THE EFFECT OF LADINO CLOVER, BIRDSFOOT TREFOIL, AND BLUEGRASS
PASTURE ON THE RATE OF CONCEPTION AMONG EWES

DISSERTATION

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INTRODUCTION

Breeding efficiency is fundamental to success in all meat animal production. Any factor or factors which tend to interfere with or impair reproductive function is of great concern to all animal husbandmen.

In the eastern farming area sheep growers have become increasingly concerned with a problem of delayed conception in many breeding flocks. This unfavorable animal response results in prolonged or strung-out lambing seasons which in turn may upset carefully designed flock management and farm operation programs. Sometimes the per cent of non-pregnant ewes is increased, thus lowering the over-all reproductive rate obtained. The end effect is that farmers are constantly asking what they can do to get their ewes to breed promptly. The question is, what promotes this irregular breeding performance?

For a number of years circumstantial evidence has been building up which indicated that the kind of pasture on which sheep graze prior to and during the breeding season affected the rate of conception. The circumstantial evidence further indicated that when the problem was encountered, it most commonly occurred on those pastures in which legume plants were dominant or in relatively pure stand.

When certain breeding troubles and infertility in sheep in Western Australia, as discussed by Bennetts et al. (1946), subsequently

was shown to be due to hormone-like substances in subterranean clover, the question arose as to whether some plant substance which produced an estrogenic effect might be involved in our own problem.

The first phase of work was undertaken to determine whether a difference in breeding performance in sheep grazed on different kinds of pasture did exist. Simultaneously a second phase of work was undertaken to develop biochemical methods for extraction of plant materials and bio-assay of these extracts for estrogenic response. This dissertation, therefore, reports the results of work undertaken to study the effect of ladino clover, birdsfoot trefoil, and bluegrass pasture on the rate of conception among ewes.

LITERATURE REVIEW

Factors Affecting Reproduction

Winter (1954) lists 12 factors as the most frequent causes of reproductive failure: (1) anatomical defects; (2) mechanical injury of the genitalia; (3) infectious diseases; (4) prenatal death of the young; (5) physiological disturbances, particularly improper hormone balances; (6) gametic incompatibility; (7) low vitality of gametes; (8) failure to mate at optimum time during the oestral period; (9) psychic incompatibility; (10) impaired nutrition; (11) miscellaneous environmental disturbances; and (12) genetic causes which may be directly or indirectly related to the above factors.

A suggestion that certain feeds may contain some component detrimental to reproduction of sheep was given by Hunt (1935) when he reported that farmers were encountering digestive and lambing problems on feeding soybean hay.

An example of the effect of a particular component in the diet in lowering fertility was reported by Bennetts et al. (1946). Ewes grazing subterranean clover in West Australia showed impaired fertility, and wethers on the same pastures showed marked changes in the urogenital tract and mammary development indicative of excessive levels of estrogens in the body. Curnow et al. (1948) reported estrogenic activity in extracts of the subterranean clover. This in turn was related to genistein in the subterranean clover by

Bradbury and White (1951). Lactation in unbred females and castrate males, prolapse of the uterus, and general relaxation of the pelvic ligaments were observed by Bennetts and Underwood (1949). The report further noted the pathological conditions of cystic glandular hyperplasia of the uterus, and squamous metaplasia of the secondary sex organs of wethers, notably the bulbo-urethral and prostate glands.

Underwood and Shier (1951) found that the infertility induced by grazing subterranean clover tended to be permanent as no measurable improvement was noted in the breeding performance of ewes of any age after 2 or 3 seasons on non-subterranean clover pasture. Ewe lambs became affected as early as 4 or 5 months of age and became progressively worse as the length of time on the pastures increased. When ovariectomized ewes were grazed on subterranean clover the uterus failed to atrophy or atrophic uteri could be restored to normal by the ingestion of subterranean clover carrying the factor producing the estrogenic effect (Bennetts and Underwood, 1951). Underwood and Shier (1952) found the breeding season of ewes to be the same whether grazed on subterranean clover pasture or non-subterranean clover pasture. The ewes on subterranean clover were only slightly irregular in estrus. Only 15 of the 20 ewes grazed on subterranean clover lambed but the failure to lamb was not due to the failure to take the ram or disturbances in the estrus cycle.

In another test Underwood et al. (1953) showed that ewes were rendered infertile by the injection of 1 mg. to 16 mg. stil-

bestrol per ewe per day over a six months period. Little recovery was noted after $2\frac{1}{2}$ years. Almost complete infertility and some dystocia were produced by doses from 0.11 mg. to 1 mg. per head per day over a six months period. In this study the estrus cycle, as judged by raddle marks imposed by teaser rams, appeared normal.

East et al. (1949, 1950, 1952) produced lesions in guinea pigs similar to those found in sheep grazing subterranean clover by treating the guinea pigs with estrogens and by feeding them subterranean clover. Androgen injection gave protection to the castrate male except that the androgen did not inhibit nipple growth induced by the estrogens or subterranean clover. In the female guinea pig injections of estradiol dipropionate interfered with normal ovarian function by deranging estrus, atresia of ovarian follicles, and lack of corpora lutea. With the feeding of subterranean clover ovulation was not impaired although 70 per cent of the female guinea pigs fed subterranean clover failed to conceive and the remaining 30 per cent failed to carry to term.

Genistein at 2 mg. per kg. of ration for mice was shown by Carter et al. (1954) to reduce the number of litters born.

There are other factors that may affect fertility level and rate of conception. Hammond (1921) has shown the number of ova shed at each estrus to be the limiting factor in sheep fertility. Marshall and Potts (1924) obtained an increase in lambing percentage from 128.7 per cent to 147.4 per cent by flushing Southdown ewes. Grain or pasture were reported equally effective with the ewes

gaining the most weight producing the largest number of lambs. Also, a difference in per cent of ewes conceiving to first service was noted with the statement that "more cases of not getting in lamb until the second or third service occurred among the flushed ewes." Similar results were obtained by Bell (1932) with Shropshires and by Wallace (1951) with Merinos. The onset of estrus was slightly earlier for ewes which were flushed but the differences were not considered of practical significance and there was no appreciable difference in the percentage of ewes which failed to conceive. A delay in conception was noted as a result of flushing which was considered important if a uniform lamb crop was desired. Although the number of lambs dropped was greater for the flushed ewes, the number surviving until weaning was seldom significantly greater for the flushed ewes than for the unflushed control ewes. Flushing was believed to induce multiple ovulation in ewes which were not naturally capable of rearing the additional lambs. Nichols (1926) supported flushing but found no effect when the flocks were continually kept in high condition. Clark (1934) confirmed this finding when he reported that the number of eggs released from the ovary was increased by flushing if the ewes were in thin condition but not if the ewes were in high condition. Clark's results show the following:

thin ewes unflushed	1.0 ovum
thin ewes flushed	1.4 ovum
high condition ewes unflushed	1.7 ovum
high condition ewes flushed	1.5 ovum

He concluded that the efficacy of flushing depends on the condition of the ewe.

An increase in ovulation rate from 1.06 to 1.15 was obtained by McKenzie and Terrill (1937) by flushing Rambouillet ewes with grain. A low plane of nutrition resulted in a shorter pre-ovulatory period, and a slightly lower ovulation rate. They state "good nutrition seems to influence reproductive phenomena only to the extent that it allows full expression of the inherited capacity."

Supplementing range forage for bred ewes was shown by Van Horn et al. (1951) to increase the lambing percentage from 80 per cent to 117 per cent. A comparable band of 1,000 ewes on a high plane of nutrition before and during the breeding season as well as through the winter had a 126 per cent lamb crop. Darroch et al. (1950) increased the lambing rate of range ewes by 10 per cent and 9 per cent by feeding supplements in the pre-breeding and during the breeding season respectively. Ewes in good condition produced 11 per cent more lambs than thin ewes; however, the thin condition ewes tended to conceive earlier than ewes in good condition but the difference was removed by flushing the ewes. The flushed ewes conceived at the later date.

