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Photographic Quantitation of DNA in Gel Electrophoresis

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A photographic procedure to quantitate the DNA in bands, obtained by gel electrophoresis after staining with ethidium bromide, is described. The relationship between the film darkening and the intensity of the light hitting the film was determined. The densities, measured in densitometric tracings of the negatives, were converted into fluorescence intensities. The fluorescence was found to be linearly proportional to the amount of DNA. Deviations due to gel overloading, to nonuniform electrophoretic migration and uv illumination, and to photodecomposition of ethidium bromide were investigated.

Restriction endonucleases (Class II), which cut the DNA at specific nucleotide sequences, are powerful tools in the study of DNA structure and function (1). Cleavage products are usually studied by gel electrophoresis, a simple, reliable, and inexpensive method. Agarose and polyacrylamide gels of various concentrations can fractionate DNA fragments in a wide range of molecular weight. DNA bands on the gels are detected either by autoradiography or by staining with methylene blue (2), toluidine blue (3,4), stains-all (4), and, most often, ethidium bromide. With radioactive DNA, the amount of DNA in the bands is estimated by slicing the gel and measuring the radioactivity. With nonradioactive DNA, the absorbance of DNA is measured on stained (3–5) and unstained gels; fluorometry of the DNA–ethidium bromide complex was also used (6); however, to our knowledge, these methods have not been studied extensively.

A photographic procedure is described which permits precise relative estimation of DNA in the bands of gels stained with ethidium bromide. This procedure, which can be applied to bands containing as little as 0.2 μg of DNA, involves (i) the determination of the relationship between the light intensity and the film darkening (darkening curve) and (ii) the conversion, using the darkening curve, of the densities obtained from tracings of the photographic negative of the gels into fluorescence intensities.

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MATERIALS AND METHODS

DNA, enzymes, and electrophoresis. SV40 DNA, restriction endonucleases Hind II + III and Hae III (7), DNA digestions, and electrophoresis conditions were as described (8). Gels were stained for 2 hr in electrophoresis buffer (20 mM sodium acetate, 2 mM EDTA, 40 mM Tris–acetate, pH 7.8) supplemented with 2 μg/ml of ethidium bromide (Sigma, St. Louis, Mo.). During staining and photography, the slab gels (0.3 × 16 × 40 cm) were left on one of the glass plates used to cast them.

Ultraviolet illumination. For photography, the gels were illuminated by two short-wave ultraviolet lamps (CS215; U.V. Products, San Gabriel, Calif.) symmetrically positioned at a 45° angle and parallel to the lanes of the gel. The uv illumination was measured *in situ* with a short-wave uv counter (J225; U.V. Products) and was found to be uniform in the full width but not in the full length of the gel; the illumination decreased 10 cm beyond the middle of the lamps. Under use, the uv filters of the CS215 lamps become progressively opaque to uv light or solarized. In later experiments not described here, they were discarded and the Wratten 23A filter of the camera was replaced by a Wratten 24.

Photography. A Polaroid MP-3 camera was used with an objective of $f = 150$ mm (Symmar-S, Schneider, Kreuznach, Germany). Reduction from the gels to the photographs was 1.6. Apertures of the objective, fitted with a Kodak–Wratten 23A red filter, varied from *f*/5.6 to *f*/11; exposure time was 3 min. Kodak Ektapan 4 × 5 in. films were developed in a daylight type tank (Combi-Plan T, Gepe, Sweden) at 21°C for 8 min in Kodak D76 developer; they were fixed for 4 min at the same temperature in Kodak Rapid Fixer, washed in deionized water, and dried at 37°C. A sensitogram was processed together with the photographic negatives.

Sensitograms. Sensitograms were prepared by exposing, to a red light, a No. 2 photographic step tablet (Eastman Kodak), containing 21 steps of regularly increasing density (0.05–3.05), and held in contact with the emulsion side of the film in a printing frame (Paterson, London, England). The tablet was illuminated by a light box situated about 2 m above the frame and supplied with a stabilized current and a Wratten 23A (or 24; see above) filter. The useful illumination, as measured with a light meter (Polycontrol; Chauvins et Arnoux, Paris, France), was uniform. Suitable neutral filters permitted a correct exposition of the film in 3 min. An identical exposure time for both sensitograms and the gel photographs avoids the reciprocity deviations (9) of the film. Many sensitograms were prepared and stored, undeveloped, at −20°C. Before developing, they were kept for about 3 hr at room temperature.

