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Determination of *De Novo* Synthesized Amino Acids in Cellular Proteins Revisited by ^{13}C NMR Spectroscopy

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^{13}C nuclear magnetic resonance spectroscopy was used to determine the absolute amounts of *de novo* synthesized amino acids in both the perchloric acid extracts and the hydrolyzed protein fractions of F98 glioma cells incubated for 2 h with 5 mmol/l [U-^{13}C]glucose. ^{13}C NMR spectra of the hydrolyzed protein fraction revealed a marked incorporation of ^{13}C-labelled alanine, aspartate and glutamate into the proteins of F98 cells within the incubation period. Additionally, small amounts of ^{13}C-labelled glycine, proline and serine could unambiguously be identified in the protein fraction. Astonishingly, approximately equal amounts of ^{13}C-labelled glutamate and aspartate were incorporated into the cellular proteins, although the cytosolic steady-state concentration of aspartate was below ^{13}C NMR detectability. Hypertonic stress decreased the incorporation of ^{13}C-labelled amino acids into the total protein, albeit their cytosolic concentrations were increased, which reflects an inhibition of protein synthesis under these conditions. On the other hand, hypotonic stress increased the amount of ^{13}C-labelled proline incorporated into the cellular proteins even though the cytosolic concentration of ^{13}C-labelled proline was largely decreased. Apparently, hypoosmotic conditions stimulate the synthesis of proteins or peptides with a high proline content. The results show that already after 2 h of incubation with [U-^{13}C]glucose there is a pronounced flux of ^{13}C label into the cellular proteins, which is usually disregarded if cytosolic fluids are examined only. This means that calculations of metabolic fluxes based on ^{13}C NMR spectroscopic data obtained from perchloric acid extracts of cells or tissues and also from *in vivo* measurements consider only the labelled 'NMR visible' cytosolic metabolites, which may have to be corrected for fast label flowing off into other compartments. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

^{13}C NMR spectroscopy has been established as a powerful tool to assess cerebral metabolism by following the fate of compounds originated from various ^{13}C-labelled substrates.[1–6] Recent work has been predominantly focused on monitoring the entry of ^{13}C label from [^{13}C]glucose and/or [^{13}C]acetate into cytosolic compounds by both *in vivo* and *in vitro* ^{13}C NMR spectroscopy.[7–12] Moreover, investigations on brain cell or tissue extracts have provided further information by studying complementarily the accumulation of ^{13}C label in both lipid components of the membrane fraction and in water-soluble molecules such as lactate and various amino acids (e.g. alanine, aspartate, glutamate, glutamine, etc.)[13–15] However, the incorporation of these *de novo* synthesized amino acids into the protein of brain cells has not yet been followed by ^{13}C NMR spectroscopy, since NMR investigations on protein mixtures are hampered by broad line-widths due to the short relaxation times of the macromolecules.

Previous investigations with radioactive-labelled substrates have shown that already 5 min after subcutaneous injection of [U-^{14}C]glucose a measurable and increasing amount of [^{14}C]-labelled glutamate is found in rat brain protein.[16–18] Since studies using ^{13}C NMR spectroscopy—limited by its relative insensitivity—require much longer incubation times with the labelled substrates, a considerable amount of ^{13}C-labelled amino acids is expected to be incorporated into the cellular proteins over the incubation period. Therefore, a quantification of the flux of ^{13}C label into the proteins is desirable to complement the calculations on metabolic fluxes which are generally based on ^{13}C NMR spectroscopic data of cytosolic fluids only.

The present work was carried out to determine the absolute amount of *de novo* synthesized amino acids in both the cytosolic and the protein fraction of glial cells supplied with a ^{13}C-labelled substrate. For this purpose, F98 glioma cells were used as a model for glial cells.[19, 20] The cells were incubated for 2 h in the presence of 5 mM [U-^{13}C]glucose and were subsequently extracted with perchloric acid (PCA). In order to obtain highly resolved NMR spectra not only for the PCA extract, but also for the protein fraction, the precipitated protein mixture was hydrolyzed with 8 M PCA at 110 °C.[21] The use of [U-^{13}C]glucose as substrate instead of [1-C^{13}]glucose facilitated or at least simplified an accurate evaluation of the ^{13}C NMR spectra, since the ^{13}C–^{13}C coupling introduced into *de novo* synthesized metabolites permitted quantification in the face of high levels of endogenous compounds with the natural abundance of ^{13}C.

The reliability of this evaluation has been examined by

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additionally investigating the effect of osmotic stress on the concentration of *de novo* synthesized amino acids in the PCA extract and the hydrolyzed protein fraction of F98 glioma cells. In previous studies of liver cells it has been reported that anisosmotic conditions strongly effect protein synthesis, proteolysis, and the cytosolic amino acid pool.[22] Hypertonic stress inhibits the synthesis of proteins,[23] whereas hypotonic stress exerts an inhibitory effect on the degradation of these macromolecules.[24] On the other hand, cellular formation and accumulation of amino acids are inhibited under hypotonic conditions and are stimulated by high ambient osmolarity in various non-mammalian tissues, in mammalian brain, and in mammalian renal medulla.[25–27] Therefore, significant differences in the distribution of ^{13}C-labelled amino acids produced from [U-^{13}C]glucose should be observable between the cytosolic and protein fraction of cells incubated under hyper-, iso- or hypotonic conditions.

