0031-3998/94/3502-0376$03.00/0
PEDIATRIC RESEARCH

Vol. 35, No. 2, 1994
Printed in U.S.A.

Effect of Diabetes Mellitus on Maternofetal Flux of Calcium and Magnesium and Calbindin$_{9K}$ mRNA Expression in Rat Placenta

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ABSTRACT. The effect of maternal diabetes mellitus on placental unidirectional maternofetal flux of calcium (Ca) and magnesium (Mg), calbindin$_{9K}$ mRNA expression, and net fetal Ca and Mg accretion has been investigated using control (C), untreated diabetic (D_O) and insulin-treated diabetic (D_I) rats. Unidirectional maternofetal flux of Ca in the D_O group was 61 and 63% of the value of the C and D_I groups; unidirectional maternofetal flux of magnesium in the D_O group was 79 and 66% of the value in the C and D_I groups. Fetal Ca and Mg content (mmol; mean ± SEM) was also significantly lower in the D_O group compared with the other two groups (0.111 ± 0.004 *versus* 0.153 ± 0.008 in C and 0.168 ± 0.007 in D_I, $p < 0.01$ D_O *versus* C and D_I for Ca; and 0.021 ± 0.001 *versus* 0.027 ± 0.001 in C and 0.031 ± 0.001 in D_I, $p < 0.01$ D_O *versus* C and D_I for Mg). However, only Ca content was significantly lower in the D_O group when normalized to fetal ash weight. Densitometric analysis of autoradiograms after Northern hybridization with cDNA probes demonstrated that the placental calbindin$_{9K}$/cyclophilin mRNA ratio was 11- to 12-fold lower in the D_O group compared with the C and D_I groups. Collectively, the data suggest that untreated maternal diabetes mellitus reduces fetal Ca and Mg accretion by an effect on the expression of placental transport components involved in the maternofetal transfer of these cations. (*Pediatr Res* 35: 376–381, 1994)

Abbreviations

Ca, calcium
Mg, magnesium
$^{Mg}J_{mf}$, unidirectional maternofetal flux of magnesium
$^{Ca}J_{mf}$, unidirectional maternofetal flux of calcium
$^{Ca}K_{mf}$, unidirectional maternofetal clearance of calcium
$^{EDTA}K_{mf}$, unidirectional maternofetal clearance of Cr-EDTA
C, control group
D_I, insulin-treated diabetic group
D_O, untreated diabetic group
Cr-EDTA, chromium EDTA

Infants of mothers with diabetes have an increased incidence of neonatal hypocalcemia (1–5) and hypomagnesemia (4, 6) and are reported to have a lower bone mineral (7) content when compared with normal infants. A reduced bone mineral content and/or retarded skeletal development are also found in the offspring of both genetically diabetic rats (8, 9) and those rendered diabetic by streptozotocin (10, 11). The cause of these disturbances is unknown but is likely to be multifactorial, including defective fetal skeletal mineralization, abnormal production and/or function of Ca- and Mg-regulating hormones, and altered placental transport of Ca and Mg. We have investigated the latter possibility in this study.

Ca is transported across the rat and human placenta by way of an energy-requiring transcellular route (12–14). By analogy with the gut (15), this route probably has three components: *1*) an energy-independent influx of Ca^{2+} from maternal plasma to trophoblast cytosol across the maternal facing plasma membrane, possibly using a facilitated carrier in human placenta (16); *2*) translocation across the trophoblast cytosol bound to a binding protein; calbindin$_{9K}$ is a likely candidate (17); and *3*) active extrusion across the fetal-facing plasma membrane by a high-affinity Ca^{2+} ATPase (18). Evidence indicates that in the rat placenta translocation bound to calbindin$_{9K}$ may be a rate-limiting step (17), as is also found in the small intestine (15). This finding is of interest because Verhaeghe *et al.* (19) have previously reported that calbindin$_{9K}$ is reduced in the placentas of diabetic rats. The primary aim of this study was to determine by direct measurement whether diabetes mellitus results in a decrease in $^{Ca}J_{mf}$ across the rat placenta and whether any such change occurs in parallel with a decreased calbindin$_{9K}$ mRNA expression.

