Review: Ethidium fluorescence assays. Part 1. Physicochemical studies

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ABSTRACT

DNA and RNA can be assayed rapidly and very sensitively by exploiting the enhanced fluorescence of ethidium intercalated into duplex regions. By assaying at different pHs and introducing a heating/cooling cycle, a great many physicochemical aspects of DNA and RNA can be studied avoiding the use of radiolabels, and often giving information not otherwise readily obtainable. Studies are described on duplex DNA which involve measurement of extinction coefficients, cross-linking by chemicals, Cot curve analysis as well as estimation of drug-DNA binding constants. The assays can be adapted to investigate multi-stranded nucleic acid structures. The use of covalently closed circular DNA also allows rapid and extremely sensitive measurements of nicking caused by irradiation or drugs.

INTRODUCTION

A fluorescence assay dependent on ethidium binding to nucleic acids was first introduced by Le Pecq and Paoletti (1). When ethidium binds in the intercalated state there is a large fluorescence enhancement (~25 fold) (1) as a result of the hydrophobic environment surrounding the ethidium molecule. This slows down proton exchange leading to a longer life time for the excited state (2). The fluorescence enhancement can be used to measure duplex (and some triplex) nucleic acids. This has formed the basis for a large number of rapid and versatile assays which have been developed in this and several other laboratories (3-7). Many of the assays provide a unique way of studying nucleic acid structure and metabolism, and they can frequently substitute for or complement radiolabel assays.

This first article will review the application of ethidium fluorescence assays to physicochemical and structural aspects of nucleic acids while the second will deal with enzymological processes. We should point out that for reasons of consistency we will concentrate on the fluorescence assays as developed in this laboratory since the methodology used by other authors is,
in some cases, slightly different.

MATERIALS

Ethidium bromide, spermine HCl, spermidine HCl, and Mechlorethamine HCl were purchased from the Sigma Chemical Co. 1,14-bis(2-methoxy-6-chloro-9-acridinyl)-1,5,10,14-tetraazatetradecane tetrahydrochloride ("Spermine Bis-acridine") was a gift of Dr. J.H. van de Sande. Bacteriophage PM2, T7 and λ DNAs and repeating sequence DNAs and RNAs were prepared as described previously (3,8,9). In general the PM2 DNA was greater than 80% covalently-closed circular* (ccc) but samples of linear or open-circular (oc)* PM2 DNA were prepared by incubating the intact phage in 1 mM CaCl₂ before isolation (10). Relaxed ccc PM2 DNA was prepared by treating PM2 DNA with calf thymus topoisomerase (11). d(AT)ₙ, d(AN·d(AN), d(TN·d(TN), rAₙ and rUₙ were purchased from either Miles Biochemicals or P-L Biochemicals.

Three ethidium-containing buffers, differing in their pH's are routinely used for the fluorescence assays. "pH 5": 1 μg/ml ethidium bromide, 20 mM Na acetate, pH 4.8, 0.2 M NaCl, 0.5 mM EDTA; "pH 8": 0.5 μg/ml ethidium bromide, 5 mM Tris-HCl pH 8.1, 0.5 mM EDTA; "pH 12": 0.5 μg/ml ethidium bromide, 20 mM K₃PO₄, pH 11.8, 0.5 mM EDTA. Adaptations of these standard solutions are sometimes used for particular assays (see below).

The following solutions are also required in many situations for providing internal controls: Calf thymus DNA, both native and heat-denatured at 1 A₂₆₀; Ribosomal RNA (from E. coli) at 1 A₂₆₀; 20 mg/ml ribonuclease A; 1 mM spermine bisacridine; and a buffer solution of 0.2 M NaOH, 0.8 M K₃PO₄ which converts the pH 8 assay to pH 12 upon addition of 50 μl of it into 2 ml of the pH 8 assay solution.

