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Cytoplasmic progestin binding in adipose tissues, and the effect of progestins on food intake, adiposity, and lipoprotein lipase activity.

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CYTOPLASMIC PROGESTIN BINDING

IN ADIPOSE TISSUES, AND THE EFFECT OF PROGESTINS

ON FOOD INTAKE, ADIPOSITY, AND LIPOPROTEIN LIPASE

ACTIVITY

A Dissertation Presented

By

JANET MARTHA GRAY

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ABSTRACT
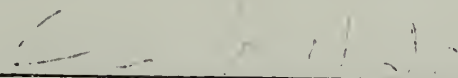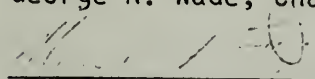
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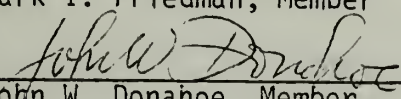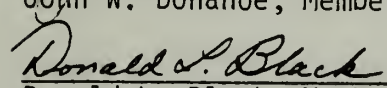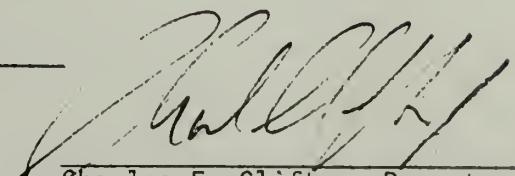Adiposity, and Lipoprotein Lipase Activity

(September, 1980)

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Ovarian hormones have multiple effects on food intake, body weight
and carcass composition in female rats. Changes in these parameters
are observed over the estrous cycle of the rat and can be induced by
experimentally manipulating hormone levels. Ovariectomy (OVX) results
in transient increases in food intake and body weight gain, with perm-
anent increases in body weight relative to gonadally-intact controls.
Estradiol benzoate (E_2B) treatment transiently decreases food intake
and body weight gain in OVX animals, with E_2B-treated animals having
lowered body weights than OVX controls. Neither progestins, progest-
erone nor R5020, had any effect on food intake when administered to
OVX animals. When administered to OVX, E_2B-treated rats, both R5020
and progesterone attenuated the estrogen-induced anorexia and weight
loss. R5020 was as effective a progestin as was progesterone at much
lower doses.

Carcass composition analyses revealed that the only carcass com-
ponent which was significantly altered by hormone treatment was
carcass lipid content. Analysis of parametrial adipose tissue

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cellularity revealed that the hormone-induced changes in lipid content were probably an effect on fat cell size, not number.

The synthetic "antiestrogen", nafoxidine, fully mimicked the effects of E_2B, i.e., nafoxidine treatment to OVX rats resulted in lowered food intake, body weight and carcass fat composition. As it does with E_2B-treated rats, progesterone attenuated these effects in nafoxidine-treated animals.

Since a major change in carcass lipid content is found following hormone administration, it was of interest to determine whether or not there were steroid binding sites in adipose tissues. If these binding sites could be demonstrated, it would indicate that hormones might have direct effects on adipose tissue metabolism. Estrogen binding sites previously had been demonstrated in adipose tissues. Using $(^3H)R5020$, progestin binding sites were demonstrated in the cytoplasm of adipose tissues from E_2B-treated rats. The E_2B-induced $(^3H)R5020$ binding was found to be high affininty (K_d=8.5 x 10^{-10}M) and progestin specific. Three progestins competed effectively for $(^3H)R5020$ binding, with the order of efficacy being R5020 > progesterone > 5α-dihydroprogesterone. An estrogen, an androgen and a glucocorticoid were effective in competing for $(^3H)R5020$ binding only when they were present in very high concentrations.

In vivo administration of progestins resulted in the depletion of cytoplasmic progestin binding sites from adipose tissues of E_2B-primed OVX rats. Again the order of efficacy was R5020 > progesterone > 5α-dihydroprogesterone.

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(^{3}H)R5020 binding sites were found in all adipose tissues studied. Regional differences in absolute levels of E_2B-induced binding were found, as were differences in the magnitude of induction of progestin binding sites. Further analyses revealed a significant correlation between estrogen receptor concentrations and the magnitude of induction of progestin receptors in specific adipose tissues.

Single injections of E_2B, CI-628 or nafoxidine depleted adipose tissue cytoplasmic estrogen binding sites and induced progestin binding sites. Three days of nafoxidine treatment resulted in the induction of about 50% (^{3}H)R5020 binding sites as compared with E_2B. In these same animals, nafoxidine lowered adipose tissue lipoprotein lipase (LPL) activity at least as much as did E_2B.

Although concurrent administration of progesterone (5 mg/day) to E_2B-treated animals increased LPL activity, no progesterone effect on LPL activity was found in nafoxidine-treated animals.

These studies demonstrate that a major effect of progestin administration is to alter body fat content. The binding data suggest that progestins might have direct effects on adipose tissues. One possible mechanism of action is progestin-induced alterations in adipose tissue LPL activity. Changes in LPL activity do reflect changes in fat content following E_2B and progestin administration. However, the data from the nafoxidine experiments indicate that the progesterone-induced changes in LPL activity are not necessary for the changes in food intake and body fat composition.

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GENERAL INTRODUCTION

Ovarian hormones have multiple effects on regulatory behaviors, body weight and body composition in female rats and other mammals (for reviews, see Wade, 1976; Wade and Gray, 1979). With cyclic variations in hormone titers over the natural estrous cycle of the female rat, changes are seen in body weight and regulatory behaviors. At proestrus, when estrogen titers are high, running wheel activity is high; food intake is low; and body weight is reduced (Brobeck, Wheatland and Strominger, 1947; ter Haar, 1972; Wang, 1923). During diestrus, when estrogen levels are reduced, food intake and body weight gain are increased (ter Haar, 1972).

Gonadectomy and Hormone Replacement Studies

Gonadectomy and hormone replacement studies indicate that the changes in food intake and body weight gain can be mimicked by experimentally altering hormone levels. Ovariectomy (OVX) results in a transient hyperphagia and body weight gain and a permanent decrease in running wheel activity. After 3-4 weeks, the increased food intake subsides and body weight gain normalizes, with body weight being maintained at about 20 percent higher than that of gonadally intact animals (Tarttelin and Gorski, 1973). Estradiol treatment, alone, reverses the effects of OVX in adult female rats, resulting in a transient hypophagia and body weight loss, and a permanent

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increase in running wheel activity and decrease in body weight relative to OVX controls (Tarttelin and Gorski, 1973; Wade, 1975; Zucker, 1969).

Treatment of OVX rats with progesterone, alone, has no effect on body weight gain, running wheel activity, or food intake (Hervey and Hervey, 1966; 1967). Concurrent treatment with both estradiol and progesterone, on the other hand, results in reversal of the estradiol-induced changes in food intake, running activity, and body weight gain (Hervey and Hervey, 1967; Roberts, Kenney and Mook, 1972; Rodier, 1971; Wade, 1975; Zucker, 1969).

The greatest change in carcass composition following the administration of ovarian hormones is observed in the total carcass fat content (Galletti and Klopper, 1964; Hervey and Hervey, 1967; Leshner and Collier, 1973). OVX doubles fat content while estradiol treatment reverses the effect of castration of adiposity. Progesterone treatment to OVX, estradiol-treated animals, in turn, reverses the effect of the estrogen, leading to an increase in the total carcass fat content.

In the first series of experiments to be described in this dissertation, some of these basic experiments demonstrating that ovarian hormones affect food intake, body weight and carcass composition were replicated and expanded. I was especially interested in the changes in these measurements which were induced by progestins in OVX and OVX, estradiol-treated animals. I examined the effects of two progestins, the natural compound, progesterone, and the synthetic compound, R5020[1], on food intake, body weight, carcass composition, adipose tissues cellularity, and adipose tissue LPL activity were studied.

R5020 was used in these metabolic studies for two reasons.
(1) R5020 has previously been shown to be a very potent progestin with
regards to inducing changes in uterine morphology (Philibert and
Raynaud, 1974) and the facilitation of female rat sexual behavior
(Blaustein and Wade, 1978). It was of interest, therefore, to see
how a highly potent progestin would affect these metabolic parameters.
(2) Biochemically, R5020 is also a highly potent progestin; it binds
to progestin receptors in uterus, vagina, pituitary and brain with a
much higher affinity than does progesterone (Blaustein and Wade,
1978; Blaustein and Feder, 1979; Leavitt et al., 1977; MacLusky and
McEwen, 1978; Raynaud, 1977). For these reasons, radioactively
labelled R5020 has been extensively used in the past few years to
characterize progestin binding in these tissues. As I will be dis-
cussing below, (^{3}H)R5020 was used to characterize progestin binding
in adipose tissues. It was of interest, therefore, to demonstrate
and study the physiological efficacy of the progestin, R5020, which
was also to be used in a series of in vitro biochemical studies.

Central Versus Peripheral Control of Hormone Effects
on Food Intake, Body Weight and Carcass Composition

Until recently, it had been widely assumed that ovarian
steroids exert their regulatory effects via the brain by directly
influencing behaviors and indirectly influencing metabolic processes
(Wade, 1976). There are steroid binding proteins (receptors) for
both estrogens and progestins in the hypothalamus (Blaustein and

Feder, 1979; Eisenfeld, 1970; Eisenfeld and Axelrod, 1965; MacLusky and McEwen, 1978), indicating that this brain area is a target organ for ovarian steroids. Implants of estradiol in the anterior hypo-thalamus-preoptic area increase running wheel activity while implants into the ventromedial hypothalamus (VMH) decrease food intake (Colvin and Kalkoff, 1971; Nunez, Gray and Wade, 1980; Wade and Zucker, 1970). The VMH is also important in metabolic regulatory processes (Powley, 1977). Effects of VMH modulation of metabolic activities may be manifested via direct efferents to peripheral target tissues such as the liver, endocrine pancreas and adipose tissues (Havel, 1965; Sawchenko and Friedman, 1979; Woods and Porte, 1974). However, the VMH is not the sole site of hormone action in controlling metabolic factors, as both OVX-induced obesity and estrogen-induced anorexia are observed in rats with large VMH lesions (Beatty et al., 1975; King and Cox, 1973).

Some data also indicate that the body weight and composition changes which are observed after ovarian steroid treatment are not always accompanied by parallel changes in behavior. Hervey and Hervey (1968) found that progesterone treatment to intact female rats resulted in increased body weights, even when the normally occurring, hormone-induced hyperphagia was prevented. Roy and Wade (1977) reported that OVX rats had to be restricted to 80 percent of the pre-surgical ad libitum food intake in order to prevent OVX-induced increases in body weight gain. These authors also reported that the decreased food intake seen during estradiol treatment was not suffi-cient to produce the normally observed, estradiol-induced decrease in

body weight gain (Roy and Wade, 1977). Pair-feeding of OVX animals to OVX animals which were injected with estradiol benzoate (E_2B) did not result in a loss in weight. MER-25, a synthetic compound which mimics the above effects of E_2B on food intake and body weight gain (Roy and Wade, 1976; 1977), was without any estrogen-like effect on running wheel activity. These data indicate that the observed differences in weight gain between the pair-fed OVX and the OVX plus E_2B animals were not merely a reflection of differences in general activity (Roy and Wade, 1977).

These data indicate that, at least under some circumstances, the changes in behavior following ovarian hormone administration may not be of primary importance for the changes in body weight and adiposity. We proposed (Wade and Gray, 1979) instead that ovarian hormones might act directly on adipose tissues and other peripheral tissues which are involved in triglyceride storage. In this way, hormones could alter the availability of circulating, utilizable (oxidizable) triglycerides, thus sparing other fuels (e.g. glucose, Randle et al., 1963). Components of triglycerides can be utilized by such tissues as muscles and the liver. Other studies have demonstrated that altering metabolic fuel availability can change food intake (Friedman and Striker, 1976). According to this hypothesis, changes in food intake following hormone manipulation might be a consequence, rather than a cause, of altered circulating metabolic fuels, and that changes in body weight gain and carcass composition might reflect direct peripheral, metabolic responses to ovarian hormones.

Estrogens increase hepatic triglyceride synthesis (Chan et al., 1976; Kim and Kalkoff, 1975; 1978). Progesterone appears to have either no additional effect (Kim and Kalkoff, 1975; 1978) or is synergistic with estradiol with regards to hepatic hypertriglyceridemia.

Lipoprotein lipase (LPL) is the enzyme involved in the hydrolysis of triglycerides for storage in adipose tissues (Scow et al., 1972). Changes in adiposity resulting from hormonal manipulations are reflected in changes in adipose tissue LPL activity. That is, OVX results in a two-fold increase in adipose tissue LPL activity while estradiol treatment reduces it (Hamosh and Hamosh, 1975). Progesterone, on the other hand, has no effect on adipose tissue LPL activity in OVX rats which have not been treated with estrogens. In gonadally intact (estrogen-secreting) rats, however, progesterone treatment signifi- cantly increase adipose tissue LPL activity (Kim and Kalkoff, 1975; 1978; Steingrimsdottir, Greenwood and Brasel, 1980).

Adipose Tissue as a Target Organ for Ovarian Hormones

This hypothesis suggests that ovarian steroids should have direct effects on peripheral tissues such as the liver and adipose tissues. Since a major component of the changes in total body weight following hormone administration are observed in carcass fat content, we have been especially interested in studying the effects of natural and synthetic hormones on adipose tissues.

If adipose tissue is to be accepted as a <u>bona</u> <u>fide</u> target organ for ovarian hormones, then it should comply with the current model of steroid action (Gorski and Gannon, 1976; O'Malley and Means, 1974; Yammamoto and Alberts, 1976). According to this model, steroid hormones passively diffuse into all cells throughout the body. In target tissues (such as uterus, pituitary, hypothalamus) the hormones bind, with high affinity and steroid specificity, to receptor proteins in the cytoplasm. The steroid-receptor complexes are translocated to the nucleus where they associate with the chromatin. These nuclear-receptor complexes cause the initiation of hormone-specific and tissue-specific responses. These responses are manifested in altered nuclear RNA synthesis followed by <u>de</u> <u>novo</u> cytoplasmic protein synthesis. For a tissue to be considered as a steroid hormone target tissue, therefore, it should contain (a) specific, high-affinity binding sites for the hormone, (b) nuclear accumulation of the hormone-receptor complex, and (c) hormone-induced changes in RNA and protein synthesis.

We have recently (Wade and Gray, 1978) demonstrated the presence of estrogen-specific, high-affinity cytoplasmic protein binding sites for 17β-estradiol in adipose tissues of female rats. We have also demonstrated that there is an accumulation of labelled steroid in the nuclei of adipose tissue cells following an injection of (^{3}H)-estradiol (Gray, Dudley and Wade, 1980). These data demonstrate that adipose tissue might be a true target tissue for estrogens.

One goal of the series of studies to be described was to demonstrate and characterize progestin binding in the cytoplasm of adipose tissues. One explanation for a lack of progestin-induced metabolic or behavioral responses in the absence of estrogen stimulation could be that estrogen pretreatment is necessary for the induction of progestin receptors. A similar prerequisite for estrogen for the induction of progestin receptors has previously been demonstrated (e.g. Leavitt et al., 1977; MacLusky and McEwen, 1970; Raynaud, 1977). Therefore, in addition to studying the affinity and specificity of these progestin binding sites, I examined the presence and induction of these binding sites under different estrogen (E_2B) priming conditions. As described earlier, (^{3}H)R5020, a potent progestin-like substance which has been previously shown to bind tightly to progestin receptors in other target tissues, was used in these binding studies.

Synthetic "Antiestrogens": Estrogen Mimetic Effects on Food Intake and Body Weight

Synthetic antiestrogens antagonize the effects of estrogen in a variety of target organs including the vagina, oviduct, uterus, mammary gland and brain (Gerall, Napoli and Cooper, 1973; Komisaruk and Beyer, 1972; Lerner, 1964; Roy and Wade, 1975). However, the synthetic antiestrogens, MER-25, clomiphene and nafoxidine, have been shown to be estrogen-like in their suppression of food intake and body weight gain (Poteat, 1977; Roy and Wade, 1976; Wade and Blaustein, 1978). In addition, Roy and Wade (1976) have demonstrated that progesterone could attenuate the food intake and body weight

suppressing effects of MER-25.

