AN ABSTRACT OF THE THESIS OF

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Title EFFECTS OF CHRONIC ESTROGEN TREATMENT ON UTERINE NUCLEIC ACIDS

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The effects of estrogen treatment on uterine weight, uterine nucleic acid content and adrenal weight were studied. Daily doses of 0.05 μg or 0.1 μg of Estradiol 17-β were administered subcutaneously to seven day castrated female rats for periods varying from 0 to 168 days. After two days of treatment uterine weight, total uterine RNA and RNA/DNA ratio showed marked increases over the castrate condition. The rapid uterine growth occurring during the first two days ceased and the uterine weights were maintained close to the levels of the two day response for periods of treatment as long as 48 days. This cessation of uterine growth was accompanied by a decline in total RNA and RNA/DNA ratio. There were no significant changes in total DNA during these periods.

The ability of the uterus to respond to changing levels of estrogen treatment was tested during the period of cessation of

growth and decline in RNA. After 36 days at a constant dosage the uterus responded to an increase in dosage with rapid growth and marked increases in total RNA and RNA/DNA ratio with no significant change in total DNA. After two days at the higher dosage these increases leveled off even though treatment was continued at the higher level. The uterus responded to a lowering of the dosage with a drop in uterine weight, total RNA and RNA/DNA ratio with no significant change in total DNA.

Beginning at about 48 days of treatment all groups, irrespective of previous treatments, showed increasing values for total RNA, RNA/DNA ratio and uterine weight. At periods of treatment under 60 days there was little evidence of a significant increase over zero time values, in total DNA. However, most of the groups autopsied after 119 days or more of estrogen treatment showed values for total DNA which were markedly higher than the total DNA value at zero time.

After prolonged treatment many of the groups showed a great deal of variability with certain individuals having very high values for uterine weight, total RNA and total DNA. Sometimes these large uteri were covered with small nodules under the uterine peritoneum. Pending histological examination these are thought to be due to endometrial glandular hyperplasia, with the bases of the endometrial glands having proliferated out through the muscularis.

EFFECTS OF CHRONIC ESTROGEN TREATMENT ON UTERINE NUCLEIC ACIDS

by

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EFFECTS OF CHRONIC ESTROGEN TREATMENT ON UTERINE NUCLEIC ACIDS

INTRODUCTION

Since the discovery, chemical isolation, and identification of estrogens, the role of these hormones in controlling female reproductive phenomena has been the object of much study. Foremost among the biological actions of estrogens, is their ability to promote growth of the female reproductive tract. The classical illustration of this is that involution of the vagina, cervix and uterus, following removal of the ovaries, is prevented by the administration of estrogen. Also, the atrophied reproductive tract of an ovariectomized female is rapidly restored by estrogens.

It has been found that the extremely rapid growth characteristic of the uterus during the first few days of estrogen treatment is not maintained during prolonged estrogen treatment. In the case of rhesus monkeys (Macaca mulatta) subjected to prolonged estrogen administration, growth of the uterus, especially the endometrium, is limited. The general morphology of such endometria treated with 10 μg of Estradiol-17β per day for 100 days is similar to that found during the follicular phase, except that the stroma may be more dense, and the cells of the glandular epithelium have large glycogen deposits between the nucleus and basement membrane. Metabolically, however, such endometria are surprisingly inactive. The activity of

oxidative enzymes, and the ratio of ribonucleic acid to deoxyribonucleic acid (RNA/DNA) are about the same as in the involuted endometria of castrated animals (16, p. 560-561).

The effects of prolonged estrogen treatment on the female reproductive tract of mice, rats, guinea pigs and rabbits, have been investigated and reviewed (7; 13; 14; 15; 20). In the vast majority of these studies, the observations have been of a morphological nature, with little being done in respect to the biochemical changes that might be taking place. In general, the morphological features of the uteri of rats or mice subjected to prolonged estrogen treatment, are as follows. The uterus is maintained in what has been termed the full estrous condition (25). Glandular hyperplasia may occur, with the bases of the uterine glands, in some cases, pushing out through the muscularis and forming nodules underneath the uterine peritoneum. The endometrium may undergo epidermidalization or squamous metaplasia with cornification, and, in some cases, endometrial pearl formation. Pyometra may be present as a result of bacterial invasion via the vagina and cervix. In a few cases, cancer has occurred.

Interest in the present investigation was prompted by the observation that the cessation of endometrial growth in monkeys subjected to prolonged estrogen treatment was accompanied by a decline in the RNA/DNA ratio. It had been shown that estrogen induced

uterine growth, in both the monkey (16, p. 560-561) and rat (28), was associated with a high RNA/DNA ratio. Studies on the mouse indicated that changes in the RNA/DNA ratio of the uterus of normally cycling female mice could be correlated with the estrus cycle. The RNA/DNA ratio is highest at estrus and lowest at diestrus (12). Additional observations in mice, rats, rabbits and humans have confirmed the concept that estrogen induced uterine growth is associated with high levels of RNA (3; 5; 6; 17; 22; 27; 29). This is in agreement with the work of many investigators linking high levels of RNA with protein synthesis (4). The significance of relating RNA to DNA by using the RNA/DNA ratio has been discussed by Davidson and Leslie (9). It has been shown that the DNA per cell nucleus in the estrogen-stimulated rat uterus is constant (1), and therefore, the RNA/DNA ratio gives a number which is directly proportional to the amount of RNA per cell. With the exception of the work referred to on the rhesus monkey, studies of the effects of estrogen treatment on uterine nucleic acids have involved short periods of treatment, but the effects of prolonged estrogen treatment have not been investigated.

The experiments in this paper were designed to determine if the nucleic acids of the rat uterus responded to prolonged estrogen treatment in a manner similar to those of the monkey. If it was found that the response was similar, then the effects of raising or

lowering the estrogen dosage, at a time when the RNA/DNA ratio was declining, could be determined. The object of the latter was to find whether or not the tissue was actually becoming refractory to the hormone, or if a steady state was being re-established at a new level, with the system still maintaining sensitivity to estrogen.

METHODS AND MATERIALS

The animals used in this investigation were sexually mature, female rats of the Wistar strain, obtained from Pacord Research Inc., Beaverton, Oregon. The estrogen used was estradiol-17β (Schering Corp.). Propylene glycol (U.S.P. Fischer Scientific Co.) was used as a carrier.

The animals were castrated under ether anesthesia, using the dorso-lumbar approach. With the exception of a few groups, the animals were about 100 days old and weighed approximately 200 grams at the time of castration. Body weight data for all groups are given in the Appendix. On the seventh day after castration, daily injections of estrogen were begun. The desired dose was administered subcutaneously in 0.1 cc of propylene glycol. The different concentrations were made by serial dilutions. The treatments were divided into five general categories:

(1) Animals receiving no injections (castrate controls)

(2) Animals receiving 0.05 μg Estradiol-17β per day

(3) Animals receiving 0.1 μg Estradiol-17β per day

(4) Animals receiving 0.05 μg Estradiol-17β per day for 36 days, after which time the dosage was raised to 0.1 μg per day

(5) Animals receiving 0.1 μg Estradiol-17β per day for 36 days, after which time the dosage was lowered to 0.05 μg per day.

These dosages were chosen because it was felt that they represented values that were well within physiological limits. Taking seven days after castration as time zero, the periods of treatment ranged from 0 to 168 days.

At the end of treatment, the rats were killed by decapitation. The entire uterus was dissected out and weighed, with portions of the uterus reweighed for dry weight, RNA, and DNA determinations. Often a portion of the uterus was fixed in Bouin's Fluid for histological purposes. The adrenals were also dissected out and weighed. All wet weights were determined to the nearest 0.1 mg. on a Roller-Smith torsion balance. The uterine portions taken for dry weight determinations were placed in tared aluminum pans and dried in an oven at 100° C for 24 hours or more. The pans, plus dried uterine segments, were then allowed to cool in a desiccator over anhydrous calcium chloride and weighed to the nearest 0.1 mg. on a Mettler balance. The uterine portions taken for nucleic acid determination were plunged immediately after weighing, into ice cold ten percent trichloroacetic acid. Duplicate samples of uterine tissue for nucleic acid determination were taken from each animal, except from the castrate controls. In the latter the uteri were so small that only one sample could be taken. A sample for nucleic acid analysis consisted of 50 to 70 mg of tissue taken from the body of a uterine horn.

