BIOLOGY OF REPRODUCTION 20, 689–698 (1979)

Uterine Luminal Proteins in the Cycling Mare

MICHAEL T. ZAVY, FULLER W. BAZER,
DAN C. SHARP and C. J. WILCOX

Department of Animal Science and Dairy Science,
University of Florida,
Gainesville, Florida 32611

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ABSTRACT

Qualitative changes in the protein content of mare uterine flushings were determined on Days 4, 8, 12, 14, 16, 18 and 20 postovulation. Acid phosphatase and leucine aminopeptidase enzymatic activities were also determined for these treatment days. All collections were obtained using a nonsurgical, transcervical technique. Sephadex G-200 column chromatography indicated that 5 distinct protein fractions existed for all days tested. The chromatographic mobility (K_{av}) of these fractions did not change during the estrous cycle. When expressed as percentage of the total protein, peaks I, IV and V were significantly affected ($P<0.005$, 0.05 and 0.025, respectively) by day of the estrous cycle, whereas, peaks II and III were not. Acid phosphatase activity was significantly affected $P<0.05$) by day of the estrous cycle and maximal values were found in the late luteal phase. Mean values ranged from 10 to 193 μM of inorganic phosphate released per h on Days 4 and 18, respectively, followed by a rapid decline on Day 20. Sephadex G-200 studies indicated that the acid phosphatase enzymatic activity was associated with low molecular weight fractions III and IV and, therefore, was probably nonlysosomal in origin. Leucine aminopeptidase activity was maximal in the midluteal phase (Day 12) and was significantly affected ($P<0.005$) by day of the estrous cycle. Sephadex G-200 chromatography indicated that this enzyme was associated with high molecular weight fractions I and II.

Over the course of this study a purple-colored protein was found in the uterine flushing of a pseudopregnant mare. Biochemical and immunological examinations indicated that similar properties existed for the equine purple protein and the porcine purple protein, previously described by Schlosnagle et al. (1974).

INTRODUCTION

Brambell (1948) and Robinson (1951) suggested that a deficiency in intermediate factors within the uterine environment could be a major cause of prenatal mortality. Bazer et al. (1969) found that the ability of the porcine uterus to support embryonic development appeared to regulate litter size early in gestation when uterine space was not a limiting factor. Murray et al. (1972) and Squires et al. (1972) reported that the protein in porcine uterine secretions changed quantitatively and qualitatively during the estrous cycle. Similar results have been reported for the mare (Zavy et al., 1978). Murray et al. (1972) described a basic glycoprotein having a purple color in uterine flushings collected from pigs between Days 12 and 16 of the estrous cycle. This protein was shown to contain iron and possess high acid phosphatase activity (Schlosnagle et al., 1974). Chen et al. (1975) found that the purple acid phosphatase was synthesized and secreted by the uterine epithelial cells under the influence of progesterone.

The oviductal and uterine fluids provide the environment in which fertilized ova develop. Uterine secretions are a product of the maternal endometrium and, in most mammals, their presence is most important at the time of early embryonic development (Amoroso, 1952). Additionally, uterine proteins have been shown to serve as enzymes and carrier molecules for hormones, vitamins and minerals (Bazer et al., 1978).

For these reasons, it was of interest to study proteins and, in some cases, their enzymatic activities within uterine secretions of the mare at different stages of the estrous cycle.

MATERIALS AND METHODS

General Procedures

Uterine flushings (42) were collected from Quarter-

Accepted October 2, 1978.
Received July 31, 1978.

horse and Thoroughbred mares by a nonsurgical transcervical technique developed in our laboratory (Zavy et al., 1978). Days of collection were 4, 8, 12, 14, 16, 18 and 20 postovulation. Ovulations were detected (day of detection = Day 0) by daily rectal palpation of the ovaries by the same individual. Mares were assigned to day of collection in ascending order within a replicate when they ovulated. Several of the mares were used for collection of more than one uterine flushing in succeeding estrous cycles but at least one estrous cycle intervened between 2 collections.

Eighty ml of sterile 0.33 M NaCl were infused through the collection device into the uterine lumen. The uterus was massaged per rectum, for a period of 5 min to insure adequate mixing of the luminal contents and flushing medium. After 5 min, the fluid containing the uterine secretions was withdrawn slowly from the uterus. Uterine flushings were placed in an ice bath, quantified, centrifuged in a refrigerated centrifuge for 20 min at 12,000 × g and the supernatant filtered through a 0.45 μm filter to remove bacterial and cellular debris. The filtrate was transferred into sterile vials and stored at −20°C until analyses were performed.

