A COLORIMETRIC MICROASSAY FOR THE DETECTION OF AGENTS THAT INTERACT WITH DNA

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ABSTRACT.—A simple microtiter assay for the detection of compounds that bind DNA is described. Agents that displace methyl green from DNA are detected spectrophotometrically by a decrease in absorbance at 630 nm. The feasibility of using the assay for detecting DNA-active compounds in fermentation extracts was assessed, and the activities of reference compounds in the methyl green assay and an ethidium bromide displacement method were compared.

Drugs that interact with nucleic acids are among the most useful cancer chemotherapeutic agents. Several biological and biochemical assays exist that are capable of specifically detecting these agents, and a commonly used technique is the lysogenic induction assay. This assay detects compounds that damage DNA (1), and the construction of *Escherichia coli* strains lysogenic for a λ-lacZ fusion phage has allowed colorimetric quantitation of prophage induction by the appearance of β-galactosidase, a product of the lacZ gene (2). Other assays involve measuring cytotoxicity in the presence and absence of exogenous DNA (3,4), monitoring topological changes in covalently closed circular DNA (5) or spectral changes in the drug resulting from the interaction (6,7). A recently described hplc assay that uses DNA as an affinity probe may be particularly powerful for identifying DNA-active compounds present in certain complex extracts (8).

The triphenylmethane dye methyl green, {4-{p-(dimethylamino)-α-[p-(dimethylamino)phenyl]benzylidene}2,5-cyclohexadien-1-ylidene} dimethylammonium chloride ethobromide, binds DNA, and the DNA/methyl green complex is commonly used as a substrate to measure DNase activity (9). Displacement of methyl green from DNA facilitates the addition to the dye of an H_2O molecule that results in formation of the colorless carbinol (10), a reaction that can be followed spectrophotometrically as a decrease in absorbance (11). In this report, we describe a simple microtiter DNA/methyl green displacement assay, determine the sensitivity of the method with reference antibiotics, and test the feasibility of the technique for detecting DNA-active agents in extracts of microbial fermentations.

Methyl green reversibly binds polymerized DNA, and the complex is stable at neutral pH, whereas free methyl green fades (9). Incubation for 24 h, in the buffer used for displacement reactions in this study, results in virtually a complete loss of methyl green absorbance (Figure 1). Also shown in Figure 1 are the structures of methyl green and the resulting colorless carbinol (10). A representative dose-response relationship for disruption of the DNA/methyl green complex by distamycin A is shown in Figure 2. The absorbance of the DNA/methyl green complex in the presence and absence of distamycin A was measured immediately after solutions were mixed and again after 24-h incubations. The decrease in absorbance observed represents the initial rapid displacement of methyl green from DNA by distamycin A, followed by the slower reaction that yields the colorless carbinol. The ability of other known DNA-active compounds to disrupt the DNA/methyl green complex was assessed in similar experiments, and results were compared with those obtained using an ethidium bromide displacement method. As shown in Table 1, agents that interact with DNA through a variety of mechanisms displaced methyl green from DNA. When tested in parallel for ability to displace ethidium bromide from DNA, a resonable correlation between potency for displace-
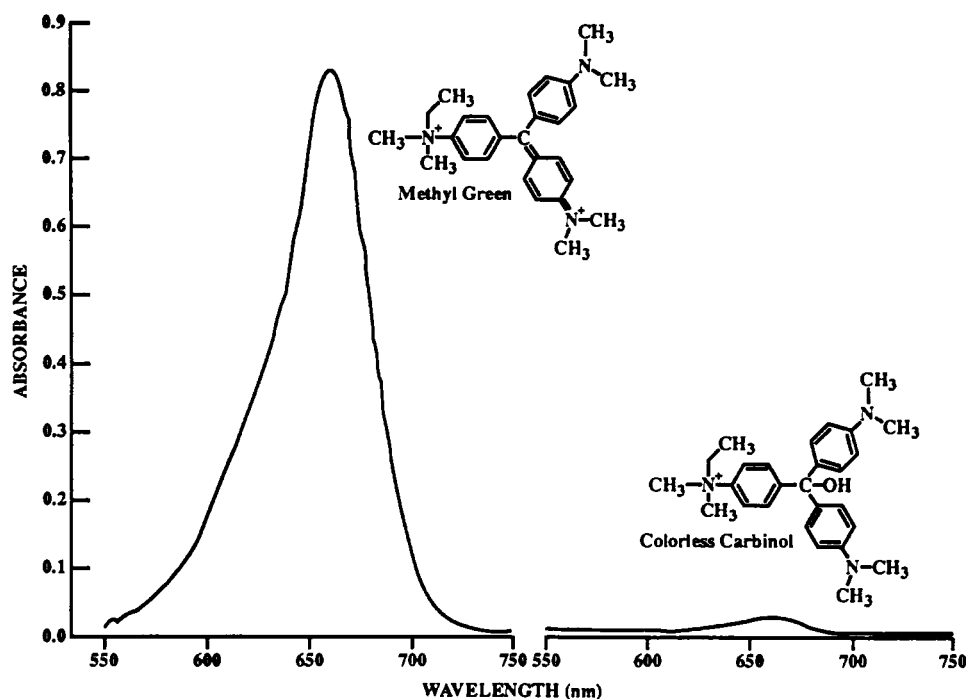
FIGURE 1.   Structure and visible spectra of methyl green and the colorless carbinol. Methyl green was dissolved in 0.05 M Tris-HCl buffer, pH 7.5, containing 7.5 mM $MgSO_4$ at a concentration of 10 μg/ml, and the absorbance spectrum was recorded immediately (left panel). The solution was then left undisturbed for 24 h, and the spectrum was recorded again (right panel).

