AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented May 11, 1966

Title EFFECTS OF CHRONIC COUMESTROL ADMINISTRATION ON FEMALE RATS

Abstract approved Redacted for Privacy
(Major professor)

The effects of coumestrol on the uterine weight, uterine fluid imbibition, uterine nucleic acid content, adrenal weight and body weight of female rats were studied. Daily doses of 20 micrograms of coumestrol per 0.1cc propylene glycol were administered subcutaneously to spayed rats following a seven day recovery period, for durations varying from zero to 123 days. Groups treated with 0.1cc propylene glycol and estradiol 17-β at 0.1 microgram per 0.1cc propylene glycol were also run for comparable studies, for periods varying from 48 to 123 days.

The results on the chronic effects of estradiol 17-β confirmed that of Rinard (1963). Coumestrol was 0.13 times as active as estradiol 17-β in promoting uterine growth, but its ability to cause fluid imbibition was nearly the same as estradiol 17-β at 123 days. Coumestrol stimulated protein synthesis and inhibited adrenal growth, while estradiol 17-β had the opposite effects. There was

a transitional period of coumestrol action at about the 48th day of treatment. The estrogenic properties of coumestrol were not fully expressed until after 100 days of treatment. The animals responded to propylene glycol with marked increase in uterine weight, uterine fluid imbibition, and the RNA/DNA ratio, with no significant changes in the adrenal weight from the 48th day. Animals treated with coumestrol, estradiol 17-β and propylene glycol had a slower rate of growth, which resulted in the animals weighing 30 to 50 grams less than the castrate controls. Upon gross histological examination, tumorigenic and pyometric conditions were found in animals chronically treated with estradiol and propylene glycol, but not in those treated with coumestrol. The effects of coumestrol, estradiol 17-β and propylene glycol on the uterine cellular mitotic rate, cellular permeability, protein synthesis and adrenal metabolism in the animals were discussed.

EFFECTS OF CHRONIC COUMESTROL ADMINISTRATION ON FEMALE RATS

by

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A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF ARTS

June 1966

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Date thesis is presented May 11, 1966

Typed by Kay Smith

ACKNOWLEDGMENT

The author wishes to express her sincere appreciation to Dr. F. L. Hisaw for his kind counselling, to Dr. E. M. Bickoff for the supply of alfalfa coumestrol, to Dr. R. W. Newburgh for the procedure of nucleic acids determination, and to many other individuals without whose encouragement this work may not have been possible.

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INTRODUCTION

For 40 years information on phytoestrogens has been accumulating. In 1926, Loewe first reported substances in plants capable of causing estrus in animals. Since the appearance of this work, a large variety of plants has been investigated, from bacteria (Pedersen-Bjergaard, 1933) to flowering plants (Coussens and Sierens, 1949). In a review on "Estrogens in plants", Bradbury and White (1954) reported that 53 different plants have been found to be estrogenic. Bickoff (1963) in a more recent and complete review on "Estrogen-like substances in plants" stated that common plant materials found to be estrogenic include garlic, milkweed, oats, coffee, licorice, sunflower, wheat, barley, apple, parsley, the fruit flesh of cherry and plum, rhubarb leaves, yeast, willow flowers, sage, rye grass, potato tubers, and a number of leguminous forage plants.

The interest in research on plant estrogens is justified by their economic importance. Infertility in animals may follow excessive ingestion of plants possessing estrogenic activity. In fact, a very significant development in the field of plant estrogens was initiated in 1941, when infertility occurred in sheep grazed on subterranean clover in Western Australia, resulting in heavy

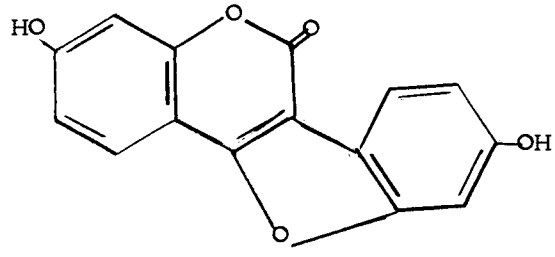
economic losses (Bennett, Underwood and Shier, 1946). The occurrence of estrogens in rapidly growing grass may probably cause "spring flush" in dairy cows (Biggers, 1958). Bickoff et al. (1960a) and Johnstons et al. (1965) recently demonstrated that carcass quality can be improved by the administration of an appropriate amount of plants rich in natural estrogens. It was also suggested (Castello and Lynn, 1950) that the estrogenic material could be isolated at a great reduction in price as compared to estrone, estriol and estradiol which are usually obtained from pregnant animals.

Thus far, the plant estrogens isolated and identified are the steroids estrone (Butenandt and Jacobi, 1933) and estriol (Skarzynski, 1933a, b) and miroestrol (Bound and Pope, 1960). The non-steroidal estrogens are beta-bitter acid (Zenisek and Bednar, 1960), and the estrogens in forage crops, genestein (Bradbury and White, 1951), formononetin, biochanin A (Pope and Wright, 1954), daidzin (Walz, 1931) and coumestrol (Bickoff et al., 1957, 1958a, b, 1964).

The estrogen coumestrol has been found in every legume so far tested (Lyman et al., 1959). In alfalfa, it appears to be the predominant estrogen (Bickoff et al., 1960b), but the stage of maturity of the alfalfa at the time of harvest is an important factor influencing the estrogenic content (Pieterse and Andrews, 1956). Studies made by Kitts et al. (1959) on legumes also show that

alfalfa displays high activity in the spring, little or none in mid-summer and an intermediate activity in the autumn.

The determination of the structure of this coumarin derivative was accomplished by a series of degradations involving alkaline methylation, decarboxylation, ozonolysis and finally hydrolysis of the ester. The structural formula suggested by Bickoff et al. (1957) is as follows:



Coumestrol
(6'7-dihydroxybenzofuro[3', 2', -3, 4] coumarin)

Thompson, Curl and Bickoff (1959) characterized and synthesized coumestrol after its isolation in pure crystalline form by means of countercurrent distribution. It was found to be 30 times as estrogenic as genistein. White (1961) studied the diacetate form of coumestrol and determined that one Astwood unit was equal to 10 micrograms of coumestrol acetate. On the basis of the Astwood assay (Astwood, 1938) estradiol is 400 times more active than coumestrol acetate (Hisaw, 1959). Its growth promoting property is 0.51 times as potent as estradiol. According to Bickoff, Livingston and Booth (1960), the estrogenic activity of the acetate form of coumestrol is equal to 85 percent of the pure compound.

The knowledge of the physiological characteristics of coumestrol as a phytoestrogen is fragmentary, White (1961) studied the ability of coumestrol acetate to promote uterine fluid imbibition and uterine growth. Lyman and Krueger (1961) compared the effects of coumestrol diacetate and estradiol on the lipid metabolism in adult male rats. But all these studies have been limited to short term treatments up to 72 hours. Observations on the prolonged coumestrol effects pertaining to reproductive physiology of animals have been restricted to commercial investigations.

The long-term response of the natural steroid hormone, estradiol 17-β, has been studied on the rhesus monkey (Macaca mulatta) by Hisaw and Hisaw (1961), and on rats by Rinard (1963). Other workers have also observed morphological changes in the female reproductive tracts of mice, rats, guinea pigs and rabbits (Burrows, 1949; Gardner, 1953a, b., 1957; Lipschutz, 1950) upon prolonged estrogen treatments. Generally, it has been found that the uterus is maintained in the full estrus condition (Selye, 1935). In some cases, glandular hyperplasia has occurred forming nodules underneath the uterine peritoneum. Endometrial pearl formation has also taken place due to squamous metaplasia. Pyometra condition has been found present, and occasionally, cancer has occurred.

Rinard (1963) emphasized the biochemical changes caused by prolonged estrogen treatment on animals. By measuring the ratio

of ribonucleic acid (RNA) to deoxyribonucleic acid (DNA), he found that estrogen induced uterine growth was accomplished mainly by hypertrophy rather than hyperplasia. His results confirmed the findings of others (Drasher, 1952; Mueller et al., 1958; Telfer, 1953; Telfer and Hisaw, 1957). They showed that a high RNA/DNA ratio in early estrogen treatment was due to the rapid synthesis of RNA. This ratio was maintained until about 48 days after the treatment, when the total RNA concentration, RNA/DNA ratio and uterine weight were increased. A significant increase in total DNA concentration was not observed until after 100 days of treatment.

The present investigation is designed in line with Rinard's work (1963), to determine if coumestrol has the same prolonged effect as estradiol 17-β on the spayed rat reproductive tract. It is hoped that the results will contribute to the knowledge of the reproductive physiology of animals fed on estrogenic plant materials over a long period of time.