The nucleic acid determinations were considered to be of primary importance, so the first two uterine portions removed were used for nucleic acid analysis. A third portion was taken for the determination of water content. It should be pointed out that the values for the percent of water obtained by this method may be slightly lower than would normally be expected. This is due to the time taken in handling and weighing the entire uterus and the first two portions, which may have allowed the third portion to become slightly more desiccated. In the standard procedure the uterus is simply weighed and immediately placed in the drying pan.

The procedure used for extraction of nucleic acids is based on those of Schneider (24), and of Ogur and Rosen (23), as modified by Burton (8). The tissue was homogenized and extracted with cold ten percent trichloroacetic acid, followed by a second extraction with five percent trichloroacetic acid. The above steps were done in a cold room at 0^{o} to 4^{o} C. The tissue was then washed twice with 95 percent ethanol, but the extraction of lipids was omitted (28). The nucleic acids were extracted according to the method of Burton (8), using two extractions in 0.5 N perchloric acid, heating each time in a water bath at 70^{o} C for 15 minutes. The two perchloric acid extracts were combined, and two sets of duplicate aliquots were taken for the colorimetric estimation of RNA and DNA.

DNA was estimated by the diphenylamine reaction of Dische

(11), as modified by Burton (8). The standard used was deoxyribonucleic acid, sodium salt, type III, highly polymerized from Salmon sperm obtained from the Sigma Chemical Company. It was dissolved in 0.5 N perchloric acid by heating at 70° C for 15 minutes. RNA was determined by the orcinol reaction of Meybaum (21). The standard used was ribose, obtained from Schwartz Laboratories Inc. It was dissolved in 0.5 N perchloric acid. Eight micrograms of ribose was considered equivalent to 30 micrograms of RNA.

The optical densities of duplicate color developments were averaged, and from these values, the concentrations of the two nucleic acids in each of the uterine portions were calculated. When two uterine portions from the same animal were analyzed, the average RNA and DNA concentrations were calculated for that animal.

Each experimental group consisted of five or six animals. In each experimental group, the mean and standard error were computed for: uterine wet weight; percentage of water in the uterus; adrenal wet weight; micrograms of RNA per milligram uterine wet weight; micrograms DNA per milligram uterine wet weight; total RNA per uterus; total DNA per uterus; and RNA/DNA ratio. The standard error was computed according to the following formula (26, p. 91-92):

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

RESULTS

All data are summarized in tabular form in the appendix. In the data for seven of the experimental groups, the bodyweights at the time of castration were markedly higher than in the other 36 groups. Since uterine weight, total RNA, and total DNA are dependent on bodyweight, data concerning these end points taken from the heavier groups, are not comparable to corresponding data from the other 36 groups. Rather than using a correction factor to fit such data into the graphs, these few points have been omitted. However, since the RNA/DNA ratio is not considered to be bodyweight dependent, the RNA/DNA ratios for the heavier groups have been included in the graphs.

It will be noticed that the curve termed "castrate control" in the graphs continues to drop during the early stages of the experiment. This is due to the fact that at time zero, seven days after castration, the uterus was still involuting. The groups comprising this curve were castrated, in most cases, at the same time that the animals receiving estrogen for the corresponding points were castrated. In order to determine how long all groups have been castrated, it is necessary to add seven days to the number of days on the graph, since these numbers refer to days of estrogen treatment.

The results of the first 48 days of treatment at 0.05 μg and

0.1 μg of estradiol per day are graphed in Figures 1, 2 and 3. During the first two days of treatment the uterine weight, total RNA, and the RNA/DNA ratio all show a marked increase. At the dosages used, the two-day responses are directly proportional to the amount of estrogen injected. With the methods employed, no significant change in total DNA could be detected during the first two days.

As the treatment was continued past two days, very little increase in uterine weight was apparent (Figure 1), so that after 48 days of treatment, the uterine weights show little increase over those at two days. Though the uterine weight is maintained at these levels, the same cannot be said for the total RNA or RNA/DNA ratio, both of which show a decline beginning on or about the second day of treatment.

At time zero the total amount of RNA per uterus (Figure 2) was 514$\pm$41 μg. After two days of treatment with 0.1 μg per day the total RNA had risen to 933$\pm$60 μg, but after 24 days of treatment this had declined to 690$\pm$26 μg. The total RNA remained very close to this level for as long as 48 days. With a treatment of 0.05 μg per day a similar pattern is produced except that the values are lower. After two days at this dosage the total RNA was 705$\pm$4 μg, but this had fallen to 607$\pm$27 μg after 24 days of treatment.

The RNA/DNA ratio (Figure 3) after two days with 0.1 μg estradiol per day was 0.71$\pm$0.048, but after 24 days of treatment it

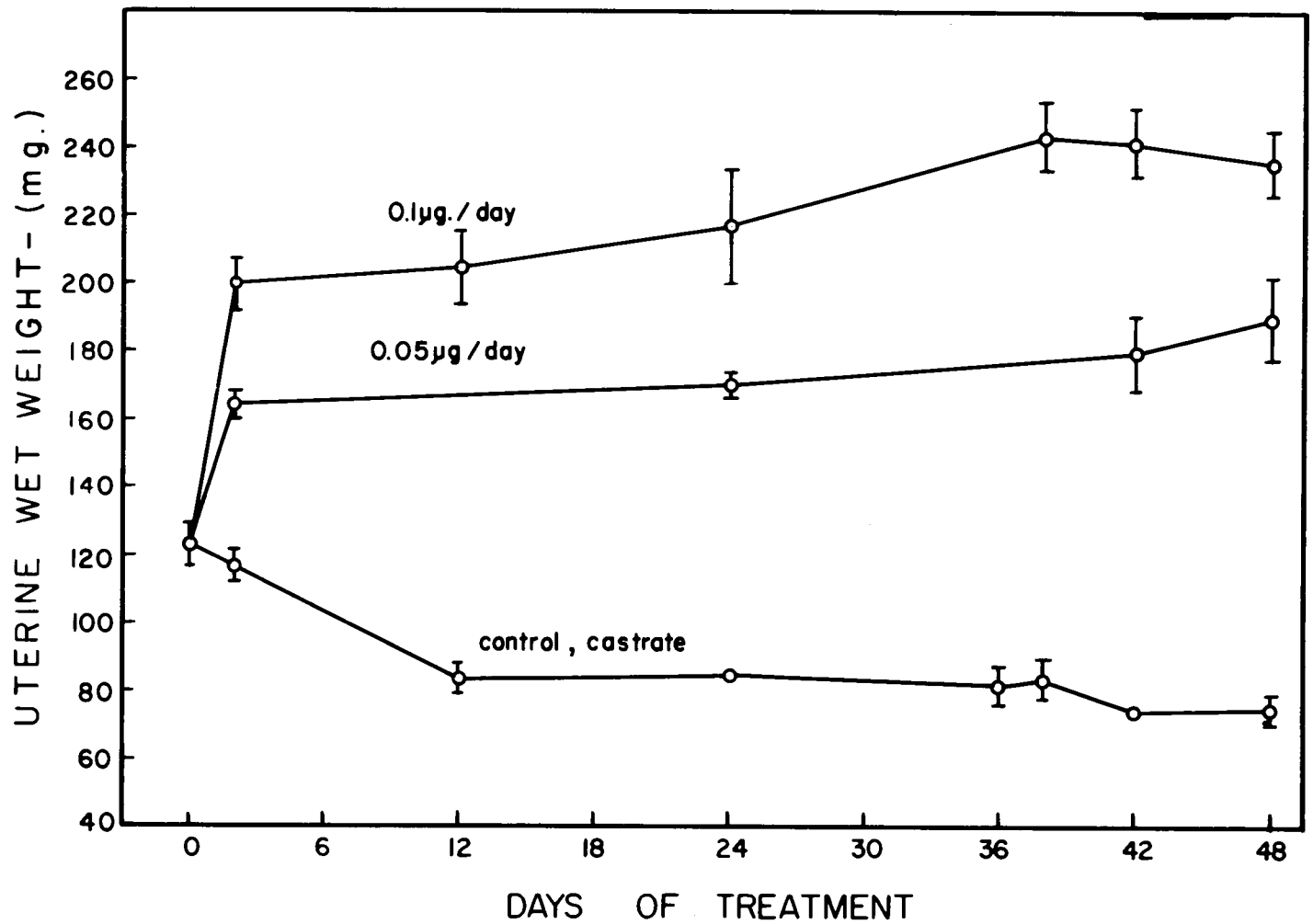

Figure 1. Uterine wet weight in response to Estradiol 17-β.

