de Metz, M., Soute, B. A. M., Hemker, H. C., Fokkens, R., Lugtenburg, J., & Vermeer, C. (1982) *J. Biol. Chem. 257*, 5326–5329.
Fasco, M. J., & Principe, L. M. (1980) *Biochem. Biophys. Res. Commun. 97*, 1487–1492.
Fasco, M. J., & Principe, L. M. (1982a) *J. Biol. Chem. 257*, 4894–4901.
Fasco, M. J., & Principe, L. M. (1982b) *Biochem. Biophys. Res. Commun. 104*, 187–192.
Fasco, M. J., Hildebrandt, E. F., & Suttie, J. W. (1982) *J. Biol. Chem. 257*, 11210–11212.
Fasco, M. J., Preusch, P. C., Hildebrandt, E., & Suttie, J. W. (1983) *J. Biol. Chem. 258*, 4372–4383.
Kanaoka, Y., Machida, M., Ando, K., & Sekine, T. (1970) *Biochim. Biophys. Acta 207*, 269–277.
Preusch, P. C., Patterson, J. L., McTigue, J. J., & Suttie, J. W. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 466 (Abstr.).
Price, P. A., Poser, J. W., & Raman, N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3374–3375.
Price, P. A., Williamson, M. K., & Lothringer, J. W. (1981) *J. Biol. Chem. 256*, 12760–12766.
Silverman, R. B. (1981a) *J. Am. Chem. Soc. 103*, 5939–5941.
Silverman, R. B. (1981b) *J. Org. Chem. 46*, 4789–4791.
Suttie, J. W. (1980) *CRC Crit. Rev. Biochem. 8*, 191–223.
Wallin, R., & Suttie, J. W. (1981) *Biochem. J. 194*, 983–988.
Wallin, R., Gebbart, O., & Prydz, H. (1978) *Biochem. J. 169*, 95–101.
Whitlon, D. S., Sadowski, J. A., & Suttie, J. W. (1978) *Biochemistry 17*, 1371–1377.
Zimmerman, A., & Matschiner, J. T. (1974) *Biochem. Pharmacol. 23*, 1033–1040.

Direct Observation of Glycine Metabolism in Tobacco Suspension Cells by Carbon-13 NMR Spectroscopy†

Dennis James Ashworth* and Irvin Jacob Mettler

ABSTRACT: Carbon-13 NMR spectroscopy has been applied to the analysis of glycine metabolism in suspension cultured tobacco cells (*Nicotiana tabacum*). Treatment of 0.5 g of cells with glycine-*2*-^{13}C, followed by NMR analysis of the intact cells, generated spectra containing seven observable resonances. Comparison of the chemical shifts to authentic samples of glycine and L-serine allowed assignment of three peaks to glycine-*2*-^{13}C, serine-*2*-^{13}C, and serine-*3*-^{13}C. The remaining four resonances, generated by one-bond ^{13}C–^{13}C coupling (J = 37.5 Hz), were ultimately assigned to serine-*2,3*-^{13}C. This was verified by two methods: ^{13}C–^{13}C spin–spin coupling and two-dimensional homonuclear (^{13}C) correlated spectroscopy. The ^{13}C–^{13}C spin-coupled spectrum of L-serine displayed a serine C_2–C_3 coupling constant of 37.4 Hz, and the 2-D spectrum, obtained from 3.5 g of tobacco cells treated with 10 mM glycine-*2*-^{13}C for 24 h, established the predicted pattern of correlation. The presence of doubly labeled serine, representing 74% of the total ^{13}C-labeled serine formed, as well as the two singly labeled serine species, allowed calculations of the intracellular glycine and serine pool sizes and the percent of serine's carbons 2 and 3, which were derived from the glycine-*2*-^{13}C added to the cells. Calculation of intracellular glycine and serine levels was achieved by integration of the individual resonances relative to an external 2.4 M sodium acetate standard. Calibration of the external standard to an internal 0.12 M acetate standard and appropriate adjustments for the individual serine, glycine, and acetate nuclear Overhauser effect values provided the basis for the semiquantitative calculations. The results demonstrate the ability of carbon-13 NMR spectroscopy to monitor the metabolism of glycine in nonphotosynthetic tobacco cells and that serine formation from glycine is the result of tight coupling between glycine decarboxylase and serine hydroxymethyltransferase.

The formation of serine from glycine is an important metabolic pathway in plant cells. In photosynthetic tissues, the glycolate pathway (glycolate → glyoxylate → glycine → serine) is responsible for the photorespiratory production of CO_2 by the decarboxylation of glycine and possibly glyoxylate (Oliver, 1979). The primary evidence for the involvement of glycine decarboxylase to produce the photorespired CO_2 and methylenetetrahydrofolic acid for serine synthesis comes from studies that have localized glycine decarboxylase and L-serine hydroxymethyltransferase in leaf mitochondria (Woo & Osmund, 1976; Woo, 1979). Studies with ^{14}C-labeled glyoxylate and glycine have suggested that glyoxylate may directly contribute to the photorespiratory CO_2 and formate (Grodzinski, 1978; Oliver, 1979). Gifford & Cossins (1982a,b) presented evidence for the production of serine from ^{3}H- and ^{14}C-labeled formate and suggested that formate may be an important source of one-carbon units utilizing the cytosolic enzyme 10-formyltetrahydrofolate synthetase.

