Calif., W. H. Freeman, p 340.
Massoulié, J., and Michelson, A. M. (1967), *Biochim. Biophys. Acta 134,* 22–26.
Michelson, A. M., Massoulié, J., and Guschlbauer, W. (1967), *Prog. Nucleic Acid Res. Mol. Biol. 6,* 83–141.
Michelson, A. M., and Pochon, F. (1966), *Biochim. Biophys. Acta 114,* 469–480.
Riley, M., and Paul, A. V. (1970), *J. Mol. Biol. 50,* 439–455.
Sigler, R. B., Davies, D. R., and Miles, H. T. (1962), *J. Mol. Biol. 5,* 509–517.
Singer, M. F., and Tolbert, G. (1965), *Biochemistry 4,* 1319–1330.
Stollar, B. D. (1973), in The Antigens, Sela, M., Ed., New York, N.Y., Academic Press, pp 1–85.
Stollar, B. D., and Raso, V. (1974), *Nature (London) 250,* 231–234.
Torrence, P. F., Bobst, A. M., Waters, J. A., and Witkop, B. (1973), *Biochemistry 12,* 3962–3972.
Torrence, P. F., De Clercq, E., Waters, J. A., and Witkop, B. (1974), *Biochemistry 13,* 4400–4408.
Torrence, P. F., De Clercq, E., Waters, J. A., and Witkop, B. (1975), *Biochem. Biophys. Res. Commun. 62,* 658–664.
Torrence, P. F., and Witkop, B. (1975), *Biochim. Biophys. Acta 395,* 56–66.

# Triple-Helical Polynucleotides. Mixed Triplexes of the Poly(uridylic acid)·Poly(adenylic acid)·Poly(uridylic acid) Class†

Paul F. Torrence, Erik De Clercq, and Bernhard Witkop*

ABSTRACT: By the techniques of interferon induction in primary rabbit kidney cells "superinduced" with metabolic inhibitors, ultraviolet absorbance–temperature profiles, sensitivity to pancreatic ribonuclease A, and sucrose velocity gradient ultracentrifugation, a number of reactions between double-helical RNA and single-stranded RNA or DNA homopolymers were investigated. The polymers involved in these studies were poly(adenylic acid), poly(uridylic acid), poly(ribothymidylic acid), poly(5-bromouridylic acid), poly(deoxythymidylic acid), poly(deoxyuridylic acid), poly(3-methyluridylic acid), poly(2′-*O*-methyluridylic acid), and poly(2′-azido-2′-deoxyuridylic acid). Two different reaction courses, both leading to the formation of triple helices, were noted: (1) poly(Ux)·poly(A) + poly(Uy) → poly(Ux)·poly(A)·poly(Uy) if the $T_m$ of poly(Ux)·poly(A) was higher than the $T_m$ of poly(Uy)·poly(A); (2) poly(Ux)·poly(A) + poly(Uy) → poly(Uy)·poly(A)·poly(Ux) if the $T_m$ of poly(Ux)·poly(A) was lower than the $T_m$ of poly(Uy)·poly(A). In these equations, the homopolymer written to the left of poly(A) implies Watson–Crick hydrogen bonding whereas the polymer to the right of poly(A) is involved in Hoogsteen hydrogen bonding.

The term "recognition" has been used largely to denote the process in which a protein combines with a specific section of a nucleic acid (Yarus, 1969). Nonetheless, it is clear that the interaction of single-stranded nucleic acid with double-stranded nucleic acid can also give rise to a recognition system based on specific affinity. For instance, hybridization studies have established that the eukaryotic genome contains significant amounts of dA-rich and poly(dA)[1] sequences and dG-rich and poly(dG) sequences (Shenkin and Burdon, 1974, and references cited therein). Just as lysine-rich histones possess a greater affinity for the (dA + dT)-rich regions in DNA (Ohba, 1966; Mazen and Champagne, 1968) and arginine-rich histones have a greater affinity for (dG + dC)-rich regions in DNA (Clark and Felsenfeld, 1972), it may be expected that a polynucleotide comprised of the base uracil (or its derivatives) would possess a specific affinity for a poly(dT)·poly(dA) sequence in the DNA duplex by virtue of the formation of a triplex helix analogous to poly(U)·poly(A)·poly(U). In this connection, it is of interest that dA-dT clusters have been found near the origin of DNA replication in *Escherichia coli* 15T⁻ cells (Baril and Kubinski, 1975).

Although our knowledge of the factors that govern formation and stability of polynucleotide triplexes is quite limited (Bloomfield et al., 1974), and in spite of the fact that no nucleic acid triple helix[2] has yet been observed to occur naturally in any biological system, a variety of hypotheses

† From the Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014 (P.F.T. and B.W.), and the Rega Institute, University of Leuven, B-3000 Leuven, Belgium (E.D.C.). *Received August 25, 1975.*

[1] Abbreviations for synthetic polynucleotides conform to the recommendations of the IUPAC–IUB Commission [*J. Mol. Biol. 55,* 299 (1971)]. Less commonly used abbreviations are: poly(br⁵U), poly(5-bromouridylic acid); poly(rT), poly(ribothymidylic acid) or poly(5-methyluridylic acid); poly(Um), poly(2′-*O*-methyluridylic acid); poly(m³U), poly(3-methyluridylic acid); poly(dUf), poly(2′-fluoro-2′-deoxyuridylic acid). Other abbreviations are: MEM, Eagle's minimal essential medium; PBS, Dulbecco's phosphate-buffered saline; poly(U*), [5-³H]poly(uridylic acid).

[2] It is important to differentiate here between a nucleic acid triple helix and a nucleic acid base triple. The former may be defined (for purposes of this paper) as the formation of a three-stranded polynucleotide for a least one helical turn. In contrast, the latter, arising from tertiary hydrogen bonding interactions, may be defined as formed from three hydrogen-bonding bases and does not result in a triple helix. Triples of this latter type have been demonstrated in yeast phenylalanine tRNA (Kim et al., 1974).

have suggested potential roles for such triple-stranded interactions. Suggestions of recent vintage have postulated nucleic acid triplexes as potential elements of control in gene regulation and chromosome structure (Britten and Davidson, 1969; Crick 1971); triple-helix formation has been invoked as a mode of interaction of a putative chromosomal RNA with DNA (reviewed by Sirlin, 1972); a triplex may be involved in stabilizing the folded chromosome of *Escherichia coli* (Pettijohn and Hecht, 1973): consistent with the earlier demonstration (Opara-Kubinska et al., 1964; Kubinski et al., 1966) of pyrimidine rich regions in eukaryotic DNA, it has recently been found that denatured bovine DNA may interact with homologous native DNA to yield some triply stranded regions (Perlgut et al., 1975).

Interferon induction in cells "superinduced" with cycloheximide and actinomycin D (Vilcek, 1970; Tan et al., 1970) provides a useful biological assay for investigating interactions among homopolyribonucleotides (De Clercq et al., 1974a). This methodology has already provided information leading to the demonstrated existence of the unique poly(U)·poly(A)·poly(I) triplex (De Clercq et al., 1975a) and has also been used to monitor polynucleotide displacement reactions (De Clercq et al., 1976). Herein, this interferon induction assay is employed in concert with uv absorbance–temperature profiles, pancreatic RNase A resistance, and sucrose velocity gradient analysis to demonstrate the formation of several nucleic acid triple helices which may be considered analogues of the poly(U)·poly(A)·poly(U) triplex. The existence and properties of such triplexes may aid in the determination of the potential role(s) of such triple-stranded nucleic acids in vivo.

## Materials and Methods

Techniques used for the measurement of interferon production in primary rabbit kidney (PRK) cell cultures "superinduced" with cycloheximide and actinomycin D have been previously described (De Clercq et al., 1975a). For this purpose, stock solutions of the homopolymers and their complexes were prepared at a concentration of 1 mg/ml in 0.1 M Tris-HCl–0.2 M NaCl (pH 7.0) and stored at 4 °C. Prior to use, the polymers were diluted in Eagle's minimal essential medium (MEM) and mixed at the appropriate stoichiometric ratios to give final concentrations of 5 μg/ml (homopolymer) or 10 μg/ml (homopolymer duplexes).

Methods employed for the determination of ultraviolet absorbance–temperature profiles have also been detailed earlier (Torrence et al., 1973a). When the triplex formation reactions were monitored by thermal profile, the general procedure was to mix the duplex homopolymer constituents at concentrations (expressed as P) of about $5 \times 10^{-4}$ M in the selected buffer. After adequate time to ensure that equilibrium has been obtained (7–14 days at 4 °C), the third homopolymer was added in the required stoichiometric quantity. The polymers were allowed to react at a concentration of approximately $5 \times 10^{-4}$ M for 7–14 days at 4 °C and then diluted (5–10×) with appropriate buffer. After an additional 1–7 days at 4 °C, the melting profile was determined. In all cases, the thermal profile was monitored simultaneously at several different wavelengths.

The procedures for the determination of sensitivity to pancreatic RNase A and for sucrose velocity gradient analysis have been described previously (De Clercq et al., 1975a). The polymer [5-$^3$H]poly(U) is presented throughout the text as poly(U*).

The polynucleotides poly(A) ($s_{20,w}$ = 9.7 S), poly(U) ($s_{20,w}$ = 7 S), poly(Um) ($s_{20,w}$ = 10 S), poly(dT) ($s_{20,w}$ = 7.0 S), and poly(dU) were products of P-L Biochemicals (Milwaukee, Wis). Poly(dUz) ($s_{20,w}$ = 12.6 S) (Torrence et al., 1973a), poly(rT) (Howard et al., 1971), poly(br$^5$U) (Riley and Paul, 1970; Torrence and Witkop, 1975), and poly(m$^3$U) (Szer and Shugar, 1961; Torrence et al., 1973b) were all prepared according to the previously published methodology. Where $s_{20}$ values were not determined, the polymers were ascertained to be of high molecular weight by one or more of the following criteria: (a) elution in the void volume of a Sephadex G-200 column (1.8 × 25 cm, 0.04 M ammonium bicarbonate (pH 7.5) buffer); (b) thermal profiles (of complementary duplexes) which showed the same cooperativity as samples of known high molecular weight (>6 S); (c) ability (as complementary duplexes) to induce the same high titers of interferon (in "superinduced" PRK cells) as samples of known high molecular weight (>6 S).