Diets which are deficient in one or more nutrients have been shown to have a marked effect on reproductive efficiency. Inadequate

protein, phosphorus, or vitamin A lowered the lambing rate in studies conducted by Miller, Hart and Cole (1942). Because the body stores of phosphorus and vitamin A were depleted slowly, deficiency symptoms did not develop rapidly; however, the effect of a protein deficiency on ewe performance was detectable in a shorter time. Slen and Whiting (1952) and Klosterman et al. (1953) reported a 10 per cent protein ration to be adequate for the breeding and pregnancy period of ewes. Protein levels from 5 per cent to 13 per cent were studied with no particular additional benefit from a level above 10 per cent. Hafez (1952) showed a substantial reduction of live weight by underfeeding did not delay onset of the breeding season if the sub-maintenance diet was started 2 months before the expected onset of the breeding season. Estrus was inhibited, however, after normal onset with silent heats which were more frequent in the yearling ewes than adults. The ewes recovered rapidly on improved diets. Also, more than one service per conception was generally required by the underfed ewes.

Uniform agreement that excessively fat rams or ewes were poorer breeders than those in vigorous, healthy condition was noted by Heape (1899) from a survey conducted in England. Though most shepherds agree, Miller, Hart and Cole (1942) stated, "We do not know of a single controlled experiment that would tell us just what degree of fatness becomes detrimental or how long an animal must be fat before its reproductive capacity is impaired." Hammond (1949) noted that stockmen blame over-fatness as a cause of sterility but

remarked that "over-fatness may be the result, as well as the cause, of infertility." He could find no experimental evidence to support the theory that fat is effective in producing infertility by absorbing oestrogens or blocking the production of progesterone. It is apparent that an excess of feed which resulted in overly fat sheep has not been considered a major problem of the sheep industry.

Estrogenic Activity of Plants

It has been established that certain plants commonly used as pasture or dry forage contain estrogenic substances as indicated by mouse bio-assay techniques. One of the first to report on a relationship between reproductive performance of sheep and the estrogenic substances in subterranean clover was Curnow et al. (1945). Since then a number of workers have studied the estrogenic activity of several common forage plants. Cheng et al. (1953b) reported that bluegrass and ladino clover showed no estrogenic activity when the extracts equivalent to 4 gm. bluegrass or 5 gm. ladino clover were injected daily into immature female mice for 4 days. However, alfalfa and red clover hay were found to be estrogenically active. The report stated, "With the purified extracts, injection was far more effective than feeding." Pieterse and Andrews (1956b) reported significant amounts of estrogenic activity in ladino clover and birdsfoot trefoil when bio-assayed by feeding alcoholic extracts to castrate female mice for 10 days. The estimated estrogenic potency of the ladino clover and birdsfoot trefoil was less than 0.5 micrograms of stilbestrol equivalent per pound of dry matter.

Alexander and Watson (1951), Bennetts and Underwood (1951), Legg et al. (1950), Cheng et al. (1953b), Dohan et al. (1951), and Pieterse and Andrews (1956b) reported seasonal variation in the estrogenic activity of the plants studied. Pieterse and Andrews (1956a) reported the estrogenic activity of alfalfa was increased

several fold by the process of ensiling. Previous work at this station, Engle (1953), indicated ladino clover and birdsfoot trefoil contained estrogenic activity but no activity was detected in bluegrass. The procedure developed for the extraction of green forage plants was considered unsatisfactory for routine bio-assay by the mouse uterine weight technique if the extracts were to be administered by injection into immature female mice. This was particularly true when attempting to bio-assay low potency plants where a dose of high equivalent dry matter was needed.

In a review of the literature of estrogenic substances in plants, Bradbury and White (1954) point out the wide distribution and variety of substances in plants which have an estrogenic effect on animals. The review was summarized in part by stating "that the known plant estrogens are estrone, estriol, and genistein and its derivatives while the Butea estrogen, $C_{19}H_{22}O_6$, is as yet unidentified. The active compounds in some 40 other plants, reported estrogenic, have not yet been isolated or characterized, and it's highly probable that many other plants contain estrogenic compounds."

Some evidence has been accumulated to indicate the activity in these plants is either the isoflavone or substances closely associated with the isoflavones.

Bradbury and White (1951) studied the estrogenic activity of formononetin and genistein isolated from subterranean clover. Additional work on genistein from subterranean clover was reported by Curnow (1954) and Bigger and Curnow (1954). A procedure for the

purification of extracts was given as well as a micro method for the qualitative detection of genistein. Formononetin was isolated from red clover by Bate-Smith et al. (1953) while Pope et al. (1953, 1954) isolated the estrogenically active isoflavones genistein, biochanin A, and formononetin from red and subterranean clover. Genistein prepared from soybean oil meal by the procedure outlined by Walter (1941) was shown by Cheng et al. (1953a) to have estrogenic activity when fed to mice. Cheng et al. (1954) assayed synthetic isoflavone derivatives and found genistein, biochanin A, and daidzein of approximately equal activity, but formononetin gave a much lower response than the other three derivatives.

Robinson (1949) reported that greater estrogenic activity was obtained when samples were extracted with basic alcohol than with alcohol but did not determine if the increase was due to more complete extraction or hydrolysis of the active substances. Hydrolysis has been shown to increase the estrogenic activity of genistein by Biggers and Curnow (1954) and also by Pieterse and Andrews (1956a).

PART I

SHEEP BREEDING EXPERIMENTS

The first phase of this work was designed to study the breeding response by sheep grazing on different kinds of pasture. The following outlines the work done and the results obtained in this area to determine the effect of grazing bluegrass, ladino clover, and birdsfoot trefoil pasture on the conception rate among ewes.

Materials and Methods

Pastures

Ladino clover (Trifolium repens var. ladino) and birdsfoot trefoil (Lotus corniculatus) pastures were seeded as pure stands in the spring of 1952 with oats as a nurse crop in adjoining fields of 8 acres each. An additional area of 3 acres for each legume pasture was seeded in 1954. An adjacent area of fertilized bluegrass (Poa pratensis) pasture containing some timothy and volunteer white Dutch clover and located nearby on the same farm was used as the grass pasture in the comparison. The 1952 seedings of ladino clover and birdsfoot trefoil became mixed with volunteer grass and weeds by 1954; also, the ladino clover pasture was severely damaged by dry weather in 1954. By the 1955 grazing season the ladino clover seeding made in 1952 contained approximately 40 per cent ladino clover and the birdsfoot trefoil approximately 50 per cent of birdsfoot trefoil. The new seeding of both ladino clover and birdsfoot trefoil were utilized to

capacity during the 1955 grazing and breeding season and were essentially pure stands with only slight weed contamination.

The exact limits of the grazing periods were prescribed by the seasonal conditions prevailing each year. The limits of the grazing seasons are shown in Table 1 and the length of the growing season in Table 2.

TABLE 1. DATE AND LENGTH OF THE PASTURE SEASON

Year	First day on pasture	Last day on pasture	Days on pasture	Date of first supplemental feeding
1953	May 6	Oct. 28	175	Oct. 9
1954	May 7*	Nov. 4	171	Aug. 4
1955	April 29	Nov. 14	199	Oct. 24

*With the exception of the late lambing ewes which went to their respective pasture lots June 8, 1954.

