Preparation of the Bifunctional Enzyme Ribonuclease–Deoxyribonuclease by Cross-Linkage†

Dalton Wang

ABSTRACT: Protease-free bovine pancreatic deoxyribonuclease (DNase) (1.6×10^{-4} mmol) was thiolated on the NH_2 groups with *N*-acetyl-DL-homocysteine thiolactone (2.4×10^{-2} mmol) at pH 10.5 with imidazole (2.4×10^{-2} mmol) as the catalyst in the presence of 4,4′-dithiodipyridine (4.2×10^{-2} mmol). The product obtained after 16 h at 4 °C, 2-acetamido-4-(4′-dithiopyridyl)butyryl-DNase, isolated by gel filtration, contained an average of 0.87 ± 0.13 mol of mixed disulfide per mol of DNase. Ribonuclease (RNase) was thiolated in a similar manner, but under N_2 in the absence of 4,4′-dithiodipyridine. The protein *N*-acetylhomocysteinyl-RNase contained on the average 0.94 ± 0.11 mol of sulfhydryl groups per mol of RNase. The coupling of RNase to DNase was accomplished by thiol–disulfide interchange at pH 6.2 and 25 °C for 90 min. The hybrid enzyme (yield 25–33%, based upon the DNase derivative used) was freed from unreacted DNase, RNase, and homodimers by gel filtration, affinity chromatography, and salting-out chromatography. The purified enzyme contained one molecule each of DNase and RNase and hydrolyzed thymus deoxyribonucleic acid (DNA) and yeast or transfer ribonucleic acid (RNA) with 75 and 40% of the efficiencies, respectively, of the parent enzymes. The RNA strand of the hybrid substrate, phage f_1 DNA·[^{3}H]RNA, prepared from phage DNA with RNA polymerase, was hydrolyzed rapidly by the hybrid enzyme but was not hydrolyzed by RNase alone. A conjugate of the two enzymes offers the possibility in vivo of delivering two enzymes that differ in size, charge, and biological function to the same site at the same time.

Interest in cross-linking RNase and DNase has grown from consideration of the chemical and biological properties of nucleases and their substrates. A single intraperitoneal injection of 100 μg of a cross-linked dimer of bovine pancreatic RNase (Wang et al., 1976) in AKR/J mice with advanced leukemia led to ~50% reduction in the enlarged lymph nodes and spleen at 24 h (Tarnowski et al., 1976), and long-term injections of pancreatic DNase have yielded favorable results in leukemic mice (Salganik et al., 1967). In leukemias, lymphomas, and sarcomas in mice (Gallo, 1976) and in man (Baxt et al., 1972; Sarngadharan et al., 1972), the reproduction of type C viruses proceeds via the viral RNA genome and an RNA-dependent DNA polymerase. The chances of interrupting the viral reproductive cycle should be enhanced by the use of an enzyme capable of acting on the RNA and the DNA strands, both alone and in combination.

The mode of synthesis of RNase cross-linked to DNase has been based upon the procedure for introduction of an –S–S– linkage between two unlike proteins (e.g., RNase and bovine serum albumin) described by King et al. (1978), with the modification that *N*-acetylhomocysteine thiolactone was used as the thiolating agent.

The present communication deals with the preparation, purification, and enzymic activity of the bifunctional enzyme. A prerequisite for this research was the availability of a protease-free preparation of pancreatic DNase (Wang & Moore, 1978); since the derivatization experiments take time, it is essential to start with a fully stable preparation of DNase.

