A ^{13}C -n.m.r. investigation of the metabolism of amino acids in renal proximal convoluted tubules of normal and streptozotocin-treated rats and rabbits

Arnold W. H. JANS* and Rudolph WILLEM†

*Max-Planck-Institut fuer Systemphysiologie, Rheinlanddamm 201, 4600 Dortmund 1, Federal Republic of Germany, and †Vrije Universiteit Brussel, AOSC Pleinlaan 2, B-1050 Brussel, Belgium

^{13}C -n.m.r. spectroscopy was used to determine the metabolic fate of alanine and aspartate in rat and rabbit kidney proximal tubules. The main purpose of the present study was to investigate the effect of streptozotocin-induced diabetes on the influx of ^{13}C label from $[3-^{13}\text{C}]$ alanine into the tricarboxylic acid cycle and through the fructose-1,6-bisphosphatase pathway. This influx was calculated from the relative enrichment of ^{13}C in the various glutamate and glutamine carbon atoms. The relative proportion of ^{13}C label which entered the tricarboxylic acid cycle via pyruvate carboxylase relative to the proportion that entered via pyruvate dehydrogenase was 1.92 ± 0.02 in fed control rats and 2.27 ± 0.04 in streptozotocin-treated rats. However, streptozotocin-induced diabetes did not significantly affect this ratio in rabbit proximal convoluted tubular cells. Only in rat proximal convoluted tubular cells did we observe an increase in flux through the fructose-1,6-bisphosphatase pathway by streptozotocin treatment compared with fed controls. The data suggest that streptozotocin-induced diabetes in rats causes the same metabolic changes as does chronic acidosis.

INTRODUCTION

^{13}C -n.m.r. spectroscopy using ^{13}C -labelled substrates has not only been used to trace metabolic pathways in hepatocytes [1,2], rat renal tubules [3] and renal epithelial cells [4,5] but also to determine metabolic fluxes in perfused liver [6,7] and renal epithelial cells in culture, such as the LLC-PK₁/Cl₄ [5]. The relative enrichment of glutamate, glucose and lactate enables the determination of the relative fluxes leading to these metabolic products.

In recent studies, Cohen [6,7] showed the effect of diabetes on the tricarboxylic acid cycle and gluconeogenesis of normal and streptozotocin-treated rat livers. Since renal proximal convoluted tubular cells are the only mammalian cells, apart from the liver [8] and the renal papilla [9], that possess gluconeogenic activity, it seemed worthwhile to investigate the effect of streptozotocin on the metabolism of rat renal proximal tubules. We therefore investigated HClO₄ extracts of rat and rabbit proximal convoluted tubular (PCT) cells incubated with ^{13}C -labelled amino acids such as alanine and aspartate with the use of ^{13}C -n.m.r. spectroscopy.

MATERIALS AND METHODS

Animals

Diabetes was produced in male Wistar rats by intravenous injection of streptozotocin at a concentration of 45 mg/kg body weight. Treated animals (3 weeks)

exhibited plasma glucose levels in excess of 300 mg/100 ml and were thus considered to be diabetic.

Isolation of rat renal PCT cells

Suspension of rat PCT cells were obtained from ten kidneys of male Wistar rats (weighing between 250 and 300 g) by the method of Balaban *et al.* [10]. A microscopic investigation showed that the PCT suspension mainly consisted of S1 and S2 rabbit proximal-convoluted-tubular segments with a purity of about 90%. This is evident from the presence of long microvilli, clear basal infoldings (basal labyrinth) and peroxisomes, all being characteristic of the S1 and S2 segments. The impurities are mainly due to blood capillaries and S3 segments (outer medulla). Viability of the cells was monitored by the ATP/ADP ratio (obtained from ^{31}P -n.m.r. data). The ATP/ADP ratio showed a value of 3.5. After isolation of the PCT, they were incubated with a medium of the following composition (in mM): NaHCO₃ (25), KCl (3.2), CaCl₂ (2.5), MgSO₄ (1.8), KH₂PO₄ (1.8), NaCl (118), lactate (0.4), adenosine (0.3), and 5 mM of the ^{13}C -labelled amino acid as required.

Isolation of rabbit renal PCT cells

Rabbit renal PCT cells were obtained from the kidneys of male New Zealand White rabbits by the method described above. Diabetes was induced by streptozotocin.

Chemicals

All ^{13}C -labelled compounds used in the present study were obtained from Merck, Sharp and Dohme Isotopes,

Abbreviations used: PCT, proximal convoluted tubular; PBS, phosphate-buffered saline (1.8 mM-KH₂PO₄/118 mM-NaCl, pH 7.4); FID, free induction decay; n.O.e., nuclear Overhauser effects; PDH, pyruvate dehydrogenase; PCB, pyruvate carboxylase; T_1 , spin lattice relaxation time; FBPase, fructose-1,6-bisphosphatase; $J_{x,y}$, scalar coupling constant between the carbon nuclei x and y ; δ , chemical shift expressed in p.p.m. (parts per million); GABA, γ -aminobutyrate; certain other abbreviations are defined in the legend to Scheme 1.

Montreal, Canada. All other chemicals were obtained from Sigma Chemie G.m.b.H., Deisenhofen, Germany.

Incubations

After being harvested and washed with phosphate-buffered saline (PBS), cells (approx. 10^6) were resuspended in a tube containing 8 ml of PBS in which the glucose was replaced by the ^{13}C -labelled amino acid at 5 mM. Incubations were performed in a water bath at 37°C , the suspensions being aerated. After 2 h, the cells were isolated by centrifugation and extracted. The supernatant was concentrated by freeze-drying and subsequently dissolved in 1.8 ml of $^2\text{H}_2\text{O}$. The pH of this solution was adjusted to 7.4. These incubations were repeated at least twice for each substrate. The number of experiments are given in the legends to the corresponding Figures.

HClO_4 extracts

After incubation of a suspension of rat PCT cells with a ^{13}C -labelled amino acid, the cell pellet and supernatant were separated by centrifugation at 4°C for 10 min. The pellet was frozen with liquid nitrogen, and 1 g of the pellet was extracted with 2 ml of HClO_4 (12%). This procedure was followed by a further centrifugation step and extraction procedure. Both extracts were titrated to pH 7.4 with 2 mM-KOH. The HClO_4 extract obtained by centrifugation was then freeze-dried and subsequently dissolved in 1.8 ml of $^2\text{H}_2\text{O}$.

