STUDIES ON THE CONVERSION OF ESTROGENS OF PLANT ORIGIN BY THE FOWL

A Thesis

by

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GENERAL INTRODUCTION

Hormones are classified according to their physiological actions rather than by their chemical structures. It is now more than 30 years since estrone was isolated from human pregnancy urine. This was the first isolation of a hormonally active steroid from natural sources. Shortly thereafter, two other estrogenic steroids, estradiol and ~~estrone~~ estriol, were isolated from human pregnancy urine, and related estrogenic steroids were isolated from horse urine. Since all these estrogens were steroids, it became customary to speak of the "naturally occurring estrogens" with the implication that such substances were steroids. The discovery of the estrogenic properties of certain stilbene derivatives did not modify this situation, because these were synthetic substances not found naturally. Both the naturally occurring estrogens and the synthetic materials have found applications in medicine and also in the livestock fattening industries.

However, the implication that all natural estrogens possess the steroid nucleus soon proved untenable. Within the past 10 years, new classes of estrogens have been isolated and characterized from plant sources. Although these estrogens have the same physiological effects as the animal estrogens, they are not steroids.

The plant estrogens that have received most attention are the estrogenic isoflavones and coumestrol. Although they are far less potent than the steroid estrogens, they have been known to cause considerable breeding difficulties in animals grazing on plants which are high in estrogen content. This is one of the main reasons why work on these plant estrogens has expanded in recent years.

This thesis describes studies on the urinary conversion products of coumestrol and of three estrogenic isoflavones in the domestic fowl, with the primary objective of determining whether or not the large amount of the inactive isoflavan, equol, which is present in the urine, is a conversion product of these plant estrogens. This work also describes the development of an efficient and reproducible technique for the routine measurement of the distribution of low levels of radioactivity on paper chromatograms.

PART I

HISTORICAL INTRODUCTION

SECTION ONE

ESTROGENIC SUBSTANCES OF PLANT ORIGIN

HISTORICAL INTRODUCTION - SECTION ONE

The first hormonally active steroid to be isolated from natural sources was estrone, which was obtained in crystalline form from human pregnancy urine more than 30 years ago (1,2). Within a few years thereafter the three primary steroid sex hormones, estradiol, progesterone, and testosterone, had been isolated and characterized, and a large number of metabolic transformation products had been recognized, some of which, like estrone, possess a certain degree of biological activity themselves. In spite of the wealth of information about what the sex hormones can do, our understanding of how they do it remains abysmally meager.

This lack of understanding is exemplified by the estrogenic hormones, substances responsible in mammals for the development and maintenance of the female reproductive tract. Estrone, estradiol, and estriol are sometimes referred to as the "classical estrogens"; all three substances are steroids formed, for the most part, in the ripening ovarian follicle. The term "classical estrogens" arises from the circumstance that for about 30 years they were the only steroid estrogens the presence of which in human urine was definitely established. However, estrogens are unusual among the various

classes of steroid hormones in that full biological activity, indistinguishable from that of the natural hormones, is exhibited by a variety of non-steroidal compounds. Since the discovery of diethylstilbestrol in 1938 (3), many synthetic estrogens have been prepared and studied. Although certain of these simple compounds, designated as proestrogens (4), may require preliminary transformation by the organism into an active hormone, others, including diethylstilbestrol and its meso-dihydroderivative hexestrol, appear to function themselves as true estrogens.

The occurrence in plants of substances capable of causing estrus in animals was first reported in 1926 (5,6,7), only 3 years after the development of the Allen-Doisy test (8) facilitated the detection of estrogenic activity and had made it possible to estimate the physiological effectiveness of different estrogens. Since then many plant extracts have been examined, and a large number of these have been reported to exhibit some estrogenic activity. Indeed, this apparently general occurrence of estrogens caused Dohrn (9) to doubt the specificity of the Allen-Doisy test for the active "product of the female reproductive glands". Subsequently, it has been demonstrated that the power to cause estrus is by no means confined to substances isolated from female

reproductive glands but is, in fact, shared by a wide variety of substances.

The interest in the plant estrogens has increased in recent years for several reasons. By 1946, Australian workers came to the realization that there were serious implications of a breeding problem in sheep grazing on subterranean clover pastures (10). The manifestations of these breeding difficulties included infertility, difficult parturition, and prolapse of the uterus some months after parturition or even in unmated ewes. Strong presumptive evidence of the presence in this pasture plant of an estrogen or a proestrogen was provided; this was confirmed by the demonstration that administration of the residue from the ether extract of the clover evoked effects qualitatively similar to those evoked by estradiol given subcutaneously (11). There have since been reports from different parts of the world of similar disturbances in the reproductive systems of cattle (12), wethers (13), and lambs (14) grazing on red clover pastures.

1. The Discovery of Estrogenic Substances in Plants

"Then Rachel said to Leah, Give me, I pray thee, of thy son's mandrakes. And she said unto her, Is it a small matter that thou hast taken my husband? and wouldest thou take away my son's mandrakes, also? And Rachel said, Therefore, he shall lie with thee tonight for thy son's mandrakes."

-- Gen. 30:14,15

The observation that some plant materials have medicinal value was realized in Biblical times. Jacob was married to the sisters Leah and Rachel. Leah was unsightly but fertile while Rachel was comely though sterile. Indifferent to Leah, he showered his attentions on Rachel. It was for this reason that Leah so readily accepted the deal offered for her son's mandrakes. Apparently, the mandrakes promoted her fertility, for later Rachel "conceived, and bare a son; and said, God hath taken away my reproach" (Gen. 30:23). Mandrakes were considered as sexual stimulants and have been referred to as love apples. In early Roman medicine, Dioscorides (lst century, A.D.) believed that the juice of the mandrake served as an aphrodisiac. Mandrakes bear a gross resemblance to Mexican yams. These yams are rich in lipids and today they are a source for the commercial synthesis of steroids (15). Mandragora officinarum, a member of the potato family, grows wild in the Holy Land,

and it was believed that its ingestion promoted fertility. However, no hormonal substance has yet been isolated from this source, and it is more likely that the aphrodisiac nature of the mandrake is a result of its tranquilizing properties. Although success in the isolation and characterization of physiologically active substances from plant sources has been attained only within the past century, the presence of these materials has been known to man throughout the ages.

In order to demonstrate the presence of estrogenic substances in plants, the first step has generally been to carry out tests with plant extracts by injecting them subcutaneously into mice or rats and observing the effects as gauged by the Allen-Doisy technique (8). An attempt can be made to compare the activities of the different plants examined by the quotation of results in terms of rat or mouse units per unit weight. A mouse or rat unit is defined as the minimal amount of an estrogenic substance required to produce full estrus response in 100 percent of the animals tested. The Allen-Doisy technique is the prototype of the more refined bioassay techniques employed today, but it is beyond the scope of this review to elaborate on this subject.

The first isolation and characterization of an

estrogen from a plant source was reported in 1933 (16). The residual cake left on pressing palm kernels was extracted; starting with 50 kilograms of press-cake, 2.4 kilograms of oil soluble in methanol was obtained. This oil had an estrogenic activity equivalent to that of 100 milligrams of estrone, or 1,000,000 mouse units per gram. Further extraction and crystallization yielded 18 milligrams of estrone. Later in the same year, the isolation of a crystalline estrogen from willow flowers was reported (17), 7 years after it was first reported that willow catkins contain an estrogen. Sixty-five kilograms of flowers yielded 7.5 milligrams of crystals which resembled estriol in microscopic appearance, solubilities, ultraviolet absorption, and melting point. Indeed, the year 1933 appears to have been a boom year for estrogenic plants, for it was at this time that the first review on the subject appeared (18). Naturally, this review reported nothing new as far as estrogenic substances specific to plants are concerned, since the plant estrogens isolated up to that date were the same as those that had already been isolated from animal sources.

The presence of estrogenic material in the tuberous roots of a woody climbing plant found in northern Thailand was first reported by Vatna in 1939 (19), after attention had been drawn to the fact that the roots were used

locally as a rejuvenating drug. An alcohol extract of the root had a potency equivalent to 500,000 mouse units per gram. Isolation from this source of a highly active, pure substance was described in 1940 (20). A preliminary chemical investigation of the compound was carried out by Butenandt (21), who reported the molecular formula as $C_{19}H_{20}O_6$ but was not able to establish the structure. The plant was at that time believed to be Butea superba, but it has since been recognized as a new species and named Pueraria mirifica (22). The structure of the compound was recently determined by X-ray crystallographic analysis (23) and it proved to be distinctly different from that of the animal estrogens (Figure 1). The compound was named miroestrol, and its chemical properties and estrogenic activity have been determined (22). When given subcutaneously in multiple doses, it is as potent as estradiol-17β, and orally it is more than three times as potent as diethylstilbestrol in producing an increase in uterine weight in the immature mouse (24). The isolation and identification of this estrogen marked an important advance in our knowledge of plant estrogens, because it was the first demonstration of the presence in a plant of a highly potent substance not identical with normal estrogens of the animal kingdom.

It has recently been reported (25) that concentrates prepared from hop extracts are estrogenic and that beta-bitter acid (Figure 1) has a high estrogenic activity. This acid is a mixture of four substances. Three of them, lupulon, colupulon, and adlupulon, have the same basic structure and differ only in the side chain. The fourth, prelupulon, is of unknown chemical constitution. Further research on the estrogenic activity of each of the four constituents of beta-bitter acid is at present being conducted.

A comprehensive review by Bradbury and White in 1954 (26) included a list of over fifty different plants that had been reported to be estrogenic to that time. However, very few of these plants have thus far yielded crystalline estrogens. High activities have been reported for garlic (Allium sativum, L.)(27), milkweed or butterfly weed (Asclepias tuberosa, L.)(28, 29), and oats (Avena sativa, L.)(7, 16, 30). Other common plant materials reported to be estrogenic included coffee (31), licorice (28), sunflower (32), wheat (7, 33), barley (18), apple (34), parsley (6, 34), the fruit flesh of cherry (6, 34, 35), sage (36), rye grass (37), and potato tubers (6, 34, 38). In none of these cases is the active principle known.

Tulip bulbs are also high in estrogenic activity (39). During the last months of World War II, there was a severe shortage of food in Holland and large quantities of tulip bulbs were eaten. As a result, many women showed manifestations of estrogen imbalance, such as uterine bleeding and abnormalities of the menstrual cycle (39). However, no attempt was made to determine the nature of the active substance.

Estrogenic activity has also been reported in bacteria (40) and in commercial animal rations (41). Soybean meal, a standard feed ingredient, contains at least two estrogenic substances.

Since the publication of the review by Bradbury and White (26), it has become apparent that erroneous conclusions can be drawn regarding the presence or absence of an estrogen in a particular species of plant. For example, these authors referred to reports that alfalfa (37, 42) and white clover (42, 43) were non-estrogenic plants, but since that time crystalline estrogens have been isolated from these two sources. It has also been demonstrated that the estrogenic potency of a particular species can vary greatly with geographic location, climatic factors, type of soil, time of year, and stage of development of the plant. This and other important

information have accrued as a result of concentrated research on two types of plant estrogen: the estrogenic isoflavones and coumestrol. This will now be discussed in some detail.

2. The Estrogenic Isoflavones

The interest in plant estrogens was stimulated by field observations in Western Australia of breeding difficulties due to estrogen excess. It was shown that these breeding difficulties were associated with the early Dwalganup strain of subterranean clover (Trifolium subterraneum, L. var. Dwalganup)(44). This clover derives its name from the fact that it buries its seed. The isolated estrogen was found to be non-volatile and ether-soluble and was extracted from ether by sodium hydroxide solution (11). The ether extract from 5 grams of dried clover was somewhat less potent per os than were 0.04 micrograms of estradiol given subcutaneously.

Further studies (43) showed that estrogens were present in the Mt. Barker as well as in the Dwalganup strain of subterranean clover and also in one sample of strawberry clover (Trifolium flagiferum, L.). Extraction, concentration, and assay of the subterranean clover estrogen showed that the leaves yielded more activity than did the roots or petioles. Later, twenty different strains were reported estrogenic (45) and it was shown (46) that 30 grams of dry subterranean clover produced an increase in nipple length in male guinea pigs similar to that caused by 2.5 micrograms of diethylstilbestrol.

It was also demonstrated (42) that the estrogen could be conveniently concentrated with the so-called "chloroplast fraction" from the plant by heating or centrifuging the press-juice. This concentration enabled the convenient preparation of extracts by means of organic solvents. These extracts were studied by methods involving saponification with alcoholic alkali (47, 48), but although potent fractions were obtained, no pure compounds were isolated or characterized.

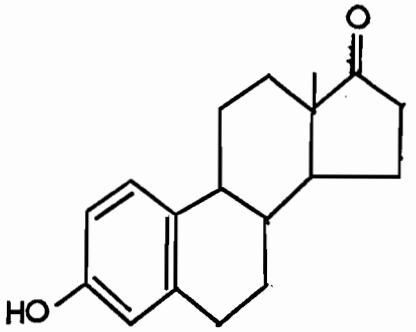
In view of the possibility that alkaline hydrolysis might lead to decomposition and the observation that such a procedure led to the presence of substantial quantities of fatty acids in the sodium hydroxide extracts, Bradbury and White (49) studied an alcoholic extract of the "chloroplast fraction" by chromatography on alumina. They isolated two crystalline products and identified them as the isoflavones formononetin (7-hydroxy-4'-methoxyisoflavone) and genistein (5,7,4'-trihydroxyisoflavone)(Figure 1). Formononetin showed no estrogenic activity but genistein was active at 1 milligram in mice and therefore seemed to be the chief culprit as far as the breeding difficulties were concerned.

Biggers and Curnow (50) decided that genistein was a proestrogen. They based this decision on reports by

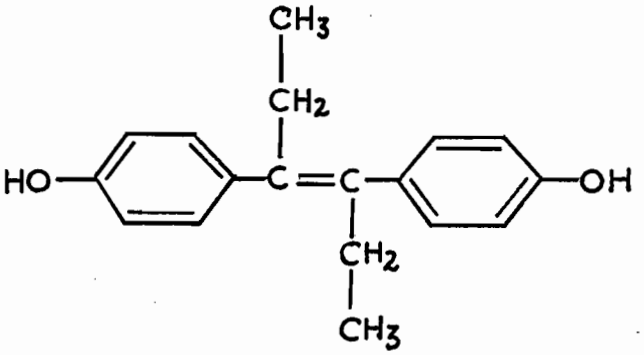
Emmens (4, 51), who classified estrogens into two groups, based on the ratio of the median effective dose required to produce vaginal cornification in ovariectomized mice when the estrogen is administered by subcutaneous injection to the dose required when given by intravaginal instillation. This ratio was called the systemic/local or the S/L ratio. Emmens examined several estrogens and two groups of substances were distinguished, one where the S/L ratio was near unity, and the other where the ratio was in the order of hundreds. Substances in the first group were called proestrogens, i.e., they were presumed to be metabolized in the body to yield a metabolic substance which acted as the true estrogen; those substances in the latter group were considered to be true estrogens. All the natural animal estrogens belonged to the group of true estrogens as thus defined. Emmens (51) examined the effects of these substances on mice whose vaginas had been surgically divided to give separate vaginal pouches. He found that a dose of a true estrogen which was just sufficient to cornify the epithelium of one pouch when placed on it failed to produce cornification of the second pouch. However, an effective dose of a proestrogen for one pouch caused cornification in both pouches. It was believed that true estrogens acted directly on the cells of the responding epithelium,

Figure 1

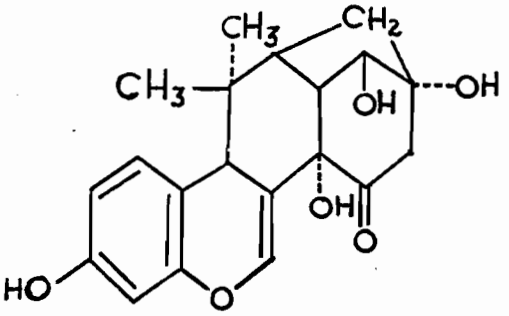
STRUCTURAL FORMULAE OF COMPOUNDS DISCUSSED



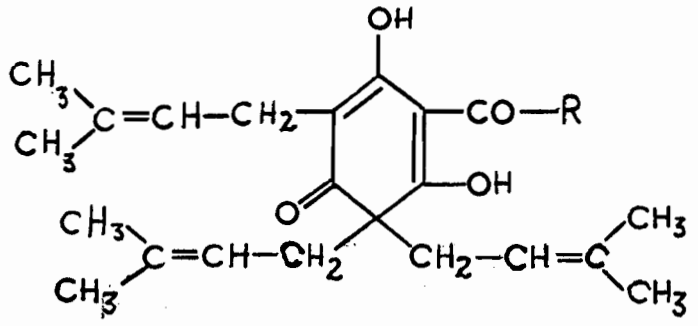
estrone



diethylstilbestrol



miroestrol



beta-bitter acid

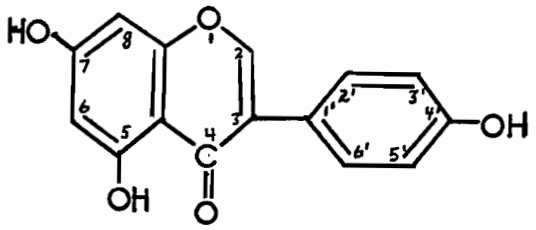
lupulon: R = $-CH-(CH_3)_2$

colupulon: R = $-CH_2-CH-(CH_3)_2$

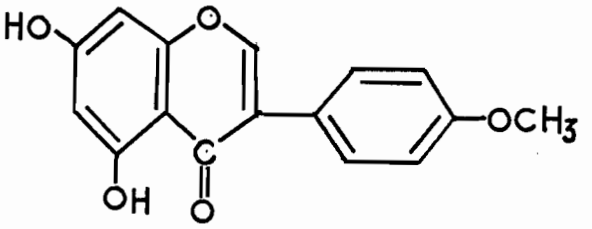
adlupulon: R = $-CH-CH_2-CH_3$ (CH_3)

prelupulon: R = ?

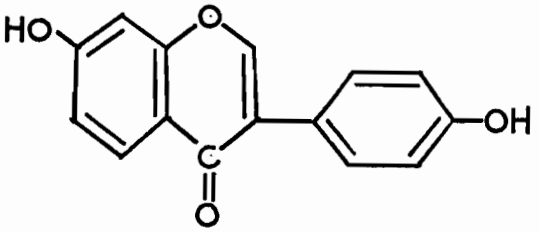
Figure 1 (Cont'd)



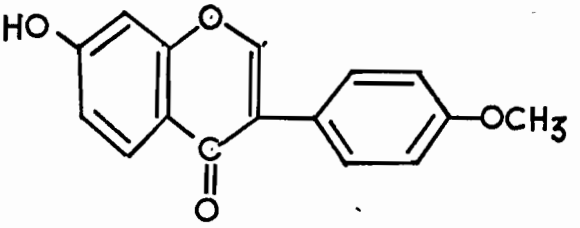
genistein



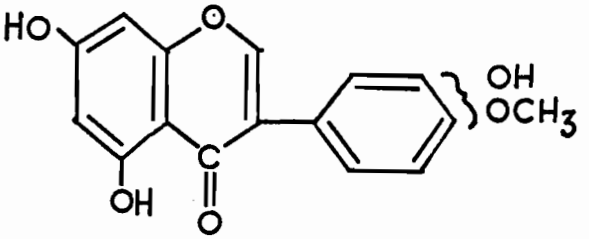
biochanin A



daidzein

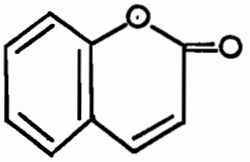


formononetin

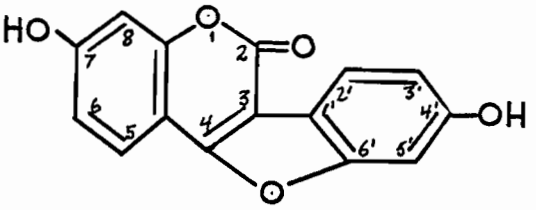


pratensein

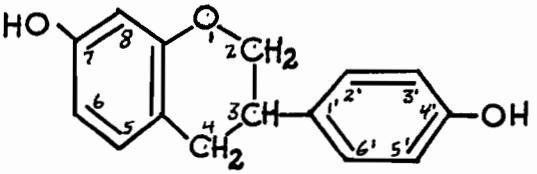
Figure 1 (Concl'd)



coumarin



coumestrol



equol

whereas the proestrogens were absorbed into the body and gave rise to true estrogenic substances during their metabolism.

Since genistein had an S/L ratio of approximately unity, and since the estrogenic potency of genistein was only 1.25-4.53 X 10^{-5} that of estradiol-17β (subcutaneous injection), Biggers and Curnow in 1954 (50) concluded that the estrogenic properties of the proestrogen genistein were caused by only a minor genistein metabolite. In view of the modern theories of estrogen action, however, this conclusion would seem to have been an unwarranted simplification. It appears that the action of estrogen on cellular processes is not stochiometric, but rather that each molecule must influence the reaction of a large number of the molecules participating in metabolic phenomena, and that it is these phenomena combined that influence the degree of estrogenic response (52). There are numerous theories as to the phenomena that result in estrogen response. The most popular view of estrogen action is that the hormone accelerates enzyme function, either by altering the activity of an existing enzyme system or by stimulating the de novo synthesis of critical enzymes. Suggestions have been put forward that the key enzymes may be those involved either in protein synthesis

(53) or in transhydrogenation, i.e., a reversible oxidation-reduction reaction between di- and triphosphopyridine nucleotides, shown to take place in placental tissue and considered to increase the cellular availability of biosynthetically useful energy (54, 55). In the latter case, it is surmised that estrogen either activates the enzyme molecule directly (54) or functions as a co-enzyme (55). Another suggestion, which perhaps should be classified as a negative type of action, is that estrogen activates enzyme synthesis and functions by the removal of some physical barrier surrounding an enzyme-template complex, so as to unmask the enzyme and release the template for repeated use (56).

Other mechanistic concepts include the enhancement by estrogen of the mobility of substrates into or within the cell, either by increased permeability of cellular membranes or blood capillaries (57, 58), or by modification of the intracellular cytoskeleton (59). Alteration of the permeability of the nuclear membrane so as to facilitate passage of messenger RNA to the sites of protein synthesis has been suggested (60). Indeed, that RNA synthesis is a major aspect of early estrogen action in the uterus of a previously ovariectomized rat is now well documented (61, 62, 63, 64, 65, 66, 67). It has been proposed that estrogens might influence membrane permeability by

participating in calcium binding (68), by acting as surface-active agents (69), or by releasing histamine bound in uterine tissue (57, 70). The possible involvement of histamine in the uterotrophic action of estrogen is especially interesting in view of the relation between histamine and rapid tissue growth in general (71).

Although many of the foregoing proposals are attractive, there is no definitive evidence to identify any of these possibilities with the actual mechanism of estrogen action. It seems advisable, therefore, that theories on the mode of action of genistein, such as the one proposed by Biggers and Curnow (50), should be regarded with reserve until the mode of action of estrogen is agreed upon by at least a substantial minority. Indeed, Biggers realized this in 1958 (72), when he conceded that although a compound may have an S/L ratio of about unity, this does not constitute direct proof that it is a proestrogen. This proof requires actual demonstration that a metabolite has sufficient estrogenic potency, and this would require an isotopically labelled compound for study. So far, this has not been done for genistein, and thus further evidence must be presented in order to define this isoflavone as a proestrogen.

Although Bradbury and White (49) isolated only

20 milligrams of genistein per kilogram of fresh subterranean clover, considerable quantities would have remained in the press-residue and in the aqueous solution after removal of the "chloroplast fraction"; thus it was considered that genistein "must be the principal estrogen in the clover" (49). Whether "principal estrogen" referred to quantity or potency of estrogen was not stated. However, the fact that the amount of genistein isolated was not nearly sufficient to account for the activity of the clover (43) caused Curnow (73) to seek improved methods for the isolation of genistein from the Dwalganup strain of subterranean clover. He succeeded in isolating 120 milligrams of genistein from the "chloroplast fraction" per kilogram of dry clover and 3.7 grams of genistein from fresh clover per kilogram of dry matter. This seemed to confirm the belief at the time that genistein was indeed the principal estrogen of the clover. An interesting result of Curnow's work was that he could find no genistein in red clover (Trifolium pratense, L.), an observation which was to be proven inaccurate.

The isolation of genistein from subterranean clover by Bradbury and White in 1951 (49) was not the first recognition that genistein is a naturally occurring isoflavone. To Finnemore (74) goes the credit of being

the first to identify any isoflavone correctly, for in 1910 he examined the bark of an unspecified species of Prunus and found a glycoside which could be hydrolyzed to a substance he called prunetin. Demethylation of prunetin to prunetol, followed by alkali fusion, gave phloroglucinol and para-hydroxyphenylacetic acid, which suggested to Finnemore that prunetol might be an isoflavone, prunetin being a methyl ether. His prunetol is now known to be identical to genistein.

The next important step might have been taken by Perkin and Everest (75) who had obtained from dyer's broom, Genista tinctoria, not only the well-known flavone, luteolin, but also a glycoside called genistin which afforded genistein when hydrolyzed by acid. Perkin and Everest realized that the aglycone was closely similar to Finnemore's prunetol but ascribed to it an incorrect empirical formula. In 1925, Bargellini (76) explored the chemistry of genistein, only to lose the right track in the mists that, in those years, still hung heavy about the means of differentiation between α-pyrones and γ-pyrones. His suggestion that genistein might be a 3-phenylcoumarin was astute for, like isoflavones, such compounds are degraded by alkali to phenols and arylacetic acid. In the same year, however, Baker and Robinson (77, 78) furnished unequivocal synthetical evidence that

genistein (prunetol) does in fact have the structure shown in Figure 1. Genistin (prunetin) was later shown to be a constituent of soybean meal (79).

The initial isolation of genistein from subterranean clover and the realization that this isoflavone was estrogenic stimulated the search for the substances responsible for the activity of other plants reported estrogenic. There were numerous reports on the preparation of estrogenic concentrates from various plant extracts, but difficulties encountered in the determination of chemical structure stalled the actual identification of the active substances. Pope and his group isolated the isoflavone biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) (80, 81) and genistein (81) from red clover, a pasture plant that had been reported to be estrogenic by several workers (37, 42, 82, 83). Pope reported that the estrogenic activity of biochanin A was 0.63 that of genistein (81). Biochanin A derives its name from the fact that it had been isolated initially from germinated chana grain, along with two unidentified compounds which were named biochanin B and biochanin C (84, 85). Biochanin A has also been isolated from the heartwood of Ferreirea spectabilis (86), a large hardwood tree found in central South America. A third isoflavone, formononetin, was also found in red clover (87), but its estrogenicity is

open to some doubt. Formononetin had been previously described mainly in connection with the most venerable of the isoflavone derivatives, ononin, a glycoside first found in the roots of Ononis spinosa, L. and discussed in 1855 (88). Ononin yields glucose and formononetin on hydrolysis (89, 90). The isoflavone content of red clover also includes pratensein (Figure 1), which was recently characterized by Wong (91). Preliminary results indicate that pratensein is less active than biochanin A but more potent than formononetin (92).

A fifth isoflavone has recently been detected not only in red and subterranean clovers, but also in alfalfa (Medicago sativa, L.) and ladino clover (Trifolium repens, L, var. Ladino) by Bickoff and his colleagues (93). The compound, daidzein (7,4'-dihydroxyisoflavone) (Figure 1), had been obtained, along with glucose, in 1931, by acid hydrolysis of daidzin, a glycoside found in Japanese "daidzu", Soja hispida (94). Daidzein has also been reported in Pueraria sp. root (95). The isoflavone has an estrogenic potency in the same order as that of genistein (96, 97).

3. Coumestrol

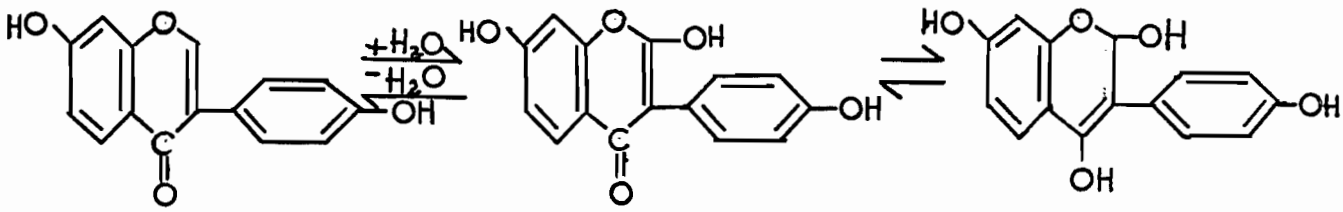
During an attempt to identify the major estrogenic component of ladino clover, Bickoff's group came to the realization that the active compound could not possibly be an isoflavone or a steroid. Furthermore, the estrogenic activity they found in several alfalfa samples as well as in a sample of fresh strawberry clover (Trifolium flagiferum, L.) appeared to be attributable to the same estrogen. Procedures involving ether extraction, alkali purification, countercurrent solvent distribution, distillation, and recrystallization resulted in the isolation of 1/12 ounce of the pure compound from 400 pounds of ladino clover (98). The compound was characterized by techniques such as absorption spectra and fluorescence measurements (99). Because of the coumarin-like structure of the molecule, the estrogen was named coumestrol (100) (Figure 1). Mouse assay tests indicated that coumestrol is at least thirty times more potent than the estrogenic isoflavones, although much less active than diethylstilbestrol. The intense blue fluorescence of coumestrol when exposed to ultraviolet light (3660^{o}A.) greatly facilitated the isolation and purification of the compound and its detection in leguminous plants such as red clover, white clover (Trifolium repens, L.), bur clover (Medicago hispida),

subterranean clover (Dwalganup variety), strawberry clover, and alfalfa (101). Sensitive fluorometric and chromatographic methods were developed for the quantitative estimation of coumestrol in these various forages (102, 103).

Bickoff and his group (104) studied the estrogenic activities of a number of synthetic compounds related to coumestrol in an attempt to correlate structure with estrogenic activity, a problem which has tantalized investigators throughout the history of estrogen research. As might be expected, these authors presented some interesting and probably significant results, but little to indicate why coumestrol itself is estrogenic and other naturally occurring compounds containing similar groups are not. Acetylation of both hydroxyl groups did not decrease activity. Etherification of both hydroxyl groups decreased estrogenic activity by 2/3. The 4'-O-methylcoumestrol was less active than the 7-O-methylcoumestrol, but neither was as active as coumestrol. Opening the lactone ring of coumestrol to form the potassium salt of the resulting O-hydroxycinnamic acid did not decrease activity. Formation of the O-methoxycinnamic acid derivative greatly decreased activity. Opening of the furan ring also greatly reduced estrogenic potency. Removal of one of the two hydroxyl

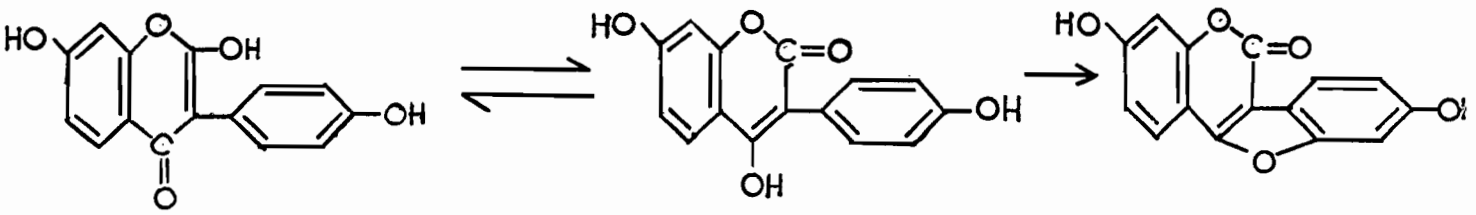
groups of coumestrol decreased activity to about 1/6 of the original, and removal of both hydroxyl groups completely eliminated the estrogenic response. The presence of additional hydroxyl groups on the molecule greatly diminished estrogenic activity.

Bradbury (105) observed the striking similarity between the naturally occurring estrogenic isoflavones, one of which is genistein, and the 3-phenyl-4-hydroxycoumarins. He pointed out that this close relationship is further emphasized when one considers the possibility of the addition of water across the double bond of an isoflavone to give 2-hydroxyisoflavone, followed by enolization to a 2,4-dihydroxyisoflav-3-en (Figure 2). In this connection, Biggers quotes Bate-Smith (72) to the effect that coumestrol may be formed in the plant by rearrangement of the isoflavanol corresponding to daidzein with ring closure to the 6' position (Figure 3). The fact that coumestrol is more than thirty times as active as the closely related estrogenic isoflavones is probably connected also with the fact that the oxygen atom at position 4 of the isoflavones is primarily ketonic, which results in a single bond in the 3,4 position. Indeed, it has been recently demonstrated that the biosynthesis of coumestrol in lucerne (Medicago sativa, L.) is related to that of the isoflavones rather



daidzein 2-hydroxyisoflavone 2,4-dihydroxyisoflav-3-en

Figure 2. Suggested possibility of interconversion of isoflavones, isoflavonones, and isoflav-3-ens (105).



isoflavonol corresponding to daidzein coumestrol

Figure 3. Suggested possibility of rearrangement of isoflavonol to coumestrol (72).

than to that of the coumarins (106, 107, 108). It was shown that radioactivity from acetic acid-1-C^{14}, DL-phenylalanine-1-C^{14}, and cinnamic acid-3-C^{14} was incorporated into coumestrol. As the C^{14} originating from cinnamic acid-3-C^{14} was essentially all in carbon-2 of coumestrol, an isoflavone-type biosynthetic pathway (108) was evidently operative. In this synthesis carbons 2, 3, and 4, according to the coumarin numbering system (Figure 1), are formed from the side chain of a phenylpropanoid compound whose aryl group migrates from C-2 to C-3. The carbons of the other benzenoid ring are derived from acetate, in a manner analogous to that by which flavonoids are formed (108, 109).

This author is unaware of any reports on the site of formation of coumestrol in the plant. However, research with other natural coumarins such as herniarin (110), scopoletin (111, 112) and umbelliferone (112) has yielded conflicting evidence as to the plant organ in which coumarins are formed. There is no doubt that both roots and aerial organs of plants can form coumarins, but the weight of evidence (113) indicates that under normal growing conditions synthesis may be predominantly in the leaves. It is not known whether this applies to coumestrol.

4. Estrogenic Potencies

In order to understand fully the importance of these forage estrogens, it will be necessary to obtain a proper perspective of their absolute potencies and of the factors that affect these estrogenic potencies. A comparison of the estrogenic activities of the forage estrogens with various steroid and synthetic estrogens is presented in Table I. The uncertainty of all determinations of true estrogenic activity must be stressed. No absolute significance can be attached to the observed potency of a substance. Many factors, besides the original chemical structure, influence the estrogenic activity of a compound. For example, mode of administration, sensitivity of the animals used, rate of absorption, rate of destruction, and various metabolic transformations can alter the estrogenic activity. In spite of these variables, Table I does give some idea as to the positions of the forage estrogens on the potency scale. For purposes of comparison, estradiol-17β has been assigned the arbitrary value of 1000.

Several workers have presented values for the relative estrogenic potencies of coumestrol and the different isoflavones. Bickoff *et al* used oral administration to

TABLE I

APPROXIMATE RELATIVE ESTROGENIC POTENCIES OF VARIOUS ESTROGENS

Compound	Mode of Administration		
	Intravaginal	Oral	Subcutaneous
Estradiol-17β	1000	-	1000
Estrone	650	30	500
Estriol	500	30	100
Diethylstilbestrol	500	400	500-1000
Miroestrol	-	1500	1000
Estrogenic isoflavones	-	0.005	-
Coumestrol	-	0.15	-

immature female mice and based his bioassay comparisons on the weight of estrogen required to produce a uterine weight of 25 milligrams (96) (Table II). The assumption was made that the initial weight of the uterus did not vary significantly from mouse to mouse. Estrone and diethylstilbestrol were employed for comparison purposes, and it will be noted that the potencies of these two estrogens were less than that which would be expected by intravaginal instillation or subcutaneous injection. Coumestrol was thirty-five times more potent than genistein. Genistein was slightly more potent than daidzein, about twice as potent as biochanin A, and four times more potent than formononetin.