Some recent data indicate that although antiestrogens mimic the effects of estradiol on food intake and body weight, these same compounds block estrogen-induced hypertriglyceridemia and do not themselves induce raised levels of serum triglycerides (Chan et al., 1976; Pageaux, et al., 1980; Ramirez, unpublished observations). These data indicate that blood triglyceride levels, per se, may not be the important factor in altering feeding behaviors following hormone treatments.

Recent studies have shown that synthetic antiestrogens can cause the induction of uterine, hypophyseal and hypothalamic cytoplasmic progestin receptors (Jordan and Prestwich, 1978; Koseki et al., 1977; Roy, MacLusky and McEwen, 1979). However, the levels of progestin receptors which are induced by the antiestrogens are far lower than those levels observed following estradiol treatment. Whether or not the progestin receptors which are induced following antiestrogen administration are physiologically effective remains to be determined. Following several days of estradiol plus antiestrogen treatment, antiestrogens inhibit the estradiol-induced synthesis of progestin receptors in the uterus (Roy, MacLusky and McEwen, 1979). Antiestrogens also block the estrogen-induced synthesis of progestin binding sites in hypothalamus-proptic area and pituitary, although, alone, the antiestrogen produced a slight induction of binding sites in these tissues (Roy, MacLusky and McEwen, 1979).

In order to more fully explore the effects of an antiestrogen on food intake and metabolic processes, and the interaction of the antiestrogen with progesterone on these measures, rats were injected with nafoxidine, with or without concurrent progesterone. Measurements were made of food intake, body weight, carcass composition, serum triglycerides, wet and dry uterine weights, adipose tissue progestin binding and adipose tissue LPL activity. These results were compared with parallel data collected from rats which were treated with E_2B plus or minus progesterone.

It was hypothesized that, if progestin-induced effects on metabolic factors such as adipose tissue LPL activity are mediated via hormone receptor complexes, there should be a correlation between the amount of progestin receptor which is induced by a specific estrogen or antiestrogen and the amount of reversal of the estrogen/antiestrogen suppression in adipose tissue LPL activity following progesterone treatment.

In summary, the general purpose of the studies which are presented in this dissertation was to examine some of the behavioral, metabolic and biochemical concomitants of progestin administration to OVX rats which had been concurrently treated with either no estrogen, E_2B, or the synthetic antiestrogen, nafoxidine. The goal of these studies was to gain a better understanding of how ovarian steroids exert effects on metabolic parameters in a mammalian model.

CHAPTER II

PROGESTIN EFFECTS ON BODY WEIGHT, FOOD INTAKE AND ADIPOSITY

As discussed in more detail in Chapter I, estradiol decreases food intake, body weight and adiposity, while progesterone antagonizes these estradiol-induced effects. One goal of the first series of studies was to replicate and extend the basic studies on the effects of ovarian hormones on food intake, body weight and adiposity.

The effects of estradiol benzoate (E_2B) and progesterone (alone and in combination) were determined with respect to food intake, body weight, carcass composition, and adipose tissue cellularity (cell size and number). The effects of administration of the potent synthetic progestin, R5020, on these measures were also determined. No previous metabolic studies have been reported using R5020, although R5020 has been previously reported to be a highly effective progestin with regard to several biochemical measures (e.g., Leavitt et al., 1977; Raynaud, 1977; Philibert and Raynaud, 1974), as well as the facilitation of female sexual behavior in rats (Blaustein and Wade, 1977).

Radioactively-labelled R5020 was used as a tag to demonstrate and characterize progestin binding in adipose tissues (see Chapter IV) and its binding efficacy relative to those of natural progestins, was determined. A second goal of these studies was to compare the behavioral and metabolic effects of the two progestins, progesterone and R5020, for later comparison with the parallel adipose tissue binding data.

General Methods

Animals. Virgin female CD strain (Charles River Breeding Laboratories) rats, weighing approximately 200 g at the start of the experiment, were housed individually in hanging wire-mesh cases (Wahman) and were maintained on a 12 h light: 12 h dark cycle. Purina laboratory chow pellets and tap water were available ad libitum throughout the experiment.

Animals were ovariectomized via bilateral, dorsolateral incisions under methoxyflurane (Metofane, Pitman-Moore) anesthesia. Care was taken to remove as little non-ovarian tissue as was possible, and to cause minimal disturbance to the parametrial adipose depot.

Body weight and food intake measurements. Approximately two weeks following surgery, food intake and body weight measurements began. Food intake, adjusted for spillage, was measured to the nearest 0.1 g while body weights were determined to the nearest g. Measurements were made either twice a week (Monday and Friday) or every three days.

After three or four baseline points were gathered, animals were grouped as necessary for the particular experiment, with groups being matched for mean body weight and food intake over the baseline period.

During the subsequent time period (2-3 weeks), hormones were administered and body weight and food intake measurements continued as were described for the baseline period. At the end of the experimental period, rats were given an overdose of sodium pentobarbitol

(Nembutal) and analyzed either for carcass composition (Experiment I or II) or for adipose cellularity (Experiment III).

Experiment I. The Effects of R5020 and Progesterone on Food Intake, Body Weight and Carcass Composition in OVX Rats

Methods. After being matched for body weight and food intake, rats were given daily injections of either oil (n=8), 100 ug R5020 (n=8) or 2 mg progesterone (n=8). Following two weeks of progestin treatment, animals were sacrificed.

Carcass analyses were performed according to a modification of the method of Leshner, Litwin and Squibb (1972). Rats were shaved and eviscerated, and then dried at 70-80 C to a constant weight in order to determine total carcass water content. Dehydrated carcasses were then ground in a Waring blender, and a homogeneous sample (approximately .5g) was taken for further analyses. Lipid was extracted with 2 x 10 ml petroleum ether; and protein was extracted from the delipidated sample with 0.3N KOH. Water and lipid content were determined by weight differences, while protein was determined by the method of Lowry et al. (1951).

Results. There were no differences in food intake as a result of administration of either progesterone or R5020 to OVX rats. Similarly, as summarized in Table I, there were no progestin-induced effects on body weight or carcass composition in these rats.

TABLE 1

THE EFFECTS OF PROGESTERONE OR R5020 ADMINISTRATION ON
BODY WEIGHT AND CARCASS COMPOSITION IN OVX RATS

(Mean $\pm$ S.E.M.)

| GROUP | BODY WEIGHT | | CARCASS ANALYSIS: PERCENT WET WEIGHT | | |
	INITIAL	FINAL	WATER	LIPID	PROTEIN
OIL (n=8)	296 $\pm$ 6	324 $\pm$ 8	59.2 $\pm$1.7	15.6 $\pm$2.0	14.2 $\pm$0.6
PROG. (n=8)	295 $\pm$ 6	315 $\pm$ 6	60.4 $\pm$1.2	15.4 $\pm$1.1	14.1 $\pm$0.7
R5020 (n=8)	296 $\pm$ 4	324 $\pm$ 6	60.7 $\pm$0.9	13.0 $\pm$0.7	14.0 $\pm$1.1

Experiment II. Effects of R5020 and Progesterone on Body Weight and Carcass Composition in Estrogen-primed OVX Rats

Methods. Two weeks following OVX, baseline body weight measurements began, with body weight data being collected twice weekly throughout the experiment. After three data points per animal had been collected, 40 OVX animals were divided into 4 matched groups of 10 animals each. Rats received daily injections for the next three weeks of either oil, 2 ug E_2B, 2 ug E_2B plus 2 mg progesterone, or 2 ug E_2B plus 100 ug R5020.

In addition, a group of oil-injected, gonadally-intact females was weighed every three days throughout the experiment. These animals were the same age as were the surgically treated animals.

At the end of three weeks of hormone or vehicle administration, rats were sacrificed and carcass analyses were performed as described in Experiment I.

Results. Analysis of the final body weights for the hormone treated OVX rats revealed a significant treatment effect ($F_{3,36}$=3.84; p < .02). Post hoc comparisons of means, using a Newman-Keuls test, revealed a significant (p < .05) decrease in body weight in E_2B-treated animals relative to oil injected controls (Table 2) and a significant (p < .05) reversal of this estrogen-induced supression of body weight by a concurrent treatment with R5020 (100 ug per day). Although daily injections of 2 mg progesterone partially reversed the E_2B-induced suppression in body weight, the effect was not statistically significant.

Carcass analyses revealed that the only component of the carcass which had a significant change in the percent of total (wet; $F_{3,36}=3.25$; $p < .05$) or dry ($F_{3,36}=3.76$; $p < .02$) carcass following hormone manipulation was that of the lipid. E_2B treatment decreased total carcass fat content, while concurrent R5020 administration increased total lipid to control (OVX) levels (Table 2). This same change in lipid content was reflected in the percent of the dehydrated body which was lipid (Table 2). No differences were observed in either water content or total protein content as a result of hormone administration to OVX rats.

Gonadally intact animals weighed less than did any of the OVX animals, both at the beginning and at the termination of the hormone administration. Ovariectomy (OVX-oil versus gonadally intact-oil) resulted in a nonsignificant increase in total lipid content and a decrease in carcass protein content (Table 2).

Experiment III. Effects of R5020 and Progesterone on Food Intake, Body Weight and Adipose Cellularity in Estrogen-primed OVX Rats

Methods. Starting two weeks following OVX, baseline food intake and body weight data were collected for 48 rats as described in Experiment I. Animals were divided into four matched groups (n=10 each), and for the next 24 days received daily subcutaneous injections of either oil, 2 ug E_2B, 2 ug E_2B plus 2 mg progesterone, or 2 ug E_2B plus 100 ug R5020. Food intake and body weight measurements were taken every three days throughout the hormone administration period.

TABLE 2

THE EFFECT OF PROGESTERONE OR R5020 ADMINISTRATION ON BODY WEIGHT
AND CARCASS COMPOSITION IN OVX, E_2B-TREATED RATS

GROUP	BODY WEIGHT		CARCASS ANALYSIS PERCENT WET WEIGHT			CARCASS ANALYSIS PERCENT DRY WEIGHT	
	INITIAL	FINAL	WATER	LIPID	PROTEIN	LIPID	PROTEIN
OVX: OIL (n=10)	269 ± 5	292* ± 9	59.6 ±1.1	14.1 +0.7	14.1 +0.9	34.9* +1.7	34.8 +1.8
OVX: E_2B (n=10)	272 ± 7	266 ± 7	61.1 +0.8	11.7 +0.7	14.5 +1.1	30.4 +1.4	37.1 +2.7
OVX: E_2B + PROG. (n=9)	273 ± 6	280 ± 7	61.2 +0.8	12.4 +0.7	15.2 +1.0	31.9 +1.2	39.4 +2.7
OVX: E_2B + R5020 (n=10)	272 ± 6	297* ± 5	60.3 +0.8	14.1* +0.7	14.6 +0.9	35.4* +1.3	36.8 +2.3
$F_{3,36}$		3.84		3.25		3.76	
P	NS	<.02	NS	<.05	NS	<.02	NS
INTACT: OIL	223 ± 4	239 ± 5	59.7 +0.9	13.2 +0.9	15.7 +0.7	32.5 +1.8	37.8 +1.5

*p < .05 versus E_2B

All data are presented as mean ± S.E.M.

An additional group of gonadally intact, oil injected female rats was run, with similar food intake and body weight data being collected as are described above. No attempt was made to monitor the hormonal status of these animals at the time of sacrifice.

At the end of the 24-day treatment period, animals were sacrificed and parametrial adipose depots were rapidly dissected and weighed. Adipose cellularity was analyzed in parametrial fat pads according to a slight modification of the photomicrographic method of Lavau et al. (1977). Adipose cells were dispersed by collagenase (Worthington) digestion, and 10 ul aliquots of the cell suspension were immediately smeared on a slide and photographed through a microscope using a yellow filter. Pictures of a standard micrometer were taken under identical conditions for use as scales in the cell diameter size determinations. To measure cell diameters, negatives of the photo-micrographs were projected onto an electronic grid and diameters were digitized using a Gar-Graf pen. Data were automatically stored and analyzed by a Nova-3 computer, with calculations being made of the mean cell diameter, volume and weight for at least 250 adipocytes per fat depot.

Lipid extractions were done on pieces of adipose tissue from the same depot as was used for the cellularity analysis. Preweighed pieces of fat were homogenized in an hexane:isopropanol (3:2; vol/vol). Na_2SO_4 was added; the samples were shaken vigorously; and the aqueous phase was decanted. The organic phase was added to preweighed vials. Lipid content was determined by drying the vials to a constant weight.

Parametrial pads were chosen for these analyses because prior studies (Wade and Gray, 1978) had demonstrated that the highest levels of cytoplasmic estrogen receptor sites were found in this adipose depot. Induction of progestin receptors, following E_2B treatment, was also highest in parametrial pads (see Chapter IV). Finally, Steingrimsdottir, Brasel and Greenwood (1980) reported that the greatest change in adipose cellularity and adipose tissue LPL activity following progesterone treatment was found in the parametrial adipose depot of gonadally intact rats. These data suggested that maximal responsiveness to hormone administration would be found in the parametrial fat pad of OVX rats.

Results. During the first week of hormone treatment to OVX rats, E_2B suppressed food intake by approximately 25% (Figure 1), while concurrent daily injections of either progesterone or R5020 attenuated these effects of E_2B ($F_{344}=5.95$; $p < .002$). Food intakes began to return to normal, for all groups, during the second week of hormone treatment and control values were found by the end of the experiment.

Changes in body weight paralleled the changes in food intake in hormone-treated, OVX rats. Again E_2B treatment suppressed body weight, while concurrent progestin (R5020 or progesterone) treatment resulted in increased body weights relative to the E_2B-treated animals (Figure 2). Hormone induced changes in body weight were not statistically significant in this experiment ($F_{3,44}=2.01$; $\underline{p} = 0.13$).

