Implantation-associated Protease in Mouse Uterine Fluid

by

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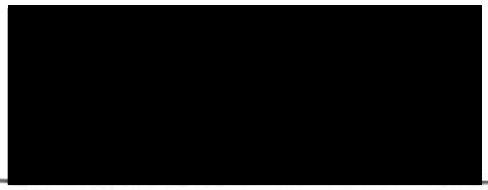

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ABBREVIATIONS

B	amount of BSA 'bound' to the uterus
BANA	α-N-benzoyl-D,L-arginine-β-naphthylamide HCl
BSA	bovine serum albumin
C	centigrade
C_e	BSA concentration in effluent
C_i	initial concentration of BSA
Ci	curie (2.22×10^{12} DPM)
DPM	disintegrations per minute
EU	enzyme unit
g	gravity
GPAN	glutaryl-L-phenylalanyl-β-naphthylamide
h	hour
IIF	Implantation Initiation Factor
I.U.	international unit
i.v.	intravenous
Km	Michaelis-Menten constant
lns	natural logs (base e)
M	Molar
min	minute
MW	molecular weight
P	probability
pH	-log of H^+ concentration
PPO	2, 5-diphenyloxazole
POPOP	ρ-bis-{2-(5-phenyloxazolyl)}-benzene
S.E.M.	standard error of the mean

s.c.	subcutaneous
Tris	tris(hydroxymethyl)aminomethane
V_i	initial volume of flush
V_o	void volume
V_t	salt volume
V_u	volume of uterine fluid
v/v	volume/volume

I. BACKGROUND

A. General

The zona pellucida is a transparent membrane secreted around the developing mammalian oocyte by the granulosa or follicle cells in the ovary (Trujillo-Cenoz and Sotelo, 1959; Bjorkman, 1962). At the time of ovulation, the egg, encased in its zona pellucida and mass of granulosa cells (cummulus mass), passes into the ampulla of the oviduct where fertilization occurs (Austin, 1961). The zygote then undergoes several cleavage divisions as it moves through the oviduct, and it enters the uterus as a blastocyst with approximately 40 cells (Lewis and Wright, 1935; Kiessling and Weitlauf, 1979) still surrounded by the zona pellucida. In mice with uncomplicated pregnancies, implantation is initiated late on the 4th and early on the 5th day following fertilization. During the early stages of this process, the zona pellucida is dissolved (Dickson, 1966; Orsini and McLaren, 1967; McLaren, 1969, 1970; Mintz, 1971, 1972), there is a marked elevation in the metabolic activity of the embryo (Greenwald and Everett, 1959; Brinster, 1967; Mills and Brinster, 1967; Weitlauf and Greenwald, 1967; Ellem and Gwatkin, 1968; Woodland and Graham, 1969; Menke and McLaren, 1970), and the blastocyst, now with approximately 120 cells (Kiessling and Weitlauf, 1979), begins to attach to the uterine epithelium (Potts, 1966, 1968; Finn and McLaren, 1967; Smith and Wilson, 1974). By late on the 5th day after fertilization, most embryos are attached securely enough that they can no longer be dislodged from the uterine epithelium by flushing a stream of saline through the uterine lumen (Mintz, 1971, 1972). It is generally assumed that attachment of mouse embryos to the uterine epithelium cannot occur

unless the zona pellucida is removed.

If mating occurs during the postpartum estrous and the resulting pregnancy is complicated by concurrent lactation, implantation of the newly conceived embryos is delayed for a variable period and is referred to as lactational delayed implantation (Daniel, 1910; Kirkham, 1916, 1918). Even though embryos become free from the zona pellucida during the prolonged free-living phase associated with the lactational delay of implantation, it is by a process of 'hatching' rather than dissolution as evidenced by the finding that empty zonae pellucidae can be recovered along with unattached blastocysts by flushing the uterine lumen (Orsini and McLaren, 1967; McLaren, 1967, 1968, 1970). Lactational delayed implantation can be terminated and implantation initiated by either the removal of the suckling young or an injection of estradiol-17β (Whitten, 1955, 1958; McLaren, 1968, 1970; Mintz, 1971, 1972). A similar delay of implantation occurs when pregnant mice are ovariectomized prior to noon of day 4 of pregnancy and injected daily with progesterone. This is referred to as experimental delayed implantation, and it can be terminated at any time by injecting estradiol-17β in addition to progesterone (Yoshinaga and Adams, 1966; Humphrey, 1967; Kirby, 1967; Smith and Biggers, 1968; Weitlauf and Greenwald, 1968). Embryos have been maintained in this delayed condition for up to 30 days before implantation was initiated (Weitlauf and Greenwald, 1968).

B. Implantation Initiation Factor

It is not known how the initial events of implantation (i.e., dissolution of the zona pellucida and attachment of embryos to the

uterine epithelium) are caused by the ovarian hormones but the Implantation Initiation Factor (IIF) proposed by Mintz (1971, 1972) provides a testable hypothesis for the cause of these events. Mintz proposed that the initiation of implantation in mice is due to a hormone dependent 'zona-lytic' factor that not only dissolves the zona pellucida but also alters glycoproteins on the surface of blastocysts resulting in their attachment to the uterine epithelium. The putative 'zona-lytic' factor is presumed to be a proteolytic enzyme or enzyme complex that is secreted by the uterus. In addition, it was proposed that absence of the 'zona-lytic' factor is the cause of delayed implantation.

The hypothesis was based on a variety of observations. 1) Dissolution of the zona pellucida does not occur when embryos are cultured in vitro (Whitten, 1957; Cole and Paul, 1965; Cole, 1967), transferred to the anterior chamber of the eye (Fawcett, Wislocki, and Waldo, 1947), or are prevented from entering the uterus by a ligature at the uterotubal junction (Orsini and McLaren, 1967). Under these conditions, the embryos 'hatch' and become free from the zona pellucida with the empty zona pellucida remaining intact. In contrast, empty zonae pellucidae in the uterine lumen, as well as those on dead morulae or unfertilized ova if present, are dissolved at the time of implantation (McLaren, 1968, 1969, 1970; Mintz, 1971, 1972). These observations support the suggestion that dissolution of zonae pellucidae at the time of implantation is due to a hormone dependent factor ('zona-lysin') in the uterine lumen rather than a factor from the embryo. 2) The close temparal relationship between dissolution of zonae pellucidae on dead morulae and attachment of blastocysts to the uterine epithelium in the same uterus

(Mintz, 1971, 1972) was used to support the suggestion that the 'zona-lytic' factor also causes attachment of embryos to the uterine epithelium. 3) Changes on the surface of the embryo occur at the time attachment to the epithelium is initiated (Hakansson et al., 1975; Nilsson et al., 1975; Jenkinson and Searle, 1977; Wu and Chang, 1978; Carollo and Weitlauf, 1979; Naeslund and Nilsson, 1979). These observations support the hypothesis that implantation is initiated by a uterine factor that works on the zona pellucida and the surface of the embryo, although conclusive evidence is lacking.

C. Characteristics of the Zona Pellucida

The exact chemical structure of the zona pellucida is not known; however, there is general agreement that it is composed of weakly acidic glycoproteins (Braden, 1952; Stegner and Wartenberg, 1961; Soupart and Noyes, 1964). It has been estimated that 96% of the hydrated zona pellucida is water and that 30% of the dry mass is protein. Carbohydrate is also present and presumably accounts for the remaining dry mass since lipid was not detected (Loewenstein and Cohen, 1964). More recently, it was shown that the mouse zona pellucida consists of 3 components with apparent molecular sizes of 200,000 daltons, 120,000 daltons, and 83,000 daltons. Each of the separate molecular entities contained carbohydrate and accounted for 36%, 47%, and 17% of the total protein, respectively (Bleil and Wassarman, 1978).

Dissolution of the zona pellucida can be obtained under a variety of conditions in vitro. Incubation of zonae pellucidae in buffer with a hydrogen ion concentration greater than pH 5 (Hall, 1935; Gwatkin,

1964; Bowman and McLaren, 1969; Inoue and Wolf, 1974), at temperatures greater than 70° C (Cholewa-Stewart and Massaro, 1972), or with guanidine HCl, sodium thiocyanate, sodium periodate, mercaptoethanol, and dithiothreitol (Domon et al., 1973; Inoue and Wolf, 1974) all lead to complete dissolution. The zona pellucida is also susceptible to enzymatic degradation, but so far only the proteases trypsin, chymotrypsin, ficin, and pronase have been effective (Smithberg, 1953; Mintz, 1962; Gwatkin, 1964; Bowman and McLaren, 1970). Enzymes that are ineffective include the proteases papain, elastase, collagenase, and the nonprotease enzymes lysozyme, α-glucosidase, β-glucuronidase, hyaluronidase, neuraminadase, lecithinase D, β-lipase, and ribonuclease (Mintz, 1962, Gwatkin, 1964; Bowman and McLaren, 1970). Thus, it appears that dissolution of zonae pellucidae in vitro occurs with conditions that interfere with either hydrogen bonds (pH, heat, thiocyanate, guanidine), sulfhydral bonds (mercaptoethanol, dithiothreitol), or peptide bonds (trypsin, chymotrypsin, ficin, pronase). These observations support the suggestion that dissolution of the zona pellucida in vivo, as well as the alterations of glycoproteins on the embryo surface, are most probably due to proteolytic enzymes (Bowman and McLaren, 1970; Pinsker et al., 1974) as envisioned in the IIF hypothesis.

D. Uterine Proteases and Peptidases

Several hormone dependent proteases and peptidases have been found in mammalian uteri and thus might act as the IIF. In early studies, hormone dependent peptidases and aminopeptidases were found in homogenates of rat, rabbit, and human uteri (Smith, 1948a, b;

Schmidt et al., 1967, 1969; Albers et al., 1961). The location of these enzymes in situ was not known. Berstrom (1970) used a gelatin film technique to test sections of mouse uteri collected on days 3, 5, and 9 of pregnancy and demonstrated that protease activity was present in the uterus on days 5 and 9 of pregnancy but not day 3. The gelatin hydrolyzing enzyme was at the site of implantation and in the adjacent epithelium. The activity was specific to the uterus since no activity was detected with either rat or mouse embryos assayed by the method (Blandau, 1949; Bergstrom, 1970).

Lammes (1963), using the substrate L-leucyl-β-naphthylamide and a histochemical technique, described an enzyme activity with levels that were higher on day 6 of pseudopregnancy than those on days 3 and 9. This enzyme was localized to the apical region of the glandular epithelium. Subsequently, the same substrate has been used to describe two different enzyme activities, one enzyme is associated with particles in the glandular epithelium and the other is associated with vacuoles both in the luminal epithelium and at the site of implantation (Bergstrom, 1972). Maximal activity for the vacuolar enzyme was found on day 5 of pregnancy, supporting the suggestion by Bergstrom (1972) that the enzyme in the luminal epithelium contributes to the breakdown of uterine secretions before the attachment reaction at the time of implantation.

