Comparison of Ribonucleic Acid–Protein Interactions in Messenger Ribonucleoproteins, Ribosomes, MS2 Virus, and Qβ Virus Examined via Phosphorus-31 Nuclear Magnetic Resonance Relaxation†

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ABSTRACT: The influence of the proteins in functional RNA–protein complexes on RNA has been investigated by using ^{31}P NMR. The ^{31}P spin–lattice relaxation time (T_1), $^{31}P\{^1H\}$ nuclear Overhauser effect (NOE), off-resonance rotating frame spin–lattice relaxation time ($T_{1\rho}^{off}$), and off-resonance intensity ratio (R) have been measured for polysomal messenger ribonucleoprotein particles and ribosomes isolated from rat liver, as well as the icosahedral bacteriophages MS2 virus and Qβ virus, and compared with those for free RNA. The results were dependent on the particular complex examined. In certain cases, the relation parameters were fitted to a motional model entailing two correlation times corresponding to a slow motion and a faster internal motion. The calculations include both dipole–dipole and chemical shift anisotropy contributions to relaxation. Analysis of the NMR relaxation data indicates that the internal motion of the RNA in intact ribosomes is restricted by a factor of about 5 relative to that of free RNA or limit-digest ribosomes. These results suggest that the "packaging" of RNA in the ribosome differs from that in either single- or double-stranded RNA and that the constraints of this packaging are removed by digesting 20–30% of the RNA in preparation of the limit-digest ribosome. Another variant is provided by the messenger ribonucleoprotein for which the T_1 value suggested substantial coupling of the phosphorus nucleus to protein protons, unlike the other RNA–protein systems studied; the protein proton–RNA phosphorus interaction was confirmed by double-resonance experiments entailing observations of the phosphorus resonance during selective irradiation of the protein proton resonances. The ^{31}P NMR results for MS2 virus and Qβ virus suggest that the RNA phosphodiester moiety in these viruses has an altered mobility (and perhaps structure) compared to that of free RNA. In addition, unlike the situation with pure RNA, the protons of water (or protons exchangeable with water) are dipolar coupled to the RNA phosphorus in the virus and ribosomes. The nature of the protein–RNA interaction clearly depends on the particular protein–RNA complex.

The nature of the interactions between nucleic acids and proteins is vital to biological processes. Consequently, this area has been subject to investigation with natural and model nucleic acid–protein systems exploring features such as ionic attractions, stacking of aromatic amino acids with nucleic acid bases, and topology. Here we will be concerned with a comparative study of the motional properties of some functional protein–ribonucleic acid systems as elucidated by ^{31}P NMR relaxation experiments. We (Bolton & James, 1979, 1980a,b) and others (Akasaka et al., 1977; Yamada et al., 1978; Tritton & Armitage, 1978; Klevan et al., 1979; Hogan & Jardetzky, 1979; Shindo, 1980; Shindo et al., 1980; Opella et al., 1981; DiVerdi & Opella, 1981; Neumann & Tran-Dinh, 1981) have utilized ^{31}P NMR relaxation to explore some facets of molecular motion in single-stranded and double-stranded nucleic acids. Similar relaxation studies, as well as selective $^{31}P\{^1H\}$ nuclear Overhauser effect (NOE)[1] experiments, have been carried out with messenger ribonucleoprotein (mRNP) complexes and with ribosomes isolated from rat liver, Qβ bacteriophage, and MS2 bacteriophage in the present investigation. The essence of this comparative study is that the nature of the protein–RNA interactions in these natural complexes varies among the complexes and is manifested in ^{31}P NMR relaxation experiments. Some of the experiments indicate variations among the complexes in terms of the extent of the protein–RNA interactions while other experiments imply a modification of the internal mobility of the RNA backbone in the complexes.

Since the ribosome is the site of protein synthesis within cells, there has been much interest in its structure and function (Cox & Bonanou, 1969; Nonomura et al., 1971). Considerable effort has been expended in determining the relative spatial distribution of the proteins within the ribosome as well as the sites of contact between the RNA and protein (Cotter et al., 1967; Lind et al., 1975). The nature of the association between protein and nucleic acid, which is basic to the structure of such a sophisticated organelle as the ribosome, remains a mystery.

The role(s) of RNA in the structure and function of ribosome is (are) mostly conjectural (Erdman et al., 1973), including the putative role of the small 5 S (or 5.8 S, depending on the source of the ribosomes) RNA (Pace et al., 1982). The two large RNA components (18 S and 28 S for eukaryotes and 16 S and 23 S for prokaryotes) comprise about two-thirds of the ribosome by weight, but their distribution and structure

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[1] Abbreviations: NOE, nuclear Overhauser effect; poly(A), poly(adenylic acid); RNP, poly(A+) mRNP, or PmRNP, poly(A) messenger ribonucleoprotein; RNase, ribonuclease; $NaDodSO_4$, sodium dodecyl sulfate; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; $T_{1\rho}^{off}$, rotating frame spin–lattice relaxation time in the presence of an off-resonance radio frequency field; $W_{1/2}$, line width; CSA, chemical shift anisotropy; poly(I), poly(inosinic acid); poly(C), poly(cytidylic acid); rf, radio frequency.

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within the ribosome and their function remain unclear. It is known that exhaustive treatment of ribosomes with pancreatic ribonuclease (RNase) leaves about 70% of the RNA in a ribonucleoprotein complex that has about the same sedimentation properties as intact ribosomes (Cox, 1969; Spencer & Walker, 1971). These residual particles are referred to as limit-digest ribosomes. The association of RNA and protein in the limit-digest ribosomes suggests that these complexes may offer some information about the interaction between protein and RNA in intact ribosomes.

In recent years, it has become clear that messenger RNA exists in the cytoplasm associated with specific proteins in ribonucleoprotein (mRNP) complexes (Lewin, 1980). An early problem associated with the credibility of these complexes was that RNP complexes can be artifacts formed by adding RNA to cell extracts (Baltimore & Huang, 1970). However, such artifact complexes are unstable at high ionic strength (above 150 mM) and are less stable if formed at 0–4 °C (Spirin, 1972). Authentic cytoplasmic mRNP complexes are therefore isolated at 0–4 °C in high ionic strength media. Most isolation techniques also utilize binding of poly(A) tracts of mRNA to oligo(dT)-cellulose (Lindberg & Sundquist, 1974). The protein components of poly(A+) mRNP isolated from many sources show a moderate degree of homogeneity [see Greenberg (1975), Jain et al. (1979), and Lewin (1980)], further suggesting that the complexes are not artifacts.

The mRNP complexes represent something of an enigma. In eukaryotes the mRNA is packaged in mRNP almost immediately after transcription. It seems likely that the packaging protects the mRNA from nucleases and allows transport through the nuclear membrane. Additional functions of the packaging are unknown (Lewin, 1980).

On a more basic level, little is known about the protein–RNA interaction in messenger RNP particles. Greenberg (1977) noted that mRNP, sedimented through CsCl gradients, retained their protein components without prior fixation, whereas ribosomes dissociated: this indicates that different types of interactions may exist in these two entities.

