THE PHYSIOLOGICAL ACTIVITY OF SOME PLANT ESTROGENS

by

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INTRODUCTION

In 1926 Loewe, Dohrn et al. and Fullner (14) demonstrated the presence of substances in plants capable of causing estrus in ovariectomized mice. Since then many plant extracts have been examined and a large number of these have been reported to show some estrogenic activity. Among the earlier workers who isolated and characterized compounds from extracts which showed estrogenic activity are Butenandt and Jacobi (16) and Skarzynski (47). Butenandt and Jacobi pressed fifty kilograms of "palm kernels" (thought to be Elaeis guineensis) and examined the remaining solid extract. From this they obtained eighteen milligrams of estrone which they thoroughly characterized by melting point, optical rotation, ultraviolet absorption, and preparations of the benzoate and semicarbazone derivatives, which were compared with authentic samples. Its biological activity was found to equal that of estrone isolated from urine.

Skarzynski (47) using sixty-five kilograms of flowers from willow catkins, obtained 7.5 milligrams of a compound thought to be estriol. It resembled estriol in crystalline appearance, solubility, ultraviolet absorption and melting point. However, it showed only one-fourth of the biological activity of estriol prepared from urine. Bradbury and White (14) state in their review article of plant estrogens that this difference may not be significant considering the limited accuracy of the Allen-Doisy technique at that time.

The work of Schoeller et al. (45), following earlier reports of estrogenic activity in Butea superba, resulted in the isolation of a compound whose activity was about midway between that of estrone and estradiol. This same compound was chemically characterized by Butenandt (15) and is particularly significant because it was the first time a compound of plant origin, shown to have estrogenic properties, was not identical with that of the normal steroid estrogens of the animal kingdom.

In the early 1940's Bennett's discovery of the reproductive disturbances caused by a well-adapted, high-yielding pasture plant of commercial importance gave new impetus to research on plant estrogens. He found that sheep raised on the Dwalganup strain of subterranean clover (Trifolium subterraneum L.) in Western Australia resulted in infertility, dystocia, prolapse of the uterus and difficult lambing. Wethers grazing similar pastures showed mammary development and sometimes milk secretion (5).

Two additional adverse effects attributed to plant estrogens have been observed in pigs following ingestion of mouldy rye. Also an investigation of a syndrome, associated with infertility in cattle, is thought to be due to estrogens in alfalfa (1).

By far the greatest problems arise during the reproduction of sheep, because of the nature of the estrus cycle and the low estrogen to progesterone ratio. Following Bennett's discovery of breeding problems caused by subterranean clover, similar reports have occurred in the United States (44, 28, 39, 30). With the increasing reports of these breeding problems the need arose for an adequate assay to

determine the estrogenisity of forage crops. According to Bickoff (8), although several chemical (21, 34) and biological (4, 19, 38) methods for the assay of these substances have been developed, there is still need for faster and more accurate procedures. It is hoped that the present study will serve as a basis for further development in this area. Curnow, Robinson and Underwood (22) developed the first assay. Although it has been modified somewhat, it has remained the most popular assay. It is based on uterine growth over a three-day period. Increasing amounts of extract, or when available the pure compound, is fed to immature mice. The uterus is then removed, weighed, and a dose response curve constructed. This is then compared to known quantities of natural estrogens and the relative activity determined.

The advent of an assay resulted in the detection of estrogenic properties in more than fifty species of plants. Besides the two steroids previously mentioned the important compounds showing estrogenic properties, thus far isolated, are the isoflavones: genistein (50), genistin (50), daidzein (40), biochanin-A (41), and formononetin (13); and a coumarin, coumestrol (7). Daidzein was isolated from soybean meal and has not been isolated from forage crops. The other plant estrogens have been found in such economically important crops as red clover, subterranean clover, ladino clover, alfalfa, and many others.

An interesting problem is concerned with correlating the occurrence of these compounds with the family of plants in which they have been discovered. Although most of the clovers tested show

estrogenicity, white clover has never shown any estrogenic properties. Recently Stob, Davis and Andrews (48) studied fifty-six different strains of alfalfa. Using immature mice they obtained uterine weights ranging from 17.00 mg to 99.96 mg. It may be concluded that no definite correlations have been established between the occurrence of estrogenic activity and the plant in which it is found.

Of even more fundamental importance is the determination of the physiological action of these compounds in the plant. The work of Bradbury and White (14) has been important in contributing to our knowledge in this field of study. By systematic rearrangement of the molecules they have been able to discover the reactive groups with respect to their action in animals.

For the most part, biological research on plant estrogens has been of an applied nature and very little basic research has been conducted to determine how these compounds cause reproductive disturbances. It has been generally concluded that these compounds act as estrogens. However, with the number of different estrogens reported, to date, and the manifold actions and interactions of these compounds, a more thorough knowledge of their physiological characteristics seems necessary. In this investigation the ability of both genistin and coumestrol to promote imbibition of fluid in the uterus and growth of the uterus were studied. The greater capacity of one estrogen over another in bringing about uterine fluid imbibition or uterine growth has been shown by Hisaw (33). It was hoped that by determining these characteristics of genistin and coumestrol a comparison could be made

to the natural-occurring estrogens. With this established it was further hoped that such data might contribute to their mode of action in reproductive disturbances.

Limited investigations have also been made into the competitive effects of genistin and coumestrol on estradiol-17B sensitized uteri. The end point in these studies was the imbibition of fluid in the uterus. Due to the limitation of both time and the supply of rats the influence these compounds may exert in altering the ability of estradiol-17 B to stimulate growth of the uterus was not undertaken. A most interesting and fruitful study of the interactions of these compounds both to each other and on the natural-occurring estrogens seems forthcoming.

METHODS AND MATERIALS

In the present investigation of the effectiveness of genistin and coumestrol to stimulate fluid imbibition and uterine growth the Wistar strain of rats was used. The rats were obtained from the laboratory of Dr. A. J. Wood at the University of British Columbia. The animals used in studying genistin were shipped by Railway Express, whereas, those used in the study of coumestrol acetate were shipped by air. In order to eliminate possible adverse effects due to dehydration brought about by the distance they traveled, care was taken to allow the animals sufficient time to rehydrate. As vegetables were always included in the shipping crates the animals were never excessively dehydrated.

The genistin used in this study was prepared by W. D. Noteboom. It was shown by ultraviolet absorption to be ninety-eight per cent pure when compared to genistin which was prepared at the Agriculture Experiment Station in Albany, California. This same laboratory, which provided the genistin standard, provided the coumestrol acetate. Inasmuch as the acetate form of this compound was used, the results presented are slightly lower than those which could be expected from pure coumestrol. Bickoff (6) states that "acetylation did not result in any marked loss of estrogenic activity." In the same publication he compares the biological activity of thirty compounds related to coumestrol. When coumestrol acetate is compared to the estrogenic activity of coumestrol (arbitrarily assigned a value of 1000) its relative activity is 850. Considering the size of the two molecules

this difference may be attributed to the molecular weight of coumestrol acetate. For purposes of convenience the compound used in this study, coumestrol acetate, will be referred to as coumestrol.

The procedure for this investigation is similar to that described by Astwood (3). The increase in uterine wet weight caused by a single injection of estrogen when plotted against time results in two peaks. The first peak is due to the imbibition of water, while the second peak is due to true uterine growth. Earlier workers using nitrogen determinations showed conclusively that the second peak was the result of growth.

In determining the ability of these plant estrogens to stimulate fluid imbibition and uterine growth, a single subcutaneous injection of various concentrations of genistin, or coumestrol was made. The concentrations used were prepared by serial dilution and unless otherwise noted they were delivered in 0.1 cc of propylene glycol (1,2-propane -diol). Some dosages were administered in a 0.2 cc aliquot because of the difficulty of getting the compound into solution at higher concentrations. The effect of each concentration was determined by using nine or more (usually ten) rats. They were approximately twenty-one days old and weighed forty to fifty grams each.

To determine the potential strength of these compounds to stimulate fluid imbibition, the animals were sacrificed at either four- or six-hour intervals following a single injection of 0.025 µg to 100 or more µg of the compound in question. A dose response histogram was constructed including both intervals of time. In studying uterine

growth, previously associated with these compounds, a single concentration, fifty micrograms, was injected. Then intervals of from four to seventy-two hours were allowed between treatment and autopsy. The results were graphed by plotting the uterine wet weight against time.

