

Buoyant Density of DNA-Hoechst 33258 (Bisbenzimidide) Complexes in CsCl Gradients: Hoechst 33258 Binds to Single AT Base Pairs

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Received September 4, 1990

Buoyant density of DNA in CsCl gradients with Hoechst 33258 (bisbenzimidide) was investigated as a function of guanine plus cytosine content of the DNA (%GC; in mole percent). A formula for calculating %GC from the refractive index (n_D) of the isopycnic CsCl/Hoechst 33258 solution over the range of 0-75 %GC was established: $\%GC = 351762.28 \times n_D - 123778.66 \times n_D^2 - 249789.47$ (the coefficients must not be rounded off). The shape of this curve indicates that under these conditions, in contrast to dilute buffers, Hoechst 33258 binds to single AT base pairs on DNA. Resolution of DNA bands in CsCl/Hoechst 33258 gradients is 1.6 to 2.1 times better than comparative CsCl gradients without the dye. Potential application to %GC determination is discussed. © 1991 Academic Press, Inc.

The most common methods for determination of guanine plus cytosine content of DNA (%GC; in mole percent)³ are buoyant density centrifugation (1,2), thermal denaturation (3), and high-performance liquid chromatography (HPLC) of DNA hydrolysate (4,5). Isopycnic banding of DNA in CsCl gradients remains unequalled by other methods with respect to speed and simplicity, as cell lysates can be used without any purification (6) and known relations between refractive index, buoyant

density, and %GC (1,7) make standardization with DNAs of known GC content unnecessary. Furthermore, DNA from complex samples is separated in gradients into components, whose %GC is determined simultaneously.

Isopycnic ultracentrifugation in CsCl solution is also a classical means for DNA purification. The use of the DNA-binding fluorescent dye Hoechst 33258 (H33258, also known as bisbenzimidide) in CsCl gradients, introduced by Garber and Yoder in 1983 (8), has become a standard procedure for purification of fungal DNA and for its separation into mitochondrial, nuclear, ribosomal, and other fractions (e.g., (9,10)). In addition to making the DNA bands visible in uv, H33258 improves the resolution power of gradients by binding preferentially to A+T rich regions on DNA (11) and thus altering its buoyant density. For example, only one single band is observed, when total *Cochliobolus heterostrophus* DNA is run in CsCl gradients with the intercalating dye ethidium, while the same DNA is separated into mitochondrial, nuclear, and ribosomal DNA in CsCl gradients with H33258 (CsCl/H33258) (8).

Although the density of fractions collected from CsCl/H33258 gradients is diagnostic for %GC of DNA which they contain, it could not be used for %GC determination until now since its relation to %GC has not been known. In this work we show that the buoyant density of DNA in CsCl/H33258 is an almost linear function of its %GC and we give an empirical equation for calculating %GC from refractive index.

MATERIALS AND METHODS

Chemicals

CsCl was from Pharmacia (purity 99.5%) and Hoechst 33258 trihydrochloride from Sigma. H33258

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³ Abbreviations: %GC, guanine and cytosine content of DNA (in mole percent); H33258, Hoechst 33258, 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)benzimidazole; CsCl/H33258, CsCl gradient with Hoechst 33258.

concentration was determined by measuring the optical density at 338 nm in 10 mM Tris, 100 mM NaCl, pH 7.0, using the molar extinction coefficient $4.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and the molecular weight 624. Double-stranded alternating copolymer poly(dA-dT) · poly(dA-dT) was purchased from Pharmacia, homopolymer poly(dA) · poly(dT) from Sigma.

Bacterial DNA and DNA fragments

Organisms used are listed in Table 1. Bacterial DNA for the calibration prepared according to Marmur (12) was purchased from Sigma (Nos. 1 and 6), kindly provided by Dr. K.-D. Jahnke (Nos. 2 and 3) or prepared in our laboratory (Nos. 4 and 5). Other bacterial and fungal DNA samples were kindly supplied by our colleagues and used without further purification. Bacteriophage λ DNA was purchased from Boehringer. Restriction fragments originated from cloned mitochondrial DNA of *Phytophthora parasitica* DSM 1829 and/or from a plasmid vector (B. Fartmann, P. Karlovsky, and H. H. Prell, unpublished work).