Extinction coefficients ($\epsilon_p$) employed in these studies were as follows ($T$ = 20°C): poly(A), $\epsilon_{max}$ 10 000 in 0.1 M NaCl–0.01 M sodium cacodylate (pH 6.85) (Sigler et al., 1962); poly(br$^5$U), $\epsilon_{max}$ 8250 in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7) (Torrence and Witkop, unpublished observations); poly(U), $\epsilon_{max}$ 9430 in 0.195 M NaCl–0.01 M sodium cacodylate (pH 7.5) (Blake et al., 1967); poly(rT), $\epsilon_{max}$ 9170 in 0.02 M sodium cacodylate (pH 7) (Howard et al., 1971); poly(m$^3$U), $\epsilon_{max}$ 10 000 in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7) (P. F. Torrence and B. Witkop, unpublished observations); poly(dT), $\epsilon_{max}$ 8700 in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.9) (Riley et al., 1966); poly(dU), $\epsilon_{max}$ 9000 in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7) (Torrence and Witkop, unpublished observations); poly(Um), $\epsilon_{max}$ 9400 in 0.05 M NaCl–0.01 M sodium cacodylate (pH 7.4) (Rottman et al., 1974).

## Results

*General Considerations.* If a polynucleotide duplex and a single-stranded homopolymer interact, the following reactions might conceivably occur (poly(Ux) and poly(Uy) represent two different modified poly(uridylic acids)):

(a) triplex formation

$$\text{poly(Ux)} \cdot \text{poly(A)} + \text{poly(Uy)} \rightarrow \text{poly(Ux)} \cdot \text{poly(A)} \cdot \text{poly(Uy)}^3 \quad \text{(a1)}$$

(b) displacement with triplex formation

$$\text{poly(Ux)} \cdot \text{poly(A)} + \text{poly(Uy)} \rightarrow \text{poly(Uy)} \cdot \text{poly(A)} \cdot \text{poly(Ux)} \quad \text{(b1)}$$

$$\text{poly(Ux)} \cdot \text{poly(A)} + \text{2-poly(Uy)} \rightarrow \text{poly(Uy)} \cdot \text{poly(A)} \cdot \text{poly(Uy)} + \text{poly(Ux)} \quad \text{(b2)}$$

$$\text{2-poly(Ux)} \cdot \text{poly(A)} + \text{2-poly(Uy)} \rightarrow \text{poly(Ux)} \cdot \text{poly(A)} \cdot \text{poly(Ux)} + \text{poly(Uy)} \cdot \text{poly(A)} \cdot \text{poly(Uy)} \quad \text{(b3)}$$

[3] Poly(Ux)·poly(A)·poly(Uy) implies that the AUx base pair has Watson–Crick hydrogen bonding while Uy is paired to A by Hoogsteen bonding. Poly(Uy)·poly(A)·poly(Ux) implies the alternate bonding scheme; that is, the AUy base pair is of the Watson-Crick type while Ux is bonded to A by Hoogsteen base pairing. Other possibilities exist (e.g., reverse Hoogsteen), but the former two schemes represent modes of pairing in the triple helix that are consistent with those determined by x-ray diffraction studies on the poly(U)·poly(A)·poly(U) triplex (Arnott and Bond, 1973).

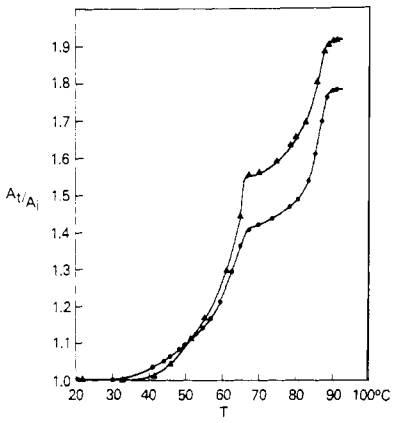


FIGURE 1: Melting profiles (in 0.05 M NaCl–0.01M sodium cacodylate (pH 7)) for the products of the reactions: poly(br$^5$U)·poly(A) + poly(U) (●) and poly(U)·poly(A) + poly(br$^5$U) (▲). The wavelength monitored is 260 nm. Changes recorded at other wavelengths (e.g., 280 nm) failed to reveal any other transitions. $A_t/A_i$ is the ratio of the absorbance at the initial temperatures (i) to that at some given temperature ($t$).

(c) displacement

$$\text{poly(Ux)} \cdot \text{poly(A)} + \text{poly(Uy)} \rightarrow \text{poly(Uy)} \cdot \text{poly(A)} + \text{poly(Ux)} \quad \text{(c1)}$$

(d) displacement with duplex and triplex formation

$$2\text{-poly(Ux)} \cdot \text{poly(A)} + \text{poly(Uy)} \rightarrow \text{poly(Uy)} \cdot \text{poly(A)} + \text{poly(Ux)} \cdot \text{poly(A)} \cdot \text{poly(Ux)} \quad \text{(d1)}$$

Still additional possibilities can be imagined, but these can be represented by summation of two or more of the above reactions, or else they are unlikely on thermodynamic grounds (e.g., the dissociation of a duplex and a single-strand into three separate homopolymers), and all are inconsistent with the data presented below.

During the course of experiments used to identify the poly(U)·poly(A)·poly(I) triplex (De Clercq et al., 1975a), a similar series of possibilities was encountered (analogous to equations a1 through d1). Mixing curves were employed in this latter instance to determine reaction stoichiometry and thus provide a means of eliminating a significant number of potential reactions. Mixing curves have not been used in the course of this present study chiefly because only limited quantities of some modified polymers were available; furthermore, mixing curves for these reactions are not so informative since most of the possibilities from which a selection must be made occur with 1:1 stoichiometry. The conclusions from the thermal profile experiments are based mainly on profiles determined in 0.05 M NaCl–0.01 M sodium cacodylate (pH 7), a buffer in which a convenient separation of the 3 → 2 (triplex to duplex) and 2 → 1 (duplex to homopolymers) transitions is witnessed during melting of the poly(A)·2poly(U) triplex (Blake et al., 1967).

*Poly(br$^5$U)·Poly(A)* + *Poly(U)*. The uv absorbance–temperature profile for a mixture equimolar in poly(br$^5$U)·poly(A) and poly(U) (or 2 mol of duplex phosphate/mol of poly(U)) is given in Figure 1. Under these conditions (0.06 M $Na^+$, pH 7), a biphasic profile is witnessed with the first transition occurring at 60 °C and the second at 85 °C. The first transition exhibits a $T_m$ which clearly distinguishes it from the melting of the poly(U)·poly(A)·poly(U) triplex ($T_{m(3\rightarrow2)}$ = 49 °C, $T_{m(2\rightarrow1)}$ = 53 °C; see also De Clercq et al., 1975a); thus, reactions corresponding to eq b2, b3, c1, and d1 (with Ux = br$^5$U and Uy = U) can be eliminated. The second transition ($T_m$ = 85 °C) corresponds to the melting of the poly(br$^5$U)·poly(A) duplex (or triplex) (data not shown). The first transition must represent the dissociation of the poly(U) strand from the poly(br$^5$U)·poly(A) helix (which may subsequently rearrange to the triplex poly(br$^5$U)·poly(A)·poly(br$^5$U). Thus poly(U) must add to the duplex to give the mixed triplex according to the equation:

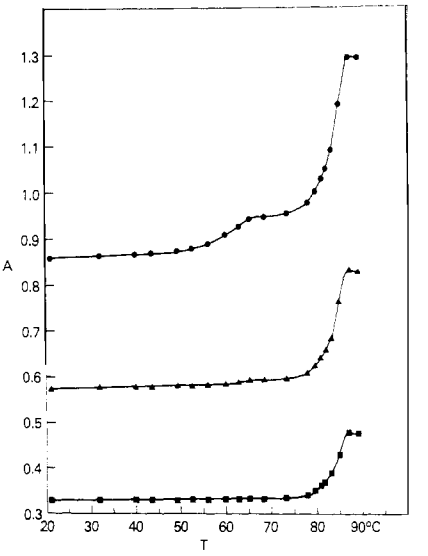


FIGURE 2: Melting profile of the reaction poly(U)·poly(A) + 2 poly(br$^5$U) determined in 0.05 M NaCl–0.01 M sodium cacodylate (pH 7). (●) 260 nm; (▲) 280 nm; (■) 290 nm.

$$\text{poly(br}^5\text{U)} \cdot \text{poly(A)} + \text{poly(U)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (1)$$

*Poly(U)·Poly(A)* + *Poly(br$^5$U)*. When poly(U)·poly(A) and poly(br$^5$U) are mixed in equimolar quantities, the melting profile (▲) shown in Figure 1 is obtained. Again this profile is biphasic and both transitions have $T_m$'s within experimental error (±1°C) of those seen in the interaction of poly(br$^5$U)·poly(A) and poly(U). Thus, the addition of poly(br$^5$U) to the duplex poly(U)·poly(A) gives rise to virtually the same melting profile as did the addition of poly(U) to poly(br$^5$U)·poly(A). The most probable explanation for this behavior corresponds to eq b1 above; that is, the poly(br$^5$U) first displaces poly(U) from the poly(U)·poly(A) duplex and then triplex formation occurs (eq 2).