METHOD AND MATERIALS

Female rats of the Wistar strain, obtained from Pacord Research Inc., Beaverton, Oregon, were used throughout this investigation. The coumestrol was kindly given by Dr. E. M. Bickoff of the Western Regional Research Laboratory. It was extracted from alfalfa and dissolved in propylene glycol at a concentration of 20μg per 0.1cc propylene glycol. This strength was chosen as it is the maximum solubility of coumestrol in propylene glycol.

The estradiol-17β was obtained in powder form from the Sigma Chemical Company. A dosage of 0.1μg estradiol-17β per 0.1cc propylene glycol was used to check the comparable response (Rinard, 1963).

Rats approximately 100 days old, weighing about 200 grams, were castrated by the dorsal lumber approach while under ether anesthesia. After a seven day recovery period, the animals were treated in the following manner:

1. Animals receiving no injection--castrate controls.
2. Animals receiving 20μg coumestrol per 0.1cc propylene glycol per day.
3. Animals receiving 0.1cc propylene glycol per day--propylene glycol controls.
4. Animals receiving 0.1μg estradiol-17β per 0.1cc propylene glycol per day.

Each experimental group consisted of six animals and the doses were administered by subcutaneous injections. Subsequent to the recovery period the experimental phase was initiated and extended for 123 days.

At the termination of each period of treatment, the animals were sacrificed by means of etherization. The entire uterus was removed by cutting across the uterine cervix and stripping away the mesentery. The adrenal glands were removed, cleared of fat, and weighed. About 50 to 70 mg. of tissue for nucleic acid analysis was taken from the body of the horn and weighed immediately on a Roller-Smith torsion balance. Another section of similar amount was taken for uterine wet and dry weight while the rest of the uterine tissue was fixed in Bouin's Fluid for histological purposes after weighing. In some cases, the uteri were so small that the last step had to be omitted. The entire uterine wet weight was obtained by adding these three values together. To determine the uterine dry weight, the uterine portion was dried in a tared aluminum pan in an oven at 100 degrees Centigrade for 24 hours or more, cooled in a desiccator over anhydrous calcium chloride and weighed on a Mettler balance. The uterine portions taken for nucleic acid determination were put into ice cold ten percent trichloacetic acid (TCA) immediately after weighing.

The method for extracting the nucleic acid followed that of Rinard (1963) as adopted from Schneider (1945), Ogur (1950) and Burton (1956). The homogenization of tissue was done in a cold room at four degrees Centigrade, using ten percent TCA followed by five percent TCA. The homogenates were subsequently washed twice with 95 percent ethanol. The nucleic acids were extracted by heating in 0.5 N perchloric acid at 70 degrees Centigrade for 15 minutes, according to Burton (1956). The colorimetric determination of nucleic acids was modified from the procedure employed by the Science Research Institute at Oregon State University.[1]

The RNA was determined by the orcinol reaction (Schneider, 1945). D(-) ribose from the Sigma Chemical Company was used as a standard. The sugar was dissolved in 0.5 N perchloric acid, with concentrations ranging from 11.8 to 94 μg per ml. Ten μg of D(-) ribose was considered equivalent to 37.6 μg RNA. The optical density was read at 670 mμ in a Beckman quartz spectrophotometer

The DNA was determined by the diphenylamine reaction (Dische, 1930). The standard used was the DNA from salmon sperm, obtained from the Sigma Chemical Company. It was dissolved in 0.005N NaOH, with concentrations ranging from 12.5 to 100 μg per ml. The optical density was read at 600 mμ.

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[1] This procedure was kindly given by Dr. R. W. Newburgh, Assistant Director of the Science Research Institute at Oregon State University.

The exact amount of RNA and DNA per mg of uterus was calculated with respect to the standards. The ratio of RNA to DNA was obtained and graphed. The animal body weight at castration and termination, total uterine weight, percentage of water in the uterus and adrenal weight were recorded in the tables in the appendix. The mean and standard error were computed according to the following formula: (Snedecor, 1946, p. 91-92)

$$\text{Standard error} = \sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}{\frac{n-1}{n}}}$$

RESULTS

Four main aspects were considered to reflect the activity of coumestrol on female rats. They were the uterine weight, uterine fluid content, adrenal weight and nucleic acid level. In order to compare the activity of coumestrol with animal estrogen, estradiol 17-β was studied alongside with the coumestrol groups from 48 to 123 days. An attempt was also made to check the chronic effect of their carrier, propylene glycol, on the animals. All data are summarized and tabulated in the appendix. While comparing the variation with the treatments, one must bear in mind that the uterine weight, total nucleic acid level and adrenal weights are dependent on the body weight. Thus, some points obtained from these animal groups which are not comparable in body weights have been omitted. However, since the RNA/DNA ratio is not affected by body weight, all points are included in the graphs for relative estimation.

Uterine Weight

Figure 1 shows the response of uterine weight to the short term coumestrol treatment. At the third day, there was an initial peak of about 13 milligrams increase from zero day. The succeeding quantities gradually dropped below the initial value. From 48 to about 100 days (Figure 2), the weight remained stable and started to

