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REPRODUCTIVE DYSFUNCTION IN DIABETES:
BEHAVIORAL, BIOCHEMICAL AND METABOLIC
ANALYSES IN FEMALE RATS

A Dissertation Presented

By

SUSAN D. DUDLEY

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 1980

Psychology

REPRODUCTIVE DYSFUNCTION IN DIABETES:
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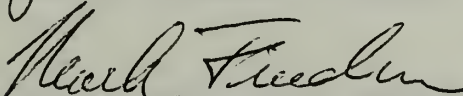
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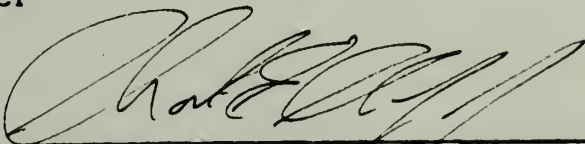
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ABSTRACT

Reproductive Dysfunction in Diabetes: Behavioral,
Biochemical and Metabolic Analyses in Female Rats
(September, 1980)

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Withdrawal of exogenous insulin for 24 hours in ovariectomized streptozotocin-diabetic rats (100 mg/kg) significantly impairs estradiol uptake in whole homogenate fractions of hypothalamus-preoptic area and pituitary gland. Significant reductions in cell nuclear fractions from the same tissues are seen after 36 hours of insulin deprivation. Subsequent reinstatement of insulin treatment does not yield full recovery of estradiol uptake after 24 hours of insulin replacement. Fat content of the diet has no effect on brain or pituitary estradiol uptake in diabetic animals deprived of insulin for 36 hours. Circulating levels of triglycerides, ketones, glucose, glycerol and free fatty acids were found to predict uptake levels to a significant extent, but no single metabolite is reliably predictive across tissues.

Chronic insulin deficiency, produced by administration of 60 mg/kg streptozotocin, leads to reduced estrous behavior and lower estradiol uptake in both whole homogenate

and nuclear fractions of hypothalamus-preoptic area and pituitary gland in ovariectomized rats. High dietary fat intake produces an ameliorative effect on both lordosis behavior and estradiol uptake, relative to animals fed a standard, carbohydrate-rich diet. Plasma glucose levels are significantly correlated with estradiol uptake levels in brain and pituitary, and with plasma radioactivity levels.

These data demonstrate that insulin dependent changes in brain and pituitary uptake of estradiol in rats are slow to develop, and they support the hypothesis that at least some of the reproductive dysfunctions observed in diabetic rats may be the result of alterations in nuclear binding of estradiol by central nervous tissue. Chronic fuel deprivation may represent an important correlate of the disruptions in normal steroid action among diabetics, and rats that are chronically deprived of insulin appear to provide a very useful metabolic model for the study of reproductive dysfunction in diabetics.

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C H A P T E R I

DEFICITS IN PITUITARY AND BRAIN CELL NUCLEAR RETENTION OF [³H]ESTRADIOL IN DIABETIC RATS DEPRIVED OF INSULIN: TIME COURSE AND METABOLIC CORRELATES

Impairment of reproductive function and performance is associated with diabetes mellitus in both humans and animals (Chieri, Pivetta & Foglia, 1969; Davis, Fugo & Lawrence, 1947; Foglia, Chiere & Peralto-Ramos, 1970; Lawrence & Contopoulos, 1960; Levi & Weinberg, 1949; Williams & Porte, 1974). In rats, this impairment includes disruption of normal estrous cycles (Davis et al., 1947; Foglia et al., 1970; Lawrence & Contopoulos, 1960; Levi & Weinberg, 1949), delayed sexual maturity (Lawrence & Contopoulos, 1960), decreased numbers of ova shed when ovulation does occur (Chiere et al., 1969), high rates of fetal resorption (Davis et al., 1947; Lawrence & Contopoulos, 1960), diminished lactational performance (Lawrence & Contopoulos, 1960) and reductions in ovarian and uterine weights (Farina, Chiere, Basabe & Foglia, 1971; Foglia et al., 1970; Lawrence & Contopoulos, 1960). The ovaries of alloxan-diabetic rats display a diminished responsiveness to exogenously administered gonadotropins, but endogenous gonadotropin levels seem to be normal (Farina et al., 1971; Howland & Zebrowski, 1974; Liu, Lin & Johnson, 1972). These deficits can be largely corrected by insulin treatment.

Work by Denari and Rosner (1972) has demonstrated that acute (24 hour) insulin withdrawal in alloxan-diabetic rats reduced [³H]estradiol uptake in anterior hypothalamus and pituitary, but not in uterus, vagina or skeletal muscle. These findings have been extended by Gentry, Wade and Blaustein (1977) who demonstrated deficits in cell nuclear uptake of [³H]estradiol in streptozotocin-diabetic female rats in hypothalamus-preoptic area and pituitary after a similar period of acute insulin withdrawal. Estrous behavior is also diminished in diabetic ovariectomized rats after 18-24 hour omission of insulin replacement during estradiol benzoate and progesterone treatment (Gentry et al., 1977; Siegel & Wade, 1979). These data suggest that decreased binding of estradiol by brain areas involved in the mediation of reproductive processes may contribute to these dysfunctions.

The goals of the present series of experiments (Chapter I) are twofold. These studies were undertaken to examine the relationship between insulin and estradiol in the central nervous system and to attempt to elucidate the mechanisms involved in the reproductive impairments of diabetic female rats.

Experiment 1: Acute Insulin Withdrawal and Estradiol Uptake: Time-Course Studies

This study was designed to examine the time-course

over which brain and pituitary estradiol uptake deficits become manifest in the absence of insulin.

Procedure. Female CD-strain rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 200-300 g were individually housed and given ad libitum access to Purina laboratory chow and tap water. A 12h:12h light-dark cycle was maintained throughout the experiment, with lights off from noon to midnight. All rats were bilaterally ovariectomized via a single midventral incision under methoxyflurane anesthesia (Metofane; Pitman-Moore, Washington Crossing, NJ).

Diabetes mellitus was induced in the ovariectomized rats by intraperitoneal injections of 100 mg/kg streptozotocin (Upjohn, Kalamazoo, MI) in citrate buffer, pH 4.8. Diabetes was confirmed by positive glycosuria (++++, TesTape, Lilly). Diabetic animals were maintained on daily injections of 3 U of U-100 Lente insulin (Lilly) administered just prior to lights out. This treatment is sufficient to restore prediabetic body weights and to prevent glycosuria. Insulin withdrawal in such animals will result in rapid weight loss and death within 3-5 days.

Approximately 72-48 hours before sacrifice, all animals were shifted to injections of 0.75 U of U-40 Regular insulin, administered every 6 hours. In the first experiment, rats received either uninterrupted insulin (control

condition) or had insulin injections omitted during the period either 12, 24, or 36 hours before [³H]estradiol (experimental condition).

All animals were sacrificed 2 hours after an intravenous injection of 40 μ Ci (0.11 μ g) of [2,4,6,7-³H]estradiol-17 β (specific activity = 98.5 Ci/mmmole; New England Nuclear, Boston, MA) in 0.1 ml 20% ethanol-saline. Rats were anesthetized with sodium pentobarbital. A blood sample was obtained by cardiac puncture in a heparinized syringe and centrifuged. The rats were perfused with cold 0.15 M cold NaCl and brains and whole pituitary glands were removed. All steps were performed at 4°C. The hypothalamus-preoptic area was dissected as previously described (Blaustein & Wade, 1977; Roy & Wade, 1977). The hypothalamic dissection was bounded by the mammillary bodies, hypothalamic fissures, and caudal edge of the optic chiasma, extending dorsally 3 mm. It included the anterior hypothalamus, preoptic area, and bed nucleus of the stria terminalis. Tissues were homogenized in Teflon-glass coaxial homogenizers, and a portion of the whole homogenate was taken. A cell nuclear fraction was isolated by the method of Zigmond and McEwen (1970) with some modifications (Blaustein & Wade, 1977; Roy & Wade, 1977), using the following solutions: 1) Nuclear Isolation I (NI): 0.32 M sucrose, 1 mM potassium phosphate, 3 mM magnesium chloride, 0.25% Triton X-100 (v/v), pH 6.5; 2) Nuclear

