

Renaturation of Alkali-Denatured T7 DNA Molecules Complexed with Ethidium Bromide

R. K. Sinha, Papiya Talapatra, Aruna Mitra, and Sandip Mazumdar

Chittaranjan National Cancer Research Centre, Calcutta

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Free and ethidium bromide (EB) complexed alkali denatured T7 DNA molecules were renatured at 58 and 62 °C respectively for 1–3 h. The structures of the renaturation products of the free candidates were as usual showing native like, branched and unrenatured DNA whereas the structures of the ethidium bromide complexed one were somewhat different, showing non-renatured loop-like and entangled regions present inbetween the renatured segments. On the basis of the linear base sequence of T7 DNA, these non-renatured parts are indicative of the inhibition of renaturation by the complexed EB molecules. Mapping of the non-renatured regions showed that they were present at some specific sites, which inturn suggested that the EB-binding has some base sequence specificity.

Introduction

Renaturation of alkali as well as heat-denatured DNA molecules from *E. coli* phage T7 has been studied extensively through spectrophotometry^{1,2}, sedimentation analysis^{3a}, viscometry⁴ and also electronmicroscopy^{5,6}. Since T7 DNA molecules have a linear (non-permutated) base sequence⁵ with slight terminal redundancy, the structures of the renatured DNA molecules, either from the intact or from the fragmented single strands, can be preconceived in the lines of the proposed models of inter-strand renaturation process^{6,7}. Some studies have been made on the renaturation of denatured DNA molecules complexed with intercalating drugs, acridine orange⁸ and nogalamycin⁹, only through spectrophotometry, which could not lead to a definite conclusion because of the fact that the hypochromism at 260 nm, a spectrophotometric measure of renaturation, is also arising from drug-drug interactions⁸ and/or drug-induced base-stacking of denatured DNA^{8,9}. We, therefore, thought that electronmicroscopy would be a potential tool for such studies. In the present paper we have investigated the renaturability of alkali-denatured T7 DNA molecules complexed with ethidium bromide (EB), a cationic, intercalating dye. Since the structural details of the free renatured T7 DNA are well-known^{5,6} it was easy to distinguish between the complexed renaturation- and the free renaturation-

products. The results indicate that the re-naturation of ethidium-bromide complexed denatured T7 DNA is partially inhibited at some specific regions only.

Materials and Methods

Purified bacteriophage T7 was obtained as a gift from Dr. W. C. Summers, U.S.A. DNA from the bacteriophage was extracted by a modified phenol method¹⁰ and was dialysed extensively at 4 °C against sterile 1 BPES (0.006 M Na₂HPO₄, 0.002 M NaH₂PO₄, 0.001 M Na₂-EDTA and 0.179 M NaCl, pH 6.8). Highly polymerised calf thymus (CT) DNA (Type I) and ethidium bromide (EB) were purchased from Sigma Chem. Co., U.S.A. DNA was denatured by alkali following an earlier method¹: to 8 parts of DNA was added 1 part of 1 M NaOH at room temperature, kept as such for 15 min and then added 1 part of 2 M NaH₂PO₄ (in order to bring down the pH from 13 to 7) placing the sample in ice-water mixture. Binding of EB with completely denatured DNA was studied spectrophotometrically following the methods of Waring¹¹ and using CT DNA, instead of T7 DNA, because being highly homogeneous a fraction of denatured T7 DNA would be converted back to the double helical state through the process of renaturation even at room temperature^{3a}. The binding constants were determined using the mass-action formula: $r/c = (n - r)/K$ in which r is the number of bound EB molecules per nucleotide, n the maximum value of r obtained as the intercept on r axis in the plot of r/c versus r (Scatchard's plot^{14,15}), and K , the apparent dissociation constant. The thermal dissociation of the bound dye molecules was followed by recording optical density (O.D.) at 460 nm at

Requests for reprints should be sent to R. K. Sinha, Chittaranjan National Cancer Research Centre, Calcutta-700026, India.



