimulated by acetylcholine, oxytocin (219, 358, 516), or prostaglandin Fα (219). These and the following observations are the basis of an hypothesis that endogenous cAMP mediates the relaxing effect of *beta*-adrenergic amines in the uterus. Epinephrine (76, 219, 358, 398, 516, 517) or isoproterenol (44, 295a, 335, 516, 517) increased adenyl cyclase activity and cAMP concentration. Also, theophylline, which inhibits enzymatic breakdown of cAMP, potentiated the relaxing effect of catecholamines (310). Theophylline and epinephrine in amounts too low to effect relaxation individually, given together inhibited oxytocin-induced contraction (358). Conversely, acetylcholine (in the intact uterus but not in homogenates) depressed the capacity of epinephrine to elevate cAMP levels, an action prevented by the cholinergic blocker, atropine (518); oxytocin inhibited accumulation of cAMP due to isoproterenol as expected (44) but not that due to epinephrine (219).

Other experiments illustrate complex interactions between effector agents, receptor sites and adenyl cyclase, which are not readily understood but which suggest the level of cAMP is not the sole determinant of drug action on motility (219, 398). For example, prostaglandins of series E and F contract the uterus (*cf.* 488); PGE, however, was found to increase cAMP levels whereas PGF was inactive in this respect (44, 219). Both prostaglandins effectively inhibited the elevation of cAMP levels due to isoproterenol (44) but PGF did not inhibit epinephrine in this regard (219). Despite these discordancies, a role for cAMP in mediating actions of certain natural effector agents on the uterus *in vitro* seems strongly supported. Far from clear, however, is the influence, if any, of estrogen and progesterone in modifying these responses. Estrogen fosters uterine accumulation (not synthesis) of epinephrine (*cf.* section III). The steroid also has been reported to elevate, acutely, cAMP levels (425, 501) in the uterus, a response found to be prevented by *beta-* (but not by *alpha-*) adrenergic antagonists (*cf.* 425, 500). However, the effect of estrogen on uterine cAMP levels has not been established (compare 425, 445, 501). In any case, an increase in cAMP by estrogen has not been correlated with changing epinephrine levels, nor reconciled with *stimulation* by estrogen of uterine motility and electrical activity.

Adenyl cyclases of the chick oviduct and rat prostate are not stimulated by estrogen and testosterone, respectively, but activity in the chick oviduct is increased by progesterone concurrent with stimulation of avidin synthesis (425). Further study of the role of estrogen in modifying the uterine response to catecholamines and other effector substances is at least of theoretical interest, in the broad context of understanding mechanisms of hormone action. But the underlying question, the normal functions of these effector agents in the

uterus, has yet to be resolved: Is uterine contractility, basically an independent myogenic phenomenon, normally subject to secondary control *in vivo* by catecholamines, acetylocholine, oxytocin, and prostaglandins?

Effects of injected *progesterone* on the myometrium generally resemble observed activity in the luteal half of the estrous cycle (349) and in pregnancy, during most of which progesterone titers are high or rising. In general, the effects of progesterone on electrical-mechanical events require estrogen priming, conforming with the estrogen priming required for progesterone action in uterine metabolism, binding to receptor protein, and other effects. Jung (*cf.* 258) found progesterone alone to be without influence on electrical properties of the spayed rat uterus, but Porter (399) obtained changes in spayed rabbits with progesterone injected systemically or locally; surprisingly little data otherwise exists on the effects of progesterone alone. In estrogen-primed subjects, when superimposed progesterone treatment has been observed to exert change over estrogen alone, there is general agreement on the following: Spontaneous contractions are weaker (104, 333, 399), irregular, and less coordinated with trains of action potentials (104, 333, 515); stimulated contractions are of shorter duration, and develop less tension with changes in length, than in estrogen-dominated muscle (451). Spikes are of mixed amplitude and generally lower (*cf.* 258, 333). Spike trains are not conducted long distances and some evidence of local conduction blockade is apparent (104, 258, 297, 333). Myometrium under estrogen-progesterone domination, furthermore, is resistant to contraction-stimulating actions of oxytocin, stretch (*cf.* 333), and norepinephrine (129).

Some negative results with progesterone have been reported. Porter could detect depression of contraction with progesterone in spayed rabbits (399) but not in guinea pigs (400). Melton and Saldiver (349) observed suppression of electrical properties and lesser coordination in the progestational half of the cycle (*v.s.*), but these workers (442) could see no change over estrogen alone, in spontaneous electrical activity or in excitability to shocks, after progesterone administration by a variety of routes to estrogen-primed animals; progesterone treatment, however, was of relatively short duration (6 to 24 hours). Schofield (451) had noted that a minimum of 18 to 24 hours of exposure *in vivo* to progesterone was required before the negative staircase effect, characteristic of progesterone domination, could be evoked by an increasing frequency of stimulation.

Some disagreement also exists in the matter of the resting potential. Kao (*cf.* 446) obtained considerable scatter in results from experiments with impaled single muscle cells, and no significant difference between rabbits treated with estrogen alone and with estrogen and progesterone (see also, 433), but other workers report increases in membrane potential after progesterone administration to estrogen-primed rabbits (186), rats (333), and guinea pigs (72). Studies which include a complete experimental series (*i.e.*, controls, estrogen-, and estrogen-progesterone-treated) (72, 186, 333), large sample sizes, and statistical analysis (*e.g.*, 333), uniformly show resting potentials to be increased after progesterone treatment and the data must be viewed as convincing. Added support for the apparent hyperpolarizing action of progesterone comes from findings that membrane potentials during pregnancy, which represents a longer term of exposure to progesterone, equal or exceed those in progesterone-treated experimental animals (72, 84, 186, 268, 333, 336). The resting potential increases progressively during pregnancy (84, 186, 258, 511), rising until the 15th day in rats, after which it decreases until day 21 and returns to normal nonpregnant levels within 1 to 3 days postpartum (84). In pregnant rabbit and rat myometria, spontaneous action potential discharge was found to be infrequent and irregular; spikes were of rela-

tively low amplitude and only occasionally accompanied by contraction (105, 268, 333), but in the rat the amplitude and number of spikes per burst increased with time during pregnancy as the resting potential increased (84, 511). Activity in pregnant rats and rabbits is generally consistent with the diminished coordination between electrical and mechanical events and the conduction block seen with progesterone treatment (84, 104, 297, 333).

During the first 25 (of 32) days of gestation in rabbits, uterine muscle does not respond to oxytocin (297). The largest response to oxytocin in rats was observed in non-pregnant animals, but pregnancy muscle responded progressively to day 15 (of 21) with increased frequency of spike discharge and duration of spike trains (511). Pregnant cat uteri exhibited a higher threshold for excitation than non-pregnant uteri, requiring electrical stimuli of greater duration to elicit a response (72). In the rat, however, Casteels and Kuriyama (84) found the threshold to be lower during pregnancy than in nonpregnant states.

Toward term, electrical-mechanical properties of the myometrium change dramatically. Within several hours of delivery in the rabbit, spontaneous action potentials, previously rare, increase in frequency and amplitude (105, 268) and the uterus becomes resensitized to oxytocin (297). In rats, the resting potential is lowered (84, 515) and the frequency of spontaneous discharge declines to a minimum on day 21, by which time, also, oxytocin no longer affects frequency (511). Recordings in larger pieces of the rat myometrium on day 1 showed "gross discoordination" in onset, frequency, and duration of action potential trains; contraction was tetanus-like made up of many local contractions only very locally coordinated with electrical discharge (299). Postpartum uteri shortly assume properties characteristic of estrogen-domination in each species.

The *basis* for these *differences* in electrical-mechanical properties of myometrium under differing hormonal states remains unknown, both from the standpoint of specific molecular events underlying excitability and contraction and (necessarily therefore) of the means by which they are modified by hormones. A continuing major difficulty is the lack of precise data on intracellular ion concentrations; traditional methods have proved inadequate for their determination in uterine muscle. The best estimates to date of ion distribution in fresh myometrium show little or no significant changes in intracellular ion concentrations between estrogen-, progesterone-, and pregnancy-dominated muscle, yet hormonal states appear to govern differences in resting potential, excitability, conduction, and contraction. Studies summarized further above on ion fluxes *in vitro* and on changes in muscle properties as a function of manipulated external ion concentrations indicate a greater selective permeability to K relative to Na in muscle from progesterone-dominated animals. The latter also seems better able to effect metabolically-dependent K accumulation and Na extrusion. These observations are theoretically consistent with the higher resting potential observed in the presence of progesterone and in pregnancy. Also consistent with theory, is the correlation (in rats) between the progressive increase in membrane potential during pregnancy and increases in spike frequency and amplitude (*cf.* 270). Present information, however, does not provide explanations for progesterone-induced conduction blocks and discoordination between electrical and mechanical activity, nor for apparent species differences during pregnancy in, for example, excitability and sensitivity to oxytocin. Even less well understood are the bases for changes in properties during pregnancy as term approaches, although some suggestion of increased Na permeability exists to explain the somewhat lower resting potential (84). Changes near term, moreover, have not clearly been associated with local tissue or circulating hormone titers, for which highly sensitive methods of measurement are available.

Studies on divalent cations point to basic roles for myometrial Ca in excitation-contraction coupling and in maintenance of normal membrane excitability (*v.s.*). Some progress has been made, additionally, in understanding possible functions for both Ca and Na as carriers of the depolarizing membrane current. From their study of rat myometrium with current-clamp and voltage-clamp techniques, Anderson *et al.* (10) conclude that the genesis of action potentials and the transient inward current are dependent on the presence of both ions; the observed dependence on Ca (10) in part involves control by Ca of membrane leakage permeability (see also, 271). Differential influences of hormones on these mechanisms have not been assessed in detail; this work, as well as most discussed earlier, was done solely on estrogenized preparations. That hormones do modify calcium function and attendant membrane properties is suggested by findings of the following kind: estrogen-dominated muscle in Ca-free medium quickly depolarizes and loses all excitability within 20 minutes; progesterone-dominated myometrium requires 90 minutes to depolarize to the same extent, thus suggesting Ca in superficial (membrane) sites is more firmly bound under progesterone influence (*cf.* 104, 186, 333, 520).

An understanding of hormonal regulation of myometrial excitation-contraction depends ultimately on the development of a suitable *in vitro* system in which hormones added directly to the medium cause changes in muscle behavior resembling hormonal effects *in vivo*. Studies of the kind have been attempted (*cf.* 104, 270, 296) but virtually all have neglected to include one or the other (or both) of two essential controls: (a) effects alone of the carrier solution used to dissolve the hormones; and (b) tests for specificity using non-gonadal and non-hormonal steroids. For example, progesterone *in vitro* hyperpolarized rabbit myometrium, reduced the frequency and amplitude of spontaneous action potentials, and depressed contractility, among responses similar although not identical with those *in vivo*. A carrier control was not applied but it was noted that estrogens, as well as several progesterone metabolites which are inactive *in vivo*, produced the same effects *in vitro* as progesterone, whereas other steroids known to be progestational *in vivo* were ineffective *in vitro* (104). The results suggest that observed effects of progesterone *in vitro* were non-specific, possibly a consequence of a general, steroid-type surface action, rather than a function of intrinsic hormonal properties of progesterone. Non-specificity is similarly indicated by the observations of Stander and Barden (493); the contractility of human myometrium was damped by an *in vitro* addition of estradiol, progesterone, or testosterone. Also, Saldiver and Melton (442) found estradiol as effective as progesterone *in vitro*, in suppressing activity of uterine muscle of estrogen-primed rats (see also 515); the results were consistent with *in vivo* observations in the same study but not consistent with work by these authors on the estrous cycle (*v.s.*, and 349). The alcoholic carrier solution itself was tested and found ineffective *in vitro* (442). However, other steroids were not tested and the behavior observed with estrogen and progesterone could as well have resulted from non-specific action than from any actual similarity of action of the two steroids as hormones.

3. ATPase activity. Hydrolysis of ATP, catalyzed specifically by adenosine triphosphatases, has been associated with active transport of ions and organic substrates across plasma membranes, and with membrane-linked packaging and extrusion of macromolecules in active secretion. Phosphatases and kinases of less specificity but which include ATP as substrate may also participate. ATPase-mediated transport of Na, K, and organic substrate has been described that requires Na and K as well as Mg for activation (Na-K-ATPase); at least one form of the enzyme is inhibited by Ca and by the

cardiac glycoside, ouabain. Other varieties include Mg-activated, ouabain-insensitive ATPase and a Ca-activated enzyme (*cf.* 467). Cellular transport of electrolytes (*v.s.*), sugars, and amino acids (*v.i.*) in virtually all accessory reproductive organs of both sexes appear to be subject to hormonal regulation and some attention has been centered, therefore, on ATPases as possible sites of action for estrogen, progesterone, and testosterone. In addition, ATPase activity of a Ca-dependent variety is intrinsic to myosin, a component of the contractile protein actomyosin of muscles including smooth muscle of reproductive tracts; myosin concentrations, in the uterus at least, vary with hormonal state.