Isolation II (NII): 0.32 M sucrose, 1 mM potassium phosphate, 3 mM magnesium chloride, pH 6.8; 3) Nuclear Isolation III (NIII): 2.39 M sucrose, 1 mM potassium phosphate, 3 mM magnesium chloride, pH 7.0. Tissues were homogenized with 20 slow up- and down-strokes in 2 ml NI. Two hundred μ l of whole homogenate was taken and the remainder was centrifuged with 1 ml NII at 1100 x gravity for 5 minutes. The supernatant was discarded, the pellet was resuspended in 1.5 ml NII, and the mixture was centrifuged at 1100 x gravity for 5 minutes. The supernatant was discarded and the pellet was resuspended in 200 μ l NII. One ml NIII was added and mixed thoroughly. This mixture was centrifuged for 10 minutes at 25000 x gravity to obtain a nuclear pellet. The supernatant was discarded, and the purified nuclear pellet was removed from the centrifuge tubes with 3 x 500 μ l 0.01 M citric acid. Radioactivity was extracted from whole homogenate and cell nuclear fractions with 3 x 4 ml of a toluene-based scintillation fluid (5.0 g 2,5-diphenyloxazole and 0.05 g 1,4-bis-2(5-phenyloxazolyl)-benzene/liter scintillation-grade toluene). A 100 μ l portion of blood plasma was pipetted into a scintillation vial containing 12 ml toluene scintillation fluid and shaken vigorously. Radioactivity was measured in a Packard model 2425 Tri-Carb liquid scintillation spectrometer at an efficiency of $55 \pm 1\%$. Counts were corrected for quenching by automatic external standardization. Protein in the

samples was precipitated with ethanol and estimated by the method of Lowry, Rosebrough, Farr and Randall (1951).

Tissue radioactivity concentrations are expressed as disintegrations per minute (DPM/mg protein) corrected for differential plasma radioactivity levels by expression as tissue: plasma ratios or DPM/mg protein: DPM/ μ l plasma (McEwen & Pfaff, 1970).

Results. Although the insulin treated rats appeared to be quite healthy, some of the insulin deprived animals were noticeably lipemic, with milky-appearing blood plasma. This lipemia was invariably associated with elevated plasma radioactivity.

The effects of insulin deprivation on brain and pituitary [3 H]estradiol uptake are summarized in Figure 1. Both whole homogenate and purified cell nuclear uptake levels decreased as the deprivation period increased. Conversely, plasma concentrations of radioactivity increased with insulin deprivation. After 24 hours of insulin withdrawal, uptake levels were significantly different from non-deprived control levels in whole homogenates from hypothalamus-preoptic area, \underline{t} (18) = 3.26, \underline{p} < .01, and pituitary, \underline{t} (18) = 2.16, \underline{p} < .05, as well as in plasma concentrations \underline{t} (19) = 3.49, \underline{p} < .01. After 36 hours of insulin deprivation, cell nuclear uptake was also significantly depressed in hypothalamus-preoptic area, \underline{t} (14) = 2.95, \underline{p} < .05, and pituitary, \underline{t} (14) = 2.29, \underline{p} < .05.

Fig. 1. Effect of acute insulin withdrawal on estradiol uptake in whole homogenates (WH) and purified nuclear pellets (NP) of hypothalamus-preoptic area (HPOA) and pituitary gland of diabetic ovariectomized rats. The deprived groups had insulin withheld for 12, 24 or 36 hours, while the control groups received uninterrupted insulin replacement. Bars represent group mean values \pm standard errors of the mean.

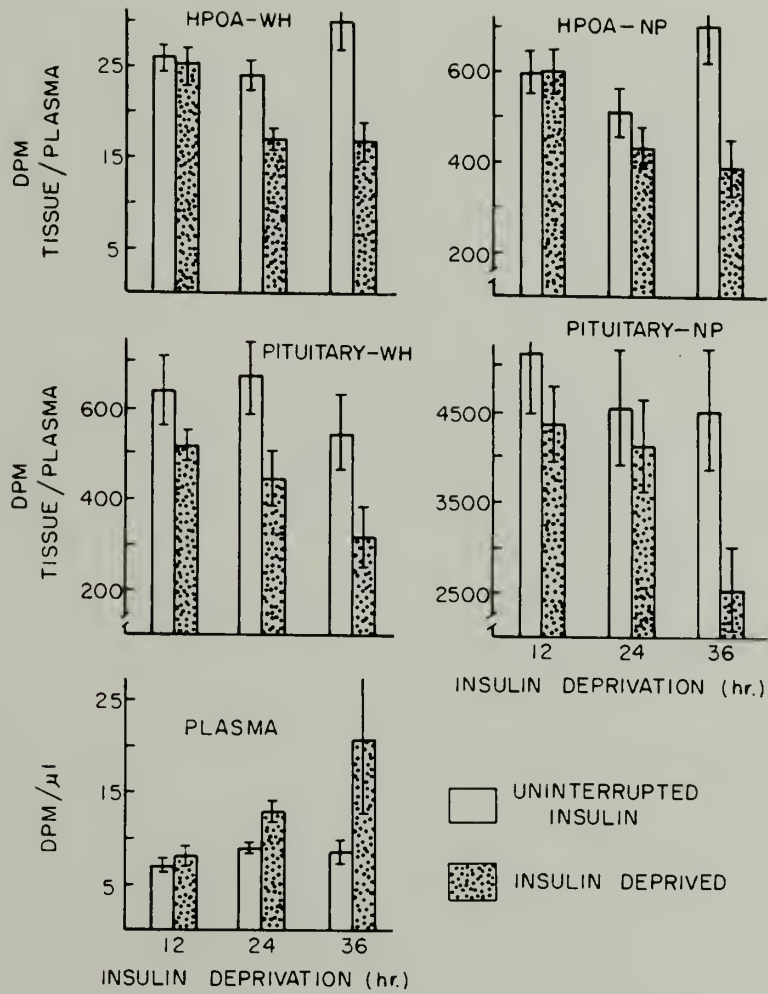


Figure 1

Experiment 2: Insulin Replacement and Estradiol Uptake: Time-Course Studies

The data from Experiment 1 established that 36 hours of insulin withdrawal are necessary before significant deficits in cell nuclear uptake are observed in neural and hypophyseal tissue. This experiment was designed to determine the time-course and effectiveness of insulin replacement in correcting these deficits.

Procedure. Female CD-strain rats weighing 200-300 g were housed and maintained as in Experiment 1. All rats were bilaterally ovariectomized and made diabetic by injection of 100 mg/kg streptozotocin, and then maintained on daily injections of 3 U of U-100 Lente insulin administered just prior to lights out. Approximately 72-48 hours before sacrifice, all animals were shifted to injections of 0.75 U of U-40 Regular insulin, administered every 6 hours. All animals were subjected to 36 hours of insulin deprivation, followed by either [³H]estradiol (control condition) or either 6, 12, or 24 hours of reinstatement of insulin treatment before [³H]estradiol (experimental condition).

All animals were sacrificed 2 hours after an intravenous injection of 40 μ Ci of [³H]estradiol in 0.1 ml 20% ethanol-saline and radioactivity levels were measured in whole homogenate and nuclear fractions of hypothalamus-preoptic area and pituitary gland as described above.

Tissue radioactivity levels are expressed as tissue: plasma ratios.

Results. The effectiveness of insulin replacement in reinstating [³H]estradiol uptake was tested by comparing uptake levels after 36 hours of insulin deprivation. Results of these experiments are summarized in Figure 2. Although a very small, general upward trend can be seen as the period of insulin replacement increased, uptake levels did not differ significantly from the depressed levels of 36-hour insulin-deprived animals in either whole homogenates or cell nuclei of hypothalamus-preoptic area or pituitary gland even after 24 hours of insulin replacement.

Fig. 2. Effect of insulin replacement on estradiol uptake in whole homogenates (WH) and purified cell nuclear pellets (NP) of hypothalamus-preoptic area (HPOA) and pituitary gland of diabetic ovariectomized rats. The replaced groups had insulin withheld for 36 hours, then received insulin replacement for 6, 12, or 24 hours. The control groups had insulin withheld for 36 hours with no subsequent replacement. Bars represent group mean values \pm standard errors of the mean.

