library and to Maxine Singer and Igor Dawid for helpful discussions during the course of this work.

References

Bedbrook, J. R., Jones, J., O'Dell, M., Thompson, R. D., & Flavell, R. B. (1980) *Cell (Cambridge, Mass.) 19*, 545.
Blattner, F. R., Williams, B. G., Blechl, A. E., Thompson, K. D., Faber, H. E., Furlong, L. A., Grunwald, D. J., Kiefer, D. O., Moore, D. D., Schumm, J. W., Sheldon, E. L., & Smithies, O. (1977) *Science (Washington, D.C.) 196*, 161.
Britten, R. J., Graham, D. E., & Neufeld, B. R. (1974) *Methods Enzymol. 29E*, 363.
Brutlag, D., Carlson, M., Fry, R., & Hsieh, T. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 1137.
Cech, T. R., & Hearst, J. E. (1976) *J. Mol. Biol. 100*, 227.
Craig, S. P., Chaudhari, N., & Steinert, M. (1979) *Biochim. Biophys. Acta 565*, 33.
Crain, W. R., Davidson, E. H., & Britten, R. J. (1976) *Chromosoma 59*, 1.
Davidson, E. H., & Britten, R. J. (1979) *Science (Washington, D.C.) 204*, 1052.
Davidson, E. H., Galau, G. A., Angerer, R. C., & Britten, R. J. (1975) *Chromosoma 51*, 253.
Denhardt, D. J. (1966) *Biochem. Biophys. Res. Commun. 23*, 641.
Donhauer, L., Furlong, C., Gillespie, D., & Kurnit, D. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2129.
Eden, F. C., & Hendrick, J. P. (1978) *Biochemistry 17*, 5838.
Eden, F. C., Graham, D. E., Davidson, E. H., & Britten, R. J. (1977) *Nucleic Acids Res. 4*, 1553.
Eden, F. C., Hendrick, J. P., & Gottlieb, S. S. (1978) *Biochemistry 17*, 5113.
Eden, F. C., Burns, A. T. H., & Goldberger, R. F. (1980) *J. Biol. Chem. 255*, 4843.
Graham, D. E., Neufeld, B. N., Davidson, E. H., & Britten, R. J. (1974) *Cell (Cambridge, Mass.) 1*, 127.
Hinnebusch, A. G., Klotz, L. C., Immergut, E., & Loeblich, A. R., III (1980) *Biochemistry 19*, 1744.
Hudspeth, M. E. S., Timberlake, W. E., & Goldberg, R. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4322.
Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K., & Efstratiadis, A. (1978) *Cell (Cambridge, Mass.) 15*, 687.
Manning, J. E., Schmid, C. W., & Davidson, N. (1975) *Cell (Cambridge, Mass.) 4*, 141.
Marx, K. A., Allen, J. R., & Hearst, J. E. (1976) *Biochim. Biophys. Acta 425*, 129.
Peacock, W. J., Appels, R., Dunsmuir, P., Lohe, A. H., & Gerlach, W. L. (1977) in *International Review of Cell Biology* (Brinkley, B. R., & Perter, K. R., Eds.) p 494, Rockefeller University Press, New York.
Pech, M., Struck, R. E., & Zachau, H. G. (1979) *Cell (Cambridge, Mass.) 18*, 833.
Rigby, P. W. J., Dickmann, M., Rhode, S. C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237.
Rimpau, J., Smith, D., & Flavell, R. (1978) *J. Mol. Biol. 123*, 327.
Rosenberg, H., Singer, M. S., & Rosenberg, M. (1978) *Science (Washington, D.C.) 200*, 394.
Shmookler-Reis, R. J., & Biro, P. A. (1978) *J. Mol. Biol. 121*, 357.
Southern, E. M. (1975) *J. Mol. Biol. 94*, 51.
Wensink, P. C., Tabata, S., & Pachl, C. (1979) *Cell (Cambridge, Mass.) 18*, 1231.

Denaturation and Renaturation of *Penicillium chrysogenum* Mycophage Double-Stranded Ribonucleic Acid in Tetraalkylammonium Salt Solutions†

James G. Wetmur,* William T. Ruyechan,‡ and Richard J. Douthart

ABSTRACT: The base composition dependence of double-stranded ribonucleic acid (RNA) melting was studied by observing the structure and widths of melting transitions for *Penicillium chrysogenum* mycophage RNA as well as differences in melting temperatures of two RNAs of different base composition. Double-stranded RNA melting is independent of base compositions in 3.5 M Et_4NCl and 4.6 M Me_4NCl, where the melting temperatures are 25 and 92 °C, respectively. Double-stranded RNA renaturation rate constants are reported in Et_4NCl solutions. The nucleation rate constant is about 10 times lower than that for double-stranded deoxyribonucleic acid. Analyses of renaturation kinetics results lead to the conclusion that each of the three similar but separable RNA segments of *Penicillium chrysogenum* mycophage is unique.

