EFFECTS OF LEGUMES ON REPRODUCTION IN MICE

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Summary. Ingestion of dried forage, incorporated into a Chow diet at a level of 15 or 30 %, induced infertility and reproductive anomalies in female mice. Conception rate in SWR/Jax mice was nil during a 22-day cohabitation with fertile males, as opposed to 57 % conception in controls. The fertility of CD-1 male mice was not affected by *Ladino*. Vaginal and behavioural oestrus in CD-1 mice was inhibited for 18 or more days during feeding of 30 % *Ladino* clover, but animals gradually adjusted whilst ingesting clover, and after 18 days oestrus reappeared and fertile matings occurred. Ingestion of 15 % prebloom *Ladino* inhibited oestrus, but oestrus was more frequent and persistent than normal in mice receiving 15 % second-growth *Ladino*. Feeding 30 % mixed-growth *Ladino* for only 3 days caused a severe reduction in number of fertilized ova recoverable and a concomitant increase in degenerate ova; longer feeding frequently inhibited ovulation and sexual receptivity in spite of vaginal cornification. No single aqueous-alcoholic, ether-alcoholic, chloroform, or acetone-chloroform extract of forages had any of the peculiar properties of the unfractionated plant, but all extracts increased the incidence of non-viable embryos.

INTRODUCTION

In recent years considerable attention has been focused on antifertility properties of forages, especially legumes. It is well established that various plants exhibit oestrogenic activity (Bradbury & White, 1954) and that forages may contain amounts large enough to affect reproduction adversely (Bennetts, Underwood & Shier, 1946; Pope, 1954). Following the isolation of genistein, East (1956) tested its effect on the fertility of mice and found that reproduction was impaired, males being more severely affected than females. Coumestrol seems to produce similar results (A. N. Booth, personal communication).

Fox, Kaufmes, Mason & Oldfield (1957) reported reproductive failure in mice ingesting 40 % red clover. Further work indicated that this reproductive disruption could not be explained in terms of oestrogenic or progestational effects or in terms of pituitary stimulation or inhibition alone. Swierstra (1958) caused breeding difficulties in mice by feeding alcoholic extracts of red clover and birdsfoot trefoil, and suggested that oestrogen-like substances were the causal agents. It is hoped that the present paper may clarify some apparent discrepancies. The work is a continuation of studies carried out by Wright

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(1960), but mice were used for comparative purposes and for further evaluation of mechanisms involved.

MATERIALS

Freshly-harvested plant material (i.e. leaves and/or bloom, but the bulk of weight consisting of petioles and leaflets) from plots in Northwood, New Hampshire, U.S.A., was dried for 12 hr at 60° C in a convection oven and pulverized in a Wiley mill. Diets were compounded by addition of powdered forage to Purina Laboratory Chow, the basal ration, plant material being thoroughly blended in the diets by means of extensive hand-mixing.

Whenever extracts of forage were administered, they were added to the Chow diet in amounts which represented a definite equivalence of fresh plant material. Various organic solvents were then removed under vacuum and final equilibrium was brought about in an air current for 24 to 48 hr, with intermittent stirring. Water-soluble fractions were either stored at 4° C and added at each feeding, or were dried with Chow in the convection oven.

All diets were fed as a wet paste in Petri dishes. Small platforms were constructed in the cages to hold the feeding dishes above the litter. This procedure afforded a minimum of contamination of the diets.

EXPERIMENTAL PROCEDURES AND RESULTS

FEEDING FORAGES IN THE DIET

Thirty-two mature SWR/Jax (Jackson Memorial Laboratory) females weighing 18 to 22 g were divided into four equal groups and housed four per cage. One group was fed on basal ration throughout and the remaining groups were fed on 15, 30 and 60 % (by weight) clover diets (5 g/mouse/day). After 7 days males were introduced, two per cage, and left with the females for 22 days, all groups being maintained on their respective rations. At the end of 22 days, the males were removed and the females continued on their respective diets through a theoretical gestation period (i.e. for an additional 22 days). Experimental females were regrouped with their respective males at the conclusion of the forage feeding period, and basal ration was substituted for the clover diets at that time.

No pregnancies occurred in experimental females during treatment with 15 and 30 % diets, as compared to 50 % pregnancies in controls. The 60 % diet proved too unpalatable to the mice during the initial 7 days (21 % weight loss), but 57 % pregnancies subsequently resulted when control ration was substituted. Return to a fertile condition required about 5 days of readjustment after cessation of the experimental diet in these animals. In experiments which incorporated lower levels of *Ladino* clover in the diets, there were body weight losses proportional to the amount of clover in the diet (16 % on the 30 % diet and 4 % on the 15 % diet). Weight gains began to appear after 18 days of feeding, indicating an adaptation to the experimental diets. Feeding of the basal diet, after 50 days of 15 or 30 % *Ladino* clover diet, allowed 75 % and 37 % pregnancies, respectively, requiring the same period of readjustment, 4 to 5 days, as the animals which were briefly put on a 60 % diet.

