Dagne, E., and Castagnoli, Jr., N. (1972b), *J. Med. Chem. 15,* 356.
DeClerq, M., and Truhaut, R. (1962), *Bull. Soc. Chim. Biol. 44,* 227.
Dietrich, L. S., Friedland, I. M. and Kaplan, L. A. (1958), *J. Biol. Chem. 233,* 964.
Guardiola, A. L., Paretsky, D., and McEwen, W. (1958), *J. Amer. Chem. Soc. 80,* 418.
Hucker, H. B., Gillette, J. R., and Brodie, B. B. (1960), *J. Pharmacol. Exp. Ther. 129,* 94.
Jacobson, K. B., and Kaplan, N. O. (1957), *J. Biophys. Biochem. Cytol. 3,* 31.
Kaplan, N. O. (1955), *Methods Enzymol. 2,* 660.
Kaplan, N. O. (1960), *Enzymes, 2nd Ed. 3,* 105.
Kaplan, N. O., and Ciotti, M. M. (1956), *J. Biol. Chem. 221,* 823.
Kaplan, N. O., Goldin, A., Humphreys, S. R., Ciotti, M. M., and Venditti, J. M. (1954), *Science 120,* 437.
Kaplan, N. O., and Stolzenbach, F. E. (1957), *Methods Enzymol. 3,* 899.
Langone, J. J., Gjika, H. B., and Van Vunakis, H. (1973), *Biochemistry 12,* 5025.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 256.
McIlwain, H. (1950), *Biochem. J. 46,* 612.
McKennis, Jr., H. (1965), *in* Tobacco Alkaloids and Related Compounds, von Euler, U. S., Ed., Oxford, Pergamon Press, p 53.
Papadopoulos, N. M. (1964), *Arch. Biochem. Biophys. 106,* 182.
Papadopoulos, N. M., and Kintzios, J. A. (1963), *J. Pharmacol. Exp. Ther. 140,* 269.
Sarma, R. H., and Mynott, R. J. (1973), *J. Amer. Chem. Soc. 95,* 7470.
Shen, W-C., and Van Vunakis, H. (1974), *Res. Commun. Chem. Path. Pharmacol.* (in press).
Windmueller, H., and Kaplan, N. O. (1962), *Biophys. Biochim. Acta 56,* 388.
Zatman, L. J., Kaplan, N. O., Colowick, S. P., and Ciotti, M. M. (1954), *J. Biol. Chem. 209,* 453.

Properties of RNA in Formamide†

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ABSTRACT: Procedures for the purification of formamide and preparation of RNA solutions are described, and details are given of buffers which function within the required range in this solvent. It is shown that for single-stranded RNA and homopolynucleotide species formamide is an effective denaturant at room temperature, and, as judged by spectroscopic criteria, annihilates both base pairing and single-stranded stacking. Further, though smaller, optical changes are observed on heating these solutions, and considerable changes in the circular dichroism, including, in the case of poly(rA), for example, inversion of the ellipticity, occur when all traces of water are removed. It is shown by proton magnetic resonance that even at a water concentration 10^{-3} that of the formamide there is preferential solvation of RNA by the water. Two-stranded RNA does not melt spontaneously in moist formamide at room temperature, but does so on warming, and the melted chain remains indefinitely stable after cooling; this represents an unusual hysteresis effect. When the formamide is completely dry the double-stranded RNA melts spontaneously on dissolution. Sedimentation studies of RNA species and fractionated poly(riboadenylic acid) samples have been performed in formamide. It is found that to a first approximation all conform to a single sedimentation coefficient-molecular weight relationship, and can therefore within these limits be regarded as conformational homologs in this solvent. This provides justification for the application of empirical methods of molecular weight determination to RNA in formamide solution, in particular polyacrylamide gel electrophoresis.

In searching for solvent conditions which would allow zone electrophoresis of RNA, but in which the chain would be devoid of all secondary structure, we found that polyacrylamide gels could be set and run in pure formamide (Staynov *et al.,* 1972). Work by Ts'o and his coworkers (Helmkamp and Ts'o, 1961, 1962; Ts'o *et al.,* 1962) had shown that DNA and single-stranded RNA were devoid of optically detectable structure in this medium, although at sufficiently high ionic strength (Ts'o *et al.,* 1963) some structure of low thermal stability appeared, which, it was suggested, might be unrelated to that present in the native state in water. There is less certainty about two-stranded, fully base-paired RNA, which is reported to be incompletely melted in formamide at room temperature (Strauss *et al.,* 1968). We have examined the conformation and other relevant properties of RNA and its constituents in formamide under the conditions used in electrophoretic experiments. The results are described below, as also is evidence for a strong preferential interaction of RNA with water.