A. FEMALE. Histochemical studies of ATPase distribution conventionally employ sections cut from frozen tissue and incubated in buffered media that include ATP and lead nitrate; enzymatically liberated terminal phosphate groups form lead phosphate which is deposited *in situ* as lead sulfide on treatment with ammonium sulfide; specificity is determined, and controlled for, by use of near-neutral pH, ion, and nucleotide substitutions, and inhibitors of non-specific phosphatases. In their study of estrous-rat uterus, Novikoff *et al.* (380) observed intense ATPase activity throughout arteriolar walls, in venule endothelium, and in fibroblasts. Myofibrils in muscle layers showed staining that appeared due to mixed phosphatases (but not alkaline phosphatase), since staining persisted to varying degrees in the absence of divalent cations and on substituting ADP or AMP for ATP as substrate. ATPase activity was strong in apical borders of the luminal epithelium but not the epithelium lining mucosal glands, whereas alkaline phosphatase activity was much stronger in glandular than in luminal epithelium. Uterine capillary endothelia showed mixed ATPase and alkaline phosphatase activities. Similar although more intense ATPase distributions were noted across the walls of mouse oviducts. In the uterus and in oviducts, staining was nowhere affected by ouabain (380).

ATPase distribution in the rat uterus after a single estradiol injection generally conforms with the pattern described for estrus (Tam and Spaziani, unpublished). Spayed control animals showed substantial ATPase activity in muscle layers and larger blood vessels (see also 230, 277), although it should be noted that uterine tissues are severely "concentrated" in the absence of ovarian hormones. Activity was absent in luminal epithelium of control animals and in glandular epithelium before and after estrogen. From 4 to 6 hours after estrogen injection, but not earlier, activity appeared on apical surfaces of the luminal epithelium and in mucosal fibroblasts closest to the epithelium. Similar staining was observed in the stimulated rabbit mucosa (230). Activity in rats was greatest in the mesometrial quadrant which is also the entry point of the uterine vascular supply. Activity in epithelial membranes was severely depressed in the absence of divalent cations but nucleoli under these conditions showed activity for the first time. Incubations without ATP totally suppressed staining. Ouabain did not affect activity in controls or in experimental preparations (Tam and Spaziani, unpublished). Twenty-four hours after a single injection of estrogen, overall staining decreased below controls, especially in muscle, but persisted in vessels and luminal epithelium (277). For periods of up to 3 days after estrogen treatment, ATPase activity did not appear in epithelial cytoplasm; the Golgi apparatus visualized with the Novikoff-Goldfischer method showed no activity with the ATPase procedure (Tam and Spaziani, unpublished; L. Loof, personal communication). Activity in the Golgi bodies was observed, however, in estrogenized mouse uterus epithelium (495).

Apical membranes of the uterine luminal epithelium in mice (377), rats (319), and guinea pigs (73) are normally thrown into cytoplasmic protrusions and microvilli. These projections into the lumen were

shown to emerge from a virtually unprofiled surface in spayed rats, to increase progressively in length with time after a single dose of estrogen (377), and to increase further in size under the influence of progesterone (319). ATPase activity, obvious only in basal portions of these membranes, was intense after estrogen treatment, moderate in progestational uteri, and absent in spayed controls (*cf.* 319).

Determined biochemically, rat uterine ATPase activity in whole homogenates, comprising the net or organellar, membranous and myosin-actomyosin enzymes was initially increased over controls three hours after estrogen injection and was further increased by 6 hours (276, 509). Since uterine weight increases markedly due to water uptake in the first several hours (*v.s.*), elevations in ATPase activity were evident when activity was expressed on a per organ basis (micromoles P_i liberated from ATP/uterus/hr) and not when expressed as a function of organ weight (509). After sedimenting actomyosin, ATPase activity appeared to fall below controls from the 7th hour onward (276).

Subcellular fractionation of ATPase activity has been attempted in homogenized uteri from animals chronically treated with estradiol and from those at an unspecified estrous cycle stage. The supernatant fraction of 9000 $\times$ *g* centrifugation contained 50%, and the mitochrondrial fraction 10%, of the total activity (174). Activity was enhanced by Ca, whereas Mg was less effective and in larger amounts was inhibitory. Drug effects suggest the presence of separate Ca- and Mg-activated enzymes; isoproterenol did not affect activity in the supernate but increased Mg- more so than Ca-dependent activity in whole homogenates, and markedly stimulated Ca- over Mg-activated ATPase activity in mitochondria; oxytocin did not affect activity in any fraction (174). A microsomal fraction of bovine endometrium contained ATPase activity that required Mg and could be stimulated by Na plus K. Activity of this preparation was reported to be stimulated maximally by 10^{-11} M estradiol added *in vitro* and inhibited by concentrations of the steroid greater than 10^{-8} M and less than 10^{-12} M (418). Results before and after freezing-thawing suggest the presence of two ATPases, one of which seems to require intact membranes for estrogen stimulation (418). A plasma membrane fraction of rat myometrium treated with estrogen was found to contain membrane fragments reformed as vesicles associated with small amounts of RNA (probably ribosomal, *v.s.*) (284). Several enzymes were identified in the preparation, including ATPase which was not further characterized; 59% of total 5′-nucleotidase activity was contained in the plasma membranes.

Actomyosin concentrations of rabbit myometrium were decreased 80% by ovariectomy and could be restored to normal levels within 7 days by estrogen treatment (103, 351). Myosins extracted from pregnant and non-pregnant rabbits have been characterized physicochemically (*cf.* 372) and their ATPase activities studied in detail (533). Monovalent cations did not activate the myosin or actomyosin ATPases (533). In contrast with that in skeletal muscle, uterine actomyosin ATPase was only weakly activated by Mg; principal activity was Ca-dependent (372, 533).

In summary, ATPase activity in normal endometrium is evident histochemically in surface membranes of epithelium lining the uterine lumen, in blood vessel walls including capillary endothelium, and in some fibroblasts; the activity in myometrium occurs both in membranes and myofibrillar cytoplasm. The activity in epithelial membranes seems absolutely dependent upon estrogen. Information on the subcellular distribution and characterization of ATPase is incomplete but activity occurs in all fractions including mitochondria, and high activity is apparent in microsomal and plasma membranes of both muscle and endometrium. Separate Ca-activated and Mg-activated enzymes are present, but the former predominates.

One or more Na plus K-activated ATPases apparently occur in the uterus, and activity, in rats at least, is insensitive to ouabain. A rise in total ATPase activity in rat uterine homogenates can first be detected 3 hours after estrogen treatment *in vivo*, a timing that coincides with maximum Na and K uptake by the organ. Increased ion uptake begins 1 to 2 hours after injection of estrogen and apparently precedes the increase in ATPase activity. It is not clear whether the early change in the enzyme, measured *in vitro* after *in vivo* treatment with estrogen, represents activation of existing protein stimulated independently by the hormone, activation stimulated secondarily by the increment in total ions, or activity of newly synthesized enzyme.

B. MALE. ATPase activity in mouse epididymis was found localized in muscle, connective tissue and capillary endothelium. The activity was low in castrates, restored by 30 days of treatment with testosterone and was Ca-Mg-dependent (9). Total ATPase activity in homogenized rat seminal vesicle or ventral prostate was substantially lowered by castration (Wieman and Spaziani, unpublished). Activity in seminal vesicles increased significantly 24 hours (but not 12 hours) after a single injection of testosterone with a second rise at 72 hours; prostatic activity did not change earlier than 72 hours after one injection but was increased at 48 hours after two injections. In both organs, substitution of ADP for ATP as substrate reduced the activity by 50% and no activity was detected with AMP. The activity was low in the absence of divalent cations in the incubation media and was stimulated more by adding Ca than Mg. Withdrawal of Na and K or addition of ouabain produced no measurable change (Wieman and Spaziani, unpublished).

That a Na-K-activated enzyme exists, nevertheless, and is probably masked in whole homogenates, is indicated by work with subcellular fractions (6, 156). An endoplasmic reticular membrane subfraction, prepared by Ahmed and Williams-Ashman (6) from microsomal fractions of normal rat prostates, contained Mg-dependent, Na plus K-activated ATPase activity, representing 5 to 10% of ATPase activity in whole homogenate. The specific activity was 50% higher than in crude microsomal preparations. Both Na and K were required for maximal activity. Ouabain inhibited the activity by 50% but affected only that portion of enzyme action due to K; this same portion was inhibited by adding Ca or by substitution of Ca for Mg. Castration reduced the Na-K-activated ATPase activity by 90 to 95% and 5 days of testosterone treatment prevented the loss. Farnsworth (156, 157) reported that uptake of K by prostatic minces, and Na-K-ATPase activity in whole microsomal fractions, could be stimulated by a brief pre-incubation with testosterone; testosterone analogous including 19-nortestosterone were ineffective or inhibitory (156). Testosterone was further observed to accelerate K-dependent dephosphorylation of the enzyme but not to affect its Na-dependent phosphorylation (157). However, Ahmed and Williams-Ashman (6) could not detect changes in Na-K-ATPase activity on *in vitro* addition of testosterone or dehydrotestosterone to microsomal fractions or to refined subfractions.

B. Sugars

1. Female. Cellular division and hypertrophy in the uterus of spayed mammals can be induced in 18 to 24 hours by a single injection of estrogen (*cf.* 17, 147, 168, 419, 481, 502). The growth effect is preceded by a general activation of uterine metabolism, as indicated by accelerated rates of oxygen consumption and synthesis of glycogen (*cf.* 419, 531 and 56, 57, 61, 68, 180, 194, 304, 371, 547), lipid (34, 489), protein, and all classes of RNA (*cf.* 184, 207, 369, 419, 502). *Net* syntheses are accompanied or preceded by marked physiological changes in the uterus, particularly edema formation with uptake of plasma solids, attributable in turn to stimulation of blood flow and of

increased capillary permeability by estrogen (see sections III and IV A,2,a). The organic solids taken up in increased amounts include substrates and potential substrates utilized as structural units in synthesis and as energy-yielding intermediates. These observations are the bases of a working assumption that estrogen stimulates anabolic metabolism, at least that of bulk syntheses linked to growth, by increasing the availability of substrates to the tissue and by controlling cellular transport. Evidence in support of this view includes findings that uterine (369) and vaginal (259, 337) segments from castrates, maintained without estrogen in the enriched environment of tissue culture media, grow (259, 337), incorporate glycine into nucleic acids, and show increased activity of amino acid-activating enzymes (369) at the higher rates normally produced by estrogen treatment *in vivo* (see section III for additional discussion).

In regard to sugars, early experiments demonstrated that uterine horns incubated aerobically after 4 hours of estrogen treatment *in vivo* took up glucose from the medium in excess of metabolic requirements. It was suggested that the hormone increased cellular permeability to glucose, possibly as an action independent of its effect on utilization (502). A second peak in glucose uptake 20 hours after injection of estrogen was not accompanied by a marked increase in oxygen consumption, which together with some lactate production suggested utilization that could account entirely for the glucose taken up. An adrenocortical hormone, cortisol, given at the same time as estrogen blocked the estrogen response both at 4 and 20 hours (502). Cortisol inhibits the increase in capillary permeability produced by estrogen and may also antagonize the action of estradiol on cell permeability generally in the uterus (see Section III). The inhibitory effect of cortisol in the above study reinforces the interpretation that estrogen normally increases glucose availability. Also, within 2 hours of injection (54, 180), estradiol increases glycogen synthesis (53, 85, 180) and the *in vivo* free glucose content of the uterus in excess of the chloride space (54, 57, 85). Cortisol caused near-total blockade of the water uptake induced by estrogen and the increment in free glucose, and partially suppressed the usual increase in glycogen content per organ (53, 85).

Uterine glycogen levels were not raised by moderate increases in blood sugar produced by an injection of glucose (498), but the marked hyperglycemia evoked with alloxan did raise the concentration of uterine glycogen (54, 306, 498) and uterine free glucose (54), increases which could further be elevated by estradiol (54, 498). Insulin alone or in combination with treatment with glucose did not alter uterine glycogen levels of castrates *in vivo*, whereas levels in liver, skeletal muscle, and heart were greatly increased; glucose or glucose plus insulin did not augment glycogen synthesis induced by estrogen in the uterus (498). Insulin added to incubations of control uterine segments did not affect the uptake of a model sugar (426) but did increase glucose uptake (21, 237); insulin inhibited glucose uptake in estrogen pretreated tissue (237). It seems that insulin does not mediate the effect of estrogen on uterine glycogenesis but some permissive action has not been ruled out.