Experimental Procedures

Materials. The commercially prepared products used were bovine pancreatic deoxyribonuclease (DNase I, DP grade) and lima bean protease inhibitor (Worthington), Sephadexes G-25, G-50, and G-75 and Sepharose 4B (Pharmacia), agarose–5′-(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate (pUp–Sepharose) and [^{3}H]tRNA (*Escherichia coli* B, 43 $\mu Ci/A_{260}$ unit) (Miles), Bio-Gel A-1.5m (Bio-Rad), *N*-acetyl-DL-homocysteine thiolactone (Pierce), 4,4′-dithiodipyridine (Aldrich), cyanogen bromide and imidazole (Eastman), polyethylene glycol 6000 (MC/B), GTP, ATP, and CTP (P-L Biochemicals), [^{3}H]UTP (New England Nuclear), and calf thymus DNA, cyclic 2′,3′-cytidylic acid, yeast RNA (type VI), bovine pancreatic ribonuclease (type IIA), and RNA polymerase (*E. coli* K 12) (Sigma). Yeast RNA was dialyzed as previously described (Wang et al., 1976). Placental RNase inhibitor was prepared according to Blackburn et al. (1977). The immobilized lima bean protease inhibitor was prepared by coupling the inhibitor to cyanogen bromide activated Sepharose 4B as described by Wang & Moore (1978), and protease-free DNase and protease-free, RNase-free DNase were prepared according to procedures described in that communication. Cultures of *E. coli* TL-K38 and phage f_1 were kindly supplied by Dr. Peter Model of The Rockefeller University.

The DNA of phage f_1 was prepared by a modification of the procedure of Model & Zinder (1974) as follows. Four 1-L flasks, each containing 300 mL of tryptone broth (10 g of tryptone, 1 g of yeast extract, 1 g of glucose, 8 g of NaCl, and water to a liter, pH 6.8–7.0), were inoculated with 5 mL of *E. coli* TL-K38 stock ($A_{300} \sim 3$) and incubated with shaking at 37 °C for ~45 min. The culture in each flask was then inoculated with phage f_1 (0.2 mL of an f_1 phage stock containing 5.5×10^{11} plaque-forming units/mL) to give ~15 phage particles/bacterium. The incubation was continued for an additional 5 h. The bacteria were removed by centrifugation (800 rpm for 15 min). Polyethylene glycol and NaCl were added to the supernatant to give final concentrations of 3% and 0.5 M, respectively. The mixture was left in a refrigerator overnight and then centrifuged at 8000 rpm for 15 min. The pellet was dissolved in 0.9% NaCl (~16 mL). The centrifugation was repeated (for 10 min). The phage in the supernatant was purified by CsCl density gradient centrifugation (Yamamoto et al., 1970). The purified phage (~4

† From The Rockefeller University, New York, New York 10021.
Received April 23, 1979; revised manuscript received July 30, 1979. The research was supported in part by U.S. Public Health Service Grant GM 07256. A preliminary report has appeared (Wang, 1979).

0006-2960/79/0418-4449$01.00/0

mL) was dialyzed, with three changes, against 4 L of 0.8% NaCl and 20 mM Tris-HCl, pH 8.0. The dialyzed phage solution was diluted with the same buffer (33.8 mg of phage per 10.5 mL). The phage was deproteinized by extraction with phenol (1:1 v/v) at pH 8.15. The resulting f_1 DNA was dialyzed against 4 L of buffer (10 mM in Tris-HCl and 0.1 mM in EDTA, pH 8.0) with two changes (yield 4.76 mg of f_1 DNA). The f_1 DNA was precipitated by the addition of 3 volumes of cold ethanol. The hybrid 1:1 f_1 DNA·[^{3}H]RNA was synthesized with RNA polymerase according to the procedure of Stavrianopoulos et al. (1972).

Sodium Dodecyl Sulfate (NaDodSO$_4$) Disc Gel Electrophoresis. Nonreductive NaDodSO$_4$ disc gel electrophoresis was performed according to the procedures described by Weber & Osborn (1969).

Assay for DNase. The activity was measured toward calf thymus DNA by the hyperchromicity assay of Kunitz (1950) as modified by Liao (1974); 1 unit of activity causes an increase in absorbance at 260 nm of 1.0 min^{-1} cm^{-1}. DNA solution was prepared according to Wang & Moore (1978).