Nuclear magnetic resonance spectroscopy has recently provided a novel method for determining biological constituents and processes. Due to its nondestructive nature, NMR[1] spectroscopy can be used to assay living tissues. These tissues can then be recovered, unharmed, for further studies. In addition, because analysis can be performed on the intact tissues, often difficult and time-consuming extraction and separation procedures are eliminated. With the advent of the pulsed Fourier-transform technique and the availability of high magnetic field instruments, NMR spectroscopy can provide a unique and sensitive method of analysis. Furthermore, the

† From the de Guigne Technical Center, Stauffer Chemical Company, Richmond, California 94804. *Received June 15, 1983.*

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; 2-D, two dimensional; T_1, spin–lattice relaxation time; THF, tetrahydrofolic acid.

0006-2960/84/0423-2252$01.50/0

commercial availability of carbon-13-enriched compounds has allowed ^{13}C NMR spectroscopy to achieve the sensitivity, once monopolized by ^{31}P and ^{1}H NMR, required for biochemical applications. Polynucleotide conformations and interactions (Ts'o & Kan, 1979; Ashworth et al., 1981), bioenergetic processes (Van Divender & Hutton, 1982), membrane organization (Bolton & Bodenhausen, 1979), and cell transformation (Krepinsky et al., 1979) are all currently being investigated by nuclear magnetic resonance spectroscopy.

The studies presented here demonstrate the application of this powerful technique to the direct observation of glycine metabolism in small samples (0.5 g) of nonphotosynthetic tobacco suspension cells. Two-dimensional homonuclear (^{13}C) correlated NMR spectroscopy has been applied to assign metabolites in intact tobacco cells. In addition, calculation of intracellular glycine levels and metabolite distributions has been undertaken following appropriate T_1, NOE, and external reference calibration experiments. This has enabled us to demonstrate, in vivo, the tight coupling of serine hydroxymethyltransferase activity with glycine decarboxylase.

Experimental Procedures

Suspension cells of tobacco (*Nicotiana tabacum*) were transferred weekly into modified Murashige-Skoog medium essentially as described by Traynor & Flashman (1981). Glycine-2-^{13}C (90%) solutions were cold sterilized by passage through a sterile 0.22-μm filter. For glycine uptake, 0.4–0.6 g fresh weight of tobacco cells was transfered aseptically into 50-mL flasks to a final volume of 5 mL. ^{13}C-Labeled glycine was then added to a final concentration of 10 mM (except where indicated). After a 24-h incubation period, the cells were collected and drained. Approximately 0.5 g (fresh weight) of cells was then rinsed 4 times with 10 mL of H_2O to remove any remaining external glycine. Washes with 10 mM unlabeled glycine did not remove additional label. The cells were then suspended in 50% D_2O, transferred to a 5-mm NMR tube, and allowed to settle. Excess water was removed, leaving a 0.95-mL volume. For quantitative analysis, a capillary tube containing a 2.4 M solution of sodium acetate was inserted into the cell suspension.

All carbon-13 spectra were obtained on a Varian XL-200 spectrometer equipped with a broad-band probe operating at 50.1 MHz with continuous broad-band proton decoupling. The 90° pulse angle, T_1 relaxation times, and nuclear Overhauser enhancement factors were obtained from an equal molar solution of sodium acetate, glycine, and L-serine. The 90° pulse angle was determined by varying the pulse width about the 360° pulse angle range. The 360° pulse angle, representing a null in peak intensity, corresponded to a pulse width of 57 μs, and thus the 90° pulse width equaled 14.25 μs. Spin–lattice relaxation times were determined by the inversion–recovery method $(\pi-\tau-\pi/2-A_t-D)_N$ with A_t (acquisition time) + D (pulse delay) equal to 15 s. The value of τ was varied from 0.07 to 9.6 s in eight increments and resulted in T_1 values of 0.54, 1.04, 1.29, and 6.72 s for C_3 and C_2 of serine, C_2 of glycine, and C_2 of acetate, respectively. The nuclear Overhauser effect values for the carbons of interest were obtained by initially acquiring a spectrum using parameters employed in the ^{13}C-enriched cellular suspension samples: pulse width = 8.8 μs, pulse delay = 0.0 s, acquisition time = 1.45 s, and 32K data points for an 11K spectral width. A spectrum was then acquired with a 58-s pulse delay inserted to remove any resonance intensity irregularities due to disproporionate spin–lattice relaxation times. NOE removal was then achieved by gated decoupling (proton irradiation only during acquisition) with a pulse delay of 58 s for the same pulse width and number of transients as in the nuclear Overhauser enhanced spectrum. These data generated NOE values of 3.07 and 2.95 for C_3 and C_2 of serine, respectively, 2.84 for C_2 of glycine, and 2.01 for the acetate methyl. The ^{13}C–^{13}C spin–spin-coupled spectrum of L-serine was obtained by utilizing a $\pi/2-\tau-\pi-\tau-\pi/2-\Delta-\pi/2$ acquisition pulse sequence described by Bax et al. (1980). A value of 6.7 μs for τ was used on the basis of a suggested serine $J_{C_2C_3}$ of 36.5 Hz (Sogn et al., 1974). Acquisition of the spectrum over a 2000-Hz spectral width for 74 500 transients provided adequate signal to noise.