TABLE 2. KILLING FROST DATES AND THE LENGTH OF THE GROWING SEASON

Year	Last killing frost date	First killing frost date	Length of growing season
1953	April 24	October 8	167 days
1954	May 9	October 7	151 days
1955	May 18	October 22	157 days

Sheep Used and Their Allotment

To test the influence of all season versus fall grazing only on the conception rate among ewes, the yearling ewes assigned for the first 2 years were divided into 6 comparable groups. One group was placed on ladino clover and one on birdsfoot trefoil pasture. A comparable group for each of the pastures was placed on bluegrass along with the bluegrass pasture ewes for summer grazing. Two weeks prior to the time breeding was to begin two lots of yearling ewes were changed from the bluegrass pasture to the legume pasture lots--one lot to ladino clover and one lot to birdsfoot trefoil. This latter pattern was followed for the aged ewes assigned during the first year. All other groups were on their respective pasture during the entire pasture season. The third year of the test the older group of ewes was removed from the test and replaced in the breeding flocks with yearling ewes produced in the test lots and grazed only in their respective lots. The number of purebred

Columbia ewes assigned per treatment and year is shown in Table 3.

These ewes were mated to purebred Columbia rams.

TABLE 3. NUMBER OF EWES ASSIGNED BY GRAZING SYSTEM, AGE, AND YEAR

Year	Ewe group and grazing period	Bluegrass	Ladino clover	Birdsfoot trefoil
1953	Aged ewes, fall only	7	7	7
	Yearling ewes, all season	14	7	7
	Yearling ewes, fall only	--	7	7
1954	Two year old ewes, all season	14	14	13
	Yearling ewes, all season	14	7	7
	Yearling ewes, fall only	--	7	7
1955	Two year old ewes, all season	12	14	13
	Yearling ewes, all season	8	14	9
Total		69	77	70

Breeding

Hand mating, using teaser rams with aprons to locate ewes in estrus, was followed in 1953 and in 1955. Field breeding was used in 1954. When hand breeding was employed, only a single service at first detection of estrus was allowed. Under field breeding, rams wearing a Jourgensen harness marked the ewes when bred and new raddle marks were recorded each day. Semen samples from all breeding rams used were examined microscopically to guard against infertility. The service of a ram was distributed equally among all lots. The

breeding date was considered as the service closest to 147 days prior to the lambing date; however, in field breeding where no raddle mark was observed the date of service was considered as the lambing date minus 147 days. In cases of premature lambs the age of the foetus was estimated and this number of days was then used as the length of gestation in determining the date of conception.

Breeding was started August 24, 1953, but was changed to September 13, 1954, and September 9, 1955, because records indicate that only a few ewes of this breed come in estrus before September 10 under the environment at Wooster, Ohio.

Feeding

Harvested feed was given each fall when weather conditions did not permit grazing or the pastures did not supply sufficient feed. In 1954 drought so reduced growth on the pastures that a short period of dry lot feeding was required in mid-August, but as soon as new growth started following rain, the sheep were returned to the respective pastures. The date on which it became necessary to begin supplemental feeding is given in Table 1 along with the date grazing was concluded for the season.

All lots were fed the same during the winter feeding and lambing season. The wintering ration for the bred ewes consisted of first cutting, light to medium mixed legume-grass hay fed according to appetite and 0.5 lb. grain (1 part corn, 1 part oats) per head per day prior to lambing. The grain was increased at the

beginning of the lambing season to 1.5 lb. of grain per head per day and 0.2 lb. of linseed oil meal per head per day was fed separately until the ewes and lambs were turned to pasture. During the first winter, one-half the roughage fed was meadow crop silage and 0.1 lb. of soybean oil meal was used in place of the 0.2 lb. of linseed oil meal. Meadow crop silage and soybean oil meal were not fed in the last two years of the test because of the estrogenic activity reported in these feeds.

The sheep had access to salt at all times. Trace mineralized salt was given during the winter feeding period. Phenothiazine was added to the salt during the grazing season to aid in the control of gastro-intestinal parasites. In addition to the use of the 9 parts salt to 1 part phenothiazine mixture the ewes were treated by drenching with phenothiazine in April, July, September, and November.

Management

The flocks were confined at night during the grazing season and were housed in adjacent 12 ft. X 24 ft. pens with 12 ft. X 24 ft. outside bedded lots during the winter. The ewes were kept under close observation during the lambing season. Each ewe and her lamb (s) were placed in a 5 ft. X 5 ft. lambing pen soon after partuition. The length of confinement varied inversely with the vigor of the lamb and the mothering and milking quality of the ewes. The entries made in the records after partuition were: ewe number, lamb number, date of birth, sex of lamb, birth weight, vigor of

lamb, condition of lamb, nature of labor, disposition of the ewe, and condition of the ewe. All individuals except lambs before weaning were weighed at monthly intervals, and lambs were weighed at 10 and 30 days and at weaning.

The lambs were docked when 10 to 15 days of age. The male lambs were left to develop as rams.

Results

Estrus

The mean date of first detected estrus, as shown in Table 4, did not differ greatly between lots. The ewes grazed on birdsfoot trefoil did not differ significantly from the other two lots but tended to be intermediate between the ewes grazed on bluegrass which had the earliest first estrus and the ewes grazed on ladino which had the latest onset of estrus. The mean date of first detected estrus for the ladino clover group during the breeding season was significantly later than for the ewes grazed on the bluegrass pasture. This delay was due primarily to delayed onset of estrus among the yearling ewes grazed all season on the ladino clover in the first two years. Figure 1 exemplifies the differences in first estrus by showing the accumulative percentage of ewes detected in estrus by date.

The pastures did not appear to affect the manifestation of heat or the regular recurrence of the next estrus period among ewes not becoming pregnant.

Conception

The ewes grazed on ladino clover and birdsfoot trefoil conceived significantly later, by 21.7 and 19.9 days respectively, than the ewes grazed on bluegrass pasture (Table 5). The ewes grazed on birdsfoot trefoil tended to conceive slightly earlier than those on ladino clover but the difference was not significant.

TABLE 4. TIME OF FIRST DETECTED ESTRUS

(Days after August 31)

Year	Ewe group and grazing period	Bluegrass		Ladino clover		Birdsfoot trefoil	
		No.	mean s.e.	No.	mean s.e.	No.	mean s.e.
1953	Aged ewes, fall only	5	17.40 ± 5.81	4	13.75 ± 4.60	6	18.83 ± 3.59
	Yearling ewes, all season	12	27.17 ± 2.85	5	50.80 ± 14.39	5	27.60 ± 6.32
	Yearling ewes, fall only			7	28.28 ± 5.53	7	29.28 ± 6.75
1954	Two year old ewes, all season	12	20.83 ± 1.84	11	30.91 ± 3.22	10	23.33 ± 2.93
	Yearling ewes, all season	13	23.46 ± 1.85	6	46.83 ± 7.46	6	45.17 ± 10.24
	Yearling ewes, fall only			5	19.40 ± 1.03	7	26.28 ± 2.81
1955	Two year old ewes, all season	12	27.75 ± 2.77	10	41.4 ± 6.49	12	29.17 ± 2.66
	Yearling ewes, all season	7	27.43 ± 2.51	13	30.92 ± 3.01	8	27.50 ± 5.72
	Total	61	24.28 ± 1.12	61	33.46 ± 2.33	61	28.10 ± 1.85

Figure 1. Date of first detected estrus. Per cent of total ewes assigned for three years by treatment.

