Volume 7 Number 3 1979 Nucleic Acids Research

Review: Ethidium fluorescence assay. Part II. Enzymatic studies and DNA-protein interactions

A.R.Morgan, D.H.Evans, J.S.Lee and D.E.Pulleyblank

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received 30 July 1979

ABSTRACT

Almost all DNA and RNA metabolizing enzymes can be assayed rapidly and very sensitively by exploiting the enhanced fluorescence of ethidium intercalated into duplex DNA or RNA. Denatured DNA and natural RNAs contain duplex regions due to intramolecular hydrogen-bonding and can also be sensitively measured. Where the product is truly single-stranded (e.g. dT_n) it can be assayed by adding the appropriate complementary strand (e.g. dA_n or rA_n). Some of the assays described provide information not readily obtained by other assay procedures. Among the enzymes readily assayed are DNA and RNA polymerases, terminal deoxynucleotidyl transferases, nucleases of all varieties (e.g. single-strand specific, endonucleases including for example AP endonucleases, exonucleases, RNase H, etc.), ligases, topoisomerases including gyrases, and indirectly enzymes such as proteases and superoxide dismutase. DNA binding proteins such as histones and helix destabilizing proteins can also be quantitatively assayed.

INTRODUCTION

In the preceding paper we have described the application of ethidium fluorescence assays to physicochemical studies of nucleic acids. This second review article will extend these applications to enzymological studies and protein-nucleic acid interactions. The assays all rely upon the enhancement of fluorescence given by ethidium on intercalating duplex nucleic acids (1). Thus enzymes (such as polymerases, nucleases, ligases, topoisomerases, gyrases and DNA binding proteins) which directly or indirectly affect the number of ethidium bindings sites can be rapidly assayed. By suitable manipulation of the basic assays, enzyme activities not obviously associated with nucleic acids such as proteases or superoxide dismutases can also be measured. Assays for topoisomerases and ligases using ethidium fluorescence have been described by other authors (2-4) but a unique feature of the assay as developed in this laboratory is the use of a high pH ethidium buffer (5-8).

METHODS

Most of the methodology has been detailed in Part I along with a description of the chemicals, DNAs and RNAs used here. A description of reaction conditions and procedures is given in the figure legends. In general these procedures were as follows:

Each reaction was carried out in a 50-100 µl volume and samples of 5-20 µl were removed at appropriate times. These were added directly to 2 ml of ethidium solution where the high dilution and EDTA stop most reactions (however, see exception below).

Synthetic single-stranded polymers (e.g. dT_n) are assayed by hybridizing them to a 1.5 to 2 fold excess of the complementary strand (e.g. dA_n or rA_n) in a small (50 µl) volume at the bottom of a 10 x 75 mm test tube. The presence of 50 mM NaCl (or KCl) encourages reannealing and brings it to completion within 10 minutes. 2 ml of ethidium solution is then added to this newly formed duplex.

The pH 8 and pH 12 ethidium assay solutions have been described in the preceding review. Table I of Part I summarizes the properties of each. Heat denaturation was accomplished as in Part I. Enzymes were prepared or obtained from the following sources: DNA ligase; the ATP dependent form from T4 infected E. coli was purchased from P-L Biochemicals (Milwaukee, Wisc.); DNA polymerase I (fraction V) was prepared from E. coli B cells (Grain Processing Co., Muscatine, Iowa) according to the procedure of Jovin et al. (9); Deoxyribonuclease I from bovine pancreas was purchased from Worthington Biochemical Corp. (Freehold, N.J.); Exonuclease III from E. coli copurifies with DNA pol I (10) and separates on Sephadex G-100 chromatography; Gene 32 protein from T4 infected E. coli was prepared according to Alberts and Frey (11); Histones - whole trout testis histones were a gift from Dr. Gordon Dixon; Polynucleotide phosphorylase was identified as a contaminant copurifying with E. coli RNA polymerase (12); Pronase, B grade, was purchased from Calbiochem (San Diego, Cal.); RNA polymerase from E. coli was prepared according to (13); Ribonuclease I "A" from bovine pancreas and Ribonuclease H from E. coli were purchased from P-L Biochemicals; S1 nuclease was prepared according to (14); Terminal deoxynucleotidyl transferase was prepared from calf thymus according to (15); Topoisomerase ("ω" protein) was prepared from calf thymus according to (7); Trypsin was from the Sigma Chemical Co. (St. Louis, Mo.).

The DNAs and chemicals not already described in Part I include the following: $d(pT)_4$ was a gift from Dr. H. van de Sande; gelatin was pur-

chased from Fisher Scientific (Pittsburgh, Pa.) and partially hydrolyzed before use by heating overnight at 96°; dithiothreitol (A grade) was purchased from Calbiochem. Buffers (sodium acetate, potassium phosphate and Tris-HCl) were prepared and titrated as 1.0 M solutions. These were then diluted as required. Other chemicals were of reagent grade or better. A. laidlawii B membranes were a gift of Dr. R. McElhaney.

RESULTS AND DISCUSSION

(a) DNA polymerases

The synthesis of duplex DNAs can be conveniently followed by the pH 8 or pH 12 fluorescence assays. These have already been described (16). In particular the pH 12 assay allows a simple determination of the amount of clc* DNA generated during replication of DNA by E. coli DNA polymerase I (17). In Fig. 1a the assay is extended to the replication of synthetic DNA polymers (pH 8 assay) containing unusual bases, $d(TC)_n \cdot d(IA)_n$ and $d(\overline{BrUC})_n \cdot d(GA)_n$. Such novel DNAs have been used in studies on multi-stranded complexes, as well as replication studies. As can be seen for the synthesis of $d(\overline{BrUC})_n \cdot d(GA)_n$ there was excellent net-fold synthesis. The reaction was initiated with $d(TC)_n \cdot d(GA)_n$ and approximately 200 fold synthesis over input template was obtained. The purified $d(\overline{BrUC})_n \cdot d(GA)_n$ showed a lower fluorescence enhancement for intercalated ethidium than the standard calf thymus DNA (52 units/10 µl at 1 A_{260} compared to 70 for calf thymus DNA in the pH 8 ethidium assay). No clc DNA (as is usual for $d(pyrimidine)_n \cdot d(purine)_n$ DNAs) was obtained as shown by lack of fluorescence after heating the samples (17). The template was also readily degraded by the exonuclease actions of DNA polymerase I as indicated by the rapid drop in fluorescence once the substrates are exhausted. The extent of synthesis can be measured almost immediately by the fluorescence readings; thus in preparative reactions where maximum synthesis is required, a small sample of the main reaction mixture is incubated a convenient time before the bulk reaction in order to determine precisely when to terminate the reaction.

Another useful feature of the fluorescence assay is that the synthesis of the contaminant $d(AT)_n$, is readily detected by a return of fluorescence after heat. DNA polymerase I will synthesize $d(AT)_n$ in the absence of a template (18) or often when a template is very slowly replicated. This is illustrated in Fig. 1a by the synthesis of $d(TC)_n \cdot d(IA)_n$. Here synthesis was slow and the level of polymerase was rate limiting. Although initially

no clc DNA was detectable, it finally appeared after 35 hours of synthesis. In general there are two ways of determining if the clc DNA is a result of replication of the polymer or due to $d(AT)_n$ synthesis taking over. The use of the pH 12 assay solution is described in Part I. A second distinguishing feature of $d(AT)_n$ is that in spite of its duplex structure it is sensitive to S_1 nuclease and exonuclease I (19).