MATERIALS AND METHODS

Materials

F98 glioma cells[19, 20] were supplied by the Max-Planck-Institut für Neurologische Forschung, Köln, Germany. Dulbecco's modified Eagle medium (DMEM), foetal calf serum (FCS) and penicillin/streptomycin were purchased from Gibco (Eggenstein, Germany), and culture dishes from Nunc (Wiesbaden-Biebrich, Germany). Perchloric acid (PCA) and (trimethylsilyl)propionic-2,2,3,3d$_4$-acid (TSP) were obtained from Aldrich (Steinheim, Germany), deuterated solvents from E. Merck (Darmstadt, Germany), and D-[U-^{13}C]glucose from Deutero GmBH (Herresbach, Germany).

Cell extracts

F98 glioma cells were grown to confluency in 15 cm culture dishes in a humidified atmosphere of 10% CO_2 in air at 37°C in DMEM, supplemented with 5% FCS and penicillin/streptomycin (100 units/ml).

For cell extracts, approximately 10^8 cells obtained from four culture dishes were incubated for 24 h with hyper-, iso- or hypotonic medium and during the last 2 h of the incubation period with fresh hyper-, iso- or hypotonic serum-free DMEM containing either 5 mmol/l [U-^{13}C]glucose or 5 mmol/l unlabelled glucose. The osmolarity of the media (hypotonic 180 mosm/l, isotonic 300 mosm/l, hypertonic 420 mosm/l) was modified by changing the NaCl concentration of the medium and was verified by freezing point depression using an osmometer from Gonotec (Berlin, Germany; Osmomat 030). After incubation, neutralized PCA extracts were prepared as previously described.[28] The residual water insoluble components were either used for the quantification of the protein content[28, 29] or they were resuspended in 4 ml H_2O, neutralized, lyophilized and freed from lipids by treatment with $CDCl_3/CD_3OD$ (2:1).[28]

The dried residue of the lipid extraction (approximately 100 mg) was crushed to a homogeneous powder and washed with 5 ml H_2O. After centrifugation, the pellet containing the cellular proteins was refluxed for 20 h with 20 ml 8 M PCA at 110 °C.[21] Thereafter, the clear brown-coloured solution was cautiously neutralized at 0 °C with 20 M KOH and centrifuged. Subsequently, the residual $KClO_4$ was washed three times with 5 ml H_2O and centrifuged. The supernatants were united and lyophilized.

Spectroscopy

NMR spectroscopy of cell extracts and quantification of metabolites were performed as previously described.[28] Lyophilized PCA extracts and lyophilized, hydrolyzed protein fractions were redissolved in 0.5 ml D_2O in each case. Spectra were recorded on a Bruker AM 360 or a Bruker AMX 360 NMR spectrometer, operating at frequencies of 360 MHz for ^{1}H and 90.5 MHz for ^{13}C measurements.

^{1}H: 5 mm ^{1}H/X inverse probe; 400 scans; flip angle of 40°; repetition time 15 s; low power water presaturation; spectral width 3600 Hz; data size 16 K; zero filling to 32 K. Chemical shifts were referenced to TSP at 0 ppm.

^{13}C: 5 mm ^{1}H/^{13}C dual probe; 20 000 scans; flip angle of 27°; repetition time 2.5 s; composite pulse decoupling with WALTZ-16;[30] spectral width 20 833 Hz; data size 16 K; zero filling to 32 K; exponential weighting resulting in a 1 Hz line broadening. Chemical shifts were referenced to C3 of alanine at 17.3 ppm. Assignments of the signals in one-dimensional ^{13}C NMR spectra were confirmed by performing additional inverse, gradient-selected two-dimensional ^{1}H, ^{13}C-correlated NMR experiments.[31–33]

Calculation of enrichment and absolute amount of ^{13}C-labelled metabolites

The pool size of the various metabolites (Table 1) has been determined from fully relaxed ^{1}H NMR spectra of extracts (i.e. PCA and hydrolyzed protein fraction, respectively), which were obtained from F98 cells incubated with unlabelled glucose. TSP was used to standardize the concentration, and the results were correlated with the protein content as previously described.[28]

The ^{13}C enrichment in C3 of alanine and lactate was determined from fully relaxed ^{1}H NMR spectra of extracts from F98 cells incubated with [U-^{13}C]glucose by evaluating the peak areas of the ^{1}H and ^{1}H–^{13}C satellite signals of the respective methyl groups.[34] The ^{1}H–^{13}C-coupled resonances were corrected for the amount of naturally abundant ^{13}C, which was derived from the experiments with unlabelled glucose. The revised values were then used to calculate the ^{13}C enrichment in these resonances as in eq. (1):

$$^{13}\text{C enrichment} = \frac{\text{area } (^1\text{H}-^{13}\text{C})}{\text{area } (^1\text{H}) + \text{area } (^1\text{H}-^{13}\text{C})} \qquad (1)$$

where area (^{1}H) and area (^{1}H–^{13}C) represent the area of the ^{1}H and ^{1}H–^{13}C resonances, respectively, measured by integration. The sum (area (^{1}H) + area (^{1}H–^{13}C)) is equivalent to the pool size, which was in good agreement with the data obtained from extracts of F98 cells incubated with unlabelled glucose.

