Phenolic Plant Compounds Functioning as Reproductive Inhibitors in *Microtus montanus*

Abstract. *Naturally occurring cinnamic acids and their related vinylphenols were found to inhibit reproductive function in* Microtus montanus. *When fed on these compounds, the rodents exhibited decreased uterine weight, inhibition of follicular development, and a cessation of breeding activity. It is suggested that these animals utilize plant compounds as a cue to terminate their reproductive effort in natural populations.*

Over 40 years ago, Friedman and his collaborators noted that extracts of alfalfa and green oats exerted an apparent gonadotrophic effect similar to that of the animal estrogens (*1*). Subsequently, Bennetts *et al.* correlated the infertility of sheep in Australia with the estrogenic effects of subterranean clover (*Trifolium subterranean*) (*2*). These findings initiated a series of investigations of "plant estrogens." Most of these studies were directed at examining the problems of infertility in livestock and concentrated on estrogenic compounds in pasture grasses (*3*). Certain nonestrogenic compounds were found to have varying degrees of inhibitory activity on reproduction. Thus, extracts of alfalfa were identified which could inhibit the estrogenic responses to natural animal estrogens such as 17-β-estradiol (*4*). Aqueous extracts of yellow pine needles were found to depress the uterine weight of immature weanling mice (*5*). Chury (*6*) succeeded in obtaining an inhibitor substance from alfalfa in crystalline form, but he failed to make any structural identification.

We have examined the interactions of *Microtus montanus*, a small herbivorous rodent, with its plant food resource, and have identified two naturally occurring compounds and their derivatives which appear to function as reproductive inhibitors of *Microtus*.

Winter wheat was sprouted under standard conditions to a height of 10 cm, harvested, and homogenized with water. The resulting pulp was subjected to continuous steam-ether extraction for 7 days (*7*). The extract was subsequently examined by gas chromatography, partitioned into three fractions, and bioassayed for reproductive activity. The assay system utilized juvenile female *M. montanus* of uniform age and weight (17 to 18 days; 13 to 14.5 g), that were obtained from a laboratory breeding colony. Plant fractions or compounds to be assayed were dissolved in ether and mixed into ground Purina Lab Chow. The solvent was then evaporated under vacuum and the resulting feed, coated with the test compound, was administered daily to the animals (*8, 9*). After 7 or 12 days the animals were killed by cervical dislocation. The uteri were removed, cleaned of fat and connective tissue, and weighed on a Mettler H20 balance. Control animals received Purina Lab Chow only. Weights of uteri of control animals were compared to those of experimental animals by means of Student's *t*-test (*10*).

When the plant fractions were assayed, one fraction was determined by uterine assay to be inhibitory (Table 1). This fraction appeared to be composed of a single compound with infrared absorptions at 2.85 μm and 11 to 13 μm, suggesting a phenolic structure, probably in the alkoxyphenol family. The nuclear magnetic resonance spectrum showed proton resonance signals at τ 4.3 to 5.1, suggesting the presence of a vinyl group, and signals at τ 2.7 to 3.6, suggesting that the phenol is disubstituted (*11*). An authentic sample of 4-vinylguaiacol (4-VG, 4-hydroxy-3-methoxystyrene) was prepared and was identical in its chemical properties and its biological activity with the inhibitor compound isolated from wheat (Table 1). The determination that 4-VG was the major phenolic compound was in accord with the findings of Steinke and Paulson (*12*). These workers determined that 4-VG along with 4-vinylphenol (4-VP, 4-hydroxystyrene) were the major phenolic compounds in steam extracts of corn, and that both compounds were produced from their parent cinnamic acids, ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) and *p*-coumaric acid (PCA, 4-hydroxycinnamic acid) by heat during the extraction process. When administered daily at dose levels of 1 mg per gram of basal diet, 4-VG and 4-VP caused a highly significant ($P < .01$) reduction in uterine weight after 7 days (Table 1). Results obtained with the parent cinnamic acids, FA and PCA, administered daily at dose levels of 4 mg per gram of basal diet were inconclusive after 7 days of treatment but induced a comparable response after 12 days (Table 1).

