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Research Article

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Sodium-Hydrogen Exchange and Glucose Transport in Renal Microvillus Membrane Vesicles from Rats with Diabetes Mellitus

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Abstract

Diabetes mellitus is associated with important changes in renal hemodynamics and transport function. Disturbances in solute transport have also been characterized in nonrenal tissues during hyperglycemia and insulinopenia. The purpose of this study was to determine if diabetes is associated with adaptive changes in function of the brush-border membrane of the proximal tubule. We studied Na^+ and glucose transport in rat microvillus membrane vesicles isolated from the renal cortex of streptozotocin-induced and BB/W autoimmune diabetic rats. Untreated diabetes was associated with an increase in pH-stimulated total and amiloride-sensitive $^{22}\text{Na}^+$ uptake into vesicles. Insulin treatment returned vesicle $^{22}\text{Na}^+$ uptake to control levels. The increased Na^+/H^+ exchange was shown to be a result of increased net renal acid production rather than a specific response to insulinopenia because treatment with NaHCO_3 also returned $^{22}\text{Na}^+$ uptake to control levels. On the other hand, Na^+ -glucose cotransport, which was depressed in vesicles from untreated diabetics, returned to control levels with insulin but not NaHCO_3 administration. This decreased Na^+ -glucose cotransport was not secondary to reduction in transport sites in untreated diabetics. These results show that in diabetes mellitus, increased Na^+/H^+ exchange activity is not the direct result of insulinopenia. However, the diabetic state appears to alter the functioning of the luminal Na^+ -glucose cotransporter.

Introduction

Insulin-dependent diabetes mellitus induces a wide range of pathophysiological alterations. Insulin serves as a modulator of many physiologic processes, and many of the abnormalities seen in diabetes result from abnormal metabolic function that is a direct result of the insulin deficiency. Other disturbances may be the result of the diabetic milieu and may be only indirectly caused by a decrease in circulating insulin.

In poorly controlled diabetes mellitus, with hyperglycemia and insulinopenia, a number of renal changes have been noted. These include increased glomerular filtration rate and renal blood flow (1), generalized renal hypertrophy (2), basement membrane thickening and glomerular damage (3), increased ammoniogenesis and gluconeogenesis (4), and decreased absolute reabsorption of fluid and Na^+ (5). Insulin has been reported to influence renal

handling of Na^+ and solutes by increasing reabsorption of Na^+ in distal nephron segments and increasing phosphate reabsorption in proximal segments (6, 7). In adipose tissue, insulin increases the concentration of plasma membrane glucose carriers (8).

The purpose of the present study was to determine the effects of diabetes mellitus on transport of glucose and Na^+ in isolated rat renal microvillus membrane vesicles. The results indicate that the increased net acid production of untreated diabetes mellitus, whether associated with systemic acidemia or not, is accompanied by an increase in Na^+/H^+ exchange activity in luminal membranes. Furthermore, Na^+ -dependent glucose transport is decreased in membrane vesicles derived from diabetic animals. These results suggest intrinsic changes in proximal tubule transport function in diabetes.

Methods

Animals. Two experimental models of diabetes mellitus were employed in these studies. In the first model, insulin-dependent diabetes was induced in male Sprague Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) by a single intravenous injection of streptozotocin (60 mg/kg). We used only animals that demonstrated hyperglycemia and glycosuria within 48 h after injection. Animals were studied 8–14 d after administration of streptozotocin. An insulin-treated subgroup received a daily subcutaneous injection of 2.5 U ultralente insulin (NOVO; Copenhagen, Denmark) in the afternoon. Another subgroup received drinking water with 75 meq/liter NaHCO_3 added, in an attempt to correct the increased endogenous acid production associated with diabetes. As a control for this group, some diabetic rats received equivalent Na^+ as NaCl in the drinking water.

The second model of diabetes employed was the BB/W autoimmune model of diabetes (9). Male rats were obtained from the breeding colony at University of Massachusetts Medical School, Worcester, MA. The animals studied either manifested insulin-dependent diabetes or were age-matched BB/W rats without evidence of diabetes. Insulin-treated animals received a single afternoon injection of protamine-zinc insulin (PZI; Eli Lilly and Co., Indianapolis, IN), calculated to prevent ketosis. Bicarbonate-treated animals received drinking water supplemented with 250 meq/liter NaHCO_3 .

All animals were fed standard 24% protein rat chow (Ralston Purina Co., St. Louis, MO). Blood gases and serum for glucose determination were obtained on the morning of study. Qualitative serum ketone determination was performed by measurement with ketostix (Miles Laboratories, Inc., Elkhart, IN). Net acid excretion rates were determined for 24-h urine collections (10).

Vesicle preparation. Animals were killed by decapitation or cervical dislocation and the kidneys were promptly removed and weighed. Renal cortical microvillus membrane vesicles were prepared by the Mg^{++} aggregation and differential centrifugation method of Booth and Kenny (11) and Aronson (12). For studies of [^3H]D-glucose uptake and [^3H]phlorizin binding, the microvillus membrane vesicles were prepared and experiments were performed in a buffered isotonic solution containing 300 mM mannitol, 16 mM Hepes, and 10 mM Tris, pH 7.50. The studies of Na^+/H^+ exchange were performed using microvillus membrane vesicles prepared in a highly buffered isotonic solution, con-

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sisting of 270 mM mannitol, 40 mM Hepes titrated to pH 7.50 with KOH. An intravesicular pH of 6.10 was obtained by preequilibration of suspensions of the membrane vesicles diluted 1:1 with a solution consisting of 220 mM mannitol, 20 mM K⁺, 40 mM 2-(*N*-Morpholino)-ethane sulfonic acid (MES), 40 mM Hepes. The brush border membrane marker enzyme, alkaline phosphatase, was assayed using *p*-nitrophenyl phosphate as substrate (Sigma bulletin #104, Sigma Chemical Co., St. Louis, MO) and Na⁺-K⁺ ATPase, a marker of basolateral membranes, was assayed as ouabain-sensitive K⁺ phosphatase (13). The homogenate specific activities for alkaline phosphatase and ouabain-sensitive K⁺ phosphatase were comparable in the membrane preparations from the different experimental groups. As shown in Table I, enzyme specific activity and purification of brush border membrane vesicles in streptozotocin treated diabetes was comparable to control values for Sprague Dawley rats. The activities and enrichment of brush border membrane vesicles in the BB/W diabetic rats was also comparable to control values for BB/W animals. Protein was determined by the method of Lowry et al. (14) or by Bradford (15).

