This is to certify that the

thesis entitled

AN ANALYSIS OF FACTORS RESPONSIBLE FOR RESORPTION OF FETUSES IN CISPLATIN TREATED RATS

presented by

Mary Lynn Bajt

has been accepted towards fulfillment
of the requirements for

Masters degree in Zoology

S. K. Agarwal
Major professor

Date September 7, 1982

AN ANALYSIS OF FACTORS RESPONSIBLE FOR RESORPTION OF FETUSES IN CISPLATIN TREATED RATS

By

Mary Lynn Bajt

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Zoology

1982

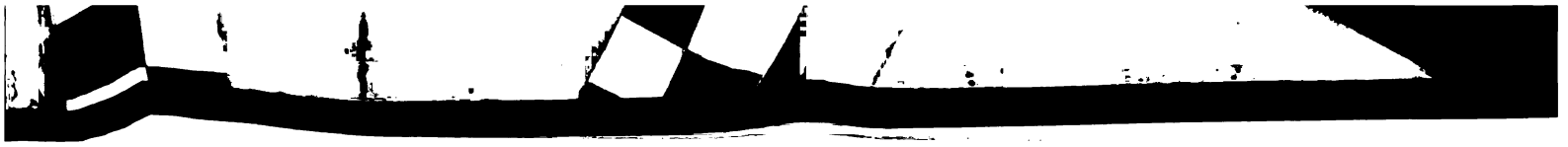
ABSTRACT

AN ANALYSIS OF FACTORS RESPONSIBLE FOR RESORPTION
OF FETUSES IN CISPLATIN-TREATED RATS

By

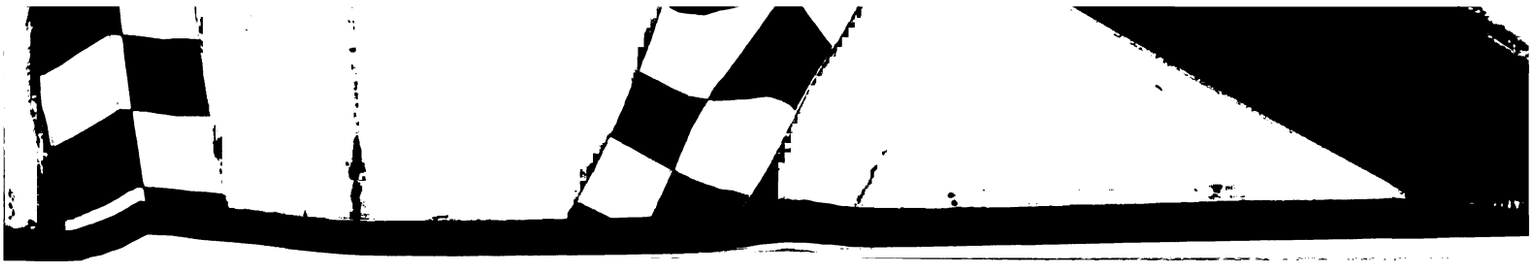
Mary Lynn Bajt

Pregnant rats were injected i.p. with 7 mg/kg cisplatin on day 6 of gestation to study its effect on fetal resorption. Serum concentrations of prolactin, luteinizing hormone (LH), and progesterone were determined by radioimmunoassay in pregnant rats, and related to the effects of cisplatin on the maintenance of pregnancy. The nocturnal prolactin surge on day 9 of gestation was abolished in cisplatin-treated rats. Within 3 days after drug injection, LH concentrations decreased 39%, while serum progesterone levels decreased 63% by day 10. A histochemical study of 20α-hydroxysteroid dehydrogenase activity revealed no enzyme activity by day 10. It is proposed that the cause of cisplatin-related fetal resorption in rats is due to decreases in pituitary hormones observed after drug treatment.



To my parents for their constant

love and support



ACKNOWLEDGEMENTS

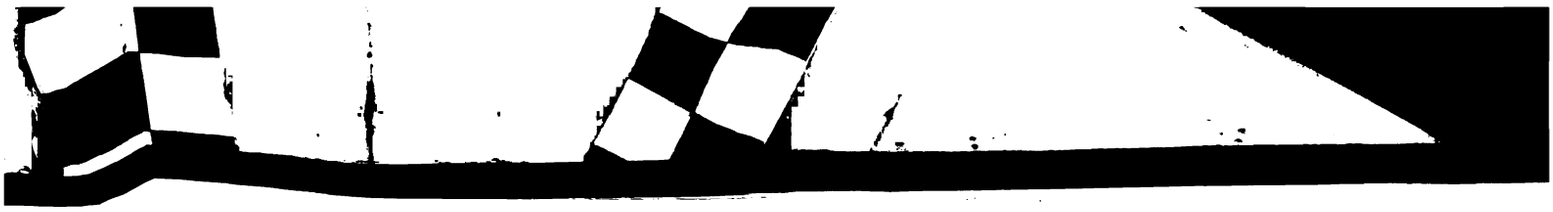
I wish to thank Dr. S.K. Aggarwal for serving as my major professor. Appreciation is also due to Drs. J. Meites and L. Clemens who served as guidance committee members. I wish to especially thank Dr. J. Meites for his generosity with regards to laboratory facilities and research materials provided.

I would also like to express appreciation to Bill Sonnteg for his technical help and friendship, and to "grumpy" Paul Sylvester and Vince Hylka.

A special thanks goes to Jim San Antonio for the technical help and advice but mostly for our eternal friendship.

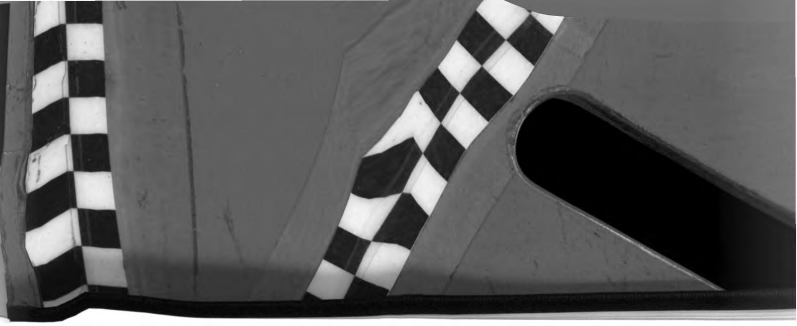
TABLE OF CONTENTS

	Page
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	v
LIST OF FIGURES	vi
INTRODUCTION	1
MATERIALS AND METHODS	4
Animals	4
Tissue Handling Procedures	5
Histochemistry	5
Radioimmunoassays (RIA)	6
Statistics	11
RESULTS	12
Maternal Weight Gain/Loss Due to Cisplatin	12
Embryolethality	12
Effects of Cisplatin on Pituitary Luteotropic Hormone	18
Serum Concentrations of Prolactin	18
Serum Concentrations of Luteinizing Hormone (LH)	21
Effects of Cisplatin on Ovarian Function	21
Ovarian Weights	21
Serum Concentrations of Progesterone	25
20α-Hydroxysteroid Dehydrogenase (20α-OHSD) Activity	25
Histology of Rat Corpora Lutea and Fetuses	28
DISCUSSION	34
BIBLIOGRAPHY	39



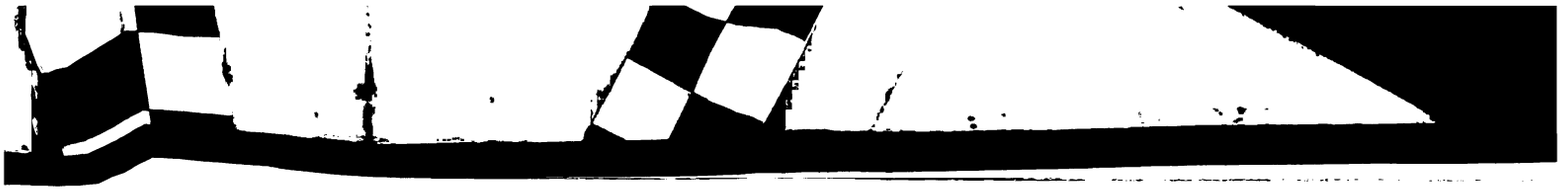
LIST OF TABLES

Table		Page
1	Effects of cisplatin (CDDP) given i.p. to pregnant rats on day 6 of gestation----------	17
2	Effects of cisplatin (CDDP) on ovarian weights during gestation----------	24



LIST OF FIGURES

Figure		Page
1	Standard curves of Prolactin RP-1 and serum from an animal treated with CDDP on day 6 of gestation---------	9
2	Maternal body weight changes for female Wistar rats following i.p. injection of saline or cisplatin (CDDP) on day 6 of gestation----------	13
3	Isolated uteri of control and cisplatin-treated pregnant rats----------	15
4	Serum concentrations of prolactin at 0800 h and 1350 h on day 9 of gestation following injection of saline or 7 mg/kg cisplatin (CDDP) on day 6----------	19
5	Serum concentrations of luteinizing hormone (LH) at 1350 h on day 9 of gestation following injection of saline or 7 mg/kg cisplatin (CDDP) on day 6----------	22
6	Serum concentrations of progesterone at 1350 h on days 9, 10, and 12 of gestation following cisplatin (CDDP) treatment on day 6----------	26
7	Light micrographs of corpora lutea on day 9 of gestation from rats treated with saline or 7 mg/kg cisplatin on day 6 of gestation----------	29
8	Light micrographs of longitudinal sections through embryos 9 days old from rats treated with saline or 7 mg/kg cisplatin on day 6 of gestation----------	32



INTRODUCTION

Cis-diamminedichloroplatinum II (CDDP or cisplatin) is the first member of a family of platinum coordination complexes to be used in cancer chemotherapy (41). Cisplatin use as a potent anticancer drug was first demonstrated by Rosenberg et al. (42). Since its introduction into clinical trials in 1972 by the National Cancer Institute (41), cisplatin has been approved primarily for clinical use against testicular and ovarian tumors (20, 25, 40). Cisplatin, when used in combination with other antitumor agents, has proven to be effective against cancers of the head, neck, bladder, prostate, lung, and cervix (20, 25, 41).

The biological activity of cisplatin on inhibition of tumor growth is still questionable (41). The primary mechanism of cisplatin at the cellular level appears to be the inhibition of DNA synthesis through intra- (19, 23) and interstrand DNA crosslinks (53). Cisplatin may interfere with division of tumor cells by causing depolymerization of microfilaments and preventing polar migration of the centrioles (1).

Therapeutic usage of cisplatin is hampered by severe dose-limiting toxic side effects including renal toxicity, nausea and vomiting, myelosuppression, and decreases in serum electrolytes (25, 41, 45). Slow intravenous infusion of cisplatin, intravenous saline

hydration, and diuresis have helped decrease the cisplatin-induced nephrotoxicity (25, 41, 51) which is no longer considered to be a dose-limiting factor (41). Antiemetic treatment slightly decreases the drug-induced nausea and vomiting (41).

Cisplatin distribution is initially highest in the excretory organs, gonads, and spleen (27, 28, 30), but after 4 days, cisplatin is concentrated mainly in the kidney, uterus, and ovaries (30). In male mice and monkeys, cisplatin treatment has been demonstrated to kill differentiated spermatogonia (35, 45). Furthermore, it has been shown to be embryotoxic in rats and mice (24, 29) and teratogenic in mice (29).

The cause of cisplatin-induced fetal resorption in the rat is unknown. Since cisplatin is used in the treatment of women of child-bearing age, it is important to study the effects of the drug on the embryo and the maintenance of pregnancy.

Hormones play a major role in maintenance of pregnancy, primarily those secreted by the pituitary, ovary, and placenta (34). Progesterone, mainly from the corpora lutea of ovaries, is one of the basic factors responsible for maintenance of pregnancy (9, 44). It is therefore essential that regression of the corpora lutea be prevented for a continual secretion of progesterone.