In Fig. 1b the replication of a natural DNA, S_1 nuclease nicked PM2 DNA, is shown using the pH 12 assay solution. Essentially all the product was clc DNA since the after-heat fluorescence paralleled the before-heat

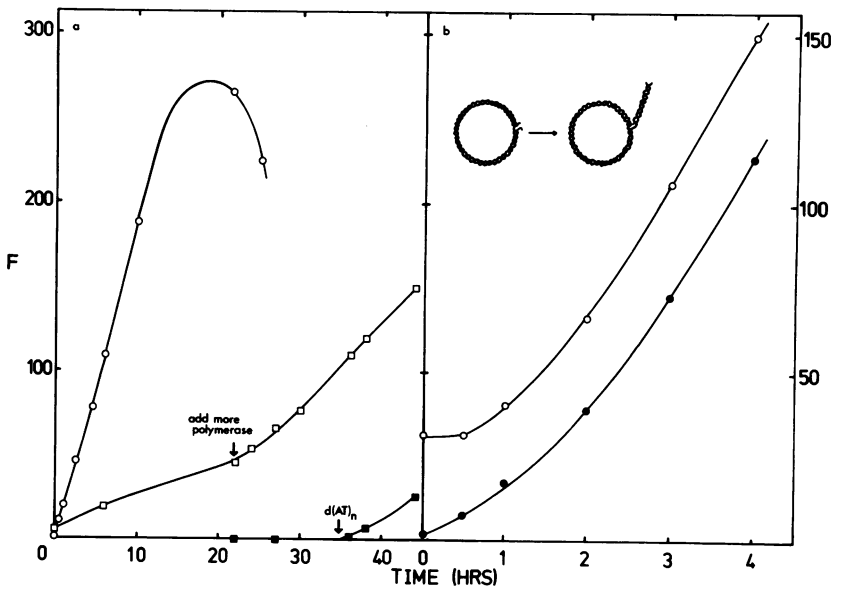

Figure 1. DNA polymerase. (a) Replication of synthetic DNAs. Reaction conditions were as described previously (16). At high levels of DNA synthesis <10 µl samples were taken in order to be within the linear range of the fluorescence assay but the readings were all normalized to the equivalent of a 10 µl sample. O = $d(\overline{BrUC})_n \cdot d(GA)_n$ synthesis with 2 mM $d\overline{BrUTP}$ substituted for dTTP and 0.1 A_{260} $d(TC)_n \cdot d(GA)_n$ as template. Samples (2-10 µl) were withdrawn and the fluorescence read in 2 ml pH 8 buffer. □ = $d(TC)_n \cdot d(IA)_n$ synthesis with 2 mM dITP substituted for dGTP and 0.4 A_{260} $d(TC)_n \cdot d(GA)_n$ as template. Polymerase levels were doubled at the time indicated. 5 µl samples were withdrawn and the fluorescence read in pH 8 ethidium buffer before (□) and after (■) heat. Appearance of after heat fluorescence represents $d(AT)_n$ synthesis. (b) Replication of natural circular DNAs. S_1 endonuclease nicked PM2 DNA was prepared as described in Fig. 7. Reactions (100 µl) contained 1.0 A_{260} DNA and 5 µl samples were taken into pH 12 buffer. O = before heat fluorescence; ● = after heat fluorescence. The inset shows a schematic illustration of this reaction.

fluorescence, except in the initial stages. This point is discussed in detail in the following papers on DNA replication, but is observed only with circular DNAs and not linear ones (see for example ref. 17, Fig. 8). This is interpreted as being due to topological constrictions which clc DNA would impose on circular DNAs limiting the amount of ethidium that can bind (see Part I and inset Fig. 1b). Thus the fluorescence assay gives additional information not so readily obtained by radiolabel assays. However fluorescence assays do not reveal nick translation (20) which can be monitored by incorporation of radiolabels. Coupling of the two types of assay can therefore be very useful.

DNA synthesis by terminal deoxynucleotidyl transferases (21) gives rise to DNAs which are not duplex and in fact are frequently homopolymers since they are usually synthesized with only one deoxynucleoside triphosphate present. Fig. 2 shows how, nevertheless, the fluorescence assay can still

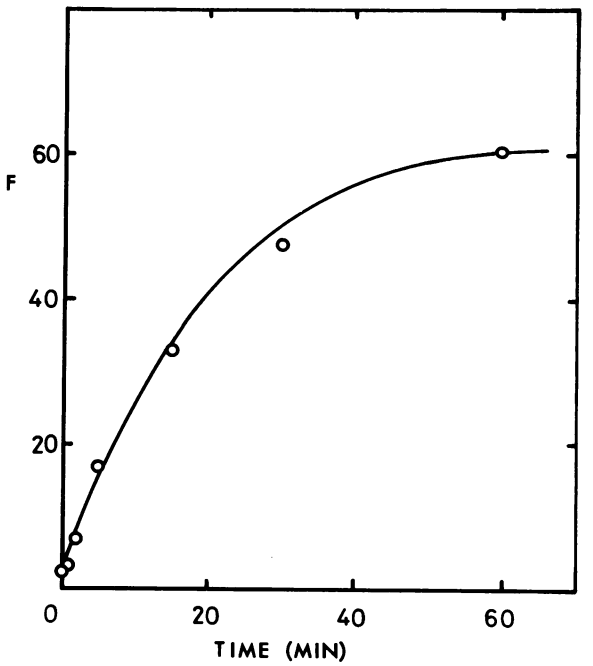

Figure 2. Terminal deoxynucleotidyl transferase. The reaction (100 µl) contained 50 µM $d(pT)_{4-5}$ and involved polymerization of thymidylate residues from dTTP onto the $d(pT)_{4-5}$ primer, using the conditions of (21), and 100 µg/ml terminal transferase. The dT_n was assayed by annealing 10 µl samples to 20 µl rA_n (1 A_{260}) + 10 µl 4 M KCl for about 5 minutes and then adding pH 8 ethidium assay solution for measuring the $dT_n \cdot rA_n$ duplex.

be utilized by adding samples of the reaction mixture to an excess of complementary strand. Using $d(pT)_4$ as the primer and dTTP as the substrate, dT_n synthesis was quantitated by adding samples to excess rA_n. The duplex $dT_n \cdot rA_n$ was then measured by ethidium fluorescence, excess rA_n giving no contribution. rA_n was used rather than dA_n as the complementary strand because it is both cheaper and also the resulting DNA·RNA duplex provides a slight increase in sensitivity over the DNA·DNA duplex (~10%).

(b) RNA polymerase

A very sensitive assay for RNA polymerase is to use $d(AT)_n$ as the template since in 0.2 M KCl many hundred rounds of transcription can be made to occur. Furthermore minute amounts of $d(AT)_n$ contaminating other DNA polymers can be quantitated (16). The product of transcription $r(AU)_n$ is of course itself self-complementary, and can be readily measured by the fluorescence assay (Fig. 3a). We have found this the most convenient way to assay for RNA polymerase during purification. The synthesis of other RNAs can also be monitored. In studies involving synthetic polymers the

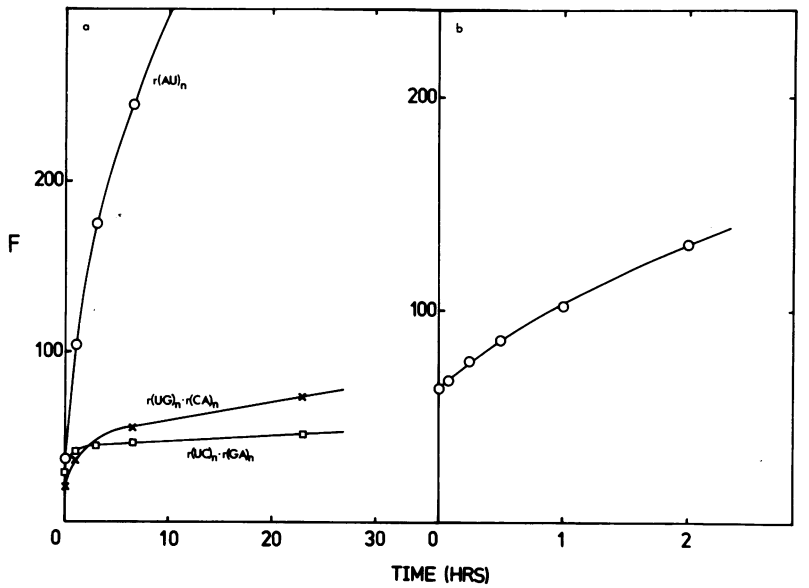

Figure 3. RNA polymerase. (a) Synthetic DNA templates. Reactions (100 µl) were as described previously (47) except that the templates were $d(AT)_n$ (1.0 A_{260}, O-O); $d(TG)_n \cdot d(CA)_n$ (1.1 A_{260}, x-x) and $d(TC)_n \cdot d(GA)_n$ (1.5 A_{260}, □-□). The ribonucleoside triphosphates were 1 mM each and only ATP and UTP were present for $r(AU)_n$ synthesis. (b) E. coli DNA. The reaction was as above except that the template was E. coli DNA (1.0 A_{260}) and RNA polymerase was at 0.18 mg/ml.

