Use of Ethidium Bromide Fluorescence Enhancement to Detect Duplex DNA and DNA Bacteriophages during Zone Sedimentation in Sucrose Gradients: Molecular Weight of DNA as a Function of Sedimentation Rate†

Philip Serwer,* Paul R. Graef,‡ and Preston N. Garrison

ABSTRACT: Duplex DNA molecules and DNA bacteriophages have been sedimented through 5–25% sucrose gradients containing ethidium bromide. The location of DNA within the gradients has been determined by illuminating gradients with ultraviolet light and observing the ethidium bromide fluorescence enhancement induced by the DNA. The relative sedimentation rates of linear, duplex DNAs from bacteriophages T4, T5, T7 and an 8.3% T7 deletion mutant have been determined. The distances sedimented by DNA have been corrected, when necessary, for a progressive decrease in sedimentation rate that occurs after the DNA has traversed 40% of the sucrose gradient. The corrected distances sedimented by two DNA molecules, r_1' and r_2', are related to the DNA molecular weights, m_1 and m_2, by the equation: $r_1'/r_2' = (m_1/m_2)^{0.38}$ when 0.025–0.70 μg of each type of DNA is sedimented. Intact bacteriophages were also sedimented in ethidium bromide–sucrose gradients and detected by fluorescence enhancement.

Zone sedimentation of DNA in sucrose gradients is a method for separating DNA molecules on the basis of their molecular weight (Burgi & Hershey, 1963; Leighton & Rubenstein, 1969). This technique can also be used for the isolation and characterization of viruses and virus precursors (for examples, see Serwer, 1974a,b, 1976). The location in the sucrose gradient of a DNA-containing macromolecule is usually determined by fractionating the gradient and assaying for radioactive molecules using liquid scintillation counting. The following problems occur: (1) In some situations it is inconvenient or impractical to label DNA with radioisotope; this occurs, for instance, when a DNA-containing structure is present in small quantities in cell lysates, a situation we have encountered with phage-related structures isolated from lysates of bacteriophage T7 infected *E. coli*. The use of a physical assay for DNA is hampered by limits on the amount of DNA that can be sedimented without distortion of DNA bands (1 μg of DNA appears to be a maximum; Burgi & Hershey, 1963). (2) Gradient fractionation and scintillation counting are time consuming, expensive processes; to accurately measure the distance sedimented, precautions must be taken to ensure that drop sizes do not vary during fractionation. To avoid the above problems it is desirable to develop a technique for observing DNA and DNA–protein complexes (including viruses) in sucrose gradients, in amounts below 1 μg of DNA and without fractionating the gradient.

The ultraviolet light induced orange fluorescence of the dye ethidium bromide is increased in the presence of DNA (Le Pecq & Paoletti, 1966) and this fluorescence enhancement has been used to detect DNA after electrophoresis in agarose gels with a detection limit of less than 0.05 μg of DNA (Sharp et al., 1973). The present communication describes the use of ethidium bromide to detect duplex DNA and DNA bacteriophages after zone sedimentation in sucrose gradients. Ethidium bromide is dissolved in the sucrose gradient and DNA within the gradient is observed as fluorescent bands after illumination of the gradient with ultraviolet light. The sedimentation rate of linear, duplex DNA as a function of molecular weight is determined in ethidium bromide–sucrose gradients using DNA from bacteriophages T4, T5, T7, and a T7 deletion mutant as standards. Intact bacteriophages have also been observed using fluorescence enhancement after sedimentation through ethidium bromide–sucrose gradients.

Materials and Methods

A. *Bacteriophage and Bacterial Strains.* Bacteriophage T7 and T7 deletion mutant C5,LG3, also containing amber mutation 342 in gene I (Simon & Studier, 1973; Studier, 1969) were received from Dr. F. W. Studier. The amber mutation was removed from T7 C5,LG3 by selecting for a revertant. Bacteriophages T4 and T5 were obtained from Dr. Eric Moody and the T4 osmotic shock mutant, Os 41, was obtained from the Caltech phage collection. The host for T4, T4Os41, T7, and T7 C5,LG3 was *E. coli* BB/1. The host for T5 was *E. coli* Bλ^s.

B. *Buffers and Reagents.* Tris/EDTA buffer contained: 0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.4. Tris/Mg buffer contained: 0.2 M NaCl, 0.01 M Tris, 0.001 M $MgCl_2$, pH 7.4. The ethidium bromide used was purchased from Calbiochem (B grade) and was filtered through a Millipore filter (0.45 μm).