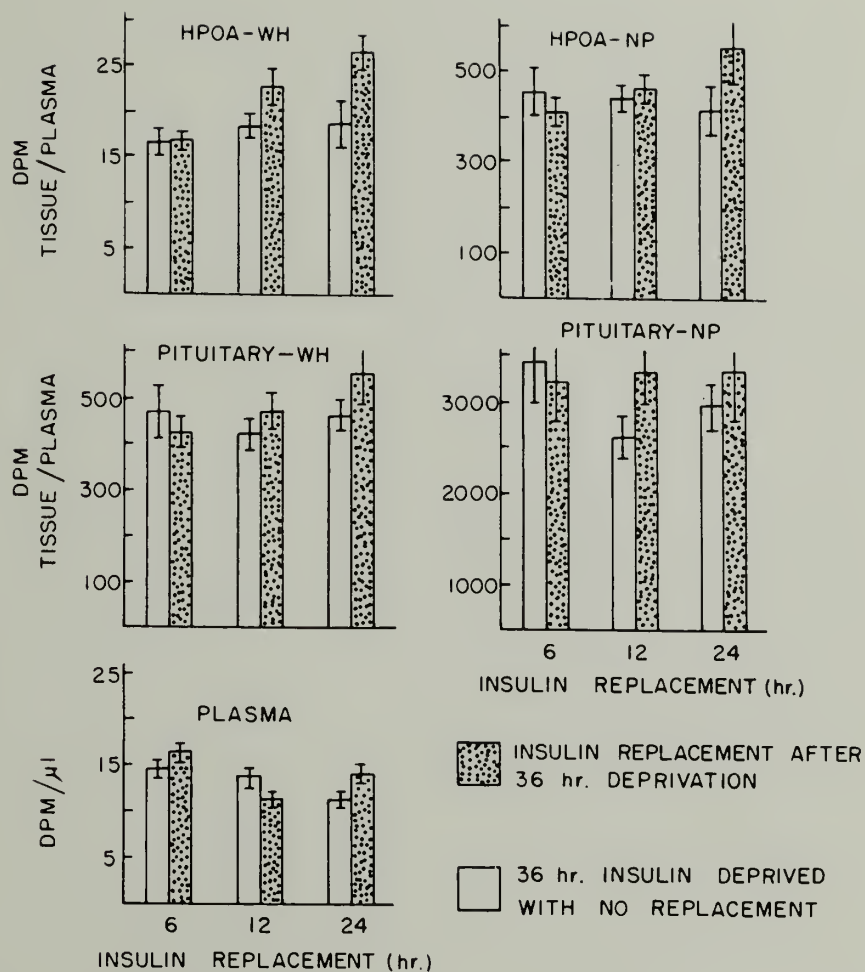


Figure 2

Experiment 3: Acute Insulin Withdrawal:
Metabolic Correlates

The diabetic organism is subject to a host of metabolic disturbances consequent to the insulin deficiency of this condition. The most obvious of these disturbances is the reduced ability to utilize dietary carbohydrate as a fuel source. It has been demonstrated that diabetic rats fed a diet rich in fat eat normal amounts of food, in contrast to the hyperphagia characteristic of untreated diabetics on a low-fat diet (Friedman, 1977). In addition, dietary supplements of olive oil to the primarily carbohydrate diet were found to normalize estrous cycles in chronically insulin deficient rats (Levi & Weinberg, 1949). This experiment was designed to examine the relationship between fuel availability and brain and pituitary estradiol uptake in diabetic rats deprived of insulin for 36 hours, and to attempt to determine which of the many metabolic effects of insulin withdrawal including lipemia, ketosis and hyperglycemia are responsible for the estradiol uptake deficits observed in these animals.

Procedure. Female CD-strain rats weighing 200-300 g were housed and cared for as described above. All animals were bilaterally ovariectomized. Diabetes mellitus was induced in half the animals by intraperitoneal injections of 100 mg/kg streptozotocin. Diabetes was confirmed by

positive glycosuria (++++, TestTape) and animals were maintained on daily injections of 3 U of U-100 Lente insulin administered just prior to lights out. Control animals were not made diabetic.

Ten days after streptozotocin treatment, all animals were given ad libitum access to either a high-fat diet (25% vegetable shortening, by weight, mixed with powdered Purina laboratory chow) or continued access to their normally high carbohydrate diet (Purina laboratory chow, 50-60% carbohydrate). Animals were given a two week period to adjust to these diets and to stabilize their food intake.

Approximately 48 hours before sacrifice, diabetic animals were shifted to injections of 0.75 U of U-40 Regular insulin, administered every 6 hours. Insulin treatments were omitted during the period 36 hours before [³H]estradiol injection. All animals were sacrificed 2 hours after an intravenous injection of 40 μ Ci of [³H]-estradiol in 0.1 ml 20% ethanol-saline. Uptake of [³H]estradiol in whole homogenate and nuclear fractions of hypothalamus-preoptic area and pituitary gland were measured and calculated as described above.

Heparinized blood samples obtained through cardiac puncture at the time of sacrifice were centrifuged to separate plasma and then stored at -17°C for up to three weeks before assay. Free glycerol was measured in samples

deproteinated with barium hydroxide-zinc sulfate (Smogyi, 1945) using Wieland's enzymatic assay. Total glycerol was measured by hydrolyzing samples in 0.5 N alcoholic KOH, neutralizing with magnesium chloride (Bucolo & David, 1973) and assaying the glycerol as above. This method of assaying total glycerol correlated $r(16) = 0.99$ with, and gave the same mean as a commercial enzymatic triglyceride assay (American Monitor Corp.). True triglyceride concentration was obtained by subtracting free glycerol from total glycerol (Bucolo & David, 1973). Ketones (acetoacetate and hydroxybutyrate) were measured by an enzymatic method (Bates, Krebs & Williamson, 1968) in samples deproteinated with barium hydroxide-zinc sulfate (Smogyi, 1945). NADH produced by the enzymatic assays was measured spectrophotometrically. Free (unesterified) fatty acids were assayed by a modification of the colorimetric method of Soloni and Sardinia (1973), using the indicator 1,5-diphenylcarbohydrazide (Mahadevanm, Dillard & Tappel, 1969). Glucose was estimated by an enzymatic method using glucose oxidase (Raabo & Terkildsen, 1960). The data from these assays were examined through multiple regression analyses.

Results. The effects of high-fat diet or high-carbohydrate diet on estradiol uptake in hypothalamus-preoptic area, pituitary gland and blood plasma are shown in Figure 3. While reduced estradiol uptake in both whole homogenate and

nuclear pellets of hypothalamus-preoptic area and pituitary gland can be seen in diabetic animals relative to nondiabetic controls, no differences are seen in either group with regard to diet. Likewise, plasma radioactivity levels are elevated in diabetic animals relative to controls, but diet has no apparent effect.

Plasma metabolite levels are shown in Figure 4. As expected, glucose, glycerol, free fatty acid, ketone and triglyceride levels are highly elevated in diabetic animals. Diet-dependent differences in circulating ketone and triglyceride concentrations are evident among diabetic animals, as are differences in free fatty acids among nondiabetic control rats. Significant negative correlations are found between whole homogenate or nuclear pellet estradiol uptake for brain and pituitary gland and both plasma triglyceride and plasma ketone levels (see Table 1). Plasma glycerol concentrations correlate negatively with plasma radioactivity levels. Stepwise multiple regression analyses of these five variables indicate an overall predictive value for estradiol uptake levels in whole homogenates of hypothalamus-preoptic area, $\underline{R} = .77$, $\underline{F} (5, 34) = 9.84$, $\underline{p} < .001$, and pituitary gland, $\underline{R} = .51$, $\underline{F} (5, 34) = 2.36$, $\underline{p} < .10$, in nuclear fractions of hypothalamus-preoptic area, $\underline{R} = .64$, $\underline{F} (5, 34) = 4.78$, $\underline{p} < .002$, and pituitary gland, $\underline{R} = .52$, $\underline{F} (5, 34) = 2.51$, $\underline{p} < .05$, and in plasma, $\underline{R} = .53$, $\underline{F} (5, 34) =$

2.66, $p < .05$. No single metabolite taken alone, however, provides a strong predictor of estradiol uptake in all tissues (see Table 1).

Fig. 3. Effect of dietary fat content on estradiol uptake in whole homogenate (WH) and purified cell nuclear pellets (NP) of hypothalamus-preoptic area (HPOA) and pituitary gland of diabetic ovariectomized rats deprived of insulin for 36 hours and of nondiabetic control rats. Bars represent group mean values \pm standard errors of the mean.

