A $^1\text{H}/^{15}\text{N}$ n.m.r. study of nitrogen metabolism in cultured mammalian cells

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1. Heteronuclear $^1\text{H}/^{15}\text{N}$ n.m.r. experiments are described in which ^{15}N labelling of cellular metabolites is detected via their proton resonances. 2. These n.m.r. experiments have been used to monitor label redistribution amongst extracellular metabolites in cultures of mammalian cells incubated with L-[2- ^{15}N]glutamine, L-[5- ^{15}N]glutamine and $^{15}\text{NH}_4\text{Cl}$. Label redistribution was monitored in two HeLa cell lines and in two CHO cell lines which showed a range of extractable activities of glutamate dehydrogenase, glutaminase and glutamine synthetase. 3. In cells incubated with L-[2- ^{15}N]glutamine the ^{15}N label was subsequently found in a number of metabolites including alanine, aspartate,

glycine and pyrrolidone-5-carboxylic acid. There was no detectable production of $^{15}\text{NH}_4^+$, showing that most of the glutamate formed in the reaction catalysed by glutaminase was subsequently transaminated rather than oxidatively deaminated by glutamate dehydrogenase. 4. Incubation of cells with L-[5- ^{15}N]glutamine showed that the ammonia in the cultures was derived predominantly from the amide group of glutamine. 5. The rate of formation of L-[5- ^{15}N]glutamine in cells incubated with $^{15}\text{NH}_4\text{Cl}$ was used to estimate glutamine synthetase flux *in vivo*. Flux in this reaction was only observable in the two CHO cell lines which express relatively high levels of the enzyme.

INTRODUCTION

Tumour cells and normal cells with proliferative potential, such as intestinal cells and cells grown in culture, show high rates of glucose and glutamine utilization. These substrates provide both energy and anabolic precursors (Reitzer et al., 1979; McKeehan, 1982). However, despite the importance of glutamine for cell growth and proliferation, relatively little is known about the control of its utilization and about the control of nitrogen metabolism in general in cultured mammalian cells (McKeehan, 1986). In part, this can be ascribed to the lack of a readily available radioactive isotope, although there have been a number of studies using the short-half-life isotope, ^{13}N ($t_{1/2} = 9.96$ min). This positron-emitting isotope has been used to study the fate of glutamine in a number of tissues including brain (Cooper et al., 1979), liver (Cooper et al., 1987) and tumour cells, in the latter case both *in vivo* and *in vitro* (Rosenspire et al., 1985; Filc-DeRicco et al., 1990). There have also been numerous studies using the stable isotope, ^{15}N , in which the label has been detected by m.s. (Nissim et al., 1985; Yudkoff et al., 1991). These include a number of recent studies in cultured mammalian cells (Yudkoff et al., 1987; Nissim et al., 1991). Both techniques require chromatographic pretreatment of the labelled sample and, in the case of m.s., derivatization, before detection of the isotope label.

N.m.r. detection of the isotope label removes the requirement for extensive sample preparation and can be used to detect the label non-invasively in an intact tissue. ^{15}N n.m.r. has been used to study nitrogen metabolism in a variety of bacteria and fungi (Legerton et al., 1981; Haran et al., 1983; Kanamori et al., 1987), in cultured mammalian cells (Fernandez et al., 1988) and in rat brain, kidney and liver (Gründer et al., 1989; Farrow et al., 1990; Kanamori et al., 1991a; Geissler et al., 1991). However, direct detection of the label using ^{15}N n.m.r. is limited, particularly *in vivo*, by the insensitivity of the technique [see, for example, Gründer et al. (1989) and Geissler et al. (1991)]. Meynial-Denis et al. (1989) used a previously described heteronuclear $^1\text{H}/^{15}\text{N}$ spin-echo n.m.r. technique (Brindle et al., 1984) to measure

leucine transamination in skeletal-muscle homogenates. Indirect detection of the label via spin-coupled protons radically enhances the sensitivity of label detection and also allows fractional labelling to be determined. Kanamori et al. (1991b) used a $^1\text{H}/^{15}\text{N}$ heteronuclear multiple quantum coherence experiment to detect ^{15}N -labelled metabolites in isolated rat brain and liver. Again the sensitivity of label detection is enhanced, although with this technique fractional labelling is not determined.

We show here how the $^1\text{H}/^{15}\text{N}$ spin-echo n.m.r. experiment (Brindle et al., 1984), and modifications of this experiment, can be used to monitor the metabolic fate of glutamine and ammonia nitrogen in cultures of mammalian cells. ^{15}N label redistribution amongst extracellular metabolites has been measured in the culture media of four different cell lines which show a range of glutaminase, glutamine synthetase and glutamate dehydrogenase activities. The labelling patterns observed have been interpreted in terms of the extractable activities of these enzymes.

