Characterization Of A Blood-uterine Lumen Barrier In Rats

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L’AVONS RÉCU
CHARACTERISATION OF A BLOOD-UTERINE LUMEN BARRIER IN RATS

by

Ann C. McRae

Department of Physiology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
October, 1981

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TO KATHARINE HINTÓN and ANNIE McRAE
ABSTRACT

Previous studies indicated that a permeability barrier between blood and the uterine lumen might exist in rats. The abilities of selected radiolabelled test substances to enter the uterine lumen were assessed by comparing radioactivity concentrations in fluid (secreted or instilled) recovered from the uterine lumen with those in serum, at fixed times after single intravenous injections of a test substance.

Initial experiments conducted in immature, ovariectomised, estradiol-treated rats revealed that after intravenous injection of \[^{3}H\] sucrose, \[^{3}H\] inulin, and \[^{125}I\] bovine serum albumin, uterine fluid radioactivity concentrations were low relative to those in serum and did not change markedly with time post-injection. These observations suggested that a barrier exists between blood and the uterine lumen which impeded the entry of these substances into the uterine lumen. Further experiments showed that this barrier is characterised by exhibiting variable restrictiveness which is hormonally modulated. Specifically, \[^{14}C\] urea was partially restricted from entering the uterine lumen in ovariectomised rats treated with vehicle only but entered without restriction in animals treated continuously with E. Progesterone appeared to increase the restrictiveness.
of the barrier to \( [^{14}C] \)-urea. \(^3\)H-Sucrose entered the uterine lumen less readily in E-treated animals relative to controls.

The relative abilities of certain substances to cross this barrier were investigated. Direct comparisons between \(^3\)H-mannitol, \(^3\)H-sucrose, and \(^3\)H-inulin (mol wt: 182, 343, =5,200, respectively) indicated that although all were markedly restricted from entering the uterine lumen, \(^3\)H-mannitol crossed the blood-uterine lumen barrier more readily than \(^3\)H-sucrose or \(^3\)H-inulin. Since these hydrophilic substances differ from each other primarily in molecular size, this observation suggested that this barrier is characterised by restricting selectively some substances according to molecular size. Similar comparisons revealed that \(^{14}C\)-antipyrene and \(^{14}C\)-barbital crossed this barrier without restriction whereas \(^3\)H-mannitol was highly impeded. Since these compounds differ from each other only slightly in molecular weight (range 182-188) but greatly in lipid solubility (chloroform/phosphate-buffered saline partition coefficients: 17.2, 0.23, 0.002, respectively), it was concluded that this barrier is characterised by restricting selectively some substances according to lipid solubility.

Uterine tissue radioactivity concentrations, following intravenous injection of certain test substances, were compared with those in serum in an attempt to ascertain the location of the blood-uterine lumen barrier. Following injection of \(^{14}C\)-urea to animals receiving any hormone regimen tested, serum and uterine tissue radioactivity concentrations did not differ greatly, even with hormone treatments associated with a barrier to this substance. With \(^3\)H-sucrose, calculated uterine extracellular fluid concentrations were approximately equal to serum radioactivity concentrations. The interpretation given to these
observations is that this barrier is located at or near the uterine epithelium. $^{3}H$-Mannitol, which was essentially restricted by the barrier, distributed primarily in uterine extracellular fluid whereas $^{14}C$-antipyrine and $^{14}C$-barbital, which were not restricted by the barrier, distributed in uterine extracellular and intracellular fluids. From these observations, it was suggested that this barrier has an extracellular locus. Hence, this barrier is characterised by being able to isolate the uterine lumen from uterine extracellular fluid by virtue of its epithelial paracellular location.

The functional significance of a blood-uterine lumen barrier is discussed in terms of the importance of the uterine luminal environment in reproduction.
ACKNOWLEDGEMENTS

As I sit, here in this "room of one's own", thinking of the people I would like to acknowledge for their contributions to this endeavour, I realise it is impossible for me to name them all. The journey to this room, along what came to be a winding road, began some time ago, and there have been many fellow-travellers. As I write this, I am thinking of them and thanking them for all they were and are to me.

However, I would be remiss if I did not extend my very special thanks to Dr. T.G. Kennedy for his excellent supervision, moral support and generosity regarding his time and research facilities; and to my family for their faith, understanding and patience throughout the years. Additionally, I wish to express my gratitude

- to the members of my Advisory Committee: Drs. D.T. Armstrong, R.L. Kline, G.J. Mogenson, and R.F. Weick for helpful advice, constructive criticism and above all, encouragement;
- to L. Lukash, A. Johnston and G. Barbe for excellent technical assistance and M. Cudmore for skillfully typing this thesis;
- and to the folks at POETS corner for making life more interesting and enjoyable.
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LIST OF ABBREVIATIONS

AIB - α-aminoisobutyric acid

cAMP - cyclic adenosine monophosphate

Ci - curie

cpm - counts per minute

C_{UF}/C_{S} - ratio of uterine fluid to serum radioactivity concentrations

C_{T}/C_{S} - ratio of uterine tissue to serum radioactivity concentrations

D - dark

dpm - disintegrations per minute

E - estradiol

L - light

NaCl - sodium chloride

P - progesterone

SEM - standard error of the mean

v - volume

wt - weight
CHAPTER 1: INTRODUCTION

Our knowledge of the uterine luminal environment, the factors which regulate its composition, and the role it plays in reproduction, is still far from complete. The available evidence indicates that this environment is regulated hormonally, particularly by estrogen and progesterone. Furthermore, there is considerable suggestive evidence that this hormonal regulation is physiologically important to various reproductive phenomena occurring in this environment. In this section, a review of some of this literature will be presented with particular emphasis placed upon studies carried out in laboratory rodents although some reference to other species will be made. To conclude this section, the evidence which suggested a blood-uterine lumen barrier might exist in rats will be presented. Briefly, previous studies indicate that rat proestrous uterine fluid has certain features in common with cerebrospinal fluid and testicular luminal fluids. Since these latter fluids are separated from blood by permeability barriers, it was hypothesized that a permeability barrier between blood and the uterine lumen might exist in rats.

1.1 Hormonal Regulation of Uterine Fluid Formation

Accumulation of copious uterine fluid in rats at proestrus and the subsequent discharge via the vagina at estrus were first reported by Long and Evans (1922). The cyclic occurrence of this fluid accumulation suggests that its appearance and disappearance during the estrous cycle are under hormonal control. Ovariotomy on the morning of the day
preceding proestrus prevented uterine fluid from accumulating at proestrus (Schwartz, 1964), suggesting that ovarian hormones are involved. Since administration of estrogen to ovariectomised, mature rats (Allen et al., 1924; Ringler, 1961; Conner and Miller, 1973a), to immature rats (Armstrong, 1968), or to ovariectomised, immature rats (Kennedy and Armstrong, 1975) resulted in uterine fluid accumulation similar in volume to that recovered from proestrus rats, it is likely that ovarian estrogen induces this fluid accumulation in the intact rat. Uterine fluid accumulation appears to result from retention of fluid within the lumen by constriction of the cervix as well as from fluid formation since insertion of cannulae into the cervix at diestrus resulted in diminished fluid accumulation at the subsequent proestrus (Genell, 1939). Furthermore, in estrogen-treated, ovariectomised rats, the withdrawal of estrogen treatment resulted in a significant loss of uterine fluid which could be prevented for up to 18 h by a tight ligation at the utero-cervical junction (Kennedy and Armstrong, 1975). The loss of fluid at estrus is considered (Kennedy and Armstrong, 1975) to be in part the result of the acute proestrus rise in plasma progesterone levels (Butcher et al., 1974) since anti-progesterone serum administered at proestrus inhibited the loss of fluid at estrus (Ferin et al., 1969). Furthermore, when progesterone was given to rats treated with estrogen to induce uterine fluid accumulation, loss of fluid occurred 12-18 h later (Armstrong, 1968; Kennedy and Armstrong, 1975).

In rats, during the remainder of the estrous cycle (Warren, 1938) and during pseudopregnancy (O'Shea, 1972), the volume of fluid present in the uterine lumen is of quantities insufficient to be measured directly. The paucity of uterine fluid at these reproductive stages may
be due to plasma estrogen levels too low to induce fluid formation and cervical constriction. Alternatively, or in addition, since progesterone administered concomitantly with estrogen inhibited the action of estrogen to induce uterine fluid accumulation when given in sufficiently high doses (the required dose of progesterone depended upon the dose of estrogen given) (Armstrong, 1968), it may be that plasma progesterone levels are sufficient to oppose this effect of circulating estrogen.

In mice, uterine fluid accumulates during proestrus (Parkes, 1929) and in response to exogenous estrogen (Homburger et al., 1955). In other species such as rabbit (Lutwak-Mann, 1962a), sheep (Iritani et al., 1969a; Wales and Restall, 1971) and cows (Olds and Van Demark, 1957), greater uterine fluid formation occurs at estrus than during other stages of the estrous cycle (or during pseudopregnancy in the case of the rabbit) suggesting that uterine fluid volume is subject to hormonal regulation in these species as well as in rats.

1.2 Hormonal Regulation of Uterine Fluid Composition

The paucity of uterine fluid present in rats at reproductive stages other than proestrus has greatly hindered investigations of the chemical composition of this fluid and the hormonal regulation of its composition. As a result of this limitation many investigators have employed the technique of flushing the uterine lumen with a volume of solution (usually a saline solution) and analysing the chemical constituents present in this uterine flushing. The primary disadvantage of this technique is that the concentrations of components cannot be known with certainty since the volume of uterine fluid is unknown. An additional difficulty implicit with this technique is that the integrity
of the tissue surrounding the uterine lumen may be disrupted by the act of flushing fluid through a lumen which is normally collapsed. Despite these limitations, this technique has afforded considerable information regarding the nature of the uterine environment.

Heap and Lamming (1962) found that the amounts of sodium and potassium present in uterine flushings obtained from intact, mature rats were lowest during diestrus, intermediate at proestrus and highest at estrus; the volume of fluid recovered being also highest at estrus. The results of a subsequent experiment (Heap and Lamming, 1962) demonstrated that the amounts of sodium and potassium present in flushings obtained from ovariectomised rats treated with progesterone or estrogen followed by progesterone did not differ from those present in untreated animals but were markedly greater in animals treated with estrogen only. This finding suggests that the cyclic variations observed in intact animals are primarily a reflection of plasma levels of estrogen.

Clemetson et al. (1972) reported that the potassium contents of uterine flushings obtained from Day 6 pregnant rats (Day 1 = vaginal spermatozoa) were several fold greater than those obtained from pregnant rats ovariectomised on Day 2 of pregnancy and treated with progesterone (0.5 or 4 mg/day) until Day 6. This finding again suggests that the potassium content of uterine fluid is modulated by estrogen since the intact animals would be exposed to the nidatory estrogen "surge" on Day 4 in contrast to the ovariectomised animals. However, whether these estrogen-induced increases in the sodium and potassium contents of uterine flushings represent hormonally regulated variations in concentration or reflect solely variations in volumes of equimolar fluids cannot be concluded since uterine fluid volumes were not directly and accurately measured.
The concentrations of most electrolytes present in pooled samples of bovine uterine fluid have been shown to exhibit variations during the estrous cycle (Schultz et al., 1971; Ibrahim et al., 1972); the predominant exception being sodium. Similar fluctuations were not observed for serum concentrations (Ibrahim et al., 1972) suggesting that the uterine fluid concentrations are regulated by the uterus and presumably in response to ovarian hormones, although this was not directly investigated. Furthermore, the finding that fluctuations in individual electrolyte concentrations were not invariably synchronous with other electrolytes (Schultz et al., 1971) suggests that each might be regulated individually. However, the object to which this regulation is directed is at present unknown. Nevertheless, the total amino acid concentration of bovine uterine fluid also exhibits cyclic variations (Fahning et al., 1967) with the period of greatest change coinciding with that for electrolytes (Schultz et al., 1971), i.e., during the transition from estrus to metestrus. Therefore, these changes in electrolyte and amino acid composition might represent increased uterine secretory activity during the luteal phase and thus might be of significance in establishing an appropriate luminal environment for embryonic development. Direct evidence of selective endocrine control of uterine fluid amino acid concentrations has been obtained for the rabbit; Gregoire et al., (1961) found that, in general but not invariably, estrogen and progesterone had opposing effects upon the uterine fluid concentrations of some amino acids and that certain amino acids (e.g., glycine) were more affected than others.

The dynamic nature of the uterine luminal environment during the interval between fertilisation and definitive attachment of the blastocyst
to the endometrium (implantation) is demonstrated by the quantitative and qualitative changes in the proteins present in uterine flushings obtained from mice (Gore-Langton and Surani, 1976; Aitken, 1977), hamster (Noske and Daniel, 1974), ferret (Daniel, 1970), rabbit (Johnson, 1972; Kulangara, 1972; Cowan et al., 1976; Eiler et al., 1977; Dunbar and Daniel, 1979), western spotted skunk (Mead et al., 1979) and sheep (Roberts et al., 1976). In the pig (Murray et al., 1972) and horse (Zavy et al., 1979), qualitative and quantitative changes in protein profiles of uterine flushings occur during the luteal phase of the estrous cycle. Maathius and Aitken (1978) demonstrated that human uterine flushings contained less protein if obtained during the secretory phase of the menstrual cycle as compared with the proliferative phase; however, this finding has not been confirmed (Hirsch et al., 1977; Sylvan et al., 1981). Analysis by acrylamide gel electrophoresis, Ouchterlony double immunodiffusion and immuno-electrophoretic techniques of peri-implantation or luteal phase uterine flushings from mice (Aitken, 1977), rats (Surani, 1977), western spotted skunks (Mead et al., 1979), pigs (Murray et al., 1972), rabbits (Beier, 1974), mares (Zavy et al., 1979) and cows (Roberts and Parker, 1974, 1976) reveal that the dynamic nature of the uterine luminal environment is due, in part, to the secretion of uterine-specific (i.e., non-serum) proteins. The hormonal regulation of the qualitative and quantitative changes in uterine proteins will be considered in more detail under the subheading "Pre-implantation Embryonic Development and the Uterine Luminal Environment."
1.3 Sperm Transport, Capacitation and the Uterine Luminal Environment

Accumulation of copious uterine fluid in rats correlates temporally with the reproductive event of mating, i.e., the deposition of sperm into the female reproductive tract. Therefore, it seems feasible that accumulation of uterine fluid at this time is functionally important for the transport of sperm to the site of fertilisation (oviduct) or for the capacitation of sperm prior to fertilisation.

Sperm transport in the rat is apparently very rapid since sperm reach the peri-ovarian space in about 4 min post coitum (Warren, 1938). A role for copious uterine fluid in this process is suggested by the finding that removal of this fluid prior to mating resulted in slower transport (Warren, 1938). However, whether this reduced rate of sperm transport was associated with diminished fertility was not determined. Nevertheless, Brown-Grant (1977) recently reported that pregnancy was induced in a high proportion of rats exposed to constant light despite the absence of detectable uterine fluid in these animals. This finding indicates that accumulated uterine fluid is not necessary for effective sperm transport. However, when the number of intromissions and ejaculations by the male was experimentally limited, only a small proportion of rats exposed to constant light became pregnant (Brown-Grant, 1977). One possible interpretation of this finding is that with suboptimal mating conditions, transport of sperm through collapsed uteri becomes a factor limiting the probability that pregnancy will result. Alternatively, pregnancy failure may have resulted from ova defective due to the prolonged anovulatory period (Brown-Grant, 1977).

Chang (1951) and Austin (1952) independently demonstrated that ejaculated sperm of rabbits and rats, respectively, were incapable of
fertilising ova immediately upon insemination into the oviduct. They found that sperm became capacitated, i.e., acquired the capacity to fertilise ova, during exposure to the female reproductive tract. The relative importance of the uterine environment as compared with that of the oviduct in this process appears to be species-specific. Several studies suggest that in the rabbit, capacitation occurs more rapidly if sperm are exposed to both environments (Chang, 1951; Adams and Chang, 1962; Bedford, 1969) providing that the endocrine status of the animal is appropriate, i.e., not under progesterone dominance (Chang, 1958). A role for uterine fluid in rabbit sperm capacitation is further suggested by the finding that in vitro incubation of sperm with whole uterine fluid from estrous rabbits resulted in partial capacitation (Kirton and Hafs, 1965). By contrast to the rabbit, capacitation of hamster sperm is considered to be primarily an oviducal phenomenon (Barros, 1974). This conclusion has been based partly upon the observation that fertilisation did not occur when epididymal or uterine-incubated sperm and ova were transferred to the uterus of estrous hamsters (Hunter, 1968). This finding has been interpreted as evidence that in the hamster, sperm do not become fully capacitated in utero and therefore the oviduct plays the primary role in this process. However, sperm known to be fully capacitated were not tested in this system so one cannot be certain whether fertilisation failure was due to uncapacitated sperm or to the presence of an environment inappropriate for fertilisation to occur. The conclusion that the oviducal environment is more crucial in hamster capacitation than that of the uterus is also based upon the observation that onset of ovum penetration following oviducal insemination of epididymal sperm or sperm incubated in utero
occurred approximately 1 h earlier with the latter relative to the former (Yanagimachi, 1966; Hunter, 1969). This slight advantage (1 h) conferred by incubation in utero has been interpreted to suggest that the uterine environment plays a minor role in capacitation. However, closer examination of the results presented in Yanagimachi's study (1966) reveals that the time required for sperm incubated in utero to penetrate 50% of the ova present was approximately 2 h less than that required by epididymal sperm. Since the entire capacitation process in situ in hamsters is considered to require 2 1/2 - 3 h for completion (Bedford, 1970), this advantage of 2 h conferred by in utero incubation represents a considerable portion of the total time requirement.

Further support for an appreciable role of the uterine environment in hamster capacitation comes from the finding that if the interval between mating and ligation of the oviducts was increased from 0.5 to 1.0 h, the proportion of fertilised eggs increased from 37.5 to 92% (Yanagimachi and Chang, 1963). These results suggest the proportion of sperm reaching the oviducts within 0.5 h that become fully capacitated is less than of those ascending to the oviduct one-half hour later. Presumably, those arriving later have spent the additional time in the lower reproductive tract. One interpretation is that the increased fertilising capacity resulted from longer exposure to the uterine environment and therefore that the uterine environment does play an appreciable role in sperm capacitation. However, since the numbers of sperm reaching the oviduct at 0.5 and 1.0 h after insemination were not examined, it is possible that greater numbers of sperm were present in the oviduct at the later time thereby increasing the probability that fertilisation would occur.
The relative importance of the uterine and oviducal environments in capacitation of rat sperm has not been investigated as extensively as that of rabbit or hamster. Noyes (1953) attempted to examine the relative fertilising capacities of epididymal and uterine-incubated sperm by inseminating estrous recipients. Since insemination was into the uterine horn and not into the oviduct directly, interpretation of these results is confounded by possible influence of the utero-tubal junction on transport to the site of fertilisation. In situ capacitation in the rat is considered to require 2 - 3 h (Bedford, 1970) whereas sperm inseminated into the peri-ovarian sac did not begin to penetrate ova until 4 - 5 h after insemination (Austin, 1951). This disparity suggests that in the rat, capacitation is at least initiated in the uterus although the oviducal environment may be necessary for completion of the process.

It is difficult to envisage an important role for the uterine environment to play in capacitation if sperm are in the uterus only for a few minutes en route to the oviduct as Warren (1938) has suggested (see above). However, Overstreet and Katz (1977) suggested that in the rabbit, the population of sperm reaching the oviduct within minutes post coitum may not be the same population ultimately participating in fertilisation. In fact, the sperm which fertilise ova may have been retained in the isthmic oviduct until ovulation or may have recently arrived in the oviduct from lower portions of the reproductive tract (Overstreet and Katz, 1977). If the small population of sperm which actually fertilise ova are those which move relatively slowly through the lower portions of the reproductive tract, then it seems more feasible that the uterine environment may play a role in modifying their function including initiating capacitation.
The factors in uterine fluid which might initiate this phase of sperm maturation are at present unknown. However, Toyoda and Chang (1974a) demonstrated that the rate of penetration of rat ova in vitro was increased and a greater proportion was penetrated when the culture medium contained a higher ratio of potassium to sodium: 0.32 vs 0.043. Since rat proestrous uterine fluid is characterised by a potassium to sodium ratio of approximately 0.3 (Howard and De Feo, 1959; Ringer, 1961), Toyoda and Chang (1974a) suggested that proestrous uterine fluid might therefore play a role in capacitation of sperm by virtue of its ionic composition. However, it should be noted that the ratio of potassium to sodium in rat seminal plasma is approximately 0.2 (Howard and De Feo, 1959) so if capacitation is initiated by an increase in the environmental potassium to sodium ratio, then this process would appear to be quite sensitive to small changes (i.e., 0.2 to 0.3). Toyoda and Chang (1974a) also observed that the addition of dibutyryl cyclic adenosine monophosphate (dibutyryl cAMP) to the high potassium to sodium ratio medium resulted in a further increase in the rate of ovum penetration. Concurrently, Rosado et al. (1974) demonstrated a similar enhancement of rabbit sperm capacitation in vitro by cAMP. Subsequently, Fraser (1981) concluded that capacitation in the mouse involves the endogenous production of cAMP by sperm since in vitro capacitation time was shortened by the presence of dibutyryl cAMP (Fraser, 1981) or the phosphodiesterase inhibitor, caffeine (Fraser, 1979) in the culture medium. It is unclear from these studies implicating cAMP in capacitation whether increased adenyl cyclase activity represents initiation of capacitation such as might occur in the uterus. Furthermore, the question of whether increased adenyl cyclase activity is a direct
response to factors present in the *in vivo* environment or results indirectly from increased sperm respiration remains to be answered. The latter possibility is intriguing because several studies have shown that sperm respiration is significantly increased following *in utero* incubation (Hamner and Williams, 1963; Mounib and Chang, 1964) possibly due to the stimulating effect of a dialysable factor present in uterine fluid (Iritani et al., 1969b). Moreover, other observations suggest that capacitation involves the removal of seminal plasma components from the surface of the sperm (Brackett and Oliphant, 1975); how this relates to increased levels of adenyl cyclase activity is unclear.

1.4 Preimplantation Embryonic Development and the Uterine Luminal Environment

Suggestive evidence that the uterine luminal environment might play a role in implantation by virtue of its varying nature comes from experiments involving embryo transfers. If the uterine luminal and/or endometrial environment is neither variant nor actively involved in implantation, then one would expect asynchronous embryo transfers to result in advanced or delayed parturition depending upon the direction of the asynchrony. However, when the gestational ages of the transferred embryos and recipient uteri were not synchronised, pregnancy failure resulted (rabbits: Chang, 1950; sheep: Moore and Shelton, 1964, Rowson and Moor, 1966; cows: Rowson et al., 1972). This pregnancy failure was at least partly due to deleterious effects of the uterine luminal environment upon asynchronous embryos since temporary residence in an asynchronous host followed by transfer to a synchronous recipient resulted in decreased pregnancy rates (Adams, 1971; Wilmut and Sales, 1981).
Furthermore, treatment of rabbits with estrogen shortly after mating resulted in pregnancy failure which was associated with several days delay in the appearance of the progestational pattern of uterine luminal proteins (Beier, 1974). When Day 4 rabbit blastocysts were transferred to a Day 8, estrogen-treated recipient, implantation occurred at the unusual time of Day 11 or 12 (Beier, 1974), suggesting that pregnancy failure in the estrogen-treated rabbit is due to asynchrony between embryo and uterine luminal environment. However, studies investigating in rabbits the effect of post coital estrogen treatment on the time course for uterine endometrial receptivity to the implanting blastocyst have not been carried out. Therefore, it is possible that the progestational development of the endometrium was delayed similarly by this treatment and that this factor may have contributed equally or solely to the resulting pregnancy failure. Nevertheless, one interpretation of these findings is that uterine secretions are important in implantation so that pregnancy failure following asynchronous embryo transfer is at least partly due to patterns of uterine luminal proteins inadequate to support embryonic development.