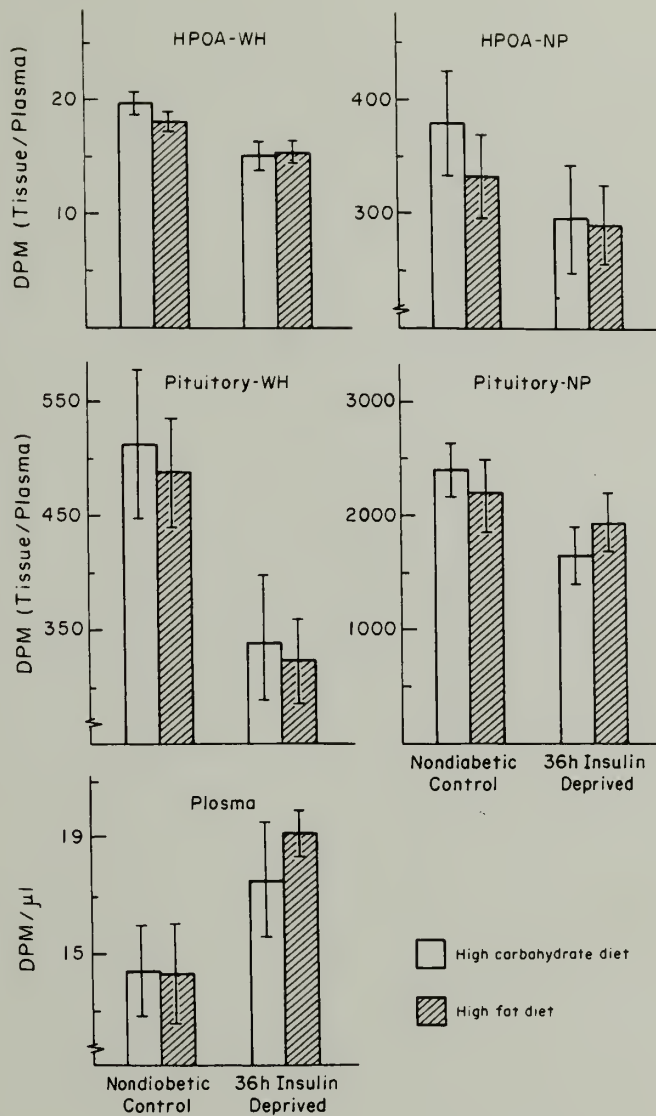


Figure 3

Fig. 4. Levels of circulating fat metabolites in ovariectomized diabetic rats deprived of insulin for 36 hours and in ovariectomized nondiabetic control animals fed diets of low- or high-fat content. Bars represent group mean values \pm standard errors of the mean.

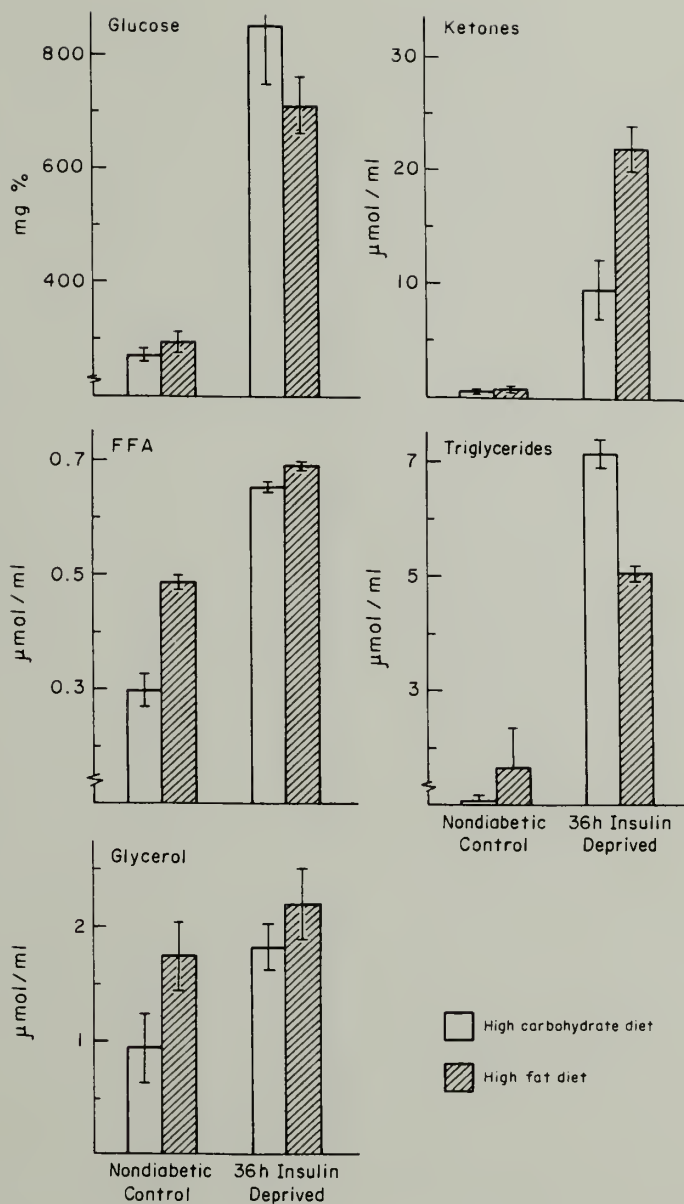


Figure 4

TABLE 1

EFFECTS OF PLASMA METABOLITE LEVELS ON [³H]ESTRADIOL UPTAKE IN
 HYPOTHALAMUS-PREOPTIC AREA, PITUITARY GLAND AND PLASMA OF NORMAL
 AND INSULIN-DEPRIVED RATS FED LOW- OR HIGH-FAT DIETS

	HPOA-WH		HPOA-NP		Pit-WH		Pit-NP		PLASMA	
	% var ^a	r ^b	% var	r	% var	r	% var	r	% var	r
Triglycerides	32	-.57***	16	-.40**	4	-.37**	12	-.34*	<1	.05
Ketones	8	-.53***	7	-.31*	15	-.39**	3	-.27*	4	.20
Glycerol	12	.11	10	.19	<1	.04	<1	.18	11	-.34*
Glucose	6	-.13	4	.01	1	-.13	1	-.06	11	.19
Free Fatty Acids	<1	-.18	4	-.12	5	-.09	11	.05	1	-.23
R ²	.59		.41		.26		.27		.28	
R	.77***		.64**		.51		.52*		.53*	

^aPercent of explained variance attributable to each metabolite as determined by stepwise multiple regression analyses, N=40.

^bPearson correlation coefficient for individual variables, N=40

*probability < .05

**probability < .01

***probability < .001

General Discussion of Chapter I

These results demonstrate that acute withdrawal of insulin can significantly impair brain and pituitary cell nuclear concentrations of [³H]estradiol-17 β in ovariectomized, streptozotocin-diabetic rats. These experiments confirm and extend the findings reported by Gentry et al. (1977) and Denari and Rosner (1972). Our data indicate that these deficits in brain and pituitary uptake of estradiol develop gradually, with the appearance of significant reductions in tissue whole homogenate uptake as much as 12 hours earlier than comparable deficits in purified cell nuclear fractions. Likewise, recovery from the effects of acute insulin withdrawal is slow and gradual. The causes for the failure to observe a clear recovery of brain and pituitary cell nuclear binding after reinstatement of insulin treatment are not clear. It is possible that the cell nuclear uptake peak is displaced in time as a result of our manipulations, but this was not investigated.

The apparent time-course discrepancy between our data and those reported by Gentry et al. (1977) can be attributed to the different metabolic consequences of withdrawal of long-acting Lente insulin or Regular insulin. Insulin effects peak at about 16-17 hours after injection of Lente and then fall during the 7-8 hours thereafter, whereas

similar declines are seen only in the last 1-2 hours of the 6 hour intervals between Regular insulin injections (Friedman, Emmerich & Gil, 1980; Dudley, unpublished observations). Therefore, 24 hour withdrawal of Lente insulin involves a longer effective period of insulin deprivation, so that the present studies represent a refined time-course evaluation relative to earlier reports.

Taken together, the results of these experiments lend further support to the hypothesis that some of the reproductive dysfunctions manifest in female diabetic rats may be the result of alterations in cell nuclear estradiol binding in the hypothalamo-hypophyseal axis. The mechanisms mediating these responses are undefined, however alterations in plasma concentrations of metabolic fuels may influence transport of estradiol to the central nervous system in some way. The correlation between noticeable lipemia and plasma radioactivity concentrations lends credence to this suggestion. Since translocation of estradiol-receptor complexes to cell nuclei is presumably temperature dependent rather than energy dependent (Shyamala & Gorski, 1969) it is not likely that insulin modulation of hypothalamic supplies of metabolic fuels could alter the translocation process directly. Our data, indicating a slowly developing deficit in estradiol uptake, with very gradual recovery after insulin reinstatement are consistent with these observations.