The mechanisms of placental Mg^{2+} transfer are less well understood, although in the rat it appears to be an active process with at least one component that is Na^+-dependent (20, 21). It has been postulated that the hypocalcemia of infants born to mothers with diabetes might be secondary to a reduction in parathyroid sensitivity to plasma Ca caused by hypomagnesemia (6, 22). We have therefore also investigated placental $^{Mg}J_{mf}$ in this study.

MATERIALS AND METHODS

All work carried out in this study was in accordance with the U.K. Animals (Scientific Procedures) Act 1986.

Animals. Female Sprague-Dawley rats ages 6–8 wk were rendered diabetic by an intraperitoneal injection of 60 mg·kg^{-1} streptozotocin (Sigma, Poole, UK) dissolved in citrate buffer (pH 6.8). C rats received an equivalent volume of citrate buffer alone. Diabetes mellitus was confirmed 2 d later by measurement of plasma glucose concentration (>15 mmol·L^{-1}) from a tail vein sample using a glucose oxidase kit (Sigma). Some diabetic rats (D_I) then received once-daily s.c. injections (4–16 units) of a

Received June 10, 1993; accepted October 14, 1993.
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Supported by an Action Research Endowment Fund.
Parts of this work have appeared elsewhere in abstract form (see References 23–25).
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heat-treated long-acting bovine insulin (Novo Nordisk; supplied by Ladybee Ltd., Horam, UK) to maintain their plasma glucose concentration below 10 mmol·L^{-1}. The remaining diabetic rats (D_O) and C rats received daily s.c. injections of equivalent volumes of sterile water. Three days later the female rats were caged with males, and the day on which a vaginal copulation plug was found was designated d 1 of gestation (term = 23 d). Maternal plasma glucose concentration in tail vein samples was measured periodically throughout gestation and the dose of insulin adjusted accordingly.

Measurement of unidirectional maternofetal clearance and flux. On d 21 of gestation the rats were initially anesthetized with ether followed by an intraperitoneal injection of 100 mg·kg^{-1} amylobarbitone sodium (Sodium Amytal, Eli Lilly, Basingstoke, UK) and the trachea, a jugular vein (for injection of radioisotopes), and a carotid artery (for maternal blood pressure monitoring and blood sampling) were cannulated. The rat was then immobilized on its back in a temperature-controlled (37°C) bath of isotonic saline, and after laparotomy and hysterotomy one fetus was delivered. The procedure for perfusing the placenta through the fetal circulation was similar to that previously described (20, 26). Briefly, the umbilical artery was cannulated with a 20-gauge steel needle attached to silastic tubing, the umbilical vein was then cannulated with a 24-gauge Abbocath catheter (Abbott Laboratories, Queenborough, UK). The cannulas in both vessels were tied together and incorporated in a suture used to occlude the vitelline vessels. The fetal circulation of the placenta was perfused (0.5 mL·min^{-1}) with Mg-free Krebs Ringer solution [to allow measurement of transfer of Mg as described previously (20)] containing NaCl (118 mM), KCl (4.7 mM), KH_2PO_4 (1.18 mM), $NaHCO_3$ (24.9 mM), $CaCl_2$ (1.4 mM), 0.2% D-glucose, 3.4% dextran (MW 40 000), pH 7.4 adjusted by continuous gassing with 95% O_2 and 5% CO_2. The solution was warmed to 37°C and perfusion pressure monitored by way of a side arm in the arterial catheter. Radioisotopes of calcium (10 μCi ^{45}Ca (0.1 mL), 0.071 mg $CaCl_2$·mL^{-1}) and Cr-EDTA (50 μCi ^{51}Cr-EDTA (0.5 mL), 0.1 mg Cr-EDTA·mL^{-1}) (Amersham International, Amersham, UK), used to measure paracellular permeability (26), were injected at time 0 into the maternal circulation, and maternal arterial blood samples were collected into heparinized tubes at 2, 12, 24, and 36 min. The samples were centrifuged immediately and the plasma separated. From 3 to 35 min consecutive 4-min collections of fetal venous effluent were made. Only one placenta was perfused in each pregnant rat.

