Maternal Diabetes in Rats

I. Effects on Placental Growth and Protein Turnover

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The developmental growth of the rat placenta was investigated between days 14 and 21 of gestation in normal control, gestational-diabetic, established-diabetic, and insulin-maintained–diabetic mothers. While established-diabetic mothers were hyperglycemic for 2 wk before and throughout the pregnancy, gestational-diabetic mothers were only hyperglycemic for the second half of pregnancy. Daily insulin replacements successfully restored normoglycemia. The wet weight and protein content of control placentas increased linearly between days 14 and 21. Although placentas from diabetic animals were initially smaller, placentomegaly was found at full term. Placental glycogen concentrations were also markedly increased in all diabetic animals. These changes were largely prevented by insulin replacement. The changes in placental size during normal development and in association with the diabetic state were explained by measuring placental rates of protein turnover (in vivo). In normal placentas, protein synthetic and degradative rates progressively declined over the last week of gestation. Because synthesis rates were unchanged in placentas of diabetic mothers, it appears that the differences in placental size primarily arise from alterations in protein degradation. *Diabetes* 37:1665–70, 1988

Placental enlargement is a feature of human diabetic pregnancies (1,2). Pregnancy in the streptozocin (STZ)-induced diabetic rat is also characterized by placentomegaly (1,3,4) in the face of varying degrees of fetal growth retardation (see Canavan and Goldspink in this issue). However, such observations in humans and animals have mainly been made at full term. Although important morphological and developmental differences exist between the placentas of primates and rodents, the rat placenta can nonetheless be a useful model for the study of placental growth and diabetic-induced placentomegaly. Structural (2,5,6) and biochemical (1,3) abnormalities have been observed in placentas derived from diabetic pregnancies of both humans and rodents and could conceivably have a causal link with the early fetal growth retardation associated with such pregnancies (5).

This study sought to establish the normal pattern of developmental growth in the rat placenta and to explain the enlargement of this organ in diabetic pregnancies. Average rates of placental protein synthesis and protein breakdown were therefore measured in vivo as a function of gestational age. The contribution of glycogen to placental size was also assessed at days 16 and 21 of gestation.

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MATERIALS AND METHODS

Female albino rats (n = 110, CD strain and originally derived from Charles River, Manston, UK) initially weighing 300 ± 10 g were used in this study. In an attempt to standardize litter and fetal sizes, only females destined for their second pregnancies were used. These were randomly assigned to either the control or diabetic group. Diabetes was induced in the appropriate group of rats by a single injection (via lateral tail vein) of 40 mg STZ/kg body wt i.v. (7). Animals in the control group received only the saline vehicle.

Blood glucose concentrations were measured 48 h after STZ injection to confirm that diabetes had been induced. Measurements were subsequently made at frequent intervals throughout pregnancy to ensure that diabetic animals remained hyperglycemic and to check that insulin therapy successfully restored and maintained normoglycemia. For these purposes, 5 μl of whole blood (in duplicate) was obtained from the tip of each animal's tail and assayed with a Sigma diagnostic kit.

The diabetic animals were then subdivided into groups designated as gestational diabetic, established diabetic,

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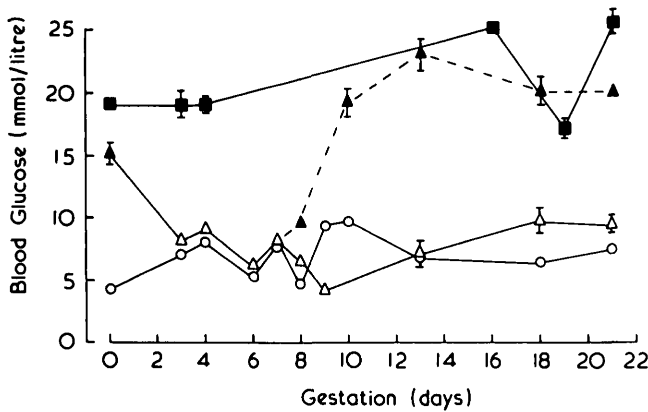

