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EFFECTS OF A 17-SPIROLACTONE ON A CIRCADIAN RHYTHM AND OTHER PHYSIOLOGICAL SYSTEMS

(Gallus Domesticus)

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ABSTRACT

1. The effect of the $\Delta 4$-androstene-3-one-17-spirolactone on the circadian rhythm of ovulation-oviposition and on anatomical changes of the adrenals, ovary, oviduct, and body weight is presented.

2. This synthetic steroid caused an exponential regression in weight of the adrenals and the ovary, a variable effect in body weight and a linear regression of the oviduct preceded by a short increase in weight.

3. Several possible sites of drug action are suggested.

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INTRODUCTION

Biological rhythms have been observed and evaluated for approximately 200 years (Bunning, 1960). However, the information collected prior to the work by Rowan (1925) on birds was primarily descriptive in nature. Many aspects of the relationship between the environment and the circadian rhythm of ovulation-oviposition have been reported (Farner, 1964; Benoit, 1961; Wolfson, 1963; Wilson and Woodard, 1958; Winget and Griffin, 1962).

The circadian rhythm of ovulation-oviposition in the bird is well established (Nalbandov, 1962), and many of the events in this cycle are documented (Nalbandov, 1958). The onset of the cycle (ovulation) and its termination (oviposition) occurs during hours of light whereas the agent inducing this cycle, luteinizing hormone (LH) is released during darkness. Morris (1961) has established a random distribution of the oviposition

cycle when birds are exposed to 24 hours of light and Wilson and Woodard (1958) have not been able to completely suppress the cycle by exposure to 24 hours of darkness. Light is the "zeitgeber" (timer) for this cycle and the stimulator for growth and development of gonadal tissue (Benoit, 1964). However, the rate of LH release is the controller for the oviposition cycle (Nalbandov, 1962).

The mechanism for control of LH release is still not completely established. Huston (1953) has been able to block LH release in the bird by the stimulation of a neurohumoral system with afferent neurons originating in the oviduct. In addition, Critchlow et al. (1963) have shown a close relationship between neuroendocrine mechanisms and the cyclic release of ACTH in mice.

The purpose of the research reported herein is to study mechanisms associated with the circadian rhythm of oviposition. A synthetic steroid $\Delta 4$-androstene-3-one-17-spirolactone with minimal estrogen-androgen properties, yet capable of interfering with the adrenal hypothalmus axis, was employed. This compound has been shown to be a competitive inhibitor to DCA (Kagawa et al., 1957a). It will block the effects of aldosterone in the rat (Kagawa and Van Arman, 1957b); and in the dog it does not possess anabolic, androgenic, or estrogenic activity (Drill and Riegel, 1958).

METHODS

Single comb white Leghorn hens, approximately 17 months old, were maintained in individual cages with food and water ad libitum. Approximately 5 foot-candles of incandescent light was supplied at the cage front from 6 a.m. to 8 p.m. To condition the birds, each bird was handled daily for 14 days prior to the start of the experiment. Daily records of lay were maintained. Two experimental groups of hens were selected from the birds which had laid an egg each day for at least three successive days immediately before drug administration. One group (96 birds) selected at random was maintained throughout the experimental period to evaluate drug effects on the primary and secondary sex organs, body weight, and the adrenals. A placebo-injected group was maintained throughout the experimental period.

The drug was suspended in corn oil, and a single injection was given in the pectoral muscle in a volume of 0.1 ml between 4 and 5 p.m. Birds were sacrificed at specific times after treatment with an overdose of sodium pentobarbitol; the organs removed, dissected, blotted, and weighed on an analytical balance. Variance and covariance methods were utilized in the analysis of the data.

RESULTS

Graded doses of a 17-spirolactone were administered to laying hens to compare the dose effects of this synthetic steroid on the circadian rhythm of ovulation-oviposition (Fig. 1) and certain organs (Table 1). From a comparison of the initial daily rate of oviposition with the daily rate of oviposition after drug injection, it is apparent that the drug is an effective inhibitor of this circadian rhythm. Furthermore, the degree of inhibition is related to the amount of the compound administered, with the two high levels causing complete cessation of the cycle.