At the end of the perfusion experiment, remaining unperfused placentas and fetuses were rapidly harvested, trimmed of membranes, and stored at either −20°C for determination of fetal Ca and Mg content or −80°C for measurement of placental calbindin$_{9K}$ mRNA. The pregnant animal was killed by an overdose of anesthetic, and harvested fetuses were killed by cervical dislocation.

Aliquots of plasma (50 μL) and perfusate collections (0.4 mL) were analyzed for ^{45}Ca (Packard Tricarb 2000CA, Canberra Packard, Pangbourne, UK) and ^{51}Cr-EDTA (Packard Autogamma 800). Maternal plasma Mg and Ca concentrations and Mg concentration in effluent perfusate were measured by atomic absorption spectrophotometry (Perkin Elmer 2380, Beaconsfield, UK) with appropriate dilution with $LaCl_3$ to obtain Mg and Ca concentration within the linear working range of the spectrophotometer. The criteria for a successfully perfused placenta were as previously described (26).

For each 4-min collection period, $^{Ca}K_{mf}$ and $^{EDTA}K_{mf}$ were calculated as (27):

$$K_{mf} = \frac{[v]\cdot Q}{[A]\cdot W}$$

where [v] is the venous effluent radiolabel concentration, Q is the perfusate flow rate (μL·min^{-1}), [A] is the maternal plasma radiolabel concentration at the midpoint of the perfusate collection period (interpolated from the disappearance curve of the radiolabel in maternal plasma), and W is the placental wet weight trimmed of membranes in grams.

The unidirectional maternofetal flux of Ca was calculated as $^{Ca}J_{mf} = {}^{Ca}K_{mf}\cdot[Ca]$ where [Ca] is the mean total Ca concentration in maternal plasma measured at the beginning and the end of the experiment. The $^{Mg}J_{mf}$ for each 4-min collection period was calculated from the efflux of Mg from placenta into perfusate as $^{Mg}J_{mf} = ([v] - [a])\cdot Q$ where [v] and [a] are Mg concentrations in perfusate outflow, and inflow and Q is as defined above. The mean $^{EDTA}K_{mf}$, $^{Ca}J_{mf}$, and $^{Mg}J_{mf}$ values during 3–35 min were taken to represent the value for each placenta studied; it has previously been shown that all three are in steady state during this period (20), and this was confirmed in the present study (data not shown).

Measurement of fetal Ca and Mg content. Four fetuses from each animal were thawed and dried at 100°C for 48 h before being ashed at 700°C for 16 h in a furnace (Gallenkamp, Loughborough, UK). Fetal ash was weighed, dissolved in 3 mL HCl, and total volume made up to 10 mL with distilled water. Ca and Mg were measured in duplicate by atomic absorption spectrophotometry as detailed above. The mean values for the four fetuses were taken to be the value of fetal Ca and Mg content for each pregnancy.

Measurement of calbindin$_{9K}$ mRNA. Calbindin$_{9K}$ mRNA was measured as described previously (17). Briefly, tissue was thawed at room temperature in guanidinium thiocyanate buffer and total RNA extracted as described by Chomczynski and Sacchi (28). The RNA pellet was dissolved in water, quantified by measuring absorbance at 260 nm, and stored at −80°C.

A 460-bp cDNA probe, complementary to an mRNA coding for rat intestinal calbindin$_{9K}$ (29), and a 680-bp cDNA probe, complementary to a mRNA coding for rat cyclophilin (30), were the generous gifts of Dr. M. Thomasset (Institut National de la Santè et de la Recherche Mèdicale, Paris, France) and Dr. K. Thornburg (Oregon Health Sciences University, Portland, OR), respectively. Cyclophilin mRNA is expressed in almost all tissues of the rat (30) and was used as a control for nonspecific differences. Both probes were labeled with ^{32}P by random priming (31) and separated from unincorporated nucleotides by chromatography on a Sephadex G-50 microcolumn.