FIG. 1. Blood glucose concentrations measured at several stages throughout pregnancy in normal control (○), gestational-diabetic (▲), established-diabetic (■), and insulin-maintained–diabetic (△) rats. Each value represents mean ± SE of 6 blood samples. In these experiments, blood glucose levels in gestational-diabetic rats were initially measured at day 3. However, from other experiments we know that normoglycemia is restored within 6 h (not 3 days) of 1st insulin injection, administered at day 0 (8).

and established diabetic maintained on insulin throughout pregnancy (insulin-maintained diabetic). Control and gestational-diabetic animals were mated immediately after their respective injections of saline or STZ. Mating took place overnight within a well-defined 12-h period (i.e., 2100–0900). Vaginal smears were taken at 0900 the next day with sperm-positive smears denoting day 0 of pregnancy. After confirming pregnancy, gestational-diabetic animals were immediately started on an insulin-replacement therapy. This consisted of 4 U of the long-acting Hypurin protamine zinc insulin (Weddel, Wrexham, UK) administered subcutaneously every 24 h (8). This insulin therapy was subsequently withdrawn from these animals on day 7 of pregnancy. Established-diabetic animals differed in that they were hyperglycemic for 2 wk before mating and throughout the 3 wk of gestation. Insulin-maintained–diabetic rats were placed on the insulin-replacement therapy once pregnancy had been confirmed, already being diabetic for 2 wk before mating.

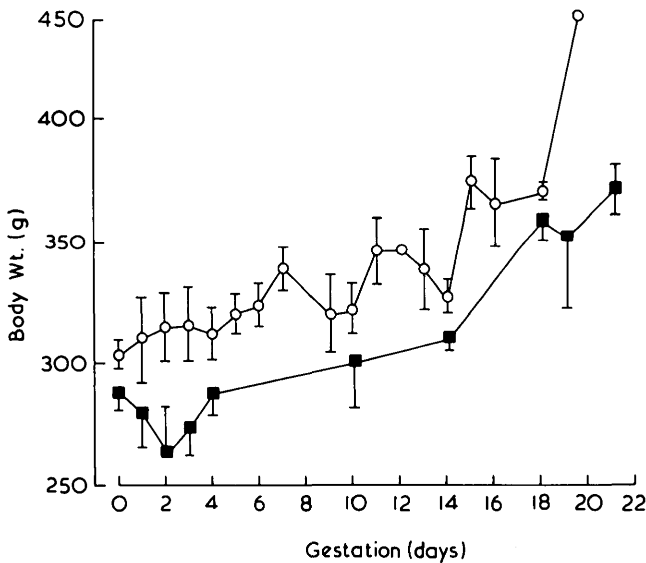

FIG. 2. Changes in body weight during gestation. Pregnant control (○) and established-diabetic (■) rats were weighed at 0900–1000 at regular intervals during the 3rd wk of pregnancy. Each value is mean ± SE of ≥6 animals.