Figure 1. Effects of progesterone
or R5020 on food intake in OVX, E_2B-
treated rats.
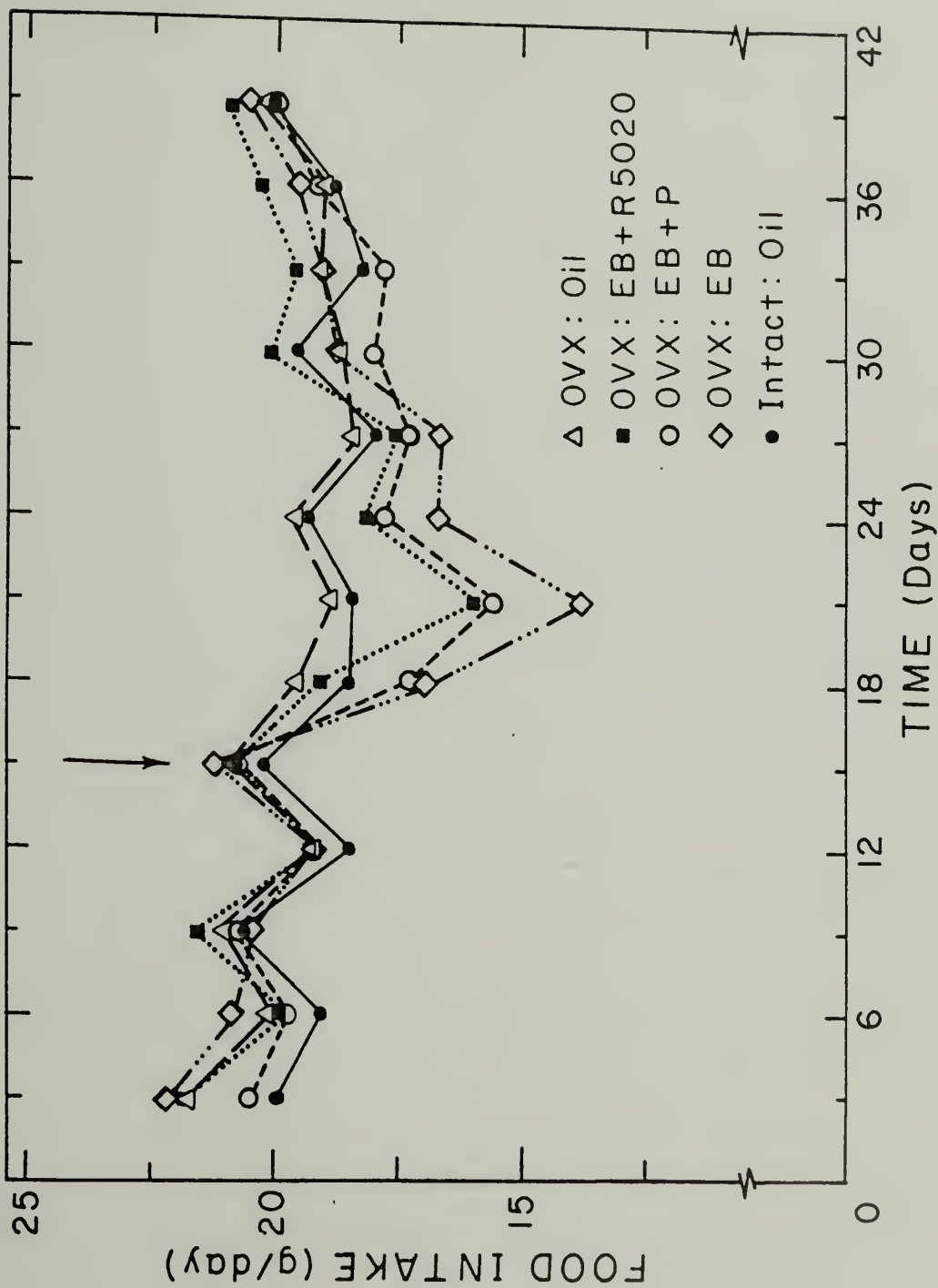
Figure 2. Effects of progesterone or
R5020 on body weight in OVX, E_2B-treated rats.
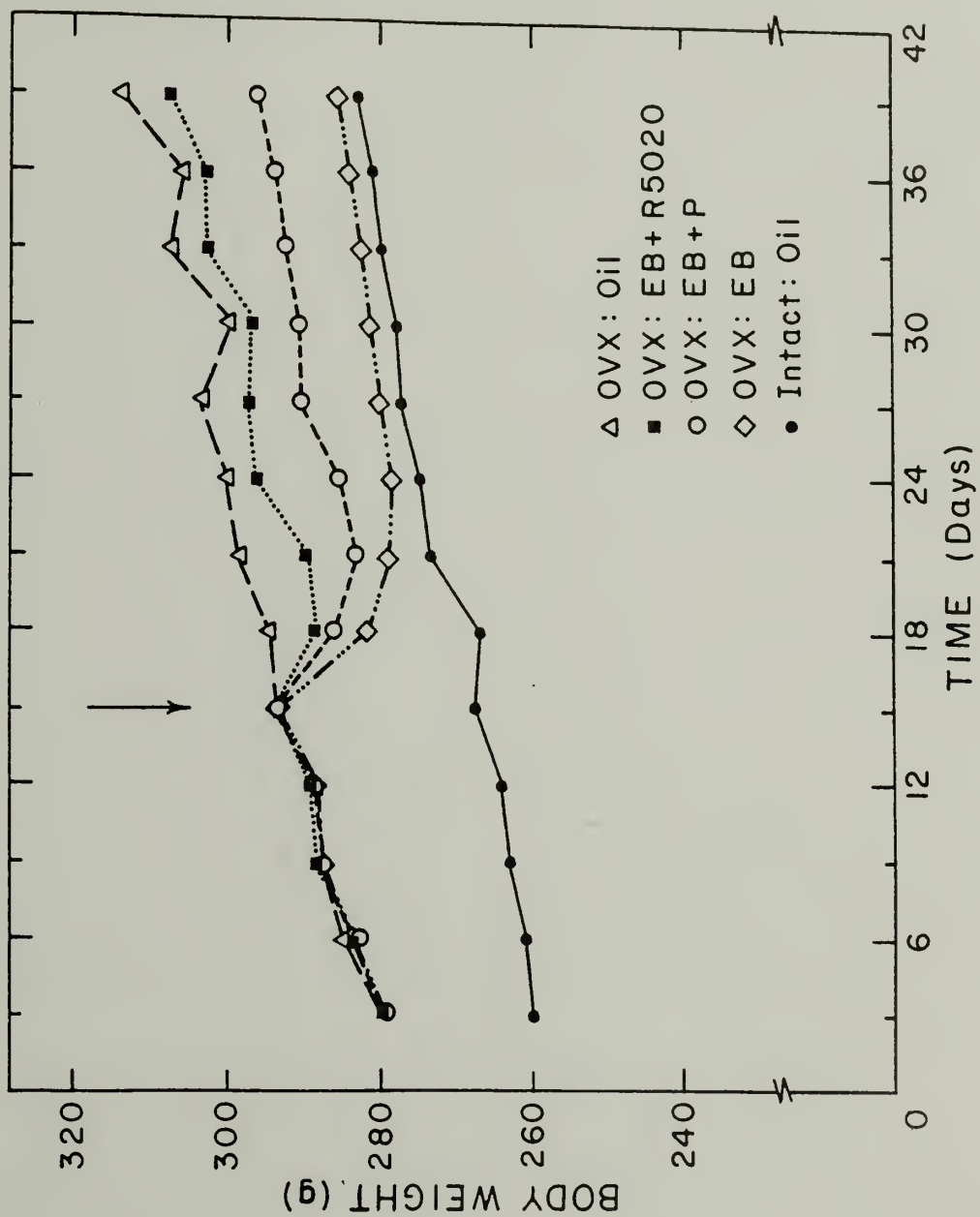
Adipose tissue cellularity data for the parametrial pad of these animals are presented in Table 3. Although no significant ($p < .05$) differences were found for any measurement, analysis of variance for the fat cell weights revealed a treatment effect approaching significance ($F_{3,36} = 2.79$; $p = .058$). Post-hoc analyses of adipose cell weights revealed no significant differences, although comparisons between oil and either E_2B or E_2B plus R5020 cells approached significance ($.05 < p < .10$).

TABLE 3

THE EFFECT OF PROGESTERONE OR R5020 ADMINISTRATION OR
PARAMETRIAL DEPOT CELLULARITY IN E_2B-TREATED, OVX RATS

			PARAMETRIAL FAT DEPOT			
GROUP	INITIAL	FINAL	PAD WT. (g)	LIPID (g)	CELL WT. (ug)	CELL NO. $\times 10^{-6}$
OVX: OIL	293 $\pm$ 9	324 $\pm$10	5.11 $\pm$0.56	3.97 $\pm$.50	1.49 $\pm$.34	5.29 $\pm$1.15
OVX: E_2B	293 $\pm$10	288 $\pm$10	4.67 $\pm$ .58	3.86 $\pm$.50	0.69 $\pm$.10	7.19 $\pm$1.26
OVX: E_2B + PROG.	293 $\pm$ 9	296 $\pm$ 8	5.01 $\pm$ .58	4.01 $\pm$.62	1.18 $\pm$.25	5.25 $\pm$ .69
OVX: E_2B + R5020	293 $\pm$10	314 $\pm$ 9	4.53 $\pm$ .59	3.42 $\pm$.44	0.74 $\pm$.10	7.16 $\pm$1.22
INTACT: OIL	268 $\pm$ 6	291 $\pm$ 4	7.21 $\pm$1.07	5.76 $\pm$.96	1.43 $\pm$.49	7.34 $\pm$1.23

Discussion

In the absence of estrogens, neither progesterone (5 mg per day) nor R5020 (100 ug per day) had any effect on food intake, body weight gain, or carcass composition. In E_2B-treated animals, however, both progestins increased food intake and body weight gain relative to E_2B-treated controls. R5020, at one-twentieth the dose of progesterone, was more effective than was progesterone in causing these changes.

As was previously reported for progesterone (Galletti and Klopper, 1964; Hervey and Hervey, 1977) when concurrently administered with E_2B, R5020 caused a significant increase in the amount of carcass lipid, both as a percent of wet (total) and dry carcass weights, when compared to E_2B-treated animals (Table 2). Across hormone treatment groups there were no differences in the amount of carcass water or protein.

The effects of treatment with either E_2B, E_2B plus progesterone, or E_2B plus R5020 on adipose cellularity in the parametrial adipose pad of OVX rats were studied. Although no significant results were found, there was a strong trend toward a decrease in fat cell size following E_2B treatment, with concurrent progesterone treatment increasing adipocyte size (Table 3). These data are in agreement with previous reports (Salans, 1974; Steingrimsdottir, Brasel and Greenwood, 1980) using intact rats in which estrogen treatment decreased and progesterone treatment increased fat cell size. As there were no differences in fat cell number, the differences in fat cell size were reflected in parallel changes in fat depot size.

As compared with the data of Steingrimsdottir, Brasel and Green-wood (1980), who used intact rats, progesterone treatment in OVX, E_2B-treated animals in these experiments had less of an effect on parametrial fat pad size or cellularity. One possible factor is the dose of progesterone used. Steingrimsdottir et al. gave daily in-jections of 5 mg. progesterone, while I gave only 2 mg per day. Also to be considered is the traumatic effect of ovariectomy on the parametrial fat depot. Even with the most careful and conservative surgical procedures, ovariectomy results in disruption of neuronal and vascular connections to the uterine-parametrial area. The con-sequences of these "inadvertant" post-surgical disruptures may include a diminished responsiveness to hormonal treatment in the parametrial fat depot.

Unlike progesterone, R5020 appeared to have no effect on para-metrial fat pad or cell size in E_2B-treated, OVX rats (Table 3). However, R5020, like progesterone, did exert its weight enhancing effects through an increase in carcass lipids.

C H A P T E R   I I I

INTERACTION OF AN "ANTIESTROGEN" AND PROGESTERONE IN AFFECTING FOOD INTAKE, BODY WEIGHT, AND CARCASS COMPOSITION

Synthetic antiestrogens, including nafoxidine, antagonize the effects of estradiol on a variety of measurements, including long-term uterine growth, female sexual behavior, binding to estrogen receptors, maximal induction of progestin receptors, and serum hypertriglyceridemia (Chan et al., 1976; Gerall, Napoli and Cooper, 1973; Komisaruk and Beyer, 1972; Lerner, 1964; Pageaux, et al., 1980; Roy, MacLusky and McEwen, 1979; Roy and Wade, 1975; Walker and Feder, 1977). However, both nafoxidine and MER-25 have been shown to be estrogen-like in their suppression of food intake (Roy and Wade, 1976; Wade and Blaustein, 1978).

The following experiment was designed to further explore some of the estradiol-agonistic and antagonistic effects of nafoxidine and to look at the effects of concurrent progesterone and nafoxidine treatment on food intake, body weight gain, carcass composition, serum triglycerides and uterine weights. These data were compared to parallel findings from animals treated either with E_2B or E_2B plus progesterone.

General Methods

Animals. Sixty female rats were ovariectomized and maintained as described in Chapter II.

Experimental measures. Body weight and food intake measurements were made twice a week as described in Chapter II.

Experiment IV. Concurrent Administration of Nafoxidine with E_2B or Progesterone: Effects on Food Intake, Body Weight, Carcass Composition and Plasma Triglycerides

Methods. Following baseline body weight and food intake measurements, rats were divided into six matched groups (n=10, each) and given daily injections of either oil, E_2B (2 ug), nafoxidine (2 mg), E_2B plus nafoxidine, E_2B plus progesterone (5 mg), or nafoxidine plus progesterone. Food intake and body weight measurements continued throughout the experiment.

At sacrifice, one animal in the E_2B plus progesterone group was found to be gonadally intact; all data from this animal were discarded in the analysis.

After three weeks of hormone administration, rats were given an overdose of Nembutal, and 2 ml of blood was taken by cardiac puncture into heparinized syringes. The blood was chilled and centrifuged, with plasma being saved for later analysis of glycerol and triglyceride levels[2]. Uteri were carefully dissected and weighed. They were then dried overnight at 70 C and reweighed. Carcasses were prepared for analysis as described in Chapter II, except that a food processor (Hamilton Beach) was used in addition to the blender in the carcass grinding steps.

Results. Hormone administration resulted in significant changes in both food intake (day 1-7 of hormone treatment, $F_{5,53}=9.33$; $p < .0001$; day 1-21 of hormone treatment, $F_{5,53}=6.42$; $p < .001$) and body weight (final day of hormone treatment, $F_{5,53}=6.04$; $p < .01$). As was expected, E_2B treatment (2 ug/day) decreased food intake (Figure 3,

Figure 3. Effects of E_2B, nafoxidine and progesterone, alone and in Combination, on food intake in OVX rats.

Table 4) and body weight gain (Figure 4, Table 4) in OVX animals, while concurrent progesterone (5 mg/day) administration attenuated these effects.

Nafoxidine (2 mg/day), alone and in combination with E_2B, had very similar weight reducing effects as did E_2B, while the depression of food intake following nafoxidine treatment was more prolonged than was the anorexia following treatment with E_2B alone (Figures 3 and 4). Progesterone treatment also significantly attenuated the food intake and body weight suppressant effects of nafoxidine (Figures 3 and 4, Table 4).

Differences in uterine weights were found with both wet ($F_{5,53}=38.70$; $\underline{p} < .0001$) and dry ($F_{5,53}=28.51$; $\underline{p} < .0001$) tissues (Table 4). All hormone treatments induce both wet and dry uterine weight increases ($\underline{p}$'s all $< .05$), relative to oil treatment. E_2B induced significantly higher ($\underline{p}$'s $< .01$ versus all other treatments) uterine weights, both wet and dry, than did any other treatment. Concurrent administration of nafoxidine blocked the magnified E_2B-induced uterine responses (Table 4). While progesterone significantly affected ($\underline{p}$'s $< .01$) uterine wet and dry weights in E_2B treated animals, it had no effect on uterine weights in nafoxidine-treated rats (Table 4).

Although there were no differences in plasma glycerol levels across hormone treatment groups, plasma triglycerides did vary significantly as a function of hormone treatment ($F_{5,53}=13.28$; $\underline{p} < .01$; Table 5). Both E_2B alone and E_2B plus progesterone treatments resulted in significant ($\underline{p} < .005$) increases in triglyceride

TABLE 4

EFFECTS OF E_2B, NAFOXIDINE AND PROGESTERONE, ALONE AND IN COMBINATION,
ON BODY WEIGHT, FOOD INTAKE, AND UTERINE WEIGHTS IN OVX RATS

GROUP	BODY WEIGHT		FOOD INTAKE (g)		UTERINE WEIGHT (mg)	
	INITIAL	FINAL	DAYS 1-7	DAYS 1-21	WET	DRY
OIL	318.1 ± 4.7	335.8[1] ± 9.0	156.4[1] ± 4.3	449.3[1] ±13.3	161.12[1] +19.40	38.85[1] ± 2.51
E_2B	318.0 ± 5.1	292.1[2] ± 4.8	115.5[2,3] ± 4.8	371.2[2] ±17.4	564.22[2] +38.43	114.12[2] ± 6.58
NAFOXIDINE	318.4 ± 4.1	296.0[2] ± 6.8	117.3[2,3] ± 4.4	351.3[2] ±11.4	249.53[4] +15.33	60.51[4] ± 7.34
E_2B + NAFOXIDINE	317.1 ± 4.6	294.9[2] ± 7.2	113.7[2] ± 4.6	344.3[2] ±12.4	238.97[4] +17.79	59.94[4] ± 2.45
E_2B + PROGESTERONE	325.0 ± 5.2	324.8[1] ± 6.9	129.6[3] ± 6.8	392.9[2] ±15.0	410.74[3] +28.92	88.41[3] ± 3.98
NAFOXIDINE + PROGESTERONE	317.4 ± 4.4	325.2[1] +10.6	126.8[2,3] ± 6.2	386.3[2] ±18.7	296.78[4] +32.16	68.69[4] ± 4.31
$F_{5,53}$		6.04	9.33	6.42	38.70	28.51
p	NS	<.001	<.0001	<.001	<.0001	<.0001

Data with the same superscripts are not significantly (< .05) different
from each other.

All data are presented as mean ± S.E.M.

