THE ESTROGEN-LIKE SUBSTANCES IN VARIOUS LEGUMES AND GRASSES, AND THE EFFECT OF THESE COMPOUNDS ON THE REPRODUCTION AND GROWTH OF CERTAIN LABORATORY ANIMALS

by

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An extensive study was undertaken on the effects of stage of maturity and frequency of cutting of alfalfa, white clover, red clover, birdsfoot trefoil and orchard grass, on the levels of estrogen-like substances in these plant species. The data of these experiments revealed that there was a great difference in estrogenic activity between samples taken from different plant species at the same time of the year. There also was a seasonal variation between first cuttings of second year growth of the different plant species.

Alfalfa and white clover were high in estrogenic activity in the spring, showed a sharp decrease in June and July, and after August 1 possessed once again considerable potency. Birdsfoot trefoil and orchard grass showed estrogenic activity only in the spring, and this was relatively small as compared to the activity of alfalfa, white clover and red clover.

Red clover differed very much from the previously mentioned species. All samples studied showed considerable potency. There was no sharp decrease during June and July as was the case with alfalfa and white clover. Data obtained by varying the number of hours of daylight (photoperiod) received by red clover seemed to indicate that estrogenic potency was decreased when the hours of daylight were reduced. Proximate analysis of the plant material revealed that estrogenic potency was not correlated with nitrogen content.

Studies on the stability of the estrogen-like compound in alfalfa, white clover and red clover showed that the activity of dried ground alfalfa and white clover plant material was easily destroyed during storage, while the potency of dried ground red clover remained fairly constant over a six month period. However, the estrogen-like compounds of these three species are much more stable after they had been extracted and the extract mixed with feed.

Red clover and birdsfoot trefoil extract interfered with the reproduction in the mouse. Females were affected more severely than males, but both recovered their reproductive ability quickly after feeding of the estrogen-like compounds ceased.

The growth rate of the female guinea pig weighing between 500-600 gm. was not significantly influenced when red clover extract was added to their diet.

II. ABSTRACT

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III. INTRODUCTION

The term estrogen has been defined as any pure chemical substance which has the ability when injected into an adult ovariectomized mouse or rat, to produce cornification of the vagina similar to that occurring during normal estrus. This definition has further been extended to apply in a collective sense to mean an estrogenic substance of unspecified chemical identity.

In 1940 Emmens (56, 57) classified the estrogens into two main groups.

1 - the true estrogens, as those compounds capable of inducing the characteristic response relative to the Allen - Doisy test (3, 4) and,

2 - the pro-estrogens, as those compounds when applied to the vagina do not give the characteristic response until they have been absorbed into the circulatory system and returned to the vagina.

The presence of estrogen-like compounds in plant material capable of inducing estrus in animals was first reported in 1926 by Loewe (85). Since that time approximately 50 plant species have been shown to possess these active compounds. The amounts and activity of the substances in the different species vary considerably.

In 1946 Bennetts et al.(18) observed that subterranean clover (Trifolium subterranean) of Western Australia contained sufficient amounts of estrogen-like substances to affect adversely the reproductive performance of grazing ewes and to cause genital and mammary abnormalities in wethers. Many other experiments have been

reported on the effect of natural and synthetic substances having estrogenic activity in livestock production. These findings have led the author to investigate the levels of estrogenic compounds in alfalfa (Medicago sativa), alsike clover (Trifolium hybridum), red clover (Trifolium pratense), white clover (Trifolium repens), birdsfoot trefoil (Lotus corniculatus), and orchard grass (Dactylis glomerata), at different stages of maturity, and to study the extent to which these levels are influenced by frequency of cutting and length of daylight.

A number of animal experiments were carried out to determine the influence of the estrogen-like substances of red clover and birdsfoot trefoil on the growth and reproduction of the mouse and the guinea pig. It seemed advisable to use laboratory animals since more fundamental knowledge was necessary before an experiment with domestic animals could be designed in order to obtain valuable results.

IV. HISTORICAL

The early work of Loewe (85) in 1926, where he reported that ether extract of willow catkins and ovaries of the water lily (Nuphare luteum) induced estrus in ovariectomized mice, has led to extensive investigations of estrogenic substances in plant and animal materials. Positive results were obtained with a group of plant materials including beet seeds, potatoes (49), cooked potatoes (72), male and female alder catkins, alder leaves, sprouted oats, rhubarb leaves (109) and soybean meal (111). In 1941 Bennetts, (17) reported the sudden appearance of a serious breeding disorder in sheep in large regions of Western Australia. The flocks were grazing on pastures which were dominated by a locally developed strain of subterranean clover (Trifolium subterranean L. var. dwalganup).

Since the economy of the country depended to a large extent on the sheep industry, intensive investigations were carried out with sheep grazing on these "affected" clover pastures. In 1946 strong evidence was presented that the breeding disorder was due to an excessive stimulation of the reproductive organs of the animals by estrogens derived from the pasture (19). This led to subsequent research programs in connection with these active compounds in different species of plants.

A. Physiological Functions of Estrogens in the Animal Body:

Since the plant estrogens appear to have similar physiological properties as the natural estrogens in the animal body, it was felt that a short discussion of their functions would be appropriate.

A more extensive review on this subject has been published by Burrows (32) while the part played by estrogens in lactation is reviewed by Folley (70).

In vertebrates, estrogens take part in the control of the estrus cycle as the name suggests. Furthermore, the growth and functions of the female accessory reproductive organs, the secondary sex characteristics and the female mating behavior are controlled by these hormones. It is interesting to note that in the female mouse all tissues and organs with the exception of the brain and striped muscle respond to estrogens by cell division. However, the intensity of the reaction depends on the organ concerned (31).

1. Control of Estrus Cycle.

In mammals the anterior pituitary secretes three hormones that directly influence reproduction. They are the follicle - stimulating hormone, luteinizing hormone and prolactin or luteotrophin. The follicle stimulating hormone stimulates the growth of the ovisacs to the point of rupture, while the actual rupturing of the ovisacs is aided by the luteinizing hormone. After rupturing,the luteinizing hormone influences the formation of the corpus luteum or yellow body. The third hormone - prolactin - causes the corpus luteum to secrete progesterone which is essential for implantation of the fertilized egg within the placental tissues.

The theca interna of the ovisacs secrete three estrogens, estradiol, estrone and estriol. The most active of these three hormones is estradiol and the least active, estriol (89, 6).

2 Control of Mammary Development and Lactation:

Mammary development is mainly if not completely, controlled by the endocrine system. At least three hormones are concerned in the growth and functioning of the mammary gland (86). Each one has its own function and they act in a special sequence. Estradiol, secreted by the Graafian follicle acts initially and causes duct development in the mammary gland. This is followed by growth of the alveoli under influence of progesterone from the corpus luteum. The third hormone, prolactin from the pituitary , initiates milk secretion. The relative amount of these three hormones may differ between species and perhaps also in their mode of action (86).

Artificial mammary development and copious lactation has been induced in intact and ovariectomized virgin goats by use of estrogens and mixtures of estrogens and progesterone. Microscopic examination of the mammary glands of these goats revealed that less abnormalities were present in animals receiving both estrogens and progesterone than in those receiving estrogens alone. Administration of synthetic estrogens to milking cows has produced controversial results. Folley (66) and Folley et al. (68) studied the influence of synthetic estrone, dihydro-follicular hormone and diethylstilbestrol (D.E.S.) in lactating cows. In some cases there was an increase in milk solids, which was not due to a secretion of less water by the mammary gland. The nitrogen partition in these cows with increased milk production was normal indicating that the experimental treatment had not caused the secretion of colostrum. This galactopoietic effect was confirmed by Spielman et al. (101) who showed that estrogens increased the lactose content of the milk. In other cases estrogens caused a considerable

inhibition of lactation in the sense of a decreased milk yield(66).

Hawkins et al. (78) fed levels of D.E.S. to lactating cows within the range of the levels of estrogen-like compounds found in forages (0.068-3.40 mcgm. per lb. of feed) and observed no significant effects on the production of fat corrected milk, the gross efficiency of milk production, changes in body weight and efficiency of feed utilization. Folley et al. (68) also noticed that synthetic estrogen administration resulted often in difficulties in conception, while in advanced pregnancy large doses of D. E. S. resulted in abortion.

3. Estrogens in the Milk

In normal cows and goats small amounts of estrogens occur in the milk during the last month of pregnancy (87), immediately after parturition (67, 108), and during estrus (87).

Human milk taken during the first 9 days of lactation was shown to induce estrus in ovariectomized mice (30), suggesting that estrogens were present. This was also recognized by Myers (88) who noticed that small amounts of milk were present in the mammary glands of a young rat 12 hours after birth suggesting that minimal amounts of hormones necessary for mammary secretion passed to the suckling young from the mother.

When D.E.S. became available for experimental use it was found that most of the administered hormone was excreted in the urine and that only very small amounts occurred in the milk (74, 81, 104).

4. Some Pathological Effects on the Female Reproductive System

Administration of estrogens to animals leads to changes in

the reproductive system. The severity of these changes depends on sex, age, species, kind of hormone used, the method and duration of administration. Emmens (59) injected male and female mice with estradiol benzoate and rendered the males sterile within six weeks in 90 per cent of the cases by injecting 10 mcgm. of the compound twice weekly. The females were more susceptible and became sterile when injected with 0.5 mcgm. twice weekly. Both groups recovered normal or nearly normal reproductive power when injections of the hormone ceased. Infertility in mice could also be produced by administering thiouracil but again the animals recovered quickly after the treatment was stopped (9).

Histological examination revealed that estrogen administration resulted first in proliferation of the epithelium followed by extension of the uterine glands which penetrated into the submucosa and became distended with fluid (32). In immature mice estrogen administration also resulted in an earlier opening of the vaginal tract.

Different species react differently to estrogens. Injections of two to five mcgm. of estrone per day for 8 to 15 days in spayed guinea pigs and rats resulted in the development of cystic glandular hyperplasia in the guinea pig uterus, while the rats were affected to a much lesser extent (113). Simultaneous administration of estrogens and androgens causes synergystic and antagonistic reactions depending on the proportions in which the hormone was supplied (32).

5. Effects on the Male Reproductive System.

It would be expected that males are more resistant to exogenous estrogens, since the androgen complement of the male exerts some

protective action. Results confirming this hypothesis were obtained by Emmens (59) with estradiol benzoate injections in mice, by East et al. (52) with entire and castrated guinea pigs receiving subterranean clover and by Bennetts (18) with intact rams grazing on subterranean clover. However, East (54) showed that the "plant estrogen", genistein, affected the male mice to a greater extent than the female.

Certain parts of the reproductive system are more susceptible to estrogens than others. In the guinea pig, for example, the seminal vesicles are more susceptible than the other accessory sex organs (52). Prolonged estrogen treatment of the male mouse results in metaplastic changes in uterus masculinus, seminal vesicles, vas deferens, prostate, bulbo-urethral glands and urethra.

6. Effects on the Skeleton.

In mammals estrogens aid in the development of the female pelvis and relaxation of the ligaments during pregnancy. Implantation of synthetic estrogens into heifers or cows resulted in loosening of the sacro-sciatic and sacro-iliac ligaments (42, 69). Furthermore, estrogen administration causes a fall in serum calcium followed by transient increase in serum phosphatase, indicating that the hormones are capable of exercising general effects on the skeletal system (66).

B. Estrogens in Forage Plants:

Recently Bradbury and White (29) reviewed the complete subject of estrogens and related substances in plants. Since this and other reviews are available it is not proposed to give here a complete review of the literature, but to point out some of the most interesting facts relating to certain forages that have been found to possess

estrogenic activity.

1. Subterranean Clover (Trifolium subterranean).

In 1941 Bennetts (17) reported the sudden appearance of a serious breeding disorder in sheep in large areas of Western Australia. The affected flocks were grazing on pastures dominated by the Dwalganup strain of subterranean clover. Three main clinical symptoms of sheep grazing on this clover were observed; dystocia, uterine prolapse, and female infertility.

The infertility had no characteristic clinical features; there was no failure of estrus. In many cases infertile sheep were served by the male, but did not conceive (10b). When the animals were sacrificed post coitus unsegmented ova were recovered in the fallopian tubes or uterus, indicating that infertility was a result of failure of fertilization. Frequently, ewes grazing on subterranean clover showed cystic glandular hyperplasia of the endometrium, and in many cases a number of cysts distributed throughout the fundus and horns of the uterus could be located by macroscopical examination (107). These cysts contained a colorless,serous fluid and varied in size from 0.1 to 1.4 cm. in diameter. Similar changes in the endometrium of guinea pigs were produced when these animals were fed fresh, air dried or artificially dried subterranean clover (18).

When ovariectomized ewes were grazed on fields of subterranean clover atrophy of the uterus did not occur (22). The same happened when ovariectomized ewes were treated with adequate estrogens, the uterus was restored almost to its normal state; thus the estrogen-like compounds in subterranean clover function as natural estrogens in preventing atrophy of the uterus of ovariectomized ewes (35). However,

when the clover reached the dry stage, atrophy of the uteri occurred. This meant that the forage had no longer any estrogenic activity (22).

A high incidence of milk secretion in virgin ewes particularly during the spring and early summer occurred. Microscopy showed normal alveolar development of the udder, and the gland yielded a secretion similar to colostrum (18). Bennetts et al. (18) noted marked mammary development in castrated rams when grazed on certain fields of subterranean clover. In some cases the nipples developed to the same size as those of ewes in full lactation. A fluid similar to sheep's milk was obtained from these extended teats.

During the same period Bennetts (19, 20) demonstrated the presence of squamous metaplasia in the sex glands of wethers grazing on subterranean clover. In most cases there was an enlarged bulbo-urethral gland and in some cases metaplasia was also observed in the prostate gland and uterus masculinus, but in these last two cases to a relatively small extent. In a few wethers a sac had developed on the dorsal wall of the pelvic urethra and was filled with urine. External rupture of the sac occurred sometimes and a permanent fistula was formed. Rams grazing on subterranean clover were unaffected and remained fertile (18, 52).

Strong presumptive evidence was given by Bennetts et al. (18) that there was an estrogen or a pro-estrogen present in subterranean clover. Curnow et al. (45) confirmed these findings and showed that artificially dried clover and the ether extracts of these samples when given per os produced changes in ovariectomized mice similar to the changes that occurred when the natural estrogen, estradiol, was fed. These physiological changes were:

a) early vaginal opening in immature mice.

b) vaginal cornification in mice.

c) uterine hypertrophy in mice and guinea pigs.

d) cystic endometrium in guinea pigs when administered over longer periods.

Further studies showed that estrogens were not only present in the Dwalganup strain of subterranean clover (97, 13) but also in the Mount Baker, the Burnerany (46), the Red Leaf (46), the Tallarook (46), and at least fifteen other strains (21). Bradbury and White (28) showed in 1951 that the estrogenic activity of subterranean clover was mainly due to genistein (5: 7: 4' trihydroxyisoflavone) and for lesser part to formononetin (7-hydroxy-4' methoxyisoflavone). The estrogenic activity of genistein has been confirmed by Carter et al. (34).

2. Red Clover (Trifolium pratense L.)

Red clover has been shown, by bio-assay procedures, to possess estrogen-like substances (10, 82, 48, 93, 94, 92). However, the levels of these substances reported in the literature vary considerably.

To date, three compounds showing estrogenic activity have been isolated from red clover. In 1953, Pope et al. (93) isolated the isoflavone, biochanin A, and in 1954 the isoflavone, genistein (94). The third isoflavone, formononetin, having very little estrogenic activity was isolated in 1953 by Bate-Smith et al. (12).

3. Alfalfa (Medicago sativa).

There are conflicting reports in the literature in connection

with the estrogen-like substances in alfalfa. Dohan et al. (48) and Legg et al. (82) reported negative results, while other (91, 92, 24) demonstrated definite estrogenic activity. In 1957 Bickoff et al. (24) isolated a new estrogen-like substance, coumestrol, from alfalfa and showed that it was ten times more active than genistein.

4. White Clover (Trifolium repens).

Many reports (97, 82, 38) state that white clover does not contain any estrogen-like substances, while others (91) state the opposite. Curnow (46) studied white clover for the presence of genistein with silica gel chromatography and spectophotometry and was unable to detect the presence of this active compound.

5. Birdsfoot Trefoil (Lotus corniculatus).

Very little literature is available in connection with estrogen-like substances in this species. However, a few reports (92, 61) state that estrogenic activity has been demonstrated in certain samples by the mouse uterine weight technique.

6. Orchard Grass (Dactylis glomerata).

Estrogenic activity of orchard grass has been demonstrated in very few samples (82). In most cases (82, 48) no estrogen-like compounds could be detected.

7. Other Forage Plants.

Other forage plants which have been reported to contain estrogen-like compounds include - strawberry clover (Trifolium frageferum) (97, 24), ladino clover (Trifolium repens, Ladino) (92, 61, 24), rye grass (Lolium perenne L.) (82, 48), oats (Aoena sativa L.) (77, 92), and wheat (Triticum aestioum) (92).

No estrogen-like substances have been detected in brome grass (Bromus inermis) (92), fescue (Festuca elatior) (92), sweet clover (Melilotus alba) (92), timothy grass (Phleum pratense) (82), veldt grass (Erharta calycina) (82), and blue grass (Poa pratense)(48, 37, 61).

8. Hay.

Hay samples of the following forages have been shown to possess estrogenic activity: alfalfa (38,92), alsike clover (38), red clover (38), and white clover (38).

Pieterse and Andrew (92) noticed that alfalfa hay had a relatively high estrogenic activity. In one case a hay sample was found to be more active than any of the alfalfa pasture samples. They suggest that there may be a compound present other than those in subterranean clover which increases in activity during the curing period.

9. Silage.

Pieterse et al. (91) state that the estrogenic activity of alfalfa silage was significantly greater than that of fresh alfalfa. Corn or brome silage did not contain any estrogenic activity. When molasses or sodium metabisulfite was added to a mixture of alfalfa, ladino clover and brome grass the estrogenic activity increased during fermentation.

C. Different Methods of Determining Estrogens:

Quantitative and qualitative determination of estrogens in plant and animal material can be accomplished by chemical and physical methods and bio-assay procedures.

1. Chemical and Physical Methods.

Chemical procedures are very tedious, especially when plant materials are involved (79). It is essential to have an accurate analytical method in order to follow the presence of the active compounds during separation of the mixtures. In many cases better results are obtained with chromatography combined with absorption measurements and spectophotometry. Chromatography enables the investigator to separate a mixture of compounds quickly so that identification of the pure substances can proceed.

When the chemical and physical properties of estrogens are unknown, a quantitative estimate of the estrogenic activity can be obtained by the use of bio-assay techniques. Since this is the case with plant estrogens, bio-assay procedures have been used extensively.

2. Bio-assay Methods.

The first bio-assay method for estrogens was developed in 1923 by Allen and Doisy (3, 4) and has been used quite extensively. In this method the estrogens are injected subcutaneously into spayed rats or mice. If the sample being assayed has estrogenic activity typical estral hyperemia, growth and hypersection in the genital tract and growth of the mammary glands occur. These changes include thickening and cornification in the vaginal wall which can be followed very easily in the living animals. Additional proof that the injected hormones are estrogenic can be obtained by mating the ovariectomized females. Copulation is normal, and this is followed by the formation of a vaginal plug, which clearly demonstrates that the animals were in estrus as a result of the injected material.

Emmens (55, 56, 58, 60) presented several modifications of the Allen - Doisy technique. The substances in this case are not injected subcutaneously but administered per vaginum into ovariectomized female mice.

