APPLIED MICROBIOLOGY, Aug. 1972, p. 179-183
Vol. 24, No. 2

Printed in U.S.A.

Fluorometric Determination of Deoxyribonucleic Acid in Bacteria with Ethidium Bromide

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Received for publication 21 March 1972

A simple, sensitive, and rapid method is presented for the determination of deoxyribonucleic acid (DNA) in both gram-positive and gram-negative bacteria. It is based upon the fluorometric determination of DNA with ethidium bromide after alkaline digestion of the bacteria to hydrolyze the interfering ribonucleic acid. The assay takes less than 2 hr. Its sensitivity is at least 0.2 μg of DNA in a final solution of 4 ml and it uses commonly available filter or double monochromator fluorometers. Judicious choice of light source and filters allows an additional 10-fold increase in sensitivity with a filter fluorometer. Turbidity caused by bacteria or insoluble polysaccharides does not interfere with the fluorescence measurements. There was no significant difference between the results obtained with this method and those obtained with the indole and diphenylamine methods when these assays were applied to *Escherichia coli* and sucrose- or glucose-grown *Streptococcus mutans*. The method was also tested by determining the specific growth rate of *E. coli*. This new procedure should be especially useful for the determination of bacterial DNA in dilute suspensions and for the estimation of bacterial growth or DNA replication where more conventional methods are not applicable or sensitive enough.

The binding of ethidium bromide (EB) to nucleic acids results in a marked increase in its fluorescence which can be used to measure pure deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) either alone or in combination (8). This method has not been extended to the measurement of DNA in microorganisms, although it is potentially about 100-fold more sensitive than the two colorimetric procedures most often used (1, 2) and has equal, or better, selectivity. We have, therefore, developed a convenient and sensitive assay for microbial DNA based on the intercalation of EB with DNA. The method consists of an alkaline digestion of the bacteria to hydrolyze the RNA and eliminate its response. The alkaline digest is then neutralized to effect partial renaturation of the DNA, EB is added, and the fluorescence is measured with a filter or double monochromator fluorometer.

MATERIALS AND METHODS

Maintenance and growth of bacteria. A laboratory strain of *Escherichia coli* and the SL-1 strain of *Streptococcus mutans* were maintained by bimonthly transfer into, respectively, Antibiotic Medium 3 (AB-3 medium, Difco) and fluid thioglycollate medium (BBL) supplemented with horse meat infusion and $CaCO_3$. After overnight growth at 37 C, the cultures were stored at 3 C. For the growth experiment, *E. coli* was grown in 50 ml of AB-3 medium in a 200-ml Erlenmeyer flask on a reciprocating shaker (120 strokes/min) at 30 C. For the comparative DNA measurements, *E. coli* was grown overnight into stationary phase on a rotary shaker (100 rev/min) and then stored at 3 C. One-half milliliter of an overnight culture grown under similar conditions was used as inoculum. *S. mutans* was grown in 30 ml of broth in test tubes (200 × 25 mm). The medium contained per liter: 5 g of Trypticase (BBL), 5 g of yeast extract (BBL), 5 g of $K_2HPO_4 \cdot 3H_2O$, 50 mg of Na_2CO_3, and 0.5 ml of a salt solution containing (per 100 ml) 800 mg of $MgSO_4 \cdot 7H_2O$, mg of $FeSO_4 \cdot 7H_2O$, and 19 mg of $MnSO_4 \cdot 4H_2O$. The *p*H was adjusted to 7.2 with HCl before steam sterilization. Afterwards, glucose or sucrose was added to give a final concentration of 0.01 M. The inoculum was grown overnight on the glucose medium, and 0.3 ml was used per tube. The cultures were incubated for 24 hr at 37 C and subsequently stored at 3 C for DNA analysis. The sucrose-grown *S. mutans* was exposed for 2 min to sonic energy from a Branson sonifier with a microtip and an output of 50 w. Microscopic inspection showed that such treatment releases most of the streptococci from the dextran aggregates.

Chemicals. Calf thymus DNA, EB, and micro-

coccal nuclease (from *Staphylococcus aureus;* EC 3.1.4.7) were dissolved in 0.1 M tris(hydroxymethyl) aminomethane-hydrochloride buffer of *p*H 8.5 (T8.5 buffer) in 0.1 mg, 0.1 mg, and 3,000-unit amounts (per ml of buffer), respectively. Yeast RNA was dissolved in 0.1 M phosphate buffer (*p*H 7.0; 0.1 mg/ml of buffer). Nuclease, DNA, and RNA were obtained from Worthington Biochemical Corp.; and EB (B grade) was from Calbiochem.