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(br}^5\text{U)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (2)$$

In this particular system, additional stoichiometries of interaction have been examined to determine if reactions like b2 or d1 can also occur. The addition of 2 mol of poly(br$^5$U) to 1 mol of poly(U)·poly(A) gave rise to the thermal profile of Figure 2 and results in a significantly different profile from that seen with the 1:1 mixture. The first transition (corresponding to the dissociation of poly(U)) is considerably reduced in proportion to the second transition which still occurs at 84 °C, characteristic of the melting of poly(br$^5$U)·poly(A)·poly(br$^5$U). This suggests that addition of a second mole of poly(br$^5$U) can lead to complete displacement of poly(U) from the triplex according to:

$$\text{poly(U)} \cdot \text{poly(A)} + 2\text{-poly(br}^5\text{U)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(br}^5\text{U)} + \text{poly(U)} \quad (3)$$

Table I: Interactions among Polynucleotides and Their Complexes as Monitored by Interferon Production in Primary Rabbit Kidney Cells Superinduced with Cycloheximide and Actinomycin D.

| System[a] | Method of Addition[b] | Fraction of Interferon Titer Compared to Control[c] |
|---|---|---|
| $(U)_n + (U)_n \cdot (A)_n$ | Mixture | 0.05 (±0.03) |
| | $(U)_n$ then $(U)_n \cdot (A)_n$ | 1.1 (±0.24) |
| | $(U)_n \cdot (A)_n$ then $(U)_n$ | 0.10 (±0.00) |
| $(A)_n + (U)_n \cdot (A)_n$ | Mixture | 1.0, 1.0 |
| | $(A)_n$ then $(U)_n \cdot (A)_n$ | 0.83 (±0.15) |
| | $(U)_n \cdot (A)_n$ then $(A)_n$ | 1.0, 0.67 |
| $(rT)_n + (U)_n \cdot (A)_n$ | Mixture | 0.01, 0.13 |
| | $(rT)_n$ then $(U)_n \cdot (A)_n$ | 0.82 (±0.23) |
| | $(U)_n \cdot (A)_n$ then $(rT)_n$ | 0.10 |
| $(rT)_n + (rT)_n \cdot (A)_n$ | Mixture | 0.06, 0.03 |
| | $(rT)_n$ then $(rT)_n \cdot (A)_n$ | 0.2, 0.53 |
| | $(rT)_n \cdot (A)_n$ then $(rT)_n$ | 0.06, 0.2 |
| $(A)_n + (rT)_n \cdot (A)_n$ | Mixture | 1.3, 0.75 |
| | $(A)_n$ then $(rT)_n \cdot (A)_n$ | 1.0, 0.53 |
| | $(rT)_n \cdot (A)_n$ then $(A)_n$ | 0.8, 1.0 |
| $(U)_n + (rT)_n \cdot (A)_n$ | Mixture | 0.20, 0.30 |
| | $(U)_n$ then $(rT)_n \cdot (A)_n$ | 0.67, 0.53 |
| | $(rT)_n \cdot (A)_n$ then $(U)_n$ | 0.30 |
| $(dUz)_n + (U)_n \cdot (A)_n$[d] | Mixture | 0.01, 0.01 |
| | $(dUz)_n$ then $(U)_n \cdot (A)_n$ | 0.007, 0.005 |
| | $(U)_n \cdot (A)_n$ then $(dUz)_n$ | 0.20, 0.12 |
| $(dUz)_n + (rT)_n \cdot (A)_n$[e] | Mixture | 0.025 |
| | $(dUz)_n$ then $(rT)_n \cdot (A)_n$ | 0.1, 0.02 |
| | $(rT)_n \cdot (A)_n$ then $(dUz)_n$ | 0.3, 0.2 |
| $(m^3U)_n + (U)_n \cdot (A)_n$ | Mixture | 0.3, 1.0 |
| | $(m^3U)_n$ then $(U)_n \cdot (A)_n$ | 0.3, 1.0 |
| $(dT)_n + (U)_n \cdot (A)_n$ | Mixture | 0.01, 0.01 |
| | $(dT)_n$ then $(U)_n \cdot (A)_n$ | 0.1, 0.1 |
| $(dU)_n + (U)_n \cdot (A)_n$ | Mixture | 0.1, 0.1 |
| | $(dU)_n$ then $(U)_n \cdot (A)_n$ | 0.33, 0.20 |
| $(Um)_n + (U)_n \cdot (A)_n$ | Mixture | <0.01, <0.01 |
| | $(Um)_n$ then $(U)_n \cdot (A)_n$ | 0.1, 0.1 |
| $(br^5U)_n + (U)_n \cdot (A)_n$ | Mixture | 0.08 (±0.04) |
| | $(br^5U)_n$ then $(U)_n \cdot (A)_n$ | 0.32 (±0.02) |
| $2.0(br^5U)_n + (U)_n \cdot (A)_n$[f] | Mixture | 0.016 |
| $0.5(br^5U)_n + (U)_n \cdot (A)_n$[g] | Mixture | 1.0 |
| $(dUf)_n + (U)_n \cdot (A)_n$[h] | Mixture | 0.03 |
| | $(dUf)_n$ then $(U)_n \cdot (A)_n$ | 0.04, 0.07 |
| | $(U)_n$ then $(dUf)_n \cdot (A)_n$ | 0.19 |

[a] The alternate abbreviation for polynucleotides is used in this table; thus, $(U)_n$ = poly(U). [b] Homopolymer and homopolymer complex either (i) mixed to give a final concentration of 5 and 10 μg/ml, respectively, and then incubated for 1 h at 37 °C in MEM before addition to the cells or (ii) added sequentially (each at 10 μg/ml in MEM) to the cells in a 1-h interval. [c] Defined as (interferon titer for homopolymer + duplex)/(interferon titer for duplex alone). Interferon titers are defined as the reciprocal of the highest dilution of the sample that reduced viral cytopathogenicity by 50%. See also De Clercq et al. (1974a). A control experiment was performed for each manner of addition to the cells; thus for the system $(U)_n + (U)_n \cdot (A)_n$, MEM was added in place of the homopolymer in the experiments that involved addition as a mixture or in experiments where the polymers were added sequentially. Titer reductions are expressed as the mean (± 1 S.D.) in those instances where 3–5 determinations (each with a different preparation of primary rabbit kidney cells) were performed. In other systems, the individual results are listed separately. [d] The interaction of $(U)_n$ with $(dUz)_n \cdot (A)_n$ was also studied. The duplex $(dUz)_n \cdot (A)_n$ is virtually inactive as an interferon inducer in this system (see also Torrence et al., 1973b) giving rise to a maximum of 10–20 units/ml at a concentration of 10 μg/ml. The addition of $(U)_n$ either mixed with $(dUz)_n \cdot (A)_n$, or in the sequential administration procedure failed to bring about any increase in interferon titer. [e] As in footnote *d* above, the addition of $(rT)_n$ to $(dUz)_n \cdot (A)_n$ either in the direct mixing experiments or in the sequential administration procedure, failed to produce any rise in interferon titer. [f] One mole of the duplex $(U)_n \cdot (A)_n$ was mixed with 2 mol of $(br^5U)_n$. [g] One mole of the duplex $(U)_n \cdot (A)_n$ was mixed with 0.5 mol of $(br^5U)_n$. [h] Taken from De Clercq et al. (1975b).

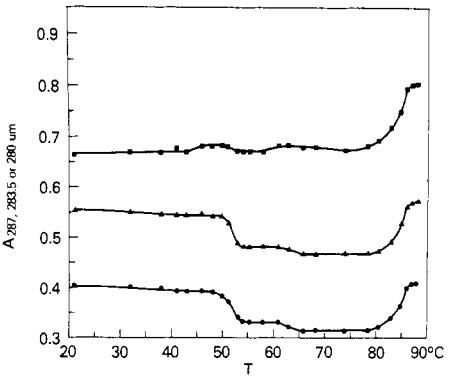


FIGURE 3: Absorbance–temperature profile for the interaction: 2-poly(U)·poly(A) + poly($br^5U$) as determined in 0.05 M NaCl–0.01 M sodium cacodylate (pH 7) at λ, 287 nm (●), 283.5 nm (▲), and 280 nm (■).

The residual transition ascribed to the strandwise melting of the poly($br^5U$)·poly(A)·poly(U) triplex may arise from experimental error, or more likely, because this system has not yet reached equilibrium (vide infra).

When the melting profile of a solution constructed from 2 mol of poly(U)·poly(A) and 1 mol of poly($br^5U$) is examined, the result of Figure 3 is obtained. At 260 nm three transitions are witnessed (data not shown). The first is the melting of poly(U)·poly(A), the second is the disproportionation of the poly($br^5U$)·poly(A)·poly(U) triplex, and the third represents the melting of the remaining poly($br^5U$)·poly(A) duplex (or triplex). This interpretation of the absorbance behavior at 260 nm is supplemented by the changes in absorbance occurring at 287, 283.5, and 280 nm (Figure 3) (Blake et al., 1967). Transitions are witnessed at 52 °C which correspond to the melting (2 → 1) of the poly(U)·poly(A) duplex. Importantly, there is no evidence for the existence of poly(U)·poly(A)·poly(U) in this mixture since a substantial hyperchromic change would be seen at 280 nm (Blake et al., 1967). These data indicate that the reaction corresponding to d1 does not occur; i.e.

$$\text{2-poly(U)·poly(A)} + \text{poly}(br^5U) \nrightarrow \text{poly}(br^5U)\text{·poly(A)} + \text{poly(U)·poly(A)·poly(U)}$$

Instead the mixed triplex is preferred:

$$\text{2-poly(U)} \cdot \text{poly(A)} + \text{poly}(br^5U) \rightarrow \text{poly(U)} \cdot \text{poly(A)} + \text{poly}(br^5U) \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (4)$$

so that no reaction occurs corresponding to a stoichiometry of 2 mol of poly(U)·poly(A) to 1 mol of poly($br^5U$).

