Doctoral Dissertations   Student Scholarship

Spring 1965

THE EFFECTS OF PLANT ESTROGENS ON THE FEMALE GUINEA PIG AND ON PARABIOTICMICE

BLAIR HENDRON CAMPBELL

Follow this and additional works at: https://scholars.unh.edu/dissertation

CAMPBELL, D.V.M., Blair Hendron, 1936–
THE EFFECTS OF PLANT ESTROGENS ON
THE FEMALE GUINEA PIG AND ON PARABIOTIC
MICE.

University of New Hampshire, Ph.D., 1965
Physiology

THE EFFECTS OF PLANT ESTROGENS

ON THE FEMALE GUINEA PIG AND ON PARABIOTIC MICE

by

BLAIR HENDRON CAMPBELL

D.V.M., Cornell University, 1960

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Zoology

June, 1965

This thesis has been examined and approved.

Paul A Wright

William L Bullock

[signature]

[signature]

Samuel C Smith

25 May 1965
_____
Date

ACKNOWLEDGEMENTS

In an expression of appreciation, the author wishes to acknowledge the help and guidance of the following people:

Dr. Paul A. Wright, for his excellent advice and liberal attitude toward the execution of this study.

My wife Marianne, for preparing the schematic anatomical drawings, for typing the manuscripts, and for her patient encouragement.

Dr. Willard E. Urban Jr., for his assistance in analyzing the data.

TABLE OF CONTENTS

TABLE OF CONTENTS (Cont.)

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

The ability of mammalian species to reproduce involves a complex coordination of many physiological functions. In view of its complexity, the reproductive process is remarkably stable, but certain phases are vulnerable to interference. Man's social, moral, and economic interests have been the cause of considerable concern about reproduction in his own and other species. Means to regulate reproduction are sought in compliance with efforts to control our environment and our burgeoning population. When existing interference with the reproductive process is considered a problem, studies are directed toward means of correction.

The present study deals with estrogens of plant origin, and includes a group of reproductive problems having effects considered detrimental to the sheep and cattle industries. Scientific association of specific plant compounds with mammalian reproduction is relatively current. It is quite possible that a more thorough knowledge of the pharmacology of plant estrogens and related compounds will provide not only immediate practical applications, but information regarding their usefulness or even their necessity.

The isolation and synthesis of estrogenic plant compounds has not been succeeded by a rush of enthusiasm on the part of investigators. The mode of action of these compounds in relation to endogenous estrogens, side effects, effects on

lactation, and potential therapeutic use in human and veter-
inary medicine represent areas that are almost totally unex-
plored. In view of this lack of information, the effects of
the plant estrogen coumestrol on the female reproductive tract
and the pituitary-gonad axis have been investigated. Labora-
tory animals were used in an effort to obtain maximal use of
a small quantity of compound available.

This study was supported in part by the National
Institute of Health Grant Number HD-00752-01, and by the
NE-41 Regional Project (Hatch Act) entitled "Factors in Forage
Affecting the Reproductive Endocrinology of Dairy Cattle".

II. REVIEW OF THE LITERATURE

1. PLANT ESTROGENS

Recently, excellent reviews of the problems connecting plants with mammalian reproduction have been published by Bickoff (1961), Leavitt (1963), and Moule, Braden and Lamond (1963). Consequently, the following review is shortened in areas not pertaining directly to the subject of the thesis. Publications concerning the general subject of plant estrogens and not mentioned by the above authors will be included.

The study of plant estrogens was given great impetus in Australia in the 1940's as a result of substantial losses in the sheep industry (Bennetts, Underwood, and Shier, 1946). A condition called "clover disease" appeared in ewes pastured on subterranean clover (<u>Trifolium</u> <u>subterraneum</u>) and was manifest by infertility, dystocia, uterine prolapse, and mammary development in virgin ewes and wethers (Bennetts <u>et</u> <u>al</u>., 1946). Pathology was characterized by endometrial cystic glandular hyperplasia in ewes, and epithelial metaplasia of the accessory sex glands in wethers. (Bennetts <u>et</u> <u>al</u>., 1946).

Since that time, estrogenic forages have been reported in Israel (Adler, 1960) New Zealand (Ch'ang, 1961), Chechoslovakia (Elghamry and Chury', 1961), France (Ferrando <u>et</u> <u>al</u>., 1963), Finland (Kallela, 1964), the USA (Pieterse

and Andrews, 1956), Great Britain (Pope, 1954), and Germany (Schoop and Klette, 1952). Schoop et al. (1952) related infertility of cattle to estrogenic pastures, as have Adler et al., (1960), Thaine (1963) and Wright (1960). The fertility of cattle may also be decreased by feeding kale (Brassica oleracea), a cabbage-like plant (Reed, 1961, and Melrose et al., 1963). Reports of estrogenic forages generally refer to legumes such as the clovers (Trifolium sp.), alfalfa (Medicago sativa), soybeans (Soja max), etc., as the source of estrogen.

In 1954, Bradbury and White cited fifty-four species of plants described in the literature as having estrogenic activity. These plants represent several orders of the Angiospermae and at least one Ascomycete (Bradbury and White, 1954). Pope et al. (1959) surveyed forages in British pastures by using an immature mouse uterine weight bioassay of ether and water extracts. They identified twenty estrogenic species.

Forages vary in estrogen content and attempts to explain this variation in relation to soil type, season, fertilizers, climate, etc., are not convincing (Alexander and Watson, 1951; Martin et al., 1958; Bickoff et al., 1960; Flux et al., 1963; and Youngman, 1963). In 1964, Loper and Hanson demonstrated significant increases in the coumestrol content of alfalfa infected with the leaf spot pathogens Pseudopeziza medicaginis and Leptosphaerulina briosiana. Estrogenic responses have been reported in swine fed moldy corn or barley (Koen and Smith, 1945; Buxton, 1927; and McNutt, 1927).

Abortions in cats, dogs, sheep and mice resulted from administration of yeast fractions (Whitney and Parfentjev, 1964).

At least six compounds with varying degrees of estrogenic activity have been isolated from plants. In 1951, Bradbury and White isolated the isoflavone genistein from subterranean clover. A glycoside of genistein, genistin, was isolated from soybean meal by Walter in 1941. Two other estrogenic isoflavones, formononetin and biochanin A, were found in red clover by Pope et al. (1953) and in subterranean clover by Beck et al. (1956) and Guggolz et al. (1961). Daidzein, another isoflavone, was isolated from four common pasture legumes by Guggolz et al. in 1961. An aglycone of daidzein, daidzin, had previously been found in soybean meal by Walz (1931). Coumestrol, an ester of a coumarin compound, was isolated from ladino clover in 1957 by Bickoff et al. This plant estrogen is approximately thirty times more potent than the isoflavones mentioned above, yet it is over 1,000 times less potent than the known estrogens found in mammals.

Four derivatives of coumestrol were isolated from the seeds of Psoralea corylifalia and all of them had estrogenic activity (Dattagupta, 1960). A compound with estrogenic potency equivalent to estradiol-17-beta was found in the roots of a plant in northern Thailand in 1939 by Vatna. The compound was later named miroestrol, and in 1960 Bounds and Pope determined its structure.

The estrogenic compounds discussed above may also be considered antifertility agents in that they disrupt the

normal sequence of the estrous cycle, conception, and par-
turition if consumed in sufficient quantities. The anti-
fertility effects of plant estrogens, however, should not be
confused with other antifertility effects of plant compounds
variously classified as anti-estrogens (Emmens et al., 1960),
estrogen inhibitors, or antigonadotrophins (Pakrashi, 1963).
In 1964, Saunders and Rorig devised a system for separating
the antifertility effects of estrogens from classic estro-
genic effects. Pregnant rats were injected with various
estrogens on post-coital days 2, 3 and 4. At the end of the
fifteenth day, the rats were killed and the number of viable
placental sites were counted and compared to those of con-
trol animals. Using this as a criterion, estradiol was ten
times as active as estrone in its antifertility action. Al-
though this system is accurate, it measures only the abortive
power of the compound, and gives no information about the
effects of estrogens on the estrous cycle or on conception.

Uterine weight bioassays are commonly used to measure
estrogenic potency. This technique has been used success-
fully for many years, but unfortunately variation in the
methods of different investigators prohibits accurate com-
parison of results. A generally accepted standard test would
have made analysis of the literature a much easier task.
The uterine weight bioassay used in the present work is dis-
cussed in the section on Materials and Methods. The methods
of other investigators are aptly described in Methods of
Hormone Research, Volume II (Dorfman, 1962).

In 1965, Aust and Broquist isolated a parasympathomimetic alkaloid from the small black fungus, <u>Rhizoctonia leguminicola</u>. A condition called "slobbering disease" developed when cattle consumed forage infected with this organism. When guinea pigs were fed mycelia, they salivated profusely. The effect of this alkaloid on glandular epithelium and smooth muscle motility of the reproductive tract has not yet been ascertained. Further investigation of the ability of fungi to produce compounds affecting reproduction may aid in controlling these forage problems.

2. OTHER EFFECTS OF PLANTS ON MAMMALIAN REPRODUCTION

Emmens <u>et al</u>. (1960) described a technique for measuring the ability of compounds to inhibit estrogens. Mice were injected intravaginally with a standardized dose of a known estrogen. Estrogenic activity was then determined by a tetrazolium reduction test for epithelial thickening. The ability of a compound to decrease vaginal thickening is a measure of its anti-estrogenic potency. Vaginal smears were used to confirm results. Using this system, anti-estrogenic activities of several steroids (Emmens <u>et al</u>., (1960) and compounds related to diethylstilbestrol (Emmens <u>et al</u>., 1964) were determined.

Search for antifertility agents has been increased in the past few years in response to concern for the human population explosion. In 1954, deLaszlo and Henshaw published a list of sixty plant materials used by various

groups of people to affect fertility. Although much of the information associated with these remedies has no scientific basis, some of the plants described have a definite effect on the reproductive process.

Breneman et al. (1960) demonstrated that isolated fractions of Lithospermum ruderale have potent inhibitory activity against pituitary gonadotrophin and oxytocin. A cold water infusion of the roots of this plant was used by the Shoshone indians of Nevada to insure sterility of women (deLaszlo et al., 1954). Pakrashi (1963) found antigonadotrophic activity in several coumarins and naphthaquinones, using a toad bioassay of human chorionic gonadotrophin (HCG) that had been incubated in vitro for one half hour with the test compound. She was able to correlate structure with activity in several cases. Anti-progestational activities have been reported for at least four plants (Kurouji, 1963). The ability of injected plant extracts to inhibit the endometrial carbonic anhydrase increase of progesterone treated rabbits was used as an index of anti-progesterone activity.

Anti-estrogenic activity has been reported in several instances with reference to forage plants. Bickoff (1961) and Adler (1962) found anti-estrogenic activity in alfalfa. This activity has also been noted in ladino clover (Wright, 1960), in birdsfoot trefoil (Lotus comiculatus) by Cook and Kitts (1960) and in yellow pine (Pinus ponderosa) by Cook and Kitts (1964). Anti-estrogenic activity can be assayed in mice by measuring the inhibition of uterine weight response to a standard dose of estrogen.

3. REPRODUCTION IN THE GUINEA PIG

a. General Reproductive Phenomena. Basic investi-
gations of the reproductive process in mammals have centered
on a limited number of species, especially rats, mice, and
man. It has been known for many years that statements about
the reproductive process of one species, based on informa-
tion about another species, are frequently wrong. Because
of this and because the guinea pig has many unique facets
to its reproductive capacity, this animal has been avoided
by many investigators. Since guinea pigs were used in this
study, pertinent information is presented in an attempt to
correlate some of these unusual characteristics.

In 1931 Haines analyzed the breeding records, taken
over a period of eighteen years, of a large colony of guinea
pigs. Data concerning litter size, birth weight, and several
other traits were analyzed and used as a basis for subsequent
studies by many investigators. A lengthy review of the
genetics and vital characteristics of this animal was pub-
lished in 1960 by Wright. In 1956, Burgos and Wislocki made
a histological and histochemical investigation of the entire
female guinea pig reproductive tract. This extensive work
was complimented by electron microscopic studies of guinea
pig oocytes (Adams et al., 1964).

The recent accumulation of basic information about
guinea pig reproduction includes studies on vaginal smears
(Leonardelli, 1961), the aging process of the ovary (Batali
and Blumenthal, 1961), and motility of the vagina and

uterus (Freund et al., 1963). Burcher et al. (1962) demonstrated an inverse relationship between the life span of corpora lutea and the quantity of viable uterine tissue remaining after partial hysterectomy. They suggest the existence of some ovarian substance capable of regulating the life span of corpora lutea via the uterus. Hermreck et al. (1964) noted a compensatory response of twice the normal number of Graafian follicles in the remaining ovary of a semi-spayed female. The removal of one ovary must be performed on or before the twelfth day of the estrous cycle in order for the response to occur.

The response of pregnant guinea pigs to injections of estrogens and progestogens is different from most mammals. When fertilized eggs are in the Fallopian tube, 10 mcg. of estradiol benzoate will terminate pregnancy (Deanesly, 1961). The "tube locking" phenomenon does not occur. However, when fertilized eggs have reached the uterus, a total of 30, and in some cases 50, mcg. of estradiol benzoate does not prevent normal implantation as it does in the rabbit, (Deanesly, 1961, 1963b). Zarrow et al. (1963) treated pregnant guinea pigs with 4 to 20 mg. of progesterone starting on or after the fifty-fifth day of pregnancy. They were unable to lengthen pregnancy, even by repeating the treatment in combination with graded doses of estradiol.

More information concerning the unique action of progesterone in the guinea pig was elicited in an interesting study of ovariectomized mothers (Deanesly, 1963a). The following are excerpts from the summary of Deanesly's paper:

In the guinea pig, an ovarian or exogenous progestogen is not required for ovo-implantation, but the lack of it in females, ovariectomized 3, 4 or 5 days after mating, affects embryonic growth and development from about day 12. Between 14 and 16 days after mating, the placenta and embryo undergo very rapid differentiation ..... From the general variability and from a study of the embryos, it appeared that arrest of embryonic development and death was not caused at any precise time or stage by uterine compression, but was more probably due to nutritional deficiencies associated with the absence of the ovarian progestogen. ..... In further experiments it was shown that pregnancy could continue after about day 21, or even earlier, in the absence of the ovaries or of exogenous hormones. Both this and the normal differentiation of some embryos in ovariectomized mothers up to day 16, indicate early production of sex hormones by the placenta.

As with rats and mice, progesterone counteracts luteinization in guinea pigs (Mardones et al., 1951). In their work with splenic ovarian transplants, Mardones and his colleagues (1951, 1956) demonstrated that the amount of progesterone necessary to prevent estrogen-induced luteinization of transplants is less than that produced by the ovary in the luteal phase. They were also able to demonstrate the high antiluteinizing potency of five derivatives of progesterone.

Information concerning the pituitary gonadotrophins of the guinea pig is scant and confusing. Smith and Engle (1929) transplanted guinea pig anterior pituitary glands into immature mice. They were able to show slight correlation between ovarian response in the mice and the stage of estrus of the guinea pigs being tested. In no case were responses as great as when rat pituitaries were used. Pituitary glands from castrated guinea pigs demonstrated definite hyperactivity.

Haller (1963), also using intrasplenic ovarian auto-transplants, noted large cystic follicles, blood-filled follicles, and large corpora lutea. He was able to prevent this hypertrophy with high doses of norethisterone acetate over a period of ninety days (10 mg. per dose at 10-day intervals). In trials with other synthetic gestogens, it became evident that the ability of steroids to inhibit castration hypersecretion in the guinea pig is much weaker than it is in rats and mice.

Aron and Marescaux (1962) present an argument favoring the existence of only one pituitary gonadotrophin in the guinea pig. They concluded that follicle development and luteinization are controlled by different concentrations of the same hormone. Perry and Roslands (1963), in working with hypophysectomized immature guinea pigs found that, "a constant feature of the ovarian response to gonadotrophins from a variety of sources consisted of a luteinization of atretic follicles and stromal tissue". They were unable to induce follicular development to an appreciable degree.

It is apparent that there is much to be learned about the pituitary-gonad axis and many other phenomena associated with the reproductive process of the guinea pig.

b. Response to Estrogens. With the exception of a few key papers, (East, 1950 and 1952; Alexander and Watson, 1951 and 1952; Chury' and Panek, 1964; Braden and Peterson, 1953) very little has been published concerning the response of guinea pigs to plant estrogens.

In using these animals for the measurement of estrogenic activity, vaginal patency is an important factor. When not under the influence of estrogen (ovarian or exogenous) the vagina is closed by a membrane in a fashion similar to that seen in wound healing. In response to estrogen, the vagina opens rapidly to what is termed "full patency". The use of this trait for estrogen assays was described by Hartman et al. in 1946. They described the degree of patency by stages numbered 1 to 5. By using immature females or ovariectomized adult females, the quantity of an estrogen necessary to cause full patency can be ascertained. The guinea pig has one major shortcoming as an estrogen assay animal in that vaginal smears cannot be taken when the vagina is closed, unless the closure membrane is mechanically disturbed.

Histological studies of the reproductive tracts of assay animals are often useful, especially in reference to vaginal and uterine epithelia and to the development of uterine glands (Burgos et al., 1956). The estrogenic responses of the vagina have also been described in detail by Stockard and Papanicolaou (1919). The guinea pig endometrium acquires a glandular hyperplasia when influenced by excessive quantities of estrogen. Epithelial cells enlarge and fill with a foamy material composed of mucin, as indicated by a positive periodic acid-Schiff reaction (Wright and Seibold, 1958).