synthesis of RNA can be followed by the fluorescence assay if both strands are transcribed to give a duplex RNA (Fig. 3a). For $r(UG)_n \cdot r(CA)_n$ and $r(UC)_n \cdot r(GA)_n$ there was poor synthesis compared to net input of DNA template. As shown from radiolabelling assays one strand is often synthesized in excess and this was also shown by the fluorescence assay by adding back either isolated single strand under annealing conditions as for the terminal deoxynucleotidyl transferase assays already described. In Fig. 3b the synthesis of RNA off a natural DNA (E. coli) is shown. The product enhances the ethidium fluorescence because it contains about 50% of self-complementary regions (22). The fluorescence at zero time is due to the E. coli DNA template. That RNA is indeed being synthesized is quickly ascertained by adding 1 µl of RNase A (20 mg/ml) directly to the pH 8 ethidium assay solution. Under these conditions the RNA is degraded within seconds to give fluorescence only due to the DNA template (see nuclease section below).

(c) Nucleases

(i) Single strand specific

S_1 nuclease is the most commonly used single-strand specific nuclease. It is readily assayed using the pH 8 ethidium assay solution since the "single-stranded" natural DNA substrates used for the assay of this enzyme contain short intra-molecular base-paired regions (see Table I, Part I). This means a loss of sensitivity over native DNA of only about 2 fold in the fluorescence assay. Fig. 4 illustrates the specificity of S_1 nuclease using native DNA and heat-denatured DNA. Note that different levels of native and denatured DNAs were used.

(ii) Exonucleases

Fig. 5a shows the action of exonuclease III on the synthetic DNA $d(TCC)_n \cdot d(GGA)_n$ using the pH 8 ethidium assay. Exonuclease III digests a duplex DNA from the 3' end of both strands simultaneously. Under the conditions of the nuclease digestion protruding complementary 5' ends rapidly reanneal so that for synthetic DNAs one would expect the digestion to continue until no duplex remained. However exonuclease III treatment of synthetic DNAs usually leads to preferential digestion of one strand (assayed by adding back the complementary strand under annealing conditions to reform a duplex) which has proved useful, for example, in isolating single-stranded $d(GA)_n$ from $d(TC)_n \cdot d(GA)_n$ (unpublished results). With natural DNAs the protruding "denatured" DNA left by exonucleolytic action can be made genuinely "single-stranded" in the pH 12 ethidium assay solution although for comparable levels of exonuclease III the reaction is much slower than

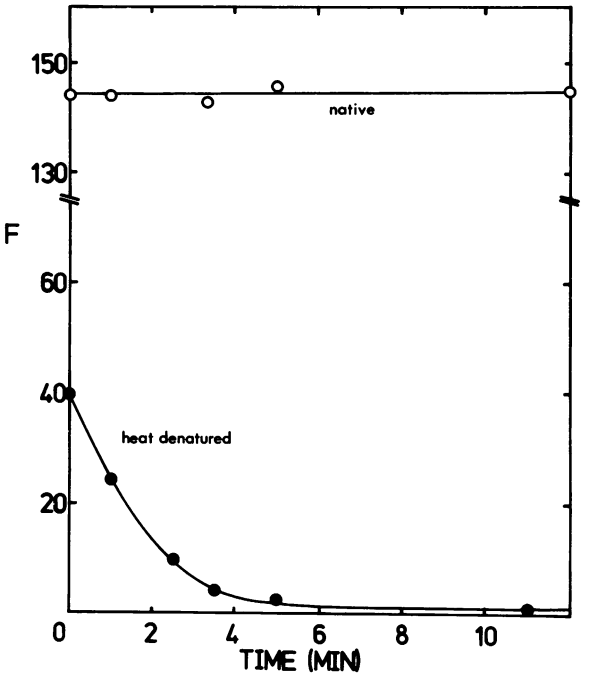

Figure 4. S_1 endonuclease susceptibility of native and heat-denatured calf thymus DNA. Reactions (100 µl) contained 1.5 A_{260} native DNA (O-O) or 1.1 A_{260} (before heating) heat-denatured DNA (●-●), 50 mM NaOAc pH 5, 50 µM $ZnCl_2$ and 7 µg/ml S_1 nuclease, with incubation at 37° and 10 µl samples were added to 2 ml of pH 8 ethidium assay solution.

with synthetic templates.

(iii) Ribonucleases

RNase A (pancreatic) is most active at low salt concentrations and in the absence of Mg^{++} since a single-stranded substrate is required. This has proved useful in rapid tests on crude cell extracts (see Part I) since the RNase A can be added directly to the pH 8 ethidium assay solution whereupon any fluorescence due to RNA rapidly disappears. A distinction can be made between "single-stranded" natural RNAs, such as phage R17 RNA and completely duplex synthetic RNAs (such as $r(UG)_n \cdot r(CA)_n$) in their sensitivities to RNase A by raising the salt in the ethidium assay solution to 0.1 M in KCl. Under these conditions essentially all the R17 RNA is rapidly degraded while $r(UG)_n \cdot r(CA)_n$ is degraded at a much slower rate. The rapid degradation of even duplex RNA in the standard fluorescence assay is due to