There is a fundamental difference between the intravaginal method and the Allen - Doisy method. In the first method the hormone action is essentially local in character and has little opportunity to elicit systemic responses, while in the Allen - Doisy test the hormone is absorbed in the blood stream and carried to the genital tract. Systemic responses then occur. Furthermore, Biggers (25) showed that the vehicle used in intra-vaginal application also influences the responses. Estrone dissolved in one per cent aqueous albumin had 2.5 to 4.9 times the activity as shown in 50 per cent aqueous glycerol when administered by the intra-vaginal route. One per cent egg albumin alone does not produce cornification of the vagina. These differences may account for the different results obtained by the two methods for the same hormone.

In 1939 Fierz and coworkers (65) developed the "LOKALEN NIPPLE TEST," (L.N.T.), involving cutaneous application of the test solution to the nipple of intact male guinea pigs. Extreme small quantities of estrogens can be tested by this method since the nipple is very sensitive to these hormones. East (51) confirmed the fact that estrogens influence teat length in guinea pigs by using castrated animals.

The "sexual skin" and nipples of female monkeys provide other areas which are sensitive to estrogenic substances (36). In the first

case there is a reddening and swelling of the skin and in the second an increase in nipple size.

Evans et al. (64) developed the uterine weight method by using immature female mice and injecting the estrogens subcutaneously. The amount of increase of the uterus was used as a measure of potency of the estrogen tested. The activity is generally expressed in terms of estrone, estradiol or D.E.S. This increase in uterine weight has been shown to be an increase in both total water and total solids (7, 33).

Alexander et al. (1, 2) determined the estrogenic activity of clover samples by oral administration to spayed guinea pigs. The resulting increase in uterine weight was used as a measure of estrogenic activity of the clover sample. The above investigators also demonstrated that the weight of the uterus of spayed guinea pigs is unaffected by injections of as much as 20 mgm. of testosterone or progesterone (1). This would indicate that these compounds do not affect the results obtained using the guinea pig as the bio-assay animal. However, it was shown (64) that testosterone and progesterone cause hypertrophy of the uterus of mice. Alexander et al. (1) further showed that much if not all of the increase in uterine weight occurs during the first two days when "estrogenic" clover was fed to guinea pigs.

Recently oral administration of the sample to be tested to intact or ovariectomized mice, and measuring uterine weight increase, has been employed frequently by United States workers. The mice are fed ad libitum (102) or ad libitum and food consumption recorded (39) or controlled feed intake (91, 92, 105), while the assay time varies between ten (102, 91, 92, 96, 105) and three days (103) which has been

shown to be sufficient for all practical purposes as far as ovariectomized mice are concerned (103).

After terminating the assay, the mice are sacrificed and the uteri removed. These are weighed directly after surplus moisture is removed with filter paper (102, 105, 91, 92) or after having been fixed in Bedouin solution for 24 hours (96). The uterine weight is directly used as an index of estrogenic activity (102, 105, 91, 92), or the weight of the uterus is expressed as a percentage of body weight and this is used as the index of hormone activity (102).

Few rats are employed in determining estrogenic activity of plant and animal material, since immature albino mice are more sensitive to gonadotrophins (75, 84, 62, 63).

3. Units Used in Expressing Estrogenic Activity.

Early workers in this field expressed the estrogenic activity of a substance in terms of the natural hormones, viz., estradiol, estrone, estriol (100). However, when synthetic hormones became available the estrogenic activity of biological material was often expressed in terms of D.E.S. (19, 92). This latter compound is relatively stable and can be obtained in pure crystalline form. In 1941 Evans et al. (64) gave the potencies for a number of natural and synthetic hormones as obtained by the mouse uterine weight technique (TABLE I). This is based on a 6 mgm. increase in uterine weight and estrone having 100 per cent activity.

TABLE I

Potencies of different natural and synthetic estrogens in terms of estrone (64)

Compound	Potency
Estrone	100
Estriol	40
α estradiol	300
Stilbestrol	250
α estradiol acetate	110
α estradiol benzoate	220
α estradiol (dipropionate)	70
β estradiol (from estrone)	7.5
β estradiol (from mare urine)	5.0
Equilin	110
Δ_6 Equilin	7.5
α dihydroquilin	200

In 1948 Pearlman (89) recorded the physiological potencies of the estrogens (TABLE II).

TABLE II

Comparative physiological potency of the estrogens

Compound	Potency					
	Effective dose levels for vaginal response in rats		μg per		Relative activity	
	Subcut., μg	Oral, μg	Rat unit	Mouse unit	Spayed rat method	Immature mouse - uterine-weight method
Estrone	0.7	20-30	1.0	1.0	100	100
β estradiol	0.3-0.4	20-30	0.08-0.125	0.05	1,000	1,000
α estradiol	-	-	3.2-12.5	1.25	10	7.5
Estriol	-	-	-	-	20	40

From these two tables one notices that different investigators report different potencies for the same hormone.

D. Location, Isolation and Activity of the Estrogen-like Substances in Plants:

Legg et al. (82) showed that in perennial rye grass (Lolium perenne L.), cocksfoot (Dactylis glomerata L.) and in red clover (Trifolium pratense) estrogen-like compounds occur in all sections of the plant, i.e., leaf, petiole, stem and inflorescence, with the highest concentration in the leaves (2). Maximal concentration did not occur at the same time in all organs of the plant and considerable seasonal variation was noticed (82). This last phenomenum has been postulated to be due to a change in the ratio of lamina and stems (2).

The chloroplasts have been suggested as the site of production, since these are present in high concentration in the leaves. It is in these parts of the plant that estrogen-like compounds occur early (82). Curnow in 1954 (46) isolated 12 mgm. of genistein per 100 gm. of dry matter from the "chloroplast" fraction, while from fresh clover 369 mg. of genistein per 100 gm. of dry matter were obtained. He stated, therefore, that only three per cent of the estrogenic compounds occur in the cloroplast fraction of the plant.

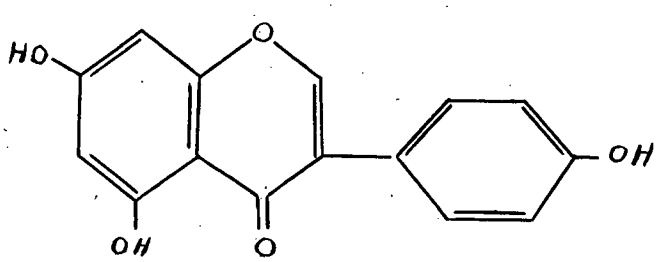
Walker (109) suggests that estrogenic activity is related to the phase of rapid growth of the plant in the spring, while others (82) state that it is associated with the reproductive growth. Spring growth precedes flowering and shows high estrogenic activity, while fall growth, when the plant is not in its reproductive state, is low in activity. A decrease in the estrogenic activity in subterranean clover,

when it reached the dry state, was also observed by Australian workers (22). Red clover on the other hand will produce flowers throughout the summer and fall and as a result is continuously high in estrogen-like substances(82).

There is considerable variation between the potency figures for the same plant estrogen as obtained by different workers. This may be partly due to the fact that investigators used different bio-assay techniques. Furthermore, the vehicles by which the estrogens are administered have been shown to influence the activity of the compound (52, 97, 25).

1. Genistein

The estrogenic isoflavone, genistein, was isolated in 1951 from subterranean clover (Trifolium subterranean) (28) and in 1954 from red clover (Trifolium pratense) (94). The compound was completely characterized by Bradbury and White (28), and has the following chemical configuration.



Genistein (5: 7: 4' - trihydroxyisoflavone)

Baker et al. (8) in 1928 and Cheng et al. (39) in 1954 synthesized this active compound. Bradbury et al. (28) state that natural and synthetic genistein have a potency 10^{-5} times that of estrone. Biggers et al. (26) revised this and showed it to be either 1.25×10^{-5} times that of estradiol when injected with propylene glycol as solvent, or

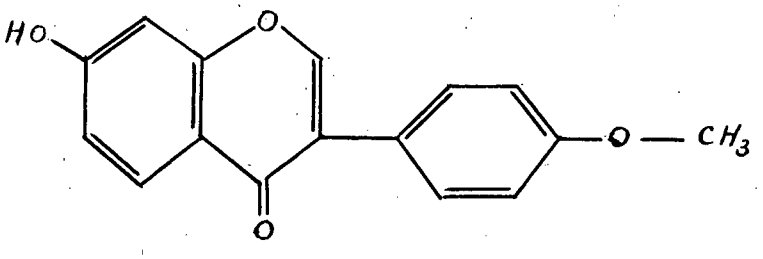
4.53×10^{-5} times that of estradiol when peanut oil was used as vehicle of administration. Pope et al. (94) estimated the potency to be 10^{-5} times that of estradiol, while Cheng and coworkers stated that it had an activity of approximately 2×10^{-5} that of D.E.S.

2. Genistin

The glucoside of genistein, genistin, is present in substantial amounts (0.1 per cent) in soybean oil meal (100) and was isolated in 1953 from this source by Cheng et al. (37). These workers reported that both genistein and genistin are estrogenic. However, the glucoside was shown to be less active than the aglucone. The estrogenic activity of genistin was confirmed by Carter et al. (34).

3. Formononetin

In 1951 Bradbury and White (28), and in 1953 Bate - Smith et al. (12) isolated a second isoflavone, formononetin, from subterranean clover (Trifolium subterranean) and red clover (Trifolium pratense) respectively.



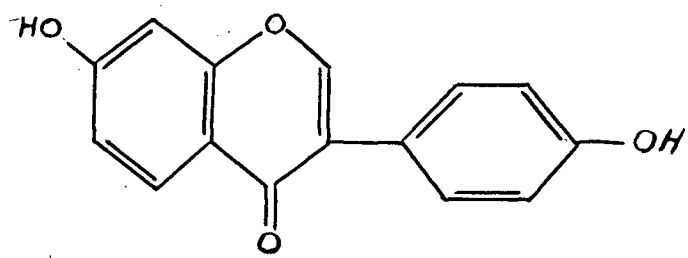
Formononetin (7 - hydroxy - 4' - methoxyisoflavone)

This compound had previously been obtained from Ononis spinosa L. where it occurs as the glucoside of the isoflavone ononin (112). The synthetic form of formononetin has been shown to exhibit low estrogenic

activity (39), while the natural compound has been reported to be inactive (94).

4. Daidzein.

A fourth compound which has been shown to possess estrogenic activity is the glucoside daidzein. This compound has been isolated from soybean meal (111). The aglucone was synthesized by Cheng et al. (39) but has not been reported to be present in nature (39).

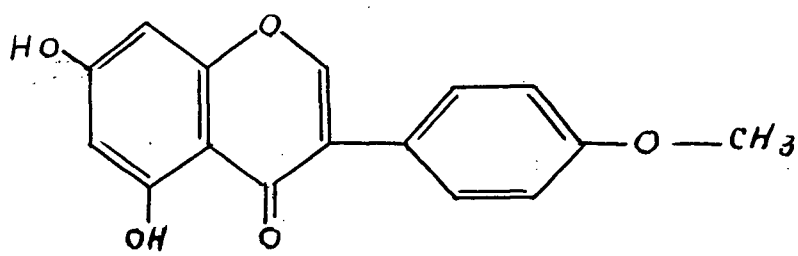


Daidzein (4', 7 dihydroxyisoflavone)

Cheng et al. (39) also reported that the synthetic aglucone was more estrogenic than genistein and biochanin A.

5. Biochanin A.

Pope et al. (93, 94) isolated another estrogenic isoflavone, biochanin A, from red clover (Trifolium pratense) and from subterranean clover (Trifolium subterranean). This compound had previously been isolated from germinated chana grain (27, 99) and from the heartwood of Ferreirea spectabilis (80).

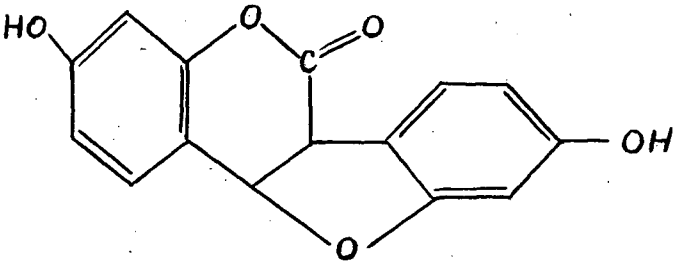


Biochanin A (5: 7 dihydroxy-4' - methoxyisoflavone)

Pope et al.(93, 94) in 1953 showed that biochanin A was estrogenic and reported in 1954 that the estrogenic activity of the synthetic form was equal to that of genistein, while the natural compound had a potency of 0.63 times that of genistein.

6. Coumestrol.

In 1957 Bickoff et al. (24) isolated an estrogenic coumarin derivative, coumestrol, from ladino clover (Trifolium repens L.).

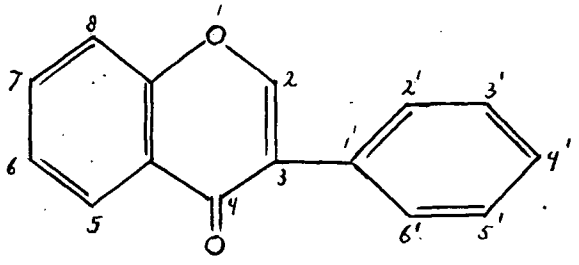


Proposed structure of coumestrol (24).

This compound is the predominant estrogen-like substance in ladino clover (Trifolium repens, ladino), strawberry clover (Trifolium fragiferum) and alfalfa (Medicago sativa). The estrogenic activity of coumestrol was shown to be higher than that of genistein but less than D.E.S. as measured by the mouse uterine weight technique.

E. Related Plant Products:

The estrogenic compounds previously mentioned belong to two groups of compounds known as isoflavones and coumarins. Genistein, genistin, diadzein and formononetin are isoflavones and have all the same basic carbon skeleton.

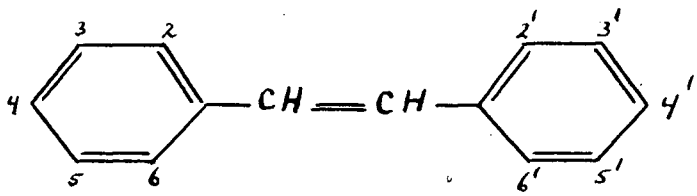


They differ in the number and location of hydroxyl groups and methoxyl groups. In many synthetic compounds the estrogenic activity is related to the number and arrangement of the hydroxyl groups. This would also explain why the synthetic isoflavone daidzein has more activity than genistein and biochanin A, and that formononetin is least active of the four (39).

To this same group of compounds belong prunitin (4', 5-dihydroxy-7-methoxy- ? -glucoside) which has been shown to be slightly estrogenic, ononin, isogenistin, methylisogenistin and methylgenistein. Closely related to the isoflavones are the flavones and flavonones.

Coumestrol belongs to the group of compounds known as coumarins. These compounds are widely distributed in nature, and are particularly abundant in the Umbelliferae and Rutaceae (73). It has been noted that numerous structurally-related coumarins may occur in the same species, genus or family (73). This may mean that in the future more estrogenic coumarins will be isolated from related species or families.

Closely related to isoflavones are the stilbenes, which have a basic carbon skeleton.



The naturally occurring stilbene derivatives vary only slightly with respect to their hydroxylation pattern (73) and are isolated from Pinaceae and Liliceae.

Synthetic stilbene derivatives have gained much attention

since 1938 when Dodds et al. (47) found that D.E.S. had similar action to estrone on the uteri of intact immature rats, rabbits and ovariectomized rats, on the vagina and the mating reaction of immature rats, and on the teat length in guinea pigs. Diethylstilbestrol and the closely resembling hexotrol have also been found to have growth promoting properties in cattle (5, 40) and sheep (15, 16), but not in swine (90, 14).

V. MATERIALS AND METHODS

A. Materials:

1. Plant Materials.

The samples of the second year growth of alfalfa (Medicago sativa var. "Rhizoma"), birdsfoot trefoil (Lotus corniculatus var. "Cascade"), orchard grass (Dactylis glomerata), alsike clover (Trifolium hybridum) and white clover (Trifolium repens) were taken from a split plot design. This split plot design was sown in the spring of 1956 and located on the agronomy fields of The University of British Columbia. These fields have an Alderwood sandy loam soil type. Each major plot was 6' x 12' and replicated four times. These 6' x 12' major plots were divided into five ultimate plots of equal size, which were assigned letters A to E at random. The plant material of the four ultimate A plots, one in each major plot, was pooled after eliminating border material. The same was done for the four B plots, the four C plots, etc.

In 1956 all plots received 200 lbs. of fertilizer (10-20-10) per acre on May 11.

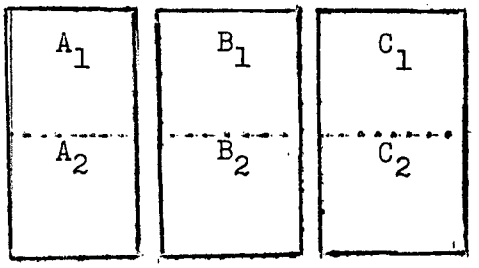
The samples of second year growth of red clover (Trifolium pratense, var. "La Salle") studied to determine the effect of stage of maturity and frequency of cutting on the estrogen-like activity were obtained from fields located at the R. Reynold's farm near Ladner, B. C. The soil type was Ladner clay.

The samples of first year growth of orchard grass and red

clover were obtained from a split plot design similar to that previously described for second year growth of alfalfa, birdsfoot trefoil, etc. This second split plot design was sown in June 1957. The red clover plots received 400 lbs. of 4-10-10 per acre on July 26, 1957 and the orchard grass plots 400 lbs. of 10-20-10 per acre on the same date.

Plant material from the first year growth of alfalfa, birdsfoot trefoil and white Dutch clover was obtained from plots measuring 12.5 by 45 feet. These plots were sown at the end of May 1957 and received 300 lbs. of fertilizer (10-20-10) per acre before seeding.

The second year growth of red clover used to study the effect of length of day on the activity of the estrogen-like substances in plants was also part of a split plot design. Each 6' x 12' major plot was replicated four times. These major plots were divided into three 3'7" x 6'6" plots, which were surrounded by a wooden frame. These three plots A, B and C were again subdivided into two ultimate plots, identified as A_1 and A_2, B_1 and B_2, C_1 and C_2 respectively, as given in the diagrams below.



The number of hours of daylight was controlled by covering them with plywood boxes measuring 6'6" x 3'7" x 1'6". All these plots received 300 lbs. of fertilizer (4-10-10) per acre at the beginning of May immediately after the first cuttings were removed.

2. Experimental Animals.

Immature female mice of the U.B.C. Swiss albino strain, 20-21 days old and weighing between 8 and 11 gm., were used in all of the bio-assays. Mice of the same strain, 60 days old and weighing from 22-30 gm., and guinea pigs weighing between 500 and 600 gm., were used to study the effect of the estrogen-like substances on their growth and reproduction.

3. Bio-assay Control Diet.

This bio-assay diet was composed of ingredients which were free from estrogen-like substances. The composition of this control diet is given in TABLE III.

TABLE III Composition of the control diet

Ingredients	Lbs.
Rolled oats	52.50
Ground wheat	26.25
Fishmeal (70%)	8.75
Meat scraps	3.75
Skim milk powder	7.50
Steamed bone meal	1.00
Iodized salt	0.25
	100.00 lbs.

During the experimental period this diet was stored at 6°C. in the dark. The proximate analysis of this control diet is given in TABLE IV.

TABLE IV The proximate analysis of the control diet

Moisture	17.91 per cent
Protein	19.00 " "
Fat	4.65 " "
Crude fiber	1.80 " "
Ash	6.86 " "
Nitrogen free extract	49.78 " "
	100.00 per cent

4. Control Guinea Pig Diet.

The control diet used for the guinea pig growth and reproduction experiment had the following composition.

TABLE V Composition of the control guinea pig diet

Ingredients	Lbs.
Ground wheat	30
Ground barley	30
Ground oats	12.5
Wheat bran	10
Fishmeal (70%)	5
Meat scraps	5
Molasses	5
Steamed bone meal	1
Iodized salt	1
Dried yeast	.5
	100 lbs.