Fluorometric DNA analysis. A sample from the bacterial suspension containing 0.2 to 10 μg of DNA was filtered over a previously wetted Metricel DM-450 membrane filter (25 mm, 0.45-μm pore size, Gelman Instrument Co.) and washed twice with an equal volume of 0.85% saline. Each filter was submerged in 2.0 ml of 0.3 N KOH in a 12-ml conical centrifuge tube, and the bacteria were resuspended by using a test tube mixer or a platinum needle. After incubation for 1 hr at 37 C, 0.5 ml of 1.0 N HCl was rapidly mixed into the digest, and 2.0 ml of the partially neutralized suspension was added to 2.0 ml of EB (4 μg/ml in T8.5 buffer). The fluorescence was measured (after equilibration to room temperature and resuspension of any precipitate) against a reagent blank and compared with that of DNA standards similarly treated. The Metricel polyvinylchloride filters do not contribute to the fluorescence, whereas certain other polyvinylchloride filters do so in a rather variable manner. Conventional cellulose ester-type filters cannot be used because they degenerate during the alkaline digestion. Fluorescence was routinely measured in round cuvettes (12 × 75 mm; Hycel, Inc.) with a Turner 111 fluorometer equipped with a general purpose lamp (General Electric F4T4.BL) and a temperature-stabilized sample holder. Primary filters were Corning 1-60 (nearest to the lamp) and Wratten 58 for the selection of 546-nm exciting light, and the secondary filter was a Wratten 23A for the passage of emitted light above 570 nm. An Aminco fluorocolorimeter was also used with the same light source and filters. For samples containing 0.02 to 0.2 μg of DNA, it is advantageous to use an EB concentration of 1 μg/ml to lower the background fluorescence and to switch to a General Electric F4T5.G green lamp to obtain sufficient response. The Aminco-Bowman, Farrand Mk-1, and Perkin-Elmer MPF-3 were used as double monochromator fluorometers. Optimal excitation and emission wavelength settings were determined with a solution containing 10 μg of DNA and 1 μg of EB per ml of T8.5 buffer in a 1-cm square cuvette and using the narrowest slits consistent with adequate response. They were, respectively, 520 nm for excitation and 585 (Farrand, Perkin-Elmer) or 600 (Aminco) nm for emission.

Colorimetric DNA analysis. For the diphenylamine method, a sample of the bacterial suspension containing at least 10 μg of DNA was filtered, and the bacteria were washed with saline and released from the filter in 2 ml of 0.5 N $HClO_4$ in a centrifuge tube. After incubation for 30 min at 70 C and cooling, 4 ml of freshly prepared reagent (1) was added, and the samples were left for 16 to 20 hr at 30 C. After centrifugation, the absorbance of the supernatant fluid was determined, at 600 nm against a reagent blank with a Gilford 300N spectrophotometer, and compared with that of DNA standards. For the indole method, a modification of the method of Ceriotti (2), the bacteria, containing at least 5 μg of DNA, were collected and washed as described and then suspended in 3 ml of reagent prepared by mixing 60 ml of 0.015% indole with 5 ml of HCl (38% w/w) before use. After 2 hr at 70 C and subsequent centrifugation, the absorbance of the supernatant fluid was measured at 490 nm against a reagent blank and compared with that of DNA standards.

Enzymatic methods. To degrade DNA, 150 units of micrococcal nuclease were added after measurement of the fluorescence of the DNA-EB complex, the samples were reincubated for 30 min at 37 C, and the fluorescence was measured again after cooling. Preliminary work showed that similar treatment of 10 μg of alkali-denatured DNA reduced the fluorescence to less than 1%, even in the presence of EB and in the absence of Ca ions. Insoluble polysaccharide was prepared in vitro by using the medium of a 24-hr culture of *S. mutans* grown with glucose as energy source. After filtration through a membrane filter (0.45-μm pore size), the filtrate was aseptically adjusted to 0.05 M sucrose and reincubated for 24 hr at 37 C. The particle size of the resultant insoluble polysaccharide was reduced by a 2-min exposure to 50-w sonic energy from a Branson sonifier. To study the effect of turbidity on fluorescence, samples were filtered off, washed, and digested with or without 10 μg of DNA added, and the fluorescence was compared with that of DNA alone. The turbidity of the final solution was also measured with a Gilford 300N spectrophotometer and expressed as apparent absorbance at 550 nm.