Materials and Methods

Escherichia coli rRNA was prepared from packed MRE 600 cells by the method of Robinson and Wade (1968). Rabbit reticulocyte rRNA was extracted by the phenol-sodium dodecyl sulfate method from purified ribosomes

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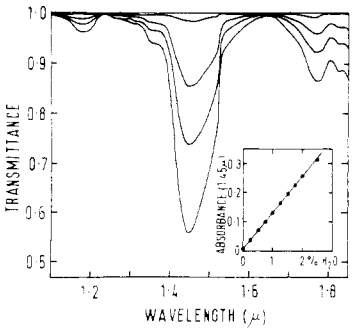

FIGURE 1: Near-infrared spectrum of water in formamide. Spectra from top to bottom correspond to 0, 0.5, 1, and 2% (v/v) added water. Reference cell contains formamide in vapor-phase contact with phosphorus pentoxide. Absorbance at 1.45 μ as a function of the concentration of added water, showing linearity of the calibration.

provided by Dr. H. J. Gould (Kirby and Parrish, 1966). Two-stranded RNA from *Penicillium cyneofulvum* was given to us by Dr. K. Buck. Synthetic polyribonucleotides were bought mainly from Miles Chemical Co. Poly(riboadenylic acid) was fractionated by salt precipitation, following Eisenberg and Felsenfeld (1967), and poly(ribouridylic acid) in the same way (Inners and Felsenfeld, 1970). Molecular weights of the fractions were estimated from their sedimentation coefficients (Pinder and Gratzer, 1974). The formamide was obtained from British Drug Houses Ltd., Eastman-Kodak, and Merck. Noticeably discolored batches were discarded. The criteria for purity appropriate to our purposes are low conductivity, neutral reaction, and high transmission in the ultraviolet. Fractional vacuum distillation and fractional crystallization by freezing were found to offer no improvement in these properties. Instead an ion-exchange method was employed. The purification of the formamide by ion exchange in a mixed bed resin (Monobed MB1 Amberlite) is described in the accompanying paper (Pinder *et al.*, 1974) about its preparation for electrophoresis.

Drying could be accomplished by distilling from a benzene–azeotrope, or by standing over Drierite molecular sieve 3A (British Drug Houses Ltd.), with or without redistillation under reduced pressure, but this procedure proved tedious because the desiccant itself had to be extensively washed with formamide and dried in order to remove ultraviolet absorbing matter extracted by the solvent, and not satisfactorily eliminated in the distillation. Consequently the method adopted was to allow the formamide, buffered as described below, to stand for a sufficient period in a desiccator over phosphorus pentoxide. The water content was estimated by spectrophotometry in the near-infrared using the overtone of the asymmetric stretching frequency at 1.45 μ in a Beckman DK-2A spectrophotometer, equipped with a lead sulfide detector. This falls in a reasonably good "window" in the formamide absorption, and it is therefore possible to make the measurements in a 1-cm Infrasil silica cell. For the reference an extensively dried formamide sample was used in a cell equipped with a standard taper into which was fitted a small side-arm tube containing phosphorus pentoxide. The use of an arrangement of this type for drying the contents of a spectrophotometer cell has been described by Christian *et al.* (1963). With formamide the desiccation process takes very much longer than in their experiments using chloroform. All sampling was done in a drybox. Spectra are shown in Figure 1. Titrations of dry formamide with known volumes of water, dispensed with a microsyringe, show that Beer's law is obeyed within the working range of our experiments.

The dissolution of RNA in very dry formamide is extremely slow, taking several days with continuous stirring. Consequently drying when required was in general carried out after dissolving the RNA, usually in the form of an air-dried ethanol precipitate, in undried formamide. It should be noted that formamide is a hygroscopic solvent, and as received the water content is in general 2–5%, and increases on contact with the atmosphere.

Sedimentation velocities were determined with a Spinco Model E analytical ultracentrifuge, using ultraviolet double-beam scanner optics with a xenon arc as source and a wavelength setting of 280 nm to avoid solvent and buffer absorption. The solvent for sedimentation contained 0.1 M potassium chloride, 0.02 M barbital (apparent pH 9.0), and a fixed content of 1.2% v/v of water. The sample absorbance was not less than 1 at 280 nm. At lower RNA concentrations it was unexpectedly found that the boundaries became unstable. The rather high density and viscosity of formamide necessitated long runs at high speed (60,000 rpm at 20°), and the precision of sedimentation velocity measurements on species of low molecular weight, such as tRNA, was inevitably somewhat low.

Ultraviolet absorption spectra and thermal melting curves were measured in a Beckman DK-2A spectrophotometer, equipped with an electrically heated cell block with a programmed heating unit. The temperature was measured in the cell with a platinum resistance thermometer. Circular dichroism was measured in a Cary 61 instrument, usually in cells of 1-mm path length. The transmission of the formamide allowed measurements down to about 265 nm. The proton magnetic resonance spectra were measured with a Perkin Elmer R10 60-MHz spectrometer, with internal lock. Some solutions were measured after addition of sodium trimethylsilylpropanesulfonate as an internal reference standard.