Total RNA (15 μg) was electrophoresed on a 1.2% agarose gel under denaturing conditions overnight [~18 h (32)] at 25 V in 1 × MOPS buffer (pH 7.0). The RNA was then transferred from the agarose gel to a nylon membrane (Hybond N, Amersham International) using 20 × standard saline citrate (33) and fixed there by exposure to UV light. The membranes were prehybridized in buffer (50% formamide, 5 × standard saline citrate, 5 × Denhardt's solution, 50 mM Na_2HPO_4 (pH 6.8), 0.2% SDS, 10% dextran sulfate, and 300 μg·mL^{-1} heat-denatured tRNA) for 18 h at 42°C. The hybridization buffer consisted of the above buffer except that only 100 μg·mL^{-1} tRNA was included plus the ^{32}P-cDNA probe. After hybridization (18 h at 42°C), the filters were washed twice at room temperature in 2 × standard saline citrate and 0.1% SDS for >30 min, followed by a final wash in the same solution at 60°C (65°C for cyclophilin) for 20 min. The blots were autoradiographed at −80°C with Fuji RX100 film. The autoradiograms were analyzed by spectrodensitometry (Shimadzu Scientific Instruments, Kyoto, Japan) and the area under the curve computed. From preliminary studies, we had determined that under these conditions the hybridization signals were within the linear range of the film used.

Statistics. All data are presented as means ± SEM, with n being the number of animals. Unpaired t test or Duncan's multiple range tests were used as appropriate to assess significance between groups.

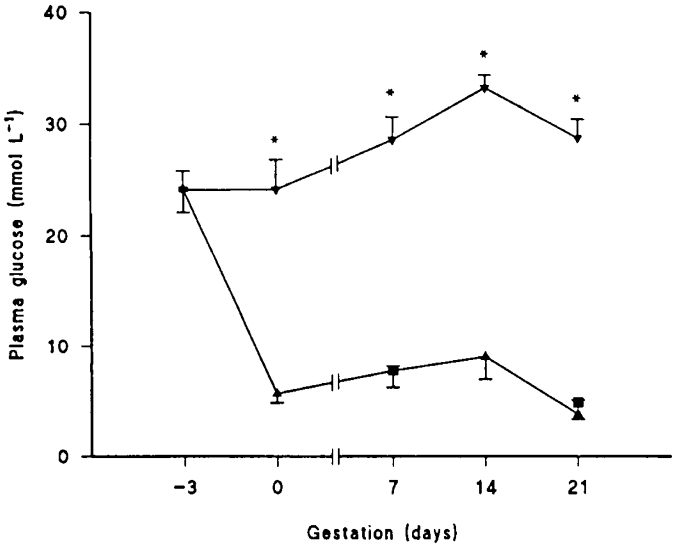

Fig. 1. Maternal plasma glucose concentration in C (■, n = 14; measured only on d 7 and 21), D_O (▼, n = 11), and D_I (▲, n = 8) rats before and during gestation. Rats were injected with streptozotocin or vehicle 5 d before being allowed to mate (d 0). Data are means ± SEM. *$p < 0.001$ *vs* C and D_I by unpaired t test on d −3, 0, and 14, and $p < 0.01$ by Duncan's multiple range tests on d 7 and 21.

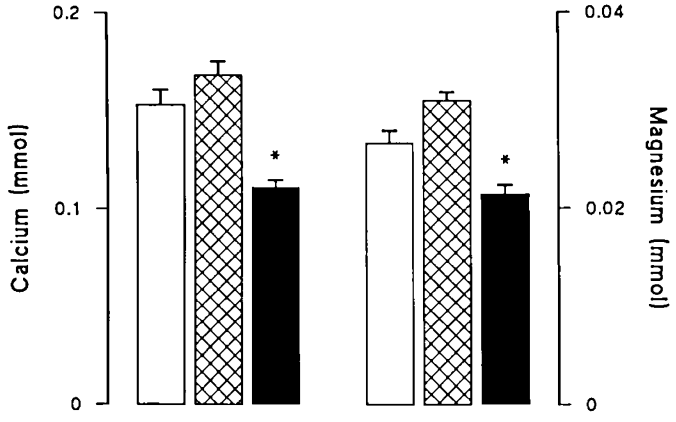

Fig. 2. Total fetal Ca and Mg content in C (*open bars*, n = 14), D_I (*hatched bars*, n = 8), and D_O (*solid bars*, n = 11) rats on d 21 of gestation. Four fetuses were used from each animal; n is the number of pregnancies. Data are means ± SEM. *$p < 0.01$ *vs* C and D_I by Duncan's multiple range tests.