The influence of estradiol on the distribution and cellular transport of sugars has been studied in rat uterus, with radiolabeled model compounds not utilized by the reproductive tract. Halkerston and colleagues (204) measured *in vivo* distribution of D-xylose-^{14}C in the uterus in which uterus plasma concentration ratios of labeled sucrose and inulin were used as estimates of the extracellular compartment. With a constant circulation time for tracers of 90 minutes, xylose distribution did not differ from control subjects 1.5 hours after injection of estradiol but the sugar occupied a significantly higher tissue volume at 6 hours. However, xylose distributions in both control and experimental subjects exceeded the extracellular

spaces, indicating that significant amounts penetrated uterine cells under all of the conditions studied, although in time estradiol further increased net penetration. It was concluded that a physiologically significant barrier to sugar permeability does not exist in the uterus, and the sugar supply therefore is not rate-limiting for metabolism (204). Spaziani and Gutman (482) compared uterine distributions of labeled arabinose, mannose, and 3-O-methylglucose against inulin and sucrose spaces as a function of time after estradiol administration. With a circulation interval of 15 minutes for all test substances, estradiol increased penetration of the pentose and hexoses into uterine cells within 1 to 2 hours of injection of hormone, while not affecting the distribution in skeletal muscle or intestine. Cellular distribution further increased at 4 hours, at which time the sucrose space also was elevated, indicating that uterine cells had also become permeable to substances normally excluded by cells. With longer (60 minutes) circulation time for the tracer substances, the monosaccharides penetrated control cells to such an extent that estradiol did not further increase uptake at 2 hours (482) but did so at 4 hours (482, 483), generally confirming observations of Halkerston *et al.* (204). These results demonstrated the existence of an estrogen-sensitive barrier to substrate-like monosaccharides that can be altered by estrogen such that there is a faster attainment of steady-state concentrations with shorter periods of hormone treatment, and increased steady-state levels with a longer (4-hour) exposure. Similar results were reported for urea-^{14}C which rapidly penetrates tissues; within 1 hour of estradiol treatment, the entry rates of urea were accelerated, thus shortening the time to steady-state distribution between the uterus and plasma (266). It has been argued (see 266, 454, 482) that stimulation of metabolism in the target tissue, if substrate availability is rate-limiting and subject to hormonal regulation, does not necessarily require that the substrate be substantially excluded from cells in the absence of the hormone and then be permitted entry to high steady-state levels (accumulation) by the hormone for metabolism to be changed. Estradiol eventually does cause accumulation of sugar but an earlier acceleration of uptake toward steady-state levels could trigger early metabolic changes in operative portions of the cell interior by subtle effect on rate of influx, efflux, or both.

Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, did not affect estrogen-stimulated 3-O-methylglucose uptake by uterine cells *in vivo* (483). Similarly, the characteristic water uptake or weight increase either was not affected (315, 483) or was only partially suppressed (376, 476, 523) by actinomycin, and in at least one instance (476) the drug given alone increased uterine weight. Prior administration of the drug did, however, substantially reduce uptake of 3-O-methylglucose *in vitro* (428). Pretreatment with puromycin, an inhibitor of protein synthesis, suppressed 3-O-methylglucose uptake *in vivo* (483) and *in vitro* (428) but *in vivo* results were regarded as inconclusive; the drug was extremely toxic, causing cyanosis, general hemoconcentration, contraction of the uterine extracellular compartment and reduction of uterine blood flow rate to below controls. It was not possible to conclude, therefore, whether effects on transport were due to blockade of early protein synthesis or to impaired local blood flow and lowered systemic blood pressure (483).

Interpretation of results with puromycin and cycloheximide, on sugar transport and glycogen synthesis, is further complicated by non-specific actions of these drugs on carbohydrate metabolism. Puromycin and cycloheximide deplete liver glycogen (*cf.* 479) and, in the uterus, cycloheximide is known to increase total free glucose, glycogen synthesis, and water uptake (*cf.* 58, 482, 524). In addition, both puromycin and cycloheximide in the uterus exert the un-

usual effect of *reversing* certain actions of estrogen after those actions have been well established (180; see also, 469). For example, pretreatment with estradiol and either drug prevents the incorporation of label from glucose-^{14}C into CO_2, glycogen, and lipid that otherwise occurs linearly 1 to 4 hours after giving the hormone alone. If injection of drug is delayed until 2 hours after the hormone, the increased incorporation up to that point is substantially or completely reversed to control levels by hour 4. Inhibitor action in the first instance is interpreted to mean that the effect of the hormone on glucose uptake and/or metabolism depends on the prior induction by the hormone of special protein species (180, 278). The reversal effect suggests that the hypothetical protein(s) turns over at a rate unusually rapid for mammalian cells but the observation opens the door to uncertainty about the specificity of action of these drugs.

The basic findings of the influence of estrogen on sugar penetration into uterine cells (482) were confirmed by Roskoski and Steiner (426) in *in vitro* studies and extended to measurements of transport kinetics. Cellular uptake of labeled 3-O-methylglucose from the medium was increased 3-fold in uterine segments from rats pretreated for 2 hours with estradiol and further increased after 4 hours of treatment, reverting to control levels by hour 12. The V_{max} for 3-O-methylglucose uptake in the 4-hour estrogen-treated group was 2-fold higher than controls, while the K_m remained unchanged. These results are consistent with a model of diffusible membrane carriers with finite numbers of saturable sites rather than simple diffusion. Experiments with segments preloaded with methylglucose showed that estradiol increased both influx and efflux rates. High concentrations of external glucose inhibited methylglucose uptake but induced its outward transport from preloaded segments. Similar counter-transport of methylglucose, indicating competition for carrier sites, was produced by D-mannose, 2-deoxy-D-glucose, and D-xylose; a degree of substrate specificity was indicated by the failure of D-galactose, L-arabinose, and D-xylose to affect counter-transport of methylglucose. These results also are consistent with a carrier-mediated transport model. Estradiol or insulin added *in vitro* to control tissue did not affect transport; NaCN, dinitrophenol and sulfhydryl inhibitors also were ineffective (426).

Neither 3-O-methylglucose nor 2-deoxyglucose is metabolized by tissues but 2-deoxyglucose undergoes initial phosphorylation normal to hexose utilization. Smith *et al.* (469, 470) have reported conversion of 2-deoxyglucose-^{14}C to 2-deoxyglucose-^{14}C-6-P by immature rat uteri *in vitro*. Phosphorylation during 30 to 60 minutes of incubation was higher than controls after only 30 minutes of estrogen treatment *in vivo*, 2-fold greater after 1 hour and increased linearly thereafter. Estradiol or diethylstilbestrol added directly to incubations had no effect. Cycloheximide administered 30 minutes before estradiol abolished the 1-hour estrogen response (469). However, the 1-hour response also was completely prevented (and reversed) when cycloheximide was given 30 minutes after estrogen, at which time some estrogen-stimulated phosphorylation had already taken place (see also 180). Glucose and non-labeled 2-deoxyglucose, but not 3-O-methylglucose, inhibited formation of labeled 2-deoxyglucose-6-P, indicating competition for common transport-phosphorylating systems only by sugars subject to phosphorylation (469, 470). Kinetic analysis, based on average velocities and doubtless reflecting activity of more than one phosphorylating enzyme, showed a higher V_{max} for the 1-hour estrogenized tissue over controls with no change in K_m (469).

Collectively, these studies demonstrate early stimulation by estrogen of one or more steps governing sugar transport in the uterus, probably centered in phosphorylation linked to transport. The findings sug-

gest that hexose entry, controlled by estradiol, may be rate-limiting in uterine carbohydrate metabolism. Further, they are consistent with the known influence of estrogen on that metabolism. For example, Nicolette and Gorski (373) found incorporation of label from glucose-^{14}C into uterine CO_2, lipid, RNA, and protein *in vitro* to be increased 1 to 2 hours (but not 30 minutes) after estradiol injection *in vivo*; similar incorporations into lipid and CO_2 were observed by Barker *et al.* (21, 22). Iodoacetate prevented the increases and actually lowered incorporation to below control levels. These results indicate that estrogen augments labeling by glucose metabolites of precursor pools common to synthesis of lipid, RNA, and protein, and suggest acceleration of a rate-limiting step in the transport or early metabolism of glucose (373). Cortisol given intraperitoneally with estradiol blocked the usual water uptake by the uterus *in vivo* but not the increased glucose-^{14}C incorporation into RNA and protein usually obtained on subsequent incubation (373); however, cortisol did partially suppress estrogen-stimulated incorporation of leucine or glycine into uterine RNA, phospholipid, and protein (374). These results with cortisol on glucose incorporation appear to disagree with previous findings that cortisol (administered intravenously) prevented increases in uterine free glucose content (54), lactate production, and oxygen consumption (419), but the two sets of experiments cannot be directly compared because of differences in procedure. Since cortisol inhibits physiological responses to estrogen (increased water uptake, blood flow, and capillary permeability (*v.s.*)), establishing whether cortisol also blocks stimulated cellular transport and metabolism of glucose will aid in deciding whether the two groups of responses are functionally linked.

In vivo pretreatment with actinomycin D blocked the estrogen-stimulated oxidation of glucose-^{14}C and incorporation into RNA and protein. Cycloheximide similarly prevented increased glucose labeling of RNA and protein but only partially blocked labeling of lipid (373). Since stimulated glucose incorporation took place before the hormone caused an increase in overall protein synthesis (206, 379), inhibition of glucose incorporation by the drugs is interpreted to mean that the effect of estrogen on glucose transport or early metabolism is a secondary response dependent on prior induction of one or more special proteins (373). The specificity of action of the inhibitors has been questioned (see discussion further above) but the matter of timing is also pertinent. An estrogen effect on hexose transport is manifest well within an hour (469); dependence of the effect on protein induction would require hormone transport, interaction with transcriptional or translational sites, and assembly and positioning of a protein within 30 minutes. Induction of a new protein and its appearance in detectable or effective amounts in that span of time is probable (*cf.* 25, 278, 343) but has not clearly been shown to precede changes in transport or blood flow that are also effected by estradiol.

Activities of several enzymes in glucose utilization pathways have been measured in the uterus after estrogen treatment (see also 397 and sections II and IV A,3). These include hexokinase and other kinases (20, 121, 134, 468, 525), glucose 6-phosphate dehydrogenase (22, 140, 308, 453, 468, 563), NAD- and NADP-linked dehydrogenases (21, 43, 94, 308, 551), mutases, isomerases (121, 463), non-specific phosphatases (65, 476), and glycogen synthetase (126, 431, 547). All are stimulated by estrogen in the uterus and, of the few measured, in the vagina (461). Consistent with the experimental studies, activities during normal cycles in the uterus (121, 126, 393, 424, 431, 448, 562) and vagina (424, 461) are highest in or near estrus. In no instance has activity been shown to increase in less than 4 to 8 hours of estrogen treatment and it must be concluded that activation of these enzymes is not a primary effect of estrogen. Progesterone alone in the short term has no effect on enzyme activa-

tion and usually interferes with estrogen when given concomitantly (121, 126, 368, 463). With longer treatment, progesterone usually enhances enzyme activity in estrogen-primed subjects, but results vary considerably with the enzyme (43, 563). Given concurrently with hormones, inhibitors of protein and RNA synthesis usually lower or prevent the increased activity (65, 121, 232, 463, 476, 494, 525, 551) but given alone are occasionally noted to *stimulate* activity (476). The increases appear when estrogen stimulates bulk protein synthesis; increments in amount and/or activity of the enzymes, therefore, cannot be separated from the general effect of estrogen on protein and do not explain the much earlier stimulation of glucose metabolism. The several enzymes are not all activated or produced at the same rate (21) but the significance of this observation is not apparent.

2. *Male.* Sugar transport in male accessory sex organs has been studied in rats (355, 513) and guinea pigs (342). Mills and Spaziani (355) measured changes in distribution of 2-deoxyglucose-^{14}C in castrates after a single intravenous dose of testosterone. Distribution of total label (2-deoxyglucose plus its 6-phosphate metabolite) exceeded inulin spaces in the prostate and seminal vesicle of both control and experimental animals, and 18 hours of testosterone treatment increased the values only in the prostate. However, seminal vesicles and prostates incubated 30 to 90 minutes with the labeled sugar showed increased intracellular concentrations over controls after 12 hours of hormone treatment *in vivo;* cellular uptake by coagulating glands *in vitro* was increased after only 3 hours of hormone pretreatment. In all tissues, glucose or phlorizin completely suppressed the uptake of 2-deoxyglucose caused by testosterone, indicating that hormone-stimulated transport involves saturable carriers and an accelerated phosphorylation step (355).