Assay for RNase. The activity toward yeast RNA was determined by the spectrophotometric assay of Kunitz (1946). The activity toward cyclic 2′,3′-cytidylic acid was determined by the method of Crook et al. (1960) and del Rosario & Hammes (1969) under the conditions defined by Lin (1970). The activity of RNase during the purification of the bifunctional enzyme RNase–DNase was monitored spectrophotometrically at 265 nm with poly(rC) as substrate [cf. Wang & Moore (1977)]. Measurements of the RNase activity of the bifunctional enzyme were made with [^{3}H]tRNA or f_1 DNA·[^{3}H]RNA hybrid as follows. The assay mixture consisted of 60 μL of buffer (0.1 M Tris-HCl, pH 7.5), 20 μL of substrate solution (~1 μg), 10 μL of $MnCl_2$ (0.1 M), and 10 μL of enzyme solution. The above reaction mixture, less enzyme, was held at 37 °C for 5 min before the reaction was initiated by the addition of enzyme. After 30 min at the same temperature, the digestion was terminated by the addition of 100 μL of bovine serum albumin (10 mg/mL) and 600 μL of cold trichloroacetic acid (12%). After 20 min on ice, the tubes were centrifuged for 30 min at 4 °C in a clinical centrifuge (~1000*g*). A 500-μL aliquot from each sample was mixed with 10 mL of Aquasol (New England Nuclear) for radioactivity measurement. Blanks were run without enzyme. The assay for the RNase activity is linear in the range of 0–50 ng of the bifunctional enzyme with ~1 μg of f_1 DNA·[^{3}H]RNA in the digestion mixture. When the inhibitory effect of placental RNase inhibitor on the hybrid enzyme was determined, the reaction was initiated by the addition of substrate instead of the enzyme.

Determination of SH Groups. The number of sulfhydryl groups introduced by thiolation was determined by the amount of 4-thiopyridone formed according to the method of Grassetti & Murray (1967) using a molar extinction coefficient of 1.98×10^4 at 324 nm.

Thiolation of RNase. In order to establish the optimum conditions for the thiolation of the NH_2 groups of RNase with *N*-acetyl-DL-homocysteine thiolactone, I studied the variables (such as pH and concentration of various reactants) in terms of the yield of a product which contained ~1 mol of sulfhydryl groups per mol of RNase. The following conditions were established. RNase (1.46×10^{-3} mmol), *N*-acetylhomocysteine thiolactone (2.13×10^{-2} mmol; Benesch & Benesch, 1958), and imidazole (2.42×10^{-2} mmol; White, 1972) were placed in a 15-mL Corex centrifuge tube which was chilled on ice. One milliliter of buffer (0.1 M in phosphate and 5 mM in EDTA, pH 10.5) which had been bubbled with N_2 for 5 min was delivered into the tube to dissolve the reactants. The reaction tube was flushed with N_2 for 5 min, sealed with Parafilm, and placed in a refrigerator to allow the reaction to proceed for 16 h. The reaction was terminated by the addition of 1.0 mL of acetic acid (0.2 M), and the products were gel filtered through a Sephadex G-75 column (0.9 × 160 cm) equilibrated with 0.1 M acetic acid. Fractions (1.16 mL) were collected, and the peak from the monomeric enzyme was located between 84 and 99 mL (yield 62%). The average number of thiol groups (for six preparations) was 0.94 ± 0.11 mol/mol of RNase. The enzyme in solution, which had 90% of the activity of the starting enzyme toward yeast RNA, could be stored in the frozen state without appreciable loss of thiol groups (less than 10% loss in 2 months). The material oxidized rapidly, however, when the lyophilized solution was stored at ~20 °C.