The use of "cystic" glandular hyperplasia as an index of estrogenic activity is open to criticism. Braden

and Peterson (1953) discussed this problem in some detail, by comparing the guinea pig with other species and by reporting experiments on the partial hysterectomy of guinea pigs being treated with estrogens. Although they cited instances (East, 1952, and others) when cystic glandular hyperplasia failed to appear in estrogen treated animals, they used this character as a measurement of estrogenicity in their study. Cystic glandular hyperplasia of the endometrium has been reported as a response to many surgical procedures involving the reproductive tract (see review by Braden et al., 1953). The condition has also been reported to occur spontaneously in intact guinea pigs (Geil and Davis, 1963). Since surgery, such as ovariectomy or hysterectomy, complicates the poorly understood phenomenon of cystic glandular hyperplasia, ovariectomized animals cannot be used for a dependable measurement of estrogenicity.

Alexander and Watson (1951, 1952) assayed the estrogenic activity of subterranean clover in ovariectomized guinea pigs. They were able to correlate graded doses of clover with a variation in uterine weight response. In comparing estradiol-diproprionate-treated intact females with intact females fed subterranean clover, East (1952) noted similar degrees of infertility and epithelial change in accessory reproductive organs. In contrast to estradiol treated guinea pigs, those fed clover continued to ovulate and have normal estrous cycles. She suggests that clover acts directly on the reproductive tract, whereas estradiol

impairs gonadotrophin activity. The guinea pigs treated with estradiol did not regain fertility for some time after cessation of treatment, but those consuming clover diets regained fertility as soon as the diet was changed.

Chury' and Panek (1964) fed alfalfa to guinea pigs for 27 to 42 days, and noted the development of cystic ovarian follicles. In experiments with castrated male guinea pigs, East (1950) found that subterranean clover caused mammary development and increased nipple length. Diethylstilbestrol treatment produced similar results, and both of the above treatments were counteracted with testosterone therapy. Wright and Seibold (1958) reported severe litter losses in a breeding colony of guinea pigs consuming pelleted feed contaminated with stilbestrol.

An interesting but perhaps inapplicable adjunct to the above discussion concerns some work by Rona and Chappel in 1963. In vitamin C deficient male guinea pigs, symptoms of scurvy were prevented by oral administration of a conjugated equine estrogen (Premarin).

4. PARABIOTIC MICE

In 1952, Finerty published an extensive review entitled "Parabiosis in Physiological Studies". He described the use of parabiotic animals with an array of approximately 200 publications. The first recorded parabiotic union of rats was performed by Bert in 1862 (Finerty, 1952). Many improvements in technique have occurred since then, and modifications of the method of Bunster and Meyer (1937) are

now used by most investigators. A discussion of the surgery
is included under the section on Materials and Methods.

Today, parabiotic rats and mice are an integral
part of the investigations of geneticists (Eichwold, 1959,
and others) cancer researchers (Borges et al., 1955; Johnson
and Witschi, 1961; and others) and biologists in many re-
lated fields. Witschi, a pioneer in work with parabiotic
amphibians, was co-author of a publication concerning the
endocrinology of ovarian tumor formation in parabiotic rats
(Johnson and Witschi, 1961).

The ability of animals to survive parabiotic union
is dependent upon histocompatability genes, which are de-
fined as, "the genes (or Loci) which determine susceptibility
and resistance to transplants of normal or tumor tissue"
(Snell, 1958). Members of many inbred strains are histo-
compatible (Roscoe B. Jackson Memorial Laboratory, 1962).
Loran et al. (1964) found that a humoral factor capable of
accelerating epithelial proliferation in a mouse with an
intestinal resection, was transported to the partner of a
parabiotic pair. This factor also accelerated epithelial
proliferation in the non-resected twin, as measured by the
uptake of tritiated thymidine in mitosing crypt cells.

Parabiotic animals are frequently used to study the
inhibitory effect of steroids and related compounds on pit-
uitary gonadotrophins. In an exhaustive study using 551
pairs of parabiotic rats, and over 1,000 pairs of parabiotic
mice, Miyake (1961a and 1961b) established graded dose re-

sponses to several commonly used steroids. Following
gonadectomy and the subsequent hypersecretion of pituitary
gonadotrophins of one partner, the gonads and accessory sex
organs of the intact partner overdevelop. The degree of
overdevelopment can be correlated with the inhibitory power
of the compound being tested.

In experiments with old female mice parabiosed to
young female mice, Takasugi (1963) demonstrated that the
atrophy of ovaries in aged mice is not due to failure of the
pituitary to produce gonadotrophins, but due to a failure of
the ovaries to respond to gonadotrophic influence. This con-
clusion is supported by many investigators on the basis of
both histological studies of the anterior pituitary basophils
and on the results of massive injections of gonadotrophins
into old female mice. Dorfman and Dorfman (1963) established
a log-dose response relationship between ovarian weight and
treatment with various doses of estrone in parabiotic rats.
The system is useful as a bioassay of the gonadotrophic in-
hibitory power of estrone within a dose range of 0.5 and 2.5
mcg.

Duckett et al. (1963) found, in studying the effects
of adrenal androgens on parabiotic mice, that nonvirilizing
doses of these compounds can inhibit pituitary gonadotrophins.
They also suppressed different individual FSH effects as in-
dicated by the ability of only selected adrenal androgens to
inhibit both uterine and ovarian weight. Two separate re-
productive functions were measured, both of which are usually
considered properties of FSH. The parabiotic union of hypo-

physectomized rats to normal or castrated males can be used in conjunction with combinations of testosterone and human chorionic gonadotrophin treatments to show that steroid inhibition is primarily relevant to LH rather than to FSH (Schuetz et al., 1964).

III. MATERIALS AND METHODS

1. MATERIALS TESTED

a. Clover. Ladino clover (Trifolium repens, Variety Ladino) is a large clover introduced into this country from Italy (Morrison, 1954). The source used for this study was a field (159 ft. x 180 ft.) on Mast Road, Durham, New Hampshire, adjacent to the Farm Department building of the University of New Hampshire. In August, 1963, the field was seeded with timothy (Phleum pratense), red top (Agrostis alba), alfalfa (Medicago sativa), winter rye (Secale cereale), red clover (T. pratense), and ladino clover (T. repens). During the summer of 1964, an estimated 93 percent of the growth in this field was ladino clover. Pure stands were easily harvested, and it is this clover which was used throughout the entire study. Except for a ten foot strip, the entire field was fertilized on May 8, 1964, with 240 pounds of 0-15-30 fertilizer (0 percent nitrate, 15 percent phosphorus, 30 percent potash). The growing season during the summer of 1964 was extremely dry and the only rain during the course of clover collection fell on 8 June, 24 June, 4 July, and 6 July. During the last week of June and the first week of July the clover became stunted and dry, so that samples had to be selected carefully in order to collect viable material. Other grasses began to predominate during the dry period, but near the end of the collection period the clover was rejuvenated by heavy

rainfall.

Clover was harvested daily from 8 May through 9 July by being cut with a hand sickle and immediately collected in a two-bushel basket. At four- to seven-day intervals, samples were set aside for moisture determination, grinding, and freezing (Table 1). Since fresh clover was fed twice daily, enough material was collected for both feedings; half was refrigerated for twelve hours prior to being fed. Refrigeration for up to twenty-four hours caused no change in percent moisture and no noticeable change in palatability or turgidity of leaves.

Samples were taken for moisture determination in sufficient quantity to fill three wire baskets lined with muslin (3 in. x 10 1/2 in. x 24 in.). No longer than one hour lapsed from the time of harvest until the samples were weighed and placed in the drying oven. The drying oven was a stainless steel "Power-O-Matic 60" mechanical convection oven (Appendix 1) adjusted to force dry air, heated to 60° C., through the clover for twenty-four hours. At the end of the drying period, the clover was again weighed, and percent dry weight was determined as $\frac{\text{total dry weight}}{\text{total fresh weight}}$, (Table 1).

Dried samples were ground in a Wiley cutting mill (Appendix 1), using a 1.0 mm mesh sifting screen. The powdered clover was placed in clean cylindrical steel cans with the lids tightly taped, and stored in a freezer at 0° C. until it was used. This material will hereafter be referred to as "preserved clover".

TABLE 1

CLOVER HARVEST DATES AND PERCENT DRY WEIGHT

Harvest Date	Percent Dry Weight	Harvest Date	Percent Dry Weight
May 8	15.3	June 14	18.3
May 12	21.5	June 19	24.4
May 18	18.2	June 25	24.6
May 26	17.3	June 29	29.5
June 2	20.0	July 4	21.8
June 9	19.0	July 7	25.3
June 11	18.8	Sept 9	16.4

The lettuce used in control diets had 6.03 percent dry weight.

Preserved clover used in guinea pig experimental diets was pooled from samples taken on 12 May, 18 May, 26 June, and 9 June, and thoroughly mixed. The blended clover was then mixed with a mash prepared from Purina Guinea Pig Chow, a standard commercial diet prepared specifically for guinea pigs (see Animals and Animal Facilities). The final diet was 60 percent preserved clover and 40 percent mash, as diets with a higher percent of clover were unpalatable.

In instances where clover was extracted and used in mouse diets, the following procedure was used. Fifty-gram samples of preserved clover were placed in small Soxhlet extractors (Appendix 1) and extracted continuously with acetone for 22 to 24 hours each, as described by Bickoff et al. (1959). Samples were then discarded and acetone extracts were concentrated in 500 ml. flasks by rotating the flasks in a 45° C.

water bath, and by removing vaporized acetone with a vacuum
pump until 10 to 20 cc of thick, dark, viscous liquid re-
mained. The extract was then mixed with 50 grams of Purina
Lab Chow mash (see Animals and Animal Facilities) and placed
in large flat petri dishes under a hood. When no odor of
acetone remained, the diets were placed in screw cap bottles
and appropriately labeled. Diets prepared in this way have a
1:1 ratio in that the extract of 50 grams of clover is con-
tained in 50 grams of diet.

    b. Coumestrol. In 1957, Bickoff et al. isolated a
crystalline estrogenic compound from ladino clover. The
same compound has also been identified in alfalfa, red clover,
and subterranean clover (Guggolz et al., 1961). The name of
this compound, 2-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofuran
carboxylic acid lactone, has been shortened to coumestrol
due to its coumarin-like structure (Figure 1), and to facil-
itate common usage. As synthesis of coumestrol is beyond
the scope of this investigation, it was prepared commercially
by Distillation Products Industries, Rochester 3, New York,
a Division of Eastman Kodak Company.

    Diets containing coumestrol were prepared in the
following two ways: 1) The total amount of compound needed
was weighed and mixed thoroughly with enough powdered dex-
trose to make a diet of 1.0 percent dextrose. The mixture
was blended into the diet with a mechanical eight quart mixer
of the type used in bakeries, and stored in one gallon screw
cap jars. 2) The total amount of compound needed was weighed,

dissolved in 95 percent ethanol, and poured over the mash. The diets were then stirred in flat dishes under a hood until no odor of alcohol remained. Blending was completed by mechanically rotating the diets in screw cap jars for one hour.

Administration of a suspension of coumestrol in carboxy methyl cellulose by oral gavage was attempted with parabiotic mice. This technique proved to be too harsh, as also noted by Miyake (1961a), and was discontinued.

Coumestrol is relatively insoluble in most injection vehicles. After experimenting with several methods, dimethyl sulfoxide (DMSO) was chosen as the injection vehicle for all experiments. DMSO dissolves coumestrol more readily than other vehicles tried, and its toxicity is negligible in small doses (Ashwood-Smith, 1961). Solubility was such that coumestrol was in complete solution at all dosage levels when mice were injected with 0.01 cc per treatment, and when guinea pigs were injected with 0.2 cc per treatment. Both mice and guinea pigs are capable of withstanding ten times this quantity of DMSO (0.1 cc and 2.0 cc respectively) daily for ten days. The preceding statement is based on 10-day treatment trials using 4 to 6 animals per trial.

c. Estradiol Benzoate and Diethylstilbestrol. These two compounds were purchased from Nutritional Biochemicals Corporation, Cleveland 28, Ohio. Both meet the standards of purity set by the United States Pharmacopia, and their structures are shown in Figure 1.

Estradiol benzoate, a salt of the natural estrogens

estradiol-17-alpha and estradiol-17-beta, was used in the
majority of cases where an estrogenic response was wanted.
In many experiments two groups of controls were used; a group
receiving no estrogens, called "negative controls"; and a
group receiving a standard dose of estradiol benzoate, called
"positive controls".

In most cases, estradiol benzoate was chosen because
of its similarity to natural estrogens and for comparison
with results of other investigators. One advantage of di-
ethylstilbestrol (DES) is its adaptability to oral adminis-
tration, and it was used in a few instances. The stability
of both compounds is excellent in crystalline form, but the
potency of estradiol benzoate decreased after being in solu-
tion in 95 percent ethanol for more than two months.

Estradiol

Estradiol Benzoate

Coumestrol

Diethylstilbestrol
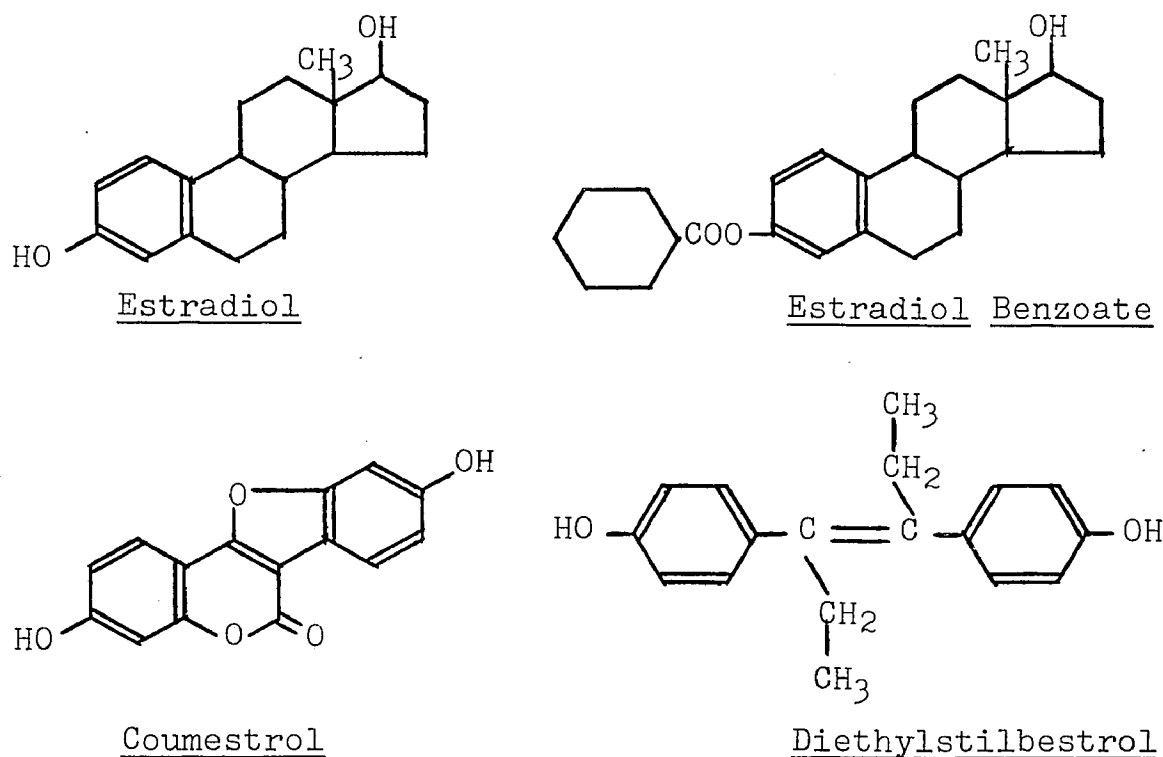
Figure 1. Structures of Selected Estrogens

2. ANIMALS AND ANIMAL FACILITIES

a. Guinea Pigs. Three general types of guinea pigs exist today and all are derived from a South American stock now living in the wild state in Peru. The three strains are: 1) Abyssinian, a short rough haired variety, 2) Peruvian, a long haired variety, and 3) Dunkin-Hartley, a strain established in England in 1926. All three varieties are members of the species Cavia porcellus, and the Dunkin-Hartley strain is used by most research workers.

For this study, virgin albino females of the Dunkin-Hartley strain were acquired from two sources: 1) Camm Research Institute, Inc., 414 Black Oak Ridge Road, Wayne, New Jersey, and 2) Albino Farms, Red Bank, New Jersey.

Guinea pigs were housed in a heated, well ventilated room (12 ft. x 34 ft.) on the first floor of Ritzman Nutrition Laboratory. The room has concrete block walls and a smooth sloped concrete floor with drains to facilitate easy cleaning. The room contains storage shelves, a refrigerator, and a sink with hot and cold running water.