RESULTS

Two days after streptozotocin injection, mean plasma glucose concentration in the diabetic rats was 24.2 ± 1.3 mmol·L^{-1}[11]. Thereafter, mean plasma glucose concentration in the D_I group was maintained below 10 mmol·L^{-1} and compared favorably with the glucose concentration in the C group, which was measured only on d 7 and 21 of gestation (Fig. 1). By contrast, D_O rats remained hyperglycemic throughout gestation (Fig. 1).

Fetal weights and Ca and Mg content. Fetal wet and ash weight, measured in grams, was significantly lower in the D_O group compared with C and D_I; in C, D_I, and D_O groups fetal wet weight was 3.51 ± 0.09 g, 3.45 ± 0.18 g, and 2.88 ± 0.10 g ($p < 0.01$ D_O *versus* C and D_I), and ash weight was 0.049 ± 0.002 g, 0.051 ± 0.001 g, and 0.039 ± 0.001 g ($p < 0.01$ D_O *versus* C and D_I), respectively. Ca and Mg content in ashed fetuses was significantly lower in the D_O group compared with the C and D_I groups ($p < 0.01$) (Fig. 2).

Fetal Ca content remained significantly lower in the D_O group compared with the other two groups even when it was normalized to ash weight (Fig. 3). However, Mg content per unit ash weight was not significantly different between the three groups.

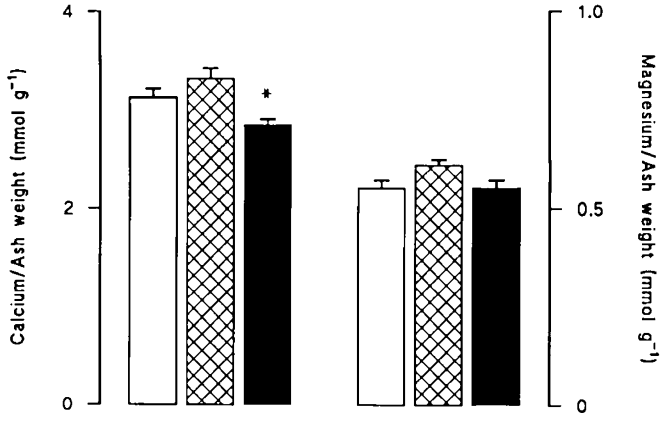

Fig. 3. Fetal Ca and Mg content normalized to ash weight in C (*open bars*, n = 14), D_I (*hatched bars*, n = 8), and D_O (*solid bars*, n = 11) rats on d 21 of gestation. Four fetuses were used from each animal; n is the number of pregnancies. Data are means ± SEM. *$p < 0.05$ *vs* C and < 0.01 *vs* D_I by Duncan's multiple range tests.

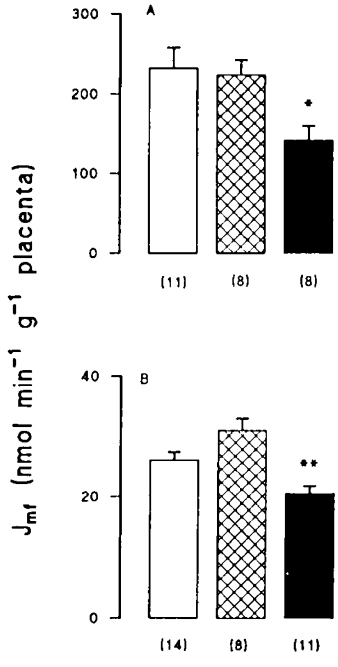

Fig. 4. Unidirectional maternofetal flux (J_{mf}) per gram placenta of Ca (*A*) and Mg (*B*) in C (*open bars*), D_I (*hatched bars*), and D_O (*solid bars*) rats on d 21 of gestation. Number of animals is shown in parentheses. Data are means ± SEM. *$p < 0.05$ *vs* C and D_I; **$p < 0.05$ *vs* C and < 0.01 *vs* D_I by Duncan's multiple range tests.