A preliminary account of this work was presented at the 10th Annual Meeting of the Society of Magnetic Resonance in Medicine (Street and Brindle, 1991).

EXPERIMENTAL

Materials

Tissue culture media were obtained from Gibco BRL (Uxbridge, Middx., U.K.). Sera were obtained from Seralab (Crawley Down, Sussex, U.K.). Other media supplements were obtained from Gibco BRL or Sigma (Poole, Dorset, U.K.). Tissue culture plastics were obtained from Falcon (Becton Dickinson, Oxford, U.K.). ^{15}N -labelled compounds were all 99 atom % and were obtained from MSD Isotopes (Croydon, Surrey, U.K.). Deuterated water (99 atom %) was obtained from Fluorochem Ltd. (Old Glossop, Derby, U.K.). Enzymes were obtained from Boehringer (Lewes, Sussex, U.K.) or Sigma. [^{14}C]Glutamate was obtained from Amersham (Little Chalfont, Bucks., U.K.). All other chemicals were of analytical grade.

Abbreviations used: TSP, 3-trimethylsilylpropionate-2,2,3,3- d_4 ; CHO, Chinese hamster ovary.

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Cell culture

Four different cell lines were used which showed a range of activities of glutaminase, glutamine synthetase and glutamate dehydrogenase (see Table 1). HeLa S3 cells (obtained from the Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.) were grown in RPMI 1640 medium supplemented with 2 mM glutamine and 10% newborn calf serum. The Chinese hamster ovary (CHO) cell lines, KG1 and KG1M, which overexpress glutamine synthetase (Sanders and Wilson, 1984) (obtained from Dr. Richard Wilson, University of Glasgow, Glasgow, Scotland, U.K.), were grown in Glasgow's minimum essential medium, supplemented with 5% dialysed fetal calf serum, 0.8 mM glutamate and 0.8 mM proline. HeLa S3 cells, adapted to grow in the presence of low concentrations of glutamine (low-glutamine HeLa), were cultured in RPMI 1640 medium supplemented with 10% newborn calf serum, 20 mM glutamate and 0.25 mM glutamine (DeMars, 1958).

Cells were seeded at 1×10^5 cells/ml in 50 ml of medium in 175 cm² flasks. Parallel incubations were seeded from the same flask to minimize any variations in growth between individual flasks. The cells were harvested for cell counting and enzyme assays by trypsin treatment.

Enzyme assays

Trypsin-treated cells were washed in PBS/EDTA (0.02%) and resuspended in an equal volume of extraction buffer containing 50 mM triethanolamine/HCl, pH 8.0, 1 mM EDTA, 5 mM MgCl₂ and 5 mM dithiothreitol. The suspension was sonicated with 4×5 s bursts at 4 °C. Enzyme assays were performed on this homogenate.

Glutamate dehydrogenase, alanine aminotransferase and aspartate aminotransferase were assayed as described by Sugden and Newsholme (1975), except that for the transaminase assays the alanine concentration was 80 mM and the aspartate concentration 60 mM. Glutaminase was assayed by the method of Heini et al. (1987) except that the glutamate produced was measured rather than ammonia. Glutamate was assayed by the method of Bernt and Bergmeyer (1974). Glutamine synthetase was assayed by the method of Tiemeier and Milman (1972), but included an ATP-regeneration system (King et al., 1983). The protein content of cell extracts was determined by the method of Bradford (1976) using a kit supplied by Bio-Rad.

Metabolite assays

Ammonia was assayed using glutamate dehydrogenase as described by Kun and Kearney (1974). Glutamine was assayed using the method described by Lund (1985).

Preparation of samples for n.m.r. spectroscopy

For the indirect detection experiments, the cell culture medium (50 ml) was decanted from the culture flasks at the designated times and the proteins present were removed by acetone precipitation. An equal volume of cold acetone was added to the media sample and left on ice for at least 20 min. The precipitated protein was removed by centrifugation and the acetone removed from the sample under vacuum using a rotary evaporator. The extract was treated with Chelex 100 (Bio-Rad) to remove paramagnetic ions present in the sample and then lyophilized. The sample was reconstituted with either 2 ml of ²H₂O or 2 ml of ¹H₂O depending on the n.m.r. experiment. In the latter case, 10% ²H₂O was added to provide a lock signal. The pH of the sample was adjusted to the designated pH with either ²HCl and

NaO²H or HCl and NaOH as appropriate and the sample placed in a 5 mm n.m.r. tube. In solutions containing ²H₂O the uncorrected pH-meter reading was used.

