J. Mol. Biol. (1980) **141**, 327–333

On the Rigidity of RNA in Tomato Bushy Stunt Virus

The motional state of RNA in tomato bushy stunt virus, both in the crystalline state and in solution, has been investigated using ^{31}P nuclear magnetic resonance methods. It has been found that the RNA is highly immobile in the native virus, and it is suggested that the lack of a high-resolution X-ray diffraction pattern for either the RNA or the N-terminal regions of the protein coat molecules (Harrison *et al.*, 1978) is due to static disorder in the crystals. Dynamic disorder has been detected in the virus after treatment with EDTA, which causes a structural change and an increase in particle size.

Tomato bushy stunt virus consists of a single molecule of RNA ($M_r \sim 1{\cdot}5 \times 10^6$), 180 coat protein molecules ($M_r \sim 41{,}000$) of identical sequence, and possibly one larger protein (Weber *et al.*, 1970; Dorne & Pinck, 1971; Ziegler *et al.*, 1972). The assembled particle is effectively spherical with a radius of about 170 Å, and X-ray diffraction studies have enabled an electron density map to be produced at a resolution of 2·9 Å (Winkler *et al.*, 1977; Harrison *et al.*, 1978). Well-resolved density is observed in the outer region of the particle (112 to 170 Å from the centre), and the coat protein molecules can be observed in three distinct packing environments. In each of these environments, however, at least 50 residues at the N-terminal region of the protein are not visible in the electron density map (Harrison *et al.*, 1978). In addition, there is a conspicuous absence of strong density attributable to the RNA, which is known to be contained in the inner portion of the virus (Chauvin *et al.*, 1978). This letter describes ^{31}P nuclear magnetic resonance experiments designed to investigate the motional character of the RNA in tomato bushy stunt virus, both in the crystalline state and in solution.

Figure 1 shows the 119 MHz ^{31}P n.m.r.† spectrum of TBSV precipitated from solution by addition of concentrated $(NH_4)_2SO_4$. Under these conditions, the virus is in an environment close to that in the X-ray crystallographic studies (Harrison *et al.*, 1978). The spectrum was obtained using proton decoupling and cross-polarization techniques (Pines *et al.*, 1973), and the shape of the spectrum arises primarily from the anisotropy of the ^{31}P chemical shift tensor. The differences in isotropic shifts of different phosphate groups of nucleic acids are small (<5 p.p.m., Gueron & Shulman, 1975) compared with the spectral width. In these and other spectra reported here we neglect these differences, and characterise each n.m.r. spectrum using a single set of parameters.

The principal values of the shift tensor can be obtained directly from the n.m.r. spectrum. These values are close to those found for small molecule phosphate esters in crystals and powders (Herzfeld *et al.*, 1978; Terao *et al.*, 1977) and for nucleic acid fibres and powders (Terao *et al.*, 1977; Cross *et al.*, 1979), as Table 1 shows. The n.m.r.

† Abbreviations used: n.m.r., nuclear magnetic resonance; TBSV, tomato bushy stunt virus; p.p.m., parts per million.

0022–2836/80/230327–07 $02.00/0

method is expected to be sensitive to internal motions occurring at rates at least comparable to the inverse of the spectral width. In the solid state, any motion faster than about 10 kHz will average the shift tensor to an extent that is determined by the degree of re-orientation of the principal axes with respect to the laboratory frame. For

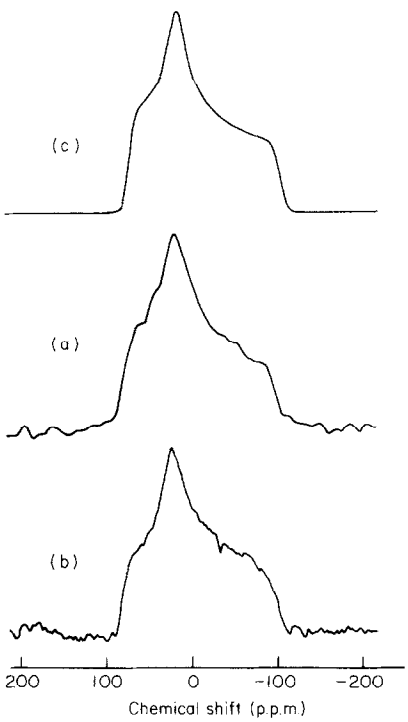

FIG. 1. (a) 119 MHz proton decoupled ^{31}P n.m.r. spectrum of TBSV in the solid state. Chemical shifts are measured in p.p.m. relative to external 85% H_3PO_4. Principal values of the shift tensor were measured directly from the spectrum.