Joshi et al. (1970) isolated an endopeptidase from the uterine fluid of proestrous rats that hydrolyzes polyalanine but not trypsin or chymotrypsin substrates. Immunological studies with antiserum developed against the enzyme activity indicated that the antigen (i.e., the enzyme) was localized in the luminal and glandular epithelium

of the rat uterus, with increased amounts present during estrus and proestrus in cyclic animals and during days 5 and 6 in both pregnant and pseudopregnant animals. This enzyme was present in the proximal portion of the rat oviduct and also in the luminal and glandular epithelium of mice uteri but was not detected in the ovary, vagina, pancreas, liver, spleen, intestine, or blood (Joshi and Murray, 1974; Rosenfeld and Joshi, 1977). In addition, enzyme activity was found in uterine flushings of mice treated with estradiol-17β for 4 days (Joshi and Rosenfeld, 1976). The finding that zonae pellucidae immunofluoresced when preincubated with the purified peptidase followed by anitpeptidase-antibody conjugated to fluorescein supports the suggestion that this enzyme may be involved with the lysis of zonae pellucidae at the time of implantation but does not prove it (Rosenfeld and Joshi, 1977).

Pinsker et al. (1974) demonstrated proteinase activity in uterine washings collected from pregnant mice. Using casein as a substrate, they found relatively little activity during diestrus and proestrus with slightly more activity at proestrus. However, the level of enzyme activity increased after mating, reached peak levels on the day of implantation, and then decreased.

Thus, there is presumptive evidence to support the hypothesis that a hormone dependent protease or peptidase activity in the uterine lumen is involved with the dissolution of zonae pellucidae and the initiation of embryo attachment. However, no investigation has clearly demonstrated that a particular enzyme activity is responsible for these events during implantation.

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II. STATEMENT OF THE PROBLEM

It has been hypothesized that a hormone dependent protease activity in the uterine lumen is essential for implantation in mice (Mintz, 1971, 1972; Pinsker et al., 1974). It is envisioned that an elevated level of enzyme activity causes dissolution of the glycoprotein zona pellucida and produces alterations of the glycoproteins on the embryo surface which result in attachment of blastocysts to the uterine epithelium. Furthermore, it is hypothesized that the level of enzyme activity is low during the prolonged preimplantation period associated with delayed implantation since neither dissolution of zonae pellucidae nor attachment of embryos to the uterine epithelium occur during the period that implantation is delayed.

Identification and a critical evaluation of the endocrine dependence of proteolytic enzyme activity in uterine fluid have not been undertaken. The purpose of the experiments presented in this thesis was to determine whether hormone dependent chymotrypsin-like and trypsin-like enzymes are present in uterine fluid of mice and, thus, could be responsible for lysis of the zona pellucida and initiation of attachment of the embryos to the uterine epithelium. This was done by determining whether:

i) the levels of enzyme activity in uterine flushings are influenced by estradiol-17β and progesterone.

ii) changes in the levels of enzyme activity in uterine flushings reflect changes in the concentration of enzyme activity in utero.

iii) lysis of zonae pellucidae and initiation of embryo attachment to the uterine epithelium are coincident with increased concentrations of enzyme activity.

iv) enzyme activity is separable from 'zona-lytic' activity in vitro using molecular seiving techniques.

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III. Manuscript 1.

The effect of estradiol-17β and progesterone on the level of amidase activity in fluid flushed from the uteri of ovariectomized mice.

ABSTRACT

The effects of estradiol-17β and progesterone on the level of chymotrypsin-like amidase activity in fluid flushed from the uteri of ovariectomized mice were estimated by hydrolysis of the peptide homolog glutaryl-L-phenylalanyl-β-naphthylamide (GPAN). Mice were ovariectomized and injected with either progesterone alone, progesterone followed by a combination of estradiol-17β and progesterone, or estradiol-17β alone. The level of amidase activity was found to be low and constant for several days in animals receiving progesterone alone; the addition of estradiol-17β to the progesterone treatment caused an increase in amidase activity that reached maximum in 18 h and then returned to the baseline level. In contrast, the level of amidase activity increased and remained elevated following the injection of estradiol-17β alone. A similar experiment was done with α-N-benzoyl-DL-arginine-β-naphthylamide HCl (BANA) to estimate the level of amidase with trypsin-like activity: no trypsin-like amidase activity was detected. These findings demonstrate that estradiol-17β is capable of increasing chymotrypsin-like amidase activity in the uterine lumen and that progesterone alters the pattern of this response. The possible implications of hormone regulation of amidase activity in the uterine lumen are discussed with respect to normal and delayed implantation.

INTRODUCTION

Mintz (1970, 1972) proposed that implantation in mice is initiated by a hormone dependent uterine factor that lyses the zona pellucida and alters the surface of blastocysts causing their attachment to the uterine wall. More recently, it was reported that the level of proteolytic activity in the uteri of mice is elevated on the fourth day of pregnancy (Pinsker et al., 1974). Because lysis of the zonae pellucidae and attachment of blastocysts to the uterine epithelium were temporally related to the peak in enzyme activity, it was suggested that a protease might be responsible for these events and therefore actually be the so-called 'implantation initiation factor' (Pinsker et al., 1974). As part of an evaluation of this possibility, the present experiments were undertaken to determine whether or not ovarian steroids can regulate the level of proteolytic enzyme activity in the uteri of mice.

MATERIALS AND METHODS

Virgin female white Swiss mice (6-8 weeks old) were selected at random stages of the estrous cycle, ovariectomized via dorso-lateral incisions, and allowed to recover for 10 days. The animals were allotted randomly to one of four groups and treated with the hormone regimens shown in Table 1. All hormones were injected s.c. in 0.1 ml of sesame seed oil at 1200 h (day 1 = first day of treatment). Animals were selected at random from each treatment group at the times indicated in Table 1 and killed by cervical dislocation. Their uteri were excised, the mesenteries and fat were removed, and ice cold Kreb's Ringer bicarbonate solution (150 µl) was flushed through the lumen of one uterine

horn by means of a blunt 27 gauge needle attached to a 1 ml syringe. A second blunt needle was inserted into the other end of the uterine horn in such a way that the effluent could be collected in a siliconized depression slide without contamination from the cut ends of the horn (Weitlauf, 1976). The fluid recovered from one uterine horn was flushed through the other horn of the same animal. Several uteri were selected at random from each group and prepared for histological examination. Flushings from the uteri of three animals from each treatment group at each time interval were pooled and centrifuged for 1 h at 59,000 x g to remove debris. All steps were carried out at 4° C. The volume of the supernatant was recorded and a portion of the fluid was immediately assayed for amidase activity by a modification of the method of Blackwood et al. (1965); although this was originally described as a method for estimating chymotrypsin-like proteases, the activity measured is more specifically that of a chymotrypsin-like amidase and is so designated in this paper. The remaining fluid was stored (-70° C) and later assayed for protein content by the method of Lowry et al. (1951).

β-naphthylamine liberated from glutaryl-L-phenylalanyl-β-naphthylamide (GPAN) (CalBiochem Lot No. 63825) was measured to estimate chymotrypsin-like amidase activity. Fifty µl of uterine flushings were added to 1.0 ml of the following solution made fresh for each assay; 2.43×10^{-3} M GPAN; 12.5% (v/v) ethylene glycol monoethyl ether, 1.25×10^{-2} M Tris-HCl buffer (pH 7.2), and 6.5×10^{-3} M $CaCl_2$. Fluorescence (excitation 338 nm, emission 410 nm) was monitored discontinuously for 30 to 40 min at 37° C with a Farrand Fluorometer

(Model 2A). All samples were analyzed in duplicate. Amidase activity/ µg protein was determined from the slope of the line generated by regression analysis of fluorescence versus time divided by the µg protein in the assay mixture and is presented as Enzyme Units/µg protein. One Enzyme Unit (EU) is defined as the increase in fluorescence detected upon hydrolysis of 50.0 ρMole GPAN/min. The assay was linear with respect to both enzyme concentration and time. Standard samples of uterine flushings with 'high' and 'low' activity as well as 3X crystallized bovine pancreatic α-chymotrypsin (Sigma, Type II) were run with each assay to assess reproducibility; intra-assay coefficient of variation was less than 15% and inter-assay coefficient of variation was less than 20% (Snedecor and Cochran, 1967).

The data were evaluated by a two-way analysis of variance with replicates (Snedecor and Cochran, 1967) after log transformation to achieve homogeneity of variance. Duncan's multiple range test (Steel and Torrie, 1960) was used to compare differences between treatments at each time point.

Chymotrypsin-like amidase activity was determined at four concentrations of GPAN (1.23×10^{-3} M, 7.41×10^{-4} M, 4.94×10^{-4} M, and 2.40×10^{-4} M) to estimate the Km (Dixon and Webb, 1964) for the enzyme in each treatment group at several collection times (day 3, 1200-2400 h; day 4, 0600 and 1200 h). Slopes (Kms) of activity vs activity/ [substrate] were compared by analysis of covariance (Snedecor and Cochran, 1967).

Trypsin-like amidase activity was estimated in uterine flushings by measurement of β-naphthylamine liberated from α-N-benzoyl-DL-

arginine-β-naphthylamide HCl (BANA, Sigma) (Blackwood and Mandl, 1961). Fluid collection and assay conditions were as outlined for the assay of chymotrypsin-like amidase with the following exceptions: 1) substrate concentration (2.27 x 10^{-3} M BANA); and 2) pH (pH 7.6).

Uterine flushings were tested for inhibition of trypsin by incubating 50 µl of the sample with 10 µl of a trypsin solution (20 µg/ml) for 10 minutes (25° C) followed by the addition of 1 ml of the BANA substrate solution. Activity was compared with that of trypsin incubated with an equal volume of Kreb's Ringer bicarbonate solution.

RESULTS

The effects of estradiol-17β and progesterone on chymotrypsin-like amidase activity in uterine fluid are summarized in Table 1. A significant treatment effect and time-treatment interaction ($P < 0.01$) were found. Following treatment with progesterone alone (group 1), the specific activity of the enzyme was low and did not change significantly over the four days of treatment. In addition, the specific activity was comparable to that in control animals injected with the oil vehicle alone. In contrast, the level of amidase activity was significantly increased at 12 h and 18 h after the injection of progesterone combined with estradiol-17β (i.e., group 2, day 3 at 2400 h and day 4 at 0600 h) and then returned to near control values within 24 h (i.e., group 2, day 4 at 1200 h). The second injection of estradiol-17β and progesterone on day 4 did not cause another increase in the level of amidase activity.

In animals given oil alone (group 3) the specific activity of chymotrypsin-like amidase was low and did not change with time.

In animals given estradiol-17β alone (group 4), amidase activity increased within 12 h and was similar to the level in animals given both estradiol-17β and progesterone (group 2). However, instead of returning to baseline values, the level of activity continued to increase and a second injection of estradiol-17β on day 4 maintained the elevated levels of enzyme activity.

The apparent Kms did not change significantly in any treatment group nor was there a significant difference between treatment groups at any interval (overall mean and S.E.M.; $5.69 \times 10^{-4} \pm 0.44$ M GPAN, n = 16). Serum was collected at each time interval for each treatment group and assayed for amidase activity with substrate specificity similar to that of chymotrypsin. The specific activity of the serum enzyme(s) was less than that in uterine fluid and was not significantly affected by either time or treatment (overall mean and S.E.M.; 0.0036 ± 0.00006 EU/μg protein, n = 72).