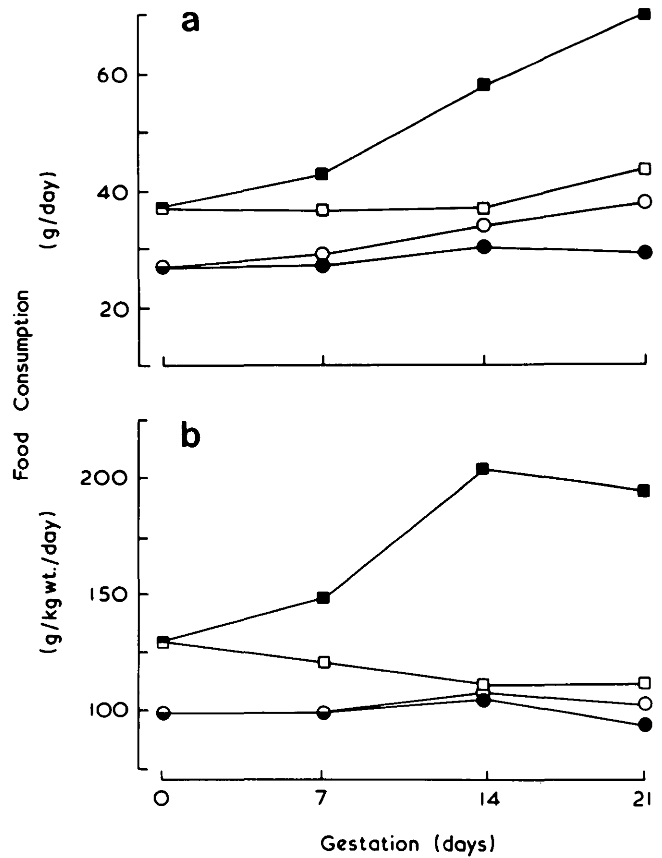

FIG. 3. Daily food intake of both control nonpregnant (●) and pregnant (○) rats, together with nonpregnant (□) and pregnant (■) established-diabetic rats, was monitored at several stages during gestation. Means ± SE, from ≥6 animals per group, are expressed as food intake per rat (*a*) and per kilogram of rat (*b*).

Average rates of protein synthesis in the placenta were measured in vivo between days 14 and 21 of gestation after an injection of 150 μmol of phenylalanine via a lateral tail vein (9,10). This injection included 65 μCi of L-[4-^{3}H]-phenylalanine (sp act 24 Ci/mmol; Radiochemical Centre, Amersham, Bucks, UK) and was administered in 1 ml of 0.9% NaCl/100 g body wt. The suitability of this method for measuring synthetic rates in body tissues has previously been described in detail (9,10). All pregnant females were killed by decapitation 10 min after the injection of phenylalanine. After exsanguination (10 s), the abdominal cavity was rapidly opened and the uterus and its contents immersed in chilled water. Four placentas per mother were then isolated, immediately frozen in liquid N_2, weighed, and stored at −20°C.

Frozen placentas were pulverized to a fine powder and samples of ~100 mg homogenized in ice-cold 0.3 M perchloric acid (1:50 wt/vol) with a ground-glass homogenizer. The specific radioactivities of [^{3}H]phenylalanine in both the free pool (S_A; i.e., perchloric acid–soluble fraction) and covalently bound in protein (S_B) were measured. The latter involved 24-h hydrolysis of the washed protein pellet in 6 M HCl at 120°C. Phenylalanine in all samples was converted to β-phenylethylamine and assayed fluorometrically (9). All measurements of radioactivity were made in a tritosol scintillant in an LKB scintillation counter (efficiency of 25% for

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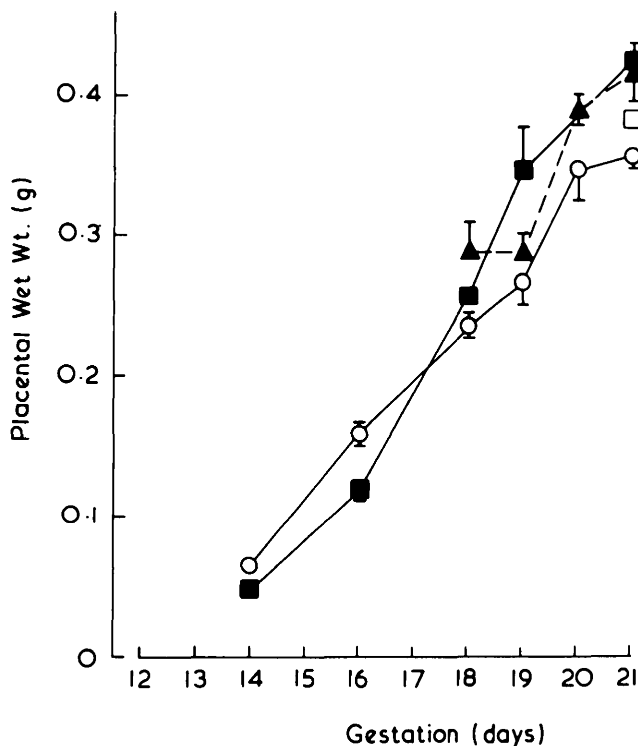