CsCl Gradients

An 8.8-g quantity of solid CsCl was added to 8.0 ml DNA solution in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. The amount of each DNA was determined empirically to produce sharp clearly visible bands (about 5–10 μg for DNA with low %GC). After dissolving CsCl, 70 μl of H33258 stock solution (1 mg/ml) in water was added. The initial density of these gradients was about 1.463 g/ml, the concentration of H33258 was 6.1 $\mu\text{g}/\text{ml}$. Tubes were centrifuged in a Beckman Ti75 rotor at 42,000 rpm for 40 h at 20°C in a preparative ultracentrifuge Beckman L8-70M. Fractions were collected by puncturing the tubes. In cases of very short restriction fragments, attempts were made to collect the middle part of rather diffuse bands.

Refractive Index (n_D) Measurements

n_D measurements were made with an Abbe refractometer (Zeiss Jena) using sodium D line at 25°C.

RESULTS

Conditions for Analytical CsCl/H33258 Gradient Centrifugation

In an attempt to use CsCl gradients with a high H33258 concentration according to Garber and Yoder (8) for analytical purposes, we encountered problems concerning a high fluorescence background and precipitation of the dye in gradients. The latter was also observed by the authors of the preparative method (8). Lowering the H33258 concentration to 6 $\mu\text{g}/\text{ml}$ ($\frac{1}{20}$ of the amount suggested) eliminated both difficulties. In order

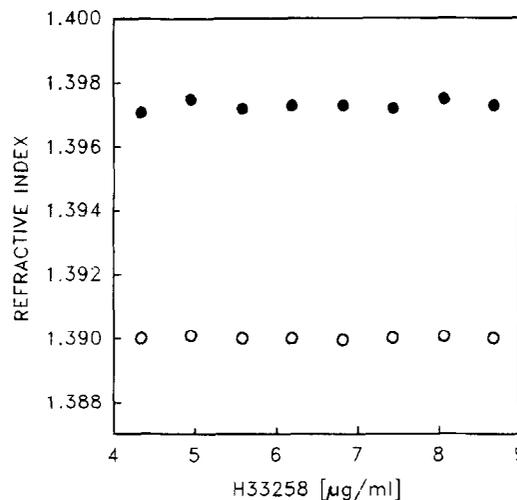


FIG. 1. Dependence of DNA buoyant density on H33258 concentration. Gradients were made as described under Materials and Methods except for the H33258 concentration. Two DNA samples were analyzed in each gradient: nuclear DNA of *Phytophthora* sp. (open circles) and poly(dA-dT) · poly(dA-dT) (filled circles).

to show that the amount of H33258 is still sufficient to saturate the DNA, we made a series of gradients with the same DNA species but with different H33258 concentrations (Fig. 1). The experiment confirmed that variations in the H33258 concentration between 4 and 8 $\mu\text{g}/\text{ml}$ do not influence the banding density of DNA.

Since density centrifugation in CsCl is an equilibrium method, centrifugation conditions are also not critical: under conditions similar to ours, an equilibrium was reached in less than 20 h (1,2). Moreover, reaching the equilibrium can be easily checked by observing narrow DNA bands. As known from (1,2) and from other classical works, altering the centrifugation speed and/or the rotor geometry would make gradients more or less steep but it would not change the absolute value of DNA buoyant density.

Using degraded DNA (DNA treated excessively with ultrasound) resulted in diffuse bands (results not shown). The same problem was encountered when working with short restriction fragments (Table 3). Since these fragments were of the order of 10^2 bp long and DNA isolated by standard procedures from prokaryotic or eukaryotic origin consists of fragments about 10^4 bp, this problem hardly occurs in a practical application.

Calibration of CsCl/H33258 Gradients

A serious problem encountered when evaluating any method for %GC determination (with the exception of HPLC) is the reliability of %GC values of DNAs used as standards. Although the strains selected for this work are those most often used in DNA studies, discrepancies exist in the literature concerning their %GC. The

TABLE 1
Bacterial and Fungal DNA

	%GC	Ref.
Bacterial strains used for calibration		
1. <i>Clostridium perfringens</i> ATCC 13124	25.5	(14)
2. <i>Cytophaga johnsonae</i> NCIB 10150	33.9	^a
3. <i>Bacillus subtilis</i> ATCC 6051	43.0	(14)
4. <i>Escherichia coli</i> K12	50.6	(5)
5. <i>Paracoccus denitrificans</i> ATCC17741	65.5	(14)
6. <i>Micrococcus lysodeikticus</i> ATCC 4698	73.1	(13)
Other bacterial and fungal strains		
7. <i>Alcaligenes eutrophus</i> H16	66.3–67.5	(14)
8. <i>Alcaligenes latus</i> DSM1123	69.1–71.1	(14)
9. <i>Aspergillus ochraceus</i> DSM824	48.3–54.0	(23, 25)
10. <i>Eubacterium acidaminophilum</i> al-2	42.8–46.0	(28) ^a
11. <i>Phytophthora infestans</i> P1.4	47.5–54.2	(23, 27)
12. <i>Phytophthora parasitica</i> DSM1829	49.0–50.5	(23)
13. <i>Pseudomonas pseudoalva</i> GA3	66.5–68.0	(14)
14. <i>Pseudomonas putida</i> Fu1	58.7–63.9	(14, 26)
15. <i>Schizophyllum commune</i> DSM1024	54.6–61.0	(23, 25)

