Vaginal Permeability and Enzymatic Activity Studies in Normal and Ovariectomized Rabbits

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Purpose. This study was initiated to develop an animal model, using ovariectomized rabbits, for the post-menopausal human, based on in vitro vaginal tissue permeability and aminopeptidase activity.

Methods. An enkephalin derivative [D-ala^2,N-methyl-phe^4-glycol^5][tyrosyl-3,5-^{3}H] enkephalin {[^{3}H] RX 783006}, which has relative enzymatic stability to aminopeptidases and dipeptidyl peptidase, was used as a model peptide drug for permeability experiments. Aminopeptidase activity in vaginal homogenates, as well as in tissue pieces, was determined using 4-methoxy-2-naphthylamides of leucine, alanine, arginine, and glutamic acid as specific substrates. In addition, histological examination of normal and ovariectomized vaginal tissues was performed.

Results. Vaginal permeability of the drug was significantly increased in the ovariectomized compared to the intact animal. The full vaginal tissue became thinner and mucosal epithelial thickness was reduced about two-fold after ovariectomization and vaginal cells from the castrated rabbit were typically immature. Aminopeptidase activity, leucine aminopeptidase, aminopeptidase B and A, was the same in vaginal tissue homogenates and whole-tissue specimens in both normal and ovariectomized rabbits whereas the activity of aminopeptidase N was significantly decreased in ovariectomized as compared to normal rabbits.

Conclusions. Based on the present data, the ovariectomized rabbit may be useful as an animal model for postmenopausal vaginal studies.

KEY WORDS: vaginal permeability; enzymatic activity; ovariectomized rabbits; enkephalin derivative.

INTRODUCTION

Vaginal drug delivery offers significant advantages over other routes including a sizeable surface area, rich vascularity, relatively high permeability to many drugs, capacity to hold an adult human female dose, and bypassing first-pass metabolism (1). Disadvantages include hormone-dependent changes in the nature of the tissue and environment of the vagina and possible periodic changes produced by sexual intercourse.

Establishing an appropriate animal model to study vaginal drug disposition and test prototype dosage forms is difficult, given the anatomical and physiological differences between animals and humans. Various animal models including rodent, rabbit, monkey, and sheep have been used in vaginal absorption studies (2). In most animals, the vagina is subject to cyclic changes which can affect its permeability to drugs. Thus, it has been reported that the in vitro permeability coefficients of vidaribine during the diestrous phase in mice and guinea pigs were 10–100 times higher than during estrous (3). Okada et al. clearly showed the effect of cyclic changes on vaginal absorption of insulin, phenolsulfonphthalein, salicylic acid, and the luteinizing hormone-releasing hormone (LH-RH) analogue (leuprolide) in rats (4). They concluded that vaginal absorption of hydrophilic compounds, which are transported mainly through intercellular channels, is highly dependent on the estrous cycle.

The ovariectomized rat model, with or without subsequent hormone treatment, has been used for absorption studies to minimize variability and standardize thickness of the vaginal epithelium. The vaginal epithelium became thin and atrophic after ovariectomy and subsequent application of hormone treatment reestablished vaginal epithelial thickness (2). It was shown that vaginal absorption of gentamicin and insulin could be significantly increased with penetration enhancers in ovariectomized rats (2, 5). Nishi et al. used ovariectomized-steroid blocked rats to investigate the effect of oral and vaginal administration of synthetic LH-RH and [D-ALA^6,DES GLY^{10}-NH$_2$]-LH-RH ethylamide on serum luteinizing hormone (LH) levels. They reported that an increase in serum LH and follicle stimulating hormone (FSH) levels with vaginal application of LH-RH and its analogs was higher than that following oral application (6). Vaginal absorption of a number of drugs, under a variety of conditions, has been reported using the rat model (2, 5, 6).

Oral, nasal, rectal, and vaginal absorption of progesterone was carried out in ovariectomized rabbits because they have a low and stable plasma progestin baseline after ovariectomy (7).

Ovariectomized animal models have also been used to investigate the effect of ovariectomy on LH-RH and FSH release, as well as development of atherosclerosis and bone loss disease state models.