A decrease in uterine weight is not conclusive evidence of reproductive inhibition. Thus, ovarian follicle development and breeding success were also studied for responsiveness to inhibitor compounds. Nulliparous adult females were fed the basal diet to which had been added 4-VG, and control animals received the basal diet of Purina Lab Chow only. After 7 days the animals were killed and the ovaries were serially sectioned and examined histologically for follicle development. In both control and experimental animals all follicles in each ovary larger than 250 μm in diameter were counted (*13*). The follicles were divided into two size classes on the basis of diameter. The large follicles (350 to 550 μm) are fully developed and would ovulate if an ovulatory stimulus were received. These follicles were unaffected by the dietary treatment. However, in the smaller size class (250 to 350 μm) there was a highly significant ($P < .001$) decrease in the number of follicles present in the ovaries of the 4-VG treated animals (Table 2). Presumably the smaller follicles are those that will undergo maturation next. *Microtus montanus* is an induced ovulator and typically has a number of mature Graafian follicles present in the ovaries

Table 1. Dietary effects of plant extracted inhibitor and authentic samples of 4-vinylguaiacol (4-VG) and 4-vinylphenol (4-VP) (1 mg/g daily), and *p*-coumaric acid (PCA) and ferulic acid (FA) (4 mg/g daily), on uterine weight in juvenile *M. montanus*. Results are means ± 1 standard deviation.

Compound	Time of treatment (days)	*N*	Mean body weight (g)	Mean uterine weight (mg)
Control	7	35	20.0 ± 2.2	13.7 ± 1.9
Extract*	7†	16	19.6 ± 1.3	10.5 ± 2.1†
4-VG	7	10	20.0 ± 1.2	9.8 ± 1.5†
4-VP	7	7	20.2 ± 2.0	9.9 ± 2.0†
PCA	7	9	20.4 ± 1.2	15.8 ± 6.1
FA	7	10	20.0 ± 1.2	12.5 ± 3.3
Control	12	8	24.3 ± 2.3	17.7 ± 3.2
PCA	12	4	23.4 ± 1.7	11.3 ± 2.0‡
FA	12	6	25.8 ± 2.9	13.6 ± 2.0‡

*Whole wheat extract purified by gas-liquid partitioning chromatography, selecting for a specific fraction. †Significantly different ($P < .01$) from controls. ‡Significantly different ($P < .05$) from controls.

Table 2. Dietary effects of 4-VG on follicle development in adult *M. montanus*. Results are means ± 1 standard deviation.

Regime	*N*	Mean body weight (g)	Mean number of follicles per female	
			350 to 550 μm*	250 to 350 μm
Control	9	29.6 ± 3.7	7.3 ± 1.9	17.7 ± 3.0
4-VG	9	29.1 ± 3.7	7.6 ± 1.7	5.7 ± 2.2†

*Size class of follicles. †Significantly different ($P < .001$) from controls.

Table 3. Dietary effects of 4-VG and PCA on breeding performance of *M. montanus* for 100 days. Results are means ± 1 standard deviation.

Regime	*N*	Mean number of litters per female	Mean number of young per female	Number breeding at 100 days (%)
Control	22	3.45 ± 0.74	15.09 ± 4.28	90.9
4-VG	19	2.79 ± 1.27*	9.79 ± 5.48†	68.4†
PCA	17	2.35 ± 1.22*	9.06 ± 5.20†	58.8†

*Significantly different ($P < .025$) from controls. †Significantly different ($P < .005$) from controls.

throughout much of its estrous cycle. Development had undoubtedly been initiated in the large follicles prior to the experimental period. The inhibitor appeared to be ineffective in halting this development or in increasing the rate of atresia in already mature follicles, but did appear to prevent the development of subsequent follicles.

The effectiveness of a reproductive inhibitor can only be determined by examining its effect on breeding performance. Approximately 80 pairs of nulliparous, 10-week-old, adult *M. montanus* were placed in separate cages and allowed to mate. They were maintained on a photoperiod of 10 hours of light and 14 hours of darkness at 19°C. Initially they received only the basal diet of Purina Lab Chow. After 14 days each female was palpated to determine if pregnancy had been initiated. Only animals that were pregnant were placed under the subsequent experimental regimes (*14*). The successfully mated pairs were placed into one of three feeding regimes: control diet alone, control diet coated with PCA, and control diet coated with 4-VG. These feeding regimes were continued for 86 days. The animals were checked twice weekly for births, and the date and number of young in each litter were recorded. The young were weaned at 17 to 18 days and removed from the experiment. At the end of the experimental period the females were examined by dissection and histology, where necessary, to determine their breeding condition at 100 days from the start of the experiment. Table 3 shows that the control animals maintained a higher level of breeding performance than the animals receiving inhibitory diets. *Microtus montanus* has a gestation period of 21 days, which is followed by postpartum mating. In 100 days the maximum number of litters that could be produced by a single female is four. In the control group (Table 3) 59 percent of the mated pairs produced four litters, but only 24 percent of the PCA-treated group and 32 percent of the 4-VG-treated group produced four litters.