Potentiometric and spectrophotometric titrations were performed with the aid of a standard glass electrode with calomel reference and an EIL pH meter. The titrant was 1 M aqueous acid or alkali, added with vigorous magnetic stirring. The total dilution with water did not exceed 1%. Since we are not concerned with the ionization constants *per se*, but only with the limits of ionization and buffering ranges, no attempt was made to take account of liquid junction potentials or to calibrate the electrode in terms of proton activities. However, we found that the same potential is recorded whether the calomel reference electrode contains a water or formamide solution of potassium chloride. Assuming constancy of junction potentials, the electrode potential E as measured will be porportional to the pH (Bates, 1973): $E = C - (RT/F) \ln a_{H+}$ where C is a constant, F the Faraday, and a_{H+} the hydrogen ion activity. Titration curves were expressed in terms of observed electrode potentials.

Results

Crude formamide before purification has an alkaline reaction and contains ionic impurities, which are responsible for a high conductivity. Such formamide will not support the polymerization reaction of acrylamide (see accompanying paper). After ion-exchange treatment, the reaction becomes neutral, the conductivity falls to 5 μmho (Figure 2), and the stability is greatly increased. Formamide is relatively unstable, especially in the dry state, and the conduc-

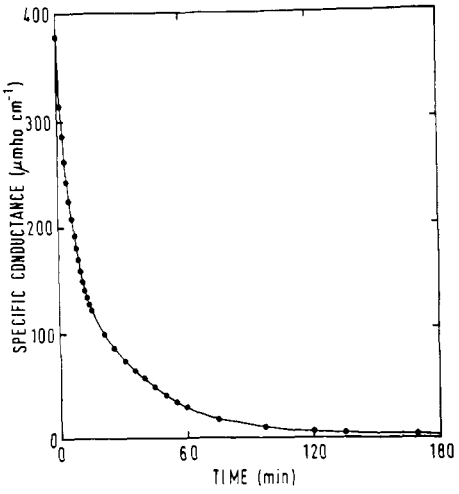

FIGURE 2: Removal of ionic impurities from formamide by mixed-bed ion exchange. The plot shows the rate of diminution of the conductance during treatment with ion exchanger.

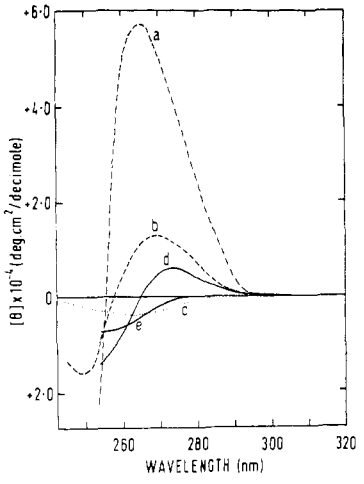

FIGURE 3: Circular dichroism of poly(riboadenylic acid) and 2',3'-adenosine monophosphate in water and formamide: (a) poly(rA) in 0.01 M aqueous Tris, pH 7.6 at 20° and (b) at 80°; (c) AMP in neutral aqueous solution; (d) poly(rA) in formamide at 20° and (e) at 80°.

tivity increases markedly after a few weeks. It is stabilized by buffer. In appreciably acid or alkaline solution deterioration occurs very rapidly, high concentrations of ionic impurities, probably predominantly ammonium formate (Verhoek, 1936), being formed, and a strong smell of ammonia is generated. The solvent, if neutralized, can, however, be used after storage for at least some days at room temperature, and when buffered with barbital for several weeks.

Many inorganic salts are soluble in formamide (Cotton and Brooker, 1958) and the behavior of a number of buffering systems was examined. The relative positions of the apparent pK's (*i.e.*, the midpoints of the potentiometric titration curves expressed in terms of the glass electrode potential) are not the same as those in water. Buffers that function in formamide in the slightly alkaline range are: diethylbarbiturate (apparent pK at 0.575 V, equivalent to a recorded pH of 9.9 in our electrode system), ethanolamine (0.480 V or 8.25), Tris (0.530 V or 9.1), and cacodylate (0.495 V or 8.5), though the use of the last two is not recommended, Tris because the titration curves are irreversible in consequence of chemical instability, and cacodylate because of a tendency to decompose with formation of the malodorous and toxic cacodyl on addition of even very little alkali. In most of the work here described a 0.02 M diethylbarbiturate buffer (apparent pH 9.0) was used. In order to ensure that the nucleic acid bases were not ionized in these conditions, spectrophotometric titrations were conducted of mononucleotides, as well as a polynucleotide, poly(rC), for comparison, and of rRNA. Because of the instability of the formamide away from the neutral region it was not possible to define the ionization curves of the bases, but it was shown that in all cases perceptible ionization (with similar spectroscopic changes to those observed in water) began only well away from the working range. In the mononucleotides small perturbations near neutrality were taken to reflect the secondary phosphate ionization. In *E. coli* rRNA there was no significant spectroscopic change between apparent pH 5 (0.29 V) and 10.5 (0.61 V).