The absolute amount of ^{13}C in the methyl groups of alanine and lactate was calculated from (^{13}C enrichment × pool size). These values were subsequently used as internal standards in the corresponding ^{13}C NMR spectra of the PCA and protein fractions, respectively. The absolute amount of ^{13}C in the resonances of the remaining metabolites was then

Table 1. Pool size of relevant metabolites in the cytosolic and the protein fraction of F98 glioma cells under control (isotonic) and anisosmotic conditions

	PCA extract (nmol/mg protein)			Protein fraction (nmol/mg protein)		
Amino acid	Hypotonic	Isotonic	Hypertonic	Hypotonic	Isotonic	Hypertonic
Alanine	1.9±0.3	26.0±1.4	54.7±4.2	423±39	424±12	410±15
Aspartate	nd	0.8±0.1	6.4±0.8	545±43	552±21	533±37
Glutamate	22.1±3.0	46.3±2.7	61.9±5.5	553±35	573±26	548±40
Glycine	6.7±1.9	29.5±2.6	68.3±6.0	400±33	370±29	387±38
Lactate	50.6±1.8	52.8±1.8	48.6±3.4	—	—	—
Proline	2.0±0.5	4.2±0.4	6.1±0.6	275±11	268±14	257±22
Serine	nd	1.1±0.3	3.7±0.5	321±20	304±11	314±27

The values are means ±SD (n=3, nd=not NMR detectable). The data for aspartate and glutamate in the protein fraction include asparagine and glutamine converted by acid hydrolysis to aspartate and glutamate, respectively.

determined as in eq. (2) by using correlation factors, which were obtained from ^{13}C NMR spectra of model solutions recorded with the same acquisition and processing parameters as the spectra of the cell extracts. The model solutions contained equimolar metabolite mixtures and 400 mmol/l KCl as well as 5 mmol/l $MgCl_2$ and were additionally saturated with $KClO_4$ in order to obtain a similar ionic strength as in the redissolved cell extracts.

$$[^{13}C]_m = \frac{[^{13}C]_s \times \text{area } (^{13}C)_m \times c_s}{\text{area } (^{13}C)_s \times c_m} \quad (2)$$

where $[^{13}C]$ represents the absolute amount of ^{13}C, area (^{13}C) represents the area of the ^{13}C resonance measured by integration, m represents the metabolite, s represents the internal standard (i.e. the C3 of alanine and/or lactate), and c represents the correlation factor determined from the integration area of the respective resonance in ^{13}C NMR spectra of the model solutions. The calculated values were corrected for the natural abundant ^{13}C present in each resonance and were correlated with the pool size of the metabolites (Table 1) to yield the ^{13}C enrichment in the individual carbons.[34]

RESULTS

^{13}C label in cytosolic and protein fraction

Typical ^{13}C NMR spectra of a PCA extract and the corresponding hydrolyzed protein fraction from F98 cells incubated for 2 h with 5 mmol/l [U-^{13}C]glucose under control conditions are illustrated in Fig. 1. PCA extract spectra (Fig. 1(a)) showed great amounts of ^{13}C label in alanine, glutamate and lactate, but also small amounts of ^{13}C label in glycine, proline and serine. As expected, all carbons of these metabolites were highly ^{13}C enriched. Nevertheless, calculations of percentage enrichment and the absolute amount of ^{13}C were performed by neglecting the carboxylic carbon signals of the various metabolites and evaluating only the carbon signals given in Table 2. For the individual carbons of a particular molecule, the sum of all ^{13}C–^{13}C spin-coupled signals resulting from the different isotopomers was considered.

The spectra of the hydrolyzed protein fractions (Fig. 1(b)) also showed a significant ^{13}C labelling of these compounds with exception of lactate, albeit ^{13}C enrichments were much lower in the cellular proteins then in the PCA extract (Table 2). Nevertheless, the absolute amount of ^{13}C label incorporated into the protein fraction over the incubation period was in a comparable order of magnitude as the amount of ^{13}C label incorporated into the cytosolic fraction (cf. Table 2). Approximately equal amounts of ^{13}C label in cytosol and protein were found for alanine and proline, whereas the amount of ^{13}C label in glycine and glutamate was about two- to three-fold greater in the PCA than in the protein fraction. However, the higher enrichment of glutamate C4 vs C3 observed in the cytosolic fraction was also found in the cellular proteins (Table 2). Furthermore, ^{13}C-labelled aspartate was unambiguously identified in the hydrolyzed protein fractions (with $[^{13}C]_{Asp} \cong [^{13}C]_{Glu}$, cf. Table 2), although the labelling of this molecule was below ^{13}C NMR detectibility in spectra of PCA extracts, which were obtained after incubation of F98 glioma cells under control conditions. A similar phenomenon was observed for the ^{13}C labelling of serine. The amount of ^{13}C-labelled serine was near the detection limit in ^{13}C NMR spectra of the cytosolic fraction (Fig. 1(a)), whereas the ^{13}C labelling of serine was clearly seen in ^{13}C NMR spectra of the hydrolyzed protein fraction (Fig. 1(b)). The amount of ^{13}C-labelled serine found in the protein fraction was even approximately twice as large than that of ^{13}C-labelled glycine or proline (Table 2).