TABLE II

RELATIVE ESTROGENIC POTENCIES OF THE PLANT ESTROGENS*

Compound	Author and Mode of Administration				
	Bickoff (96)	Nilsson (97)			Cheng (116)
	Oral	Oral	Subcut.	Intraper.	Oral
Diethyl-stilbestrol	100,000	100,000	100,000	100,000	100,000
Estrone	6,900	-	-	-	-
Coumestrol	35	-	-	-	-
Genistein	1.00	0.80	1.10	0.71	1.20
Daidzein	0.75	0.92	0.12	1.04	1.68
Biochanin A	0.46	0.52	-	-	1.32
Formononetin	0.26	0.00	-	-	0.36

*Results in each column are expressed in terms of an arbitrarily assigned value of 100,000 for diethylstilbestrol.

Nilsson used three bioassays for her comparisons of the potencies of the estrogenic isoflavones (97) (Table II). She found formononetin to be inactive orally. This is an interesting divergence from Bickoff's results, since she has shown that formononetin is demethylated to the active estrogen daidzein (and formaldehyde) in the rumen fluid of sheep (114) and by microsomal liver enzyme systems of the rabbit, rat, mouse, chicken, pig, cattle, and sheep (115). These observations seem to indicate that ingested formononetin would be demethylated to active daidzein, yet Nilsson has not offered any explanation for the apparent lack of estrogenic potency of formononetin. Another discrepancy is that Nilsson's daidzein was more active orally than genistein. Since this difference was relatively small, this may indicate real differences in laboratory techniques and the strains of mice employed in the different laboratories. Nilsson's results also showed that, when given subcutaneously, genistein was still active while daidzein had almost no effect. The difference in action of daidzein, depending upon mode of administration, was probably due to its low solubility which resulted in the development of crystals in the skin after subcutaneous injection, a phenomenon observed during the investigation.

The bioassay used by Cheng and co-workers (116) was the same as that of Nilsson, i.e., the measurement of the weight of mouse uteri after ingestion of a measured amount of estrogen incorporated in the diet. His results again emphasized the disagreement among authors as to the relative estrogenic strengths of these compounds. Cheng's results (Table II) were generally the highest; daidzein was the most potent isoflavone, genistein and biochanin A had approximately equal activities, and formononetin was weakly estrogenic.

5. Variation in Estrogenic Activity

After it had been realized that some forages contain estrogenic substances, workers embarked on supposedly routine quantitative determinations of the estrogenic potencies of these forages by means of standard bioassay procedures. They soon came to the realization that there were enormous variations in estrogenic activity as a result of different varieties, different stages of growth, seasons of the year, number of cuttings, geographic location, type of soil, and climatic conditions. In order to control the estrogenic activity, it will be necessary to develop a complete understanding of normal variations in potency that can be expected to occur as a result of these factors.

Andrews and his co-workers at Purdue University have worked on several phases of this problem (117, 118, 119, 120). They showed that estrogen content of alfalfa varies greatly during the growing season, with the number of cuttings, and with stage of development of the plant. In one phase of their study, fifty-six different strains of alfalfa were assayed (120). The samples assayed were second cuttings from individual clones harvested in the bud stage. Varieties of alfalfa studied included Ranger,

Grimm, Hardigan, Cossack, Buffalo, and Viking. As expected, a wide variation in the estrogenic activity of various samples was found. The increase in uterine weight which resulted from feeding the alfalfa extracts ranged from 1.6 to 83.0 milligrams. Differences in the degree of estrogenic activity occurred in all of the varieties studied, which indicated a lack of varietal specificity in terms of estrogen content.

Kitts and his co-workers at the University of British Columbia have also studied variation in estrogen activity in forages by mouse bioassay (121, 122, 123). Their results confirm the findings of those of the group at Purdue that extreme variations in estrogen content occur during the growing season. They found that estrogenic content was highest at the vegetative stage and tended to decrease until full bloom, when the plants were partially in the dough stage. They concluded that estrogenic activity was not associated solely with rapid growth nor with the reproductive phase of the plant. In a related portion of their study, four successive cuttings of alfalfa were made throughout the summer, all plants being harvested at almost the same stage of development. Estrogenic content was high in the spring, decreased in the summer, but built up again in the fall. This differs from the findings of the Purdue group that alfalfa cuttings subsequent to the

first tended to be lower in estrogenic activity. However, it should be pointed out that these divergent results may indicate real differences due to such factors as climate, locality, and soil conditions.

In studies on the mode of action of the plant estrogens, Kitts has found that oral administration of red clover extracts as well as of diethylstilbestrol to rats increased the thyroid uptake of radioactive iodine (123). He believes that the apparent stimulation of the thyroid gland by red clover extracts as well as by diethylstilbestrol may be used to explain, in part, the various physiological responses attributed to the presence of the estrogenic substances in forages ingested by livestock.

Bickoff and his co-workers also studied estrogen variability as influenced by stage of growth, cutting, and season of the year (124, 125, 126). As in the work reported above, wide differences in estrogen content were found between different samples. In general, estrogen content increased with the age of the plant. In a cooperative study with the Arizona Experimental Station (124), a series of sun-cured alfalfa meals was prepared and bioassayed. Samples were taken from different fields and represented average cuttings during the period involved. The first two cuttings showed almost no activity. Estrogen

content was extremely high during January and then gradually tapered off. These results may indicate effects of temperature and length of daylight on estrogenic content in plants.

Bickoff has also undertaken a cooperative study with the Crops Research Division of the Agricultural Research Service of the United States Department of Agriculture, and the American Dehydrators Association (127, 128). Samples of five different varieties of alfalfa (Bullalo, Ranger, Dupuits, Vernal, and Lahontan) were grown at seven USDA Crops Research stations in locations ranging from Pennsylvania to California. Successive cuttings were sampled at known stages of growth and subjected to bioassay and chemical analysis. Although all the results have not been coordinated as yet, it has been reported that differences range from zero to over six hundred parts per million of coumestrol in samples cut at the same stage ("one-tenth bloom") from different locations. Another phase of this investigation relates to estrogen content of commercially dehydrated alfalfa as determined by chemical assay. Commercially grown alfalfa was sampled during the growing period and then harvested and dehydrated at the peak estrogen level (127, 128). As the alfalfa matured, the coumestrol content rose progressively until

a peak value was obtained well beyond the normal harvest period. At this time the alfalfa was harvested and dehydrated. A slight loss of coumestrol activity resulted from the dehydration process. The 12 tons of highly estrogenic alfalfa meal obtained in this way was used in studies on growth promotion of animals, a topic which will be discussed in the next section.

The estrogenic activity of red clover has been the subject of investigations in many parts of the world. Red clover has been subjected to bioassay in Sweden (129), Finland (130, 131), Great Britain (132, 133), New Zealand (92, 134), U.S.A. (135), and Canada (121). With due allowance for objections to the bioassay methods, the estrogenic activities of red clover samples from different parts of the world are of the same order, i.e., they correspond to 2-5 micrograms of diethylstilbestrol activity per 100 grams of dry matter. As with alfalfa, the estrogen levels of red clover differed between varieties and between sampling dates (92). The preparation of ensilage did not alter the estrogenicity of the forage (129, 131, 136). Field-drying lowered the estrogenic activity of red clover whereas artificial drying of small quantities of forage had no effect on its activity (129). In general, it was found that the leaf or leaf and petiole

of the clover had the highest activity; small and large stem fractions were both relatively less active, and blossom and seed head were inactive (134). The variations in estrogenicity that have been reported for red clover have been similarly found in subterranean clover (Trifolium subterraneum, L.) (92, 137).

6. Some Physiological Effects of Plant Estrogens

a. Breeding Difficulties

The effects of plant estrogens on animals can be desirable or undesirable, depending on how much forage is to be used, its potency, the type of animals, etc. As mentioned previously, current interest in forage estrogens was stimulated by serious reproductive difficulties in sheep grazed on subterranean clover pastures in western Australia (10). The types of problems included infertility, uterine prolapse, difficult lambing, stillbirth, and loss of young. Transfer of ewes to good non-clover grazing areas for three or more successive seasons did not restore fertility. It has since been found that this problem could be controlled to a considerable extent by limiting the intake of clover and by increasing the intake of grasses, which are known to be low in estrogens.

In the United States, there has been an increasing interest in forage estrogens as a result of field observations of breeding difficulties in sheep and cattle on legume forage pastures. It has been reported from the Ohio Agricultural Experimental Station (138) that ladino clover interfered with the reproductive process of sheep grazed on this clover in a manner somewhat similar to

that found earlier with sheep on subterranean clover in Australia. This report stated that ewes grazed on ladino clover pasture took longer to conceive than did comparable animals grazed all season on blue grass. In addition, the average fertility of the animals grazed on ladino clover was considerably lessened.

Workers at the Oregon State University have reported breeding difficulties in sheep fed on red clover (139). They fed some of the suspected clover to mice and obtained confirmatory evidence that both the fresh clover and the hay made from it inhibited reproduction. Pasture estrogens have been implicated in dairy cow breeding in the United States as well as in Israel. Reproduction disorders have also been reported in New Zealand (13, 14) and in Germany (12). It is to be anticipated that as more researchers investigate the relation of forage estrogens to breeding difficulties, more such cases will be found.

It is apparent, therefore, that for breeding animals, it would be desirable to obtain low-estrogen forages either by development of special strains of legumes or by controlling environment, stage of growth, etc.

b. Rate of Growth

On the positive side of the estrogen feeding picture is the effect of the synthetic estrogen, diethylstilbestrol. It was reported in 1955 (140) that feeding 5 to 10 milligrams of diethylstilbestrol per animal daily to cattle resulted in an increase of about 20 percent in rate of gain and in increased feed efficiency. This finding led immediately to intensive study of the use of synthetic hormones to stimulate rate and efficiency of gain in animals and the practice has become widely accepted. It is estimated that more than 80 percent of all steers fattened in the United States are now treated with synthetic estrogen preparations either by implantation or by means of special dietary supplements. The economic implications of the practice for the livestock industry have been highly significant.

It would be of considerable theoretical interest, and might well also be of economic importance, to ascertain the extent to which forage estrogens may also be capable of accelerating the rate of growth of animals. The very fact that some forages contain enough natural estrogen to produce negative effects in breeding animals suggests a positive potential in fattening steers, wethers, poultry, and other livestock. As Andrews (119) has suggested, if

a level of estrogenic activity could be obtained in forage that would stimulate growth or other functions, great benefits would accrue to the forage producer, processor, and user. The importance of developing alternative sources of growth promoters and fatteners is underlined by the fact that the use of diethylstilbestrol for poultry was banned by the Federal Drug Authority when residues were found in the tissues of treated birds; certain restrictions have also been placed upon its use for larger animals.

From the reported diethylstilbestrol equivalence of the various forage crops, it may be difficult to understand the magnitude of estrogenic potency with respect to animal growth promotion sometimes attributed to them. Kitts (123) reported diethylstilbestrol equivalences of alfalfa samples as ranging from almost nil up to a level of 55 micrograms per pound of dry matter. Assuming that a useful daily intake of diethylstilbestrol for the beef steer is 10 milligrams, it can be calculated that to obtain this amount of estrogenic activity, a steer would have to consume almost 200 pounds of dry matter daily from this alfalfa.

Nevertheless, this may still be of great importance, since the mouse test may not accurately reflect the relative estrogenicity of diethylstilbestrol versus plant estrogen

to the beef animal. Fermentative changes in the rumen may increase the activity of plant estrogens (118). Another possibility is that the estrogenlike compound may be metabolized within the animal body into a more potent growth-promoting substance (127). Cumulative effects in the animal body might thus be greater with plant estrogens than with diethylstilbestrol.

Because of the lack of large enough supplies of plant estrogens for feeding experiments with large animals, their effectiveness in the promotion of growth of steers and wethers and in fattening poultry has yet to be proven by direct experiment. However, sufficient indirect evidence is available to provide a fairly convincing picture. For example, investigators at Iowa State University (141) fed a crude estrogenic extract from clover hay to fattening lambs. The fortified ration contained about 2 to 3 micrograms of equivalent diethylstilbestrol activity per pound of ration. The clover extract promoted a rate of gain of approximately 10 percent over the control. In an attempt to substantiate this effect more conclusively, these workers also performed similar experiments on a limited scale with the purified plant estrogen glycoside, genistin, obtained from soybean meal, and again obtained increased growth rates. The addition of 200 milligrams of genistin per pound of ration was equivalent in estrogenic

activity to 3 micrograms of diethylstilbestrol per pound. The addition of the pure genistin to the diet resulted in a 15 percent increase in rate of gain.

One of the most positive evidences of weight gain stimulation in steers fed alfalfa was obtained at the University of Nebraska (142), where it was shown that a daily intake of 5 pounds of this alfalfa produced the same daily weight gain in pounds (2.46) as did the addition of diethylstilbestrol to the diet (2.47). Lower amounts of alfalfa were also effective but to a lesser extent. When diethylstilbestrol and dehydrated alfalfa were combined as a supplement, rates of gain were only slightly better than when either was administered alone.

In another study, two samples of dehydrated alfalfa meal which showed potencies of 111 and 23 milligrams of coumestrol per kilogram were fed to growing lambs (143). When fed the high-estrogen meal, wether lambs gained significantly faster than those fed the low-estrogen meal and ewe lambs gained more slowly. Federal grades at slaughter indicated little difference in the lambs on the high- and low-estrogen meals, although there was a tendency toward greater fatness in the high-estrogen-fed group. Organoleptic tests conducted by a trained taste

panel showed significant differences in tenderness, juiciness, and texture between the two treatments, all in favour of the high-estrogen-fed lambs.

The high-estrogen meal prepared during the USDA-ADA cooperative study, as described in a previous section, was used in an interesting experiment (127). Three meals were employed: low-estrogen alfalfa containing 20 parts per million coumestrol, high-estrogen alfalfa containing 210 parts per million coumestrol, and a blend of equal parts of these meals that contained about 110 parts per million coumestrol. Each meal was fed at a level of 65 percent of the diet to groups of six lambs each. In addition, the high- and low-estrogen meals were fed to diethylstilbestrol-implanted animals. The results are presented in Table III. It is difficult to explain why blended material produced better gains than did the high-estrogen meal. When a diethylstilbestrol implant was used with the low-estrogen meal or high-estrogen meal, the gain rose in proportion to total estrogen content. It is to be noted that animals on blended alfalfa meal were doing almost as well as animals on low-estrogen meal plus diethylstilbestrol. While these experiments were positive for a growth-promoting response, it is not certain at present whether coumestrol or the other forage

TABLE III

FEEDING TRIALS WITH LAMBS (127)

Diet Description	Coumestrol (p.p.m.)	Average Daily Gain (pounds)
Low-estrogen alfalfa	20	0.292
Low-estrogen alfalfa + diethylstilbestrol implant	20	0.486
High-estrogen alfalfa	220	0.367
High-estrogen alfalfa + diethylstilbestrol implant	220	0.533
Blended high- and low-estrogen alfalfa	110	0.478

estrogens were the only active agents in the alfalfas involved.

Some rather curious observations were recently reported by Magee (144), who fed young rats diets containing pure genistein and genistin in order to determine the biological responses. A dietary level of 0.5 percent of genistin or genistein resulted in significant decreases in weight gain and in the weights of the kidneys and spleens. Rats fed diets containing either plant estrogen had liver and spleen iron levels that were significantly higher than those of the controls, but hemoglobin and tissue copper levels were not affected by the feeding of

either genistin or genistein. Levels of zinc in the bones and livers of the estrogen-fed rats were higher than the corresponding zinc levels of the control. Genistin and genistein enhanced the deposition of calcium, phosphorus, and magnesium in the bones of the young rats. The bone Ca/P ratios were lower in the animals receiving diets containing the plant estrogens. Not only do the effects of genistein and genistin on weight gain in young mice differ from those on larger animals, but the overall actions of these plant estrogens on the criteria studied are not the same as would be expected to occur with either the naturally occurring animal estrogens or synthetic estrogens. It is not within the scope of this report to discuss the biological effects of the various classes of estrogen. It should, however, be pointed out that authors other than Magee have likewise noted that the effects of genistin and genistein on such phenomena as weight gain and development of the kidneys, ovaries and testes of the mouse are not the same as the effects of estradiol and diethylstilbestrol (145, 146). Indeed, the plant estrogens may imitate animal and synthetic estrogens in the manner in which their biological effects on various organisms varies with the species of animals.

c. Lactation

Lush, green pasture has long been recognized as having a stimulating effect on milk production. The possibility that plant estrogens are associated with the effect was first suggested by British workers in 1948 (82). They pointed out that a progressive decline in the solids-not-fat content of milk occurred during the winter months, followed by a substantial and immediate rise when cows were turned out to pasture in the spring. These workers also pointed out that on "going out to grass", cows often showed an increase in milk yield greater than the amount that can be ascribable to the extra nutrients ingested. These workers assayed samples of forage cut from a number of pastures during the period of active growth and found significant estrogenic activity.

More recently, workers at the New Zealand Department of Agriculture attempted to establish a possible correlation between lush pastures, high milk production, and estrogenic activity in the forage. Lactation curves of cows maintained under two different types of grazing management on a typical New Zealand dairy pasture showed that a sharp rise in milk production occurred during the period of lush growth (147). A large number of samples of the pasture was taken at weekly intervals through the period

of lush spring growth and assayed for estrogenic activity. No positive responses were obtained. This would seem to indicate that there is no correlation between high milk yield and estrogenic content in the pasture. However, it has since been demonstrated that the assay technique employed was satisfactory for the isoflavonoid estrogens, but tended to destroy the coumestrol (127). Therefore, if coumestrol were the dominant estrogen in the white clover examined, misleading assays would result.

Many workers have used diethylstilbestrol or related compounds to induce udder growth and lactation in dairy animals. In a typical study, identical twin cows were employed and milk response was measured during complete lactations (148). Each cow treated with diethylstilbestrol was approximately 6 percent more efficient in milk production and gained less weight during the test period than did the controls.

d. Speculations on the Overall Significance of the Plant Estrogens

It has become apparent in recent years that the presence in plants of estrogenic substances is by no means a scientific curiosity. There is no doubt that as more plants are examined, it will be found that these estrogens are of widespread occurrence. It is a striking fact that many foods commonly consumed by man contain these estrogens. Whether they are beneficial, detrimental, or neither is unknown. The function of these substances in plants has been open to much speculation. Possibly they have no function at all; they may simply represent stored products of metabolism since they seem to increase as the plant gets older (127).

Another interesting speculation is that possibly the animal kingdom acquired estrogens from the plant world by one method or another (127). Estrogenic substances are widely distributed among plants. Since animals evolved in the presence of plants, they must have been closely associated with estrogenic substances throughout time. Possibly animals acquired estrogens from plants by adaptive means. The first step in this direction might have been the utilization of the estrogenic substances in foods

by animals in certain of their metabolic processes, similarly to vitamins as they are now known. Also, it seems evident that, at present, animals are constantly subjected to estrogenic substances from plant sources to which they have become adapted; this adaptation may indeed have developed into a dependence.

Theories on the overall significance of the plant estrogens should not exclude the possible effects of anti-estrogenic substances of plant origin. The presence in plants of substances capable of inhibiting estrus has been known for some time. It was reported in 1937 (149) that extracts of the white bean (Phaseolus vulgaris, L.) inhibited estrus. More recently, anti-estrogenic substances have been reported in alfalfa (150), birdsfoot trefoil (151), and yellow pine needles (Pinus ponderosa, L.) (152, 153, 154). However, the picture is complicated by the fact that weak estrogens can antagonize the uterotrophic effect of strong estrogens (155, 156, 157, 158), i.e., they act as weak estrogens in small doses but have a selective anti-estrogenic effect in large doses. It is possible, therefore, that a physiological balance exists between estrogenic and anti-estrogenic substances which are consumed by animals whose survival is dependent upon the plants containing these substances.

7. Metabolic Studies on the Plant Estrogens

Very few experiments have been performed as to the metabolic transformations of plant estrogens in animals. So far as this author is aware, the only studies that have been conducted are those undertaken by Nilsson of the Royal Agricultural College of Sweden; her work was confined mainly to the metabolic fates of biochanin A and formononetin. This will now be reviewed briefly.

In vitro experiments with rumen fluid indicated that biochanin A (159) and formononetin (114) were to some extent transformed in the rumen into the more potent estrogens, genistein and daidzein respectively. In the rat, genistein was isolated as a metabolite of biochanin A in both urine and feces (160). Incubation of biochanin A with rat liver slices demonstrated that this demethylation reaction occurred in the liver (160). A further study on this metabolic transformation (115) showed that biochanin A was O-demethylated by a microsomal enzyme system belonging to the so-called "mixed ~~fraction~~ function oxidases" (161), which require free oxygen and a reduced pyridine nucleotide for activity (162). It was demonstrated that both DPNH and TPNH were required for this transformation (115). The process was similar for formononetin (115). O-demethylation

of biochanin A and formononetin was also demonstrated in the liver of mouse, rabbit, chicken, sheep, and cattle. Several other methoxylated isoflavones were found to be demethylated at a varying rate by liver enzyme systems.

Experiments on the excretion of biochanin A metabolites have shown that biochanin A and genistein are excreted in both the conjugated and non-conjugated form in the rat (163). An attempt was made to differentiate between the conjugation of the isoflavones with sulphate and glucuronide respectively. This was done with the aid of addition of phosphate as an inhibitor of sulphatase in the hydrolysis of the conjugates isolated from the urine. Only in the case of genistein did the sulphate conjugation seem to be of any importance. A greater percentage of total conjugates present as glucuronides was indicated.

8. Equol

The discovery by Zondek in 1930 (164) that the urine of pregnant mares contained large amounts of estrone resulted in widespread adoption of this material as the starting-point for the large scale manufacture of the hormone. It soon became apparent, however, that the methods available at the time for the isolation of crystalline estrogens from human urine resulted in the production of crystals which were contaminated with substances of lower melting points when the methods were applied to pregnant mare's urine (165, 166, 167). However, the hormone was separated by repeated crystallizations.

As a result of their analyses of the total phenolic fraction of pregnant mare's urine, Marrian and Haslewood in 1932 (168) succeeded in the isolation of a substance which had the empirical formula $C_{15}H_{14}O_3$. It exhibited no estrus-producing action when injected into ovariectomized mice even in relatively large doses. Some chemical analyses were performed, but no structural formula was presented at the time. In view of the source of this new phenolic compound, it was named "equol".

The isolation of equol was facilitated by the observation that it could be separated directly from the

ether-soluble phenolic fraction of toluene extracts of the acid-hydrolyzed urine by means of crystallization from chloroform. Marrian and Beall soon discovered that stallions and non-pregnant mares likewise excreted large amounts of equol in the urine (169), and therefore its excretion was not associated with the presence of large amounts of urinary estrogen nor with the reproductive cycle of the animals. During the autumn, the amounts isolated from urine extracts steadily decreased and in the winter months it was not possible to isolate it at all. At the time, it was not possible to find any dietary factor which caused this variation. Fusion of equol with potassium hydroxide yielded resorcinol, β-resorcylic acid, para-ethylphenol, para-hydroxybenzoic acid, and an unidentified phenol (169). On the basis of this information, it was suggested that equol must be 7-hydroxy-2-(4'-hydroxyphenyl)chroman, 7-hydroxy-3-(4'-hydroxyphenyl)chroman, or 6-hydroxy-2-(4'-hydroxybenzyl)coumaran.

An investigation into the constitution of equol by Wessely et al (170, 171) resulted in the identification of the unknown phenol produced by the fusion of equol with potassium hydroxide. Their results narrowed down the possible structures of equol to two, namely

7-hydroxy-3-(4'-hydroxyphenyl)chroman, and 6-hydroxy-2-(4'-hydroxyphenyl)-2-methyl coumaran. Almost simultaneously, Anderson and Marrian (172) in 1939 reported that equol must be 7-hydroxy-3-(4'-hydroxyphenyl)chroman (Figure 1).

Except for the publication in 1940 of a non-specific colour reaction for the presence of equol in mare's urine (173), no work had been done with equol until the year 1957. At that time, Klyne and Wright reported the presence of equol in the urine of pregnant goats (174) and later in the urine of pregnant cows (175). More recently, equol was detected in the urine and faeces of the domestic fowl (176) and was subsequently isolated from the urine in crystalline form (177).

Examination of Figure 1 will readily reveal the structural similarities of the isoflavones, coumestrol, and equol. First, they all have the same basic carbon skeleton. Second, they all have two benzenoid rings. Third, they have an oxygen function at position-1. Fourth, they have a hydroxyl group at position-7. Finally, they all have oxygenated substituents at the 4'-position. It does not seem unlikely, therefore, that the significant amounts of equol that have been detected in the urine of the domestic fowl may arise from the estrogenic isoflavones and possibly from coumestrol which are present in the diet.

In the case of the isoflavones, equol might arise by reduction of the 2:3 double bond and of the 4-oxo group, and by the removal of the hydroxyl group when genistein and biochanin-A are being considered. In the case of coumestrol, reduction might occur after opening of the furan ring in association with the removal of the ketonic group at position-2, the latter being an α,β-unsaturated ketone. It was decided, therefore, to inject H^3- and C^{14}-labelled starting materials into non-laying hens and to attempt to identify radioactive equol and other metabolites in the phenolic extracts of the collected urine.

SECTION TWO

ASPECTS OF THE MEASUREMENT OF RADIOACTIVITY DISTRIBUTION ON H^3- AND C^{14}-LABELLED PAPER CHROMATOGRAMS

HISTORICAL INTRODUCTION - SECTION TWO

The major portion of the work reported in this thesis involved the injection into non-laying hens of H^3- and C^{14}-labelled starting materials and the location and identification of urinary radioactive metabolites on paper chromatograms. In many instances, the amounts of radioactivity in the various chromatographic zones were extremely low. As a result, it was necessary to employ a method which would be sensitive enough to measure low levels of radioactivity on small pieces of chromatographic paper and yet sufficiently reproducible to be within the acceptable limits of variation. A technique was developed which involved the use of liquid scintillation spectrophotometry. In view of the often conflicting reports in the literature concerning methods of measurement of radioactivity on paper chromatograms, this section will be devoted to a brief review of the literature on the aspects of liquid scintillation spectrophotometry and the measurement of radioactivity on paper chromatograms which are directly relevant to this investigation.

Since the appearance of the comprehensive review by Davidson and Feigelson on liquid scintillation counting in 1957 (178), the field has expanded greatly. Important

advances have been made in instrument design and, more important, in instrument usage. Numerous publications devoted to the liquid scintillation technique have appeared and texts and symposia have been devoted to the subject (179, 180, 181, 182). Applications include not only new uses of conventional instrumentation, but approaches that have encouraged design of new instruments and accessories which, in turn, have broadened significantly the scope of liquid scintillation counting. A recent review by Rapkin (183) illustrates the trend toward the wider application of this technique.

One of the main reasons for the increasing popularity of liquid scintillation counting is the development of numerous methods of sample preparation. This has resulted in the use of this method for the measurement of radioactivity in a wide variety of samples, including solids, liquids, gases and vapours, suspensions, materials with two radioactive isotopes, and paper strips. Frequently, different approaches to the solution of the same general problem are described, since it has been found that subtle differences in sample characteristics necessitate changes in preparative methods. Workers in the field must be prepared to evaluate alternative approaches in order to find the most suitable method for working with any particular sample.

1. Paper-Strip Counting

If one is faced with the problem of determining the distribution of radioactivity on a paper chromatogram, several assay techniques are available. The chromatogram may be scanned in a strip counter and the radioactivity estimated by measuring areas under each peak or by use of an electronic integrator (184). The paper strip can also be cut into appropriate sections and the radioactivity of each section determined under a Geiger counter. Depending upon the thickness of the paper, the counting efficiency of these techniques is in the range of 3 to 10 percent. More serious than the inefficiency is the variation in efficiency which renders quantitation difficult. If the radioactive compounds are eluted from the paper, the efficiency and precision of counting are greatly improved. However, such elution is tedious and may not be quantitative.

In 1957, Roucayrol et al (185) first described liquid scintillation counting of radioactivity on paper strips. Strips containing C^{14}, P^{32}, or I^{131} were moistened with toluene/phenylbiphenyloxadiazole (PBD) and then placed on the window of a photomultiplier tube which in turn was connected to an appropriate amplification and recording system. Counting efficiencies of all three isotopes were

satisfactory; in addition, it was reported possible to measure C^{14} in the presence of either P^{32} or I^{131}. However, the mechanical procedure required was somewhat tedious. Another very similar technique was employed whereby dried paper strips which had previously been impregnated with a solution of anthracene and benzene were placed on the surface of a similar photomultiplier tube and amplification set up (186). Neither of these reports made any attempt to count papers in the presence of solvent nor did they pay particular attention to the geometry problems which would probably arise from such a relatively crude counting system.

Wang and Jones (187) were the first to describe routine measurement of paper strips using the liquid scintillation method. Strips were placed in counting vials and counted with toluene/2,5-diphenyloxazole (PPO). The samples were counted automatically using a coincidence system. Their experiments were also concerned with the effect of varying types of commercially available filter papers on the counting rates; they observed that conventional filter papers did not scatter or absorb a significantly large number of photons. Therefore, counting efficiencies in the presence of paper were comparable to those obtained in the absence of paper, or, in other words, the paper was

transparent to the emitted photons. The only limitation appeared to be due to whatever self-absorption effect was exerted by the paper, which was found to be quite small, and to whatever quenching effect might be exerted by the various types of compounds or solutes that would be counted in such a manner. Proceeding on the conclusion that the paper was essentially transparent to the emitted light, Wang and Jones were able to demonstrate that the counting efficiency for C^{14}-labelled compounds directly from the paper chromatogram was approximately 55 percent. This referred to compounds insoluble in their scintillation solvent, toluene; for compounds eluted completely from the paper by the solvent, the efficiencies were in the order of 85 percent. The counting efficiency for insoluble tritium labelled compounds was in the order of 1.5 percent. It was also noted that the position of the sample between the two photomultipliers was not particularly important for C^{14} counting but was a significant factor when H^3 was being measured. These findings on C^{14} counting were confirmed by other workers (188) who found the variation to be 5 percent or less; thus it was unnecessary for the paper to be held in a fixed orientation relative to the photomultiplier tube window. This view has been upheld by other investigators (189).

Loftfield (190), however, believed that reproducible orientation was far more important in the counting of C^{14}-labelled chromatograms than had been previously suggested. He overcame problems of reproducible orientation by rolling the paper strips lengthwise into closed cylinders which completely lined the counting vial. The size of the paper strips were 2.3 X 5.0 centimeters, and thus the strip just fitted into a 10 millimeter test tube filled with the scintillation solvent. Although a 20 percent loss in counting efficiency was noted when compared to measuring the same amount of activity on a paper square, Loftfield reported that reproducibility was significantly increased. A comparison between thin-window Geiger and liquid scintillation counting was also given which indicated that the latter method was five to ten times as efficient as the former.

Davidson (191) preferred to cut the paper into circular discs and place them flat at the bottom of the counting vial; the average variation was 1.5 percent for repetitive counts. He observed a count loss comparable to that reported by Loftfield. Davidson also reported that the observed count rate was independent of solvent volume from 5 to 15 milliliters.

Ekins (192) immersed paper strips containing tritium

in a toluene solution of the plastic scintillator Naton-136 (Nash and Thompson, Ltd.) or, more usually, placed between two pieces of Kleenex soaked in the plastic solution. The strip of "Kleenex sandwich" was allowed to dry slowly and then counted in the dried state in a conventional liquid scintillation counter. Counting efficiencies between 10 percent and 25 percent were achieved.

There are numerous reports in the literature on developments of new methods or refinements of previous ones in order to solve specific problems involved in the measurement of radioactivity in particular samples on filter papers (193, 194, 195, 196, 197). Nilsson's evidence on the demethylation of biochanin A to genistein (159) comprised measurements of radioactivity on chromatograms of a maximum of 15 centimeters in length which had been cut into 1 centimeter bands; each band was eluted with 80 percent ethanol and aliquots of the eluates were analyzed for radioactivity. In this procedure, the problem of incomplete elution was neglected. The variation of techniques reveals a wide diversity of opinion in this field of paper strip counting. However, the consensus of current opinion seems to be that paper orientation is not critical for the counting of either carbon, sulfur, or phosphorus labelled material but may

be quite significant when tritium counting is required. It should be noted that this opinion is based entirely on investigations with relatively large pieces of filter paper. A typically complicated procedure for the attainment of maximum efficiency involves placing a narrow test tube in the center of the standard counting vial, and supporting this test tube by drilling or punching a hole in the cap of the vial (198). The paper strips to be counted may then be wedged into this test tube and covered with the scintillation solvent. The counting vial merely acts as a support for the test tube which contains both the paper strip and the solvent.

In spite of the controversy about the mode of application, the use of automatic liquid scintillation spectrometry for the assay of radioactive materials on paper chromatograms has distinct advantages over the conventional paper scanning systems that had been previously employed. For example, Osinski (199) has described a 4π windowless Geiger counter for paper strips; with tritium activity on Whatman No. 1 filter paper, a maximum of only 1 percent counting efficiency was reported. Wilson (200) has combined radioautography with liquid scintillation counting in an attempt to improve tritium measurement. He immersed a two-dimensional chromatogram in a

toluene/terphenyl-diphenyl hexatriene solution and then prepared a radioautograph. He reported increased sensitivity; with a 50 hour exposure, a maximum of 0.1 μC/cm was detected. In contrast, the increased high efficiency and the low background inherent in the use of the liquid scintillation technique has resulted in sensitivity of at least twenty times those of other reported results.

2. Quenching

Although liquid scintillation counting is ideal for many applications, there is one substantial disadvantage which must be kept in mind. Quenching, either by virtue of the light-filtering effect of coloured substances or interference by colourless substances with the liquid scintillation process, may cause reduction of counting efficiencies. This problem is more severe when working with the less energetic isotopes and is of little consequence when working with highly energetic nuclides.

Quenching which results from colour can be easily overcome by decolourization (201, 202, 203), but it is often more difficult to eliminate quenching by certain colourless substances. Frequently it is difficult to be assured that quenching is completely absent and so it is necessary to be able to accurately determine absolute counting efficiencies. Kerr et al (204) described an internal standardization method wherein a known amount of benzoic acid-C^{14} was added to a sample once it had been counted. Recounting indicated the efficiency for counting the added sample. It was assumed that the added sample was counted with the same efficiency as was the original sample, thereby permitting a simple estimate of the original counting efficiency.

Peng (205) has described an extrapolation method for the determination of quenching which, while it may be somewhat more accurate than the internal standard method, is more time-consuming. He repetitively counted the same material after the addition of larger and larger quantities. A plot of specific count rate versus sample weight was constructed and was extrapolated to zero weight in order to determine the specific counting rate for an unquenched sample. This figure could then be converted to absolute efficiency by determining the performance of the instrument with a non-quenching standard.

The problem of overcoming quenching seems to be similar to those involved in paper strip counting in one respect: each experiment with a different sample should be treated individually in order to find the best method to overcome any possible quenching problem. Numerous solutions to problems of quenching have been published (206, 207, 208, 209, 210). They all serve to illustrate that there are many general techniques to deal with quenching, but that preliminary experiments are recommended for each individual investigation to determine which is most suitable.

3. Decomposition of Tritium-Labelled Compounds

The high specific activities which can be obtained with tritium compounds, the short range (0.7 mg/cm^2) (211) of the weak β-radiation (maximum energy between 17.5 and 19.4 keV (212) and mean energy of 5.52 keV (213)) and the long half-life (12.3 years) (214) of tritium, enable compounds labelled with this isotope to have a very wide variety of uses. The half-life of tritium, although conveniently long for most experimental uses, represents a decomposition rate of approximately 5 percent per annum in the storage of labelled compounds, due to the natural decay of tritium to helium. This primary, or internal decomposition, represents only a minor problem when compared to the secondary radiation effects and chemical decomposition of tritium-labelled compounds. These considerations have been reviewed recently by Evans and Stanford (215).

The decomposition or transformation due to the reaction of a labelled molecule with a reactive species, for example, free radicals or the excited decomposition fragments of primary decay, presents the most serious problem in the storage of tritium compounds. Such secondary radiation effects give rise to radiochemical impurities which can often present a serious disadvantage to the user of tracer

compounds. This is especially true for experiments, for example, on the metabolism or some analytical use of tritium compounds, the results of which can only be deemed reliable if the tracer-labelled compounds are of a high radiochemical purity. Fortunately for many uses, the presence of a few percent impurity is not necessarily an embarrassment, but it is obviously most desirable to know the exact radiochemical purity of the labelled compound before use.

All organic compounds are thermodynamically unstable, but chemical decomposition at room temperature, for many compounds, is usually very slow and can be slowed even further by storing the compound at low temperatures. In radiochemical investigations, the presence of chemical non-radioactive impurities is not normally a handicap, but for work involving the absorption or reaction rates of a particular labelled compound, these chemical impurities may prove embarrassing. As chemical decomposition can give rise to radiochemical impurities, compounds are often stored at low temperatures to reduce this type of decomposition to a minimum.