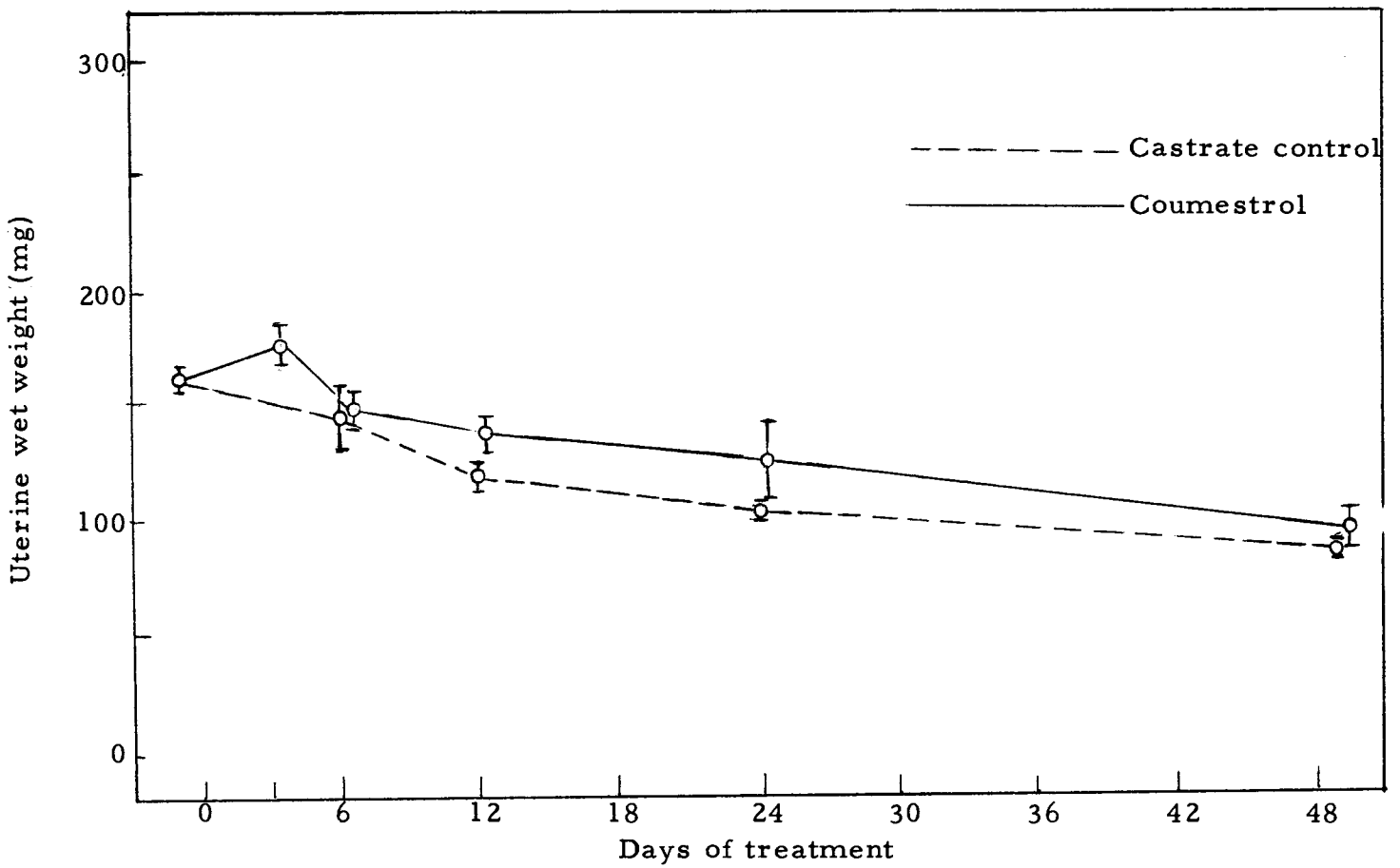


Figure 1. Variation in uterine weight as a response to coumestrol treatment

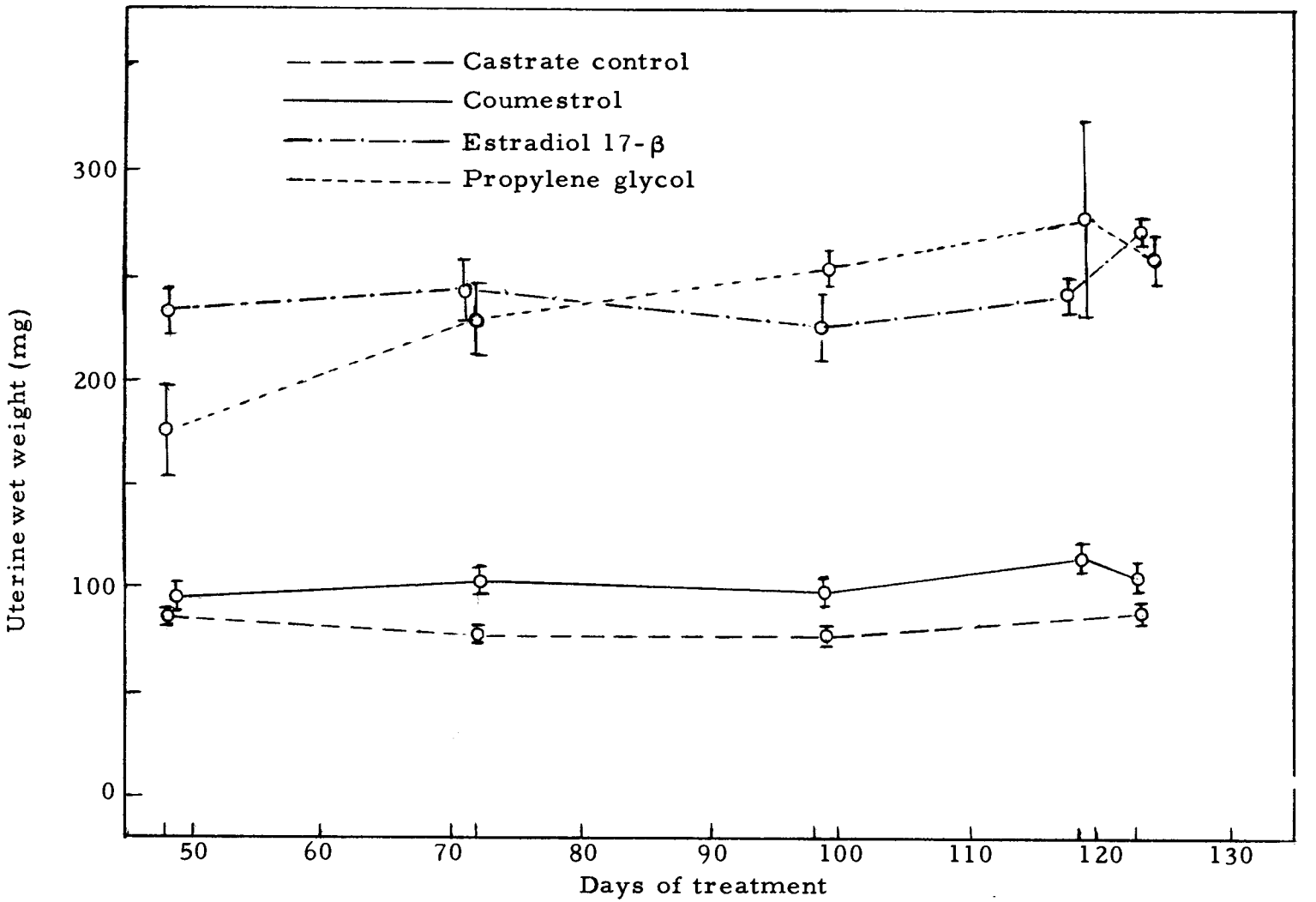


Figure 2. Variation in uterine weight as a response to prolonged treatments with coumestrol, estradiol 17-β and propylene glycol

rise again at 100 days until the end of the treatment.

The control group also showed a slow decrease in uterine weight from zero to about 48 days, then it stayed at nearly the same level up to 123 days. Throughout the experiment, the coumestrol-induced animals retained a higher total uterine weight of about 10 to 12 milligrams over the corresponding uterine weight of the control animals.

The response of the uterus to estradiol 17-β treatment was similar to Rinard's finding (1963). There was a threefold increase of uterine weight over the control animals. Compared with coumestrol, its activity to cause uterine growth was about 7.5 times as great.

The results obtained from the group treated with propylene glycol alone was most surprising. At 48 days, the uterine weight was 175 ± 20.2 milligrams as compared to 80.5 ± 2.85 milligrams for the castrated control uterus. On the basis of average uterine weight increase over the control, propylene glycol was 6.4 times as active as its solute coumestrol. Although there was a high individual variation among the animals, propylene glycol showed an effect approaching that of estradiol 17-β during the final periods of treatment. While coumestrol and estradiol 17-β maintained a rather stable level in the uterine weight, propylene glycol continued to stimulate the growth of the uterus, with only a slight decrease

toward the end of the treatment.

RNA:DNA Ratio

Both the control and coumestrol groups decreased sharply in the RNA/DNA ratio during the early periods of treatment (Figure 3). The ratio of the control group started to rise slowly from the 12th day. After staying at a stable level until the 48th day the ratio started to decline slowly until 123 days (Figure 4). The drop in coumestrol was more pronounced, but at the sixth day it increased sharply to above the control value. At 48 days, there was a slight drop in the nucleic acid ratio, but subsequently it rose rather steeply until reaching a value of 0.354 ± 0.021 at 123 days (Figure 4).

With the estradiol 17-β treatment, the ratio decreased from 48 to 72 days, and subsequently stayed at approximately the same level. Contrarily, the ratio of the propylene glycol group increased at a constant rate. The abrupt rise from 119 to 123 days brought the ratio to as high as 0.733 ± 0.021. The ratio of RNA to DNA was closely related to the actual concentration of RNA and DNA per milligram of uterus. The data show that propylene glycol has caused an increase in the amount of RNA accompanied with a decrease in DNA. This indicated that the cells in the uterus have grown in size while their mitotic rate has slowed down. The significance of the RNA/DNA ratio with regards to the relationship between RNA and

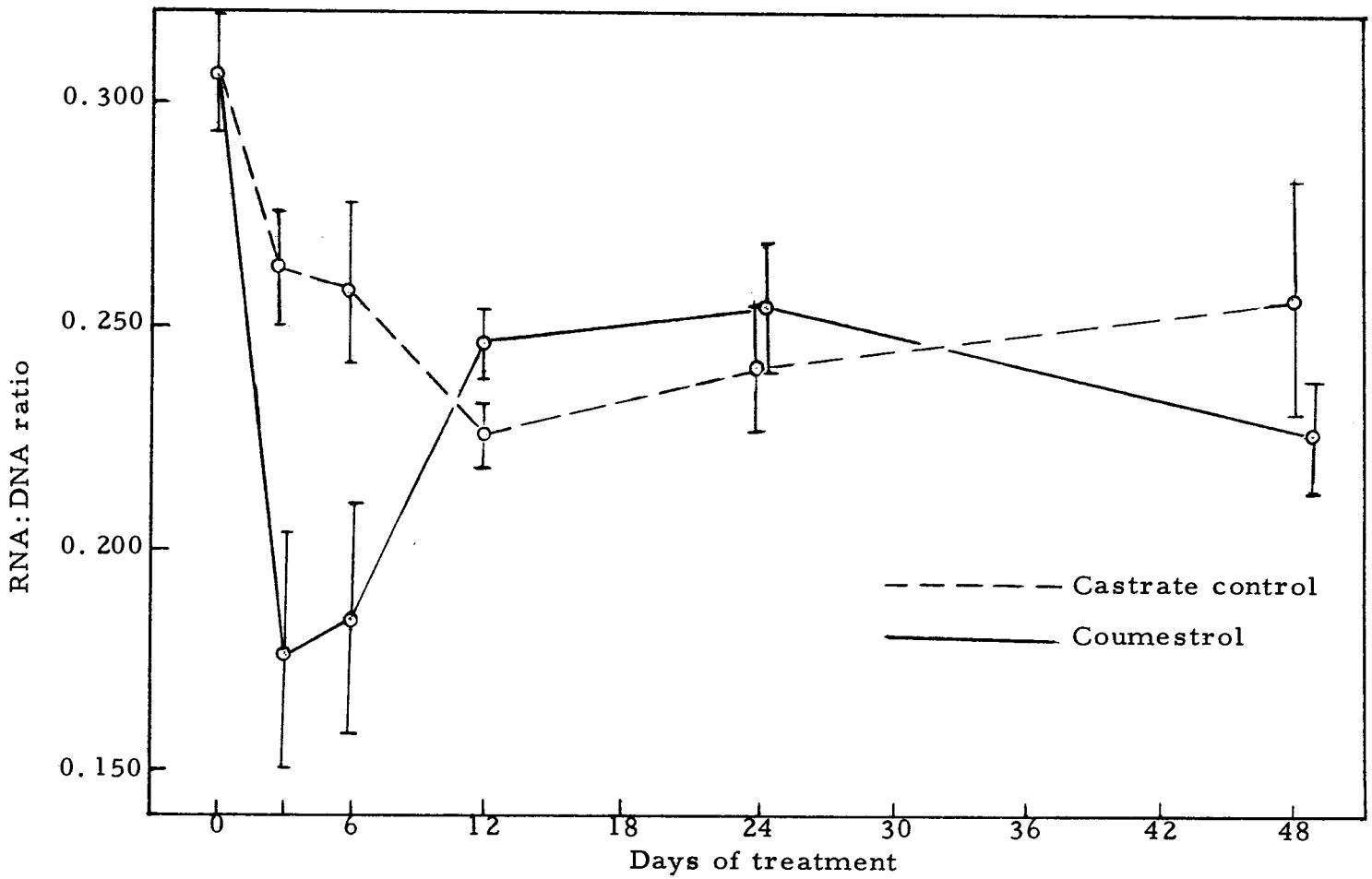


Figure 3. Variation in the level of nucleic acids as a response to coumestrol treatment