At the prescribed time following treatment the animals were killed by cervical dislocation. The uterus was removed by cutting across the uterine cervix, stripping away the mesentaries, then freeing the uterine horns by cutting on the ovarian side of the tubal sphinctor. After removal from the animal it was immediately weighed on a Roller-Smith balance, placed in a tared aluminum pan and dried for twenty-four hours in an oven ranging in temperature from 100 to 115 degrees centigrade. From the dry weights, the percentage of water was calculated. The average variation (standard error) occurring in the dry weight, wet weight, percentage water, and body weight was computed by the following formula:

$$\text{Standard Error} = \sqrt{\frac{Ex^2 - \frac{(Ex)^2}{n}}{\frac{n-1}{n}}}$$

RESULTS

The present investigation of two of the physiological properties of genistin and coumestrol acetate has resulted in further establishing their action as estrogenic. Although they had been shown to induce cornification of the vaginal epithelium, stimulate growth of the uterus and cause certain anomalies characteristic of estrogens, little else is known about the physiology of these compounds. Previous work has shown that the natural occurring steroidal estrogens vary markedly in their ability to stimulate fluid imbibition and growth (33). It was hoped in the present study that some foundation for more detailed studies into the physiology, biochemistry and enzymology might be established by the detailed characterization of genistin and coumestrol acetate with respect to these same two properties.

UTERINE FLUID IMBIBITION

The action of estrogens on the uterus is responsible for a rapid dilation of the blood vessels and a disturbance in the electrolyte balance in the endometrial cells. The hyperemic condition in phase with the altered electrolyte balance results in an increase in the fluids in the intracellular spaces, and as the response becomes maximal, deposition of fluids occurs in the lumen. This response has served as an assay for estrogens since the initial work of Astwood (3) and has been employed in the present study of genistin and coumestrol acetate.

The assay previously used to evaluate the estrogenicity of plants

was established by Curnow et al. (22). This was based on the increased uterine wet weights resulting from forage or extracts of forage introduced into a purified diet. An assay of this type was used because of the difficulty of injecting extracts, sometimes quite concentrated, and because of the difficulty of obtaining purified compounds. Little is known of the possible changes which may occur in the digestive tract. It was felt that a more direct comparison to the natural-occurring steroidal estrogens could be obtained by using the Astwood assay. This assay involves obtaining a dose response curve at six hours for the compound in question. The amount of estrogen which will produce a thirty-three per cent increase in the average uterine wet weight of a group of rats is the resulting Astwood Rat Unit.

Genistin

It has been noted in the present investigation that not only is genistin a poor promoter of growth (14), but also, that it is only a weak stimulator of fluid imbibition (Tables I and II, Figure 1). The maximum weight produced by genistin was 23.9 mg. (Table I). This was a twenty-eight per cent increase which occurred at only four hours and with a dosage of 500 ug. The maximum increase that occurred at six hours was twenty-three per cent. This percentage increase was obtained with a dosage of two hundred ug (Table II). As it was impossible to determine an Astwood unit for this compound, no further effort was made to quantitate the relationship between genistin and the other known estrogens.

TABLE I

GENISTIN FOUR-HOUR DOSE RESPONSE

Dosage in micrograms	No.	Body Wt	Uterine Wet Wt	Dry Wt	% Water
Non-injected control	45	43.3 ±0.3	18.7 ±0.3	3.3 ±0.1	82.4 ±0.3
Propylene glycol control	10	43.9 ±0.9	19.9 ±0.8	3.6 ±0.3	81.8 ±1.0
0.025	9	44.4 ±1.1	19.3 ±0.2	3.4 ±0.03	82.5 ±0.1
0.05	9	44.7 ±1.1	19.5 ±0.2	3.2 ±0.03	83.4 ±0.1
0.1	9	45.8 ±0.9	18.9 ±0.2	3.6 ±0.02	80.9 ±0.2
0.312	10	42.8 ±0.7	18.0 ±0.1	3.1 ±0.04	82.9 ±0.2
0.625	10	45.9 ±0.8	16.5 ±0.1	3.1 ±0.02	81.5 ±0.1
0.25	9	43.7 ±1.0	18.4 ±0.2	3.4 ±0.04	81.4 ±0.1
2.5	9	44.5 ±1.1	17.4 ±0.2	3.4 ±0.03	80.5 ±0.3
5	10	43.6 ±1.0	16.7 ±0.1	3.3 ±0.03	80.5 ±0.1
10	10	48.6 ±1.8	19.3 ±0.3	3.6 ±0.04	81.4 ±0.1
25	10	40.8 ±1.2	18.1 ±0.3	3.4 ±0.03	81.3 ±0.1
50	9	45.1 ±1.5	19.2 ±0.4	2.9 ±0.1	84.8 ±0.2
100	10	45.7 ±0.8	18.2 ±0.8	3.3 ±0.1	81.7 ±0.4
200	10	46.8 ±0.9	21.1 ±0.5	3.1 ±0.1	85.4 ±0.1
500	10	42.2 ±0.6	23.9 ±1.7	3.5 ±0.3	85.1 ±0.8

TABLE II

GENISTIN SIX-HOUR DOSE RESPONSE

Dosage in micrograms	No.	Body Wt	Uterine Wet Wt	Dry Wt	% Water
Non-injected control	45	43.3 ±0.3	18.7 ±0.3	3.3 ±0.1	82.4 ±0.3
Propylene glycol control	10	43.9 ±0.9	19.9 ±0.8	3.6 ±0.3	81.8 ±1.0
0.5	10	43.8 ±0.9	19.4 ±0.3	3.5 ±0.04	81.9 ±0.1
0.1	8	46.0 ±1.0	18.8 ±0.1	3.6 ±0.02	80.8 ±0.2
0.312	10	42.8 ±0.6	17.9 ±0.2	3.1 ±0.02	82.6 ±0.1
0.625	7	42.4 ±1.2	16.4 ±0.3	3.0 ±0.04	81.6 ±0.1
1.25	9	44.1 ±0.7	19.4 ±0.2	3.3 ±0.02	82.8 ±0.2
2.5	10	44.4 ±1.1	18.8 ±0.2	3.1 ±0.01	83.6 ±0.1
5	20	42.9 ±0.8	21.7 ±0.5	3.7 ±0.2	82.2 ±0.7
10	10	43.5 ±0.8	18.2 ±0.1	3.5 ±0.1	80.8 ±0.1
25	10	47.4 ±1.2	20.2 ±0.4	3.8 ±0.1	81.2 ±0.2
50	10	41.1 ±1.4	20.6 ±0.4	3.7 ±0.1	81.8 ±0.1
100	10	46.0 ±0.8	21.1 ±1.5	3.4 ±0.2	83.9 ±0.5
200	10	43.7 ±0.7	22.5 ±0.5	3.2 ±0.1	85.9 ±0.2
500	10	43.2 ±0.6	22.1 ±1.6	3.6 ±0.02	83.5 ±0.5