General Methods

The pH 5, pH 8 and pH 12 fluorescence assay solutions are made up in 1 litre quantities and stored at room temperature in light-proof Repipet Ltd. dispensing containers. This allows for the rapid and accurate delivery of the 2 ml volumes required for the spectrofluorimeter. The pH 5 and pH 8 solutions are stable for several months but the pH 12 buffer has to be prepared fresh about once a week since the pH slowly drops. The reliability of the pH 12 buffer can be easily checked by the addition of heat-denatured calf thymus DNA which in fresh solution gives rise to 2-5% of the fluorescence of native calf thymus DNA (see below).
On a daily basis fresh blanks and standards are first prepared for the pH 8 and pH 12 assay solutions. The standard contains 10 µl of calf thymus DNA at 1 A_{260} (0.5 µg DNA) added to 2 ml of the ethidium assay solution. Using the Turner spectrofluorimeter 430, excitation is at 525 nm and emission at 600 nm with the slit widths on their maximum settings. The fluorescence scale is arbitrary, the scale being set at X100 normally and the sensitivity adjusted so that, having set the pH 8 ethidium assay solution at 0 for the blank, a standard containing 10 µl of calf thymus DNA at 1 A_{260} (0.5 µg) reads 70. For the pH 12 ethidium assay solution the blank has to be readjusted (there is a small difference in the free ethidium fluorescence in the various buffer systems), and the standard is set at 50. The pH 12 ethidium assay solution shows a decrease in sensitivity compared to pH 8 (mainly due to the increase in ionic strength). Thus only small adjustments to the sensitivity control are required on changing from pH 8 to pH 12. Because nearly all assayed samples are also heat-denatured it is convenient to have a temperature block available. A temp-block (Lab-line Instruments), drilled to take 10x75 mm tubes (3 ml) is kept at 96° and stored in a styrofoam box to minimize temperature variations. All tubes used are first heated with pH 12 assay solution for >10 minutes to make sure no fluorescing material contaminates them. These tubes are then constantly recycled being rinsed twice with distilled water before reuse. Samples (1-200 μl) are added to the ethidium solution in 10x75 mm tubes and can be read immediately or left till a convenient number have accumulated. If samples containing ccc DNA are left to stand for a while, they must be kept in the dark otherwise the ethidium gradually nicks the DNA in a light-catalyzed reaction (12). The fluorimeter cell compartment and circulating water-bath (for cooling heated samples) are thermostated at 25°. Significant changes in fluorescence readings can be obtained if the temperature changes by ±3° since the DNA is not saturated with ethidium. After the "before-heat" fluorescence readings are taken the solutions are heated in the 10x75 mm tubes in the temp-blok for 2 minutes. The tubes are then cooled in the circulating water bath for a further two minutes. The heating time is not critical and tubes are best left for longer than two minutes if in doubt. ccc PM2 DNA can be left for 15 minutes at 96° in the pH 12 ethidium solution without detectable nicking but longer times do result in a gradual loss of fluorescence. This puts an upper limit on the accurate assay of ccc DNAs of very high molecular weight (i.e. >50x10^6), if the assumption is made that the rate of nicking is proportional to molecular weight.
Because the xenon lamp light intensity tends to drift over a period of hours, the sensitivity scale is routinely checked before a series of readings with the appropriate blank and standard (pH 8, 70 units; pH 12, 50 units). When using the pH 5 solution the standard was set as for the pH 8 solution at 70. On this scale all three different pH assay solutions give a linear response (±1%) to nucleic acid concentration up to a fluorescence reading of approximately 150 units (13 and Fig. 1).

RESULTS AND DISCUSSION

The original stimulation to develop a fluorescence assay was derived from the difficulty of measuring clc* DNA which forms on in vitro replication of DNA, particularly synthetic DNAs such as d(TG)ₙ·d(CA)ₙ. The synthetic polymers with repeating sequences renature very rapidly under conditions normally used to test for double-strandedness (e.g. S₁ nuclease), and alkaline buoyant density gradients were initially the only method for detecting clc synthetic DNAs. At sufficiently low salt concentrations (< 10 mM in ionic strength) even homopolymers such as dₐ·dₜₙ will not renature after heat denaturation although they are stable when added as the duplex to the low ionic strength solution. However clc DNA (a product of DNA replication or resulting from chemical cross-linking of DNA) spontaneously renatures at the low ionic strength after heat denaturation. Presumably an electrostatic barrier to renaturation exists for the separable complementary strands which is penetrated by clc DNA. A very rapid and sensitive assay for clc DNA was thus developed using the enhanced fluorescence of intercalated ethidium. A bonus was that at the low ionic strengths required, the binding constant of ethidium to DNA is maximized being close to \(10^7\) M⁻¹. The assays with synthetic polymers were all carried out at pH 8 in 5 mM Tris HCl buffer.

When attempts were made to measure clc DNA occurring in natural DNAs as a result of replication, the pH 8 ethidium assay solution was not suitable (see Table I), due to the random short intramolecular hairpin loops which exist in a random sequence of the 4 nucleotides (14). An increase in pH to >12 was found to virtually abolish all intramolecular hairpins in denatured DNA (except long palindromic sequences) while duplex DNA was intact in the presence of ethidium which stabilizes duplex DNA. It thus became possible to extend the fluorescence assay to natural polymers containing clc sequences.

A further expansion of the use of fluorescence assays became evident when ccc DNAs were studied. The complementary strands while not being co-
valently linked are topologically linked. At lower pH's (<8) it is possible
to get a denatured form of ccc DNA (15) but under the pH 12 assay conditions
ccc DNA spontaneously renatures after heat denaturation in contrast to oc DNA.
This has proved to have many valuable applications, as will become evident
in the following examples.