^a K.-D. Jahnke, personal communication.

seriousness of the problem increases with the precision of the method concerned. We selected the following data, which evenly span the range 25–75% GC: *Escherichia coli*, the weighted average of results obtained in (5) with a help of a very precise variant of HPLC was used; *Cytophaga johnsonae*, data obtained with the same method were kindly supplied by K.-D. Jahnke (DSM Braunschweig, Germany); *Micrococcus lysodeikticus*, the average of three independent determinations compiled in (13) was used. In the other cases, we decided to take the mean of the range of %GC listed in the Bergey's compendium (14) instead of arbitrarily selecting values from individual reports. These %GC values are summarized in Table 1. Since no natural DNAs with defined very low %GC are available, we included a synthetic double-stranded homopolymer and an alternating copolymer of deoxyadenylic acid and thymidylic acid in the analysis. The mean of their densities was used in the calibration, since natural DNAs with very low %GC must possess a distribution of A/T base pairs somewhere between these two extremes, under the assumption of operational randomness exactly in the middle.

The relationship between buoyant density in CsCl/H33258 gradients and %GC of the DNAs tested is shown in Fig. 2. Refractive indices measured were converted to buoyant densities according to (7). Every point in Fig. 2 represents an average of results of at least eight gradients. The points lie on a curve described by the equation

$$\%GC = 351762.28 \times n_D - 123778.66 \times n_D^2 - 249789.47, \quad [1]$$

where n_D is the refractive index. This formula was obtained by fitting the data by the standard least-squares method. Since the result obtained with [1] is a small difference between large numbers, the coefficients in [1] must not be rounded off and the calculation must be performed to a sufficient number of significant places. For example, rounding the coefficients to whole numbers produces errors of about 2% in GC content and rounding to one place more further increases the errors considerably.

How accurate is %GC determination using this method? The simplest approach to this question would be an analysis of other DNAs with known %GC and comparison of the results with published values. Unfortunately, no known %GC values of genomic DNAs are reliable enough (the most reliable ones already being selected for the standard curve) to make a meaningful interpretation of the comparison possible. Considerable discrepancies in %GC exist in the literature for all repeatedly analyzed species between the results of different methods and often even when the same method was used with the same strain. Furthermore, the possible differences between our results and published results could be interpreted as a consequence of inaccuracy associated with our method, with methods used by other authors, or both. The problem was recently encountered by Sanders *et al.*, who tried to assess the accuracy of their dual-laser flow cytometry method in this way (15). They found both agreements and differences between their own values and published values, but they could not say anything about the cause of the differences. For

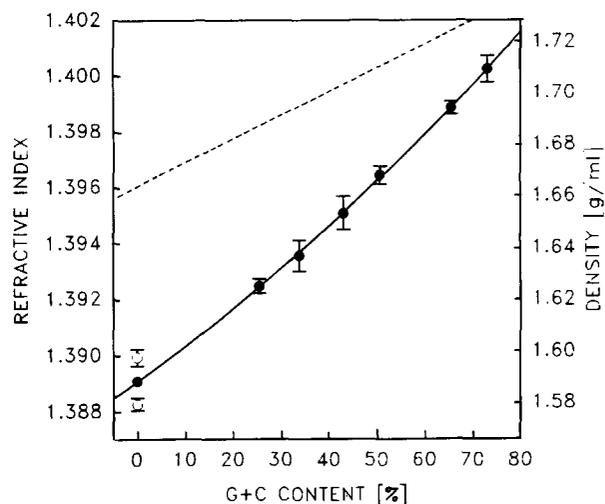


FIG. 2. Buoyant density of DNA in CsCl/Hoechst 33258 gradients. Gradients were made as described under Material and Methods. Bacterial DNAs used (filled circles with vertical bars) are listed in Table 1, bars indicate standard errors. Open circles represent synthetic double-stranded copolymers poly(dA-dT) · poly(dA-dT) (higher density) and poly(dA) · poly(dT) (lower density), their mean was used for the regression curve. Density of DNA in gradients without a dye (1) is indicated by a broken line.

