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Ovarian Dysfunction in Streptozotocin-Induced Diabetic Rats (41714)

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Abstract. The effect of streptozotocin diabetes on some ovarian functions in adult rats was examined. Diabetic diestrus animals showed reduced ovary weight and lower circulating levels of progesterone. Scatchard plots of binding data derived from ovarian particulate fractions of normal and streptozotocin diabetic rats revealed the presence of one class of binding sites with high affinity for ^{125}I-hCG. The apparent association constant of the hCG receptors of diabetic ovaries was comparable to that of normal gonads. However, a marked decrease (42%) in the number of hCG binding sites was found in diabetic animals. With isolated luteal cells similar results were obtained, and the administration of insulin to streptozotocin diabetic rats restored to normality the number of hCG binding sites. The maximal response of progesterone production by luteal cells from control ovaries was obtained with 10^{-10} *M* hCG. A 100-fold higher concentration of hCG was required for the maximum stimulation of cAMP synthesis. The cAMP response of cells from diabetic rats was significantly higher than that of control cells. However, luteal cells from diabetic rats showed some loss of sensitivity in the synthesis of progesterone during incubation with hCG. Most of the alterations seen in diabetic female rats could be restored with insulin therapy, indicating that insulin plays an important role in the regulation and maintenance of normal reproductive functions. It is suggested that the diminution of the LH receptor population causes the disruption of normal luteal cell function. This fact could be responsible for some of the reproductive alterations in the diabetic female rat.

Sexual disturbances which follow the onset of diabetes are well-known not only in clinical practice but also in laboratory animals. In addition to subtotal pancreatectomy and alloxanization, a new diabetogenic agent, streptozotocin (1), made it possible to obtain in a few days severe and uniform diabetes in albino Wistar rats (2). Knowledge of the molecular mechanisms involved in the infertility of the diabetic male rat has advanced considerably in the past few years. Diabetes has been implicated in reduced androgen biosynthetic activity, decreased uptake of testosterone by peripheral androgen target tissues, reduced testosterone-to-dihydrotestosterone conversion in ventral prostate, and low circulating levels of LH (3–6). Moreover, several papers from our laboratory have shown that in the diabetic male rat there is: (a) reduced number of LH binding sites in Leydig cells (7); (b) reduced response of Leydig cells to hCG as judged by the *in vitro* production of testosterone, likely due—at least in part—to diminished activity of NADPH-generating enzymes (8); (c) enhanced conversion of testosterone to 5α-reduced metabolites in seminiferous tubules (9); (d) reduced levels of the androgen binding protein (ABP) in epididymis (9); (e) greatly reduced cytosol and nuclear androgen binding activity in the prostate (10); and (f) diminished 5α-reductase and 3α- and 3β-hydroxysteroid dehydrogenase activities in prostate (manuscript in preparation). The majority of these altered parameters in the diabetic rat were restored to normality when exogenous replacement therapy with insulin was used (7–9). In few cases only testosterone therapy was capable of recovering normal function (10).

On the other hand, studies on the infertility of the female diabetic rat have dealt with problems involving ovulation, fecundation, nidation, and pregnancy evolution (11, 12). Accordingly, in this paper, we report some molecular alterations which occur after female rats have been rendered diabetic with streptozotocin treatment.

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Materials and Methods. *Materials.* Carrier free ^{125}I (100 mCi/ml) was purchased from New England Nuclear (Boston, Mass.) or the Radiochemical Centre (Amersham, England). Streptozotocin (lot No. 60140) was from Upjohn (Kalamazoo, Mich.) and protamine-zinc insulin was from Eli Lilly Company (Buenos Aires, Argentina). Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (Endocrion) used for pseudopregnancy induction and highly purified hCG (11,500 IU/mg) were kindly donated by Dr. Eduardo Passeron from Laboratorios Elea (Buenos Aires). [1,2,6,7-^{3}H(N)]progesterone (98.3 Ci/mmole) and [2,8-^{3}H]adenosine-3',5'-cyclic phosphate (cyclic AMP, 34.6 Ci/mmole) were purchased from New England Nuclear. Unlabeled steroids, cyclic AMP, and bovine serum albumin, fraction V (BSA), were from Sigma Chemical Company (St Louis, Mo.); medium 199 was from Difco Laboratories (Detroit, Mich.); collagenase type 4176 CLS II 49 A 087 (153 U/mg) was from Worthington Biochemical Company (Freehold, N.J.). Other reagents and chemicals were of the highest quality available.