The rates of decrease in the circadian rhythm after drug treatment are given in Table 2. Polynomials were fitted to the response curves by the method of least squares. The control group fits a linear regression, whereas the treated groups were best described by second degree polynomials. In all cases the regression coefficients were highly significant ($p < 0.001$). This indicates a single-phase response for all levels of the spirolactone investigated.

The effect of the spirolactone on ovum formation, measured as a function of ovary weight, has been calculated for each group of birds in terms of a pretreatment weight (Fig. 2). The data suggest a dose gradient effect with the maximum level approximately 50 percent more effective in causing ovary regression than the

minimum level. The ovary weights (Table 1) show a significant dose response ($p < 0.01$) at all levels. However, a comparison of the various levels indicates that the minimum level has significantly different effect than the other levels ($p < 0.01$).

The change in ovary weight following treatment is logarithmically related to the dosage of the drug administered and can be expressed as:

$$\text{Per cent change in ovary weight} = 62e^{-0.04D}$$

where 62 is an extrapolated constant; D is the dosage in mg/kg body weight; and -0.04 is the dose response constant.

The oviducal response curves for all drug levels investigated are similar (Fig. 3) and the oviducal weights (Table 1) show a drug effect over time ($p < 0.01$). Days 2, 5, and 9 are significantly different ($p < 0.01$) from the other days observed and from each other. There was no significant difference in response for the other experimental days. The rate of decrease in oviducal weight per day for the maximum drug level ($k = -0.25$) is somewhat greater than the rate of decrease for the two intermediate levels ($k = -0.18$; $k = -0.16$) but it is smaller in comparison to the lowest drug level ($k = -0.31$); however, the duration of the response to the minimum drug level is shorter than the others. These involution rates are smaller than those reported for "normal" pigeons (Riddle, 1938) undergoing seasonal sexual cycles. On the other hand, they are larger than the rates of regression reported by Smith et al. (1957) for progesterone treated SCWL fowl.

To further elucidate the mechanisms involved in the drug effect on the oviduct, the change in weight of the uterus and magnum were measured. The uterus and magnum appear to have a response similar to the oviduct for this spirolactone. Further, the magnum and shell gland show the same significant variation over time ($p < 0.01$) as the oviduct.

The kinetics of the adrenal response are similar to those observed in the ovary and apparently are also of the first order, and follow the relationship:

$$D = 0.23e^{-0.04T}$$

where 0.23 is an extrapolated constant; D is the adrenal weight (mg) at time T; T is the time (in days) elapsed since drug treatment; -0.04 is the rate constant for adrenal involution; and e is the Naperian number, the base of the natural logarithms. The drug effect may be somewhat larger than the data indicate since the experiment was performed in the summer months (June and July) when the adrenal weight would be increasing (Kornfeld, 1957). The variability (±0.05) of the adrenal weight is larger than for other organs because the left adrenal is embedded in the ovary (Biswal, 1954) and therefore a "clean" dissection is difficult.

An analysis of variance of these adrenal data for the birds treated with a spirolactone showed that the drug has a highly significant effect ($p < 0.01$) at all levels (Fig. 4). Level 1 did not vary significantly from the other levels; level 2 was significantly different from 3 and 4 ($p < 0.05$); and level 3 showed no difference from level 4.

The drug has some general physiological effects since there is a decrease in mean body weight in all animals treated. However, this decrease in body weight is not statistically significant. The average body weight response to the drug is illustrated in Fig. 5. The body weight decreases at approximately the same rate for all drug levels through day 13, after which only those animals receiving the maximum level continue to decrease in weight. Whether this decrease can be attributed to the increase in metabolic rate reported to accompany forced decrease of sexual activity (Rice et al., 1908) is not apparent from the data. However, the body weight decrease is similar to the weight losses reported for Enheptin (Smith et al., 1957). The variability in weight loss observed in progesterone-treated birds (Adams, 1955), as well as norethandrolone treated birds (Winget and Griffin, 1962) is also apparent in these experiments (CV ≈ 13 per cent).

DISCUSSION

The experiments described indicate that a $\Delta 4$-androstene-3-one-17-spirolactone has an effect on the adrenals, ovary, oviduct, and the circadian rhythm of oviposition. The data do not indicate a single mechanism response to this synthetic steroid. A probable site of drug action is in the preoptic area of the diencephalon because a "block" in this area can explain most of the drug effects observed (i.e., apparent decrease in FSH, LH, and ACTH secretion

rates). Such a "block" may be similar to certain other steroids (Drill and Riegel, 1958) and act as a ganglionic "blocker" in the preoptic area.

