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References

Alexeev, D. G., Lipanov, A. A., & Skuratovskii, I. Y. (1987) *J. Biomol. Struct. Dyn. 4*, 989–1012.

Arnott, S., Chandrasekaran, R., Birdsall, D. L., Leslie, A. G. W., & Ratliff, R. L. (1980) *Nature (London) 283*, 743–745.

Arnott, S., Chandrasekaran, R., Hall, I. H., Puigjaner, L. C. (1983) *Nucleic Acids Res. 11*, 4141–4152.

Broido, M. S., James, T. L., Jon, G., & Keepers, J. W. (1985) *Eur. J. Biochem. 150*, 117–128.

Coll, M., Frederick, C. A., Wang, A. H.-J., & Rich, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8385–8389.

Gupta, G., Sarma, M. H., & Sarma, R. H. (1985) *J. Mol. Biol. 186*, 463–469.

Gupta, G., Sarma, M. H., Sarma, R. H., Bald, R., Engelke, U., Oei, S. L., Gessner, R., & Erdmann, V. A. (1987) *Biochemistry 26*, 7715–7723.

Hagerman, P. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4632–4636.

Hagerman, P. J. (1986) *Nature (London) 321*, 1149–1150.

Keeper, J. W., & James, T. L. (1984) *J. Magn. Reson. 57*, 404–429.

Kitchin, P., Klein, V. A., Ryan, K. A., Gann, K. L., Rauch, C., Kang, D. S., Wells, R. D., & Englund, P. T. (1986) *J. Biol. Chem. 261*, 11301–11310.

Levene, S. D., & Crothers, D. M. (1983) *J. Biomol. Struct. Dyn. 1*, 429–435.

Marini, J. C., Levene, S. D., Crothers, D. M., & Englund, P. T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7664–7668.

Matteuci, M. D., & Caruthers, M. H. (1981) *J. Am. Chem. Soc. 103*, 3185–3191.

Nelson, H. C. M., Finch, J. T., Luisi, B. F., & Klug, A. (1987) *Nature (London) 330*, 221–226.

Saenger, W. (1984) in *Principles of Nucleic Acid Structures*, Springer-Verlag, New York.

Sarma, M. H., Gupta, G., & Sarma, R. H. (1985) *J. Biomol. Struct. Dyn. 2*, 1057–1084.

Sarma, M. H., Gupta, G., & Sarma, R. H. (1986) *Biochemistry 25*, 3659–3665.

Sarma, M. H., Gupta, G., & Sarma, R. H. (1987a) *J. Biomol. Struct. Dyn. 1*, 1423–1455.

Sarma, M. H., Gupta, G., Sarma, R. H., Bald, R., Engelke, U., Oei, S. L., Gessner, R., & Erdmann, V. A. (1987b) *Biochemistry 26*, 7707–7714.

Sarma, M. H., Gupta, G., & Sarma, R. H. (1988) *Biochemistry 27*, 3423–3432.

States, D. J., Haberkorn, R. H., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.

Trifonov, E. N. (1986) *CRC Crit. Rev. Biochem. 19*, 89–106.

Ulanovsky, L. E., & Trifonov, E. N. (1987) *Nature (London) 326*, 720–722.

Zahn, K., & Blattner, F. R. (1987) *Science (Washington, D.C.) 236*, 416–422.

Fluorescence Measurement of the Kinetics of DNA Injection by Bacteriophage λ into Liposomes†

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ABSTRACT: Bacteriophage λ attaches to Gram-negative bacteria using the outer membrane protein LamB as its receptor. Subsequently, DNA is injected by the bacteriophage into the host cell for replication and expression. The mechanism of DNA injection, however, is poorly understood. In order to begin to characterize DNA injection, a quantitative kinetic assay to detect injection into reconstituted LamB liposomes is described. The technique involves monitoring the increase in fluorescence of liposome-encapsulated ethidium bromide, which occurs as DNA enters the aqueous compartment of the vesicles. The data indicate that injection is several times faster than indicated by earlier studies and is complete within 1 min. Such assays which allow direct observation of this process are necessary first steps toward a mechanistic understanding.

Bacteriophage λ is an important tool in molecular biology and biochemistry due to its ease of laboratory manipulation. Little is known, however, about the molecular mechanisms by which it functions. Bacteriophage λ is a temperate, double-stranded DNA-containing phage which infects Gram-negative bacteria, using the outer membrane maltose porin LamB as its receptor (Szmelcman & Hofnung, 1975). LamB exists in the membrane as an integral trimer of 47-kDa subunits (Schwartz, 1975, 1983). In addition to its role in bacteriophage attachment, LamB is also the channel through which phage DNA passes as it enters the host cell (Roessner & Ihler, 1986).

