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Studies on the Chemical and Biological Properties of Coumestrol and Related Compounds

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Contents

Studies on the Chemical and Biological Properties of Coumestrol and Related Compounds

By E. M. Bickoff, R. R. Spencer, S. C. Witt, and B. E. Knuckles, *chemists,*
Western Utilization Research and Development Division,
Agricultural Research Service

Introduction

Natural estrogens in plants have assumed considerable importance since their connection with infertility in grazing sheep was demonstrated in 1946 by Bennetts and co-workers (*19*)[1] and in 1948 by Curnow and co-workers (*33*). These reports were followed in 1951 by the isolation from subterranean clover of the isoflavone, genistein (*56*), which was shown to be estrogenically active in mice (*49, 82*). Subsequently, the estrogenically active isoflavones, formononetin, biochanin A, daidzein, and pratensein, were found in both subterranean and red clovers (*39, 68, 100, 221*). At first genistein was believed to be the factor that interfered with the breeding of sheep (*83*). However, more recent work suggests that formononetin may be more important (*84, 182, 184*).

Until 1953, alfalfa and Ladino clover were considered to be among the nonestrogenic forages (*54*). However, experiments in the Western Utilization Research and Development Division (WURDD) as well as reports from Cheng and co-workers at Iowa State (*66, 67*), Engle and co-workers at Ohio State (*98*), and Pieterse and Andrews at Purdue (*197*) definitely established that both alfalfa and Ladino clover frequently display significant estrogenic activity. In 1955, work was begun at WURDD on the occurrence, biological properties, and isolation of the Ladino clover estrogen(s). Surprisingly, the major compound, which was named coumestrol (*30*), was not an isoflavone as were all previously reported forage estrogens. Instead, it turned out to be a benzofurocoumarin derivative belonging to an entirely new class of compounds, which were later named coumestans (*85*). These compounds have subsequently been shown to be biogenetically related to the isoflavones (*118*). Coumestrol has a close structural relationship not only to the isoflavones, but also to the natural estrogen, estradiol,

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[1] Italic numbers in parentheses refer to Literature Cited, p. 83.

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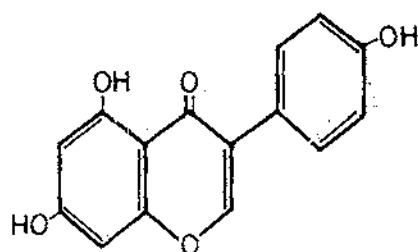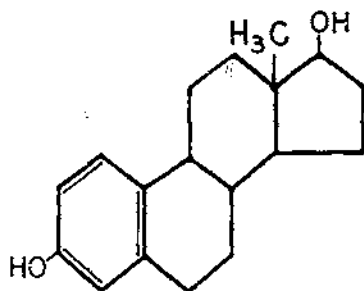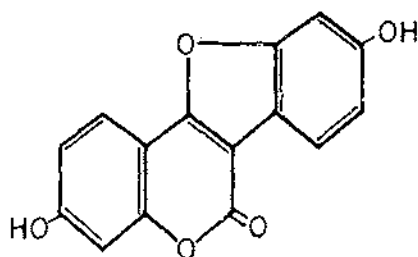
FIGURE 1.—Structural formulas of genistein, estradiol, coumestrol, and
diethylstilbestrol (22).

and the synthetic estrogen, diethylstilbestrol (fig. 1). In fact, Whalley (48) proposed that the estrogenic activity of coumestrol can be attributed to its stilbene-like structure, which is analogous to that of diethylstilbestrol.

Studies established that coumestrol was the dominant estrogen of alfalfa (39, 122), Ladino clover (39, 122), and the annual medics (18, 104, 183). Although the isoflavones account for most of the estrogenic activity of red and subterranean clovers, their contribution to the estrogenicity of alfalfa and Ladino clover is small (122). Subsequently, coumestrol was found in a wide variety of clovers, e.g., red (167, 222), white (105, 167), bur (167), subterranean (18, 167, 183, 184), and strawberry (18, 105, 167), as well as meadow flora (179), Chinese milk vetch (217), soybeans (217), soybean sprouts (217), pea silage,[2] and commercial samples of frozen peas and beans (2). Studies on the variation in coumestrol content of alfalfa and Ladino clover, as related to environmental factors (36, 37, 127), indicated that coumestrol content was greatly affected by disease (43, 164) and insect infestation (163).

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[2] Personal communication from J. H. Adler, animal physiologist, Hadassah Medical School, Israel, 1967.

The discovery of coumestrol stimulated a number of studies related to its physiological effects on animals. Coumestrol has been shown to be considerably more potent estrogenically for both mice (39) and sheep (57) than has genistein. Reproductive problems attributed to alfalfa and Ladino clover, such as those reported by Engle and co-workers (98), Wright (224), and Adler and Trainin (6, 7, 8), can be explained by the presence of coumestrol. Estrogenically active alfalfa was shown to beneficially affect the rate of growth of cattle (176) and sheep (103, 194). Although later results suggested that coumestrol was not the causative agent, coumestrol did improve the quality of the meat obtained from sheep (131).

In addition to coumestrol, seven other coumestans have been isolated from alfalfa (Medicago sativa) (41) and Ladino clover (Trifolium repens) (38). They are trifoliol (41, 160), 4'-O-methylcoumestrol (42), medicagol (161), lucernol (205), sativol (205), 3'-methoxycoumestrol (45), and 11,12-dimethoxy-7-hydroxycoumestan (206). This type of compound has also been isolated from other plants. The first compound of this basic structure was wedelolactone, which Govindachari and co-workers (111, 112) isolated from Wedelia calendulacea in 1956. Other naturally occurring coumestans are erosnin isolated from Pachyrrhizus erosus (88), psoralidin from Psoralea corylifolia (65, 86, 141), and norwedelolactone from Eclipta alba (148). The structural formulas of these naturally occurring coumestans are given in figure 2. Coumestans are also structurally similar to the naturally occurring isoflavonoids inermin (77), trifolirhizin (59), pisatin (81, 196), pterocarpin (60, 64, 198), and homopterocarpin (64, 171) (fig. 3). This similarity in structure was further demonstrated by Bowyer and co-workers (53), who converted homopterocarpin into coumestrol dimethylether.

This technical bulletin summarizes and correlates 13 years of research on coumestrol and related compounds in this laboratory as well as at other research institutions throughout the world. It also includes some of the studies on other forage estrogens as they relate to coumestrol.

Analytical Procedures

An early method of determining estrogenic activity in plant material was the Allen-Doisy test (96), which is based on a determination of the onset of estrus in the test animal by the appearance of cornified epithelial cells in vaginal smears. In most of the work on the isolation of estrogenic constituents from forages, estrogenic activity has been measured by the increase in uterine weight of ovariectomized or immature female mice or rats (49, 56, 83, 197). Once the estrogenic compound is identified, chromatographic methods (156) can often be used for its quantitative determination.
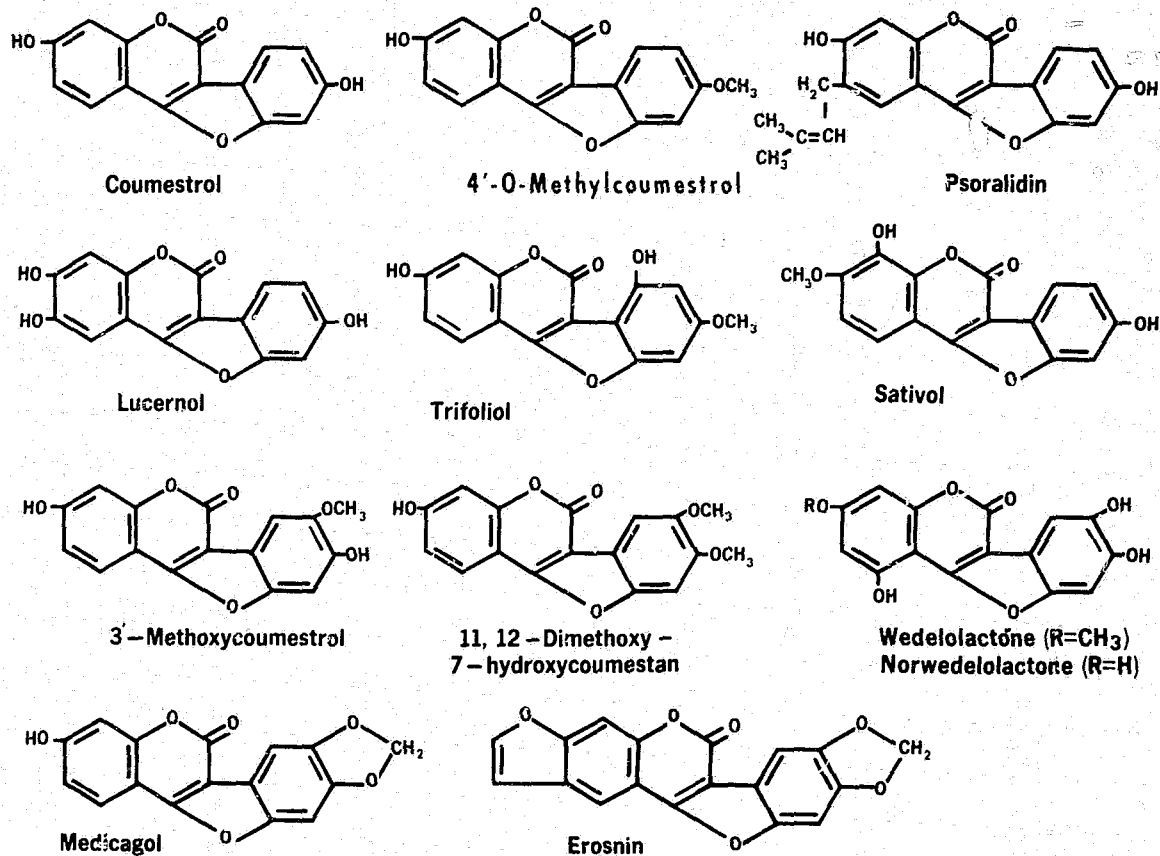
FIGURE 2.—Naturally occurring coumestans (45).
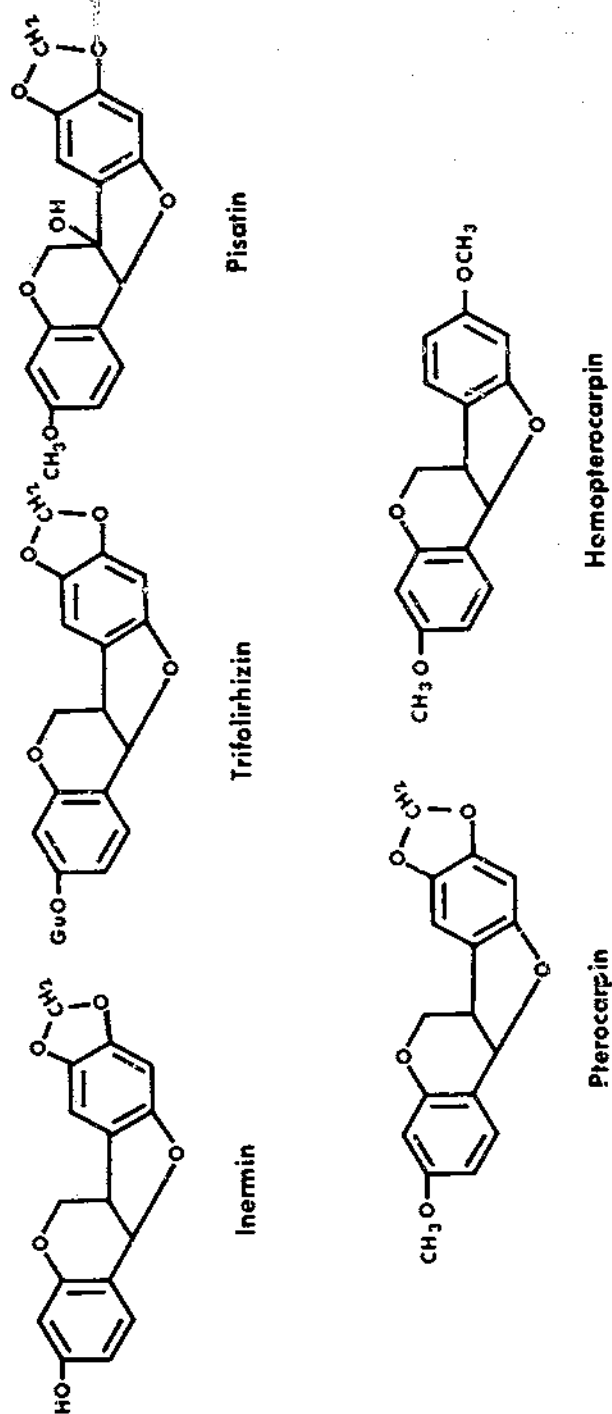
FIGURE 3.—Naturally occurring isoflavonoids (Gu = glucose).

Biological procedures

During the isolation of the plant estrogen, coumestrol, from Ladino clover, estrogenic activity was measured by a mouse-uterine-weight bioassay (29). This bioassay was also employed for our initial studies on the occurrence of coumestrol in plants. Booth and co-workers (51) observed that a number of commonly used vegetable oils were themselves capable of producing an estrogen-like response in mice. Therefore, test materials were administered orally to eliminate any complications that might have arisen from subcutaneous injections in oil. This procedure more closely duplicated the normal route of ingestion by livestock and, also, eliminated the need for a laborious purification procedure (199). Sufficient sample or extract was incorporated into the test diet to cause at least a doubling of the uterine weight during the test period.

Since a graded uterine response could not be obtained from feeding the samples of whole alfalfa or clover, it was necessary to prepare extracts for assay. Fresh forages were mascerated in acetone in a mechanical blender. Acetone was the preferred solvent because it extracted more estrogenic activity and less total solids than the other solvents tested (table 1.). After concentration of the aqueous-acetone extract, the estrogenic substances were reextracted into ether. This eliminated the presence of water-soluble factors that sometimes reduce uterine response. Dried forages having moderate estrogenic activity, i.e., requiring less than 10 grams of plant material for assay, were Soxhlet-extracted with acetone for 24 hours. Dried forages having low estrogenic activity, i.e., requiring more than 10 g. of plant material for assay, received a preliminary purification by extracting with chloroform. This removed a large amount of nonestrogenic material. The residual meal was then

TABLE 1.—*Relative effectiveness of organic solvents for extracting estrogenic activity from Ladino clover meal*

Solvent	Average uterine weight at different Soxhlet extraction times		
	12 hours	16 hours	24 hours
	Milligrams	*Milligrams*	*Milligrams*
Control	9	9	9
Acetone	51	62	78
Alcohol	38	63	49
Benzene-alcohol (2:1)[1]	38	64	59
Chloroform	9	9	9
Ether	--	--	17
Petroleum ether	9	9	9

[1] Proportion by volume.

Source: Bickoff, E. M., Booth, A. N., Livingston, A. L., and others (29).

TABLE 2.—*Uterine response to graded levels of estrogen*

Diet	Quantity fed per mouse	Uterine weight ± S.E.[1]
	Micrograms	*Milligrams*
Control	....	9.73 ± 0.33
Coumestrol	100	13.74 ± .73
	200	18.74 ± .97
	300	25.68 ± 1.05
	400	34.39 ± 1.23
Diethylstilbestrol	.025	11.64 ± .41
	.050	17.61 ± .83
	.075	22.80 ± .95
	.100	33.83 ± .99

[1] S.E. = standard error mean.

Source: Bickoff, E. M., Booth, A. N., Livingston, A. L., and others (*29*).

treated in the manner described for the moderately active samples. The various fractions prepared during the isolation of coumestrol as well as the pure compounds were dissolved in acetone or alcohol and incorporated into the mouse diet.

Estrogenic activity was evaluated by feeding the test diets (10 g./mouse) to immature female mice. The mice, caged in groups of five, were fed the test diet *ad libitum* until they had consumed about 50 g. of ration. Each assay included a negative control (basic ration only) and a positive control (basic ration plus diethylstilbestrol, usually 0.1 mg./10 g.-diet). The mice were sacrificed just prior to exhaustion of the food supply. The freshly excised uteri were carefully trimmed and weighed without blotting or slitting. Since there was a lack of correlation between body weight and uterine weight, the assay results were expressed as the average uterine weight per mouse rather than per gram of body weight. Results could also be expressed in terms of diethylstilbestrol or coumestrol equivalency by constructing dosage-response curves from data such as those given in table 2.

Chromatographic procedures

Since alfalfa contained at least five substances which give a response in the mouse bioassay (*122*), a more selective test was required for the determination of coumestrol. Chromatographic techniques were investigated as they are simple, rapid, and permit the screening of a large number of samples. In addition, the characteristic blue fluorescence of coumestrol under ultraviolet (UV) light (*44*) lent itself to chromatographic techniques. Initially, a qualitative procedure (*167*) for detecting small amounts of coumestrol in plant material was developed, which was refined to a rapid, quantitative paper chromatographic procedure (*158, 159*).

TABLE 3.—*Sequence of chromatographic solvent systems for the identification of coumestrol*

Type of chromatography	Solvents and proportions by volume	Average R_t values for coumestrol [1]
Filter paper _____	Chloroform-acetic acid-water (2:1:1)	0.51
	Acetic acid-water (1:1) _____	.57
Silicic acid _____	Chloroform-ether (1:1) _____	.56
	Ethyl acetate-Skellysolve B (3:1) ___	.74
	Acetone-Skellysolve B (1:3) _____	.24
	Ethyl ether-Skellysolve B (7:3) ____	.36

[1] As measured from the leading edge of the fluorescent spot produced by a reference coumestrol sample.

Source: Lyman, R. L., Bickoff, E. M., Booth, A. N., and Livingston, A. L. (*167*).

Qualitative procedure

In our procedure, an acetone extract of dry plant material was chromatographed sequentially in two solvent systems on heavy filter paper, then in four systems on silicic acid chromatostrips (*167*). The solvent systems in order of use are summarized in table 3. An empirical visual estimate of the amount of coumestrol was made by observing the relative intensity of fluorescence eluted from the fourth chromatostrip.

Recently, Churý (*69*) discussed extraction procedures for both fresh and dry plant materials and gave R_t values for coumestrol in 10 solvent systems (table 4). He reported that systems 1, 2, 8, and 9 gave the best results with plant extracts. Where impurities tended to mask the coumestrol spot, the spot was eluted and rechromatographed in a different solvent system to verify the presence of coumestrol. Although chromatographic procedures are not exact when compared to the bioassay, they are more specific and give a reasonably good estimate of the relative amount of coumestrol.

TABLE 4.—*Churý's solvent systems* [1]

Solvent system No.	Solvents and proportions by volume	R_t values for coumestrol
1 _____	Acetone-acetic acid-water (3:1:6) ____	0.088–0.10
2 _____	Acetone-water (3:7) _____	.10 – .16
3 _____	Chloroform-acetic acid-water (2:1:1) __	.23 – .40
4 _____	Benzene-acetic acid-water (2:2:1) ____	.19 – .23
5 _____	Butanol-acetic acid-water (4:1:5) ____	.91 – .93
6 _____	Methanol-benzene (10:40) _____	.77 – .80
7 _____	Acetic acid-water (30:20) _____	.55 – .60
8 _____	Acetic acid-water-HCl (50:35:15) ____	.55 – .60
9 _____	Benzene-acetone (9:1) _____	0
10 _____	Methanol-chloroform (11:89) _____	.91 – .95

[1] Ascending chromatography.

Source: Churý, Jiří (*69*).

Quantitative procedure

A sensitive quantitative paper chromatographic procedure, using a fluorometer, was developed. The design of the instrument (14) provided for the insertion of paper chromatograms between a UV radiation source-filter combination and a detector-filter combination. Intensity of fluorescence was proportional to galvanometer readings. The method for the quantitative measurement of coumestrol on paper chromatograms (159), using a fluorometer, involved spotting known quantities of pure coumestrol on sheets of Whatman No. 1 paper; developing the chromatograms in 50 percent acetic acid, ascending; and preparing a standard curve from the galvanometric readings. Measurements from at least four spots of the same concentration were averaged to obtain each point on the standard curve. Typical fluorometric measurements are shown in table 5. Using this technique, quantities of coumestrol in the range of 0.2 to 1.0 micrograms could be estimated with a standard deviation of about 7 percent.

TABLE 5.—*Typical fluorometric measurements for coumestrol*

Coumestrol concentration	Galvanometer reading [1]	
	Operator A	Operator B
Micrograms	*Millimeters*	*Millimeters*
0.2	62.2	62.0
.6	40.7	40.4
1.0	16.2	15.7

[1] Average of 10 separate spots. Full-scale deflection is 100 millimeters.

Source: Livingston, A. L., Bickoff, E. M., Guggolz, Jack, and Thompson, C. R. (159).

Extracts were prepared from the forage samples for assay (158). Fresh samples were rapidly mascerated with 95 percent alcohol in a mechanical blender. During cooperative studies with various laboratories throughout the United States on the factors that influence the coumestrol content of alfalfa, this procedure was modified. Fresh material was extracted with methanol by standing for at least 3 days (127, 213). Samples treated in this manner could be stored for at least 3 months with no appreciable loss of coumestrol. Dried samples were first rehydrated, then extracted with either 95 percent ethanol or methanol. Recently, Churý (69) reported that a higher yield could be obtained if an enzymatic hydrolysis was carried out prior to alcohol extraction. Extracts of samples low in coumestrol had to be further purified to remove the waxes and fatty materials that interfered with the paper chromatographic phase of the analysis. In the earlier work, these impurities were removed by extraction with petroleum ether. However, later we found that chloroform was a more satisfactory solvent for extracting the impurities. In this procedure, the alcohol had to be removed from the original extract. The aqueous phase was then made basic with sodium carbonate. After the green color was removed with chloroform, the aqueous phase was acidified. Coumestrol and the other phenolic compounds were then reextracted into ether, and a concentrate representing a tenfold increase over the original extract was prepared. Extracts were analyzed on paper chromatograms and coumestrol

concentration was reported on a dry weight basis, in parts per million. This method is sensitive to about 2 p.p.m. of coumestrol, with a maximum error of about ±5 percent.

Recently the paper chromatographic procedure was adapted for the quantitative measurement of coumestans and flavones in alfalfa samples using two-dimensional paper chromatography (43). Samples were spotted two to four times heavier than in the coumestrol analysis and developed in 50 percent acetic acid, ascending for the first dimension, followed, after drying, by isopropyl alcohol-concentrated ammonium hydroxide (2:1), descending for the second dimension. Fluorometric measurements were made on each compound using two templates to eliminate fluorescence from surrounding spots (fig. 4). The relative concentration of each compound was determined from a coumestrol calibration curve. After a correction had been made for the difference between the fluorescence intensity of coumestrol and that of an individual compound, the concentration of the compound was calculated in the same manner as for coumestrol.

Other chromatographic procedures

Wong (222) developed a two-dimensional paper chromatographic method for the separation and detection of coumestrol as well as the known estrogenic isoflavones in red clover. A purified alcohol extract of the fresh red clover was chromatographed on Whatman 3MM paper, using the descending technique. The single phase system, benzene-acetic acid-water (125:72:3), was used for the first dimension. This system had the advantage of moving contaminating green pigments and lipoid material near the solvent front and gave a very good separation of the polyphenols. Aqueous ammonia (2N) was found to be the most suitable solvent for the second dimension. After the above separation, the concentrations of the individual isoflavones were determined by spectrophotometric measurement. The level of coumestrol was too low to measure by UV absorption. Its concentration was estimated visually based on the intensity of its fluorescence. As little as 0.1 μg. of coumestrol was detected by this technique.

While studying plant estrogens in sheep, Lindner (155) developed a procedure for determining microgram quantities of coumestrol and the estrogenic isoflavones in plasma and depot fat of sheep. The assay procedure consisted essentially of (1) extraction and preliminary fractionation of estrogens by solvent partition; (2) paper chromatographic separation of the estrogens; (3) spectrophotometric determination of the isoflavones after elution with ethanol; and (4) fluorometric determination of 5-deoxy-isoflavones and coumestrol, both on paper strips and after elution with aqueous methanol. Figures 5 and 6 represent flow-sheets for the determination of these compounds in plasma and in body fat, respectively. Steps (3) and (4) could be satisfactorily replaced by vapor phase chromatography of their trimethylsilyl ether derivatives. Losses during the extraction and purification procedures were determined by the use of radioactive internal standards and averaged 35 percent.