**Formation and Expansion of Blastocysts**

In the rabbit, the uterine-specific protein, uteroglobin, accounts for up to 60% of the total protein present in uterine flushings obtained from pregnant and pseudopregnant rabbits (Johnson, 1972). The synthesis and secretion of this protein (Nieto and Beato, 1980) is progesterone-dependent (Urzua et al., 1970; Arthur and Daniel, 1972; Rahman et al., 1975). Uteroglobin first appears in rabbit uterine flushings on Day 3 post coitum, reaches maximal amounts on Day 5 and declines thereafter.
until Day 9 (Krishnan and Daniel, 1967). Therefore, its appearance in uterine flushings coincides with the arrival of embryos into the uterus and incipient blastulation whereas its maximal content correlates with blastocyst expansion (Beier and Maurer, 1975). Although several studies have shown that blastulation and blastocyst expansion occurred in vitro in the absence of uteroglobin (Van Blerkom et al., 1973; Beier and Maurer, 1975); other in vitro studies demonstrated that the presence of uteroglobin or unfractionated uterine flushings was associated with marginally enhanced blastocyst expansion (Krishnan and Daniel, 1967; El-banna and Daniel, 1972; Maurer and Beier, 1976) and increased [³H]-uridine uptake or incorporation (Gulyas et al., 1969; El-banna and Daniel, 1972). Taken together, these findings suggest that uteroglobin (and other uterine fluid components) is not required for blastocyst development but might facilitate this process. Nevertheless, these experiments conducted in vitro to determine the effects on blastocyst development of supplementation of culture medium with dialysed whole uterine fluid or high molecular weight uterine fluid fractions are based upon the assumption that culture medium without supplementation is an appropriate control. However, at present we do not know the precise micromolecular composition of uterine fluid during the pre-implantation period (or at any reproductive stage); therefore, the possibility exists that culture medium does not accurately represent uterine fluid. Interactions between micro- and macromolecular components might exert profound effects upon blastocyst development in utero which have not yet been observed in vitro due to the composition of culture medium employed. Additionally, in none of these in vitro studies mentioned above was the reproductive potential of the blastocysts formed in vitro determined by
transferring them to appropriately prepared recipients. In this regard, it is notable that those embryos retained in the oviduct by tubal ligation which progress to later developmental stages in the oviduct result in a lower proportion of successful pregnancies following transfer to synchronised pseudopregnant recipients than less advanced embryos (Adams, 1973). One interpretation of this finding is that prolonged exposure to the oviducal environment is detrimental to embryos. Alternatively, blastocyst development in the absence of some essential uterine factor may be associated with decreased reproductive potential.

Embryonic blastulation in other species is also temporally correlated with the transient appearance in uterine flushings of particular proteins. Noske and Daniel (1974) reported an α-globulin fraction which was present in hamster uterine flushings only on the third day post coitum and suggested that since the presence of this fraction coincided with embryonic blastulation, it might play a role in this process. A similar suggestion was made by Roberts and Parker (1974) regarding a uterine-specific protein present in bovine uterine flushings only until blastulation has occurred. Roberts and Parker (1974) noted that two additional uterine-specific fractions appeared coincident with subsequent blastocyst elongation. In the pig, fractionation of uterine flushings during the estrous cycle revealed the presence of a low molecular weight, non-serum fraction (uteroferrin) in fluid obtained between Day 9 through 16 post ovulation which comprised approximately 20% of the total protein present (Murray et al., 1972). During porcine pregnancy, blastulation occurs between Day 6 and 8 and therefore precedes the appearance of this protein; however, blastocyst elongation begins between Day 8 and 10 and initial placentation occurs about Day 11.
(Murray et al., 1972) and are therefore coincident with the appearance of this fraction in uterine fluid. However, correlative data such as these should be interpreted with caution since temporal coincidence does not necessitate a direct causal relationship. Blastulation of hamster embryos occurs in the oviduct if embryos are retained there by a uterostubal ligature (Orsini, 1965) and therefore occurs presumably in the absence of the α-globulin fraction of hamster uterine fluid described above. Since the reproductive potential of blastocysts formed in the oviduct was not determined (Orsini, 1965), a role for this uterine fluid fraction in blastulation cannot be ruled out. A role for uterine proteins including uteroferrin in porcine embryonic development is supported to a limited extent by the finding that exogenous progesterone given to pregnant gilts was associated with increased total protein in uterine fluid on Day 15 of pregnancy and enhanced placental development on Day 40 (Knight et al., 1974). However, the precise relationship between enhanced uterine secretory activity and placental development remains to be elucidated.

Embryo Reactivation

The duration of the interval between fertilisation and implantation varies considerably among species being a few days in hamsters, rabbits, sheep and ferrets or several months as in roe deer, European badgers and western spotted skunks (Finn and Porter, 1975). Obligate delayed implantation is said to occur when every fertile cycle is characterised by a prolonged interval between fertilisation and implantation. When this interval is extended only by an intervention such as lactation (McLaren, 1968), the resulting delay is considered to be
facultative. In rats and mice, facultative delayed implantation can be imposed experimentally by removing the ovaries on Day 2 or 3 of gestation (Chambon, 1949), maintained by progesterone treatment (Cochrane and Meyer, 1957) and terminated by administration of small doses of estrogen (Psychoyos; 1963). During this delay, the blastocysts undergo developmental arrest (embryonic diapause) which is characterised by reduced cellular mitoses (Sherman and Barlow, 1972), RNA (Gulyas and Daniel, 1969) and protein synthesis (Weitlauf and Greenwald, 1968). During termination of delayed implantation, quiescent embryos are reactivated and exhibit increased rates of metabolism and macromolecular synthesis. Embryonic reactivation is the indirect result of modified uterine luminal conditions (Torbit and Weitlauf, 1975).

Surani (1976) confirmed an earlier finding (Daniel and Krishnan, 1969) that uterine flushings obtained from pregnant rats during delayed implantation contained considerably less protein than those obtained from pregnant rats (Days 5 to 7 of pregnancy). Surani (1975a) demonstrated electrophoretically that estrogen-induced termination of delayed implantation was associated with the appearance in uterine flushings of a new protein at 1 h and two additional proteins at 18 h post estrogen. Radiolabelling of uterine luminal proteins following instillation of [35S]-methionine into Day 5 pregnant and ovariectomised rats treated with appropriate hormones indicated that the proteins appearing at 18 h post estrogen were probably synthesised by the uterus and secreted into the lumen in response to estrogen (Surani, 1977). Although zona-free blastocysts in vitro apparently bind specifically iodinated rat uterine luminal proteins (Tzartos and Surani, 1979), whether this binding is functionally

1 Hereafter, zona refers to zona pellucida.
coupled to embryonic cellular activities has not been determined. Thus, the functional significance of the appearance of proteins in uterine flushings in response to nidatory estrogen remains unclear. However, in mice, estrogen-induced termination of delayed implantation is also associated with a marked increase in total protein in uterine flushings within 4 hr post estrogen (Aitken, 1977) which corresponds to a period of increased uterine protein synthesis and secretion into the uterine lumen (Pratt, 1977). Furthermore, Aitken (1974) demonstrated that seasonal termination of obligate delayed implantation in the roe deer was correlated temporally with a distinct rise in not only total protein but also total hexose and calcium in uterine flushings and with a rise in plasma estrogen levels. Moreover, termination of delayed implantation in the western spotted skunk was associated with increased uterine protein synthesis and secretion into the uterine lumen, and, in this species, with a rise in plasma progesterone levels (Mead et al., 1979). These correlations between increased protein content in the uterine luminal environment and release of embryos from diapause observed in several species are suggestive of a causal relationship between these events. In rats and mice, the appearance of proteins in uterine flushings within 4 hr after termination of delayed implantation (Surani, 1975; Aitken, 1977; Pratt, 1977) makes it feasible for them to play a role in the embryo reactivation which occurs during this interval (Pratt, 1977; Van Blerkom et al., 1979); however, this has not been demonstrated definitively. Nevertheless, Fishel (1979) observed in mice that when estrogen is given without concomitant progesterone to terminate delay of implantation, a uterine protein secretion pattern occurs which differs markedly from that appearing with concomitant progesterone and
implantation does not occur. Unfortunately, Fishel (1979) did not
determine whether this hormone regimen is sufficient to induce
a) embryo reactivation and b) uterine endometrial receptivity to
implanting blastocysts.

Loss of the Zona Pellucida

Dickmann and Noyes (1961) concluded that timing of zona pellucida
loss from embryos was determined by progestational age of embryos rather
than by appearance of zona lytic factors in the uterine luminal environ-
ment on the basis that 24 h after transfer of Day 4 or Day 5 embryos to
Day 4 pseudopregnant recipients, Day 4 embryos were still encased in
zonae whereas Day 5 embryos were not. However, these authors did not
test further this conclusion by transferring Day 4 or 5 embryos to
Day 5 pseudopregnant recipients 24 h prior to determining whether the
embryos were zona-intact. Subsequently, Orsini and McLaren (1967)
obtained evidence that the uterine environment is also important in
determining the timing of zona loss since embryos retained in the ovi-
duct by a tight ligature at the utero-tubal junction did not shed their
zonae at the same time as those allowed to enter the uterus. McLaren
(1970) suggested that there are two mechanisms for zona loss; one which
involves lysis of the zona and is dependent upon appropriate conditions
in the uterine luminal environment and another which involves shedding
(as compared by lysing) of the zona and is independent of the uterine
environment but dependent upon blastocyst development. The finding that
the zonae of morulae of the lethal, homozygous genotype (t^{12}/t^{12}) are
lysed in utero at the same time as heterozygous (+/t^{12}) blastocysts
(Mintz, 1972) confirms the zona lytic role of the uterine luminal
environment. Zona lysis does not occur during delay of implantation
(McLaren, 1967; Surani, 1975b) suggesting that this form of zona loss is estrogen-dependent. It has been suggested that nidatory estrogen acts upon the uterus to induce secretion into the uterine lumen of a proteolytic enzyme or enzyme complex which then lyses the zonae pellucidae (Mintz, 1972; Hoversland and Weitlauf, 1978; Rosenfeld and Joshi, 1981). Rosenfeld and Joshi (1977) hypothesised that the endopeptidase present in rat proestrous uterine fluid may play a role in implantation and were able to demonstrate that intrauterine (luminal) instillation of antiserum raised against this endopeptidase to Day 4 pregnant rats was associated with a decreased implantation rate at Day 10. However, they were unable to demonstrate in vitro that the zonae of rat morulae and blastocysts are lysed in the presence of this endopeptidase (Rosenfeld and Joshi, 1981). Hoversland and Weitlauf (1978, 1981) demonstrated that estrogen treatment given to ovariectomised, progesterone-treated mice is associated with a marked increase in amidase activity in uterine flushings obtained from these animals and with increased uterine zona-lytic activity (as assessed by loss of zona from transferred blastocysts). Although these findings suggest that this amidase activity might be zona-lytic, estrogen given to ovariectomised mice (not treated with progesterone) results in increased enzyme activity in uterine flushings and no increase in uterine zona-lytic activity. Therefore, the estrogen-dependent, uterine luminal, zona lytic factor remains unidentified at present.

1.5 **Modulation of the Uterine Luminal Environment by Embryos**

There is appreciable evidence suggesting that the uterine luminal environment during the peri-implantation period is influenced not only
by the endocrine status of the animal but also by the presence of embryos themselves. For example, exogenous progesterone treatment given to ovariectomised western spotted skunks induces a pattern of uterine protein synthesis and secretion into the uterine lumen which is qualitatively similar but quantitatively less than that occurring during termination of obligate delayed implantation (Mead et al., 1979). Although it is possible that this difference is due to the absence of ovarian estrogen, this is unlikely since concomitant estrogen treatment virtually abolished the effect of progesterone to induce uterine synthesis and secretion (Mead et al., 1979). The possibility that the presence of embryos in the uterine lumen enhances endometrial secretory activity cannot be ruled out and could be tested by transferring embryos to progesterone-treated, ovariectomised animals. In addition, nidatory estrogen given to ovariectomised pregnant and pseudopregnant mice treated with a progestin results in patterns of proteins in uterine flushings obtained from these animals which are qualitatively similar but are quantitatively greater in pregnant mice (Aitken, 1977); again suggesting that the presence of embryos in the uterine lumen might be stimulating greater secretory activity. Furthermore, ultrastructural examination of the uterine lumen of mice 24 h after estrogen-induced termination of delayed "implantation" revealed that the density of a uterine secretion is greatest in the presence of a blastocyst, intermediate in the presence of a sepharose bead and least in the absence of either (Nilsson, 1977). These findings suggest that blastocysts may produce a signal which (directly or indirectly) results in increased endometrial secretion. In the pig, Knight et al. (1973a) observed that experimental conditions producing uterine crowding of embryos
(superovulation and unilateral ovariectomy-hysterectomy) resulted in greater total amounts of protein in uterine flushings relative to that in control animals but the proportion of protein secreted per corpus luteum was similar. Furthermore, Knight et al (1973b) reported that treatment with progesterone alone or in combination with estrogen resulted in similar qualitative patterns of proteins in uterine flushings but the latter treatment resulted in a two-fold increase in total protein content relative to that with progesterone only. These findings combined with the fact that porcine blastocysts in vitro are capable of converting androgens to estrogens (Perry et al., 1976) suggest that endometrial secretory activity may be modulated locally by blastocyst estrogen. This possibility is supported somewhat by the observation—that Day 60 porcine endometrial explants obtained from gravid uterine horns secrete greater quantities of protein relative to non-gravid horns (Basha et al., 1980).

1.6 A Blood-Uterine Lumen Barrier in Rats

Rat proestrus uterine fluid (Ringler, 1961) resembles mammalian cerebrospinal fluid (Davson, 1967); rete testis fluid (Voglmayr et al., 1966; Johnson and Setchell, 1968) and seminiferous tubule fluid (Turner et al., 1979) in having similarly lower protein concentrations relative to that of plasma. With the exception of cerebrospinal fluid, these fluids resemble each other also in having potassium concentrations in excess of, and sodium concentrations less than those in plasma (Tuck et al., 1970; Levine and Marsh, 1971; Voglmayr et al., 1966, 1967; Howard and De Feo, 1959; Ringler, 1961; Lévin and Edwards, 1968); the potassium concentration of cerebrospinal fluid is less than that of plasma (Davson,
1967). Seminiferous tubular fluid (Tuck et al., 1970; Levine and Marsh, 1971) has higher potassium but lower sodium concentrations than rete testis fluid (Voglmayr et al., 1966, 1967) or rat proestrous uterine fluid (Howard and De Feo, 1959; Ringler, 1961; Levin and Edwards, 1968).

The cerebrospinal fluid is formed at the choroid plexuses probably as a result of active transport of salts with associated water movement (Davson, 1976) since application to the choroid plexus of a transport inhibitor, ouabain, reduces fluid formation (Vates et al., 1964; Ames et al., 1965), and net sodium flux across this epithelium (Wright, 1972). However, there is not complete agreement that cerebrospinal fluid is a secretion in contrast to a passive filtrate (Rapaport, 1976). The rete testis fluid probably also originates as an active secretion and not as a filtrate since fluid formation is reduced during testicular ischemia or hypoglycemia (Linzell and Setchell, 1969). Furthermore, rete testis fluid formation in rams did not vary as testicular perfusion pressure was experimentally manipulated (Linzell and Setchell, 1969). However, the possibility that the alterations in perfusion pressure were not of sufficient magnitude or duration to induce changes in capillary filtration pressure, and thereby in rete testis fluid formation, cannot be ruled out; concurrent measurement of testicular lymph formation might resolve this uncertainty. Nevertheless, since rete testis fluid formation is not reduced within 24 h of efferent duct ligation (Setchell, 1967), which presumably elevates intratubular hydrostatic pressure, it is unlikely that fluid formation occurs as a passive filtration. Evidence based upon measurement of seminiferous tubular potential differences and calculation of electrochemical...
gradients suggests that potassium and bicarbonate ions are actively secreted into seminiferous tubule fluid against relatively steep concentration gradients (Tuck et al., 1970). However, this does not constitute proof of active transport; such proof is obtained by measuring unidirectional fluxes of the solute in question under conditions in which electrochemical gradients have been abolished. Nevertheless, the finding that bicarbonate may be transported actively into the seminiferous tubule, combined with observations that a carbonic anhydrase inhibitor, acetazolamide infused intravenously decreased rete testis fluid flow rate (Setchell and Linzell, 1968), provides further suggestive evidence that this fluid is formed by active secretion, and that secretion of bicarbonate is involved in this process. Rat proestrous uterine fluid may also be formed as a result of active secretion since there is a potential difference across the endometrium which is dependent upon aerobic metabolism and is ouabain-sensitive (Levin and Edwards, 1968). Furthermore, it would appear that potassium and chloride enter the uterine fluid against electrochemical gradients, suggesting that they are actively transported into this fluid (Levin and Edwards, 1968). Therefore, it seems likely that proestrous uterine fluid is formed by the active secretion of salts (potassium chloride) into the uterine lumen with confluent movement of water as an osmotic consequence (Levin and Edwards, 1968); however, this mechanism has not been proven directly. Taken together, these findings suggest that rat proestrous uterine fluid, seminiferous tubule fluid and cerebrospinal fluid are similar in that they are formed as a result of active secretion.

The concept of a blood-brain barrier arose early in this century as a result of the observations that, although the majority of body
tissues were stained following systemic administration of certain synthetic dyes, those of the central nervous system remained "colourless". Ehrlich (1902; cited by Bradbury, 1979) considered this differential effect to result from lack of affinity of nervous tissue for these agents. However, the unlikeness of this as the sole explanation was demonstrated by Goldmann (1909, 1913; cited by Davson, 1976) who found that one dye, trypan blue, which did not stain the central nervous system when injected intravenously, did so when injected into the cerebrospinal fluid. This inability of certain blood-borne dyes to stain the central nervous system was taken to indicate that there is a barrier which prevents the entry from blood into the central nervous system of certain substances. Stern and Gautier (1921) proposed that this phenomenon be referred to as the blood-brain barrier ("barri~re hémato-encéphalique"). These early studies of the distribution of dyes demonstrated that, in addition to the central nervous system, the testicular seminiferous tubules were unstained following systemic administration of certain dyes (Ribbert, 1904; Bouffard, 1906; Goldmann, 1909; and Pari, 1910; cited by Setchell et al., 1969). This finding has been confirmed more recently (De Bruyn et al., 1950; Körmanto, 1967) and suggested that a blood-testis barrier might also exist. However, Davson (1967) has pointed out dyes are "treacherous" materials to use in the study of the blood-brain barrier (and presumably any other barrier) because of their tendency to form aggregates, to adsorb to proteins, to change colour with oxidation or reduction and finally, because of their toxicity.

To circumvent these limitations of dyes, many investigators have examined the abilities of a wide variety of non-dye substances to
penetrate brain tissue and cerebrospinal fluid. For example, Davson (1955) confirmed the existence of the blood-brain barrier by demonstrating that low molecular weight substances such as thiocyanate, iodide, creatinine, sucrose and p-amino-hippurate penetrated the cerebrospinal fluid very slowly; the ratios of cerebrospinal fluid to plasma concentrations were less than 0.1 despite the fact that constant plasma concentrations had been established for a period of 2 h by intravenous infusion. Furthermore, the restricted entry of iodide, sucrose and p-amino-hippurate from blood into brain tissue was demonstrated by the observation that these substances distributed in less than 5% of the chloride "space" of brain after 2 h, whereas they equilibrated in the chloride "space" of diaphragm within 6 min (Davson and Spaziani, 1959). Such studies as these have formed the basis of several full-length monographs (Davson, 1967; Rapaport, 1976; Bradbury, 1979) and have led to general acceptance of the existence of a blood-brain barrier.

As noted above, the inability of certain dyes to penetrate the seminiferous tubule from blood suggested the existence of a blood-testis barrier. Further suggestive evidence for such a barrier comes from the observations that the brain and testis share certain anomalous behaviours which have been attributed in the case of the brain to the blood-brain barrier. The first of these observations is that radioactive sodium, following systemic administration, exchanged with endogenous sodium more slowly in the brain and testis than in other tissues, such as liver, skin, muscle and kidney (Manery and Bale, 1941). The second is that whereas most tissues exhibited constancy of potassium or rubidium uptake from blood during the first minute following intravenous injection, the brain
(Sapirstein, 1958) and testis (Waites and Setchell, 1966) did not. Subsequently, Setchell et al. (1969) provided direct evidence for the existence of a blood-testis barrier by demonstrating that certain substances infused intravenously either did not enter rete testis fluid or did so at slow rates; all substances tested passed readily into testicular lymph. Additionally, a primary barrier to penetration of the seminiferous tubule lumen by electron-opaque substances such as ferritin, peroxidase and lanthanum, at the level of the peritubular contractile cells, and a secondary, more restrictive barrier at the junctional complexes between Sertoli cells (Fawcett et al., 1970; Dym and Fawcett, 1970) have been demonstrated by means of the electron microscope. These findings confirmed the existence of a blood-testis barrier. However, as noted above, recent studies indicate that rete testis fluid does not correspond exactly to seminiferous tubule fluid in regards to sodium and potassium concentrations, and additionally to protein profiles (Koskimies and Kormano, 1979; Turner et al., 1979). These compositional differences suggest that the blood-testis barrier might not exhibit uniform restrictiveness along the length of the testicular tubules. The finding that urea was partially restricted from entering the seminiferous tubule fluid (Howards et al., 1976) but entered the rete testis fluid without restriction (Setchell et al., 1969) suggests that the blood-semiferous tubule barrier might be more restrictive than the blood-rete testis barrier.

The similarities noted above between cerebrospinal, rete testis and seminiferous tubule fluids and rat proestrus uterine fluid suggested that a permeability barrier between blood and the uterine lumen might exist in rats. The tight junctions of the junctional complexes between
cells in the choroid plexus epithelium (Brightman and Reese, 1969) and in the seminiferous epithelium (Dym and Fawcett, 1970) are considered to function as barriers separating respective luminal compartments from underlying tissues. The epithelial cells lining the uterine lumen are joined by similar junctional complexes (Finn and Porter, 1975). Therefore, in addition to compositional similarities amongst these various fluids, there are also structural similarities amongst the epithelia, further suggesting the possible existence of a blood-uterine lumen barrier.
CHAPTER 2: AIMS AND OBJECTIVES

The aim of this research was to further our understanding of the factors influencing the ability of blood-borne substances to enter the uterine lumen. In accordance with the evidence presented above, it was postulated that one such factor might be the existence of a permeability barrier which restricts the passage of substances from blood into the uterine lumen. The specific aims of the studies presented in this thesis were:

1) to determine whether a blood-uterine lumen permeability barrier exists in rats and to characterise further this barrier by:

2) investigating possible factors which might influence the restrictiveness of this barrier

3) investigating whether this barrier selectively restricts some substances

4) examining the location within the uterus of this barrier.
CHAPTER 3: GENERAL METHODS

Details of experimental designs and procedures as well as collection of samples are given in subsequent chapters. This chapter outlines the general procedures employed.

A permeability barrier does not necessarily completely restrict the passage of substances between two fluids. Instead, it may result in substances entering one fluid from another only at a relatively slow rate (Setchell et al., 1969). Therefore, to investigate the characteristics of a blood-uterine lumen permeability barrier in rats, the abilities of certain radiolabelled test substances to enter the uterine lumen from blood were assessed by comparing the levels of radioactivity in fluid from the uterine lumen with those in serum at several times after intravenous injection of a test substance. The location of the blood-uterine lumen barrier was investigated by employing two approaches. With the first, the distribution of certain test substances within the uterus following intravenous injection of a radiolabelled test substance was assessed by comparing uterine tissue radioactivity concentrations with those in serum. Secondly, the ability of the electron-opaque tracer, lanthanum, to penetrate the uterine epithelial junctional complexes was examined with the electron microscope.

For all experiments, immature, Sprague-Dawley female rats were obtained from either Bio-Breeding Laboratories, Ottawa, Canada or Canadian Breeding Farm and Laboratories, St. Constant, Canada. They were housed under controlled light (14h L:10h D) and temperature (22°C)
conditions with free access to food and water. All animals were ovariectomised under ether anaesthesia by midventral approaches and tight ligatures (5-0 silk) were tied at the utero-tubal junctions to prevent loss of fluid from the uterine lumen. In the experiments described in Chapter 4.2, all animals were treated with hormones prior to and after ovariectomy; the regimen used is given in detail in Chapter 4.2. In the remaining experiments, hormone treatments were initiated several days after ovariectomy and consisted of estradiol (E) or progesterone (P) given in various regimens. E and P were dissolved in sesame oil and given subcutaneously in daily doses of 0.5 μg and 2 mg, respectively. These doses were selected because they are sufficient to induce maximal uterine growth in immature rats (Armstrong, 1968) and to maintain pregnancy in ovariectomised rats when combined with small doses of estrogen (Yochim and Zarrow, 1961), respectively. In certain experiments, E was given by means of Silastic capsule implants (Kennedy and Armstrong, 1975) containing estradiol:cholesterol (3:1, wt/wt) which were inserted subcutaneously.

