

## Stabilization of Two-Stranded Ribohomopolymer Helices and Destabilization of a Three-Stranded Helix by Ethidium Bromide

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Thermal-denaturation profiles of helical polynucleotides have been measured in the presence of increasing concentrations of ethidium bromide. The poly(A)·poly(U) helix is strongly stabilized by binding of ethidium, to much the same extent as is DNA, but the stabilizing effect on poly(I)·poly(C) is much smaller. In the poly(A)·2poly(U) system the drug selectively destabilizes and eventually destroys the triple helix, leaving only the double-helix-to-coil transition.

Ethidium bromide is a trypanocidal drug which readily forms complexes with nucleic acids *in vitro* (Waring, 1965, 1966). There is abundant evidence that its trypanocidal activity, as well as its effects in other biological systems, can be ascribed to its binding to DNA with consequent impairment of nucleic acid synthesis (reviewed by Waring, 1972, 1974). In recent years it has acquired importance as an experimental probe of conformational properties of circular DNA (Crawford & Waring, 1967) and tRNA (Cantor *et al.*, 1971; Tritton & Mohr, 1973), as an aid to the assay of the activity of nucleic acid-associated enzymes (Le Pecq, 1971) and in a density-gradient procedure for the isolation of closed circular duplex DNA from natural sources (Radloff *et al.*, 1967). For these reasons it is important to gain as much information as possible about the nature and specificity of the interaction between ethidium bromide and nucleic acids.

Binding of ethidium to DNA occurs by a strong primary process attributed to intercalation (Fuller & Waring, 1964) followed by a much weaker secondary process believed to involve stacking of the drug molecules on the outside of the DNA helix (Waring, 1965). Only very weak interaction occurs when ethidium is added to single-stranded synthetic homopolyribonucleotides *in vitro*, but with natural RNA or double-helical structures formed by mixing complementary pairs of homopolyribonucleotides the strong intercalative binding predominates again (Waring, 1965, 1966; Douthart *et al.*, 1973). In the present paper it is shown that double-helical structures are stabilized by binding of ethidium, but to different extents depending on the nature of the nucleotide pairs, whereas the formation of a triple-stranded helix is impeded and eventually abolished in the presence of the drug.

### Materials and methods

Ethidium bromide was kindly provided by Dr. G. Woolfe of the Boots Co., Nottingham, U.K. Solutions were freshly prepared and maintained in

the dark. All buffers were made up in glass-distilled water with A.R.-grade chemicals. DNA from *Escherichia coli* was extracted by the method of Marmur (1961); calf thymus DNA (highly polymerized) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Both preparations were further purified by phenol extraction and reprecipitation as previously described (Waring, 1965). Polyribonucleotides were purchased from Miles Chemical Co., Elkhart, Ind., U.S.A. They were extracted with phenol, dialysed and stored frozen, the procedures and precautions outlined by Chamberlin & Patterson (1965) being used to minimize degradation. Helical complexes of complementary polymers were allowed to form in the appropriate buffer as previously described (Waring, 1966).

Thermal-denaturation profiles ('melting' curves) were measured in a Unicam SP. 500 series II spectrophotometer with Teflon-stoppered quartz cuvettes of 10mm light-path placed in a water-jacketed cell holder. One of the cuvettes contained a thermistor probe, the resistance of which was continuously monitored to provide a direct measurement of the temperature of the liquid inside the cuvettes. Water was circulated through the cell holder from a Haake thermostatically controlled water bath at a flow rate of 10 litres/min. The temperature of the circulating water was increased at the rate of 0.5°C/min by coupling the contact thermometer of the water bath to a synchronous motor revolving at 1 rev./min. Readings of absorbance were taken at intervals of 30-60s, providing an essentially continuous trace of absorbance versus temperature. The wavelength of measurement was 260nm, except for the experiments with poly(I)·poly(C) where, the absorbance was monitored at 250nm to take advantage of the larger hyperchromicity at this wavelength. Actual hyperchromicity values (i.e. fractional increase in absorbance above the starting value measured at room temperature) for the various polymer systems in the absence of drug were as follows: poly(A)·poly(U), 56%; poly(I)·poly(C), 69% (at 250nm); poly(A)·2poly(U), 72%; *E. coli* DNA, 25%; calf thymus

DNA, 37%. In all experiments the polymer concentration was  $50\ \mu\text{M}$  with respect to nucleotides. Values of  $T_m$  (mid-point of the hyperchromic transition) were determined as the temperature at which the absorbance attained a value half-way between the values immediately before and after the transition, which was characteristically sharp for the synthetic polymers; consequently no correction for cuvette expansion was necessary. No adsorption of ethidium on the walls of the quartz cuvettes was observed.

