Glutamate-glutamine metabolism in the perfused rat liver

^{13}C-NMR study using (2-^{13}C)-enriched acetate

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^{13}C-NMR has been used te follow the metabolism of ^{13}C-enriched substrates in isolated perfused rat liver. The fate of 90% enriched [2-^{13}C]acetate has been studied in the perfused liver in order to investigate mitochondrial metabolism and the interrelations between cytosolic and mitochondrial pools of metabolites. Some compounds of the intermediary metabolism where found to be extensively labelled, e.g. glutamate, glutamine, acetoacetate and β-hydroxybutyrate. Under our experimental conditions, labelling of glutamate reached a steady-state within 30 min after the onset of perfusion of 20 mM acetate. In addition, the observed incorporation of ^{13}C into glutamine can be linked to the operation of the glutamate-glutamine antiport and to the high activity of cytosolic glutamate synthetase. The finding of both active glutaminase and glutamine synthetase activity in the same liver cells is evidence of the existence of an active glutamine-glutamate futile cycle.

NMR *Perfused liver* *Glutamate metabolism* *Acetate metabolism*

1. INTRODUCTION

The metabolism of glutamine and glutamate remains imperfectly understood despite 25 years of active research. Many reviews [1–4] have recently confirmed the renewed interest in the study of these important metabolic pathways; they have also revealed a large number of unanswered questions. Among the most relevant issues which are still pending, one can quote the control of glutamine and glutamate metabolism, the intracellular compartmentation and physiological role of glutamine in the metabolism of normal and tumor cells.

Most of the studies on hepatic metabolism to date have been conducted on isolated hepatocytes, mitochondria and perfused liver by using the conventional method of biochemical analysis of the perfusate or of freeze-clamped extracts. Recently, it has become clear that NMR can give precise information on the variation of metabolite levels and fluxes through biochemical pathways in a non-invasive manner and in real time [5,6]. ^{31}P- and ^{13}C-NMR have been applied to study hepatic metabolism on various biological systems including isolated rat hepatocytes [7,8] and mitochondria [9,10], perfused mouse and rat livers [11,12] and liver of whole rat [13,14]. In relation to the relatively low sensitivity and low natural abundance of ^{13}C, the majority of the ^{13}C-NMR studies on hepatocytes and perfused liver are based on the use of ^{13}C-enriched substrates such as glycerol, alanine and pyruvate [8,11].

Here, we have applied ^{13}C-NMR to follow the fate of 90% enriched [2-^{13}C]acetate in perfused rat liver in order to investigate the mitochondrial metabolism and the interrelations between the cytosolic and mitochondrial pools of metabolites. As a result of the operation of the Krebs cycle, some compounds of the intermediary metabolism

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were found to be extensively labelled, e.g., glutamate, glutamine, acetoacetate and β-hydroxybutyrate. Under our experimental conditions we have observed a transfer of the label from glutamate to glutamine consistent with an operative glutamate-glutamine antiport and a high activity of cytosolic glutamate synthetase. In addition, the finding of both active glutaminase and glutamine synthetase in the same liver is in agreement with the previously reported glutamine-glutamate futile cycle [14].

2. MATERIALS AND METHODS

2.1. *NMR spectroscopy*

Experiments were performed on a Nicolet NT200-WB spectrometer operating at 4.7 T. ^{13}C spectra were recorded at 50.3 MHz from rat liver preparations perfused in 20-mm diameter tubes. Each spectrum corresponds to the Fourier transform of 720 free induction decays resulting from 45° radiofrequency pulses repeated every 0.5 s. Gated bilevel broad-band proton decoupling was used with high-level power (5 W) applied only during the acquisition time.

2.2. *Liver perfusion*

Rats of the Wistar strain (60–80 g) were used as liver donors and were fed ad libitum. The liver was excised under intraperitoneal pentobarbital anesthesia (50 mg/kg) and perfused at the rate of 2–3 ml/min per g liver wet wt. Perfusion was carried out in the retrograde mode (caval to portal vein). The perfusion system consisted of two low pulsing peristatic pumps (Gilson), a membrane oxygenator (Scimed), a pHM73 unit (Radiometer) for monitoring the pH, pO_2 and pCO_2 of the perfusate and a multichannel chart recorder (Sefram). The perfusion medium was a Krebs-Henseleit bicarbonate buffer gassed with 95% O_2/5% CO_2. The perfusate was delivered to the liver at 37°C by a water-jacketed system connected to a thermoregulating bath.