The decomposition of tritium-labelled compounds can be minimized by storing the compounds dissolved in a suitable

solvent rather than maintaining the compound in the solid state (215). Owing to the very short range of the β-particles, it is reasonable to assume that all the energy of the radiation will be self-absorbed. In the solid state, therefore, decomposition due to primary (external) radiation, i.e., radiation from other molecules, is likely to be a major contributing source. Interaction between radioactive and unlabelled molecules will be much smaller in solution and primary decomposition will be minimized, most of the β-radiation being absorbed by the surrounding solvent. The decomposition should be minimal at infinite dilution, but the gross effects of variation in concentration have yet to be studied in detail.

In numerous cases there does not seem to be any great advantage in storing the compound at $-40^{o}C$ rather than at $0^{o}C$. Tritium compounds having specific activities less than 500 mC/millimole when stored under the best-known conditions suffer no serious decomposition for at least 1 year. For compounds with higher specific activities, the rate of decomposition increases with increased specific activity and depends on the actual type of compound.

In 1957, Wilzbach (216) described a simplified technique for labelling organic compounds with tritium that

has come to be called the Wilzbach gas-exposure method and undoubtedly is responsible for the increased popularity of tritium as a tracer. The technique requires relatively few man-hours and relatively simple and inexpensive equipment, and results in labelled compounds of very high specific activities. The major disadvantage of the method is the formation of labelled byproducts which may be present in very great amounts, depending upon the tritiated compound in question. The isolation of a radiochemically pure product is frequently extremely difficult because the contaminants usually have molecular weights of the same order as that of the parent compound. Another point which must be kept in mind is that the labile tritium must be removed before the compound can be used as a tracer.

The removal of labile tritium is accomplished readily by repeatedly dissolving the sample in hydrolytic solvents, such as water or alcohol, and then removing the solvent by evaporation or distillation (217, 218, 219, 220, 221). Gas-liquid chromatography has been used extensively for further purification (222, 223, 224, 225, 226). Other techniques of purification that have been used include paper chromatography (227, 228, 229), column chromatography (230, 231), counter current distribution (216, 227),

fractional distillation (216), and recrystallization (216, 224, 232, 233). It is apparent from all these reports that the type of compound and extent of contamination dictates the mode of purification.

PART II

AN INVESTIGATION OF THE MEASUREMENT OF RADIOACTIVITY DISTRIBUTION ON H^3- AND C^{14}-LABELLED PAPER CHROMATOGRAMS

INTRODUCTION

It was necessary at the outset of this investigation to develop an efficient technique for the measurement of radioactivity distribution on H^3- and C^{14}-labelled paper chromatograms. Since the results of the metabolic experiments of the subsequent investigations were based primarily on the use of a relatively large number of chromatograms, it was of the utmost importance that the method developed for the measurement of radioactivity on these chromatograms should not be as laborious as those reported in the literature. For this purpose, efficiency could be sacrificed to some extent in favour of reproducibility, since radioactivity distribution rather than absolute radioactivity was to be measured. The primary criterion of reliability to be considered, therefore, was reproducibility combined with a reasonably high degree of efficiency. It must be pointed out that individual estimations of radioactivity were performed on paper bands of much smaller size (¾ X 2 cm or ½ X 2 cm) than those used by other investigators. It was thought that reduction in size of the bands might of itself lessen the effects of orientation of the bands in the scintillation vials on the recorded counts per minute. It was decided, therefore, to investigate the effects of

scintillation solvent volume, quenching of extraction solvent (methanol), presence of filter paper bands (¾ X 2 cm), filter paper thickness, and paper orientation on the efficiency and reproducibility of the measurement of radioactivity on small bands (¾ X 2 cm) of filter paper containing H^3 or C^{14}.

EXPERIMENTAL

1. Materials

(a) Scintillation Fluid

2,5-Diphenyloxazole (PPO) (Packard Instrument Company, Inc., La Grange, Illinois, U.S.A.) (3 gm) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) (Packard Instrument Company, Inc.) (0.1 gm) were dissolved in toluene (Scintillation Grade, Nuclear Enterprises, Winnipeg, Canada) (1000 ml). Because of the relatively low solubility of POPOP in toluene, it was necessary to heat the mixture to about 35°C to aid solution. This solution of PPO and POPOP in toluene has been used with much success for a wide variety of scintillation counting techniques (eg. 183, 234, 235, 236).

(b) Methanol

Methanol (Spectranalyzed grade, Fisher Scientific Company) was distilled before use.

(c) Filter Papers

Whatman No. 42 (3 X 22.5") filter paper was extracted (72 h) in a Soxhlet extractor with methanol and chloroform

(50:50, v:v). Whatman No. 3MM filter paper was first extracted with methanol and benzene (50:50, v:v).

(d) Standard Coumestrol-H^3

Purified coumestrol-H^3 (approx. 1 mg), the preparation of which is described in a subsequent section, was dissolved in methanol (10 ml).

(e) Standard Biochanin A-C^{14}

Synthetic biochanin A-4-C^{14} (10 mg) was dissolved in methanol (10 ml).

2. Methods and Results

Each of the tests herewith reported was performed in triplicate on both standard coumestrol-H^3 and standard biochanin A-C^{14}. These two compounds were employed in the later metabolic studies. The methanolic solutions of these two substances will subsequently be called the "standard solutions". The preparation of samples was performed in standard screw-top glass scintillation vials and each sample was counted five times for 1 minute at $-4^{o}C$ in a Packard Tri-Carb Liquid Scintillation Counter Model 500B.

a. Effect of Methanol

(i) The standard solution (0.1 ml) was evaporated to dryness in the scintillation vial and scintillation fluid (10 ml) was then added to the residue. Coumestrol and biochanin A are insoluble in toluene.

(ii) Scintillation fluid (10 ml) was added directly to the standard solution (0.1 ml).

(iii) To the standard solution (0.1 ml) were added methanol (0.1 ml) and scintillation fluid (10 ml).

(iv) To the standard solution (0.1 ml) were added methanol (0.2 ml) and scintillation fluid (10 ml).

(v) To the standard solution (0.1 ml) were added methanol (0.3 ml) and scintillation fluid (10 ml).

The quenching effects of different concentrations of methanol on the observed counts per minute of the standard solutions of coumestrol-H^3 and biochanin A-C^{14} are presented in Table IV and Table VIII respectively. The values reported are the average observed counts per minute (corrected for background) of three samples counted five times each, and thus each value represents the mean of fifteen observations. The efficiencies of the scintillation counter for the measurement of H^3 and C^{14} were not taken into consideration. Each value is accompanied by the standard deviation of the mean and the coefficient of variability per sample.

A summary of the analysis of variance of the averages in Table IV is presented in Table V. The analysis of variance was carried out according to the

procedure outlined by Bennett and Franklin (237), which simplifies the computation for machine calculation. Subsequent analyses of variance were performed in the same manner and only the actual variances are given; these are to be found in the same tables as are the average counts per minute. Further statistical analysis of the data is presented in Tables VI, VII, IX, and X. Abbreviations used in these and subsequent tables are as follows: b.c. = variance between classes; w.c. = variance within classes; + = significant; - = not significant.

TABLE IV

EFFECT OF AMOUNT OF METHANOL ON THE OBSERVED COUNTS PER MINUTE OF STANDARD COUMESTROL-H^3

Test Number	Volume of Methanol (ml)	Average C.P.M.	S.D. of Mean	C.V. per Sample
a(i)	0	129,000	±3980	5.34
a(ii)	0.1	129,800	±1690	2.26
a(iii)	0.2	129,500	±1850	2.48
a(iv)	0.3	124,700	±1910	2.65
a(v)	0.4	131,100	±2240	3.43

TABLE V

ANALYSIS OF VARIANCE OF AVERAGES PRESENTED IN TABLE IV

Test Number	Source of Variation	Sum of Squares X 10^{-6}	D.F.	Mean Square X 10^{-6}	Variance X 10^{-6}
a(i)	between classes	470	2	$235.0(\sigma^2+n\sigma_3^2)$	$46.7(\sigma_3^2)$
	within classes	19	12	$1.6(\sigma^2)$	$1.6(\sigma^2)$
	total	489	14		
a(ii)	between classes	30	2	$15.0(\sigma^2+n\sigma_3^2)$	$2.78(\sigma_3^2)$
	within classes	13	12	$1.08(\sigma^2)$	$1.08(\sigma^2)$
	total	43	14		
a(iii)	between classes	104	2	$52.0(\sigma^2+n\sigma_3^2)$	$10.35(\sigma_3^2)$
	within classes	3	12	$0.25(\sigma^2)$	$0.25(\sigma^2)$
	total	107	14		
a(iv)	between classes	109	2	$54.50(\sigma^2+n\sigma_3^2)$	$10.75(\sigma_3^2)$
	within classes	9	12	$0.75(\sigma^2)$	$0.75(\sigma^2)$
	total	118	14		
a(v)	between classes	411	2	$205.50(\sigma^2+n\sigma_3^2)$	$37.33(\sigma_3^2)$
	within classes	226	12	$18.83(\sigma^2)$	$18.83(\sigma^2)$
	total	637	14		

TABLE VI

F TESTS: COMPARISONS OF VARIANCES PRESENTED IN TABLE V

Numerator			Denominator			Significance	
Parameter	Value	D.F.	Parameter	Value	D.F.	5%	1%
b.c., a(i)	46.7	2	b.c., pld. a(ii-v)	15.3	8	-	-
b.c., a(i)	46.7	2	b.c., pld. a(ii-iv)	7.96	6	+	-
b.c., a(v)	37.33	2	b.c., pld. a(ii-iv)	7.96	6	-	-

TABLE VII

t TESTS: COMPARISONS OF AVERAGES PRESENTED IN TABLE IV

Comparison	D.F.	t	P
a(iv) vs. pooled a(i-iii)	10	0.687	<0.35
a(v) vs. pooled a(i-iv)	13	1.903	<0.05

TABLE VIII

EFFECT OF AMOUNT OF METHANOL ON THE OBSERVED COUNTS PER MINUTE OF STANDARD BIOCHANIN A-C^{14}

Test Number	CH_3OH (ml)	Average C.P.M.	S.D. of Mean	C.V. per Sample	Variances	
					B.C. (σ_b^2)	W.C. (σ^2)
a(i)	0	8320	±171	3.56	8.73×10^4	0.33×10^4
a(ii)	0.1	8250	± 98	2.06	2.85×10^4	0.25×10^4
a(iii)	0.2	8210	± 67	1.41	1.47×10^4	0.17×10^4
a(iv)	0.3	7870	± 71	1.57	1.33×10^4	0.33×10^4
a(v)	0.4	7570	± 59	1.35	2.95×10^4	0.75×10^4

TABLE IX

F TESTS: COMPARISONS OF VARIANCES PRESENTED IN TABLE VIII

Numerator			Denominator			Significance	
Parameter	Value	D.F.	Parameter	Value	D.F.	5%	1%
b.c., a(i)	8.73	2	b.c., pld. a(ii-v)	2.15	8	-	-
b.c., a(i)	8.73	2	b.c., pld. a(ii-iv)	1.88	6	-	-
b.c., a(v)	2.95	2	b.c., pld. a(ii-iv)	1.88	6	-	-

TABLE X

t TESTS: COMPARISONS OF AVERAGES PRESENTED IN TABLE VIII

Comparison	D.F.	t	P
a(iv) vs. pooled a(i-iii)	10	1.233	<0.15
a(v) vs. pooled a(i-iv)	13	1.652	<0.10
a(v) vs. pooled a(i-iii)	10	1.946	<0.05

b. Effect of Scintillation Fluid

(i) Scintillation fluid (5 ml) was added to the standard solution (0.1 ml) and methanol (0.1 ml).

(ii) Scintillation fluid (15 ml) was added to the standard solution (0.1 ml) and methanol (0.1 ml).

The effects of scintillation fluid volume on the observed counts per minute of the standard solutions of coumestrol-H^3 and of biochanin A-C^{14} in methanol (0.2 ml) are presented in Table XI and Table XIII respectively. The values reported are the average observed counts per minute of three samples counted five times each. Statistical analyses of the data are summarized.

TABLE XI

EFFECT OF AMOUNT OF SCINTILLATION FLUID ON THE OBSERVED COUNTS PER MINUTE OF STANDARD COUMESTROL-H^3

Test Number	Scint. Fluid (ml)	Average C.P.M.	S.D. of Mean	C.V. per Sample	Variances	
					B.C.(σ_3^2)	W.C.(σ^2)
b(i)	5	114,500	$\pm$1780	2.37	9.67×10^6	0.17×10^6
a(iii)	10	129,500	$\pm$1850	2.48	10.35×10^6	0.25×10^6
b(ii)	15	130,700	$\pm$ 780	1.03	1.50×10^6	5.50×10^6

TABLE XII

F TEST: COMPARISON OF VARIANCES PRESENTED IN TABLE XI

Numerator			Denominator			Significance	
Parameter	Value	D.F.	Parameter	Value	D.F.	5%	1%
b.c., pooled b(i) & a(iii)	10.01	4	b.c., b(ii)	1.50	2	-	-

TABLE XIII

EFFECT OF AMOUNT OF SCINTILLATION FLUID ON THE OBSERVED COUNTS PER MINUTE OF STANDARD BIOCHANIN A-C^{14}

Test Number	Scint. Fluid (ml)	Average C.P.M.	S.D. of Mean	C.V. per Sample	Variances	
					B.C. (σ_3^2)	W.C. (σ^2)
b(i)	5	7930	$\pm$46	1.00	0.43 X 10^4	0.33 X 10^4
a(iii)	10	8210	$\pm$67	1.41	1.47 X 10^4	0.17 X 10^4
b(ii)	15	8290	$\pm$57	1.07	0.68 X 10^4	0.08 X 10^4

TABLE XIV

t TESTS: COMPARISONS OF AVERAGES PRESENTED IN TABLE XI AND TABLE XIII

Comparison	D.F.	t	P
b(i) vs. pooled a(iii) & b(ii):			
Coumestrol-H^3 (Table XI)	7	2.756	<0.025
Biochanin A-C^{14} (Table XIII)	7	1.737	<0.10
b(ii) vs. a(iii):			
Coumestrol-H^3 (Table XI)	4	0.480	<0.35
Biochanin A-C^{14} (Table XIII)	4	0.371	<0.40

c. Effect of Filter Paper

(i) A band of filter paper (¾ X 2 cm) was spotted with the standard solution (0.1 ml), air-dried and placed in a scintillation vial. Scintillation fluid (10 ml) was added directly to the vial. Both Whatman No. 42 and Whatman No. 3MM filter papers were tested.

(ii) A filter paper band was spotted with the standard solution (0.1 ml), dried and placed in a vial. Methanol (0.2 ml) was added and the contents were shaken occasionally for 5 minutes. Scintillation fluid (10 ml) was added. Both Whatman No. 42 and Whatman No. 3MM filter papers were tested.

(iii) A filter paper band was spotted with the standard solution (0.1 ml), air-dried, cut into small pieces, and extracted twice with methanol (1 ml each) in a centrifuge tube. Both extracts were transferred to a scintillation vial, the methanol was evaporated under nitrogen, and methanol (0.2 ml) was added to the residue. The contents were shaken and flooded with scintillation fluid (10 ml). This procedure was carried out on both Whatman No. 42 and Whatman No. 3MM filter papers.

(iv) This test was the same as c(iii) except that methanol was not added to the residue in the scintillation vial. The residue was flooded directly with the scintillation fluid (10 ml). This test was similarly performed on both Whatman No. 42 and Whatman No. 3MM filter papers.

The effects of various treatments of filter paper bands impregnated with standard solutions of coumestrol-H^3 and of biochanin A-C^{14} on the average observed counts per minute are presented in Table XV and Table XVII respectively. Each value reported is the average for three samples counted five times and is accompanied by the results of appropriate statistical analyses.

TABLE XV

EFFECT OF TREATMENT OF FILTER PAPER BAND IMPREGNATED WITH STANDARD SOLUTION OF COUMESTROL-H^3

Test Number	Filter Paper Number	Average C.P.M.	S.D. of Mean	C.V. per Sample	Variances	
					B.C. (σ_3^2)	W.C. (σ^2)
c(i)	42	64,700	±5814	15.57	95.88 X 10^6	0.97 X 10^6
	3MM	56,800	±2444	7.45	17.58 X 10^6	0.80 X 10^6
c(ii)	42	102,600	±1964	3.41	11.41 X 10^6	0.78 X 10^6
	3MM	99,200	± 863	1.49	0.64 X 10^6	0.42 X 10^6
c(iii)	42	111,400	±4889	7.60	71.84 X 10^6	0.90 X 10^6
	3MM	105,500	±2408	3.94	62.73 X 10^6	0.63 X 10^6
c(iv)	42	89,600	±3320	6.42	32.74 X 10^6	1.00 X 10^6
	3MM	83,900	±3265	6.74	31.91 X 10^6	1.60 X 10^6

TABLE XVI

F TESTS: COMPARISONS OF VARIANCES PRESENTED IN TABLE XV

Numerator			Denominator			Significance	
Parameter	Value	D.F.	Parameter	Value	D.F.	5%	1%
b.c., c(i), No. 42	95.88	2	b.c., c(ii), No. 42	11.41	2	-	-
b.c., c(iii), No. 42	71.84	2	b.c., c(ii), No. 42	11.41	2	-	-
b.c., c(iv), No. 42	32.74	2	b.c., c(ii), No. 42	11.41	2	-	-
b.c., c(i), No. 3MM	17.58	2	b.c., c(ii), No. 3MM	0.64	2	+	-
b.c., c(iii), No. 3MM	62.73	2	b.c., c(ii), No. 3MM	0.64	2	+	-
b.c., c(iv), No. 3MM	31.91	2	b.c., c(ii), No. 3MM	0.64	2	+	-
b.c., No. 42, pooled c(i-iv)	52.97	8	b.c., No. 3MM, pooled c(i-iv)	28.21	8	-	-

TABLE XVII

EFFECT OF TREATMENT OF FILTER PAPER BAND IMPREGNATED WITH STANDARD SOLUTION OF BIOCHANIN A-C^{14}

Test Number	Filter Paper Number	Average C.P.M.	S.D. of Mean	C.V. per Sample	Variances	
					B.C. (σ_3^2)	W.C. (σ^2)
c(i)	42	6780	$\pm$114	2.91	3.93×10^4	0.10×10^4
	3MM	6490	$\pm$115	3.07	3.79×10^4	0.57×10^4
c(ii)	42	7730	$\pm$ 46	1.04	0.49×10^4	0.53×10^4
	3MM	7540	$\pm$ 74	1.69	0.88×10^4	0.53×10^4
c(iii)	42	7600	$\pm$335	7.65	32.73×10^4	0.32×10^4
	3MM	7730	$\pm$191	4.30	11.19×10^4	0.32×10^4
c(iv)	42	7350	$\pm$145	3.41	6.29×10^4	0.22×10^4
	3MM	7190	$\pm$ 67	1.60	0.44×10^4	0.63×10^4

TABLE XVIII

F TESTS: COMPARISONS OF VARIANCES PRESENTED IN TABLE XVII

Numerator			Denominator			Significance	
Parameter	Value	D.F.	Parameter	Value	D.F.	5%	1%
b.c., c(i), No. 42	3.93	2	b.c., c(ii), No. 42	0.49	2	-	-
b.c., c(iii), No. 42	32.73	2	b.c., c(ii), No. 42	0.49	2	+	-
b.c., c(iv), No. 42	6.29	2	b.c., c(ii), No. 42	0.49	2	-	-
b.c., c(i), No. 3MM	3.79	2	b.c., c(ii), No. 3MM	0.88	2	-	-
b.c., c(iii), No. 3MM	11.19	2	b.c., c(ii), No. 3MM	0.88	2	-	-
b.c., c(iv), No. 3MM	0.44	2	b.c., c(ii), No. 3MM	0.88	2	-	-
b.c., No. 42, pooled c(i-iv)	10.86	8	b.c., No. 3MM, pooled c(i-iv)	4.08	8	-	-

TABLE XIX

t TESTS: COMPARISONS OF AVERAGES PRESENTED IN TABLE XV AND TABLE XVII

Comparison	D.F.	t	P
c(ii) vs. c(i)(Whatman No. 42):			
Coumestrol-H^3	4	2.289	<0.05
Biochanin A-C^{14}	4	2.608	<0.05
c(ii) vs. c(i)(Whatman No. 3MM):			
Coumestrol-H^3	4	6.117	<0.005
Biochanin A-C^{14}	4	2.665	<0.05
c(ii) vs. c(iii)(Whatman No. 42):			
Coumestrol-H^3	4	0.613	<0.35
Biochanin A-C^{14}	4	-	-
c(ii) vs. c(iii)(Whatman No. 3MM):			
Coumestrol-H^3	4	1.005	<0.20
Biochanin A-C^{14}	4	0.413	<0.40
c(ii) vs. c(iv)(Whatman No. 42):			
Coumestrol-H^3	4	1.204	<0.15
Biochanin A-C^{14}	4	0.858	<0.35
c(ii) vs. c(iv)(Whatman No. 3MM):			
Coumestrol-H^3	4	1.733	<0.10
Biochanin A-C^{14}	4	1.112	<0.20
c(ii) vs. a(iii)(Coumestrol-H^3):			
Whatman No. 42	4	4.071	<0.01
Whatman No. 3MM	4	6.060	<0.005
c(ii) vs. a(iii)(Biochanin A-C^{14}):			
Whatman No. 42	4	2.412	<0.05
Whatman No. 3MM	4	2.742	<0.05
pooled c(i-iv)(Whatman No. 42) vs. pooled c(i-iv)(Whatman No. 3MM):			
Coumestrol-H^3	16	3.176	<0.005
Biochanin A-C^{14}	16	1.597	<0.10

d. Effect of Filter Paper Orientation in the Scintillation Vial

The samples prepared for test c(ii) were employed in this experiment. Each vial was placed above the counting well in the scintillation counter in such a manner that the paper band, which rested flat at the bottom of the vial, would enter the well from four different orientations on four successive trials. For this purpose, a mark was made at the side of the well and the four positions, in relation to this mark, were designated as follows: aligned, transverse, and the two diagonals. It was realized that the vials would undergo a certain degree of rotation as they entered the well, but it was assumed that this rotation would be constant from sample to sample. For replicate determinations, the samples were not allowed to remain in the well but were released and then permitted to re-enter from the same orientation.

When the results of this experiment were being analyzed, it was realized that the foregoing procedure did not provide complete randomness of counting. Accordingly, the experiment was repeated but with the following modification: instead of counting five times in one position, followed by five times in a second position, etc., the order of the

positions were chosen by lot until the counts with each orientation had been measured five times. Although the samples were prepared in the same way as were those for test c(ii), it will be noted that the actual counts were higher than those recorded in test c(ii). This can be ascribed to evaporation of some of the solvent of the standard solutions during the interval (18 months) between the performance of test c(ii) and the repetition of test d. The results are presented in the tables which follow. The average counts per minute represent the mean of the four different orientations, each counted five times.

TABLE XX

EFFECT OF FOUR DIFFERENT POSITIONS IN SCINTILLATION VIAL OF FILTER PAPER IMPREGNATED WITH STANDARD SOLUTION OF COUMESTROL-H^3

Test Number	Filter Paper Number	Sample Number	Average C.P.M.	S.D. of Mean	C.V. per Sample	Variances	
						B.C.(σ_3^2)*	W.C.(σ^2)
		1	122,700	±603	0.70	1.99×10^6	0.90×10^6
d(i)	42	2	127,500	±558	0.64	1.28×10^6	0.54×10^6
		3	129,800	±805	0.97	2.59×10^6	0.38×10^6
		1	113,900	±502	0.69	0.99×10^6	0.24×10^6
d(ii)	3MM	2	120,700	±549	0.71	2.01×10^6	0.34×10^6
		3	115,200	±521	0.70	1.59×10^6	0.30×10^6

*Each variance between classes represents the variance between the four different orientations of the filter paper band for one sample.

TABLE XXI

F TESTS: COMPARISONS OF VARIANCES (COUMESTROL-H^3)

Numerator			Denominator			Significance	
Parameter	Value	D.F.	Parameter	Value	D.F.	5%	1%
b.c., c(ii), No. 42	11.41	2	b.c., pld. d(i)	1.95	9	+	-
b.c., pld. d(ii)	1.86	9	b.c., c(ii), No. 3MM	0.64	2	-	-

TABLE XXII

EFFECT OF FOUR DIFFERENT POSITIONS IN SCINTILLATION VIAL OF FILTER PAPER IMPREGNATED WITH STANDARD SOLUTION OF BIOCHANIN A-C^{14}

Test Number	Filter Paper Number	Sample Number	Average C.P.M.	S.D. of Mean	C.V. per Sample	Variances	
						B.C. (σ_3^2)*	W.C. (σ^2)
		1	8860	$\pm$54	0.94	1.20×10^4	0.33×10^4
d(iii)	42	2	8500	$\pm$49	0.88	0.84×10^4	0.37×10^4
		3	8290	$\pm$35	0.79	0.81×10^4	0.30×10^4
		1	8280	$\pm$63	1.19	1.65×10^4	0.35×10^4
d(iv)	3MM	2	8000	$\pm$42	0.82	0.65×10^4	0.61×10^4
		3	8480	$\pm$ 3	0.51	0.24×10^4	0.27×10^4

*Each variance between classes represents the variance between the four different orientations of the filter paper band for one sample.

TABLE XXIII

F TESTS: COMPARISONS OF VARIANCES (BIOCHANIN A-C^{14})

Numerator			Denominator			Significance	
Parameter	Value	D.F.	Parameter	Value	D.F.	5%	1%
b.c., pld. d(iii)	0.95	9	b.c., c(ii), No. 42	0.49	2	-	-
b.c., c(ii), No. 3MM	0.88	2	b.c., pld. d(iv)	0.85	9	-	-

DISCUSSION

The results of test (a) (Table IV and Table VIII) show that up to 0.2 milliliters of methanol can be present in 10 milliliters of scintillation fluid without any noticeable quenching. When methanol was present in concentrations higher than 2 percent, quenching occurred with both coumestrol-H^3 and biochanin A-C^{14}. The amount of quenching was not significant when the concentration of methanol was 3 percent ($P<0.35$ for coumestrol-H^3; $P<0.15$ for biochanin A-C^{14}), but was significant when the concentration was 4 percent ($P<0.05$ for both isotopes). It was noted that the variances between classes were highest in the triplicate determinations when no methanol was present in the scintillation vial; this difference was significant with coumestrol-H^3 (at the 5% level) but not significant with biochanin A-C^{14}. Since coumestrol and biochanin A are both insoluble in the scintillation solvent toluene, then possibly these compounds became dispersed throughout the solvent to a variable degree, which may have resulted in a certain degree of quenching caused by the compounds themselves, the extent of which would depend upon the manner of this dispersal throughout the scintillation solvent. Since the difference was

significant with coumestrol-H^3 and not with biochanin A-C^{14}, it is suggested that either biochanin A was slightly more soluble in toluene (thus less particle interference), or that the low energy of emission of the β-particles of H^3 resulted in a greater degree of quenching than would be observed with the stronger β-emitter, C^{14}.

It was also observed that the standard deviations and coefficients of variability were relatively high in replicate determinations of the same sample when no methanol was present in the scintillation fluid ($P<0.10$ for both isotopes). One possible explanation for this observation is that the insoluble coumestrol or biochanin A settled to the bottom of the vial during the time required for counting each sample five times; such a progressive change in the physical makeup of each sample may have resulted in quenching if the insoluble particles were close enough to each other to cause some self-absorption. This explanation is supported by the fact that, for the most part, the observed counts per minute for each of these samples decreased with time after they were placed in the scintillation counter. In order to substantiate this view further, some of the samples were agitated slightly after completion of the counting period and recounted immediately; the observed counts per minute were again high at first,

and decreased until a more or less constant level was attained. Reproducible results can be obtained, therefore, if the coumestrol or biochanin A is first dissolved in a small volume of methanol, a procedure which would afford a constant dispersal of the solute throughout the scintillation fluid.

Test (b) (Tables XI to XIV) confirms the conclusion that quenching is not observed unless the concentration of methanol in the scintillation fluid exceeds 2 percent; 0.2 milliliters of methanol in 5 milliliters of scintillation fluid resulted in significant quenching ($P<0.025$ for coumestrol-H^3; $P<0.10$ for biochanin A-C^{14} (Table XIV)). There was no significant difference in the observed counts per minute when 10 or 15 milliliters of scintillation fluid were added to 0.2 milliliters of methanol ($P<0.35$ for coumestrol-H^3; $P<0.40$ for biochanin A-C^{14} (Table XIV)), although the coefficients of variability were less when 15 milliliters of scintillation fluid were used. However, since the coefficients of variability with the use of 10 milliliters of scintillation solvent were themselves well below those of most scintillation counting techniques, it was decided to use 10 instead of 15 milliliters of scintillation solvent in subsequent investigations in order to spare the use of the expensive scintillation grade toluene.

The results of test (c) (Table XV and Table XVII) show that Whatman No. 3MM filter paper absorbed more radioactivity than did the thinner Whatman No. 42 filter paper and that the difference was significant for both isotopes (Table XIX). A comparison of tests a(iii) and c(ii) reveals that neither filter paper was completely transparent to the emitted photons, as had been proposed by Wang and Jones (187), since the observed counts per minute were significantly lower (Table XIX) in the presence of filter paper than in the absence of filter paper. The degree of difference was greater with coumestrol-H^3 than with biochanin A-C^{14}; this was most likely due to the higher energy of the C^{14}-labelled compound, which would have resulted in less absorption by the filter paper band of any C^{14}-activity (as compared to H^3-activity) which remained on the paper after methanolic extraction. This conclusion is supported by the observation that the absence of methanol resulted in efficiencies far lower (test c(i)) than those observed when the radioactive material was partially extracted with 0.2 milliliters of methanol (test c(ii)) ($P<0.05$ and less (Table XIX)). These results are summarized in Table XXIV. This table compares the efficiencies of counting both Whatman No. 42 and Whatman No. 3MM filter paper bands impregnated with the standard solution of either

TABLE XXIV

THE EFFECTS OF METHANOL AND FILTER PAPER THICKNESS ON THE EFFICIENCIES OF COUNTING COUMESTROL-H^3 AND BIOCHANIN A-C^{14} ON FILTER PAPER BANDS*

Compound	Filter Paper Number	With Methanol Extraction		Without Methanol Extraction	
		C.P.M.	Efficiency (%)	C.P.M.	Efficiency (%)
Coumestrol-H^3	42	102,600	79.2	64,900	50.1
	3MM	99,200	76.6	56,800	43.9
Biochanin A-C^{14}	42	7,730	94.1	6,780	82.6
	3MM	7,540	91.8	6,490	79.0

*The observed counts per minute in test a(iii) (129,500 c.p.m. for coumestrol-H^3 and 8210 c.p.m. for biochanin A-C^{14}) were taken as 100% efficient.

coumestrol-H^3 or biochanin A-C^{14}. It also compares the efficiencies of counting with and without extraction with methanol. For this purpose, the counts observed in test a(iii) were taken as 100 percent efficient. For these comparisons, the vials contained the standard solution (0.1 ml), methanol (0.1 ml), and the scintillation fluid (10 ml); no filter paper bands were present. It was not necessary to consider the efficiencies of the scintillation counter for H^3 and C^{14} because these values would remain the same for all samples. Table XXIV shows that the

efficiencies were higher with biochanin A-C^{14} than with coumestrol-H^3. Prior extraction with methanol greatly increased the efficiencies of counting of both isotopes. Separate extraction of the filter paper bands with 2 milliliters of methanol, evaporation of the solvent in the counting vial, and addition of methanol (0.2 ml) and scintillation fluid (10 ml) resulted in slightly higher efficiencies (test c(iii)) as compared with extraction of the paper band in the vial (test c(ii)), but this difference was not significant (Table XIX). Furthermore, the standard deviations and coefficients of variability were higher in test c(iii). It is concluded, therefore, that the procedure outlined in test c(ii) was at once the most satisfactory and convenient. It will be observed that the variances between classes were lowest in test c(ii) and that in some instances the differences between these variances and the variances between classes of the other tests in this group were significant (Table XVI and Table XVIII).

The results of test (d) (Tables XX-XXIII) demonstrate that orientation of the small filter paper bands in the counting vial had no significant effect on the observed counts per minute. The coefficients of variability were less than 1 percent (except in one instance), and the standard deviations and variances between classes were

generally low in comparison with those of the other tests in this series of experiments. The variances between classes of test (d) and test c(ii) were compared in order to determine whether the variances which resulted from paper orientation were significantly greater than the variances of sample replicates. The results are presented in Table XXI and Table XXIII. It will be noted that, in two comparisons, the variances which occurred as a result of paper orientation were less than those of sample replicates, one of these being significant at the 5 percent level (coumestrol-H^3, Whatman No. 42). In the other two comparisons, the variances of sample replicates were slightly greater than those of paper orientation, but these differences were not significant for either comparison.

On the basis of the foregoing results, it was concluded that the most favourable method of measuring radioactivity distribution on H^3- and C^{14}-labelled paper chromatograms is to cut a pilot strip 2 centimeters wide into ¾ centimeter (or less) bands, to extract the bands with methanol (0.2 ml) in the scintillation vials, and to flood the contents of each vial with scintillation fluid (10 ml). As long as the paper bands are flat on the bottom of the scintillation vials, the effect of variable paper orientation with bands

of this size is negligible. Since the efficiencies of the scintillation counter used were 18.0 percent for H^3 and 68.3 percent for C^{14}, then, from the results of Table XXIV, the efficiencies that can be expected from this technique are as follows:

Isotope	Filter Paper	Efficiency
H^3	No. 42	79.2 X 0.18 = 14.3%
H^3	No. 3MM	76.6 X 0.18 = 13.8%
C^{14}	No. 42	94.1 X 0.683 = 64.4%
C^{14}	No. 3MM	91.8 X 0.683 = 62.7%

These efficiencies are higher, in general, than those reported in the literature in connection with studies of scintillation counting of paper chromatograms.

PART III

INVESTIGATIONS ON THE URINARY CONVERSION PRODUCTS OF GENISTEIN, COUMESTROL, BIOCHANIN A, AND FORMONONETIN IN THE FOWL

SECTION ONE

MATERIALS AND METHODS

1. Materials

(a) Equol: Samples of equol were donated by Dr. William Klyne, Westfield College, London, England, and by Dr. D. S. Layne, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, U.S.A.

(b) Genistein: Genistein was donated by Dr. Wilfred Lawson, Courtauld Institute of Biochemistry, London, England, and was tritiated by the Wilzbach gas-exposure method (216) by Picker X-Ray Engineering Limited, Montreal, Canada.

(c) Coumestrol: Samples of coumestrol were presented by Dr. E. M. Bickoff, Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, California, U.S.A., and was tritiated by means of the Wilzbach gas-exposure method (216) by Picker X-Ray Engineering Limited, Montreal, Canada.

(d) Biochanin A: A sample of reference biochanin A was presented by Dr. H. Grisebach, Chemical Laboratory, University of Freiburg, Germany. Biochanin A-4-C^{14} was synthesized by Mr. Gregory Tang, Department of Agricultural Chemistry, Macdonald College, Quebec, Canada, by a procedure based on the methods of Yoder et al (238) and Nilsson et al (239).

(e) Formononetin: Reference samples of formononetin were presented by Dr. H. Grisebach and Dr. E. M. Bickoff. Formononetin-4-C^{14} was synthesized by Mr. Gregory Tang by a procedure based on the methods of Yoder et al (238) and Nilsson et al (239).

(f) Daidzein: A sample of reference daidzein was donated by Dr. E. M. Bickoff.

(g) Organic Solvents:

N-hexane and spectranalyzed grade methanol, both Fisher certified reagents, were distilled before use.

Analar benzene and cyclohexane (laboratory reagent), both obtained from British Drug Houses, were distilled before use.

Propylene glycol (laboratory reagent), from British Drug Houses, and formamide, a Fisher Certified

laboratory reagent, were used without further treatment.

Chloroform (laboratory reagent), from Nichols Chemical Company Limited, isopropanol (A.C.S. reagent), from Anachemia Chemicals Limited, and ethyl acetate (analytical reagent), from Mallinckrodt Chemical Works, were distilled before use.

Ethanol was freed from reducing materials by the method outlined by Bauld (240). Absolute ethanol was refluxed for 12 hours with zinc dust (5%) and sodium hydroxide (5%), distilled and redistilled.