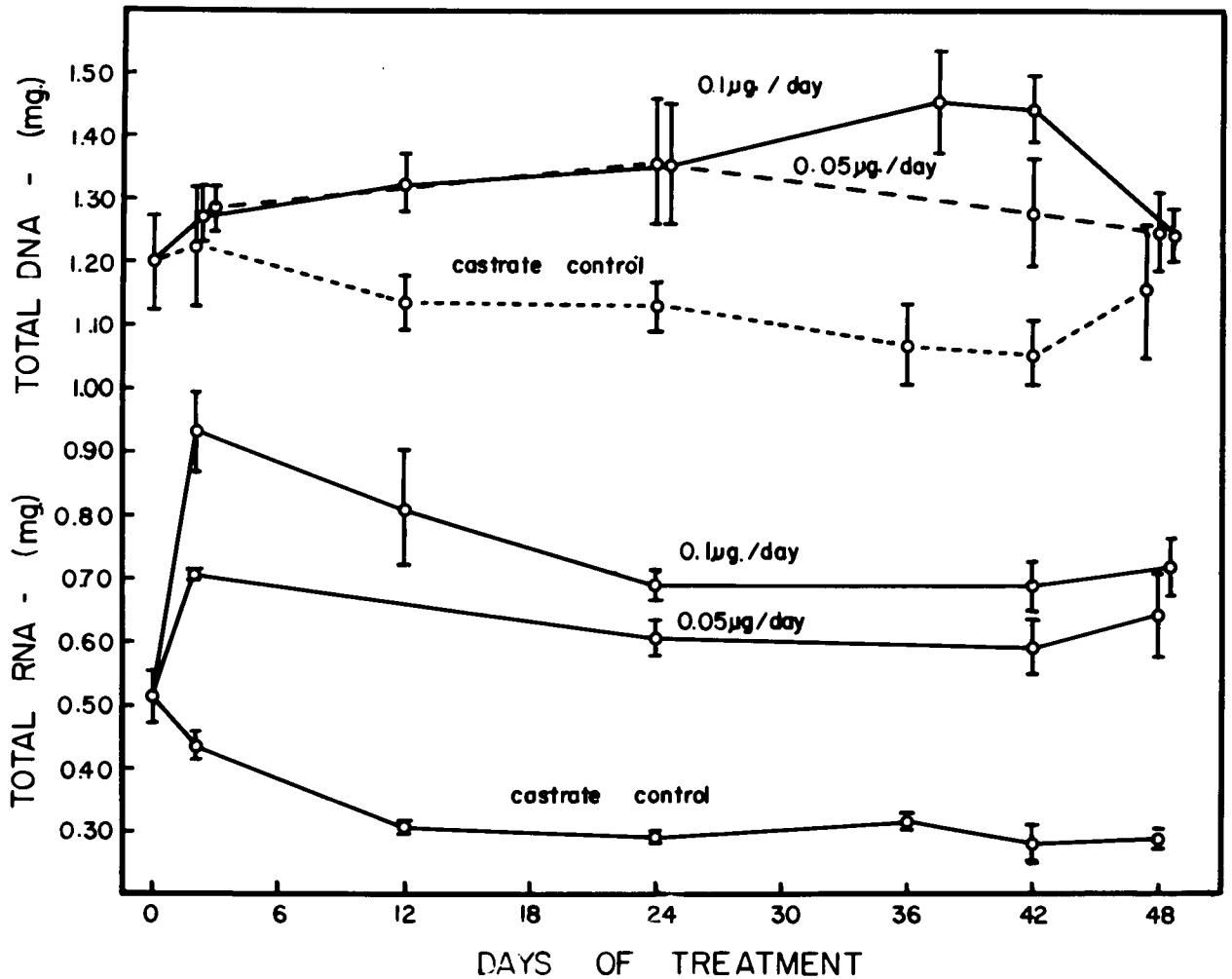

Figure 2. Total RNA per uterus and total DNA per uterus in response to Estradiol 17-β.

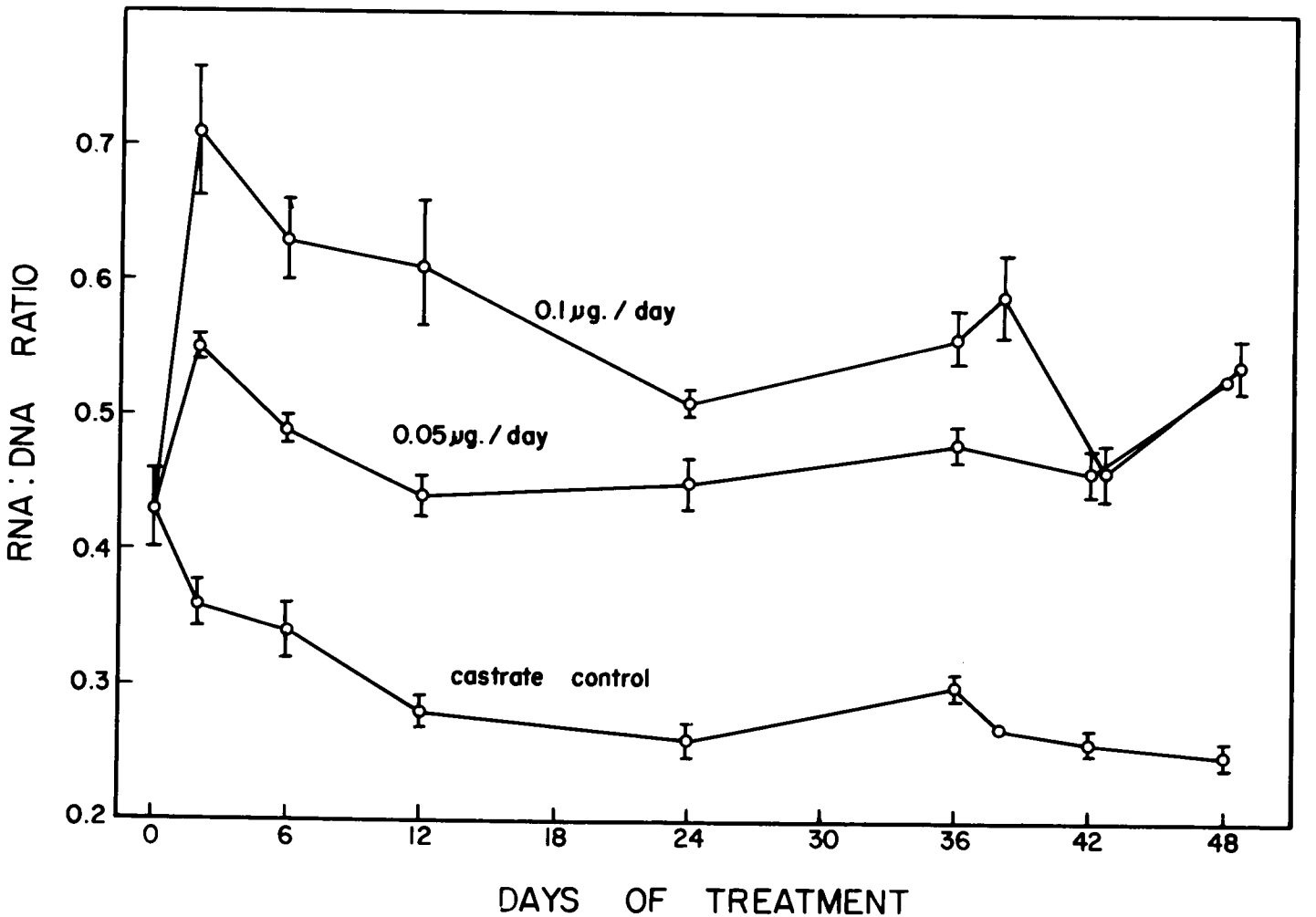

Figure 3. RNA/DNA ratio in response to Estradiol 17-β.

was only 0.51±0.01. After two days with 0.05 μg per day the ratio was 0.55±0.008, but ten days later this had fallen to 0.44±0.01. The ratio at zero time was 0.43±0.029. The RNA/DNA ratio became more or less stabilized at the lower levels until about 42 days of treatment, having only a slight tendency to rise.