The interferon induction data presented in Table I are in total agreement with the above observations and with eq 2–4. If poly($br^5U$) is added to the cells as a mixture with poly(A)·poly(U), the interferon titer is significantly reduced. Similarly, when poly($br^5U$) is added to the cells first, a smaller, yet still significant reduction in titer is witnessed. These results can be related to formation of the poly($br^5U$)·poly(A)·poly(U) (eq 2) triple helix, since it, like other triplexes (De Clercq et al., 1974a, 1975a), is expected to be inactive as an inducer. Consistent with and supportive of eq 3 is the observation that addition of a mixture of 2 mol of poly($br^5U$) and 1 mol of poly(U)·poly(A) to the cells also leads to a major decrease in interferon titer as would be expected if poly($br^5U$)·poly(A)·poly($br^5U$) is a reaction product. This latter triplex has also been shown to be incapable of significant interferon production (De Clercq et al., 1974a). Finally, the occurrence of eq 4 is also verified by

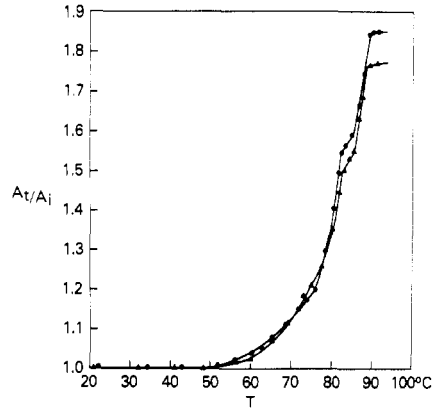


FIGURE 4: Melting profiles (in 0.05 M NaCl–0.01 M sodium cacodylate (pH 7)) for the reactions poly(rT)·poly(A) + poly(br$^5$U) (▲) and poly(br$^5$U)·poly(A) + poly(rT) (●). The wavelength monitored in both cases was 260 nm.

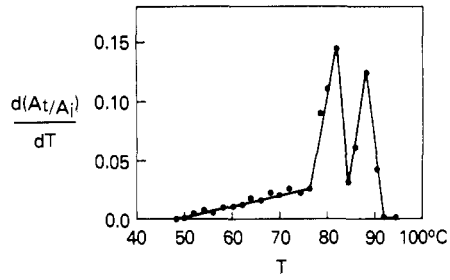


FIGURE 5: Differential melting curve (from best fit integral) for the product of the reaction poly(br$^5$U)·poly(A) + poly(rT).

the interferon data. When 0.5 mol of poly(br$^5$U) is mixed with 1.0 mol of poly(U)·poly(A), there is no reduction in interferon titer since 50% of the input poly(U)·poly(A) is still available for induction. In our interferon assay conditions, a decrease in poly(U)·poly(A) concentration from 10 to 5 μg/ml does not lead to an appreciable change in interferon titer (De Clercq, Torrence, Waters and Witkop, unpublished observations). If the alternative reaction (d1) occurred in this case, the products poly(br$^5$U)·poly(A) and poly(U)·poly(A)·poly(U) would lead to a large decrease in interferon production (De Clercq et al., 1974a).

*Poly(br$^5$U)·Poly(A)* + *Poly(rT)*. A mixture consisting of equimolar amounts of poly(rT) and poly(br$^5$U)·poly(A) (or 1 mol of poly(rT) phosphate/2 mol of duplex phosphate) gave the absorbance–temperature profile shown in Figure 4 (●). As seen in the interaction of poly(br$^5$U)·poly(A) with poly(U), the present system gave a biphasic profile which is especially evident in the differential profile of Figure 5. These data prove that there is no transition which can be ascribed to the melting of the poly(rT)·poly(A) double helix or the poly(rT)·poly(A)·poly(rT) triple helix (Figure 6) (see also Howard et al., 1971), and that the reactions corresponding to eq b2, b3, c1, and d1 may therefore be eliminated. Furthermore since the second transition corresponds to the melting of poly(br$^5$U)·poly(A), the first transition ($T_m$ 80 °C) must represent the melting out of the poly(rT) strand from the poly(br$^5$U)·poly(A)·poly(rT) triplex. Thus when poly(rT) is added to poly(br$^5$U)·poly(A), the single strand adds to the outside of the double helix:

$$\text{poly(br}^5\text{U)} \cdot \text{poly(A)} + \text{poly(rT)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(rT)} \quad (5)$$

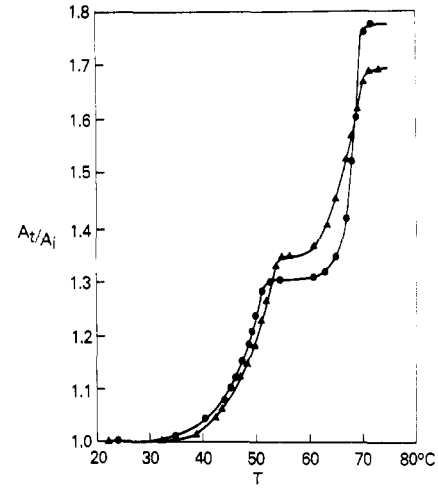


FIGURE 6: Melting profiles (in 0.05 M NaCl–0.01 M sodium cacodylate (pH 7)) for the reactions: poly(U)·poly(A) + poly(rT) (▲) and poly(rT)·poly(A) + poly(U) (●), λ 260 nm.

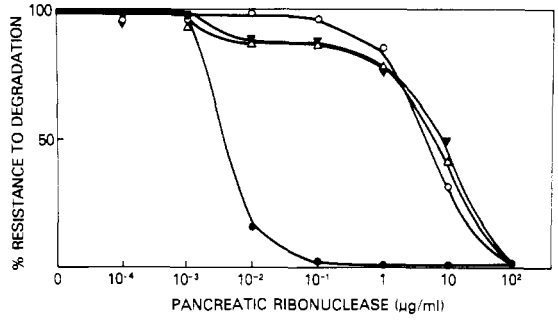


FIGURE 7: Pancreatic ribonuclease A sensitivity of the products of the reactions poly(U)·poly(A) + poly(U*) (△) and poly(rT)·poly(A) + poly(U*) (▼). For comparison purposes, the sensitivity of poly(U*) (●) and poly(U*)·poly(A) (○) is also included. The reaction mixtures were incubated at 37 °C for 1 hr then 1 week at 4 °C before analysis. Incubation for only 1 h left a substantial quantity of nuclease sensitive poly(U*).

*Poly(rT)·Poly(A)* + *Poly(br$^5$U)*. An equimolar mixture of the poly(rT)·poly(A) duplex and poly(br$^5$U) homopolymer showed the behavior (Figure 4, (▲)) identical with reaction 5, demonstrating that in this instance, poly(br$^5$U) first displaces poly(rT) from the duplex to give poly(br$^5$U)·poly(A) to which poly(rT) then adds in triplex formation.

$$\text{poly(rT)} \cdot \text{poly(A)} + \text{poly(br}^5\text{U)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(rT)} \quad (6)$$

*Poly(rT)·Poly(A)* + *Poly(U)*. Determination of the melting profile of a mixture equimolar in poly(U) and poly(rT)·poly(A) gives the biphasic melting curve pictured in Figure 6. The shape and midpoint of the first transition are not consistent with the presence of any significant quantity of poly(U)·poly(A)·poly(U) or poly(U)·poly(A); nonetheless, small quantities of the latter complexes would be most difficult to detect. The second transition in this profile corresponds to the melting of the poly(rT)·poly(A) duplex (or poly(rT)·poly(A)·poly(rT) triplex) (data not shown), suggesting the following:

$$\text{poly(rT)} \cdot \text{poly(A)} + \text{poly(U)} \rightarrow \text{poly(rT)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (7)$$

The results of pancreatic ribonuclease A sensitivity experiments are depicted in Figure 7. Radiolabeled [5-$^3$H]-

poly(U) was mixed in a 1:1 stoichiometric ratio with poly(rT)·poly(A) (unlabeled). In one experiment (data not shown), the mixture was incubated for only 1 h at 37 °C. Alternatively (Figure 7), the reaction was allowed to proceed for 1 h at 37 °C and then 1 week at 4 °C before analysis. For comparison purposes a similar experiment was performed with cold poly(U)·poly(A) and [5-$^3$H]poly(U). It was clear that after 1 h at 37 °C, a significant portion of the labeled poly(U) was still available for degradation by RNase, whereas after 1 week longer at 4 °C both systems (poly(U)·poly(A) + poly(U*) and poly(rT)·poly(A) + poly(U*)) reached a state wherein the poly(U) was just as resistant to degradation as it was when a part of the poly(U)·poly(A) duplex.

Sucrose gradient ultracentrifugation data also give a result consistent with the above (Figure 8). Again, with the poly(U)·poly(A) + poly(U*) system as a control, when poly(U*) was mixed with poly(rT)·poly(A) and the mixture incubated for 1 h at 37 °C before centrifugation, a significant portion of the label was found in a peak that sedimented faster than poly(U*) (Figure 8). In addition this label was found at a position that corresponded to the same peak formed in the interaction of poly(U*) with poly(U)·poly(A) and both of these peaks sedimented faster than poly(U*)·poly(A) itself. In both cases, some poly(U*) remained in a peak corresponding to free poly(U) indicating that as in the pancreatic RNase experiments, equilibrium had not yet been obtained. When longer reaction times (1 week at 4 °C) were used in these instances, there was a disappearance of the peaks due to free poly(U), but this increase was not matched by an increase of label in the faster sedimenting fractions, suggesting loss of labeled poly(U) during these longer incubation times (data not shown).