The absorption spectra of the nucleotides in formamide show only minor differences from those in water, as already reported by Ts'o *et al.* (1962). The circular dichroism curves show larger quantitative differences. The effects of formamide on the structure of homoribopolynucleotides are also seen in circular dichroism. Poly(rA) (Figure 3) in formamide containing low proportions of water (the conditions corresponding in practice to electrophoresis experiments, and most other applications of formamide as a solvent) has a very low ellipticity, much lower even than aqueous poly(rA) at high temperature, where there is only small residual single-stranded stacking (Brahms *et al.*, 1966). The ellipticity at the maximum is still positive, however, whereas that of monomeric AMP is negative. When the formamide is heated, however, the ellipticity undergoes a further change to approach that of the monomer.

Poly(rU) is totally unstacked in aqueous solution at room temperature (Simpkins and Richards, 1967; Inners and Felsenfeld, 1970), and the effect of formamide on the circular dichroism is accordingly small. Nevertheless there is a considerable drop in ellipticity on heating, though still not to the value for the mononucleotide. The origin of this effect is unknown, and its existence indicates that the temperature effect observed in poly(rA) in formamide solution does not necessarily imply residual stacking interactions in this solvent. Poly(rC) behaves similarly to poly(rA): formamide causes a larger decrease in circular dichroism but heating engenders a further (reversible) diminution, still not completely to the level of the monomer. Poly(rG) presents a more complex problem, because this polymer forms a highly refractory multistranded structure in aqueous solution (Pochon and Michelson, 1965; Small and Peticolas, 1971), which does not melt at 100°. Wet formamide does not melt this structure, but on heating complete melting ensues. The process is not instantly reversible, since on cooling only a small proportion of the structure returns. The long-wavelength Cotton effect is totally absent in this form, and we observe that it also disappears when the polymer is heated in aqueous solution, though with little change in absorbance. Minor changes in poly(rG) on heating have been reported in the Raman spectrum (Small and Peticolas, 1971), and it therefore seems that transitions occur which involve more than one base-paired form. In the formamide system hysteretic effects are evidently present.

In considering RNA we take first the case of a typical partly base-paired species, *E. coli* rRNA (Figure 4). Form-

[1] Abbreviations used are: poly(rA), poly(riboadenylic acid); poly(rG), poly(riboguanylic acid); poly(rU), poly(ribouridylic acid); poly(rC), poly(ribocytidylic acid).

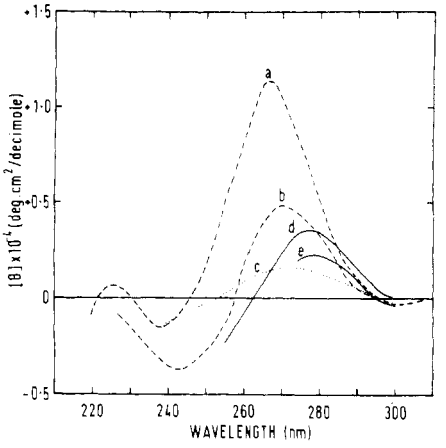

FIGURE 4: Circular dichroism of *E. coli* rRNA in water and formamide: (a) RNA in 0.01 M Tris (pH 7.6) at 20°, and (b) at 70°; (c) mixture of mononucleotides corresponding to base composition of RNA in same solvent; (d) RNA in formamide at 20°, and (e) at 70°.

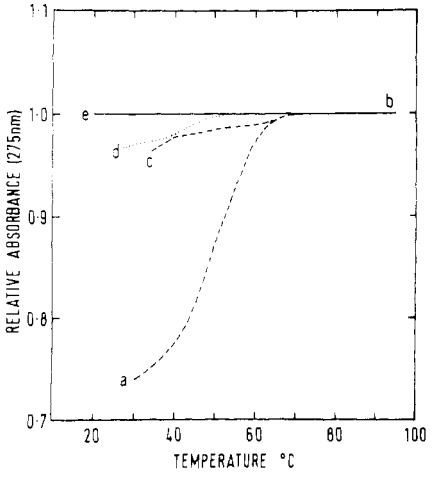

FIGURE 5: Temperature-absorbance profiles (275 nm) in formamide solution containing 0.05 M potassium chloride of *P. cyaneofulvum* double-stranded RNA; heating transition a → b; cooling b → c; reheating c → b; *E. coli* rRNA, heating d → b and cooling, b → d; calf thymus DNA and *E. coli* RNA in the absence of salt show no change with temperature (e → b heating, b → e cooling).

amide causes the ellipticity to drop to a level below that in hot aqueous solution. On heating there is a further, though very small, change. The ellipticity is still higher, however, than that of the sum of the corresponding mononucleotides. The temperature-absorbance profile in formamide in the absence of salt is completely flat over the entire range, and it is safe to conclude that in such RNA species both base pairing and single-stranded stacking are completely annihilated in formamide. Ts'o *et al.* (1963) observed that the addition of potassium chloride at 0.1 M and above generated a small hypochromic effect of doubtful origin, melting out in the 30° region. We see a similar effect in 0.02 M barbital buffer with 0.05 M potassium chloride. We consider next a double-stranded viral RNA. Such molecules have an extremely stable structure and in aqueous solution melt only near 100° (see, *e.g.*, Billeter *et al.*, 1966; Guschlbauer *et al.*, 1968). *P. cyaneofulvum* double-stranded RNA (Banks *et al.*, 1969) at room temperature is not melted in formamide (Figure 5). A sharp melting transition occurs at 50°, and the absorbance neither increases any more on further heating, nor reverses on cooling. There is thus a large hysteresis effect, for the RNA remains in its melted state in-