Some studies have been conducted to investigate the phage–receptor interaction in vitro and in well-characterized model membranes such as phospholipid vesicles. These studies focused on phage λ interactions with LamB extracted from *Escherichia coli* K-12. The complexes are formed reversibly (Schwartz, 1975) and require addition of chloroform to trigger irreversible binding and DNA injection (Schwartz, 1975; Mackay & Bode, 1976a; Zgaga et al., 1973). λ host range mutants (λh) which bind LamB from *E. coli* K-12 irreversibly and inject their DNA in the absence of chloroform have been identified (Randall-Hazelbauer & Schwartz, 1973). In ad-

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dition, the *Shigella* receptor binds wild-type phage λ irreversibly (Schwartz & Le Minor, 1975). *E. coli* pop154 expresses the LamB-encoding region of *Shigella sonnei* 3070 (Roa & Scandella, 1976), and the extracted receptor causes DNA injection by the phage (Schwartz & Le Minor, 1975; Roa & Scandella, 1976; Roessner et al., 1983).

Previous kinetic studies of λ DNA injection in vitro have employed indirect assays for injection (Schwartz, 1975; Roessner & Ihler, 1986; Mackay & Bode, 1976a; Zgaga et al., 1973). These include a plaque inhibition assay, analysis of nuclease sensitivity of free DNA, and separation of labeled solutes leaking from LamB-containing liposomes upon DNA injection. Studies of wild-type λ interactions with LamB from strain K-12 required chloroform addition to trigger DNA injection, a condition which would seem to greatly affect the measurement being made. Kinetic measurements in these systems have indicated that injection takes minutes to hours to occur. The plaque inhibition assay assumes that none of the steps between phage–receptor binding and DNA injection are rate limiting. Phage may also bind irreversibly without subsequent injection. These limitations restrict the conclusions which can be drawn from data collected using indirect assays.

In the present study, we report a direct method for measurement of the kinetics of bacteriophage λ DNA injection into LamB-containing liposomes. Ethidium bromide is entrapped inside the liposomes, and the fluorescence enhancement is monitored continuously as the fluorophore binds to injected DNA (Le Pecq & Paoletti, 1966). Fluorescence spectroscopy combines the advantages of fast data acquisition and high sensitivity and is well-suited to kinetic studies. The injection step is much slower than either phage–receptor attachment or ethidium bromide–DNA binding (Schwartz, 1975; Delbrück, 1940; Bresloff & Crothers, 1975), so that the apparent rate reflects that of DNA injection. Use of this direct assay in the wild-type λ–*Shigella* LamB system shows that DNA injection is complete within 1 min, much faster than has been shown thus far with indirect assays.

Materials and Methods

Reagents. Octyl β-D-glucopyranoside and Triton X-100 were purchased from Aldrich. λ DNA and sodium [^{125}I]iodide were purchased from Amersham. Egg yolk phosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL) as the chloroform solution and was stored at −20 °C. Bio-Beads SM-2 and Enzymobeads radioiodination reagent were obtained from Bio-Rad. 5(6)-Carboxyfluorescein was purchased from Eastman (Rochester, NY). Cholesterol, ethidium bromide, Sephadex G25-50, and Sepharose 4B were all purchased from Sigma. Spectrapor-2 dialysis tubing was obtained from Spectrum Medical Industries (Los Angeles). Cholic acid was obtained from Sigma and was recrystallized from ethanol/water prior to use. *E. coli* strain C600-(λcI857Sam7) was used as the source of bacteriophage; strain pop154 was used for production of LamB; strain Ymel was used as the lysable indicator for plaque assays.

Phage Preparation. The procedure for phage growth and purification is essentially the same as described previously (Roessner et al., 1983; Maniatis et al., 1982) with minor modifications in the phage precipitation step (Kaslow, 1986; Latchman & Brickell, 1986). A 2-L culture of *E. coli* C600(λcI857Sam7) was grown to approximately 5×10^8 cells/mL, and phage growth was induced by heating at 43 °C for 20 min, with an additional 3 h of growth at 37 °C. The cells were centrifuged at 5000 rpm for 5 min and were resuspended in 20 mL of λ dil buffer [10 mM tris(hydroxymethyl)aminomethane (Tris)[1] (pH 7.5)/2 mM $MgSO_4$]. Cells were lysed by adding 2 mL of $CHCl_3$. The mixture was vortexed and allowed to sand for 30 min at room temperature. To reduce the viscosity due to cellular nucleic acids, 10 mg of crystalline pancreatic DNase I and 30 mg of crystalline RNase A were added, and the mixture was allowed to stand 10 min at room temperature. After centrifugation at 10000 rpm for 10 min to remove cell debris, the phage-containing supernatant was decanted. Solid NaCl and PEG6000 were added to 1 M and 10%, respectively, to precipitate phage. The solutes were dissolved by slow stirring on a magnetic stirrer. The solution was cooled in ice water and allowed to stand for at least 1 h on ice. Phage were pelleted by centrifuging at 10000 rpm for 10 min at 4 °C, resuspended in 0.7 g/mL CsCl, and allowed to stand overnight at 4 °C. The solution was centrifuged at 30000 rpm for 16 h at 4 °C, and the phage band was removed in 1–1.5 mL and stored at 4 °C. λ ghosts were prepared according to the method of Roessner and Ihler (1986). Phage particles were dialyzed against 10 mM Tris (pH 7.5)/5 mM EDTA and then heated to 50 °C for 30 min. $MgSO_4$ was added to 10 mM, and the mixture was incubated at 37 °C for 30 min with 10 mg/mL DNase I, followed by dialysis against 10 mM Tris (pH 7.5)/10 mM $MgSO_4$. Phage particles were titered with the plaque assay (Randall-Hazelbauer & Schwartz, 1973), plating on *E. coli* Ymel. Typically, a titer of 1.4×10^{12} pfu/mL (±50%) was obtained from a starting 2-L culture of the C600 lysogen.