That some time may be required for this and other similar systems to approach equilibrium was also indicated by melting profile experiments. If the $T_m$ of the reaction of poly(U)·poly(A) + poly(rT), or poly(br$^5$U)·poly(A) + poly(U) was determined immediately after mixing or even as late as 48 h after mixing, the presence of large quantities of poly(U)·poly(A) was revealed. Reaction mixtures allowed to stand for 2–3 weeks at 4 °C, however, showed no evidence of free poly(U)·poly(A).

Poly(rT)·poly(A) has been demonstrated to be an effective inducer of interferon in primary rabbit kidney cells (De Clercq et al., 1974a). When poly(U) is added to the cells together with or 1 h after poly(rT)·poly(A), a significant reduction in interferon titer occurs (Table I). If poly(rT)·poly(A) is added to the cells 1 h after the addition of poly(U), a smaller, barely significant titer reduction occurs. For comparison purposes, poly(rT), added to the cells together with or 1 h after poly(rT)·poly(A), or poly(U), added together with or 1 h after poly(U)·poly(A) also effect large reduction in interferon titer due to the formation of poly(rT)·poly(A)·poly(rT) (Howard et al., 1971) and poly(U)·poly(A)·poly(U), respectively. On the other hand, when these homopolymers were administered to the cells 1 h before addition of their respective duplexes, there was little, if any, reduction in interferon titer. Finally it is clear that the shifts in interferon titer, as noted above, may be considered as specific, since poly(A) did not significantly affect the titer of either poly(U)·poly(A) or poly(rT)·poly(A) when added before, together with, or after the duplexes (Table I) (vide infra and De Clercq et al., 1974a).

To summarize, the reaction of poly(rT)·poly(A) with poly(U) produces (a) a partial but not complete reduction in interferon titer; (b) a product in which the poly(U) is in a form resistant to ribonuclease A; (c) a complex incorporating poly(U) that sediments faster than poly(U)·poly(A) and at about the same position as poly(U)·poly(A)·poly(U); (d) a melting profile that shows no evidence of poly(U)·poly(A) or poly(U)·poly(A)·poly(U) but is biphasic with the second transition corresponding to the melting of poly(rT)·poly(A). These data eliminate all possibilities corresponding to eq b1 through d1 and leave as the only alternative the reaction expressed by equation 7, formation of the poly(rT)·poly(A)·poly(U) triple helix.

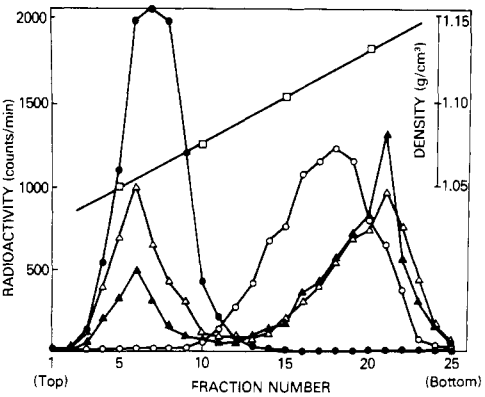


FIGURE 8: Sucrose velocity gradient ultracentrifugal analysis of the products of the reactions: poly(U)·poly(A) + poly(U*) (▲) and poly(rT)·poly(A) + poly(U*) (△). For comparison, the patterns for free poly(U*) (●) and poly(U*)·poly(A) (○) are included. The reactions were incubated for 1 h at 37 °C before they were layered onto the sucrose gradient (De Clercq et al., 1975a).

*Poly(U)·Poly(A) + Poly(rT)*. The addition of 1 mol of poly(rT) to 1 mol of the poly(U)·poly(A) double helix (2 mol of P) results in the formation of a triple helix which is the same as that seen in the interaction of poly(rT)·poly(A) with poly(U) according to the equation:

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(rT)} \rightarrow \text{poly(rT)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (8)$$

The evidence for this conclusion is as follows. (1) Poly(rT), when added together with, or 1 h after poly(U)·poly(A), causes a 10–100 drop in interferon titer in "superinduced" PRK cells (Table I). (2) Both RNase sensitivity experiments (at 1 h or 1 week reaction time) as well as sucrose velocity gradient ultracentrifugation show that no free poly(U) is released in this interaction (data not illustrated). Furthermore, the labeled poly(U) product sediments at a position expected for a double or triple helix. (3) The melting profile of an equimolar mixture of poly(rT) + poly(U)·poly(A) is nearly identical with that obtained in the interaction of poly(rT)·poly(A) + poly(U) (Figure 6 (▲)).

*Poly(A)·Poly(U) + Poly(dUz) and Poly(dUz)·Poly(A) + Poly(U)*. Before continuing with additional systems in which compelling evidence for mixed triplexes exists, the factors that determine whether or not a nucleic acid will melt in a stepwise fashion (i.e., 3 → 2, then 2 → 1) should be discussed. A biphasic melting profile for a nucleic acid will be obtained if (1) the extinction of the products of the following reaction is significantly different from the extinction of the reactants: poly(Ux)·poly(A)·poly(Uy) → poly(Ux)·poly(A) + poly(Uy); (2) a second requirement is that there must be an inversion of the relative stabilities of the double- and triple-helical forms; i.e., stepwise melting of the triplex simply implies that at the $T_m$ for the dissociation of the third strand, the double helix becomes more stable

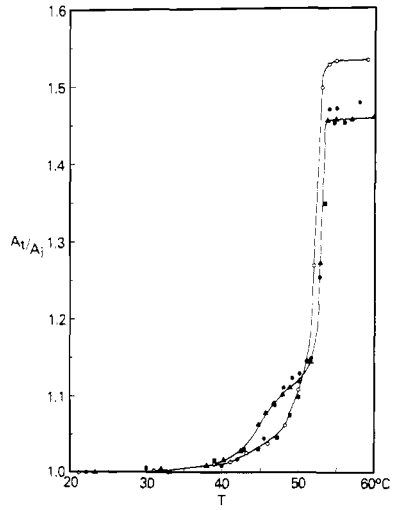


FIGURE 9: Melting profiles for the duplexes poly(U)·poly(A) (○) and poly(dUz)·poly(A) (■), and the products of the reactions poly(U)·poly(A) + poly(dUz) (▲) and poly(dUz)·poly(A) + poly(U) (●). All profiles were determined in 0.05 M NaCl–0.01 M sodium cacodylate (pH 7) at 260 nm. No other transitions were revealed at a variety of other wavelengths.

than the triple helix. The most thoroughly studied triplex, poly(U)·poly(A)·poly(U), undergoes separate 3 → 2 and 2 → 1 transitions in solutions of low salt (e.g., Figure 2). This melting behavior does not hold for a number of previously studied systems: poly(rT)·poly(A)·poly(rT) (Howard et al., 1971); poly(br$^5$U)·poly(A)·poly(br$^5$U) (Riley and Paul, 1970) and poly(dUz)·poly(A)·poly(dUz) (Torrence et al., 1973a; see also Figure 9). All undergo direct 3 → 1 melting even at low ionic strength. Figure 9 shows the melting profiles obtained for poly(U)·poly(A) and poly(dUz)·poly(A), the interactions of poly(dUz) with poly(U)·poly(A), and the interaction of poly(U) with poly(dUz)·poly(A). While the differences are less pronounced than in the previous examples, the following observations may be made. (1) The profile for the mixture of poly(dUz) with poly(U)·poly(A) is identical with the profile of the poly(U) + poly(dUz)·poly(A) system. (2) Two transitions appear to occur in the poly(U) + poly(dUz)·poly(A) system (or the poly(dUz) + poly(U)·poly(A) system). The second higher melting transition is identical with the melting of poly(dUz)·poly(A) and is 1 °C higher than the melting of the poly(U)·poly(A) duplex. (3) There is no evidence for the existence of the poly(U)·poly(A)·poly(U) triplex in either of the two interacting homopolymer duplex systems. Furthermore neither poly(dUz) nor poly(U) could be detected by thermal profile in an equimolar mixture of poly(dUz) + poly(U)·poly(A) (0.15 M NaCl–0.01 M sodium cacodylate–0.01 M $Mg^{2+}$ (pH 7)) (data not illustrated). We have previously shown that free poly(U) can be detected in mixtures when such a buffer is employed (De Clercq et al., 1975a). In addition, while in the same buffer, no free poly(dUz) can be detected in mixtures of poly(A) and poly(dUz) when the ratio of poly(dUz) to poly(A) is 1:1 or 2:1, when the stoichiometry is increased to 3:1, poly(dUz) is easily detected (absorbance change of 0.1 $A_{260}$ unit at $T_m$ for poly(dUz)) (data not illustrated, see also Torrence et al., 1973a).

From such considerations (absence of poly(U), poly(dUz), poly(U)·poly(A)·poly(U), and the same $T_m$ (same as poly(dUz)·poly(A)) for both reaction mixtures), all reactions corresponding to b2, b3, c1, and d1 can be reasonably eliminated leading to the conclusions that:

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(dUz)} \rightarrow \text{poly(dUz)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (9)$$

$$\text{poly(dUz)} \cdot \text{poly(A)} + \text{poly(U)} \rightarrow \text{poly(dUz)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (10)$$

The conclusion that reaction 9 proceeds via the displacement of poly(U) from the poly(U)·poly(A) duplex by poly(dUz) followed by triplex formation in the addition of poly(U) to the poly(dUz)·poly(A) helix is based on several factors including observations 1 and 2 above, and the previous findings that poly(dUz)·poly(A) has a higher $T_m$ (2 °C) than poly(U)·poly(A) (0.21 M NaCl (pH 7), Torrence et al., 1973a) and so the former helix should be the product of a simple displacement reaction (Sigler et al., 1962; Michelson et al., 1967; De Clercq et al., 1976).

