THE INDUCTION OF CONSECUTIVE PSEUDOPREGNANCIES

BY ESTROGEN-PROGESTERONE INJECTIONS IN THE

MATURE ALBINO RAT, Rattus norvegicus (Berkenhout).

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Master of Arts in Biology

by

Michael Reed Garrett

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DEAN OF THE GRADUATE SCHOOL

John O. Reynolds

Respectfully Dedicated To

My Parents,

Mr. and Mrs. Harry L. Garrett

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ABSTRACT

Michael Reed Garrett. THE INDUCTION OF CONSECUTIVE PSEUDOPREGNAN-
CIES BY ESTROGEN-PROGESTERONE INJECTIONS IN THE MATURE ALBINO RAT,
Rattus norvegicus. (Under the direction of Everett C. Simpson)
Department of Biology, August, 1967.

In 10 untreated control rats of the Holtzman strain, two consec-
utive periods of pseudopregnancy were not induced by cervical stim-
ulation.

The administration of estrogen-progesterone injections to 30
experimental rats was begun on the twelfth or thirteenth day of the
first pseudopregnancy, and this pseudopregnancy was extended. The
longest extended first pseudopregnancy was exhibited in rats that
received hormonal injections beginning on day 12.

Consecutive pseudopregnancies were induced only when first pseu-
dopregnancies were dependent upon intervals at which hormones were
given during second pseudopregnancies.

Investigation of ovaries of rats in a first pseudopregnancy as
compared to those in a second consecutive pseudopregnancy, showed no
significant difference between the numbers of corpora lutea observed
in each group. There was however, a significant reduction in the
corpora lutea of control rats which were not induced into a second
consecutive pseudopregnancy. This was thought to have resulted in
an insufficiency of gonadal hormones, and it was hypothesized that
this insufficiency prevented the induction of a second consecutive
pseudopregnancy in the control animals.

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INTRODUCTION

In laboratory mammals which have very short estrous cycles, an induced neural condition known as "pseudopregnancy" can occur. This condition may be induced by procedures that prolong the secretory function of the corpora lutea of ovulation, thus holding in abeyance the onset of the next estrus. Since there are indications that neural mechanisms are involved, any method of cervical stimulation may elicit afferent nerve impulses. It is thought that upon reaching the hypothalmus this stimulus promotes the release of neurohumoral agents that are transported through the hypophyseal portal venules to the anterior pituitary where they are discharged into the blood. These agents cause the corpora lutea of the last ovulation to persist and function for a period lasting in excess of one-half the length of the normal gestation period of the animal.

Previous workers have shown that, in the albino rat, Rattus norvegicus, the pseudopregnant condition terminates usually after the thirteenth or fourteenth day following cervical stimulation.

It has been reported that a second consecutive pseudopregnancy in rats cannot be induced by cervical stimulation, and this was verified in our laboratory. The purpose of this research was to induce two consecutive periods of pseudopregnancy in the albino rat without the usual five-day estrous cycle interval. It was postulated that there is an insufficiency of gonadal hormones which prevents the onset of the second pseudopregnancy. Therefore, various dosages of estrogen and progesterone were administered in an attempt to correct

this insufficiency, and facilitate the induction of the second con-
secutive pseudopregnancy.

REVIEW OF LITERATURE

In order to analyze the neuroendocrine mechanism, investigators have employed various methods of maintaining the activity of the corpus luteum which controls the initiation of the pseudopregnant condition. The most popular of these methods include electrical stimulation, chemical stimulation with the use of tranquilizing drugs such as reserpine and chloropromazine (Barraclough, 1956; and Jacobson et al., 1960), coital stimulation using vasectomized males, lutienizing hormone and prolactin injections (Jacobson, 1960), and mechanical stimulation with the use of a glass rod. Defeo (1966) has improved the glass rod technique by attaching this to a battery operated vibrator. According to Malkowa (1956), daily exogenous dosages of estrogen can activate corpora lutea in both normal and spayed females by stimulating the anterior hypophysis to secrete prolactin. Apparently cervical trauma causes the release of prolactin also. This then stimulates extensive growth of the mammary system at a rate equivalent to pregnancy. As a result of prolactin secretion, progesterone is secreted for about 14 days (Velardo, 1958).

It has been recognized since the original experiment by Leo Loeb (1908) that the response of the uterus of the rat to stimuli is by a hyperplasia of stromal components which histologically resemble the maternal portion of the placenta. This structure is referred to as the "deciduoma" or sometimes the "placentoma", and according to Zarrow (1964) who cites work done by Rothchild (1940) and Yochim (1962), its maintenance is dependent upon synergizing ratios of estrogens and progesterones.

Although pseudopregnancy may occur in the absence of decidual formation, earlier investigators used the presence of these growths as one criterion for the verification of the pseudopregnant state. Thus it is considered appropriate to review some of the work done in this area.

In the pseudopregnant female rat, the deciduoma reaches maximum growth about the fifth day following traumatization and declines after about the seventh or eighth day. At the point of maximum size there can be observed an increase of glycogen and water content of the uterus, which accounts for the enlarged appearance of the deciduoma (Zarrow, 1964).

The original experiment of Loeb in 1908, indicates that there are three major factors necessary for the formation of the deciduoma: 1. chemical sensitization of the uterus by estrogen from the stratum granulosum and theca interna of the ovary (Bengt, 1957), and progesterone from the corpus luteum; 2. mechanical stimulation of the uterus; 3. the proper condition of the body to sustain the deciduous tissue (Velardo, 1958).

Studies involving the effects of hormonal therapy upon the deciduoma, and consequently pseudopregnancy, have shown that estradiol-17β has the greatest effect on progesterone-maintained deciduomata when estradiol is administered at $1.0\mu g$ and progesterone is at 2.0 mg (Zarrow, 1964). In a comparative study of the biological effects of estrogens, it was reported by Overbeek (1958), that estradiol-17β has 100 to 1,000 times greater effect on the prevention of cervical and vaginal cornification in the rat than does estrone and estriol.

It should be mentioned at this point that according to Malkowa (1956), no deciduomal growth will follow cervical stimulation when daily exogenous dosages of estrogen are administered. Continuous administration of estrogen causes marked lutienization and in turn prevents vaginal cornification in mature female rats. It is probable that the secretion of prolactin and lutienizing hormone is stimulated.

Rothchild (1963), found that if pseudopregnancy is induced in a rat by a single injection of progesterone (10.0 mg), no deciduomal reaction will occur. Although functional corpora lutea are assumed to be present in all pseudopregnant animals regardless of the species (Turner, 1963), the actual endocrine condition must differ, for it has been observed by both this investigator and others (Banik, 1965) that hormonal injections do not cause deciduomal reactions.