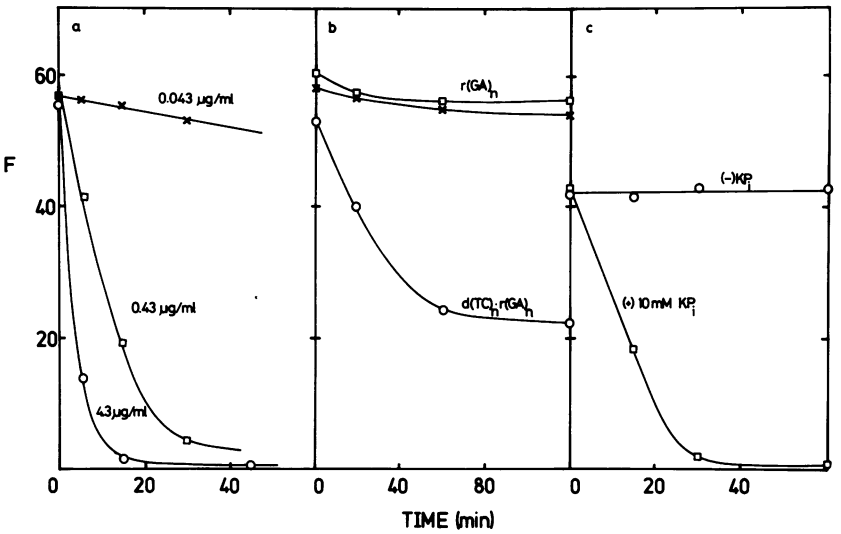

Figure 5. Nuclease assays. (a) Exonuclease III. Reactions (50 µl) contained 1.4 A_{260} $d(TCC)_n \cdot d(GGA)_n$, 50 mM Tris -HCl pH 7.6, 10 mM DTT, 10 mM $MgCl_2$, 1 mg/ml gelatin and exonuclease III at 4.3, (O-O); 0.43 (□ - □) and 0.043 (x-x) µg/ml, at 37° and 10 µl samples were added to the ethidium pH 8 buffer. (b) Ribonuclease H. Reactions (50 µl) were those of ref. 48 and contained 0.5 units of enzyme (as defined by PL Biochemicals) at 37°, and 10 µl samples were added to the pH 8 ethidium buffer. The $r(GA)_n \cdot d(TC)_n$ template was prepared by annealing 20 µl of $r(GA)_n$ (3.9 A_{260} ε = 8,800) to 25 µl $d(TC)_n$ (2.7 A_{260}, ε = 7,500) by cooling from 50° slowly in 10 mM Tris-HCl pH 8, 0.1 mM EDTA. $r(GA)_n \cdot d(TC)_n$ was present at 140 µM (P) in the reaction (O-O). Two controls to show that the $r(GA)_n$ strand was uniquely degraded in the DNA·RNA hybrid are shown. $r(GA)_n$ was substituted for $r(GA)_n \cdot d(TC)_n$ in the reaction (□ - □) at 70 µM (P) and it was detected fluorimetrically by annealing 10 µl samples with excess $d(TC)_n$ (5 µl, 360 µM (P) + 5 µl 4 M KCl) and then adding the ethidium pH 8 buffer. Also the specific loss of $r(GA)_n$ in the original reaction mixture was demonstrated by adding 10 µl samples to excess $r(GA)_n$ (3 µl, 440 µM (P) + 5 µl 4 M KCl) (x-x) giving essentially constant fluorescence with time. (c) Polynucleotide phosphorylase. The enzyme was assayed by the phosphorolysis of rA_n. The reaction (100 µl) contained rA_n (0.5 A_{260}) and crude RNA polymerase (fraction 4 of ref. 49, 3.8 µg) with (□ - □) or without (O-O) 10 mM KP_i, pH 7.5. Samples (10 µl) of the reaction were annealed to excess rU_n (10 µl, 1 A_{260}) in the presence of 20 µl of 2 M KCl, before adding the pH 8 ethidium buffer.

more extensive breathing and possibly slippage which can occur at very low ionic strengths.

RNase H activity (specific for RNA in a DNA·RNA duplex) is assayed as in Fig. 5b. The synthetic hybrid $d(TC)_n \cdot r(GA)_n$ is readily degraded by RNase H whereas $r(GA)_n$ is unaffected (assayed by adding back excess $d(TC)_n$). As

a further control excess $r(GA)_n$ was added back to the RNase H treated duplex $d(TC)_n \cdot r(GA)_n$ to show that the original fluorescence is recovered and therefore the $r(GA)_n$ strand was specifically degraded, not the $d(TC)_n$ strand.

(iv) Polynucleotide phosphorylase

Polynucleotide phosphorylase can act synthetically or degradatively. In Fig. 5c contaminating polynucleotide phosphorylase in an RNA polymerase preparation was detected by the phosphorolysis of rA_n. The loss of rA_n was followed by a prior annealing to rU_n before the ethidium assay solution was added. The strict dependence of degradation on phosphate characterized the contaminant as polynucleotide phosphorylase.

(v) Endonucleases

These are most sensitively assayed by the ethidium fluorescence assay using circular DNAs. The method has already been described (2,6) and is illustrated further here to emphasize some of the unique aspects of the assay. First its sensitivity is extremely high; pg quantities of pancreatic DNase are readily detected. The assay depends as detailed in Part I, on the renaturability of ccc DNA in the pH 12 ethidium assay, due to its unique topological properties, i.e. that the complementary strands are topologically linked and behave like clc DNA. A single phosphodiester bond break allows the strands to separate. The advantage of the pH 12 assay is that two independent readings are obtained for each sample (before and after heat) giving a redundancy of information. In Fig. 6a DNase at 0.2 ng/ml was readily detected using PM2 DNA ($\sim 6 \times 10^6$ daltons). For λ DNA circles ($\sim 3 \times 10^6$ daltons) the sensitivity would be 5 times higher. For comparison a hyperchromicity assay is shown in Fig. 6b which is about 5,000 fold less sensitive.

The second unique aspect of the assay already mentioned is the redundancy of information. In Part I the usefulness of this fact was illustrated by the ability to measure simultaneously both cross-linking and nicking by mitomycin. With nucleases one use of this redundancy is that the volume of sample assayed does not have to be known. This can be especially useful in assaying extremely small volumes, for example less than 1 μl. A further feature of this redundancy of information is that if the increase in fluorescence before heat does not match the decrease after heat (for accurately measured volumes) then one must suspect that more than one enzyme activity is present. In ref. 23 for example the action of both topoisomerases and nucleases were detected in the same reaction.

A third useful feature of using ccc DNAs for assaying endonucleases is

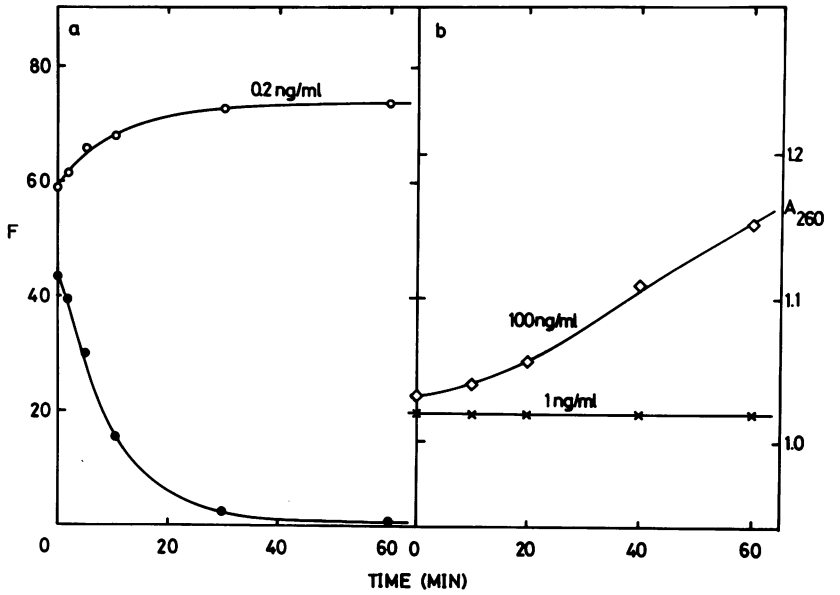

Figure 6. A comparison of hyperchromicity and fluorescence nuclease assays. (a) The fluorescence assay. The reaction (100 µl) contained 50 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 1.2 A_{260} ccc PM2 DNA, 1 mg/ml gelatin and 0.2 ng/ml DNase I. Incubation was at 23° and 10 µl samples were taken into 2 ml pH 12 buffer. O = before heat; ● = after heat. (b) Hyperchromicity assay. Reactions (500 µl) were as above except 1.0 A_{260} calf thymus DNA was used instead of PM2 DNA. Incubation was at 23° and the absorbance followed at 260 nm. ◇ = 100 ng/ml; x = 1 ng/ml.

that flexibility can be introduced into the assay by appropriately modifying the substrate DNA. These will not be detailed here but some examples will be mentioned. The depurination of ccc DNA has been followed by the susceptibility of the depurinated site to alkaline cleavage. Using the fluorescence assay we have prepared ccc DNAs containing at least one depurinated site per DNA molecule (24). These have proved useful and sensitive substrates for looking at AP endonucleases, although in this case only the fluorescence increase could be utilized to measure AP endonuclease activity since the substrate is sensitive to heat in alkali. The sensitivity was increased by using topoisomerase-relaxed ccc PM2 DNA (see Part I). N-glycosidases (25) can in principle also be assayed by coupling such enzyme activities with AP endonucleases. Other nucleases have been found specific for carcinogen modified DNA (26) and again the fluorescence assay should be useful in screening as well as the characterization of such enzymes. More recently it

has been found that nucleosides can be enzymatically inserted into AP sites on DNA (27) which would now become insensitive to AP endonuclease action (or alkali). With the proliferation of enzymes of known action and by suitable modification of the DNA substrate, the fluorescence assay can be used in almost any conceivable situation involving nucleases, and should be of help in studying such complex phenomena as repair and recombination.