3. RESULTS

The excised rat liver was perfused in the non-recirculating mode with a perfusion medium containing 10 mM glucose during 30 min to allow for temperature equilibration and optimization of magnetic field homogeneity. During this period, the energetic status of the liver was assessed by recording sequential ^{31}P-NMR spectra. A background natural abundance ^{13}C spectrum was also recorded prior to the addition of the enriched substrate. A solution of [2-^{13}C]acetate (1 ml) carefully adjusted to pH 7.4 was then added to the buffer reservoir to give a concentration of 20 mM

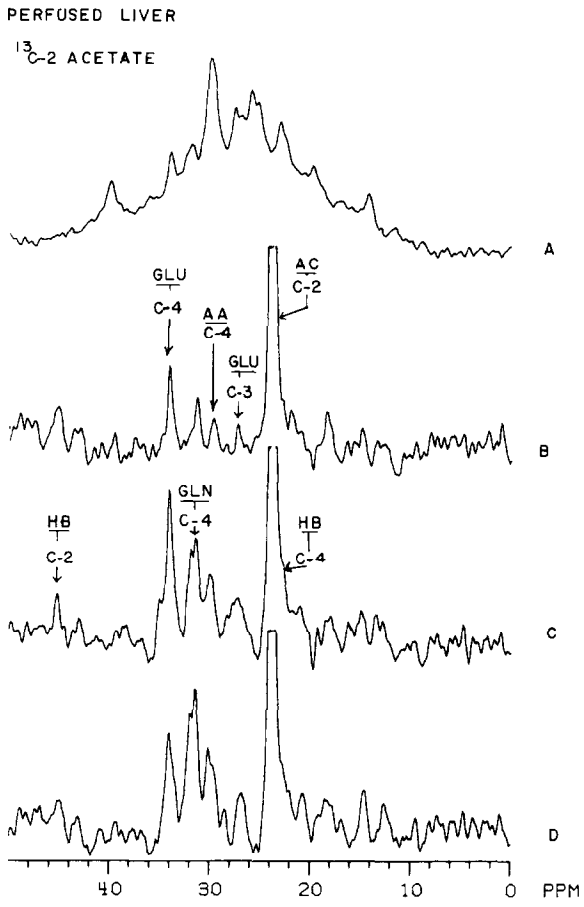

Fig.1. ^{13}C-NMR spectra of isolated rat liver perfused with [2-^{13}C]acetate. The liver was perfused with a Krebs-Henseleit medium containing 10 mM glucose. (A) 0–50 ppm region of the natural abundance ^{13}C-NMR spectrum from perfused liver. (B) Difference spectrum obtained by subtracting the background spectrum (A) from the spectrum recorded 6 min after switching the perfusate from glucose to glucose plus acetate. (C and D) Same as (B) but 36 min and 1 h after the onset of acetate perfusion, respectively. GLU, glutamate; GLN, glutamine; AC, acetate; AA, acetoacetate; HB, β-hydroxybutyrate.

acetate in the perfusate. The perfusion system was then switched to the recirculating mode, and the time course of acetate utilization was monitored by serially recording ^{13}C-NMR spectra at 6-min intervals. An illustration of acetate metabolism as followed by ^{13}C-NMR is given in fig.1. This figure shows the natural abundance ^{13}C-NMR spectrum of perfused liver from 0 to 50 ppm (fig.1A) and the difference spectra obtained 6 min, 36 min and 1 h after the onset of acetate perfusion (fig.1B–D).

The signals appearing in the difference spectra correspond to carbon resonances arising from labelled metabolites in the liver. They have been assigned to specific carbons of metabolites on the basis of their chemical shifts and the analysis of liver extracts. In the 6 min difference spectrum (spectrum B), the intense line at 23.8 ppm corresponds to the signal of [2-^{13}C]acetate, whereas the signals at 27.7 and 34.2 ppm arise from C-3 and C-4 of labelled glutamate, respectively. Accumulation of acetoacetate which is expected