Fluorometer
light
source

Solvent front

Template A

Template B

Sativol

1st Dimension

50% ACETIC ACID

7, 4'-Dihydroxyflavone

Medicagol and
4'-O-Methylcoumestrol

Coumestrol

Trifoliol

3'-Methoxycoumestrol

2nd Dimension   ISOPROPYL ALCOHOL-Conc. NH$_4$OH (2:1)
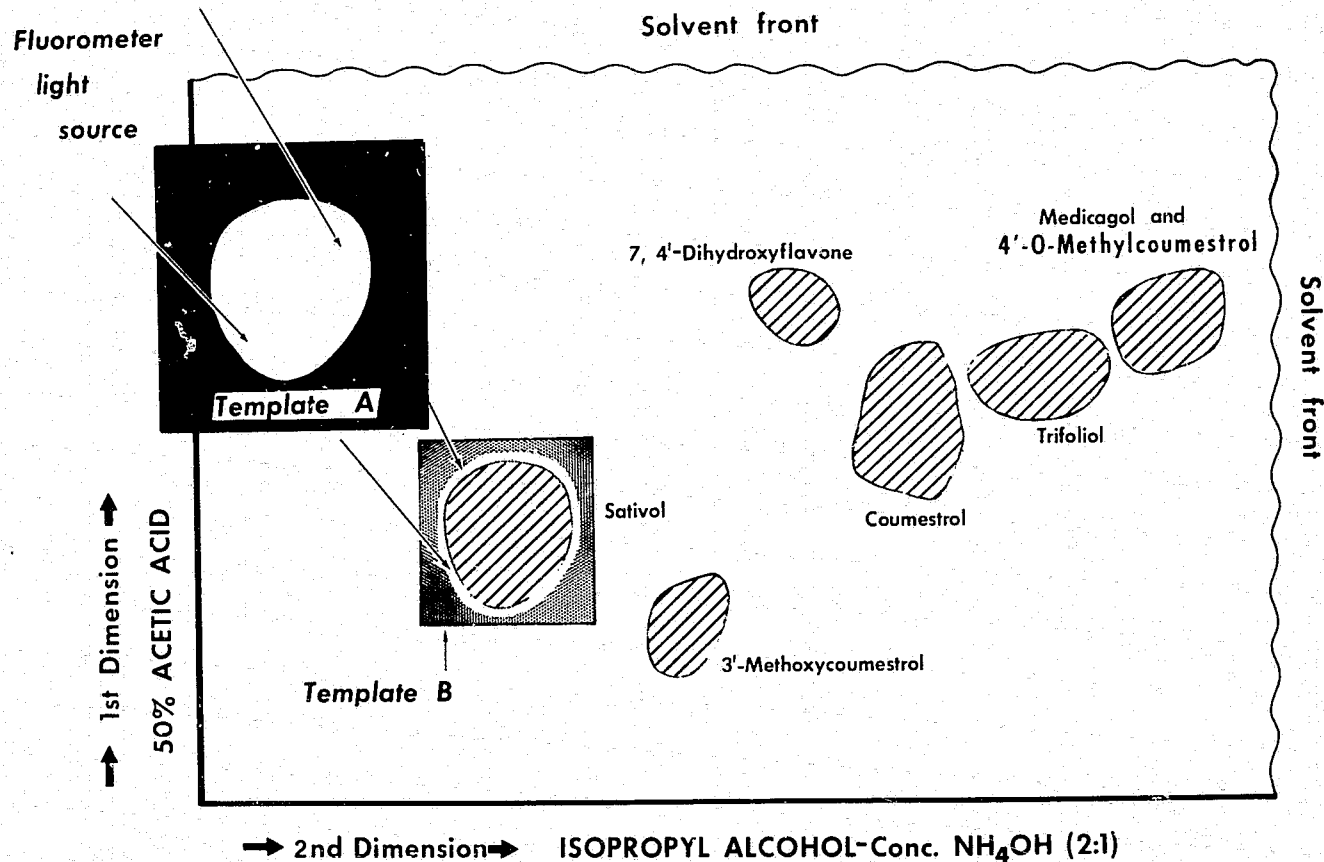
FIGURE 4.—Chromatographic technique used for coumestan analysis (43).

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                        ┌─────────────────────┐
                        │    BLOOD PLASMA      │
                        └─────────────────────┘
                                  │
                                  │  BROUGHT TO pH 9.9 WITH 1N NaOH.
                                  │  EXTRACTED WITH ETHYL ACETATE( 4 X 2 VOLUMES)
                  ┌───────────────┤
                  │               │
    INCUBATED WITH β-GLU-   ┌──────────────┐  ┌──────────────────────┐
    CURONIDASE AT pH 4.5    │  EXHAUSTED   │  │  CRUDE ETHYL ACETATE  │
    AND RE-EXTRACTED FOR    │   PLASMA     │  │      EXTRACT E        │
    ESTIMATION OF CONJUGATED└──────────────┘  └──────────────────────┘
    ISOFLAVONES                                      │
                                                     │  WASHED TWICE WITH 1/15 VOLUME CARBONATE BUFFER
                                                     │  (pH 9.9) AND TWICE WITH 1/20 VOLUME WATER. ADDED DROP
                                                     │  OF GLACIAL ACETIC ACID. SOLVENT REMOVED UNDER REDUCED PRESSURE.
                                              ┌──────────────┐
                                              │  DRY RESIDUE │
                                              └──────────────┘
                                                     │  DISSOLVED IN 15 ML. 70% AQUEOUS METHANOL.
                                                     │  STORED AT –12°C. FOR 12 HOURS AND CENTRIFUGED
                                                     │  IN A REFRIGERATED CENTRIFUGE.
                              ┌──────────────────────┤
                              │                      │
    DISCARDED          ┌──────────────┐      ┌──────────────────────┐
                       │  PRECIPITATE │      │    SUPERNATANT S      │
                       └──────────────┘      └──────────────────────┘
                                                     │  TAKEN TO DRYNESS IN VACUO. RESIDUE
                                                     │  DISSOLVED IN 1 ML. ETHANOL + 25 ML. BENZENE + 25
                                                     │  ML. LIGHT PETROLEUM ( b.p. 40°-60°C.)
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FIGURE 5.—Schematic flowsheet for the concurrent determination of isoflavones, coumestrol, and hormonal steroids in the blood plasma of sheep. System B-1 is toluene-light petroleum (boiling point 80°–100° C.)-methanol-water (5:5:7:3) and system B-5 is benzene-methanol-water (10:5:5) (155).
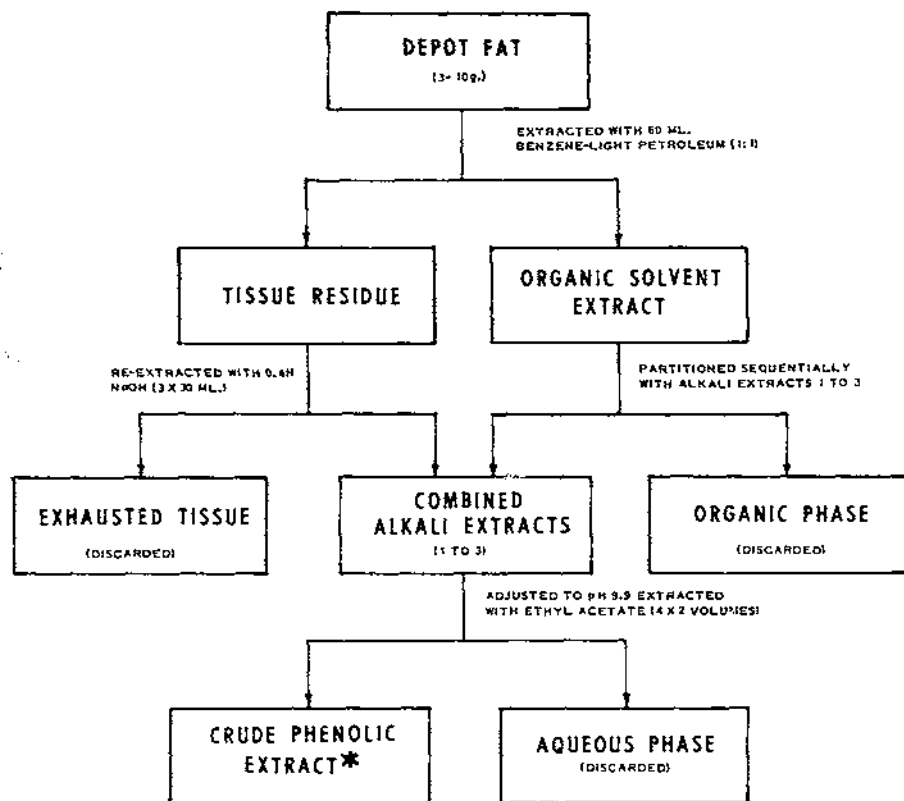
FIGURE 6.—Schematic flowsheet for the extraction of isoflavones from the body fat of sheep (155).

Adler and co-workers (9) modified Lindner's method (155) for determining both free and conjugated coumestrol in whole bovine blood. A crude ethyl acetate extract of the blood was purified by sequential thin layer chromatography. Chloroform-methanol-water (9:10:1) was used as the first developer and cyclohexane-ethyl acetate-methanol (45:45: 10), the second. Coumestrol was eluted from the second chromatoplate and its concentration estimated fluorometrically by Lindner's technique (155). Conjugated coumestrol was converted to free coumestrol by enzymatic hydrolysis and its concentration estimated in the same manner as coumestrol.

Isolation

Isolation procedures for naturally occurring phenolic compounds are generally based upon successive extraction with solvents of increasing polarity. Once the crude compound is extracted

from the plant tissue, it may be purified by fractional crystallization, by solvent partition, or by separations employing any of the commonly used chromatographic techniques. Final purification is generally accomplished by recrystallization, sublimation, or both.

Isolation of coumestrol from Ladino clover

Coumestrol was first isolated from Ladino clover in 1956 (*26*, *31*). The mouse-uterine-weight bioassay was employed to guide the various steps of the isolation. Reground Ladino clover meal, high in estrogenic activity, was extracted with hot water to remove the water soluble materials, including saponins. This was followed by a Skellysolve C extraction to remove fats, waxes, chlorophylls, and carotenoids. The crude coumestrol was separated from the residual meal by ether extraction, which gave a dark tarry material upon evaporation of the solvent. Coumestrol and the other phenolic compounds were further purified by taking advantage of their weakly acidic properties. They are preferentially soluble in organic solvents when in their normal or lactone form. However, in their open ring form (basic salt), they are preferentially soluble in water.

The materials that did not exhibit acidic properties were removed by partitioning between chloroform and dilute alkali (pH 10-11). The aqueous layer was acidified (pH 6) and re-extracted with ether, which, after evaporation of the other, gave a yellow product.

Coumestrol was separated from the other phenolic and flavonoid-type compounds by a series of six countercurrent distributions (CCD). The solvent systems and the order in which they were employed are presented in table 6. The number of transfers and the position of coumestrol at the end of each distribution are also given. The results of the estrogenic assays of the various fractions obtained during the isolation procedure are summarized in table 7. For final purification, coumestrol was repeatedly recrystallized from methanol-chloroform and sublimed.

TABLE 6.—*Solvent systems employed in purification of coumestrol by countercurrent distribution*

Solvents and proportions by volume	No. of transfers	Coumestrol location
		Tubes [1]
Acetone-ether-water-Skellysolve B (10:5:5:2)	100	69–90
Chloroform-carbon tetrachloride-methanol-water (2:2:3:2)	100	56–80
Methanol-benzene-ether-water (4:4:1:1)	100	30–60
Skellysolve B-ethyl acetate-methanol-carbon tetrachloride-water (1:1:1:1:1)	100	25–58
Acetone-carbon tetrachloride-water (2:1:1)	100	40–76
Acetone-carbon tetrachloride-water-methanol (10:5:5:1)	280	30–60

[1] Refers to location in 100 tubes of the Craig countercurrent distribution instrument.

Source: Bickoff, E. M., Booth, A. N., Lyman, R. L., and others (*31*).

TABLE 7.—*Biological potency of fractions obtained during the isolation of coumestrol from Ladino clover*

Material tested	Amount fed per mouse	Uterine weight
	Milligrams	*Milligrams*
Control (basal ration) _____	_____	10
Ladino clover meal _____	3,000	15
Residue after water extraction _	3,000	27
Residue after Skellysolve extraction _____	3,000	65
Concentrate from ether extraction _____	200	36
After extraction with alkali, and transfer back to ether _____	10	30
Coumestrol _____	.67	95
Diethylstilbestrol _____	.0004	72

Source: Bickoff, E. M., Booth, A. N., Lyman, R. L., and others (*31*).

Large-scale processing of alfalfa for coumestrol

For large-scale isolation (*32*), alfalfa was used as the starting material because of its commercial availability (*145*). The procedures for extracting coumestrol from Ladino clover were employed with several modifications in order to facilitate the handling of the large quantity of meal (45 tons) (*32*). Acetone instead of ether was employed to extract the defatted meal. The acetone extracted 2.8 percent of the solids and 37 percent (6,200 g.) of the coumestrol originally present in the meal. The acetone concentrate was distributed between chloroform and alkali in a manner similar to that described for Ladino clover. After acidification, a dark gummy mass was collected instead of a yellow crystalline solid. This was purified by partitioning between methanol-water-ether-hexane. Coumestrol and a number of other phenolic compounds co-crystallized from the upper phase as greenish-yellow solids (about 70 percent coumestrol). Recrystallization, first from isopropyl alcohol and then dimethylformamide (DMF), removed most of the green contaminants and left a light yellow product containing 85 percent coumestrol. This crude product was purified by acetylation and recrystallization of its acetate from chloroform. Basic hydrolysis regenerated pure coumestrol. Paper chromatography was employed to follow this large-scale isolation of coumestrol.

Portions of this coumestrol as well as some of the cruder fractions were supplied to several cooperating agencies to evaluate their potential growth-promoting properties with sheep (*193*) and cattle (*209*).

Isolation of compounds closely related to coumestrol

The chloroform filtrate from the final purification of coumestrol acetate contained several hundred grams of crystalline material. This was combined with the crude coumestrol acetate obtained by reworking various fractions of the large-scale processing. After further recrystallization of these combined acetates from chloroform, deacetylation yielded over a kilogram of material containing about 60 percent coumestrol. Two-dimensional paper chromatograms of this crude coumestrol preparation showed the presence of at least 15 other blue or yellow fluorescing compounds. A number of these were isolated by CCD (41). Fifty-eight distributions employing four solvent systems (table 8) were required to separate coumestrol and 11 of the other phenolic compounds. By using dimethylformamide (DMF) as a carrier solvent, over 200 g. of material were distributed in certain solvent systems with adequate separations being made. Figure 7 shows a schematic chromatogram of a typical CCD separation. For preliminary identification, a Roman numeral was assigned to an individual compound after it was isolated. Figure 8 presents the sequence of these distributions.

A number of compounds were present in minute amounts and were not detected by paper chromatography in the original preparation or in the initial fractions. However, as the major components were removed by CCD, the minor ones became visible on the developed chromatograms. A total of 12 compounds were isolated. These included coumestrol, trifoliol, salicylic acid, tricin, and eight compounds which at that time were of unknown structure. These have been subsequently characterized as medicagol (compound I), 4'-O-methylcoumestrol (compound II), 3'-methoxycou-

TABLE 8.—*Solvent systems employed in countercurrent distributions of alfalfa phenolics*

Solvent system	Solvents and proportions by volume	Starting material	Total solids	Solids per run
			Grams	*Grams*
A	Skellysolve B-ether-methanol-water-DMF [1] (2:5:3:1:0.4).	Crude coumestrol preparation.	1,000	40
B	Ethyl acetate-Skellysolve B-ether-methanol-water-DMF (12:12:10:5:4:8).	Fraction 2 _____	860	215
		Fraction 11 _____	56	28
		Fraction 12 _____	96	24
C	Acetone-Skellysolve B-ether-water (2:1:1:1).	Combined fractions 4 and 7.	200	10
D	Water-DMF-acetone-carbon tetrachloride (2:4:3:5).	Fraction 19 _____	36	12

[1] DMF = dimethylformamide.
Source: Bickoff, E. M., Livingston, A. L., Witt, S. C., and others (41).

SOLVENT FRONT
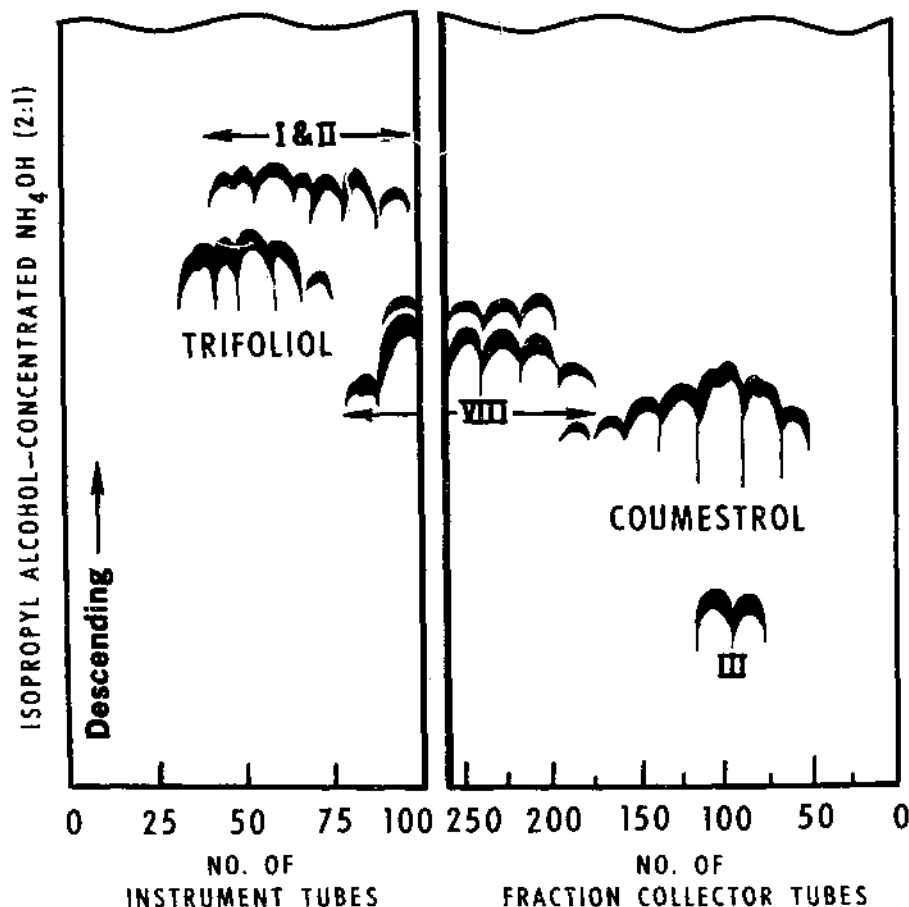
FIGURE 7.—Example of a paper chromatogram of a typical countercurrent distribution separation (41). See page 17 for names of numbered compounds.

mestrol (compound III), 4',7-dihydroxyflavone (compound IV), 3', 4', 7-trihydroxyflavone (compound V), lucernol (compound VI), sativol (compound VII), and 11,12-dimethoxy-7-hydroxycoumestan (compound VIII). Figure 2 presents the structures of the coumestans that were isolated.

Characterization of Coumestans

The trivial name coumestan was proposed by Mentzer (85) for the skeletal structure of the heterocyclic, four ring system having the systematic name 6H-benzofuro [3,2,c] [1] benzopyran-
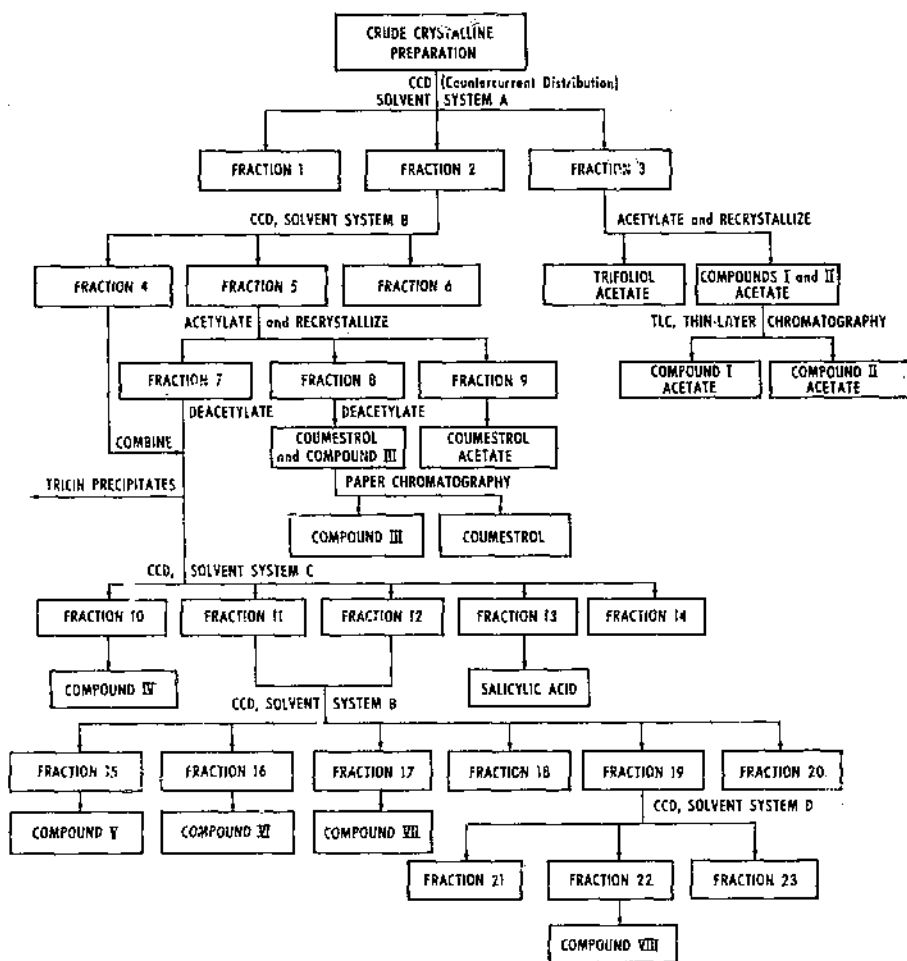
FIGURE 8.—Sequence of countercurrent distributions for the isolation of coumestans and flavones from alfalfa (41).

6-one (44) and has been accepted by Chemical Abstracts. The names coumarino-c o u m a r o n e (113), c o u m a r i n o-benzofuran (133), and benzofurocoumarin (132) have also been applied to this class of compounds, of which coumestrol is representative (113). The name coumarinocoumarone was not acceptable according to the Ring Index published by the American Chemical Society (160).

Both physical and chemical means have been employed in the characterization of the coumestans. Derivatives such as acetates and methyl ethers are useful to indicate the number of hydroxyl groups. Fusion studies and stepwise degradation to known compounds have been employed to

determine the ring structure as well as the location of some of the functional groups. Both UV and proton magnetic resonance (p.m.r.) spectral analyses have been used in pinpointing the location of functional groups. Synthesis is usually used for final confirmation of structure. Table 9 summarizes the physical data for these compounds.

Coumestrol

The presence of two free hydroxyl groups was indicated by the formation of diacetoxyl and dimethoxyl derivatives (31). The empirical formula, $C_{15}H_8O_5$, differed from that of a typical flavonoid by the absence of two hydrogen atoms. Fusion studies yielded only resorcinol and β-resorcylic acid, giving an indication of the number and position of the hydroxyl groups on the two rings. The UV absorption spectrum of coumestrol (fig. 9) differed markedly from spectra of known isoflavones and flavones having the hydroxyl pattern indicated by the fusion products.

The structure of coumestrol as 3,9-dihydroxy-6H-benzofuro[3,2,c][1]benzopyran-6-one (fig. 10, Ia) (44) was established by stepwise degradation, following a procedure similar to that described for wedelolactone (111). Methylative ring opening of coumestrol gave a trimethylether methyl ester (fig. 10, IIa), which was readily saponified to the corresponding 2-(2,4-dimethoxyphenyl)-6-methoxybenzofuran-3-carboxylic acid (fig. 10, IIb). Heating decarboxylated the acid to 2-(2,4-dimethoxyphenyl)-6-benzofuran (fig. 10, III). Ozonolysis of this product, followed by treatment with peroxide, gave 4-methoxy-2(2,4-dimethoxybenzoyl)-benzoic acid (fig. 10, IV), which was hydrolyzed to give the 2,4-dimethoxy- and 2-hydroxy-4-methoxybenzoic acids (fig. 10, V and VI). These were the breakdown products expected for a compound having the same basic ring structure as wedelolactone. The structure of coumestrol (7,12-dihydroxycoumestan, fig. 10, Ia) was then confirmed by its unequivocal synthesis (94).

Trifoliol

Trifoliol was originally isolated from Ladino clover (38) and later from alfalfa (41). Elemental analysis showed that trifoliol was a monomethoxyl compound having the empirical formula $C_{16}H_{10}O_6$. The presence of two phenolic hydroxyl groups was indicated by the formation of diacetoxyl and dimethoxyl derivatives. UV spectral measurements indicated that trifoliol was structurally related to coumestrol and its 12-methoxyl derivative. This suggested that the methoxyl group was probably in the 12-position, and that one of its hydroxyl groups was in the 7-position. Trifoliol was degraded to its benzofuran in the same manner as described for coumestrol. Comparison of the p.m.r. spectra of trifoliol and its benzofuran derivative showed that the D-ring (fig. 10) was substituted at the 10- and 12-posi-

TABLE 9.—*Physical data for naturally occurring coumestans from alfalfa*

Common name	Trivial name	Parent compound		Acetate derivative		Methylether derivative		Reference [1]
		Empirical formula	Melting point	Empirical formula	Melting point	Empirical formula	Melting point	
			°C.		°C.		°C.	
Coumestrol _____	7,12-Dihydroxy-coumestan	$C_{15}H_8O_5$	385 d.[2]	$C_{19}H_{12}O_7$	237	$C_{17}H_{12}O_5$	198	(31)
Trifoliol _____	7,10-Dihydroxy-12-methoxycoumestan	$C_{16}H_{10}O_6$	332 d.	$C_{20}H_{14}O_8$	243	$C_{18}H_{14}O_6$	255–58	(38)
Medicagol _____	7-Hydroxy-11,12-methylenedioxycoumestan	$C_{16}H_8O_6$	324–25	$C_{18}H_{10}O_7$	262–63	$C_{17}H_{10}O_6$	269–70	(161)
4'-O-methylcoumestrol ___	7-Hydroxy-12-methoxy-coumestan	$C_{16}H_{10}O_5$	331–32	$C_{18}H_{12}O_6$	240	$C_{17}H_{12}O_5$	198	(42)
3'-Methoxycoumestrol ___	7,12-Dihydroxy-11-methoxycoumestan	$C_{16}H_{10}O_6$	329	$C_{20}H_{14}O_8$	282–83	$C_{18}H_{14}O_6$	205–207	(45)
Lucernol _____	6,7,12-Trihydroxy-coumestan	$C_{15}H_8O_6$	>350	$C_{21}H_{14}O_9$	253–54	$C_{18}H_{14}O_6$	255	(205)
Sativol _____	8,12-Dihydroxy-7-methoxycoumestan	$C_{16}H_{10}O_6$	303	$C_{20}H_{14}O_8$	256–57	$C_{18}H_{14}O_6$	209–210	(205)
	11,12-Dimethoxy-7-hydroxycoumestan	$C_{17}H_{12}O_6$	306	$C_{19}H_{14}O_7$	213–15	$C_{18}H_{14}O_6$	246	(206)

[1] Italic numbers in parentheses refer to Literature Cited at end of report.
[2] d. = decomposes on melting.