N.m.r. spectroscopy

All ^{13}C -n.m.r. spectra were recorded at 100.61 MHz and 125.75 MHz on a Bruker AM 400 WB spectrometer and a Bruker AM 500 spectrometer (values in parentheses) respectively, both equipped with an Aspect 3000 computer. Typical acquisition parameters were as follows: spectral width 21 800 Hz (28 000 Hz), pulse width $12.0\ \mu\text{s}$ ($1.8\ \mu\text{s}$), data size 16 K (32 K), relaxation delay 1.7 s (3 s). Proton decoupling was carried out using a standard composite pulse sequence.

The ^{13}C -n.m.r. spectra of HClO_4 extracts were measured in 10 mm tubes at 5°C , and free induction decays (FID) consisting of 20 000 scans were accumulated. Chemical shifts (δ , in p.p.m.) were referenced to L-[3- ^{13}C]alanine at 17.11 p.p.m. [10] or to [3- ^{13}C]lactate at 20.95 p.p.m. [11]. Both compounds were present in HClO_4 extracts and supernatants, and their chemical shifts were determined relative to dioxan [11]. Assignments were made on the basis of the chemical shifts and comparison with values given in the literature [11–13]. In those cases where no reference data were available, chemical shifts were obtained by recording ^{13}C -n.m.r. spectra of the authentic compounds in solutions of ionic composition and pH similar to those of the extracts and supernatants.

Since the relative ^{13}C enrichment for distinct ^{13}C resonances in the spectra of HClO_4 extracts strongly depends on accurate intensity measurements, we corrected the integrated intensities for T_1 (spin-lattice relaxation time) and nuclear Overhauser effects (n.O.e.) by using a 0.1 M model solution of glutamate. Theoretically the ratio of the integrated intensities for the natural-abundance carbon atoms in glutamate would be 1. Under our experimental conditions (which included a Gaussian multiplication of the FID) the intensity ratios of glutamate were: C-2/C-3 = 0.68, C-2/C-4 = 0.82 and

C-3/C-4 = 1.2. The ratios obtained from HClO_4 extract spectra were corrected by using the above values.

A simple model for the calculation of stationary metabolic fluxes from ^{13}C -n.m.r. data

^{13}C -labelled substrates such as alanine, lactate and pyruvate induce a label influx into the tricarboxylic acid cycle via pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PCB).

The ^{13}C label which scrambles through the tricarboxylic acid cycle can leave the cycle at the level of oxoglutarate and oxaloacetate. Depending on the metabolic characteristics of the material under investigation, the enrichment of intermediates of the tricarboxylic acid cycle in the ^{13}C -n.m.r. spectrum is mainly reflected in ^{13}C -n.m.r. resonances of glutamate and glucose. In some cases minor resonances attributable to labelled citrate, β -hydroxybutyrate and succinate can also be observed.

Scheme 1. Network of metabolic reactions used for the determination of metabolic fluxes in renal PCT cells

Abbreviations: P, C, K, G, S, F, M, O, PCB, PDH, PEP, PK, and Glc represent pyruvate, citrate, α -oxoglutarate, glutamate, succinate, fumarate, malate, oxaloacetate, pyruvate carboxylase, pyruvate dehydrogenase, phosphoenolpyruvate, pyruvate kinase, and α - β -glucose respectively. a , flux via PDH; b , flux via PCB; $a(1-x)$, flux via glutamate dehydrogenase; x , fraction of α -oxoglutarate which scrambles through the tricarboxylic acid cycle (rate of the cycle); $(1-y)$, the fraction of oxaloacetate leaving the tricarboxylic acid cycle via phosphoenolpyruvate carboxykinase; and y corresponds to the fraction of oxaloacetate which scrambles through the tricarboxylic acid cycle. The fraction of oxaloacetate leaving the cycle towards aspartate is taken as negligible for simplicity in model interpretation.

Scheme 2. Fate of the ^{13}C label of $[3-^{13}\text{C}]$ alanine and subsequent scrambling by an influx (a) via PDH (sequence 1) and (b) via PCB (sequences 2 and 3)

In (a) and (b) abbreviations are as in Scheme 1. (b) This scheme includes complete scrambling due to very rapid equilibrations through the fumarase pathway before oxaloacetate enters either the pyruvate kinase pathway or the citrate synthase one.

These pathways are represented in Scheme 1. From $3-^{13}\text{C}$ -labelled substrates such as $[3-^{13}\text{C}]$ alanine, the following label scrambling in the tricarboxylic acid cycle occurs during an influx via pyruvate dehydrogenase: $[3-^{13}\text{C}]$ pyruvate (P3) → $[2-^{13}\text{C}]$ acetyl-CoA → C4 → K4 → S2 → [F2 + F3] → [M3 + M2] → [O2 + O3] (sequence 1; see Scheme 2a).

In addition, a label influx of $3-^{13}\text{C}$ -labelled substrate via PCB into the tricarboxylic acid cycle gives rise to the following label scrambling: (P3) → O3 → C2 → K2 → S1 → [F1 + F4] → [M1 + M4] → [O1 + O4] (sequence 2)

Arising from the activity of malate dehydrogenase and fumarase, an additional label exchange and subse-

quent scrambling occurs: (P3) → O3 → M3 → F3 → M2 → O2 → K3 → S2 → [F2 + F3] → [M2 + M3] → [O2 + O3] and M3 → F2 → M3 → O3 → K2 → S1 → [F1 + F4] → [M1 + M4] → [O1 + O4] (sequence 3; see Scheme 2b)

In ^{13}C -n.m.r. spectra of cells and tissues (incubated with $3-^{13}\text{C}$ -labelled substrates) with both PCB and PDH activities, such as the liver [6,7], a ^{13}C -labelling pattern appears which is the consequence of a scrambling according to sequences 1–3.

The theoretical representation of Scheme 1 and sequences 1–3 prompted Cohen [6,7] to postulate that the ratio of the proportion of ^{13}C label (derived from $3-^{13}\text{C}$ -labelled substrates) which enters the tricarboxylic acid

cycle via PCB relative to the proportion which enters via PDH can be estimated by the following equation:

$$[\text{PCB}]/[\text{PDH}] = \frac{[G^x2 + G^x3]}{[G^x4]} = \beta \quad (1)$$

where G^xi ($i = 2, 3, 4$) is the sum of the relative enrichments of observed glutamate and glutamine resonances, measured from the integrated resonance intensities, which are corrected for T_1 and n.O.e. effects.