TABLE 5. TIME OF CONCEPTION

(Days after August 31)

Year	Ewe group and grazing period	Bluegrass		Ladino clover		Birdsfoot trefoil	
		No.	mean s.e.	No.	mean s.e.	No.	mean s.e.
1953	Aged ewes, fall only	5	31.40 ± 14.06	4	26.75 ± 13.55	6	68.67 ± 8.69
	Yearling ewes, all season	12	40.92 ± 6.21	5	78.40 ± 17.19	5	52.80 ± 16.61
	Yearling ewes, fall only			7	68.71 ± 12.19	7	61.86 ± 13.40
1954	Two year old ewes, all season	12	25.75 ± 2.71	11	40.91 ± 5.07	10	47.20 ± 8.90
	Yearling ewes, all season	13	34.23 ± 4.94	6	64.50 ± 7.51	6	51.50 ± 9.55
	Yearling ewes, fall only			5	51.80 ± 11.38	7	57.57 ± 8.01
1955	Two year old ewes, all season	12	41.50 ± 7.85	10	60.90 ± 6.00	12	51.42 ± 4.37
	Yearling ewes, all season	7	34.85 ± 6.50	13	60.54 ± 5.59	8	55.88 ± 8.75
	Total	61	35.15 ± 2.70	61	56.92 ± 3.28	61	55.03 ± 3.21

Age of ewe, length of grazing season, and between year differences in time of conception were noted, but the effect of pastures on these differences was not significant for the small number of animals used in this test. Figure 2 exemplifies the differences in date of conception by showing the accumulative percentage of ewes conceiving by date. A difference in the promptness of conception is indicated by the number of services required per pregnancy (Table 6). The ewes grazed on ladino clover averaged

TABLE 6. AVERAGE NUMBER OF SERVICES PER PREGNANCY

(Based on ewes lambing)

Pasture	1953	1954	1955	Three year average
Bluegrass	1.6	1.4	1.6	1.5
Ladino clover	2.0	1.8	2.4	2.1
Birdsfoot trefoil	2.7	1.9	2.4	2.3

0.6 and the ewes grazed on birdsfoot trefoil averaged 0.8 more services per pregnancy than the ewes grazed on bluegrass. Sixty-six per cent, forty-one per cent, and thirty-one per cent of the ewes grazed on bluegrass, ladino clover, and birdsfoot trefoil, respectively, lambed to first service.

Figure 2. Date of conception. Per cent of total ewes assigned for three years by treatment.

Lambing

The lambing percentages (Table 7) differed by 6.5 per cent or less between the averages for the lots or years. Larger differences, up to 24.9 per cent, were found between lots within a year and 26.1 per cent between years within a lot.

TABLE 7. LAMBING RATE AND SURVIVAL BY KIND OF PASTURE AND YEAR

Kind of pasture	1953	1954	1955	Treatment, three-year average
Lambing percentage*				
Bluegrass	147.1	136.0	121.0	134.4
Ladino clover	143.7	136.4	134.8	137.7
Birdsfoot trefoil	122.2	130.4	140.0	131.2
Year average	137.2	134.3	132.3	134.4
Per cent lambs surviving to weaning				
Bluegrass	92.0	88.2	82.6	87.8
Ladino clover	91.3	80.0	74.2	81.0
Birdsfoot trefoil	86.4	96.7	78.6	87.5
Year average	90.0	88.3	78.0	85.4

*Based on ewes lambing

No consistent differences were found in lamb survival (Table 7). However, the average was lower in the lot where the ewes had been grazed on ladino clover.

Weight of Ewes

The weight of the ewes just prior to the breeding season is given in Table 8. The ewes grazed all season on ladino clover and birdsfoot trefoil were 8.4 per cent to 23.6 per cent heavier than the ewes which grazed bluegrass.

TABLE 8. PREBREEDING WEIGHT OF THE EWES

(pounds)

Year	Ewe group and grazing period	Bluegrass		Ladino clover		Birdsfoot trefoil	
		No.	mean s.e.	No.	mean s.e.	No.	mean s.e.
1953	Aged ewes, fall only	5	118.4 ± 8.2	4	138.0 ± 9.9	6	129.3 ± 6.2
	Yearling ewes, all season	12	121.5 ± 2.1	5	143.2 ± 5.2	5	147.8 ± 7.5
	Yearling ewes, fall only			7	128.8 ± 6.8	7	125.6 ± 2.3
1954	Two year old ewes, all season	12	125.3 ± 3.6	11	137.2 ± 7.9	10	145.7 ± 6.3
	Yearling ewes, all season	13	124.7 ± 2.4	6	135.2 ± 5.2	6	137.2 ± 4.5
	Yearling ewes, fall only			5	115.8 ± 6.5	7	119.8 ± 5.0
1955	Two year old ewes, all season	12	118.0 ± 2.3	10	139.5 ± 6.3	12	141.8 ± 5.0
	Yearling ewes, all season	7	111.6 ± 4.7	13	128.7 ± 3.3	8	138.0 ± 6.6
Total		61	120.8 ± 1.4	61	133.4 ± 2.4	61	136.4 ± 2.2

Discussion

The kind of pasture on which ewes graze prior to and during the breeding season influenced the rate of conception among ewes even though the pastures did not affect the manifestation of estrus or the regular recurrence of the next estrus period. The regularity of the estrus cycle of ewes which fail to conceive indicates that the interference with conception takes place prior to implantation. The failure to conceive could be due to failure of ovulation, fertilization, or implantation.

The temporary nature of the infertility is indicated by the late conception of the ewes in the test lots. The per cent of ewes not lambing was consistently higher in the lot grazed on ladino clover with no practical difference between the lots grazed on bluegrass and birdsfoot trefoil. Ewes, though, that did not lamb one year did lamb in subsequent years. While the percentages of barren ewes for the 3 years--11.6 per cent for bluegrass, 20.8 per cent for ladino clover, and 12.9 per cent for birdsfoot trefoil--are all high, only the ewes grazed on ladino clover had a higher percentage of dry ewes than the 14 year average for the purebred Columbia ewes maintained at this station.

Part of the delay in conception, particularly in the ladino clover lot, may be attributed to the delay in coming in estrus, but the delay in conception was 22 days compared with 9 days for the delay in first estrus. Only 41 per cent of the ewes grazed on ladino

clover conceived to first service and 2.1 services were required per pregnancy. The largest number of repeat breedings occurred in the lot grazed on birdsfoot trefoil where 31 per cent of the ewes conceived to first service. The ewes grazed on bluegrass tended to come in estrus earlier and required the least number of services per pregnancy.

An influence of weight and condition of the ewe on estrus and conception is suggested since the ewes grazed all season on the legumes were 8.4 per cent to 23.6 per cent heavier than ewes grazed all season on bluegrass. The ewes transferred from bluegrass to the ladino clover and birdsfoot trefoil in mid-August remained at a weight and finish similar to those which remained on bluegrass. Although they were lighter in weight, the ewes put on the birdsfoot trefoil in mid-August did not conceive appreciably earlier than those grazed on birdsfoot trefoil all season. Likewise, those ewes grazed on ladino clover from mid-August conceived only slightly later than those grazed all season on this crop. However, the yearling ewes placed on the pastures in mid-August did come in estrus earlier than those grazed all season on the legumes. The short period of grazing before breeding did not influence the onset of estrus but did influence the date of conception. All lots gained weight during the breeding season and as a result the late breeding ewes were heavier when they did conceive than at first mating.

The pastures differed markedly in protein content (Kjeldahl nitrogen times 6.25). The samples analyzed showed bluegrass to

contain 11.7 to 14.8 per cent; ladino clover, 27.3 to 29.2 per cent; and birdsfoot trefoil, 20.3 to 26.2 per cent of protein on a dry matter basis. The bluegrass should have provided a digestible protein level near the allowance recommended by the National Research Council (Pearson et al. 1949), but the protein in the legumes was much in excess of the recommendation of 5 per cent digestible protein. No attempt was made in this study to evaluate critically the influence the higher protein levels had on the rate of conception among the ewes.