C. *Growth and Purification of Bacteriophages.* For all bacteriophages the bacterial host was grown in 6 L of M9 medium (Kellenberger & Séchaud, 1957) at 30 °C to a concentration of 2–3 × 10^8. The cultures were infected at a multiplicity of 0.05–0.1 and were allowed to lyse spontaneously; lysis was completed by the addition of chloroform in the case of T4 and T4 Os41. Bacteriophages in the lysates were precipitated using Carbowax 6000, were then purified on a cesium chloride step gradient, and were finally subjected to buoyant density banding in a cesium chloride density gradient as described in Serwer (1976) for phage T7. The viscosity of T5 and T4 preparations was reduced prior to the cesium chloride step

† From the Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284. *Received October 18, 1977.* Supported by National Institutes of Health Grants S01 RR 05654 and GM-24365-01.

‡ Present address: The University of Texas Health Science Center at Houston, Texas.

0006-2960/78/0417-1166$01.00/0

gradient by incubating the preparation with 2.5 μg/mL DNase I (Worthington) at 37 °C for 1 h. Even though the salt concentration at this stage is high for optimal DNase I activity (0.5 M NaCl, 0.01 M Tris, 0.001 M $MgCl_2$, pH 7.4, are present), a sufficient reduction in viscosity occurs.

Phage T5 produces a series of bands in cesium chloride gradients some of which were described by Saigo (1976). The contents of the densest band is a phage envelope with packaged DNA and no tail (Saigo, 1976; we have confirmed this observation). This lowest band, whose contents will be referred to as a full head, was the most intense in our preparations and DNA from this band is used in all experiments using T5 DNA. DNA from the T5 full head cosediments with DNA from T5 phage.

All phage preparations were dialysed into Tris/Mg buffer. Phages T4 and T5 were sequentially dialyzed into buffers containing 4, 2, 1, 0.5, and 0.2 M NaCl in 0.01 M Tris, pH 7.4, 0.001 M $MgCl_2$ to avoid osmotic shock damage. Nonetheless, some increase in viscosity occurred during dialysis of phage T4. DNA from burst T4 phage was eliminated by shearing the preparation 15× through a 22-gauge needle followed by pelleting and resuspension of the phage. DNA from T40s41 sediments at the same rate as DNA from wild-type T4 and was sometimes used in place of wild-type DNA. The concentration of DNA in bacteriophage preparations was determined from their OD_{260} as described in Bancroft & Freifelder (1970).

D. *Release of DNA from Bacteriophages and Sedimentation through Ethidium Bromide–Sucrose Gradients.* DNA was released from bacteriophages by diluting the phage by at least a factor of three into Tris/EDTA buffer + 0.1% Sarkosyl NL 97 (Geigy) and heating for 15 min at 75 °C. Ethidium bromide was added to the sample and the sample (150 μL) was layered on a linear 5–25% w/v sucrose gradient in Tris/EDTA or Tris/Mg buffer with ethidium bromide. The gradient volume was 5.0 mL in the SW50.1 rotor and was 12.2 mL in the SW 41 rotor; cellulose nitrate centrifuge tubes were used. DNA can also be released using sodium dodecyl sulfate instead of Sarkosyl, but sodium dodecyl sulfate induces ethidium bromide fluorescence and produces a bright band at the top of the centrifuge tube. To avoid shear-mediated DNA degradation, released DNA samples that contained T5 or T4 DNA were pipetted with disposable 1-mL glass pipets by removing the cotton plug and using the plugged end as the pipet tip (the inner pipet diameter was 2.5 mm). Pipets with a diameter as small as 1.0 mm could be used to load T4 DNA without degradation, but only if the DNA was pipetted very slowly. DNA was sedimented through sucrose gradients at 18 ± 2 °C; the time and rotor speed are in the figure legends.

E. *Photography and Scanning of Fluorescent DNA Bands.* After centrifugation, centrifuge tubes were photographed by placing them between an ultraviolet transilluminator (Ultra-Violet Products, Inc.) and a camera. Photographs were taken on 4 × 5 in. Kodak Tri-X Pan film using a glass Kodak 23A orange filter in front of the camera lens. Prints were made on Kodabrome RC medium paper.