Animals. Female, 60-day-old Wistar rats fed Purina rat chow *ad libitum* and kept in an air-conditioned, light-controlled environment (lights on from 0700–1900 hr) were used throughout. The estrous cycle was carefully followed through vaginal smears. Two experiments were performed. In both experiments, rats were given or not given a single, intravenous injection of streptozotocin (70 mg/kg body wt) on Day 60 of age. Starting on Day 75 of age, diabetic rats received either 2 IU of protamine-zinc insulin subcutaneously or saline for the remainder of the experiment. In the first experiment there were no additional treatments. The rats were killed on or about Day 90 of age at diestrus, in part because few female diabetic rats could be found in other stages. In the second experiment the rats were primed with subcutaneous injections of PMSG (100 IU) on Day 80 of age and hCG (50 IU) on Day 82 of age per rat and were killed on Day 90 of age. The assessment of the pseudopregnant state was made by visual inspection of the ovaries at the time of sacrifice and further by optical microscopy (see below). Rats from both experiments were sacrificed by decapitation, trunk blood was collected, and the ovaries were removed, weighed, and placed in buffer. In the first experiment, ovarian weight, serum glucose, serum progesterone, and binding of ^{125}I-hCG to membranes prepared from whole ovaries were performed. In the second experiment, dispersed cell preparations were made from the ovaries (highly luteinized, superovulated) which were used to measure luteal cell binding of hCG, and the response of luteal cells to hCG as assessed by progesterone production and cAMP production.

In each experiment the diabetic state was evaluated by following glycemia, polydipsia, and polyuria. For rapid measurements of blood and urine glucose, Test-tape (Eli Lilly Co., Buenos Aires) was used; otherwise, blood glucose was determined using the Glucostat kit method (Worthington Biochemical Co., Freehold, N.J.).

Preparation of collagenase-dispersed luteal cells. Luteal cells from pseudopregnant rats, were isolated as described by Conti *et al.* (13). In brief, ovaries which had been placed in medium 199 containing 0.1% bovine serum albumin (BSA) were finely minced and washed to remove blood cells. The minced tissue was suspended in medium 199 containing 2 mg/ml of collagenase, using 1 ml per ovary. The tubes were shaken at 37°C for 30 min. The supernatant containing the cells was withdrawn. The cell suspension was passed through Nytex into a plastic bottle on ice. The residual particles were washed with medium and the supernatant was clear. The combined supernatants were centrifuged at 250*g* for 5 min at 4°C. The cell pellet was resuspended in the same medium in a ratio of 1 ml/ovary and counted in a Levy ultraplane counting chamber after being stained with methylene blue. The effectiveness of the treatment with PMSG/hCG was verified by a yield of approximately 99% for luteal cells in the sample. The viability of the cells was not less than 90% as determined with trypan blue staining. Final dilutions were made in medium 199 to give 10^6 or 10^7 cells/ml.

Membrane preparation for binding assays. Ovaries from diestrus animals were minced and homogenized at 0°C in phosphate-buffered saline (PBS) in a ratio 1:6 (w/v) using an Ultra-Turrax (IKA-WERK). The homoge-

nates were filtered through nylon cloth (Nitex 50) and centrifuged at 12,000*g* for 20 min at 2–4°C. The resulting membrane pellet was resuspended in the same volume of PBS and used in binding studies. Appropriate aliquots were taken for protein determination by the method of Lowry *et al.* (14).