5. Plant Material Used in the Mouse Reproduction Experiment No. I.

The red clover used for this experiment was a first cutting of second year growth. It was cut on May 3, 1957, in the vegetative stage and after drying at 150°F. for 24 hours had the composition as given in TABLE VI.

TABLE VI Composition of the red clover used for the mouse reproductive experiment No. I.

Total dry matter	13.2 per cent
Protein	23.56 " "
Fat	5.92 " "
Fiber	12.55 " "
Ash	8.54 " "
Nitrogen free extract	49.43 " "

The birdsfoot trefoil used was also a first cutting of second year growth. It was cut on July 2, 1957, in the late bloom stage, and

contained 26.4 per cent dry matter and 18.45 per cent protein after drying at 150°F. for 24 hours.

The method used in extracting the estrogen-like substances from the plant material is given elsewhere under the section headed "Methods." The red clover extract was mixed with mouse control diet so that one gram of the experimental diet corresponded with one gram of dried plant material, and the same procedure was followed for the birds-foot trefoil extract.

The D.E.S. diet used in the mouse reproduction experiments contained 0.068 mcgm. of D.E.S. per gram of mouse control diet. The estrogenic potency of this ration corresponded to that of the first cutting of red clover previously described.

The feed-extract mixtures and the D.E.S. ration were stored during the experimental period in "seal-tight" containers at 6°C.

6. Plant Material for the Guinea Pig and Mouse Reproduction Experiment No. II.

A second cutting of second year growth of red clover was used for this experiment. The clover was harvested in the early bloom stage from the Agronomy field on July 12, 1957. It contained 13.2 per cent dry matter, 12.51 per cent protein (dry weight) and had an estrogenic activity per lb. of dry matter equivalent to 12 mcgm. of D.E.S.

B. Methods:

1. Drying of Plant Material.

Immediately after cutting, the fresh material was weighed,

spread one inch thick on wooden drying trays and dried at 150°F. for approximately 20 hours. After this period the dry weight and the per cent dry matter of the material were recorded.

2. Grinding of Dried Plant Material.

Immediately after drying the plant material was ground in a Wiley mill through a 1/16 inch screen.

3. Storage of Ground Samples.

All red clover samples and the first cuttings of second year growth of alfalfa, birdsfoot trefoil, orchard grass, alsike and white clover were stored in one gallon glass jars with metal lids. The rest of the samples were stored in one half gallon and in one pint "seal tight" cardboard containers.

All samples were stored at room temperature and those in the one gallon glass jars were exposed to daylight.

4. Extraction Procedure.

One hundred and fifty grams of each dried sample were placed with 800 ml. of 95 per cent ethanol in a 3000 ml. flask and refluxed for one hour. After cooling, the ethanol was removed by suction filtration using No.1 Whatman filter paper. The plant material was returned to the flask and refluxed for an additional hour with 700 ml. of ethanol. Again after cooling,the alcohol was removed by suction filtration and the two alcoholic extracts were combined. These combined extracts were concentrated in a flash-evaporator at 49°C. to approximately 150 ml.

This concentrate was mixed with approximately 140 gm. of the

control mouse diet and the remaining ethanol was evaporated by drying the feed-extract mixture at 85°C. for 15 hours. After drying, the mixture was ground, using a mortar and pestle and the weight adjusted with control diet, so that one gram of experimental diet corresponded to one gram of dried plant material.

The feed-extract mixtures were stored in one pint "seal tight" cardboard containers and their estrogen-like activity determined as soon as possible. The diet remaining after the bio-assay was left in the containers and kept at room temperature for subsequent use.

5. Proximate Analysis of Samples.

A complete proximate analysis was carried out on all red clover samples used in the periodicity experiment. From all other samples the moisture and protein content were determined. The analyses were done according to the A.O.A.C. methods (83), and the results recorded on a dry weight basis.

6. Housing and Care of Bio-assay Animals.

In all cases the animals were housed in 4" x 6" x 12" enamel lined crisper dishes. In all cases wood shavings were used for bedding. Each dish housed a group of 10-12 immature female mice for an 80 hour period.

All experimental diets for bio-assay purposes were fed in finely ground form. Each animal received five grams of feed per 72 hours, divided into three equal portions over the three day period. The diets were offered in four ounce glass jars placed within the crisper dishes. After having been on the diet for 72 hours, the animals were

fasted for eight hours. Water was supplied fresh daily ad libitum in one half pint jars fitted with rubber stoppers and glass delivery tubes.

7. Sacrifice and Dissection of Bio-assay Mice.

After having been on the experiment for 80 hours the animals were sacrificed with ether anaesthesia and weighed individually. The uteri were then removed (dissected at the uterine side of the cervix), trimmed of extraneous tissue, pressed between filter paper to remove free moisture and immediately weighed on a Roller - Smith balance.

The weight of the uterus was expressed as a per cent of body weight.

8. Housing and Feeding of the Mature Mice on Production Experiments Nos. I and II.

The mice used in this experiment were approximately 60 days old at the beginning of the experiment. The males weighed 24-27 gm. and the females 22-30 gm. These animals were also housed in crisper dishes. During the pre-mating period five females were housed in one cage, to which was added one male during the mating period. After termination of the mating period the females were separated and housed individually. Throughout the pre-mating and mating period animals received their experimental diets in finely ground form. The diets were offered ad libitum in open six ounce glass jars placed within the cages, and the feed consumption recorded. Fresh water was supplied daily ad libitum in one half pint bottles as described previously.

After the breeding period all animals received the mouse control ration (TABLE III) in the form of 3/16 inch pellets.

9. Housing and Feeding of Guinea Pigs.

During the 48 day pre-mating period the guinea pigs were housed individually in wooden 18" x 9" x 6.5" boxes. At the start of the pre-mating period the animals received the control guinea pig ration (TABLE V). This period was designed to get the animals adjusted to their new surroundings and ration. The feed was supplied in the form of 3/16 inch pellets during this preliminary period. The remaining 33 days of the pre-mating time was used to study the influence of the estrogen-like substances of red clover on the growth of the guinea pig. During this period and during the mating period the animals received the feed - red clover extract mixture in finely ground form. During the post mating period all guinea pigs received the control diet (TABLE V) in the form of 3/16 inch pellets. Throughout the experiment ascorbic acid was added to the drinking water, at a level of 1.8-2 mgm. per 100 gm. of body weight. This level is approximately three times greater than the amount required by the animal (29A). The reason for this high level was that ascorbic acid is broken down in the water. During the entire experiment, feed consumption was recorded. The water - ascorbic acid mixture was made fresh daily and supplied in the previously described one half pint bottles.

10. Mating Plan of Mice of Reproduction Experiment No. I.

Twelve days prior to mating, the male and the female mice were fed the particular experimental diets. On the 12th day mating was initiated and the females were continued on the same diets during the 15 day mating period. The males which received the same ration as the females stayed with the females during the whole breeding period while males receiving a different ration than the females were rotated. In the last case two males were used for each group of females. After

having been with the females for two days the male was removed and put back on its particular ration for two days. The second male which had been on its particular ration for two days was then put with the same group of females.

TABLE VII Design of mating plan

Group	Mating
1	5 control females x 1 control male.
2	5 control females x 2 red clover males.
3	5 control females x 2 birdsfoot trefoil males.
4	5 control females x 2 D.E.S. males.
5	5 red clover females x 1 red clover male.
6	5 red clover females x 2 control males.
7	5 birdsfoot trefoil females x 1 birdsfoot trefoil male.
8	5 birdsfoot trefoil females x 2 control males.
9	5 D.E.S. females x 1 D.E.S. male.
10	5 D.E.S. females x 2 control males.

After mating was completed, all animals, male and female, received the control ration in pelleted form. Those females which did not have a litter three weeks after termination of the breeding period, were mated again to their original males, in order to check if males and females had recovered from their particular treatments. This second mating period was also 15 days.

11. Mating Plan of Mice of Reproduction Experiment No.II.

These mice received the guinea pig control diet plus red clover extract during the eight day pre-mating and the 13 day mating

period. The mating plan differed slightly from that of Experiment I. In Experiment II the males were not rotated every two days but the first male stayed with the females for the first six days and the second for the remaining seven.

TABLE VIII Design of the mating plan

Group	Mating
1	5 control females x 2 red clover males.
2	5 red clover females x 1 red clover male.

After the mating period all animals received the mouse control ration in pelleted form.

Those females which did not litter within three weeks after mating were bred again to the same males. This second mating period lasted for 14 days and was designed to see if both sexes had recovered from the previous treatment.

12. Mating Plan of Guinea Pigs.

Ten females and two males were used in this experiment. The females were divided into two groups of five, one receiving control ration and the other control ration plus red clover extract (see Materials). One male received control diet and the other control plus red clover extract ration.

After having been on their particular ration for 48 days the control females were mated to the control male and the "red clover" females to the "red clover" male.

The mating period was terminated after thirty-one days.

VI. RESULTS AND DISCUSSION

A. Development of the Bio-assay Procedure:

Since the Permanent Commission on Biological Standardization has not yet established an international standard for plant estrogens the author has expressed the potency of these substances in terms of once recrystallized D.E.S. This compound has been used widely in the animal industry and is relatively stable. In developing a new bio-assay procedure it is interesting to note Hartley's (76) comments when he reviewed the work of the Permanent Commission on Biological Standardization.

"Improvement in existing methods of assay and the devising of new ones, and the progress of research, are more likely to be advanced by leaving to individual workers freedom of choice as to the method by which assays are carried out, rather than by insistence upon the details of a particular method which, on the one hand, may be difficult to describe adequately, and, on the other, may appear to give an air of finality in a field of biological standardization in which every encouragement should be given for improvement and advance."

In an analytical dilution assay the biological system plays an analogous role to that of a balance in weighing an object. The biological system is an instrument and not a factor influencing the result. Wood (114) gives the following description of this kind of a bio-assay.

"(a) that the response supposed to be produced by the known amounts of 'factor X' (i.e. the effective constituent of the standard preparation) is actually due to the factor itself and not to some other substance associated with it, e.g., an impurity; and (b) that the response produced by the material to be analyzed is also due solely to the presence in it of 'factor X', without augmentation, diminution, or modification by any other substance also present. In other words, if we use the terms 'Standard Preparation' and 'Test Preparation' to denote respectively the solution of allegedly pure 'factor X' and the solution prepared from the material to be analyzed, we assume that the Standard Preparation contains no substance, other than factor X itself, contributing to the response we measure, and that the Test Preparation behaves for the purpose of the analysis so similarly to the Standard Preparation that it may be regarded simply as a dilution of the Standard Preparation in a completely inert diluent."

In establishing a suitable bio-assay procedure for the quantitative determination of estrogen-like substances in plant materials a number of preliminary experiments were carried out.

1. The Determination of Feed Intake of Immature Female Mice over a Three Day Period.

TABLE IX shows that 21 day old female mice weighing 9-11 gm. consumed 7.9-9.2 gm. of control ration over a three day period.

TABLE IX Feed intake of 21 day old Swiss albino mice receiving control ration and water ad libitum.

Group	Number of Animals	Average Body Weight (gm.)	Average Feed Intake Over 3 Day Period
1	8	9.54	7.9 gm.
2	7	10.63	9.2 gm.
3	7	9.28	7.9 gm.

Control rations containing plant extracts having high estrogenic activity tend to lower palatability. This was also observed by Alexander et al. (2) when dried "estrogenic" subterranean clover was fed to guinea pigs. From these observations it was decided not to feed at maximum intake level, but five grams of diet per animal for the three day assay period. In doing so, all bio-assay animals consumed approximately the same amount of diet.

2. The Time of Feed Passage in 21 Day Old Swiss Albino Mice.

The time of feed passage was determined for two groups of three mice. One group received the control ration with ferric oxide added as an indicator at the 0.1 per cent level, and the other group received

the control ration with chromic oxide added at the 0.1 per cent level. The results are shown in **TABLE X.**

TABLE X The time of feed passage in 21 day old mice fed control ration and receiving water ad libitum

Animal	Indicator	Time of Feed Passage
	Ferric oxide	
1		8.30 hrs.
2		7.55 hrs.
3		8.10 hrs.
	Chromic oxide	
4		8.45 hrs.
5		8.15 hrs.
6		8.20 hrs.

The average time of passage for the ferric oxide group was 7.98 hours and for the chromic oxide group 8.27 hours when control ration and water were offered ad libitum. However, this difference is not significant.

3. The Influence of Time of Fasting on Uterine Weight of Intact Immature Female Mice.

To obtain maximum values for uterine weight, when plant extracts were fed, it was felt necessary to study the effect of time of fasting on the ultimate uterine weight of the assay females. From the results shown in TABLE XI it seemed reasonable to fast all bio-assay animals for eight hours. This was approximately the same number of hours as necessary for the control ration to pass through the

TABLE XI

The influence of "time of fasting" on uterine weight

(Animals were fed 5 gm. of feed per 3 days and water ad libitum)

Ration	Group	Number of Animals	Hours of Fasting	Av. Body Wt. at Zero hrs. Fasting (gm.)	Av. Body Wt. at end of Fasting (gm.)	Average Uterine Wt. (mgm.)	Per cent Body Wt.
Control	1	12	0	9.99	9.99	19.90	0.199±0.008*
	2	12	8	10.24	9.51	16.93	0.178±0.010
	3	12	24	10.44	8.66	17.93	0.206±0.007
Control Ration containing 0.05 mcgm. D.E.S. per gm. of feed.	4	12	0	10.80	10.80	40.09	0.368±0.027
	5	12	8	10.77	9.62	36.94	0.385±0.027
	6	12	24	10.63	8.58	28.64	0.333±0.014

* Standard error

digestive tract of the mouse.

4. The Length of the Bio-assay Period.

An experiment was undertaken to study the number of hours required for the uterus to reach its maximum size when the mice were fed a ration containing estrogenic compounds. One level of D.E.S. and two red clover extract mixtures having different estrogenic activity were fed to three groups of immature female mice. TABLE XII shows that in all three cases the uteri reached their maximum weight at 72 hours. Stob et al. (103) showed that a three day assay period was sufficient in overiectomized mice in order to measure maximum activity of an estrogen-like substance. Alexander et al. (1) observed that in spayed guinea pigs much if not all of the increase in uterine weight occurred over the first two days when they were fed "estrogenic" subterranean clover. A short bio-assay period, using intact 21 day old female mice, also has the advantage that normal estrus does not easily mask the experimental results. From the results of TABLE XII it was concluded that a 72 hours assay period would be used for all subsequent bio-assays.

5. The Effect of Dehydration of the Uteri on the Accuracy of the Bio-assay.

A comparison of wet uterine weight, dry uterine weight and alcohol dehydrated uteri was studied. TABLE XIII shows that there is an increase in both total water and total solids of the uterus when estrogenic substances are fed to mice. These results confirm the work of Astwood (7) and Carroll (33) who observed the same phenomenum.

TABLE XII

The influence of "assay length" on the uterine weight
(Animals were fed 1.67 gm. of feed per day and water ad libitum)

Ration	Group	Number of Animals	Hours on Ration	Hours of Fasting	Av. Body Wt. at end of Fasting (gm.)	Average Uterine Wt. (mgm.)	Per cent Body Wt.
Control	1	12	24	8	10.35	17.65	0.171±0.007
	2	10	48	8	10.45	20.50	0.195±0.009
	3	12	72	8	9.51	16.93	0.178±0.010
Control Ration	4	12	12	8	10.45	26.37	0.252±0.008
containing 0.05 (mcgm.)	5	12	24	8	10.35	27.65	0.268±0.006
D.E.S. per gm. of feed	6	12	48	8	10.43	37.13	0.354±0.016
	7	12	72	8	10.02	40.28	0.400±0.017
	8	12	96	8	10.40	36.18	0.349±0.014
Control Ration	9	12	12	8	11.90	40.30	0.340±0.016
containing red clover	10	12	24	8	10.00	30.97	0.310±0.011
extract (1st cutting;	11	12	48	8	9.69	44.54	0.464±0.023
May 3, 1957)	12	12	72	8	10.34	51.58	0.501±0.021
	13	12	96	8	10.27	50.76	0.494±0.010
Control Ration	14	8	48	8	9.88	44.00	0.452±0.031
Containing red clover	15	8	72	8	9.23	46.00	0.492±0.034
extract (2nd cutting;	16	8	96	8	9.53	43.60	0.460±0.033
July 18, 1956)							

TABLE XIII A comparison of wet uterine weights, dry uterine weights and uterine weights of alcohol dehydrated uteri (2 hrs. in 95% ethanol)

	Control Ration	Control Ration	0.02 mcgm. D.E.S. per gm. of Feed	0.07 mcgm. D.E.S. per gm. of Feed
Number of Animals*	12	12	10	12
Mean Body Weight in Grams	10.34	9.21	9.57	10.35
Mean Wet Uterine Wt. Mgm.	18.70	14.90	26.90	51.60
Mean Uterine Weight after ethanol Dehydration Mgm.	-	5.70	-	-
Mean Dry Uterine Wt. in Mgm.	4.70	3.00	6.20	9.00
Wet Uterine Wt. per Body Wt. x 100	0.180	0.164	0.281	0.498
Et. OH Dehydrated Uterine Wt. per Body Weight x 100	-	0.062	-	-
Dry Uterine Wt. per Body Wt. x 100	0.045	0.033	0.064	0.086

* All animals consumed 5 grams of feed per three days and received water ad libitum.

For the control ration the standard errors of wet uterine weight, dry uterine weight and alcohol dehydrated uterine weight as a per cent of body weight were 5.8, 5.2 and 5.2 per cent respectively. For the ration containing 0.02 mcgm. of D.E.S. per gram of feed the per cent standard error for wet uterine weight as a per cent of body weight was 4.3, and for dry uterine weight 5.9. Corresponding figures for the ration containing 0.07 mcgm. of D.E.S. per gram of feed were 3.0 and 3.0 per cent. These results indicate that neither drying nor alcohol dehydration of the uteri improve the accuracy of the procedure. Since both are time consuming, it was decided to use wet uterine weight expressed as a percentage of body weight as a measurement of

estrogenic activity.

From the results obtained in the preliminary experiments it was decided to feed five grams of diet per three days, to have an assay period of 72 hours, to fast the animals for eight hours, and to use wet uterine weight expressed as a per cent of body weight as the measurement of estrogenic activity of the experimental diets. In all cases water was supplied ad libitum.

B. Construction of the Dose Response Curve:

The potency of the estrogen-like substances in the plant material studied was determined relative to the activity of once recrystallized D.E.S.

Known quantities of this compound, dissolved in ethanol, were added to the standard mouse diet and fed to groups of randomly selected immature female mice. Groups of 10 mice were used for the first dose response curve and groups of 12 for the second.

Two dose response curves were constructed since a significant difference was established between the three lots of control ration.

TABLE XIV Bio-assay results of the three lots of mouse control ration.

Lot	Date of Assaying	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 Days (gm.)
1	22-4-57	10	9.72	22.0	0.222±0.016*	5
2	22-7-57	12	9.51	16.93	0.178±0.010	5
3	9-12-57	12	10.34	18.70	0.180±0.007	5

* $P < 0.05$

The first lot of mouse control diet was only used to construct dose response curve 1 (FIGURE I), and to assay the second year growth of red clover harvested in 1956. The data obtained for the construction of this dose response curve are given in TABLE XV. From these data a regression line was calculated by the "method of least squares" (43).

$$Y = 0.206 + 6.139x$$
$$S_{ys} = 0.035$$

x = mcgm. of D.E.S./gm. of feed
y = uterine weight/body weight x 100

The second dose response curve and all other bio-assays and experiments were based on the second and third lot of ration. Bio-assays of these two lots gave similar results (TABLE XIV). The data for dose response curve II (FIGURE II) are shown in TABLE XVI. The equation of the regression line again was calculated by the "method of least squares" and was

$$Y = 0.172 + 4.843x$$
$$S_{ys} = 0.017$$

This second dose response curve was used to estimate the estrogenic activity of all samples with the exception of the previously mentioned red clover.

