*THE USE OF AN ETHIDIUM ANALOGUE IN THE DYE-BUOYANT DENSITY PROCEDURE FOR THE ISOLATION OF CLOSED CIRCULAR DNA: THE VARIATION OF THE SUPERHELIX DENSITY OF MITOCHONDRIAL DNA**

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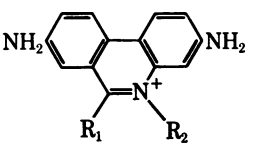
Abstract.—The separation between open and closed circular DNA in buoyant CsCl gradients containing intercalating dyes depends on the superhelix density of the closed form. These separations are about 1.8 times larger with propidium iodide than with ethidium bromide. The superhelix densities of mitochondrial DNA from HeLa cells and *Lytechinus pictus* eggs appear to be about two thirds that of mitochondrial DNA from rat and rabbit liver.

The dye–buoyant density method[1] has proved to be a reliable and efficient procedure for the detection and isolation of closed circular DNA. This communication reports the results of a study of variations in the method designed to increase the resolution of closed circular species from open species, while at the same time preserving other useful features of the method, particularly reproducibility and sensitivity for the detection of DNA by inspection or photography of fluorescent bands. The method previously described employed ethidium bromide (EB) I with $R_1 = C_6H_5$ and $R_2 = C_2H_5$ as the "dye" component. We have found that an ethidium analogue with $R_2 = C_3H_6N(C_2H_5)_2CH_3$, which we refer to as propidium iodide (PI), enhances the resolution between closed and open DNA by a factor of approximately 1.8 relative to ethidium bromide. The increased resolution appears to be independent of the molecular weight and only slightly dependent on the superhelix density of the DNA. The superhelix density σ_0 is defined as the number of superhelical turns per ten base pairs under the assumption that the angle of the duplex is the same in the closed and open forms.[2]



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The magnitude of the separation between the closed and open (nicked or linear) DNA should be sensitive to the superhelix density of the DNA.[3] This has been found to be the case. We have concluded from the results obtained in this study that there are differences in superhelix densities between mitochondrial DNA's from various sources. The superhelix densities of mitochondrial DNA's from HeLa cells and sea urchin eggs appear to be about two thirds as large as those from rat and rabbit liver.

Materials and Methods.—Analogues of ethidium bromide: The analogues of 3,8-diaminophenanthridine used in this study were kindly provided by Dr. T. I. Watkins of Boots Pure Drug Co., Ltd. We have successfully repeated the straightforward synthesis of 3,8-diamino-5-diethylaminopropyl-6-phenylphenanthridinium iodide[4] from 3,8-dinitro-6-phenylphenanthridine. Propidium was used as the diiodide salt of the N′ methyl derivative of the above quaternary compound.

Photography and measurement: After centrifugation, the dye-CsCl gradients were illuminated with ultraviolet light as described previously[1] and photographed through a Wratten 16 filter either with a Polaroid camera and type-46L film, *ca.* 10 sec at f11, $5^3/_4$ in., or with a single-lens reflex camera and Ektachrome film, *ca.* 1 sec at f1.4, 6 in. The resulting transparencies were measured in a Nikon 6 projection comparator.[5] A separation on the film could be reproducibly measured to ± 0.005 cm. The magnification factor for the fixed photographic arrangement was determined by photography of a rule and by measurement of the centrifuge tube width.

Fluorescence measurements: Fluorescence intensities were measured on a Farrand fluorimeter. The uncorrected values for different dyes were compared.

Preparation of DNA's: Viral SV40[2] and the mitochondrial DNA's[6, 7] were prepared as described elsewhere.