For ¹⁵N n.m.r. experiments the untreated tissue culture medium was decanted directly from the tissue culture flask into a 10 mm n.m.r. tube and 10% ²H₂O added to provide a lock signal.

N.m.r. spectroscopy

¹H and ¹⁵N n.m.r. spectra were obtained using a Varian Unity 400 MHz wide-bore spectrometer operating at 400.0 MHz and 40.5 MHz respectively. ¹⁵N chemical shifts were referenced to H¹⁵NO₃ contained in a coaxial capillary, and ¹H chemical shifts were referenced to trimethylsilylpropionate-2,2,3,3-d₄ (TSP), which was added as an internal standard. For both sets of experiments the sample temperature was maintained at 30 °C.

¹⁵N n.m.r. spectra were acquired with a sweep width of 20 kHz using a 90° pulse, WALTZ-16 composite pulse ¹H decoupling and a recycle delay of 7 s. A total of 10000 scans was usually acquired.

Pulse-and-collect ¹H spectra were acquired with a sweep width of 6 kHz using a 90° pulse and a recycle delay of 15 s. Under these conditions the ¹H signals were fully relaxed and the concentrations of metabolites present could be determined by comparison of their resonance intensities with that of the TSP standard. Indirect detection of ¹⁵N labels was carried out using two different experiments. Detection of ¹⁵N labels via three-bond ¹H-¹⁵N coupling (see the Results section) was carried out using the following experiment:

$$\begin{array}{ll} ^1\text{H} & 90^\circ - T - 180^\circ - T - \text{Acquire} \\ ^{15}\text{N} & 90^\circ_x 180^\circ_y 90^\circ_{\pm x} \end{array}$$

This experiment is similar to that described previously (Brindle et al., 1984) except that non-selective ¹H pulses were used and the 180° ¹⁵N pulse was a composite pulse in which the phase of the final 90° pulse was alternated by 180° to give either a 180° or 0° ¹⁵N pulse. Spectra acquired with or without a 180° ¹⁵N pulse were interleaved during the experiment (see Figure 1). The optimal delay (*T*) used in these experiments, where this was defined as the shortest delay which gave maximal peak inversion, was determined empirically (see the Results section). Labelled alanine and glutamate and amine-labelled glutamine were detected with *T* set to 130 ms. Amide-labelled glutamine was detected with a delay of 270 ms. The spectra were acquired with a sweep width of 6 kHz and the free induction decay was zero-filled to 32768 data points before Fourier transformation. The experiments were performed in ²H₂O and the residual water signal was suppressed by selective irradiation. For the amine detection experiment the repetition rate was 2 s and 1024 transients were collected for each spectrum ($\pm$ the 180° ¹⁵N pulse). For the amide-detection experiment, the repetition rate was 4 s and 512 transients were collected for each spectrum.

Amide-labelled glutamine was also detected via the directly bonded amide protons using the following pulse sequence:

$$\begin{array}{ll} ^1\text{H} & [90^\circ - \tau/2 - 90^\circ] - T - [90^\circ - \tau - 90^\circ]_{\text{Ex.}} - T - \text{Acquire} \\ ^{15}\text{N} & 90^\circ_x 90^\circ_{\pm x} \end{array}$$

where Ex. indicates that this pulse sequence element is phase-cycled according to the EXORCYCLE scheme. Since the amide protons are solvent-exchangeable the experiment was performed in ¹H₂O, the solvent resonance being suppressed by the semi-selective proton pulses (Von Kienlin et al., 1988). The solvent was placed on resonance and τ was set to 0.575 ms. The ¹⁵N pulses were applied at the midpoint of the τ delay. The phase of the second 90° pulse was alternated to give either a 0° or 180° ¹⁵N

Figure 1 Indirect ^{15}N label detection using a heteronuclear $^1\text{H}/^{15}\text{N}$ spin-echo difference experiment

The spectra labelled (i) and (ii) were acquired with a 0° ^{15}N pulse and a 180° ^{15}N pulse respectively (see the Experimental section). The difference spectra (i—ii) show signal only from the ^{15}N -labelled component. (a) β -proton resonances of ^{15}N -alanine; (b) β -proton resonances of L-[2- ^{15}N]glutamine; (c) γ -proton resonances of L-[5- ^{15}N]glutamine. (d) Spectra were obtained in $^1\text{H}_2\text{O}$ using semiselective ^1H pulses and show the upfield proton resonance from the directly bonded solvent-exchangeable amide protons of L-[5- ^{15}N]glutamine. These spectra were ^{15}N -decoupled during acquisition. The pure labelled compounds were used at a concentration of 20 mM. In experiments in (a) and (b) the pH was 1.0, in (c) the pH was 12.0 and in (d) the pH was 7.0.

pulse. Spectra acquired with or without the 180° ^{15}N pulse were interleaved. The optimal delay, T , for this experiment, i.e. the delay which gave maximal inversion of the ^{15}N -coupled amide protons, was determined to be 5.6 ms (see Figure 1). The amide protons were ^{15}N -decoupled during the acquisition using WALTZ-16 composite pulse decoupling.