The castrated animals receiving no estrogen show involution of the uterus accompanied by a marked decrease in uterine weight, total RNA and RNA/DNA ratio (Figures 1, 2 and 3). These end points show a rapid decline during the first 19 days after castration (equivalent to the 12-day points on the graphs). After this initial rapid decline a basal level is reached. Here again, changes in the total DNA are not significant.

Immediate Effect of Changing Dose

The results of experiments in which the dosage was either raised or lowered after 36 days of treatment at a constant dosage, are summarized in Tables 1, 2 and 3. Table 1 shows the effects of raising the dosage from 0.05 μg per day to 0.1 μg per day. Table 2 shows the effects of raising the dosage from 0.1 μg per day to 0.2 μg per day. The uterine weight, total RNA and RNA/DNA ratio of groups in which the dosage was raised were markedly increased over corresponding groups maintained on the same dosage. There was no significant change in total DNA.

Table 1. Effect of raising dosage of Estradiol-17β from 0.05 μg per day to 0.1 μg per day on the 37th day of treatment.

	Low Dosage for 36 Days	2 Days after Raising Dosage	High Dosage for 36 Days
Uterine weight (mg)	244.4 ±14.2	303.2 ±17.9	323.2 ± 7
Total RNA (μg)	894 ±57	1230 ±47	1081 ±49
Total DNA (μg)	1847 ±87	2040 ±94	1912 ±28
RNA/DNA	0.48± 0.013	0.61± 0.005	0.56± 0.087
Bodyweight-start	246 ± 5.3	236 ± 5.5	224 ± 8
Bodyweight-finish	318 ± 6.9	315 ± 7	296 ±10.8

Table 2. Effect of raising dosage of Estradiol-17β from 0.1 μg per day to 0.2 μg per day on the 36th day of treatment.

	Low Dosage for 35 Days	2 Days after Raising Dosage
Uterine weight (mg)	243.8 ± 9.88	288.9 ± 7.5
Total RNA (μg)	853 ±20	1092 ±70
Total DNA (μg)	1460 ±78	1435 ±33
RNA/DNA	0.59± 0.031	0.76± 0.038
Bodyweight-start	207 ± 7.7	187 ± 1.9
Bodyweight-finish	293 ±12.2	248 ± 4.5

Table 3. Effect of lowering dosage of Estradiol-17β per day to 0.05 μg per day on the 37th day of treatment.

	High Dosage for 38 Days	2 Days after Lowering Dosage	6 Days after Lowering Dosage
Uterine weight (mg)	243.8 ± 9.88	223.4 ± 3.5	204 ±34
Total RNA (μg)	853 ±20	691 ±52	541 ±36
Total DNA (μg)	1460 ±78	1318 ±87	1429 ±73
RNA/DNA	0.59± 0.031	0.52± 0.023	0.35± 0.008
Bodyweight-start	207 ± 7.7	200 ± 4.6	204 ± 4.4
Bodyweight-finish	293 ±12.2	292 ± 8.3	277 ±10.5

Table 3 shows the results of lowering the dose from 0.1 μg per day to 0.05 μg per day. Lowering the dose caused a reduction in uterine weight, total RNA and RNA/DNA ratio. This reaction was readily apparent after two days and became very pronounced after six days, especially the RNA/DNA ratio.

Chronic Effects

The results of experiments in which estrogen treatment was continued for periods of 48 days or more, are graphed in Figures 4, 5 and 6. Some time after 48 days of treatment there occurs a resumption of uterine growth. Two days after zero time, at 0.1 μg of estradiol per day, the average uterine weight is 199.7$\pm$7.2 mg. After 48 days at the same dosage the weight has increased to 236.6$\pm$9.4 mg, an increase of 37 mg or roughly 0.75 mg per day. In contrast, assuming a constant rate of growth between 48 and 119 days, a growth rate of roughly two milligrams per day would have to be maintained for the average uterine weight at 119 days, 320$\pm$ 13.4 mg, to be reached. Similarly on a treatment of 0.05 μg per day the uterine growth rate between 48 and 119 days would have to be roughly twice the rate between 2 and 48 days.

More striking than the resumption of uterine growth, however, is the increase in RNA under these prolonged treatments (Figures 5 and 6). This is a complete reversal of the trend observed between

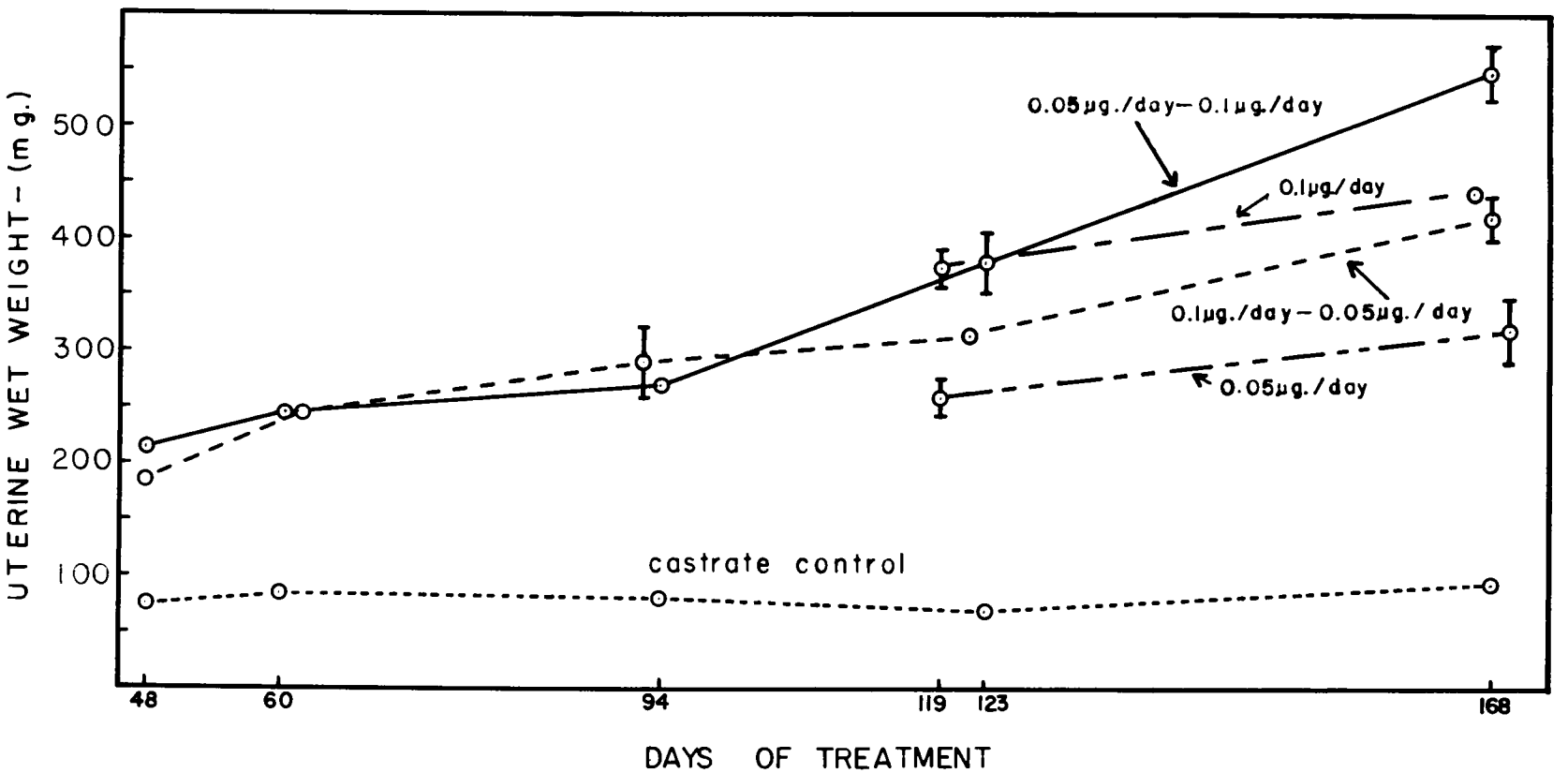

Figure 4. Chronic effects of Estradiol 17-β on uterine wet weight.