As described in Chapter 1, only small volumes of uterine fluid are present except in animals treated continuously with E only. Therefore, to facilitate recovery of radioactivity from the uterine lumen and to allow concentrations of radioactivity to be calculated, uterine injections of 15 or 50 μl of phosphate-buffered saline were administered intraluminally to all animals except those treated continuously with E only. Under ether anaesthesia, immediately prior to the intravenous injection of a test substance, the cervical end of one uterine horn was exposed by midline laparotomy and an intraluminal injection of phosphate-buffered saline was given through a 30-gauge needle. Before withdrawing
the needle, a tight ligature was tied just anteriorly to the site of injection to prevent loss of fluid through the cervix (Armstrong, 1968) or the puncture wound.

All β-emitting test substances were obtained from New England Nuclear, Amersham Corp. or ICN Pharmaceuticals. [125I]-Bovine serum albumin was prepared by the chloramine-T method of Greenwood et al. (1963). When [125I]-bovine serum albumin was used, radioactivity in samples was determined with an automatic gamma counting system (Searle Instrumentation). In the experiments using β-emitting substances, the composition of the scintillation fluid added to serum, cisterna chyli lymph and uterine fluid was 0.5% PPO (2,5-diphenyloxazole); 0.05% POPOP (1,4-bis-(5-phenyloxazol-2-yl) benzene) in Triton X-100:toluene, 1:3, v/v. That added to uterine tissue samples consisted of 0.6% PPO and 0.005% POPOP in toluene. Radioactivity was determined with an Isocap liquid scintillation counter (Searle Instruments). For each sample, disintegrations per minute were calculated from counts per minute using appropriate quench correction equations.

The hormones used in the treatment of animals were obtained from the following sources: progesterone and human chorionic gonadotropin from Sigma Chemical Company; estradiol from Steraloids, Inc. and pregnant mare serum gonadotropin as Equinex from Ayerst Laboratories.

The statistical significance of experimental effects was determined by analysis of variance. Where significant interactions were indicated, Duncan's new multiple range tests (Steel and Torrie, 1960) were used for group comparisons between animals and paired t tests were used for within animal comparisons. All data obtained were first tested for heterogeneity of variance by Bartlett's test (Snedecor and Cochran, 1967). Where significant heterogeneity was indicated and eliminated by logarithmic
transformation, further statistical analyses were carried out on logarithmically transformed data. In all experiments, each treatment group consisted of 5 to 7 animals, unless specified otherwise.
CHAPTER 4: EVIDENCE FOR A BLOOD-UTERINE LUMEN BARRIER IN RATS

4.1 IMMATURE, OVARIECTOMISED RATS TREATED WITH ESTRADIOL

Introduction

The existence of a blood-uterine lumen barrier was investigated initially in estradiol-treated rats for two reasons. First, in rats, the uterine fluid which accumulates at proestrus (Long and Evans, 1922) and in ovariectomised or immature animals treated with estrogen (Armstrong, 1968; Conner and Miller, 1973a) differs from plasma in composition having, for example, lower sodium ion (Howard and De Feo, 1959) and protein (Ringler, 1961) concentrations. These concentration differences suggested that the passage of some substances from blood into the uterine lumen of these rats may be restricted by a permeability barrier. Secondly, attempts to recover test substances from the uterine lumen are facilitated by the presence of copious uterine fluid which is secreted (Levin and Edwards, 1968) by the uterus in response to estrogen.

Methods

Animals

At the time of ovariectomy and by midline laparotomy, a ligature was tied at the utero-cervical junction of each animal. To induce uterine fluid accumulation, estradiol (E) implants (Chapter 3) were inserted subcutaneously under light ether anaesthesia approximately 72 h prior to the experiment.
Experimental Design

The effect of time of autopsy after an intravenous injection of one of the five radioactive compounds on the radioactivity concentrations in uterine fluid, lymph and serum was determined. Under light ether anaesthesia, each animal was given a single injection of the test substance via a lateral tail vein. The animals were sacrificed at one of four time intervals post-injection, as indicated in Results. The following radioactive compounds were used: tritiated water (90 μCi/mmole), [14C]-urea (50 mCi/mmole), [3H]-sucrose [fructose-1-3H(N)] (380 mCi/mmole), [3H-(G)]-inulin (1.8 Ci/mmole) and [125I]-bovine serum albumin (approximately 240 Ci/mmole). Radio-labelled urea was chosen because it distributes in both intracellular and extracellular fluids in most tissues (Schloerb, 1960); radio-labelled sucrose and inulin because of their extracellular distribution (Kruhoffer, 1946) and tritiated water as a positive control. Iodinated bovine serum albumin was selected as a relatively high molecular weight test substance. The radioactive solutions were diluted with 0.9% NaCl to a concentration of 10 μCi/ml and each animal was injected with 0.3 ml/100 g body weight.

Collection of Samples

Rats were killed by cervical dislocation and blood collected by cardiac puncture. After the blood was allowed to clot, the serum was separated by centrifugation and a 100 μl aliquot was taken for radioactivity determination. Lymph was obtained from the cisterna chyli (Hebel and Stromberg, 1976) by aspiration using a glass capillary tube drawn to an outer diameter of approximately 200 μ. A 10 μl Hamilton syringe was used to measure the volume of lymph obtained. To collect
uterine fluid, the uterus was removed, stripped of adhering mesentery and blotted on filter paper in an attempt to prevent contamination of uterine fluid by blood. The balloononed horns were punctured and the accumulated fluid drained into preweighed vials which were subsequently reweighed.

Results

\[ ^{14}\text{C}]\text{-urea (mol wt 60)} \]

Analysis of variance of the data obtained when tritiated water was injected (Fig. 1) indicated a significant \((p < 0.05)\) interaction between time of autopsy and site of sampling. This was brought about because whereas the radioactivity concentrations in serum and in lymph did not change with time, those in uterine fluid increased with time. For any given time of autopsy, the radioactivity concentrations did not differ significantly \((p > 0.05)\) between sites of sampling.

\[ ^{3}\text{H}]\text{-urea (mol wt 342)} \]

The data obtained following the intravenous injection of \([^{14}\text{C}]-\text{urea}\) are presented in Fig. 2. Analysis of variance indicated that the only significant \((p < 0.005)\) effect was that of time (linear) indicating that the radioactivity concentrations at all sites sampled were not different for any given time of autopsy and decreased with time in a manner which did not depart significantly \((p > 0.05)\) from linearity.

\[ ^{3}\text{H}]\text{-Sucrose (mol wt 342)} \]

When \([^{3}\text{H}]-\text{Sucrose}\) was injected (Fig. 3), analysis of variance of the radioactivity concentrations in uterine fluid, lymph and serum indicated a significant \((p < 0.025)\) interaction between time of autopsy.
FIGURE 1

Time course for radioactivity concentrations in serum (●), cisterna chyli lymph (■), and uterine fluid (dpm/mg) (▲) after intravenous injection of tritiated water into ovariectomised, E-implanted rats (n = 5). In this and all succeeding figures, each point represents mean ± SEM for a specified number of animals and where no error bars are shown, the SEM falls within the symbol.
FIGURE 2

Time course for radioactivity concentrations in serum (●), cisterna chyli lymph (■), and uterine fluid (dpm/mg) (▲) after intravenous injection of [14C]-urea into ovariectomized, E-implanted rats (n = 5).
and site of sampling. The interaction was brought about because although the radioactivity concentrations at all sites sampled changed significantly \((p < 0.001)\) with time, they did so at different rates. The radioactivity concentrations at all sites were significantly different for any given time of autopsy except at 60 min at which time the concentrations in lymph and serum were not different and at 120 min at which time the concentrations at all sites sampled were not different. Calculation of confidence intervals from means and standard deviations of uterine fluid radioactivity concentrations indicated that these radioactivity concentrations were not significantly \((p > 0.05)\) different from zero for any given time of autopsy.

\(^{3}H\)-inulin (mol wt approx. 5,200)

Analysis of variance of the data obtained when \(^{3}H\)-inulin was injected (Fig. 4) indicated a significant \((p < 0.001)\) interaction between time of autopsy and site of sampling brought about because whereas the radioactivity concentrations in uterine fluid did not change with time, those in serum and lymph decreased with time. The radioactivity concentrations in uterine fluid were different from those in serum at any given time of autopsy except at 120 min, and the levels in lymph differed from those in serum at 60 and 120 min. Calculation of confidence intervals from means and standard deviations of uterine fluid radioactivity concentrations indicated that the radioactivity concentrations in uterine fluid differed significantly \((p < 0.05)\) from zero at 30 and 120 min.
FIGURE 3

Time course for radioactivity concentrations in serum (●), cisterna chyli lymph (■), and uterine fluid (dpm/mg) (▲) after intravenous injection of [3H]-sucrose into ovariectomised, E-implanted rats (n = 5).
FIGURE 4

Time course for radioactivity concentrations in serum (●), cisterna chyli lymph (■), and uterine fluid (dpm/mg) (▲) after intravenous injection of [3H]-inulin into ovariectomised, E-implanted rats (n = 5).
[125I]-Bovine Serum Albumin (mol wt approx. 60,000)

The data obtained when [125I]-bovine serum albumin was injected are presented in Fig. 5. Analysis of variance of the radioactivity concentrations in uterine fluid, lymph and serum indicated a significant (p < 0.001) interaction between time of autopsy and site of sampling. The interaction was brought about because the radioactivity concentrations in serum decreased with time; those in lymph increased initially, followed by a decrease, and those in uterine fluid increased. The radioactivity concentrations at all sites of sampling differed at any given time of autopsy.

Discussion

The results of the experiments using [3H]-sucrose, [3H]-inulin and [125I]-bovine serum albumin provide evidence for a blood-uterine lumen barrier in rats because in each case the levels of radioactivity in the uterine fluid were significantly lower than those in serum. By contrast, tritiated water and [14C]-urea entered the uterine fluid without restriction. In addition, the levels of radioactivity in the uterine fluid either did not differ from zero ([3H]-sucrose) or did not change with time ([3H]-inulin), further suggesting a permeability barrier. With [125I]-bovine serum albumin, the times of autopsy were extended and, although increases in the uterine fluid radioactivity concentrations with time were observed, the levels were still lower than those in serum 8 h after injection, again suggesting a permeability barrier.

When [3H]-sucrose and [3H]-inulin were injected, the concentrations of radioactivity in serum rapidly decreased and approached the
FIGURE 5

Time course for radioactivity concentrations in serum (●), cisterna chyli lymph (■), and uterine fluid (cpm/mg) (▲) after intravenous injection of $^{125}$I- bovine serum albumin into ovariectomised, E-implanted rats (n = 5).
uterine fluid levels, presumably due to renal clearance. If constant serum radioactivity concentrations had been maintained (either by constant infusion or perhaps nephrectomy), the levels of radioactivity in uterine luminal fluid may have increased until equilibration between serum and uterine fluid radioactivity concentrations was achieved. However, this presumed requirement of maintenance of constant serum levels over time for equilibration to be achieved in itself suggests the existence of a permeability barrier.

Following injection of tritiated water, the uterine fluid radioactivity concentrations were significantly higher at 80 min than at 10 min. This difference might represent increased uterine fluid radioactivity concentrations occurring over time as uterine fluid radioactivity concentrations approached those in serum. However, the absence of significant differences between serum and uterine fluid radioactivity concentrations at all times post-injection examined suggests that equilibration was achieved before the earliest time studied (10 min). The statistically significant difference in uterine fluid radioactivity concentrations between 10 and 80 min post-injection might have resulted from sampling error.

The results of the present experiments suggest that the blood-uterine barrier might be selective according to the molecular weight of substances present in the blood. The lower molecular weight substances (tritiated water and $[^{14}C]$-urea) entered the uterine fluid without restriction, whereas entry of the higher molecular weight substances ($[^{3}H]$-sucrose, $[^{3}H]$-inulin and $[^{125}I]$-bovine serum albumin) was slower, presumably because of the permeability barrier.
The restricted passage of some substances from blood into the uterine lumen might result from relative impermeability of uterine capillaries, the uterine endometrial stroma, the endometrial epithelium, or to an unstirred water layer adjacent to the luminal surface of the epithelium. Measurement of the radioactivity concentrations in uterine lymph in the present experiments would have indicated whether the radioactive substances used were able to escape from the uterine vascular system. As it was not technically feasible to collect uterine lymph directly, radioactivity concentrations in lymph obtained from the cisterna chyli were determined. Lymph present in this reservoir is formed in the organs of the abdominal cavity including uterus, intestine and liver (Hebel and Stromberg, 1976). When $[^3\text{H}]-\text{sucrose},[^3\text{H}]-\text{inulin}$ and $[^{125}\text{I}]-\text{bovine serum albumin}$ were injected, the radioactivity concentrations in lymph were significantly lower than those in serum, indicating that at least some capillary beds were relatively impermeable to these substances. However, whether these include the uterine capillaries could not be determined from the present experiments.

In the rat, the endometrial stromal cells normally do not make structural contact (Finn and Porter, 1975); therefore, it seems unlikely that the stroma restricts the passage of substances between blood and uterine fluid. In contrast, the epithelial cells lining the uterine lumen are joined at their apical portions by junctional complexes consisting of zonulae occludens, zonulae adherens and maculae adherens (Finn and Porter, 1975). The zonula occludens (tight junction) is common to the epithelium of many cavity organs and is thought to function as a seal between cells, thus isolating the underlying tissue from the lumen. This seal varies in its ability to restrict the passage
of substances; in some epithelia only large molecules such as hemoglobin are restricted from passing through the zonula occludens, whereas in others even water and small ions are partially restricted (Staehelin, 1974). The epithelium of the uterus is suited, by virtue of the zonula occludens, to function as a permeability barrier. It is likely that the radioactivity concentrations measured in uterine fluid represented primarily those in the bulk luminal water phase rather than in an unstirred water layer. Therefore, the contribution of this layer to the total resistance encountered by some substances is unknown in the present study. However, it is unlikely that the restricted entries into the uterine lumen by \(^{3}H\)-sucrose and \(^{3}H\)-inulin are due entirely to the unstirred water layer; this layer contributes negligibly to the permeability to these substances of other epithelia (Smutthers and Wright, 1974).

4.2 IMMATURE RATS TREATED WITH HORMONES TO MIMIC EARLY PSEUDOPREGNANCY

Introduction

A permeability barrier which restricts the passage of certain substances from blood into the uterine lumen has been demonstrated to exist in immature, ovariectomised rats treated with estradiol. This treatment simulates the hormonal environment to which the uterus is exposed during early proestrus of the estrous cycle. Since it was not known whether a permeability barrier exists between blood and the uterine lumen during other reproductive conditions, the existence of such a barrier was investigated under conditions which simulate a time other than early proestrus.
A blood-uterine lumen barrier may function to maintain a special environment within the uterine lumen. Setchell (1980) has attributed such a role to the blood-testis barrier in relation to the lumen of the seminiferous tubule. A requirement of a special intrauterine environment for normal embryonic development during the peri-implantation period of gestation is suggested by the observations that development beyond the blastocyst stage does not occur in ectopically-transplanted embryos (Fawcett, 1950; Kirby, 1962). One mechanism through which a special environment could be maintained in the uterine lumen during this period is by the presence of a blood-uterine lumen barrier. Therefore, experiments have been performed to investigate whether such a barrier exists in rats treated with hormones to mimic the implantation period. In rats, developing embryos enter the uterus as morulae early on Day 4 (Day 1 = presence of spermatozoa in vaginal smear) and transform into blastocysts shortly thereafter (Brinster, 1973). By late afternoon or early evening of Day 5, the free-lying blastocysts have lost their zonae pellucidae and have begun to adhere to the uterine wall (Surani, 1975b; Enders and Schlafke, 1967). At this time, blastocysts can be flushed from the uterine lumen without damaging them. However, by the following day, the blastocysts are firmly attached and attempts to flush them from the uterus result in low recovery rates of undamaged blastocysts (Enders and Schlafke, 1967). Thus, the implantation period in rats can be considered to occur between Days 4 and 6 of pregnancy.
Methods

Animals

All animals were treated hormonally so that they were the equivalent of Days 4, 5 or 6 of pseudopregnancy (Day 1 = day of induced ovulation) on the days the experiments were performed. A hormone regimen similar to that described by Armstrong and King (1971) was used to achieve this. On 3 consecutive days, groups of animals received subcutaneous injections of 5 I.U. of pregnant mare serum gonadotropin followed approximately 56 h later by intraperitoneal injections of 7.5 I.U. human chorionic gonadotropin to ensure the occurrence of ovulation. On the morning after ovulation (equivalent of Day 2 of pseudopregnancy), the rats were ovariectomised under ether anaesthesia and given 2 mg progesterone in 0.2 ml sesame oil. Thereafter, until sacrifice, the animals received daily subcutaneous injections of 0.5 mg estrone and 2 mg progesterone. This hormone regimen mimics appropriately the endocrine milieu of early pregnancy, since the decidual cell response of these hormone treated animals to a deciduogenic stimulus exhibited a biphasic pattern with maximal sensitivity on Day 5 (Kennedy, 1980), which was similar to that of pseudopregnant animals (De Feo, 1963).

Experimental Design and Procedure

The effect of time of autopsy after an intravenous injection of one of three radiolabelled test substances on the levels of radioactivity in fluid from the uterine lumen, uterine tissue (except when tritiated water was injected) and serum was determined in rats at the equivalent of Day 4, 5 or 6 of pseudopregnancy. Between 10:00 and 14:00 h and under ether anaesthesia, each animal received a single injection of the
test substance via a lateral tail vein. Immediately prior to this injection, an intraluminal injection of 50 µl of phosphate-buffered saline (0.01 M phosphate in 0.054 M NaCl, pH 7.4, osmolality 300 mOsm/kg) warmed to 37°C was given into one uterine horn. Details of the intrauterine injection procedure are given elsewhere (Chapter 3). The following radioactive compounds were used: tritiated water (18 µCi/mmol), [14C]-urea (53.5 mCi/mmol) and [3H]-sucrose ([3H]-fructose-1-3H(N)) [380 mCi/mmol]. Each compound was diluted with 0.9% NaCl to a concentration of 10 µCi/ml and each animal was injected with 0.3 ml/100 g body weight.

Collection of Samples

Each animal was killed by decapitation and blood collected from the severed trunk vessels. Duplicate, 100 µl aliquots of serum were taken for radioactivity determinations. The uterus was removed, stripped of adhering mesentery, rinsed in 0.9% NaCl and blotted on filter paper in an attempt to prevent contamination of uterine fluid by blood. Except when tritiated water was the test substance, the uterine fluid was expressed onto a small, preweighed piece of filter paper which was immediately reweighed and placed into a test tube. After the water was allowed to evaporate, the radioactivity was eluted from the filter paper by vigorous mixing with 2 x 5 ml methanol. The methanol was decanted into a scintillation vial and evaporated to dryness. Radioactivity recovery from the filter paper was determined for each experiment and was greater than 90%. Uterine fluid radioactivity data were corrected for this procedural loss. A uterine tissue sample taken from the same horn as the fluid was weighed and placed into a scintillation vial. To solubilize this tissue, 400 µl of NCS reagent
(Amersham Corp.) were added.

When tritiated water was the test substance, the uterine fluid was drawn into a glass capillary tube. This fluid was transferred to a preweighed scintillation vial containing 450 μl of water which was then reweighed.

Results

[^14C]-Urea

The data obtained when[^14C]-urea was injected are presented in Table 1. Serum radioactivity concentrations decreased significantly \( (p < 0.01) \) with time in a manner which did not differ significantly \( (p > 0.05) \) between the equivalent of Days 4, 5 or 6 of pseudopregnancy. There was no significant \( (p > 0.05) \) effect of time of autopsy on uterine fluid radioactivity concentrations. Furthermore, uterine fluid radioactivity concentrations were significantly \( (p < 0.01) \) higher on Day 6 than on Day 4. In all groups of animals, except those killed on Day 6, the uterine fluid radioactivity concentrations were significantly \( (p < 0.01) \) lower than those in serum. Uterine tissue radioactivity concentrations did not differ significantly \( (p > 0.05) \) from those in serum in any group and, in addition, decreased significantly \( (p < 0.01) \) with time in a manner which did not vary significantly \( (p > 0.05) \) between the equivalent of Days 4, 5 or 6 of pseudopregnancy.

[^3H]-Sucrose

When[^3H]-sucrose was injected (Table 2), both serum and uterine tissue radioactivity concentrations decreased significantly \( (p < 0.01) \) with time but at different rates. By contrast, uterine fluid radioactivity...
<table>
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<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
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<tbody>
<tr>
<td></td>
<td>20 min</td>
<td>60 min</td>
<td>20 min</td>
</tr>
<tr>
<td>serum&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>85.6 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.8 ± 1.9</td>
<td>88.2 ± 1.0</td>
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<tr>
<td>uterine tissue</td>
<td>76.9 ± 2.5</td>
<td>56.1 ± 1.9</td>
<td>79.0 ± 1.4</td>
</tr>
<tr>
<td>uterine fluid</td>
<td>32.1 ± 2.7*</td>
<td>39.2 ± 3.6*</td>
<td>37.3 ± 2.1*</td>
</tr>
</tbody>
</table>

<sup>a</sup> dpm/μl

<sup>b</sup> Mean ± SEM, 6 animals per group

<sup>c</sup> significantly (p < 0.01) different between 20 and 60 min post-injection

<sup>d</sup> significantly (p < 0.01) different between Day 4 and Day 6

<sup>*</sup> significantly (p < 0.05) different from serum radioactivity concentrations
concentrations did not change significantly \( p > 0.05 \) with time. Both uterine tissue and uterine fluid radioactivity concentrations were significantly \( p < 0.05 \) higher on the equivalent of Day 6 of pseudopregnancy than on Day 4, although serum concentrations did not vary significantly \( p > 0.05 \) between Days 4, 5 or 6. Uterine fluid radioactivity concentrations were significantly \( p < 0.01 \) less than those in serum in all groups of animals except those killed on Day 6 at 60 min post-injection. Uterine tissue radioactivity concentrations were significantly \( p < 0.05 \) less than serum radioactivity concentrations in all groups except those killed on Day 5 or 6 at 60 min post-injection.

**Tritiated Water**

Nested analysis of variance of the data obtained, following intravenous injection of tritiated water, indicated that the concentrations of radioactivity in uterine fluid were significantly \( p < 0.01 \) greater than those in serum in all groups of animals (Table 3). In addition, both serum and uterine fluid radioactivity concentrations were significantly \( p < 0.01 \) higher on the equivalent of Day 6 of pseudopregnancy than on Day 4.