To quantitate the fluorescence in a peak, a Helena Quick Scan Fluor-Vis scanning densitometer was turned on its side; the centrifuge tube was inserted in the sample holder and was scanned directly in the fluorescence mode using a Kodak 23A Wratten filter. This procedure will be referred to as fluorescence scanning.

F. *Measurement of Distances Sedimented.* Distances of the peak of a DNA band from the sedimentation origin were measured on photographs of the sucrose gradient using a Nikon Microcomparator. The ratio of the distance sedimented to the gradient length is referred to as r.

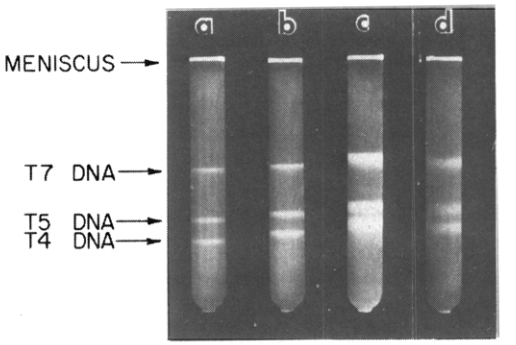

FIGURE 1: Sedimentation of DNA from bacteriophages T4, T5, and T7. Two mixtures both containing 0.35 μg of DNAs extracted from bacteriophages T4, T5, and T7 were prepared; similar mixtures containing 0.18 and 1.4 μg of each DNA were prepared. One of the 0.35-μg mixtures, the 0.18-μg mixture, and the 1.4-μg mixture were sedimented through ethidium bromide–sucrose gradients in Tris/EDTA buffer (SW 41 rotor, 4.0 h, 35K). The second 0.35-μg mixture was sedimented for a total of 3.75 h at 35K, but the rotor was stopped and restarted three times during this period: (a) 0.18 μg; (b) 0.35 μg (continuous run); (c) 1.4 μg; (d) 0.35 μg (interrupted run).

G. *Collection of Fractions and Infectivity Assays.* Material in sucrose gradients to be assayed for infectivity was collected by pipetting fractions from the top of the gradient. The fractions which contained fluorescent bands were determined by visual observation during the fractionation procedure. The phage were diluted into 0.5 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M $MgCl_2$, 1 mg/mL gelatin. For experiments in which 1% β-mercaptoethanol was present in the sucrose gradient, it was also included in the dilution buffer.

Results

A. *Detection of DNA in Sucrose Gradients Using Ethidium Bromide.* The concentration of ethidium bromide required to observe DNA in 5–25% sucrose gradients was determined by sedimenting 0.2 μg of phage T7 DNA through sucrose gradients in Tris/EDTA buffer containing different amounts of ethidium bromide. A single fluorescent DNA band was observed using ethidium bromide concentrations between 0.1 and 40 μg/mL. The ratio of the peak fluorescence to background fluorescence was determined by fluorescence scanning to be highest with ethidium bromide concentrations between 1 and 2 μg/mL. A concentration of 2 μg/mL of ethidium bromide is used in all future experiments. The least amount of phage DNA that has been detected photographically is 0.025 μg. However, below 0.07–0.10 μg the bands are not visible to the eye.

Sedimentation profiles of DNAs from bacteriophages T4, T5, and T7 using 0.18, 0.35, and 1.4 μg of each DNA are in Figures 1a–c, respectively. The three DNAs migrate as separate bands. The T7 DNA is skewed to the fast sedimenting side. The amount of skewing decreases with DNA amount between 1.6 μg and 0.2 μg of DNA[1] and phage T7 DNA isolated from the leading edge of a sedimentation peak sediments at the same rate relative to T5 DNA as T7 DNA isolated from the trailing edge of the same peak ± 3%. Thus, the skewing is probably not the result of DNA heterogeneity and probably results from interactions between DNA molecules.

[1] The amount of skewing is defined as the ratio of the integrated fluorescence intensity between the band peak and the trailing edge of the DNA band to the integrated intensity between the peak and the leading edge. This ratio is 0.4 when 1.6 μg of DNA are used and increases to 0.8 for 0.2 μg of DNA.