The regulatory effect of progesterone on pseudopregnancy appears to be that ovarian follicles are capable of responding to an acute burst of lutienizing hormone secretion only at very limited times during the cycle (estrus). The exogenous progesterone either prevents or reduces the secretions of lutienizing hormone during the treatment period (Nallar, 1966).

One fact further verified by Velardo's research was that physiological dosages of estrogen (estradiol-17β) and progesterone can prolong pseudopregnancy beyond the normal thirteen or fourteen day period (Velardo, 1958).

Normally estrogens and progesterone cause persistence of the corpora lutea. According to Asdell (1946), a minimum of 50.0 IU of estrogen daily is needed. However, corpora lutea are not maintained

indefinitely but undergo atrophy at day twenty, thus terminating
pseudopregnancy. Estrogen is used as a primer. In similar work by
Olsen (1951), it was also shown that the presence of estrogen-pro-
gesterone-maintained deciduomata can prolong the pseudopregnant con-
dition. The extent of prolongation of this pseudopregnancy can be
pre-determined by the number of deciduomata present (Velardo, 1953).
Further investigation in this area showed that the ovaries during
prolonged pseudopregnancy have corpora lutea of similar appearance
to those seen in normal pseudopregnancy, and histochemical evidence
shows that there is a continuation of luteal function (Velardo, 1953).

According to Turner (1960), when female rats are coming out of
pseudopregnancy after the normal thirteen or fourteen day period, the
corpora lutea are becoming nonfunctional, therefore reducing the
secretions of progesterone. The injection of physiological or thera-
peutic dosages of estrogen and progesterone can extend this condi-
tion by providing the necessary hormonal levels, although the corpora
lutea have begun to undergo atrophy.

In another investigation, functional corpora lutea were main-
tained for months by autografts of rat hypophyses (Everett, 1956).
This appears to further verify the work of Malkowa (1956), since pro-
lactin and lutienizing hormone are released from hypophyseal tissue
and thus could maintain the functional corpora lutea.

At this point in the review, it can be seen that pseudopregnancy
and deciduomata can be hormonally induced and/or prolonged by exclu-
sive injections of only progesterone, injections of both estrogen and
progesterone, and by hypophyseal grafts.

Austin (1956) and Fluhmann (1955) reported that if a female rat
is given 22.0 to 65.0 μg of estrogen (Stillboestrol) daily in its
drinking water, the animal expresses continuous vaginal cornification
and a capacity for repeated coitus at intervals corresponding to those
between the estrous periods of a normal cycling rat.

These reports are in accord with work done on the effects of
exclusive dosages of estrogen on pseudopregnancy. The pseudopregnant
condition in rats can be induced by a single large injected dosage
(Alloiteau, 1957). There was, however, no mention of an extension or
prolongation of pseudopregnancy by this injection. Recently Reed
(1966) performed an investigation using albino hamsters, in which
daily injections of Nilevar (17α ethy-19-nortestosterone) began on
the cycle day when the vaginal membrane of the animal underwent clo-
sure. As a result of this hormonal administration, the return to
estrus was prohibited for two months. The closure of the vaginal mem-
brane in the hamster is analogous to pseudopregnancy in rats, in that
neither animal will permit coitus nor exhibit vaginal cornification
during this period. Nilevar, like estradiol, acts by blocking gonad-
otropins in their control of initiation of follicular development and
action on mature follicles, thus preventing ovulation.

Bogandove (1966) recently reported that if daily injections of
50.0 μg of estrogen are administered to rats, the pseudopregnant
condition can be induced and maintained up to 46 days. Direct evi-
dence that a sustained vaginal diestrus reflects the preservation of
a single set of corpora lutea was provided by tagging two to three
week-old corpora with micro injections of India ink and allowing them

to survive for an additional 17 to 27 days. These corpora lutea were not maintained indefinitely, however, since they degenerated after four to six weeks.

Another method of inducing pseudopregnancy in estral rats is by permitting them to suckle foster young. With the onset of suckling, the female enters a period of pseudopregnancy which lasts for approximately 18 days. Finally the animal exhibits estrus; but if suckling is maintained, another pseudopregnancy follows (Turner, 1955). During suckling pseudopregnancy produced in this manner, the continuous neural stimulation of the nipples produces a longer luteal phase than is produced by the single nervous stimulus which initiates copulation pseudopregnancy (Turner, 1955; and Scharrer et al., 1960). This suckling pseudopregnancy is the only report in the literature of consecutive pseudopregnancies in rats of which this investigator is aware.

In comparison, Asdell (1946) reported that the suckling stimulus can also prolong pregnancy in rats. Asdell stated that if a female rat is suckling six or more of her young, implantation in a new consecutive pregnancy is delayed, and the pregnancy is consequently prolonged. In the absence of pregnancy, lactation causes the corpora lutea of post-parturition ovulation to persist. This seems to further re-inforce the statements previously made by Turner concerning the effects of hormonal administration on the extension of pseudopregnancy. He stated that injecting physiological dosages of estrogen and progesterone on and after day 12 or 13 in pseudopregnant rats, would extend this pseudopregnancy. These corpora lutea apparently act similar to the lactation-maintained corpora lutea.

In summary, it may be seen that there are several methods of initiating pseudopregnancy in the white rat. However, a second consecutive pseudopregnancy has been proven to be much more difficult to obtain. It seems reasonable to postulate that this condition can be induced by increasing the levels of gonadal hormones. This postulation is apparently supported by three statements made by previously cited investigators: (1) Some corpora lutea are still functional at the termination of the first extended pseudopregnancy (Velardo, 1953). (2) Continuous administration of estrogen prevents vaginal cornification by prolonging the luteal phase (Malkowa, 1956), and seemingly this may act like the continuous stimulation of the nipples in rats that entered a second successive pseudopregnancy. (3) Exogenous progesterone administered during the second pseudopregnancy may prevent the return to estrus, thus simulating a second consecutive pseudopregnancy. The return to estrus may be brought about by reducing the secretions of lutienizing hormone (Nallar, 1966).

The present investigation is confined to the use of exogenous gonadal hormones in an attempt to initiate a second consecutive pseudopregnancy.

MATERIALS AND METHODS

Animals

Mature female albino rats of the Holtzman strain were used in this investigation. These rats were subjected to daily light and dark periods of fourteen and ten hours respectively in order to maintain consistent photoperiodic estrous cycles throughout the investigation. They were fed Purina Laboratory Chow, _ad lib_.