In addition to tissue thickness changes with hormonal fluctuation, enzymatic activity can also change, which may be of concern for metabolically labile drugs such as peptides and proteins. Fishman and Mitchell demonstrated that the activity of β-glucuronidase, acid phosphatase, alkaline phosphatase, and esterase in vaginal tissue varied in premenopausal and postmenopausal women (8). It has also been reported that rat vaginal smears have trypsin-like activity, which reaches a maximum during the proestrus stage (9). Kashi and Lee studied the mucosal metabolism of various enkephalins and suggested that at least three peptidases, including aminopeptidases, dipeptidyl peptidase and dipeptidyl carboxypeptidase, play an important role in hydrolysis of the enkephalins. Among these enzymes, aminopeptidases were the major enzymes for methionine and leucine enkephalin; whereas dipeptidyl carboxypeptidase was important for the D-ala-met-enkephalin. The highest activity of dipeptidyl carboxypeptidase was found to be in the order of buccal>rectal>vaginal>nasal (10). In another study, aminopeptidase activity in rabbit vaginal mucosal homogenates was determined to be 87.1% ∓ 23% of ileal activity (11).

Although a number of vaginal permeability and enzyme activity studies have been performed in either intact or ovariectomized animals, no comparative study has been reported. Therefore, the objectives of the present study were to compare the vaginal permeability characteristics and specific aminopeptidase activity in intact vs. ovariectomized rabbits. The rabbit was chosen as an animal model because of its lack of cyclic changes, hence consistency of vaginal tissue thickness; its volume capacity is similar to that of the human; and it is a small, inexpensive test animal. Potential disadvantages of the rabbit

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model is the absence of correlation to the human vagina relative to drug permeability and the tissue is histologically different from primates.

MATERIALS AND METHODS

Materials

An enkephalin derivative [D-ala^2,N-methyl-phe^4,-gly-col^5][tyrosyl-3,5-^{3}H] enkephalin {[^{3}H]RX 783006}, Amersham Life Science Inc., Arlington Heights, IL, with a specific activity of 58.0 Ci/mmol was used in diffusion cell studies. The lability of the tritium label on RX 783006 was tested.

Aminopeptidase substrates, 4-methoxy-2-naphthylamides of L-leucine, L-alanine, L-arginine, and L-glutamic acid were purchased from Sigma Chemical Co., St. Louis, MO.

All other materials and solvents were of analytical grade.

Animals and Treatment

Female New Zealand White Rabbits, ex-breeders, weighing 4.5–5 kg (Bakkom's Rabbitry, Viroqua, WI), maintained under a cycle of 12 h light: 12 h darkness with food and water available ad lib were used throughout the study. The rabbits were ovariectomized under xylazine/ketamine anaesthesia. The animals were allowed to recover for at least four weeks before being used in studies. Luteinizing hormone (LH) level of the rabbits was measured by a radioimmunoassay method[4] in blood samples taken before and after ovariectomy.

Histological Studies

Rabbits were sacrificed by a lethal injection of sodium pentobarbital into a marginal ear vein and harvested vaginal tissues were fixed in Bouin's Fixative Solution for 24 h. For light microscopy, a 2 mm thick cross section of proximal and distal tissue samples was routinely processed and embedded in paraffin and then stained with hematoxylin and eosin. The mucosal epithelial thickness of vaginal tissues was measured using an eye piece graticule calibrated with a stage micrometer. The data were evaluated for each rabbit to express the mean mucosal epithelial thickness.

Diffusion Studies

Six side-by-side diffusion cells (Precision Instrument Design, Los Altos, CA) were used for the diffusion studies. The diffusional area was 0.785 cm^2 and temperature was maintained at 37°C. The following buffer systems were employed in the diffusion experiments: pH 4.5 $\mp$ 0.3 Walpole Acetate buffer and pH 7 $\mp$ 0.3 Sorensen Phosphate buffer. Tonicity of the buffers was adjusted with NaCl (295 mOsm). The solutions were aerated by a gas mixture (O_2/CO_2, 95/5) throughout the study.

After animal sacrifice, vaginal tissue was removed and placed in ice-cold saline solution. Tissue samples were mounted in the diffusion cells with the mucosal surface facing the donor cell. Diffusion experiments were performed for not more than 4 hr. Tissue integrity during the course of the study was monitored with electrophysiology measurements and ATP levels. The apparent permeability coefficient (P) of RX 783006 was calculated using the following equation

$$P = (V/AC_o][dC/dt]$$

where V is the volume of the chamber (7 ml), A is the diffusional area of the tissue, C_o is the initial concentration of the donor cell (100%), and dC/dt is the steady state slope of the plot of the percent drug transported vs. time.

Statistical analysis of the data was done using student t test.