For further comparison, we have chosen to examine two icosahedral bacteriophages, Qβ and MS2, which contain single-stranded RNA. These two bacteriophages have a diameter of about 250 Å; the Qβ phage has a particle weight of 4.2×10^6 daltons containing 1.5×10^6 dalton RNA, and MS2 phage has a particle weight of 3.6×10^6 daltons containing 1.3×10^6 dalton RNA (Boedtker & Gesteland, 1975). The manner of RNA packaging in viruses has been a subject of considerable interest. In fact, Min Jou et al. (1972) have proposed a "flower model" for the structure of the bacteriophage MS2 RNA, comprising some base-paired and some unpaired regions; the structure is supported by partial ribonuclease T1 digestion fragmentation. As pointed out by Kaper (1975), the particular secondary or tertiary structure of the RNA in bacteriophage Qβ is also important for ribosomal binding, translation, and replication.

Materials and Methods

Male, Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, DE) and were starved overnight before use.

Isolation of Ribosomes. Rat livers were homogenized in Teflon–glass homogenizers in 3 volumes of buffer [150 mM RNase-free sucrose, 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2.5 mM KCl, and 5 mM 2-mercaptoethanol]. The homogenate was centrifuged at 14500*g* for 10 min at 4 °C in an SS-34 rotor in a Sorval centrifuge. The supernatant was withdrawn and centrifuged at 144000*g* for 90 min at 4 °C in a 60Ti rotor in a Beckman ultracentrifuge. The pellet was resuspended in the above buffer (20 mL/liver), and $^1/_{10}$ volume of 10% sodium deoxycholate was added. The mixture was rehomogenized, 10 mL of the resulting suspension was layered over 25 mL of the above buffer containing 1.0 M sucrose, and the discontinuous gradient was centrifuged at 144100*g* for 90 min at 4 °C in a 60Ti rotor. The supernatant was discarded, the sides of the tubes were wiped clean, and the pellets containing the ribosomes were stored at −80 °C until use. The NMR samples were prepared by suspending up to 70 mg of the pellet in 2 mL of ribosome buffer [50 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, and 100 mM potassium chloride], lyophilized, and redissolved in D_2O. ^{31}P NMR spectra were obtained with the ribosome samples at 20 °C. After overnight incubation in buffer the ribosomes showed partial degradation apparently by endogenous nucleases (see Results). Digestion of rat-liver ribosomes with pancreatic RNase (at 1 mg/mL) was performed in NMR tubes.

Isolation of Polysomal Poly(A+) mRNP (PmRNP$^+$). PmRNP$^+$ were isolated from rat liver by a method derived from that of Jain et al. (1979). Livers were homogenized in 250 mM sucrose–TKMED buffer [10 mM Tris-HCl (pH 7.6), 250 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM dithiothreitol]. The homogenate was centrifuged at 10000*g* for 10 min at 4 °C. The supernatant was carefully decanted; sodium deoxycholate and Triton X-100 detergents were added to a final concentration of 1% (each). The detergent-containing homogenate was centrifuged at 105000*g* for 1 h at 4 °C in an SW27 rotor. The pelleted polysomes (from six to eight rat livers) were resuspended in TKMED buffer (but containing 500 mM KCl); this polysomal suspension was layered over a sucrose gradient consisting of 10-mL layers of 1.35, 1.6, and 2.0 M sucrose–TKMED buffer (all containing 500 mM KCl), and the gradient was centrifuged at 177000*g* for 16 h at 4 °C in a 60Ti rotor.

The KCl-washed polysomes were then dissociated by resuspension in buffer containing 10 mM Tris-HCl (pH 7.6), 250 mM KCl, and 20 mM EDTA. The dissociated polysomes were dialyzed against 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 400 mM NaCl. One gram of oligo(dT)-cellulose (T_3, Collaborative Research, Waltham, MA) was added to this dialyzed solution, and the mixture was stirred for 5 h at 0 °C. After incubation, the oligo(dT)-cellulose was pelleted by centrifugation at 2000*g* for 10 min, and the oligo(dT)-cellulose was resuspended in low-salt buffer [10 mM Tris-HCl (pH 7.6) and 10 mM NaCl], incubated for 10 min at 4 °C, and centrifuged as before, followed by a second cycle of resuspension and centrifugation. The poly(A+) mRNP was then eluted from the oligo(dT)-cellulose by resuspension in low-salt buffer and incubation at 45 °C for 10 min; the oligo(dT)-cellulose was then pelleted, resuspended, and subjected to a second 10-min incubation at 45 °C (residual mRNP was eluted from the oligo(dT)-cellulose with 70% formamide in low-salt buffer). The mRNP fraction (which eluted at 45 °C) was then centrifuged at 255000*g* for 16 h at 4 °C in a 60Ti rotor.

Characterization of RNA from Ribosomes and PmRNP$^+$ Particles. RNA was characterized after NMR experiments. For RNA extraction, RNP particles were resuspended in buffer [0.3% $NaDodSO_4$, 100 mM NaCl, 50 mM sodium acetate (pH 5.2), and 10 mM EDTA], an equal volume of buffer-saturated phenol (recystallized) was added, and the mixture was shaken at 60 °C for 5 min. The phases were separated by centrifugation, and RNA was precipitated from the aqueous phase by addition of 2 volumes of 70% ethanol.

Table I: ^{31}P NMR Relaxation Parameters for RNA, Messenger Ribonucleoprotein, Ribosome, and Virus Samples

sample	T_1 (s)[e]	NOE[f]	R[g,h]	$T_{1\rho}^{off}$ (s)[e,g]	$W_{1/2}$ (Hz)
rat-liver messenger[a] ribonucleoprotein (40.5 MHz)	0.46[i]	1.5	0.11 (4 kHz) 0.29 0.60 (16 kHz)	0.051 (4 kHz) 0.13 0.28 (16 kHz)	140
rat-liver ribosomes[b] (40.5 MHz)	2.35	1.1	0.33	0.77	80
rat-liver ribosomes[b] (81 MHz)	2.45	1.1	ND	ND	90
E. coli ribosomes[c] (36.4 MHz)	2.3	1.08	ND	ND	208
limit digest of rat liver ribosomes[b] (40.5 MHz)	2.5	1.4	0.4	1.0	60
MS2 virus[b] (81 MHz)	3.1[j] 4.2	1.4[j] 1.15	ND	ND	80
Qβ virus[b] (81 MHz)	3.1[j]	1.4[j]	ND	ND	80
poly(I)·poly(C)[d] (40.5 MHz)	2.0	1.4	0.37	0.75	90
poly(A)[d] (40.5 MHz)	1.54	1.29	0.95	1.46	<10

[a] Samples were prepared as described in the text. Data were obtained at 5 °C. [b] Samples were prepared as described in the text. Spectra were obtained for samples at 20 °C. [c] Data of Tritton & Armitage (1978) for ribosomes in deuterated solvent at 23 °C. [d] Data of Bolton & James (1979) for samples in 0.1 M NaCl and 10 mM cacodylate buffer (deuterium oxide) at pH 7.0 at 20 °C. [e] Accuracy is estimated to be ±0.1 s except as noted. [f] Accuracy is estimated to be ±0.05. [g] Experiment performed with an rf field of strength 0.48 G applied 8.0-kHz off-resonance unless noted otherwise. [h] Accuracy is estimated to be ±0.03. [i] Accuracy is estimated to be ±0.05 s. [j] Sample prepared in H_2O rather than D_2O as with all other samples.