(a) Determination of extinction coefficients

As indicated above the pH 8 ethidium assay solution is used primarily
for synthetic polymers or where denatured natural DNAs are being studied.
Particularly with synthetic polymers in which unusual bases have been in-
corporated for structural or enzymatic studies, characterization becomes a
problem if only small amounts of the DNA are available. This is best
illustrated by the characterization of \( d(\text{BrUG})_n \cdot d(\text{CA})_n \), a density-labelled
analogue of \( d(TG)_n \cdot d(\text{CA})_n \), required for studies on DNA replication. The
strands were separated by alkaline CsCl density gradients. The molar
extinction coefficient of \( d(\text{CA})_n \) was known but those of \( d(\text{BrUG})_n \cdot d(\text{CA})_n \)
and \( d(\text{BrUG})_n \) were not. In order not to utilize most of the polymer in con-
tventional methods for determining extinction coefficients, mixing curves
(the method of continuous variation) were obtained as illustrated in Fig. 1.
Increasing amounts of \( d(\text{CA})_n \) were added to a fixed amount of \( d(\text{BrUG})_n \) or
vice versa. As can be seen a linear increase in fluorescence was obtained
as one strand was added to the other until the equivalence point was reached,
at which a sharp break was obtained with no further increase in fluorescence,
since the excess single-stranded polymer is not intercalated by ethidium.
In both cases the equivalence point was reached at the same \( A_{260} \) ratio of
\( d(\text{CA})_n \) to \( d(\text{BrUG})_n \) (1.10 and 1.12 for Fig. 1(a) and (b), respectively;
average = 1.11), demonstrating the accuracy of the method. The extinction
coefficient of \( d(\text{CA})_n \) was found to be \( 10.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \) at 260 nm by measuring
the hyperchromicity upon complete digestion with \( N. \ crassa \) endonuclease and
snake venom phosphodiesterase. Thus the extinction coefficient of \( d(\text{BrUG})_n \)
is calculated to be \( \frac{10.1}{1.11} \times 10^3 = 9.1 \times 10^3 \text{ M}^{-1} \) at 260 nm. The extinction
coefficient of the duplex \( d(\text{BrUG})_n \cdot d(\text{CA})_n \) can then be determined from the
fluorescence (using an identical fluorescence scale as in Fig. 1) given by
a small sample (\( \approx 0.6 \mu \text{g}) of the duplex of accurately known optical density.
It was found that 5 \( \mu \text{l} \) of the duplex at 2.526 \( A_{260} \) gave a fluorescence
reading of 113.7 (average of three readings). From Fig. 1(a) 0.0036 OD/ml
\( d(\text{CA})_n \) (in 2 ml of the pH 8 assay solution) which is equivalent to \( 0.7129 \times 10^{-9} \text{ moles} \) (from the known extinction coefficient) gave on average 87.5
fluorescence units when annealed to an excess of \( d(\text{BrUG})_n \). Thus 87.5 units
Figure 1. Mixing curves for (a) d(BrUG)n added to d(CA)n and (b) d(CA)n added to d(BrUG)n. The two strands (0-20 μl aliquots) were allowed to reanneal for 10 min in 20 μl of 0.1 M NaCl before addition of 2 ml of the pH 8 fluorescence solution. In (a) the d(CA)n concentration was kept constant (0.0036 A260/ml in the ethidium solution) and in (b) the d(BrUG)n concentration was kept constant (0.00269 A260/ml). The "breakpoint" occurs at a d(BrUG)n A260/d(CA)nA260 ratio of 1.10 and 1.12 for (a) and (b) respectively.

would be given by $2 \times 0.7129 \times 10^{-9} = 1.426 \times 10^{-9}$ moles of duplex d(BrUG)n · d(CA)n. Since the fluorescence assay is linear over this range 113.7 fluorescence units is equivalent to 1.853 $\times 10^{-9}$ moles of duplex ($= \frac{113.7}{87.5} \times 1.426 \times 10^{-9}$) which must be equal to 5 μl of duplex at 2.526 A260. Thus the extinction coefficient of d(BrUG)n · d(CA)n at 260 nm is

$$\frac{5 \times 2.526}{10^6 \times 1.853 \times 10^{-9}} = 6.8 \times 10^3 \text{ M}^{-1}.\text{cm}^{-1}.$$ An identical extinction coefficient can be calculated from the data of Figure 1(b).

The main advantages of the fluorimetric assay compared with the spectrophotometric method of continuous variations depending on hypochromicity, are the sensitivity and speed. The spectrophotometric method requires at least 10 fold more DNA for equal accuracy and moreover requires very careful positioning of cuvettes and weighing of samples added if meaningful values are to be attached to changes of 0.001 absorbance. A mixing curve as shown
in Fig. 1 can be done in 15 minutes with the fluorimeter.