The two-dimensional heteronuclear multiple-quantum coherence spectra were obtained using the multiple-bond heteronuclear multiple-quantum coherence (HMBC) experiment described by Summers et al. (1986). Δ_1 was set to 11.1 ms and Δ_2 was set to 100 ms. The experiment was performed in $^2\text{H}_2\text{O}$ and the residual solvent signal suppressed by selective irradiation. A total of 512 t_1 increments were collected with 16 scans per increment and a recycle delay of 4 s. The t_2 domain was zero-filled to 1024 data points before the second Fourier transformation. Sine-bell filtering was applied in both dimensions.

RESULTS

Label detection

Figure 1 illustrates how an ^{15}N label can be detected in the proton spectra of labelled metabolites using a heteronuclear $^1\text{H}/^{15}\text{N}$ spin-echo difference experiment. Application of a 180° ^{15}N pulse results in phase modulation of the ^{15}N -coupled proton resonances (see also the Experimental section). Therefore subtraction of this spectrum from the spectrum acquired with a 0° ^{15}N pulse gives a difference spectrum representing signal from the ^{15}N -labelled compound only. The fractional labelling of the molecule can thus be determined. The optimal delays used in these experiments were determined empirically with the pure labelled compound under conditions of pH and ionic strength similar to those present in the cell extracts. The value employed for label detection was the shortest value that gave maximal peak

Figure 2 Detection of L-[5-¹⁵N]glutamine in cell culture medium

The CHO cell line KG1M was grown for 48 h in media in which 10 mM ¹⁵NH₄Cl had been added. The regions of the spectra containing the glutamine amide proton resonances are shown. Spectra were acquired using the semiselective ¹H pulse sequence described in the text and Experimental section (see also Figure 1d). Spectrum (i) was acquired with a 0° ¹⁵N pulse and (ii) was acquired with a 180° ¹⁵N pulse. Each spectrum is the sum of 256 scans. (i—ii) is the difference spectrum.

inversion after application of the ¹⁵N pulse. These measurements with the pure labelled compounds were used to calibrate the measurements on cell extracts. In some cases a cell extract was spiked with a known quantity of the labelled compound in order to check the calibration. L-[¹⁵N]Alanine was detected via phase inversion of its methyl protons (Figure 1a) (Brindle et al., 1984). L-[2-¹⁵N]Glutamine and L-[2-¹⁵N]glutamate were detected via phase inversion of their β-protons at pH 1 (Figure 1b). This pH was used in order to minimize overlap with adjacent resonances in the spectra of media extracts. The β-protons of glutamine and glutamate were not resolved, although in some cases it was possible to quantify the two independently (see below). L-[5-¹⁵N]Glutamine was detected by either phase inversion of the γ-protons at pH 12 (Figure 1c) or phase inversion of the directly bonded amide protons at pH 7.0 (Figure 1d). Detection via the γ-protons was conducted at pH 12 in order to minimize overlap of the γ-proton resonances with those from pyrrolidone-5-carboxylic acid, which is a spontaneous decomposition product of glutamine (Tritsch and Moore, 1962) and which was labelled in cells incubated with L-[2-¹⁵N]glutamine (see Figure 4). Although these experiments were carried out at pH 1 and pH 12 respectively this was not essential and indeed some experiments were carried out at pH 8.0. However, there was some variation

Table 2 Measurements of NH₄⁺ production by ¹⁵N n.m.r. and enzyme assay

HeLa cells were grown for the specified time in media to which 2 mM labelled glutamine had been added. The culture medium was then decanted and assayed for ammonium ion. This value has been corrected for the ammonium ion present in the medium at *t* = 0. The ¹⁵NH₄⁺ present was assayed by ¹⁵N n.m.r. The ¹⁵N n.m.r. experiment was calibrated by the addition of a known amount of ¹⁵NH₄Cl to the extract.