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increasing temperatures in a Carl Zeiss (East Germany) Spectrophotometer fitted with a thermostated water circulating device. The effect on O.D. due to volume expansion at high temperatures was duly taken into account. The renaturation experiments were carried on at 58 and 62 °C for the normal and the complexed denatured T7 DNA respectively in a thermostated water bath for 1–3 h. The samples for electronmicroscopy were prepared according to the protein monolayer technique¹² on parlodion (2% in amyl acetate) coated, 400 mesh copper grids. The necessary contrast was obtained by staining with Uranyl acetate¹³. Electronmicrographs were recorded in a Siemens Elmiskop I. The magnification of the electronmicroscope was routinely checked by means of a cross-grating replica (54800 lines/inch, Polaron Equip. Ltd.). The length measurements of the DNA molecules and the non-renatured zones were made by a map-measurer after enlarging the micrographs to 80000 \times . The entangled nonrenatured parts were measured carefully assuming that the length of the thick portions had to be doubled in order to convert them into a single-stranded length.

Results and Discussions

(1) Spectrophotometric studies on the binding of EB with denatured DNA

EB interacts with denatured DNA in the same manner as with native DNA, showing an optimal red shift of its absorption maximum by 30 nm and an isobestic point at 512 nm. The Scatchard's plot (Fig. 1) indicates that there are two types of binding sites for EB in denatured DNA. The binding constants for these two types are: $n_1 = 0.121$, $n_2 = 0.177$, $K_1 = 2 \times 10^{-7} \text{ M}^{-1}$ and $K_2 = 4 \times 10^{-6} \text{ M}^{-1}$. The higher value of K_2 reveals the weakness of the type 2 binding as compared to the type 1. However, the present value of K_1 is in nice agreement with that ($4 \times$ to $7 \times 10^{-7} \text{ M}^{-1}$, ref. 11) of the process I¹⁵ (intercalation) binding of EB with native DNA. The process I binding is not unlikely here since there can be considerable number of intra-strand base-paired zones^{3b} in denatured DNA at room temperature under a solvent condition comparable with that of the present samples. The type 2 would be the process II or the electrostatic binding between the cationic dye molecules and the anionic surface of the phosphodiester back-bone of DNA. However, this binding is favoured in low molar solvent¹⁶. As the environment of denatured

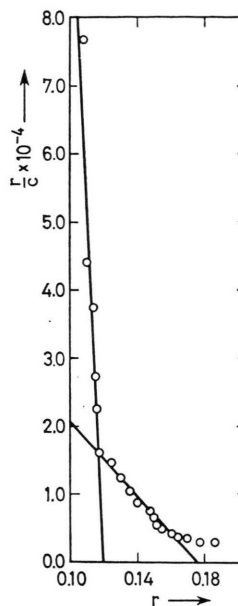


Fig. 1. The Scatchard's plot of binding of ethidium bromide to alkali denatured calf-thymus DNA in BPES buffer (final Na^+ -concentration after alkali denaturation was 0.4 M).

DNA in the present samples is a high molar (0.4 M Na^+) solvent the electrostatic binding is less probable. But like proflavine¹⁶ as well as 9-aminoacridine^{16, 8}, or daunomycin¹⁷ (an intercalating, cationic antibiotic), EB may bind in the mono-strands of DNA, through partial intercalation, each dye molecule placing itself in between the adjacent bases of the same strand, which is consistent with the binding model of Pritchard *et al.*¹⁸. That this may be true is apparent in the value of K_2 which is considerably larger than that for usual process II (K_2 for the process II binding of EB is in the order of 10^{-5} M^{-1} , unpublished results) binding.

Most efficient inter-strand renaturation occurs at elevated temperatures^{19, 20}. In the present experiments the dye-complexed and the free denatured T7 DNA were electromicroscopically found to show optimal renaturation at 62 and 58 °C respectively. It was then necessary to see what fraction of the bound-EB would dissociate at such a high temperature of renaturation. The thermal absorbance profiles (Fig. 2), drawn from O.D.₄₆₀ values, showed gradually increasing hyperchromicity (curve 1), an indication of heat-induced dissociation of the bound-EB molecules. It is evident that the mode of release of EB from the bound to the free state is non-cooperative and may be distinguished from the release of the intercalated ligands, which is cooperative as well as concomitant with helix-coil transition of the ligand-DNA complex^{21, 22}. At 62 °C the complexed