Production of cyclic AMP and progesterone by luteal cells. Isolated luteal cells obtained from pseudopregnant control, diabetic, and diabetic insulin-treated rats were incubated under constant shaking with increasing concentrations of hCG in medium 199 containing 0.1 m*M* 1-methyl-3 isobutylxanthine (Aldrich Chem. Co., Milwaukee, Wisc.) at 34°C under an atmosphere of 95% O_2–5% CO_2 for 4 hr. Each incubation had 2×10^6 cells in a final volume of 2.2 ml. Incubation was terminated by rapidly transferring the vials to crushed ice; 1-ml aliquots of the incubates were heated at 100°C for 15 min and stored at −20°C until the determination of total cyclic AMP by means of a protein binding competition assay (15). Other 1-ml aliquots were centrifuged at 800*g* for 10 min at 4°C and the supernatants kept frozen at −20°C for determination of progesterone by RIA (see below).

Preparation of iodinated hormones. ^{125}I-labeled hCG, was prepared using the lactoperoxidase technique according to Thorell and Johansson (16), except that the reaction was allowed to proceed in 0.5 *M* phosphate buffer, pH 7.4, for 2 min. The reaction was stopped by dilution with 0.05 *M* phosphate buffer, pH 7.0, and the mixture was layered on top of a 0.5 × 20-cm column of Sephadex G-75 equilibrated with phosphate buffer, 50 m*M*, pH 7.5, containing 0.2% BSA and 100 m*M* NaCl. The peak corresponding to the iodinated hormone was stored frozen at −70°C in aliquots. Prior to use, hormones were further purified by chromatography on Ultrogel ACA-54 (LKB, Sweden) columns. The biological activity of the labeled hormones was tested in radioligand assays using fresh rat liver or testis membrane preparations, as described elsewhere (7, 10).

Maximal binding capacity and precise specific activity calculations were performed by binding at large excesses of receptors and self-displacement assays were performed as described by Moyle *et al.* (17). In each case never less than 40% of the radiolabeled hormone was bound to an excess of reference membrane preparations. Specific activities of typical preparations of ^{125}I-hCG averaged 55 μCi/μg.

Binding assays. The binding activity of collagenase-dispersed luteal cells was studied as follows. Aliquots of the cell suspension containing 3×10^6 cells were incubated in medium 199 with increasing concentrations of ^{125}I-labeled hCG in a final volume of 0.25 ml during 3 hr at 34°C. Parallel incubation of ^{125}I-hCG plus 5 μg of non-radioactive hCG were performed in order to assess the non-specific binding. The reaction was stopped by adding 2 ml of cold medium 199 and the tubes were centrifuged at 2000*g* for 20 min at 4°C. The pellets were washed twice with the same medium and the radioactivity in the final pellets was determined using a Beckman Auto-Gamma 4000 spectrometer with 66% efficiency.

For binding assays using the membrane-rich fraction of ovary homogenates, aliquots of the membrane suspension (400–600 μg protein) were incubated in PBS medium with increasing concentrations of ^{125}I-labeled hCG in a final volume of 0.25 ml during 16 hr at 20°C. Nonspecific binding was assessed by parallel incubates containing 5 μg of unlabeled hormone. PBS was used for reaction arrest and washing procedures, as above.

Specific binding data were studied using the Scatchard analysis (18).

Other methods. All results are given as the means ± SEM. The statistical significances were calculated using the multiple comparisons Dunnet's *t* test (19). Results obtained in RIA procedures were calculated by linear regression analysis. For both purposes a 9815-A Hewlett–Packard desk calculator was used.

