

# Amino Acid Transport in Isolated Hepatocytes from Streptozotocin-diabetic Rats

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## SUMMARY

The enhanced ability of liver to extract glucogenic precursors from plasma in insulinopenic diabetes was investigated at the cellular level by measuring amino acid transport in isolated hepatocytes from streptozotocin-diabetic rats. The concentrative  $\text{Na}^+$ -dependent uptake of  $\alpha$ -amino[1- $^{14}\text{C}$ ]isobutyric acid (AIB), a non-metabolizable analog of alanine, was increased in hepatocytes from diabetic rats compared with controls. This increase was observed both at a low (0.1 mM) and high (30 mM) extracellular concentration of the amino acid. Analysis of the relationship between AIB influx and AIB concentration revealed that the increased uptake in diabetic rat hepatocytes was due to an enhanced capacity (i.e., a twofold increase in the  $V_{\text{max}}$ ) of the transport system whereas the apparent affinity ( $K_m$ ) for AIB was unaltered.

The increased amino acid transport activity in diabetic rat hepatocytes was due mainly to an increased transport through system A (alanine-preferring) and, to a much lesser extent, through system ASC (alanine, serine, cysteine). System L (leucine-preferring) and AIB entry through simple diffusion were not affected in diabetic rat hepatocytes compared with controls. Increased AIB transport of diabetic rat hepatocytes was returned to normal by insulin treatment. **DIABETES** 29:996-1000, December 1980.

The liver plays a central role in the maintenance of blood glucose homeostasis by three processes: glycogenolysis, glycogen synthesis, and gluconeogenesis. In normal conditions, one effect of insulin on the liver is to inhibit gluconeogenesis.<sup>1</sup> Accordingly, insulinopenic diabetes is characterized by an in-

crease in gluconeogenesis. This increase occurs in the face of diminished circulating levels of alanine and other glucogenic amino acids;<sup>2,3</sup> this and other observations in vivo in human diabetics<sup>2,3</sup> strongly suggested that the liver extraction of amino acids is enhanced in insulinopenic diabetes.

Relatively little direct information is available about the effect of diabetes on the hepatic transport of amino acids. It has recently been reported that in alloxan-diabetic rats, hepatic amino acid uptake is increased both in vivo and in the isolated perfused liver in vitro.<sup>4</sup> However, the question of whether or not an increased hepatic extraction of amino acids in diabetes is specifically related to an alteration in the kinetic properties of the transport step has not been examined at the cellular level.

The present study was consequently undertaken to investigate the effect of insulinopenic diabetes on amino acid transport in isolated rat hepatocytes. In a previous study from this laboratory,<sup>5</sup> it was found that freshly isolated rat hepatocytes take up neutral amino acids through the major transport systems described for eukaryotic cells,<sup>6</sup> namely, systems A (alanine), ASC (alanine, serine, cysteine), and L (leucine). The present experiments have been designed to investigate whether the kinetic properties of transport of AIB,\* a nonmetabolizable analog of alanine, are altered in isolated hepatocytes from streptozotocin-diabetic rats.

## MATERIALS AND METHODS

**Animals.** Male Wistar rats (140-150 g) were used throughout this study. They were maintained in a constant temperature (22°C) animal room, with a fixed 12-h artificial light cycle (8 a.m.-8 p.m.) and fed ad libitum with a standard laboratory chow (U.A.R., Villemoisson, Epinay/Orge, France) consisting of (kcal/100 kcal) 63% carbohydrate, 26% protein, and 11% fat. After an overnight fast (with free access to water) rats were randomly divided into two

Part of this work has been presented in abstract form (International Diabetes Federation meeting, Vienna 1979).

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\* The abbreviations and trivial names used are: AIB,  $\alpha$ -aminoisobutyric acid; N-Me-AIB,  $\alpha$ -(methylamino)isobutyric acid; and cycloleucine, 1-aminocyclopentane-1-carboxylic acid.

groups. Animals from one group received streptozotocin<sup>7</sup> (80 mg/kg body weight, dissolved in isotonic saline acidified to pH 4.5 with citric acid) through the tail vein. Rats from the control group were injected with the medium alone. All animals were then fed ad libitum until killing. Diabetic (streptozotocin) rats were not treated with insulin except for three that were injected subcutaneously with 6 U of Novo Lente insulin each evening (5 p.m.) commencing on the tenth day after streptozotocin administration. The diabetic state was considered reversed when all of the glycemia remained below 250 mg/dl in individual samples collected from the tail vein at 8 a.m., noon, and 6 p.m. under slight ether anesthesia; under these conditions, glycosuria had also disappeared.