The secretion of a luteotropic complex is largely responsible for the prolongation of the life span of the corpora lutea (21, 34). During the first half of gestation, the luteotropic complex in rats consists of pituitary hormones luteinizing hormone (LH) and prolactin (PRL) (21). LH and PRL play an indespensable role during gestation, since hypophysectomy before day 12 terminates pregnancy (39).

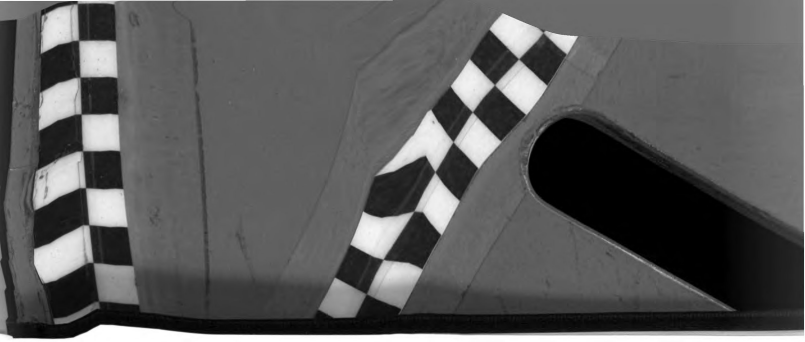
The soluble enzyme 20α-hydroxysteroid dehydrogenase (20α-OHSD) in corpora lutea is responsible for the conversion of progesterone to the hormonally inactive derivative, 20α-hydroxypregn-4-en-3-one (26). The induction of 20α-OHSD has been used as an index of luteolysis in the rat (21, 26).

The aim of this study was to determine the effects of cisplatin on serum concentrations of PRL, LH, and progesterone because of the importance of these hormones in the maintenance of pregnancy. The appearance of histochemically demonstrable 20α-OHSD enzyme activity was studied because of its importance in steroidogenesis.

MATERIALS AND METHODS

Animals

Laboratory bred Wistar virgin female rats (Charles River Breeding Lab, Willington, MA) weighing between 250-300 grams and approximately 3 months of age were used in these experiments. Rats were maintained under a controlled 12:12 h light/dark schedule (lights on 0900 h to 2100 h) and temperature (23°C), and were given Wayne laboratory animal food (Allied Mills, Inc., Chicago) and tap water ad libitum. During pair-fed control experiments, intake of food by the control animals was limited to that consumed by drug-treated animals. This was done because drug treatment is known to decrease food intake in rats (24). Estrous cycles were monitored by daily vaginal smears between 1000 h and 1300 h and only rats showing regular 4 day cycles were included in experiments. Rats in proestrus were placed with males overnight; if spermatozoa were found in vaginal smears on the following day, this was considered day 1 of pregnancy. Animals were weighed daily during the length of the experiment. Rats exhibiting daily diestrous smears 6 days after mating were assigned to control or drug-treated groups so that equal numbers of animals from each group were sacrificed on the same day. Control and drug-treated animals were housed separately with 4 animals per cage.



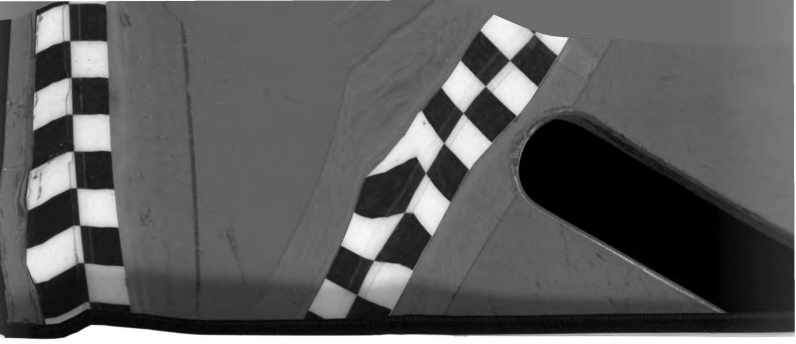
Cisplatin (Johnson Matthey Research Laboratories, Sonning, U.K.) in powder form was dissolved in physiological saline prepared just before use. Rats received a single i.p. injection of 4 or 7 mg/kg of cisplatin in saline on day 6 of gestation and control animals received saline only. Animals were sacrificed on day 9 at 0800 h and 1350 h, while on day 10 or 12 they were sacrificed at 1350 h. Rats were decapitated within 20 seconds after removal from their cages. Trunk blood was collected and allowed to clot overnight at 4°C. The following day clots were removed and samples were centrifuged at 5000 rpm for 10 minutes. Serum was stored frozen at -20°C until assayed.

Tissue Handling Procedures

Uteri and ovaries were dissected from one third of the rats and fixed in Bouin's fluid for routine histological examination. Tissue samples were serially sectioned at 8 μm and stained with hematoxylin and eosin. In sections of ovaries, luteal cells were measured by use of an ocular micrometer. The uteri and left ovaries from the rest of the animals were placed in 25 ml of saline and widths of embryonic swellings were recorded. The ovaries were blotted on a filter paper and weighed to nearest 0.001 g. The right ovaries were removed and prepared for histochemical evaluation of 20α-hydroxysteroid dehydrogenase activity.

Histochemistry

Ovaries were placed on a microtome stub covered with O.C.T. compound (Miles Laboratories, Ill.) and immediately frozen by immersion



for 20 seconds in liquid nitrogen. Frozen sections were cut at 10 μm in a freezing microtome. Sections were placed on coverslips and thawed at room temperature for 30 minutes. Sections were treated according to a modified procedure of Balagh (6). Coverslips were transferred by forceps into the incubation medium. The incubation medium was composed of 5 mg of nitro-blue tetrazolium, 5 mg B-nicotinamide adenine dinucleotide phosphate (TPN), and 10 mg of disodium ethylenediaminetetraacetate dissolved in 2 ml of tris HCl at pH 9.0. Two ml of 50% polyvinylpyrrolidine K-30 (PV) solution (in tris-HCl buffer pH 9.0) and 1 ml of N,N-dimethylformamide containing 5 mg of 20α-hydroxypregn4-en-3-one was then pipetted into the medium. Control incubation medium was prepared identically with the exclusion of the substrate, 20α-hydroxypregn-4-en-3-one. Tissues were incubated at 37°C for 60 minutes. Sections were then fixed for 1 hour in neutral 10% formalin at room temperature, rinsed in saline, and mounted on microscope slides with glycerin jelly. All compounds were obtained from Sigma (St. Louis, Mo.) and solutions were mixed fresh for each experiment. Slides were examined by light microscopy.

Radioimmunoassays (RIA)

Serum levels of PRL and LH were measured using the RIA method originally developed by Niswender et al. (37, 38). Material for RIA were provided by NIAMDD (Bethesda, Md.). A non-equilibrium assay was used for PRL and LH RIA and the protocols were essentially the same. The reference preparation was serially diluted and the various concentrations were used to determine a standard curve. Antiserum raised in rabbits was incubated with the standard or serum sample at

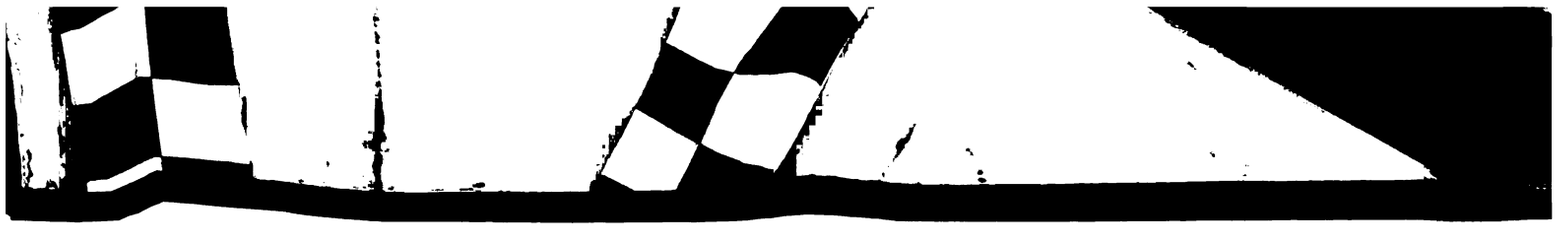
4°C for approximately 24 hours. Hormone iodinated by chloramine T (17) was then added to all tubes, and tubes were allowed to incubate for another 72 hours at 4°C. Total count tubes (iodinated hormone only), total bound tubes (no cold hormone added), and nonspecific bound tubes (excess amount of cold hormone added) were also included in the assay.

Protein A (IgG SORB) of 2.0% cell suspension was then added to separate antibody-bound hormone from free hormone. After 30 minutes incubation at 4°C, 2 ml of ice cold saline was added and tubes were centrifuged at 25,000 rpm for 20 minutes. The supernatants were decanted and the precipitates counted on a tracer analytic gamma spectrometer.

For prolactin RIA, reference preparation Rat Prolactin RP-1 and antiserum Anti-Rat Prolactin #4-10 (gift from Dr. D. Chen) was used. The minimum detectable dose was calculated to be 0.05 ng/tube and 50% inhibition of tracer binding was achieved at 0.7 ng/tube. The intra-assay coefficient of variation was 4%.

For LH RIA, reference preparation NIAMDD Rat LH RP-1 and antiserum NIAMDD Anti-Rat LH S-5 was used. The minimum detectable dose was calculated to be 0.8 ng/tube and 50% inhibition of tracer binding was achieved at 3.95 ng/tube. The intra- and inter-assay coefficients of variation were 4% and 7%.

Since cisplatin within serum samples might interfere with RIA, serum samples from rats treated on day 6 of gestation with CDDP and Prolactin RP-1 were quantitated for standard curves (47). The slopes of the linear curves were not significantly different



(Figure 1). Therefore, no interference due to cisplatin within serum samples was observed.

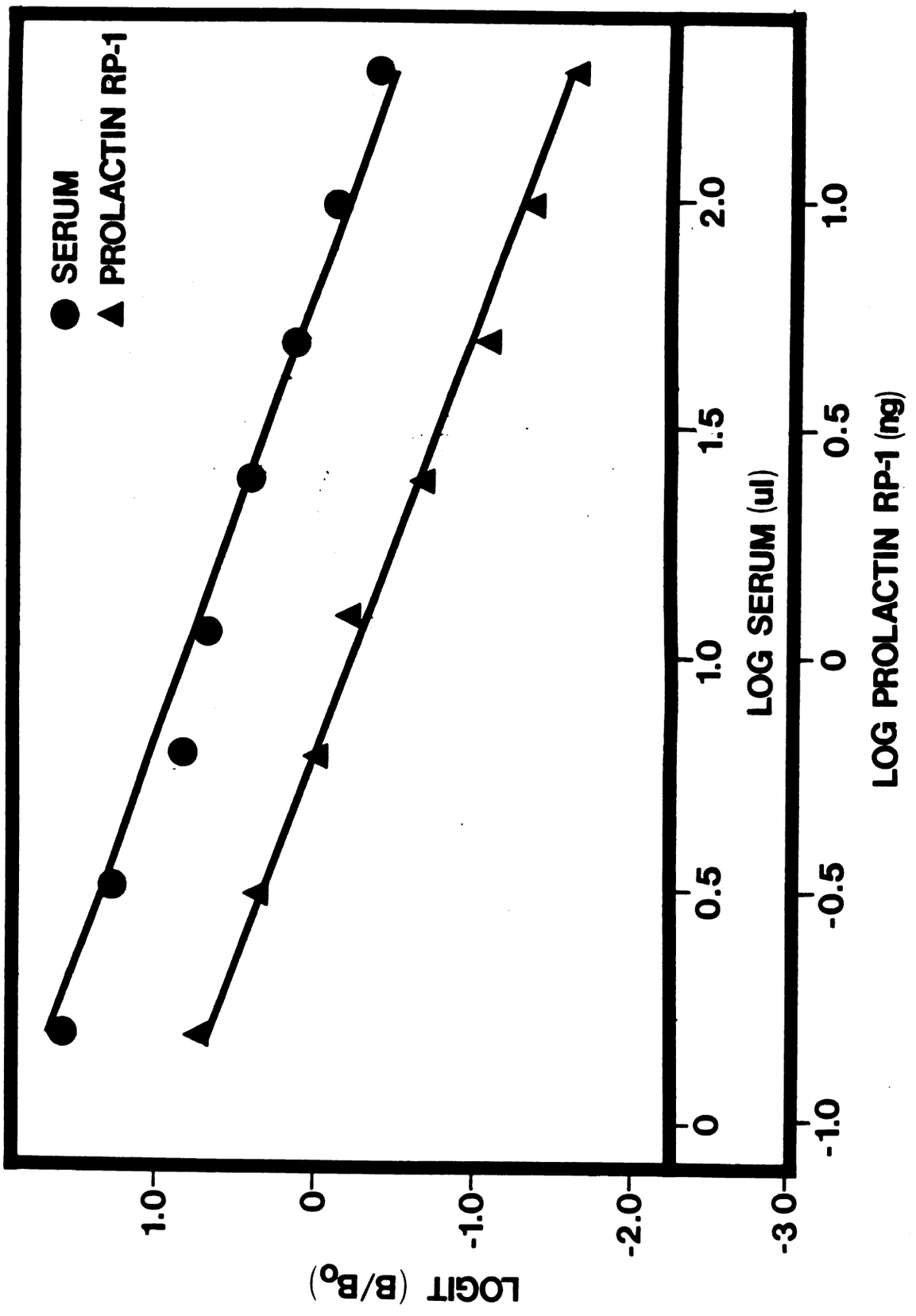
Serum progesterone levels were determined by a non-chromatographic radioimmunoassay method. Serum samples were diluted 1:20 in assay buffer in duplicate. Radiorecovery solution, 20% of radioactive 1, 2, 6, 7, ^{3}H-progesterone (New England Nuclear, Mass.) was added to one set of duplicates to correct for extraction procedural losses. Serum was extracted twice with distilled petroleum ether and dried extracts were resuspended in assay buffer.