In early work, batteries of six wooden framed wire cages (20 in. x 32 in. x 14 in.) were used. These were replaced by stainless steel cages made by Great Falls Products Company, Rochester, New Hampshire. The stainless steel cages have removable slatted floors, overlap catch pans, removable doors, large protected external feeding hoppers, and solid side walls. They are 18 inches wide, $23\frac{1}{2}$ inches deep, $14\frac{1}{2}$ inches high, and are suspended in movable batteries of 12 cages (Figure 2).
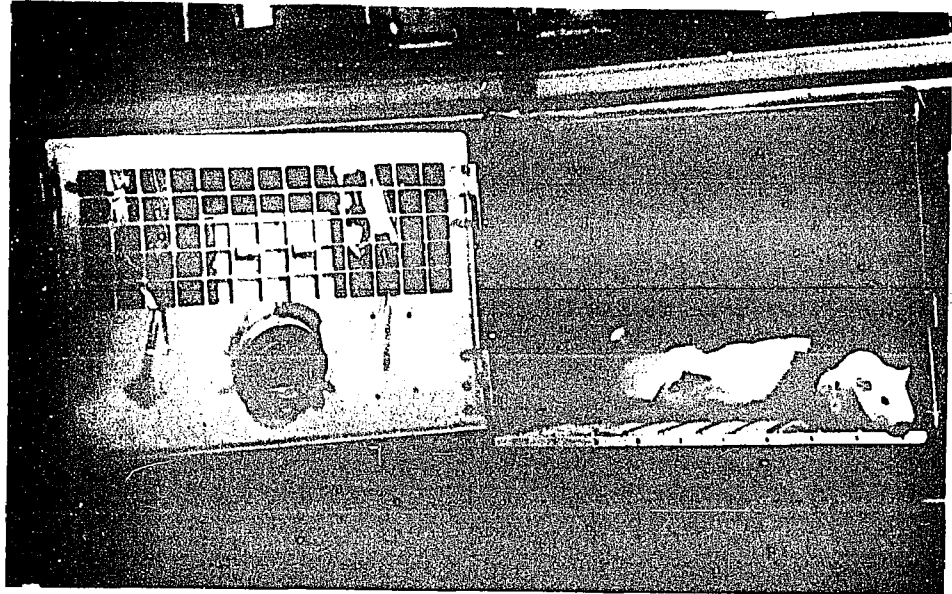
Figure 2.   Photograph of the Interior of a
            Stainless Steel Guinea Pig Cage

Experimental and control diets other than fresh

clover and lettuce were prepared from either pelleted or

mash forms of Purina Guinea Pig Chow.   Animals not on ex-

periment were also fed this ration.   The Purina Company gives

a complete feed analysis in the Purina Laboratory Manual

(published annually).   Each gram of feed contains 0.33 mg

of vitamin C to compensate for the guinea pig's inability

to synthesize this compound.   The mash form was prepared by

grinding the pellets in a Wiley cutting mill with a 2.0 mm

mesh screen.

Guinea pigs were watered from eight ounce bottles

fitted with rubber stoppers and glass watering tubes. Bottles

were refilled twice daily, and every twenty-four hours they were replaced by others that had been washed and disinfected in a 1:5000 solution of Roccal (alkyl dimethylbenzyl-ammonium chloride, by Winthrop Laboratories, New York 18, New York).

General sanitation included scrubbing and disinfecting the floor twice weekly, washing and disinfecting cage floors twice weekly, and daily removal and replacement of soiled shavings in catch pans.

The general health of all guinea pigs was closely watched and unhealthy animals were removed from the colony, killed, and autopsied prior to disposal. Other than isolated cases of shipping fever type pneumonia (Merck Veterinary Manual, 1961), the colony remained in excellent health. As a protective measure, one group of newly-arrived guinea pigs was treated with intramuscular injections of 20,000 units of penicillin G and 25 mg. of dihydrostreptomycin sulfate per animal. This treatment exceeded the guinea pig's toxicity tolerance for penicillin, and the majority of the group was lost. The diagnosis was made at the Department of Veterinary Pathology, Cornell University, Ithaca, New York.

The numbering system used was a color code of dots and dashes applied to the hair of the dorsal midline. The coloring agents employed were saturated aqueous picric acid, 1 percent aqueous methylene blue, 1 percent aqueous orange G, and periodic acid-Schiff reagent.

Several techniques for quick killing were investigated, and the method adopted for all guinea pig and mouse work was disarticulation of cervical vertebrae. This

technique was chosen because it produced instantaneous un-
consciousness, and eliminated the pooling of blood in ab-
dominal viscera, as is often the case when anesthetics are
used. After removal of the necessary organs, carcasses
were cremated.

b. Mice. For parabiosis experiments, 15- to 17-
gram C57BL/10J female mice (Mus musculus) were purchased
from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.
A high degree of histocompatability in this inbred strain
makes these mice superior subjects for transplant and par-
abiosis work (Snell, 1958).

Mice used in estrogen and gonadotrophin bioassays
were 19 to 20 day old random-bred albino females, designated
as type "CD-1" by the supplier (Charles River Farms, North
Wilmington, Massachusetts). Immature mice of this age are
hardy enough to survive series of injections and are not
under the influence of significant quantities of estrogens
from their own ovaries.

Mice were housed in a heated, well-ventilated room
used solely for that purpose. Cages made of 3/8-inch ply-
wood with sheet aluminum bottoms were large enough (8 in.
wide x 11 in. deep x $5\frac{1}{2}$ in. high) to hold 6 to 8 immature
mice or 4 adult mice comfortably. Covers for the cages were
made of $\frac{1}{4}$-inch hardware cloth mounted on a wooden frame, and
supported a simple feed basket and a water bottle. Cages
were arranged on wooden shelves built flush with a wall fac-
ing the outside windows (Figure 3).
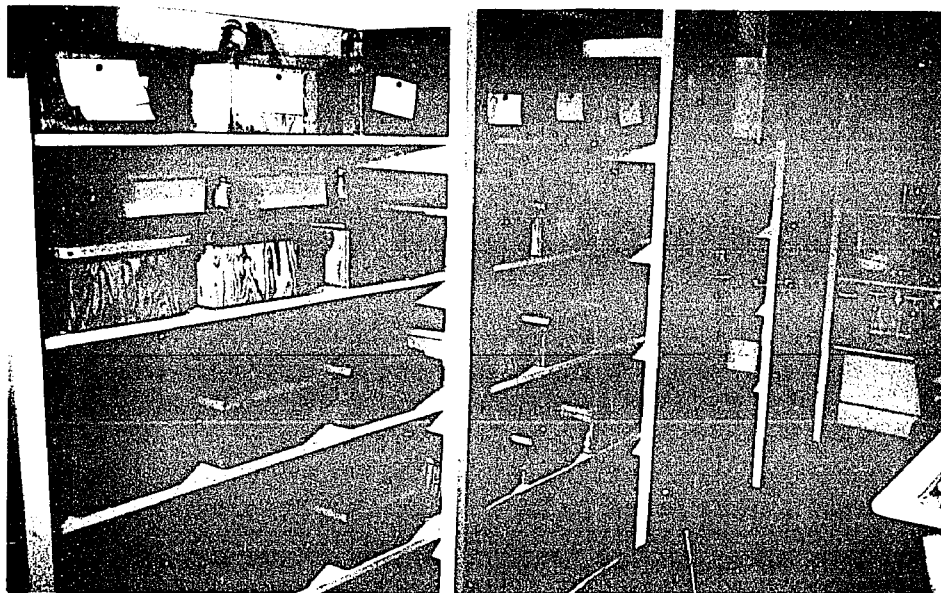
Figure 3.   Photograph of Mouse Room

All mouse diets consisted of either pellet or mash forms of Purina Lab Chow, a product designed specifically for laboratory animals. Diets containing additives were mixed as they were for guinea pigs. A personal letter from the Purina Research Division described a mouse uterine weight bioassay control procedure used on all laboratory diets to check for estrogenic activity. According to Paul E. Kifer, Manager, both Lab Chow and Guinea Pig Chow have "less than 7 parts per billion of total estrogen content".

Mice were watered from 4 ounce glass bottles with rubber stoppers and glass watering tubes. The bottles were washed and refilled every two days. Cages were cleaned twice

weekly, and all bedding (hard wood shavings) was replaced. At the end of each mouse experiment, cages were disinfected in a 1:2500 solution of Roccal, and allowed to air dry for at least two weeks. The health of the mouse colony was excellent throughout the course of this study. The numbering system was a coded hole pattern punched into the right and left ears with a 2 mm steel punch. Quick killing and disposal methods were the same as those used for guinea pigs.

3. SURGICAL TECHNIQUES

a. <u>Anesthesia</u>. Both mice and guinea pigs were anesthetized with Nembutal (Sodium Pentobarbital by Abbott Laboratories, North Chicago, Illinois) supplied in 50 ml. vials of sterile solution for injection. The dosage used was 29 mg. per kg, as recommended by Wright (1957). Nembutal stock solution was diluted with sterile saline so that intra-peritoneal injection volumes were approximately 0.5 to 0.9 cc for mice and 1.5 to 2.5 cc for guinea pigs. Anesthesia for both mice and guinea pigs was supplemented by ethyl ether inhalation when necessary.

b. <u>Guinea Pig Ovariectomy</u>. Ovaries were removed through two incisions, one in each paralumbar fossa, in the following manner. When surgical anesthesia had been produced, each paralumbar fossa was clipped with number 40 clipper blades (Appendix I). The operative area was thoroughly cleaned with 70 percent ethyl alcohol, and the animal was placed on a clean paper towel. A ½ inch skin incision was

made over the area directly lateral to the ovary. The ab-
dominal musculature was elevated with thumb forceps and in-
cised, opening the abdominal cavity. The ovary was withdrawn
through the incision and removed adjacent to its hilus with
Ferguson Angiotrobe forceps (Appendix I), eliminating the
need for ligatures. One interrupted suture of number OO
chromic gut was used to close the abdominal cavity, and the
skin incision was held in apposition with a single 1 mm
wound clip. The same procedure was repeated on the opposite
side, and the animal was placed in a warm enclosure to re-
cover from the anesthetic.

c. Parabiosis. Mice were placed in parabiotic
union by a modification of the technique of Bunster and
Meyer (1937), briefly described as follows: The two animals
to be parabiosed were anesthetized and clipped, one on the
right side and one on the left side, from the base of the
ear to the base of the tail (Figure 4). The operative areas
were cleaned with 70 percent ethyl alcohol and the two
animals were placed on a clean paper towel with their sur-
gery cites upward. The skin was incised from the base of
the tail to the base of the ear on each mouse. The mice
were then turned over, and the ventral sides of their in-
cisions were solidly united with 1 mm wound clips. They were
then returned to their original position, and 3/4 inch in-
cisions were made into each abdominal cavity. At this time,
the left ovary of the right partner was exteriorized, and
removed with hemostatic forceps. The peritoneal cavities
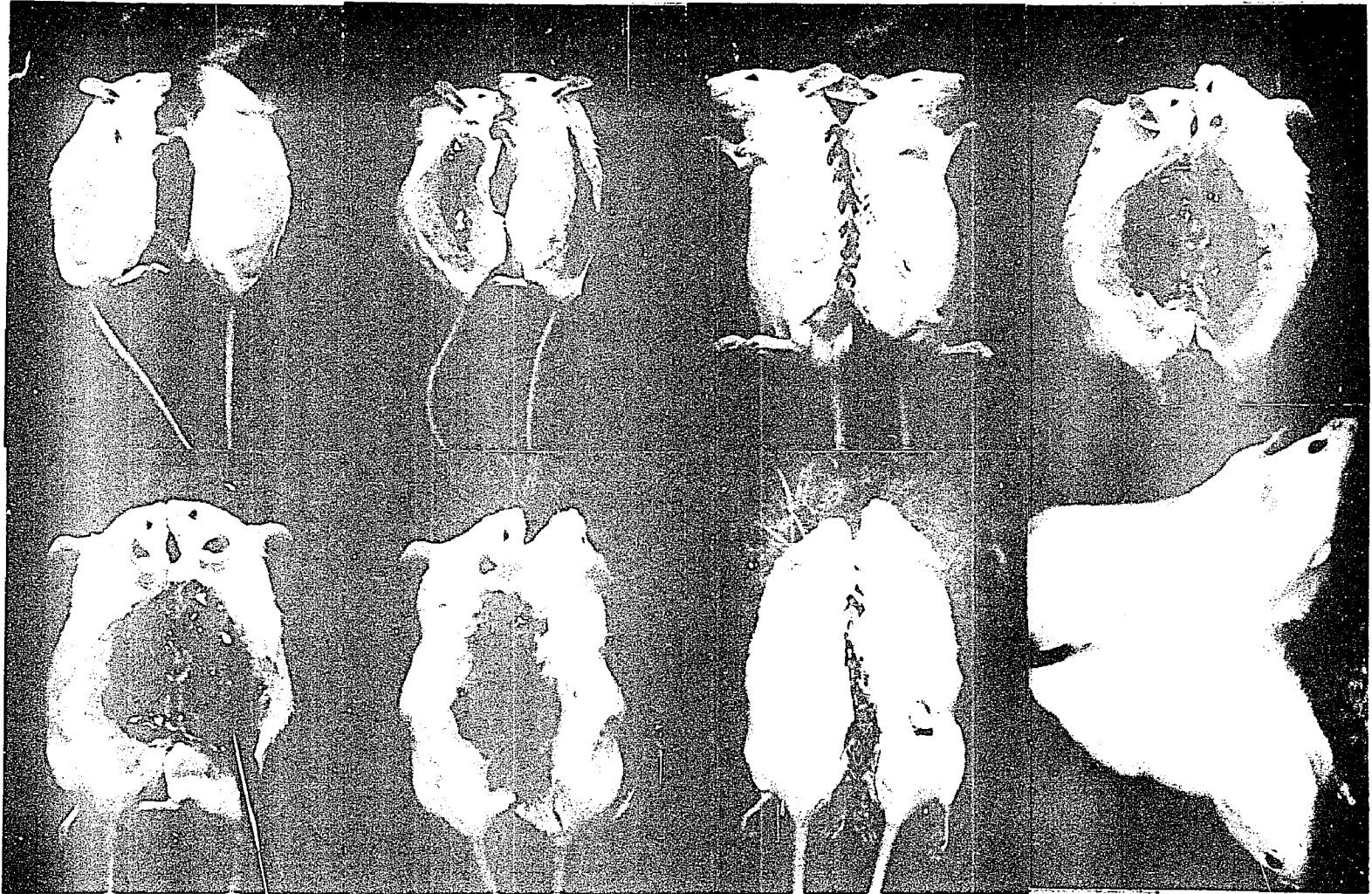
Figure 4. Photographs of the Steps Involved in the
Parabiotic Union of Mice (1 through 8)

were then united by joining the edges of the two abdominal
incisions with a continuous suture of number 000 chromic
gut. The exposed shoulders of the two animals were held in
apposition with a single gut suture through the opposing in-
fraspinatus muscles. Continuation of the series of wound
clips around the entire perimeter of the two skin incisions
completed the parabiosis. Removal of the right ovary of the
right partner was performed as in the guinea pig. The major
steps in the procedure are illustrated in Figure 4.

    <u>d</u>. <u>Facilities</u>. The surgery was a 6 ft. x 13 ft.
room with hot and cold running water, sloped concrete floors,
floor drains, and excellent overhead illumination. Two
large storage cabinets with doors and two operating tables
were mounted on the walls. Equipment included a dry heat
sterilizer, a tissue grinder, a binocular dissecting micro-
scope, an illuminated 6 inch magnifying glass, an Oster
animal clipper, and a surgery lamp (Appendix I). Stainless
steel surgical instruments, glass and disposable syringes,
hypodermic needles, sutures, etc. were supplied in ample
quantity for all procedures.

    Instruments were sterilized prior to surgery and
placed in a container of 70 percent ethyl alcohol when not
being used during an operation. After being used, they were
thoroughly cleaned and stored in the wall cabinets. The
floors, tables, and other equipment used were cleaned and
disinfected immediately after surgery was complete. Some
of the facilities are illustrated in Figure 5.

a
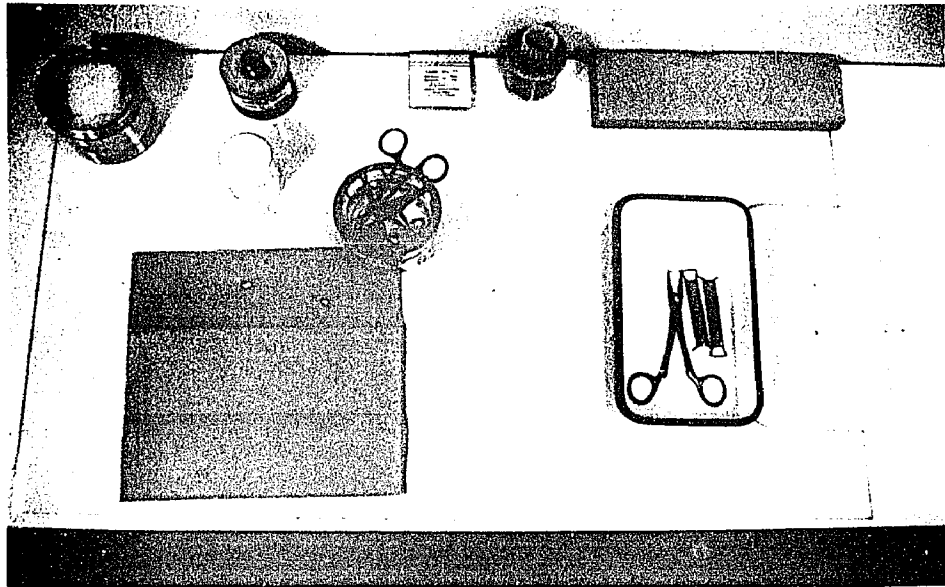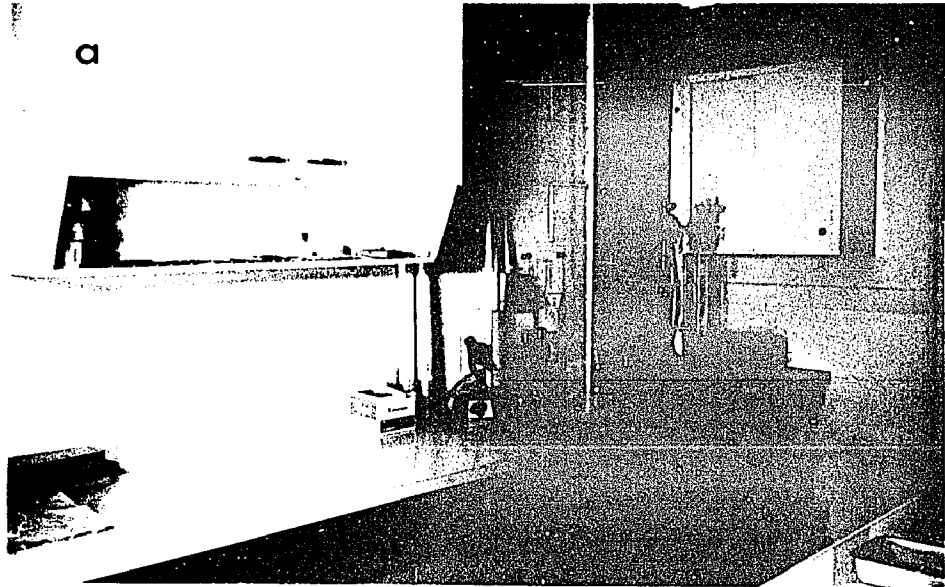
Figure 5a.   Photograph of the Surgery.