Some characteristics of the experimental animals are presented in Table 1. Rats injected with streptozotocin became rapidly hyperphagic, but gained less weight than controls (2 g/day versus 7 g/day). Water intake and diuresis (not shown) increased in parallel to reach a plateau 8–10 days after the streptozotocin injection. Glycosuria (but not ketonuria) was observed throughout the study in non-insulin-treated animals. Insulin-treated diabetic rats gained weight again at the same rate as controls (7 g/day). After 15 days of insulin therapy, food intake, water intake, and diuresis were similar or very close to those of controls (not shown).

**Assays.** Blood samples were collected from the tail vein into heparinized tubes under slight ether anesthesia. After centrifugation at 4°C, plasma glucose was determined by the glucose oxidase method (Boehringer kit, Mannheim, West Germany). On the third day after streptozotocin administration all of the animals injected with the drug had plasma glucose levels above 400 mg/dl. Glycosuria and ketonuria were estimated by colorimetric methods (Tes-Tape urine sugar analysis paper, Eli Lilly; Acetest, Ames). On the experimental day, i.e., between 5 and 10 days after the injection of streptozotocin, blood samples (1.0 ml) were collected from the aorta in a mixture (0.05 ml) of EDTA (2.5 mg/ml) and Trasylol (10,000 KIU/ml) and centrifuged at 4°C; plasma aliquot samples were stored at -20°C.

Plasma insulin was measured by radioimmunoassay<sup>8</sup> using porcine <sup>125</sup>I-insulin as tracer, a guinea pig antiporcine

insulin serum, and crystalline rat insulin (controls and diabetics) or porcine insulin (insulin-treated diabetics) as standards. Plasma glucagon was radioimmunoassayed without prior extraction of plasma, using porcine glucagon as <sup>125</sup>I tracer and unlabeled standard, and 30 K antiglucagon serum.<sup>9</sup> Free insulin and glucagon were separated from bound hormone by adsorption on magnesium silicate.<sup>10</sup>

At the time of killing, the diabetic rats were hypoinsulinemic and hyperglucagonemic compared with controls; insulin-treated diabetic animals also exhibited high plasma glucagon levels concomitant with low plasma glucose concentrations (Table 1).

**Preparation of isolated hepatocytes.** Hepatocytes were isolated between 8 and 9 a.m. by collagenase dissociation of the liver as previously described.<sup>5,11</sup> The proportion of parenchymal cells (i.e., hepatocytes) in the purified<sup>11</sup> cell suspension exceeded 95%. The viability of the cell suspensions, estimated by the cell membrane refractoriness in phase contrast microscopy,<sup>5,11</sup> ranged between 85 and 95% in both control and diabetic rat hepatocytes. Freshly isolated cells were used in suspension in a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 mg/ml bovine serum albumin (fraction V), and gassed with a mixture of 5% CO<sub>2</sub>:95% O<sub>2</sub>. All experiments were done at 37°C, and hepatocytes were used within 1 h after isolation.

**Measurement of ATP, glycogen, and protein contents in hepatocytes.** The intracellular ATP content was measured by a spectrophotometric UV method (Boehringer kit, Mannheim, West Germany). Glycogen content was measured as described elsewhere.<sup>12</sup> Cell protein was determined by the method of Lowry et al.<sup>13</sup> with bovine serum albumin as standard. ATP and protein contents of hepatocytes from control and diabetic rats did not differ significantly, whereas glycogen content was decreased fivefold in hepatocytes from diabetic rats compared with controls (Table 2).