Reference preparation, 4-pregnene-3,20-dione (Sigma, St. Louis, Mo.), from a stock solution in ethanol was added to standard tubes at various concentrations to determine a standard curve. Serum samples which contained radiorecovery 1, 2, 6, 7 ^{3}H-progesterone were used to calculate percent recovery of progesterone extracted.

Hormone specific antibody raised in rabbits (gift from Dr. Niswender) and radioactive 1, 2, 6, 7 ^{3}H-progesterone were added to all tubes. A reference preparation or serum sample was then added to tubes and tubes were incubated at 4°C for approximately 24 hours. Total count, total bound, nonspecific bound, and radiorecovery tubes were included in the assay.

A suspension of activated charcoal (Matheson Coleman and Bell, Norwood, Ohio) coated dextran T-70 (Pharmacia, Uppsala, Sweden) was added to separate free hormone from the soluble antibody hormone complex for 10 minutes at 4°C. Tubes were spun down at 3000 rpm for 10 minutes in a refrigerated centrifuge. The supernatant was decanted into scintillation vials and 10 ml of Formula 963, aqueous

Figure 1. Standard curves of Prolactin RP-1 and serum from an animal treated with 7 mg/kg cisplatin on day 6 of gestation and sacrificed on day 9. Curves plotted in terms of logit Y (where Y = Bound/B_0 and B_0 equals the fraction of tracer bound when only tracer is present) plotted against the log of hormone concentration or volume.

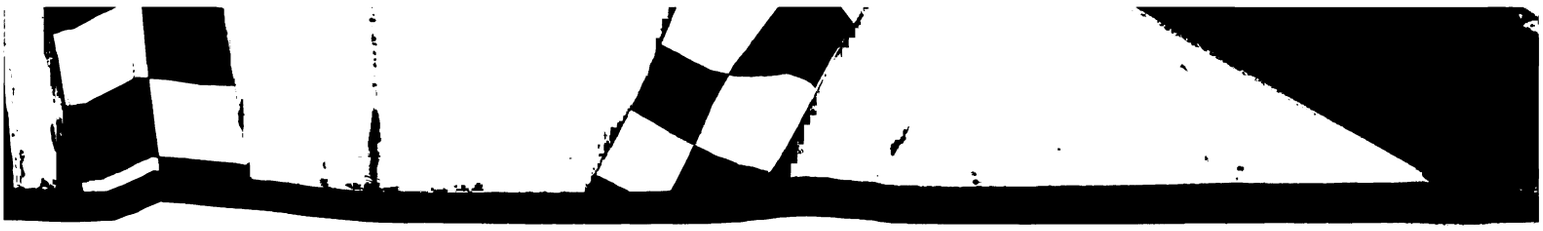

SERUM
PROLACTIN RP-1
LOGIT (B/B$_o$)
1.0
0
-1.0
-2.0
-3.0
LOG SERUM (ul)
2.0
1.5
1.0
0.5
0
-0.5
-1.0
LOG PROLACTIN RP-1 (ng)

counting cocktail, was added. Sample radioactivity was determined by use of a scintillation counter. Correction for procedural losses during extraction was accomplished by calculation of the percent recovery of 1, 2, 6, 7 ^{3}H-progesterone added to each serum sample. Minimum detectable dose was calculated to be 0.88 pg/tube and 50% inhibition of tracer binding was achieved at 51.3 pg/tube. The intra-assay coefficient of variation was 2%.

Serum concentrations of PRL, LH, and progesterone were determined by comparison of the amount of labeled hormone precipitated in the presence of the serum sample to that observed in a standard curve prepared with the appropriate reference preparation. A computer program using logit and log transformations and linear regression analysis was used for calculation of hormone levels in the sample. Serum from normal females, castrated males, and charcoal washed serum were included in the assays to serve as controls.

Statistics

All data were statistically analyzed by use of the student's "t" test when a comparison between control and CDDP treatment was made. Data from experiments including controls, pair-fed controls, and CDDP-treated animals was analyzed by use of analysis of variance followed by Duncan's New multiple range test.



RESULTS

Maternal Weight Gain/Loss Due to Cisplatin

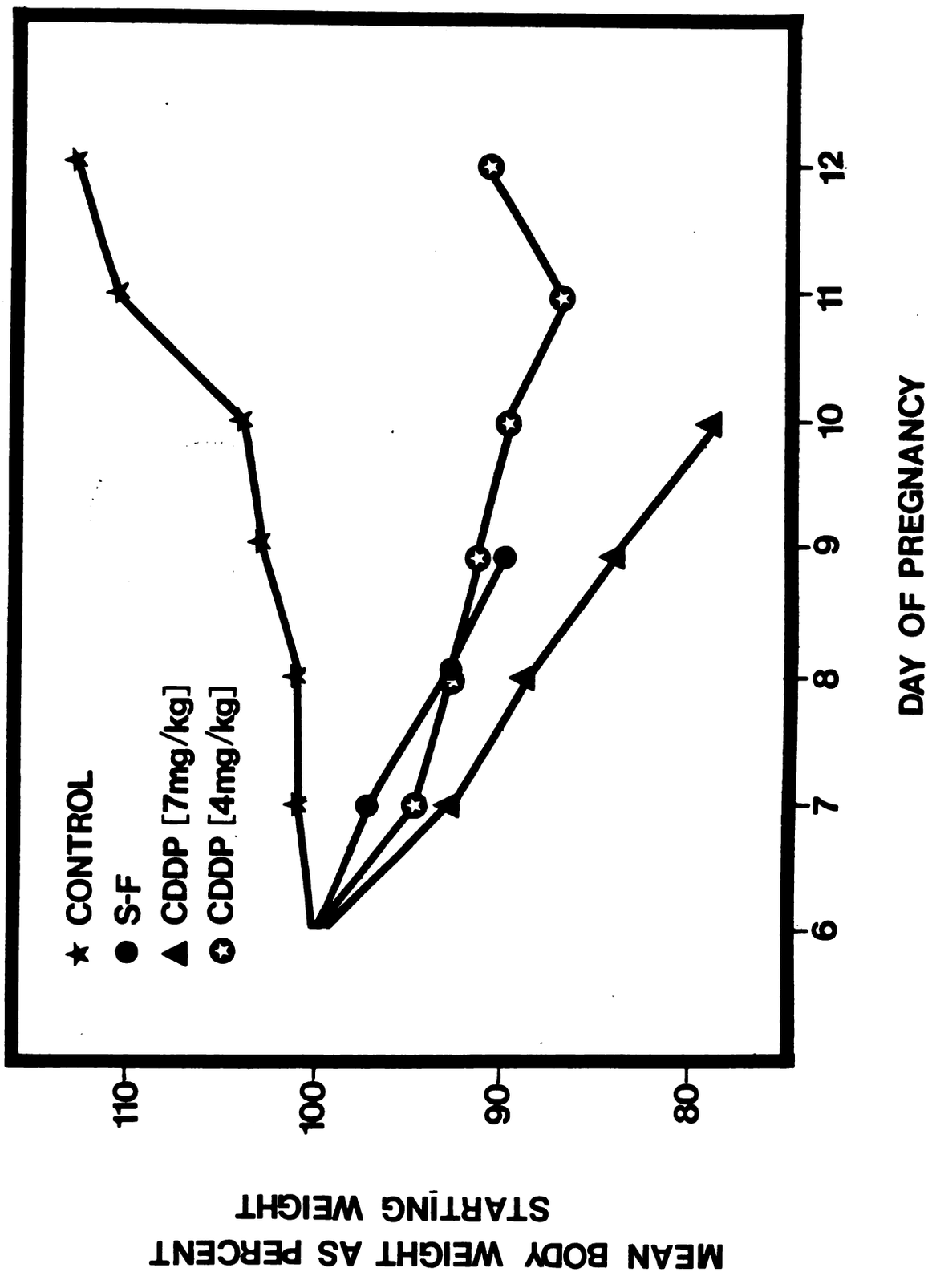
Changes in the maternal weight of cisplatin-treated rats were compared to that of controls in order to determine if the appetite-suppressant effects of cisplatin could be partly responsible for the drug-related maternal toxicity. Drug-treated and control dams were injected on day 6 of gestation (Figure 2). A 19% weight loss occurred within three days following treatment with 7 mg/kg cisplatin. Pair-fed controls with the 7 mg/kg cisplatin-treated dams exhibited a 13% weight reduction by day 9 of gestation. Maternal weight of dams treated with 4 mg/kg cisplatin decreased 12% by day 9 and 24% by day 11 compared to controls. By day 12, however, maternal weight increased 4% from day 11.

Cisplatin caused a decrease in maternal weight which can be partly attributed to decreased food intake as indicated by pair-fed controls. With a lower dosage of cisplatin, 4 mg/kg, maternal weight decreased during the first five days following treatment but an increase in weight was apparent by the sixth day.

Embryolethality

Cisplatin was highly embryolethal in rats, causing 100% resorption when administered on day 6 of gestation (Figure 3). Table 1

Figure 2. Maternal body weight changes for female Wistar rats following i.p. injection of saline or cisplatin (CDDP) on day 6 of gestation. Mean scatter about each point > 7.0%. Each point based on an average of 8 animals. S-F, saline treated animals whose food intake was limited to that consumed by 7.0 mg/kg cisplatin-treated animals.