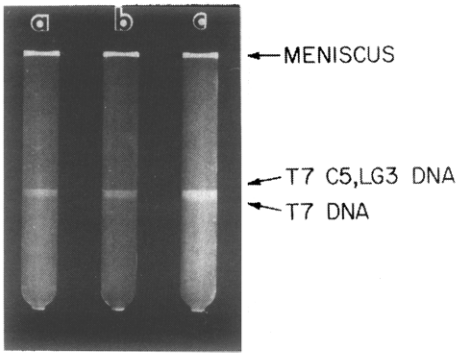

FIGURE 2: Sedimentation of T7 DNA and DNA from an 8.3% deletion mutant. Mixtures of DNA extracted from bacteriophages T7 and T7 C5,LG3 were made that had 0.10, 0.18, and 0.34 μg of each DNA. These mixtures were sedimented through sucrose gradients in Tris/EDTA buffer (SW 41 rotor, 5.0 h, 35K): (a) 0.10 μg; (b) 0.18 μg; (c) 0.34 μg. The C5,LG3 deletion was measured to be 9.2% in the electron microscope (Simon & Studier, 1973), but a more recent analysis using restriction enzyme digests of DNA from this mutant indicates that it has an 8.3% deletion (Studier, personal communication).

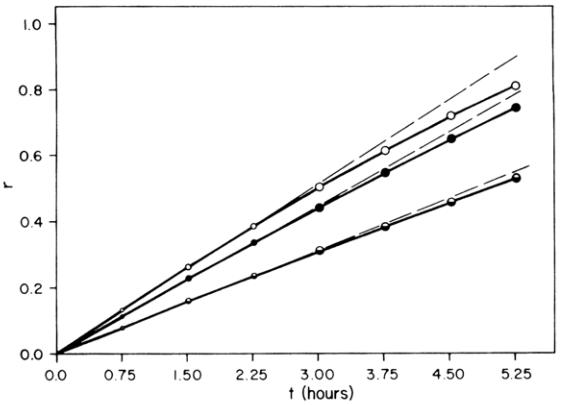

FIGURE 3: Sedimentation of DNA as a function of time. A mixture of DNAs from bacteriophages T4, T5, and T7 containing 0.18 μg of each DNA was sedimented as in Figure 1, except that the centrifuge was stopped at intervals of 0.75 h and photographs of the centrifuge tubes were taken. The distance migrated by each DNA is plotted as a function of time. (○—○) T4 DNA; (●—●) T5 DNA; (◒—◒) T7 DNA. The diameter of the data points represents the experimental error on the r axis.

To test the resolving power of this technique, wild type T7 DNA was cosedimented with DNA that is 8.3% shorter, obtained from a T7 deletion mutant. Sedimentation profiles obtained using 0.10, 0.18, and 0.35 μg of DNA are shown in Figures 2a–c, respectively. The two DNAs are resolved from each other, the resolution improving as the amount of DNA is decreased.

It should be possible to develop an empirical formula for determining the molecular weight of any linear, duplex DNA molecule from its sedimentation rate relative to a phage DNA of known molecular weight. To do this accurately sedimentation distance as a function of centrifugation time was determined.

B. *Sedimentation Distance of DNA as a Function of Time.* To measure the sedimentation distance as a function of time and DNA amount, mixtures of DNA from bacteriophages T7, T5, and T4 were made up that had 0.10, 0.18, 0.35, and 0.70 μg of each DNA. These mixtures were sedimented, the centrifuge was stopped at 0.75-h intervals and the fluorescent DNA bands were photographed. The position of each DNA was measured as a function of time.[2] During this procedure DNA bands broaden considerably more than they do when DNA is centrifuged for a comparable time without stopping the centrifuge (compare Figures 1b and 1d). The cause of the broadening is not known; the centrifuge vacuum was kept below 100 μm mercury at the beginning of each sedimentation to minimize convection due to frictional heating of the buckets. The band position was assumed to be at the band peak and sedimentation distance (r) was measured as a function of time (t) for all DNA bands. However, DNA, present in amounts of 0.10 μg or less, becomes invisible at late times because of band smearing and could not be measured.