Melchior & von Hippel (1973) found that aqueous solutions of small tetraalkylammonium ions eliminated base composition effects on the helix–coil transition in deoxyribonucleic acid (DNA). All DNAs tested melted at 63 °C in 2.4 M tetraethylammonium chloride (Et_4NCl) and at 94 °C in 3.2 M tetramethylammonium chloride (Me_4NCl). The width of the observed melting transition in these two solvents is generally less than or equal to 1 °C. Tetraalkylammonium salt solutions, especially Et_4NCl, have been applied to the differentiation of melting temperature effects caused by compositional differences from those attributable to base-pair mismatches (Britten et al., 1978). Thermodynamic parameters involved in DNA melting in Me_4NCl and Et_4NCl and in Et_4NCl binding to native DNA may be found in Klump (1977) and Anderson et al. (1978). Orosz & Wetmur (1977) ex-

† From the Department of Microbiology, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029 (J.G.W. and W.T.R.), and the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46206 (R.J.D.). *Received December 23, 1980.* This work was supported by U.S. Public Health Service Research Grants GM22029 and AI14094 from the National Institutes of Health.

‡ Present address: Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, MD 20014.

0006-2960/81/0420-2999$01.25/0

tended the investigations of Melchior & von Hippel (1973) to include RMe_3NCl and REt_3NCl where R is a linear hydrocarbon chain up to six carbon atoms in length. Different alkyl groups led to different DNA melting temperatures for the same salt concentration. However, the same concentrations of salts affected the removal of the base composition dependence of melting no matter what the alkyl group. These results implied a stereospecific interaction between tetraalkylammonium salts and DNA. The results below suggest the explanation may be more complicated.

Work by Chang et al. (1974) and Wetmur (1975) has shown that under optimum conditions the renaturation rates obtained in 2.4 M Et_4NCl and 3.2 M Me_4NCl are 1.4–1.5 times as rapid as those seen with 0.4 M NaCl. The optimum renaturation temperatures in the presence of the two salts are 45 and 70 °C, respectively. The nucleation rate constant, k_N', for DNA renaturation in 2.4 M Et_4NCl was determined to be 2.25×10^5 L/mol·s. Wetmur (1975) has also shown that for complex eukaryotic DNAs with very long renaturation half-times anionic polymers can be added to Et_4NCl solutions to achieve a marked increase in rate. As indicated in a review concerning the methodology and nomenclature of DNA renaturation reactions (Wetmur, 1976), the low melting and renaturation temperatures, coupled with the lack of compositional dependence and relatively rapid renaturation rate, obtained in 2.4 M Et_4NCl make it an ideal standard solvent for DNA renaturation. No solvent with all of these properties has been reported for ribonucleic acid (RNA).

Double-stranded RNA tends to be much more stable thermally than double-stranded DNA. Kallenbach (1968) lists the melting temperatures of eight double-stranded RNAs from animal virus, plant virus, and bacterial virus sources. All of the melting temperatures were determined in 0.2 M Na^+ solutions, and all are near or above 100 °C. Thus, standard melting and renaturation studies in high salt would be difficult. Burnett et al. (1975) have shown that double-stranded RNA (dsRNA) isolated from *Penicillium chrysogenum* mycophage (PCM) is some 12 °C more thermally stable than DNA of a similar (51% G + C) base composition in dilute $NaClO_4$ solutions. The melting temperature observed at 0.045 M Na^+ was 93 °C. Chan et al. (1976) reported that PCM dsRNA denatured at 60 °C in 6.4 M $NaClO_4$. Again, RNA is more thermally stable than DNA. The melting transitions were extremely broad. $NaClO_4$ appears to affect the base composition dependence of the melting profile in the same manner as found for DNA.

We report below our studies of double-stranded RNA melting and renaturation in Et_4NCl and Me_4NCl. No single salt solution is as ideal for RNA as is 2.4 M Et_4NCl for DNA. However, we have found that is is possible either to eliminate the base composition dependence of melting (confirmed by P. H. von Hippel, unpublished experiments) or to obtain good, reproducible renaturation rates at low renaturation temperatures by using one or another tetraalkylammonium salt solutions.

