Vol. 97, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1980 Pages 1053-1059

# $^{13}C$ ENRICHMENT OF TOBACCO MOSAIC VIRUS

J.L. de Wit, N.C.M. Alma, T.J. Schaafsma

Department of Molecular Physics, Agricultural University,
de Dreijen 6, P.O. Box 8091, 6700 EP Wageningen
The Netherlands

Received October 14,1980

SUMMARY: $^{13}C$ NMR spectra of Tobacco Mosaic Virus with enhanced signal-to-noise ratio have been obtained by isotope enrichment up to 15 %, using $^{13}CO_2$ as the carbon source, obtained from Ba $^{13}CO_3$ 90 % enriched, and tobacco leaves as the substrate.

Determination of the enrichment percentage of TMV is made by IR spectroscopy of the $CO_2$ mixture resulting from complete combustion. Four days of controlled growth and 10 grams of Ba $^{13}CO_3$ yield 100 mg 15 % enriched TMV.

Analysis of $^{13}C$ NMR spectra of denatured TMV protein both natural abundance and $^{13}C$ enriched show that enrichment is statistically random. Different strains of TMV (Vulgare and U2) can be distinguished by their $^{13}C$ NMR spectra.

## INTRODUCTION

*Enrichment with stable isotopes:* Enrichment with stable isotopes, e.g. $^{2}H$, $^{13}C$, and $^{15}N$ has been widely applied in NMR (1). Isotopic enrichment can be carried out rather easily in algae, yeast and bacteria (2). In plants, only $^{13}C$ and $^{15}N$ can be incorporated to a high degree ($\sim$ 90 %) without adverse effects (3, 4), but high enrichment cannot be easily obtained due to isotope dilution. $^{2}H$ enrichment can be attained up to $\sim$ 50 % isotope content, but growth is severely inhibited, similar to what is observed for animals (5, 6). Some strains of algae and yeast must first be adapted to increasing $^{2}H_2O$ concentrations before a sufficient growth rate is reached in 99 % $^{2}H_2O$ (7).

Selective enrichments with $^{2}H$, $^{13}C$ and $^{15}N$ are suitable for tracer studies, and for various NMR applications (8, 9, 1, 2). Non-selective enrichment, e.g. via biosynthesis, with $^{13}C$ and $^{15}N$ can be used to obtain NMR spectra with enhanced signal-to-noise ratio. Here, we report non-selective isotope enrichment of Tobacco Mosaic Virus (TMV) to about 12 %. Tobacco plants infected with TMV represent a very efficient system to incorporate $^{13}C$ with minor isotope dilution. This paper describes the enrichment procedure, a simple method for determining the $^{13}C/^{12}C$ ratio, and some typical $^{13}C$ NMR results.

0006-291X/80/231053-07$01.00/0

## MATERIALS AND METHODS

*$^{13}C$ enrichment:* 90 % enriched $Ba^{13}CO_3$ was purchased from Bio Rad; aureomycine and kinetine from Serva and fungizone from Squibb. For illumination we used 400 Watt SON 400 W, BSN 400 LO2, S50 Na lamps (Philips).

*TMV purification and protein preparation:* TMV strain Vulgare was purified according to Leberman (10). The protein preparation procedure used is similar to that described by Durham et al. (11), with minor changes as described elsewhere (12). Protein preparations in 0.12 M Tris-HCl, pH 8.6, 5 °C were checked and characterized with polyacrylamide gelelectrophoresis showing a single band (13), sedimentation analysis (11) yielding a single sedimentation coefficient ∿ 3.8 S and spectrometrically for Vulgare yielding $E_{280}/E_{250} > 2.5$.

*$^{13}C$ NMR spectra at 90.5 MHz:* $^{13}C$ NMR spectra at 90.5 MHz have been obtained with a Bruker SPX 360 supercon spectrometer equipped with quadrature detection using 10 mm sample tubes and ∿ 5 W continuous wave broadband $^{1}H$ decoupling power. Sample pH was directly measured in the tube with a long 5 mm diameter pH electrode (Radiometer). Sample temperature was maintained with a modified version of the Bruker temperature accessory (12) and was measured with a thermometer inserted into the tube when it was positioned inside the probe. After equilibration of sample temperature, the tube with the thermometer was quickly removed from the probe with an air lift for taking thermometer readings. The error expected for measuring sample temperature in this manner is estimated to be $\pm$ 2 °C.

