NATIVE AND DENATURED DNA, CROSS-LINKED AND PALINDROMIC DNA AND CIRCULAR COVALENTLY-CLOSED DNA ANALYSED BY A SENSITIVE FLUOROMETRIC PROCEDURE*

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SUMMARY: Ethidium bromide intercalates duplex DNA with a 25 fold enhancement of its fluorescence. At pH 8 denatured DNA shows about 50% the fluorescence enhancement of duplex DNA due to intramolecular hydrogen-bonding. By raising the pH to 12 short intramolecular duplex regions can be selectively destabilized without altering long duplex DNA. This forms the basis for a sensitive assay for duplex DNA in the presence of denatured DNA. Cross-linked and palindromic DNA differ from normal duplex DNA by their spontaneous renaturation after a heat step with return of fluorescence. Covalently-closed circular DNA is similarly distinguished from open circular DNA.

INTRODUCTION

Le Pecq and Paoletti (1967) showed that ethidium intercalates duplex structures with a 25 fold fluorescence enhancement. We have exploited this property of ethidium to measure DNA synthesis *in vitro* (Coulter *et al.*, 1974) and also the formation of covalently-linked complementary (clc)[1] DNA (Morgan and Paetkau, 1972). In the case of synthetic polymers of repeating sequence having no possibility for intra-molecular base-pairing, the assay for clc DNA depended on heating the DNA-ethidium solution at pH 8. Only clc DNA showed return of fluorescence. Even homopolymer DNAs such as $dA_n \cdot dT_n$ did not reanneal at sufficiently low ionic strength, unless a covalent link existed between the strands. However natural polymers gave a 50% return of fluorescence enhancement at pH 8 as expected for a random polymer (Gralla and De Lisi, 1974). Of various methods used for destabilization of the intramolecular

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[1]Abbreviations used are: clc DNA for covalently-linked complementary DNA, ccc DNA for covalently-closed circular DNA and oc DNA for open circular DNA.

base-pairing of denatured DNA without affecting duplex DNA pairing, raising the pH was the best. At pH 12 after heating and cooling the fluorescence enhancement due to duplex DNA was completely lost unless the DNA was cross-linked or palindromic in which case there was a quantitative return of fluorescence. Also ccc^1 DNA with topologically linked strands behaves like cross-linked DNA with respect to heating in contrast to oc^1 DNA in which the strands can separate with total loss of fluorescence. These results are summarized in Fig. 1.

MATERIALS AND METHODS

DNAs: T7 DNA, extracted from purified T7 phage by phenol extraction (Thomas and Abelson, 1966) sedimented in alkali with $S^{o}_{20,\omega}$ of 40, characteristic of intact T7 DNA. Purified PM2 phage banded in a CsCl gradient (Salditt, Braunstein, Camerini-Otero and Franklin, 1972) was the source of ccc DNA. The phage band, 20 mM in EDTA was extracted twice with phenol containing 1% sodium dodecyl sulfate and then with chloroform. The aqueous phase was then dialized against 10 mM Tris pH 8, 0.1 mM EDTA. The DNA was usually 90% ccc DNA (see Results) and further purification if necessary was by alkali denaturation at pH 12.5 followed by neutralization with citric acid and passage over a nitrocel-S column in 0.5 M KCl (Armstrong and Boezi, 1965).

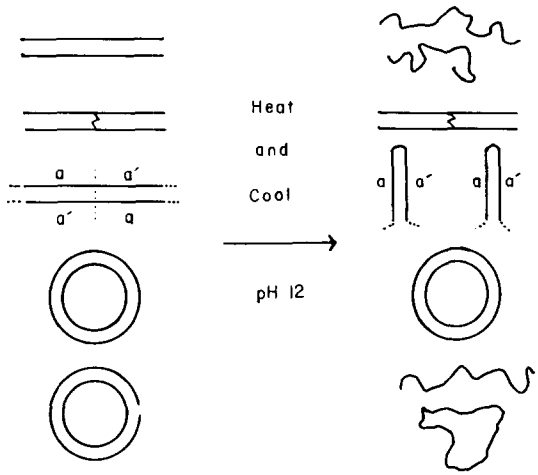

Fig. 1 A schematic representation of the effects of heat treatment on various DNAs in ethidium pH 12 solution.