Labelled substrate	Period of incubation (h)	[NH ₄ ⁺] (mM)	
		Measured by enzyme assay	Measured by n.m.r.
L-[5- ¹⁵ N]Glutamine	46	1.10	0.97
L-[5- ¹⁵ N]Glutamine	70	1.61	1.83
L-[2- ¹⁵ N]Glutamine	56	1.08	Not detected
L-[2- ¹⁵ N]Glutamine	81	1.28	Not detected
L-[2- ¹⁵ N]Glutamine	71	1.88	Not detected
+ 0.6 mM amino-oxyacetate			

in the optimal delay values with changes in pH (Street, 1992). The long delay used in the amide-label detection experiment (270 ms) considerably degrades the sensitivity of label detection and, furthermore, the degree of peak inversion is relatively small. Therefore the label was also detected by phase inversion of the directly bonded amide protons. At pH 7.0, in media extracts, the upfield amide proton of glutamine was in slow exchange with a line width of approx. 7 Hz (Figure 1d) (Kanamori et al., 1991b). The pulse sequence used to acquire the spectra shown in Figure 1(d) is a modification of that described previously (Brindle et al., 1984). The 1331-2662 ¹H pulse sequence element has been replaced with a [90°-τ/2-90°]-T-[90°-τ-90°]_{EX.}-T-acquire pulse sequence which avoids the frequency-dependent phase distortion associated with the earlier sequence but which retains good solvent elimination properties (Von Kienlin et al., 1988). The third and fourth pulses are phase-cycled according to the EXORCYCLE scheme.

The sensitivity of amide proton detection of ¹⁵N amide-labelled glutamine is illustrated in Figure 2, which shows spectra acquired from a concentrated medium sample taken from CHO cells (KG1M) incubated with 10 mM ¹⁵NH₄Cl. The total glutamine concentration in this sample was 15.5 mM and the ¹⁵N-labelled concentration 2.5 mM. The spectra are the sum of 256 scans and took a total of 25 min to acquire. Spectra with good signal-to-noise ratio, however, could be acquired from this sample with only 16-scan acquisitions. The difference spectrum shows ¹⁵N-

Table 1 Enzyme activities measured in cell extracts

The low-glutamine HeLa cells were a HeLa cell line adapted to grow on low concentrations of glutamine (see the Experimental section). KG1 and KG1M are CHO cell lines which were grown in the absence of glutamine and which have elevated levels of glutamine synthetase (Sanders and Wilson, 1984). The values shown are the means ± S.D. of determinations made on three different batches of cells.

Cell line	Enzyme activities (nmol/min per mg of cell protein)				
	Glutamate dehydrogenase	Alanine aminotransferase	Aspartate aminotransferase	Glutaminase	Glutamine synthetase
HeLa	25 ± 6	7 ± 2	206 ± 10	5 ± 1	Not detected
Low-glutamine HeLa	42 ± 2	31 ± 4	133 ± 10	14 ± 3	0.8 ± 0.4
KG1	134 ± 5	12 ± 1	219 ± 31	51 ± 4	9 ± 3
KG1M	259 ± 48	14 ± 1	224 ± 113	49 ± 11	32 ± 4

Figure 3 Glutamine consumption and ammonia production in HeLa cells

HeLa cells were incubated in RPMI 1640 supplemented with 10% newborn calf serum and 2 mM glutamine. Samples of the medium were taken periodically and assayed enzymically for glutamine (□) and ammonia (■) as described in the Experimental section.

Figure 4 Time-dependence of the labelling of glutamate (■) and alanine (□) in a HeLa cell culture

Parallel incubations, seeded at the same cell density from the same stock culture, were incubated for up to 72 h in media to which 2 mM $^{15}\text{NH}_4\text{Cl}$ had been added. Each time point shows the data obtained from the medium supernatant taken from a single flask. (a) Absolute concentrations of labelled alanine and glutamate in the media; (b) fractional labelling.

Table 3 ^{15}N label redistribution in cells incubated with L-[2- ^{15}N]glutamine, L-[5- ^{15}N]glutamine or $^{15}\text{NH}_4\text{Cl}$