PCM dsRNA has been studied by Wood & Bozarth (1972), Douthart et al. (1973), Nash et al. (1973), Burnett et al. (1975), and Yazaki & Miura (1977). The RNA is found to consist of three different but quite similar size classes with identical terminal nucleotides for at least two to three residues. The melting transition has a great deal of structure. We have found that this structure is due to base composition heterogeneity within the RNA strands which may be eliminated in a base composition independent solvent. In fact, we have used this heterogeneity, as well as the melting transition width of PCM dsRNA and the difference in melting temperature between PCM dsRNA and double-stranded sendai RNA (Kolakofsky et al., 1974), to determine concentrations of Et_4NCl and Me_4NCl which eliminate the base composition dependence of RNA melting. The base compositions of PCM and sendai RNAs have been determined by Burnett et al. (1975) and Blair & Robinson (1968) to be 50.6% and 46.3% G + C, respectively. Finally, using renaturation kinetics results reported below, we are able to conclude that each of the three RNA segments of PCM RNA is unique.

Materials and Methods

Reagents. Et_4NCl and Me_4NCl were purchased from Eastman and purified by repeated treatment with Norit A (Eastman) and filtration until the absorbance profile of the solution between 350 and 210 nm was less than 0.01 unit of all wavelengths. The molarity of Et_4NCl solutions was often determined by using the relationship between concentration and refractive index obtained by Chang et al. (1974). All other chemicals were reagent grade.

Sendai Virus. Sendai virus was grown in the allantoic cavities of embryonated 10-day-old eggs at 37 °C for 48 h following inoculation with approximately 10^4 egg-infective doses. Allantoic fluid from eggs with viable embryos was pooled and clarified by sedimentation at 5000 rpm for 30 min in a Sorvall GSA rotor. The supernatant was layered into a discontinuous 30–60% (w/w) sucrose gradient and spun for 2 h at 19 000 rpm in a Beckman SW27 rotor. The virus was collected from the interface between the two densities and either dialyzed against or diluted with 0.15 M NaCl, 0.001 M EDTA, and 0.01 M Tris-HCl (pH 7.6). Virus was then pelleted, the supernatant was discarded, and the pellets were stored at −70 °C until use.

Sendai Double-Stranded RNA. The plus and minus strands of sendai virus RNA were extracted by using a modification of the method of Palese & Schulman (1976) which allowed formation of double-stranded RNA. The frozen virus pellets were resuspended in 0.45 mL of a solution containing 0.1 M NaCl, 0.025 M Tris-HCl (pH 7.2), 0.001 M EDTA, and 500 μg/mL proteinase K (E. Merck). The proteinase K solution had been preincubated at 56 °C for 5 min. The pellets were manually dispersed with a stirring bar and incubated at room temperature for 15 min. Sodium dodecyl sulfate was then added to a final concentration of 0.5% (w/v), and the resulting mixture was incubated at 56 °C for 10 min. The reaction was ice quenched, and distilled water, CsCl, and ethidium bromide were added to give a final ethidium bromide concentration of 500 μg/mL and a final density of 1.75 g/mL. The RNA was banded at 40 000 rpm for 48 h at 15 °C. Removal and examination of the centrifuge tubes showed two widely separated bands. Examination by electron microscopy indicated that the upper band contained full-length double-stranded molecules of RNA. The lower band contained primarily single-stranded RNA with some double-stranded material present, presumably due to trapping. It should be noted that the isolation method above is not completely reproducible. Occasionally, most of the double-stranded material was found to be trapped in the lower band. Double-stranded RNA fractions were dialyzed against 2.4 M Et_4NCl, extracted 1:1 with butanol, and redialyzed against fresh Et_4NCl. The RNA was then stored at −70 °C until use.

Penicillium chrysogenum Mycophage (PCM) and Its Double-Stranded RNA. The PCM dsRNA was extracted from mycelium as previously described (Nash et al., 1973). The RNA was dialyzed against 2.4 M Et_4NCl and stored at −70 °C until use.