MEAN BODY WEIGHT AS PERCENT
STARTING WEIGHT
110
100
90
80
6
7
8
9
10
11
12
DAY OF PREGNANCY
★ CONTROL
● S-F
▲ CDDP [7mg/kg]
✪ CDDP [4mg/kg]

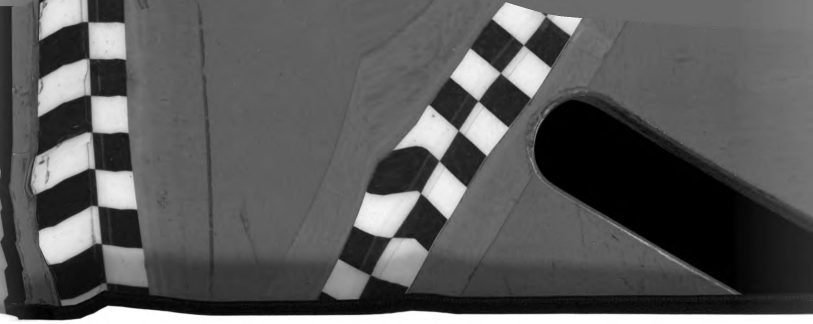
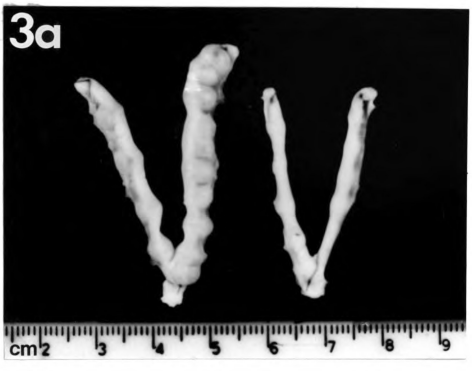


Figure 3. a. Isolated uteri of control (left) and 7 mg/kg cisplatin-treated (right) pregnant rats. Rats were 9 days pregnant and cisplatin treatment was given on day 6 of gestation.

b. Isolated uteri from control (left) and 4 mg/kg cisplatin-treated (right) pregnant rats. Rats were 12 days pregnant and cisplatin treatment was given on day 6 of gestation.


3a
cm 2 3 4 5 6 7 8 9

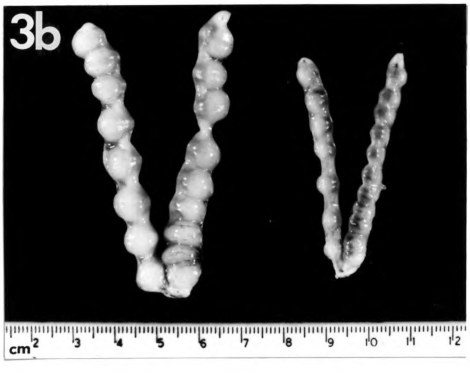

3b
cm 2 3 4 5 6 7 8 9 10 11 12

Table I. Effects of cisplatin (CDDP) given i.p. to pregnant rats on day 6 of gestation.

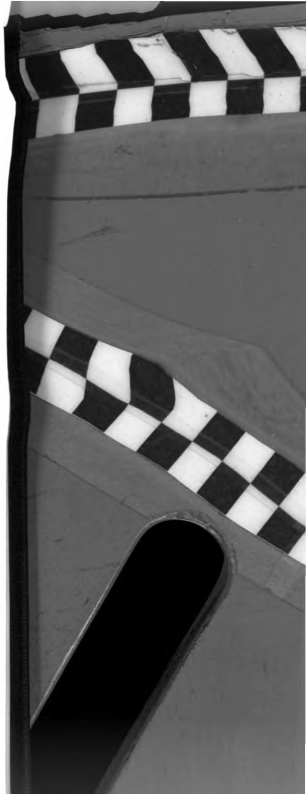
Treatment (mg/kg)	Day of Gestation Sacrificed	Number of Animals	Number of Embryonic Swellings	Average Widths of Embryonic Swellings[a] (mm)	State of Pregnancy[b]
Saline	9	5	76	5.4 ± .4	n
S-F[c]	9	4	56	5.3 ± .6	n
7.0 CDDP	9	9	124	3.0 ± .4[d]	res
Saline	12	2	31	8.0 ± .5	n
4.0 CDDP	12	4	54	4.3 ± .6[d]	res

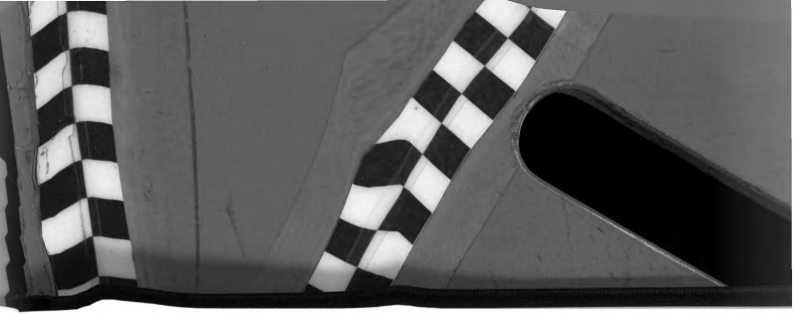
[a]Results given as mean ± S.D.

[b]N = normal fetuses, res = resorbed fetuses.

[c]Saline treated animals whose food intake was limited to that consumed by 7.0 CDDP-treated animals.

[d]Significantly different ($p<0.01$) from control.





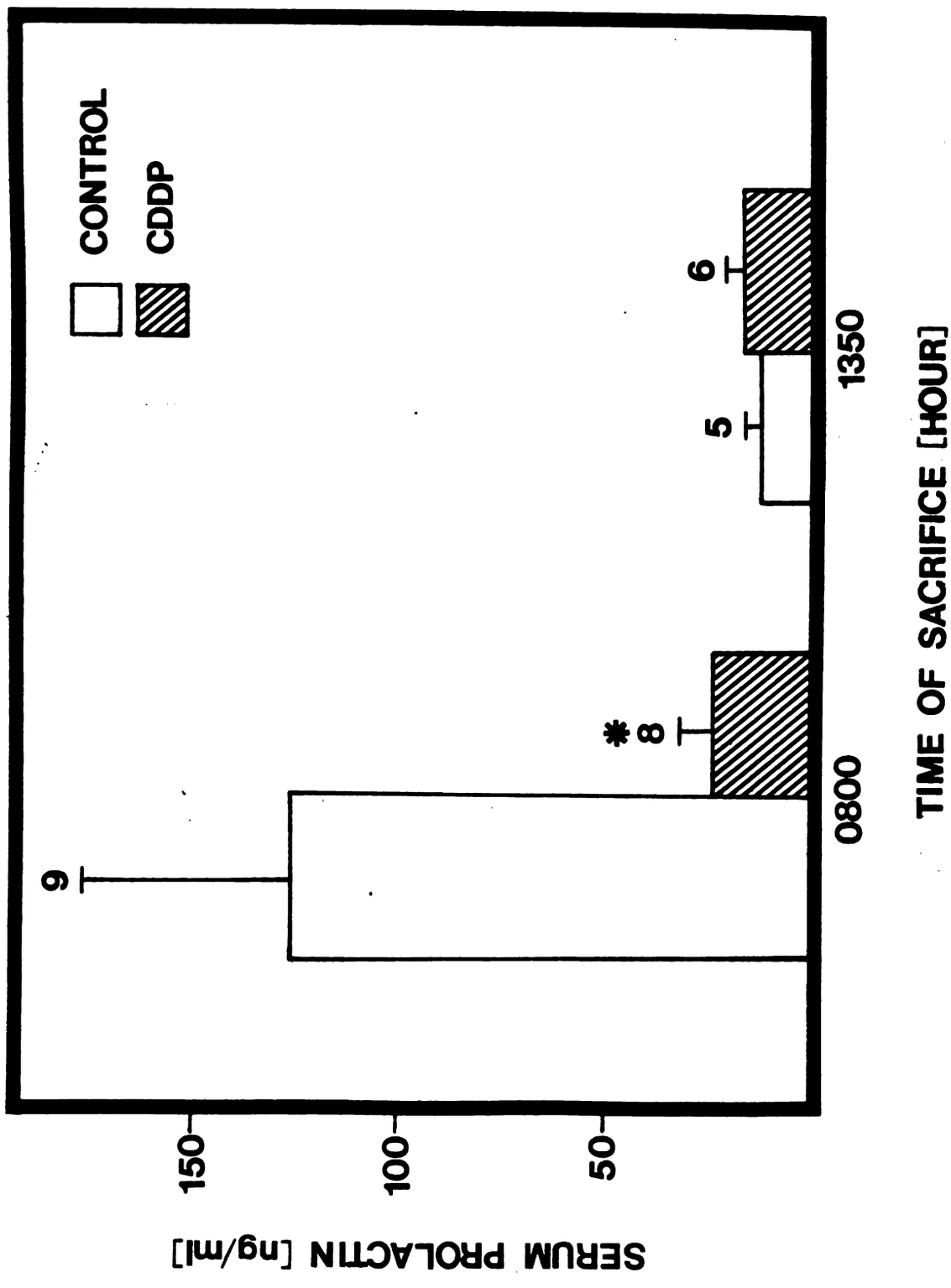
summarizes the effects of a single i.p. injection of cisplatin on fetal destruction in pregnant rats. Cisplatin (7 mg/kg) caused a significant reduction in widths of embryonic swellings by day 9 of gestation ($p<0.01$). Pair-fed control dams did not demonstrate any significant difference from the controls. Dams treated with 4 mg/kg cisplatin showed a significant ($p<0.01$) reduction in widths of embryonic swellings by day 12 of gestation compared to controls.

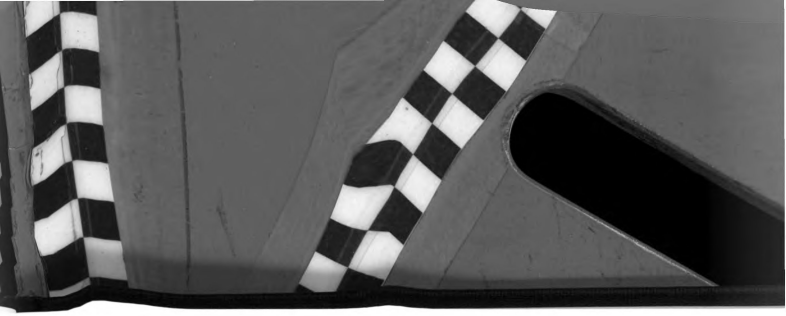
The appetite-suppressant effects of cisplatin alone cannot account for the resorption of fetuses, since pair-fed control dams did not demonstrate the same embryolethal effects as cisplatin-treated dams.

Effects of Cisplatin on Pituitary Luteotropic Hormone

Serum concentrations of prolactin: To investigate the possibility that cisplatin affects surges of prolactin during pregnancy, rats were decapitated on day 9 of gestation to obtain blood for prolactin analysis (Figure 4). Rats were sacrificed at 0800 h to obtain concentrations of prolactin during the nocturnal surge and at 1350 h for baseline levels of prolactin. Mean concentrations of prolactin in control animals at 0800 h indicated the presence of the nocturnal surge of prolactin with a 9-fold increase above baseline levels obtained at 1350 h. Cisplatin treatment (7 mg/kg) on day 6 of gestation abolished the nocturnal surge of prolactin on day 9. Baseline levels of prolactin at 1350 h were not affected by cisplatin treatment as compared to control prolactin concentrations.

Figure 4. Serum concentrations of prolactin at 0800 h and 1350 h on day 9 of gestation following injection of saline or 7 mg/kg cisplatin (CDDP) on day 6. Results are mean + S.D. with number of rats used in parenthesis. *, significantly different ($p<0.01$) from control.


CONTROL
CDDP
SERUM PROLACTIN [ng/ml]
150
100
50
9
*8
6
5
0800
1350
TIME OF SACRIFICE [HOUR]



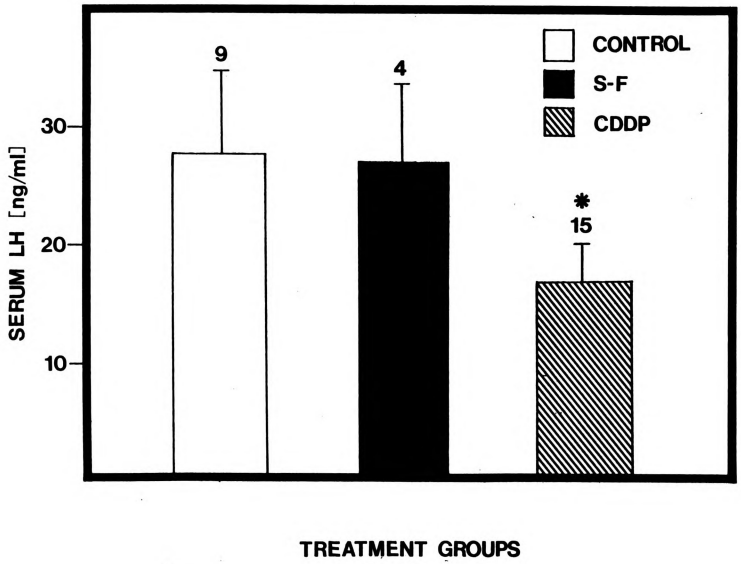
Serum concentrations of luteinizing hormone (LH): In order to determine if cisplatin affects LH levels during pregnancy, trunk blood was collected from pregnant rats at 1350 h on day 9 of gestation (Figure 5). Dams treated with cisplatin (7 mg/kg) showed a significant decrease ($p<0.05$) in circulating LH levels as compared to controls.