Serum progesterone radioimmunoassay was performed using an anti-4-pregnen-3,20-dione 3-CMO:BSA antiserum kindly supplied by Dr. Pirke and Dr. Doerr (Max-Planck Institut, Munich, West Germany) according to Doerr (20) with the modifications which are described in detail by Barañao *et al.* (submitted for publication). In brief, sera (0.1 ml) were extracted twice with 2 ml of ethyl ether (Merck) and the dried extracts resuspended in 0.5 ml of 50 m*M* phosphate buffer, pH 7.0, containing 155 m*M* NaCl, 0.1% sodium azide,

and 0.1% gelatin (GP buffer). Tubes containing 200 μl of the extracts, 100 μl GP buffer, 100 μl antibody (1:25,000 final dilution), and 10,000 cpm [^{3}H]progesterone were incubated overnight at 4°C. Separation of free and bound hormones was achieved by using a dextran–charcoal suspension (0.05% dextran and 0.5% charcoal in GP buffer) as described earlier (10). Radioactivity was measured in a Beckman LS-100 C scintillation counter with 50% efficiency; scintillation fluid consisted of 0.5% PPO and 0.05% POPOP in toluene. In our conditions, recoveries averaged 90%; the within-assay and between-assay variations were 8.0 and 14.2%, respectively. Progesterone production by luteal cells was evaluated by the same RIA procedure directly in suitable dilutions (1:25 v/v) of the incubation media (without ether extraction). The scintillation fluid used for measurements of radioactivity in the cyclic AMP determinations contained 30% Triton X-100.

Results. Presumed diestrous diabetic animals showed reduced ovary weight and lowered circulating levels of progesterone. These parameters could be restored to normal upon treatment with exogenous insulin (Table I).

Scatchard plots (Fig. 1) of binding data derived from diestrous ovarian particulate binding fractions of normal (group C) and streptozotocin diabetic rats (group D) revealed the presence of one class of binding sites with high affinity for ^{125}I-hCG. The apparent association constant of the hCG receptors of diabetic ovaries ($1.57 \times 10^{10}\ M^{-1}$) was comparable with that of receptors in normal gonads ($1.13 \times 10^{10}\ M^{-1}$). However, a marked decrease in the number of hCG binding sites was found in diabetic animals: 8.1 ± 0.05 (N = 10) vs 14.0 ± 1.3 (N = 10) fmole/mg protein in the control group ($P < 0.01$).

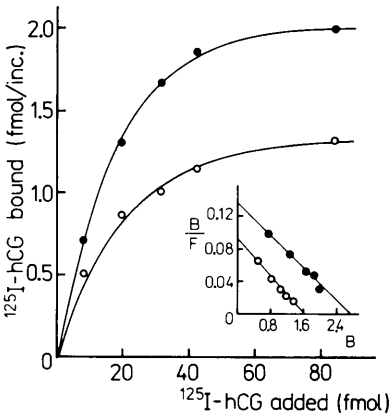

FIG. 1. Binding of ^{125}I-hCG to membrane-rich fraction of ovaries from control and streptozotocin diabetic rats. Aliquots of the ovarian membrane suspension (400–600 μg protein) were incubated in PBS medium with increasing concentrations of ^{125}I-hCG and a constant concentration of unlabeled hCG in a final volume of 0.25 ml during 16 hr at 20°C. PBS was used for reaction arrest and washing procedures. Data were converted to a saturation curve and the *inset* shows the Scatchard plot of specific binding. Values are expressed as fentomoles of hCG bound per incubation in the saturation curve. Each point is the mean of duplicate samples. ●, control rats (N = 10); ○, diabetic rats (N = 10).