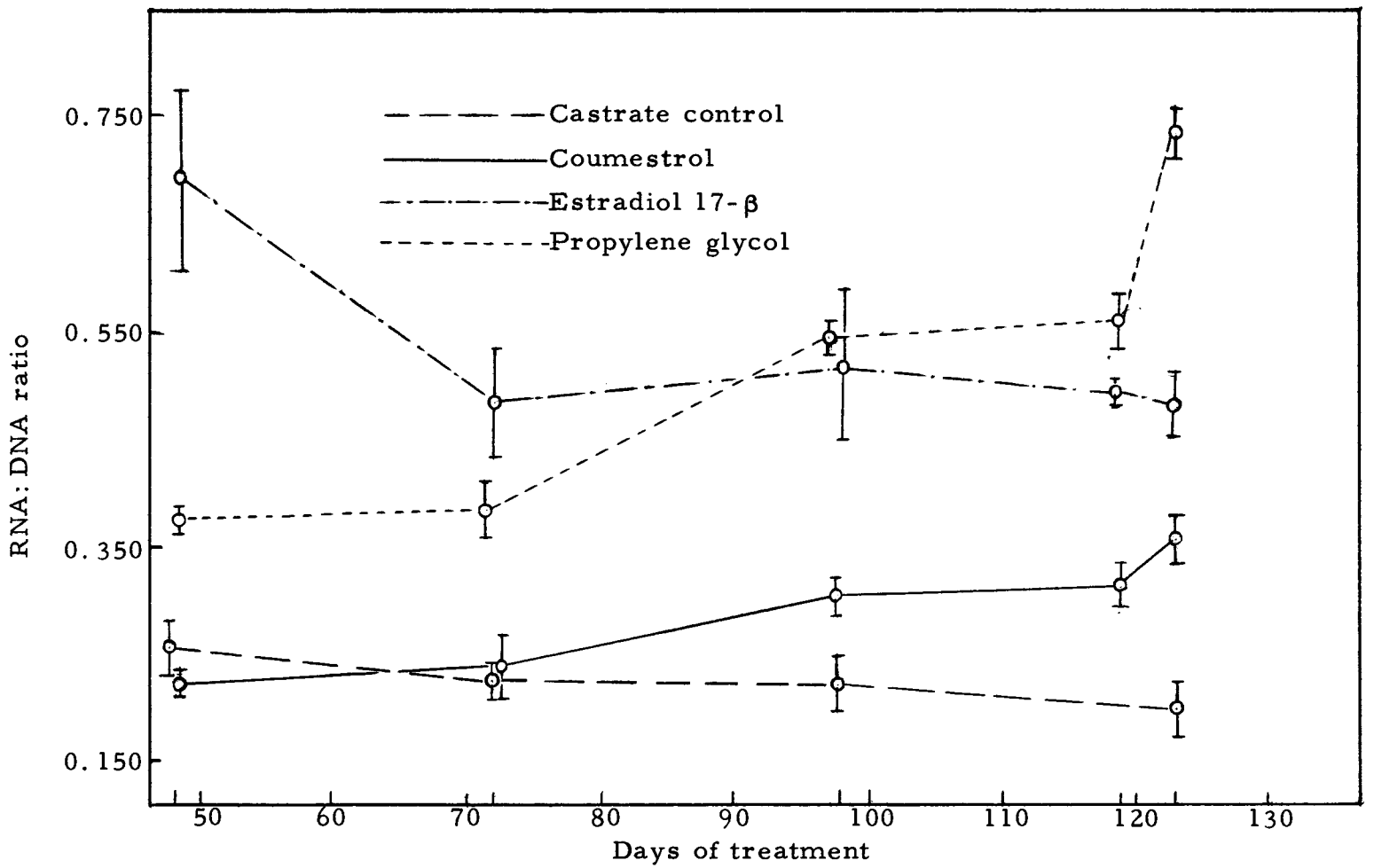


Figure 4. Variation in the level of nucleic acids as a response to prolonged treatments with coumestrol, estradiol 17-β and propylene glycol

DNA will be considered more extensively in the discussion.

Uterine Fluid Content

In general, there were no pronounced differences in the uterine fluid content among the four groups in both short term and prolonged periods of treatment (Figures 5 and 6). The control uteri decreased continuously in the percentage of water from 80.8 ± 0.437 percent at zero day to 78.0 ± 0.761, with only a slight rise at 72 days, when the fluid content returned to that of the twelfth day.

The curve of the early periods of coumestrol treatment (Figure 5), shows the fluctuation in water content with marked individual variations. The fluid content was below that of control animals up to the twelfth day. At the 24th day of treatment, the uterine fluid content returned to the initial value while that of the control group continued to decrease. At 48 days, however, the fluid percentage of the coumestrol group fell to approximately the same value as that of the control group. As the treatment with coumestrol continued, the fluid imbibition activity causing an increase in uterine fluid content augmented, and persisted until the percentage reached a value of 80.1 ± 0.294 at 123 days of treatment. This value was close to that of estradiol 17-β at the corresponding period of time.

The animals treated with estradiol 17-β maintained a rather constant level of fluid in the uteri throughout the experiment, with