Characterization of Proteins, Acid Phosphatase and Leucine Aminopeptidase Enzymatic Activities in Uterine Fluid of Cycling Mares

Sephadex G-200 column chromatography was used to determine qualitative protein differences for different treatment days. Throughout this study, the term "total" refers to the concentration of the substance measured multiplied by the recovery volume of the flushing. When the volume of flushing medium recovered was less than 80.0 ml (100%), a value of 80.0 ml was used as the recovery volume, but when the recovered volume was greater than 80.0 ml, the actual volume collected was used.

Protein concentrations of uterine flushings were estimated using the procedure described by Lowry et al. (1951). Acid phosphatase activity of uterine flushings was determined by the method described in Sigma Technical Bulletin No. 104 (1971) and leucine aminopeptidase activity was determined by the procedure described in Sigma Technical Bulletin No. 251 (1972).

Sephadex G-200 protein profiles were constructed for the different treatment days and for equine blood plasma. Sephadex chromatography was carried out according to the procedure described by Reiland (1971). Approximately 60 ml of uterine flushings were concentrated 10-fold by ultrafiltration and dialyzed against 0.05 M phosphate citrate buffer, pH 7.4; 5 μl of blood plasma were chromatographed without prior concentration. Between 2.0 and 10.0 mg of protein were loaded onto columns of Sephadex G-200. All samples were chromatographed on 1.5 × 90 cm columns with bed heights of 80−85 cm. The eluant used was 0.05 M phosphate-citrate buffer, pH 7.4 and the flow rate was approximately 10 ml/h. Gel filtration was carried out at 4°C. Fractions were collected every 15 min and each contained approximately 2.0−3.0 ml. Aliquots of each fraction were analyzed for protein content. The optical density of each fraction was determined and plotted against elution volume of the fraction, so that a protein profile was constructed. Protein peaks were assigned K_{av} values based on their elution volume. Since it was not feasible to load a standard quantity of protein from each sample on the Sephadex column, the optical densities obtained from the fractions were not directly comparable among samples. In order to compensate for this, the amount of protein in each peak was estimated by determining the mass under each peak and expressing it as a percentage of the total protein. Additionally, acid phosphatase and leucine aminopeptidase enzymatic activities were located on Sephadex G-200 protein profiles for Days 8, 12 and 16 postovulation of the estrous cycle.

Characterization of a Purple Acid Phosphatase in the Uterine Flushing of a Pseudopregnant Mare

Carboxymethyl cellulose (CMC) was used to partition acidic proteins and basic proteins. CMC was equilibrated with 0.01 M sodium acetate (NaAC) for 48 h and packed in 1.0 × 30 cm columns. Mare uterine protein which had previously been dialyzed against 0.01 M NaAc was then placed on the column and eluted with NaAc buffer. The column was washed with several bed volumes of buffer to elute all acidic proteins. Following this step, the column was washed with 1.0 M NaAc to elute the basic proteins retained on the CMC column.

Ouchterlony gel immunodiffusion analysis (Kwapinski, 1965) was used to discern the relationship between porcine purple protein (Chen et al., 1973) and the purple protein from the uterine flushing of the pseudopregnant mare. Day 16 mare uterine flushing from a cycling mare was also included in the analysis. Antiserum to the porcine purple protein previously prepared and validated in our laboratory (Chen et al., 1973) was used in this study. Twenty-five μl of antibody were placed in the center well. Well I contained 25 μl of porcine uterine fluid (high in porcine purple protein); well II contained 25 μl of uterine fluid from the pseudopregnant mare (purple colored); well III contained 25 μl of Day 16 mare uterine fluid and well IV contained 25 μl of saline as a control.

Statistical Analysis

Least squares procedures (Harvey, 1960) were used to evaluate effects of day of estrous cycle and mare on the constituents measured on the different days of the estrous cycle. The statistical model used in the analysis of variance of total acid phosphatase activity, total leucine aminopeptidase activity and Sephadex G-200 profiles is presented in Table 1. Variability among days was partitioned into single degrees of freedom by weighted least squares in order to detect curvilinearity in trends and aid in interpretation of results.