With intact luteal cells, similar results were obtained (Fig. 2) and the administration of insulin to streptozotocin diabetic rats (group D + I) restored the binding of labeled hCG: C = 5.7 ± 0.3 (N = 6), D = 2.8 ± 0.07 (N

TABLE I. EFFECTS OF STREPTOZOTOCIN DIABETES AND FURTHER INSULIN TREATMENT ON OVARIAN WEIGHT, SERUM GLUCOSE, AND SEXUAL STEROIDS IN FEMALE RATS

Parameter	Experimental group		
	Control (12)	Diabetic (12)	Diabetic + insulin (12)
Ovaric weight (mg)	44.0 ± 2.3	29.8 ± 1.2*	41.3 ± 2.5
Serum glucose (mg/100 ml)	98 ± 12	420 ± 39*	110 ± 29
Serum progesterone (ng/ml)	4.8 ± 0.5	2.4 ± 0.2*	4.2 ± 0.6

Note. Results are given as means ± SEM. Figures in parentheses indicate the number of animals. Statistical comparisons were made in relation to the control values (* $P < 0.001$). Animals were killed 4 weeks after streptozotocin or vehicle injection. Ovaries were removed and weighed. Trunk blood was collected to determine serum glucose and progesterone (see Methods).

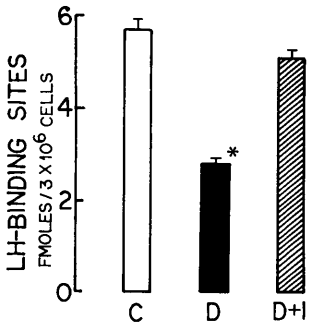

FIG. 2. Binding of ^{125}I-hCG to isolated luteal cells from control (C), streptozotocin-diabetic (D) and insulin-treated diabetic rats (D + I). Luteal cells obtained from pseudopregnant rats were prepared by collagenase dispersion. Aliquots of the luteal cell suspension containing 3×10^6 cells were incubated in medium 199 as described under Material and Methods. Specific binding data were calculated through the Rosenthal reasoning of Scatchard analysis. Each bar represent the mean ± SEM. Each group consists of 12 animals, and the ovaries were collected in two separate pools for every group. The statistical significances were calculated using the multiple comparisons Dunnet's t test. *, $P < 0.001$; C, control; D, diabetic; and D + I, insulin-treated diabetic rats.

= 6), and D + I = 5.11 ± 0.2 (N = 6) fmole/3×10^6 cells; C versus D and D versus D + I, $P < 0.001$. The Ka (1.1×10^{10} M^{-1}) was similar in all the groups. Calculations of number of binding sites and Ka were made using Scatchard analysis.

Since the changes in ^{125}I-hCG binding were detected in both the ovarian particulate fraction and the collagenase-dispersed luteal cells from diabetic rats, it was of interest to determine whether the differences in ^{125}I-hCG binding could be correlated with changes in cyclic AMP and progesterone production.

Figure 3 shows the dose–response curve to hCG for cAMP production. The maximal response by luteal cells from control rats was obtained with 10 ng hCG/tube (1.26×10^{-10} M). At hCG concentrations in which cAMP stimulation was observed, the response of the cells from diabetic rats was significantly higher than the controls. In our conditions, the treatment of diabetic animals with insulin was not able to restore the normal cyclic AMP response to luteal cells.