The failure to find an amelioration of estradiol uptake deficits in fat-fed, 36-hour insulin-deprived animals, taken together with the fact that no individual circulating fat metabolite is strongly predictive of estradiol uptake levels across tissues suggests that the differences in fat metabolism between diabetic and normal rats are related in a general way to the overall diabetic condition of the animal rather than to the direct influence of the availability of any single fuel source. However, while exogenous insulin is available, carbohydrate components of the diet may be utilized in a more or less normal way. Acute, 36-hour insulin deprivation might then produce a severe fuel deficiency in an animal enzymatically unprepared to shift rapidly to fat utilization to meet its metabolic demands. This preparation might be compared to nondiabetic, food deprived animals, that are also severely fuel deficient. It seems clear that rats that are chronically deprived of insulin provide a more useful metabolic model for the study of reproductive dysfunction in diabetics. We have found that mildly diabetic female rats with chronic insulin deficiencies show deficits in estradiol uptake in brain and pituitary, as well as deficits in sex behavior, that are ameliorated by high dietary fat (Dudley, Ramirez & Wade, 1980b; see Chapter II). The mechanism by which insulin deficiency modulates estradiol uptake in the central nervous system, and whether simple starvation affects estradiol

uptake in brain and pituitary in nondiabetic animals remains unclear (see Chapter III). We can conclude from the present results that the estradiol uptake deficits in brain and pituitary gland in acutely insulin deprived rats seem to result indirectly from the general alterations in the metabolic condition of the animal and that these deficits seem likely to be related to reproductive dysfunction.

C H A P T E R I I
ESTROUS BEHAVIOR AND PITUITARY AND BRAIN CELL NUCLEAR
RETENTION OF [³H]ESTRADIOL IN CHRONICALLY
INSULIN-DEFICIENT FEMALE RATS

Previous research has implicated the central nervous system as one of the possible sites of functional disruption leading to diabetic infertility in rats, with the demonstration that acute insulin deficiency leads to reduced estradiol uptake in hypothalamus-preoptic area and pituitary gland (Dudley, Ramirez & Wade, 1980a; Gentry et al., 1977), reduction of cell nuclear estrogen receptor levels in hypothalamus-preoptic area, and reduction in lordosis behavior (Gentry et al., 1977; Siegel & Wade, 1979).

Deficits in brain estradiol uptake develop slowly in rats, with whole homogenate fractions showing depressed uptake after 24 hours of insulin withdrawal, and nuclear fractions showing similar reductions after 36 hours (Dudley et al., 1980a). These data suggest that chronic insulin deprivation might provide a more useful metabolic model for the study of reproductive dysfunction in diabetics. This idea is supported by data demonstrating that dietary supplements of olive oil to a carbohydrate-rich diet can normalize estrous cycles (Levi & Weinberg, 1949), and that increasing dietary fat content can eliminate hyperphagia

(Friedman, 1978) among chronically insulin deficient rats. High dietary fat does not, however, ameliorate brain estradiol uptake deficits in female rats acutely deprived of insulin (Dudley et al., 1980a).

Experiment 4: Estrous Behavior in Chronically Insulin-Deprived Rats

The present experiments were designed to examine estrous behavior in female rats that are chronically insulin deficient. We have also attempted to determine the effects of dietary fat and fuel availability on this behavioral measure.

Procedure. Female CD-strain rats weighing 200-300 g were individually housed and cared for as described above. All rats were bilaterally ovariectomized. Mild diabetes mellitus was induced in half of the rats by intraperitoneal injections of 60 mg/kg streptozotocin in citrate buffer, pH 4.8. This dose of streptozotocin produces weight loss and glycosurea (++++, TesTape) detectable within 24-48 hours after administration, but exogenous insulin is not required for survival of rats so treated. Plasma glucose analysis at the time of sacrifice confirmed diabetes mellitus.

Ten days after streptozotocin treatment, animals were given ad libitum access to either a high-fat diet (25% vegetable shortening, by weight, mixed with powdered Purina laboratory chow), or continued access to their normally

high-carbohydrate, low-fat diet (Purina laboratory chow). Animals were given a two-week period to adjust to these diets and to stabilize food intakes.

Sexual receptivity was induced in ovariectomized rats with sequential injections of estradiol benzoate (EB, 2 μ g in 0.1 ml sesame oil) and progesterone (P, 0.1 mg in 0.1 ml sesame oil). Injections of EB were given 24 hours before progesterone treatment, and behavioral testing was done 6-8 hours after progesterone injection. All tests for lordosis behavior took place during the dark, under dim red illumination, in a 76 cm diameter round testing arena with 25 cm high walls. Wood chips covered the floor of the arena. Three sexually experienced Sprague-Dawley male rats were adapted to the arena for approximately 15 minutes prior to the introduction of the first female. Females were tested one at a time. The responses to 10 vigorous mounts with thrusting by the males were rated as 0, 1, 2, or 3 (no, slight, moderate, and full dorsiflexion, respectively) after the method of Powers and Valenstein (1972). Tests were done with the experimenter blind as to the diabetic and diet conditions of the experimental animals.

Results. Lordosis ratings for chronically insulin-deficient rats were reduced relative to nondiabetic controls, with fat-fed diabetics intermediate to chow-fed diabetics and

nondiabetic control rats, Kruskal Wallis $H(3) = 10.89$, $p < .05$ (see Figure 5). Mann-Whitney U tests reveal no differences between the nondiabetic groups. Diabetics eating low-fat diets show significantly lower lordosis ratings than do nondiabetics eating high-fat diets, $U(4, 9) = 2$, $p < .02$, and fat-fed diabetics display lower levels of lordosis than chow-fed control rats, $U(6, 9) = 9.5$, $p < .05$. Among diabetic animals, a high-fat diet increased lordosis ratings, $U(4, 6) = 5$, $p < .05$ (one tail).

Fig. 5. Effect of dietary fat content on estrous behavior in mildly diabetic (60 mg/kg streptozotocin) and nondiabetic control rats, ovariectomized and primed with 2 μ g estradiol benzoate and 1 mg progesterone. Bars represent group mean lordosis ratings \pm standard errors of the mean.

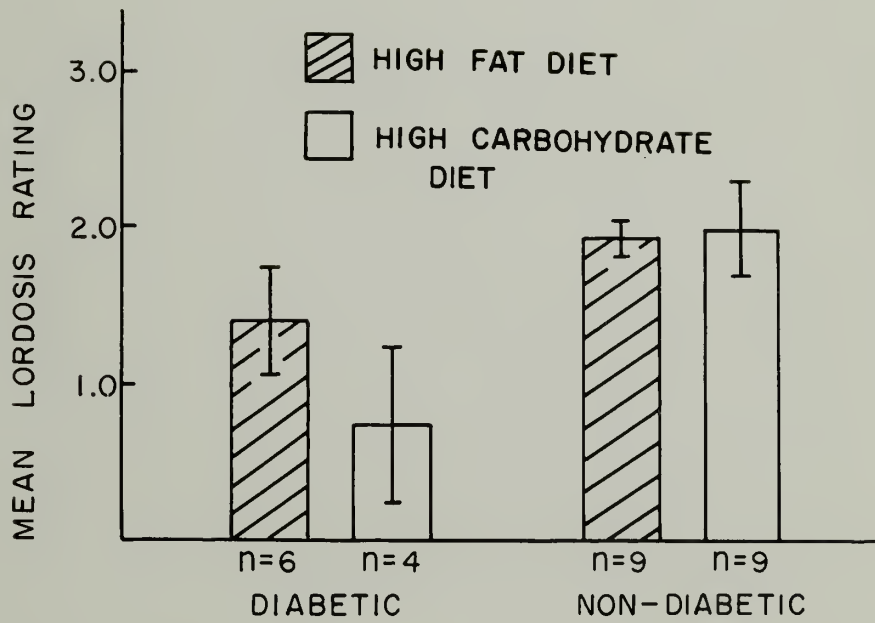


Figure 5

Experiment 5: Estradiol Uptake and Metabolic
Correlates of Chronic Insulin Deficiency

The results of Experiment 4 demonstrated that chronically insulin deficient rats fed high-fat diets showed less severe deficits in lordosis behavior than do diabetics fed a standard laboratory diet which is high in carbohydrate and low in fat content. This experiment was designed to determine the effects of dietary fat and fuel availability on brain and pituitary estradiol uptake, and to examine some of the metabolic correlates of chronic insulin deprivation.

Procedure. The same animals used in Experiment 4 were used in this experiment. Within seven days after completion of behavioral testing, all animals were sacrificed 2 hours after an intravenous injection of 40 μ Ci of [3 H]estradiol in 0.1 ml 20% ethanol-saline. Estradiol uptake was measured in whole homogenate and nuclear fractions of hypothalamus-preoptic area and pituitary gland as described above. Tissue radioactivity concentrations are expressed as tissue: plasma ratios.

Plasma samples were stored at -17°C for up to 3 weeks before assay for circulating metabolites. Total glycerol, triglyceride, ketone (acetoacetate and hydroxybutyrate), free fatty acid and glucose concentrations were determined as described above. The data from these assays were

examined through multiple regression analyses and Pearson correlation.