Fluorescence assay: The standard pH 12 fluorescing solution was 20 mM KP_i pH 12, 0.2 mM EDTA, 0.5 µg/ml ethidium bromide. It is stored in the dark and 5 to 40 µl samples (1 A_{260} in DNA) from reaction mixtures are added to 2 ml of the solution to be within the linear range of the assay. The fluorescence was measured using a blank without added sample on the Turner Spectrofluorometer 430 with excitation at 525 nm and emission at 600 nm. Cuvettes (1 cm sq.) and cell compartment were thermostated at 23°. For the heat denaturation, the solutions were heated for two minutes at 96° on a temp-blok and cooled to 23°. Solutions stored for periods of several hours should be kept in the dark since DNA strand scission can occur.

RESULTS AND DISCUSSION

The effect of pH on ethidium fluorescence enhancement with DNA. Heat denatured synthetic polymers with no possibility for intramolecular hydrogen-bonding gave no fluorescence enhancement with ethidium (Morgan and Paetkau, 1972) confirming the original work of Le Pecq and Paoletti (1967) that an ordered duplex (or triplex, unpublished data) was necessary for ethidium intercalation leading to fluorescence enhancement. However heat-denatured natural polymers gave about 50% as much fluorescence enhancement as native DNA at pH 8 and room temperature, but this disappeared at 50° as expected for short intra-molecular duplexes. In order to circumvent the inconvenience of high temperatures for the assays, the effect of pH was one of the parameters varied and proved to be the most successful for distinguishing between duplex and denatured DNA. Fig. 2 shows that at a pH of 12 using phosphate buffer, denatured calf thymus DNA gave virtually no fluorescence enhancement whereas duplex DNA could still be measured with a sensitivity of about ± 1% for one microgram of DNA. Thus by choosing the correct conditions for the ethidium assay native or denatured DNA may be measured. This has suggested that the assay may be convenient for measuring DNA from crude extracts. The major source of interference is expected to be RNA which may be removed by a prior incubation in alkali. As shown in Fig. 3 the DNA content of a crude E. coli

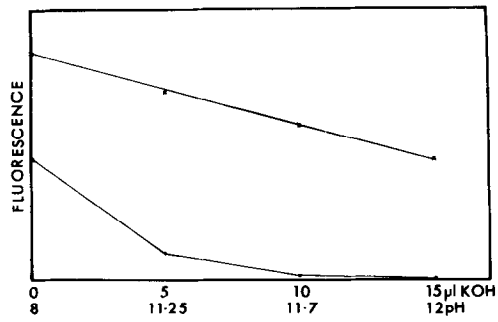

Fig. 2 To 4 ml of ethidium solution containing 20 mM KP_i pH 8 instead of 12 (Methods) was added 20 µl of native (x-x-x) or denatured (·-·-·) calf thymus DNA (1 A_{260} when native). 5 µl samples of 5 M KOH were added and the pH and fluorescence were measured. The decrease in fluorescence for native DNA on adding KOH is identical if KCl is substituted for KOH and is a salt effect.

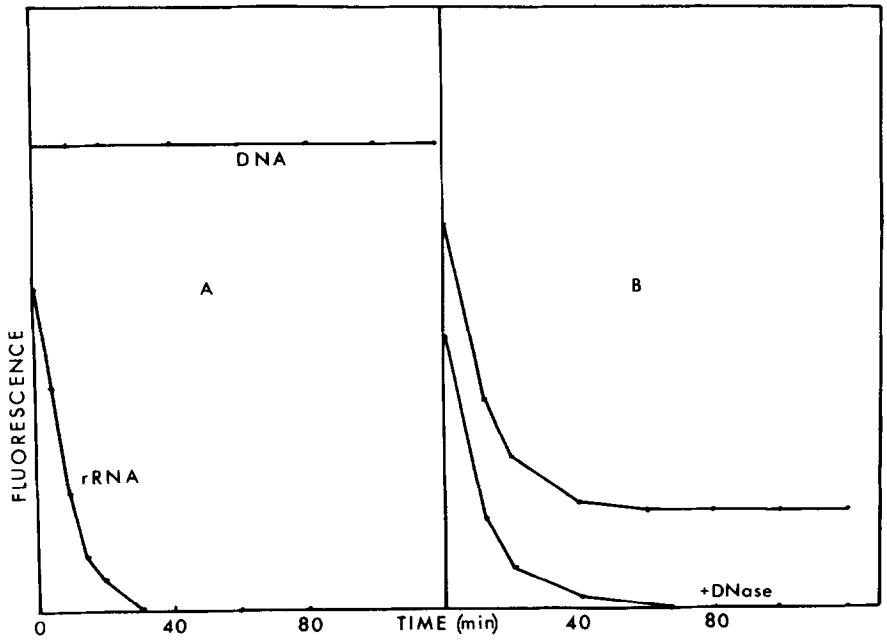

Fig. 3 A. Calf thymus DNA (1.5 A_{260}) or rRNA (2.5 A_{260}) were incubated at 37° in 0.2 M NaOH. Samples (10 µl) were added to ethidium solution containing 5 mM Tris pH 8 instead of KP_i (Methods).