In Figure 3 the distances sedimented by DNA from bacteriophages T4, T5, and T7 are plotted as a function of time for a DNA amount of 0.18 μg per band. The plots are linear for r values below 0.35–0.40 and gradually become convex for higher r values. To eliminate this nonlinearity a correction factor $Q(r)$ has been determined: $Q(r)$ is the ratio of the distance the DNA would have traveled if it had continued at the rate it moved during the linear portion of the $r(t)$ plot to the measured r. When DNA is sedimented beyond $r = 0.4$ one corrects r for nonlinearity by multiplying r by $Q(r)$ to obtain r'. The data from T4 DNA in Figure 2 have been used to obtain $Q(r)$; for $0.4 < r < 0.75$, $Q(r)$ can be presented by the equation:

$$Q(r) = 0.89 + 0.34r \qquad (1)$$

Values of $Q(r)$ are independent of DNA amount within experimental error between 0.18 and 0.70 μg of DNA. In an experiment not shown $r(t)$ was measured for times up until 9.75 h. It was found that $Q(r)$ was the same for T4, T5, and T7 DNA within experimental error for r values below 0.75.

C. *Sedimentation of Linear, Duplex DNA as a Function of Molecular Weight.* The molecular weights of DNAs from bacteriophages T4 and T5 have been shown to be 110×10^6 and 67.9×10^6, respectively (reviewed in Freifelder, 1970). The molecular weight of DNA from the Studier T7 strain is 26.5×10^6.[3] Values of $r'/r'(T7)$ have been determined for DNAs from bacteriophages T7, T5, and T4 after sedimentation through regions of the sucrose gradient for which $r(t)$ is linear ($Q(r) = 1$). This ratio has also been determined for T7C5,LG3 from the data in Figure 2a. Even though the DNA in Figure 2a is in a region of the gradient for which $r(t)$ is nonlinear, the DNA bands are so close to each other that the effect of nonlinearity on $r/r(T7)$ is insignificant. Values of $r'/r'(T7)$ have been plotted as a function of DNA molecular weight. On a double logarithmic scale the data fall on a straight line and fit the following equation:

$$\frac{r'}{r'(T7)} = \left(\frac{m}{m(T7)}\right)^{0.38} \qquad (2)$$

For any two linear duplex DNAs with molecular weights m_1 and m_2, the respective r's, r_1' and r_2', are related by the equation:

$$\frac{r_1'}{r_2'} = \left(\frac{m_1}{m_2}\right)^{0.38} \qquad (3)$$

[2] The distance sedimented is not affected by the exposure to ultraviolet irradiation necessary to photograph DNA bands. Bacteriophage T7 DNA that was preirradiated in the presence of ethidium bromide cosediments with unirradiated DNA.

[3] Slightly lower values have also been obtained (Freifelder, 1970), but it has been shown that the T7 strains used to obtain these lower values had small DNA deletions relative to the Studier strain used here (Studier, personal communication).

The exponent given is the average of ten independent determinations using a least-squares program to determine the exponent. The uncertainty in the exponent, estimated from the uncertainty in measuring r (about ± 0.006) and the uncertainty in the DNA molecular weights (assumed to be $\pm 2\%$), is ± 0.015. This exponent is independent of DNA amount within experimental error for DNA amounts between 0.025 and 0.70 μg of DNA. If DNA is sedimented as in Figure 1, into a region of the gradient where $r(t)$ is nonlinear, and $Q(r)$ is used to convert r into r', the exponent determined is equal to 0.38 within experimental error.

To determine the effect of ethidium bromide on the sedimentation rate of DNA, 0.35 μg each of the bacteriophage DNAs were sedimented as in Figure 1, but without ethidium bromide in the density gradient. To observe the DNA a micropipet with 5 μL of 5 mg/mL ethidium bromide was submerged to the bottom of the centrifuge tube and the ethidium bromide was released. After allowing the ethidium bromide to diffuse for 2–3 h the DNA bands were observable using fluorescence enhancement. The phage DNAs sedimented 16–20% further than DNA sedimented simultaneously through a sucrose gradient containing 2 μg/mL ethidium bromide (two independent experiments).

D. *Sedimentation of Intact Bacteriophages.* Ethidium bromide fluorescence enhancement can also be used to detect intact bacteriophages after sedimentation in sucrose gradients. In Figure 4a is a sedimentation profile of bacteriophages T7 and T4. The single fluorescent band in Figure 4a contains phage T7; phage T4 does not produce a fluorescent band, probably because the phage T4 envelope is not penetrated by the ethidium bromide. The gradient in Figure 4a was slowly heated to 75.0 °C to eject DNA from the phage capsids and then slowly cooled (see the legend to Figure 4). After heating, DNA from phage T4 becomes visible by fluorescence (Figure 4b) and the fluorescence of the T7 band increases. Fluorescence scanning of the centrifuge tubes before and after heating reveals that the intensity of the phage T7 band increases by a factor of 4–5 after heating and that the background fluorescence increases by a factor of 1.5–2.0. The intensity of the phage T4 band after heating is less than the intensity of the T7 band, even though the DNA amounts are the same. This is probably because not all T4 particles have ejected their DNA; further heating of the centrifuge tube at 75 °C results in equal intensities for phage T4 and T7, but also results in poorer visualization of both bands because of an increase in background fluorescence and a decrease in fluorescence of the T7 band. Bacteriophage T5 full heads sediment about 20% faster than phage T4 when analyzed on similar gradients and have fluorescent properties indistinguishable from those described above for phage T7.