Results. While estradiol uptake levels in whole homogenate and purified nuclear fractions of both hypothalamus-preoptic area and pituitary gland are similar for non-diabetic rats eating either diet, a clear reduction can be seen among diabetic animals maintained on a standard chow diet, as shown in Figure 6. The deficits in estradiol uptake in insulin-deficient rats appear to be ameliorated among diabetic animals given high-fat diets, with estradiol uptake levels for this group falling between those of chow-fed diabetics and nondiabetic control rats in nuclear fractions of hypothalamus-preoptic area and both whole homogenate and nuclear fractions of pituitary gland. Plasma radioactivity levels are elevated in the low-fat fed, diabetic group relative to the other experimental groups.

Levels of circulating fuel metabolites are shown in Figure 7. Elevations in plasma glucose, ketone, free fatty acid, triglyceride and glycerol concentrations are evident among diabetic rats relative to nondiabetic control rats. While dietary fat content has no effect on plasma levels of these fuels in nondiabetic animals, significant, diet-dependent differences can be seen among insulin-deficient rats in plasma glucose, ketones, and triglycerides. Glucose and triglyceride levels are elevated in chow-fed diabetics,

and ketones are elevated in fat-fed diabetics. Significant negative correlations are found between plasma glucose concentrations and estradiol uptake levels in whole homogenate and nuclear fractions of both hypothalamus-preoptic area and pituitary gland (see Table 2) and between plasma ketone levels and estradiol uptake in whole homogenate fractions of both tissues. Plasma triglyceride concentrations correlate negatively with estradiol uptake in hypothalamus-preoptic area whole homogenate and pituitary nuclear fractions. Plasma radioactivity levels are positively correlated with both triglyceride and glucose concentrations.

Stepwise multiple regression analysis of these five variables indicates an overall predictive value for estradiol uptake levels in hypothalamus-preoptic area whole homogenate, $R = .70$, $F(5, 21) = 4.10$, $p < .01$, with plasma glucose concentrations accounting for the greatest percentage of explained variance (see Table 2). Multiple regression coefficients were not significant in analyses of these circulating metabolites and estradiol uptake in hypothalamus-preoptic area nuclear pellet, nor in whole homogenate or nuclear fractions of pituitary gland, nor in analysis of plasma radioactivity.

Fig. 6. Effect of dietary fat content on estradiol uptake in whole homogenates (WH) and purified nuclear pellets (NP) of hypothalamus-preoptic area (HPOA) and pituitary gland of mildly diabetic and nondiabetic, ovariectomized rats. Bars represent group mean values \pm standard errors of the mean.

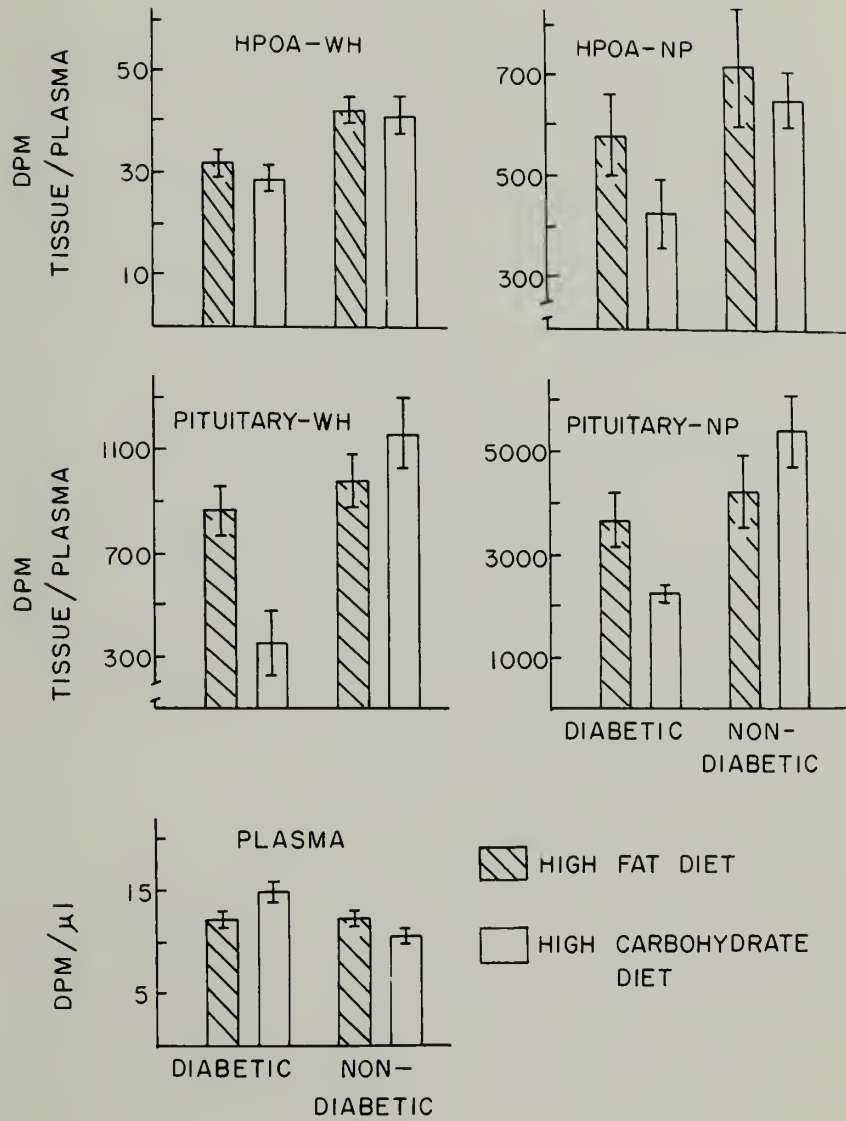


Figure 6

Fig. 7. Levels of circulating fuel metabolites in ovariectomized, mildly diabetic and nondiabetic rats fed diets of low- or high-fat content. Bars represent group mean values \pm standard errors of the mean.

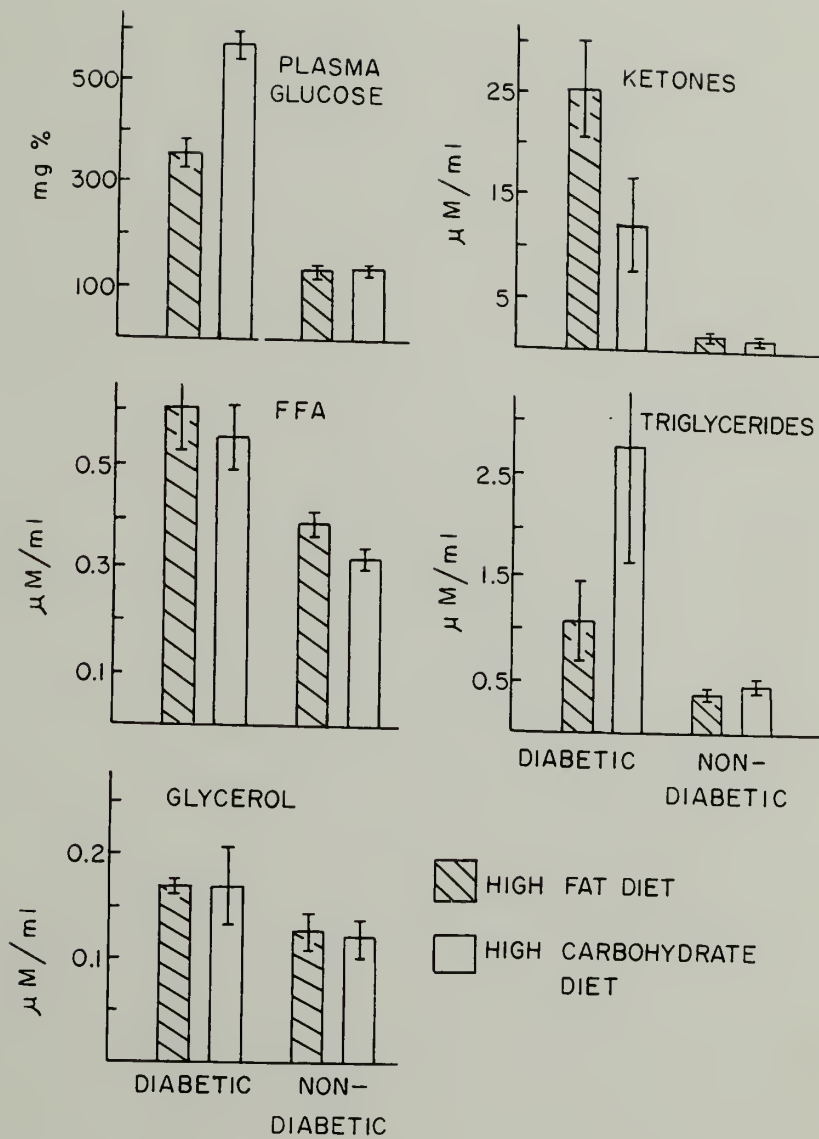


Figure 7

General Discussion of Chapter II

These results demonstrate that chronic insulin deficiency can lead to significantly impaired lordosis responding as well as to reductions in brain and pituitary cell nuclear binding of [³H]estradiol in ovariectomized, streptozotocin diabetic rats. These data are consistent with reports of similar deficits among acutely insulin deficient rats (Dudley et al., 1980a; Gentry et al., 1977; Siegel & Wade, 1979), and they support the hypothesis that at least some of the reproductive dysfunctions manifest in diabetic rats are the result of alterations in cell nuclear binding of estradiol in the central nervous system (Gentry et al., 1977; Siegel & Wade, 1979).