(d) Ligases

The ligase assay is the reverse of the above endonuclease assay; oc DNA is converted to ccc DNA. Therefore the before heat fluorescence drops as ccc DNA is formed due to topological constraints on ethidium binding whilst the after heat fluorescence increases as expected of ccc DNA. When all the oc DNA is converted to ccc DNA then (Fig. 9) the before and after heat fluorescence readings should be identical. Since there is approximately a 100% increase in fluorescence on nicking relaxed ccc DNA, the drop in fluorescence before heat should match the rise in fluorescence after heat. Fig. 7 illustrates the ligase assay and bears out the above predictions, except that the DNA was not converted fully to relaxed ccc DNA, probably because the substrate contained some unrepairable gaps. The substrate for the ligase was prepared by S_1 nuclease treatment of ccc PM2 DNA. S_1 nuclease cuts supercoiled DNA much more rapidly than relaxed ccc DNA because supercoiled molecules contain regions which are partially denatured by the positive free energy of supercoiling (28). The moment the supercoiled molecule is nicked by S_1 nuclease, it immediately relaxes and becomes a poor substrate for the further action of the nuclease. Fig. 7 shows that about 70% of the substrate could be closed by ligase at the highest level of ligase used. The remaining 30% of the DNA probably contained both gaps (DNA polymerase I repair increased the final level of ccc DNA) and other structures not fully characterized. For example, S_1 nuclease can also introduce double-strand breaks (29). The sensitivity of the assay compares very favorably with that of Modrich and Lehman (30) whose units are used in the legend to Fig. 7. Their assay depends on the formation of $d(AT)_n$ circles resistant to exonuclease III. The other commonly used assay for T4 ligase measures the exchange of $^{32}PP_i$ with ATP (31). Only those ligases requiring ATP as the cofactor can be assayed in this manner. Also this fluorescence assay is more enzymologically relevant in the sense that other enzymatic reactions can give rise to $^{32}PP_i$ exchange; e.g. starch synthetase in plants (32).

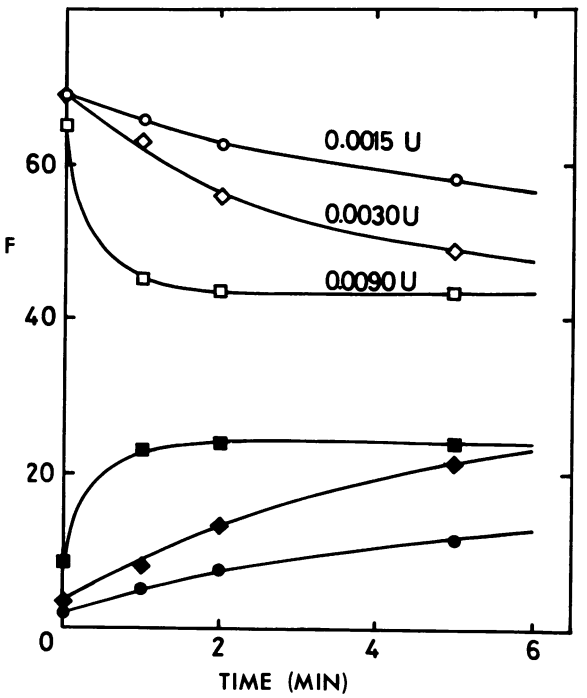

Figure 7. Ligase. The substrate, S_1 nicked oc PM2 DNA, was prepared as follows: the reaction (200 µl) contained 50 mM NaOAc pH 5, 10 mM $ZnCl_2$, 0.3 M KCl, 5 A_{260} PM2 DNA (80% ccc) and 7 µg/ml S_1 nuclease, incubated at 4° for 36 hours (till no fluorescence remained after heating in the pH 12 ethidium buffer, incidating all the ccc DNA had been nicked). The oc DNA was purified by gel exclusion chromatography on Biogel A 1.5 m, 50-100 mesh. The ligase reaction (50 µl) contained 20 mM Tris-HCl pH 7.6, 10 mM DTT, 1 mg/ml gelatin, 10 mM $MgCl_2$, 5 mM ATP, 1.4 A_{260} S_1-treated PM2 DNA and ligase at 0.0015 (O-O), 0.003 (◇-◇) and 0.009 (□-□) units (30). Incubation was at 30° with 10 µl samples added to the pH 12 ethidium buffer. The filled symbols show the fluorescence remaining after heating.

(e) Topoisomerases and Gyrases

An ethidium fluorescence assay has been used extensively in this and other laboratories for the purification and characterization of topoisomerases and gyrases (3,4,7,23,34-37). Since these enzymes change the topological winding number of ccc DNAs, they also affect the amount of ethidium that can be bound. The more negatively supercoiled a DNA is, the greater the amount of ethidium bound (33). Fig. 8 is a typical example of a fluorescence assay in which native ccc PM2 DNA is relaxed by partially purified calf thymus topoisomerase. Since eucaryotic topoisomerases are

active in the presence of EDTA (38), they can be assayed in crude extracts from nuclei (or whole cells) without interference from contaminating nucleases. It is possible to detect topoisomerase activity in as few as 10^4 HeLa cells using the fluorescence assay. For native ccc PM2 DNA there is a fluorescence decrease of about 30% on complete relaxation by topoisomerase. In the absence of contaminating oc DNA the before and after heat fluorescence readings for any particular time point would be identical (Fig. 9). However in practice two parallel curves (Fig. 8) are obtained due to contaminating oc DNA which remains constant throughout the time course of the reaction.

A similar assay can also be used for DNA gyrases. Gyrases insert negative supercoils into relaxed or partially relaxed ccc DNAs by utilizing the free energy of hydrolysis of ATP. Topologically, this is the reverse of the topoisomerase reaction and results in an increase in ethidium

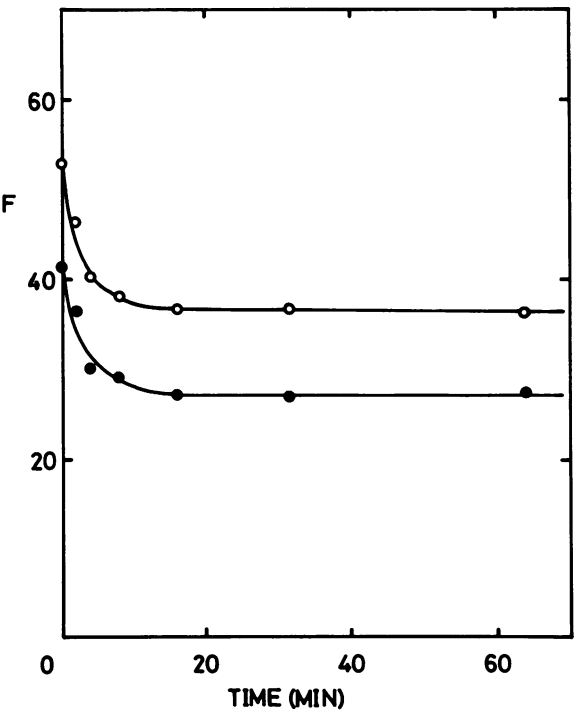

Figure 8. Topoisomerase. The reaction (100 µl) contained 0.2 M KCl, 0.1 mg/ml gelatin, 10 mM Tris-HCl pH 8, 1.5 A_{260} PM2 DNA, 2 mM EDTA and 11 µg/ml crude calf thymus topoisomerase (7), at 37° with 10 µl samples added to pH 12 ethidium buffer. The filled symbols show the fluorescence after heating.

fluorescence. The assay has been used by others and typically with relaxed DNA a 50% increase in fluorescence is obtained (37).

Fig. 9 summarizes the 4 assays involving ccc DNA. It should be stressed that the endonuclease and ligase assays both give a redundancy of information which can be exploited as described above. This is not the case for the topoisomerase and gyrase assays. However if there are other contaminating activities, especially nucleolytic, there are readily detected by an increasing divergence in the before and after heat fluorescence readings.