The RNA was examined on 2.5% polyacrylamide [0.125% bis(acrylamide)] gels; electrophoresis was performed at room temperature in $NaDodSO_4$-containing buffer [36 mM Tris-HCl (pH 7.6), 30 mM NaH_2PO_4, 1 mM EDTA, and 0.2% $NaDodSO_4$] for 3 h at 5 mA/tube according to Loening (1967). RNA samples were melted at 70 °C for 5 min prior to application. Gels were scanned for absorbance at 260 nm in a Gilford 240 equipped with linear transport and servograph recorder.

Characterization of Protein from PmRNP$^+$. Proteins were quantitated by the method of Lowry et al. (1951) and were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis on 12% slab gels according to Laemmli (1970). Standards were obtained from Bio-Rad Laboratories ($NaDodSO_4$–polyacrylamide gel electrophoresis standards). Gels were stained for 30 min with 0.2% Coomassie blue in methanol–acetic acid–water (5:1:5) and destained in 7% acetic acid. Slab gels were air-dried on slab gel backing (Bio-Rad Laboratories, Richmond, CA), cut into strips of 0.75-cm width, and scanned at 550 or 600 nm in a Gilford 240. Ribosomal proteins were examined under similar conditions.

Viruses. Bacteriophage MS2 was obtained from Miles Laboratories (code no. 21-791, lot no. 17A and no. 18A). Bacteriophage Qβ was also obtained from Miles Laboratories (code no. 21-794, lot no. 6C).

RNase Precautions. All glassware was autoclaved and acid-washed in sulfuric acid–dichromic acid solution. Solutions were made to 0.1% diethyl pyrocarbonate and subsequently incubated at 90 °C for 1 h to remove diethyl pyrocarbonate.

NMR Relaxation Measurements. ^{31}P NMR spectra were obtained at 40.5 MHz by using a Varian XL-100 equipped with a Nicolet Multiple Observe Nuclei Accessory. Spectra at 81 MHz were obtained by using a Nicolet 200 spectrometer at the Magnetic Resonance Laboratory of the University of California at Davis and a Varian XL-200 at Wesleyan University. The relaxation experiments were carried out as described previously (Bolton & James, 1979, 1980; James et al., 1978). Unless noted otherwise, all samples contained D_2O as the solvent.

For some of the samples, a series of selective $^{31}P\{^1H\}$ nuclear Overhauser effect experiments were carried out as described previously for $^1H\{^1H\}$ NOE (James, 1976). For these experiments a region of the proton NMR spectrum covering about 50–60 Hz was irradiated and the NOE observed for the ^{31}P resonance. This experiment was repeated by using a series of 50-Hz windows in the proton spectrum in order to differentiate the chemical shifts of the protons that were producing the ^{31}P NOE.

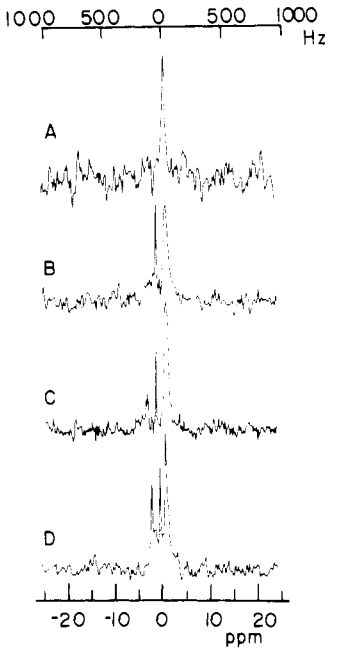

FIGURE 1: 40.5-MHz phosphorus-31 NMR spectra of intact rat-liver ribosomes (A), partially degraded rat-liver ribosomes (B), rat-liver ribosomes that were more extensively degraded than those that gave the spectrum in (B), resulting in spectrum C, and limit-digest ribosomes (D). The samples that gave the spectra in (B) and (C) were degraded by endogenous factor(s). The limit-digest ribosomes were prepared as described in the text.

Results

Ribosomes. Ribosomes were isolated from rat liver as described (Materials and Methods). ^{31}P NMR spectra (Figure 1) and relaxation parameters (Table I) were obtained from intact ribosomes. The spin–lattice relaxation time (T_1) and $^{31}P\{^1H\}$ nuclear Overhauser effect (NOE) results obtained here for rat-liver ribosomes are similar to those obtained with *Escherichia coli* ribosomes in D_2O (Tritton & Armitage, 1978; see Table I). It is noted that the NOE for the *E. coli* ribosomes in H_2O is larger.

Ribosomes resuspended in buffer for long periods (overnight or longer) showed a progressive partial degradation that increased with time. This slow degradation, apparently by endogenous factors, provided ribosomal populations that were

degraded to varying extents (see Figure 1). RNase digestion of intact ribosomes produced limit "core" particles; these core ribosome particles contain most of the ribosomal proteins and have approximately the same sedimentation value as the intact 80S ribosomes, even though 20–30% of the RNA has been digested (Cox, 1969; Spencer & Walker, 1971). Two satellite peaks appear downfield in NMR spectra of degraded ribosomes at 0 and −3 ppm; these can be assigned to oligonucleotides and mononucleotides, respectively. These assignments are supported by the pH dependence of the chemical shift of the peak occurring at −3 ppm at pH 7.5 (Tritton & Armitage, 1978). Further, in partially degraded ribosomal preparations, the oligonucleotide peak was larger than the mononucleotide peak, but decreased in intensity as further degradation occurred, while the mononucleotide peak increased (the sum of the peak intensities did not change). Results obtained with the resonance peak for the partially degraded ribosomes exhibited NMR parameters reflective of RNA that was unrestricted in internal motion but that was tumbling with the ribosomes, and similar results were obtained with the core particles produced by RNase digestion (Table I).

The line width estimated for rat-liver ribosomes on the basis of off-resonance rotating frame spin–lattice relaxation time ($T_{1\rho}^{off}$) data is about 50 Hz; this is in good agreement with the observed line width of 80 Hz if the chemical shift inequivalence of pure RNA is assumed, i.e., 20 Hz at 40.5 MHz (Bolton & James, 1979). This indicates that the dispersion in the chemical shifts of ^{31}P nuclei of ribosomal RNA is less than about 40 Hz. Since the chemical shift of the ^{31}P of nucleic acids is sensitive to diester bond angle (Gorenstein et al., 1976), and since the chemical shift of the ^{31}P peak of intact ribosomes is characteristic of free, helical RNA, the small range of chemical shifts observed (less than 1 ppm) suggests that the majority of the ribosomal RNA is in a conformation closely approximating the A form of RNA.

Characterization of ribosomes was performed after NMR experimentation. The intact ribosomes sedimented at approximately 80 S on sucrose gradients. RNA extracted from 80S ribosomes migrated as 28S and 18S (and a small amounmt of 5.8S and 5S) RNA on 2.5% polyacrylamide gels. The proteins present in the ribosomal preparations exhibited a complex pattern after polyacrylamide gel electrophoresis, with major stainable bands in the low molecular weight range (15 000–25 000) and in the range of 40 000–60 000 (data not shown).