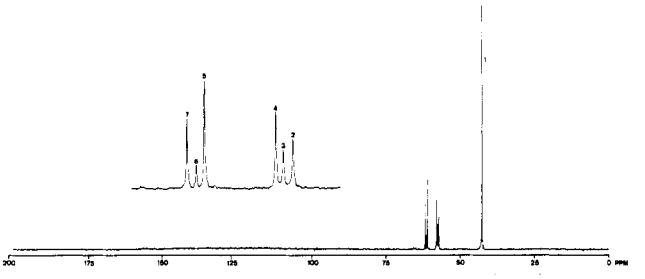
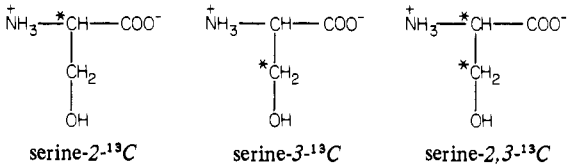


FIGURE 1: Carbon-13 spectrum of a 0.5-g tobacco cell suspension (D_2O) following treatment with 10.0 mM glycine-2-^{13}C for 24 h. 1 = glycine-2-^{13}C; 3 = serine-2-^{13}C; 6 = serine-3-^{13}C; 2, 4, 5, and 7 = serine-2,3-^{13}C.

The two-dimensional homonuclear correlated spectrum was obtained from a 3.5-g cellular fresh weight (4.0-mL packed cell volume). A $\pi/2-t_1-\pi/2$ acquisition pulse sequence ($\pi/2$ = 16.5 μs) was employed with appropriate phase cycling for quadrature detection in the second domain and continuous ^{1}H decoupling. An 1800-Hz spectral width was used in both dimensions with the evolution period (t_1) incremented over 256 equidistant values to finally produce a zero-filled 512 × 512 data matrix. Five hundred transients were accumulated for each evolution period increment requiring 63 h to acquire the 2-D spectrum. A 0.14-s acquisition time and a 1.5-s pulse delay were employed.

Results

The control spectrum of the tobacco cells following treatment with 10.0 mM glycine-2-^{13}C for 24 h is shown in Figure 1. Essentially seven lines appear in the spectrum. By comparison of the chemical shifts of these signals to previously obtained shifts of glycine and serine, resonance 1 (42.5 ppm) was assigned to glycine-2-^{13}C and resonances 3 (57.7 ppm)



serine-2-^{13}C serine-3-^{13}C serine-2,3-^{13}C

and 6 (61.5 ppm) were assigned to singly enriched serine with resonance 3 corresponding to serine-2-^{13}C and resonance 6 to serine-3-^{13}C. The remaining four signals were ultimately considered to be derived from ^{13}C–^{13}C coupling between adjacent ^{13}C carbons in the 2- and 3-positions of serine.

This possibility was confirmed by two methods: ^{13}C–^{13}C spin–spin coupling and two-dimensional homonuclear correlated NMR spectroscopy. The ^{13}C–^{13}C spin–spin-coupled (inadequate) spectrum obtained from a natural abundance sample of L-serine is shown in Figure 2 and was obtained to establish the C_2–C_3 coupling constant in L-serine. Clearly observed at C_3 is the antiphase doublet resulting from direct C_2–C_3 coupling ($J_{2,3}$ = 37.4 Hz). At C_2, again this doublet is observed. In addition, C_2–C_1 coupling ($J_{1,2}$ = 52.9 Hz) can be seen. The residual resonance centered at C_2 results from

Table I: Typical Calculation of Intracellular Glycine and Serine Concentrations from Carbon-13 Integral Data

resonance	serine-3-^{13}C (61.5 ppm)	serine-2-^{13}C (57.7 ppm)	serine-2,3-^{13}C (C_3 doublet)	glycine-2-^{13}C (42.5 ppm)	acetate (standard) (24.5 ppm)
integral (1.45-s pulse delay)	32.98	50.35	288.77	639.18	100.00 (108.70)[a]
integral (59.5-s pulse delay)	33.06	66.46	290.61	965.17	212.12 (230.56)[a]
NOE	3.07	2.95	3.07	2.84	2.01
integral without NOE	10.77	22.53	94.66	339.85	105.53 (9524.73)[b]
μmol of compound[c] (0.5 g of cells)$^{-1}$ (1.0 mL)$^{-1}$	0.15	0.32	1.33	4.77	120.00

[a] Includes 2.4 M external acetate/0.12 M internal acetate calibration factor of 0.92. [b] Includes $^{13}C \rightarrow {}^{12}C$ acetate integral conversion (1.108% natural abundance ^{13}C). [c] Includes 10% contribution from glycine-2-^{12}C in 90% enriched glycine-2-^{13}C.