Fig. 1 also demonstrates that truly single-stranded polymers do not enhance the fluorescence of ethidium. This is in contrast to heat-denatured natural DNAs (which are often confusingly called "single-stranded") and all natural RNAs which contain up to 50% duplex regions caused by hairpin loops (14). Thus they also give rise to considerable fluorescence at pH 8. As would be expected, d(AT)\textsubscript{n} and d(GC)\textsubscript{n} spontaneously reform the original duplex after heat-denaturation so that the fluorescence at pH 8 remains unchanged (see Table I).

| Nucleic Acid                        | pH of the assay solution | Approximate Relative Fluorescence Before Heat | Approximate Relative Fluorescence After Heat |
|-------------------------------------|--------------------------|---------------------------------------------|---------------------------------------------|
| Calf Thymus DNA                    | 8                        | 70                                          | 35                                          |
| d(AT)\textsubscript{n}             | 8                        | 100                                         | 100                                         |
| d(TC)\textsubscript{n} \cdot d(GA)\textsubscript{n} | 8                        | 70                                          | 0                                           |
| rRNA (E. coli)                     | 8                        | 35                                          | 35                                          |
| Calf Thymus DNA                    | 12                       | 50                                          | 0-2                                         |
| 100% ccc Native PM2 DNA*           | 12                       | 36                                          | 36                                          |
| Nicked or Linear PM2 DNA           | 12                       | 50                                          | 0-2                                         |
| Topoisomerase-relaxed PM2 DNA      | 12                       | 26                                          | 26                                          |
| rRNA (E. coli)                     | 12                       | 0                                           | 0                                           |
| Calf Thymus DNA                    | 5                        | 30                                          | -                                           |
| d(TC)\textsubscript{n} \cdot d(GA)\textsubscript{n} | 5                        | 30                                          | -                                           |
| d(TC)\textsubscript{n} \cdot d(GA)\textsubscript{n} \cdot d(CT)\textsubscript{n} | 5                        | 0                                           | -                                           |
| dT\textsubscript{n} \cdot dA\textsubscript{n} | 5                        | 3                                           | -                                           |
| dT\textsubscript{n} \cdot dA\textsubscript{n} \cdot dT\textsubscript{n} | 5                        | 20                                          | -                                           |

* See section (e)

Values for the approximate fluorescence both before and after heat are tabulated for 10 \textmu l of various nucleic acids at 1 A\textsubscript{260} in the three assay solutions.
The effects of pH - the pH 12 assay solution.

Although the pH 8 assay solution can be used to measure nucleic acid concentrations it cannot accurately distinguish between single and double-stranded naturally-occurring polymers since both give rise to some fluorescence. Thus the pH 12 assay solution was developed since the high pH destabilizes short hairpin loops without affecting native double-stranded polymers. The effects of increasing the pH by adding KOH to a solution at pH 8 are shown in Fig. 2. Thus heat-denatured calf thymus DNA ceases to give significant fluorescence at pH's greater than 11.7 whereas native calf thymus DNA and all other DNAs except dA\textsubscript{n}\cdotdT\textsubscript{n} only show a slight decrease. This decrease can be attributed to increases in the ionic strength which reduces the affinity of ethidium for DNA (16). The duplex dA\textsubscript{n}\cdotdT\textsubscript{n} is denatured at pHs greater than 11.7 and thus gives no fluorescence. d(AT)\textsubscript{n} also behaves anomalously in the "pH 12" ethidium buffer. In freshly prepared solutions the d(AT)\textsubscript{n} shows fluorescence but after heat readings show no fluorescence (in contrast to the pH 8 buffers). However on aging of the

![Figure 2. Effect of pH on the enhancement of fluorescence given by various polymers.](image_url)
assay solutions and a slight decrease in pH, the after heat reading can equal the before heat reading indicating renaturation. Evidently this is a critical pH range for d(AT)$_n$ renaturation and internal controls with bona fide d(AT)$_n$ are always recommended. At any rate by the application of these simple tests d(AT)$_n$ and dA$_n$$\cdot$dT$_n$ can be distinguished from each other and all other (G.C) containing DNAs.

After a heating and cooling step (see methods) all DNAs except those mentioned below give no return of fluorescence. The important exceptions, all of which give 100% return of fluorescence after heating at pH 12, include covalently-linked complementary (arising by replication and chemical cross-linking), palindromic and covalently-closed circular DNAs (3). These DNAs renature spontaneously even in the pH 12 assay solution because they contain a nucleation point which overcomes the repulsion between the negatively-charged strands. Examples of assays for these DNAs will appear below. The approximate fluorescence values expected for various polymers under a variety of conditions are summarized in Table I, as well as results for the pH 5 assay solution which are described in the next section.

(c) Multistranded structures - the pH 5 assay solution

On lowering the pH below 6 (G.C) containing d(purine)$_n$$\cdot$d(purine)$_n$$^+$ DNAs of repeating sequence dismutate to form a triplex together with a free d(purine)$_n$ (17). Since neither a (C.G-C) containing triplex nor a d(purine)$_n$ enhance the fluorescence of ethidium, this structural change can be followed fluorimetrically. A typical experiment is shown in Fig. 3 where it can be seen that increasing the temperature produces a large increase in the rate of the dismutation. The pH 5 assay solution must, of course, fulfil the condition that the triplex is stable but also duplex DNAs must only slowly dismutate. The low pH ensures the triplex stability and the high dilution, high ionic strength and presence of ethidium all ensure that the rate of dismutation is sufficiently slow that accurate readings can be taken. The high ionic strength reduces the binding constant of ethidium so that the pH 5 assay solution requires approximately 2.5 times more DNA to reach the same sensitivity as the pH 8 assay solution (see Table I). Other techniques to monitor triplex formation, for example thermal denaturation profiles or buoyant density studies (18), are extremely cumbersome compared with this fluorimetric method and they cannot be used to follow the kinetics of dismutation.

