The Binding of Small Cations to Deoxyribonucleic Acid. Nucleotide Specificity*

Jewel T. Shapiro, Beth S. Stannard, and Gary Felsenfeld

ABSTRACT: The relative affinities of a number of small weakly bound cations for deoxyribonucleic acids of varying base composition have been measured. Dialysis equilibrium measurements were carried out by allowing two deoxyribonucleic acids of different base compositions to compete with each other for a strongly bound "test" cation, such as Ca^{2+} or spermine, in the presence of excess concentrations of the weakly bound cation under investigation. The binding properties of the weakly bound cation were deduced from its effect upon the distribution of strongly bound cation. The method does not involve a complicated theoretical analysis of binding data, and the use of direct competition experiments reduces the error in the computation of relative binding affinities. We find that Na^+, Li^+, Cs^+, and K^+, as well as arginine, lysine, and tetralysine, are bound equally tightly to deoxyribonucleic acids of any base composition. Tetramethyl- and tetraethylammonium ions, as well as some other small tetraalkylammonium ions, are bound more tightly to adenine-thymine-rich deoxyribonucleic acids than to guanine-cytosine-rich deoxyribonucleic acids.

In the preceding paper (Shapiro *et al.*, 1969), it was shown that polylysine can react preferentially with DNA richer in A·T pairs, and that the reaction is reversible and cooperative, resulting in almost complete selective precipitation of A·T-rich DNA when it is in competition with DNA of lower A·T content. It was also shown that although polylysine is selective for A·T-rich DNA in the presence of sodium, lithium, or cesium chlorides, the addition of TMA^+ results in reversal of this preference, so that polylysine in 2 M TMA^+ interacts selectively with DNA richer in G·C pairs.

The purpose of the investigation described in this paper was to measure the strength of binding of small cations to DNA as a function of the base composition of the DNA, in the expectation that this would provide some evidence concerning the chemical basis of the selective reaction of polylysine, and the effect of TMA^+ in reversing the selectivity. Earlier studies of the binding of oligolysines of varying size to synthetic polyribonucleotides, and of the effect of a series of cations on the binding, have been carried out by Latt and Sober (1967). We will show that the selective binding of cations to DNA differs considerably from their behavior toward polyribonucleotides.

The experiments and analysis of data have been carried out in such a way as to minimize the dependence of the results of binding experiments upon specific assumptions about electrostatic and statistical corrections or the nature of the binding sites. Dialysis equilibrium techniques have been used; the critical measurements were made by placing DNAs of different base composition on opposite sides of a membrane, and allowing them to compete for cations. In most cases we made use of a relatively tightly bound cation, either Ca^{2+} or the aliphatic tetraamine, spermine (charge +4), that could be shown to bind with equal affinity to DNAs of all base compositions. When salts of weakly bound cations were added in excess, the displacement of the tightly bound Ca^{2+} or spermine to one DNA sample or the other was a measure of the selectivity of the weakly bound cation.

We find that basic amino acids and their derivatives, as well as tetralysine, are bound equally tightly to all DNAs regardless of base composition. On the other hand, tetramethyl- and tetraethylammonium ions are bound more tightly to A·T-rich DNA than to G·C-rich DNA.

Materials and Methods

Dialysis Equilibrium. Dialysis experiments were carried out in Lucite cells; the two chambers, separated by a cellulose acetate membrane, were accessible for addition or withdrawal of solution through holes which were plugged during equilibration by tapered Teflon stoppers. Membranes were cut from standard dialysis tubing (Union Carbide Corp.) that had been boiled in sodium carbonate and washed in distilled water. In a typical competition experiment, DNA samples of different base compositions were introduced into the two chambers using a hypodermic syringe with a Teflon needle. The quantity of DNA added was determined by weighing the cell. Other materials, such as the supporting electrolyte and radioactive Ca^{2+} or spermine, using were added microsyringes (Hamilton Co.) with stainless steel needles. In competition experiments, DNA concentrations were adjusted to be equal within about 2%. Equilibration was carried out either at 4 or 23°, using mechanical rotors. In most cases the equilibration time was 16–24 hr. Attainment of equilibrium was verified for most systems studied either by measuring the distribution of radioactive ions at 24 and 48 hr, and showing that no change had occurred, or by using two cells and approaching equilibrium from both directions by adding radioactive ion to only one of the two sides in each case. At the end of most experiments, the DNA concentration on each side of at least several cells in each series was determined to ascertain that no losses had occurred. Recovery of radioactivity was 90% or better and of DNA was 95% or better.

