THE ESTROGEN-LIKE ACTIVITY AND IDENTIFICATION OF
certain isoflavones present in red clover

by

ROBERT WILLIAM HOGG

B. S. A., The University of British Columbia, 1960

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
Master of Science in Agriculture

in the
Division of Animal Science

We accept this thesis as conforming to the required standard

Members of the Division

THE UNIVERSITY OF BRITISH COLUMBIA
April, 1962.
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Animal Science

The University of British Columbia,
Vancouver 8, Canada.

Date April 10, 1962
ABSTRACT

A study was carried out to determine the relative estrogen-like activity attributed to the isoflavone compounds present in red clover plants (*Trifolium pratense*). The pure compounds were fed as additives to a non-estrogenic basal diet at total dosages ranging from zero to fifteen milligrams per mouse. The biological assay used for the determination of the estrogen-like activity was a slight modification of that outlined by Kitts et al. in 1959 (63). The isoflavone found to possess the greatest estrogen-like activity was Genistein followed by Daidzein, Biochanin A and Formononetin. A non-isoflavone compound, Coumestrol, was found to possess a greater estrogen-like activity than any of the isoflavones considered. Mixtures of the pure isoflavones were bioassayed to observe any inhibitory or synergistic characteristics of one upon another. Biochanin A appeared to exert a slight inhibitory action and formononetin a synergistic character.

An electrophoretic technique was developed which facilitated the detection of the isoflavones in plant extracts of monthly harvested red clover. An attempt was made to associate the biological activity of the red clover samples and the compounds which were observed in these same samples using electrophoresis.
ACKNOWLEDGMENT

The author wishes to express his thanks to Dr. W. D. Kitts, Associate Professor in the Division of Animal Science for his direction and criticism throughout the course of this study.

Thanks is also extended to the staffs of the Division of Plant Science and the Central Animals Depot for their cooperation in this investigation.

The author is grateful to Dr. K. Venkataraman of the National Chemical Laboratories at Poona, India for supplying samples of synthetically prepared Formononetin and Biochanin A. Thanks is also extended to Dr. E. M. Bickoff of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. D. A. in California for supplying samples of naturally isolated isoflavones. Dr. H. J. McLean of the Forest Products Research Laboratory, University of British Columbia, is thanked for the infrared analysis, and aid in interpretation of the results obtained, on the unidentified substance isolated from red clover.
# TABLE OF CONTENTS

**ACKNOWLEDGMENT**

**ABSTRACT**

**INTRODUCTION** 1

**LITERATURE REVIEW** 4

The Isoflavones 4

The Identification of Isoflavones 12

The Isolation of Isoflavones 15

Coumestrol 17

The Biological Significance 19

The Effect of Estrogens on the Female Reproductive Tract 19

Plant Estrogens 21

Estrogenic Activity and Structure 26

Non-estrogenic Effects of the Isoflavones 27

**MATERIALS AND METHODS** 29

I. The Experimental Compounds 29

II. The Bioassay Procedure 32

III. The Preparation of Diets Containing the Pure Isoflavones 33

IV. The Preparation of Rations Containing Two or More Isoflavones 36

V. The Preparation of Red Clover Extracts for Bioassay 36

VI. The Electrophoresis Procedure 38

**RESULTS AND DISCUSSION** 44

I. The Bioassay of Genistein, Biochanin A, Daidzein, Formononetin and Coumestrol 44
II. The Results of the Bioassay of Isoflavone Mixtures  

III. The Results of the Bioassay of Red Clover  

IV. The Electrophoresis Procedure  

V. The Estrogen-like Activity versus Electrophoresis Analysis of Red Clover  

SUMMARY AND CONCLUSIONS  

BIBLIOGRAPHY
INTRODUCTION

Within the large group of compounds referred to as flavonoids, there exists a minor subdivision of naturally occurring and synthetically prepared substances designated as isoflavones. To date fourteen isoflavones are known to occur in plant material, however their functional role in these biological systems has not been clearly defined. The isoflavones do not take part, to any discernable extent, in the process of photosynthesis and as they are of common occurrence in most higher plants it is thought that they may in some way be associated with the oxidation-reduction systems of plant tissue (97). Isoflavones occur in plant tissue as aglycones and as glycosides, rhamnose and glucose being the carbohydrates most commonly found. The first natural isoflavone was isolated from Prunus bark, characterized and called prunetin (56). The work of Baker and Robinson in 1925 (6), in which 7 methoxyisoflavone was synthesized, was rapidly followed by additional synthesis procedures by which other chemically pure isoflavones were obtained.

The fact that these compounds were of biological significance to a system other than that from which they were isolated was realized when they were identified in plant material which was thought to have caused reproductive disorders in grazing sheep. The compounds appeared to exert an action similar to that of the natural estrogens in the grazing animals. The presence of such estrogen-like compounds in plants was first reported in 1926 by Loewe (73). However it was not until 1944 when extensive reproductive failures occurred in ewes of some Australian flocks grazing on subterranean clover, that serious consideration was given to their presence. Bennetts (14), at this time, reported such reproductive disturbances as dystocia, uterine prolapse and general female infertility resulting in low
lambing rates.

Biological assays developed as modifications of the original Allen and Doisey test (1) have classified the isoflavones present in the clover as possessing an estrogen-like effect in laboratory animals as well as in sheep. The extent of this activity varies between the different isoflavones (14). The isoflavones possessing an estrogen-like activity which can be isolated from red clover are Biochanin A, Daidzein, Formononetin, Genistein, Pratensein and the coumarin-like derivative Coumestrol (61, 84, 101).

Variations in the reported activity levels of these compounds resulting from the use of differing bioassay procedures has led the author to investigate as many of the estrogen-like compounds, of plant material as possible using one technique over a range of values such that their relative intensities could be determined. The compounds used in these studies were naturally isolated or obtained by chemical conversion of available synthetic compounds. The substances studied were genistein, daidzein, biochenin A, formononetin and coumestrol.

The first study involved the biological assay of the individual compounds at increasing levels to determine their estrogenic intensity relative to one another. Associated with this, a study considering mixtures of the compounds was carried out to determine the possibility of a synergistic or inhibitory effect of one upon the other(s).

The second study involved the development of an electrophoretic technique for the identification of these active compounds in plant extracts. Such a technique was advisable owing to inadequate results obtained by the use of chromatography.
The third study involved an attempt to associate the estrogenic activity of red clover with the compounds identified electrophoretically in the extracts at various stages of the growing period of the plant.
The Isoflavones

The chemical class of the Isoflavones is a minor subdivision of the larger group of compounds referred to as flavonoids. The designation "flavonoid" encompasses a large group of naturally occurring compounds which consist of two benzene rings joined by a propane bridge of form C₆-C-C-C₆. Such compounds include: chalcones, aurones, flavanones, flavones and isoflavones, flavonals, flavanones, anthocyanidins and catechins. However, as the name indicates isoflavones are natural isomers of the parent structure and are of the form C₅-C-C₆.

Isoflavone compounds are synthesized in greater or lesser amounts by most higher plants and on the basis of their distribution in the plant kingdom it is thought that they may serve some useful function in plant tissue, perhaps in some association with the plant's oxidation-reduction system (97). No positive role can be assigned to such compounds in photosynthetic processes other than that they are resulting endproducts.

Fourteen isoflavones are known to occur in plant material. This number excludes glycosides which may be present in a number of forms for any one of the given compounds. Table I gives the common name, substitution and natural source of the known isoflavones (97). It would appear that isoflavones occur in all portions of the plant tissue -- roots, rhizomes, bark, heartwood, sapwood, leaves, flowers, seeds, fruits and resin secretions. They are present as free entities or glycosides and the occurrence of several differing glycosides of one compound in any plant is not uncommon. An example of two forms of one compound which exist simultaneously would be genistin, the 7 glucoside of genistein, and sophoricoside, the
Table I
Natural Isoflavones and their Occurrence

<table>
<thead>
<tr>
<th>Parent compound</th>
<th>Name</th>
<th>Substitution</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daidzein</td>
<td>7,4'-OH</td>
<td>Soyabean as 7-glucoside, daidzin</td>
</tr>
<tr>
<td></td>
<td>Formononetin</td>
<td>7-OH-4'-OCH$_3$</td>
<td>Ononis spinosa as glucoside ononin; subterranean and red clover</td>
</tr>
<tr>
<td></td>
<td>Pseudobaptigenin</td>
<td>7-OH-3',4'-CH$_2$O$_2$</td>
<td>Baptisia tinctoria as 7-rhamnoglucoside pseudobaptisin.</td>
</tr>
<tr>
<td></td>
<td>Maximin</td>
<td>7-OCH$_2$CH=O(CH$_3$)$_2$-3',4'-CH$_2$O$_2$</td>
<td>Root of Tephrosia maxima</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td>5,7,4'-OH</td>
<td>Genista tincteria and soyabean as 7-glucoside-genistin; Sophora japonica as 4'-glucoside and 4'-rhamnoglucoside; Trifolium species</td>
</tr>
<tr>
<td></td>
<td>Prunetin</td>
<td>5,4'-OH</td>
<td>Prunus bark as 4'-glucoside, prunitrin</td>
</tr>
<tr>
<td></td>
<td>Biochanin A (Olmelin)</td>
<td>5,7-OH</td>
<td>Clove, germinated grain of Cicer arietinum</td>
</tr>
<tr>
<td></td>
<td>Orobol (Norsantal)</td>
<td>5,3',4'-OH</td>
<td>Orobus tuberosus as the glucoside, oroboside</td>
</tr>
<tr>
<td></td>
<td>Santal</td>
<td>5,3',4'-OH</td>
<td>Santalwood, Barwood</td>
</tr>
<tr>
<td></td>
<td>Tectorigenin</td>
<td>5,7,4'-OH</td>
<td>Rhizome of Iris tectorum Max as 7-glucoside tectorigin</td>
</tr>
<tr>
<td></td>
<td>Muningin</td>
<td>6,4'-OH</td>
<td>Heartwood of Pterocarpus angolensis</td>
</tr>
<tr>
<td></td>
<td>Tlatlancuayin</td>
<td>5,2'-(OCH$_3$)$_2$-6,7-CH$_2$O$_2$</td>
<td>Iresine celosioides L.</td>
</tr>
<tr>
<td></td>
<td>Irigenin</td>
<td>5,7,3'-OH</td>
<td>Root of Iris florentina</td>
</tr>
<tr>
<td></td>
<td>Pratensein</td>
<td>5,7,4'-(OH)$_3$,x'0CH$_3$</td>
<td>Red clover (structure proposed)</td>
</tr>
</tbody>
</table>

Taken from Venkataraman, K. "Flavones and Isoflavones" p.56 (97).
(Pratensein, 101)
4' glucoside of the same compound (100). The sugar residues of the iso-
flavones are found most commonly at the 7 or 4' position of the structure,
glucose and rhamnose being of most common occurrence. The occurrence of
isoflavones as the aglycone is more common than the corresponding form for
flavones (97). Figure I gives the basic chemical structure for flavones
and isoflavones as well as the individual structures for those compounds
which will be considered in detail.

The majority of the work in the field has been concerned with the
study of pure isoflavones and to a lesser extent with the glycosides. It
is unfortunate that removal of the sugar residue by mineral acid hydrolysis
leaves no indication as to the site of attachment (100). The glycosides
are usually water and alcohol soluble and ether insoluble whereas the
aglycones are alcohol and ether soluble and unless highly hydroxylated
show no water solubility. Methylation of the hydroxyl groups eliminates
water solubility. The isoflavones and their glycosides are normally con-
sidered as pigments and range from pale cream to rust in intensity. Those
compounds which are isolated from red clover and considered in this study
were all pale cream or colorless.

In 1910 Finnermore (56) assigned the first isoflavone structure to a
compound of natural origin. This compound was prunetin and had been solvent
extracted from prunus bark. Since this time isoflavones have been isolated
from a large variety of plant species. The parent compound and the mono-
and penta- hydroxy isoflavones have not been shown to occur naturally.
Three of the compounds are polyhydroxy and the remaining possess one or
more ether groups. No naturally occurring isoflavone is known in which
the B ring is unsubstituted. The 7 position is always occupied, eight
compounds having hydroxyl groups and three possessing methoxy groups at
**Figure 1**

The Chemical Structure of Compounds Discussed in the Context

- **Basic flavone structure**
- **Basic isoflavone structure**
- **Daidzin** $C_{21}H_{20}O_9$
  7 Glucose of Daidzein
- **Genistin** $C_{21}H_{20}O_{10}$
  7 Glucose of Genistein
- **Coumestrol** $C_{15}H_{8}O_5$
  3,9 Dihydroxy-6H-benzofuro-[3,2-C][1]benzopyran 6-one
- **Biochanin-A** $C_{16}H_{12}O_5$
  5,7 Dihydroxy-4'methoxy isoflavone
- **Formononetin** $C_{16}H_{12}O_4$
  7 Hydroxy-4'methoxy isoflavone
- **Daidzein** $C_{15}H_{10}O_4$
  7,4' Dihydroxy isoflavone
- **Genistein** $C_{15}H_{10}O_5$
  5,7,4' Trihydroxy isoflavone
- **Pratensein** $C_{16}H_{12}O_6$ (proposed)
  5,7,3' Trihydroxy-4'methoxy isoflavone
this site (97). The recently isolated compound Pratensein (101) has not been included in the above figures as its substitution in the B ring is not definite. This compound would appear to be a trihydroxy methoxyiso-flavone.