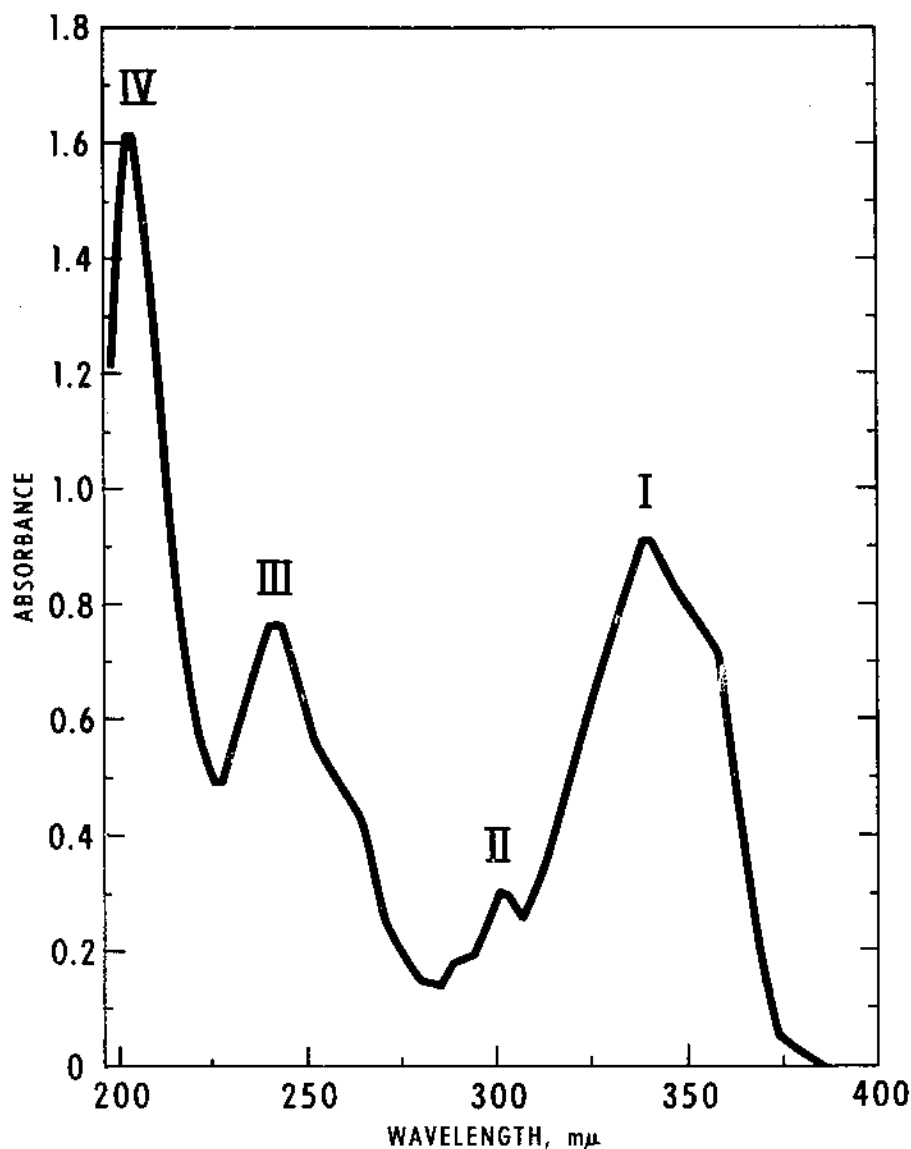FIGURE 9.—Ultraviolet absorption spectrum of coumestrol in methanol (*31*).

tions. Degradation of trifoliol benzofuran to 2, 4-dimethoxybenzoic acid and 2-hydroxy-4, 6-dimethoxybenzaldehyde established the second hydroxyl group at the 10-position. The structure of trifoliol (7, 10-dihydroxy-12-methoxycoumestan) was confirmed unequivocally by its synthesis.
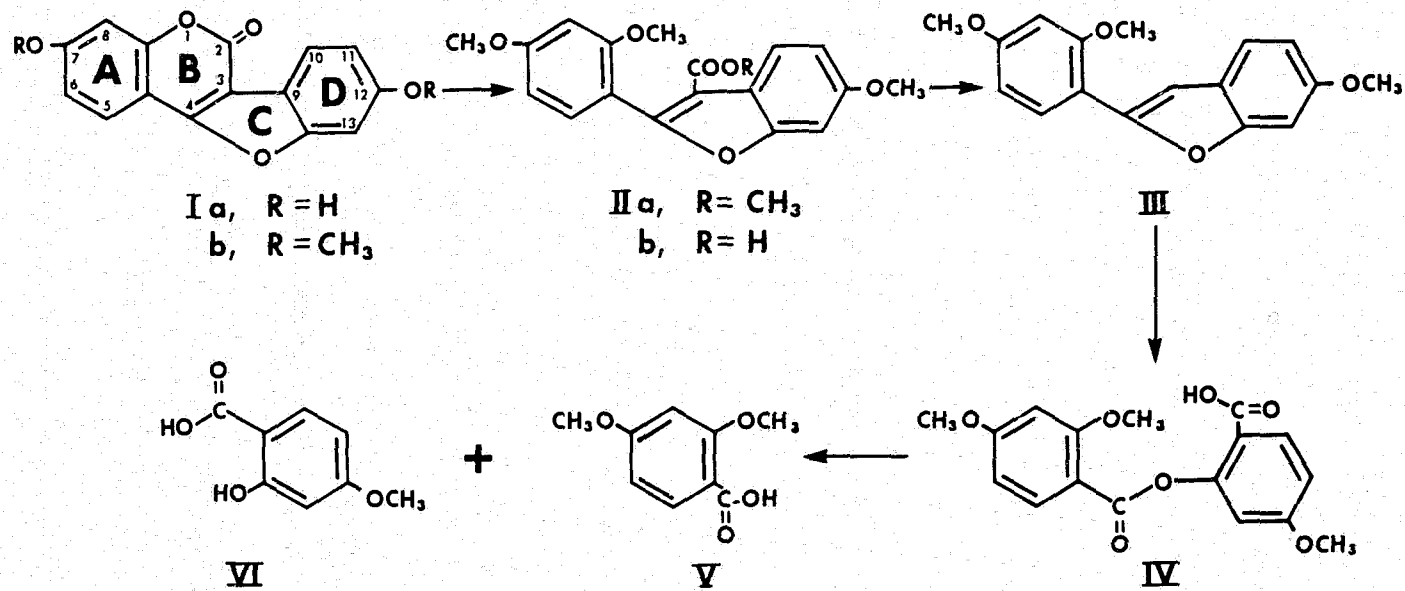
FIGURE 10.—Stepwise degradation of coumestrol (44).

Medicagol

Medicagol was isolated from alfalfa as a mixture with a second compound (4'-O-methylcoumestrol) from which it could not be separated by standard techniques. Although thin-layer chromatography (TLC) afforded a slight separation, its use was impractical for isolating sufficient quantities for characterization studies. UV spectral studies of the mixture suggested that each compound had a hydroxyl group in the 7-position. The two compounds were separated by fractional crystallization of their mixed benzofuran derivatives. Elemental analysis of medicagol benzofuran gave an empirical formula of $C_{17}H_{14}O_5$, indicating that the parent compound could have been derived from a coumestan containing a methylenedioxyl and a hydroxyl group. The presence of the methylenedioxyl group was further indicated by a positive Hansen test (124) and by p.m.r. and infrared (IR) spectroscopy. Strong acid hydrolysis of the medicagol acetate mixture gave 7, 11, 12-trihydroxycoumestan and 4'-O-methylcoumestrol. This further indicated that medicagol must be 7-hydroxy-11, 12-methylenedioxy coumestan, which was confirmed by its synthesis (161).

4'-O-Methylcoumestrol

4'-O-methylcoumestrol was isolated as a mixture with medicagol. Thin-layer chromatographic comparison of the mixture with known compounds suggested that it was 4'-O-methylcoumestrol. Definite location of the hydroxyl group on the unknown compound was established by preparation of the benzyloxybenzofuran derivatives of the mixture. The benzofurans were separated by fractional crystallization. Elemental analysis of the benzyloxybenzofurans substantiated that one of the compounds had been a monomethoxyl derivative of coumestrol. Comparison with authentic 2-[2-methoxy-4-(benzyloxy) phenyl]- 6-methoxylbenzofuran (the benzyloxybenzofuran of 4'-O-methylcoumestrol) confirmed its identity. A small amount of natural 4'-O-methylcoumestrol was subsequently obtained by treatment of the mixture with 86 percent sulfuric acid. Under these conditions, medicagol was completely converted to 7, 11, 12-trihydroxycoumestan, while 4'-O-methylcoumestrol remained unchanged. These were separated by CCD, and the natural product was compared with an authentic sample of 4'-O-methylcoumestrol (135), with which it was identical (42).

3'-Methoxycoumestrol

3'-Methoxycoumestrol was isolated as a mixture with coumestrol from which it could not be easily separated. Limited quantities of the pure compound were obtained by paper chromatography. Elemental analysis indicated that it was a monomethoxyl compound having the empirical formula $C_{16}H_{10}O_6$. Forma-

tion of diacetoxyl and dimethoxyl derivatives demonstrated the presence of two hydroxyl groups. UV spectral studies indicated that the two hydroxyls were not *ortho* to each other (*136*) and that one was in the 7-position (*135*).

To establish the ring substitution pattern of 3'-methoxycoumestrol, it was desirable to degrade the compound to its benzofuran derivative. Since it was difficult to obtain sufficient material for this purpose by paper chromatography, the mixture of coumestrol and 3'-methoxycoumestrol was taken to their *ortho*-methoxycinnamic acid derivatives via methylative ring opening, followed by hydrolysis. These derivatives were separated by CCD and the *ortho*-methoxycinnamic acid derivative of 3'-methoxy-coumestrol was decarboxylated to the benzofuran.

Comparison of the p.m.r. spectra of the methylether and benzofuran derivatives of 3'-methoxycoumestrol indicated that the compound was substituted in the 7-, 11-, and 12-positions. This functional group assignment was confirmed by comparison of the methylated derivative of 3'-methoxycoumestrol with 7, 11, 12-trimethoxy coumestan (*161*), with which it was identical. Comparing the UV spectrum of 3'-methoxycoumestrol with those of known coumestans indicated that the methoxyl group was in the 11-position.

Definite assignment of the methoxyl group to the 11-position and confirmation of the structure of 3'-methoxycoumestrol were accomplished by its unequivocal synthesis (*45*).

Lucernol

The UV spectrum of lucernol was similar to that of the coumestans. Analytical data indicated that no methoxyl groups were present. The formation of triacetoxyl and trimethoxyl derivatives confirmed the presence of three free hydroxyl groups. Degradation by alkaline fusion gave resorcinol and hydroxyhydroquinone, which suggested the number and possible positions of the hydroxyl groups on the two rings. UV spectral studies indicated a hydroxyl group at the 7-position as well as the presence of an *ortho*-dihydroxyl grouping. The p.m.r. spectral studies indicated that lucernol was either 7,11,12-trihydroxycoumestan or 6, 7, 12-trihydroxycoumestan. Comparison of the physical properties of lucernol with those of 7, 11, 12-trihydroxycoumestan (*161*) proved them to be different. Thus, the structure of lucernol had to be 6, 7, 12-trihydroxycoumestan (*205*). This structure was later confirmed by its synthesis by Kalra and co-workers (*138*).

Sativol

Analysis of sativol, its acetoxyl and methoxyl derivatives, indicated that it was a monomethoxyl compound containing two hydroxyl groups. Degradation by alkaline fusion gave a mixture of four compounds that were identified as re-

sorcinol, β-resorcylic acid, pyrogallol, and pyrogallol carboxylic acid. As with lucernol, these products suggested the presence of one oxygen-containing functional group on one ring and two on the other. Systematic degradation using the same procedure as described for coumestrol indicated that sativol had substituents at the 7-, 8-, and 12-positions. This substitution pattern was confirmed by p.m.r. studies.

UV spectral studies indicated that sativol did not contain a hydroxyl group at the 7-position or an ortho-dihydroxyl grouping.

Therefore, the lone methoxyl group had to be at the 7-position and the hydroxyl groups at the 8- and 12-positions. The location of the lone methoxyl group was confirmed by p.m.r. spectroscopy through a comparison of the ether-acetate shift of the aromatic protons of sativol with those observed for several coumestans of known structure. Thus, the structure of sativol is 7-methoxy-8, 12-dihydroxycoumestan (205). Kalra and co-workers (133) confirmed this substitution pattern by the synthesis of sativol methylether.

11,12-Dimethoxy-7-hydroxycoumestan

Elemental analysis of 11, 12-dimethoxy-7-hydroxycoumestan and its acetoxyl and methoxyl derivatives indicated that it was a dimethoxylcoumestan containing one hydroxyl group. Its methoxyl derivative was identical with authentic 7, 11, 12-trimethoxycoumestan (161). UV spectral studies indicated that the hydroxyl group must be located at the 7-position. The structure of this compound as 11,12-dimethoxy-7-hydroxycoumestan was confirmed by synthesis (173, 206).

4',7-Dihydroxyflavone

4',7-Dihydroxyflavone was isolated from both alfalfa and Ladino clover (40). The empirical formula, $C_{15}H_{10}O_4$, was typical of a flavonoid compound. The presence of two hydroxyl groups was demonstrated by the formation of a diacetoxyl derivative. Color reactions and UV spectral data indicated that the compound was a flavone. Alkaline fusion studies suggested that the compound was 4',7-dihydroxyflavone. This was confirmed by comparison of the natural compound with an authentic sample of 4', 7-dihydroxyflavone.

3', 4',7-Trihydroxyflavone

3',4',7-Trihydroxyflavone, isolated from both alfalfa (46) and Ladino clover (157), had an empirical formula of $C_{15}H_{10}O_5$, which suggested that it was a trihydroxyflavone. Color tests and UV spectral studies confirmed the basic flavone structure. Formation of a triacetoxyl derivative confirmed the presence of three hydroxyl groups. The UV spectral shifts suggested that the compound was 3',4',7-trihydroxyflavone. This was confirmed by comparison with an authentic sample of 3',4',7-trihydroxyflavone.

Synthesis of Coumestans

Four approaches have been employed for the synthesis of coumestans. They are: (1) cyclization of appropriately substituted 4-hydroxy-3-phenylcoumarins, (2) oxidation of flavylium salts, (3) condensation of catechol with substituted 4-hydroxycoumarins, and (4) preparation of a new coumestan from one prepared by any of the above procedures. The four methods will be discussed separately.

Cyclization of 4-hydroxy-3-phenylcoumarin derivatives

The first synthesis of coumestrol was accomplished by Emerson and Bickoff (94). They condensed 2,4-dimethoxyphenylacetonitrile (fig. 11, I) with resorcinol (fig. 11, II) in a Hoesch-type reaction to give α-(2,4-dimethoxyphenyl) - 2, 4-dihydroxyacetophenone (fig. 11, III). This product was then condensed with methyl chloroformate using the procedure described by Gilbert and co-workers (109) to give a methylformate derivative (fig. 11, IV). Hydrolysis with alkali gave 3-(2,4-dimethoxyphenyl) -4,7-dihydroxycoumarin (fig. 11, V), which was converted to coumestrol (fig. 11, VI) by demethylation with simultaneous ring closure. The crude product was purified through its diacetoxy derivative.

Kawase (139) shortened the synthesis of coumestrol by starting with 2,4-dimethoxybenzoic acid methyl ester instead of resorcinol (fig. 11, II), which gave 2,4-dimethoxybenzoyl-(2,4 - dimethoxyphenyl) acetonitrile as the intermediate. This was converted to coumestrol by demethylation and simultaneous ring closure. Other coumestans have also been synthesized using similar procedures. They include wedelolactone trimethylether by Govindachari and co-workers (114), lucernol and sativol dimethylether by Kalra and co-workers (138), as well as the basic unsubstituted parent coumestan by Deschamps-Vallet and Mentzer (85).

Oxidation of flavylium salts

In connection with a study of anthocyanins and related compounds, Jurd (133, 134) found that coumestan-type compounds could be prepared by the oxidation of flavylium salts. For the synthesis of coumestrol (fig. 12, V), 2,4-dihydroxybenzaldehyde was condensed with ω-methoxy-2, 4-dibenzyloxyacetophenone (fig. 12, I) to give the benzyloxyflavylium salt (fig. 12, II). Acid hydrolysis gave 2',4',7-trihydroxy-3-methoxyflavylium chloride (fig. 12, III). This flavylium salt was oxidized to a carbomethoxybenzofuran (fig. 12, IV), which rapidly lactonized on acidification to coumestrol (fig. 12, V). The synthetic coumestrol was identical with the natural compound in all respects.

A number of other naturally occurring coumestans have been synthesized using Jurd's procedure. They include the following: trifoliol by Livingston and co-workers (160), medicagol by Jurd (132), 3'-methoxycoumestrol by Bickoff and co-
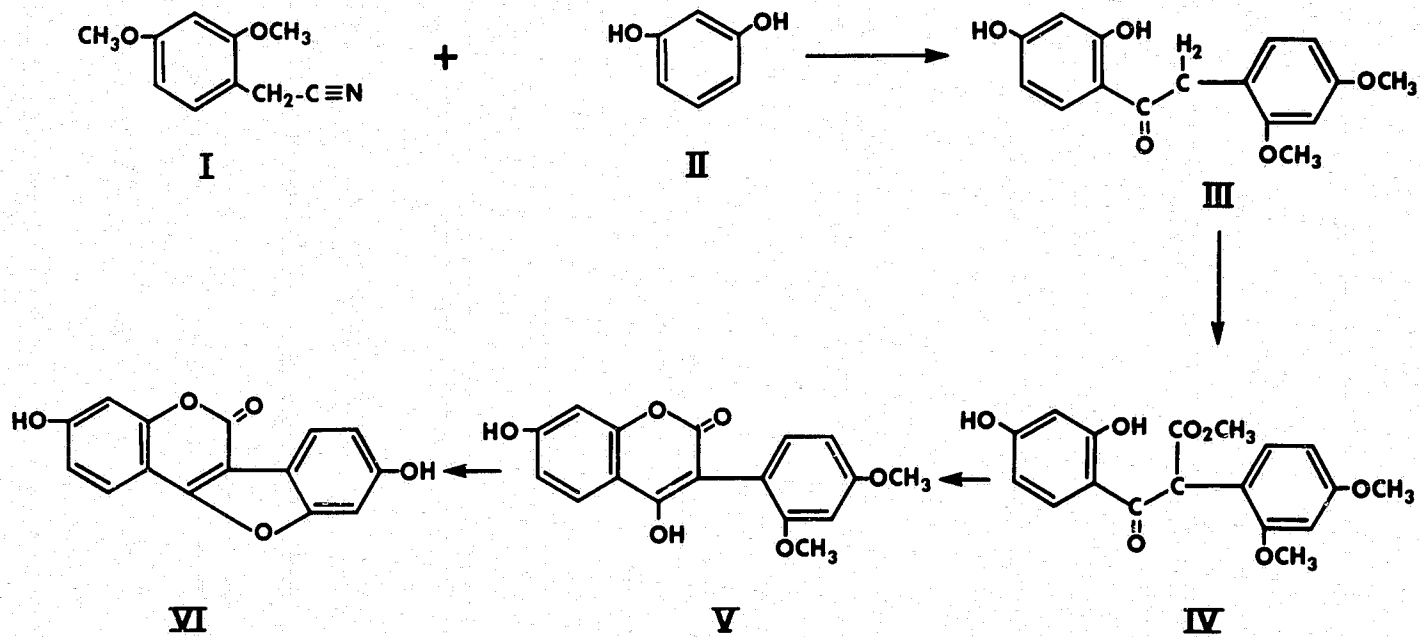
FIGURE 11.—Synthesis of coumestrol by cyclization of 3-(2',4'-dimethoxyphenyl)-4,7-dihydroxycoumarin (94).
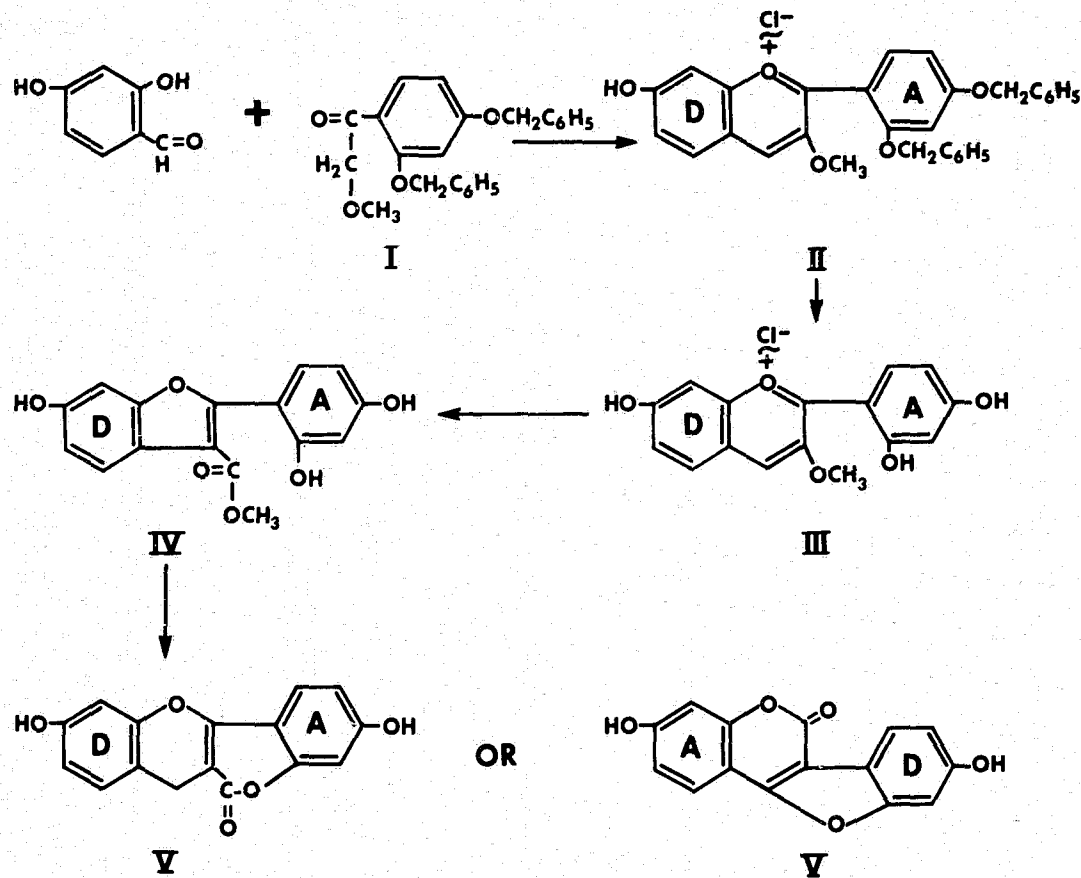
FIGURE 12.—Synthesis of coumestrol from a flavylium salt (the Jurd technique) (*185*).

TABLE 10.—*Physical data for synthetic coumestans*

Compound	Figure 13, III	Melting point		
		Parent compound	Acetate derivative	Methylether derivative
		° C.	° C.	° C.
7,10-Dihydroxycoumestan _____	e	278-79	247-48	230-31
7-Methoxy-10-hydroxycoumestan ____	c	234-35	236	230-31
7-Hydroxy-10-methoxycoumestan ____	f	333-34	288-89	230-31
7,11-Dihydroxycoumestan _____	g	>350	253	211.5
7-Methoxy-11-hydroxycoumestan ____	d	281-82.5	229-31	211.5
7,13-Dihydroxycoumestan _____	b	>325	214	¹204
7-Hydroxy-13-methoxycoumestan ____	a	²303	214-15	¹204

[1] Reported melting point is 195° C. (*133*).
[2] Reported melting point is 289° C. (*133*).

Source: Spencer, R. R., Knuckles, B. E., and Bickoff, E. M. (*207*).

workers (*45*), and 4'-O-methyl-coumestrol by Jurd (*133*).

Jurd's procedure has been used to synthesize a number of coumestans that are presently not known to occur in nature. In addition to 7,10,12-trihydroxy-coumestan (the parent of trifoliol) (*160*), a series of isomers of coumestrol, which have a different substitution pattern in the D-ring (*207*), have been prepared. An appropriately substituted *ortho*-hydroxybenzaldehyde was condensed with ω-methoxy-2, 4-dibenzyloxyacetophenone to form the benzyloxyflavylium salt,

which was debenzylated with acid to give the desired flavylium salt. The flavylium salt was then oxidized to give the coumestan (fig. 13, III). The orientation pattern of the D-ring (fig. 13, I) is governed by the aldehyde. An aldehyde (fig. 13, II) substituted at R_1 ($R_2 = R_3 = H$) will produce substitution at the 13-position in the D-ring. Similarly, R_2 ($R_1 = R_3 = H$) substituted aldehydes produce the 11-series and R_3 ($R_1 = R_2 = H$), the 10-series. Table 10 gives a summary of the coumestans that were prepared in this study.