FIG. 4. Changes in placental weights in control and diabetic pregnancies. Placentas were isolated at intervals between days 14 and 21 of gestation from control (○), gestational-diabetic (▲), and established-diabetic (■) animals. Placentas from insulin-maintained–diabetic rats (□) were only obtained on day 21. Each value represents mean ± SE of 12 organs removed from 3 mothers.

tritium), by use of the external-channels–ratio method of quench correction. The fractional rate of synthesis (K_s), defined as the percentage of the protein mass synthesized per day, was calculated from

$$K_s = \frac{S_B}{S_A \times t} \times 100$$

where t is the time in days. Placental growth rates (K_g; defined as the percentage of change in protein mass per day) were determined as the net accumulation of protein between two time points. This was usually 2 days, spanning the point at which the synthesis rate was measured. Meaningful measurements of protein degradation are technically difficult to obtain in vivo due to efficient recycling of amino acids and the nonexponential decay of prelabeled proteins (11). Therefore, because growth arises from an imbalance between the rates of protein synthesis and breakdown (K_b), the latter was calculated by subtracting the two measured parameters, i.e., $K_b = K_s - K_g$. Because the indirect rates of degradation are calculated from mean values, no standard errors or statistical analyses can be presented with these data.

The protein and glycogen content of each placenta was measured by the method of Lowry et al. (12) and Siu et al. (14), respectively. Placental RNA and DNA were also extracted and measured as described elsewhere (15). Student's two-tailed t test was used to analyze the statistical differences between mean values.

RESULTS

Except those designated to be normal controls, all female rats were rendered diabetic by injection of 40 mg STZ/kg body wt i.v. We have previously shown that this dose of the diabetogenic agent induces a threefold increase in blood glucose concentrations within 2 days of its administration (6,7). At the same time, all diabetic animals survived >6 wk and were maintained in a healthy state throughout, which are essential prerequisites for the experimental design used here. Approximately 10% of the diabetic animals spontaneously reverted back to normal during the course of the investigations and were excluded from the study. This potential problem, and the need to monitor the efficiency of the insulin-replacement therapy, necessitated frequent measurements of blood glucose concentrations in all animals.

Of the diabetic animals, established-diabetic rats possessed significantly ($P < .01$) higher blood glucose levels, i.e., three- to fourfold above normal (4–9 mM), for 2 wk before mating and then throughout the entire 3 wk of pregnancy (Fig. 1). In a second group of established-diabetic rats, normoglycemia was restored throughout the 3 wk of gestation by administering daily injections of a long-acting insulin (Fig. 1). The latter animals served as additional controls to the established-diabetic rats. In another group, the rats were mated within 6 h of receiving STZ, and successful matings were immediately followed by insulin replacement for the first 7 days of pregnancy. Normoglycemia was achieved within 6 h of the first insulin injection (at day 0) (8). Seven days later the insulin was withdrawn, rendering these gestational-diabetic rats significantly ($P < .01$) hyperglycemic from 10 days of pregnancy onward (Fig. 1).

As expected, the normal pregnant rats gained weight (40%, $P < .01$; Fig. 2) and increased their food intake (Fig. 3), particularly over the 3rd wk of gestation. The established-

TABLE 1
Nucleic acid contents of placentas from control and diabetic pregnancies

	Total DNA-P (μg)				Total RNA-P (μg)			
	Day 14	Day 16	Day 19	Day 21	Day 14	Day 16	Day 19	Day 21
Control	13.1 ± 0.8	29.8 ± 1.7	33.3 ± 1.7	36.7 ± 1.1	27.8 ± 2.2	71.1 ± 3.7	100 ± 7.2	95.3 ± 2.3
Gestational diabetic			33.2 ± 1.4	35.8 ± 1.7			96.9 ± 4.1	106 ± 4.4
Established diabetic	9.9 ± 0.8	22.7 ± 1.2*	38.5 ± 1.9†	39.4 ± 2.0	22.5 ± 1.7	49.7 ± 3.4‡	106 ± 4.6	112 ± 5.0

Nucleic acids of 12 control and 12 established-diabetic placentas (see Figs. 4 and 5) were extracted and assayed as described previously (15).
*$P < .01$, †$P < .05$, and ‡$P < .001$, determined with Student's t test.