Unidirectional flux and clearance measurements. Both $^{Ca}J_{mf}$ and $^{Mg}J_{mf}$ were significantly lower in the D_O group than in C or D_I groups (Fig. 4). $^{Ca}J_{mf}$ in the D_O group was 61 and 63% of the values in C and D_I groups, respectively, and $^{Mg}J_{mf}$ in the D_O group was 79 and 66% of the values in C and D_I groups, respectively. By contrast, $^{EDTA}K_{mf}$ was not significantly different between the groups (Fig. 5). Furthermore, flux measurements normalized to fetal weight were also not different between the three groups (data not shown).

No significant difference was observed between the three groups in terms of mean maternal plasma Ca and Mg concentrations on d 21 of gestation (Table 1). The mean wet weight of the *in situ* perfused placentas in the D_O group was significantly

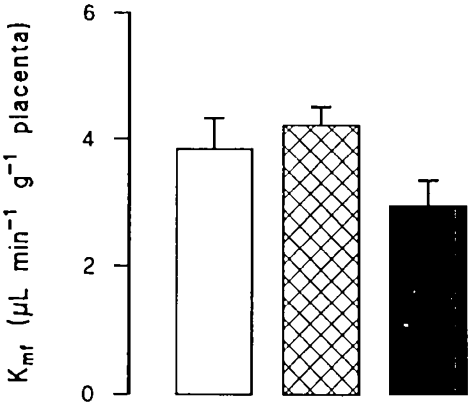

Fig. 5. Unidirectional maternofetal clearance (K_{mf}) of ^{51}Cr-EDTA per gram placenta in C (*open bars*, n = 14), D_I (*hatched bars*, n = 8), and D_O (*solid bars*, n = 11) rats on d 21 of gestation. Data are means ± SEM; n is the number of animals.

Table 1. *Plasma Ca and Mg concentration and placental weight on d 21 of gestation**

	C	D_I	D_O
Ca (mmol·L^{-1})	2.01 ± 0.04 (11)	2.06 ± 0.05 (8)	2.03 ± 0.05 (8)
Mg (mmol·L^{-1})	0.73 ± 0.02 (14)	0.73 ± 0.02 (8)	0.73 ± 0.03 (11)
PW (g)	0.47 ± 0.02 (14)	0.41 ± 0.03 (8)	0.48 ± 0.02† (11)

* Maternal plasma Ca and Mg concentration and weight of perfused placenta (PW) is shown in C, D_I, and D_O groups on d 21 of gestation. Number of animals is shown in parentheses. Values are means ± SEM.
† $p < 0.05$ *vs* D_I (Duncan's multiple range tests).

greater than that in the D_I group but was not different to the value in the C group (Table 1).

Calbindin$_{9K}$ mRNA levels. Northern-blot hybridization of rat placental RNA with calbindin$_{9K}$ cDNA revealed a single transcript of 0.44 kb in all three groups. Densitometric analysis showed a 12- to 15-fold reduction of calbindin$_{9K}$ mRNA in the D_O group compared with the C and D_I groups (Fig. 6*A*). Reprobing with cyclophilin cDNA revealed a single transcript of 0.69 kb in all three groups. As with calbindin$_{9K}$, cyclophilin expression was lower in the D_O group but only by 1- to 2-fold (Fig. 6*B*). However, when calbindin$_{9K}$ mRNA expression was normalized to cyclophilin mRNA expression, an 11- to 12-fold reduction still existed in the D_O group compared with the other two groups (Table 2). Similar results were obtained using a β-actin cDNA probe instead of cyclophilin (data not shown).