Figure 5b.   Photograph of the Operating Table.

4. VAGINAL PATENCIES AND SMEARS

The degree of vaginal patency of all guinea pigs on experiment was recorded daily according to the method of Hartman et al. (1946). The vagina is patent, in the guinea pig, when under the influence of estrogen. In intact cycling animals the source of estrogen is the ovary, but in ovariectomized animals estrogen must be administered to elicit this effect. When not under the influence of estrogen the vagina is "healed" closed. The degree of patency is estimated by numbers from 0 to 5, 0 representing the closed stage and 5 representing full patency (Hartman et al., 1946).

Vaginal patency of mice was recorded by using a modification of the above method, since the vagina is closed prior to puberty only. This information is of value when treating immature mice with estrogens or gonadotrophins, either of which will hasten vaginal patency. Mice retain some degree of patency after puberty, even when ovariectomized, however, ovariectomized mice have a decreased degree of vaginal patency when not treated with estrogen.

In instances where vaginal smears were used, they were taken daily by flushing the vagina with saline solution. After drying the suspension of exfoliated vaginal epithelium and exudate on a slide, the smear was stained in 1 percent methylene blue. The stage of the estrous cycle, as indicated by vaginal smear, was recorded according to the method of Allen and Doisy (1920).

5. COLLECTION AND HANDLING OF TISSUES

Tissues to be used for histological studies and organ weight determinations were collected in the same manner for mice and guinea pigs. After the animal was killed and its abdominal cavity had been opened, the reproductive tract was immediately dissected free. Excess tissue was carefully trimmed from uteri and ovaries (when present) prior to their immersion in separate vials of Bouin's fixative. After a twenty-four hour fixing period, organ weights were taken on a Mettler analytical balance accurate to 0.1 mg.

Guinea pig pituitary glands to be used in immature mouse bioassay experiments were removed aseptically after the dorsal portion of the cranium was removed and the brain had been allowed to drop out. The gland was dissected free from its attachment to the sella turcica with a sharp scalpel and aseptically transferred to a sterile tissue grinding vial immersed in ice water or to a sterile freeze drying vial immersed in an acetone and dry ice bath.

Pituitary glands pooled in tissue grinding vials were ground into a homogeneous cell suspension with enough sterile saline solution so that each 0.6 cc of suspension contained one gland. This material was stored in a refrigerator for use during the following 72 hours.

Pituitary glands to be freeze-dried were pooled in vials representing one treatment group. Immediately after killing was complete, the vials containing quick frozen

glands were transferred to a refrigerated vacuum pump. When air and moisture had been sufficiently removed the vials were heat sealed, leaving a permanent vacuum inside.

6.   HISTOLOGICAL PROCEDURES

<u>a</u>.   <u>Embedding Sectioning and Staining</u>.   Tissues to be sectioned were removed from fixative solutions and embedded as described by Gray (1954), with slight modifications in some instances.   The embedding material used (Tissuemat by Fisher Scientific Company) has a melting point of 55.0°C. Embedded tissues were stored in a refrigerator if sectioning was not begun at once.

Sections of ovaries were taken in ribbons 8 micra thick, and transferred to a sheet of paper to be cut into short lengths.   Mayer's albumen solution was used to float sections on slides placed on a slide warmer.   Sections were allowed to flatten before they were drained, positioned, and dried.   Sections of guinea pig uteri were treated similarly except that they were cut in ribbons 12 micra thick and were taken from the end of the uterine horn nearest the body of the uterus.

Sections of reproductive tracts from guinea pigs and mice were stained by the same hematoxylin-eosin procedure, briefly outlined below:

1.   Xylene - 5 min.
2.   Xylene - 3 min.
3.   100% alcohol - 5 min.
4.   95% alcohol - 5 min.
5.   85% alcohol - 5 min.
6.   70% alcohol - 3 min. - place in 70% alcohol, saturated with lithium carbonate, for 1

      min. to remove picric acid used in Bouin's
      fixative.

7. 60% alcohol - 3 min.
8. 50% alcohol - 3 min.
9. Distilled water - 3 min.
10. Ehrlich's hemotoxylin (Gray, 1954) - 12 to 15
     min.
11. Running water - 15 min.
12. 50% alcohol - 3 min.
13. 60% alcohol - 3 min.
14. 70% alcohol - 3 min.
15. 85% alcohol - 3 min.
16. .02% Eosin Y in 95% alcohol - 15 to 60 seconds.
17. 95% alcohol - immerse 4 to 5 times.
18. 100% alcohol - 2 to 3 min.
19. Xylene - 3 min.
20. Xylene - 5 min.

Stained sections removed from the xylene bath were
immediately coated with Permount mounting medium (Fisher
Scientific Company) and covered with 24 mm x 50 mm cover
glasses. Slides were labeled with respect to experiment,
animal, treatment, type of tissue, date and stain used.
Completed slides were stored in covered plastic trays hold-
ing 100 slides each, and tissue blocks were stored in card-
board boxes designed for that purpose.

Slides were examined with a binocular Bausch and
Lomb research microscope mounted on an Ortho illuminator
(Appendix I).

    b. Photomicrography. The microscope and illu-
minator described above were used for photomicrography pro-
cedures, except that the binocular apparatus was replaced
with a monocular unit. A Leitz camera adapter with a focus-
ing eye piece was placed on the monocular tube, and a 35 mm
Beseler Topcon Super D camera was mounted on the adapter.
The Beseler camera contains a light meter capable of measur-
ing incoming light on a mirror behind the lens. This system

permits automatic exposure control with each photograph, and eliminates the use of a separate meter and conversion tables.

Colored photographs were taken with Kodachrome II Professional Film - Type A, and black and white photographs were taken with Kodak Plus-X-Pan Film. Developing and printing were handled through a local camera shop.

IV. EXPERIMENTAL PROCEDURES AND RESULTS

1. GUINEA PIG STUDIES

a. Fresh Ladino Clover Feeding Experiment. This experiment was designed to investigate the response of the normal adult female to a diet composed primarily of fresh ladino clover. Forty-eight adult female guinea pigs were divided into two equal groups. The experimental group was fed fresh ladino clover ad libitum for 63 days, with a supplement of 15 grams of Guinea Pig Chow each day. Controls were given Guinea Pig Chow ad libitum (average consumption was 34.5 grams of pellets per animal per day), and fresh lettuce at a rate of 50 grams per animal per day. Clover was fed twice daily and lettuce was fed once daily. It was thought that lettuce would supply control animals with some of the benefits of fresh vegetation, and thereby eliminate this factor as a difference in the diets. Water was available to all animals continuously. Vaginal patency estimates were taken daily, and body weights were determined weekly.

After 63 days, the experiment was terminated and ovaries and uteri were removed for further study. On the following day, weights of fixed ovaries and uteri were taken and recorded. Because these animals were normal adult females, both groups contained members at various stages of the estrous cycle when uteri were removed. The uterine weights of both groups, therefore, varied substantially due

to differences in the quantity of endogenous estrogen. The large number of animals used offsets this individual variation, when the mean uterine and ovarian weight differences between the two groups are compared.

No significant differences in ovarian weight, uterine weight, body weight or uterus expressed as percent body weight, were noted in comparing the two groups (Table 2). Histological examination of uterine cross-sections showed a small degree of cystic glandular hyperplasia in the endometrium of only one experimental animal and in none of the control animals. In each of the two groups of animals, isolated uterine glands were occasionally found to be enlarged. This condition is not considered to be a variation from normal in that it had a low random distribution in the several hundred uteri from guinea pigs undergoing a wide spectrum of treatment. Ovarian sections appeared normal in all cases.

Estrous cycles, recorded as the degree of vaginal patency at twenty-four hour intervals (Hartman et al., 1946), appeared normal in both groups. Four guinea pigs in the clover treatment group and two in the control group demonstrated irregular estrous cycles, but this was attributed to the onset of puberty.

Other than the expected normal variation, estrous cycle data of the clover treatment group were identical to those of the control group. An analysis of several aspects of this comparison is shown in Table 3. Total "estrous days"

TABLE 2

COMPARISON OF REPRODUCTIVE ORGAN WEIGHTS OF GUINEA
PIGS FED LADINO CLOVER TO REPRODUCTIVE ORGAN
WEIGHTS OF CONTROL GUINEA PIGS

	Clover	Control
Number of Animals	24	24
Mean Body Weight(g)	708.4	704.8
Mean Uterine Weight $\pm$ S.E.* (g)	10^{-2}x 125.8 $\pm$ 7.5	10^{-2}x117.6 $\pm$ 6.9
Mean Ovarian Weight $\pm$ S.E. (g)	10^{-2}x 9.84 $\pm$ 0.506	10^{-2}x11.06 $\pm$ 0.489
Mean Uterine Weight as Percent Body Weight $\pm$ S.E.	10^{-2}x 17.8 $\pm$ 1.06	10^{-2}x 16.7 $\pm$ 0.910

* $\pm$ S.E. = plus or minus standard error of the mean.

TABLE 3

COMPARISON OF ESTROUS CYCLES OF GUINEA PIGS FED LADINO
CLOVER TO ESTROUS CYCLES OF CONTROL GUINEA PIGS

	Clover	Control
Number of Animals	24	24
Total Complete Estrous Cycles for Group	69	72
Mean Number of Estrous Cycles per Guinea Pig	2.9	3.0
Mean Estrous Cycle Length (Days)	16.2	16.6
Total Number of Estrous Days*	380	360
Percent of Total Days Spent as Estrous Days	25.1	23.8
Mean Number of Days in Estrus per Guinea Pig	15.8	15.0

* See text for explanation.

refers to the total number of days of full patency experienced by all twenty-four animals in the group. Uterine weight expressed as percent body weight was determined for each animal by the fraction: $\frac{\text{uterine weight}}{\text{body weight}}$.

Guinea pigs were placed on experiment when their body weights and ages approximated those of females at the onset of puberty. Data were collected from newborn and growing females to establish these and other values (Appendix II).

Variation in uterine weight and vaginal patency, due to the influence of cyclic ovarian estrogen secretion, complicates analysis of the response of normal guinea pigs to exogenous estrogens. Consequently, ovariectomized females were used in subsequent studies. The ovariectomized female is also better adapted to study of the ability of estrogens to inhibit pituitary gonadotrophins.

b. Preserved Clover Feeding Experiment. A second study of the effect of crude clover was designed using ovariectomized guinea pigs. This procedure provided the advantages of increased uterine sensitivity to estrogen, and an increased consumption of clover as measured by dry weight. Ovariectomy and clover preservation techniques were performed as previously described.

All guinea pigs were ovariectomized during a twenty-four hour period and allowed to recover for the following six days. On the sixth post-operative day none of the animals demonstrated any degree of vaginal patency. At this

time, 5.0 mcg of estradiol benzoate in 0.25 cc of sterile corn oil was administered intramuscularly into each guinea pig to determine the ability of individuals to respond to exogenous estrogen. The estradiol benzoate had been stored in solution in 95 percent alcohol for over two months, and only a partial response was noted. This "priming" dose of estradiol benzoate was repeated in four days with freshly mixed compound and produced a more consistent estrogenic response as measured by complete vaginal patency in all animals.

Fifty ovariectomized guinea pigs showing positive response to the priming treatment with estrogen were divided into five equal groups. Treatment for each of the five groups was as follows:

1) Intramuscular injections of 5.0 mcg of estradiol benzoate in 0.25 cc of sterile corn oil were administered every 48 hours to each animal for a total of five treatments. The diet consisted of Guinea Pig Chow in mash form, and was termed "basal diet". These animals constituted a positive control group, and were expected to develop full vaginal patency and greatly increased uterine weights.

2) This group was given a diet composed of 60% preserved ladino clover and 40% basal diet. Mock intramuscular injections of 0.25 cc of sterile corn oil were given every 48 hours for 5 treatments. These guinea pigs were used to detect the presence of plant estrogens in the clover.

3) The only treatment given these animals consisted of mock intramuscular injections of 0.25 cc of sterile corn

oil every 48 hours for 5 treatments. They were fed the basal diet and used as negative controls. The expected response was low uterine weight and absence of vaginal patency.

4) As in the clover treatment group, these animals received a diet of 60% ladino clover and 40% basal diet. Intramuscular injections of 1.0 mcg of estradiol benzoate in 0.25 cc of sterile corn oil were administered every 48 hours for 5 treatments, as in group 5. This group was used to measure any effects that clover might have on concurrent low level estrogen treatment. The expectation was that an estrogen inhibitor would decrease the response to estradiol benzoate and that a plant estrogen would have either an additive or a synergistic relationship to estradiol benzoate. If neither effect occurred, the clover could be considered inert with respect to the measured responses, and the group would closely resemble group 5.

5) Guinea pigs in this group were given intramuscular injections of 1.0 mcg of estradiol benzoate in 0.25 cc of sterile corn oil every 48 hours for 5 treatments, and were fed a basal diet. These animals served to create the standard dose response necessary for interpretation of the results in group 4. They were also useful in determining the degree of response of guinea pigs to two graded doses of estradiol benzoate, thereby helping to define either maximal or minimal responses.

During the ten day treatment period, vaginal patencies were recorded daily, and body weights were recorded on days

TABLE 4

THE RESPONSE OF OVARIECTOMIZED GUINEA PIGS TO PRESERVED
LADINO CLOVER AND ESTRADIOL BENZOATE

Treatment	Number of Animals	Mean Body Weight(g)	Mean Uterine Weight(g)	Mean Uterine Weight Expressed as Percent Body Weight $\pm$ S.E. $(\text{x } 10^{-2})$	Percent Vaginal Patency
Estradiol Benzoate- 5.0 mcg per 48 hours	10	436.4	1.68	38.7 ± 1.82	100
Preserved Ladino Clover 60% of Diet	9	454.2	0.38	8.2 ± 0.59	0
Control	10	486.3	0.41	8.4 ± 0.65	0
Preserved Ladino Clover 60% of Diet and Estradiol Benzoate-1.0 mcg per 48 hours	8	443.9	1.41	31.7 ± 1.73	100
Estradiol Benzoate-1.0 mcg per 48 hours	10	469.8	1.45	31.0 ± 1.88	100

1, 3, 6, and 9. On day 10, all guinea pigs were killed. Their uteri were removed, carefully trimmed, and fixed in individual vials of Bouin's fluid. Uteri were weighed after a twenty-four hour fixing period, and subsequently embedded for sectioning and staining. Pituitary glands were removed aseptically and immediately prepared for gonadotrophin bioassay in immature female mice (see Mouse Gonadotrophin Bioassay Experiment). Uterine weight and vaginal patency data are given in Table 4.

The groups of guinea pigs treated with 1.0 and 5.0 mcg of estradiol benzoate had mean uterine weights between three and four times as great as untreated control animals. This response is probably maximal in that the animals receiving 1.0 mcg of estradiol benzoate had uterine weights only slightly less than those receiving 5.0 mcg doses. It is probable that the lower of the two doses is at the higher end of the expected linear dose response and may be too high to measure inhibitory activity. It is clear, however, that clover did not exhibit inhibitory activity in the form of decreased uterine weight or increased vaginal patency when given to guinea pigs receiving estradiol benzoate at the 1.0 mcg level, by comparison to others receiving the same dose of estradiol benzoate in the absence of a clover diet.

The mean uterine weight and percent vaginal patency of animals receiving a clover diet and no estradiol benzoate were almost identical to those of untreated control animals. Neither of these gross measurements, therefore, provides evidence for the existence of estrogenic substances in ladino

clover.

Cross-sections of uteri were taken from the junction
of a uterine horn with the body of the uterus. It is this
area of the endometrium in guinea pigs that is most likely to
contain endometrial cysts if any are present (Braden et al.,
1953). Uterine sections were taken from all guinea pigs,
stained with hematoxylin-eosin, and carefully examined.
Cystic glandular hyperplasia of the endometrium was noted in
a mild form in at least three animals in each of the three
groups receiving estradiol benzoate. The negative control
group demonstrated no evidence of this condition. However,
small endometrial cysts were noted in two of the guinea pigs
fed clover without supplementary estrogen treatment. Accur-
ate description of the occurrence of cystic glandular hyper-
plasia involves complete sectioning of the entire uterus of
each guinea pig. The value of this information in relation
to the excessive amount of work involved was not considered
adequate, and only 8 to 10 sections were examined from each
uterus.

The uterine epithelium in groups receiving estradiol
benzoate was composed of tall columnar cells filled with
large quantities of clear cytoplasm. The control animals and
clover treatment animals failed to show this response, in
that the epithelial cells were much smaller and lower, and
contained more densely stained cytoplasm.

c. Coumestrol Experiment. An extensive experiment
was designed to study the plant estrogen coumestrol by varying

the dosage and route of administration, and by comparing
certain responses to those elicited by estradiol benzoate.
Eleven treatment groups having from eight to twenty-one ani-
mals each were used to obtain information relative to a var-
iety of questions concerning plant estrogens. The description
of each group includes an explanation for its use.