The avian pituitary-gonad relationship is similar to the mammalian counterpart, except FSH and LH are secreted continually with observed peaks approximately every 24 hours (Nalbandov, 1958). FSH is not under estrogen control and progesterone or progesterone-like substances can cause ovulation (Fraps, 1955). Further, relatively large doses of ACTH produce premature ovulations in laying hens (van Tienhoven, 1961). The data herein indicate that a 17-spirolactone can produce a "blockage" of the circadian cycle of ovulation-oviposition. However, they do not permit a definite statement as to which specific mechanism is involved.

Blockage of a mechanism in the adrenohypophysis or at a higher level, the hypothalamus, is suggested by the drug effect on the ovaries, since ovarian involution is drug-dependent and immediately follows drug administration. One possible explanation is that the spirolactone causes an increase in corticosterone release which has been shown to block ovulation (van Tienhoven, 1961). Progesterone is an intermediate in the biosynthesis of corticosterone and causes premature ovulation. Since premature ovulations were not observed this does not seem to be a plausible answer.

Three hormones (estrogens, adrogens, and progesterones) are necessary for growth and maintenance of the oviduct. Estrogens stimulate growth and development of the entire oviduct, whereas progesterones and androgens are necessary for physiological activity of the anterior portion (Nalbandov, 1959). The observations indicate that the rate of estrogen secretion is insufficient to maintain the oviduct. However, immediately after drug treatment an increase in estrogen must have resulted because of the increase in weight of both the magnum and uterus prior to atrophy. Otherwise an increase in the magnum weight independent of the uterus would have been observed if there had been an increase in progesterone.

This experiment demonstrates the ability of a 17-spirolactone to cause atrophy of the adrenal glands in the bird. Although the mechanisms involved are not clear, several possibilities are evident. The synthetic steroid has its effect at the adrenohypophysis or the hypothalamus, by inhibiting the secretion of ACTH, the controller of adrenal growth and development (Zarrow et al., 1961). The hypothalamus is most likely the site of the drug action since it has been shown that hypophysectomy causes only slight adrenal atrophy (Newcomer, 1959); whereas hypothalamic blocks result in atrophy similar to that obtained in these experiments.

A second possible mechanism of drug action on the adrenals is a decrease in estrogen secretion rate. Adrenal hypertrophy is estrogen dependent in rats (Bernstein, 1964). Kornfeld (1957)

has shown that the weight of the birds' adrenals is dependent on estrogen levels. The data clearly show a decrease in estrogen levels. Further, the rate of ovary atrophy is approximately equal to the rate of the adrenal atrophy; and it is well established that the ovaries are the major source of estrogens in birds (Marshall, 1961). Furthermore, these data do not rule out the direct effect of the spirolactone on the adrenals or ovaries.

SUMMARY

The effect of a steroid lacking both androgenic and estrogenic activity ($\Delta 4$-androstene-3-one-17-spirolactone) on the circadian rhythm of ovulation-oviposition, and on anatomical changes of the adrenals, the ovary, and the oviduct appeared to be one of hypothalamic inhibition. The influence of the steroid upon body weight was variable.

Following drug administration, the rate of decrease in the circadian cycle, ovary weight, and adrenal weight was exponential. The rate of oviduct regression was linear preceded by a short increase in oviduct weight.

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TABLE 1 - WEIGHT OF CERTAIN ORGANS AT SPECIFIC TIMES AFTER DRUG INJECTION. EQUAL NUMBERS (4) WERE SACRIFICED AT SAMPLING TIMES AND ARE GIVEN AS MEAN VALUES.