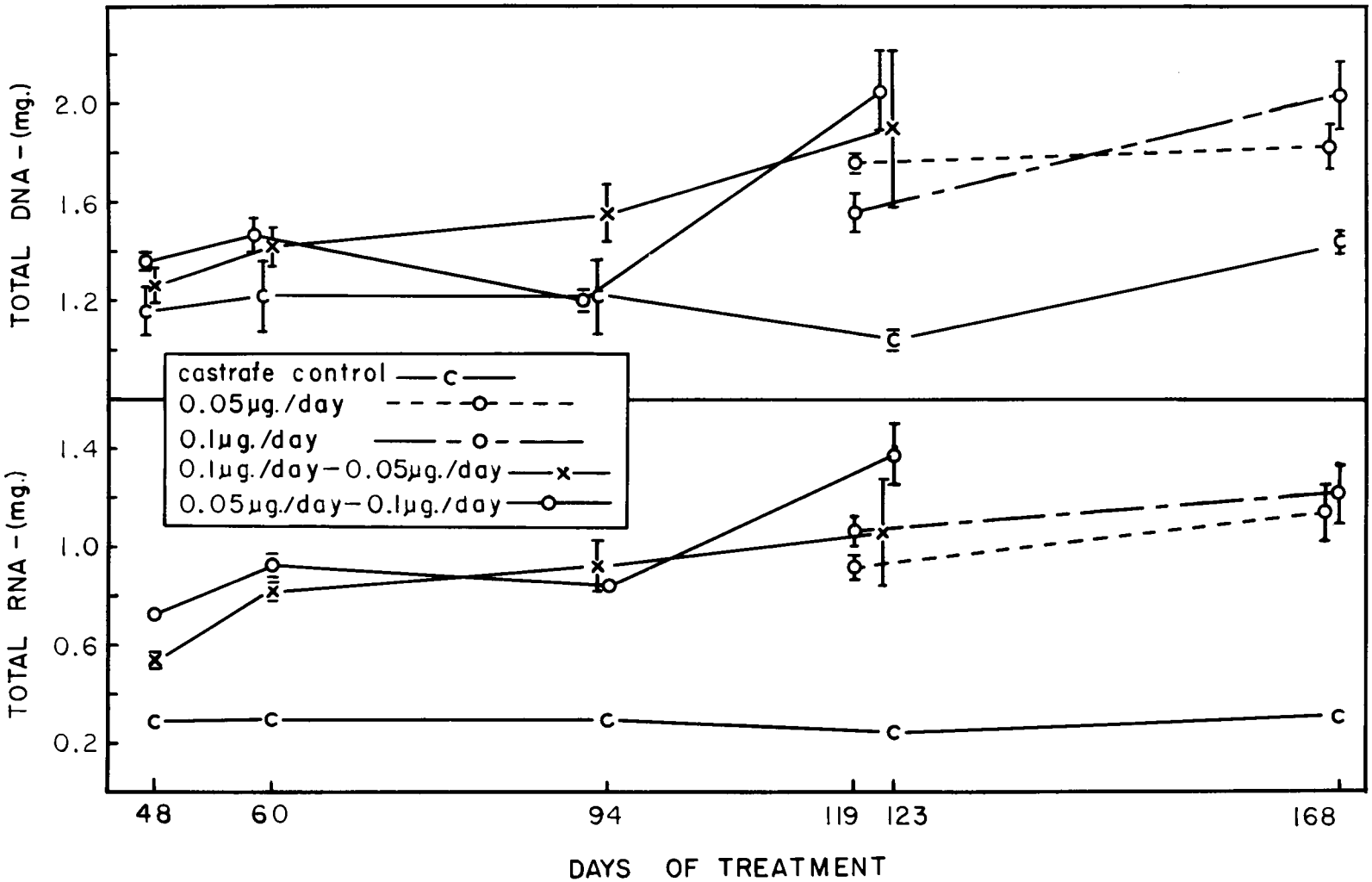

Figure 5. Chronic effects of Estradiol 17-β on total RNA per uterus and total DNA per uterus.

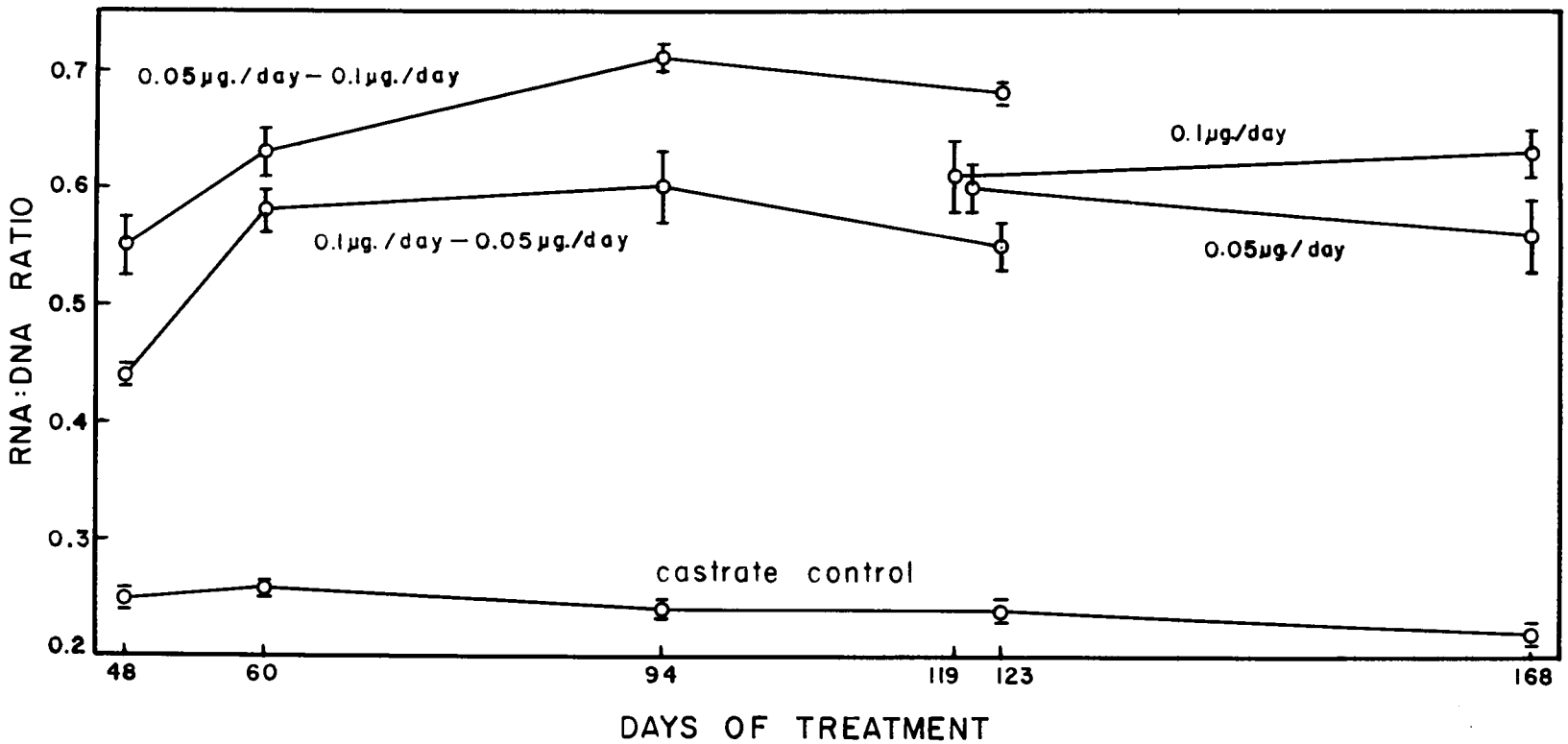

Figure 6. Chronic effects of Estradiol 17-β on RNA/DNA ratio.