Commercially obtained female albino guinea pigs were
ovariectomized at a time when their ages and body weights
were suggestive of the onset of puberty (Appendix II). After
a post-operative recovery period of nine or ten days, each
guinea pig was treated with an injection of 5.0 mcg of estra-
diol benzoate to establish the ability of the animal to re-
spond to estrogen. Only ovariectomized guinea pigs responding
to this priming treatment with full vaginal patency were used
in the experiment. A second ten day waiting period provided
ample time for all animals to recover from the priming dose,
so that no evidence of vaginal patency was observed.

During the post-operative waiting period, all diets
were prepared using the mash form of Purina Guinea Pig Chow.
The vehicle used to blend small quantities of coumestrol into
diets was powdered dextrose in sufficient amount to make each
diet 1.0 percent dextrose. All eleven diets contained the
blended vehicle, but those not containing coumestrol were
alike and are referred to as "basal diet". These diets were
fed during the same ten day period that injected treatments
were administered.

The injection vehicle for both coumestrol and estra-
diol benzoate was dimethyl sulfoxide (DMSO) in sub-cutaneous

doses of 0.2 cc every 48 hours. The use of this compound as
an injection vehicle is discussed in the section on Materials
and Methods. All guinea pigs not receiving parenteral doses
of estrogen were given mock-injections of DMSO, using the
same volume, route, and 48 hour time interval. For the ten
day treatment period, all guinea pigs were given diets con-
taining 1.0 percent dextrose, and injections of DMSO at 48
hour intervals for a total of five injections. The only dif-
ference in the treatment of the eleven groups was the estrogen
content of the diet and/or the injections.

Vaginal patency was determined and recorded daily for
each animal, as previously described, beginning on the day
of ovariectomy and continuing to the termination of the ex-
periment. Body weights were taken and recorded every three
or four days for the same length of time, ending on the day
before the termination of the experiment.

The group descriptions are arranged in a logical se-
quence; however, in the experiment they were placed randomly
in batteries of cages. Total feed consumption was measured
by weighing the feed every twenty-four hours for each group.
The oral intake of coumestrol was based on the mean diet con-
sumption for all groups (27.5 grams per guinea pig per day)
and is a close approximation of actual dosage. Treatments
and their rationale are described as follows:

1) Treatment consisted of mock injections of DMSO
and basal diet. These animals served as negative controls,
and were expected to show low uterine weights, no vaginal

patency, and quiescent uterine histology in respect to estrogen treatment.

2) Treatment consisted of 0.1 mcg injections of estradiol benzoate in DMSO and basal diet. The primary function of this group was comparison with the group receiving the same dose of estradiol benzoate plus coumestrol. It was also used to obtain data for low estrogen dosage response. Slight increase in uterine weight was expected, but no supposition was made regarding the degree of vaginal patency.

3) Treatment consisted of 1.0 mcg injections of estradiol benzoate in DMSO and basal diet. The function of the group was to establish an intermediate standard dosage response to estrogen, to aid in determining the estrogenic potency of coumestrol.

4) Treatment consisted of 5.0 mcg injections of estradiol benzoate in DMSO and basal diet. These guinea pigs were expected to demonstrate maximum responses, with high uterine weights and full vaginal patency in all cases. Foamy cytoplasm of uterine glands (Wright et al., 1958), and cystic glandular hyperplasia were anticipated to some degree in the uterine cross-sections.

5) Treatment consisted of mock injections of DMSO and 25 mcg of coumestrol per gram of diet. It was hoped that this dosage would be near the minimum requirement for a detectable response in at least one of the measurements used. Since no information is available concerning the effect of coumestrol on guinea pigs, this dosage estimate was based on

a simple mouse uterine weight bioassay described below. Total consumption of coumestrol per guinea pig was approximately 1.38 mg per 48 hours.

6) Treatment consisted of mock injections of DMSO and 200 mcg of coumestrol per gram of diet. A definite response to this dosage was expected, but the character of the response in comparison to estradiol benzoate was to be determined. It was conceivable that the relative effects of coumestrol on uterine weight, vaginal patency, and uterine histology would vary from the effects of endogenous estrogens. Total consumption of coumestrol per guinea pig was approximately 11.0 mg per 48 hours.

7) Treatment consisted of mock injections of DMSO and 400 mcg of coumestrol per gram of diet. On the basis of plant estrogen potency determination by Bickoff et al. (1960), substantial uterine weight increases were anticipated in this group. Since it is unlikely that greater amounts of coumestrol would be found under field conditions, this was the maximum oral dosage used. Total consumption of coumestrol per guinea pig was 22.0 mg per 48 hours.

8) Treatment consisted of 1.0 mg injections of coumestrol in DMSO and basal diet. The function of this group of guinea pigs was two-fold. First, they were to be compared to the animals in groups 2 and 11 for determination of the combined activity of coumestrol and estradiol benzoate. Secondly, they were part of an injection dosage range trial to be used in comparison with the oral administration of coumestrol.

9) and 10)   Treatments consisted of 2.5 mg and 7.5 mg injections of coumestrol in DMSO respectively, and basal diet.   These two groups, in conjunction with group 8, were used to establish a dose response range for comparison to guinea pigs treated with various doses of estradiol benzoate. Since many compounds demonstrate more predictable responses if given by injection rather than orally, it was expected that animals in these groups would have more consistant uterine weights, uterine histology, and vaginal patencies than those given coumestrol in their diet.

11)   Treatment consisted of injections containing 0.1 mcg of estradiol benzoate and 1.0 mg of coumestrol, and basal diet.   Effects of this treatment were to be compared to the effects of the same dosages of the two compounds given separately.   In this way, it could be determined if coumestrol and estradiol benzoate were mutually inhibitory, additive, or synergistic in eliciting a combined response.

On the tenth day of the experiment, all guinea pigs were killed.   Uteri were removed as in other studies, carefully trimmed, and immediately placed in individual vials of Bouin's fluid.   After a twenty-four hour fixing period, they were removed from the solution, blotted on paper towels, and weighed.   A portion of one uterine horn adjacent to the uterine body was removed, embedded in paraffin, and prepared in stained cross-sections as previously described.

Uterine weight responses in animals receiving estradiol benzoate increased from the lowest to the highest dosage,

TABLE 5

THE RESPONSE OF OVARIECTOMIZED GUINEA PIGS
TO THE PLANT ESTROGEN COUMESTROL

Treatment (Injected, Unless Otherwise Indicated)	Number of Animals	Mean Body Weight	Mean Uterine Weight	Mean of Uterus as Percent Body Weight $\pm$ Standard Error $(\times 10^{-2})$	Percent Vaginal Patency
Control (Negative)	17	430.5	.3654	8.65 $\pm$ 0.685	0
0.1 mcg Estradiol Benzoate	12	410.3	.4951	12.32 $\pm$ 0.778	8.3
1.0 mcg Estradiol Benzoate	8	425.6	1.4058	33.48 $\pm$ 2.20	100
5.0 mcg Estradiol Benzoate	11	423.0	1.5880	37.39 $\pm$ 1.69	100
25 mcg Coumestrol per Gram of Diet	10	429.1	.3016	7.09 $\pm$ 0.569	0
200 mcg Coumestrol per Gram of Diet	21	399.0	.3712	9.39 $\pm$ 0.609	52.4
400 mcg Coumestrol per Gram of Diet	11	401.0	.5604	14.26 $\pm$ 1.35	77.8
1.0 mg Coumestrol	11	410.5	.6830	16.62 $\pm$ 0.676	100
2.5 mg Coumestrol	11	404.1	1.1471	28.61 $\pm$ 2.43	100
7.5 mg Coumestrol	11	381.1	1.4364	37.72 $\pm$ 2.18	90.0
0.1 mcg Estradiol Benzoate Plus 1.0 mg Coumestrol	11	423.3	.9582	22.72 $\pm$ 1.26	100

and all of these groups had a mean uterine weight above that
of the negative control group. Contrary to what was expected,
uterine weight response of guinea pigs consuming 25 mcg of
coumestrol per gram of diet was below that of negative con-
trols, and in those consuming 200 mcg of coumestrol per gram
of diet it was about equal to that of negative controls. The
group receiving coumestrol orally at a rate of 400 mcg per
gram of diet was the only group receiving dietary treatment
that demonstrated an increase in uterine weight. The guinea
pigs treated with injections of coumestrol had uterine weights
that increased with increased dosage, as expected. The in-
crease in uterine weight for animals receiving a combined
treatment of coumestrol and estradiol benzoate was additive.
When the increase above the negative control response for
each compound given separately is added to the response of
the negative control group, the value is equal to that noted
in the combined treatment group, within the limits of stan-
dard error (Table 5).

Guinea pigs treated with the two higher doses of es-
tradiol benzoate demonstrated 100 percent vaginal patency,
whereas only 8.3 percent of those treated at the 0.1 mcg
level responded with full patency. Orally administered coum-
estrol at levels of 25, 200, and 400 mcg per gram of feed
produced vaginal patency in 0.0, 52.4, and 77.8 percent of
the guinea pigs respectively. Injected coumestrol produced
vaginal patencies in more guinea pigs than when included in
the diet, but one animal at the 7.5 mg dose level failed to

Figure 6a. Uterine Cross-Section from an Ovariectomized Guinea Pig in
the Control Group.
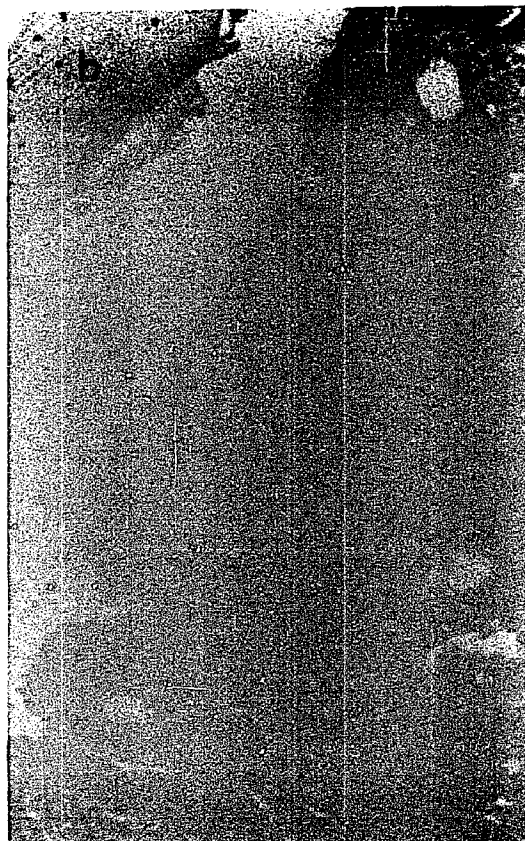
Figure 6b. "Foamy" Response of Uterine Epithelium to Estrogen Treatment.
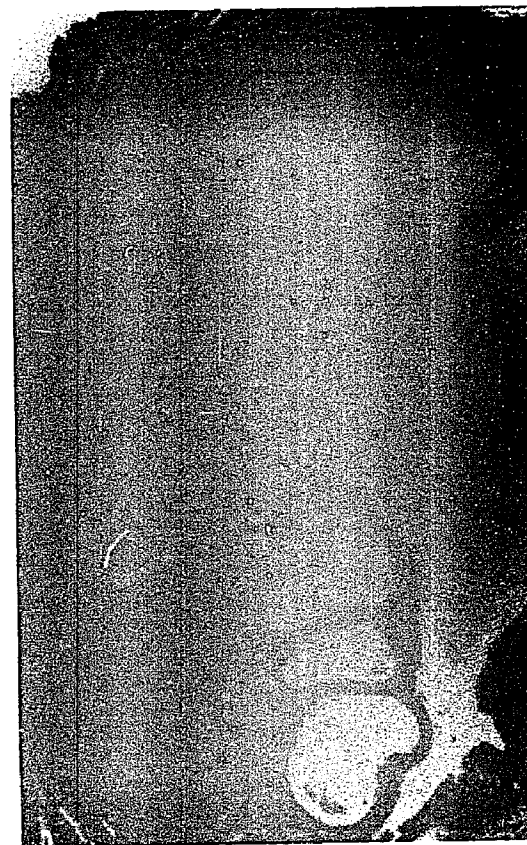
Figure 6c. Severe Cystic Glandular Hyperplasia.

respond. The combination treatment of estradiol benzoate and coumestrol caused the entire group to become fully patent (Table 5).

The two prominent phenomena noted in hematoxylin-eosin stained uterine cross-sections were cystic glandular hyperplasia (Figure 6c) and increased height and cytoplasmic vacuolation of epithelial cells (Figure 6b). Both observations were associated primarily with groups receiving enough coumestrol or estradiol benzoate to elicit other estrogenic responses. As noted by other investigators (Geil, 1962), cystic glandular hyperplasia may appear spontaneously even in the normal cycling female. It occurred to a very mild degree in two guinea pigs from the negative control group, and extensively in most estrogen treated animals. Although the "foamy" character of uterine epithelium (Wright et al., 1958) is also a response to estrogen treatment noted in this study, no correlation was made between a particular dose of estrogen and epithelial development. Figure 6a is a photograph typical of those taken of uterine cross-sections from ovariectomized guinea pigs not being treated with estrogen. It is taken from a guinea pig in the negative control group.

2. COUMESTROL STUDIES USING PARABIOTIC MICE

The fact that coumestrol is capable of causing increased uterine weight in immature mice has been known since soon after the compound was discovered by Bickoff et al. in 1957. It has been suggested by some authors that plant

estrogens bring about this and other responses only by direct
action on target organs (East, 1952; Carter et al., 1960).
The following experiment was designed to test that hypothesis.
An alternative possibility is that plant estrogens might act
through the pituitary-gonad axis.

The subjects for this study were C57 Bl/10J parabiotic
mice. Parabiotic union offers a unique situation to study
the effect of compounds on pituitary gonadotrophin activity.
When two histocompatible mice are surgically united so that
a large area of tissue from each animal is involved in the
permanently healed union, capillary beds from the two partners
intermingle to the extent that there is an exchange of blood
fluids. As in other gonadectomized rodents, pituitary pro-
duction of gonadotrophins is greatly increased in a similarly
treated parabiotic mouse. When one female partner remains
intact, excessive blood-borne gonadotrophins from the castrate
are transported to the intact partner and cause enlargement
of the ovaries and uterus. Under these circumstances, repro-
ductive organs may increase in weight by as much as 300 per-
cent above normal.

Gonadotrophin hypersecretion in the ovariectomized
mouse can be blocked by many steroids, including the endoge-
nous estrogens. The subsequent failure of ovarian and uterine
weights to increase in the intact partner of an ovariectomized
steroid treated mouse is termed "steroid inhibition". If
coumestrol (a non-steroid) possesses some capability of ster-
oid inhibition, one may safely conclude that its reproductive
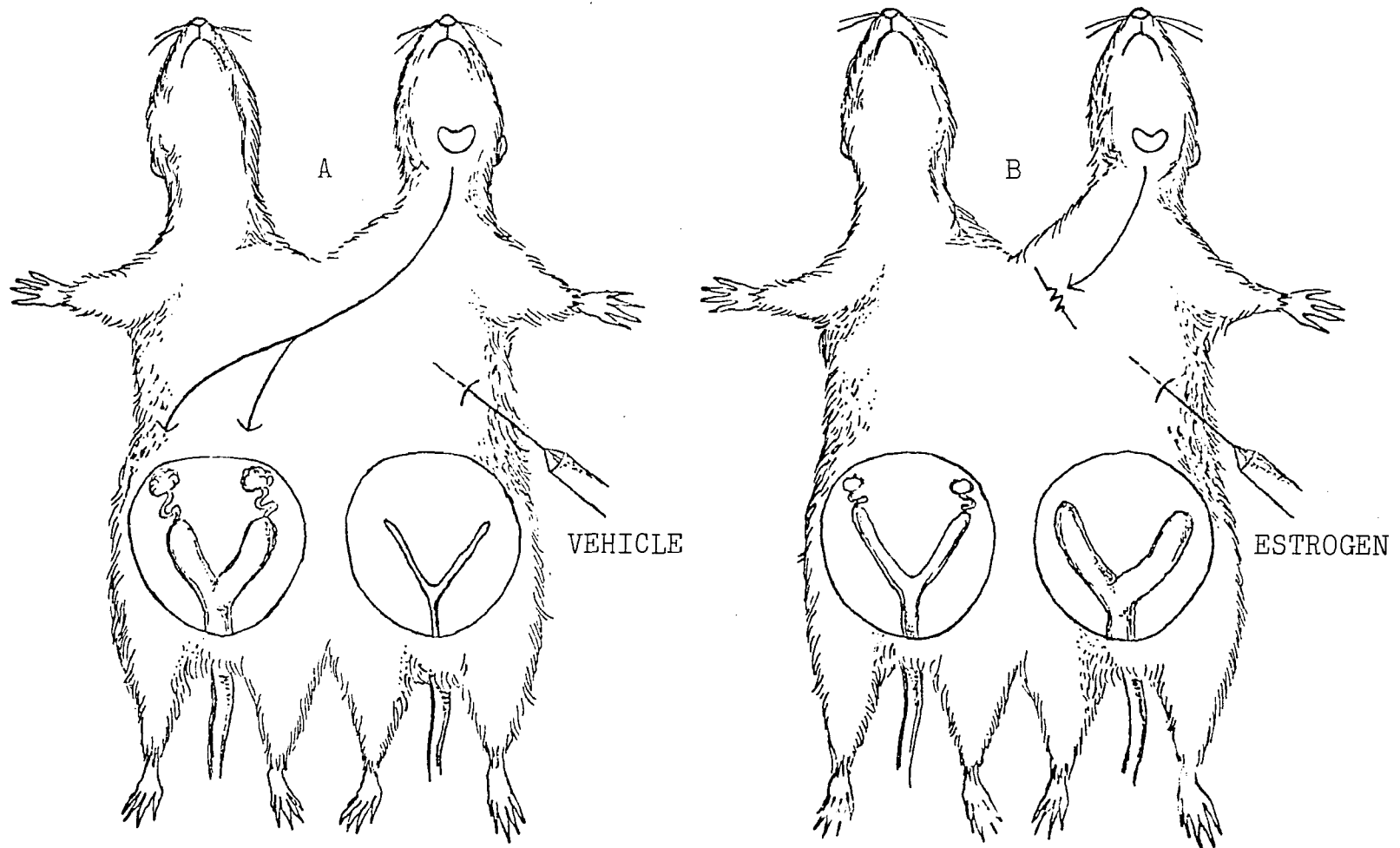
Figure 7.   Schematic Illustration of Parabiotic Mice

A.   Response of female reproductive tracts to control injection of vehicle.

B.   Response of female reproductive tracts to injection of an estrogen.

effects are mediated at least in part through the pituitary-gonad axis.