Throughout the experiments the general body condition of each animal was assessed. It could be speculated that the effects of the plant compounds on reproduction were due to toxicity. Tables 1 and 2 indicate that the experimental animals were able to maintain normal body weight compared to control animals, suggesting that toxicity was not the cause of the reduced reproductive activity.

Microtus montanus is a short-lived herbivore living in unpredictable environments in the northern latitudes of North America. In such environments these animals must be able to time their reproductive effort to coincide with the period of maximum food availability. The arrival of snow in the fall and its melting in the spring cannot be accurately signaled by precisely timed factors in the environment such as photoperiod. The most reliable cue to the favorableness of the environment could, perhaps, reside in the food resource itself. If *M. montanus* could use chemical cues from the plant to initiate as well as terminate its reproductive effort, it would have evolved maximum efficiency in the timing of its breeding cycle. Chemical cues from the plant would have to signal in the one case the onset of vegetational growth for the initiation of reproduction and in the latter case the termination of vegetational growth for the termination of reproduction. It has been demonstrated in laboratory experiments (*15*) that the addition of supplements of green plant food to the diet enhances reproductive performance in *M. montanus*. In addition, field studies indicate that the timing of the reproductive effort in *M. montanus* coincides with the initiation of plant growth after the melting of the snow in the spring. These observations suggest that some chemical substance is present in the vegetatively growing plant that enhances, if not initiates, reproduction in *M. montanus*.

The data herein suggest that reproduction in *M. montanus* may be terminated in response to chemical substances present in the plant food resources. If these chemical inhibitors signal the qualitative decline of the food resource they should become abundant in the food grasses at the stage that these grasses enter senescence.

To test this hypothesis and determine whether the naturally occurring plant foods of *M. montanus* contain inhibitors, a sample of salt grass (*Distichlis stricta*) was collected in the winter from Timpie Springs, Utah. This grass is the dominant food item of *M. montanus* in the Timpie Springs populations and represents over 90 percent of their diet throughout the year. At the time of sampling (January 1975) the grass had flowered, fruited, dried, and browned. The dried grass was extracted with methanol and the extract coated onto ground Purina Lab Chow (*16*). Animals ($N = 12$) fed with the coated Lab Chow for 7 days had significantly ($P < .001$) reduced uterine weights (10.0 ± 1.4 mg) compared to control weights (13.7 ± 1.9). These data indicate that inhibitors are present in the natural food of *M. montanus*. Related work also suggests that FA and PCA are most abundant in plants during the later stages of the growth cycle. El-Basouni and Towers (*16*) found that concentrations of these acids in wheat remained below 1 mg/g of dry plant material during the period of active vegetative growth (*17*). As the wheat matured and entered senescence, the amount of PCA increased to 3 mg/g. Kuwatsuka and Shindo (*9*) determined that the PCA content of rice straw (rice that had flowered, fruited, and browned in the field) was 4 mg/g. On the basis of these data it appears that the cinnamic acids are most abundant in the food resource when the grasses have flowered, fruited, and dehydrated at the end of the vegetative growing season. This stage of the plant growth cycle coincides with the end of the breeding season for *M. montanus* in

its natural habitat. These compounds could thus represent reliable cues signaling the termination of a high-quality food supply.

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References and Notes

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8. The amount of compound coated on the feed was determined in an arbitrary fashion. Initially, approximately 1 mg of crude material was extracted per gram of fresh wheat. The crude material was administered in the diet at a dosage of 1 mg per gram of chow. When the crude material was identified as 4-VG, the pure compound was also administered at the level of 1 mg/g, but the parent cinnamic acids, PCA and FA, were administered at the level of 4 mg/g on the basis of results obtained by Kuwatsuka and Shindo (*9*). These workers reported that rice straw contained 4 mg of PCA per gram. While rice and wheat are probably not comparable, this was the only available result. Because *M. montanus* consumes approximately 0.2 g of Purina Lab Chow per gram of body weight per day, the animals were probably receiving a relatively low amount of the various compounds.
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13. It is sometimes difficult to distinguish between atretic and normal follicles. Because there were so few obviously atretic follicles, no attempt was made to count them.
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16. The dried grass was homogenized with methanol, heated for 30 minutes at 80°C, and filtered. The resulting filtrate was evaporated to small volume. This filtrate was subsequently coated onto ground Purina Lab Chow at the level of 1 g of dried grass per gram of Chow.
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Concomitant Elevations in Serum Sialyltransferase Activity and Sialic Acid Content in Rats with Metastasizing Mammary Tumors