Amidase with activity similar to that of trypsin could not be detected; if a trypsin-like enzyme is present in uterine fluid of mice, it is below the sensitivity of the present assay (i.e., less than the equivalent of 40 ng bovine pancreatic trypsin per uterus).

Identical activities were obtained for trypsin preincubated with uterine flushings and trypsin preincubated with an equal volume of Kreb's Ringer bicarbonate solution; thus, inhibitors to trypsin were not demonstrated in the uterine flushings.

DISCUSSION

The present results demonstrate that estrogen and progesterone regulate the level of activity of an amidase, with substrate specifi-

city similar to that of chymotrypsin, in the uteri of ovariectomized mice. The level of enzyme activity is low in the fluid flushed from the uteri of animals injected with progesterone alone; it increases, but only transiently, if estradiol-17β treatment is superimposed on progesterone treatment; and it increases, and remains elevated, in animals injected with estrogen alone. Thus, it appears that although estradiol-17β is capable of increasing amidase activity in the uterus, the response is significantly modified by progesterone. Although these results are compatible with the observations on pregnant mice reported previously by Pinsker et al. (1974), it is not yet known whether or not the enzyme(s) measured in the two studies are the same.

Changes in the activity of chymotrypsin-like enzyme (i.e., expressed as activity/μg protein) in uterine fluid could be the result of either: 1) changes in inhibitors to the enzyme (Van Winkle et al., 1973); 2) changes in the amount of other uterine proteins relative to the enzyme; or 3) changes in the amount of enzyme. Since the apparent Michaelis-Menten constant (Km) for the chymotrypsin-like amidase did not change significantly with either hormone regimens or time, it seems unlikely that changes in competitive inhibitors of chymotrypsin-like enzyme(s) are responsible for the different levels of activity observed. Furthermore, analysis of the present results on the basis of activity/uterus (not shown) rather than as activity/μg protein gave essentially the same pattern as that shown for specific activity, suggesting that differences in amidase activity are the result of changes in the amount of enzyme in the uterus and not due to changes in the relative amount of other uterine proteins.

Since serum levels do not change with either time or treatment, it seems unlikely that the effect is a generalized or systemic response to the hormones.

The source of the enzyme responsible for the hydrolysis of GPAN in the uterine fluid cannot be determined from the present experiment. It seems improbable that it is from disrupted epithelial cells because histologic examination of the uteri revealed that the epithelium was intact. However, since no attempt was made to characterize either the serum or uterine enzyme activities beyond the ability to hydrolyse GPAN, it is not known if the uterine lumenal amidase is derived from a transudate of serum or secreted by the endometrium.

Various kinds of hormone dependent peptidase or protease activity have been found in uterine fluid. Rosenfeld and Joshi (1976), using an antibody developed against a rat uterine peptidase, reported that a similar peptidase appears in mouse uterine fluid following four days of treatment with estradiol-17β but not with progesterone. Treatment with a combination of estradiol-17β and progesterone resulted in a suppression of this enzyme. In contrast, treatment with either estradiol-17β or progesterone lowers the level of mouse uterine fluid plasminogen-dependent fibrinolytic activity (Harpel et al., 1966; Harpel, 1968). Bergstrom (1970), using sections from 5 to 9 day pregnant mouse uteri, reported that the epithelium caused areas of lysis when laid in a gelatin film, whereas, sections from day 3 pregnant mouse uteri showed no lysis suggesting the presence of a protease associated with hormone changes of early pregnancy. In these experiments, as in the present case, it has not been possible to demonstrate that

the enzymes are actually involved in any specific way with implantation.

Pinsker et al. (1974) stated that protease in the uterus might act as an implantation initiating factor. In their view, increased levels of proteolytic activity could be related to changes on the blastocyst surface and thus induce attachment of embryos to the uterine wall. The present results with fluid from animals given progesterone alone or estradiol-17β combined with progesterone are compatible with such a possibility. They demonstrate that blastocysts would be exposed to little chymotrypsin-like amidase activity during the prolonged preimplantation phase associated with delayed implantation and would be exposed briefly to high levels of this amidase activity a few h before implantation. However, amidase activity is also elevated in animals given estradiol-17β alone and this treatment is not sufficient to cause attachment of the embryos (unpublished results). Therefore, if chymotrypsin-like amidase activity is involved in initiating implantation, as envisioned by Pinsker et al. (1974), the process is more complex than a simple increase in the level of enzymatic activity.

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Table 1

Effect of Estrogen and Progesterone on the Specific Activity of

Chymotrypsin-like Amidase in Fluid Flushed from the Uteri of Ovariectomized Mice*

Hormone Treatment Group**	Time of Fluid Collection Day: 1 Hour: 1200	2 1200	3 1200	3 1800	3 2400	4 0600	4 1200	4 2400	5 1200
1	$0.34 \pm 0.08^{a***}$ (4)	0.36 ± 0.02^{a} (6)	0.29 ± 0.05^{a} (6)	0.27 ± 0.05^{a} (6)	0.22 ± 0.07^{a} (5)	0.30 ± 0.06^{a} (5)	0.22 ± 0.07^{a} (5)	0.26 ± 0.11^{a} (5)	0.12 ± 0.04^{c} (4)
2			0.29 ± 0.05^{a} (6)	0.44 ± 0.08^{a} (6)	0.75 ± 0.18^{b} (6)	1.38 ± 0.38^{b} (5)	0.45 ± 0.15^{a} (5)	0.58 ± 0.23^{a} (5)	0.56 ± 0.23^{a} (4)
3	0.45 ± 0.17^{a} (4)	0.40 ± 0.11^{a} (5)	0.40 ± 0.10^{a} (4)	0.34 ± 0.04^{a} (5)	0.23 ± 0.06^{a} (5)	0.27 ± 0.09^{a} (4)	0.42 ± 0.22^{a} (4)	0.46 ± 0.18^{a} (4)	0.24 ± 0.04^{ac} (3)
4			0.50 ± 0.09^{a} (7)	0.32 ± 0.04^{a} (7)	0.92 ± 0.22^{b} (7)	1.23 ± 0.40^{b} (6)	1.58 ± 0.55^{b} (6)	1.25 ± 0.28^{b} (6)	1.87 ± 0.44^{b} (7)

*Enzyme units/μg protein, one Enzyme Unit is defined as the increase in fluorescence detected upon hydrolysis of 50.0 ρMole GPAN/min.

**Hormones injected s.c. in 0.1 ml sesame seed oil daily at 1200 hrs (i.e., days 1,2,3, & 4).

Group 1: Progesterone (2.0 mg) on days 1-4.

Group 2: Progesterone (2.0 mg) on days 1 and 2 and progesterone (2.0 mg) combined with estradiol-17β (25.0 ng) on days 3 and 4.

Group 3: Sesame seed oil only (0.1 ml) on days 1-4.

Group 4: Sesame seed oil (0.1 ml) on days 1 and 2 and estradiol-17β (25.0 ng) on days 3 and 4.

***Mean ± S.E.M. (number of replicates). Duncan's multiple range test was used to detect significant differences between treatments at each collection time: superscripts with a letter in common designate means that are not statistically different ($p > 0.05$); conversely, the lack of a common letter in the superscript indicates means that are statistically different ($p < 0.05$).

IV. Manuscript 2.

The volume of uterine fluid in 'implanting' and 'delayed implanting' mice.

ABSTRACT

A variation of the dye dilution technique was used to determine the volume of uterine fluid in 'implanting' and 'delayed implanting' mice. The method used involves rinsing Kreb's Ringer bicarbonate buffer containing $^{14}C(CH_3-)$BSA through the uterine lumen and using the resulting decrease in the concentration of BSA to calculate the volume of uterine fluid. The results indicate that the volume of uterine fluid is essentially the same (i.e., between 300 and 400 nl/pair of uterine horns) and, therefore, that the total amounts of a substance recovered by rinsing the uteri of 'implanting' and 'delayed implanting' mice provides an estimate of relative concentrations in situ.

INTRODUCTION

It has been suggested that humoral factors in uterine fluid are involved in regulation of the metabolic activity of blastocysts (Daniel and Krishnan, 1969; Mintz, 1970, 1972; McLaren, 1973; Aitken, 1977b; Enders and Given, 1977; Surani, 1977). In attempts to test this hypothesis, several investigators have compared the amounts of various substances in fluid rinsed from the uteri of 'delayed implanting' animals (i.e., where the embryos are metabolically dormant) and 'implanting' animals (i.e., where the embryos are metabolically active). For example, it has been shown that the total amount of protein in uterine fluid during delayed implantation is small and that it increases as the embryos are activated to implant in mice (Aitken, 1977a), rats (Surani, 1975, 1976), roe deer (Aitken, 1974a), mink (Daniel, 1971), western spotted skunks (Mead, Rourke, and Swannack, 1979), northern fur seals (Daniel and Krishnan, 1969), and tammar wallabys (Tyndale-Biscoe, Hearn, and Renfree, 1974). Similar observations have been made with enzymes that are present in uterine fluid, including endopeptidase in the rat (Joshi and Rosenfeld, 1976; Rosenfeld and Joshi, 1977) and protease (Pinsker, Sacco, and Mintz, 1978) as well as chymotrypsin-like amidase activity (Hoversland and Weitlauf, 1978) in the mouse. In addition, Aitken (1974b, 1976) reported similar increases in the total amount of hexose, fructose, alpha-amino nitrogen, and calcium as 'delayed implantation' is terminated in the roe deer.

However, before such data can be used to support the hypothesis that a change in the amount of a particular substance in uterine

fluid might influence embryonic metabolism, it must be shown that the change actually reflects a different concentration in situ; this cannot be done without an estimate of the original volume of uterine fluid. Therefore, the present experiments were undertaken to determine the volume of uterine fluid in 'delayed implanting' and 'implanting' mice.

MATERIALS AND METHODS

Virgin female white Swiss mice (Lab Supply, 6 - 8 weeks old) were selected at random stages of the estrous cycle and ovariectomized via a dorso-lateral incision. They were allowed to recover for 10 days, divided into 3 groups, and treated with ovarian hormones according to the schedule in Table 1: Group 1 - progesterone alone (i.e., conditions for delayed implantation); Group 2 - progesterone alone followed by a combination of estradiol-17β and progesterone (i.e., conditions for implantation, Weitlauf and Greenwald, 1965); Group 3 - estradiol-17β alone. Animals were killed by cervical dislocation at the times indicated in Table 1. Their uteri were removed, trimmed free of mesenteries and fat, and each horn was separated from the cervix.

The volume of uterine fluid in each pair of uterine horns was estimated by the dilution of $^{14}C(CH_3-)$BSA (20.0 μCi/mg BSA, New England Nuclear) in a Kreb's Ringer bicarbonate buffer that was rinsed through the uteri. The volume of fluid in each pair of uterine horns (V_u) was calculated from the following formula:

$$V_u = \frac{V_i C_i - (V_i C_e + B)}{C_e}$$

where V_i is the original volume of buffer instilled into the uterine

lumen; C_i is the concentration of $^{14}C(CH_3-)$BSA in the original buffer solution; C_e is the concentration of $^{14}C(CH_3-)$BSA in the buffer solution after dilution by uterine fluid (i.e., effluent from the uterine rinse); B is the amount of $^{14}C(CH_3-)$BSA 'bound' to the uterine horns.