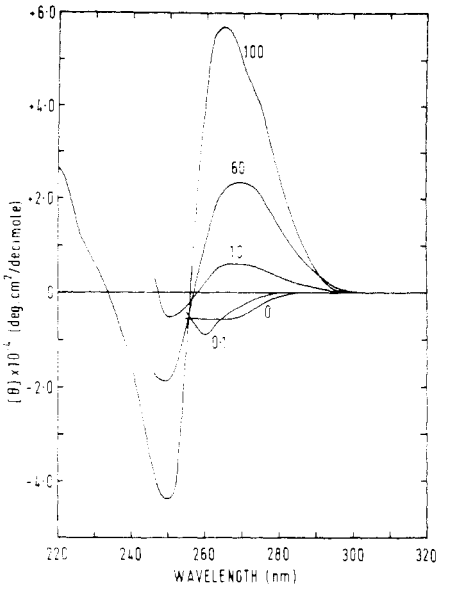

FIGURE 6: Effect of added water on circular dichroism of poly(riboadenylic acid) in formamide solution. Volume percentages of water as indicated.

definitely (or at least for weeks), whereas when returned to aqueous solution by dissolution in aqueous buffer after precipitation from the formamide with ethanol, the native two-stranded state is fully regained, as judged by absorbance and circular dichroism. This RNA may again be transferred, after recovery with ethanol, to formamide, in which it remains two stranded by the same criteria indefinitely at room temperature. The cycle may then be repeated. (Dilution of the formamide solution with water leads to substantial but not complete return of secondary structure.) In practical terms the relevant feature is the stability of the melted form in formamide solution at room temperature. Unlike the two-stranded RNA, DNA (calf thymus) melts spontaneously in formamide at room temperature (Figure 5), and exhibits a flat absorbance-temperature profile.

We have examined in more detail the effect of water concentration on the properties of the polynucleotide chain in formamide. With both RNA and synthetic polynucleotides the conformation changes progressively between the extremes of a partly ordered and a full disordered state. The transition is not a simple smooth function, however, for significant changes occur when minute proportions of water are added to the dry formamide solutions. In the case of poly(rA), for example (Figure 6), an effect can be perceived even when the mole fraction of water is of the order of 10^{-3}, and with progressive increase in water content the ellipticity changes from negative to positive. In poly(rU) and poly(rC) there is little effect at these very low concentrations of water, but in poly(rG) there is a quite striking effect (Figure 7), with a progressive change in the sign of both extrema. With double-stranded RNA, dehydration of the formamide causes the collapse of the native conformation (Figure 8). The ellipticity increases perceptibly at a water concentration in the 0.1% range, and thereafter more rapidly. It is interesting to note that at high proportions of water, where the magnitude of the principal Cotton effect approaches that of the native aqueous state, the small negative Cotton effect at the long-wavelength edge of the absorption band is not yet discernible.

The effect of water at very low levels is unexpected, and carries the implication that there is very strong preferential

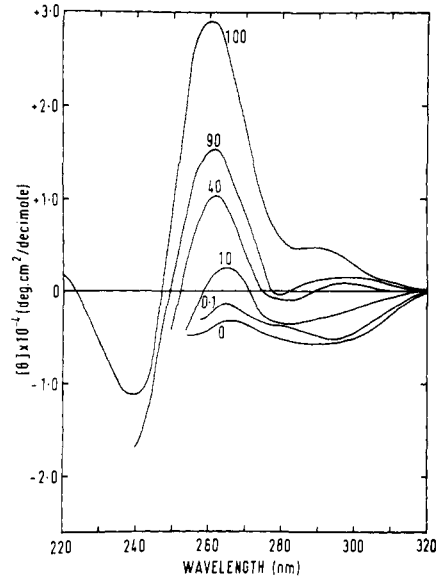

FIGURE 7: Effect of added water on circular dichroism of poly(riboguanylic acid) in formamide solution. Volume percentages of water as indicated.

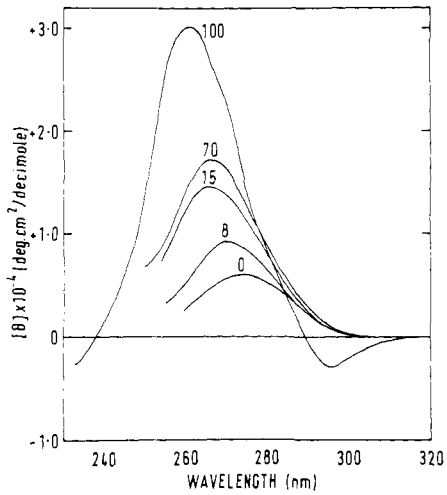

FIGURE 8: Effect of added water on circular dichroism of formamide-denatured *P. cyaneofulvum* double-stranded RNA in formamide solution. Volume percentages of water as indicated. The curve for 100% water corresponds to the original two-stranded RNA, or RNA after precipitation from formamide, redissolved in aqueous buffer.