In order to explore these results in more detail, males and females were

tested separately. Since SWR/Jax mice are highly inbred, fertility and number of offspring per litter were low. Therefore, CD-1 mice originating from an initial stock of gnotobiotic animals were used for comparative purposes and for further evaluation of the mechanism of effect.

Sixteen mature CD-1 males, ranging in age from 3 to 7 months, were divided into two equal groups. Each male was housed in an individual cage. One group was fed on a 30 % *Ladino* diet and the other group on the control ration. After 5 days, each male was proffered, in the evening, an oestrous female (not of proven fertility) until a positive mating occurred or until four different females had been tried. Matings were determined by the presence of a copulation plug in the vagina or spermatozoa in a vaginal smear. Seven of eight *Ladino*-fed males mated with a record of 0·54 matings per trial. Six of the seven matings were fertile. All control males mated with a record of 0·67 matings per trial, and seven of the eight matings were fertile. The control males were subsequently fed on 30 % *Ladino* for 5 days and mating trials were carried out as before. All eight mated with 0·62 matings per trial, and seven of the matings were fertile.

Vaginal smears were taken daily on mature CD-1 females for 12 days to check for regularity of oestrous activity. A 30 % *Ladino* diet was then administered to eight of these, and upon the appearance of an oestrous smear, the female was placed with a male (of proven fertility) overnight. Copulation was determined as previously described. The first oestrous smear after beginning the *Ladino* diet occurred on Day 18 and all females had achieved oestrus by Day 23. The majority of animals did not mate immediately upon reappearance of vaginal oestrus. Six positive matings did eventually occur, and all were fertile. These resulted from thirteen trials at vaginal oestrus.

Ninety immature females were weaned at 18 days of age and started then on various 15 % forage diets. A cellulose diet was included for comparison, as it was reasoned that the relatively large intake of vegetable matter was unnatural for the laboratory mouse and might constitute a type of dietary stress. Animals were caged at random in groups of five, and fed twice daily (6 g/mouse/day), in the early morning and evening. Body weights were recorded every other day. The animals were checked daily for vaginal introitus and vaginal smears were taken to determine the time of first oestrus and cyclic oestrous activity. One-half of the animals in each group were killed at 45 days of age, and the weights of both ovaries and adrenals were determined on a direct-reading analytical balance.

The data (Table 1) indicate a variety of effects from 15 % forage diets. None of the diets significantly influenced the time of onset of vaginal patency, which averaged 31·0 days. It is interesting to note the trend within the *Ladino* groups. No significant oestrogenic activity was detected by separate bioassay in any of the samples at the levels used. A potentiation of endogenous oestrogenic response was manifested by more frequent and more persistent vaginal cornification in groups receiving second growth *Ladino*. Prebloom *Ladino* clearly inhibited oestrous activity, but bloom forage had neither stimulatory nor inhibitory properties. No useful data could be obtained from adrenal weights, but ovarian weight at Day 45 and log growth rate showed high correlation

(Table 2). Neither ovarian weight nor growth rate were significant when considered individually. Whether or not these data indicate an effect on pituitary function must be determined by more critical experimentation.

Fifty-one CD-1 females 2 to 4 months old were selected for more detailed study of the effect of a 30% *Ladino* diet on reproductive physiology. Twenty-

TABLE 1

EFFECT OF 15% FORAGE DIETS ON VAGINAL SMEARS IN YOUNG CD-1 MICE

Diet*	No. smears	No. oestrous smears	No. oestrous cycles†	Oestrous smears / No. smears	Oestrus occurring by 44 days (%)
Timothy *Phleum pratense*	95	18	15	0·190	80
Alfalfa‡ *Medicago sativa*	176	24	16	0·136	80
Cellulose§	157	16	12	0·102	90
Ladino prebloom *Trifolium repens*	125	6	5	0·048	44
Ladino bloom	144	19	11	0·132	70
Ladino second growth July	121	21	16	0·174	100
Ladino second growth late August	155	35	25	0·226	100
Control	283	42	31	0·149	95

* *Ladino* prebloom = nine animals; Control = twenty animals; All other groups = ten animals.
† In some cases oestrous smears occurred on consecutive days. The value given here is the number of vaginal oestrous cycles.
‡ Oestrogenic as measured by immature mouse bioassay.
§ Purified ashless powder.