Figure 4. Effects of E_2B, nafoxidine and progesterone, alone and in combination, on body weight in OVX rats.
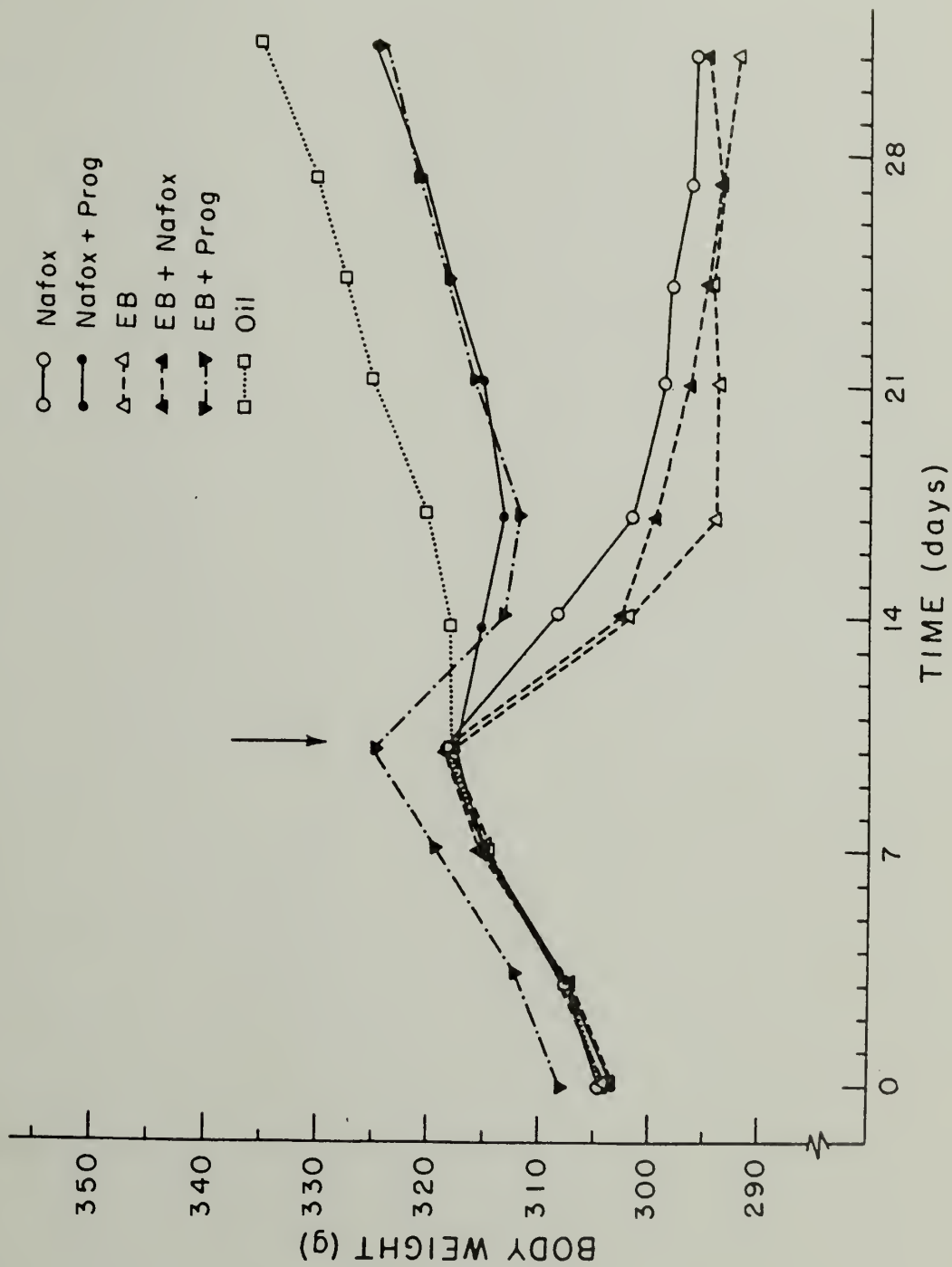
levels, with concurrent progesterone treatment significantly adding to the estrogen-induced hypertriglyceridemia (Table 5).

Carcass analysis (Table 6) revealed significant treatment effects in both water ($F_{2,27}$=6.69; p < .01) and lipid ($F_{2,27}$=5.94; p < .01) content when oil, nafoxidine and nafoxidine plus progesterone animals were compared. Post hoc analysis revealed significant decreases in carcass lipid and increases in carcass water content following nafoxidine treatment. Relatively large variances precluded statistically significant differences from being found between nafoxidine and nafoxidine plus progesterone groups. Further analysis, however, shows more dramatically the lipogenic effects of progesterone following nafoxidine treatment. If total carcass lipid is presented in grams, rather than as a percent of body weight, it becomes more apparent that a major portion (28%) of the difference between the nafoxidine and the nafoxidine plus progesterone-treated animals is in fat content (Table 6).

Discussion

Nafoxidine was "antiestrogenic" with regard to uterine growth (both wet and dry uterine weights) in these animals. However, like E_2B, nafoxidine treatment decreased food intake and body weight gain (Figures 3 and 4). These data support those previously reported which indicate that nafoxidine (Wade and Blaustein, 1978) as well as other so-called antiestrogens (Poteat, 1977; Roy and Wade, 1976) are estrogen-like in their suppression of food intake and body weight regulation.

TABLE 5

EFFECT OF E_2B, NAFOXIDINE AND PROGESTERONE, ALONE AND IN COMBINATION,
ON SERUM GLYCEROL AND TRIGLYCERIDE LEVELS IN OVX RATS

GROUP	GLYCEROL (umol/ml)	TRIGLYCERIDES (umol/ml)
OIL	.134 $\pm$.015	.735[1] $\pm$.124
E_2B	.123 $\pm$.019	1.026[1] $\pm$.230
NAFOXIDINE	.124 $\pm$.015	.552[3] $\pm$.104
E_2B + NAFOXIDINE	.073 $\pm$.013	.336[3] $\pm$.059
E_2B + PROGESTERONE	.108 $\pm$.010	1.890[2] $\pm$.288
NAFOXIDINE + PROGESTERONE	.107 $\pm$.022	.552[3] $\pm$.104
$F_{5,53}$	1.76	13.283
$\underline{p}$	NS	<.005

Data points with the same superscript in the triglycerides column
are not significantly ($\underline{p}$ < .05) different from each other.

TABLE 6

EFFECTS OF NAFOXIDINE AND PROGESTERONE ON CARCASS COMPOSITION
IN OVX RATS

GROUP	CARCASS COMPOSITION PERCENT WET WEIGHT			TOTAL LIPID (g)
	WATER	LIPID	PROTEIN	
OIL	62.0 $\pm$0.7	14.7 $\pm$1.1	13.5 $\pm$0.3	44.6 $\pm$4.0
NAFOXIDINE	65.5 $\pm$0.6	9.8 $\pm$0.9	13.7 $\pm$0.4	26.0 $\pm$2.5
NAFOXIDINE + PROGESTERONE	64.4 $\pm$0.7	11.6 $\pm$1.1	13.3 $\pm$0.4	34.2 $\pm$3.9
$F_{2,27}$	6.69	5.94		6.88
p	<.01	<.01	NS	<.01

All data are presented as means $\pm$ S.E.M.

The return of food intake toward control values appears to be slower in nafoxidine than E_2B treated animals (Figure 3). Wade and Blaustein (1978) found that a single injection of nafoxidine (4 mg) caused food intake to be depressed by at least 30% for 6 days, much longer than would be expected with E_2B treatment alone.

As had been previously shown with E_2B (Chapter II), nafoxidine decreased carcass lipid content in OVX rats. Concurrent progesterone treatment partially reversed this estrogen-like effect on total carcass fat content (Table 6). These results were found despite the fact that nafoxidine, either alone or in combination with progesterone, did not induce serum hypertriglyceridemia as was found following either E_2B or E_2B plus progesterone treatment (Table 5). These serum triglyceride data support those previously reported by Chan et al., (1976) in which nafoxidine was shown to inhibit estrogen induced hypertriglyceridemia in birds. These data also show that levels of serum triglycerides, per se, can not be the only important factor in regulating food intake and body weight gain following hormone manipulation.

CHAPTER IV

CHARACTERIZATION OF CYTOPLASMIC PROGESTIN BINDING
IN ADIPOSE TISSUES OF OVX-ADX RATS

The studies which were reported in Chapter II, as well as those
which have been reported by other authors (Galletti and Klopper, 1964;
Hervey and Hervey, 1967; Leshner and Collier, 1973), demonstrate that
the major change in carcass composition following hormone treatment is
found in the total carcass fat content. We have suggested (Wade and
Gray, 1979) that hormones might be having direct effects on peripheral
metabolic tissues such as adipose tissues. In support of this
hypothesis, we have demonstrated the presence of high affinity,
estrogen specific, macromolecular binding sites for 17β-estradiol
in the cytoplasm of adipose tissues from OVX rats (Wade and Gray, 1978).
In agreement with the current dogma of the mechanism of action for
steroid hormones, we also demonstrated specific in vivo nuclear
accumulation of radioactivity in adipose tissues after injection of
(^{3}H)17β-estradiol (Gray, Dudley and Wade, 1980).

The purpose of the following series of studies was to demonstrate
and to characterize cytoplasmic progestin binding in adipose tissues
of OVX-ADX rats. One explanation for a lack of progestin-induced
metabolic or behavioral response in the absence of estrogens is
that estrogens are necessary for the induction of cytoplasmic progestin
receptors. Therefore, in addition to studying the affinity and the
specificity of these progestin binding sites, I examined the induction
of these binding sites under different estrogen (E_2B) priming
conditions.

R5020, the synthetic progestin used in the food intake and adiposity studies reported in preceding chapters, has been used to demonstrate specific progestin binding sites in the uterus, vagina, pituitary, and brain (Blaustein and Feder, 1979; Leavitt et al., 1977; MacLusky and McEwen, 1978; Raynaud, 1977). R5020 binds tightly to the progestin receptor, with an association constant approximately four times that of the natural progestin, progesterone (Philibert and Raynaud, 1974). Since R5020 has been used successfully in describing progestin binding sites in other tissues, I chose to use this compound in examining progestin binding in the cytoplasm of adipose tissues.

General Methods

Animals. Multiparous CD strain rats (Charles River Breeding Laboratories), weighing 350-450 g were housed 7-9 per cage and were maintained under a 12 h light: 12 h dark cycle. Animals were ovariectomized and adrenalectomized (ADX) via bilateral dorsolateral incisions under methoxyflurane (Metofane; Pitman Moore) anesthesia. They were maintained on Purina Rodent chow and 0.9% saline ad libitum.

Tissue preparation. Adipose tissues were dissected, as previously described (Wade and Gray, 1978), and homogenized in ground-glass tissue grinders (Duall size 23; Kontes) at a concentration of 400 mg tissue per ml buffer containing 10mM Tris-HCl, 1.5 mM EDTA, 12 mM monothioglycerol, and 10% (vol/vol) glycerol, pH. 7.4 (TEMG). This and all subsequent steps were performed at 4 C. Homogenates were centrifuged at 48,000 x g for 30 minutes.

Assay conditions. Cytoplasmic progestin binding in adipose tissue was assayed according to the method of MacLusky and McEwen (1978) as modified by Blaustein and Feder (1979). Aliquots (250 ul) of the high speed supernatant were incubated with (^{3}H)R5020 (New England Nuclear) for 4 hours at 0 C with or without the addition of various competitors or enzymes. Bound and free (^{3}H)R5020 were separated by gel filtration on 5 x 60 mm. Sephadex LH-20 columns, equilibrated with TEMG, as described by Ginsburg et al. (1974) but using TEMG as the eluent. Two hundred microliters of the incubate were pipetted into the column and washed in with 200 ul TEMG. Thirty minutes later, the protein peak was eluted into a scintillation vial with 800 ul TEMG, and 12 ml toluene-based scintillation fluid were added. Samples were vigorously shaken and counted to a standard deviation of less than 1% in a Packard model 2425 liquid scintilation spectrometer at an efficiency of 55±1%.

To determine the protein concentrations of the cytoplasmic fractions, 100 ul aliquots of the high speed supernatants were pre-cipitated with 5 ml ethanol. The precipitate was dried, dissolved in 0.3 N KOH, and assayed for protein by the method of Lowry et al. (1951). Because significant amounts of the R5020 seemed to be absorbed to either plastic or glass incubation tubes, the total (bound plus free) (^{3}H)R5020 concentration of the incubates was determined by counting 20 ul aliquots at the end of the incubation period.

Experiment V. Saturation Analyses of (^{3}H)R5020 Binding in Cytoplasm of Parametrial Adipose Tissue

<u>Methods</u>. Saturation analyses were conducted on high speed super-natans of parametrial fat pads from unprimed OVX-ADX rats or from OVX-ADX rats which had been primed with 2 ug E_2B per day for 7 days. Pooled (within priming condition) high speed supernatant was incubated with increasing concentrations (0.05 - 4.0 nM) of (^{3}H)R5020 in the presence or absence of 1 uM unlabelled R5020. Scatchard analyses (Scatchard, 1949) of the specifically bound fractions were performed and dissociation constants (K_d's) were calculated.

<u>Results</u>. Only very low levels of (^{3}H)R5020 binding were measurable in the cytoplasmic fractions of parametrial pads from untreated OVX-ADX rats (data not shown). Priming with E_2B induced a substantial quantity of cytoplasmic progestin binding sites (Figure 5). Scatchard analysis of the specifically bound (^{3}H)R5020 yielded a K_d of 8.5 x 10 $^{-10}$M (Figure 5) in E_2B-primed rats.

Experiment VI. In Vitro Specificity of (^{3}H)R5020 Binding in Cytoplasmic Fractions of Parametrial Adipose Tissue

<u>Methods</u>. To determine the specificity of (^{3}H)R5020 binding in the cytoplasm of parametrial adipose tissue from estrogen-primed, OVX-ADX rats, high speed supernatant from pooled parametrial fat pads of rats pretreated for 3 days with 2 ug E_2B were incubated with 2nM (^{3}H)R5020 with either no competitor or with 20, 200, or 2000 nM R5020, proges-terone, 5α-dihydroprogesterone, 5α-dihydrotestosterone, estradiol, or corticosterone. Duplicates were run for each concentration of

Figure 5. Saturation and Scatchard plots of (^{3}H)R5020 binding in cytoplasm of parametrial adipose tissues from E_2B-treated, OVX-ADX rats.
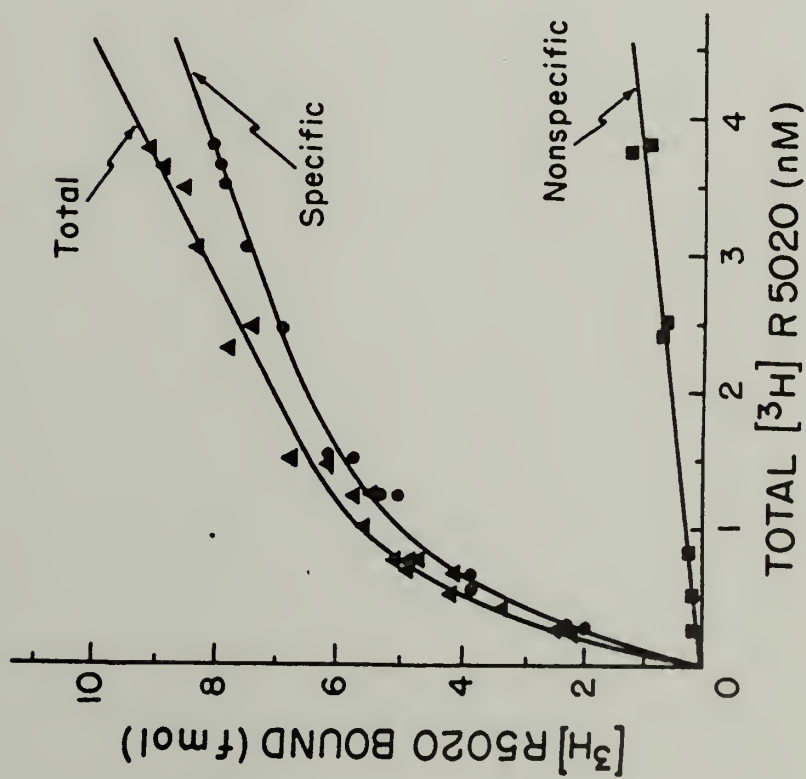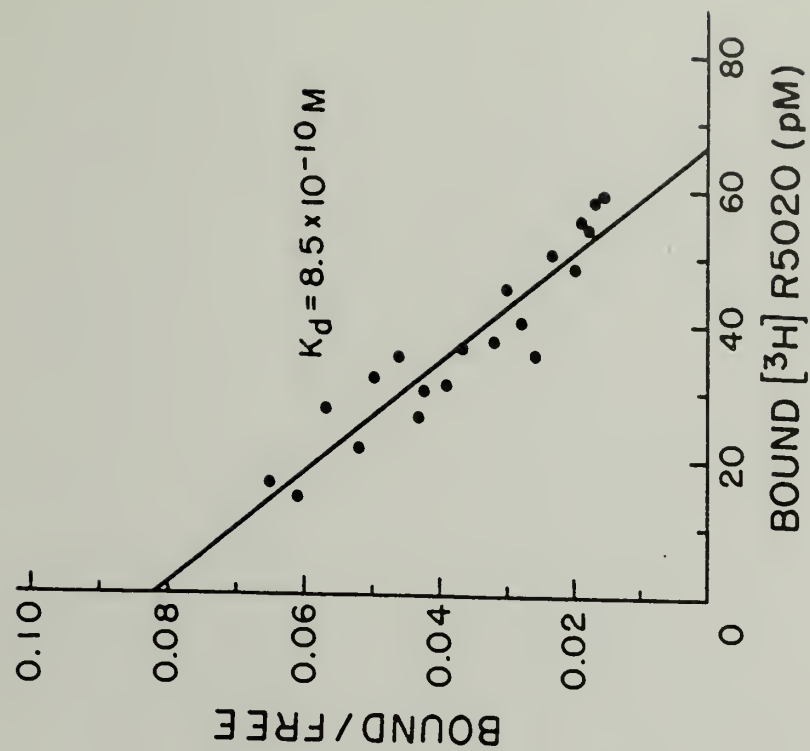
competitor.