Preparation of 2-Acetamido-4-(4′-dithiopyridyl)butyryl-DNase. The optimum conditions for the formation of the mixed disulfide, enzyme–S–S–pyridine, were first established with RNase as the model protein. In a typical experiment, the thiolation of DNase with *N*-acetyl-DL-homocysteine thiolactone in the presence of 4,4′-dithiodipyridine was performed as follows. Protease-free DNase (3.23×10^{-4} mmol in 1 mL) as previously prepared (Wang & Moore, 1978) was first dialyzed against 2 L of buffer (0.1 M in triethanolamine, 10 mM in $CaCl_2$, and 10% in glycerol, pH 10.5) for 24 h at 4 °C. The enzyme solution was quantitatively transferred with 1 mL of wash buffer into a test tube (13 × 100 mm) which contained *N*-acetylhomocysteine thiolactone (4.16×10^{-2} mmol) and imidazole (4.83×10^{-2} mmol). Dithiodipyridine (8.32×10^{-2} mmol) in 200 μL of acetonitrile was added. The reaction was allowed to proceed for 15 h at 4 °C. The reaction was terminated by passing the solution through a column (0.9 × 65 cm) of Sephadex G-25 (coarse) equilibrated with 0.01 M HOAc, 10 mM $CaCl_2$, and 10% glycerol. The effluent fractions (1.3 mL) that contained the protein (no. 10–19) were pooled. The yield was 75% of DNase based on enzyme activity. The product (average for five preparations) contained 0.87 ± 0.13 mol of mixed disulfide per mol of DNase. The derivative, which had 95% of the activity of the parent enzyme toward thymus DNA, could be stored at −20 °C either in solution or in the lyophilized form without appreciable loss of the mixed disulfide (measured by assay for 4-thiopyridone after adding a mercaptan).

Preparation of the Bifunctional Enzyme by Thiol–Disulfide Interchange. The coupling of RNase–SH[1] to DNase–S–S–pyridine was accomplished by thiol–disulfide interchange at pH 6.2. A molar ratio of RNase–SH to DNase–S–S–pyridine of ~4 was necessary to achieve a maximum yield (25% of the DNase used) of the hybrid enzyme. A typical coupling experiment was performed as follows. DNase–S–S–pyridine (9.56×10^{-2} mM) in a buffer of 0.1 M Tris–OAc, pH 6.6, 10 mM $CaCl_2$, and 10% glycerol was mixed with RNase–SH (4.6×10^{-1} mM) in 0.01 M acetic acid and 1 mM EDTA. The reaction mixture was titrated to approximately pH 6.2 with 0.1 N NaOH. The interchange reaction was allowed to proceed at 25 °C for 90 min. The reaction mixture was added to a Sephadex G-75 column (0.9 × 160 cm) equilibrated with 10% glycerol and 10 mM $CaCl_2$. The elution pattern and the enzymic activities are shown in Figure 1. Fractions which

[1] Abbreviations used: LBI, lima bean inhibitor of trypsin and chymotrypsin; RNase–SH, ribonuclease thiolated by reaction with *N*-acetylhomocysteine thiolactone; DNase–S–S–pyridine, thiolated DNase with the –SH group blocked by –S–S formation with thiopyridine.

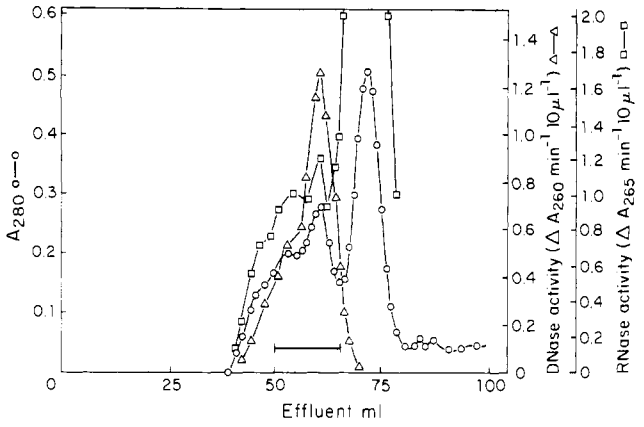

FIGURE 1: Gel filtration profile of a product mixture obtained from thiol–disulfide interchange between RNase–SH and DNase–S–S–pyridine on a Sephadex G-75 column (0.9 × 160 cm) equilibrated with 10% glycerol and 10 mM $CaCl_2$. The bar indicates the fractions which were pooled and which contained the 1:1 hybrid enzyme plus other products of the reaction.