From the extrapolation of eqn. (1), Cohen [6,7] assumed that no full label cycling occurs and that the enrichment of C-2 and C-3 carbon atoms is only due to PCB activity (which is only true if no intense label cycling in the tricarboxylic acid cycle occurs). However, the utilization of $[3-^{13}\text{C}]$ alanine can result in glutamate and glutamine labelled at the C-1 carbon atoms. This C-1 enrichment is both the consequence of a full scrambling from the C-4 carbon atom of α -oxoglutarate, which arises from an influx of the ^{13}C label via PDH and/or from the C-2 atom of α -oxoglutarate originating from PCB influx.

Therefore, according to Schemes 2(a) and (b), an estimation of PCB to PDH activity by eqn. (1) overestimates the PCB activity, because G^x2 and G^x3 are not only due to a label influx via PCB (sequences 2 and 3), but also to a scrambling of the ^{13}C label, which entered the tricarboxylic acid cycle via PDH (sequence 1).

For experiments with $2-^{13}\text{C}$ -labelled substrates, label influx into the tricarboxylic acid cycle can be described by the following sequences:

PDH

$[2-^{13}\text{C}]\text{Pyruvate (P2)} \rightarrow [1-^{13}\text{C}]\text{acetyl-CoA} \rightarrow \text{C5} \rightarrow \text{K5} \rightarrow \text{S4} \rightarrow [\text{F1} + \text{F4}] \rightarrow [\text{M1} + \text{M4}] \rightarrow [\text{O1} + \text{O4}] \rightarrow [\text{C6} + \text{C1}] \rightarrow \text{CO}_2 + \text{K1} \rightarrow \text{CO}_2$ (sequence 4; see Scheme 3).

PCB

$\text{P2} \rightarrow \text{O2} \rightarrow \text{C3} \rightarrow \text{K3} \rightarrow \text{S2} \rightarrow \text{F2} \rightarrow [\text{M2} + \text{M3}] \rightarrow [\text{O2} + \text{O3}]$ (sequence 5)

Scheme 3. Fate of the ^{13}C label of $[2-^{13}\text{C}]\text{alanine}$ by an influx into the tricarboxylic acid cycle via PCB and PDH (sequences 4 and 6)

Abbreviations are as in Scheme 1.

Owing to the activity of malate dehydrogenase and fumarase, an additional label exchange and subsequent scrambling occurs: $\text{O2} \rightarrow \text{M2} \rightarrow \text{F2} \rightarrow \text{M3} \rightarrow \text{O3} \rightarrow \text{K2} \rightarrow \text{S1} \rightarrow [\text{F1} + \text{F4}] \rightarrow [\text{M1} + \text{M4}] \rightarrow [\text{O1} + \text{O4}]$ (sequence 6; see Scheme 3).

From the above sequences, the relative influx via PCB compared with PDH can be estimated by the following equation:

$$[\text{PCB}]/[\text{PDH}] = \frac{[G^x2 + G^x3]}{[G^x5]} = \alpha \quad (2)$$

An estimation of the ratio of the influx via PCB relative to the influx via PDH according to eqn. (2) would give no overestimation of the PCB activity, owing to scrambling of labelled oxoglutarate, as can be observed by experiments with $3-^{13}\text{C}$ -labelled substrates. We prefer a model which considers label efflux from the tricarboxylic acid cycle by glutamate dehydrogenase ($1-x$) and phosphoenolpyruvate carboxykinase ($1-y$) and label scrambling through the tricarboxylic acid cycle (x and y). This model is based on experiments with both $2-^{13}\text{C}$ - and $3-^{13}\text{C}$ -labelled substrates.

More precisely, $1-x$ represents the fraction of labelled oxoglutarate leaving the tricarboxylic acid cycle by glutamate dehydrogenase activity, whereas x is the remaining fraction that cycles further through the tricarboxylic acid cycle each time the oxoglutarate pool is traversed. Similarly, $1-y$ represents the fraction of labelled oxaloacetate, after rapid scrambling through fumarase activity, leaving the tricarboxylic acid cycle by phosphoenolpyruvate carboxykinase, whereas y is the remaining fraction that continues cycling at the level of the oxaloacetate pool.

This model is derived from the network represented in Scheme 1 and uses experimental data from experiments with $[2-^{13}\text{C}]$ - and $[3-^{13}\text{C}]\text{alanine}$. For the experiment with $[3-^{13}\text{C}]\text{alanine}$, the fate of its ^{13}C label by an influx into the tricarboxylic acid cycle via PDH is represented in Scheme 2(a) (sequence 1).

From Scheme 2(a), the following expressions can be derived for the isotopomer fluxes:

$$G4 = a(1-x) \quad (3)$$

$$G3 = G2 = \frac{axy}{2} (1-x) \left(1 + \frac{xy}{2} + \frac{x^2y^2}{4} + \dots \right) = \frac{axy(1-x)}{2-xy} \quad (4)$$

$$\begin{aligned} [\text{PEP-C-2}] &= [\text{PEP-C-3}] \\ &= \frac{ax}{2} (1-y) \left(1 + \frac{xy}{2} + \frac{x^2y^2}{4} + \dots \right) \\ &= \frac{ax(1-y)}{2-xy} \end{aligned} \quad (5)$$

where a is the flux via PDH, and PEP represents the phosphoenolpyruvate pool that becomes viable through its subsequent metabolites, namely glucose, alanine and lactate.

Influx of the ^{13}C label arising from $[3-^{13}\text{C}]\text{alanine}$ via PCB with subsequent rapid label exchange via malate dehydrogenase and fumarase is represented in Scheme

2(b). From this schematic representation the following expressions can be derived (where *b* is the flux via PCB).

$$G4 = 0 \quad (6)$$

$$G2 = G3 = \frac{b}{2}y(1-x) \left[1 + \frac{xy}{2} + \frac{(xy)^2}{4} + \dots \right] = \frac{by(1-x)}{2-xy} \quad (7)$$

$$\begin{aligned} [\text{PEP-C-2}] = [\text{PEP-C-3}] &= \frac{b}{2}(1-y) \left[1 + \frac{xy}{2} + \frac{(xy)^2}{4} + \dots \right] \\ &= \frac{b(1-y)}{2-xy} \end{aligned} \quad (8)$$

and from Schemes 1 and 3 it can be determined that:

$$[\text{PEP-C-2}] = [\text{Glc2} + \text{Glc5} + \text{Ala2} + \text{Lac2}] \quad (9)$$

where Glc is glucose, Ala is alanine and Lac is lactate.