Radioactivity was measured by removing aliquots from the

* From the Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland 20014. *Received January 15, 1969.*

cells with a hypodermic syringe and weighing them onto a glass fiber filter paper (Whatman GF/C) in a scintillation vial. After oven drying, radioactivity was determined in a liquid scintillation counter using a toluene-based scintillation fluid ("Liquiflor," Nuclear Chicago Corp.). Using an analytical balance and approximately 100-μl aliquots, 1% reproducibility could be obtained.

Materials. DNA was prepared from bacterial sources according to a modification of methods described by Marmur and Doty (1962) and by Berns and Thomas (1965). Cells were generally lysed with sodium dodecyl sulfate and incubated overnight at 37° with Pronase (Calbiochem) at a concentration of 1 mg/ml. The suspension was then extracted with chloroform–isoamyl alcohol, and ethanol was precipitated, redissolved, and treated with pancreatic ribonuclease and β-amylase. A series of extractions with chloroform–isoamyl alcohol and phenol was followed by two ethanol precipitations and an isopropyl alcohol precipitation from 0.3 M NaAc. Occasionally samples of DNA from any of the sources we used were found to contain small amounts of an unknown impurity which bound spermine very tightly both in NaCl and tetramethylammonium chloride. The impurity gave a distinctly nonlinear shape to the graph of $\bar{\nu}$ *vs.* $\bar{\nu}/C$ at low values of $\bar{\nu}$. We have found that treatment with DEAE-cellulose removes this impurity, and our procedure now routinely includes such a step. The DNA, after the above procedures, is dialyzed into 0.001 M NaCl–0.001 M cacodylate buffer (pH 7). About 10% by volume of packed DEAE-cellulose previously equilibrated against the same solvent is mixed thoroughly with the DNA. The DEAE-cellulose is centrifuged off, and the DNA is precipitated with isopropyl alcohol as before, redissolved, and dialyzed into the desired buffer. In general, each sample was analyzed for protein (Lowry *et al.*, 1951), for RNA by measurement of the non-acid-precipitable material present after alkaline hydrolysis (Cohn, 1957), for molecular size by sedimentation velocity, and for the presence of denatured DNA by cesium chloride density gradient centrifugation (Meselson *et al.*, 1957) and hyperchromism (Hirschman and Felsenfeld, 1966). The latter two methods also served to verify the base composition of the DNA. DNA concentrations were determined from the appropriate molar extinctions for DNA from each source (Felsenfeld and Hirschman, 1965). Typical DNA preparations had less than 1% protein, less than 0.5% RNA, sedimentation coefficients, s_{20}, in 1 M NaCl in the range 19–22 S units, and had no detectable denatured DNA.

Tetramethylammonium chloride was prepared from the commercial product (Eastman) by treatment with activated charcoal and recrystallization from ethanol–water. A commercially available Polarographic grade product (Southwestern Chemicals, Austin) was also used without further treatment. The two materials gave identical results. TMA^+ concentration was determined by refractive index measurement calibrated by nitrogen analysis. Tetraethylammonium chloride was either Southwestern Chemicals highly purified grade, or was prepared as above from the Eastman product, as were tetrabutylammonium and phenyltrimethylammonium chlorides.

Lysine hydrochloride, arginine hydrochloride, and histidine were obtained from Eastman Kodak Co. and were used without further purification. *N*-α-Acetyllysine methyl ester hydrochloride was obtained from Cyclo Chemical Co., as mentioned in the preceding paper (Shapiro *et al.*, 1969); its purity was checked by thin-layer chromatography as was that of histidine methyl ester. ^{3}H-Labeled tetralysine was prepared as described in the preceding paper.

Spermine tetrahydrochloride was obtained from Mann Laboratories and the uniformly ^{14}C-labeled compound from New England Nuclear Corp.; $^{45}Ca^{2+}$ was supplied by the same company.