The progesterone response to increasing

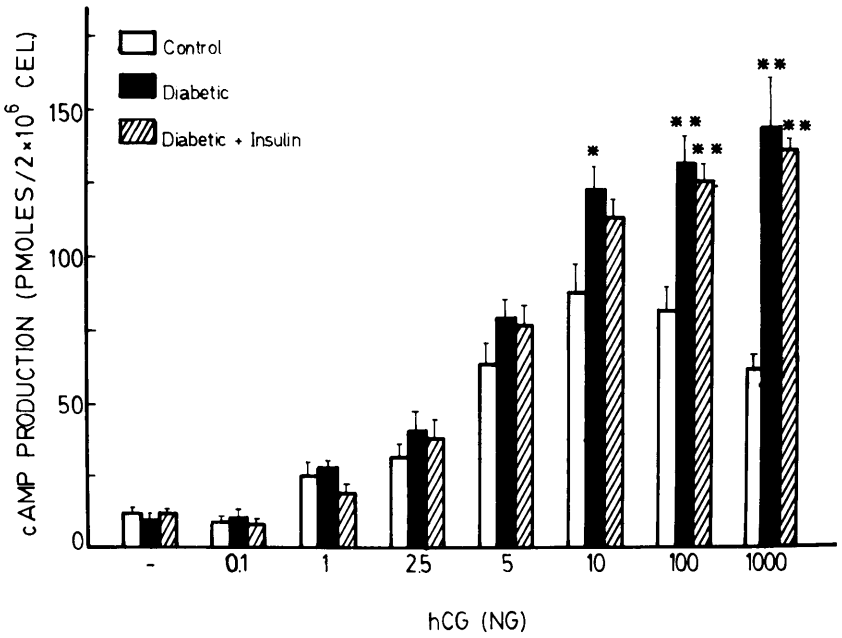

FIG. 3. Effect of increasing doses of hCG on the production of cyclic AMP in isolated luteal cells from control, diabetic, and insulin-treated diabetic rats. The preparation of luteal cells and the incubation and assay of cyclic AMP are described under Materials and Methods. The data are expressed as picomoles of cAMP formed per 2×10^6 cells. Each bar represents the mean ± SEM of triplicate determinations. Statistical comparisons were made in relation to the control values (*$P < 0.05$, **$P < 0.01$).

doses of hCG in luteal cells isolated from ovaries of control, diabetic, and insulin-treated diabetic animals is shown in Fig. 4. Although no significant changes in maximum stimulation of progesterone by hCG were detected among the three groups, some loss of sensitivity of cells from diabetic animals was indicated by the significantly lower response seen.

Discussion. Ovaries from streptozotocin-treated rats have provided an opportunity to analyze the biological responses of the affected target cells to hormonal stimulation during the onset of experimentally induced diabetes.

Previous studies in our laboratory demonstrated that whole ovaries from diabetic rats showed a diminished response to exogenous hCG measured as progesterone production under *in vitro* incubations (data not shown). These results are in accordance with the lower levels of serum progesterone observed (Table I). In addition, the diabetic state caused loss of over 40% of the number of LH/hCG receptors measured in ovarian particulate fractions from diestrus rats. This decrease in binding capacity was not accompanied by any detectable change in binding affinity, the *Ka* remaining at about $10^{10}\ M^{-1}$. Since the diabetic females do not ovulate normally, their ovaries might have a different population of luteal cells. This fact could be responsible for the observed changes. We found, however, that in luteal cells isolated from highly luteinized ovaries of PMSG/hCG-treated rats, the number of LH/hCG receptors per cell was diminished by 50% in the diabetic state; insulin treatment increased these values to normality.

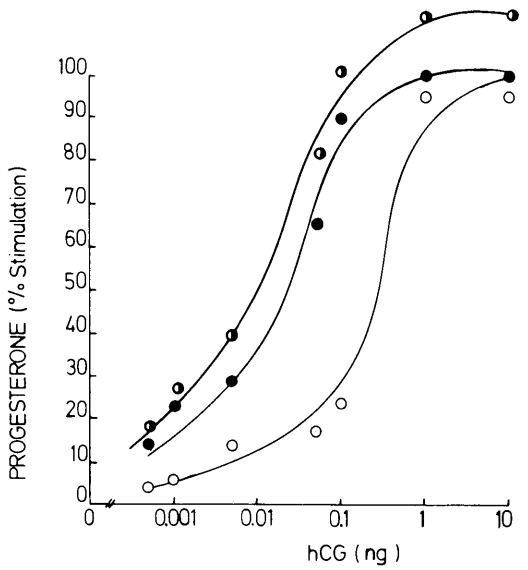

FIG. 4. Effect of increasing doses of hCG on the progesterone production in isolated luteal cells from control, diabetic, and insulin-treated diabetic rats. The experimental details are the same as described in the legend to Fig. 3. Data are expressed as percentage of progesterone stimulation referred to increase over basal values obtained in the absence of hCG. Basal values were: control, 10.9 ± 0.1; diabetic, 11.7 ± 0.4; insulin, 10.4 ± 0.2 ng progesterone formed per 2×10^6 cells. Data are expressed as percentage of progesterone stimulation. ●, Control rats ($N = 8$); ○, diabetic rats ($N = 8$); ◑, insulin-treated diabetic rats ($N = 8$).