**Discussion**

A blood-uterine lumen barrier apparently exists in immature rats treated with hormones to mimic the endocrine conditions of early pseudopregnancy because both \( ^{14}\text{C} \)-urea and \( ^{3}\text{H} \)-sucrose were at least partially restricted from the uterine lumen. It is evident that these substances did not enter readily the uterine lumen from blood since following intravenous injection of either, the uterine fluid radioactivity concentrations did not change significantly with time of
TABLE 2: EFFECTS OF THE EQUIVALENT OF DAY OF PSEUDOPREGNANCY AND TIME OF AUTOPSY POST-INJECTION OF $[^{3}H]$-SUCROSE ON RADIOACTIVITY CONCENTRATIONS (dpm/mg)

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<th>Day 4</th>
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<td>60 min</td>
<td>20 min</td>
<td>60 min</td>
<td>20 min</td>
<td>60 min</td>
</tr>
<tr>
<td>serum</td>
<td>117.2 ± 3.2$^b$</td>
<td>21.5 ± 1.7</td>
<td>113.7 ± 3.4</td>
<td>21.2 ± 2.3</td>
<td>109.1 ± 4.0</td>
<td>20.0 ± 1.7</td>
</tr>
<tr>
<td>uterine tissue</td>
<td>61.6 ± 2.0$^*$</td>
<td>15.2 ± 1.5$^*$</td>
<td>61.4 ± 4.1$^*$</td>
<td>17.4 ± 1.2</td>
<td>65.3 ± 2.4$^*$</td>
<td>20.3 ± 1.6</td>
</tr>
<tr>
<td>uterine fluid</td>
<td>6.2 ± 0.7$^*$</td>
<td>10.3 ± 2.2$^*$</td>
<td>13.1 ± 3.5$^*$</td>
<td>9.3 ± 1.0$^*$</td>
<td>20.6 ± 3.9$^*$</td>
<td>19.9 ± 3.9</td>
</tr>
</tbody>
</table>

$^a$ dpm/μl

$^b$ Mean ± SEM, 6 animals per group

$^c$ significantly ($p < 0.01$) different between 20 and 60 min post-injection

$^d$ significantly ($p < 0.01$) different between Day 4 and Day 6

$^*$ significantly ($p < 0.05$) different from serum radioactivity concentrations
<table>
<thead>
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<th>Day 4</th>
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<th>Day 5</th>
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<td>20 min</td>
<td>60 min</td>
</tr>
<tr>
<td>serum&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>93.2 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>89.7 ± 2.0</td>
<td>95.8 ± 0.5</td>
<td>97.9 ± 1.2</td>
<td>97.7 ± 1.6</td>
<td>100.5 ± 1.6</td>
</tr>
<tr>
<td>uterine&lt;sup&gt;c,d&lt;/sup&gt; fluid</td>
<td>93.8 ± 1.6</td>
<td>92.9 ± 1.3</td>
<td>97.0 ± 1.0</td>
<td>98.5 ± 0.7</td>
<td>102.3 ± 1.4</td>
<td>104.3 ± 2.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> dpm/µl
<sup>b</sup> Mean ± SEM, 6 animals per group
<sup>c</sup> significantly (p < 0.01) different between Day 4 and 6
<sup>d</sup> significantly (p < .01) different from serum radioactivity concentrations
autopsy on any equivalent day of pseudopregnancy examined. Furthermore, these radioactivity concentrations were significantly less than those in serum at both autopsy times on Days 4 and 5. On Day 6, after intravenous injection of $^{14}\text{C}$-urea, the uterine fluid radioactivity concentrations did not differ significantly from those in serum; however, with $^{3}\text{H}$-sucrose, they were significantly less than those in serum at 20 min post-injection. By contrast, following intravenous injection of tritiated water, the uterine fluid radioactivity concentrations were significantly higher than those in serum in all groups, suggesting that this substance entered the uterine lumen without restriction. It was determined experimentally that the concentration of radioactivity in serum water was 6.5% greater relative to that in whole serum. Analysis of variance of serum water and uterine fluid radioactivity concentrations indicated that the former were slightly but significantly ($p < 0.01$) greater than the latter (data not shown).

This finding of partial restriction to $^{14}\text{C}$-urea was unexpected since urea is a small, highly permeant molecule which entered the uterine lumen in E-implanted rats apparently without restriction. However, permeability barriers to urea exist between blood and seminiferous tubule fluid (Howards et al., 1976) and cerebrospinal fluid (Johanson and Woodbury, 1978). The restricted entry of $^{14}\text{C}$-urea observed in the present experiments indicates that under the endocrine conditions of early pseudopregnancy, the blood-uterine lumen barrier is more restrictive than it is under some other conditions, e.g., three days of estradiol treatment.
In this study, uterine tissue radioactivity concentrations were determined in an attempt to ascertain the location within the uterus of the barrier to penetration into the uterine lumen. Since the lumina of the uterine horns were not flushed with a solution prior to sample collection, the possibility that the uterine tissue radioactivity concentrations were overestimated due to adsorption of radioactivity to the luminal surfaces cannot be eliminated. No attempt was made to account for the amount of radioactivity retained in the uterine vasculature following decapitation of the animal; it was known that uterine blood volume in mature, ovariectomised rats treated acutely with E had been determined to be 0.05 μl/mg uterine tissue (Cole, 1950). Therefore, it was considered that only 5%, at most, of the uterine tissue radioactivity would have been due to that present in residual blood. Following intravenous injection of [14C]-urea, the uterine tissue radioactivity concentrations did not differ significantly from those in serum under any test condition, suggesting that the majority of the uterine tissue was located on the blood side of the permeability barrier. This observation, combined with the fact that the epithelium comprises only 5-10% of the uterus (Finn and Porter, 1975), suggests that the restricted entry of [14C]-urea into the uterine lumen was due to relative impermeability of the uterine epithelium. By contrast to [14C]-urea, following injection of [3H]-sucrose, the uterine tissue radioactivity concentrations were significantly lower than those in serum at 20 min post-injection on all days tested and at 60 min on the equivalent of Day 4 of pseudopregnancy. This disparity between
serum and uterine tissue radioactivity concentrations might suggest that the uterine capillaries and/or stroma were relatively impermeable to this substance. However, on the assumption that the extracellular fluid compartment in which sucrose distributes comprises at most 60% of the uterine tissue mass (NoALL and Allen, 1961; Spaziani and Suddick, 1967), the extracellular fluid radioactivity concentrations were approximately equal to or greater than serum radioactivity concentrations in all groups. Furthermore, Parr (1980) observed in Day 5 pregnant rats injected intravenously with horseradish peroxidase that by 20 min post-injection, peroxidase-reaction product was localised in the extracellular spaces between stromal and epithelial cells up to, but not beyond the zonula occludens of the latter. Since $[^3\text{H}]$-sucrose is a much smaller molecule than horseradish peroxidase, it is likely that $[^3\text{H}]$-sucrose was able to permeate throughout the extracellular spaces of the stroma and between the epithelial cells, and if so, that the entry of $[^3\text{H}]$-sucrose into the uterine lumen was restricted by epithelial zonulae occludens. Thus, it is likely that the restricted entries into the uterine lumen of both test substances were due to relative impermeability of the epithelial layer of cells.

Following intravenous injection of either $[^{14}\text{C}]$-urea or $[^3\text{H}]$-sucrose, the uterine fluid radioactivity concentrations were significantly higher on the equivalent of Day 6 than Day 4 of pseudopregnancy suggesting that the ability of the epithelium to restrict the passage of some substances from blood into the uterine lumen decreases during this period. Because sucrose distributes primarily extracellularly, one explanation for the increased entry of this substance is that paracellular solute permeation through the epithelium was increased.
Enhanced paracellular permeability would also account for the increased entry of $^{14}$C-urea which was observed since it distributes partly in the extracellular fluid. The physiological significance of decreased barrier restrictiveness during the equivalent of the implantation period is unknown. However, it is possible that the increased permeability to $^{3}$H-sucrose and $^{14}$C-urea was the result of damage inflicted upon the uterus by the introduction of fluid into the uterine lumen which is normally collapsed (O'Shea, 1972). The damage may have increased in severity between the equivalent of Day 4 and 6 of pseudopregnancy since during this period opposing luminal surfaces become closely apposed (uterine closure)(Finn and Porter, 1975).
CHAPTER 5: EFFECTS OF ESTRADIOL AND PROGESTERONE ON THE RESTRICTIVENESS OF THE BLOOD-UTERINE LUMEN BARRIER

Introduction

The sodium ion and protein concentration differences between plasma and estrogen-induced uterine fluid described earlier (Chapter 1) suggested that there may be a blood-uterine lumen barrier, the existence of which was subsequently demonstrated (Chapter 4). The quantity of uterine fluid present throughout the estrous cycle excluding proestrus (Warren, 1938), or in estrogen-treated rats receiving progesterone concomitantly or sequentially (Armstrong, 1968; Kennedy and Armstrong, 1975) is so small that accurate determinations of various fluid constituents have not been made. Thus, it was not known whether the permeability of the blood-uterine lumen barrier is similar under these conditions relative to those in estradiol-treated animals and in animals treated hormonally to mimic early pseudopregnancy. However, the results obtained from rats treated to the equivalent of early pseudopregnancy suggested the possibility that the restrictiveness of the barrier may be subject to hormonal modulation.

The present experiments were designed to investigate whether the restrictiveness of a blood-uterine lumen barrier in rats is influenced by the endocrine status of the animal. The effects of estradiol (E) and progesterone (P) treatments on the abilities of certain radioactively-labelled test substances to enter the uterine lumen from blood were determined.
Methods

Animals

All animals were ovariectomised as described previously. Beginning two days after ovariectomy, all rats received daily hormone injections for three days. The experiments were performed on the fourth day.

Experimental Procedure

The ability of each test substance to enter the uterine lumen from blood was assessed by comparing the levels of radioactivity in fluid from the uterine lumen with those in serum following intravenous injection of the radiolabelled test substance. This injection was given via a lateral tail vein. Additionally, the levels of radioactivity in uterine tissue and serum were compared, except when tritiated water was the test substance. Under ether anaesthesia, immediately prior to this intravenous injection, the cervical end of one uterine horn was exposed and, to all animals except those treated continuously with E only, an intraluminal injection of 15 µl of saline (see below) was given into one uterine horn. This volume was chosen (in contrast to 50 µl as in the experiments described above) because any attempt to instill a larger volume into the uteri of ovariectomised animals untreated or treated with P only, resulted in rupture of the uterine horn and loss of fluid. For animals treated with E only, a tight ligature was tied around one horn just anteriorly to the utero-cervical junction. Details of the intruterine injection procedure were given in Chapter 3. The solution injected intraluminally (warmed to 37°C) was 0.9% NaCl titrated to pH 7.4 with 0.01 M Na₂HPO₄ and was approximately 275 mOsm/kg.
The following radioactive compounds were used: tritiated water (18 μCi/mmole), [¹⁴C]-urea (8.6 mCi/mmole) and [³H]-sucrose [fructose-1-³H(N)] (380 mCi/mmole). Each compound was diluted with 0.9% NaCl to a concentration of 10 μCi/ml and each animal was injected with 0.3 ml/100 g body weight.

Experimental Design

Each test substance was tested in two, 2 x 2 x 2 factorial experiments. In the first series, the factors were as follows:

Factor 1: time of autopsy: 15 vs 60 min
Factor 2: estradiol treatment: none vs 0.5 μg/day for three days
Factor 3: progesterone treatment: none vs 2 mg/day for three days.

The factors in the second series were as follows:

Factor 1: time of autopsy: 15 vs 60 min
Factor 2: estradiol pretreatment: none vs 0.5 μg/day for two days
Factor 3: progesterone treatment: none vs 2 mg for one day.

This additional hormone regimen was investigated because P normally acts upon an estrogen-primed uterus (Steinetz, 1973).

Collection of Samples

Details of these procedures have been described previously (Chapter 4). In brief, each animal was killed by decapitation and trunk blood collected for radioactivity determinations. The uterus was
removed, cleaned of extraneous tissue and, except when tritiated water was the test substance, the uterine fluid, injected or secreted (one horn only), was expressed onto a small, preweighed piece of filter paper which was immediately reweighed. The methanolic elution used to recover radioactivity from this filter paper resulted in recoveries greater than 90%. Recovery was determined for each experiment and uterine fluid radioactivity data were corrected for this procedural loss. A uterine sample taken from the same horn as the fluid was weighed and solubilized with 400 μl of NCS reagent (Amersham Corp.). When tritiated water was the test substance, uterine fluid, injected or secreted (one horn only), was drawn into a glass capillary tube and transferred to a preweighed vial which was then reweighed.

Results

Effects of E and P Treatments

[14C]-Urea

The data obtained when [14C]-urea was injected intravenously into rats treated for three days with or without E and P and killed 15 or 60 min post-injection are presented in Fig. 6. Serum radioactivity concentrations decreased significantly (p < 0.05) with time, but they did so at slightly but significantly [p < 0.05] slower rates in P-treated animals. In those animals receiving E, the uterine fluid radioactivity concentrations either did not differ significantly (p > 0.05) from those of serum (E only—at 15 min) or were significantly (p < 0.01) higher. In addition, for both serum and uterine fluid, the decreases
in the radioactivity concentrations which occurred between 15 min and 60 min were similar (p > 0.05).

By contrast, in the absence of E, the uterine fluid radioactivity concentrations were significantly (p < 0.01) less than those in serum at 15 min post-injection and at 60-min in P only-treated animals. Additionally, the uterine fluid radioactivity concentrations increased with time, in contrast to the decreases observed to occur in serum radioactivity concentrations. For uterine fluid radioactivity concentrations, there was a significant (p < 0.01) interaction between E and P treatments; P alone resulted in lower concentrations, E alone in higher, and combined E plus P in even higher concentrations.

Uterine tissue radioactivity concentrations decreased significantly (p < 0.01) with time in a manner which did not vary significantly (p > 0.05) between hormone treatments. Serum and uterine tissue radioactivity concentrations differed significantly (p < 0.05) only in E plus P-treated animals, where serum levels were slightly higher.

[^3H]-Sucrose

Fig. 7 summarises the data obtained when[^3H]-sucrose was injected intravenously into rats treated for three days with or without E and P and killed 15 or 60 min post-injection. Serum radioactivity concentrations decreased significantly (p < 0.01) with time in a manner which did not vary significantly (p > 0.05) between hormone treatments. By contrast, there was no significant (p > 0.05) effect of time on uterine fluid radioactivity concentrations. In addition, uterine fluid radioactivity concentrations were significantly (p < 0.01) lower than serum concentrations with all treatments at both times of autopsy except for
FIGURE 6

Effects of estradiol and progesterone treatments on radioactivity concentrations in serum (dpm/μl) (●), uterine tissue (■), and uterine fluid (▲) at two autopsy times after intravenous injection of [14C]-urea. Immature, ovariectomised rats were treated with vehicle (SO), progesterone (P), estradiol (E) or progesterone plus estradiol (P + E), for three days and sacrificed on the fourth day (n = 7).
vehicle and P only-treated animals killed at 60 min post-injection. Furthermore, there was a significant \((p < 0.05)\) interaction between E and P treatments. This interaction was brought about because treatment with E alone resulted in lower uterine fluid radioactivity concentrations, P alone had no effect but with combined treatment, P partially reversed the estradiol effect.

Uterine tissue radioactivity concentrations decreased significantly \((p < 0.01)\) with time in a manner which did not vary significantly \((p > 0.05)\) between hormone treatments and were significantly \((p < 0.001)\) lower than serum concentrations with all treatments at 15 min post-injection and in P-treated animals at 60 min. Additionally, the uterine tissue radioactivity concentrations were lower at each time of autopsy in animals receiving E.

Tritiated Water

When tritiated water was injected intravenously into rats treated for three days with or without E and P and killed 15 or 60 min post-injection, the concentrations of uterine fluid radioactivity were significantly \((p < 0.05)\) higher than those of serum in all groups at both times of autopsy (Fig. 8). Additionally, uterine fluid radioactivity concentrations were significantly \((p < 0.01)\) greater at each time of autopsy in animals receiving P. Furthermore, with all hormone treatments tested, the uterine fluid radioactivity concentrations were significantly \((p < 0.05)\) higher at 60 min than at 15 min.
FIGURE 7

Effects of estradiol and progesterone treatment on radioactivity concentrations in serum (dpm/µl) (○), uterine tissue (■), and uterine fluid (▲) at two autopsy times after intravenous injection of [3H]-sucrose. Immature, ovariectomised rats were treated with vehicle (SO), progesterone (P), estradiol (E) or progesterone plus estradiol (P + E) for three days and sacrificed on the fourth day (n = 7).
FIGURE 8

Effects of estradiol and progesterone treatments on radioactivity concentrations in serum (dpm/μl) (●) and uterine fluid (▲) at two autopsy times after intravenous injection of tritiated water. Immature, ovariectomised rats were treated with vehicle (SO), progesterone (P), estradiol (E) or progesterone plus estradiol (P + E) for three days and sacrificed on the fourth day (n = 6).
Effects of P Treatment, With and Without E Pretreatment

$[^{14}\text{C}]-\text{Urea}$

The results obtained when $[^{14}\text{C}]-\text{urea}$ was injected intravenously into rats pretreated for two days with or without E, treated for one day with or without P, and killed 15 or 60 min post-injection are summarized in Fig. 9. Serum radioactivity concentrations decreased significantly ($p < 0.01$) with time in a manner which did not vary significantly ($p > 0.05$) between hormone treatments. By contrast, the uterine-fluid radioactivity concentrations were higher at 60 min than at 15 min for all treatments. In addition, uterine fluid radioactivity concentrations were significantly ($p < 0.05$) lower than those of serum in all groups at 15 min and in P-treated animals at 60 min. For uterine fluid radioactivity concentrations, there was a significant ($p < 0.01$) interaction between E pretreatment and P treatment; P treatment resulted in lower concentrations, E pretreatment alone had no effect, but E pretreatment partially reversed the P treatment effect.

Uterine tissue radioactivity concentrations decreased significantly ($p < 0.01$) with time in a manner which did not vary significantly ($p > 0.05$) between hormone treatments and were significantly ($p < 0.05$) lower than serum concentrations in all groups except vehicle-only-treated animals killed 60 min post-injection. These differences between uterine tissue and serum concentrations contrast with the results obtained in the preceding $[^{14}\text{C}]-\text{urea}$ experiment perhaps because in the present experiment, the uterine tissue samples were not completely solubilized prior to radioactivity determinations.
FIGURE 9

Effects of progesterone treatment with or without estradiol pre-treatment on radioactivity concentrations in serum (dpm/μl) (●), uterine tissue (■) and uterine fluid (▲) at two autopsy times after intravenous injection of [14C]-urea. Immature, ovariectomised rats pre-treated for two days with vehicle (S0) or estradiol (E) were treated on the following day with vehicle (S0) or progesterone (P) and sacrificed on the fourth day (n = 7).
[\textsuperscript{3}H]-Sucrose

Fig. 10 summarises the results obtained when [\textsuperscript{3}H]-sucrose was injected intravenously into rats pretreated for two days with or without E, treated for one day with or without P, and killed 15 or 60 min post-injection. Serum radioactivity concentrations decreased significantly (p < 0.01) with time in a manner which did not vary significantly (p > 0.05) between hormone treatments. By contrast, the uterine fluid radioactivity concentrations at 15 min post-injection were similar to those at 60 min in animals receiving any given hormone treatment. In addition, uterine fluid radioactivity concentrations were significantly (p < 0.01) lower than those of serum in all groups killed at 15 min post-injection. For uterine fluid radioactivity concentrations, there was a significant (p < 0.05) three-way interaction between E pretreatment, P treatment and time of autopsy; although E pretreatment resulted in lower concentrations, this effect of pretreatment with estradiol depended upon time of autopsy and upon whether P treatment followed E pretreatment.

Uterine tissue radioactivity concentrations decreased significantly (p < 0.01) with time in a manner which did not vary significantly (p > 0.05) between hormone treatments. There were significant (p < 0.01) differences between serum and uterine tissue radioactivity concentrations in all groups at 15 min, and in vehicle only-treated animals at 60 min. Uterine tissue concentrations were lower at each time of autopsy in those animals pretreated with E.
FIGURE 10

Effects of progesterone treatment with or without estradiol pretreatment on radioactivity concentrations in serum (dpm/μl) (●), uterine tissue (■), and uterine fluid (▲) at two autopsy times after intravenous injection of [3H]-sucrose. Immature, ovariectomised rats pretreated for two days with vehicle (SO) or estradiol (E) were treated on the following day with vehicle (SO) or progesterone (P) and sacrificed on the fourth day (n = 5).
Tritiated Water

When tritiated water was injected intravenously into rats pre-treated for two days with or without E, treated for one day with or without P; and killed 15 or 60 min post-injection, the concentrations of uterine fluid radioactivity were higher than those in serum in all groups at both times of autopsy (Fig. 11).

Discussion

The results of the present experiments provide further evidence for the existence of a blood-uterine lumen barrier because $^{3}H$-sucrose was essentially restricted from the uterine lumen in ovariectomised rats treated with various regimens of E and P. It is evident that $^{3}H$-sucrose did not enter the uterine lumen readily in these animals since the uterine fluid radioactivity concentrations did not change significantly with time and were significantly lower than serum radioactivity concentrations at 15 min post-injection. However, the restrictiveness of this barrier is apparently hormonally regulated since the permeability to $^{3}H$-sucrose was decreased further by E, given either as treatment or pretreatment, as indicated by the lower uterine fluid radioactivity from animals receiving this hormone compared with those receiving only vehicle (Figs. 7 and 10). This conclusion is further substantiated by the results obtained with $^{14}C$-urea. $^{14}C$-Urea entered the uterine lumen apparently without restriction in animals treated continuously with E since uterine fluid and serum concentrations of radioactivity decreased with time in manners which did not vary significantly from each other and the radioactivity concentrations in uterine fluid were either not significantly different from or were significantly higher than those of serum.
FIGURE 11

Effects of progesterone treatment with or without estradiol pre-treatment on radioactivity concentrations in serum (dpm/μl) (●) and uterine fluid (▲) at two autopsy times after intravenous injection of tritiated water. Immature, ovariectomised rats, pretreated for two days with vehicle (SO) or estradiol (E) were treated on the following day with vehicle (SO) or progesterone (P) and sacrificed on the fourth day. (n = 7).
[\textsuperscript{3}H]-OH

RADIOACTIVITY (dpm/mg).

TIME (min)

TREATMENT

SO, SO

SO, P

E, SO

E, P
However, in animals receiving any of the other hormone treatments tested, including vehicle alone, P alone and E pretreatment with or without P treatment, $^{14}$C-urea was partially restricted from the uterine lumen as indicated by radioactivity concentrations in uterine fluid that were less than those of serum at 15 min post-injection and that increased significantly with time. Moreover, P treatment (without E) appeared to increase the restrictiveness of the barrier to $^{14}$C-urea as indicated by uterine fluid radioactivity concentrations from these animals that were less than those from vehicle only-treated animals.

Following injection of tritiated water, the uterine fluid radioactivity concentrations were significantly higher than serum radioactivity concentrations, irrespective of hormone treatment, suggesting that this substance entered the uterine lumen without restriction. Furthermore, the uterine fluid radioactivity concentrations were also significantly ($p < 0.01$) greater than serum water radioactivity concentrations (data not shown). One interpretation of this finding is that tritiated water was retained in uterine fluid against a concentration gradient by a mechanism which might be associated with a blood-uterine lumen barrier. Furthermore, the present finding that uterine fluid radioactivity concentrations were higher in animals receiving P for three days suggests that this presently unknown mechanism might be modulated by P.

Except for animals receiving E alone, the uteri of the rats were collapsed (Armstrong, 1968; Conner and Miller, 1973a) and it is therefore possible that the apparently restricted entries of $^{14}$C-urea and $^3$H-sucrose may have resulted entirely from injected fluid distending uteri normally collapsed. However, when $^{14}$C-urea was injected, higher
uterine fluid radioactivity concentrations were recovered from E plus 
P-treated animals receiving an intraluminal injection than from animals 
with uteris distended due to fluid secretion (E only-treated). Likewise, 
following injection of \(^{3}\text{H}\)-sucrose, higher radioactivity concentrations 
were recovered from vehicle only- and P only-treated animals than from 
animals treated with only E. Thus, it is likely that the observed 
exclusions of \(^{14}\text{C}\)-urea and \(^{3}\text{H}\)-sucrose were the result of permeability 
barriers to these substances rather than an artifact produced by uterine 
distention. However, this is not to say that distention of uteri nor-
mally collapsed did not affect the entries of \(^{14}\text{C}\)-urea and \(^{3}\text{H}\)-sucrose 
into the lumina of these uteri. In this regard it is notable that dis-
tension of seminiferous tubules due to accumulation of luminal fluid 
following efferent duct ligation has been shown to result in reduced 
restrictiveness to lanthanum of the epithelial tight junctions (Neaves, 
1973). It is possible in the present experiments that instillation of 
fluid into uteris normally collapsed resulted in increased permeability 
to \(^{14}\text{C}\)-urea and \(^{3}\text{H}\)-sucrose. If so, then the apparent blood-uterine 
lumen barriers to these substances would be, in fact, more restrictive 
than was indicated by the results obtained.

