THE EFFECTS OF CERTAIN EXTRACTS OF BIRDSFOOT TREFOIL
(LOTUS CORNICULATUS) AND YELLOW PINE NEEDLES (PINUS
PONDEROSA) ON THE REPRODUCTIVE PROCESSES
OF THE LABORATORY MOUSE AND RAT

by

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Members of the Division

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Date July 26, 1962
The anti-estrogenic properties of birdsfoot trefoil (Lotus corniculatus) and yellow pine (Pinus ponderosa) needles were studied. Oral administration of a water-soluble fraction of an acetone extract of birdsfoot trefoil was found to decrease uterine weight of immature female mice. The estrous cycles of rats receiving this extract in the ration, were not disturbed. Specially prepared aqueous fractions of an acetone extract of yellow pine needles decreased the uterine weight of mice. In further experiments, immature female mice were fed 0.040 mcgms. and 0.020 mcgms. diethylstilbestrol (D.E.S.) per gram of feed. When a water-soluble fraction of pine needle extract was administered, it did not affect uterine weight significantly in the 0.040 mcgm. D.E.S. group, but markedly and significantly decreased uterine weight in the 0.020 mcgm. D.E.S. group. This pine needle extract also interrupted the estrous cycles of mature rats, causing a prolonged diestrus. Chromatographic studies indicated that the pine extracts probably do not owe their anti-estrogenic activity to pinosylvin or its mono-methyl ether.
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III INTRODUCTION

An estrogen can be defined as any substance which can cause uterine enlargement or vaginal cornification in ovariectomized or intact, mature or immature female mice. A true estrogen is capable of inducing the characteristic positive response upon intravaginal application while a pro-estrogen does not give a response until it has been absorbed into the blood stream (119). While the endogenous estrogens are well known and characterized, the exogenous estrogens, both of synthetic and plant origin, are still not fully known.

An anti-estrogen can be defined in general terms as any substance which can cause a decrease in uterine weight or prevent vaginal cornification. Dorfman (33) defines anti-estrogenic substances as compounds which interfere with any action of an estrogen at a peripheral level. He further states that inhibition of estrogen action has been shown for androgenic substances, progestational substances, estrogens, and corticoids. Although Dorfman (33) does not mention substances which interfere with the normal gonadotropin levels, such substances are able to interfere with estrogen secretion so that the end result is of a similar nature.

For the past few years, the estrogen-like activity possessed by the extracts of certain of the legumes and grasses has been the object of research in this laboratory. These, as well as other investigations have shown that estrogenic compounds present in a number of forages can be the cause of reproductive disorders in mice (77,119), rats (77,119), and sheep (5,98). A recent summary by Jackson (53) has shown
that many substances of plant origin have various effects on the reproductive processes of animals. The species which have attracted most attention are Lithospermum ruderale, Pisum sativum, and Polygonum hydropiper.

Work in this laboratory (56, 77, 119) has shown that birdsfoot trefoil (Lotus corniculatus) obtained in an early first cutting, possessed estrogenic activity. Later in the season the estrogenticity disappeared, in fact, several times a significant anti-estrogenic activity could be observed. Whenever anti-estrogenic properties were noted, they were found either in the acetone extract of the forage, or in the water soluble fraction of this previously prepared acetone extract. It is interesting to note that the anti-fertility substances found in Lithospermum ruderales and the "inhibitor" of uterine growth in alfalfa as mentioned by Bickoff (11, 13) are both water soluble. Little is known about the nature of the anti-estrogenic compounds which may be present in forage legumes, and further investigations in this direction are necessary.

Work at this laboratory (2, 83) has shown that a specially prepared aqueous fraction of the acetone extract of yellow pine (Pinus ponderosa) needles contains a factor, or factors, which depress the uterine weight of immature mice. It was suggested that these factors may have anti-estrogenic activity or some other action that would interfere with the reproductive processes of the animal. Ranchers from the yellow pine areas of British Columbia, Washington, Oregon, and Idaho have long been of the opinion that pregnant cows will abort after the consumption of sufficient quantities of yellow pine needles. MacDonald (63), in 1952, stated that the ingestion of pine needles and buds appears
to be a cause of abortion and the birth of weak calves. Little work
has been done to isolate and characterize the compounds, in pine needles,
that could cause these difficulties. Lindstedt (59, 60) has isolated
several stilbene derivatives from pine heartwoods, but not from the
needles, and has given chromatographic methods to separate these com-
pounds. Pinosylvin (3, 5-dihydroxystilbene) and its mono-methyl ether
(3-hydroxy-5-methoxystilbene) because of their structure, should be
further investigated as possible causes of the reproductive disorders
which occur after the consumption of pine needles.

Birdsfoot trefoil is an important legume forage for cattle.
The losses through abortion caused by pine needle consumption are also
of considerable importance in British Columbia. For these reasons it
was decided to further investigate the effects of extracts of birdsfoot
trefoil and yellow pine needles on the reproductive system of laboratory
mice and rats.
IV LITERATURE REVIEW

A. General

Many species of plants appear to contain compounds which have an anti-fertility activity. DeLaszlo and Henshaw (32) in 1954 published a list of sixty plant species which have been used by primitive people to decrease fertility. This list was collected from folklore, from popular medicine of western cultures, from 19th century books on medical botany, and from old materia medica. While the effects of many of these plants have not been substantiated, some of these species have been investigated and found to have a fertility depressing action. Some of the species which are known to contain substances that influence reproduction will be discussed in this literature survey. The presence of these anti-fertility substances in plants would not seem impossible because other physiologically active materials such as digitalis, quinine, and antibiotics have also been isolated from plants. The anti-fertility substances in plants may affect reproductive processes in a variety of ways. As different plants which are known or suspected to affect fertility are discussed, the mode of action, if known, will be described.

The complicated processes of reproduction have been described in detail by Cole and Cupps (27), Long and Evans (62), Marshall and Parkes (64), Nalbandov (70), Selye (112), Stockard and Papanicalaou (118), and Turner (123). Some aspects of these processes will be described in brief form to aid in the discussion of the influence of some plant materials on animal reproduction.
Much evidence points to the fact that a major part of the reproductive processes is governed by the gonadotropin secretion of the anterior pituitary. Although there are two gonadotropic hormones, the follicle stimulating hormone (F.S.H.) and the luteinizing hormones (L.H.), some aspects of their function will be described under the collective term "gonadotropins". The gonadotropins have great control over both the hormone secretion and the gametogenesis which occurs in the gonads of both sexes.

In the male, both L.H. and F.S.H. have important effects. The L.H. stimulates the Leidig (interstitial) cells to secrete the male hormones. Although the F.S.H. is known to enlarge the seminiferous tubules, it seems certain that both L.H. and F.S.H. are needed for complete spermatogenesis. It is possible that L.H. exerts its effects through the androgen secretion stimulated by it. Thus, both L.H. and F.S.H. are needed if the male is to carry out the two functions of spermatogenesis and testicular hormone production.

As the young female animal matures, the anterior pituitary begins to influence the ovary through the mediation of the gonadotropic hormones. F.S.H. causes the growth of follicles in the ovary. As these follicles grow, they secrete an increasing amount of estrogen which causes growth of secondary sex characteristics, cornification of the uterus and vagina, increases in uterine size, and plays other vital roles in the processes of reproduction. L.H. causes luteinization, or rupture, of the follicles, and thus causes the release of the ova. After luteinization, the ruptured follicle begins to produce progesterone as well as continuing the estrogen secretion. As the animal matures, the develop-
ment and rupture of follicles occurs regularly as part of the estrous cycle in connection with the rise and wane of F.S.H. and L.H. production. Thus F.S.H. governs estrogen production while L.H. production is correlated with progesterone output. The variation in levels of the hormones relative to each other gives rise to the phenomenon of the estrous cycle. The estrous cycle manifests itself in several ways, and may be observed by several methods. The estrogen secretion is highest at time of estrus and causes a desire for mating; in the non-primate, estrus is the only time at which the female permits coitus. Temperature changes or the hormone excretion patterns have also been used to determine the stages of the estrous cycle. However, vaginal smear examination is used most frequently to follow the changes which occur in the histology of the uterus and vagina during the estrous cycle.

The estrous cycle, with its associated histological changes, was described for the guinea pig as early as 1917 by Stockard and Papanicalaou (117, 118). Other early publications described the estrous cycle in the rat (62), mouse (1, 26, 90), dog (46, 47), ewe (97), and woman (78). Most of these research papers were very detailed and thus provided a useful background for further experimental work. The estrous cycle of rodents is usually divided into four distinct stages, namely proestrus, estrus, metestrus, and diestrus. Proestrus is characterized by the presence of nucleated epithelial cells. Estrus, the stage at which the follicle is almost mature and is secreting large amounts of estrogen, is marked by large numbers of cornified epithelial cells. During the metestrus stage, leucocytes appear among the cornified epithelial cells. Diestrus can show varying proportions of nuc-
leated epithelial cells and leucocytes; at some stages of diestrus, leucocytes are very numerous, and no other cell types are present. Emmens has given numerical values to the stages of the estrous cycle so that it may be plotted or treated statistically. Many aspects of vaginal smear assays are discussed in two reviews by Emmens (42, 43). Thus the vagina reflects accurately the changes which occur in the pituitary gland, ovary, and endometrium of the uterus.

Most of the estrogen assays depend either on the assessment of vaginal cornification or the increase in uterine weight which occurs in a rodent when an estrogen is administered orally, intravaginally, or by injection. Uterine weight can be increased by androgens and progestational compounds (43); in fact the traditional standard Clauberg assay (25) for progesterone is based on the uterine proliferation caused by this steroid. For this reason, Biggers (15) has suggested that vaginal cornification is a more suitable assay for estrogens than the increase in uterine weight. Other workers (48, 68) have suggested that for initial screening purposes, uterine weight increase is more satisfactory because it is likely to show a response with low doses of estrogens. Uterine weight increase has been commonly used to detect estrogenic substances in plants (11, 24, 48, 55, 56, 68).

Several papers report that the vaginal cornification caused by estrogens can be inhibited by the administration of other hormones (43, 44, 92, 93, 94). However, for the assay of inhibitors in plant fractions this may not be very suitable. If the response to the administered estrogen is inhibited, a diestrous condition is usually the result. Not only is it difficult to express this response in quanti-
tative terms, but with some plant fractions it is difficult to determine whether the diestrus produced by the fraction is due to its hormone content or its general irritating properties.