Transport. Timed uptake of radiolabeled solutes at 20°C was assayed by Millipore rapid filtration methods (Millipore Corp., Bedford, MA) (16). Timed incubations were terminated by the addition of 4 ml of an ice cold MgSO₄ solution and then filtered immediately through a pre-wetted 0.65-micron filter (DAWP; Millipore Corp.). The filter was washed with an additional 12 ml of the MgSO₄ solution. Filters were then dissolved in 4.5 ml of Aquasol (New England Nuclear, Boston, MA) and radioactivity was determined by liquid scintillation spectrometry. Nonspecific retention of radioactivity to the filters was subtracted from total counts of the sample. All incubations were performed in triplicate using fresh membrane preparations. Tracer [³H]D-glucose uptakes were performed in the presence of 0.35 μM [³H]D-glucose. Uptake of [³H]phlorizin was assayed by the method of Aronson (12). 10 μl of microvillus membrane suspension was incubated at 20°C with 40 μl of a mannitol solution buffered with 10 mM Tris, 16 mM Hepes, pH 7.50, containing 50 mM Na₂SO₄ and [³H]phlorizin concentrations of 0.05–100 μM (4 × 10⁴ cpm). Preliminary experiments revealed that equilibrium binding of 0.05 μM phlorizin in the presence of 50 mM Na₂SO₄ occurred between 10 and 30 min and remained stable for at least 60 min. Therefore, incubations for binding assays were performed for 40 min. Incubations

were terminated by addition of an ice cold solution containing 150 mM NaCl, 10 mM MgSO₄, 1 mM Tris, 1 mM Hepes, pH 7.50. The rapid filtration process described above was employed.

Statistics. Results are expressed as mean ± SEM for at least three separate experiments on different membrane preparations. Student's unpaired *t* test was used. In multiple group comparisons, one way analysis of variance and the Bonferroni *t* test were employed.

Materials. ²²Na⁺, [³H]D-glucose (31 Ci/mmol), [³H]L-alanine (108 Ci/mmol), and [³H]phlorizin (56 Ci/mmol) were obtained from New England Nuclear. Amiloride was a gift of Merck, Sharp, and Dohme, Inc. (West Point, PA).

Results

In early (7–14 d) streptozotocin-induced diabetes, animals that were not treated with insulin experienced weight loss and profound hyperglycemia (Table II). Treatment with the ultralente insulin preparation significantly reduced the blood glucose levels (474 ± 24 vs. 186 ± 24 mg/dl, *P* < 0.005), although not completely to control. In addition, insulin treatment prevented the weight loss noted in the untreated diabetics. Untreated diabetic rats developed significant renal hypertrophy. Whole kidney weight increased from an average control value of 3.14 ± 0.06 g to 3.69 ± 0.11 (*P* < 0.005). Kidney weight as a percentage of body weight was also increased in diabetics (1.07 ± 0.03% vs. 0.82 ± 0.02; *P* < 0.005). Insulin-treated rats sustained smaller but significant increases in whole kidney weight; values corrected for body weight were slightly greater than control values (0.87 ± 0.02% vs. 0.82 ± 0.02). NaHCO₃-treated diabetic rats underwent less gain of renal weight than untreated diabetics (3.35 ± 0.12 g vs. 3.69 ± 0.11, *P* < 0.05). Although untreated diabetic rats developed significant ketonemia, metabolic acidosis was not present (pH 7.43 ± 0.02 in control vs. 7.42 ± 0.03 in diabetic rats). Urinary excretion rates of net acid were increased in the streptozotocin-treated animals (4.90 ± 0.9 meq/dl vs. 1.50 ± 0.38, *P* < 0.005).

Table III provides similar data for the BB/W rats. These rats

Table I. Enzyme Activity and Purification of Brush Border Membrane Vesicle Preparations of the Experimental Groups

	Control	Streptozotocin-induced diabetes	
		No treatment	+ insulin
Alkaline phosphatase (n)			
Specific activity (μmol pNPP/mg protein per min)	0.52 ± 0.06	0.63 ± 0.07	0.58 ± 0.06
Enrichment	10.1 ± 0.8 } (14)	9.4 ± 0.9 } (14)	10.5 ± 0.6 } (8)
Ouabain-sensitive ATPase (n)			
Specific activity (μmol pNPP/mg per min)	0.0039 ± 0.0007	0.0024 ± 0.0004	0.0054 ± 0.0008
Enrichment	0.66 ± 0.08 } (14)	0.50 ± 0.05 } (14)	0.71 ± 0.13 } (8)
BB/W			
	Control	No treatment	+ insulin
Alkaline phosphatase (n)			
Specific activity (μmol pNPP/mg per min)	0.30 ± 0.02	0.30 ± 0.02	0.30 ± 0.03
Enrichment	7.3 ± 0.3 } (9)	8.1 ± 0.8 } (7)	7.9 ± 0.8 } (9)
Ouabain-sensitive ATPase (n)			
Specific activity (μmol pNPP/mg per min)	0.0031 ± 0.0010	0.0030 ± 0.0008	0.0023 ± 0.0007
Enrichment	0.52 ± 0.07 } (9)	0.57 ± 0.03 } (7)	0.38 ± 0.09 } (9)

Specific activity in vesicle preparation is expressed as μmol *p*-nitrophenyl phosphate (pNPP) hydrolyzed/mg membrane protein per min at 20°C for alkaline phosphatase and 37°C for ATPase. Enrichment factor refers to activity in vesicle preparation compared with activity in the whole cortex.