From eqns. (1), (3), (4) and (7) the following can be derived:

$$\frac{G2 + G3}{G4} = \beta = \frac{2y}{2-xy} \left(\frac{b}{a} + x \right) \quad (10)$$

β can be determined experimentally from the integrated area ratio of the labelled glutamate and glutamine pool sizes.

The following expression, which is derived from eqns. (4), (7), (8) and (9), can be obtained from n.m.r. experiments:

$$\frac{\text{Glc2} + \text{Glc5} + \text{Ala2} + \text{Lac2}}{G2} = \frac{1-y}{y(1-x)} = \gamma \quad (11)$$

The fate of the ¹³C label arising from [2-¹³C]alanine is represented in Scheme 3.

From the representation of Scheme 3, the following expressions can be derived:

$$\frac{G2 + G3}{G5} = \alpha = \frac{2b}{a} \cdot \frac{y}{(2-xy)} \quad (12)$$

Substitution of eqn. (10) in eqn. (12) gives:

$$\beta = \alpha + \frac{2xy}{(2-xy)} \quad (13)$$

With $\epsilon = \beta - \alpha$ we can calculate *y* from eqn. (14), obtained by combining eqns. (11) and (13).

$$y = \frac{\epsilon(2\gamma + 1) + 2}{(\gamma + 1)(\epsilon + 2)} \quad (14)$$

With the value of *y* obtained from eqn. (14) we can calculate *x* from eqn. (11). Hence, with *x* and *y* numerically determined the following expression for the estimation of an influx into the tricarboxylic acid cycle via PCB relative to the influx via PDH can be derived from eqn. (11).

$$\frac{\text{PCB}}{\text{PDH}} = \frac{b}{a} = \frac{\beta(2-xy)}{2y} - x \quad (15)$$

According to Cohen [6], the rate of pyruvate kinase flux relative to the flux through fructose-1,6-bisphosphatase (FBPase) can be estimated by the following equation:

$$\begin{aligned} \frac{\text{Pyruvate kinase flux}}{2(\text{FBPase flux})} &= \\ \frac{[\text{Ala (C-2/C-3)}] [1 + \text{Glc (C-2/C-1)}]}{1 - [\text{Glc (C-2/C-1)}] [\text{Ala (C-2/C-3)}]} \end{aligned} \quad (16)$$

In this equation, the ratios Ala (C-2/C-3) and Glc (C-2/C-1) are chosen to take label exchange into account at the stage of oxaloacetate. The above eqn. (16) is derived from the consideration that the ¹³C-labelled oxaloacetate pool gives rise to labelled phosphoenolpyruvate (C-2 and C-3) by an efflux from the tricarboxylic acid cycle via phosphoenolpyruvate carboxykinase. Labelling of the intermediate formed PEP (C-2 and C-3) is reflected in labelled alanine and glucose, which is represented in Scheme 4. It should be noted that eqns. (3)–(15) do not express the PCB/PDH ratio if substrates like D,L-[3-¹³C]-aspartate are used, because in this case the influx into the tricarboxylic acid cycle is a consequence of aspartic deamination, resulting in C-3-labelled oxaloacetate, which scrambles through the tricarboxylic acid cycle (Scheme 1).

RESULTS

Incubation of rat PCT cells with [3-¹³C]alanine

Fig. 1 represents the ¹³C-n.m.r. spectrum of an HClO₄ extract of rat PCT cells derived from control animals and those incubated with 5 mM-[3-¹³C]alanine. The most intense resonance in Fig. 1 corresponds to C-3 of alanine, which has been taken up into the cells, but has not yet been metabolized by rat PCT (alanine is also additionally labelled at C-2). The resonances at 69.8 p.p.m. and 20.9 p.p.m. assigned to the C-2 and C-3 carbon atoms of lactate indicate that lactate has a labelling pattern similar to that of alanine.

The labelling of α -oxoglutarate by label influx in the tricarboxylic acid cycle via PCB (EC 6.4.1.1) and PDH

Scheme 4. Correlation between the labelling (*) pattern of oxaloacetate (OA), PEP and $\alpha\beta$ -glucose (Glc)

Abbreviations are as given in Scheme 1.

Fig. 1. 125.7 MHz ^{13}C -n.m.r. spectrum of an HClO_4 extract of rat PCT cells incubated with 5 mM-[3- ^{13}C]alanine for 60 min

This spectrum is the result of 10000 accumulations ($n = 3$). Definitions: 1, citrate C-3; 2, β -glucose C-2; 3, α -glucose C-3; 4, α -glucose C-2,5; 5, malate C-2; 6, Gln C-2; 7, acetoacetate C-2; 8, malate C-3; 9, *cis*-aconitate C-4; 10, γ -amino butyrate C-4; 11, GABA C-3. G3P, L-glycerol 3-phosphate; L, resonances due to lipids; X, unassigned, but probably due to choline and betaine. The insets show the carboxy region, the glucose region and a Lorentzian-to-Gaussian resolution-enhanced expansion of the Glu C-3 resonance, exhibiting small, but significant, splitting, which is due to scalar coupling (J).

(EC 1.2.4.1, EC 2.3.1.12) is reflected in the presence of glutamate and glutamine, both being labelled at C-2, C-3 and C-4 in the proportion of the resonance line intensities of 1:1.1:1.39. In addition, we calculated from eqn. (2) a value of $1.75 \pm 0.02 = \beta$ for the ratio $[\text{G2} + \text{G3}]/[\text{G4}]$.

Minor resonances indicate that the glutamate residue in glutathione (GSH) is also labelled, which is in agreement with a similar observation on LLC-PK₁/Cl₄ cells [5]. Other less intense, but significant, resonances belong to γ -aminobutyrate (GABA) (C-2, C-3 and C-4), which is probably derived from labelled glutamate by the high levels of activity of the cytoplasmic glutamate decarboxylase (EC 1.4.1.2) present in the kidney [14,15].

