

A $^1\text{H}/^{13}\text{C}$ inverse 2D method for the analysis of the polyamines putrescine, spermidine and spermine in cell extracts and biofluids†

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ABSTRACT: The polyamines putrescine, spermidine and spermine are involved in the regulation of various metabolic processes. It is therefore desirable to detect and quantify the polyamines with NMR. We present the proton and carbon assignments for all polyamine signals obtained from PCA extracts of F98 glioma cells with high resolution using a semi-selective HSQC 2D-experiment. The biosynthesis of the polyamines in cell culture was examined using the labeled substrates $[\text{U}-^{13}\text{C}]$ glucose and $[\text{U}-^{13}\text{C}]$ glutamate. In such studies the high resolution of the semi-selective HSQC experiment at very high magnetic fields (14–19 T) allows the analysis of carbon-carbon couplings, and isotopomer patterns. The different effects of osmotic stress on the concentrations of polyamines and amino acids are also reported. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: polyamines; F98 glioma cells; selective HSQC; HSQC-TOCSY; $[\text{U}-^{13}\text{C}]$ glucose; $[\text{U}-^{13}\text{C}]$ glutamate; osmotic stress

INTRODUCTION

The naturally occurring polyamines spermidine and spermine and their metabolic precursor putrescine have gained increasing interest for tumour therapy. They are involved in the regulation of various metabolic processes including cell growth, differentiation and nucleic acid and protein biosynthesis.^{1–3} The NMDA receptor has a polyamine binding site and polyamines enhance the binding of glycine and glutamate to this receptor.^{4,5} Interestingly, some neurotoxins which act on the NMDA receptor have a polyamine-like chemical structure.⁶ The biosynthesis of the polyamines and their interconversion is depicted in Fig. 1. Changes in ODC activity and polyamine concentration have been found as response to brain injury and stress, for example cerebral ischaemia.⁷ The role of polyamines in these processes is still not fully understood.

The NMR detection of polyamines in cell extracts is difficult because the intracellular concentrations are typically 10- to 50-fold lower than those of the amino acids. So far no detailed analysis of polyamines in cell

extracts has been done by NMR. Spm has been detected with NMR in seminal fluid⁸ and fluorine-labeled polyamines have been studied with *in vivo* NMR.⁹ In a recent publication Moreno *et al.* reported the ^1H NMR detection of Spm in a 2D-COSY spectrum¹⁰ of a cell extract. However, the detection of the polyamines in homonuclear correlation spectra is equivocal because of signal overlap. For example, Moreno *et al.*⁸ failed to identify Spd. To overcome this problem our aim was to identify and quantify the carbon signals of the polyamines with NMR. The rather low concentrations of these compounds require the application of the highest magnetic field strengths and 2D inverse techniques for optimum sensitivity. PCA extracts of F98 glioma cells fed with $[\text{U}-^{13}\text{C}_6]$ glucose and $[\text{U}-^{13}\text{C}_5]$ glutamate for 24 h were analyzed.

EXPERIMENTAL METHODS

Spectroscopy

All experiments were performed on Bruker DRX 600 and 800 MHz spectrometers at 300 K using a 5-mm H,C,N inverse triple resonance probe with actively shielded field gradient coils. Gradients were shaped by a waveform generator and amplified by a Bruker Acustar amplifier. Sinusoidal z-gradients of 1-ms duration and a recovery time of 100 μs were used for the echo/antiecho gradient

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Abbreviations used: DMEM, Dulbecco's modified Eagle medium; HSQC, heteronuclear single quantum correlation; NMDA, N-methyl-D-aspartate; ODC, ornithine decarboxylase; PCA, perchloric acid; Put, putrescine; Spd, spermidine; Spm, spermine; TOCSY, total correlation spectroscopy.