Organ / Drug level	Oviduct weight (Gms.)						Ovary weight (Gms.)						Adrenals weight (Gms.)					
	Time of kill (days)						Time of kill (days)						Time of kill (days)					
	2	5	9	13	19	30	2	5	9	13	19	30	2	5	9	13	19	30
1.65/mg/kg body weight	58.0	56.7	59.4	41.7	46.2	47.3	46.9	53.3	36.1	38.6	30.3	30.8	0.223	0.191	0.170	0.156	0.120	0.109
2.85/mg/kg body weight	56.6	60.6	43.3	38.4	46.7	38.1	47.5	47.1	28.8	19.6	29.4	27.6	.264	.266	.177	.161	.133	.091
5.10/mg/kg body weight	62.4	59.1	45.5	29.1	25.9	49.5	51.2	32.9	28.8	13.7	14.5	40.5	.235	.232	.154	.118	.109	.101
12.60/mg/kg body weight	72.7	67.3	41.6	21.2	9.4	23.2	42.9	25.9	15.3	7.5	2.9	20.2	.205	.169	.133	.123	.129	.093
Pooled standard deviation of cell means	±8.8						±8.7						±0.003					

TABLE 2 - RATES OF DECREASE IN THE CIRCADIAN RHYTHM AFTER DRUG TREATMENT, AND DURATION OF MINIMUM CIRCADIAN ACTIVITY

Drug level (mg/kg body weight)	Per cent change day^{-1} in oviposition	Duration of maximum effect in days
0 (-0-) (Placebo)	0.5	0
1 (1.65)	3.2	4
2 (2.85)	13.6	3
3 (5.10)	16.8	1
4 (12.60)	28.0	7

$k \times 100$ = per cent change per day where k is the calculated rate constant.

FIGURE LEGENDS

Fig. 1. Data showing the effect of a 17-spirolactone on oviposition. Day zero is the day of drug administration.

Per cent oviposition = [number of eggs laid/number of animals per group]×100

Fig. 2. Decrease in ovary weight, expressed as a ±Δ per cent change, after giving a 17-spirolactone.

Fig. 3. The change in weight of the oviduct after the administration of a 17-spirolactone.

Fig. 4. The response curve of the adrenals to the drug.

Fig. 5. The effect of a 17-spirolactone on body weight as compared to pretreatment body weight.

$$\pm\Delta \text{ per cent body weight} = \left[\frac{\text{kill day weight} - \text{day zero weight}}{\text{day zero weight}}\right]\times 100$$