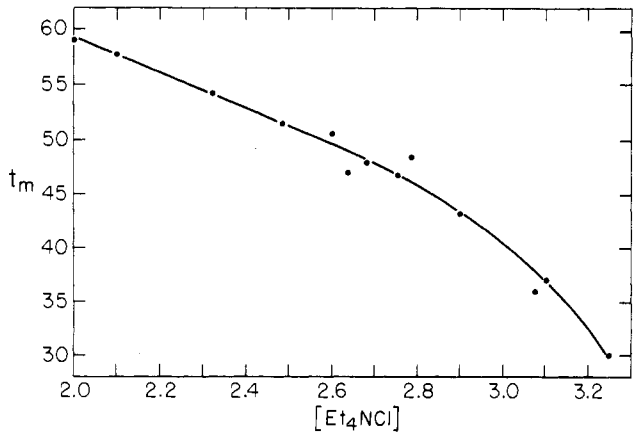

FIGURE 1: Melting temperature, t_m, for *Penicillium chrysogenum* mycophage (PCM) RNA shown as a function of the concentration of Et_4NCl.

Melting of Double-Stranded RNA. RNA samples were dialyzed into the desired buffers in the presence of 0.001 M EDTA. Melting curves of double-stranded RNA were obtained by using either a Gilford 2400 multiple-sample spectrophotometer or a Beckman Model 25 single-sample spectrophotometer. The Gilford 2400 was equipped with a thermostated sample compartment with a thermistor temperature sensor. The Beckman Model 25 spectrophotometer was equipped with a thermostated sample compartment. The temperature was monitored with a YSI Tele-thermometer (Yellow Springs Instruments, Inc.), the temperature probe of which had been fixed to the sample holder. The output of the Tele-thermometer was displayed on a strip chart recorder. Temperatures were regulated with either a recirculating Tamson (Neslab) or a Lauda V bath. Temperature gradients were generated with a Neslab TP2 temperature programmer. The heating rate was generally 0.3 °C/min. The helix–coil transition was monitored at 260 nm throughout the melt.

Renaturation Kinetics. The kinetics of renaturation of double-stranded PCM and sendai RNA were followed in the Beckman spectrophotometer described above. Two recirculating Lauda V baths, one a few degrees above the melting temperature and the other at the renaturation temperature, were used to regulate the temperature. Temperature and absorbance at 260 nm were monitored as described above.

Other Methods. Electron microscopy of the RNAs was carried out by using the modified, high-salt Kleinschmidt technique of Davis et al. (1971). Band velocity sedimentation of double-stranded PCM RNA through 3 M CsCl was carried out in a Beckman Model E analytical ultracentrifuge equipped with ultraviolet optics. Electrophoresis of PCM RNA in 0.7% agarose gels was carried out according to Helling et al. (1974).

Results and Discussion

Double-Stranded RNA Melting in Tetraalkylammonium Salt Solutions. Double-stranded PCM RNA was melted in various Me_4NCl and Et_4NCl solutions. The melting temperatures (t_m) in Me_4NCl were only weakly dependent upon Me_4NCl concentration. t_m values of 93.3, 92.7, and 91.8 °C were found in 3.4, 4.0, and 4.6 M Me_4NCl, respectively. t_m values in Et_4NCl solutions ranging from 2.0 to 3.3 M are shown in Figure 1. Et_4NCl appears to be an even more effective double-stranded RNA denaturant than DNA denaturant.

The melting profile of PCM RNA in 0.02 M Na^+ has been published (Nash et al., 1973). A small hyperchromic transition is seen some 14 °C below the major transition (ΔT_i = 14 °C). Burnett et al. (1975) observed up to three moderately distinguishable transitions in dilute $NaClO_4$ solutions. The last transition showed ΔT_i = 12–14 °C. In both cases, the premelts were completely reversible, indicating that the change observed was intramolecular and probably due to preferential melting of (A + U)-rich regions of the molecules. The transition width, σ, of the main melting transition in 0.02 M Na^+ is 7–8 °C. σ refers to the entire transition and not to the 30–70% portion only. We have investigated ΔT_i and σ for PCM RNA in Me_4NCl and Et_4NCl solutions. The results of these measurements as a function of Et_4NCl concentration are shown in Figures 2 and 3 for ΔT_i and σ, respectively. ΔT_i continuously decreases, approaching 0 at about 3.5 M Et_4NCl. σ also decreases linearly with increasing Et_4NCl concentration until it levels off at about 2 °C. Extrapolation to σ = 0 leads to about 3.5 M Et_4NCl. These results agree with the conclusion that the RNA base composition dependence of melting can be eliminated by using 3.5 M Et_4NCl. Unfortunately, t_m in 3.5 M Et_4NCl, as seen in Figure 1, would be near or below room temperature and is too low for many types of experiments. We have also found that the base composition dependence of RNA melting can be eliminated in Me_4NCl solutions. Extrapolation of ΔT_i and σ values to 0, as shown for Et_4NCl in Figures 2 and 3, leads to 4.6 M Me_4NCl. This concentration is achievable and corresponds to a double-stranded RNA t_m of about 92 °C. Such a high melting temperature is consistent with denaturation and renaturation measurements. A solvent which eliminates the base composition dependence of melting and lowers t_m significantly below 100 °C, such as 2.4 M Et_4NCl for DNA, has not been demonstrated for RNA. Other alkyltrimethylammonium salts (Orosz & Wetmur, 1977) are reasonable candidates.