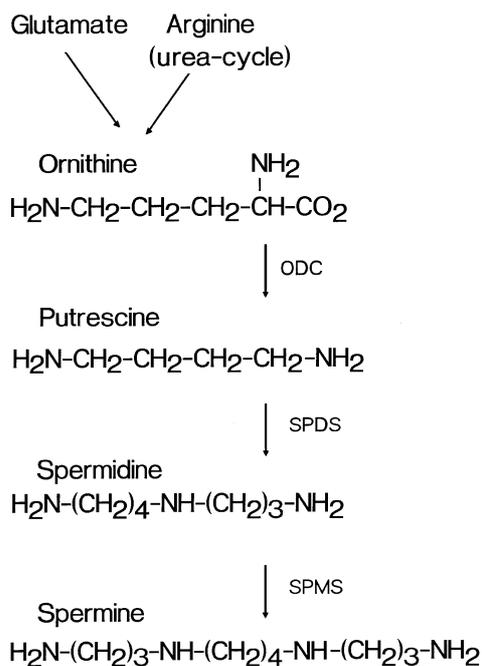


Figure 1. Biosynthetic pathway of polyamine synthesis. ODC = Ornithine Decarboxylase, SPDS = spermidine synthase, SPMS = spermine synthase

selection. Fine-tuning of the gradient amplitude ratios (40:10.08) resulted in optimum signal intensities. Low

power adiabatic composite pulse decoupling with WURST¹¹ has been used for ¹³C-decoupling. A sensitivity-improved HSQC^{12,13} in the version described in¹⁴ with a relaxation delay of 1 s was used. A non-selective sensitivity-improved HSQC was used for acquiring the spectrum in Fig. 2. 512 *t*₁-increments have been acquired with 64 scans per increment, resulting in a resolution of 30 Hz/pt in F1(¹³C).

The semi-selective HSQC experiments used a Gaussian 180° pulse of 1 ms length to excite a range of ca 15 ppm in the ¹³C dimension and with 512 or 1024 *t*₁-increments a digital resolution in the carbon dimension of 4 or 2 Hz/pt was obtained. An acquisition time of 285 ms was used to acquire 1024 data points resulting in a spectral width of 3 ppm in the proton dimension. The suppression of folded signals has been done using digital quadrature detection (DQD, Bruker). For each cell extract two semi-selective HSQC spectra were performed with different ¹³C frequency offsets to get one spectrum of the high field polyamine B-signals and one of the low field A-signals. For details of the semi-selective HSQC see^{14,15}. All spectra are pure absorption mode spectra and were processed with a $\pi/2$ shifted squared sine bell in F2 (¹H) and a $\pi/4$ shifted squared sine bell in F1 (¹³C). Following osmotic stress experiments the polyamines were quantified via signal integrals in the HSQC-spectra. A model solution with known concentrations was used for calibration of individual correction factors for all signals.

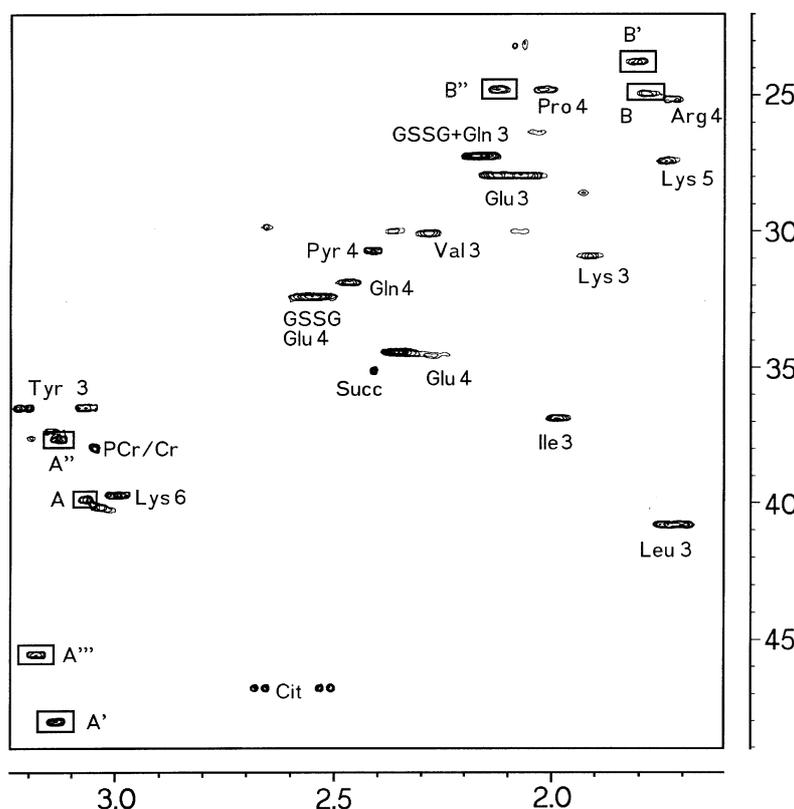


Figure 2. 600 MHz non-selective HSQC spectrum of an F98 glioma cell extract with natural abundance of ¹³C. The region containing the polyamine signals is shown (enclosed in rectangles). Polyamine signals are labeled as defined in Table 1