Estrogenic activity was demonstrated in the ladino clover and birdsfoot trefoil pastures used in this study by Engle (1953) but no estrogenic activity was detected in bluegrass. Further laboratory studies on the estrogenic activity of the three pastures are reported in Part II of this dissertation. Udder palpation of the yearling ewes on this study indicates that the udders of the ewes grazed on ladino clover and birdsfoot trefoil were more developed than the udders of the yearling ewes grazed on bluegrass. The technique was not developed to the extent whereby a critical analysis could be made. More positive evidence that the ewes grazed on the ladino clover and birdsfoot trefoil were actually under the influence of estrogens was provided by studies of vaginal smears by Sanger (1957). Anestrus vaginal smears from ewes on birdsfoot trefoil and ladino clover showed high levels of cornification of epithelial cells which is consistent with the presence of excessive estrogens. This is in contrast to finding only small

numbers of normal basophilic epithelial cells in anestrus smears from control ewes not on these pastures.

The dates of conception plotted in Figure 2 point out the individual variation observed in this test. While some of the ewes grazed on the ladino clover and birdsfoot trefoil conceived in the early part of the season, they were not grouped as were those grazed on bluegrass but tended to conceive at a uniform rate throughout the season. The majority of the ewes grazed on the legume pastures conceived after the pastures had been subjected to a killing frost. From these results no particular difference would be expected if breeding was delayed until late October or November. The dystocia which was a prominent aspect of grazing subterranean clover was not observed here, but the ewes were off the pastures from the early part of gestation until after lambing. Under the Australian system of subterranean clover grazing the ewes were continued on the pastures throughout the gestation and lambing periods.

It is also to be noted that a group of yearling ewes grazed on birdsfoot trefoil until mid-August and then transferred to the bluegrass conceived at an intermediate date between the ewes grazed all season on bluegrass and those grazed all season on birdsfoot trefoil. This suggests the birdsfoot trefoil pasture could be used until mid-summer with only a minor delay in conception resulting. Further studies will be needed to determine the best method to make maximum use of the increased forage production of the ladino clover and birdsfoot trefoil and avoid their adverse effect on reproduction.

The factors of ovulation, fertilization, histology of the uterus, and sperm transport and survival in the reproductive tract of the ewe were not studied directly in this experiment. A study of these factors is indicated to provide data on the mechanism of the infertility, and particularly in relation to "low level" exogenous estrogens. Additional feeding of ewes grazing bluegrass could be used to equalize the weight and nitrogen intake for studies of these two factors and to reduce the weight difference between lots used for studying the effect of estrogens.

Summary

The reproductive performance of purebred Columbia ewes grazed on bluegrass, ladino clover, and birdsfoot trefoil pasture was studied during three grazing, breeding, and lambing seasons.

The ewes grazed all season on bluegrass conceived three weeks earlier, on the average, than those grazed all season on ladino clover or birdsfoot trefoil pasture. Sixty-six per cent of the ewes grazed on bluegrass lambed to first service, while only 41 per cent of the ewes grazed on ladino clover and 31 per cent of the ewes grazed on birdsfoot trefoil lambed to first service.

The ewes grazed on bluegrass tended to have an earlier mean date of first detected estrus than those grazed on birdsfoot trefoil and significantly earlier than those grazed on ladino clover.

The three year average lambing percentages were 134.4, 137.7 and 131.2 for the ewes grazed on bluegrass, ladino clover, and birdsfoot trefoil, respectively.

PART II

EXTRACTION AND BIO-ASSAY OF PLANT MATERIAL FOR ESTROGENIC ACTIVITY

The analysis of the bluegrass, ladino clover, and birdsfoot trefoil by chemical extraction and bio-assay for the presence of substances with estrogenic activity was the objective of this phase of the study.

Materials and Methods

Sampling Procedure

Fresh cut green bluegrass, ladino clover, and birdsfoot trefoil from the fields used for the grazing experiments reported in Part I were brought to the laboratory and preserved by drying in a forced draft oven at 65° C. A portion of the sample was used for a moisture determination. The dried samples were stored in the cold room at -5° C. until extracted.

Incidental to the planned experiments, meadow crop silage samples were obtained from the silage in use at the sheep center during the winter feeding period of 1955-56 and were handled in the same manner as the pasture samples.

Fermented samples of bluegrass and birdsfoot trefoil were obtained by covering 200 gm. of the dry plant material with water in an Erlenmeyer flask and incubating it for 96 hours after 1.5 gm. of glucose and 3 ml. saturated Na_2CO_3 were added. The fermentation

mixture was saturated with CO_2 by bubbling the gas through the mixture at the beginning and twice each day. At the end of the fermentation a liter of ethanol was added and the extraction carried out in the same manner as with dry samples.

Purification of Plant Extracts

In preliminary work a procedure comparable to that developed by Robinson (1949) was used for extracting estrogenic substances from the plant material. This procedure yielded extracts which were often toxic and upon injection caused ulceration at the site of injection and kidney damage. Also, the black tarry product obtained was difficult to handle making it necessary to modify the extraction procedure to obtain a satisfactory product.

The procedure ultimately adopted for the preparation of extracts is shown in Figure 3. The fractions obtained at each step are designated by Roman numerals. The procedure differs from that previously used by Engle (1953) primarily in the extent to which the extract to be assayed is purified. Ethyl alcohol (95 per cent) was used to extract the plant material and the extraction was repeated twice. The alcoholic extracts were bulked, concentrated by vacuum distillation, and the residue partitioned between ethyl ether and saturated aqueous NaHCO_3 . The aqueous phase was kept to the minimum amount necessary to wash the ether phase until a clear aqueous phase was obtained. After the ether phase was concentrated to a thick sirup acetone was added until precipitation ceased as suggested

* These fractions were bio-assayed and found to contain activity.

** These fractions were bio-assayed and found to contain little or no activity.

Figure 3. A fractionation procedure for the isolation of substances with estrogenic activity from plant material.

by Walter (1941) for the purification of genistein from soybean oil meal. The precipitate was thoroughly washed with acetone and the washings bulked with the precipitating solution. The acetone phase was reduced to dryness in the steam hood. Trial assays with this produce showed that the black tarry extracts were difficult to inject at the dilution apparently necessary to obtain a response thus making their bio-assay by injection unsatisfactory. Attempts at assaying the material by incorporating it in the diet of the mice were not highly satisfactory because of the lack of palatability of the extracts.

The product obtained from the removal of the acetone was partitioned between benzene and 60 per cent ethanol as suggested by Biggers and Curnow (1954) for the purification of subterranean clover extracts by dissolving the residue from the previous step in a minimum of benzene. It was then transferred to a separatory funnel and washed with 60 per cent ethanol until the 60 per cent ethanol layer was clear. The 60 per cent ethanol phase was bulked and back-extracted with benzene until the benzene layer became clear. Emulsions were encountered during this separation but the emulsions would break on standing. The 60 per cent ethanol phase was reduced to dryness in the forced draft oven at 65° C. and the benzene phase in the steam hood.

The residue from the 60 per cent ethanol phase was further purified by precipitation with lead acetate as outlined by Roberts et al. (1956) for the purification of flavonols of tea. The residue

from the 60 per cent ethanol phase was transferred to a 70 ml. test tube with a minimum of water, 50 ml. of saturated aqueous lead acetate were added, and the tube contents were mixed thoroughly. The precipitate was removed by centrifuging and decanting the supernatant liquid. This precipitate was washed once with 40 ml. of water and the washing discarded. A methanol soluble portion of the lead precipitate was separated by washing the precipitate with methanol until the methanol was clear after centrifuging. The remaining precipitate was suspended in methanol and the lead compounds in the suspension and in the methanol solution decomposed with hydrogen sulfide gas. The lead sulfide was removed by filtering and the solutions reduced to dryness in the forced draft oven at 50° C.