An independent proof that 3.5 M Et_4NCl is a base composition independent solvent for RNA was obtained by simultaneous melting of PCM and sendai double-stranded RNAs dialyzed into the same solvents. At 2 M Et_4NCl, the melting temperatures differ by 2.3 °C while the base compositions are known to differ by 4.3%. The difference in

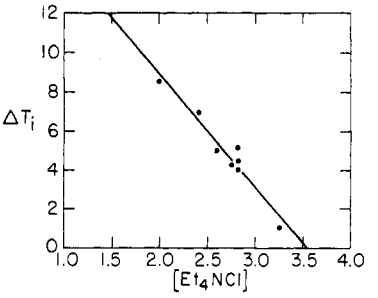

FIGURE 2: Difference between the melting temperatures of the low melting initial hyperchromic transition and the major transition, ΔT_i, for PCM RNA shown as a function of the concentration of Et_4NCl.

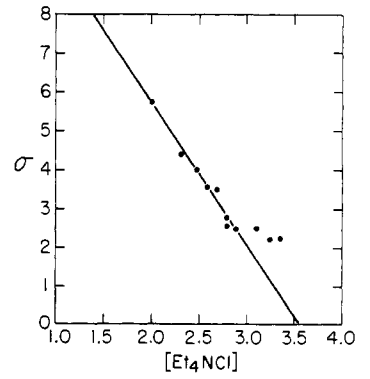

FIGURE 3: Width of the major melting transitions of PCM RNA, σ, shown as a function of the concentration of Et_4NCl.

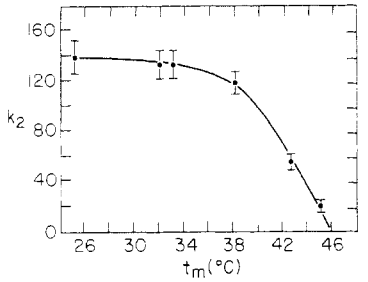

FIGURE 4: Renaturation rate constant, k_2, for a PCM RNA sample in 2.6 M Et_4NCl shown as a function of temperature.

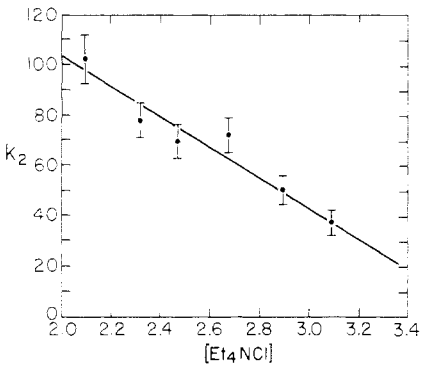

FIGURE 5: Renaturation rate constant, k_2, at 20–25 °C below t_m for a sample of PCM RNA shown as a function of Et_4NCl concentration.

melting temperatures between the RNAs decreased with increasing Et_4NCl concentration. Extrapolation to zero melting temperature difference again led to an Et_4NCl concentration of approximately 3.5 M.

Double-Stranded RNA Renaturation in Et_4NCl Solutions. Although Et_4NCl solutions which completely eliminate the base composition dependence of RNA melting are not very compatible with renaturation, less concentrated Et_4NCl solutions are of interest. A rate constant vs. temperature profile for PCM dsRNA in 2.6 M Et_4NCl is shown in Figure 4. Et_4NCl solutions are stable and optically pure and permit a full range of options for t_m and renaturation temperatures. A rate constant vs. Et_4NCl concentration profile for PCM dsRNA is shown in Figure 5. These results are analogous to those obtained by Chang et al. (1974) for DNA. The difference in k_2 values at 2.6 M Et_4NCl in Figures 4 and 5 can be attributed to length differences. The lengths obtained by electron microscopy after denaturation and renaturation were 0.71 and 0.21 μm for the RNAs used in Figures 4 and 5. Calculation of the change in k_2 due to the decrease in length (L_S), using the equation (Wetmur, 1976) $k_2 = k_N' L_S^{0.5}/N$, yields a value within the experimental error of the k_2 observed.