Topoisomerases have also been used in an assay for the determination of the superhelix density of a ccc DNA or the unwinding angle caused by an intercalated drug (39). Since large quantities of calf thymus topoisomerase can be rapidly prepared it is a useful, inexpensive reagent. Fig. 10

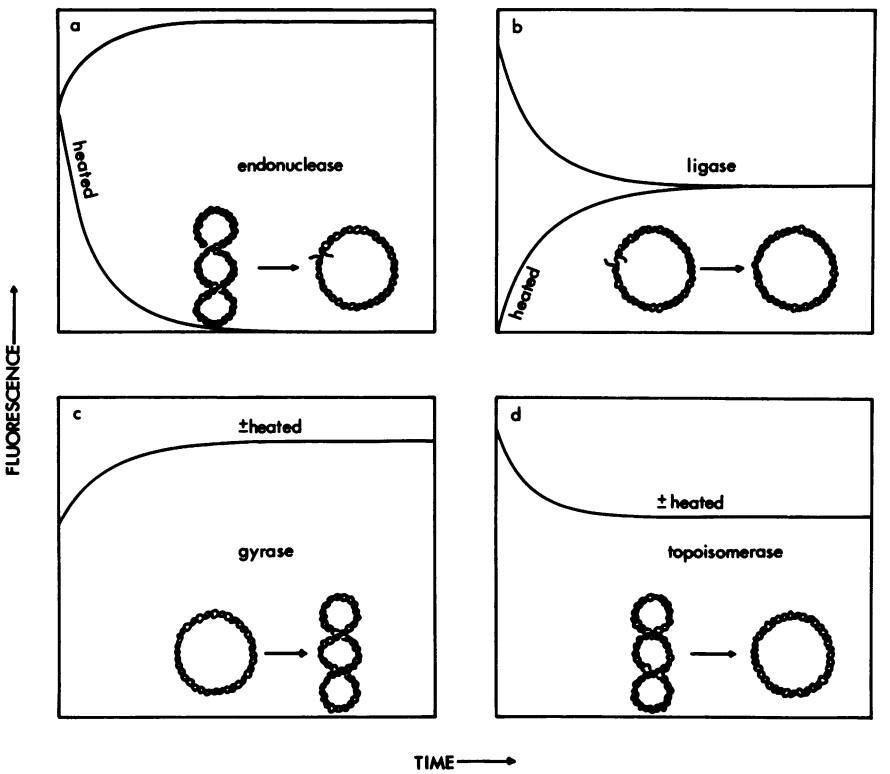

Figure 9. Idealized curves summarizing fluorescence assays that use circular DNAs as substrates. (a) Endonuclease nicking of superhelical DNA. (b) Ligase closure of nicked oc DNA. (c) Gyrase insertion of negative superhelical turns into relaxed ccc DNA. (d) Topoisomerase relaxation of negative superhelical DNA.

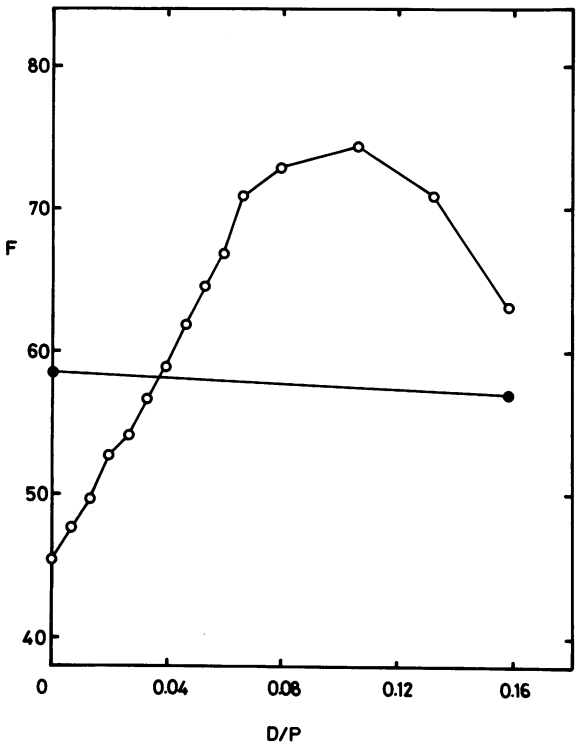

Figure 10. Superhelix densities and unwinding angles of intercalators. Reactions (50 µl) contained 0.1 M NaCl, 0.3 mg/ml gelatin, 10 mM Tris-HCl pH 8, 2 mM EDTA and 1.5 A_{260} ccc PM2 DNA (∿10% contaminated with oc DNA). To these 50 µl solutions were added 1 µl of varying concentrations of spermine bisacridine and 2 µl of crude calf thymus topoisomerase (shown in Fig. 8 to completely relax the DNA) with incubation at 37° for 30 min. D/P represents the molar ratio of drug to DNA phosphate. The fluorescence of duplicate 20 µl samples were read in the pH 12 ethidium buffer after heating (0-0). The filled circles are controls to which no topoisomerase had been added, without drug and at the highest concentration of drug. The superhelix density or unwinding angle can be calculated from the cross-over point as described in (39).

shows the titration of the superhelical turns of PM2 DNA with spermine bisacridine. The open circles are data points derived from reaction mixtures to which topoisomerase has been added. It can be seen that the fluorescence of ethidium in the pH 12 buffer after heat (and thus the superhelix density of the DNA) increases with increasing concentrations of spermine bisacridine in the reaction mixture. The filled circles are data points derived from "control" reaction mixtures to which no topoisomerase

has been added. The line joining these two points has a slight negative slope due to the binding of a small amount of spermine bisacridine even in the pH 12 assay solution. The point at which the two lines cross represents the equivalence point of the titration since at this point the ccc DNA has been relaxed by the binding of the drug and the topoisomerase has had no effect. Further details are given in ref. (39) but it is evident that if the unwinding angle of the drug is known the superhelix density of the ccc DNA can be determined from the D/P ratio at the "cross-over", or conversely if the superhelix density of a standard DNA is known the unwinding angle of the drug can be estimated. The assays can be done within about three hours with less than 1 OD_{260} of DNA. The decrease in fluorescence at very high levels of drug is due to topoisomerase inhibition. The initial linearity shows that the amount of ethidium bound in the fluorescence assay is directly proportional to the superhelix density. A word of caution with respect to the superhelix densities of ccc DNAs; for PM2 phage DNA, different preparations can sometimes give quite different superhelix densities (unpublished data). Therefore it is dangerous to assume any DNA has a literature value superhelix density until it has been titrated against a standard drug such as ethidium, most simply as described above.

(f) DNA Binding Proteins

We have found that many DNA binding proteins can be studied by fluorescence assays. Histones block ethidium binding to duplex DNAs while gene 32 protein stabilizes single-stranded regions. Protein binding in both cases can be followed because of the resulting loss of fluorescence and this is illustrated in Fig. 11. The stoichiometry of histone binding agrees well with that reported by other methods (40).

In the case of gene 32 much more protein is bound her nucleotide residue than for the histone complex (∿10x) again as reported in the literature (11). We have found these interactions particularly useful in assaying gene 32 during purification. Aliquots of column fractions are added to denatured DNA, and then the ethidium pH 8 assay solution is added and the fluorescence measured. A dip in the fluorescence plot indicates the presence of gene 32 or nucleases. These are readily distinguished by heating the ethidium solution. For gene 32 the fluorescence increases to the control level whereas for nucleases it may even decrease further.