RESULTS

Intrauterine total acid phosphatase activity was significantly affected ($P<0.05$) by day of the estrous cycle. Acid phosphatase activities for the different days of the estrous cycle are summarized in Table 2. Day means for total

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TABLE 1. Model of least squares regression analysis of recovery volume, total protein, total acid phosphatase, total leucine aminopeptidase and Sephadex G-200 fractions (K_{av} and *PTP).

Source	df	Expected mean square	Error term
Day	6	$\sigma^2_\Sigma + k_2\theta^2 D$	Remainder
Linear	1	$\sigma^2_\Sigma + k_2\theta^2 D_{linear}$	Remainder
Quadratic	1	$\sigma^2_\Sigma + k_2\theta^2 D_{quadratic}$	Remainder
Cubic	1	$\sigma^2_\Sigma + k_2\theta^2 D_{cubic}$	Remainder
Quartic	1	$\sigma^2_\Sigma + k_2\theta^2 D_{quartic}$	Remainder
Quintic	1	$\sigma^2_\Sigma + k_2\theta^2 D_{quintic}$	Remainder
Residual	1	$\sigma^2_\Sigma + k_1\theta^2 D_{residual}$	Remainder
Mare	A	$\sigma^2_\Sigma + k_1\theta^2 M$	Remainder
Remainder	B	σ^2_Σ	

This statistical model is the same for all the above parameters with the exception of df for mare[A] and remainder[B] which differ as follows:

	df (A)	df(B)
Recovery volume	14	21
Total protein	14	21
Total acid phosphatase	14	21
Total leucine aminopeptidase	13	11
Sephadex G-200 fractions (K_{av} and PTP)	11	5

*Percent of total protein.

acid phosphatase activity increased from 10.3 to 193.8 μM pi released/h on Days 4 and 18, respectively, followed by a sharp decline on Day 20.

Total leucine aminopeptidase activity (LAP) was significantly affected ($P<0.005$) by day of the estrous cycle. Treatment means are expressed on a concentration, specific activity and total basis in Table 3. Total LAP activity, expressed in modified sigma units (MSU) was shown to range from 72.3 MSU on Day 4 to a peak value of 4867.4 MSU on Day 12.

Representative Sephadex G-200 profiles for each of the treatment days are shown in Fig. 1. On each of the treatment days, 5 protein peaks were observed. Changes in the amount of protein in each peak throughout the estrous cycle are shown in Fig. 2. Molecular weights

TABLE 2. Acid phosphatase activity in mare uterine flushings.

Day	No. of observations	Total acid phosphatase activity (μM Pi/h)[a]	Acid phosphatase specific activity (μM Pi/mg protein/h)[a]
4	5	10.26 ± 50.04	0.26 ± 0.71
8	6	16.70 ± 41.80	0.30 ± 0.59
12	7	60.10 ± 42.00	1.06 ± 0.61
14	6	60.41 ± 47.08	1.74 ± 0.67
16	4	180.67 ± 50.95	2.21 ± 0.72
18	7	193.83 ± 39.73	2.49 ± 0.56
20	7	14.23 ± 39.08	0.39 ± 0.55

Total acid phosphatase activity (overall mean ± SEM = 76.60 ± 16.47; coefficient of variation = 135.65%).

Acid phosphatase specific activity (overall mean ± SEM = 1.21 ± 0.23; coefficient of variation = 102.62%).

[a]LSM ± SEM.

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TABLE 3. Leucine aminopeptidase in mare uterine flushings.

Day	No. of observations	LAP concentration MSU/ml[a,b]		Total LAP activity MSU/flushing[a,c]		LAP specific activity MSU/mg protein[a,d]	
4	4	0.14	1.02	72.3	789.9	0.80	0.14
8	4	0.99	0.97	508.8	946.5	0.81	0.14
12	6	5.03	0.87	4867.4	849.8	0.46	0.12
14	3	4.62	1.12	3991.1	1092.5	0.51	0.16
16	3	0.38	1.20	296.0	1127.2	0.34	0.16
18	6	0.22	0.72	133.8	706.6	0.04	0.10
20	5	0.06	0.88	271.3	875.0	0.75	0.12

[a]Least squares mean ± SEM.

[b]LAP concentration (overall mean ± SEM) 1.45 ± 0.32; coefficient of variation = 123.51%.

[c]LAP total activity (overall mean ± SEM) 1227.6 ± 309.6; coefficient of variation = 130.4%.

[d]LAP specific activity (overall mean ± SEM) 0.53 ± 0.04; coefficient of variation = 138.39%.