All solvents were stored in brown glass bottles.

(h) Scintillation Fluid: The scintillation fluid was the same as the one used in Part II of this thesis.

(i) Thin-Layer Gel: Merck Silica Gel G (30 gm) in water (60 ml) was shaken vigourously for 10 minutes and was applied immediately to the standard glass plates. Merck Silica Gel H was prepared in the same manner. The latter preparation contains no calcium sulphate binder and was found to retain less radioactive material after the gels were eluted with methanol.

(j) Colour Reagents: Diazotized sulphanilic acid reagent (DSA) (241) was prepared immediately before use. A

reagent prepared by dissolving para-nitrobenzenediazonium fluoroborate (1 gm) in 50 percent acetic acid (100 ml) (DFB) was also employed. The former reagent proved superior for use with paper chromatograms, whereas the latter reagent proved the more sensitive for use with thin layer chromatograms and also gave a lower background colouration.

(k) Experimental Birds: In order to permit collection of urine without fecal contamination, two operative procedures were performed. In the case of the genistein experiment, the recta of non-laying White Leghorn hens were exteriorized according to the method of Richardson et al (242). In the other experiments, the ureters of non-laying White Leghorn hens were exteriorized by the method developed by Dixon and Wilkinson (243). The urine was collected through a latex rubber tube (artificial vagina reliners), the lower end of which was tied over the back of the bird. Urine collections were made at 12 hour intervals after injection. The birds had been maintained on an alfalfa-free diet for at least 1 month prior to injection. The urine was stored in plastic bottles in the deep freeze (-20^{o}C) pending analysis.

2. Methods

(a) Paper Chromatography

(i) Chloroform-Formamide System

The chloroform (mobile phase) - formamide (stationary phase) paper chromatographic system (244) has been successfully employed in this laboratory for the identification of equol and various steroid estrogens (176), and it was decided to examine the possibility that equol and the plant estrogens could be separated from each other by the use of this system. Chromatograms were prepared in the following manner. Whatman No. 42 (3 X 22.5") filter paper was extracted for 72 hours in a Soxhlet extractor with methanol and chloroform (50:50, v:v). If more than one component was to be run on a single chromatogram, the paper was cut longitudinally into the appropriate number of strips of approximately the same width in such a manner that the paper was left intact for 3½ inches from one end. The line of application was drawn 4 inches from this end. In this way, there would be no possibility of contamination of the material on one strip by that on another during the chromatographic run. A chromatogram split in this manner will subsequently be called a "split

chromatogram". The paper was impregnated with the stationary phase by immersion in a mixture of methanol and formamide (2:1, v:v), blotting with filter paper between two sheets of plate glass, and drying in a horizontal position at 37°C for 45 minutes. In order to maintain the evaporation of formamide at a minimum, the chromatograms were run on the same day as their impregnation. Saturation of the chromatographic chamber was accomplished by placing redistilled chloroform in the chamber to a level of ½ - 1 inch from the bottom.

(ii) Benzene - Acetic Acid - Water (2:2:1, v:v:v) System

The ascending biphasic benzene - acetic acid - water (2:2:1, v:v:v) paper chromatographic system has been commonly used for the chromatography of phenolic substances (245, 246). The use of an ascending paper chromatographic technique presents difficulties in the addition of solvent after the chamber and paper have been equilibrated; if the solvent is run through the hole in the glass top, splattering and wetting of the paper may occur before the solvent reaches the trough at the bottom of the chamber. The following technique was developed to circumvent this problem. Whatman No. 3MM filter paper (3 X 22.5") was extracted for 72 hours in a Soxhlet extractor with a

mixture of methanol and benzene (50:50, v:v). The chromatographic chamber used was a rectangular glass jar, 12 inches square and 24 inches high. The line of application was 6 centimeters from the end of the paper. A piece of glass tubing was placed through a rubber stopper in one of the two holes in the glass cover of the jar and was bent in such a manner so that the other end of the tubing dipped into the lower aqueous layer of the biphasic solvent mixture in a dessicator dish at the bottom of the chamber without touching the chromatogram (Figure 4). A small glass cylinder was filled with benzene (the upper layer) and placed in the dessicator. The upper end of the glass tubing was widened to facilitate entry of solvent and was covered with a piece of aluminum foil. After the chamber was equilibrated, the aqueous solution of acetic acid (saturated with benzene) was added from a separatory funnel to the bottom layer. The glass tubing was then manipulated in such a way that the benzene layer (saturated with acetic acid and water) was added to the glass cylinder until the paper was dipped sufficiently far into the benzene layer (1 - 2 cm). This method of solvent addition minimized agitation and thus prevented temporary mixing of the two layers. It was necessary to calculate the volumes of the solvents such that the initial and final concentrations of

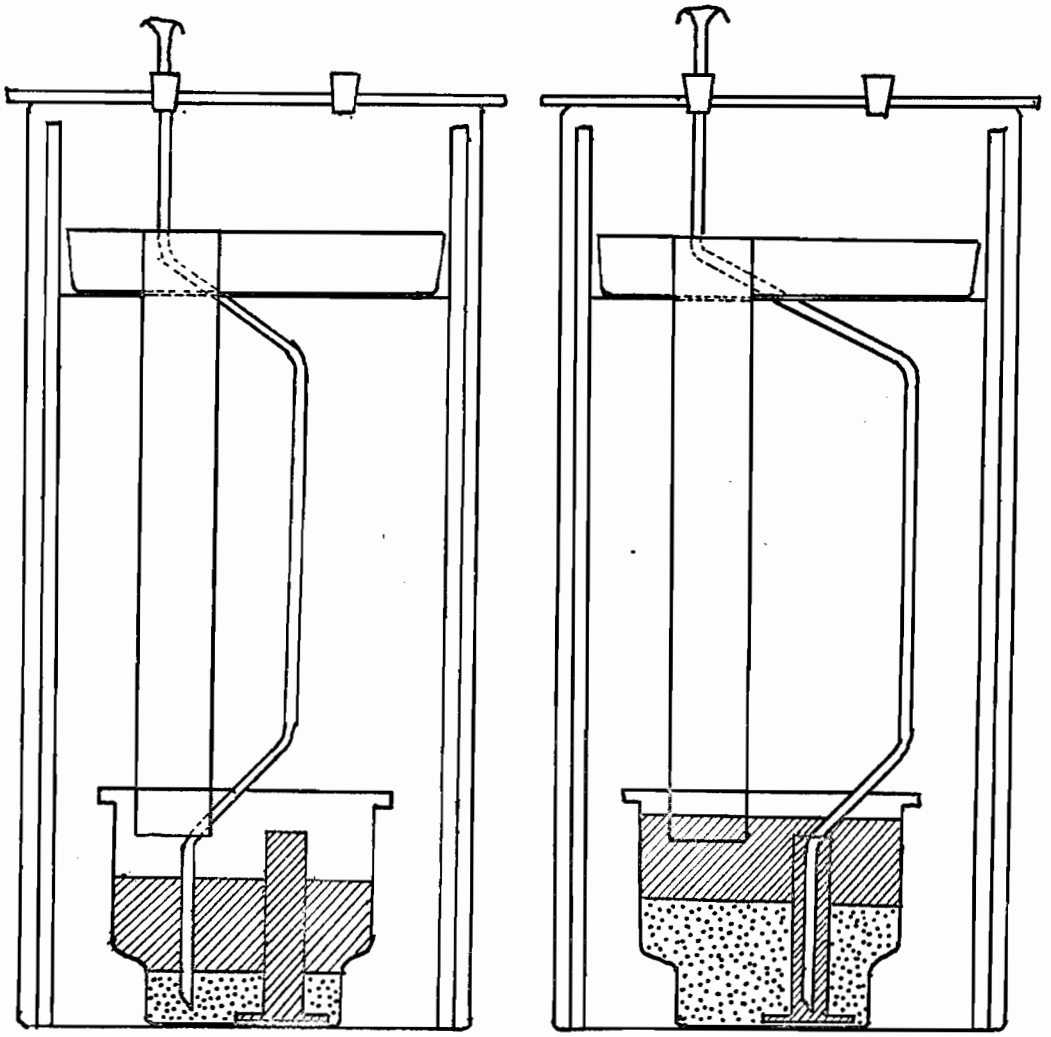


Figure 4. Diagram of chromatographic chamber modified for use with the benzene - acetic acid - water (2:2:1, v:v:v) system. The chamber with paper was equilibrated (A) and acetic acid - water (2:1, v:v) was added. The glass tubing was placed into the cylinder (B), benzene was added and the chromatogram was run in the upper phase.

= benzene

= acetic acid - water

benzene, acetic acid, and water were in the ratio of 2:2:1. In this way, equilibrium was not disturbed during the final addition of solvent.

Because of its high volatility, this solvent system was very sensitive to temperature in that the paper became wet when equilibrated for 24 hours during the summer months. When this occurred, it became difficult to observe the position of the solvent front during the chromatographic run. Consequently, equilibrium time was allowed to vary from 6 to 24 hours, according to the room temperature. Room temperature was also a factor in determining the time necessary for the solvent front to migrate over the workable length of the paper; this period varied from 12 to 24 hours. On completion of the chromatographic run, the paper was air-dried at room temperature.

(iii) Benzene - Acetic Acid - Water (125:72:3, v:v:v) System

The descending monophasic benzene - acetic acid - water (125:72:3, v:v:v) paper chromatographic system has been used for the separation of flavonoid compounds by Wong and Taylor (247). It was decided to employ this system in an attempt to identify the radioactive conversion products of

formononetin. Whatman No. 1 (3 X 22.5") filter paper was extracted for 72 hours with benzene and methanol (50:50, v:v). The line of application was 4 centimeters from the end of the chromatogram. The high volatility of the solvents led to difficulties in that the entire paper became wet with solvent if equilibration was carried to full saturation of the chamber. In order to meet this difficulty, equilibration was not carried to full saturation during the summer months. The presence of solvent in the chamber to a level of ½ - 1 inch from the bottom was sufficient for at least partial equilibration and the solvent front could be seen clearly during the entire chromatographic run. It is not feasible to secure reproducible Rf values under such conditions but this circumstance did not introduce any difficulty when split chromatograms were run with standards. The time of run varied from 3 to 6 hours. It is suggested that trial chromatograms be run when this system is to be used in order to determine the most efficient method of equilibration at a given room temperature.

(iv) Acetic Acid - Water - Hydrochloric Acid (50:35:15, v:v:v) System

The ascending acetic acid - water - hydrochloric acid (50:35:15, v:v:v) paper chromatographic system has been employed by Livingston et al (103) for the quantitative determination of coumestrol in fresh and dried alfalfa, and it was therefore decided to use this system for the chromatographic separation of coumestrol and equol that might be present in the urinary extracts. Whatman No. 3MM filter paper (3 X 22.5") was extracted for 72 hours with a mixture of methanol and benzene (50:50, v:v). A rectangular glass jar, 12 inches square and 24 inches high, was the chromatographic chamber. The line of application was 6 centimeters from the end of the paper. Attempts to equilibrate chamber and paper for any length of time, even during the winter months, resulted in dampening of the paper to such an extent that it was impossible to observe solvent migration during the chromatographic run. As a result, chromatograms run in this system were not equilibrated; in this way, the solvent front remained visible during the entire run. On completion of the run, the paper was air-dried at room temperature.

(v) Isopropanol - Water Systems

The ascending isopropanol - water (22:78, v:v) paper chromatographic system has been used by Livingston et al (102) in connection with the fluorometric estimation of coumestrol. Whatman No. 3MM filter paper (3 X 22.5") was extracted for 72 hours with methanol and benzene (50:50, v:v). A rectangular glass jar (12" square and 24" high) was the chromatographic chamber. Attempts to equilibrate the chamber and paper for any length of time resulted in dampening of the paper to such a degree that the solvent front could not be observed at any time during the chromatographic run. Furthermore, even without equilibration, the paper became so saturated with isopropanol during the run that the solvent front became obscure in about 2 hours and disappeared completely after about 3 hours. Lowering the amount of solvent in the trough at the bottom of the chamber to the minimum workable amount did not alter this paper-saturation effect. Indeed, it is difficult to understand how Livingston et al (102) achieved success with this solvent system under the conditions outlined in their paper. It was decided to examine this solvent system in an open system. The paper was suspended by means of a clothes pin in 200 milliliters of solvent in a 2-liter glass cylinder. The line of application was 6 centimeters from

the end of the paper. The solvent front remained clearly visible throughout the entire run and ran smoothly up the paper; the time of run was limited to 24 hours, during which time the solvent rose approximately two-thirds the length of the chromatogram. Admittedly, such a technique is not in accord with the classic principles of paper chromatography, since the proportions of the components of the solvent mixture would change with increased evaporation of the more volatile isopropanol. Yet if reproducibility is not a necessary parameter, this system can be used for the identification of compounds provided that standards are run simultaneously with each chromatogram, or, where applicable, by means of fluorescence. Coumestrol fluoresces with a blue-white colour under ultraviolet light (100, 101) and can thus be located on such chromatograms provided that it is the only fluorescent compound present. Under these conditions, the Rf value of coumestrol averaged 0.14. It was found that this Rf value could be altered by changing the proportions of isopropanol and water. When the ratio was 42:58 (v:v), the Rf value of coumestrol was 0.60-0.65; this ratio was found to be most suitable and convenient for the later purification of tritiated coumestrol.

(b) Thin-Layer Chromatography

Thin-layer chromatography was performed at various stages of the experimental work as a supplementary means of determining the purity of radioactive starting materials, or as a means of preliminary identification of the components of a urinary extract. A typical chromatogram was prepared in the following manner. Preliminary experiments (248) indicated that the most efficient solvent system for the separation, on thin-layer chromatoplates, of genistein and equol is the monophasic benzene - acetic acid - water (125:72:3, v:v:v) system (245, 247). Thin-layer chromatography was carried out according to Stahl (249) using Merck Silica Gel G or Merck Silica Gel H as the thin layer. The following technique was employed to locate the radioactive zones. Reference genistein, reference equol, and the purified urinary fraction in question were run and stained with 1 percent para-nitrobenzenediazonium fluoroborate in 50 percent acetic acid (DFB) on both sides of the chromatogram in order to correct for the possibility of an uneven migration of the solvent front. The urinary fraction, which was chromatographed simultaneously in a small section in the center of the plate, was left unstained. The unstained portion of the chromatogram was divided into 1 centimeter

sections in such a manner as to include the entire genistein or equol spot in one section. This was sometimes facilitated by the inclusion of ½ centimeter bands. The sections were scraped from the plates with suitable spatulas and each section was quantitatively transferred to 15 milliliter centrifuge tubes. Methanol (5 ml) was added to each tube, the contents were shaken vigourously and allowed to stand overnight at room temperature. The following morning, the tubes were centrifuged and the supernatants transferred to other 15 milliliter centrifuge tubes. Two successive 1 milliliter volumes of methanol were added to the gel and the contents were shaken vigourously. The supernatants were combined, evaporated to about 1 milliliter under a stream of nitrogen, and quantitatively transferred to standard scintillation counting vials. The solutions were evaporated to dryness, methanol (0.2 ml) was added, the contents were shaken and then flooded with scintillation fluid (10 ml). Each sample was counted five times for 3 minutes in the liquid scintillation spectrophotometer.

(c) Radioactivity Measurements

Since the radioactive materials in this study were all insoluble in the scintillation solvent toluene, these compounds were counted in a small amount of methanol

(10 - 100 µl) in the usual manner, i.e., after the addition of scintillation fluid (10 ml). When the radioactive sample was in aqueous solution, the addition of scintillation fluid would result in a biphasic mixture. To overcome this problem, the following method of sample preparation was used (250). Ethanol (6 ml) was added to an aliquot (0.2 ml) of the aqueous solution, followed by the addition of scintillation fluid (10 ml). These concentrations resulted in a monophasic solution. The sample was counted in the scintillation counter. Internal standard (0.05 ml methanolic solution) was then added in order to determine the amount of quenching. This internal standard had a high activity and was the starting material of the experiment in question, for example, coumestrol-H^3 in the coumestrol experiment or biochanin A-C^{14} in the biochanin A experiment. The sample was recounted and the quenching factor for each sample was calculated by means of the following equation:

$$F = \frac{B - A}{C}$$

where: A = observed counts per minute of the sample.
B = observed counts per minute of sample with added standard (0.05 ml).
C = true counts per minute of standard (0.05 ml).
(All values are uncorrected for counter efficiency).

The true counts per minute of the sample (uncorrected) were then determined by dividing the sample counts per minute (A) by the correction factor (F).

(d) Densitometry

The DSA-stained chromatograms were scanned with a densitometer obtained from Photovolt Corporation, New York, N.Y. (Model 52C). The slit-width was 1 millimeter.

(e) Preparation of Urinary Extracts

The method described by Bauld (251) for the extraction and purification of steroid estrogen fractions from human urine was used for the isolation of the conversion products of coumestrol and the plant isoflavones from the urine of the hen, since this technique is essentially a separation and purification of the phenolic components of urine. The urine was first treated in the following manner. Each of the 24-hour urine samples was filtered through a Büchner funnel with the aid of suction. The solid material which was separated was ground up with distilled water (50 ml) and filtered. The combined filtrates were made up to 500 milliliters with distilled water and the Bauld procedure was carried out.

(f) Melting Points

Melting points were determined with a Fisher-Johns Melting Point Apparatus (Fisher Scientific Co., Montreal, Canada). The values given are uncorrected for emergent stem.

SECTION TWO

STUDIES ON THE URINARY CONVERSION PRODUCTS OF GENISTEIN IN THE FOWL

1. Introduction

The purpose of this experiment was to determine whether or not genistein is a precursor of urinary equol in the domestic fowl. It was decided to employ genistein-H^3 rather than genistein-C^{14} in this investigation for two reasons: first, a product of higher specific activity can be obtained with the use of tritium, and, second, genistein-C^{14} would have to be synthesized whereas the tritium-labelled compound can be readily prepared by means of the Wilzbach gas-exposure technique.

2. Experimental

(a) Purification of Genistein-H^3

Genistein (50 mg) was tritiated by the method of Wilzbach (216). The product had a specific activity of 0.38 mC/mg. The genistein-H^3 was allowed to stand in methanol for 24 hours, and the methanol was evaporated in order to remove the more labile tritium. This procedure was repeated until constant specific activity was attained. Attempts to recrystallize the material were unsuccessful. The crystals obtained from the mother liquors were therefore recombined with the final crystals, dissolved in methanol, and an aliquot was chromatographed on a split chromatogram, with reference genistein on the second strip, in the benzene - acetic acid - water (2:2:1, v:v:v) paper chromatographic system. Genistein can readily be identified by means of its red fluorescence under ultraviolet light. Each component strip of the split chromatogram exhibited a single fluorescent band, and the two bands lined up perfectly with each other, thus indicating that the tritiated sample contained at least some genistein. Measurement of the radioactivity distribution on this chromatogram showed that other major radioactive zones were present, the majority of these being concentrated near the

solvent front; genistein-H^3 accounted for only 11.6 percent of the total radioactivity in the sample. It was decided, therefore, to purify the material chromatographically.

The entire sample was chromatographed on 62 paper strips of Whatman No. 3MM filter paper in the benzene - acetic acid - water (2:2:1, v:v:v) system. The genistein zones were located by means of fluorescence, and the zones were cut from the rest of the chromatograms. Genistein was eluted from the papers with methanol by forming the paper sections into cylinders, and attaching to one corner a No. 20 syringe needle shaped into a hook. The needles were fitted to 5 milliliter syringes which were filled with methanol. The entire system was enclosed in a large inverted glass trough. The methanol diffused slowly through the papers and was collected in small beakers. The four corners of each paper section were each similarly eluted. The eluates were not concentrated until the entire sample had been chromatographed and eluted; this high dilution lessened spontaneous decomposition of the tritiated material. Each band was re-examined under ultraviolet light in order to determine if all the genistein-H^3 had been removed; no fluorescence was observed on any of the bands.

When the eluates were finally concentrated, the solution was light brown in colour. Since a methanolic solution of genistein is colourless, contaminants were obviously still present. Chromatography of an aliquot of this solution in benzene - acetic acid - water showed that some radioactive impurities were still present. Genistein-H^3 comprised 63 percent of the total radioactivity in the sample. Accordingly, the entire sample was rechromatographed in this solvent system.

The second chromatographic purification required 38 chromatograms. Genistein was eluted in the manner described above and the concentrated eluate was clear and colourless. The radioactivity distribution of an aliquot of the resultant product in the benzene - acetic acid - water system showed that further purification had been effected. Genistein-H^3 accounted for 88 percent of the total radioactivity of the sample. In the chloroform - formamide system, the radioactivity distribution gave similar results; the genistein-H^3 band contained 87 percent of the total radioactivity on the chromatogram, with the majority of the radioactive impuritites being concentrated at the solvent front. It was decided to use this material immediately, keeping in mind the fact that about 13 percent of the radioactivity in the sample was not attributable to genistein-H^3.

(b) Preparation of the Urinary Extracts for Chromatography

Purified genistein-H^3 (4.21 mg containing 1.20 mC; specific activity, 0.28 mC/mg) was dissolved in propylene glycol and injected into the breast muscles of a suitably operated non-laying hen. The urine for the succeeding 8 days was collected every 12 hours and bulked into two 4-day lots. The daily urinary volumes ranged from 30 milliliters to 120 milliliters, and averaged 75 milliliters. The radioactivity in the second 4-day lot represented only about 10 percent of the total radioactivity of the crude urine; this lot was therefore discarded. The first 4-day lot was worked up into hydrophilic and lipophilic fractions by the procedure of Bauld (251). The recovery of counts in the initial ether extract of the acid-hydrolyzed urine was 14.5 percent. The purified hydrophilic and lipophilic fractions were each diluted to 10 milliliters with methanol. The hydrophilic fraction contained 3.5 percent of the originally injected counts and the lipophilic fraction contained 3.8 percent of the original counts.

(c) Chromatography of the Urinary Extracts

The hydrophilic and lipophilic fractions were both chromatographed in the chloroform - formamide and benzene - acetic acid - water (2:2:1, v:v:v) paper chromatographic

systems. A portion of each chromatogram was stained with DSA and the intensities of the positive stains were scanned on the densitometer. The radioactivity distribution was measured on another pilot strip of each chromatogram. Chromatography was also carried out on thin-layer chromatograms using the monophasic benzene - acetic acid - water (125:72:3, v:v:v) system; these chromatograms were stained with DFB and the radioactivity distributions were measured according to previously described methods.

3. Results

(a) Chromatography of the Urinary Extracts in the Chloroform - Formamide System

An aliquot of the hydrophilic fraction was chromatographed for 6 hours in the chloroform - formamide system. A portion of the chromatogram was stained with DSA (Figure 5, densitometer readings). Comparisons with reference equol and reference genistein indicated that the presumptive equol and unaltered genistein appeared as one broad zone with two densitometric peaks, the second of which had a distinct shoulder. This broad zone was the only region that gave a positive stain. The radioactivity distribution on the chromatogram (Figure 5) also showed incomplete separation of genistein from equol, although the results suggested that both these components were radioactive. Additional radioactive zones were present which did not stain with DSA.

In view of the incomplete separation of presumptive genistein from presumptive equol, an aliquot of the hydrophilic fraction was run four times each for 6 hours. Densitometer readings and the radioactivity distribution showed a clearer separation of the presumptive genistein and

equol zones than in the single run, but the zones were not completely separate from each other. The equol zone exhibited two densitometric peaks. The failure to separate genistein from equol under these conditions may have been due to partial losses of the stationary phase (formamide) in connection with the successive chromatographic runs.

Recourse was taken, therefore, to running an aliquot of the hydrophilic fraction for 24 hours in the chloroform - formamide system so that the solvent front, along with the radioactive degradation products and/or additional genistein metabolites, were allowed to run off the paper. Complete separation of genistein and equol was attained (Figure 6), both zones being radioactive. The equol zone did not exhibit a shoulder.

The purified lipophilic fraction of hens' urine normally contains some brown material. This material runs just behind the solvent front in most paper chromatographic systems and its brown colour is intensified by treatment with the DSA reagent, as is exemplified by the densitometer readings for the chromatogram of the lipophilic fraction run for 6 hours in the chloroform - formamide system (Figure 7). Genistein and equol appeared again as one zone with two densitometric peaks, with the presumptive equol zone again displaying a distinct shoulder. The two

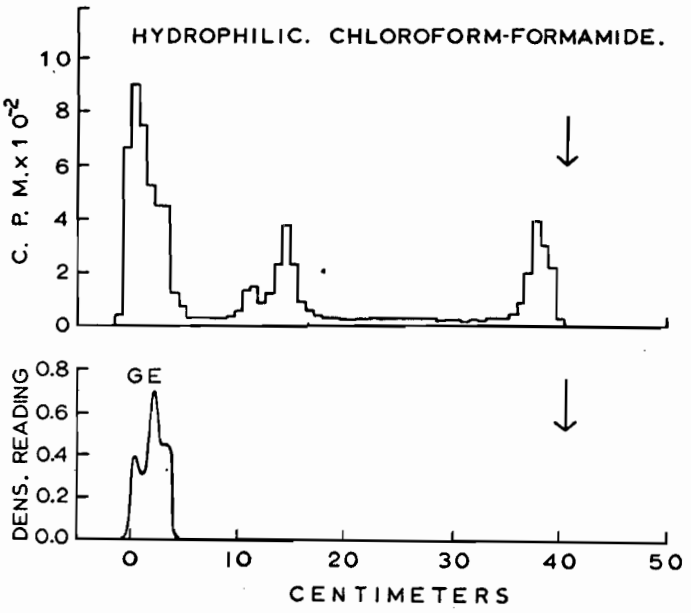


Figure 5. Distribution of radioactivity and staining (DSA) on paper chromatogram of the hydrophilic fraction of the urinary extract run in the chloroform - formamide system for 6 hours. G = genistein; E = equol; vertical arrows indicate the position of the solvent front.

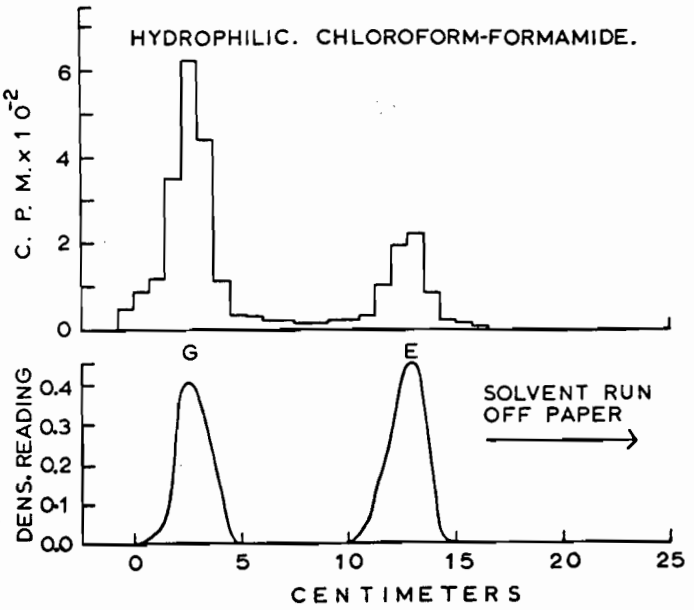


Figure 6. Distribution of radioactivity and staining (DSA) on paper chromatogram of the hydrophilic fraction of the urinary extract run in the chloroform - formamide system for 24 hours. G = genistein; E = equol.

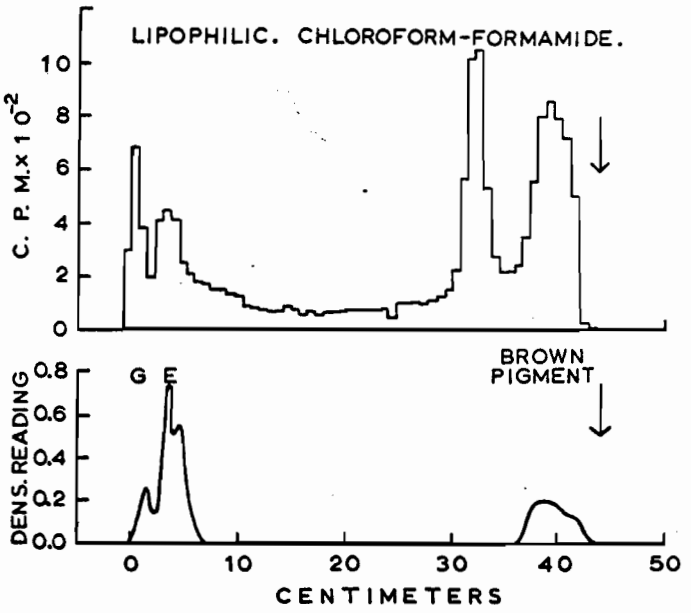


Figure 7. Distribution of radioactivity and staining (DSA) on paper chromatogram of the lipophilic fraction of the urinary extract run in the chloroform - formamide system for 6 hours. G = genistein; E = equol; vertical arrows indicate the position of the solvent front.

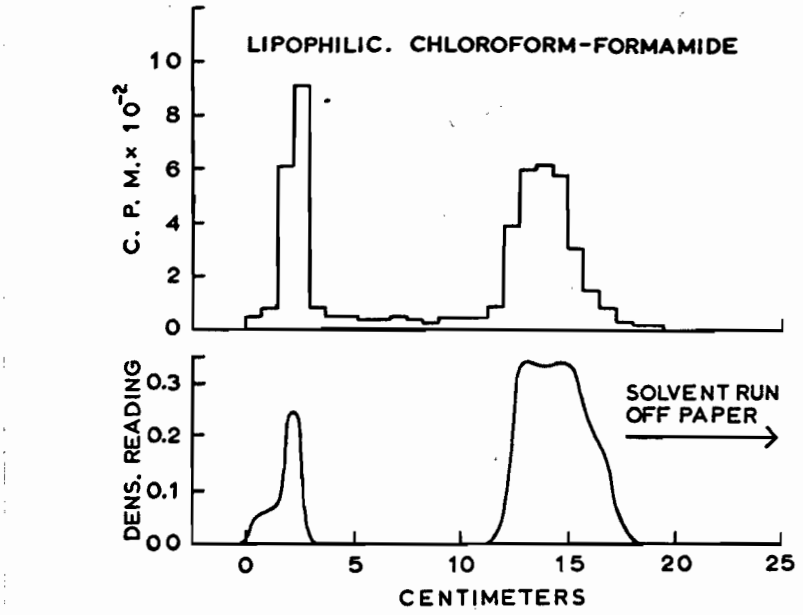


Figure 8. Distribution of radioactivity and staining (DSA) on paper chromatogram of the lipophilic fraction of the urinary extract run in the chloroform - formamide system for 24 hours. G = genistein; E = equol.

zones were radioactive. In addition, two unidentified highly radioactive zones were present which were DSA-negative; it is assumed that the brown pigment did not contribute significantly to this radioactivity, although its position corresponded with that of the second of these radioactive peaks. When chromatography was extended to 24 hours so that the solvent front was run off the paper (Figure 8), the results were similar to those given by the hydrophilic fraction in that radioactive genistein was separated from radioactive equol.

(b) Chromatography of the Urinary Extracts in the Benzene - Acetic Acid - Water (2:2:1, v:v:v) Paper Chromatographic System

Use of the benzene - acetic acid - water system made it possible to effect a clean separation of genistein from equol in both the hydrophilic and lipophilic fractions without the necessity of running the solvent off the paper (Figure 9 and Figure 10, respectively). The shoulder of the equol band noted in the chloroform - formamide system was not obtained with the benzene - acetic acid - water system. In the latter system, the hydrophilic fraction contained some DSA-positive material that ran just behind the solvent front. Thus it seems highly

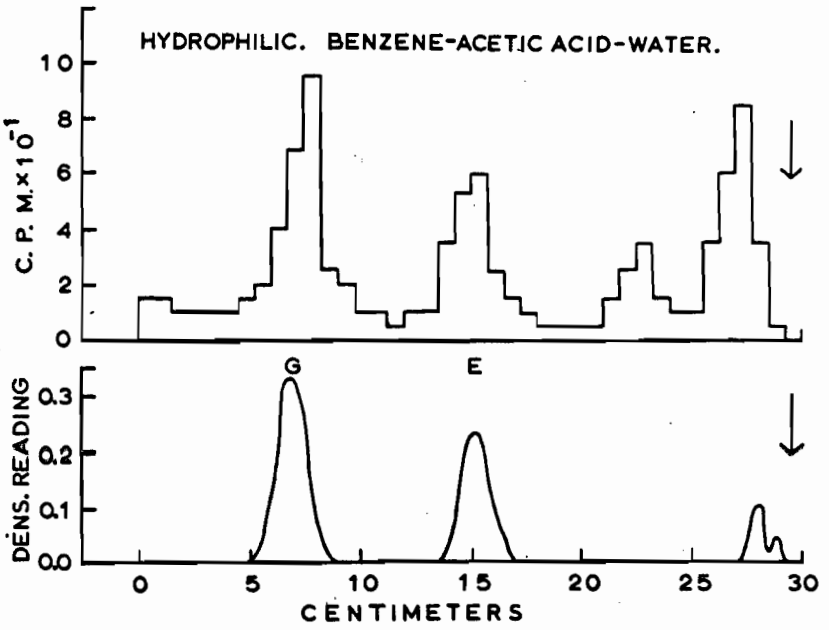


Figure 9. Distribution of radioactivity and staining (DSA) on paper chromatogram of the hydrophilic fraction of the urinary extract run in the benzene - acetic acid - water (2:2:1, v:v:v) system. G = genistein; E = equol; vertical arrows indicate the position of the solvent front.

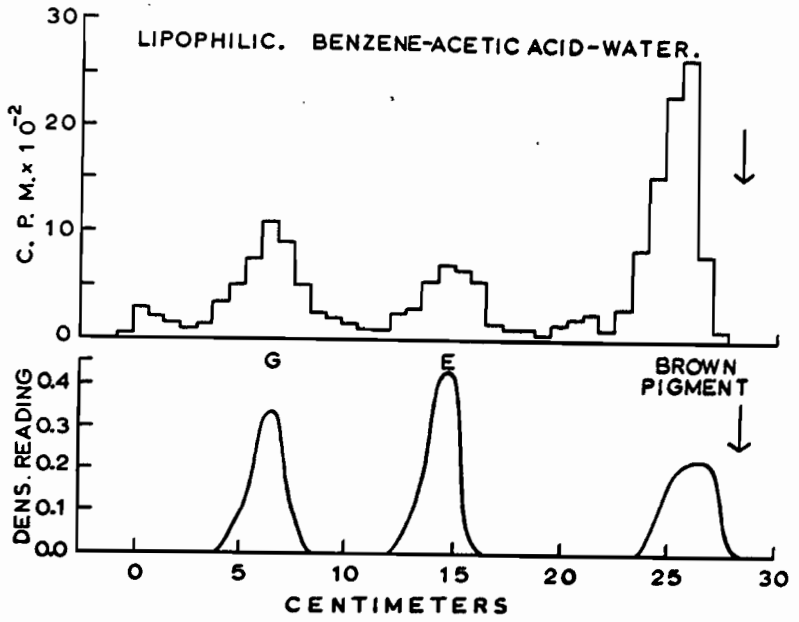


Figure 10. Distribution of radioactivity and staining (DSA) on paper chromatogram of the lipophilic fraction of the urinary extract run in the benzene - acetic acid - water (2:2:1, v:v:v) system. G = genistein; E = equol; vertical arrows indicate the position of the solvent front.

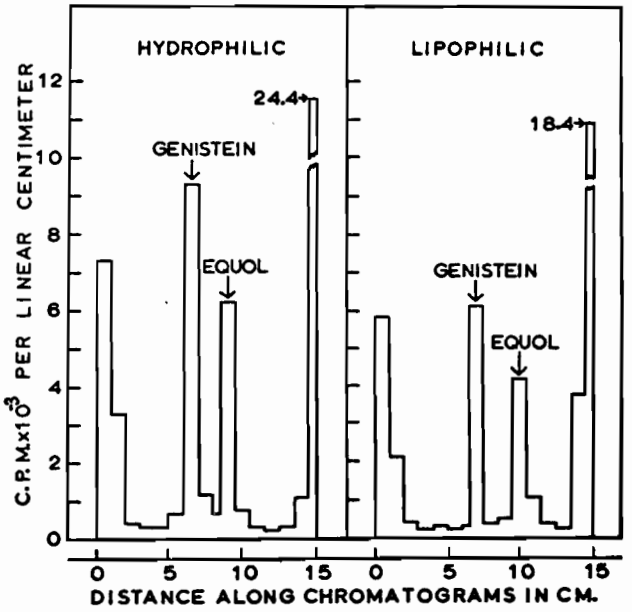


Figure 11. Distribution of radioactivity on thin-layer chromatograms of the hydrophilic (left) and lipophilic (right) fractions of the urinary extract run in the benzene - acetic acid - water (125:72:3, v:v:v) system. G = genistein; E = equol.

probable that the greater resolving power of the benzene - acetic acid - water system moved the material responsible for the shoulder to a position just behind the solvent front. In view of the high radioactivity of this zone, it is presumed that this DSA-positive material was mixed with some DSA-negative substance(s).