Experimental Procedures

Vaginal Lavages

For a period of two weeks prior to the onset of pseudopregnancy, estrous cycles were checked daily by vaginal lavages at 8:30 A.M. This was done in order to be certain that each animal was consistently exhibiting a normal five-day cycle. The vaginal lavage was performed by introducing approximately 1/4 cc of water into the vagina by means of a small pipette, and this was aspirated three or four times to obtain the maximum amount of sloughed cells. One drop of water containing these sloughed cells was then placed on a clean, numbered microscope slide (since the rats were numbered, several smears were placed on the same slide). Each lavage was examined microscopically at 100x and the daily results were recorded. The daily vaginal lavages were made throughout the period of this research. The criterion for each stage of the estrous cycle is listed in Table 1.

Table 1. Different stages in the rat estrus cycle and corresponding cell types in the vaginal lavage (Turner, 1960)

Stage of Cycle	Type of Cells
Diestrus	almost entirely all leucocytes
Proestrus	rounded, nucleated epithelial cells
Estrus	masses of cornifies epithelial cells, (degenerate nuclei)
Metestrus	many leucocytes, with a few cornified epithelial cells

Induction of Pseudopregnancy

Pseudopregnancy was initiated by glass rod stimulation of the cervix on the day of estrus.

Administration of Hormones

Hormonal therapy consisted of a concentration of 2.0μg of estradiol benzoate and 4.0 mg of progesterone, administered in combination or individually. These substances were dissolved in corn oil (Mazola) and the injections were made interperitoneally.

In attempting to induce the second consecutive pseudopregnancy, all injections were administered either at 24-hour or 48-hour intervals, commencing at the time of cervical stimulation.

Procedure For The Surgical Detection of Deciduomata

The animal was anesthetized with ethyl ether and placed on the table with its ventral surface up and nose exposed to an ether cone.

The pelvic region of the abdomen was then sheared with animal clippers and swabbed with 70% alcohol.

A mid-ventral longitudinal incision about 2.5 cm in length was made slightly anterior to the pelvic region. After opening the body cavity the uterine horns were exposed. It was necessary to exterior-eze each uterine horn to locate any possible deciduomal enlargements.

The exposed viscera was periodically swabbed with physiological saline (.85%) throughout the operation in order to prevent drying and adhesions of the exposed tissues.

The uterine horns and their associated fat were replaced into the abdominal cavity and several interrupted sutures were taken in the muscle wall. These sutures were placed close enough to prevent herniation, and the skin incision was closed with surgical clamps (9 mm). These clamps were removed after one week to insure proper healing.

Ovariectomy Procedure

The animals were killed with an overdose of ether and placed on the table. A 1.5 cm long incision was then made immediately posterior to the last rib. After opening the body cavity, the mass of fat containing the ovary was exposed. The fat was then withdrawn and the ovary was separated from the uterus.

The associated fat and oviduct were then removed from the ovary, along with the ovarian bursa, in order to have optimal conditions for the observation of the corpora lutea. The ovaries were then placed in a neutral buffered formalin solution until the number of corpora lutea could be counted. A 10 x binocular dissecting microscope was

used in determining the number of corpora present.

Design of Experiment

Part I - Untreated Controls

Group A - Deciduomal Formation

After the initial two weeks of observation, the 10 rats used in this group were made pseudopregnant. Five days after the onset of pseudopregnancy, these rats were laporotomized and checked for possible deciduomal formation.

Group B - First and Second Pseudopregnancies

The 10 rats in this group were made pseudopregnant and used as controls for other groups in this investigation. The normal pseudopregnancy lengths exhibited by these animals were recorded for comparison with any modification of pseudopregnancy lengths exhibited by the animals in other groups receiving hormonal treatment.

Upon termination of this pseudopregnancy, these animals were restimulated in an attempt to induce a second consecutive pseudopregnancy without exogenous hormones.

Group C - Ovarian Analysis of Rats In An Initial Pseudopregnancy

This group of 10 rats was induced into an initial pseudopregnancy and ovariectomized six days after this stimulation. This was a control group designed to determine the number of corpora lutea seen in those rats in a second consecutive pseudopregnancy.

Group D - Ovarian Analysis At Termination of First Pseudopregnancy

Pseudopregnancy in this group of five rats was induced and allowed

to complete its normal 13 or 14 day duration. Upon the first day of estrus, (pseudopregnancy termination), they were re-stimulated and on day four, following re-stimulation, they were ovariectomized. No injections were administered at any time. This was an attempt to compare the number of ovulations (corpora lutea) in those rats that were not induced into a second consecutive pseudopregnancy, with those which entered this condition.

Part II - Treated Rats

This part of the investigation involved 60 rats. Upon the first exhibited estrus, these rats were made pseudopregnant. On either the twelfth or thirteenth day of this pseudopregnancy, hormonal administration for each rat began. With the exception of Group A, the injections were administered until the twenty-fourth day of pseudopregnancy, or until the animal returned to estrus, whichever occurred earlier. All injections were combined dosages of estrogen and progesterone. This group was divided as follows:

Group A

Hormonal treatment for the 10 animals in Group A began on day 12 of the first pseudopregnancy and was administered at 48-hour intervals.

Group A_1

Upon the first estrus onset following the first pseudopregnancy, six of these rats were again cervically stimulated and the injections were resumed at that time. These injections were continued until the termination of the second consecutive pseudopregnancy.

Group A_2

The remaining four rats of Group A were ovariectomized on day six of the second consecutive pseudopregnancy. The number of corpora lutea observed in the ovaries of these animals was compared to the number found in the ovaries of rats in Groups C and D. This was an attempt to show the relationship between the number of corpora lutea produced and the length of the second consecutive pseudopregnancy. The six-day time interval was chosen, since it was considered that these animals would have returned to estrus earlier if pseudopregnancy had not been induced.

Group B

In this group of ten rats, hormonal treatment began on day 13 of the first pseudopregnancy, and the injections were administered at 24-hour intervals. As these animals returned to estrus, they were re-stimulated and the injections were resumed at the same intervals.

Group C

The ten rats in this group began receiving the estrogen-progesterone injections on day 13 of the first pseudopregnancy, at 48-hour intervals. As they returned to estrus, they were re-stimulated and the injections were resumed at the same intervals. The data from these rats were then compared with the data from the rats in Groups A and B in an attempt to discover any similarities and/or dissimilarities between the length of the extended first pseudopregnancy and the length of the second consecutive pseudopregnancy. This would compare the length of pseudopregnancy with regard to

initial injections and the frequency of these injections.

Group D

For the 10 rats used in this group, hormonal treatment began on day 12 of the first pseudopregnancy and was repeated at 48-hour intervals. The first pseudopregnancy was then extended to 20 days or the first exhibited estrus, and the injections were halted. Upon the animals' return to estrus, the rats were stimulated for a second consecutive pseudopregnancy, but hormonal treatment was not resumed.