Complexity of PCM RNA. The PCM and sendai double-stranded RNAs were observed by electron microscopy and found to be 0.71 and 3.3 μm long, respectively. In both cases, the number average length was within 10% of the weight average length. Band velocity sedimentation in 3 M CsCl led to $s_{20,w}$ values of 13.6 and 23.6 S for PCM and sendai double-stranded RNAs. These results are consistent with double-stranded sendai RNA being about 4.5–5 times larger than any of the PCM dsRNA segments, in agreement with Kolakofsky et al. (1974) and Nash et al. (1973). If each of the PCM dsRNA segments were nearly the same and were able to renature with each other, then using the equation above, one would predict k_2 for intact PCM dsRNA would be approximately $(4.8)^{1/2}$ times larger than k_2 for sendai RNA. In fact, k_2 for sendai RNA was 185 under the conditions where k_2 for PCM RNA was 140 L/mol·s. Examination of the renatured products in the electron microscope after renaturation revealed no significant length degradation. Thus, the complexity (N) of PCM RNA must be larger than indicated by the number of base pairs in any one of the segments which may be separated by gel electrophoresis. The simplest explanation of the kinetic results is that each of these segments contains unique information and that the complexity of PCM dsRNA is 3 times its length. We also find that k_N', consistent with the results above for double-stranded RNAs in 2.6 M Et_4NCl, is 2×10^4 L/mol·s, a value approximately 10-fold lower than k_N' for double-stranded DNA in 2.4 M Et_4NCl. This difference in nucleation rate constants for RNA and DNA in tetraalkylammonium salt solutions is larger than, but qualitatively similar to, results with homopolymers in NaCl solutions (Wetmur, 1976).

Acknowledgments

We thank Dr. V. Racaniello for his contributions to initial measurements of double-stranded RNA melting in Et_4NCl. We also thank N. Fauer for technical assistance.

References

Anderson, C. F., Record, M. T., Jr., & Hart, P. A. (1978) *Biophys. Chem. 7*, 301–316.
Blair, C. D., & Robinson, W. S. (1968) *Virology 35*, 537–549.
Britten, R. J., Cetta, A., & Davidson, E. H. (1978) *Cell (Cambridge, Mass.) 15*, 1175–1186.
Burnett, J. P., Frank, B. H., & Douthart, R. J. (1975) *Nucleic Acids Res. 2*, 759–771.
Chan, H.-C., Ruyechan, W. T., & Wetmur, J. G. (1976) *Biochemistry 15*, 5487–5490.
Chang, C.-T., Hain, T. C., Hutton, J. R., & Wetmur, J. G. (1974) *Biopolymers 13*, 1847–1858.
Davis, R. W., Simon, M., & Davidson, N. (1971) *Methods Enzymol. 21*, 413–428.
Douthart, R. J., Burnett, J. P., Beasley, F. W., & Frank, B. H. (1973) *Biochemistry 12*, 214–220.
Helling, R. B., Goodman, H. M., & Boyer, H. W. (1974) *J. Virol. 14*, 1235–1244.
Kallenbach, N. R. (1968) *J. Mol. Biol. 37*, 445–467.
Klump, H. (1977) *Biochim. Biophys. Acta 475*, 605–610.
Kolakosfsky, D., de la Tour, E. B., & Bruschi, A. (1974) *J. Virol. 14*, 33–39.
Melchior, W. B., Jr., & von Hippel, P. H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 298–302.
Nash, C. H., Douthart, R. J., Ellis, L. F., Van Frank, R. M., Burnett, J. P., & Lemke, P. A. (1973) *Can. J. Microbiol. 19*, 97–103.
Orosz, J. M., & Wetmur, J. G. (1977) *Biopolymers 16*, 1183–1199.
Palese, P., & Schulman, J. L. (1976) *J. Virol. 17*, 876–884.
Wetmur, J. G. (1975) *Biopolymers 14*, 2517–2524.
Wetmur, J. G. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 337–361.
Wood, H. A., & Bozarth, R. F. (1972) *Virology 47*, 604–609.
Yazaki, K., & Miura, K.-I. (1977) *Virology 82*, 14–24.