(Fraction I, >275,000; II, 275,000; III. 170,000; IV, 56,000 and V, 15,000 daltons) were determined from the calibration curve seen in Fig. 3. As a means of comparison, gel filtration was carried out on equine blood plasma collected from the experimental animals. Results indicated that 3 major protein peaks were found when chromatographed on Sephadex G-200. The last inset of Fig. 1 indicates the protein pattern for equine plasma. The elution characteristics of these fractions are similar to those of fractions I, II and III from uterine flushings. However, chromatography of plasma proteins did not reveal elution patterns similar to fractions IV and V from uterine flushings.

Leucine aminopeptidase and acid phosphatase enzymatic activities were identified relative to Sephadex G-200 protein profiles for Days 8, 12 and 16 of the estrous cycle (Fig. 4). Results indicated that, in all cases, the majority of LAP activity cochromatographed with protein(s) in fraction I, with a small amount of activity associated with fraction II. Acid phosphatase activity was associated with an area between fractions III and IV.

A purple colored protein was found in the uterine flushing of a pseudopregnant mare, 66 days postovulation. The protein concentration of the pseudopregnant uterine flushing was 2.96 mg/ml of flushing or 266.1 mg total recoverable protein in the flushing. Acid phosphatase activity was 571.2 μM pi released/h, which is 3 times greater than the least squares mean acid phosphatase peak on Day 18 of the estrous cycle. Sephadex G-200 gel filtration resulted in a profile with 4 protein fractions (Fig. 5) which corresponded roughly in K_{av} value to fractions I, III, IV and V found in normally cycling mares. In addition, the estimated molecular weights of these 4 fractions were similar to molecular weight estimates obtained from normally cycling mares for fractions I, III, IV and V, respectively. The apparent absence of fraction II may reflect poor chromatographic resolution of fraction III observed in this case and fraction II may actually be represented as part of fraction III. Acid phosphatase activity was determined for each fraction collected from Sephadex G-200 gel filtration columns. Results indicated that all of the acid phosphatase activity was located in an area which corresponded to fraction IV (0.587). As previously noted, the K_{av} of fraction IV (0.587) from pseudopregnant mare uterine flushings compared closely with the K_{av} value of fraction IV (0.611) from the uterine flushings of cycling mares. The small difference in K_{av} values may be partially explained by poor resolution of fraction III (0.308).

When the lavender colored, pseudopregnant mare uterine flushing was chromatographed on a CMC column, a dense purple band was retained at the top of the column. This CMC+ fraction was found to contain high acid phosphatase activity, whereas the CMC− fraction contained no appreciable activity. When the CMC+ fraction was concentrated and chromatographed on Sephadex G-200, a profile with 1 predominant peak was observed (Fig. 6). The K_{av} value for this peak was 0.689 which is comparable to fraction IV (0.611) in the profiles from cycling mares and indicates a