Results. Unlabelled R5020 was approximately 8 times more effective in competing for binding sites than was progesterone, which in turn was about 8 times as effective as the progesterone metabolite, 5α-dihydro-progesterone (Figure 6). All three progestins tested were more potent inhibitors than were 17β-estradiol, 5α-dihydrotestosterone, or corticosterone (Figure 6).

Experiment VII. Regional Differences in (^{3}H)R5020 Binding in Adipose Tissues

Methods. Regional differences in the binding of progestins was measured in the cytoplasms of parametrial, retroperitoneal, omental, inguinal and interscapular brown fat of E_2B-primed (2 ug E_2B/day for 3 days) or unprimed rats. Specific progestin binding in this experiment, and in all further experiments, was determined by incubating the high speed supernatant with 2nM (^{3}H)R5020 in the presence or absence of 0.5uM unlabelled R5020.

Results. Very low levels of progestin binding were observed in high speed supernatants from the various pads of unprimed animals (Figure 7). Daily E_2B induced additional progestin binding sites in all adipose tissues studied. The greatest induction (26-fold) was found in parametrial pads, followed by inguinal, retroperitoneal, omental and brown fat (Figure 7); the differences in estrogen-priming across fat pads were highly significant ($F_{4,40}=34.9$; $p < .001$). We have previously reported (Wade and Gray, 1978) significant regional

Figure 6. <u>In vitro</u> specificity of
(^{3}H)R5020 binding in cytoplasm of parametrial
adipose tissues in E_2B-treated, OVX-ADX rats.

SPECIFICITY OF ADIPOSE TISSUE CYTOPLASMIC R5020 BINDING

48
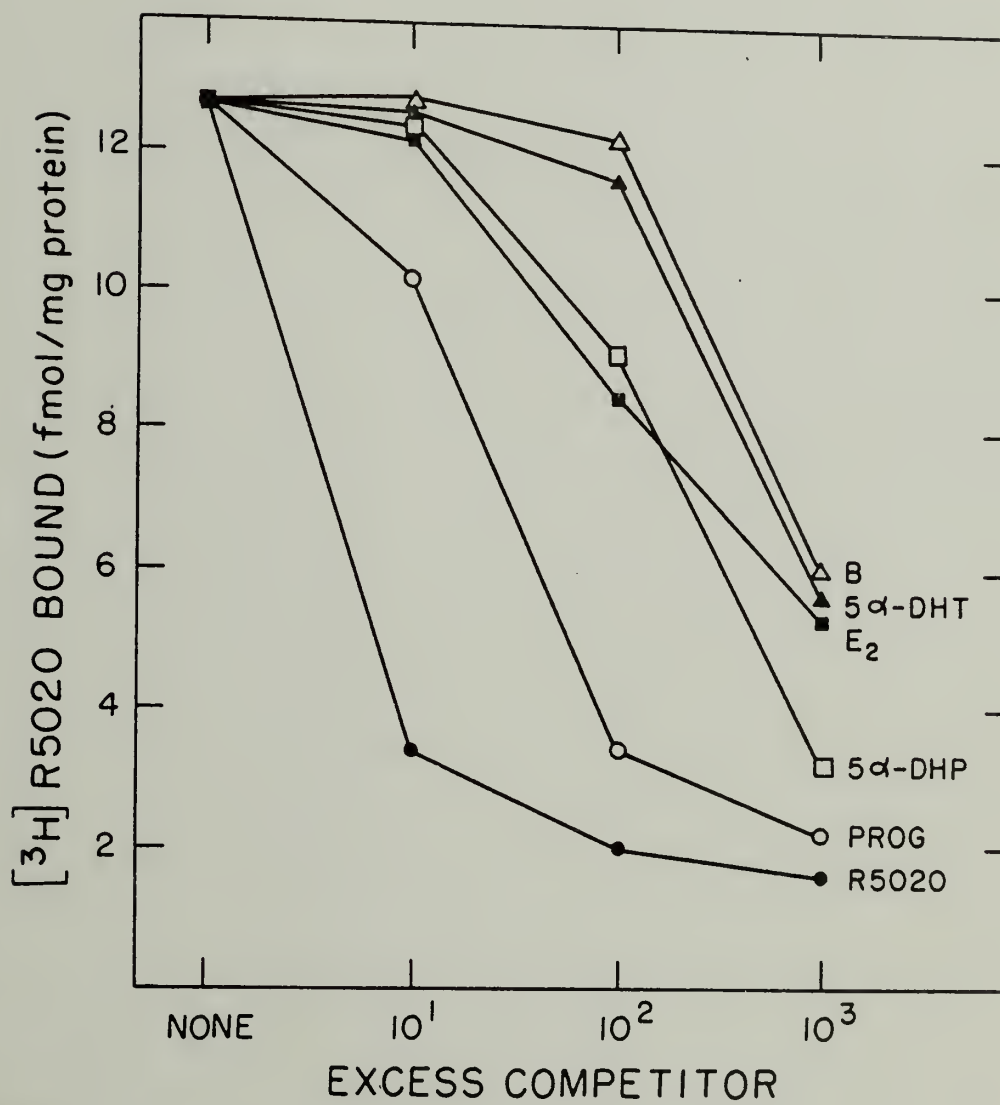
Figure 7. Regional differences in binding
of (^{3}H)R5020 in cytoplasm of various adipose
depots in E_2B-primed, OVX-ADX rats.

REGIONAL DIFFERENCES IN ADIPOSE
TISSUE CYTOPLASMIC R5020 BINDING
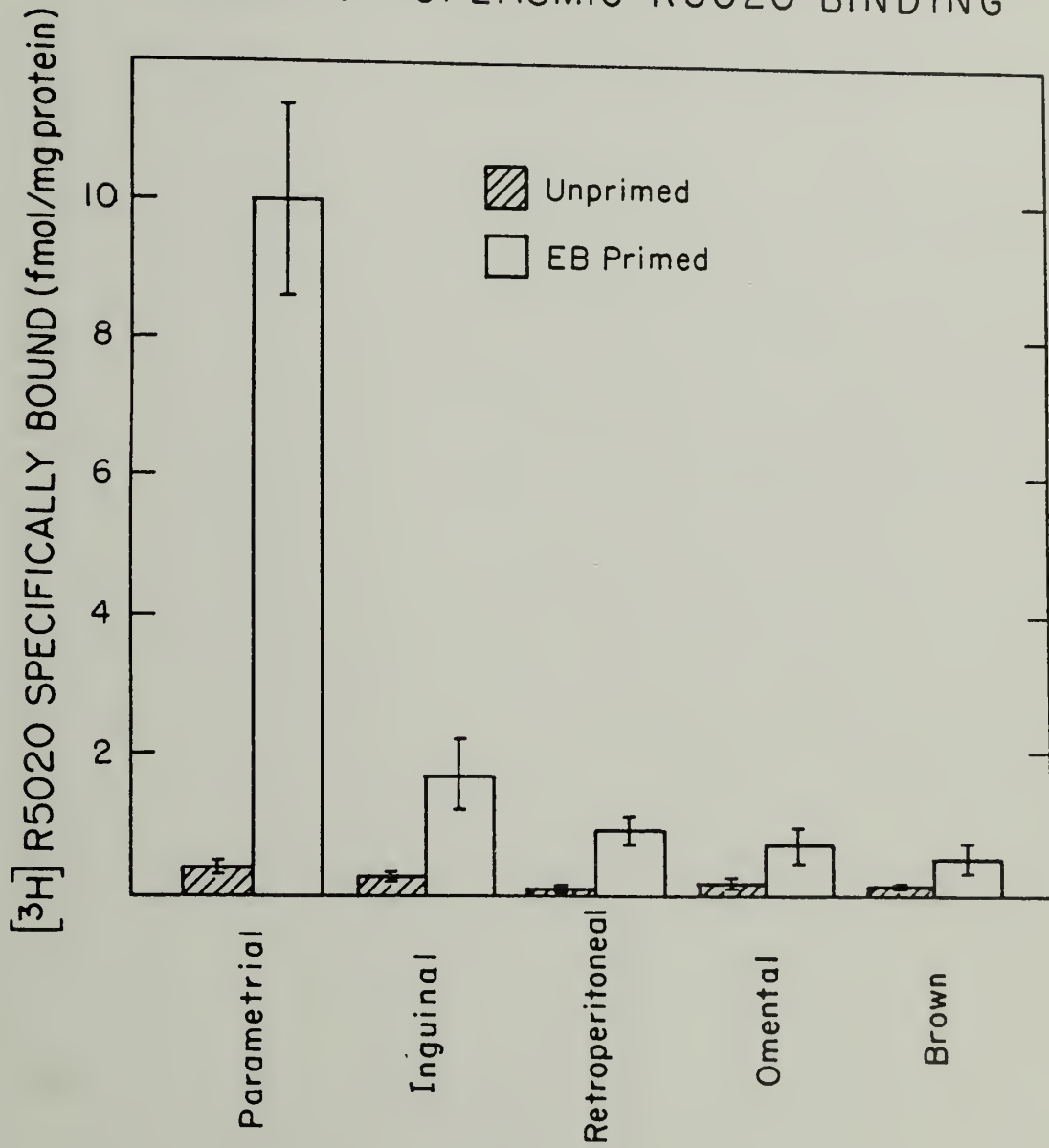
differences in 17β-estradiol binding in adipose tissues (parametrial > retroperitoneal > brown > omental > inguinal). Further analyses of these binding data show that, given the basal (unprimed) level of progestin receptors and the concentration of estrogen receptors in a specific pad (Table 7), the absolute level of induced (from E_2B-primed animals) progestin receptors can be predicted by multiple linear regression (R=.998, $F_{2,4}$=204.0; $\underline{p}$ <.0001). Significant predictions can also be made from these data of the magnitude of induction of progestin binding sites (induced level/basal level; R=.990, $F_{2,4}$=47.5, $\underline{p}$ <.002). There is also a highly significant correlation between fat pad estrogen receptor concentration and magnitude of progestin receptor induction (induced progestin receptor/basal progestin receptor; r=.96, $\underline{t}_3$=5.87, $\underline{p}$ < .01).

Experiment VIII. Time Course of Induction of (^{3}H)R5020 Binding in Adipose Tissues Following a Single Pulse of E_2B

Methods. The time course of induction of progestin binding after E_2B administration was determined by assaying (^{3}H)R5020 binding in high speed supernatant from parametrial fat pads of animals which are sacrificed 0, 6, 12, 24, 36, 48, or 60 hours after a single injection of 2 ug E_2B.

Results. Although no significant induction of progestin binding was observed 6 hours after injection of E_2B, cytoplasmic binding rose rapidly between 6 and 12 hours post-injection (Figure 8). Peak binding

TABLE 7

CYTOPLASMIC ESTROGEN AND PROGESTIN BINDING IN VARIOUS ADIPOSE DEPOTS OF OVX RATS

ADIPOSE DEPOT	A CYTOPLASMIC* $(^3H)-E_2$ BINDING	B CYTOPLASMIC (^3H)R5020 BINDING $-E_2B$	C CYTOPLASMIC (^3H)R5020 BINDING $+E_2B$	D MAGNITUDE OF INDUCTION(C/B)
PARAMETRIAL	11.4	0.38	9.99	26.3
RETROPERITONEAL	7.3	0.07	0.90	12.9
OMENTAL	3.8	0.13	0.70	5.4
SUBCUTANEOUS (INGUINAL)	1.8	0.24	1.71	7.1
BROWN	4.7	0.08	0.48	6.0

Multiple Linear Regression:

1) A,B,C
   R = .9976
   $F_{2,4}$=203.96
   $\underline{p}$ <.0001

2) A,B,D
   R = .9895
   $F_{2,4}$=47.5
   $\underline{p}$ < .002

*From Wade and Gray, 1978

All binding data (A, B, and C) are presented as fmol radioactive steroid specifically bound/mg protein.

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Figure 8. Time course of induction of parametrial cytoplasmic binding sites for (^{3}H)R5020 following E_2B administration to OVX-ADX rats.

ADIPOSE TISSUE CYTOPLASMIC R5020
BINDING AFTER A SINGLE INJECTION OF EB
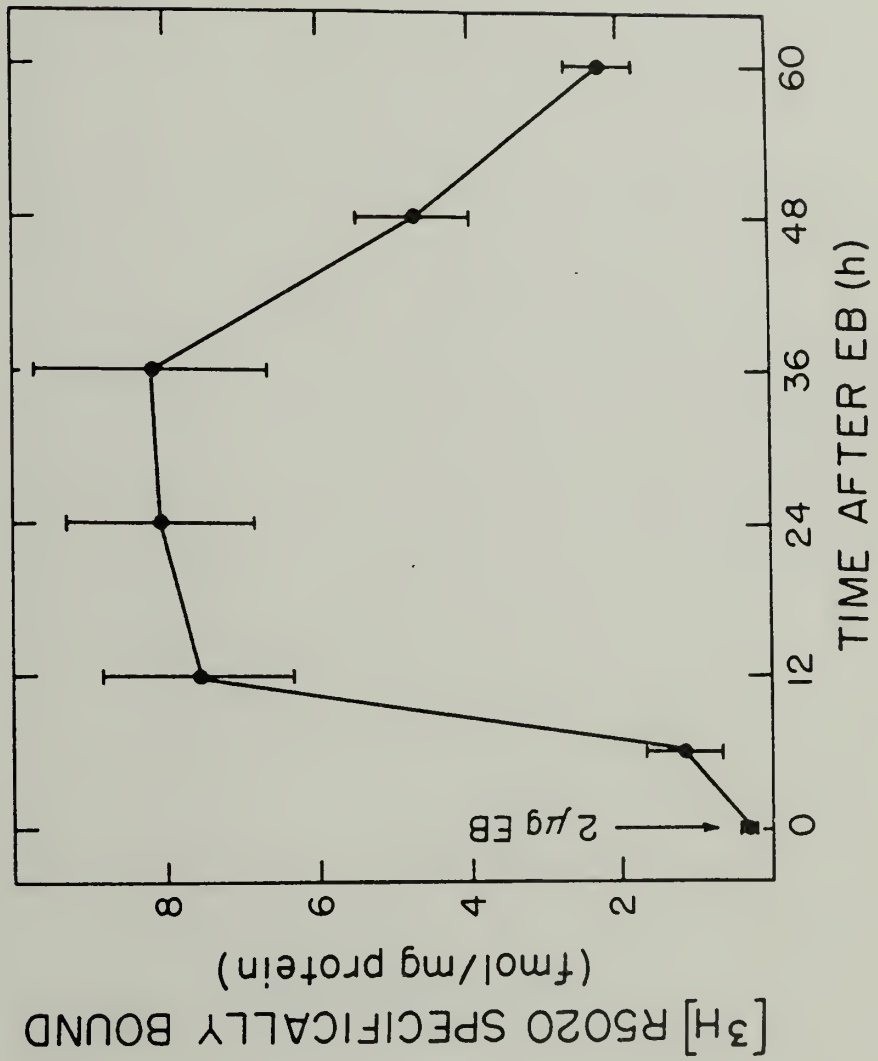
was observed between 12 and 36 hours, and binding diminished to about 25% of maximal levels of 60 hours (Figure 8).