*Computer simulations:* Computer simulations of $^{13}C$ spectra of TMV-protein were performed using the known amino acid composition of the TMV strain Vulgare. The starting positions for the chemical shifts were basically those of James (14) and were shifted between + or -0.4 ppm from these positions for maximum overlap with the denatured TMV-P spectrum. A DEC 10 computer program allows convolution of each individual carbon with a line shape function.

## RESULTS AND DISCUSSION

*The preparation of 10-15 % enriched TMV:* The enrichment of TMV proceeds through assimilation of 90 % $^{13}C$ enriched carbon dioxide by green leaves of the Samsun NN' variety of Nicotiana tabacum, infected with TMV. The amount of 90 % $^{13}C$ enriched barium carbonate was always approximately 1 g/10 g wet leaf, so that the final enrichment of TMV always ends up between 10-15 %. At this isotope content, the relative amount of $^{13}C$-$^{13}C$ pairs is ∿ 1 % and sufficiently low to prevent a decrease of resolution and signal-to-noise ratio by spin-spin splittings, without improving the spectral information content (1). Tobacco plants of about six weeks age were given a dark period of one night in a temperature controlled room (25 °C) in order to reduce the sugar content. The middle leaves of the plant are inoculated with TMV about two hours starting the dark period and are transferred in the dark to sterilized 100 ml beakers containing

sterilized nutrient solution which were placed in a closed, gas--impermeable system of desiccators interconnected by butyl tubing and connected to a Kipp apparatus. The composition of the nutrient solution is equivalent to that of the Hoagland mineral salt solution and is completed with aureomycine and fungizone to a concentration of 1 and 2 µg/ml, respectively, preventing bacterial and mold growth; ferricitrate (0-5 µg/ml) is used as the iron source. In the desiccators high humidity is maintained through a water film on the bottom. After a second dark period of twelve hours, necessary for prolonged carbohydrate exhaustion, kinetine to a concentration of 1 µg/ml is added to the nutrient solution to inhibit ageing of leaves. Immediately after installing the leaves in the growth system, the desiccators are covered so that a maximum humidity is reached in the shortest possible time. For about 15 minutes the system is then flushed with a carbon dioxide-free mixture of 80 % nitrogen and 20 % oxygen. After closing the system from air, $^{13}CO_2$ (∿ 90 % enriched) is admitted to the system by release from $Ba^{13}CO_3$, by dropwise addition of hydrochloric acid (5 ml 2.5 N hydrochloric acid per gram barium carbonate) to the Kipp apparatus. After completely filling the desiccator-system with $^{13}CO_2$, the plants are illuminated by two 400 W sodium lamps at approximately 50 cm distance from the bottom of the desiccators. To prevent rapid ageing it is important to keep temperature constant at ∿ 25 °C, especially during the change from the dark to the light period. Growth of TMV is continued as long as no leaf degeneration occurs (large brown spots) and leaves are stored at -20 °C. The average growth period is about four days and the average yield is 1-1.5 mg/g wet leaf.

Approximately 10 g barium carbonate (∿ 90 % enriched) is needed to obtain ∿ 100 mg enriched TMV. The relatively low TMV yield compared to that for growth under normal conditions is caused by the short TMV multiplication period. TMV yield is found to be an optimum when 400 W sodium lamps are used. Both assimilation and dissimilation rates (20 mg $CO_2/h/dm^2$ leaf surface (15) and 4 mg $CO_2/h/g$ dry leaf weight (35)) are high, so that after the addition of $^{13}CO_2$, a $^{13}CO_2/^{12}CO_2$ equilibrium is reached very quickly, the $^{12}CO_2$ originating from residual endogenous starch. The $^{13}CO_2/^{12}CO_2$ ratio in the system can be easily determined at any moment during the enrichment process, allowing control of the final TMV isotope content. A glass system is favourable since it is gas-impermeable, in contrast with most plastic chambers.