Aminopeptidase Activity Studies

After euthanasia, vaginal tissue was removed and rinsed with ice-cold saline solution. Either homogenate or chopped whole vaginal tissue specimens were used in the experiments. The vaginal tissue was cut into small pieces and frozen in liquid nitrogen to prepare mucosal homogenates. These frozen tissue pieces were then crushed with a mortar and pestle and the powder homogenized in cold 0.05 M Tris-maleate buffer, pH 7.4, using a Teflon homogenizer for 1 min. The supernatant was separated by centrifuging at 3000× g for 2 min. The homogenate and tissue samples were kept on ice and used within 4 h of preparation. Protein concentration of the supernatant was determined using a dye-binding assay with rabbit serum albumin as the standard (12) and aminopeptidase activity was determined using the method of Stratford and Lee (11). For this purpose, stock solutions of 4-methoxy-2-naphthylamides of L-leucine, L-alanine, L-arginine, and L-glutamic acid were prepared in dimethylformamide (3 mM). 100 μl of substrate was added to the reaction mixture consisting of tissue homogenate or tissue pieces and 2.8 ml of 0.05 M Tris-maleate buffer pH 7.4. Concentration of the substrates were determined from the preliminary maximal hydrolytic velocity (V_{max}) measurements. The reaction mixture was preincubated at 37°C for 15 min. Fluorescence intensity was monitored at an excitation wavelength of 342 nm and an emission wavelength of 426 nm for 5 min (Hitachi F-3010, Fluorescence Spectrophotometer). Boiled tissue homogenates or pieces of tissue were used as the blank. Duplicate or triplicate incubations were performed for each sample.

Initial velocities were calculated from the standard curves for fluorescence intensity vs. moles β-naphthylamine and from plots of fluorescence intensity vs. time. Activity was expressed in μmoles of substrate hydrolyzed per min per mg protein for homogenates and nmoles of substrate hydrolyzed per min per mg tissue for tissue pieces.

RESULTS AND DISCUSSION

Histological Studies

Histological studies of normal rabbit vagina showed that the vaginal wall (mucosa to serosa) had a well-developed tunica muscularis. In the upper and the middle part of the vaginal tract (2/3), the mucosa has complex primary and secondary folds lined by a simple high columnar mucinous epithelium. The mucosal epithelium cytoplasm is strongly positive for mucosubstance as shown PAS-Periodic acid schiff stain. The lamina propria is moderately expanded by edema. The lower part of the vagina (1/3) is lined by non-keratinized stratified squamous epithelium consisting of about 5–7 cells. In the case of the ovariectomized

[4] Radioimmunoassay developed by the University of Wisconsin Primate Center.

rabbit, vaginal wall thickness is attenuated. The tunica muscularis was atrophic as evidenced by markedly reduced muscle fiber diameter with a relative increase in cellularity of muscle bundles. The mucosa had a variable and marked loss of primary folding with large segments having no folds. Intermittent foci with short primary folds had only residual secondary folds. The mucosal epithelium is a simple, low columnar, non-mucinous epithelium. The mean mucosal epithelial thicknesses of the upper part of the vagina in intact rabbits was measured as 30.3 (∓ 4) μm (Table I). After ovariectomization, the entire tissue became thinner and mean mucosal epithelial thickness was reduced to 14.9 (∓ 2.5) μm. The mean mucosal epithelial thickness of the lower vagina was also reduced from 57.5 (∓ 0.1) μm to 30.6 (∓ 2.9) μm. The difference between the mean mucosal epithelial thickness of the intact and ovariectomized rabbit vagina was statistically significant ($p < 0.005$). It was interesting to note that the upper mucosal epithelium thickness of two of the ovariectomized rabbits was reduced, but the appearance of the epithelial lining was similar to that of the intact rabbit, i.e., primary and secondary folding. It has been reported that plasma LH and FSH levels markedly rise after ovariectomy (13). In order to evaluate a potential relationship between tissue vs. hormonal changes, plasma LH levels of the ovariectomized and control rabbits were measured. In intact rabbits, plasma LH levels were almost the same. However, LH levels reached a maximal value of 1.4–60 fold above intact levels 24–54 days after ovariectomy in the ovariectomized rabbits. Upon examining the increase in LH level of the ovariectomized rabbits, it can be seen that an increase in LH level ratio of these two rabbits (Ovariectomized rabbits number 4 and 5) was lower than the other three ovariectomized rabbits (Table 1).