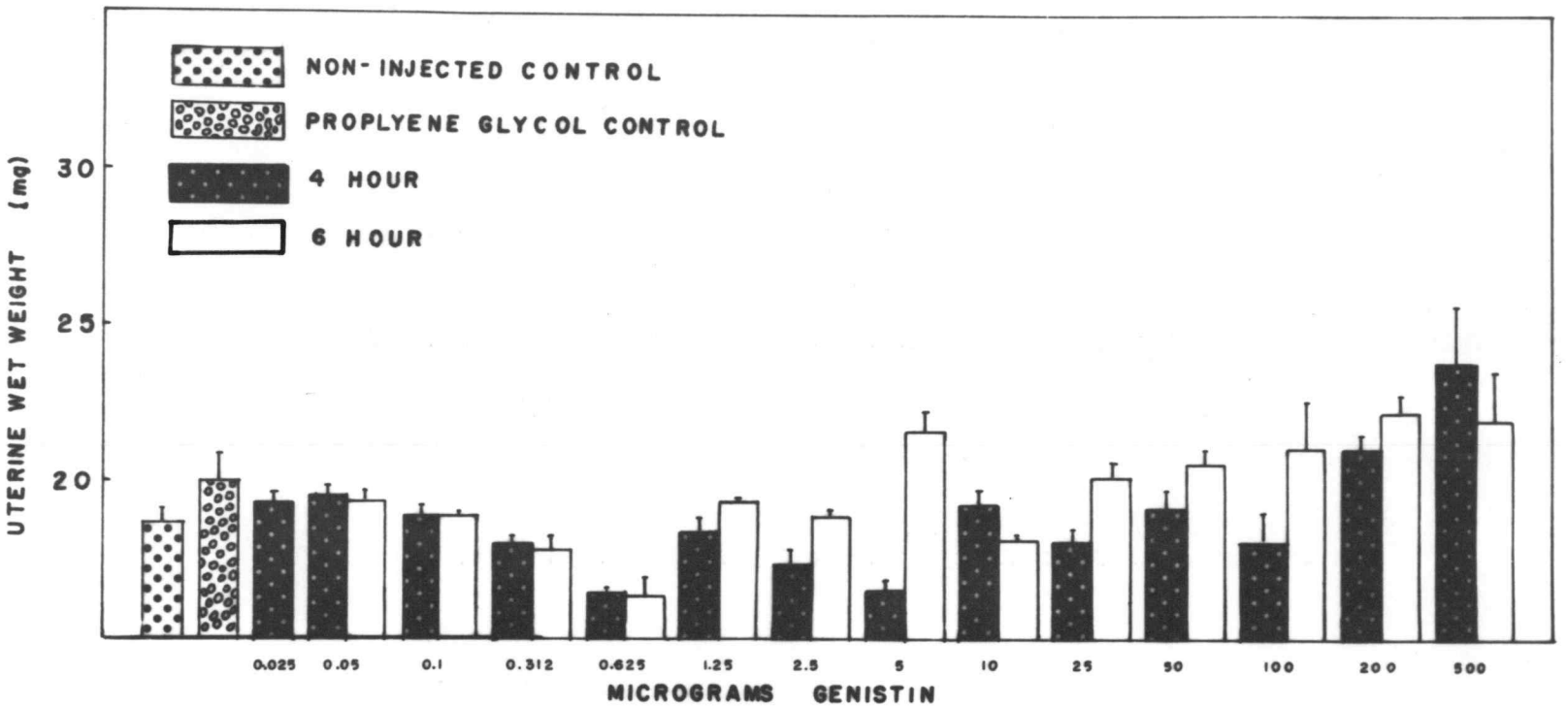

Fig. 1 Four- and Six-Hour Dose Response with Genistin

Coumestrol

Coumestrol, diethylstilbesterol and the natural-occurring steroidal estrogens can be compared on the basis of micrograms or one Astwood Rat Unit.[1] In order of estrogenic potency from strongest to weakest they are as follows: estradiol-17 B, 0.025; estriol, 0.029; diethylstilbesterol, 0.078; equilin, 0.312; equilenin, 0.468; and coumestrol, 10.

Relating the Astwood units of these compounds it is found that estradiol is 400 times stronger than coumestrol acetate. In the same manner of comparison, estriol is 348, diethylstilbesterol, 128; equilin, 32; and equilenin, 21.

UTERINE GROWTH

Growth of the uterus in rats is characterized by an increase in mitotic activity at about the fourteenth hour after a single injection of estrogen. The maximal growth induced, as indicated by uterine dry weights, may occur at varying times depending on the estrogen administered. The duration of the growth period is also variable. Hisaw (33), in correlating the two responses, states that the maximal growth with fifty micrograms was greater for long-acting estrogens, while those acting for a shorter time produced a maximal effect earlier in

[1]The Astwood units and other data concerning the natural-occurring steroidal estrogens and diethylstilbesterol used in this thesis were taken from a Ph.D. thesis presented by F. L. Hisaw Jr. to Harvard University. His thesis contained detailed tables and additional information not included in his publication so often referred to in this thesis.

TABLE III

COUMESTROL ACETATE FOUR-HOUR DOSE RESPONSE

Dosage in micrograms	No.	Body Wt	Uterine Wet Wt	Dry Wt	% Water
Non-injected control	45	43.3 ±0.3	18.7 ±0.3	3.3 ±0.1	82.4 ±0.3
Propylene glycol control	10	43.9 ±0.9	19.9 ±0.8	3.6 ±0.3	81.8 ±1.0
0.025	8	44.0 ±0.9	18.7 ±0.6	3.6 ±0.07	80.7 ±0.4
0.05	9	47.2 ±1.0	18.2 ±0.8	3.4 ±0.2	81.1 ±0.7
0.1	10	46.0 ±1.1	16.9 ±0.2	3.2 ±0.1	81.1 ±0.8
0.312	10	46.4 ±1.2	19.5 ±0.8	3.1 ±0.1	83.8 ±0.6
0.625	8	43.5 ±1.4	21.9 ±1.1	3.6 ±0.2	83.5 ±0.9
1.25	10	48.3 ±0.7	21.0 ±0.6	3.4 ±0.1	84.0 ±0.2
2.5	10	43.8 ±0.8	21.8 ±1.3	3.5 ±0.1	83.9 ±0.5
5	9	43.9 ±1.6	23.6 ±2.0	3.8 ±0.2	83.2 ±1.0
10	10	42.4 ±0.9	24.2 ±2.7	3.4 ±0.2	85.2 ±1.0
25	10	45.4 ±1.4	33.7 ±2.0	4.6 ±0.3	86.3 ±0.4
50	10	44.3 ±1.0	31.6 ±1.5	3.6 ±0.1	88.5 ±0.9
100	10	42.8 ±0.7	33.9 ±2.0	3.6 ±0.2	89.4 ±0.3

TABLE IV

COUMESTROL ACETATE SIX-HOUR DOSE RESPONSE

Dosage in micrograms	No.	Body Wt	Uterine Wet Wt	Dry Wt	% Water
Non-injected control	45	43.3 ±0.3	18.7 ±0.3	3.3 ±0.1	82.4 ±0.3
Propylene glycol control	10	43.9 ±0.9	19.9 ±0.8	3.6 ±0.3	81.8 ±1.0
0.025	10	44.5 ±1.0	18.8 ±0.9	3.4 ±0.1	81.7 ±0.5
0.05	20	42.8 ±2.4	20.5 ±0.6	3.4 ±0.1	83.2 ±0.6
0.1	19	44.9 ±0.7	20.0 ±0.7	3.6 ±0.1	81.7 ±0.4
0.312	10	44.7 ±1.0	18.7 ±0.5	2.9 ±0.1	84.7 ±0.6
0.625	10	47.4 ±1.1	20.7 ±1.3	3.4 ±0.1	83.6 ±0.4
1.25	10	47.0 ±0.5	20.7 ±1.4	3.5 ±0.3	83.2 ±0.4
2.5	19	43.7 ±1.0	18.8 ±0.6	3.1 ±0.2	82.2 ±0.5
5	20	44.4 ±0.7	21.7 ±0.8	4.2 ±0.2	80.6 ±0.7
10	10	43.5 ±1.3	26.2 ±2.0	3.9 ±0.2	84.7 ±0.8
25	10	41.7 ±1.7	30.7 ±1.9	4.2 ±0.2	86.4 ±0.8
50	19	44.3 ±0.7	30.3 ±0.9	3.6 ±0.2	88.5 ±0.3
100	10	45.7 ±0.9	30.2 ±1.2	3.7 ±0.1	87.7 ±0.7