Table 1. Chemical Shifts of the Polyamines at pH 7, 300 K. $\Delta^{13}\text{C}$ is the separation in ppm between the corresponding carbons in Put/Spd or Spd/Spm, respectively. Chemical shift reference: Glc 1 β : 96.8 ppm (^{13}C), 4.66 ppm (^1H)

Carbon	Put	Spd	Spm	$\Delta^{13}\text{C}/10^{-3}$ ppm
A	3.062/39.823	3.061/39.792	—	31
B	1.771/24.800	1.786/24.855	—	55
A'	—	3.133/47.993	3.131/47.945	48
B'	—	1.786/23.697	1.805/23.750	53
A''	—	3.124/37.595	3.124/37.595	0
B''	—	2.110/24.758	2.110/24.749	9
A'''	—	3.175/45.498	3.175/45.489	9

The carbon and proton notation, respectively, is as follows: Putrescine, $\text{H}_2\text{N}-\text{A}-\text{B}-\text{B}-\text{A}-\text{NH}_2$; Spermidine, $\text{H}_2\text{N}-\text{A}''-\text{B}''-\text{A}'''-\text{NH}-\text{A}'-\text{B}'-\text{B}'-\text{A}'-\text{NH}_2$; Spermine, $\text{H}_2\text{N}-\text{A}''-\text{B}''-\text{A}'''-\text{NH}-\text{A}'-\text{B}'-\text{B}'-\text{A}'-\text{NH}-\text{A}'''-\text{B}''-\text{A}''-\text{NH}_2$.

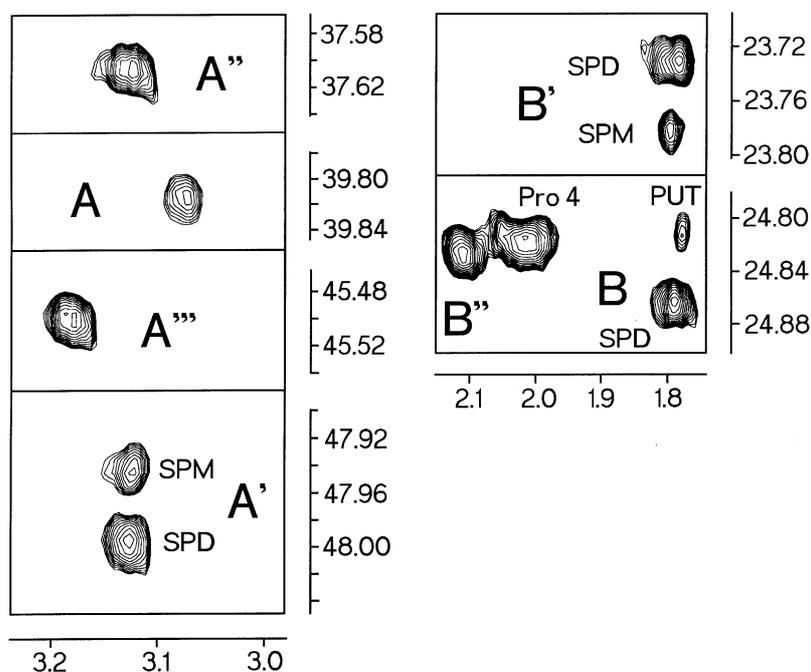
Alanine, which was quantified from the 1D ^1H spectrum was used as internal standard, for quantification details see¹⁶. The glucose 1 β signal (96.8/4.66 ppm) was used as chemical shift reference.

Cell Culture and Extraction

F98 glioma cells were grown to confluency in culture dishes 15-cm in diameter in a humidified atmosphere of 10% CO_2 in air at 37°C in DMEM containing 8 mM $[\text{U}-^{13}\text{C}_6]$ glucose, supplemented with 5% FCS and penicillin/streptomycin (100 units/mL). Another experiment was done with 1 mM $[\text{U}-^{13}\text{C}_5]$ glutamate and 8 mM unlabeled glucose. A control experiment was done with DMEM containing 5mM unlabeled glucose only. Inoculation was performed at a density of 1×10^4 cells/mL, and the medium was routinely changed 3 days later.