C. Plant Extract Bio-assays:

After developing a suitable bio-assay technique and constructing a dose response curve for the determination of estrogen-like substances in forage samples an experiment was designed to study the effect of length of time from cutting the forage in the field to drying on these estrogenic substances. The results of this study are shown in

TABLE XV Tabulated results for the construction of dose response curve I

Group	Mcgm. of D.E.S.* per gm. of Feed	Number of Animals	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.
1	0.000	10	9.72	22.0	0.222
2	0.0025	10	8.94	19.6	0.218
3	0.005	10	9.45	23.3	0.246
4	0.010	10	9.68	26.5	0.274
5	0.015	10	9.72	30.9	0.318
6	0.020	10	9.70	33.6	0.344
7	0.025	10	9.75	30.9	0.317
8	0.030	10	9.74	37.0	0.379
9	0.035	10	9.47	38.6	0.410
10	0.040	9	9.48	39.6	0.416
11	0.045	9	9.31	45.9	0.495
12	0.050	9	9.91	46.6	0.468
13	0.055	10	10.16	58.5	0.578
14	0.060	10	9.74	55.4	0.576
15	0.065	10	10.33	69.6	0.673
16	0.070	10	9.86	65.2	0.670
17	0.075	10	10.36	67.1	0.649
18	0.090	9	9.89	72.1	0.726

* All animals consumed 5 gm. of feed per three days and received water ad libitum.

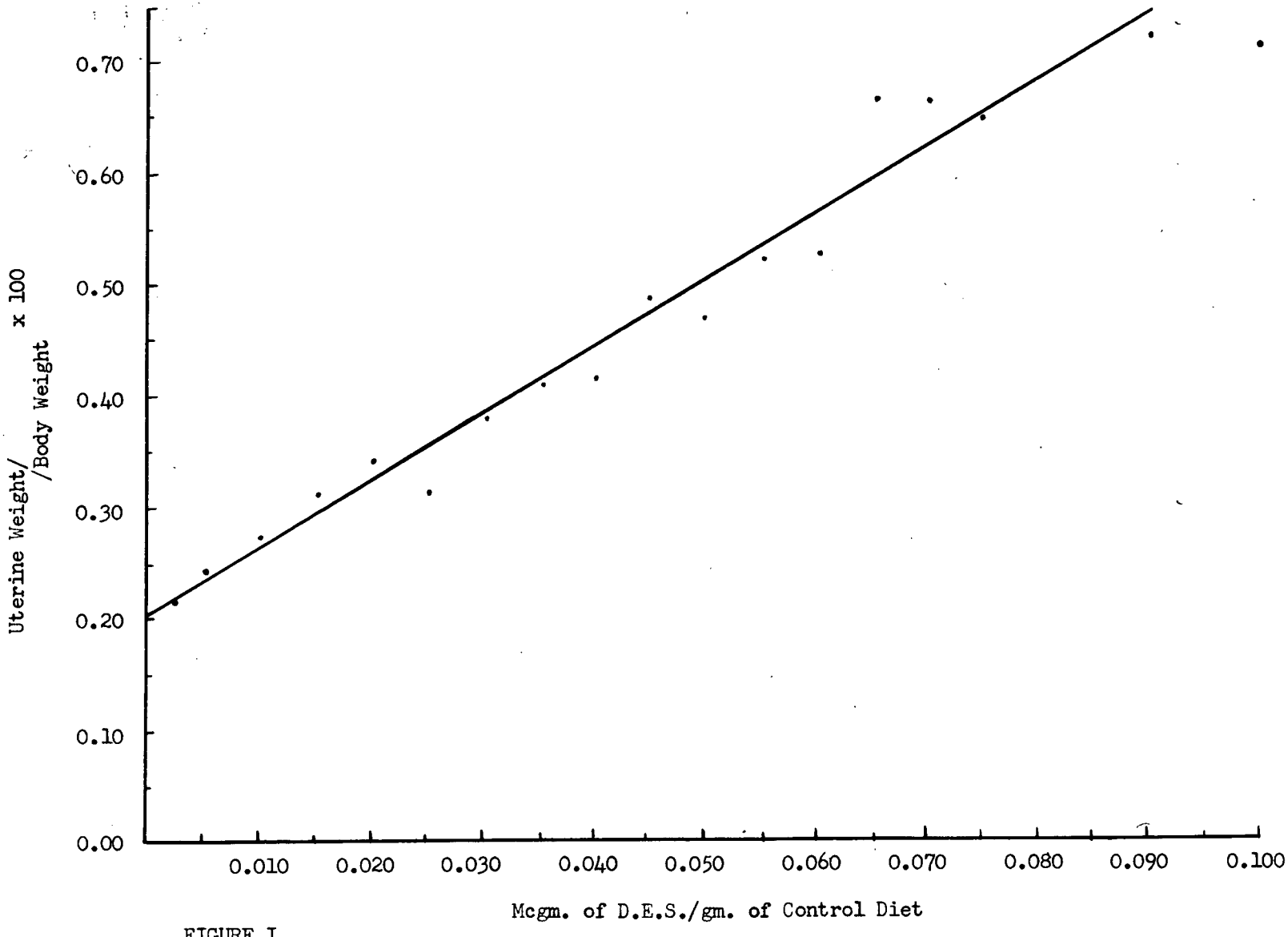


FIGURE I

DOSE RESPONSE CURVE I

TABLE XVI Tabulated results for the construction of dose response curve II

Group	Mcgm. of D.E.S.* per gm. of Feed	Number of Animals	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.
1	0.000	12	9.51	16.93	0.178
2	0.0025	12	9.61	18.82	0.194
3	0.005	12	9.09	18.03	0.198
4	0.010	12	9.13	19.66	0.214
5	0.015	12	9.57	22.49	0.236
6	0.020	12	9.60	24.68	0.256
7	0.025	12	9.60	27.82	0.288
8	0.030	12	9.39	30.92	0.328
9	0.035	12	9.95	32.87	0.330
10	0.040	12	9.83	39.08	0.399
11	0.045	12	10.00	39.70	0.397
12	0.050	12	10.02	40.28	0.400
13	0.060	12	9.70	43.87	0.451
14	0.070	12	9.83	51.42	0.520
15	0.080	12	9.58	52.05	0.557
16	0.090	12	9.91	56.75	0.571
17	0.100	12	9.95	56.95	0.581

* All animals consumed 5 gm. of feed per three days and received water ad libitum.

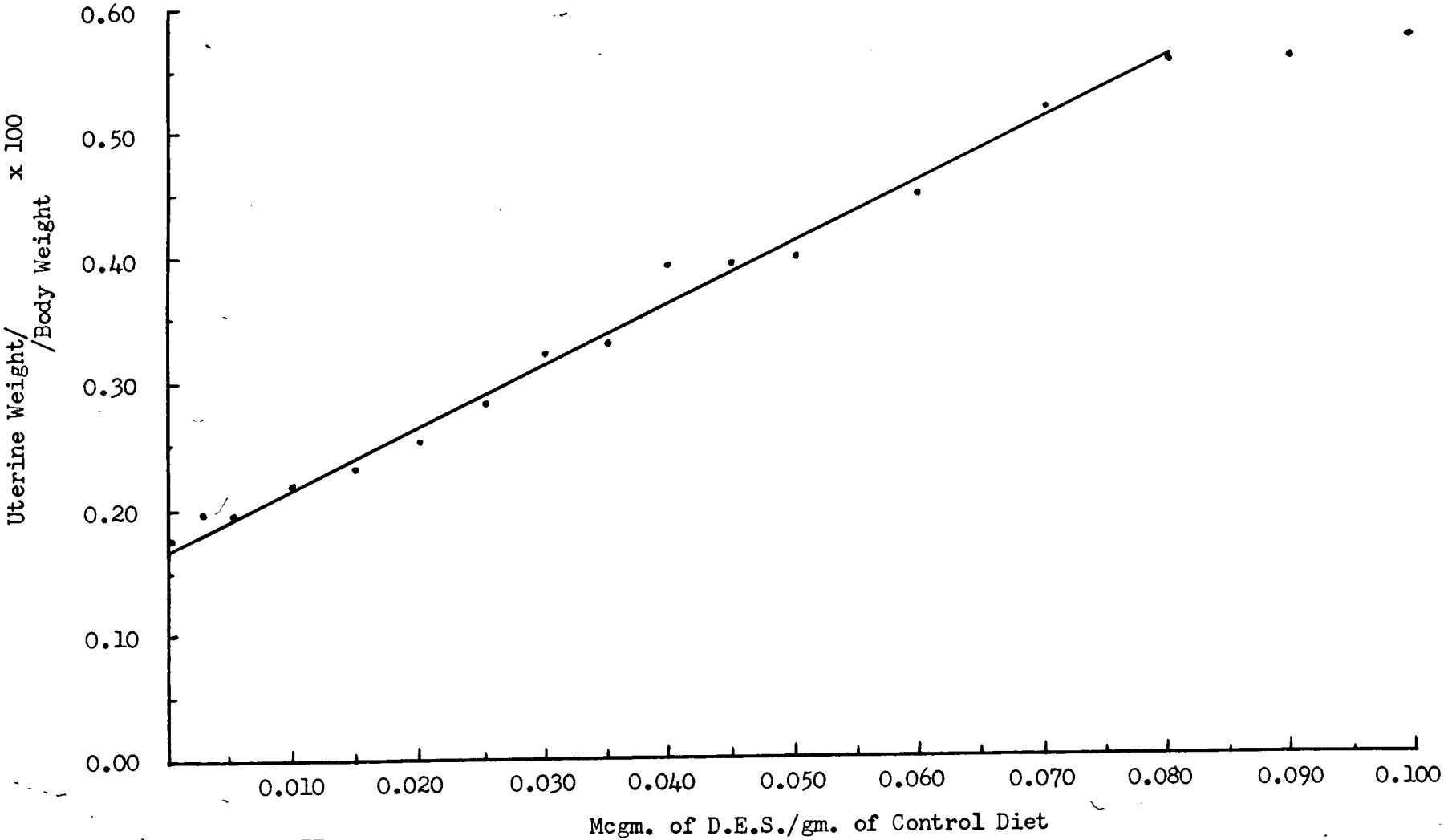


FIGURE II

DOSE RESPONSE CURVE II

TABLE XVII. An analysis of variance revealed that the estrogenic activity of red clover was not significantly influenced ($P > 0.05$) by leaving the plant material on the field for 48 hours. Alexander et al. (2) made a similar observation for the estrogenic activity of subterranean clover when the plant material was stored for 48 hours at 16 to 21°C.

Next the effects of drying time and drying temperature on the estrogenic substances in the feed-extract mixtures were studied. The results obtained by varying drying time between 7 and 24 hours and keeping the drying temperature constant at 65°C. are shown in TABLE XVIII, while those obtained by varying drying temperature between 65 and 102°C. (drying time constant at 19 hours) are reported in TABLE XIX. An analysis of variance revealed that the estrogenic activity of red clover was not significantly influenced by either of the two treatments.

After these experiments were completed an extensive study of the effects of stage of maturity and frequency of cutting on the estrogenic substances of first and second year growth of certain forages was undertaken. The results of this study are given in TABLES XX to LI.

1. The Effect of Stage of Maturity and Frequency of Cutting on the Estrogenic Activity of Alfalfa (TABLES XX TO XXVI And FIGURE III).

Walker et al. (109) postulated that the highest concentrations of estrogen-like substances in forage plants occurred during the phase of rapid growth in the spring, while other investigators (82) correlated the activity with the reproductive growth of the plant. The results in TABLE XX show that second year growth of alfalfa is not only high

TABLE XVII The influence of time of curing on estrogen-like activity of red clover (first cutting of 1957 seeding, cut Sept. 24, 1957)

Group	Time between cutting and the beginning of drying at 65°C.	Number of Animals	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption per animal per 3 days (gm.)	Estimated Potency per lb. of Dry Matter (mcgm.)
1	12 minutes	12	9.14	33.48	0.365±0.019	5.0	18
2	1.30 hrs.	11	9.46	34.11	0.361±0.017	5.0	18
3	26.00 hrs.	10	8.85	33.67	0.382±0.025	4.4	20
4	48.00 hrs.	12	9.38	28.63	0.306±0.023	5.0	13

TABLE XVIII The effect of drying time on the level of estrogen-like substances in "control ration - red clover extract" mixtures (Red clover - first cutting May 3, 1957)

Sample Treatment						Mouse Bio-assay		
Sample	Drying Time	Temperature	No. of Animals	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption per 3 days (gm.)	Estimated Potency per lb. of Dry Matter (mcgm.)
1A	7 hrs.	65° C.	11	9.84	40.62	0.413±0.007	4.3	26
1B	19 hrs.	65° C.	11	9.99	42.51	0.425±0.015	4.3	27
1C	24 hrs.	65° C.	10	10.54	45.05	0.426±0.017	4.8	25

TABLE XIX The effect of drying temperature on the level of estrogen-like substances in "control ration - red clover extract" mixtures (red clover = first cutting May 3, 1957)

Sample Treatment						Mouse Bio-assay		
Sample	Drying Time	Temperature	Number of Animals	Mean Body Weight (gm.)	Mean Uterine Weight (mgm.)	Per cent Body Weight	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. of Dry Matter (mcgm.)
1 B	19 hrs.	65°C.	11	9.99	42.51	0.425±0.015	4.3	27
1 D	19 hrs.	82°C.	12	9.94	39.62	0.400±0.014	4.7	23
1 E	19 hrs.	102°C.	12	9.38	35.30	0.378±0.026	43	22

TABLE XX The estrogen-like activity of first cuttings of alfalfa (Rhizoma) at different stages of maturity (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A	May 3	Vegetative	84.5	22.15	12	10.18	74.62	0.733±0.016*	4.7	55
B	June 3	Pre-bloom	74.8	18.71	12	10.08	30.17	0.298±0.011	5.0	12
C	July 2	Full Bloom	66.0	13.72	12	9.96	21.85	0.218±0.011	5.0	4
D	Aug. 1	Late Bloom	70.1	16.43	12	9.90	37.35	0.388±0.028	4.8	21
E	Sept. 3	Past Bloom	63.6	7.93	12	9.81	34.27	0.350±0.020	5.0	17

* Standard error

TABLE XXI The estrogen-like activity of 1st, 2nd, 3rd and 4th cuttings of Alfalfa (Rhizoma) of plots A (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cuttings	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	May 3	Vegetative	84.5	22.15	12	10.18	74.62	0.733±0.016	4.7	55
2	Jan. 2	Vegetative	81.2	22.34	12	10.46	33.54	0.322±0.021	5.0	14
3	July 2	Vegetative	76.2	22.75	11	9.71	21.95	0.226±0.007	4.5	6
4	Aug. 15	Vegetative	79.5	18.53	12	9.92	59.34	0.598±0.024	4.6	43

TABLE XXII

The estrogen-like activity of 1st, 2nd and 3rd cuttings of alfalfa (Rhizoma) of plots B (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	June 3	Pre-bloom	74.8	18.71	12	10.08	30.17	0.298±0.011	5.0	12
2	July 2	Vegetative	80.0	22.12	12	9.74	19.51	0.169±0.008	4.3	0
3	Aug. 15	Vegetative	78.2	25.40	12	8.79	29.14	0.332±0.032	4.3	18

TABLE XXIII — The estrogen-like activity of 1st, 2nd and 3rd cuttings of alfalfa (Rhizoma) of plots C (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	July 2	Full Bloom	66.0	13.72	12	9.96	21.85	0.218±0.011	5.0	4
2	Aug. 1	Vegetative	81.8	26.31	12	9.06	17.04	0.187±0.010	4.3	2
3	Sept. 3	Vegetative	76.6	22.75	11	9.17	17.64	0.194±0.012	4.6	2

TABLE XXIV — The estrogen-like activity of 1st and 2nd cuttings of alfalfa (Rhizoma) of plots D (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	Aug. 1	Late Bloom	70.1	16.43	12	9.90	37.35	0.388±0.028	4.8	2
2	Sept. 3	Vegetative	76.9	19.60	12	9.28	18.54	0.199±0.010	4.8	3

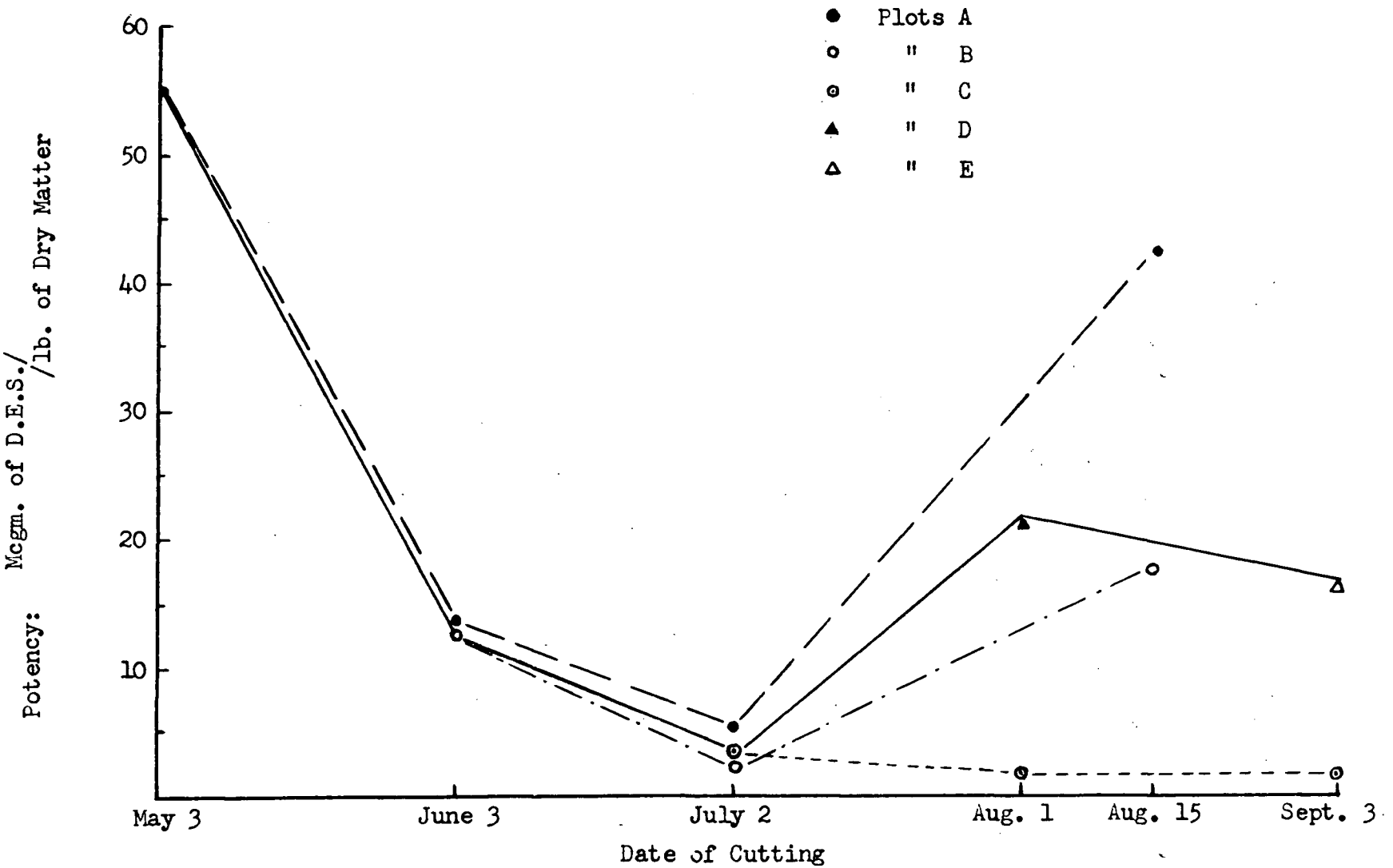


FIGURE III

The Estrogenic Activity in Terms of D.E.S. of Second Year Growth of Alfalfa at Various Stages of Maturity, and at Successive Cutting.