Diffusion Studies

Enkephalins are hydrolyzed primarily by three peptidases including aminopeptidase, dipeptidyl peptidase, and dipeptidylcarboxypeptidase (10). The aminopeptidases are the major enzymes responsible for degradation of enkephalins. Replacement of Gly^2 in methionine enkephalin by D-Ala makes the peptide more stable against aminopeptidases. An enkephalin derivative, (D-Ala^2-$Mephe^4$-$glyol^5$-tyrosyl-3,5) enkephalin, was used as a model peptide drug for all permeability experiments. This drug was chosen because the structure of this enkephalin is based on the tetrapeptide sequence Tyr-D-Ala-Gly-MePhe, which shows enzymatic stability, particularly to aminopeptidases and di-peptidyl peptidase and also because Kashi and Lee reported vaginal tissue metabolism data in homogenates from a normal rabbit in an earlier study. They reported that the half-life of D-Ala-met-enkephalinamide, which has a similar sequence to RX 783006, was 183.7 min in vaginal homogenates whereas the half-life of leucine and methionine enkephalin was 27.8 min and 22.2 min, respectively (10). The activity of aminopeptidase N was pH dependent with maximum enzyme activity in the range of pH 7.5–8 and reduced activity below pH 6.5 (14).

Diffusion experiments were performed at pH 4.5 and 7.0. These pHs were used to represent the extreme pre- and postmenopausal conditions in the human female. Figure 1A and B show the result of the permeability experiments at pH 4.5 and

Table I. Mucosal Epithelial Thicknesses of Vaginal Tissues of Intact and Ovariectomized Rabbits (Mean ∓ SEM) and LH Level Ratio in Ovariectomized Rabbits

Rabbit	X (μm)(Upper)[a]	X (μm)(Lower)[b]	LH Level Ratio[c]
Control 1	29.0 (∓0.3)	—	—
Control 2	35.3 (∓0.5)	57.1 (∓1.1)	—
Control 3	27.7 (∓0.3)	57.6 (∓0.5)	—
Control 4	28.2 (∓0.4)	57.9 (∓0.7)	—
Control 5	31.1 (∓0.3)	—	—
Mean	**30.3 (∓1.4)**	**57.5 (∓0.1)**	
Ovariect. 1	12.9 (∓0.1)	27.3 (∓0.8)	28.3
Ovariect. 2	10.7 (∓0.2)	23.7 (∓0.4)	7.79
Ovariect. 3	9.1 (∓0.2)	26.9 (∓0.5)	11.6
Ovariect. 4	20.8 (∓0.2)	37.8 (∓1.0)	4.12
Ovariect. 5	21.0 (∓0.2)	37.3 (∓0.7)	1.41
Mean	**14.9 (∓2.5)**	**30.6 (∓2.9)**	

[a] n = 300 counting sites/rabbit.
[b] n = 100 counting sites/rabbit.
[c] ratio represents LH level before ovariectomy/after ovariectomy.