This group was intended to show that exogenous hormones do have an effect on inducing and maintaining the second consecutive pseudopregnancy.

Group E

The 10 rats in this group were made pseudopregnant and carried up to the point where they approached estrus (proestrus to estrus). The injections were then initiated at 48-hour intervals, but there was no cervical stimulation when the rat came into estrus. This was an attempt to determine if the second consecutive pseudopregnancy (as obtained in Groups A-C) is induced by the effects of exogenous hormones, cervical stimulation, or both.

Group F

This group of 10 rats was used in an attempt to induce a second consecutive pseudopregnancy after cervical stimulation with the use of exogenous dosages of estrogen alone. Hormonal treatment began on day 12 of the first pseudopregnancy, and the dosages were administered at 48-hour intervals. Upon the next exhibited estrus, these

rats were re-stimulated and the injections were resumed at the same intervals.

Part III - Vaginal Lavage Controls

In the previous trials, all changes in the estrus cycle were determined by vaginal lavages. It was possible that these hormones may have had a direct effect on the vagina and thus may not have been a true indication of pseudopregnancy. Therefore, it was considered appropriate to check the effect of these hormones on the normal cycling rat. The dosage level injected was similar to the previous treatments.

Group A

This group consisted of three rats which were administered a single dosage of progesterone on the day of estrus.

Group B

The three rats in this group were injected with a single dosage of progesterone on the day of diestrus. This hormonal treatment was maintained at 48-hour intervals.

Group C

This group consisted of three rats which were administered a single injection of estrogen on the day of estrus.

RESULTS

Part I - Untreated Control Rats

Most of the rats showed relatively consistent estrus cycles. A majority exhibited a complete cycle in five or six days. Due to extreme inconsistency in cycling, various rats initially used in this investigation were later excluded, since data from these rats would be difficult to assess.

Surgical investigation for deciduomata on the fifth day of pseudopregnancy (Part I, Group A) showed no conclusive evidence of their formation. Due apparently to increases in circulation, glycogen, and water, all of the uteri were red and slightly enlarged. However, there were no enlarged areas in the horns which confirm decidual formation. Thus, it can be concluded that the formation of deciduomata is not necessary for pseudopregnancy.

The normal pseudopregnancy lengths for the control rats used in Part I-B of this experiment showed a mean of 12.6 days, ± .84 (Table 2). These results are consistent with the observations of several other investigators.

At this point it should be mentioned that throughout this investigation, animals which failed to enter pseudopregnancy when stimulated on the day of estrus, were re-stimulated on the following estrus. Although the glass rod method of cervical stimulation is considered by many workers as a highly effective means of inducing pseudopregnancy, it is not completely effective. This was not a significant factor in this investigation, since relatively few of the rats required a second or third stimulation.

Table 2. Duration of first pseudopregnancies in untreated control rats

Days

Rat Number
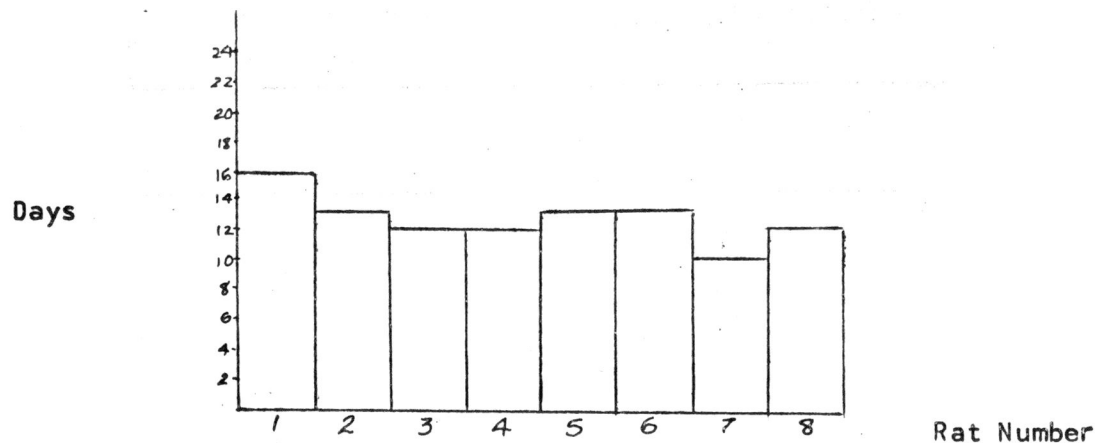
None of the animals which were stimulated in an attempt to induce a second consecutive pseudopregnancy without hormonal treatment could be induced into this condition (Part I-B). All of the rats exhibited estrus within six days after stimulation.

The rats of Part I-C were stimulated during estrus and ovariectomized on day six of pseudopregnancy. As previously mentioned, these were control rats to be used in comparing the number of corpora lutea produced in the first pseudopregnancy with those produced in the second consecutive pseudopregnancy if it could be induced. Each pair of ovaries in these rats exhibited a mean of 13.0 corpora lutea, with a range from 10 to 16 (Table 3). This is considered to be within the normal range for ova in both normal and pseudopregnant rats.

Table 3. Corpora lutea produced in the ovaries of control pseudo-pregnant rats

Rat Number	Corpora Lutea
1	16
2	10
3	13
4	13
5	11
6	15
7	13
8	15
9	12
10	12

The rats used in Part I-D (Table 4) were ovariectomized on the fourth day following the termination of the first pseudopregnancy. No injections were administered at any time. These rats showed a mean of 8.5 corpora lutea. This reduction in the number of corpora lutea may in part account for the inability of these animals to maintain a second consecutive pseudopregnancy.

Table 4. Corpora lutea in ovaries of rats not induced into a second consecutive pseudopregnancy

Rat Number	Corpora Lutea
41	7
33	9
44	8
45	10

Part II - Treated Rats

Hormonal treatment for the rats in Part II-A began on day 12 of the first pseudopregnancy, and the injections were administered at

48-hour intervals. Pseudopregnancy was definitely lengthened beyond
the normal 13 or 14 day period due to the effects of estrogen and
progesterone, but marked individual variations arose in the extension
of this period (Table 5). The extended lengths of pseudopregnancy
showed a mean of 26.1 days. This was the longest mean period of the
extended first pseudopregnancies in all the groups involved in this
experiment. When the mean length of these pseudopregnancies was com-
pared to the mean of the control pseudopregnancies, the results were
significant (p < 0.05).