Densitometry. Negatives were scanned with a Joyce–Loebl (Gateshead on Tyne, England) MK III C microdensitometer. Since the instrument magnification used was 20, enlargement from the gels to the tracings was

12. Suitable grey wedges allowed optimum pen deflections for each band of the pattern. To avoid making allowances for the slopes of the gray wedges and their nonlinearity, the sensitograms and the gel negatives were scanned with the same wedges; for each wedge, a zone of appropriate density in the sensitogram was chosen as a common reference of pen deflection.

In the Joyce–Loebl microdensitometer, the light spot, which hits the negative, is projected through an objective of magnification 5 onto a slit of dimensions 11 × 0.4 mm. The useful dimensions of the spot on the negative are 1 × 0.036 mm, if account is taken of a projection factor of 2.2. This spot of larger dimension parallel to the bands, moved in a direction parallel to the bands' electrophoretic migration. The width, 0.036 mm, was small enough to give excellent resolution of all bands and to avoid peak integration. In fact, larger or smaller slit width did not change the height of the peaks, even with the sharpest bands. After addition of a Wratten 23A filter (or 24; see above), the densities (or transmissions) of the steps of the tablet were measured with a digital densitometer (TD 504; Macbeth Products Division, Newburg, N.Y.), which uses scattered light. This was necessary because the sensitograms were prepared with the film in contact with the tablet. These densities were slightly different from the ones measured with the Joyce–Loebl microdensitometer, which uses directed light.

Film darkening curve. If I_i is the intensity of the incident light on the tablet, and I_t the intensity of the light transmitted to the film through the step of transmission T, then $I_t/I_i = T$. I_i is a constant whose value is not required for relative measurements. Therefore, T is directly proportional to the intensity of the light striking the film.

A film darkening curve (Fig. 1) is obtained by plotting the density of the sensitogram zones, in terms of pen deflection, against the transmission, on a logarithmic scale, of the corresponding steps of the tablet. This curve is linear in the intermediate range of light intensity. The curvatures at high and low intensities are respectively related to the saturation and sensitivity thresholds of the film (9).

Fluorescence measurement. The fraction of fluorescent radiation from the gel bands that actually impinges on the film decreases if the distance of the bands from the optical axis of the objective increases. This phenomenon (or vignetting) can be neglected in the central part of a negative, that part included in a circle with a diameter equal to 70% of the length of the negative, as indicated by the following experiment: A uniformly illuminated (as checked with a light meter; see above) white paper was photographed; tracings of the negative show constant pen deflections only inside the circle described above and decreased deflections outside. Therefore, only the central part of the negatives, showing no vignetting, was measured.

Each peak of the tracing was fitted with a baseline and subdivided by an odd number of vertical lines at 0.5-cm intervals; deflection values, at their

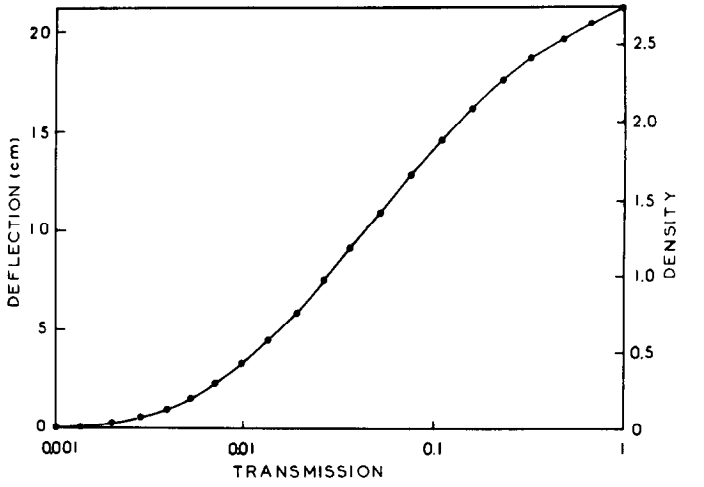

FIG. 1. Plot of microdensitometer pen deflections, obtained in the tracing of a sensitogram, vs transmissions of the tablet steps on a logarithmic scale. The points refer to the steps. Densities were calculated from the slope of the gray wedge used (0.13 OD units/cm).

intercept with the peak and the baseline, were converted into light intensities with the film darkening curve (Fig. 1). The differences between peak and baseline intensities were integrated over the entire peak, giving the fluorescence intensity of the DNA present in the corresponding band. It is important to note that such an integrated intensity cannot be obtained from the area under the peak in the densitometer tracing, even if the film darkening lies within the linear part of the curve of Fig. 1.