(c) Thin-layer Chromatography of the Urinary Extracts

When aliquots of both the hydrophilic and lipophilic fractions were run on thin-layer chromatograms in the monophasic benzene - acetic acid - water system, only the equol and genistein zones stained with DFB, except for the brown pigment of the lipophilic fraction which ran with the solvent front. Both the equol and genistein zones exhibited considerable radioactivity (Figure 11). There was also highly active material near the origin and at the solvent front.

(d) Identification of Radioactive Genistein and Radioactive Equol

After preliminary chromatographic identification, enough hydrophilic fraction was not available for further experimentation. Consequently, only the lipophilic fraction

could be used for recrystallization experiments. The remainder of the lipophilic fraction was chromatographed on a number of split chromatograms for 24 hours (solvent front run off the paper) in the chloroform - formamide system and the eluates of the zones containing genistein and equol were collected in separate pools. After addition of about 40 milligrams each of the appropriate reference carrier, constant specific activities were obtained for both these compounds after three recrystallizations from aqueous methanol (Table XXV and Table XXVI). The melting points of the purified compounds corresponded to those reported in the literature (Table XXVII).

TABLE XXV

CRYSTALLIZATION OF URINARY GENISTEIN TO CONSTANT SPECIFIC ACTIVITY

Purification Step	C.P.M.	Weight (mg)	Specific Activity (c.p.m./mg)
Original pool	416,000	41.11	10,119
1st Crystallization	184,000	19.02	9,674
Mother liquor	206,000	17.79	11,804
2nd Crystallization	82,000	9.20	8,913
Mother liquor	79,000	7.97	9,917
3rd Crystallization	41,400	4.81	8,607
Mother liquor	31,000	3.63	8,540

TABLE XXVI

CRYSTALLIZATION OF URINARY EQUOL TO CONSTANT SPECIFIC ACTIVITY

Purification Step	C.P.M.	Weight (mg)	Specific Activity (c.p.m./mg)
Original pool	379,000	48.39	7,832
1st Crystallization	211,000	22.94	9,198
Mother liquor	134,000	20.51	6,533
2nd Crystallization	79,000	8.09	9,765
Mother liquor	88,000	9.20	9,565
3rd Crystallization	34,400	3.52	9,773
Mother liquor	36,800	3.77	9,761

TABLE XXVII

MELTING POINTS OF PURIFIED URINARY GENISTEIN AND EQUOL

Compound	Melting Point (^{o}C)	
	Experimental	Reference
Genistein	294.0-296.0 (decomp.)	300.0-301.0 (49)
Equol	188.0-189.5 (decomp.)	189.0-190.5 (168)

4. Discussion

The results demonstrate that the isoflavone genistein is converted *in vivo* to equol by the domestic fowl. However, radioactive substances other than genistein-H^3 and equol-H^3 were also present in the urinary extracts. Accordingly, attention was directed to the possibility that the additional radioactive chromatographic zones found in both urinary fractions may have been due to decomposition of the tritium-labelled genistein itself during the interval (8 weeks) between the last stage of purification of genistein-H^3 and the chromatographic analysis of the urinary extracts. In order to examine this possibility, a sample of the originally injected genistein that had been stored in methanol at 5°C. was analyzed chromatographically in the benzene - acetic acid - water (2:2:1, v:v:v) system. However, this methanolic solution of genistein-H^3 was not necessarily decomposing at the same rate as the genistein-H^3 that had been injected, excreted, extracted, and purified by the techniques described in this experiment. Accordingly, an arbitrary time of 24 weeks was chosen for chromatographic analysis of the methanolic solution of genistein-H^3, which is presumed to have remained relatively stable as compared to the genistein-H^3 that had been employed in this experiment (215). The additional radioactive zones which

were observed must have represented decomposition products, although it was established that none of these decomposition products corresponded chromatographically with equol. The results were also compared quantitatively with those for the initial purified genistein-H^3 and of the urinary extracts. These values are presented in Table XXVIII. It will be observed that the percentage of radioactive decomposition products (all DSA-negative) in the 24-week genistein-H^3 sample accounted for most of the total percentage of additional radioactive zones in the hydrophilic fraction, but not of the lipophilic fraction. However, the peak of radioactivity observed at 15 centimeters for both the hydrophilic and lipophilic fractions as run in the benzene - acetic acid - water system (Figure 9 and Figure 10, respectively) was not present on the chromatograms of the decomposed genistein. It may be concluded, therefore, that this peak represented an additional metabolite of genistein rather than a decomposition product. It will be noted that the peaks of radioactivity between 20 centimeters and 30 centimeters accounted for 35.8 percent of the total radioactivity of the hydrophilic fraction (Figure 9) and for 50.9 percent of the radioactivity of the lipophilic fraction (Figure 10). Since the decomposition material of the 24-week genistein-H^3 was present entirely within this

zone (30.2 percent), it can be presumed that the corresponding peaks of both urinary fractions contained some decomposition products, possibly in association with genistein metabolites.

Attention may now be given to the percentage conversion of administered genistein to urinary equol under the conditions of this experiment. Here it must be remembered that the value observed is unlikely to be representative of the percentage values that may be obtained under normal conditions, and this for three reasons. First, the genistein-H^3 was administered by injection and at a dosage level far in excess of any likely normal dietary intake of isoflavone material by the fowl. Under such conditions of flooding the organism with genistein, relatively low percentage conversion values might be expected. Second, the formation of radioactive degradation products from tritiated genistein (and possibly from equol-H^3) during the course of the experimental work would tend also to lower the observed values. Third, the rigorous purification procedures of Bauld's technique most likely resulted in significant losses of equol during the preparation of the urinary extracts for chromatography. The estimation of the percentage conversion for the present experiment is set out in Table XXIX. It will be noted that 0.87 + 0.72 = 1.59 percent of the injected genistein radioactivity was

TABLE XXVIII

DISTRIBUTION OF RADIOACTIVITY BETWEEN THE MAJOR ZONES OF THE URINARY EXTRACTS AS OBTAINED IN THE BENZENE - ACETIC ACID - WATER SYSTEM

Material Chromatographed	Genistein (%)	Equol (%)	Other Zones (%)
Purified genistein-H^3	88	-	12
Lipophilic fraction	25.8	19.0	55.2
Hydrophilic fraction	35.2	24.9	39.9
24-Week genistein-H^3	69.8	-	30.2

TABLE XXIX

PERCENTAGE CONVERSION OF INJECTED GENISTEIN TO URINARY EQUOL

	In Purified Hydrophilic Fraction	In Purified Lipophilic Fraction
Percentage recovery of total counts injected	3.5	3.8
Percentage of recovered counts present as equol	24.9	19.0
Percentage conversion	0.87	0.72

recovered from the urine as equol radioactivity. The percentage conversion by weight may be presumed to be of the same order. A second noteworthy feature of the results presented in Table XXIX is the relatively even distribution of equol between hydrophilic and lipophilic fractions. The results of this experiment have been published (252).

SECTION THREE

STUDIES ON THE URINARY CONVERSION PRODUCTS OF COUMESTROL IN THE FOWL

1. Introduction

The purpose of this experiment was to determine whether or not coumestrol is a precursor of urinary equol in the domestic fowl. Although coumestrol is a coumarin derivative, its structure contains the same carbon skeleton as do genistein and equol. Indeed, coumestrol behaves more as an isoflavone than a coumarin as regards the course of its biosynthesis (106, 107, 253). It was decided to use coumestrol-H^3 rather than coumestrol-C^{14} in this study. Although rigorous and time-consuming procedures are required for the purification of the tritium-labelled compound, the synthesis of C^{14}-labelled coumestrol would probably be more time-consuming in view of the fact that the compound contains both a furan and a coumarin nucleus.

2. Experimental

(a) Purification of Coumestrol-H^3

Coumestrol (50 mg) was tritiated by the method of Wilzbach (216). The product had a specific activity of 0.35 mC/mg. The coumestrol-H^3 was allowed to stand in methanol (100 ml) for at least 24 hours, and the methanol was evaporated in a rotary evaporator in order to remove the more labile tritium. The use of a rotary evaporator made it possible to measure the radioactivity in the methanol removed. Although this procedure was repeated ten times, appreciable radioactivity was still present in the tenth lot of evaporated solvent. A total of 2.61 percent of the original activity was removed in the methanol, and thus as labile tritium. It seemed futile to continue this process, and thus it was decided to advance to the next stages of purification, with the hope that these subsequent purification procedures would remove all the labile tritium.

Since attempts in the previous experiment to recrystallize genistein-H^3 had been unsuccessful, it was decided to dispense with this technique for the purification of coumestrol-H^3 and to move on directly to chromatographic purification.

Both the chloroform - formamide and benzene - acetic acid - water (2:2:1, v:v:v) paper chromatographic systems were found in trial runs to be unsatisfactory for the purification of coumestrol-H^3; with the former, coumestrol migrated only very slightly from the line of application; with the latter, the coumestrol band was spread over the lower half of the chromatogram. It was decided, therefore, to purify coumestrol-H^3 by means of the chromatographic systems described by Livingston et al (102, 103): the acetic acid - water - hydrochloric acid (50:35:15, v:v:v) system and the isopropanol - water system.

The Rf value of coumestrol in the acetic acid - water - hydrochloric acid system was found to be 0.63. Before chromatographic purification, the coumestrol zone elicited a barely perceptible peak in the radioactivity distribution of impure coumestrol-H^3 in this solvent system, and comprised only 4.75 percent of the total radioactivity of the sample. Purification of the material in this system required 388 chromatograms, as compared to only 62 chromatograms necessary for the initial purification of approximately the same weight (45 mg) of impure genistein-H^3. It was necessary to limit the weight of coumestrol-H^3 to approximately 100 micrograms per chromatogram in order to prevent tailing of the coumestrol band. Chromatography

was carried out for 16 to 24 hours, depending upon the rate of migration of the solvent front. The coumestrol bands were located by fluorescence and were eluted for 24 hours in Soxhlet extractors with methanol. This method of elution was as efficient but less laborious than the one employed for the elution of genistein-H^3. The bands were examined under ultraviolet light after elution and those which fluoresced were re-eluted. The radioactivity distribution in this system of an aliquot of the final concentrated eluate showed that coumestrol comprised 48.3 percent of the total radioactivity in the sample. It was necessary, therefore, to rechromatograph the material.

For the second chromatographic purification, the isopropanol - water (42:58, v:v) system was employed. Chromatography was carried out for 24 hours, and the fluorescent coumestrol bands were eluted as described above. The number of chromatograms required was 96. Aliquots of the combined eluates were chromatographed in four solvent systems and the radioactivity distributions showed the percentage coumestrol to be as follows: (1) isopropanol - water (42:58, v:v), 90.7 percent; (2) chloroform - formamide, 88.0 percent; (3) benzene - acetic acid - water (2:2:1, v:v:v), 81.4 percent; (4) acetic acid - water - hydrochloric acid (50:35:15, v:v:v), 78.8 percent. The majority of the radioactive

impurities in all four systems was concentrated at the solvent front. In spite of the obvious limitation that the sample contained radioactive impurities to the extent of at least 20 percent, this material was injected immediately, but with the knowledge that some conversion products may possibly arise from the impurities rather than from coumestrol.

(b) Preparation of the Urinary Extracts for Chromatography

Purified coumestrol-H^3 (3.89 mg containing 0.45 mC; specific activity, 0.12 mC/mg) was dissolved in propylene glycol and injected into the breast muscles of a suitably operated non-laying hen which had been fed an alfalfa-free diet for 2 months prior to the experiment. The urine for the succeeding 5 days was collected every 12 hours, and the urine for each day was worked up separately into hydrophilic and lipophilic fractions by Bauld's procedure (251), but with one alteration. Instead of partitioning the residue of the purified ether extract between benzene and water, a mixture of n-hexane and benzene (50:50, v:v) was substituted for pure benzene. This procedure provided a means of concentrating any equol present in the hydrophilic fraction (254), rather than effecting a more or less even distribution of equol between the two fractions, as was

observed in the previous experiment. The recovery of counts in the various fractions during the purification of the urine is listed in Table XXX. It will be noted that the urinary recovery of counts totalled 44.5 percent. The ether extract of the hydrolyzed urine contained 14.3 percent of the originally injected counts. The hydrophilic and lipophilic fractions contained 0.29 percent and 0.06 percent, respectively, of the counts injected. These counts are considerably lower than those observed in the experiment with genistein-H^3, in which the recoveries in the purified extracts were 3.5 percent and 3.8 percent respectively. In view of the relatively low activities of the lipophilic fractions and of the hydrophilic fractions of days 4 and 5, subsequent chromatographic analyses were performed on the hydrophilic fractions of days 1, 2, and 3.

(c) Chromatography of the Urinary Extracts

The hydrophilic fractions were each chromatographed in four solvent systems: chloroform - formamide, benzene - acetic acid - water (2:2:1, v:v:v), isopropanol - water (22:78, v:v), and acetic acid - water - hydrochloric acid (50:35:15, v:v:v). A portion of each chromatogram was stained with DSA, and the radioactivity distribution was measured on another pilot strip.

TABLE XXX

COUMESTROL: PERCENTAGE OF TOTAL INJECTED COUNTS RECOVERED IN THE VARIOUS URINARY FRACTIONS

Day	Percent of Injected Counts in					
	Crude Urine	Ether Extract	Initial Hydr. Fraction	Initial Lip. Fraction	Final Hydr. Fraction	Final Lip. Fraction
1	20.38	5.72	0.236	0.144	0.117	0.022
2	8.42	2.54	0.187	0.032	0.076	0.013
3	7.19	3.13	0.367	0.012	0.056	0.011
4	4.75	1.84	0.169	0.019	0.028	0.007
5	3.80	1.10	0.141	0.013	0.017	0.007
Total	44.54	14.33	1.100	0.220	0.294	0.060

3. Results

(a) Chromatography of the Hydrophilic Fractions in the Chloroform - Formamide System

Each hydrophilic fraction was chromatographed for 5, 24, and 48 hours in the chloroform - formamide system. The solvent ran off the paper with the 24 hour and 48 hour chromatograms; this facilitated the separation of any coumestrol and equol, both of which ran close to the origin when chromatography was halted at 5 hours. None of the chromatograms run in this solvent system gave a positive DSA stain, but presumptive coumestrol zones were radioactive on all chromatograms. No radioactivity whatever was detected in the positions corresponding to equol on any of the chromatograms (24 hours and 48 hours) on which the equol zone was separated from the coumestrol zone. Sample results for day 2 run for 5 hours and 48 hours are presented in Figure 12 and Figure 13 respectively. Most of the radioactivity (Figure 12) was confined to a position near the origin; the rest of the radioactivity ran just behind the solvent front. In this chromatogram, coumestrol and equol ran too close to the origin to be present in the form of distinct zones. When chromatography was extended to 48 hours (Figure 13), the non-radioactive equol zone

was removed from the more polar radioactive zones. Similar results were obtained with chromatograms of the urinary extracts of day 1 and day 3. A summary of the distribution of radioactivity between relevant zones on the various chromatograms is presented in Table XXXI. In addition to the major observation of the absence of detectable radioactivity in the presumptive equol zones, it will be noted that the ratio of the amount of radioactivity at the origin to the amount at the solvent front increased from day to day, i.e., the proportion of more polar radioactive material in the extracts increased. The results also show that the coumestrol recovered in the urine represented something in the order of 12 percent of the total radioactivity of the hydrophilic fraction as analyzed by the chloroform - formamide system.

(b) Chromatography of the Hydrophilic Fractions in the Benzene - Acetic Acid - Water (2:2:1, v:v:v) System

Chromatography was carried out for 16 hours. The radioactivity distribution of a typical chromatogram is presented in Figure 14. There was no radioactivity in the equol zone. The proportion of more polar substances increased from day to day (Table XXXI) in a similar fashion as for the chloroform - formamide system. The percent of

TABLE XXXI

COUMESTROL: RADIOACTIVITY IN THE VARICUS ZONES AS PERCENT OF TOTAL ON CHROMATOGRAM

Chromatographic System	Day, Duration of Run (h)	Zone				
		Origin	Solvent Front	Coumestrol	Presumptive Equol	Other
Chloroform - formamide	1, 5	51.2	27.1	*	*	21.7
	1, 24	51.2	-	*	0	48.8**
	1, 48	38.3	-	12.9	0	48.8**
"	2, 5	86.1	13.9	*	*	0
	2, 24	86.1	-	*	0	13.9**
	2, 48	55.8	-	12.0	0	32.2**
"	3, 5	89.5	10.5	*	*	0
	3, 24	89.5	-	*	0	10.5**
	3, 48	76.9	-	12.6	0	10.5**
Benzene - acetic acid - water (2:2:1, v:v:v)	1, 16	0.8	79.0	9.4	0	10.8
	2, 16	13.9	64.6	7.8	0	13.7
	3, 16	32.4	54.8	5.4	0	7.4
Isopropanol - water (22:78, v:v)	1, 24	25.3	55.8	*	0	18.9
	2, 24	73.4	19.9	*	0	6.7
	3, 24	83.6	13.9	*	0	2.5
HAc-H_2O-HCl (50:35:15, v:v:v)	1, 16	8.4	69.0	6.6	+	16.0
	2, 16	22.7	60.2	(9.8)+§	+	17.1
	3, 16	52.3	33.4	5.5	+	8.8
	4, 16	67.0	18.2	6.2	+	8.5

* Any radioactivity in this zone is included at the origin.

** Includes some radioactivity which had been run off the paper.

\+ Any radioactivity in this zone is included at the solvent front.

§ The coumestrol zone on this chromatogram was not distinctly separable from the solvent front.

radioactivity present as coumestrol in the hydrophilic fractions was somewhat lower than with the chloroform - formamide analyses and appeared to decrease with time.

(c) Chromatography of the Hydrophilic Fractions in the Isopropanol - Water (22:78, v:v) System

In this paper chromatographic system, the coumestrol zone ran near the origin and thus it was not possible to calculate the activity due to coumestrol since this was mixed with the radioactive polar substances. However, it was possible to separate the presumptive equol zone from the radioactive components of the extract. As in the other systems, the presumptive equol zone exhibited no radioactivity (Figure 15). An increase in the proportion of more polar radioactive substances from day to day was also observed in this chromatographic system (Table XXXI).

(d) Chromatography of the Hydrophilic Fractions in the Acetic Acid - Water - Hydrochloric Acid (50:35:15, v:v:v) System

Equol ran close to the solvent front along with the less polar radioactive substances and thus it was not possible to determine the presence or absence of radioactive equol in this solvent system (Figure 16). The percent of

radioactivity present as coumestrol in the hydrophilic fraction was again low (Table XXXI). The proportion of more polar radioactive materials was observed to increase with time.

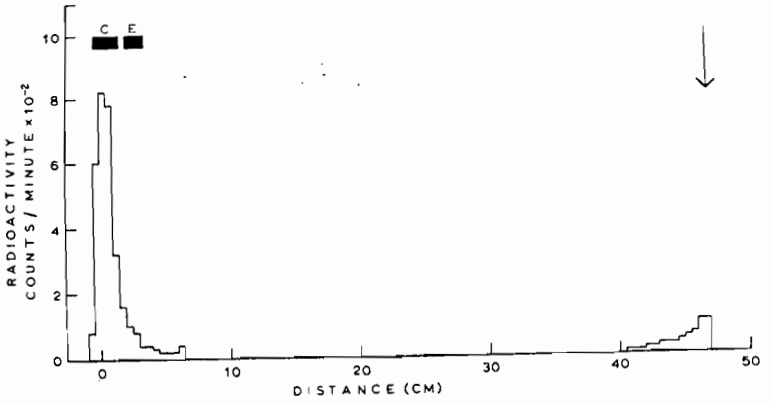


Figure 12. Distribution of radioactivity on paper chromatogram of hydrophilic fraction of urinary phenolic extract for day 2, run in the chloroform - formamide system for 5 hours. Positions of coumestrol (C) and of equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

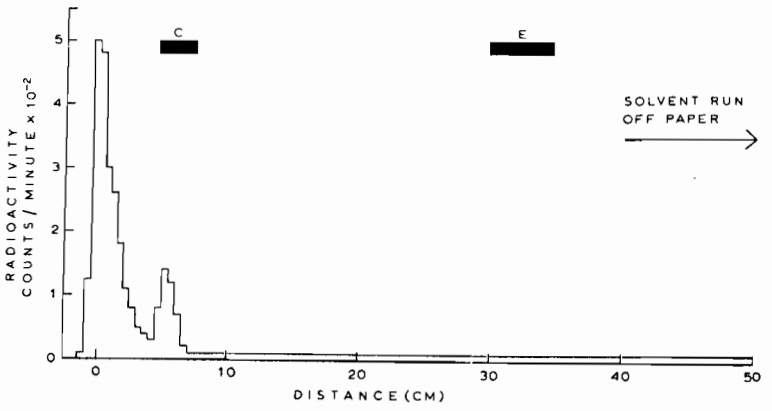


Figure 13. Distribution of radioactivity on paper chromatogram of hydrophilic fraction of urinary extract for day 2, run in the chloroform - formamide system for 48 hours. Positions of coumestrol (C) and of equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars.

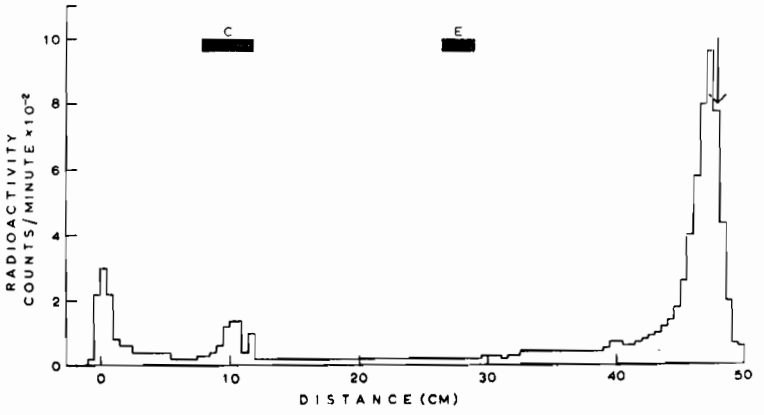


Figure 14. Distribution of radioactivity on paper chromatogram of hydrophilic fraction of urinary extract for day 2, run in the benzene - acetic acid - water (2:2:1, v:v:v) system. Positions of coumestrol (C) and of equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the observed position of the solvent front. Note radioactivity in advance of observed position of solvent front in this system; this was probably due to lateral movement of solutes during the relatively slow air-drying of this chromatogram.

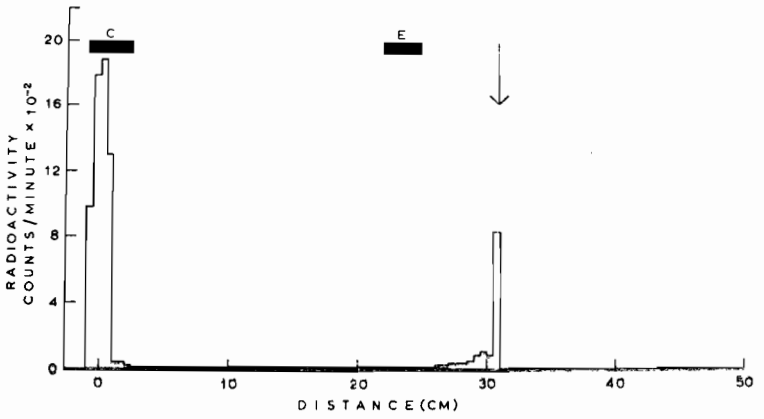


Figure 15. Distribution of radioactivity on paper chromatogram of hydrophilic fraction of urinary extract for day 2, run in the isopropanol - water (22:78, v:v) system. Positions of coumestrol (C) and of equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

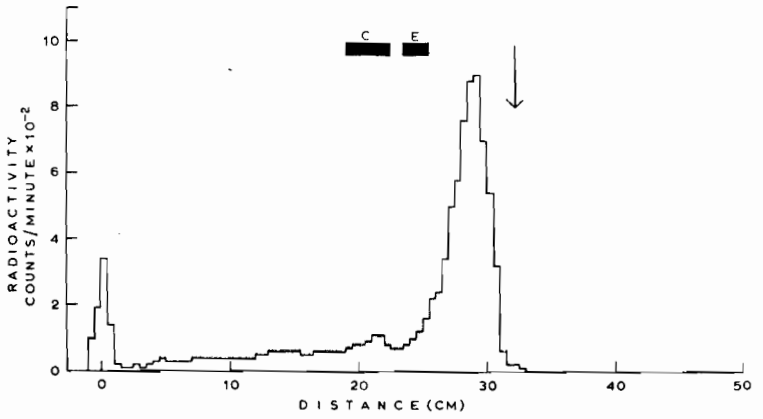


Figure 16. Distribution of radioactivity on paper chromatogram of hydrophilic fraction of urinary extract for day 2, run in the acetic acid - water - hydrochloric acid (50:35:15, v:v:v) system for 16 hours. Positions of coumestrol (C) and of equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

4. Discussion

The experiments described in Part III, Section Two, have shown that equol is a conversion product of genistein in the fowl, and that when equol is thus formed, some of it will appear in the urine. The absence of any evidence whatsoever for the presence of radioactive equol in the urine following administration of radioactive coumestrol demonstrates that equol is almost certainly not one of the *in vivo* conversion products of coumestrol. It may be surmised, therefore, that coumestrol is degraded in the fowl along pathways quite different from those along which genistein is degraded, despite the superficial resemblance of its structure to the isoflavonoid structure of genistein and despite the evidence that the biosynthetic pathways of coumestrol in the plant resemble those of the isoflavones (106, 107, 253).

The percent recovery of counts in the ether extract of the hydrolyzed urine was the same (14.33%) as in the experiment with genistein-H^3 (14.5%). However, losses of counts during the purification of the urinary fractions were greater in the coumestrol experiment; this would indicate that the amount of radioactive phenolic materials excreted in the urine was very low. The urinary recoveries

of radioactivity as coumestrol and as phenolic conversion products demonstrate that coumestrol was subject to extensive in vivo conversion in the fowl. Some of these conversion products were less polar and some were more polar than either coumestrol or equol in the chromatographic systems used; no appreciable proportion of the conversion products had polarity intermediate between the polarities of coumestrol and equol.

A progressive increase in the ratio of more polar to less polar degradation or conversion products in the urinary hydrophilic fractions was observed consistently with all four solvent systems. It is possible that the more polar materials represent later steps in the degradation of the less polar materials, which themselves represent earlier steps in the degradation of coumestrol, but much further work will be necessary before these steps can be defined. The results of this experiment are being published (255).

SECTION FOUR

STUDIES ON THE URINARY CONVERSION PRODUCTS OF BIOCHANIN A IN THE FOWL

1. Introduction

The purpose of this experiment was to determine whether or not biochanin A is a precursor of urinary equol in the domestic fowl. Because of the difficulties incurred with the purification of the tritium-labelled starting materials in the previous experiments, it was decided to use biochanin A-C^{14} rather than biochanin A-H^3 for the present study. Although the specific activity of the C^{14}-labelled compound would be lower than that of the H^3-labelled material, it was calculated from the percentage recoveries of the two previous experiments that the amount of radioactivity in the final purified urinary hydrophilic and lipophilic fractions would be sufficiently high to be able to carry out appropriate radiochemical analyses.

2. Experimental

(a) Preparation of Biochanin A-C^{14}

Biochanin A-4-C^{14} was synthesized by Mr. Gregory Tang by a procedure based on the methods of Yoder et al (238) and Nilsson et al (239). The purity of the compound was tested chromatographically. The following systems were used: chloroform - formamide, benzene - acetic acid - water (2:2:1, v:v:v), and isopropanol - water (42:58, v:v). One DSA-positive spot, which corresponded to standard biochanin A, appeared on all chromatograms. Radioactivity distributions showed that the material was chromatographically pure: on all chromatograms there was one radioactive zone which corresponded to standard biochanin A. The melting point of the synthesized compound corresponded to the one reported in the literature (239); when the material was mixed with unlabelled reference standard, the melting point remained the same.

(b) Preparation of the Urinary Extracts for Chromatography

Biochanin A-4-C^{14} (138.5 mg containing 0.00976 mC; specific activity, 0.704×10^{-4} mC/mg) was dissolved in propylene glycol and injected into the two breast muscles and the two thigh muscles of a suitably operated non-laying

hen every 24 hours over a 3 day period. Approximately equal quantities were administered each day. The urine for 12 days was collected every 12 hours and stored in 24-hour lots in the deep-freeze in plastic bottles pending analysis. The urines for the first 6 days were worked up separately into hydrophilic and lipophilic fractions by the procedure of Bauld (251) modified as outlined in Part III, Section Three. The ether extracts of the hydrolyzed urine for days 6 to 12 were discarded since the levels of radioactivity in these extracts were so low that further purification was deemed unnecessary. The daily recoveries of counts in the various fractions during the purification procedures are listed in Table XXXII. The biochanin A-C^{14} was administered as three injections over a 3-day period, and it will be noted that the percentage of counts recovered began to decrease on day 4. The recovery of counts in the crude urine was lower in this experiment (30.6%) than in the one with coumestrol-H^3 (44.5%). It will also be observed that the total count in the initial ether extract of the hydrolyzed urine was higher in the biochanin A experiment (20.6%) than in either of the experiments with genistein-H^3 (14.5%) or coumestrol-H^3 (14.3%). The final hydrophilic and lipophilic fractions contained 0.471 percent and 0.030 percent, respectively, of the counts injected; these values are of the same order as those found

TABLE XXXII

BIOCHANIN A: PERCENTAGE OF TOTAL INJECTED COUNTS RECOVERED IN THE VARIOUS URINARY FRACTIONS

	Percent of Injected Counts in					
Day	Crude Urine	Ether Extract	Initial Hydr. Fraction	Initial Lip. Fraction	Final Hydr. Fraction	Final Lip. Fraction
1	7.25	5.15	1.100	0.310	0.057	0.0098
2	6.15	4.53	0.967	0.312	0.114	0.0063
3	7.67	5.61	0.961	0.550	0.128	0.0108
4	3.85	2.65	0.629	0.200	0.078	0.0016
5	1.84	1.31	0.355	0.127	0.063	0.0009
6	1.41	0.66	0.195	0.077	0.031	0.0008
7	0.65	0.28	-	-	-	-
8	0.65	0.19	-	-	-	-
9	0.57	0.09	-	-	-	-
10	0.32	0.09	-	-	-	-
11	0.16	0.00	-	-	-	-
12	0.09	0.00	-	-	-	-
Total	30.61	20.56	4.207	1.576	0.471	0.0302

with coumestrol-H^3 but are considerably lower than those observed in the experiment with genistein-H^3. In view of the relatively low activity of the lipophilic fractions, subsequent analyses were restricted to the hydrophilic fractions. The six hydrophilic fractions were bulked into one lot.

(c) Chromatography of the Urinary Extracts

The combined hydrophilic fraction was chromatographed in the chloroform - formamide system and in the benzene - acetic acid - water (2:2:1, v:v:v) system. The acetic acid - water - hydrochloric acid (50:35:15, v:v:v) and isopropanol - water (42:58, v:v and 22:78, v:v) paper chromatographic systems were not used because it was found that the Rf values of genistein and biochanin A were almost identical in these systems, and thus it would not be possible to detect radioactive genistein in the presence of radioactive biochanin A should the former have been demethylated to the latter. A portion of each chromatogram was stained with DSA and the radioactivity distribution was measured on another pilot strip.

3. Results

(a) Chromatography of the Hydrophilic Fraction in the Chloroform - Formamide System

Aliquots of the hydrophilic fraction were chromatographed for 4, 24, and 48 hours in the chloroform - formamide system. There was not enough phenolic material on any of the chromatograms to elicit a positive stain with DSA. On the chromatogram run for 4 hours (Figure 17), biochanin A appeared as a single radioactive peak with an Rf value of 0.85. There was no radioactivity at the solvent front. A single radioactive peak at the origin was the only other radioactive zone on the chromatogram; this peak corresponded to both genistein and equol. On the chromatograms run for 24 hours and 48 hours (Figure 18 and Figure 19, respectively), both presumptive genistein and presumptive equol appeared as separate radioactive zones. Radioactive biochanin A ran off the paper with both these chromatograms. A small amount of radioactivity remained at the origin. A summary of the distribution of radioactivity between the relevant zones on the various chromatograms is presented in Table XXXIII. It will be observed that over half of the radioactivity corresponded to genistein and that presumptive biochanin A-C^{14} accounted for only 11.6 percent of the radioactivity on the chromatograms.

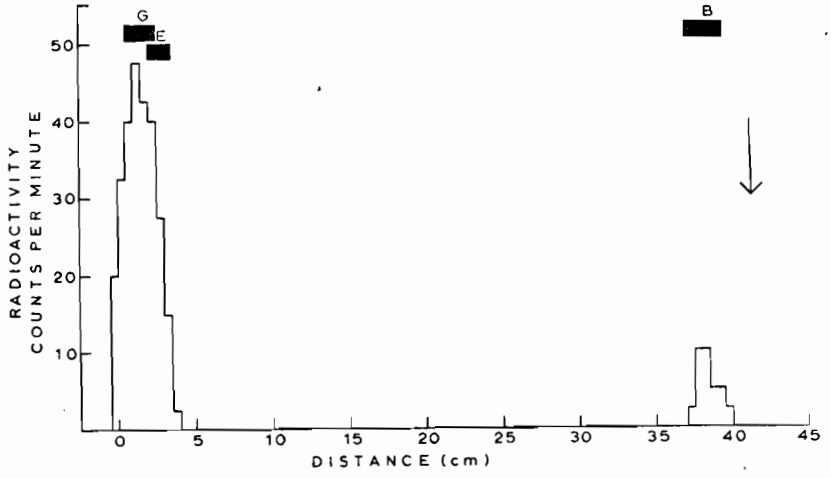


Figure 17. Distribution of radioactivity on paper chromatogram of hydrophilic fraction of urinary extract run in the chloroform - formamide system for 4 hours. Positions of genistein (G), equol (E), and biochanin A (B), as located by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

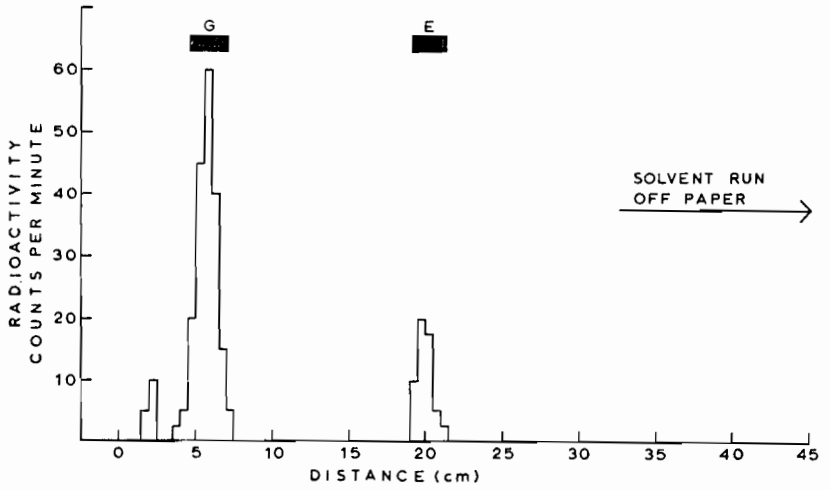


Figure 18. Distribution of radioactivity on paper chromatogram of hydrophilic fraction of urinary extract run in the chloroform - formamide system for 24 hours. Positions of genistein (G) and equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars.