B. 41 mg E. coli cells were sonicated in 1 ml 10 mM Tris pH 8, 0.1 mM EDTA. 20 µl of the supernatant (120 A_{260}) was directly incubated with 0.2 M NaOH after 5 fold dilution and another 20 µl was made 10 mM in $MgCl_2$ and treated with pancreatic DNase (0.1 mg/ml) for 1 hour at 37° before alkaline digestion. 10 µl samples were assayed as for A.

extract could be determined rapidly and very sensitively in a denatured state. That the residual fluorescence was all due to DNA was shown by pretreating the extract with DNase. The calculated amount of DNA in E. coli was 1.2% by weight in good agreement with other quoted values (Watson, 1970). Because of the high pH and very large dilution into the assay mixture interference by nucleases or complex formation is minimized. This was confirmed by adding known amounts of DNA and obtaining the expected increase in fluorescence. (It should also be possible to obtain DNA/RNA ratios from the alkali sensitive fluorescence). The only sources of interference with the assay were high concentrations of phospholipids and histone I artificially added (to be published).

Cross-linking of DNA and Palindromic DNA. Assays for cross-linking of DNA depend on the unimolecular rapid renaturation of such DNA, and the separation of duplex from denatured DNA by column chromatography or equilibrium centrifugation (Waring, 1966). Duplex and denatured DNA existing within the same molecule complicate the assays. However using the fluorescence assay at

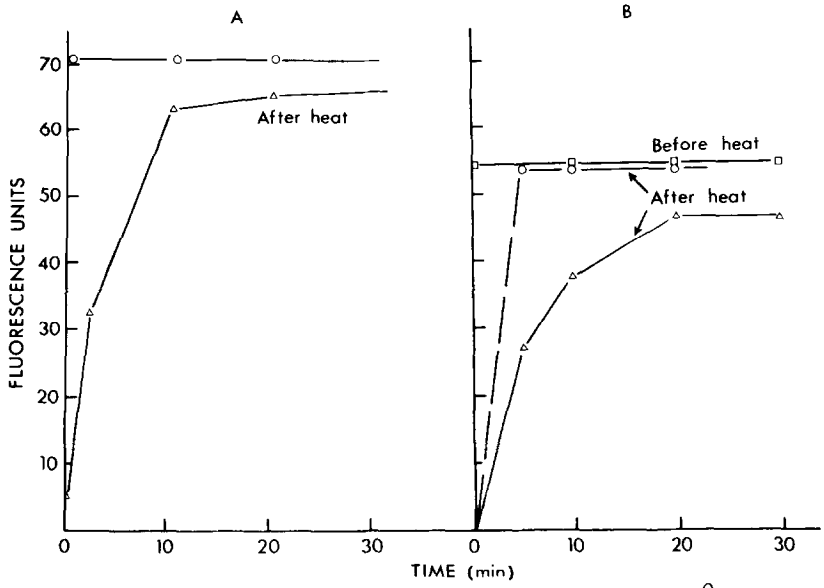

Fig. 4 T7 DNA (1.3, A or 0.86, B, A_{260}) was incubated at 37° in 0.1 M Na acetate pH 4.5, 0.2 M Na nitrite (A) or 50 mM Tris pH 8, 5 µg/ml mechlorethamine HCl (Δ-Δ-Δ) and 20 mM KP_i pH 12, 50 µg/ml mechlorethamine HCl (o-o-o) (B). 10 µl samples were assayed and the fluorescence measured before and after heating.