Following intravenous injection of \(^{14}\text{C}\)-urea and \(^{3}\text{H}\)-sucrose, 
the uterine tissue radioactivity concentrations relative to serum radio-
activity concentrations were comparable to those observed in animals 
treated with hormones to mimic early pseudopregnancy (Chapter 4.2). 
Thus, for similar reasons as were given previously (Chapter 4.2), it is 
likely that the restricted passages of these substances observed in the 
present experiments were due to relative impermeability of the uterine 
epithelium.
Murphy et al. (1981) concluded, on the basis of freeze-fracture studies, that uterine epithelial tight junctions in ovariectomised rats treated with E for three days were morphologically less complex than those of control animals or animals treated with P for three days. They suggested that this alteration indicated reduced effectiveness of the tight junctions as a barrier. This morphological effect of E coincides with the present finding that the entry of $[^{14}\text{C}]$-urea into the uterine lumen was not restricted in E-treated animals, in contrast to the partial restriction of $[^{14}\text{C}]$-urea observed with other hormone treatments. Furthermore, these authors determined that the depth of tight junctions in P-only-animals was significantly greater than in control animals. In the present experiments, P-only treatment appeared to increase the restrictiveness of the barrier to $[^{14}\text{C}]$-urea. Therefore, the hormone-induced changes in tight junction structure and in permeability of the blood-uterine lumen barrier to $[^{14}\text{C}]$-urea correspond closely. Taken together, these findings suggest that the enhanced entry of $[^{14}\text{C}]$-urea into the uterine lumen in E-treated animals may have been due to increased permeability of a paracellular route through the epithelium. However, the permeability of $[^{3}\text{H}]$-sucrose was not increased by E treatment. Nevertheless, increased permeability of a paracellular pathway would not necessarily enhance the entry of $[^{3}\text{H}]$-sucrose in conjunction with $[^{14}\text{C}]$-urea if molecular sieving according to molecular size occurs since sucrose has a larger molecular radius than urea (Boyd et al., 1976). Furthermore, direct evidence of a causal relationship between uterine epithelial tight junction structure and permeability has not been obtained to date. Therefore, the enhanced entry of $[^{14}\text{C}]$-urea observed with E treatment may have resulted from increased uterine blood flow.
rather than greater permeability; transplacental diffusion of urea in sheep has been shown to be flow- and permeability-limited (Meschia et al., 1967). Moreover, uterine blood flow in rats is highest at proestrus when plasma estrogen levels are high (Harvey and Owen, 1976).

By contrast to $^{14}$C-urea, the permeability to $^{3}$H-sucrose was decreased further by E, as indicated by the lower uterine fluid concentrations of radioactivity from animals receiving this hormone compared with those receiving only vehicle. This decrease may have been due to E-induced changes in the uterine extracellular matrix (Fainstat, 1962) since an increased density of collagen fibres, particularly in the subepithelial region could retard the passage of $^{3}$H-sucrose. Alternatively, E increases the thickness of the endometrial epithelium (Nilsson and Wirsen, 1963) and this could impede the entry of $^{3}$H-sucrose by increasing the length of the diffusional pathway between epithelial cells.

The results of the present study can be interpreted as indicating that the restrictiveness of the blood-uterine lumen barrier is hormonally regulated. With E treatment, the barrier becomes less restrictive to $^{14}$C-urea but more restrictive to $^{3}$H-sucrose. With P treatment (in the absence of E), the barrier becomes slightly more restrictive to $^{14}$C-urea. In addition, the present findings indicate that the blood-uterine lumen barrier is located at or near the uterine luminal epithelium, suggesting that the permeability effects of E and P are mediated by changes in these cells.
CHAPTER 6: EFFECT OF INTRAUTERINE HYPERTONICITY ON THE PERMEABILITY OF THE BLOOD-UTERINE LUMEN BARRIER TO $^{14}$C-UREA

Introduction

Studies in various epithelia such as frog skin (Mandel, 1975) and toad urinary bladder (Dibona and Civan, 1973) have shown that passive solute permeation through these epithelia is increased by application of hypertonic solutions to the mucosal surfaces. Furthermore, this increase is considered to occur due to opening of the tight junctions between epithelial cells (Dibona and Civan, 1973). Estradiol treatment has been shown to increase the entry of $^{14}$C-urea into the uterine lumen (Chapter 5) and to decrease the morphological complexity of uterine epithelial tight junctions (Murphy et al., 1981). These findings suggest that the hormone-induced increase in permeability of the blood-uterine lumen barrier to urea may result from decreased resistance of the epithelial tight junctions. To test this possibility, the following experiment, which investigated an effect of intrauterine hypertonic mannitol on the ability of $^{14}$C-urea to enter the uterine lumen from serum, was carried out.

Methods

Animals

All rats were ovariectomised as described previously. Beginning three days after ovariectomy, these animals received daily injections of P (2 mg/day) for 3 days and the experiment was performed on the fourth
day. This hormone treatment was chosen because previous experiments (Chapter 5) revealed that under this condition, $^{14}$C-urea is partially restricted from entering the uterine lumen.

**Experimental Design and Procedure**

The time course for an effect of intravaginal hypertonic mannitol on the ability of $^{14}$C-urea to enter the uterine lumen from blood was investigated. Groups of animals received intravenous injections of $^{14}$C-urea at 0, 30, 60 or 120 min following intravaginal administration of hypertonic mannitol and were killed 14 min after the intravenous injection. Each animal was given, into one uterine horn, an intraluminal injection of 15 μl of 500 mM mannitol in phosphate-buffered saline (0.01 M phosphate in 0.154 M NaCl). Details of this procedure have been given previously. $^{14}$C-Urea (53.5 mCi/mmol) was diluted with 0.9% NaCl to a concentration of 5 μCi/ml and each animal was injected with 0.3 ml/100 g body weight.

**Collection of Samples**

Details of these procedures have been described previously (Chapter 4). In brief, each animal was killed by decapitation and blood collected for radioactivity determinations. The uterus was removed, cleaned of extraneous tissue and the uterine fluid was expressed onto a small, preweighed piece of filter paper which was immediately reweighed. The methanolic elution used to recover radioactivity from this filter paper resulted in recoveries greater than 90%. The uterine fluid radioactivity data were corrected for this procedural loss. A sample from the injected uterine horn was weighed and solubilised with 400 μl of NCS reagent (Amersham Corp.).
Results

The results obtained when $^{14}$C-urea was injected intravenously 14 min prior to autopsy of rats treated with intraperitoneal hypertonic mannitol for increasing time intervals are presented in Table 4. Serum radioactivity concentrations did not differ significantly ($p > 0.05$) between animals treated with hypertonic mannitol for 0, 60 or 120 min and were slightly but significantly ($p < 0.05$) greater in animals treated for 30 min. The uterine tissue radioactivity concentrations did not differ significantly ($p > 0.05$) between treatment groups. By contrast, there was a significant ($p < 0.05$) effect of duration of treatment to increase the volumes of uterine fluid recovered. Furthermore, the radioactivity contents (dpm) in uterine fluid recovered from animals treated with hypertonic mannitol for 30 or 60 min were significantly ($p < 0.05$) greater than those in animals treated for 0 min. However, the uterine fluid radioactivity concentrations (dpm/mg) did not differ significantly ($p > 0.05$) between groups treated with hypertonic mannitol for 0, 30, or 60 min but were significantly ($p < 0.05$) lower in animals treated for 120 min.

Discussion

The volumes of uterine fluid recovered from animals treated with hypertonic mannitol for 120 min prior to receiving an intravenous injection of $^{14}$C-urea were nearly twice as great as from animals receiving hypertonic mannitol immediately prior to the intravenous injection. This observation suggests that the presence of 500 mM mannitol in the uterine lumen created an osmotic gradient of sufficient magnitude to draw fluid into the uterine lumen from the surrounding tissue. Furthermore,
TABLE 4: TIME COURSE FOR AN EFFECT OF INTRAUTERINE HYPERTONIC MANNITOL ON RADIOACTIVITY LEVELS AND UTERINE FLUID VOLUMES IN OVARIECTOMISED, PROGESTERONE TREATED RATS

<table>
<thead>
<tr>
<th>Time of $[^{14}\text{C}]$-urea injection$^{a}$</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>serum radioactivity (dpm/μl)$^{b}$</td>
<td>28.9 ± 0.2$^{c,1}$</td>
<td>31.7 ± 0.8$^{2}$</td>
<td>30.2 ± 1.0$^{1,2}$</td>
<td>28.3 ± 0.7$^{1}$</td>
</tr>
<tr>
<td>uterine tissue radioactivity (dpm/mg)$^{b}$</td>
<td>33.1 ± 1.3$^{1}$</td>
<td>35.3 ± 1.4$^{1}$</td>
<td>33.5 ± 3.6$^{1}$</td>
<td>29.7 ± 0.5$^{1}$</td>
</tr>
<tr>
<td>uterine fluid radioactivity (dpm/mg)$^{b}$</td>
<td>7.9 ± 0.7$^{1}$</td>
<td>8.3 ± 0.9$^{1}$</td>
<td>8.1 ± 1.4$^{1}$</td>
<td>4.4 ± 0.3$^{2}$</td>
</tr>
<tr>
<td>uterine fluid radioactivity (dpm)$^{b}$</td>
<td>151.0 ± 12.0$^{1}$</td>
<td>224.4 ± 25.0$^{2}$</td>
<td>216.4 ± 36.0$^{2}$</td>
<td>166.0 ± 9.0$^{1,2}$</td>
</tr>
<tr>
<td>uterine fluid volume (mg)$^{b}$</td>
<td>19.5 ± 0.5$^{1}$</td>
<td>27.1 ± 0.6$^{2}$</td>
<td>27.5 ± 2.9$^{2}$</td>
<td>37.7 ± 1.0$^{3}$</td>
</tr>
</tbody>
</table>

$^{a}$ Time between intraluminal injection of hypertonic mannitol and intravenous injection of $[^{14}\text{C}]$-urea

$^{b}$ Radioactivity levels 14 min post-intravenous injection of $[^{14}\text{C}]$-urea

$^{c}$ Mean ± SEM for seven animals per group

Values within individual rows having differing numerical superscripts are significantly (p < 0.05) different.
it suggests that the luminal surface was exposed to a hypertonic solution. This hypertonic condition has been shown to result in increased permeability to urea of frog skin (Mandel, 1975), rat choroid plexus (Johanson et al., 1974) and to horseradish peroxidase of rat brain capillaries (Sterret et al., 1974). Therefore, it was anticipated that this condition of intrauterine hypertonicity would induce increased permeability of the uterine epithelium to $^{14}\text{C}$-urea and that this change would result in higher uterine fluid radioactivity concentrations in animals exposed to this condition for longer periods. However, the uterine fluid radioactivity concentrations did not differ significantly between exposure periods of 0, 30 and 60 min but were significantly lower at 120 min. The lower concentrations of uterine fluid radioactivity observed at 120 min might be due, in part, to increased volumes of uterine fluid recovered at 120 min because the uterine fluid radioactivity contents did not differ between 30 or 60 min and 120 min, whereas the volumes of uterine fluid recovered did differ. However, another interpretation of these results is that the uterine epithelium becomes more restrictive to $^{14}\text{C}$-urea with prolonged (120 min) exposure to hypertonicity. Alternatively, it may be that under this condition, the efflux of $^{14}\text{C}$-urea from the uterine lumen is enhanced relative to the influx with the result of reduced net entry into the uterine lumen and lower uterine fluid radioactivity concentrations.

The design of this experiment was such that all animals were treated with hypertonic mannitol for at least 30 min prior to autopsy. Hence, it is possible that permeability to $^{14}\text{C}$-urea was increased within 30 min in all animals and that no further change occurred until
between 60 and 120 min. To test this possibility, another experiment was carried out in which ovariectomised, P only-treated rats received either isotonic or hypertonic (500 mM mannitol) phosphate-buffered saline intraluminally 30 min prior to intravenous injections of $\text{[14C]}$-urea and were killed 15 min after the intravenous injection. The uterine fluid radioactivity concentrations did not differ significantly ($p > 0.05$) between treatments. (26.7 ± 4.1 dpm/mg for control and 20.3 ± 1.7 dpm/mg for treated animals). Therefore, it seems unlikely in the first experiment that the permeability to $\text{[14C]}$-urea was affected by intrauterine hypertonic mannitol prior to 30 min.

The observed changes in uterine fluid volumes suggest that osmotic equilibrium between uterine fluid and plasma was reached (or nearly reached) in animals injected with $\text{[14C]}$-urea at 120 min following treatment with hypertonic mannitol. This conclusion is based upon the assumption that the osmolarity of 500 mM mannitol in phosphate-buffered saline was approximately 800 mOsm/kg. Therefore, if the osmolarity of 15 μl of this solution (the volume injected intraluminally) was 800 mOsm/kg, then the osmolarity of 40 μl would be 300 mOsm/kg or approximately that of plasma. The uterine fluid volume of animals injected with $\text{[14C]}$-urea at 120 min following treatment with hypertonic mannitol was 37.7 ± 1.0 mg which approximates 40 μl. Therefore, on the assumption that there was no loss of solute from the uterine lumen during the experiment, this observation suggests that osmotic equilibrium was achieved by 120 min. This finding that osmotic equilibrium was reached relatively slowly (between 1 and 2 h were required) contrasts with the rapid entry into the uterine lumen of tritiated water observed in other experiments (Figs. 1, 8, 11). One possible explanation for this apparent difference is that in the
present experiment the osmotic pressure gradient which drew water into the uterine lumen was opposed by a hydrostatic pressure gradient which decreased in magnitude. This hydrostatic pressure gradient might have resulted from a tendency of the uterine tissue to oppose temporarily stretch induced by increasing volumes of fluid in the uterine lumen.

In summary, the results of these experiments do not support the hypothesis that intrauterine hypertonicity increases the permeability of the uterine epithelium to urea. In fact, they support the opposite, i.e., that permeability to urea is decreased by this treatment. This finding was unexpected since hypertonicity has been shown to increase the permeability of the blood-brain (Sterret et al., 1974), the blood-cerebrospinal fluid barrier (Johanson et al., 1974) and of various epithelia (Mandel, 1975; Dibona and Civan, 1973).
CHAPTER 7: EVIDENCE FOR SELECTIVE RESTRICTION BY THE BLOOD-UTERINE LUMEN BARRIER

Introduction

The results of the experiments described in Chapter 5 suggest that one characteristic of the blood-uterine lumen barrier is that the restrictiveness of this barrier is dependent upon the ovarian hormone (E and P) milieu, with E being particularly influential. Another characteristic of this barrier may be that it is selective according to the molecular weight of substances present in the blood since lower molecular weight substances, such as tritiated water and $^{14}$C-urea, entered the uterine lumen without restriction, whereas higher molecular weight substances ($^{3}$H-sucrose, $^{3}$H-inulin, and $^{125}$I-bovine serum albumin) were restricted (Chapter 4.1). However, in another study (Conner and Miller, 1973b), the distribution of substances into rat uterine fluid was found to be related generally to lipid solubility. Furthermore, the blood-cerebrospinal-brain (Mayer et al., 1959; Brodie et al., 1960) and blood-testis (Okumura et al., 1975) barriers are considered to be selective towards exogenous substances primarily on the basis of lipid solubility. Therefore, experiments were carried out to investigate the selectivity of the blood-uterine lumen barrier. This was done by examining the abilities to enter the uterine lumen from blood a) of substances of similar lipid solubilities (hydrophilic substances) but with increasing molecular weights, and b) of substances of similar molecular weights (182-188) but with increasing lipid solubilities.
In addition, the effect of E treatment in relation to the importance of lipid solubility was investigated.

Methods

Animals

Beginning several days after ovariectomy, all rats received hormone treatments for three days. On the morning of the fourth day, between 2 and 5 h prior to the experiment, all animals were anaesthetised with ether. Both kidneys of each animal were exposed by midventral approaches and the renal pedicles ligated with 3-0 silk. The kidneys were then rinsed with 0.9% NaCl and returned to the abdominal cavity. Thus, all animals were effectively nephrectomised in an attempt to achieve relatively constant serum levels of radioactivity over time by preventing renal clearance of the test substances.

The animals used in experiments designed to investigate the influence of molecular size were treated with estradiol by means of subcutaneous insertion of Silastic capsule implants (E-implanted animals) as described previously (Chapter 3). In the experiments designed to investigate the influence of lipid solubility, all animals received daily injections of either estradiol (0.5 μg/day) plus progesterone (2 mg/day) (E plus P-treated animals) or progesterone only (2 mg/day) (P only-treated animals).

Experimental Designs and Procedures

Molecular Weight

A preliminary series of experiments was carried out to determine the time course for serum, uterine tissue and uterine fluid radioactivity
concentrations following intravenous injection, via a lateral tail vein, of a test substance. The animals were killed at 1, 2 or 4 h post-injection of D-[\textsuperscript{3}H(N)]-mannitol (22.4 Ci/mmol) (mol wt 182.2), [fructose-\textsuperscript{3}H(N)]-sucrose (11.2 Ci/mmol) (mol wt 342.3) or [\textsuperscript{3}H]-inulin (2.1 Ci/mmol) (mol wt approximately 5,200). These tritiated saccharides were chosen as test substances because of their hydrophilic nature and differing molecular weights. These substances also differ in lipid solubility (as indicated by isobutanol/phosphate-buffered saline partition coefficients of 0.019, 0.007, and 0.0011, respectively). However, this factor was disregarded since lipid solubility of substances is primarily important physiologically in predicting ability to traverse cell membranes (Bradbury, 1979), which are considered to be impermeable to these test substances (Manery, 1954).

An additional experiment was carried out in which the relative abilities of these three saccharides to enter the uterine lumen from blood were directly compared. All animals were killed 2 h post-injection of one of three tritiated saccharides (2.1 Ci/mmol).

The radioactive solutions were diluted with 0.9% NaCl to a concentration of 10 μCi/ml and each animal was injected with 0.3 ml/100 g body weight.

Lipid Solubility

To investigate the influence of lipid solubility on the abilities of test substances to enter the uterine lumen from blood, a 3 x 3 x 2 factorial experiment was carried out. The factors were:

Factor 1: test substance: [\textsuperscript{14}C]-antipyrine vs [\textsuperscript{14}C]-barbital vs [\textsuperscript{3}H]-mannitol
Factor 2: time of autopsy: 5 vs 20 vs 80 min

Factor 3: hormone treatment: E plus P vs P only.

Immediately prior to intravenous injection of a test substance, via a lateral tail vein, an intraluminal injection of 15 μl phosphate-buffered saline (0.01 M phosphate in 0.154 M NaCl, pH 7.4) was given into one uterine horn. Details of the intrauterine injection procedure were given previously (Chapter 3: General Methods). The radioactive compounds used in this experiment were [N-methyl-\(^{14}\text{C}\)]-antipyrine (mol wt 188.2), [2-\(^{14}\text{C}\)]-barbital (mol wt 184) and D-[\(^{3}\text{H}\)]-mannitol (mol wt 182.2) (15.5 mCi/mmol for all test substances). These compounds were selected because of their similar molecular weights and differing lipid solubilities. Their partition coefficients between equal volumes of chloroform and phosphate-buffered saline (pH 7.4) expressed as cpm/ml chloroform ÷ cpm/ml buffer were found to be 17.2 for [\(^{14}\text{C}\)]-antipyrine; 0.23 for [\(^{14}\text{C}\)]-barbital and 0.002 for [\(^{3}\text{H}\)]-mannitol (the chloroform was evaporated to dryness prior to radioactivity determination). [\(^{14}\text{C}\)]-Antipyrine was chosen despite its relatively short metabolic half life of 90 min in the rat (Bakke et al., 1974). However, Bakke et al. (1974) reported that chromatographic separation of a chloroform extract of serum obtained several hours after [\(^{14}\text{C}\)]-antipyrine administration revealed only one peak, which was not distinguishable from that of [\(^{14}\text{C}\)]-antipyrine. Therefore, in the present study, serum and uterine fluid obtained from animals injected with this compound were extracted with chloroform prior to radioactivity determination. Uterine tissue samples from these animals were not processed in this manner due to an oversight. The radioactive solutions were diluted with 0.9% NaCl to a concentration of 10 μCi/ml and each animal was injected with 0.2 ml/100 g body weight.
Collection of Samples

Each animal was killed by decapitation and blood collected from the severed trunk vessels. Duplicate, 100 µl aliquots of serum were taken for radioactivity determinations. Serum obtained from animals injected intravenously with $^{14}\text{C}$-antipyrine was extracted by vigorous mixing with 2 x 3 ml chloroform. The chloroform was aspirated into a pipette, transferred into a scintillation vial and evaporated to dryness. The uterus was removed, stripped of adhering mesentery, rinsed in 0.9% NaCl and blotted on filter paper in an attempt to prevent contamination of uterine fluid by blood. In the experiments using E-implanted animals, both horns of each uterus were punctured and the accumulated fluid drained into a preweighed vial which was then reweighed. Additionally, a uterine tissue sample was taken from either horn. In the experiment with E plus P- and P only-treated animals, the uterine fluid was expressed onto a small, preweighed piece of filter paper which was immediately reweighed, as described previously (Chapter 4.2). For uterine fluid samples obtained from animals given $^{14}\text{C}$-barbital or $^{3}\text{H}$-mannitol, methanolic elution was used to recover radioactivity from the filter paper; recoveries were greater than 90%. The chloroformic elution used for the remaining samples also resulted in recoveries greater than 90%. Recovery was determined for each compound and uterine fluid radioactivity data were corrected for this procedural loss. In addition, a uterine tissue sample was taken from the injected horn. All uterine tissue samples were weighed, placed into scintillation vials and, to solubilise the tissue, 400 µl of NCS reagent (Amersham Corp.) were added.
Results

Time courses for serum, uterine tissue and uterine fluid radioactivity concentrations following intravenous injection of tritiated saccharides


Serum radioactivity concentrations (Fig. 12) did not differ significantly ($p > 0.005$) between animals killed at 1 and 2 h post-injection and were significantly ($p < 0.01$) greater than those in animals killed at 4 h. Both uterine tissue and uterine fluid radioactivity concentrations were slightly but significantly ($p < 0.05$) greater in animals killed at 4 h than at 1 h post-injection.


Following injection of $[^3]H$-sucrose (Fig. 13), serum radioactivity concentrations decreased significantly ($p < 0.01$) with time. By contrast, the uterine fluid and uterine tissue radioactivity concentrations did not differ significantly ($p > 0.05$) between animals killed at 1 and 4 h post-injection.

$[^3]H$-mannitol

Serum radioactivity concentrations (Fig. 14) were significantly ($p < 0.001$) less in animals killed at 4 h than at 1 h post-injection, whereas uterine tissue radioactivity concentrations were slightly but significantly ($p < 0.01$) greater. The uterine fluid radioactivity concentrations did not differ significantly ($p > 0.05$) between animals killed at 1 and 4 h post-injection.

In addition, serum radioactivity concentrations were significantly ($p < 0.01$) greater than those in uterine tissue or uterine fluid,
FIGURE 12

Time course for radioactivity concentrations in serum (dpm/μl) (●), uterine tissue (■), and uterine fluid (▲) after intravenous injection of [3H]-inulin into ovariectomised, E-implanted rats with ligated renal pedicles (n = 8).
FIGURE 13

Time course for radioactivity concentrations in serum (dpm/µl), (●), uterine tissue (■), and uterine fluid (▲) after intravenous injection of [3H]-sucrose into ovariectomised, E-implanted rats with ligated renal pedicles (n = 9).
FIGURE 14

Time course for radioactivity concentrations in serum (dpm/μl) (●), uterine tissue (□), and uterine fluid (▲) after intravenous injections of [3H]-mannitol into ovariectomised, E-implanted rats with ligated renal pedicles (n = 7).
\[ {^{3}H\text{-MANNITOL}} \]

**Radioactivity**

- (dpm/\mu{l} or mg)

**Time (h)**

- 1
- 2
- 4
irrespective of tritiated saccharide injected or time of autopsy.


The results obtained when $[^3]H$-mannitol, $[^3]H$-sucrose or $[^3]H$-inulin was injected intravenously into E-implanted rats killed 2 h post-injection of the test substance are presented in Table 5. The serum radioactivity concentrations following injection of all test substances were significantly (p < 0.05) different, with those for animals injected with $[^3]H$-inulin being greatest, those with $[^3]H$-sucrose intermediate, and those with $[^3]H$-mannitol lowest. By contrast, the uterine fluid radioactivity concentrations following injection of $[^3]H$-inulin and $[^3]H$-sucrose did not differ significantly (p > 0.05), but both were significantly (p < 0.01) lower than those with $[^3]H$-mannitol. There were no significant (p > 0.05) differences between uterine tissue radioactivity concentrations following injection of the tritiated saccharides.