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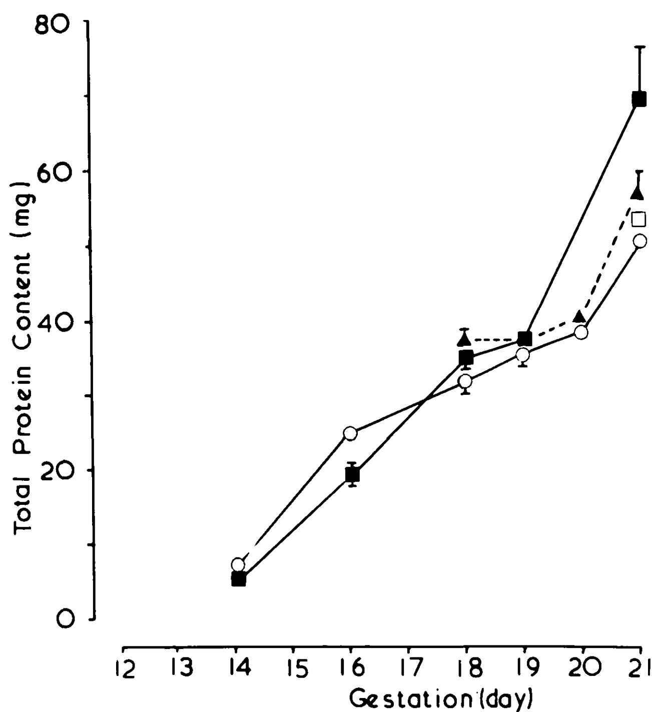

FIG. 5. Protein mass of the same control (○), gestational-diabetic (▲), established-diabetic (■), and insulin-maintained–diabetic (□) placentas as in Fig. 4 determined by method of Lowry et al. (12).

diabetic rats were 12% lighter at the commencement of pregnancy ($P < .05$). Although they too gained weight by full term (24%, $P < .05$), they were consistently smaller than the controls. Despite this, food intake was significantly ($P < .01$) greater in pregnant diabetic rats, even when adjusted for differences in body weight.

Significant differences were noted in the wet weights of the placentas isolated from the 4 experimental groups (Fig. 4). The placentas of the established-diabetic mothers at days 14 and 16 were significantly ($P < .001$) lighter (24%) than those obtained from normal controls. However, from day 18 on, this situation was reversed. That is, placentas from both established-diabetic and gestational-diabetic mothers were significantly ($P < .001$) heavier (16%) and possessed more nucleic acids than those derived from normal pregnancies at full term (Table 1). Continuous insulin therapy for 21 days was only partially successful in preventing this placentomegaly (Fig. 4).

The larger diabetic placentas, however, were not edematous, because no significant differences were found in their water contents at 20 days (range 84.3 ± 0.5 to 86.1 ± 0.5%). In addition, the protein composition, at 13.2 ± 1.2% of the wet weight, was unchanged in all placentas. Hence, the changes in the protein content (Fig. 5) closely followed the differences in tissue wet weight (Fig. 4) and indicate genuine differences in placental growth. If, as appears to be the case, most of the cells in the placenta are mononucleate, then the difference in the DNA content suggests that fewer cells are found in the diabetic placentas up to day 16 (Table 1). However, as with the later reversal in overall placental size (Fig. 4), more DNA and hence a greater number of cells are present in the placentas of diabetic rats from day 18 on (Table 1). Both the glycogen content and concentration of the placentas of established-diabetic mothers were significantly greater than those from control pregnancies at days 16 and 21 (Table 2). Despite the greater placental storage of glycogen in hyperglycemic mothers, this difference accounted for <0.5% of the increase in organ wet weight (Fig. 4).