Under these conditions the cultures reached confluency within 5 days. The osmolarity of the media (hypotonic 180 mosm/L, isotonic 300 mosm/L, hypertonic 420 mosm/L) was modified by variation of the NaCl concentration and was verified by freezing point depression using an osmometer from Gonotec (Berlin, Germany; Osmomat 030). Experiments were performed after the cells had reached confluency. Approximately 10^8 cells were obtained from four culture dishes. After removal of the medium the cells were immediately washed twice with 4 mL ice-cold 0.9% NaCl solution, frozen in liquid nitrogen and extracted with 2×2 mL PCA.

The suspension was centrifuged and the aqueous layer was removed, neutralized and lyophilized. The residual water insoluble components were either resuspended in 4 mL H_2O , neutralized and lyophilized for the NMR-spectroscopic study of the lipid components or they were

**Figure 3.** Semi-selective 600 MHz HSQC spectrum of an F98 glioma cell extract at natural abundance ^{13}C . The digital resolution in $F_1(^{13}\text{C})$ is about 2 Hz/pt

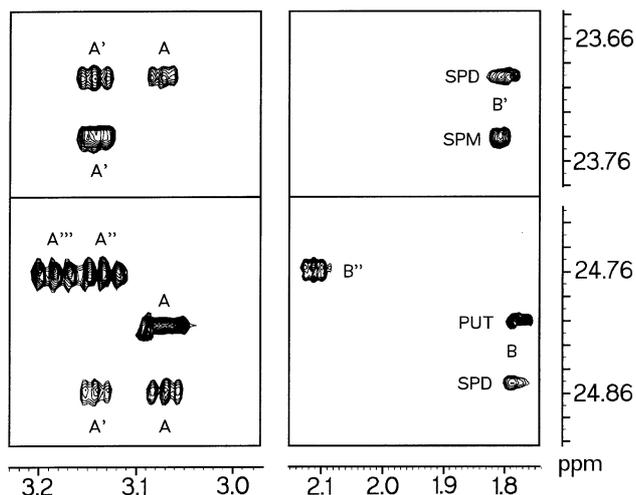


Figure 4. Semi-selective 600 MHz HSQC-TOCSY (left panels) and semi-selective HSQC (right panels) spectrum of a polyamine model solution. Higher resolution compared to the spectra of PCA extracts can be obtained. Even the B'' signals for Spd and Spm are partially resolved. Spd is the only polyamine molecule which shows magnetization transfer from B' to both A and A'

used for the quantification of the protein content. The protein content of the cells was obtained by a slightly modified Biuret reaction¹⁷. For this the water insoluble components were resuspended in 3 ml H₂O. Of the suspension, 150 μ L ($n = 6$) were taken, and 350 μ L H₂O, 500 μ L 6% NaOH and 250 μ L of the Biuret reagent were added. After the reagent had acted for 20 min, the UV-absorption of the samples was determined at 540 nm.