Buffered isotope solution was prepared by adding $^{14}C(CH_3-)$BSA (1 mg/ml) Kreb's Ringer bicarbonate buffer (pH 7.4) containing 1 mg/ml of nonlabelled BSA (final specific activity of 1.0 - 1.2 µCi/mg BSA). The solution was then divided into aliquots and stored at -20° C until used. The concentration of the $^{14}C(CH_3-)$BSA (C_i) in the buffer was confirmed before the rinse procedure by pipetting 4 or 5 µl (± 1%, Dade pipettes) of the buffer into a scintillation vial containing 0.5 ml of distilled water followed by 5 ml of scintillation cocktail containing 0.4% PPO, 0.004% POPOP, and 25.0% Triton X-100; radioactivity was estimated with a Beckman LS-230 Scintillation Counter (90% efficiency). The intra-assay coefficient of variation for these determinations was 1.5% (n = 4) and the inter-assay coefficient of variation was 3% (n = 4).

Each uterine horn was flushed with between 3 and 4 µl of the buffer followed by a 45 µl bolus of air to purge the lumen. Buffer and air were injected into the lumen near the utero-tubal junction with a 50 µl Hamilton syringe and 25 gauge needle. The effluent buffer solution, diluted by uterine fluid, was collected via a cannula (made from a blunt hypodermic needle) inserted into the cervical end of the uterine horn and held in place by a modified hemostat (notched to accommodate the cannula) and bibulous paper cushion: total time in utero was 5 - 6 seconds. The effluent from both uterine horns

of each animal were pooled, 4 or 5 µl of the effluent was pipetted into a scintillation vial, and the amount of $^{14}C(CH_3-)$BSA per µl of effluent (C_e) was determined.

To estimate the amount of $^{14}C(CH_3-)$BSA 'bound' to the uterus (B), 400 µl of PBS (0^{o}-4^{o} C, pH 7.4, containing 1 mg/ml of nonlabelled BSA) was first flushed through each horn to remove unbound residual $^{14}C(CH_3-)$BSA in the lumen. The residual collected from both horns of one animal was placed in a scintillation vial and the amount of radioactivity determined. Each pair of uterine horns were then dissolved in 2 ml of NCS ('tissue solubilizer', Amersham) and the amount of radioactivity 'bound' to the epithelium (B) was determined.

For purposes of calculation, the volume of buffer rinsed through each uterus (V_i) was assumed to be equal to the total radioactivity instilled into the uterine lumen (summation of the $^{14}C(CH_3-)$BSA in the effluent, the residual, and that 'bound' to the uterus) divided by the original concentration of $^{14}C(CH_3-)$BSA in the buffer (C_i).

Calculated volumes of uterine fluid were transformed to lns, analyzed for heteroscedasticity of variance by Bartlett's test, then evaluated for significant differences with a 2-way analysis of variance (Sokol and Rohlf, 1969) followed by Duncan's multiple range test (Steel and Torrie, 1960). A similar procedure was used to determine significant differences in the amount of BSA that was 'bound' to the uterine epithelium.

RESULTS

In preliminary experiments undertaken to confirm that measurement of the dilution of $^{14}C(CH_3-)$BSA would detect small differences in

the volume of diluent, a standard curve was made by pipetting 0.5, 1.0, 2.5, 5.0, 10.0, and 15.0 µl of diluent into 50 µl aliquots of buffer containing $^{14}C(CH_3-)$BSA. The concentration of $^{14}C(CH_3-)$BSA was then determined by measuring the radioactivity in 5 µl aliquots of each dilution. It was found that 1.0 µl of diluent resulted in a significant decrease in the concentration of $^{14}C(CH_3-)$BSA ($P < 0.05$), whereas 0.5 µl of diluent did not ($P > 0.05$). Thus, a change in the concentration of BSA of less than 1.9% represents the lower limit of sensitivity for the assay.

The volume of uterine fluid in 'delayed implanting' mice was found to be unchanged during the 3 days of treatment. Mean volumes ranged from 277 to 369 nl of uterine fluid per pair of uterine horns (Table 1, Group 1, $P > 0.05$). Furthermore, the volume of uterine fluid was found to be unchanged as the dormant phase of 'delayed implantation' was terminated by the injection of estradiol-17β (Table 1, Group 2, $P > 0.05$); the volumes of uterine fluid in 'delayed implanting' and 'implanting' animals were not significantly different ($P > 0.05$). In contrast, the volume of uterine fluid increased significantly in animals injected with estradiol-17β alone. The increase in volume was statistically significant within 18 hours after the first injection of estradiol-17β (Table 1, Group 3, compare Day 3 1200 h to Day 4 0600 h, $P < 0.05$). A second injection of estradiol-17β caused a further increase in the volume of uterine fluid (Table 1, Group 3, Day 4 1200 h to Day 5 1200 h). Data collected on Day 5 1200 h is shown in Table 1 but was not included in the statistical analysis due to heteroscedasticity that could not be corrected by

transformation to lns.

As shown in Table 2, the amount of BSA that was 'bound' to the uterus increased in all three treatment groups ($P < 0.05$): treatment with estradiol-17β, with or without progesterone (Groups 2 and 3), resulted in an increase by Day 4, whereas treatment with progesterone (Group 1) resulted in an increase between Days 4 (0600 h) and 5 (1200 h). In addition, there was a greater amount of BSA 'bound' following treatment with a combination of progesterone and estradiol-17β (Group 2, $P < 0.05$) than with estradiol-17β alone (Group 3).

DISCUSSION

The results of the present experiments demonstrate that although exogenous estradiol-17β and progesterone influence the volume of fluid in the uteri of ovariectomized mice the volume is not different in 'implanting' and 'delayed implanting' mice.

In the present isotope dilution assay, the limiting sensitivity of the measurement of the concentration of $^{14}C(CH_3-)$BSA and loss of isotope by nondilution mechanisms were examined. The assay would detect changes in the concentration of BSA of less than 1.9% and thus, was sensitive to less than 152 nl of uterine fluid when 8 µl of isotope solution was instilled into the uterus. To correct for the effect of isotope 'binding' to the uterus and resulting in an overestimate of dilution, the amount of $^{14}C(CH_3-)$BSA 'bound' to each pair of uterine horns was determined and accounted for in the calculation of the volume of uterine fluid. Even if such a procedure was omitted, the estimates of the volume of fluid in the uteri of 'delayed implanting' and 'implanting' animals would be increased

by approximately 125 nl for every 100 ng of BSA 'bound' and this difference would not alter the conclusions.

The amount of albumin that was 'bound' increased in each of the three hormone treatment groups (Table 2) and was hormone dependent, with significantly less albumin 'bound' following treatment with estradiol-17β alone (Group 3) than with a combination of progesterone and estradiol-17β (Group 2). From the present experiments, there was insufficient information to determine whether the increase in albumin 'bound' was due to the incorporation of tracer into pinopods on the surface of the epithelium (Enders and Nelson, 1974; Parr and Parr, 1977), changes in the morphology of the luminal surface (Nilsson, 1966; Smith and Wilson, 1974), changes in the molecular structure of the epithelial cell surface (Hewitt, Beer, and Grinnell, 1979), or a combination of these hormone dependent changes.

The volume of fluid is low in mice treated with vehicle alone (Group 3, Day 3 1200 h), progesterone alone (Group 1), or with progesterone followed by a combination of estradiol-17β and progesterone (Group 2). By contrast, the volume of fluid in the uterine lumen increased following treatment with estradiol-17β alone (Group 3). Thus, it appears that although estradiol-17β is capable of increasing the volume of fluid in the uteri of mice, this effect does not occur in the presence of progesterone. Similar results were obtained by Armstrong (1968) in prepuberal rats. In those experiments, the decrease in the weight of the uterus after the luminal contents were blotted onto filter paper indicated that treatment with estradiol-17β resulted

in an increase in the volume of fluid, whereas treatment with a combination of estradiol-17β and progesterone did not.

Kulangara (1972) measured the volume of fluid in pregnant and nonpregnant rabbit uteri using radial immunodiffusion to determine the change in concentration of a BSA solution after its passage through the uterus. The results indicated the importance of measuring the volume of fluid in the uterine lumen before interpreting data on the total amount of any substance recovered in rinses of the uterine lumen. For example, nonpregnant and lactating rabbits were found to have nearly equal amounts of protein per uterine horns, however, the volumes of fluid in those two conditions were different indicating that the protein concentrations in situ were also different.

The present experiments demonstrate that the volume of fluid in the uterine lumen of mice does not change as 'delayed implantation' is terminated and the process of 'implantation' initiated. Therefore, it appears that the differences in the total amount of various substances found in rinses of the uterine lumen of either 'delayed implanting' or 'implanting' mice reflect actual differences in the concentrations in situ. Thus, the increases in the total amount of protein (Aitken, 1974a), protease (Pinsker, Sacco, and Mintz, 1974), and chymotrypsin-like amidase (Hoversland and Weitlauf, 1978) reported to occur in uterine fluid as embryos are metabolically activated for implantation reflect increases in the concentrations. Such findings are compatible with the possibility that one or more of these increases are involved with either activation of the embryo or other aspects of the implantation process.

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Table 1: The volume of uterine fluid in 'delayed implanting' and 'implanting' mice*

Hormone Treatment Schedule**			Day of Experiment			
Hormone Treatment Groups	Progesterone 2.0 mg/day	Oestradiol-17β 25.0 ng/day	Day 3 1200 h	Day 4 0600 h	Day 4 1200 h	Day 5 1200 h
1	days 1-4	none	277 ± 30[a]****	360 ± 45[a]	369 ± 74[a]	285 ± 29
			n = 12	n = 12	n = 5	n = 8
2	days 1-4	days 3-4	301 ± 40[a]	335 ± 45[a]	307 ± 30[a]	246 ± 30
			n = 12	n = 11	n = 7	n = 11
3	none***	days 3-4	360 ± 28[a]	652 ± 106[b]	1513 ± 252[c]	3735 ± 1077
			n = 13	n = 12	n = 7	n = 14

*Volume reported as nanoliters per pair of uterine horns (i.e., from one animal).
**Hormones injected s.c. in 0.1 ml of sesame seed oil daily at 1200 h (i.e., days 1,2,3, and 4). Day 1 is the first day of hormone treatment and is ten days after ovariectomy.
***Animals in group 3 were injected with only the sesame seed oil vehicle on days 1 and 2.
****Mean ± S.E.M., n is the number of replicates (animals). Data was transformed to lns and evaluated by 2-way ANOVA followed by Duncan's multiple range test to detect significant differences: superscripts with a letter in common designate differences that are not significant ($P > 0.05$); conversely, the lack of a common letter in the superscripts designates differences that are significant ($P < 0.05$). Data collected on day 5 1200 h was not included in the statistical analysis. See text for details.