Table II. Physiologic Parameters in Streptozotocin-induced Diabetes Mellitus

	Control	Diabetes		
		No treatment	+ insulin	+ NaHCO ₃
Body weight (g)	386±6 (30)	343±7‡ (25)	391±12 (30)	353±13 (14)
Kidney weight (g)	3.14±0.06 (30)	3.69±0.11‡ (25)	3.51±0.08‡ (30)	3.34±0.12‡ (14)
Kidney weight, % Body weight	0.82±0.02	1.07±0.03‡	0.87±0.02*	0.96±0.03‡
Blood				
Glucose (mg/dl)	131±6 (7)	474±24‡ (25)	186±24 (30)	364±70* (14)
pH	7.43±0.02 (7)	7.42±0.03 (8)	7.43±0.01 (6)	7.38±0.03 (9)
pCO ₂ (mmHg)	39±3	36±3	42±2	48±5
HCO ₃ (mEq/liter)	25±2	24±2	28±1	28±2
Ketones (mg/dl)	0±0 (11)	12±3‡ (10)	0±0 (4)	14±5‡ (5)

Body weight, kidney weight, and glucose concentrations were determined for all diabetic animals used in transport studies. Arterial blood gases, and ketone measurements were performed on a subset of control and diabetic rats used in vesicle studies. The number of animals, *n*, is indicated in parenthesis.
 * *P* < 0.05 compared with control.
 ‡ *P* < 0.005 compared with control.

were diabetic for 79±14 d, a significantly longer period than were the streptozotocin-treated animals. They were maintained on insulin after the onset of hyperglycemia. Insulin was withheld from the animals in the untreated and bicarbonate-treated groups for 48–72 h before sacrifice. These two subgroups both experienced significant weight loss (untreated, 14±2%; bicarbonate-treated, 9±2%). Kidney weights in the untreated and bicarbonate-treated groups were not significantly different from control. However, note that the animals in these two subgroups were smaller to begin with than the animals in the control and insulin-treated groups. Thus, kidney weight corrected for body weight was markedly increased in all hyperglycemic animals. The daily insulin treatment was insufficient to control blood glucose concentrations. The average values were 130±6 mg/dl in control; 324±9 in the untreated diabetic, and 360±30 in the insulin-treated animals. Withholding insulin resulted in a profound acidemia (pH 6.82±0.08). Administration of NaHCO₃ in the drinking water corrected the acidemia (pH 7.41±0.02) as did insulin treatment (pH 7.43±0.01).

Na⁺ transport. Previous studies have revealed that in the presence of an inside acid pH gradient, the majority of 1 mM ²²Na⁺ uptake into brush border membrane vesicles is mediated by Na⁺/H⁺ exchange (17). As indicated in Table IV, in the streptozotocin-induced diabetics, 5- and 10-s ²²Na⁺ influx into vesicles isolated from untreated animals was significantly greater than for control rats (5 s, 1.78±0.10 nmol/mg protein vs. 1.31±0.06, *P* < 0.005; 10 s, 2.70±0.11 vs. 1.94±0.08, *P* < 0.005). To determine the component of Na⁺ flux occurring via Na⁺/H⁺ exchange, the effect of the pyrazine diuretic amiloride was tested. In microvillus membrane vesicles, 1 mM amiloride inhibits electroneutral Na⁺/H⁺ exchange (18). As shown in Fig. 1, the ²²Na⁺ flux inhibitable by amiloride was significantly greater in

Table III. Physiologic Parameters in BB/W Rats

	Control	Diabetes		
		No treatment	+ insulin	+ NaHCO ₃
Body weight (g)	383±25 (6)	266±6* (4)	374±26 (6)	247±24* (3)
Kidney weight (g)	2.49±0.17 (6)	2.70±0.07* (4)	2.87±0.16* (6)	2.41±0.28 (3)
Kidney weight % Body weight	0.65±0.02	1.01±0.01‡	0.78±0.04	0.97±0.03‡
Blood				
Glucose (mg/dl)	130±6 (6)	324±9‡ (4)	360±30‡ (6)	324±35‡ (3)
pH	7.45±0.05 (6)	6.82±0.08‡ (4)	7.43±0.01 (6)	7.41±0.02 (3)
pCO ₂ (mmHg)	40±7	18±3‡	40±1	50±4
HCO ₃ (mEq/liter)	29±2	4±1‡	26±1	32±3
Ketones (mg/dl)	0±0 (3)	37±14‡ (3)	3.5±3 (3)	18±4‡ (3)

Body weight, kidney weight, glucose concentrations, and arterial blood gases were determined for all control and diabetic rats used in vesicle studies. Ketones were measured in a subset of these animals. The number of animals in each group, *n*, is given in parenthesis. * *P* < 0.05 compared with control. ‡ *P* < 0.005 compared with control.

Table IV. 1 mM ²²Na⁺ Uptake into Renal Microvillus Membrane Vesicles

Time	Streptozotocin-induced diabetes				
	Control	No treatment	+ insulin	+ NaHCO ₃	+ NaCl
5 s (n)	1.31±0.06 (11)	1.78±0.10* (7)	—	1.40±0.12 (5)	1.77±0.12* (5)
10 s	1.94±0.08 (14)	2.70±0.11* (10)	2.10±0.02 (4)	2.09±0.14 (5)	2.64±0.16* (5)
2 h	2.53±0.09 (14)	2.98±0.14 (10)	2.50±0.19 (4)	2.64±0.22 (5)	3.06±0.23 (5)
<i>+1 mM Amiloride</i>					
10 s	0.35±0.02 (5)	0.41±0.06 (10)	0.38±0.17 (3)	0.26±0.04 (5)	0.31±0.04 (5)
BB/W autoimmune diabetes					
	Control	No treatment	+ insulin	+ NaHCO ₃	
5 s (n)	0.77±0.07 (4)	1.48±0.06‡ (3)	0.93±0.08 (3)	0.92±0.12 (3)	
10 s	1.34±0.13	2.05±0.14‡	1.37±0.02	1.43±0.06	
2 h	2.20±0.10	2.71±0.21	2.35±0.16	2.52±0.14	
<i>+1 mM Amiloride</i>					
10 s	0.25±0.11	0.28±0.04	0.19±0.06	0.27±0.03	

Values given in nmol/mg protein. Membrane vesicles were preincubated for 120 min to contain at equilibrium 224 mM mannitol, 24 mM K⁺ 40 mM Hepes, 40 mM 2 (*N*-morpholino) ethane sulfonic acid (MES), pH 6.1. Influx of 1 mM ²²Na⁺ was measured after the addition of 10 μl of vesicle suspension to 40 μl of a solution containing 264 mM mannitol, 24 mM K⁺, 40 mM Hepes, pH 7.5 to give final external pH 7.2.