Osmotic stress

Figure 2, on the left, shows ^{13}C NMR spectra of PCA extracts from F98 cells incubated for 24 h under control (isotonic) and anisosmotic conditions, respectively (and only during the last 2 h of the incubation period in the presence of 5 mmol/l [U-^{13}C]glucose). Pronounced alterations in ^{13}C signal intensity were detected for all carbons of alanine, glutamate, glycine and proline as compared to control (isotonic) conditions. Hypertonic stress increased the incorporation of ^{13}C label into cytosolic metabolites (Fig. 3). Moreover, additional resonance signals for aspartate were detected under these conditions (cf. Fig. 2(a), on the left). In contrast, hypotonic conditions largely decreased the absolute amount of ^{13}C in cytosolic compounds produced from [U-^{13}C]glucose (Fig. 3). However, since the pool size of the various molecules was also increased (decreased) under hypertonic (hypotonic) stress (Table 1), ^{13}C enrichment in the individual resonances of the metabolites was almost unaffected under anisosmotic as compared to isosmotic conditions (data not shown). Steady-state

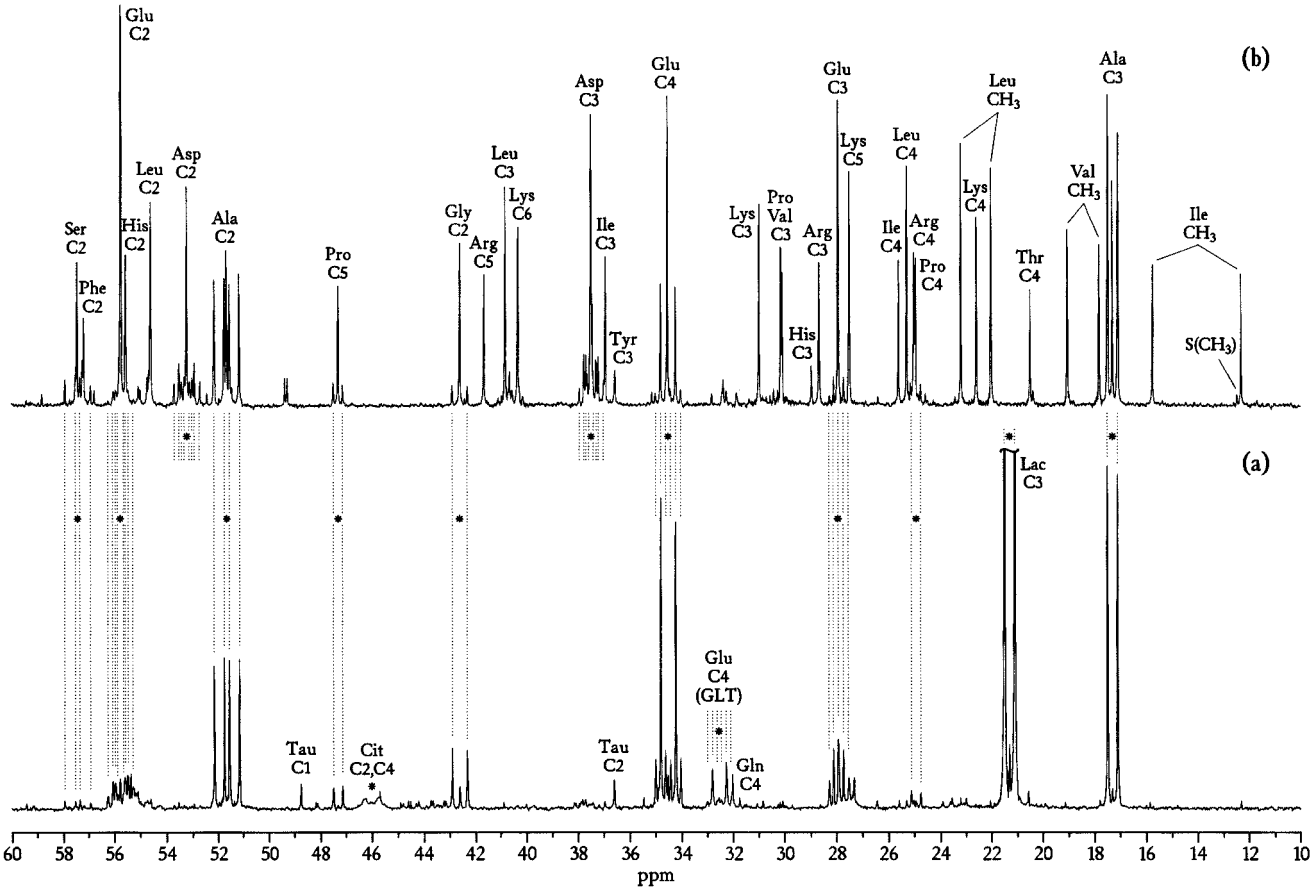

Figure 1. Sections of ^{13}C NMR spectra from (a) a PCA extract and (b) a hydrolyzed protein fraction of F98 cells incubated for 2 h with 5 mmol/l [U-^{13}C]glucose. The asterisks/dotted lines indicate ^{13}C–^{13}C spin-coupled signals arising from *de novo* synthesized metabolites. Assignments: Ala, alanine; Arg, arginine; Asp, aspartate; Cit, citrate; Gln, glutamine; GLT, glutathione; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Lac, lactate; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Ser, serine; Tau, taurine; Thr, threonine; Tyr, tyrosine; Val, valine.

concentrations of cytosolic [U-^{13}C]glucose and ^{13}C labelling of intracellular lactate also revealed no significant differences for hypertonic compared to hypotonic stress (data not shown).