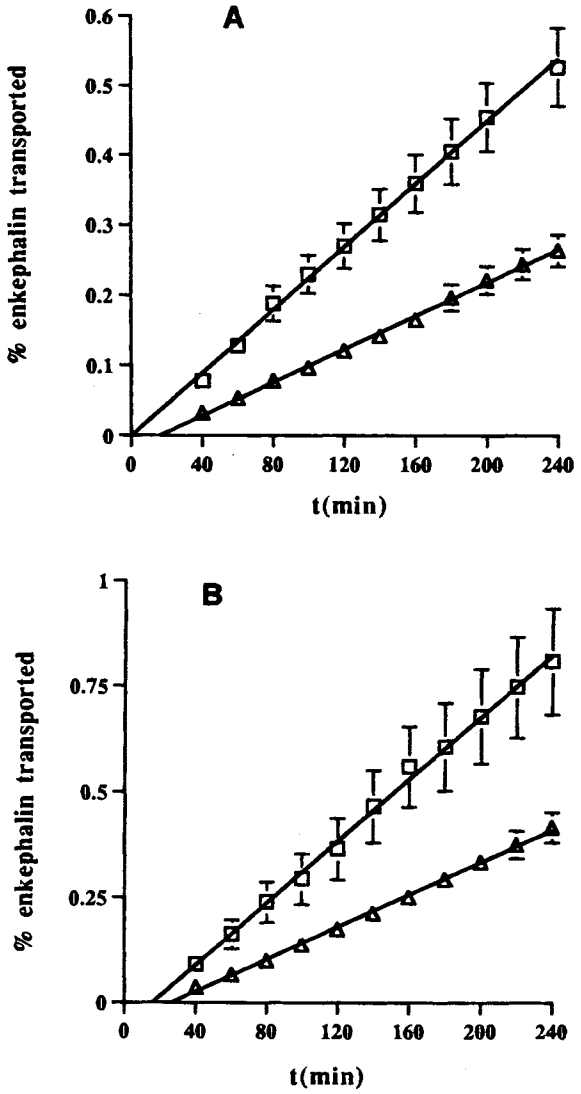

Fig. 1. Permeability of RX 783006 in rabbit vagina in vitro. A—pH 4.5; B—pH 7.5. △—intact; □—ovariectomized.

7, respectively. The calculated permeability coefficients of RX 783006 are summarized in Table II. The data reveals that the permeability coefficients of the drug were significantly increased in the ovariectomized compared to the normal animal ($p < 0.005$). Specifically, the permeability coefficients of the drug in the ovariectomized rabbit were 1.92 and 1.90 times higher than those of the intact rabbit at pH 4.5 and 7, respectively. It was noted that pH had a significant effect on permeability of the drug. The enkephalin molecule has three dissociable groups; therefore, the pH of the solution affects ionization and hence permeability of the drug. Although the permeability coefficient of the drug at pH 4.5 is significantly lower than that at pH 7.0 ($p < 0.005$), the incremental ratio remained constant. On the other hand, variation in the permeability data of the ovariectomized rabbits was equal to or greater than normal animals. It appears that vaginal tissue changes due to hormonal fluctuations affects permeability of the rabbit vagina and can differ from rabbit to rabbit. Examination of the permeability vs. increase in LH level ratio data leads to no direct relationship.

Earlier work has reported that estrogen absorption from the vagina improved in postmenopausal women due to a reduced epithelial thickness (15). Simple thinning of the epithelium will not lead to an increase in permeability but rather a change in the tissue is needed to alter permeability. Although the rabbit does not exhibit an estrous cycle, increased permeability of drug after ovariectomy must be attributed to tissue changes related to a reduced epithelial thickness in ovariectomized rabbits. Due to the anatomical structure of the rabbit, the upper and middle part of the vaginal tract can be easily removed and used for diffusion experiments. The primary and secondary foldings in this part of the vagina can increase the surface area of the vagina and it was thus expected that permeability of the drug would be enhanced in intact vs. ovariectomized rabbit vagina. However, the area issue was masked by the overall increase in permeability in the ovariectomized rabbit. Epithelial tissue changes, related to thickness, are important for altering drug permeability. Indeed, when mucosal epithelial thickness was reduced about 2-fold, the permeability coefficient increased 1.9 times.

Aminopeptidase Activity Studies

Aminopeptidases, one of the major enzymes for peptide and protein degradation, are distributed throughout the cell. The aminopeptidase substrates, 4-methoxy-2-naphthylamides of L-leucine, L-alanine, L-arginine, and L-glutamic acid are relatively specific for leucine aminopeptidase, aminopeptidase N, aminopeptidase B, and aminopeptidase A, respectively. Aminopeptidase N and A are membrane bound peptidases whereas aminopeptidase B and leucine aminopeptidase are cytosolic enzymes. The presence of all of the aminopeptidases except aminopeptidase B in vaginal homogenates was reported by Stratford and Lee (11). In the present experiments, whole vaginal tissue, including mucosa and serosa, was used so that all aminopeptidase activity could be studied. Considering that the homogenization procedure may destroy enzymes, both homogenate and whole tissue pieces were used for specific aminopeptidase activity determinations. Figure 2A and B depict the result of the specific enzyme activity in intact and ovariectomized rabbits. In both cases the same pattern was obtained, i.e., aminopeptidase N activity appears to be the highest both in homogenate and whole tissue pieces followed by leucine

Table II. Mean Permeability Coefficients (P) of RX 783006 in Normal and Ovariectomized Rabbit Vaginal Mucosa *in Vitro* at pH 4.5 and pH 7.0

	pH 4.5		pH 7.0	
Rabbit	N[a]	P (cm/sex × 10^{-6})	N	P (cm/sec × 10^{-6})
Control	23/4	1.76 (∓0.16)[b]	23/4	2.84 (∓0.26)
Ovariectomized	23/5	3.34 (∓0.31)	25/5	5.45 (∓0.80)

[a] Number of experimental determinations (experiment/animal).

[b] Number in parentheses represents the standard error of the mean (SEM).