TABLE XXV The estrogen-like activity of first cuttings of alfalfa (Rhizoma) at different stages of maturity (first year growth, 1957)

Sample Description					Mouse Bio-assay					
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A	Aug. 15	Vegetative	84.1	24.28	12	9.40	15.75	0.168±0.008	5.0	0
B	Sept. 25	Vegetative (not many leaves present)	64.1	6.58	12	9.12	15.90	0.171±0.009	5.0	0

TABLE XXVI

The estrogen-like activity of 1st and 2nd cuttings of alfalfa (Rhizoma) of plots A (first year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	Aug. 15	Vegetative	84.1	24.28	12	9.40	15.75	0.168±0.008	5.0	0
2	Sept. 25	Vegetative	75.1	22.85	12	9.09	19.99	0.220±0.015	5.0	5

in estrogenic substances in the spring but that it also contains substantial amounts after August 1 when the plants are past the period of rapid growth. The third cutting of plots B and the fourth cutting of plots A, both cut on August 15 also showed considerable activity. The last two samples were in the vegetative and not in the reproductive state. The results also contradict a third hypothesis stating that seasonal variation is due to a change of lamina to stem ratio (2). Furthermore, all samples of second year growth of alfalfa cut in July showed little or no estrogenic activity.

The plots of first year growth of alfalfa were heavily infected with leaf spots, and, as a result of this, most plants lost their leaves. First cuttings of these affected plots did not show any estrogenic activity, but a second cutting of plot A showed some activity. This second cutting was affected to a much lesser degree by the above mentioned disease and hence the plants lost few leaves.

2. The Effect of Stage of Maturity and Frequency of Cutting on the Estrogenic Activity of White Clover (TABLES XXVII to XXXII and FIGURE IV).

The growth pattern of white clover differs considerable from that of alfalfa. This clover produces flowers throughout the summer and fall, i.e., it is continuously in its reproductive stage. However, the estrogenic pattern of white clover is very similar to that of alfalfa. The estrogen-like activity was again highest in the first cutting of second year growth, cut on May 3. The potency was lowest in the samples cut in June and July, but not as low as the estrogenic activity of alfalfa samples cut on the same date. An exception to this result

TABLE XXVII The estrogen-like activity of first cuttings of white clover at different stages of maturity (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A	May 3	Vegetative	87.7	26.69	12	9.65	70.00	0.720±0.023*	5.0	57
B	June 3	¾ Full Bloom	84.0	23.43	12	9.53	25.94	0.271±0.012	5.0	9
C	July 2	Full to Late Bloom	75.4	20.65	12	9.77	21.53	0.221±0.010	5.0	5
D	Aug. 1	Late Bloom	73.4	20.93	12	9.77	38.69	0.392±0.025	5.0	20
E	Sept. 3	Past Bloom (seed)	59.1	18.63	12	9.46	36.25	0.381±0.031	5.0	20

* Standard error

TABLE XXVIII The estrogen-like activity of 1st, 2nd, 3rd and 4th cuttings of white clover of plots A (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	May 3	Vegetative	87.7	26.69	12	9.65	70.00	0.720±0.023	5.0	57
2	June 3	$\frac{3}{4}$ Bloom	84.2	26.47	12	9.81	26.90	0.273±0.017	5.0	10
3	July 2	5/6 Bloom	79.7	22.47	11	9.88	33.51	0.339±0.020	5.0	15
4	Aug. 1	Past Full Bloom	73.4	25.44	12	10.21	52.92	0.518±0.025	5.0	30

TABLE XXIX

The estrogen-like activity of 1st, 2nd and 3rd cutting of white clover of plots B (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	June 3	¾ Full Bloom	84.0	23.43	12	9.53	25.94	0.271±0.012	5.0	9
2	July 2	½ Full Bloom	78.3	23.34	12	9.80	41.82	0.426±0.029	5.0	24
3	Aug. 1	1/5 Bloom	81.2	25.50	12	10.67	65.73	0.616±0.021	5.0	42

TABLE XXX The estrogen-like activity of 1st and 2nd cutting of white clover of plots C (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	July 2	Full-Late Bloom	95.4	20.65	12	9.77	21.53	0.221±0.010	5.0	5
2	Aug. 1	1/8 Bloom	80.2	23.00	12	9.44	26.52	0.286±0.017	5.0	11

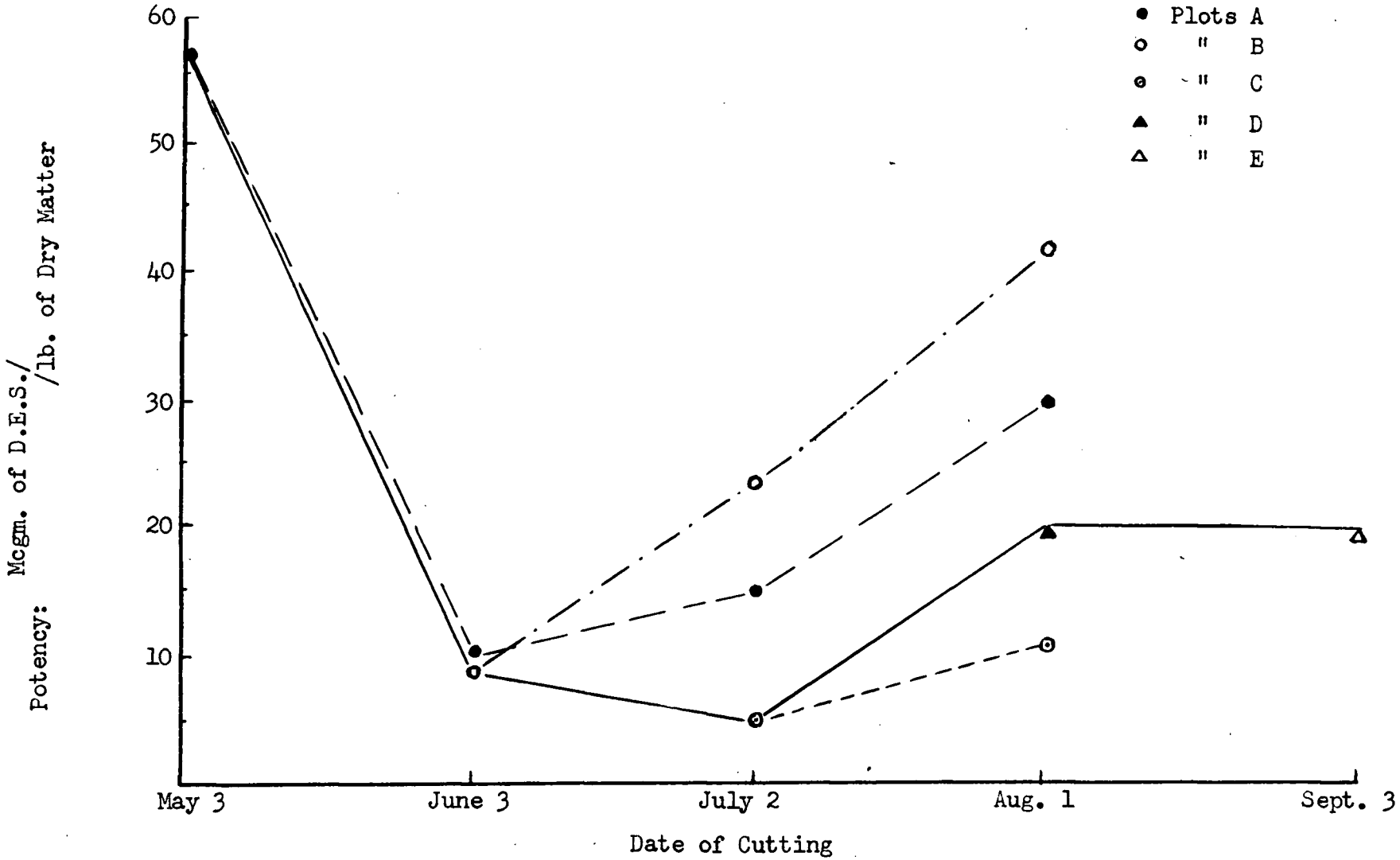


FIGURE IV

The Estrogenic Activity in Terms of D.E.S. of Second Year Growth of White Clover at Various Stages of Maturity and at Successive Cuttings.

TABLE XXXI The estrogen-like activity of first cuttings of white clover at different stages of maturity (first year growth, 1957)

Sample Description					Mouse Bio-assay					
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A	Aug. 15	Vegetative	90.4	26.50	12	9.60	16.74	0.174±0.003	5.0	0
B	Sept. 25	Vegetative	88.1	22.68	11	8.93	10.69	0.120±0.005	5.0	0

TABLE XXXII The estrogen-like activity of 1st and 2nd cuttings of white clover of plots A (first year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	Aug. 15	Vegetative	90.4	26.50	12	9.60	16.74	0.174±0.003	5.0	0
2	Sept. 25	Vegetative	87.1	25.10	12	9.51	15.01	0.158±0.003	5.0	0

was the second cutting of plots B, cut on July 2. After August 1 all samples showed an increase in estrogenic activity. This increase was in most cases of a greater magnitude than the increase in estrogen-like substances in corresponding alfalfa samples. The samples from first year growth which were in the vegetative state and cut between August 15 and September 25 did not show any estrogenic activity.

3. The Effect of Stage of Maturity and Frequency of Cutting on the Estrogenic Activity of Red Clover (TABLES XXXIII to XXXVII and FIGURE V).

Red clover produces flowers throughout the summer and fall similar to white clover. However, its estrogenic pattern differs considerably from the latter. All samples of first and second year growth showed considerable estrogenic activity with highest potency in the spring and decreasing towards the fall. Samples of first year growth were relatively higher in activity than the samples from second year growth cut at the same time. However, the former samples were in the reproductive state, while the latter were past bloom. Legg et al. (85) made similar observations on red clover in Great Britain. These workers postulated that the presence of estrogenic substances was associated with reproductive growth. This hypothesis may hold true for red clover but certainly not for the other forages studied.

4. The Effect of Stage of Maturity and Frequency of Cutting on the Estrogenic Activity of Birdsfoot Trefoil (TABLES XXXVIII to XLIV).

The only sample of birdsfoot trefoil that showed considerable

TABLE XXXIII The estrogen-like activity of first cuttings of red clover at different stages of maturity (second year growth, 1956)

Sample Description				Mouse Bio-assay					
Plot	Date of Cutting	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Weight (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption per 3 days (gm.)	Estimated Potency per lb. of Dry Matter (mcgm.)
A	May 2	82.6	22.00	8	8.46	47.0	0.56±0.02*	3.8	34
B	May 25	73.8	19.52	8	10.62	62.6	0.59±0.01	3.9	36
C	June 18	84.2	16.03	8	8.72	41.7	0.48±0.01	3	33
D	July 2	73.2	15.87	8	9.09	44.1	0.49±0.02	3.5	30
E	July 18	69.5	14.62	8	9.63	37.6	0.39±0.02	5	14
F	Aug. 18	58.8	13.26	8	-	-	0.28±	5	6
G	Sept. 2	56.9	14.65	8	9.36	23.0	0.25±0.01	5	<5

* Standard error

TABLE XXXIV The estrogen-like activity of first, second and third cuttings of red clover of plots B (second year growth, 1956)

Sample Description				Mouse Bio-assay					
Cutting	Date of Cutting	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Weight (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption per 3 days (gm.)	Estimated Potency per lb. of Dry Matter (mcgm.)
1	May 25	73.8	19.52	8	10.62	62.6	0.59±0.01	3.8	36
2	July 18	75.9	17.71	8	11.11	61.9	0.57±0.04	3.9	34
3	Sept. 2	57.9	14.87	8	10.36	43.8	0.42±0.02	5.4	15

TABLE XXXV The estrogen-like activity of first, second and third cuttings of red clover of plots[C] (second year growth, 1956)

Sample Description				Mouse Bio-assay					
Cutting	Date of Cutting	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Weight (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption per 3 days (gm.)	Estimated Potency per lb. of Dry Matter (mcgm.)
1	June 18	84.2	16.03	8	8.72	41.7	0.48±0.01	3	33
2	July 18	75.6	17.90	8	9.53	43.6	0.49±0.03	5	21
3	Aug. 18	70.2	15.76	8	9.60	31.1	0.33±0.02	5	9

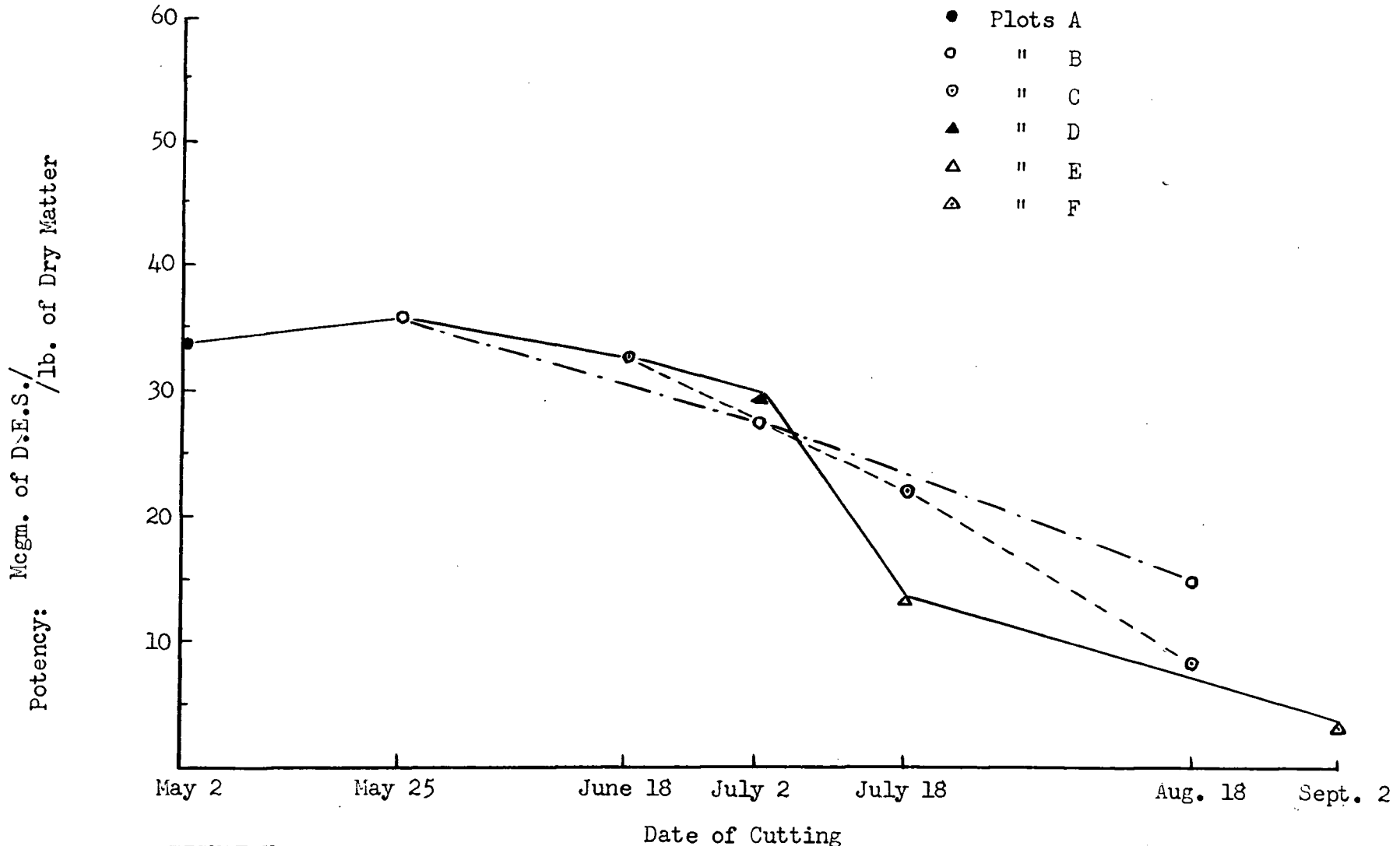


FIGURE V

The Estrogenic Activity in Terms of D.E.S. of Second Year Growth of Red Clover at Various stages of Maturity, and at Successive Cuttings.

TABLE XXXVI The estrogen-like activity of first cuttings of red clover at different stages of maturity (first year growth, 1957)

Sample Description					Mouse Bio-assay				
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A	Aug. 15	Early Buds	86.7	21.75	12	10.18	36.87	0.362±0.019	4.8	18
B	Sept. 25	Full Bloom	68.1	15.74	11	9.46	34.11	0.361±0.017	5.0	18

TABLE XXXVII. The estrogen-like activity of first and second cuttings of red clover of plots A (first year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. of Dry Matter (mcgm.)
1	Aug. 15	Early Buds	86.7	21.75	12	10.18	36.87	0.362 ± 0.019	4.8	18
2	Sept. 25	Few Flowers	76.4	21.45	11	8.79	28.23	0.321 ± 0.016	4.7	15

TABLE XXXVIII The estrogen-like activity of first cuttings of birdsfoot trefoil at different stages of maturity (second year growth, 1957)

Sample Description					Mouse Bio-assay				
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A	May 3	Vegetative	87.7	24.81	11	9.26	32.71	0.364 ± 0.026	4.1	22
B	June 3	1/3 Bloom	77.5	17.62	10	10.10	16.44	0.163 ± 0.006	4.8	0
C	July 2	Late Bloom	73.6	18.45	11	9.59	17.85	0.188 ± 0.006	4.8	1
D	Aug. 1	Late Bloom (second wave of flowering)	67.6	16.34	11	8.83	14.38	0.163 ± 0.006	3.6	0
E	Sept. 3	Past Bloom	61.4	11.31	9	8.73	17.53	0.201 ± 0.011	3.9	4

TABLE XXXIX The estrogen-like activity of 1st, 2nd, 3rd, 4th and 5th cuttings of birdsfoot trefoil of plots A (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	May 3	Vegetative	87.7	24.81	11	9.26	32.71	0.364±0.026	4.1	22
2	June 3	Early Budding	84.1	27.37	10	10.08	18.15	0.179±0.009	4.8	1
3	July 2	Vegetative	82.3	20.25	11	8.58	13.78	0.161±0.008	4.5	0
4	Aug. 1	Early Bud	83.9	26.25	11	8.75	12.26	0.154±0.006	4.6	0
5	Sept. 3	Vegetative	67.6	17.63	9	9.20	12.30	0.133±0.007**	4.7	0

* Significantly decreased $P < 0.05$
** " " $P < 0.01$

TABLE XL

The estrogen-like activity of 1st, 2nd, 3rd and 4th cuttings of birdsfoot trefoil of plots B (second year growth, 1957)

Sample Description					Mouse Bio-assay				
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	June 3	1/3 Bloom	77.5	17.62	10	10.10	16.44	0.163 ± 0.006	4.8	0
2	July 2	Very Early Bud	81.9	21.19	11	9.37	14.39	$0.154 \pm 0.003^{*}$	4.9	0
3	Aug. 1	Late Budding Early Flowering	82.2	26.25	11	9.03	13.35	$0.146 \pm 0.006^{*}$	4.3	0
4	Sept.	Vegetative	69.4	17.22	10	9.71	13.21	$0.137 \pm 0.004^{**}$	5.0	0

* Significantly decreased $P < 0.05$
** " " $P < 0.01$

TABLE XLI The estrogen-like activity of 1st, 2nd and 3rd cuttings of birdsfoot trefoil of plots C (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	July 2	Late Bloom	73.6	18.45	11	9.59	17.85	0.188 ± 0.006	4.8	1
2	Aug. 1	Budding	83.4	27.62	10	8.56	14.80	0.171 ± 0.010	4.1	0
3	Sept. 3	Vegetative	78.6	20.22	12	8.80	14.18	0.161 ± 0.008	4.2	0

TABLE XLII

The estrogen-like activity of 1st and 2nd cutting of birdsfoot trefoil of plots D (second year growth, 1957)

Sample Description						Mouse Bio-assay				
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	Aug. 1	Late Bloom (second wave of flowering)	67.6	16.34	11	8.83	14.38	0.163±0.006	3.6	0
2	Sept. 3	Vegetative	77.7	17.71	11	9.17	13.95	0.151±0.007*	4.2	0

* Significantly decreased $P < 0.05$
** " " $P < 0.01$

TABLE XLIII The estrogen-like activity of first cuttings of birdsfoot trefoil at different stages of maturity (1st year growth, 1957)

Sample Description					Mouse Bio-assay				
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency Per lb. Dry Matter (mcgm.)
A	Aug. 15	Vegetative (few buds)	86.6	16.32	12	9.44	14.97	0.157±0.007	4.8	0
B	Sept. 25	Vegetative (few pods)	78.0	10.82	9	10.20	15.77	0.154±0.007	5.0	0

TABLE XLIV The estrogen-like activity of 1st and 2nd cuttings of birdsfoot trefoil of plots A (1st year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	Aug. 15	Vegetative (few buds)	86.6	16.32	12	9.44	14.97	0.157±0.007	4.8	0
2	Sept. 25	Vegetative	87.1	18.18	12	9.84	14.44	0.148±0.008*	4.5	0

* Significantly decreased $P<0.05$
** " " $P<0.01$

estrogenic activity was the first cutting of second year growth, cut May 3. None of the other samples analyzed showed significant amounts of estrogen-like substances as measured by the increase in uterine weight of immature female mice. However, it is interesting to note that in many bio-assays the average uterine weight and the average feed intake of the mice were significantly reduced. This decrease in uterine weight may be explained by assuming that birdsfoot trefoil contains a compound or series of compounds which lower directly or indirectly the weight of the uteri of immature mice, since starvation itself does not lead to a decrease in uterine weight as great as resulted from the feeding of birdsfoot trefoil extract.