Studies by Thomas *et al.* (513) suggest that phosphorylation is rate-limiting for hexose transport in rat ventral prostate. Increasing the external concentration of non-labeled 2-deoxyglucose in incubation media greatly depressed intracellular phosphorylation of 2-deoxyglucose-^{14}C while only slightly affecting intracellular concentrations of the labeled free sugar. Uptake of 3-O-methylglucose (which cannot be phosphorylated) as a function of the external concentration was similar to that of free 2-deoxyglucose. Castration for 7 days doubled the prostatic inulin space over normal controls but did not affect the uptake of 3-O-methylglucose or 2-deoxyglucose, nor phosphorylation of the latter. Prostates incubated after 12 hours of testosterone pretreatment showed significant elevations of intracellular free and phosphorylated, 2-deoxyglucose, the latter predominating. Similar results were obtained in guinea pig seminal vesicles and anterior prostates (513).

Hexose transport in the prostate and seminal vesicle thus seems to be first elevated 10 to 12 hours after administration of testosterone, well before the hormone stimulates cell division and associated DNA synthesis (36 to 48 hours; 74, 97, 136, 171, 417, 542). Increased transport in seminal vesicles also precedes measurable increases in activation or production (397) of hexokinase and glucose-6-phosphate dehydrogenase (16–24 hours; 447, 462), but roughly coincides with elevated respiration (432) and synthesis of fructose (432, 512) and glycogen (464). However, these effects occur several hours after the hormone is known to stimulate synthesis of at least two classes of RNA in seminal vesicles (193, 543, 544) and the activity of RNA polymerase in the prostate (314, 323), responses that are detectable within 90 minutes. Increased hexose transport seems not to be the earliest effect of testosterone but it has not been determined whether transport results from an independent primary action of the hormone or is dependent on prior RNA production.

The levator ani muscle, part of the male

sexual apparatus and attached to the perineum, is testosterone-sensitive to a degree more characteristic of the accessory reproductive glands than of other skeletal muscles. Sugar and amino acid transport and metabolism in the levator ani has been studied (*cf.* 13, 38, 350, 355, 519) but the muscle is not part of the reproductive tract and is ruled outside the limits of this review.

C. Amino Acids

1. Female. Amino acid uptake and distribution in accessory sex organs were originally studied in the immature rabbit uterus by Noall and coworkers (*cf.* 378) with the non-metabolizable amino acid, α-aminoisobutyric acid (AIB). Uterine uptake of AIB *in vivo* required at least 10 hours to reach a steady-state distribution between intra- and extracellular water in nephrectomized controls, but in estrogen-treated animal less than 4 hours were required, at which time the levels were 9-fold higher than controls. In incubations with AIB-^{14}C, uterine horns removed from rabbits after only 30 mintues of estrogen treatment *in vivo* took up 150% more label than control horns. Estradiol added *in vitro* to control horns had no effect (378). Halkerston *et al.* (204) and Coulson and Gorski (101) could not find significant changes in AIB (204), cycloleucine or putrescine (101) uptake earlier than 2 hours after estrogen injection, although averages were higher than controls from 1 hour onward. However, with careful control of factors contributing to variability, Riggs and colleagues (*cf.* 411, 412) and Roskoski and Steiner (427, 428) have confirmed the original observation that estradiol stimulates cellular uptake of AIB within 1 hour. The estrogen also causes early accumulation of L-alanine, L-proline and aminocyclopentanecarboxylic acid (411).

Uptake of AIB by the uterus conforms with all criteria for active transport. Penetration occurred against a gradient of the free amino acid and was inhibited 50% or more by anaerobiosis, low temperature, dinitrophenol and cyanide; iodoacetate or fluoride inhibited less effectively (411). Uptake in both control and estrogen-treated uteri (the latter more so) was sensitive to sulfhydryl agents (*e.g.*, *N*-ethylmaleimide), being inhibited by 5×10^{-4} M but stimulated by 5×10^{-5} M (161). Omitting glucose did not interfere with AIB uptake during incubation (427) but a possible influence of endogenous cellular glucose was not assessed. Accumulation of ^{14}C-AIB *in vitro* was inhibited by natural amino acids, but to differing degrees: methionine, unlabeled AIB, and proline (high inhibition); alanine, serine, and cysteine (moderate); glycine, valine, phenylalanine, arginine, and glutamate (1–45% inhibition) (411, 427). Histidine and tyramine were without effect (427), and lysine *stimulated* AIB uptake (411). Kinetic analysis gave approximations of constants to indicate that estradiol doubled the V_{max} without altering K_m (411).

These data demonstrate the presence of active transport mediated by saturable carriers. However, the kinetic data are interpreted with caution by Riggs and coworkers (411) who observed that entry rates of AIB and other amino acids do not follow simple Michaelis-Menton kinetics. Complicating factors include an apparent requirement for Na ion. The uptakes were inhibited by the presence of K or the absence of Na (161, 411) and were sensitive to ouabain (161, 410), suggesting mediation by Na-K-dependent ATPase activity. Another factor, potentially important in understanding uterine transport mechanisms, is the preincubation effect; if uteri were first incubated in the usual Krebs-Ringer bicarbonate medium for 3 to 5 hours, and the test amino acid then added, uptakes of AIB, proline, alanine, or aminocyclopentanecarboxylic acid (but not valine, phenylalanine, or leucine) were enhanced, mimicking the effect of 1 hour of estrogen pretreatment and a short incubation time (411, 413). Moreover, the increase in uptake showed the same kinetics, sensitivity to inhibitors, and Na dependence as hormone stimulated tissue (161, 413). With the prolonged incubation technique, Riggs

et al. (410, 411) may also have uncovered the means that permits estradiol to affect transport when added *in vitro*. Uteri incubated for 6 hours in the presence of 10^{-6} M estradiol took up more AIB than was usually effected by a 6-hour incubation alone, and the same as obtained in uteri pretreated *in vivo* for 1 hour and incubated for 6 hours. Controls for steroid specificity, however, were apparently not applied. The influence on amino acid transport of progesterone alone or in combination with estrogen has not been studied in the usual animals but Curet and Caton (108) have reported both hormones are required to stimulate amino acid uptake by uteri of spayed ewes.

Pretreatment of rats with actinomycin D, in doses that inhibit uterine RNA synthesis by 90%, substantially reduced but did not prevent the AIB uptake *in vitro* due to prior estrogen administration (428); cycloheximide (428) or puromycin (413) completely blocked the response. In the experiments of Riggs and colleagues (411, 413), puromycin was added *in vitro* and was observed to block not only AIB uptake due to 3 hours of estrogen pretreatment but also that uptake in untreated control uteri stimulated by prolonged incubation. If puromycin was omitted from preincubation and added in the final hour concurrent with addition of AIB-^{14}C, uptake of the amino acid was not inhibited (413). These data suggest that carrier proteins for amino acids are synthesized in response to estrogen or to prolonged incubation. Taken together, the experiments described on uterine amino acid transport clearly show an estrogen effect on transport well before the hormone stimulates general protein synthesis, but cause-effect relationships that may exist (a) between transport and induced synthesis of carrier or other special protein, and (b) between transport and bulk protein synthesis, have not been clarified.

Uterine amino acid transport is apparently carrier-mediated but evidence suggesting that transport is dependent on *de novo* synthesis of carrier is based entirely on the use of inhibitors. Some uncertainty exists as to the specificity of action of the inhibiting drugs and is discussed above in the section on sugars. Carrier activation by estrogen, an equally probable mechanism, has only minimally been explored although activation more plausibly explains the stimulated transport that occurs within the first hour after estrogen administration. Carrier activation in this early period, as opposed to induction, is supported also by evidence that uterine protein synthesis is transiently depressed by estrogen 30 minutes after injection; estradiol lowered incorporation of amino acids into nuclear, mitochondrial, and microsomal protein (347). However, evidence also exists that estradiol induces at least one specific protein in the immature rat uterus; electrophoresis of uterine proteins shows the presence of a new product 30 to 40 minutes after injection of hormone, which increases to a maximum in 1 to 2 hours and declines by hour 4 (25, 343). The protein further appears to be inducible by estradiol added *in vitro* (278).

Events in later periods after estrogen injection are similarly complex. Uterine total free amino acid content was found to be lower than controls at 4 hours and to increase linearly thereafter (265) in conjunction with increased bulk synthesis of protein (379); amino acid content of the vagina follows a similar time course (300). Studies in our laboratory (Peterson and Spaziani, unpublished) confirm that uterine total free amino acid levels are lower than controls at 4 hours, but showed the levels at that time were actually increasing from an earlier decline; the amino acid pool size was depressed as early as 1 hour after estrogen, continued downward to a loss minimum of 50% at hour 3, and was restored to control levels by the 5th hour. Thus, in the earliest period after estrogen injection (within 1 hour) increases in amino acid transport can first be detected during, or immediately after, a short period when incorporation into general protein is depressed. A period follows (1–3 hours) when transport and incorporation increase

while total pool size decreases. Finally, a period ensues in which all three parameters are rising; incorporation now reflects an increase in net amount of total protein being synthesized per organ. Whether these events are interlocked causally or represent separate and independent primary hormone actions is not known. Also, possible contributions to amino acid pools by plasma proteins has not been fully assessed. Quantities of plasma protein enter uterine tissue during the first 4 hours when the uterine capillary bed is rendered more permeable by estrogen (267, 395, 396) and evidence has been presented that 20% or more of the total may be taken up by cells (395, 396). Being labile species, plasma proteins may be broken down in whole or part and incorporated into structural or other protein.

2. *Male.* The influence of testosterone on amino acid transport in male accessory reproductive organs has been studied utilizing AIB (355). Under apparent steady-state conditions *in vivo*, a single intravenous dose of testosterone raised cellular concentrations of AIB-^{14}C significantly by the 6th hour in seminal vesicles and ventral prostates of castrate rats. Uptakes increased continually such that by hour 18 the amounts were 65% and 120% above controls in prostates and seminal vesicles, respectively, and seminal vesicles were concentrating AIB against a plasma: tissue gradient (355). Results were similar with pretreated tissue incubated in the presence of AIB-^{14}C, except that changes were not significant before 12 hours of hormone treatment. The testosterone-stimulated increase in AIB-^{14}C uptake *in vitro* was completely prevented by addition of 20 μM alanine, indicating competition with natural amino acids for carrier sites in common. The increase in transport also was reduced or abolished by anoxia, dinitrophenol, and low temperature; Q_{10} values for uptake at these low temperatures were 1.8 and 1.9 for ventral prostates and seminal vesicles, respectively. Further, transport was reduced by ouabain and lowered below control levels by removing external Na (355). In sum, testosterone stimulates penetration of amino acids into cells of the prostate and seminal vesicle by a process that appears to be carrier-mediated, energy-requiring, and Na-dependent. In seminal vesicles, these changes seem to precede increased incorporation of tyrosine and valine into vesicular protein (552), and to coincide with incorporation of leucine (77). Possible cause-effect relationships between amino acid transport and protein synthesis or induction have not been ascertained.

D. Nucleic Acid Precursors

1. *Female.* Contents per uterus of individual free (acid-soluble) nucleotides have been determined as a function of hormonal state. In general for the rat uterus, adenosine triphosphate (ATP) predominates but principal nucleotides also include adenosine diphosphate (ADP), uridine di- and triphosphates (UDP, UTP), uridine diphosphoglucose (UDPG), uridine diphospho-N-acetylglucosamine (UDPAG) and nicotinamide adenine dinucleotide (NAD) (181, 369, 381, 385, 532). Lesser amounts of adenosine monophosphate (AMP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), nicotinamide adenine dinucleotide phosphate (NADP), and the reduced forms of the coenzymes (NADH, NADPH) have been detected (381, 385, 532). Additionally, in the rabbit uterus, hormonal alteration of GDP-mannose, UDP-galactose, UDP-N-acetylgalactose, and UDP-glucuronic acid concentrations have been studied (149).

Longer-term to chronic estrogen treatment of spayed animals (10 hours to several days) increases the uterine (80, 149, 352, 532) and vaginal (36) content of acid soluble nucleotides, including coenzymes (36, 381); uridine and guanine nucleotides (36, 532) and their sugar derivatives (149) seem especially affected. Limited information indicates progesterone generally antagonizes estrogen-stimulated nucleotide metabolism (149). With acute estrogen treatment, changes in uterine nucleotide biosynthesis and pool size, as well as pre-

cursor transport, have been studied in relation to hormone-stimulated RNA synthesis. Accordingly, findings on uterine RNA metabolism are briefly summarized as background before proceeding to discussion of precursor dynamics.