The products obtained at this stage were miscible with propylene glycol (Bigger and Curnow, 1954) but tended to separate from corn oil. Therefore, propylene glycol was adopted as the preferred suspending vehicle.

The extracts thus obtained were bio-assayed by uterine weight technique, administering the extract by the injection of 0.05 ml. twice daily for 3 days. Suckling rats were utilized for the preliminary test of the products.

Bio-assay Procedures

The increase in uterine weight of immature mice or rats was used as the assay for the determination of estrogenic activity. The

mice were allotted according to weight and the rats were assigned to a treatment randomly by litter. The materials assayed by injection were given by two subcutaneous injections daily of 0.05 ml. for 3 or 5 days in accordance with the trial. The materials assayed by feeding were incorporated in the diet which was offered the mice for 84, 104, or 128 hours in accordance with the trial. At the close of the trial, 24 hours after the final injection or 12 hours after the removal of the test diet, the animals were killed by bleeding and weighed to the nearest 0.1 gm. The uterus of each mouse was removed by dissection with the aid of a binocular dissecting microscope. The uterus was pressed between blotting paper and weighed immediately on a Roller-Smith balance to the nearest 0.1 mg.

Results

The results of the bio-assay of bluegrass, ladino clover, and birdsfoot trefoil extracts purified to the fraction VII (acetone soluble) stage are summarized in Table 9 and the response to known estrogens is summarized in Table 10. The complete results of these particular bio-assays are given in Tables 11, 12, 13, 14, and 15.

TABLE 9. A SUMMARY OF THE BIO-ASSAYS OF BLUEGRASS, LADINO CLOVER, AND BIRDSFOOT TREFOIL

Table No.	Bluegrass		Ladino clover		Birdsfoot trefoil	
	Dry matter equiv. gm.	Response to controls per cent	Dry matter equiv. gm.	Response to controls per cent	Dry matter equiv. gm.	Response to controls per cent
11			6.5*	120.0	7.8*	108.7
11					5.0*	355.5
12					7.0	130.7
12					7.5	141.6
12					5.1	150.2
12					6.3	119.9
13	15.6	51.2	17.2	224.5		
14	15.6	64.6	17.2	149.5		
15	15.2	92.6	15.0	113.6	14.4	125.0

* Administered by injections. All others were administered by feeding.

TABLE 10. A SUMMARY OF THE RESPONSE OBTAINED FROM THE BIO-ASSAY OF ESTRADIOL AND STILBESTROL

Estradiol			Stilbestrol		
Table No.	Dose administered micrograms	Response to control per cent	Table No.	Dose administered micrograms	Response to control per cent
11	.009*	119.2	12	.007	84.3
11	.021*	197.7	13	.021	162.2
13	.020*	150.1	12	.022	110.2
13	.040*	284.3	14	.022	124.3
13	.043	143.7	15	.033	120.8
			12	.040	192.1
			13	.042	116.6
			14	.046	171.6
			12	.058	173.7
			15	.061	160.8
			13	.064	130.1
			14	.074	136.4
			11	.082	396.4
			14	.098	137.1
			15	.141	400.2
			11	.164	615.8
			15	.192	584.0
			11	.264	778.4

* Administered by injection. All others were administered by feeding.

TABLE 11. DOSE RESPONSE RELATIONSHIP OF IMMATURE FEMALE MICE TO ESTRADIOL, STILBESTROL, AND PLANT EXTRACTS

Lot No.	Treatment by injection*	No. of mice	Ave. bled weight gm.	Mean fresh uterine weight mg. s.e.	Response to control per cent
1	Controls	10	9.5	7.9 ± 0.5	100.0
2	Estradiol 0.009 µg.	5	9.4	9.4 ± 0.6	119.2
3	Estradiol 0.021 µg.	5	8.5	15.5 ± 1.7	197.7
4	Genistein 3.0 mg.	5	9.0	7.6 ± 0.6	96.9
5	Ladino clover 6.5 gm. dry matter equiv.	5	8.5	9.4 ± 1.3	120.0
6	Birdsfoot trefoil 7.8 gm. dry matter equiv.	5	9.3	8.5 ± 0.6	108.7
7	Birdsfoot trefoil 5.0 gm. dry matter equiv.	5	9.3	27.9 ± 2.2	355.5
8	Commercial grass juice 15.0 gm. equiv.	5	9.5	13.5 ± 0.6	171.5
Treatment by feeding**					
9	Stilbestrol 0.082 µg.	5	9.3	31.2 ± 1.2	396.4
10	Stilbestrol 0.164 µg.	5	10.1	48.4 ± 2.4	615.8
11	Stilbestrol 0.246 µg.	5	10.1	61.2 ± 1.9	778.4

* Administered in 6 injections of 0.05 ml. each using corn oil as the suspending agent.

** Administered by incorporating the stilbestrol in the test diet. The test diet was offered the mice during an 84 hour period.

TABLE 12. DOSE RESPONSE RELATIONSHIP OF IMMATURE FEMALE MICE TO STILBESTROL AND PLANT EXTRACTS

Lot No.	Treatment by feeding*	No. of mice	Ave. bled weight gm.	Mean fresh uterine weight mg. s.e.	Response to control per cent
1	Control	10	9.9	10.5 $\pm$ 1.5	100.0
2	Stilbestrol 0.007 μ g.	10	10.0	8.9 $\pm$ 0.9	84.3
3	Stilbestrol 0.022 μ g.	10	10.0	11.6 $\pm$ 0.7	110.2
4	Stilbestrol 0.040 μ g.	10	9.1	20.2 $\pm$ 2.3	192.1
5	Stilbestrol 0.058 μ g.	10	9.6	18.3 $\pm$ 1.1	173.7
7	Birdsfoot trefoil 7.0 gm. dry matter equiv.	5	9.6	13.8 $\pm$ 2.0	130.7
6	Birdsfoot trefoil 7.5 gm. dry matter equiv.	5	10.0	14.9 $\pm$ 1.4	141.6
8	Birdsfoot trefoil 5.1 gm. dry matter equiv.	9	9.2	15.8 $\pm$ 1.7	150.2
9	Birdsfoot trefoil 6.3 gm. dry matter equiv.	10	9.6	12.6 $\pm$ 0.5	119.9

* Administered by incorporating the extract or stilbestrol in the diet. Test diet was offered the mice during an 84 hour period.

TABLE 13. DOSE RESPONSE RELATIONSHIP OF IMMATURE FEMALE MICE TO ESTRADIOL, STILBESTROL AND PLANT EXTRACTS

Lot No.	Treatment by injection*	No. of mice	Ave. bled weight gm.	Mean fresh uterine weight mg. s.e.	Response to control per cent
1	Control	6	9.9	10.3 ± 1.7	100.0
2	Estradiol 0.020 µg.	5	11.0	15.4 ± 1.8	150.1
3	Estradiol 0.040 µg.	5	10.7	29.2 ± 2.6	284.3
4	Ladino seed 6.0 gm. dry matter equiv.	5	10.6	13.5 ± 0.9	131.6
5	Birdsfoot trefoil seed 6.0 gm. dry matter equiv.	5	10.7	9.9 ± 1.1	96.4
Treatment by feeding**					
6	Estradiol 0.043 µg.	5	11.0	14.8 ± 1.6	143.7
7	Stilbestrol 0.021 µg.	5	11.1	16.7 ± 4.5	162.2
8	Stilbestrol 0.042 µg.	5	10.5	12.0 ± 1.3	116.6
9	Stilbestrol 0.064 µg.	5	10.6	13.4 ± 0.8	130.1
10	Ladino clover 17.2 gm. dry matter equiv.	5	10.1	23.1 ± 3.7	224.5
11	Ladino clover seed 17.6 gm. dry matter equiv.	5	10.7	12.2 ± 2.5	118.4
12	Birdsfoot trefoil seed 17.2 gm. dry matter equiv.	5	10.3	12.5 ± 1.8	121.5
13	Bluegrass 15.6 gm. dry matter equiv.	5	8.8	5.3 ± 0.4	51.2
14	Meadow crop silage 15.2 gm. dry matter equiv.	5	9.4	24.7 ± 2.2	240.5

* Administered in 10 injections of 0.05 ml. each using corn oil as the suspending agent.