Polynucleotide ligase:[8-10] The enzyme was prepared from *Escherichia coli* K12 strain 1100 by a modification of the method of Gefter, Becker, and Hurwitz,[10] assayed by the adenylate binding method of Hurwitz[11] and Little *et al.*,[12] using the units of Hurwitz.[11]

Closure of SV40 DNA by ligase: Purified SV40 I DNA (54 μg/ml) was converted to SV40 II DNA with DNase I (bovine pancreas, Sigma Chemical Co.) at a concentration of 1.63×10^{-5} μg/ml in 0.015 *M* NaCl, 0.012 *M* $MgCl_2$, 0.01 *M* tris(hydroxymethyl)-aminomethane (Tris), 0.007% BSA, 0.001 *M* ethylenediaminetetraacetate (EDTA), pH 8, for 30 min at 30°. SV40 II DNA was purified by the dye-CsCl buoyant method. SV40 II DNA was closed with polynucleotide ligase in essentially the medium described by Olivera and Lehman[8] for 20 min at 30°C. SV40 L1 DNA was prepared with SV40 II DNA at 24 μg/ml with 2 units/ml of ligase. SV40 L2 DNA was prepared with SV40 II DNA at 26 μg/ml and 5 units/ml of ligase with 6.8 μg/ml of ethidium bromide added.[14] SV40 L1 DNA was purified by the dye-CsCl buoyant method and SV40 L2 DNA by sedimentation through a sucrose gradient.

Results and Discussion.—The buoyant density of a DNA species at equilibrium in a cesium chloride gradient containing a gradient of a reacting solute is approximately equal to the mass of the buoyant complex divided by its volume.[2]

$$\theta = \frac{1 + \Gamma' + \nu'}{\bar{v}_3 + \Gamma' \bar{v}_1 + \nu' \bar{v}_4}. \tag{1}$$

In equation (1), Γ' is the preferential hydration of the Cs DNA in grams water per gram Cs DNA, ν' is the mass of dye bound per gram Cs DNA, and the $\bar{v}$'s are the partial specific volume of water (1), Cs DNA (3), and the dye (4). To an approximation of about 2 per cent for the case where the partial specific volume of the dye is about equal to that of water, $\Delta\theta$, the difference in buoyant density between closed (I) and open (II) DNA is given by

$$\Delta\theta = \frac{\Delta\Gamma' (\bar{v}_3 - \bar{v}_1) + \Delta\nu' (\bar{v}_3 - \bar{v}_4)}{(\bar{v}_3 + \bar{\Gamma}'\bar{v}_1 + \bar{\nu}'\bar{v}_4)^2}, \tag{2}$$

where $\Delta\Gamma' = \Gamma_I' - \Gamma_{II}'$, and $\bar{\Gamma}' = (\Gamma_I' + \Gamma_{II}')/2$, $\bar{\nu}' = (\nu_I' + \nu_{II}')/2$, and $\Delta\nu' = \nu'_I - \nu'_{II}$. The quantity $\Delta\nu'$ is not zero at high levels of an intercalating dye because of the *restricted binding*[2] of the dye to closed circular DNA. It is this effect that gives rise to the buoyant density difference between the closed and open forms of a circular DNA.

Analogues of ethidium: A variety of analogues of ethidium were tested for an increase in $\Delta\theta$. Table 1 presents the results obtained with SV40 DNA. The individual separations have been normalized with the value for ethidium bromide (EB) measured in the same experiment in order to cancel out small variations due to time, temperature, speed, and radial distance. The ratio $\Delta r_X/\Delta r_E$ is designated χ.

TABLE 1. *Effects of substituents in the diaminophenanthridinium ring system (I) on the buoyant separation between closed and open viral SV40 DNA in dye-CsCl density gradients.*

R_1	R_2	$\chi = (\Delta r_X/\Delta r_E)$
C_2H_5	C_2H_5	0.70
ϕ	CH_3	0.92
ϕ	C_2H_5	1.00
ϕ	C_3H_7	1.01
$pNH_2\phi$	C_2H_5	1.00
ϕ	$C_3H_6N(C_2H_3)_2CH_3$	1.80

The dye concentrations were 300–500 μg/ml, and the initial density was 1.55–1.58 gm/ml. The samples were centrifuged in an SW50 rotor at 43 krpm for 48 hr at 20°.