solvation of polynucleotides with water compared with formamide. The preferential solvation, which is generally defined as $(\partial m_3/\partial m_2)_{\mu_3}$ at $m_2 \rightarrow 0$, where m is the molality of each component in the mixture, the suffix 2 referring to the solute species (RNA), 3 to the added solvent (here water), and μ_3 the corresponding chemical potential, must be as high as about 10^3. To demonstrate that binding of the water to RNA indeed occurs in formamide solution we have examined the effect of added RNA on the line width of the water proton signals in formamide solution. The results are shown in Figure 9. There is a marked difference between the line widths in the presence and absence of water, which reflects the condition

$$\left(\frac{1}{T_2}\right)_{\text{free}} < \left(\frac{1}{T_2}\right)_{\text{bound}} < \frac{1}{T_{\text{exch}}}$$

where the $(1/T_2)$ are the spin–spin relaxation rates for the free and RNA-bound water, and the last term is the exchange rate between free and bound molecules.

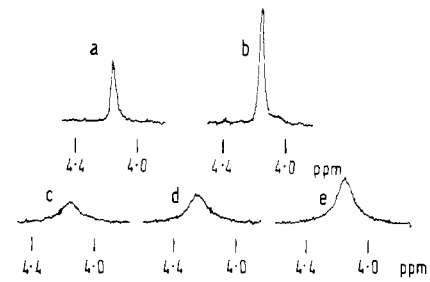

FIGURE 9: Proton magnetic resonance spectra of water in formamide in the absence and presence of RNA (*E. coli* ribosomal): (a) 0.2% and (b) 0.5% in absence of RNA; (c) 0.2%, (d) 0.3%, and (e) 0.5%, all in presence of 5 mg/ml of RNA.

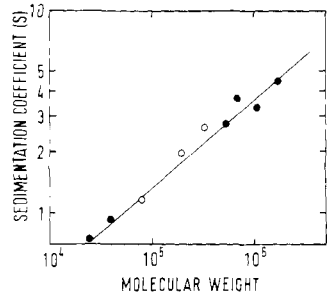

FIGURE 10: Relation between sedimentation coefficient (s^0_{20} uncorrected for solvent) in formamide (0.1 M potassium chloride, 0.02 M barbital containing 1.2% water, apparent pH 9.0) and molecular weight for RNA and poly(riboadenylic acid) fractions.

The relation $1/T_2 = \pi\Delta\nu_{1/2}$ represents the observed time-averaged relaxation rate from the free and bound water in this system. Compared with a relaxation rate of some 4 sec^{-1} for water in formamide in the absence of RNA, water at a concentration of 0.2% (v/v) in an RNA solution of 6 mg/ml gives a relaxation time of about 14 sec^{-1}, which is perceptibly diminished when the concentration is raised to 0.5% at the same RNA concentration. The data bear out the inference of strong binding of water in the presence of a large molar excess of formamide.

The coil characteristics of RNA in formamide will determine the relation between sedimentation coefficient and molecular weights. This relation is shown in Figure 10 for a series of RNA species and poly(rA) fractions. Literature values for the RNA molecular weights have been used, and the assumption, implicitly justified by earlier work (Eigner and Doty, 1965; Boedtker, 1968; Dawid and Chase, 1972), is made that any variation of partial specific volume with base composition can be neglected. It appears from Figure 10 that all RNA species, as well as poly(rA), are conformationally homologous in these solutions. The corresponding equation is

$$s = 0.0089M^{0.43}$$

The value of the exponent may be compared with that of 0.49 found for poly(rU) in Θ conditions in water at high ionic strength (Inners and Felsenfeld, 1970) and 0.43 for the same unstructured polymer in dilute aqueous salt solution (Richards *et al.*, 1963). The latter value is typical for a somewhat expanded coil in a good solvent, which may therefore be the state of RNA in formamide. We are not, however, concerned with the hydrodynamic nature of the polynucleotide chain in this solvent, except to the extent that we wish to be reassured that all structure has been sensibly eliminated, and that there is no significant depen-

dence of the hydrodynamic characteristics of the chain on base composition. The treatment (and the linearity of the plot in Figure 10) imply that the partial specific volume is also not in important degree a function of base composition, at least for the range of compositions of the molecules employed.

Discussion

Formamide can be classed as a weakly basic, essentially aprotic, dipolar solvent. It has a very high dielectric constant of 109.5, and its solutions of salts have high conductivity. (For a summary of properties of ionic solutions in formamide, see Robinson and Stokes, 1969.) In the closely similar solvent, dimethylformamide, it has been shown that ionic conductivities are unaffected by small concentrations of added water (Juillard, 1970). Despite the high dielectric constants of formamide and similar amides—much higher in fact than that of water—deviations from Onsager's limiting law indicate some ion-pair formation by simple electrolytes. There are few studies of the dissociation behavior of weak electrolytes in formamide. Mandel and Decroly (1960), however, have shown that carboxylic acids ionize more weakly in formamide than in water. We have shown here that a number of organic buffer ions give satisfactory buffering in formamide, within a range of hydrogen ion activity in which this solvent is stable, and the nucleic acid bases are not ionized. Assumptions unsupported by experiment, regarding the buffering range of weak electrolytes in formamide, are inadvisable.