Because the serum radioactivity concentrations differed significantly between test substances, the ratios of uterine fluid to serum radioactivity concentrations ($C_{UF}/C_S$) and the ratios of uterine tissue to serum radioactivity concentrations ($C_T/C_S$) were calculated and are presented in Table 5. The ratio of uterine tissue to serum radioactivity concentrations provides a measure of the extent of distribution in a tissue of a substance (Johanson and Woodbury, 1978). The $C_{UF}/C_S$ and $C_T/C_S$ values obtained following injection of $[^3]H$-inulin and $[^3]H$-sucrose did not differ significantly (p > 0.05) and both were significantly (p < 0.001) lower than those for $[^3]H$-mannitol.
### TABLE 5: RADIOACTIVITY LEVELS AT 2 h AFTER INTRAVENOUS INJECTION OF TRITIATED SACCHARIDES INTO OVARIETOMISED, E-IMPLANTED RATS WITH Ligated RENAL PEDICLES

<table>
<thead>
<tr>
<th>tritiated saccharide</th>
<th>serum (dpm/μl)</th>
<th>uterine fluid (dpm/mg)</th>
<th>uterine tissue (dpm/mg)</th>
<th>$C_{UF}/C_{S}$ $^a$</th>
<th>$C_{T}/C_{S}$ $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>inulin</td>
<td>222.6 ± 2.8 $^c$</td>
<td>8.2 ± 2.6 $^1$</td>
<td>84.0 ± 2.7 $^1$</td>
<td>0.037 ± 0.012 $^1$</td>
<td>0.378 ± 0.011 $^1$</td>
</tr>
<tr>
<td>sucrose</td>
<td>193.9 ± 4.3 $^2$</td>
<td>7.4 ± 2.1 $^1$</td>
<td>82.9 ± 3.5 $^1$</td>
<td>0.038 ± 0.011 $^1$</td>
<td>0.43 ± 0.026 $^1$</td>
</tr>
<tr>
<td>mannitol</td>
<td>167.0 ± 2.5 $^3$</td>
<td>19.9 ± 2.8 $^2$</td>
<td>90.0 ± 2.9 $^1$</td>
<td>0.118 ± 0.015 $^2$</td>
<td>0.54 ± 0.02 $^2$</td>
</tr>
</tbody>
</table>

$^a$ Uterine fluid radioactivity concentrations ÷ serum radioactivity concentrations

$^b$ Uterine tissue radioactivity concentrations ÷ serum radioactivity concentrations

$^c$ $\bar{X}$ ± SEM for seven animals per group

Values within individual columns having differing numerical superscripts are significantly (p < 0.05) different
Relative abilities of \(^{14}\text{C}\)-antipyrene, \(^{14}\text{C}\)-barbital and \(^{3}\text{H}\)-mannitol to enter the uterine lumen from serum.

The data obtained when \(^{14}\text{C}\)-antipyrene, \(^{14}\text{C}\)-barbital or \(^{3}\text{H}\)-mannitol was injected intravenously into E plus P-treated or P only-treated rats killed 5, 20 or 80 min post-injection of the test substance are presented in Fig. 15-19. Fig. 15 summarises the serum radioactivity concentrations determined for each group of animals. For those animals receiving \(^{14}\text{C}\)-antipyrene, the data presented are the radioactivity concentrations in chloroform extracts of whole serum. Irrespective of test substance or time after injection, the serum radioactivity concentrations from E plus P- and P only-treated animals did not differ significantly \((p > 0.05)\). However, at each time post-injection, the serum radioactivity concentrations differed significantly \((p < 0.01)\) between test substances. These radioactivity concentrations following injection of \(^{3}\text{H}\)-mannitol were greater than those of \(^{14}\text{C}\)-barbital which were greater than those of \(^{14}\text{C}\)-antipyrene. Additionally, the magnitude of these differences varied significantly \((p < 0.001)\) between times post-injection because, following injection of \(^{14}\text{C}\)-antipyrene or \(^{3}\text{H}\)-mannitol, the serum radioactivity concentrations were significantly \((p < 0.001)\) less in animals killed at 80 min than at 5 min post-injection, whereas they did not vary significantly \((p > 0.05)\) following injection of \(^{14}\text{C}\)-barbital.

The uterine fluid radioactivity data are presented in Fig. 16. For those animals receiving \(^{14}\text{C}\)-antipyrene, the data presented are the radioactivity concentrations in chloroform extracts of the recovered uterine fluid. Analysis of variance of the effects of test substance, hormone treatment and time post-injection on uterine fluid radioactivity...
FIGURE 15

Serum radioactivity concentrations at three autopsy times after intravenous injection of $[^{14}\text{C}]$-antipyrene, $[^{14}\text{C}]$-barbital or $[^{3}\text{H}]$-mannitol into animals with ligated renal pedicles. Ovariectomised rats were treated with estradiol plus progesterone (■—■) or progesterone only (■—■) for three days ($n = 6$).
concentrations indicated a significant (p < 0.01) interaction between test substance and time post-injection. Although this analysis did not reveal a significant effect of hormone treatment, the 3-way interaction (test substance x time x hormone treatment) tended towards significance (p = 0.1) as did the interaction for time x hormone treatment (p = 0.1). The significant interaction between test substance and time post-injection was brought about because the uterine fluid radioactivity concentrations differed significantly between test substances at certain times post-injection only. At 5 min post-injection to animals receiving the same hormone treatment, the uterine fluid radioactivity concentrations following injection of [14C]-antipyrene were significantly (p < 0.01) greater than those following injection of either [14C]-barbital or [3H]-mannitol. Additionally, the uterine fluid radioactivity concentrations following injection of [14C]-barbital were significantly (p < 0.001) greater than those with [3H]-mannitol. Following injection of either [14C]-antipyrene or [14C]-barbital, the uterine fluid radioactivity concentrations from animals treated with E plus P were significantly (p < 0.001) greater than those from animals treated with P only. The magnitude of these differences did not vary significantly (p > 0.05) between these test substances. At 20 min post-injection to animals receiving either hormone treatment, the uterine fluid radioactivity concentrations following injection of either [14C]-antipyrene or [14C]-barbital did not differ significantly (p > 0.05). However, both were significantly (p < 0.001) greater than those following injection of [3H]-mannitol. By 80 min post-injection to animals receiving either hormone treatment, the uterine fluid radioactivity concentrations following injection of [14C]-barbital or [3H]-mannitol did not differ significantly
FIGURE 16

Uterine fluid radioactivity concentrations at three autopsy times after intravenous injection of $^{14}$C-antipyrene, $^{14}$C-barbital or $^{3}$H-mannitol into animals with ligated renal pedicles. Ovariectomised rats were treated with estradiol plus progesterone (●—●) or progesterone only (■—■) for three days ($n = 6$).
UTERINE FLUID

ANTIPYRENE

BARBITAL

MANNITOL

RADIOACTIVITY (dpm/mg)

Time (min)

5 20 80

5 20 80

5 20 80
(p > 0.05) and both were significantly (p < 0.05) greater than those following injection of $[^{14}\text{C}]-\text{antipyrine}$. For all test substances at 20 or 80 min post-injection, the uterine fluid radioactivity concentrations did not vary significantly (p > 0.05) between hormone treatments.

Fig. 17 summarises the data obtained when ratios of uterine fluid to serum radioactivity concentrations ($C_{UF}/C_S$) were calculated; this was done because of the significant differences in serum radioactivity concentrations between test substances. Significant (p < 0.05) interactions between the effects of hormone treatment and time post-injection and between test substance and time post-injection were indicated. These interactions were brought about because the $C_{UF}/C_S$ differed significantly between hormone treatments and test substances at certain times post-injection only. At 5 min post-injection of either $[^{14}\text{C}]-\text{antipyrine}$ or $[^{14}\text{C}]-\text{barbital}$, the $C_{UF}/C_S$ were significantly (p < 0.001) greater in those animals receiving E plus P as compared to those receiving P only. By contrast, following injection of $[^3\text{H}]-\text{mannitol}$, the $C_{UF}/C_S$ did not vary significantly (p > 0.05) between treatments. In addition, at 20 and 80 min post-injection for all substances tested, the $C_{UF}/C_S$ did not differ significantly (p > 0.05) between treatments. In animals receiving the same hormone treatment and at 5 min post-injection, the $C_{UF}/C_S$ differed significantly (p < 0.01) between all test substances; with those of $[^{14}\text{C}]-\text{antipyrine}$ being greatest and those with $[^3\text{H}]-\text{mannitol}$ least. At 20 and 80 min post-injection to animals receiving the same hormone treatment, the $C_{UF}/C_S$ following injection of $[^{14}\text{C}]-\text{antipyrine}$ or $[^{14}\text{C}]-\text{barbital}$ did not differ significantly (p > 0.05) and were significantly (p < 0.001) greater than those of $[^3\text{H}]-\text{mannitol}$. \footnote{\textit{Note:} These results are preliminary and require further investigation.}
FIGURE 17

Ratios of uterine fluid to serum radioactivity concentrations \( C_{UF}/C_S \) at three autopsy times after intravenous injection of \(^{14}C\)-antipyrene, \(^{14}C\)-barbital or \(^{3}H\)-mannitol into animals with ligated renal pedicles. Ovariectomised rats were treated with estradiol and progesterone \( \bullet-\bullet \) or progesterone only \( \square-\square \) for three days \( n = 6 \).
The uterine tissue radioactivity data are presented in Fig. 18. In contrast to serum and uterine fluid, the uterine tissue samples obtained from animals injected with $[^{14}\text{C}]$-antipyrene were not extracted with chloroform. At each time post-injection for all substances tested, the uterine tissue radioactivity concentrations did not differ significantly ($p > 0.05$) between hormone treatments. However, at all given times post-injection, the uterine tissue radioactivity concentrations differed significantly ($p < 0.01$) between test substances. Following injection of $[^{3}\text{H}]$-mannitol, these radioactivity concentrations were greatest, whereas they were least with $[^{14}\text{C}]$-antipyrene. In addition, the magnitude of the differences of the radioactivity concentrations between those of $[^{3}\text{H}]$-mannitol and the other test substances was significantly ($p < 0.05$) greater at 80 min than at 5 min post-injection. In Fig. 19 the data obtained when the ratios of uterine tissue to serum radioactivity concentrations ($C_T/C_S$) were calculated are summarised. This data manipulation was carried out partly because of the significant differences in serum radioactivity concentrations between test substances but also to provide a measure of the distribution of these substances in uterine tissue. In the case of $[^{14}\text{C}]$-antipyrene, these are the ratios of non-chloroform-extracted uterine tissue radioactivity to chloroform-extracted serum radioactivity. At 5 and 20 min post-injection of $[^{14}\text{C}]$-antipyrene and $[^{14}\text{C}]$-barbital, the $C_T/C_S$ did not differ significantly ($p > 0.05$) between test substances and were significantly ($p < 0.001$) greater than those obtained following injection of $[^{3}\text{H}]$-mannitol. By 80 min post-injection the $C_T/C_S$ following injection of $[^{14}\text{C}]$-antipyrene were significantly ($p < 0.001$) greater than those of $[^{14}\text{C}]$-barbital which were significantly
FIGURE 18

Uterine tissue radioactivity concentrations at three autopsy times after intravenous injection of \(^{14}\text{C}\)-antipyrene, \(^{14}\text{C}\)-barbital or \(^{3}\text{H}\)-mannitol into animals with ligated renal pedicles. Ovariectomised rats were treated with estradiol plus progesterone (•••) or progesterone only (–––) for three days (n = 6).
FIGURE 19

Ratios of uterine tissue to serum radioactivity concentrations (C_T/C_S) at three autopsy times after intravenous injection of \(^{14}\text{C}\)-antipyrene, \(^{14}\text{C}\)-barbital or \(^{3}\text{H}\)-mannitol into animals with ligated renal pedicles. Ovariectomised rats were treated with estradiol and progesterone (●—●) or progesterone only (■—■) for three days (n = 6).
(p < 0.001) greater than those of $[^3H]$-mannitol.

Discussion

General Comments

In the present experiments, the renal pedicles of all animals were ligated prior to intravenous injection of a test substance in an attempt to achieve relatively constant serum radioactivity concentrations over time by preventing renal clearance of the test substances. Investigation of the time courses for radioactivity concentrations following injection of the tritiated saccharides (Fig. 12-14) indicates that relatively constant serum radioactivity concentrations could be achieved for the period between 1 and 4 h post-injection. However, in other experiments, the serum radioactivity concentrations following injection of $[^{14}C]$-antipyrine and $[^3H]$-mannitol (Fig. 15) were significantly less in animals killed at 80 min than at 5 min post-injection. It is likely that the decreased concentrations of chloroform-extracted radioactivity following injection of $[^{14}C]$-antipyrine were, in part, due to metabolism of antipyrine to compounds not extracted by chloroform (Bakke et al., 1974). When $[^3H]$-mannitol was injected (Fig. 15), serum radioactivity concentrations decreased rapidly between 5 and 80 min post-injection, perhaps because of more complete penetration of extracellular fluid at 80 min post-injection.

The time courses for uterine fluid radioactivity concentrations post-injection of tritiated saccharides were used to determine an
appropriate time of autopsy post-injection for comparing directly the relative abilities of these substances to enter the uterine lumen. The uterine fluid radioactivity concentrations either did not differ significantly between animals killed at 1 and 4 h post-injection ([\(^3\)H]-sucrose and [\(^3\)H]-mannitol) or were slightly (10.1 ± 0.9 vs 6.9 ± 1.0 dpm/mg) greater at 4 h compared with 1 h ([\(^3\)H]-inulin). Therefore a time of autopsy of 1, 2 or 4 h post-injection would have been appropriate and 2 h was chosen.

The results of these experiments also provide further evidence for the existence of a blood-uterine lumen barrier in E-implanted rats. When [\(^3\)H]-sucrose and [\(^3\)H]-inulin were injected intravenously into E-implanted rats with intact kidneys (Chapter 4.1), the serum radioactivity concentrations rapidly decreased between 15 and 120 min post-injection. It is possible that the low radioactivity concentrations in uterine fluid observed in those experiments were due to the decreasing serum radioactivity concentrations rather than to a permeability barrier. However, the results obtained using rats with ligated renal pedicles indicate that the passages of [\(^3\)H]-mannitol, [\(^3\)H]-sucrose and [\(^3\)H]-inulin from blood into the uterine lumen were restricted since uterine fluid radioactivity concentrations were much less than those in serum at all times of autopsy post-injection. This suggests that the low uterine fluid radioactivity concentrations observed in animals with intact kidneys were primarily due to the presence of a permeability barrier.
Does molecular size influence the ability of a substance to cross the blood-uterine lumen barrier?

The results obtained when E-implanted rats with ligated renal pedicles were injected intravenously with one of three tritiated saccharides and killed 2 h post-injection suggest that molecular size is one factor influencing the ability of some substances to cross the blood-uterine lumen barrier. This conclusion is based upon the finding that when $[^3\text{H}]$-mannitol was injected, the uterine fluid radioactivity concentrations were significantly greater (by a factor of 2-3 times) than when either of the other saccharides was injected. Additionally, the $C_{UF}/C_S$, which were calculated to take into account serum radioactivity concentration differences, were also significantly greater for $[^3\text{H}]$-mannitol. However, it is possible that this apparent effect of molecular size on ability to enter the uterine lumen was secondary to differences in uterine volumes of distribution between saccharides since the $C_{UF}/C_S$ for $[^3\text{H}]$-mannitol was significantly greater than for the other saccharides. Nevertheless, the $[^3\text{H}]$-mannitol volumes of distribution (0.54 ± 0.02 μl/mg) were slightly greater than those for $[^3\text{H}]$-sucrose (0.43 ± 0.02 μl/mg) and for $[^3\text{H}]$-inulin (0.378 ± 0.011 μl/mg) and were not 2-3 times greater as were the uterine fluid radioactivity concentrations for $[^3\text{H}]$-mannitol compared with those for $[^3\text{H}]$-sucrose and $[^3\text{H}]$-inulin. Thus, it is unlikely that a larger volume of distribution within the uterus can account entirely for the greater ability of $[^3\text{H}]$-mannitol to enter the uterine lumen. These results indicate that of the saccharides tested, $[^3\text{H}]$-inulin and $[^3\text{H}]$-sucrose were less able to cross the blood-uterine lumen barrier than $[^3\text{H}]$-mannitol. Since these substances differ from $[^3\text{H}]$-mannitol by having larger molecular
sizes, these results suggest that this barrier impedes selectively some substances according to molecular size.

The finding that the uterine fluid radioactivity concentrations and $C_{UF}/C_S$ following injection of $[^3H]$-sucrose and $[^3H]$-inulin did not differ significantly is surprising, given the difference between their molecular weights (342 vs 5,200, respectively) and hydrodynamic molecular radii (4.6 vs 13.1 Å, respectively; Amtorp, 1980). Amtorp (1980) found that the brain capillary permeability coefficients for sucrose and inulin differed only slightly and suggested this indicated that these substances cross the blood-brain barrier in part by a "non-discriminatory" pathway involving pinocytotic vesicles. Direct evidence for a transcellular, vesicular pathway for the movement of substances across the uterine epithelium has not been presented to date. However, following intravenous injection of horseradish peroxidase to immature, E-treated rats (Anderson et al., 1975) or to Day 5 pregnant rats (Parr, 1980), peroxidase reaction product was located in pinocytotic vesicles in the basolateral membranes and in apical vesicles. Therefore, a transcellular pathway for movement of substances such as sucrose and inulin across the uterine epithelium may exist in rats.

In view of these findings, the effects of hormone treatment on the abilities of $[^3H]$-sucrose and $[^14C]$-urea to enter the uterine lumen can be given further consideration. It was suggested (Chapter 5) that if molecular sieving according to molecular size occurs, then one mechanism by which the permeability to $[^14C]$-urea could be increased relative to that of $[^3H]$-sucrose was reduced resistance of a paracellular pathway. The present finding that the entry into the uterine lumen of $[^3H]$-sucrose and of a much larger saccharide, $[^3H]$-inulin, did
not differ significantly whereas that of a smaller one, $[^3H]$-mannitol, was greater suggests that molecular sieving does occur. Therefore, increased permeability of a paracellular pathway (which could allow urea to enter more readily) would not necessarily result in increased permeability to $[^3H]$-sucrose. However, if sucrose is transported to the uterine lumen in vesicles, then the finding that the ability of $[^3H]$-sucrose to enter the uterine lumen was decreased in estradiol-treated animals would suggest that this transcellular pathway is subject to hormonal control. Parr (1980) observed fewer basal pinocytotic invaginations per uterine epithelial cell in Day 1 pregnant rats (Day 1 = estrus) than in Day 5 and 6 pregnant ones. This increase may have been induced by the presence of blastocysts in the uterus or by endocrine conditioning of the uterus for implantation. If the latter is the case, then these observations would suggest that the pinocytotic activity of the epithelial basolateral membranes is hormonally regulated and that estradiol treatment depressed this activity thereby decreasing the ability of $[^3H]$-sucrose to enter the uterine lumen via pinocytotic vesicles. Furthermore, these findings would also suggest that the increased entry of $[^3H]$-sucrose observed between the equivalent of Days 4 and 6 of pseudopregnancy may have resulted, at least in part, from increased pinocytotic activity.

Does lipid solubility influence the ability of a substance to cross the blood-uterine lumen barrier?

As an attempt to answer this question, an experiment was carried out in which the radioactivity concentrations in serum, uterine tissue and uterine fluid were determined at 5, 20 or 80 min post-injection of
[\textsuperscript{14}C]-antipyrene, [\textsuperscript{14}C]-barbital or [\textsuperscript{3}H]-mannitol into E plus P- or P only-treated rats. The results obtained suggest that [\textsuperscript{14}C]-antipyrene and [\textsuperscript{14}C]-barbital entered the uterine lumen from serum apparently with minimal restriction since the $C_{UF}/C_S$ for these compounds ranged from 0.67 to 0.89 in animals killed at 20 or 80 min post-injection. By contrast, [\textsuperscript{3}H]-mannitol was apparently restricted from entering the uterine lumen readily since the $C_{UF}/C_S$ for this compound ranged from 0.14 to 0.31 in groups of animals killed at the same times post-injection. Because these compounds differ only slightly in molecular weight (range from 182 to 188), it is unlikely that this disparity in ability to enter the uterine lumen was due to differences in molecular size. Since [\textsuperscript{3}H]-mannitol differs from [\textsuperscript{14}C]-antipyrene and [\textsuperscript{14}C]-barbital in lipid solubility (as measured by chloroform/phosphate-buffered saline partition coefficients), these results suggest that lipid solubility is an important factor determining whether a substance will be restricted from entering the uterine lumen by a permeability barrier. Some endogenous compounds are weak acids and bases. For these substances, it is the lipid solubility of the non-ionised molecule which will be important in determining the permeability of the barrier to these compounds. Furthermore, the proportion of ionised molecules to non-ionised molecules depends upon pH. Therefore, the distribution across the blood-uterine lumen barrier of weak acids and bases will depend upon the pH of uterine extracellular fluid and the fluid within the uterine lumen (Bradbury, 1979). Thus, a compound which is highly lipid soluble in the non-ionised state but largely dissociated at uterine pH, would presumably be restricted from entering the uterine lumen.
Following injection of $[^{14}C]$-antipyrine and $[^{14}C]$-barbital, the $C_T/C_S$ for these compounds ranged between 0.78 and 0.93 μl/mg for animals killed at 5 or 20 min post-injection, whereas for $[^{3}H]$-mannitol, they ranged between 0.4 and 0.47 μl/mg. Hence, a similar pattern of differences between test substances was observed for $C_T/C_S$ as for $C_{UF}/C_S$. The $C_T/C_S$ for $[^{3}H]$-mannitol probably represents distribution primarily in the uterine extracellular fluid compartment since Spaziani and Gutman (1965) estimated the size of this compartment to be 0.456 μl/mg in ovariectomised rats. Therefore, the $C_T/C_S$ obtained for $[^{14}C]$-antipyrine and $[^{14}C]$-barbital suggest that these compounds were able to distribute, at least partially, in the extracellular and intracellular fluid compartments of the uterus because they were approximately twice as great as those for $[^{3}H]$-mannitol. This correlation between volume of distribution within the uterus ($C_T/C_S$) and the ability to enter the uterine lumen ($C_{UF}/C_S$) suggests that the importance of lipid solubility may be related to the distribution of the substance within the uterus. Taken together, these results suggest that substances which penetrate both fluid compartments of the uterus, also enter the uterine lumen without restriction and imply that the blood-uterine lumen barrier has an extracellular or paracellular locus.

Lipid solubility may influence the rate of entry into the uterine lumen of substances not restricted by the blood-uterine lumen barrier because at 5 min post-injection (the earliest time studied), the uterine fluid radioactivity concentrations and $C_{UF}/C_S$ following injection of $[^{14}C]$-antipyrine were significantly greater than those following injection of $[^{14}C]$-barbital. Since these compounds differ more in lipid solubility than in molecular weight, it seems likely that this enhanced
entry of $[^{14}C]$-antipyrene was due to greater lipid solubility. The uterine fluid radioactivity concentrations and $C_{UF}/C_{S}$ obtained from E plus P-treated animals were significantly greater than those from P only-treated animals at 5 min post-injection of $[^{6}S]$-antipyrene and $[^{14}C]$-barbital. This significant effect of E treatment was apparent only at 5 min post-injection and not at later times suggesting that the rates of entry of these compounds were increased by estradiol, and that the steady-state equality relationship between serum and uterine fluid radioactivity concentrations was not affected. Thus, for some substances, lipid solubility and hormone treatment may be factors involved in determining rates of entry from blood into the uterine lumen.

There are at least two explanations for how E treatment could increase the rates of entry from blood into the uterine lumen of $[^{14}C]$-antipyrene and $[^{14}C]$-barbital without a similar increase in that of $[^{3}H]$-mannitol. Firstly, this enhanced rate of entry may have resulted from increased uterine blood flow as was suggested previously (Chapter 5) for the increased entry of $[^{14}C]$-urea observed in E-treated animals. Transplacental diffusion of antipyrene in sheep has been shown to be flow-limited and not permeability-limited (Meschia et al., 1967) as has diffusion of antipyrene into cerebrospinal fluid (Johanson and Woodbury, 1977). Increased blood flow would not alter the rate of entry of $[^{3}H]$-mannitol if its diffusion into the uterine lumen is permeability-limited rather than flow-limited. Secondly, for reasons discussed above, it is likely that $[^{14}C]$-antipyrene and $[^{14}C]$-barbital diffused across uterine cell membranes to distribute in intracellular as well as extracellular spaces. If estradiol treatment induced/ changes in uterine cell membranes which render them more permeable to
lipophilic solutes, then these substances may have diffused across the epithelial cell membranes more readily, thereby resulting in enhanced rates of entry into the uterine lumen. $[^3\text{H}]-\text{Mannitol}$, because of its inability to diffuse across cell membranes, would not be similarly influenced by E treatment.