The fluorescence present before heating is coming from intact phage structures because the fluorescent bands continue to sediment at the rate of phage particles when resedimented after photography. The sedimentation rate of T7 phage is the same in the presence as in the absence of ethidium bromide $\pm 5\%$. The detection limit for T7 phage after heating (0.05–0.10 μg of phage DNA or $5\text{–}10 \times 10^8$ infective units) is a factor of 5–10 lower than the detection limit for visualizing the phage using light scattering from the sucrose gradient.

In order to use ethidium bromide–sucrose gradients as a preparative technique for viruses, it should be possible to recover the virus in an infective form. The recovery of T7 phage infectivity has been measured after sedimentation through ethidium bromide–sucrose gradients and fluorescence photography. Gradients were fractioned by pipetting from the top and the phage band was visualized during collection of the gradient by fluorescence. Because short wavelength ultraviolet light (<300 Å) inactivates bacteriophages (reviewed in McLaren & Shugar, 1964), the amount of short wavelength ultraviolet light during fractionation was reduced by placing a $\frac{1}{8}$-in. glass plate between the transilluminator and the centrifuge tube; the glass plate causes no detectable reduction in fluorescence. Recovery of T7 phage infectivity was 0.02–1%. The recovery was increased to 50–100% by including in the sucrose gradient 1% of the reducing agent β-mercaptoethanol. Experiments with and without β-mercaptoethanol were conducted simultaneously under the same room lighting conditions (overhead fluorescent lights). The β-mercaptoethanol may decrease ethidium bromide-mediated visible light inactivation of the phage (photoinactivation). Photoinactivation of bacteriophages occurs in the presence of other multicyclic basic dyes (Yamamoto, 1958) and is blocked by removing oxygen or adding cysteine, a reducing agent (Welsh & Adams, 1954; Appleyard, 1967).

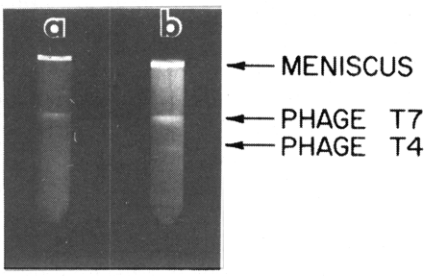

FIGURE 4: Sedimentation of intact bacteriophages T4 and T7. A sample containing intact bacteriophages T4 and T7 in Tris/Mg buffer (2.0 μg of DNA from each phage) was sedimented through an ethidium bromide–sucrose gradient in Tris/Mg buffer (SW 50.1 rotor, 0.33 h, 21K, 18 °C). The centrifuge tube was subsequently covered with parafilm, submerged in a water bath at 25 °C to within 2–3 mm of the tube top, and then heated continuously to a temperature of 75 °C over a period of 20 min. The water bath was turned off as soon as the temperature reached 75 °C and was allowed to cool (the cooling rate was roughly 1 °C every 1.2 min between 75 and 65 °C). (a) Before heating; (b) after heating.

Discussion

The relationship between distance sedimented in ethidium bromide–sucrose gradients and the molecular weight of linear, duplex DNA is described by equation (3). The exponent in this equation, 0.38, is close to the exponent 0.35 derived using sedimentation of radiolabeled DNA (Burgi & Hershey, 1963) and revised to 0.38 by more recent molecular weight determinations (Freifelder, 1970). This exponent is empirically obtained and no attempt has been made to interpret it in terms of the physical properties of DNA.