The most obvious of the many metabolic disturbances characteristic of diabetes mellitus is the reduced ability to utilize dietary carbohydrate as a primary fuel source. The present data demonstrate that the effects of chronic insulin deprivation on lordosis behavior and brain and pituitary estradiol uptake can be ameliorated by dietary supplements of fat, which provide the animal with an alternate and more readily utilizable fuel source. There are other data which support this hypothesis. The extreme hyperphagia associated with experimental diabetes when animals are fed standard laboratory diets can be abolished with diets rich in fat, despite persisting impairments in

glucose utilization, fat storage and liver glycogen deposition (Friedman, 1978). Thus, feeding diabetic rats a high-carbohydrate, low-fat diet may be similar to feeding normal rats a diet diluted with nonnutritive material such as cellulose.

If the reproductive impairments of diabetic rats fed standard laboratory diets are due in part to a lack of utilizable metabolic fuels, then we would expect the deficits to be ameliorated by increasing dietary fat content. This is, in fact, the case. Levi and Weinberg (1949) found that the addition of olive oil to the Purina laboratory chow diet of their diabetic rats normalized estrous cycles, but did not restore fertility. In the present experiments, diabetic rats on a high-carbohydrate, low-fat diet showed significant decrements in estrous behavior and in neural and hypophyseal [^3H]estradiol binding, but the diabetic rats eating high-fat diets showed less severe deficits. Although supplementing the diabetics' diet with fat may ameliorate some of the reproductive impairments, the high-fat diet does not reverse the metabolic derangements such as hyperglycemia, hyperlipemia, glycosuria, ketonuria, depleted liver glycogen and reduced carcass fat content, other than to normalize caloric intake (Friedman, 1978). These data are consistent with the suggestion that it is a general lack of utilizable fuels, rather than any single metabolic derangement per se, that is responsible for the reproductive deficits of diabetics.

C H A P T E R I I I
CHRONIC FOOD DEPRIVATION AND PITUITARY AND BRAIN
CELL NUCLEAR RETENTION OF [³H]ESTRADIOL IN
NONDIABETIC FEMALE RATS

The results of experiments with both acutely and chronically insulin deprived female rats (Dudley et al., 1980a, 1980b; Gentry et al., 1977; Siegel & Wade, 1979) have supported the hypothesis that some of the reproductive dysfunctions manifest in female diabetic rats may be the result of alterations in cell nuclear estradiol binding in the hypothalamo-hypophyseal axis (Gentry et al., 1977; Siegel & Wade, 1979). We have also demonstrated that dietary supplements of fat ameliorate at least some of the reproductive deficits observed in chronically insulin deprived rats (Dudley et al., 1980b; see Chapter II).

It has been previously reported that olive oil added to the standard high-carbohydrate diet of diabetic rats normalized estrous cycles but did not restore fertility (Levi & Weinberg, 1949), and that high-fat diets prevent diabetic hyperphagia without reversing the metabolic derangements of diabetes such as hyperglycemia, hyperlipemia, glycosuria and ketonuria (Friedman, 1978). These data are consistent with our hypothesis that it is a general lack of utilizable fuels, rather than any single metabolic derangement per se that is responsible for the reproductive

deficits of diabetics (Dudley et al., 1980a, 1980b; see Chapter I and Chapter II). Chronic fuel deprivation may represent an important correlate of the disruptions in normal steroid action among diabetics. The present experiments were designed to examine the relationship between chronic underfeeding and pituitary and brain cell nuclear estradiol retention in nondiabetic female rats.

Experiment 6: Estradiol Uptake in Food Deprived Rats

Caloric restriction is inhibitory to gonadal activity in many species (Walker & Bethea, 1977). In female rats, chronic underfeeding leads to cycle disruptions and ovarian atrophy (Howland, 1972b; Mulinos & Pomerantz, 1940; Piacsek & Meites, 1967; Rinaldini, 1949) and to decreased levels of circulating LH (Howland, 1972a; Howland & Ibrahim, 1971, 1973). These reproductive dysfunctions have been attributed to aberrant neural control of the pituitary-gonadal axis rather than to a simple lack of nutrients available during the period of food restriction (Piacsek & Meites, 1967; Sorrentino, Reiter & Schalch, 1971). The present experiment was designed to examine cell nuclear estradiol uptake levels in hypothalamus-preoptic area and pituitary gland in chronically food deprived female rats in order to assess the effects of fuel deficiency on steroid binding in neural and pituitary tissues.

Procedure. Female CD-strain rats weighing 150-200 g were housed and cared for as described above. All animals were bilaterally ovariectomized. Food intake and body weights of all rats were monitored daily. Four weeks after surgery, animals were divided into 2 groups, with control and experimental rats paired. Yoked individuals were closely matched for body weight and food intake.

The control group was given ad libitum access to Purina laboratory chow throughout the experiment. Experimental animals were pair-fed 50% of the control ad libitum food intake each day for 21 days. After this period of food restriction, animals from each group were sacrificed 2 hours after an intravenous injection of 40 μCi [^3H]-estradiol, and brain and pituitary whole homogenate and cell nuclear uptake of estradiol were measured as described above. Tissue radioactivity levels are expressed as tissue: plasma ratios.

Plasma samples obtained at the time of sacrifice were frozen and later assayed for glucose, triglyceride, free fatty acid, ketone (acetoacetate and hydroxybutyrate), and glycerol levels as described above, except that NADH produced by the enzymatic assays was measured fluorometrically in this experiment.

Results. Body weights of food deprived animals fell steadily throughout the 21 day restriction period (Figure 8) so that

there was a 100 g mean difference between groups at the time of sacrifice.

Estradiol uptake levels for matched pairs of animals are shown in Figure 9. Plasma radioactivity levels are significantly elevated following chronic caloric restriction, paired t (7) = 4.14, p < .005. Estradiol retention in the whole homogenate fraction of hypothalamus-preoptic area is consistently reduced in food deprived animals, paired t (7) = 2.18, p < .10, but significant differences were not found in whole homogenate or nuclear fractions of brain or pituitary gland.

Plasma glucose levels were significantly depressed in food restricted animals, paired t (7) = 4.0, p < .01, but there were no differences between groups in ketone, tri-glyceride, glycerol, or free fatty acid levels.

Fig. 8. Body weights of ovariectomized rats fed Purina laboratory chow ad libitum and of paired animals restricted to 50% of the daily ad libitum quantities.

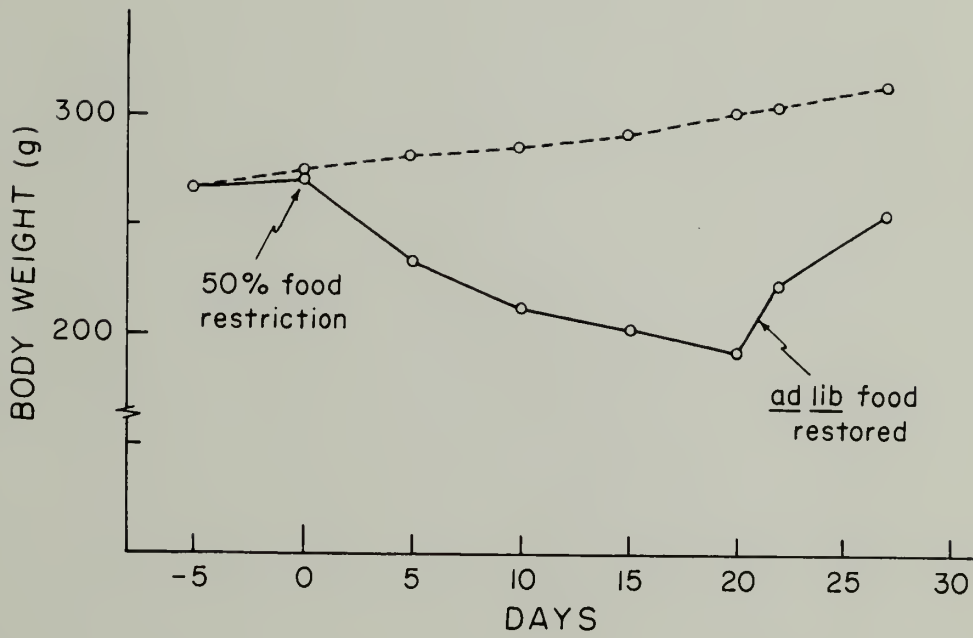


Figure 8

Fig. 9. Effects of chronic food restriction on estradiol uptake in whole homogenate (WH) and purified nuclear pellets (NP) of hypothalamus-preoptic area (HPOA) and pituitary gland of ovariectomized rats. The food restricted animals were given 50% of the daily ad libitum quantities eaten by matched control animals.