The lack of a significant effect of hormone treatment on the entry of $[^3\text{H}]-\text{mannitol}$ is surprising considering the effect of estradiol on the entry of a similar compound, $[^3\text{H}]-\text{Sucrose}$, as well as on $[^{14}\text{C}]-\text{urea}$. If the permeability of the uterine epithelial tight junctions is increased by estrogen as freeze fracture studies suggest (Murphy et al., 1981), then an increased ability of $[^3\text{H}]-\text{mannitol}$ to enter the uterine lumen in the E-treated animals would be expected. However, the absence of a significant effect could be due to failure to detect a change, rather than the absence of a change.

Characteristics of the blood-uterine lumen barrier in terms of selectivity

The results obtained from the experiments described in preceding chapters, combined with the results of the present experiments can be taken together to characterise the blood-uterine lumen barrier in terms of its selectivity. For small compounds, molecular size is apparently at least as important as lipid solubility in determining their ability to enter the uterine lumen since tritiated water (mol wt 18) and $[^{14}\text{C}]-\text{urea}$ (mol wt 60) are small, hydrophilic compounds which were able to enter the uterine lumen from blood without restriction, whereas $[^3\text{H}]-\text{mannitol}$, a slightly larger hydrophilic compound (mol wt 182) was essentially restricted. However, $[^3\text{H}]-\text{mannitol}$ was restricted not only
because of its molecular size since similar-sized compounds, \[^{14}\text{C}]\text{-antipyrine and }[^{14}\text{C}]\text{-barbital, apparently entered the uterine lumen without restriction. Thus, for slightly larger compounds, lipid solubility is apparently also important in determining ability to enter the uterine lumen. Since substances larger and less hydrophilic than \[^{3}\text{H}]\text{-mannitol were not tested, further conclusions regarding the relative importance of molecular size and lipid solubility in determining penetration of the blood-uterine lumen barrier cannot be drawn. However, since }[^{3}\text{H}]\text{-mannitol crossed this barrier more readily than }[^{3}\text{H}]\text{-sucrose, it may be that molecular size influences ability to enter the uterine lumen of hydrophilic compounds with hydrodynamic molecular radii between those of urea and sucrose.}

Localisation of the Blood-Uterine Lumen Barrier

Two approaches were employed in the present study to investigate the locus of the blood-uterine lumen barrier. The first was to evaluate the distribution of certain test substances within the uterus following intravenous injection of the radiolabelled test substance by comparing the radioactivity concentrations in samples of uterine tissue with those in serum. As noted in Chapters 4, 5 and this chapter, the results obtained with this approach indicated that the blood-uterine lumen barrier is located at or near the uterine epithelium and has an extracellular or paracellular locus. In an attempt to confirm this conclusion, a second approach was employed. This was to examine, with the electron microscope, the ability of an electron-opaque tracer, lanthanum, to penetrate the uterine epithelial intercellular spaces and tight junctions. For uncertain reasons, the results obtained with this
approach were inconclusive. Thus, they neither confirmed nor contra-
dicted the hypothesis that the blood-uterine lumen barrier is due, in
part, to relative impermeability of the uterine epithelial tight junctions.
Therefore, they are not reported in any detail.
CHAPTER 8: TRANSPORT OF AN AMINO ACID ANALOGUE
ACROSS THE BLOOD-UTERINE LUMEN BARRIER

Introduction

The free amino acid composition of rat uterine fluid has not been investigated directly to date. Nevertheless, uterine flushings obtained from intact, cyclic rats have been found to contain microgram amounts of acid-soluble nitrogen (Heap and Lamming, 1962); however, the sources of this nitrogen were not determined. Since rat blastocysts in utero accumulate radioactivity following maternal intravenous injection of \( ^{14}C \)-\( \alpha \)-aminoisobutyric acid (an amino acid analogue) (Lindqvist et al., 1978), it is likely that at least some amino acids are able to enter the uterine lumen and therefore that a portion of the acid-soluble nitrogen was derived from free amino acids present in the uterine flushings. Uterine fluids obtained from pregnant rabbits (Jaszczak and Hafez, 1972) or from cyclic cows (Fahning et al., 1967) have been found to contain free amino acids, some of which were present in concentrations several times greater than those in serum. However, in both species, the concentrations of certain amino acids (glutamine and asparagine, rabbit; arginine, rabbit and cow) were greater in serum than in uterine fluid. Therefore, it would seem that the entry of amino acids into the uterine lumen in some species is both concentrative and selective. The following experiments were carried out to investigate the ability of \( \alpha \)-aminoisobutyric acid (AIB), an amino acid analogue, to enter the uterine lumen from blood in estradiol-treated, ovariectomised immature rats.
In the first experiment, the relative entries of $[^3\text{H}]$-AIB and $[^3\text{H}]$-mannitol into the uterine lumen from blood were directly compared in an attempt to distinguish between passive diffusion and specific transport of AIB; mannitol fluxes between uterine tissue and lumen are considered to occur by passive diffusion only (Walters et al., 1975, 1979, 1981). The uptake of AIB by uterine tissue in vitro has been shown to occur by an active transport mechanism which is inhibited by the pharmacological agent, ouabain (Riggs et al., 1972; Walters et al., 1979). Therefore, the possibility existed that AIB might be transported into the uterine lumen in vivo by a similar transport mechanism. To test this, the effect of ouabain treatment on the entry of $[^3\text{H}]$-AIB into the uterine lumen from blood was investigated in a second experiment.

**Methods**

**Animals**

Beginning three days after ovariectomy, all rats received twice daily injections of 0.25 μg estradiol (E) in 0.1 ml sesame oil for three days. On the morning of the fourth day, between 2 and 5 h prior to the experiment, all animals were anaesthetised with ether. Both kidneys of each animal were exposed by midventral approaches and the renal pedicles ligated with 3-0 silk. The kidneys were then rinsed with 0.9% NaCl and returned to the abdominal cavity. Ligation of renal pedicles was undertaken in an attempt to prevent renal clearance of the test substances.
Experimental Designs and Procedures

The first experiment was a 2 x 2 factorial experiment in which the animals were given single intravenous injections, via a lateral tail vein, of either \(^3\text{H}\)-AIB (mol wt 103) or \(^3\text{H}\)-mannitol (mol wt 182) and were killed either 1 or 4 h post-injection. The radioactive compounds used in this experiment were \(\alpha\)-methyl \(^3\text{H}\)-aminoisobutyric acid (10 Ci/mmol) and D-[1-\(^3\text{H}\)]-mannitol (22.4 Ci/mmol) and were diluted with 0.9% NaCl to concentrations of 2.6 nmol/ml and 0.34 nmol/ml, respectively. Each animal was injected with 0.3 ml/100 g body weight. The results of preliminary experiments indicated that the whole body volumes of distribution of \(^3\text{H}\)-AIB in effectively nephrectomised rats were 5-8 times larger than those of \(^3\text{H}\)-mannitol, presumably because of active uptake of the former substance by various tissues in the body. Therefore, as an attempt to ensure that serum concentrations would be similar at 1 and 4 h post-injection, those animals given \(^3\text{H}\)-AIB received 7.6 fold more mass of test substance than the remaining animals.

The second experiment was a 2 x 3 factorial experiment, with the factors being:

Factor 1: time of autopsy: 35 vs 120 min post-injection of \(^3\text{H}\)-AIB

Factor 2: ouabain treatment: none vs ouabain intravenously vs ouabain intraluminally.

Ouabain or its vehicle (0.01 M phosphate in 0.154 M NaCl) was given immediately prior to the intravenous injection, via a lateral tail vein, of \(^3\text{H}\)-AIB. Details of the unilateral, intrauterine injection procedure used to administer ouabain intraluminally were given previously (Chapter 3). The radioactive solution was \(\alpha\)-methyl \(^3\text{H}\)-AIB (10 Ci/mmol)
diluted with 0.81 M phosphate in 0.154 M NaCl to a concentration of 15 μCi/ml. Ouabain octahydrate was diluted with phosphate-buffered saline to a concentration of 1 mg/ml for intravenous injection and 0.036 mg/ml for intraluminal injection. All intravenous injections were 0.3 ml/100 g body weight whereas intraluminal injections were 10 μl irrespective of body weight.

Collection of Samples

Each animal was killed by decapitation and blood collected from the severed neck vessels. Duplicate, 100 μl aliquots of serum were taken for radioactivity determinations. The uterus was removed, stripped of adhering mesentery, rinsed in 0.9% NaCl and blotted on filter paper in an attempt to prevent contamination of uterine fluid by blood. In the first experiment ([3H]-AIB vs [3H]-mannitol), both horns of each uterus were punctured and the accumulated fluid drained into preweighed vials which were then reweighed. Additionally, a uterine tissue sample was taken from either horn. In the second experiment (effects of ouabain treatment), the uterine horn receiving an intraluminal injection was punctured and the accumulated fluid collected into a preweighed vial. In addition, a tissue sample from each uterine horn was taken. All uterine tissue samples were weighed, placed into scintillation vials and, to solubilise the tissue, 400 μl of NCS reagent (Amersham Corp.) were added.

Results

The data obtained when [3H]-AIB or [3H]-mannitol was injected intravenously into E-treated rats killed 1 or 4 h post-injection are presented in Fig. 20. Because the specific activities of [3H]-AIB and
[\textsuperscript{3}H]-mannitol differed and to allow direct comparisons between test substances, radioactivity determinations were converted to concentrations (mnoles/g or \(\mu\)l) using respective specific activities. In doing so, it has been assumed that neither test substance was metabolised appreciably in vivo.

Serum concentrations of \([\textsuperscript{3}H]\)-AIB and \([\textsuperscript{3}H]\)-mannitol were significantly \((p < 0.001)\) lower at 4 h than at 1 h post-injection. In addition, at each time post-injection, the serum concentrations of \([\textsuperscript{3}H]\)-AIB were significantly \((p < 0.001)\) lower than those of \([\textsuperscript{3}H]\)-mannitol. The magnitude of this difference did not vary significantly \((p > 0.05)\) between animals killed at 1 and 4 h post-injection. Following injection of either test substance, the uterine fluid concentrations were slightly but significantly \((p < 0.05)\) greater at 4 h than at 1 h post-injection. Additionally, at each time post-injection, the uterine fluid concentrations of \([\textsuperscript{3}H]\)-AIB were significantly \((p < 0.01)\) greater than those of \([\textsuperscript{3}H]\)-mannitol. Furthermore, with each test substance, at each time post-injection, the uterine fluid concentrations were significantly \((p < 0.01)\) lower than serum concentrations. The uterine tissue concentrations of \([\textsuperscript{3}H]\)-AIB were significantly \((p < 0.01)\) greater in animals killed at 4 h than at 1 h post-injection, whereas those of \([\textsuperscript{3}H]\)-mannitol did not vary significantly \((p > 0.05)\) between animals killed at 1 h and 4 h post-injection. At each time post-injection, the uterine tissue concentrations of \([\textsuperscript{3}H]\)-AIB were significantly \((p < 0.01)\) greater than those of \([\textsuperscript{3}H]\)-mannitol and were significantly \((p < 0.01)\) greater than serum concentrations of \([\textsuperscript{3}H]\)-AIB. By contrast, uterine tissue concentrations of \([\textsuperscript{3}H]\)-mannitol were significantly \((p < 0.01)\) less than those in serum.
Ratios of uterine fluid concentrations to serum concentrations for $[^3\text{H}]$-AIB and $[^3\text{H}]$-mannitol at each time post-injection were calculated and are presented as an inset in Fig. 20. At each time post-injection, the ratios for $[^3\text{H}]$-AIB were significantly ($p < 0.001$) greater than those for $[^3\text{H}]$-mannitol. The magnitude of this difference did not vary significantly ($p > 0.05$) between animals killed at 1 and 4 h post-injection.

The data obtained when E-treated rats receiving vehicle, ouabain intravenously or ouabain intraluminally, were injected intravenously with $[^3\text{H}]$-AIB and killed 35 or 120 min post-injection are presented in Fig. 21. Serum radioactivity concentrations were significantly ($p < 0.01$) lower at 120 min than at 35 min post-injection of $[^3\text{H}]$-AIB, with any given ouabain treatment. At both times post injection of $[^3\text{H}]$-AIB, the serum radioactivity concentrations in animals treated intraluminally with ouabain did not differ significantly ($p > 0.05$) from those in animals treated with vehicle only. By contrast, the serum radioactivity concentrations in those animals treated intravenously with ouabain were significantly ($p < 0.01$) greater at both times post-injection than those in animals treated with vehicle only.

Uterine fluid radioactivity concentrations in animals receiving either ouabain intraluminally or vehicle only did not vary significantly ($p > 0.05$) between animals killed at 35 and 120 min post-injection of $[^3\text{H}]$-AIB whereas they were significantly ($p < 0.05$) greater at 120 min than at 35 min post-injection of $[^3\text{H}]$-AIB in animals receiving ouabain intravenously. At each time post-injection of $[^3\text{H}]$-AIB, the uterine fluid radioactivity concentrations in animals receiving ouabain intraluminally were significantly ($p < 0.05$) greater than those in animals
FIGURE 20

Concentrations of $[^3\text{H}]-\text{AIB}$ (open symbols) and $[^3\text{H}]-\text{mannitol}$ (closed symbols) in serum (mmol/l x 10$^{19}$) $(\bullet \circ)$, uterine tissue $(■ □)$, and uterine fluid $(▲ △)$ at two autopsy times after intravenous injection into ovariecotomised, E-implanted rats with ligated renal pedicles $(n = 6)$.

**Insert:** Ratios of uterine fluid to serum concentrations of $[^3\text{H}]-\text{AIB}$ (open symbols) and $[^3\text{H}]-\text{mannitol}$ (closed symbols).
receiving vehicle only. By contrast, in animals receiving ouabain intravenously, the uterine fluid radioactivity concentrations at 120 min post-injection were significantly \( p < 0.05 \) greater than those in vehicle only-treated animals at either 35 or 120 min post-injection.

In any group of animals, the uterine tissue radioactivity concentrations did not differ significantly \( p > 0.05 \) between uterine horns of a given animal. In addition, the uterine tissue radioactivity concentrations at each time post-injection of \( ^3\text{H} \)-AIB did not differ significantly \( p > 0.05 \) between ouabain treatments. However, for all ouabain treatments given, including vehicle only, they were significantly \( p < 0.01 \) greater at 120 min than at 35 min post-injection of \( ^3\text{H} \)-AIB.

**Discussion**

The ability of \( ^3\text{H} \)-AIB to enter the uterine lumen from blood was assessed in E-treated rats by comparing the levels of radioactivity in uterine fluid with those in serum following intravenous injection of this test substance. In an attempt to ascertain whether the entry of \( ^3\text{H} \)-AIB was at least partly due to specific transport mechanisms, the relative abilities of \( ^3\text{H} \)-AIB and \( ^3\text{H} \)-mannitol to enter the uterine lumen from blood were compared. In doing so, the assumptions that \( ^3\text{H} \)-mannitol would enter the uterine lumen by passive diffusion only and that the diffusion coefficients of \( ^3\text{H} \)-mannitol and \( ^3\text{H} \)-AIB in water were similar despite the substances' slight differences in molecular weight were required (Walters et al., 1979). Thus, a greater ability of \( ^3\text{H} \)-AIB to enter the uterine lumen, relative to that of \( ^3\text{H} \)-mannitol, would suggest that \( ^3\text{H} \)-AIB was, in part, transported by a specific carrier mechanism.
FIGURE 21

Radioactivity concentrations in serum (dpm/μl) (●), uterine tissue (■, □), and uterine fluid (△) at two autopsy times after intravenous injection of [3H]-AIB into ovariectomised, E-implanted rats with ligated renal pedicles. Animals were treated with a) phosphate-buffered saline, b) intrauterine ouabain, and c) intravenous ouabain. Uterine tissue radioactivity concentrations were determined for the injected (■) and non-injected (□) horns. (n = 4)
From the results obtained (Fig. 20), it is apparent that neither 
$[^3]H\text{-AIB}$ nor $[^3]H\text{-mannitol}$ entered the uterine lumen readily since
following intravenous injection of either, the uterine fluid concentra-
tions were significantly less than those in serum at 1 and 4 h post-
injection. In addition, although the uterine fluid concentrations
increased significantly between 1 and 4 h post-injection, these in-
creases were slight ($4.3 \pm 0.4$ to $5.0 \pm 0.7 \text{ mmol/mg} \times 10^{13}$ for $[^3]H$
-AIB and $2.3 \pm 0.2$ to $3.8 \pm 0.5 \text{ mmol/mg} \times 10^{13}$ for $[^3]H\text{-mannitol}$).
However, the data obtained suggest that $[^3]H\text{-AIB}$ entered the uterine
lumen more readily than $[^3]H\text{-mannitol}$ since the uterine fluid concentra-
tions of $[^3]H\text{-AIB}$ were significantly greater than those of $[^3]H\text{-mannitol}$
at each time post-injection. This enhanced ability of $[^3]H\text{-AIB}$ to
enter the uterine lumen is emphasised by the ratios of uterine fluid to
serum concentrations, from which it would appear that the entry of
$[^3]H\text{-AIB}$ was approximately twice that of $[^3]H\text{-mannitol}$. Whether this
difference was due to active transport or facilitated diffusion of
$[^3]H\text{-AIB}$ cannot be concluded from the results of this experiment. However,
the uterine tissue concentrations of $[^3]H\text{-AIB}$ were 2-4 times greater
than those in serum confirming the presence of concentrative, amino
acid uptake mechanisms (Riggs et al., 1972; Walters et al., 1975;
Lindqvist et al., 1977). Furthermore, Walters et al. (1979) have
reported evidence obtained in vitro demonstrating that a ouabain-
sensitive, active transport mechanism for amino acids exists on or near
the uterine luminal surface. Moreover, Walters et al. (1979) suggested
that this transport mechanism exhibits influx and efflux properties but
that the efflux (uterine tissue to lumen) mechanism has a higher
affinity (lower $K_m$) so net transport is from uterine tissue into the lumen.
Thus, it may be that the low uterine fluid concentrations of $[^3H]$-AIB observed in the present study were due to low net transport of $[^3H]$-AIB into the uterine lumen and not due to low efflux. To investigate this possibility, the effects of ouabain treatment on the ability of $[^3H]$-AIB to enter the uterine lumen from blood were examined in the second experiment.

Ouabain treatment, at the doses given either intraluminally or intravenously, apparently enhanced the ability of $[^3H]$-AIB to enter the uterine lumen from serum since with both treatments, the uterine fluid radioactivity concentrations were significantly greater than in vehicle-treated animals. This effect could have resulted from a ouabain-induced inhibition of the active uptake from the uterine lumen of $[^3H]$-AIB transported into the uterine lumen thereby increasing the net transport of $[^3H]$-AIB into the uterine lumen and resulting in greater uterine fluid radioactivity concentrations. Alternatively, since serum radioactivity concentrations were significantly elevated in those animals receiving ouabain intravenously, as compared with control animals, it is possible that the higher uterine fluid radioactivity concentrations were due to increased passive diffusion of $[^3H]$-AIB into the uterine lumen. However, intraluminal ouabain treatment resulted in significantly greater uterine fluid radioactivity concentrations in the absence of significantly elevated serum levels. Furthermore, the presence of a ouabain-sensitive, active transport mechanism for AIB at or near the uterine-luminal surface has been demonstrated (Walters et al., 1979). Moreover, Conner and Miller (1973b) have shown that $[^3H]$-ouabain injected intravenously was able to enter the uterine lumen of estrogen-treated rats. Thus, it is likely that the effect of ouabain
on [$^3$H]-AIB entry from blood into the uterine lumen was a direct one at or near the luminal surface.

Taken together, the results of the present experiments suggest that the apparently restricted entry of [$^3$H]-AIB into the uterine lumen observed in the absence of ouabain treatment was due, in part, to the active removal of [$^3$H]-AIB from the uterine lumen. Hence, it appears that some amino acids can be transported across the blood-uterine lumen barrier. This barrier presumably maintains concentrations of some substances in the uterine lumen which are at variance with those in blood and the surrounding tissue. If so, then the concentrative active transport of substances out of the uterine lumen, as observed for [$^3$H]-AIB in the present experiments, or into the uterine lumen, might be considered to be a component of the blood-uterine lumen barrier.
CHAPTER 9: GENERAL DISCUSSION

The existence of a permeability barrier which restricts or impedes the passage of some substances from blood into the uterine lumen has been demonstrated in ovariectomised, immature rats and in ovariectomised, immature rats treated with various regimens of E and P. Furthermore, the results of certain experiments suggest that this blood-uterine lumen barrier is characterised by exhibiting variable permeability (restrictiveness), modulated by E and P. With E treatment, this barrier becomes less restrictive to $^{14}\text{C}$-urea but more restrictive to $^{3}\text{H}$-sucrose. With P treatment (in the absence of E), the barrier becomes slightly more restrictive to $^{14}\text{C}$-urea. The blood-uterine lumen barrier is characterised further by restricting selectively the passage into the uterine lumen of some substances according to molecular size and/or lipid solubility. For small molecules (mol wt less than 200) it would appear that lipid solubility (as indicated by chloroform/phosphate-buffered saline partition coefficients) is an important factor determining whether their passage into the uterine lumen will be impeded by this barrier. In contrast, for substances of a hydrophilic nature, molecular size (as indicated by molecular weight) is apparently a critical factor involved in determining the influence of this barrier on their abilities to gain access to the uterine lumen. In addition,
suggestive evidence that this barrier is characterised by being able to isolate the uterine lumen from uterine extracellular fluid by virtue of its epithelial, paracellular location has been presented.

Whether a blood-uterine lumen barrier exists in other species has not been investigated directly; however, it is likely, for several reasons, that one does in rabbits. First, Kulangara (1976) demonstrated that following intravenous injection of human serum albumin and human gamma globulin to non-pregnant rabbits, the uterine fluid concentrations of these proteins were 10% or less of those in serum at 12 or 18 h post-injection. This finding is similar to the observation in the present study that $^{125}$I-bovine serum albumin was restricted from entering the uterine lumen following intravenous injection (Fig. 5). Secondly, rabbit uterine fluid obtained shortly after human chorionic gonadotropin treatment resembles rat proestrous uterine fluid and seminiferous tubule fluid by having a higher potassium to sodium ratio than plasma (Lutwak-Mann, 1962a); permeability barriers in rats between blood and the uterine lumen (this study) and seminiferous tubule lumen (Howards et al., 1976) have been demonstrated. Lastly, glucose concentrations in uterine fluid 24 h post-coitum were lower than those in plasma (Lutwak-Mann, 1962b), suggesting that the entry of glucose into the uterine lumen might be restricted by a permeability barrier. However, the possibility that low glucose levels in rabbit uterine fluid were due to net transport out of the lumen or, to utilisation by sperm (Leese et al., 1981) since this measurement was made in post coital rabbits cannot be ruled out. Nevertheless, taken altogether, these observations suggest that a blood-uterine lumen barrier exists in rabbits as well as in rats. For reasons given below, it seems likely that blood-uterine lumen barriers
exist in other species, in addition to rats and rabbits, since the epithelial cells lining cavitary organs such as uterus are characteristically joined by junctional complexes which include tight junctions (Farquhar and Palade, 1963).