Note: Differences between RX 783006 permeability measurements were significant to $p < 0.005$.

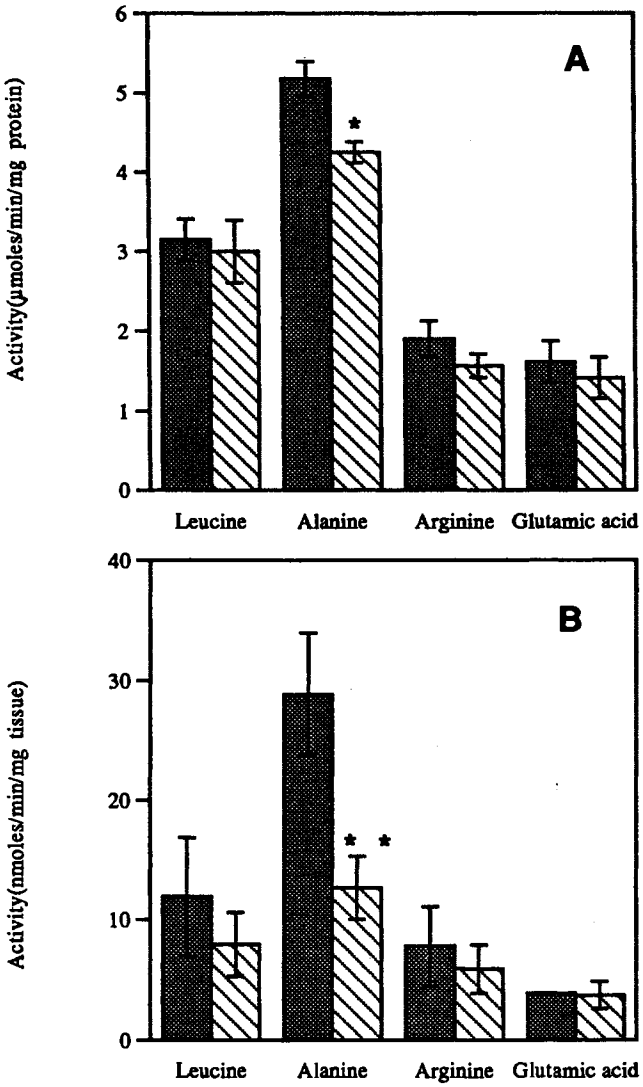

Fig. 2. Aminopeptidase activity in the intact and ovariectomized rabbit vagina against 4-methoxy-2-naphthylamide substrates using vaginal homogenates (A) and whole tissue pieces (B). ▩ —intact; ▨ —ovariectomized Each bar represents the mean + of three to five rabbits. * $p < 0.005$. ** $p < 0.01$.

aminopeptidase, aminopeptidase B, and aminopeptidase A. Specific aminopeptidase activity values in vaginal homogenates was found higher than those values reported by Stratford and Lee (11). This may be due to use of the whole tissue (mucosa-serosa) samples in the present experiments. It was noted that the aminopeptidase activity, except aminopeptidase N, was not statistically different between the intact and ovariectomized rabbit. In the case of aminopeptidase N, a significant decrease in activity was noted in both vaginal homogenates and whole tissue specimens of the ovariectomized rabbit (Figure 2). Variability in metabolism, at least with this class of enzymes, was no less, and in some case greater in the ovariectomized versus the normal rabbit. It would appear that having a consistent tissue thickness by surgical castration of the test animal does not insure, and in fact makes worse, consistency in metabolic rates or in permeability.

In summary vaginal permeability of a peptide drug, an enkephalin derivative, differed markedly after ovariectomy in the rabbits. The vaginal epithelia changed and the thickness of the entire vaginal epithelia was significantly reduced. Surprisingly, the ovariectomized animal does not give less variability and, indeed, in the case of pH 7.0 it is considerably higher. Thus, variability in tissue thickness may be reduced in the ovariectomized animal, but the increase in permeability as a result of this tissue change remain variable. Metabolically, the activity of leucine aminopeptidase, aminopeptidase A, and aminopeptidase B remained unchanged both in intact and ovariectomized rabbits whereas aminopeptidase N activity was reduced in the ovariectomized rabbits.

It was concluded that although the rabbit has no estrus cycle it can simulate the postmenopausal human female by ovariectomization. Although the quantitative relationship between the ovariectomized rabbit and postmenopausal human, relative to permeability and metabolism, is not yet known, qualitative similarities are apparent.

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