A. RNA METABOLISM. Beginning 4 to 8 hours after estrogen treatment, total RNA content per uterus increases as net synthesis of all RNA classes takes place dominated by ribosomal and ribosomal precursor species (48, 49, 192, 208, 352, 369, 385). However, before RNA begins to accumulate, estrogen causes increased RNA turnover. All uterine RNA classes (182, 183, 320) show augmented labeling and specific activity within 1 to 2 hours after hormone administration (182, 183, 191, 352), concurrent with increased activity (not synthesis, 27) of RNA polymerase I (the nucleolar, "low-salt" enzyme; 176, 179, 208, 375). RNAs of all subcellular fractions are affected by estrogen, each subcellular fraction in proportion to its relative labeling rate in controls; nuclear RNA of both control and experimental tissues incorporate the largest amount of labeled precursor (46, 183). Analysis of still shorter periods indicates that the increase in rapid labeling of RNA occurs first in the nucleus approximately 30 minutes after injection of hormone, shortly after which label appears in cytoplasmic RNA fractions (207, 359); some disagreement exists that concerns RNA labeling occurring *in vivo* earlier than 30 minutes (*cf.* 190, 191, 207, 252, 287). The earliest labeling seems to affect ribosomal-ribosomal precursor RNA, then transfer and mature-ribosomal RNAs, and eventually and to a lesser extent DNA-like RNA (48, 49, 207). However, this sequence may be reevaluated if a recent observation is confirmed, namely that the activity of extranucleolar RNA polymerase (RNA polymerase II) increases 15 minutes after injection of estrogen (176); the activity was detected in an *in vitro* system containing uterine nuclei, nucleotides, and high salt which generates RNA with U/G ratios more like DNA than rRNA. The data suggest that estrogen stimulates synthesis of messenger-like RNA earlier than ribosomal species (*cf.* 176, 386). Where tested, effects described above on RNA metabolism could not be obtained by adding estradiol directly to the incubations (*e.g.*, 207, 456; however, see 386).

After 30 to 60 minutes of treatment with hormone *in vivo*, isolated nuclear chromatin from the rat uterus exhibits greater template activity for RNA synthesis than the controls (23, 92, 176, 207), but that of mouse uterus does not respond to estrogen treatment (119). The chromatin preparations require *in vitro* addition of homologous or bacterial RNA polymerase for expression of increased template activity (23, 92, 119, 207). Chromatin isolated from control or experimental uteri contains only negligible polymerase activity, and levels intrinsic to whole uterine nuclei have been reported as very low (*cf.* 92). These observations, together with the lack of demonstration that uterine RNA polymerase I activity is increased by less than 1 hour of treatment with hormone (*v.s.*), suggest uterine chromatin rapidly incurs a capacity for expanded transcription but its expression in the form of increased RNA production is not immediate, and *in situ* may be delayed approximately 1 hour.

It is clear that estrogen eventually raises the uterine protein synthesizing apparatus to new levels of activity through production of RNAs. Consideration of timing is appropriate in determining whether the earliest RNA labeling represents the primary direct action of the hormone from which all other metabolic and physiological effects derive, whether the effect represents only one in an array of direct and independent actions, or whether it is secondary to another, more primary event. One theory (*cf.* 207, 386) proposes that the primary action of estrogen in the uterus is to activate genetic transcription in conjunction with hormone-chromatin binding, followed by sequential stimulation of nuclear and cytoplasmic genetic translation. Evidence cited

in support, largely indirect, includes observed binding of estradiol to chromatin *in vitro* (207, 283, 341), the early increase in chromatin template activity, and a surge in RNA labeling in isolated nuclei observed within approximately 20 to 30 minutes of hormone treatment. The resulting ribosomal-type RNA, rapidly produced through an increase in some combination of nucleolar and extranucleolar chromosomal activity, is thought to be available for protein synthesis after transport to the cytoplasm; the ribosomal units assembled into polyribosomes after transport (510), are presumed to carry messenger RNA (207).

A direct effect on genetic transcription as *the* primary action of estrogen suffers from a number of difficulties; *e.g.*, the theory is not consistent with the known availability and timing of changes in RNA polymerase activities, although one possible exception has been noted (*v.s.*). Also, evidence has been introduced that suggests the apparent template activity of isolated uterine chromatin is not a reliable measure of its gene activation (119); mouse chromatin activity was found lowered after estradiol injection, an effect that could be attributed to higher amounts of intrinsic ribonuclease, whereas elevated template capacity of rat chromatin was associated with lowered ribonuclease activity (119). In addition, the function of an early surge in production and transport of RNAs, which in mammalian uterus are apparently quite unstable and which have not yet been shown to include messenger RNA, is not clear. Increased production of relatively stable RNAs of all types eventually occurs and doubtless "underwrites" much of the synthesis of structural and other proteins accompanying growth. Estrogen may well prove to activate directly nuclear machinery for these purposes. However, the primacy of such action as sole determinant and rate-limiting for all known early responses to estrogen in the uterus, including early synthesis of RNA and protein, has not been established and, indeed, can be challenged on the basis of physiological data alone. Very early effects of the hormone on the uterine vasculature, on *in vivo* transport of sugars, and other parameters described elsewhere in this review seem to be independent of new RNA synthesis. Increased amino acid transport is an early effect of estrogen that may result from a separate, direct action of the hormone and may be rate-limiting for general and specific protein synthesis (see section IV C). Evidence has been introduced, and will be discussed below, that the apparent synthesis of rapidly labeled RNAs may be controlled by separate effects of estrogen on transport, endogenous uterine synthesis, or phosphorylation of nucleotide precursors.

B. NUCLEOTIDE POOLS. Gorski and Mueller (181) observed that estrogen increased the overall pool size of acid-soluble nucleotides *in vivo*, beginning 1 to 2 hours after injection and continuing over the 4-hour experimental period. Incorporation studies with ^{32}P indicated that the pool expanded without significant change in turnover. Among individual compounds, total amounts and labeling of uridine derivatives were markedly affected, principally UTP and to lesser extent UDPG and UDPAG, whereas ATP content and specific activity were only slightly increased by estrogen treatment. The main effect of the hormone was to cause an absolute expansion of UTP pools, an apparent result of some *de novo* synthesis but otherwise of cause unknown; breakdown exceeding synthesis of short-lived RNA was ruled out as contributing to pool size since significant increases in free guanosine and cytidine compounds did not occur (181).

There is uniform agreement (*v.s.*) that virtually all measurable uterine free nucleotides increase in amount beyond 8 to 10 hours of estrogen treatment. Data on the extent and nature of pool changes in the 1- to 6-hour period, however, are in conflict. The ATP content is a case in point (see ref. 36 for older literature). Gorski and Mueller (181) found slight increases in ATP over the first 4 hours, whereas Oliver and Kellie (385) and Aaronson *et al.* (1) observed a

linear decrease to 50 to 60% of control levels over the same period; the decline could be prevented by actinomysin but not by puromycin (1). Billing *et al.* (47) and Miller and Baggett (352) saw essentially no change in uterine total acid-soluble nucleotide pool size over the first 6 hours of estrogen treatment, whereas Oliver and Kellie (385) observed a general decline and Gorski and Mueller (181) a slight increase. Oliver and Kellie (385) analyzed totals of individual nucleotides and nucleotide classes by utilizing rapid freezing techniques that preserve large amounts of ATP apparently lost with other methods. The progressive decline in total pool size after hormone treatment observed by these workers was shown to reflect a decrease in the purine nucleotide fraction (ATP, ADP) while amounts of pyrimidines (mainly UTP, UDP) remained constant. These results have indirectly been confirmed by Miller and Baggett (352).

In view of differences in findings, the precise effects of estrogen on sizes of nucleotide pools over the first 4 hours of treatment have not been established. Once clarified however, conclusions on the biological significance of any change or lack of change in pools would be premature without more knowledge of hormone influences on nucleotide synthesis. A decline in pool size, for example, however well correlated with the increase in RNA labeling that occurs, does not necessarily reflect solely the demands of RNA synthesis. Should the observed decline in pool size be proved correct, and confirmed as taking place at the expense of ATP but not UTP, a differential effect on nucleotide biosynthesis would be indicated. Some data on uterine nucleotide biosynthesis have been reported and is discussed next.

C. NUCLEOTIDE SYNTHESIS. Estrogen effects on uterine purine- and pyrimidine-riboside synthesis and phosphorylation have been studied but information on the critical early periods of hormone action is limited. Mueller and colleagues and others (*cf.* 181, 369, 384) established that estrogen treatment in the 1- to 6-hour range stimulates incorporation of one- and two-carbon units into purine and pyrimidine bases and nucleotides. Factors affecting size of the formate pool appeared especially sensitive. RNA purines were observed more heavily labeled than pyrimidines from one-carbon sources, an effect attributed to a differential estrogen action on the sizes of the intermediate nucleotide pools. Estrogen also increased the label in RNA adenine and guanine from adenine-^{14}C given by injection, indicating activation of interconversion and salvage pathways (369). Hagerman (203) found that 1 hour of estrogen treatment increased ^{32}P incorporation into the nucleotide pool of uterine nuclei before any effect could be seen on cytoplasmic pools or on incorporation into phospholipid; nuclear:cytoplasmic distribution of inorganic phosphate was not affected. The results suggest direct hormone action on nuclear nucleotide synthesis. Labeled uridine or cytidine administered to mice in 20-minute pulses by Miller and Baggett (352) was incorporated into uterine-free nucleotide and RNA fractions at greatly increased rates 1 to 2 hours after injection of estrogen, and labeling continued to increase up to 4 hours. Labeling of the fractions by orotic acid was much slower. The amount of label in uterine cytidine nucleotides relative to that in uridine nucleotides increased with time after estrogen, regardless of whether the source of label was cytidine, uridine, or orotic acid (352). The activity of ribose-5-P-pyrophosphokinase was observed to be low in uterine extracts and to increase rapidly after estrogen. These and other data led Oliver (384) to the conclusion that hormone activation of pathways of purine nucleotide synthesis, both *de novo* and from preformed precursors, may be due to increased availability of the common rate-limiting substrate 5-phosphoribosyl-1-pyrophosphate, mediated by activation of ribose-5-P-pyrophosphokinase (384).

D. NUCLEOSIDE TRANSPORT. Labeling of nucleotide pools with radionucleosides also

has been studied in relation to transport of the precursors and to the synthesis of RNA. One hour after injection of estrogen nucleosides administered individually or as mixtures in 20- to 30-minute pulses were incorporated in larger amounts into whole-uterine free nucleoside, nucleotide, and RNA fractions; labeling then sharply increased during the next 2 to 3 hours (47, 191, 352). Meanwhile, total RNA content and nucleotide pool sizes remained constant. With the nucleoside mixtures, appearance of label in the acid-soluble fraction after estrogen followed the same time course as labeling of RNA, and percentage increases in the two fractions were equivalent (47). By injecting cytidine-^{3}H alone, Greenman (191) found that 2 hours or more (but not 30 minutes) of exposure to estrogen caused a percentage increase in labeling of free CTP that exceeded the increase into RNA. Since in control tissue distribution of labeled cytidine did not exceed the uterine inulin space, it was concluded that recoverable nucleoside was extracellular and that label in CTP represented rapid conversion to nucleotide without accumulation of cytidine as it entered cells. Uterine inulin space does not change during 2 hours of treatment with estrogen when expressed as a function of wet weight volume, although in absolute terms the extracellular volume has expanded through edema formation (482). The observation by Greenman (191) of a small increase over control in free cytidine volume per 100 g of wet weight at 2 hours represents an increase over the inulin space and indicates, therefore, some cytidine accumulation intracellularly; his data clearly show larger cellular accumulations at later time periods. However, at 2 hours, a sharp increase in labeled CTP was observed (191), which strongly suggests that estrogen stimulated cytidine penetration far in excess of the small amount apparently accumulated. Since cytidine nucleotide concentrations in the uterus are normally quite low (181, 385), it was suggested that the rate of cellular penetration by cytidine is rate-limiting for the synthesis of CMP and its incorporation into RNA (191). These results imply that the early increases in RNA labeling observed after estrogen treatment (*e.g.*, 182, 208, 347, 348, 523) are more the result of a hormonal effect on the cellular entry of the labeled nucleoside than a direct action on RNA synthesis (191). Billing *et al.* concur (47) and Miller and Baggett (352) reached essentially the same conclusion in their study of the mouse uterus after administration of cytidine or uridine, although different results and conclusions emerged from their use of orotic acid. Hamilton and colleagues (208, 347, 348) regularly observed increases in nucleoside uptake along with RNA labeling in uterine subcellular fractions. These workers interpret their findings to mean that early increases in RNA labeling resulted from direct estrogen action on transcription, but concede the possibility that increased nucleoside entry determined the rate of labeling.