TABLE 2

EFFECT OF 15% FORAGE DIETS ON OVARIAN WEIGHT AND GROWTH RATE IN CD-1 MICE

Diet	Ovarian weight (mean ±s.d.) (mg)	Growth rate in log growth phase (Average g gained/day)
Timothy	5·8 ± 1·1	0·65
Alfalfa	6·7 ± 1·6	0·69
Cellulose	7·2 ± 1·4	0·79
Ladino, August (second growth)	7·4 ± 1·5	0·81
Control	8·4 ± 2·1	0·99

eight of these were used for egg counts performed 34 to 37 hr *post coitum* in the manner described by Lewis & Wright (1935). Two experimental procedures were devised: (1) Five animals were started on clover diets at early pro-oestrus and mating trials were attempted at oestrus approximately 1½ days later. (2) Thirteen mice were fed a clover diet for 11 to 22 days, with mating trials commencing at the first oestrus after 11 days of treatment.

Ladino's influence on fertilization and survival of ova is depicted in Table 3. Under temporary treatment (Group A), the number of abnormal ova increased, resulting in only 53 % fertilized ova being recovered. After prolonged treatment

TABLE 3

EFFECT OF *Ladino* ON OVA OF CD-1 MICE

Ova from	Condition of ova		Control	Ladino A*	Ladino B*
Receptive females in oestrus (mated)	Normal	2-cell†	49 (8)‡	26 (4)	31 (6)
		1-cell	5	4	12
	Abnormal		2	19	4
Non-receptive females in oestrus	Normal 1-cell		16 (2)	0 (1)	12 (7)§
	Abnormal		1	10	20

* Diet A given for 36 hr prior to mating, Diet B given for 11 to 22 days prior to mating.
† Two-cell ova were considered fertilized. No data were collected to indicate the number of non-viable ova represented by these figures.
‡ Number of animals in parentheses.
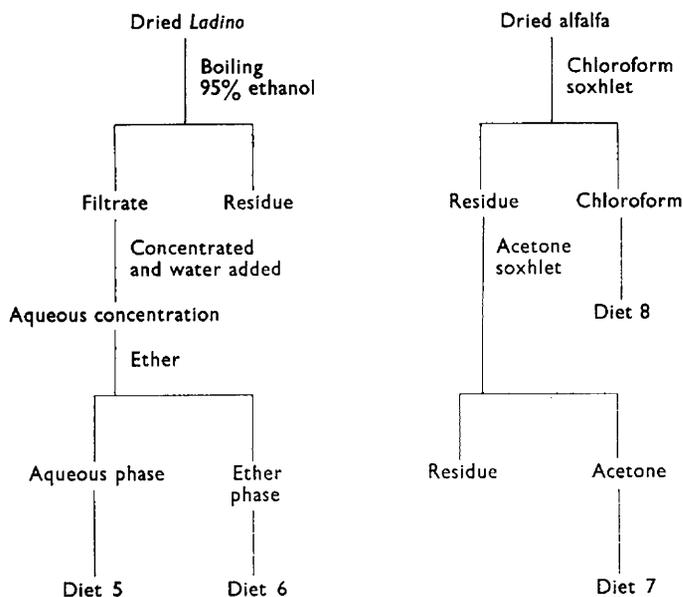§ Three of these animals did not ovulate.
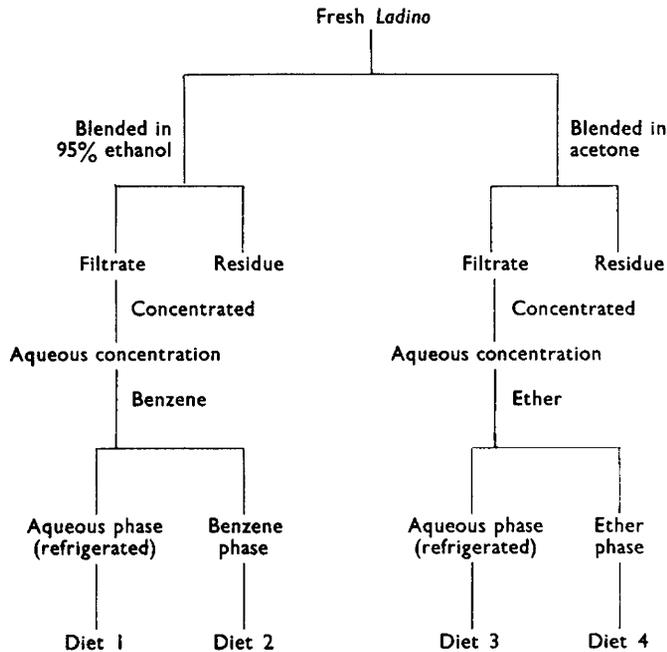
TEXT-FIG. 1. Outline for Experiment 1 diet preparation.