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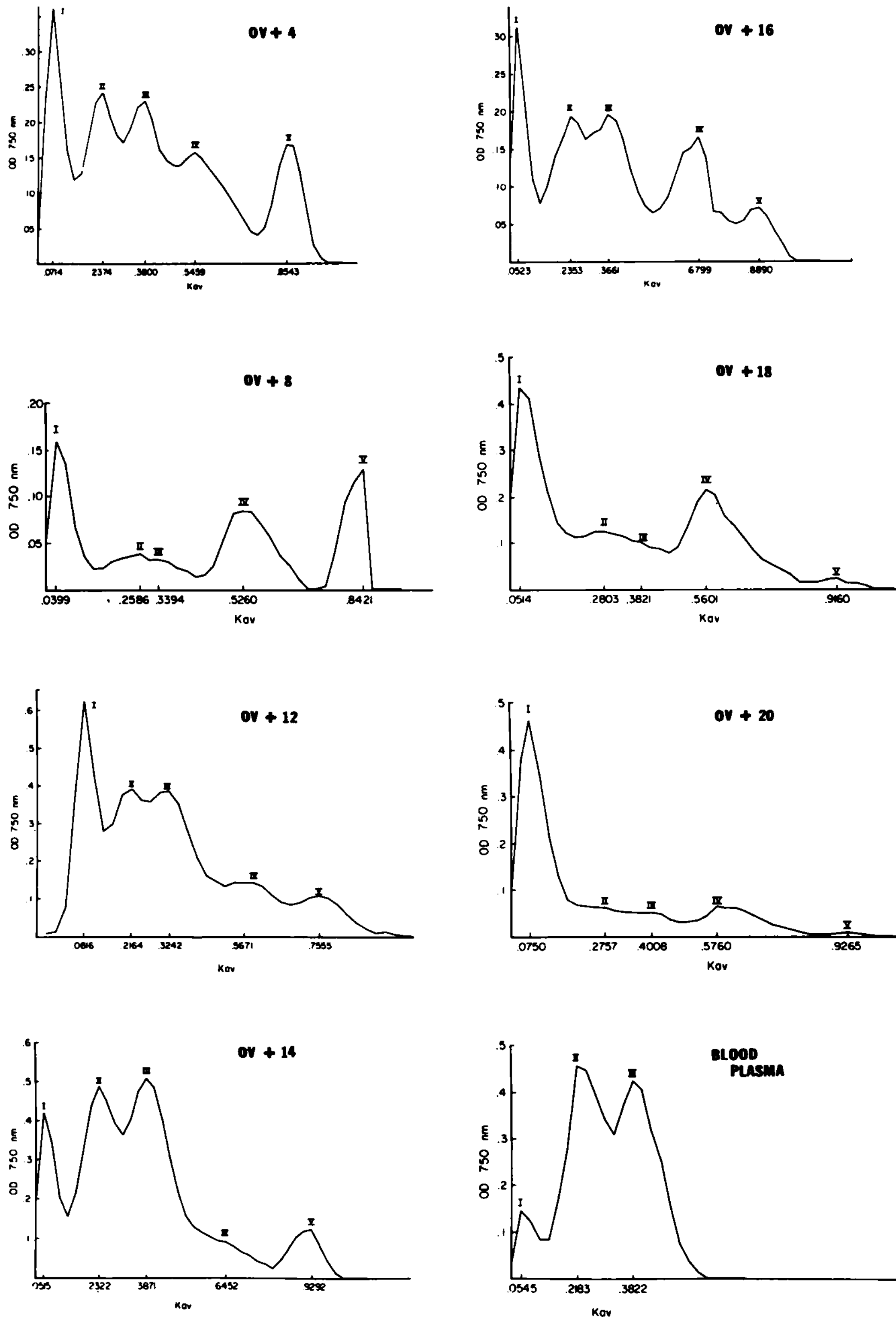

FIG. 1. Representative Sephadex G-200 protein profiles for each of the treatment days and for equine blood plasma. Numerals above peaks designate fraction number.

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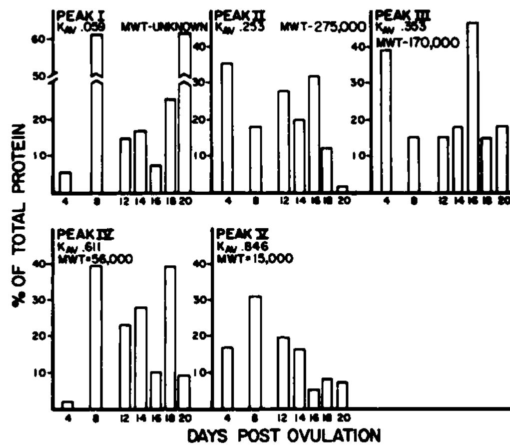

FIG. 2. Least squares means histogram of fractions I, II, III, IV and V as a percent of total recoverable protein on Days 4, 8, 12, 14, 16, 18, and 20 postovulation.