2 and 48 days of treatment. After 168 days of treatment at 0.1 μg per day the total RNA per uterus (Figure 5) is 1231±115 μg. This value is nearly double the level at 48 days of treatment (671±46) and is markedly higher than the peak value of 933±60 μg obtained after two days of treatment. The animals on a treatment of 0.05 μg show a similar pattern.

This resumption of uterine growth and increase in RNA appears to be related more to length of treatment than dosage. The data for periods of treatment in excess of 48 days, from groups which were subjected to changes in dosage level, are plotted in Figures 4, 5 and 6. Despite the immediate abrupt changes which occurred in response to changes in dosage (Tables 1, 2 and 3) the trend of these groups from 48 days on, is toward increases in uterine weight and RNA. The highest average uterine weight recorded in these experiments, 551±24 mg was from a group which had received 0.05 μg of estradiol per day for 36 days followed by 0.1 μg per day for 129 days, a total period of 165 days. The tendency toward a recovery of uterine growth is especially surprising in the case of those animals subjected to a drop in the daily dose of estrogen. The initial response to the lower dosage was a drop in uterine weight, and RNA (Table 3). However, this did not persist. Twenty-four days after the dose was lowered, the uterine weight had returned to the same level as before the dose was lowered. Not

only was this weight regained, but the data show increasing uterine weights, so that after a total period of treatment of 165 days, an average uterine weight of 422.6±28 mg was recorded. The increases in uterine weight are also accompanied, in these groups, by increases in RNA.

The RNA/DNA ratios at periods of treatment of 60 days or more (Figure 6), also show general increases over the values for the low points recorded earlier during the period of decline. These values tend to maintain a more uniform level, with differences between the treatments being maintained more distinctly. It is interesting to note that in three of the four treatments, RNA/DNA ratios are recorded which are as high or higher than those recorded for the two day treatments at the same dose. The only exception is the 0.1 μg per day treatment where the ratios, though high, do not reach the level of those on the second day.

The data concerning total DNA are plotted in Figures 2 and 5. At periods of treatment up to 60 days, it cannot be said that there is any consistant trend toward a change in total DNA from that at zero time. However, values of total DNA on treatments of 119 days or longer, with the exception of the group on 0.05 μg per day for 119 days, are all definitely higher than either the controls castrated at the same time as the animals on treatment, or the total DNA values at zero time.

In some of the groups on prolonged treatment, there was more variability than usual, especially in uterine weight, total RNA, and total DNA. It was noticed at autopsy of these groups that the uteri of some individuals were especially large and studded with numerous small nodules which appeared to be pushing out from the center of the uterus. When these uteri were opened and examined grossly, the surface of the endometrium possessed many small nodules which were tender and ruptured easily to exude a brown pussy material. It is thought that the external swellings may have been due to glandular hyperplasia. In this condition the bases of the endometrial glands proliferate down through the muscularis and appear as protrusions under the surface of the uterine peritoneum (2). Tissue from these uteri has been fixed, and the above diagnosis is pending histological verification. The pussy condition of the inner part of the uterus may have been due to pyometra caused by bacterial infection via the vagina (2; 32). Groups in which individuals showing these conditions appeared, are marked in the tables in the appendix.

Complete data for adrenal weights are tabulated in the appendix. In the castrate controls the adrenals gradually lost weight. All adrenal weights from animals castrated 43 days or longer are 50 milligrams or less. In the estrogen treated animals this atrophy was prevented. Most of the adrenal weights from animals receiving

0.05 μg per day are about 57 mg. The adrenal weights of the groups receiving 0.1 μg per day, tended to be heavier, with most values over 60 mg. A notable exception to this is the two longest treatments, 119 and 168 days, where the average adrenal weights are only 54.1 and 56.6 mg. A similar trend is seen in the 168 day treatment at 0.05 μg per day. This trend is again seen in the groups which were subjected to a change in dosage level. Here the adrenal weights at 123 and 165 days of treatment are lighter than any of the previous adrenal weights in the series. The more immediate adrenal response to a rise in dosage was an increase in weight. The adrenals tended to lose weight when the dose was lowered. None of the adrenal weight changes were very pronounced. However, these adrenal changes do give some hint that an estrogen-adrenal interplay may be related to some of the changes observed in the uterus.

DISCUSSION

The results of this investigation confirm the work of others (12; 22; 28;29). They showed that the early rapid growth response of the uterus to estrogen treatment is accompanied by rapid synthesis of RNA leading to high levels of total RNA and RNA per cell. The data confirm the conclusion of these workers that estrogen induced uterine growth is accomplished mainly by hypertrophy rather than hyperplasia.

The results further show that in the rat, as well as the monkey, the high uterine RNA/DNA ratio reached in the early stages of estrogen stimulation is not maintained, even though estrogen treatment is continued. In the rat this decline is correlated with the cessation of rapid uterine growth. A similar situation has been observed in testosterone induced growth of the prostate, seminal vesicles, and kidney (19). In this case it has been postulated that this decline in RNA is due to a negative feedback mechanism which serves to limit the bio-synthetic activity of the system.

The controlling mechanisms involved in the limitation of estrogen induced uterine growth, and the concommitant decline in RNA may be such cellular regulatory mechanisms as negative feedback inhibition of enzyme activity or repression of enzyme formation (10). Data from the work of many investigators indicate that

high levels of RNA are linked with protein synthesis (4). Thus, it can be said that the decline in RNA occurs because the metabolism of the cell is shifting from the rapid synthesis of protein necessary in rapid growth, to maintenance of a nearly steady state.

Factors other than regulatory mechanisms within the tissue must be considered in respect to their possible importance in causing this decline in RNA. Lipschutz has put forth the idea of auto-defense mechanisms against excess estrogens which act in preventing the formation of estrogen induced tumors. He presents evidence that excess estrogen may increase the rate of hepatic inactivation of estrogens (20, p. 194-195). It has been shown that the adrenal corticoids inhibit uterine growth (30; 31). It has been recently demonstrated that low levels of estrogen within the normal range for female rats, stimulate adrenal cortical activity. At higher levels estrogens inhibit adrenal function (18). The doses of estrogen used in the present investigation are not high enough to cause inhibition of adrenal function but, rather, are probably stimulatory.

The data presented in respect to the reaction of the uterus to changing doses after 36 days of treatment clearly demonstrate that the uterus retains sensitivity to estrogen at this stage. The drop in uterine weight, total RNA, and RNA/DNA ratio when the dosage was lowered is similar in principle to the observation by the

Hisaws that the uteri of monkeys on prolonged estrogen treatment will readily show withdrawal bleeding if estrogen treatment is stopped (16, p. 561). The marked increases in uterine weight, total RNA and the RNA/DNA ratio, when the dosage is increased, also indicate that the uterus retains the ability to respond to estrogen. These data support the interpretation that the decline in RNA, observed in studies of prolonged estrogen treatment, does not indicate that the tissue is actually losing its responsiveness to estrogen, but rather, the decline is merely a reflection of the adjustment of cellular metabolism from growth to maintenance.