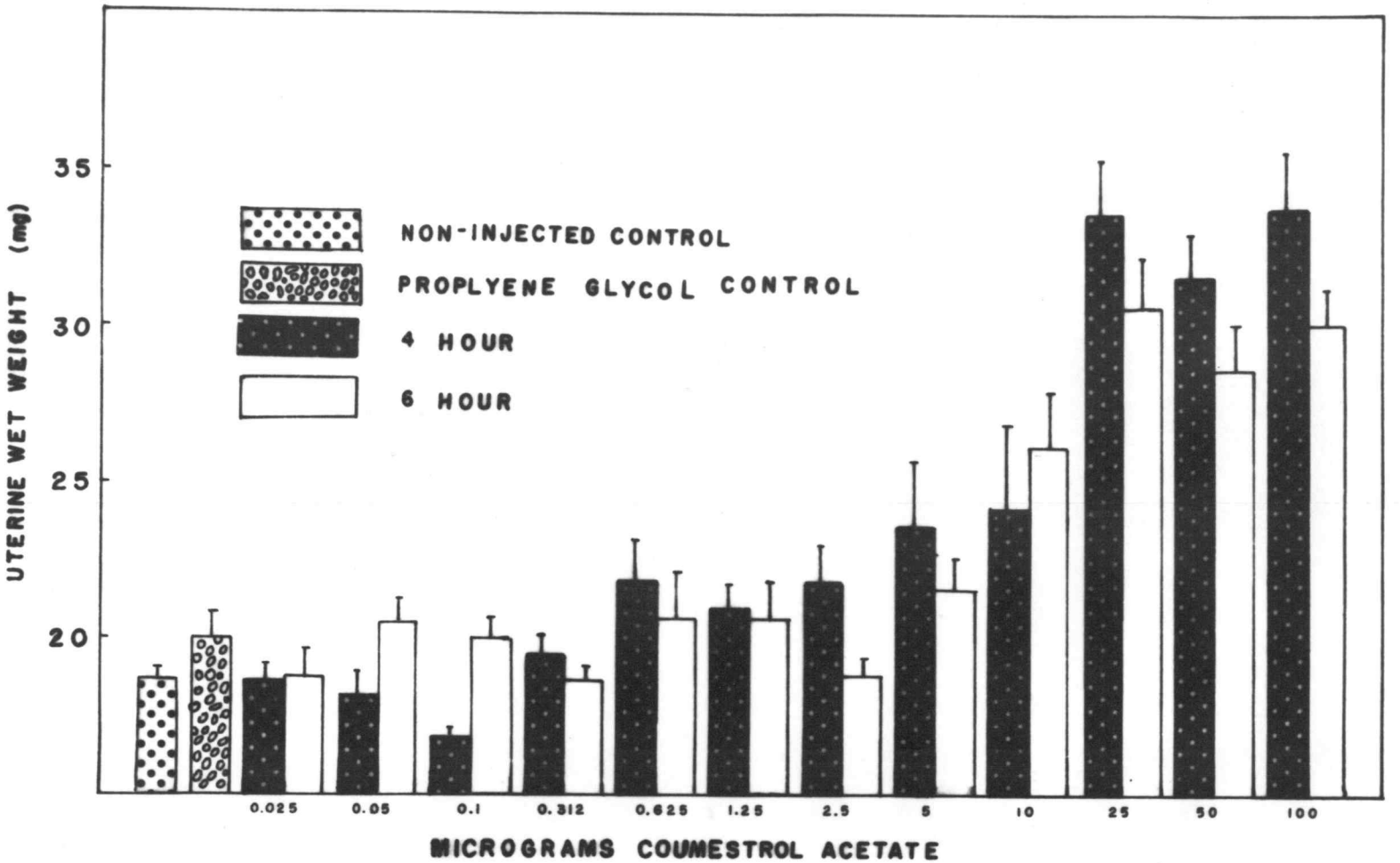

Fig. 2 Four- and Six-Hour Dose Response with Coumestrol Acetate

the growth phase with a subsequent decline that was more rapid. Although these effects may be due, in part, to solubility differences, a distinct individual difference in these steroids is present.

Genistin

An extreme increase in the ability of the natural steroidal estrogens and one synthetic estrogen (33) to stimulate growth over genistin was observed. Cheng et al. (19), using the assay he established based on uterine growth, compared genistein to diethylstilbesterol and determined genistein to be 1/50,000 as active as diethylstilbesterol. On a molecular basis he found genistin to be equal to genistein. The growth promoting property of diethylstilbesterol at 0.1 ug is sufficient to produce a seventy-two per cent increase in uterine wet weight at thirty hours (33). Thus, comparing these two authors' work, the dose of genistin required to stimulate growth would be approximately 2-5 mg. This is in accord with the work of Curnow (22), Biggers and Curnow (11), and Carter et al. (18).

Research with genistin by investigators in animal husbandry has been significant, but, at the rather high dosages examined, its physiological significance is questioned. One of the fundamental characteristics of hormones is the fact that they exist in minute quantities at physiological levels. As a result an attempt has been made to examine the action of genistin nearer physiological dosages (micrograms in contrast to milligrams).

In determining the growth-promoting properties of genistin,

levels as high as 500 micrograms were administered. As noted in Table V and Figure 3, a slight amount of growth occurred. A comparison of genistin to other estrogenic compounds can be made on the basis of maximal growth produced. Even at a dosage of genistin ten times as great (500 µg) as that of the other estrogenic compounds (50 µg), the following percentage increase in uterine wet weight over genistin occurred: coumestrol, 8.5; estrone, 64.5; equilenin, 67.3; estradiol, 73.8; equilin, 117.0; diethylstilbesterol, 368.4. Calculations of the relative potency of genistin to diethylstilbesterol and the natural occurring steroidal estrogens is found in Table VIII.

Coumestrol

Considering maximal growth responses at fifty micrograms without respect to time, the natural and synthetic estrogens produced the following percentage increases in uterine wet weight over coumestrol: estriol, 18.6; estrone, 51.6; equilenin, 54.2; estradiol-17 B, 60.1; equilin, 100; and diethylstilbesterol, 331. Calculations of the relative potency of coumestrol, as compared to what are believed to be physiological levels of the other known estrogens, may be found in Table VIII.

Noting the time at which growth occurred for coumestrol (Figure 4) a marked variation is shown among estrogens. In plotting uterine wet weight against time, following a single injection of an estrogen, the first peak associated with fluid imbibition is generally reached by the sixth to ninth hour. Following the reabsorption of fluid, a decrease in uterine wet weight occurs. The second peak is usually

TABLE V

GENISTIN 50 MICROGRAMS GROWTH RESPONSE

Hours after Injection	No.	Body Wt Start	Body Wt End	Uterine Wet Wt	Dry Wt	% Water
Non-injected control	45	43.3 ± 0.3	----	18.7 ± 0.3	3.3 ± 0.1	82.4 ± 0.3
Non-injected 15-hr control	7	42.6 ± 0.9	----	18.4 ± 1.9	3.3 ± 0.2	81.8 ± 0.4
Non-injected 40-hr control	10	44.4 ± 0.3	46.6 ± 1.3	20.9 ± 0.7	3.2 ± 0.1	84.6 ± 0.5
Non-injected 72-hr control	9	38.1 ± 1.7	47.9 ± 1.5	22.3 ± 0.4	3.9 ± 0.2	82.1 ± 0.3
4.5	9	45.1 ± 1.5	----	19.2 ± 0.4	2.9 ± 0.1	84.8 ± 0.2
6	10	41.1 ± 1.4	----	20.6 ± 0.4	3.7 ± 0.1	81.8 ± 0.1
9	9	44.5 ± 1.6	----	18.5 ± 0.3	3.5 ± 0.04	81.1 ± 0.2
30	9	44.9 ± 0.9	49.1 ± 0.9	20.0 ± 1.1	3.2 ± 0.1	83.9 ± 0.3
48	11	45.0 ± 0.8	48.0 ± 1.0	20.7 ± 0.2	3.2 ± 0.1	84.6 ± 0.2
72	9	36.6 ± 1.2	45.3 ± 1.9	19.2 ± 0.3	3.8 ± 0.1	80.2 ± 0.3

TABLE VI

GENISTIN 500 MICROGRAMS GROWTH RESPONSE

Hours after Injection	No.	Body Wt Start	Body Wt End	Uterine Wet Wt	Dry Wt	% Water
Non-injected control	45	43.3 ±0.3	----	18.7 ±0.3	3.3 ±0.1	82.4 ±0.3
Non-injected 15-hr control	7	42.6 ±0.9	----	18.4 ±1.9	3.3 ±0.2	81.8 ±0.4
Non-injected 40-hr control	10	44.4 ±0.3	46.6 ±1.3	20.9 ±0.7	3.2 ±0.1	84.6 ±0.5
Non-injected 72-hr control	9	38.1 ±1.7	47.9 ±1.5	22.3 ±0.4	3.9 ±0.2	82.1 ±0.3
3	10	52.0 ±0.6	----	19.0 ±0.3	3.6 ±0.04	80.9 ±0.1
4	10	42.2 ±0.6	----	23.9 ±1.7	3.5 ±0.3	85.1 ±0.8
6	10	43.2 ±0.6	----	22.1 ±1.6	3.6 ±0.02	83.5 ±0.5
30	10	46.6 ±0.8	47.2 ±1.0	28.2 ±0.7	4.3 ±0.1	84.5 ±0.2
48	10	44.7 ±1.0	50.6 ±1.0	24.8 ±0.8	4.1 ±0.1	83.0 ±0.2