5. The Effect of Stage of Maturity and Frequency of Cutting on the Estrogenic Activity of Orchard Grass (TABLES XLV to L).

The first cutting of orchard grass harvested on May 3 was the only sample of orchard grass analyzed that showed significant estrogenic activity ($P < 0.01$). This confirms the results given in the literature. Estrogenic activity of orchard grass has been reported in very few cases (82); most investigators (82, 48) did not detect any estrogen-like compounds in this plant species.

6. The Effect of Stage of Maturity on the Estrogenic Activity of the Second Year Growth of Alsike Clover.

From May 13 to August 1 different plots of the second year growth of alsike clover were cut at two week intervals.

The estrogen-like activity of the samples is recorded in TABLE LI. As compared to white and red clover samples, the alsike samples showed very little or no estrogenic activity. However, it is

TABLE XLV The estrogen-like activity of first cuttings of Orchard Grass at different stages of maturity (second year growth, 1957)

Sample Description					Mouse Bio-assay				
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A	May 3	Vegetative	81.6	11.75	12	10.35	23.15	0.224±0.008*	5.0	5
B	June 3	½ Bloom	73.6	11.47	12	9.84	16.95	0.171±0.010	5.0	0
C	July 2	Full Bloom	63.9	11.14	12	8.93	14.05	0.158±0.010	5.0	0
D	Aug. 1	Late Seed (much undergrass)	59.8	6.22	11	9.17	15.17	0.164±0.008	5.0	0
E	Sept. 3	Seed (much undergrass)	45.9	5.81	10	8.80	17.56	0.201±0.007	5.0	3

* Standard error

TABLE XLVI The estrogen-like activity of 1st, 2nd and 3rd cuttings of Orchard Grass of plot A (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	May 3	Vegetative	81.6	11.75	12	10.35	23.15	0.224±0.008	5.0	5
2	June 3	Vegetative	80.4	13.90	11	9.82	18.27	0.186±0.008	5.0	1
3	July 22	Vegetative	71.7	8.43	12	9.34	16.19	0.173±0.010	5.0	0

TABLE XLVII The estrogen-like activity of 1st and 2nd cuttings of Orchard Grass of plots B (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	June 3	½ Bloom	73.6	11.47	12	9.84	16.95	0.171±0.010	5.0	0
2	July 22	Vegetative	71.9	7.62	12	9.19	15.64	0.169±0.006	5.0	0

TABLE XLVIII The estrogen-like activity of 1st and 2nd cuttings of Orchard Grass of plots C (second year growth, 1957)

Sample Description					Mouse Bio-assay				
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	July 2	Full Bloom	63.9	11.14	12	8.93	14.05	0.158±0.010	5.0	0
2	Aug. 1	Vegetative	76.5	8.25	12	8.68	15.93	0.183±0.005	5.0	1

TABLE XLIX The estrogen-like activity of first cuttings of Orchard Grass at different stages of maturity (first year growth, 1957)

Sample Description					Mouse Bio-assay					
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A	Aug. 15	Vegetative	-	13.25	12	9.94	17.13	0.172±0.007	5.0	0
B	Sept. 25	Vegetative	66.9	9.50	10	10.32	19.85	0.189±0.009	5.0	2

TABLE L

The estrogen-like activity of 1st and 2nd cuttings of Orchard Grass of plots A (first year growth, 1957)

Sample Description					Mouse Bio-assay					
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	Aug. 15	Vegetative	-	13.25	12	9.94	17.13	0.172±0.007	5.0	0
2	Sept. 25	Vegetative	68.1	10.30	12	9.62	15.21	0.157±0.008	5.0	0

TABLE LI

The estrogen-like activity of first cuttings of alsike clover at different stages of maturity (second year growth, 1957)

Sample Description					Mouse Bio-assay					
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. of Dry Matter (mcgm.)
A	May 13	Vegetative	84.9	19.97	12	9.86	25.23	0.257±0.010*	5	8
B	June 4	Full Bloom	82.5	18.06	12	10.43	20.20	0.194±0.006	5	2
C	June 18	Little Past Full Bloom	80.4	16.00	10	9.38	14.74	0.158±0.010	5	0
D	July 2	Late Bloom	69.1	15.91	10	9.25	13.43	0.145±0.006	5	0
E	July 22	Past Bloom (moldy)	48.9	14.00	10	10.32	21.11	0.205±0.010	5	3
F	Aug. 1	Past Bloom (moldy)	28.7	16.75	10	9.99	25.38	0.256±0.018	5	8

* Standard error

interesting to note that the last sample cut on August 1 showed significant amounts of these compounds to be present. The plant material in this sample was in the drought stage, had a dark brown color and was moldy.

7. The Influence of Length of Daylight on the Estrogenic Activity of Red Clover.

An experiment was designed to study the effect of exposure to different lengths of daylight (Photoperiod) on the level of estrogen-like compounds in second year growth of red clover. The results obtained in this investigation are given in TABLES LII to LIX. On May 3 all plots were cut and samples for analysis were obtained by pooling the plant material of the four ultimate A_1 plots, A_2 plots, B_1 plots, etc. (See "Materials.") Proximate analysis of these samples were carried out (TABLE LIII). The data reveal no significant difference in composition and estrogenic potency between the samples.

From May 3 to June 4 all B and C plots were covered with wooden boxes (See "Materials.") from 4:30 P.M. to 8:30 A.M.; in other words, these plants received only eight hours of daylight per day. On June 4 the ultimate plots designated with odd numbers were harvested. Duplicate samples were obtained by pooling plant material of two corresponding odd numbered ultimate plots. Six samples were obtained in total, two of which had received full daylight and four of which had been restricted to eight hours of daylight per day. The proximate analyses of these samples are given in TABLE LIV and the bio-assay results in TABLE LV. The plant material from the control plots A_1 was physiologically older than that of the plots (B_1 and C_1) which had been under reduced

TABLE LII

The estrogen-like activity of first cuttings of red clover (second year growth, 1957)

Sample Description						Mouse Bio-assay				
Plots	Cutting*	Length of Daylight	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A_1+A_2	1	Full Day	Vegetative	86.8	23.47	10	10.08	49.82	0.495±0.026	5.0	36
A_1+A_2	1	Full Day	Vegetative	86.8	23.56	10	9.97	50.82	0.512±0.025	4.3	37
B_1+B_2	1	Full Day	Vegetative	86.7	23.25	10	10.27	54.60	0.532±0.019	4.4	39
B_1+B_2	1	Full Day	Vegetative	87.3	23.28	12	10.34	51.58	0.501±0.021	4.3	36
C_1+C_2	1	Full Day	Vegetative	86.7	23.31	11	10.21	50.94	0.500±0.021	4.2	37
C_1+C_2	1	Full Day	Vegetative	87.2	23.53	11	9.72	46.51	0.480±0.025	4.3	34

* Cut on May 3, 1957.

TABLE LIII

Proximate analyses of first cuttings of red clover

(second year growth, 1957)

Sample Description				Proximate Analyses						
Plots	Cutting*	Length of Daylight	Stage of Maturity	Per cent Dry Matter	Per cent Protein	Per cent Fat	Per cent Crude Fiber	Per cent Ash	Per cent N-free Extract	Estimated Potency per lb. of Dry Matter (mcgm.)
A_1+A_2	1	Full Day	Vegetative	13.2	23.5	6.0	12.7	8.5	49.3	36
A_1+A_2	1	Full Day	Vegetative	13.2	23.6	6.0	13.8	8.2	48.4	37
B_1+B_2	1	Full Day	Vegetative	13.3	23.3	5.3	13.5	9.1	48.8	39
B_1+B_2	1	Full Day	Vegetative	12.7	23.3	5.9	12.6	9.0	49.2	36
C_1+C_2	1	Full Day	Vegetative	13.3	23.3	5.3	12.5	8.5	50.4	37
C_1+C_2	1	Full Day	Vegetative	12.8	23.5	5.3	13.4	8.7	49.1	34

* Cut on May 3, 1957.

TABLE LIV Proximate analyses of second cuttings of plots of red clover which were exposed to different periods of daylight (second year growth, growing period - May 2 to June 4, 1957)

Sample Description				Proximate Analyses						
Plots	Cutting	Length of Daylight	Stage of Maturity	Per cent Dry Matter	Per cent Protein	Per cent Fat	Per cent Crude Fiber	Per cent Ash	Per cent N-free Extract	Estimated Potency per lb. of Dry Matter (mcgm.)
A_1	2	Full Day	Very Early Bud	15.9	21.4	5.1	14.0	8.3	51.2	38
A_1	2	Full Day	Very Early Bud	14.2	22.2	5.4	14.9	8.5	49.0	36
B_1	2	8hr. Day*	Vegetative	14.7	29.3	5.1	13.3	9.4	42.9	34
B_1	2	8 hr. Day	Vegetative	15.3	28.3	5.1	14.2	9.8	42.6	29
C_1	2	8 hr. Day	Vegetative	-	27.2	5.5	13.2	9.1	45.0	25
D_1	2	8 hr. Day	Vegetative	15.5	28.9	5.7	13.2	9.9	42.3	35

* Daylight from 8:30 A.M. to 4:30 P.M.

TABLE LV

The estrogen-like activity of second cuttings of plots of red clover which were exposed to different periods of daylight (second year growth, growing period - May 2 to June 4, 1957)

Sample Description						Mouse Bio-assay				
Plots	Cutting	Length of Daylight	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A_1	2	Full Day	Very Early Bud	84.1	21.37	12	9.39	51.04	0.545±0.014	4.6	38
A_1	2	Full Day	Very Early Bud	85.8	22.22	12	10.09	53.14	0.526±0.016	4.6	36
B_1	2	8 hr. Day*	Vegetative	85.3	29.25	12	10.68	56.98	0.535±0.023	5.0	34
B_1	2	8 hr. Day	Vegetative	84.7	28.31	11	10.04	48.47	0.483±0.025	5.0	29
C_1	2	8 hr. Day	Vegetative	81.0	27.16	12	10.53	45.74	0.397±0.024	5.0	25
C_1	2	8 hr. Day	Vegetative	84.5	28.90	11	10.30	54.28	0.530±0.026	4.8	35

* Daylight from 8:30 A.M. to 4:30 P.M.

daylight. The fiber content of the samples of the former plots was higher and the protein content lower. The average potency of the control samples corresponded to 37 mcgm. of D.E.S., and that of the samples taken from plots which had been restricted to eight hours of daylight to 31 mcgm. Since the difference between 37 mcgm. and 31 mcgm. can be accounted for by chance, no definite conclusion can be reached as to the effect of length of daylight on the subsequent estrogenic activity.

From June 4 to June 20 only the C plots were restricted to eight hours of daylight. On the last mentioned date the plant material from the ultimate plots designated with even numbers was harvested. By that time the plants on plots B_2 had reached approximately the same physiological age as those of plots A_1 on June 4. Again, duplicate samples were obtained by pooling plant material of two corresponding ultimate plots. Proximate analyses of these samples are given in TABLE LVII. The estrogenic potency of the control plots had dropped from 37 to 29.5 mcgm. of D.E.S., while that of the C_2 plots, which had been restricted in daylight over the whole growing period, had dropped from 31 to 28.5 mcgm. of D.E.S. Restoring plots B_2 to full daylight for the last 16 days of the growing period resulted in an increase from 31 to 33 mcgm. of D.E.S. per lb. of dry matter. However, this decrease is not statistically significant. The physiological age of the plant decreased from plots A_2 to C_2, while the protein content increased in the same order.

The third part of this experiment was designed to study the effect of restricting the daylight received by the plants over the first part of the growing period as compared to restricting them over the

TABLE LVI

Proximate analyses of second cuttings of plots of red clover which were exposed to different periods of daylight (second year growth, growing period - May 2 to June 20, 1957)

Sample Description				Proximate Analyses						
Plots	Cutting	Length of Daylight	Stage of Maturity	Per cent Dry Matter	Per cent Protein	Per cent Fat	Per cent Crude Fiber	Per cent Ash	Per cent N-free Extract	Estimated Potency per lb. of Dry Matter (mcgm.)
A_2	2	May 4-June 20 Full Day	1/4 Bloom	18.5	17.9	4.7	20.0	7.3	50.1	28
A_2	2	May 4-June 20 Full Day	1/5 Bloom	18.1	20.3	4.1	19.0	7.1	49.5	31
B_2	2	May 4-June 4 8 hr. Day*	Very Few Buds	14.7	22.3	5.2	14.4	9.5	48.6	30
B_2	2	May 4-June 4 8 hr. Day	Vegetative	14.2	23.3	5.2	13.9	7.9	49.7	36
C_2	2	May 4-June 20 8 hr. Day	Vegetative	15.5	24.7	5.2	14.5	9.4	46.2	27
C_2	2	May 4-June 20 8 hr. Day	Vegetative	15.2	27.8	6.1	14.3	8.8	43.0	30

* Daylight from 8:30 A.M. to 4:30 P.M.

TABLE LVII

The estrogen-like activity of second cuttings of plots of red clover which were exposed to different periods of daylight (second year growth, growing period - May 2 to June 20, 1957)

Sample Description						Mouse Bio-assay				
Plots	Cutting	Length of Daylight	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A_2	2	May 4 - June 20 Full Day	$\frac{1}{4}$ Bloom	81.5	17.99	12	9.80	42.90	0.444±0.033	4.5	28
A_2	2	May 4 - June 20 Full Day	1/5 Bloom	81.9	20.28	12	9.06	45.82	0.508±0.031	4.5	31
B_2	2	May 4 - June 4 8 hr. Day*	Very few buds	85.3	22.29	11	9.41	46.57	0.492±0.027	5.0	30
B_2	2	May 4 - June 4 8 hr. Day	Vegetative	85.8	23.13	12	9.03	46.30	0.516±0.025	4.5	36
C_2	2	May 4 - June 20 8 hr. Day	Vegetative	84.5	24.74	11	9.93	45.16	0.460±0.030	5.0	27
C_2	2	May 4 - June 20 8 hr. Day	Vegetative	84.8	27.75	12	10.26	50.68	0.494±0.022	5.0	30

* Daylight from 8:30 A.M. to 4:30 P.M.

TABLE LVIII — Proximate analyses of third cuttings of plots of red clover which were exposed to different periods of daylight. (second year growth, growing period - June 4 to July 3, 1957)

Sample Description				Proximate Analysis						
Plots	Cutting	Length of Daylight	Stage of Maturity	Per cent Dry Matter	Per cent Protein	Per cent Fat	Per cent Crude Fiber	Per cent Ash	Per cent N-free Extract	Estimated Potency per lb. of Dry Matter (mcgm.)
A_1	3	June 4-July 3 Full Day	Budding	15.1	22.3	6.1	13.7	8.5	49.4	29
A_1	3	June 4-July 3 Full Day	Budding	15.9	21.1	5.4	13.9	8.0	51.6	25
B_1	3	June 4-June 20 Full Day June 20-July 3 8 hr. Day*	Budding	13.1	26.8	5.9	12.3	8.4	46.6	28
B_1	3	June 4-June 20 Full Day June 20-July 3 8 hr. Day	Budding	12.5	26.7	5.1	15.7	9.3	43.2	23
C_1	3	June 4-June 20 Vegetative 8 hr. Day June 20-July 3 Full Day		15.2	26.0	5.9	12.3	8.4	47.4	32
C_1	3	June 4-June 20 Vegetative 8 hr. Day June 20-July 3 Full Day		13.8	26.6	5.6	12.5	9.0	46.3	22

* Daylight from 8:30 A.M. to 4:30 P.M.

TABLE LIX

The estrogen-like activity of third cuttings of red clover which were exposed to different periods of daylight (second year growth, growing period - June 4 to July 3, 1957)

Sample Description						Mouse Bio-assay					
Plots	Cutting	Length of Daylight	Stage of Maturity	Per cent Moisture	Per cent Protein	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A_1	3	June 4 - July 3 Full Day	Budding	84.9	22.25	12	9.46	42.39	0.447±0.027	4.5	29
A_1	3	June 4 - July 3 Full Day	Budding	84.1	21.12	12	9.21	39.08	0.425±0.016	4.8	25
B_1	3	June 4 - June 20 Full Day June 20 - July 3 8 hr. Day*	Budding	86.9	26.76	12	10.00	46.59	0.463±0.019	4.8	28
B_1	3	June 4 - June 20 Full Day June 20 - July 3 8 hr. Day	Budding	87.5	26.72	12	9.51	39.48	0.418±0.019	5.0	23
C_1	3	June 4 - June 20 8 hr. Day June 20 - July 3 Full Day	Vegetative	84.8	26.00	12	9.60	45.13	0.470±0.021	4.3	32
C_1	3	June 4 - June 20 8 hr. Day June 20 - July 3 Full Day	Vegetative	86.2	26.56	12	10.26	41.40	0.406±0.025	5.0	22

* Daylight from 8:30 A.M. to 4:30 P.M.

last part of the growing period. Plots C_1 were restricted in daylight from June 4 to June 20, while the B_1 plots had eight hours of daylight from June 20 to July 3. On July 3 the plots were harvested (third cutting) and the samples analyzed (TABLES LVIII AND LIX). Since there is so much difference between duplicates as far as the estrogenic activity is concerned, no reliable conclusions can be reached on this part of the experiment. The proximate analyses show, as expected, that the greater the physiological age of the plant, the higher the fiber content and the lower the protein content.

TABLES LIV, LVI and LVIII show that there is no correlation between estrogenic activity on the one hand and on the other hand protein, fat, fiber, ash or nitrogen free extract content of the samples.

D. The Stability of the Estrogen-like Substances of Plant Origin:

Little literature is available on the stability of the estrogen-like substances in plant materials. Alexander et al. (2) observed that storage of subterranean clover over a period of 48 hours at 16-21°C. did not affect the estrogenic activity. Prolonged storage at room temperature in air, and in the dark also did not affect the potency of dehydrated clover. However, the dehydration process itself lowered estrogenic activity of the clover sample.