Experiment IX. In Vivo Depletion of Cytoplasmic Progestin Binding in Adipose Tissues

Methods. To determine the relative potencies of various progestins in depleting available cytoplasmic progestin receptors in vivo, cytoplasmic (^{3}H)R5020 binding was determined one hour following injection of vehicle (.1 cc EtOH), 500 ug progesterone, 500 ug unlabelled R5020, or 500 ug 5α-dihydroprogesterone.

Results. Following treatment with three progestins, a significant treatment effect was found in the effect on the depletion of cytoplasmic progestin binding inparametrial adipose tissues ($F_{3,9}$=12.92; p <.01). The order of efficacy of depletion was R5020 > progesterone > 5α-dihydroprogesterone (Figure 9).

Experiment X. Effects of Hydrolytic Enzymes on (^{3}H)R5020 Binding in Cytoplasm of Parametrial Adipose Tissues

Methods. The effects of various hydrolytic enzymes were determined by measuring the binding of (^{3}H)R5020 in pooled high speed supernatant from parametrial fat pads of E_2B-primed animals incubated with RNase (0.5 mg/ml; bovine pancreas; Worthington), DNase (0.5 mg/ml; bovine pancreas/Worthington), pronase (0.5 mg/ml; Sigma), or no enzyme. All conditions were run in duplicate.

Figure 9. *In vivo* depletion of parametrial (^{3}H)R5020 binding sites following administration of different progestins to OVX-ADX rats.

IN VIVO DEPLETION OF ADIPOSE TISSUE R5020 BINDING SITES
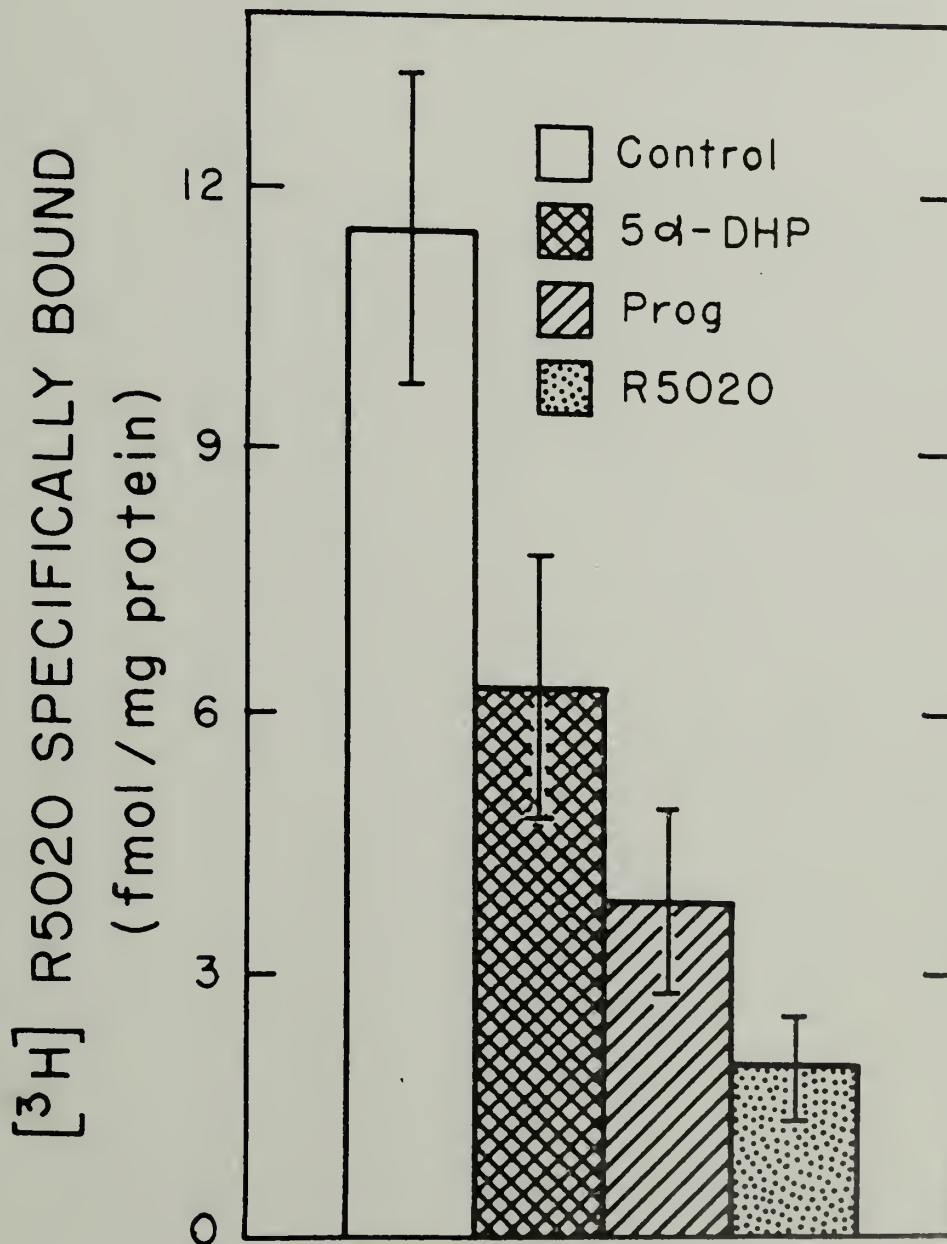
Results. Incubation of cytoplasmic fractions from parametrial adipose tissues with either DNase or RNase had no effect on (^{3}H)R5020 binding. Pronase, on the other hand, caused a 95% inhibition of specific progestin binding (Figure 10).

Discussion

The (^{3}H)R5020 binding to high speed supernatant from adipose tissues of OVX-ADX rats is comparable to progestin binding previously reported in other tissues (Blaustein and Feder, 1979; Leavitt et al., 1977; MacLusky and McEwen, 1978; Raynaud, 1977). The binding is of high affinity, with a calculated K_d of 8.5 x 10^{-10}M. Specificity was demonstrated, with the progestins R5020, progesterone and 5α-dehydroprogesterone being more effective competitors for binding sites than were an estrogen, an androgen or a corticosteroid. The nearly total inhibition of binding in the presence of pronase and the lack of effect of either RNase or DNase on progestin binding indicate that the binding macromolecule is probably a protein.

Only very low levels of (^{3}H)R5020 binding were found in adipose tissues from animals which had not been previously treated with E_2B. Following estrogen priming, a significant induction of progestin binding sites was observed in all adipose tissues studied. The effects of the progestins, progesterone and R5020, on food intake, body weight and adiposity are not observed in animals lacking estrogens, although they are seen in gonadally-intact and estrogen-primed, OVX rats (Chapter II; Wade, 1976). This lack of a progestin effect in the absence of estrogens could therefore be due to the absence of

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Figure 10. Effect of hydrolytic enzymes
on parametrial cytoplasmic (^{3}H)R5020 binding
in E_2B-primed, OVX-ADX rats.

EFFECT OF ENZYMES ON
ADIPOSE TISSUE R5020 BINDING
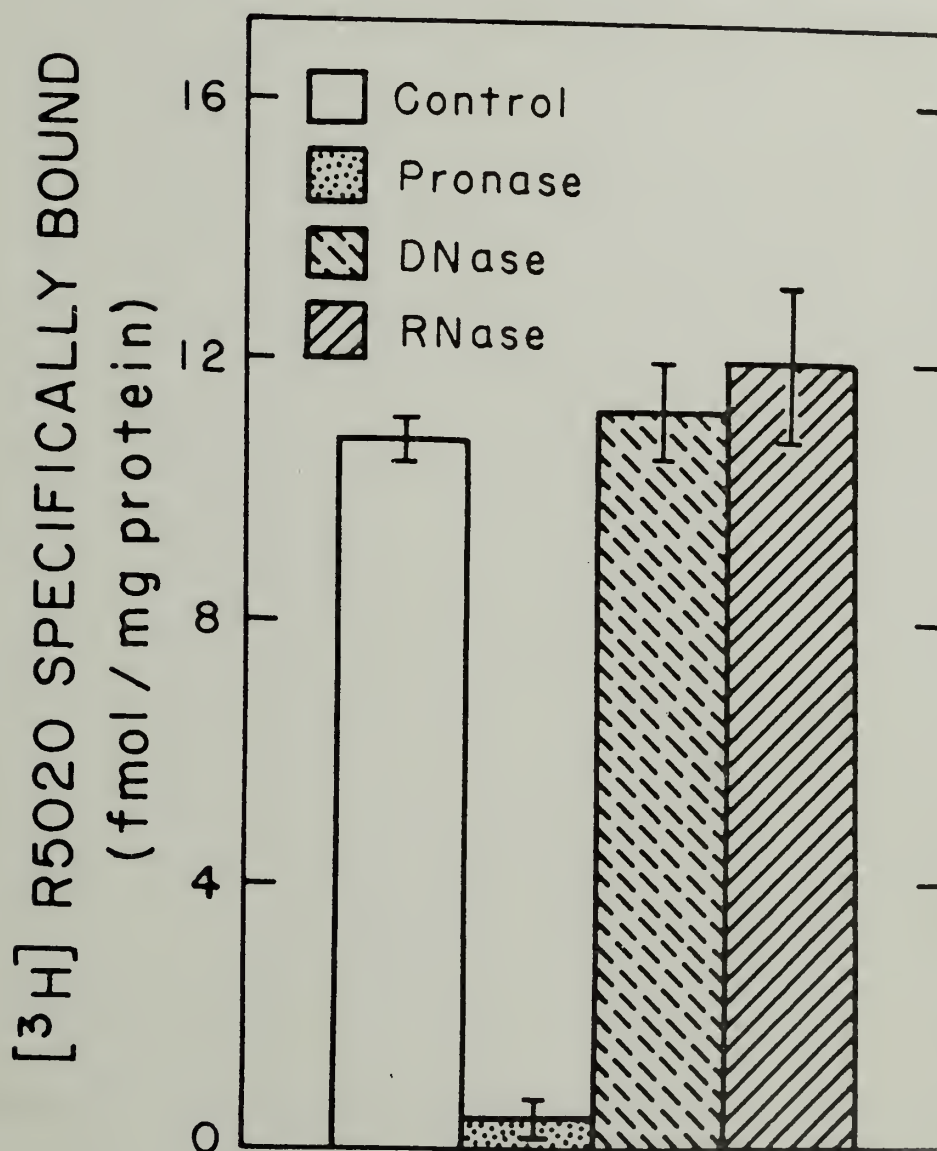
progestin receptors in target tissues. Estrogens have been demon-
strated to induce progestin receptor formation in other progestin
sensitive tissues, such as hypothalamus, pituitary, uterus and vagina
(Blaustein and Feder, 1979; Leavitt et al., 1977; Leavitt et al.,
1978; MacLusky and McEwen, 1978; Raynaud, 1977).

Regional differences have been found for absolute levels of
estrogen (Wade and Gray, 1978), basal (unprimed) and induced (E_2B-
primed) progestin receptor, as well as in the magnitude of induction
of progestin receptors (induced binding/basal binding) in adipose
tissues. Regression analyses of these data indicate that the magni-
tude of the induction of progestin receptors is directly related to
the tissue estrogen receptor concentration and basal levels of
progestin receptor. This highly significant correlation between
estrogen receptor levels and a physiological response in adipose
tissue supports the contention (Wade and Gray, 1978, 1979) that adipose
tissues are bona fide target organs for estrogens.

In vivo depletion of available cytoplasmic progestin receptors
by R5020, progesterone and 5α-dihydroprogesterone was directly
related to their effectiveness in competing for cytoplasmic progestin
binding sites in vitro (R5020 > progesterone > 5α-dihydroprogesterone).
Efficacy in depleting cytoplasmic binding sites is also directly
related to the in vivo effects on body weight. R5020 is more effective
than progesterone (Chapter II), and progesterone is more effective
than 5α-dihydroprogesterone (Wade, 1975) in increasing food intake
and body weight gain in E_2B primed rats.

C H A P T E R V

THE EFFECTS OF PROGESTINS ON ADIPOSE TISSUE LPL ACTIVITY IN VARIOUS FAT DEPOTS IN OVX, E_2B-TREATED RATS

In the absence of estrogens, neither progesterone nor R5020 has any effect on food intake, body weight or body composition (Experiment I; Hervey and Hervey, 1966; 1967). However these two progestins effectively increase food intake and body weight gain in E_2B-treated, OVX rats (Experiments II and III; Hervey and Hervey, 1967; Roberts, Kenney and Mook, 1972). Carcass analysis revealed that the major body constituent which was affected by progestin treatment was body fat (Experiment II; Galletti and Klopper, 1964; Leshner and Collier, 1973).

Lipoprotein lipase (LPL) is the enzyme involved in triglyceride transport into adipose tissues. Previous studies have shown that estrogen treatment decreases, and concurrent progesterone treatment increases, adipose tissue LPL activity (Hamosh and Hamosh, 1975; Kim and Kalkoff, 1975; 1978; Steingrimsdottir, Brasel and Greenwood, 1980). Steingrimsdottir and colleagues (Steingrimsdottir, Brasel and Greenwood, 1980) have also shown that progesterone exerts its greatest effect on LPL activity in parametrial depots, followed by retroperitoneal and omental tissues. In all of the above studies, hormones were administered to gonadally intact rats.

In the previous chapter, the presence of progestin binding sites in several adipose depots of OVX, E_2B-primed rats was demonstrated. R5020 was shown to be a more potent competitor (i.e. competed for binding at lower concentrations) for the progestin binding sites in fat tissues.

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The following experiment was designed to study the effects of the two progestins, progesterone and R5020, on adipose tissue LPL activity in three different fat depots from OVX, E_2B-treated rats. The study addresses three points: a) Do progestins affect adipose tissue LPL activity in OVX, E_2B-primed rats (as opposed to gonadally intact animals), b) does R5020 affect the activity of the enzyme in the same manner as does progesterone, and at a dose which is significantly less than the effective dose of progesterone, and c) are there regional differences in changes in LPL activity following progestin administration, and if so, do these differences reflect regional differences in cytoplasmic progestin receptors?

Experiment XI. Effect of Progesterone or R5020 on LPL Activity of Parametrial, Retroperitoneal and Inguinal Adipose Depots of OVX, E_2B-primed Rats

Methods. Four days after ovariectomy, 15 female rats were divided into 3 equal groups and began receiving injections of either E_2B (2 ug), E_2B plus progesterone (5 mg) or E_2B plus R5020 (200 ug). Daily injections continued for 3 days. On the day after the last injection, animals were sacrificed by decapitation and inguinal, parametrial and retroperitoneal adipose depots were dissected and chilled on ice.

Lipoprotein lipase activity was assayed according to a modification (Hietanen and Greenwood, 1977) of the method of Schotz et al., (1970). Tissues were homogenized at a concentration of 200 mg per ml medium containing 0.25M sucrose and 1mM EDTA, pH 7.4. Post-mitochondrial supernatants were incubated with a substrate emulsion

containing (carboxyl-^{14}C) triolein (S.A. 84.1 mCi/mmol; New England Nuclear), radioinert triolein, lysolecithin, fasted human serum, fatty acid poor bovine serum albumin, and Tris-HCl buffer, pH 7.4. Nonspecific hydrolase activity was assayed by adding NaCl to the incubation tubes to inhibit LPL activity. The reaction was stopped by addition of a chloroform:methanol:heptane (2.3:2.5:1.8;v/v/v) mixture. Phase separation was facilitated by addition of carbonate buffer, pH 10.5. Samples were centrifuged and aliquots of the upper phase containing free fatty acids (FFA) were pipetted into scintillation vials; toluene based scintillation fluid containing 33% Triton X-114 was added; and samples were counted at an efficiency of 85%.

Protein concentration were analyzed in aliquots of the post-mitchondrial supernatants by the method of Lowry et al. (1951) and data are presented as umol FFA released/hr/mg protein.

Results. Three days of administration of either progesterone (5 mg) or R5020 (200 ug) elevated adipose tissue activity in inguinal, para-metrial, and retroperitoneal fat depots of E_2B-primed, OVX rats (Table 8). However, the only statistically significant difference was found in the parametrial adipose depots of animals treated with R5020 (p=.05; E_2B vx. E_2B plus R5020; t-test).