DISCUSSION

Maternofetal flux of Ca and Mg and placental calbindin$_{9K}$ mRNA expression. In this study, the values obtained for $^{Ca}J_{mf}$, $^{Mg}J_{mf}$, and $^{EDTA}K_{mf}$ in the C group are in agreement with previous observations (12, 20, 26). In the presence of untreated maternal diabetes mellitus, both $^{Ca}J_{mf}$ and $^{Mg}J_{mf}$ were reduced by between 20 and 40% (Fig. 4) to values significantly lower than in the C group. These alterations in placental transport of Ca and Mg were prevented by control of the diabetes with insulin. Because unidirectional maternofetal clearance values for Ca and Mg are several-fold greater than $^{EDTA}K_{mf}$ whereas their diffusion coefficients in water are similar (20), it is unlikely that any difference in paracellular permeability between the three groups (Fig. 5) could explain the effect of maternal diabetes mellitus on $^{Ca}J_{mf}$ and $^{Mg}J_{mf}$. Furthermore, maternal plasma total Ca and Mg concentrations were not different between the three groups (Table 1), making it unlikely that reduced cation availability in the D_O group led to a lower unidirectional maternofetal flux. Placental wet weight was significantly greater in the D_O group compared with the D_I group, but we found that placental protein and DNA content was not different between the three groups (data not shown). When the data were analyzed by expressing maternofetal flux or clearance per placenta, maternofetal flux of Ca in the D_O group was not different compared with the D_I group but was still significantly lower than in the C group. Calculated in this way, Mg flux was still significantly lower in D_O compared with either C or D_I, and Cr-EDTA clearance was not different between the three groups.

The D_O group of placentas had a 12- to 15-fold lower calbindin$_{9K}$ mRNA expression, whereas cyclophilin mRNA (coding for a protein unrelated to transport) was only 1- to 2-fold lower than C or D_I groups. This effect on calbindin$_{9K}$ expression confirms and extends the data of Verhaeghe *et al.* (19), who measured placental calbindin$_{9K}$ protein and found a 20% reduction in diabetic rats compared with controls. Taken together, these data suggest that diabetes mellitus in the rat leads to a reduction in expression of components of placental Ca and Mg transporting mechanisms.

Fetal Ca and Mg content. Both fetal wet weight and ash weight were lower in the D_O group compared with C and D_I groups. This effect of diabetes mellitus on fetal growth is a consistent finding in the rat irrespective of whether the disease is induced with streptozotocin or is genetic (8–11). It is thought to be caused by fetal hyperglycemia leading to a depletion of insulin from the fetal pancreas and therefore a lack of this growth factor (34). This situation is analogous to the pancreatectomized fetal sheep, which is also growth retarded (35). Whatever the cause of this phenomenon, it does contrast with the situation in the pregnant woman with diabetes, whose offspring tend to be overgrown at term (36), although there is a suggestion that in the first trimester of such pregnancies, the fetus is growth retarded and more similar to the condition in rats (37).

Nevertheless, even allowing for the reduced weight of the fetuses, the Ca content, but not the Mg content, of the fetuses of the D_O group was significantly lower than that of the other two groups (Fig. 3). This finding is consistent with previous reports of a lower bone mineral content of fetuses of women and rats with diabetes (7–9, 11). Thus, the net flux of Ca across the diabetic rat placenta is reduced to a greater degree than that expected from the growth retardation alone. This situation is quite different from that in which growth retardation is induced in the rat by reducing uterine blood flow where net Ca flux is appropriate for fetal size (13).

Net flux of any solute across the placenta is equal to the sum of the unidirectional maternofetal and fetomaternal fluxes (27). Although unidirectional maternofetal flux for Ca appears to be larger than unidirectional fetomaternal flux in the rat, the latter is still significant (12). Therefore, the decreased Ca content of the diabetic rat fetus (Fig. 2) could be a result of either a decreased unidirectional maternofetal flux or an increased unidirectional fetomaternal flux. The unidirectional maternofetal flux was significantly lower in this group when expressed per gram placenta (Fig. 4). However, when expressed per gram fetal wet or ash weight a significant difference no longer existed (data not shown). This finding suggests that unidirectional maternofetal flux was appropriate for the size of the fetus in the diabetic group and that the decrease in the normalized Ca content was due to an increase in unidirectional fetomaternal flux, which was not measured in the present experiments. However, it is also possible that the precision of measurement of unidirectional maternofetal flux was not adequate to measure the small additional decrease required to explain the lower normalized Ca content.