Other determinations. The growth of *E. coli* was measured by determining the apparent absorbance of 1-ml samples at 600 nm. The samples were diluted with saline to keep the readings below 0.4 to preserve linearity between the readings and the dry weight (7). Dry weights were determined in triplicate by collecting the cells from a 2-ml sample, on a previously weighed Metricel DM-450 filter, and washing the bacteria with 5 ml of water. The filters were then transferred to aluminum pans and dried for a few hours at 100 C until constant weight was obtained. Control filters were treated similarly to determine the weight loss. The filters were weighed on the 20-mg scale of a Cahn Gram Electrobalance after equilibration over $CaCl_2$ in a desiccator.

RESULTS

Because EB intercalates with the double-stranded regions of both DNA and RNA, it was necessary to eliminate the RNA binding by a 1-hr hydrolysis in 0.3 N KOH at 37 C (10, 13, 18). The data in Fig. 1 confirm that this procedure virtually eliminates the RNA response. The fluorescence of the denatured DNA after this treatment is about 45% of that of native DNA. This lower value does not only

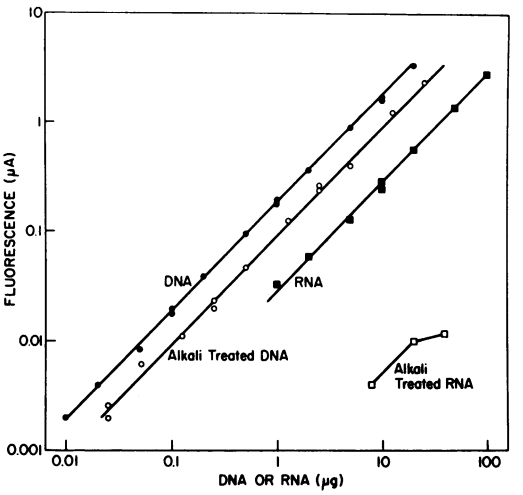

FIG. 1. *Fluorescence of native and alkali-treated DNA and RNA after reaction with ethidium bromide. The fluorescence was measured against a reagent blank on a Farrand Mk-1 spectrofluorometer and is expressed as the photomultiplier current. The extinction and emission wavelengths were 520 and 585 nm, respectively. Slits with a bandpass of 20 nm were used. To preserve linearity, the amount of EB should at least equal the amount of nucleic acid in micrograms.*

result from the alkaline conditions to which the DNA has been subjected, but also from the presence of 0.1 M KCl in the final assay mixture (9). Furthermore, these data suggest that a certain amount of order is restored in the DNA after adjustment of the *p*H to 8.5. Figure 1 also shows that the response for the DNA after this treatment is linear over a range of at least 0.1 to 10 μg of DNA as measured with a double monochromator fluorometer. Similar results were obtained with the other fluorometers tested. We found that the time period between the partial neutralization with acid and the addition of EB did not critically effect the final fluorescence. In addition, the complex formation between EB and DNA is very rapid compared with the time required for temperature equilibration prior to the fluorescence measurements.

When this method was applied to *S. mutans*, the bacteria were collected and washed on polyvinylchloride membrane filters which withstood the alkaline digestion. After the digestion and partial neutralization, the final solution was slightly turbid because of particulate matter from the bacteria. This turbidity could be removed by membrane filtration or centrifugation, but both methods resulted in considerably lower fluorometer readings, probably as a result of the adherence of the DNA to the streptococcal debris (14).

To confirm that the fluorometric response observed was, indeed, due to streptococcal DNA and not to other fluorescent compounds, micrococcal nuclease was used to degrade the DNA after measurement of the fluorescence. This treatment reduced the fluorescence to less than 5% of the earlier values, even though the conditions used are suboptimal for enzyme activity.

Variations in the length of the alkaline digestion between 0.5 and 2 hr did not affect the final result significantly. A period of 1 hr was, therefore, adopted for this resulted in hydrolysis of at least 100 μg of RNA under the conditions used.