*Infrared determination of the $^{13}CO_2/^{12}CO_2$ ratio:* The gas mixture resulting from complete combustion of TMV at 1100 °C is passed through solid

potassium chlorate and carbon dioxide is precipitated as barium carbonate by reaction with $Ba(OH)_2$ solution. HCl is added dropwise and the $^{13}CO_2/^{12}CO_2$ ratio of the released carbon dioxide is determined from the IR gaseous carbon dioxide spectrum taken on a Hitachi (EPI-G3) IR spectrometer against carbon dioxide free nitrogen/oxygen mixture. The centers of the absorption peaks of $^{12}CO_2$ and of $^{13}CO_2$ are located at 2349 and 2284 $cm^{-1}$ respectively, and both peaks are split into two. A relation between the $^{13}CO_2/^{12}CO_2$ ratio and the ratio of the IR $^{13}CO_2$ and $^{12}CO_2$ peak heights has been determined using the reference mixtures of known amounts of enriched and normal barium carbonate for which the same procedure is followed for the release of the $^{13}CO_2$ and $^{12}CO_2$ mixture from the enrichment experiment.

We have derived the following empirical relation:

$$^{12}CO_2/^{13}CO_2 = 2.25 \times \frac{h_C}{h_A} + \frac{h_D}{h_B} \qquad [1]$$

where $h_A$ and $h_B$ represent the heights of the two IR peaks of $^{13}CO_2$; $h_C$ and $h_D$ are the corresponding peak heights for $^{12}CO_2$.

*$^{13}C$ NMR spectra of TMV-protein:* In Fig. 1A the 6 M urea denatured spectrum is shown of natural abundance $^{13}C$ TMV. Fig. 1B represents the same spectrum of 12 % $^{13}C$ enriched TMV. Fig. 2C is the best fit of a computer simulation based on the known amino acid composition. The line widths used for this simulation are 45 Hz (primary carbons); 50 Hz (secondary carbons); 60 Hz (tertiary carbons); 70 Hz (CA); 60 Hz (quarternary); 50 Hz (carbamyl) and the resonance positions of the amino acids are essentially similar to those published (14), with slight deviations necessary for an optimum fit. Such deviations ($< \pm 0.4$ ppm) are attributed to residual chemical shift inequivalences and pH effects. The aliphatic resonances shown in the spectra of Figs. 1A and 1B correspond to fully relaxed carbons. Yet, a comparison of Fig. 1A resonances with the computer-simulated pattern of Fig. 1C reveals small intensity differences. However, given the simple simulation procedure, the overall resemblance between the two aliphatic spectral regions is satisfactory. Intensity differences between aliphatic resonances arise from linewidth and NOE variations, due to a spread in rotational correlation times of the carbon-proton dipole-dipole vectors.

Proceeding from Fig. 1A to 1B or from Fig. 1C to 1B the intensity increase of the aliphatic resonances is non-uniform. The increase for at least three resonances (labeled 1 to 3) is larger than that of the remaining aliphatic resonances. These labeled resonances have been