To determine whether the observed changes in the number of LH receptors resulted in altered biological responses to LH, isolated luteal cells were prepared from control, diabetic, and insulin-treated diabetic animals to measure the production of cyclic AMP and progesterone in response to hormonal stimulation. In cells prepared from diabetic rats the cyclic AMP response was increased, while the progesterone response was reduced.

Changes in cyclic AMP production were inversely correlated with changes in hCG binding activity. This fact could be due to a specific regulation by insulin of the adenyl cyclase and phosphodiesterase activities enzymes which regulate cyclic AMP concentration in the cell. These studies are being pursued in our laboratory.

We were unable to measure any changes in cyclic AMP levels at doses between 0 and 1 ng of hCG, the doses at which differences in progesterone synthesis are found. The discrepancy between the dose–response curves for cyclic AMP and steroid production has been attributed to changes in specific pools of cyclic AMP (21) or cyclic AMP bound to protein kinase (22) in which minor increases are not readily detected by the current methods.

Liu and co-workers (23) demonstrated that alloxan-diabetic female rats have sufficient levels of circulating gonadotropins but, unexplained at the time, lack normal ovarian response to the gonadotropic stimulus. Our results showing reduction of luteal hCG binding sites indicate that the diminished responsiveness of the ovaries to gonadotropin stimulation

may be due to the reduction of hCG receptor population in the ovaries of diabetic animals. Moreover, the decrease in circulating levels of progesterone (Table I) would be an expectedly normal consequence of this alteration.

Direct measurements of peripheral LH levels, however, failed to show any significant difference between diabetic and *ad lib* fed controls (23), but an important question to be answered is whether the diabetic rat has gonadotropins immunologically indistinguishable from those of the intact controls, but biologically different.

In a previous work (7) we have demonstrated that both pancreatectomy- and streptozotocin-induced diabetes reduced the number of Leydig cell LH receptors without affecting the association constant or the sedimentation coefficients of the LH/hCG-receptor complexes. It was postulated that a change in the carbohydrate moiety of the gonadotropin receptor might result from the markedly altered carbohydrate metabolism of the diabetic animal. As a consequence the receptor might be at least in part inactivated, or its synthesis diminished. The present results showing similar effects in luteal cell LH receptors of diabetic animals would be indicative that similar mechanisms could take place in both sexes.

However, it can not be excluded that the diabetic state could produce other alteration at the hypothalamic or hypophyseal level. Kirchick *et al.* demonstrated that the cause of anovulation in alloxan diabetic rats is the loss of the preovulatory LH surge which is not due to insufficient secretion of estradiol (24) or decreased release/secretion of Gn RH from the hypothalamus (25). They showed that there is an attenuated pituitary response to Gn RH in the diabetic rats which is incapable of yielding the LH levels required for ovulation although LH synthesis and storage are normal (25).

In spite of the known changes in the glycemic state after the insulin injection (with hypo, normo and hyperglycemic peaks) this treatment was successful in normalizing most of the parameters studied. This suggests that insulin plays an important role in the regulation and maintenance of normal female reproductive functions. It is quite obvious that some of the alterations should not be directly attributed to insulin deficiency, but to indirect consequences of altered carbohydrate metabolism. It is suggested that disturbances in the reproductive functions of diabetic female rats appeared to be based at least in part on lowered luteal cell responsiveness to gonadotropin stimulation which may be due to diminished LH receptor population in ovarian target cells.

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