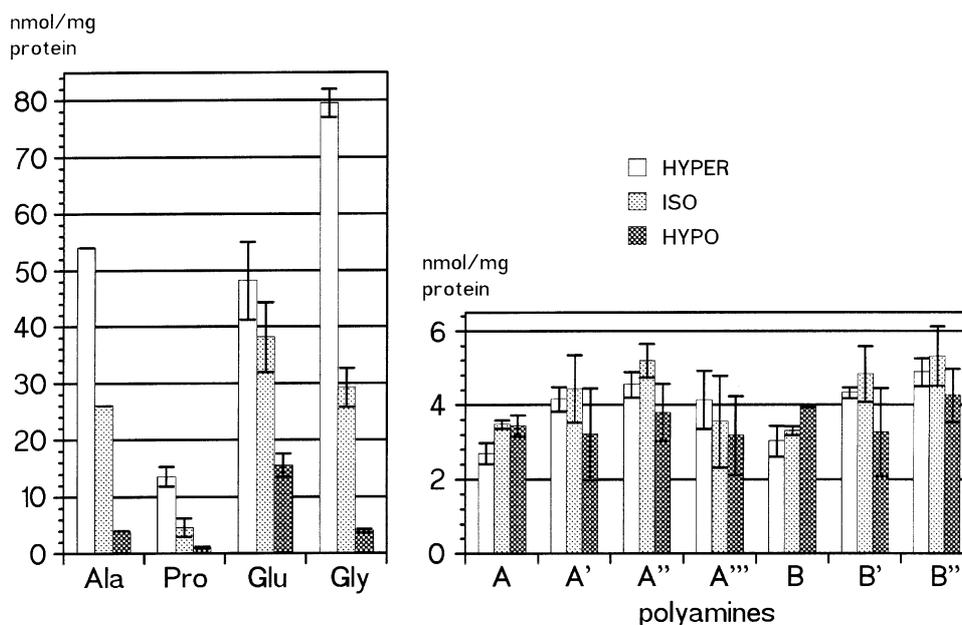


Figure 5. Concentration of amino acids and polyamines in F98 glioma cells exposed to hyper-, iso- and hypo-tonic osmolarity. The error bars indicate the standard deviation for three experiments. Alanine, which was quantified from the 1D ¹H spectrum has been used as an internal concentration standard

These data were correlated with standard protein measurements. Lyophilized PCA extracts were redissolved in 0.6 mL D₂O. All NMR samples were adjusted to pH values of 7–8. It is possible to adjust the pH to an exact value, for example pH 7.2, but this value shifts within the measuring time (1 day) in the range of ± 0.3 units. This might be one reason for the poor resolution of the cell extract spectra with respect to model solutions.

RESULTS

Figure 2 shows a non-selective HSQC spectrum of a PCA extract of F98 glioma cells. Seven polyamine signals are easily distinguished. They can be divided into two groups, the carbons bound to nitrogen (A–A''', 37–48 ppm) and the central carbons bound to other carbons only (B–B'', 23–25 ppm). The signals are labeled as indicated in Table 1. The resonance assignments for the other molecules were obtained previously¹⁸. Three of the polyamine signals can be further resolved with the high resolution of the semi-selective HSQC^{14,15} as shown in Fig. 3. Even at 800 MHz the proton chemical shifts are almost identical for Spm and Spd, which means that these two polyamines cannot be resolved in H,H-COSY spectra¹⁰. The small differences in the carbon frequencies allow the resolution of the A' and B' signals for Spm and Spd and the B signals for Spd and Put. All other signals, e.g. A for Put and Spd, or A'' for Spd and Spm, are separated by less than 4 Hz and could not be resolved further in our experiments with cell extracts (limitations due to viscosity, high salt content and paramagnetic ions). All signals except A'' can be resolved, however, for