(g) Other Applications

The fluorescence assay can also be used to measure some enzyme activities which are not obviously associated with nucleic acids. Figure 12 shows

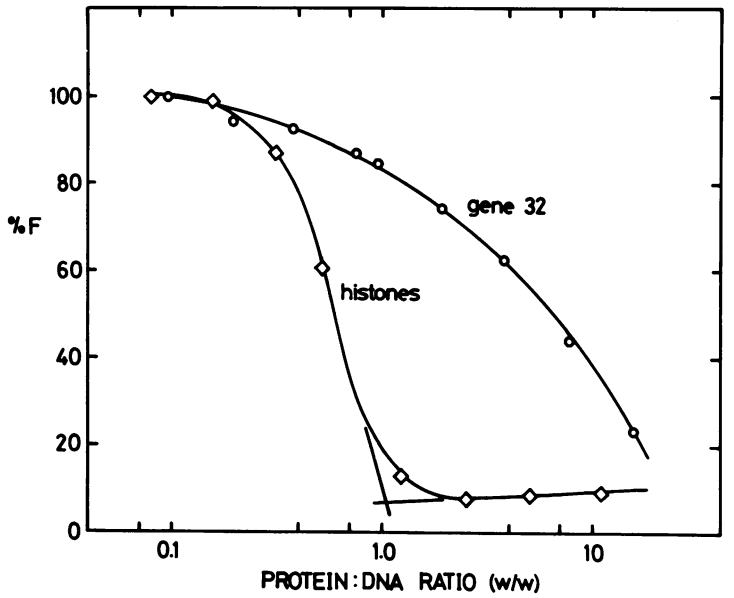

Figure 11. Histone and gene 32 binding to calf thymus DNA. Whole histones were mixed with DNA in 25 µl reaction mixtures containing 84 µg/ml calf thymus DNA, 10 mM Tris-HCl pH 8, 0.1 mM EDTA, 20 µg/ml gelatin and whole histones at the weight ratio given. These were incubated at 37° for 30 min with 10 µl aliquots added to 2 ml of pH 8 fluorescence buffer (◇- ◇). The inter section represents 1 mole of histone complex (MW ∿130,000) per 190 base pairs (MW ∿660). Gene 32 protein was mixed with heat denatured DNA in 30 µl reaction mixtures containing 33 mM Tris-HCl pH 8, 40 mM KCl, 35 µg/ml calf thymus DNA and protein at the weight ratio given. These were incubated at 37° for 90 min and the entire sample (30 µl) transferred to 2 ml pH 8 buffer. The loss of fluorescence (O-O) represents gene 32 destabilization of short intramolecular duplexes in the denatured DNA.

how it can be converted into a novel protease assay. The digestion of histone/DNA complexes by trypsin or pronase causes a return of ethidium fluorescence. Note that these proteolytic assays were carried out directly in the ethidium assay solution, i.e. under very dilute substrate conditions. For an increase in sensitivity the assays should be done on complexes with DNA at around 1 A_{260} and 10 µl samples are then added to the ethidium assay solution. It also should be noted that many proteolytic substrates could be generated with different DNA binding proteins which bind either duplex or denatured DNA or RNA.

The measurement of the activities of xanthine oxidase and superoxide dismutase (8) provides a final example of the wide applicability of these

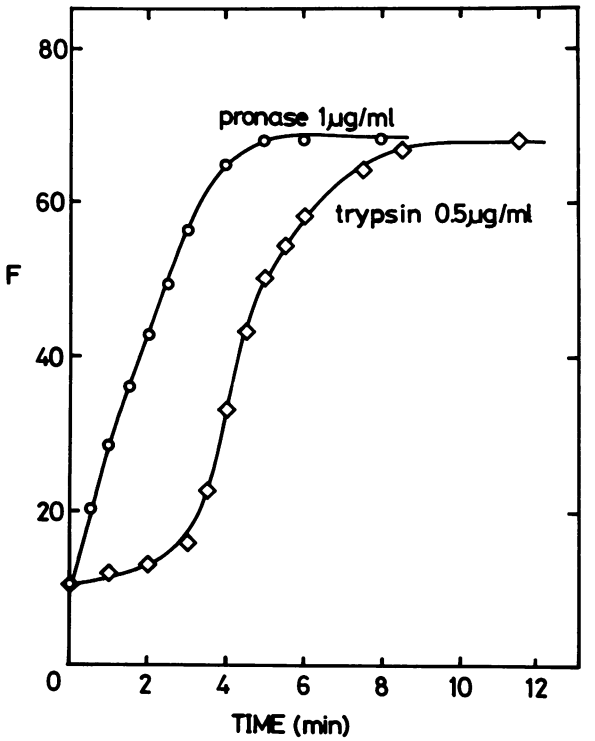

Figure 12. Proteases. The reactions were carried out directly in the pH 8 fluorescence buffer to which had been added 15 µl of histone/DNA complex (1.3:1, w:w) prepared as described in Fig. 11. To this was added the indicated levels of protease and the reaction followed directly in the fluorimeter at 25°. O = pronase, 1 µg/ml; ◇ = trypsin, 0.5 µg/ml.

assays. Xanthine oxidase in the presence of xanthine generates superoxide radicals which will cause breaks in DNA. Using ccc DNA as a substrate the cleavage reaction is readily followed in the pH 12 assay solution (see nuclease section above). Superoxide dismutase inhibits the cleavage reaction by dismutating superoxide radicals and thus it, too, can be rapidly assayed. Further details of this coupled enzyme assay are given in ref. (8).

(h) Controls and Interferences

Before concluding it is appropriate that sources of error and interference be discussed. These can be divided into two broad categories, interferences that directly affect fluorescence measurements and sources of error peculiar to the use of ccc DNAs.

TABLE I

Material[a]	% change in pH 8 fluorescence	% change in pH 12 fluorescence
Lipids and detergents		
10% sarcosyl	not sig.[c]	not sig.
10% SDS	not sig.	ppt's
10% SDS[b]	+ 30%	ppt's
3.5 mg/ml A. laidlawii membranes	+120%	+40%
Organic solvents		
acetone	+ 5%	not sig.
EtOH	+ 4%	not sig.
Proteins		
10 mg/ml gelatin	not sig.	not sig.
1 mg/ml RNA polymerase[b]	- 8%	not sig.
Salts		
4 M NaCl	-11%	-12%
1 M NaCl	- 2%	- 4%
1 M $MgCl_2$	-76%	ppt's
sat. CsCl	-30%	-23%
Miscellaneous		
1 mM spermine bisacridine (1 µl)	-100%	not sig.

[a] 10 µl samples were added to 2 ml of ethidium buffer containing 70 F_8 or 50 F_{12} units of calf thymus DNA.

[b] Sample + DNA mixed first before adding buffer.

[c] not significant.

Table I summarizes a variety of materials that could potentially enhance or inhibit fluorescence. Lipids and detergents can cause problems especially if detergents are being used in high concentrations to lyse cells. The amount of spurious fluorescence depends on the order of addition. This indicates that where micellar formation can occur precise controls are difficult to perform and caution in interpretation should be exercised. It is most severe in the case of biological membranes.

Spurious fluorescence of this sort can be easily distinguished from that due to duplex nucleic acids by adding spermine bisacridine (see Part I). Spermine bisacridine quantitatively displaces intercalated ethidium at pH 8 and does not fluoresce in the spectral region used here. Any residual fluorescence (after adding spermine bisacridine) can be ascribed

to material other than DNA or RNA. Note that at pH 12 the spermine chain is not positively-charged and thus the drug no longer inhibits the binding of ethidium.

Another source of interference is organic solvents which cause a small increase in fluorescence in the pH 8 ethidium assay solution due to inhibition of proton exchange (41). If the problem is considered severe enough the blank can be suitably adjusted.

Proteins, other than the binding proteins discussed earlier, have little effect. High levels of RNA polymerase, for example, cause only a small drop in fluorescence. It is possible but rare for proteins themselves to interfere, e.g., the fluorescence of spermine oxidase. This is probably due to a bound flavin moiety. Thus care should be taken when working with proteins that may carry fluorescent ligands.