To assure that the decrease in concentration of LH was not due to the decreased food intake caused by cisplatin treatment, LH concentration in pair-fed control animals was determined. Pair-fed controls had no significant difference in serum LH concentrations as compared to controls, indicating that the decrease in LH levels observed in cisplatin-treated dams was due to the effect of the drug alone.

Effects of Cisplatin on Ovarian Function

Ovarian weights: Weights of the left ovaries from control and drug-treated animals were recorded to determine if cisplatin affects ovarian luteinization (Table 2). Weights of ovaries from cisplatin-treated (7 mg/kg) dams were significantly lower ($p<0.05$) than ovaries from saline-treated animals, on day 9 of gestation. Ovaries from pair-fed control dams did not show any significant difference compared with the ovaries of control dams fed ad libitum. Ovaries obtained from cisplatin-treated (7 mg/kg) dams on day 10 of gestation showed decreased ovarian weights as compared with controls, but the decrease was not statistically significant. Rats treated with 4 mg/kg cisplatin and sacrificed on day 12 of gestation demonstrated a

Figure 5. Serum concentrations of luteinizing hormone (LH) at 1350 h on day 9 of gestation following injection of saline or 7 mg/kg cisplatin (CDDP) on day 6. Results are mean + S.D. with number of rats used in parenthesis. *, significantly different ($p<0.05$) from control. S-F, saline treated animals whose food intake was limited to that consumed by 7 mg/kg cisplatin-treated rats.


CONTROL
S-F
CDDP
9
4
15
*
30
20
10
SERUM LH [ng/ml]
TREATMENT GROUPS

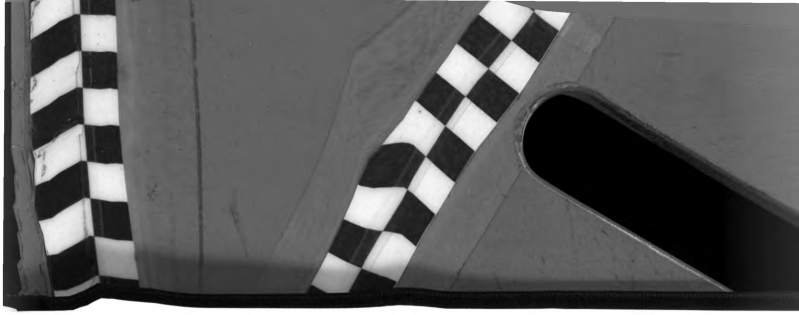


Table II. Effects of cisplatin (CDDP) on ovarian weights during gestation.

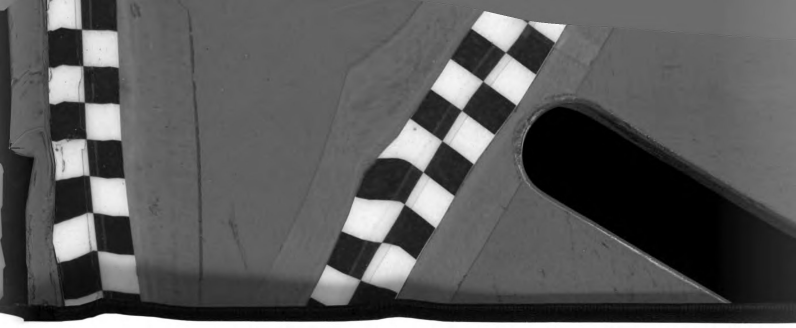
Treatment[a] (mg/kg)	Day of Gestation Sacrificed	Number of Dams	Average Weight of Left Ovary[b] (mg)
Saline	9	5	48 ± 5
7.0 CDDP	9	9	40 ± 3
S-F[c]	9	4	45 ± 5[d]
Saline	10	5	45 ± 8
7.0 CDDP	10	3	37 ± 2
Saline	12	2	52 ± 3
4.0 CDDP	12	4	41 ± 4[d]

[a]Injected i.p. on day 6 of gestation.

[b]Results given as mean ± S.D.

[c]Saline treated animals whose food intake was limited to that consumed by 7.0 mg/kg CDDP-treated animals.

[d]Significantly different ($p<0.05$) from control.

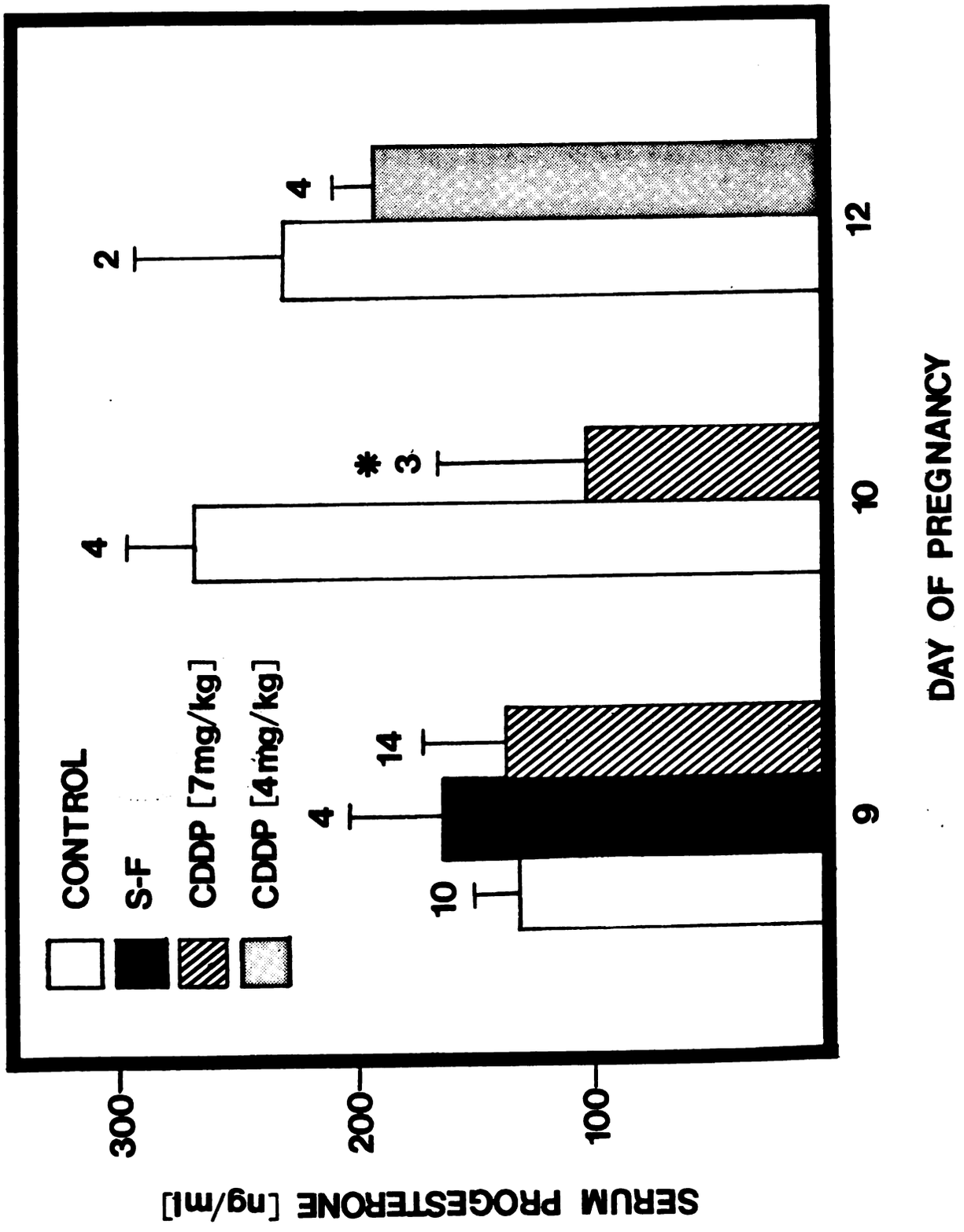


significant decrease ($p<0.05$) in ovarian weights as compared to ovaries from controls. In general, a decrease in ovarian weights was demonstrated following cisplatin treatment.

Serum concentrations of progesterone: To determine the effects of cisplatin on ovarian secretion of progesterone, trunk blood was analyzed for progesterone following cisplatin treatment on day 6 of gestation (Figure 6). There was no difference in progesterone levels between cisplatin-treated, pair-fed controls, and saline-treated rats on day 9 of gestation. Four days after cisplatin treatment a significant decrease ($p<0.01$) in serum progesterone occurred in 7 mg/kg cisplatin-treated dams as compared with controls. Cisplatin dosage was lowered from 7 to 4 mg/kg to obtain a greater percent survival of cisplatin-treated dams, and rats were sacrificed on day 12 of gestation. A decrease in concentration of progesterone was apparent in 4 mg/kg cisplatin-treated animals, as compared to controls although it was not a significant difference. In summary, serum progesterone decreased by day 10 of gestation, 4 days after 7 mg/kg cisplatin treatment.

20α-Hydroxysteroid dehydrogenase (20α-OHSD) activity: Histochemical appearance of 20α-OHSD activity in the corpus luteum was studied as an index of luteolysis (21, 26) in drug-treated animals. Histochemical reaction was taken to be dark blue tissue staining as confirmed by controls (no substrate). Results from this experiment indicated that cisplatin treatment did not induce 20αOHSD enzyme activity in corpora lutea by day 9, 10, or 12 of gestation.

Figure 6. Serum concentrations of progesterone at 1350 h on days 9, 10, and 12 of gestation following cisplatin (CDDP) treatment on day 6. Results are mean ± S.D. with number of rats used in parenthesis. *, significantly different ($\overline{p}<0.01$) from control. S-F, saline treated animals whose food intake was limited to that consumed by 7 mg/kg cisplatin-treated animals.


CONTROL
S-F
CDDP [7mg/kg]
CDDP [4mg/kg]
SERUM PROGESTERONE [ng/ml]
300
200
100
DAY OF PREGNANCY
9
10
12
10
4
14
4
*
3
4
2
4

In untreated animals, the large corpora lutea of pregnancy do not demonstrate 20α-OHSD activity on day 9, 10, or 12 of gestation. Involuting corpora lutea from previous cycles gave intense histochemical reaction. In cisplatin-treated rats, no increase in 20α-OHSD activity in the corpora lutea of pregnancy was apparent as in control animals. Only involuting corpora lutea readily demonstrated enzyme activity.

Histology of Rat Corpora Lutea and Fetuses

The corpora lutea of cisplatin-treated (7 mg/kg) animals were studied at the light microscope level in order to determine if structural luteolysis was occurring by day 9 of gestation. The luteal cells of the corpora lutea from control animals exhibited characteristic histological features (Figure 7a). The large polyhedral luteal cells were pale and appeared slightly vacuolated, due to lipid droplets which leached out during tissue preparation. Luteal cells from cisplatin-treated animals demonstrated signs of structural luteolysis (Figure 7b). The luteal cells were smaller and less uniform in size, and appeared more highly vacuolated, a characteristic of disintegrating luteal cells.