The immature mouse uterine weight technique which is widely employed for the assay of estrogen-like substances in plants can also be used to assay anti-estrogenic substances (33). Because the uterine weight of immature mice is relatively small, the uteri can be "primed", i.e. increased in size, with the aid of an estrogen such as diethylstilbestrol or estradiol. The anti-estrogenic effect of the plant fraction, administered orally or by injection, can then be assessed by measuring the weight decrease of the "primed" uteri. The immature mouse uterine weight technique, both with and without priming, has been widely used in this work.

It can be understood from this brief presentation of the processes concerned with reproduction, that the conception and development of mammalian young is an intricate process which is vulnerable to interference in several places. These vulnerable points in the reproductive processes and some of the substances which may interfere have been reviewed by Casey (20), Chang (21, 22), Henshaw (52), Jackson (53), Millman and Hartman (67), Nelson (71), and Parkes (82).

B. Plants Which Affect Reproductive Processes

1. Lithospermum ruderale

In 1941, Train, Hendricks and Archer (122) published a list of over 300 species of plants used for medicinal purposes by the Shoshone
Indians of Nevada. These workers reported that one of these species, Lithospermum ruderale (lithosperm) was used by these Indians as a means of preventing conception. Cranston (28) reported on some of the first laboratory work with this species in 1945, and since then many other papers from other laboratories have followed. To date, lithosperm, or a closely related species, has not been reported as the cause of reproductive disorders in grazing domestic animals.

Cranston (28) reported that a 50 per cent ethanol extract decreased frequency of littering in mice, caused prolonged diestrus in mice with previously regular estrous cycles, and caused decreased weights of sex organs, thymus, and pituitary gland. Drasher and Zahl (36) confirmed the cessation of estrous cycles after feeding 25 per cent lithosperm in the diet, and also noted that strains of mice differed in the persistence of the diestrus condition. The estrous cycles returned immediately when the experimental diet was replaced by control diet (128). The anestrus condition of mice was accompanied by atrophy of ovaries and uterus, and atresia of follicles (36). The observed anestrus was not due to inanition (28), a deficiency of the vitamins thiamin, niacin, riboflavin, pyridoxine, or pantothenic acid (34), or an inhibition of the thyroid (34, 128). The facts seem to indicate that lithosperm inhibits the production of gonadotropins (28) though no observable changes in the anterior pituitary are induced (128). A decrease in gonadotropin potency of the pituitaries of normal adult female mice was demonstrated by Cranston and Robinson (30). However, 30 days after castration, there was no significant difference in pituitary weights or gonadotropin potency between animals fed lithosperm in the diet and animals fed normal
diet (34). Gonadotropin potency was diminished significantly in lithosperm fed mice 40 to 50 days after castration.

Early reports on the action of lithosperm were concerned with the material being incorporated in the diet of mice (28, 29, 34, 35, 36, 128, 129). Incorporation of high levels of plant materials may lead to questionable results due to the lowering of caloric intake (76). Noble and co-workers (76, 87) found that lithosperm extract disturbed the estrous cycles of rats similarly to that of mice although rats seemed more resistant to this change. Since incorporation of higher levels of plant materials seemed inadvisable, the plant extract was administered by subcutaneous injection. This method of administration was found to be at least ten times more effective than oral administration (76, 87).

The injection of plant extracts into rats led to several interesting findings (75, 76, 87). Lithosperm did not inhibit the action of administered estradiol. The ovaries and uterus failed to mature, follicular and luteal development being absent. In males, the prostate and seminal vesicles failed to mature or became atrophic. Because the testes remained fairly normal, it was suggested that androgen production was affected. The trophic action of pregnant mare serum on the ovary was also reduced. From these facts it was suggested that lithosperm acts by antagonizing or neutralizing the action of gonadotropic hormones, or rendering the target organ insensitive to their action. The luteinizing hormone (L.H.) is more affected than the follicle stimulating hormone (F.S.H.). Injection of extracts of lithosperm into laying hens stopped egg production and caused atrophy of the reproductive tract and ovary (130).
Lithosperm was found to lower the incidence of mammary tumors in mice (29, 129). The mode of action and possible effects of diet restriction were not determined. More work is needed on this phase of the lithosperm problem as information is incomplete.

When gonadotropins, prolactin, thyrotropin and pregnant mares' serum gonadotropin (P.M.S.), are incubated in vitro with aqueous extract of lithosperm, these hormones are inactivated. A.C.T.H., growth hormone, and chorionic gonadotropins are unaffected by this treatment (49, 73). Quinone compounds, some of which will be mentioned in connection with the work of Sanyal with Pisum sativum, such as 2,6-dimethyl-hydroquinone and many others, were found to possess the ability to inactivate P.M.S. The substances that have this ability do not inhibit chorionic gonadotropin and may have an inhibitory effect on estrous cycles when injected. In this respect they are similar in action to lithosperm extracts (74).

Noble (73) suggests that there are at least two substances present in lithosperm which are capable of exerting effects on reproduction. The active in vitro substance is believed to be identical with the substance which inactivates the anterior pituitary hormones. In addition, another substance in the plant is believed to be capable of interfering with the estrous cycle. These two factors are located mainly in the roots of the plant (75, 76, 87). The leaves of the plant may also contain a water soluble estrogen-like substance (75, 87).

Several plants related to Lithospermum ruderale have been found to contain similar active principles (49, 54, 126). Lithospermum
**Lithospermum officinale**, or Gromwell, was found to inactivate pituitary hormones in a similar manner to *Lithospermum ruderale*. For this reason Kemper and Loeser (54) recommended this species as a pituitary inhibitor for clinical use. It is more easily grown under cultivation than *Lithospermum ruderale*. Wiesner and Yudkin (126) found that an aqueous extract of dried, ground *Lithospermum officinale* also inhibited estrus in mice. It caused no ill effects when fed to human subjects.

Although some workers feel that the effects of lithosperm extracts are caused by non-specific toxic substances (113), most other work indicates that there are factors present in lithosperm which specifically affect reproduction. Isolation of the active principles has not been accomplished. While the action of certain known quinones is similar to a lithosperm fraction (74), the presence of one or more of these compounds has not been demonstrated. An orange colored flavanol or isoflavanol has been isolated and partially characterized (57), but this was probably not the active compound. Thus, while the hormonal properties of the substance (or substances) are fairly well understood, the isolation of these substances still remains a problem. The literature concerning lithosperm has been reviewed by Noble (73), Meyer (65), and Jackson (53).

2. *Pisum sativum*

The effect of the common field pea (*Pisum sativum*) on the reproductive efficiency of certain laboratory animals has received much attention and is the object of some controversy. Nag and Pain (69) in 1934 found that the reproductive ability of rats was greatly reduced
when *P. sativum* was included in the diet; however, when the animals
were offered a normal diet containing *P. artemeium*, their previous level
of fertility returned. Sanyal further found that the ingestion of the
peas, or injection of the oil derived from the legume, caused sterility
in rats (99, 105), and infertility and postponement of menstruation in
women (102, 106). Later work by Sanyal (101) showed that *P. sativum*
is a natural source of 2,6-dimethylhydroquinone (m-xylohydroquinone or
D.M.H.Q.). From this work, he and his co-workers developed methods by
which D.M.H.Q. could be synthesized in the laboratory (105, 109). Sanyal
and his co-workers also reported on several human clinical trials in
which pure D.M.H.Q. was used (100, 104, 107, 108). It is of interest
to note that the hydroquinone has been used in India as a biological

Sanyal has postulated several mechanisms of action for the
anti-fertility compound, D.M.H.Q. In one instance he noted that the
administration of vitamin E could prevent the sterility caused by the
hydroquinone. He has suggested also that the compound interferes with
the action of progesterone, or that the compound may inactivate the es-
trogens (104, 110) and gonadotropins (104) so necessary in the natural
functions concerned in reproduction. Much of the recent work carried
out in Sanyal's laboratory has been discussed by him in a recent review
(105).

Several other workers have reported on the results from re-
search with D.M.H.Q. but their findings appear to conflict not only
with those presented by Sanyal but also with each other. Thiersch (121)
reported that he has been unable to detect any significant effect of
the compound on successful mating or implantation, and could not observe any effect on a single fetus or litter. Pincus (85, 86) found that in rats, when the compound was administered orally or by subcutaneous injection, it delayed successful mating but did not influence the number of ova released per ovulation. Although the percentage of ova which implanted was not significantly reduced, the percentage of embryos developed to the time of parturition appeared to be reduced. Batra and Hakim (4) working with rats and mice, confirmed the increase in rates of resorption, abortion and still births, but these workers could not confirm the delay in ovulation time. In the discussion on lithosperm, papers were quoted which showed that D.M.H.Q. was similar in action to lithosperm because this quinone had the ability to inactivate several gonadotropins (73, 74). Rosen and Millman (96) substantiated this observation and it may well be that this is the main mode of action of the hydroquinone compound. It is possible that the differences observed between laboratories are due to differences in the amount of the hydroquinone administered, differences in species, strains, and weights of laboratory animals used, and differences in vitamin E levels of the feeds.

It is felt that Sanyal must be given credit for doing the extensive work with *P. sativum* and its active compound, but his explanations of the mechanisms of action need further investigation. Jackson (53), summarized the present situation well in his review:

"In the face of conflicting evidence from reputable sources, it is difficult to assess the validity of the data. At least it would appear that the activity of *m*-xylohydroquinone is undramatic and somewhat difficult to demonstrate."
3. *Polygonum hydropiper*

According to Train, Hendricks and Archer (122), another plant species used by the Nevada Indians to prevent conception was the water-pepper herb, *Polygonum hydropiper*. Zadina and Geisler (127) confirmed this, with laboratory findings, indicating that the addition of dry powdered *P. hydropiper* to the diet produced immediate, though temporary anestrus in mice. East (37) administered 20 per cent dried *P. hydropiper* in the diet and found temporarily impaired fertility in male and female mice and sterility in female, but not in male, guinea pigs. Assays showed no evidence of estrogentic or androgenic activity, and it was concluded that this species exerted its effect by interfering with the gonadotropin secretion of the anterior pituitary. Although East quoted literature concerning several compounds found in this plant species, it was not possible to identify the compound which caused the effects noted.