(b) Spectrum of fibrous DNA from calf thymus obtained under identical conditions.

(c) Simulated rigid lattice powder pattern for TBSV. Each crystallite orientation is presumed to correspond to a Gaussian line with a width of 6 p.p.m.

TABLE 1

Principal values of chemical shift tensors[a]

	σ_{11}	σ_{22}	σ_{33}	σ_{iso}
TBSV[b]	78	19	−100	−1
tRNA[c]	82	21	−101	−1
DNA[d]	81	20	−103	−1

[a] Values are ±3 p.p.m., relative to 85% H_3PO_4. $\sigma_{iso} = \frac{1}{3}(\sigma_{11} + \sigma_{22} + \sigma_{33})$.
[b] Precipitated using $(NH_4)_2SO_4$.
[c] From yeast, precipitated using ethanol.
[d] Fibrous, from calf thymus.

example, fast continuous rotation about any axis would lead to an axially symmetric shift tensor (Mehring *et al.*, 1971). Straightforward considerations show that any oscillation or rotation of the phosphate group of more than a few degrees should be detected from the n.m.r. spectrum, though the exact spectral effects depend in detail on the nature of the motion. The similarity of the values for TBSV to those for crystalline phosphate esters indicates that very little averaging occurs, and that as far as the n.m.r. experiment is concerned, the phosphate groups are rigidly held. Similar conclusions have been drawn from ^{31}P spectra of RNA in a filamentous bacterial virus (Cross *et al.*, 1979).

It is possible, however, that the phosphate groups of the RNA in TBSV do not all have the same motional state. In this case, the measured shift tensor values would represent the least mobile groups, and other less-constrained groups would give rise to averaged components contributing to the central region of the spectrum only. In order to investigate this possibility, the spectral lineshape was examined. In Figure 1, the spectrum of dry, fibrous DNA is shown recorded under the same conditions as the virus spectrum. Also in Figure 1 is shown a simulated rigid lattice spectrum calculated (Bloembergen & Rowland, 1953) using the experimental principal values of the TBSV shift tensor. In order to resemble the experimental spectrum, the theoretical powder pattern was convoluted with a Gaussian function corresponding to a standard deviation of 6 p.p.m. The experimental linewidth includes contributions not only from T_2 effects but also from the inhomogeneity of the magnet (about 2 p.p.m.) and from the small variation in the chemical shifts of the phosphate groups. The close similarity between the spectral lineshape of the virus, that of fibrous DNA and the simulated rigid lattice spectrum shows that under these conditions all of the phosphate groups in the virus RNA give rise to spectra corresponding to those of a rigid lattice without internal motion.

For the virus in solution, molecular tumbling partially averages the shift tensor, but the extent of averaging for a spherical particle of known radius, such as TBSV, under given conditions can be calculated fairly accurately (Debye, 1929). Any internal motion of significant extent will reveal itself as a further line-narrowing. For nucleic acids such as DNA in solution, a highly averaged spectrum is observed with a linewidth much narrower than that observed here, because of extensive internal flexibility (Cross *et al.*, 1979; Hanlon *et al.*, 1976). By contrast, the ^{31}P n.m.r. spectrum of TBSV in solution recorded at 109·3 MHz and 19°C corresponds to a line of Lorentzian shape with a full width at half maximum of nearly 2 kHz (Fig. 2). The linewidth is, however, highly dependent upon the frequency at which the measurement is made. The linewidth behaviour at once shows that the dominant transverse relaxation mechanism is due to chemical shift anisotropy (Abragam, 1961; Hull & Sykes, 1975). This relaxation is proportional to the square of the observing frequency, whilst the other mechanisms that are likely to be of importance (chiefly the dipolar coupling) are expected to be frequency-independent. In Figure 3, a plot of linewidth against the square of the observing frequency can be seen to be closely linear, and enables the contribution to the linewidth from mechanisms other than chemical shift anisotropy to be evaluated at a temperature of 292 K as 190 ± 20 Hz. The chemical shift anisotropy contribution to the half-height linewidth ($\Delta\nu_{\frac{1}{2}}$) is $1{\cdot}54 \pm 0{\cdot}10$ kHz at a spectrometer frequency (ν) of 109·3 MHz; more generally, $\Delta\nu_{\frac{1}{2}}/\nu^2 = 1{\cdot}29$ $(\pm 0{\cdot}08) \times 10^{-13}$ s.