LamB Preparation. A 1-L culture of *E. coli* pop154 was grown in tryptone broth containing 0.4% maltose to OD_{600nm} = 1.0. Cells were pelleted by centrifugation at 5000 rpm for 10 min and resuspended in 60 mL of 10 mM EDTA/2% sodium cholate/10 mM Tris (pH 7.5). The solution was shaken for 30 min at 37 °C and then probe sonicated for 3 min in ice with a Heat Systems–Ultrasonics W-225R sonicator operated at 50% duty cycle and an output setting of 5. Insoluble material was removed by centrifugation at 10000 rpm for 10 min. The supernatant was further clarified by centrifugation at 50000 rpm for 1 h. A 10-fold volume of butanol was added and the mixture allowed to stand for 30 min in ice. The precipitate was collected by centrifugation at 10000 rpm for 10 min. Residual butanol was removed by lyophilization overnight. The powder was solubilized in 1 M NaCl/2% sodium cholate/10 mM EDTA/10 mM Tris (pH 7.5) by probe sonicating for 30 s. After being cooled to room temperature, insoluble material was pelleted by centrifuging at 10500 rpm for 5 min. The supernatant, containing LamB, was lyophilized and stored at 4 °C. Receptor activity was determined by using the plaque inhibition assay (Roessner et al., 1983).

Liposome Preparation. Egg PC (10 mg) and 4.9 mg of cholesterol in chloroform solution were dried under a stream of nitrogen, and residual chloroform was removed by vacuum. The dry lipid film was hydrated in 1 mL of PBS (pH 7.4) and vortexed to solubilize the lipid. The aqueous dispersion was sonicated to clear opalescence in a Laboratory Supplies (Hicksville, NY) bath sonicator. Octyl glucoside was then added in a 10:1 detergent to lipid molar ratio to form mixed micelles and the mixture clarified immediately. LamB (40 μg) in 10 mM Tris (pH 7.5)/2% sodium cholate was added, and the mixture was dialyzed for 24 h in Spectrapor-2 dialysis tubing against a 100-fold volume of PBS (pH 7.4) containing

[1] Abbreviations: Tris, tris(hydroxyethyl)aminomethane; PEG6000, poly(ethylene glycol), molecular weight 6000; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; NTA, nitrilotriacetic acid; ACAC, acetylacetone; CF, 5(6)-carboxyfluorescein; PAC, perturbed angular correlation spectroscopy; ATP, adenosine 5′-triphosphate.

1 mM ethidium bromide, to which prewashed Bio-Beads had been added (Holloway, 1973; Mimms et al., 1981; Philippot et al., 1983), for 24 h. The Bio-Bead:detergent ratio used was 1 mg of beads/0.1 μmol of octyl glucoside. Unincorporated solute was removed by gel filtration on a Sephadex G25-50 spin column. Lipid concentration was determined with the Böttcher modification of the Barlett assay (Böttcher et al., 1961). Protein concentration was determined with the Peterson modification of the Lowry assay (Peterson, 1977).

Efficiency of LamB Reconstitution. A solution containing approximately 75 ng of LamB was radioiodinated using Enzymobead reagent (Bio-Rad) and 20 μL of 100 mCi/mL sodium [^{125}I]iodide solution, yielding 2.8 Ci/μmol labeled protein. Reconstitution of LamB was conducted as described above. Reconstitution efficiency was determined by gel filtration of the dialysate on Sepharose 4B followed by counting of eluted fractions in a Beckman Biogamma II counter. Column packing was also dried, sliced into 1-cm increments, and counted to check for sticking of unincorporated protein to the column.

Carboxyfluorescein Release Studies. The relief of self-quenching of the fluorophore carboxyfluorescein is a common method of measurement of liposome integrity and has been reviewed by Weinstein et al. (1984). Vesicles with and without LamB were prepared as described previously by dialysis against PBS (pH 7.4) containing 100 mM carboxyfluorescein and Bio-Beads SM-2. Carboxyfluorescein was purified prior to use by the method of Weinstein et al. (1984). Leakage was monitored as a function of time and phage binding, with excitation and emission wavelengths of 492 and 520 nm, respectively. Triton X-100 was added to 1% as a control to rupture the vesicles. Fluorescence measurements were made with an SLM 4800 spectrofluorometer.