A label efflux from the tricarboxylic acid cycle at the stage of intermediate oxaloacetate by aspartate aminotransaminase (EC 2.6.1.1) and phosphoenolpyruvate carboxykinase (EC 4.1.1.32) is confirmed by the presence of labelled aspartate (C-2 and C-3) and α , β -glucose (C-1, C-2, C-3, C-4, C-5 and C-6) respectively. Here, however, the glutamate and glutamine resonance only exhibit less intense splitting, owing to a scalar carbon-carbon coupling in multiple-labelled species. An intense cycling of the ^{13}C label in the tricarboxylic acid cycle is evident from the presence of glutamate and

glutamine C-1 resonances and the labelling of all α , β -glucose carbon atoms. In addition, a label exchange giving rise to the C-2 enrichment of alanine and lactate is a consequence of label cycling and an efflux of ^{13}C -labelled oxaloacetate via phosphoenolpyruvate carboxykinase (EC 4.1.1.32).

Incubation of rat PCT cells with [2- ^{13}C]alanine

The ^{13}C -n.m.r. spectrum of an HClO_4 extract obtained from rat PCT cells isolated from control animals and incubated with 5 mM-[2- ^{13}C]alanine confirms the results presented in Fig. 1, although the most intense resonances are to be seen at the C-2 carbon atoms of alanine and lactate. The main difference seen between incubation with [3- ^{13}C]- and [2- ^{13}C]alanine is that, in the latter experiment ([2- ^{13}C]alanine), the ratio of the line intensities of the C-2, C-3 and C-4 glutamate atom is 1:1.15:0.53, reflecting the different labelling patterns of the substrate. The low-field region of the ^{13}C -n.m.r. spectrum (Fig. 2) of the HClO_4 extract of rat PCT cells (incubated with [2- ^{13}C]alanine) demonstrates that glutamate and glutamine are also labelled at the C-5 and C-1 carbon atoms, which confirms a full cycling of the tricarboxylic acid cycle. By the use of eqns. (10), (11) and (13)–(15), we calculated a ratio of 1.92 ± 0.02 for the influx via PCB versus the influx PDH.

Fig. 2. Carboxy region of a 100.6 MHz ^{13}C -n.m.r. spectrum of the HClO_4 extract of rat PCT cells incubated with 5 mM- $[\text{3-}^{13}\text{C}]$ alanine for 60 min

The ^{13}C spectrum is the result of 30 000 accumulations ($n = 3$).

Incubation of rat PCT with DL- $[\text{3-}^{13}\text{C}]$ aspartate

As has been described previously for LLC-PK₁/Cl₄ cells [5], DL- $[\text{3-}^{13}\text{C}]$ aspartate is an interesting substrate

for the investigation of renal metabolism because its label does not enter the tricarboxylic acid cycle by PCB or by PDH, but enters after a deamination via C-3-labelled oxaloacetate in the tricarboxylic acid cycle.

The ^{13}C -n.m.r. spectrum of the HClO_4 extract obtained from the pellet of a suspension of rat renal PCT cells, incubated with 5 mM-DL- $[\text{3-}^{13}\text{C}]$ aspartate, is shown in Fig. 3. The most intense resonance is located at 37.4 p.p.m. and can be assigned to DL- $[\text{3-}^{13}\text{C}]$ aspartate, which was transported into the cells, but not utilized. In addition, the spectrum shown in Fig. 3 exhibits a labelling pattern similar to, but not identical with, that seen in the experiment with $[\text{3-}^{13}\text{C}]$ alanine. The pattern differs as follows: (a) the ratio of the glutamate C-4 intensities to the glutamine C-4 line intensities is 3.1, whereas for alanine a ratio of 5.5 was demonstrated; (b) in contrast with the incubation with $[\text{3-}^{13}\text{C}]$ alanine, the ^{13}C -n.m.r. spectrum of rat PCT cells incubated with DL- $[\text{3-}^{13}\text{C}]$ aspartate does not show any multiplicity of the glutamate resonances; and finally (c) from the data of Fig. 3, the calculated ratio of the glutamate C-2:C-3:C-4 resonances is 1:1.1:0.75, which indicates the different possible influxes of the ^{13}C label into the tricarboxylic acid cycle.

Effect of streptozotocin

To examine the effect of streptozotocin on the metabolism of amino acids in rat PCT cells, we incubated suspensions of rat PCT cells, derived from streptozotocin-

Fig. 3. 100.6 MHz ^{13}C -n.m.r. spectrum of an HClO_4 extract of rat PCT incubated with 5 mM-DL- $[\text{3-}^{13}\text{C}]$ aspartate

Definitions and conditions are as in Fig. 1 ($n = 3$).

Table 1. Relative intensities of the ^{13}C -n.m.r. resonances in ^{13}C -n.m.r. spectra (measured at 100.61 MHz) of HClO_4 extracts of rat renal PCT cells incubated with various ^{13}C -labelled substrates

Substrate	Streptozotocin	relative intensities in the glutamate pool (C-2/C-3/C-4)	Ratio of the relative intensities of $\alpha\beta$ -glucose C-1 to C-4 of glutamate
[2- ^{13}C]Alanine	—	1:1.15:0.53	0.35
	+	1:1.12:0.38	1.75
[3- ^{13}C]Alanine	—	1:1.10:1.39	0.32
	+	1:0.99:0.73	2.80
[3- ^{13}C]Aspartic acid	—	1:1.10:0.75	0.39
	+	1:1.04:1.16	1.57

Table 2. Metabolic fluxes in rat renal PCT cells derived from 100.6 MHz ^{13}C -n.m.r. data

An improvement of the signal-to-noise ratio was obtained by a 5 Hz line broadening. Definitions and abbreviations: $\gamma = [\text{Glc2} + \text{Glc5} + \text{Ala2} + \text{Lac2}]/\text{G2}$; x and y are defined in the legend to Scheme 1.

Substrate	Streptozotocin	PCB/PDH = $\frac{b^*}{a}$	$\frac{\text{Pyruvate kinase flux}}{\text{FBPase flux}}$	γ^*	x^*	y^*
[2- ¹³ C]Alanine	—	1.92±0.02	—	0.49	0.16	0.77
	+	2.27±0.04	—	0.97	0.55	0.77
[3- ¹³ C]Alanine	—	1.92±0.02	0.78±0.04	0.49	0.16	0.77
	+	2.27±0.04	0.33±0.03	0.97	0.55	0.77
[3- ¹³ C]Aspartic acid	—	—	0.74±0.03	—	—	—
	+	—	0.32±0.03	—	—	—

* Determined by eqns. (3)–(15) from experiments with both [2- ^{13}C]– and [3- ^{13}C]alanine.