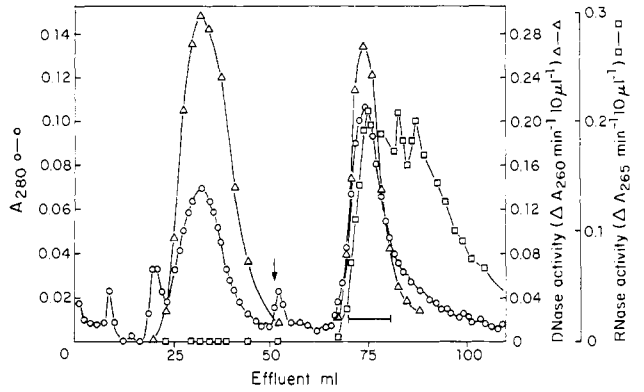

FIGURE 2: Affinity chromatography of the pooled material from Figure 1 on a pUp–Sepharose 4B column to remove free DNase from the hybrid enzyme and RNase homooligomers. The column (0.4 × 72 cm) was equilibrated with 0.02 M sodium acetate, 0.01 M $CaCl_2$, and 10% glycerol, pH 5.2. The eluent was changed to 4 M NaCl at the point indicated by the arrow. The bar indicates the fractions which were pooled.

contained the 1:1 hybrid enzyme were pooled.

Chromatography on pUp–Sepharose to Remove Free DNase. The pooled material obtained above, which contained free DNase, was concentrated by ultrafiltration through dialysis tubing (0.7 in.) at 100 mmHg pressure. The concentrated solution was dialyzed overnight at 4 °C against 500 mL of 0.02 M sodium acetate, 0.01 M $CaCl_2$, and 10% glycerol, pH 5.2. The dialyzed material was passed through a pUp–Sepharose affinity column [0.4 × 72 cm; cf. Wang & Moore (1978)] equilibrated with the same pH 5.2 buffer. The free DNase passed through slightly retarded, while the hybrid enzyme as well as the RNase homooligomers was retained on the column and subsequently eluted with 4 M NaCl. A typical elution curve is shown in Figure 2. The fractions that contained the hybrid enzyme were pooled, concentrated, and dialyzed overnight against 500 mL of 0.1 M Tris-HCl, 10 mM $CaCl_2$, and 4 M NaCl, pH 7.0.

Chromatography on LBI–Sepharose. Free RNase and homooligomers of RNase were separated from hybrid enzyme by salting-out adsorption on an LBI–Sepharose column (Wang & Moore, 1978); DNase, but not RNase, is retained through adsorption on the ligand (lima bean trypsin inhibitor). The dialyzed sample obtained as described above was placed on an LBI–Sepharose column (0.4 × 60 cm) equilibrated with Tris–$CaCl_2$–NaCl buffer (0.1 M, 10 mM, and 4 M, respectively). RNase which was not coupled to DNase passed through the column unretarded, as shown in Figure 3, while the hybrid enzyme was retained on the column until it was eluted with the same buffer but without NaCl. The efficacy of these chromatographic procedures was confirmed by nonreductive $NaDodSO_4$ gel electrophoresis of the product which showed no detectable free RNase or DNase. When reductive $NaDodSO_4$ gel electrophoresis was employed, the 1:1 hybrid enzyme gave rise to two components, the thiolated RNase and DNase in equal proportions, as judged by the intensity of the bands.