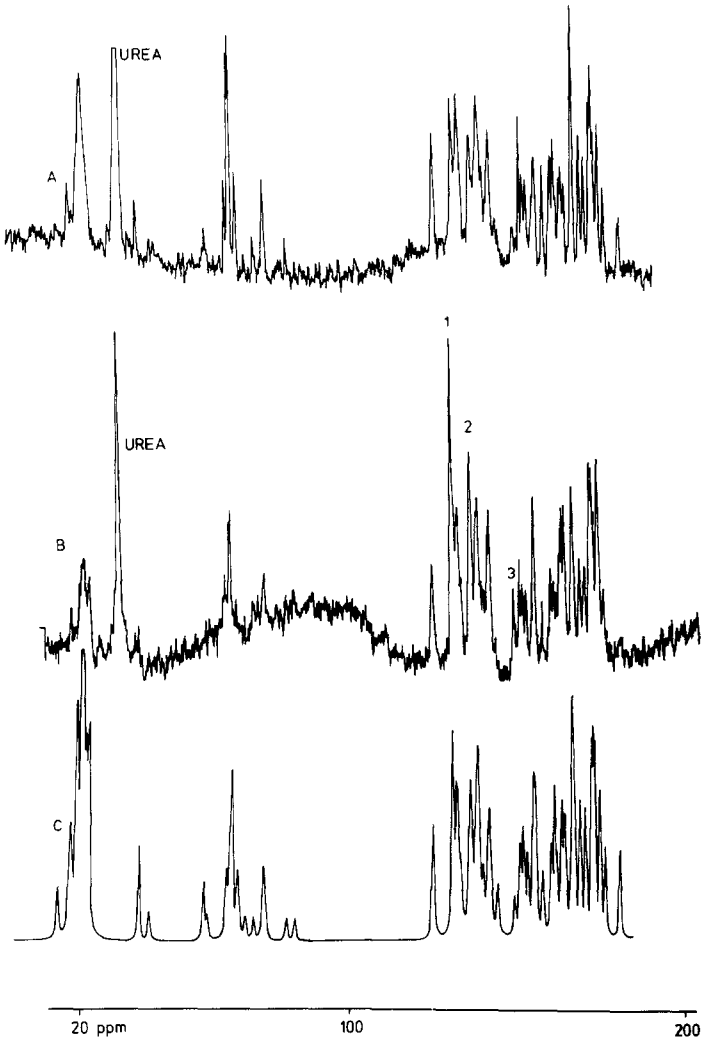


Fig. 1A, B: Broadband $^{1}H$ decoupled $^{13}C$ spectra at 90.5 MHz of TMV-protein using quadrature detection with an acquisition plus delay time of 0.4 s; solution: 0.1 M NaCl; pH 11.7; temperature 7 °C; 6 M urea; sensitivity enhancement: 17 Hz. Other conditions for Fig. 1A: natural abundance TMV; concentration: 120 mg/ml accumulations: 70,000. For B: 15 % $^{13}C$ enriched TMV; concentration: 30 mg/ml; accumulations: 4,700. Three of the strongest enriched resonances have been labeled by numbers 1 - 3.
Fig. 1C: Natural abundance $^{13}C$ computer simulation of TMV protein NMR spectrum using the known amino acid composition of TMV strain Vulgare. Linewidths are given in the text and spectral positions are of James (14). The ppm scale is referenced to $CS_2$ taking ß-Thr at 125.8 ppm.

assigned to Ser $C_{\beta}^{1}$; Phe/Tyr $C_{\alpha}^{2}$; Ser $C_{\alpha}^{2}$; Gly $C_{\alpha}^{3}$. During plant biosynthesis, Ser and Gly are known to be in enzymatic equilibrium involving serin-hydroxymethyltransferase; Tyr is known to be synthesized from Phe. Our

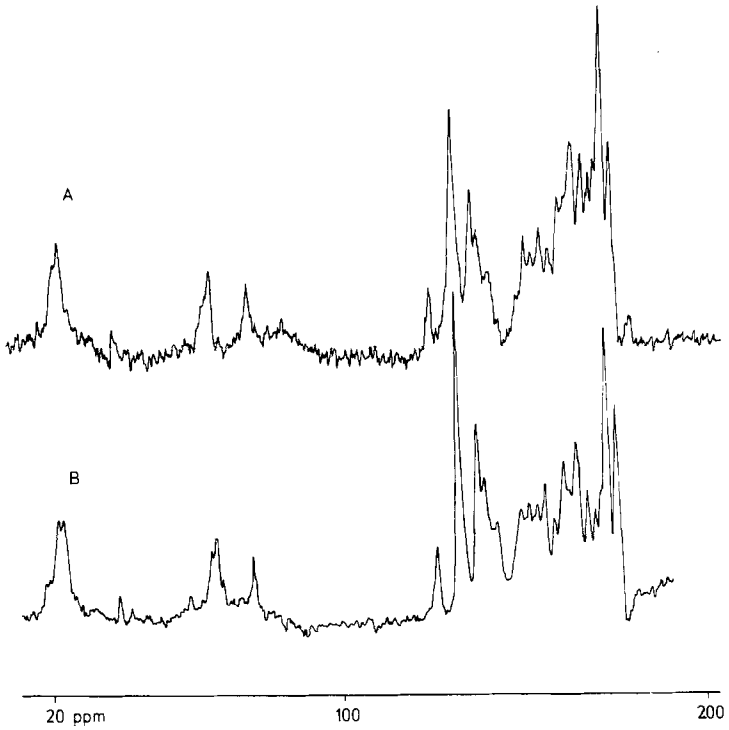


Fig. 2: Broadband $^{1}H$ decoupled 90.5 MHz $^{13}C$ spectra of (A) TMV-protein Vulgare and (B) TMV protein $U_2$ strain. Experimental conditions: quadrature detection with acquisition and delay time: 0.4 s; sensitivity enhancement: 17 Hz; accumulation: 17,000; sample 0.1 M NaCl, pH 11.7, temperature 7 °C, concentration (A) 30 mg/ml, (B) 15 mg/ml. The ppm scale is referred to $CS_2$, taking β-Thr at 125.8 ppm.