TABLE 2
Accuracy and Reproducibility of %GC Determination in CsCl/H33258 Gradients

%GC value from the literature	Mean %GC from gradients \pm standard error	No. of determinations
0.0	-0.18 ^a	10, 7
25.5	25.02 \pm 0.63	8
33.9	32.59 \pm 1.25	9
43.0	42.64 \pm 1.13	11
50.6	51.08 \pm 1.95	8
65.5	65.10 \pm 1.27	8
73.1	72.42 \pm 0.78	10

^a The mean of averages for both polynucleotides with 0% GC was used for the calculation. Application of the formula to the individual polynucleotides would yield 6.43 and -6.89% GC, respectively.

this reason, we decided to restrict ourselves to the analysis of data obtained with the strains used to construct the standard curve. We calculated %GC from n_D for every single measurement and compared them with theoretical values (Table 2). All averages were accurate within 1.3 %GC or less, demonstrating consistency of theoretical values of standards selected.

Determination of %GC of Bacterial and Fungal Genomic DNA

As discussed in the previous section, a comparison of experimentally determined %GC with published values for arbitrarily selected DNAs cannot give a measure of the accuracy of the method, but it can provide information about its predictive capacity and reveal a systematic error. We applied the CsCl/H33258 method to five bacterial DNAs not used for the calibration and to four fungal nuclear DNAs. The results (Fig. 3) demonstrate

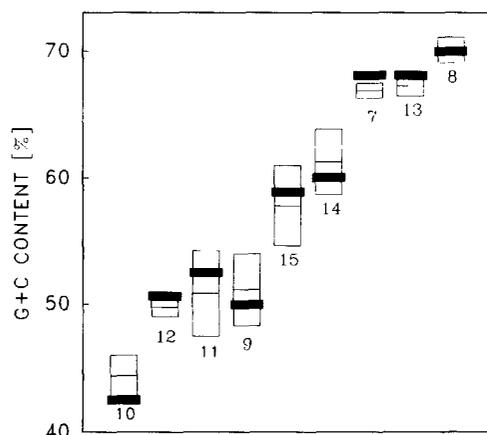


FIG. 3. Determination of %GC of bacterial and fungal DNA by the means of CsCl/H33258 gradients. Organisms are identified by numbers assigned in Table 1. Boxes demonstrate %GC range reported in the literature with the mean indicated by a thin horizontal lines; thick lines designate values obtained in this work.

TABLE 3
Analysis of DNA with Known Nucleotide Sequences in CsCl/H33258 Gradients

Fragment	Length (bp)	%GC from the sequence	%GC from CsCl/H33258
pARSII-20/ <i>Bam</i> HI+ <i>Kpn</i> I	375	10.9	14.9
pARSII-20/ <i>Pvu</i> II+ <i>Hind</i> III	463	19.0	28.5
pARSEQ/ <i>Bam</i> HI+ <i>Kpn</i> I	3696	48.8	50.7
Bacteriophage λ DNA	49502	49.8	45.1

a good correlation between experimental and published values. The results of our determination fell within the range of published values in five cases and they deviated from the literature values by no more than 1% in the remaining four cases.

Application of CsCl/H33258 Gradients to %GC Determination of Low Complexity DNA

DNA fragments with known nucleotide sequence are excellent standards for %GC determination by HPLC. Since H33258 seems to bind to single A/T pairs in concentrated CsCl solutions (see Discussion), we wanted to know, whether CsCl/H33258 gradients are applicable to %GC determination of short DNA fragments with nonrandom sequences. We selected three sequenced restriction fragments with various %GC (Tab. 3) and the chromosome of the bacteriophage λ for the analysis. The results show clearly that the influence of the nucleotide sequence on H33258 binding is so large that %GC of restriction fragments can be estimated only very approximately by this method (Table 3). This observation agrees with the results of the analysis of synthetic polynucleotides containing only A and T (Fig. 2).

DISCUSSION

The addition of H33258 to CsCl gradients improves their resolving power by spreading the banding scale (11). By comparing the tangent of the curve in Fig. 1 with the slope of the line for gradients without H33258 (1), we can quantify this improvement as follows: the same difference in %GC corresponds to about two times greater difference in buoyant density in CsCl/H33258 as compared with CsCl alone. General advantages of buoyant density centrifugation in CsCl gradients also apply for CsCl/H33258: simple probe preparation, separation of DNA into fractions, and the possibility of preparative use. The influence of nucleotide sequence on H33258 binding, as shown in experiments with defined restriction fragments, is the most serious disadvantage of the method. It precludes it from using with small replicons like plasmids or mitochondrial DNAs.