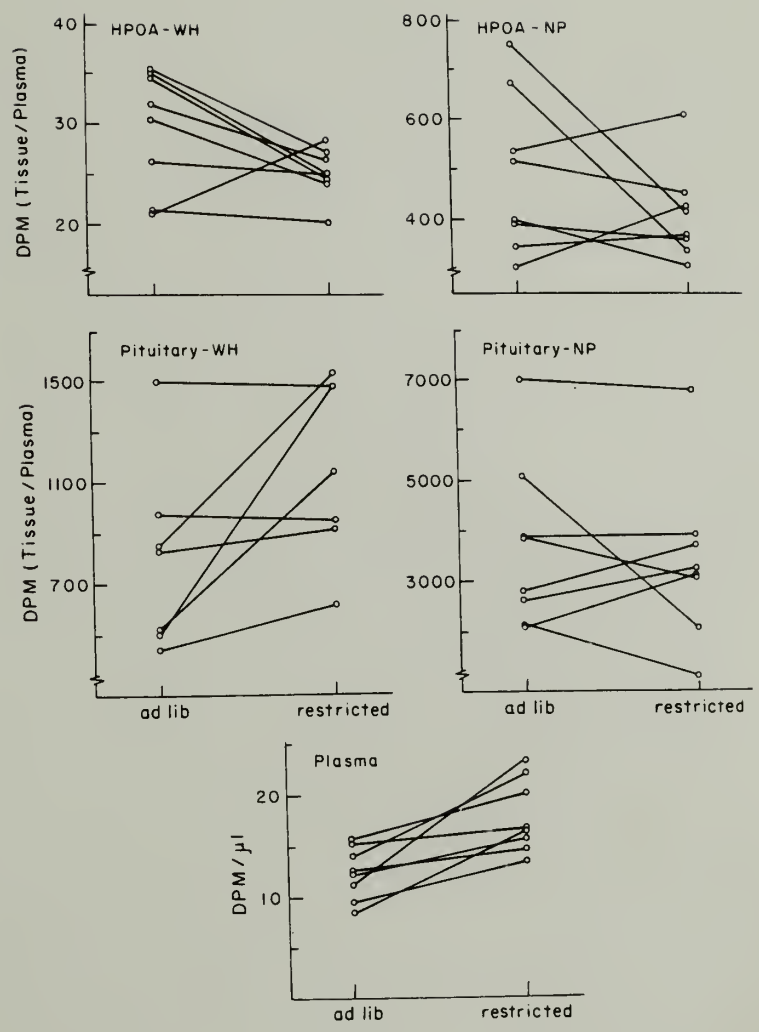


Figure 9

Experiment 7: Estradiol Uptake After Refeeding

The abnormal estrous cycles observed in chronically underfed rats can be restored to normal after 3-5 days of ad libitum food intake (Nakanishi, Mori & Nagasawa, 1976). This experiment was designed to determine the effects of refeeding on estradiol uptake in brain and pituitary after 21 days of food restriction.

Procedure. Ovariectomized CD-strain rats were housed and cared for as described above. Body weights and food intake were monitored daily, and half of the animals were restricted to 50% of the daily ad libitum food intake of yoked control rats, matched for initial body weight and food intake. After 21 days of food restriction, deprived animals were restored to ad libitum food intake for 10 days.

After this period of refeeding, paired animals were sacrificed 2 hours after an intravenous injection of 40 μCi [^3H]estradiol, and brain and pituitary whole homogenate and cell nuclear uptake of estradiol were measured as described above. Plasma samples obtained at the time of sacrifice were frozen and later assayed for circulating glucose, triglyceride, ketone (acetoacetate and hydroxybutyrate), glycerol and free fatty acid levels as described above, with NADH produced by the enzymatic assays measured fluorometrically.

Results. Body weights of food-restricted rats increased daily during refeeding, and were only 50 g below non-restricted controls at the time of sacrifice (Figure 8). Estradiol retention in neural and hypophyseal tissue, and plasma radioactivity levels were similar for refed and control animals. There were no differences between groups in plasma glucose, ketone, free fatty acid, triglyceride or glycerol concentrations.

General Discussion of Chapter III

Although equivocal, the results of these experiments are suggestive of a starvation-induced depression in brain and pituitary estradiol uptake in ovariectomized, non-diabetic rats. The strongest evidence supporting this interpretation comes from our plasma measurements. Plasma radioactivity levels are clearly elevated in food deprived rats relative to nondeprived control animals, and this indicates that there is some change in the dynamics controlling the transport of estradiol to the central nervous system. The relationship between these plasma changes and estradiol uptake in the hypothalamo-hypophyseal axis is not immediately clear because of the inconsistent results observed in these experiments.

Certain methodological problems probably account for a great deal of the variability in these data. All of these animals were subjected to a 2 hour automobile trip with

consequent disruptions of the normal circadian signals during the 24 hour period immediately preceding assay, and this stress may well have had differential effects on the hypothalamo-hypophyseal-adrenal axes of the rats. Coupled with rather small sample sizes, these factors could obscure the effects of the experimental manipulations.

For purposes of the present discussion, a demonstration that chronic food deprivation leads to diminished estradiol uptake in brain and pituitary gland of nondiabetic rats would provide strong support for the hypothesis that a shortage of utilizable fuel is responsible for the estradiol uptake deficits observed in acutely- and chronically-insulin-deprived rats. A failure to demonstrate such a relationship, however, might indicate that there are additional factors affecting diabetic animals that are not present in a food-deprived rat with a normally functioning pancreas. Although we can draw no firm conclusions from the present data, the elevated radioactivity levels observed in plasma of the food-deprived rats suggest that additional investigation of this hypothesis would prove fruitful. Little can be said about the implications of the refeeding data until these questions are explored more fully.

C H A P T E R I V

CONCLUSIONS

Taken together, the results of the experiments reported here support a number of conclusions with regard to reproductive functioning in diabetic rats and estradiol action in neural and hypophyseal tissue. It is clear from these and other studies that deficits in brain and pituitary uptake and retention of estradiol do exist in rats that are deprived of insulin, and that these deficits are associated with deficits in lordosis response. In contrast to the fact that many of the metabolic responses accompanying insulin deficiency develop quite rapidly, whole homogenate estradiol uptake in brain and pituitary gland is significantly reduced after 24 hours of insulin deprivation, and similar reductions in estradiol retention in cell nuclear fractions of these tissues are not evidenced until 36 hours after insulin is withdrawn. The slow development of these changes suggests that they are not directly dependent on the altered levels of circulating metabolic products.

We have found that the plasma concentrations of glucose, triglycerides, ketone, glycerol and free fatty acids are inter-correlated with one another and that taken together, levels of these metabolites are moderately predictive of estradiol uptake levels in brain and pituitary gland. No single metabolic product, however, is reliably

predictive across tissues. This finding also indicates that a more general factor of the diabetic conditions is responsible for the changes observed in estradiol uptake among diabetic rats.

Comparison of the acutely-insulin-deprived rat and the chronically-insulin-deprived rat indicates that the latter probably represents a more useful metabolic model in which to study reproductive deficiency in diabetes. The effect of insulin deprivation appears to be in some ways similar to feeding a normal animal a diet diluted with a nonnutritive substance. Increasing the proportion of utilizable fuel by providing the animals with fat enriched diets is effective in ameliorating both behavioral and steroid uptake deficits in chronically-insulin-deficient rats that have made the necessary enzymatic and metabolic shifts that would favor fat utilization. The present work supports the conclusion that at least some of the reproductive dysfunctions seen in diabetic rats are the result of alterations in estradiol actions at the level of the cell nucleus in hypothalamus and pituitary, and suggests that these alterations are related to the availability of a utilizable fuel.

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