#### Studies of AIB transport and expression of results.

Transport assays were carried out as previously described.<sup>5,14–16</sup> Results have been corrected to account for the viability of the cell suspension (estimated as mentioned above). The Na<sup>+</sup>-dependent part of transport was determined by subtracting, at each AIB concentration, the values obtained in a Na<sup>+</sup>-free medium from total velocities measured in the presence of Na<sup>+</sup>. The relationship between the initial rate (influx) of the Na<sup>+</sup>-dependent transport (*v*) and the substrate (AIB) concentration was analyzed according to the graphical method of Woolf-Augustinsson-Hofstee, where *v* is plotted against the *v*/[AIB] ratio.

TABLE 1  
Characteristics of experimental animals

	Control	Diabetic	Treated diabetic
Weight (g)*			
Day 0	141 ± 1 (4)	148 ± 2 (6)	144 ± 1 (3)
Day 10	220 ± 6 (4)	167 ± 4 (6)	169 ± 5 (3)
Day 25	—	—	269 ± 5 (3)
Plasma†			
Glucose (mg/dl)	183 ± 4 (8)	533 ± 20 (7)	73 ± 30 (3)‡
Insulin (μU/ml)	86 ± 18 (8)	13 ± 1 (7)	75 ± 40 (3)
Glucagon (pg/ml)	222 ± 22 (8)	369 ± 45 (7)	500 ± 91 (3)

\* Weights were recorded at day 0 (immediately before streptozotocin administration), day 10 (10 days after streptozotocin administration), and day 25 (25 days after streptozotocin administration and 15 days after initiation of insulin therapy).

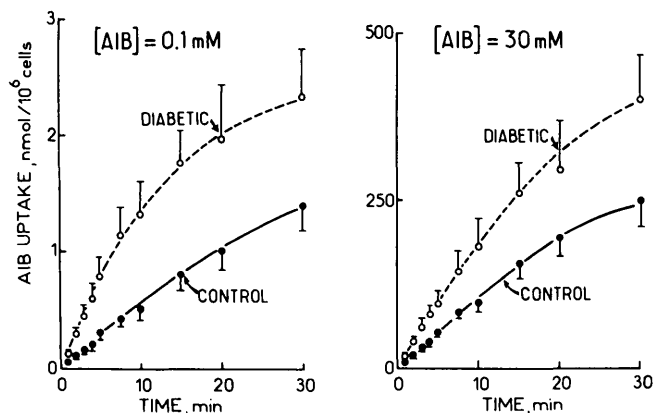
† Plasma glucose, insulin, and glucagon were determined from blood samples collected on the day of killing. Values are means ± SEM of (N) individual animals.

‡ Individual plasma glucose concentrations (mg/dl) were 30, 132, and 58.

TABLE 2  
ATP, glycogen, and protein contents in isolated hepatocytes were from control and diabetic rats

	ATP (nmol/10 <sup>6</sup> cells)	Glycogen (μg glucose/ 10 <sup>6</sup> cells)	Protein (mg/10 <sup>6</sup> cells)
Control	19 ± 1 (5)	219 ± 15 (3)	1.53 ± 0.11 (4)
Diabetic	16 ± 2 (5)	42 ± 13 (3)	1.82 ± 0.16 (4)

ATP, glycogen, and protein contents in isolated hepatocytes were determined 5–10 days after streptozotocin (Diabetic) or saline (Control) administration. Values are means ± SEM for the number of separate experiments given in parentheses. Each experiment was performed with hepatocytes from one animal.



**FIGURE 1.** Time course of AIB uptake. Hepatocytes isolated from control and diabetic rats were incubated with [<sup>14</sup>C]AIB at 0.1 mM (left panel) and 30 mM (right panel). At the indicated time points, cells were isolated by centrifugation, washed, and counted for <sup>14</sup>C radioactivity as described under METHODS. Each point is the mean  $\pm$  SEM of 4 separate experiments. Each experiment was performed with hepatocytes from one animal.