For investigating triplexes containing only (A-U) or (A-T) base-pairs a low pH is unnecessary but there is still a requirement for a high ionic
Figure 3. Effect of temperature on the rate of dismutation of d(TC)$_n$·d(GA)$_n$. The DNA at 2.4 A$_{260}$ was incubated at 25° (○), 37° (■) or 50° (▲) in 50 mM Na acetate pH 5 and 10 µl samples were pipetted at various times into 2 ml of the pH 5 assay solution.

strength or divalent metal ions (19). Thus addition of 2 mM Mg$^{2+}$ to the pH 8 assay solution allows other triplexes to be studied. Representative mixing curves are shown in Fig. 4. Under these conditions the triplex rU$_n$·dA$_n$·rU$_n$ is the only stable structure formed by dA$_n$ and rU$_n$ whereas rA$_n$ and rU$_n$ form both the duplex and triplex depending upon the composition of the mixture. The addition of Mg$^{2+}$ to the pH 8 assay solution again reduces the binding of ethidium so that more polymer must be added but this method is still many times more sensitive than the spectrophotometric one (18).

The fluorescence upon ethidium binding given by rU$_n$·dA$_n$·rU$_n$ and rU$_n$·rA$_n$·rU$_n$ (as well as dT$_n$·dA$_n$·dT$_n$ and dT$_n$·dA$_n$·rU$_n$ - data not shown) is of considerable interest since, as mentioned above (C·G·C) containing triplexes do not enhance the ethidium fluorescence. It is not clear whether this is due to repulsion of the ethidium molecule by the positively charged cytosine or whether these triplexes have a rather different conformation. However this does illustrate that ethidium is a sensitive probe of secondary structure.
Figure 4. Mixing curves for the addition of (a) dAn and (b) rAn to rUn. The polymers were added to 2 ml of the pH 8 assay solution supplemented with 2 mM MgCl₂ and allowed to anneal for one hour before reading the fluorescence. The total polymer concentration was kept constant at 7.28 μM and 2.26 μM for (a) and (b) respectively.

(d) C₀t curves and renaturation kinetics

Up to this point our major concern has been to describe the basic functions and properties of the three assay solutions. However many of the applications of the fluorescence assays are not at all obvious, and therefore we shall now consider specific examples in more detail.

DNA renaturation kinetics (C₀t curves) have been used to study the complexity of genomes, repetitive DNA sequences and gene amplification (20,21). The extent of renaturation is often followed by retention of duplex molecules on hydroxyapatide but the pH 12 fluorescence assay provides a much simpler and more accurate procedure. Since only renatured molecules give rise to fluorescence at pH 12 then one rapid measurement leads directly to a value for the percentage renatured. This is demonstrated in Fig. 5 for PM2, T7 and λ DNAs. As expected the C₀t₂₀ values increase with increasing genome size. The values are not directly comparable with those of Britten and Kohne (20) since very different renaturation conditions have been used. However the utility of the method is quite clear.
Figure 5. $C_0t$ curve analysis for various bacteriophage DNAs. After heat-denaturation at low ionic strength, the reannealing was started by incubating at 37°C in a buffer containing 50 mM NaCl, 10 mM Tris-HCl pH 8 and 0.1 mM EDTA. At various times 10 μl samples were pipetted into 2 ml of the pH 12 assay solution and the fluorescence measured. The % reannealed was calculated with reference to the fluorescence given by samples of the DNAs which had not been heat-denatured. $O$ = linear PM2 DNA at 320 μM; $A$ = T7 DNA at 268 μM; $\bullet$ = λ DNA at 243 μM.

(e) Covalently-closed circular DNA and superhelix density

Because of its peculiar topological properties ccc DNA has been a popular tool for the study of drugs which bind to DNA (22) but with the advent of recombinant DNA research the study and analysis of circular DNAs has acquired new significance. The applications of the fluorescence assay to ccc DNAs is illustrated in Fig. 6. Here γ-irradiation has been used to nick the ccc PM2 DNA (both native and topoisomerase-relaxed) and the extent of reaction has been followed with the pH 12 assay solution both before and after heat-denaturation. As mentioned in section (b) only ccc DNA will spontaneously renature after heat-denaturation, and thus the after heat fluorescence gives the percentage ccc remaining (see later). Before heat, however, the fluorescence increases as nicking proceeds with the topoisomerase-relaxed ccc DNA giving the larger increase.
Figure 6. Effect of γ-irradiation on the fluorescence of native ccc and topoisomerase-relaxed PM2 DNA. 30 μl samples of DNA at 1 A_{260} were irradiated for various times with the caesium-137 source of a gammacell 40 (28). 20 μl samples were then pipetted into 2 ml of the pH 12 assay solution. The fluorescence readings have been normalized so that the initial fluorescence was 100%. O, ● = before and after heat readings for native PM2 DNA; □, ■ = before and after heat readings for topoisomerase-relaxed PM2 DNA. To avoid confusion only one line has been drawn through both sets of after heat readings.