A histological study of 9 day-old fetuses was done to determine if signs of fetal destruction were apparent after cisplatin treatment (7 mg/kg). A light microscopy study on the process of fetal destruction in rats has been described by Clabaut (8). Embryonic detachment and cellular lysis within the decidual and fetal tissues

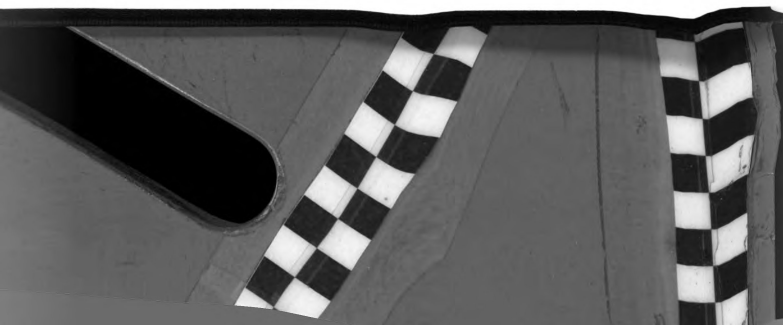
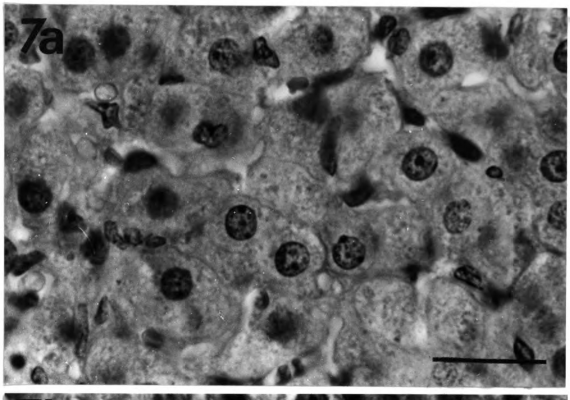
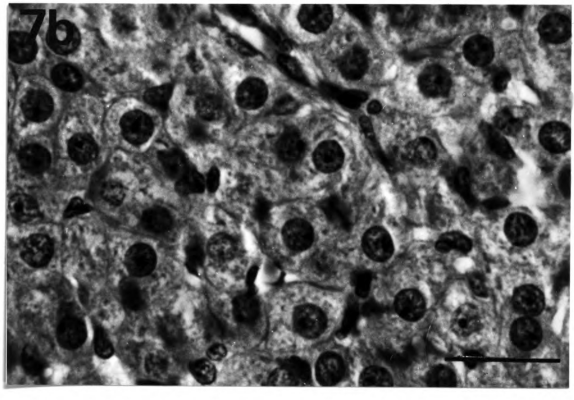
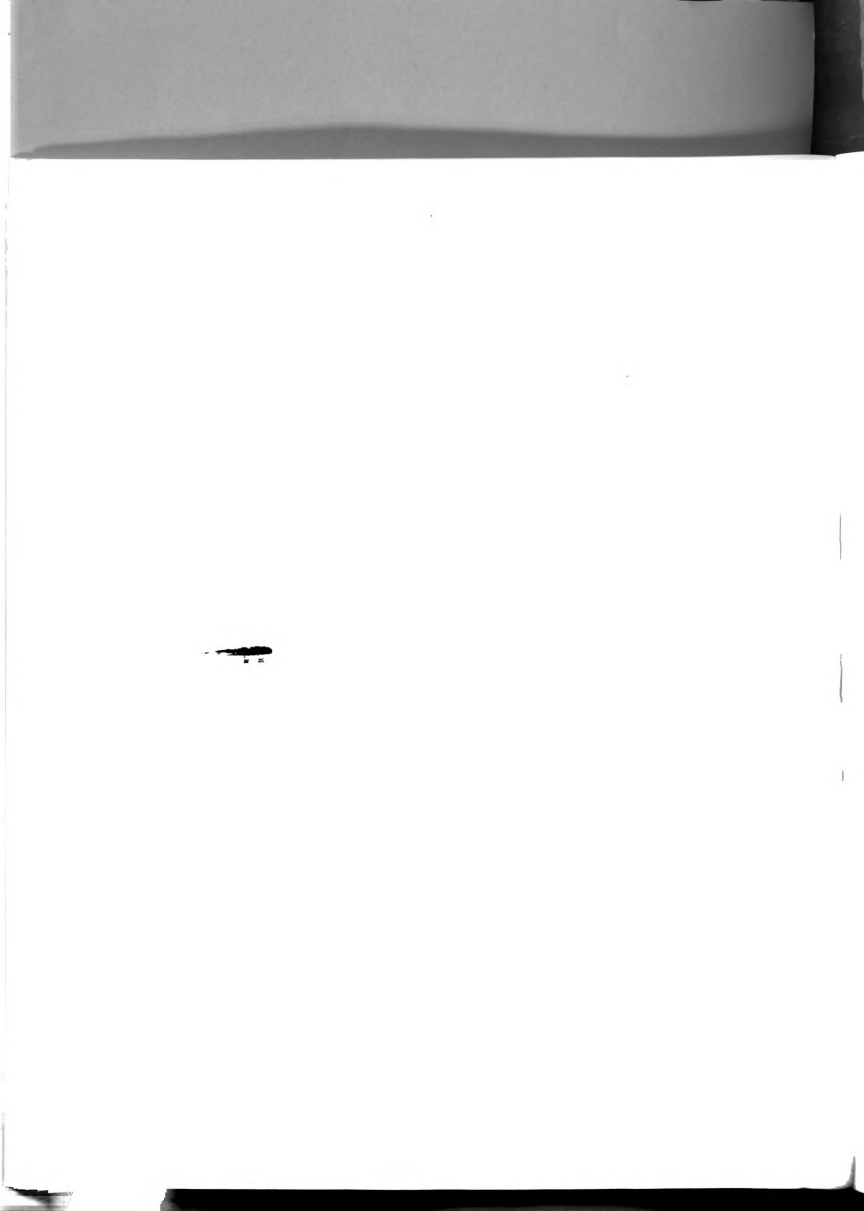


Figure 7. Light micrographs of corpora lutea on day 9 of gestation from rats treated with (a) saline or (b) 7 mg/kg cisplatin on day 6 of gestation. Note signs of structural luteolysis in cisplatin-treated animals. Haematoxylin and eosin. Original manigification x56. Bar = 0.1 mm.

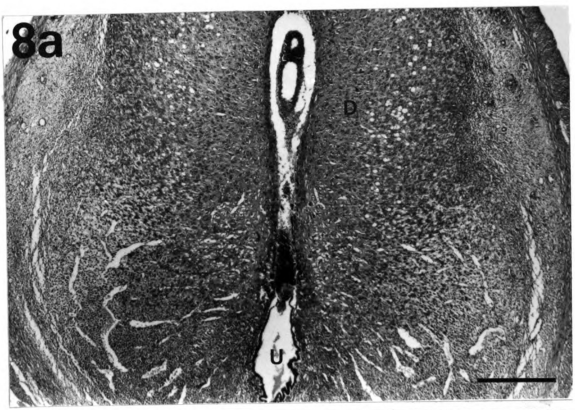

7a

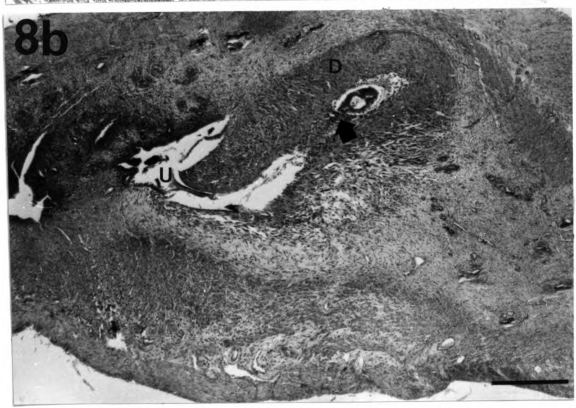

7b

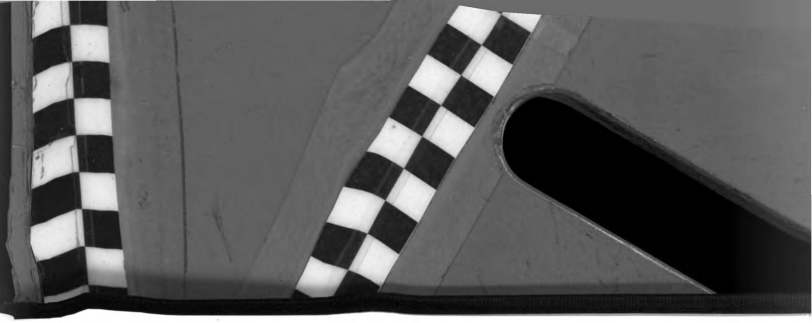


were exhibited in fetuses from drug-treated animals (Figures 8a and 8b). Hemorrhaging was observed at implantation sites and infiltration of polynuclear leucocytes were predominant. The uterine cavity appeared more narrow than controls by visual inspection, and signs of fetal compression were apparent.

Figure 8. Light micrographs of longitudinal sections through embryos 9 days old from rats treated with (a) saline or (b) 7 mg/kg cisplatin on day 6 of gestation. Note cell degeneration of decidua (D) and hemorrhaging (arrow) in cisplatin-treated animals. Remnant of uterine lumen (U). Haematoxylin and eosin. Original magnification x22. Bar = 0.2 mm.


8a
D
U

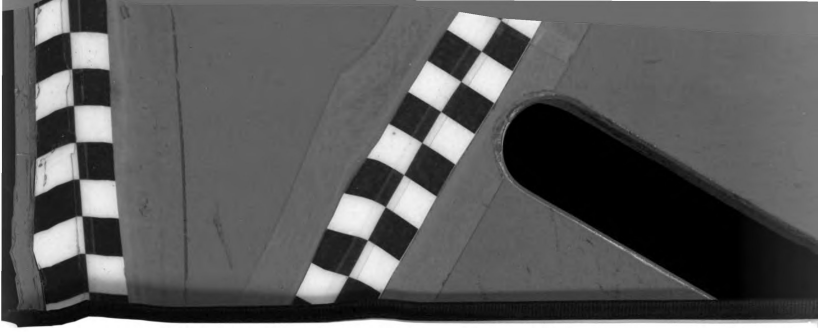

8b
D
U



DISCUSSION

No studies have been reported thus far concerning the effects of cisplatin on the endocrine environment during pregnancy in the rat. In the present study, cisplatin treatment on day 6 of gestation causes 100% fetal resorption. On day 9 of gestation, the nocturnal PRL surge is suppressed and LH concentrations decrease in cisplatin-treated rats. Cisplatin treatment also decreases progesterone levels significantly and causes structural luteolysis of corpora lutea. Decreases in serum concentrations of LH and PRL are due to cisplatin treatment and it is proposed that these decreases in pituitary luteotropic hormones are the cause of fetal resorption in cisplatin-treated animals.

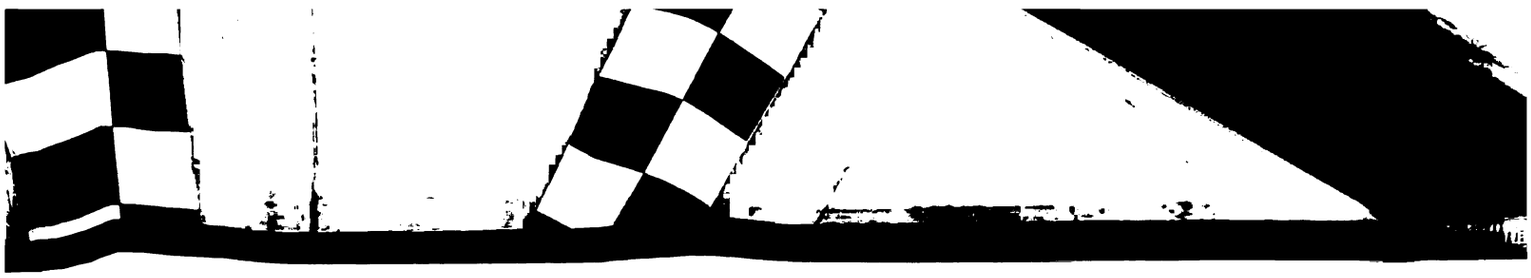
Secretion patterns of prolactin during pregnancy are composed of two daily surges, one diurnal and one nocturnal (7). Nocturnal surges during pregnancy occur until day 11 of gestation, while diurnal surges last until day 9 (48, 52). These PRL surges are necessary for maintenance of pregnancy because they stimulate progesterone secretion from the corpus luteum (4, 10). Maintenance of pregnancy is correlated with the concentration of progesterone secreted by the corpus luteum which is its primary source (9). Bilateral ovariectomy performed in rats before day 14 of gestation leads to abortion (9).