To explain both the developmental growth of the normal placenta and the differences in its size in the diabetic pregnancies, protein turnover was measured in vivo with the most reliable method available (9). Rates of protein synthesis can be expressed in two ways. First, as the total amount of protein synthesized in the whole organ. Although these rates increased with gestational age (data not shown), this mainly reflected the age-related increases in placental size (Fig. 4). This becomes particularly apparent when protein synthesis is expressed as a fractional rate, thereby eliminating differences in organ size (Fig. 6). In fact, the fractional synthetic rate in normal placentas progressively decreased between days 14 (73 ± 2.7%/day) and 21 (20 ± 1.3%/day). Similar developmental changes were found in the diabetic pregnancies with no significant differences in the synthetic rates of placentas from control, established-diabetic, or gestational-diabetic (data not shown for clarity) mothers (Fig. 6). Hence, the observed differences in placental size (Figs. 4 and 5) do not appear to result from alterations in the rate of protein synthesis. This suggests that the hyperglycemic state must have induced changes in protein degradation.

It is technically difficult to measure meaningful rates of protein breakdown in vivo (11). However, because placental growth arises from the imbalance between the rates of protein synthesis and breakdown, indirect rates of protein degradation can be calculated by substracting the two measured rates, i.e., growth from synthesis (see MATERIALS AND METHODS). As such, in normal placentas, these rates explain

TABLE 2
Glycogen content of placentas from normal and diabetic pregnancies

	Glycogen content on day 16		Glycogen content on day 21	
	mg/organ	mg/g	mg/organ	mg/g
Control	0.54 ± 0.08	3.5 ± 0.4	0.59 ± 0.01	1.7 ± 0.9
Established diabetic	0.78 ± 0.02 (+43)*	5.1 ± 0.5 (+44)*	1.68 ± 0.3 (+187)*	5.0 ± 0.4 (+192)*
Gestational diabetic			1.82 ± 0.3 (+211)*	4.5 ± 0.9 (+164)*
Insulin maintained			0.75 ± 0.06 (+28)†	1.8 ± 0.2 (+5)

Placentas obtained from normal control, gestational-diabetic, established-diabetic, and insulin-maintained–diabetic mothers were analyzed for their glycogen content (mg/organ) and concentrations (mg/g placenta) by Siu et al. (14). Values are means ± SE of 6 placentas. Values in parentheses indicate percentage of change.
*$P < .001$ and †$P < .02$ vs. control values, determined with Student's t test.

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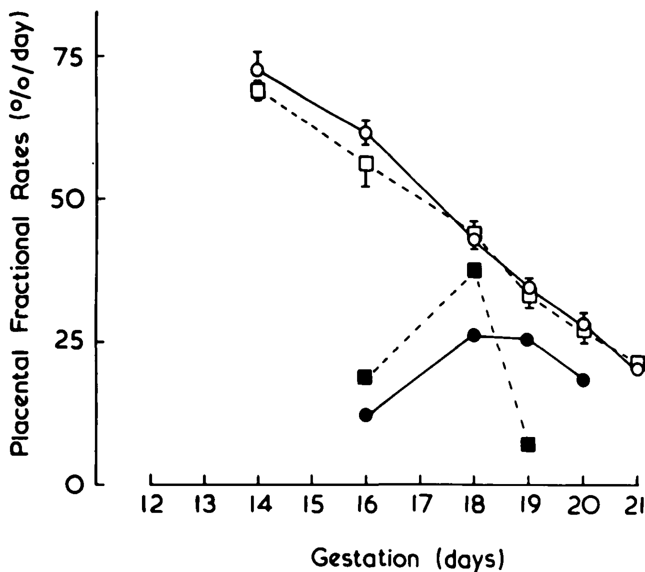

FIG. 6. Rates of protein synthesis and breakdown in placentas derived from control and diabetic pregnancies. Fractional rates of protein synthesis were measured in vivo in same placentas studied in Figs. 4 and 5, i.e., those of control (○) and established-diabetic (□) animals (8). Where possible, degradative rates were also calculated in the control (●) and established-diabetic (■) placentas (see MATERIALS AND METHODS).

the slowing of placental growth toward full term. The situation was, however, different in the diabetic pregnancies. Initially, when the placentas from diabetic pregnancies were smaller than those of the controls (Figs. 4 and 5), the rate of degradation was higher than control values (Fig. 6). This situation was subsequently reversed at day 19 (Fig. 6). It would appear, therefore, that changes in the rate of protein breakdown are probably responsible for the differences in placental size as observed in normal and diabetic pregnancies.