Choice of experimental conditions: The properties of propidium iodide (PI) ($R_1 = \phi$, $R_2 = C_3H_6N(C_2H_5)_2CH_3$) relative to EB were studied in more detail. The separation between closed and open SV40 DNA in an ethidium chloride–cesium chloride density gradient is approximately constant at high dye levels. The separation becomes smaller, however, if the free dye concentration in the region of the bands falls below about 75 μg/ml. This can occur in preparative ultracentrifuges because of the large redistribution of the dye in the gradient. We have found that the originally described conditions,[1] $\rho = 1.55$ gm/ml and 100 μg/ml EB, are barely adequate to maintain the needed free dye concentration at the bands. Raising the initial EB concentration from 200 to 500 μg/ml has no observable effect on the separation of SV40 DNA components. In the case of PI, it was found that the separation increased upon raising the concentration from 100 to 300 μg/ml, but then remained constant to 500 μg/ml. At these high dye levels, however, the sensitivity for detection by fluorescence is somewhat reduced because of the fluorescence of free dye.

The optimum choice of an initial dye concentration also depends on the initial density. Low dye concentrations can be used if the density is sufficiently high that the bands form near the top of the cell where the dye is concentrated. The final choice was 300–500 μg/ml of dye, either EB or PI, and density of 1.58 gm/ml. Centrifugation for 24 hours at 43 krpm in a 3.0-ml volume in an SW50 rotor is sufficient for preparative purposes, although 48 hours was routinely used in this study. The details of the centrifugation are unimportant and a wide variety of rotors (including angle rotors) has been used in this laboratory. A tube containing a reference DNA in the open and closed form was always included in the same rotor for purposes of quantitative comparison.

The effect of molecular weight and superhelix density on the separation enhancement of PI over EB: The increased separation of SV40 DNA's in PI as compared with EB is shown in Figure 1*a, b*. The same result (Table 2) was obtained in a similar experiment with HeLa cell mitochondrial DNA (M-DNA), which has a molecular weight approximately three times higher than SV40 DNA. The ratio of separations with PI over EB, χ, is approximately 1.8 in each case. This is the expected result, since all of the properties that determine the buoyant density of a DNA-dye complex are intensive, and there should be no effect of molecular weight.

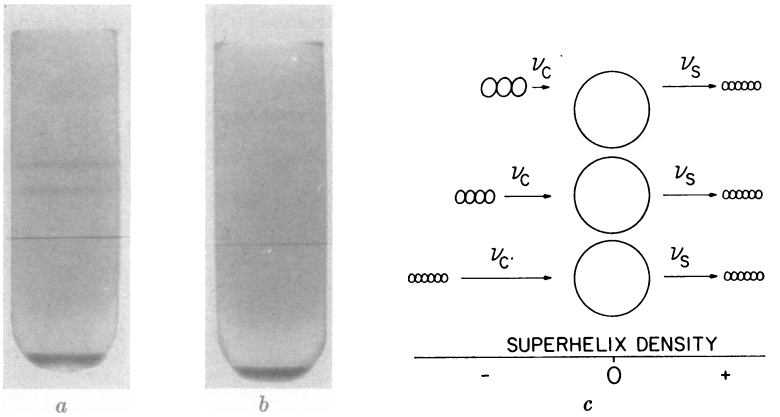

FIG. 1.—Fluorescent bands of open (*upper*) and closed (*lower*) SV40 DNA in a buoyant CsCl density gradient containing (*a*) ethidium bromide and (*b*) propidium iodide. The initial densities were 1.58 gm/ml. The tubes were centrifuged in the same rotor under standard conditions (Table 2). (*c*) Diagrammatic representation of the binding of an intercalating dye to three closed circular DNA's with differing initial superhelix densities. The dye-free molecule is shown at the left, the relaxed molecule in the center, and a highly twisted one at the right.

We have prepared two artificial forms of closed SV40 DNA to test the effect of the superhelix density on χ. A preparation of SV40 I DNA was treated with pancreatic DNase at very low levels to produce some nicked SV40 II with 3'-hydroxyl groups at the hydrolyzed bond. Species II was then purified and closed with the enzyme polynucleotide ligase.[8-10] The first product, SV40 L1 DNA, was obtained by enzymatic closure in moderately low salt. The second product, SV40 L2 DNA, was obtained similarly except for the presence of ethidium bromide during the action of the ligase.