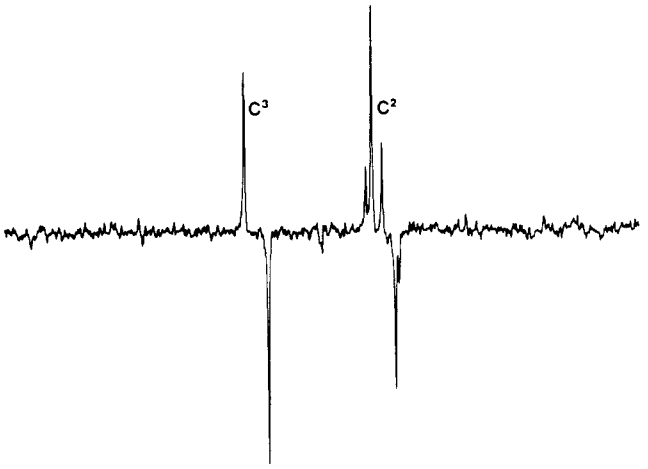


FIGURE 2: Natural abundance ^{13}C–^{13}C spin–spin-coupled spectrum of L-serine (1000-Hz expansion of C_2/C_3 region). Antiphase doublets at C_2 and C_3 verify serine C_2–C_3 coupling of 37.4 Hz. Also observed at C_2 is a 52.9-Hz C_2–C_1 coupling.

imperfect transfer of magnetization into double-quantum coherence for $J_{1,2}$; $\tau = {}^1/_4 J_{C\text{-}C}$ (Bax et al., 1980). Comparison of the C_2–C_3 coupling obtained here (37.4 Hz) to the value obtained in the cellular experiment (37.5 Hz) strongly indicated that indeed direct C_2–C_3 coupling was being observed in the in vivo generated serine.

Final confirmation of this coupling was obtained from the cellular two-dimensional homonuclear (^{13}C) correlated spectrum. The experiment generates a square matrix, and the data are represented as carbon chemical shift plotted against carbon chemical shift (Bax et al., 1981). Thus, the normal one-dimensional spectrum appears along the diagonal. Coupled carbons were then assigned as resonances found at the X–Y intersection off the diagonal. The contour representation (Figure 3) clearly shows the coupling occurring between C_2 and C_3 of serine. By observation of the off-diagonal resonances, the predicted pattern of correlation was established. Also observed is the strong resonance of glycine occurring at 400 Hz with no off-diagonal coupling to the adjacent carboxyl group due to the low abundance (1.1%) of ^{13}C in this resonance.

Calculation of intracellular glycine and serine levels was achieved by using an external aqueous solution of sodium acetate (2.4 M). The external reference was used due to the potential nonuniform distribution of an internal acetate reference in the cell suspension. The external acetate solution was then calibrated to an internal 0.12 M acetate solution in the presence of a D_2O-filled capillary. To achieve consistent experimental conditions throughout the course of this investigation, all tobacco cell NMR samples were weighed (fresh weight following removal of culture medium) and adjusted consistently to a volume of 0.95 mL with D_2O. The weight of cells used in each experiment, approximately 0.5 g, ensured a consistent cell density about the receiver and transmitter coils

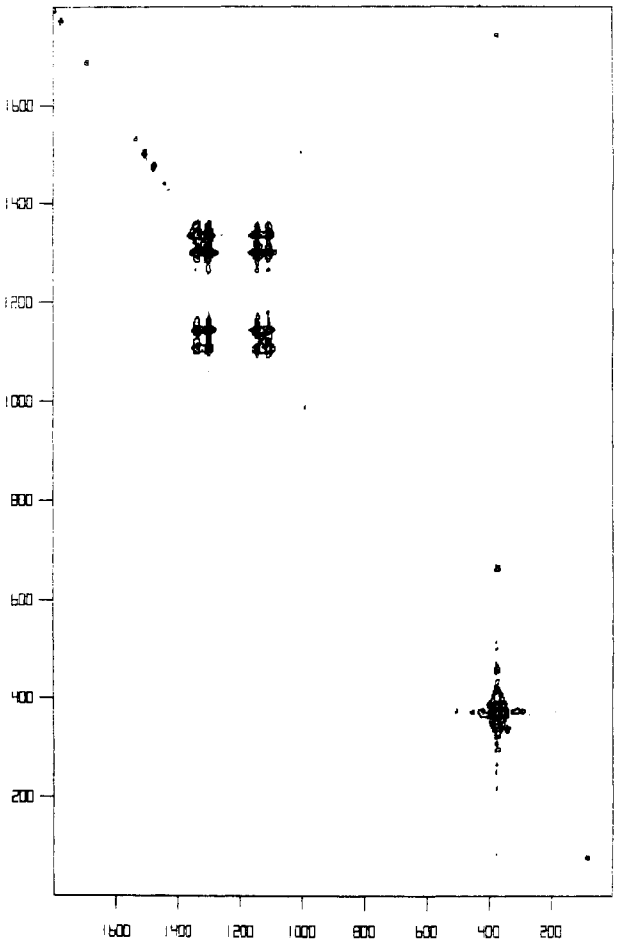


FIGURE 3: Two-dimensional homonuclear (^{13}C) correlated spectrum of a tobacco cell suspension following 24-h glycine-2-^{13}C uptake and metabolism. Square data matrix is represented as carbon chemical shift plotted against carbon chemical shift. The one-dimensional spectrum appears along the diagonal. Off-diagonal cross peaks (1100–1300 Hz) verify coupling between C_2 and C_3 of serine. Glycine-2-^{13}C appears at 400 Hz.