Table 2: The amount of albumin 'bound' to the uteri of 'delayed implanting' and 'implanting' mice*

Hormone Treatment Schedule**			Day of Experiment			
Hormone Treatment Groups	Progesterone 2.0 mg/day	Oestradiol-17β 25.0 ng/day	Day 3 1200 h	Day 4 0600 h	Day 4 1200 h	Day 5 1200 h
1	days 1-4	none	59 ± 8^{ab}****	58 ± 11^{a}	72 ± 12^{ab}	93 ± 16^{b}
			n = 12	n = 12	n = 5	n = 8
2	days 1-4	days 3-4	49 ± 10^{a}	115 ± 21^{b}	108 ± 23^{b}	110 ± 16^{b}
			n = 12	n = 11	n = 7	n = 11
3	none***	days 3-4	27 ± 3^{a}	44 ± 8^{ab}	46 ± 3^{b}	46 ± 5^{b}
			n = 13	n = 12	n = 7	n = 14

*Amount of albumin 'bound' reported as nanograms of albumin per pair of uterine horns (i.e., from one animal).
**Hormones injected s.c. in 0.1 ml of sesame seed oil daily at 1200 h (i.e., days 1,2,3, and 4). Day 1 is the first day of hormone treatment and is ten days after ovariectomy.
***Animals in group 3 were injected with only the sesame seed oil vehicle on days 1 and 2.
****Mean ± S.E.M., n is the number of replicates (animals). Data was transformed to lns and evaluated for significant differences between collection times within each treatment: superscripts with a letter in common designate means that are not statistically different ($P > 0.05$); conversely, the lack of a common letter in the superscript designates means that are statistically different ($P < 0.05$).

V. Manuscript 3.

Lysis of the zona pellucida and attachment of embryos to the uterine epithelium in ovariectomized mice treated with estradiol-17β and progesterone.

ABSTRACT

Preimplantation mouse embryos with intact zonae pellucidae were transferred into the uteri of ovariectomized females that were treated with either progesterone, estradiol-17β in combination with progesterone, or estradiol-17β alone; the disappearance of zonae from the uterine lumen was used to determine the presence of 'zona-lytic' activity in situ. Lysis of zonae did not occur in animals treated with either progesterone or estradiol-17β alone. However, lysis did occur when estradiol-17β was combined with progesterone, 'zona-lytic' activity reached peak levels within 12 to 24 h, then decreased. In addition, it was found that attachment of embryos to the uterine epithelium occured only in animals treated with estradiol-17β in combination with progesterone and was initiated at about the time of peak 'zona-lytic' activity. Because of the similarity between the endocrine regulation of 'zona-lytic' activity and initiation of embryo attachment reported here, and the control of chymotrypsin-like enzyme activity in uterine fluid reported previously (Hoversland & Weitlauf, 1978), it is suggested that the chymotrypsin-like enzyme activity in uterine fluid is involved with the initiation of implantation.

INTRODUCTION

The mechanism that is responsible for removal of zona pellucida in mice at time of implantation has not been established, but most evidence suggests that a 'zona-lytic' factor from the uterus is involved (Orsini & McLaren, 1967; McLaren, 1969, 1970; Mintz, 1971). In addition, it has been hypothesized that the factor responsible for lysis of the zona pellucida, possibly a proteolytic enzyme or enzyme complex (Bowman & McLaren, 1970; Mintz, 1972; Pinsker, Sacco, & Mintz, 1974), is also responsible for altering the surface of the blastocyst and thereby initiating attachment of embryos to the uterine epithelium (Mintz, 1972; Pinsker & Mintz, 1973).

Recently it has been demonstrated that the concentration of a chymotrypsin-like enzyme in the uterine lumen of ovariectomized mice is influenced by treatment with both estradiol-17β and progesterone (Hoversland & Weitlauf, 1978, 1980). The concentration was found to be low following treatment with progesterone alone (i.e., conditions for delayed implantation) and was elevated transiently following the injection of estradiol-17β in combination with progesterone (i.e., conditions for termination of delayed implantation). Although it was suggested that the enzyme might be involved in zona lysis and initiation of embryo attachment, one of the questions that remained unanswered was whether changes in the concentration of the chymotrypsin-like enzyme activity follow the same temporal course as 'zona-lytic' activity and the initiation of embryo attachment in utero. The following experiments were undertaken to examine this question.

MATERIALS AND METHODS

The egg transfer technique was used in the first two experiments. These deal with the effects of estradiol-17β and progesterone on lysis of zonae pellucidae in situ. The third experiment deals with the effects of these hormones on attachment of embryos to the uterine epithelium.

Experiment I

Embryo Donors Virgin female white Swiss mice (6 - 8 weeks old) were selected at random stages of the estrous cycle and induced to ovulate with intraperitoneal injections of pregnant mare's serum gonadotrohin (5 I.U.) followed 48 h later with human chorionic gonadotropin (5 I.U.) (Fowler and Edwards, 1957) and were immediately placed with fertile males. Mating was confirmed the following morning by the presence of a vaginal plug (designated day 1 of pregnancy). Embryo donors were killed on the fourth day of pregnancy by cervical dislocation, the uterine horns were excised, and embryos with intact zonae pellucidae were recovered by flushing a stream of Eagle's basal medium (GIBCO, containing Earle's salts in addition to glutamine 280 ng/ml, bovine serum albumin 1 mg/ml, penicillin-G 6 µg/ml, and streptomycin 50 µg/ml) from a blunt hypodermic needle through the uterus. Embryos were pooled and rinsed twice in fresh medium before transfer into recipient mice.

Recipients Virgin white Swiss mice were selected at random stages of the estrous cycle, bilaterally ovariectomized via dorsolateral incisions, and ligatures were placed, avoiding uterine vein and artery, at the cervical end of each uterine horn via a midventral

incision. Counting the day of ovariectomy as day 1, animals were allowed to recover for 10 days and allotted randomly to one of three hormone treatment groups as shown in Table 1: Group 1, progesterone only (i.e., conditions for delayed implantation); Group 2, progesterone followed by the combination of estradiol-17β and progesterone (i.e., conditions for implantation); and Group 3, sesame seed oil vehicle followed by estradiol-17β.

Five to 6 embryos with intact zonae pellucidae were transferred into the uteri of recipients at 1200 h on day 13 as described previously (Weitlauf & Greenwald, 1968). At various times between 12 and 24 h after the transfer (see Table 2), recipients were killed and the embryos and zonae pellucidae were recovered by flushing the uteri with a stream of normal saline from a blunt needle and syringe. The resultant flushings were stained with 5 µl of 1% toluidine blue in normal saline, to enhance the visibility of zonae, and examined under a compound microscope to determine the number of embryos and zonae recovered. Differences in the ratios of recovered zonae to embryos at the various times were tested for statistical significance with contingency tables (Snedecor and Cochran, 1967).

Experiment 2

Embryo Donors and Recipients were treated as described in Experiment 1 with the exception that all recipients were injected with progesterone followed by estradiol-17β in combination with progesterone (Table 1, Group 2). For purposes of reference, 1200 h on day 13 (i.e., the time of the first injection of estradiol-17β combined with progesterone) is considered 0 hour. Five to 6 embryos with intact

zonae pellucidae were transferred into recipients at various times with respect to the injection of the estradiol-17β in combination with progesterone (i.e., -24 h, -12 h, 0 h, +12 h, +18 h, +21 h, or +24 h) and recovered 24 h after the transfer procedure.

Experiment 3

Virgin white Swiss mice were induced to ovulate and mate. They were bilaterally ovariectomized and ligatures were placed at the cervical end of each uterine horn on the fourth day of pregnancy. The animals were then allowed to recover for 10 days and randomly alloted to either hormone treatment groups 2 or 3 (Table 1). Counting the time of the hormone injection on day 13 as 0 h, the animals were killed at 0 h, 18 h, 24 h, 30 h, or 36 h. Each animal was injected with 0.2 ml of 1% Evan's blue in normal saline (tail vein, i.v.) ten min prior to the time it was killed. The number of blueing reactions (Psychoyos, 1966) were counted and then each uterine horn was flushed with a stream of normal saline to recover unattached embryos.

RESULTS

Experiment 1 As shown in Table 2, the ratios of recovered zonae pellucidae to embryos were not significantly different from 1.00 between 12 h and 24 h following transfer into recipients treated with progesterone alone (group 1, Table 2, $P > 0.05$) or with estradiol-17β alone (Table 2, group 3, $P > 0.05$). In contrast, there was a significant decrease in the ratio of recovered zonae pellucidae to embryos following transfer into recipients treated with both estradiol-17β and progesterone in combination (Table 2, group 2, $P < 0.001$). Thus, lysis of zonae pellucidae occurred only in animals treated with estradiol-

17β in combination with progesterone, and this process was essentially complete by 18 h.

Experiment 2 The ratios of recovered zonae pellucidae to embryos were significantly different for the various 24 h-intervals that zonae were incubated in situ (Table 3, $P < 0.001$). The ratio of zonae recovered in the -24 h to 0 h interval (i.e., the 24 h-interval before the injection of estradiol-17β and progesterone) was 0.87. The ratio decreased to 0.38 in the -12 h to +12 h-interval and this was followed by a further decrease to 0.00 and 0.05 in the 0 h to +24 h and +12 h to +36 h-intervals, respectively. In contrast, the ratio increased to 0.34 in the +18 h to +42 h-interval followed by a further increase to 0.50 and 0.68 in the +21 h to +45 h and +24 h to +48 h-intervals, respectively. Thus, the level of 'zona-lytic' activity appeared to increase transiently with peak levels of activity occurring during the 0 h to +24 h and +12 h to +36 h-intervals.

Experiment 3 The mean number of embryos recovered per blue positive horn (uterine horns with one or more blue reactions) decreased significantly with time in animals injected with estradiol-17β in combination with progesterone (Table 4, group 2, $P < 0.001$) indicating attachment of embryos. The difference between the mean number of blue reactions and the mean number of embryos recovered (Table 4, group 2) was due to the retention of attached embryos in the uterus. Approximately 4 embryos were recovered per positive horn initially and essentially no embryos recovered 36 h after the addition of estradiol-17β to the progesterone regimen. In contrast, the mean number of embryos recovered did not change with time in mice treated with

estradiol-17β alone (Table 4, group 3, $P > 0.05$). Thus, attachment of embryos occurred only in mice treated with the combination of both hormones; this process was initiated by 18 h to 24 h after the addition of estradiol-17β to the progesterone regimen and was essentially completed by 36 h.

Failure of embryos to attach in animals treated with estradiol-17β alone is not due to the presence of zonae since all embryos were free of zonae.

DISCUSSION

The present experiments demonstrate that lysis of zonae pellucidae occurs in the uteri of ovariectomized mice following treatment with estradiol-17β combined with progesterone and that treatment with either hormone alone is insufficient for this effect. Peak levels of 'zonalytic' activity were found between 12 h and 24 h after the injection of estradiol-17β combined with progesterone, and this time coincided with the initiation of embryo attachment to the uterine epithelium.