* *P* < 0.005 compared with control. ‡ *P* < 0.05 compared with control.

the untreated diabetics than in controls (10 s, 2.29±0.12 nmol/mg protein vs. 1.67±0.15, *P* < 0.05). In addition, there were no differences in the amiloride insensitive ²²Na⁺ flux in the untreated group compared with control (Table IV). In the insulin-treated group, total ²²Na⁺ flux (10 s, 2.10±0.02 nmol/mg protein) and the amiloride sensitive component (1.69±0.19) were not significantly different from control (Table IV and Fig. 1).

The streptozotocin-induced diabetic rat developed significant ketosis (12±3 mg/dl) without systemic metabolic acidosis (pH

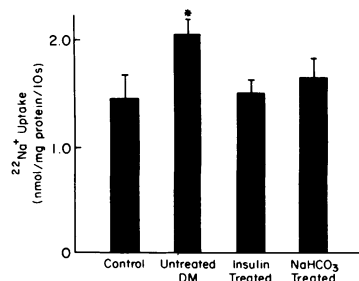


Figure 1. ²²Na⁺ uptake in streptozotocin-induced diabetes mellitus inhibitable by 1 mM amiloride. Vesicles were preincubated for 120 min to contain at equilibrium 224 mM mannitol, 24 mM K⁺, 40 mM Hepes, and 40 mM MES, pH 6.1. 10-s uptakes of 1 mM ²²NaCl were performed af-

ter addition of 10 μl of vesicle suspension to 40 μl of external medium containing 1.25 mM ²²NaCl, 264 mM mannitol, 24 mM K⁺, 40 mM Hepes, pH 7.5, with or without amiloride (1 mM final concentration). Values represent the mean±SEM of from four to 10 experiments in each group. **P* < 0.05

7.42±0.03). Insulin treatment abolished the ketosis and corrected the hyperglycemia. To determine if the stimulated Na⁺/H⁺ exchange was due to hyperglycemia and/or insulinopenia or a manifestation of increased production of hydrogen during ketosis, a separate group of animals was given NaHCO₃ in the drinking water. Each animal ingested at least 50 ml water per day. As shown in Table II, bicarbonate treatment did not reduce hyperglycemia (364±70 mg/dl) or prevent ketosis. Arterial pH and plasma [HCO₃⁻] were not significantly different compared with control. As demonstrated in Table IV, bicarbonate treatment was associated with rates of Na⁺ uptake similar to control values (2.09±0.14 nmol/mg at 10 s vs. 1.94±0.08). As shown in Fig. 1, amiloride-sensitive Na⁺ flux in the bicarbonate-treated group was also not significantly different from control (1.82±0.12 nmol/mg per 10 s vs. 1.67±0.15). Since this last result occurred despite persistently elevated glucose concentrations and insulinopenia, a role for ketosis or acid load in stimulation of Na⁺/H⁺ exchange was suggested. Diabetic animals receiving NaCl instead of NaHCO₃ in the drinking water did not differ in amiloride-sensitive Na⁺ flux when compared with the untreated diabetics (2.34±0.16 nmol/mg per 10 s vs. 2.29±0.12).

Na⁺/H⁺ exchange was also studied in the BB/W autoimmune diabetic rat. Animals maintained on sufficient insulin to prevent ketonemia and metabolic acidosis (Table III) had ²²Na⁺ uptake rates that were not different from control animals despite hyperglycemia. For example, as shown in Table IV, in the presence

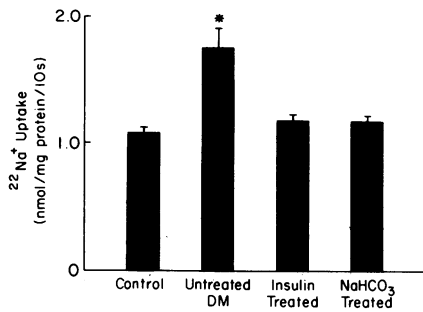


Figure 2. $^{22}\text{Na}^+$ uptake in BB/W autoimmune diabetes mellitus inhibited by 1 mM amiloride. Vesicles were prepared and transport assays performed as described for Fig. 1. Values represent the mean \pm SEM of from three to four experiments in each group. * $P < 0.05$

of an inside acid pH gradient, 1 mM $^{22}\text{Na}^+$ uptake at 10 s was 1.37 ± 0.02 nmol/mg protein in the insulin treated group vs. 1.34 ± 0.13 in control. On the other hand, withdrawal of insulin for 48–72 h resulted in a significant increase in the 10-s flux rate (2.05 ± 0.14 nmol/mg protein vs. 1.34 ± 0.13 ; $P < 0.05$). When these insulinopenic animals were simultaneously treated with sufficient NaHCO_3 to suppress metabolic acidosis, the increases in Na^+ transport were also prevented (Tables III and IV). As shown in Fig. 2 untreated diabetics had increased amiloride-sensitive Na^+ uptake, studied at 10 s (1.76 ± 0.17 nmol/mg protein vs. 1.08 ± 0.02 in control; $P < 0.05$). As with the streptozotocin-induced diabetics, insulin treatment also prevented the increased amiloride-sensitive uptake (1.18 ± 0.14 nmol/mg protein vs. 1.08 ± 0.02 in control) as did NaHCO_3 treatment (1.18 ± 0.03).

Glucose transport. Na^+ -glucose cotransport was also studied in brush border membrane vesicles in the streptozotocin induced model of early diabetes. As shown in Table V, glucose transport rates studied in the absence of Na^+ (K^+ substitution) were not different in control and experimental groups. However, Na^+ -dependent glucose uptakes, in the presence of an inwardly directed Na^+ gradient, were significantly decreased at each early time point in untreated diabetic animals. Thus, at 5 s, glucose uptake averaged 0.70 ± 0.09 pmol/mg protein in diabetics vs. 1.15 ± 0.14 in control ($P < 0.005$). Also shown in Table V, insulin treatment of diabetic animals completely prevented the decrease in glucose transport (1.11 ± 0.06 pmol/mg protein/5 s). The full time course of this effect is shown in Fig. 3.