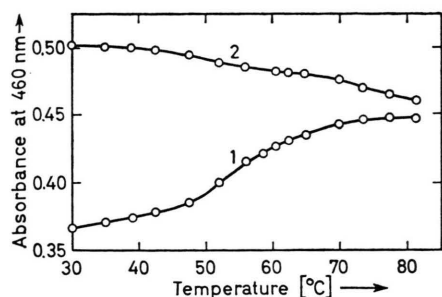


Fig. 2. Thermal absorbance profiles of ethidium bromide (curve 2) and of the complexes of ethidium bromide with alkaline denatured CT DNA at $P/D=4$ (curve 1). P , molar concentration of nucleotide phosphate; D , molar concentration of ethidium bromide.

fraction of the drug molecules is 41% of that at the room temperature. The actually bound EB per nucleotide of denatured DNA at the renaturing temperature is calculated out from these curves and with the help of a Scatchard's plot, as $r=0.045$. It is the dissociation of all the weakly bound and also some of the partially intercalated dye molecules that contributed to the lowering of r value at 62 °C. That the weakly bound cationic ligands are dissociated out in a similar, non-cooperative fashion has also been clearly shown elsewhere^{21, 22}.

(2) Electronmicroscopy

Electronmicroscopic pictures showed that each unit of alkali denatured (Fig. 5 a) T7 DNA, either free or complexed, was highly folded up, compact structures, as displayed elsewhere^{6, 2, 3}. The foldings arise out of random intrastrand base-base associations^{3b}. The renaturation-products of the free denatured T7 DNA molecules were of three types: type 1 — the linear DNA molecules indistinguishable in length as well as form from native T7 DNA molecules, constituting about 60% of the total products, type 2 — the branched DNA molecules (30%) and type 3 — the completely unrenatured, folded up structures of DNA (10%). As alkali-denatured T7 DNA consists of both intact (60–90%) and fragmented (10–40%) single strands^{24, 3b}, the combination of the former gives rise to type 1 products whereas the repeated combinations of the latter lead to type 2 ones. This has been explained in details in earlier papers^{1, 5, 6, 3}. The electronmicrographs are shown in Figs 3 a, b and 4 *. The renaturation-products of the EB-complexed renatured

T7 DNA were of the similar types, but with a very remarkable difference that in most of them (types 1 and 2) were present unrenatured loop-like and entangled regions (Figs 5b–d and 6 **) in between the renatured parts. It has been clearly shown and explained earlier^{5, 7} that since T7 DNA molecules have linear base sequence a successful nucleation (forming a stable minimum length⁷ of duplex), that is formed between any two complementary sites, would help the rest of the bases in the two interacting strands to zipper up automatically, thus leaving no scope for any unrenatured part or parts in whatsoever form (looplike or entangled) to be present in between the renatured segments. The presence of such unrenatured parts exclusively in the renaturations-products of EB-complexed denatured T7 DNA is therefore indicative of a partial inhibition of renaturation in them.

The renaturation may be inhibited by some conformational changes that do not favor Watson-Crick type base-pairing. As the number of actually bound EB molecules at 62 °C with denatured T7 DNA is only 0.045, (that is one EB molecule binds per 20–25 nucleotides) the overall conformation of denatured T7 DNA is not expected to be altered significantly. Some local conformational changes, particularly at the sites of non-renatured regions, where the effect of EB-complexing is prominently manifested, is not unlikely. Such changes may arise if EB binding has some base sequence specificity. Evidences are increasingly accumulating in favor of the base sequence specificity of binding of intercalating dyes and drugs. Acridine orange as well as proflavine has been found²¹ to confer greater thermal stability to A–T rich DNA. That these two drugs bind more at the A–T rich parts has been supported by fluorescence studies^{25, 26} and also by theoretical calculations²⁷. Acriflavine too has A–T specificity of binding, whereas binding of Actinomycin D to DNA is G–C specific²⁹. Nogalamycin, an intercalating antibiotic, binds specifically to dAdT³⁰. Recent co-crystallization experiments³¹ with mono- and dinucleotides showed that EB binding has A–T sequence specificity. In order to ascertain whether the non-renatured regions occur at some specific sites in the EB-complexed renatured T7 DNA molecules the mapping method was followed. The branched renaturation products were excluded from this mapping. The non-renatured

* Figs 3–4 see Plate on page 96 a.