Condensation of catechol with substituted 4-hydroxycoumarins

With his synthesis of wedelolactone, Wanzlick (*219*) developed a novel method of synthesizing the coumestan structure. Wedelolactone (fig. 14, III) was formed by the condensation of 4,5-dihydroxy-7-methoxycoumarin (fig. 14, I) with catechol (fig. 14, II) in an aqueous-acetone mixture of potassium ferrocyanide and sodium acetate. Catechol is the only compound that

has been found to react with a 4-hydroxycoumarin in this reaction. Therefore, the only substitution that can be obtained in the D-ring is at the 11- and 12-positions. However, any substitution can be effected in the A-ring depending on the 4-hydroxycoumarin used in the coupling reaction. Employing this reaction, Livingston and co-workers (*161*) synthesized medicagol by conden-
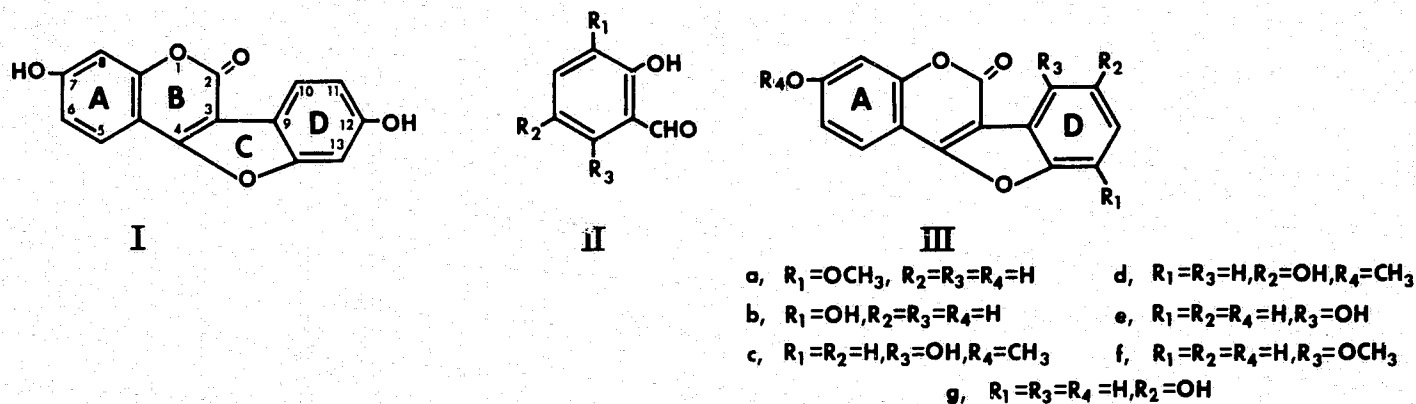
FIGURE 13.—Relationship of aldehyde substitution pattern to the resulting coumestan (207).

a, R_1=OCH$_3$, R_2=R_3=R_4=H   d, R_1=R_3=H,R_2=OH,R_4=CH$_3$

b, R_1=OH,R_2=R_3=R_4=H   e, R_1=R_2=R_4=H,R_3=OH

c, R_1=R_2=H,R_3=OH,R_4=CH$_3$ f, R_1=R_2=R_4=H,R_3=OCH$_3$

g, R_1=R_3=R_4=H,R_2=OH

FIGURE 14.—Synthesis of wedelolactone by the Wanzlick technique (219).

sing 4,7-dihydroxycoumarin with catechol to give 7,11,12-trihydroxycoumestan. This was then converted to medicagol by direct methylenation with di-iodo-methane. Similarly, medicagol dimethylether was synthesized from 4-hydroxy-7-methoxycoumarin by Fukui and co-workers (108), 11, 12 - dimethoxy-7-hydroxycoumestan from 7-benzyloxycoumarin by Malleshawar and co-workers (173), and erosnin from 5–hydroxypsoralene by Fukui and Nakayama (107).

Synthesis from previously formed coumestans

Jurd (135) synthesized the 4'-O-methyl and 7-O-methyl derivatives of coumestrol by selective alkylation of coumestrol diacetate. The 7-O-methyl derivative was prepared directly from coumestrol diacetate by selective methylation followed by hydrolysis. In order to prepare the 4'-O-methyl derivative, however, it was necessary to block the 7-position with a base stable group. Thus, selective benzylation of coumestrol diacetate under similar conditions gave 7-O-benzylcoumestrol monoacetate. After hydrolysis, this compound was methylated, forming 7-O-benzyl-4'-O-methylcoumestrol. Debenzylation of the latter compound gave 4' - O - methylcoumestrol, which was identical with the natural 4'-O-methylcoumestrol. Using this procedure, 10-hydroxy-7-methoxycoumestan (fig. 13, IIIc) and 11-hydroxy-7-methoxy-coumestan (fig. 13, IIId) were prepared from their respective diacetates by Spencer and co-workers (207). They also prepared 7,13-dihydroxycoumestan (fig. 13, IIIb) by demethylation of 7-hydroxy-13-methoxycoumestan (207).

Spencer and co-workers (206) synthesized 11,12-dimethoxy-7-hydroxycoumestan from 7,11, 12-trihydroxycoumestan (fig. 15, Ia). The 11- and 12-positions were first blocked by the formation of the diphenylmethylenedioxyl derivative (fig. 15, IIa). Benzoylation (fig. 15, IIb), followed by acid hydrolysis, regenerated the 11,12-dihydroxyl derivative (fig. 15, Ib). Methylation of the 11- and 12- positions then formed 7-O-benzoyl-11,12-dimethoxycoumestan (fig. 15, Ic), which upon basic hydrolysis yielded 11, 12-dimethoxy-7-hydroxycoumestan (fig. 15, Id).

Physical Properties of Coumestans

The physical properties of coumestans, such as UV and p.m.r. spectroscopy, and chromatography can be used as aids for structural determination. These properties also assist in determining the substitution pattern and type of ring substituents.
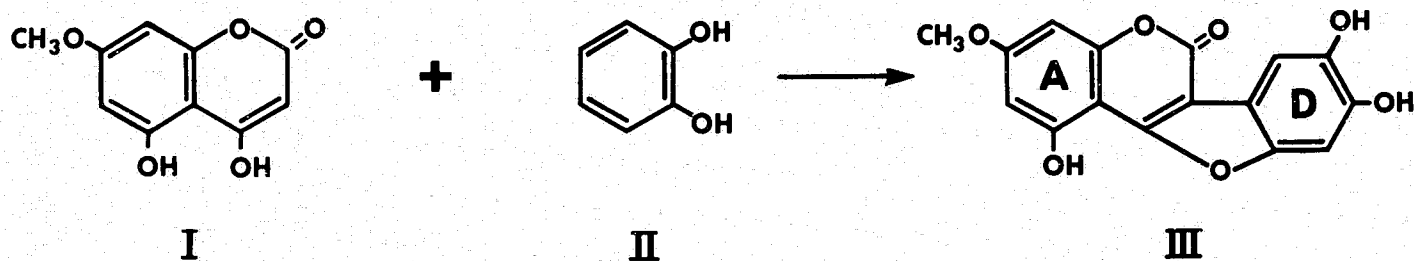
Ultraviolet absorption spectral measurements

Coumestans generally exhibit high intensity UV absorption in the 340 to 355 millimicron region (band I), in the 230 to 250 milli- micron region (band III) and the 200 to 215 millimicron region (band IV) along with low intensity absorption in the 300 to 320
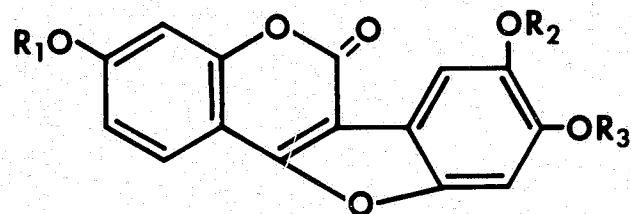
I
a, $R_1=R_2=R_3=H$
b, $R_1=B_z, R_2=R_3=H$
c, $R_1=B_z, R_2=R_3=CH_3$
d, $R_1=H, R_2=R_3=CH_3$
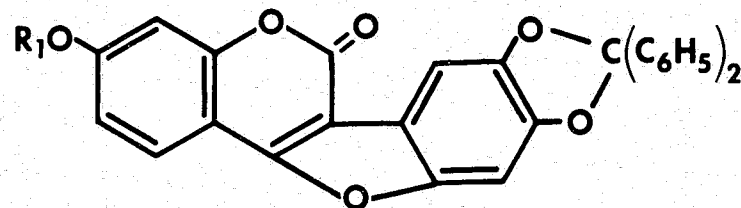
II
a, $R=H$
b, $R=B_z$

FIGURE 15.—Synthesis of 11,12-dimethoxy-7-hydroxycoumestan (206).

millimicron region (band II) (fig. 9). A shift in band I of a coumestan indicates the presence of a hydroxyl gruup at the 7-position. For example, in the spectrum of lucernol (6,7,12-trihydroxycoumestan) a shift with sodium acetate is observed (fig. 16). However, when the 7-hydroxyl is blocked, as in sativol (fig. 17), this shift is not observed. This can be explained by the fact that the hydroxyl group in the 7-position is conjugated with the lactone carbonyl and, therefore, is strongly acidic. In confirmation of this observation, band I of coumestrol (λ_{max}. 344 millimicron, table 11) is shifted to 387 millimicrons in sodium ethylate but only to 362 millimicrons in sodium

acetate (135), indicating that of the two hydroxyl groups only one (the 7-hydroxyl) is sufficiently acidic to be ionized by the weakly basic sodium acetate.

In the presence of sodium acetate, boric acid chelates with phenolic compounds, including coumestans, which contain *ortho*-dihydroxyl groups to cause a bathochromic shift. The spectra of coumestans that do not contain this grouping are not appreciably affected. For example, a 5 to 10 mμ shift is observed in the spectrum of lucernol (fig. 16) but not in the spectrum of sativol (fig. 17). A summary of the peak locations and shifts of representative coumestans is given in table 11.
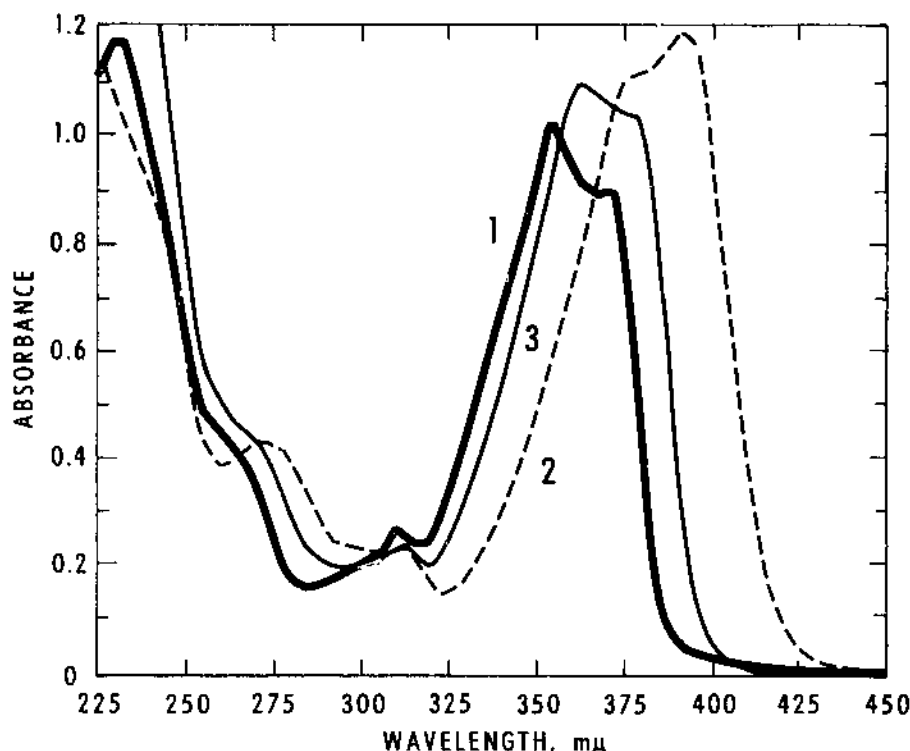
FIGURE 16.—Ultraviolet absorption spectra of lucernol in ethanol: (1) neutral, (2) sodium acetate, (3) boric acid-sodium acetate.
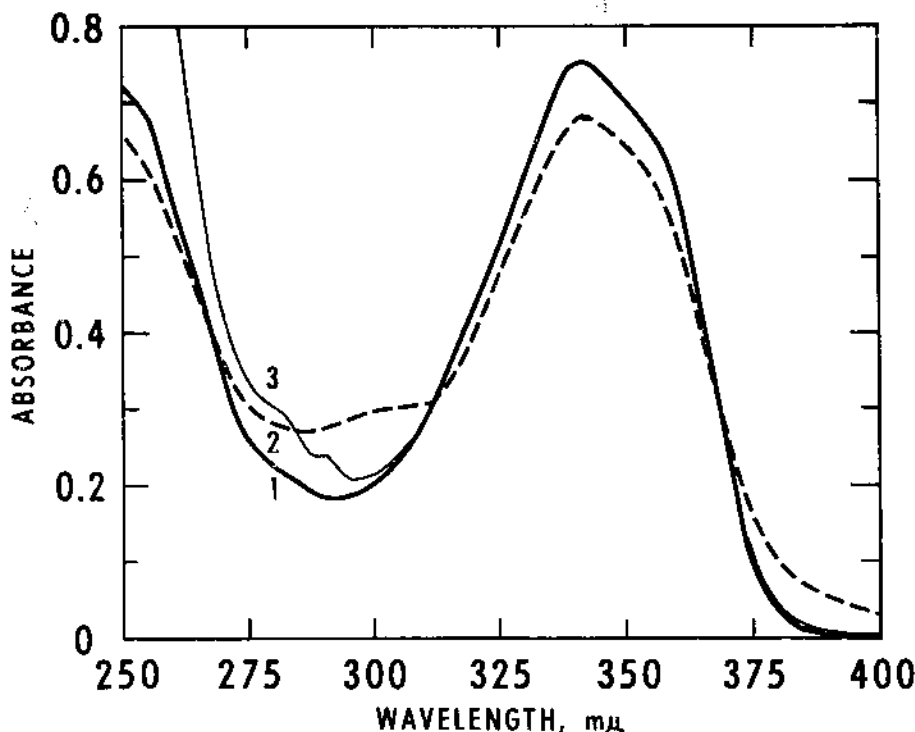
FIGURE 17.—Ultraviolet absorption spectra of sativol in ethanol: (1) neutral, (2) sodium acetate, (3) boric acid-sodium acetate.

Proton magnetic resonance spectral measurements

The p.m.r. spectra of coumestans, like their UV spectra, have several characteristic features. Although most of the proton resonances occur in the range of $\tau = 2.50$ to 3.50 of the aromatic region, the signals from two of the protons (H-5 and H-10) are located significantly downfield from the other aromatic protons. Figure 18, spectrum 1, shows these peaks in sativol dimethylether. Decarboxylation of a coumestan has a major effect on the position of H-10, due to the removal of the paramagnetic shift caused by the nearby carbonyl group, but does not have an appreciable effect on the shielding of the H-5 proton. In spectrum 2 of figure 18, one of the low field ortho-doublets has remained unchanged while the other has shifted upfield. The unshifted doublet is assigned to H-5 and the shifted resonance to H-10. A β-furanal proton is also produced as a result of decarboxylation. Its signal is found in the region $\tau = 2.8$ to 2.9. Since the only proton that is known to couple to H-3 is H-13, a 1.0 Hz splitting of the H-3 signal provides direct evidence for the absence of substitution at H-13. This doublet is clearly evident in spectrum 2 of

TABLE 11.—*Maximum values for the ultraviolet spectra of coumestans in ethanol*

Compound	Band I			Band II			Band III			Band IV		
	Neutral	Sodium acetate	Sodium acetate + boric acid	Neutral	Sodium acetate	Sodium acetate + boric acid	Neutral	Sodium acetate	Sodium acetate + boric acid	Neutral	Sodium acetate	Sodium acetate + boric acid
	$M\mu$	$M\mu$	$M\mu$	$M\mu$	$M\mu$	$M\mu$	$M\mu$	$M\mu$	$M\mu$	$M\mu$	$M\mu$	$M\mu$
Coumestrol	344	362	-------	304	313	-------	244	244	-------	208	208	-------
Trifoliol	350	372	-------	303	312	-------	268	273	-------	212	212	-------
Lucernol	[1]372 355	377 [1]392	378 [1]362	310	312	312	230	272	238	208	208	208
Sativol	342	342	-------	-------	300	-------	242	248	-------	-------	-------	-------
4'-O-methylcoumestrol	342	363	-------	303	312	-------	243	243	-------	208	210	-------
3'-Methoxycoumestrol	350	373	-------	307	315	-------	247	249	-------	210	210	-------
Medicagol	347	362	-------	309	318	-------	245	245	-------	210	210	-------
11,12-Dimethoxy-7-hydroxycoumestan	348	367	-------	306	294 314	-------	247	249	-------	210	210	-------
7-Methoxycoumestrol	342	342	-------	303	303	-------	243	243	-------	208	208	-------
7,11,12-Trihydroxy-coumestan	352	358	372	309	309	316 295	248	250	255	207	208	207

[1] Shoulder (i.e., not a separate peak).

figure 18. Table 12 summarizes similar results obtained with trifoliol and coumestrol.

The position of methoxyl groups on the coumestan ring can be determined by p.m.r. spectroscopy through a comparison of the ether-acetate shift of the aromatic protons. An *ortho* shielding constant (referred to unsubstituted benzene) of 0.21 p.p.m. for the acetate and 0.45 p.p.m. for the methoxyl group has been suggested from a study of disubstituted benzenes. Substitution of an acetoxyl for a methoxyl group, while both structure and solvent are otherwise held constant, will cause a downfield shift of roughly 0.24 p.p.m. for the resonance of a proton located *ortho* to a methoxyl group. To obtain these values, the parent compound is methylated and acetylated. The resonance of protons located *para* to a methoxyl group shifts less, while an even smaller shift occurs for protons located *meta* to a hydroxyl group. These values are obtained by subtracting the τ values of the acetate from the τ values of the methylether for a given proton, solvent, and compound. The results for a series of coumestans are shown in table 13. In general, the resonance of protons located *meta* to a methoxyl group on a coumestan will shift downfield by 0.12 to 0.14 p.p.m.; those located *para* to a methoxyl will show a resonance shift of 0.22 to 0.28 p.p.m, while the resonance of protons located *ortho* to a methoxyl will shift 0.28 to 0.35. These shifts are additive, e.g., for protons located *ortho* to one methoxyl group and *meta* to another, as shown by lucernol (H-5, H-8) in table 13.

Mass spectral measurements

Although not a new technique, mass spectrometry has only recently been applied to the study of the naturally occurring oxygen heterocyclics. Barnes and Occolowitz (*15*) reported the mass spectra of a number of compounds containing the coumarin structure to serve as a guide to more complex systems. The characteristic fragmentation under electron impact is the ready loss of CO from the pyrone ring to form an ion having the benzofuran structure followed by a further loss of the remaining oxygen atoms, again as CO.

Only preliminary studies have been carried out on coumestans.[3,4] Apart from the breakdown of methoxyl functions, the only major fragmentation results from the loss of CO from the coumarin carbonyl. Coumarins that are unsubstituted on the pyrone ring show this fragmentation to a more marked extent.[3]

Coumestrol shows an intense molecular ion that is so stable that the loss of CO is the only significant fragmentation (fig. 19). The monomethyl derivatives of coumestrol show the loss of one CH_3 and two CO functions that results in the formation of a benzofuran in which the D-ring has also been converted to a 5-member ring (fig. 20).

[3] Personal communications from C. S. Barnes and J. L. Occolowitz, research chemists, C. S. R. Research Laboratories, Australia, 1965.

[4] Personal communication from Chris Falshaw, chemist, Massachusetts Institute of Technology, Boston, 1964.
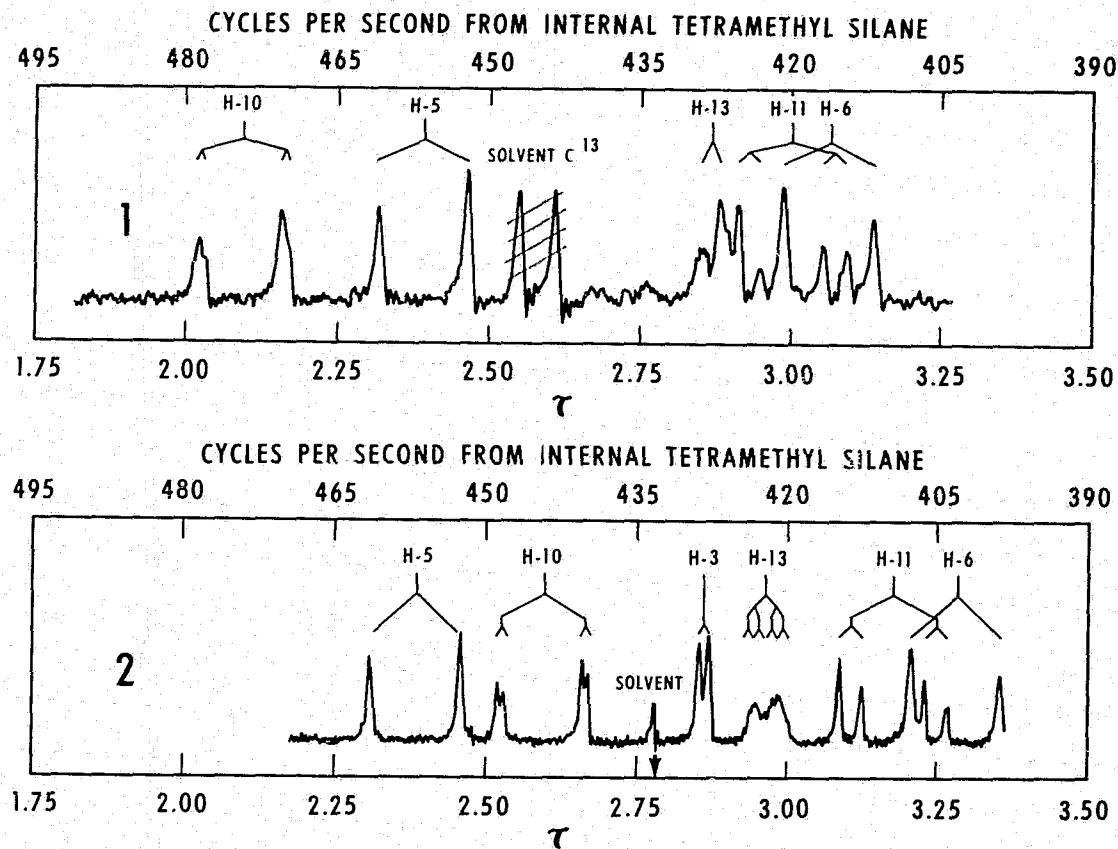
FIGURE 18.—The 60 Hz proton magnetic resonance spectra: (1) sativol dimethylether in 1,1,2,2-tetrachloroethane, (2) the benzofuran of sativol in deuterochloroform (*205*).

TABLE 12.—*Shielding values, in τ units determined at 60 Hz, of trifoliol and coumestrol* [1]

[Values outside parentheses are shielding values; values inside parentheses are coupling constants]

Compound	Aromatic Protons						
	H–3	H–5	H–6	H–3	H–10	H–11	H–13
Trifoliol _____	_____	[2]2.17 (9.0)	2.97 (6.2)	3.03	_____	3.58 (1.5)	3.20 (1.5)
Coumestrol _____	_____	2.10 (9.5)	([3])	([3])	2.22 (9.0)	([3])	([3])
Trifoliol benzofuran ____	2.83 (1.0)	2.10 (9.5)	3.42 (6.5, 2.0)	3.49	_____	3.70 (2.0)	3.36 (2.0, 1.0)
Coumestrol benzofuran__	2.90 (1.0)	2.10 (9.5)	3.40 (8.0, 2.5)	3.48	2.60 (8.5)	3.20 (8.0, 2.5)	3.00

[1] Hz is an abbreviation for megacycles per second.
[2] 9.0 = first order approximation in cycles per second of spin-spin coupling constants of aromatic protons.
[3] Values are too complex in the 2.80–3.00 τ region to identify H-6, H-8, H-11 or H-13.

Source: Livingston, A.L., Bickoff, E. M., Lundin, R. E., and Jurd, Leonard (*160*).

TABLE 13.—*Shielding of ring protons [1] for acetate and methoxyl derivatives of coumestans*

[(O), (M), (P), refer to location of substituting hydroxyl., *i.e., ortho, meta, or para*]

No.	Compounds	H-5	H-6	H-8	H-10	E-11	H-13
1	Coumestrol dimethylether (7, 12-dimethoxycoumestan)	2.17	(²)	(²)	2.09	(²)	(²)
2	Coumestrol diacetate	2.05	2.82	2.74	1.97	2.87	2.52
	1 minus 2	− .12(M)			− .12(M)		
3	4'-O-methylcoumestrol acetate (7-acetoxy-12-methoxy-coumestan)	2.07	2.89	2.77	2.07	3.00	2.87
	1 minus 3	− .10(M)					
	3 minus 2					− .13(O)	− .35(O)
4	Trifoliol dimethylether (7, 10, 12-trimethoxycoumestan)	2.24	3.27	3.09	(³)	3.27	3.55
5	Trifoliol diacetate (7, 10-diacetoxy-12-methoxycoumestan)	2.12	2.87	2.79	(³)	2.97	3.27
	4 minus 5	− .12(M)	− .40(O)	− .30(O)		− .30(O)	− .28(P)
6	Sativol dimethylether	2.38	3.04	(³)	2.08	2.98	2.84
7	Sativol diacetate	2.15	2.97	(³)	1.94	2.82	2.50
	6 minus 7	− .23(P)	− .07(M)		− .14(M)	− .16(O)	− .34(O)
8	Lucernol trimethylether	2.66	(³)	3.01	2.08	2.96	2.82
9	Lucernol triacetate	2.18	(³)	2.60	1.94	2.80	2.51
	8 minus 9	− .48(O,M)		− .41(O,M)	− .14(M)	− .16(O)	−.31(O)
10	7, 11, 12-Trimethoxycoumestan	2.15	3.05	3.03	2.48	(³)	2.82
11	7, 11, 12-Triacetoxycoumestan	2.02	2.82	2.69	2.11	(³)	2.41
	10 minus 11	− .13(M)	− .23(O)	− .34(O)	− .37(O,M)		− .41(O,M)

[1] Measured from tetramethylsilane (TMS) as internal standard at 60 Hz in τ units. All spectra run in 1,1,2,2-tetrachloroethane at 120° C. Negative value indicates resonance at lower field in acetate.
[2] Unassigned because of spectral complexity.
[3] Substituted position.