Table 5. Duration of individual first pseudopregnancies (estro-
gen-progesterone injections administered at 48-hour intervals)

Days

Rat Number
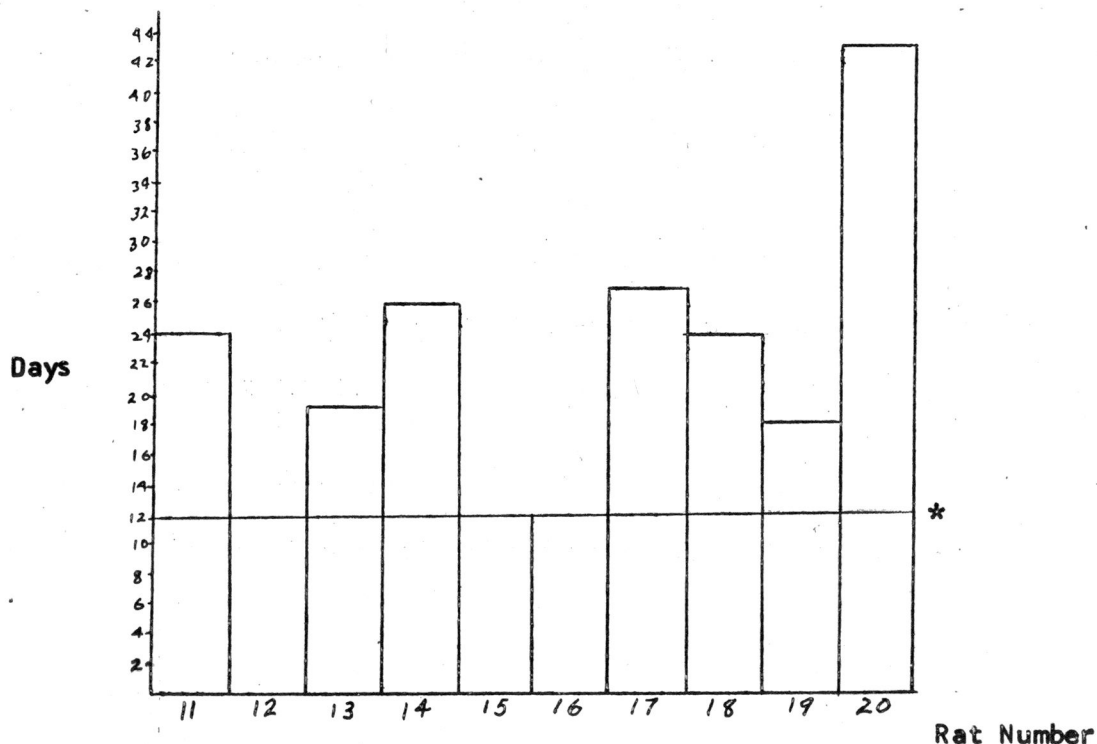
* Red color indicates initial day of hormonal treatment

It can be seen in Table 5 that for three rats the initial pseudo-pregnancy terminated on the day in which hormonal treatment was to begin. These three rats were re-stimulated and injected in an attempt to see if they would still enter a consecutive pseudopregnancy. Each of these rats exhibited estrus within six days following the re-stimulation and initiation of hormonal treatment.

The seven rats in this group that entered an extended first pseudopregnancy were re-stimulated and the injections resumed at 48-hour intervals (Part II-A). Five of these rats definitely entered a second successive pseudopregnancy with a mean length of 16.6 days (Table 6).

Table 6. Duration of Consecutive pseudopregnancies (estrogen-progesterone injections administered at 48-hour intervals)

Rat Number	Length in Days
11	17
13	19
18	10
19	20
20	17

The results of ovariectomy on day six (Part II-A$_2$) of the second consecutive pseudopregnancy (Table 7) resulted in no significant difference between the number of corpora lutea found in the ovaries of rats in an initial pseudopregnancy (Part I-C) and those found in rats in a second consecutive pseudopregnancy ($\bar{x}$ = 12.7) when under hormonal treatment. This is in contrast to the significant reduction of corpora lutea formed in the control second pseudopregnancy of Part

I-D, (p< 0.01).

Table 7. Corpora lutea in rats in a second consecutive pseudo-pregnancy

Rat	Corpora Lutea
a	12
b	11
c	13
d	15

The rats in Part II-B were given the estrogen-progesterone dosages on day 13 of the first pseudopregnancy and at 24-hour intervals. There was a marked decrease in the number of animals that entered an extended pseudopregnancy as well as a decrease in the length of the extended pseudopregnancy. It can be seen in Table 8 thay only three of 10 rats entered an extended pseudopregnant condition. The mean length of this condition was 21.0 days as compared to the 26.1 days in the rats used in Part II-A. Although these rats entered a pseudopregnancy which was much longer than the control pseudopregnancies, the significance of this group is reduced, since only three out of 10 entered an extended pseudopregnancy. A comparison of Part II-A and B shows that the time of initial injections of estrogen and progesterone combination is more important than the dosage interval upon extension of the first pseudopregnancy. This will be discussed in more detail later.

Table 8. Duration of individual first pseudopregnancies (estrogen-progesterone injections administered at 24-hour intervals)
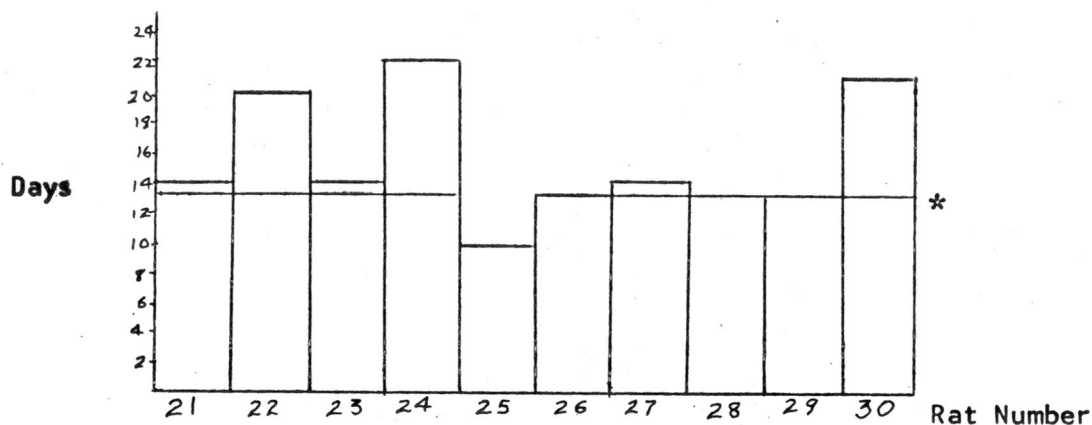
* Red color indicates initial day of hormonal treatment