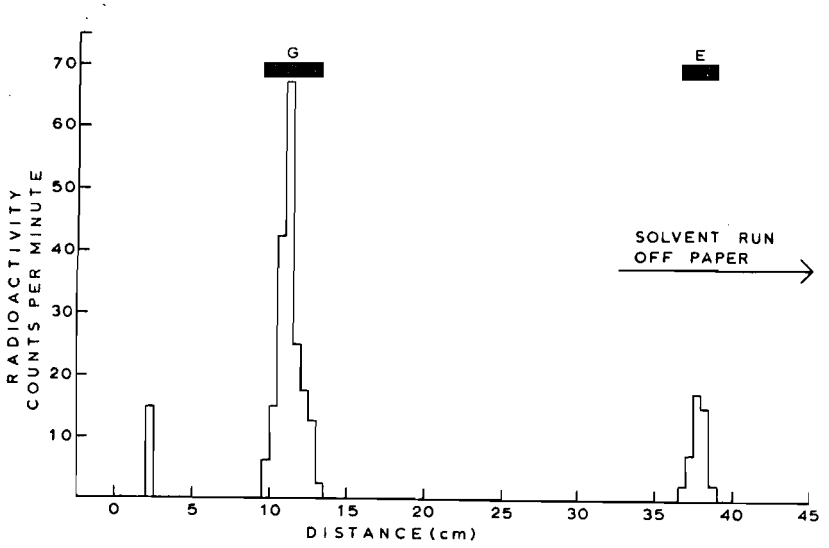


Figure 19. Distribution of radioactivity on paper chromatogram of hydrophilic fraction of urinary extract run in the chloroform - formamide system for 48 hours. Positions of genistein (G) and equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars.

TABLE XXXIII

BIOCHANIN A: RADIOACTIVITY IN THE VARIOUS ZONES AS PERCENT OF TOTAL ON CHROMATOGRAM

Chromatographic System		Zone				
		Origin	Genistein	Equol	Biochanin A	Solvent Front
Chloroform - formamide:	4h	88.4	*	*	11.6	0
	24h	5.0	58.3	25.1	11.6**	0**
	48h	6.4	66.5	15.5	11.6**	0**
Benzene - acetic acid - water (2:2:1, v:v:v)		17.2	48.6	23.8	7.6	2.8

* Any radioactivity in this zone is included at the origin.

** This zone was run off the paper.

(b) Chromatography of the Hydrophilic Fraction in the Benzene - Acetic Acid - Water (2:2:1, v:v:v) System

An aliquot of the hydrophilic fraction was chromatographed for 12 hours in the benzene - acetic acid - water (2:2:1, v:v:v) system. Presumptive genistein, equol, and biochanin A each appeared as distinct radioactive zones (Figure 20). There was also some radioactivity at the origin and at the solvent front. The distribution of radioactivity between the various zones followed a similar pattern as with the chloroform - formamide system (Table XXXIII).

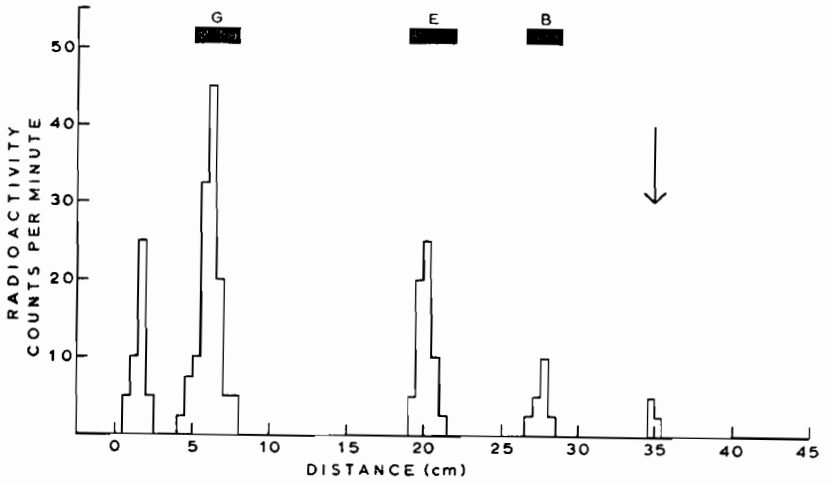


Figure 20. Distribution of radioactivity on paper chromatogram of hydrophilic fraction of urinary extract run in the biphasic benzene - acetic acid - water (2:2:1, v:v:v) system. Positions of genistein (G), equol (E), and biochanin A (B), as located by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

(c) Identification of Radioactive Genistein, Equol, and Biochanin A

For the recrystallization experiments, it was decided to use the chloroform - formamide system rather than the benzene - acetic acid - water (2:2:1, v:v:v) system for the chromatographic separation of the radioactive components of the hydrophilic fraction for the following reasons. First, it was found in previous chromatographic runs that, on occasion, equol and biochanin A ran so close to each other that the two zones overlapped to a very slight degree; this would render difficult separate elution of the two compounds. Second, although the benzene - acetic acid - water system has the greater resolving power, its use is time-consuming and laborious, especially when a substantial number of chromatograms are to be run. The following method, which was developed for the separation of genistein, equol, and biochanin A by use of the chloroform - formamide system, was found to be satisfactory. The remainder of the hydrophilic fraction was chromatographed on a number of split chromatograms in the chloroform - formamide system for a period of time 1 hour longer than the time required for the solvent front to reach the end of the paper. The chromatogram was removed from the chamber and, after 5 minutes had elapsed (time required for evaporation of

chloroform), the arm with standard biochanin A was removed and stained immediately with DSA. It was found that biochanin A had almost reached the end of the paper. The area corresponding to biochanin A in the hydrophilic fraction was cut off and the rest of the chromatogram was returned immediately to the chamber and was rechromatographed for an additional 18 hours. When the arms containing standard genistein and standard equol were later stained with DSA, it was found that a clean separation of these two compounds had been effected. It should be emphasized that these steps were carried out in rapid succession in order to reduce evaporation of the formamide of the stationary phase. The three zones were separately eluted with methanol for 24 hours in a Soxhlet extractor. After addition of about 40 milligrams of the appropriate reference carrier, constant specific activities were obtained for the three compounds after three recrystallizations from aqueous methanol (Tables XXXIV - XXXVI, incl.). The melting points of the purified compounds corresponded to those reported in the literature (Table XXXVII).

TABLE XXXIV

CRYSTALLIZATION OF URINARY GENISTEIN TO CONSTANT SPECIFIC ACTIVITY

Purification Step	C.P.M.	Weight (mg)	Specific Activity (c.p.m./mg)
Original pool	27,600	34.74	794
1st Crystallization	19,000	18.98	1002
Mother liquor	5,780	11.89	486
2nd Crystallization	9,800	10.79	908
Mother liquor	2,660	4.28	621
3rd Crystallization	4,700	4.92	955
Mother liquor	3,480	3.67	948

TABLE XXXV

CRYSTALLIZATION OF URINARY EQUOL TO CONSTANT SPECIFIC ACTIVITY

Purification Step	C.P.M.	Weight (mg)	Specific Activity (c.p.m./mg)
Original pool	12,200	39.92	306
1st Crystallization	8,100	21.81	371
Mother liquor	2,360	9.47	249
2nd Crystallization	4,100	9.40	436
Mother liquor	2,580	9.08	284
3rd Crystallization	1,780	4.00	445
Mother liquor	1,460	3.48	425

TABLE XXXVI

CRYSTALLIZATION OF URINARY BIOCHANIN A TO CONSTANT SPECIFIC ACTIVITY

Purification Step	C.P.M.	Weight (mg)	Specific Activity (c.p.m./mg)
Original pool	3,420	47.66	72
1st Crystallization	1,940	24.31	80
Mother liquor	820	15.29	54
2nd Crystallization	920	10.35	89
Mother liquor	800	10.58	76
3rd Crystallization	380	4.23	90
Mother liquor	320	3.57	90

TABLE XXXVII

MELTING POINTS OF PURIFIED URINARY COMPOUNDS

Compound	Melting Point (^{o}C)	
	Experimental	Reference
Genistein	296.5-298.0 (decomp.)	300.0-301.0 (49)
Equol	187.5-189.5 (decomp.)	189.0-190.5 (168)
Biochanin A	207.0-208.0 (decomp.)	210.0-211.0 (239)

4. Discussion

The results of this experiment demonstrate that biochanin A-4-C^{14} is converted to both genistein-C^{14} and equol-C^{14} in the domestic fowl. It may be presumed that biochanin A is first demethylated in the 4' position to genistein, which is subsequently reduced in the 2,3 position and dehydroxylated in the 5 position to give rise to equol. This demethylation has previously been shown to occur in animals other than the domestic fowl (115).

A comparison of the distributions of radioactivity on the appropriate chromatograms shows that more unidentified radioactive materials were present in the hydrophilic fraction in the experiment with genistein than in the one with biochanin A. This suggests that additional conversion products were formed from genistein which were not formed from biochanin A and/or that the tritium-labelled starting material (genistein-H^3) produced radioactive degradation products which appeared in the hydrophilic fraction

From the results of Table XXXII, it can be calculated that the total counts of the final lipophilic and hydrophilic fractions represented only 1.6 percent of the total counts

recovered in the crude urine. It seems unlikely that the losses incurred during purification of the urine were due entirely to removal of non-phenolic radioactive materials. The procedure of Bauld was designed for the isolation and purification of steroid estrogens, and it is possible that losses of isoflavonoid material were significant at various stages of the purification process, e.g., during saponification or in the aqueous solution which remained after the saponified hydrophilic fraction was extracted with ether when the pH was lowered to 9.3 to 9.5. Although losses of counts occurred at virtually every step of Bauld's procedure, no attempt was made to determine whether or not these radioactive materials were isoflavonoid compounds (or equol).

It is evident from the results reported in Table XXXIII that biochanin A-C^{14} underwent considerable degradation in the fowl; the extent of degradation was of the same order as with coumestrol-H^3, the difference being that the degradation products of coumestrol were not identified. A comparison of this Table with Table XXVIII reveals that the percent radioactivity present as equol in the final hydrophilic fractions was approximately the same with both genistein and biochanin A.

Estimations of the percentage recoveries of counts as genistein, equol, and biochanin A in the hydrophilic fraction are presented in Table XXXVIII. The calculations are based on the following considerations. First, the percentage recovery of counts in the hydrophilic fraction of the total counts injected was 0.471 (Table XXXII). Second, the best available estimates of the percent of recovered counts in the hydrophilic fraction present as genistein, equol, and biochanin A are the average values of the percent radioactivity in the various chromatographic zones as outlined in Table XXXIII. The product of the two values would give the percentages of the total counts injected which were recovered as genistein, equol, or biochanin A. It will be noted that the percentage recovery of equol in the hydrophilic fraction was lower in the biochanin A experiment (0.101%) than in the experiment with genistein (0.87%, Table XXIX). This was a consequence of the fact that the percentage of the originally injected counts recovered in the hydrophilic fraction from the biochanin A experiment was lower (0.471%) than in that from the genistein experiment (3.5%).

TABLE XXXVIII

PERCENTAGE OF TOTAL COUNTS INJECTED RECOVERED IN HYDROPHILIC FRACTION AS GENISTEIN, EQUOL, AND BIOCHANIN A*

	Genistein	Equol	Biochanin A
Average percentage of counts present in hydrophilic fraction	57.8	21.5	10.3
Net percentage recovery	0.272	0.101	0.049

* Calculations based on percentage recovery in hydrophilic fraction of total counts injected as 0.471.

SECTION FIVE

STUDIES ON THE URINARY CONVERSION PRODUCTS OF FORMONONETIN IN THE FOWL

1. Introduction

The purpose of this experiment was to determine whether or not formononetin is demethylated (to daidzein) and subsequently reduced to equol in the domestic fowl in a manner analogous to that exhibited by biochanin A. C^{14}-Labelled formononetin was used in this study. It was decided to analyze chromatographically the final purified ether extract of the hydrolyzed urine in order to determine whether the losses of radioactivity which result from the subsequent partitioning and purification procedures are due to the removal of radioactive isoflavonoid material or to the removal of radioactive impurity.

2. Experimental

(a) Preparation of Formononetin-C^{14}

Formononetin-4-C^{14} was synthesized by Mr. Gregory Tang by a procedure based on the methods of Yoder *et al* (238) and of Nilsson et al (239). The melting point of the compound corresponded to the one reported in the literature (238); no depression of melting point occurred when the material was mixed with unlabelled reference standard. The results of the radioactivity distributions of the synthesized compound in the chloroform - formamide system and in the monophasic benzene - acetic acid - water (125:72:3, v:v:v) system indicated that the material was radiochemically pure; in the isopropanol - water (42:58, v:v) system, a small amount of radiochemical impurity appeared at the solvent front, but this zone comprised less than 2 percent of the total radioactivity on the chromatogram.

(b) Preparation of the Urinary Extracts for Chromatography

Formononetin-4-C^{14} (74.04 mg containing 0.0205 mC; specific activity, 2.77×10^{-4} mC/mg) was dissolved in propylene glycol. It was found that the compound was less

soluble in the solvent than coumestrol and the other isoflavones employed in these studies; a total of 33 milliliters of propylene glycol was required to dissolve completely the formononetin, as compared, for example, to the 10 milliliters required to dissolve 138.5 milligrams of biochanin A-4-C^{14}. It was intended to spread the injections over a 4-day period. A dose of approximately 8 milliliters of solution was injected each day for 3 days into the two breast muscles and the two thigh muscles of a suitably operated non-laying hen. However, on the morning after the third series of injections, the bird could not stand in an upright position and she tended to rest on her side. This appeared to be due to cramp of the leg muscles consequent on the flooding with the propylene glycol. The bird would neither eat nor drink of her own accord and she had to be massaged and nursed for the following 2 days; she ate and drank only if held at the feed bins. It was estimated that approximately half the urine was lost on both these days as a result of the fact that the urine was not passing directly into the collection tube but tended to trickle out to one side. By the morning of day 5, the bird had recovered, was able to stand upright, and ate and drank normally. The proposed fourth series of injections was not administered. The weight of formononetin-4-C^{14} injected was 61.06 milligrams (0.0169 mC).

The urine for 12 days after the first injection was collected every 12 hours. Each day's urine was hydrolyzed and the ether extracts of the hydrolyzed urine were purified according to the procedure of Bauld (251). At this stage of the procedure, the purified ether extracts were each divided into approximately equal portions. The exact number of counts in each portion was measured. One portion of each day's extract was taken and the twelve portions thus obtained were bulked into one lot; the other parts were bulked into six lots (2 consecutive days per lot) and the procedure of Bauld was carried out to the final purification of the hydrophilic and lipophilic fractions.

The daily recoveries of counts in the various urinary fractions are presented in Table XXXIX. The values presented for the percentage recoveries after the extracts were partitioned between water (hydrophilic fraction) and benzene-hexane (lipophilic fraction) have been corrected for the portions which were removed, and thus represent percentage recoveries of the theoretically complete extracts. It will be noted that the recovery of counts in the crude urine dropped sharply on day 3; this circumstance was undoubtedly due to the fact that part of the urine was lost on day 3 and on day 4. In spite of this occurrence, the total recovery of counts in the crude

TABLE XXXIX

FORMONONETIN: PERCENTAGE OF TOTAL INJECTED COUNTS RECOVERED IN THE VARIOUS URINARY FRACTIONS

Day	Percent of Injected Counts in					
	Crude Urine	Initial Ether Extract	Initial Hydr. Fraction	Initial Lip. Fraction	Final Hydr. Fraction	Final Lip. Fraction
1	14.98	12.84	5.974	1.253	1.793	0.208
2	15.94	13.54				
3	3.73	3.24	1.596	0.376	0.480	0.056
4	3.71	3.03				
5	2.04	1.64	1.113	0.254	0.275	0.018
6	2.56	2.15				
7	2.08	1.59	0.684	0.247	0.111	0.014
8	1.12	0.88				
9	1.12	0.81	0.331	0.076	0.059	0.007
10	0.87	0.53				
11	0.50	0.17	0.076	0.040	0.016	0.000
12	0.31	0.08				
Total	48.96	40.50	9.774	2.246	2.734	0.303

urine (48.9%) was higher than in any of the previous experiments. Another noteworthy feature of the results in this table is the fact that the total recovery of counts in the initial ether extract of the hydrolyzed urine was much higher (40.5%) than in any of the other experiments and did not decrease to the same degree from the total percentage recovery of counts in the crude urine. The hydrophilic fractions were combined into one lot.

(c) Chromatography of the Urinary Extracts

The combined final ether extracts of the hydrolyzed urines and the combined final hydrophilic fractions were analyzed chromatographically in the chloroform - formamide system, the biphasic (upward) benzene - acetic acid - water (2:2:1, v:v:v) system, and in the monophasic (downward) benzene - acetic acid - water (125:72:3, v:v:v) system. The isopropanol - water paper chromatographic systems were found to be unsatisfactory for this experiment; when the ratio of the solvents was 42:58 (v:v), the Rf values of formononetin (0.72) and daidzein (0.63) were too close for this system to be useful; when the ratio was 22:78 (v:v), both formononetin and daidzein were spread out from the origin. A portion of each chromatogram was stained with DSA and the radioactivity distribution was measured on

another pilot strip. Thin-layer chromatography of the extracts was carried out in the benzene - acetic acid - water (125:72:3, v:v:v) system and in the cyclohexane - ethyl acetate (50:50, v:v) system.

3. Results

(a) Chromatography of the Ether Extract of the Hydrolyzed Urine

Aliquots of the combined ether extracts of the hydrolyzed urines were chromatographed in the chloroform - formamide system (4, 12, 24 and 60 h), the biphasic (upward) benzene - acetic acid - water (2:2:1, v:v:v) system, and in the monophasic (downward) benzene - acetic acid - water (125:72:3, v:v:v) system. The location of formononetin on these and on subsequent chromatograms was found by means of its intense white fluorescence under ultraviolet light; the compound does not stain with DSA. There was not enough equol on any of the chromatograms to elicit a positive stain when the compound was separated from the isoflavonoid components of the extract.

Radioactive formononetin ran just behind the solvent front (Rf = 0.87) in the chloroform - formamide system (Figure 21); when chromatography was extended over a period longer than 4 hours, formononetin ran off the paper. On the chromatograms run for 4 hours (Figure 21) and 12 hours (Figure 22), the equol zones were radioactive and were completely separate from the other radioactive

components of the urinary extract. It is interesting to note that in the previous experiments, the equol zone overlapped with genistein on chromatograms run for 6 hours or less; this difference in resolving power may be associated with seasonal differences in ambient temperatures.

The zones corresponding to daidzein were radioactive on all four chromatograms run in the chloroform - formamide system (4, 12, 24 and 60 h). Aside from presumptive radioactive daidzein, equol, and formononetin, an additional major radioactive zone was present on all chromatograms. The polarity of this zone was similar to that of genistein. When standard genistein was present on an additional pilot strip, it was found that this radioactive zone corresponded to genistein on the chromatogram run for 4 hours (Figure 21); this zone, which overlapped with the daidzein zone, gave a positive stain with DSA. (There was also some DSA-positive material at the solvent front, but this area was not radioactive). On the chromatogram run for 12 hours (Figure 22), this additional radioactive zone was slightly less polar than genistein, but standard genistein did overlap to some extent with this zone. The combined daidzein - genistein area was DSA-positive. On the chloroform - formamide chromatograms run for 24 hours and 60 hours (Figure 23), this radioactive zone was less polar than

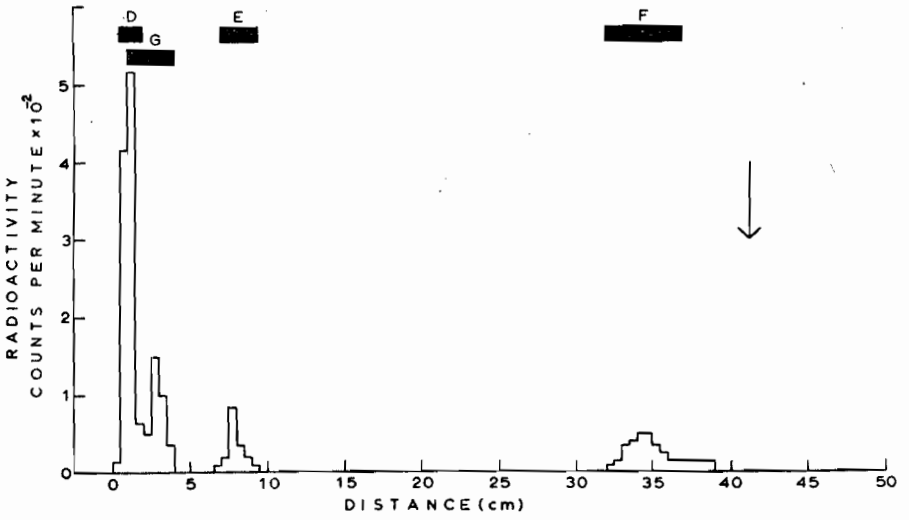


Figure 21. Distribution of radioactivity on paper chromatogram of the purified ether extract of the hydrolyzed urine run in the chloroform - formamide system for 4 hours. Positions of daidzein (D), genistein (G), equol (E), and formononetin (F), as located by fluorescence and by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

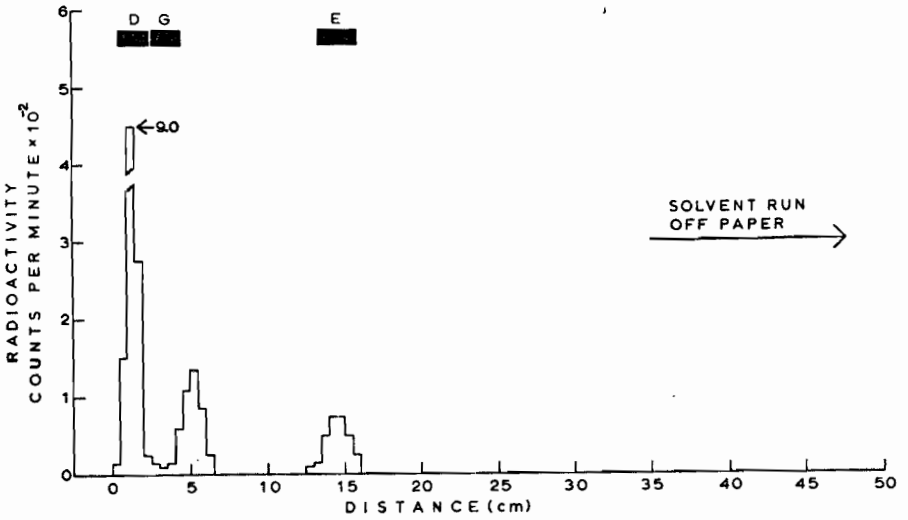


Figure 22. Distribution of radioactivity on paper chromatogram of the purified ether extract of the hydrolyzed urine run in the chloroform - formamide system for 12 hours. Positions of daidzein (D), genistein (G), and equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars.

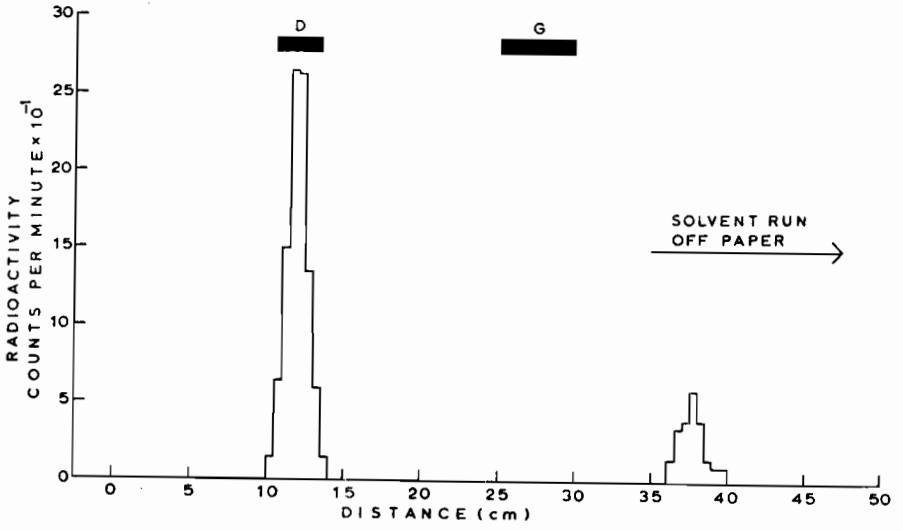


Figure 23. Distribution of radioactivity on paper chromatogram of the purified ether extract of the hydrolyzed urine run in the chloroform - formamide system for 60 hours. Positions of daidzein (D) and genistein (G), as located by staining of standard pilot strips with DSA, are indicated by black bars.

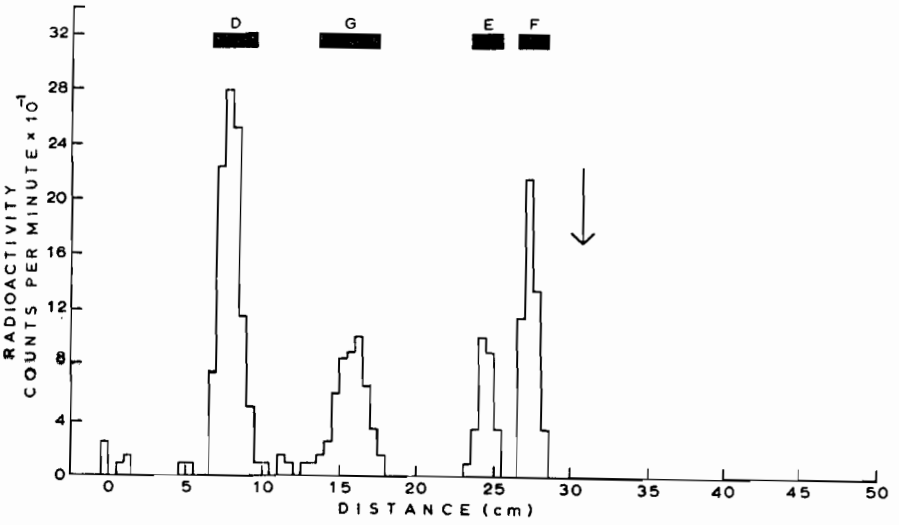


Figure 24. Distribution of radioactivity on paper chromatogram of the purified ether extract of the hydrolyzed urine run in the biphasic benzene - acetic acid - water (2:2:1, v:v:v) system. Positions of daidzein (D), genistein (G), equol (E), and formononetin (F), as located by fluorescence and by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

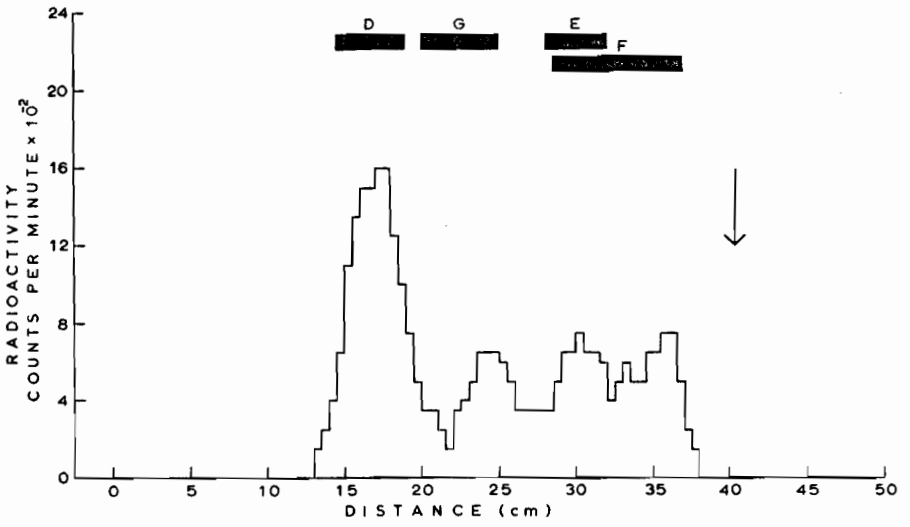


Figure 25. Distribution of radioactivity on paper chromatogram of the purified ether extract of the hydrolyzed urine run in the monophasic benzene - acetic acid - water (125:72:3, v:v:v) system. Positions of daidzein (D), genistein (G), equol (E), and formononetin (F), as located by fluorescence and by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

reference genistein and the two zones did not overlap. The zone corresponding to daidzein gave a very faint positive stain with DSA; this was the only DSA-positive area on the two chromatograms.

When the purified ether extract of the hydrolyzed urine was chromatographed in the biphasic benzene - acetic acid - water (2:2:1, v:v:v) system (Figure 24), daidzein, equol, and formononetin appeared as distinct separate radioactive zones. The additional major radioactive zone which was present on chromatograms run in the chloroform - formamide system was found to correspond almost exactly with standard genistein in this system. A small amount of radioactivity was present near the origin. There was some DSA-positive material at the origin and the solvent front and at positions corresponding to those of daidzein and formononetin. The material responsible for the DSA stain corresponding to formononetin must have been some other phenolic compound because formononetin is DSA-negative.

Little information was gained from the results of chromatography of the ether extract of the hydrolyzed urine in the monophasic benzene - acetic acid - water (125:72:3, v:v:v) system (Figure 25). The radioactivity distribution showed that there was a single broad large radioactive region comprising five peaks. One of these peaks

corresponded in position with daidzein, another with genistein, and a third with equol; the three less polar peaks fell within the region occupied by formononetin. Some DSA-positive material appeared opposite daidzein, genistein, and at the solvent front.

A summary of the distribution of radioactivity between the relevant zones on the various chromatograms is presented in Table XL. The results of the monophasic benzene - acetic acid - water system (Figure 25) were not included in this table since it was difficult to delineate clearly the various chromatographic zones.

(b) Chromatography of the Hydrophilic Fraction

Aliquots of the combined final hydrophilic fractions were chromatographed in the chloroform - formamide system (4, 12, 24, and 48 h), and in both the monophasic and biphasic benzene - acetic acid - water systems. On only two chromatograms was there sufficient concentration of phenolic material to elicit positive DSA stains: one DSA-positive band corresponded to the daidzein-genistein zone at the origin of the chromatogram run in the chloroform - formamide system for 4 hours; the other band, which was barely visible, corresponded to daidzein and genistein

TABLE XL

FORMONONETIN: RADIOACTIVITY IN THE VARIOUS ZONES AS PERCENT OF TOTAL ON CHROMATOGRAM (PURIFIED ETHER EXTRACT OF HYDROLYZED URINE)

Chromatographic System	Zone				
	Daidzein	Additional Zone	Equol	Formononetin	Other
Chloroform - formamide: 4h	53.8*	48.8*	13.3	18.7	0
12h	40.6	29.2	11.5	18.7**	0
24h	51.5	17.4	12.4***	18.7**	0
60h	52.7	16.2	12.4***	18.7**	0
Biphasic benzene - acetic acid - water	42.5	21.3	11.3	20.9	4.0

*These two zones overlapped.

**This zone was run off the paper.

***This zone was run off the paper. The percentage value given is the average percentage equol as found in chloroform - formamide chromatograms run for 4 hours and 12 hours.

on the monophasic benzene - acetic acid - water chromatogram. No DSA-positive material was present on any of the other chromatograms.

In the chloroform - formamide system (Figures 26-29,

incl.), it will be noted that, in general, the Rf values of the reference standards run with the hydrophilic fraction were less than when they were run with the ether extract of the hydrolyzed urine. It is possible that temperature changes which occurred from the time the ether extract chromatograms were run to the time when the hydrophilic fraction was chromatographed may have been sufficient enough to alter significantly the Rf values. This difference in Rf values is exemplified by the migration of equol; equol ran off the paper when the ether extract was chromatographed for a period 24 hours or longer, but stayed on the papers when the hydrophilic fraction was chromatographed for periods up to 48 hours.

Presumptive equol was radioactive on all chromatograms run in the chloroform - formamide system. Formononetin was radioactive on the chromatogram run for 4 hours (Figure 26) but ran off the other chromatograms. At least some radioactivity appeared opposite daidzein on all chromatograms. The additional radioactive peak which was present in the ether extract was the most radioactive zone in the hydrophilic fraction; the zone corresponded to genistein on all chromatograms except in the one run for 48 hours (Figure 29). A small radioactive zone remained at the origin (Figure 28 and Figure 29).

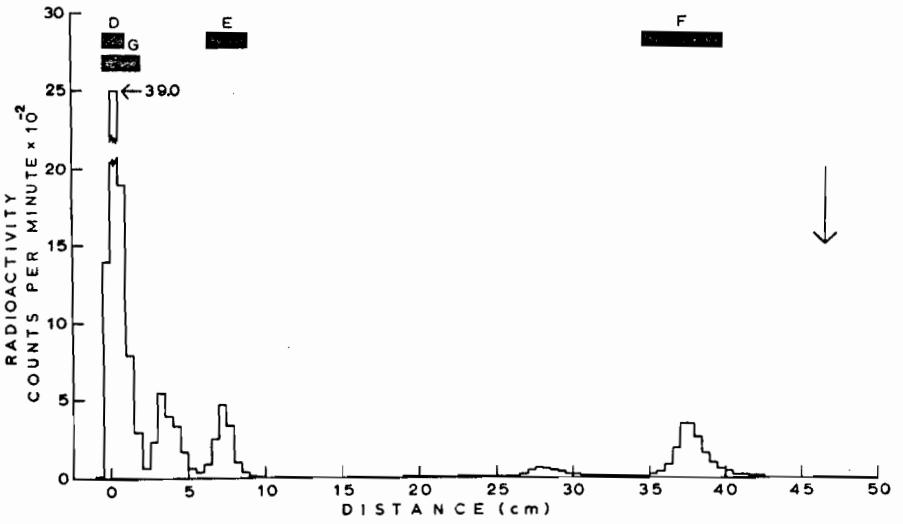


Figure 26. Distribution of radioactivity on paper chromatogram of the hydrophilic fraction of the urinary extract run in the chloroform - formamide system for 4 hours. Positions of daidzein (D), genistein (G), equol (E), and formononetin (F), as located by fluorescence and by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

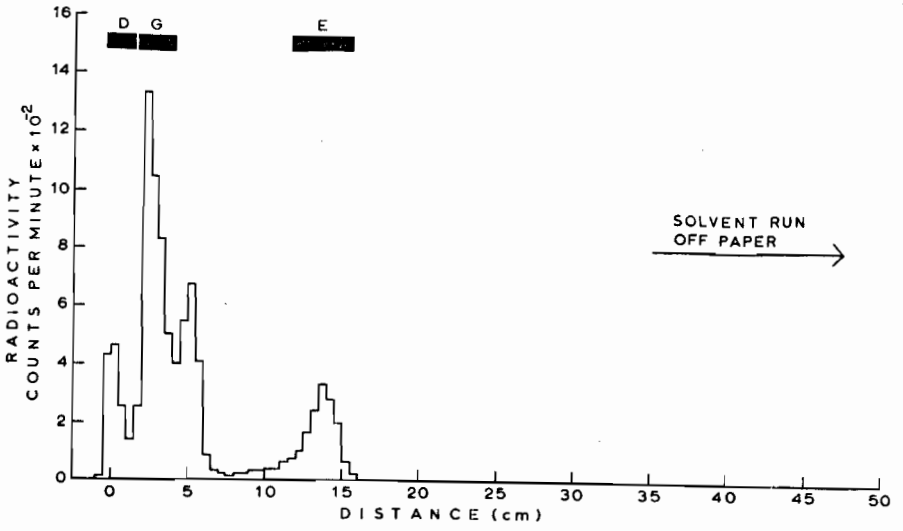


Figure 27. Distribution of radioactivity on paper chromatogram of the hydrophilic fraction of the urinary extract run in the chloroform - formamide system for 12 hours. Positions of daidzein (D), genistein (G), and equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars.

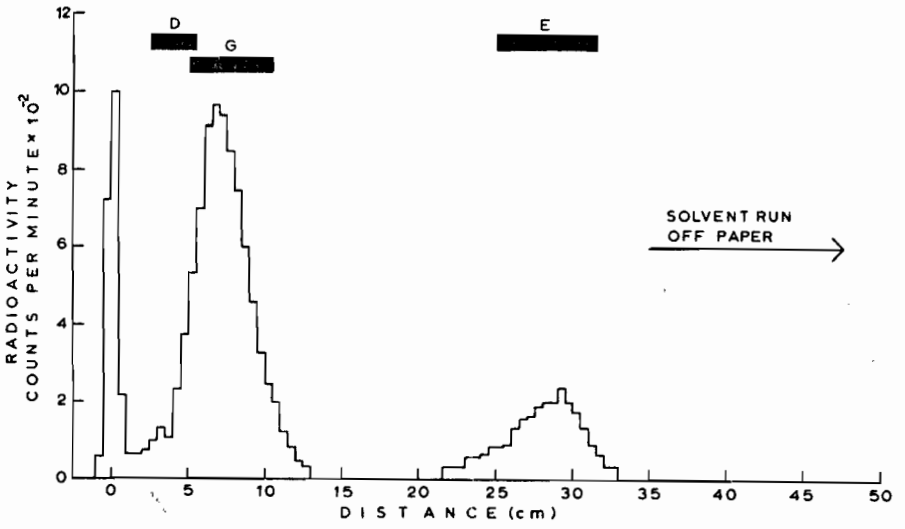


Figure 28. Distribution of radioactivity on paper chromatogram of the hydrophilic fraction of the urinary extract run in the chloroform - formamide system for 24 hours. Positions of daidzein (D), genistein (G), and equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars.