Source: Spencer, R. R., Bickoff, E. M., Lundin, R. E., and Knuckles, B. E. (205).
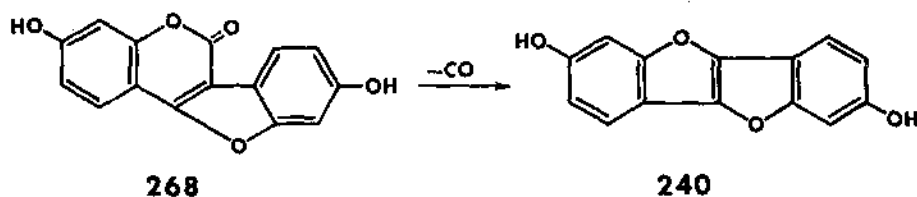
FIGURE 19.—The mass spectral fragmentation pattern of coumestrol.
(See text footnote 3, page 37.)
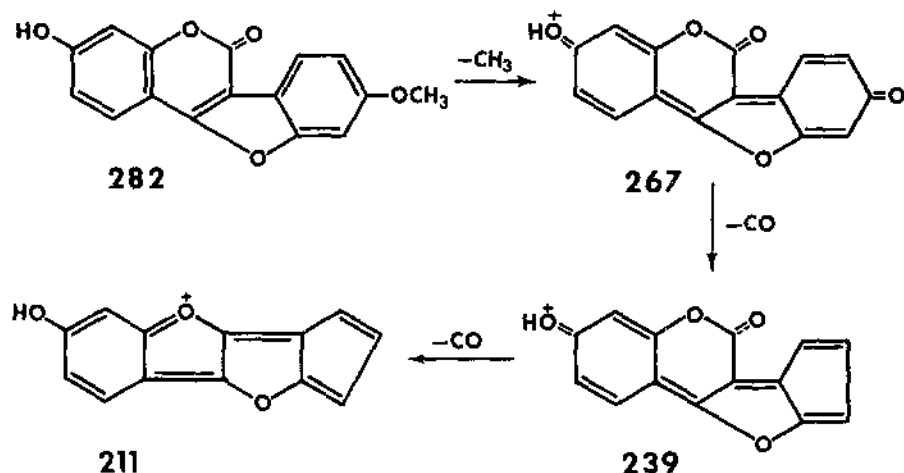
FIGURE 20.—The mass spectral fragmentation pattern of 4'-O-methylcoumestrol.
(See text footnote 3, page 37.)

Trifoliol behaves more like coumestrol and shows the loss of one CH_3 and one CO function

$$(298 \xrightarrow{-CH_3} 283 \xrightarrow{-CO} 255).$$

Its monomethyl derivatives lose one CH_3 and two CO functions, similar to the monomethyl coumestrol derivatives

$$(312 \xrightarrow{-CH_3} 297 \xrightarrow{-CO} 269 \xrightarrow{-CO} 241).$$
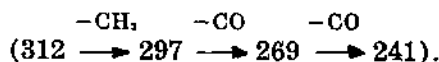
Although these measurements do not give as much information as p.m.r. spectroscopy about the molecular structure of these compounds, they are useful in determining the molecular weights of the compounds and the presence of the pyrone ring.

Chromatography

Coumestans characteristically fluoresce when exposed to UV light. This property makes them very adaptable to chromatographic studies. It also offers a tool to assist in their separation and purification. Exposing developed chromatograms to ammonia vapor or spraying them with dilute base usually intensifies the fluorescence and, in some cases, changes the color of the spots (table 14). In general, coumestans containing only hydroxyl-

TABLE 14.—*Fluorescence and R_f values of phenolic compounds of alfalfa on two-dimensional paper chromatograms* [1]

Compound	R_f values [2]		Color of fluorescence [2]		Relative intensity of fluorescence [3]
	First dimension [4]	Second dimension [5]	Untreated	Exposed to NH^3 vapor	
Coumestrol	0.50	0.52	Purple-white	Yellow-white	1.0
Trifoliol	.66	.57	Dull blue-gray	Blue-gray	([6])
Medicagol	.75	.60	Blue	Intense blue	1.4
4'-O-methyl-coumestrol	.75	.60	Blue	Intense blue	.3
3'-Methoxy-coumestrol	.36	.36	Bluish-white	White	2.1
Lucernol	.16	.34	Gray	Yellow-gray	.6
Sativol	.26	.43	Violet	Pink	.2
11, 12-Dimethoxy-7-hydroxy-coumestan	.58	.42	Blue	Intense blue	2.6

[1] Data obtained using Whatman No. 1 filter paper.
[2] Data obtained from reference (*41*) in Literature Cited section.
[3] Data obtained from reference (*43*) in Literature Cited section. Coumestrol arbitrarily assigned a value of 1.00 as standard.
[4] Isopropyl alcohol-concentrated ammonium hydroxide (2:1), descending.
[5] Fifty percent glacial acetic acid, ascending.
[6] Undetermined.

functional groups exhibit white to gray fluorescence. The introduction of a single methoxyl group at the 7-position changes the fluorescence to violet or pink. A blue fluorescence is generally exhibited by coumestans with other substitution patterns. The relative fluorescence intensity of the coumestans, as compared to coumestrol, is given in table 14.

At this laboratory, the solvent systems that gave the best resolution of these compounds were 50 percent acetic acid, ascending, and isopropyl alcohol-concentrated ammonium hydroxide (2:1), descending. These systems have been used both for one- and two-dimensional chromatography. The R_f values for the coumestans in these solvents are given in table 14. These values represent a ratio between the distance the compound travels and the distance the solvent travels. In general, coumestans containing two free hydroxyl groups, the remaining substituted with methoxyl or methylenedioxyl groups, have R_f values in 0.4 to 0.6 region. Those coumestans with one free hydroxyl group have R_f values in the 0.6 to 0.8 region, and those containing three or more hydroxyl groups have R_f values below 0.3 in these solvent systems. Thus, by employing the combination of R_f values and color under UV light, paper chromatography can be an aid in determining the structure of a coumestan.

Although, the best resolution of these compounds has been obtained with paper chromatography, thin-layer chromatography (TLC) gave better resolution of their methylated and acetylated derivatives. Silicic acid chromatostrips have also been used for separating their degradation products (169). Ether-Skellysolve B (7:3) gave the best separation of methylether derivatives, while chloroform was the best for acetylated derivatives. Other workers have applied both paper chromatographic and TLC techniques to the separation of coumestans. The solvent systems they used are discussed under "Analytical Procedures" and "Coumestrol in Plants."

Coumestrol in Plants

Estrogenic activity of forages is extremely variable (36, 142, 143, 197, 211) and has been shown to be related to such factors as cuttings, stages of growth, diseases, and genetic and environmental differences. Since 1950, many studies have attempted to correlate estrogenic activity with one or more of these variables, with varying degrees of success. However, because of great environmental variations occurring under normal field conditions, it has been difficult to correlate estrogenic activity or coumestrol content with plant growth and development. Therefore, recent work on coumestrol content of alfalfa has been carried out under controlled environmental conditions.

Variations as affected by cuttings and stages of growth

Cheng and co-workers (66, 67), studying estrogenic substances in alfalfa and clover hays, obtained evidence that estrogenic activity increased with number of cuttings. In a more ex-

tensive study on alfalfa, Pieterse and Andrews (197) found that estrogenic activity increased with maturation, although the estrogenic pattern varied from cutting to cutting. In later studies, Bickoff and co-workers (23, 28) found that the estrogenic activity of alfalfa increased as the plant matured and usually reached its highest level at full bloom or seedhead stage (table 15). Similar results were also obtained with white clover clones, including the Ladino variety (37). In cooperative studies by WURDD with commercial dehydrators (144, 145), the coumestrol content of alfalfa during the growing season was shown to vary greatly with number of cuttings as well as with stage of plant development. By allowing the plants to mature to the seed pod stage, far beyond the normal harvest age, dehydrated alfalfa meals containing from 340 to 560 p.p.m. coumestrol were obtained. Generally, commercially prod-

uced alfalfa meals contain less than 100 p.p.m. coumestrol (145). Loper (162), studying alfalfa grown under controlled environmental conditions, found evidence that senescence may cause the accumulation of coumestrol.

Francis and Millington (104) found that the coumestrol content of annual medic species increased with maturation and subsequent senescence. Although coumestrol levels were comparatively low at all growth stages up to flowering, they reached considerable proportions in the naturally dried standing plants, especially Medicago littoralis Harbinger (335 p.p.m.) (table 16). In the flowering plants, coumestrol was concentrated in the mature leaves. However, in the dried standing plants, the stems and pods contained the majority of the coumestrol. Loper (162) was unable to find any coumestrol in either the immature or mature seed pods of barrel medic plants (Medicago littoralis Rhode).

TABLE 15.—Variation in estrogenic activity with stage of growth of alfalfa, 1958

Stage of maturity	Amount of coumestrol[1] as determined from the following cuttings					
	First	Second	Third	Fourth	Fifth	Sixth [2]
	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.
Vegetative	13	0	6	11	7	12
Early bud	0	0		1	20	11
Full bud	0	0	111		17	10
1/10 bloom	7	0	92	49		23
¼ bloom	9	20		47		27
½ bloom	33	63	93	43		17
Full bloom	77	262	98	103		44
Dough	120	94	181	93		41
Seedhead	123	187	210	198		

[1] As determined by mouse-uterine-weight bioassay and reported on a dry-weight basis.
[2] First cutting of 1959 crop.

Source: Bickoff, E. M., Booth, A. N., Livingston, A. L., and Hendrickson, A. P. (28).

TABLE 16.—*Variation with stage of growth in the coumestrol content of some Medicago species*

Species	Variety or local designation	Cotyl-edons [1]	Unifol-iate	First or Second trifoliate	Winter growth	First flowers	Full-late flowers	Senescence obvious	Dry		
		June 22	July 3	Aug. 3	Aug. 25	Sept. 3	Oct. 3	Nov. 3	Dec. 3	Jan. 3	Feb. 3
		P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.
M. littoralis	Harbinger	1	3	2	18	42	40	125	180	240	256	335
M. truncatula	Cyprus	1	2	2	13	40	20	104	90	170	205	210
M. truncatula	Commercial	1	1	2	15	38	46	116	85	95	100	200
M. truncatula	Mtr 173[2]	1	1	1	9	26	28	48	50	48	60	120
M. scutellata	Snail medic	1	1	1	1	3	2	12	12	18	24	52
M. polymorpha	Burr medic	2	2	2	5	7	7	16	14	18	24	46
M. sativa	Hunter river[3]	1	1	1	3	3	4	6	8	*9	7	9

[1] Values are on a dry-weight basis.
[2] This is strain 173 of *M. truncatula*.
[3] Did not flower.
[4] Not naturally dried.

Source: Francis, C. M., and Millington, A. J. (*104*).

Furthermore, he found that senescence by itself did not lead to large amounts of coumestrol. However, he found coumestrol concentrations as high as 2,362 p.p.m. in the mature leaves of medic plants exhibiting severe physiogenic leaf spotting. Working with soybeans, Wada and Yahara (*217*) reported that coumestrol was higher in the sprouts than in the bean itself.

Variations as affected by location and climatic conditions

In cooperative studies with Crops Research Division of ARS, Bickoff and co-workers (*37*) found considerable differences between the estrogenic activity of Ladino clover grown in South Carolina and that grown in California. Similarly, Ochi and co-workers (*189*) in Japan attributed the variation in estrogenic potency among Ladino clover samples to differences in site of sample collection. Millington and co-workers (*183*) found that the coumestrol content of Cyprus and commercial barrel medic varieties grown in adjacent plots in a number of Australian localities varied considerably (table 17). Cooperative studies on alfalfa by WURDD with commercial dehydrators in California, South Dakota, Pennsylvania, and Kansas further confirmed that geographic location can influence coumestrol content (*145*).

Britten,[5] in Hawaii, studied the coumestrol content of the Moapa variety of alfalfa grown under different climatic conditions (table 18). The alfalfa grown at 5,000 feet was under almost continuous moisture conditions from rain and fog. Consequently, these plants had much less sunshine than those grown at sea level or 2,000 feet. The plants that had the least sunshine and the most moisture contained three times the level of coumestrol.

Loper and Hanson (*164*) studied the coumestrol content of Ranger alfalfa grown under controlled environmental conditions and found essentially no differences between plants grown at different temperature regimes. Hanson and co-workers (*127*) were unable to find any correlation between light intensity and coumestrol content in field tests.

[5] Personal communication from E. J. Britten, agronomist, University of Hawaii, 1961.

TABLE 17.—*Variation in coumestrol content of barrel medics grown at different localities in Australia*

[Mean of 10 estimates; measured in parts per million of coumestrol ± standard error]

Variety	Mendel	Perth	Cunderdin	Merredin	Toodyay
Cyprus	50 ± 3.8	55 ± 2.6	40 ± 4.6	180 ± 14.2	160 ± 12.6
Commercial	45 ± 3.4	45 ± 2.6	100 ± 6.9	150 ± 14.8	210 ± 13.8

Source: Millington, A. J., Francis, C. M., and McKeown, N. R. (*183*).

TABLE 18.—*Variation in coumestrol content of alfalfa grown at different experiment stations in Hawaii*

Station elevation (feet)	Rainfall	Average temperature	Source of moisture	Coumestrol
	Inches/year	° *F.*		*P.p.m.*
Sea level _____	12	67-85	Irrigation	39.5
2,000 _____	75	61-73	Rain	31.5
5,000 _____	100	49-63	Rain and fog	99.0

Source: See text footnote 5, page 46.

Variations as affected by varietal and genetic differences

Francis and Millington (*104*) found wide differences in the coumestrol content of a number of medic species and v a r i e t i e s grown under similar conditions. Wide variations in the estrogenic content of 56 strains of alfalfa, all harvested in the bud stage, have been reported by Stob and co-workers (*211*). Ladino clover has been shown to contain a moderate amount of coumestrol (*37*, *122*), w h i l e strawberry (*105*), red (*222*) white (*105*), and subterranean clovers (*183*) and *Trifolium nigrescens* (*105*) contain only small amounts.

Hanson and co-workers (*127*) found that nearly all of the variation in coumestrol content of five alfalfa varieties in the one-tenth bloom stage was nongenetic and t h e r e f o r e concluded that breeding only for high or low coumestrol content did not appear promising. However, the extent to which coumestrol content can be changed by selection depends also on variation among individual plants, which was not m e a s u r e d. Stuthman and co-workers (*213*) studied the coumestrol content of four alfalfa clones that previously had been reported to differ in estrogenic activity. C o u m e s t r o l content

ranged from 6 to 25 p.p.m., while estrogenic activity ranged from 106 to 182 percent of the control. The alfalfa clone with the lowest estrogenic activity contained the least coumestrol, while the clone with the greatest estrogenic activity contained the most coumestrol. H o w e v e r, the coumestrol concentration d i d not coincide with the estrogenic activity for the intermediate clones. With six clones, including the above four, they conducted crossbreeding experiments (*214*). U s i n g estrogenic activity as a guide, they concluded (on the basis of these six clones) that it should be possible to develop alfalfa of either high or low estrogenic activity.

An e x t e n s i v e study on the magnitude and nature of the variability of coumestrol content of alfalfa (*125, 126, 127*) confirmed the earlier observations that coumestrol content is influenced by stage of growth, variety, and location. For this study, five varieties, Buffalo, Du Puits, Lahontan, Ranger, and Vernal, repre-s e n t a t i v e of the germ plasm found in principal alfalfa varieties grown in the United States, were grown in replicated plots in California, Iowa, K a n s a s, Ne-

braska, North Carolina, Pennsylvania, and Utah. More than 99 percent of the plot variation was estimated to be environmental.

The most significant correlation between variables and coumestrol was between defoliation scores and coumestrol content.

Variations as related to disease

While studying estrogenic activity in white clover, Bickoff and co-workers (37) found that a virus-infected sample of the Louisiana 6 strain contained considerably more estrogenic activity than the comparable virus-free sample (table 19). The high level of activity in the diseased sample suggested a possible correlation between estrogenic activity and virus infection. Later, based on the results of their extensive studies, Hanson and co-workers (127) concluded that most of the differences attributed to the other variables could result, for the most part, from their relationship with disease. Thus, the trend toward increased coumestrol content with successive stages of growth is consistent with the common observation that disease incidence increases with advancing maturity. Some

of the differences in coumestrol content among varieties can be explained by varietal differences in resistance to foliar pathogens. Lahontan, for example, had the highest coumestrol content, the greatest leaf loss, and was the most susceptible to foliar disease. DuPuits and Vernal, the most resistant of the varieties, had the lowest coumestrol content. Similarly, coumestrol was lowest in forages grown in California and Utah. Incidence of foliar disease in these two States is generally lower than in the other States studied. Bennett and co-workers (18) in Australia have consistently found very low levels of coumestrol (usually less than 5 p.p.m.) in alfalfa free of obvious signs of pathogenic attack.

The effect of fungicidal control of foliar diseases on the accumulation of coumestrol was also

TABLE 19.—*Varietal distribution of estrogenic activity in white clover harvested at Clemson, S. C., 1957*

Clover line	Date of harvest	Flower density	Coumestrol[1]
		No. per square foot	*Parts per million*
269	May 6	10	0
2682	May 6	15	0
4304	May 7	12	0
4306	May 8	10	7
Louisiana 21	May 9	9	3
Louisiana 6 (virus-free)	May 8	6	13
Louisiana 6 (with virus)	May 9	2	105

[1] Determined by mouse-uterine-weight bioassay.

Source: Bickoff, E. M., Livingston, A. L., Booth, A. N., and others (37).
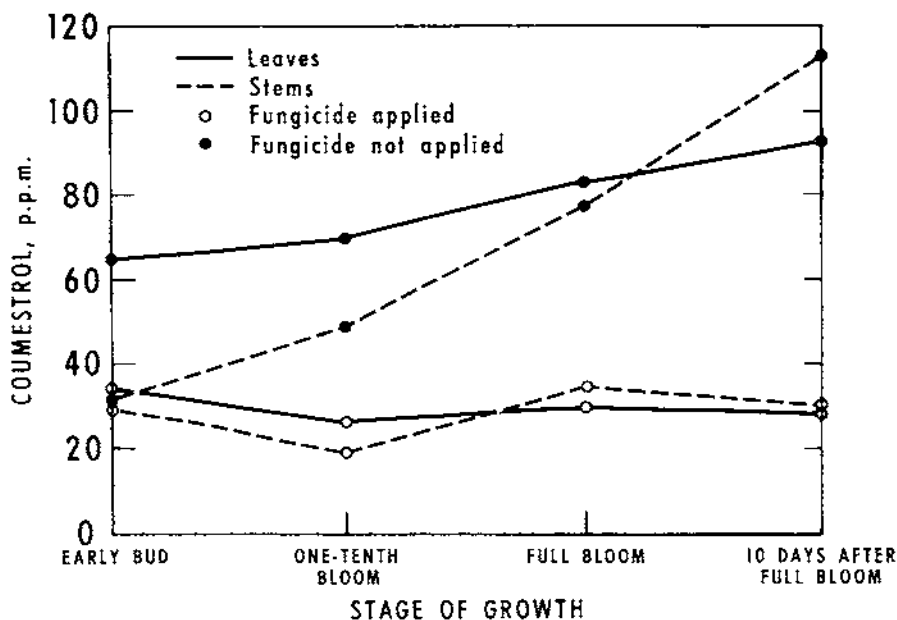
FIGURE 21.—Coumestrol content of leaves and stems of alfalfa at four stages of growth, with and without fungicide application to control foliar diseases (127).

s t u d i e d (127). The coumestrol c o n t e n t of alfalfa grown in sprayed plots was found to be relatively low and constant over all stages of growth (fig. 21). In contrast, coumestrol l e v e l s increased with successive stages of growth in alfalfa grown in unsprayed plots. On the last samp l i n g date, average coumestrol content of leaves and stems from sprayed plots was 29 p.p.m. as compared with 103 p.p.m. for unsprayed plots, indicating that the increased coumestrol content was primarily due to increased infection by foliar pathogens. Simultaneous controlled inoculation experiments (127), in which greenhouse and field-grown a l f a l f a plants were infected with each of four fungi and one virus, substantiated these results. In general, infection caused a marked increase in coumestrol content of the plants (table 20).

In studies with alfalfa grown under controlled environmental conditions, the coumestrol content was found to i n c r e a s e sharply after the foliage was infected with either Pseudopeziza medicaginis (43, 164) or Leptosphaerulina briosiana (164), two leaf spotting organisms (t a b l e 21). Significant amounts of coumestrol were present only in infected tissue, and the level of coumestrol was positively correlated with the number of lesions and the severity of i n f e c t i o n. Further studies (165) confirmed that coumestrol accumulated in i n f e c t e d leaflets and was very high in the necrotic lesions. In lesions with a radius of 2.5 millimeters or less, 85 percent of the coumestrol was located within a

TABLE 20.—*Effect of five foliar pathogens on coumestrol content of alfalfa in greenhouse experiments, 1963*

Pathogen	Alfalfa variety or clone	Stage of growth	Part of foilage analyzed	Coumestrol content	
				Inoculated with pathogen	Healthy check
				Parts per million	*Parts per million*
Phoma herbarum var. *medicaginis*	Buffalo	Prebud	Whole	{ 219.1 181.9	{ 0 1.4
Pseudopeziza medicaginis	do	One-quarter bloom	do	{ 59.5 74.2	{ 0 0
	Vernal	Prebud	do	{ 33.2 48.4	{ 0 0
	Clone R–5	Full bloom	do	9.1	2.9
Leptosphaerulina briosiana	Vernal	Prebud	do	{ 0 0	{ 0 0
	Clone R–5	Late bud	do	30.9	0
	do	do	Leaves	84.9	0
	do	do	Stems	0	0
Stemphylium botryosum	Buffalo	Prebud	Whole	{ 30.0 41.5	{ 0 0
Yellow mosaic virus	Clone R–5	One-half bloom	do	{ 0 0	{ 0 0
	Ranger	One-tenth bloom	do	29.5	19.0
	do	do	Leaves	32.7	----------
	do	do	Stems	18.8	----------

Source: Hanson, C. H., Loper, G. M., Kohler, G. O., and others (*127*).

TABLE 21.—*Coumestrol content of the leaves and stems of Ranger alfalfa infected with two foliar pathogens*

Pathogen	Description of tissue analyzed	Coumestrol
		Parts per million
Pseudopeziza medicaginis	Leaves with two or more lesions per leaflet	183.7
	Leaves with one lesion per leaflet	40.4
	Stems (no lesions apparent)	5.7
Healthy check	Leaves	1.1
	Stems	.6
Leptosphaerulina briosiana	Leaves heavily infected [1]	71.7
	Leaves lightly infected [2]	28.5
	Stems with occasional lesions	7.2
Healthy check	Leaves	1.0
	Stems	.5

[1] Lesions necrotic or yellow.
[2] Leaves with either few large or many small lesions that were not yellow.
Source: Loper, G. M., and Hanson, C. H. (*164*).

radius of 1.0 mm. from the center of the lesion. The portion of the leafspot within a 0.5-mm. radius contained as high as 2,600 p.p.m. coumestrol, while only t r a c e s (less than 3 p.p.m.) were found in the healthy-appearing tissue between lesions.

Common - leafspot (*Pseudopeziza medicaginis*) infection has also been shown to cause the accumulation of other coumestans and of flavones in alfalfa (*43*). The concentrations of these compounds increased several-fold as the stage of infection increased (table 22). The coumestan, sativol, increased the most with increased disease development. These phenolics reached higher concentrations in the Atlantic variety, which is more susceptible to common leafspot than the Cayuga variety. This is in agreement with the findings of Loper and co-workers (*165*) that recurrent selection of alfalfa for increased resistance to common leafspot resulted in fewer leafspots and lower coumestrol content.

Francis (*104*) reported t h a t leaf rust could produce a threefold to fourfold increase in the coumestrol content in the leaves of common burr medic (*Medicago polymorpha*). Smart and Sherwood [a] showed that the coumestrol content of rust-infected alfalfa leaflets was inversely proportional to level of resistance and that the urediospores of the rust pathogen contained up to 868 p.p.m. coumestrol. Studies on alfalfa (*165*) infected with *Uromyces striatus*, the alfalfa rust fungus, showed that in all but the most susceptible plants coumestrol content w a s positively associated with lesion size and number. This relationship could not be shown for the most susceptible plants because of the massive discharge of urediospores that contained over 400 p.p.m. coumestrol.

The interrelationship between coumestrol levels and disease is

[a] Listed as a private communication of W. W. G. Smart and R. T. Sherwood in (*165*) of Literature Cited.

TABLE 22.—*Coumestan and flavone levels in two alfalfa varieties as affected by infection stage of common leafspot pathogen*

[E. I. = early infection stage; L. I. = late infection stage]

Alfalfa variety and days after inoculation	Sativol	4',7-Dihydroxy flavone	Trifoliol	Medicagol and 4'-O-methyl-coumestrol	3'-Methoxy-coumestrol	Coumestrol	Leafspot infection score [1]
	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.	
Atlantic:							
Check	([2])	([3])	([2])	([2])	([2])	2	0.0
12 (E. I.)	17	4	14	4	([2])	49	1.5 ± 0.27
18 (L. I.)	191	20	48	35	1	219	4.5 ± .60
Cayuga:							
Check	([2])	([3])	([2])	([2])	([2])	2	.0
12 (E. I.)	8	3	9	4	([2])	50	1.7 ± .27
18 (L. I.)	68	5	20	6	([2])	137	3.6 ± .60

[1] Scored from 0 to 6; 0 = no evidence of infection, 6 = heavily infected. Values are means ± standard error.
[2] Undetected.
[3] Less than 1 p.p.m.
Source: Bickoff, E. M., Loper, G. M., Hanson, C. H., and others (43).

not limited to bacterial and fungal diseases. Infestation by pea aphids and spotted alfalfa aphids has been f o u n d to cause the buildup of coumestrol (127, 163). The aphid damaged portions of the plants contained elevated levels of coumestrol, while undamaged plant t i s s u e and uninfected plants contained less than 1 p.p.m. In addition, the more aphid-resistant alfalfa varieties accumulated less coumestrol than the susceptible ones. Loper (162)

demonstrated t h a t coumestrol concentrations increased in the leaves of barrel medic (Medicago littoralis Rhode) with the incidence of physiogenic leaf spotting. The spots were considered to be physiogenic because they were free of pathogenic f u n g i and bacteria. High levels of coumestrol were found in the affected leaves (up to 2,362 p.p.m.), with the major amount concentrated in the spotted areas, i.e., the damaged tissue.

Mechanism of biosynthesis

Disease resistance in plants has been correlated with the metabolism of phenolic compounds (128), whose occurrence at infection and wound sites suggests that they are associated with the plant's resistance mechanism. The similar effects of foliar pathogens, aphid infestation, and physiogenic leaf spotting on the accumulation of coumestrol suggest that disease triggers some nonspecific mechanism in the plant that causes the rapid accumulation of this type of compound.