Messenger Ribonucleoprotein. PmRNP$^+$ particles were isolated by a method derived from that of Jain et al. (1979); the major difference was our use of Triton X-100 and deoxycholate detergents in place of heparin (phenylmethanesulfonyl fluoride was generally not used). The PmRNP$^+$ particles we isolated (examined before and after NMR experimentation) had an A_{260}/A_{280} ratio of 1.42 and a protein:RNA ratio of 3:1. These results match characteristics of the postpolysomal poly(A+) mRNP (CmRNP$^+$) particles isolated by Jain et al. (1979) but differ from those of their PmRNP$^+$ particles, which had a much lower protein content. The RNA extracted from PmRNP$^+$ was heterogeneously distributed on polyacrylamide gels near 18 S (Figure 2), and the polypeptides present in our PmRNP$^+$ particles (Figure 3) were quite similar to those found by Jain et al. (1979) for CmRNP$^+$ particles of rat liver. The major proteins have molecular weights of 78 000 and 52 000. We found a protein of molecular weight 128 000 that was not found in the preparations of Jain et al. A similar component has been reported in PmRNP$^+$ preparations by Barrieux et al. (1976) in Ehrlich ascites cells, by Lindberg & Sundquist (1974) in KB cells, by Kish & Pederson (1976) in HeLa cells, and by Cardelli & Pitot (1977) in rat-liver cells.

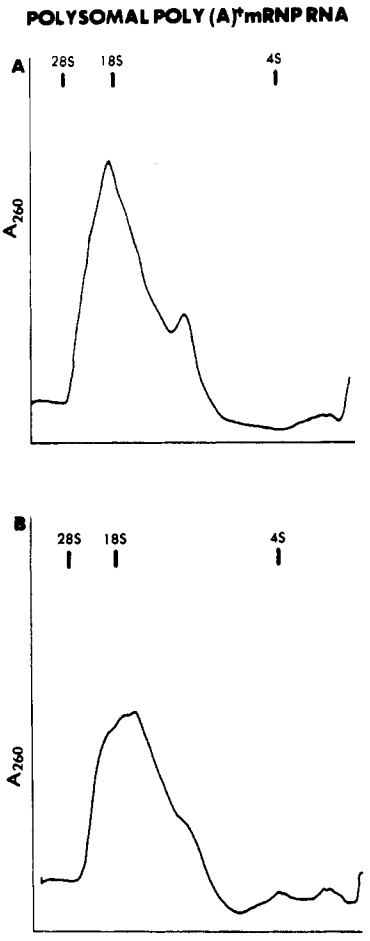

FIGURE 2: Densitometric profiles of mRNP RNA following polyacrylamide gel electrophoresis. Messenger RNP particles were isolated, and RNA was purified as described by Clawson & Smuckler (1980) and examined on 2.5% polyacrylamide tube gels as described by Loening (1967) following heating at 80 °C for 5 min. The gels were then scanned for absorbance at 260 nm by using the Gilford 240 spectrophotometer. (A) and (B) represent two separate preparations.

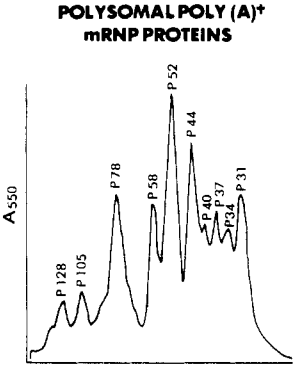

FIGURE 3: Densitometric profile of mRNP proteins following polyacrylamide gel electrophoresis. Messenger RNP particles were isolated as described in the text, and RNP proteins were electrophoresed on 12% polyacrylamide gels as described by Laemmli (1970), after boiling in a water bath for 2 min. Gels were stained with Coomassie brilliant blue dye and were destained by diffusion in 7% acetic acid. The gels were placed on backing sheets, dried, cut into 1 cm wide strips, and scanned at 550 nm with a Gilford 240 spectrophotometer equipped with linear transport and servograph recorder and a 50-μm filter.

The ^{31}P spectrum of the messenger ribonucleoprotein particles (15 mg/mL) in deuterium oxide buffer (0.3% $NaDodSO_4$, 0.1 M NaCl, 0.05 M sodium acetate, and 0.01 M EDTA,

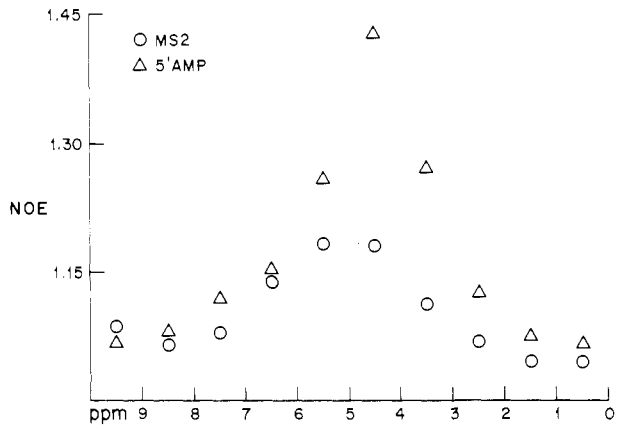

FIGURE 4: The data from selective $^{31}P\{^1H\}$ NOE experiments for 5′-AMP and MS2 virus are shown. The two samples were run by using identical experimental conditions. The proton rf field used had a strength of 175 Hz and was applied to the sample for 10 s to allow buildup of the NOE. The MS2 sample was in a normal water solution containing 10% ^2H_2O as was the 5′-AMP sample. Both experiments were performed on at least three independent samples with reproducible results. The selective $^{31}P\{^1H\}$ NOE of MS2 is too small to be observed in solutions containing 90% or more ^2H_2O. ^{31}P resonance frequency = 81 MHz.

pH 5.2) consists of a single line of width 140 Hz. The ^{31}P relaxation parameters for PmRNP$^+$ were measured and are listed in Table I.

It is apparent that the T_1 value of PmRNP is much lower than that of any other RNA–protein complex; indeed, it is lower than one would expect for a ^{31}P nucleus relaxed by chemical shift anisotropy (CSA) and dipolar coupling solely to protons on the RNA. Consequently, selective $^{31}P\{^1H\}$ nuclear Overhauser effect experiments were carried out to discover the chemical shifts (and therefore identities) of the protons dipolar coupled to the RNA phosphorus nuclei. The selective NOE experiment differs from the usual nonselective NOE experiment, in which all proton resonances are strongly irradiated, by selective irradiation of only a small range (~50 Hz) of proton resonance frequencies. Selective irradiation of the resonances of protons near enough to be dipolar coupled to the phosphorus can cause a change in the ^{31}P resonance intensity. As well as being coupled to the ribose protons resonating at about 4.2 ppm, selective NOE experiments indicated that the ^{31}P nuclei in PmRNP$^+$ are sufficiently close to protons on the protein moiety that protein protons resonating near 1.2 ppm are also coupled to the phosphorus.