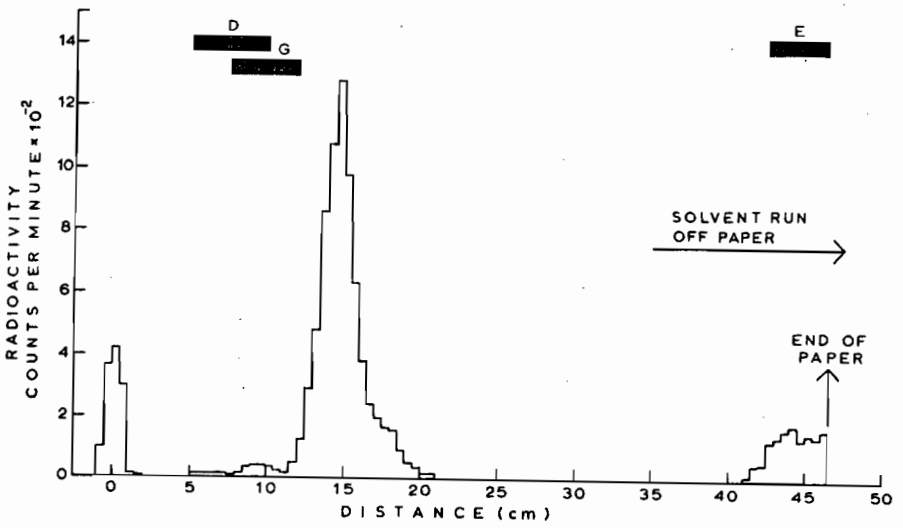


Figure 29. Distribution of radioactivity on paper chromatogram of the hydrophilic fraction of the urinary extract run in the chloroform - formamide system for 48 hours. Positions of daidzein (D), genistein (G), and equol (E), as located by staining of standard pilot strips with DSA, are indicated by black bars.

In the biphasic benzene - acetic acid - water system, daidzein, equol, and formononetin were separated as distinct radioactive zones (Figure 30). Some radioactivity was present opposite reference genistein, but the major additional unidentified conversion product was slightly less polar than genistein.

The radioactivity distribution of the hydrophilic fraction in the monophasic benzene - acetic acid - water system (Figure 31) gave results very similar to those given by the ether extract of the hydrolyzed urine. Radioactivity corresponded to daidzein, genistein, equol, and formononetin, but each of these zones overlapped with one or more of the other three. The most highly radioactive zone appeared directly opposite to reference genistein.

The distribution of radioactivity between the various chromatographic zones is summarized in Table XLI. Again, the results of the monophasic benzene - acetic acid - water system are not included because it was difficult to delineate the zones.

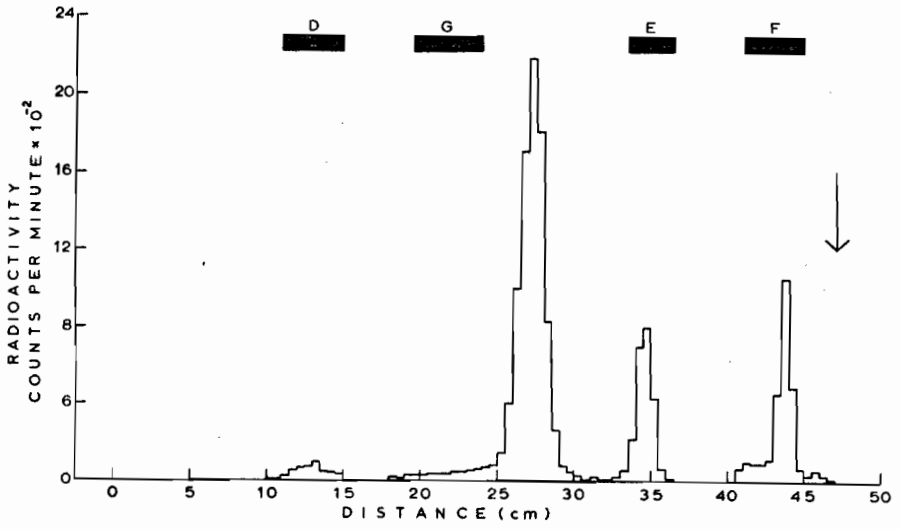


Figure 30. Distribution of radioactivity on paper chromatogram of the hydrophilic fraction of the urinary extract run in the biphasic benzene - acetic acid - water (2:2:1, v:v:v) system. Positions of daidzein (D), genistein (G), equol (E), and formononetin, as located by fluorescence and by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

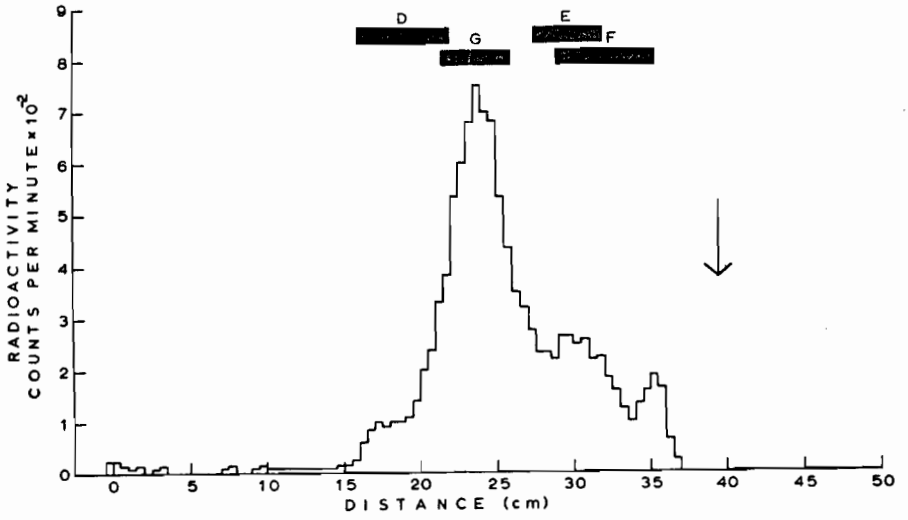


Figure 31. Distribution of radioactivity on paper chromatogram of the hydrophilic fraction of the urinary extract run in the monophasic benzene - acetic acid - water (125:72:3, v:v:v) system. Positions of daidzein (D), genistein (G), equol (E), and formononetin (F), as located by fluorescence and by staining of standard pilot strips with DSA, are indicated by black bars. Vertical arrow indicates the position of the solvent front.

TABLE XLI

FORMONONETIN: RADIOACTIVITY IN THE VARIOUS ZONES AS PERCENT OF TOTAL ON CHROMATOGRAM (HYDROPHILIC FRACTION)

Chromatographic System	Zone					
	Daidzein	Genistein	Additional Zone	Equol	Formononetin	Other
Chloroform - formamide: 4h	60.4[a]	60.4[a]	9.3	9.3	12.6	8.4
12h	12.3[b]	35.9[b]	21.6[b]	16.3	12.6[c]	1.3
24h	62.2[a]	62.2[a]	62.2[a]	19.0	12.6[c]	16.2[d]
48h	3.0[a]	3.0[a]	62.3	11.6[e]	12.6[c]	10.5[d]
Biphasic benzene - acetic acid - water	3.6	3.8	59.2	15.3	18.1	0

[a]Same zone.

[b]Because of overlapping, the distribution of radioactivity between these three zones is an approximation only.

[c]This zone was run off the paper.

[d]Consisted of a small zone at the origin.

[e]Part of this zone may have run off the chromatogram.

(c) Thin-layer Chromatography of the Urinary Extracts

In a further attempt to determine the chromatographic nature of the unidentified radioactive conversion product, the ether extract of the hydrolyzed urine and the hydrophilic fraction were run on thin-layer chromatoplates using the ethyl acetate - cyclohexane (50:50, v:v) system and the benzene - acetic acid - water (125:72:3, v:v:v) system. Standard daidzein and formononetin did not stain with DFB and were located by means of fluorescence. Although partial separation of the reference standards was attained in both solvent systems (as shown by staining and fluorescence), it was impossible to delineate the radioactive zones according to the radioactivity distributions. The results were similar to those obtained with paper by use of the monophasic benzene - acetic acid - water system in that the entire area which contained daidzein, genistein, equol, and formononetin was radioactive.

(d) Identification of Radioactive Daidzein, Equol, and Formononetin

The remainder of the ether extract of the hydrolyzed urine was chromatographed for 4 hours in the chloroform - formamide system on a number of split chromatograms. After drying (5 min), the formononetin zone was cut from the end

of the chromatograms and the papers were returned immediately to the chamber and rechromatographed for an additional 24 hours. At the end of this period, reference daidzein, genistein, and equol had not been run off the paper and were completely separate from each other. The zones which corresponded to the reference standards were cut out and all four zones were eluted with methanol in Soxhlet extractors for 24 hours. After addition of about 40 milligrams of the appropriate reference carrier, constant specific activities were obtained for daidzein, equol, and formononetin after recrystallizations from aqueous methanol (Tables XLII - XLIV incl.). The melting points of the purified compounds corresponded to those reported in the literature (Table XLV). Attempts to recrystallize genistein to constant specific activity were unsuccessful; after four recrystallizations, no radioactivity whatsoever was detectable in the 2 milligrams of crystals which remained.

In order to preclude the possibility that equol might be formed from urinary isoflavone during the course of analysis, ad hoc experiments were performed in which radioactive daidzein was added to normal urine samples which were then analyzed in the same way as were the samples of urine from the birds injected with radioactive isoflavones. When the hydrophilic fraction of the urine was analyzed chromatographically, no radioactivity was observed in the chromatographic positions corresponding to equol. Therefore equol was not produced as an analytical artifact.

TABLE XLII

CRYSTALLIZATION OF URINARY DAIDZEIN TO CONSTANT SPECIFIC ACTIVITY

Purification Step	C.P.M.	Weight (mg)	Specific Activity (c.p.m./mg)
Original pool	106,800	41.06	2,601
1st Crystallization	68,400	21.77	3,142
Mother liquor	19,600	15.25	1,284
2nd Crystallization	41,000	12.39	3,309
Mother liquor	19,200	6.40	3,000
3rd Crystallization	24,100	6.81	3,539
Mother liquor	12,500	3.59	3,482

TABLE XLIII

CRYSTALLIZATION OF URINARY EQUOL TO CONSTANT SPECIFIC ACTIVITY

Purification Step	C.P.M.	Weight (mg)	Specific Activity (c.p.m./mg)
Original pool	68,900	47.76	1,442
1st Crystallization	41,800	26.49	1,578
Mother liquor	19,400	15.01	1,292
2nd Crystallization	22,700	13.22	1,641
Mother liquor	15,900	10.05	1,584

TABLE XLIV

CRYSTALLIZATION OF URINARY FORMONONETIN TO CONSTANT SPECIFIC ACTIVITY

Purification Step	C.P.M.	Weight (mg)	Specific Activity (c.p.m./mg)
Original pool	70,700	46.45	1,522
1st Crystallization	46,500	24.75	1,878
Mother liquor	20,300	18.39	1,104
2nd Crystallization	22,800	11.10	2,054
Mother liquor	17,500	9.83	1,780
3rd Crystallization	11,800	5.43	2,173
Mother liquor	9,800	4.82	2,033

TABLE XLV

MELTING POINTS OF PURIFIED URINARY DAIDZEIN, EQUOL, AND FORMONONETIN

Compound	Melting Point (°C)	
	Experimental	Reference
Daidzein	322.5-325.5 (decomp.)	325.0-330.0 (114)
Equol	187.0-188.5 (decomp.)	189.0-190.5 (168)
Formononetin	253.0-254.0 (decomp.)	255 (238)

4. Discussion

The results demonstrate that formononetin-4-C^{14} is demethylated to daidzein-C^{14} and reduced to equol-C^{14} in the domestic fowl. The fate of formononetin in the hen thus parallels that of biochanin A. However, a few intriguing side results of the formononetin experiment merit further attention.

The first consideration involves the relative polarities of equol and the various isoflavones. According to the broad classical concepts of partition chromatography, hydroxyl groups decrease and methoxyl groups increase the Rf value of a substance. Thus it was found that the methoxylated isoflavones (formononetin and biochanin A) were less polar than equol, which, in turn, was less polar than the isoflavones in which the only substituents were hydroxyl groups (daidzein and genistein). However, it was observed that genistein, which has three hydroxyl groups, was consistently less polar than the di-hydroxylated daidzein; similarly, biochanin A (two hydroxyl groups) was less polar than formononetin (one hydroxyl group). These results can be explained on the basis of reports by Simpson and co-workers (256, 257), who studied the formation of hydrophobic chelate ring systems in 3- and 5-hydroxylated flavones with the 4-carbonyl group. They found that the

carbonyl-5-hydroxyl chelate system can derive part of its charge from another hydroxyl group in either of the benzene rings of the molecule, such as in the 4' position. Such a molecule (5,4'-dihydroxyflavone), therefore, would be less polar than 4'-hydroxyflavone. In an analogous manner, the 5,7-hydroxylated isoflavones (genistein, biochanin A), which would contain this chelate ring system, will be partially stabilized by the 7-hydroxyl group. The 7-hydroxyisoflavones, which are not substituted in the 5 position, would not have their charges modified by such a chelate ring system. In their investigation of the monophasic benzene - acetic acid - water paper chromatographic system, Wong and Taylor (247) found that the relative Rf values of various flavones, isoflavones, and flavonones were in accord with this principle.

The second consideration concerns the presence of an additional radioactive zone which did not correspond to reference daidzein, equol, or formononetin. This zone appeared in both the ether extract of the hydrolyzed urine and in the hydrophilic fraction. It was noted that this material had a polarity similar to that of genistein and it was first suspected that, in addition to being demethylated and reduced, formononetin might also have been hydroxylated. However, whenever reference genistein was run on additional

pilot strips, it was observed that, on some chromatograms, the radioactive zone corresponded to genistein, but that on other chromatograms, the material was less polar than genistein. The failure to attain constant specific activity when this radioactive zone was repeatedly recrystallized with genistein reference carrier proved, however, that the radioactivity was not attributable to genistein and hence that genistein was not a conversion product of formononetin. One may speculate as to the structure of this unidentified metabolite, but no evidence can be presented to support any of these speculations. Judging from the polarity of the material, one might surmise that possibly hydroxylation did take place in either of the benzene rings to yield a catechol derivative. However, it is doubtful that opening of the pyran ring occurred to produce a chalcone; such a compound would most likely be more polar than the isoflavones in the paper chromatographic systems employed. With the meager information which is available at the present time, the most reasonable speculation would seem to be that this metabolite has retained the isoflavonoid nucleus.

The third interesting result of the formononetin experiment is the fact that a radioactive zone remained at the origin when the hydrophilic fraction was chromatographed

in the chloroform - formamide system for 24 hours and 48 hours; this zone was not present in the purified ether extract of the hydrolyzed urine, thus it cannot be said that this zone represents an additional metabolite of formononetin. This occurrence leads immediately to the question of the merit of the procedure of Bauld for the isolation and purification of these compounds. This method was employed because it was by means of this technique that equol was first identified and isolated from the urine of the domestic fowl. The fact that an additional radioactive peak was present in the hydrophilic fraction which was not present in the ether extract suggests that this zone represents an artifact formed during the rigourous purification of the hydrophilic fraction, possibly during saponification. If this be the case, then hasty conclusions should not be drawn from observations that the unidentified radioactive zones found in the hydrophilic fractions in the coumestrol and genistein experiments were decomposition products or additional metabolites. (No substantial unidentified radioactive material was present in the hydrophilic fraction of the biochanin A experiment). From the results of the formononetin experiment, it would seem that no advantage accrued from the partitioning of the purified ether extract of the hydrolyzed urine, for the

major unidentified radioactive zone was carried along to the final hydrophilic fraction. Indeed, one may carry this argument further by suggesting that radiochemical analysis of the initial ether extract of the urine may be sufficient or, indeed, preferable for the chromatographic identification of isoflavone metabolites and possibly also for recrystallization experiments. It is, of course, true that the numerous non-radioactive urinary materials with polarities similar to those of equol and the isoflavones might interfere with uniform migration of the radioactive materials along the paper chromatograms. On the other hand, it seems probable that the losses of radioactivity which were observed during the various purification procedures may have been due to destruction or removal of equol and of isoflavonoid material. Thus it must be remembered that the yield of equol and other metabolites which have been reported in these experiments represent the percentage recoveries under the specified experimental conditions. All that is claimed for the quantitative results is that they at least represent the minimum percentage conversion from administered starting materials.

Estimations of the percentage recoveries of counts as daidzein, equol, and formononetin in the purified ether extract of the hydrolyzed urine and in the final hydrophilic fraction are presented in Table XLVI and Table XLVII respectively.

TABLE XLVI

PERCENTAGE OF TOTAL COUNTS INJECTED RECOVERED IN THE PURIFIED ETHER EXTRACT OF THE HYDROLYZED URINE AS DAIDZEIN, EQUOL, AND FORMONONETIN*

	Daidzein	Equol	Formononetin
Average percentage of counts present in final ether extract	46.8	13.0	19.8
Net percentage recovery	5.63	1.56	2.38

*Calculations based on percentage recovery in ether extract of total counts injected as 12.02.

TABLE XLVII

PERCENTAGE OF TOTAL COUNTS INJECTED RECOVERED IN THE HYDROPHILIC FRACTION AS DAIDZEIN, EQUOL, AND FORMONONETIN*

	Daidzein	Equol	Formononetin
Average percentage of counts present in hydrophilic fraction	3.6	12.0	15.3
Net percentage recovery	0.098	0.328	0.468

*Calculations based on percentage recovery in hydrophilic fraction of total counts injected as 2.734.

The values were calculated on the basis of the fact that the percentage recoveries of counts in the ether extract and in the hydrophilic fraction were 12.02 and 2.734 respectively (Table XXXIX). The calculations were based on the considerations discussed in the section on biochanin A. An examination of these two tables will reveal that most of the daidzein was lost during purification of the hydrophilic fraction. It will reveal also that the percentage (13.0) of the counts in the purified ether extract of the hydrolyzed urine that was present as equol was approximately the same as the percentage (12.0) present as equol in the hydrophilic fraction. Since the corresponding percentage values for daidzein and formononetin dropped considerably from the ether extract to the hydrophilic fraction, it can be concluded that equol is more resistent to saponification and the subsequent purification of the hydrophilic fraction than are the isoflavones.

SUMMARY

An analytical technique was developed for the routine measurement of radioactivity distribution on paper chromatograms which contained low levels of H^3- and C^{14}-activity. The method involved the use of liquid scintillating counting. The radioactive materials were insoluble in the scintillation solvent toluene. A pilot strip 2 centimeters wide was cut into ½ or ¾ centimeter bands and each band was eluted with 0.2 milliliters of methanol in a standard screw-top liquid scintillation counting vial. Both paper and methanol were flooded with 10 milliliters of scintillation fluid. The effect of paper orientation in the counting vial was negligible provided that the filter paper band rested flat on the bottom of the vial. Methanol at this concentration did not quench the solution. This method provided a means of dispersing the methanol-soluble radioactive materials throughout the scintillation fluid and thus reduced the effect of self-absorption by the filter paper band. The technique was found to be more efficient, more reproducible, and less laborious than several other methods tested.

The possibility that equol in the urine of the domestic fowl may represent a conversion product of the

isoflavone genistein was investigated. Genistein was randomly tritiated by the Wilzbach process and purified by repeated crystallization and paper chromatography. The purified material was injected intramuscularly into a non-laying hen. Phenolic extracts were prepared from the urine for the first 4 days. The radioactivity in these extracts was distributed between genistein and equol together with several less polar conversion and breakdown products which were not identified. The amount of radioactivity recovered from the urine in the form of equol represented 1.59 percent of the radioactivity injected as genistein.

In a similar experiment, randomly tritiated coumestrol was purified by paper chromatography and injected intramuscularly into a non-laying hen. The radioactivity in the phenolic extracts of the urine was distributed between coumestrol and conversion or breakdown products which were not identified. No radioactive equol was detectable. The percent of radioactive conversion products in the hydrophilic fraction which were more polar than coumestrol increased from day to day.

Synthetic biochanin A-4-C^{14} was injected intramuscularly into a non-laying hen. The radioactivity in the hydrophilic fraction was distributed between genistein, equol, and

biochanin A. This suggested that biochanin A is demethylated in the 4' position to genistein, which is subsequently reduced in the 2,3 position and dehydroxylated in the 5 position to give rise to equol. A small amount of radioactivity remained at the origin on all chromatograms and was not associated with any of the reference compounds tested. The amount of radioactivity recovered in the hydrophilic fraction in the form of genistein and equol represented 0.272 percent and 0.101 percent, respectively, of the radioactivity injected as biochanin A.

Formononetin-4-C^{14} underwent a similar conversion in the fowl in that it was demethylated to daidzein-C^{14} and reduced to equol-C^{14}. A highly radioactive unidentified zone, which had a polarity very close to that of genistein, was also present in both the purified ether extract of the hydrolyzed urine and in the final hydrophilic fraction. It was shown that this radioactivity was not due to genistein. The preparation and purification of the hydrophilic and lipophilic fractions from the ether extract of the hydrolyzed urine resulted in substantial losses of radioactive isoflavones and of radioactive equol and also encouraged the formation of radioactive degradation materials. It is suggested that appropriate radiochemical analyses can be carried out on the purified ether extract without noticeable interference from any non-radioactive impurities which may

also be present. The amount of radioactivity recovered in the purified ether extract of the hydrolyzed urine in the form of daidzein and equol represented 5.63 percent and 1.56 percent, respectively, of the radioactivity injected, and in the hydrophilic fraction, the amount represented 0.098 percent and 0.328 percent, respectively, of the radioactivity injected as formononetin.

These experiments have demonstrated that the fowl can demethylate and reduce administered isoflavonoid material. They also demonstrate that estrogenic isoflavones are precursors of urinary equol in the fowl, but that the coumarin-like estrogen, coumestrol, is not a precursor of urinary equol and must, therefore, be degraded *in vivo* along conversion pathways different from those along which the isoflavones are degraded.

CLAIMS TO ORIGINAL RESEARCH

1. The development of a convenient and relatively efficient technique for the routine measurement of radioactivity distribution on paper chromatograms containing low amounts of H^3- and C^{14}-labelled compounds which are insoluble in the scintillation solvent toluene.

2. The definition of paper chromatographic methods which are specially suitable for the separation of isoflavones and coumestrol from equol.

3. The demonstration that genistein gives rise to equol in the domestic fowl. This constitutes the first direct experimental proof, so far as the author is aware, for the conversion of an isoflavone to equol in any animal species.

4. The demonstration that coumestrol failed to give rise to detectable amounts of equol in the fowl. So far as the author is aware, this constitutes the first information that has been obtained about the *in vivo* conversion of coumestrol.

5. The demonstration that biochanin A is demethylated to genistein and reduced to equol in the domestic fowl. Demethylation of biochanin A in various species has been reported previously, but the present work constitutes

the first evidence for its conversion to equol in any species.

6. The demonstration that formononetin is demethylated to daidzein and reduced to equol in the domestic fowl. So far as the author is aware, this represents the first information obtained regarding the *in vivo* conversion products of formononetin in any animal species.

LITERATURE CITED

1. Doisy, E.A., Veler, C.D., and S.A. Thayer.
   Folliculin from Urine of Pregnant Women.
   Amer. J. Physiol., 90: 329-330 (1929).

2. Butenandt, A.
   Progynon, a Crystalline Female Sexual Hormone.
   Naturwiss., 17: 879 (1929).
   Cited in Chem. Abstr., 24: 870 (1930).

3. Dodds, E.C., Goldberg, L., Lawson, W., and R. Robinson.
   Oestrogenic Activity of Certain Synthetic Compounds.
   Nature, 141: 247-248 (1938).

4. Emmens, C.W.
   Precursors of Estrogens.
   J. Endocrinol., 2: 444-458 (1941).

5. Loewe, S.
   Female Sexual Hormones from Vegetable Starting Materials.
   German Patent 517,761 (1926).
   Cited in Chem. Abstr., 25: 2815 (1931).

6. Dohrn, M., Faure, W., Poll, H., and W. Blotevogel.
   Tokokinine, Stoffe mit Sexualhormonartiger Wirkung aus Pflanzenzellen.
   Mediz. Klinik, 22: 1417-1419 (1926).

7. Fellner, O.O.
   Zuckerstoffwechsel, Sexualorgane und Insulin.
   Mediz. Klinik, 22: 1886-1888 (1926).

8. Emmens, C.W.
   "Hormone Assay".
   Academic Press, New York, N.Y., 1950.

9. Dohrn, M.
   Is the Allen-Doisy Test Specific for the Female Sex Hormone?
   Klin. Wochschr., 6: 359-360 (1927).
   Cited in Chem. Abstr., 21: 1838 (1927).

10. Bennetts, H.W., Underwood, E.J., and F.L. Shier.
    A Specific Breeding Problem of Sheep on Subterranean Clover Pastures in Western Australia.
    Austral. Vet. J., 22: 2-12 (1946).

11. Curnow, D.H., Robinson, T.J., and E.J. Underwood.
Estrogenic Action of Extracts of Subterranean Clover.
Austral. J. Exp. Biol. Med. Sci., 26: 171-180 (1948).

12. Schoop, G. and H. Klette.
Fruchtbarkeitsstörungen durch Pflanzenoestrogene.
Fortpflanzung und Besammung der Haustiere, 2: 73-74 (1952).

13. New Zealand Department of Agriculture Report (1950), p. 37.
Cited in Nilsson, A.
Studies on Occurrence, Estrogenic Activity and Metabolism of Plant Estrogens, Especially Biochanin A.
Acta Universitatis Upsaliensis, 29: 1-15 (1963).

14. Ch'ang, T.S.
Reproductive Disturbances of Romney Ewe Lambs Grazed on Red Clover (Trifolium pratense) Pastures.
Nature, 182: 1175 (1958).

15. Greenblatt, R.B.
"A Physician's Quest for Answers in the Bible".
Les Frères des Ecoles Chrêtiennes, Montreal, 1962.

16. Butenandt, A.
Zur Charakterisierung des Oestrogen Wirksamen Tokokinins aus Butea superba.
Naturwiss., 28: 533 (1940).

17. Skarzynski, B.
An Oestrogenic Substance from Plant Material.
Nature, 131: 766 (1933).

18. Loewe, S.
"Klein's Handbuch der Pflanzenanalyse".
Springer, Vienna, 4: 1034-1041 (1933).

19. Vatna, S.
Preliminary Report on the Presence of an Oestrogenic Substance and a Poisonous Substance in the Storage Root of Butea superba Roxb.
Thailand Sci. Bull., 4: 3-9 (1939).
Cited in Chem. Abstr., 34: 2929 (1940).

20. Schoeller, W., Dohrn, M., and W. Hohlweg.
Über eine Oestrogene Substanz aus der Knolle der Siamesischen Schlingpflanze Butea superba.
Naturwiss., 28: 532-533 (1940).

21. Butenandt, A.
Zur Charakterisierung des Oestrogen Wirksamen Tokokinins aus Butea superba.
Naturwiss., 28: 533 (1940).

22. Bounds, D.G. and G.S. Pope.
Light-absorption and Chemical Properties of Miroestrol, an Oestrogenic Substance from Pueraria mirifica.
J. Chem. Soc., 3696-3705 (1960).

23. Taylor, N.E., Hodgkin, D.C., and J.S. Rollett.
The X-Ray Crystallographic Determination of the Structure of Bromomirestrol.
J. Chem. Soc., 3685-3695 (1960).

24. Jones, H.E.H. and G.S. Pope.
A Study of the Action of Miroestrol and other Oestrogens on the Reproductive Tract of the Immature Female Mouse.
J. Endocrinol., 20: 229-235 (1960).

25. Zenisek, A. and J. Bednar.
Contribution to the Identification of the Estrogenic Activity of Hops.
American Perfumer, 61-62 (May, 1960).

26. Bradbury, R.B. and D.E. White.
Estrogens and Related Substances in Plants.
Vitamins and Hormones, 12: 207-233 (1954)

27. Glaser, E. and R. Drobnik.
Active Ingredients of Garlic.
Arch. exptl. Pathol. Pharmakol., 193: 1-9 (1939).
Cited in Chem. Abstr., 34: 3283 (1940).

28. Costello, C.H. and E.V. Lynn.
Estrogenic Substances from Plants.
J. Amer. Pharm. Assoc., Sci. Ed., 39: 177-180 (1950).

29. Costello, C.H. and C.L. Butler.
The Estrogenic and Uterine-Stimulating Activity of Asclepias tuberosa. A Preliminary Investigation.
J. Amer. Pharm. Assoc., Sci. Ed., 39: 233-237 (1950).

30. Walker, B.S. and J.C. Janney.
Estrogenic Substances. II. Analysis of Plant Sources.
Endocrinol., 14: 389-392 (1930).

31. Chakravorty, P.N., Wesner, M.M., and R.H. Levin.
Cafesterol. II.
J. Amer. Chem. Soc., 65: 929-932 (1943).

32. Wehefritz, E. and E. Gierhake.
Über die Spezifität des Weiblichen Sexualhormons.
Zentr. Gynäkol., 55: 16-21 (1931).

33. Levin. E., Burns, J.F., and V.K. Collins.
Estrogenic, Androgenic, and Gonadotropic Activity in Wheat-germ Oil.
Endocrinol., 49: 289-301 (1951).

34. Schering, E.
Swiss Patent 129,124 (1928).
Cited in Ref. 25.

35. Dingemanse, E. and E. Laqueur.
The Existence of Menformone in the Animal and Vegetable Kingdoms in Different Circumstances. The Vital Activity of Menformone.
Arch. néerland. physiol., 14: 271-276 (1929).
Cited in Chem. Abstr., 24: 5832 (1930).

36. Kroszczynski, S. and M. Bychowska.
Estrogenic Action of Sage (Salvia officinalis).
Compt. rend. soc. biol., 130: 570-571 (1939).
Cited in Chem. Abstr., 33: 4299 (1939).

37. Dohan, F.C., Richardson, E.M., Stribley, R.C., and P. Gyorgy.
Estrogenic Effects of Extracts of Spring Rye Grass and Clover.
J. Amer. Vet. Med. Assoc., 118: 323-324 (1951).

38. Wadehn, F.
Sexual Hormones, Especially Feminin.
Z. angew. Chem., 41: 352-355 (1928).
Cited in Chem. Abstr., 23: 889 (1929).

39. Coussens, R. and G. Sierens.
Oestrogenic Properties of Tulip Bulbs.
Arch. int. Pharmacodyn., 78: 309-312 (1949).

40. Pederson-Bjergaard, K.
De l'Action Folliculinique d'un Lipoïde Isolé des Bacilles Tuberculeux.
Compt. rend. soc. biol., 112: 103-105 (1933).

41. Zarrow, M.X., Lazo-Wasem, E.A., and R.L. Shogor.
Estrogenic Activity in a Commercial Animal Ration.
Science, 118: 650-651 (1953).

42. Legg, S.P., Curnow, D.H., and S.A. Simpson.
The Seasonal and Species Distribution of Oestrogen in British Pasture Plants.
Biochem. J., 46: Proc. XIX-XX (1950).

43. Robinson, T.J.
Estrogenic Potency of Subterranean Clover: Preparation and Assay of Extracts.
Austral. J. Exptl. Biol. Med. Sci., 27: 297-305 (1949).

44. Bennetts, H.W.
Two Sheep Problems on Subterranean Clover Dominant Pastures. 1. Lambing Trouble (Dystokia) in Merinos. 2. Prolapse of the Womb (Inversion of the Uterus).
J. Dept. Agr. Western Australia, 21: 104-109 (1944).

45. Bennetts, H.W. and E.J. Underwood.
The Oestrogenic Effects of Subterranean Clover (Trifolium subterraneum).
"Plant and Animal Nutrition in Relation to Soil and Climatic Factors".
Proceedings of the Specialist Conference in Agriculture, Melbourne, Australia, 1949, pp. 329-334.

46. East, J.
Oestrogenic Effects of Subterranean Clover (T. subterraneum L., var. Dwalganup): Mammary Development in the Castrate Male Guinea-pig.
Austral. J. Exptl. Biol. Med. Sci., 28: 449-458 (1950).

47. Curnow, D.H.
Studies on an Oestrogenic Factor in Subterranean Clover (Trifolium subterranean L., var. Dwalganup).
Ph.D. Thesis, University of London, 1950.

48. Beck, A.B. and A.W. Braden.
Studies on the Estrogenic Substance in Subterranean Clover: (Trifolium subterraneum var. Dwalganup).
Austral. J. Exptl. Biol. Med. Sci., 29: 273-279 (1951).

49. Bradbury, R.B. and D.E. White.
The Chemistry of Subterranean Clover. Part I. Isolation of Formononetin and Genistein.
J. Chem. Soc., 3447-3449 (1951).

50. Biggers, J.D. and D.H. Curnow.
Oestrogenic Activity of Subterranean Clover. I. The Oestrogenic Activity of Genistein.
Biochem. J., 58: 278-282 (1954).

51. Emmens, C.W.
The Differentiation of Estrogens from Pro-estrogens by the Use of Spayed Mice Possessing Two Separate Vaginal Sacs.
J. Endocrinol., 3: 174-177 (1942).

52. Jensen, E.V.
On the Mechanism of Estrogen Action.
Perspectives Biol. Med., 6: 47-60 (1962).

53. Mueller, G.C., Gorski, J., and Y. Aizawa.
The Role of Protein Synthesis in Early Estrogen Action.
Proc. Nat. Acad. Sci., 47: 164-169 (1961).

54. Hagerman, D.D. and C.A. Villee.
A Mechanism of Action for Estrogenic Steroid Hormones.
"Mechanism of Action of Steroid Hormones".
C.A. Villee and L.L. Engel, eds., Int. Series of Monographs on Pure and Applied Biology, Pergamon Press, New York, N.Y., 1: 169-187 (1961).

55. Talalay, P. and H.G. Williams-Ashman.
Participation of Steroid Hormones in the Enzymic Transfer of Hydrogen.
Recent Progress Hormone Res., 16: 1-47 (1960).

56. Mueller, G.C., Herranen, A.M., and K.F. Jervell.
Studies on the Mechanism of Action of Estrogens.
Recent Progress Hormone Res., 14: 95-139 (1958).

57. Spaziani, E. and C.M. Szego.
The Influence of Estradiol and Cortisol on Uterine Histamine of the Ovariectomized Rat.
Endocrinol., 63: 669-678 (1958).

58. Roberts, S. and C.M. Szego.
Steroid Interaction in the Metabolism of Reproductive Target Organs.
Physiol. Rev., 33: 593-629 (1953).

59. Hechter, O.M. and G. Lester.
Cell Permeability and Hormone Action.
Recent Progress Hormone Res., 16: 139-186 (1960).

60. Jensen, E.V. and H.I. Jacobson.
Basic Guides to the Mechanism of Estrogen Action.
Recent Progress Hormone Res., 18: 387-414 (1962).

61. Hamilton, T.H.
Isotopic Studies on Estrogen-Induced Accelerations of Ribonucleic Acid and Protein Synthesis.
Proc. Nat. Acad. Sci., 49: 373-379 (1963).

62. Wilson, J.D.
The Nature of the RNA Response to Estradiol Administration by the Uterus of the Rat.
Proc. Nat. Acad. Sci., 50: 93-100 (1963).

63. Talwar, G.P. and S.J. Segal.
Prevention of Hormone Action by Local Application of Actinomycin D.
Proc. Nat. Acad. Sci., 50: 226-230 (1963).

64. Noteboom, W.D. and J. Gorski.
An Early Effect of Estrogen on Protein Synthesis.
Proc. Nat. Acad. Sci., 50: 250-255 (1963).

65. Ui, H. and G.C. Mueller.
The Role of RNA Synthesis in Early Estrogen Action.
Proc. Nat. Acad. Sci., 50: 256-260 (1963).

66. Gorski, J. and J.A. Nicolette.
Early Estrogen Effects on Newly Synthesized RNA and Phospholipid in Subcellular Fractions of Rat Uteri.
Arch. Biochem. Biophys., 103: 418-423 (1963).

67. Hamilton, T.H.
Sequences of RNA and Protein Synthesis during early Estrogen Action.
Proc. Nat. Acad. Sci., 51: 83-89 (1964).