A separate subgroup of diabetic rats were given 75 meq NaHCO_3 /liter in their drinking water. In diabetics given NaHCO_3 , Na^+ glucose cotransport was still decreased in the presence of an inwardly directed Na^+ gradient, compared with simultaneously measured uptakes in a control group (Fig. 4 A). Compared with controls, glucose transport was also decreased in vesicles from diabetic rats given daily low doses of insulin (0.5 U NOVO ultralente insulin subcutaneously) sufficient to prevent ketosis but not hyperglycemia and glycosuria. Since Na^+/H^+ exchange was increased in diabetic animals, it was possible that glucose flux was decreased due to greater dissipation of the Na^+ gradient, via Na^+/H^+ exchange. Arguing against this interpretation are the results shown in Fig. 4 A, in which NaHCO_3 treatment in amounts sufficient to prevent increases in Na^+/H^+ exchange did not prevent decreases in glucose transport. Additional experiments were performed under Na^+ preequilibrated conditions in which Na^+ -dependent glucose transport can be evaluated in the absence of a Na^+ gradient. In results given in

Table V. Streptozotocin-induced Diabetes [^3H]D-Glucose Uptake

Time	Diabetes		
	Control	No treatment	+ insulin
$\text{KCl}_{\text{out}} = 150 \text{ mM}; \text{KCl}_{\text{in}} = 0 \text{ mM}$			
15 s	0.11 ± 0.01	0.15 ± 0.02	0.11 ± 0.03
2 h	0.65 ± 0.07	0.57 ± 0.05	0.64 ± 0.04
(n)	(5)	(6)	(5)
$\text{NaCl}_{\text{out}} = 150 \text{ mM}; \text{NaCl}_{\text{in}} = 0 \text{ mM}$			
5 s	1.15 ± 0.14	$0.70 \pm 0.09^*$	1.11 ± 0.06
15 s	2.95 ± 0.20	$1.69 \pm 0.21\ddagger$	2.98 ± 0.22
60 s	5.67 ± 0.28	$3.46 \pm 0.41\ddagger$	5.83 ± 0.10
2 h	0.72 ± 0.05	0.71 ± 0.07	0.75 ± 0.02
(n)	(6)	(7)	(5)
$\text{NaCl}_{\text{out}} = \text{NaCl}_{\text{in}} = 80 \text{ mM}$			
15 s	0.25 ± 0.04	0.20 ± 0.02	0.21 ± 0.03
60 s	0.37 ± 0.06	0.31 ± 0.03	0.32 ± 0.02
2 h	0.38 ± 0.05	0.34 ± 0.05	0.36 ± 0.04
(n)	(8)	(4)	(4)

Values given in pmol/mg protein. Membrane vesicles were prepared and assays performed as described for Fig. 3.

* $P < 0.05$.

‡ $P < 0.005$.

Table V, initial rates of Na^+ -dependent glucose uptake under the nongradient condition, $\text{Na}_{\text{in}}^+ = \text{Na}_{\text{out}}^+ = 80 \text{ mM}$, revealed a statistically insignificant decrease in diabetic compared with control vesicles (0.20 ± 0.02 pmol/mg protein per 15 s vs. 0.25 ± 0.04). It is also apparent from Table V that non- Na^+ -dependent transport might contribute importantly to total glucose uptake studied under the Na^+ preequilibrated condition, thereby possibly obscuring a significant decrease in transport in diabetic animals. To accentuate the component of Na^+ -dependent glucose transport under the nongradient condition, initial rates of glucose transport were determined at an increased ambient tem-

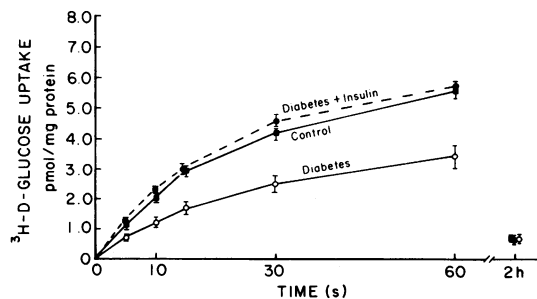


Figure 3. Time course of Na^+ gradient-dependent [^3H]D-glucose uptake in streptozotocin-induced diabetes mellitus. Vesicles were prepared in 300 mM mannitol, 16 mM HEPES, and 10 mM Tris. Influx of $0.35 \mu\text{M}$ [^3H]D-glucose was assayed at 20°C after the addition of $40 \mu\text{l}$ of an external solution containing 150 mM NaCl, 16 mM HEPES, and 10 mM Tris. Insulin was given in amounts sufficient to correct hyperglycemia (Table II). Values represent the mean \pm SEM of from five to seven experiments in each group.

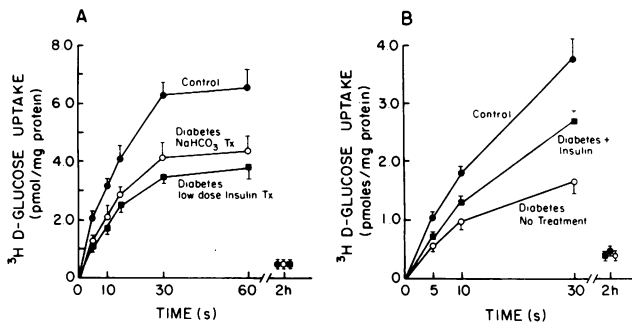


Figure 4. Time course of Na^+ gradient dependent $[^3\text{H}]\text{D}$ -glucose uptake in vesicles from diabetic rats: effect of treatment with NaHCO_3 or low dose insulin with continued hyperglycemia. Time course in (A) represents results for streptozotocin-induced diabetes; (B), for BB/W autoimmune diabetes. Vesicles were prepared as described in Fig. 3 and transport studied in the presence of 150 mM NaCl in the external solution. Values represent mean \pm SEM of three or four experiments in each group.

perature. The effects of temperature on glucose flux are shown in Table VI. Non- Na^+ -dependent glucose flux, studied in the presence of an inwardly directed gradient of 50 mM KCl , was 0.056 ± 0.04 pmol/mg protein per 10 s at 20°C and 0.057 ± 0.04 at 37°C ($n = 3$). In control animals, Na^+ -dependent uptake under the nongradient conditions ($\text{Na}_{\text{in}}^+ = \text{Na}_{\text{out}}^+$) increased from 0.148 ± 0.01 pmol/mg protein per 10 s at 20°C to 0.251 ± 0.011 at 37°C . At 37°C , nongradient Na^+ -dependent glucose uptake was significantly decreased in vesicles from diabetic animals

Table VI. Effects of Temperature on Na^+ -dependent Glucose Uptake in Streptozotocin-induced Diabetes