of the spectrometer. The sealed external reference capillary tube (1.6 × 100 mm) was then positioned, via a Teflon plug, within the cell mass. All spectra were obtained under attempted identical conditions, i.e., identical number of transients, pulse width and delay, etc. Quantitation was then achieved by integration of the individual resonances followed by appropriate acetate and NOE conversions (Table I), which allowed a semiquantitative computation of the amount of ^{13}C in each carbon of serine. On the basis of the percent of labeled and unlabeled carbon in serine and the 90% ^{13}C enrichment of added glycine, the source of carbon used for serine formation could be determined (Table II) as well as the size of the internal glycine and serine pools. The C_2 of serine was derived almost entirely from the glycine-2-^{13}C added to the cells (96–99%). The C_3 of serine was also heavily labeled (80–89% from added glycine).

Table II: Incorporation of Carbon-13 into C_2 and C_3 of Serine by Tobacco Suspension Cells As Determined by ^{13}C NMR[a]

concn of glycine-2-^{13}C (mM)	% serine carbon labeled		% serine carbon derived from added glycine	
	C_3	C_2	C_3	C_2
25	78.5	87.6	87.2	97.4
10	80.4	88.0	89.3	97.8
4	75.1	89.8	83.5	99.7
1.5	87.6	94.6[b]	97.4	105.1[b]

[a] Tobacco suspension cells (0.5 g) were treated with glycine-2-^{13}C (90%) for 24 h. [b] Due to the low levels of serine formation, the integration error may account for ^{13}C contents of greater than 90%.

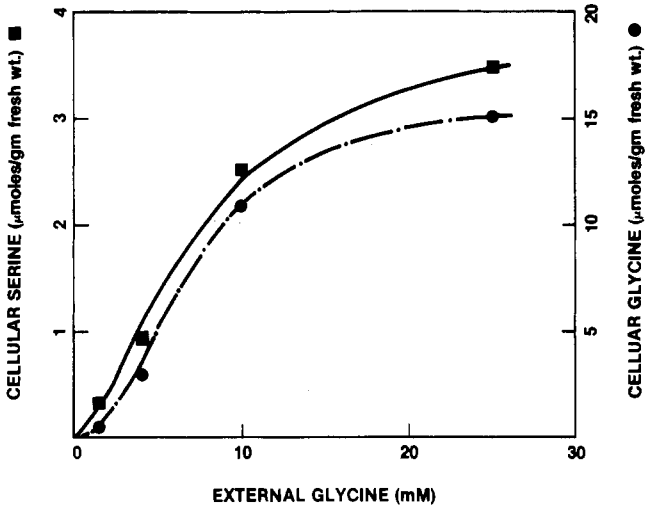


FIGURE 4: Cellular glycine content and serine formation as a function of external glycine concentration in tobacco suspension cultured cells treated with glycine-2-^{13}C for 24 h and determined by ^{13}C NMR. Hyperbolic curve fit for serine data: $Y = 4X^{1.57}/(X^{1.57} + 24.5)$ (r = 0.998). Hyperbolic curve fit for glycine data: $Y = 16X^{2.23}/(X^{2.23} + 78.1)$ (r = 0.998).

The kinetics of serine formation was investigated by varying the amount of glycine added to the external media. Serine production was not a simple Michaelis–Menten function of the added glycine (Figure 4). The cellular concentration of glycine (determined by ^{13}C NMR) showed similar complex kinetics as a function of external glycine concentration as observed for serine formation. Plotting serine formation against the cellular glycine content produced the linear relationship shown in Figure 5 (correlation coefficient r = 0.998). In an effort to detect the formation of labeled compounds other than serine, tobacco cells were incubated with glycine-2-^{13}C for longer periods (up to 4 days). No significant changes in the NMR spectra were observed.

Discussion

Carbon-13 NMR has only recently been recognized as a useful tool for studying metabolism in vivo. Studies have been reported with whole animals (Alger et al., 1981), bacteria (Halpin et al., 1981), and yeast (den Hollander et al., 1979; Thevelein et al., 1982), and limited work has been done with plant tissues (Schaefer et al., 1975; Skokut et al., 1982).

The results presented here demonstrate the ability of carbon-13 NMR spectroscopy to monitor glycine metabolism in intact plant cells. Not only did the NMR spectra allow us to identify the formation of serine from glycine but also it allowed us to approximate the amount of carbon in each of the three observed products, serine singly labeled at C_2 or C_3 and the doubly labeled serine. The formation of a primarily