### Results and discussion

Fig. 1(a) shows a series of 'melting' curves for poly(A)·poly(U) determined in the presence of increasing concentrations of ethidium. It is clear that this double-helical structure is strongly stabilized by binding of the drug. When similar experiments were performed with the poly(I)·poly(C) helix, stabilization was again observed, but the magnitude of the effect was much smaller (Fig. 2a). In both cases the hyperchromic transitions at high drug ratios were sharp, like the control, but at low ratios they were perceptibly broadened and even showed signs of heterogeneous 'melting' behaviour (cf. also the upper portions of the curves in Fig. 1b, which also corre-

spond to the double-helix-to-coil transition). This broadening no doubt reflects redistribution of bound drug molecules among remaining helical segments as the secondary structure collapses, which could be anticipated from the known rapidity of the association and dissociation reactions with RNA (Bittman, 1969; Tritton & Mohr, 1973). The increase in  $T_m$  seen with the poly(A)·poly(U) system is of the same order as that occurring with DNA (Fig. 2b) and naturally occurring helical RNA (Douthart *et al.*, 1973). The anomaly, then, lies in the meagre stabilization of the poly(I)·poly(C) helix. It was not entirely unexpected, since binding measurements performed at room temperature had revealed considerably weaker interaction with poly(I)·poly(C) than with poly(A)·poly(U) (Waring, 1966). If this difference in binding energy persists at elevated temperatures it could well account for the difference. In terms of structure the explanation may reside in the precise geometrical arrangement of the two ribopolymer helices; both are known to adopt A-type helical conformations different from the tenfold B-helical form of DNA (Arnott *et al.*, 1968), and there is evidence that the poly(I)·poly(C) helix exists in the 12-fold A' form in solution (Bram, 1971). It is also noteworthy that simple aliphatic diamines generally have a more

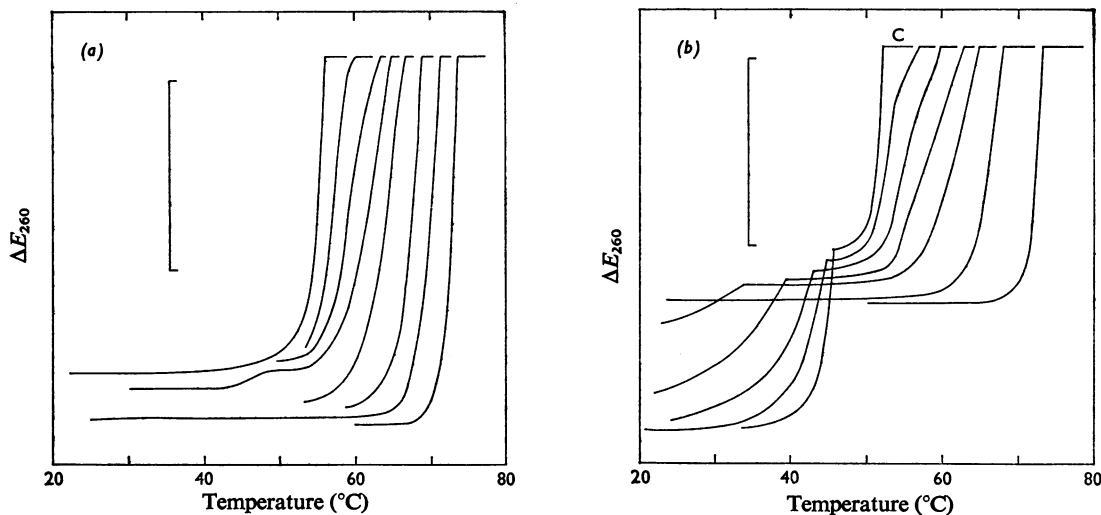


Fig. 1. Effect of ethidium bromide on the thermal-denaturation profile of (a) poly(A)·poly(U) in 90 mM-NaCl-10 mM-Tris-HCl-0.1 mM-EDTA (pH 7.9 at 20°C), and (b) poly(A)·2poly(U) in 50 mM-NaCl-10 mM-Tris-HCl-0.1 mM-EDTA (pH 7.9 at 20°C)

Bars represent an absorbance change of 0.1 unit. In each plot the curves have been normalized to the same absorbance above the hyperchromic transition; the small differences in total hyperchromicity in (a) and in the upper portions of curves in (b) are due to release of bound ethidium, which absorbs significantly at 260 nm. In (a) the control curve is at the extreme left; proceeding to the right the curves correspond to complexes having drug/nucleotide ratios of 0.014, 0.036, 0.072, 0.107, 0.215, 0.358 and 0.572. In (b) the control curve (C) is in the centre, showing a triple-to-double-helix transition at 44.3°C and a double-helix-to-coil transition at 51.5°C. Successive curves show the denaturation profiles of complexes having drug/nucleotide ratios of 0.014, 0.029, 0.050, 0.072, 0.145 and 0.434.