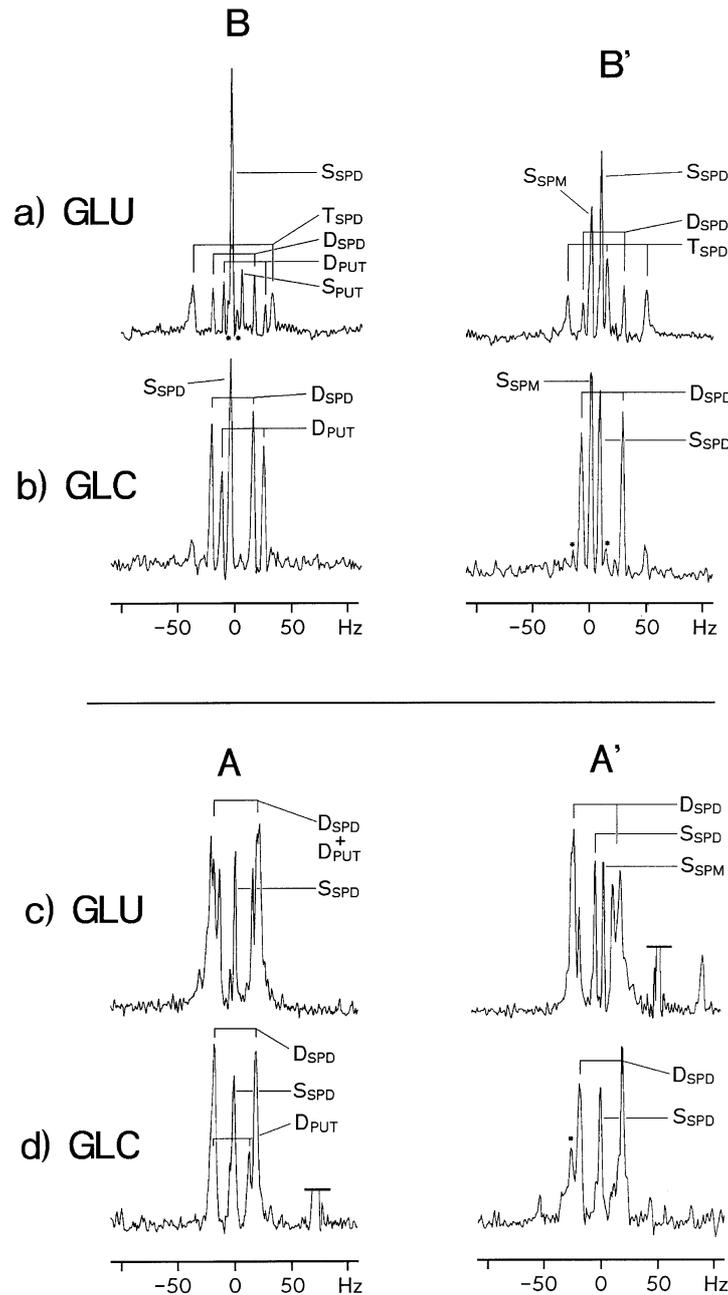


Figure 6. Plots of ^{13}C columns from semi-selective HSQC spectra (800 MHz) of F98 glioma cell extracts obtained after 24-h incubation with $[\text{U-}^{13}\text{C}]$ glutamate (a, c) or $[\text{U-}^{13}\text{C}]$ glucose (b, d). S = singlet, D = doublet, T = (pseudo)triplet. The one-bond ^{13}C - ^{13}C -coupling is 35.5 ± 1 Hz. A distinction of A-B and B-B' coupling constants was not possible due to insufficient resolution. Artifacts distinguished in the 2D-plots are labeled with an asterisk

the pure compounds in a reference solution (Table 1). The assignment was confirmed by measuring the individual compounds and by using HSQC-TOCSY spectra. Figure 4 shows details of an HSQC-TOCSY spectrum obtained from a polyamine mixture. Complete resolution of all three polyamines relies on the ^{13}C shift difference of ca 0.05 ppm between B' in Spd and Spm and between B in Spd and Put. If resolution in the carbon dimension is insufficient, Spd can still be detected in the presence of Spm and Put on the basis of the unique

TOCSY transfers from B' to A (3.06 ppm) and from B to A' (3.14 ppm), respectively. The absence of Spm or its quantification when present is a problem when Spd is present.

Recently we have studied the adaption of glial cell metabolism to anisotonic stress¹⁶. The synthesis of small and osmotically active organic molecules is reduced during hypotonic stress and stimulated under hypertonic conditions. This study is extended to polyamines in this paper. Figure 5 shows that, in contrast to