Although the biosynthetic pathway of coumestrol in the alfalfa-pathogen relationship is unknown, it has been shown that its biosynthesis in healthy plants is related to that of the flavones

and isoflavones. These compounds are synthesized in plants through a combination of two pathways responsible for aromatic ring formation (121). One route involves a poly-β-keto-acid intermediate produced by head to tail condensation of acetate units. The other route, which is responsible for coumarin biosynthesis (61, 146, 220), involves the intervention of C_n-intermediates associated with the shikimic-prephenic acid pathway.

Detailed studies on the biosynthesis of flavones, isoflavones, and coumestrol have been made by Grisebach and co-workers (116, 117, 118, 121). Acetic acid was shown to be a very effective source of the C_6-units comprising the A-ring of flavones (129,
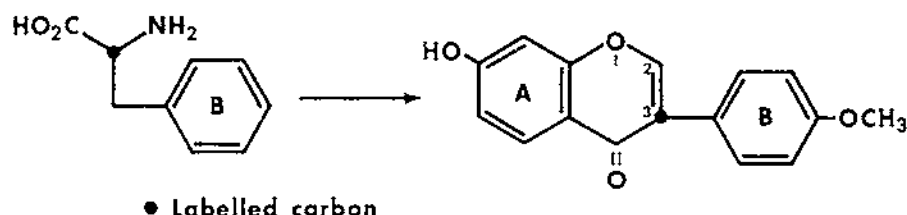

• Labelled carbon

FIGURE 22.—Incorporation of labeled phenylalanine into the B-ring of formononetin (121).
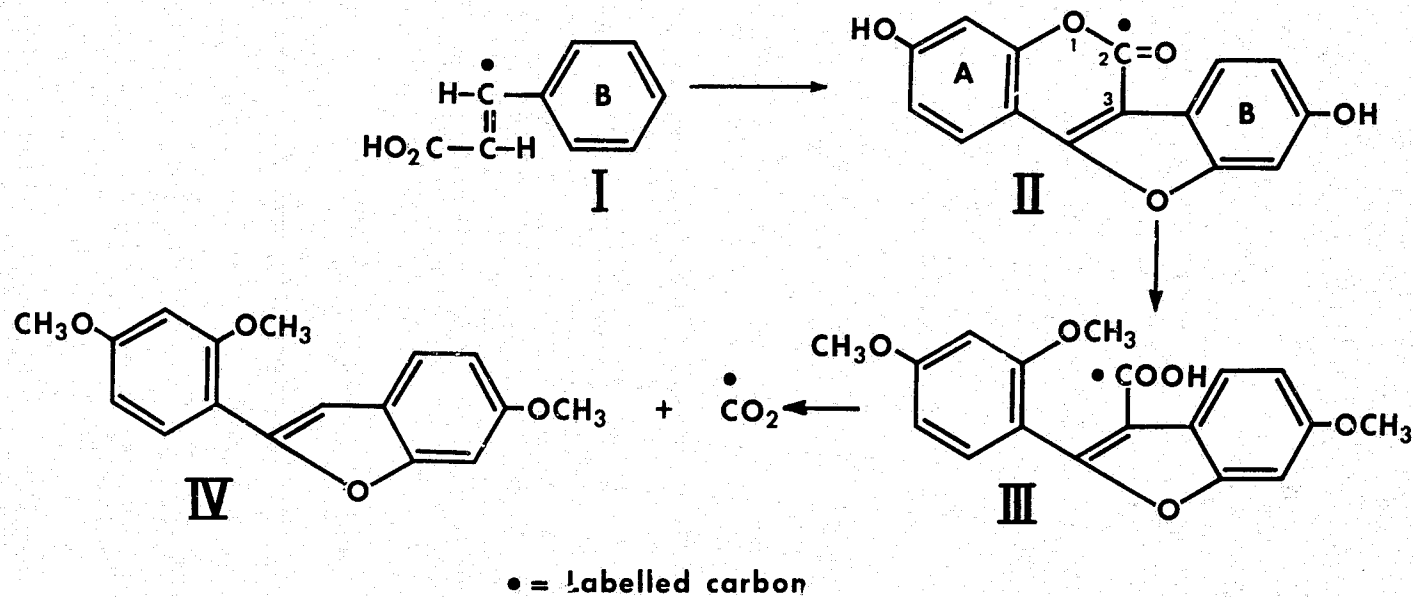
● = Labelled carbon

FIGURE 23.—Incorporation of labeled cinnamic acid into the lactone ring of coumestrol (117).

202). The remaining C_9-units, which are responsible for the formation of the B-ring (fig. 22), are formed from precursors such as shikimic acid, phenylalanine, and cinnamic acid.

The isoflavones are synthesized in the plant in a manner similar to the flavones, except that an aryl migration occurs. Grisebach and Doerr (*115, 119, 120*) have studied this migration in the biosynthesis of formononetin by red clover. Using labeled phenylalanine-2-¹⁴C as the starting material for the biosynthesis, they found that the formononetin produced by the plant was labeled at the 3-position instead of the 2-position (fig. 22). This could only be explained by the migration of the B-ring from the 2-carbon to the 3-carbon at some stage during the biosynthesis of formononetin.

According to Grisebach and Barz (*117, 118*), if coumestrol were biosynthesized in a manner similar to the coumarins, aromatic ring A and the three carbon atoms of the lactone ring would arise from an intact phenylpropane unit and the B-ring from either acetate or glucose (fig. 23). If, on the other hand, the biosynthesis were analogous to that of the isoflavones, the A-ring would arise from the condensation of three acetate units, while the B- and lactone rings would arise from a phenylpropane unit. Also, a rearrangement involving an aryl migration would occur at some point during

the course of the biosynthesis. Preliminary feeding experiments showed that acetic acid-(1-¹⁴C) and D,L-phenylalanine-1-¹⁴C were incorporated into coumestrol (*117*). Experiments with cinnamic acid-(3-¹⁴C) provided unequivocal proof for the occurrence of an aryl migration (*118*). The labeled carbon was located by degradation of coumestrol to its benzofuran derivative (fig. 23) in the manner described under "Characterization of Coumestans." Decarboxylation of the acid (fig. 23, III) to the benzofuran (fig. 23, IV) revealed that the total radioactivity had been located in the carbonyl group of the lactone ring, i.e., the 2-position. If a migration had not occurred, the labeled carbon would have been located in the 3-position. Therefore, coumestrol must be biogenetically related to the isoflavones rather than the coumarins.

Grisebach (*115*) proposed a series of reactions to show how the isoflavones and coumestrol could be synthesized in the plant (fig. 24). This relationship was proved by feeding 2',4,4'-trihydroxychalcone-4'-glucoside (β-¹⁴C) to alfalfa plants (*116*). After 72 hours, labeled coumestrol, daidzein, and formononetin were isolated from an ether extract of the dried roots following the scheme shown in figure 25. Thus, the chalcone shown in figure 24 is a common intermediate for the biosynthesis of these compounds in alfalfa.

Utilization of Coumestrol by the Animal

Interest in naturally occurring plant estrogens stems from their effects on reproduction in farm animals. These compounds may cause an estrogenic response, an infertility syndrome, or effect the
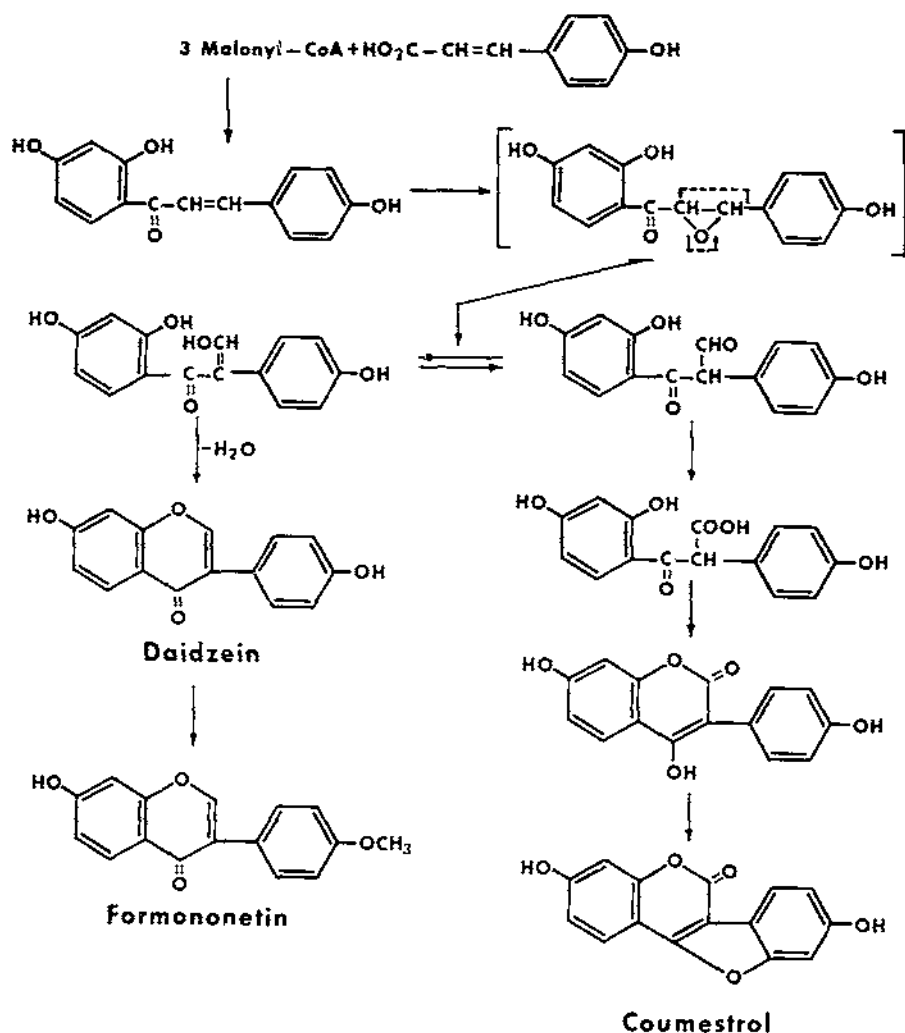
FIGURE 24.—Proposed biosynthetic pathway for the isoflavones and coumestrol in plants (*115*).

growth rate of animals. In an attempt to understand these effects, the metabolic role of these compounds has been studied. This present discussion is primarily concerned with studies on plants in which coumestrol may be the active constitutent as well as on coumestrol itself.
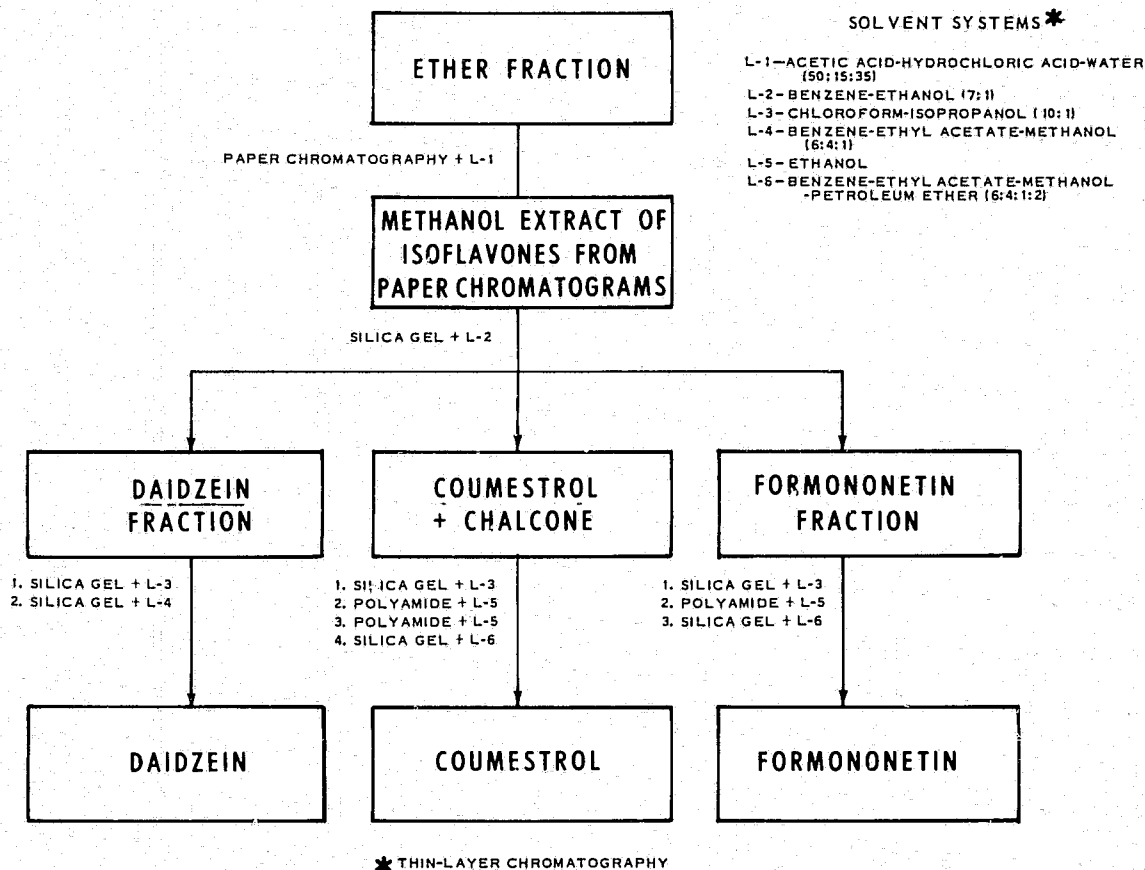
FIGURE 25.—Schematic flowsheet for the isolation of coumestrol, daidzein, and formononetin from dried alfalfa roots (116).

The figure contains the following text elements:

ETHER FRACTION

SOLVENT SYSTEMS *

L-1—ACETIC ACID-HYDROCHLORIC ACID-WATER
(50:15:35)
L-2—BENZENE-ETHANOL (7:1)
L-3—CHLOROFORM-ISOPROPANOL (10:1)
L-4—BENZENE-ETHYL ACETATE-METHANOL
(6:4:1)
L-5—ETHANOL
L-6—BENZENE-ETHYL ACETATE-METHANOL
-PETROLEUM ETHER (6:4:1:2)

PAPER CHROMATOGRAPHY + L-1

METHANOL EXTRACT OF ISOFLAVONES FROM PAPER CHROMATOGRAMS

SILICA GEL + L-2

DAIDZEIN FRACTION

COUMESTROL + CHALCONE

FORMONONETIN FRACTION

1. SILICA GEL + L-3
2. SILICA GEL + L-4

1. SILICA GEL + L-3
2. POLYAMIDE + L-5
3. POLYAMIDE + L-5
4. SILICA GEL + L-6

1. SILICA GEL + L-3
2. POLYAMIDE + L-5
3. SILICA GEL + L-6

DAIDZEIN

COUMESTROL

FORMONONETIN

* THIN-LAYER CHROMATOGRAPHY

Metabolism

Although a considerable number of reports have been published in recent years on the metabolism of forage estrogens, in an attempt to clarify their role in the infertility syndrome, most of these studies have been conducted with the estrogenic isoflavones. Metabolic studies on the isoflavones have been recently reviewed by Bickoff (20).

It has been suggested (22, 99) that plant estrogens are proestrogenic in character, i.e., they are changed into more active forms in the animal body. For example, methylated coumestans or isoflavones might be demethylated to more "potent" compounds.[7] If this is true, this raises the question of the nature of the compounds formed, as well as the site at which this change occurs. It is assumed that the main process of activation and inactivation of the plant estrogens takes place in the liver (137, 168). This creates difficulties in investigation, and the processes involved may differ considerably between laboratory and farm animals. At the same time, additional problems arise from the presence of substances in plants, as yet largely unknown, which may increase or diminish the potency of forage estrogens (27, 36).

Lindner (155) studied the presence of the forage estrogens in the plasma and depot fat of sheep and was able to detect trace amounts of coumestrol in the plasma of ovariectomized ewes grazing on estrogenic clover pastures that contained trace amounts of coumestrol. Adler and co-workers (9) found both free and conjugated coumestrol in the blood of cows that were fed a ration containing estrogenic alfalfa meal. Two groups of animals received 2.56 and 4.8 mg. coumestrol per day, respectively. The concentration of free coumestrol was higher in the blood of the cows on the higher coumestrol ration, while the conjugated coumestrol level was similar in both groups (table 23).

Estrogenic isoflavones are extensively degraded by the animal to simple acids and phenols. Equol is one of the major detectable metabolites. Taken together, all of these metabolites account for only a small percentage of the original quantity of isoflavone consumed (20). Adler [8] was unable to find any coumestrol in the urine of mice fed coumestrol. Braden [9] was unable to find any obvious metabolic products in the urine or blood plasma of sheep given coumestrol intraruminally. Cayen and Common (63) injected tritiated coumestrol intramuscularly into nonlaying hens and reported that the radioactivity in the phenolic extracts of the urine was distributed between coumestrol and unidentified conversion or breakdown products. As they were unable to detect any radioactive equol in these extracts, they concluded that coumestrol must be degraded in the fowl along pathways quite different from those along which the isoflavones are degraded. Their find-

[7] Personal communication from David Bennett, Commonwealth Scientific and Industrial Research Organization, Australia, 1967.

[8] See footnote 2, page 2.

[9] Personal communication from A. W. H. Braden, Commonwealth Scientific and Industrial Research Organization, Australia, 1967.

TABLE 23.—*Fluorometric determination of free and
conjugated coumestrol in whole bovine blood*

Form	Coumestrol concentration	
	High coumestrol ration	Low coumestrol ration
	Micrograms/liter	*Micrograms/liter*
Free coumestrol _____	1.21	0.35
Conjugated coumestrol _____	.50	.55
Total _____	1.71	.90

Source: Adler, J. H., Weitzkin, G., and Marinov, U. (*9*).

ings are very interesting when considered in the light of evidence that the biosynthetic pathway of coumestrol in alfalfa resembles that of the isoflavones (*113*). This difference in metabolic routes for coumestrol and the isoflavones may account for the much higher relative estrogenic potency of coumestrol (*39, 57*).

Lyman and Krueger (*168*) were unable to show any effects due to coumestrol on plasma lipid distribution or on the glyceride fraction of the cholestrol-induced fatty liver of either normal or castrated male rats. Noteboom and Gorski (*187*) found in ovariectomized rats that coumestrol stimulated the incorporation of labeled precursors into protein, phospholipid, and ribonucleic acid in uterine cells and also increased the acid soluble uridine triphosphate in the uterus. The pattern of metabolic stimulation was similar to that of estradiol, differing only in the degree of stimulation. Since the metabolic responses studied were indicative of typical estrogen activity, Noteboom and Gorski considered them to be sufficient to establish that coumestrol was similar to estradiol in its action on metabolic pathways in the uterus. Furthermore, their observation that a combination of estradiol and coumestrol gave no additive effects supported the hypothesis that forage estrogens perform the same function as natural estrogens in triggering anabolic responses.

Estrogenic potency

Until recently, the most commonly used method for quantitative bioassay has been the measurement of increase in uterine weight of immature female mice. However, difficulties have often been encountered when attempting to correlate results obtained with mice and other laboratory animals with the results obtained under field conditions with farm animals (*184*). Consequently, a number of workers, particularly in Australia, (*17, 18, 57, 104*) have gone directly to sheep as the test animal. Criteria for estrogenic response in sheep include milk secretion (*76, 172*), teat length (*58, 182*), and vaginal and uterine increases (*17, 57, 149*).

TABLE 24.—*Dose-response data and relative potency of forage estrogens vs. diethylstilbestrol and estrone*

Compound	Quantity fed per mouse	Uterine weight ± S.E. [1]	Quantity to produce 25-mg. uterus	Relative potency [2]
	Micrograms	*Milligrams*	*Micrograms*	
Control	0	9.6 ± 0.3	....	....
Diethylstilbestrol	.025	11.8 ± .2		
	.050	18.1 ± .7		
	.075	23.7 ± .8	0.083	100,000
	.100	29.2 ± .4		
	.200	78.1 ± 6.2		
Estrone	.50	14.7 ± .9		
	.75	16.0 ± 1.3		
	1.00	23.8 ± 2.5	1.20	6,900
	1.50	36.1 ± 2.3		
	2.00	45.3 ± 6.7		
Coumestrol	100	13.8 ± .2		
	200	24.2 ± .2		
	300	29.2 ± 1.3	240	35
	400	40.7 ± 1.9		
	500	76.0 ± 6.0		
Coumestroldiacetate	250	19.0 ± 1.2		
	300	22.3 ± 1.6		
	400	26.7 ± 1.4	340	24
	500	42.4 ± 3.3		
	1,000	87.0 ± 6.2		
Genistein	5,000	19.4 ± .9		
	7,500	28.0 ± 5.0		
	8,000	27.0 ± 1.6	8,000	1.00 [3]
	12,000	32.4 ± 1.9		
	15,000	36.6 ± .6		
	20,000	52.7 ± 1.9		
Daidzein	5,000	17.3 ± 1.3		
	7,500	18.5 ± 2.6		
	10,000	24.8 ± 1.3	11,000	.75
	15,000	31.2 ± 1.4		
Biochanin A	10,000	20.3 ± 2.5		
	20,000	27.9 ± 2.1	18,000	.46
	30,000	27.9 ± 2.6		
	40,000	45.5 ± 3.8		
Formononetin	15,000	16.8 ± .8		
	20,000	17.9 ± 1.4		
	25,000	23.2 ± 1.0	32,000	.26
	30,000	27.5 ± 2.3		
	40,000	26.1 ± 2.0		

[1] S.E. = standard error.
[2] Measured at the dosage required to produce a 25-mg. uterus.
[3] Genistein was arbitrarily assigned a value of 1.00.
Source: Bickoff, E. M., Livingston, A. L., Hendrickson, A. P., and Booth, A. N. (*39*).

Bickoff and co-workers (39) compared the estrogenic potencies of orally fed coumestrol, genistein, daidzein, biochanin A, formononetin, diethylstilbestrol, and estrone by the mouse-uterine-weight bioassay method (table 24). For coumestrol, estrone, and diethylstilbestrol, the logarithms of the uterine weights were linear with dosage. With the isoflavones, however, a linear relationship existed between the mean uterine weight and dosage. To obtain a uniform basis of comparison, relative potencies were based on the dosage required to produce a uterine weight of 25 mg. These were determined from dosage-response curves, such as those that can be plotted from the data given in table 24. Coumestrol was found to be from 30 to 100 times more potent than the isoflavones under the conditions of the assay. Estrone and diethylstilbestrol were respectively about 200 and 3,000 times more active than coumestrol.

Booth and Bickoff [10] observed that the potency of coumestrol relative to diethylstilbestrol was about the same, whether both were administered orally or subcutaneously. However, estrone was more potent subcutaneously, being about 1,000 times more potent than coumestrol (table 25). Pincus and Merrill [11] also compared coumestrol with estrone administered subcutaneously and obtained results similar to the above.

Leavitt (151) reported that, by slope-ratio analysis, estradiol administered subcutaneously was 33,000 times more active than coumestrol administered orally on the uterus of ovariectomized mice and 120,000 times more active in blocking pituitary gonadotropic function.

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[10] Booth, A. N., and Bickoff, E. M. Unpublished data.
[11] Personal communication from Gregory Pincus and A. P. Merrill, The Worchester Foundation for Experimental Biology, Massachusetts, 1968.

TABLE 25.—*Dose-response data for coumestrol, estrone, and diethylstilbestrol injected subcutaneously in corn oil over a 4-day period*

Compound	Total dose	Uterine weight ± S.E.[1]
	Micrograms	*Milligrams*
Control (basal ration plus corn oil)	.	15.10 ± 1.25
Coumestrol	200	22.80 ± 2.12
	300	40.04 ± 5.69
Estrone	.1	19.32 ± 2.11
	.2	23.00 ± 2.78
Diethylstilbestrol	.05	34.48 ± 4.54
	.1	39.84 ± 2.54

[1] S.E. = standard error.
Source: Booth, A. N., and Bickoff, E. M. Unpublished data.

As early as 1959, Jennings and Dow (*130*) suggested employing sheep as the test animals since they are highly sensitive to clover estrogens. They reported that uterine and vaginal morphology of sheep on estrogenic pastures differed from that of sheep maintained on other pastures. Oldfield and co-workers (*194*) reported that after 3 weeks on test diets, growing lambs fed a high coumestrol alfalfa meal (119 p.p.m.) showed increased udder and vulval development over lambs fed a low coumestrol meal (22 p.p.m.). It was even possible to draw milk from some of the ewe lambs on the high coumestrol diet. Using arbitrarily assigned values for progressive udder development, three independent scorers evaluated the ewe lambs at 1.25 on the high meal and 0.52 on the low meal. Similar evaluation for vulva development gave values of 2.04 and 1.07 for the high and low meals, respectively.

In measuring the estrogenic activity of annual medic pastures, Francis and Millington (*104*) found increases in teat length of wethers to correlate with coumestrol content. The most active varieties produced teat lengths equivalent to those resulting from injections of 8 micrograms of diethylstilbestrol daily (table 26). If coumestrol alone were responsible for the activity of the pastures, then the potency of injected diethylstilbestrol for wether sheep was about 8,500 times as great as that of orally administered coumestrol. This value may be compared to the 3,000-fold increase obtained

by mouse-uterine weight bioassay (*39*).

Braden and co-workers (*57*) used the increase in uterine weight of ovariectomized ewes to compare estrogenic potencies of coumestrol, genistein, biochanin A, and formononetin. The activity of these compounds in relationship to diethylstilbestrol varied depending on the mode of administration (table 27). Their results indicated that, when given parenterally, synthetic genistein, biochanin A, and coumestrol exhibited estrogenic activities in sheep comparable with those observed in mice (*39, 49, 223*). When administered intramuscularly, however, diethylstilbestrol was about 100,000 times more active than genistein and biochanin A, while coumestrol was about 100 times more active than these two isoflavones. The negligible activity of formononetin either intraperitoneally or intramuscularly was thought to be due possibly to its low solubility, although when injected intraruminally, it had estrogenic activity of the same order of magnitude as genistein and biochanin A. Coumestrol was still some 15 times more active intraruminally than the isoflavones. These results are summarized in table 27.

Despite lower potencies of the forage estrogens when compared with diethylstilbestrol, they are present in certain forages in sufficient quantities to be influential in animal nutrition and physiology. Furthermore, the less potent isoflavones are present in Australian subterranean clover (*56*) in sufficient quantities to cause reproductive problems in sheep.