Oliver (383) directly approached the question of estrogen influence on transport of nucleotide precursors by using the non-metabolizable cytidine analogue, cytosine arabinoside (CA). During incubations of immature rat uteri for periods up to 1 hour, CA equilibrated in the total water volume of the tissue within 15 minutes in both control and estrogen pretreated (4-hour) samples, and no differences were seen in initial rates of uptake. To determine whether CA is in fact transported like natural nucleosides, CA was incubated with potential competitors for transport sites. Uridine lowered initial rates of CA uptake. In uteri preloaded with CA to equilibrium, addition of natural nucleosides caused efflux (countertransport) of CA, whereas purine or pyrimidine bases, sugars, or amino acids were without effect. The data indicates competition between CA and other nucleosides for common carrier sites specific for nucleosides. It was concluded that nucleosides are transported by a facilitated diffusion process which is not sensitive to estrogen (383).

The mechanism seems complex; data reported by Oliver (383) show CA itself and cytidine to be noticeably more effective in stimulating CA countertransport than uridine, adenosine, and inosine in descending order of competition. The significance of these data is otherwise difficult to evaluate in the absence of *in vivo* study of initial rates of uptake and of distribution of CA with time and as a function of estrogen treatment. A single experiment *in vivo* (383) showed counts per uterus from injection (route not mentioned) of Ca-^{3}H to be 25% above control samples 2 hours after estrogen administration, and to level off at 4 to 6 hours to about 40% above the baseline. Counts from uridine in the same experiments were increased to 150% above controls 2 hours after estrogen, but no attempt was made to determine tissue distribution of counts nor the proportion that remained free as nucleoside.

In vivo studies discussed further above indicate that natural nucleosides, delivered to the uterus by vascular routes, are transported into uterine cells by an estrogen-sensitive process. That estrogen controls nucleoside transport receives some added support from experiments of Billing *et al.* (47, 48). Two hours after estrogen injection, *in vivo* labeling from a mixture of nucleosides into both RNA and the tissue acid-soluble fraction was greatly increased above controls. Actinomycin D suppressed 80% of RNA synthesis but labeling of the acid-soluble fraction was only minimally affected, being higher than controls by 212% (47). Unfortunately, cellular-extracellular distribution of the acid-soluble label and distribution of the label within the acid-soluble fraction were not determined, but other experiments in the same study (47) suggest that much of the nucleoside precursor had been converted to a pool of free nucleotides. The degree to which estrogen affected nucleoside transport *vs.* nucleotide biosynthetic pathways is not clear, but Billing *et al.* (47, 48) conclude that the increased incorporation of labeled nucleosides into RNA over the first 5 hours of estrogen exposure is mainly the result of increased specific activity of precursor pools due to transport. The results with actinomycin D indicate that estrogen can affect nucleoside transport-phosphorylation independently of any effect on RNA synthesis.

E. COMMENT. From the studies surveyed, the general conclusion may be drawn that estrogen alters pool sizes and turnover of uterine nucleotides. Evidence that pool changes are effected through some combination of altered cellular penetration of precursors and altered nucleotide synthesis is sufficiently strong that further study of their dynamics is justified. Establishing whether estrogen effects on precursor transport and nucleotide synthesis are direct actions or instead are secondary to stimulated synthesis of a specific RNA would help to clarify the following issues: (a) does estradiol have more than one primary site and mechanism of action in the uterus; and (b) is the accelerated synthesis of RNAs, observed shortly after estrogen administration and detected by pulse-labeling, an "artifact" of increased availability of label through greater cellular transport and/or synthesis of precursors? Nucleotides available for synthesis of macromolecules in cells are largely synthesized within those cells, and pool sizes most likely reflect rates of local nucleotide synthesis, degradation and utilization. However, it remains to be determined whether precursor nucleosides, bases or smaller units are available outside uterine cells in *absolute* amounts sufficient to affect nucleotide synthesis; *i.e.*, whether precursor transport could be rate limiting for net changes in nucleotide pools and RNA synthesis. In any case, transport of purine and pyrimidine compounds requires further study at least for the reason that availability of injected *labeled* precursors seems to be estrogen-sensitive and to have an important bearing on specific activities of newly synthesized products. Possible estrogen regulation of nucleotide synthetic pathways also warrants further study *in vivo*

and in cell-free systems since ribonucleotides are involved in hormone-sensitive, energy-requiring processes in addition to synthesis of macromolecules.

Published observations of several workers in this field make it clear that experiments designed to explain hormonal regulation of protein and nucleic acid syntheses should routinely include estimations of labeling effects due to change in precursor pools. These and studies of concentration changes of individual nucleotides should include procedures that insure against rapid degradation of the more labile compounds before and during extraction. Also, analyses by Greenman (190) and Knowler and Smellie (287) illustrate the need for care in such mundane matters as choice of injection route for labeled precursors; the popular intraperitoneal route was shown to produce substantial variability and biasing of results in the uterus, sufficient to account in part for discordant findings between laboratories. Finally, a plea is entered, often expressed but seldom heeded, for study of these hormone effects separately in myometrium and endometrium. Whole-organ studies may be obscuring qualitative and quantitative differences in effects related to the stimulated growth that is normally hypertrophic in the muscle (40, 62, 168, 430) and a sequence of hypertrophy and hyperplasia in endometrium (*cf.* sections III C and IV A, 1).

2. *Male.* Nucleic acid metabolism in male accessory tissues has received intensive study and samples of data from the large number of imaginative experiments, employing in particular isolated prostatic nuclei and nuclear components, have been mentioned in preceding sections. Discussion of this research in detail would be appropriate here in the context of precursor transport but is not attempted since (a) RNA metabolism in male accessories has been reviewed extensively (313, 382, 549, 550) and (b) information on pool sizes and synthesis of nucleotides and on cellular transport of labeled precursors, as functions of androgen treatment and stimulated RNA synthesis, is surprisingly meager.

In brief, the earliest changes in RNA metabolism of the prostate and seminal vesicles in response to testosterone are similar to those in the uterus responding to estradiol. As in the uterus (*v.s.*), male structures show increased labeling of RNA from administered inorganic phosphate, nucleosides or nucleotides well within the 1st hour after injection of hormone (210, 544). The activity of RNA polymerase(s) is elevated by 1 hour in prostatic and uterine nuclei, but in both cases has not clearly been shown to be associated with earliest RNA synthesis, nor with observed increases in template activity of isolated nuclear chromatin (*cf.* 313). Also similar to findings in the uterus, the rapidly-labeled RNA first produced appears to be largely ribosomal in character and a product, in part at least, of activated nucleolar sites (*cf.* 312, 313). Indirect evidence suggests the presence also of messenger RNA (*cf.* 312, 550); however, in no instance in either sex has the production of new mRNA been proved by association with synthesis of a specific protein. Administered to castrates, testosterone eventually increases the amounts per prostatic cell of all classes of RNA (169, 170, 193, 288) as repair is effected of significant net losses in the cytoplasm (*cf.* 97, 288, 417).

Labeling experiments with ^{32}P indicate testosterone markedly increases the acid-soluble phosphate pool in seminal vesicles, the earliest detectable change occurring 30 minutes after labeling increases in RNA (544). As in the uterus (*v.s.*), ATP levels appear first to fall then rise over the first few hours after injection of testosterone, concurrent with increased RNA synthesis (96, 416); meanwhile, NAD and NADH concentrations rise according to Ritter (416), or do not change according to Coffey *et al.* (96). Consistent with the earliest effect on ATP levels *in vivo*, androgen added to isolated prostatic nuclei inhibited incorporation of ^{32}P into ATP (29).

Prostate glands and seminal vesicles in

castrates incorporated higher than normal amounts of tritiated orotic acid, but subnormal amounts of labeled uridine (139a, 289) and cytidine (169), into acid-soluble and RNA fractions. Since RNA turnover did not increase after castration, the results with orotic acid apparently reflected the rise in the specific activity of the precursor (acid-soluble) pool. Testosterone treatment (12 hours) depressed incorporation of orotic acid, and increased incorporation of the nucleosides, toward normal control levels. Raising the amount of uridine injected alone tended to mimic the effect of testosterone. These results suggest testosterone affects RNA synthesis by stimulating salvage pathways in precursor formation (139a). Testosterone seems to stimulate nucleotide biosynthesis directly but its precise action is unknown. The mechanism may involve accelerated precursor transport, which would affect labeling analyses of nucleotide pools and RNA, but this possibility has not adequately been examined.

E. Cellular Transport of Sex Hormones

Gonadal and adrenal steroid hormones penetrate their respective target cells, in contrast with peptide hormones and catecholamines that appear to initiate effects by binding to plasma membranes. Steroids are transported in the circulation largely bound to plasma proteins but the manner of their subsequent passage from capillaries through tissue matrix and into cells is not known. The hormones may be in free form at one or more of these transit points but the possibility that they remain bound may be considered since, for example, plasma proteins cross capillary walls, are taken up in quantity by tissues during the early stages of hormonal stimulation and enter responsive cells (*v.s.*). In any case, on reaching target cytoplasm, hormonal steroids become bound to proteins highly specific to each; specificity is defined in terms of recognition-binding characteristics and that the binding ("receptor") proteins occur only in target cells. After binding in cytoplasm, steroid-protein complexes are found in nuclei. Hormonal binding and intracellular transfer are reasonably assumed to be essential for hormone action and the field has advanced to the point of testing hypotheses that attempt to link formation and movement of steroid-protein complexes with known target organ responses. This section presents a brief overview of current information and hypotheses relative to binding of estrogen, progesterone, and androgen. Detailed coverage is not attempted since a comprehensive treatise (284a) and several reviews of the subject (33, 185, 248, 250, 251, 280, 344, 370, 386, 545, 549, 550) have appeared.

1. Estrogens. Several workers involved in earlier investigations of mechanisms of hormone action had surmised that action at the molecular level may depend on accumulation or retention of a hormone by its target cells. The concept was based on still older (and current) pharmacological theory that drug-responsive cells contain receptor sites that specifically recognize and bind effector substances. Direct testing of the concept was hampered by technical difficulties of localizing and measuring physiological amounts of effector in tissues, until Jensen and colleagues (*cf.* 250) developed and employed tritiated estradiol of high specific activity. They were able to show that estradiol (a) is rapidly taken up and retained by estrogen-responsive tissues (*e.g.*, uterus, vagina, pituitary), and (b) exerts its action without being converted chemically; other tissues rapidly took up, but did not retain, the hormone. Initial stages of estradiol uptake-retention by uterine cells involve binding to cytoplasmic proteins. The most significant of these is an oligomer, the steroid-bound complex of which sediments in sucrose gradients with a coefficient of approximately 8 S. Isolated in higher salt concentrations the complex reversibly disaggregates into steroid-associated units of coefficient of close to 4 S. Binding to these subunits can take place spontaneously in cell-free uterine

cytoplasm, provided the tissue is from estrogen deprived animals. Further, the binding is non-covalent in character and has a high free energy of interaction; association constants (at low temperature) are in fact among the highest known for biological systems.

Exacting radioautography and fractionation studies of uterine tissue show that, with the passage of time after an *in vivo* injection of labeled estradiol, most of the bound steroid resides in nuclei. At low salt concentrations the nuclear complex sediments with a coefficient of 8 S but in high salt concentrations disaggregates to distinctive 5S subunits. These and the following observations have led to the concept that bound estradiol enters nuclei and does so through a binding process involving at least two steps: initial binding in cytoplasm as an aggregate of 4 S subunits, then transformation to an aggregate of 5 S subunits which allows nuclear penetration. The sequence is most clearly brought out by manipulating temperature during incubations of uterine tissue. At 2°C nearly all bound ^{3}H-estradiol occurs, and remains, in the cytoplasmic 8 S form (extractable as 4 S units). On brief warming to 36°C the bulk of radioactivity is mostly nuclear and extractable in 5 S form. Data from *in vivo* and *in vitro* experiments indicate the normal time course of change. In the first 4 hours after administration of physiological amounts of estradiol, the nuclear protein appears while cytoplasmic binder steadily falls. Beyond 4 hours, the cytoplasmic protein is gradually restored. The latter process is sensitive to cycloheximide while depletion is not, thereby suggesting that restoration of cytoplasmic binding capacity (but not its transformation) involves synthesis of new protein. The depletion of cytoplasmic binder has also been observed to be 4- to 5-fold greater than can be accounted for by the increase in nuclear binding. These and other data suggest the nuclear complex is labile or undergoes inactivation. Also, a given estrogen molecule may repeatedly enter and leave the nucleus transforming several protein units in maintaining nuclear levels.

Occurrence of the 5 S nuclear estroprotein complex appears to depend on prior formation of the cytoplasmic counterpart. Thus, 5 S protein cannot be found in nuclei unless the uterus is first exposed to estrogen, but cannot be formed by adding estradiol directly to isolated nuclei. It readily forms, however, on adding estradiol to whole homogenates or to isolated nuclei supplemented with cytoplasm. Further, it appears the 5 S complex is formed from 4 S units *prior* to entering nuclei. In the presence of estrogen, but in the absence of nuclei, the uterine cytoplasmic fraction forms 4 S complex in the cold and transforms to 5 S on warming. Finally, accumulated evidence strongly indicates that uterine nuclei do not take up bound estradiol unless transformation from 4 S to 5 S subunits has taken place.