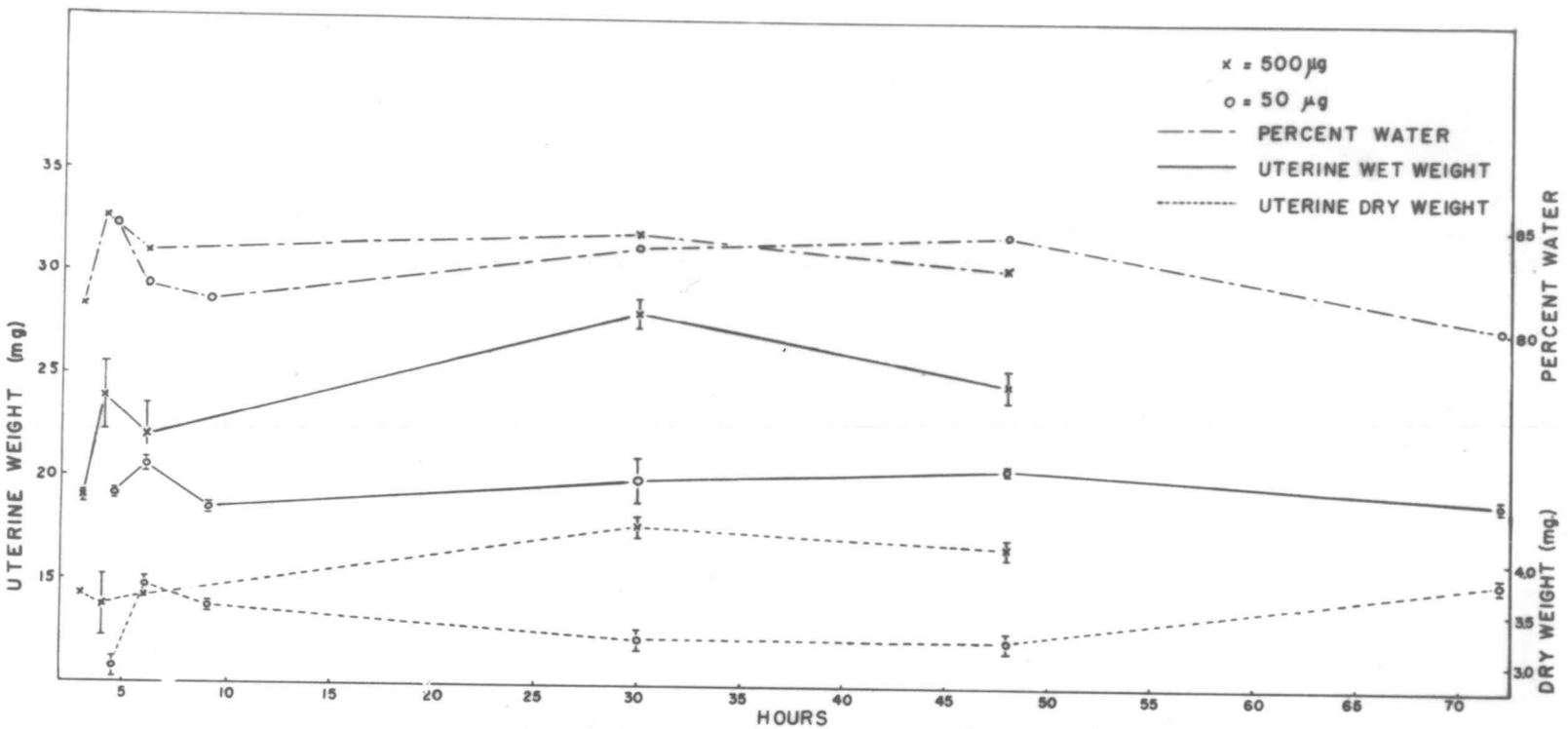

Fig. 3 Growth Response with Genistin

TABLE VII

COUMESTROL ACETATE 50 MICROGRAMS GROWTH RESPONSE*

Hours after Injection	No.	Body Wt Start	Body Wt End	Uterine Wet Wt	Dry Wt	% Water
Non-injected control	45	43.3 ± 0.3	----	18.7 ± 0.3	3.3 ± 0.1	82.4 ± 0.3
Non-injected 15-hr control	7	42.6 ± 0.9	----	18.4 ± 1.9	3.3 ± 0.2	81.8 ± 0.4
Non-injected 40-hr control	10	44.4 ± 0.3	46.6 ± 1.3	20.9 ± 0.7	3.2 ± 0.1	84.6 ± 0.5
Non-injected 72-hr control	9	38.1 ± 1.7	47.9 ± 1.5	22.2 ± 0.4	3.9 ± 0.2	82.1 ± 0.3
2	10	43.3 ± 0.8	----	20.0 ± 0.7	3.7 ± 0.1	81.6 ± 0.5
4	10	44.3 ± 1.0	----	31.6 ± 1.5	3.6 ± 0.1	88.5 ± 0.9
6	9	43.3 ± 0.9	----	28.7 ± 1.3	3.6 ± 0.4	87.6 ± 0.9
9	10	45.1 ± 0.4	----	26.2 ± 1.0	3.6 ± 0.2	86.1 ± 0.5
15	10	44.9 ± 1.0	45.8 ± 1.1	26.0 ± 0.7	4.2 ± 0.1	83.7 ± 0.2
30	10	45.6 ± 1.0	44.6 ± 1.1	25.1 ± 0.9	4.6 ± 0.2	81.6 ± 0.2
40	10	45.4 ± 0.9	45.7 ± 0.8	24.1 ± 1.1	4.0 ± 0.2	83.3 ± 0.4
48	9	45.7 ± 1.0	51.1 ± 1.3	30.6 ± 1.1	5.2 ± 0.4	83.5 ± 0.3
60	10	41.5 ± 0.5	51.7 ± 0.8	28.3 ± 2.5	5.2 ± 0.5	81.7 ± 0.6
72	10	45.2 ± 0.9	55.8 ± 1.1	28.5 ± 1.9	4.7 ± 0.03	83.3 ± 0.4

*Injected as 50 micrograms per 0.2 cc propylene glycol.

