Table V. Relative Effectiveness of Glasses

Glass			Ratio of Borax to Glass Application at Equal Response in Boron Content of Crop						Maximum Change in Relative Effectiveness, Ratio of 5th/1st	
Reactivity	Number	Borax equivalent, lb./acre	1st	2nd	3rd	4th	5th	6th	Single treatment	Material mean
High	176-B	10	1.49	1.30	1.65	1.23	1.84	1.23	1.2	1.3
		20	1.28	1.34	1.21	1.24	1.66	0.70	1.3	
		40	0.96	1.10	1.26	1.33	1.33	0.88	1.4	
High	3134	10	1.11	0.89	1.17	1.06	1.65	1.46	1.5	1.3
		20	1.22	1.12	1.22	1.32	1.19	0.84	1.0	
		40	1.00	0.85	1.02	1.23	1.52	1.12	1.5	
Moderate	176-E	10	0.72	0.80	1.09	1.31	2.20	0.94	3.1	3.1
		20	0.73	0.88	1.07	1.18	2.20	1.01	3.0	
		40	0.58	0.69	1.00	1.04	1.79	1.08	3.1	
Moderate	176-F	10	0.44	0.67	0.81	0.46	1.85	1.05	4.2	3.9
		20	0.48	0.57	0.63	0.60	1.57	0.89	3.3	
		40	0.34	0.49	0.63	0.87	1.47	0.88	4.3	
Low	176-C	10	0.19	0.19	0.21	0.27	0.36	0.47	1.9	3.0
		20	0.19	0.23	0.39	0.24	0.72	0.69	3.8	
		40	0.19	0.24	0.38	0.33	0.63	0.48	3.3	
Very low	215-A	10	0.05	0.02	0.02	0.07	0.27	0.04	5.4	4.4
		20	0.04	0.01	0.05	0.08	0.16	0.07	4.0	
		40	0.03	0.02	0.03	0.04	0.11	0.06	3.7	

important with respect to application than a small difference at low levels of response. In order to circumvent this inherent difficulty, the performance of glasses must be considered in terms of relative effectiveness which may be expressed numerically as the ratio of borax application to glass application at equal response in boron content of the crop (Table V).

Maximum change in relative effectiveness with respect to time, shown in the right hand columns as a ratio of effectiveness in the fifth harvest relative to that of the first, divides the glasses into two main groups. The ratio for highly reactive glasses was 1.3 ± 0.2, while for other glasses it was 3.6 ± 0.6. As the value is greater than one in each case, a similarity in behavior is indicated. Thus, release from the highly reactive glasses may not be regarded as immediately complete, though it was too rapid to offer any real improvement over readily soluble boron. The constancy of the ratio for the other glasses shows that their ability to damp cyclic seasonal fluctuation is not altered substantially as the scale of reactivity is descended to very low values.

Relative effectiveness of boron contained in the moderately reactive glasses was reduced to about one half at the first harvest, but increased to become as much as double that of a readily soluble form at the fifth harvest. At the lower reactivities of glasses 176-C and 215-A, the magnitude of effectiveness was about one fifth and one twentieth that of the reference material, respectively. Accordingly, their effect relative to season was somewhat less important.

Effect on Range of Application. The use of moderately reactive boron glasses in lieu of borax for fertilization of soils would extend the limits of application by virtue of the reduction of maximum values and the increase of minimum values for boron content of the crop. By measure of relative effectiveness of these glasses at uniform particle size of 48- to 100-mesh, the indicated effect on range of application was to approximately double the upper limit and to reduce the lower limit to about one half.

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PLANT ESTROGENS

Isolation of a New Estrogen from Ladino Clover

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ESTROGENIC COMPOUNDS, including genistein, biochanin A, and formononetin, have been isolated from subterranean clover (*Trifolium subterraneum*) (*4*) and red clover (*Trifolium pratense*) (*12*). In certain strains of subterranean clover, the estrogenic content, mainly genistein, has been sufficient to affect reproductive performance of grazing ewes adversely and to cause genital and mammary stimulation in wethers (*7*).