The most significant characteristic regarding the determination of the chemical structure of isoflavones is their susceptibility to alkaline hydrolysis and the resulting products. Five per cent ethanolic potassium hydroxide and mild heat hydrolyzes an isoflavone to formic acid and desoxybenzoin which can be recrystallized for accurate melting point confirmation. More severe conditions cause the breakdown of desoxybenzoin to phenol and phenylacetic acid. This pathway of breakdown is characteristic of all isoflavones and was first observed by Finnemore (56) and considered later by Venkataraman (97). Substitution positions in the isoflavone series occur in the A ring at the 5', 5,7; or 5,6,7 positions and in the B ring at 3', 3', or 3', 5'. The ultraviolet absorption spectra of isoflavones indicate maxima at wave lengths of 320 and 260 millimicrons. Table II lists the ultraviolet absorption spectra maxima for several naturally occurring isoflavones.

The color reactions associated with the isoflavone complex will be discussed under means of identification of these compounds at a later point.

The first synthesis of an isoflavone was carried out in 1925 by Baker and Robinson (6) who at this time synthesized 7 methoxyisoflavone, and later in 1928 synthesized genistein (7). It was not until 1934 that the parent compound, isoflavone, was synthesized (61). Ten known glycosides have been synthesized by reacting the isoflavone in question, for example,
Table II

The Ultraviolet Absorption Spectra of some Naturally Occurring Isoflavones in Ethanol (97)

<table>
<thead>
<tr>
<th>Name</th>
<th>$\lambda_{max}^{\text{mu}}$</th>
<th>log E</th>
<th>$\lambda_{max}^{\text{mu}}$</th>
<th>log E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formononetin</td>
<td>300'</td>
<td>4.05</td>
<td>250</td>
<td>4.44</td>
</tr>
<tr>
<td>Genistein</td>
<td>325*</td>
<td></td>
<td>263</td>
<td>4.63</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>325*</td>
<td></td>
<td>263</td>
<td></td>
</tr>
<tr>
<td>Prunetin</td>
<td>325*</td>
<td></td>
<td>263</td>
<td></td>
</tr>
<tr>
<td>Orobol</td>
<td>-</td>
<td></td>
<td>287</td>
<td></td>
</tr>
<tr>
<td>Santal</td>
<td>-</td>
<td></td>
<td>263</td>
<td></td>
</tr>
<tr>
<td>Tlatlancuayin (in chloroform)</td>
<td>320</td>
<td>3.86</td>
<td>245</td>
<td>4.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>278*</td>
<td>4.12</td>
</tr>
</tbody>
</table>

* Inflection

Taken from Venkataraman, K. "Flavones and Isoflavones" p.58 (97).
genistein in acetone with aqueous potassium hydroxide and acetobromoglucose to form genistin (97). It has been previously mentioned that removal of the sugar residue is facilitated by mineral acid hydrolysis. The interconversion of the isoflavones is also possible as demonstrated by the removal of the 4'-methyl ether group of formononetin to produce daidzein (31). Advances in chemical synthesis have reduced much of the ambiguity of certain compounds. An example of this is Bose's synthesis of formononetin and its identification with biochanin B (29), and also the proof that pratensol was equivalent to biochanin A (97). The interrelations of many of these compounds is self evident from their structures, biochanin A being the 4'-methyl ether of genistein as formononetin is the 4'-methyl ether of daidzein. Similarly prunetin is the 7 methyl ether of genistein (8). Other compounds in the series also demonstrate these close similarities and before an accumulation of literature developed many compounds bore different names on similar structures which led to some confusion. In many cases elimination of impurities in what were thought to be new compounds resulted in their identification with those already known. A large volume of literature has accumulated on various methods for the synthesis of isoflavones (7, 8, 9, 10, 11, 29, 44, 59, 97, 100).

The possible means of the biogenesis of these compounds has been considered by Geissman (57). He states that one must consider that all carbon is originally derived from carbon dioxide and the relationships between amino acids and carbon moieties must be remembered. As very little conclusive proof is available which would indicate definite paths of formation much is based on speculation regarding chemical pathways which would be mechanistically feasible. One possibility would be the rearrange-
ment of a common precursor (not identified) into a number of differing flavonoid moieties. Geissman has also suggested that isoflavones may be formed in the plant from a C_6-C-C-C fragment, the aromatic ring present forming the B ring. Although they are very similar in the positioning of hydroxyl and other substituting groups no biogenic evidence is available to support the transformation of a flavonone to a flavone or isoflavone. In 1959 Grisebach (60) using C^{14} labelled D,L-phenylalanine found that the carbon skeleton of phenylalanine is incorporated into 7 hydroxy 4'-methoxyisoflavone in red clover and a rearrangement involving either the migration of a phenyl or benzoyl group occurs in the biogenesis. This work outlines possible biogenic pathways for flavones, isoflavones and coumestrol in red clover. Additional information regarding the possibilities of the biogenic origin of isoflavones is considered by Geissman (58) and Seshadri (88).

Isoflavones with additional furan or pyran rings are relatively common in plants and form the basis of many large complex compounds in these systems. These forms have not been considered here but have been referred to in the review by Venkataraman (97).

The compounds which were specifically studied in this program are: Biochanin A, 5,7 dihydroxy 4'-methoxyisoflavone; Formononetin, 7 hydroxy 4'-methoxyisoflavone; Daidzein, 7,4'-dihydroxyisoflavone, and Genistein, 5,7,4'-trihydroxyisoflavone. These compounds have all been isolated from Red clover. A fifth isoflavone has recently been isolated from red clover by Wong (101); and called Pratensein. It is present at very low levels and preliminary structural determinations would indicate that it is 5,7,3'-trihydroxy 4'-methoxy isoflavone. The individual character of the above
mentioned compounds will be given when identification is discussed.

The Identification of Isoflavones

In general the isoflavones are thought to be pigments ranging in intensity from colorless to a soft rust color. The compounds considered in this study were all colorless or very pale yellow in nature. It has been mentioned that the determination of chemical structure can be readily facilitated by hydrolytic fission (alkaline hydrolysis) and by observation of the maxima in the ultraviolet absorption spectra (Table II). A number of specific color reactions are also characteristic of the isoflavones. Briggs (34) has shown that the presence of a free hydroxyl group at the 3,5 or 8 position is required for the characteristic rust color in alcoholic or aqueous ferric chloride. Pope et al. (84) has reported this color reaction for the compound Biochanin A and observed a red color when the isoflavone was in excess in methanolic ferric chloride and a green color when the ferric chloride was in excess. Addition of hydrochloric acid resulted in the green color while sodium hydroxide caused the red color. This would indicate that the color observed is dependent on the pH of the solution. Pope also observed varying shades of peach to rust when a solution of the compounds spotted on paper was sprayed with diazotized p-amino phenyl-2-diethyl aminoethyl sulphone. The color reaction associated with a diazotized salt is normally indicative of the presence of an aromatic structure. It has also been observed that certain isoflavones exhibit a weak visible fluorescence in concentrated sulfuric acid. Isoflavones are recognized as giving similar color reactions to flavones and thus reduction with sodium amalgan followed by concentrated hydrochloric acid produces a pink color which is indicative of a flavonoid type com-
pound (101). Walters (98) has stated that a change in color from red to purple when genistin is placed in concentrated sulfuric acid indicates the presence of impurities in the sample.

A considerable amount of work has been done involving the chromatographic separation and identification of these compounds. Bate-Smith et al. have studied the chromatographic characteristics of formononetin using the solvent system butanol-acetic acid-water as well as several others. Pope and Elcoate (82) carried out similar studies on genistein. In 1954 Pope and Wright (84) reported the character of genistein, biochanin A, and formononetin in a number of different solvent systems with resulting Rf values. Because of variations resulting in Rf values depending on the specific conditions, no mention will be made of them. A number of the more common solvents used are: acetic acid:water (1:1), butanol:acetic acid:water (4:1:5), acetone:water (3:7), acetic acid:water:hydrochloric acid (3:6:1), five per cent potassium carbonate, benzene:acetic acid:water (2:2:1), and isopropanol:water (6:4). Guggolz, in 1961 (61) outlined an elaborate repetitive chromatographic separation and identification of the above-mentioned compounds including daidzein from forages. The procedure involves the elution and rerunning of the compounds through several different solvent systems, the final run being completed on a silicic acid strip. The resulting Rfs for the compounds in the various solvent systems have been reported (61).

The determination of the position of the isoflavones on a chromatogram is readily facilitated by the fluorescent character of the compounds in ultraviolet light. This fluorescence occurs at a wave length of 253 millimicrons and to a lesser extent at 366 millimicrons. At a wave length
of 253 millimicrons formononetin produces a fluorescent character (pale bright blue) as does daidzein. This fluorescence is markedly increased by exposure of the paper to ammonia fumes or in the presence of sodium hydroxide. Genistein and biochanin A can be observed as shadowed or dark spots due to the ultraviolet light absorbing character of these compounds. A number of developing sprays have also been used. These include diazotized salts, ferric chloride, magnesium and hydrochloric acid, and neutral or basic lead acetate (97). A composite table of the common characteristics of identification has been prepared.

Table III.

Common Characteristics used in the Identification of Isoflavones

<table>
<thead>
<tr>
<th>Compound</th>
<th>Melting Point</th>
<th>Crystals in U.V. light</th>
<th>Spray*</th>
<th>Ferric chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochanin A</td>
<td>214°C</td>
<td>--</td>
<td>dark orange</td>
<td>rust/green</td>
</tr>
<tr>
<td>Daidzein</td>
<td>325°C</td>
<td>colorless</td>
<td>blue cream</td>
<td>--</td>
</tr>
<tr>
<td>Formononetin</td>
<td>262°C</td>
<td>--</td>
<td>blue cream</td>
<td>--</td>
</tr>
<tr>
<td>Genistein</td>
<td>296°C</td>
<td>white 6-sided rods</td>
<td>dark peach</td>
<td>rust/green</td>
</tr>
</tbody>
</table>

*A diazotized spray

In 1952 Hashimoto (62) reported briefly on the electromigration of flavonoids and sugars in a buffer solution of two per cent borax. Identification of the spots was carried out by immersion in a five per cent ethereal solution of lithium aluminum hydride followed by rapid drying and a dilute hydrochloric acid spray. The spots appeared red to dark green in color. Developments in the use of electrophoresis as an identi-
fication technique led the author to investigate its possibilities for the isoflavone series.

The Isolation of Isoflavones

The isolation of prunetin by Finnemore in 1910 \(^\text{[56]}\) was the first isolation of an isoflavone from a natural source. The compound was extracted from *Prunus* bark with "hot industrial spirits" and purified by ether and ammonium carbonate extractions followed by recrystallization from alcohol. Other early extractions and studies were carried out by Walz \(^\text{[99]}\). This procedure is still the basis for the extraction of isoflavones and hormone-like compounds from plant sources. Burnham et al. in 1930 \(^\text{[35]}\) and Skarzynski in 1933 \(^\text{[91]}\) used this basic procedure for the analysis of estrogenic compounds from plant sources, and though no mention is made to them, these compounds could well have been isoflavones.

The basic procedure involves the extraction of the plant material, if dry with alcohol, if wet with acetone. The extract is then concentrated and the chlorophyll and extraneous matter removed by benzene extraction followed by extraction of the estrogens with ether and/or a dilute base. Various slight modifications have been developed and will be mentioned below.

In 1941 Walters \(^\text{[98]}\) outlined a method for isolating genistin from soybeans and obtained a 0.1 per cent yield. This method will be discussed in detail at a later point as it was used as an isolation technique in this study.