If solutions containing concentrated salts are added to the ethidium assay solution there is a reduction in fluorescence due to a decrease in binding constant of ethidium. However because of the usual 200 fold dilution into the assay solution, even CsCl density gradients give only a small decrease in fluorescence. Divalent metal ions such as Mg^{++} are more serious but it is unusual to have >10 mM concentrations of Mg^{++} and the EDTA present in the standard ethidium solution is sufficient to complex the Mg^{++} up to 10 mM for 10 µl samples. If necessary the EDTA concentrations can be raised appropriately. As a last precaution, marking pens often contain highly fluorescent inks. In our experience it is inadvisable to mark tubes since contamination of the ethidium solutions, even with careful washing, becomes a problem.

With respect to the assays using ccc DNAs, their great sensitivity to any agent causing nicking warrants stressing the following precautions. Thiols in the presence of oxygen generate superoxide radicals (42) which we have shown to break DNA (8). Some plasmids such as col E1 ccc DNA are sometimes prepared from cells treated with chloramphenicol, conditions which allow many-fold replication of the plasmid. However the resulting DNA contains some ribonucleotides (43) and therefore is unsuitable for assays involving heating in the pH 12 ethidium solution. Also many plasmids exist as "relaxation" complexes in which a bound protein will nick the DNA in the presence of a variety of perturbants, alkali being one of them for some complexes (44). Therefore care in interpretation must be exercised in following a ccc DNA in some purification schemes from crude extracts. Finally, the ethidium solutions themselves will slowly nick ccc DNA in a

light and oxygen dependent reaction (45) and it is therefore advisable to keep the solutions in the dark if the fluorescence readings are not read immediately.

CONCLUSION

The above examples of the use of ethidium fluorescence assays in studying polynucleotide metabolism attest to the flexibility, sensitivity and speed with which such assays can be carried out. After eight years of using and extending the possibilities for the fluorescence assays we are convinced any research group interested in polynucleotide research would benefit from their use. Perhaps the most exciting area for the future lies in the use of bis-intercalating drugs which have binding constants $>10^4$ times as high as ethidium's (46). In the assays described here the limitations on sensitivity are the binding constant of ethidium and the noise level of the fluorimeter. The sensitivity of the latter can be extended by several orders of magnitude using an argon laser light source with photon-counting detectors (private communication from Dr. W. Lown). Since picogram levels of pancreatic DNase can be detected ($\sim 6 \times 10^7$ molecules) with the present assays, one might anticipate the detection of DNase activity in single cells (and like-wise for the other assays using ccc DNA) when bis-intercalators and an argon laser light source are used. The present assays have great utility but one can look forward to their future extension to increasing levels of sensitivity.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada.

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). "AP endonuclease" refers to a nuclease activity specific for apurine or apyrimidine sites.

REFERENCES

1 LePecq, J.-B. and Paoletti, C. (1967) J. Mol. Biol. 27, 87-106.

2 Paoletti, C., LePecq, J.-B. and Lehman, I.R. (1971) J. Mol. Biol. 55, 75-100.
3 Vosberg, H.-P., Grossman, L.I. and Vinograd, J. (1975) Eur. J. Biochem. 55, 79-93.
4 Champoux, J.J. and McConaughy, B.L. (1976) Biochemistry 15, 4638-4642.
5 Morgan, A.R. and Paetkau, V. (1972) Can. J. Biochem. 50, 210-216.
6 Morgan, A.R. and Pulleyblank, D.E. (1974) Biochem. Biophys. Res. Commun. 61, 396-403.
7 Pulleyblank, D.E. and Morgan, A.R. (1975) Biochemistry 14, 5205-5209.
8 Morgan, A.R., Cone, R.L. and Elgert, T.M. (1976) Nucl. Acids Res. 3, 1139-1149.
9 Jovin, T.M., Englund, P.T. and Bertsch, L.L. (1969) J. Biol. Chem. 244, 2996-3008.
10 Richardson, C.C. and Kornberg, A. (1964) J. Biol. Chem. 239, 242-250.
11 Alberts, B.M. and Frey, L. (1970) Nature 227, 1313-1318.
12 Burgess, R.R. (1976) in RNA Polymerase, Losick, R. and Chamberlin, M. eds., p. 88. Cold Spring Harbour.
13 Chamberlin, M. and Berg, P. (1962) Proc. Natl. Acad. Sci. U.S.A. 48, 81-94.
14 Vogt, V.M. (1973) Eur. J. Biochem. 33, 192-200.
15 Johnson, D. and Morgan, A.R. (1976) Biochem. Biophys. Res. Commun. 72, 840-849.
16 Morgan, A.R., Coulter, M.B., Flintoff, W.F. and Paetkau, V.H. (1974) Biochemistry 13, 1596-1603.
17 Coulter, M., Flintoff, W., Paetkau, V., Pulleyblank, D. and Morgan, A.R. (1974) Biochemistry 13, 1603-1609.
18 Burd, J.F. and Wells, R.D. (1970) J. Mol. Biol. 53, 435-459.
19 Lehman, I.R. and Nussbaum, A.L. (1964) J. Biol. Chem. 239, 2628-2636.
20 Kornberg, A. (1969) Science 163, 1410-1418.
21 Bollum, F.J. (1962) J. Biol. Chem. 237, 1945-1949.
22 Gralla, J. and De Lisi, C. (1974) Nature 248, 330-332.
23 Burrington, M.G. and Morgan, A.R. (1976) Can. J. Biochem. 54, 301-306.
24 Futcher, A.B. and Morgan, A.R. (1979) Can. J. Biochem. 57, 932-937.
25 Tye, B.-K., Nyman, P.-O., Lehman, I.R., Hochauser, S. and Weiss, B. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 154-157.
26 Hecht, R. and Thielman, H.W. (1978) Eur. J. Biochem. 89, 607-618.
27 Deutsch, W.A. and Linn, S. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 141-144.
28 Dasgupta, S., Allison, D.P., Snyder, C.E. and Mitra, S. (1977) J. Biol. Chem. 252, 5916-5923.
29 Germond, J.-E., Vogt, V.M. and Hirt, B. (1974) Eur. J. Biochem. 43, 591-600.
30 Modrich, P. and Lehman, I.R. (1970) J. Biol. Chem. 245, 3626-3631.
31 Weiss, B., Jacquemin-Sublon, A., Live, T.R., Fareed, G.C. and Richardson, C.C. (1968) J. Biol. Chem. 243, 4543-4555.
32 Hassid, W.Z. (1969) Science 165, 137-144.
33 Bauer, W. and Vinograd, J. (1968) J. Mol. Biol. 33, 141-171.
34 Kung, U.T. and Wang, J.C. (1977) J. Biol. Chem. 252, 5398-5402.
35 Eskin, B. and Morgan, A.R. (1978) Can. J. Biochem. 56, 89-91.
36 Burrington, M.G. and Morgan, A.R. (1978) Can. J. Biochem. 56, 123-128.
37 Liu, L.F. and Wang, J.C. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2098-2102.
38 Champoux, J.J. and Dulbecco, R. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 143-146.
39 Lee, J.S. and Morgan, A.R. (1978) Nucl. Acids Res. 5, 2425-2439.
40 Honda, B.M., Baillie, D.L. and Candido, E.P. (1975) J. Biol. Chem. 250, 4643-4647.

41 Olmsted III, J. and Kearns, D.R. (1977) Biochemistry 16, 3647-3654.
42 Misra, H.P. (1974) J. Biol. Chem. 249, 2151-2155.
43 Blair, D.G., Sherratt, D.J., Clewell, D.B. and Helinski, D.R. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 2518-2522.
44 Clewell, D.B. and Helinski, D.R. (1969) Proc. Natl. Acad. Sci. U.S.A. 62, 1159-1166.
45 Deniss, I.A. and Morgan, A.R. (1976) Nucl. Acids Rs. 3, 315-324.
46 Markovits, J., Roques, B.P. and LePecq, J.-B. (1979) Anal. Biochem. 94, 259-264.
47 Morgan, A.R. (1970) J. Mol. Biol. 52, 441-466.
48 Berkower, I., Leis, J. and Hurwitz, J. (1973) J. Biol. Chem. 248, 5914-5921.
49 Burgess, R.R. (1969) J. Biol. Chem. 244, 6160-6167.