These results are in agreement with earlier reports that dissolution of zonae pellucidae in mice does not occur during experimental delayed implantation (Orsini & McLaren, 1967; McLaren, 1971), lactational delayed implantation (McLaren, 1967, 1968), or in prepuberal mice (Mintz, 1971, 1972). These findings also agree with reports that lysis of zonae is induced within 18 hours after removal of the suckling young or an injection of estradiol-17β into mice with lactational delay (McLaren, 1968, 1970; Mintz, 1971). In addition, premature lysis of zonae occurs when pregnant mice are given an injection of estradiol-17β prior to the fourth day of pregnancy (Mintz,

1971). These previous observations have led to the hypothesis that the appearance of 'zona-lytic' activity in the uterus is estrogen dependent. Although this hypothesis is supported in the present experiments, the finding that lysis of zonae does not occur following treatment with either hormone alone suggests that, although estradiol-17β may act as a stimulus for the appearance of 'zona-lytic' activity, actual lysis of zonae is dependent upon both hormones.

Although the first experiment reported here demonstrates that there is a progressive loss of zonae pellucidae following treatment with estradiol-17β in combination with progesterone, and thus the appearance of 'zona-lytic' activity, the design of the experiment does not allow the distinction between two possible explanations; i) that there are increasing concentrations of 'lytic' activity and zonas are lost when the concentration reaches an effective level, or ii) there are constant levels of 'lytic' activity and zonas are lost with increasing lengths of incubation. In the second experiment, the length of time that zonae were exposed to the uterine environment was held constant so that comparisons of the level of 'zona-lytic' activity could be made between different or overlapping intervals. Because the length of exposure was constant, differences in the ratio of zonae recovered to embryos could only represent differences in the level of 'zona-lytic' activity. Comparison of the data obtained at intervals -12 h to +12 h, 0 h to +24 h, and +12 h to +36 h clearly indicates that maximal activity occurs later than +12 h and comparison of the data obtained at intervals 0 h to +24 h and +24 h to +48 h demonstrates that peak levels of activity occur prior to +24 h.

Thus, from the data in Table 3, it appears that peak levels of activity are between +12 and +24 h and most probably maximum at about +18 h. It is of interest that a second injection of estradiol-17β in combination with progesterone at 24 h did not result in further increases of 'zona-lytic' activity.

Results of the third experiment reported here demonstrate as expected that there is a significant effect of hormone treatment on attachment of embryos to the uterine epithelium. Attachment of embryos occurred only in mice treated with both hormones, it was initiated by 18 to 24 h and essentially completed by 36 h as indicated by the decrease in the number of embryos that could be recovered by flushing the uterus. Attachment of embryos was confirmed since the total number of embryos per uterine horn (i.e., mean number of blue reactions per 'positive' uterine horn, Table 4) did not decrease during this interval. In contrast, the mean number of embryos recovered per 'positive' uterine horn remained constant (Table 4) following treatment of estradiol-17β alone, indicating that this treatment is insufficient to support attachment of embryos. Fifty-four to 68% of the uterine horns examined from females treated with estradiol-17β in combination with progesterone contained embryos as shown by at least one blue reaction. Similarly, 50 to 68% of the uterine horns from females treated with estradiol-17β alone contained at least one embryo. Thus, the proportion of fertile horns in the two treatments were not different, indicating that comparisons between the two treatment are valid.

In this experiment, all embryos were free of zonae pellucidae at the time animals were killed and empty zonae were recovered from

animals treated with estradiol-17β alone. This confirms the observation that embryos in ovariectomized mice escape the zona by a nonlytic process ('hatching', Orsini & McLaren, 1967). Because zona-free embryos were recovered, it is clear that attachment is dependent upon more than simply the removal of the zona. It is possible that an enzyme dependent alteration in the glycoproteins on the surface of the embryo is required as suggested by Pinsker and Mintz (1973). Mintz (1971, 1972) suggested that 'zona-lytic' activity in the uterine lumen is involved in implantation and it is envisioned that the factor responsible for lysis of zonae is also responsible for altering the surface of embryos and thus initiating embryo attachment. The factor responsible for these events is thought to be a proteolytic enzyme or enzyme complex in uterine fluid (Bowman & McLaren, 1970; Mintz, 1972; Pinsker, Sacco & Mintz, 1974).

The similarity in the endocrine regulation of 'zona-lytic' activity and initiation of embryo attachment reported here, and that reported for chymotrypsin-like enzyme activity in uterine fluid (Hoversland & Weitlauf, 1978) suggests that this enzyme may be involved in lysis of zonae and/or initiation of embryo attachment. Before the chymotrypsin-like enzyme activity in uterine fluid can be implicated in these events, however, it will be necessary to demonstrate both 'zona-lytic' activity and an ability to alter the surface of the embryo by purified enzyme in vitro.

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Table 1. Schedule of Hormone Treatments.

Group Number	Progesterone* (2.0 mg/day)	Estradiol-17β* (25.0 ng/day)
1 'delayed implantation'	days 11 - 14**	none
2 'implantation'	days 11 - 14	days 13 - 14
3	none***	days 13 - 14

*All hormones dissolved in 0.1 ml sesame seed oil vehicle and injected s.c. at 1200 h of days indicated above.
**Day of ovariectomy is designated day 1 of the experiment.
***Animals allotted to group 3 were injected with 0.1 ml sesame seed oil vehicle on days 11 and 12.

Table 2. The Effect of Estradiol-17β and Progesterone on 'Zona-lytic' Activity in the Uteri of Ovariectomized Mice.

Hormone Treatment Group Number*	Hours in utero	Transferred: Total Embryos with Zonae Pellucidae	Recovered: Total Zonae Pellucidae	Recovered: Total Embryos	Ratio of: Zonae Recovered to Embryos
1	12**	91	41	45	0.93
	24	45	27	31	0.87
2	12	45	15	22	0.68
	15	72	13	32	0.40
	18	56	1	25	0.04
	24	63	0	25	0.00
3	12	45	23	26	0.88
	24	50	22	24	0.91

*See Table 1 for decription of treatment groups and treatment schedule.
**Embryos with intact zonae pellucidae were transferred into recipients at 1200 h on day 13, the time of the third hormone injection.

Table 3. The Level of 'Zona-lytic' Activity during Different 24 Hour Intervals in Mice Treated with Estradiol-17β in Combination with Progesterone.

Time of:	Transferred:	Recovered:		Ratio of:	
Transfer : Recovery	Total Embryos with Zonae Pellucidae	Total Zonae Pellucidae	Total Embryos	Zpmae Recovered to Embryos	Zonae 'Lysed' to Embryos
-24 h : 0 h*	72	34**	38	0.87	0.13
-12 h : +12 h	107	9	24	0.38	0.62
0 h : +24 h	63	0	25	0.00	1.00
+12 h : +36 h	50	1	18	0.05	0.95
+18 h : +41 h	117	15	44	0.34	0.66
+21 h : +45 h	124	26	51	0.50	0.50
+24 h : +48 h	150	25	35	0.68	0.32

*0 h is 1200 h on day 13, the time of the first injection of estradiol-17β in combination with progesterone (see Table 1). Negative hours refer to hours prior to injection and positive hours to those after.

**Includes zonae pellucidae that had become free fwee from embryos.

Table 4. The Effect of Estradiol-17β and Progesterone on Attachment of Embryos to Uterine Epithelium

Treatment Group Number	Hours after Estradiol-17β	Total Number of: Horns Examined	Horns +Blue***	Horns +Embryos***	Mean Number of: Blue Reactions per +Blue Horn	Embryos Recovered per +Embryo Horn
2*	0 h**	36	0	18 (50%)	0	4.6 ± 0.6****
	18 h	28	16 (57%)	16 (58%)	3.7	3.6 ± 0.7
	24 h	46	25 (54%)	20 (43%)	3.6	2.7 ± 0.4
	30 h	32	22 (68%)	13 (40%)	5.3	1.6 ± 0.3
	36 h	26	15 (57%)	2 (7%)	4.4	0.2 ± 0.1
3*	0 h	24	0	16 (66%)	0	4.5 ± 0.5
	18 h	28	0	18 (67%)	0	4.6 ± 0.6
	24 h	35	0	19 (54%)	0	3.4 ± 0.5
	30 h	30	0	18 (60%)	0	4.1 ± 0.7
	36 h	42	0	21 (50%)	0	4.7 ± 0.4

*See Table 1 for description of treatment groups and treatment schedule.
**0 h is the time of the hormone injection on day 13 (1200 h).
***+Blue designates uterine horns containing one or more blue reaction, +Embryo designates uterine horns from which one or more embryo was recovered.
****Mean ± S.E.M.

VI. Manuscript 4.

In vitro 'zona-lytic' activity in uterine fluid from ovariectomized mice treated with estradiol-17β and progesterone.

ABSTRACT

To determine whether mouse uterine fluid contains a hormone dependent 'zona-lysin', preimplantation stage embryos were incubated in vitro for 24 h with fluid rinsed from the uteri of ovariectomized females injected with either progesterone, estradiol-17β in combination with progesterone, or estradiol-17β alone. Although none of the zonae were completely dissolved, it was found that those incubated in fluid from animals treated with the combined hormones were subsequently more soluble in NaSCN than those incubated similarly in control buffer and thus, had undergone a sublytic change. Zonae incubated in fluid from animals injected with either hormone alone did not undergo such a change. Hence, 'zona-lytic' activity was observed in vitro only with fluid from animals in which dissolution of zonae would also occur in utero.

In addition, because the activity of a chymotrypsin-like enzyme in mouse uterine fluid also reaches maximum concentrations following the injection of estradiol-17β combined with progesterone, the fluid was chromatographed with a Sephadex G-200 column and the effluent fractions were assayed for both chymotrypsin-like enzyme and 'zona-lytic' activities. The finding that both activities were excluded from the gel is compatable with the suggestion that they are the same.

INTRODUCTION

It has been suggested that dissolution of zonae pellucidae in mice is due to a hormone dependent lytic factor of uterine origin (Orsini and McLaren, 1967; McLaren, 1969, 1970; Mintz, 1972; Pinsker, Sacco, and Mintz, 1974). Although the presumptive lysin has not been identified, the recent finding that the level of a chymotrypsin-like enzyme in uterine fluid of ovariectomized mice follows the same temporal pattern as 'zona-lytic' activity in utero after an injection of estradiol-17β in combination with progesterone suggests that this enzyme may be the lysin (Hoversland and Weitlauf, 1978; 1980b). However, it is equally possible that the increased levels of enzyme activity and 'zona-lytic' activity are only coincidental. One step in the evaluation of these possibilities, is to determine whether chymotrypsin-like enzyme activity and 'zona-lytic' activity in uterine fluid are separable. In the present experiments, uterine fluid was collected from ovariectomized mice treated with estradiol-17β and progesterone, concentrated, and tested for 'zona-lytic' activity in vitro by the method of Domon, Pinsker, and Mintz (1973) both before and after fractionation with Sephadex G-200 to determine if 'zona-lytic' and chymotrypsin-like enzyme activities are separable on the basis of molecular size.

METHODS AND MATERIALS

Experiment I. In vitro 'zona-lytic' activity in uterine fluid.