** Figs 5–6 see Plate on page 96 b.

sites of considerable length were found to be invariably present at a distance of $2\ \mu\text{m}$ from one end of the EB complexed renatured DNA molecules. This end was taken as the left end and was used as marker, for convenience of mapping. The length of a complexed renatured T7 DNA was, however, affected in two opposite directions. The presence of

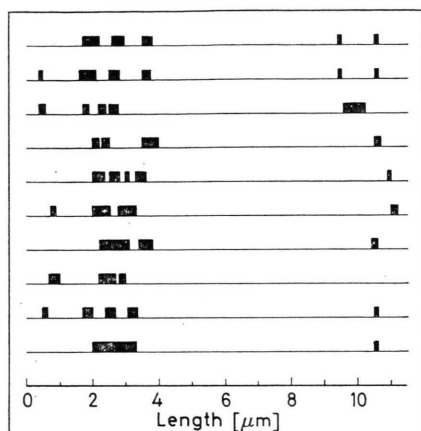


Fig. 7. Schematic representations of the mapping of the non-renatured regions of the complexed renatured T7 DNA. Each line is an average representation of at least three molecules.

the loops and of the intercalated EB molecules in them would lead to the increment in length whereas the entangled, folded up regions would decrease the length drastically. For the purpose of mapping their lengths were, however, normalized to the average length ($11.5\ \mu\text{m}$) of a free native T7 DNA (the average length of native T7 DNA was identical with that of the free renatured T7 DNA). The map (Fig. 6) shows that most of the non-renatured sites were present in the region of 1.6 to $4.0\ \mu\text{m}$ from the left end. Comparing with the denaturation map³³ of T7 DNA we find that the map of non-renatured zones almost coincided with that of A–T rich regions (the regions that were most vulnerable to denaturation) of T7 DNA. This encourages us to conclude that the renaturation of EB-complexed denatured T7 DNA was inhibited at some specific sites that were perhaps rich in A–T base pairs.

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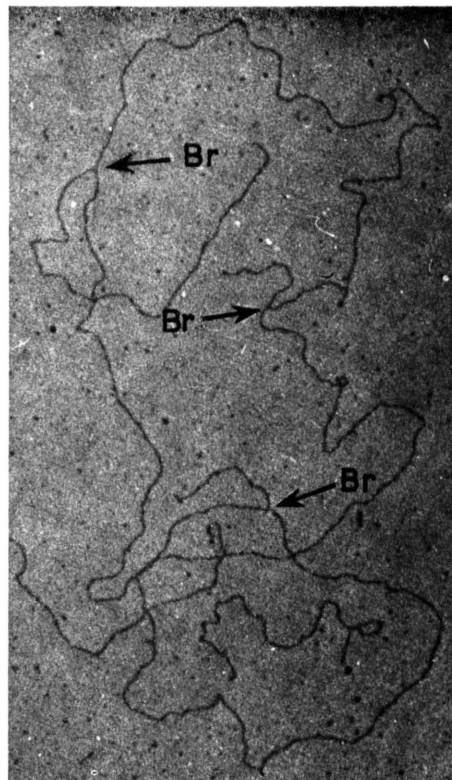
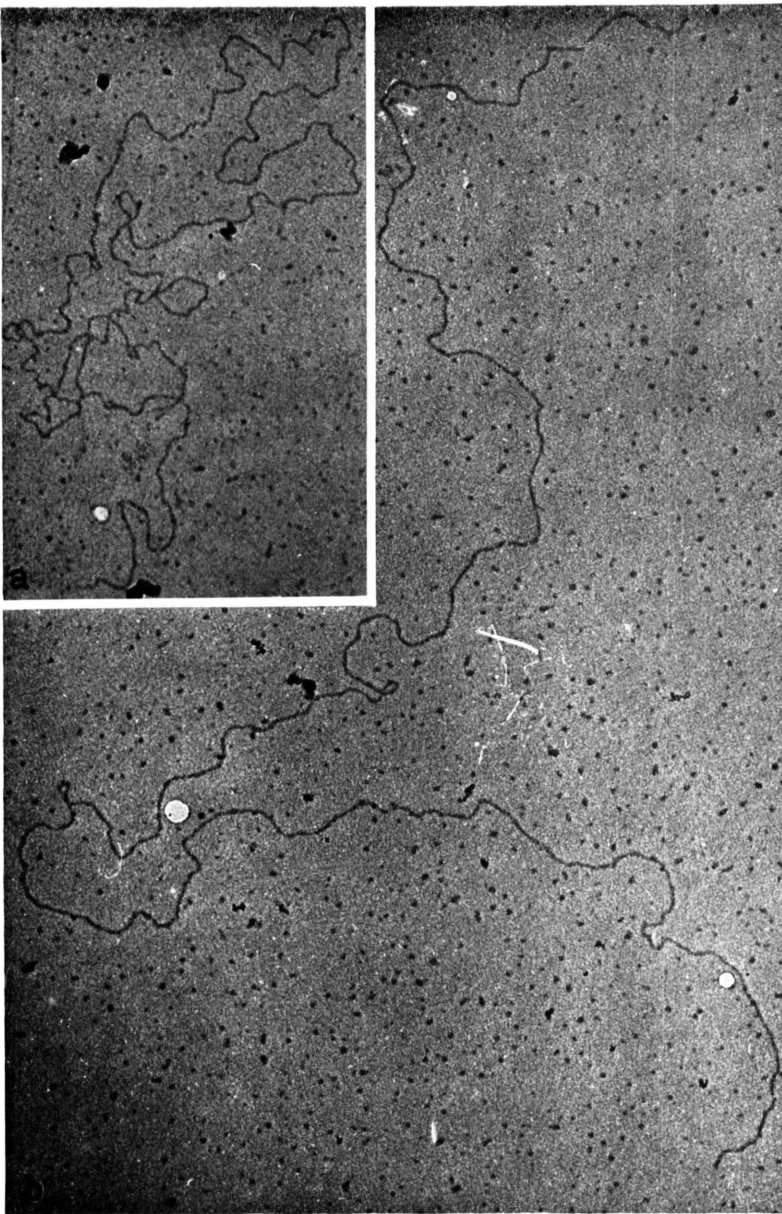