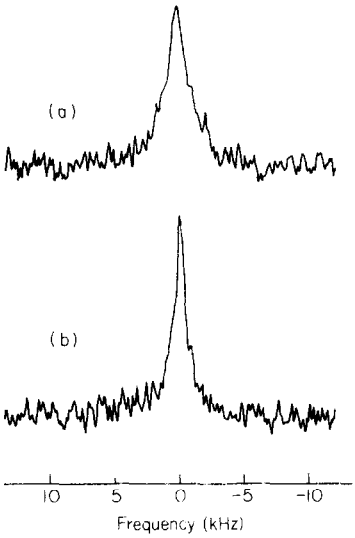

FIG. 2. (a) 109·3 MHz spectrum of native TBSV in solution at 19°C. The spectrum consists of a single Lorentzian line with a full width at half maximum of 1730 Hz.
(b) 109·3 MHz spectrum of an expanded form of TBSV in solution. The line is narrowed to ~800 Hz as a result of increased internal freedom of the RNA.

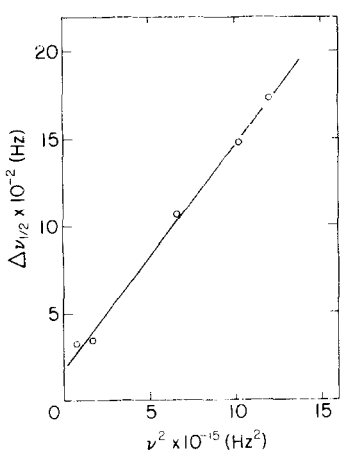

FIG. 3. A plot of linewidth *versus* the square of the observing frequency for native TBSV in solution. The linearity of the plot indicates that chemical shift anisotropy is a dominant relaxation mechanism. Extrapolation to zero frequency provides an estimate of the contribution to the linewidth from frequency-independent mechanisms.

Assuming a radius of 170 Å for the particle, the correlation time for isotropic tumbling in solution at 19°C was calculated to be $5{\cdot}3 \times 10^{-6}$ s by using the Debye (1929) relationship. The shift anisotropy contribution to the linewidth ($\Delta\nu_{\frac{1}{2}}$) was calculated to be $1{\cdot}73 \pm 0{\cdot}18$ kHz at 109·3 MHz, or $\Delta\nu_{\frac{1}{2}}/\nu^2 = 1{\cdot}45\ (\pm 0{\cdot}15) \times 10^{-13}$ s.

The error limits are based on the accuracy of the measurements of the temperature (± 1 deg.), the radius (± 1 Å), and of the shift tensor (± 3 p.p.m.). The differences in the calculated and experimental linewidths are insignificant in view of the fact that it is difficult to define the radius of the virus particle in the absence of a completely uniform surface. Assumption of a radius of 164 Å rather than 170 Å would put calculated and experimental linewidths in exact agreement.

The linewidth of the spectrum decreases as the temperature increases, and the behaviour gives a linear Arrhenius plot over the range from 7 to 27°C with an activation energy of 6 ± 1 kcal mol^{-1}. This value is close to that expected from the change with temperature of the solution viscosity (4·6 kcal mol^{-1}). Internal motion is expected to reduce the spectral linewidth and increase its temperature dependence. Whilst the experimental data deviate from the theory in the direction expected for internal motion, the difference is insignificant. The conclusion is, therefore, that the spectrum of TBSV in solution can be described adequately by a model with immobile phosphate groups. Finally, we note that the spectrum of turnip crinkle virus (TCV) in solution is indistinguishable from that of TBSV. TCV differs slightly from TBSV in protein and RNA sequences (Symons *et al.*, 1963), but has the same radius and consequently is expected to have the same hydrodynamic properties.

The effect of internal motion can be seen in an expanded form of TBSV, which is obtained by treating the native virus with EDTA at pH values of 7·0 or above. This treatment causes removal of divalent metal ions, and an increase in the radius of the particle results from a change in the interaction between the protein coat subunits (Robinson *et al.*, unpublished results). This increase in radius, about 10%, corresponds to a volume change in the TBSV cavity of about 30%. The expanded virus has been crystallized and a 7 Å structure determination is in progress. The ^{31}P n.m.r. spectrum of expanded TBSV in solution (Fig. 2) is considerably narrower than that of the native virus; it appears to be a superimposition of narrow and broad components, perhaps reflecting the presence of phosphate groups with different mobilities. The expansion of the virus particle would be expected to cause a reduction in overall tumbling rate, and an increase in linewidth of about 30% would result if RNA in the expanded virus were rigidly held. An explanation for the observed narrowing is that only a fraction of the RNA is now immobile, whilst the rest has significant internal motion. The full relevance of this observation to an understanding of the RNA–protein interactions remains to be established, but it does indicate that linewidth changes resulting from internal motion in TBSV can be readily detected.