Fig. 4. 100.6 MHz ^{13}C -n.m.r. spectrum of an HClO_4 extract of rabbit PCT cells after incubation with 5 mM-[3- ^{13}C]alanine ($n = 3$)
Abbreviation: AA, acetoacetate; J is defined in Fig. 1.

treated rats with 5 mM-[2-¹³C]- and -[3-¹³C]alanine and 5 mM-DL-[3-¹³C]aspartate respectively.

The changes in amino acid metabolism after streptozotocin treatment can be seen by comparing the ¹³C-n.m.r. spectra of the HClO₄ extracts of PCT cells (incubated with 5 mM-¹³C-labelled amino acids) derived from streptozotocin-treated animals with the corresponding spectra of normal animals. Changes in metabolism can be obtained from the relative intensities of the glutamate pool and the ratio of the relative intensities of α,β-glucose C-1 to C-4 of glutamate. These intensities are compiled in Table 1.

Biochemically more relevant information can be obtained from the influx into the tricarboxylic acid cycle (i.e. the PCB/PDH ratio) and the efflux from the tricarboxylic acid cycle (i.e. the ratio of the flux via pyruvate kinase relative to the flux via FBPase). These data for normal and streptozotocin-treated PCT cells are compiled in Table 2.

Incubation of rabbit PCT with [3-¹³C]alanine

To examine the differences in L-alanine metabolism in different species, we also incubated rabbit renal PCT cells with 5 mM-[3-¹³C]alanine. The ¹³C-n.m.r. spectrum of the corresponding HClO₄ extract is shown in Fig. 4, which shows some differences from the ¹³C-n.m.r. spectrum obtained for rat PCT cells (Fig. 1). The main differences between rabbit and rat PCT are as follows: (a) after utilization of 5 mM-[3-¹³C]alanine, rabbit renal PCT cells exhibit higher levels of labelled glutamine than do rat PCT cells; (b) the relative levels of labelled glucose are higher in rat PCT cells than in rabbit PCT cells; (c) only the ¹³C-n.m.r. spectrum of rat PCT cells shows resonances due to acid GABA and to (unlabelled) lipids.

For rabbit renal PCT cells we determined the flux through PCB relative to that through PDH of untreated and streptozotocin-treated rabbits. These results are compiled in Table 3. These data show that PCT cells of untreated rabbits possess a slightly higher activity of PDH than do rat renal PCT, whereas the PCB/PDH ratio is only slightly, but significantly, affected by streptozotocin treatment. We did not determine the rate of the flux through pyruvate kinase relative to the flux through FBPase in rabbit PCT cells, as the relative levels

of labelled glucose are too low. The ¹³C-n.m.r. spectrum of rabbit PCT cells derived from streptozotocin-treated animals showed a decrease in the levels of labelled lactate with increasing incubation time.

DISCUSSION

The present study was undertaken to determine the metabolic changes in renal PCT cells caused by streptozotocin treatment of rats and rabbits, as we are unaware of any study which describes the effect of streptozotocin treatment on metabolic fluxes in renal PCT cells. Therefore we incubated tubular cells of rat and rabbit with [3-¹³C]alanine and [3-¹³C]aspartic acid and estimated the fluxes through different metabolic pathways from modified equations which were first proposed by Cohen [6] for the determination of metabolism from ¹³C-n.m.r. spectra.

¹³C-n.m.r. spectra of HClO₄ extracts

The ¹³C-n.m.r. spectra of the HClO₄ extracts of rat and rabbit PCT cells incubated with three ¹³C-labelled amino acids contain different types of information which can be obtained either directly or after some calculations from the spectra.

Firstly, the chemical shift of each resonance line (i.e. peak position) directly indicates which carbon atom of the different metabolic intermediates is labelled. Thus chemical shifts enable us to trace out the metabolic pathways of ¹³C-labelled amino acids. Secondly, the line intensities of the various carbon resonances are an indirect indication for the qualitative analysis of the spectra. However, line intensities can be directly obtained from the ¹³C-n.m.r. spectra (Table 1). They cannot be used for the determination of concentrations because they also correspond to a natural-abundance contribution of ¹³C, derived from carbon sources like fatty acids, and a line intensity is also affected by the n.O.e. and *T*₁ of a certain carbon atom. So far, significant differences in intensities indicate a different influx of the ¹³C label into the tricarboxylic acid cycle.

A comparison of the intensities of the C-2, C-3 and C-4 in HClO₄ extracts of rat PCT cells incubated with [2-¹³C]alanine and [3-¹³C]alanine indicates that the labelling of C-4 occurs in both cases by two different pathways, as indicated in Schemes 2 and 4. Thirdly, the relative enrichments of two carbon atoms in the same molecule can be derived from the following equation:

$$[C^{xi}/C^{xj}] = \frac{[C_i^{\text{exp}}/C_j^{\text{exp}}]}{[C_i^{\text{mod}}/C_j^{\text{mod}}]}$$

where *C^{xi,j}* is the relative enrichment of the carbon atoms *Ci* and *Cj*, *C_{i,j}^{exp}* are the integrated intensities of *Ci* and *Cj* resonances in the ¹³C-n.m.r. spectrum of the extract, and *C_{i,j}^{mod}* are the integrated intensities determined in a 0.1 M solution of the molecule of interest. The relative enrichment of pools of ¹³C isotopomers allows us to determine metabolic fluxes by using eqns. (5)–(15). These fluxes are compiled in Table 2.

An interesting feature of ¹³C-n.m.r. spectroscopy using ¹³C-labelled substrates is that this technique allows the determination of fluxes into the tricarboxylic acid cycle from the resonance line-splittings of glutamate C-3 [16] and from the relative enrichments of the glutamate

Table 3. Effect of streptozotocin on the ratio of PCB to PDH activity in rabbit PCT cells

The data are derived from 100.6 MHz ¹³C-n.m.r. spectra to enhance the signal-to-noise ratio; a 5 Hz line broadening was applied to the FID. The substrate was [3-¹³C]alanine throughout.