Uterine Fluid Virgin female white Swiss mice (6 - 8 weeks old)

were selected at random stages of the estrous cycle and ovariectomized via dorso-lateral incisions. The animals were allowed to recover for 10 days, randomly allotted to 3 groups, and treated as indicated in Table 1: Group 1, received progesterone alone (i.e., 'delayed implantation'); Group 2, received progesterone in combination with estradiol-17β (i.e., 'implantation'); and Group 3, received estradiol-17β alone. All hormones were dissolved in 0.1 ml of sesame seed oil and injected s.c. at 1200 h on the days indicated. Animals were killed by cervical dislocation 18 h after the last injection and their uteri were excised and cleaned of mesenteries and fat. The uterine horns were separated at the cervix, then flushed with 5 µl of dilute Kreb's Ringer bicarbonate buffer (diluted 1:24 with H_2O) followed by a bolus of air to to purge the lumen. The buffer and air were injected into the lumen near the utero-tubal junction with a 50 µl Hamilton syringe and 25 gauge hypodermic needle. The entire experiment was repeated 5 times. Samples of uterine fluid, each being the combined effluents collected from 5 - 9 animals, as well as buffer controls (i.e., dilute Kreb's Ringer bicarbonate) were centrifuged at 9,000 x g for 10 minutes and the supernatant fluid was frozen, lyophilized, and stored at -20° C until used.

Zonae Pellucidae Virgin female mice were selected at random stages of the estrous cycle and induced to ovulate with injections of gonadotrophins and placed with fertile males (Fowler and Edwards, 1957). Mating was confirmed the following morning by the presence of a vaginal plug (designated as Day 1 of pregnancy). Animals were

killed by cervical dislocation on day 4 of pregnancy, the uterine horns were excised, and embryos with intact zonae pellucidae were recovered by flushing each horn with a stream of Eagle's basal medium (GIBCO, containing Earle's salts with the addition of glutamine 280 ng/ml, bovine serum albumin 1 mg/ml, penicillin-G 6 µg/ml, and streptomycin 50 µg/ml) from a blunt hypodermic needle and syringe. Embryos with intact zonae were pooled and rinsed twice in fresh medium then transfered into reconstituted uterine fluid or control buffer.

Assay of Lytic Activity in Uterine Fluid Samples of uterine fluid from each treatment group, as well as the buffer controls, were reconstituted with distilled water so that the final volume was equivalent to 400 nl/uterus. Thus, the concentration of the components of uterine fluid from animals treated with either estradiol-17β combined with progesterone or progesterone alone was similar to that in utero, while uterine fluid from animals treated with estradiol-17β alone was over-concentrated (Hoversland and Weitlauf, 1980a). Zonae pellucidae (3 - 6) and a small volume of medium (1 - 2 µl) were placed in a depression slide and the medium was removed with a micropipette and replaced with reconstituted uterine fluid or control buffer. The droplet of uterine fluid was covered with paraffin oil and allowed to incubate with zonae for 24 hours at 37° C in a humidified incubator. The embryos and zonae were then recovered and placed in a 1.3 M NaSCN solution containing 0.25% gelatin and 0.05 M $NaPO_4$, (pH 7.4, 25° C; Domon, Pinsker, and Mintz, 1973). The zonae were examined with a compound microscope at 5 - 10

minute intervals (more often as dissolution approached) and the time required for complete dissolution (up to 400 minutes) was determined. Data were expressed as the percentage of the time required for dissolution of zonae that were preincubated in control buffer. A decrease in the time required (i.e., less than 100%) indicated that the zonae were more sensitive to NaSCN because of a sublytic change during the preincubation step and hence, that there was 'zona-lytic' activity in the sample of uterine fluid.

Experiment II. Comparison of in vitro 'zona-lytic' activity and chymotrypsin-like enzyme after fractionation of uterine fluid with Sephadex G-200.

Uterine Fluid Virgin female mice were ovariectomized, allowed to recover for 10 days, then treated with estradiol-17β in combination with progesterone as indicated for Group 2 in Experiment I. The animals were killed 18 h after the injection of estradiol-17β combined with progesterone. Their uteri were removed and the uterine horns separated at the cervix. Five µl of 0.05 M NH_4HCO_3 buffer (pH 7.8) was flushed through each uterine horn and the effluent from 21 - 23 animals were combined as described in Experiment I. This procedure was repeated four times resulting in four different samples of uterine fluid. Each sample was centrifuged at 9,000 x g for 10 minutes and then the supernatant fluid was chromatographed with Sephadex G-200 that was equilibrated with 0.05 M NH_4HCO_3 (50 cm x 1 cm column) and previously calibrated with the molecular weight markers blue dextran ($2x10^6$ MW), ferritin ($4.5x10^5$ MW), albumin ($6.8x10^4$ MW), and uridine ($2.4x10^2$ MW). The samples were eluted at room temperature

with a flow rate of approximately 6 ml/hr and collected in 1 ml fractions. Each fraction was analyzed for protein by absorption spectrophotometry (Waddel, 1956; Murphy and Kies, 1960) and chymotrypsin-like enzyme activity by hydroloysis of glutaryl-L-phenylalanyl-β-naphthylamide as described previously (Hoversland and Weitlauf, 1978). The fractions were then combined into 7 Pools (designated Pools A - G, see Fig. 1) on the basis of protein content and, along with buffer controls, were frozen, lyophilized, and stored at -20° C until used.

Assay of Lytic Activity in Pools Pools A - G and buffer controls were reconstituted with 9 µl of 0.05 M Tris-HCl with 0.02 M $CaCl_2$ (pH 7.4) and assayed for 'zona-lytic' activity as described in Experiment I. Data were expressed as a percentage of the control, transformed to the arcsine of the square root of the percentage (Sokal and Rolfe, 1969), and homogeniety of variance was examined with Bartlett's test (Snedecor and Cochran, 1967). Data were then analyzed with a one-way analysis of variance with significant differences between pooled samples and controls determined by the least significant differences method (Snedecor and Cochran, 1967). The intraassay coefficient of variation for zonae preincubated in control buffer was 10.8% (n = 4).

RESULTS

Results of preliminary experiments indicated that neither samples of whole uterine fluid nor fractions of uterine fluid would completely dissolve zonae pellucidae in vitro. For this reason, the assay described by Domon, Pinsker, and Mintz (1973) was used to detect

sublytic modification of zonae in vitro and thus determine whether 'zona-lytic' activity was present in the samples of uterine fluid.

Experiment I.

It was found that following preincubation of zonae in fluid from animals treated with the combination of estradiol-17β and progesterone, the time required for their dissolution in NaSCN was decreased (i.e., 87.7% of that required for dissolution after preincubation in control buffer, Table 1, Group 2) indicating that 'zona-lytic' activity was present in the uterine fluid. In contrast, there was no decrease in the time necessary for dissolution of zonae that were preincubated in fluid from animals treated with progesterone alone (109.9% of control, Table 1, Group 1) indicating that this fluid did not contain 'zona-lytic' activity. The time necessary for dissolution of zonae that were preincubated in fluid from animals treated with estradiol-17β alone was found to be increased (155.7% of controls, Table 1, Group 3) indicating the presence of a factor(s) that renders the zona less susceptible to NaSCN and thus, no inferences can be made about 'zona-lytic' activity in fluid from animals treated with estradiol-17β alone.

Experiment II.

The fractionation of uterine fluid with Sephadex G-200 with respect to protein, chymotrypsin-like enzyme activity, and 'zona-lytic' activity is summarized in Fig. 1. Three peaks of protein were found: one was associated with the void volume (Pool B), one co-chromatographed with albumin (Pool D), and a minor peak appeared at the salt volume (Pool F, Fig. 1a). Chymotrypsin-like enzyme activity was found

primarily in the void volume (Pool B), followed by a broad minor zone of activity (Pools C & D, Fig. 1b). Although zonae pellucidae that were preincubated in pooled fractions A, B, or G appeared to dissolve more rapidly in NaSCN than those preincubated in control buffer, the difference was statistically significant only for those in pool B ($p < 0.05$, Fig. 1c). The amount of lytic activity in pool B was roughly equivalent to that previously observed in whole uterine fluid (compare Table 1, Group 1 to Fig. 1c, Pool B).

DISCUSSION

The present results demonstrate in vitro the presence of a hormone dependent 'zona-lytic' activity in fluid collected from the uteri of ovariectomized mice. The lytic activity was found in fluid recovered from animals treated with a combination of estradiol-17β plus progesterone but not in fluid from animals treated with either hormone alone and, thus, corresponds to situations with maximum zona lysis in utero (Hoversland and Weitlauf, 1980b) and maximum concentrations of chymotrypsin-like enzyme activity in uterine fluid (Hoversland and Weitlauf, 1978). Furthermore, it was found that the 'zona-lysin' and the chymotrypsin-like enzyme are similar in that they were both excluded from Sephadex G-200.

The observation that uterine fluid from animals receiving a combination of estradiol-17β and progesterone increased the susceptability of zonae to NaSCN, but did not cause complete dissolution, suggests that conditions in vitro were not the same as those in utero. Two possible explanations are that the concentration of 'zona-lysin' was insufficient to be completely effective, or that

the lytic factor was damaged by the lyophilization process. The actual concentrations of various components of uterine fluid in vivo are not known because it cannot be collected without dilution. However, the volume of uterine fluid, in mice given the same hormone treatments used here, has been estimated (Hoversland and Weitlauf, 1980a) and in the present experiments the fluid was reconstituted to that volume. Therefore, it seems unlikely that the attenuated effect in vitro was due to a low concentration of 'zona-lysin'. The more likely explanation is that activity of the factor was reduced by the process of lyophilization.

The finding that both chymotrypsin-like and 'zona-lytic' activities were excluded from Sephadex G-200 indicates that both activites have molecular sizes of 800,000 daltons or greater. This similarity, in conjunction with the fact that their hormone control is identical, argues in favor of the possibility that both activites are due to the same enzyme or enzyme complex. However, before it can be proven that the chymotrypsin-like enzyme in uterine fluid is responsible for zona lysis it will be necessary to show that the two activities are the same size by molecular sieving with a gel that has an appropriate pore size, and that they share other similarities such as electrophoretic mobility, susceptibility to inhibitors, and immunologic cross reactivity. The crucial test will be to show that highly purified enzyme is capable of lysing zonae in vitro.

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Table 1: Dissolution of Zonae Pellucidae in 1.3 M NaSCN after Preincubation in Reconstituted Uterine Fluid from Ovariectomized Mice Treated with Estradiol-17β and Progesterone.

Treatment Group	Treatment Schedule: Progesterone (2.0 mg/day)*	Treatment Schedule: Estradiol-17β (25.0 ng/day)*	Time Required for Dissolution in 1.3 M NaSCN (% of controls)**
1	days 11 - 14***	none	109.9 ± 4.9***** (n=5)
2	days 11 - 14	days 13 - 14	87.7 ± 4.8 (n=5)
3****	none	days 13 - 14	155.7 ± 17.3 (n=4)

*All hormones dissolved in 0.1 ml sesame seed oil.
**Control zonae were incubated in buffer rather than uterine fluid.
***Hormones injected s.c. at 1200 h on days indicated above. Day 1 is the day of ovariectomy.
****Animals in group 3 were injected with sesame seed oil vehicle on days 11 and 12.
*****Mean ± S.E.M., n is the number of samples of uterine fluid assayed, see text for details.