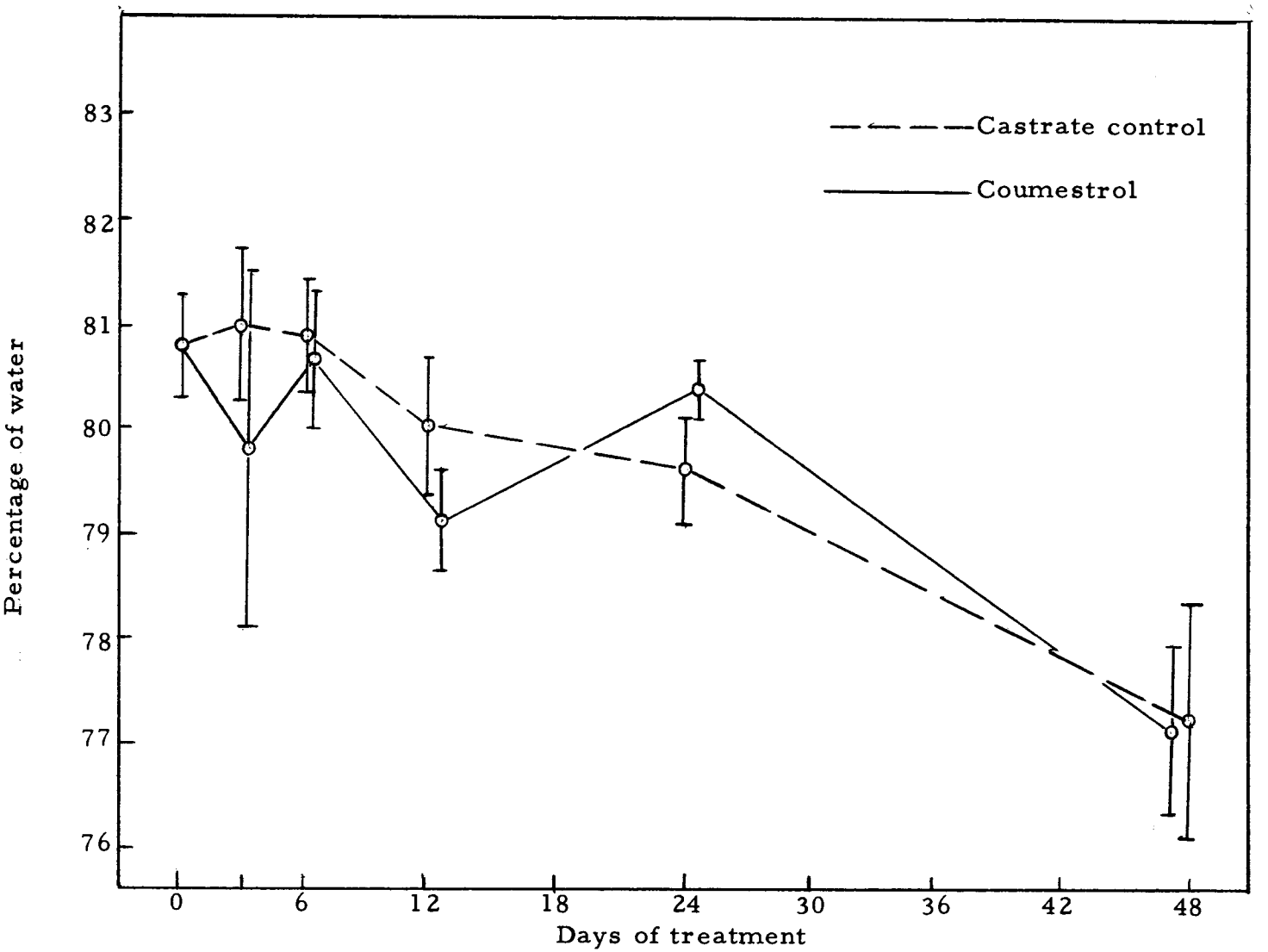


Figure 5. Variation in uterine fluid content as a response to coumestrol treatment

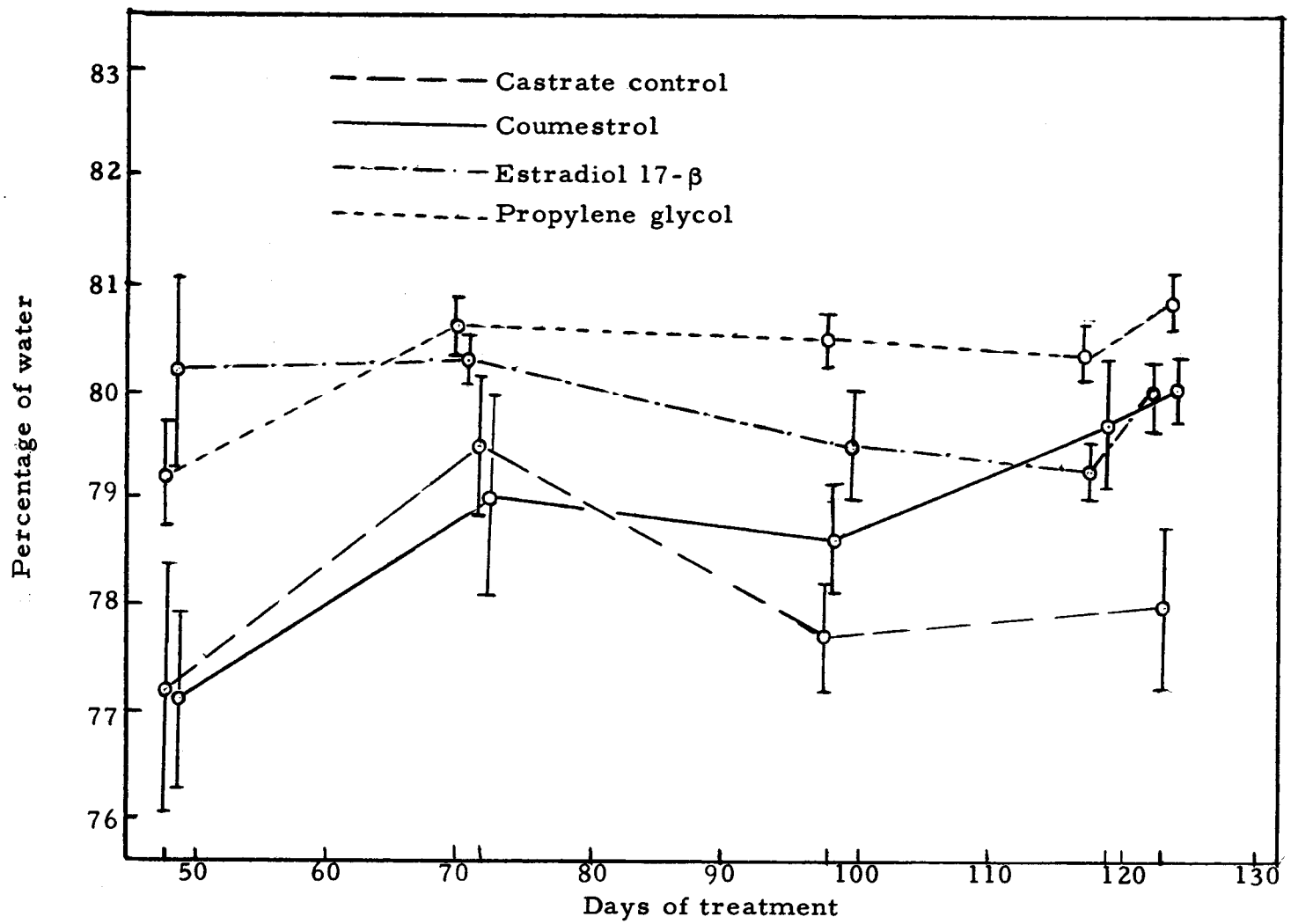


Figure 6. Variation in uterine fluid content as a response to prolonged treatments with coumestrol, estradiol 17-β and propylene glycol

only a slight decline at 118 days. This level was close to that of the zero day value, indicating the ability of estradiol to bring back the normal activity of fluid imbibition during the prolonged treatment.

Again, propylene glycol exerted a great influence on the activity of the uterus, in causing a high value of the percentage of uterine fluid. The curve (Figure 6) shows an increase at 48 days of treatment from a value below that of the estradiol 17-β group to a plateau much higher in the percentage of water.

Adrenal Weight

During the early periods of treatment, animals in both control and coumestrol groups responded with a reduction in adrenal weight (Figure 7). The weight of the adrenal glands in the control group had reached a stable level and was maintained until the end of the treatment at 123 days (Figure 8). However, in the coumestrol group, there was an increase of adrenal weight at 24 days of treatment. This point appeared to be striking in the graph (Figure 7) compared with the corresponding point of the control group. However, it should be noted that the data for adrenal weight have been calculated against 100 grams of body weight which in this case was unusually low, 230 ± 17.4 grams as compared to 280 ± 15.6 grams for the control animals at the same period. There was actually only a slight increase in adrenal weight from 63.8 ± 6.65 milligrams at 12 days