TABLE 8

THE EFFECT OF PROGESTERONE OR R5020 ON LPL ACTIVITY IN INGUINAL, PARAMETRIAL, OR RETROPERITONEAL FAT DEPOTS OF OVX, E_2B-TREATED RATS

LPL Activity (umol FFA released/hr/mg protein)

HORMONE TREATMENT	FAT DEPOT		
	INGUINAL	PARAMETRIAL	RETROPERITONEAL
E_2B	.038 $\pm$.009	.080 $\pm$.013	.065 $\pm$.011
E_2B + PROGESTERONE	.073 $\pm$.026	.137 $\pm$.036	.164 $\pm$.044
E_2B + R5020	.061 $\pm$.011	.140* $\pm$.020	.105 $\pm$.045

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*p = .05, vx. E_2B, two-failed t-test

All data are presented as mean $\pm$ S.E.M.

Discussion

Statistical significance was only found in parametrial pads of R5020-treated animals, although group means were elevated for all adipose depots from animals treated with either R5020 or progesterone, as compared with depots from animals treated only with E_2B (Table 8). Other experiments (e.g. Gray and Wade, 1980; Steingrimsdottir, Brasel and Greenwood, 1980; see also Experiment 14) have demonstrated significant elevations in parametrial adipose tissue LPL activity following progesterone administration to either intact rats or to OVX animals treated with E_2B. The exact reasons for the inability to replicate these results in this experiment are unclear; further investigation of relevant experimental factors are required.

Following progestin administration, Steingrimsdottir, Brasel and Greenwood (1980) have shown that, in gonadally intact animals, progesterone treatment affects parametrial, but not retroperitoneal or omental, tissue LPL activity. No test was made of inguinal tissue LPL activity, although inguinal pads had the second highest amount of progestin binding sites in adipose tissues (Experiment VII). That the largest effect on enzyme activity is found in parametrial tissue (above and Steingrimsdottir, Brasel and Greenwood, 1980) indicates that there is a parallel between the amount of progestin binding sites and physiological responsivity to progestin administration. Such a parallel between adipose tissue hormone receptor levels and cellular response level was shown, in Experiment VII for estrogen-induced synthesis of progestin receptors and estrogen receptor levels.

PROGESTINS AND ANTIESTROGENS: THEIR EFFECTS ON ADIPOSE TISSUE (^{3}H)R5020 BINDING AND LPL ACTIVITY

The results of Chapter III indicate that the so-called anti-estrogen, nafoxidine, is estrogen-like in its suppression of food intake and body weight gain. Similar results have been reported previously for both nafoxidine (Wade and Blaustein, 1978) and for another antiestrogen, MER-25 (Roy and Wade, 1976). Concurrent progesterone treatment to animals treated with either MER-25 (Roy and Wade, 1977) or nafoxidine (Chapter III) increased food intake and body weight in a manner similar to that seen following progesterone administration to estrogen-treated rats (Chapter III).

Carcass analysis revealed that, as with E_2B-treated animals, progesterone administration to nafoxidine-treated rats resulted in an increase in total carcass fat content (Chapter III). These data indicate that in animals which have been administered nafoxidine, progesterone might act directly on adipose tissues to alter metabolic processes.

Recent studies have demonstrated that synthetic antiestrogens, including nafoxidine, can induce the synthesis of uterine and brain cytoplasmic progestin receptors, although much less effectively (approximately 50% induction) than is found with the true estrogens (e.g. estradiol, E_2B, estriol) (Jordan and Prestwich, 1978; Koseki et al. 1977; Leavitt et al., 1977; Roy, MacLusky and McEwen, 1979). In the previous series of experiments (Chapter IV), the presence of

high affinity, E_2B-induced cytoplasmic progestin binding in adipose tissues was demonstrated. The following series of experiments was designed to examine the effects of various synthetic antiestrogens, especially nafoxidine, on the depletion of estrogen receptors and the induction of progestin binding sites in the cytoplasm of parametrial adipose tissues (Experiment XII).

In addition, I studied the effects on adipose tissue LPL activity of antiestrogen treatment, with or without concurrent administration of progesterone. I was interested to see whether or not antiestrogens which mimic estradiol's anorexic actions (at least with regard to food intake and body weight gains) had similar suppressant effects on adipose tissue LPL activity (Experiment XIII). When decreases in LPL activity were found following nafoxidine treatment, it was of interest to see whether or not progesterone could reverse this effect in a tissue with lowered amounts of progestin receptors (Experiment XIV).

General Methods

Animals. Female rats were housed and maintained as described in previous chapters. Rats used in studies in which progestin binding was assayed (Experiments XII & XIII) were both ovariectomized and adrenalectomized; when only LPL activity was assayed (Experiment XIV), OVX rats were used.

Assays. Cytoplasmic progestin binding and LPL activity were determined in parametrial adipose tissues as described in earlier chapters.

In Experiment XII, cytoplasmic estrogen binding was determined in the same fraction as was used for the progestin binding assay. To determine estrogen binding, high speed supernatant was incubated at 24 C with 1 nM 17β-(^{3}H)estradiol (S.A. 115 Ci/mmol; New England Nuclear) plus or minus 1×10^{-7}M radioinert estradiol. Bound and free (^{3}H)estradiol were separated by gel filtration as described on page 3 of Chapter IV, except that only 700 ul TEMG was used to elute the protein fraction. Counting of radioactivity and analysis of the protein content were determined in the same manner as was described for the progestin binding assay (Chapter IV).

Experiment XII. Antiestrogen Administration: Depletion of Estrogen Binding and Induction of Progestin Binding in the Cytoplasm of Parametrial Adipose Tissue

Methods. The depletion of cytoplasmic estrogen receptors and the concurrent induction of cytoplasmic progestin receptors in parametrial adipose depots were measured after a single injection of various estrogens or antiestrogens. Animals were sacrificed 24 h after injection of E_2B (2 ug in sesame oil; n=6), estrone benzoate (E_1B; 2 ug in sesame oil; n=3), CI-628 (50 ug in saline; n=7), nafoxidine (50 ug in saline, n=7) or MER-25 (500 ug in saline; n=7), or no injection.

Results. Cytoplasmic (^{3}H)R5020 and 17β(^{3}H)estradiol binding were measured in parametrial fat depots after injection of various estrogens and antiestrogens. E_2B (2 ug), CI-628 (50 ug), and nafoxidine (50 ug) were effective in significantly depleting free cytoplasmic estrogen receptors, while E_1B (2 ug) and MER-25 (500 ug) were without effect (Figure 11). Significant induction of progestin receptors compared with control values were found following the administration of E_2B, CI-628, and nafoxidine (each $\underline{p}$ < .01 by $\underline{t}$ test). Again, E_1B and MER-25 were without effect.

Experiment XIII. Nafoxidine Administration: Induction of Adipose Tissue Progestin Binding and Effect on Adipose Tissue LPL Activity

Methods. Starting 4 days after OVX, rats received 3 daily injections of either oil (n=9), E_2B (2 ug/day; n=8), nafoxidine (2 mg/day; n=7), or a combination of E_2B and nafoxidine (n=8). Approximately 24 h after the last injection, animals were sacrificed by decapitation and parametrial adipose depots were rapidly dissected. The left parametrial pad was assayed for LPL activity and the right pad was assayed for cytoplasmic progestin ((^{3}H)R5020) binding.

Results. Both E_2B and nafoxidine, alone and in combination, significantly induced progestin binding sites in the cytoplasm of a parametrial fat tissue (Figure 12). At the doses used in this study, nafoxidine was only half as effective as was E_2B (16.4 vs. 30.4 fmol (^{3}H)R5020 specifically bound per mg protein, respectively) in the induction of progestin receptors. Nafoxidine also significantly

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Figure 11. Effect of administration of
various estrogens or antiestrogens of depletion
of cytoplasmic (^{3}H)-estradiol binding sites and
induction of (^{3}H)R5020 binding sites in parametrial
adipose depots of OVX-ADX rats.

INDUCTION OF ADIPOSE TISSUE R5020
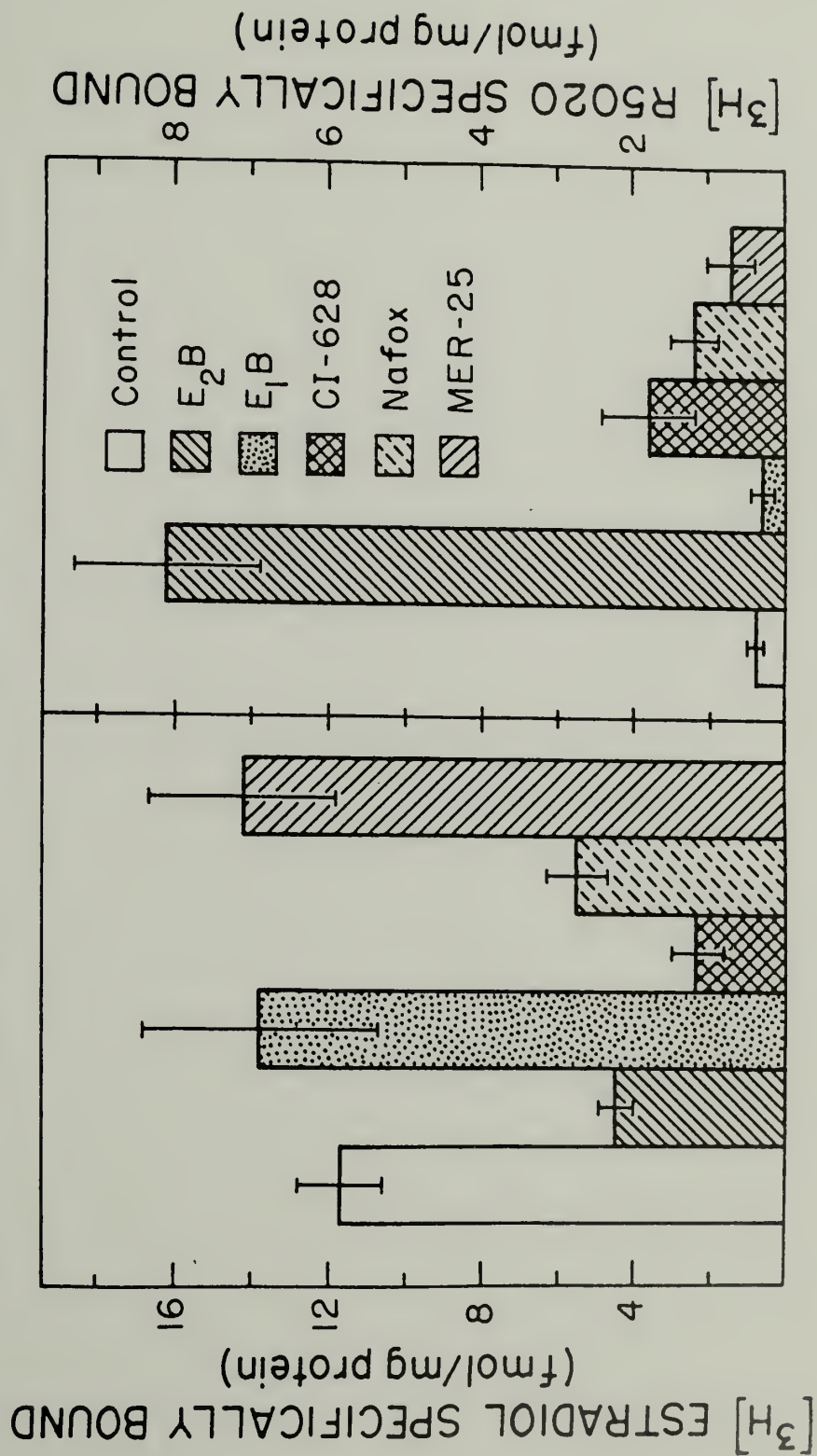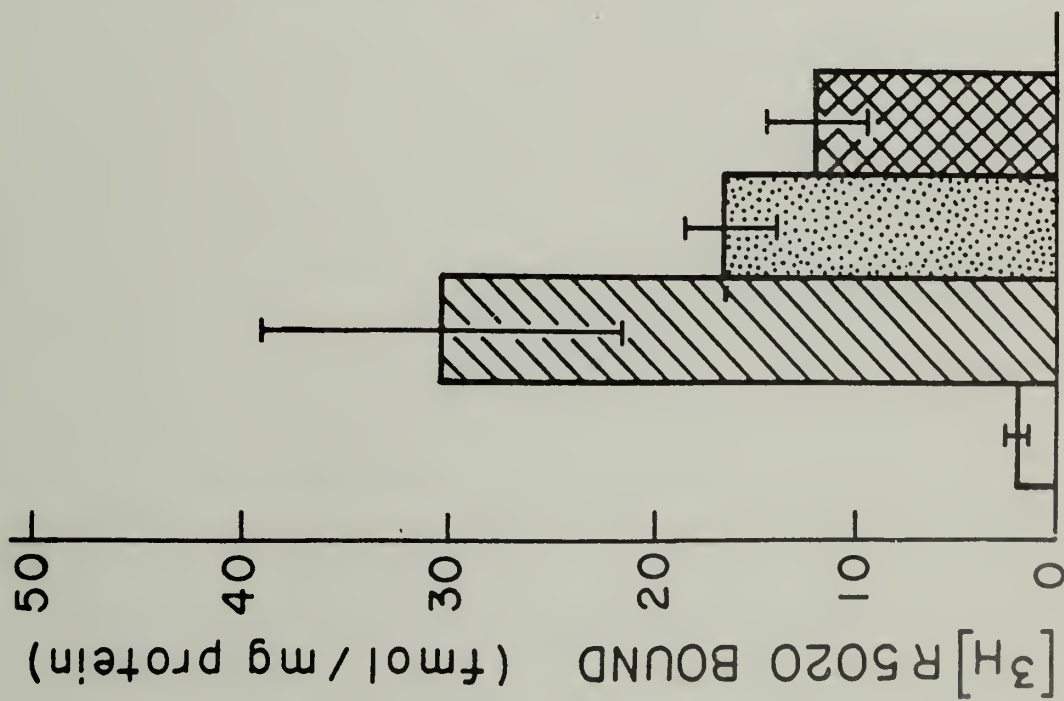BINDING BY ESTROGENS AND ANTIESTROGENS

Figure 12. Effect of nafoxidine on the
induction of parametrial cytoplasmic (^{3}H)R5020
binding sites and on parametrial adipose tissue
LPL activity.
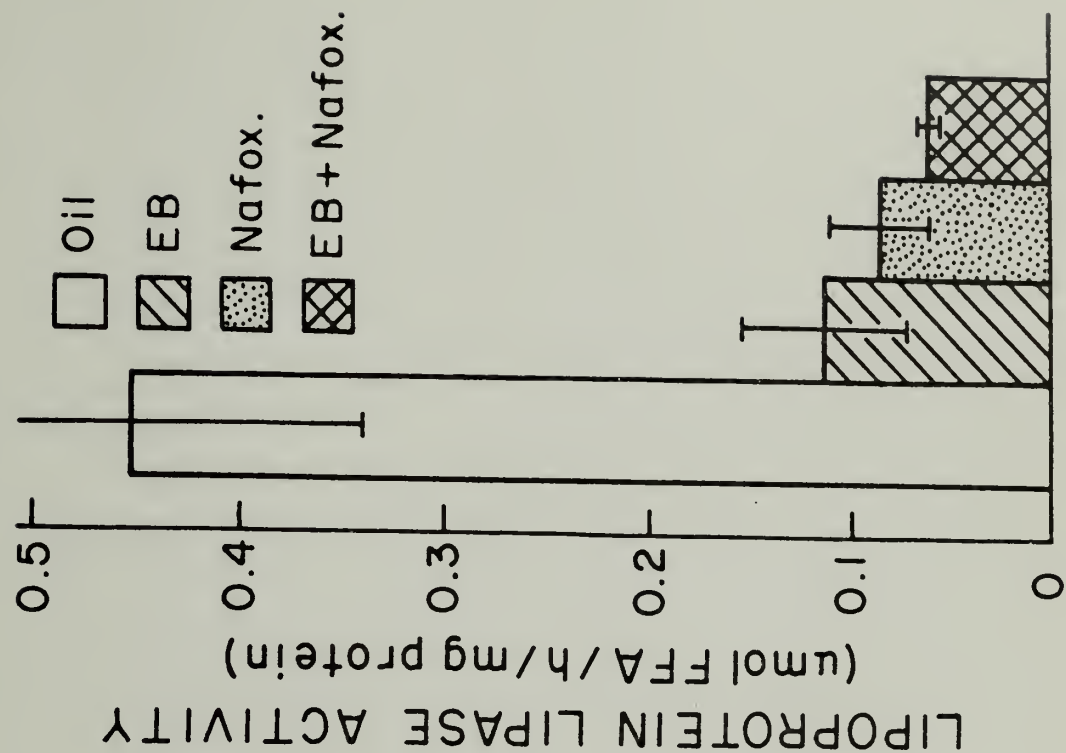
suppressed E_2B induction of progestin receptors ($\underline{p}$ < .05, E_2B vs. E_2B plus nafoxidine, $\underline{t}$ test).