Bacteriophage. The ^{31}P spectra of Qβ bacteriophage and MS2 bacteriophage are single-line spectra. The relaxation parameters for these viruses are listed in Table I. The ^{31}P T_1 and NOE values are invariant over the temperature range 22–45 °C. Measurements were made in both H_2O and D_2O solutions for MS2 virus. Selective $^{31}P\{^1H\}$ NOE experiments were carried out for the MS2 virus. The results of the selective NOE experiments for MS2 virus are compared with the results for 5′-AMP in Figure 4. The solutions contained 90% H_2O–10% D_2O to provide for deuterium field-frequency locking on the NMR spectrometer. It is apparent that the three ribose protons that resonate at 4.1 ppm (5′ and 5″) and 4.3 ppm (3′) are coupled to the phosphorus in each case. However, in the case of MS2 virus, additional dipolar interactions with protons resonating downfield (between 5 and 6 ppm) are evident. It is probable that the additional coupling derives from water protons or other exchangeable protons. It is interesting that experimental results show that water protons contribute to the ^{31}P relaxation in nucleosomes (Klevan et al., 1979) and ribosomes (Tritton and Armitage, 1978) but not in pure nucleic acids (Klevan et al., 1979; Bolton & James, 1980a,b).

Discussion

The single-line ^{31}P spectrum obtained for each of the RNA–protein complexes is a composite arising from many individual nuclei. The measured line widths probably reflect chemical shift heterogeneity on the order of 0.4–0.8 ppm (Shindo et al., 1980; Bolton & James, 1979). This chemical shift inequivalence is not large, reflecting similar environments for all the ^{31}P nuclei. The relaxation data were also consistent with the notion that each phosphorus experienced about the same molecular motion as the other phosphorus nuclei in a particular RNA–protein complex.

Although motional properties of the different RNA–protein complexes can be compared qualitatively, we have chosen to describe the motional behavior quantitatively with the same basic motional model we have previously used to describe the motions of different DNAs and RNAs using ^{31}P and ^{13}C NMR (Bolton & James, 1979, 1980a,b). Considerably more complicated and time-consuming calculations, which take into account P–H distance and orientation fluctuations with molecular motion as well as chemical shift anisotropy (CSA) contributions to the ^{31}P relaxation, are in substantial agreement with our earlier correlation time determinations (Keeper & James, 1982).

We have recently presented the mathematical details for the motional model presented here, including contributions from the CSA mechanism to relaxation (Bolton et al., 1981). Briefly, the motional aspects of the model used here entail an isotropic, long-range motion of RNA with correlation time τ_0 that can be ascribed to bending of double-stranded polynucleotides but probably entails molecular tumbling for single-stranded species and protein–RNA complexes. Additionally, internal motions strongly influence ^{31}P relaxation; these can be identified as ribose puckering motions and bond rotations in the phosphodiester moiety of the RNA backbone (Keepers & James, 1982). We will characterize the internal motion with a correlation time τ_i. The more sophisticated models employed in the investigation of Keepers & James (1982) were not used in the present comparative study since (a) the simple model yields similar motional frequenices and (b) computer time would be more efficiently utilized by calculations requiring seconds rather than hours.

The dipolar contribution to ^{31}P relaxation arises from the three (3′, 5′, and 5″) ribose protons that are at an average distance of 2.86 Å (Zhurkin et al., 1978; Keepers & James, 1982) from the phosphorus; the angle for internal rotation was taken as 40° as previously (Bolton & James, 1979, 1980a).

Our analysis of the field dependence of T_1 suggested to us that CSA contributions were small in our earlier work (Bolton & James, 1980a), but a more detailed analysis indicated that CSA contributions to ^{31}P relaxation should be included (Keepers & James, 1982). Calculations for the present study included the CSA contribution to ^{31}P relaxation. The chemical shift anisotropy parameters of $\delta_z = 103$ and $\eta = -0.63$ ppm and the suggested orientation of the CSA tensor in a DNA fiber reported in the publication of Nall et al. (1981) were used in calculations of the CSA contribution to relaxation. The CSA parameters are similar to those reported by Terao et al. (1977), Shindo (1980a), and Opella et al. (1981).

The motional dependence of the spin–lattice relaxation times, T_1, nuclear Overhauser effect, NOE, off-resonance rotating frame spin–lattice relaxation time, $T_{1\rho}^{off}$, and off-resonance intensity ratio, R (which experimentally is the ratio of the intensity of the NMR signal in the presence to that in the absence of an off-resonance rf field), are given in Figure

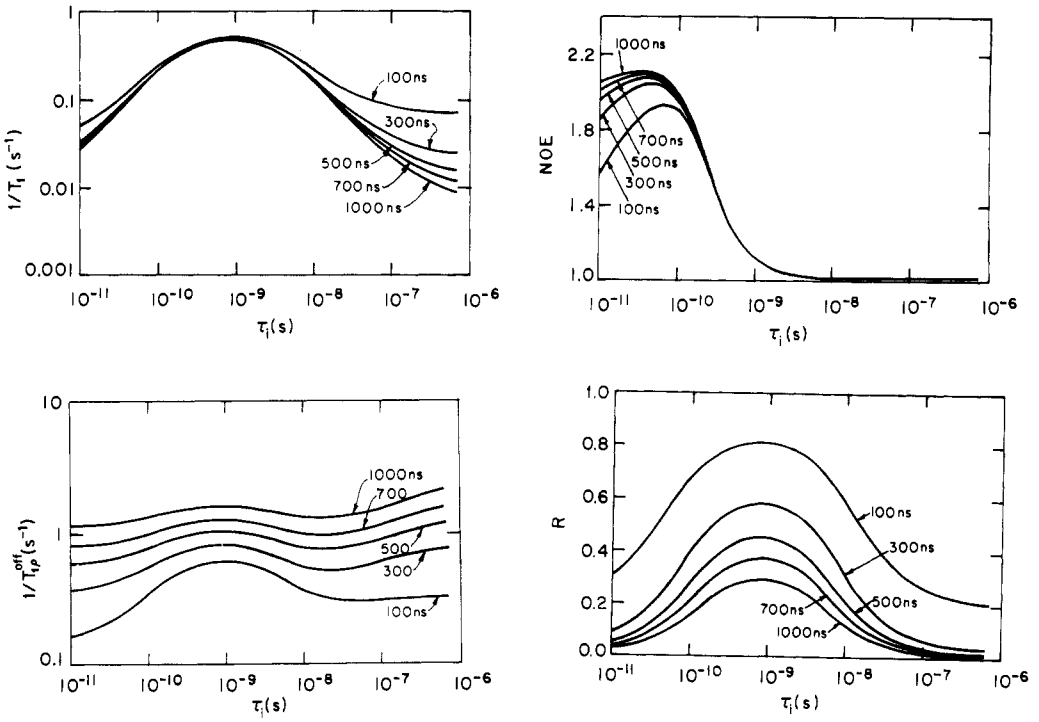

FIGURE 5: Theoretical values of the spin–lattice relaxation rate ($1/T_1$), nonselective nuclear Overhauser effect (NOE), off-resonance rotating frame spin–lattice relaxation rate ($1/T_{1\rho}^{off}$), and off-resonance intensity ratio (R) for ^{31}P at 40.5 MHz. The relaxation parameters were calculated by assuming the two correlation time model presented in the text with an internal motion correlation time τ_i and an isotropic slower motion correlation time τ_o. The calculations include contributions from chemical shift anistropy as well as dipolar contributions from three protons at a distance of 2.86 Å from the phosphorus. The dipolar interaction is modulated by internal rotation with an angle of 40° between P–H vectors and the axis of internal motion. The CSA contributions utilized the following values: chemical shift anisotropy δ_z = 103 ppm, asymmetry parameter η = −0.63 ppm, and Euler angles β = 90° and γ = 0°. The off-resonance NMR relaxation parameters $T_{1\rho}^{off}$ and R were calculated by assuming an rf field of strength of 0.48 G applied 8.0-KHz off-resonance. The various curves were calculated by assuming τ_o values listed next to the curve.