In comparison with the large alterations in the absolute amount of ^{13}C label incorporation into the cytosolic amino acids under osmotic stress, ^{13}C NMR spectra of the corresponding hydrolyzed protein fractions (Fig. 2, on the right) exhibited less pronounced but nonetheless sizeable changes in the ^{13}C labelling of the amino acids in the cellular proteins under anisosmotic conditions. Hypertonic stress decreased the incorporation of ^{13}C label into the amino acids of the protein fraction, which especially holds for alanine, glycine, proline and serine (Fig. 3). In contrast, the ^{13}C labelling of glutamate and particularly proline was increased in the protein fraction under hypotonic conditions

Table 2. Absolute amount and ^{13}C enrichment of *de novo* synthesized compounds in the cytosolic and the protein fraction of F98 glioma cells incubated for 2 h with 5 mmol/l [U-^{13}C]glucose under control conditions

	Absolute amount (nmol/mg protein)		Enrichment (%)	
Amino acid	PCA extract	Protein fraction	PCA extract	Protein fraction
Alanine (C2, C3)	18.9±1.0	15.1±0.4	72.7±2.9	3.56±0.14
Aspartate (C2, C3)	nd	6.4±0.5	nd	1.16±0.12
Glutamate (C3)	7.2±0.5	1.8±0.2	15.5±1.2	0.34±0.04
Glutamate (C4)	20.9±0.9	6.9±0.3	45.2±3.6	1.12±0.07
Glycine (C2)	2.3±0.2	1.0±0.2	7.8±1.7	0.27±0.03
Lactate (C2, C3)	38.8±1.3	—	73.4±2.5	—
Proline (C4, C5)	1.0±0.2	1.1±0.1	23.8±4.8	0.41±0.02
Serine (C2, C3)	0.4±0.1	1.8±0.1	36.4±9.0	0.59±0.03

The values are means ±SD (n=3, nd=not NMR detectable). Data were averaged over the carbons indicated in parentheses. For the individual carbons of a particular molecule, the sum of all ^{13}C–^{13}C spin-coupled signals resulting from the different isotopomers was considered. The values for aspartate and glutamate in the protein fraction include asparagine and glutamine converted by acid hydrolysis to aspartate and glutamate, respectively.