4. *Sanguisorba officinale*

Zadina and Geisler (127) also reported that *Sanguisorba officinale* (garden burnet) interfered with the estrous cycles of mice when it was dried and fed as 25 per cent of the diet. From this, these workers concluded that the plant material exerted a direct anti-gonadotropic effect on the hypophysis cerebri. Subsequent work by East (39) showed that the estrous cycle was not stopped but that the diestrous phase was prolonged in adult mice. Lactation was also affected adversely. The plant material showed no estrogentic activity, and it was suggested that:

"Although some modification of pituitary function is suggested by these results, they do not prove
conclusively that *Sanguisorba* exerts a direct anti-gonadotropic effect on the pituitary as stated by Zadina and Geisler (1950).

5. **Capsella bursa pastoris**

East (38) reviewed the literature on this plant species as follows:

"Zadina & Geisler (1950) reported that dried *Capsella bursa pastoris* suppressed the oestrus cycles of female mice when given as 40% of the diet, but showed little effect at a concentration of 20%. The authors concluded that the plant material had a specific anti-gonadotropic effect and discounted, theoretically, that the high dosage level employed might itself be a complicating factor."

Further work by East (38) showed that 40 per cent *Capsella* in the diet impeded ovulation, and produced temporary infertility in males and females. No evidence of estrogenic activity was found. East was led to conclude that:

"The infertility produced was probably due to the high degree of dilution of the diet rather than to specific anti-fertility activity, but the latter possibility has not been excluded entirely."

6. **Lathyrus odoratus**

Although this species is related to the common pea, the specific anti-fertility effect of *Lathyrus odoratus*, the sweet pea, is of an entirely different nature. Stamler (116) has reviewed the most important investigations concerning this species. When the peas are incorporated in the diet of pregnant female rats, death of the feti is caused, especially late in gestation. Young rats, if fed the peas, are also susceptible and may become deformed (23). The active principle of the sweet pea has been identified as (β-L-glutamyl-amino) propioni-
trile (124). Other species within the Lathyrus genus have been shown to have similar effects. Stanler (116) found that 50 per cent L. odoratus peas in the diet of rats did not affect male or female fertility, and no toxic substance could be detected in the milk of nursing females. Certain aminonitrile compounds chemically related to the active principle of the pea, when added to the diet of pregnant rats, also caused death of the fetal rat. Aminoacetonitrile was the most toxic of the compounds tested.

7. Plants possessing estrogen-like substances

As early as 1926, Loewe et al. (61) reported the presence in plants of substances capable of producing effects on the reproductive system, similar to those induced by animal estrogens. Since that time, many plant species have been found to contain compounds with estrogenic activity. These species are mentioned in several reports and reviews (13, 18, 48, 84, 88, 89, 119). The legumes most commonly mentioned as possessing estrogenic activity are red clover (Trifolium pratense) (48, 55, 84), alfalfa (Medicago sativa) (12, 56, 89), white clover (Trifolium repens) (11, 14, 45), and birdsfoot trefoil (Lotus corniculatus) (56, 98). Many other forage plants such as strawberry clover (Trifolium fragiferum) (91), rye grass (Lolium perenne) (58), oats (Avena sativa) (84), rye (Secale cereale) (84), and wheat (Triticum aestivum) (84) have also been reported to contain estrogen-like compounds. Other investigators have been unable to find estrogenic activity in some of these species, but this disagreement may be due to differences in climate, soil, time of cutting, or stage of maturity (14, 56).
The breeding disorders of sheep grazing on subterranean clover in Western Australia illustrate the impairment of fertility which can occur in extreme cases when grazing animals consume plants containing estrogen-like substances. Bennetts (5), in 1944, reported that flocks grazing on pastures dominated by the Dwalganup variety of subterranean clover exhibited dystocia, uterine prolapse and female infertility. This variety of subterranean clover is of considerable economic importance in Western Australia and research on this problem was initiated. Uterine atrophy caused by ovariectomy was prevented when sheep were grazed on the subterranean clover pastures (8). This atrophy could also be prevented when adequate estrogens were administered. It was further observed that the dried clover had little estrogenic potency. Milk secretion in virgin ewes, mammary development in wethers (9), and aberrations of the male accessory glands of wethers (6, 7) further suggested the presence of an estrogen-like compound(s) in the forage. Other Australian investigators (16, 91) have shown that some plant extracts could produce changes in ovariectomized mice similar to effects observed when estradiol was fed. Other strains of subterranean clover also exhibited estrogenic activity. Bickoff et al. (10) has confirmed the estrogenic content of this species with California-grown clover. The activity seems to be due mainly to the isoflavones genistein and formononetin (17, 31).

The subject of the bioassay procedures used to determine the presence and quantity of estrogens in biological specimens has been comprehensively reviewed by Emmens (42, 43). Most assays depend on the change in vaginal histology or uterine weight which occurs in fe-
male rodents when estrogens are administered orally or parenterally.
The uterine weight techniques have been used extensively for assaying
plant estrogens (119, 77). Some of the assays for estrogens can also
be used for the assay of compounds which possess anti-estrogenic acti-
vity. These are discussed later.

8. *Lotus corniculatus*

It has been shown that in some cases birdsfoot trefoil (*Lotus
corniculatus*) has estrogenic activity (45, 84, 98) while in other cases
no activity could be demonstrated (48). Previous work at this labora-
tory (56, 77, 119) has shown that birdsfoot trefoil cut on the Agronomy
fields of the University of British Columbia possessed estrogenic acti-
vity early in the year. This activity disappeared later in the year
and subsequent mouse uterine weight assays (119) and rat uterine histo-
logy studies (77) demonstrated an anti-estrogenic effect. The reduction
of uterine weight was most marked when second year growth of the plant
material was fed. Whenever anti-estrogenic properties were observed,
they were found in the acetone extract of the forage or in the water
soluble fraction derived from this extract. It is interesting to note
that the antifertility substances found in *Lithospermum ruderale* are
also water-soluble. The fact that the "inhibitor" of uterine growth
present in alfalfa (11, 19) is also water-soluble, is noteworthy because
this suggests that both birdsfoot trefoil and alfalfa are leguminous
species which may contain ether soluble estrogenic compounds and a water
soluble anti-estrogenic compound or compounds. However, at this time
the identity of the anti-estrogenic compound in birdsfoot trefoil cannot
be assumed to be similar to anti-fertility substances in any other plant
species because experimental evidence to support such assumptions has not been found.

9. *Pinus ponderosa*

In Canada, the yellow pine (*Pinus ponderosa*) occurs only in the dry areas of the southern interior of British Columbia. Usually known as Western yellow pine, it is also known as bull pine, yellow pine, British Columbia pine, or jack pine. Under favorable conditions it may grow to height of 160 to 170 feet but under usual range conditions it reaches a height of 70 or 80 feet. Yellow pine usually has three needles in a bundle, but this is not always the case. The needles are from seven to eleven inches long and are dark green in color (51).

Ranchers from the yellow pine areas of British Columbia, Washington, Oregon and Idaho have long been of the opinion that pregnant cows will abort after the consumption of sufficient quantities of yellow pine needles. Bruce (19) stated that several reliable reports from stockmen indicated that needles from newly cut pine, if eaten, could cause abortion in cattle. However, Gunn (50) stated that other nutritional imbalances could lead to the observed abortions and he was not prepared to admit that pine needle consumption was the cause of the abortion. Some reported abortions occurred in areas with brucellosis, phosphorus deficiency, and vitamin A deficiency, each of which are capable of causing abortion. Because accurate scientific data was not available on this subject, the B. C. Beef Growers Association requested that the Canada Range Experiment station in Kamloops, B. C. undertake studies to determine whether pine needles can cause abortion
in cattle. As a result of this experimental work, MacDonald (63) re-
ported that consumption of yellow pine needles and buds can cause abor-
tion and the birth of weak calves. He further stated that pine needles
and buds are palatable to wintering stock and that pregnant range cows
will consume quantities of needles and buds even though adequately fed.

Previous preliminary work at this laboratory showed that the
ethanol extract of dried yellow pine needles decreased uterine weight
of immature mice if this extract was incorporated into the diet of the
animals. Further work by Allen and Kitts (2) has shown that the factor
which decreases uterine weight is present throughout the year in the
water soluble part of the acetone extract while the ether soluble frac-
tion contains toxic factors which are most concentrated in the winter.
She has also shown that both fractions reduced fetus weight although
litter sizes were not affected. The acetone extract decreased radio-
active iodine uptake. Some of the effects caused by the consumption
of yellow pine fractions were also observed when douglas fir (Pseudotsuga
taxifolia) was administered.

Lindstedt (59, 60) has isolated several stilbene derivatives
from pine heartwoods but not from the needles, and has given chromoto-
graphic methods to separate and identify these compounds. Pinosylvin
(3,5-dihydroxystilbene) and its mono-methyl ether (3-hydroxy-5-metho-
xystilbene), and also some of the other stilbenes have been identified
by Linstedt as being present in the heartwood. Since other stilbenes
such as diethylstilbestrol or dimethylstilbestrol have marked effects
on reproductive processes, and it would not seem impossible that some
of the pine heartwood stilbenes may be responsible for some of the
effects of pine needles in reproductive processes.
V MATERIALS AND METHODS

A. Materials

1. Plant materials
   a. Birdsfoot trefoil (*Lotus corniculatus*)
   b. Yellow pine (*Pinus ponderosa*)

2. Experimental animals

3. Control ration

4. Experimental chemical compounds

B. Methods

1. Preparation of plant materials
   a. General
   b. Birdsfoot trefoil
      i drying and grinding
      ii alcohol extraction of dried birdsfoot trefoil
      iii acetone extraction of fresh birdsfoot trefoil
   c. Yellow pine needles

2. Preparation of experimental rations

3. Assay methods employed
   a. Mouse uterine weight bioassay
   b. Modified Astwood assay
   c. Estrous cycle experiments

4. Chromatographic techniques
V MATERIALS AND METHODS

A. Materials

1. Plant materials
   a. Birdsfoot trefoil (Lotus corniculatus)

   First year growth of birdsfoot trefoil of a narrowleaf strain was obtained from a plot located on the agronomy fields of the University of British Columbia. The dry matter content of the plant material used for Experiments BT1 to BT4, and also the material used for the experiments on the estrous cycle of the animals, was 19.4 and 20.1 per cent respectively.

   b. Yellow pine (Pinus ponderosa)

   The samples of yellow pine needles were collected at Cherry Creek, approximately ten miles west of Kamloops, B.C. This area had been logged, and at the time of sampling it supported an open grown, second growth stand of yellow pine and douglas fir. Most of the samples were obtained from trees no less than 20 feet tall. All the samples were obtained from heights below seven feet from the ground. In order to acquire a representative sample, not more than a dozen tips were taken from any one tree.