Fig. 4. One branched DNA molecule renatured from free, alkali-denatured T7 DNA. Magnification: 35 000 \times .

Fig. 3 a, b and 4 do not contain any loops or entangled regions.

Fig. 3. (a) One native T7 DNA molecule. Magnification: 35 000 \times .
(b) One T7 DNA molecule renatured from free alkali-denatured T7 DNA. Magnification: 37 00 \times .



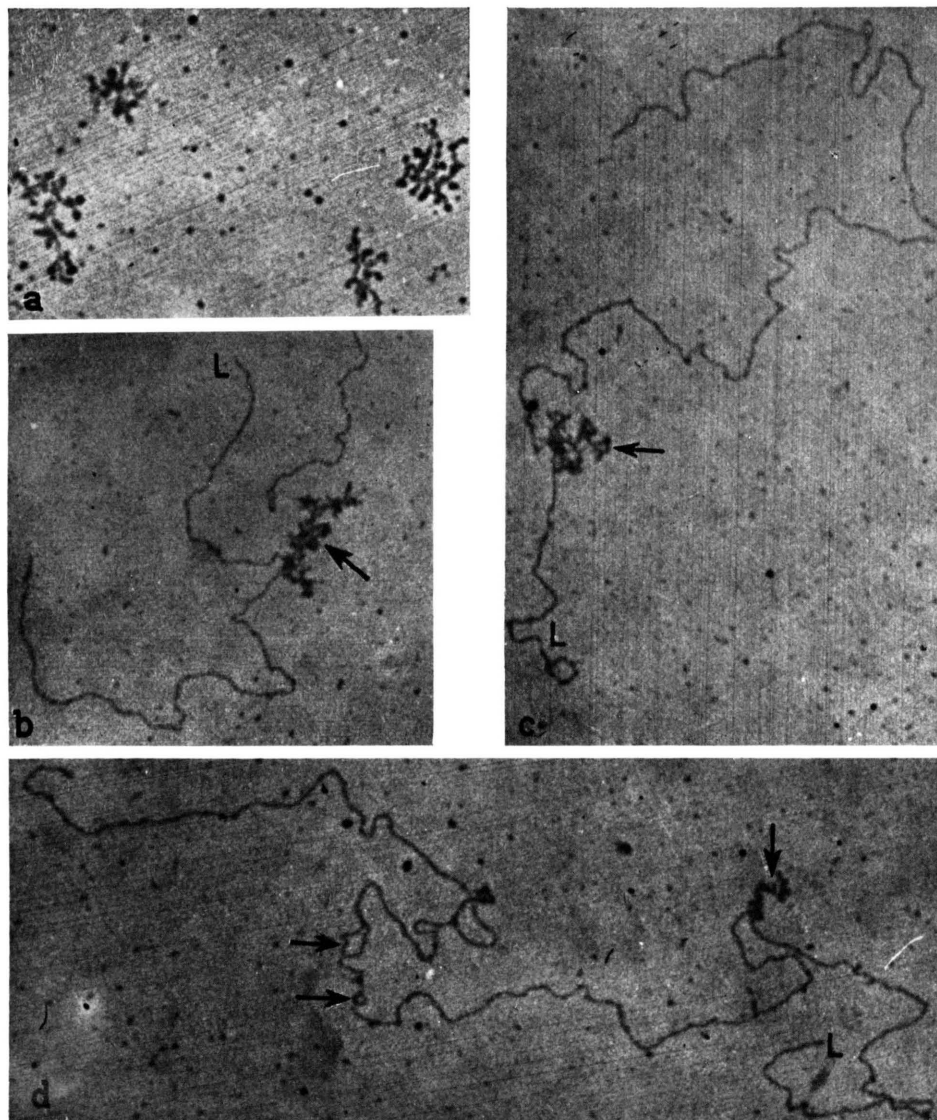


Fig. 5. (a) Alkali-denatured T7 DNA molecules showing folded up structures. Magnification: $36\,000\times$, (b), (c) and (d) Linear (unbranched) DNA molecules renatured from EB-complexed alkali denatured T7 DNA, showing loops and entangled regions (arrow marks), L denotes the left end, arbitrarily taken for convenience of mapping of the unrenatured sites. Magnifications: b - $30\,000\times$, c - $35\,000\times$, d - $35\,000\times$.

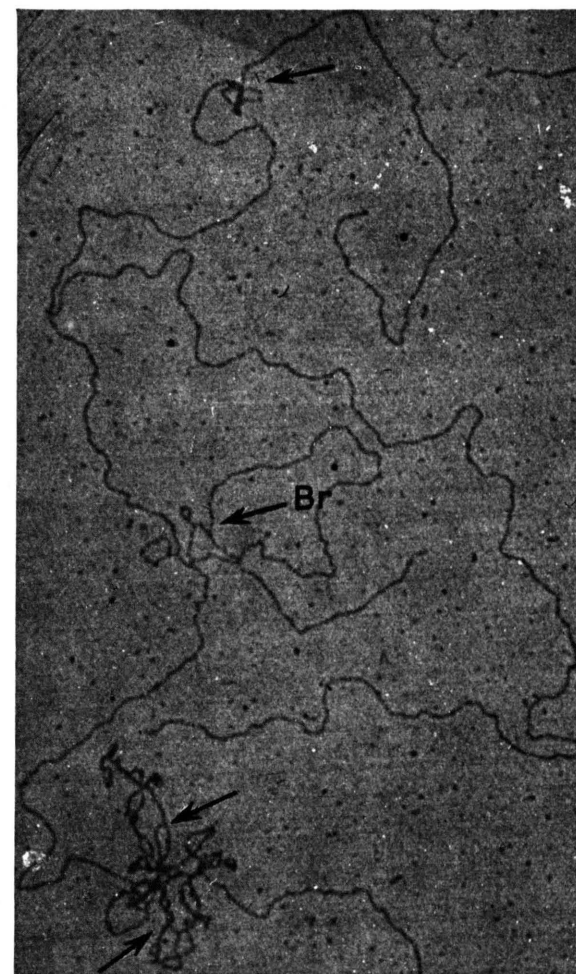


Fig. 6. One branched DNA molecule renatured from EB-complexed alkali denatured T7 DNA, showing entangled regions (arrow marks) and branching (Br). Magnification: $36\,000\times$.