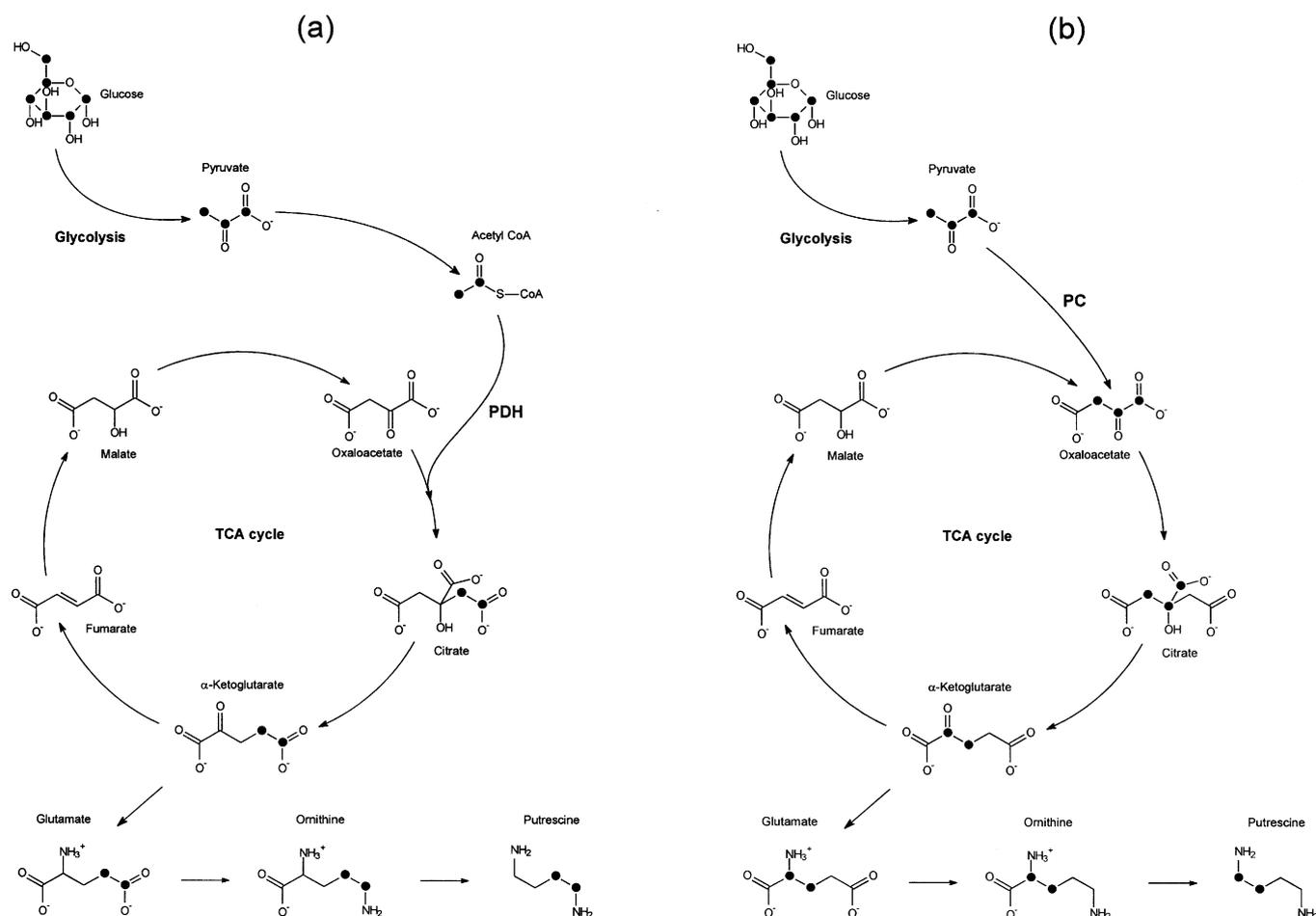


Figure 7. Biosynthetic pathways for the formation of putrescine from uniformly labeled glucose (a) *via* pyruvate dehydrogenase (PDH) or (b) *via* pyruvate carboxylase (PC). Pathway (c) shows the formation of doubly labeled Put from uniformly labeled glutamate

the behaviour of several amino acids as organic osmolytes, the concentrations of the polyamines remain essentially unaffected under osmotic stress. This is a strong indication that polyamines are not immediately involved in cell volume regulation. The concentrations of the polyamines are 3–5 nmol/mg protein.

To follow the synthesis of the polyamines, F98-glioma cells were fed with [U- ^{13}C]glucose and [U- ^{13}C]glutamate for 24 h. The biosynthesis of the polyamines is rather slow. Figure 6 shows columns extracted from the HSQC matrix for the polyamine signals A, B, A' and B' obtained upon [U- ^{13}C]glucose and [U- ^{13}C]glutamate feeding, respectively. Uniformly ^{13}C -labeled glucose leads to doubly labeled Put and Spd. Both doubly labeled A-B and A'-B' fragments are present. In Fig. 6(b) a doublet is observed for the Put B-signal, the Spd B-signal and the Spd B'-signal. No natural-abundance ^{13}C -singlet is detected for Put, of which the intracellular concentration is about one tenth of the concentration of Spd and Spm. The rather intense doublet to singlet ratio for Put indicates a higher amount of ^{13}C -enrichment compared to Spd, while Spm shows the natural abundance singlet

only. The corresponding A and A' signals [Fig. 6(d)] show doublets too. The A- and A'-signals are broader because of faster relaxation, chemical exchange and/or ^{14}N -couplings. A distinction of Put and Spd is made based on the intensity ratios obtained for the B- and B'-carbons.