Table II: Correlation Times and Fit of ^{31}P NMR Relaxation Parameters for RNA and RNA–Protein Complexes[a]

sample	τ_o (ns)[b]	τ_i (ns)	T_1 (s) exptl	T_1 (s) calcd	NOE exptl	NOE calcd	$T_{1\rho}^{off}$ (s) exptl	$T_{1\rho}^{off}$ (s) calcd	R exptl	R calcd
ribosomes, 20 °C: 40.5 MHz	700	2.0	2.35	2.35	1.1	1.05	0.77	0.82	0.33	0.35
81 MHz			2.45	2.34	1.1	1.01	ND		ND	
limit digest ribosomes, 20 °C, 40.5 MHz	700	0.42	2.5	2.27	1.4	1.34	1.0	0.82	0.4	0.36
MS2 virus, 20 °C, 81 MHz	700	4.5	4.2	4.2	1.15	1.0	ND		ND	
poly(I)·poly(C), 20 °C, 40.5 MHz	700	0.42	2.0	2.27	1.4	1.34	0.75	0.82	0.37	0.36
poly(A), 20 °C, 40.5 MHz	10	0.42	1.54	1.55	1.29	1.29	1.46	1.52	0.95	0.98

[a] The experimental relaxation data were fit by using the mathematical model described under Discussion. Conditions are as listed in Table I. [b] The calculated T_1 and NOE parameters are negligibly affected by the particular τ_o value chosen as long as τ_o is >100 ns.

5. It is seen that T_1 and NOE are primarily sensitive to the rate of the internal motion. The T_1 shows a weak dependence on the internal motion correlation time in the range of about 0.3–4 ns. The NOE is not informative about internal motions with correlation times longer than about 2 ns except that it can be used to obtain a lower limit on the correlation time. The R value is sensitive to both the internal and overall motion correlation times, and $T_{1\rho}^{off}$ is primarily sensitive to the slowest motion with correlation time τ_o. Taken together the NMR relaxation parameters can typically be used to determine the overall and internal motion correlation times within a factor of 2 by assuming that the model for the motion of the RNA is reasonable.

The line width of the ^{31}P peak was not used in the motional analysis of the NMR data. The line width data are considered unreliable for quantitative purposes due to the unknown magnitude of the chemical shift inequivalence of the different phosphorus-31 nuclei of RNA.

Our previously published ^{31}P data (Bolton & James, 1979) for double-stranded poly(I)·poly(C) and single-stranded poly(A) were analyzed with the present model. As shown in Table II, the data for poly(I)·poly(C) indicate τ_o = 700 ns and τ_i = 0.42 ns, i.e., modified slightly from our previously reported values of 1000 ns and 0.5 ns, respectively. For poly(A), the fit in Table II indicates τ_o = 10 ns and τ_i = 0.42 ns compared with our previous report of 10 ns and 0.5 ns, respectively.

Ribosomes. The correlation times determined for the RNA in ribosomes are listed in Table II. The τ_i value quantitates the influence of ribonuclease treatment on the ribosomal RNA, but a qualitative examination of the relaxation data provides nearly as much information. Limit digestion of the rat-liver ribosomes induces a change in the NOE values (cf. Table I). Examination of the dependence of the NOE on the rate of internal motion, as illustrated by the theoretical curve in Figure 5, shows that an increase in the NOE is indicative of an increase in the rate of local motion. Thus, the NMR data

suggest that the RNA that remains undigested and complexed with ribosomal proteins after limit digestion of rat-liver ribosomes exhibits less restricted local motion than observed in intact ribosomes. The correlation time values quantitate the change in internal motion.

Comparison of the correlation times in Table II reveals that the rate of internal motion in limit-digest ribosomes, double-stranded RNA, and single-stranded RNA is about the same. However, the correlation time for the internal motion of RNA in intact ribosomes is about 5 times larger. This implies that the RNA in intact ribosomes is restricted in its local motion relative to free RNA but the RNA of limit-digest ribosomes is not.

An estimate of the percentage of RNA in intact ribosomes that is restricted can be obtained from the NMR relaxation data. The NOE observed for intact rat-liver ribosomes approaches the theoretical minimum. If it is assumed that the RNA is either restricted or free, the simplest two-state model, then no more than about 20% of the ribosomal RNA is in an environment that allows unrestricted motion. It is quite plausible that there exists a range of states with the RNA on the average being restricted by ribosomal proteins and with individual nucleotides units having a range of correlation times.

It is of interest to note that limit digestion of ribosomes leaves a ribonucleoprotein complex with approximately the same sedimentation value as intact ribosomes even though 20–30% of the RNA is digested (Cox, 1969; Spencer & Walker, 1971). This implies that the limit-digest ribosome is a "core" particle containing most of the ribosomal proteins and the undigested RNA from both subunits. The RNA of the core particle exhibits local motion similar to that of free RNA, indicating that limit digestion relieves the strain (due in part to the continuity of the RNA chain) inhibiting local motion in the intact ribosome. We might speculate that the RNA is folded in the ribosome in a manner unlike that in either single- or double-stranded RNA. RNase treatment cleaves the RNA in the ribosomes, thus modifying the RNA "packaging". Alternatively, the nature of the protein–nucleic acid interactions could constrain the local motion in the intact ribosome. Limit digestion with RNase would thus alter the properties of the protein–RNA interactions. Further, this alternative would imply that the nature of the protein–nucleic acid interactions are dependent on the presence of undegraded RNA and not just that portion protected from nuclease digestion. This second alternative leaves us to explain how a limit digest ribosome with 70–80% of the RNA and with the same macroscopic structure as intact ribosome can be formed by disruption of those peculiar protein–RNA interactions which influence τ_i. Our present prejudice is in favor of the former alternative, i.e., that packaging the RNA with the proteins in ribosomes induces strain that inhibits local motion. In addition to the proteins influencing RNA folding in ribosomes, the methylated nucleosides may alter RNA packaging (Fellner et al., 1972). In contrast to the results obtained for ribosomes, it appears that the local motion of DNA in nucleosomes is not restricted relative to the free DNA (Kallenbach et al., 1978; Klevan et al., 1979; Feigon & Kearns, 1979). A basis for such a difference may be suggested to arise from the formation of hydrogen bonds between the cationic groups of amino acids with both the 2′-OH and phosphate groups of RNA (Bolton & Kearns, 1978, 1979). A 2′-OH interaction cannot occur with DNA.