   The pine needles for Experiment P1, and those used for Experiment P2 to P8 had a dry matter content of 52 and 50.6 per cent respectively.

2. Experimental animals

   Immature female mice of the U.B.C. Swiss albino strain were
used for the mouse bioassay procedure. These mice were 20 or 21 days of age and weighed 8 to 11 grams. Other studies at this laboratory have utilized mice of similar strain, age, and weight (2, 55, 56, 77). Immature female rats of the U.B.C. Wistar strain were used for the six hour uterine weight assay modified from Astwood (3, 43). These rats weighed 35 to 45 grams and were 19 to 22 days of age. Female rats of the U.B.C. Sprague-Dawley strain, weighing 175 to 200 grams, were used for the experiments involving the effect of the extracts of plant material on the estrous cycle, using the vaginal smear technique.

3. Control ration

The composition and proximate analysis of the control ration (G-56) used in all of the reported experiments are given in Tables I and II. This ration was fed to the experimental animals in a finely ground form.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolled oats</td>
<td>52.50</td>
</tr>
<tr>
<td>Ground wheat</td>
<td>26.25</td>
</tr>
<tr>
<td>Fish meal</td>
<td>8.75</td>
</tr>
<tr>
<td>Meat scraps</td>
<td>3.75</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>7.50</td>
</tr>
<tr>
<td>Steamed bone meal</td>
<td>1.00</td>
</tr>
<tr>
<td>Iodized salt</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>

Between experiments, the ration was stored in plastic containers at room temperature.
Table II: Proximate analysis of the G-56 ration (119)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>17.91</td>
</tr>
<tr>
<td>Crude protein (N×6.25)</td>
<td>19.00</td>
</tr>
<tr>
<td>Fat extract</td>
<td>4.65</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>1.80</td>
</tr>
<tr>
<td>Ash</td>
<td>6.86</td>
</tr>
<tr>
<td>Nitrogen free extract</td>
<td>49.78</td>
</tr>
<tr>
<td></td>
<td>100.00</td>
</tr>
</tbody>
</table>

4. Experimental chemical compounds

Estrone and methyl testosterone were obtained from commercial sources. Pinosylvin (3,5-dihydroxystilbene) and its mono-methyl ether (3-hydroxy-5-methoxystilbene) were obtained from Dr. E. Swann, Forest Products Laboratories, Vancouver, B.C.

B. Methods

1. Preparation of plant material

a. General

Most of the studies using birdsfoot trefoil and all the studies using yellow pine needles were conducted with an acetone extract of the fresh plant material. However some of the experiments conducted with birdsfoot trefoil also involved an alcohol extract of the dried and ground plant material.

b. Birdsfoot trefoil

i. drying and grinding

The forage was weighed immediately after harvesting, and a
sample was taken for dry matter determination. The plant material was
dried in a tunnel drier at 55° to 60° C. The dried plant material was
ground in a Wiley mill through a 1/16 inch screen.

ii alcohol extraction of the dried birdsfoot trefoil

The method used to extract the active principles from the forage
was similar to that reported by Pieterse and Andrews (84), and later by
Swierstra (119) and Kitts et al. (55). One hundred grams of ground plant
material were placed with 800 mls. of 95 per cent ethanol in a 3000 ml.
round bottom flask and refluxed for one hour. After some cooling, the
ethanol was removed from the ground forage by suction filtration through
a large Buchner funnel with a Whatman No. I filter paper. The plant
material was returned to the flask and refluxed for an additional hour
with 700 mls. of clean ethanol. Again after some cooling, the alcohol
was removed by suction filtration. The two ethanolic extracts were com­
bined and concentrated in a flash evaporator at 49° C to approximately
150 mls. This extract is referred to as the "whole alcohol extract".

This extract was added directly to the feed for one animal group in the
first birdsfoot trefoil experiment (BT1), while further fractionation
of the alcohol extract was carried for the other groups of this experi­
ment. The fractionation procedure carried out was as follows: One
hundred and fifty mls. of the whole alcohol extract were combined with
75 mls. of distilled water, and the mixture concentrated in the flash
evaporator until the alcohol was removed. The resulting solution was
shaken four times with approximately 75 mls. of diethyl ether. The
erther washings were combined and concentrated to a volume of 75 mls.
This fraction is referred to as the "ether fraction of the alcohol extract".
This solution contained all the chlorophyll, and probably most of the estrogen-like substances along with other impurities. The remaining aqueous solution, which was light brown in color, is referred to as the "aqueous fraction of the alcohol extract".

iii acetone extraction of fresh birdsfoot trefoil

The method of acetone extraction was similar to the procedure suggested by Bickoff et al. (11). After the forage was harvested the material was cut into one inch lengths. Three hundred grams of the chopped forage were macerated in approximately 600 ml. of acetone in a large Waring Blender for three minutes. The acetone extract was removed from the forage by suction filtration through a large Buchner funnel with Whatman No. filter paper. The filter cake was washed with an additional 200 ml. of acetone. The filtrate was concentrated in a flash evaporator at 49°C, thus removing the acetone. The aqueous solution remaining is referred to as the "whole acetone extract". Some of this extract was added directly to the ration; another portion was washed four times with diethyl ether in a similar manner to the treatment of the alcohol extract described previously. This produced two birdsfoot trefoil fractions, the "ether fraction of the acetone extract", and the "aqueous fraction of the acetone extract". The acetone extraction was carried out on the same day the forage was cut. The fractions were refrigerated to 50°C until required.

c. Yellow pine needles

A modification of the extraction technique of Bickoff et al. (11), as described by Allan and Kitts (2), was used for the initial step
of the fractionation and purification procedure. Pine needles were stripped from their stems and ground in a Hobart N-50 grinder. The ground needles were extracted with acetone in a large Waring blender for three minutes. The acetone extract was removed from the ground needles by suction filtration through a larger Buchner funnel with a Whatman No. 1 filter paper. The filter cake was washed with an additional 200 mls. of acetone. The acetone extract was concentrated in a flash evaporator, to leave essentially an aqueous concentrate. In all cases, this first step was carried out within one week of the arrival of the pine needles. This procedure was repeated in order to provide the required amounts for the experiments reported.

This crude acetone extract was fractionated further as shown in Figure 1. The crude extract was washed four times with diethyl ether in a separatory funnel. An ether washed water soluble fraction was then collected and stored. The crude acetone extract was also washed twice with ether, then twice with benzene, in a separatory funnel, to provide a second water soluble fraction. All benzene and ether fractions were discarded since these do not cause significant reductions in uterine weights (2). The resulting two aqueous fractions were studied using the immature mouse uterine weight bioassay procedure. The ether washed water soluble fraction was also studied further in several experiments.

The ether washed aqueous fraction from the crude acetone extract was further fractionated. This fraction was dried onto celite.  

\[^{1}\text{celite, a diatomaceous earth - trademark of Johns-Manville Co. Ltd.}\]
at a temperature below 60° C.

Figure 1. The Fractionation Procedure for the Crude Acetone Extract of Yellow Pine Needles

The dried mixture was then refluxed with either 95 per cent ethanol or ethyl acetate. The ethanol and ethyl acetate eluents were collected, and stored at 5° C.
2. Preparation of experimental rations

The extracts were concentrated under vacuum and added to the control ration so that one gram of the experimental diet contained the extractives from a predetermined weight of forage dry matter. For example, a ration designated as "1.5:1" implies that one gram of the experimental diet contained the extractives from 1.5 grams of forage dry matter. In all cases the extract was added to a small amount of control ration; the rest of the ration was added after the previous mixture was completely dried. After all of the extract was added to the ration and the mixture dried, the resulting experimental ration was reground before being offered to the animals.

3. Assay methods employed

a. Mouse uterine weight bioassay

The mice used in the fraction bioassays were housed in round metal pans with a base diameter of 9 inches, a top diameter of 14 inches and a height of 5 inches. One group was housed per pan. Fine wood shavings were used as bedding. Water was offered fresh daily from inverted half pint bottles fitted with No. 8 rubber stoppers with 8 cm. glass delivery tubes. The arrangement has been further described by Miller and Wood (66).

Three day and four day mouse bioassay techniques were used. In each case the groups of animals under study were fed a definite amount of ration each day. Total feed consumption was recorded. After the feeding period, the animals were fasted for eight hours, then killed
by ether inhalation. The uterus was removed by cutting at the proximal side of the ovaries and cervix. After the extraneous tissue was trimmed from the organ and the free moisture was removed with the use of filter paper, the uterus was weighed on a precision spring balance. The weight of each uterus was expressed as a percentage of the body weight (55, 119).

b. Modified Astwood assay

The aqueous fraction of the acetone extract of the plant material was injected subcutaneously into the experimental rats (Exp. P6). Previous to injection, the extract was neutralized with NaHCO₃ to pH7.0. Each of the control animals was injected subcutaneously with one mcgm. of estrone² in 0.5 ml. physiological saline. Each of the experimental animals was injected with one mcgm. of estrone in 0.5 ml. of the neutralized pine needle extract. This volume of extract contained 0.52 gm. forage dry matter extractives.

The animals were not fed during the assay period. Six hours after injection, the animals were killed by ether inhalation. The uterus was excised and its weight was expressed as a percentage of body weight.

c. Estrous cycle experiments

The rats were housed individually in wire cages with sawdust on the dropping boards under the wire floors. Water was provided ad libitum. The control and experimental rations were placed in four ounce glass jars. Fifteen grams were offered daily to each animal. The con-

² Estrone - supplied as Theelin (aqueous suspension) by Parke, Davis & Co. Ltd.
control group of animals was restricted to the amount of the ration consumed by the experimental group. This provided an isocaloric intake.

Vaginal washings, or "smears" were taken daily by the lavage (pipette) method. The smears were examined in the unstained condition. Prior to the experiment, vaginal smears of the rats were examined daily to ascertain that the estrous cycles were regular.

Four groups of two animals each were used in this experiment. Group 1 animals served as the control and were offered the control ration. Group 2 received in their diet methyl testosterone, an orally active progestational compound, at a level of five mcgms. per gm. of feed. Group 3 animals, fed the control ration, were exposed during early estrus to a male. One of these females became pseudopregnant and its vaginal washings were taken every day, while the other was discarded as it did not become pregnant. Group 4 animals were fed a 2:1 mixture of the aqueous fraction of the pine needle extract in the control ration.