ment of ethidium bromide and methyl green was observed. However, for daunomycin and pluramycin, the DNA/methyl green assay was more than 10-fold less sensitive that the ethidium method. An exception was 4,6-diamidino-2-phenylindole, which displaced methyl green but did not alter ethidium binding to DNA. Since the DNA binding footprints of compounds are different, it is not surprising that differences in ethidium and methyl green displacement curves were observed. The nature of competitive interactions between ethidium and methyl green with a given compound will result in different IC_{50} values; however, either assay detects a wide range of DNA-active compounds.

To determine if the methyl green assay would be useful for the detection of DNA-active compounds produced in microbial fermentations, the minor-groove binder berenil [4,4'-(diazoamino)dibenzamidine diaceturate] was added to selected extracts. As shown in Figure 3, the selected extracts did not displace methyl green from DNA, and as expected, reduced absorbance was observed when 50 μg/ml berenil was added to each extract. The statistics for the distribution of absorbance values obtained when extracts alone (mean ± SD = 100 ± 6) or berenil-spiked extracts (50 ± 11) were tested indicated that the assay was not subject to excessive variability. Although the extracts tested did not significantly alter the initial absorbance of the DNA-methyl green complex, colored or turbid samples may result in increases in absorbance at 630 nm. In these cases, correction of final absorbance values with initial readings may be necessary to measure displacement of methyl green from DNA specifically.