In a recent review of plant estrogens,

Procedures are described for the isolation of an estrogenic compound from ladino clover. These involve ether extraction, alkali purification, countercurrent solvent distribution, molecular distillation, and recrystallization. The pure isolated material crystallized from methanol as clumps of small needles. Elemental analysis indicated an empirical formula of $C_{15}H_8O_5$. Formation of the diacetate and dimethylation established two free hydroxyl groups. Ultraviolet absorption spectra and paper chromatography showed the compound to be distinct from any previously described estrogenic isoflavones. By mouse-uterine–weight assay, the compound was at least 30 times more estrogenic than genistein.

ladino clover (*Trifolium repens*) is listed among the nonestrogenic plants (*5*). This conclusion has been reported also by a number of investigators (*6, 9, 10, 14*). However, within the last 2 years, it has been definitely established that ladino clover displays significant estrogenic activity, although the nature of the estrogen was not determined (*8, 11*). Engle, Bell, and Davis at Ohio State University recently reported (*8*) that ewes, grazed on ladino clover, conceived 3 weeks later than comparable animals grazing all season on blue grass. In addition, their fertility was considerably lessened. Mouse-uterine–weight assays demonstrated that estrogenic activity was present in clover but not in grass.

Because of the widespread use of ladino clover as a forage and feedstuff for animals in this country, more information was desirable on the occurrence and nature of the estrogen found in this forage. In a preliminary note, evidence for the existence of this estrogen was presented, and the name Coumestrol was proposed for the substance (*2*).

This communication presents details of the isolation and partial characterization of the compound.

Isolation

The estrogen was isolated from a commercial dehydrated, pelleted ladino-clover meal. One half ton of pellets was purchased and reground to a finely divided powder.

As no information regarding the chemical nature of the estrogen was available, a bioassay technique was employed to guide the various steps. This method was a modification of the mouse-uterine–weight technique as employed by Preston *et al.* (*13*). The basal diet of the mice consisted of corn meal 75%, casein 10%, linseed meal 10%, cod liver oil 3%, bone meal 1.5%, and sodium chloride 0.5%. To evaluate the various fractions, the test substance was incorporated into the standard diet and fed to immature female mice. The mice employed were of the Dal-Swiss strain, 18 to 20 days of age and selected to weigh from 8 to 10 grams. After 6 to 8 days, during which time each animal was allowed to eat 10 grams of test material, the animals were sacrificed, and the weights of the freshly excised uteri were determined. A positive estrogenic response was manifested by an increased uterine weight response over the controls.

In the course of the isolation, many purification techniques were attempted. Some did not effect a concentration of estrogen. Others, although effecting a concentration, resulted in relatively large losses of total activity. Saponification, for example, resulted in loss of most of the activity. Distribution of the activity between benzene and 60% alcohol, successful in the purification of genistein (*7*), was relatively ineffective as in these solvents the ladino estrogen had a more equal distribution. Still other techniques were effective in concentrating the activity but were either too laborious or applicable only on a small scale. Included in the latter category were paper chromatography and molecular distillation. However, by evaluation of the relative efficiency of the various purification techniques, the following extraction procedure was finally developed. Application of countercurrent distribution was of utmost importance in finally obtaining a pure crystalline product. Three countercurrent distribution instruments, a 100-tube 20-ml.-per-tube manually operated model, a 200-tube 20-ml.-per-tube, and a 100-tube 200-ml.-per-tube robot-operated model were available for this study.

Extraction Procedure

Step 1. The reground meal (200 kg.) with enough hot water (90° C.) to make a total volume of 400 gallons was mechanically stirred overnight. The next day the mixture was filtered and the filtrate was discarded. A second extraction was then carried out in the same manner. The filtered meal was dried in a forced-draft oven (70° C.), yielding 120 kg. of dried solid material.

Step 2. The dried product from Step 1 was extracted with Skellysolve C in a large steel, funnel-shaped extractor. The dried meal was covered with about 50 gallons of solvent and allowed to steep overnight. A tightly fitted wooden cover on the extractor prevented excessive evaporation of the solvent. After about 16 hours, the extract was withdrawn, concentrated to small volume in a steam-jacketed still and the recovered solvent was returned to the extractor. The meal was repeatedly extracted in this manner until the percolate was almost colorless. Total solids, 11 kg., were recovered from the Skellysolve extract. These showed no estrogenic activity and were discarded.