Use of the term "receptor" for steroid binders derives in part from the very high specificity shown for steroid-protein interactions. Initial uptake by cells is not saturable even at high hormone concentrations but binding is saturable at physiological concentrations, indicating a relatively small number of binding sites. Testosterone, progesterone, or adrenal steroids do not compete with labeled estradiol for binding sites, whereas unlabeled estradiol and other estrogens do compete. Remarkably consistent with expectations, estrogens regardless of structure (estrone, hexestrol, diethylstilbestrol) compete with estradiol and exhibit binding affinities in rough proportion to their potencies as estrogens. Similarly, synthetic anti-estrogens (Mer 24, U-11100, Clomiphene, CI-628) block estrogen uptake and binding. These agents suppress growth responses to estrogen and it is assumed they do so by preventing hormone-receptor binding.

2. Androgens and progesterone. A sequence of protein binding and nuclear transfer occurs similar to that for estrogens. However, events are less well understood in part because they are somewhat

more complex; testosterone is metabolized by male target cells to dihydrotestosterone (DHT), the apparent active unit (30, 69, 553), and the action of progesterone depends heavily on estrogen priming. In general, both hormones are taken up by respective target cells against gradients and are subjected to high affinity binding. Isolated nuclei do not bind hormone but do so when the cytoplasmic fraction is added. Prostatic cytoplasm contains at least two DHT-binding proteins, including 3.5 S and 8 S forms that may represent different aggregation states of the same complex. The 3.5 S prostatic protein exhibits some affinity for testosterone and cortisol but more for estradiol and progesterone. A DHT-protein unit with 3 S coefficient has been isolated from prostatic nuclei and is assumed to derive from the 3.5 S cytoplasmic unit. The evidence is insufficient to conclude, however, that binding proteins for DHT (or for progesterone in uterus) is altered in the process of nuclear transfer.

Progresterone is metabolized extensively by many tissues including the uterus but evidence is strong that progesterone itself rather than a metabolite is the effective hormone. It is, in any case, the only progestin extensively bound. Binding is highly specific although degrees of binding competition with adrenal steroids is evident in some species. Labeled progesterone given to castrates is found radioautographically in nuclei of virtually every tissue layer of oviducts, uterus, and vagina, but nuclear concentrations are more intense after estrogen priming and are reduced at least 50% if castrates are also treated with unlabeled progesterone or dihydroprogesterone. Determined as extractable protein-bound radioactivity, small amounts of bound progesterone, confined to cytoplasm, occur in uteri of castrates. After estrogen treatment, uptake and binding of ^{3}H-progesterone increases as much as 7-fold and the bound hormone occurs in both cytoplasm and nucleus. Protein bound progesterone extracted 6 hours after an injection of estrogen sediments in sucrose gradients predominantly with coefficients of 4 to 5 S, representing a mix of binders probably including plasma proteins. Extracted 20 hours after estrogen injection, complexes are largely 6 to 7 S, representing aggregates of at least three kinds of subunits. This shift is sensitive to cycloheximide indicating its dependence on new protein synthesis.

In intact animals, concentrations of bound progesterone increase in sites undergoing progesterone-dependent events (blastocyst implantation, decidual formation, inhibition of myometrial contraction). Yet during estrous cycles, the total number of progesterone binding sites per cell increase from diestrus to proestrus and fall rapidly through estrus to early diestrus. Thus, the concentration of progesterone binding sites increases with rising estrogen titers and falls at times of maximum progesterone secretion. Both changes largely involve the cytoplasmic, high affinity 6 to 7 S fraction. Experimental evidence (*v.s.*) attributes the follicular phase rise in binding capacity to a positive influence of estrogen. The fall is effected by progesterone. For example, 20 hours after a single estrogen injection to castrates, the number of progesterone binding sites increases 8-fold and then slowly decreases over several days. If progesterone is given by injection at the 20th hour, binding sites fall to below basal castrate levels within the next 24 hours. Shift of bound hormone to nuclei does occur under the influence of progesterone but the fall in 6 to 7 S sites cannot be accounted for simply by transfer. Evidence from a limited number of experiments suggests progesterone causes some form of binder inactivation. The mechanism is unknown but does not appear to involve synthesis of new protein.

3. Hypothetical significance of intracellular binding and transport. Great interest in the subject of intracellular steroid-protein interaction is sustained by the universal working assumption that hormone binding in cytoplasm and nucleus is causally linked to characteristic target cell

responses. The hypothesis is supported by recent work that relates binding with established effects of steroids in stimulating RNA synthesis. RNA polymerase activity of uterine nuclei *in vitro* and incorporation of precursors into RNA are enhanced under conditions that generate the 5 S estrogen-protein complex, but not under conditions that prevent formation of the complex. RNA production in non-target nuclei, already high, is not further enhanced by estrogen, indicating only uterus and related targets are specifically deficient in synthesizing capacity when estrogen is absent. However, little data yet exist that bear on the central questions raised by research on binding, namely, (a) by what means do steroid hormone-protein complexes affect the genome and RNA synthesis, and (b) what is the relation of steroid binding to hormonal effects described elsewhere in this review that are not, or seem not to be, dependent on synthesis of new RNA and protein?

Principally two working models have been advanced to explain the influence of bound hormone on the genome. Experimental evidence is still too meager to allow a choice between them. Both models accept that the hormone, on binding, effects a change in the receptor protein (assumed to be conformational) that permits passage of the complex (by means unknown) across the nuclear membrane. (i) The hormone is the genome effector and binding is the means of transfer to the nucleus. It is not known whether the hormone is free at any point during nuclear uptake. In this model binding has been viewed as a means of stabilizing the hormone supply. (ii) The protein itself affects the genome and combination with hormone causes the change(s) necessary or its nuclear entry. Whether the altered protein has an action of its own free of steroid remains an open question. The protein has been visualized as (a) a gene repressor until it should bind to hormone, (b) an activating component in a second messenger system leading to RNA synthesis, or (c) essential for enhancing the activity of RNA polymerase. In view of high association constants between steroid and cytoplasmic protein binder, it is unlikely that the protein is an enzyme and the hormone its activating agent. Either of the above models could accommodate observations indicating that (a) estrogen turns over rapidly in and out of the nucleus, repeatedly transferring altered cytoplasmic binder, (b) nuclear steroid binders are rapidly inactiviated, and (c) RNA chains synthesized by isolated nuclei from estrogen-treated rats are greater in length than in number, and greater in total amount than necessary for synthesizing mRNAs for a small number of specific proteins.

A somewhat more elaborate form of model (ii), above, has been proposed, in which the hormone, through binding, controls the availability or the state of specific proteins that control in turn both gene expression and "extragenomic" effects (*cf.* 370). This model is based on impressions that estrogen receptors are a family of proteins with dissimilar subunits. Dissimilarity is apparent from observations that some estrogen binding requires the presence of purine nucleotides while other binding seems to require only elevated temperature. Binding seems to be accompanied by a loss of small molecular weight components of the original complex. Estrogen is considered to destabilize native 8 to 9 S protein aggregates; a subunit (conformationally altered) with estrogen attached is taken up by the nucleus for use at RNA polymerase sites, while other subunits released may be available for regulating other portions of the cell or for diffusion and extracellular regulation.

F. Summary and Conclusions

1. Gonadectomy in either sex drastically reduces the size and weight of accessory reproductive organs. Absolute decreases occur in both cellular and extracellular volumes. In relative terms, reduction in

cell volume is greater than that of the extracellular compartment, resulting in an apparent "expansion" of extracellular space. Hormone replacement ultimately reverses this pattern as tissues rehydrate, net synthesis occurs in both cellular and extracellular areas, complex organization of protoplasm is reestablished in both compartments and growth or increased cell turnover ensues. Cellular hypertrophy is marked compared with expansion of extracellular spaces, leading to relative "reduction" of the latter to normal. An exception to this general pattern occurs in the uterus; shortly after an injection of estrogen (and normally in proestrus) water uptake rapidly takes place causing edema and a disproportionately large but temporary expansion of the extracellular compartment.

2. In castrate females, an injection of estrogen raises the total amount of Na, Cl, K, P, Ca and Mg in the uterus, and does so prior to the onset of growth (true hypertrophy and accelerated cell division). That is, ion contents increase in disproportion to organ dry mass. Much of the rise accompanies the early edematous uptake of water. Net cellular influx occurs, raising intracellular concentrations of Na, Cl, K, and Mg. With growth, the ion contents increase further but the growth-related rise of solid tissue mass and redistribution of water gradually reestablish the ion concentrations normal for uteri of intact animals. Data on *in vivo* ion distributions between endometrium and myometrium in a given species, and effects of hormones thereon, are insufficient to permit generalization.

3. The ions in myometrium alone have been analyzed, mostly in conjunction with the use of the tissue as a model for studies of smooth muscle excitation and contraction. Whole myometrium *in vivo* (*i.e.*, analyzed fresh) is always higher in total monovalent ion concentration, and much lower in ionic Ca, than is serum. Treatment with estrogen increases Mg and the total amount of monovalent ions but does not noticeably affect Ca. Species differences exist in relative ion concentrations and in hormone effects. Thus, K in rabbit muscle is higher than Na under all conditions but highest after estrogen treatment. Cat and rat muscle is usually higher in Na than in K but the reported effects of estrogen vary with laboratory.

In terms of *distribution* in fresh myometrium, estrogen treatment raises the intracellular concentrations of all monovalent ions, particularly Na which is further increased when treatment is supplemented with progesterone. Values are usually expressed in relative terms. Reliable information is lacking on absolute values for intracellular ion concentrations. Most measurements have been made *in vitro* and differences in handling, means and length of incubation and methods of determining extracellular space have led to wide variance in reported intracellular concentrations. Little can be said therefore about hormonal control of intracellular concentrations or of transmembrane distribution. Add to this that most studies compare muscle from estrogen-treated against estrogen plus progesterone-treated animals, and only rarely against castrate control animals. The most careful of the *in vitro* studies show little or no differences in intracellular values between estrogen- and progesterone-dominated muscle.

Nevertheless, these preparations show distinct differences between them in ion-related physiological behavior, and subtle hormonal effects on ion distribution and transport may be confidently assumed. Examples of differences in properties and behavior are included in sections 4 to 7, below.

4. Incubated myometrium rapidly takes up excess Na and loses K. With time, recovery occurs in which the Na excess is expelled while lost K is regained. Muscles from estrogen-progesterone treated animals extrude Na and regain K faster than after estrogen alone. Other experiments indicate the basic capacity for Na pumping and its hormonal modification are active

processes linked to glycolysis. The degrees to which K movements are independently driven or linked to Na pumping are not clear; some species differences in mechanism are indicated.

5. Ca distribution and transport have been studied in relation to the mechanism of myometrial excitation-contraction. Depletion of Ca abolishes both spontaneous and drug-induced electrical-mechanical activity. Drug-induced relaxation is also Ca-dependent. Experimental data generally conform to models which propose that (a) contraction-relaxation cycles in myometrium involve direct interaction of Ca with contractile elements, (b) Ca is resident in superficial sites in or on the plasma membrane and regularizes membrane function in spontaneous (depolarization-induced) activity by controlling permeability, and (c) Ca is resident in deeper "sequestering" sites, resistant to depletion to the outside but still accessible to the contractile machinery; mobilization of this Ca may explain contraction induced by drugs in muscle that is experimentally depolarized and deprived of action potentials. The deep sites may be one or a combination of the inner surface of the plasma membrane, the smooth endoplasmic (sarcoplasmic) reticulum, or other myoplasmic organelles.

Ca transport is active and involves a mechanism that tends to prevent excess Ca entry. The role(s) of estrogen and progesterone in Ca distribution and transport is not known. Determining effects of hormone on transport is complicated by cyclic changes in cell structure. During estrus or after treatment with estrogen, plasma membranes of myometrial cells ruffle, vesiculate, and actually interdigitate with markedly enlarged endoplasmic reticular and Golgi systems.

6. In myometrium isolated from castrate or immature females, contraction cycles are rare, weak, and uncoordinated along the organ; accompanying action potentials, if any, are of low amplitude; electrical excitability to inducing stimuli is low or absent. Similar properties and lack of activity are exhibited by uteri from intact adults in diestrus. In contrast, proestrus-estrus uteri, and these from castrates given injections of estradiol, show large, coordinated spontaneous contractions accompanied by synchronous bursts of action potentials of high amplitude conducted the full length of the organ; excitability is high and some laboratories report an increase in the resting membrane potential. Fallopian tubes respond similarly. Progesterone action requires estrogen priming and its effects on muscle activity resemble those in the luteal part of the cycle. Generally, spontaneous contractions are weaker, irregular, and less coordinated with action potentials than in muscle dominated by estrogen alone; action potentials are lower in amplitude and are not conducted long distances; there is evidence of local conduction blockade.