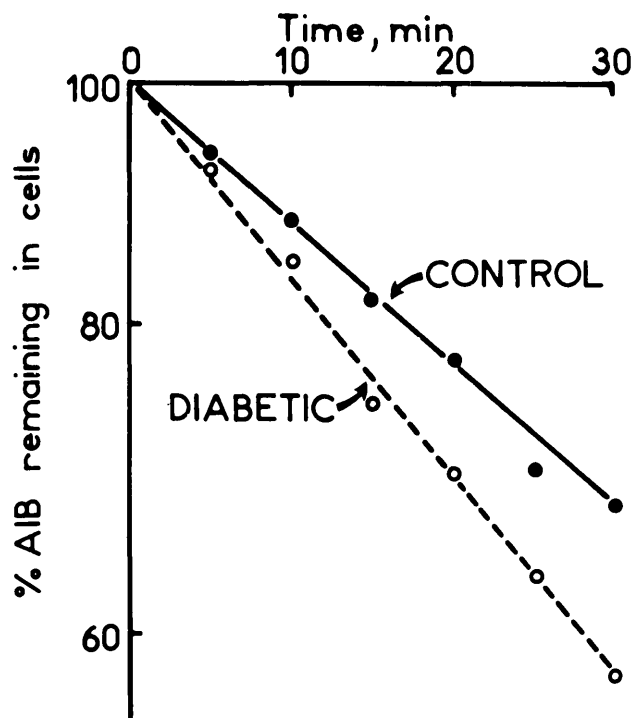
**Chemicals.** [<sup>14</sup>C]AIB (specific activity, 60 mCi/mmol) and [<sup>14</sup>C]cycloleucine\* (specific activity, 60 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. The unlabeled nonmetabolizable amino acids, AIB, N-Me-AIB,\* and cycloleucine, as well as bovine serum albumin (fraction V), were from Sigma Chemical Co., St. Louis, Missouri. The antiglycagon serum (30 K) was obtained from Dr. R. H. Unger, Dallas, Texas. The following were gifts: streptozotocin, from Upjohn Co., Kalamazoo, Michigan; porcine monocomponent insulin, rat insulin, and porcine glucagon, from Novo Research Institute, Copenhagen, Denmark; and anti-insulin serum from Dr. P. H. Wright, Indianapolis, Indiana. Other chemicals were of the best commercial grade available.

## RESULTS

**Time course of AIB uptake.** The time course of AIB uptake by hepatocytes from control and diabetic rats was studied with extracellular AIB at 0.1 mM and 30 mM, which represent the two extreme concentrations used in subsequent kinetic analyses. As shown in Figure 1, in hepatocytes from diabetic animals both the initial rate of AIB uptake (first 7 min) and the amount of AIB taken up at later time points were increased compared with controls; this increase was observed at both AIB concentrations. The uptake of AIB increased linearly with time for at least 7 min in all four conditions under study (Figure 1).

Distribution ratios for AIB (i.e., the ratio of intracellular to extracellular concentrations of the amino acid<sup>5</sup>) after 30 min of uptake were 5 (controls) and 8 (diabetics) with AIB at 0.1 mM, and 2.5 (controls) and 4.5 (diabetics) with AIB at 30 mM.

**Time course of AIB efflux.** To test the possibility that the amino acid efflux might also have been altered in diabetic rat hepatocytes, cells were first preloaded for 10 min with [<sup>14</sup>C]AIB at 0.1 mM. The efflux of [<sup>14</sup>C]AIB was then measured (Figure 2). Semilogarithmic plots of the fractional efflux yielded straight lines, indicating that efflux followed first order kinetics in both control and diabetic rat hepatocytes. Efflux proceeded at a slightly faster rate in cells from diabet-



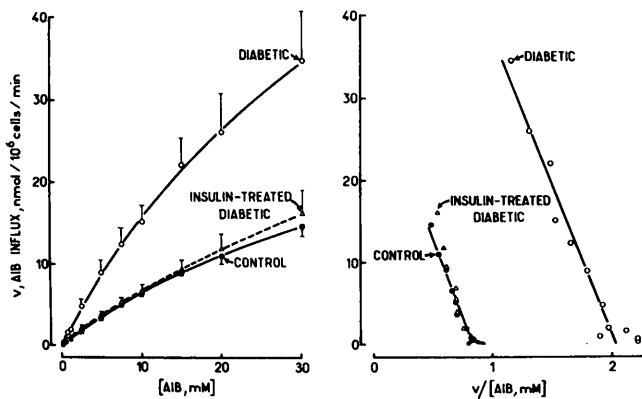
**FIGURE 2.** Time course of AIB efflux. Hepatocytes isolated from control and diabetic rats were preloaded for 10 min with 0.1 mM [<sup>14</sup>C]AIB. Cells were washed once and immediately resuspended in a fivefold excess of AIB-free medium to minimize reuptake. The amount of AIB remaining in cells was determined at the times indicated, and is expressed as the percentage of the amount of AIB present in cells at time zero.