The fluorescence increase on nicking ccc DNA is due to the removal of topological constraints. The concentration of ethidium in the assay solutions is sufficient that positive supercoils are driven into closed-circular DNAs. This process is energetically unfavorable so that less ethidium becomes bound compared to a linear or oc DNA which has no topological constraints (23). Native PM2 DNA is negatively supercoiled initially so that many ethidium molecules must be bound before positive supercoils inhibit further binding. For topoisomerase-relaxed PM2, positive supercoils are immediately introduced on binding ethidium and consequently upon nicking this DNA the fluorescence increase is much larger than upon nicking native PM2 DNA. From the data of Fig. 6, the % increase on nicking native or topoisomerase-relaxed PM2 (100% ccc) can be calculated. First, let us consider native PM2 DNA. Before any irradiation the before and after heat readings
are 100 and 79 units respectively so that 21 units are due to oc DNA. After irradiation the before heat reading reaches a maximum of 130 units and therefore, \( 130 - 21 = 109 \) units are due to DNA which was originally ccc. Thus the \% increase in fluorescence is \( \frac{109 - 79}{79} = 38\% \). Similarly for topoisomerase-relaxed PM2 DNA the before and after heat readings are initially 100 and 74 units respectively so that 26 units are due to oc DNA. The maximum before heat value after irradiation is 171 units, 145 units of which \( (171 - 26) \) is due to DNA which was originally ccc. Therefore the \% increase in fluorescence is \( \frac{145 - 74}{74} = 96\% \). Originally we reported (3) that there was a 30% increase on nicking native PM2 DNA which is significantly different from the present value of 38%. We believe that this discrepancy is due to different preparations of PM2 DNA having slightly different superhelix densities. For example the temperature during lysis of the host may be critical. For this reason topoisomerase-relaxed PM2 DNA (provided the ionic strength and temperature are kept constant (24, 25)) may provide a more consistent standard.

Knowing the \% increase in fluorescence on nicking a 100% ccc DNA allows one to calculate the \% ccc DNA in any sample from the before and after heat readings. Thus again using the data of Fig. 6, the before and after heat readings for native PM2 DNA were 100 and 79 units. Therefore the \% oc DNA in the original sample is \( \frac{21 \times 100}{21 + (79 \times 1.38)} = 16.2\% \). Similarly for the topoisomerase-relaxed DNA the \% oc in the original samples is \( \frac{26 \times 100}{26 + (74 \times 1.96)} = 15.2\% \). These calculations neglect the small \% remaining fluorescence (probably due to palindromes) when 100% oc DNA is heat denatured. However as long as the \% oc DNA is small (as in the examples above) then the error caused by this approximation is insignificant. It can be seen that the \% increase in fluorescence upon nicking a ccc DNA gives an approximate measure of the super helix density (although a more accurate method will be described in the accompanying paper and also see ref. 26).

There is considerable redundancy of information in Fig. 6, since, upon nicking a ccc DNA containing a known amount of oc DNA, both the before heat or after heat readings can individually be used to calculate the \% ccc DNA remaining. This is a very useful property of the pH 12 assay solution since it effectively provides an internal control; that is any discrepancy between the expected before and after heat values suggests that some other process (e.g. cross-linking) is occurring simultaneously with the nicking. An example of this will be given in the next section. Also the redundancy means that the sample volume used for the assay need not be known accurately
since knowing the before and after heat fluorescence values (and also the superhelix density) the DNA concentration can be calculated as well as the % nicked. This has proved useful in cases where reactions need to be performed on the 1-2 µl scale.

The fluorescence assay, therefore, provides an alternative method for the analysis of circular DNAs. It compares favourably with gel electrophoresis and sedimentation velocity techniques since not only is it much quicker but also quantitation of the % ccc can be performed more accurately. Examples of the use of ccc DNA in conjunction with drugs or DNA-metabolizing enzymes will be found below and in the accompanying paper. Topologically closed DNA also occurs in nucleoids (27,28) and Cook and Brazell have successfully used an ethidium fluorescence assay in their studies of the HeLa nucleoid.

(f) Cross-linking and other chemical interactions

As with ccc DNA, cross-linked DNA will spontaneously renature since the cross-link provides a nucleation site for renaturation. Thus chemically-induced cross-links which are stable at pH 12 can be assayed rapidly by measuring the return of fluorescence after heating at pH 12. This is illustrated in Fig. 7 where mechloretamine has been used to cross-link T7 or linear PM2 DNA. T7 DNA is vulnerable to shear breakage (29) and thus a broken Pedersen pipette was used to take aliquots after various times of incubation. The before heat values are unaffected by this level of drug and the after heat fluorescence can be accurately corrected for any volume errors using the before heat values as standards. Therefore, in Fig. 7, the before heat readings have all been normalized to 100%. It can be seen that, as expected, T7 DNA is cross-linked more rapidly than linear PM2 DNA since the latter is of lower molecular weight and only one cross-link is needed to make the whole intact genome renatable (3). Thus with an agent that only cross-links DNA the initial rate of reaction would be directly proportional to the molecular weight of the DNA. In this case, however, the initial rates are in the ratio of 1:2 instead of 1:4 which would be expected on the basis of their molecular weights. Presumably mechloretamine also causes nicking and this is born out by the fact that at longer incubation times while the before heat fluorescence still remains constant, the after heat fluorescence actually decreases.