(Group B), 66 % were fertilized, and abnormal ova had returned to a normal level. Attention should be drawn to the fact that three of seven mice in Group B (non-copulating, oestrous animals) did not ovulate. A higher incidence of abnormal ova was noted in non-receptive animals from both groups.

FEEDING FORAGE EXTRACTS IN THE DIET

Diets were prepared with various fractions of legume, as shown in Text-figs. 1 and 2. Experiment 1 was designed to investigate fresh material and to compare fractionation procedures. Two different dried legumes were compared in Experiment 2. The *Ladino* meal sample contained no significant oestrogenic activity as measured by an immature mouse bioassay. The alfalfa sample had an activity equal to 0·02 µg diethylstilboestrol/g. Since all diets were prepared with doses of extracted material equivalent to levels in the original source (i.e. fresh or dried legume), those tested in Experiment 2 contained higher concentrations of extract that those in Experiment 1. Each diet was tested on eight or more mice with proven oestrous activity. Vaginal smears, and mating trials with males known to be fertile, were carried out as before with CD-1 females.

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    Dried Ladino                              Dried alfalfa
         |                                         |
      Boiling                                  Chloroform
      95% ethanol                              soxhlet
       _____|_____                            _____|_____
      |           |                          |           |
   Filtrate    Residue                    Residue    Chloroform
      |                                      |           |
 Concentrated                             Acetone        |
 and water added                          soxhlet        |
      |                                      |          Diet 8
 Aqueous concentration                       |
      |                                       |
    Ether                                     |
   ___|___                               _____|_____
  |       |                             |           |
Aqueous  Ether                       Residue     Acetone
phase    phase                          |           |
  |        |                            |           |
  |        |                            |           |
Diet 5   Diet 6                                   Diet 7
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TEXT-FIG. 2. Outline for Experiment 2 diet preparation.

Seven days were allowed for adjustment at the onset of experimental feeding, with matings attempted at each oestrus during treatment (22 to 35 days). Conceptions resulting in Experiment 1 were not disturbed, whereas pregnancies in Experiment 2 were terminated 15 days *post coitum*. Viable embryos were weighed at autopsy and the characteristics of non-viable embryos were noted.

None of the extracts in Experiment 1 exhibited any of the peculiar properties of whole *Ladino*. One animal on Diet 2 and one on Diet 4 became pseudopregnant, but no pseudopregnancies were noted in the other twenty-eight animals tested in this part of our research. Again in Experiment 2 there was no suppression of oestrous cycles, but one pseudopregnancy resulted from Diet 6 (*Ladino*) and three were produced by Diet 8 (alfalfa). No repeat breeders were noted among the remaining animals. In both extract experiments there was no significant difference in number of conceptions or in litter size. Feed

consumption data indicated that Diets 5 and 8 were unpalatable but not enough to cause weight losses. Table 4 shows that there was an increase in number of non-viable embryos in all females on extract diets in Experiment 2.

Bickoff, Livingston, Booth, Hendrickson & Kohler (1960) have pointed out that the oestrogenic response obtained when a crude forage extract is assayed represents a summation of effects of oestrogen inhibitors and potentiators,

TABLE 4

RESULTS OF EXTRACT EXPERIMENT 2 AT 15TH DAY OF PREGNANCY

Diet*	Wt. mice before treatment (g)	Wt. gain at Day 15 pregnancy (g)	No. viable embryos	Wt. viable embryos (g)	No. non-viable embryos†
Basal	36·4	11·0	10·9	0·256	0·75
5	36·4	10·1	11·0	0·245	1·49
6	37·5	11·2	11·6	0·299	1·25
7	34·2	9·8	9·8	0·285	1·20
8	35·5	9·0	10·1	0·262	1·36

* Eight animals per diet. All figures are group averages.
† Includes resorptions and embryos dead at time of autopsy.