Incubation time (min)	Streptozotocin	PCB/PDH = $\frac{b^*}{a}$	<i>x</i> *	<i>y</i> *
60	—	1.55 ± 0.03	0.42	0.85
30	+	1.29 ± 0.03	0.33	0.96
60	+	1.28 ± 0.03	0.32	0.96
90	+	1.29 ± 0.03	0.33	0.96
120	+	1.28 ± 0.03	0.33	0.96

* Determined from eqns. (3)–(15) and similar incubations with [2-¹³C]alanine.

carbon atoms [6]. In a first approximation, the percentage of $[2-^{13}\text{C}]$ acetyl-CoA entering the tricarboxylic acid cycle can be directly determined from the ^{13}C -n.m.r. spectrum of the HClO_4 extracts derived from the incubations with $[3-^{13}\text{C}]$ alanine. This percentage can be calculated from the ratio of the glutamate C-3 doublet intensity to the glutamate C-3 singlet intensity [16]. From the incubation of rat PCT cells with $[3-^{13}\text{C}]$ alanine, we calculated that about 20% of the acetyl-CoA entering the tricarboxylic acid cycle was labelled at C-2, which is reflected in the enrichment of the glucose carbon atoms (15–20%). This is similar to the observations of Krebs *et al.* [17], who demonstrated that the specific radioactivity of glucose carbon atoms was only 29% in the rat kidney cortex incubated with L-[U- ^{14}C]lactate when taking into account an additional influx of ^{13}C label via PCB in our experiments. For rabbit PCT we found that about 55% of the acetyl-CoA entering the tricarboxylic acid cycle was labelled at C-2, indicating that rabbit PCT possess lower levels of endogenous carbon sources than do rat PCT.

The determination of the relative proportion of pyruvate entering the tricarboxylic acid cycle by the competing PDH and PCB routes is the ^{13}C -n.m.r. analogue, for the first time used by Cohen [6,7], of pioneering ^{14}C methods of Freedman *et al.* [18] and Koeppe *et al.* [19].

In the present study we developed a mathematical model [eqns. (3)–(15)] based on the above-mentioned studies [6,7,18,19], which allows not only the determination of the PCB/PDH ratio from ^{13}C -n.m.r. data, but in addition, the estimation of the flux through the tricarboxylic acid cycle (' x ') and the fraction of intermediate oxaloacetate (' y '), which is used for the citrate synthase reaction. The data of Tables 2 and 3 indicate that our measurements were performed under steady-state conditions.

The expressions (3)–(15) derived in this work and resulting in values for the PCB/PDH ratio, as compiled in Tables 2 and 3, reduce to the expression (1), originally proposed by Cohen [6] for the case that $x = 0$ and $y = 1$. The calculation of the PCB/PDH ratio in control rat renal PCT cells incubated with $[3-^{13}\text{C}]$ alanine, as described by Cohen [6], i.e. by the application of eqn. (1), results in a PCB/PDH ratio of 1.75 ± 0.04 , whereas the estimation of the PCB/PDH ratio from the data of incubations with $[2-^{13}\text{C}]$ alanine (eqn. 2) results in a value of 1.41 ± 0.02 .

The difference between both calculations mentioned above for the PCB/PDH ratio in control-rat renal PCT cells can be explained by a scrambling of the ^{13}C label [arising from $[3-^{13}\text{C}]$ alanine, which enters the tricarboxylic acid cycle via PDH (sequence 1)]. This label scrambling does not occur if $[2-^{13}\text{C}]$ alanine is used as ^{13}C -labelled substrate (sequence 4; Scheme 4).

Estimations by eqns. (1) and (2) do not give a 'true' PCB/PDH ratio, because the equations are only valid if ' x ' and ' y ' in Fig. 1 have values of 0 and 1 respectively. This can be excluded, because $x = 0$ and $y = 1$ would result in the absence of labelled glucose, malate and C-2 alanine in the n.m.r. spectrum of Fig. 1.

Taking account of all major influxes into and effluxes from the tricarboxylic acid cycle, as indicated in Scheme 1, subsequent calculation of the PCB/PDH ratio from expressions (3)–(15), using n.m.r. data of the incubation with both $[2-^{13}\text{C}]$ alanine and $[3-^{13}\text{C}]$ alanine results in a value of 1.92 ± 0.02 for normal-rat renal PCT cells. The

discrepancies between the values for the PCB/PDH ratio by the expressions derived in the present work and the values obtained from the model of Cohen [6] can only be explained by the fact that the present model takes into account, in contrast with Cohen [6], all important effluxes from the tricarboxylic acid cycle as well as label scrambling. Similar discrepancies can be found for experiments with streptozotocin-treated animals (results not shown).

Metabolic pathways

We used two different strategies to trace out the metabolic pathways in renal PCT cells. Firstly, we performed incubations with $[2-^{13}\text{C}]$ alanine and $[3-^{13}\text{C}]$ alanine, the ^{13}C labels of which enter the tricarboxylic acid cycle via PCB and PDH, which is reflected in the enrichment of glutamate and glutamine resonances and to a minor extent in the labelling of intermediates of the tricarboxylic acid cycle.

In addition, we performed incubations of rat renal PCT cells with DL-[$3-^{13}\text{C}$]aspartate, as in this experiment the ^{13}C label enters the tricarboxylic acid cycle via a deamination of aspartate and is subsequently scrambled through the tricarboxylic acid cycle. In addition, a direct efflux from labelled oxaloacetate via phosphoenolpyruvate carboxykinase occurs and results in an additional enrichment of $\alpha\beta$ -glucose and products that are a consequence of pyruvate kinase activity.

The renal metabolic pathways observed are in agreement with the general pathways described by Wirthensohn & Guder [20] and with a recent ^{13}C -n.m.r. study [3]. The main metabolic differences between rat and rabbit PCT cells are the lower glutamine synthase activity in rat PCT cells and the higher gluconeogenic activity of rat PCT cells, which results in higher intensities of the $\alpha\beta$ -glucose resonances in the ^{13}C -n.m.r. spectrum of rat PCT cells (Fig. 1) than in that of rabbit PCT cells (Fig. 4). This observation is in agreement with the work of Gullans *et al.* [21], who found that gluconeogenesis of rabbit PCT cells from lactate was $34 \text{ mmol} \cdot \text{h}^{-1} \cdot \text{g dry wt.}^{-1}$, and the results concerning similar investigations on PCT cells from starved rats, where gluconeogenesis from lactate was found to be $388 \text{ mmol} \cdot \text{h}^{-1} \cdot \text{g dry wt.}^{-1}$ [22].