PmRNP$^+$ Particles. An additional variation in the properties of RNA in a protein–RNA complex is provided by the messenger ribonucleoprotein particles from rat liver. The very low ^{31}P T_1 value of PmRNP compared with the others listed in Table I was indicative of additional dipolar coupling of the phosphorus to protons; ^{31}P{^{1}H} NOE experiments with selective proton irradiation revealed that the additional dipolar relaxation was due to coupling to protons on the proteins (see Results). The character of the protein–RNA interaction in PmRNP is therefore substantially different from that found in the other protein–RNA complexes. The r^{-6} distance dependence of the phosphorus–proton dipolar interaction indicates that a substantial fraction of the phosphorus nuclei in the RNA is within 2–3 Å of at least one proton on the proteins of PmRNP (James, 1976). With a steady-state NOE experiment such as carried out in the present study, the identification of the responsible protein protons is not possible due to spin diffusion among the protein protons. Transient NOE experiments in the future may provide more detail about the identity of the protons affecting the phosphorus NOE. Earlier selective intermolecular NOE experiments illustrated that the lysine ϵ-CH_2 protons (i.e., adjacent to the ϵ-ammonium) would produce an NOE in an anion bound at the phosphoryl attachment site in the enzyme creatine kinase (James & Cohn, 1974). It is tempting to speculate that the anionic phosphoryls of RNA are likewise interacting with positively charged amino acyl side chains in the proteins of PmRNP; such an interaction could bring the nuclei into sufficiently close proximity to effect an NOE. This is consistent with the observation by Greenberg (1977) that PmRNP retains its protein components while ribosomes dissociate with CsCl density gradient centrifugation. The different character of the protein–RNA interaction in PmRNP may confer additional stability to the complex.

To a first approximation, the nuclear Overhauser effect does not depend on the number of protons or proton–phosphorus internuclear distances. The measured ^{31}P NOE value of 1.5 for PmRNP$^+$ is similar to that of poly(I)·poly(C), suggesting that the internal motions of mRNA have not been restricted by interactions with proteins.

Bacteriophages. The results for the two nearly spherical bacteriophages, MS2 virus and Qβ virus, in H_2O solution are identical (see Table I). The ^{31}P{^{1}H} NOE and T_1 were measured for a sample of MS2 virus in D_2O solution as well (see Table I). These results were used to calculate the τ_o and τ_i values, which are listed in Table II. It should be noted that T_1 and NOE are rather insensitive to τ_o in this motional range. The existence of secondary (and possibly tertiary) structure, as well as protein interactions, may have some effect on the internal motions of RNA in these viruses as compared to those of pure RNA (see Table II). As indicated in Table II, the τ_i value calculated for RNA in MS2 virus is 1 order of magnitude larger than in pure RNA. The correlation time needed to fit the experimental T_1 value for the MS2 virus resulted in a NOE somewhat smaller than the experimental value, however. Although this could be attributed to experimental error, it should be noted that a change in RNA conformation, such that phosphorus–proton distances increase, would give similar results; the T_1 would increase and the NOE would decrease (a greater fraction of the relaxation would be due to the CSA mechanism). The lack of any chemical shift difference, however, argues against any substantial structural variation of the viral RNA compared with free RNA. The unequivocal point, however, is that either the internal motion or the structure or both have been modified for RNA in MS2 virus (and presumably Qβ virus). A definitive delineation must await experiments using other nuclei. The existence of secondary (and possibly tertiary) structure, as well as protein interactions, may have some effect on the internal motions of

RNA in these viruses as compared to those of pure RNA.

As noted under Results, the protons of water (or possibly other protons exchangeable with water) are apparently coupled to the RNA phosphorus of the viruses, ribosomes, and nucleosomes, unlike the case with pure RNA. The reason for the lack of influence with pure RNA was considered by Bolton & James (1980a) and attributed to the rapid exchange ($\sim 10^6$/s) of bound water with bulk water together with the rapid reorientation ($\sim 10^{10}$/s) of the bound water molecules. This situation could be altered with protein–nucleic acid complexes in that specific interactions of the phosphate with the proteins could decrease water exchange and reorientation rates. An even stronger possibility is that protein groups such as lysine NH_3^+ bring protons sufficiently close to the RNA phosphorus to affect its relaxation: (a) the NH_3^+ protons exchange with water so they have the same chemical shift as water protons; (b) the lifetime of the NH_3^+–phosphate interaction should be long enough for the proton–phosphorus coupling to be important; (c) substitution of D_2O for H_2O will effectively eliminate this contribution by formation of $-ND_3^+$, which will contribute only 2% as much as $-NH_3^+$ to ^{31}P relaxation.

The present investigation has shown that ^{31}P NMR relaxation experiments can be used to distinguish different motional properties of functional protein–nucleic acid complexes as well as to demonstrate that different types of interactions occur between protein and nucleic acid moieties in these complexes. Future studies will attempt to uncover more details of the protein–nucleic acid interactions as well as ascertaining changes in those interactions induced by drugs or other perturbations.

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References

Akasaka, K., Yamada, A., & Hatano, H. (1977) *Bull. Chem. Soc. Jpn. 56*, 2858–2862.

Baltimore, D., & Huang, A. S. (1970) *J. Mol. Biol. 47*, 263–273.

Barrieux, A., Ingraham, H. A., Nystul, S., & Rosenfeld, M. G. (1976) *Biochemistry 15*, 3523–3528.

Boedtker, H., & Gesteland, R. F. (1975) in *The RNA Phages*, pp 1–28, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Bolton, P. H., & Kearns, D. R. (1978) *Nucleic Acids Res. 5*, 1315–1324.

Bolton, P. H., & James, T. L. (1979) *J. Phys. Chem. 83*, 3359–3366.

Bolton, P. H., & Kearns, D. R. (1979) *J. Am. Chem. Soc. 101*, 479–484.

Bolton, P. H., & James, T. L. (1980a) *J. Am. Chem. Soc. 102*, 25–31.

Bolton, P. H., & James, T. L. (1980b) *Biochemistry 19*, 1388–1392.

Bolton, P. H., Mirau, P. A., Shafer, R. H., & James, T. L. (1981) *Biopolymers 20*, 435–449.

Cardelli, P. J., & Pitot, H. C. (1977) *Biochemistry 16*, 5127–5134.

Clawson, G., & Smuckler, E. A. (1980) *Biochem. Biophys. Res. Commun. 96*, 812–816.

Cotter, R. I., McPhie, P., & Gratzer, W. B. (1967) *Nature (London) 216*, 864–868.

Cox, R. (1969) *Biochem. J. 114*, 753–767.

Cox, R., & Bonanou, S. (1969) *Biochem. J. 114*, 769–774.

DiVerdi, J. A., & Opella, S. J. (1981) *Biochemistry 20*, 280–284.

Erdman, V. A., Sprinzl, M., & Pongs, O. (1973) *Biochem. Biophys. Res. Commun. 54*, 942–948.

Feigon, J., & Kearns, D. R. (1979) *Nucleic Acids Res. 6*, 2327–2337.

Fellner, P., Ehresmann, C., Stiegler, P., & Ebel, J.-P. (1972) *Nature (London), New Biol. 239*, 1–5.

Gorenstein, D. G., Findlay, J. B., Mommii, R. K., Luxon, B. A., & Kar, D. (1976) *Biochemistry 15*, 3796–3803.

Greenberg, J. R. (1975) *J. Cell Biol. 64*, 269–288.

Greenberg, J. R. (1977) *J. Mol. Biol. 108*, 403–416.

Hogan, M. E., & Jardetzky, O. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6341–6345.

Jain, S. K., Pluskal, M. G., & Sarkar, S. (1979) *FEBS Lett. 97*, 84–90.

James, T. L. (1976) *Biochemistry 15*, 4724–4730.

James, T. L., & Cohn, M. (1974) *J. Biol. Chem. 249*, 2599–2604.

James, T. L., Matson, G. B., & Kuntz, I. D. (1978) *J. Am. Chem. Soc. 100*, 3590–3594.