Hypophysectomy before day 12 of gestation results in termination of pregnancy (18, 39); prolactin has been reported to maintain the corpora lutea in such rats (5, 11). Blocking the prolactin surges causes abortion of fetuses, demonstrating the necessity for PRL during pregnancy until day 11 (46, 52). Therefore, the suppression of the nocturnal prolactin surge on day 9 of gestation due to cisplatin treatment could be responsible for the resorption of fetuses.

Prolactin regulates and maintains LH and estrogen (E) receptors in corpora lutea (14, 15, 22). Additionally, PRL and E are able to maintain progesterone secretion in hypophysectomized and hysterectomized rats above those stimulated by PRL alone (16). PRL and estrogen are thought to act synergistically along with LH as the luteotropic complex during the first half of gestation in the rat (12, 16). PRL increases and maintains LH receptors in the corpora lutea, possibly allowing LH to stimulate the synthesis of androgens. Androgens are then converted to estrogen in the corpus luteum. Also PRL induces formation of estrogen receptors, which might enable estrogen to act within the luteal cell to maintain structural and functional luteolysis (13, 15). Therefore, lack of PRL support in cisplatin-treated rats could ultimately prevent secretion of progesterone from the corpus luteum.

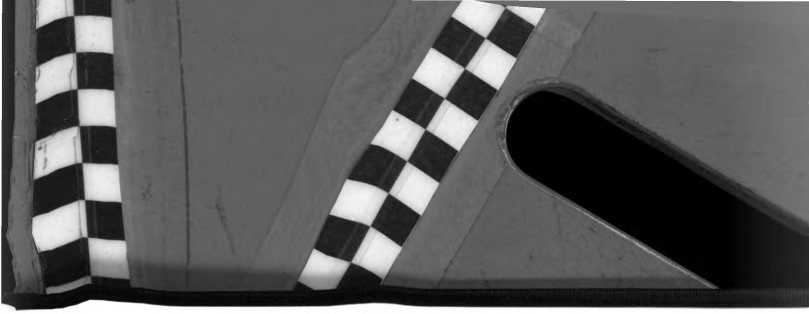
The corpora lutea of pregnancy in the rat become highly dependent upon LH from days 8 to 12 of gestation (2, 31, 34) for the secretion of progesterone (3, 43). Injection of an antiserum to LH (LH-AS) during this period results in a decrease in progesterone levels (43) and termination of pregnancy (2, 36). However,



progesterone administered along with LH-AS has been shown to be able to maintain pregnancy (49, 50). Morishige et al. (36) report that a high dosage of LH-AS injected on day 6 results in abortion of 2/3 of the fetuses. The authors propose that due to the high LH-AS concentrations injected, circulating levels of LH-AS still capable of neutralizing LH are present on days 8 and 9, a time when LH is critically necessary for progesterone secretion.

On day 9, a 39% decrease in serum LH concentration is observed in cisplatin-treated rats whereas the cisplatin injection was given on day 6 of gestation. However, it has been demonstrated in dogs injected with 1 mg/kg cisplatin, that measurable plasma concentrations of platinum are still detectable 12 days after treatment (30). In the present study a much higher dosage of CDDP was used. It is therefore proposed that cisplatin treatment on day 6 causes the decreased serum levels of LH due to high levels of cisplatin still present in the serum on days 8 and 9. Support for this proposal comes from studies performed by Keller (24). Cisplatin injections were given on day 6, 8, 11, or 14 of gestation. A dosage of 3 mg/kg injected on day 6, 8, or 11 caused fetal mortality of 79.63%, 100%, and 100%, respectively. Cisplatin injection on day 14 failed to induce fetal resorption. Embryolethality was highest when cisplatin was injected on day 8 or 11, which corresponds to the period when the corpora lutea are highly LH dependent.

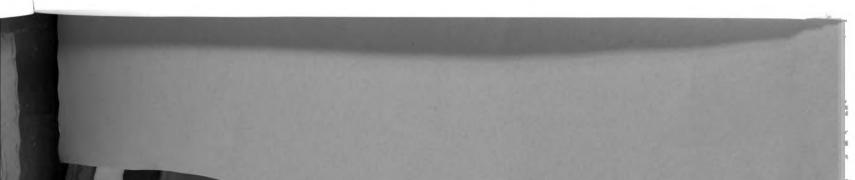
Since the luteotropic hormone complex in the rat regulates and maintains corpora lutea (4), withdrawal of these hormones leads to regression (18). Evidence of structural luteolysis is apparent in

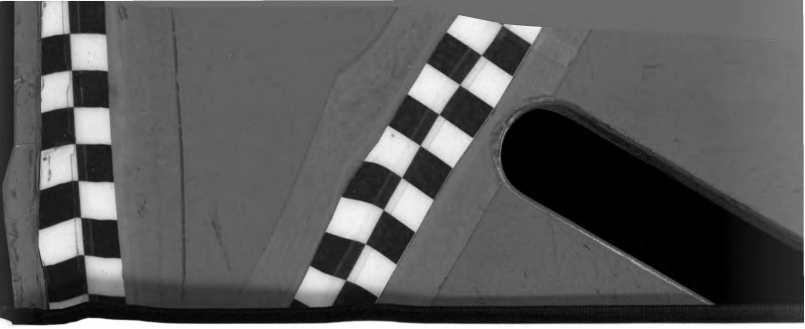


cisplatin-treated rats. Ovarian weights are decreased and histological studies of the corpora lutea show signs of structural luteolysis.

In CDDP-treated animals, no appearance of 20α-OHSD enzyme activity is apparent in the corpora lutea on day 9 or 10, although progesterone levels are decreased on day 10 of gestation. Progesterone is converted into 20α-hydro-derivative by 20α-OHSD, though the actual control of 20α-OHSD synthesis is still questionable. Loewit et al. (32, 33) postulate that gonadotropic dependent progesterone levels regulate 20α-OHSD activity. The authors have demonstrated that fetal destruction by LH-AS, and decreased progesterone levels, clearly precede the appearance of 20α-OHSD. Progesterone in some way might be involved in the regulation of this enzyme. Therefore it is probable that no appearance of 20α-OHSD enzyme activity was apparent by day 10 in cisplatin-treated rats, since progesterone levels only start to decrease on the same day. With 4 mg/kg CDDP, no enzyme activity is demonstrable by day 12. However, no significant decrease in progesterone levels were yet apparent.

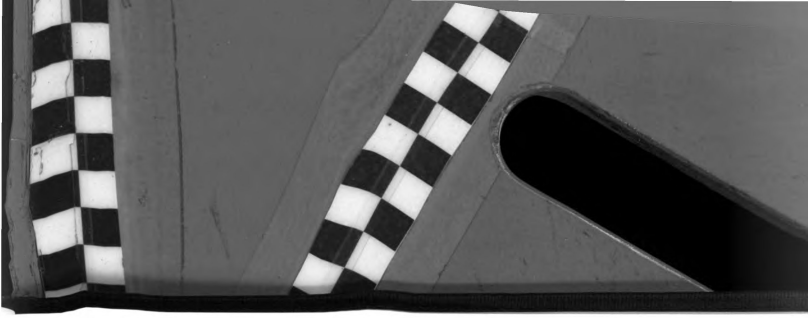
In conclusion, decreases in serum hormone levels are due to cisplatin treatment in pregnant rats. It is proposed that fetal resorption in cisplatin-treated animals is caused by the decreases in the pituitary luteotropic hormones, PRL and LH. These decreases occur during the time when the maintenance and regulation of the corpora lutea is highly dependent upon the pituitary luteotropic complex. With the loss of these hormones, pregnancy is no longer maintained, and serum concentrations of progesterone are decreased.





It is possible, however, that cisplatin directly kills the fetuses which could cause the decreases in concentrations of hormones as were observed in the present study. Further studies using pseudopregnant rats need to be done to determine if the fetal death is a direct cause of cisplatin, or whether it results from the effects of the drug on hormone concentrations.

BIBLIOGRAPHY



BIBLIOGRAPHY

1. Aggarwal, S.K. (1974). Inhibition of cytokinesis in mammalian cells by cis-dichlorodiammine-platinum (II). Cytobiol. 8: 395-402.

2. Akaka, J., O'Laughlin-Phillips, E., Antozak, E., and Rothchild, I. (1977). The relation between the age of the corpus luteum (CL) and the luteolytic effect of an LH-antiserum (LH-AS): Comparison of hysterectomized pseudopregnant rats with intact pregnant rats for their response to LH-AS treatment at four stages of CL activity. Endo. 100: 1334-1340.

3. Armstrong, D.T., O'Brien, J., and Greep, H.O. (1964). Effects of luteinizing hormone on progestin biosynthesis in the luteinized rat ovary. Endo. 75: 488-500.

4. Armstrong, D.T., Miller, L.S., and Knudsen, K.A. (1969). Regulation of lipid metabolism and progesterone production in rat corpora lutea and ovarian interstitial elements by prolactin and luteinizing hormone. Endo. 85: 393-401.

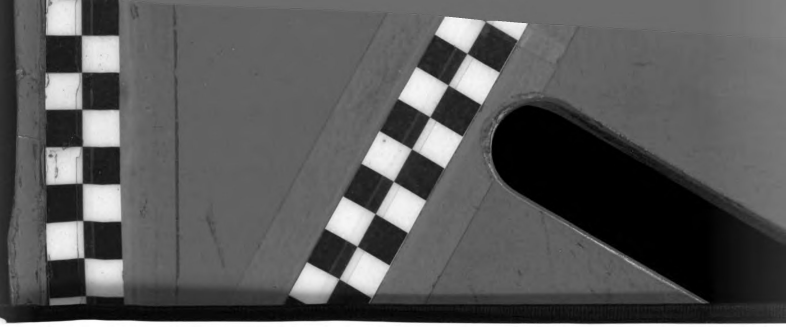
5. Astwood, E.B. (1941). The regulation of corpus luteum function by hypophysial luteotropic. Endo. 28: 309-320.

6. Balogh, K. (1964). A histochemical method for the demonstration of 20α hydroxysteroid dehydrogenase activity in rat ovaries. J. Histochem. Cytochem. 12: 670-673.

7. Butcher, R.L., Fugo, N.W., and Collins, W.E. (1972). Semicircadian rhythm in plasma levels of prolactin during early gestation in the rat. Endo. 90: 1125-1127.

8. Clabaut, M. (1972). Effets de la biovariectomie sur la jonction embryomaternelle chez la ratte gestante avant le relais placentaire. Ann. Endocr. (Paris). 33: 221-230.

9. Csapo, A.I. and Wiest, W.G. (1969). An examination of the quantitative relationship between progesterone and the maintenance of pregnancy. Endo. 85: 735-746.



10. Day, S.L., Kirchick, H.J., and Birnbaumer, L. (1980). Effect of prolactin on luteal functions in the cyclic rat: Positive correlation between luteinizing hormone-stimulated adenylyl cyclase activity and progesterone secretion; role in corpus luteum rescue of the morning surge of prolactin on day 3 of pseudopregnancy. Endo. 106: 1265-1270.