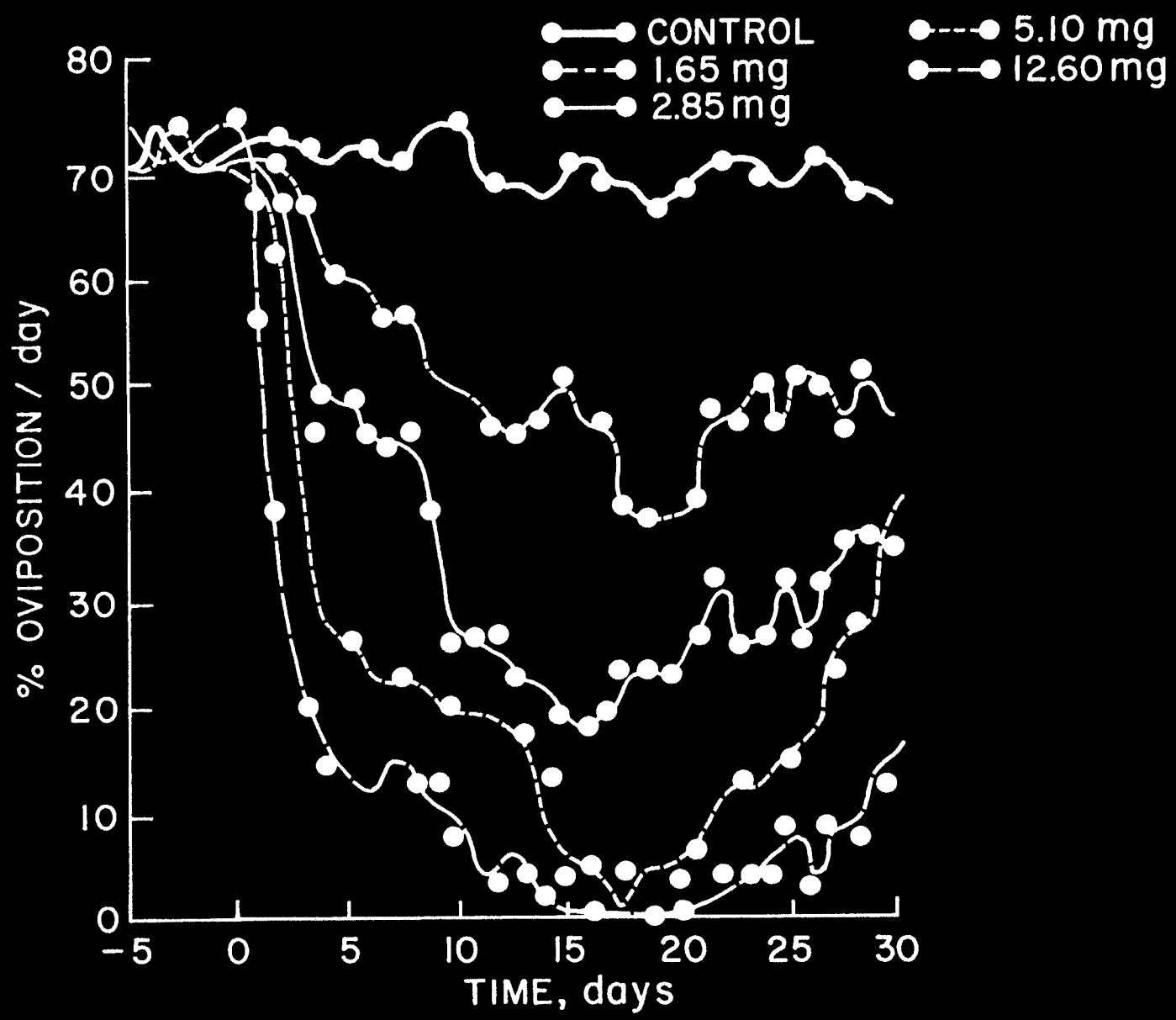

Figure 1.

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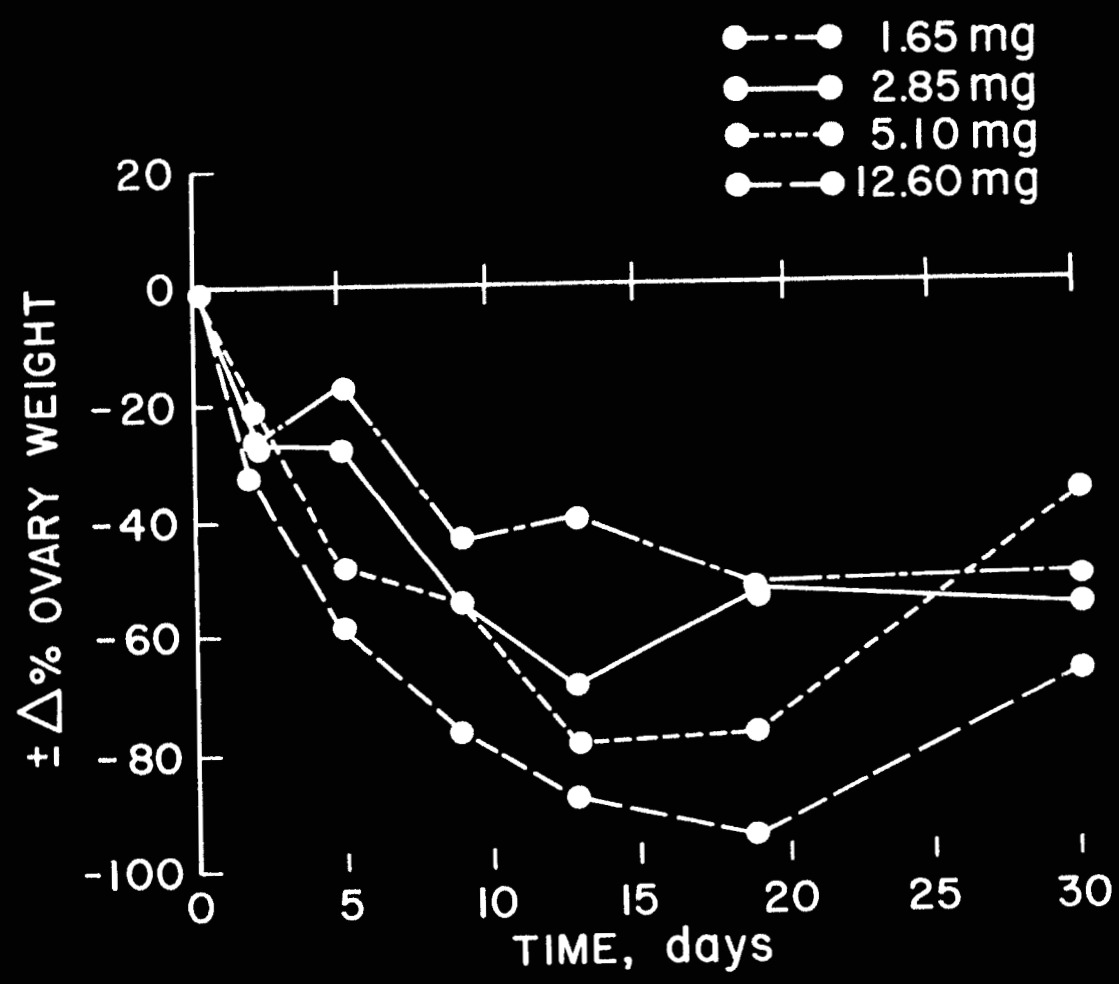