** Administered by incorporating the stilbestrol or extract in the diet. The test diets were offered the mice during a 128 hour period.

TABLE 14. DOSE RESPONSE RELATIONSHIP OF IMMATURE FEMALE MICE TO STILBESTROL AND PLANT EXTRACTS

Lot No.	Treatment by feeding*	No. of mice	Ave. bled weight gm.	Mean fresh uterine weight mg. s.e.	Response to control per cent
1	Dissected at outset of trial	5	8.7	6.6 ± 0.2	62.9
2	Control	7	12.1	10.5 ± 1.1	100.0
3	Stilbestrol 0.022 µg.	4	12.0	13.0 ± 1.1	124.3
4	Stilbestrol 0.046 µg.	4	13.1	18.0 ± 5.3	171.6
5	Stilbestrol 0.074 µg.	5	13.0	14.3 ± 1.7	136.4
6	Stilbestrol 0.098 µg.	4	12.4	14.4 ± 0.2	137.1
7	Genistein 14.5 mg.	4	12.4	32.3 ± 2.7	307.6
8	Ladino clover 17.2 gm. dry matter equiv.	5	11.3	15.7 ± 2.5	149.5
9	Bluegrass 15.6 gm. dry matter equiv.	5	10.4	6.8 ± 0.9	64.6
10	Bluegrass 15.6 gm. dry matter equiv. plus Stilbestrol 0.039 µg.	5	11.1	9.3 ± 0.7	88.8
11	Wheat germ oil 1.65 gm.	4	13.0	19.0 ± 2.0	180.5
12	Meadow crop silage 16.8 gm. dry matter equiv.	5	10.8	31.5 ± 3.6	300.0

* Administered by incorporating the stilbestrol or extract in the diet. The test diets were offered the mice during a 128 hour period.

TABLE 15. DOSE RESPONSE RELATIONSHIP OF IMMATURE FEMALE MICE TO STILBESTROL AND PLANT EXTRACTS

Lot No.	Treatment by feeding*	No. of mice	Ave. bled weight gm.	Mean fresh uterine weight mg. s.e.	Response to control per cent
1	Dissected at outset of trial	8	6.4	6.8 $\pm$ 0.3	71.1
2	Control	9	10.2	9.5 $\pm$ 1.1	100.0
3	Stilbestrol 0.033 μ g.	5	11.8	11.5 $\pm$ 0.4	120.8
4	Stilbestrol 0.061 μ g.	5	10.8	15.3 $\pm$ 1.4	160.8
5	Stilbestrol 0.141 μ g.	4	11.8	38.0 $\pm$ 8.3	400.2
6	Stilbestrol 0.192 μ g.	5	10.8	55.5 $\pm$ 4.8	584.0
7	Bluegrass 15.2 gm. dry matter equiv.	10	10.4	8.8 $\pm$ 0.5	92.6
8	Ladino clover 15.0 gm. dry matter equiv.	10	10.5	10.8 $\pm$ 1.1	113.6
9	Birdsfoot trefoil 14.4 gm. dry matter equiv.	10	10.2	11.9 $\pm$ 1.5	125.0
10	Fermented birdsfoot trefoil 7.2 gm. dry matter equiv.	5	7.8	17.0 $\pm$ 1.6	179.2
11	Fermented bluegrass 14.4 gm. dry matter equiv.	5	10.5	11.3 $\pm$ 1.5	119.2

* Administered by incorporating the stilbestrol or extract in the diet. The test diets were offered the mice during a 104 hour period.

Extracts of ladino clover and birdsfoot trefoil produced a positive response upon bio-assay. The response as a per cent of the controls ranged from 113.6 per cent to 224.5 per cent, and 108.7 per cent to 355.5 per cent for ladino clover and birdsfoot trefoil, respectively. However, in three bio-assays of bluegrass the uterine weights were less than the controls, being only 51.2 per cent, 64.6 per cent, and 92.6 per cent of the controls. While these bio-assays are not directly comparable because variation was noted between extracts and bio-assay methods, estrogenic activity is indicated for the ladino clover and birdsfoot trefoil.

The feeding of stilbestrol produced variable results, particularly below 0.1 microgram total dose. A greater response was obtained from injection than from feeding of estradiol, and the administration of the estradiol or stilbestrol in three days produced a greater response than when an equal total dose was given over a four or five day period.

Meadow crop silage extracts consistently produced a significant response upon bio-assay as shown in Tables 13 and 14. An extract of the fermented birdsfoot trefoil produced a greater response than a similar extract from the unfermented birdsfoot trefoil (Table 15). The extract of fermented bluegrass produced a positive response, whereas the extracts of unfermented bluegrass produced a negative response (Table 15).

Other products bio-assayed were commercial grass juice, a product of Cerophyl Laboratories, Inc. (Table 11), wheat germ oil,

a product of Vio Bin Corporation (Table 14), and genistein, provided by Dr. Wise Burroughs and Dr. E. Cheng of the Iowa Agricultural Experiment Station, at 3.0 mg. and 14.5 mg. total dose (Tables 11 and 14). All produced a positive response except the low level of genistein. Progesterone alone did not increase the uterine weight, but when added to low levels of stilbestrol and response was greater than from stilbestrol alone (Table 16).

The purification of extracts beyond the fraction VII (acetone soluble) stage greatly reduced the volume of extract of the estrogenically active fraction. The assay reported in Table 16 indicates the estrogenic substance can be extracted from benzene by 60 per cent ethanol with only a small portion remaining in the benzene.

The final steps in purification, lead precipitation, reduced the estrogenically active fraction of the extract to approximately 0.2 per cent of the dry ladino clover and 0.5 per cent of the ladino clover seed. This permitted the administration of a larger equivalent dose by injection than had been possible at previous steps in the purification.

The results of the preliminary bio-assay of these products using suckling rats are shown in Table 17. A significant response was obtained from fraction XVII (methanol soluble) of both ladino clover seed extract and ladino clover plant extract. No response was obtained from fraction XV (methanol soluble after decomposition of the lead precipitate) indicating the partition of the estrogenic substances into fraction XVII (methanol soluble).

TABLE 16. DOSE RESPONSE RELATIONSHIP OF IMMATURE FEMALE MICE TO STILBESTROL, PROGESTERONE, AND PLANT EXTRACTS

Lot No.	Treatment by feeding*	No. of mice	Ave. bled weight gm.	Mean fresh uterine weight mg. s.e.	Response to control per cent
1	Dissected at outset of trial	8	6.4	6.8 ± 0.3	71.1
2	Control	9	10.2	9.5 ± 1.1	100.0
3	Stilbestrol 0.067 µg. plus progesterone 6.7 µg.	5	11.1	21.0 ± 3.3	221.0
4	Stilbestrol 0.059 µg. plus injected progesterone 6.24 µg.	5	10.3	20.3 ± 1.6	213.2
5	Progesterone 6.24 µg.	5	10.1	10.4 ± 1.8	109.9
6	Bluegrass 10.0 gm. dry matter equiv. fraction IX	5	9.3	7.3 ± 1.1	77.3
7	Bluegrass 12.0 gm. dry matter equiv. fraction VIII	5	9.6	7.9 ± 1.1	83.6
8	Meadow crop silage 16.8 gm. dry matter equiv. fraction IX	5	10.9	20.6 ± 1.1	217.3
9	Meadow crop silage 12.8 gm. dry matter equiv. fraction VIII	5	10.3	14.0 ± 1.1	146.9

* Administered by incorporating in the diet. The test diets were offered the mice during a 104 hour period.