Step 3. The residue from Step 2 was repeatedly extracted with ether in the same manner until the extract was almost colorless. About 1000 gallons of ether was employed, and extraction continued for about 30 days. Even after this time, some estrogenic activity still remained in the meal. Although alcohol is a more efficient extractant for estrogen, ether is preferred because alcohol removes many more of the impurities which the ether extraction leaves behind. These alcohol extractives make subsequent purification steps considerably more difficult.

The ether extract was evaporated to a solid residue weighing 5 kg. The ether-extracted meal was discarded.

Step 4. The solids from the ether extract obtained in Step 3 were dissolved in warm chloroform employing 1 liter of the latter per 300 grams of concentrate. This chloroform solution was extracted with 5% aqueous sodium carbonate adjusted to pH 10 to 11 with sodium hydroxide. Occasionally, stable emulsions formed which could be broken only by centrifugation.

Step 5. The resulting aqueous alkaline extract was quickly separated, acidified to pH 6.0 to 6.5 with hydrochloric acid, and then extracted five times with 1-liter portions of ether. The ether extracts were combined and evaporated, leaving 150 grams of product.

Step 6. The product from Step 5 was next subjected to countercurrent distribution. Six separate solvent systems were required to obtain the material in sufficiently pure form to permit further purification by recrystallization. Quantities as large as 100 grams of total solids could be distributed in the larger instrument (200-ml. capacity per tube) by adding the starting material to the first 10 tubes. The smaller instrument was employed when the quantity of starting material was less than 10 grams.

The solvent systems and the order in which they were employed are presented in Table I. Also presented are the number of transfers and the position of the estrogen at the end of the distribution. For the 280-tube transfer, the 200-tube instrument with fraction collector was employed so that the 280 transfers

required no recycling. At the end of 280 transfers, the estrogen was found in tubes 30 to 60.

The contents of selected tubes taken at intervals were removed and evaporated to dryness, and a weighed aliquot was added to the standard diet and fed to mice with the results shown in Figure 1. Subsequently, under ultraviolet light, an intense blue-white fluorescence was found and associated with the estrogenic fractions. The estrogenic factor had an R_f of 0.50 on a paper chromatogram when developed with 50% acetic acid and was readily observable under ultraviolet light. This property greatly facilitated the work of collecting and combining the fractions.

Step 7. The contents of tubes 30 to 60 of the sixth countercurrent distribution (Table I, solvent system F) were combined and evaporated to dryness under vacuum. The resulting solids consisted predominantly of the estrogen, but still contained a small amount of impurity. After eight recrystallizations from a methanol-chloroform solution, almost all of the occluded impurities were removed and the estrogenic material had a light-tan color. Coumestrol crystallized with difficulty, but was obtained as a mass of birefringent granules when allowed to crystallize from a solution of chloroform and methanol, brought to a boil, and allowed to cool slowly.

Step 8. For final purification the estrogen was sublimed at 175° C. under a pressure of 7 microns, whereby the estrogen was obtained as a light-yellow crystalline material, in the shape of minute needles. The crystalline estrogen melted at 385° C. with slight decomposition. During the course of the isolation process, various fractions were subjected to estrogenic assays to determine the activity of the material as it was rendered increasingly free from impurities. A summary of the results of these assays is presented in Table II.

Evaluation of Estrogenic Activity

Table III shows the relative estrogenic activity of ladino-clover estrogen when compared with the estrogenic isoflavone, genistein. Ladino estrogen is at least 30 times as effective as genistein when measured by the uterine-weight assay.

Partial Characterization of Coumestrol

Elemental analysis resulted in an empirical formula, $C_{15}H_8O_5$. Analysis of coumestrol: Calculated for $C_{15}H_8O_5$: C, 67.1 and H, 3.09. Found: C, 67.1; H, 2.98; OMe, none; and acetyl, none.