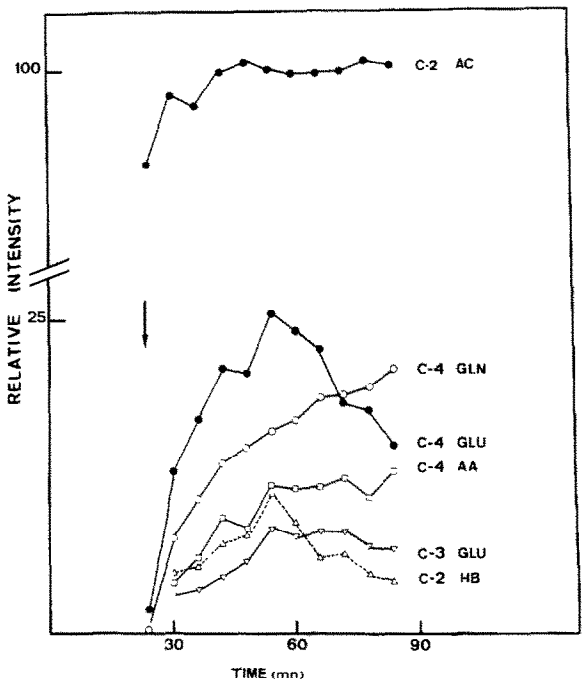

Fig.2. Time course of ^{13}C incorporation into intermediary metabolites in isolated rat liver perfused with [2-^{13}C]acetate. Experimental conditions and abbreviations as in fig.1. Ordinates give the relative intensities of the signals corresponding to labelled metabolites. The arrow indicates the time of acetate injection.

under these experimental conditions is reflected in the signal at 30.1 ppm (acetoacetate C-4). As the perfusion time with the acetate-containing glucose medium increased, other metabolites were labelled, giving rise to well-defined resonances in the difference spectra (fig.1C and D). The signals have been assigned to glutamine C-4 resonance (32.1 ppm) and β-hydroxybutyrate C-2 (45.4 ppm) and C-4 (22.1 ppm) resonances.

Assuming that the values for the T_1 relaxation time of all the observed metabolites do not differ significantly, the intensities of the signals can be considered as directly proportional to the ^{13}C enrichment of the molecule. The rate of label incorporation calculated on the basis of the area of the signals is given in fig.2. The data show that glutamate at the C-4 position is the first labelled. The glutamate C-4 labelling reaches a maximum value after 30 min acetate perfusion, and then decreases linearly with time back to 60% of its maximum value after 1 h perfusion. The same profile is observed for C-2 and C-4 of β-hydroxybutyrate. In contrast, the labelling of glutamine C-4 increases with time. After 1 h acetate perfusion, the enrichment of glutamine C-4 is found to be higher than that of the homologous carbon in the glutamate molecule.

4. DISCUSSION

Transient and steady-state changes induced by acetate utilization in perfused rat liver can be directly monitored by ^{13}C-NMR as illustrated in fig.2. The rapid isotope enrichment of glutamate at the C-4 position can be related to an increase in intracellular glutamate concentration subsequent to enhanced Krebs cycle activity. In fact, the total content of Krebs cycle intermediates increases after the perfusion of acetate, as a result of the transamination of part of the aspartate pool into oxaloacetate, under the control of aspartate aminotransferase. The rate of glutamate labelling can then be interpreted in terms of exchange rate across the aspartate-glutamate transamination process. It is noteworthy that aspartate is not labelled, indicating a very low activity of the malate-aspartate shuttle under our experimental conditions.

The fall in isotopic enrichment of glutamate C-4 after 30 min acetate perfusion can be accounted

for in terms of isotopic dilution rather than corresponding to a decrease in intracellular glutamate concentration. The concomitant labelling of glutamine is direct evidence of the existence of an electrogenic glutamate-glutamine antiporter, the efflux of labelled glutamate through the mitochondrial membrane being coupled with the mitochondrial influx of unlabelled cytosolic glutamine. The decrease in isotope enrichment of glutamate is linked to the activity of the intramitochondrial glutaminase leading to deamidation of glutamine and a release of unlabelled glutamate into the matrix space.

The kinetics of ^{13}C incorporation presented in fig.2 is consistent with a transfer of the (4-^{13}C) from glutamate to glutamine indicating a high glutamine synthetase activity, located in the cytosolic compartment [15]. These results further support the idea of the existence of an active glutamate-glutamine cycle in the liver [16,17]. The relative contributions of the intracellular vs the reported intercellular pathway [18] is now under investigation.

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