In a computer program,[3] the darkening curves were represented by series of third degree polynomes, and values were integrated by the use of Simpson's rule.

Correction of nonuniformity of uv illumination. As reported above, only the central 20 cm of the gels (lengthwise) are uniformly illuminated. The decrease of illumination at the two extremities can, however, be overcome by scanning a lane of the gel free from DNA; the tracing (not shown) has constant pen deflections only in its central part corresponding to the uniformly illuminated region. If I_1 is the fluorescence of the DNA-containing lane and I_2 is that of the lane free from DNA at the same abscissa, the corrected DNA fluorescence, or the DNA fluorescence which should be obtained with a uniform illumination, is $(I_1 - I_2)/I_2$. Such a correction was, however, not useful here, since only the bands located in the uniformly illuminated region of the gel were taken into account.

RESULTS AND DISCUSSION

Figure 2 shows the electrophoretic patterns on 2% polyacrylamide–0.5% agarose gels of SV40 DNA digested with Hind II + III (Lanes 1–3) and Hae III (Lanes 4–7). Faint bands (unlettered) may be seen in addition

[3] This program is available upon request.

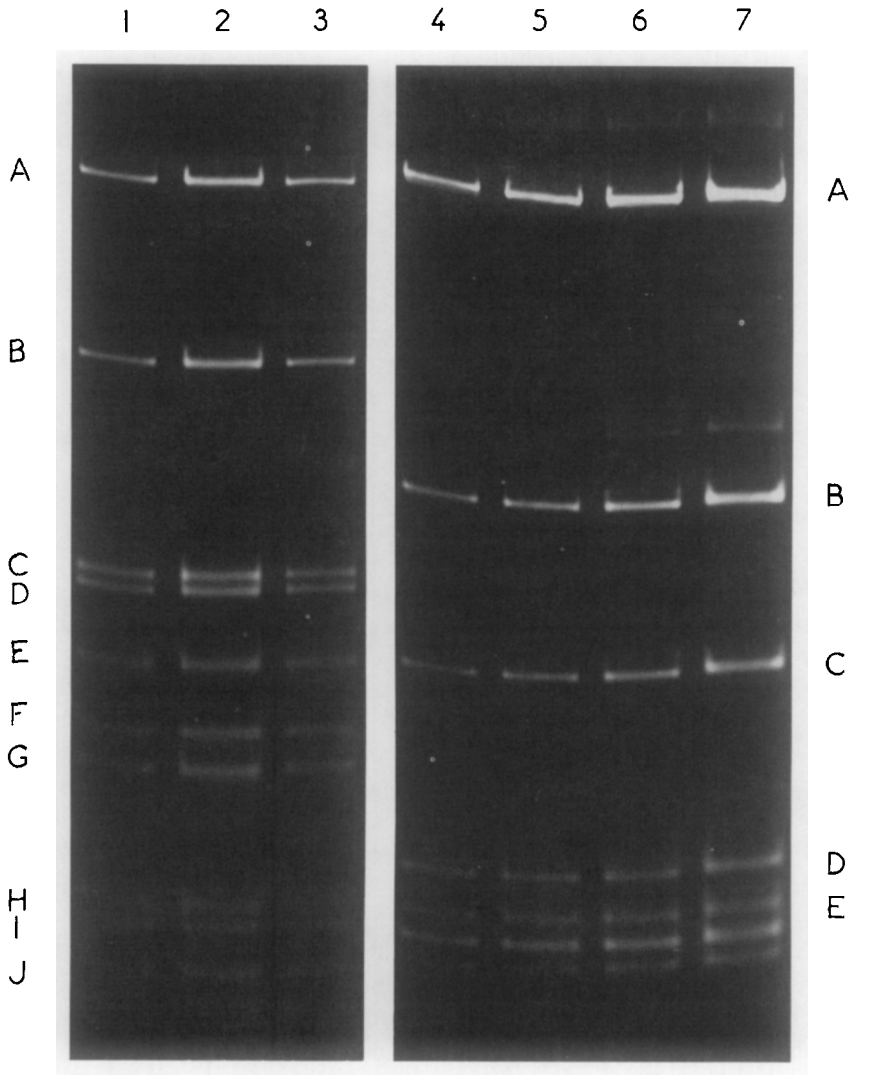

FIG. 2. Electrophoretic patterns of SV40 DNA restriction digests on 2% polyacrylamide–0.5% agarose slab gels. In Lanes 1, 2, and 3 were loaded 10, 20, and 10 μl, respectively, of a Hind II + III digest containing 0.2 μg of DNA/μl. In Lanes 4, 5, 6, and 7, respectively, were loaded 10, 15, 20, and 30 μl of a Hae III digest also containing 0.2 μg of DNA/μl. Hind II + III and Hae III fragments, respectively, were indicated by letters according to Refs. (12) and (16).