The response of an intact female mouse in parabiotic union with an ovariectomized partner, as well as the phenomenon of steroid inhibition are diagramed in the schematic drawing in Figure 7. The relative size of the reproductive organs has been exaggerated to emphasize the expected results.

A pilot experiment was conducted using thirteen pairs of parabiotic female albino mice, with one partner ovariectomized. This experiment was used to perfect surgery technique and to demonstrate steroid inhibition with estradiol-17-beta. Estradiol-17-beta injected daily for ten days in 0.1 cc doses of sterile corn oil produced the expected results, but oil deposits collected at the injection sites in rather large quantities.

The experiment designed to study steroid inhibition properties of coumestrol involved C57 Bl/10J female mice in parabiotic union, the right partner being ovariectomized and the left partner intact. Surgical procedure was performed as previously described (Figure 4). Of the forty pairs of mice used in the experiment, thirty-four (85 percent) survived and are included in the tabulation of the results. Four groups of ten parabiotic pairs were treated for ten days with daily sub-cutaneous injections of 0.01 cc of DMSO used as a mock injection or as a vehicle for one of the estrogens. For this degree of accuracy it was necessary to use a Hamilton microsyringe. In all cases, only the left (ovariectomized)

partner received the injection, and the right (intact) part-
ner was not treated. Body weights of the pairs were taken
every other day, and the degree of vaginal patency was re-
corded daily. Diet consisted of Purina Lab Chow pellets, and
general care was performed in the manner set forth in the
section pertaining to that subject. Treatments and their
rationale are described as follows:

1) Treatment consisted of daily injections of 100
mcg of coumestrol for each ovariectomized partner. This
dosage level was slightly above that needed to increase uter-
ine weight in ovariectomized adult female mice, as described
below. The group was expected to demonstrate a low but defin-
ite response to estrogenic activity as measured by uterine
weight of the ovariectomized partners. Properties of steroid
inhibition were in question and to be determined.

2) Treatment consisted of daily injections of 400
mcg of coumestrol for each ovariectomized partner. This
dosage was expected to be high enough to produce a substantial
increase in the uterine weight of its recipient. If coumestrol
possesses any significant power of steroid inhibition, it
should have appeared in this group.

3) These animals constituted the negative control
group and were treated with daily 0.01 cc mock injections of
the vehicle, DMSO. Ovarian and uterine weights of the intact
mice were expected to weigh two or three times more than those
of intact mice in the estradiol benzoate treated group. It
was anticipated that uterine weights of the ovariectomized

partners would be comparatively low.

4) Treatment consisted of daily injections of 0.1 mcg of estradiol benzoate for each ovariectomized partner. These animals were considered a positive control group in that they were expected to evoke the phenomenon of steroid inhibition. Coumestrol treated animals were expected to duplicate the response of either negative or positive control animals, or aquire a value somewhere between. The uterine weights of ovariectomized partners were expected to show substantial increase above those of their counterparts in the negative control group.

All mice were killed on the tenth day of the experiment, and the reproductive tracts of both partners were dissected free of the abdominal cavity. Ovaries and uteri were then carefully trimmed and fixed in individual vials of Bouin's fluid for twenty-four hours prior to being weighed. Tissues were weighed to the nearest 0.1 milligram after being gently blotted three times between two paper towels. The ovaries were embedded in pairs, serially sectioned, and stained with hematoxylin-eosin.

Mean uterine weights of the ovariectomized partners receiving 0.1 mcg of estradiol benzoate or 400 mcg of coumestrol per day were both more than four times greater than the mean uterine weight of respective animals in the negative control group. Mice receiving 100 mcg of coumestrol per day had mean uterine weights only twice as great as respective negative control animals. This measurement can be considered a direct

TABLE 6

THE EFFECTS OF COUMESTROL AND ESTRADIOL BENZOATE
ON PARABIOTIC MICE

Treatment Group	Number of Pairs	Mean Ovarian Weight(mg) $\pm$ S.E.*	Mean Left Uterine Weight (mg) $\pm$ S.E.	Mean Right Uterine Weight (mg) $\pm$ S.E.	Percent Vaginal Patency	
					Left Partner	Right Partner
Coumestrol- 100 mcg per day	8	2.54 $\pm$ .083	12.5 $\pm$ 0.88	31.1 $\pm$ 1.80	0.0	100
Coumestrol- 400 mcg per day	10	2.23 $\pm$ 0.15	15.0 $\pm$ 1.17	79.2 $\pm$ 4.39	0.0	100
Control	8	5.39 $\pm$ 1.02	52.2 $\pm$ 10.2	15.4 $\pm$ 0.86	87.5	0.0
Estradiol Benzoate- 0.1 mcg per day	8	2.09 $\pm$ .097	15.9 $\pm$ 0.80	68.9 $\pm$ 2.67	0.0	100

* $\pm$ S.E. = plus or minus standard error of the mean.
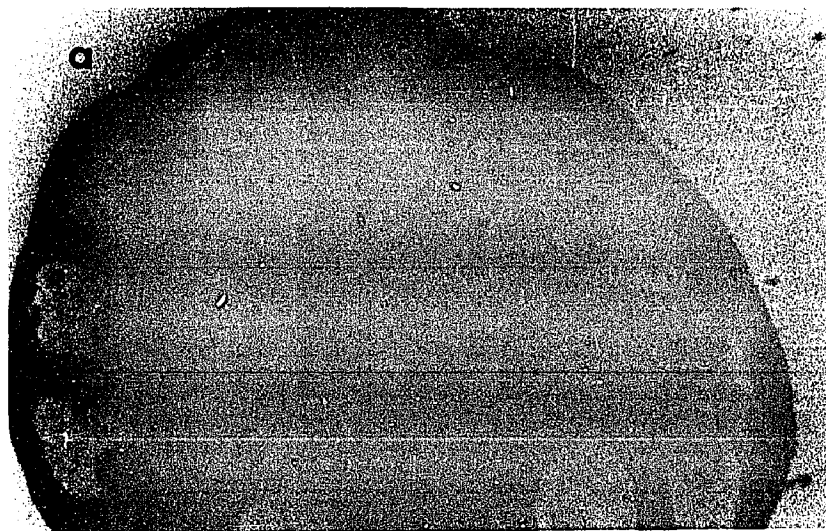
Figure 8a. Ovarian Section from Intact Parabiotic
            Mouse in Negative Control Group.

Figure 8b. Ovarian Section from Intact Parabiotic
            Mouse in Estrogen Treatment Group.

estrogen bioassay, and the results are apparently not affected by parabiotic union to an intact female (Table 6).

Mean uterine weights of intact females parabiosed to ovariectomized negative control mice were more than three times as great as the mean uterine weights of the three groups of intact mice in parabiotic union with ovariectomized partners receiving either coumestrol or estradiol benzoate. In terms of uterine weight response, there was no significant difference in the steroid inhibition activity of the three estrogen treatments (Table 6).

The mean ovarian weight of each group of intact females attached to partners receiving estrogen treatment was less than one-half that of intact females in the negative control group (Table 6). Ovaries from the negative control group demonstrated excessive follicle development with no luteinization (Figure 8a). Ovaries from groups receiving both doses of coumestrol and from the group receiving estradiol benzoate demonstrated sub-normal development (Figure 8b). Steroid inhibition as measured by ovarian weight response appeared equal in degree with all three estrogen treatments.

Vaginal patency was used as an index of estrogen activity only when it was maximal. All ovariectomized partners receiving estrogen treatment demonstrated 100 percent vaginal patency, and no cases of full patency were noted in their intact partners. In the control group, no cases of full vaginal patency occurred in ovariectomized mice, whereas it was noted in 87.5 percent of their intact partners (Table 6).

3. OTHER STUDIES

a. Pituitary Gonadotrophin Bioassay in Immature Mice.
Pituitary gonadotrophin content is known to increase after
gonadectomy of males or females in a great many species.
Bioassay of crude anterior pituitary tissue has been used by
other investigators to evaluate gonadotrophin content, and
much of this work is reviewed by Dorfman (1962). The bio-
assay of mouse and rat anterior pituitary tissue has been
rather successful, but reports on similar work with guinea
pigs are limited. In 1929, Smith and Engle transplanted en-
tire pituitary glands into immature mice, and attempted to
measure gonadotrophin activity by uterine and ovarian weight
increase.

Both endogenous estrogens and coumestrol will inhibit
gonadotrophin hypersecretion in ovariectomized mice, as shown
in the parabiosis studies. Attempts were made to find evi-
dence of a relationship between coumestrol and pituitary ac-
tivity in the guinea pig by selective staining of pituitary
basophils (Elftman, 1959; Purves et al., 1954; Pearse, 1950,
1952; Jubb et al., 1955), but the results were variable and
cell counts were inaccurate.

A procedure was developed for the bioassay of gonad-
otrophin activity in guinea pig pituitary glands. The glands
were macerated in sterile saline solution with a mechanical
tissue grinder (Appendix I) and injected into immature female
mice in six doses evenly spaced over a period of seventy-two
hours. The mice were then killed, and uterine and ovarian

weights were used as an index of gonadotrophin content.
Pilot work indicated that total doses of 1/20, 1/10, and
1/5 of a gland per assay mouse were too small for the sensitivity of the test, but that one entire gland was adequate.

On the basis of these findings, two experiments were
designed to assay the pituitary gonadotrophin content of
guinea pigs on clover, estradiol benzoate, coumestrol, and
control treatments. Description and results of the two experiments will be combined, as they were executed in the same
manner and the only notable variable was time.

Pituitary glands were removed aseptically from guinea
pigs immediately after they were killed, at the end of an
experiment. The glands were either freeze-dried for later
use, or cooled in grinding vials for use during the following
three days. Those to be used that day were pooled, macerated,
and suspended in sterile saline so that 0.6 cc of saline contained one pituitary gland. Six doses of 0.1 cc of suspension would then give a total dose of one entire gland. The
injection material was refrigerated in sterile screw-cap
vials, and sub-cutaneous injections were made with 1.0 cc
tuberculin syringes calibrated to 0.01 cc and fitted with
1-inch, 26-gauge hypodermic needles.

Albino female mice were ordered to arrive so that
they were twenty days old on the day that guinea pigs were
killed. They were then weighed, numbered, and placed in
cages containing five or six animals (one treatment group).
Starting on the evening of the day pituitary glands were

prepared, sub-cutaneous 0.1 cc injections were given at twelve hour intervals for six treatments. At the end of seventy-two hours, all mice were killed, reproductive tracts were removed, and ovaries and uteri were carefully trimmed and placed in individual vials of Bouin's fluid. After a twenty-four hour fixation period, the organs were weighed. The degree of vaginal patency had been recorded daily for each animal, and body weights were taken again at the end of the experiment.

Freeze-dried glands were reconstituted in sterile saline, macerated, refrigerated, and injected in the same manner as fresh glands. Freeze-drying would eliminate the need of immediate bioassay in future studies if the two methods gave comparable results. This bioassay method was used to determine the pituitary gonadotrophin content of six ovariectomized guinea pigs being treated with injections of 5.0 mcg of estradiol benzoate every forty-eight hours, and for five ovariectomized untreated quinea pigs.

A group of six intact female guinea pigs in various stages of the estrous cycle were bioassayed using fresh pituitary tissue. This group served to demonstrate, by comparison, the degree of castration hypersecretion one could expect under the existing conditions.

Control groups of bioassay mice were given mock injections of sterile saline using the same volume that was used for treatment groups.

The treatment groups of ovariectomized guinea pigs

(Table 7) produced remarkably similar uterine weight responses in bioassay mice. The only mouse groups with low mean uterine weights were those treated with pituitary glands from intact cycling female guinea pigs, and those given mock saline injections. It is apparent from mouse bioassay results that pituitary glands from ovariectomized guinea pigs have sufficient gonadotrophin to produce approximately 500 percent increase in mean uterine weight response. This response is not inhibited by relatively large doses of estradiol benzoate, coumestrol, or clover given to the guinea pigs over a period of ten days.

Mean mouse ovarian weight response to pituitary glands from ovariectomized guinea pigs given various estrogen treatments is less sensitive than mean uterine weight response. A definite tendency of glands from estradiol benzoate treated guinea pigs to increase ovarian weight response more than those from control guinea pigs (Table 7) is puzzling since the reverse is true with mice and rats (Dorfman, 1962). More study of this response is indicated before an accurate interpretation can be made.

Results obtained from the bioassay of pituitary glands from intact female quinea pigs were similar to those obtained from mice receiving mock saline injections. It must be concluded that this test is too insensitive to be used in the bioassay of pituitary glands from intact, cycling female guinea pigs.

Freeze-drying of pituitary glands with subsequent

TABLE 7

IMMATURE MOUSE BIOASSAY OF GUINEA PIG
PITUITARY GONADOTROPHIN CONTENT

Treatment of Guinea Pigs Being Assayed*	Number of Mice	Mean Body Weight (g)	Mean Uterine Weight (mg)	Mean Ovarian Weight (mg)
Control (negative)	5	14.0	35.2	3.8
5 mcg Estradiol Benzoate	5	14.8	44.6	5.8
1 mcg Estradiol Benzoate	11	13.8	41.6	5.4
1 mcg Estradiol Benzoate + Clover	5	14.5	42.6	5.7
Clover	5	13.3	39.5	4.1
25 mcg Coumestrol	6	14.2	45.7	4.6
200 mcg Coumestrol	6	14.1	48.2	4.0
Intact, Untreated Females	6	13.1	7.6	3.1
Control, Freeze-Dried	5	13.3	41.4	5.6
5 mcg Estradiol Benzoate, Freeze-Dried	6	13.2	43.8	7.0
-----No Tissue Assayed- Saline Injections	22	12.7	8.4	3.3

* For detailed description of treatment, consult
  appropriate section of text.

storage for up to two months had no degradation effect on gonadotrophin content. The elevated responses produced by these two groups were probably due to the larger pituitary glands and longer post-ovariectomy waiting periods (Table 7).

The failure of estradiol benzoate and coumestrol to demonstrate steroid inhibition may be explained by some recent work by Haller (1963). He was able to inhibit gonadotrophin hypersecretion in ovariectomized guinea pigs using oral administration of progesterone for up to six months. It is possible that the ten day steroid treatment period used in this study was too short.

b. Mouse Bioassay of Preserved Ladino Clover. Acetone extracts of preserved ladino clover (Bickoff et al., 1957) were incorporated into diets as previously described, and bioassayed for estrogenic activity. Groups of five or six 20-day-old female albino mice were fed the prepared diets for three days, and then killed. Mean uterine weight increase above the value for negative control animals was used as the index of estrogen content. Ovarian weight and vaginal patency were measured but did not vary significantly (Table 8). Positive control animals were fed a diet containing 0.02 mcg of diethylstilbestrol per gram. Clover samples collected at intervals during the growing season were assayed separately in an effort to detect any change. Except for the expected response of the positive control group, no signs of estrogenic activity were observed. The results are shown in Table 8.

A thirty-day feeding experiment was used to evaluate

TABLE 8

MOUSE BIOASSAY OF LADINO CLOVER FOR ESTROGENIC ACTIVITY

Treatment	Number of Mice	Mean Body Weight (g)	Mean Ovarian Weight (mg)	Mean Uterine Weight (mg)	Mean of Uterus as Percent Body Weight
Clover Extract 8 May	5	12.7	3.2	12.4	.097
Clover Extract 19 June	5	12.5	3.8	11.9	.095
Clover Extract 8 July	5	12.3	2.8	13.5	.110
Clover Extract 9 September	5	12.9	3.4	15.0	.116
Lettuce Extract	5	13.0	3.3	13.0	.100
Negative Control	16	12.9	3.4	14.3	.111
0.02 mcg Diethyl-stilbestrol per Gram of Diet, Positive Control	6	13.3	3.6	22.8	.171

TABLE 9

MOUSE UTERINE WEIGHT BIOASSAY COMPARING GRADED DOSES
OF COUMESTROL TO DIETHYLSTILBESTROL

Treatment: Expressed as mcg per Gram of Diet	Number of Mice	Mean Body Weight (g)	Mean Uterine Weight (mg)	Mean of Uterus as Percent Body Weight
0.01 mcg Diethylstilbestrol	5	25.1	37.1	.149
0.02 mcg Diethylstilbestrol	5	23.3	61.9	.263
50 mcg Coumestrol	5	24.1	20.8	.086
100 mcg Coumestrol	5	23.6	33.1	.140
200 mcg Coumestrol	5	24.3	54.6	.223
400 mcg Coumestrol	5	24.1	68.0	.284
Control	5	23.6	17.1	.073
Control	5	24.6	21.4	.086

the effect of ladino clover on the mouse estrous cycle.
Mature albino female mice were fed a mash diet containing
20 percent preserved ladino clover by weight. Vaginal smears
from each mouse were taken daily, stained with 1 percent
methylene blue, and the stage of the estrous cycle was re-
corded. Of the sixteen mice treated, none demonstrated a
significant variation from the normal estrous cycle sequence.

c. Mouse Uterine Weight Bioassay of Coumestrol.
A simple mouse uterine weight bioassay was used to determine
the approximate estrogenic activity of the coumestrol used
in these studies. Six-week-old albino CD-1 female mice were
ovariectomized while under Nembutal anesthesia, as described
previously. Eight groups of five mice were treated for ten
days as described in Table 9. Estrogens were blended in a
mash diet of Purina Lab Chow and fed ad libitum, and at the
end of the ten-day treatment period, all mice were killed.
Uteri were dissected free, carefully trimmed, and fixed in
Bouin's fluid for twenty-four hours prior to being weighed.
Higher doses of coumestrol in the diet caused larger in-
creases in uterine weight as was expected. Positive control
animals fed diethylstilbestrol, and untreated negative con-
trol animals were used for comparison.