Perturbed Angular Correlation Measurements of Solute Leakage. The perturbed angular correlation (PAC) technique allows quantitation of liposomal integrity by monitoring the tumbling rate of $^{111}In^{3+}$ (Hwang & Mauk, 1977), which is entrapped in the liposomes with the chelator nitrilotriacetic acid (NTA). Measurements of the angular correlations of the two γ rays emitted in a cascade from the nucleus allow calculation of rotational correlation times [for a review, see Hwang (1984)]. Inside intact vesicles, $^{111}In^{3+}$ tumbles rapidly in the chelated complex. When vesicles leak or rupture, $^{111}In^{3+}$ binds to large serum proteins and has a slow tumbling rate. According to this technique, the effect of incorporation of LamB into liposomes on solute leakage was assayed in 50% serum.

The NTA was encapsulated by performing the LamB reconstitution as desribed earlier, in buffer containing 1 mM NTA. Unencapsulated NTA was removed over a Sephadex G25-50 spin column. The $^{111}In^{3+}$ was loaded into liposomes using the ionophore acetylacetone (ACAC) (Beaumier & Hwang, 1982). $^{111}InCl_3$ was evaporated to dryness under a heat lamp and redissolved in a minimum volume of 3 mM HCl. The solution was diluted 10-fold in 30 mM ACAC/10 mM Tris (pH 7.4). This loading buffer was then added to a 5-fold volume of liposomes, in three aliquots while vortexing. After incubation at room temperature for 1 h, unencapsulated $^{111}In(ACAC)_3$ was separated from liposomes on a Sephadex G25-50 spin column. Liposome stability was measured as a function of time in 50% fetal calf serum.

Fluorescent Measurement of DNA Injection. Ethidium bromide was encapsulated in liposomes by dialyzing against Bio-Beads SM-2 in buffer containing 1 mM ethidium bromide. Unencapsulated solute was removed on a Sephadex G25-50 spin column. Fluorescence was measured in an SLM 4800 spectrofluorometer with a Schott Glass Technologies 450-nm high-pass cutoff filter in the emission channel (Model GG-495). Excitation and emission wavelengths used were 295 and 598 nm, respectively. Zero fluorescence was taken as the background fluorescence due to ethidium bromide in the liposomes. Bacteriophage was added to the sample in a quartz cuvette in a thermostated 37 °C sample chamber equipped with a magnetic stirrer, and fluorescence was monitored continuously as a function of time.

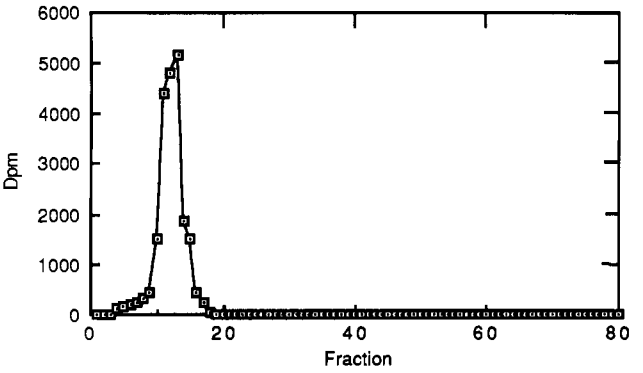

FIGURE 1: Gel filtration chromatographic profile of ^{125}I-LamB liposomes. Radioiodinated LamB was reconstituted into vesicles composed of egg PC/cholesterol, as described under Materials and Methods. Liposomes and free protein were separated on Sepharose 4B equilibrated in PBS, pH 7.4. The same buffer was used as eluant. Fractions were counted in a Beckman Biogamma II counter. The column packing was sliced into 1-cm increments and counted after elution.

RESULTS

Vesicle Size. Vesicles with and without LamB were sized by negative-stain electron microscopy on a Philips EM201 electron microscope operated at 80 kV, using 1% phosphotungstic acid. Mean vesicle diameters were 159 ± 44 nm without LamB and 118 ± 52 nm with LamB, from populations of 300 vesicles each.

Efficiency of LamB Reconstitution. Radioiodinated LamB was reconstituted as described under Materials and Methods. The dialysate was subjected to gel filtration chromatography on Sepharose 4B and was eluted with PBS (pH 7.4). The elution profile is shown in Figure 1. All of the labeled protein was associated with the liposomal fraction eluting at the void volume. Free protein, which normally elutes at the included volume (not shown), was not present, indicating essentially quantitative reconstitution efficiency. This finding is consistent with that of Roessner et al. (1983), who observed 98% retention of phage-inactivating capacity in reconstituted LamB liposomes using the plaque inactivation assay for LamB receptor activity.