ics ( $t_{1/2} \cong 37$  min) than in hepatocytes from controls ( $t_{1/2} \cong 51$  min) (Figure 2).

**Kinetic analysis of influx in hepatocytes from control and diabetic rats.** To determine whether the increase in AIB uptake observed in diabetic rat hepatocytes was due to an increased  $V_{max}$ , or to an altered  $K_m$ , or both, the initial velocity ( $v$ ) of AIB uptake (i.e., the influx observed over a 4-min period of uptake) was measured at varying AIB concentrations from 0.1 to 30 mM (Figure 3). Over the entire range of AIB concentrations, the uptake of AIB was greater in hepatocytes from diabetic rats than in cells from controls (Figure 3, left). With hepatocytes from both control and diabetic animals, plots of  $v$  against  $v/[AIB]$  were linear over the major part of the range of AIB concentrations (Figure 3, right), with only a slight deviation from linearity at AIB  $\leq 0.5$  mM, indicating that in both situations AIB transport was accounted for mainly by one homogeneous component.

Table 3 gives the values of the kinetic parameters derived from these experiments. Whereas the apparent affinity of the transport component for AIB ( $K_m$ ) was similar in hepatocytes from both controls and diabetics, the capacity of the transport system ( $V_{max}$ ) was increased about twofold in hepatocytes from diabetic rats compared with control animals. Therefore, it appears that the increased AIB uptake observed in diabetic rat hepatocytes is due to an increase in the  $V_{max}$  of transport with no detectable change in  $K_m$ .

**Reversion with insulin treatment.** When diabetic rats were treated with insulin for 2–3 wk as indicated under METHODS, the increase in AIB transport was no longer observed (Figure 3) and the capacity ( $V_{max}$ ) of the transport system was similar to that of controls (Table 3).



**FIGURE 3.** Concentration dependence of AIB influx. Hepatocytes isolated from control, diabetic, and insulin-treated diabetic rats were incubated for 4 min with AIB concentrations ranging from 0.1 to 30 mM. The Na<sup>+</sup>-dependent part of transport was determined as indicated under METHODS. Left panel: each point is the mean  $\pm$  SEM of 6 separate experiments for control and diabetic rats and 3 separate experiments for insulin-treated diabetic rats. Right panel: the data have been plotted according to Woolf-Augustinsson-Hofstee.

### Specificity of the increased AIB transport in diabetic rat hepatocytes.

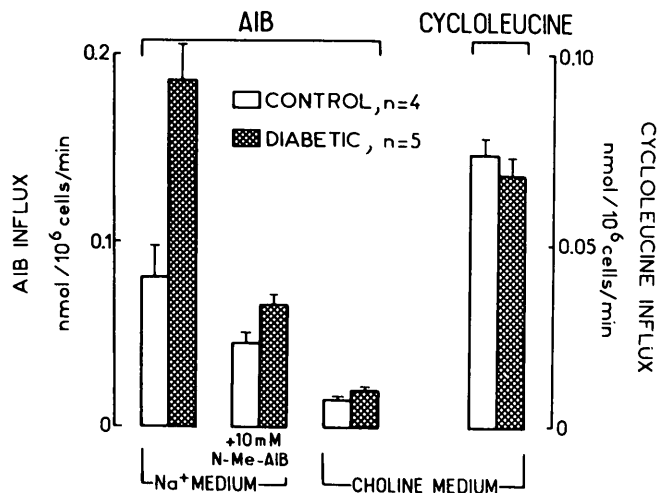
In isolated rat hepatocytes, the Na<sup>+</sup>-dependent transport of AIB occurs through the A (alanine-preferring) and ASC (alanine, serine, cysteine) systems.<sup>5</sup> To characterize the specificity of the system(s) involved in the increased amino acid transport in diabetic rat hepatocytes, the influx of [<sup>14</sup>C]AIB (0.1 mM) was measured in a Na<sup>+</sup>-containing medium (Krebs-Ringer bicarbonate buffer) in the absence and presence of N-Me-AIB (10 mM), a specific substrate for system A; in the latter condition, the entry of AIB through system A is largely inhibited,<sup>5</sup> and the remaining portion of the Na<sup>+</sup>-dependent influx is accounted for mainly by system ASC. Figure 4 shows that the increase in AIB influx in diabetic rat hepatocytes was mainly due to an enhanced transport through system A since this increase was markedly reduced in the presence of N-Me-AIB. However, AIB transport through system ASC (measured in the presence of N-Me-AIB) was still slightly increased in diabetics compared with controls. By contrast, the entry of AIB by simple diffusion (measured in a Na<sup>+</sup>-free, choline medium) was not significantly different in control and diabetic rat hepatocytes.