Robinson \(^\text{[86]}\) carried out extractions on the different structural portions of the plants and reported that the estrogen-like compounds
(possibly isoflavones) appeared in greatest intensities in the leaves followed by the roots and petioles. Bradbury and White (31) in isolating genistein and formononetin from subterranean clover did not extract the plant material in the usual manner but rather expressed the juice from the plants, heated it and on cooling collected a residue which separated out and was termed the "chloroplast" fraction. The dried residue was then used for solvent extraction. This method was used later by Beck (13) and Pope and Elcoate (82) using the basic solvent extractions isolated biochanin A from red clover. Purification in this case was carried out by fractionation in a Celite column. Bate-Smith et al. (12) also used this method to isolate formononetin from red clover. Many extractions have been accelerated by using a Waring blender instead of extended refluxing and result in very similar yields. Cheng using naturally isolated compounds reported that synthetics exerted the same estrogenic activity as did those isolated from the plant source (43). Curnow (47) and Pope (84) have both reported on the isolation of various isoflavones from red and subterranean clover. Virtanen (97a) isolated formononetin readily by the ether soluble fraction being further extracted with petroleum ether and sublimed the undissolved residue in vacuo at 260°C to 280°C. Thompson (96) observed a one hundred times concentration of the estrogenic components of plants by carrying out a preliminary extraction with dilute alkali, acidifying the aqueous extract and re-extracting the now acid fraction with ether. Extraction procedures used by Kitts et al. (65) do not involve the use of dilute alkali. Guggolz in 1961 (61) outlined a long chromatographic procedure for the identification and isolation of genistein, daidzein, formononetin and biochanin A from red clover using an additional preliminary extraction with Shellysolve to remove fats and extraneous
matter. Rehydration of dried clover samples before extraction was reported by Livingston in 1961 (71). There would appear to be some controversy as to whether dried samples possess similar or slightly lower levels of estrogen-like compounds than do fresh extractions (22). This loss is attributed to failing respiration during drying, however, in many cases the decrease in potency is negligible.

Goumestrol

The compound coumestrol is a recently observed factor which appears to be present in certain varieties of alfalfa, ladino and strawberry clover and possibly other forage plants. A discussion of the compound has been separated from those mentioned previously as it is not of an isoflavone nature. However, as this compound bears some structural similarity, and a definite similarity in biological action it was considered in the basic research program. The structure of the compound coumestrol is given in Figure I.

In 1957 Bickoff et al. (17) reported the identification and subsequent isolation of a crystalline substance from alfalfa which possessed an estrogen-like activity similar to, though more intense than, the reported activity for known isoflavones. The compound was solvent extracted from the alfalfa meal, purified by countercurrent distribution and recrystallization from methanol. The isolate was shown to be a coumarin derivative and was called coumestrol. Coumestrol exhibited a brilliant blue fluorescence in acid or neutral solution, a green-yellow fluorescence in strong alkali, and melted with slight decomposition at 385°C. The methanolic ultraviolet spectra showed maxima at wave lengths of 208, 243 and 343 millimicrons and the empirical formula was determined to be C_{15}H_{20}O_{5}. Additional confirmation
of the compound's character was obtained later in 1957. This original isolation of the compound from a natural source was rapidly followed by a chemical synthesis and the assignment of the systematic name, 3,9 Di-hydroxy-6H-benzofuro(3,2-C)(1) benzopyran 6 one (19,52).

Subsequent studies have demonstrated the presence of coumestrol in other forage crops (18, 21, 71) and also the fact that in alfalfa particularly, the concentration of the active compound appears to increase and/or remain relatively high throughout the growing season (22,23,25). Some controversy has developed regarding this point and other plant estrogen-like compounds.

Coumestrol may be isolated from both fresh and dried forages, the biological activity being higher in the fresh clover, the decrease probably resulting from decomposition on drying (22). As the extraction procedure for wet, fresh forage is similar to that outlined for the isoflavones only the dry extraction will be briefly considered. The dried ground forage was initially extracted with water and Skellysolve C before ether extraction for removal of the estrogen-like substance. These initial extractions plus the use of ether as the preferred solvent eliminated many impurities and facilitated subsequent purification using five per cent sodium carbonate, countercurrent distribution and recrystallization from methanol. Bickoff et al. (18) has reported details regarding the countercurrent solvents and the purification technique.

The identification of coumestrol was readily facilitated when it was realized that the brilliant fluorescence, visible on chromatographic papers, was associated with the compound. This fact associated with advancing silicic acid chromatographic techniques led to the development by Bailey (5) of an apparatus which measured the fluorometric intensity quantitatively
of the compound on chromatography strips. Livingston et al. (70) proved this apparatus accurate for the quantitative range necessary and demonstrated that storage of the papers led to a decrease in the fluorescent character of the compound over a one hundred day period. The solvent used in the chromatographic studies was acetic acid and water (1:1) which was found to give an Rf value of approximately 0.5.

In 1960 Bickoff et al. (24) carried out rather extensive studies regarding the effect of altering the coumestrol molecule and the relationship between various molecular characteristics and its biological activity. Acetylation of both hydroxyl groups did not significantly reduce the estrogenic activity nor did opening of the lactone ring. Decreases in activity were noted when the compound was esterified, the furan ring was opened, one hydroxyl group was removed, and when additional hydroxyl groups were placed on the molecule. Removal of both hydroxyl groups completely eliminated the estrogenic activity. Coumestrol would appear to be thirty times as estrogenically active as genistein. Bickoff et al. (24) has stated that the lower activity of the isoflavones may result from the fact that the oxygen at position four is ketonic and results in a single bond in the 3,4 position while coumestrol possesses a double bond at this site.

The Biological Significance
The Effect of Estrogens on the Female Reproductive Tract

The estrogens of the mammalian body are, δ and α estradiol, estriol and estrone. Hereafter these compounds will be referred to inclusively in the term "estrogens". The estrogens control and regulate the female sexual behavior and the development of primary and secondary sex characteristics (2). The major source of estrogens in the female is the ovary, particularly the
Graafian follicle. A secondary source, of little significance in the normal animal is the adrenals. Estrogens are specifically involved in the control of the female sexual cycle, changes in which can be observed externally by variations in the uterine and vaginal cytology of most small laboratory animals. The following events may be observed during the sexual cycle of the normal mature intact animal and also following the injection of estrogen into an immature or ovariectomized mature animal. Reference is made primarily to laboratory rats and mice in which the injection of estrogens cause the typical changes in the endometrial cytology of the uterus encountered in the normal intact animal. Estrogens have been found to cause increased vascularity and accelerated mitotic activity in the uterine tissue, resulting in an increased weight of this organ. In castrate mice and rats the injection of estrogens also results in the accumulation of water in the uterine lumen which contributes to the increased weight. In other species the uterine interstitium becomes very edematous. It has also been shown that the increase in uterine weight is proportional to the amount of estrogen administered. These conditions occur in the normal animal at "estrus", identified by the presence of cornified cells and some leucocytes in prepared vaginal smears. In the immature or castrate animal such conditions may be observed following administration of an estrogen or estrogen-like compound.

Injection of estrogenic compounds into an immature rodent results in an immediate increase in the uterine water content followed by a later increase in the dry weight. The water uptake appears to be related to vasodilation and changes in permeability of the uterine vessels, however, the water increase appears to be largely of an extracellular nature. The
increase in dry weight at the later phase is associated with some protein synthesis (72). Astwood (3) developed a six hour bioassay for the determination of estrogenic compounds based on the accumulation of water in the mucosal stroma and the resulting increase in organ weight. He also found that the water increase in noncastrates was maximal when injection occurred during proestrus and minimal in the early stages of diestrus (4). Later work states that the rapid influx of water within six hours increases the intra and extracellular moisture from 79.6 to 85.3 per cent. This high level gradually decreases to 82 per cent as cell division occurs (37). The endometrium and musculature appeared to be similarly affected and following a single injection normal water levels return approximately 50 hours post-injection. Prior to the work of Astwood, Allen and Doisy (1) had developed a technique which gave relatively conclusive results and allowed the analysis of a compound to determine its estrogenicity non-quantitatively. The criterion of estrogenicity is satisfied when injection of the compound into a mature ovariectomized rat results in vaginal cornification similar to that observed during the "estrus" phase of the sexual cycle. Ostrovsky (78) has shown that the plant estrogens possess a similar contact character to the natural estrogens. This means that both plant and natural estrogens are contact hormones and that surface application will result in similar effects as caused by the same compounds being transported via the blood stream to the contact organ.

Plant Estrogens

Although estrogen-like compounds had previously been observed in plant material by Loewe in 1926 (73) little significance was attached to their presence until 1944 when extensive reproductive failures were observed in
some Australian sheep flocks (14). Such disorders as dystocia and uterine prolapse as were reported were of such significance that extensive research was carried out to ascertain the causal agent. The flocks primarily affected had been grazed on pastures consisting predominantly of a locally developed strain of subterranean clover var. dwalgamp (15). Transfer of the affected ewes to other forages did not restore the lost fertility (100). Investigation of the forage led to the conclusion that the isoflavone constituents, particularly genistein were causing the adverse effects.

Extracts of willow and alder were among the first plant materials found to demonstrate an estrogenic effect. At this time workers found that the greatest activity appeared to coincide with the rapid spring growth period and following this stage the green parts of the plant demonstrated little or no activity (35). Studies of subterranean clover have shown that the leaves possess the highest concentrations of estrogenic substances followed by the roots and petioles (86). It has been noted that application of estrogens to plants appears to stimulate the vegetative growth (69). As the plant estrogens are of highest concentration in the leaves (probable site of production) in the early spring, the possibility of them being growth stimulants in the plant cannot be disregarded. Skarzynski in 1933 (91) demonstrated the presence in willow of an estrogenic compound, the crystalline character and melting point of which was identical to estriol but possessing a much greater estrogenic activity. At this same time true estrogens (estrone and estriol) were isolated from plant sources (36).

In 1941 the mouse uterine weight assay was elaborated by Evans (54) and with minor modifications is the assay still employed. Immature female mice weighing between six and eight grams were injected twice daily with
the compound of question for 3 days. Eighteen hours following the last injection the mice were sacrificed and the uteri dissected and weighed. The increase in weight in excess of the control, if any, is proportional to the estrogenic potency of the compound. Present day techniques usually use mice heavier (up to eleven grams) and the unknown compound is fed in a non-estrogenic basal diet at various levels. Sacrifice of the animals occurs from six to twelve hours after removal of or last consumption of the experimental feed. Injection procedures are still used, the unknown compound being suspended in such media as aqueous egg albumin (26) and peanut oil (38, 95). The guinea pig has been found to be impractical as an experimental animal because of its susceptibility to uterine cysts (46). However this animal is still used in some cases as its digestive tract approximates that of the grazing animals. Drasher (48) has observed strain differences in the response of mice to estrogens and consequently work in several laboratories using similar assay procedures cannot really be compared. The use of the mouse uterine assay procedure feeding the dry clover as an additive, i.e. not extracting the substances, can be erroneous in that feeding high levels of dried clover tends to lower the feed intake, results in lower body weights and subsequently inaccurate percentages of uterine weight to body weight.

Other assay procedures have been based on the time interval required for vaginal opening in immature animals (53,55). Opening of the vaginal tract is indicative of reproductive maturity and is hastened by the presence of estrogens (87). Carter (39) observed vaginal opening six days following the addition of genistein to the diets of immature female mice. This worker also reported that inclusion of genistein in the diet resulted
in decreases in the number of young born, the percentage of females
dropping litters, the average number of young per litter, and the average
weight of the litters. Such results are all indicative of reproductive
interruptions. Genistin fed to male mice resulted in a linear decrease
in growth with increasing levels of the compound and at the higher levels
causd a loss of weight. Similarly, groups fed at the higher levels
demonstrated greater than normal death rates. Increases in the level of
genistin fed resulted in linear decreasing testicular weights in which
workers were unable to observe spermatozoa (75). East (50) working with
guinea pigs fed subterranean clover observed extended estrus periods and
up to 70 per cent failures in conception as well as epithelial changes in
the accessory reproductive organs. In this case withdrawal of the clover
resulted in immediate recovery. Using mice this same worker reported
sterility or impairment of reproductive function in males and a lack of
mating in females. When matings occurred high percentages of stillbirths
resulted. Withdrawal in this case resulted in recovery of only some of
the animals (50). Injection of androgen into male castrate sheep appears
to provide some protection against the adverse affects of ingested sub-
terranean clover (49). Pope (83, 85) has reviewed the importance of
pasture plant estrogens in the reproduction and lactation of grazing
animals. Suomalainen (95) noted increases in the non-fat solids in milk,
produced by animals on spring pasture, which he felt resulted from stimu-
lation by estrogenic compounds in the forage. It appears that certain
silages possess an even greater estrogenic activity than the fresh plant
material (81). This along with the fact that moldy corn possesses estrogenic
activity (80) would suggest that microbiological fermentation increases
concentration of, or transforms to a more active form, the plant estrogen-like compounds. Seasonal variations in the concentrations of such compounds in certain legumes and grasses as well as the effects of harvesting frequencies have been studied by Kitts et al. in 1959 (65,66). The estrogenicity of a wide variety of American forages, from varying geographical locations have been determined by Bickoff et al. (21), as well as a number of associated studies regarding isoflavones, coumestrol and their estrogenic significance.