V. DISCUSSION

1. LADINO CLOVER

Ladino clover used in this experiment is a leguminous forage widely found in New England. It was handled in a manner to cause minimum alteration of chemical composition both in fresh feeding work and in the process of preservation and storage (Bickoff et al., 1959; Alexander et al., 1951).

Intact female guinea pigs fed a diet of fresh ladino clover were unaffected in terms of estrous cycle length, estrus duration, uterine weight, ovarian weight, and body weight when compared to control animals. This would indicate an absence of estrogenic and anti-estrogenic activity in that none of the measurements taken were significantly altered by dietary clover. Guinea pigs have relatively large capacity for forage consumption, and estrous cycles similar in many ways to those of grazing ruminants. It is believed that the natural relationship of forage to the female reproductive system was closely simulated. Mouse estrous cycles were not noticeably affected when mature intact females were fed a diet composed of 20 percent preserved ladino clover (dry weight) for thirty days.

Ovariectomized guinea pigs were fed preserved clover diets containing up to 60 percent clover by dry weight. Those receiving a treatment of clover diet demonstrated no evidence of estrogen activity in terms of uterine weight, vaginal

patency, or character of endometrial cytology. Those re-
ceiving the same clover diet and low dosage of estradiol
benzoate responded as did those receiving the same dosage of
estradiol with no dietary clover. This would indicate an
absence of anti-estrogenic potency. The dry weight of clover
consumed by these guinea pigs was approximately equal to the
dry weight of clover consumed by guinea pigs fed fresh mate-
rial.

A mouse uterine weight bioassay was performed to
detect the presence of estrogen activity by using acetone
extracts of ladino clover in a 1:1 ratio with basal mash
diet. Samples of the clover fed to guinea pigs were assayed
at different stages of the growing season, and none of the
samples produced an increase in uterine weight. A positive
control group of mice receiving 0.02 mcg. of diethylstilbes-
trol per gram of diet demonstrated substantial increase in
uterine weight when compared to the negative control group
and to the groups treated with clover extract. It is appar-
ent from the experiments described that ladino clover used
.in these studies was inert in relation to its ability to
impose reproductive change on female mice or guinea pigs.

The plant estrogen coumestrol was isolated from
ladino clover by Bickoff et al. (1957) and its estrogenic
activity was measured by the method used above. The plant
estrogens genistein (Guggolz et al., 1961) and daidzein
(Guggolz et al., 1961; Wong, 1962) have also been isolated
from ladino clover. It is clear that under certain cir-
cumstances, ladino clover can be estrogenic, but that its

estrogenic activity is variable and in some instances absent. Recent work by Loper et al. (1964) associating the estrogenic activity of forages with fungus infections of the plants examined may lead to a better understanding of the entire problem. It is tempting to use his work to explain marked seasonal, growth stage, and geographic variations in activity (Pieterse et al., 1956; Kitts et al., 1959; Bickoff et al., 1960).

A degree of infertility has been reported in rabbits (Wright, 1960) and sheep (Engle, 1957; Sanger et al., 1961) consuming ladino clover for periods of several weeks. Current work indicates that preserved ladino clover used in the present investigations has no direct effect on conception rate or breeding efficiency of mice. It is difficult to make a positive correlation between purely estrogenic activity as measured by studies on immature or ovariectomized females, and decreased fertility of intact females. The possibility that there are other factors working alone or in conjunction with plant estrogen needs further investigation.

2. COUMESTROL

The literature contains many reports of estrogenic activity in various plant extracts and fractions as reported in reviews by Moule et al., (1963) and Bickoff (1961). The chemistry of isolated plant estrogens has been described in detail, and their "estrogenic potency" has been ascertained using uterine weight bioassays. The similarity of plant estrogens to endogenous estrogens, beyond their ability to

stimulate increased uterine weight, (Bickoff et al., 1960; East, 1955; Carter et al., 1960) has not been thoroughly investigated. The need for more detailed information on the mode of action of plant estrogens and variations in the response of animal species is a major problem of this study.

The difficulty in obtaining sufficient quantities of relatively pure plant estrogens has hampered research in this area for a long time. Fortunately, enough coumestrol was obtained from the Eastman Kodak Company to permit some detailed studies using laboratory animals. It is expected that supplies will become available for work with ruminants in the near future.

The potencies of some plant estrogens have been estimated (Bickoff, 1961; Leavitt, 1963; and others) by comparing mouse uterine weight response to plant estrogen treatment with the same response in mice treated with diethylstilbestrol. Relative estrogenic potency of plant compounds can also be measured by comparison with certain responses (vaginal patency, uterine histology, species variation, vaginal exfoliative cytology, etc.) to endogenous estrogens. In many cases, variation in the estrogen assay technique produces substantially different results. Cheng and Burroughs (1959) found diethylstilbestrol to be 83,000 times as potent as genistein. Bickoff (1961) states that coumestrol is approximately thirty times more potent than genistein, giving diethylstilbestrol a potency approximately 2,780 times greater than coumestrol when measured by uterine weight increase. Bickoff et al. (1960) state that variation

in the methods used by different investigators casts confusion on relative potency claims for plant estrogens. The present studies substantiate this by the following findings regarding estrogenic potency of coumestrol:

1. When orally administered coumestrol was compared to diethylstilbestrol at levels of 0.01 and 0.02 mcg. per gram of diet and fed to ovariectomized adult female mice, the diethylstilbestrol was respectively 11,000 and 14,500 times more potent than coumestrol.

2. When injected coumestrol was compared to estradoil benzoate injected at levels of 1.0 and 5.0 mcg. per treatment into ovariectomized adult female guinea pigs, the estradiol benzoate was respectively 3,500 and 1,300 times more potent than coumestrol.

3. When orally administered coumestrol was compared to estradiol benzoate injected at a level of 0.1 mcg. per treatment into ovariectomized adult female guinea pigs, the estradiol benzoate was 87,000 times more potent than coumestrol.

4. When injected coumestrol was compared to estradiol benzoate injected at a level of 0.1 mcg. per treatment into ovariectomized partners of a parabiotic union (direct bioassay), estradiol benzoate was 3,300 times more potent than coumestrol.

It should be noted that in none of these cases did approximate dose-response curves of coumestrol and those of reference estrogens coincide. The validity of relative potency claims would seem questionable when based on the

crossing point of two very different response curves, unless exact dosages and conditions are indicated. There is, however, little question that coumestrol has estrogenic activity but is much weaker than most endogenous estrogens.

Coumestrol is capable of producing estrogen responses other than increased uterine weight. As noted in the section on Procedures and Results, some responses of ovariectomized guinea pigs to coumestrol are cystic glandular hyperplasia, full vaginal patency, and vacuolation and increased height of endometrial epithelium. All of these responses show variability when the dose level or route of administration is changed. Several attempts were made to evaluate the effect of coumestrol on pituitary activity in guinea pigs. Selective staining of pituitary gonadotroph cells met with partial success, but the results were not dependable enough for accurate interpretation. Pituitary gonadotrophin bioassays in immature female mice demonstrated that neither coumestrol nor estradiol benzoate was able to inhibit castration hypersecretion in ovariectomized guinea pigs at the treatment levels used.

No conclusive information has been available concerning the ability of coumestrol to affect pituitary function. Parabiotic mice were used in our experiments to determine if coumestrol possesses anti-gonadotrophic activity similar to that of steroidal estrogens. Results indicate that doses of 400 mcg. of coumestrol (a non-steroid) per day were capable of strong "steroid inhibition", and that doses of 100 mcg.

of coumestrol per day were capable of weak "steroid inhibition" of castration hypersecretion in mice.

It is concluded that the plant estrogen coumestrol is capable of interfering with reproductive processes at the pituitary level as well as through direct action on reproductive tissues. Although the effects on reproductive phenomena are similar to those produced by endogenous estrogens, the degree to which certain tissues and functions respond is variable.

VI. SUMMARY

Recent publication of three excellent reviews pertaining to the general problem of forage estrogens has directed the present Review of the Literature to specific areas of study.

Fresh and preserved ladino clover were harvested by hand and treated in a manner to cause minimum decomposition of inherent compounds that might affect reproduction. Preserved samples were dried in a convection oven, ground into a mash, and immediately sealed in cans for storage at $0°$ C or below. The estrogens used in this study, coumestrol (a plant. estrogen), estradiol benzoate, estradiol-17-beta, and diethylstilbestrol, were commercially obtained and of excellent quality.

An experiment was designed to study the effects of fresh ladino clover on the reproductive tract of the intact mature female guinea pig. Forty-eight guinea pigs were divided into two equal groups, one treated with fresh clover diet, and the other serving as a control. The two groups demonstrated no significant difference in the following measurements:

1. ovarian weight and histology
2. uterine weight and histology
3. duration of estrus
4. duration of estrous cycles
5. body weight

Five groups of ten ovariectomized guinea pigs receiving various treatment combinations of preserved clover and estradiol benzoate were arranged so that the presence of both estrogenic and anti-estrogenic properties could be demonstrated. No evidence of either of these properties was found to be a result of clover treatment alone. Expected responses to estrogen were noted in all groups receiving estradiol benzoate treatments.

An extensive experiment was designed to study the plant estrogen coumestrol by varying the dosage and route of administration, and by comparing certain responses to those elicited by estradiol benzoate. Eleven groups of from eight to twenty-one ovariectomized guinea pigs were used to obtain information relative to a variety of questions concerning plant estrogens. Coumestrol produced significant increase in uterine weight when fed in the diet at a rate of 400 mcg. per gram and when injected at 1.0, 2.5, and 7.5 mg. levels. Vaginal patency response to coumestrol develops at a lower dosage than does an increase in uterine weight. Cystic glandular hyperplasia of the endometrium and enlargement of cells of the uterine epithelium developed in response to both coumestrol and estradiol benzoate in higher doses. The combined estrogenic effects of coumestrol and estradiol were additive rather than synergistic or inhibitory, at the levels tested.

A study involving C57 Bl/10J mice in parabiotic union, with one partner ovariectomized and one partner intact, was used to determine the ability of coumestrol to affect

pituitary function. The ability of certain steroid compounds to inhibit castration hypersecretion can be measured by failure of the reproductive tract in the intact female to enlarge. The phenomenon, termed "steroid inhibition", is based on the ability of steroidal compounds to interfere with the production or release of excess gonadotrophin in treated subjects, and is a measurement of direct pituitary response to the test compound. Coumestrol (a non-steroid) demonstrates the same ability, and forces the conclusion that this plant estrogen relates itself to mammalian reproduction via the central nervous system as well as by direct action on reproductive target organs.

Bioassays of pituitary gonadotrophin content in guinea pigs from the above experiments were performed by measuring mouse uterine and ovarian weight responses to pituitary gland suspensions. At the treatment levels used, neither coumestrol nor estradiol benzoate was capable of inhibiting the substantial increase in pituitary gonadotrophin content of ovariectomized guinea pigs.

Immature mouse uterine weight bioassay of acetone extracts from preserved clover samples collected periodically during the growing season demonstrated no evidence of estrogenic activity at any of the growth stages. A similar bioassay of commercially obtained coumestrol provided a dose response curve for use in other experiments. Mouse uterine weight increase began when coumestrol was included in the diet at levels between 50 and 100 mcg. per gram.

It is concluded that ladino clover used in these

studies was inert in relation to its ability to impose reproductive change on female mice or guinea pigs. It is also concluded that the plant estrogen coumestrol is capable of interfering with reproductive processes at the pituitary level as well as through direct action on reproductive tissues. Although the effects on reproductive phenomena are similar to those produced by endogenous estrogens, the degree to which certain tissues and functions respond is variable.

BIBLIOGRAPHY

Adams, E. C., and A. Hertig. 1964. Studies of guinea pig oocytes. J. Cell. Biol. 21: 397.

Adler, J. H., and D. Trainin. 1960. Hyperoestrogenic syndrome in cattle. Refuah Veterinarith, 17: 108.

Adler, J. H. 1962. Anti-oestrogenic activity in alfalfa. Vet. Record. 74: 1148.

Alexander, G., and R. H. Watson. 1951. The assay of oestrogenic activity of Trifolium Subterraneum L. by increase in uterine weight in the spayed guinea pig. I. Characteristics of the dose-response relationship. Austral. J. Agr. Res. 2: 457.

Alexander, G., and R. H. Watson. 1952. The assay of oestrogenic activity of Trifolium Subterraneum L. by increase in uterine weight in the spayed guinea pig. II. Assays. Austral. J. Agr. Res. 3: 480.

Allen, E., and E. A. Doisy. 1923. An ovarian hormone; a preliminary report on its localization, extraction and partial purification, and action on test animals. J. Am. Med. Assoc. 81: 819.

Aron, M., and J. Marescaux. 1962. New argument in favour of a single pituitary gonadotropin. J. Physiol. (Paris) 54: 276.

Ashwood-Smith, M. J. 1961. The use of dimethyl-sulfoxide as a protective agent in mice receiving high doses of x-radiation. Int. J. Radiation Biol. 3: 41.

Aust, S. D. and H. P. Broquist. 1965. Isolation of a parasympathomimetic alkaloid of fungal origin. Nature. 205: 204.

Batali, M., J. B. Rogers, and H. T. Blumenthal. 1961. The aging process in the guinea pig ovary. J. Gerontol. 16: 230.

Beck, A. B. 1964. The oestrogenic isoflavones of subterranean clover. Austral. J. Agr. Res. 15:223.

Bennetts, H. W., E. J. Underwood, and F. L. Shier. 1946. A specific breeding problem of sheep on subterranean clover pastures in western Australia. Austral. Vet. J. 22:2.

Bickoff, E. M. 1961. Estrogen-like substances in plants. Physiol. of Reprod. Proceedings of Twenty-Second Biology Colloquium. Corvallis, Ore.

Bickoff, E. M., A. N. Booth, R. L. Lyman, A. L. Livingston, C. R. Thompson, and F. De Eds. 1957. Coumestrol, a new estrogen isolated from forage crops. Science 126: 969.

Bickoff, E. M., A. N. Booth, R. L. Lyman, A. L. Livingston, C. R. Thompson, and G. O. Kohler. 1958. Isolation of a new estrogen from ladino clover. J. Agr. Food Chem. 6: 536.

Bickoff, E. M., A. L. Livingston, A. N. Booth, A. P. Hendrickson, and G. O. Kohler. 1960. Estrogenic activity in dehydrated and sun-cured forages. J. Animal Sci. 19: 189.

Borges, P. R. F., and G. E. Forester. 1955. Study of C57 Bl/10-H-2^b and C57 Bl/10-H-2^d mice joined in parabiosis, and their reaction to a transplantable leukemia. Acta Un. Cancer 11: 610.

Bounds, D. G., and G. S. Pope. 1960. Light-absorption and Chemical properties of miroestrol, the estrogenic substance of _Pueraria mirifca_. J. Chem. Soc. 3696-3705.

Bradbury, R. B., and D. E. White. 1951. The chemistry of subterranean clover. Part I. Isolation of formononetin and genistein. J. Chem. Soc. 3447-3449.

Bradbury, R. B., and D. E. White. 1954. Estrogens and related substances in plants. Vitamins and Hormones 12: 207.

Braden, A. W. H., and J. E. Peterson. 1953. The persistence of endometrial cysts induced by oestrogen in guinea pigs. Austral. J. Biol. Sci. 6: 520.

Breneman, W. R., M. Cormack, D. E. Overack, R. O. Creek, and R. Shaw. 1960. Inhibition of anterior pituitary gonadotrophins and oxytocin by extracts of _Lithospermum ruderale_. Endocrinology 67: 583.

Bunster, E., and R. K. Meyer. 1937. An improved method of parabiosis. Anat. Rec. 57: 339.

Burcher, R. L., K. Y. Chu, and R. M. Melampy. 1962. Uteroovarian relationships in the guinea pig. Endocrinology 71: 810.

Burgos, M. H., and G. B. Wislocki. 1956. The cyclical changes in the guinea pig's uterus, cervix, vagina and sexual skin, investigated by histological and histochemical means. Endocrinology 59: 93.

Buxton, J. B. 1927. Mycotic vaginitis in gilts. Vet. Med. 22: 428.

Carter, M. W., G. Matrone, and W. W. G. Smart. 1960. The effect of genistein and its aglycone on uterine weight gain in the mouse. Brit. J. Nutr. 14: 301.

Ch'ang, T. S. 1961. Reproductive performance of New Zealand Romney sheep grazed on red clover (Trifolium pratense) pastures. J. Agr. Sci. 57: 123.

Cheng, E., and W. Burroughs. 1959. Estrogenic substances in forages. In Grasslands, H. B. Sprague, ed. Amer. Assoc. for the Adv. of Sci. Pub. 53, pp. 195-202.