Figure 1. Comparison of in vitro 'Zona-lytic' Activity and Chymotrypsin-like Enzyme after Fractionation of Uterine Fluid with Sephadex G-200.

Uterine fluid recovered from ovariectomized mice 18 h after the injection of estradiol-17β and progesterone was fractionated on a Sephadex G-200 column. The elution of molecular weight standards are indicated by the arrows in 1a. Representive elution profiles are shown for protein (1a), chymotrypsin-like enzyme activity (1b), and 'zona-lytic' activity (1c). See text for details.

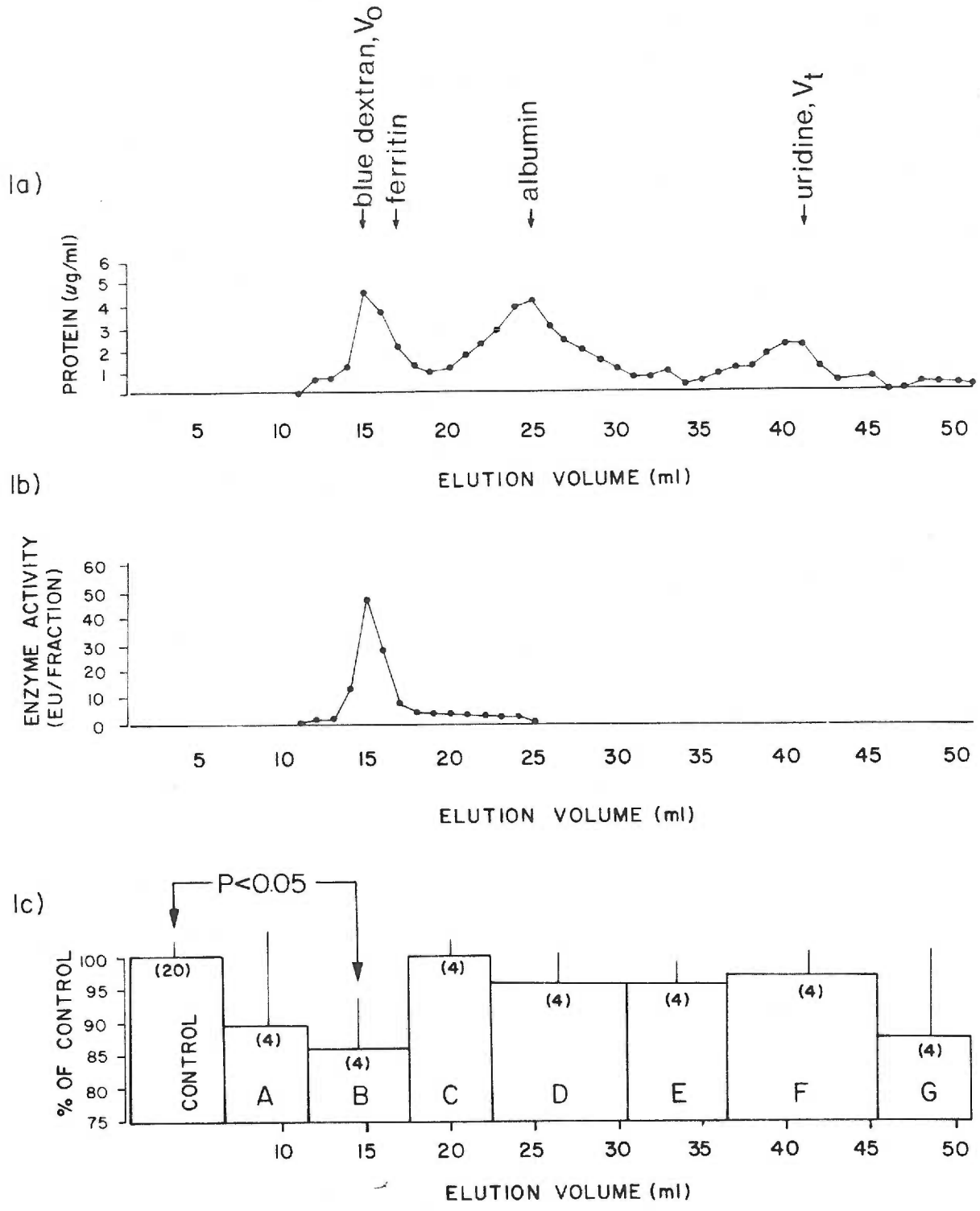
Ia)
←blue dextran, V_o
←ferritin
←albumin
←uridine, V_t
PROTEIN (ug/ml)
6
5
4
3
2
1
5 10 15 20 25 30 35 40 45 50
ELUTION VOLUME (ml)
Ib)
ENZYME ACTIVITY (EU/FRACTION)
60
50
40
30
20
10
0
5 10 15 20 25 30 35 40 45 50
ELUTION VOLUME (ml)
Ic)
P<0.05
% OF CONTROL
100
95
90
85
80
75
(20)
CONTROL
(4)
A
(4)
B
(4)
C
(4)
D
(4)
E
(4)
F
(4)
G
10 15 20 25 30 35 40 45 50
ELUTION VOLUME (ml)

Figure 1

VII. SUMMARY

The effect of estradiol-17β and progesterone on the level of both trypsin-like and chymotrypsin-like enzyme activities in uterine fluid were evaluated with synthetic peptide homologs as substrates. The level of chymotrypsin-like enzyme activity was found to be low in the fluid from the uteri of animals treated with progesterone alone (condition for delayed implantation) and it increased transiently when estradiol-17β was superimposed on the progesterone treatment (conditions for implantation). These findings indicate that the amount of chymotrypsin-like enzyme activity in the uterine lumen was influenced by ovarian steroids. Small amounts of enzyme activity were present during the prolonged free-living phase associated with delayed implantation and increased amounts at the time of implantation. Thus, it is possible that this enzyme serves as an Implantation Initiation Factor according to the hypothesis of Mintz (1972). However, the observation that the amount of chymotrypsin-like enzyme activity was also elevated after treatment with estradiol-17β alone is inconsistent with this interpretation since implantation does not occur in animals given this treatment. Trypsin-like enzyme activity was not found in uterine fluid collected from animals in any treatment group, so it was not considered further.

To evaluate the question of whether the above changes in chymotrypsin-like enzyme activity reflect changes in the concentration of enzyme activity in utero, the volume of uterine fluid was determined using an isotope dilution technique. The volume was found to be 300 - 400 nl/uterus in mice treated with progesterone alone and did not change when

estradiol-17β was added to the treatment regimen. Furthermore, it was found that the volume of uterine fluid increased (up to 2,000 nl/uterus) in mice treated with estradiol-17β alone. The observation that the volume of uterine fluid was the same during the prolonged preimplantation phase associated with delayed implantation and at the time of implantation indicates that the total amount of enzyme activity in fluid flushed from 'delayed implanting' and 'implanting' mice does indeed reflect the relative concentration of chymotrypsin-like enzyme activity in utero. Thus, an increase in the concentration of chymotrypsin-like enzyme activity occurs in utero at the time implantation is initiated. Conversely, the finding that the volume of uterine fluid increased markedly and remained elevated following treatment with estradiol-17β alone indicates that in this case the increase in the total enzyme activity does not reflect an increase in the concentration of enzyme activity in utero. Therefore, changes in the concentration of enzyme activity in utero are compatible with the hypothesis that a chymotrypsin-like enzyme is involved with the initiation of implantation.

The temporal association of maximum 'zona-lytic' activity in utero and the attachment of embryos to the uterine epithelium with times of maximum concentrations of chymotrypsin-like enzyme activity were evaluated using the embryo transfer technique. These experiments demonstrate that lysis of zonae pellucidae and attachment of embryos to the uterine epithelium occurs in mice treated with a combination of progesterone and estradiol-17β but not in those treated with either hormone alone. Peak levels of 'zona-lytic' activity, as determined by the dissolution of zonae pellucidae in the uterine lumen, were found between 12 h and

24 h after the injection of estradiol-17β and progesterone. Thus, it was shown, that times of peak 'zona-lytic' activity and the initiation of embryo attachment coincided with elevated concentrations of chymotrypsin-like enzyme activity. The coincidence of these three events supports the hypothesis that chymotrypsin-like enzyme activity is the cause of lysis of zonae pellucidae and attachment of embryos to the uterine epithelium at at the time of implantation.

The ability of uterine washings to cause lysis, or more precisely sublytic modification, of zonae pellucidae was shown in vitro after the washings were concentrated to a volume that approximated that present in utero (400 nl/uterus). It was found that zonae pellucidae were not completely lysed after a 24 h incubation in whole uterine fluid. However, after preincubation of zonae pellucidae in uterine fluid collected from animals treated with the combination of progesterone and estradiol-17β (18 h), it was found that less time was required for the dissolution of zonae pellucidae in NaSCN as compared with zonae preincubated in control buffer. This did not occur with zonae preincubated in uterine fluid from animals treated with either hormone alone. Thus, detectable 'zona-lytic' activity in vitro, as inferred from the sublytic changes, was found only in uterine fluid from animals subjected to a treatment that leads to lysis of zonae pellucidae in utero. Chromatographic separation of chymotrypsin-like enzyme and 'zona-lytic' activities on the basis of molecular size (with Sephdex G-200) demonstrated that both activities eluted with the void volume. These findings suggest that the molecular size of the substance(s) responsible for each of these activities is 800,000 daltons or greater and that by this method they are not separ-

able. The finding that these two activities were not separable under these conditions is consistent with the hypothesis that chymotrypsin-like enzyme activity in mouse uterine fluid is involved in the lysis of zonae pellucidae at the time of implantation.

The experiments in this thesis examined the hypothesis that chymotrypsin-like enzyme activity in the uterine fluid of mice is involved with the initiation of implantatation by dissolving the zona pellucida and initiating the attachment of embryos to the uterine epithelium. The results of all experiments presented here support this hypothesis but do not provide conclusive proof. Several interesting questions remain to be answered. 1) Do the chymotryspin-like enzyme and 'zonalytic' activities have physio-chemical characteristics in common other than the same endocrine regulation and apparently large molecular size? Characteristics of interest would include eletrophoretic mobility, isoelectric point, thermal stability, subunit identity, susceptibility to inhibitors, and anitgenic determinants. 2) Do inhibitors of chymotrypsin-like enzyme activity prevent lysis of the zona pellucida in utero? 3) Does purified chymotrypsin-like enzyme activity cause alterations of glycoproteins on the surface of the embryo in vitro? 4) Do inhibitors of chymotrypsin-like enzyme activity prevent the attachment of embryos to the uterine epithelium in utero?

Answers to these questions will determine whether chymotrypsin-like enzyme activity in the uterine fluid of mice has a role in the implantation process and is the putative Implantation Initiation Factor envisioned by Mintz (1972).

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