The sedimentation rate of a linear, duplex DNA molecule relative to one of the phage DNAs can be used with eq 3 to determine the molecular weight of the DNA molecule. A heterogeneous mixture of duplex DNA molecules can be fractionated on the basis of molecular weight by sedimentation in ethidium bromide–sucrose gradients. Fluorescent DNA bands are collected with a high degree of precision by pipetting from the top of the centrifuge tube while observing visually the fluorescence of material being collected. The amount of DNA that can be fractionated is limited by band broadening that occurs as the amount of DNA sedimented is increased. The upper limit to the amount of DNA sedimented depends on the range of sedimentation rates of the DNA molecules in the sample. For instance, 3–4 μg of DNA per band is the upper limit for resolving phage T4 from T5 DNA; 0.3–0.4 μg DNA

per band is the upper limit for resolving phage T7 DNA from T7, C5 LG3 DNA.

The only biological macromolecules known to induce the enhanced fluorescence of ethidium bromide are nucleic acids (Le Pecq & Paoletti, 1966). The successful use of ethidium bromide fluorescence enhancement to detect DNA and DNA bacteriophages during zone sedimentation therefore suggests the use of this technique for the isolation from cell lysates of viruses and DNA-containing viruses precursors. For instance, we have used zone sedimentation in ethidium bromide–sucrose gradients combined with buoyant density sedimentation in ethidium bromide–sodium iothalamate gradients (Serwer, 1975) to purify the DNA–protein complexes and fast sedimenting DNA isolated from lysates of bacteriophage T7-infected *E. coli* by Serwer (1974a,b). A description of this work will be the subject of a future communication.

The fluorescence technique is more sensitive than visible light scattering for detecting T7 phage and possibly for detecting other viruses. Some cellular structures that scatter visible light do not induce ethidium bromide fluorescence enhancement (Le Pecq & Paoletti, 1966), suggesting that virus bands in sucrose gradients may be less readily obscured by other material if the fluorescence enhancement procedure is used rather than light scattering. Ethidium bromide–sucrose gradients may be of use in isolating viruses for which a biological assay has not yet been developed.

Acknowledgments

For technical assistance during these experiments, we thank Mary E. Pichler. We also thank Helena Laboratories, Inc., for the loan of the densitometer used in this study.

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Specific Binding of a Cardiotoxin from *Naja mossambica mossambica* to Charged Phospholipids Detected by Intrinsic Fluorescence†

Jean Dufourcq* and Jean-Francois Faucon

ABSTRACT: The fluorescence of intrinsic tryptophan in cardiotoxin II from *Naja mossambica mossambica* is a sensitive probe of interactions between the toxin and phospholipid vesicles. The formation of the lipid–protein complexes leads to more than a threefold increase in the fluorescence intensity and a blue shift of 10–15 nm. Cardiotoxin II does not bind to neutral or zwitterionic phospholipids, but interacts specifically with negatively charged phospholipids such as phosphatidylserine, phosphatidylinositol, and phosphatidic acid. The association constant of the lipid–protein complex is $>10^6\ M^{-1}$, and its stoichiometry is 7 ± 1 lipid molecules per protein molecule, when only one negative charge is borne by the lipid molecule. Results obtained with lipid mixtures can be explained only by assuming that cardiotoxin induces the formation of clusters or lateral phase separations between negative and neutral phospholipids. The binding is reversible and is mainly due to electrostatic interactions between the basic residues of cardiotoxin and the phosphate and/or carboxylic groups of the phospholipids. The complexes can be dissociated either by an increase in ionic strength or by pH effects. Cations compete with cardiotoxin, with an efficiency that increases in the order $Na^+ = K^+ \ll Mg^{2+} < Ca^{2+} < Mn^{2+}$. These results can account for the known actions of cardiotoxin on cells or biological membranes, and we propose that negatively charged phospholipids are the binding site of cardiotoxin on natural membranes.

Snake venom toxins can be separated according to their pharmacological effects into neuro- and cardiotoxins. Both types of toxins have potent effects on membranes, but the neurotoxins are highly specific for only a few cell targets, whereas cardiotoxins display a general action on many kinds of membranes (Yang, 1974). Cardiotoxins are described as direct lytic factors, due to their ability to break down the plasmic membrane. They are generally nonenzymatic proteins, although several toxins have recently been characterized as phospholipases, the major example being β-bungarotoxin (Strong et al., 1976; Howard & Truog, 1977).

Lipids have been implicated in the binding of cardiotoxin

† From the Centre de Recherche Paul Pascal, Domaine Universitaire, 33405 Talence, France. *Received July 29, 1977.*

0006-2960/78/0417-1170$01.00/0