Although it remains to be proven, the minor apparent transition that precedes the major higher melting transition in both the poly(U)·poly(A) + poly(dUz) and the poly(dUz)·poly(A) + poly(U) systems may represent a 3 → 2 transition that is partly superimposed on the second transition.

Perfectly consistent with eq 9 and 10 are the results obtained in interferon induction experiments (Table I). When poly(dUz) was added to the PRK cells either before, together with, or after poly(U)·poly(A), there resulted a dramatic drop in the interferon-inducing ability of the active helix. On the other hand, poly(U), added either before, together, with, or after poly(dUz)·poly(A), brought about no increase in interferon titer. Poly(dUz)·poly(A) has been previously shown to be inactive as an inducer of interferon (Torrence et al., 1973b; E. De Clercq, P. F. Torrence, and B. Witkop, unpublished observations). These data provide further evidence against the possibility of reactions corresponding to c1 and d1.

Pancreatic RNase sensitivity and sucrose gradient ultracentrifugation experiments provide additional corroborative evidence for reactions 9 and 10. When poly(dUz) was mixed with poly(U*)·poly(A) the radioactive label remained resistant to RNase degradation (Figure 10a) and did not appear in the fractions corresponding to free poly(U) but instead appeared in fractions sedimenting faster than poly(U*)·poly(A) (Figure 11a). The results obtained with the poly(U*) + poly(dUz)·poly(A) system were also unambiguous. The added poly(U*) label became more resistant to RNase degradation than poly(U*) alone (Figure 10b), and achieved a level of resistance similar to that of the system poly(dUz) + poly(U*)·poly(A). Similarly, the added label sedimented in fractions typical of triple-helical structures with little remaining in the free poly(U*) position (Figure 11b). These results were obtained only when the polymers were incubated for 1 h at 37 °C, then 1 week at 4 °C. Incubation for 1 h at 37 °C alone left much of the added label sensitive to RNase or sedimenting as free poly(U*).

*Evidence for the Poly(rT)·poly(A)·poly(dUz) and the Poly(br$^5$U)·poly(A)·poly(dUz) Triplexes.* The following four interactions have also been examined by interferon-induction experiments and/or uv absorbance–temperature profile measurements:

$$\text{poly(dUz)} + \text{poly(rT)} \cdot \text{poly(A)} \quad (11)$$

$$\text{poly(rT)} + \text{poly(dUz)} \cdot \text{poly(A)} \quad (12)$$

$$\text{poly(dUz)} + \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \quad (13)$$

$$\text{poly(br}^5\text{U)} + \text{poly(dUz)} \cdot \text{poly(A)} \quad (14)$$

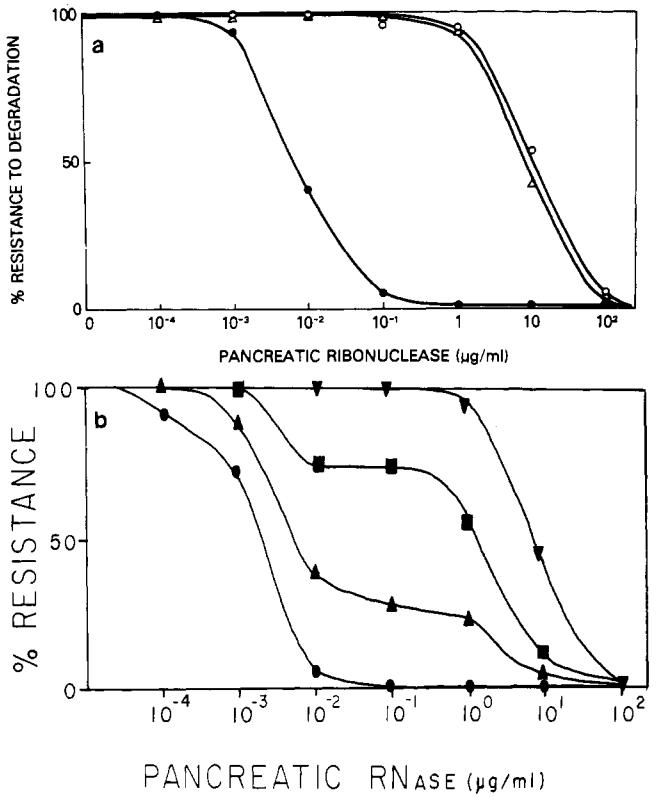


FIGURE 10: (a) Pancreatic ribonuclease A sensitivity of the product of the reaction of poly(U*)·poly(A) with poly(dUz) (▽). The sensitivities of poly(U*) (●) and poly(U*)·poly(A) (○) are included as controls. (b) Pancreatic ribonuclease A sensitivity of the product of the reaction of poly(U*) with poly(dUz)·poly(A) after 1 h reaction time at 37 °C (▲) and after an additional week at 4 °C (■). The sensitivities of poly(U*) (●) and poly(U*)·poly(A) (▼) were determined as controls.

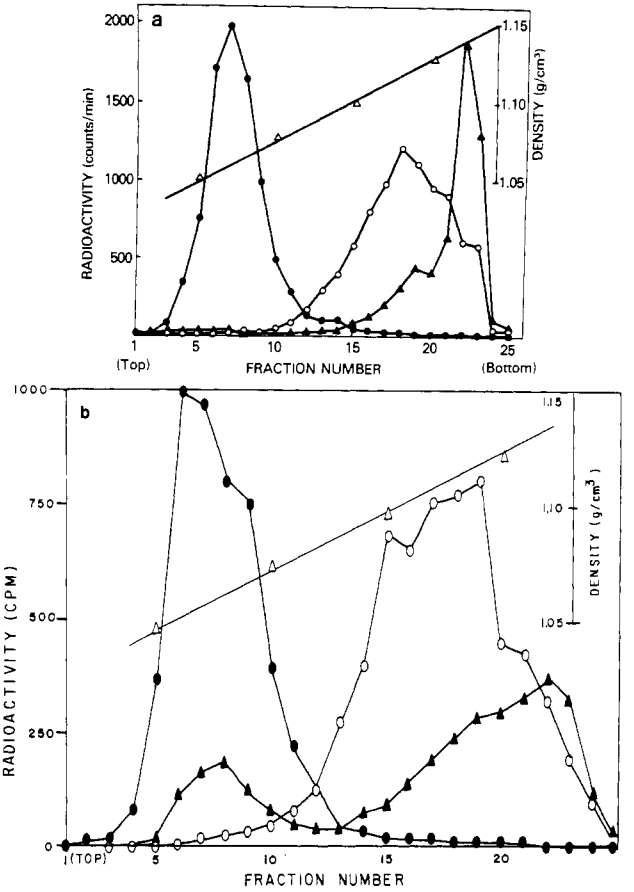


FIGURE 11: (a) Sucrose gradient ultracentrifugal analysis of the interaction of poly(U*)·poly(A) + poly(dUz) (▲) with poly(U*) (●) and poly(U*)·poly(A) (○) as controls. Mixtures were incubated for 1 h at 37 °C before analysis. (b) Ultracentrifugal analysis of the reaction between poly(U*) and poly(dUz)·poly(A) after 1-h incubation at 37 °C followed by a further storage at 4 °C for 1 week (▲). The profiles of poly(U*) (●) and poly(U*)·poly(A) (○) are included as controls. If the centrifugation was carried out after only 1 h incubation at 37 °C, nearly all the poly(U*) label sedimented as free poly(U*).

Poly(dUz), added before, together with, or after the active poly(rT)·poly(A) helix, caused large reductions in the interferon titer (Table I). The addition of poly(rT), following the same protocol, to poly(dUz)·poly(A) produced no rise in interferon titer. Not one of these four systems shows a biphasic melting profile (in 0.06 M $Na^+$ (pH 7)); instead all four systems melt monophasically at the $T_m$ of the higher melting duplex (poly(rT)·poly(A) or poly(br$^5$U)·poly(A)). Thermal profiles for the latter two systems (in 0.01 M $Mg^{2+}$) fail to reveal the presence of either poly(dUz) or poly(br$^5$U). These data are consistent with the formation of the poly(rT)·poly(A)·poly(dUz) and poly(br$^5$U)·poly(A)·poly(dUz) triplexes, but demonstrate that, most probably, under the conditions investigated, the triple helices are always thermodynamically more stable than the corresponding double helices.

*Poly(U)·Poly(A) + Poly(m$^3$U).* Poly(m$^3$U) (Szer and Shugar, 1961) in which hydrogen-bonding capability at the N-3 atom is blocked, should not be able to form a triple helix with poly(U)·poly(A) and therefore should not reduce the interferon titer of poly(U)·poly(A). As is evident in Table I, no significant reduction in titer was obtained when poly(m$^3$U) was added prior to, together with or after the active poly(U)·poly(A)helix.