observations are consistent with these equilibria and synthetic pathways. Although selective $^{13}C$ isotope effects are not unknown in enzymatic reactions (16), the selective enrichment of the abovementioned carbons cannot satisfactorily be explained.

The intensity decrease of the aromatic and carbonyl carbon resonances as a whole, in Fig. 1B w.r.t. those in Fig. 1A, despite the large Phe and Tyr $C_{\alpha,\beta}$ enrichment, arises because these carbons have $T_1$'s which are longer than the corresponding $T_1$'s of the same carbons in Fig. 1A. The $T_1$'s in both cases (Figs. 1A and B) are larger than the 0.4 s acquisition time. The longer $T_1$'s of Fig. 1B is caused by the 4 times smaller sample concentration and, by consequence, a lower viscosity. The absence of the Ileu $C_\delta$ resonance in Fig. 1B (18.1 ppm) does not find a simple explanation.

Figs. 2A and B represent the $^{13}C$ NMR spectra of 12 % $^{13}C$ enriched TMV-P from strains Vulgare and $U_2$, respectively. The reproducible differences

between both spectra demonstrate that $^{13}C$ NMR has sufficient sensitivity and resolution to distinguish these two strains with different amino acid composition.

ACKNOWLEDGEMENTS

The technical assistance of Mr. H. Jongejan of the Department of Organic Chemistry and of Miss N. Boerjan is gratefully acknowledged. We are indebted to the Department of Virology for providing laboratory facilities.

The NMR investigations at high magnetic field (8.5 T) were supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

REFERENCES:

1. Wüthrich, K., NMR in Biological Research: peptides and proteins (1976) North Holland Publishing Company, Amsterdam.
2. Proc. of the first int. conf. on stable isotopes in chem., biol. and med. (1973) Argonne (eds. Klein, P.D. and Peterson, S.V.). Available through Nat. Techn. Info. Service U.S. Department of Commerce.
3. Siegel, S.M., Halpern, L.A. and Giumarro, C. (1964) Nature 201, 1244-1245.
4. Kollman, V.H., Gregg, C.T., Hanners, J.L., Whaley, T.W. and Ott, D.G., in ref. 2.
5. Manson, L.A., Carp, R.I., Defendi, V., Rothstein, E.L., Hartzell, R.W. and Kritchevsky, D. (1960) Ann. New York Acad. Sci. 84, 685-694.
6. Morowitz, H.J. and Brown (1953) Natl. Bur. Standards Rept. No. 2179.
7. Crespi, H.L., Conrad, S., Uphans, R.A. and Katz, J.J. (1960) Ann. New York Acad. Sci. 84, 648-666.
8. MacInnes, A.G., Walter, J.A., Wright, J.L.C. and Vining, L.C. (1976) in Topics in carbon-13 NMR spectroscopy, 125-172 (ed. Levy, G.C.) John Wiley and Sons, New York.
9. Blomberg, F., Maurer, W. and Rüterjans, H. (1976) Proc. Nat. Acad. Sci. U.S.A. 73, 1409-1413.
10. Leberman, R. (1966) Virology 30, 341-347.
11. Durham, A.C.H. (1972) J. Mol. Biol. 67, 289-305.
12. De Wit, J.L., Hemminga, M.A. and Schaafsma, T.J. (1978) J. Magn. Resonance, 31, 97-107.
13. Ornstein, L. (1964) Ann. New York Acad. Sci. 121, 321-349.
14. James, T.L. (1975) Nuclear Magnetic Resonance in Biochemistry, p. 246, Academic Press, New York.
15. Zaitlin, M. and Hesketh, J.D. (1965) Ann. Appl. Biol. 55, 239-243.
16. Hegazi, M.F., Borchardt, R.T. and Schowen, R.L. (1979) J. Am. Chem. Soc. 101, 4359-4365.