As these rats exhibited estrus, they were re-stimulated and the injections were resumed at the same intervals. Each of the three rats that entered extended first pseudopregnancies (rats number 22, 24, 30), also entered a second consecutive pseudopregnancy which lasted for a mean of 24.0 days (Table 9).
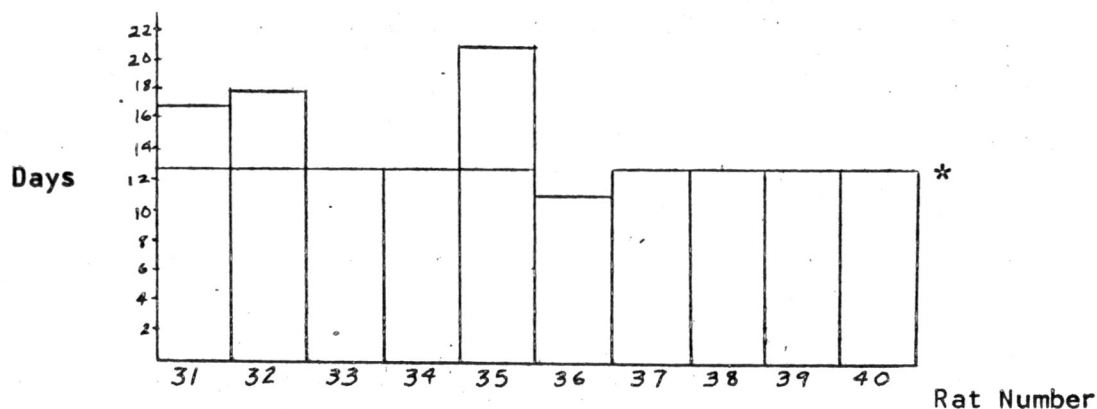
Table 9. Duration of consecutive pseudopregnancies when injections were initiated on day 13 of the first pseudopregnancy, and given at 24-hour intervals in the second consecutive pseudopregnancy.

Rat Number	Days
22	21
24	26
30	25

The rats used in Part II-C were given the injections at 48-hour intervals, beginning on day 13 of the first pseudopregnancy. The extended pseudopregnancy periods of these rats showed a mean of 18.6

days (p < 0.01), which was the shortest mean period for any group of treated rats used in this investigation (Table 10).

Table 10. Duration of individual first pseudopregnancies (estro-gen-progesterone injections administered at 48-hour intervals)

*Red color indicates initial day of hormonal treatment

As rats which entered an extended first pseudopregnancy were re-stimulated at estrus-onset, injections were resumed in an attempt to induce a second consecutive pseudopregnancy. Each of these three rats (31, 32, 35) entered a second consecutive pseudopregnancy which lasted for a mean of 16.0 days. This was the shortest mean duration of second consecutive pseudopregnancies in any group of estrogen-progesterone-treated rats in this investigation.

Table 11. Durations of the second consecutive pseudopregnancies in rats injected on day 13 of the first pseudopregnancy, and at 48-hour intervals in the second consecutive pseudopregnancy

Rat	Days
31	14
32	13
35	21

Hormonal treatment for the 10 rats used in Part II-D began on
day 12 of the first pseudopregnancy and was given at 48-hour inter-
vals until day 20. The injections were then stopped. As soon as
estrus was exhibited the animals were re-stimulated, but no further
injections were administered. None of the rats entered a consecu-
tive pseudopregnancy as a result of this treatment.

Table 12. Durations of the first pseudopregnancies in rats used
in Part II-D in which no consecutive pseudopregnancy was induced
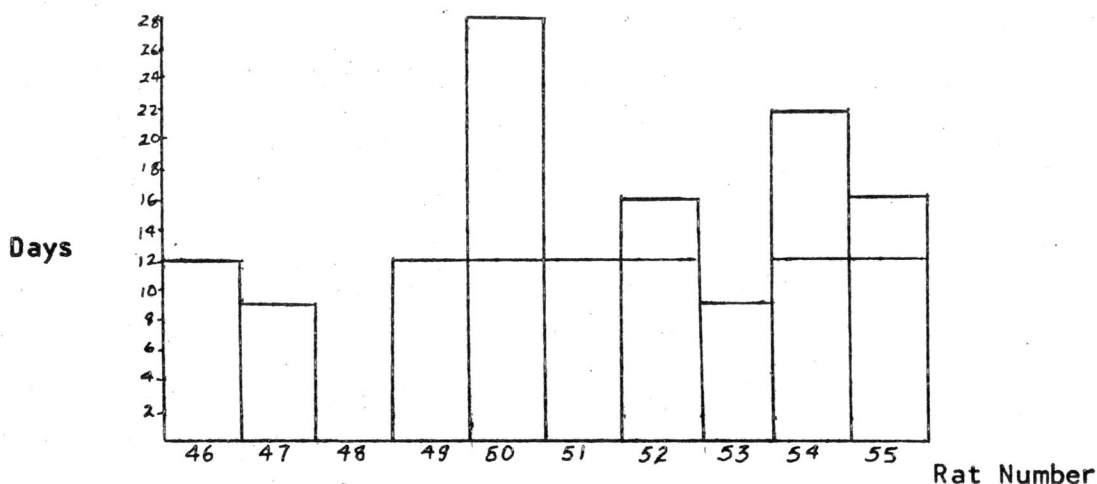
The 10 rats involved in Part II-E were used in an attempt to
determine if a second pseudopregnancy could be induced by a single
estrogen-progesterone injection on the day when the first pseudopreg-
nancy was near termination. Although no table is presented, all of
these animals entered an extended first pseudopregnancy as indicated
by the fact that no typical estral smears were observed. It was
assumed that this prolongation was an extension of the first pseudo-
pregnancy and not the initiation of a second consecutive pseudopreg-
nancy. Due to the extended first pseudopregnancy, this experiment

gave no conclusive support for the postulation that a second consecutive pseudopregnancy could be induced by hormonal treatment in the absence of cervical stimulation.

The ten rats used in Part II-F were given dosages of estrogen alone at 48-hour intervals, beginning on day 12 of the first pseudopregnancy (Table 13). This extended the first pseudopregnancy to a mean of 17.4 days. A second consecutive pseudopregnancy was induced by stimulating and resuming the hormonal treatment during the estrus which terminated the extended first pseudopregnancy.