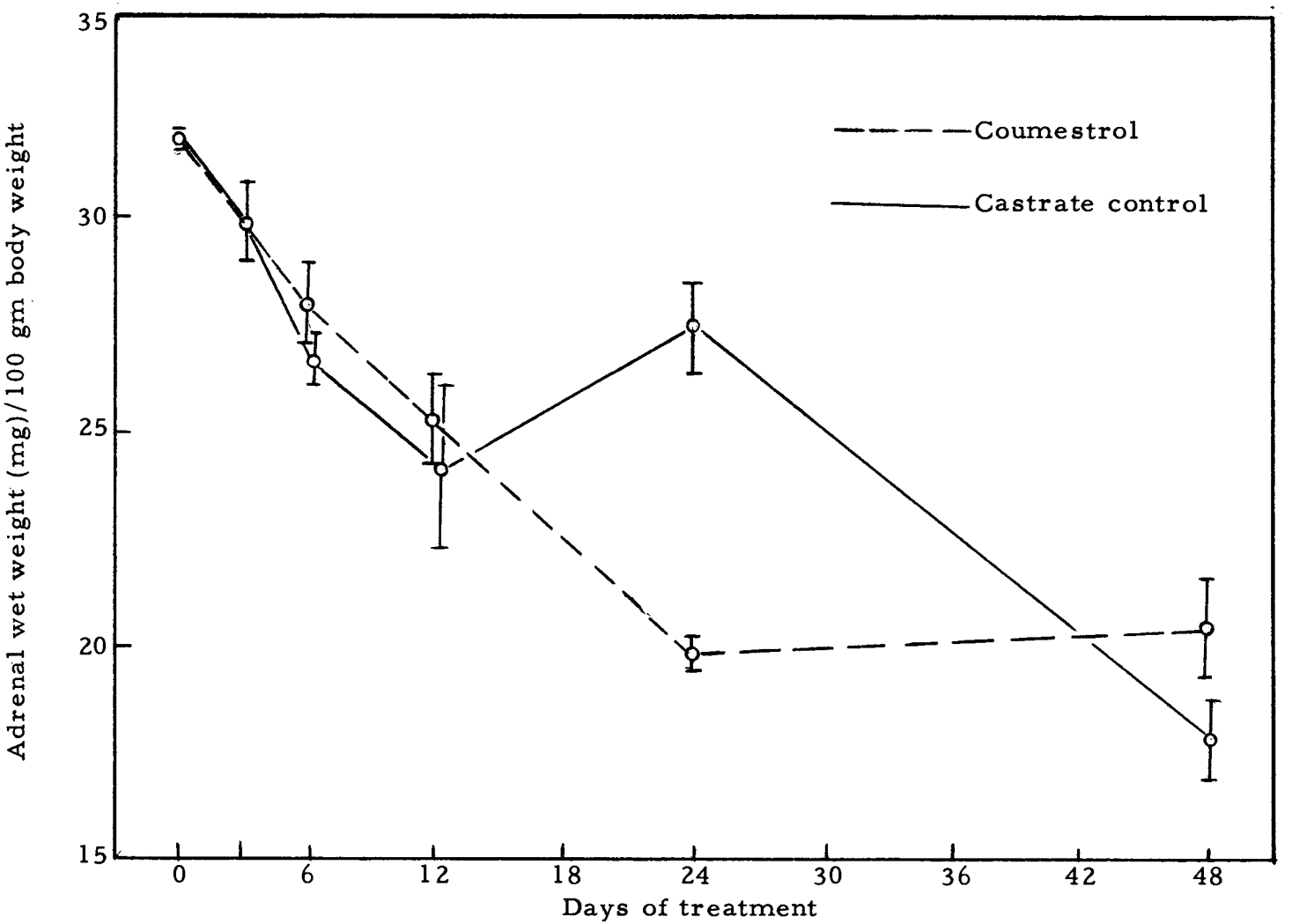


Figure 7. Variation in adrenal weight as a response to coumestrol treatment

to 66.2 ± 4.20 milligrams at 24 days. Nevertheless, compared with the control animal adrenals, which were 55.5 ± 1.99 milligrams, there was still ten milligrams difference. The significance of this difference is considered later in the discussion. Despite the discrepancy in body weight, this point was retained on the graph to show a transition between the increase and the sharp decrease of the adrenal weight. From 48 to 123 days, the weight of the glands stayed at about the same level with only a slight increase at 72 days and a slight decrease at 123 days (Figure 8). The inhibitory effect of prolonged coumestrol treatment on the adrenal growth was expressed by the curve below that of the control group.

The response of the adrenal glands in animals treated with estradiol 17-β has fluctuated between about 20.0 to 24.0 milligrams per 100 grams of body weight, with a tendency to decrease as the treatment continued. The adrenal glands of the animals treated with propylene glycol were smaller than those treated with estradiol 17-β. The individual variability was great among the animals of this group, however, the average level of the adrenal weight per 100 grams of body weight remained approximately the same.

During the termination of each experimental group, it was noticed that the animals injected with coumestrol, estradiol 17-β and propylene glycol had lighter body weights than those of the control animals. At 123 days, the difference in body weight between the

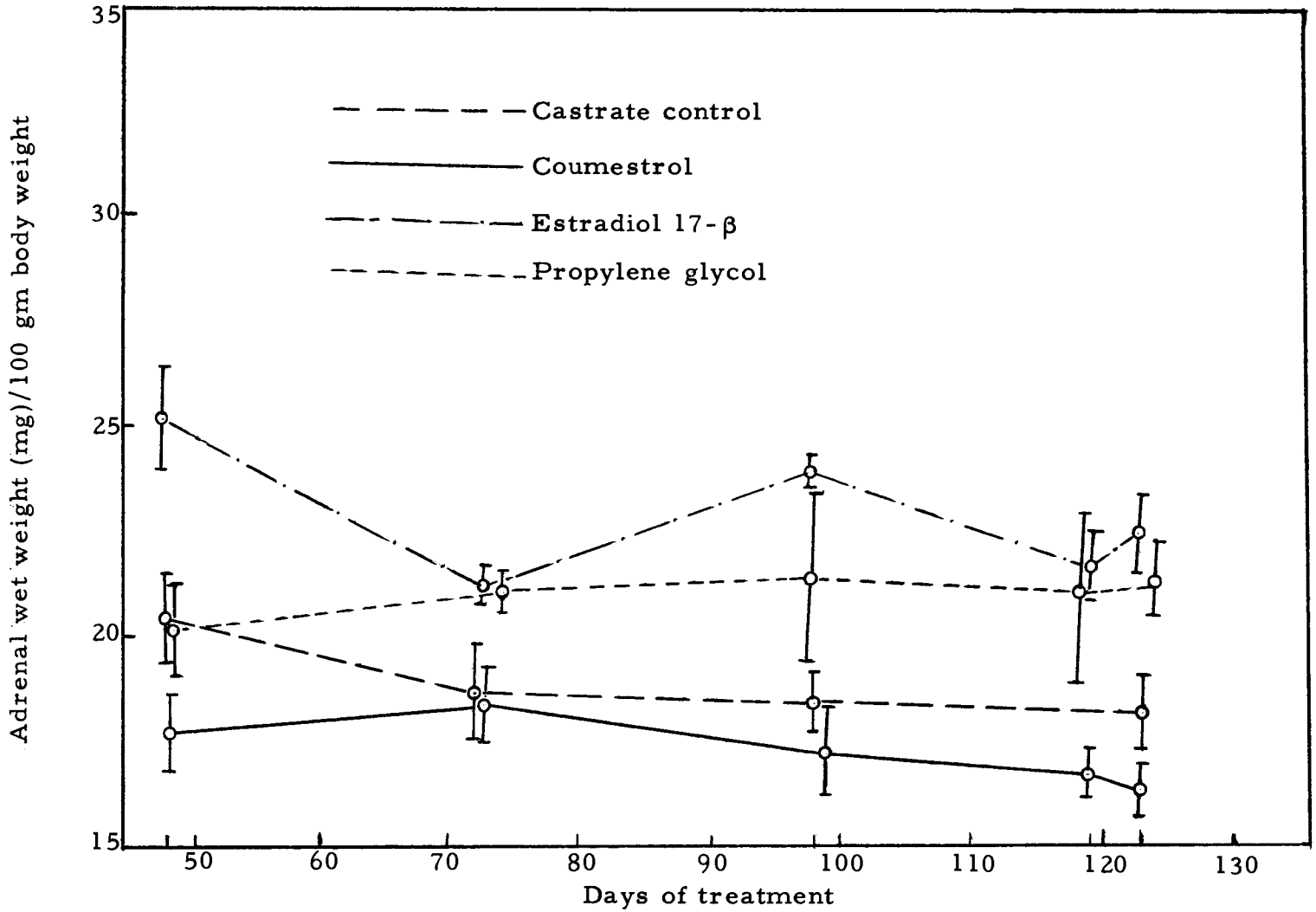


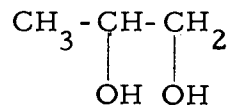
Figure 8. Variation in adrenal weight as a response to prolonged treatments with coumestrol, estradiol 17-β and propylene glycol

animals of the estradiol 17-β group and the control group was about 50 grams, that between the coumestrol and the propylene glycol group and the control group was about 30 grams.

A gross histological examination of the uteri showed that some uteri treated with propylene glycol and estradiol 17-β for prolonged periods had pearl-like nodules filled with clear fluid. In the propylene glycol group, this was observed as early as 72 days. A condition of pyometra also occurred in one case in an animal treated with estradiol 17-β for 123 days.

DISCUSSION

Many questions have arisen as a result of the surprising data obtained from the propylene glycol treatment. Both the uterus and the adrenal gland responded strikingly to the compound. A look at the literature shows that this hygroscopic, viscous solution with a slight acrid taste is 1, 2-propanediol:



It has a high boiling point, is miscible with water and chemically behaves like alcohol (Morrison and Boyd, 1961. p. 128, 153, 650). It is non-toxic when taken internally and is not irritating to the skin of animals (Riddick and Toops, 1955). It is widely used as an anti-freeze, aerosol antiseptic, inhibitor of fermentation and mold growth as well as a good solvent for pharmaceuticals and various medicaments (Stecher, 1960. p. 863).

Although propylene glycol is harmless and useful, its high estrogen-like activity cannot be overlooked. Its ability to cause an increase in fluid imbibition and the RNA/DNA ratio has exceeded that of estradiol 17-β from about 80 days up to the end of the treatment. The data reveal that the DNA level of the uterus induced with propylene glycol has slightly decreased during the prolonged periods of treatment while the RNA has increased. This shows that the

uterine growth caused by propylene glycol has been due to hypertrophy rather than hyperplasia (Brody and Wiqvist, 1961).

Davidson and Leslie (1950) have stated that the differentiation of tissue is accompanied by the increase in protein content, and protein synthesis is associated with the high RNA level (Brachet, 1955; Brody, 1958). The increase in protein synthesis, therefore, accounts for the increase in uterine size, with a concomitant increase in the RNA/DNA ratio. Furthermore, since propylene glycol, upon oxidation in the body of the animals, produces pyruvic and acetic acid (Stecher, 1960. p. 863), it may further augment protein synthesis through the Kreb's Cycle during intermediary metabolism.