Solute Leakage from Reconstituted LamB Liposomes. It was necessary to determine the effect of LamB on membrane permeability to solutes, since the fluorescence asssay depends on solute retention in the liposomes. Liposome stability in both buffer and serum was measured by using entrapped carboxyfluorescein and $[^{111}In(NTA)_2]^{3-}$. In PBS at 37 °C, no leakage of CF was observed either before or after addition of bacteriophage (Figure 2). After 1000 s, Triton X-100 was added to rupture the vesicles as a control. This result is consistent with the finding that no ion leakage accompanies DNA injection by bacteriophage T5, a phage also having a noncontractile tail (Filali Maltouf & Labedan, 1983). The lack of solute leakage upon phage binding is not consistent, however, with observation of ATP release by Roessner et al. (1983) or the conductance measurements of Benz et al. (1986), both

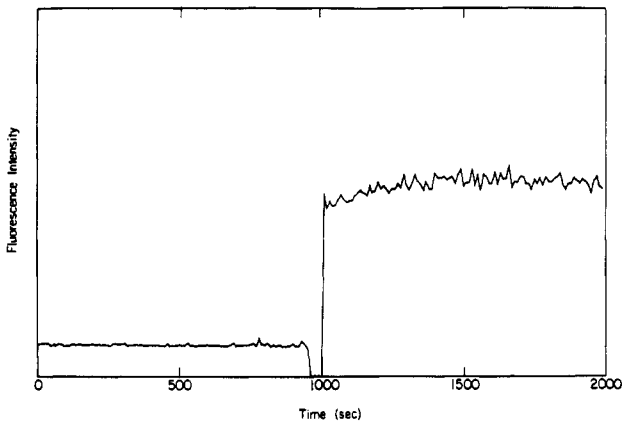

FIGURE 2: Carboxyfluorescein assay of solute retention by LamB-containing liposomes. Carboxyfluorescein (100 mM) was entrapped by dialysis (see Materials and Methods) in buffer containing the same concentration of solute. After removal of unentrapped solute on Sephadex G-25, NaCl was added to the liposomes, so that the buffer and liposome contents would be isotonic. Carboxyfluorescein release was monitored continuously at 37 °C. Excitation and emission wavelengths used were 492 and 520 nm, respectively. Bacteriophage (12 pM) was added at 100 s. Triton X-100 was added to 1% after 1000 s as a control to rupture the liposomes. All measurements were made on an SLM 4800 spectrofluorometer.

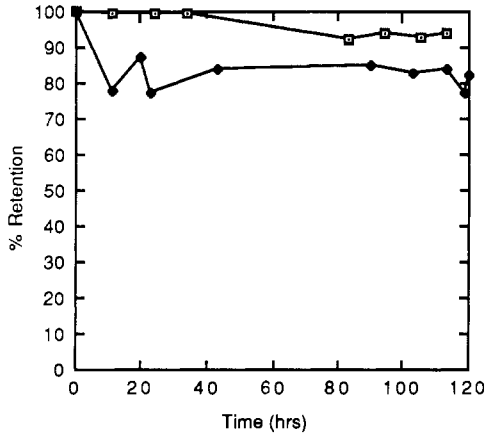

FIGURE 3: Perturbed angular correlation assay of serum stability of LamB-containing liposomes. NTA (1 mM) was entrapped in liposomes by dialysis in buffer containing the same concentration of solute. Unentrapped NTA was removed by gel filtration chromatography on a Sephadex G25-50 spin column, and $^{111}In^{3+}$ was loaded into liposomes using the ionophore acetylacetone (ACAC; see Materials and Methods). Free $^{111}In(ACAC)_3$ was also removed by gel filtration. Liposomal retention of $[^{111}In(NTA)_2]^{3-}$ was monitored by correlation counting in the PAC spectrometer at 37 °C in 50% fetal calf serum. The percentages of solute retention by vesicles composed of only lipid PC (upper curve) and reconstituted LamB vesicles at an average of one receptor per vesicle (lower curve) were determined and plotted as a function of time.

indicating that the DNA pore may remain open after injection.

Solute leakage in 50% serum was assayed by the PAC assay (see Materials and Methods). Figure 3 shows that approximately 95% of the vesicles composed of only lipid and 85% of the reconstituted LamB vesicles remained intact after 120 h. The large initial drop in the stability of LamB-containing vesicles is probably due to the early rupture of vesicles containing many receptors (an average of one receptor per vesicle was used).