SV40 L1 DNA has a low superhelix density. Viral λ DNA closed under the incubation conditions and used to prepare SV40 L1 DNA has been calculated to

TABLE 2. *The buoyant separation Δr between the closed and open forms of four DNA's in cesium chloride containing ethidium iodide (EI) and propidium bromide (PB).*

DNA	Rotor	Δr_{PI} (cm)	Δr_{EB} (cm)	$\chi = \Delta r_{PI}/\Delta r_{EB}$	$\bar{\chi}$
SV40 L1	SW50.1†	0.822	0.429	1.91	1.91
	SW50.1*	0.895	0.470	1.90	
SV40 I	SW50	0.532	0.300	1.77	1.80
	SW41	0.570	0.310	1.84	
	SW50.1†	0.619	0.345	1.79	
	SW50.1*	0.669	0.370	1.81	
SV40 L2	SW50.1†	0.347	0.201	1.72	1.72
	SW50.1*	0.389	0.228	1.71	
HeLa M-DNA	SW50	0.608	0.345	1.76	1.76

The dye concentration for all experiments was 330 μg/ml and the initial density was 1.58 gm/cm.[3] Three-ml volumes were used in all experiments except in the SW41 rotor, where 6 ml were used. All runs were 20°C for 48 hr except for the SW41, which was run for 72 hr. The nominal rotor speed for the three rotors used was 43 krpm for the SW50, 35 krpm for the SW41, and 40 krpm for the SW50.1. The measured speed in the experiment marked with an asterisk was 39 krpm as compared with 40.5 krpm in the experiment marked with a dagger. This difference accounts for the variation of Δr in the two experiments.

have about 15 per cent of the superhelix density of SV40 I.[13] SV40 L1 DNA should have a similarly low superhelix density. SV40 L2 DNA, on the other hand, has a high superhelix density because it was closed in an underwound condition. The extent of this underwinding, and the final superhelix density, depends on the amount of dye bound to the DNA at the instant of closure.[14] Rough calculations demonstrate that the superhelix density of SV40 L2 DNA should be substantially higher than that of SV40 I DNA.

The separations of these forms of SV40 DNA have been determined in EB and PI in order to see if there was any effect of the initial superhelix density on χ (Table 2). There seems to be no large effect, although a trend may be indicated.

Fluorescence: EB and PI were compared for fluorescence enhancement[15] at 365 mμ and found to be the same. This implies that the detection sensitivity of PI and EB should also be equal. However, in practice it seems that PI is somewhat less sensitive than EI for detecting small amounts of DNA.

Removal of dye: Several methods are available for removal of EB and PI. Removal is greatly facilitated by high salt because of the reduced binding affinity of DNA for dye. Dialysis against low ionic strength medium is inefficient. The dye is easily removed from the DNA in the 4.5 *M* CsCl by passage through a Dowex 50 column,[1] or by extraction with isopropanol[16] or isoamyl alcohol followed by ether extraction. The extraction methods have the advantage that they are quick and can be used on small samples with little dilution.

Variation of the buoyant separation with superhelix density: Consider the three forms of SV40 DNA which have been made as described above. SV40 L1 has a low superhelix density, SV40 I has an intermediate superhelix density, and SV40 L2 has a high superhelix density. The binding of an intercalative dye to these molecules is shown schematically (Fig. 1*c*), in which the horizontal coordinate represents the superhelix density. The figures at the left represent the molecules before addition of dye. A certain amount of dye, ν_c, binds to the molecule and converts it to the open conformation. This amount depends on the initial superhelix density, since $\nu_c = (\pi/10\phi)\sigma_0$,[2, 17] where ϕ is the unwinding angle of the base pairs upon binding one molecule of the intercalating dye. The relaxed closed circular molecules bind further dye, ν_s, and are eventually "saturated" in that the free energy of the dye binding is effectively counterbalanced by the free energy of superhelix formation.[2] If ν_s is a constant with respect to σ_0, the amount of dye bound at saturation, $\nu = \nu_c + \nu_s$, should depend on σ_0, and closed circular molecules with high (negative) superhelix densities would exhibit small separations in a dye–buoyant density system. A high initial superhelix density results in increased binding at saturation and a decreased buoyant density. There is, however, a limit for the sum $\nu_c + \nu_s$ which cannot exceed $\nu_{II,\,max}$, the maximum number of binding sites, expressed in moles dye per phosphate, on the nicked circular form. As the limit is approached for molecules with high superhelix density, the assumption that ν_s is independent of σ_0 may no longer be valid.