Differences between selected literature values and average experimental values of %GC were at most 1.3%

(Table 2 and Fig. 3). The reproducibility was better than 2% while that of the standard density gradients (2) as well as of the melting temperature and flow cytometry methods (3,15) were about 1%. It can be further improved by using more sophisticated methods for gradient fractionation (16). For the most accurate measurements, HPLC will likely become a standard in the future (5). It is the only direct method for %GC determination, but it requires special instrumentation and laborious sample preparation (enzymatic degradation to nucleosides and dephosphorylation). Special equipment and optically pure samples are required for the thermal denaturation method. A new method based on measurement of the ratio of chromomycin A3 fluorescence to H33258 fluorescence (15) allows work with whole bacterial cells, but the reliability of determination is affected by instrument adjustment and alignment and a new standard curve must be prepared just before every set of analyses. Moreover, the instrumentation is inaccessible to the majority of biologists.

Determination of %GC using CsCl/H33258 gradients has a great application potential in taxonomical research where it can be combined with studies for which separation of nuclear DNA from other fractions (mitochondrial, ribosomal, or plasmid DNA) is necessary. Only a small part of the nuclear DNA fraction from a gradient is required for the measurement of %GC. The method should be especially advantageous if a large number of samples is to be processed. A typical potential application of the formula [1] can be found in a recent taxonomical study on *Pythium* (10), in which positions of DNA bands in CsCl/H33258 gradients (measured in mm) and %GC (determined by another method) were used as independent markers in cluster analysis.

Our results provide interesting information on the interaction of H33258 with DNA in a concentrated salt solution. The binding of one H33258 molecule to DNA is known to require at least three consecutive A/T base pairs. This requisite was identified already in 1975 by spectroscopic methods (11) and confirmed in recent fingerprinting and DNA damage studies on defined DNA sequences (17–19) and by X-ray crystallography of H33258–oligonucleotide complexes (20–21). Since the DNA buoyant density difference between CsCl gradients with and without H33258 is nearly proportional with (100 – %GC) (Fig. 2), an average binding site in a concentrated CsCl solution contains only one A/T pair. This untypical binding mode can be accounted for by the effect of an extremely high ionic strength. This reduces the electrostatic component of intermolecular interactions and hence the contribution of a positive charge of the H33258 molecule to DNA binding. On the contrary, hydrophobic forces are enhanced in a high dielectric environment. The interior of the minor groove in A/T pairs was shown to be quite nonpolar with a local

dielectric constant of ≈ 20 D (22). We hypothesize that H33258 in very high salt concentrations binds to DNA primarily through its hydroxyphenyl moiety which slides into the minor groove of an A/T pair and is stabilized here by hydrophobic and van der Waals' interaction with the groove walls and by displacement of water from this microenvironment. Both these factors are more important in a highly concentrated CsCl solution than in dilute buffers normally used in H33258–DNA interaction studies. The complex formation can be additionally promoted by hydrogen bonding between the hydroxyl group of H33258 and N-3 of adenine or O-2 of thymine, which is expected to contribute a substantial stabilization energy in the low dielectric environment of the minor groove (22).

The fact that buoyant densities of DNA in the presence and in the absence of H33258 appears to extrapolate to the same value with raising %GC (Fig. 2) suggests that no H33258 binds to DNA without AT pairs. This contradicts findings in dilute buffers and eliminates some modes of binding anticipated in such conditions. In the terminology of Loontjens *et al.* (24), these are charge-mediated and structure-mediated binding modes. Only sequence-mediated (AT-specific binding of H33258 to DNA) and perhaps dye-mediated binding (binding of a free dye molecule to H33258 already bound to DNA) seems to be significant in highly concentrated CsCl solutions.

ACKNOWLEDGMENTS

We thank Klaus-Dieter Jahnke for providing DNA and unpublished data used in the calibration, Bärbel Buchholz, Meike Lübbers, and Ute Windhövel for supplying bacterial DNA samples and Gabriele Nordsiek for a *P. denitrificans* culture. We are grateful to J. Auer for skillful technical assistance, H. H. Prell and unknown reviewers for valuable comments on the manuscript, and D. Yarrow for correcting the English text. The work was partially supported by a fellowship of the Alexander von Humboldt Foundation to AdC.

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