to the usual pattern (lettered). They are not likely to be due to incomplete digestion since they remain unchanged with increasing amounts of enzyme and incubation times. They could be due to the presence of defective genomes (10,11); with Hae III, some of them could originate from a slight contamination by Hae II. Figure 3A shows the fluorescences, at two apertures, of bands A to G in Lanes 1 and 2 in Fig. 2 (Hind II + III

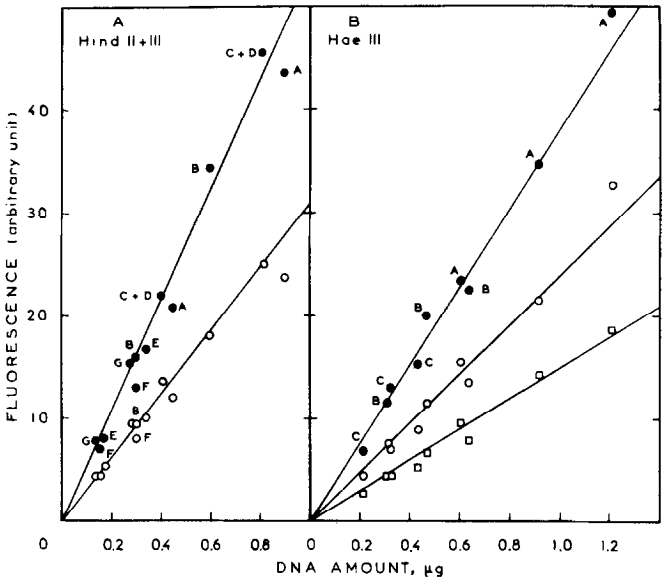

FIG. 3. Plots of fluorescence against the amount of DNA in the bands of Fig. 2. Objective apertures were *f*/5.6 (●), *f*/8 (○), and *f*/11 (□). The amount of DNA (in micrograms) in the bands was calculated from the size of the corresponding fragments, expressed as a fraction of the SV40 genome size [see Refs. (12) and (16)] and multiplied by the amount of DNA (in micrograms) loaded in the lanes. (A) Data from bands A to G in Lanes 1 and 2 of Fig. 2. (B) Data from bands A to C in Lanes 4–6 of Fig. 2.

patterns) as a function of the DNA amount (see legend of Fig. 3). Figure 3B shows the same plot with bands A to C in Lanes 4–6 in Fig. 2 (Hae III patterns) at three apertures. The fact that most of the points in Fig. 3 are on straight lines passing through the origin indicates that the fluorescence is directly proportional to the amount of DNA. Some points are not on the lines; their relative positions, similar at all apertures, indicate that deviations are not due to errors in the photographic procedure. In the particular cases of bands A and F in Fig. 3A, the deviations, similar in both lanes, probably correspond to wrong estimations of the amount of DNA. This could originate from (i) wrong estimations of fragment molecular weights, possibly because large plaque SV40 DNA was used here whereas the molecular weights were determined for small plaque SV40 DNA (12); or (ii) the presence of defective genomes contributing inequally to the bands. A differential binding of ethidium bromide to the fragments is unlikely since this stain binds nonspecifically to DNA (13,14).

A fluorescence artifact which affects the measurements is the photodecomposition of ethidium bromide. This was shown in Fig. 4A where fluorescences obtained at the same aperture in two successive photographs of Lanes 4–7 in Fig. 2 were plotted against each other. The gel was exposed to uv illumination for 6 min between the two photographs. The fluores-