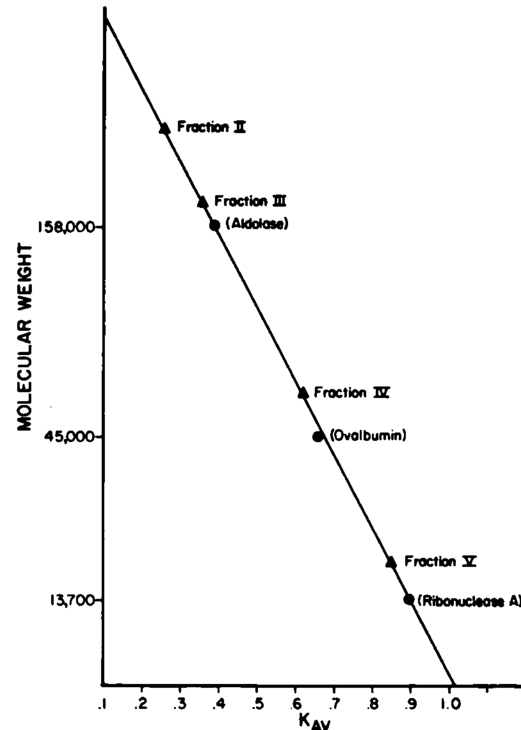

FIG. 3. A comparison of K_{av} vs molecular weight, plotted on semilogarithmic paper, of standard proteins (aldolase = 158,000 MWT; ovalbumin, 45,000 MWT and ribonuclease A, 13,700 MWT) and uterine protein fractions II–V. Fraction I eluted with the void volume; therefore, no accurate estimate of the molecular weight could be made.

molecular weight in the range of 40,000 daltons. Additionally, 3 minor higher molecular weight fractions were found.

Ouchterlony gel immunodiffusion results are shown schematically in Fig. 7. This plating is interpreted as indicating a cross reaction between well I (porcine Day 15 uterine flushing) and well II (equine pseudopregnant uterine flushing) and a reaction of identity between wells II and III (equine Day 16 uterine flushing) with no reaction concerning the saline control in well IV.

DISCUSSION

Zavy et al. (1978) have previously described quantitative changes in uterine protein of cycling mares throughout the estrous cycle. Data from this study indicated a trend in which recoverable protein reached maximal levels in the late luteal phase (Day 16) of the estrous cycle then declined to minimal levels on Day 20. Similar results have been reported for the gilt (Murray et al., 1972). This increase in protein is of importance since the luteal phase of the estrous cycle is classically thought of as a preparatory stage of pregnancy.

Analysis of the present data indicates that acid phosphatase activity was also greater late in the luteal phase of the estrous cycle. A similar pattern of acid phosphatase activity has been reported for the gilt by Chen et al. (1975). In the pig, uterine luminal protein peaks on Day 15 of the estrous cycle, which is coincidental with the time of maximum purple acid phosphatase secretion. Recently, Ducsay (1977) indicated that the purple acid phosphatase may act as an iron transport protein in the pig. From the present study it is not known if peak synthesis of the purple protein occurs on Day 18 or if the increased levels at this time are due to accumulation of the protein. It is of interest to note the rapid decline in acid phosphatase activity from Days 18–20 (8-fold decrease in specific activity). This decline may be explained by a uterine "wash-out," in which the protein leaves the lumen of the uterus either via the cervix as has been demonstrated in the rat (Warren, 1938) or into the stroma surrounding the uterine glands. Bazer and Thatcher (1977) postulated that the uterine glands may secrete in either an exocrine or endocrine fashion. In the estrous cycle, products of endometrial secretion would be transferred into the uterine vasculature (endocrine secretion) whereas if pregnancy were established, secretion would be

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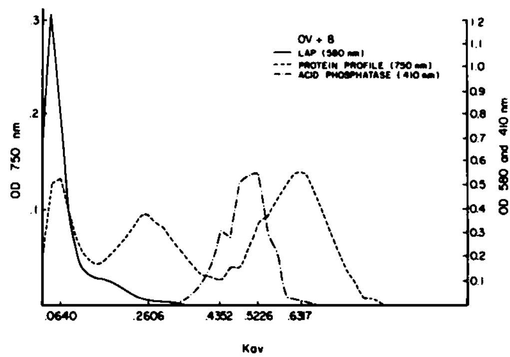

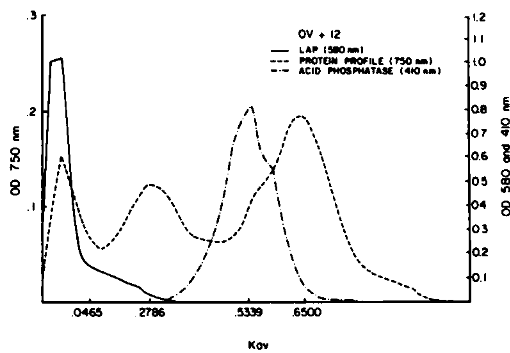

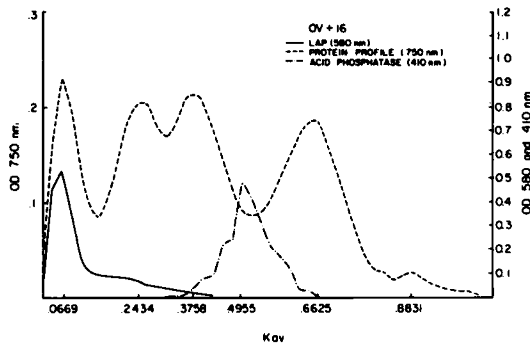

FIG. 4. Leucine aminopeptidase and acid phosphatase enzymatic activities of mare uterine flushings located on Sephadex G-200 protein profiles for Days 8, 12 and 16 postovulation.