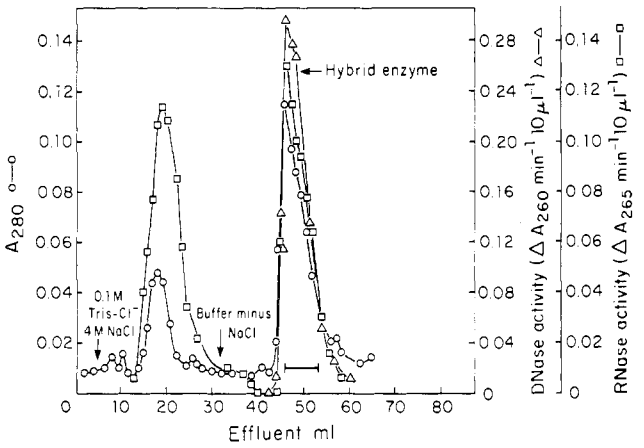

FIGURE 3: Salting-out chromatography of the pooled material from Figure 2 on an LBI–Sepharose 4B column (0.4 × 60 cm) to remove free RNase and RNase homooligomers from the hybrid enzyme RNase–DNase. The bar indicates the fractions which were pooled.

Table I: Effect of Metal Ions on the Enzymic Activities of RNase–DNase and DNase and/or RNase toward f_1 DNA·[^{3}H]RNA

metal ion[a]	RNase–DNase (cpm)	DNase[b] (cpm)	DNase + RNase (cpm)	RNase (cpm)
Mn^{2+}	7370	140	12 520	280
Mg^{2+}	760		8 640	279
none	0	0	109	98

[a] The concentration of metal ion was 10 mM. Assay conditions were as defined under Experimental Procedures. Enzyme concentrations per 100 μL: RNase–DNase, 50 ng; DNase, 33 ng; RNase, 17 ng; DNase + RNase, 33 ng + 17 ng, respectively. [b] Protease-free, RNase-free DNase (Wang & Moore, 1978).

Results

Comparison of the Enzymic Activities of the Bifunctional Enzyme and Its Parent Enzymes. The DNase in the bifunctional enzyme, RNase–DNase, hydrolyzed thymus DNA 75% as rapidly as the parent enzyme, DNase, under the same conditions. The RNase in the hybrid enzyme hydrolyzed yeast RNA or tRNA only 40% as rapidly as RNase. cCMP and poly(rC) were hydrolyzed at 45 and 56% of the rate of the parent enzyme, RNase.

The RNA strand of the double-stranded hybrid substrate, f_1 DNA·[^{3}H]RNA, was hydrolyzed very slowly by RNase alone, but was hydrolyzed rapidly by the bifunctional enzyme. Table I shows the effect of metal ions on the enzymic activities (as measured by counts per minute) of RNase–DNase and DNase and/or RNase toward f_1 DNA·[^{3}H]RNA. Similar to its parent enzyme DNase, the bifunctional enzyme showed an absolute requirement for activation by bivalent metal ions, Mn^{2+} or Mg^{2+}. However, with Mg^{2+} the nucleolytic activity of the hybrid enzyme was only 10% of that with Mn^{2+}. Neither RNase nor the admixture of DNase and RNase in

the absence of metal ions could hydrolyze the complementary RNA strand of the hybrid substrate to a significant extent. These results support the conclusion that the complementary DNA strand in the f_1 DNA·[^{3}H]RNA must be hydrolyzed before the complementary RNA strand can be acted upon by the hybrid enzyme.

Effect of Human Placental RNase Inhibitor on the Hybrid Enzyme. The RNase inhibitor isolated by Blackburn et al. (1977) needed to be used at ~20 times the concentration required with the parent RNase to effect 50% inhibition of the RNase moiety of the hybrid enzyme.