To determine if the turbidity that was observed with the bacterial samples interfered with the measurements by scattering either the exciting (primary) or the emitted (secondary) light, several experiments were performed. The method was first applied to increasing amounts of streptococci, with and without an internal DNA standard added. The results of this reconstruction experiment are shown in Fig. 2. Without added DNA, the relationship between bacteria and response is described by a straight line through the origin over the

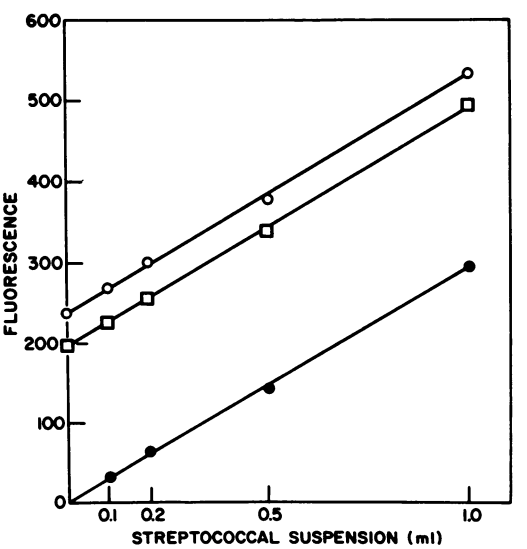

FIG. 2. *Determination of DNA in S. mutans with ethidium bromide. After measurement of the fluorescence (●), 5 μg of DNA was added and the tubes were measured again (○). In the other set of tubes, 10 μg of DNA was added to the streptococci before the digestion (□). Fluorescence was measured on a Turner 111 fluorometer and is expressed in arbitrary units.*

range studied. The line obtained with 10 μg of DNA added to the samples is also straight and parallel to the one without the internal standards. This shows that the backscatter of the primary light due to the bacterial debris does not significantly decrease the intensity of the primary light, and hence the fluorescence, over the range studied. Figure 2 also shows that when 5 μg of DNA was added after the measurement of the streptococcal samples, the recovery was 100%. In a second experiment (data not shown), different amounts of insoluble polysaccharide were added to 10 μg of DNA, and both the fluorescence and turbidity were measured after the procedure. We found that an increase in the apparent absorbance at 550 nm from 0 to 0.2 did not significantly alter the fluorescence, which proves that the amount of primary light that reaches the photomultiplier because of scatter does not contribute significantly to the observed fluorescence.

The EB method was developed by using streptococci but can also be used to measure the DNA in a gram-negative organism such as *E. coli*. Figure 3 shows that the DNA measurements correlate well with the absorbance data. The doubling time derived from either the absorbance or the DNA data was 40 min.

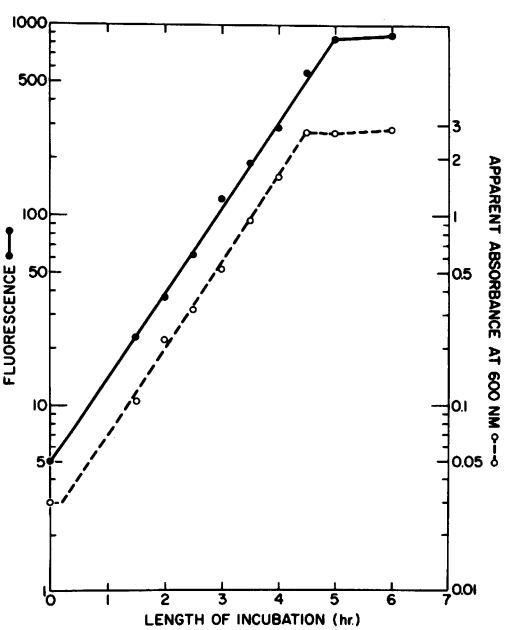

FIG. 3. *Growth of E. coli on Antibiotic Medium 3. Turbidity* (O) *was measured with a Gilford 300N spectrophotometer after suitable dilution with saline. DNA* (●) *was measured by the ethidium bromide method. A Turner 111 fluorometer was used, and the fluorescence is expressed in arbitrary units.*

Finally, we compared this new method with the diphenylamine and indole assays for DNA. The data in Table 1 show that there was no significant difference between the three methods when applied to *E. coli* or *S. mutans*. The DNA content of the stationary-phase *E. coli* and glucose-grown *S. mutans* was 4.3 and 3.0%, respectively. The sucrose-grown *S. mutans* culture also contained an appreciable amount of insoluble polysaccharide, but the results confirm that this did not interfere significantly with the method. Nuclease treatment of the final solution reduced the fluorescence to less than 5% with pure DNA or glucose-grown *S. mutans*. But with sucrose-grown *S. mutans* and with *E. coli* only a 10 to 20% reduction in fluorescence was observed. In the latter cases, the denatured and partially insoluble DNA is apparently more resistant towards enzymatic degradation.