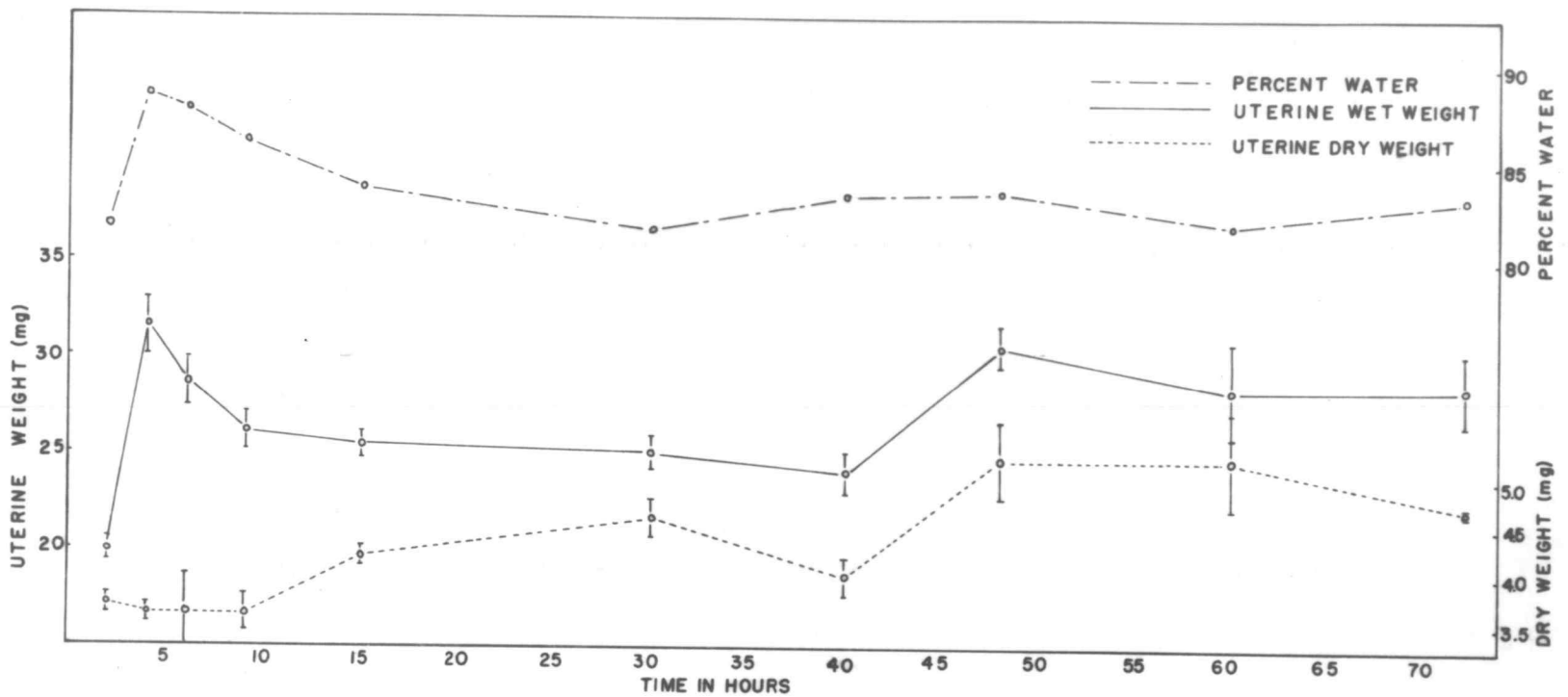

Fig. 4 Growth Response with Coumestrol Acetate

TABLE VIII

RELATIVE POTENCY OF GENISTIN AND COUMESTROL ACETATE IN STIMULATING GROWTH AS COMPARED TO OTHER ESTROGENS*

Estrogen	Dose (µg)	Max Uterine Weight (mg)	Potency of Genistin	Potency of Coumestrol Acetate
Diethyl-stilbesterol	0.1	43.8	1.29×10^{-4}	1.40×10^{-3}
Estradiol	50	60.0	4.70×10^{-2}	5.10×10^{-1}
Equilenin	1.25	25.4	2.77×10^{-3}	3.01×10^{-2}
Equilin	1.25	34.5	2.04×10^{-3}	2.22×10^{-2}
Estrone	1.25	26.4	2.67×10^{-3}	2.90×10^{-2}
Estriol	5	30.8	9.16×10^{-3}	9.94×10^{-2}
Coumestrol	50	30.6	9.22×10^{-2}	----------
Genistin	500	28.2	----------	1.09×10^{1}

*The uterine wet weights, with the exception of coumestrol acetate and genistin, used in this table were taken from a Ph.D. thesis presented to Harvard University.

culminated by the fortieth hour. Cessation of growth and involution then results in uteri that are comparable to the non-injected control.

Hisaw (33) reported that with equilenin and estrone maximal growth occurred as early as 18.5 and 24.5 hours respectively. A rather unique characteristic of the coumestrol growth curve appears to be the delay in growth. As noted in Figure 4, the reabsorption of luminal and intracellular fluid, prior to growth, did not take place until the fortieth hour. This is in contrast to not only the natural-occurring steroidal estrogens and diethylstilbesterol but also to genistin (Figure 3). This difference may be due, in part, to solubility differences, but it is the author's belief that this is not the complete answer.

The possible interactions a newly isolated compound may undergo in exerting its effect is of interest. To this end, limited studies have been conducted to ascertain a possible relationship between estradiol, and either genistin or coumestrol. Tables IX and X and Figures 5 and 6 summarize the uterine wet weight and the body weight ratios of estradiol-sensitized uteri to genistin or coumestrol. Inasmuch as a six-hour test is employed, the end effect studied is that of fluid imbibition.

ESTROGEN INTERACTIONS

Progress, to date, has shown genistin to decrease the uterine wet weight of estradiol-sensitized uteri by 7.8 per cent. This was at a dosage of 0.00625 µg. Similar low dosages (0.0125 µg and 0.025 µg) also resulted in decreased uterine wet weights, but high dosages of genistin have consistently given increases. The maximum percentage

TABLE IX

SIX-HOUR RESPONSE OF GENISTIN ON ESTRADIOL SENSITIZED UTERI

Micrograms Genistin	No.	Body Wt	Uterine Wet Wt	Dry Wt	% Water	% Body*
Control 0.05 µg estradiol	25	45.2 ± 0.6	31.4 ± 0.8	4.0 ± 0.1	87.3 ± 0.4	69.69 ± 1.87
0.00625	10	46.1 ± 0.9	29.3 ± 0.3	3.6 ± 0.04	87.6 ± 0.1	63.53 ± 2.24
0.0125	10	43.5 ± 0.8	28.8 ± 0.4	3.7 ± 0.04	87.2 ± 0.3	66.22 ± 2.01
0.025	10	44.0 ± 1.2	29.0 ± 0.3	3.7 ± 0.03	87.1 ± 0.1	66.27 ± 2.50
0.1	10	47.6 ± 0.9	34.1 ± 1.5	4.7 ± 0.3	86.2 ± 0.8	71.84 ± 3.02
1.25	10	42.9 ± 0.8	31.4 ± 0.5	3.9 ± 0.04	87.1 ± 0.1	73.28 ± 3.54
10	10	45.5 ± 1.2	36.9 ± 1.4	4.4 ± 0.2	88.1 ± 0.5	81.11 ± 3.91
50	5	46.0 ± 1.0	33.2 ± 1.6	3.8 ± 0.6	88.5 ± 0.6	72.32 ± 3.34

*% Body is equal to the uterine percentage of body weight, in grams, multiplied by 1000.