For extracts of microbial fermentations prepared and tested as described in the Experimental section, compounds with affinities for DNA similar to those of distamycin or berenil would be detected by a 50% reduction in absorbance if present in fermenta-
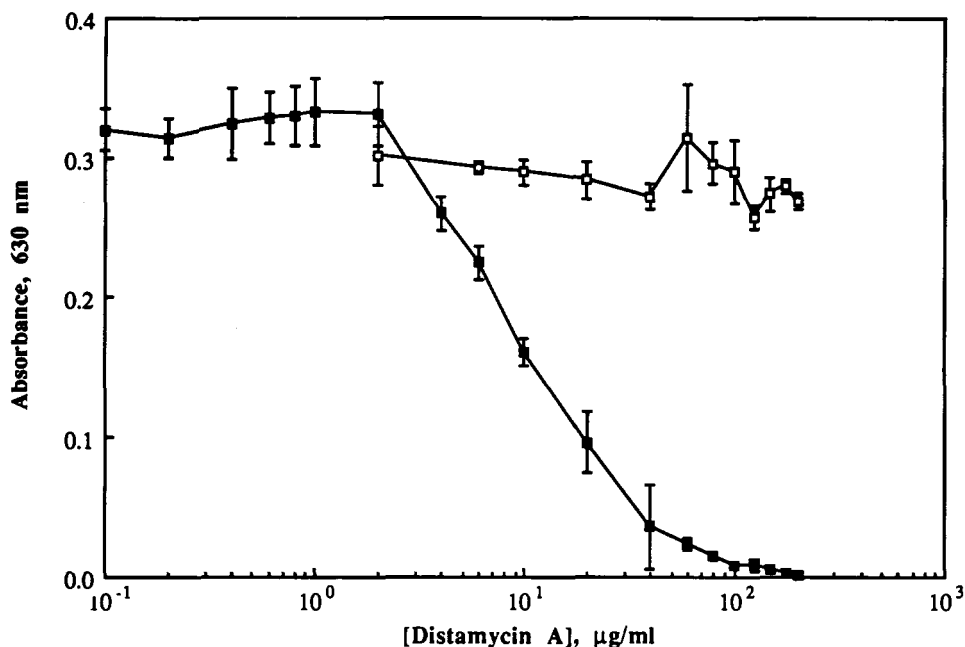
FIGURE 2. Displacement of methyl green from DNA by distamycin A. Results represent the initial (□) and final absorbance (■) readings (mean ± SD, $n = 3$) of solutions of DNA/methyl green in the presence of distamycin A at the concentrations indicated. In the absence of distamycin A, the initial absorbance of the DNA/methyl green complex was 0.296 ± 0.006 and the final absorbance was 0.326 ± 0.012 (mean ± SD, $n = 6$). Values were corrected for absorbance not associated with the DNA/methyl green complex (0.090 absorbance units, as determined after hydrolysis of DNA with DNase I).

tion broths at concentrations of 3 to 13 µg/ml. This sensitivity is in the range necessary to detect production of DNA-active antibiotics, provided that the compounds are quantitatively recovered by solid phase extraction with XAD or by a comparable recovery procedure. For example, *Streptomyces insignis* ATCC 31913 produces daunomycin at 55 to 75 µg/ml (12), and peak levels of the altromycins in the fermentation beer were 110 µg/ml (13).

TABLE 1. Activity of Reference Compounds in the Methyl Green/DNA and Ethidium Bromide Displacement Assays.[a]

DNA-active agents	DNA/methyl green IC_{50}, µg/ml	Ethidium Bromide/DNA IC_{50}, µg/ml
Altromycin B	67 ± 2	44 ± 10
Berenil	67 ± 17	59 ± 6
CC 1065	62 ± 15	26 ± 3
Daunomycin	30 ± 7	2.0 ± 0.4
4,6-Diamidino-2-phenylindole	15 ± 4	>300
Distamycin A	18 ± 7	4.3 ± 0.3
Hoechst 33342	37 ± 4	33 ± 4
Pluramycin	51 ± 3	3.6 ± 0.2
Rubiflavin	17 ± 1	13 ± 1