The low-glutamine HeLa cell line was a HeLa cell line adapted to grow on low concentrations of glutamine (see the Experimental section). KG1 and KG1M are CHO cell lines which were grown in the absence of glutamine and which have elevated levels of glutamine synthetase (Sanders and Wilson, 1984) (see Table 1). The cells were incubated in media containing 2 mM glutamine and 2 mM NH_4Cl . The molecule, or position in the molecule, in which the label was located is indicated. In all other respects the media were identical with those used to grow the cells except that for the low-glutamine HeLa cells there was no added glutamine (see the Experimental section). The absolute concentrations of the metabolites and their fractional labellings at the end of the incubation were determined by n.m.r. measurements on extracts of the media (see the Experimental section). The glutamine concentrations shown are total concentrations. HeLa, KG1 and KG1M cells were seeded at a cell density of 1×10^5 cells/ml and incubated for 48 h to give final cell densities of 4.4×10^5 cells/ml, 4.5×10^5 cells/ml and 2.7×10^5 cells/ml respectively. HeLa cells adapted to grow on 0.25 mM glutamine and 20 mM glutamine (low-glutamine HeLa cells) were seeded at 1×10^5 cells/ml and incubated for 24 h in the same media as the control HeLa cells to give a final cell density of 1.8×10^5 cells/ml.

Labelled substrate	Metabolite	HeLa		Low-glutamine HeLa		CHO cell line KG1		CHO cell line KG1M	
		Concn. (mM)	Labelling (%)	Concn. (mM)	Labelling (%)	Concn. (mM)	Labelling (%)	Concn. (mM)	Labelling (%)
L-[2- ^{15}N]Glutamine	Alanine	0.25	38	0.21	15	0.56	36	0.31	20
	Glutamate	0.64	48	0.64	34	0.81	55	0.34	33
	L-[2- ^{15}N]Glutamine	0.58	100	1.28	100	0.57	100	0.77	100
L-[5- ^{15}N]Glutamine	L-[5- ^{15}N]Glutamine	0.21	0	0.16	0	0.49	0	0.27	0
	Alanine	0.83	0.8	0.68	0.6	0.96	1.2	0.61	5
	Glutamate	0.44	0.6	0.99	1.2	0.74	0.4	0.90	4
$^{15}\text{NH}_4\text{Cl}$	L-[2- ^{15}N]Glutamine	0.18	100	0.18	100	0.55	100	0.29	100
	Alanine	0.65	2.9	0.56	8	0.83	8	0.87	19
	Glutamate	0.55	2.5	1.08	9	0.94	0	0.92	13
	L-[2- ^{15}N]Glutamine		0		0		0		0
	L-[5- ^{15}N]Glutamine		0		2.5		6		6

Figure 5 Two-dimensional multiple-bond heteronuclear multiple-quantum coherence spectrum

Part of the absolute value mode ^1H - ^{15}N multiple-bond shift correlation spectrum of a medium supernatant taken from a culture of HeLa cells grown for 48 h in medium to which 2 mM L-[2- ^{15}N]glutamine had been added. Pyr represents pyrrolidone-5-carboxylic acid. The ^1H chemical-shift scale is referenced to TSP at 0.0 p.p.m. and the ^{15}N chemical-shift scale is referenced to alanine at -332.3 p.p.m.

labelled glutamine amide proton resonances at 7.49 and 6.87 p.p.m. The smaller upfield resonance in this spectrum at 6.81 p.p.m. has not been assigned.

Activities of enzymes involved in nitrogen metabolism

The activities of some of the key enzymes involved in nitrogen metabolism in the cells used here are shown in Table 1. Elevated levels of glutamine synthetase were obtained in the CHO cells by culturing them in the absence of glutamine (KG1) and in the absence of glutamine and the presence of methionine sulfoximine (KG1M) (Sanders and Wilson, 1984). Some increase in glutamine synthetase activity was also obtained in HeLa cells by culturing them in the presence of low glutamine concentrations (cell line designated low-glutamine HeLa) (DeMars, 1958).

Origin of ammonia in the cultures

HeLa cells were incubated with glutamine, ^{15}N -labelled in the amine or amide positions, and the resultant ammonia measured by enzyme assay and by ^{15}N n.m.r. (Table 2). In cells incubated with amide-labelled glutamine there was good agreement between the ammonia measured by enzymic assay and that measured by ^{15}N n.m.r. However, in cells incubated with amine-labelled glutamine, there was no detectable ^{15}N -labelled ammonia produced. Therefore most or all of the ammonia produced in the cultures was derived from the amide group of glutamine. There was no detectable production from the amine group. This result was supported by measurements of a 1:1 relationship between glutamine consumed and ammonia produced (Figure 3). Similarly, incubation of the CHO cell line, KG1M, with amine-labelled glutamine resulted in no detectable production of ^{15}N -labelled ammonia (results not shown).

^{15}N label incorporation into cellular metabolites

HeLa cells incubated with $^{15}\text{NH}_4\text{Cl}$ showed progressive in-

corporation of ^{15}N label into alanine and glutamine over a period of 72 h (Figure 4). In subsequent experiments label detection was restricted to single time points of between 40 and 80 h of incubation.