The results of estrogen treatment for periods greater than 60 days was unexpected. After a period during which the uterus shows little growth and the high RNA levels associated with rapid growth subside, this trend is reversed. Total RNA, RNA/DNA ratio and uterine weight begin to increase again. The rate of increase is not as rapid as during the first two days of treatment but it is definitely a different rate than is observed at periods of treatment from 2 to 42 days in length. This trend began at about 48 days of estrogen treatment irrespective of the previous dosages to which the animals had been subjected. It would seem that this response is more dependent on length of treatment than dosage. This is especially striking in the case of the groups in which the estrogen treatment was lowered at 36 days. The initial response was a drop

in uterine weight and RNA, but this trend is abruptly reversed. Between 48 and 94 days of treatment, these end points show a marked increase.

The cause of this resumption of growth could be attributed to a reversal of the same mechanism which brought about the earlier decline. Alternatively it could be due to a shift in a mechanism other than the one bringing about the decline, but which compensates for the original inhibition of growth and thus allows growth to resume. On the cellular level this change could be attributed to a "derepression" or failure in negative feedback. Until more information concerning the mechanism of action of estrogens on the molecular level is available, the possible interactions between estrogens and metabolic regulation will probably remain obscure. On the systemic level, these changes could be related to changes in adrenal function or hepatic inactivation of estrogens as discussed earlier.

The data on adrenal weights in the appendix give some indication that the adrenals may play a role in the resumption of uterine growth. It was noted in the Results that the adrenals from certain groups on treatments 119 days and longer were lighter than adrenals from animals on the same dosage but shorter treatment periods. These differences would probably be more pronounced if the adrenal weights were expressed as a percentage of body weight. This would make the adrenals of the groups on the longer periods of treatment

appear lighter than those on the shorter periods of treatment. From this, one could put forth the following hypothesis: In the longer periods of treatment the normal effect of these doses of estrogen in stimulating adrenal function (18) wears off, hence the decline in adrenal weight. As adrenal production of corticoids declines, the normal antagonism between the corticoids and estrogen, in respect to uterine growth, is relaxed, and uterine growth is resumed. Gardner has stated that prolonged exposure to estrogens may induce adrenal deficiency (14, p. 207).

Additional information which has a bearing on this, is the fact that estrogens induce thymic regression in mice for about the first ten weeks of continuous estrogen treatment (13, p. 231). After ten weeks, however, the thymus begins to regenerate. It is well known that glucocorticoids cause thymic regression. If the action of estrogens in causing thymic regression is brought about by stimulation of the secretion of adrenal glucocorticoids, which then act on the thymus, then the failure of thymic regression to be maintained after about ten weeks of treatment may be associated with a decline in adrenal secretion under these conditions.

The total DNA data indicate that at periods of treatment under 60 days, the methods of analysis show no detectable increase in total DNA. The total amount of DNA in the uterus is directly proportional to the number of cells in the uterus (1). Therefore, these data are

interpreted as meaning that the predominant type of growth response elicited during this time is hypertrophy rather than hyperplasia. Certain groups subjected to longer periods of treatment, however, show marked increases over zero time and control values. This indicates that part of the growth response of prolonged treatment is due to mitosis, with the production of more cells per uterus.

These growth responses occurring after chronic estrogen treatment are probably related to the tumorigenic and/or carcinogenic properties of estrogens. In view of this, it was not surprising that conditions which appeared to be endometrial glandular hyperplasia, appeared in some of the animals. It has been suggested that bacterial products associated with the septic conditions common in mice on prolonged estrogen treatment are responsible for cervical tumors found in such mice (13). Since conditions resembling pyometra occurred in some of the animals in this study, a possible effect of bacterial products in inducing some of the abnormal conditions seen here cannot be ruled out.

SUMMARY

The effects of estrogen treatment on uterine weight, uterine nucleic acid content and adrenal weight were studied. Daily doses of 0.05 μg or 0.1 μg of Estradiol 17-β were administered subcutaneously to seven day castrated female rats for periods varying from 0 to 168 days. After two days of treatment, uterine weight, total uterine RNA and RNA/DNA ratio showed marked increases over the castrate condition. The rapid uterine growth occurring during the first two days ceased and the uterine weights were maintained close to the levels of the two day response for periods of treatment as long as 48 days. This cessation of uterine growth was accompanied by a decline in total RNA and RNA/DNA ratio. There were no significant changes in total DNA during these periods.

The ability of the uterus to respond to changing levels of estrogen treatment was tested during the period of cessation of growth and decline in RNA. After 36 days at a constant dosage the uterus responded to an increase in dosage with rapid growth and marked increases in total RNA and RNA/DNA ratio with no significant change in total DNA. After two days at the higher dosage these increases leveled off even though treatment was continued at the higher level. The uterus responded to a lowering of the dosage with a drop in uterine weight, total RNA and RNA/DNA ratio with no

significant change in total DNA.

Beginning at about 48 days of treatment all groups, irrespective of previous treatments, showed increasing values for total RNA, RNA/DNA ratio and uterine weight. At periods of treatment under 60 days there was little evidence of a significant increase over zero time values, in total DNA. However, most of the groups autopsied after 119 days or more of estrogen treatment showed values for total DNA which were markedly higher than the total DNA value at zero time.

After prolonged treatment many of the groups showed a great deal of variability with certain individuals having very high values for uterine weight, total RNA and total DNA. Sometimes these large uteri were covered with small nodules under the uterine peritoneum. Pending histological examination these are thought to be due to endometrial glandular hyperplasia, with the bases of the endometrial glands having proliferated out through the muscularis.

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APPENDIX

Appendix Table 1. Noninjected castrate controls.

Days of treatment+	Body weight at start	Body weight at finish	Uterine wet weight	Percent H_2O	Adrenal wet weight	Total RNA in uterus	Total DNA in uterus	μg RNA per mg uterine wet weight	μg DNA per mg uterine wet weight	RNA/DNA
0	190±1.7	209± 2.7	122.6±5.7	75.63	54.0 ±2.34	514±41	1205± 76	4.2±0.19	9.8±0.40	0.43±0.029
2	194±0.81	223± 4.9	117.3±4.6	76.17±0.492	56.8 ±2.77	438±23	1226± 98	3.7±0.087	10.4±0.57	0.36±0.017
6	*227±6.6	*260± 7.1	*120.8±16	77.85±0.421	*61.8 ±3.7	*442±73	*1293± 81	3.7±0.34	11.1±1.2	0.34±0.02
12	191±3.1	247± 9.3	84.5±4	74.4 ±0.05	56.5 ±3.94	317±16	1137± 47	3.7±0.10	13.1±0.40	0.28±0.012
24	199±3.1	280±12.6	84.6±2.5	73.08±1.32	58.98±2.51	290±12	1132± 40	3.4±0.10	13.4±0.71	0.26±0.012
36	187±1.9	268±11.4	82.3±5.2	75.85±1.57	47.8 ±2.97	314±16	1070± 64	4.0±0.19	13.5±0.27	0.30±0.009
38	196±2.2	277± 5.7	84.2±5.6	77.25±0.592	47.0 ±1.38	320±14	1164± 43	3.8±0.13	14.0±0.37	0.27±0.006
42	190±1.4	288± 5.8	74.2±2.2	76.18±1.41	45.72±2.73	281±23	1058± 59	3.7±0.23	14.2±0.56	0.26±0.008
48	202±3.6	291± 9.3	74.6±4.1	77.29±0.345	47.5 ±2.23	289±14	1162±109	3.9±0.075	15.5±0.71	0.25±0.01
60	208±1.4	292±14.2	84.8±6.3	75.80±0.105	45.9 ±1.62	301±14	1162± 48	3.6±0.14	13.9±0.71	0.26±0.004
94	202±3.2	328±20.2	81.5±7.6	77.52±0.415	50.2 ±3.10	291±29	1213±146	3.3±0.14	14.5±0.44	0.24±0.008
123	206±4.1	324±17.6	70.3±3.6	74.12±0.584	48.6 ±1.36	247±12	1037± 40	3.5±0.13	14.8±0.36	0.24±0.01
168	197±1.5	330± 5.9	95.4±7.5	78.18±1.55	49.6 ±2.14	325±21	1442± 54	3.4±0.13	15.4±0.63	0.22±0.013

* Body weights were heavy at start of experiment.