Abstract. *Rats with transplantable spontaneously metastasizing mammary tumors have elevated levels of both serum sialoglycoconjugate and serum sialyltransferase activity compared with normal female rats or rats with various nonmetastasizing mammary tumors. A direct relationship was observed between the amount of serum protein–bound sialic acid and serum sialyltransferase activity in all rats studied. Serum sialyltransferase activity in rats with a representative metastasizing mammary tumor, SMT-2A, was also correlated with tumor age. Microsomes prepared from the SMT-2A tumor have a sixfold higher sialyltransferase activity than do microsomes prepared from the nonmetastasizing mammary tumor MT-W9B. Normal rat liver microsomes have the same level of activity as microsomes prepared from livers of animals with either SMT-2A or MT-W9B tumors. The data indicate that spontaneously metastasizing mammary tumor cells have an increased production and release, perhaps through cell surface shedding, of a sialyltransferase. It is suggested that this sialyltransferase may increase the serum half-life of certain tumor-specific circulating glycoconjugates by increasing the content of protein-bound sialic acid and may thereby play a role in the immune escape mechanism of metastasizing tumor cells.*

Cell surface glycoconjugates and glycosyltransferases have been implicated in controlling cell division and intercellular association in a variety of normal cell types. Alteration of such components may lead to loss of growth control (*1*). Increases in specific sialyltransferases have been observed in rat (*2*) and human (*3*) mammary tumor tissue as compared to normal breast tissue. Kim and Chatterjee observed that galactosyltransferase, another specific glycosyltransferase, is higher in spontaneously metastasizing rat mammary tumors than in nonmetastasizing tumors (*4*). These authors also found that nonmetastasizing mammary tumors have a thick ruthenium red–stainable glycocalyx surface coat, while metastasizing mammary tumor cells lack this stainable surface material. It has been suggested that this difference is due to increased turnover and shedding of membrane glycoconjugate rather than to lack of production of the material in the metastasizing tumors. Once shed, this material may provide the tumor with an immune escape mechanism by interfering with the immune response of the host to the tumor (*5*).

Besides containing surface glycoconjugates, mammary tumor cells may also contain surface glycosyltransferases. Bernacki (*6*) and Porter and Bernacki (*7*) reported biochemical and electron microscopic evidence for the presence of an ectosialyltransferase on the surface of murine leukemic cells. Thus, an increase in plasma membrane shedding might result in an increase in serum sialyltransferase activity. Elevated serum sialyltransferase activities have already been observed in animals with a variety of tumors, including mammary tumors (*8*), especially in women with malignant breast tumors (*9*). In this report we compare sialyltransferase (E.C. 2.4.99.1) activities in the serum, liver, and tumors of W/Fu rats with nonmetastatic and metastatic mammary tumors. We conclude that the increased serum sialyltransferase found in rats with metastasizing mammary tumors is provided by the tumor cells, probably through tumor plasma membrane shedding.

The induction of spontaneously metastasizing and nonmetastasizing mammary tumors by 3-methylcholanthrene and the biological and biochemical properties of the resulting induced tumors have been described (*5*). The representative non- or weakly immunogenic metastasizing mammary carcinoma (SMT-2A) and the nonmetastasizing, immunogenic mammary carcinoma (MT-W9B), both induced with 3-methylcholanthrene and maintained in the same strain of W/Fu female rats, were selected for comparative studies since they are similar in their degree of structural differentiation and growth rate.

At appropriate times, normally after the transplanted tumors have reached 1 to 2 cm in average diameter, or as otherwise stated, the rats were anesthetized with ether and the tissues (blood and liver or tumor or both) removed and placed on ice for later assessment of sialyltransferase activity. Blood was allowed to clot and serum was obtained by centrifugation. The serum was frozen at −20°C and later assayed for sialyltransferase activity by using desialylated (*10*) fetuin (GIBCO, Buffalo, N.Y.) or desialylated human α_1-acid glycoprotein as an accep-