The effect of variations of the superhelix density is demonstrated by the results obtained with closed SV40 DNA's. Figure 2*a*, *b* presents the experiment in which the three closed forms and the open form were banded in dye–cesium chloride gradients containing either EB or PI. The experiments have also been con-

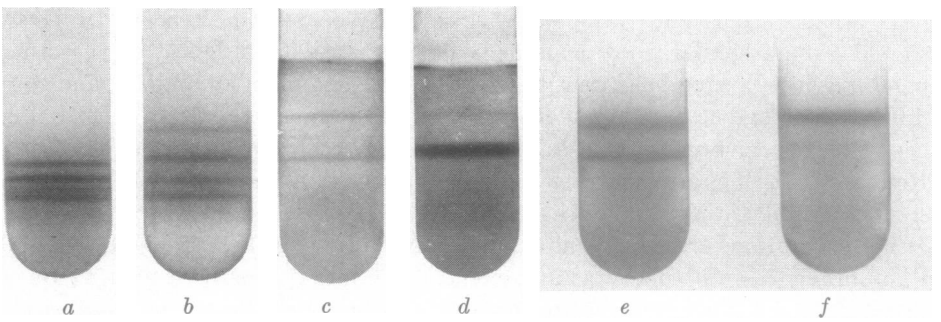

FIG. 2.—The effect of superhelix density on the buoyant density in dye-CsCl gradients. Fluorescent bands of SV40 DNA in buoyant CsCl containing (*a*) ethidium bromide and (*b*) propidium iodide. In order of increasing densities, the bands are SV40 II DNA, SV40 L2 DNA, SV40 I DNA, and SV40 L1 DNA. A comparison of the buoyant separations of (*c*) HeLa cell M-DNA and (*d*) SV40 I DNA in propidium iodide–CsCl density gradients. A comparison of the buoyant separations of (*e*) HeLa cell M-DNA and (*f*) chicken liver M-DNA in ethidium bromide–CsCl density gradients.

ducted with each of the DNA's separately to be sure of the assignment of the bands. The result is that SV40 L2 DNA, the high superhelix density DNA, shows a very small buoyant separation from the open form. The native DNA, SV40 I DNA, shows an intermediate separation and SV40 L1 DNA, the low superhelix density DNA, shows a large buoyant separation. The trend of these data is in the direction expected; Δr varies inversely with σ_0. The sensitivity of the separation to a given change in the superhelix density is about 1.8 times as large for PI as for EB.

The buoyant separation between SV40 I and II in an EB-CsCl gradient was measured six times under standard conditions (Table 3) and found to be 0.297 $\pm$ 0.004 cm (SE). The separation is thus very reproducible. In three experiments with HeLa M-DNA in EB, it was found that the separation 0.340 $\pm$ 0.005 cm was slightly but significantly larger than the SV40 separation. This was also the case in PI, where there was no doubt about the larger separation for HeLa cell M-DNA, 0.608 cm, as compared with 0.545 cm for SV40 DNA (Fig. 2*c*, *d*). An experiment with a mixture of SV40 I, SV40 II, HeLa M I, and HeLa M II DNA's in EB showed a slight splitting of the bottom bands, but not of the top bands. The obvious interpretation of these results is that HeLa cell M-DNA has a lower superhelix density than SV40 I DNA.