In order to facilitate a comparison between the patterns of label incorporation from glutamine and ammonia, experiments were carried out using media that were identical with respect to the starting concentrations of ammonia and glutamine, i.e. 2 mM for each. The site of the ^{15}N label, however, was rotated between the amide position of glutamine, the amine position of glutamine and the ammonium ion. Thus differences in labelling could be attributed to the source of the label and were not complicated by the possible effects of different concentrations of ammonia and glutamine on the levels and kinetic properties of the enzymes involved in label redistribution (Stamatiadou, 1972; Tiemeier and Milman, 1972; Soni et al., 1991). The results of applying this experimental protocol to the four different cell lines used in this study are shown in Table 3. For each cell type the three experiments shown were run in parallel on the same batch of cells at the same cell density. With L-[2- ^{15}N]glutamine there was substantial labelling of alanine and glutamate but no detectable labelling of the amide position of glutamine in any of the cell lines tested. As with the experiment shown in Figure 2, the fractional labelling of alanine and glutamate were comparable. Incubations with L-[5- ^{15}N]glutamine resulted in a low level of labelling of alanine and glutamate in all four cell lines. Again the fractional labelling was comparable in the two molecules. The ^{15}N -coupled proton resonances of glutamate and glutamine could not be resolved in media extracts, so in both of these experiments the glutamate fractional labelling was calculated by assuming that the glutamine amine group was either 100% labelled or unlabelled respectively. This is a valid assumption for the HeLa cell lines since incubations with $^{15}\text{NH}_4\text{Cl}$ showed that there was insignificant glutamine synthesis. The CHO cell lines, however, which express relatively high levels of glutamine synthetase, did show a small amount of glutamine synthesis as evidenced by low levels of ^{15}N label incorporation

from $^{15}\text{NH}_4\text{Cl}$ into the amide position of glutamine (Table 3, see also Figure 2). Furthermore, this was higher in the KG1M cells, which express a higher level of glutamine synthetase, than in the KG1 cells.

Incorporation of ^{15}N label into aspartate could not be detected using the spin-echo difference experiment. Pulse-and-collect n.m.r. experiments showed that the aspartate concentration declined throughout the incubations and was undetectable at 60 h. However, in HeLa cells incubated with L-[2- ^{15}N]glutamine, ^{15}N -labelled aspartate was detected using a two-dimensional heteronuclear multiple-quantum coherence experiment (Figure 5). Labelled glycine was also observed in this experiment as well as some, as yet, unassigned peaks. Comparison of the glutamate and aspartate peaks in the projected ^1H spectrum (see Figure 5) with their concentrations determined in the pulse-and-collect spectra suggested that the percentage labelling of aspartate was quite high.

DISCUSSION

The source of ammonia in cultures of proliferating cells has not been extensively studied (McKeehan, 1986). The data presented in Table 2 show that in the HeLa cell line most or all of the culture ammonia is derived from the amide group of glutamine in the reaction catalysed by glutaminase. There was no detectable production of ammonia from the amine group of glutamine in the consecutive reactions catalysed by glutaminase and glutamate dehydrogenase. This was also the case in the CHO cell line, KG1M (results not shown), which has tenfold higher activities of both glutaminase and glutamate dehydrogenase (see Table 1). The transaminase inhibitor, amino-oxyacetate (Rognstad and Clark, 1974), which stimulates ammonia production via glutamate dehydrogenase in the kidney (Schoolwerth and LaNoue, 1980; Nissim et al., 1991), had no effect on ammonia production in the HeLa cell line (see Table 2).

In all of the cell lines studied most of the glutamine amine nitrogen was subsequently found in glutamate and a variety of other metabolites including alanine, glycine, aspartate and pyrrolidone-5-carboxylic acid (see Table 3 and Figure 5). The latter is formed through the spontaneous decomposition of glutamine which results in loss of the amide group as ammonia (Tritsch and Moore, 1962). The fractional labelling of the glutamate produced from L-[2- ^{15}N]glutamine was considerably lower than 100% reflecting exchange of the glutamate nitrogen with other amino acids in transamination reactions. The fractional labelling of glutamate and alanine were comparable regardless of the source of the ^{15}N label (Table 3) and, in cells incubated with L-[2- ^{15}N]glutamine, there was also detectable and significant labelling of aspartate (see Figure 5 and the Results section). These data suggest that the alanine and aspartate aminotransferases are catalysing reactions that are near to equilibrium. This pathway for glutamine utilization, where glutamate produced from glutamine is metabolized by transaminases rather than by glutamate dehydrogenase, is similar to that found in lymphocytes (Ardawi and Newsholme, 1985). However, whereas in lymphocytes aspartate was found to be a much more significant end product of glutamine metabolism than alanine, in the cell lines used here alanine was quantitatively the most important end product. In lymphocytes the production of aspartate rather than alanine was attributed to the much higher maximal activity of aspartate aminotransferase compared with alanine aminotransferase (Ardawi and Newsholme, 1985). However, in the cell lines used here, where the activity of aspartate aminotransferase is also much higher than that of