The activity of system L (leucine-preferring), a Na<sup>+</sup>-independent transport process with the properties of facilitated diffusion, was investigated by measuring the influx of [<sup>14</sup>C]cycloleucine in a Na<sup>+</sup>-free, choline medium.<sup>5</sup> The influx of cycloleucine was not significantly different in control and diabetic rat hepatocytes (Figure 4). Therefore, in contrast to

**TABLE 3**  
Kinetic parameters for AIB transport in isolated hepatocytes from control, diabetic, and treated diabetic rats

	Control	Diabetic	Treated diabetic
K <sub>m</sub> (mM)	38 $\pm$ 2	35 $\pm$ 5	42 $\pm$ 5
V <sub>max</sub> (nmol/10 <sup>6</sup> cells/min)	31.8 $\pm$ 3.3	70.1 $\pm$ 11.9	34.9 $\pm$ 2.7

Values are means  $\pm$  SEM of 6 separate experiments for control and diabetic, and 3 separate experiments for treated diabetic. Each experiment was performed with hepatocytes from one animal.



**FIGURE 4.** Amino acid transport in hepatocytes from control and diabetic rats: discrimination of transport systems. Hepatocytes were incubated for 4 min with [<sup>14</sup>C]AIB (0.1 mM) in a Na<sup>+</sup> medium or in a Na<sup>+</sup>-free (choline) medium, and in the absence or presence of N-Me-AIB (10 mM) as indicated. The influx of [<sup>14</sup>C]cycloleucine (0.2 mM) was measured in a Na<sup>+</sup>-free (choline) medium. Results are means  $\pm$  SEM of 4 separate experiments for control rat hepatocytes, and 5 separate experiments for diabetic rat hepatocytes.

system A, system L is not altered in diabetic rat hepatocytes.

### DISCUSSION

While in insulinopenic diabetes muscle protein catabolism is increased, plasma alanine levels are decreased,<sup>2,17,18</sup> suggesting an increased hepatic extraction of the gluconeogenic precursor consistent with an enhanced gluconeogenesis. It has indeed been shown that in insulin-dependent, nonketotic diabetic patients, splanchnic uptake of alanine and other glucogenic amino acids is 1.5–2 times greater than in control subjects.<sup>3</sup> Furthermore, isolated perfused livers from alloxan-diabetic rats accumulate about twice as much [<sup>14</sup>C]N-Me-AIB as do livers from control animals.<sup>4</sup>

The results of the present study, performed at the cellular level, have confirmed that the uptake of AIB is enhanced in isolated hepatocytes from streptozotocin-diabetic rats. Moreover, the use of isolated cells has allowed determination of the kinetic parameters of transport. Kinetic analyses indicate that in diabetic rat hepatocytes the increase in AIB uptake is accounted for by a twofold increase in the capacity (V<sub>max</sub>) of the transport system with no detectable change in affinity (K<sub>m</sub>). In agreement with observations made by Kilberg and Neuhaus<sup>4</sup> in the perfused liver from alloxan-diabetic rats, our results in isolated hepatocytes show that the increased AIB transport in diabetic animals predominantly involves system A, a Na<sup>+</sup>-dependent, concentrative transport system for neutral amino acids, whereas the activity of system L is unaltered.