Effect of streptozotocin on metabolism in PCT cells

In rat renal PCT cells, streptozotocin did not significantly affect the general pathways, i.e. the same carbon atoms in the ^{13}C -n.m.r. spectra of normal and streptozotocin-treated PCT cells were observed. But streptozotocin clearly increased the relative levels of enriched $\alpha\beta$ -glucose, which is evident from the ratio of the $\alpha\beta$ -C-1 glucose resonances to the intensities of the glutamate C-4 resonances (Table 1). A similar increase of the glucose levels in rat PCT cells has been observed by Nissim *et al.* [3] in the case of NH_4^+ -induced acidosis.

A more quantitative estimation of the biochemical implications on rat PCT-cell metabolism of streptozotocin-induced diabetes can be obtained from the data of Table 2. Streptozotocin causes an increase in the PCB/PDH ratio of 1.92 ± 0.02 to 2.27 ± 0.04 and decreases the ratio of the flux through FBPase from 0.78 ± 0.04 to 0.33 ± 0.03 . These data are consistent with the higher levels of labelled $\alpha\beta$ -glucose in the HClO_4 extract (Table 1) and strongly indicate a higher activity of FBPase in PCT cells derived from streptozotocin-treated rats. This increase in gluconeogenic activity also

implies a higher activity of PCB, because an influx of pyruvate in the tricarboxylic acid cycle via this enzyme causes an increase of the oxaloacetate pool, which is a requirement for a higher rate of gluconeogenesis [7]. Consistent with the above data is the observed increase in the flux through the tricarboxylic acid cycle (x) in PCT cells derived from streptozotocin-treated rats, because a higher turnover of the tricarboxylic acid cycle results in an increased oxaloacetate pool. The present results with rat renal PCT are consistent with the results of Cohen [6] obtained from ¹³C-n.m.r. studies with perfused rat livers. Cohen [6] found a PCB/PDH ratio of 1.2 ± 0.1 in the liver of fed control rats. This ratio increased to 2.6 ± 0.3 in liver from diabetic animals [7]. The rate of pyruvate kinase flux was found to be 0.74 ± 0.04 of the gluconeogenic rate in liver from 24 h fasted control rats [6], whereas in diabetic liver a value of 0.34 ($n = 1$) was found. In contrast with the experiments with rat PCT cells, it was found that streptozotocin treatment in rabbits decreased the level of $\alpha\beta$ -glucose by about 10%, an observation which was in agreement with a decrease of the PCB/PDH ratio from 1.55 ± 0.03 to 1.30 ± 0.03 upon streptozotocin treatment. This also indicates that PCB controls the gluconeogenic activity in renal PCT cells. These observations of the effect of streptozotocin treatment on gluconeogenesis in rabbit PCT cells are similar to the results of investigations on the effect of acidosis in rabbit PCT cells described by Richterich & Goldstein [23].

We thank Dr. R. W. Grunewald, Mrs. P. Glitz and Mrs. J. Luig for technical assistance and Dr. D. M. Scott and Professor R. Kinne for stimulating discussions.

REFERENCES

1. Cohen, S. M., Ogawa, S. & Shulman, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1603–1607
2. Cohen, S. M., Rognstad, R., Shulman, R. G. & Katz, J. (1981) *J. Biol. Chem.* **256**, 3428–3432
3. Nissim, I., Yudkoff, M. & Segal, S. (1987) *Biochem. J.* **241**, 361–370
4. Jans, A. W. H. & Kinne, R. K. H. (1988) *Biochim. Biophys. Acta* **969**, 194–197
5. Jans, A. W. H. & Leibfritz, D. (1988) *Biochim. Biophys. Acta* **970**, 241–250
6. Cohen, S. M. (1987) *Biochemistry* **26**, 573–580
7. Cohen, S. M. (1987) *Biochemistry* **26**, 581–589
8. Gstraunthaler, G., Pfaller, W. & Kotanko, P. (1985) *Am. J. Physiol.* **248**, C181–C183
9. Jans, A. W. H., Grunewald, R. W. & Kinne, R. K. H. (1988) *FASEB J.* **2**, A704 (abstr. no. 2369)
10. Balaban, R. S., Soltoff, S. P., Storey, J. M. & Mandel, L. J. (1980) *Am. J. Physiol.* **238**, F50–F59
11. Bárány, M., Arus, C. & Chang, Y.-C. (1985) *Magn. Reson. Med.* **2**, 289–295
12. Canioni, P., Alger, J. R. & Shulman, R. G. (1983) *Biochemistry* **22**, 4974–4986
13. Santos, C., Buldain, G., Frydman, B., Cannata, J. J. B. & Cazzulo, J. J. (1985) *Eur. J. Biochem.* **149**, 421–429
14. Nissim, I., Yudkoff, M. & Segal, S. (1985) *J. Biol. Chem.* **260**, 13955–13967
15. Lancaster, G., Mohyuddin, F., Scriver, C. R. & Whelan, D. T. (1973) *Biochim. Biophys. Acta* **297**, 229–246
16. Cohen, S. M. (1983) *J. Biol. Chem.* **258**, 14294–14308
17. Krebs, H. A., Hems, R., Weidemann, M. J. & Speake, R. N. (1966) *Biochem. J.* **101**, 242–249
18. Freedman, A. D. & Graff, S. (1958) *J. Biol. Chem.* **233**, 292–295
19. Koeppe, R. E., Mourihedes, G. A. & Hill, R. J. (1959) *J. Biol. Chem.* **234**, 2219–2222
20. Wirthensohn, G. & Guder, W. G. (1986) *Physiol. Rev.* **66**, 469–497
21. Gullans, S. R., Brazy, P. C., Dennis, V. W. & Mandel, L. J. (1984) *Am. J. Physiol.* **246**, F859–F869
22. Guder, W., Wiesner, W., Stukowski, B. & Wieland, H. O. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1319–1328
23. Richterich, R. W. & Goldstein, L. (1958) *Am. J. Physiol.* **195**, 316–320

Received 4 August 1988/31 March 1989; accepted 5 April 1989