[a]Values represent the concentration (mean ± SD, $n = 3$ to 5 separate determinations) required for a 50% decrease in the initial absorbance of the DNA methyl green solution or in the initial fluorescence of the ethidium bromide solution.
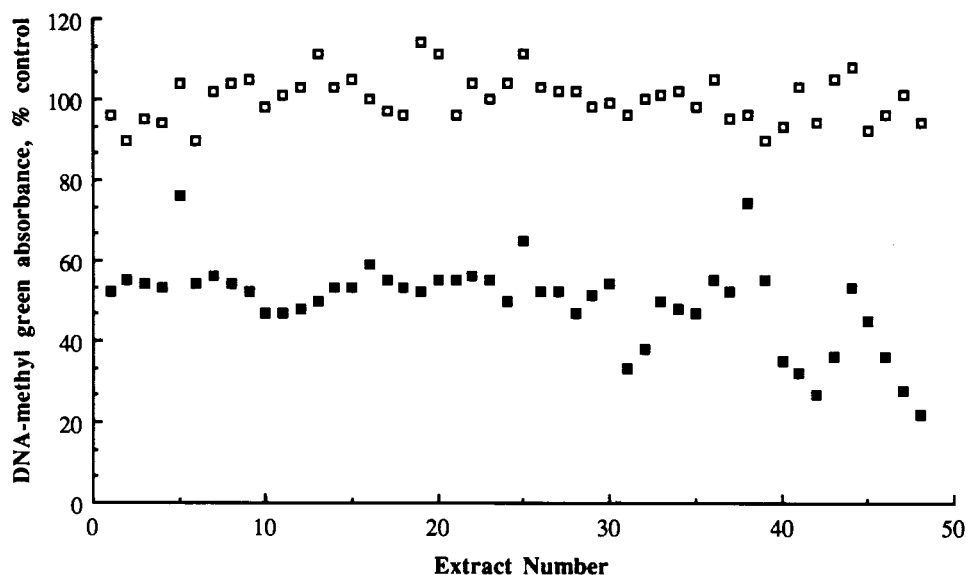
FIGURE 3.   Detection of a DNA-active compound in extracts of microbial fermentations with the DNA/ methyl green assay. Samples were tested in parallel in the absence (□) or presence (■) of 50 μg/ml berenil (final concentration in the reaction).

To date, 10,000 fermentation extracts have been tested for ability to displace methyl green from DNA, and 0.6% of those tested were active (50% reduction in absorbance). The extract of a submerged fermentation of an uncharacterized *Streptomyces* species displaced methyl green from DNA, and subsequent purification resulted in the isolation of chrysomycin B. The isolated chrysomycin B displaced methyl green from DNA with an IC_{50} of 20 μg/ml. Chrysomycins have been previously detected using the biochemical prophage induction assay (BIA), an activity that is associated with the ability of a compound to initiate DNA damage (14). A direct interaction between chrysomycin A (chrysomycins A and B differ by the presence of a vinyl or methyl group, respectively, at position C-8 of the chromophore) and DNA has been shown by ability to relax covalently closed circular DNA (15).

In conclusion, the described microtiter DNA/methyl green assay is useful for the detection of DNA-active antibiotics present in crude fermentation extracts. Comparison of the activity of reference compounds in the DNA/methyl green and ethidium bromide displacement assays indicates that a variety of DNA-active antibiotics can be detected with either assay. Since colorimetric plate readers are widely available, the DNA/methyl green assay may be easily adapted for use without the need of a fluorometer to measure ethidium displacement. However, highly colored or turbid samples may be difficult to assay with the colorimetric DNA/methyl green assay. Advantages of the DNA/methyl green method include the commercial availability of all reagents, easy automation of the assay, no requirement for microbiological or tissue culture skills or facilities, lack of interference by fluorescent compounds present in samples, and the relative ease of the test in comparison to DNA-selective assays previously described.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Altromycin B (16) was isolated and characterized by spectral methods at Abbott Laboratories. CC-1065 was a gift from Dr. J.P. McGovern, The Upjohn Company, Kalamazoo, MI. Pluramycin and rubiflavin were obtained from the National Cancer Institute. Un-

identified microorganisms were isolated from soil samples and grown (100 ml cultures). The medium consisted of glucose monohydrate 1.0%, Staclipse JUB starch 1.5% (Staley), yeast extract 0.5% (Difco), NZ amine type A 0.5% (Humko Sheffield), and $CaCO_3$ 2 0.1% (pH 7.0) in shake flasks for 5 days, before 10 ml of a slurry of XAD-16 resin in H_2O (50:50) was added. After 30-min incubations, the fermentation beer was decanted, and the resin was washed with H_2O and steeped in 20 ml MeOH for 30 min. The MeOH extract was dried under vacuum and resuspended in 2 ml of EtOH-H_2O (50:50), and 20-μl samples were dispensed into wells of a 96-well microtiter tray.