Discussion

The present results demonstrate the enzymic properties of RNase cross-linked to DNase in equimolar proportions. We initially considered the cross-linking of the two enzymes by dimethyl suberimidate, and one of the purposes in preparing protease-free DNase (Wang & Moore, 1978) was to obtain a sample of the enzyme that would be suitable for such experiments by being fully stable in the absence of divalent cations. In the absence of Ca^{2+}, DNase is highly susceptible to proteolysis, but Ca^{2+} would precipitate with PO_4^{3-} if the latter were present to prevent inactivation of RNase by amidination of lysine-41 by dimethyl suberimidate (Wang et al., 1976). Preliminary experiments showed that when RNase and protease-free DNase were cross-linked in phosphate buffer at pH 10 by a reagent such as dimethyl suberimidate, which is capable of reacting with one or more lysine residues in each protein species, the result is a very complex mixture of hybrids, dimers, and oligomers in which the yield of the desired product was very small. The thiolation approach (King et al., 1978), in which one especially reactive group per monomer is introduced, has afforded a stepwise pathway toward a better yield of a hybrid enzyme. In the formation of the reactive intermediate, protein–S–S–pyridine, the use of a thiolating agent that does not contain an –SH group is important to the yield. Otherwise, a very slowly interchanging mixed disulfide with the reagent would be a side product. We have preferred to use *N*-acetylhomocysteine thiolactone rather than the 2-iminothiolane used by King et al. (1978) because the former has advantages in availability and solubility.

A disadvantage of the –S–S– cross-link is that it is less stable than one involving amidination; stability of RNase–S–S–DNase in vivo will bear upon experiments on the biological properties of this hybrid enzyme.

Acknowledgments

The author is indebted to T. P. King for consultation on the chemistry of the cross-linking reaction, to Stanford Moore for counsel in the course of this research, to Grace Dean for skilled technical assistance, to Gerald Vovis for his advice on the production of phage f_1, and to Barbara Curtopelle for preparation of the typescript.

References

Baxt, W., Hehlmann, R., & Spiegelman, S. (1972) *Nature (London), New Biol. 240*, 72–75.

Benesch, R., & Benesch, R. E. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 848–853.

Blackburn, P., Wilson, G., & Moore, S. (1977) *J. Biol. Chem. 252*, 5904–5910.

Crook, E. M., Mathias, A. P., & Rabin, B. R. (1960) *Biochem. J. 74*, 234–238.

del Rosario, E. J., & Hammes, G. G. (1969) *Biochemistry 8*, 1884–1889.

Gallo, R. C. (1976) in *Modern Trends in Human Leukemia II* (Neth, R., Gallo, R. C., Mannweiler, K., & Maloney, W. C., Eds.) pp 431–455, J. F. Lehmann Verlag, München, Germany.

Grassetti, D. R., & Murray, J. F., Jr. (1967) *Arch. Biochem. Biophys. 119*, 41–49.

King, T. P., Li, Y., & Kochoumian, L. (1978) *Biochemistry 17*, 1499–1506.

Kunitz, M. (1946) *J. Biol. Chem. 164*, 563–568.

Kunitz, M. (1950) *J. Gen. Physiol. 33*, 349–362.

Liao, T. H. (1974) *J. Biol. Chem. 249*, 2354–2356.

Lin, M. C. (1970) *J. Biol. Chem. 245*, 6726–6731.

Model, P., & Zinder, N. D. (1974) *J. Mol. Biol. 83*, 231–251.

Salganik, R. I., Martynova, R. P., Matienko, N. A., & Ronichevskaya, G. M. (1967) *Nature (London) 214*, 100–102.

Sarngadharan, M. G., Sarin, P. S., Reitz, M. S., & Gallo, R. C. (1972) *Nature (London), New Biol. 240*, 67–72.

Stavrianopoulos, J. G., Karkas, J. D., & Chargaff, E. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2609–2613.

Tarnowski, G. S., Kassel, R. L., Mountain, I. M., Blackburn, P., Wilson, G., & Wang, D. (1976) *Cancer Res. 36*, 4074–4078.

Wang, D. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 487.

Wang, D., & Moore, S. (1977) *Biochemistry 16*, 2937–2942.

Wang, D., & Moore, S. (1978) *J. Biol. Chem. 253*, 7216–7219.

Wang, D., Wilson, G., & Moore, S. (1976) *Biochemistry 15*, 660–665.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

White, F. H., Jr. (1972) *Methods Enzymol. 25B*, 541–546.

Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L., & Treiber, G. (1970) *Virology 40*, 734–744.