That the change in amino acid transport activity in diabetic rat hepatocytes predominantly affected system A (with a small apparent contribution of system ASC) is in keeping with the general observation that the A mediation is the sole system subject to regulation (hormone and nonhormone) in a variety of cell types,<sup>19</sup> including isolated hepatocytes from adult rats.<sup>14–16</sup> The predominant increase in system A, and its reversion after insulin therapy, rule out the possibility that

some toxic damage of streptozotocin in liver might have been implicated in the change observed. This conclusion is also supported by the lack of increase in the  $\text{Na}^+$ -independent entry of AIB through simple diffusion, which excludes a nonspecific increase in cell membrane permeability.

Insulinopenic diabetes and fasting share a number of common hormone and metabolic alterations, such as hypoinsulinemia, hyperglucagonemia, increased muscle protein catabolism, and increased liver capacity to extract glucogenic substrates from plasma. Isolated hepatocytes from 48-h-fasted rats exhibit an increased ability to transport neutral amino acids.<sup>14</sup> As observed with diabetic rat hepatocytes, this increase occurs through system A. However, whereas in diabetic rat hepatocytes the enhanced transport results from an increase in the  $V_{\text{max}}$  of a homogeneous, low affinity transport component whose  $K_m$  (about 40 mM) is similar to that of transport in hepatocytes from normal fed rats,<sup>14</sup> the increased transport in fasting rat hepatocytes stems from the emergence of a high affinity ( $K_m \cong 1$  mM) transport component<sup>14</sup> that is virtually not expressed (or detectable) in hepatocytes from normal fed animals and from diabetic rats. Therefore, whereas in insulinopenic diabetes the hepatocyte increases its ability to extract amino acids from the extracellular milieu by increasing the  $V_{\text{max}}$  of a low affinity transport component (the predominant if not unique species in the basal state), after a 48-h fast this is achieved by the emergence of a high affinity transport component.

Glucagon is an effective inducer of the high affinity amino acid transport component in hepatocytes, both *in vitro*<sup>16</sup> and *in vivo* (data not shown). One possible explanation for the lack of emergence of the high affinity component in diabetic rat hepatocytes would be that the degree of the hyperglucagonemia (a 65% increase above controls) was not high enough in the relatively mild type of diabetes observed in the present study. Under our usual experimental conditions for the induction of insulinopenic diabetes, diabetic animals exhibited a markedly reduced weight gain compared with controls at the same stage of development, but they did not lose weight. However, when rats were made diabetic with a higher dose of streptozotocin (120 instead of 80 mg/kg body weight), they became ketotic and plasma glucagon levels were increased three to fourfold compared with controls. Of nine animals in this group, only 2 lost weight and only in hepatocytes from those 2 rats was the high affinity component of amino acid transport observed (not shown). Therefore, it is likely that *in vivo* factors other than hyperglucagonemia, as yet undefined but possibly related to the intensity of the catabolic state, are implicated in the induction of the high affinity amino acid transport. This is also suggested by the reversal, with insulin therapy, of the increased AIB transport in diabetic rat hepatocytes, which occurred despite the fact that these animals were still hyperglucagonemic at the time of the study, presumably due to insulin-induced hypoglycemia. It should be noted that *in vitro* glucose (up to 30 mM) failed to significantly alter glucagon induction of the high affinity transport (not shown), making it unlikely that, in diabetic rats, hyperglycemia might have impeded the expression of this high affinity component.

In conclusion, these studies have shown that in insulinopenic diabetes hepatocytes have an enhanced capability to

transport amino acids, owing mainly to an increased capacity of system A, a  $\text{Na}^+$ -dependent, concentrative amino acid transport. This change is reversed by insulin therapy. These observations provide a cellular basis to explain the increased liver extraction of circulating amino acids in insulinopenic diabetes, which may play a role in the enhanced gluconeogenesis observed in this situation. The exact mechanism(s) and factors (hormone and nonhormone) whereby such a change occurs remain to be elucidated.

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