*Poly(U)·Poly(A) + Poly(dT), Poly(U)·Poly(A) + Poly(dU), and Poly(U)·Poly(A) + Poly(Um).* Previous studies have provided ample evidence for the existence of a nucleic acid triple helix derived from the interaction of poly(dT)·poly(A) and poly(U) (Riley et al., 1966; Stollar and Raso, 1974). The melting profile for an equimolar mixture of poly(dT) with poly(U)·poly(A) (data not shown) exhibited a minor transition at 52.5 °C (corresponding to melting of poly(U)·poly(A) duplex probably due to experimental error or failure of this system to attain equilibrium) and two major transitions: the second corresponded to the melting of poly(dT)·poly(A), and the first may represent the poly(dT)·poly(A)·poly(U)→poly(A)·poly(dT) + poly(U) transition that was not observed in earlier studies since a high salt concentration (0.5 M NaCl) was employed. This behavior indicates that in this instance, as in previous ones, a displacement reaction first occurs to form the more stable duplex and then the poly(U) strand adds in subsequent triplex formation:

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(dT)} \rightarrow \text{poly(dT)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (15)$$

Again, as with many other mixed triplex systems, the melting curve for the interaction of poly(dU) with poly(U)·poly(A) gave rise to a biphasic profile with the second transition occurring at the $T_m$ of the poly(U)·poly(A) (higher melting) duplex. The first transition at $T_m$ = 40 °C cannot be due to the melting of poly(U)·poly(A), since this duplex (and analogous triplex) melts at 47 °C under comparable conditions (Zmudzka et al., 1969). The most reasonable interpretation of this profile is that it represents the biphasic

(3 → 2, 2 → 1) melting of the poly(U)·poly(A)·poly(dU) triplex. Thus,

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(dU)} \rightarrow \text{poly(U)} \cdot \text{poly(A)} \cdot \text{poly(dU)} \quad (16)$$

The reaction of poly(U)·poly(A) with poly(Um) also gave a biphasic melting profile (data not illustrated). The second transition corresponded to the melting of the more stable poly(Um)·poly(A) duplex (e.g., see Zmudzka and Shugar, 1970) so that the lower melting transition is most likely the dissociation of the poly(U) strand from the poly(Um)·poly(A) duplex. Thus

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(Um)} \rightarrow \text{poly(Um)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (17)$$

The interactions of poly(dT), poly(dU), and poly(Um) with poly(U)·poly(A) were also investigated using interferon induction in "superinduced" PRK cells. All three homopolymers, poly(dT), poly(dU), and poly(Um), were able to bring about a large reduction in the cell's interferon response to poly(U)·poly(A) whether the polymers were added to the cells mixed with poly(U)·poly(A) or 1 h before poly(U)·poly(A) (Table I). Poly(dU) was somewhat less effective in this regard than was poly(dT) or poly(Um), but this might be expected since, of the three triple helices, poly(U)·poly(A)·poly(dU) has the lowest $T_m$ (3 → 2) and may exist only partially as a triplex under physiological conditions.

## Discussion

Evidence has been presented herein to support the occurrence of the following reactions and resultant polynucleotide triple helices:

$$\text{poly(br}^5\text{U)} \cdot \text{poly(A)} + \text{poly(U)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (1)$$

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(br}^5\text{U)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (2)$$

$$\text{poly(A)} \cdot \text{poly(U)} + 2\text{poly(br}^5\text{U)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(br}^5\text{U)} + \text{poly(U)} \quad (3)$$

$$\text{poly(br}^5\text{U)} \cdot \text{poly(A)} + \text{poly(rT)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(rT)} \quad (5)$$

$$\text{poly(rT)} \cdot \text{poly(A)} + \text{poly(br}^5\text{U)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(rT)} \quad (6)$$

$$\text{poly(rT)} \cdot \text{poly(A)} + \text{poly(U)} \rightarrow \text{poly(rT)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (7)$$

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(rT)} \rightarrow \text{poly(rT)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (8)$$

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(dUz)} \rightarrow \text{poly(dUz)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (9)$$

$$\text{poly(dUz)} \cdot \text{poly(A)} + \text{poly(U)} \rightarrow \text{poly(dUz)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (10)$$

$$\text{poly(rT)} \cdot \text{poly(A)} + \text{poly(dUz)} \rightarrow \text{poly(rT)} \cdot \text{poly(A)} \cdot \text{poly(dUz)} \quad (11)$$

$$\text{poly(dUz)} \cdot \text{poly(A)} + \text{poly(rT)} \rightarrow \text{poly(rT)} \cdot \text{poly(A)} \cdot \text{poly(dUz)} \quad (12)$$

$$\text{poly(br}^5\text{U)} \cdot \text{poly(A)} + \text{poly(dUz)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(dUz)} \quad (13)$$

$$\text{poly(dUz)} \cdot \text{poly(A)} + \text{poly(br}^5\text{U)} \rightarrow \text{poly(br}^5\text{U)} \cdot \text{poly(A)} \cdot \text{poly(dUz)} \quad (14)$$

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(dT)} \rightarrow \text{poly(dT)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (15)$$

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(dU)} \rightarrow \text{poly(U)} \cdot \text{poly(A)} \cdot \text{poly(dU)} \quad (16)$$

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(Um)} \rightarrow \text{poly(Um)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (17)$$

Two analogous reactions were demonstrated in a separate study (De Clercq et al., 1975b):

$$\text{poly(U)} \cdot \text{poly(A)} + \text{poly(dUf)} \rightarrow \text{poly(dUf)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (18)$$

$$\text{poly(dUf)} \cdot \text{poly(A)} + \text{poly(U)} \rightarrow \text{poly(dUf)} \cdot \text{poly(A)} \cdot \text{poly(U)} \quad (19)$$

From these reactions, the generalization may be made that when 1 mol of the homopolymer poly(Uy) interacts with 1 mol (2 mol of P) of the duplex poly(Ux)·poly(A), one of two reactions occurs:

$$\text{poly(Ux)} \cdot \text{poly(A)} + \text{poly(Uy)} \rightarrow \text{poly(Ux)} \cdot \text{poly(A)} \cdot \text{poly(Uy)} \quad (a)$$

if the $T_m$ of poly(Ux) · poly(A) is higher than the $T_m$ of poly(Uy) · poly(A); or

$$\text{poly(Ux)} \cdot \text{poly(A)} + \text{poly(Uy)} \rightarrow \text{poly(Uy)} \cdot \text{poly(A)} \cdot \text{poly(Ux)} \quad (b)$$

if the $T_m$ of poly(Ux)·poly(A) is lower than the $T_m$ of poly(Uy)·poly(A). This assumes that both the homopolymers poly(Ux) and poly(Uy) are capable of double-helix formation with the complementary homopolymer, poly(A).

Two properties of triple-helical nucleic acids are of special interest with regard to hypothetical roles such triplexes may assume in the structure or expression of genes, or other phenomena. (a) For instance, a poly(dA)·poly(dT) segment of double-helical DNA can interact with a poly(U) stretch in an RNA *without disrupting the DNA double helix*. The poly(U)-RNA would simply add via Hoogsteen hydrogen bonding to the outside of the DNA duplex. (b) Depending on the composition of the triple helix, conditions (e.g., of ionic strength, divalent metal ion, etc.) may be found *wherein a triplex of one composition may retain a triple-stranded structure, whereas a triplex of another composition would exist as a free duplex and free homopolymer*. The potential effect of modifications or fraudulent nucleosides on the above properties is obvious.

As with the case of the poly(U)·poly(A)·poly(I) (De Clercq et al., 1975a) triplex and polynucleotide displacement reactions (De Clercq et al., 1976), interferon induction as measured in PRK cells, "superinduced" with cycloheximide and actinomycin D, provides a valuable technique for detecting triple-helix formation among polynucleotides. Unfortunately, due to the requirement of the interferon induction system for intact 2′-OH groups in both strands of the double helix (De Clercq, 1974), the interferon induction assay cannot be employed to monitor triple-helix formation of the reacting double helix in either a DNA duplex or a DNA–RNA hybrid; however, it is highly sensitive for the

detection of triple-helix formation between RNA double helices and other polymers whether they be ribo- or deoxyribopolynucleotides. It is of interest to examine the probable course of events during the three different procedures used to assess triple-helix formation; that is, homopolymer added to the cells 1 h before, together with, or 1 h after the active interferon-inducing duplex. Various studies have established that synthetic polynucleotide homopolymers and duplexes become cell-associated when incubated with the cells either at 0 or 37 °C. The amount of cell-associated polymer varies from 0.2 to 1.5% depending on input concentration, conditions, and cell type (Bausek and Merigan, 1969; Pitha and Carter, 1971; Bachner et al., 1975; De Clercq and De Somer, 1972, De Clercq et al., 1972). Furthermore, it has not been unequivocally established whether the hypothetical receptor for interferon inducers is intracellular or membrane associated. It has been previously established that homopolymers, such as poly(I) and poly(C), administered sequentially to the cells, reunite at the cellular level, and induce interferon (De Clercq and De Somer, 1972). In light of the above data several assumptions may be made to rationalize the events occurring during the three different experimental protocols.

Situation 1. Active duplex + homopolymer mixed, then applied to cells. Result: mixed triplex forms which no longer induces interferon.

Situation 2. Active duplex is added to cells first. Cells are then washed removing the greater percentage of added duplex. Now the added homopolymer is in excess and

$$\text{poly(Ux)} \cdot \text{poly(A)} + 2\text{-poly(Uy)} \rightarrow \text{poly(Uy)} \cdot \text{poly(A)} \cdot \text{poly(Uy)}$$

if and only if the $T_m$ of poly(Uy)·poly(A) is greater than the $T_m$ of poly(Ux)·poly(A); otherwise, the poly(Ux)·poly(A)·poly(Uy) mixed triplex forms. Both possible products are inactive as interferon inducers.