Figure 2.

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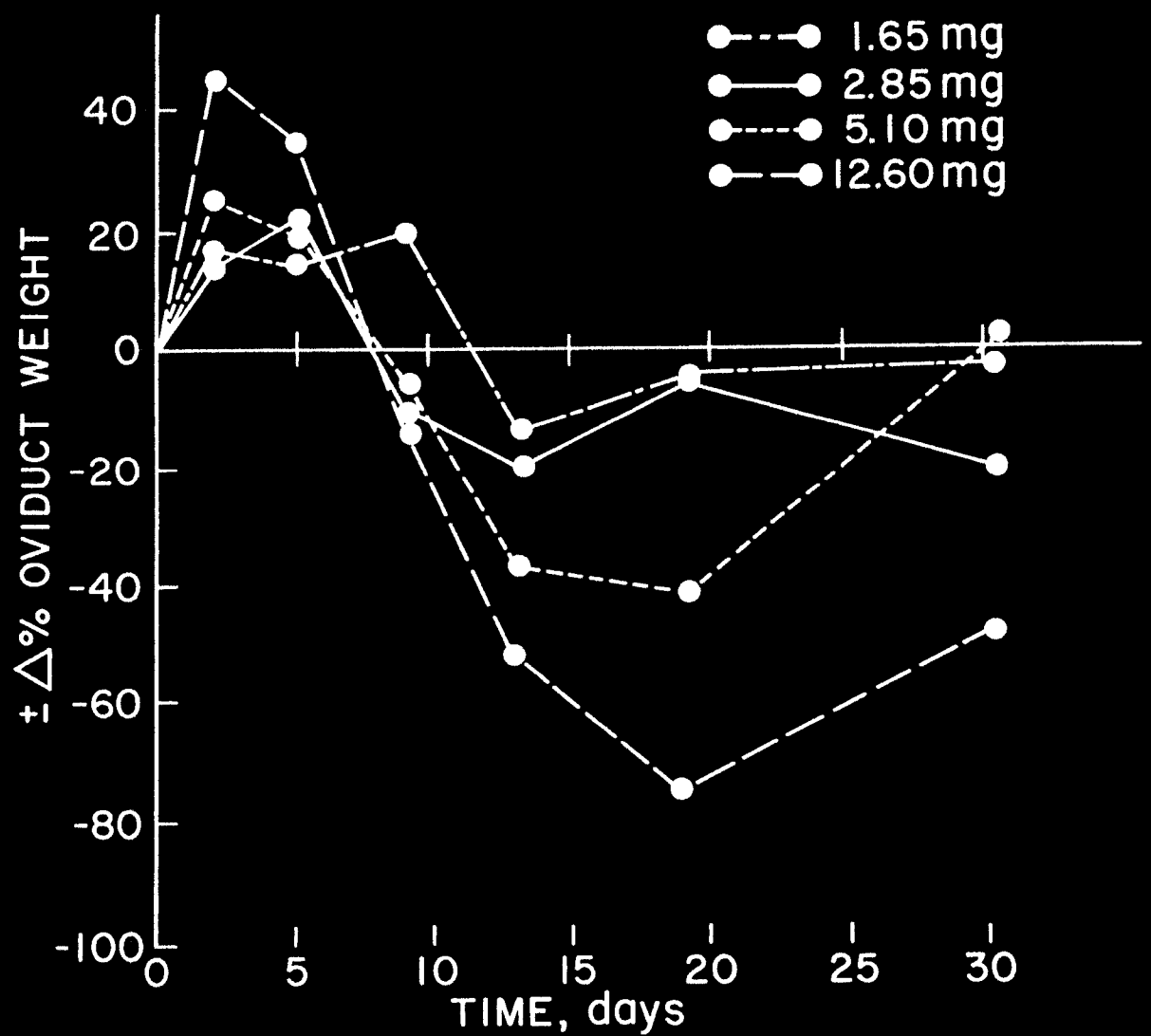