The stability of estrogenic-like substances in dried ground alfalfa, red clover and white clover, as well as the stability of these compounds in feed extract mixtures was studied. Samples of plant material taken at different stages of maturity and samples of sequential cuttings were studied. The results of this investigation are recorded in TABLES LX to LXV.

TABLE LX shows the stability of the estrogen-like sugstances in first cuttings of ground dry alfalfa and in dry feed extract mixtures of the same samples. The sample of plot A was cut on May 3, extracted on May 20 and the feed-extract mixture assayed on May 22 and November 14. On May 22 the sample had an estrogenic activity corresponding to 55 mcgm. of D.E.S. and on November 14 a potency of 42 mcgm. of D.E.S. This shows that the estrogenic activity of the feed-extract mixture decreased when it was stored for six months in the dark and at room temperature. The dry ground sample of the plant material was stored in glass bottles with metal lids, at room temperature and in daylight during this six month period. On November 7 part of this sample was extracted and assayed for estrogenic activity on November 10. The bio-assay revealed that no estrogenic substances were present at that time. Similar results were obtained when sequential cutting of alfalfa were assayed in the above manner (TABLE LXI).

A study on the stability of the estrogen-like compounds in white clover gave similar results to those from the alfalfa samples. the longer the samples were stored the lower the hormone-like activity. However, here again the breakdown of the active compound is much more rapid in the dried ground plant material than in the dried feed extract mixtures (TABLES LXII and LXIII).

The stability of the active compound in red clover differs considerably from that of alfalfa and white clover. The estrogen-like substances in red clover are much more stable, little breakdown occurs when the ground dry material is stored for a five months' period at room temperature and subjected to daylight. Here the breakdown of the estrogenic compounds in the feed-extract mixture was of a

TABLE LX

The stability of the estrogen-like compounds in 1st cuttings of ground dry alfalfa (RHIZOMA) and in the dry free-alcohol extract mixtures of the same samples (second year growth, 1957)

Sample Description					Time of Storage		Mouse Bio-assay				
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	Date of Extraction	Date of Assaying	No. of Mice	Mean Body Wt. (gms)	Mean Uterine Wt. (mgms)	Per cent Body Wt.	Feed consumption 13 days (gm.)	Estimated Potency per lb. Dry Matter (mcgms.)
A	May 3	Vegetative	84.5	22.15	May 20	May 22	12	10.18	74.62	0.733±0.016	4.7	55
						Nov. 14	10	10.49	61.98	0.591±0.018	5.0	42
					Nov. 7	Nov. 10	12	9.92	16.93	0.170±0.011	5.0	0
B	June 3	Pre-Bloom	74.8	18.71	June 14	June 25	12	10.08	30.17	0.298±0.011	5.0	12
						Dec. 4	10	9.72	31.22	0.318±0.029	4.7	15
					Oct. 7	Oct. 15	11	9.64	22.96	0.237±0.015	5.0	6
					Nov. 7	Nov. 11	12	9.20	18.08	0.196±0.008	4.9	2
C	July 2	Full Bloom	66.0	13.72	July 9	July 20	12	9.96	21.85	0.218±0.011	5.0	4
						Dec. 5	10	9.80	22.48	0.230±0.010	5.0	6
					Nov. 11	Nov. 13	12	8.35	13.39	0.161±0.009	5.0	0
D	Aug. 1	Late Bloom	70.1	16.43	Aug. 27	Aug. 28	12	9.90	37.35	0.388±0.028	4.8	21
						Dec. 3	11	8.81	27.40	0.307±0.022	3.9	16
					Nov. 7	Nov. 17	12	10.29	17.08	0.168±0.007	5.0	0
E	Sept. 3	Past Bloom	63.6	7.93	Sept. 5	Sept. 9	12	9.81	34.27	0.350±0.020	5.0	17
						Dec. 4	9	10.81	37.27	0.345±0.027	4.8	17
					Nov. 10	Nov. 17	12	10.39	22.07	0.210±0.023	5.0	2

TABLE LXI The stability of the estrogen-like compounds in sequential cuttings of dry ground alfalfa (RHIZOMA), and in the dry feed-alcohol extract mixtures of the same samples. (plots A, second year growth, 1957)

Sample Description					Time of Storage		Mouse Bio-assay				
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	Date of Extraction	Date of Assaying	No. of Mice	Mean Body Wt. (gms.)	Mean Uterine Wt. (mgms.)	Per cent Body Wt. Consumption 3 days (gms.)	Feed	Estimated Potency per lb. Dry Matter (mcgms.)
1	May 3	Vegetative	84.5	22.15	May 20	May 22	12	10.18	74.62	0.733±0.016	4.7	55
						Nov. 14	10	10.49	61.98	0.591±0.018	5.0	42
					Nov. 7	Nov. 10	12	9.92	16.93	0.170±0.011	5.0	0
2	June 3	Vegetative	81.2	22.34	June 14	July 2	12	10.46	33.54	0.322±0.021	5.0	14
						Dec. 26	12	9.40	19.30	0.207±0.010	5.0	3
					Dec. 27	Jan. 2	11	8.98	18.49	0.209±0.015	4.7	4
3	July 2	Vegetative	76.2	22.75	July 20	Aug. 2	12	9.71	21.95	0.226±0.007	4.5	6
						Dec. 26	12	9.61	19.91	0.209±0.010	5.0	3
					Dec. 27	Jan. 2	9	9.20	18.32	0.201±0.015	4.1	3
4	Aug. 15	Vegetative (border effect)	79.5	18.53	Aug. 28	Sept. 2	12	9.92	59.34	0.598±0.024	4.6	43
						Dec. 26	12	9.39	4 .20	0.469±0.018	5.0	28
					Dec. 27	Jan. 2	12	9.50	34.96	0.371±0.019	4.3	22

TABLE LXII

The stability of the estrogen-like compounds in first cuttings of dry ground white clover, and in the dry feed-alcohol extract mixtures of the same samples (second year growth, 1957)

Sample Description					Time of Storage			Mouse Bio-assay				
Plots	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	Date of Extraction	Date of Assaying	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
A	May 3	Vegetative	87.7	26.69	May 16	May 22	12	9.65	70.00	0.720±0.023*	5.0	57
						Dec. 5	12	10.42	55.03	0.527±0.018	5.0	33
					Nov. 11	Dec. 2	12	10.57	18.65	0.176±0.011	5.0	0
B	June 3	Nearly Full Bloom	84.0	23.43	July 19	July 30	12	9.53	25.94	0.271±0.012	5.0	9
						Dec. 5	10	10.29	19.10	0.185±0.011	5.0	1
					Oct. 7	Oct. 15	10	9.10	12.80	0.151±0.008	4.6	0
					Nov. 11	Nov. 18	12	9.84	15.76	0.161±0.006	5.0	0
C	July 2	Full-Late Bloom	75.4	20.65	July 29	Aug. 12	12	9.77	21.53	0.221±0.010	5.0	5
						Dec. 6	11	10.17	22.37	0.220±0.009	5.0	4
					Nov. 12	Dec. 2	10	9.76	19.94	0.204±0.013	5.0	3
D	Aug. 1	Late Bloom	73.4	20.93	Sept. 6	Sept. 17	12	9.77	38.69	0.392±0.025	5.0	20
						Dec. 6	11	10.33	30.10	0.291±0.013	5.0	11
					Nov. 12	Nov. 30	11	10.01	25.30	0.253±0.011	5.0	8
E	Sept. 3	Past Bloom (seed)	59.4	18.63	Sept. 7	Sept. 17	12	9.46	36.25	0.381±0.031	5.0	20
						Dec. 9	12	10.22	21.96	0.215±0.010	5.0	4
					Nov. 13	Dec. 2	12	9.83	25.00	0.252±0.015	5.0	8

* Standard error

TABLE LXIII

The stability of the estrogen-like compounds in sequential cuttings of dry ground white clover, and in the dry feed-alcohol extract mixtures of the same samples (plots A, second year growth, 1957)

Sample Description					Time of Storage		Mouse Bio-assay				
Cutting	Date of Cutting	Stage of Maturity	Per cent Moisture	Per cent Protein	Date of Extraction	Date of Assaying	No. of Mice	Mean Body Wt. (gm.)	Mean Uterine Wt. (mgm.)	Per cent Body Wt.	Feed Consumption/3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
1	May 3	Vegetative	87.7	26.69	May 16	May 22	12	9.65	70.00	0.720±0.023	5.0	57
						Dec. 5	12	10.42	55.03	0.527±0.018	5.0	33
					Nov. 11	Dec. 2	12	10.57	18.65	0.176±0.011	5.0	0
2	June 3	¾ Bloom	84.2	26.47	July 8	July 10	12	9.81	26.90	0.273±0.017	5.0	10
						Dec. 26	12	9.42	20.18	0.218±0.012	5.0	4
					Dec. 27	Jan. 2	10	9.93	18.33	0.185±0.009	5.0	0
3	July 2	5/6 Bloom	79.7	22.47	July 30	Aug. 27	11	9.88	33.51	0.339±0.020	5.0	15
						Dec. 26	12	9.96	20.50	0.229±0.010	5.0	5
					Dec. 27	Jan. 2	10	10.12	15.14	0.149±0.006	5.0	0
4	Aug. 1	1/5 Bloom	73.4	25.44	Aug. 29	Sept. 2	12	10.21	52.92	0.518±0.025	5.0	30
						Dec. 27	12	10.78	49.79	0.460±0.022	5.0	27

TABLE LXIV The stability of the estrogen-like compounds in ground dry red clover and in the feed-extract mixture of the same sample (first cutting of 1956 seeding, cut May 3, 1957)

Sample Description			Time of Storage		Mouse Bio-assay					
Stage of Maturity	Per cent Moisture	Per cent Protein	Date of Extraction	Date of Assaying	Number of Animals	Mean Body Weight (gm.)	Mean Uterine Weight (mgm.)	Per cent Body Wt.	Feed Consumption/ 3 days (gm.)	Estimated Potency per lb. Dry Matter (mcgm.)
Vegetative	86.8	23.44	May 15	May 15	10	10.08	49.82	0.495±0.026	5.0	36
				Dec. 2	9	9.75	39.52	0.406±0.024	4.2	26
			Oct. 21	Oct. 24	12	9.69	48.50	0.503±0.018	5.0	31

greater magnitude than that in the dry ground plant material, (TABLE LXIV). In other feed - red clover extract mixtures stored over longer periods of time , the breakdown was less than that which occurred in the above sample.

Cheng et al. (38) demonstrated the presence of estrogenic substances in alsike, red and white clover and alfalfa hay. Pieterse et al. (92) also found estrogen-like substances in certain samples of alfalfa hay. However, no information was given in regard to time of cutting, stage of maturity and time of storage of these samples. Pieterse et al. (91) further showed that the estrogenic activity of alfalfa silage was significantly greater than that of fresh alfalfa. This type of storage seems to increase the potency as a result of fermentation.

E. The Effect of Plant Estrogens on Certain Laboratory Animals:

Apart from the publications on sheep, grazing on subterranean clover in Western Australia, little literature is available on the effect of plant estrogens on reproduction and growth. Engle (61) studied the conception rate in three groups of Columbia ewes grazing on ladino clover, birdsfoot trefoil and blue grass. The ewes grazing on blue grass conceived on the average three weeks earlier than those grazing on the other two pastures. Sixty-six per cent of ewes grazed on blue grass lambed to first service, while on ladino clover the figure was 41 per cent and on lotus 31 per cent. The majority of the ewes on the legume pasture conceived after it had been subjected to a killing frost. Mouse bio-assays revealed the presence of estrogenic compounds in ladino clover and birdsfoot trefoil but not in blue grass.

In humans, Coussens and Sierens (41) reported that a war-time

diet of tulip bulbs caused aberrations in the reproductive cycle of Dutch women. Estrogenic substances were shown to be present with the aid of a mouse bio-assay technique. The influence of estrogenic substances on milk production in the cow has been postulated (10, 98) but no direct proof has been obtained.

Mouse reproduction experiments Nos. I and II and the guinea pig experiment were designed to obtain more detailed information on the effects of the plant estrogens on the reproduction and growth of animals.

1. Mouse Reproduction Experiment No. I.

The plant material used in this experiment is described under "Material" while the feeding and mating procedures are fully discussed under "Methods". The mean initial body weight, the mean body weight at mating and the average food consumption per day are shown in TABLE LXV. The feed consumption of the mice receiving control ration with red clover extract was significantly decreased. The same was the case with mice receiving control ration plus birdsfoot trefoil extract. As a result of this reduced feed intake most mice on these rations decreased in body weight.

The effects of the different rations on the reproduction in the mouse are given in TABLE LXVI. In the discussion of the results the different groups of animals will be identified by the rations they were receiving, e.g., "red clover females" are a group of female mice receiving the control diet containing red clover extract. "Control females" bred to "control males" showed 100 per cent fertility, indicating that the control diet did not interfere with reproduction in the

TABLE LXV

Mean initial weight, mean body weight at mating and mean daily feed consumption of mice on reproductive experiment 1

	Females			Males		
	Mean Initial Body Weight (gm.)	Mean Mating Body Weight (gm.)	Mean Feed Consumption per day (gm.)	Initial Body Weight (gm.)	Mating Body Weight (gm.)	Mean Feed Consumption per day (gm.)
Control	26.0	25.2	7.55	25.3	26.4	7.12
Control + Red Clover Extract	27.1	23.8	4.02**	25.3	21.0	5.61*
Control + Birdsfoot Trefoil Extract	26.2	22.3	3.45**	26.3	22.7	4.88**
Control + D.E.S.	26.1	25.9	7.41	26.7	26.7	6.97

* $P < 0.05$
** $P < 0.01$

TABLE LXVI

Experimental data of female mice on reproductive experiment 1

Group	Mating	First Mating					Second Mating				
		No. of Females Bred	No. of Females Littering	No. of Young Born Alive	Mean No. per Litter	Per cent Fertility	No. of Females Bred	No. of Females Littering	No. of Young Born Alive	Mean No. per Litter	Per cent Fertility
1	Control Females x Control Male	5	5	48	9.6	100	0	0	0	0	0
2	Control Females x Red Clover Male	5	3	28	9.3	60	0***	0	0	0	0
3	Control Females x Birdsfoot Trefoil Male	5	3	27	9.0	60	2	1	10	10	50
4	Control Females x D.E.S. Male	5	5	47	9.4	100	0	0	0	0	0
5	Red Clover Females x Control Male	5	0	0	0	0	5	4	41	10.3	80
6	Birdsfoot Trefoil Females x Control Male	4*	1	9	9.0	25	3	3	25	8.3	100
7	D.E.S. Females x Control Male	5	2	17	8.5	40	3	3	32	10.7	100
8	Red Clover Females x Red Clover Male	5	0	0	0	0	5	5	35	7.0	100
9	Birdsfoot Trefoil Females x Birdsfoot Trefoil Male	3**	0	0	0	0	3	3	34	11.3	100
10.	D.E.S. Female x D.E.S. Male	5	3	17	5.7	60	2	2	21	10.5	100

* One animal died during the pre-mating period as a result of the ration.
** Two animals died during the pre-mating period as a result of the ration.
*** Remaining two animals of Group II had a skin infection and were removed from the experiment.

mouse. Three groups of five "control females" bred to "red clover males," "birdsfoot trefoil males" and "D.E.S. males" showed 60, 60 and 100 per cent fertility, respectively. This shows that the males receiving the "D.E.S. ration" were less affected by their diet than males on "red clover" or "birdsfoot trefoil rations."

Mating "control males" to groups of "red clover females," "birdsfoot trefoil females" and "D.E.S. females" resulted in 0, 0 and 40 per cent fertility. This indicates that the reproduction in the females was affected to a much greater extent than that of the males.

By mating "red clover females" to "red clover males," "birdsfoot trefoil females to birdsfoot trefoil males" and "D.E.S. females" to D.E.S. males, the per cent fertility of the groups was 0, 0 and 60, respectively. Bio-assays of the feed - red clover extract mixture and the ration containing D.E.S. resulted in the same increase in uterine weight. However, the red clover extract mixture interfered with the reproduction in the mouse to a much greater extent than did the ration containing 0.068 mcgm. of D.E.S. per gram of diet. This result is doubly interesting in that the estrogenic activity of the red clover extract expressed in terms of D.E.S. was 0.068 mcgm. per gram dry matter.

Those females which did not have a litter three weeks after the mating period was terminated were bred again to the same males in order to determine if both males and females had recovered from the previous treatment. With the exception of one "control female" of group 3 and one "red clover female" of group 6, all females and males recovered their reproductive ability.

Similar results were obtained by Fox et al. (71) who incorporated clover hay and fresh red clover in a mouse diet at the 40 per cent dry matter level, and found that females and males failed to reproduce on these diets. East (54) showed that when genistein, the estrogenic compound of red clover, was added to a mouse diet at the 0.2 per cent level it lowered the number of litters born, but not the number of young per litter. The present results in connection with red clover extract confirm these observations. However, East (54) also stated that genistein affected fertility in the males to a greater degree than in the females. This contradicts the results obtained in this experiment. Emmens (59) injected estradiol benzoate into male and female mice and observed that females were much more susceptible than males to this synthetic estrogenic hormone. These observations and those of Bennetts (18) in connection with rams grazing on "estrogenic" subterranean clover are more in line with the author's results. The results of this study also confirm observations made by Emmens (59) and Fox et al. (71) that male and female mice recover normal reproductive power after the hormone administration stops.

2. Mouse Reproduction Experiment No. II.

The clover used in this experiment had a potency corresponding to 12 mcgm. of D.E.S. This was approximately 60 per cent lower than that of the red clover used in mouse reproductive experiment No.I. The control ration and the control ration containing red clover extract were the same as those used in the guinea pig experiment. This was done to determine if different species of animals reacted differently to the same level of estrogen-like substances. Housing, feeding and the mating plan of the animals are fully discussed under "Methods."

The mean initial body weight, the mean body weight at mating and the mean daily feed consumption over this period are given in TABLE LXVII while the results on the reproductive performance of these animals are given in TABLE LXVIII.

Mating "control females" to "red clover males" resulted in 60 per cent fertility, while "red clover females" mated to "red clover males did not reproduce at all. These results are identical to those obtained in mouse reproduction experiment No. I. It should be noted that even though the results are the same, the relative potency of the red clover was 60 per cent lower than in the first experiment. Again the males were affected to a lesser extent than the females. The mean daily feed consumption of the animals receiving red clover extract was significantly lowered, as was the case in mouse reproduction experiment No. 1.

After the animals were returned to control diet they recovered quickly as is shown by the number of litters produced from the second mating (TABLE LXVIII).

3. The Guinea Pig Experiment.

Little literature is available on the influence of the plant estrogens on the growth of animals. Certain effects of estrogens in pasture plants on fattening lambs have been postulated (38) but no direct proof has been given. Carter et al. (34) showed that the growth of mice from three to seven weeks was not affected by either soybean oil meal or genistein. This last compound is the main source of estrogenic activity in red clover.