alanine aminotransferase (see Table 1), this cannot be the case and presumably reflects the relative availabilities of pyruvate and oxaloacetate for transamination, pyruvate being readily available from glycolysis. Significant production of alanine in cultures of mammalian cells has been noted previously [see for example Lanks (1987) and Eagle (1959)].

The low activity of glutamate dehydrogenase in the oxidative deamination of glutamate observed in lymphocytes (Ardawi and Newsholme, 1985) has also been observed in tumour cells (Filic-DeRico et al., 1990). In both cases it was attributed to the relatively low concentration of the enzyme. This could explain the low activity of the enzyme in the HeLa cell line used here since the maximal glutamate dehydrogenase activity measured in extracts of these cells was similar to that measured in lymphocytes (Ardawi and Newsholme, 1985).

The low activity of glutamate dehydrogenase in oxidative deamination of glutamate was also observed in the reductive amination of 2-oxyglutarate catalysed by the enzyme. In cells incubated with $^{15}\text{NH}_4\text{Cl}$ there were only very low levels of label incorporation into extracellular glutamate and alanine. The degree of label incorporation, however, did correlate with the maximal glutamate dehydrogenase activities measured in the cell extracts (Table 1), the labelling being higher in the two CHO cell lines than in the two HeLa cell lines.

Incubations with $^{15}\text{NH}_4\text{Cl}$ also showed that there was very little glutamine synthesis in all four cell lines. The only cells that showed detectable glutamine amide labelling were the two CHO cell lines which express relatively high levels of glutamine synthetase.

The presence of glutaminase and glutamine synthetase means that there is the potential for a futile ATP-requiring cycle catalysed by the two enzymes. Such a cycle has been demonstrated previously in liver (Häussinger and Sies, 1979; Häussinger et al., 1983), in isolated liver cells (Vincent et al., 1989) and in kidney tubules (Dugelay and Baverel, 1991). However, in these systems it seems likely that the two enzymes are in different cell types within the tissue and therefore the cycle takes place at the level of the whole organ. In the CHO cell lines used here there is clearly some futile cycling since there is both glutamine synthesis and degradation. Assuming that the cell population is homogeneous, this must be taking place in individual cells. However, the degree of futile cycling appears to be quite small despite the relatively high levels of glutamine synthetase in these cells compared with other tissues such as liver (14 nmol/min per mg of protein), brain (14 nmol/min per mg of protein) and kidney (5 nmol/min per mg of protein) (Rowe, 1985). For example, a lower estimate of the glutamine synthetase flux *in vivo* can be made from measurements of the rate of incorporation of label from $^{15}\text{NH}_4\text{Cl}$ into the amide group of extracellular glutamine. Note that in these cultures the intracellular volume is a negligible fraction of the total culture volume (approx. 0.04% in the HeLa cultures) and thus virtually the entire amino acid content of the incubation will be in the extracellular medium. In the KG1M cells, making no corrections for glutamine breakdown or changes in the fractional labelling of the ammonia pool during the incubation, the estimated glutamine synthetase flux was only 6% of the glutamine consumption rate, although this could be increased by increasing the ammonia concentration in the culture (see Figure 2). Futile cycling in the reactions catalysed by glutaminase and glutamine synthetase has been proposed previously as a possible contributor to the maintenance energy requirements of mammalian cells in culture (Glacken, 1988). The experiments described here have shown that this is unlikely to be significant in most cell types.

This study has shown how indirect detection of ^{15}N labels

using $^1\text{H}/^{15}\text{N}$ n.m.r. spin-echo difference spectroscopy can be used to monitor cellular nitrogen metabolism *in vivo*. This approach should also be applicable to intact biological systems such as perfused cells (Ugurbil et al., 1981; Sri-Pathmanathan et al., 1989; Gillies et al., 1991), perfused organs (Geissler et al., 1991) and the intact animal (Gründer et al., 1989) where it would allow continuous monitoring of label redistribution and have the added advantage that intracellular labelled metabolites could be detected. The increased sensitivity of indirect detection also offers the prospect of spatially localized ^{15}N -labelling measurements.

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