4. Chromatographic techniques

Descending chromatography was employed for all the chromatography procedures. The following three solvent systems were used:

1. A two per cent acetic acid solution
2. A 4:1:5 solution of butanol: acetic acid: water ($V/V$). The top (organic) layer was used as the solvent.
3. Lindstedt's solvent (59)
### Constituent Vols.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Vols.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>50</td>
</tr>
<tr>
<td>Petroleum ether (65°-110°)</td>
<td>50</td>
</tr>
<tr>
<td>Methanol</td>
<td>1</td>
</tr>
<tr>
<td>Water</td>
<td>50</td>
</tr>
</tbody>
</table>

The upper organic layer was used.

Methods employed for the preparation of the chromatograms, and placement in the chromatographic jars were according to those outlined by Smith (114). Location of the compounds separated from the various extract solutions was done by the use of ultraviolet light (3660 and 2537Å) and/or by the use of diazotized sulfanilic acid-potassium carbonate as a chromogenic spray.

The experimental solutions which were studied by chromatography were:

1. The aqueous fraction of the acetone extract of pine needles.
2. The ethyl acetate eluent of this aqueous fraction adsorbed onto celite.
3. The aqueous fraction of the acetone extract of pine needles after it was refluxed for six hours with the Dowex 50W cationic exchange resin.
4. An ethanolic solution of pinoresin.
5. An ethanolic solution of pinoresin monomethyl ether.
VI RESULTS AND DISCUSSION

A. Preliminary Experiments

1. The effect of various levels of feed intake of the control diet on the ultimate uterine weight of immature female mice

This preliminary experiment was conducted because it was found that some experimental groups in several assays showed a lower feed intake and/or a lower final body weight than the control group. Although it is possible to restrict the feed intake of the control group, it is not desirable to restrict the feed intake of all the experimental groups to the intake of the slowest eating group since the response desired depends on an adequate feed intake. For this reason it was decided to determine the effect of feed intake levels and variations in final body weight on uterine weight expressed as a percentage of body weight.

The results from the preliminary experiment which was conducted to determine the effect of various levels of feed intake on the uterine weight expressed as a percentage of body weight are tabulated in Table III. Four groups of mice were fed four levels of control ration. It may be observed that reduced feed intake caused a lower final body weight, as would be expected, and also caused a lower final uterine weight. However, when uterine weight was expressed as a percentage of body weight, no significant difference between the four groups was present. Since the levels of feed intake and final body weight are approximately paralleled in the experimental work reported in this report, it may be concluded that uterine weight expressed as a percentage of body weight is
### Table III  The effect of four levels of feed intake on body weight and uterine weight

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of feed eaten per mouse in 3 days (gms.)</td>
<td>6.8</td>
<td>5.5</td>
<td>4.5</td>
<td>3.5</td>
</tr>
<tr>
<td>No. of mice</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Average body weight when sacrificed (gms.)</td>
<td>10.7</td>
<td>9.9</td>
<td>9.2</td>
<td>8.5</td>
</tr>
<tr>
<td>Average uterine wt. in mgms.</td>
<td>15.38</td>
<td>14.27</td>
<td>13.34</td>
<td>12.31</td>
</tr>
<tr>
<td>Average uterine wt. as a percentage of body wt.</td>
<td>0.142</td>
<td>0.144</td>
<td>0.144</td>
<td>0.144</td>
</tr>
<tr>
<td>Standard Error</td>
<td>±0.006</td>
<td>±0.007</td>
<td>±0.007</td>
<td>±0.007</td>
</tr>
</tbody>
</table>

- a 3 day assay

- Analysis of variance of the uterine weight percentages revealed no significant differences at P > 0.10.
not affected by variation in intake of control ration under the conditions of these experiments.

Expression of the uterine weight as a percentage of body weight for each animal has been used in these and the following experiments in order to correct for differences in body weight as has been used in other reports (2, 55, 56). Correction by means of a method based on covariance would theoretically be a more precise adjustment, but, for routine assays it is doubtful if the additional accuracy would be worth the more elaborate calculations involved.

B. The Effect of Chemical Extracts of Birdsfoot Trefoil on the Reproductive System of the Laboratory Mouse and Rat

1. The effect of various chemical extracts of birdsfoot trefoil on the uterine weight of immature female mice (3 day bioassay)

The first birdsfoot trefoil experiment (BT1) compared the effects of the acetone and alcohol extracts of birdsfoot trefoil, and also the water soluble fractions and the ether soluble fractions of these two extracts, by means of a uterine weight bioassay. The groups were small and, probably as a result, the standard errors of the uterine weight percentages were quite large. The results of this preliminary experiment are tabulated in Table IV.

It may be observed that the uterine weight percentages of the mice fed the aqueous fractions of the extracts are lower than those fed the whole extracts or the ether fraction. Although these results sugges-
Table IV  The effect of six chemical extracts of birdsfoot trefoil on the uterine weight of immature female mice

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Control (basal) ration</td>
<td>Whole acetone extract</td>
<td>Whole alcohol extract</td>
<td>ether fraction of the alcohol extract</td>
<td>aqueous fraction of the alcohol extract</td>
<td>ether fraction of the acetone extract</td>
<td>aqueous fraction of the acetone extract</td>
</tr>
<tr>
<td>Number of mice per group</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Feed consumption per mouse in three days (grms.)</td>
<td>8.3</td>
<td>8.3</td>
<td>8.3</td>
<td>8.3</td>
<td>8.3</td>
<td>8.3</td>
<td>8.3</td>
</tr>
<tr>
<td>Average body weight when sacrificed (gms.)</td>
<td>12.0</td>
<td>11.2</td>
<td>11.6</td>
<td>11.3</td>
<td>12.9</td>
<td>12.9</td>
<td>11.2</td>
</tr>
<tr>
<td>Average uterine weight in mgms.</td>
<td>16.27</td>
<td>17.92</td>
<td>18.99</td>
<td>17.74</td>
<td>15.22</td>
<td>22.31</td>
<td>13.69</td>
</tr>
<tr>
<td>Mean uterine weight as a percentage of body weight</td>
<td>0.132</td>
<td>0.157</td>
<td>0.163</td>
<td>0.155</td>
<td>0.118</td>
<td>0.175</td>
<td>0.124</td>
</tr>
<tr>
<td>± standard error</td>
<td>±0.016</td>
<td>±0.016</td>
<td>±0.012</td>
<td>±0.018</td>
<td>±0.011</td>
<td>±0.019</td>
<td>±0.010</td>
</tr>
</tbody>
</table>

- The birdsfoot trefoil was cut on June 3, 1960. The fractions were fed 1:1 in a 3 day assay.
- Analysis of variance revealed significant treatment differences.

Duncan's test at P.05

<table>
<thead>
<tr>
<th>(5)</th>
<th>(7)</th>
<th>(1)</th>
<th>(4)</th>
<th>(2)</th>
<th>(3)</th>
<th>(6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.118</td>
<td>0.124</td>
<td>0.132</td>
<td>0.155</td>
<td>0.157</td>
<td>0.163</td>
<td>0.175</td>
</tr>
</tbody>
</table>
ted that an anti-estrogenic substance was present in the birdsfoot trefoil material, more conclusive results were needed. Although some of the differences observed were statistically significant, it might be expected that higher concentration of extracts, larger groups of animals, and possibly a longer bioassay period would give more conclusive results.

2. The effect of the ether fraction and the water fraction of the acetone extract on the uterine weight of immature female mice (4 day bioassay)

An experiment was designed with a longer feeding period, higher fraction concentrations, and larger animal groups, to compare the effects of the ether soluble fraction and the water soluble fraction of the acetone extract of birdsfoot trefoil on the uterine weight of immature mice. The feeding period was prolonged to four days, the concentration of extracts in the feed was raised to 1.5:1, and the group size was increased to ten mice. The results of this experiment, BT2, are given in Table V.

The results of this second experiment tended to confirm those of experiment BT1. Although there was no significant difference between the group fed the ether fraction and the control group, the group fed the aqueous fraction again showed significantly lower uterine weight percentages than the control group and the group fed the ether soluble fraction.

3. The effect of the acetone extract and its ether and water fraction on the uterine weight of immature mice (4 day bioassay - individually fed)
Table V  The effect of the ether and the water fraction of the acetone extract of birdsfoot trefoil on the uterine weight of immature mice

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Control (basal) ration</td>
<td>Ether soluble fraction of the acetone extract</td>
<td>Aquous fraction of the acetone extract</td>
</tr>
<tr>
<td>Number of mice in group</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Feed consumption per mouse in four days (gms.)</td>
<td>11.0</td>
<td>11.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Average body weight when sacrificed (gms.)</td>
<td>14.8</td>
<td>14.5</td>
<td>15.1</td>
</tr>
<tr>
<td>Average uterine weight (mgms.)</td>
<td>21.79</td>
<td>19.38</td>
<td>15.96</td>
</tr>
<tr>
<td>Mean (uterine weight expressed as a percentage of body weight) ± standard error</td>
<td>0.146±0.009</td>
<td>0.135±0.006</td>
<td>0.106±0.008</td>
</tr>
</tbody>
</table>

Analysis of variance revealed highly significant differences at P0.005.

Duncan's test at P.01

(2) 0.106 (1) 0.135 (3) 0.146

The birdsfoot trefoil was cut on June 3, 1960. The fractions were fed at a concentration of 1.5:1.
The third birdsfoot trefoil experiment, BT 3, was similar in design and results, to the previous two experiments. The crude acetone extract and its ether and water soluble fraction were assayed using the immature mouse uterine weight technique. The mice were individually fed, a refinement which probably did not increase the precision of the experiment and which may account for the high mortality observed. The concentration of the extracts in the rations was raised to 2:1, and the bioassay was continued for four days. The results of the experiment are tabulated in Table VI.

The water soluble fraction caused significantly lower uterine weights in comparison with the control group or the ether fraction-fed group. Evidence of slight estrogen stimulation was again observed in the ether fraction-fed group. The crude acetone extract also caused a lowering of uterine weight, but not to a significant extent. It seems probable that the ether extraction technique makes it possible to separate the estrogen-like substances which are mainly ether soluble, and the anti-estrogenic factor(s) which is mainly water soluble.