Formamide is evidently a good denaturant for nucleic acids, as Ts'o and coworkers have shown (though other amides have been shown to be more effective, when added to aqueous solutions of DNA (Herskovits, 1962, 1963; Levine *et al.*, 1963)). Our results indicate that the formamide not only destroys base pairing, at least when the double helices are not very long (though this reservation does not hold for DNA), but also eliminates nearest neighbor stacking in such single-stranded polymers as poly(rA) and poly(rC). Very stable structures, such as that of poly(rG) and two-stranded RNA, are destroyed only on heating, and at least in the case of the latter the melted state is indefinitely stable after heating. Hysteresis effects in RNA conformations have often been observed and their basis analyzed by Neumann and Katchalsky (1970) and Revzin *et al.* (1973). The cycle which we have described in formamide represents a twofold hysteresis of a type previously observed by Geiduschek and Herskovits (1961) in DNA dissolved in methanol–water mixtures. The work of Elson and Record (1974) on the thermal transitions of phage DNA in formamide–water mixtures reveals that the hysteresis is associated with the strand-separation equilibrium, which follows on the melting of the double helix. Elson and Record infer that renucleation of the two-stranded structure is kinetically forbidden in the formamide–water solution. The stability of the melted form after cooling is fortunate from the viewpoint of electrophoretic analysis of RNA in formamide. We cannot of course be certain that double-stranded species very rich in G-C base pairs would behave in the same manner. Other solvents exist which may be more universal denaturants, notably perhaps dimethyl sulfoxide, which has been reported (Helmkamp and Ts'o, 1961; Herskovits, 1962; Strauss *et al.*, 1968) to be highly effective for nucleic acids.

Despite the elimination of RNA structure in formamide there are further, though generally small, optical changes on heating. It would not be warranted to identify these with elimination of residual stacking. They may rather reflect changes in preferred backbone torsional angles with changes in solvation, for example, and the effects of water at very low concentrations can probably also be explained in some such way. The strong preferential binding of water to the polynucleotide chain in the presence of a massive excess of a solvent of much higher polarity is remarkable. We can find no precedent for such a situation. The absence of chemical shifts in addition to the line broadening of the water proton signals in the nuclear magnetic resonance (nmr) experiments suggests that the mechanism of competitive solvation does not involve strong hydrogen bond formation.

The adherence of all the samples which we have examined (Figure 10) to the same molecular weight–sedimentation coefficient law suggests that to a first approximation all melted polynucleotide chains in formamide may be configurational homologs, with similar values of the coil parameters. The value of the exponent in the logarithmic relation is 0.43, which is lower than the 0.5 expected for a true coil, and found (Inners and Felsenfeld, 1970) for poly(rU) under Θ conditions. An explanation in terms of solvent immobilization might be sought, though light scattering studies would be required to determine the conformational character with certainty. It may be noted that the value of the exponent for formaldehyde-treated RNA is 0.40 (Boedtker, 1968), though this may be affected by stacking, which is absent in formamide solution. The value of the exponent in the latter does not approach the remarkably low value of 0.31 found for RNA in dimethyl sulfoxide solution (Strauss *et al.*, 1968; Dawid and Chase, 1972). The scatter of our data is greater than that shown in the last-mentioned paper, we assume because of the high density and viscosity of formamide, in consequence of which sedimentation experiments take a long time, and this leads to diffuse boundaries. We cannot be certain for instance that poly(rA) adheres to more than a first approximation to the same law as RNA.

We show in the accompanying paper (Pinder *et al.*, 1974) that the characteristics of nucleic acids in formamide, as well as other features to be described, render this solvent very suitable for electrophoretic analyses of RNA, and molecular weight determination from the observed mobilities.

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References

Banks, T. T., Buck, K. W., Chain, E. B., Darbyshire, J. E., and Himmelweit, F. (1969), *Nature (London) 223,* 155.

Bates, R. G. (1973), *in* Determination of pH: theory and practice, New York, N. Y., Wiley.

Billeter, M. A., Weissman, C., and Warner, R. C. (1966), *J. Mol. Biol. 17,* 145.

Boedtker, H. (1968), *J. Mol. Biol. 35,* 61.

Brahms, J., Michelson, A. M., and van Holde, K. E. (1966), *J. Mol. Biol. 15,* 467.

Christian, S. D., Affsprung, H. E., Johnson, J. R., and Worley, J. D. (1963), *J. Chem. Educ. 40,* 419.