Diacetate Derivative. To 50 mg. of estrogen, 1.5 ml. of acetic anhydride and 200 mg. of sodium acetate were added. The solution was brought to boiling over a microburner and immediately poured into a small beaker of ice water. After filtering, the acetate crystallized from methanol and acetone in small white needles (50 mg.)—melting point, 237° C. (All melting-point determinations were made on a Kofler block.)

Analysis of diacetate: Calculated for $C_{15}H_6O_5(C_2H_3O)_2$: C, 64.7; H, 3.56; and acetyl, 24.1%. Found: C, 64.7; H, 3.41; and acetyl, 24.1%.

Methyl Ether Derivatives. To an acetone solution of 100 mg. of estrogen was added 4.5 grams of sodium carbonate. While the solution gently refluxed during 4.5 hours, 4 ml. of dimethyl sulfate was added dropwise. The mixture was filtered and acidified, and the acetone was evaporated. Water was added to the residue which was extracted with ether. Concentration of ether and addition of Skellysolve B precipitated a solid which crystallized from methanol. However, a solution of the crystals when spotted on silicic acid chromatostrips and viewed under ultraviolet light showed the presence of two fluorescent compounds, which could not be separated by further crystallization. As a result, 78 mg. of the mixture was subjected to a 278-tube Craig countercurrent distribution in a solvent system consisting of Skellysolve B, ether, acetone, and water (1:1:2:1). A 200-tube instrument was used and the last 78 fractions were collected separately in a fraction collector. The tubes appearing in the

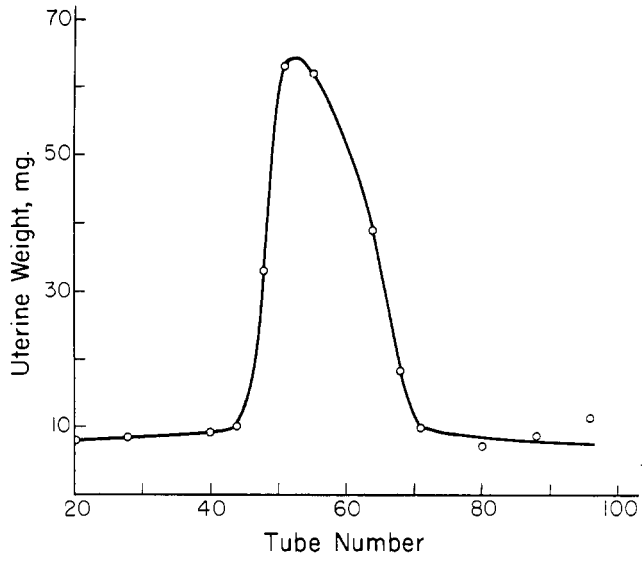

Figure 1. Countercurrent distribution of estrogen concentrate in solvent B

Table I. Solvent Systems Employed in Countercurrent-Distribution and the Position of the Estrogen

Solvent System	Solvents and Proportions by Volume	No. of Transfers	Position of Estrogen in Tubes
A	Acetone:ether:water:Skellysolve B (10 : 5 : 5 : 2)	100	69–90
B	Chloroform:carbon tetrachloride:methanol:water (2 : 2 : 3 : 2)	100	56–80
C	Methanol:benzene:ether:water (4 : 4 : 1 : 1)	100	30–60
D	Skellysolve B:ethyl acetate:methanol:carbon tetrachloride:water (1 : 1 : 1 : 1 : 1)	100	25–58
E	Acetone:carbon tetrachloride:water (2 : 1 : 1)	100	40–76
F	Acetone:carbon tetrachloride:water:methanol (10 : 5 : 5 : 1)	280	30–60

Table II. Biological Potency of Fractions Obtained during Fractionation Scheme

Step	Material Tested	Amount Fed per Mouse, Mg.	Uterine Weight, Mg.
	Control (basal ration)	...	10
	Ladino clover meal	3000	15
1	Residue after water extn.	3000	27
2	Residue after Skellysolve extn.	3000	65
3	Concentrate from ether extn.	200	36
5	After extn. with alkali, and transfer back to ether	10	30
7	Recrystallized estrogen	0.67	95
	Diethylstilbestrol	0.0004	72