Table 13. Extended lengths of the first pseudopregnancies using estrogen-treated rats
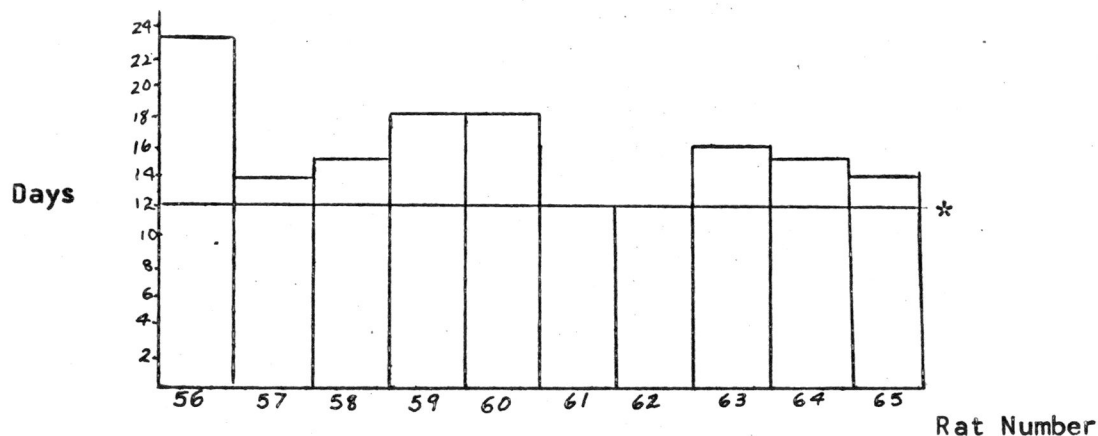
Days

*Red color indicates initial day of hormonal treatment

Six of the eight rats entered a second consecutive pseudopregnancy and were carried for a mean of 12.7 days (Table 14).

Table 14. Durations of the second consecutive pseudopregnancies in estrogen-treated rats

Rat Number	Day Length
56	7
57	16
58	15
59	12
60	-
63	13
64	-
65	13

Part III - Vaginal Lavage Controls

The rats in Part III-A which received a single injection of progesterone on the day of estrus, exhibited no detectable change in the length of their estrus cycle. These rats came into estrus five days after the injection was given.

Rats in Part III-B were given an injection of progesterone on the first day of diestrus. Each of these rats exhibited a normal five-day cycle following the injection.

The rats in Part III-C exhibited continuous vaginal cornification for a mean of 8.0 days as a result of the single injection of estrogen on the day of estrus.

DISCUSSION

The results of the investigation for deciduomata show that the
three main factors necessary for the formation of these growths (see
Review of Literature) may be present in the animal; however, this
does not always insure deciduomal formation since none of the untreated
experimental rats exhibited these growths. Malkowa (1956) has also
shown that deciduomal formation is not necessary for the pseudopregnant
condition.

None of the untreated control animals were induced into a second
consecutive pseudopregnancy. The corpora lutea of the previous estrus
period would have undergone atrophy by the termination of the first
pseudopregnancy. Normally a five day estrus period must occur between
pseudopregnancies. This would allow corpora lutea of the interval
ovulation to produce additional amounts of gonadal hormones, thus re-
sulting in an increase in the endogenous hormonal levels and re-estab-
lishing an environment in which another pseudopregnancy would be
possible.

Rats in Part II-A which received estrogen-progesterone treat-
ment at 48-hour intervals beginning on day 12, had the longest ex-
tended pseudopregnancy lengths of any of the groups in this inves-
tigation. This appears to confirm the work by Asdell (1946) and
Turner (1960) in which physiological dosages of estrogen and pro-
gesterone extend the pseudopregnant state for nine days. However,
most of the rats used in this group and in others showed an extended
pseudopregnant condition which was beyond the nine days reported by
these two investigators. Since the normal length of pseudopregnancy

in this strain of rats is 14 days, any extension beyond this time is considered to be due to the effects of the hormones used. Deciduomata are formed between the fifth and seventh day of the pseudopregnant condition, and any injections of exogenous hormones later in the period would have no effect on deciduomal formation. Therefore, it can be seen that the presence of deciduomata is not a physiological prerequisite for the extension of pseudopregnancy, since no deciduomata were found when the control rats were laporotomized.

When rats received injections at 48-hour intervals beginning on day 12, there were proportionally more that entered a second consecutive pseudopregnancy than any other group used in this study. However, it was shown in Part II-B that consecutive pseudopregnancies can be induced and maintained by the administration of injections at intervals other than 48 hours. Thus, it seems apparent that the induction of pseudopregnancies is dependent upon exogenous amounts of estrogen and progesterone which are quantitatively similar to the endogenous amounts normally produced in the rat.

When the rats that received no hormonal treatment during the first pseudopregnancy were re-stimulated and injected in an attempt to induce a second consecutive pseudopregnancy, they all failed to enter this condition. It is possible that the induction and length of the second consecutive pseudopregnancy is dependent upon the effects of exogenous hormones given during the first pseudopregnancy.

Previous to the ovariectomy of rats in a second consecutive pseudopregnancy, it was postulated by this worker that possibly the induction and maintenance of a second consecutive pseudopregnancy would

be controlled by accessory corpora lutea formed sometime between the
first and second pseudopregnancies. This idea was disproved by the
ovariectomy. Apparently, the exogenous dosages of estrogen and pro-
gesterone suppressed the gonadotropic activity. It seemed that this
tended to arrest the production of any accessory corpora lutea.

The results obtained from Parts II-A through II-C indicate that
the rats injected on day 12 of the first pseudopregnancy remained in
this extended pseudopregnancy longer than any of the other estrogen-
progesterone-treated rats. It is apparent that the extended length
of the first pseudopregnancy is dependent upon the day of initiation
of hormonal treatment and not necessarily the various intervals used
in this investigation. The fact that only three out of 10 rats entered
an extended pseudopregnancy when the treatment was initiated on day 13
also seems to confirm this. Further statistical support is given to
this concopt in that there was no significant difference between the
extended pseudopregnancy lengths of the groups in which treatment be-
ban on day 13 at 24-hour intervals and the groups in which treatment
began on day 13 at 48-hour intervals. It is thought that since these
rats were injected on the day in which pseudopregnancy was close to
termination, the effects of the exogenous hormones were not exhibited.