Whether it was related to the reduced size of the fetus, the lower fetal mineral content in the untreated diabetic group, or both, the lower placental calbindin$_{9K}$ expression in this group is of considerable interest. It is the 2nd time we have found a relationship between the placental flux of Ca and its calbindin$_{9K}$

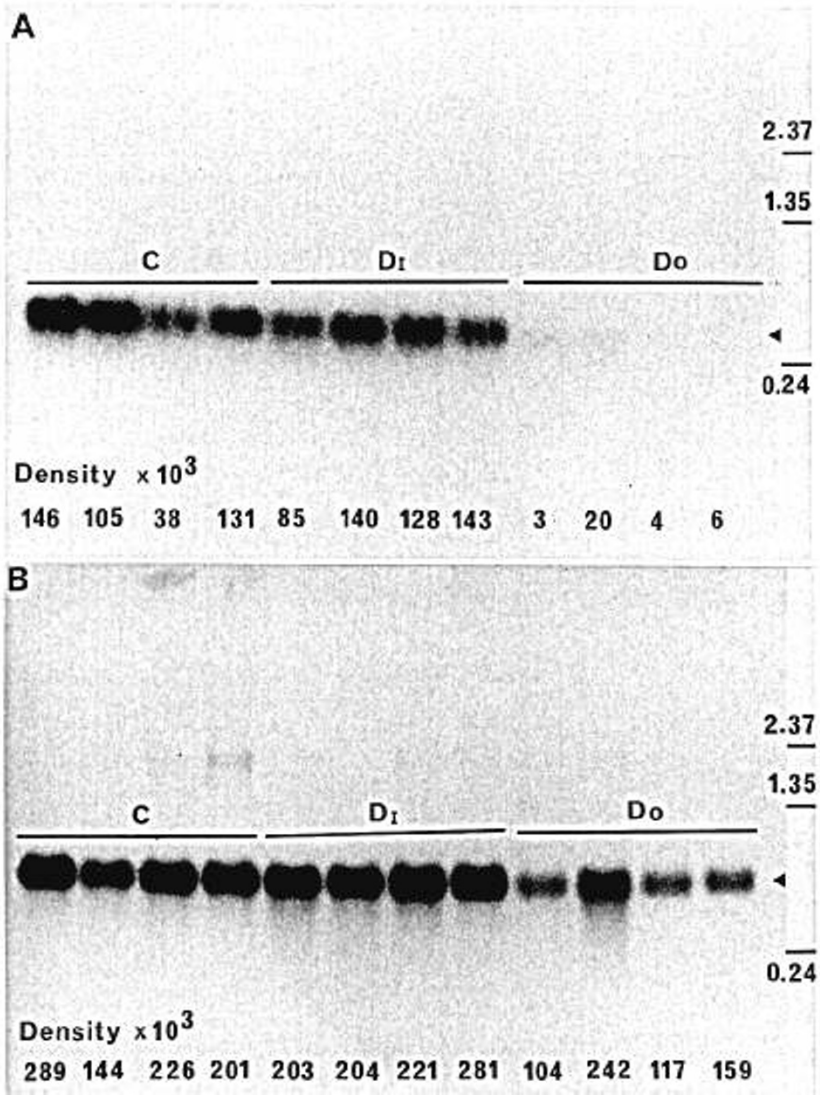

Fig. 6. Northern hybridization of rat placental RNA with ^{32}P-cDNA probes. Single Northern blot probed with calbindin$_{9K}$ (*A*) and cyclophilin (*B*). Equal amounts of RNA (15 μg) were loaded in lanes 1–4 (C), 5–8 (D_I), and 9–12 (D_O). Position of RNA size markers (in kilobase) is shown on right. *Arrows* indicate major RNA species at 0.44 kb (*A*) and 0.69 kb (*B*). Transcript density peak area values (arbitrary units) are given beneath each lane.

Table 2. *Placental calbindin$_{9K}$/cyclophilin mRNA ratio**

	C	D_I	D_O
Ratio	0.51 ± 0.12	0.62 ± 0.12	0.05 ± 0.01†

* Placental calbindin$_{9K}$ mRNA expression normalized to cyclophilin mRNA expression in C, D_I, and D_O groups on d 21 of gestation ($n = 4$ in each group). Values are means ± SEM.

† $p < 0.01$ *vs* C and D_I (Duncan's multiple range tests).

mRNA content (17). The control of placental calbindin$_{9K}$ is not understood. Possibly insulin is involved because it is increasingly recognized as a regulator of gene expression (38) as well as a growth factor (39).

Acknowledgment. The authors thank Professor R. D. H. Boyd for reviewing the manuscript and for his helpful suggestions.

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