Cotton, E., and Brooker, R. E. (1958), *J. Phys. Chem. 62,* 1595.
Dawid, I. B., and Chase, J. W. (1972), *J. Mol. Biol. 63,* 217.
Eigner, J., and Doty, P. (1965), *J. Mol. Biol. 12,* 549.
Eisenberg, H., and Felsenfeld, G. (1967), *J. Mol. Biol. 30,* 17.
Elson, E. L., and Record, M. T. (1974), *Biopolymers 13,* 797.
Geiduschek, E. P., and Herskovits, T. T. (1961), *Arch. Biochem. 95,* 114.
Guschlbauer, W., Courtois, Y., Bové, C., and Bové, J. M. (1968), *Mol. Gen. Genet. 103,* 100.
Helmkamp, G. K., and Ts'o, P. O. P. (1961), *J. Amer. Chem. Soc. 83,* 138.
Helmkamp, G. K., and Ts'o, P. O. P. (1962), *Biochim. Biophys. Acta 55,* 601.
Herskovits, T. T. (1962), *Arch. Biochem. 97,* 474.
Herskovits, T. T. (1963), *Biochemistry 2,* 335.
Inners, L. D., and Felsenfeld, G. (1970), *J. Mol. Biol. 50,* 373.
Juillard, J. (1970), *J. Chim. Phys. Physicochim. Biol. 67,* 691.
Kirby, K. S., and Parrish, J. H. (1966), *Biochim. Biophys. Acta 139,* 554.
Levine, L., Gordon, J. A., and Jencks, W. P. (1963), *Biochemistry 2,* 168.
Mandel, M., and Decroly, P. (1960), *Trans. Faraday Soc. 55,* 29.
Neumann, E., and Katchalsky, A. (1970), *Ber. Bunsenges. Phys. Chem. 74,* 868.
Pinder, J. C., and Gratzer, W. B. (1974), *Biochim. Biophys. Acta, 349,* 47.
Pinder, J. C., Staynov, D. Z., and Gratzer, W. B. (1974), *Biochemistry 13,* 5367.
Pochon, F., and Michelson, A. M. (1965), *Proc. Nat. Acad. Sci. U. S. 53,* 1425.
Revzin, A., Neumann, E., and Katchalsky, A. (1973), *Biopolymers 12,* 2853.
Richards, E. G., Flessel, C. P., and Fresco, J. R. (1963), *Biopolymers 1,* 431.
Robinson, H. K., and Wade, H. E. (1968), *Biochem. J. 106,* 897.
Robinson, R. A., and Stokes, R. H. (1959), *in* Electrolyte Solutions. London, Butterworths.
Simpkins, H., and Richards, E. G., (1967), *Biopolymers 5,* 551.
Small, E. W., and Peticolas, W. L. (1971), *Biopolymers 10,* 1377.
Staynov, D. Z., Pinder, J. C., and Gratzer, W. B. (1972), *Nature (London), New Biol. 235,* 108.
Strauss, J. H., Kelly, R. B., and Sinsheimer, R. (1968), *Biopolymers 6,* 793.
Ts'o, P. O. P., Helmkamp, G. K., and Sander, C. (1962), *Biochim. Biophys. Acta 55,* 584.
Ts'o, P. O. P., Helmkamp, G. K., Sander, C., and Studier, F. W. (1963), *Biochim. Biophys. Acta 76,* 54.
Verhoek, F. H. (1936), *J. Amer. Chem. Soc. 58,* 2577.

Electrophoresis of RNA in Formamide†

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ABSTRACT: The technique of acrylamide gel electrophoresis of nucleic acids in formamide is described. The method affords high resolution, and, within error, electrophoretic mobilities are independent of the base composition over the range of the materials studied. Fractionated poly(riboadenylic acid) and poly(ribouridylic acid) samples show mobilities lying on the same molecular weight–mobility curve as RNAs. It thus appears that polynucleotide chains in general are all effectively homologous in formamide, and this provides the basis for a reliable and convenient empirical method for molecular weight determination on unknown nucleic acids alone or in mixtures. The technique is analogous in scope to sodium dodecyl sulfate gel electrophoresis for proteins. Limitations have been explored and are discussed. Mobility–molecular weight relationships are shown for a series of acrylamide concentrations. Possibilities for the use of other nonaqueous solvent systems have been explored.

Early work in this laboratory (Richards *et al.,* 1965; McPhie *et al.,* 1966) showed that for a series of RNAs the electrophoretic mobility in polyacrylamide gel electrophoresis bore a smooth relation to the molecular weight within reasonable limits, and could therefore be used to provide estimates of the molecular weights of unknown species, singly or in a mixture. The molecular weight range of the method could be extended both upwards (Hadjiolov *et al.,* 1966; Tsanev *et al.,* 1969; Loening, 1967; Bishop *et al.,* 1967) and downwards (Philippsen and Zachau, 1972) by changing the gel concentration, or using agarose or agarose–acrylamide mixtures at the high-molecular weight end. Subsequently, it became apparent that a mobility–molecular weight calibration based on the readily available bacterial and cytoplasmic eukaryotic rRNA species did not necessarily hold good for all other RNAs (Loening, 1969; Groot *et al.,* 1970; Grivell *et al.,* 1971), and the latter authors showed that by changing the temperature the apparent molecular weights of mitochondrial rRNA based on

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