The cause of the increase in uterine fluid content was probably due to the high miscibility of propylene glycol with water. Similar to other hormonal action, the surface of the uterine cell may have been orientated by propylene glycol to allow the water to permeate into the cell (Szego and Roberts, 1953).

The adrenal weight of the animals induced with propylene glycol remained stable throughout the experiment. This shows that either propylene glycol did not have any significant effect on the organ like the other hormones, or the effect had already reached a steady state at 48 days.

The problem with propylene glycol is intriguing, but since

it was not recognized until after the experiment had been started, no attempt was made to test its immediate effect on the animals. How then, can we account for the picture with coumestrol, if its carrier, propylene glycol alone, can show such a seemingly hormonal effect?

The data obtained from coumestrol and estradiol 17-β treated animals are obviously independent of those of the propylene glycol group. The response of the animals to coumestrol is much lower than to propylene glycol. This shows that in the presence of coumestrol, the effect of propylene glycol is suppressed.

Moreover, the same reagent has been used to carry estradiol 17-β by Rinard (1963). His work has confirmed the results of many workers. In the present investigation, the prolonged estradiol 17-β treatment has produced similar results to those of Rinard. These considerations may support the reliability of propylene glycol used in this investigation, and the following account on the estrogenic activity of coumestrol.

In general, coumestrol causes a slight but evident stimulatory or inhibitory effect on animals. The uterine growth response is closely correlated with the RNA/DNA ratio. This observation confirms that of Davidson and Leslie (1950), Alfert and Bern (1951), and Brody and Wiqvist (1961), who have indicated that the DNA increase is due to cellular multiplication, while RNA increase is

due to cellular growth. When DNA remains constant, the RNA/DNA ratio gives a number directly proportional to the RNA amount per cell.

As a response to early coumestrol treatment, the uterus rapidly increases in weight and size. The low RNA/DNA ratio during this period shows that the uterine growth is largely due to the increase in mitotic activity rather than the enlargement of the cell. This growth stimulation is not maintained. At the sixth day, the uterine growth declines to a steady state approximately 20 milligrams above the control uterus. This cessation in uterine growth and concomitant decline in RNA content may be due to a negative feedback mechanism which serves to limit the biosynthetic activity of the system, as proposed by Rinard (1963). Enzyme activity or repression of enzyme formation may also be inhibited (Davis, 1961). Since RNA is associated with protein synthesis (Brachet, 1955), it is thought that the decline in RNA at about 24 days with coumestrol treatment, indicates the shift of cellular metabolism from protein synthesis necessary for growth, to the maintenance of the steady state. Starting from about 100 days of treatment, coumestrol expresses its estrogenic activity by causing an increase in RNA with no significant change in DNA. This shows that after prolonged coumestrol treatment, the uterine growth is due to hypertrophy rather than hyperplasia.

Astwood (1938) has indicated that estrogen is capable of bringing about water uptake in the uterus by changing the permeability of the capillaries in the uterus. The percentage of fluid content fluctuates during the first 12 days of treatment, showing an irregular response of the uterus to the vasodilatory effect of coumestrol. After 72 days of treatment, the percentage of water rises sharply, until at 119 days it reaches the zero day value. During this period, the uterus may have gradually adjusted to the effect of coumestrol, allowing it to bring back the original cellular permeability.

In general, coumestrol has an inhibitory effect on the adrenal. Since the mechanism of the adrenal-gonadal relationship is very complicated and involved, the responses of the adrenal gland to estrogen treatment are inconsistent (Gorbman and Bern, 1962. p. 325). Kitay (1963) has demonstrated that low levels of estrogen stimulate adrenal cortical activity whereas at high levels, estrogen inhibits adrenal function. Since the dosage of coumestrol used here is by no means high as compared to estradiol which shows a stimulatory effect, it can only be concluded that the adrenal atrophy of the coumestrol treated animals is caused by a different mechanism. The rise of the adrenal weight at 24 days may be correlated with the high RNA/DNA ratio in the uterus. This point demonstrates an interplay between the uterus and the adrenal gland. The shift of the cellular metabolism from RNA synthesis to the maintenance of

a steady state of growth requires greater activity of the adrenal gland, resulting in its hypertrophy. The great reduction in the body weight of the animals during this period may also be due to the greater usage of metabolic reserves necessary to adjust to the physiological changes.

Considering the complex physiological actions of estrogens on the animals, it is not surprising that both prolonged estradiol and coumestrol cause them to have a delayed growth response. Szego and Roberts (1953. p. 454, 455) have suggested one possible mechanism for delayed growth, in that the hormones exert their influences on growth and metabolism by modifying specific enzyme systems and by altering the permeability of the target cell.

Rinard (1963) noticed tumorigenic growth on the uteri upon chronic estradiol treatment. Several similar nodules have been observed in this investigation, on some uteri treated with estradiol 17-β and propylene glycol, but not with coumestrol. In one case a nodule had brownish pus which exuded upon rupturing. This pyometric condition has been explained by Allen and Gardner (1941) that this may be caused by bacterial infection via the vagina. In some other cases pearl-like enlargements containing clear fluid have been seen protruding from the head of the uteri. This condition may be due to glandular hyperplasia. The bases of the endometrial

glands proliferate down through the muscularis and push under the surface of the uterine peritoneum (Allen and Gardner, 1941).

CONCLUSION

The present investigation shows that, although coumestrol has only a small effect on rats compared to the action of estradiol 17-β, a closer analysis of the data reveals its most interesting mode of action in the animals. The estrogenic property of coumestrol is most fully expressed after the prolonged treatment of 100 days. The changes in mitotic rate, protein synthesis, cellular permeability and adrenal metabolism have reached a steady direction of action. Since all these physiological activities are intimately concerned with reproduction and growth, the reproductive disturbances caused by animals feeding on plants containing coumestrol can thus be explained.

This investigation on the mechanism of the activity of coumestrol as a phytoestrogen, is by no means complete. But let it serve as a stimulation for further studies. A few suggestions along this line of research will be given in the following:

1. The study of the chronic interaction between coumestrol and steroidal estrogen: Since animals fed on phytoestrogens are usually normal, it would appear seemly to study the effect of coumestrol in the presence of natural estrogen.

2. Since the period around the 48th day of treatment appears to be a transition point of the action of coumestrol, a detail study from 24 to 72 days may give a clearer pattern of the activity of the plant estrogen.

3. Many significant changes occur at 119 and 123 days. It may be profitable to check on the activity of coumestrol beyond 123 days.

4. The surprising results on the effect of propylene glycol on the uterus makes it urgently necessary to study more carefully its hormone-like activity in animals.

SUMMARY

1. The purpose of this investigation was to study the effects of coumestrol on the uterine weight, uterine fluid imbibition, uterine nucleic acid concentration, adrenal weight and animal body weight of female rats.
2. Daily doses of 20 micrograms of coumestrol in 0.1cc propylene glycol were administered subcutaneously to spayed rats following a seven day recovery period, for various durations from zero to 123 days. Groups treated with 0.1cc propylene glycol and estradiol 17-β at 0.1 microgram per 0.1cc propylene glycol were also run alongside, from periods of 48 days to 123 days.
3. The animals responded to propylene glycol with marked increases in uterine weight, uterine fluid imbibition, and the RNA/DNA ratio, with no significant changes in adrenal weight from the 48th day.
4. The results of the chronic effect of estradiol confirmed those of Rinard (1963). Compared with coumestrol, it was 7.5 times more active in promoting uterine growth, but its ability to stimulate fluid imbibition was nearly the same as coumestrol at 123 days of treatment. Coumestrol stimulated protein synthesis and inhibited adrenal growth,

while estradiol 17-β had the opposite effects.

5. There was a transitional period of the coumestrol activity at 48 days of treatment. The estrogenic properties of coumestrol were most fully expressed after 100 days of treatment.
6. Animals treated with coumestrol, estradiol 17-β and propylene glycol had a slower rate of growth, which resulted in the animals weighing 30 to 50 grams less than the castrate controls.
7. Tumorigenic and pyometric conditions were found on the uteri of rats treated with estradiol 17-β and propylene glycol for long periods of time.

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APPENDIX

Appendix Table 1. Data obtained from noninjected castrate control animals, showing changes in body weight, uterine weight, adrenal weight, percentage of water, and nucleic acid level.