TABLE 17. DOSE RESPONSE RELATIONSHIP OF SUCKLING FEMALE RATS TO ESTRADIOL AND PLANT EXTRACTS*

Lot No.	Treatment by injection**	No. of rats	Ave. bled weight gm.	Mean fresh uterine weight mg. s.e.	Response to control per cent
1	Control	4	35.2	17.0 $\pm$ 0.9	100.0
2	Propylene glycol 0.3 ml.	4	35.9	18.8 $\pm$ 1.6	110.6
3	Estradiol 0.12 μ g.	4	37.1	23.0 $\pm$ 2.7	135.6
4	Ladino seed 20.5 gm. dry matter equiv. fraction IVI4	4	35.9	51.8 $\pm$ 1.5	305.0
5	Ladino seed 20.5 gm. dry matter equiv. fraction IV	4	36.2	16.0 $\pm$ 0.8	93.8
6	Ladino clover 23.2 gm. dry matter equiv. fraction IVI4	4	36.0	41.6 $\pm$ 2.7	244.7

* Administered in 6 injections of 0.05 ml. each using propylene glycol as the suspending agent.

** Four litters, one rat per litter on each treatment. Rats were 12 days of age at the start of the test.

Discussion

Ladino clover and birdsfoot trefoil contain substances which produce an estrogenic response equivalent to from 4 to 10 parts per billion of estradiol or stilbestrol. A critical evaluation of the estrogenic activity in terms of estradiol or stilbestrol equivalence cannot be made. One of the main reasons was that the response to the plant extracts was near the response obtained from a minimal effective dose of the standard reference estrogens where the reliability of the assay was low. However, of equal interest is the finding that bluegrass extracts inhibited normal uterine development. Whether this effect is due to a specific substance in the bluegrass extracts or the general effect of the extracts has not been determined.

Other investigators have suggested estrogenic activity for ladino clover and birdsfoot trefoil, but only a part of the samples assayed contained enough activity to obtain an estimation of potency. Thus, they were considered non-estrogenic, probably because the level of activity was below the sensitivity of the bio-assay. Subterranean clover, red clover, alfalfa, spring growth of rye, and alfalfa silage are among the plant materials which have been shown to contain several times more estrogenic activity than ladino clover or birdsfoot trefoil; therefore, they could be bio-assayed with the administration of a smaller equivalent dose.

The steps taken to increase the administrable amount of the

"low potency" extracts obtained at fraction VII (acetone soluble) stage of purification were not too successful. The bulk of the extracts limited concentration and the quantity that could be injected; and palatability limited the quantity that would be consumed when the extracts were mixed in the diet. A longer feeding period, 104 or 128 hours, increased the dose administered, but the decreased sensitivity and increased variation of the bio-assay made the longer trials impractical.

Since the 3 day injection technique was the preferred method of administering the extracts, it was necessary to extend the extraction and purification procedure to obtain a product suitable for injection at the desired dose.

The active product recovered from the lead precipitate has not been studied critically but appears to be suitable for routine bio-assay by the injection technique. It may be possible to introduce the lead precipitation in an earlier step of the extraction procedure. The black tarry material, which makes up a large portion of the extract obtained at fraction IV (ether soluble) or fraction VII (acetone soluble), is not precipitated by the lead acetate; but the removal of the black tarry material is difficult. The black tarry material can be effectively separated by careful use of benzene (emulsions develop easily) and mechanical transfer. The earlier use of lead in the procedure would avoid the benzene and 60 per cent ethanol separation which is the most troublesome step of the procedure because of emulsification and poor separation of the active

substances. Further purification by chromatographic separation of the products recovered from the lead precipitate seems possible. Attempts to chromatograph extracts either on paper or on celite 545 columns before the final purification step were not successful due to the large amount of contaminating pigment.

In these studies meadow crop silage was one of the more active plant materials bio-assayed, and a fermented sample of birds-foot trefoil produced a greater response than the birdsfoot trefoil without fermentation. Fermented bluegrass gave a slightly positive response while in all other bio-assays of bluegrass extracts the uterine weights were less than the controls. The fermented samples yielded extracts with less dark pigment than the unfermented samples. A more efficient extraction and bio-assay may have been obtained from the fermented samples or an inhibiting substance may have been removed by the fermentation. Bacterial synthesis, modification of existing compounds to more active analogs, and degradation of complex compounds are possible explanations of the increase in estrogenic activity from fermentation and ensiling.

Animal modification of the plant substances may be of great importance in the ultimate effect the substances may have on the animal. The possible modification of the estrogenic substances in plants to a more active form by rumen action is suggested by the increase in activity resulting from fermentation and ensiling. The results of feeding the estrogenic substances of plants to ruminants may not be the same as feeding to the monogastric bio-assay rice

where a smaller response was obtained by feeding than by injection.

Another important factor in the interpretation of the effect of estrogenic substances from plants was pointed out by Biggers and Curnow (1954) in relation to the estrogenic activity of subterranean clover. Genistein, the estrogenically active substance of subterranean clover, was shown to be a "pro estrogen" and the estrogenically active compound in the animal was considered a metabolite of the genistein.

Additional qualitative and quantitative studies of the estrogenic substances in plants and the modification of these substances by the animal are needed.

Summary

A study was made of methods for extraction of estrogenically active substances from plant material and the bio-assay of such extracts, using the increase in uterine weight of immature female mice and rats as the assay criterion.

The extraction procedures reported in the literature were modified in order to obtain extracts of sufficient potency for bio-assay which would not be toxic to the animals.

Plant material was extracted with 95 per cent ethanol, then followed by the extraction of the estrogenic material with ether from a NaHCO_3 solution of the residue of the plant extract. The residue of the ether phase was taken up in acetone and the precipitate discarded. The acetone was removed by evaporation and the residue partitioned between 60 per cent ethanol and benzene. The residue from the 60 per cent ethanol was further purified by precipitation with lead acetate, recovering the methanol soluble portion of the precipitate from which the lead was removed by filtering after decomposition with hydrogen sulfide. The residue after evaporation of the methanol was made to the desired concentration for injection with propylene glycol.

Attempts to bio-assay ladino clover, birdsfoot trefoil, and bluegrass extracts at the acetone stage of purification were only partially successful because of the small amount of active material in relation to the large amount of inactive material which prevented

increasing the total dose administered either by injection or feeding.

A greater dose of estrogenic material was required to obtain a response by feeding than by injection. Lengthening the period of administration of the extracts increased the variation and decreased the sensitivity of the bio-assays.

Positive indications of estrogenic activity on bio-assay were obtained with the following extracts: ladino clover plant and seed, birdsfoot trefoil plant and seed, and meadow crop silage. Extracts of bluegrass appeared to inhibit normal uterine development of the test mice. However, an extract of a sample of fermented bluegrass did not show the inhibition and an extract of fermented birdsfoot trefoil gave a greater response than the unfermented birdsfoot trefoil.

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I, Paul Howland Engle, was born in Fayette County, Ohio, February 15, 1924. I received my secondary school education in the public schools of Washington Court House, Ohio. After three years' service with the United States Navy I entered The Ohio State University in 1946, where I was granted the Bachelor of Science degree in 1950 and the Master of Science degree in 1953. While preparing for the latter degree I was a research assistant at the Ohio Agricultural Experiment Station.

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