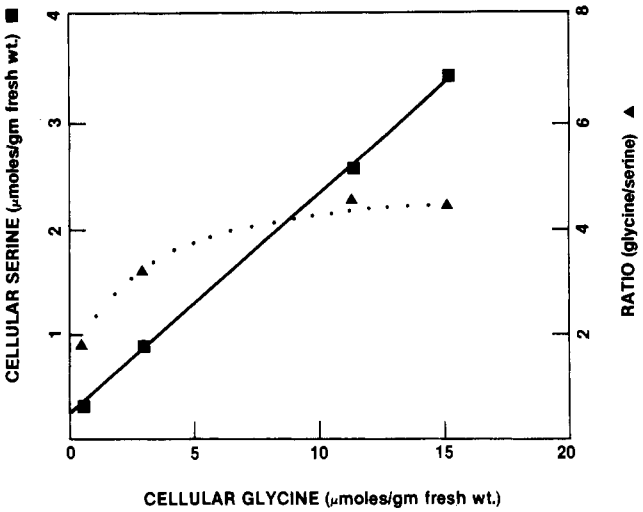


FIGURE 5: Serine formation and ratio of cellular glycine to serine as a function of cellular glycine content in tobacco suspension cells treated with glycine-2-^{13}C for 24 h. Equation of best linear fit: $Y = 0.235 + 0.209X$ (r = 0.998).

dual-labeled product allowed exceptionally powerful conclusions to be made about the size of the serine and glycine pools and the source of carbon used for serine synthesis. This information would be extremely difficult or impossible to obtain with ordinary radioactive tracers, which often require tedious cell constituent separation procedures prior to analysis. The carbon-13 spectrum provides a window for the total observation of the ^{13}C-labeled substrate's metabolism. Given a large enough cellular accumulation of product (in our case 0.02 μmol/0.5 g of cells), no unexpected principal metabolite need go undetected. Then, by comparison of the chemical shifts of the various products to those of authentic reference compounds, assignments can very often be made.

The carbon-13 NMR spectrum following 24-h glycine uptake and metabolism (Figure 1) revealed three different labeled serine species. Wang and co-workers (Wang & Burris, 1963a; Wang & Waygood, 1963b) were one of the first groups to suggest that serine was derived from glycine in plants. They suggested, at that time, that both the C_2 and C_1 carbons of glycine gave rise to the C_3 carbon of serine. Rabson et al. (1962), using $^{14}CO_2$, as well as Marker & Whittingham (1967), using glycine-2-^{14}C, ultimately showed that it is the C_2 carbon of glycine that becomes the C_2 and C_3 carbons of serine. These findings are confirmed by the pair of doublets resulting from one-bond C_2–C_3 coupling (J = 37.5 Hz) observed in Figure 1 and represent 74% of the total ^{13}C-labeled serine formed. The remaining two serine species, serine-2-^{13}C and serine-3-^{13}C, observed in the spectrum are derived from selective carbon-13 enrichment.

The choice of 24 h as the length of incubation time for the experiments was determined by incubating parallel cultures with 10 mM glycine-2-^{13}C for periods up to 4 days. The intensities of the serine resonances steadily increased with increasing incubation time and reached maximum values at 24 h. Longer treatment periods did not appreciably increase the intensities of the resonances or result in spectra displaying new signals. Therefore, a 24-h incubation time was utilized in all subsequent experiments.

The two-dimensional homonuclear (^{13}C) correlated spectrum obtained here (Figure 3) may represent the first application of this technique to the assignment of coupled carbons in living cells. The experiment, first proposed by Jeener (1971), has recently been applied to the identification of scalar proton and phosphorus coupling patterns in a number of biological

molecules (Bax et al., 1981; Prestegard et al., 1982; Van Divender & Hutton, 1982). In the present investigation, this technique has aided in the unambiguous assignment of resonances generated by direct $^{13}C-^{13}C$ coupling in serine.

Three primary difficulties faced in the calculation of cellular glycine/serine levels are resonance intensity irregularities resulting from spin–lattice (T_1) relaxation times, nuclear Overhauser effects, and nonuniform distributions of cells in the NMR tube relative in the external standard. Since the experiments presented here were carried out under continuous proton decoupling with a 1.45-s acquisition time/pulse delay, signals arising from carbons with long spin–lattice relaxation times (e.g., acetate CH_3 = 6.7 s) will integrate erroneously low. Furthermore, under proton decoupling conditions the saturation of proton transitions leads to a redistribution in populations of the individual ^{13}C energy levels (Solomon, 1955; Kuhlmann & Grant, 1968). This redistribution (nuclear Overhauser effect) causes certain carbon resonance intensities to be greater than the normal equilibrium intensity. To obtain accurate nuclear Overhauser enhancement factors, Harris & Newman (1976) have suggested pulse delays of ≥ 9 T_1 for gated decoupling. The longest spin–latice relaxation time (T_1) observed here, 6.7 s for the sodium acetate standard, was used for calculation of the 59.5-s pulse delay in the gated decoupled NOE experiment. To minimize the nonuniform distribution of cells in the NMR tube, a sufficient number of cells (0.5 g/5-mm tube) were added to each tube such that the density of cells throughout the tube was approximately uniform. The Teflon plug that supported the external standard capillary then provided a "stopper" to prevent any significant vortexing of the cell suspension. The calculation of glycine/serine levels would, however, probably be more accurately described as semiquantitative due to irregular distributions of the spinning cell suspensions relative to the external reference as well as other potential unknown factors.