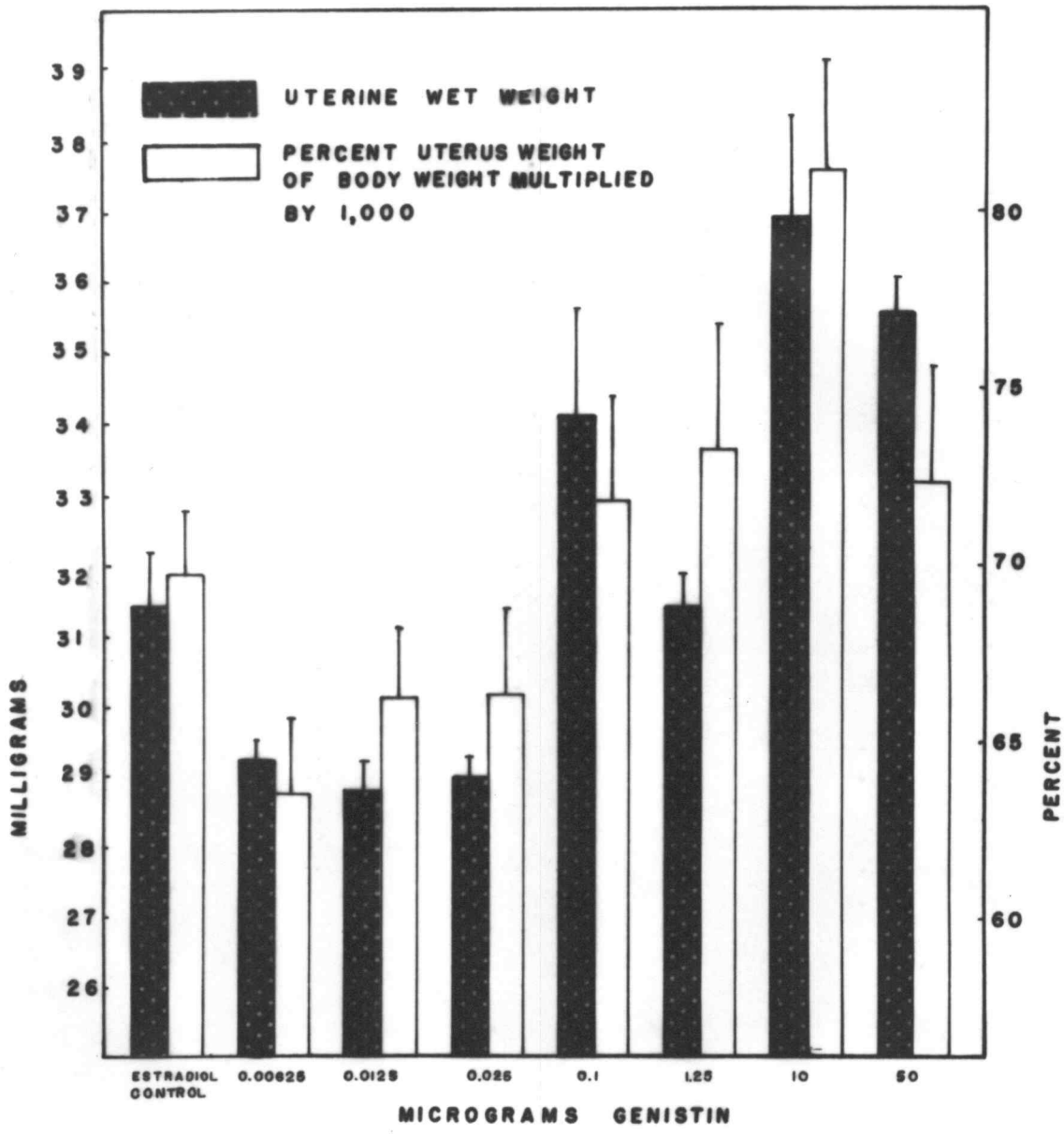

Fig. 5 Interaction of Varying Amounts of Genistin with 0.05 µg Estradiol

TABLE X

SIX-HOUR RESPONSE OF COUMESTROL ACETATE ON ESTRADIOL SENSITIZED UTERI

Micrograms Coumestrol Acetate	No.	Body Wt	Uterine Wet Wt	Dry Wt	% Water	% Body*
Control 0.05 µg estradiol	25	45.2 ± 0.6	31.4 ± 0.8	4.0 ± 0.1	87.3 ± 0.4	69.69 ± 1.87
0.00625	10	45.0 ± 0.4	35.5 ± 1.2	4.4 ± 0.02	87.6 ± 0.4	79.07 ± 2.48
0.0125	9	44.5 ± 1.0	35.2 ± 2.3	4.5 ± 0.02	86.9 ± 0.8	79.48 ± 6.05
0.025	9	44.9 ± 0.8	33.6 ± 1.1	4.0 ± 0.2	88.2 ± 0.4	74.70 ± 1.79
0.1	10	46.2 ± 1.2	36.9 ± 1.3	4.5 ± 0.2	87.7 ± 0.3	79.86 ± 2.48
1.25	10	44.2 ± 1.0	38.7 ± 1.4	5.1 ± 0.2	86.9 ± 0.6	87.92 ± 3.55
10	10	43.7 ± 0.9	31.1 ± 1.6	3.8 ± 0.2	87.7 ± 0.5	71.44 ± 1.16
50	10	44.7 ± 1.0	33.8 ± 0.8	4.5 ± 0.2	86.5 ± 0.4	76.06 ± 2.74

*% Body is equal to the uterine percentage of body weight, in grams, multiplied by 1000.

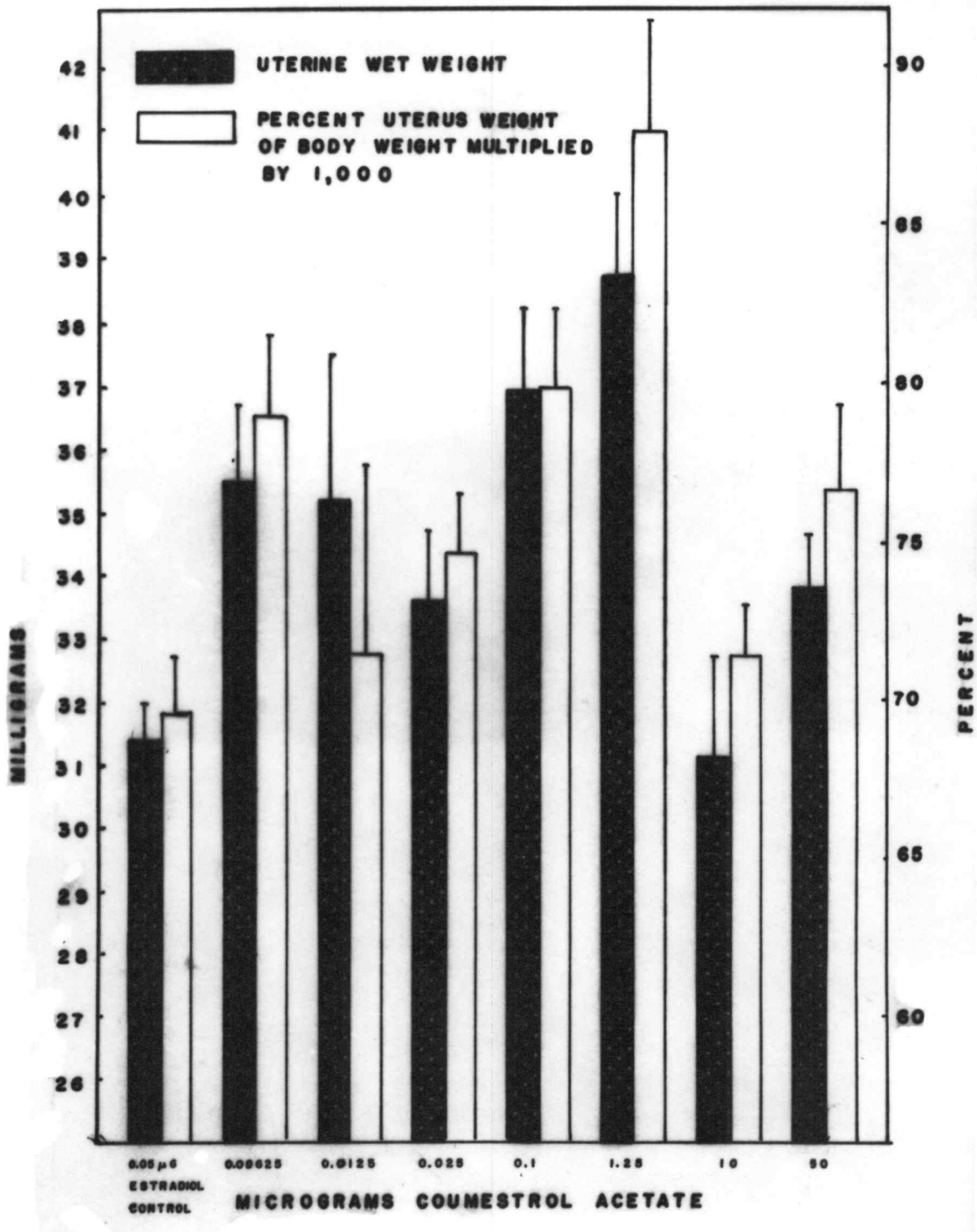

Fig. 6 Interaction of Varying Amounts of Coumestrol Acetate with 0.05 µg Estradiol

increase over the estradiol control was 17.5 at 1.25 micrograms.

In the case of coumestrol a consistent increase in uterine wet weight has been shown. However, there is no correlation between the increase in uterine wet weight and the amount of coumestrol added.

DISCUSSION

Current research on estrogens is establishing them as a family of compounds possessing individual characteristics. Although they all bring about the typical response of vaginal cornification, uterine growth, uterine fluid imbibition and a diverse number of other responses, their ability to do so varies. Individual variations of the ability to stimulate growth and fluid imbibition have been studied by Hisaw (33). He has shown that some that are strong promoters of fluid imbibition are relatively weak for growth (equilenin and estriol), while others (estradiol and equilin) are equally effective for both. In the present study, two economically important plant estrogens (genistin and coumestrol) were found to be poor for both fluid imbibition and growth. A careful comparison of the four- and six-hour dose response curves and the growth curves of genistin and particularly of coumestrol to similar curves obtained by Hisaw (33) show some rather marked differences. Although the present study has been an effort to determine the morphological changes occurring in the uterus, some speculation will be made on their mode of action.

Genistin

In 1941 Emmons (26) introduced the hypothesis that certain compounds similarly related in structure to the natural-occurring steroidal estrogens exert their action by undergoing metabolic changes to estrogens. He bases this theory on the fact that some compounds require a relatively larger dosage to bring about a response when

given locally as compared to those given systemically.

The ratio of the minimal amount of compound required to elicit vaginal cornification systemically to the minimal amount of compound required to elicit a local response is referred to as the S/L ratio. The S/L ratio of natural-occurring steroidal estrogens, as well as a number of other compounds, is approximately 50 to 400, whereas the S/L ratio of a proestrogen is approximately 1. Emmons states that as no gradations occur between the two ratios it is difficult to explain these data on the basis of solubility differences. He concludes that the resulting low ratio is due to its conversion in some other part of the body before it can act on the site of application.