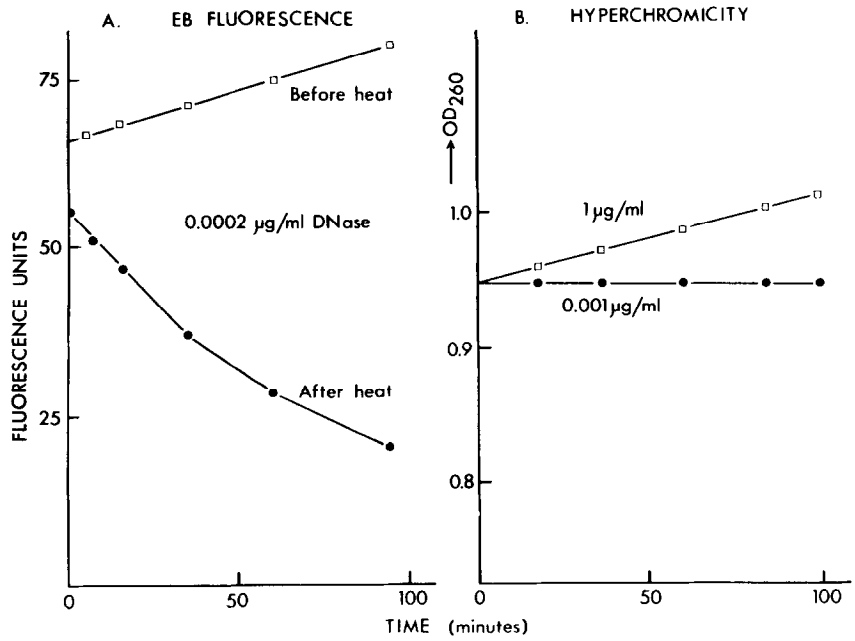

Fig. 5 PM2 DNA (1.1 A_{260}) in 50 mM Tris pH 8, 5 mM $MgCl_2$ at 23° was treated with the indicated levels of pancreatic DNase. At low levels of DNase, heated-denatured gelatin (0.1 mg/ml) was added to prevent non-specific losses. The assays required 10 μl samples for the fluorescence assay (A) and 2 ml of solution for the hyperchromicity assay using a Gilford Spectrophotometer 2400.

pH 12 a very rapid, sensitive assay for cross-linking is obtained as illustrated in Fig. 4, by the nitrous acid induced cross-linking of T7 DNA. The assay was checked by conventional techniques (banding in CsCl and nitrocellulose columns) as well as sensitivity to S_1 endonuclease. A wide variety of cross-linking agents has been studied including nitrogen mustards and antibiotics such as mitomycins. Although depurinated sites will give rise to strand cleavage in alkali, the 7-alkylated purines ring-open immediately at pH 12 preventing strand scission (Lawley and Brookes, 1963). Palindromic DNA has the same properties with respect to denaturation as cross-linked DNA. Calf thymus DNA contains about 1.4% clc[1] DNA as assayed at pH 12. From its sensitivity to pH and sonication and resistance to S_1 endonuclease it has been characterized as duplex DNA (details to be published), presumably palindromic (Wilson and Thomas, 1974).

Circular DNAs. PM2 ccc[1] DNA was characterized by its neutral and alkaline sedimentation velocity and also by banding in ethidium CsCl density gradients. On heating ccc DNA in ethidium at pH 8 and cooling a denatured form of ccc DNA is obtained (Pouwels, van Rotterdam and Cohen, 1969) with a large but not complete loss in fluorescence. However at pH 12 there is a quantitative return of fluorescence presumably because of the increased mobility of the two strands relative to one another. The topological constraints permit renaturation despite the electrostatic repulsion which prevents duplex formation with homopolymers. Fig. 5 shows how ccc DNA converted to oc DNA by pancreatic DNase is readily assayed. The 30% increase in fluorescence before heat is due to the removal of topological constraints on ethidium binding (Paoletti, Le Pecq and Lehman, 1971). This assay for nuclease is about 10^5 times as sensitive as the hyperchromicity assay. Saucier and Wang (1973) and Beard and Berg (1974) have also developed microanalytical methods for DNA circles but they depend on radio-labelled DNA and are more complicated than the assay we describe which requires submicrogram amounts of unlabelled DNA. PM2 DNA is readily obtained and a 30 l culture provides enough DNA for 10^4 assay points. The instrumentation is cheap and there is virtually no cost in chemicals.

These assays have many other applications of which the following have proved useful: $C_o t$ curves, multistranded structures e.g. triplexes, DNA strand cleavage by radiation, vitamin C, antibiotics and nucleases, polynucleotide ligase and unswivelling proteins.

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