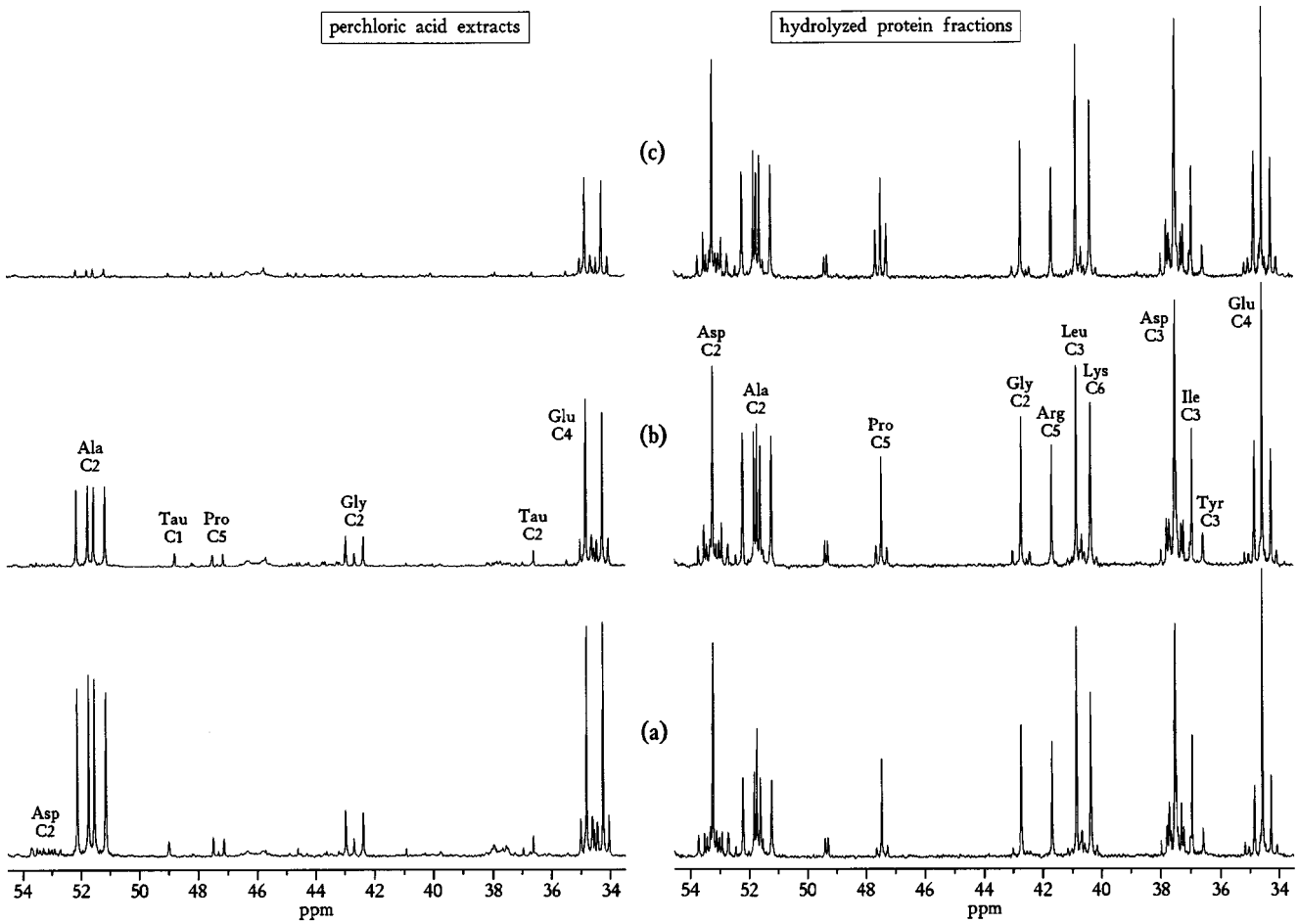

Figure 2. Sections of ^{13}C NMR spectra from PCA extracts (on the left) and hydrolyzed protein fractions (on the right) of F98 cells incubated for 2 h with 5 mmol/l [U-^{13}C]glucose under (a) hypertonic, (b) control (isotonic), and (c) hypotonic conditions. Assignments: Ala, alanine; Arg, arginine; Asp, aspartate; Glu, glutamate; Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Pro, proline; Tau, taurine; Tyr, tyrosine.

(Fig. 2(c), on the right, and Fig. 3). However, ^{13}C label incorporation into alanine and aspartate of the protein fraction was almost unaffected under these conditions, whereas the absolute amount of ^{13}C label in glycine and serine of the cellular proteins was decreased under hypo-osmotic stress (cf. Fig. 3).

DISCUSSION

Amino acid composition of the hydrolyzed protein fraction

The quantification of the amino acid content in the hydrolyzed protein fraction by NMR spectroscopy is in good agreement with data collected from previous studies of protein amino acid composition. Table 3 shows only small differences between the relative amino acid content of the total hydrolyzed protein of F98 cells calculated from the pool sizes given in Table 1 and the average values for alanine, aspartate, glutamate, glycine, proline and serine, which were extracted from a database of nonredundant proteins containing approximately 300 000 residues.[35] The greatest deviation is seen for the content of serine, which is underestimated by about 20%. This can be put down to a partial destruction of this amino acid under the conditions chosen for protein hydrolysis.[21]

Distribution of ^{13}C-labelled amino acids between cytosolic and protein fraction

^{13}C NMR spectra of the hydrolyzed protein fraction revealed a marked incorporation of ^{13}C-labelled alanine, aspartate and glutamate into the proteins of F98 cells within 2 h of incubation with 5 mmol/l [U-^{13}C]glucose. Despite the large amounts of natural abundant ^{13}C present in the resonance of each amino acid, small quantities of ^{13}C label could additionally be identified in glycine, proline and serine of the protein fraction due to the appearance of ^{13}C–^{13}C spin-coupled carbon signals (Fig. 1). Astonishingly, approximately equal amounts of ^{13}C-labelled glutamate and aspartate were incorporated into the cellular proteins, although the cytosolic steady-state concentration of the latter was below ^{13}C NMR detectibility. Obviously, individual amino acids have different relative fluxes of their labelled species from cytosol to protein within the incubation period.

This is supported by additional comparisons regarding the distribution of the *de novo* synthesized amino acids between PCA extract and protein fraction (Table 2): (i) the cytosolic concentrations of ^{13}C-labelled aspartate and serine were much lower than of ^{13}C-labelled glycine and proline, but in contrast larger amounts of ^{13}C-labelled aspartate and