11. Evans, H.M., Simpon, M.E., Lyons, W.R., and Turpeinen, K. (1941). Anterior pituitary hormones which favor the production of traumatic uterine placentomata. Endo. 28: 933-945.

12. Gibori, G., Rodway, R., and Rothchild, I. (1977). The luteotropic effect of estrogen in the rat: Prevention by estradiol of the luteolytic effect of an antiserum to luteinizing hormone in the pregnant rat. Endo. 101: 1683-1689.

13. Gibori, G., Keyes, P.L., and Richards, J.S. (1978). A role for intraluteal estrogen in the mediation of luteinizing hormone action on the rat corpus luteum during pregnancy. Endo. 103: 162-169.

14. Gibori, G. and Richards, J.S. (1978). Dissociation of two distinct luteotropic effects of prolactin: Regulation of luteinizing hormone-receptor content and progesterone secretion during pregnancy. Endo. 102: 767-774.

15. Gibori, G., Richards, J.S., and Keyes, P.L. (1979). Synergistic effects on prolactin and estradiol in the luteotropic process in the pregnant rat: Regulation of estradiol receptor by prolactin. J. Reprod. 21: 419-423.

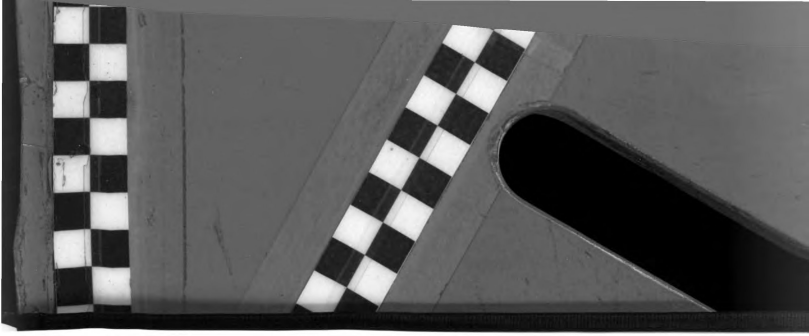
16. Gibori, G. and Keyes, L. (1980). Luteotropic role of estrogen in early pregnancy in the rat. Endo. 106: 1584-1588.

17. Greenwood, F.C., Hunter, W.M., and Glover, J.S. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89: 114-123.

18. Greenwald, G.S. and Johnson, D.C. (1968). Gonadotropic requirements for the maintenance of pregnancy in the hypophysectomized rat. Endo. 83: 1052-1064.

19. Harder, H.C. and Rosenberg, B. (1970). Inhibitory effects of anti-tumor platinum compounds on DNA, RNA, and protein synthesis in mammalian cells in vitro. Int. J. Cancer 6: 207-216.

20. Hayat, M., Brule, G., Cappelaera, P., Cattan, A., Chauvergne, J., Clavel, B., Guerren, J., Misset, J.L., Pommataue, E., Ribaud, P., Muggia, F.M., Rozencweig, M., and Mathe, G. (1980). Cis-platinumdiamminodichloride (CDDP) in chemotherapy of cancers: A phase II therapeutic trial. In Cancer Chemo- and Immunopharmacology (G. Mathe and F.M. Muggia, eds.). pp. 139-145, Springer-Verlag Berlin Heidelberg, New York.



21. Heap, R.B. (1972). Role of hormones in pregnancy. In Hormones in Reproduction (C.R. Austin and R.V. Stort, eds.). pp. 73-105, Cambridge at the University Press, England.

22. Holt, J.A., Richards, J.S., Midgley, Jr., A.R., and Reichert, Jr., L.E. (1976). Effect of prolactin on LH receptor in rat luteal cells. Endo. 98: 1005-1013.

23. Howle, J.A. and Gale, B.R. (1970). Persistant and selective inhibition of deoxyribonucleic acid synthesis in vivo. Biochem. Pharmacol. 19: 2757-2762.

24. Keller, K.A. (1982). Embryotoxicity of cisplatin in rats and mice. Master Thesis of Science. Michigan State University.

25. Knoff, M.K., Lewis, K.P., Fischer, D.S., Schneider, W.R., and Welch, D. (1981). Cancer Chemotherapy Treatment and Care (M. Morra, ed.). pp. 33-34, G.K. Hall Medical Publishers, Massachusetts.

26. Lamprecht, S.A., Lindner, H.R., and Strauss, J.F. (1969). Induction of 20α hydroxysteroid dehydrogenase in rat corpora lutea by pharmacological blockage of pituitary prolactin secretion. Biochim. Biophys. Acta 187: 133-143.

27. Lange, R.C., Spencer, R.P., and Harder, H.C. (1972). Synthesis and distribution of a radiolabeled antitumor agent: Cisdiamminedichloroplatinum (II). J. Nucl. Med. 13: 328-330.

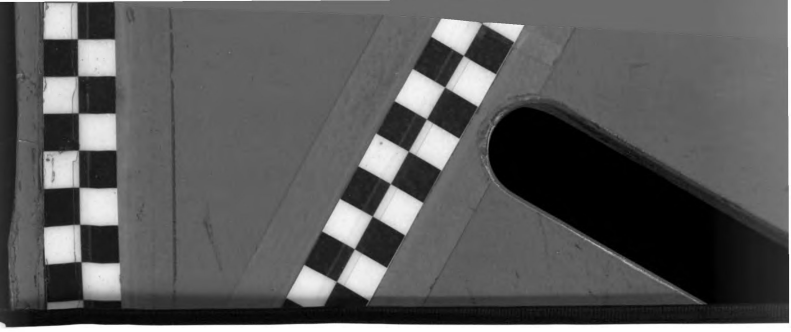
28. Lange, R.C., Spencer, R.P., and Harder, H.C. (1973). The antitumor aggent cis-PT$(NH_3)_2CL_2$: Distribution studies and dose calculation for ^{193n}Pt and ^{195n}Pt. J. Nucl. Med. 14: 191-195.

29. Lazar, R., Conran, P.C., and Damjanov, I. (1979). Embryotoxicity and teratogenicity of cis-diamminedichloroplatinum. Experientia. 35: 648-649.

30. Litterst, C.L., Gram, T.E., Dedrick, R.L., Leroy, A.F., and Guarina, A.M. (1976). Distribution and disposition of platinum following intravenous administration of cis-diamminedichloroplatinum (II) (NSC 119875) to dogs. Cancer Res. 36: 2340-2344.

31. Loewit, S., Badawy, S., and Laurence, K. (1969). Alteration of corpus luteum function in the pregnant rat by anti-luteinizing serum. Endo. 84: 244-251.

32. Loewit, K. and Zambelis, N. (1979). Progesterone and 20α hydroxysteroid dehydrogenase regulation in the corpus luteum of the pregnant rat. Acta Endo. 90: 176-184.



33. Loewit, K., Kofler, R., Tabarelli, M., and Schwarz, S. (1981). Effect of bromocryptine on 20α hydroxysteroid dehydrogenase regulation in the corpus luteum of the pregnant rat. Acta Endo. 98: 133-136.

34. Madhwa Raj, H.G. and Moudgal, N.R. (1970). Hormonal control of gestation in the intact rat. Endo. 86: 874-889.

35. Meistrich, M.L., Finch, M., Cunha, M.F., Hacker, U., and An, W.W. (1982). Damaging effects of fourteen chemotherapeutic drugs on mouse testis cells. Cancer Res. 42: 122-131.

36. Morishige, W.K. and Rothchild, I. (1974). Temporal aspects of the regulation of corpus luteum function by luteinizing hormone, prolactin and placental luteotrophin during the first half of pregnancy in the rat. Endo. 95: 260-273.

37. Niswender, G.D., Midgley, Jr., A.R., Monroe, S.E., and Reichert, Jr., L.E. (1968). Radioimmunoassay for rat luteinizing hormone with antiovine LH serum and ovine LH ^{131}I. Proc. Soc. Exp. Biol. Med. 128: 870-811.

38. Niswender, G.D., Chen, C.L., Midgley, Jr., A.R., Meites, J., and Ellis, S. (1969). Radioimmunoassay for rat prolactin. Proc. Soc. Exp. Biol. Med. 130: 793-797.

39. Pencharz, R.I. and Long, J.A. (1933). Hypophysectomy in the pregnant rat. Am. J. Anat. 53: 117-135.

40. Pinedo, H.M. (1981). Cancer Chemotherapy Annual 3. pp. 317-336, Elsevier North-Holland, Inc., New York.

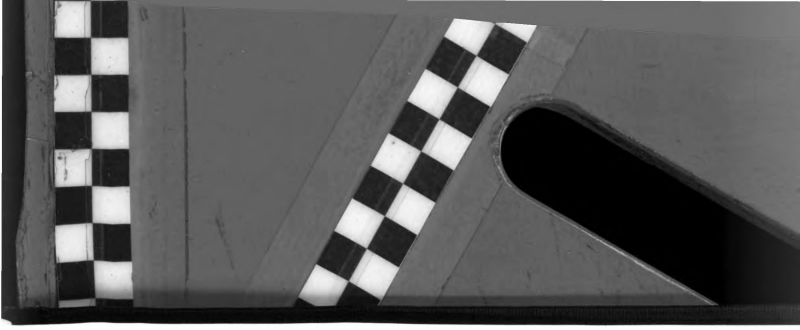
41. Prestayko, A.W., Crooke, S.T., and Carter, S.K. (1980). Cisplatin: Current Status and New Developments. Academic Press, New York.

42. Rosenberg, B., Van Camp, L., Trosko, J., and Mansour, V.H. (1969). Platinum compounds: A new class of potenti antitumor agents. Nature 222: 385-386.

43. Rothchild, J., Pepe, G.J., and Morishige, W.K. (1974). Factors affecting the dependency on LH in the regulation of corpus luteum progesterone secretion in the rat. Endo. 95: 280-287.

44. Sanyal, M.K. (1978). Secretion of progesterone during gestation in the rat. J. Endo. 79: 179-190.

45. Schaeppi, U., Heyman, I.A., Fleischman, R.W., Rosenkrantz, H., Ilievski, V., Phelan, R., Cooney, D.A., Davis, R.D. (1973). Cis-dichlorodiammineplatinum (II) (NSD - 119875): Preclinical toxicologic evaluation of intravenous injection in dogs, monkeys and mice. Toxicol. Appl. Pharmacol. 25: 230-241.



46. Shelesnyak, M.C. (1958). Maintenance of gestation in ergotoxine-treated pregnant rats by exogenous prolactin. Acta Endo. 27: 99-109.

47. Skelly, D.S., Brown, L.P., and Besch, P.K. (1973). Radioimmunoassay. Clin. Chem. 19: 146-186.

48. Smith, M.S. and Neill, J.D. (1976). Termination at midpregnancy of the two daily surges of plasma prolactin initiated by mating in the rat. Endo. 98: 696-701.

49. Terranova, P.F. and Greenwald, G.S. (1981). Effects of an antiserum to luteinizing hormone and steroid replacement therapy on maintenance of pregnancy in the rat: Serum and luteal levels of progesterone, testosterone and oestradiol. J. Endo. 90: 9-18.

50. Terranova, P.F. and Greenwald, G.S. (1981). Acute effects of an antiserum to luteinizing hormone on ovarian steroidogenesis in the pregnant rat. J. Endo. 90: 19-30.

51. Vogl, S.E., Zaravinos, T., Kaplan, B.H., and Wollner, D. (1981). Effective two hour outpatient regimen of hydration and diuresis for the administration of cis-diamminedichloroplatinum II. Eur. J. Cancer 17: 345-350.

52. Voogt, J.L. (1980). Regulation of nocturnal prolactin surges during pregnancy in the rat. Endo. 106: 1670-1676.

53. Zwelling, L.A., Kohn, K.W., Ross, W.E., Ewig, R.A.G., and Anderson, T. (1978). Kinetics of formation and disappearance of a DNA cross-linking effect in mouse leukemia L1210 cells treated with cis- and trans-diamminedichloroplatinum (II). Cancer Res. 38: 1762-1768.