A review of the biologically active compounds by Kohler (67) states that the estrogenic potency of red clover results primarily from the presence of the compounds genistein and formononetin, and that of subterranean clover from the compounds genistein and biochanin A. Using the chloroplast isolation developed by Bradbury and White (31), Gurnow (47) isolated two mgm of genistein per 100 grams of fresh clover and twelve mgm per 100 grams of dry clover. Cheng et al. (41) have stated that one pound of mixed clover is equivalent to two micrograms of diethylstilbestrol. Both Cheng (43) and Biggers (27) have found that the synthetically prepared isoflavones possess similar estrogenic activities to those which have been naturally isolated. Increases in uterine weights have been obtained with genistein fed at a two and one-half mgm level and based on extrapolation of this value genistein is said to be 1/50,000 as estrogenically potent as diethylstilbestrol (42). The order of estrogenic intensity for the isoflavones has been stated to be: daidzein (greatest), genistein equal to biochanin A, and formononetin (least), when fed at the two and one-half mgm level (43). Contrary to the work of Cheng, Bradbury and White (32,33) have reported that genistein exerts an estrogenic response at the one mgm level. Several values for the estrogenic potency of bio-
chanin A have been reported (28,30).

Comparisons of genistein and diethylstilbestrol show that dose response curves of the two give similar slopes between the range of three to six mgm for genistein and 0.015 to 0.03 ugm for diethylstilbestrol. The activity of genistein can be increased four fold by heating with one and one-half per cent potassium hydroxide (20). This increase probably results from opening of the heterocyclic ring in the molecule and results in a more favorable orientation of the hydroxyl groups. The compound coumestrol has been found to be thirty times as estrogenically active as genistein and the other isoflavones (24).

The results obtained in this research program may clarify the relative estrogenic intensities of these compounds particularly those observed at higher levels of intake than have been previously studied.

Estrogenic Activity and Structure

Improved synthesis techniques and a greater chemical understanding of such compounds, with particular reference to synthetics (for example, diethylstilbestrol) has led to some attempts of interpreting the relationship between chemical structure and estrogenic activity. Low values of estrogenic potency is a relatively common characteristic for a large number of simple phenols and phenolic stilbenes (92). It was originally thought that estrogenic activity depended on a structural similarity between the tested compound and natural estrogens. This is in part true, however the primary requirement is the presence and spatial arrangement of two free hydroxyl groups (92). The studies of Bickoff et al. regarding the compound coumestrol and its activity to structure relationship have been previously discussed in this review (24).
Spatial manipulation of the isoflavone molecule results in an arrangement similar to that of the natural estrogens. Bradbury and White (32) have demonstrated that the presence of a 2 alkyl substituent reduces the activity of the isoflavone probably resulting from distortion of the coplanarity of the 3 phenyl ring with the chromone ring. Proof of this distortion is indicated by the ultraviolet absorption shift when a 2 alkyl group is added. Isoflavinol has been shown to possess a relatively high activity which drops markedly after introduction of a methoxyl group (68). Warburton (100) states that the presence of a 5 hydroxyl group is required before significant activity will be observed. It is possible that certain compounds undergo hydroxylation or some other conversion in the organism from an inactive to an active form. Such compounds are referred to as "pro-estrogens".

Non-estrogenic Effects of the Isoflavones

Several additional biological effects have been associated with a few of the isoflavone compounds considered here. Formononetin isolated from red clover has been found to possess certain antifungal properties, particularly against the strain Sclerotinia trifoliorum (97a). This information may lead to some further understanding of the role of such compounds in the plant system. Japanese workers have found that daidzein, found in many Japanese and Chinese crude drugs possesses an anti-spasmodic effect but does not possess an atropine-like character (89,90). A possible relationship may exist between such an effect and the uterine musculature producing the observed increase in size and weight.

While studying the effect of genistin on growth it was found that moderate levels of the compound caused increases in the weight of the
adrenal gland while high levels caused a decrease (40,75). Because of
the close association between the ovary and the adrenal (a secondary
source of estrogens) this effect may be considered as resulting from
stimulation and finally replacement of the organ as source of estrogen.
Very little work has been carried out on the isoflavones other than that
associated with pure chemistry and the estrogen-like character of such
compounds. Other studies are just beginning to take form.
MATERIALS AND METHODS

I. The Experimental Compounds

A. Genistein (5,7,4'-trihydroxyisoflavone)

The method used for the isolation of genistein was that outlined by Walters in 1941 (98) with slight modification. Three kilograms of commercial, solvent extracted soybean meal were placed in a cheese cloth sack within a six liter round bottom flask to which was added five liters of methanol. The mixture was refluxed at the lowest possible temperature for twelve hours, the methanol decanted and an additional four liters of methanol added, after which refluxing was continued for an additional twelve hours. The combined extracts were concentrated to approximately 300 milliliters in a flash evaporator.

Approximately two liters of hot acetone were added to the concentrate until the resulting precipitation of acetone insoluble material ceased. The acetone precipitation removed many impurities such as saponins and carbohydrates. The resulting acetone solution was then filtered. The clear acetone solution or filtrate was then concentrated to a thin syrup, a final volume of approximately 40 milliliters. Two volumes of water added to the concentrate resulted in the precipitation of genistin. The small amount of oil that had accumulated on the surface of the liquid was removed with hexane. The crude precipitate of genistin, the glucone of genistein, was collected by centrifugation, dissolved in hot 80 percent ethanol, treated with charcoal, and filtered. The pure compound was crystallized at room temperature, and recrystallized three times or until no purple color was obtained when several crystals were placed in con-
centrated sulfuric acid, as described by Walters (98). The final crystals were air dried and found to have a melting point of 255°C, similar to that reported for genistin by Walters (98).

Four grams of genistin were placed in a round bottom flask with 90 milliliters of methanol and 20 milliliters of concentrated hydrochloric acid. The mixture was refluxed for six hours or for one hour after all solid material had disappeared from the solution. The solution was cooled and one volume of water added. Upon sitting at room temperature overnight genistein precipitated. This precipitate was collected by ether extraction in a separatory funnel and subsequent evaporation of the solvent. The crude genistein precipitate was recrystallized from 60 per cent ethanol three times to ensure purity and the final product air dried. The crystals appeared to be white six-sided rods as reported by Walters (98). The determined melting point using a Kofler Micro Hot Stage was found to be 287°C. The isolated genistein was found to contain a slight impurity of daidzein, also present in soybeans. Using this procedure a number of extractions totaling seventeen pounds of soybean meal yielded two grams of relatively pure genistein.

The genistein used for the biological assays (500 milligrams) was continually recrystallized from 60 per cent ethanol until no daidzein could be detected. At this time the melting point was found to be 292°C. (Reported 296°C) (98). The crude and purified products were retained for future use.

B. Biochanin A. (5,7 dihydroxy 4' methoxyisoflavone)

Two grams of synthetically prepared biochanin A were obtained from Dr. Venkataraman of the National Chemical Laboratory at Poona, India.
The sample used for the bioassay was recrystallized twice from 80% per cent ethanol at which time a melting point of 212°C was observed. (Reported 214°C) (82). No impurities could be detected in the sample.

C. Formononetin (7 hydroxy 4′ methoxyisoflavone)

Formononetin was obtained from the same source as Biochanin A. The bioassay sample was recrystallized and the melting point determined to be 263°C. (Reported 262°C.) (31). No impurities were detected in the sample.

D. Daidzein (7,4′ dihydroxyisoflavone)

Daidzein was obtained from formononetin by means of a chemical conversion outlined by Bradbury and White in 1951 (31). One gram of formononetin was placed in a small round bottom flask with 20 milliliters of hydriodic acid (d. 1.7) and the mixture refluxed for two hours. Extreme care was used to avoid bumping. The mixture was cooled and poured into 100 milliliters of water. Sulfur dioxide gas was then passed through the solution to decolorize it by removing residual iodine. This reaction involves the removal of the methyl group from the 4′ position of formononetin and its replacement with a hydroxyl group to yield daidzein. Approximately 400 milligrams of daidzein were obtained, however it was contaminated with formononetin. The reaction was repeated and the resulting product showed no formononetin after crystallization from 80 per cent ethanol. The crystals formed were colorless needles melting at 321°C. (Reported 325°C.) (31). By this method 270 milligrams of daidzein was obtained and subsequently used for the biological assay.
E. Coumestrol (3,9 dihydroxy-6H-benzofuro (3,2-0)(1) benzopyran 6 one)

A sample of coumestrol (as coumestrol acetate) was obtained from Dr. E. M. Bickoff of the Western Regional Laboratory in California. The sample was recrystallized once from a mixture of 80 per cent ethanol and acetone after which no impurities could be observed.

The use of the synthetic isoflavones and coumestrol in this program is justified because there exists no difference in the estrogenic activity of natural versus synthetic compounds (43).

II. The Bioassay Procedure

The biological assay used to estimate the estrogenic activity of the compounds considered was that outlined by Kitts et al. in 1959 (65). In this assay the compound in question is fed at a predetermined level as an additive to a non-estrogenic control ration (Table IV). The experimental diet (control plus compound or extract) is then fed to immature female Swiss albino mice weighing between 8 and 11 grams and 20 to 21 days of

<table>
<thead>
<tr>
<th>Table IV</th>
<th>The Composition of the Control Ration (G-56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolled oats</td>
<td>52.50 pounds</td>
</tr>
<tr>
<td>Ground wheat</td>
<td>26.25 pounds</td>
</tr>
<tr>
<td>Fishmeal (70%)</td>
<td>8.75 pounds</td>
</tr>
<tr>
<td>Meat Scraps</td>
<td>3.75 pounds</td>
</tr>
<tr>
<td>Skim Milk powder</td>
<td>7.50 pounds</td>
</tr>
<tr>
<td>Steamed bone meal</td>
<td>1.00 pounds</td>
</tr>
<tr>
<td>Iodized salt</td>
<td>0.25 pounds</td>
</tr>
<tr>
<td></td>
<td>100.00</td>
</tr>
</tbody>
</table>
The ration is fed at a level of 1.67 grams per mouse per day for a three day period giving a total consumption of five grams of feed per mouse. At the end of the 72 hour period the feed is removed and any remaining weighed. Eight hours following the removal of the experimental diet the mice are sacrificed, the body weights recorded, the uteri dissected, blotted gently between two pieces of filter paper and finally weighed on a precision balance. The presence of estrogen-like stimulation is indicated by an increase in the weight of the uteri and consequently represents a higher percentage of the body weight.

Several slight modifications of this procedure were carried out. The range in initial body weights of the female mice was restricted to 8.5 to 10 grams to increase uniformity. It was found that at the end of 72 hours, and very often sometime prior to this all feed had been consumed and animals housed in groups of six often exhibited cannibalistic tendencies, particularly during the eight hour fasting period. To alleviate this situation the feed consumption per mouse was increased to 5.5 grams for the three day period and the fasting time reduced from eight to six hours. Such steps eliminate the occurrence of cannibalism and produced similar results to those obtained with less feed and the longer fast period. Thus the groups of experimental animals remained complete and a certain degree of uniformity retained.

III. The Preparation of Diets Containing the Pure Isoflavones

Stock solutions of the pure compounds to be added at varying amounts to the control ration were prepared as follows.

a. Biochanin A, Formononetin and Genistein

Five hundred milligrams of the individual compounds were dissolved
in a mixture of 80 per cent ethanol and acetone (3:1). Because of the expansive sensitivity to temperature changes of the solutions they were not brought to exact volumes until use and were held at a constant temperature throughout measurement and transfer to the diet.

b. Daidzein

Because of the limited amount of the compound available a lesser volume of this solution was prepared. The same solvent was employed. Two hundred fifty milligrams were dissolved in 125 milliliters of solvent and 20 milligrams in 10 milliliters for a total of 135 milliliters containing 270 milligrams of compound.

The concentrations of the stock solutions was thus two milligrams per milliliter or 2000 micrograms per milliliter.

c. Coumestrol

Due to the low solubility of this compound it was necessary to use 1000 milliliters of the solvent to dissolve 607.3 milligrams of coumestrol acetate (equivalent to 500 milligrams of coumestrol). This solution therefore contained only 500 micrograms of coumestrol per milliliter.

Preparation of the experimental diets and the concentration of the tested compounds ranging from 0.5 to 15.0 milligrams per mouse per three day period are summarized in Table V. The volume of the stock solution reported was added to 30 grams of the experimental diet.

In cases where the amount of stock solution to be added to the diet was less than 10 milliliters additional solvent was added to bring the volume to between 10 and 20 milliliters which ensured adequate dispersion of the compound in the ration. The solvent was removed by evaporation and the dry ration brought to 33 grams with control diet. The rations were well mixed and stored in a cool dark place until used.
Table V.

Values Used for the Preparation of the Experimental Diets.