Of course if it is suspected that the heat-denaturation at pH 12 is itself the cause of nicking then an alternative procedure can be followed. Two samples are taken for each time point and one of them is heat-denatured
Figure 7. Cross-linking of linear PM2 and T7 DNAs by mechlorethamine. The DNAs at 1.4 A260 were incubated at 37° in 10 mM Tris-HCl pH 8, 0.1 mM EDTA with 1 µg/ml of mechlorethamine. At various times 10 µl samples were pipetted into 2 ml of the pH 12 assay solution and the fluorescence measured both before and after heat. The after heat readings have been normalized with respect to before heat readings set at 100%. See Text. ■ = T7 DNA; ○ = linear PM2 DNA.

before addition of the pH 12 solution. With many alkylating reagents, however, depurination of alkylated bases followed by nicking at pH 12 is not a problem since the high pH leads to immediate opening of the imidazole ring which is then resistant to further attack (30).

Elucidation of the mechanism of action of drugs and antibiotics which have multiple effects on DNA is considerably simplified by the use of ccc DNA. For example mitomycin C (activated by sodium borohydride) is primarily considered to be a cross-linking agent (31) but Fig. 8 elegantly demonstrates that it also causes strand cleavage, since the before heat fluorescence increases (32). The cross-linking is also noticeable since there is little loss of fluorescence after heat. Moreover the effect of sodium benzoate (a radical scavenger) gives a clear insight into its mode of action. Many antibacterial and antitumor agents cause strand cleavage and/or cross-linking as their primary mode of action so that this fluorescence assay has

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Figure 8. Interaction of activated mitomycin C with ccc PM2 DNA. The data is taken from ref. 28. ccc PM2 DNA at 1.1 A_{260} was incubated at room temperature with 3 \times 10^{-4} M mitomycin C in 50 mM KPi, pH 7.2. At various times 10 μl samples were pipetted into 2 ml of the pH 12 assay solution and the fluorescence measured both before (open symbols) and after heat (filled symbols). O, ● = 5.3 mM sodium borohydride; □, ■ = 5.3 mM sodium borohydride and 50 mM sodium benzoate.

found widespread use in some laboratories (32-35).

(g) Estimation of drug-DNA binding constants

Binding constants of drugs to DNA can be estimated and compared by measuring the loss of ethidium fluorescence as a function of added drug. This is illustrated in Fig. 9 for spermidine, spermine and spermine bis-acridine. The drug concentration which produces 50% inhibition of fluorescence is approximately inversely proportional to the binding constant. (This relationship takes no account of differences in binding site size or cooperative effects. Moreover for drugs with binding constants which are orders of magnitude larger than that of ethidium it invariably leads to a minimum value for the binding constant.) From Fig. 9 the drug concentrations producing 50% inhibition were found to be 35, 2 and 0.1 μM for spermidine, spermine and the spermine bisacridine respectively. (Controls were performed to show that the drugs themselves did not fluoresce nor did they interfere
Figure 9. Inhibition of ethidium binding to calf thymus DNA by the addition of polyamines. Small aliquots of concentrated polyamine solutions were added sequentially to 2 ml of the pH 8 assay solution containing 1.35 µM calf thymus DNA and the fluorescence was measured after equilibration for a few minutes. The abscissa shows the final drug concentration in the assay solution (note the logarithmic scale). O = spermine bisacridine, □ = spermine, △ = spermidine.

with the fluorescence of unbound ethidium.) Taking the binding constant of ethidium to be $10^7$ M$^{-1}$ and the ethidium concentration to be 1 µM then binding constants of $3 \times 10^5$, $5 \times 10^6$ and $>10^8$ M$^{-1}$ can be calculated for spermidine, spermine and the spermine bisacridine. For spermidine and spermine these values will be slight overestimates (2 or 3 fold) because these polyamines occupy more potential binding sites on the DNA than ethidium does. For the spermine bisacridine on the other hand the calculated value is an underestimate since the binding constant is higher than that of ethidium. Indeed under these conditions it appears that the spermine bisacridine binds quantitatively to the DNA as expected from its very high binding constant (36). (Use will be made of this property in the next section to measure nucleic acid concentrations in the presence of interfering substances.) We envisage that this technique will prove extremely useful for the rapid screening of a series of related compounds, especially since only very small quantities
are needed. If more accurate binding constants are required they can be calculated using the method of Falkenburg and Baguley (37).