In the estrogenized uterus, epinephrine and isoproterenol depress electrical-mechanical activity, whereas oxytocin, acetylcholine, and certain of the prostaglandins are stimulatory. Epinephrine and isoproterenol increase the synthesis of uterine cyclic AMP; consistent with effects of epinephrine and isoproterenol, cyclic AMP itself depresses uterine contractile activity. The role of estrogen in epinephrine-like actions in the uterus is mysterious. By most tests, the two hormones work in physiological opposition. Yet, estrogen causes accumulation (apparently, not synthesis) of epinephrine by the uterus. Whether estrogen also stimulates synthesis of cyclic AMP is a matter of controversy.

7. Hydrolysis of ATP, catalyzed by membrane-associated ATPases, is involved in active transport of ions and organic metabolites. The uterus contains separate Ca- and Mg-dependent forms of the enzyme and one or more Na plus K-activated forms. Activity of the latter, measured in uterine homogenates of rats at least, is peculiarly insensitive to ouabain. Active extrusion of Na by the intact uterus (see 4, above) also is insensitive to the drug.

Estrogen increases ATPase activity in uterine luminal epithelium, mucosal fibroblasts, and blood vessels. Measured biochemically in homogenates, an increase in total ATPase activity is initially apparent after estrogen pretreatment has caused substantial organ uptake of Na and K. A cause-effect relation between increased enzyme activity and excess ion uptake is likely but has not been studied.

Castration of males markedly reduces ATPase activity in all accessory organs examined. Testosterone therapy ultimately restores full activity. Changes are initially apparent after a time at which testosterone is known to increase transport of sugars and amino acids (see 8, 9, below). Testosterone-stimulated ATPase activity in whole homogenates of the prostate and seminal vesicle is dependent on Ca and Mg but not on Na and K; ouabain is without effect. However, fractionation of the prostate reveals an Mg-dependent, Na-K-ATPase that is associated with the endoplasmic reticulum, stimulated by testosterone and partially inhibited by ouabain.

8. Estrogen stimulates one or more steps governing sugar transport into uterine cells, an effect apparently centered in transport-linked phosphorylation. The action is consistent with effects of estrogen on carbohydrate metabolism, which include elevations in free glucose concentration, glycogen synthesis, and incorporation of label from glucose into CO_2, lipid, RNA, and protein. *In vivo* experiments with non-metabolizable 5- and 6-carbon sugars show that estrogen increases cellular penetration leading to a more rapid attainment of steady-state levels. Tests with 2-deoxyglucose, which undergoes phosphorylation but not further metabolism, demonstrate that estrogen increases uterine capacity to transport and phosphorylate hexoses; the capacity is 50% above controls 30 minutes after an estrogen injection and 2- to 3-fold higher at 1 hour.

Kinetic analyses *in vitro* demonstrate that hexose transport (and phosphorylation) in the uterus is mediated by membrane carriers with a limited number of saturable sites. Under the conditions of short-term incubation required for analysis, estrogen pretreatment doubles the maximum influx of test sugar attainable by the system (V_{max}) while the concentration of external sugar required to attain half-maximal influx (K_m) is not different from controls. No change in K_m suggests that estrogen does not alter the affinity of the carrier system for the test sugar. With increasing time of estrogen exposure, affinity appears to lessen suggesting the stimulation of a non-saturable entry process. Countertransport occurs, but flux in either direction is not diminished by oxidative inhibitors. Thus, under the special conditions and assumptions of analysis, uterine hexose transport may be characterized as passive facilitated diffusion. Estrogen somehow accelerates the process. The existence of an active component *in vivo* has not been ruled out, and possible ion-dependence has not been studied. In view of the net ion uptake produced by estrogen (see 2 and 3, above), the possibility of active co-transport linked to Na should be examined.

Inhibitors of RNA synthesis do not affect estrogen-stimulated hexose transport *in vivo*; results with inhibitors of protein synthesis are not interpretable due to severely adverse effects on the vascular system. Pretreatment of the animal with cycloheximide blocks estrogen-stimulated protein synthesis and accelerated early transport *in vitro*. These and other data suggest estrogen controls assembly of a specific protein of rapid metabolic turnover which in turn controls the transport effect of estrogen. The observed increase in V_{max} for transport after estrogen would be consistent with activation of existing protein or positioning of new protein in membranes. In any case, the hexose transport-phosphorylation steps controlled by estrogen in the uterus are rate-limiting in the utilization of glucose. For example, glucose and 2-deoxyglucose are mutually competitive for transport sites; 2-deoxyglucose is

not metabolized beyond transport and phosphorylation but when present with glucose severely depresses glucose oxidation and incorporation into lipid and protein (470). Other supporting lines of evidence are reviewed.

Testosterone stimulates cellular transport of hexoses by a saturable carrier system in the prostate, seminal vesicles, and coagulating gland. The increase is abolished by phlorizin. These and other data suggest the presence of a rate-limiting phosphorylation step sensitive to the hormone. Uptake appears to be active since accumulation occurs against large concentration gradients. Testosterone also stimulates RNA and protein metabolism. It is not clear whether accelerated transport represents a separate primary action of testosterone.

9. Estrogen increases amino acid transport into uterine cells within 30 minutes of injection. Conclusions from kinetic studies parallel those with glucose; transport is mediated by a saturable membrane carrier system and the hormone increases the V_{max} of transport without changing the K_m. In contrast with glucose transport, facilitated diffusion of amino acids is demonstrably active. Uptake occurs against a concentration gradient and is sensitive to anoxia and metabolic inhibitors. Moreover, the system is Na-dependent and inhibited by ouabain, strongly suggesting involvement of cotransport linked to active extrusion of Na. A system with these same characteristics has been reported for amino acid transport in the seminal vesicle and prostate as affected by testosterone.

In the uterus, estrogen produces the following sequence: Amino acid transport is accelerated in 30 minutes, at about which time incorporation into general protein is depressed. At the 1- to 3-hour intervals, transport continues to increase while the pool size of total amino acids decreases, presumably as amino acids are utilized for general protein synthesis which is on the rise. After 3 hours, all parameters increase. Timing of these events suggest that they are interlocked causally but precise information is lacking. Estrogen-stimulated transport is reduced but not abolished by amounts of actinomycin D that inhibit 90% of RNA synthesis. Cycloheximide completely blocks the increase in transport. Interestingly, the effect of estrogen on amino acid transport can be mimicked *in vitro* simply by prolonging incubation of uterine horns 3 to 6 hours, an effect also inhibited by cycloheximide. Indications are strong that new synthesis or activation of membrane carrier protein is involved in the estrogen response.

10. Estrogen alters metabolism of all nucleotide classes in the uterus and vagina. Progesterone generally antagonizes the longer-term effects of estrogen. The subject has not been studied systematically and data on the early time course and direction of change in the size of individual pools often are in conflict. Nevertheless, it is clear that estrogen promptly increases turnover of certain nucleotides and eventually increases size of all pools. Evidence is strong that major increases are effected directly through a combination of accelerated transport of precursors and *de novo* synthesis, not secondarily through degradation of short-lived RNAs. The action of estrogen on synthesis is discriminate, not affecting nucleotide classes uniformly. Incorporation of 1- and 2-carbon precursor units, as well as interconversion and salvage pathways, are stimulated. Increased incorporation of label from exogenous nucleosides into uterine free nucleotide fractions is not hindered by amounts of actinomycin D that suppress 80% of RNA synthesis. Lines of evidence are reviewed that indicate estrogen influences nucleotide pool labeling also by accelerating transport of precursors. Data from several studies imply that the resulting increases in specific activity of nucleotide pools are rate-limiting for the observed early increase in synthesis (labeling) of RNA.

Stimulation of RNA metabolism in accessory male organs by testosterone has been studied extensively and shown to

resemble that in the uterus responding to estrogen. Little is known of androgen influences on precursor synthesis but isolated studies suggest nucleotide pools are turned over more rapidly and salvage pathways are stimulated.

11. Sex steroid hormones bind to cytoplasmic proteins of target organs in a highly specific manner and are transported into nuclei where they are again observed to be bound. A vast literature is briefly summarized and models are described that attempt to link intracellular binding and transport phenomena with known biological effects of the hormones.

V. Concluding Remarks

In any organ, factors governing the availability of water, ions, and organic substrates and rates of exchange between blood and cells are important determinants of metabolic rate. Such factors include hydrostatic pressures, blood flow rate, functional capillary volume, capillary permeability, and electrochemical gradients. Also, diffusion rates are affected by intercellular matrices which, in a given tissue, can be altered in density and composition to present differing properties as a barrier or capacities for binding. At the cellular level are added structural and metabolic features built into, or linked with, plasma membranes that regulate diffusion rates and actively maintain gradients.

Generally in organs, one or another of these factors are subject to small, short-term homeostatic adjustments by hormones and local controls (*e.g.*, pH, temperature, neural activity), and to progressive adjustments over the long term during somatic growth and aging. Thus, the means of access of molecules to cells, as well as growth and function, are maintained more or less in a steady-state. This condition appears to be general for hormonal target organs normally exposed to relatively constant amounts of controlling hormones. Accessory glandular organs of the male reproductive tract (seminal vesicles, prostate) are typical, although the male accessories are at the same time absolutely dependent on a hormone (testosterone) for the structural integrity required for normal function including transport. The dependence can be "unmasked" by experimental withdrawal and replacement of hormone. In female accessories by contrast (notably the uterus), it is the usual condition that basic maintenance adjustments are dominated or overridden by estrogen and progesterone, hormones that are alternately secreted and withdrawn. Regular cycles of growth and subsidence result. Information assembled in this review documents that all factors governing tissue and cell transport are substantially altered in the process. These findings compel a fresh consideration of at least two questions relating to hormonal control of reproductive target organs.

First, do changes of the kind described, grouped as effects on connective tissue, hemodynamics and cell transport, mediate hormone-stimulated increases in metabolism and growth? To the physiologist, it would appear intuitively obvious that they do and, for female sex accessory organs at least, the literature abounds with supportive circumstantial evidence. For example, experimental blockade of the early increase in uterine blood flow (and, incidentally, in temperature) due to estrogen severely depresses accelerated cell division that would otherwise occur in the organ. Inhibition of the capacity of estrogen to accelerate glucose transport suppresses the usual increase in glucose utilization in carbohydrate, lipid, protein, and nucleic acid pathways. Other apparent cause-effect relationships are described, together with several that suggest themselves but have not been explored. For example, an early estrogen effect is to increase the total salt concentration in the uterus particularly in regard to Na. Possibilities that this response is linked to the observed increase in Na-K-dependent transport of amino acids, to changes in myometrial excitation-contraction cycles and to hormone binding to

cytoplasmic receptor proteins, phenomena known to be sensitive to changes in intracellular ion concentration, are especially intriguing. It must be reemphasized that instances of apparent cause-effect relationships are based on circumstantial evidence and require confirmation with several lines of experimental approach.

Second, do all changes evoked by sex steroids in reproductive target organs result from the activation of the genetic machinery by the hormones? Twenty years of productive research, in which knowledge of transcriptional and translational events in microorganisms has been applied to the hormone field, have established that sex hormones activate the genome, increasing net synthesis of RNAs and protein. This work also has given rise to an assumption that gene activation accounts for all known effects of hormone. The working assumption has been changed to an assertion in at least one recent review (207). This position suffers from a number of difficulties, in addition to being premature. Arguments detailed in appropriate sections of the text, above, are represented in brief here. The direct action of steroids on transcriptional events has yet to be established, although recent evidence strongly supports the likelihood of such interaction. Next, claims that all effects are secondary to genome changes, ultimately secondary to limited production of special proteins, are based entirely on results with inhibitors of RNA and protein synthesis. These data are persuasive but not conclusive until supplemented with direct supporting evidence. Also, hormonal effects have been reported that proceed normally in the presence of inhibitors. For example, actinomycin D does not interfere with stimulation by estrogen of hexose transport *in vivo*, blood flow, or nucleotide synthesis. A large number of steroid effects, some of which are reviewed here for the first time, have not yet been tested *vis a vis* inhibitors and may prove to be independent hormone actions. Finally, certain of the effects (*e.g.*, on blood flow, cell transport) occur with sufficient promptness after hormone injection that they are not likely explained as dependent on transcription of mRNA followed by assembly and positioning of special proteins. A causal relationship of the kind is not consistent with observed lag-times for appearance of inducible proteins in animal cells. In sum, certain effects of gonadal steroid hormones may be characterized as "extragenomic." For purposes of future experimental work, it may be useful to assume that the rich variety of responses produced by gonadal hormones in complex targets like the uterus do not emanate from a single primary hormone action.

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