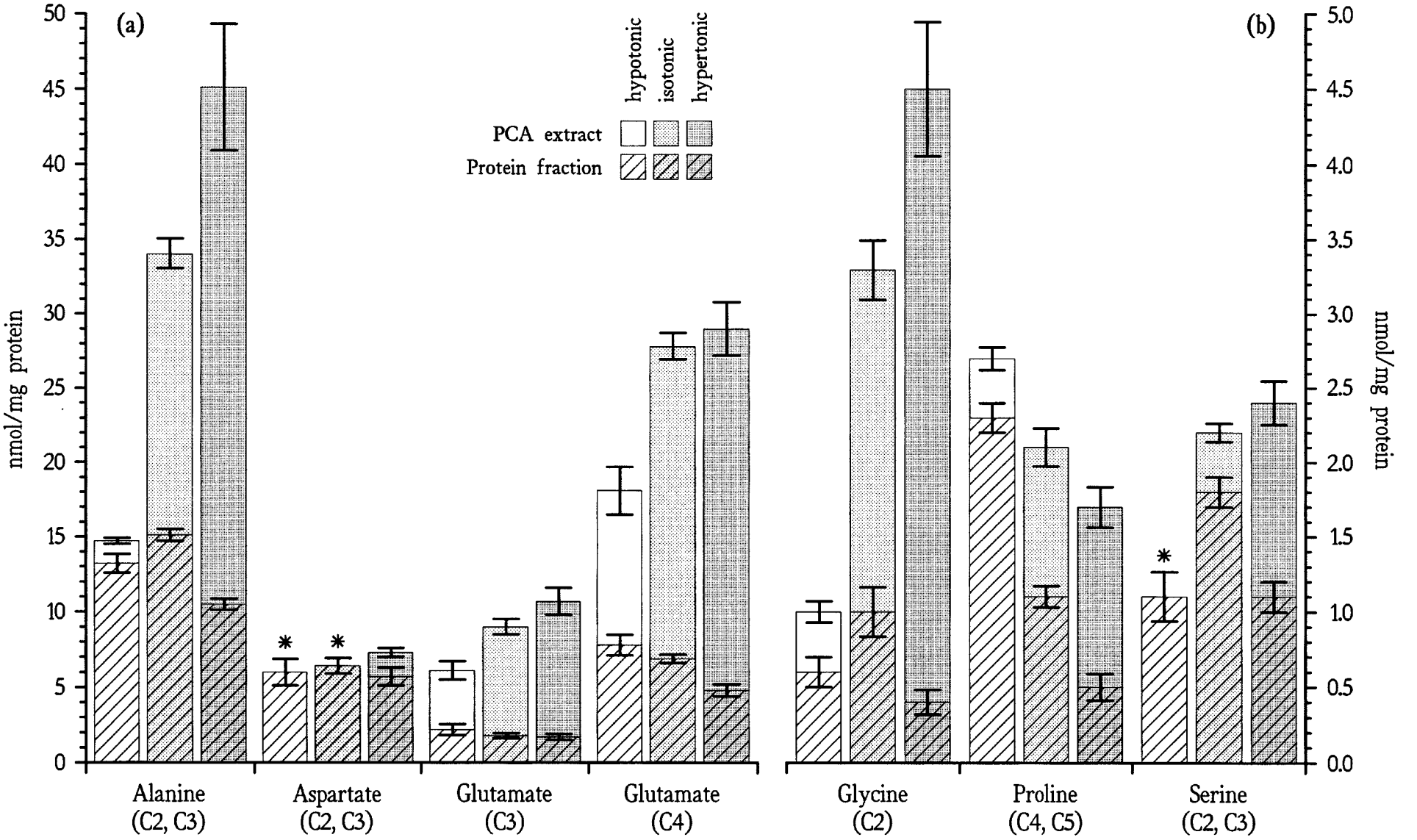

Figure 3. Absolute amount of ^{13}C label in amino acids of the cytosolic and protein fraction from F98 cells incubated for 2 h with 5 mmol/l [U-^{13}C]glucose under hypertonic, control (isotonic), and hypotonic conditions. The scale in (b) is 10-fold vertically enlarged vs (a). The values are means ± SD (n=3, ∗=cytosolic concentration below ^{13}C NMR detectibility). Data were averaged over the carbons given in parentheses. For the individual carbons of a particular molecule, the sum of all ^{13}C–^{13}C spin-coupled signals resulting from the different isotopomers was considered. The values for aspartate and glutamate in the protein fraction include asparagine and glutamine converted by acid hydrolysis to aspartate and glutamate, respectively.

Table 3. Relative occurrence of some amino acids in proteins

Amino acid	Relative occurrence in proteins F98 cells	Average
Alanine	1.6	1.5
Aspartate	2.0	1.9
Glutamate	2.1	2.0
Glycine	1.4	1.4
Proline	1.0	1.0
Serine	1.1	1.3

The data for F98 cells were calculated from the pool sizes determined by NMR spectroscopy (Table 1). The average values were extracted from a database of nonredundant proteins containing approximately 300 000 residues.[35] The values for aspartate and glutamate for F98 cells include asparagine and glutamine converted by acid hydrolysis to aspartate and glutamage, respectively. In order to take this into account, the average values for aspartate and glutamate were correspondingly calculated from the sum (asparagine + aspartate) and the sum (glutamine + glutamate), respectively.

serine than ^{13}C-labelled glycine or proline were detected in the protein fraction; and (ii) the concentrations of ^{13}C-labelled alanine and glutamate C4 were approximately equal in the PCA extract, whereas the amount of ^{13}C-labelled glutamate incorporated into cellular protein was only about one-third of the quantity of ^{13}C-labelled alanine found in the protein fraction.

It is noteworthy that the ^{13}C–^{13}C-coupled signal patterns for the individual carbons of glutamate did not show any significant differences between spectra of the cytosolic and the protein fraction (with the exception of the large amounts of natural abundant ^{13}C present in the latter). Moreover, the higher enrichment in cytosolic glutamate C4 vs C3 caused by the predominant entry of ^{13}C label into the tricarboxylic acid (TCA) cycle via pyruvate dehydrogenase[36, 37] was also found in the cellular proteins. Obviously, the glutamate of the hydrolyzed protein fraction exhibits a comparable isotopic pattern as in the PCA extract indicating a direct use of the cytosolic glutamate pool for protein biosynthesis and gives no evidence for different glutamate pools in F98 glioma cells.[18, 38]