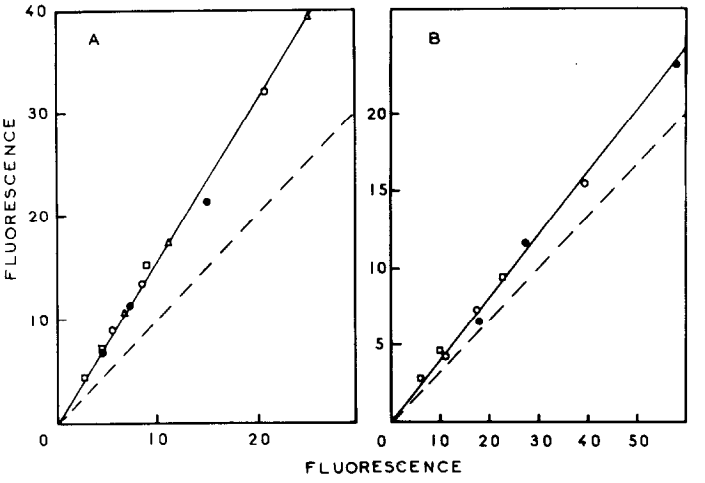

FIG. 4. (A) Plot of the fluorescences (given in Fig. 3B), at *f*/8 aperture in the first photograph (ordinate), against the fluorescences obtained at the same aperture in the second photograph (see text). Symbols refer to bands A to C in Lanes 4 (□), 5 (●), 6 (○), and 7 (△). The slope of the dashed line is 1. (B) Plot of the fluorescences of bands A to C in Lane 4 of Fig. 2 (ordinate) against the corresponding fluorescences in Lane 7 at apertures *f*/5.6 (●), *f*/8 (○), and *f*/11 (□). The slope of the dashed line, 0.33, is equal to the ratio between the DNA amounts loaded in Lanes 4 and 7.

cences in the second photograph decreased by 36% as compared to those of the first one. The straight line shows that relative values between bands in the second photograph were unaffected. Photodecomposition is probably responsible for the fact that the fluorescence ratios (*f*/5.6)/(*f*/8) and (*f*/8)/(*f*/11) in Fig. 3 were not equal to 2, as expected, but were lower, since photographs with smaller apertures were taken first.

A nonuniform electrophoretic migration of DNA could modify the width of the bands or cause the bands not to stay perpendicular to their migration direction; this changes not only their width but also their apparent thickness in the scanning. The bias resulting from the first artifact is shown when the fluorescences of bands A to G in Lane 1 (given in Fig. 3A) are plotted against the corresponding fluorescences in Lane 3. The points obtained (not shown) are on a line whose slope does not equal 1, as expected from the equal amounts of DNA loaded in these lanes, but equals 0.88, the ratio between bandwidths in Lanes 3 and 1. (These widths, approximately constant within a lane, were measured with a profile projector, Model 6C; Nikon, Japan.)

All DNA bands are homogeneous along their width as indicated by the scans of different parts of them because peak dimensions do not change. Trailing and greater amounts of DNA were observed, however, on both sides of the bands. These "side effects" do not appear to bias the results shown in Fig. 3. Overloading increased the side effects. This is shown in

Fig. 4B where the fluorescences of bands A to C in Lane 4 (given in Fig. 3B) were plotted against the corresponding ones in Lane 7; the points, obtained at three apertures, are on a line whose slope does not equal 0.33, as expected from the respective loadings of the lanes (see legend of Fig. 2), but equals 0.40. This indicates a 17% decrease of the fluorescences in Lane 7, where the amount of DNA was higher. These increased side effects are apparent in Lane 7 of Fig. 2 and, much more so, in another lane (not shown) of the same gel where a greater volume of the Hae III digest was loaded (40 μl instead of 30 μl; see legend to Fig. 2). The absence of curvature in the plot of Fig. 4B indicates that the fluorescence decrease in Lane 7 is not due to a concentration quenching occurring for bands containing large amounts of DNA; it also shows that side effects are constant for all bands within a lane. Relative measurements of DNA amounts in bands are, therefore, more accurate within a lane than between lanes.

This method has been used in our laboratory to estimate the yield of the larger fragments present in the restriction digests of yeast mitochondrial DNAs and to compare it to the yield expected from a random degradation of the DNAs during their preparation (8). The method has also been used to measure fragment size distribution of the restriction digests of eucaryotic DNAs for comparison with the distributions predicted from a random location of the restriction sites in the genomes (manuscript in preparation). In this latter case, the fluorescence was measured on about 30 cm of the gels and was corrected for the nonuniformity of the uv illumination (see above).

Recently, a similar photographic procedure was reported, with little detail, by De Leys and Jackson (15). These authors fractionated SV40 DNA molecules differing in superhelical density. They found that the amount of DNA in the bands was linearly proportional to the height of the peaks of the densitometer tracings. Obviously, such a simple relationship can only hold under their particular conditions where the molecular weight corresponding to the different bands is constant. In fact, a decrease in peak heights, not related to decreased amounts of DNA, would be expected for bands of smaller molecular weight, as they will diffuse more.

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