The use of ^{13}C-labeled compounds requires much higher concentrations of tracer than is used normally with radioactive tracers. In the experiments described here, the concentration of [^{13}C]glycine added to the cells determined the size of the cellular glycine and serine pools (Figure 4). This result suggests that the initial cellular pool sizes of glycine and serine were probably less than 1 mM. Although 10 mM external glycine was routinely used to optimize the NMR signals, the source of carbon for serine formation did not change as a function of glycine concentration (Table II), indicating that the observed coupling of glycine decarboxylase and serine hydroxymethyltransferase was not artificially caused by the expanded glycine pool. The linear relationship between serine formation and cellular glycine concentration indicates that glycine to serine conversion was essentially at equilibrium. The fact that the line does not pass through the origin is probably the result of a minor bias in the integration of the NMR spectra perhaps due to a slight drift in the base line. This is noticeable with faint signals where negative integrals will sometimes be indicated. At normal concentrations, however, this error is considered to be less than 5%.

The biochemical pathway for the formation of serine from glycine in C_3 plants is highly active during photosynthesis and is thought to occur via serine hydroxymethyltransferase located in the mitochondria. Glycine is the direct source for carbons 1 and 2 of serine. The third carbon is transferred by the folate cofactor methylenetetrahydrofolate. Methylenetetrahydrofolate can be produced from the C_2 carbon of glycine by glycine decarboxylase also located in the mitochondria or alternatively from formate by the cytosolic enzyme 10-formyltetrahydrofolate synthetase. Using [^3H]formate to label serine, Gifford & Cossins (1982a,b) showed inhibition of label incorporation into serine with the folate analogue aminopterin and with isonicotinic acid hydrazide. They proposed a flow of carbon from glycolate to 10-formyl-THF via glyoxylate and formate in greening barley leaves. The results presented here with tobacco suspension cells demonstrate that glycine is actively metabolized to serine in nonphotosynthetic cells and that serine hydroxymethyltransferase is tightly coupled to glycine decarboxylase for the supply of methylenetetrahydrofolate.

The discovery that serine was the only metabolite formed in significant quantity from the labeled glycine was initially somewhat surprising since the cellular concentration of labeled glycine and serine was shown to be quite high (15 and 3–5 μmol/g fresh weight, respectively). This result was consistently observed and did not vary as a function of incubation time, concentration of labeled glycine, or stage of suspension cell growth. The lack of any other significantly labeled products of one-carbon metabolism suggests that the mitochondrial pool of methylene-THF derived from glycine decarboxylase may have limited exchange with the cytoplasmic pools of tetrahydrofolate. Furthermore, the absence of any other observed products in the NMR spectra suggests that either other potential metabolic routes from glycine and serine apparently do not produce significant enough labeled product pools to be detected (e.g., tryptophan, cysteine, threonine, purines, or glycolytic intermediates) or these metabolic routes were not sufficiently active in these tissues to be detected. Localization of metabolic steps and product pools may also have been involved in our observed specificity of serine formation.

The work presented here demonstrates the ability of NMR spectroscopy to monitor specific biochemical reactions in intact plant cells. Because the analysis can be performed on the intact tissue in one NMR experiment, the need for time-consuming extraction, separation, and bioassay procedures is almost completely eliminated. These results and future experiments may provide new insight into the photorespiratory pathway and its regulation in photosynthetic cells.

Acknowledgments

We thank Drs. D. Bowler, L. Chang, and C. Tseng at Stauffer for their help and Dr. G. Gray at Varian Associates for suggesting the 2-D experiment.

Registry No. Glycine, 56-40-6; serine, 56-45-1; serine-2-^{13}C, 89232-76-8; serine-3-^{13}C, 89232-77-9; serine-2,3-^{13}C, 89232-78-0; glycine-2-^{13}C, 20220-62-6.

References

Alger, J. R., Sillerud, L. O., Behar, K. K., Gillies, R. J., Shulman, R. G., Gordon, R. E., Shaw, D., & Hanley, P. E. (1981) *Science (Washington, D.C.) 214*, 660–662.

Ashworth, D. J., Chang, C., Unger, S. E., & Cooks, R. G. (1981) *J. Org. Chem. 46*, 4770.

Bax, A., Freeman, R., & Kempsell, S. P. (1980) *J. Am. Chem. Soc. 102*, 4849.

Bax, A., Freeman, R., & Morris, G. (1981) *J. Magn. Reson. 42*, 164.

Bolton, P. H., & Bodenhausen, G. (1979) *J. Am. Chem. Soc. 101*, 1080.

den Hollander, J. A., Brown, T. R., Ugurbil, K., & Shulman, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6096.

Gifford, D. J., & Cossins, E. A. (1982a) *Phytochemistry 21*, 1479.

Gifford, D. J., & Cossins, E. A. (1982b) *Phytochemistry 21*, 1485.

Grodzinski, B. (1978) *Planta 144*, 31.