Both E_2B and nafoxidine, alone and in combination, depressed adipose tissue LPL activity to 25% or less of control values (Figure 12). Nafoxidine was at least as effective as was E_2B in lowering LPL activity in this tissue. In combination with E_2B, nafoxidine further lowered, although not significantly (.10 < $\underline{p}$ < .05, E_2B vs. E_2B plus nafoxidine, $\underline{t}$ test), LPL activity in parametrial adipose tissues (Figure 12).

Experiment XIV. Concurrent Administration of Nafoxidine and Progesterone: Effect on Adipose Tissue LPL Activity

<u>Methods</u>. Beginning 4 days following surgery (OVX), animals received 3 daily injections of either oil (n=5), progesterone (5 mg/day; n=6), E_2B (2 ug/day; n=5), E_2B plus progesterone (n=6), nafoxidine (2 mg/day; n=6), nafoxidine plus progesterone (n=6), E_2B plus nafoxidine (n=6), or E_2B plus nafoxidine plus progesterone (n=6). On the day following the third injection, animals were sacrificed by decapitation; parametrial fat tissue was removed; and LPL activity was assayed.

<u>Results</u>. As was shown in the previous experiment, E_2B nafoxidine and the two compounds in combination significantly depressed adipose tissue LPL activity (Figure 13). Although progesterone had no effect on adipose tissue LPL activity when it was administered to oil-treated animals, it reversed the E_2B-induced suppression of LPL

Figure 13. Effect of concurrent admin-
istration of nafoxidine and progesterone on
parametrial adipose tissue LPL activity.

LIPOPROTEIN LIPASE ACTIVITY
(μmol FFA / h / mg protein)
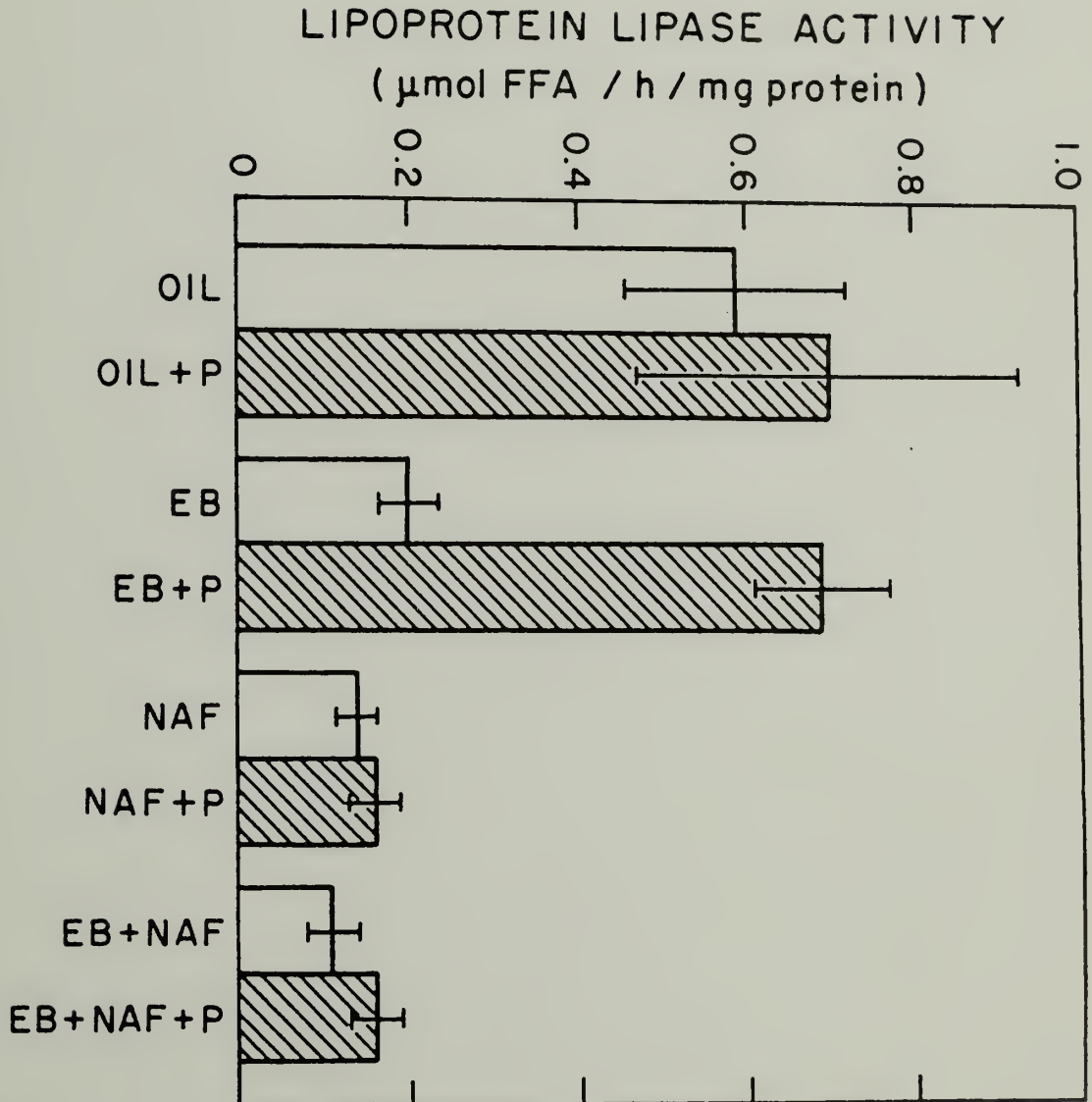
activity (Figure 13). Progesterone had no effect on LPL activity in nafoxidine-treated animals, and nafoxidine blocked the progesterone reversal of E_2B-induced suppression of LPL activity (Figure 13).

Discussion

The efficacy of various estrogens and antiestrogens in depleting cytoplasmic estrogen receptors and inducing cytoplasmic progestin binding sites was examined in parametrial adipose tissues (Experiment XII). Those compounds which are known to mimic the effects of estradiol on body weight (E_2B, CI-628, nafoxidine; see Chapter III; King and Cox, 1976) were found both to deplete available cytoplasmic estrogen receptors and induce progestin binding sites (Figure 11). Note that, while a single pulse of E_2B, CI-628, and nafoxidine all depleted estrogen receptors, E_2B was much more effective than the other compounds in inducing progestin receptors. The extent of cytoplasmic receptor depletion, therefore, is not necessarily a good indicator of the potency of an estrogen-like compound in inducing a physiological response, such as the induction of progestin receptors.

In the doses used in this study, MER-25 (500 ug) and E_1B (2 ug) were ineffective in changing either estrogen or progestin binding levels from control values (Figure 11). At these doses, E_1B and MER-25 are ineffective in causing changes in estrogen receptor distribution in other tissues (Cidlowski and Muldoon, 1976) or in altering body weight or feeding behaviors, although at higher doses both compounds do induce estrogen-like behaviors (Roy and Wade, 1976; Wade, 1975).

The effect of nafoxidine on the induction of progestin binding sites was further examined, and another physiological endpoint, i.e. changes in adipose tissue LPL activity, was also examined in nafoxidine-treated animals (Experiment XIII). Three days of E_2B (2 ug/day) treatment resulted in a higher induction of progestin binding sites than did 3 days of nafoxidine (2 mg/day) treatment, alone or in combination with E_2B. The amount of induced progestin receptors seen in adipose tissues following nafoxidine, i.e. approximately 50% of that seen in E_2B-treated animals, is similar to data previously reported for uterine progestin receptors (Leavitt et al., 1977). That is, nafoxidine induce, in both uterine and adipose tissues, approximately half the progestin receptors induced by E_2B. In the same animals in which adipose tissue progestin binding was measured, however, nafoxidine was found to be at least as effective as E_2B in suppressing adipose tissue LPL activity (Figure 12).

Concurrent progesterone treatment increased LPL activity in E_2B-treated animals, although it had no effect in animals which were treated with either nafoxidine, alone, or with nafoxidine plus E_2B (Figure 13). These data suggest that the progestin receptors which were induced following nafoxidine treatment were either qualitatively or quantitatively insufficient to promote progestin-induced responses following progesterone administration. That is,

either the receptors which were induced by nafoxidine were abnormal, or there is a threshold number of receptors needed before a physiological response can be manifested, and this amount was not reached. Which hypothesis is the more valid one remains to be elucidated.

CHAPTER VII

GENERAL DISCUSSION

The effects of two progestins (i.e. the natural compound, pro-
gesterone, and the synthetic compound, R5020) were examined with
regard to food intake, body weight and carcass composition regulation.
In all cases examined, R5020 was at least as effective in inducing
progestin-typical responses, and R5020 exerted its effects at much
lower (1/50 the dose required of progesterone) doses than progesterone.
Responses to the two compounds were qualitatively similar, although at
the doses used, R5020 sometimes induced greater effects. For the
purpose of further discussion, the actions of progesterone and R5020
will be considered to be similar and will be discussed in terms of
progestin-induced effects.

Progestin Effects on Carcass Composition and Adiposity

Ovarian hormones have multiple effects on regulatory behaviors
and metabolic processes. Estrogens decrease and, in the presence of
estrogens, progestins increase food intake, body weight gain and adi-
posity. These results were replicated in Experiments I-III (Chapter
II). It was expected that the changes in total carcass fat content
following hormone manipulation would be reflected in changes in para-
metrial adipose pad and cell size. Such changes had previously been
reported in the parametrial depots of intact animals which had been
treated with progesterone (Steingrimsdottir, Brasel and Greenwood,
1980) or estradiol benzoate (Salens, 1971). Upton (1980), using OVX

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rats, was unable to demonstrate hormone induced changes in parametrial adipose tissue cellularity. Reasons for the discrepancy in data are unclear, although two possibilities should be considered. First there were methodological differences between the studies. Upton (1980) and I both used OVX, as opposed to gonadally intact,animals. We also both used the modified photomicrography method for determining adipose cell diameters as described in Experiment III. Steingrimsdottir and colleagues, on the other hand, used an electronic counting system of osmium fixed cells, a method which is supposedly more sensitive. A second possibility is that hormone treatment altered cell size distribution, not uniformly altering cell size alone. Faust et al. (1978) have demonstrated that after adipose cells reach a certain maximum size, they stop growing and further adipose tissue expansion is manifested by the maturation and filling of smaller "pre-adipocytes". In this way, factors which increase adipose pad weight might result in increases in both very large and very small cells, thus not altering the mean cell size. The result of this sort of change would be non-significant changes in mean cell size, but large within pad cell size variances. In order to verify or disprove this possibility, it would be necessary to measure the distribution of cell diameters, not just the mean cell size within hormone treatments.

Progestin Effects on Adipose Tissue LPL Activity and Progestin Binding in Adipose Depots

Both estrogens and estrogen plus progesterone treatment induce serum hypertriglyceridemia (Experiment IV; Kim and Kalkoff, 1975; 1978). Much of the hormone induced increase in circulating triglyceride levels is the result of greatly enhanced hepatic production of hepatic triglycerides, particularly very low density lipoprotein (VLDL)-triglyceride (Chan et. al., 1977; Heimberg et al., 1978; Watkins, Fizette and Heimberg, 1972; Weinstein, Seltzer and Belitsky, 1974). Progestin administration to gonadally intact rats also results in enhanced activities of several lipogenic enzymes (Dahm, Miagawa and Jellinek, 1977; Dahm et al., 1978).

In parallel with their effects on adiposity, estrogens (e.g. E_2B) decrease and progestins increase adipose tissue LPL activity (Experiments XI and XIV; Hamosh and Hamosh, 1975; Kim and Kalkoff, 1975; 1978; Steingrimsdottir, Brasel and Greenwood, 1980). These changes in LPL activity might be regulated by apoprotein cofactors of hepatic origin (Blum and Levy, 1975; Tan, 1978). Hormone administration alters the ratio of inhibitory and activating apoproteins which are produced by the liver (Kim and Kalkoff, 1978). LPL activity might also be directly influenced by ovarian steroids at the level of the adipocyte. High affinity, hormone specific binding sites for both estrogens (Wade and Gray, 1978) and progestins (Chapter IV) were demonstrated in parametrial adipose depots. The receptors are depleted following hormone administration, presumably reflecting translocation of steroid-receptor complexes into the nucleus. We

have demonstrated, with (^{3}H)-estradiol, that following an injection of radioactively labelled steroid, there is nuclear accumulation of radioactivity. These data suggest that adipose tissues are true target organs for steroid hormones. If so, one would expect that steroids would have direct effects on the activities of adipose tissue enzymes, perhaps including LPL.

Steingrimsdottir, Brasel and Greenwood (1980) have shown that progesterone has no effect on the rate of adipose tissue lipolysis (glycerol release). Of the three parameters which have been studied, therefore, i.e. adipose tissue lipolysis, adipose tissue LPL activity and serum triglyceride levels, the direction of clearance of triglycerides into adipose tissues most closely reflects the behavioral and gross metabolic changes which are observed following hormone administration.

The greatest change in LPL activity following progestin treatment appears to be in the parametrial fat depot, the pad in which there is the highest induction of progestin-specific, high affinity binding sites (Chapter IV). Hormone-induced changes in LPL activity are found prior to changes in food intake after E_2B (Ramirez, unpublished data) and progesterone (Steingrimsdottir, Brasel and Greenwood, 1980). These data indicate that changes in adipose tissue LPL activity might be of primary importance in initiating the chain of metabolic and behavioral events which follow hormone manipulation.

Antiestrogen-Progesterone Interactions

However, changes in adipose tissue LPL activity appear not to be necessary for the changes in food intake, body weight gain and carcass lipid content following progesterone treatment. Although the "antiestrogen", nafoxidine, mimics the effects of estradiol and lowers adipose tissue LPL activity (Experiments XIII, XIV), concurrent progesterone treatment did not reverse LPL activity, as is normally seen with progesterone when it is given concurrently with estrogens. However, progesterone still manifested its full lipogenic effects when it was injected into nafoxidine-treated rats (Experiment IV), despite the fact that it did not alter parametrial adipose tissue LPL activity (Experiment XIV). While it is possible that progesterone exerted its effects on LPL activity in other fat depots of nafoxidine-treated animals, this is highly unlikely. Parametrial pads have the highest capacity for induction of progestin binding sites (Experiment VII), and regional studies in estrogen treated animals indicate that the parametrial depot LPL activity is far more responsive to progestin treatment than are other depots (Experiment XI; Steingrimsdottir, Brasel and Greenwood, 1980).

Nafoxidine also prevented the normally seen rise in serus triglyceride levels. Progesterone treatment did not increase triglyceride levels over nafoxidine-treated rats' values as it did after E_2B treatmeant (Experiment IV). Yet progesterone was completely effective in increasing body weight and carcass lipid content in nafoxidine-treated rats.

To date, no one has studied the effects of ovarian hormones or antiestrogens on adipose tissue lipogenesis. Adipose tissue is a major site of fatty acid synthesis and esterification into trigly-cerides (Newsholme and Start, 1973). Presumably if progesterone is altering the amount of lipid in the adipose depots without changing the amount of lipid which is entering the pad (as is found under the nafoxidine plus progesterone condition) as demonstrated by a lack of an effect on tissue LPL activity, then the hormone must be exerting some effect on de novo fatty acid/triglyceride synthesis within the pad. Further research is needed to test this hypothesis.

FOOTNOTES

1. Trivial names used in this report, and their proper chemical names, are: R5020, 17α, 21-dimethyl-19-norpregna-4,9-diene-3,20-dione; nafoxidine, 1-(1(p-3,4-dihydro-6-methoxy-2 phenylnaphth-1-61)-phenoxy)ethyl-pyrrolidine; MER-25,1-yl (p-2-diethylamino-ethoxyphenyl)-1-phenyl-2-methoxy-phenylethanol; CI-628, α-(4-pyrrolidinethoxy)phenyl-4-methoxy-α-nitrostilbene.

2. Serum triglyceride and glycerol levels were generously assayed by Dr. Israel Ramirez.

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