A guinea pig experiment was designed to acquire more information

TABLE LXVII Mean initial weight, mean body weight at mating and mean daily feed consumption of mice on reproductive experiment II

Ration	Females			Males		
	Mean Initial Body Wt.	Mean Mating Body Wt.	Mean Feed Consumption per day (gm.)	Initial Body Wt. (gm.)	Mating Body Wt. (gm.)	Mean Feed Consumption per day (gm.)
Control	23.9	25.2	7.60	25.2	26.4	7.20
Control + Red Clover Extract	23.6	23.2	4.20	28.3	27.7	5.04*

* P 0.05
** P 0.01

TABLE LXVIII

Experimental data of female Swiss albino mice fed control ration containing red clover extract (mouse reproductive experiment II)

Group	Mating	First Mating					Second Mating				
		No. of Females Bred	No. of Females Littering	No. of Young Born Alive	Mean No. per Litter	Per cent Fertility	No. of Females Bred	No. of Females Littering	No. of Young Born Alive	Mean No. per Litter	Per cent Fertility
1	Control Females x Control Male	5	5	48	9.6	100	0	0	0	0	0
2	Control Females x Red Clover Male	5	3	34	11.3	60	2	2	15	7.5	100
3	Red Clover Females x Red Clover Male	5	0	0	0	0	5	5	58	11.6	100

.026 mg per gm

on the effects of plant estrogens on growth and reproduction of animals having a digestive tract comparable to that of a ruminant. Growth curves obtained during the pre-mating period of the females receiving control ration are given in FIGURE VII and those of the animals receiving control ration containing red clover extract in FIGURE VIII. Statistical analysis revealed that the growth of guinea pigs weighing 500 to 600 grams was not significantly influenced by adding red clover extract to the ration. Feed consumption was a little increased as compared to the animals receiving control ration (TABLE LXIX). However, this increase was not significant.

TABLE LXIX Mean initial body weight, mean body weight at mating, and mean feed consumption per day of female guinea pigs over pre-mating period (33 days).

Group	Ration	Mean Initial Body Wt. (gm.)	Mean Body Weight at Mating (gm.)	Mean Feed Consumption per day (gm.)
1	Control	548	565	22.8
2	Control Containing Red Clover Extract	553	575	24.7

Five"control females" were mated to a "control male", and five "red clover females to a "red clover male." In the first group two out of five females conceived and in the second group three out of five were pregnant, (TABLE LXX).

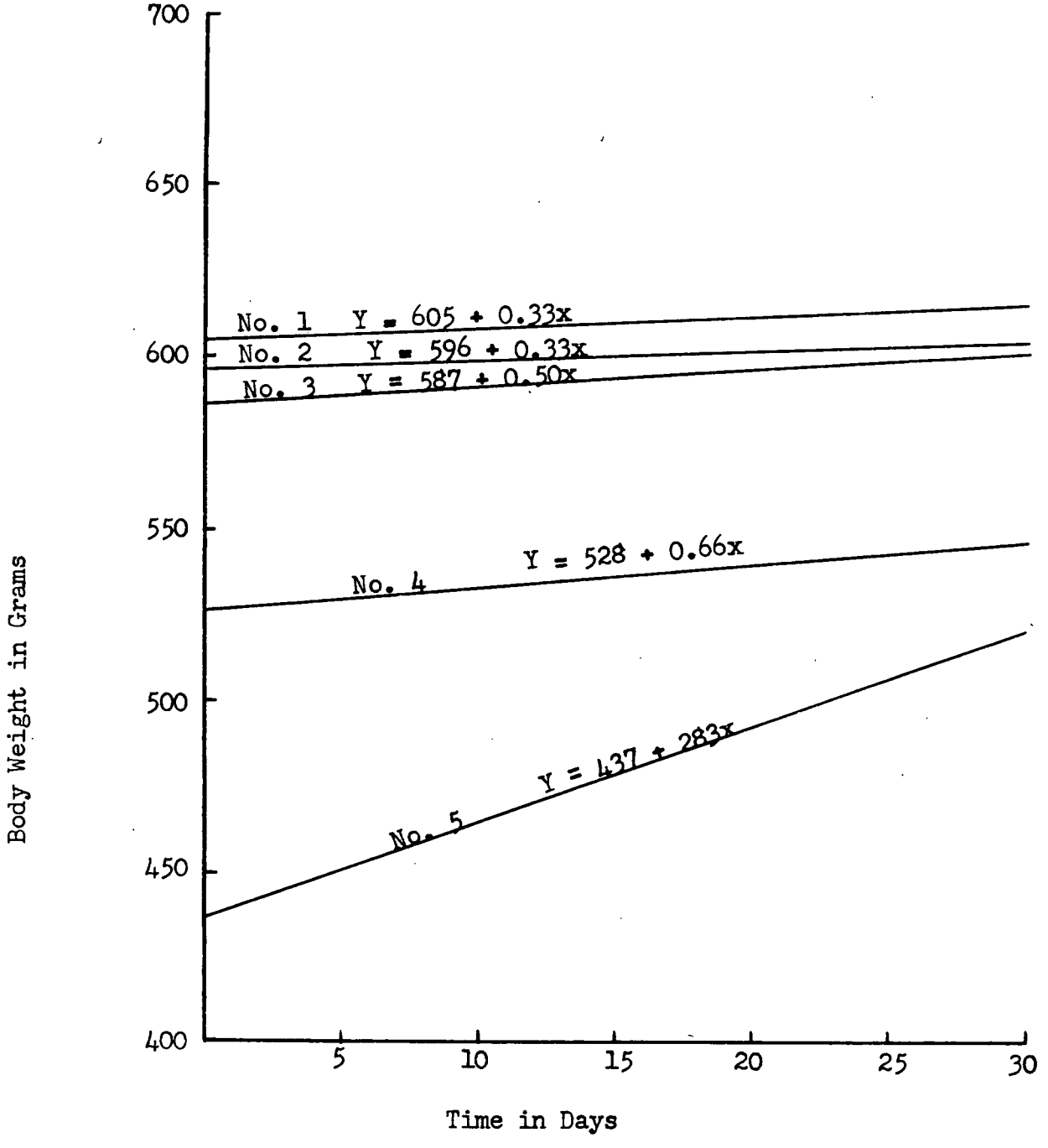


FIGURE VII

Growth Curves of Female Guinea Pigs Receiving Control Diet Containing Control Diet.

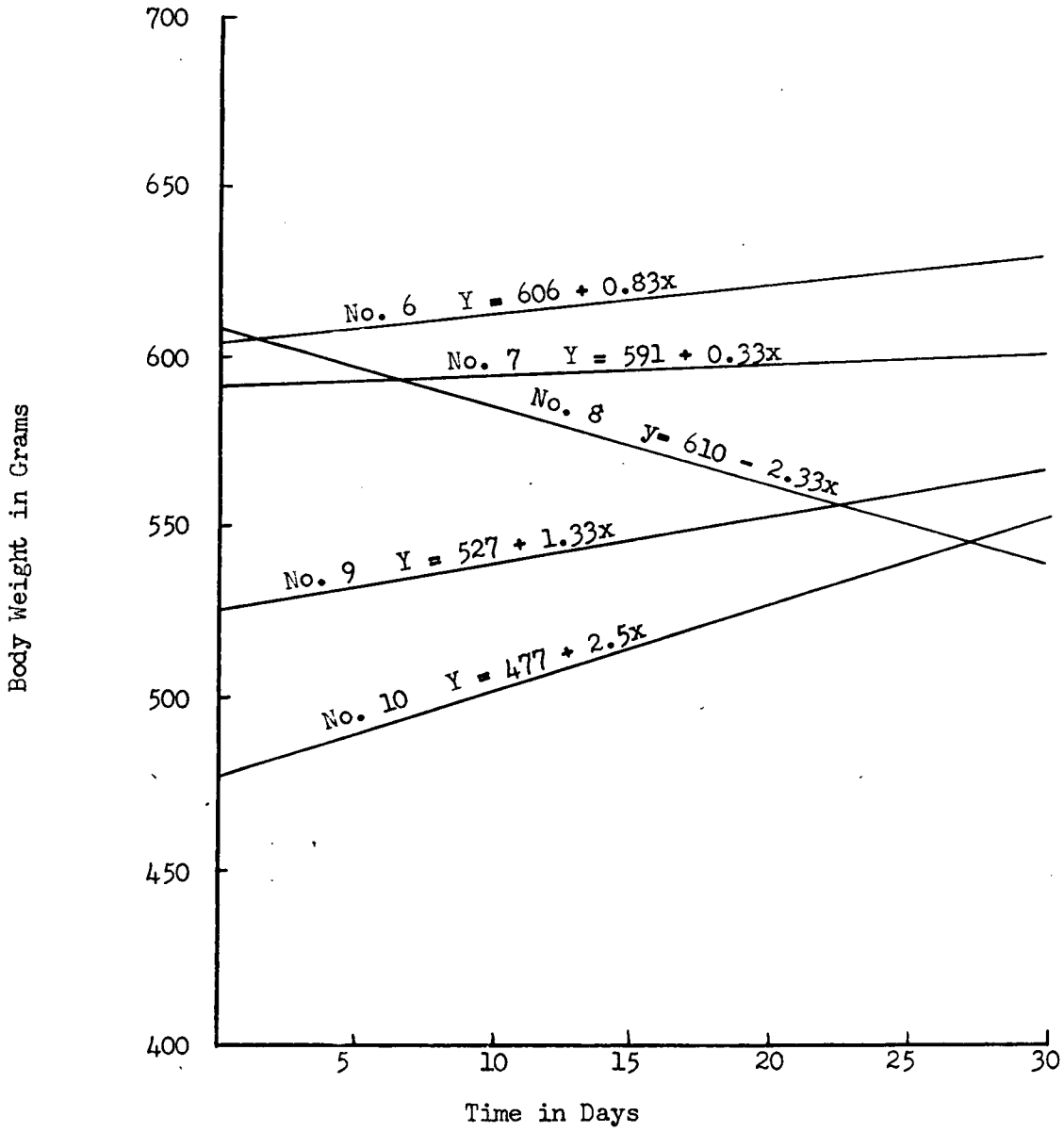


FIGURE VIII

Growth Curves of Female Guinea Pigs Receiving Control Diet Containing Red Clover Extract.

TABLE LXX Conception rate of guinea pigs

	Ration	Initial No. of Females	Number of Females			No. of Fetuses	Mean No. per Female
			Alive at Time of Mating	Alive at end of Mating Period	Pregnant		
1	Control	5	5	4	2*	1	1
2	Control + Red Clover Extract	5	5	5	3	9	3

* One early abortion

Since the fertility in the control group was poor and that of the "red clover group" below average no definite conclusion can be reached in regard to the effect of red clover extract on reproduction in the guinea pig. East (53) showed that ingestion of subterranean clover caused infertility in female guinea pigs, and that this was due to failure of conception rather than to failure of implantation.

However, the results of this experiment show that the reproduction in the guinea pig is affected to a much lesser extent if not at all, by red clover extract as compared to that of female mice receiving the same ration.

VII. SUMMARY AND CONCLUSION

To determine the estrogenic activity in certain forage samples a suitable mouse bio-assay procedure was developed. This procedure consisted of having an assay period of 72 hours during which each animal received five gm. of diet, of fasting the animals for eight hours, and of using uterine weight expressed as a per cent of body weight as the measurement of estrogenic activity of the experimental diets. A dose response curve was then constructed using once recrystallized diethylstilbestrol (D.E.S.) as the reference compound. From this curve the estrogenic potency of the samples of plant material was estimated, and expressed in terms of D.E.S.

Before studying the effect of stage of maturity, frequency of cutting and length of daylight (photoperiod) on the estrogen-like compounds of plant material, a number of preliminary experiments were carried out. The data of these experiments revealed that estrogenic activity of red clover was not significantly influenced by leaving the plant material on the field for 48 hours, before it was dried at 150°F. for 18 hours. Furthermore, the estrogenic activity of feed - red clover extract mixtures was not significantly influenced by either varying the drying time between 7 and 24 hours (drying temperature constant at 65°C.), or by varying drying temperature between 65 and 102°C. (drying time constant at 19 hours).

The studies of the effects of stage of maturity and frequency of cutting of alfalfa, white clover, red clover, birdsfoot trefoil and orchard grass on the estrogen-like compounds in these species, revealed

that there was a great difference between samples taken from different plant species at the same time of the year (PLATE I).

A seasonal variation in the estrogenic activity occurred in the first cuttings of second year growth of the different plant species (FIGURE VIII). Alfalfa and white clover possessed high estrogenic activity in the spring (May), showed a sharp decrease in June and July, while after August 1st, the active substances were present once again in considerable quantities. The only samples of birdsfoot trefoil, orchard grass and alsike clover that showed significant estrogenic activity were the first cuttings of second year growth harvested on May 3. An exception to this was the sample of alsike clover cut on August 1st. This plant material was in the dough state and appeared moldy. However, it is possible that in this case the estrogen-like substances were produced by the molds, since certain micro-organisms are capable of producing these estrogenic compounds.

Red clover differed greatly from the previously mentioned species. Again samples taken in May were highest in potency, but there was no sharp decrease during June and July as was the case with alfalfa and white clover. Nevertheless there was a gradual decrease towards the fall. Samples of first year growth were relatively higher in estrogenic activity than the samples from second year growth cut at the same time. Here again red clover differed considerably since none of the samples of first year growth of the other species studied showed significant amounts of estrogen-like substances. The hypothesis that estrogenic activity is associated with reproductive growth (82) holds true for the samples of red clover studied. However, this hypothesis is not valid for alfalfa and white clover, since these

THE INFLUENCE OF ESTROGENIC EXTRACT OF DIFFERENT SPECIES OF FORAGE ON UTERINE WEIGHT

CONTROL *
Uterine weight 19.80 mgm.
% Body weight .179

ORCHARD GRASS
1st cutting, 3rd May, 1957
Vegetative
Uterine weight: 21.00 mgm.
% Body weight .195

LOTUS
1st cutting, 3rd May, 1957
Vegetative
Uterine weight: 38.35 mgm.
% Body weight .372

RED CLOVER
1st cutting, 3rd May, 1957
Vegetative
Uterine weight: 54.05 mgm.
% Body weight: .524

ALFALFA
1st cutting, 3rd May, 1957
Vegetative
Uterine weight: 60.15 mgm.
% Body weight: .581

WHITE CLOVER
1st cutting, 3rd May, 1957
Vegetative
Uterine weight: 63.05 mgm.
% Body weight: .612

* All animals received 5 gm. of feed mixture/3 days and were fasted for 8 hours.

PLATE I

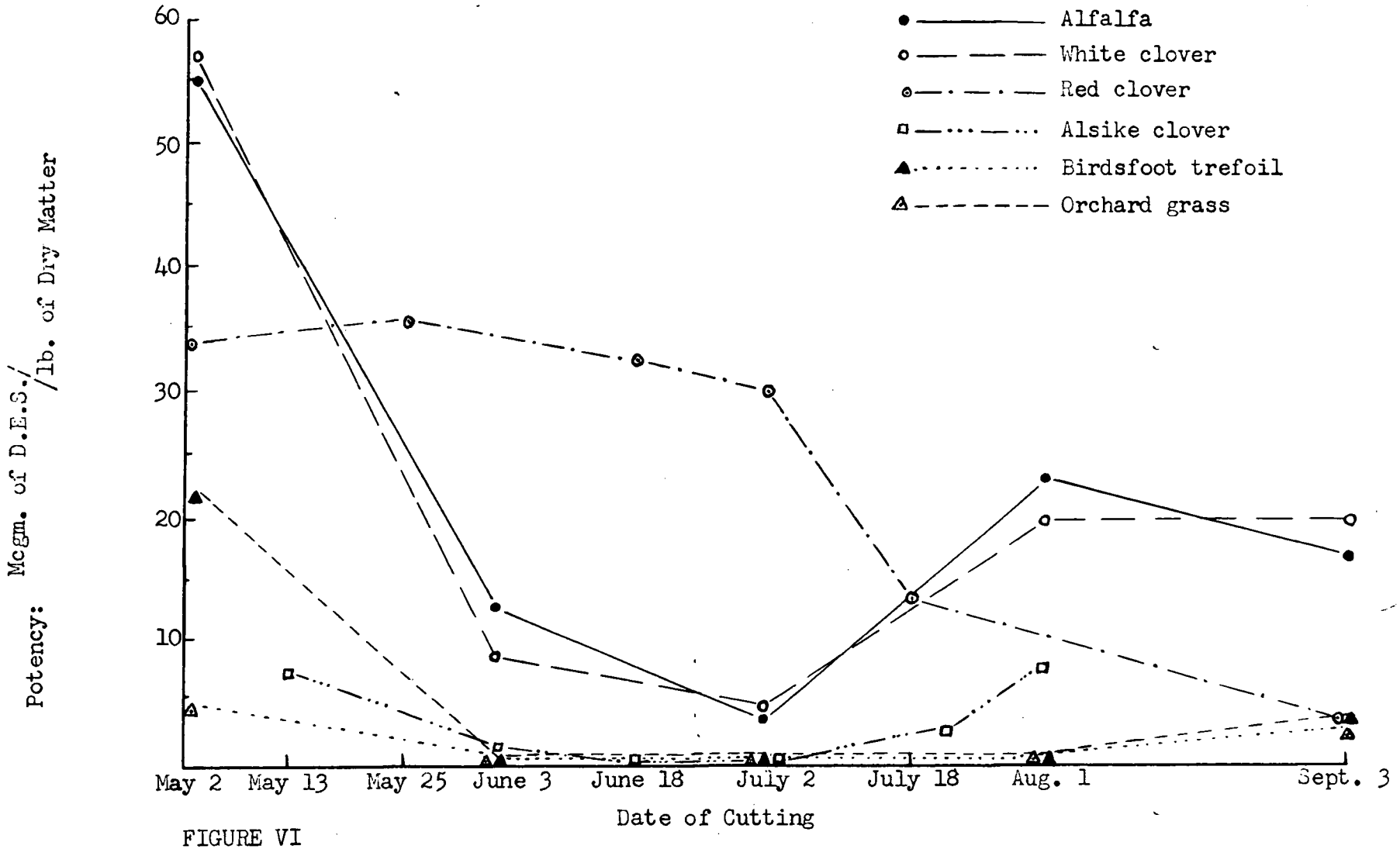


FIGURE VI

The Estrogenic Activity of Second Year Growth of Different Species of Plants at Various Stages of Maturity.

species were low in estrogenic activity when they were in the reproductive stage (June and July) and showed an increase in the "late bloom" and "past bloom" stages.

The results also indicate that estrogenic activity is not solely associated with the phase of rapid growth, since the samples of first cuttings of second year growth of alfalfa and white clover harvested in August and September showed higher estrogenic activity than second, third and fourth cuttings taken at the same time. Furthermore, first cuttings of first year growth of alfalfa and white clover did not contain any estrogen-like substances. The results also disprove a third hypothesis stating that seasonal variation is due to a change in lamina to stem ratio (2).

The data obtained by varying the number of hours of daylight (photoperiod) received by the plant seem to indicate that the estrogenic activity in red clover decreased when the photoperiod was shortened.

Proximate analysis of the samples revealed further that estrogenic activity was not correlated with the nitrogen content of the sample.

The study on the stability of the estrogen-like compounds in alfalfa, white clover and red clover suggested that the estrogenic activity of alfalfa and white clover was due to a different compound than that of red clover. This observation has been proven to be correct by Bickoff et al. (24) who isolated coumestrol from ladino clover, alfalfa and strawberry clover. This compound seems to be easily destroyed during storage (TABLES LX to LIV) while genistein of red clover is much more stable (TABLE LXIV). This may be due to the

fact that coumestrol contains in its chemical configuration an oxygen bridge which could be easily destroyed. It is interesting to note that estrogen-like compounds of alfalfa and white clover are not as easily destroyed in the feed - extract mixtures as in the dried ground forage sample. The reason for this may be due to the presence of a compound(s) which degrades the active substance.

The animal reproduction experiments showed clearly that red clover and birdsfoot trefoil extract interfered with the reproduction in the mouse. In both cases the females were more severely affected than the males. Furthermore, it was interesting to note that the reproduction in the mouse was influenced to a greater extent when red clover extract was fed than when a corresponding quantity of D.E.S. as measured by the uterine weight technique, was added to the diet. In nearly all cases both males and females recovered their reproductive ability quickly when the feeding of estrogen-like compounds ceased. In female guinea pigs weighing between 500 and 600 gm. the growth rate was not significantly influenced by the addition of red clover extract to the diet.

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