Comparable studies of the utilization of *de novo* synthesized amino acids for protein synthesis have been carried out using radioactive-labelled substrates.[16–18, 39] These investigations have been predominantly focused on the determination of labelled alanine, aspartate and glutamate in the cytosolic and the protein fraction, whereas the metabolic fate of labelled glycine, proline and serine in this connection has not yet been elucidated in any detail. In a study of isolated glial cell fractions,[39] the ratio between the radioactivity found in individual cytosolic amino acids and the radioactivity found in total protein has been shown to be 0.84 for glutamate and 0.25 for alanine after 2 h of incubation with 10 mmol/l [U-^{14}C]glucose. Using the NMR data given in Table 2 to calculate the ratio between the amount of ^{13}C found in individual cytosolic amino acids and the amount of ^{13}C found in total protein, values of 0.82 for glutamate and 0.55 for alanine are determined. Even if it is not possible to draw definite conclusions from this rough comparison, it can be stated that the quantitative results derived from our NMR experiments agree at least in the order of magnitude with the data obtained from the radioactive tracer study.

The results are furthermore in line with an investigation on rat brain,[18] where the ratio between the specific radioactivity of glutamate in the brain protein and the specific radioactivity of the cytosolic glutamate has been found to be 0.008 for glutamate 1 h after subcutaneous injection of [U-^{14}C]glucose. In our study the corresponding ratio between the percentage ^{13}C enrichment of glutamate in the protein fraction and the percentage ^{13}C enrichment of glutamate in the PCA extract (cf. Table 2) is found to be 0.022 and 0.025 for glutamate C3 and C4, respectively, after 2 h of incubation with [U-^{13}C]glucose. Interpolating the values of the rat brain study to 2 h, which will increase this ratio almost linearly[18] and taking into account the systematic differences between investigations on rat brain and brain cell cultures, our results are in good agreement with the data of this previous study.

Effect of osmotic stress

^{13}C NMR spectra of PCA extracts from F98 cells (Fig. 2, on the left) showed significant alterations in the absolute amount of ^{13}C found in cytosolic alanine, glutamate, glycine and proline under anisosmotic conditions, whereas steady-state concentrations of glucose and lactate remain nearly unchanged (data not shown). With regard to the changes in metabolite concentrations determined by ^{1}H NMR spectroscopy (cf. Table 1), the ^{13}C NMR data show that the production of cytosolic compounds from [U-^{13}C]glucose is decreased under hypotonic stress and is inversely increased under hypertonic stress as compared to control (isotonic) conditions. This is hardly surprising, since the generation of small, osmotically active molecules is expected to enhance intracellular osmolarity and therefore supports the accomodation to the changed extracellular osmolarity.[22, 40, 41]

^{13}C NMR spectra of the corresponding hydrolyzed protein fractions (Fig. 2, on the right) exhibited a decreased incorporaton of ^{13}C-labelled amino acids into total protein under hypertonic stress, although their cytosolic concentration was largely increased as compared to control (isotonic) conditions (cf. Fig. 3). This indicates an inhibition of protein synthesis under hypertonic conditions, which was also observed in previous studies of various mammalian cell types.[23, 42,43] In contrast, hypoosmotic stress induced an increased incorporation of *de novo* synthesized glutamate and especially proline into the cellular protein, even though their cytosolic concentrations were greatly decreased as compared to control conditions (Fig. 2). It should be noted that despite the depressed concentration of proline in the cytosol, the total amount of ^{13}C-labelled proline produced from [U-^{13}C]glucose is increased under hypotonic stress (Fig. 3(b)). Therefore, alterations in ^{13}C labelling of a cytosolic metabolite does not necessarily imply similar changes in the total labelling of this compound, i.e. the sum of ^{13}C label in the cytosolic and the protein fraction.

However, the amount of the other ^{13}C-labelled amino acids found in the hydrolyzed protein fraction was either almost unaffected (alanine, aspartate) or decreased (glycine, serine) by hypotonic conditions (cf. Fig. 3). These irregular effects of hypoosmotic stress on the utilization of the individual amino acids for protein biosynthesis suggest that one particular protein/polypeptide or one definite group of related proteins/polypeptides with an exceptionally high proline content is synthesized/expressed under these conditions. Whether this represents an adaptive response of particular proline-rich proteins of the cytoskeleton[44–47] and/or individual ion channels[48–51] to cell volume changes or alterations in the synthesis, expression and metabolism of other proteins induced by hypotonic stress[23, 52–55] cannot be decided on the base of these results.

CONCLUSIONS

De novo synthesized amino acids are immediately used to a large extent for the protein biosynthesis of F98 glioma cells. Hypertonic stress decreases the incorporation of ^{13}C-labelled amino acids into the cellular protein, although their cytosolic concentrations are increased, which reflects an inhibition of protein synthesis under these conditions. On the other hand, hypotonic stress increases the amount of ^{13}C-labelled proline incorporated into the total protein, even though the cytosolic concentration of ^{13}C-labelled proline is largely decreased. Apparently, hypoosmotic conditions stimulate the synthesis of proteins/polypeptides with a high proline content.

The results can also be of interest for quantitative flux measurements through the TCA cycle, because spin off metabolites of the TCA cycle with low steady-state concentrations in the cytosol (e.g. aspartate) show sizeable label enrichment in the protein fraction. Additionally, the kinetic data for fractional enrichments of glutamate or glutamine, usually the target metabolites for TCA cycle flux measurements, may be affected if the flux of ^{13}C label into the amino acids of the protein fraction is taken.

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