Initial condition	$[^3\text{H}]\text{glucose uptake}$ (pmol/mg protein per 3 s)		
	14°C	20°C	37°C
$\text{Na}_{\text{out}}^+ = 50 \text{ mM}; \text{Na}_{\text{in}}^+ = 0 \text{ mM}$			
Control	0.15 ± 0.01	0.21 ± 0.03	0.31 ± 0.02
Diabetic	0.12 ± 0.03	0.13 ± 0.04	$0.17 \pm 0.02^*$
	$[^3\text{H}]\text{glucose uptake}$ (pmol/mg protein per 10 s)		
	14°C	20°C	37°C
$\text{Na}_{\text{out}}^+ = \text{Na}_{\text{in}}^+ = 50 \text{ mM}$			
Control	0.12 ± 0.05	0.15 ± 0.01	0.25 ± 0.01
Diabetic	0.10 ± 0.01	0.13 ± 0.02	$0.14 \pm 0.04^\ddagger$
	$^{22}\text{Na}^+$ uptake (nmol/mg protein per 3 s)		
	14°C	20°C	37°C
$\text{Na}_{\text{out}}^+ = 50 \text{ mM}; \text{Na}_{\text{in}}^+ = 0 \text{ mM}$			
Control	5.98 ± 0.32	8.41 ± 0.69	10.00 ± 1.22
Diabetic	5.95 ± 1.47	9.51 ± 2.65	7.72 ± 1.94

* $P < 0.05$ compared with control.

‡ $P < 0.005$ compared with control. $n = 3$ for all groups.

(0.137 ± 0.038 pmol/mg protein per 10 s vs. 0.251 ± 0.011 in control; $P < 0.05$). Also shown in Table VI, Na^+ gradient stimulated glucose uptake was decreased in diabetics at 37°C (0.172 ± 0.023 pmol/mg protein per 3 s vs. 0.311 ± 0.014 in control; $P < 0.05$). This effect was not due to greater dissipation of the Na^+ gradient in diabetic vesicles since 50 mM Na^+ uptake was not significantly different between the groups. In another set of diabetic animals, rates of $0.1 \mu\text{M}$ L-alanine cotransport studied in vesicles under the condition of Na^+ preequilibration at 37°C were not statistically different from values in control animals (0.029 ± 0.003 pmol/mg per 20 s vs. 0.020 ± 0.003 , $n = 3$).

Na^+ -dependent glucose uptake was also depressed in vesicles derived from the BB/W rats. As shown in Table VII, initial rates of $[^3\text{H}]\text{D}$ -glucose influx were decreased in the diabetic rats (0.60 ± 0.07 pmol/mg protein per 5 s) compared with control (1.04 ± 0.06 , $P < 0.05$). Insulin treatment prevented the metabolic acidosis but not hyperglycemia in these animals (Table III) and as shown in Table VII only partially prevented the fall in glucose transport. These results suggest that the diabetic state per se, not the metabolic acidosis, results in decreased glucose transporter activity. Fig. 4 B shows the full time course of Na^+ -dependent glucose uptake into vesicles derived from BB/W rats.

Phlorizin binding studies were performed to determine if brush border glucose carrier concentration was altered by diabetes. Previous studies (12) have indicated evidence for a high affinity, Na^+ -dependent phlorizin binding site in the renal brush border. In the presence of 100 mM Na^+ , a single high affinity binding site is present in the range of phlorizin concentrations of 0.05 – $1 \mu\text{M}$. In the present studies, removal of Na^+ from the incubation medium inhibited $0.02 \mu\text{M}$ phlorizin binding by 85%. There were no differences in this Na^+ independent phlorizin binding in vesicles from control, untreated streptozotocin induced diabetic and insulin treated diabetic rats.

Scatchard analysis (Fig. 5) of the high affinity binding site revealed no differences in binding sites or affinity for Na^+ -dependent phlorizin binding in microvillus vesicles from controls, untreated diabetics, and insulin-treated diabetics. There was a numerical but not statistically significant increase in the concentration of sites (R_0) in the untreated diabetics, 176 ± 6 pmol/mg protein, compared with controls, 138 ± 13 , while the insulin treated group had an R_0 of 156 ± 16 ($n = 3$ for all groups). Similarly, the binding affinity (K_D) was $0.76 \pm 0.02 \mu\text{M}$ in the controls, 0.84 ± 0.08 in the diabetic, and 0.79 ± 0.05 in the insulin-treated animals.

Table VII. BB/W Autoimmune Diabetes $[^3\text{H}]\text{D}$ -Glucose Uptake

Time	Control	Diabetes	
		No treatment	+ insulin
$\text{NaCl}_{\text{out}} = 150 \text{ mM}; \text{NaCl}_{\text{in}} = 0 \text{ mM}$			
5 s	1.04 ± 0.06	$0.60 \pm 0.07^*$	$0.75 \pm 0.04^*$
10 s	1.84 ± 0.09	$0.97 \pm 0.09^*$	1.31 ± 0.09
30 s	3.78 ± 0.38	$1.69 \pm 0.21^*$	2.72 ± 0.21
2 h	0.48 ± 0.06	0.44 ± 0.08	0.53 ± 0.08
(n)	(4)	(3)	(4)

Values given in pmol/mg protein. Membrane vesicles were prepared and assays were performed as described in Fig. 3.

* $P < 0.05$ compared with control.

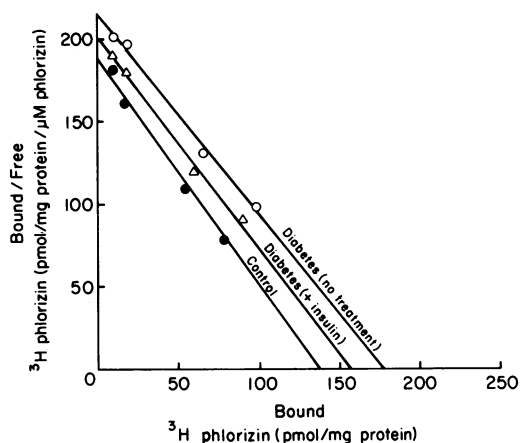


Figure 5. Scatchard transformation of equilibrium ^3H phlorizin binding in streptozotocin-induced diabetes. Microvillus membrane vesicles were prepared in 300 mM mannitol, 16 mM Hepes, and 10 mM Tris. Binding assays were performed for 40 min at 20°C after the addition of 10 μl of vesicle suspension to 40 μl of a solution containing 62.5 mM Na_2SO_4 , 113 mM mannitol, 16 mM Hepes, 10 mM Tris, and ^3H phlorizin to give final concentrations of 0.05–1.0 μM ^3H phlorizin. Values represent mean \pm SEM for three experiments in each group.