Fluorescent Measurement of DNA Injection Kinetics. Kinetics of DNA injection by bacteriophage λ into reconstituted LamB liposomes containing ethidium bromide are shown in Figure 4. The most striking feature of these data is the rapid kinetic behavior. Injection is essentially complete within 1 min. This is several times faster than the kinetics observed

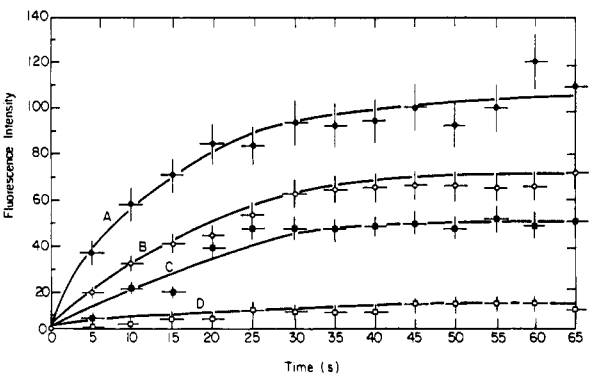

FIGURE 4: Fluorescence measurement of DNA injection kinetics into LamB-containing liposomes. Ethidium bromide (1 mM) was entrapped in liposomes by dialysis in buffer containing the same concentration of solute. Unentrapped fluorophore was removed by gel filtration on Sephadex G25-50. Fluorescence was monitored continuously, with the preinjection fluorescence taken as zero. Excitation and emission wavelenths used were 295 and 598 nm, respectively, and a 450-nm high-pass cutoff filter was used in the emission channel. Phage concentration was (A) 12 pM; (B) 110 pM; (C) 61 pM; and (D) 12 pM. Concentration of receptor (at an average of one receptor per liposome) was 840 nM in curves A, B, and C and 85 nM in curve D. The rate law was calculated by the method of initial rates.

in studies in which indirect methods of detecting DNA injection have been employed (Schwartz, 1975; Mackay & Bode, 1976a; Zgaga et al., 1973). Using the method of initial rates, the second-order rate constant measured for injection was 4.9×10^3 M^{-1} s^{-1} (±50%). The reaction was first order with respect to both phage and receptor, consistent with earlier findings (Schwartz, 1975; Mackay & Bode, 1976a; Delbrück, 1940). The observed rate law was $R_0 = k[\lambda]^{0.84\pm0.20} \times [\text{LamB}]^{1.05\pm0.26}$, where R_0 is the measured initial rate, [λ] and [LamB] are the molar concentrations of phage particles and LamB-containing liposomes, respectively, and k is the measured rate constant. The effect of bacteriophage concentration is shown in curves B and C, where the 1.5-fold higher concentration in curve B results in a corresonding increase in the final level of fluorescence, compared with curve C. Similarly, the 10-fold higher concentration of receptor carrying liposomes in curve A compared with curve D causes an approximately 10-fold increase in the measured extent of injection. The level of fluorescence in curve A corresponds to injection by approximately 20% of the phage in the sample, consistent with the finding that only a fraction of the particles in a given sample inject their DNA (Roa, 1981). The technique showed sensitivity to approximately 800 μg of injected DNA in a sample.

However, in spite of increasing phage concentrations from curve A to curve C to curve B, the extent of fluorescence does not continue to increase linearly, due to increasing amounts of light scattering by the dense phage particles at higher concentrations, causing an inner filter effect. Such behavior is a common interference in optical spectroscopy, in which scattering of both exciting and emitted light within the sample greatly reduces the observed fluorescence (Lakowicz, 1983). Thus, while clearly a useful kinetic technique, the overall extent of injection can be measured by this method over only a limited phage concentration range.

As a control to verify that fluorescence was due to DNA injection into the liposomes and not due to ethidium bromide diffusion into the bacteriophage, phage ghosts were incubated with LamB-carrying liposomes containing ethidium bromide. DNA was added to the external aqueous medium. Ethidium bromide diffuses easily through the phage capsid (Hall & Schellman, 1982). If leakage into the phage was occurring,

the ethidium would have bound the exogenous DNA and fluoresced. No fluorescence was observed, indicating that ethidium bromide does not diffuse out of the liposome upon binding by λ. Upon Triton X-100 addition, the vesicles ruptured, and fluorescence due to binding of the ethidium bromide to exogenous DNA was observed. If phage were added to vesicles at 4 °C, at which temperature injection does not occur (Roessner et al., 1983), no fluorescence occurred on additon of exogenous DNA to the sample after allowing binding to occur for 30 min (S. L. Novick and J. D. Baldeschwieler, unpublished observation). This also supports the notion that phage–receptor binding does not induce solute leakage.

Discussion

A direct assay was used to measure the kinetics of DNA injection by bacteriophage λ into reconstituted LamB liposomes, based on fluorescence of liposome-entrapped ethidium bromide. The data presented show that DNA injection is a rapid one-step process, occurring much faster than had been previously shown (Schwartz, 1975; Mackay & Bode, 1976a; Zgaga et al., 1973). An advantage of this technique is that it allows direct observation of the phenomenon of interest. With the proper choice of phage and bacteria, the need for chloroform addition is obviated (Roessner et al., 1983). Its use in earlier studies (Schwartz, 1975; Mackay & Bode, 1976a; Zgaga et al., 1973) probably was responsible for a great deal of conformational change, perturbing both the system and the measurements.