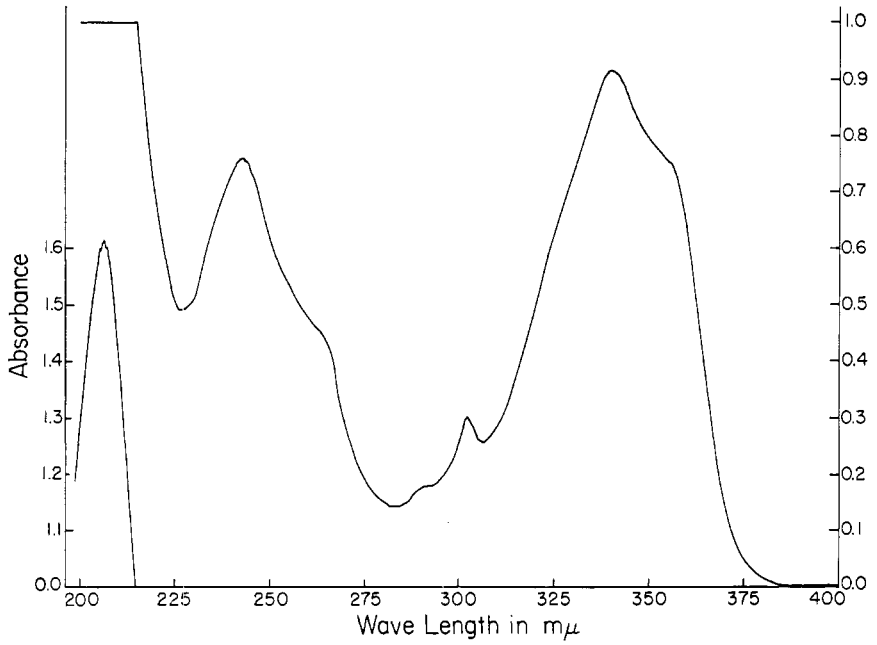

Figure 2. Ultraviolet absorption spectrum of crystalline coumestrol in methanol

Table III. Comparison of Estrogenic Activity of Ladino Clover Estrogen with Genistein

Substance Fed	Amount per Mouse, Mg.	No. of Mice	Mouse Uterine Weight after 7 Days, Mg.
Normal control	0	5	10
Ladino clover estrogen	0.50	5	34
	0.75	5	61
Genistein	10	2	18
	15	5	44

fraction collector as 11 to 24 contained a single compound that was a monomethyl ether of the estrogen—melting point, 278° C.

Analysis of monomethylether: Calculated for $C_{15}H_7O_4(OCH_3)$: C, 68.1; H, 3.54; and OMe, 11.1%. Found: C, 68.1; H, 4.00; and OMe, 11.0%. The tubes numbered 36 to 79 contained the dimethyl ether melting point, 198° C.

Analysis of dimethyl ether: Calculated for $C_{15}H_6O_3(OCH_3)_2$: C, 68.9; H, 4.05; and OMe, 20.9%. Found: C, 68.9; H, 4.52; and OMe, 19.3%. These results indicate two free hydroxyl groups in the compound.

Absorption Spectrum. The ultraviolet absorption spectrum of coumestrol in methanol is presented in Figure 2. As coumestrol has strong ultraviolet absorption peaks at 208, 243, and 343 mμ, the possibility that the compound is an isoflavone similar to genistein, biochanin A, or formononetin was considered unlikely, because isoflavones generally show no prominent absorption peaks above 300 mμ. These latter compounds are the only known estrogens previously isolated from forage plants.

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PHOSPHORUS AVAILABILITY

Effect of Particle Size on Availability to Plants of Phosphorus in Phosphate Rock from Various Sources

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FOR THE YEAR ENDING June 30, 1955, 605,000 tons of ground phosphate rock were marketed in the United States and territories to use as a fertilizer medium for direct application (*5*). The main ore-producing areas are located in the southeastern and western United States. In a recent experiment, Armiger and Fried (*7*) compared the relative agro-