Figure 3.

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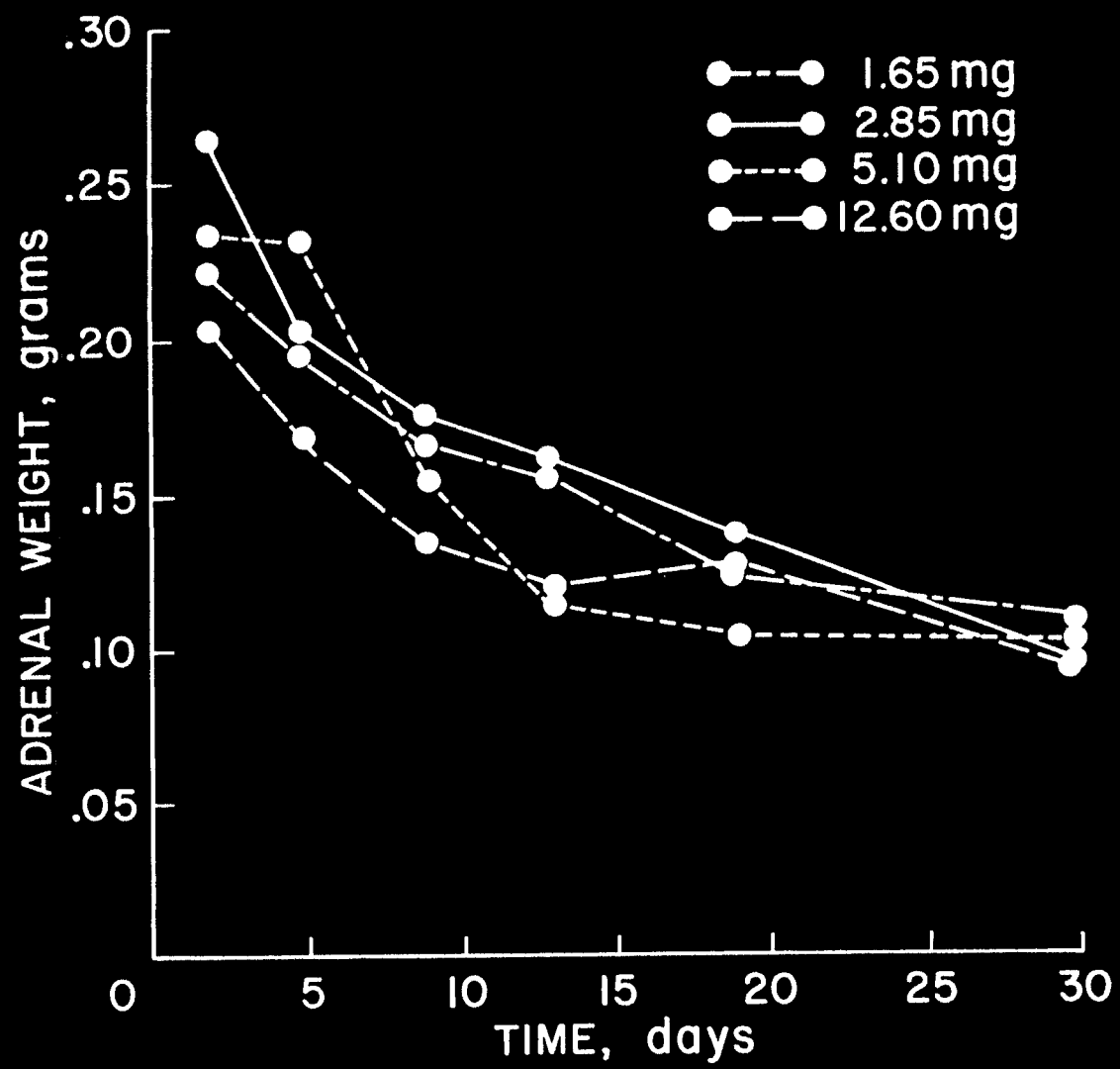

Figure 4.