<table>
<thead>
<tr>
<th>mgm comp'd /gm.</th>
<th>comp'd at 1.67 gm/day</th>
<th>mcgm consumed /3 days</th>
<th>mgm comp'd /6 mice</th>
<th>mgm consumed /3 days</th>
<th>ml stock sol'n /ration comp'd at 2000 prepared comp'ds</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>167.00</td>
<td>501.00</td>
<td>0.501</td>
<td>3.006</td>
<td>1.503</td>
</tr>
<tr>
<td>150</td>
<td>250.50</td>
<td>751.50</td>
<td>0.751</td>
<td>4.509</td>
<td>2.254</td>
</tr>
<tr>
<td>200</td>
<td>334.00</td>
<td>1,002.00</td>
<td>1.002</td>
<td>6.012</td>
<td>3.006</td>
</tr>
<tr>
<td>250</td>
<td>417.50</td>
<td>1,252.50</td>
<td>1.252</td>
<td>7.515</td>
<td>3.757</td>
</tr>
<tr>
<td>300</td>
<td>500.00</td>
<td>2,505.00</td>
<td>2.505</td>
<td>15.030</td>
<td>7.515</td>
</tr>
<tr>
<td>350</td>
<td>583.00</td>
<td>3,757.50</td>
<td>3.757</td>
<td>22.545</td>
<td>11.272</td>
</tr>
<tr>
<td>400</td>
<td>660.00</td>
<td>5,010.00</td>
<td>5.010</td>
<td>30.060</td>
<td>15.030</td>
</tr>
<tr>
<td>450</td>
<td>737.50</td>
<td>6,262.50</td>
<td>6.262</td>
<td>37.575</td>
<td>18.787</td>
</tr>
<tr>
<td>500</td>
<td>815.00</td>
<td>7,515.00</td>
<td>7.515</td>
<td>45.090</td>
<td>22.545</td>
</tr>
<tr>
<td>550</td>
<td>892.50</td>
<td>8,767.50</td>
<td>8.767</td>
<td>52.605</td>
<td>26.302</td>
</tr>
<tr>
<td>600</td>
<td>970.00</td>
<td>10,020.00</td>
<td>10.020</td>
<td>60.120</td>
<td>30.060</td>
</tr>
<tr>
<td>650</td>
<td>1,047.50</td>
<td>11,272.50</td>
<td>11.272</td>
<td>67.635</td>
<td>33.817</td>
</tr>
<tr>
<td>700</td>
<td>1,125.00</td>
<td>12,525.00</td>
<td>12.525</td>
<td>75.150</td>
<td>37.575</td>
</tr>
<tr>
<td>750</td>
<td>1,202.50</td>
<td>13,777.50</td>
<td>13.777</td>
<td>82.665</td>
<td>41.332</td>
</tr>
<tr>
<td>800</td>
<td>1,280.00</td>
<td>15,030.00</td>
<td>15.030</td>
<td>90.180</td>
<td>45.090</td>
</tr>
</tbody>
</table>

* As the stock solution of coumestrol contained only 500 mcgm/ml it is necessary to multiply the reported values for this compound times four.

(1) B-biochanin A, C-coumestrol, D-daidzein, F-formononetin, G-genistein.

N.B. Omission of several levels of daidzein was necessary because of the limited amount available. Two additional levels of formononetin were added to substantiate the graph at intermediate points.
IV. The Preparation of Rations Containing Two or More Isoflavones

Due to the insufficient quantities of daidzein it was necessary to exclude this compound from the study of the effect of two or more of the isoflavones fed simultaneously. The following combinations were added to the control diet as before, each at a level of 2,500 mcgm per gram of ration or 37.57 milliliters of each of the stock solutions. This high level was chosen because all compounds at this point had displayed some evidence of an estrogen-like activity.

a. Genistein (2500 mcgm/gm) plus Biochanin A (2500 mcgm/gm)
b. Biochanin A (2500 mcgm/gm) plus Formononetin (2500 mcgm/gm)
c. Formononetin (2500 mcgm/gm) plus Genistein (2500 mcgm/gm)
d. Biochanin A (2500 mcgm/gm) plus Formononetin (2500 mcgm/gm) plus Genistein (2500 mcgm/gm)

All bioassays involved groups of six immature female mice. The animals were housed in circular aluminum pans and water provided ad libitum. The experimental diet was fed in three equal lots once daily for three days.

V. The Preparation of Red Clover Extracts for Bioassay

Throughout the 1961 growing season a plot consisting of seven rows of red clover in the second year of growth was maintained in the Agronomy fields of the Division of Plant Science at the University of British Columbia. The plot was irrigated regularly but did not receive any fertilizer during the experimental period. The rows were 20 feet long and a foot and a half apart. Each row contained approximately 40 plants. At monthly intervals a row was harvested with hand shears and a representative sample of the fresh forage taken. A small sample of the fresh
clover was removed and a dry weight determination carried out. The sample size listed in Table VI was immediately chopped into short lengths and macerated for three minutes with a total of five liters of acetone in a Waring Blender. It was found that this step is best carried out in three lots each utilizing 1.5 liters of acetone and a portion of the red clover sample. The progressively decreasing sample size takes into account the increase in dry weight of the plant. The acetone solution was removed by suction filtration and the filter pack discarded. The extract was then concentrated under vacuum to approximately 200 to 250 milliliters. The concentrated solution was transferred to a separatory funnel and repeatedly extracted with 200 milliliters portions of benzene.

<table>
<thead>
<tr>
<th>Date</th>
<th>Row number</th>
<th>Sample size (wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 10, 1961</td>
<td>5</td>
<td>1,000 grams</td>
</tr>
<tr>
<td>June 12, 1961</td>
<td>2</td>
<td>500 grams</td>
</tr>
<tr>
<td>July 11, 1961</td>
<td>1</td>
<td>400 grams</td>
</tr>
<tr>
<td>August 9, 1961</td>
<td>3</td>
<td>350 grams</td>
</tr>
<tr>
<td>September 11, 1961</td>
<td>4</td>
<td>300 grams</td>
</tr>
</tbody>
</table>

This step removed the chlorophyll fraction and other extraneous material leaving a clear brown solution. The benzene layer has been shown to contain no estrogenic material and consequently may be discarded (79). The benzene extracted solution was further concentrated to a volume of approximately 50 milliliters and stored in the refrigerator prior to use.
Based on the percentage dry weight of the fresh sample the concentrated extract was added to the control diet such that one gram of feed contains the extract of one gram of the dried clover. The diet was dried, mixed, brought to 33 grams and stored in a cool dark place prior to use. The remainder of the concentrated extract was retained for studies involving the electrophoretic identification of isoflavones in plant extracts.

VI. The Electrophoresis Technique

Advances in the use of electrophoresis as a means of identification led the author to investigate the possibilities of such in the isoflavone series.

A. The Power Supply and Electrophoresis Cabinet

The power supply used is a commercially produced unit obtained from the Research Specialties Limited Company of Richmond, California, model 1911, "Electrophoresis Power Supply". The unit is operated from a 115 volt A.C. outlet and has an output range of 0 to 750 volts D.C. with 0 to 200 milliamperes.

The electrophoresis cabinet is constructed entirely of 1/4 and 3/16 inch plexiglass. The water cooled bed (13 x 18 inches) is quarter-rounded at the ends such that the paper drapes into a multicompartment buffer trough (13 x 3 1/2 x 2 1/2 inches) in which rests a 1/4 inch carbon plate electrode. The design of the cabinet is given in Figure II. The outer protective cover is fitted onto the base at a limit switch connection such that removal of the cover immediately disconnects the power flow irregardless of the state in the power source.

B. The Electrophoresis Paper

Several weights of paper were considered in this study in an attempt
FIGURE II

The Electrophoresis Cabinet

Horizontal view

Vertical view
Scale 1 inch = 4 inches
to determine which gave optimum compound movement, wet strength, ease of handling and minimal resistance. The size of sheet used was 9 x 21 inches, the weights considered, Whatman Number 1, 3 and 4.

C. The Buffer Systems

A number of buffers were prepared and preliminary experiments were designed to determine which produced the optimum movement and separation of the isoflavone compounds. Two liters of each of the following solutions were prepared:

a. Acetate buffer (45)
   8.8 ml of 0.2M acetic acid plus 11.2 ml of 0.2M sodium acetate diluted to 100 ml. Final pH 5.3.

b. Tris (hydroxymethyl)aminomethane maleate (Tris-maleate) buffer (45)
   50 ml of 0.2M Tris acid maleate (24.2 gm Tris plus 23.2 gm maleic acid per 1000 ml) plus 54 ml of 0.2M sodium hydroxide diluted to 200 ml. Final pH 7.4.

c. Ammonium carbonate
   15.7 gm per liter. Final pH 8.8.

d. Boric acid-borax (45).
   50 ml of 0.2M boric acid plus 115 ml of 0.5M borax (0.2M in terms of sodium borate) diluted to 200 ml. Final pH 9.2.

e. Borax-sodium hydroxide (45)
   50 ml of 0.05M borax (0.2M in terms of sodium borate) plus 46 ml of 0.2M sodium hydroxide diluted to 200 ml. Final pH 10.1.

f. Carbonate-bicarbonate buffer (45)
   45 ml of 0.2M anhydrous sodium carbonate plus 5 ml of 0.2M sodium bicarbonate diluted to 200 ml. Final pH 10.7.
g. Borax-sodium hydroxide

50 ml of 0.05M borax plus 50 ml of 0.2M sodium hydroxide diluted to 200 ml. Final pH 11.6.

D. The Biological Material

The biological material used in this study was that prepared for the biological assay of red clover. The extract remaining after the biological assay will hereafter be referred to as the crude extract.

The crude extract was further concentrated under vacuum to a volume such that one milliliter contained the extract of four grams of dried clover. This preparation was then refrigerated. Upon extended refrigeration, or temporary placement in a deep freeze, extraneous matter of a wax-like consistency settled out and accumulated on the sides and bottom of the vessel. A cold one milliliter sample of the extract was carefully removed from the center of the preparation by pipette, transferred to a test tube and extracted by shaking with one milliliter of diethyl ether. The ether layer was removed and the procedure repeated. The combined ether layers were concentrated to 0.5 milliliter containing the extract from four grams of clover. This preparation constituted a "pure extract" and was used as such in the electrophoresis studies. Extracts for samples of May, June, July, August and September forages were prepared.

It was felt that electrophoretic comparisons would best be made with samples of isoflavones isolated from red clover and not with those of largely synthetic origin used in the bioassay studies. It was possible to obtain such a set of samples and the following standards were prepared. The solvent in all cases was 80 per cent ethanol-acetone (4:1).

(a) Genistein, 4.5 mg/ml; (b) Biochanin A, 4.5 mg/ml; (c) Daidzein,
4.0 mgm/10 ml; (d) Formononetin, 10.0 mgm/10 ml; (e) Coumestrol, 4.0 mgm/10 ml. A mixed standard was prepared by combining one milliliter of each of the above.

E. The Electrophoresis Procedure

The standard solutions were applied onto the filter paper using capillary tubes, with a 0.4 millimeter inside diameter, at a line marked at one-half the length of the sheet. At a later point, once direction of movement had been determined, the origin was moved to a line 1 1/4 inches from the end of the page to allow a greater distance for spot migration and separation. The applied standard solutions were dried by means of a forced hot air drier. A five millimeter diameter spot of the applied solutions was used as nearly as possible throughout the study. The paper was then dipped in a dish containing the buffer solution in such a manner that the applied spots were moistened by capillary movement of the buffer in the paper. The moistened sheet was then placed on the water cooled bed of the electrophoresis cabinet. Papers with the origin 1 1/4 inches from the end were placed such that the applied compounds were proximal to the negative pole, spot migration being towards the positive electrode. Contact with the electrolytes in the buffer troughs was made by dipping the overhanging ends of the paper directly into the solution. The paper was then blotted, using a second sheet of filter paper and a tissue pad, to remove excess moisture and dissipate air pockets which alter the pattern of flow. It is important to maintain a relatively constant degree of wetness over the paper and consequently the blotting pressure must be evenly applied. The cabinet cover was placed in position and the current applied. A number of varying voltages and running periods were tried to obtain the optimum of separation between the standards.
Upon completion of the run the power was turned off and the ends of the sheet in contact with the buffer cut away from the rest of the paper. Removal of the wet ends eliminated dripping and possible spot distortion during drying. The paper was hung in a forced air drying oven and dried at 100°C for 25 minutes.

F. Detection of the Position of the Compounds

Trials were carried out with a number of chromogenic sprays which have been reported to give distinctive color reactions with flavonoid type compounds. The sprays considered were as follows:

a. Aqueous sodium hydroxide and heat
b. Ethanolic aluminum chloride
c. Perchloric acid and ferric chloride
d. Ferric chloride in sulfuric acid
e. Ethanolic one per cent sodium nitrite
f. Five per cent perchloric acid and sodium nitrite
g. Five per cent methanolic ferric chloride
h. Neutral lead acetate
i. Basic lead acetate
j. Five per cent etherial lithium aluminum hydride and dilute hydrochloric acid
k. Diazotized sulfanilic acid in ten per cent sodium carbonate

The sprays were applied to the dried electrophoresis papers using a "Universal Aerosol Spray Kit" prepared by the Nutritional Biochemicals Corporation of Cleveland, Ohio.