TABLE 5

BIOASSAYS WITH IMMATURE CD-1 MICE OVARIECTOMIZED AT 18 DAYS AND TREATED FOR 6 DAYS

Treatment	No. animals	Vaginal patency at 25 days	Average uterine weight (mg)	P value	Average weight at 25 days (g)
Basal diet	10	0	7·79		17·3
'Alfalfa'*	6	6	12·66	<0·05	17·8
Diet 5	6	0	7·72		17·0
Diet 6	6	0	7·81		17·0
Diet 7	6	2	10·20	<0·05	17·0
Diet 8	6	0	9·28		12·9†
0·01 µg DES‡/g	7	0	11·08	<0·05	17·1
0·03 µg DES/g	6	3	24·73	<0·01	17·0
0·06 µg DES/g	6	6	59·90	<0·01	17·3

* Acetone Soxhlet extract similar to Diet 8 preparation.
† Significance, $P = 0.01$.
‡ DES = diethylstilboestrol.

together with that of the oestrogenic compounds in the forage. With this in mind only raw data from bioassays are presented in Table 5. Records of premature vaginal opening can be regarded as additional evidence of oestrogenic response when taken in conjunction with other results (East, 1956), and are included. Only Diet 7 produced significant oestrogenic response. Feed consumption and weight gain were significantly reduced by chloroform extract (Diet 8). In contrast, the acetone extract of the same alfalfa sample exhibited none of these effects.

DISCUSSION

From the above results it is clear that whole legume may influence reproduction in several ways, but at this stage of experimentation only trends and not detailed explanations can be presented. A report by McClure (1958) on temporary nutritional stress and infertility in mice may be pertinent. Pituitary gonadotrophins have been shown to be very sensitive to the effects of malnutrition (Davies, 1960). Suppression of oestrous cycles by *Ladino* was similar to the effects of a 15 % *Lithospermum* diet (Drasher & Zahl, 1946). In this latter study, strain differences were found, namely, C3H females were thrown into persistent anoestrus whilst the Rockland strain of animals developed varying degrees of refractoriness to treatment. It would seem, therefore, that the 'infertility' produced by feeding unfractionated legume in the diet is not a true manifestation of hormone-like substances present in the plant, but possibly a nutritional stress mainly influencing pituitary function. Results substantiating this concept are weight losses upon initial treatment, suppression of oestrous cycles, production of abnormal ova, and a reduction in mating behaviour.

Whether or not oestrogenic activity in *Ladino* and alfalfa is responsible for impaired reproductive performance is still questionable. The preliminary work presented in this paper indicates that the minimal oestrogenic activity usually found in these legumes (Diet 7) does not significantly influence reproduction in female mice. Pseudopregnancy occurred with low but consistent frequency in animals consuming extracts prepared with organic solvents. No pseudopregnancies were noted in animals receiving basal ration or diets containing aqueous extracts. It is felt that this observation is worthy of further experiment and evaluation. It would seem likely that other materials of unknown identity, but soluble in several organic solvents, are responsible for these results. Work along this line is in progress.

Ershoff, Hernandez & Matthews (1956) suggested the presence of an oestrogen inhibitor in dried alfalfa. The results of feeding prebloom *Ladino* to weanling female mice also indicate the existence of an oestrogen inhibitor in this species of plant, but other samples of *Ladino* acted as potentiators of endogenous oestrogenic activity, even though they did not show oestrogenic activity in bioassay. Bickoff *et al.* (1960) have noted oestrogen inhibitors and potentiators in crude forage extracts. It is possible that the repeated occurrence of pseudopregnancies in the experiments with extracts could have resulted from the action of such substances and not from any oestrogenic substances *per se*.

Ostrovsky & Kitts (1962) point out that no single oestrogenic compound can be regarded as accurately representing the oestrogenicity of a forage with respect to potency or to spectrum of activity. The results in Table 5 show alfalfa extracts to be much more effective in hastening vaginal patency than in stimulating uterine weight increases. It is still possible that hormone-like substances exist which are not effectively measured by uterine weight assay but are active in influencing reproductive phenomena.

While seeming to answer some controversial points, our studies have at the same time left many unanswered questions. Problems relating to (1) interrelationships between nutritional factors and endogenous and exogenous oestrogens, (2) digestibility and relative nutritional value of diets and extracts,

and (3) effects of pituitary cytology, hormone content, and function are receiving current attention. Srebnik & Nelson (1962) have demonstrated clear effects on pituitary function by restriction of dietary protein. Could forage diets be guilty of a similar lack or imbalance? We also know nothing of the vitamin content of diets and extracts; could some of the reproductive anomalies which have been observed be due to vitamin deficiencies and imbalance, as suggested by Lutmak-Mann (1958)? We have also to bear in mind the possibility of wide variation among different samples of forage crop resulting from varying climatic conditions, soil factors, illumination, or even rainfall.

ACKNOWLEDGMENTS

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