68. Csapo, A.
The *In Vivo* and *In Vitro* Effects of Estrogen and Progesterone on the Myometrium.
"Mechanism of Action of Steroid Hormones".
C.A. Villee and L.L. Engel, eds., Int. Series of Monographs on Pure and Applied Biology, Pergamon Press, New York, N.Y., 1: 126-147 (1961).

69. Mueller, G.C.
Effects of Estrogens and Anti-estrogens *In Vitro*.
Cancer Res., 17: 490-506 (1957).

70. Spaziani, E. and C.M. Szego.
Further Evidence for Mediation by Histamine of Estrogenic Stimulation of the Rat Uterus.
Endocrinol., 64: 713-723 (1959).

71. Kahlson, G.
New Approaches to the Physiology of Histamine.
Perspectives Biol. Med., 5: 179-197 (1962).

72. Biggers, J.D.
Plant Phenols Possessing Oestrogenic Activity.
"Pharmacology of Plant Phenolics".
J.W. Fairbairn, ed., Academic Press, London, 1958, pp. 51-69.

73. Curnow, D.H.
Oestrogenic Activity of Subterranean Clover. 2. The Isolation of Genistein from Subterranean Clover and Methods of Quantitative Estimation.
Biochem. J., 58: 283-287 (1954).

74. Finnemore, H.
Examination of a Species of Prunus.
Pharm. J., 85: 604 (1910).

75. Perkin, A.G. and A.E. Everest.
"The Natural Organic Coloring Matters".
Longmans, Green, and Co., New York, N.Y., 1918.

76. Bargellini, G.
Phenylcoumarins.
Gazz. chim. ital., 55: 945-951 (1925).
Cited in Chem. Abstr., 20: 1987 (1926).

77. Baker, W. and R. Robinson.
Synthetical Experiments in the Isoflavone Group. Part I.
J. Chem. Soc., 127: 1981-1986 (1925).

78. Baker, W. and R. Robinson.
Synthetical Experiments in the Isoflavone Group. III. Synthesis of Genistein.
J. Chem. Soc., 3115-3118 (1928).

79. Walter, E.D.
Genistin (an Isoflavone Glucoside) and its Aglucone, Genistein, from Soybeans.
J. Amer. Chem. Soc., 63: 3273-3276 (1941).

80. Pope, G.S., Elcoate, P.V., Simpson, S.A., and D.G. Andrews.
Isolation of an Estrogenic Isoflavone (Biochanin A) from Red Clover.
Chem. & Ind., 1092 (1953).

81. Pope, G.S. and H.G. Wright.
Oestrogenic Isoflavones in Red Clover and Subterranean Clover.
Chem. & Ind., 1019-1020 (1954).

82. Bartlett, S., Folley, S.J., Rowland, S.J., Curnow, D.H., and S.A. Simpson.
Oestrogens in Grass and their Possible Effects on Milk Secretion.
Nature, 162: 845 (1948).

83. Evans, I.A. and W.C. Evans.
Effect of Young Grass in the Diet on the Onset of Sexual Maturity in Mice.
Nature, 163: 908-909 (1949).

84. Siddiqui, S.
Constituents of Chana. I. Isolation of Three New Crystalline Products from the Chana Germ.
J. Sci. Indust. Res. India, 4: 68-70 (1945).

85. Bose, J. and S. Siddiqui.
The Constituents of Chana. II. Constitution of Biochanin A.
J. Sci. Indust. Res. India, 4: 231-235 (1945).

86. King, F.E., Grundon, M.F., and K.G. Neill.
The Chemistry of Extractives from Hardwoods. Part IX. Constituents of the Heartwood of Ferreirea spectabilis.
J. Chem. Soc., 4580-4584 (1952).

87. Bate-Smith, E.C., Swain, T., and G.S. Pope.
The Isolation of 7-Hydroxy-4'-methoxyisoflavone (Formononetin) from Red Clover (Trifolium pratense, L.) and a Note on the Identity of Pratol.
Chem. & Ind., 1127 (1953).

88. Hlasiwetz, 1855.
Cited by F.M. Dean, "Naturally Occurring Oxygen Ring Compounds", Butterworths (London), 1963, p. 366.

89. Hemmelmayr, F. von.
Über das Ononin.
Monatsh. für Chemie, 23: 133-164 (1902).

90. Baker, W., Ollis, W.D., Binns, P.J.L., Chadderton, J., Dunstan, I., Harborne, J.B., and D. Weight.
New Synthesis of Isoflavones.
Nature, 169: 706 (1952).

91. Wong, E.
A New Isoflavone from Red Clover.
Chem. & Ind., 1963-1964 (1961).

92. Wong, E.
Isoflavone Contents of Red and Subterranean Clovers.
J. Sci. Food Agr., 6: 376-379 (1963).

93. Guggolz, J., Livingston, A.L., and E.M. Bickoff.
Detection of Daidzein, Formononetin, Genistein, and Biochanin A in Forages.
Agr. & Food Chem., 9: 330-332 (1961).

95. Shibata, S., Murakami, T., and Y. Nishikawa.
Constituents of Japanese and Chinese Crude Drugs.
I. Constituents of Pueraria Root.
Yakugaku Zasshi, 79: 757-760 (1959).
Cited in Chem. Abstr., 53: 18387 (1959).

96. Bickoff, E.M., Livingston, A.L., Hendrickson, A.P., and A.N. Booth.
Relative Potencies of Several Estrogen-like Compounds found in Forages.
Agr. & Food Chem., 10: 410-412 (1962).

97. Nilsson, A.
Estrogenic Activity of some Isoflavone Derivatives.
Acta Physiol. Scand., 56: 230-236 (1962).

98. Bickoff, E.M., Booth, A.N., Lyman, R.L., Livingston, A.L., Thompson, C.R., and G.O. Kohler.
Isolation of a New Estrogen from Ladino Clover.
Agr. & Food Chem., 6: 536-539 (1958).

99. Bickoff, E.M., Lyman, R.L., Livingston, A.L., and A.N. Booth.
Characterization of Coumestrol, a Naturally-Occurring Plant Estrogen.
J. Amer. Chem. Soc., 80: 3969-3971 (1958).

100. Bickoff, E.M., Booth, A.N., Lyman, R.L., Livingston, A.L., Thompson, C.R., and F. DeEds.
Coumestrol, a New Estrogen Isolated from Forage Crops.
Science, 126: 969-970 (1957).

101. Lyman, R.L., Bickoff, E.M., Booth, A.N., and A.L. Livingston.
Detection of Coumestrol in Leguminous Plants.
Arch. Biochem. Biophys., 80: 61-67 (1959).

102. Livingston, A.L., Bickoff, E.M., Guggolz, J., and C.R. Thompson.
Fluorometric Estimate of Coumestrol on Paper Chromatograms.
Anal. Chem., 32: 1620-1622 (1960).

103. Livingston, A.L., Bickoff, E.M., Guggolz, J., and C.R. Thompson.
Quantitative Determination of Coumestrol in Fresh and Dried Alfalfa.
Agr. & Food Chem., 9: 135-137 (1961).

104. Bickoff, E.M., Livingston, A.L., and A.N. Booth.
Estrogenic Activity of Coumestrol and Related Compounds.
Arch. Biochem. Biophys., 88: 262-266 (1960).

105. Bradbury, R.B.
Some Oestrogenic 4-Phenyl-substituted Isoflav-3-enes.
Austral. J. Chem., 6: 447-449 (1953).

106. Grisebach, H. and W. Barz.
Biosynthesis of Coumestrol.
Chem. & Ind., 690-691 (1963).

107. Grisebach, H. and W. Barz.
Über die Biogenese des Cumöstrols in der Luzerne. (Medicago sativa, L.).
Z. Naturforsch., 18b: 466-470 (1963).

108. Grisebach, H. and W.D. Ollis.
Biogenetic Relationships between Coumarins, Flavonoids, Isoflavonoids, and Rotenoids.
Experientia, 17: 4-12 (1961).

109. Neish, A.C.
Biosynthetic Pathways of Aromatic Compounds.
Ann. Rev. Plant Physiol., 11: 55-80 (1960).

110. Brown, S.A.
Biosynthesis of Coumarins. IV. The Formation of Coumarin and Herniarin in Lavender.
Phytochemistry, 2: 137-144 (1963).

111. Reznik, H. and R. Urban.
Über den Metabolismus ^{14}C-Markierter Ferulasäure in Pflanzenversuch.
Naturwiss., 44: 13 (1957).

112. Mothes, K. and H. Kala.
Die Wurzel als Bildungsstätte für Cumarine.
Naturwiss., 42: 159 (1955).

113. Brown, S.A.
Recent Studies on the Formation of Natural Coumarins.
Lloydia, 26: 211-222 (1963).

114. Nilsson, A.
Demethylation of the Plant Estrogen Formononetin to Daidzein in Rumen Fluid.
Arkiv för Kemi, 19: 549-550 (1962).

115. Nilsson, A.
O-Demethylation of Biochanin A and some other Isoflavones and Methylated Estrogens by Microsomal Liver Enzymes.
Arkiv för Kemi, 21: 97-121 (1963).

116. Cheng, E., Yoder, L., Story, C.D., and W. Burroughs.
Estrogenic Activity of some Isoflavone Derivatives.
Science, 120: 575-576 (1954).

117. Pieterse, P.J.S. and F.N. Andrews.
The Estrogenic Activity of Legume, Grass, and Corn Silage.
J. Dairy Sci., 39: 81-89 (1956).

118. Pieterse, P.J.S. and F.N. Andrews.
The Estrogenic Activity of Alfalfa and other Feedstuffs.
J. Animal Sci., 15: 25-36 (1956).

119. Andrews, F.N.
The Estrogenic Activity of Alfalfa and other Feedstuffs.
Feedstuffs, 30(5): 34a-35a (Feb. 1, 1958).

120. Stob, M., Davis, R.L., and F.N. Andrews.
Strain Differences in the Estrogenicity of Alfalfa.
J. Animal Sci., 16: 850 (1957).

121. Kitts, W.D., Swierstra, E., Brink, V.C., and A.J. Wood.
The Estrogen-like Substances in certain Legumes and Grasses. I. The Quantitative Determination of such Substances in Red Clover and Oats.
Can. J. Animal Sci., 39: 6-13 (1959).

122. Kitts, W.D., Swierstra, E., Brink, V.C., and A.J. Wood.
The Estrogen-like Substances in certain Legumes and Grasses. II. The Effect of Stage of Maturity and Frequency of Cutting on the Estrogenic Activity of some Forages.
Can. J. Animal Sci., 39: 158-163 (1959).

123. Kitts, W.D.
Estrogenic Activity of Forage Crops.
Feedstuffs, 32(5): 18,23 (1960).

124. Bickoff, E.M., Livingston, A.L., Booth, A.N., Hendrickson, A.P., and G.O. Kohler.
Estrogenic Activity in Dehydrated and Sun-cured Forages.
J. Animal Sci., 19: 189-197 (1960).

125. Bickoff, E.M., Booth, A.N., Livingston, A.L., and A.P. Hendrickson.
Observations on the Effect of Drying on Estrogenic Activity of Alfalfa Samples of Varying Maturity.
J. Animal Sci., 19: 745-753 (1960).

126. Bickoff, E.M., Livingston, A.L., Booth, A.N., Thompson, C.R., Hollowell, E.A., and E.G. Beinhart.
Some Variation in Estrogenic Activity in Fresh and Dried White Clover Clones and the Ladino Variety.
J. Animal Sci., 19: 1143-1149 (1960).

127. Bickoff, E.M.
Estrogen-like Substances in Plants.
"Physiology of Reproduction".
Proceedings of the Twenty-Second Biology Colloquium, Oregon State University, 1961, pp. 93-118.

128. Kohler, G.O. and E.M. Bickoff.
Coumestrol and other Biologically Active Compounds in Forages.
Feedstuffs, 33(5): 46, 55-56 (1961).

129. Nilsson, A.
The Oestrogenic Activity of Hay and Silage from some Swedish Forage Plants.
Kungl. Lantbrukshögskolans Annaler, 26: 19-31 (1960).

130. Kallela, K.
Estrogen Content of Forage Plants in Finland.
Soumen Kemistilehti, B35: 116-118 (1962).
Cited in Chem. Abstr., 57: 17077 (1962).

131. Kallela, K. and L. Vasenius.
Plant Estrogens and their Occurrence in Finnish AIV Silage.
Nordisk Veterinaermedicin, 14: 192-199 (1962).

132. Pope, G.S.
The Importance of Pasture Plant Oestrogens in the Reproduction and Lactation of Grazing Animals.
Dairy Sci. Abstr., 16: 334-355 (1954).

133. Pope, G.S., McNaughton, M.J., and H.E.H. Jones.
Oestrogens in British Pasture Plants.
J. Dairy Res., 26: 196-202 (1959).

134. Flux, D.S., Munford, R.E., and G.F. Wilson.
Biological Estimation of Oestrogenic Activity in Red Clover (Trifolium pratense): Relative Potencies of Parts of Plant and Changes with Storage.
J. Dairy Res., 30: 243-249 (1963).

135. Cheng, E., Story, C.D., Payne, L.C., Yoder, L., and W. Burroughs.
Detection of Estrogenic Substances in Alfalfa and Clover Hays fed to Fattening Lambs.
J. Animal Sci., 12: 507-514 (1953).

136. Stob, M., Walker, B.J., and F.N. Andrews.
Factors Affecting the Estrogenic Content of Alfalfa Silage.
J. Dairy Sci., 41: 438-439 (1958).

137. Davies, H.L. and D. Bennett.
Studies on the Oestrogenic Potency of Subterranean Clover (Trifolium subterraneum L.) in South-Western Australia.
Austral. J. Agr. Res., 13: 1030-1040 (1962).

138. Ershoff, B.H.
Protective Effects of Alfalfa in Immature Mice fed Toxic Doses of Glucoascorbic Acid.
Proc. Soc. Exptl. Biol. Med., 87: 134-136 (1954).

139. Fox, C.W., McKensie, F.F., and J.E. Oldfield.
The Effects of Red Clover on Reproduction in Sheep.
J. Animal Sci., 18: 1178 (1959) (abstract).

140. Hale, W.H., Homeyer, P.G., Culbertson, C.C., and W. Burroughs.
Response of Lambs Fed Varied Levels of Diethylstilbestrol.
J. Animal Sci., 14: 909-918 (1955).

141. Story, C.D., Hale, W.H., Cheng, E.W., and W. Burroughs.
The Effect of Low Levels of Diethylstilbestrol and Plant Estrogens upon Performance of Fattening Lambs.
Proc. Iowa Acad. Sci., 64: 259-266 (1957).

142. Matsushima, J.
Dehydrated Alfalfa as a Protein Supplement (with and without Stilbestrol) for Fattening Cattle.
47th Annual Feeders Day, Nebraska Agri. Exp. Sta., April 17, 1959, pp. 29-32.

143. Oldfield, J.E., Fox, C.W., and E.M. Bickoff.
Effects of Estrogenic Activity in Alfalfa on Growing Lambs.
J. Animal Sci., 19: 1281 (1960).

144. Magee, A.C.
Biological Responses of Young Rats fed Diets Containing Genistin and Genistein.
J. Nutrition, 80: 151-156 (1963).

145. Matrone, G., Smart Jr., W.W.G., Carter, M.W., Smart, V.W., and H.W. Garren.
Effect of Genistin on Growth and Development of the Male Mouse.
J. Nutrition, 59: 235-241 (1956).

146. Carter, M.W., Matrone, G., and W.W.G. Smart Jr.
The Effect of Genistin and its Aglycone on Weight Gain in the Mouse.
British J. Nutrition, 14: 301-304 (1960).

147. Bassett, E.G. and E.P. White.
Oestrogens and New Zealand Dairy Pastures.
New Zealand J. Sci. Technol., Sec. A., 36(5): 485-492 (1955).

148. Browning, C.B., Fountaine, F.C., Marion, G.B., and F.W. Atkeson.
Milk Response from Feeding Diethylstilbestrol to Identical-twin Cows during Complete Lactations.
J. Dairy Sci., 40: 1590-1598 (1957).

149. Belak, S. and J. Szathmary.
Die Wirkung der Wassen Bohne auf den Oestrus der Mus. I.
Biochem. Z., 291: 259-262 (1937).

150. Alder, J.H.
Anti-oestrogenic Activity in Alfalfa.
Vet. Record, 74: 1148-1150 (1962).

151. Ostrovsky, D. and W.D. Kitts.
The Effect of Estrogenic Plant Extracts on the Uterus of the Laboratory Rat.
Can. J. Animal Sci., 43: 106-112 (1963).

152. Macdonald, M.A.
Pine Needle Abortion in Range Beef Cattle.
J. Range Management, 5: 150-155 (1952).

153. Allen, M.R. and W.D. Kitts.
The Effects of Yellow Pine (Pinus ponderosa Laws) Needles on the Reproductivity of the Laboratory Female Mouse.
Can. J. Animal Sci., 41: 1-8 (1961).

154. Cook, H. and W.D. Kitts.
Anti-oestrogenic Activity in Yellow Pine Needles (Pinus ponderosa).
Acta Endocrinologica, 45: 33-39 (1964).

155. Hisaw, F.L., Velardo, J.T., and C.M. Goolsby.
Interaction of Estrogens on Uterine Growth.
J. Clin. Endocrinol. Metab., 14: 1134-1143 (1954).

156. Velardo, J.T. and S.H. Sturgis.
Interaction of 16-epiestriol and 17β-Estradiol on Uterine Growth.
Proc. Soc. Exptl. Biol. Med., 90: 609-610 (1955).

157. Emmens, C.W. and R.I. Cox.
Dimethylstilbestrol as an Estrogen Inhibitor.
J. Endocrinol., 17: 265-271 (1958).

158. Lerner, L.J., Turkheimer, A.R., and A. Borman.
Phloretin, a Weak Estrogen and Estrogen Antagonist.
Proc. Soc. Exptl. Biol. Med., 114: 115-117 (1963).

159. Nilsson, A.
On the In Vitro Metabolism of the Plant Estrogen Biochanin A in Rumen Fluid.
Arkiv för Kemi, 17: 305-310 (1961).

160. Nilsson, A.
Demethylation of the Plant Oestrogen Biochanin A in the Rat.
Nature, 192: 358 (1961).

161. Mason, H.S.
Mechanisms in Oxygen Metabolism.
Science, 125: 1185-1188 (1957).

162. Brodie, B.B.
"Enzymes and Drug Action".
Ciba Foundation Symposium, J. & A. Churchill, Ltd., (London), 1962, p. 137.

163. Nilsson, A.
Fractionation of some Plant Estrogens and their Animal Excretion Metabolites on Dextran Gels.
Acta Chem. Scand., 16: 31-40 (1962).

164. Zondek, B.
Hormonal Reaction for Pregnancy with the Urine of Humans and Animals.
Klin. Wochschr., 9: 2285-2289 (1930).
Cited in Chem. Abstr., 25: 1853 (1931).

165. de Jongh, S.E., Kober, S., and E. Laqueur.
The Identity of Menformone from Urine of Pregnant Women with that from Urine of Pregnant Horses.
Biochem. Z., 240: 247-262 (1931).
Cited in Chem. Abstr., 26: 1026 (1932).

166. Nieuwenkamp, W. and S. Kober.
The Crystal Forms of Menformone. A Contribution to the Problem of the Identity of the Estrus Hormone from Urine of Women with that from Urine of Horses.
Biochem. Z., 240: 263-264 (1931).
Cited in Chem. Abstr., 26: 1026 (1932).

167. Dingemanse, E., Kober, S., Reerink, E.H., and A. van Wijk.
Absorption Spectrum of Menformone Crystals from Different Sources.
Biochem. Z., 240: 265-267 (1931).
Cited in Chem. Abstr., 26: 1026 (1932).

168. Marrian, G.F. and G.A.D. Haslewood.
Equol, a New Inactive Phenol Isolated from the Ketohydroxyoestrin Fraction of Mare's Urine.
Biochem. J., 26: 1227-1232 (1932).

169. Marrian, G.F. and D. Beall.
The Constitution of Equol.
Biochem. J., 29: 1586-1589 (1935).

170. Wessely, F., Hirshel, H., Schlögl-Petziwal, G., and F. Prillinger.
Constitution of Equol.
Monatsh. Chem., 71: 215-228 (1938).
Cited in Chem. Abstr., 32: 3414 (1938).

171. Wessely, F. and F. Prillinger.
Constitution of Equol.
Ber. 72B: 629-633 (1939).
Cited in Chem. Abstr., 33: 4991 (1939).

172. Anderson, E.L. and G.F. Marrian.
The Identification of Equol as 7-Hydroxy-3-(4'-hydroxyphenyl) chroman, and the Synthesis of Racemic Equol Methyl Ether.
J. Biol. Chem., 127: 649-656 (1939).

173. Dirscherl, W. and K. Schodder.
A Simple Colour Reaction of the Equol in Mare's Urine.
Z. physiol. Chem., 264: 57-63 (1940).
Cited in Chem. Abstr., 34: 4758 (1940).

174. Klyne, W. and A.A. Wright.
Steroids and other Lipides of Pregnant Goat's Urine.
Biochem. J., 66: 92-101 (1957).

175. Klyne, W. and A.A. Wright.
Steroids and other Lipids of Pregnant Cow's Urine.
J. Endocrinol., 18: 32-45 (1959).

176. MacRae, H.F., Dale, D.G., and R.H. Common.
Formation In Vivo of 16-Epiestriol and 16-Ketoestradiol-17β from Estriol by the Laying Hen and Occurrence of Equol in Hen's Urine and Feces.
Can. J. Biochem. Physiol., 38: 523-532 (1960).

177. Common, R.H. and L. Ainsworth.
Identification of Equol in the Urine of the Domestic Fowl.
Biochim. Biophys. Acta, 53: 403-404 (1961).

178. Davidson, J.D. and P. Feigelson.
Practical Aspects of Internal-sample Liquid-scintillation Counting.
Int. J. Appl. Radiation & Isotopes, 2: 1-18 (1957).

179. Bell, C. and F.N. Hayes (Eds.).
"Liquid Scintillation Counting".
Pergamon Press, 1958.

180. Daub, G.H., Hayes, F.N., and E. Sullivan (Eds.).
"Proceedings of the University of New Mexico Conference on Organic Scintillation Detectors".
U.S. Atomic Energy Commission Document, TID 7612, 1960.

181. Rothchild, S. (Ed.).
"Advances in Tracer Methodology".
Plenum Press, New York, 1963.

182. Schram, E.
"Organic Scintillation Detectors".
Elsevier, New York, 1963.

183. Rapkin, E.
Liquid Scintillation Counting 1957-1963: A Review.
Int. J. Appl. Radiation & Isotopes, 15: 69-87 (1964).

184. Ludwig, H., Potter, V.R., Heidelberger, C., and C.H. de Verdier.
Automatic Direct Quantitation of Radioactivity on Paper Chromatograms.
Biochim. Biophys. Acta, 37: 525-527 (1960).

185. Roucayrol, J.C., Oberhousen, E., and R. Schussler.
Liquid Scintillators in Filter Paper - a New Detector.
Nucleonics, 15(11): 104-108 (1957).

186. Seliger, H.H. and B.W. Agranoff.
Solid Scintillation Counting of Hydrogen-3 and Carbon-14 in Paper Chromatograms.
Anal. Chem., 31: 1607-1608 (1959).

187. Wang, C.H. and D.E. Jones.
Liquid Scintillation Counting of Paper Chromatograms.
Biochem. Biophys. Res. Comm., 1: 203-205 (1959).

188. Geiger, J.W. and L.D. Wright.
Liquid Scintillation Counting of Radioautograms.
Biochem. Biophys. Res. Comm., 2: 282-283 (1960).

189. Bousquet, W.F. and J.E. Christian.
Quantitative Radioassay of Paper Chromatograms by Liquid Scintillation Counting. Application to C-14-Labeled Salicylic Acid.
Anal. Chem., 32: 722-723 (1960).

190. Loftfield, R.B.
Scintillation Counting of C^{14} Labeled Paper Chromatograms.
Atomlight No. 13: 1-5 (1960).

191. Davidson, J.D.
Some Recent Developments in Liquid Scintillation Counting of Biochemical Samples.
"Proceedings of the University of New Mexico Conference on Organic Scintillation Detectors".
U.S. Atomic Energy Commission Document, TID 7612, 1960, pp. 232-238.

192. Ekins, R.P.
Fifth Annual Symposium on Advances in Tracer Methodology, Washington, D.C., October, 1961.

193. Blair, A. and S. Segal.
Isolation of Blood Glucose as Potassium Gluconate.
J. Lab. Clin. Med., 55: 959-964 (1960).

194. Mans, R.J. and G.D. Novelli.
Measurement of the Incorporation of Radioactive Amino Acids into Protein by a Filter-paper Disk Method.
Arch. Biochem. Biophys., 94: 48-53 (1961).

195. Blair, A. and S. Segal.
Use of Filter Paper Mounting for Determination of the Specific Activity of Gluconate-C^{14} by Liquid Scintillation Assay.
Anal. Biochem., 3: 221-229 (1962).

196. Chiriboga, J.
Radiometric Analysis of Metals by using Chelates Labeled with Carbon-14 and Liquid Scintillation Counting Procedures.
Anal. Chem., 34: 1843 (1962).

197. Willenbrink, J.
On the Quantitative Assay of Radiochromatograms by Liquid Scintillation Counting.
Int. J. Appl. Radiation & Isotopes, 14: 237-238 (1963).

198. Davidson, E.A.
Techniques for Paper Strip Counting in a Scintillation Spectrometer.
Packard Technical Bulletin No. 4: 1-10 (1962).

199. Osinski, P.A.
Detection and Determination of Tritium-labeled Compounds on Paper Chromatograms.
Int. J. Appl. Radiation & Isotopes, 7: 306-310 (1960).

200. Wilson, A.T.
Detection of Tritium on Paper Chromatograms.
Biochim. Biophys. Acta, 40: 522-526 (1960).

201. Langham, W.H., Eversole, W.J., Hayes, F.N., and T.T. Trujillo.
Assay of Tritium Activity in Body Fluids with Use of a Liquid Scintillation System.
J. Lab. Clin. Med., 47: 819-825 (1956).

202. Werbin, H., Chaikoff, I.L., and M.R. Imada.
Rapid Sensitive Method for Determining H^3-water in Body Fluids by Liquid Scintillation Spectrometry.
Proc. Soc. Exp. Biol. Med., 102: 8-12 (1959).

203. Herberg, R.J.
Determination of Carbon-14 and Tritium in Blood and Other Whole Tissues.
Anal. Chem., 32: 42-46 (1960).

204. Kerr, V.N., Hayes, F.N., and D.G. Ott.
Liquid Scintillators. III. The Quenching of Liquid Scintillator Solutions by Organic Compounds.
Int. J. Appl. Radiation & Isotopes, 1: 284-288 (1957).

205. Peng, C.T.
Quenching of Fluorescence in Liquid Scintillation Counting of Labeled Organic Compounds.
Anal. Chem., 32: 1292-1296 (1960).

206. Baillie, L.A.
Determination of Liquid Scintillation Counting Efficiency by Pulse-height Shift.
Int. J. Appl. Radiation & Isotopes, 8: 1-7 (1960).

207. Toporek, M.
Liquid Scintillation Counting of C^{14}-labeled Plasma Proteins using a Standard Quenching Curve.
Int. J. Appl. Radiation & Isotopes, 8: 229-230 (1960).

208. Bruno, G.A. and J.E. Christian.
Determination of Carbon-14 in Aqueous Bicarbonate Solutions by Liquid Scintillation-counting Techniques. Application to Biological Fluids.
Anal. Chem., 33: 1216-1218 (1961).

209. Packard, L.E.
"Liquid Scintillation Counting".
C. Bell and F.N. Hayes, Eds., Pergamon Press, 1958, p. 50.

210. Takahashi, H., Hattori, T., and B. Maruo.
Liquid Scintillation Counting of C^{14} Paper Chromatograms.
Anal. Biochem., 2: 447-462 (1961).

211. Glenderin, L.E.
Determination of the Energy of β-Particles and Photons by Absorption.
Nucleonics, 2(1): 12-32 (1948).

212. Hollander, J.M., Perlman, I., and G.T. Seaborg.
Table of Isotopes.
Rev. Mod. Physics, 25: 469-651 (1953).

213. Popov, M.M., Gagarinskii, Y.V., Senin, M.D., Mikhalenko, I.P., and Y.M. Morozov.
Average β-Particle Energy and Decay Constant of Tritium.
Soviet J. Atomic Energy, 4: 393-396 (1958).
Cited in Chem. Abstr., 53: 12873 (1959).

214. Jones, W.M.
Half-Life of Tritium.
Phys. Rev., 100: 124-125 (1955).

215. Evans, E.A. and F.G. Stanford.
Decomposition of Tritium-labelled Organic Compounds.
Nature, 197: 551-555 (1963).

216. Wilzbach, K.E.
Tritium-labeling by Exposure of Organic Compounds to Tritium Gas.
J. Amer. Chem. Soc., 79: 1013 (1957).

217. Steinberg, D., Vaughan, M., Anfinsen, C.B., and J. Gorry.
Preparation of Tritiated Proteins by the Wilzbach Method.
Science, 126: 447-448 (1957).

218. Jellinck, P.H. and D.G. Smyth.
Molecular Changes in Exchange Labelling with Tritium.
Nature, 182: 46 (1958).

219. Gordon, M.P., Intrieri, O.M., and G.B. Brown.
Nucleosides Labeled with Tritium in the Ribosyl Group.
J. Amer. Chem. Soc., 80: 5161-5164 (1958).

220. Jones, E.P., Mason, L.H., Dutton, H.J., and R.F. Nystrom.
Labeling Fatty Acids by Exposure to Tritium Gas.
II. Methyl Oleate and Linoleate.
J. Org. Chem., 25: 1413-1417 (1960).

221. Misra, A.L., and L.A. Woods.
Preparation of Radiochemically Pure Tritium Nuclear-labelled Morphine.
Nature, 185: 304-305 (1960).

222. Ahrens, R.W., Sauer Jr., M.C., and J.E. Willard.
Hydrogen Labelling of Hydrocarbons using Ionizing Radiation.
J. Amer. Chem. Soc., 79: 3285-3286 (1957).

223. Wilzbach, K.E. and P. Riesz.
Isotope Effects in Gas-Liquid Chromatography.
Science, 126: 748-749 (1957).

224. Dorfman, L.M. and K.E. Wilzbach.
Tritium Labeling of Organic Compounds by means of Electric Discharge.
J. Phys. Chem., 63: 799-801 (1959).

225. Cacace, F., Guarino, A., and G. Montefinale.
Labelling of Organic Compounds by Mercury-photosensitized Reaction with Tritium Gas.
Nature, 189: 54-55 (1961).

226. Whisman, M.L.
Preparation of Tritium-labeled 1-Hexene and 1-Octene.
Anal. Chem., 33: 1284-1285 (1961).

227. Rosenblum, C.
Chemistry and Application of Tritium Labeling.
Nucleonics, 17(12): 80-83 (1959).

228. Crane, R.K., Drysdale, G.R., and K.H. Hawkins.
Some Experience with Labeling Sugars by Tritium Gas Exposure.
Atomlight No. 15: 4-6 (1960).

229. Nilsson, A.
The Quantitative Determination of Biochanin A in Red Clover Samples by means of an Isotope Dilution Method.
Arkiv för Kemi, 21: 87-91 (1963).

230. Dutton, H.J., Jones, E.P., Mason, L.H., and R.F. Nystrom.
The Labelling of Fatty Acids by Exposure to Tritium Gas.
Chem. & Ind., 1176-1177 (1958).

231. Rajam, P.C. and A. Jackson.
In Vitro Labelling of Antibody Globulin by Tritium Exchange.
Nature, 184: 375 (1959).

232. Cameron, G.G., Grassie, N., and S.J. Thomson.
Incorporation of Tritium into Trans-stilbene by the Gas Exposure Method.
J. Chem. Soc., 1411-1412 (1960).

233. Jackson, F.L., Kittinger, G.W., and F.P. Krause.
Efficient Tritium Labeling with an Electric Discharge.
Nucleonics, 18(8): 102-105 (1960).

234. Hayes, F.N.
Solutes and Solvents for Liquid Scintillation Counting.
Packard Technical Bull. No. 1: 1-7 (1963).

235. Horrocks, D.L.
Liquid Scintillation Counting of Inorganic Radioactive Nuclides.
Packard Technical Bull. No. 2: 1-8 (1962).

236. Rapkin, E.
Liquid Scintillation Measurement of Radioactivity in Heterogeneous Systems.
Packard Technical Bull. No. 5: 1-7 (1963).

237. Bennett, C.A. and N.L. Franklin.
"Statistical Analysis in Chemistry and the Chemical Industry".
John Wiley & Sons, Inc., New York, N.Y., 1954, pp. 196-200, 319-330.

238. Yoder, L., Cheng, E., and W. Burroughs.
Synthesis of Estrogenic Isoflavone Derivatives.
Proc. Iowa Acad. Sci., 61: 271-277 (1954).

239. Nilsson, A., Gronowitz, S., and R. Ekman.
Synthetic Estrogenic Isoflavonoids. II. The Synthesis of $C^{14}_{(4)}$-Labelled Biochanin A.
Arkiv för Kemi, 17: 179-182 (1961).

240. Bauld, W.S.
Some Errors in the Colorimetric Estimation of Oestriol, Oestrone and Oestradiol by the Kober Reaction.
Biochem. J., 56: 426-434 (1954).

241. Block, R.J. and D. Bolling.
"The Amino Acid Composition of Proteins and Foods".
Charles C. Thomas, Springfield, Illinois, U.S.A., 1951, p. 51.

242. Richardson, C.E., Watts, A.B., Wilkinson, W.S., and J.M. Dixon.
Techniques used in Metabolism Studies with Surgically Modified Hens.
Poultry Sci., 39: 432-440 (1960).

243. Dixon, J.M. and W.S. Wilkinson.
Surgical Technique for the Exteriorization of the Ureters of the Chicken.
Amer. J. Vet. Res., 18: 665-667 (1957).

244. Layne, D.S. and G.F. Marrian.
The Isolation of 16 β-Hydroxyoestrone and 16-Oxooestradiol-17β from the Urine of Pregnant Women.
Biochem. J., 70: 244-248 (1958).

245. Smith, I.
"Chromatographic and Electrophoretic Techniques".
Heinemann, London, England, Vol. 1, 1960, p. 292.

246. Ibrahim, R.K. and G.H.N. Towers.
The Identification, by Chromatography, of Plant Phenolic Acids.
Arch. Biochem. Biophys., 87: 125-128 (1960).

247. Wong, E. and A.O. Taylor.
The Chromatography of Flavonoid Aglycones in the Solvent System Benzene - Acetic Acid - Water.
J. Chromatog., 9: 449-454 (1962).

248. MacKenzie, R.E.
The Application of Thin Layer Chromatography to the Separation of Plant Isoflavones.
Unpublished Results, Macdonald College, Quebec, April, 1963.

249. Stahl, E.
Dünnschicht-chromatographie in der Pharmazie.
Pharmaz. Rundschau, 1(No. 2): 1-6 (1959).

250. Flood, C., Layne, D.S., Ramcharan, S., Rossipal, E., Tait, J.F., and S.A.S. Tait.
The Urinary Metabolites and Secretion Rates of Aldosterone and Cortisol in Man. Methods of Measurement.
Acta Endocrinol., 36: 237-264 (1961).

251. Bauld, W.S.
A Method for the Determination of Oestriol, Oestrone, and Oestradiol-17β in Human Urine by Partition Chromatography and Colorimetric Estimation.
Biochem. J., 63: 488-495 (1956).

252. Cayen, M.N., Carter, A.L., and R.H. Common.
The Conversion of Genistein to Equol in the Fowl.
Biochim. Biophys. Acta, 86: 56-64 (1964).

253. Grisebach, H. and W. Barz.
The Biosynthesis of Coumestrol in Lucerne.
Int. Union Biochem., 32: 442 (1964).

254. Hertelendy, F.
Personal Communication, Macdonald College, Quebec, 1963.

255. Cayen, M.N. and R.H. Common.
An Investigation of Urinary Conversion Products of Coumestrol in the Fowl.
Biochim. Biophys. Acta, (in press).

256. Simpson, T.H. and L. Gordon.
Chelate Systems. Part I.
J. Chem. Soc., 4638-4644 (1952).

257. Shaw, B.L. and T.H. Simpson.
Chelate Systems. Part II.
J. Chem. Soc., 5027-5032 (1952).