Biggers and Curnow (11) suggest the aglucoside of genistin, genistein, is a proestrogen. In establishing a compound as a proestrogen or estrogen, a positive response of vaginal cornification is determined by taking vaginal lavages seventy-two hours after treatment. To date, no response as early as four hours has been examined for genistin. The question is raised as to the possibility of genistin becoming converted and eliciting a response within such a limited length of time. It would seem likely that if this compound was being metabolized to another structural form a latent period might exist prior to fluid imbibition. The six-hour response in general was greater than that of the four-hour response. This, it would seem, might support a conversion hypothesis. Direct evidence, such as an intermediate compound, will be required before any definite statement may be made concerning the active structural form of genistin.

Coumestrol

The characteristic of the dose response curve of coumestrol at four and six hours is similar to those reported for the natural-occurring steroidal estrogens. As the dosage increased, the time required for maximal fluid imbibition decreased. This results in a greater response at four hours than at six hours at higher dosages. This point is of particular importance in explaining the growth curve obtained for this compound. With the natural-occurring steroidal estrogens maximal uterine wet weights are usually established by the fortieth hour. This is followed by a cessation of growth and involution. In the case of coumestrol the lowest uterine wet weight, obtained following fluid imbibition and just prior to true growth, occurred at the fortieth hour. As mentioned, evidence from the dose response curve tends to discount the possibility that this is due to the solubility properties of this compound.

It may be that coumestrol acts in a manner similar to that of the natural-occurring steroidal estrogens. Due to its dissimilar structural formula, it elicits a delayed growth response. In considering the complex physiology and action of hormones on the uterus, this may be possible. It is generally held that hormones exert their influence on growth and metabolism by modifying specific enzyme systems (49, p.454) and by altering the permeability of the target cell (49, p.455). Our knowledge of the action of estrogens on the uterus is still too limited to determine whether a difference in structure could alter these mechanisms in a manner that would delay growth. The further

biological characterization of coumestrol by Lyman and Krueger (35) offers some evidence that different estrogens may vary markedly in their specific effects. They compared the effects of estradiol and coumestrol diacetate on the lipid metabolism in adult male rats. It was found that coumestrol was not similar to estradiol. Coumestrol did not depress growth, alter the testis and adrenals, increase plasma cholesterol or induce a lipotropic effect on the glyceride fraction of the cholesterol-induced fatty liver. The discovery of such a wide range of dissimilar biological characteristics seems to make it more probable that coumestrol is another estrogen with somewhat marked differences in its ability to stimulate growth.

A second possibility is that this may be a proestrogen (26) and that the delayed growth may be due to the time interval required for its conversion. The fluid imbibition which occurred at four hours makes this seem unlikely, even in view of the difference in the characteristics of the two responses. Fluid imbibition is associated with an enhancement of the uterine blood supply and an increase in the permeability of the uterine capillaries. Uterine growth is associated with early alterations in tissue composition and enzymatic activity (49, p.420). Further research will be required before the mode of action of this non-steroidal estrogen may be determined. Perhaps of the most immediate interest would be the determination of its S/L ratio.

Synthetic, natural-occurring steroids and natural-occurring non-steroidal estrogens may be compared on their ability to stimulate

uterine growth. Listed in order of potency from strongest to weakest they are diethylstilbesterol, the natural-occurring steroidal estrogens, coumestrol and genistin. Although genistin is weak in estrogenic activity and the dosage required for it to elicit a dramatic response is in the milligram range, its concentration in plants may be quite high. Charter et al. (17) reported soybean meal to contain 0.1 per cent genistin. Curnow has reported genistein concentrations in forage crops to be as high as 740 mg per 100 g dry matter (21). Coumestrol, although more active, seems to be less concentrated in plants. Bickoff (8) recently reported ladino clover to have a concentration of coumestrol equal to 0.02 per cent. The significance of these reports are hampered by the extreme variation which can occur in the concentration of these compounds. The concentration may be influenced by differences in varieties, stage of growth, season of year, climatic conditions, etc. Sanger and Bell (43) speculate that reproductive disturbances of economic importance could occur from their estrogenic properties. Courrier (20), studying the interactions between estrogens and progesterone in the rabbit, found that as little as 2.5 µg of estradiol interrupted pregnancy when given daily for three days beginning twenty-four hours post coitum. He concluded that estrogens appear to interfere with the "progesterone dominant mechanisms" in the uterus. Considering the low estrogen to progesterone ratio of ruminants, it seems conceivable that genistin or coumestrol could disrupt the reproductive physiology in sheep in the same manner.

A disturbance of normal reproductive physiology may also be due

to what appears to be anti-estrogenic properties of genistin. This would not be surprising in light of the discovery of Martin, Emmons and Cox (36) that dimethylstilbesterol is both a proestrogen and an anti-estrogen. It has been shown, in addition, that inhibition occurs between the natural-occurring steroidal estrogens (32). From Martin, Emmons and Cox's limited study they concluded that the interruption of pregnancy is correlated with anti-estrogenic potency.

Sanger and Bell (43) determined by flushing the fallopian tubes of ewes grazed on blue grass (non-estrogenic) and ladino clover (determined to be estrogenic) that ovulation occurred without incident on both forages, but fertilization (implantation) did not follow as consistently. Shelesnyak (46) has related the action of estrogens and implantation in showing that implantation appears to depend on an estrogen-induced release of histamine in the uterus, which in turn causes a decidual reaction. There has been some degree of success in inhibiting this reaction with both anti-estrogens and antihistamine drugs.

In recent personal communications with Bickoff concerning inhibition (antiestrogenicity) that he reported occurring in an extract of alfalfa meal (9), he stated that he met with no success in isolating an inhibitor. The possibility seems likely that genistin present, in concentrations too low to demonstrate estrogenic activity, was acting as an antiestrogen.

Studies in the near future are planned to determine the effect genistin has on the decidual response. Further study is also planned

to determine a possible antagonistic effect of genistin as well as coumestrol on estradiol-stimulated uterine growth in addition to that already shown on fluid imbibition.

Experimental evidence on the interaction of even the natural-occurring steroidal estrogens is lacking. In view of the important roll of estrogens in uterine physiology their interactions should in the near future give rise to fruitful investigations. It is hoped that this study will serve other investigators in the final elucidation of the action of the natural-occurring non-steroidal estrogens in reproductive physiology.

SUMMARY

Since the classical work of Bennett in which he implicated compounds of plant origin with reproductive disturbances in sheep, little has been done to determine the physiological characteristics of these compounds. Inasmuch as these compounds have been generally considered to be estrogens, the purpose of the present study has been to determine the ability of genistin and coumestrol acetate to stimulate uterine fluid imbibition and uterine growth. Preliminary studies have been made to determine their possible interactions with the natural-occurring steroid, estradiol-17 B.

It was determined that genistin was a poor promoter of both uterine growth and fluid imbibition. The maximal per cent increase in uterine wet weight was only twenty-eight. It was, therefore, not possible to determine an Astwood unit for this compound. In determining the growth promoting properties of genistin, levels as high as 500 micrograms were administered. No growth occurred at the level (50 micrograms) used to compare coumestrol acetate with the natural-occurring estrogens. Comparing the growth promoting properties of genistin to other estrogens, it is found to range from 1.29×10^{-4} times as potent as diethylstilbesterol to 9.16×10^{-3} times as potent as estriol.

Coumestrol acetate stimulates some fluid imbibition of the uterus. One Astwood unit was determined to be equal to ten micrograms of coumestrol acetate. On the basis of this assay other estrogens are from 21 (equilenin) to 400 (estradiol) times more active than

coumestrol acetate. The growth promoting properties of coumestrol acetate range from 1.40×10^{-3} times as potent as diethylstilbesterol to 5.10×10^{-1} times as potent as estradiol.

Limited studies on the interactions of genistin and coumestrol acetate with estradiol may indicate that genistin inhibits fluid imbibition at low dosages and increases fluid imbibition at higher dosages. Coumestrol acetate increased both the uterine wet weight and the uterus to body weight ratio (per cent body weight) in estradiol-sensitized rats. No correlation could be drawn between the dosage of coumestrol acetate administered and the increase in either uterine wet weight or the uterus to body ratio (per cent body weight).

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