Although the extension of the first pseudopregnancy seemed to be
primarily dependent upon the day of initiation of hormonal treatment,
the length of the consecutive pseudopregnancy was more dependent upon
the injection intervals during the second pseudopregnancy. This was
supported by the results obtained from Parts II-A through II-C.
These rats, in which hormonal treatment was initiated on day 13 and

given at 24-hour intervals during the second consecutive pseudopreg-
nancy, exhibited the longest consecutive pseudopregnancy of any group
used in this investigation. When this hypothesis was statistically
tested it was seen that there was a high degree of significance be-
tween the 24-hour and 48-hour injected rats ($p < 0.01$). There was,
however, no significant difference between the various 48-hour inject-
ed groups. It was more important, however, that the first pseudopreg-
nancy be extended in order for a second consecutive pseudopregnancy
to be induced. There were more corpora lutea produced during the one
day estrus interval in those animals that entered an extended first
pseudopregnancy. The increased number of corpora lutea produced under
this condition did not vary significantly from those produced in the
control animals.

When the rats in Part II-D which were administered hormones only
during the first pseudopregnancy failed to enter a second consecutive
pseudopregnancy, the results tended to give support to two previous
postulations: (1) The neural stimulus of cervical trauma alone is
not capable of inducing a second pseudopregnancy. (2) The induction
of the second pseudopregnancy appears to be correlated with hormonal
treatment involved in the extension of the first pseudopregnancy.

Since hormonal treatment for each rat in Part II-E began on
either day 13 or when there was a vaginal smear of proestrus (which-
ever came first), it was expected that the exogenous hormones would
not have prevented or delayed the on-coming estrus due to the time
factor. However, each of these rats entered an extended pseudopreg-
nancy. Since this extended the first pseudopregnancy, it was of no

value in determining a second consecutive pseudopregnancy. Apparently this injection extended the functional life of the corpora lutea present.

The mean extended length of the first pseudopregnancy in rats receiving exogenous dosages of estrogen alone was 17.4 days. This was shorter than the shortest extended pseudopregnancy of the estrogen-progesterone treated rats (Part II-C). Since the estrogen-progesterone-treated rats had a significantly longer extended first pseudopregnancy, it is possible that progesterone acted synergistically with estrogen in the prolongation of the first pseudopregnancy.

A shorter second consecutive pseudopregnancy ($p < 0.01$) was produced by the injection of estrogen alone (Part II-E) than the shortest estrogen-progesterone-treated rats (Part II-A). This suggests that there is a synergistic action between estrogen and progesterone in maintaining a second consecutive pseudopregnancy. It was also seen that the lengths of the second pseudopregnancy among the estrogen-treated rats were more uniform than in the estrogen-progesterone treated groups. Due to the potency of small amounts of exogenous estrogen, it is thought that the reason for the uniformity in the lengths of pseudopregnancy is that the exogenous estrogen suppressed any endogenous effects of circulating progesterone. The results from this estrogen-treated group are supported by similar work done by Reed (1966), Bogandove (1966) and Malkowa (1956). They reported that small dosages of estrogen extend pseudopregnancy. It is thought that the extended first pseudopregnancies which were induced in this investigation were brought about by prolonging the corpora lutea. This prolongation was

the result of estrogen and progesterone injections administered at various intervals. This is based upon the previously mentioned report by Velardo (1953) that histochemical evidence indicates that corpora lutea of prolonged pseudopregnancy are functional.

When the functional lives of corpora lutea were not prolonged, they underwent complete atrophy by day 13 or 14. It is thought that when the untreated controls were re-stimulated for a second consecutive pseudopregnancy, the relatively few corpora lutea observed (8.5), were not enough to produce the necessary amounts of estrogen and progesterone to cause a second consecutive pseudopregnancy. This is based on results obtained from the three groups which were ovariectomized in this investigation. There is no significant difference between the numbers of corpora lutea found in the ovaries of the untreated control rats in the first pseudopregnancy (Part I-C), and those in Part II-A$_2$ which entered a second consecutive pseudopregnancy, 13.0 and 12.7 respectively. Both groups had the same number of corpora lutea as were found in normal pseudopregnant rats. This suggests that the number of corpora lutea may be a significant factor in obtaining the second consecutive pseudopregnancy.

The dosages without cervical stimulation apparently were not enough to induce a second consecutive pseudopregnancy. It appears that the induction of the second consecutive pseudopregnancy is brought about by a simulation of the natural physiological hormone levels by estrogen and progesterone from the injections as well as additional amounts produced by the corpora lutea.

Two consecutive pseudopregnancies were induced in the specified

groups in this investigation. No consecutive pseudopregnancies were induced in the untreated control rats which did not receive hormonal treatment. Since estrogen and progesterone can cause changes in uterine and vaginal tissues in the absence of pseudopregnancy, it is possible the continuous diestrus smears were not a true indication of pseudopregnancy. Vaginal lavages of the three control groups of Part III (which received a single dosage of progesterone on the day of estrus, a single dosage of progesterone on the first day of diestrus, and a single dosage of estrogen on the day of estrus), showed no direct effect on the vaginal epithelium. Therefore, the extended non-cyclic condition is considered to be the result of pseudopregnancy and not an extended diestrus period. The previously cited work of Austin (1956) and Fluhmann (1955), gives support to the results obtained from the animals in Part III-C which received a single dosage of estrogen on the day of estrus, in that the rats expressed a continuous vaginal cornification for 8.0 days.

SUMMARY

(1) Results from untreated control rats showed that the formation of deciduomata was not necessarily a physiological prerequisite for the induction or extension of pseudopregnancy.

(2) A second consecutive pseudopregnancy was not induced in the control rats which received no estrogen-progesterone injections. It was thought that an insufficiency of gonadal hormones existed in these animals during the estrus which terminated the first pseudopregnancy.

(3) Results obtained from rats in ovariectomy experiments indicated that there was no significant difference between numbers of corpora lutea in those rats which were in first pseudopregnancies and hormonally-treated rats in second consecutive pseudopregnancies. However, there was a significant reduction of corpora lutea in control animals that did not enter a second consecutive pseudopregnancy.

(4) Injections of estrogen and progesterone combined and of estrogen alone on day 12 or 13 prolonged first pseudopregnancies. Results also indicated that the hormonal treatment had a greater effect on extending the first pseudopregnancy when injections were initiated on day 12.

It was demonstrated that a second consecutive pseudopregnancy could be induced and maintained in treated groups. Estrogen-progesterone injections had a greater inducing and maintaining effect on second consecutive pseudopregnancies when administered at 48-hour intervals. Second consecutive pseudopregnancies were initiated only following extended first pseudopregnancies.

Injections of estrogen, when initiated on day 12 of first pseudo-pregnancy and administered at 48-hour intervals, prolonged this pseu-dopregnancy and facilitated induction of a second consecutive pseu-dopregnancy.

(5) Results from controls which were administered estrogen and pro-gesterone indicated that continuous diestrus-like vaginal cells seen in non-control animals were the effects of pseudopregnancy and did not result from direct histological effects of hormones on uterine and vaginal tissues.

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