DNA/METHYL GREEN ASSAY.—DNA Methyl Green (20 mg) obtained from Sigma (St. Louis, MO) was suspended in 100 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 7.5 mM $MgSO_4$ and stirred at 37° with a magnetic stirrer for 24 h. Unless otherwise indicated, samples to be tested were dissolved in EtOH and dispensed into wells of a 96-well microtiter tray. Solvent was removed under vacuum, and 200 μl of the DNA/methyl green solution was added to each well. The absorption maxima for the DNA/methyl green complex is 642.5–645 nm (9), yet sufficient absorbance at 630 nm allows use of the standard 630 nm interference filter available for plate readers. The initial absorbance of each sample was measured at 630 nm with a Bio-Tek El-320 microplate reader (Winooski, VT), and samples were incubated in the dark at ambient temperature. After 24 h, the final absorbance of samples was determined as above. Readings were corrected for initial absorbance and normalized as a percentage of the untreated DNA/methyl green absorbance value. IC_{50}'s were determined by linear regression of data plotted on a semi-log scale.

ETHIDIUM BROMIDE DISPLACEMENT ASSAY.—DNA (calf thymus type I, Sigma, 100 μg/ml) was dissolved in Dulbecco's phosphate buffered saline containing magnesium and calcium (0.8% NaCl, 0.22% Na_2HPO_4, 0.02% KCl, 0.1% $CaCl_2$, and 0.1% $MgCl_2 \cdot 6H_2O$ w/v) and dialyzed against a 100-fold volume of the same buffer. Samples to be tested were dissolved in EtOH, dispensed into wells of MicroFLUOR "W" trays (Dynatech, Chantilly, VA), and solvent removed under vacuum. To initiate reactions, DNA (100 μg/ml) and ethidium bromide (10 μM) were added to each well (total reaction volumes were 225 μl). After 4-h incubations, fluorescence (excitation 295 nm, emission 590 nm, 10 nm slit widths) was measured with a Perkin-Elmer LS50 fluorometer with a plate reader attachment (Norwalk, CT). Readings were normalized as a percentage of the untreated fluorescence of the DNA-ethidium bromide complex. IC_{50}'s were determined by linear regression of data plotted on a semi-long scale.

ISOLATION AND CHARACTERIZATION OF CHRYSOMYCIN B.—Chrysomycin B was produced in submerged fermentation by an uncharacterized *Streptomyces* species. The crude antibiotic was isolated by extracting the filtered mycelial cake from 10 liters of beer with EtOAc. Evaporation of the solvent afforded 2.9 g of an oil. This material was analyzed by hplc diode-array (C-8 Adsorbosphere, MeCN/0.1% H_3PO_4 gradient, 1 ml/min) and found to contain several peaks with uv chromophores similar to chrysomycins A and B (248, 285 nm and 244, 272 nm, respectively). An apparent major component eluting at a retention time of 9.7 min possessing the B-type chromophore was isolated by preparative hplc. The preparative method was carried out on 250 g of C-18 Rsil (10 μ) packed in a 5 cm dynamic axial compression column (Prochrom). The 2.9 g sample above was processed in 8 runs in an MeCN/H_2O gradient (50 ml/min) to give a total of 69 mg of chrysomycin B, which was characterized by eims (molecular ion = 496) and ^{13}C nmr. The results obtained were consistent with the empirical formula ($C_{27}H_{28}O_9$) and ^{13}C-nmr chemical shifts previously published for chrysomycin B (17).

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