The functional significance of a blood-uterine lumen barrier is uncertain, at least partly because what physiological effects perturbations to this barrier might have are unknown. However, the existence of a blood-uterine lumen barrier provides indirect evidence that the uterine luminal environment in rats differs significantly in composition from uterine extracellular fluid since in this species the cerebrospinal (Davson, 1967), rete testis (Voglmaier et al., 1966), and seminiferous tubular (Levine and Maish, 1971; Tuck et al., 1970) fluids all differ significantly from plasma in composition and are separated from plasma by permeability barriers (Davson, 1976; Setchell et al., 1969; Howards et al., 1976, respectively). Because the quantity of uterine fluid present during reproductive stages other than proestrus is so small (Warren, 1938; O'Shea, 1972), accurate determinations of the concentrations of various constituents have not been made, and thus the composition cannot be compared directly with plasma. However, the concept of a special environment (i.e., one which differs from the surrounding milieu) in the uterine lumen of rats is supported by the presence of uterine specific proteins in uterine flushings obtained from pregnant and pseudopregnant animals (Surani, 1975, 1977) and by the composition of proestrus uterine fluid which differs from plasma (Howard and De Feo, 1959; Ringler, 1961). A permeability barrier capable of separating the uterine lumen from the surrounding extracellular milieu might function to maintain a special intraluminal environment.
The question of functional significance of this barrier might be approached by considering the evidence that reproductive events normally occurring in the uterine lumen require a special environment. The finding that rat epididymal sperm incubated in vitro in chemically defined culture medium (designed to resemble extracellular fluid) with ova penetrate the majority of ova within 5 to 6 h and the resulting embryos will develop to term following transfer to appropriately prepared recipients (Toyoda and Chang, 1974b) suggests that sperm capacitation and fertilisation do not require a special environment to proceed. However, Barros (1974) suggested that the time course for sperm capacitation in vivo may differ from that in vitro due to interaction of sperm with complex molecules in the female reproductive tract, and that appropriate timing of this event may be important in regulating the chronology of events leading to fertilisation. Special uterine luminal factors are not required for early embryonic development since eight-cell (in contrast to one-cell; Whittingham, 1975) rat embryos develop to the blastocyst stage in chemically-defined culture medium and will progress to mature fetuses following transfer as blastocysts to recipients (Folstad et al., 1969). However, embryos cultured in vitro exhibit reduced rates of development (Bowman and McLaren, 1970; Van Blerkom et al., 1973; Beier and Maurer, 1975) and decreased viability following transfer to appropriately prepared recipients (Maurer et al., 1970). In addition, altered patterns of carbohydrate (Ozias and Stern, 1973) and protein (Van Blerkom and Manes, 1974) synthesis, and more variable but on average lower carbon dioxide production (Menke and McLaren, 1970) are exhibited by embryos cultured in vitro relative to embryos in utero. Furthermore, two- or four-cell mouse embryos...
cultured *in vitro* for 72 or 96 h appear grossly similar to normal blastocysts but examination with the electron microscope reveals marked ultrastructural differences (McReynolds and Hadek, 1972). Taken altogether, these observations indicate that although development of pre-implantation embryos from morulae to blastocysts can occur *in vitro*, the process is not identical to that occurring *in utero*. One possible interpretation is that specific differences exist between the *in vitro* culture systems employed and the uterine luminal environment and therefore that the uterine environment, by virtue of its composition, might play a facultative role in early embryonic development.

The influence which can be exerted by the uterine luminal environment on reproductive processes is possibly best exemplified by the phenomenon of embryonic quiescence during delayed implantation. Embryos become quiescent when intrauterine conditions are "non-permissive" due to the presence of inhibitory substances (Psychoyos and Bitton-Casimiri, 1969; Weitlauff, 1976, 1978) and/or the absence of required factors (Surani, 1975; Pratt, 1977), and remain so until the appropriate environmental conditions are re-established. Changes in the micromolecular composition of the uterine environment might be of importance in producing permissive conditions since omission of glucose (Naesiund, 1979; Van Blerkom et al., 1979) or leucine and arginine (Gwatkin, 1966) from culture medium containing dialysed serum results in quiescent embryos remaining in growth arrest. Furthermore, dormant and activated embryos incubated for 25 h in 124 mM Na medium and activated embryos in 55 mM Na medium exhibit similar levels of amino acid uptake, whereas inactive embryos incubated in 55 mM Na medium exhibit lower levels which do not differ markedly from those of inactive blastocysts freshly
obtained from uteri (Van Winkle, 1975). These findings suggest that the concentrations of these micromolecules in uterine fluid during delay of implantation might be suboptimal for embryo development and, since quiescent embryos do not remain dormant when incubated in standard culture medium (Torbit and Weitlauf, 1975), that these concentrations might be less than those in plasma. Therefore, embryo activation following termination of delay of implantation might result, in part, from increased concentrations of certain micromolecules in uterine fluid. Presumably, such low molecular weight substances are present in uterine extracellular fluid in concentrations similar to those in plasma. Thus, if changes in their intraluminal concentrations are to play a regulatory role in implantation, then their entry into the uterine lumen must be controlled. One mechanism to accomplish this would be to restrict their entry by passive diffusion into the uterine lumen thereby necessitating specific transport mechanisms controlled by cellular processes and subject to hormonal regulation. Restricted diffusion into the uterine lumen could be achieved by a blood-uterine lumen barrier such as has been demonstrated to exist. Direct evidence for the existence of this barrier during delay of implantation has not been obtained, but the present finding that the barrier exists in animals treated for three days with progesterone only suggests that it probably does. Walters et al. (1981) demonstrated that uptake of \(^{3}H\)3-O-methyl-D-glucose from the uterine lumen into tissue in vitro was increased at 4 and 24 h after in vivo estrogen treatment given to ovariectomized rats. This finding indicates that luminal glucose transport is subject to hormonal control, and suggests that nidatory estrogen might increase the rate of entry of glucose into the uterine lumen. However, this
suggestion is not supported by the observation that, in rats, the rate of entry of glucose into saline recirculated through the uterine lumen did not differ between stages of the estrous cycle (Leese et al., 1979).

In many species, the uterine luminal environment changes during the pre-implantation period of gestation as the total protein content increases and uterine-specific proteins appear (see Chapter 1 for references). This increase in total protein content is partly due to the presence of greater quantities of serum proteins, at least in mice (Aitken, 1977). The results obtained in the present study indicate that a blood-uterine lumen barrier to sucrose exists in rats throughout the endocrine equivalent of this period (Days 4 to 6 of pseudopregnancy). Since this barrier has been found to be selective according to molecular weight and proteins are much larger than sucrose, it is likely that the entry of serum proteins by passive diffusion into the uterine lumen is restricted by this barrier. Therefore, those proteins present in uterine fluid which are not synthesised and secreted by the uterine epithelial cells probably enter by way of a transcellular, pinocytotic mechanism. Parr (1980) observed a three-fold increase in the number of basal pinocytotic invaginations in uterine epithelial cells between Days 4 and 5 of pregnancy which corresponds temporally with the increased protein content of uterine flushings obtained on Day 5 of pregnancy (Surani, 1975). Surani (1977) suggested that the protein with a molecular weight of approximately 70,000 appearing in uterine flushings 1 h after E treatment to rats exhibiting delayed implantation (Surani, 1975) might be derived from serum. The possibility that such a large macromolecule is able to gain access to the uterine lumen from serum (or uterine extracellular fluid) within this short period of
time is unlikely since lower molecular weight substances, such as urea and sucrose, are restricted from entering readily the uterine lumen. Thus, it is more likely that this protein is synthesised by the uterine epithelium and, in response to E, is released into the uterine lumen.

The precise nature of the uterine fluid of rats is not known; however, results obtained in the present study afford some speculation regarding its nature. The finding that low-molecular weight substances, lipophilic in nature, ([\(^{14}\)C]-antipyrine and [\(^{14}\)C]-barbital) are able to enter the uterine lumen without restriction suggests that similar compounds might behave correspondingly. Although not tested, it seems likely that some steroid hormones (estadiol, progesterone, testosterone) might enter the uterine lumen with only minimal restriction due to their lipophilic natures. In support of this are the findings that the blood-brain barrier, like the blood-uterine lumen barrier, selectively restricts some substances according to lipid solubility (Mayer et al., 1959; Brodie et al., 1960), and that steroid hormones with high partition coefficients cross this barrier without restriction (Pardridge and Meitus, 1979). However, several studies have suggested that the actual concentrations of steroids in the cerebrospinal fluid depend not only upon their lipid solubility but also upon the degree of binding to plasma proteins (Backstrom et al., 1976; Marynick et al., 1976; Pardridge and Meitus, 1979). It seems reasonable that the same would apply to the concentrations of steroid hormones in uterine fluid. Setchell et al. (1978) have reported that testosterone crosses the blood-testis barrier in part by facilitated diffusion and is retained in testicular fluids at high concentrations by the presence of a high affinity androgen binding protein (Hansson et al., 1975). A progesterone
binding protein (putatively uteroglobin) has been shown to be present in uterine flushings obtained from rabbits (Cowan et al., 1976; Fowler et al., 1977). Thus, it appears that the actual concentrations of steroids in uterine fluid might be determined by other factors in addition to ability to cross the blood-uterine lumen barrier and serum concentrations. The poor correlation between plasma concentrations and steroid content of uterine flushings following luteinising hormone injection or mating in estrous rabbits (Eiler et al., 1977) bears this out.

Considerable interest has arisen recently (Heap et al., 1979) in the possibility in some species (e.g., pig) that estrogens produced by the blastocysts in utero might be an important factor in the maternal recognition of pregnancy; i.e., the transformation of the corpus luteum of the estrous cycle to the one of pregnancy (Short, 1969). Fundamental to this hypothesis is the demonstration that these hormones are capable of passing from the uterine lumen to their sites of action (endometrium and/or extrauterine sites). On the assumption that the lipophilic test substances employed in this study ([¹⁴C]-antipyrine and [¹⁴C]-barbital) entered the uterine lumen from blood only by passive diffusion down concentration gradients, it seems likely that passage from the uterine lumen to the surrounding tissue along a concentration gradient would be similarly unrestricted. If so, it is probable that unconjugated estrogens present in the uterine lumen are capable of passively diffusing from the lumen into the surrounding medium. However, presumably only unconjugated steroids would have this capacity since conjugation would result in reduced lipid solubility and therefore they might not cross the blood-uterine lumen barrier as readily by virtue of
their molecular size. The present results also suggest that estrogen acting locally at or near the uterine epithelium would enhance its own rate of transfer from the lumen to the surrounding tissue since systemic E treatment was associated with enhanced rates of entry into the uterine lumen of [14C]-antipyrene and [14C]-barbital.

The existence of the blood-uterine lumen barrier is not dependent upon the endocrine milieu; however, its restrictiveness, as indicated by the entrance of urea and sucrose, is apparently subject to hormonal regulation. E only-treatment given to ovariectomised rats is associated with increased permeability of the barrier to urea, reduced morphological complexity of the tight junctions between uterine epithelial cells (Murphy et al., 1981) and the secretion of a copious uterine fluid (see Chapter 1 for references). The fact that these activities occur concurrently is suggestive that they are functionally interrelated. In this regard, Levin and Edwards (1968) hypothesised that, under the influence of E, potassium ions are actively secreted into the uterine lumen and movement of fluid into the lumen occurs as an osmotic consequence of this secretion. The morphological changes in tight junction structure suggest reduced resistance of the paracellular pathway through these junctions (Bentzel et al., 1980; Murphy et al., 1981) which might enhance the entry of urea in addition to facilitating the osmotic fluid flow into the uterine lumen.

A corollary of this proposed mechanism of fluid formation is that the fluid entering the uterine lumen will be primarily a filtrate of uterine extracellular fluid and as such, will have a lower potassium to sodium ratio relative to the known composition of proestrous uterine fluid. This disparity might be accounted for in three ways. First, a
sodium/potassium exchange pump on the luminal surface might reabsorb sodium and secrete potassium thereby increasing the potassium to sodium ratio. Although the existence of a ouabain-sensitive transport mechanism for amino acids at or near the luminal surface has been demonstrated (Walters et al., 1979), instillation of ouabain into the uterine lumen does not affect the sodium or potassium concentrations in uterine fluid (Conner and Miller, 1973c). However, these findings do not prove or disprove that such a pump is involved in determining the composition of proestrous uterine fluid. Secondly, the paracellular pathway through tight junctions might exhibit cationic selectivity (Diamond, 1971) such that potassium enters the uterine lumen more readily than sodium; the ionic selectivity of the uterine epithelium has not been investigated to date. Thirdly, the ratio of potassium to sodium in proestrous uterine fluid might represent dilution by extracellular fluid of a fluid present in the uterine lumen having an even higher potassium to sodium ratio prior to increased conductivity of the epithelial tight junctions. In this regard, it is notable that Linzell and Peaker (1974) proposed that the fluid secreted by goat mammary glands prior to lactation (colostrum) has a lower potassium to sodium ratio than true milk at least partly because the tight junctions of the mammary epithelium are more permeable prior to lactation, thereby allowing partial equilibration between extracellular fluid and the secreted fluid. Whether a similar mechanism is involved in the formation of proestrous uterine fluid is not known.

Permeability of the barrier to urea might, in general, reflect the conductivity of the uterine epithelial tight junctions. If so, the finding that permeability to urea increases during the implantation
period, perhaps due to nidatory estrogen, suggests that the epithelial tight junctions become progressively more conductive. The physiological significance of this change is unknown; however, Martin (1980) postulated that the collapse of the uterine lumen around the blastocyst which occurs prior to attachment might be the result of reabsorption of fluid from the lumen. This process might be facilitated by greater conductivity of the tight junctions.

One interpretation of this study is that the existence of a blood-uterine lumen barrier is incidental to the morphology of the uterine epithelium; that is, this barrier exists by virtue of the tight junctions of the junctional complexes joining the epithelial cells at their apices. The epithelial cells of other organs and tissues such as stomach, intestine, exocrine pancreas, kidney, gall bladder (Farquhar and Palade, 1963), choroid plexus (Brightman and Reese, 1969), seminiferous tubule (Dym and Fawcett, 1970) and mammary glands (Linzell and Peaker, 1971) are also joined by junctional complexes with tight junctions, and in several instances, a permeability barrier across the epithelium has been demonstrated to exist. It seems likely that the same will be true for the others as well, although the characteristics of each might differ in certain aspects. The uterine epithelium shares with these other epithelia the characteristic of active solute secretion coupled by some mechanism to water flow, in addition to the structural similarity of tight junctions. As such, the uterine epithelium can be properly considered to be a transporting epithelium as are the others (Berridge and Oschman, 1972). Our knowledge of the transport physiology of these other epithelia is considerable compared to that of the uterine epithelium. Considerable insight into the
physiology of the uterine epithelium might be afforded by applying to this epithelium, the electrophysiological techniques employed in the study of properties and mechanisms of other transporting epithelia. For example, it has been stated several times in this thesis that during certain reproductive states the nature of the uterine fluid is unknown at least partly due to the paucity of fluid available for analysis. Perhaps this limitation could be overcome in part by accurate analyses of solute fluxes across the epithelium in vitro. Such information might allow reasonable extrapolations regarding the nature of the uterine environment. Once the mechanisms of uterine epithelial secretion are more fully understood, the possibility of applying perturbations to them becomes more feasible; such experiments might provide greater understanding of the functional significance of the uterine luminal environment.

The uterine and seminiferous tubule epithelia, as transporting epithelia, differ dramatically from other epithelia such as gall bladder, intestinal epithelia, etc. in that their transport phenomena are directed towards the creation and maintenance of milieux suitable for the complex biology of developing embryos and sperm, respectively. On the assumption that these milieux differ in composition from the whole organism's internal milieu (extracellular fluid) (studies of the composition of rat proestrous uterine fluid and seminiferous tubule fluid would suggest they do) and that these milieux have evolved to represent near optimal conditions for sperm and embryo development, it follows that these milieux might be homeostatically regulated. That is, there might be homeostatic mechanisms operating to regulate the composition of these luminal environments in the face of dynamic extra-luminal environments.
In support of this concept is the finding that glucose concentration in rabbit uterine fluid does not vary appreciably following intravenous infusion of glucose despite marked changes in systemic blood levels (Lutwak-Mann, 1962b). This concept of homeostatic regulation of the uterine luminal environment seems particularly relevant when considering species of animals which exhibit the reproductive strategy of obligate delay of implantation. In these species, the uterine luminal environment imposes dormancy upon the developing blastocysts which is maintained presumably until such time as delivery will occur during a season favourable to the survival of young (Finn and Porter, 1975). During this period, the uterine luminal environment is obliged to maintain the dormancy but viability of the embryos. Also during this period, the maternal organism presumably is exposed to numerous environmental fluctuations which challenge her own homeostatic mechanisms. It might be that, at times, the degree of regulation achieved by these mechanisms is not sufficient to maintain an appropriate uterine luminal environment for embryonic diapause in which case further mechanisms, possibly at the level of the uterus, would be required.

In conclusion, the physiology of the uterine epithelium and its luminal environment represents a challenge not only to the reproductive physiologist but also to others who are intrigued by problems of transport and/or homeostatic mechanisms.
CHAPTER 10: SUMMARY AND CONCLUSIONS

1. Previous studies indicated that rat progesterous uterine fluid has certain features in common with cerebrospinal fluid and testicular luminal fluids. Since these latter fluids are separated from blood by permeability barriers, it was hypothesised that a permeability barrier between blood and the uterine lumen might exist in rats.

2. The existence of a blood-uterine lumen barrier in rats was investigated by assessing the abilities of selected radiolabelled test substances to enter the uterine lumen from blood. This assessment was accomplished by comparing, at various times post-intravenous injection of a test substance, radioactivity concentrations in serum with those in fluid recovered from the uterine lumen. Prior to the intravenous injections, all animals, except those treated continuously with estradiol (E) only, were given intraluminal injections of phosphate-buffered saline into one uterine horn to facilitate recovery of radioactivity from the uterine lumen.

3. Initial investigations were carried out in immature rats treated with E only or with hormones to mimic early pseudopregnancy. With both hormone treatment regimens, uterine fluid radioactivity concentrations, following intravenous injection of $[^3]$H-sucrose, were low relative to those in serum and did not change markedly with time post-injection. Similar patterns of serum and uterine fluid radioactivity concentrations were observed in E only-treated rats following injection of $[^3]$H-inulin and $[^{125}]$I-bovine serum albumin and in "early pseudopregnant" rats.
following injection of $[^{14}\text{C}]$-urea. Rapid equilibration between serum and uterine fluid radioactivity concentrations was observed with tritiated water with both hormone regimens and with $[^{14}\text{C}]$-urea in E only-treated animals. These findings were interpreted to indicate that the passage from blood into the uterine lumen of some substances (sucrose, inulin or bovine serum albumin) is restricted in E only-treated animals and in "early pseudopregnant" animals by a blood-uterine lumen barrier. Furthermore, it was concluded that this barrier is more restrictive in animals treated with hormones to mimic early pseudopregnancy than in E only-treated animals because the entry of $[^{14}\text{C}]$-urea into the uterine lumen was partially restricted with the former hormone regimen and not with the latter.

4. Further experiments were carried out to investigate the effects of estradiol (E) and progesterone (P) on the restrictiveness of the blood-uterine lumen barrier. The results obtained indicated that the permeability of the blood-uterine lumen barrier is hormonally modulated. Estradiol treatment was associated with increased permeability to $[^{14}\text{C}]$-urea but decreased permeability to $[^{3}\text{H}]$-sucrose; decreased permeability to $[^{14}\text{C}]$-urea was observed with P treatment. In accordance with previous studies, it was suggested that E-induced increased permeability to $[^{14}\text{C}]$-urea might have resulted from decreased restrictiveness of uterine epithelial tight junctions.
5. The time course for an effect of intrauterine (intraluminal) hypertonicity on the permeability of the blood-uterine lumen barrier to $[^{14}\text{C}]$-urea was investigated in $P$ only-treated rats. The uterine fluid radioactivity concentrations, 14 min post-intravenous injection of $[^{14}\text{C}]$-urea, did not differ significantly between groups of animals receiving intrauterine hypertonic mannitol 0, 30, or 60 min prior to the intravenous injection. However, they were significantly lower in animals exposed to hypertonic mannitol for 120 min prior to the intravenous injection. Thus, intrauterine hypertonicity was associated with decreased permeability to $[^{14}\text{C}]$-urea. This finding did not support the hypothesis that the $E$-induced increased permeability to $[^{14}\text{C}]$-urea was due to decreased resistance of uterine epithelial tight junctions.

6. The relative abilities a) of substances of similar lipid solubilities (hydrophilic substances) but with increasing molecular weights and b) of substances of similar molecular weights (range 182-188) but with increasing lipid solubilities to cross the blood-uterine lumen barrier were investigated. a) When $[^{3}\text{H}]$-mannitol (mol wt, 182) was injected intravenously 2 h prior to autopsy, the uterine fluid radioactivity concentrations were significantly greater (by a factor of 2-3) than when either $[^{3}\text{H}]$-sucrose (mol wt, 342) or $[^{3}\text{H}]$-inulin (mol wt, approx. 5,200) was injected. Since the latter substances differ from $[^{3}\text{H}]$-mannitol primarily in having larger molecular sizes, this observation suggested that the blood-uterine lumen barrier impedes selectively the entry into the uterine lumen of some substances according to molecular size.

b) Direct comparisons between $[^{14}\text{C}]$-antipyrine, $[^{14}\text{C}]$-barbital and $[^{3}\text{H}]$-mannitol in $E$ plus $P$- or $P$ only-treated animals showed that $[^{14}\text{C}]$-antipyrine and $[^{14}\text{C}]$-barbital entered the uterine lumen with apparently
minimal restriction whereas $[\text{H}]$-mannitol was essentially restricted. These compounds differ only slightly in molecular weight (range 182-188) but greatly in lipid solubility (chloroform to phosphate-buffered saline partition coefficients: 17.2, 0.23, 0.002, respectively). Hence, this observation suggested that lipid solubility is an important factor determining whether a substance will be restricted from entering the uterine lumen from blood by a permeability barrier. Furthermore, at 5 min post-injection, the uterine fluid radioactivity concentrations were significantly greater with $[\text{C}]$-antipyrine than with $[\text{C}]$-barbital, and with both test substances, in E plus P-treated animals than in P only-treated animals. These observations suggested that lipid solubility and hormone treatment influence rate of entry into the uterine lumen of some substances.

In several experiments, uterine tissue radioactivity concentrations, following intravenous injection of particular test substances, were compared with those in serum in an attempt to ascertain the location within the uterus of the blood-uterine lumen barrier. It was concluded that this barrier is located at or near the uterine epithelium since following intravenous injection of $[\text{C}]$-urea and $[\text{H}]$-sucrose, the serum and uterine tissue radioactivity concentrations (uterine extracellular fluid radioactivity concentrations in the case of $[\text{H}]$-sucrose) did not differ markedly even though, with some hormone treatments, both substances were restricted from the uterine lumen. The ratios of uterine tissue to serum radioactivity concentrations following intravenous injection of $[\text{C}]$-antipyrine and $[\text{C}]$-barbital suggested that these substances, which entered the uterine lumen from blood without restriction, distributed in uterine extracellular and intracellular fluid
compartments. By contrast, [3H]-mannitol, which was essentially restricted from the uterine lumen, distributed primarily in uterine extracellular fluid. The interpretation given to these observations was that the blood-uterine lumen barrier has an extracellular locus. Hence, it was hypothesised that the restricted entry into the uterine lumen of certain substances was due, at least partly, to relative impermeability of the tight junctions joining uterine epithelial cells at their apices. This hypothesis was tested by attempting to visualise with the electron microscope, the ability of lanthanum, a small, electron-opaque tracer, to penetrate into the uterine lumen. The results obtained neither confirmed nor contradicted this hypothesis.

8. The relative abilities of an amino acid analoge, [3H]-α-aminoisobutyric acid ([3H]-AIB), and [3H]-mannitol to enter the uterine lumen from blood were directly compared in a further experiment. [3H]-AIB entered the uterine lumen approximately twice as readily as [3H]-mannitol. The interpretation given to this finding was that [3H]-AIB is transported into the uterine lumen by a carrier mechanism. Hence, an effect of an amino acid transport inhibitor, ouabain, given intravenously or into the uterine lumen, on transport of [3H]-AIB from blood into the uterine lumen was investigated. The results obtained indicated that some of the [3H]-AIB entering the uterine lumen is subsequently transported from the lumen into surrounding tissue by a ouabain-sensitive mechanism.

9. The results obtained in the present study, taken altogether, suggest the following conclusions:

1. A blood-uterine lumen barrier exists in rats.
2. This barrier is characterised by:
   a) exhibiting variable permeability which is hormonally 
      \((E,P)\) modulated
   b) restricting selectively the passage into the uterine 
      lumen of some blood-borne substances according to 
      molecular size and/or lipid solubility
   c) being able to isolate the uterine lumen from uterine 
      extracellular fluid by virtue of its epithelial 
      paracellular location.
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