DISCUSSION

In late gestation and at full term, the placentas of diabetic rats were found to be significantly larger (Fig. 4), possessing more protein (Fig. 5), RNA and DNA (Table 1), and glycogen (Table 2) compared with age-matched controls. Placentas from both established-diabetic and gestational-diabetic mothers were essentially similar in these respects, despite the shorter period of hyperglycemia in the latter group. As such, these observations confirm earlier studies describing placentomegaly and a greater glycogen deposition within the basal zone of both diabetic humans (16) and experimental animals (4,6). However, no investigations have reported on earlier stages of placental growth, either in situations of normal or disturbed blood glucose concentrations.

This study revealed that earlier in gestation the placentas of diabetic rats were significantly smaller than controls, possessing less protein (Fig. 5) and RNA and DNA (Table 1) and probably a reduced cell number. The only exception to this consistent trend concerned the storage of glycogen, which was already elevated in the diabetic placentas at day 16 (Table 2). Furthermore, the glycogen concentration did not progressively decline with advancing gestation as normally occurs (4). At 16 days of gestation, protein synthesis was slightly, but not significantly, decreased while protein breakdown increased (Fig. 6). Such changes in protein turnover are similar to those observed in other tissues of the body, e.g., muscle, when the anabolic effects of insulin are removed (17,18). Although insulin receptors have been identified in the placenta, their precise function(s) is unknown. For example, insulin does not appear to influence the transport of either amino acids or glucose across the placenta. The question therefore arises as to whether placental growth can be regulated via these insulin receptors. Although the induced changes in protein turnover described at day 16 of pregnancy could be interpreted as being consistent with an insulin-related action on placental growth, the events at day 19 (i.e., the reverse) are not. Yet, clearly at both stages of gestation, the diabetic mothers are deficient in insulin. Hence, the functional significance of the insulin receptors remains uncertain.

In previous studies, little mention has been made of how the widely varying doses (2–8 U/day) of long-acting insulins were chosen and how effective they were in restoring and maintaining normoglycemia. In this study, throughout the 3 wk of pregnancy the insulin-replacement therapy proved successful in restoring normal blood glucose levels, this being achieved within 6 h of the initial administration (8; Fig. 1). Further indicating good control, glycosylated hemoglobin (8) and placental glycogen concentrations (Table 2) were maintained at levels similar to those found in normal controls. Despite this, the insulin treatment was only partially successful in preventing the placentomegaly (Figs. 4 and 5).

Several lines of evidence have been presented pointing to genuine differences in the growth and overall size of the placentas from diabetic pregnancies. Although trapped plasma components would inevitably influence the wet weight and protein content of the placentas, these trapped elements would presumably be in proportion to the changes in organ size. This conclusion is supported by the fact that the protein composition of the placentas was unchanged at 13% in both the control and diabetic placentas. The differences in placental DNA, and hence cellular hyperplasia, also provide further evidence to support genuine differences in organ size, especially because little of this DNA could have been derived from humeral components (Table 1).

Few studies have measured protein turnover in the placenta (19,20), and none have measured it during the developmental growth of this organ. The progressive decline in the placental rates of protein synthesis and protein degradation is, however, similar to that found in many other tissues of the body during prenatal or postnatal life or both (10,11,21; Fig. 6). Presumably these changes represent a gradual decline in tissue differentiation as well as a slowing of growth per se as the two rates converge. The primary differences in placental size in the diabetic pregnancies appear to reside in the alterations of the degradative rates, i.e., initially higher (day 16) and then lower (day 19) than in the control organs. Precisely what factor(s) associated with the diabetic state might be responsible for these interesting changes is not clear at this time.

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