Low affinity [^3H]phlorizin binding in the range of 5–100 μM phlorizin gave an R_0 of 1,946 pmol/mg protein and a K_D of 78 μM in controls. In untreated diabetics, R_0 was 1,816 pmol/mg protein and K_D was 75 μM and in insulin-treated diabetics, R_0 was 1,773 pmol/mg protein and K_D was 51 μM .

Discussion

Studies in renal microvillus membrane vesicles have shown increases in Na^+/H^+ exchange activity in response to ammonium chloride-induced metabolic acidosis, glucocorticoid administration, renal ablation, and high protein feeding (19–23). In a preliminary report by El-Seifi et al. (24), Na^+/H^+ exchange was increased in untreated diabetes. In the present study, untreated streptozotocin-induced diabetic animals demonstrated a significant increase in total and amiloride-sensitive 1 mM $^{22}\text{Na}^+$ uptake. Insulin treatment prevented the increased Na^+/H^+ exchange activity seen in the uncontrolled diabetics. The increased exchanger activity occurred without systemic acidemia, but was associated with ketosis and increased net acid excretion, consistent with an increase in endogenous H^+ production. Bicarbonate treatment of these animals normalized Na^+/H^+ exchange without correcting the hyperglycemic state. The dose of streptozotocin given to the animals in the present study may not induce ketoacidosis since all functioning islet tissue may not be destroyed (25).

The results in the BB/W autoimmune rats provide further evidence that acid-base disturbances rather than insulinopenia per se were responsible for increased Na^+/H^+ exchange. These animals were severely acidemic when deprived of insulin, and compared with the streptozotocin-treated animals, demonstrated a greater percentage increase in antiporter activity. Correction of the metabolic acidosis normalized Na^+/H^+ exchange. These results provide the first evidence for increases in Na^+/H^+ exchange activity secondary to an endogenously induced metabolic acidosis. In metabolic acidosis, increased antiporter activity, by

stimulating proton secretion, may be an important adaptation of the nephron to enhance proximal bicarbonate reabsorption (26, 27). However, in severe acidosis, the corresponding decrease in filtered substrate may limit overall bicarbonate reabsorption. Although proximal acidification has not been studied in the diabetic model, ammonium chloride acidosis is associated with increased bicarbonate absorption in the proximal tubule perfused in vivo (28). The increased absolute bicarbonate absorptive rate was observed when perfusion rate and luminal concentrations were similar in control and acidotic animals. Although a decrease in intracellular pH in acidotic animals could explain an increase in H^+ secretion, the finding is also consistent with the increase in vesicle Na^+/H^+ exchange noted in that model (19–21). The mediating factors of increased Na^+/H^+ exchange in untreated diabetes are at present undetermined. Kinsella et al. (20) have shown the importance of glucocorticoids in mediating increased Na^+/H^+ exchange activity in ammonium chloride-induced acidosis. In untreated diabetes, the corticosteroid levels, as well as those of glucagon and growth hormone, increase, especially in the presence of ketoacidosis (29).

In muscle, insulin has been shown to exert a direct modulating effect on Na^+/H^+ exchange (30). Although insulin receptors exist on both the luminal and basolateral membranes of the proximal tubule, the role of insulin in regulation of metabolic and transport functions in these cells is unclear (31). In vivo studies in dogs have suggested that acute infusion of insulin may decrease proximal tubule Na^+ reabsorption (3). Pretreatment of a suspension of proximal tubule fragments with insulin before preparation of microvillus membrane vesicles induced increases in Na^+/PO_4 cotransport without change in $\text{Na}^+/\text{glucose}$ cotransport or total Na^+ uptake (7).

One of the most intriguing possibilities concerning the increase in Na^+/H^+ activity in early streptozotocin-induced diabetes is the possible relationship between this exchanger and renal hypertrophy. In poorly controlled diabetes significant renal hypertrophy occurs in man and experimental animals (2). The initiating factors and mediators of this hypertrophic response are at present undetermined. Studies in a variety of cultured cells have demonstrated that one of the early responses to mitogenic stimuli involves cytoplasmic alkalinization mediated by Na^+/H^+ exchange (32). Recent work from our laboratory has suggested a close correlation between the renal hypertrophy induced by contralateral nephrectomy or a high protein diet and increased brush border membrane vesicle Na^+/H^+ exchange activity (23). In the present studies, increased antiporter activity was noted in the untreated streptozotocin-induced diabetics. In addition to increased acid production rates and hyperglycemia, these animals had significant whole kidney and renal cortical hypertrophy. When the streptozotocin-induced diabetic animals were treated with a long-acting insulin preparation, in an attempt to provide good control of the diabetes, they demonstrated much less renal hypertrophy. In addition, Na^+/H^+ exchange activity was only minimally elevated above control values. The effect of diabetes on renal mass was difficult to assess in the autoimmune model because of the disparity in body weight in the experimental groups. It remains possible that either systemic or intracellular acidosis is a common feature to states associated with increased Na^+/H^+ exchange activity and stimulated renal growth. Whether Na^+/H^+ exchange increases in untreated diabetes simply in response to decreased intracellular pH of the proximal tubule cell or in response to hormonal or hypertrophic stimuli remains to be determined.