Kallenbach, N. R., Appelby, D. W., & Bradley, C. H. (1978) *Nature (London) 272*, 134–138.

Kaper, J. N. (1975) in *The Chemical Basis of Virus Structure, Dissociation and Reassembly*, pp 218–221, North-Holland Publishing Co., Amsterdam.

Keepers, J. W., & James, T. L. (1982) *J. Am. Chem. Soc. 104*, 929–939.

Kish, V. M., & Pederson, T. (1976) *J. Biol. Chem. 251*, 5888–5894.

Klevan, L., Armitage, I. M., & Crothers, D. M. (1979) *Nucleic Acids Res. 6*, 1607–1616.

Laemmli, U. K. (1970) *Nature (London) 222*, 680.

Lewin, B. (1980) in *Gene Expression 2*, 2nd ed., pp 728–760, Wiley-Interscience, New York.

Lind, A., Villams, R., & Saarma, M. (1975) *Eur. J. Biochem. 51*, 529–536.

Lindberg, U., & Sundquist, B. (1974) *J. Mol. Biol. 86*, 451–468.

Loening, U. E. (1967) *Biochem. J. 113*, 131–138.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Min Jou, W., Haegeman, G., Ysebaert, M., & Fiers, W. (1972) *Nature (London) 237*, 82–88.

Nall, B. T., Rothwell, W. P., Waugh, J. S., & Rupprecht, A. (1981) *Biochemistry 20*, 1881–1887.

Neumann, J. M., & Tran-Dinh, S. (1981) *Biopolymers 20*, 89–109.

Nonomura, Y., Blobel, G., & Sabatini, D. (1971) *J. Mol. Biol. 60*, 303–323.

Opella, S. J., Wise, W. B., & DiVerdi, J. A. (1981) *Biochemistry 20*, 284–290.

Pace, B., Matthews, E. A., Johnson, K. D., Cantor, C. R., & Pace, N. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 36–40.

Shindo, H. (1980) *Biopolymers 19*, 509–522.

Shindo, H., McGhee, J. D., & Cohen, J. S. (1980) *Biopolymers 19*, 523–537.

Spencer, M. E., & Walker, I. O. (1971) *Eur. J. Biochem. 19*, 451–456.

Spirin, A. S. (1972) in *The Mechanism of Protein Synthesis and its Regulation*, Vol. 27, pp 515–537, North-Holland Publishing Co., Amsterdam and London.

Terao, T., Matsui, S., & Akasaka, K. (1977) *J. Am. Chem. Soc. 99*, 6136–6138.
Tritton, T. R., & Armitage, I. M. (1978) *Nucleic Acids Res. 5*, 3855–3869.
Yamada, A., Akasaka, K., & Hatano, H. (1978) *Biopolymers 17*, 749–757.
Zhurkin, V. B., Lysov, Y. P., & Ivanov, V. I. (1978) *Biopolymers 17*, 377–412.

Nuclear Overhauser Effect Study of Yeast Aspartate Transfer Ribonucleic Acid†

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ABSTRACT: Nuclear Overhauser effect studies are described for yeast $tRNA^{Asp}$ in 0.1 M NaCl, pH 7.0. A primary aim is to develop a general method for attacking the problem of assignment in transfer ribonucleic acids (tRNAs). Previously, we have demonstrated the utility of the nuclear Overhauser effect (NOE) between protons on adjacent base pairs combined with C8 deuterium substitution, by assigning the imino protons of the dihydrouridine stem and the two reverse-Hoogsteen base pairs T54-A58 and U8-A14. Here, we extend that approach to other parts of the molecule. We also describe several NOE-connected patterns for, e.g., m^5CG and Ψ55 N3H imino protons which may be of general utility. For the first time, a purine-15–pyrimidine-48 base pair (in this case A15-U48) has been assigned. A total of 13 of 25 base pairs from all parts of the molecule and several noninternally bonded imino protons have now been assigned unambiguously. This is a general method for assigning resonances in tRNA and perhaps in all double-stranded nucleic acids. This, and the distance information inherent in NOE measurements, should make NMR more generally applicable to nucleic acids.

Nuclear magnetic resonance is being applied to an increasing variety of problems of biological interest. We are interested in studying the conformation, dynamics, and structure–function relationship of transfer ribonucleic acid (tRNA)[1] by NMR. We have focused our attention on proton NMR because of its sensitivity and the abundance of protons. In the tRNA NMR spectra, the imino protons resonate considerably downfield from the rest of the protons and form a relatively uncluttered set of lines [reviewed by Schimmel & Redfield (1980) and Reid (1981)]. Observable imino protons are from guanosines and uridines, for which the exchange with the solvent has been slowed down by internal hydrogen bonding or inaccessibility to solvent. Thus, at physiological temperature and solution conditions, most of the imino protons, which are part of the secondary and tertiary structure, contribute resonances in this region. In addition, some imino protons which are not internally hydrogen bonded, but are perhaps partially shielded from solvent, also contribute resonances in the same region. Since each AU and GC pair contributes one resonance to this region, and each GU contributes two, the imino proton spectra collectively reflect contributions from most parts of the molecule. Also, the exchange rates of the imino protons with water are important indicators of the segmental mobility of the molecule in solution and an aid to studies of the effect of different physical parameters on the conformation of the molecule (Johnston & Redfield, 1981b). Despite increased attention and a number of attempts to understand the spectra on the basis of chemical shift theories and fragment studies, assignments of the imino proton spectra remained disputed. The conclusions of earlier papers have been challenged, and serious doubts have been raised about the validity of such procedures in tRNA (Sánchez et al., 1980; Roy & Redfield, 1981).

The nuclear Overhauser effect (NOE) can be viewed as transfer of saturation to a proton nearby in space [see, for example, Johnston & Redfield (1981a)]. The magnitude of transfer is inversely proportional to the sixth power of the distance between the protons and becomes too small to observe beyond 4 Å in most situations. Thus, NOE gives us an important avenue to find the spatial relationship between protons.

NOE is relatively useless unless pairs of resonances it connects can be identified on the basis of uniqueness, chemical modification, isotope labeling, or previously identified NOEs. The first such marker in tRNA was the GU pair (Johnston & Redfield, 1978). GU pairs contain two imino protons in close proximity and hence show a relatively large and unique transfer of saturation pattern between these exchangeable protons. The study of GU pairs has been extended to other tRNAs.

Many other markers were subsequently developed. There are relatively few methyl groups in tRNA, and many of them can be assigned by comparison between tRNAs. Thus, NOE from identified methyl groups to nearby imino protons can identify some important resonances (Tropp & Redfield, 1981; Johnston & Redfield, 1981a). Each AU pair has a purine aromatic proton near in space to the uridine imino proton, so that AU imino protons characteristically show relatively large and sharp NOEs to the aromatic region (Johnston & Redfield, 1981a). In Watson–Crick type AU pairs, the C2 proton of adenosine is close to the uridine imino proton, whereas in

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[1] Abbreviations: tRNA, transfer ribonucleic acid; NOE, nuclear Overhauser effect; EDTA, ethylenediaminetetraacetic acid; T, ribothymidine; m^5C, 5-methylcytidine; Ψ, pseudouridine; ppm, parts per million; BD, benzoylated diethylaminoethyl.

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