In an analogous manner loss of ethidium fluorescence has also been used to measure the alkylation of DNA (38). It can be shown that methylated DNAs have an unaltered binding constant for ethidium but a reduction in the number of binding sites. Thus the loss of ethidium fluorescence is directly proportional to the extent of alkylation (38). This technique may prove particularly attractive for situations where radioactive labelling is impractical.

(h) General assays for DNA and RNA

In the above sections specific applications of the fluorescence assays have been described. In this final section we would like to suggest two other broader areas of research where these assays are extremely useful. The first of these concerns assaying for DNA and RNA in crude cell lysates.

For example we have found while preparing DNA and RNA polymerases that it is essential to remove the majority of the nucleic acids before DEAE cellulose chromatography (39,40). Both DNA and RNA can be estimated in the following manner:- An aliquot of the crude lysate (1-200 μl) is pipetted into 2 ml of the pH 8 assay solution in order to give a measurable fluorescence reading. Then 1 μl of RNase A at 20 mg/ml is added and the solution is mixed. Any RNA is immediately digested resulting in a loss of fluorescence from which the RNA concentration can be calculated. The DNA in the solution can then be estimated by adding 1 μl of a 1 mM solution of spermine bisacridine. As shown in section (g) addition of this amount of drug will obliterate any ethidium fluorescence due to binding to DNA. Again the loss of fluorescence is a measure of the amount of DNA. Any residual fluorescence is due to light scattering caused by insoluble matter in the crude lysate. This determination can be performed in under five minutes and so it rapidly gives the information required as to whether or not this lysate is ready for DEAE cellulose chromatography.

DNA concentrations in whole cells can be measured using a more direct method (3). A concentrated suspension of cells is heated at 96° in 0.2 M NaOH for five minutes. This lyses the cells and hydrolyses all the RNA. After neutralizing the solution with HCl, aliquots can be pipetted directly into the pH 8 assay solution. Only the DNA (which will be denatured) will give rise to any fluorescence so that its original concentration in the cell suspension can be calculated. Again any interference from cell debris can be detected as fluorescence remaining after addition of the spermine bisacridine (which also abolishes ethidium binding to denatured DNA).
method is sufficiently accurate that cell numbers can be estimated if the DNA content per cell is known or this latter quantity can be estimated if the number of cells is known. Other authors have developed similar techniques (41,42).

The pH 12 assay can also be used to follow the purification of ccc DNA either from bacterial plasmids or bacteriophage. For example during the preparation of PM2 DNA we routinely take aliquots and measure the fluorescence before and after heat. The high pH disrupts the phage so that ethidium can bind to the DNA and thus phage concentrations can be estimated (at high phage concentrations there is a small amount of interference due to the phage lipid). The after heat measurement is used to calculate the % ccc DNA in the sample.

Finally we have found the fluorescence assays to be useful in many cases where it would otherwise be necessary to use radioactive labelling. An example is given in Fig. 10 where the nucleoid of E. coli (27) has been isolated on a sucrose gradient. The nucleoid peak is readily identified by pipetting 50 µl aliquots into the pH 8 assay solution. The RNA present in the nucleoid and at the top of the gradient can also be detected on the same samples by adding RNase to the assay solutions. Thus the DNA:RNA ratio is calculated without recourse to double-labelling experiments. The fluorescence assay has other advantages over labelling methods. Firstly it is quicker since there is no need to acid precipitate all samples to remove unincorporated labels. Secondly, for efficient incorporation of labels auxotrophs are required (which also necessitates a more complex and expensive growth medium) while using the fluorescence assay wild type cells can be studied.

CONCLUSION

In this review we have presented a range of examples of the applicability of the fluorescence assays to the study of nucleic acids. In all cases we feel that the assay compares very favourably with alternative techniques when evaluated in terms of convenience, cost, accuracy and sensitivity. In some cases the assays provide unique methods for studying polynucleotides. The kinetics of triplex formation and the mechanism of drug action, for example, would be extremely difficult to elucidate by any other technique. These points will be discussed further in the accompanying paper where applications of the fluorescence assays to nucleic acid metabolizing enzymes will be considered.
Figure 10. Identification of the E. coli nucleoid after centrifugation through a sucrose gradient. An E. coli lysate was prepared essentially according to the procedure of Worcel and Burgi (43). 0.6 ml of this lysate was layered onto a 6 ml 10-30% sucrose gradient containing 1 M NaCl, 10 mM Tris- HCl pH 8, 1 mM EDTA and 1 mM DTT. After centrifugation at 13,800 g for 1 hour at 0°, 250 µl fractions were collected from the bottom of the tube. O = 50 µl aliquots assayed in the pH 8 assay solution; ● = 50 µl aliquots assayed in the pH 8 assay solution after incubation for a few minutes with 1 µl of 20 mg/ml RNase A.

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ABBREVIATIONS

*ccc DNA is covalently-closed circular DNA; oc DNA is open-circular (i.e., nicked) DNA, and clc DNA is covalently-linked complementary DNA (i.e., spontaneously renatures after heat denaturation).
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