in an exocrine fashion (toward the uterine lumen) in order to supply histotrophe for the developing conceptus. At the present time it is not known if the acid phosphatase in the gilt and mare are similar or if they serve similar roles. However, Sephadex G-200 protein profiles and Ouchterlony results from this study lend support to the supposition that a

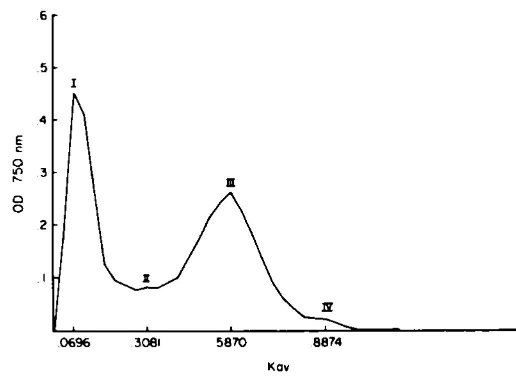

FIG. 5. Sephadex G-200 protein profile of a Day 66 (pseudopregnant) mare uterine flushing.

similar protein exists in the mare and gilt.

Leucine aminopeptidase activity was greatest in the midluteal phase of the estrous cycle. The peak occurred at a time when progesterone in the peripheral plasma is elevated according to data from Ganjam et al. (1975) and Sharp and Black (1973). Data from Beier (1974) indicate that LAP in rabbit uterine flushings is modulated by progesterone of maternal origin. Roberts et al. (1976) demonstrated that LAP in uterine flushings of nonpregnant ovariectomized gilts was under the influence of progesterone. These authors indicated that the enzyme accumulated in allantoic and amniotic fluids of pregnant gilts. The role of LAP in the nonpregnant uterine environment is not known. However, in the pregnant animal it seems likely that it functions to hydrolyze small peptides.

Results from gel filtration chromatography of mare uterine flushings indicated that 5 protein peaks existed for each of the treatment

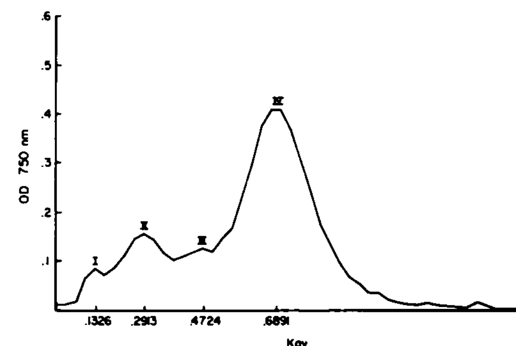

FIG. 6. Sephadex G-200 protein profile of a Day 66 CMC+ (pseudopregnant) mare uterine flushing.

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OUCHTERLONY PLATING RESULTS

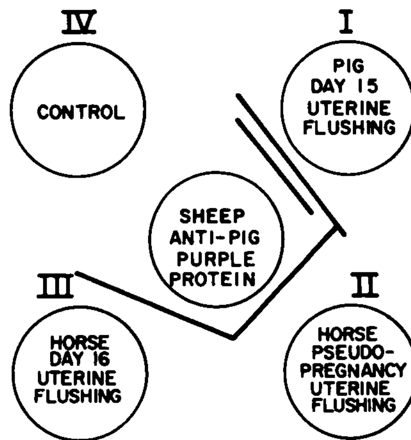

FIG. 7. Ouchterlony plating, indicating relationships between porcine Day 15 uterine flushings (high in purple protein) in well I, mare pseudopregnant uterine flushings (lavender colored) in well II, Day 16 mare uterine flushings (colorless) in well III and a saline control in well IV. A smooth continuous line between antigen wells indicates the presence of an identical protein (i.e., wells II and III), a spur indicates the presence of similar proteins (i.e., as in wells I and II). The absence of a precipitin line between the antibody and antigen wells indicates the absence of protein for which the antibody is specific.