The high efficiency of LamB reconstitution (Figure 1) is consistent within experimental error with the finding of Roessner et al. (1983), who reported 98% retention of plaque inhibition activity in LamB reconstituted into egg phosphatidylcholine liposomes. The binding of phage may be independent of membrane fluidity, then, since the liposomes in the present study contained equimolar egg PC and cholesterol, in contrast to the egg PC membrane used in the former study.

Solute leakage from the reconstituted vesicles was negligible (Figure 2). No carboxyfluorescein leakage was observed either before or after addition of bacteriophage. The lack of solute leakage upon phage binding is consistent with a similar finding for phage T5 (Filali Maltouf & Labedan, 1983). The difference in leakage results of Roessner and Ihler (1986) and Benz et al. (1986) may be due to the large hydrophobic character of the solutes employed in the present work relative to ATP or ions involved in single-channel conductance used in the earlier studies, or to a different source of small ion leakage in those studies. In the control experiment for ethidium leakage in the present study, binding of phage ghosts did not induce leakage, lending further support to the notion that the transmembrane channel does not remain open after injection, although Roessner and Ihler (1986) did not observe ATP leakage upon ghost binding. If the pore were to remain open, the lack of fluorescence due to ethidium bromide binding to exogenous DNA after phage–receptor binding at 4 °C suggests that it is not open between the binding and injection steps.

Vesicle stability in 50% serum was quite high, with 85–95% of the entrapped ^{111}In complex remaining entrapped after 120 h (Figure 3). Such stability is desirable in applications of a reconstituted DNA injection system to liposomal delivery of DNA to cells, as suggested by Roessner et al. (1984).

The rapid kinetics of DNA injection shown in Figure 4 are much faster than those obtained with indirect methods. The absence of a lag phase before injection is consistent with in vitro observations of λ–wild-type receptor interactions (Mackay & Bode, 1976a; Zgaga et al., 1973) and bacteriophage T5–receptor interactions (Zarybnicky et al., 1973). The reaction was first order with respect to both reactants, also consistent with earlier findings (Schwartz, 1975; Mackay & Bode, 1976a; Delbrück, 1940). The bimolecular rate law seen suggests that injection may be a rate-limiting step in plaque inactivation assays used in earlier kinetic studies, although this type of reaction would still apply to binding even if injection had not been rate limiting. The fact that only about 20% of the phage inject their DNA is consistent with earlier observations that 15–30% of phage inject their entire DNA complement immediately (Roa, 1981), as shown by nuclease sensitivity of the DNA. This result does not indicate that injection into liposomes is substantially less efficient than injection into bacteria. The efficiencies of injection into bacteria have been characterized by plaque inhibition assays, which measure injection efficiency only in the lysable indicator strain. Binding and injection are measured as an aggregate in the target bacteria, as an irreversible binding event inactivates the phage, and the assay is a measure only of inactivation. In one study, in which cellular incorporation of radiolabeled DNA was used to measure in vivo injection efficiency, only 50% of bound phage particles injected their DNA at 37 °C (Mackay & Bode, 1976b). It therefore remains unclear whether injection in vitro is markedly less efficient than the in vivo process.

The observation that DNA injection both by λ (Mackay & Bode, 1976b) and by T5 (Zarybnicky et al., 1973) into Gram-negative bacteria occurs in two steps suggests an additional transport step in vivo, during which DNA injected into the periplasmic space is carried into the cytoplasm. The mechanism by which this occurs is unclear, however (Labedan & Letellier, 1984), and remains the subject of continuing study. Spectroscopic assays based on the conformational dynamics of bacteriophage DNA during injection are also currently under investigation.

The direct fluorescence method described here will allow much greater insight into the process of DNA transfer across membranes in a wide range of systems. The ability to monitor this process directly provides the exciting opportunity to begin to obtain a molecular understanding of DNA injection, in both artificial and biological systems. Such an understanding could allow extensions of the use of bacteriophage in molecular biology and biochemistry.

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References

Beaumier, P. L., & Hwang, K. J. (1982) *J. Nucl. Med. 23*, 810–815.

Benz, R., Schmid, A., Nakae, T., & Vos-Scheperkeuter, G. (1986) *J. Bacteriol. 165*, 978–986.

Böttcher, C. J. F., Van Gent, C. M., & Pries, C. (1961) *Anal. Chim. Acta 24*, 203–204.

Bresloff, J. L., & Crothers, D. M. (1975) *J. Mol. Biol. 95*, 103–123.

Delbrück, M. (1940) *J. Gen. Physiol. 23*, 631–642.

Filali Maltouf, A., & Labedan, B. (1983) *J. Bacteriol. 153*, 124–133.