4. The effect of increasing levels of the aqueous fraction of the acetone extract of birdsfoot trefoil on the uterine weight of immature female mice

Experiment BT4 was designed to show the effects of increasing levels of the aqueous fraction of the acetone extract. This fraction was fed at the levels of 1.5:1, 3:1, and 4.5:1. The results of the experiment are presented in Table VII.
Table VI  The effect of the whole acetone extract, and its ethers and water soluble fractions on the uterine weight of immature mice

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Control (basal) ration</td>
<td>The whole acetone extract</td>
<td>The ether soluble fraction of the acetone extract</td>
<td>The water soluble (aqueous) fraction of the acetone extract</td>
</tr>
<tr>
<td>Number of mice in group</td>
<td>8</td>
<td>9</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Feed consumption per mouse in four days in gms.</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Average body weight when sacrificed in gms.</td>
<td>13.2</td>
<td>11.1</td>
<td>12.4</td>
<td>11.7</td>
</tr>
<tr>
<td>Average uterine weight in mgms.</td>
<td>21.72</td>
<td>14.97</td>
<td>22.45</td>
<td>14.21</td>
</tr>
<tr>
<td>Mean (uterine weight as a percentage of body weight) ( \pm ) standard error</td>
<td>0.163( \pm )0.018</td>
<td>0.134( \pm )0.010</td>
<td>0.178( \pm )0.012</td>
<td>0.121( \pm )0.007</td>
</tr>
</tbody>
</table>

Analysis of variance revealed highly significant treatment differences.

Duncan's test at P.05 at P.01

<table>
<thead>
<tr>
<th>(4)</th>
<th>(2)</th>
<th>(1)</th>
<th>(3)</th>
<th>(4)</th>
<th>(2)</th>
<th>(1)</th>
<th>(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.121</td>
<td>0.134</td>
<td>0.163</td>
<td>0.178</td>
<td>0.121</td>
<td>0.134</td>
<td>0.163</td>
<td>0.178</td>
</tr>
</tbody>
</table>

The birdsfoot trefoil was cut June 3, 1960. The fractions were fed 2:1 in a four day assay.
Table VII  The effect of various concentrations in the feed of the aqueous fraction of the acetone extract of birdsfoot trefoil on the uterine weight of immature mice

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Control ration</td>
<td>Control ration plus the aqueous fraction of the acetone extract 1:5:1</td>
<td>Control ration plus the aqueous fraction of the acetone extract 3:1</td>
<td>Control ration plus the aqueous fraction of the acetone extract 4:5:1</td>
</tr>
<tr>
<td>Number of mice in group</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Feed consumption per mouse in four days (gms.)</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Average body weight when sacrificed (gms.)</td>
<td>13.2</td>
<td>14.0</td>
<td>14.3</td>
<td>12.6</td>
</tr>
<tr>
<td>Average uterine weight (mgms.)</td>
<td>24.50</td>
<td>21.28</td>
<td>17.26</td>
<td>18.42</td>
</tr>
<tr>
<td>Mean (uterine weight expressed as a percentage of body weight) + standard error</td>
<td>0.185±0.011</td>
<td>0.150±0.018</td>
<td>0.119±0.011</td>
<td>0.143±0.011</td>
</tr>
</tbody>
</table>

An analysis of variance revealed significant treatment differences.

Duncan's test

<table>
<thead>
<tr>
<th>P.05</th>
<th>P.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>0.119</td>
<td>0.143</td>
</tr>
</tbody>
</table>

A four day assay. The birdsfoot trefoil was cut on June 3, 1960.
From the results of experiment BT4, it would seem probable that increasing levels of the aqueous fraction would cause an increased uterine weight depression. Group four, although fed the highest concentration of extract in the ration, did not exhibit the lowest uterine weight percentage. This may be due to the fact that its feed consumption was lower than the other groups, and consequently the consumption of the extract was not as high as would seem from the 4.5:1 concentration in the feed. The small group sizes may account for the large standard errors observed.

5. The effect of the aqueous fraction of the acetone extract of birdsfoot trefoil on the estrous cycle of mature female rats

Estrous cycle studies were also conducted using the vaginal smear technique. Rats received the aqueous fraction of the acetone extract of birdsfoot trefoil in the feed. The estrous cycles of the rats were not disturbed by the birdsfoot trefoil extracts under the conditions of the experiment. The length of the cycle (i.e. the frequency of the occurrence of estrus) or the characteristics of the cells in the vaginal smear did not seem to be affected by the administration of the plant extract. However, the plant material for the feed was cut on June 22, 1961, early in the summer when the anti-estrogenic content was probably not very high. A mouse bioassay of the same feed revealed that it was only moderately active in its ability to decrease uterine weight. While these data tend to indicate that birdsfoot trefoil does not interrupt the estrous cycle, it would seem necessary to conduct more experimental work on this phase of the problem before a conclusion
can be reached.

6. **General discussion of the results obtained from the birdsfoot trefoil experiments conducted**

From the data presented, it seems highly probable that birdsfoot trefoil contains an anti-estrogenic factor which is present in the water soluble fractions of the acetone and the alcohol extracts. The work reported was carried out with plant material cut in June of 1960 and 1961. This is not the season of optimum anti-estrogenic activity (56). Both the work of Ostrovsky (77) and these data, suggest the possibility that the factor(s) affects the secretion of the pituitary gonadotropins. However, interference with the action of estrogens, perhaps by the peripheral inactivation of circulating estrogens, is also a possibility which should be further investigated. More experimentation with the anti-estrogenic factor of birdsfoot trefoil is necessary before its mode of action can be given with certainty. The ether extraction technique makes it possible to separate the estrogen-like substances reported (45, 56, 98, 119) and the anti-estrogenic factor(s). However, the content of estrogen-like substances is not high in this plant species during its growing season (56, 119).

In general, the nature and occurrence of the anti-estrogenic substance in birdsfoot trefoil may be of significance as this plant material is used widely for feeding purposes. Since the work reported suggests the presence of an anti-estrogen, it could be postulated that this substance(s) has economic importance in livestock production. However, more work with this plant species is needed before this postulate is proven. Whether the anti-estrogenic factor is identical with
Bickoff's inhibitor, or one of the other anti-fertility factors mentioned in the literature review, is not known. Certainly, the inhibitor of uterine growth in alfalfa is of interest because of the economic importance of alfalfa.

C. The effect of Chemical Extracts of Yellow Pine Needles on the Reproductive System of the Laboratory Mouse and Rat

1. The effect of chemical extracts of yellow pine needles on the uterine weight of immature female mice

a. Experiment P1

Previous work (2, 83) showed that the anti-estrogenic factor was present mainly in the water soluble fraction. In this work, the crude extract was washed four times with ether. This method was followed again to provide one of the groups in the first pine needle experiment (P1). A second fractionation of the crude extract which was carried out was the washing twice with benzene and twice with ether. The effect of the two aqueous fractions on the uterine weight of intact immature mice is shown in the results of Experiment P1 in Table VIII.

The results of Experiment P1 show that the anti-estrogenic activity of the fractions is similar if the whole acetone extract is washed twice with ether and twice with benzene, or if it is extracted four times with ether. The aqueous fractions caused significantly lower uterine weight percentages than the control group in spite of the small groups and the large standard errors in uterine weight percentages. Since
Table VIII  The effect of two extraction methods on the anti-estrogenic activity of the water soluble fraction of yellow pine needles

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Control (basal) ration</td>
<td>Aqueous fraction of the acetone extract after washing twice with ether then twice with benzene</td>
<td>Aqueous fraction of the acetone extract after washing four times with ether</td>
</tr>
<tr>
<td>No. of mice in group</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Feed consumption per mouse in four days (gms.)</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Average body weight when sacrificed (gms.)</td>
<td>11.9</td>
<td>11.2</td>
<td>11.5</td>
</tr>
<tr>
<td>Average uterine weight in mgms.</td>
<td>20.33</td>
<td>13.69</td>
<td>13.59</td>
</tr>
<tr>
<td>Mean uterine weight as a percentage of body weight ± standard error</td>
<td>0.173±0.015</td>
<td>0.122±0.013</td>
<td>0.119±0.011</td>
</tr>
</tbody>
</table>

- Analysis of variance revealed significant treatment differences in uterine weight percentages.
- The pine needles were cut on July 21, 1960.
- The fractions were fed 1.5:1 in a four day assay.

Duncan's Test
P.05
(1) 0.173 (2) 0.122 (3) 0.119
P.01
(1) 0.173 (2) 0.122 (3) 0.119
there is no difference between the two aqueous fractions, the ether extraction was used for further work because it is more convenient and less time consuming.

b. Experiment P2

As was described previously, a further purification procedure of the aqueous fraction of the acetone extract of pine needles was attempted by drying onto, and eluting from, celite. The two solvents used were ethyl acetate and 95 per cent ethanol. The results of the mouse bioassay of the two eluents are given in Table IX. It may be seen that the two solvents were almost equally successful in eluting the anti-estrogenically active compound(s). Both eluents could be crystalized to a mixture of white crystals and brown amorphous precipitates which was very soluble in water.

Elution from celite was also attempted with ether as a solvent. Although some lowering of uterine weight was noted when this eluent was fed in the ration, this anti-estrogenic effect was not as marked as obtained when ethyl acetate or alcohol was used as a solvent.

c. Experiments P3, P4, P5, and P6

The aqueous fractions of Experiment P1 were assayed with intact immature mice. The ovary of an immature mouse produces only very small amounts of estrogen to stimulate uterine development. It is for this reason that immature mice have been used for estrogen assays almost as extensively as ovariectomized mice. However, the uterus of an immature mouse, if under no estrogenic stimulation, cannot be expected
Table IX  The effect of the ethyl acetate eluent and the 95 per cent ethanol eluent from the aqueous fraction of the acetone extract of pine needles adsorbed and dried onto celite

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Control (basal) ration</td>
<td>95 per cent ethanol eluant of the aqueous fraction of the acetone extract adsorbed onto celite</td>
<td>ethyl acetate eluant of the aqueous fraction of the acetone extract adsorbed onto celite</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of mice in group</th>
<th>10</th>
<th>10</th>
<th>10</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Feed consumption per mouse in three days (gms.)</th>
<th>6.7</th>
<th>6.7</th>
<th>6.7</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Average body weight when sacrificed (gms.)</th>
<th>10.8</th>
<th>10.0</th>
<th>9.6</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Average uterine weight in mgms.</th>
<th>18.62</th>
<th>13.29</th>
<th>12.32</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Mean (uterine weight expressed as a percentage of body weight ± standard error</th>
<th>0.172±0.008</th>
<th>0.133±0.006</th>
<th>0.128±0.004</th>
</tr>
</thead>
</table>

Analysis of variance revealed highly significant differences between the uterine weight percentages.