From these results we deduce that after 24 h [U- ^{13}C]glucose incubation approximately 10% of Put, 1–2% of Spd and negligible amounts of Spm are labeled. The formation of doubly labeled putrescine isotopomers through the TCA cycle may occur *via* pyruvate dehydrogenase (PDH) and *via* pyruvate carboxylase (PC) activity [Fig. 7(a) and 7(b), respectively]. The contribution of the two in principle indistinguishable isotopomers can be deduced indirectly from the isotopomer patterns of the ^{13}C glutamate signals. The *de novo* synthesis of the polyamines *via* PDH and PC is 1.6/1 by comparing the glutamate C4,C5-doublet with the C2,C3-doublet. Incubation with uniformly ^{13}C -labeled glutamate leads first to uniformly labeled Put (cf. Fig. 1) and subsequently to Spd, also with a uniformly labeled C₄ subunit. While Put shows only a doublet for A and B

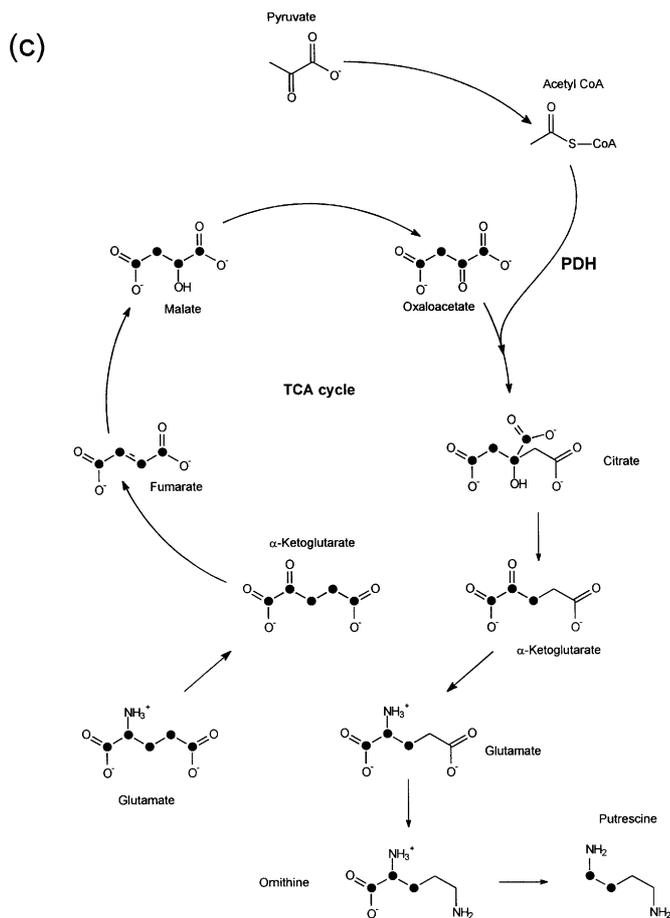


Figure 7. Continued.

because of symmetry, Spd reveals triplet splittings for B and B' and doublets for A and A'. The latter show in addition partially resolved long range ^{13}C - ^{13}C couplings. In addition to uniformly labeled Put and its derivatives, doubly labeled Put is also formed, if $[\text{U-}^{13}\text{C}]$ glutamate is desaminated and enters the TCA cycle as α -ketoglutarate (Fig. 7). After one passage through the TCA cycle doubly labeled α -ketoglutarate leaves the TCA-cycle, is converted to doubly labeled glutamate and subsequently Put. Spd derived from the latter isotopomer will be doubly labeled as well and will exhibit doublet splittings. The amount of fully labeled Spd is about twice the amount of doubly labeled Spd.

CONCLUSIONS

The individual polyamines Put, Spd and Spm can be unequivocally detected and quantified in PCA cell extracts by use of HSQC spectra, provided that chemical shift differences of 0.05 ppm can be resolved in the carbon dimension. This can be achieved with the semi-selective HSQC experiment. Several normally superimposed signals can be resolved, and in studies with ^{13}C -labeled precursors ^{13}C - ^{13}C spin couplings can be

observed in the F1 domain, whereby the analysis of the different isotopomers allows one to distinguish between different metabolic pathways. However, quantitative analysis is limited by the low synthesis rate. Incubation with uniformly labeled glucose or glutamate results in similar amounts of labeled Put and Spd, but different isotopomer patterns. $[\text{U-}^{13}\text{C}]$ glucose incubation results in doubly labeled species only, whereas $[\text{U-}^{13}\text{C}]$ glutamate also leads to polyamines with uniformly labeled C_4 subunits. This can be used to distinguish the formation of Put *via* the TCA-cycle from the direct formation *via* ornithine.

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