As well as possible chromogenic developers, the fluorescent character of the compounds in ultraviolet light was observed in a chromatoview cabinet at wavelengths of 253 and 366 millimicrons.
RESULTS AND DISCUSSION

I. The Bioassay of Genistein, Biochanin A, Daidzein, Formononetin and Coumestrol

The fact that different workers, interested in the estrogen-like substances in plants, have in many cases developed and advocated the use of a particular assay procedure, has led to some lack of uniformity in the reported estrogen-like potencies for different compounds. There is also an evident lack in the literature of work relating to a number of compounds, as studied by one procedure, over any extended range of dosages. In many cases a particular compound, for example genistein, has been reported in the literature as simply being active or inactive at a given level (for example active at the one milligram level) (32). It would appear that no one has considered any one compound over an extended range of values to determine if the response of the uterus to the estrogen-like stimulation increases as concentration increases or if such an effect bears some relation to that produced by other isoflavones over the same dosage range.

In this study the estrogen-like activity of genistein, biochanin A, daidzein, formononetin and coumestrol was determined at total dosages of 0.5 to 15.0 milligrams per mouse.

The Control Diet

Immature female mice fed the control ration were found to have an average uterine weight of 0.17± expressed as a per cent of body weight. The standard error of estimate was ± 0.010. This value is similar to that observed by Ostrovsky in 1960 (78) and Kitts et al. in 1959 (65).
both of whom used the same strain of mice and the same control ration. As this value has been shown to vary slightly by other workers at this laboratory the author felt that the average uterine weight would best be expressed in terms of a range existing between 0.16 and 0.19 per cent (Figures I, II and III). During the present study animals of this strain (Swiss Albino), age and weight did not exhibit values above or below this range.

Genistein

It has been reported that genistein elicits an estrogen-like response when administered per orum at a level of one milligram to the immature female mouse (32). This study would confirm this fact in that the first signs of uterine stimulation, as measured by an increase in weight, was observed at the one milligram level and thereafter increased as the dosage was increased to the 15 milligram level. However when a dose response equation is derived for the present data the first definite stimulation appears to occur at the 1.75 milligram level. The response has been expressed in terms of uterine weight as a per cent of body weight because this affords an opportunity to correct for the slight fluctuations arising from differences in body weights among the assay animals. For example, the dose response line is constructed on the basis of dose per mouse rather than dose per unit mouse, hence some of the variation in the assay values is probably due to the fact that mice above the mean weight were receiving relatively less of the effective agent and those below relatively more; this is assuming feed consumption was equal within the group. Data obtained from the bioassay of genistein is presented in Table VII. The calculated equation was found to be $Y=0.0209x + 0.1651$ between 0 and 15 milligram total dosages,
when \( x \) equals the milligrams per mouse and \( Y \) the uterine weight as a percentage of body weight. It is realized that it may be somewhat erroneous to force the observed increases to the conformity of an arithmetic relationship. The response may in fact be other than arithmetic. The correlation coefficient, \( r = 0.973 \), is highly significant \((P < 0.01)\) which may tend to indicate that the arithmetic relationship is valid, however, the possibility of the response not being arithmetic can not be disregarded. The equation and points from which it was calculated have been expressed graphically in Figure III.

Biochanin A

Biochanin A has been reported as possessing an estrogen-like potency similar to that of genistein (\( \text{II}\)). The results of this study would indicate that the estrogen-like effect of biochanin A begins to exert itself at the five milligram level and continually increases as the dosage increases to the 15 milligram level, though to a much lesser extent than the increase observed for genistein. Consequently this study would indicate that biochanin A and genistein do not exert a quantitatively similar estrogenic response and is contrary to the results reported by Cheng et al. in 1955 (\( \text{II}\)). The data obtained from the bioassay is presented in Table VIII, the calculated equation being \( Y = 0.0093x + 0.1414 \), between 3.76 and 15.0 milligram total dosages, when \( x \) equals the milligrams per mouse and \( Y \) the uterine weight as a percentage of body weight. Here again the imposition of an arithmetic relationship on the data may be a misrepresentation. The correlation coefficient, \( r = 0.738 \), \((0.01 < P < 0.05)\) however would still indicate that an arithmetic relationship may be valid. The equation and points from which it was calculated has been graphically presented in Figure III.
Table VII.

Results of the Bioassay of Genistein

<table>
<thead>
<tr>
<th>Level mgm/gm</th>
<th>Mgm/mouse/3 day assay</th>
<th>No. of mice/group</th>
<th>Av. body wt. (gm)</th>
<th>Av. uterine wt. (mgm)</th>
<th>Uterine wt. as % B.W.</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.50</td>
<td>6</td>
<td>10.70</td>
<td>17.38</td>
<td>0.163</td>
<td>0.010</td>
</tr>
<tr>
<td>150</td>
<td>0.75</td>
<td>6</td>
<td>10.41</td>
<td>17.08</td>
<td>0.164</td>
<td>0.017</td>
</tr>
<tr>
<td>200</td>
<td>1.00</td>
<td>6</td>
<td>11.10</td>
<td>21.27</td>
<td>0.191</td>
<td>0.009</td>
</tr>
<tr>
<td>250</td>
<td>1.25</td>
<td>5</td>
<td>10.24</td>
<td>16.94</td>
<td>0.166</td>
<td>0.015</td>
</tr>
<tr>
<td>500</td>
<td>2.51</td>
<td>6</td>
<td>10.75</td>
<td>24.52</td>
<td>0.228</td>
<td>0.013</td>
</tr>
<tr>
<td>750</td>
<td>3.76</td>
<td>6</td>
<td>10.67</td>
<td>24.40</td>
<td>0.228</td>
<td>0.021</td>
</tr>
<tr>
<td>1,000</td>
<td>5.01</td>
<td>6</td>
<td>10.26</td>
<td>30.12</td>
<td>0.293</td>
<td>0.014</td>
</tr>
<tr>
<td>1,250</td>
<td>6.26</td>
<td>5</td>
<td>10.75</td>
<td>32.53</td>
<td>0.302</td>
<td>0.028</td>
</tr>
<tr>
<td>1,500</td>
<td>7.52</td>
<td>6</td>
<td>11.13</td>
<td>36.65</td>
<td>0.329</td>
<td>0.011</td>
</tr>
<tr>
<td>1,750</td>
<td>8.77</td>
<td>6</td>
<td>10.56</td>
<td>42.53</td>
<td>0.403</td>
<td>0.016</td>
</tr>
<tr>
<td>2,000</td>
<td>10.02</td>
<td>6</td>
<td>10.76</td>
<td>39.86</td>
<td>0.371</td>
<td>0.017</td>
</tr>
<tr>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>11.33</td>
<td>44.98</td>
<td>0.397</td>
<td>0.018</td>
</tr>
<tr>
<td>3,000</td>
<td>15.03</td>
<td>6</td>
<td>11.24</td>
<td>51.99</td>
<td>0.462</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Calculated equation \( Y = 0.0209x + 0.1651 \) (94)
\( (x = \text{mgm/mouse/3 days} \ Y = \text{uterine wt. as } \% \text{ B.W.}) \)

\( r = 0.973 \) (\( P < 0.01 \))
Table VIII

Results of the Bioassay of Biochanin A.

<table>
<thead>
<tr>
<th>Level mgm</th>
<th>Mgm/mouse/3 day assay</th>
<th>No. of mice/group</th>
<th>Av. body wt. (gm)</th>
<th>Av. uterine wt. (mgm)</th>
<th>Uterine wt. as % B.W.</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>750</td>
<td>3.76</td>
<td>6</td>
<td>11.06</td>
<td>17.77</td>
<td>0.161</td>
<td>0.010</td>
</tr>
<tr>
<td>1,000</td>
<td>5.01</td>
<td>6</td>
<td>10.42</td>
<td>21.25</td>
<td>0.164</td>
<td>0.012</td>
</tr>
<tr>
<td>1,250</td>
<td>6.26</td>
<td>5</td>
<td>10.35</td>
<td>17.73</td>
<td>0.163</td>
<td>0.013</td>
</tr>
<tr>
<td>1,500</td>
<td>7.52</td>
<td>6</td>
<td>10.93</td>
<td>25.69</td>
<td>0.236</td>
<td>0.018</td>
</tr>
<tr>
<td>1,750</td>
<td>8.77</td>
<td>6</td>
<td>11.30</td>
<td>26.18</td>
<td>0.231</td>
<td>0.013</td>
</tr>
<tr>
<td>2,000</td>
<td>10.02</td>
<td>6</td>
<td>11.14</td>
<td>28.26</td>
<td>0.253</td>
<td>0.033</td>
</tr>
<tr>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>10.93</td>
<td>32.87</td>
<td>0.301</td>
<td>0.021</td>
</tr>
<tr>
<td>3,000</td>
<td>15.03</td>
<td>6</td>
<td>11.09</td>
<td>26.17</td>
<td>0.235</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Calculated equation \( Y = 0.0093x + 0.1441 \) (94).

\( x = \text{mgm/mouse/3 days} \), \( Y = \text{uterine wt. as % B.W.} \)

\( r = 0.738 \) \((0.01 < P < 0.05)\)
FIGURE III

The Uterine Response to Increasing Levels of Genistein and Biochanin A in the Laboratory Mouse

Uterine weight as a % of body weight

Genistein

Biochanin A

Control range

mgms/mouse/3 day assay period
Coumestrol

In the case of coumestrol the uterine response to the estrogen-like stimulation was not arithmetic, this response being the greatest of all substances considered. The data obtained has been reported in Table IX and graphically presented in Figure IV. The calculated equation was found to be \( Y = 0.133\% \log x + 0.137 \), between 0 and 15.0 milligrams total dosage, when \( x \) equals the milligrams per mouse and \( Y \) the uterine weight as a per cent of body weight. The correlation coefficient, \( r = 0.96 \), \( P < 0.01 \), would tend to verify this relationship. Coumestrol has been previously reported as being 30 times more estrogenically potent than genistein. The results of this study would indicate that such a relationship does in fact exist but only at the lower dosage levels. As the concentration of the compounds increases the uterine response to genistein increases linearly to a uterine weight as a per cent body weight of 0.46, while that to coumestrol tends to plateau at 0.52. At the 10 milligram level coumestrol exerts twice the estrogenic potency of genistein while at the 15.0 milligram level the potency is only 1.25 times that of genistein. Therefore as the amount of genistein administered increases, the uterine response approaches that observed for coumestrol at the same level. It would appear that the limiting response observed with dosages greater than eight milligrams (a uterine weight as per cent of body weight of 0.54) is probably the result of the limited expansive character of the uterine epithelium and not resulting from a decreased sensitivity to coumestrol.

Daidzein

Daidzein has been previously reported as being the isoflavone exhibiting
Table IX.

Results of the Bioassay of Coumestrol

<table>
<thead>
<tr>
<th>Level mgm/gm</th>
<th>Mgm/mouse/3 day assay</th>
<th>No. of mice/group</th>
<th>Av. body wt. (gm)</th>
<th>Av. uterine wt (mgm)</th>
<th>Uterine wt. as % B.W.</th>
<th>Standard error ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.50</td>
<td>6</td>
<td>10.04</td>
<td>31.90</td>
<td>0.347</td>
<td>0.031</td>
</tr>
<tr>
<td>150</td>
<td>0.75</td>
<td>6</td>
<td>10.29</td>
<td>43.52</td>
<td>0.422</td>
<td>0.023</td>
</tr>
<tr>
<td>250</td>
<td>1.25</td>
<td>6</td>
<td>10.09</td>
<td>61.50</td>
<td>0.411</td>
<td>0.026</td>
</tr>
<tr>
<td>500</td>
<td>2.51</td>
<td>6</td>
<td>10.36</td>
<td>67.56</td>
<td>0.458</td>
<td>0.019</td>
</tr>
<tr>
<td>750</td>
<td>3.76</td>
<td>6</td>
<td>10.17</td>
<td>52.90</td>
<td>0.520</td>
<td>0.027</td>
</tr>
<tr>
<td>1,000</td>
<td>5.01</td>
<td>6</td>
<td>10.31</td>
<td>51.48</td>
<td>0.499</td>
<td>0.030</td>
</tr>
<tr>
<td>1,250</td>
<td>6.26</td>
<td>6</td>
<td>11.04</td>
<td>58.25</td>
<td>0.527</td>
<td>0.027</td>
</tr>
<tr>
<td>1,500</td>
<td>7.52</td>
<td>6</td>
<td>9.84</td>
<td>52.03</td>
<td>0.528</td>
<td>0.013</td>
</tr>
<tr>
<td>1,750</td>
<td>8.77</td>
<td>6</td>
<td>10.13</td>
<td>54.65</td>
<td>0.539</td>
<td>0.020</td>
</tr>
<tr>
<td>2,000</td>
<td>10.02</td>
<td>6</td>
<td>9.82</td>
<td>54.93</td>
<td>0.559</td>
<td>0.031</td>
</tr>
<tr>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>11.14</td>
<td>59.55</td>
<td>0.534</td>
<td>0.021</td>
</tr>
<tr>
<td>3,000</td>
<td>15.03</td>
<td>6</td>
<td>11.30</td>
<td>65.04</td>
<td>0.575</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Calculated equation $Y = 0.1334 \log x + 0.4137 \ (g/l).$

($x = \text{mgm/mouse/3 days} \ Y = \text{uterine wt. as \% B.W.}$)

$r = 0.96 \quad (P < 0.01)$
the greatest estrogen-like potency (IU). The results obtained indicate that daidzein does in fact elicit an estrogen-like response at concentrations lower than that of the other isoflavones, however as the dosage administered increases, the uterine response does not increase to the extent of that obtained with genistein. The slope of the calculated arithmetic equation for daidzein is less than that of genistein. Therefore daidzein demonstrates a greater estrogen-like activity than genistein, which decreases in extent to the 3.5 milligram level and thereafter shows progressively less potency than does genistein. The data obtained from the bioassay of daidzein are presented in Table I and have been graphically presented in Figure IV. The calculated equation was found to be 
\[ Y = 0.0151x + 0.1872, \]
between 0 and 12.5 milligram total dosages, when \( x \) equals the milligrams per mouse and \( Y \) the uterine weight as a per cent of body weight. The correlation coefficient, \( r = 0.974, P < 0.01 \), would indicate an arithmetic relationship exists but here again such an interpretation may be a misrepresentation of the true response.