Studies of glucose transport in intestinal microvillus membrane vesicles from diabetics have indicated increased Na⁺-glucose cotransport, postulated to be secondary to decreased rates of nonglucose-dependent Na⁺ flux (33). In preliminary reports, Whiting et al. (34) and El-Seifi et al. (24) found evidence for decreased Na⁺-dependent glucose transport in renal brush border membranes. Since the latter group also demonstrated increased Na⁺/H⁺ exchange, it was suggested that the decrease in vesicle glucose transport was secondary to this increase. By contrast, Northrup (35) found no alterations in Na⁺ glucose cotransport although he reported decreased Na-PO₄ cotransport, and Wong et al. (36) found increased Na glucose cotransport. In the present study, Na⁺-dependent glucose transport was studied in both the presence and the absence of an inwardly directed Na⁺ gradient. In the presence of an inwardly directed gradient, initial rates of [³H]D-glucose were decreased in microvillus vesicles from untreated diabetics compared with controls and were not different from control in membrane vesicles from diabetics given sufficient insulin to control hyperglycemia. Besides an intrinsic decrease in Na⁺-dependent glucose transport capabilities, these findings could also be attributed to competition by other Na⁺ transport sites for the Na⁺ gradient. Previous studies have demonstrated competition among Na⁺-glucose, Na⁺-alanine, and Na⁺-phosphate cotransport in microvillus vesicles and decreased Na⁺ glucose cotransport when Na⁺/H⁺ exchange was increased (37, 38). To exclude this possibility, glucose transport was studied in the presence of Na⁺ but under nongradient conditions. Glucose uptake studied at 20°C in microvillus vesicles in the absence of a net driving force for Na⁺, (Na_{in}⁺ = Na_{out}⁺), was numerically but not statistically lower in untreated diabetics than controls. However, in this preequilibrated condition glucose uptake is markedly reduced, although still increased above flux rates in the absence of Na⁺. Therefore, possible differences in transport rate among groups may not be easily detected. To provide a more sensitive test, transport assays were also performed at 37°C, a condition expected to increase Na⁺-dependent glucose uptake. At 37°C, glucose transport was significantly decreased in diabetic animals, in the presence and absence of the Na⁺ gradient, an effect abolished by insulin treatment. Furthermore, decreased Na⁺ glucose transport in brush border membrane vesicles prepared from diabetic animals treated with NaHCO₃, which normalized Na⁺/H⁺ exchange, suggests an intrinsic impairment of Na⁺-glucose transport function. In both streptozotocin-induced and autoimmune diabetics, insulin-treated groups that still manifested marked hyperglycemia and glycosuria continued to exhibit decreased glucose transport, even though Na⁺/H⁺ exchange was not different from control values. There was a further decrease in glucose transport in the acidotic BB/W rats, who did have increased Na⁺-H⁺ exchange rates. In the absence of acidosis Na⁺ transport was not altered by diabetes. Na⁺-L-alanine cotransport was also not significantly different although a small effect cannot be excluded. Other Na⁺ cotransporters not evaluated in the present study may have decreased function in diabetes (24, 35).

The significance of decreased Na⁺-glucose cotransport function in the microvillus membrane to glucose handling in the intact proximal tubule deserves consideration. Net proximal glucose reabsorption, determined by multiple factors, has been shown to increase proportionately with glomerular filtration rate (GFR)¹ (39, 40) and delivery rate (41) and to increase with renal

hypertrophy (42). In the streptozotocin-treated diabetic rat, net glucose reabsorption has been shown to increase (43), although in that study GFR and filtered glucose load also increased markedly. As a result, the decrease in intrinsic carrier-mediated transport may be more than offset by the increment in filtered glucose load. This is analogous to the situation in metabolic acidosis in which an increase in Na⁺/H⁺ exchange and proton secretory capacity is offset by a decrease in filtered bicarbonate load so that absolute bicarbonate reabsorption is diminished (26).

Alterations in GFR in diabetes might induce changes in membrane transport function. However, in a previous report, changes in GFR were not associated with differences in Na-dependent glucose transport in microvillus membrane vesicles (23). Severe hyperglycemia and ketoacidosis are associated with a reduced GFR (1). In the rat model of streptozotocin-diabetes, moderate hyperglycemia of the magnitude observed in the present study is associated with an increased GFR (1). It remains possible that changes in GFR may directly regulate carrier activity but it is more likely that glomerular-tubular balance for glucose in the intact proximal nephron is related to effects on glucose delivery and peritubular forces (39, 40).

An inverse relationship between ambient glucose concentration and glucose carrier function has been noted previously. Studies in LLC-PK₁ cells, a porcine renal cell line with many characteristics of proximal tubule cells, have shown that the concentration of phlorizin binding sites increases in cells grown in a low glucose medium and decreases when the cells are grown in a high glucose medium (44). In adipose cells, insulin has been shown to exert a direct effect upon glucose transport (8). Insulin administration induces a translocation of glucose transporters to the plasma membrane from an intracytoplasmic pool (45), and insulin seems to regulate the number of cellular glucose transporters available, since streptozotocin-induced diabetes decreases the number of glucose transporters (46). In brain tissue, decreased activity of glucose carriers pertains in the setting of increased net glucose uptake by the cells during hyperglycemia (47). The independent role of insulin or the diabetic state in modifying proximal glucose reabsorption or glucose carrier activity remains to be determined.

To further characterize the adaptive decrease in glucose transport in diabetic animals, we performed phlorizin binding studies. ³H phlorizin binding revealed that there were no significant differences in either concentration of binding sites or substrate affinity among controls, untreated diabetics, and insulin treated diabetics. In fact, there was a numerical but not statistically significant increase in binding sites in untreated diabetics. Therefore, any decreased glucose transport in untreated diabetics cannot be attributed to a decrease in the number of transporting sites available.

Besides a decrease in the number of sites, alteration of the mobility of the transporter in its lipid domain may alter transport activity (48). Phospholipid analysis of plasma membranes isolated from untreated streptozotocin-induced diabetic rats has shown decreased unsaturated fatty acids in phosphatidylcholine and phosphatidylethanolamine, with an increase in linoleic acid and a decrease in arachidonic acid (49). Decreases in the unsaturability index are known to alter membrane fluidity (50). Therefore, an intrinsic change in membrane composition may be involved in decreased rates of Na⁺ gradient dependent glucose uptake noted in untreated diabetics. The alteration in temperature dependence of glucose transport noted in the diabetic brush border membrane vesicles is consistent with a change in mem-

1. Abbreviations used in this paper: GFR, glomerular filtration rate.

brane lipid content. Alternatively, changes in the protein composition of the glucose carrier may be secondary to nonenzymatic glycosylation (51).

In conclusion, we have shown increased activity of microvillus membrane Na^+/H^+ exchange in untreated diabetic rats, related to increases in endogenous H^+ production. Decreased rates of Na^+ -glucose cotransport, also associated with the diabetic state, did not require metabolic acidemia or increased acid production but were responsive to insulin treatment. The decreased Na^+ glucose cotransport is related to decreased functioning of existing carriers rather than a decrease in the number of carriers.

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