Halpin, R. R., Hegeman, G. D., & Kenyon, G. L. (1981) *Biochemistry 20*, 1525.
Harris, R. K., & Newman, R. H. (1976) *J. Magn. Reson. 24*, 449.
Jeener, J. (1971) Ampere International Summer School, Basko Polje, Yugoslavia.
Krepinsky, J., Carver, J. P., Rajalakshmi, S., Rao, P. M., & Sarma, D. S. R. (1979) *Chem.-Biol. Interact. 27*, 381.
Kuhlmann, K. F., & Grant, D. M. (1968) *J. Am. Chem. Soc. 90*, 7355.
Marker, A. F. H., & Whittingham, C. P. (1967) *J. Exp. Bot. 18*, 732.
Oliver, D. J. (1979) *Plant Physiol. 64*, 1048.
Prestegard, J. H., Koerner, T. A. W., Demov, P. C., & Yu, R. K. (1982) *J. Am. Chem. Soc. 104*, 4993.
Rabson, R., Tolbert, N. E., & Kearney, P. C. (1962) *Arch. Biochem. Biophys. 98*, 154.
Schaefer, J., Stejskal, E. O., & Beard, C. F. (1975) *Plant Physiol. 55*, 1048.
Skokut, T. A., Varner, J. E., Schaefer, J., Stejskal, E. O., & McKay, R. A. (1982) *Plant Physiol. 69*, 314.
Sogn, J. A., Craig, L. C., & Gibbons, W. A. (1974) *J. Am. Chem. Soc. 96*, 4694.
Solomon, I. (1955) *Phys. Rev. 99*, 559.
Thevelein, J. M., den Hollander, J. A., & Shulman, R. G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3503.
Traynor, P. L., & Flashman, S. M. (1981) *Theor. Appl. Genet. 60*, 31.
Ts'o, P. O. P., & Kan, L. S. (1979) in *Chromatin Structure and Function* (Nicolini, C. A., Ed.) Part A, p 217, Plenum Press, New York.
Van Divender, J. M., & Hutton, W. C. (1982) *J. Magn. Reson. 48*, 272.
Wang, D., & Burris, R. H. (1963a) *Plant Physiol. 38*, 430.
Wang, D., & Waygood, E. R. (1963b) *Plant Physiol. 37*, 826.
Woo, K. C. (1979) *Plant Physiol. 63*, 783.
Woo, K. C., & Osmond, C. B. (1976) *Aust. J. Plant Physiol. 3*, 771.

Sequence-Specific Recognition of DNA: NMR Studies of the Imino Protons of a Synthetic RNA Polymerase Promoter†

Shan-Ho Chou, David E. Wemmer, Dennis R. Hare, and Brian R. Reid*

ABSTRACT: We have synthesized both strands of a DNA duplex containing the consensus Pribnow promoter sequence TATAATG, flanked by GC base pairs to stabilize the ends of the helix. The stability of this duplex has been studied by using ^1H nuclear magnetic resonance. The imino protons have been assigned by using the sequential nuclear Overhauser effect approach. Exchange rates have been monitored by using selective inversion recovery measurements. The helix is relatively unstable in the center of the AT-rich region even when surrounded by GC base pairs, and there is considerable asymmetry in the melting of the helix.

We have begun a systematic study of the structural and dynamic characteristics of short DNA duplexes containing operator and promoter sequences that function in the regulation of DNA expression (Chou et al., 1983). Promoters are short regulatory DNA sequences, located upstream of structural genes, that function in transcriptional control of DNA by binding RNA polymerase such that RNA synthesis is correctly initiated. The promoter sequence most proximal to the site of initiation is the well-known Pribnow sequence, or "TATA" box, located upstream at about position −10 from the first transcribed residue (Rosenberg & Court, 1979; Siebenlist et al., 1980; Hawley & McClure, 1983). Several different promoter sequences occur naturally, and these variations probably govern the efficiency of transcription at a given gene locus. We have chosen to first examine the prototype or consensus sequence

CG*T*T*A*T*A*A*T*GCG
GC*A*A*T*A*T*T*A*CGC

in which the Pribnow octamer is embedded in two GC pairs at each end of the helix. The boldface letters represent base pairs that are strongly conserved in promoters whereas the italic letters are only moderately conserved (Rosenberg & Court, 1979). The central ten base pairs of this sequence constitute the actual promoter sequence of the *spc* ribosomal protein operon (Post et al., 1978), and the central eight base pairs, including those that are highly conserved, are the specific sequence for a promoter in the SV40 virus (Dhar et al., 1974). The remaining letters thus represent structural support and were introduced into the sequence to reduce the effects of end fraying rather than being in the promoter sequence per se. We will henceforth refer to this sequence as the consensus Pribnow sequence. In this paper, we describe the assignment of the hydrogen-bonded imino proton spectrum and discuss the dynamics of this sequence as probed by imino proton exchange. In the accompanying paper (Wemmer et al., 1984) we assign most of the nonexchangeable protons and discuss the structural features of this dodecamer.

NMR[1] spectroscopy is one of the few methods that can provide both structural and dynamic information on molecules in solution and has seen increasing application in the study of nucleic acids in recent years. Imino proton assignment and dynamic studies on several tRNAs and small DNA sequences have been carried out including two operator DNA sequences (Johnston & Redfield, 1981; Hare & Reid, 1982a,b; Early

† From the Department of Chemistry and the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received July 25, 1983.* We gratefully acknowledge NIH Grant R01 GM32302 and instrumentation grants from the Murdock Foundation, the National Institutes of Health (GM2874-01S1), and the National Science Foundation (PCM80-18053).

[1] Abbreviations: DMT, dimethoxytrityl; NMR, nuclear magnetic resonance; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; NOE, nuclear Overhauser effect; FID, free-induction decay.

0006-2960/84/0423-2257$01.50/0