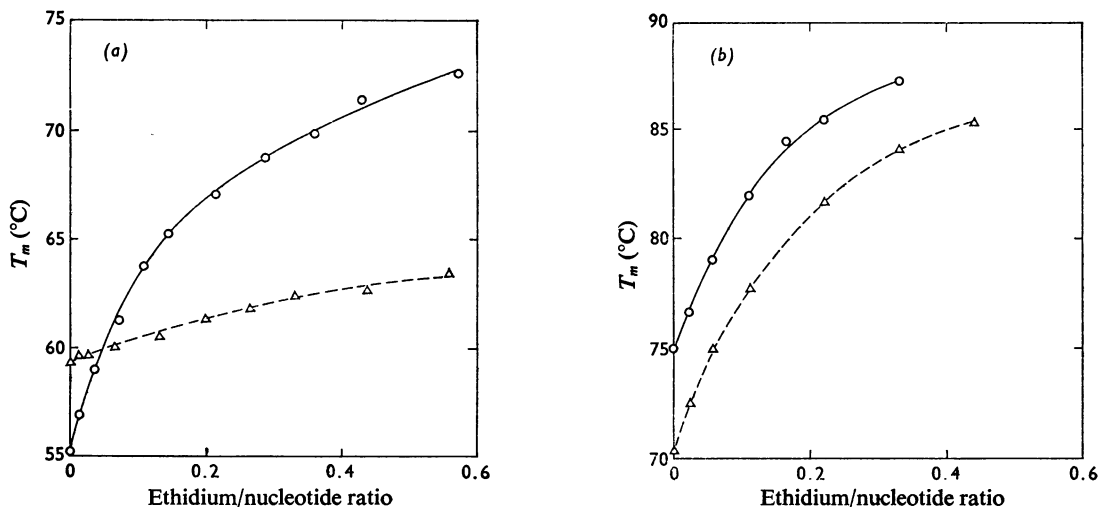


Fig. 2. Elevation of the transition mid-point ('melting' temperature) of double-helical polymers by ethidium bromide

(a) Poly(A)·poly(U) (○) and poly(I)·poly(C) (△) measured in 90mM-NaCl-10mM-Tris-HCl-0.1mM-EDTA (pH 7.9 at 20°C). (b) *E. coli* DNA (○) and calf thymus DNA (△) measured in 10mM-NaCl-10mM-Tris-HCl-1mM-EDTA (pH 7.9 at 20°C).

marked stabilizing effect on poly(A)·poly(U) than on poly(I)·poly(C) (Gabbay, 1966).

Additional experiments were performed with the poly(A)·poly(I) system, to which ethidium binds quite strongly (Waring, 1966). Stabilization was occasionally observed, but not in a consistently reproducible fashion. The reason for this variability is unknown at present.

The most clear-cut evidence of selectivity in the effects of ethidium on the stability of helical polymers was obtained with the poly(A)·2poly(U) system. Fig. 1(b) shows that under ionic conditions where the break-up of the triple helix occurs in two distinct phases, with an intermediate plateau corresponding to the double helix plus a displaced poly(U) strand (Stevens & Felsenfeld, 1964), ethidium specifically destabilizes the three-stranded helix while stabilizing the two-stranded form as before. Above a drug/nucleotide ratio of approximately 0.1 it promotes essentially complete loss of the third strand at room temperature, so that only the double-helix-to-coil transition remains. There have been previous reports that intercalating aminoacridines appear to shift the triple-helix-double-helix equilibrium in favour of the two-stranded form (Lerman, 1964), and that the fluorescence of ethidium is enhanced to a greater extent by poly(A)·poly(U) than by poly(A)·2poly(U) (Le Pecq, 1971), but this is the first time that a specific destabilization of the triple helix and disproportionation to yield poly(A)·poly(U) plus a displaced poly(U) strand has been demonstrated. It makes interesting

comparison with the report of Pohl *et al.* (1972) that, above a critical salt concentration, binding of ethidium to poly(dG-dC) is highly co-operative and results in an allosteric change in the polymer structure from the high-salt *L*-form to the low-salt *R*-form. In both cases the presence of ethidium seems to trigger a change in the polymer structure to a form with which it interacts strongly at the expense of the original structure [*L*-form of poly(dG-dC) or triple ribopolymer helix], which probably does not bind the drug at all.

Since the effects of aliphatic diamines and polyamines on poly(A)·2poly(U) differ markedly from those of ethidium [strong stabilization and disproportionation from double helix to triple helix have been reported (Glaser & Gabbay, 1968; Gabbay & Glaser, 1970)] it is possible that the clear differentiation between two- and three-stranded helices seen in Fig. 1(b) may be characteristic of intercalative binding. Similar effects (unpublished) have been observed with the schistosomicidal drug hycanthone, which also binds to DNA by intercalation (Waring, 1970, 1972; cf. also Heller *et al.*, 1974), and it would clearly be of interest to extend the measurements to other known intercalating agents. A case in point is acriflavine, for which Chan & Van Winkle (1969) reported binding constants using the same polymers as in the present work. Their values for interaction between acriflavine and poly(A)·2poly(U) would be consistent with partial rearrangement to poly(A)·poly(U) under the experimental conditions used.

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