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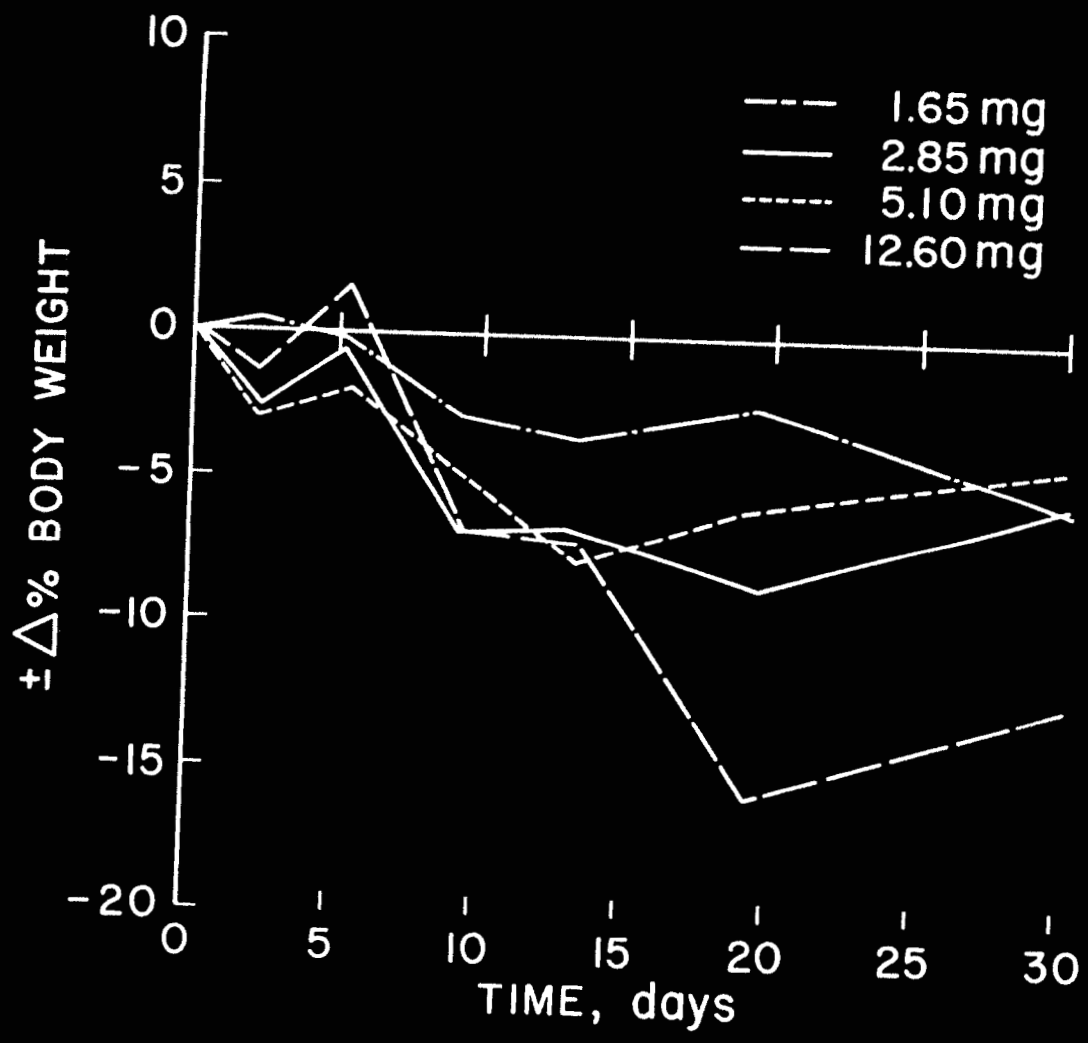

Figure 5.

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