For the intact particle, we conclude that the n.m.r. data eliminate the possibility of large-scale internal motion that would produce fluctuations of more than a few degrees in the orientation of phosphate groups with respect to the whole particle. The lack of well-defined electron density in the X-ray diffraction study cannot therefore be explained satisfactorily by internal fluctuations in the RNA, but must arise from disorder within the virus crystals. The following picture is consistent with all that we currently know. The RNA is highly structured in the native particle, and it probably interacts strongly and specifically with the N-terminal portions of the coat subunits, which are presumed to penetrate into the virus cavity (Harrison *et al.*, 1978; Chauvin *et al.*, 1978). The RNA is not of regularly repeating sequence, however, and the interaction with each protein subunit must be different. The subunits are

then equivalent as far as the outer surface of the virus particle is concerned, but not with respect to their configurations and interactions with RNA inside the protein shell. The lack of a high-resolution X-ray diffraction pattern for either the RNA or the N-terminal region of the protein coat molecules is therefore due to static rather than dynamic disorder in the crytals.

This study has demonstrated the application of n.m.r. methods to the definition of the motional character of a macromolecule, both immobilized in crystals and free in solution. Extension of this work to study the assembly of virus particles, and the interaction of nucleic acids and proteins could be made.

The n.m.r. experiments were carried out at the Francis Bitter National Magnet Laboratory at the Massachusetts Institute of Technology. The work was supported by the National Science Foundation and by the National Institutes of Health.

Department of Chemistry
Harvard University
12 Oxford Street
Cambridge, Mass. 02138
U.S.A.

M. G. MUNOWITZ
C. M. DOBSON

Francis Bitter National Magnet Laboratory
Massachusetts Institute of Technology
Cambridge, Mass. 02139
U.S.A.

R. G. GRIFFIN

Gibbs Laboratory
Harvard University
12 Oxford Street
Cambridge, Mass. 02138
U.S.A.

S. C. HARRISON

Received 16 April 1980

REFERENCES

Abragam, A. (1961). *Principles of Nuclear Magnetism*, Oxford University Press, Oxford.
Bloembergen, N. & Rowland, T. J. (1953). *Acta Met.* **1**, 731–753.
Chauvin, C., Witz, J. & Jacrot, B. (1978). *J. Mol. Biol.* **124**, 641–651.
Cross, T. A., Diverdi, J. A., Wise, W. B. & Opella, S. J. (1979). In *NMR and Biochemistry* (Opella, S. J. & Lu, P., eds), pp. 67–74, Dekker, New York.
Debye, P. (1929). *Polar Molecules*, The Chemical Catalog Co., New York.
Dorne, B. & Pinck, L. (1971). *FEBS Letters*, **12**, 241–243.
Gueron, M. & Shulman, R. G. (1975). *Proc. Nat. Acad. Sci., U.S.A.* **72**, 3482–3485.
Hanlon, S., Gloneck, T. & Chan, A. (1976). *Biochemistry*, **15**, 3869–3875.
Harrison, S. C., Olson, A. J., Schutt, C. E., Winkler, F. K. & Bricogne, G. (1978). *Nature (London)*, **276**, 368–373.
Herzfeld, J., Griffin, R. G. & Haberkorn, R. A. (1978). *Biochemistry*, **17**, 2711–2718.
Hull, W. E. & Sykes, B. D. (1975). *J. Mol. Biol.* **98**, 121–153.
Mehring, M., Griffin, R. G. & Waugh, J. S. (1971). *J. Chem. Phys.* **55**, 746–755.
Pines, A., Gibby, M. G. & Waugh, J. S. (1973). *J. Chem. Phys.* **59**, 569–590.
Symons, R. H., Rees, M. W., Short, M. N. & Markham, R. (1963). *J. Mol. Biol.* **6**, 1–15.
Terao, T., Matsui, S. & Akasaka, K. (1977). *J. Amer. Chem. Soc.* **99**, 6136–6138.

Weber, K., Rosenbush, J. & Harrison, S. C. (1970). *Virology*, **41**, 763–765.
Winkler, F. K., Schutt, C. E., Harrison, S. C. & Bricogne, G. (1977). *Nature (London)*, **265**, 509–513.
Ziegler, A., Harrison, S. C. & Leberman, R. (1972). *Virology*, **59**, 509–515.