Days of Treatment +	Body weight at start (gm)	Body weight at end (gm)	Total uterine wet weight (mg)	mg uterine weight per gm body weight	Percent water	Adrenal weight (mg)	mg adrenal per 100 gm body weight	μg RNA per mg uterine wet weight	μg DNA per mg uterine weight	RNA/DNA
0	196 ± 9.10	226 ±11.8	159 ± 5.10	0.659 ± 0.344	80.8 ± 0.437	71.1 ± 2.46	31.8 ± 0.093	2.86 ± 0.014	9.33 ± 0.435	0.307 ± 0.014
3	*228 ±10.5	250 ±11.4	183 ± 6.69	0.740 ± 0.067	81.0 ± 0.700	68.4 ± 1.94	27.7 ± 1.71	3.25 ± 0.485	13.0 ± 0.736	0.263 ± 0.013
6	203 ± 7.50	240 ± 8.85	142 ±15.5	0.586 ± 0.535	80.9 ± 0.519	67.2 ± 3.21	28.0 ± 0.981	3.42 ± 0.356	14.6 ± 1.22	0.258 ± 0.018
12	206 ± 8.20	264 ± 7.75	115 ± 7.89	0.443 ± 0.019	80.0 ± 0.705	65.4 ± 4.35	25.3 ± 1.07	2.48 ± 0.065	11.0 ± 0.387	0.226 ± 0.007
24	197 ± 7.15	280 ±15.6	103 ± 2.42	0.370 ± 0.026	79.6 ± 0.529	55.5 ± 1.99	19.8 ± 0.354	2.61 ± 0.131	10.9 ± 0.502	0.241 ± 0.017
48	184 ± 4.75	284 ± 2.96	80.5 ± 2.85	0.312 ± 0.031	77.2 ± 1.18	55.0 ± 4.18	20.4 ± 1.12	2.75 ± 0.533	11.1 ± 2.60	0.256 ± 0.027
72	185 ± 7.51	294 ±13.3	78.8 ± 3.30	0.270 ± 0.005	79.5 ± 0.632	54.3 ± 2.40	18.6 ± 1.12	2.65 ± 0.232	11.9 ± 0.188	0.223 ± 0.017
98	188 ± 8.27	292 ±10.8	78.2 ± 2.86	0.269 ± 0.012	77.7 ± 0.575	53.5 ± 2.92	18.3 ± 0.725	3.06 ± 0.296	13.7 ± 0.960	0.227 ± 0.022
123	194 ± 6.85	333 ± 5.51	87.2 ± 5.45	0.263 ± 0.019	78.0 ± 0.761	60.6 ± 2.22	18.2 ± 0.800	2.02 ± 0.167	10.4 ± 0.835	0.198 ± 0.030

+Zero time equals seven days after castration.

*Body weight heavy at the start of the experiment.

Appendix Table 2. Data obtained from animals treated with coumestrol at 20 micrograms per 0.1cc propylene glycol per day, showing changes in body weight, uterine weight, adrenal weight, percentage of water and nucleic acid level.

Days of Treatment	Body weight at start (gm)	Body weight at end (gm)	Total uterine wet weight (mg)	mg uterine weight per gm body weight	Percent H_2O	Adrenal weight (mg)	mg adrenal per 100 gm body weight	μg RNA per mg uterine wet weight	μg DNA per mg uterine wet weight	RNA/DNA
3	187 ± 6.12	220 ± 5.29	172 ± 8.45	0.785 ± 0.049	79.8 ± 1.69	54.4 ± 3.34	29.9 ± 0.915	2.83 ± 0.342	16.4 ± 1.25	0.177 ± 0.028
6	202 ± 8.35	235 ± 5.50	149 ± 8.04	0.629 ± 0.040	80.7 ± 0.630	63.2 ± 6.19	26.6 ± 0.600	2.49 ± 0.048	13.8 ± 1.52	0.182 ± 0.026
12	218 ± 7.66	263 ± 8.90	136 ± 9.40	0.515 ± 0.019	79.1 ± 0.516	63.8 ± 6.65	24.2 ± 1.92	2.67 ± 0.153	10.8 ± 0.556	0.247 ± 0.007
24	200 ±10.1	230 ±17.4	123 ±17.1	0.583 ± 0.039	80.4 ± 0.296	66.2 ± 4.20	27.5 ± 1.14	2.48 ± 0.125	9.73 ± 0.869	0.255 ± 0.014
48	171 ± 6.24	281 ± 7.53	94.6 ± 2.42	0.338 ± 0.014	77.1 ± 0.855	49.7 ± 2.38	17.7 ± 0.882	2.63 ± 0.123	11.8 ± 0.572	0.225 ± 0.012
72	193 ± 6.25	295 ± 8.85	101 ± 5.35	0.347 ± 0.031	79.0 ± 0.937	54.1 ± 2.38	18.4 ± 0.930	2.32 ± 0.165	10.2 ± 1.13	0.242 ± 0.029
98	177 ± 4.55	296 ±12.7	97.2 ± 5.55	0.339 ± 0.040	78.6 ± 0.454	50.1 ± 1.87	17.1 ± 1.14	2.97 ± 0.068	9.81 ± 0.564	0.307 ± 0.018
119	193 ± 5.74	300 ± 7.33	115 ± 5.16	0.385 ± 0.035	79.7 ± 0.589	49.3 ± 2.96	16.6 ± 1.49	2.92 ± 0.250	9.41 ± 0.387	0.311 ± 0.024
123	191 ± 9.83	308 ± 3.02	106 ± 6.15	0.344 ± 0.019	80.1 ± 0.294	50.1 ± 1.89	16.3 ± 0.630	2.98 ± 0.425	8.34 ± 0.704	0.354 ± 0.021

Appendix Table 3. Data obtained from animals treated with estradiol 17-β at 0.1 micrograms per 0.1cc propylene glycol per day, showing changes in body weight, uterine weight, adrenal weight, percentage of water and nucleic acid level.

Days of Treatment	Body weight at start (gm)	Body weight at end (gm)	Total uterine wet weight (mg)	mg uterine weight per gm body weight	Percent H_2O	Adrenal weight (mg)	mg adrenal per 100 gm body weight	μg RNA per mg uterine wet weight	μg DNA per mg uterine wet weight	RNA/DNA
48	193 ± 4.45	268 ± 4.86	234 ± 12.8	0.875 ± 0.048	80.2 ± 1.06	67.2 ± 2.78	25.2 ± 1.23	2.65 ± 0.064	3.72 ± 0.386	0.693 ± 0.086
72	194 ± 7.78	291 ± 7.71	243 ± 18.9	0.834 ± 0.064	80.3 ± 0.203	60.9 ± 0.795	21.0 ± 0.445	2.90 ± 0.244	6.20 ± 0.615	0.488 ± 0.052
98	184 ± 6.33	292 ±18.6	226 ± 17.7	0.796 ± 0.105	79.5 ± 0.525	68.3 ± 6.49	23.8 ± 3.08	3.00 ± 0.305	5.88 ± 0.301	0.520 ± 0.073
119	192 ± 5.00	288 ±19.0	241 ± 8.85	0.766 ± 0.051	79.3 ± 0.291	62.5 ± 5.50	21.6 ± 0.844	2.51 ± 0.051	5.12 ± 0.140	0.492 ± 0.014
123	190 ± 6.94	286 ±10.6	**271 ± 6.42	0.957 ± 0.055	80.0 ± 0.306	63.7 ± 2.31	22.4 ± 0.965	2.42 ± 0.104	5.08 ± 0.276	0.482 ± 0.028

**Tumorigenic nodules present.

Appendix Table 4. Data obtained from animals treated with 0.1cc propylene glycol per day, showing changes in body weight, uterine weight, adrenal weight, percentage of water and nucleic acid level.

Days of Treatment	Body weight at start (gm)	Body weight at end (gm)	Total uterine wet weight (mg)	mg uterine weight per gm body weight	Percent H_2O	Adrenal weight (mg)	mg adrenal per 100 gm body weight	μg RNA per mg uterine wet weight	μg DNA per mg uterine wet weight	RNA/DNA
48	195 ± 3.45	290 ± 6.91	175 ±20.2	0.602 ± 0.066	79.2 ±0.480	58.6 ± 4.56	20.2 ±1.21	2.94 ± 0.133	7.87 ± 0.411	0.376 ± 0.014
72	189 ± 8.20	284 ±11.6	**228 ±17.7	0.819 ± 0.042	80.6 ±0.283	59.6 ± 2.56	21.0 ± 0.499	2.06 ± 0.056	5.43 ± 0.372	0.386 ± 0.026
98	180 ± 2.90	296 ± 5.55	**254 ± 9.51	0.860 ± 0.040	80.5 ±0.250	63.1 ± 6.10	21.4 ± 2.26	2.80 ± 0.503	5.12 ±0.522	0.546 ± 0.017
119	193 ± 5.72	286 ±10.7	279 ±47.4	0.978 ± 0.160	80.4 ±0.292	59.4 ± 4.04	21.0 ± 2.09	3.12 ± 0.153	5.27 ± 0.414	0.596 ± 0.021
123	177 ± 3.86	302 ± 9.59	***259 ±11.0	0.861 ± 0.049	80.9 ±0.272	64.5 ± 3.98	21.3 ± 0.996	3.10 ± 0.348	4.22 ± 0.382	0.733 ± 0.021

** Tumorigenic nodules present.

*** Pyometric and tumorigenic nodules present.