Formononetin

This compound has been reported in the literature as possessing little or no estrogen-like character (IU). In this study it was found that no estrogen-like stimulation occurred until the compound was administered at the 10.68 milligram level and thereafter this response increased gradually as the dosage increased to the 15 milligram level. Consequently the compound does possess an estrogen-like character which is of very low potency and observed only when the dosage is relatively high. The data from this assay are presented
Table X

Results of the Bioassay of Daidzein

<table>
<thead>
<tr>
<th>Level mgm/gm</th>
<th>Mgm/mouse/3 day assay</th>
<th>No. of mice/group</th>
<th>Av. body wt. (gm)</th>
<th>Av. uterine wt. (mgm)</th>
<th>Uterine wt. as % B.W.</th>
<th>Standard error ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.50</td>
<td>6</td>
<td>10.24</td>
<td>18.42</td>
<td>0.179</td>
<td>0.013</td>
</tr>
<tr>
<td>150</td>
<td>0.75</td>
<td>6</td>
<td>10.76</td>
<td>21.25</td>
<td>0.197</td>
<td>0.016</td>
</tr>
<tr>
<td>200</td>
<td>1.00</td>
<td>6</td>
<td>10.62</td>
<td>22.89</td>
<td>0.216</td>
<td>0.009</td>
</tr>
<tr>
<td>250</td>
<td>1.25</td>
<td>6</td>
<td>10.45</td>
<td>23.09</td>
<td>0.221</td>
<td>0.020</td>
</tr>
<tr>
<td>500</td>
<td>2.51</td>
<td>6</td>
<td>10.32</td>
<td>21.63</td>
<td>0.209</td>
<td>0.010</td>
</tr>
<tr>
<td>750</td>
<td>3.76</td>
<td>6</td>
<td>10.64</td>
<td>28.60</td>
<td>0.268</td>
<td>0.019</td>
</tr>
<tr>
<td>1,000</td>
<td>5.01</td>
<td>6</td>
<td>10.07</td>
<td>25.62</td>
<td>0.254</td>
<td>0.018</td>
</tr>
<tr>
<td>1,500</td>
<td>7.52</td>
<td>6</td>
<td>10.49</td>
<td>29.67</td>
<td>0.282</td>
<td>0.011</td>
</tr>
<tr>
<td>2,000</td>
<td>10.02</td>
<td>6</td>
<td>9.89</td>
<td>34.37</td>
<td>0.347</td>
<td>0.026</td>
</tr>
<tr>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>10.39</td>
<td>39.67</td>
<td>0.381</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Calculated equation \( Y = 0.0151x + 0.1872 \) (94).

\( x = \text{mgm/mouse/3 days} \) \( Y = \text{uterine wt. as % B.W.} \)

\( r = 0.974 \) \( (P < 0.01) \)
Table XI

Results of the Bioassay of Formononetin

<table>
<thead>
<tr>
<th>Level mgm/gm</th>
<th>Mgm/mouse/3 day assay</th>
<th>No. of mice/group</th>
<th>Av. body wt. (gm)</th>
<th>Av. uterine wt. (mgm)</th>
<th>Uterine wt. as % B.W.</th>
<th>Standard error</th>
<th>Calculated equation: ( Y = 0.0119x + 0.0502 ) (94).</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,250</td>
<td>11.27</td>
<td>5</td>
<td>10.68</td>
<td>20.42</td>
<td>0.190</td>
<td>0.014</td>
<td>(x = mgm/mouse/3 days ( Y = ) uterine wt. as % B.W.)</td>
</tr>
<tr>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>11.15</td>
<td>21.55</td>
<td>0.193</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>2,750</td>
<td>13.78</td>
<td>6</td>
<td>9.77</td>
<td>20.49</td>
<td>0.209</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>3,000</td>
<td>15.03</td>
<td>5</td>
<td>11.26</td>
<td>26.47</td>
<td>0.235</td>
<td>0.015</td>
<td></td>
</tr>
</tbody>
</table>

\( r = 0.95 \) \( P = 0.05 \)
The Uterine Response to Increasing Levels of Coumestrol Daidzein and Formononetin in the Laboratory Mouse

FIGURE IV

Uterine weight as a % of body weight

Coumestrol

Daidzein

Formononetin

Control range

Mgm/mouse/3 day assay period
in Table XI and have been graphically presented in Figure IV. The
calculated equation was found to be \( Y = 0.0119x + 0.0502 \), between 11.27
and 15.03 milligram total dosages, when \( x \) equals the milligrams per mouse
and \( Y \) the uterine weight as a per cent of body weight. The correlation
coefficient, \( r = 0.95 \), \( P = 0.05 \), would indicate an arithmetic relationship,
however this may again not be a true representation of the response.

Discussion-general

The response of the five compounds considered is illustrated in
Figure V for relative comparisons. The results of this study appear to
contradict several statements which exist in the literature pertaining
to the estrogenicity of the isoflavones. It is evident that all of the
compounds considered possess an estrogen-like activity to greater or
lesser extents over the range of the dosages considered in this study.
Contrary to the work of Cheng et al. in 1955, genistein possesses
a markedly greater estrogenic potency than does biochanin A. These
workers stated that the two compounds exhibited highly similar estrogen-
like potencies, and that daidzein possessed the greatest estrogenic
potency within the isoflavones considered. The present study indicates
that the conclusions made by Cheng and coworkers are only valid at low
dosages (< 3.5 milligrams) and that at higher levels genistein is by far the
more potent of the four isoflavones.

It would have been desirable to assay each individual compound out
to a dose level at which the dose line had reached its approximate
asymptote in order to establish the upper limit of the response. Unfortu-
nately the quantities of these relatively rare compounds were not
adequate to permit such detailed studies of the dose response relationships.
The response of the mouse uterus to increasing levels of estradiol-like compounds is illustrated in the figure. The graph depicts the uterine weight as a percentage of body weight over time. The equations for the lines are given as follows:

- Formononetin: $y = 0.0119x + 0.0502$
- Biochanin A: $y = 0.0093x + 0.141$
- Genistein: $y = 0.015x + 0.1872$
- Conjugestrol: $y = 0.1344 \log x + 0.4137$

These equations were calculated based on observed data.
II. The Results of the Bioassay of Isoflavone Mixtures

Since the estrogen-like activity of forage plants consumed by grazing animals may possibly be due to the presence of one or more isoflavones in the forage it is of great importance to determine if inhibitory or synergistic effects exist between the various compounds. As mentioned earlier the supply of compounds available precluded the conduct of the extensive bioassays necessary to precisely determine the nature and extent of such effects if they exist. As a preliminary to future work a screening assay was conducted with combinations of genistein, biochanin A and formononetin. An arbitrary selection of dosage level had to be made as no existing literature permitted a reasoned selection of dosage levels.

Stock solutions were mixed together in the experimental diets such that each compound considered in the mixture was present at a dosage of 12.5 milligrams per mouse per three day assay. This level was chosen as all compounds had demonstrated a definitive estrogen-like activity at this concentration in the individual assay studies. The results of this study are presented in Table XIX.

The mixture of genistein and biochanin A, at the amounts used, produced an estrogen-like response which was approximately midway between the responses observed for the individual compounds. This would indicate either, that biochanin A exerts an inhibitory effect on the extent of the uterine response normally observed after administration of genistein, or that the combined dosage was so far in excess that the response was partially reversed. This latter possibility seems unlikely. Biochanin A and formononetin fed simultaneously produced a uterine response greater
Table XII

Results of the Bioassay of Pure Isoflavone Mixtures

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Level mcgm/gm</th>
<th>Mgm/mouse 3 day assay</th>
<th>No. of mice/group</th>
<th>Av. body wt. (gm)</th>
<th>Av. uterine wt. (mgm)</th>
<th>Uterine wt. as % B.W.</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>10.28</td>
<td>33.93</td>
<td>0.330</td>
<td>0.011</td>
</tr>
<tr>
<td>B</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>10.70</td>
<td>33.95</td>
<td>0.317</td>
<td>0.032</td>
</tr>
<tr>
<td>B</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>10.70</td>
<td>33.95</td>
<td>0.317</td>
<td>0.032</td>
</tr>
<tr>
<td>F</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>10.56</td>
<td>46.68</td>
<td>0.423</td>
<td>0.019</td>
</tr>
<tr>
<td>B</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>11.33</td>
<td>44.98</td>
<td>0.397</td>
<td>0.018</td>
</tr>
<tr>
<td>B</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>11.33</td>
<td>44.98</td>
<td>0.397</td>
<td>0.018</td>
</tr>
<tr>
<td>F</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>11.15</td>
<td>21.55</td>
<td>0.193</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Individual Response

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Level mcgm/gm</th>
<th>Mgm/mouse 3 day assay</th>
<th>No. of mice/group</th>
<th>Av. body wt. (gm)</th>
<th>Av. uterine wt. (mgm)</th>
<th>Uterine wt. as % B.W.</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>11.33</td>
<td>44.98</td>
<td>0.397</td>
<td>0.018</td>
</tr>
<tr>
<td>B</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>10.93</td>
<td>32.87</td>
<td>0.301</td>
<td>0.021</td>
</tr>
<tr>
<td>F</td>
<td>2,500</td>
<td>12.53</td>
<td>6</td>
<td>11.15</td>
<td>21.55</td>
<td>0.193</td>
<td>0.012</td>
</tr>
</tbody>
</table>

G = genistein, B = Biochanin A, F = formononetin.
than that observed when either of the compounds were fed individually. Such a result suggests that formononetin possesses a synergistic character. Supporting evidence to this would be that formononetin (of individual low potency) when fed with genistein produced a response which was slightly greater than that observed when genistein was fed alone. The mixture of genistein, formononetin and biochanin A produced a response slightly below that of pure genistein but well above that observed for pure formononetin; pure biochanin A; the mixture of formononetin and biochanin A; and the mixture of genistein and formononetin. This result would appear to indicate that formononetin exerts a synergistic effect which is great enough to overcome or counteract the inhibitory effect of biochanin A and produce a result approximating that observed for pure genistein. These preliminary results must be interpreted with a great deal of reservation and should be supported by much more extensive assays than have been possible in the present work.

III. The Results of the Bioassay of Red Clover

The results of the bioassay of the red clover, harvested monthly, were similar to those previously observed by workers at this laboratory (66). The estrogen-like activity was greatest in the early summer and remained relatively high until July, after which a decrease was observed until September at which time insignificant estrogen-like potency existed in the sample material. The results of this study have been presented in Table XIII and graphically presented in Figure VI. The association of electrophoretic studies to this study have been considered at a later point.
Table XIII

Results of the Bioassay of Red Clover and the Compounds Identified in the Monthly Extract*

<table>
<thead>
<tr>
<th>Date</th>
<th>No. of mice/group</th>
<th>Av. body wt. (gm)</th>
<th>Av. uterus wt. (mgm)</th>
<th>Uterine wt. as % B.W.</th>
<th>Standard error ±</th>
<th>Compds in extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 10</td>
<td>6</td>
<td>10.93</td>
<td>51.6b</td>
<td>0.472</td>
<td>0.017</td>
<td>F,D,G</td>
</tr>
<tr>
<td>June 12</td>
<td>6</td>
<td>10.85</td>
<td>39.6b</td>
<td>0.365</td>
<td>0.013</td>
<td>F,D,G</td>
</tr>
<tr>
<td>July 11</td>
<td>6</td>
<td>10.22</td>
<td>48.52</td>
<td>0.574</td>
<td>0.025</td>
<td>F,D,G</td>
</tr>
<tr>
<td>Aug. 9</td>
<td>4</td>
<td>11.21</td>
<td>26.30</td>
<td>0.234</td>
<td>0.013</td>
<td>F,D</td>
</tr>
<tr>
<td>Sept. 11</td>
<td>6</td>
<td>10.53</td>
<td>21.32</td>
<td>0.202</td>
<td>0.018</td>
<td>F,D,G</td>
</tr>
</tbody>
</table>

* Extracts assayed such that one gram of feed contained the contents of one gram of dry clover.