Duncan's test at P<0.01

(1)  (2)  (3)

0.172  0.133  0.128

The pine needles were cut on July 3, 1961. The fractions were fed 1.5:1 in a 3 day assay.
to reduce in size very significantly if an anti-estrogen is administered. For this reason, the bioassay of the aqueous fractions of the acetone extract of yellow pine was also carried out with immature mice receiving diethylstilbestrol (D.E.S.) in the feed. Such "priming" increases the size of the uterus and makes a decrease caused by the plant extract more likely and more meaningful. A decrease of the uterine weight in "primed" mice would tend to complement the findings of the bioassays in which immature "non-primed" mice were employed. For these reasons, it was decided to determine the effect of the water soluble fraction of the acetone extract on the uteri of immature "primed" mice. From previous data (55) it seemed that 0.040 mcgms. D.E.S. per gram of feed was a suitable dosage for the desired 100 per cent increase in uterine weight. However, when this dosage was administered in Experiments P3 and P4 (Tables X and XI), the uteri increased to a much greater extent than was desired, or was expected from the data of Kitts et al. (55). This may be attributed to the fact that P3 and P4 were four day assays while the data of Kitts et al. were obtained from a three day assay. Also, the feed consumption per day was higher in these experiments. Thus the total amount of D.E.S. ingested during the assay period, especially in Experiment P3, was much higher than in the experiments reported by Kitts et al., although the concentration of D.E.S. per gram of ration was the same.

In Experiment P3, where the aqueous fraction was fed in a concentration of 1:1, no effect on uterine weight was noted. The fraction was fed in a higher concentration in Experiment P4, but, although a decrease in uterine weight could be observed, this difference was
Table X  The response of immature female mouse uterine weights to oral administration (in diet) of the aqueous fraction of pine needles after D.E.S. "priming"

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Basal (G-56) ration plus 0.040 mcgms. D.E.S. per gram of feed</td>
<td>Basal (G-56) ration plus 0.040 mcgms. D.E.S. per gram of feed, plus plant extract added at a concentration of 1:1</td>
</tr>
<tr>
<td>Number of mice in group</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Feed consumption per mouse in four days (gms.)</td>
<td>8.1</td>
<td>8.1</td>
</tr>
<tr>
<td>Average body weight when sacrificed (gms.)</td>
<td>11.2</td>
<td>9.8</td>
</tr>
<tr>
<td>Average uterine weight (mgms.)</td>
<td>82.72</td>
<td>76.51</td>
</tr>
<tr>
<td>Mean (uterine weight as a percentage of body weight) ± standard error</td>
<td>0.754±0.078</td>
<td>0.761±0.052</td>
</tr>
</tbody>
</table>

Statistical analysis (t-test) revealed that there were no significant differences between the uterine weight percentages of the two groups. The pine needles were cut on July 3, 1961. A four day assay.
Table XI

The response of immature female mouse uterine weights to oral administration (in diet) of the aqueous fraction of pine needles after D.E.S. "priming"

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Basal G-56 ration plus 0.040 mcgms. D.E.S. per gram of feed</td>
<td>Basal G-56 ration plus 0.040 mcgms. D.E.S. per gram of feed, plus plant extract added at a concentration of 2:1</td>
</tr>
<tr>
<td>Number of mice in group</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Feed consumption per mouse in four days (gms.)</td>
<td>6.1</td>
<td>5.7</td>
</tr>
<tr>
<td>Average body weight when sacrificed (gms.)</td>
<td>9.6</td>
<td>8.2</td>
</tr>
<tr>
<td>Average uterine weight in mgms.</td>
<td>62.03</td>
<td>47.52</td>
</tr>
<tr>
<td>Mean (uterine weight as a percentage of body weight) ± standard error</td>
<td>0.636±0.056</td>
<td>0.575±0.069</td>
</tr>
</tbody>
</table>

Statistical analysis (t-test) revealed that there were no significant differences between the uterine weight percentages of the two groups. The pine needles were cut on July 3, 1961, and the fractions fed in a four day assay.
was not significant. Because of the large size of the uteri, it was thought possible that the D.E.S. was present in excess of the amount required for a maximal response, and that the pine needle extract could not exert an effect while the stilbestrol was in excess.

The D.E.S. dosage was lowered to 0.020 mcgms. per gram of feed for Experiments P5 and P6. The plant fractions were again administered at a concentration of 1:1 and 2:1 as in the previous two experiments. As is shown in the results in Tables XII and XIII, the response to the lower D.E.S. dose was significantly reduced by the pine needle extracts. The greatest reduction in response occurred with the high (2:1) pine extract concentration in Experiment P6. The results from these four experiments indicate that, at a suitable physiological level of estrogen administration, the pine needle fraction exerted a definite anti-estrogenic effect. Three possibilities for the mode of action of the pine needle extract are suggested:

1. The anti-estrogenic compound(s) is an anti-gonadotropin or any other substance which causes a reduction of the estrogen secretion of the ovary.

2. The compound(s) inactivates estrogens circulating in the blood, and

3. The effect is directly on the uterus, altering the size of the uterus quite independently of estrogenic effects, or reducing the response of the uterus to a certain level of circulating estrogens.
Table XII  The response of immature female mouse uterine weights to oral administration (in diet) of the aqueous fraction of pine needles after D.E.S. "priming"

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Basal G-56 ration plus 0.020 mcgms. D.E.S. per gram of feed</td>
<td>Basal G-56 ration plus 0.020 mcgms. D.E.S. per gram of feed, plus pine extract added at a concentration of 1:1</td>
</tr>
<tr>
<td>Number of mice in group</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Feed consumption per mouse in four days (gms.)</td>
<td>8.9</td>
<td>8.3</td>
</tr>
<tr>
<td>Average body weight when sacrificed (gms.)</td>
<td>10.4</td>
<td>9.3</td>
</tr>
<tr>
<td>Average uterine weight in mgms.</td>
<td>39.79</td>
<td>30.90</td>
</tr>
<tr>
<td>Mean (uterine weight as a percentage of body weight) ± standard error</td>
<td>0.378±0.030</td>
<td>0.328±0.022</td>
</tr>
</tbody>
</table>

Statistical analysis by t-test revealed that there were highly significant differences (P > .001) between the uterine weight percentages of the two groups. The pine needles were cut on July 3, 1961, and the fractions were fed in a four day assay.
Table XIII  The response of immature female mouse uterine weights to oral administration (in diet) of the aqueous fraction of pine needles after D.E.S. "priming"

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of feed</td>
<td>Basal G-56 ration plus 0.020 mcgms. D.E.S. per gram of feed</td>
<td>Basal G-56 ration plus 0.020 mcgms. D.E.S. per gram of feed, plus pine extract added at a concentration of 2:1</td>
</tr>
<tr>
<td>Number of mice in group</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Feed consumption per mouse in four days (gms.)</td>
<td>4.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Average body weight when sacrificed (gms.)</td>
<td>10.9</td>
<td>9.1</td>
</tr>
<tr>
<td>Average uterine weight in mgms.</td>
<td>31.63</td>
<td>18.64</td>
</tr>
<tr>
<td>Mean (uterine weight as a percentage of body weight) ± standard error</td>
<td>0.292±0.018</td>
<td>0.205±0.012</td>
</tr>
</tbody>
</table>

Statistical analysis by t-test revealed that there were highly significant differences (P < 0.001) between the uterine weight percentages of the two groups.

The pine needles were cut on July 3, 1961 and the fractions tested in a four day assay.
2. **The effect of subcutaneous injection of the aqueous fraction of pine needles on the uterine weight of immature rats (Experiment P7)**

The original Astwood method (3, 43) is an assay method for estrogens using immature female rats. The estrogenic content of a substance is assayed by injecting it into groups of 21 to 23 days old rats and measuring the uterine response. The test has been standardized with estradiol or estrone. Astwood showed that 1 mcgm. estrone would cause approximately a 60 per cent increase in uterine weight in six hours.

A difficulty often encountered in the immature mouse three or four day assay is the reduced feed intake of the experimental groups. In order to make the feed intake of the control group comparable to the experimental group, the former's feed intake needed to be severely reduced, or a sizeable difference in body weights between the groups would be observed. A shorter assay not dependent on feed intake was needed to confirm the biological action of some of the plant fractions. It was also desirable to note if an injection of the plant extract suspected to contain an anti-estrogenic factor(s) would decrease the response of the uterus to an estrogen injection. Further, it was of interest to note the effect of injected plant fraction, and to compare this effect with the response obtained when the plant material is administered orally. The Astwood assay was modified so that the experimental group received a plant extract injection along with the estrogen injection. All groups, control and experimental, received one injection of
1 mcgm. estrone. A minor modification from Astwood's original method was that the uterine weights of the rats were expressed as a percentage of the individual body weights. Astwood expressed all uterine weights as a percentage of the average body weight.

An advantage of this six hour assay is that it is almost independent of nutritional effects during the assay period. This may help to clarify some aspects of the mode of action of the fraction to be studied. However, the fraction is injected, and this should be taken into account when interpreting the results of Experiment P7.

The results of Experiment P7 obtained from the modified Astwood assay, are given in Table XIV. Again the water soluble fraction of the pine needle extract caused a significant decrease in uterine weight (expressed as a percentage of body weight). The results of this experiment suggest that it is unlikely that the effect is on the pituitary or ovary. Any effect on the pituitary or ovary would probably require longer than six hours before a significant decrease in uterine weight would be noted. Thus, the decrease of the response to an injected estrogen, caused by an injected extract, in a short period of time, would suggest that the pine needle extract inactivates estrogens, or has a direct effect on the uterus.