TABLE 26.—*Response in wether teat length to intake of green medic and injection of diethylstilbestrol*

Medic variety	Mean coumestrol content of medic ± standard error [1]	Coumestrol consumed during 9-day test	Mean teat length increase	Diethyl-stilbestrol
	Parts per million	*Milligrams per sheep*	*Millimeters*	*Micrograms per day*
M. *littoralis*	132.0 ± 25	714	2.90	
M. *truncatula* (Cyprus)	104.0 ± 24	617	2.73	
M. *truncatula* (commercial)	101.0 ± 24	519	1.94	
M. *truncatula* (Mtr 173)	25.8 ± 5.4	172	.89	
M. *sativa*	15.8 ± 5.0	90	.29	
M. *polymorpha*	11.4 ± 1.1	56	− .13	
M. *scutellata*	9.2 ± 1.9	50	− .25	
Control, oats		...	− .54	0
		...	2.12	4
		...	2.65	8

[1] Dry-weight basis.

Source: Francis, C. M., and Millington, A. J. (*104*).

Antifertility effects

The estrus cycle of female animals is initiated when the brain, via the hypothalamus, stimulates the pituitary gland to secrete the gonadotropic hormones. Each of these hormones regulates specific functions in the female animal. The follicle-stimulating hormone (FSH) influences the development of the ovaries during puberty and later acts directly on them in promoting the development and maturation of the follicles. The interstitial cell-stimulating or luteinizing hormone (LH) depresses the first part of the ovarian cycle and encourages the second or luteal phase, thereby causing the uterine changes associated with pseudopregnancy or with gestation. Under its influence, the follicles (ova surrounded by a sac of follicular cells) produce estrogen, ovulation occurs, i.e., the follicles rupture and eggs are released, and the follicular cells become the corpus luteum; The corpus luteum secretes progesterone, which prepares the uterine lining for implantation. The processes of the uterus are regulated by estrogens. The naturally occurring estrogens are estradiol, estrone, and estriol. They are primarily responsible for the regular course of the estrus cycle. Upon fertilization and implantation, the estrogens and progesterone inhibit the secretion of FSH and LH, thereby preventing ovulation.

The estrus cycle is regulated by the alternating stimulation of the ovary by the pituitary gland hormones and the stimulation of

TABLE 27.—*Estrogenic activity of isoflavones and coumestrol in ovariectomized ewes*

[i/r = intraruminally; i/m = intramuscularly; i/p = intraperitoneally]

Treatment	Total dose in 4 days	Route	Mean uterine weight
			Grams
Control	......	.....	11.7
Genistein .... grams	9	i/r	[1]13.3
	1	i/m	[1]21.4
	3	i/m	[1]21.6
Diethylstilbestrol ..... micrograms	15	i/m	[1]22.3
	45	i/m	[1]20.7
Control	......	.....	12.2
Formononetin .... grams	1	i/m	12.9
Genistein .... do	6	i/r	12.6
	0.5	i/m	[1]16.4
Control	......	.....	14.7
Biochanin A .... grams	20	i/r	15.3
	1	i/m	[1]18.4
Control	......	.....	5.9
Diethylstilbestrol ..... micrograms	10	i/m	7.1
	20	i/m	[1]14.3
Biochanin A .... grams	24	i/r	[1]13.4
Genistein .... do	20	i/r	[1]16.4
Control	.....	.....	10.4
Formononetin .... grams	1	i/p	10.9
	24	i/r	[1]17.4
Diethylstilbestrol ... micrograms ...	15	i/m	[1]14.5
	30	i/m	[1]22.6
Control	......	.....	7.8
Diethylstilbestrol ... micrograms	20	i/m	[1]15.2
Coumestrol ...... grams	1.4	i/r	[1]14.0
	0.03	i/m	[1]18.6
	0.10	i/m	[1]19.3
Control	......	.....	6.2
Coumestrol ...... grams	.012	i/m	[1]13.9
Biochanin A .... do	1	i/m	[1]14.0
Diethylstilbestrol ..... micrograms .	15	i/m	[1]13.9
	30	i/m	[1]17.6

[1] From Fisher's one-sided test, these values are found significantly greater ($P < 0.05$) than the control values.

Source: Braden, A. W. H., Hart, N. K., and Lamberton, J. A. (57).

the pituitary gland by the ovarian hormones. Factors that contribute to either higher or lower than normal hormone levels can interfere with this cycle. These antifertility factors may function by inhibiting the secretion of, or response to, the endogenous animal estrogens or other hormones effecting reproduction. They may block ovulation and stimulate pseudopregnancy or cause anovulatory estrus, as in the case of birth control pills. They may also effect the secretion mechanism in the Fallopian tubes, thereby effecting egg movement; change the uterine lining in such a manner as to prevent implantation; render the

mucus composition in the cervix unfavorable to sperm transport; or cause a degenerate effect on the reproductive organs.

Hyperestrogenic syndrome

Excessive estrogen stimulation caused by long periods of grazing estrogenic pastures is well established. However, most of these studies have dealt with red and subterranean clovers, wherein the isoflavones are the predominant estrogens and coumestrol is either absent or present in very small quantities. Reviews (20, 185) covering this work are available. Relatively few cases of reproductive disorders attributable to estrogenic alfalfa and Ladino clover have been reported. In these cases, coumestrol is undoubtedly the causative agent.

As early as 1952, Foltin (102), in Israel, suspected that a relationship existed between heavy alfalfa feeding and seasonal sterility in cattle. Similar reports began to appear in this country. For example, in one small dairy herd in New England,[12] 19 out of 32 cows were treated for cystic ovaries one or more times during a single year. Six of the cows had to be treated from four to six times. Since most of the cystic cows were not in "heat," it was suggested that the alfalfa hay fed the animals might contain enough estrogens to interfere with the estrus cycle. Adler and Trainin (6, 7, 8, 216), in Israel, were the first to clearly associate reproductive problems in dairy cattle with the feeding of large quantities of alfalfa. They reported a hyperestrogenic syn-

drome in the cattle, which was characterized by irregular estrus cycles, cystic ovaries, and decreased fertility. Other hyperestrogenic effects included precocious mammary and genital development in heifer cows. A study of these disturbances, in relation to the feeding program, revealed that their appearance roughly coincided with the availability of fresh alfalfa. In some cases, the alfalfa was found to contain as much as 52 micrograms equivalents of estradiol per kg. (on a dry-weight basis). After alfalfa was excluded from the rations of two different dairy herds, there was an increase in conception rates and a decrease in the frequency of cystic ovaries. Later studies by Lotan and Adler (166) confirmed that increased feeding of estrogenic alfalfa increased the percentage of cows with irregular estrus cycles and decreased conception rate.

Recently Ayalan,[13] also in Israel, isolated coumestrol from alfalfa fed to a herd of dairy cattle with complaints of infertility. Adler [14] has since found coumestrol as well as 4'-O-methylcoumestrol, daidzein, and genistein in pea silage fed to dairy cattle experiencing cystic ovaries and irregular heat cycles.

Dairy cattle fed Ladino clover hay have sometimes been found to require more services per conception than comparable animals fed timothy hay.[15] Ochi and co-workers (188) reported a

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study on reproductive conditions of dairy cows fed Ladino clover. In the animals on clover, estrus seemed to recur earlier after parturition. More cows showed distinct signs of estrus and a proper duration of the estrus period than animals not fed clover. Even though no particular changes were observed in their estrus cycle, the occurrence of reproductive disorders was a little more frequent and the rate of conception a little lower in cows fed clover. These trends were especially pronounced in animals fed more than 30 kg. Ladino per day.

Engle and co-workers (98) studied the reproductive performance of ewes during three grazing, breeding, and lambing seasons. Ewes grazed on Ladino clover came into heat and conceived an average of 21.7 days later than ewes grazed on bluegrass all season. They also required more services per conception and underwent longer lambing seasons. Based on mouse-uterine-weight bioassay, the Ladino clover was found to be estrogenically active while the bluegrass was inactive. Sanger and co-workers (201), using virgin ewes, confirmed the presence of estrogen-like substance in the above Ladino clover pastures. Extensive cornification and desquamation of the vaginal cells was observed in anestrus smears from the ewes on Ladino. They were similar in appearance to smears from anestrus controls treated with estradiol, as well as to the metestrus and diestrus smears of controls undergoing their normal estrus cycle. In studies on the nature and cause of the delay in conception, Sanger and Bell (200) found that ovulation occurred normally, but that fertilization did not follow as consistently. Of the total eggs recovered from ewes on Ladino clover, only 59 percent were fertile, as compared to 75 percent for the ewes on bluegrass. There is probably a connection between this observation and that of Coop and Clark (79), in Australia, who reported that ewes mated on alfalfa exhibited a 10 to 12 percent reduction in lambing percentage that was due mainly to reduction in the number of twins.

Infertility problems have also been reported in nonruminant animals. Bornstein and Adler (52) found that an alfalfa extract that supplied the equivalence of 0.21 μg. of estradiol per kg. feed significantly increased oviduct weights and caused ovarian hyperemia in female chicks. Churý and Panek (73) found follicular cysts and cystic hyperplasia of the uterine lining in guinea pigs fed large amounts of alfalfa. These effects were associated with the estrogenic substances in the plants. Elghamry (90) has shown that estrogenic extracts of Ladino clover exerted two types of influence on the uterus of growing female rats. Initially, during the first 2 weeks of administration, there was a stimulatory effect, as shown by an increase in uterine weight. When the estrogenic feeding was continued for 3 or 4 weeks, an inhibitory effect occurred. The uteri lost weight as compared to the controls and began to degenerate. Elghamry concluded that his latter effect offered a logical explanation for breeding disturbances in domestic animals as well as clinical manifestations of infertility resulting from long continued ingestion of plant estrogens.

Crouse (*80*) reported continuous heat cycles as well as uterine and vaginal diseases in chinchillas. He attributed these problems to the alfalfa in the feed and suggested that they might be due to estrogenic substances present in the alfalfa. Once alfalfa was eliminated from the feed, the conditions corrected themselves. Negus and co-workers (*186*) studied the population size of colonies of the rice rat on an isolated island. They suggested that a sharp change in the quality of the green plants, including their estrogen contents, could have immediate effects on the reproductive potential of and eventual influence on the population size of the rodent colonies in the field.

Leavitt and Meismer (*152*) found that injecting coumestrol subcutaneously at 10 mg./kg. or at 100 mg./kg. into 5-day-old female rats caused persistent anovulatory estrus. At 100 days of age, ovaries from animals treated with coumestrol were entirely follicular but only weighed half as much as the controls. On the other hand, Bierwagen [16] found that when coumestrol was administered orally at 10 mg./kg. to adult female mice, it did not prevent pregnancy. Deanesly [17] reported that coumestrol acetate given to female guinea pigs subcutaneously at levels of 2 to 6 mg. did not influence ovulation, the duration of the corpora lutea, or their normal cycle.

There have been only a very few reports of substances in forages that affect the male animal.

Bornstein and Adler (*52*) reported that cockerels fed a diet containing 10.3 percent alfalfa, equivalent to 1.65 microgram of estradiol per kg., had significantly retarded testicular development as shown by reduced testes weight and by inhibition of sperm production. Comb growth was only slightly inhibited. Recently Churý (*70*) reported the isolation of a crystalline antigonadal substance from alfalfa that decreased the weight of the seminal vesicles in male rats. However, this substance had no effect on male mice.

Pincus and Merrill [18] compared coumestrol with testosterone and found that coumestrol also had androgen-like activity in castrated male rats. When administered alone, coumestrol caused the weight of the seminal vesicles of the rats to increase, but had no effect on the weights of the ventral prostrate gland or the levator ani muscle. When coumestrol was combined with testosterone, an additive effect was obtained on all three tissues (table 28). Lyman and Krueger (*168*) reported that doses of coumestrol diacetate ranging from 20 to 300 mg. per animal given orally over a 21-day feeding period did not inhibit testicular development or adrenal size in either normal or castrated male rats. They suggested that the absence of any physiological effects might be due to the rapid degradation of coumestrol diacetate by the liver in the adult male rat, similar to certain other estrogens (*203*).

Oldfield and co-workers (*193*) found that coumestrol stimulated

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[14] Personal communication from M. E. Bierwagen, Director of Pharmacologic Research, Bristol Laboratories, 1968.
[17] Personal communication from Ruth Deanesly, University of Cambridge, England, 1968.

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[18] See footnote 11, page 61.

TABLE 28.—*Effect of coumestrol diacetate injected subcutaneously on castrated male mice*

Treatment	Total dose in 7 days	Average body weight	Seminal vesicles (SV)[1]			Ventral prostate (VP)			Levator ani muscle (LA)		
			Average weight	SV/body	Standard error	Average weight	VP/body	Standard error	Average weight	LA/body	Standard error
		Grams	*Grams*			*Grams*			*Grams*		
Control _____	----	73	5.8	0.079	0.002	8.2	0.112	0.006	18.4	0.252	0.013
Sesame oil _____ milliliters____	1.4	72	5.2	.073	.005	9.3	.130	.009	18.7	.259	.016
Testosterone _____ milligrams ____	.14	76	13.3	.177	.014	22.5	.300	.020	27.8	.368	.013
	.35	78	16.2	.208	.025	23.7	.306	.039	31.3	.402	.020
	.70	80	19.1	.240	.027	36.3	.455	.070	34.5	.433	.016
Coumestrol diacetate _____ do _____	[2]1	77	6.4	.084	.008	8.0	.104	.005	18.0	.235	.014
	[3]5	68	12.1	.179	.012	9.0	.132	.009	20.1	.294	.025
Testosterone _____ do _____	.35										
Testosterone + coumestrol diacetate _____ do _____	[2]1	86	23.6	.276	.011	43.5	.508	.016	38.2	.447	.018
Testosterone _____ do _____	.35										
Testosterone + coumestrol diacetate _____ do _____	[3]5	66	30.4	.455	.047	36.9	.552	.074	34.0	.513	.029

[1] Fluid expressed from seminal vesicle before weighing.
[2] Since coumestrol is not soluble, a suspension was made containing 5 mg. and diluted 1 to 5 for the 1 mg. dose.
[3] Heavy suspension.
Source: Pincus, Gregory, and Merrill, A. P. (*101*).

TABLE 29.—*Teat lengths and glandular weights*

Item	Trial 1		Trial 2	
	Control ration	Coumestrol ration [1]	Control ration	Coumestrol ration [2]
Teat length (centimeters)				
3 weeks			1.56	2.21
5 weeks			1.46	2.49
7 weeks			1.48	2.53
Pituitary weight .... grams	0.940	1.258	.835	.933
Pituitary				
final body weight .... mg./kg	15.460	19.710	15.75	17.81
Seminal vesicle weight ... grams			.80	7.05
Seminal vesicle				
final body weight .... mg./kg			15.09	134.54

[1] Coumestrol added to ration = 112 p.p.m.
[2] Coumestrol added to ration = 145 p.p.m.
Source: Oldfield, J. E., Fox, C. W., Bahn, A. V., and others (*193*).

mammary growth and increased the weights of the seminal vesicles and the pituitary gland in wether lambs (table 29). Moreover, one of the wethers suffered a rectal prolapse, which was considered suggestive of an estrogenic response. Based on a report (*174*) that genistin depressed testicular development and sperm production in male mice, Booth [19] investigated the possibility that coumestrol would cause similar effects. High levels of coumestrol diacetate (1.5 g./kg. diet) completely inhibited reproduction in mice when both sexes were fed coumestrol. When attempts were made to ascertain whether one or both sexes were involved, the results were inconclusive. However, the data suggested that the reproductive failure was due to sterility in the male and that the effect was reversible. These effects were similar to those caused by feeding the males diethylstilbestrol (1 mg./kg. diet). Booth

(*50*) found that coumestrol did not inhibit reproduction in rats. The reasons for the differences in the effects of coumestrol on various male animals are not known. Differences among species are very likely contributing factors.

Hypoestrogenic syndrome

The concept of a hyperestrogenic syndrome caused by long periods of grazing estrogenic pasture is well established. However, the concept that forages may contain factors that interfere with the plant estrogens or with the endogenous animal estrogens and produce a hypoestrogenic syndrome is not as widely accepted. But, evidence for such an antiestrogenic effect is growing. It is conceivable that forage with a high level of detectable estrogen by chemical assay might have no apparent estrogenic effect on the animal due to the presence of a sufficiently high level of an antiestrogenic substance. The situation in which antiestrogens would mask or coun-

[19] See footnote 10, page 61.

teract the effect of the plant estrogens would help to explain the frequent lack of correlation between chemical and biological assays for forage estrogens. In seeking an explanation for the actions of antiestrogenic substances, Cook and Kitts (78) suggested that they may function by inactivating the natural estrogens in the blood, or by making the uterus nonresponsive to estrogen stimulation.

It is well established that the prolonged administration of massive doses of estrogens inhibit ovarian development in the immature rat (225). In these cases, Ershoff and co-workers (99) have shown that the concurrent feeding of alfalfa, presumably rich in antiestrogenic substances, could largely counteract this effect when the estrogen was estradiol. Similarly, Churý (71) demonstrated that feeding of alfalfa minimized the effects of small doses of estradiol benzoate on the ovaries and pituitary glands of rats. Working with rats, rabbits, and guinea pigs, Churý and Panek (74) reported detrimental effects of alfalfa on ovarian and uterine functions, resulting in depression of fertility, which were not associated with the estrogen in the plant and presumably due to antiestrogens. In later studies, they decided that the weight changes observed in the pituitary glands, uteri, and ovaries of guinea pigs fed only alfalfa were probably the result of the mutual effect of estrogenic and antiestrogenic factors (73).

Churý (71) and Ershoff (99) both observed that different batches of alfalfa varied markedly in their antiestrogenic activity (table 30). Biely and Kitts (47), who assayed 13 legumes and grasses, including alfalfa and Ladino clover, showed that antiestrogenic activity varied widely depending on species, stage of growth, and date of harvest.

Bickoff (36) found that some samples of alfalfa, which entirely lacked estrogenic activity on the basis of effect on uterine weight

TABLE 30.—*Comparative effects of dried alfalfa on the ovarian weight of rats fed massive doses of alpha-estradiol*

Supplements fed with basal ration	Alpha-estradiol per kilogram of ration	Body weight		Average ovarian weight ± S.E.[1]
		Initial	Final	
	Milligrams	*Grams*	*Grams*	*Milligrams*
Basal	10	41.4	170	17.3 ± 1.1
20 percent alfalfa meal:				
1	10	41.2	185	19.4 ± 1.8
2	10	41.0	179	28.1 ± 1.7
3	10	41.2	184	34.2 ± 3.3
4	10	41.4	184	38.3 ± 3.9
5	10	41.4	185	38.1 ± 4.0
6	10	41.1	189	43.3 ± 2.1
Basal ration without alpha-estradiol	0	41.9	209	53.4 ± 3.0

[1] S.E. = standard error.
Source: Ershoff, B. H., Hernandez, H. J., and Matthews, J. H. (99).

TABLE 31.—*Demonstration of presence of an estrogen inhibitor in alfalfa meal*

Equivalent amount of alfalfa meal in control diet (grams/mouse)	Average uterine response to supplemental coumestrol	
	None	0.3 mg./mouse
	Milligrams	*Milligrams*
0	9.0	31.1
5	10.4	13.4
10	9.2	9.3

Source: Bickoff, E. M., Livingston, A. L., Booth, A. N., and others (*36*).

of treated mice, contained a substance that was capable of inhibiting the estrogenic response of coumestrol. When an extract of one of these meals was incorporated into a control diet, the normal response to coumestrol was depressed (table 31). Using the Astwood test with rats, Adler (*4, 5*) confirmed this observation and reported additional experiments in which the antiestrogen reduced the effectiveness of coumestrol to approximately one-tenth of its actual potency. He further showed that the antiestrogenic substance in alfalfa also inhibited the uterine response to estradiol and diethylstilbestrol (*4*). This inhibition was quite persistent and lasted 5 days after the antiestrogenic treatment under conditions of daily estradiol treatment. [20]

Antiestrogenic activity has been reported in other forages, such as fahli hay and oat hay (*3*). MacDonald (*170*) reported that beef cows tend to abort or give birth to weak calves when they consume yellow pine needles. Kitts and co-workers (*10, 11*) fractionated yellow pine needles and found a factor that depressed

the uterine weight of immature mice and antagonized the effects of both diethylstilbestrol and estrone (*78*). Ostrovsky and Kitts (*195*) have suggested that birdsfoot trefoil contains an antiestrogen similar to that found in alfalfa. The triterpene, glycyrrhetinic acid, which is a constituent of crude licorice extract, has been shown to have antiestrogenic activity, but no estrogenic activity (*147*). It is interesting to note the structural similarity of this compound to some of the saponin aglycones known to be present in alfalfa and clover (*218*).

Effects such as those produced by the antiestrogens in plants may also be induced by estrogenic, androgenic, and progestatic substances (*87*). Working with synthetic estrogens, Emmens (*95*) proved that, depending on the level administered, a given compound may act either as an estrogen or as an estrogen inhibitor. In accordance with this observation, Folman and Pope (*101*) found that at certain dose levels both coumestrol and genistein could markedly inhibit the uterovaginotropic action of estradiol, estrone, and diethylstilbestrol. Similar results on the in-

teraction between coumestrol and estrone have been reported by the U.S. Cancer Chemotherapy National Service Center, (CCNSC) [21] confirming that plant estrogens can also exhibit antiestrogenic properties.

Antigonadotropic factors

Some evidence exists that plant estrogens, at levels normally encountered in the field, may sometimes not be as significant in reproductive problems as are certain presently unknown estrus and estrogen inhibitors. Wright (224) reported that feeding Ladino clover to female rabbits caused infertility. Although the clover was estrogenically active by rat-assay, it did not cause any premature stimulation of the reproductive tract of newly-weaned rabbits even though they ingested the equivalent of approximately 30 micrograms of diethylstilbestrol daily. However when female rabbits, 5 to 12 months old, were fed clover containing the equivalent of 60 micrograms of diethylstilbestrol daily both prior to and during gestation, it interfered with ovulation and implantation. Since the clover did not produce any estrogenic effects in the immature rabbits, but did cause infertility problems in the mature rabbits, it was suggested that the observed infertility might be entirely unrelated to the estrogenic content of the Ladino.

In a continuation of these stud-

ies employing mice as the test animal, Leavitt and Wright (154) showed that Ladino clover samples, having no significant estrogenic activity by bioassay, caused reproductive anomolies and almost completely stopped conception in mice. No single aqueous-alcohol, ether-alcohol, chloroform, or acetone-chloroform extract of the Ladino had any of the properties of the unfractionated plant. However, all of the extracts increased the incidence of nonviable embryos in the pregnant mice.

Churý and Crha (72) investigated the effects of alfalfa and alfalfa extracts on induced ovulation in rabbits. An unbalanced alfalfa diet caused a considerable decrease in ovulation and in the number of fertilized eggs. The alfalfa extracts completely inhibited ovulation for varying lengths of time, usually at least 10 days and, in a few cases, over a month. They felt that egg degeneration was mainly caused by the changed physiochemical conditions in the fallopian tubes. Since the maximum suppression of ovulation occurred when the estrogenic content of the alfalfa was lowest, they concluded that these phenomena were probably caused by an unknown antigonadotropic factor, rather than an estrogen.

It is interesting that coumestrol itself also exhibits antigonadotropic activity. Leavitt and Wright (153) compared the role of coumestrol with that of estradiol in the feedback mechanism of the anterior pituitary gland of mice. Histologically, the ovaries of the mice receiving coumestrol or estradiol had become luteinized, whereas those of the controls were still in the various stages of follicular development.

The gonadotropes (hormone-producing cells) in the anterior lobe of the pituitary gland of the controls were abundant. Few gonadotropes were left after estradiol treatment, showing that the dose used was capable of inhibiting production of gonadotropic hormones. Coumestrol was shown to block release of the gonadotropic hormones rather than their production since the gonadotropes were plentiful in the coumestrol-fed mice. Dietary coumestrol at several lower levels produced normal estrogenic effects in the mice. However, ovarian growth was not depressed and they concluded that no significant inhibition of hormone release occurred at these feeding levels. In general, their studies indicated that coumestrol has physiological effects on pituitary function with relatively large doses being required for antigonadotropic effects. In continued studies, Leavitt (151) and Leavitt and Meismer (152) confirmed that coumestrol influenced the release of the gonadotropic hormones but, unlike estradiol, caused uterine growth before it affected pituitary gonadotropic function.

Effects on rate of growth

During the 1950's it became clear that the growth rate, the efficiency of feed utilization, and the chemical composition of meat in animals could be altered by hormone administration (12, 13, 123, 180, 215). In 1955, Burroughs and co-workers (62) reported that feeding 5 to 10 mg. of diethylstilbestrol to beef cattle daily resulted in an increase of about 20 percent in rate of gain and also increased feed efficiency. The practice of using synthetic hormones to stimulate rate and efficiency of gain in animals has since become widely accepted. Synthetic estrogens are now being fed to or implanted in more than 80 percent of fattening steers in this country (22).

The fact that forage estrogens could cause infertility problems in breeding animals suggested that they might be also capable of producing the same beneficial effects that are obtained with synthetic estrogens. This possibility was stimulated by a report from Iowa State University (212) in 1957 that genistein and a crude estrogenic extract of clover hay caused increased rate of growth in fattening lambs.

Booth found that coumestrol caused measurable increases in the growth of male rats (50). O'Dell and co-workers at the University of Missouri,[22] studying growth factors for guinea pigs, found that an alcohol extract of alfalfa was active, particularly in males. As this suggested that the factor might be a plant estrogen, coumestrol was fed to male guinea pigs at a level of 2 mg./100 g. of diet. In the first trial, they obtained a remarkable response, e.g., a gain of 8.7 g./day during a 4-week period compared with 7.5 g./day for the controls. In two subsequent trials, however, the gains were less than the controls, and they concluded that coumestrol was not the active factor.

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[22] Personal communication from B. L. O'Dell, Professor of Agricultural Chemistry, University of Missouri, 1968.