Chur'y, J., and K. Panek. 1964. The effect of unbalanced lucerne ration on the ovaries and uterus. Vet. Med. (Praha) 9: 99 (Cz.)

Cook, H., and W. D. Kitts. 1964. Anti-oestrogenic activity in yellow pine needles (Pinus ponderosa). Acta Endocrinologica 45: 33.

Dattagupta, P. C., H. N. Khastgir, and P. Shengupta. 1960. Investigations on the structure of psoralidin. Chem. and Ind. 1960: 48.

Deanesly, R. 1961. The effects of oestrogen on tubal transport and ovo-implantation in the guinea pig. Proc. of Fourth Intern. Cong. of Animal Reprod., The Hague 371-374.

Deanesly, R. 1963a. Early embryonic growth and progestagen function in ovariectomized guinea pigs. J. Reprod. Fertil. 6: 143.

Deanesly, R. 1963b. Further observations on the effects of oestradiol on tubal eggs and implantation in the guinea pig. J. Reprod. Fertil. 5: 49.

De Laszlo, H., and P. S. Henshaw. 1954. Plant materials used by primitive peoples to affect fertility. Science 119: 626.

Dorfman, R. I. ed. 1962. Methods in hormone research. Vol. II. Academic Press, New York. 774 p.

Dorfman, R. I., and A. S. Dorfman. 1963. Pituitary gonadotropic inhibitory action of estrone in parabiotic rats. Endocrinology 72: 115.

Duckett, G. E., H. H. Varon, and J. J. Christian. 1963. Effects of adrenal androgens on parabiotic mice. Endocrinology 72: 403.

East, J. 1950. Oestrogenic effects of subterranean clover; Mammary development in the castrate guinea pig. Austral. J. Exp. Biol. Med. Sci. 28: 449.

East, J. 1952. Oestrogenic effects of subterranean clover; Studies on the female guinea pig. Austral. J. Sci. Res. 5: 472.

East, J. 1955. The effect of genistein on the fertility of mice. J. Endocrin. 13: 94.

Eichwald, E. J. 1959. Genetic factors in parabiosis. J. Natl. Cancer Inst. 23: 1193.

Elftman, H. 1959. Combined aldehyde-fuchsin and periodic acid-Schiff staining of the pituitary. Stain Tech. 34: 77.

Elghamry, M. I., and J. Chur'y. 1961. Determination of the lactogenic principle of hypophysis of male rats after treatment with phytoestrogens. Ceskoslov. Akod. Zemedel. Ved., Vet. Med. 34: 273. (Cz)

Emmens, C. W., R. I. Cox, and L. Martin. 1960. Oestrogen inhibition by steroids and other substances. J. Endocrin. 20: 198.

Emmens, C. W., R. I. Cox, and L. Martin. 1964. The oestrogenic and anti-oestrogenic activity of compounds related to diethylstilbestrol. Acta Endocrinologica 90: 61.

Engle, P. K., D. S. Bell, and R. P. Davis. 1957. The effect of ladino clover, birdsfoot trefoil, and bluegrass pasture on the rate of conception among ewes. J. Animal Sci. 16: 703.

Ferrando, R., M. M. Builleux, and V. A. Guerillot. Estrogen content of wheat, carrots, and grass hay. Nutr. Abstr. and Rev. 34: No. 2.

Finerty, J. C. 1952. Parabiosis in Physiological Studies. Physiol. Rev. 32: 277.

Flux, D. S., R. E. Munford, and G.F. Wilson. 1963. Biological estimation of oestrogenic activity in red clover (Trifolium pratense): Relative potencies of parts of plant and changes with storage. J. Dairy Res. 30: 243.

Freund, M., J. Wiederman, and A. Saphier. 1963. A method for the simultaneous recording, in vitro, of the motility of the vagina, of the body of the uterus, and of both uterine horns in the guinea pig. Fertil. Steril. 14: 416.

Geil, R. G., and C. L. Davis. 1962. Spontaneous endometrial hyperplasia in guinea pigs. Am. J. Vet. Res. 23: 362.

Gray, P. 1954. The microtomist's formulary and guide. The Blakiston Co., New York. 794 p.

Guggolz, J., A. L. Livingston, and E. M. Bickoff. 1961. Detection of daidzein, formononetin, genistein, and bio-chanin A in forages. J. Agr. Food Chem. 9: 330.

Haines, G. 1931. A statistical study of the relation between various expressions of fertility and vigor in the guinea pig. J. Agr. Res. 42: 123.

Haller, J. 1963. Reversibility of gestagen-induced gonado-trophin inhibition in guinea pigs. J. Reprod. Fertil. (abstr.) 5: 297.

Hartman, C. G., J. L. Littrell, and J. Tom. 1946. The Weanling guinea pig as a test object for estrogen assays. Endocrinology 39: 120.

Hermreck, A. S., and G. S. Greenwald. 1964. The effects of unilateral ovariectomy on follicular maturation of the guinea pig. Anat. Rec. 148: 171.

Johnson, D. C., and E. Witschi. 1961. Endocrinology of ovarian tumor formation in parabiotic rats. Cancer Res. 21: 783.

Jubb, K. V., and K. McEntee. 1955. Observations on the bovine pituitary gland. Cornell Vet. 45: 576.

Kallela, K. 1964. The incidence of plant oestrogens in Finnish pastures and fodder plants with special reference to their possible effects in cases of sterility in ruminants. Helsinki Col. Vet. Med. Pub. 131 p.

Kitts, W. D., E. Swierstra, V. C. Brink, and A. J. Wood. 1959. The estrogen-like substances in certain legumes and grasses. I. The quantitative determination of such substances in red clover and oats. Canad. J. Animal Sci. 39: 396.

Koen, J. G., and H. C. Smith. 1945. An unusual case of genital involvement in swine associated with eating moldy corn. Vet. Med. 40: 131.

Kurouji, K. 1963. Anti-progestational activities of certain plant preparations on the uterine endometrium. Tohoku J. Exp. Med. 79: 335.

Leavitt, W. W. 1963. Influences of legumes on the repro-ductive physiology of mice. Ph.D. Thesis. Univ. of N. H. 174 p.

Leonardelli, J. 1961. Investigation of vaginal smears during the oestrous cycle of the guinea pig. C. R. Soc. Biol. 155: 2156 (Fr.)

Loper, G. M., and C. H. Hanson. 1964. Influence of controlled environmental factors and two foliar pathogens on coumestrol in alfalfa. Crop Sci. 4: 480.

Loran, M. R., and J. V. Carbone. 1964. The humoral effects of intestinal resection on cellular proliferation and maturation in parabiotic rats. Fed. Proc. 23: 407. (abstr.)

Mardones, E., J. Bruzzone, R. Iglesias, and A. Lipschutz. 1951. The non-concomitancy of the progestational and anti-luteinizing action of steroids. Endocrinology 49: 417.

Martin, L., C. W. Emmens, and R. I. Cox. 1960. The effects of oestrogens and anti-oestrogens on early pregnancy in mice. J. Endocrin. 20: 299.

McNutt, S. H. 1927. Vaginitis in gilts. Vet. Med. 22: 452.

Merk Veterinary Manual. 1961. 2nd ed. Merk and Co., Rahway, N. J.

Miyake, T. 1961a. The use of parabiotic mice for the study of pituitary gonadotrophin hypersecretion and its inhibition by steroids. Endocrinology 69: 447.

Miyake, T. 1961b. Inhibitory effect of various steroids on gonadotrophin hypersecretion in parabiotic mice. Endocrinology 69: 534.

Morrison, F. B. 1954. Feeds and feeding. The Morrison Publishing Co., Ithaca, N. Y. 1207 p.

Moule, G. R., A. W. H. Braden, and D. R. Lamond. 1963. The significance of oestrogens in pasture plants in relation to animal reproduction. Animal Breeding Abstr. 31: 139.

Pakrashi, A. 1963. Endocrinological studies on plant products. Part IV: Effect of some coumarins on the biological potency of human chorionic gonadotrophin. Ann. Biochem. Exp. Med. 23: 357.

Pearse, A. G. E. 1950. Differential stain for the human and animal anterior hypophysis. Stain Technol. 25: 95.

Pearse, A. G. E. 1952. The cytochemistry and cytology of the normal anterior hypophysis investigated by the tri-chrome-periodic acid-Schiff method. J. Pathol. Bacteriol. 64: 811.

Perry, J. S., and I. W. Roslands. 1963. Hypophysectomy of the immature guinea pig and the ovarian response to gonadotrophins. J. Reprod. Fertil. 6: 393.

Pieterse, P. J. S., and F. N. Andrews. 1956. The estrogenic activity of legume, grass, and corn silage. J. Dairy Sci. 39: 81.

Pope, G. S. 1954. The importance of pasture plant oestrogens in the reproduction and lactation of grazing animals. Dairy Sci. Abstr. 16: 333.

Pope, G. S., P. V. Elcoate, S. A. Simpson, and D. G. Andrews. 1953. Isolation of an oestrogenic iso-flavone (Biochanin A) from red clover. Chem. Ind. 1953: 1092.

Pope, G. S., M. J. McNaughton, and H. E. H. Jones. 1959. Oestrogens in British pasture plants. J. Dairy Res. 29: 196.

Purina Laboratory Manual. 1963. Pub. Ralston Purina Co., St. Louis, Mo.

Purves, H. P., and W. E. Griesbach. 1954. The site of FSH and LH production in the rat pituitary. Endocrinology 55: 785.

Reed, H. C. B. 1961. The relationship between kale and fertility in dairy cattle. Proc. 54th. Intern. Congr. Animal Reprod., The Hague.

Rona, G., and C. I. Chappel. 1963. Protection of scorbutic bone lesions of the guinea pig by equine estrogen. Endocrinology 72: 1.

Sanger, V. L., and D. S. Bell. 1961. Comparative effect of ladino clover and bluegrass pasture on fertilization of ova in sheep. Cornell Vet. 51: 204.

Saunders, F. J., and K. Rorig. 1964. Separation of antifertility and classic estrogenic effects. Fertil. Steril. 15: 202.

Schoop, G., and H. Klette. 1952. Gehaufte Sterilitat durch oestrogene Stoffe in Weidegras. Rep. II. Int. Congr. Physiol. Path. Animal Reprod. Vol. II: 87.

Schuetz, A. W., D. B. Sager, and R. K. Meyer. 1964. Studies on the control of FSH secretion in parabiotic and single rats. Fed. Proc. 23: 410 (abstr.).

Smith, P. E., and E. T. Engle. 1929. Evidence of a correlation between the amount of gonadal stimulating hormone present in the pituitary of the guinea pig and the stage of the reproductive cycle. Anat. Rec. 42: 38.

Snell, G. D. 1958. Histocompatability genes of the mouse. J. Natl. Cancer Inst. 20: 787.

Stockard, C. R., and G. N. Papanicolaou. 1917. The existence of a typical oestrous cycle in the guinea pig with a study of its histological and physiological changes. Amer. J. Anat. 22: 225.

Takasugi, N. 1963. Gonadotropic activity of the anterior hypophysis of old female mice as demonstrated by parabiosis with young partners. J. Fac. Sci. Univ. Tokyo, Sec. 4, 10: 193.

Thaine, R. I. 1963. Bovine infertility syndrome possibly involving pasture oestrogens. Austral. Vet. J. 39: 37.

Vatna, S. 1939. Preliminary report on the presence of an oestrogenic substance and a poisonous substance in the storage root of Butea superba. Roxb. Thai. Sci. Bull. No. 4: 3.

Walter, E. D. 1941. Genistin (an isoflavone glucoside) and its aglucone, genistein, from soybeans. J. Am. Chem. Soc. 63: 3273.

Walz, E. 1931. Isoflavone and saponin glucosides in Soja hispida. Justus Leibigs Annalen, 439: 118.

Whitney, L., and I. A. Parfentjev. 1964. Use of yeast fraction for birth control in animals. Fed. Proc. 23: 292 (abstr.).

Wong, E., and D. S. Flux. 1962. The oestrogenic activity of red clover isoflavones and some of their degradation products. J. Endocrin. 24: 341.

Wright, J. F., and N. R. Seibold. 1958. Estrogen contamination of pelleted feed for laboratory animals - Effect on guinea pig reproduction. J. Am. Vet. Med. Assoc. 132: 258.

Wright, J. G. 1957. Veterinary anesthesia. Williams and Wilkins Co., Baltimore, Md. 248 p.

Wright, P. A. 1960. Infertility in rabbits induced by feeding ladino clover. Proc. Soc. Expt. Biol. Med. 105: 428.

Wright, S. 1960. The genetics and vital characters of the guinea pig. J. Cell. Comp. Physiol. 56: 123.

Youngman, V. E. 1963. Investigation on the estrogenic activity of selected forage crops. Dist. Abstr. 23: No. 2286.

Zarrow, M. S., N. C. Anderson, and M. R. Callantine. 1963. Failure of progestogens to prolong pregnancy in the guinea pig. Nature 198: 690.

APPENDIX I

EQUIPMENT

To avoid prolonged descriptions of commercially ob-
tained equipment used in this study, the following list is
included. A brief description, immediate source, and catalog
number for each item are given as a reference for more de-
tailed information.

	Description	Source	Catalog Number
1.	Microscope Illuminator,A-O Spencer, Ortho-Illuminator	Fisher Scientific Company	12-408V4
2.	Microscope, Bausch and Lomb, Dynazoom, Binocular-Style A	Fisher	12-314
3.	Microscope, Stereoscopic, Wide Field, Binocular, Bausch and Lomb	Fisher	12-328-5
4.	Evaporator, Rotating Vacuum type, Rinco	Fisher	9-548V4
5.	Extraction Apparatus, with Friedrich Condenser, Pyrex	Fisher	9-571-B
6.	Centrifuge, International Model HN, Electric	Fisher	5-111-W1
7.	Head, Horizontal, Six-place 50 ml.	Fisher	5-111-11
8.	Head, Angle, 24-place, for 15 ml Shields, No.5-160	Fisher	5-111-12
9.	Vacuum Oven, National	Fisher	13-262X2
10.	Electric Tissue Grinder	Bodine Electric Co. Chicago, Illinois	
11.	Paraffin Embedding Oven, large model	Aloe	V64720

Description	Source	Catalog Number
12. Extraction Apparatus, Soxhlet Modified, Large	Aloe	VC3885
13. Lamp, Aloe-Dozor	Aloe	P9415
14. Laboratory Stools, Swivel	Aloe	V51965
15. Chromatography Cabinet, Chromatocab, Large Size, Stainless Steel	Aloe	V29202 B
16. Chromatography Assembly-Square	Aloe	V29204 SA
17. Desicators-Scheibler, Tubulated Cover	Aloe	V36210 C
18. Balances, Triple Beam, Ohaus Model 1750S	Fisher	2-034
19. Balance, Analytical, Semi-Micro, Mettler, Model H-16	Fisher	1-908-20X2
20. Oven, Mechanical Convection, Blue M "Power-O-Matic 60" Model 256	Fisher	13-258-20W2
21. Extractor, Glass, Side-Arm, with Condenser	Ace Glass,Inc. Vineland,N.J.	6740-B
22. Autoclave, Sterilizing, Electrically Heated, 3 Heat	Fisher	1-801 W4
23. Autotemp Heaters, Thermostatically Controlled	Fisher	11-466-100
24. Lab Wagon, Chemicart, Stainless Steel	Fisher	11-926
25. Cutting Mill, Wiley, Standard Model No. 3, Motor Driven	Fisher	8-336-5W1
26. Fluorometer, Automatic, Turner Model 111	Fisher	7-118-5V2
27. Water Bath, Utility, Constant Temperature Precision	Fisher	15-457 large
28. Water Bath Covers, Size 170	Fisher	15-454-5

Description	Source	Catalog Number
29. Clipper, Animal	Fisher	1-305V4
30. Balance, Animal, Triple Beam, Ohaus, Model 730	Fisher	2-034-25
31. Air Pump, Vacuum, Welch Dist-O-Pump with Motor	Fisher	1-103
32. Microtome, AO Spencer, Model 815, Open Rotary Type	Fisher	12-606
33. Microtome Knife Sharpener, AO Spencer Sutomatic	Fisher	12-643-50V1
34. Slide Warmer, Fisher Improved	Fisher	12-594-5V3
35. Topcon Super D 35 mm Camera, with Microscope Adapter, Carrying Case and 1.4 Lens	Chas. Beseler Co. E. Orange, N.J.	
36. Ventilated Fume Hood		
37. Refrigerator-Freezer Combination	Sears Roebuck and Co.	
38. Monroe Calculator, Model 8F-213	Monroe Calculating Machine, Portland, Maine	
39. Calab Flash Evaporator, Cold Finger Assembly	Calab Company Berkeley, California	

Assorted glassware, surgery instruments, chemicals, small equipment and animal facilities are not included.

APPENDIX II

GUINEA PIG PUBERTY DATA

Table 10 contains data describing the ages and body weights of female albino quinea pigs on the first day of "full vaginal patency", considered the onset of puberty. Duration of the first estrus (in days) and birth weights are included. This information was used in determining the starting date for the fresh ladino clover feeding experiment and for choosing an opportune time for ovariectomy of young females in other studies. Mean values are close approximations.

TABLE 10

DATA TAKEN FROM TEN FEMALE ALBINO GUINEA PIGS
AT ONSET OF PUBERTY

Guinea Pig Number	1	2	3	4	5	6	7	8	9	10	Mean
Approximate Weight at Birth (grams)	102	102	103	111	111	111	107	107	122	122	109.8
Weight at First Vaginal Patency (grams)	278	280	339	330	374	310	323	280	364	355	323.3
Age at First Vaginal Patency (days)	25	21-22	30	27	31-34	26	31	23	26	33	27.6
Duration of First Estrus (days)	7	6-7	4-5	4+	—	5+	4	6	3	7	5.3