The positive results obtained from the modified Astwood test removes the doubt caused by nutritional influences, and it seems certain that, whatever the mode of action may be, under the conditions of these experiments, pine needles contain a specific anti-estrogenic factor.
Table XIV  The effect of injecting the aqueous fraction of pine needles, on the uterine weight of immature female rats

<table>
<thead>
<tr>
<th>Group number</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description of injection</td>
<td>Control group</td>
<td>Experimental group</td>
</tr>
<tr>
<td></td>
<td>1 mcgm. estrone in aqueous suspension in physiological saline (per rat)</td>
<td>1 mcgm. estrone in aqueous suspension and the aqueous fraction of the acetone extract of 0.52 gms. of pine needle dry matter in physiological saline (per rat)</td>
</tr>
<tr>
<td>Number of rats per group</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Average body weight when sacrificed in gms.</td>
<td>39.0</td>
<td>42.4</td>
</tr>
<tr>
<td>Average uterine weight when sacrificed (mgms.)</td>
<td>76.54</td>
<td>70.30</td>
</tr>
<tr>
<td>Mean (uterine weight as a percentage of body weight) ± standard error</td>
<td>0.195±0.007</td>
<td>0.165±0.004</td>
</tr>
</tbody>
</table>

Statistical analysis by t-test revealed highly significant differences between uterine weight percentages of the two treatments. The pine needles were cut on July 3, 1961.
In the literature survey it was noted that Noble (73) found an injection of lithosperm to be much more potent in its physiological properties when injected. This may also be the case with the aqueous fraction of the yellow pine extract. The injections of the experimental group contained the extract of only 0.52 grams of pine needle dry matter. Because this approach seems promising, further research in this direction would be interesting.

3. The effect of chemical extracts of yellow pine needles on the estrous cycle of mature rats (Experiment P8)

As was suggested in the literature survey, the estrous cycle reflects changes in hormone level accurately and quickly. It was further stated that the vaginal cytology is an accurate index of the estrous cycle, a fact which has led to the adoption of the vaginal smear technique for many species. Several reports suggest, however, that frequency and methods of taking the smears can affect the results obtained. Taking the cells with a cotton swab may lead to a smear similar to one obtained at time of estrus (41, 95). If smears are taken with a cotton swab often enough (e.g. four times daily), an estrus type smear may be obtained from ovariectomized or hypophysectomized animals (125). Frequent smearing with a curette or spatula may also bring about irregularities in the cycle (40). These experimental results suggest that the lavage (pipette) method of obtaining smears is the most reliable. Because increased frequency of smearing may also tend to cause cornification of the vagina (40), it was decided that, for the experiments involving vaginal smear observation, smears
would be taken once daily by the lavage method.

Smears may be stained with methylene blue, haematoxylin, or Papanicalaou's (79, 80) stain, among others. However, smears can easily be assessed without the aid of staining. Since many smears would be taken for these experiments, it was decided to observe the smears in the unstained condition.

The results from the vaginal smear experiment with pine needles (Experiment P8), are given in Figure II. The occurrence of each period of estrus is marked by a notch on the line of the animal concerned. The control animals were quite regular throughout the observation period, indicating that the animal material, the basal ration, the caging arrangement, and the management conditions were satisfactory. The vaginal smear pattern of the animals receiving methyl testosterone was characterized by the elongation of the diestrus stage. The long periods of diestrus were marked by excessive numbers of leucocytes or by the presence of "plug" material consisting of mucus containing numerous small cells, probably leucocytes. The presence of plug material was also observed in the pseudopregnant animal. It is believed that this material emanates from the cervical plug which protects the uterus from infection during diestrus, anestrus, pseudopregnancy, and pregnancy. The pseudopregnant animal showed leucocytes or plug material in its smear from the breeding exposure on for a period of twenty days, at the end of which the rat was killed. When put on the ration containing the water soluble fraction of the acetone extract of pine needles, the rats underwent a prolonged period of diestrus which could be termed a period of anestrus. The cessation of estrus cycles continued as
Figure II. The estrous cycle experiments.

- Control ration
- Time in days: 10, 20, 30, 40, 50, 60, 70
- 5 mcgms. methyl testosterone per gram of feed
- The aqueous fraction of pine needle extract (2:1)
- ■ denotes first observation of a heat.
- ○ denotes return to control ration
long as the animals received the experimental ration, a period of 21 days. As soon as the animals were returned to basal ration, the normal estrous cycle returned. During the period of prolonged diestrous, leucocytes were numerous, and plug material was observed on several days. Other rats were obtained for vaginal smear experiments, but because the animals receiving the ration containing the plant extract lost weight and appeared to be in poor condition, no interpretation of the data from these experiments was made.

The limited data reported in Figure II need supplementation before conclusions can be drawn. Adverse conditions, if serious enough, can cause anestrus in animals which were cycling normally. However, there is a suggestion that the extract contained factors which stopped the estrous cycles of rats. These factors do not cause a smear containing cornified epithelial cells typical of estrogen stimulation, but seem to have rather the opposite effect, stopping the cycle in the diestrous phase.

4. General discussion of the results obtained from the yellow pine needle experiments conducted

The results from the experiments conducted using the yellow pine needles indicate that the needles contained a factor(s) which had a definite effect on the reproductive processes of laboratory mice and rats. This compound(s) was water soluble. Further fractionation procedures, perhaps similar to those initiated, may help to purify the extract enough so that isolation and identification may be accomplished.
Although the results from the pine needle experiments did not reveal definitely the mode of action of the anti-estrogenic compounds, several observations may be made. The mouse D.E.S. experiments (P3, P4, P5, and P6) suggest that the response to administered estrogens can be reduced by the pine needle extracts. If the estrogens are administered by injection, as in the Astwood assay, injected pine needle extract can partly prevent the increase in uterine size which normally occurs in six hours. This shows further that the action of the compound(s) is very rapid. If an inactivation of gonadotropins, or an effect on the pituitary which decreased the gonadotropin secretion, would be the mode of action, then it is probable that more than six hours would be required before an effect on uterine size could be noted. It is more likely that the mode of operation is one of the following: 1. The compound(s) inactivates estrogens circulating in the blood, 2. The compound(s) makes the uterus non-responsive to estrogenic stimulation, or 3. The compound(s) decreases uterine size directly without any interference with estrogen action.

Whether the compound which is responsible for the action on the uteri of mice and rats is identical to the compound causing the abortion of cattle on yellow pine ranges is not known. This should be investigated by further experiments. Also, it is not known whether any compounds present in yellow pine are similar to the compound or compounds present in birdsfoot trefoil, lithosperm, \textit{P. sativum}, or any other plant material. It is hoped that these experiments will be an aid to further investigations in this interesting and important field of study.
D. Chromatographic Studies of Chemical Extracts of Yellow Pine Needles

The chromatographic studies of the pine needle fractions and the two reference compounds, yielded interesting results. Under the butanol:acetic acid:water solvent system, the pinosylvin and the pinosylvin mono-methyl ether moved almost at the solvent front. In the two per cent acetic acid, the two compounds did not move far from the origin, both having \( R_f \) values close to 8.6 per cent. These two compounds also appeared very similar if viewed under ultraviolet light or if sprayed with the diazotized sulfanilic acid spotting reagent. With these two solvent systems, the three plant fractions appeared to have many spots, none of which could be identified as pinosylvin or its mono-methyl ether. Lindstedt's solvent (59) has been reported to separate pinosylvin and its mono-methyl ether. With this solvent, pinosylvin was found to have an \( R_f \) of 3.3 per cent while the pinosylvin mono-methyl ether gave an \( R_f \) of 63.7 per cent.

Three pine needle fractions, the aqueous fraction of the acetone extract, the ethyl acetate eluent (from celite), and the fraction refluxed with Dowex 50-W cation exchange resin, were also chromatographed using Lindstedt's solvent, together with pinosylvin and pinosylvin mono-methyl ether as reference compounds. The aqueous fraction of the acetone extract did not show any spots resembling those of the reference compound. However, the ethyl acetate eluent and the fraction refluxed with Dowex 50-W cation exchange resin, when chromatographed using Lindstedt's solvent the chromatograms viewed under ultraviolet light, showed spots very similar, in location and
fluorescence, to pinosylvin. The $R_f$ values of these spots were found to be as follows:

<table>
<thead>
<tr>
<th>Solution</th>
<th>$R_f$ in per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinosylvin</td>
<td>3.3</td>
</tr>
<tr>
<td>Ethyl acetate eluent</td>
<td>2.8</td>
</tr>
<tr>
<td>Dowex 50-W solution</td>
<td>3.0</td>
</tr>
</tbody>
</table>

When these chromatograms were sprayed with the diazotized sulfanilic acid spotting reagent, the pinosylvin spot became yellow in color. The spots from the two plant solutions became a dark pink, indicating that these spots were not caused by the presence of pinosylvin.

The similarity of the spots to pinosylvin, both in mobility and fluorescence suggests the possibility that these spots were caused by the presence of a compound similar in structure to pinosylvin. Because the color reaction with diazotized sulfanilic acid yielded a darker color than pinosylvin, it is possible that the compound is a stilbene with more substitutions in the rings than pinosylvin. Although its structure is not exactly known, the compound was probably released by hydrolysis in the ethyl acetate refluxing process, and the Dowex 50-W cationic exchange resin. As would be expected, most hydrolysis occurred in the solution refluxed with ion exchange resin. A glycosidic linkage is a likely possibility. Although it is not known whether these spots were caused by the compound causing the effects on reproduction in cattle and laboratory animals, further research on this aspect of the problem would be of interest.
VII CONCLUSIONS

1. Birdsfoot trefoil extracts were found to contain one or more water-soluble factors which decreased uterine weight (expressed as a percentage of body weight) of immature female mice. The identity or mode of action of this compound was not determined. It is not known to what extent the anti-estrogenic forage influences the basic processes of reproduction, lactation, and growth.

2. Yellow pine needle extracts were found to contain one or more water-soluble factors which had anti-estrogenic effects on the reproductive system of mice and rats. Oral administration of a specially prepared aqueous extract of pine needles decreased the uterine weight (expressed as a percentage of body weight) of immature mice, and disrupted the estrous cycles of adult female rats. When diethylstilbestrol was added to the feed at a level of 0.040 mcgms. per gram of feed, the addition of pine needle extract did not decrease uterine weights (expressed as a percentage of body weight), however, at a level of 0.020 mcgms. of diethylstilbestrol per gram of feed, the addition of the aqueous fraction of the acetone extract significantly decreased uterine weight percentages. When injected subcutaneously into female rats, this extract reduced the uterine response to an estrogen injection. This reduction occurred in less than six hours. The factor(s) is believed to act by the inactivation of estrogens circulating in the blood, or by acting on the uterus directly. The identity of the compound was not determined although chromatography provided some data which may help in the solution of this problem. Whether the compound which is responsible for the action on the uteri of mice and rats is identical to the compound causing the abortion of cattle on yellow pine ranges is not known.


35. DRASHER, M.L., "Further observations on the inhibition of the production of luteinizing hormone by lithosperm", Endocrinology, 47:399-413, 1950.