From 1959 through 1964, Old-field and co-workers (1, 103, 190, 191, 192, 193, 194) conducted a series of feeding trials with lambs to investigate the possible growth-promoting effects of coumestrol. The trials progressed from feeding alfalfa meals to feeding coumestrol concentrates from alfalfa and, finally, to feeding isolated coumestrol. In general, a trend toward a positive growth response was obtained with wether lambs, but not with ewes. Weight differences were significant for wethers fed crude alfalfa and the coumestrol concentrates, but not for wethers fed isolated coumestrol. Organoleptic test consistently demonstrated improved tenderness and juiciness scores for lamb roasts from animals fed high-coumestrol diets (193, 194).

Johnston and co-workers (131) studied the effects of five levels of dietary coumestrol ranging from 18 to 151 p.p.m. on the organoleptic quality of lamb. The tests were conducted both in the presence and absence of diethyl-stilbestrol implantation. They found that tenderness and juiciness values of lambs were enhanced by increased levels of coumestrol in the ration (fig. 26). Based on taste tests and shear-force measurements, meat
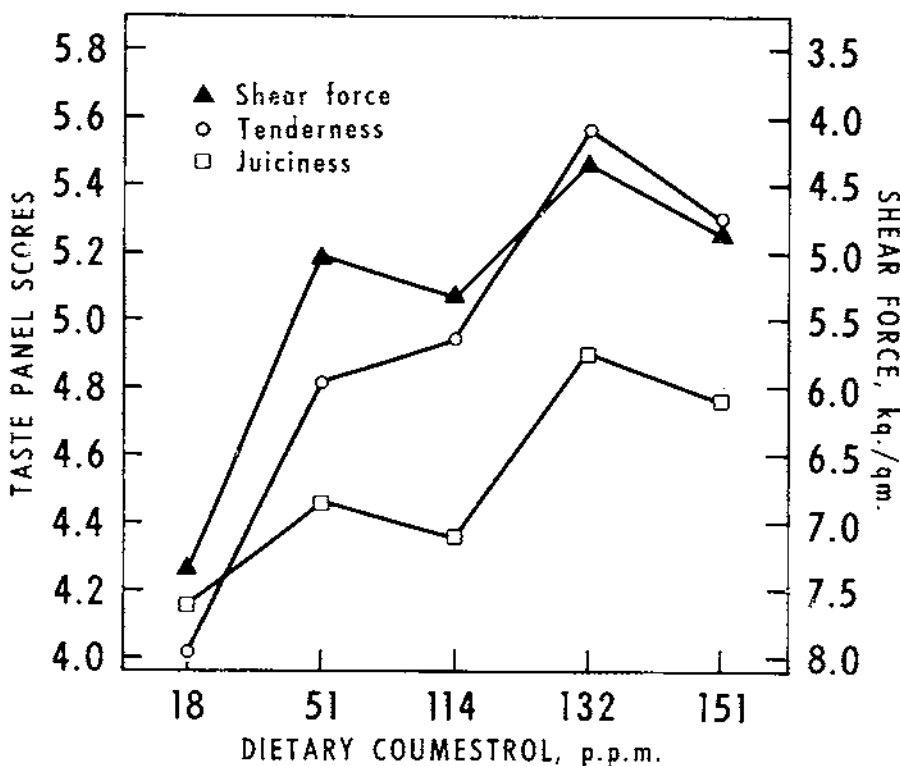

FIGURE 26.—Effects of five levels of dietary coumestrol on the meat quality of lamb. (Reproduced from a graph by Johnston and co-workers (131).)

TABLE 32.—*Average daily gains of steers in two feeding tests*

Protein supplement	Daily rate of gain		Increase in gain by adding diethyl-stilbestrol
	With diethyl-stilbestrol in ration	Without diethyl-stilbestrol in ration	
	Pounds	*Pounds*	*Percent*
1 lb. soybean oil meal	2.07	2.47	19.3
0.5 lb. soybean oil meal plus 1.25 lb. dehydrated alfalfa.	2.30	2.59	12.6
2.5 lb. dehydrated alfalfa	2.41	2.64	9.5
5.0 lb. dehydrated alfalfa	2.46	2.64	7.3

Source: Matsushima, J. K., Clanton, D. C., and Arthaud, V. H. (*177*).

from diethylstilbestrol-implanted lambs that received a diet containing 114 p.p.m. coumestrol was rated juicy and the most tender. Furthermore, these values for tenderness and juiciness were only slightly better than those obtained from lambs that received 132 p.p.m. coumestrol without diethylstilbestrol.

Matsushima and co-workers at Nebraska (*175, 176, 177*) have reported positive evidence of weight gain stimulation in steers fed alfalfa naturally high in estrogenic activity (table 32). They found that 5 pounds of this alfalfa in the daily ration produced the same daily weight gain in pounds (2.46) as did the addition of diethylstilbestrol to the protein-equivalent basal diet (2.47). Lower amounts of alfalfa were also effective but to a lesser extent. When diethylstilbestrol and dehydrated alfalfa were combined, rates of gain were only slightly better than when either was administered alone. In later experiments, Matsushima and co-workers (*178*) were unable to find any significant differences in gains among steers receiving 4 pounds of dehydrated alfalfa pellets having either low (0 p.p.m.), medium (122 p.p.m.), or high

(245 p.p.m.) coumestrol content in their daily ration. They attributed these results to either or all of the following reasons: (1) that coumestrol was not the factor in dehydrated alfalfa that contributed to greater gains in cattle; or (2) levels of coumestrol ranging from 122 to 245 p.p.m. were too high; or, (3) the quantity of the major nutrients in the three samples of dehydrated alfalfa was so variable that the estrogenic effects were masked. Further tests (*175*) with alfalfa containing 25, 100, and 250 p.p.m. coumestrol also showed no significant differences in gain or feed efficiency among the cattle.

Elam (*89*) compared the digestibility and nitrogen utilization of high (209 p.p.m.) and low (23 p.p.m.) coumestrol alfalfa. There were no significant differences between the two meals in nitrogen retention. In later studies (*21*) employing pure crystalline coumestrol, Elam and Putnam again obtained negative results for increased nitrogen retention.

Beeson at Purdue (*16*) conducted feeding experiments with steers in which they found that a daily intake of 0.5 pounds of

dehydrated alfalfa meal per steer increased daily gain 8 percent. They also found that it improved feed efficiency 7 percent and increased the concentration of vitamins A and E, carotene, and cholesterol in the blood and vitamin A and carotene in the liver (table 33). However, Stob and co-workers (210), in experiments in which high (560 p.p.m.) and low (50 p.p.m.) coumestrol dehydrated alfalfa pellets were fed, were unable to find a growth-promoting effect due to coumestrol. In fact, daily gains of steers fed the low coumestrol alfalfa were significantly greater than those of steers fed the high coumestrol ration.

Stob and co-workers (209) also compared the growth-promoting effects of a coumestrol concentrate with that of diethylstilbestrol on steers. Daily feeding of diethylstilbestrol (10 mg.) improved the daily gain and feed efficiency 12 percent. However, feeding the coumestrol concentrate (equivalent to 840 mg. coumestrol) resulted in an improvement of only 4 percent. The differences in daily gain and feed efficiencies resulting from these treatments were not statistically significant. Neither treatment had any effect on carcass grade, dressing percentage, or transit shrink of the steers when compared to the controls. In further experiments,[23] three sets of identical twin calves were fed a coumestrol concentrate equivalent to 1.36 g. coumestrol per head daily. No improvement in daily gain or feed efficiency was found. In fact in most instances, a slight depression occurred. Their latest studies[24] were conducted with pure crystalline coumestrol. Four sets of identical twin heifer calves were fed differing levels of coumestrol. Fortification of the high-energy ration with 50 and

[23] Personal communication from W. M. Beeson, Lynn Professor of Animal Nutrition, Purdue University, 1963.
[24] W. M. Beeson, Martin Stob, and M. T. Mohler. EFFECT OF CRYSTALLINE COUMESTROL ON THE PERFORMANCE OF IDENTICAL TWIN HEIFERS. Amer. Dehy. Res. Council, final rpt., 3 pp. 1966.

TABLE 33.—*Effect of dehydrated alfalfa meal on steers*

Item		No dehydrated alfalfa meal	One-half pound dehydrated alfalfa meal	Increase
				Percent
Daily gain	pounds	2.05	2.21	8
Feed per hundred weight of gain.	do	910	842	7
Blood analysis:				
Vitamin A	micrograms per 100 milliliters	22	34	55
Carotene	do	72	163	126
Vitamin E	do	38	48	26
Cholesterol	do	110	136	24
Liver values:				
Vitamin A	micrograms per gram	4.6	11.0	139
Carotene	do	10.4	12.0	15

Source: Beeson, W. M. (16).

100 p.p.m. coumestrol did not cause any significant change in the daily gain, feed comsumption, or feed efficiency. Higher levels of coumestrol (150 and 200 p.p.m.) depressed daily gain. From their results, Beeson and co-workers concluded that coumestrol is not a growth-stimulant for cattle. Thus, although coumestrol is similar to diethylstilbestrol in its estrogenic effects in animals, it must have a different metabolic function, since it has little effect on their rate of growth or feed efficiency.

Other biological effects

Coumestrol and several of the other coumestans have been screened for a variety of physiological effects other than those already discussed.

Coumestrol was tested by the CCNSC [25] in their routine anticancer screening tests for activity against three mouse tumors. As it did not cause any statistically significant reduction in tumor weight in the Sarcoma 180 or Adenocarcinoma 755 tumor systems, nor any significant increase in lifespan in the Leukemia L-1210 system, it was considered inactive. In addition, coumestrol was tested by Parke, Davis & Co., for its effect on the normal serum cholestrol levels in rats. It was found to have no effect on either atherosclerotic plaques or the serum lipids of hyperchloresteremic rats.[26] Jacobs also found that coumestrol had little effect on the deformability of erythrocytes (red blood corpuscles).[27]

Antifungal screening tests have been performed on coumestrol, 4'-O-methylcoumestrol, medicagol, and trifoliol at several different laboratories. Monsanto compared them with Captan as a spore germination inhibitor for *Venturia ineaqualis* and *Phytophthora infestans* and with PCNB and Dixon, which are soil fungicides, as a mycelial growth inhibitor for *Rhizoctonia solani* and *Pythium ultimum*.[28] Although the reference compounds were active at concentrations as low as 10 p.p.m., the coumestans were inactive at even 1,000 p.p.m. Cruickshank [29] compared these same four coumestans with pisatin for toxicity against mycelial growth of *Monilinia fructicola* and found them to have no fungitoxic activity. The relationship of coumestrol to antifungal activity in red clover was studied by Berkenkamp of Canada Department of Agriculture.[30] His results showed that coumestrol was not inhibitory to fungi attacking Canadian red clover. The Pesticide Chemicals Research Branch of ARS [31] tested medicagol as a residue for insecticidal properties. Medicagol

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[25] See footnote 21, page 72.
[26] Personal communication from M. L. Black, G. Rodney, and F. Armstrong, Parke, Davis & Co., 1959.
[27] Personal communication from H. R. Jacobs, M.D., Evanston Hospital Association, 1968.

[28] Personal communication from E. G. Jaworski, Monsanto Chemical Co., 1964.
[29] Personal communication from I. A. M. Cruikshank, Principal Research Scientist, Commonwealth Scientific and Industrial Research Organization, Australia, 1965.
[30] Personal communication from Bill Berkenkamp, plant pathologist, Canada Department of Agriculture, 1968.
[31] Personal communication from S. A. Hall, J. H. Fales, and O. F. Bodenstein, USDA, 1965.

was not found to be toxic to adult face flies, to DDT-resistant houseflies, or to adult male chlordane resistant German cockroaches.

Frank and co-workers (106) found a definite relationship between the consumption of alfalfa and the incidence of clinical mastitis in small herd of dairy cows. Over a 2-year period more than twice the number of attacks were recorded for animals receiving alfalfa and bromograss as silage than for those fed almost entirely nonlegumes. Animals fed alfalfa-bromograss silage, made when the estrogen content of the plants were relatively high, exhibited 3.6 attacks of mastitis per cow as compared to 0.8 attacks per cow when the estrogen content was low. However, they were unable to establish any definite correlations between mastitis incidence and estrogenic activity of the feed as determined by mouse bioassay. The milk production and breeding efficiency did not appear to be effected by the different feeding programs. Kesler and co-workers at Pennsylvania State University [32] conducted preliminary studies on the relationship between plant estrogens and the solids-not-fat (SNF) content of milk. The percent SNF in the milk dropped when the cows went from winter feeding to pasture. However, no coumestrol was found in the pasture and the study was discontinued.

Correlation of Structure and Activity

Robertson and Whalley (198) suggested that the estrogenic activity of a molecule is dependent upon certain molecular characteristics. These include the presence of oxygen-contaning functional groups capable of forming hydrogen bonds and positioned at a given distance from each other (140) as well as the presence of at least one functional phenolic group of high hydrogen-bond-forming capacity (75). Both coumestrol and genistein contain phenolic groups that may satisfy the configurational and electrostatic requirements necessary for an estrogenic molecule.

Bradbury and White (55) studied the estrogenic activity of a series of isoflavones, isoflavanones, and isoflavenes related to genistein and formononetin. The removal of the 5-hydroxyl group from genistein (forming daidzein) decreased its activity (39). While the 5-hydroxyl group appeared to be important for activity, the introduction of an alkyl group at the 2-position greatly reduced the activity of genistein. In contrast, Bradbury and White (54, 55) found the isoflav-3-enes to be active only when they had a 2- or 4-alkyl substituent, and they were then more active than genistein. The isoflavenes appear to be more comparable to the stilbene estrogens than to the isoflavone estrogens, since the effects of substituent groups on their activity is similar to the effects of similar groups in the stilbene series (204). The isoflavenes have stilbene-like structures, e.g., 4', 7-dihydroxy-4-ethyl-2-methylisoflav-3-ene (fig. 27, IIa), which has the same carbon skeleton as diethylstilbestrol (fig. 27, Ia) and is the closest approach to it that
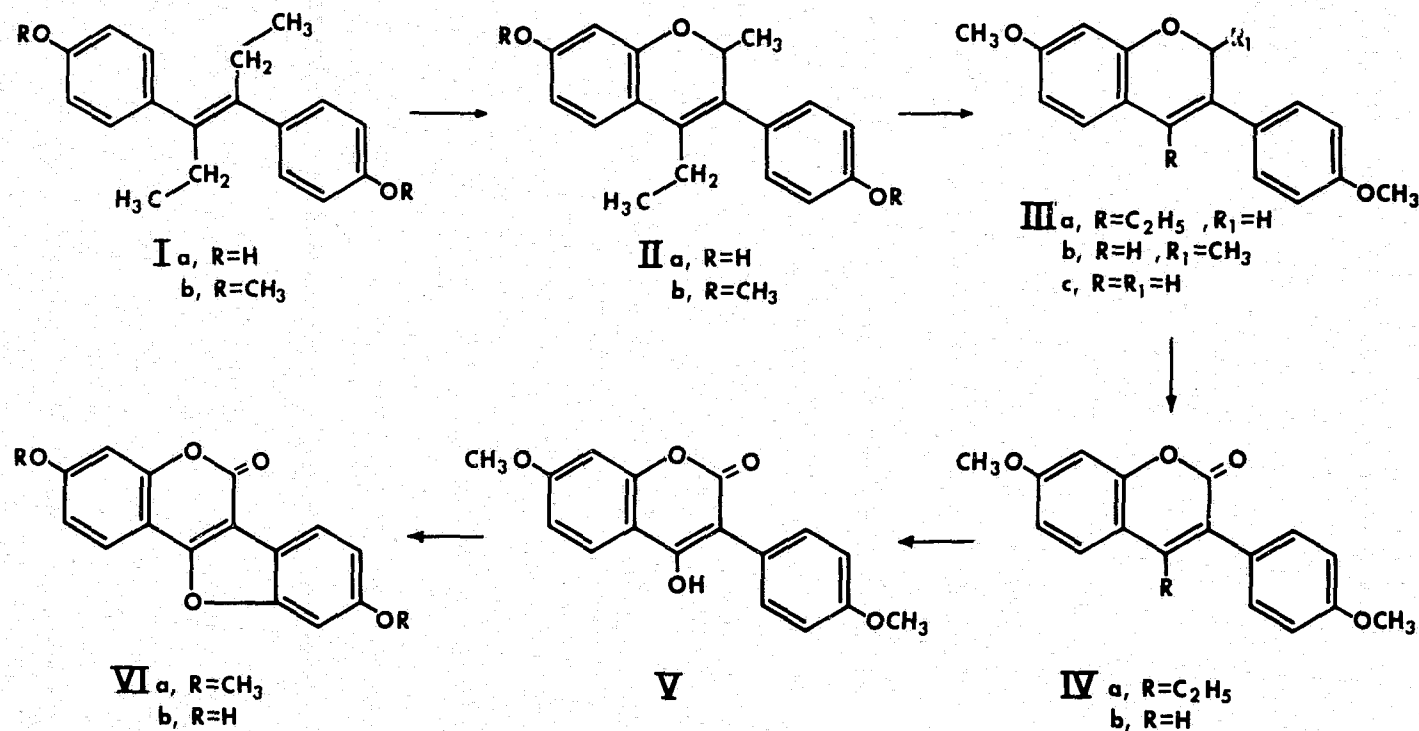
FIGURE 27.—Structural changes in estrogenic molecules in going from diethylstilbestrol to coumestrol.

still retains the oxygen in the heterocyclic ring (54). Lawson (150) prepared the dimethylether of this compound (fig. 27, IIb) and found it to have estrogenic activity of the same order of magnitude as diethylstilbestrol dimethylether (fig. 27, Ib). Micheli and co-workers (181) prepared the diacetates of Ia and IIa (fig. 27) and found them to compare closely in activity, each being about three times as active as their methylethers.

In a review article by Biggers (48), a suggestion was made by Whalley that coumestrol also derives its estrogenic activity by virtue of its stilbene-like structure. This possibility was investigated by Bickoff and co-workers (35) and Micheli and co-workers (181), who studied the activity of a series of compounds related to coumestrol, including the above-type compounds. By comparing the variations in estrogenic activity caused by gradual structural changes starting with 4',7-dimethoxy-4-ethyl-2-methylisoflav-3-ene (fig. 27, IIb) and ending with coumestrol dimethylether (fig. 27, VIa), the importance of alkyl substituents and the 3,4-double bond, i.e., the stilbene-like structure, for estrogenic activity can be demonstrated.

Replacement of both the 2-methyl and 4-ethyl groups on the isoflaven (fig. 27, IIb) with hydrogen to from 4',7-dimethoxyisoflav-3-ene (fig. 27, IIIc) decreases the estrogenic activity of the isoflavene (fig. 27, IIb) more than 10,000-fold (55). However replacing only the methyl group at the 2-position with hydrogen (fig. 27, IIIa) or the ethyl group at the 4-position with hydrogen

(fig. 27, IIIb) only reduces the activity about a hundredfold (181). The introduction of a carbonyl group at the 2-position appears to have little effect on the resultant activity, since the activity of the 3-phenylcoumarin (fig. 27, IVa) is of the same order of magnitude as the 2-dihydro derivative (fig. 27, IIIa). When the 4-ethyl group on the phenylcoumarin (fig. 27, IVa) is replaced with either a hydrogen (fig. 27, IVb) or a hydroxyl group (fig. 27, V), the entire activity is lost. Lack of activity in 4-hydroxycoumarins is general and has been attributed to destabilization of the 3,4-double bond and, therefore, of the stilbene system, which is now free to exist in tautomeric keto-enol forms (208). Formation of the furan ring by introduction of a 4,6'-oxygen bridge to give coumestrol dimethylether (fig. 27, VIa), stabilizes the 3,4-double bond and maintains the stilbene-like structure. This restores a considerable amount of the lost activity as coumestrol dimethylether is about one-twentieth as active as 4-ethyl-7-methoxy-3-(4-methoxyphenyl) coumarin (fig. 27, IVa) and about one-two thousandth as active as diethylstilbestrol dimethylether. Coumestrol (fig. 27, VIb) itself is about three times as active as its dimethylether (fig. 27, VIa).

Modification in the structure of coumestrol, such as the addition or removal of nuclear substituents and blocking of the hydroxyl groups, reduces the activity of coumestrol (35). The effect of substituent groups on the activity of coumestrol is similar to the effects of similar groups in the stilbene series (204). The fact that coumestrol is from 30 to

100 times more active than the closely related estrogenic isoflavones (110) is probably due to the carbonyl group at the 4-position of the isoflavones, which is primarily ketonic, but which can exist in keto-enol forms. This results in a single bond in the 3,4-position of the isoflavone and, consequently, the partial destruction of the estrogenic stilbene system.

Patents

Eight patents were issued as a result of studies on coumestrol performed at Western Regional Research Laboratory. These described the isolation of coumestrol and its estrogenic activity (26), its purification (92), its synthesis (91, 93), and the preparation and estrogenic activity of several of its derivatives (24, 25, 33, 34).

The first patent by Emerson (91) described the synthesis of dialkyloxyphenyl-2-thiopyruvic acids from dialkyloxybenzal rhodanines using aqueous alkali in the presence of an alkali metal sulfide. This method afforded a means of preparing these compounds in greater yields than previously possible. The use of (2,4-dimethoxyphenyl) thiopyruvic acid to prepare intermediates for the synthesis of coumestrol was also described. The second patent was by Emerson and Bickoff (93) and was on the synthesis of coumestrol from 3-(2,4-dimethoxyphenyl)-4,7-dihydroxycoumarin by heating in the presence of a hydrohalic salt of an aromatic amine, such as aniline hydrochloride. The reaction sequence was described earlier under "Synthesis of Coumestans" (fig. 11). The preparation of coumestrol diacetate was also described.

A patent on estrogenic factors, by Bickoff and Booth (26) gave details of methods for isolating coumestrol from plants and described the preparation and utilization of feeds containing coumestrol. In addition, the physical characteristics and estrogenic properties of coumestrol were discussed. Purification of coumestrol via its acetate derivative and regeneration of coumestrol from its acetate by alkaline hydrolysis were the subject of the fourth patent (92).

Patents were obtained by Bickoff and Booth on the preparation and uses of ester (25) and ether (24) derivatives of coumestrol. These compounds were shown to exhibit estrogenic activity similar to that of coumestrol and to be more stable and more readily produced in pure, crystalline form. The preparation of coumarone derivatives, i.e., salts of 2-(2,4-dihydroxyphenyl)-6-hydroxycoumarone-3-carboxylic acid, from coumestrol by mild alkaline hydrolysis (34) and use of these salts as estrogenic agents (33) were the subjects of the last two patents.

The authors assigned a non-exclusive, irrevocable, royalty-free license on these patents to the Government of the United States of America for their use throughout the world. Power to grant sublicenses on these patents was also given to the United States Government.

Summary

The result of extensive studies on the occurrence and the chemical, physical, and biological properties of the plant estrogen, coumestrol, and the seven other alfalfa and Ladino clover coumestans are described in this bulletin. Methods for their isolation from and determination in these plants are described in detail. Coumestrol and the other coumestans were separated from other plant constituents by solvent extraction and countercurrent distribution. Both physical and chemical means were employed for their structural elucidations, with synthesis generally being used for confirmation.

The occurrence of coumestrol in nature is widespread. It is the dominant estrogen of alfalfa, Ladino clover, and the annual medics. Both genetic and environmental factors have been shown to influence the buildup of coumestrol in these forages. Present evidence indicates that coumestan levels, including coumestrol, are quite low in alfalfa free of disease, but that they rapidly increase to very high levels shortly after pathogen attack or aphid infestation. In barrel medic, physiogenic leaf spotting has been shown to cause the accumulation of coumestrol. In all cases, coumestrol is concentrated in the immediate vicinity of the damaged tissue. The pathogen-host relationship has not been established for the other coumestrol-containing forages.

The similar effects of foliar pathogens, aphid infestation and physiogenic leaf spotting on the accumulation of coumestrol suggest that disease "triggers" some nonspecific mechanism in the plant that causes the rapid accumulation of this type of compound. Although the biosynthetic pathway for coumestrol in diseased plants has not been studied, it has been shown that healthy alfalfa biosynthesizes coumestrol in a manner analogous to the isoflavones. Radioactive tracer studies proved that an aryl migration occurs at some point during the biosynthesis of coumestrol, and that coumestrol, daidzein, and formononetin can arise from the same intermediate chalcone.

Although there is a biosynthetic interrelationship between coumestrol and the isoflavones, they do not appear to be metabolized along the same pathways. The differences in metabolic routes may account for the much higher relative estrogenic potency of coumestrol. Equally important may be structural differences. There are certain configurational and electrostatic requirements for a molecule to be estrogenic. The most important ones, as far as diethylstilbestrol is concerned, are the alkyl substituents and the 3,4-double bond, i.e., the stilbene structure. The furan ring in coumestrol stabilizes this 3,4-double bond. However, the carbonyl at the 4-position of the isoflavones can exist in tautomeric forms, which results in a single bond between the 3,4-positions and, consequently, the partial destruction of the estrogenic stilbene system.

The problem of infertility in farm animals caused by the ingestion of estrogenic alfalfa and Ladino clover is probably more severe than is generally recognized. Moderate cases of in-

fertility frequently go undetected or are only transient and, in most cases, are not associated with the feed being ingested by the animal. It is becoming increasingly evident that forages can also contain antiestrogenic and antigonadotropic substances that may act to partly nullify the expected response to the ingested coumestrol or even to that of the normal animal estrogens. Moreover, it has been shown that under certain conditions, coumestrol itself can interfere with the endogenous estrogens and pituitary hormones.

Most growth studies have indicated that coumestrol has no effect on the rate of growth of either cattle or sheep. However, evidence exists which suggests that coumestrol may have beneficial effects on carcass quality in lambs. Additional work is necessary to confirm this observation.

The role of coumestrol in the pathogen-host relationship should be elucidated. It is possible that coumestrol is either elaborated by the plant in response to attack or by the pathogen itself. Detailed studies of the coumestrol-plant-pathogen interrelationship will provide a better understanding of the mechanism of disease resistance. Genetic studies have shown that, by recurrent selection for resistance to disease, alfalfa very low in coumestrol can be produced. The reduction of coumestrol content by development of new varieties or by control of pathogens should eliminate reproductive problems in farm animals caused by the ingestion of high estrogen forages.

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