The separation between closed and open DNA's in a dye-CsCl density gradient should also depend on the buoyant density of the DNA in the absence of dye, and hence on the G + C content of the DNA. This effect has been estimated to result in about a $^1/_2$ per cent increase in the closed to open separation of DNA per 1 per cent increase in G + C content[3] for the case in which the binding constant is independent of G + C and $\bar{\nu}_4 \approx 1$.

We have measured the buoyant separations of rat and rabbit liver M-DNA in EC and have found them to be about the same as or slightly less than SV40 DNA (Table 3). The separation for chicken liver M-DNA is smaller than that for HeLa M-DNA (Fig. 2*e*, *f*). The separation measured for sea urchin (*L. pictus*)

M-DNA is considerably larger than for rat and rabbit liver M-DNA's, but less than for HeLa M-DNA (Table 3). This implies that the superhelix densities of HeLa cell and sea urchin M-DNA are lower than that of the liver M-DNA's. The results are in agreement with those of Smit and Ruttenberg[18] in that there appears to be little difference in superhelix density between SV40 DNA and rat liver DNA. Furthermore, these results confirm the expectation that if two DNA's have equal superhelix densities, they will have an equal separation in these systems.

TABLE 3. *The buoyant separation between the open and closed forms of six DNA's relative to the comparable separation for SV40 viral DNA measured in each experiment.*

		$\Omega = \Delta r/\Delta r_{SV40\ I}$	
Expt.	Material	Ethidium bromide[1]	Propidium iodide
1.	SV40 L1 DNA	1.26 ± 0.01 (2)	1.33 ± 0.01 (2)
2.	HeLa M-DNA	1.12 ± 0.005 (2)	1.12 (1)
3.	*L. pictus* M-DNA		1.07 (1)
4.	SV40 I	1.00	1.00
5.	Rabbit liver M-DNA	0.97 (1)	
6.	Rat liver M-DNA	0.96 (1)	
7.	SV40 L2 DNA	0.60 ± 0.02 (2)	0.57 ± 0.01 (2)

Typical values of Δr_{SV40} are given in Table 1. The average value of Δr_{SV40} was 0.297 ± 0.004 (SE) in six experiments in an SW50 rotor at 43 krpm. The number of determinations of each ratio is given in parentheses. For details of composition and centrifugation see text.

The superhelix density of the *in vitro* closed SV40 L1 DNA should be about the same as that measured for *in vitro* closed λ DNA made under the same conditions. The latter value is 15 per cent of the value of viral SV40 DNA. The superhelix density of HeLa cell M-DNA can be estimated to be 61 per cent or 69 per cent of that of SV40 viral DNA if a linear interpolation is made with the data for EB or PI, respectively. A similar estimate with the data for PI gives a value of 82 per cent for sea urchin egg DNA. These numbers indicate the magnitude of the variations in superhelix densities and should not be considered as accurate determinations. The linearity of superhelix density with separation has not yet been established, nor has the effect of base composition been examined. It has been shown in this laboratory that the minimum in an EB–sedimentation velocity titration of HeLa cell M-DNA occurs at a much lower free-dye concentration than in a titration with SV40 I DNA,[19] confirming the above interpretation.

All closed circular DNA molecules isolated to date contain superhelical turns. Chicken[20] and rat[18] liver mitochondrial DNA's, papilloma viral DNA's,[21,22] SV40 viral DNA,[2] and polyoma DNA[5, 17] have superhelix densities which are approximately equal, although there are small but definite differences between SV40 and polyoma DNA's.[19] The superhelix densities of HeLa cell and sea urchin egg mitochondrial DNA's are considerably lower. The biological problem of the origin of superhelical turns[23] has not been solved. Wang[13, 24] has shown that about 15 per cent of the superhelix density of SV40 DNA can be accounted for by changes in the average angle between base pairs due to changes in ionic strength, and that temperature has a small effect on superhelix density. Since the internal physical-chemical environment of cells is unknown, the relative con-

tribution of the former effect cannot be assessed. Any proposed mechanism for the origin of superhelical turns must include the potential for a variability.

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