Hall, S. B., & Schellman, J. A. (1982) *Biopolymers 21*, 2011–2031.

Holloway, P. W. (1973) *Anal. Biochem. 53*, 304–308.

Hwang, K. J. (1984) in *Liposome Technology*, (Gregoriadis, G., Ed.) Vol. 3, pp 247-262, CRC, Boca Raton, FL.
Hwang, K. J., & Mauk, M. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4991-4995.
Kaslow, D. C. (1986) *Nucleic Acids Res. 14*, 6767.
Labedan, B., & Letellier, L. (1984) *J. Bioenerg. Biomembr. 16*, 1-9.
Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp 44-47, Plenum, New York.
Latchman, D. S., & Brickell, P. M. (1986) *Nucleic Acids Res. 14*, 9220.
Le Pecq, J.-B., & Paoletti, C. (1966) *Anal. Biochem. 17*, 100-107.
Mackay, D. J., & Bode, V. C. (1976a) *Virology 72*, 167-181.
Mackay, D. J., & Bode, V. C. (1976b) *Virology 72*, 154-166.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp 76-84, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., & Reynolds, J. (1981) *Biochemistry 20*, 833-840.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346-356.
Philippot, J., Mutaftschiev, S., & Liautard, J. P. (1983) *Biochim. Biophys. Acta 734*, 137-143.
Randall-Hazelbauer, L., & Schwartz, M. (1973) *J. Bacteriol. 116*, 1436-1446.
Roa, M. (1981) *FEMS Microbiol. Lett. 11*, 257-262.
Roa, M., & Scandella, D. (1976) *Virology 72*, 182-194.
Roessner, C. A., & Ihler, G. M. (1986) *J. Biol. Chem. 261*, 386-390.
Roessner, C. A., Struck, D. K., & Ihler, G. M. (1983) *J. Biol. Chem. 258*, 643-648.
Roessner, C. A., Struck, D. K., & Ihler, G. M. (1984) *Bio-Techniques 2*, 140-146.
Schwartz, M. (1975) *J. Mol. Biol. 99*, 185-201.
Schwartz, M. (1983) *Methods Enzymol. 97*, 100-112.
Schwartz, M., & Le Minor, L. (1975) *J. Virol. 15*, 679-685.
Szmelcman, S., & Hofnung, M. (1975) *J. Bacteriol. 124*, 112-118.
Weinstein, J. N., Ralston, E., Leserman, L. D., Klausner, R. D., Dragsten, P., Henkart, P., & Blumenthal, R. (1984) in *Liposome Technology* (Gregoriadis, G., Ed.) Vol. 3, pp 183-204, CRC, Boca Raton, FL.
Zarybnicky, V., Zarybnicka, A., & Frank, H. (1973) *Virology 54*, 318-329.
Zgaga, V., Medic, M., Salaj-Smik, E., & Novak, D. (1973) *J. Mol. Biol. 79*, 697-708.

Interparticle Interactions and Structural Changes of Nucleosome Core Particles in Low-Salt Solution†

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ABSTRACT: The structural behavior of the nucleosome core particles in the range of solvent Na^+ concentration from 10.45 to 0.45 mM has been studied by small-angle neutron and synchrotron radiation X-ray scattering, sedimentation, atomic absorption spectroscopy, density measurements, and circular dichroism. With decreasing salt concentration, the appearance of a scattering peak that is assignable to interparticle interactions, an intraparticle structural transition, a decrease in the sedimentation velocity of the particle, and a release of bound Na^+ ions from the particle are all observed concurrently when the ratio of solvent Na^+ ions per particle is below ~1000. These observations are interpreted to indicate that a release of bound Na^+ ions from the particle brings about structural rearrangements and weakens the electrostatic shielding of the particle, and this introduces long-range repulsive ordering of the particle in low-salt solution. Analyses of the scattering data indicate that the rearrangement within the core particle in low-salt solution is slight, changing the particle's shape slightly from cylindrical to a more spherical form by moving the center of the mass of the DNA somewhat inward with accompanying small decreases in the radii of gyration of both the DNA and the histones.

The basic structural features of the nucleosome have been established. The recent results of X-ray diffraction studies of crystals of nucleosome core particles (Richmond et al., 1984; Überbacher & Bunick, 1985) and histone–DNA cross-linking studies (Bavykin et al., 1985) afford a detailed knowledge of the three-dimensional structure of the core particle. However, the dynamical structural properties of nucleosomes in solution are indeterminate and are a subject of current interest because of their possible relation to different conformations that nucleosome might adopt during transcription and replication.

Many investigators have addressed this problem by examining the response of nucleosome core particles as polyelectrolytes in solution to reduction of ionic strength. The methods that have been applied include hydrodynamics (Gordon et al., 1978, 1979; Burch & Martinson, 1980; Libertini & Small, 1980; Harrington, 1981), fluorescence (Zama et al., 1978;

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