Situation 3. The homopolymer is added to the cells first, the cells are washed, and active duplex is added. (Assumption: greater part of homopolymer is washed away, only a small percentage remaining cell-associated.) The active duplex is therefore in excess. Even in the excess of duplex, the mixed triple helix would form, but enough of the duplex should remain to trigger the interferon response. In order to account for the significant reduction in interferon titer obtained when polymers, such as poly(dUz), poly(dT), and poly(Um), are added to the cells first it may be hypothesized that (a) such polymers are bound to the cells more effectively than double-helical polymers, such as poly(A)·poly(U) and poly(I)·poly(C), and/or that (b) the triple helices that do form inhibit the induction of interferon by the active duplexes. It has been demonstrated earlier (De Clercq et al., 1974a) that certain triplexes (e.g., poly(U)·poly(A)·poly(U) and poly(rT)·poly(A)·poly(rT)) are capable of inhibiting the interferon response of active inducers. It is important that these homopolymers that are most effective in reducing the interferon titer of the active complexes, when added to the cells before the active complex, are those which are virtually totally resistant to degradation by pancreatic ribonuclease A (e.g., poly(dUz), poly(dUf), poly(dT), poly(Um), and poly(dU)), whereas those polymers that are ineffective in reducing the interferon titer under these conditions are those which are markedly more sensitive (poly(U), poly(rT), and poly(br$^5$U)) to degradation by RNase A. To carry the analogy further, poly(I), also resistant to RNase A, is nearly as effective in reducing the titer of poly(A)·poly(U) when the former is added to the cells 1 h prior to the active duplex as it is when added as a mixture with poly(A)·poly(U) (De Clercq et al., 1975a). Whatever the exact modus operandi might be, it is clear that the dramatic reduction in interferon titer, obtained after sequential administration of poly(I), poly(dUz), poly(dT), etc., and poly(A)·poly(U) and poly(A)·poly(rT), stems from the formation of a mixed triple helix at the cellular level. The results further imply that polymers such as poly(dUz), poly(dT), poly(I), etc., once added to the cells, become firmly associated with the cells, and are not degraded (by cell associated nucleases). Triplex formation with the subsequently added poly(A)·poly(U) or poly(A)·poly(rT) would most likely occur at the cell surface.

## References


Arnott, S., and Bond, P. J. (1973), *Nature (London), New Biol. 244,* 99–101.

Bachner, L., De Clercq, E., and Thang, M. N. (1975), *Biochem. Biophys. Res. Commun. 63,* 476–483.

Baril, B., and Kubinski, H. (1975), *Nature (London) 255,* 252–253.

Bausek, G. H., and Merigan, T. C. (1969), *Virology 39,* 491–498.

Blake, R. D., Massoulié, J., and Fresco, J. R. (1967), *J. Mol. Biol. 30,* 291–308.

Bloomfield, V. A., Crothers, D. M., and Tinoco, Jr., I. (1974), Physical Chemistry of Nucleic Acids, New York, N.Y., Harper and Row, p 331.

Britten, R. J., and Davidson, E. H. (1969), *Science 165,* 349–357.

Clark, R. J., and Felsenfeld, G. (1972), *Nature (London), New Biol. 240,* 226–229.

Crick, F. (1971), *Nature (London) 234,* 25–27.

De Clercq, E. (1974), *Top. Curr. Chem. 52,* 173–208.

De Clercq, E., and De Somer, P. (1972), *J. Virol. 9,* 721–731.

De Clercq, E., Janik, B., and Sommer, R. G. (1975b), *Chem.-Biol. Interact.,* submitted for publication.

De Clercq, E., Torrence, P. F., De Somer, P. and Witkop, B. (1975a), *J. Biol. Chem. 250,* 2521–2531.

De Clercq, E., Torrence, P. F., and Witkop, B. (1974a), *Proc. Natl. Acad. Sci. U.S.A. 71,* 182–186.

De Clercq, E., Torrence, P. F., and Witkop, B. (1976), *Biochemistry,* preceding paper in this issue.

De Clercq, E., Torrence, P. F., Witkop, B., Stewart II, W. E., and De Somer, P. (1974b), *Science 186,* 835–837.

De Clercq, E., Wells, R. D., and Merigan, T. C. (1972), *Virology 47,* 405–415.

Howard, F. B., Frazier, J., and Miles, H. T. (1971), *J. Biol. Chem. 246,* 7073–7086.

Kim, S. H., Sussman, J. L., Suddath, F. L., Quigley, G. J., McPherson, A., Wang, A. H. J., Seeman, N. C., and Rich, A. (1974), *Proc. Natl. Acad. Sci. U.S.A.,* 4970–4974.

Kubinski, H., Opara-Kubinska, Z., and Szybalski, W. (1966), *J. Mol. Biol. 20,* 313–329.

Mazen, A., and Champagne, M. (1968), *Bull. Soc. Chem. Biol. 50,* 1601–1613.

Michelson, A. M., Massoulié, J., and Guschlbauer, W. (1967), *Prog. Nucleic Acid Res. Mol. Biol. 6,* 83–141.

Ohba, Y. (1966), *Biochim. Biophys. Acta 123,* 84–90.

Opara-Kubinska, Z., Kubinski, H., and Szybalski, W. (1964), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3684–3688.

Perlgut, L. E., Byers, D. L., Jope, R. S., and Khamvinwathna, V. (1975), *Nature (London) 254,* 86-87.
Pettijohn, D. E., and Hecht, R. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 31-41.
Pitha, P. M., and Carter, W. A. (1971), *Virology 45,* 777-781.
Riley, R., Maling, B., and Chamberlin, M. J. (1966), *J. Mol. Biol. 20,* 359-389.
Riley, M., and Paul, A. V. (1970), *J. Mol. Biol. 50,* 439-455.
Rottman, F., Frederici, K., Comstock, P., and Khurshed-Khan, M. (1974), *Biochemistry 13,* 2762-2771.
Shenkin, A., and Burdon, R. H. (1974), *J. Mol. Biol. 85,* 19-39.
Sigler, R. B., Davies, D. R., and Miles, H. T. (1962), *J. Mol. Biol. 5,* 509-517.
Sirlin, J. L. (1972), Biology of RNA, New York, N.Y., Academic Press, pp 130-132.
Stollar, B. D., and Raso, V. (1974), *Nature (London) 250,* 231-234.
Szer, W., and Shugar, D. (1961), *Acta Biochim. Polon. 8,* 235-248.
Tan, Y. H., Armstrong, J. A., Ke, Y. H., and Ho, M. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 464-471.
Torrence, P. F., Bobst, A. M., Waters, J. A., and Witkop, B. (1973a), *Biochemistry 12,* 3962-3972.
Torrence, P. F., Waters, J. A., Buckler, C. E., and Witkop, B. (1973b), *Biochem. Biophys. Res. Commun 52,* 890-898.
Torrence, P. F., and Witkop, B. (1975), *Biochim. Biophys. Acta 395,* 56-66.
Vilcek, J. (1970), *Ann. N.Y. Acad. Sci. 173,* 390-403.
Yarus, M. (1969), *Annu. Rev. Biochem. 38,* 841-880.
Zmudzka, B., Bollum, F. J., and Shugar, D. (1969), *J. Mol. Biol. 46,* 169-184.
Zmudzka, B., and Shugar, D. (1970), *FEBS Lett. 8,* 52-54.

# Synchronous Digestion of SV40 DNA by Exonuclease III†

Ray Wu,* George Ruben, Benjamin Siegel, Ernest Jay, Paul Spielman, and Chen-pei D. Tu

ABSTRACT: We have established an optimal condition for the synchronous digestion of SV40 DNA with *Escherichia coli* exonuclease III. Electron microscopy and polyacrylamide gel electrophoresis were used to obtain accurate measurements on the lengths of DNA before and after exonuclease III digestion. Based on this finding, a new method for determining the sequence of long duplex DNA can be realized. It involves (a) the synchronous digestion of the DNA from the 3′ ends with exonuclease III, followed by (b) repair synthesis with labeled nucleotides and DNA polymerase, and (c) sequence analysis of the repaired DNA.

*Escherichia coli* exonuclease III catalyzes the sequential hydrolysis of mononucleotides from the 3′ termini of duplex DNA (Richardson et al., 1964). At 5 °C, and using a high concentration of salt, exonuclease III catalyzes the synchronous hydrolysis of approximately six nucleotides from each 3′ terminus of the duplex DNA (Donelson and Wu, 1972). The use of this enzyme to produce single-stranded 5′ ends, followed by repair synthesis to label the ends, constitutes a method for the analysis of short sequences from the ends of a duplex DNA (Wu et al., 1972; Ghangas et al., 1973; Bambara and Wu, 1975).

Based on the above principles, a method is being developed for determining long sequences from the ends of a duplex DNA. In this method, exonuclease III is used to remove a large number of nucleotides (e.g., from 40 to 400 nucleotides) to produce long single-stranded ends (Figure 1, step 1). This DNA molecule can then be labeled with radioactive nucleotides (step 2), digested with a restriction enzyme so that the two labeled ends can be separated (step 3), and sequenced with one of the current methods, such as ribo substitution, for DNA sequence analysis (see review articles by Murray and Old, 1974; Salser, 1974; Wu et al., 1974), or with a new method to be perfected. A crucial factor in the success of this method is the degree to which the exonuclease III digestion proceeds synchronously under the optimal condition of incubation (step 1). In other words, for ease of sequence analysis, all DNA molecules should be digested to approximately the same extent.

In this communication, we describe our establishment of an optimal condition for the synchronous digestion of SV40 DNA with exonuclease III so that the method depicted in Figure 1 can now serve to determine long DNA sequences from each 3′ terminus of any duplex DNA.

## Experimental Section

### *Materials*

*Cell Lines and Virus.* Two lines of African green monkey kidney cells, CV-1 and TC-7, were kindly supplied by J.

† From the Department of Biochemistry, Molecular and Cell Biology, (R.W., G.R., E.J., P.S., and C.T.), and the Department of Applied Physics (G.R. and B.S.), Cornell University, Ithaca, New York 14853. *Received September 29, 1975.* This work was supported by Research Grant CA-14989, awarded by the National Cancer Institute, DHEW to R.W., Grant GM-16195 (awarded to B.S.) from the National Institutes of Health, and BMS 73-01859 A01 (R.W.) and GB-30593X (B.S.) from the National Science Foundation. This is paper XXIV in a series on Nucleotide Sequence Analysis of DNA. Paper XXIII is by Marians, Padmanabhan, and Wu (1975).