+ Zero time equals seven days after castration.

Appendix Table 2. 0.05 μg Estradiol 17-β per day.

Days of Estrogen treatment	Body weight at start	Body weight at finish	Uterine wet weight	Percent H_2O	Adrenal wet weight	Total RNA in uterus	Total DNA in uterus	μg RNA per mg uterine wet weight	μg DNA per mg uterine wet weight	RNA/DNA
2	197±1.8	226 ± 1.9	163.0± 4.7	77.19±0.320	57.2±1.59	705± 4	1284± 33	4.3±0.10	7.9 ±0.15	0.55±0.008
6	*222±4.2	*246 ±11.6	*177.3± 6	78.71±0.241	*64.4±2.25	* 722± 25	*1459± 35	4.1±0.13	8.25±0.22	0.49±0.01
12	*242±2.2	*290 ± 5.04		78.37±0.213	*65.1±1.38			3.6±0.10	8.2 ±0.13	0.44±0.015
24	195±3	258 ± 4.8	172.7± 3.8	76.55±1.05	58.4±1.66	607± 27	1359±110	3.5±0.16	7.9 ±0.54	0.45±0.019
36	246±5.3	318 ± 6.9	*244.4±14.2	78.46±0.197	*61.4±2.05	* 894± 57	*1847± 87	3.7±0.087	7.6 ±0.18	0.48±0.013
42	196±1.7	287 ± 4.8	179.5±11	76.29±0.240	57.4±3.49	592± 45	1282± 86	3.3±0.09	7.1 ±0.15	0.46±0.018
48	188±0.8	297 ± 4.6	189.7±12	77.4 ±0.406	57.9±3.41	643± 64	1244± 49	3.4±0.17	6.7 ±0.48	0.53±0.07
119	209±5.5	351 ±10.3	261.9±17	77.85±0.285	57.0±2.18	**923± 47	1555± 79	3.6±0.12	6.0 ±0.25	0.60±0.02
168	195±1.7	353 ±12.2	329.0± 6	76.97±0.673	55.6±2.6	1145±115	2044±143	3.5±0.15	6.3 ±0.22	0.56±0.03

* Body weights were heavy at start of experiment.
**Nodules present on some uteri from this group.

Appendix Table 3. 0.1 μg Estradiol 17-β per day.

Days of Estrogen treatment	Body weight at start	Body weight at finish	Uterine wet weight	Percent H_2O	Adrenal wet weight	Total RNA in uterus	Total DNA in uterus	μg RNA per mg uterine wet weight	μg DNA per mg uterine wet weight	RNA/DNA
2	195±2.1	226± 6.6	199.7± 7.2	77.69± .175	57.6 ±2.4	933± 60	1271±11	4.7±0.11	6.4 ±0.24	0.71±0.048
6	*215±7.4	*244± 9.4	231.7±15.6	78.45±0.301	*65.6 ±2.44	*1025± 66	*1629±71	4.4±0.12	7.1 ±0.18	0.63±0.03
12	199±2.6	235± 9.3	205 ±10	79.29±0.311	59.1 ±2.77	807± 90	1322±51	3.9±0.26	6.4 ±0.1	0.61±0.047
24	196±2.2	261± 6	217.4±17	76.44±0.4	61.6 ±1.79	690± 26	1355±96	3.2±0.03	6.25±0.13	0.51±0.01
36	*224±8	*296±10.1	*323.2± 7	76.54±0.291	*63.2 ±3.67	*1081± 49	*1912±28	3.3±0.11	5.9 ±0.087	0.56±0.02
38	207±7.7	293±12.2	243.8± 9.88	79.68±0.86		853± 20	1460±78	3.5±0.21	5.9 ±0.04	0.59±0.031
42	191±1.5	278± 9.1	242.3±11	76.19±0.4	62.01±1.66	659± 42	1445±49	2.7±0.11	6.0 ±0.14	0.46±0.023
48	191±0.8	288± 4.5	236.3± 9.4	77.10±0.397	67.1 ±4.11	671± 46	1250±65	2.9±0.11	5.4 ±0.27	0.54±0.02
119	**204±3.3	320±13.4	373.2±17.5	78.42±0.516	54.1 ±2.95	**1071± 62	1765±40	2.9±0.066	4.8 ±0.19	0.61±0.03
168	194±1.7	363±10.5	443 ±37	80.06±0.579	56.6 ±0.75	1231±115	1933±79	2.8±0.071	4.4 ±0.16	0.63±0.02

* Body weights were heavy at start of experiment.

**Nodules present on some uteri from this group.

Appendix Table 4. Dosage lowered from 0.1 μg Estradiol 17-β per day to 0.05 μg Estradiol 17-β per day on the 37th day of treatment.

Days of Estrogen treatment	Body weight at start	Body weight at finish	Uterine wet weight	Percent H_2O	Adrenal wet weight	Total RNA in uterus	Total DNA in uterus	g RNA per mg uterine wet weight	g DNA per mg uterine wet weight	RNA/DNA
38	200±4.6	292± 8.3	223.4± 3.5	77.3 ±0.316	61.7±3.32	691± 52	1318± 87	3.9±0.12	5.9±0.13	0.52±0.023
42	204±4.4	277±10.5	204 ±34	75.55±0.245	59.9±3.41	541± 36	1429± 73	2.4±0.06	7.0±0.18	0.35±0.0077
48	187±0.8	294± 4.7	184 ± 8.5	76.51±0.083	58.3±0.82	553± 32	1259± 72	3.0±0.06	6.8±0.14	0.44±0.008
60	208±1.6	308±11.4	245.8± 7.4	75.77±0.185	60.3±1.57	820± 38	1420± 82	3.3±0.059	5.8±0.25	0.58±0.017
94	**205±4.5	343±13.5	293.9±33.2	78.1 ±0.608	59.6±2.56	927± 95	1562±125	3.2±0.05	5.3±0.21	0.60±0.032
123	**201±2.2	350±11	312.8±49.6	76.02±0.342	56 ±2.52	1065±212	1905±322	3.3±0.19	6.0±0.20	0.55±0.02
165	**218±5.9	342± 8.1	422.6±28	79.52±0.381	55.8±2.62					

* Body weights were heavy at start of experiment.
**Nodules present on some uteri from this group.

Appendix Table 5. Dosage raised from 0.05 μg Estradiol 17-β per day to 0.1 μg Estradiol 17-β per day on the 37th day of treatment.

Days of Estrogen treatment	Body weight at start	Body weight at finish	Uterine wet weight	Percent H_2O	Adrenal wet weight	Total RNA in uterus	Total DNA in uterus	g RNA per mg uterine wet weight	g DNA per mg uterine wet weight	RNA/DNA
38	236±5.5	*315± 7	*303.2±17.9	79.68±0.86	*64.4±3.33	*1230± 47	*2040± 94	4.1±0.11	6.8±0.18	0.61±0.005
48	189±1.8	285± 7.4	215 ± 7.6	76.4 ±0.907	67.1±3.06	734± 27	1360± 39	3.4±0.092	6.4±0.23	0.55±0.025
60	208±7.7	282±21.7	245.8± 7.4	77.18±0.159	63 ±1.33	933± 55	1474± 69	3.6±0.12	5.7±0.17	0.63±0.02
94	194±1.5	321±10.2	270.7± 7.4	77.82±0.752	60.2±4.56	853± 27	1200± 43	3.2±0.05	4.4±0.11	0.71±0.012
123	**201±4.1	336± 6.2	382.7±27.2	76.27±1.85	55.4±1.88	1383±130	2051±220	3.6±0.16	5.3±0.27	0.68±0.009
165	**220±4.1	336± 5.1	551 ±24	80.83±0.67	57.7±1.49					

* Body weights were heavy at start of experiment.

**Nodules present on some uteri from this group.