days. The first 3 protein peaks (mean K_{av} values: 0.059, 0.253 and 0.353, respectively). However, none of the major plasma protein fractions had elution patterns similar to fractions IV (0.611) or V (0.846) from uterine flushings. Since fractions I–III were similar in both blood plasma and uterine flushings, the possibility exists that these proteins are present in blood and appear in uterine secretions by transudation. Relative to this, it appears that the quantity of protein fraction V (0.846) may be modulated by stage of the estrous cycle (Fig. 2) and thus reflect the influence of ovarian steroids. Least squares analysis of peak V indicated a significant change ($P<0.025$) throughout the estrous cycle, when expressed on a percentage of total protein basis. Additionally, fractions I and IV were also significantly affected ($P<0.005$ and $P<0.05$, respectively) by day of the estrous cycle. However, throughout the estrous cycle there appeared to be no clear relationship between the pattern of change in fractions I and IV and the known endocrinology of the uteroovarian axis. This may best be explained by the large variation associated with mares and the low number of observations on some of the days (Days 8 and 20).

Data from this study indicate that acid phosphatase activity was associated with an area between fractions III and IV when chromatographed on Sephadex G-200. This indicates that the equine acid phosphatase is probably nonlysosomal in origin, since lysosomal acid phosphatases are high molecular weight proteins which would ordinarily be associated with fractions I and II. The characteristic is shared with the purple acid phosphatase isolated from porcine uterine flushings by Schlosnagle et al. (1974) and may be unique among this class of enzymes. Acid phosphatase from porcine uterine flushings has a purple coloration. This is not always the case with the equine acid phosphatase from the cycling mare. Whether the absence of color is due to lack of iron binding potential or a difference in reduction state is not known. Since acid phosphatase from the pig and mare share similar physical, enzymatic and immunological characteristics, it is possible that they also serve a similar role(s).

On the basis of Sephadex G-200 protein profiles, LAP was associated with the higher molecular weight fraction I and II. No estimate of the molecular weight of fraction I could be made in that it eluted with the blue dextran marker and was beyond the inclusion limits of the column. Basha et al. (1978) found that LAP from pig uterine flushings has an estimated molecular weight of 480,000 daltons which would associate with fraction I on Sephadex G-200 similar to mare LAP. Beier (1974) reported that in the rabbit the presence of uterine LAP in early pregnancy was associated with time of implantation. The equine embryo lies free within the uterine lumen until initial attachment is made after about 36 days of pregnancy (Allen et al., 1973). Due to the noninvasive nature of the mare's placenta, a more plausible explanation for the role of LAP would be to hydrolyze small peptides for subsequent uptake by the trophoblast at a time when histotrophic nutrition is most important (Basha et al., 1978).

The presence of a purple colored protein in a pseudopregnant mare's uterine flushing was of interest since a similar protein had been previously described in the pig by Schlosnagle et al. (1974). Throughout the course of this study the purple protein was not visualized in the uterine flushings of mares during the cycle. This

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protein was shown to bind to CMC, possess high acid phosphatase activity and cross react with antibody specific for porcine purple protein. From these observations it is evident that the protein is similar in the sow and mare.

Additional results from Ouchterlony plating indiated that a Day 16 mare uterine flushing had a decreased amount of this protein, as judged by a less dense precipitin band. These data indicate that a colorless form of the purple protein may exist in the uterine flushings of luteal phase mares. Comparable levels of acid phosphatase activity are present in the uterine flushings of the gilt and mare. Since acid phosphatase activity of pseudopregnant mare uterine flushing was greater than for Day 16 uterine flushings, the possibility exists that the noncolored form of purple protein found in the luteal phase is an altered form of the protein seen in the pseudopregnant mare. This may partially explain why there is decreased acid phosphatase activity during the estrous cycle when compared with the uterine flushing from pseudopregnancy.

The finding that the sow and mare share a similar protein within their uterine secretions is not surprising in that both species possess a diffuse epitheliochorial type of placentation. Therefore, it would not be inconsistent to assume that similar maternal-fetal transfer systems for nutrient exchange exist in the two species. Amoroso (1952) suggested that in species which possess this type of placentation, histotrophe plays an important role in nurturing the conceptus.

ACKNOWLEDGMENTS

The authors wish to thank D. R. J. Collier for reviewing this manuscript. This research was supported by NIH Grant HD08560 to FWB and a grant from the Morris Animal Foundation to DCS. Florida Agricultural Experiment Station. Journal Series No. 1298.

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RECOMMENDED REVIEWS

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