DISCUSSION

The method presented here is based on the binding of EB to double-stranded DNA and, therefore, should be insensitive to interferences encountered with the indole and diphenylamine procedures for DNA, which depend on the deoxyribose moiety. The good agreement observed between the three methods indicates that each one can be used reliably for *E. coli* and *S. mutans* and, furthermore, that the pool of low-molecular-weight compounds that might interfere with the colorimetric tests is relatively small in these bacteria.

The RNA response with EB is most conveniently eliminated by an alkaline hydrolysis (13). The length of this digestion was found to be relatively unimportant, but it should be checked whenever the method is applied to a new class of organisms which might be more

TABLE 1. *Comparison of the diphenylamine, indole, and ethidium bromide assays for bacterial DNA*

Assay method	DNA (μg/ml)		
	Escherichia coli[a]	*Streptococcus mutans*	
		Glucose[b]	Sucrose[c]
Diphenylamine	54.3	12.5	14.1
Indole	50.8	12.3	15.4
Ethidium bromide ...	54.9	12.2	13.2

[a] *E. coli* culture had a dry weight of 1.24 mg/ml.
[b] Glucose-grown *S. mutans* culture had a dry weight of 0.41 mg/ml.
[c] Sucrose-grown *S. mutans* culture had a dry weight of 0.75 mg/ml and contained insoluble polysaccharides.

resistant. The quantitative response of the alkali-denatured DNA was found to be the same as that of heat-denatured DNA (9). Most of the streptococcal DNA remains insoluble after the alkaline digestion, but the results indicate that this does not interfere with the EB binding or fluorescence. It has been reported that extraction of *Streptococcus faecalis*, *Lactobacillus leichmanni*, or *Euglena gracilis* with 1 N KOH solubilized only 20 to 35% of their total DNA (4, 14). To determine the possible interference of the bacterial debris with the fluorescence measurements, various experiments were performed, but no significant effects were encountered. This agrees with the findings of others as reviewed by Udenfriend (17).

The main advantages of the EB method are its ease and sensitivity. The latter is at least 50 ng per ml of final solution and can be extended further by using a light source with a higher intensity around 520 nm. The sensitivities of the indole and diphenylamine methods in the modifications used here were 1 and 2 μg per ml of final solution, respectively. Another fluorometric assay for DNA is based on the reaction of deoxyribose with 3,5-diaminobenzoic acid (6). Its disadvantage is that lipids have to be removed by extraction, which requires great care, especially with small samples, to prevent the loss of DNA (15). Particular attention also has to be paid to the hot-acid hydrolysis of the DNA to expose the purine-bound deoxyribose and to the purity of the reagent (5, 12). A third fluorometric procedure is based on the degradation of thymine with bromine water to yield acetol, which is subsequently reacted with *o*-aminobenzaldehyde to give the fluorescent 3-hydroxyquinaldine (11). The latter two methods are about equally as sensitive as the EB method but, to our knowledge, have only been tested with animal tissues.

One promising application for the method presented here is to measure growth in situations where solids interfere with the more conventional absorbance or dry-weight assays. Such a case arises when *S. mutans* is grown with sucrose as energy source (16). Another possible application is in the detection of very low amounts of bacteria. Assuming 10^{-6} μg of dry weight per bacterial cell and 4% DNA, it can be estimated that, with the higher intensity light source, the EB method can be used to measure DNA in a 10-ml sample containing 50,000 cells per ml. The EB method is, therefore, as sensitive for the detection of bacteria as nephelometry with a laser light-scattering photometer (Science Spectrum, Santa Barbara, Calif., 93105), about 10 times more sensitive than direct turbidimetry, but at least an order of magnitude less sensitive than the method using the firefly bioluminescence assay for ATP (3), a method dependent on the efficient extraction of ATP from the cells and special instrumentation.

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