F - formononetin, D - daidzein, G - genistein.
FIGURE VI

The Uterine Response of the Laboratory Mouse to Monthly Harvested Red Clover Samples

Date of Harvest

Uterine weight as a % of body weight

Control range

May 10  June 12  July 11  August 9  September 11
IV. The Electrophoresis Studies

The use of paper electrophoresis as a method for the identification of isoflavones as an alternative to the use of chromatography was investigated and the following observations made.

The standard solutions of naturally isolated isoflavones, described under the section Materials and Methods, were used to develop the optimum conditions for the procedure. The standard solutions were genistein, daidzein, biochanin A, formononetin, coumestrol and a composite mixture of these five compounds. Buffer solutions ranging in pH from 5.3 to 11.6 (Materials and Methods) were tested to determine which would produce the optimum separation and rate of movement. The compounds migrated towards the positive electrode, the migration rate and separation improving as the alkalinity of the buffer systems increased. Migration was extremely restricted at pH values below nine. The optimum migration was observed using a Borax-sodium hydroxide buffer of pH 11.6 containing 0.1M sodium borate and 0.1M sodium hydroxide. In the course of a number of trials it was found that further dilution of the buffer to 0.05M sodium borate and 0.05M sodium hydroxide produced a slower and more even migration of the compounds. This dilution was not associated with any changes of the pH. Voltages ranging from 625 to 750 D.C. were utilized. This variation did not alter the relative separation of the isoflavones but it was found that at lower voltages the streaking often observed in mixed plant extracts was reduced and more definitive spots observed following migration. During the course of a run the milliamperage increased from 15 to 20 mA. The fluorescent character of the compounds, which was used as a means of detection, was enhanced by the presence of sodium hydroxide.
in the buffer solution.

Whatman number three filter paper was found to be most suitable as this grade allowed good migration of the compounds and possessed superior wet strength which facilitated handling of the moistened sheets.

Detection of the Spot Position

The electrophoresis sheets were dried and placed in a "chromatoview" ultraviolet viewing apparatus for observation. At a wavelength of 253 millimicrons daidzein and formononetin produced a white fluorescence which was enhanced by the presence of sodium hydroxide from the buffer solution. Coumestrol similarly produced a brilliant blue fluorescence. Genistein and biochanin A possessing an ultraviolet light absorbing character were not clearly visible on the sheets. Indistinct shadows were present at their positions. The three fluorescent compounds were marked and the paper sprayed with a number of possible developing solutions for the detection of genistein and biochanin A and the possibility of chromogenic reactions occurring with the compounds which could be observed with ultraviolet light.

The developing sprays which have been mentioned in the literature were found to be inadequate as the faint color reactions produced were hidden in the total discoloration of the paper or produced no visible color reaction. Diazotized sulfanilic acid however did produce a significant color reaction with all of the compounds and was particularly good with genistein and biochanin A. This substance was later reported in the literature by other workers (61) and had been developed during the same period at both laboratories.

The diazotized sulfanilic acid was dissolved in 10 per cent sodium
carbonate and sprayed onto the electrophoresis sheets. Genistein and biochanin A produced a deep rust colored spot, formononetin, daidzein and coumestrol appearing as very pale yellow-orange spots. Observation of the papers under ultraviolet light now resulted in deeply shadowed areas at the positions of genistein and biochanin A, however these compounds were now clearly visible without the use of the ultraviolet light. Consequently the combination of ultraviolet light and the diazotized sulfanilic acid spray permitted the accurate determination of spot locations following migration. The use of an ultraviolet hand lamp allowed the spot migration to be observed in a darkened room during the period of migration. Periods of current application from six to eight hours produced excellent separation of the pure isoflavones and coumestrol. The separation observed between the pure standards and a composite mixture is illustrated in Figure VII. It can be seen that daidzein migrated most rapidly and was followed by genistein, formononetin, biochanin A and coumestrol in that order. Diffusion of the spots was minimized by making the initial applications at the origin as compact as possible.

The Electrophoresis of Red Clover

Following the development of the electrophoresis procedure, the extracts of the red clover samples were analyzed for the presence of genistein, formononetin, daidzein, biochanin A and coumestrol. The extracts were prepared as outlined in "Materials and Methods" and the five monthly samples and standards applied to the electrophoresis sheet. These results have been presented in Figure VIII. Coumestrol and biochanin A were not observed in any of the monthly samples considered, which may have resulted from an inadequate extraction or alteration of these substan-
ces during extraction. This would appear unlikely as the remaining iso-
flavones were observed and the extraction involved only mild solvents.
Daidzein and formononetin were observed in all samples and the absence of
genistein from the August sample probably resulted from a slight inadequacy
of extraction in this case. The samples appeared to contain a number of
substances as can be seen from the additional spots marked on the paper
and the occurrence of streaking in the extracts. The compounds existing
at the positions of coumestrol and biochanin A in the extracts were not
considered as being these two, a decision based on differences in fluores-
cent and chromogenic character of these compounds as compared to the
standards. The movement of genistein in the plant extracts appeared to
be retarded by the presence of extraneous material. The absence of
coumestrol from the samples is in keeping with the results of chromatogra-
phic studies previously carried out at this laboratory. Coumestrol has
not been identified in samples of red clover grown in this locality, to date.

The extracted samples were found to contain a number of unidentified
compounds. Figure IX demonstrates the existence of five such substances
(numbered one to five). In every case these substances demonstrated
either a fluorescent or chromogenic character differing from the known
compounds which occurred at or near these locations on the electrophoresis
paper. Unknowns "four" and "five" preceded the migrating isoflavones.
The substance at position "five" displayed a distinct pink coloration
following the application of diazotized sulfanilic acid.

From the results obtained it would appear that electrophoresis can
be considered as a highly suitable technique for the identification
and possible separation of isoflavones in plant extracts.
FIGURE VII. Separation of Standard Solutions of Isoflavones by Paper Electrophoresis. (7/31/61)

FIGURE VIII. Observations of Isoflavones in Monthly Harvested Red Clover Samples. (9/28/61).

FIGURE IX. The Presence of Unidentified Compounds (No. 1, 2, 3, 4, 5) in Red Clover Samples. (9/20/61).
ELECTROPHORESIS OF STANDARDS

Time  7 1/2 hrs.  NaOH-BORAX  pH 11.6
750v  20m.a.  Spray-Diazotized-
R.W.H. 7/31/61  Sulfanilic acid
ELECTROPHORESIS OF SAMPLES

- **Time:** 8 hrs.
- **NaOH-BORAX pH 11.6**
- **V:** 625v
- **20m.a.**
- **Spray-Diazotized**
- **R.W.H. 3/28/61**
- **Sulfanilic acid**
ELECTROPHORESIS OF SAMPLES

Time 7 1/2 hrs.
625v 20 m.a.
9/20/61
NaOH - BORAX pH 11.56
Spray - Diazotized
Sulfanilic acid
R.W.H.
V. The Estrogen-like Activity versus Electrophoresis Analysis of Red Clover

The electrophoresis analysis demonstrated that daidzein, formononetin and genistein were present throughout the summer period in the red clover plant. This procedure was not quantitative but as approximately similar amounts of the extract were applied to the paper it would appear that these compounds are present at a relatively constant amount throughout the season. This may in fact not be true and varying proportions may exist at different periods. However the estrogen-like activity of the samples as determined by the bioassay changed considerably over the period considered. The extract of the September sample demonstrated little or no estrogen-like activity yet was shown to contain daidzein, formononetin and genistein. Daidzein and genistein are both relatively active substances as shown earlier, and formononetin possibly possessing a synergistic action as measured by the mouse uterine response. Figure X depicts the demonstrated activity and the compounds present in both red clover and the pure mixtures. It is possible that daidzein, not considered in the mixture studies, also possesses a synergistic character.

Consequently one is forced to consider the possibility that daidzein, formononetin and genistein appear to be present throughout the season at insignificant levels to cause a biological effect. The estrogen-like response of red clover extracts may in fact be due, at least in part, to some other substance present in the plant or some substance which is capable of activating the existing isoflavones. On this surmise several samples were run and the compound which appeared at position "five" (Figure IX) was cut from the sheet and eluted with warm acetone. A very small amount of the substance was obtained and a preliminary
FIGURE X

The Uterine Response of the Laboratory Mouse to Mixed Standards and Red Clover Associated with the Compounds Present

<table>
<thead>
<tr>
<th>Date</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 11</td>
<td>Formononetin, Genistein</td>
</tr>
<tr>
<td>May 10</td>
<td>Daidzein</td>
</tr>
<tr>
<td>Jun 11</td>
<td>Formononetin, Genistein, Daidzein</td>
</tr>
<tr>
<td>Aug 9</td>
<td>Formononetin, Daidzein</td>
</tr>
<tr>
<td>Sept 11</td>
<td>Formononetin, Genistein, Daidzein</td>
</tr>
</tbody>
</table>

Control range

Mixed samples of 12.5 mgm level and Red clover response and compounds observed in the samples.
examination indicated that the compound may possess an estrogen-like character. It was also noted that this substance could be identified in the forage samples of May, June and July, associated with a relatively high estrogen-like activity, but did not appear in the extracts of August and September, both of which possessed a low estrogen-like action. A large number of samples containing the compounds at locations "four" and "five" were run, the spots cut from the sheets and eluted with acetone. A brown oil-like substance was obtained which would not crystallize from the common solvents or solvent mixtures. The two compounds ("four" and "five") were subjected to infrared analysis and found to be the same substance. However the distinctiveness of the recording indicated some degree of purity in the samples. The fact that both were the same probably resulted from the excessively heavy spots and the predominance of heavy streaking on the papers, plus possibly, the elution of a third overall spot present but not readily visible. The infrared absorption spectra indicated that the substance possessed no aromatic character, no hydroxyl groups, at least one carbonyl group, appeared to be of a chain structure and was possibly an aliphatic fatty acid ester.

The substance was added to the diet at the 12.5 milligram level and produced no estrogen-like activity. Inclusion in the diet at the 30 milligram level produced a uterine weight of 0.2580, as a per cent of body weight, and definitely indicated an estrogen-like response. However, this is considered as a very high level and the response normally observed in the active red clover samples is considerably higher. There is a possibility that this substance in association with the isoflavones present could result in a synergistic activation to produce the observed
activity. More extensive studies than were carried out are necessary before definite conclusions can be drawn.
SUMMARY AND CONCLUSIONS

The study indicated fairly conclusively a number of points regarding several of the estrogen-like substances present in red clover and other forage plants.

Coumestrol possesses the greatest estrogen-like activity of the substances considered, the response of the mouse uterus to this substance being of a logarithmic nature (Figures IV, V).

The order of the estrogen-like potency of the isoflavones considered was found to be genistein, greatest, followed by daidzein, biochanin A and formononetin, in that order. Daidzein produced a uterine response at the lowest dosage but was surpassed by the response to genistein which produced an initial activity at a slightly greater dosage (Figure V).

Biochanin A appeared to exert a slight inhibitory effect on the response of genistein while formononetin appeared to cause a synergistic action (Figure X). These results should be interpreted with some reservation as the experiment was of a preliminary nature.

The estrogen-like potency of monthly harvested red clover samples decreased as the growing season progressed until by September only a slight estrogen-like activity was observed (Figure VI).

An electrophoresis technique using a Borax-Sodium hydroxide buffer system (pH 11.6) was developed for the identification of isoflavones in red clover samples.

The monthly harvested red clover samples were found to contain genistein, daidzein and formononetin. Biochanin A and coumestrol were not observed in any of the samples.
The estrogen-like activity of red clover was greater in the early season than would be expected from the compounds found to be present and less than the expected in the late season. This would indicate the presence of a substance in the plant in the early season which produced a great estrogen-like effect or was capable of activating the isoflavones present to produce the observed response. An unidentified substance was observed in the May, June and July extracts which was isolated in a crude form and found to possess a slight estrogen-like effect when administered at high dosages. Infrared analysis of this substance indicated that it was not of aromatic character and did not contain hydroxyl groups, both characteristics thought to be necessary for a compound to exert an estrogen-like activity.
BIBLIOGRAPHY


12. Bate-Smith, E. C., Swain, T., and Pope, G. S. The isolation of 7 hydroxy-4'-methoxyisoflavone (formononetin) from red clover (Trifolium pratense, L) and a note on the identity of pratol. Chem. and Ind. 1127. 1953.


82. Pope, G. S. and Elcoate, P. V. Isolation of an oestrogenic isoflavone (Biochanin A) from red clover. Chem. Ind. 1092. 1953.