All dialysis experiments, except where noted, were carried out in 0.001 M cacodylate (pH 7) in addition to other salts.

Results and Interpretation

In a typical dialysis competition experiment, one of the chambers of the dialysis cell was filled with *Micrococcus lysodeikticus* (ML) DNA, the other with *Proteas mirabilis* (PM) DNA, to a final concentration of about 10^{-5} M (nucleotides). The base compositions of these DNAs differ by 34% in A·T content. As described in the preceding section, the DNA concentrations were carefully matched, as were the total fluid volumes on each side. The desired "supporting electrolyte," such as NaCl, LiCl, CsCl, KCl, or TMACl, was added to a final concentration between 0.05 and 0.15 M depending upon the experiment, and then ^{14}C-labeled spermine or $^{45}Ca^{2+}$ was added to the desired final concentration, which was always much lower than the DNA concentration. Each set of experiments usually involved a series of cells with a fixed concentration of the supporting electrolyte and varying concentrations of the radioactive ion.

Results of a variety of such experiments are given in Table I. We find that in the presence of NaCl, LiCl, KCl, or CsCl, the concentrations of spermine or Ca^{2+} on both sides of the membrane are the same. As we shall show, a considerable fraction of the spermine or Ca^{2+} ions is bound to the DNA under the conditions chosen. We conclude that in all of these supporting electrolytes, spermine and Ca^{2+} are bound with equal strength to both DNAs involved in the competition, since otherwise the concentrations of spermine or Ca^{2+} would not be equal. In contrast, if the supporting electrolyte is tetramethyl-, tetraethyl-, methyltriethyl-, or phenyltrimethylammonium chloride, there is a marked displacement of spermine or Ca^{2+} to the cell chamber containing the DNA richer in G·C base pairs. Finally, in the presence of tetrabutylammonium chloride, the distribution of radioactive ion is uniform.

In order to interpret these results quantitatively, it is necessary to know the fraction of the total Ca^{2+} or spermine actually bound to DNA in each case. This requires the direct measurement of the binding of Ca^{2+} or spermine to at least one of the DNAs used in the competition experiments. In typical dialysis equilibrium experiments, ML DNA was equilibrated with solvent containing the appropriate concentration of supporting electrolyte, and the distribution of Ca^{2+} or spermine was determined. The data were treated by well-known methods (Tanford, 1961) as shown in Figure 1, where $\bar{\nu}/c$ is plotted against $\bar{\nu}$ for a typical set of binding data. (Definitions of symbols are contained in the caption to Figure 1.) Data were collected only at low values of $\bar{\nu}$. Under such conditions, all of the data to a good approximation fall on a straight line. This line defines two parameters: K, the apparent equilibrium constant, and n, the apparent number of sites possessing this equilibrium constant. The x intercept of the line gives n, while nK is given by the y intercept. We are

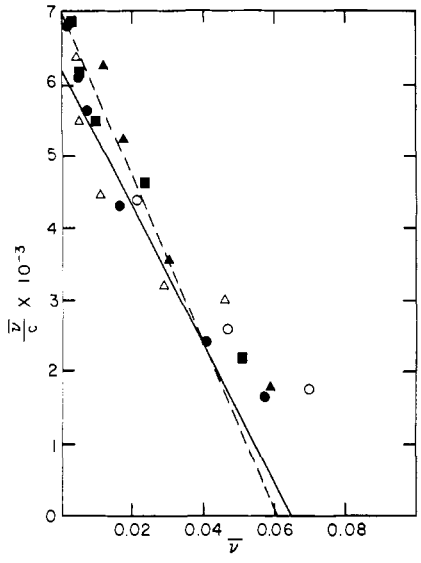

FIGURE 1: The binding of spermine to ML DNA in 0.143 M TMA-Cl. $\bar{\nu}/c$ is plotted as a function of $\bar{\nu}$, where $\bar{\nu}$ is the concentration of bound spermine divided by the total nucleotide concentration, and c is the concentration of free spermine. Open symbols show data from two experiments at 4°; closed symbols are for experiments at 23°. The dashed line is the one used to represent the 23° data at low $\bar{\nu}$; the solid line represents the 4° data.

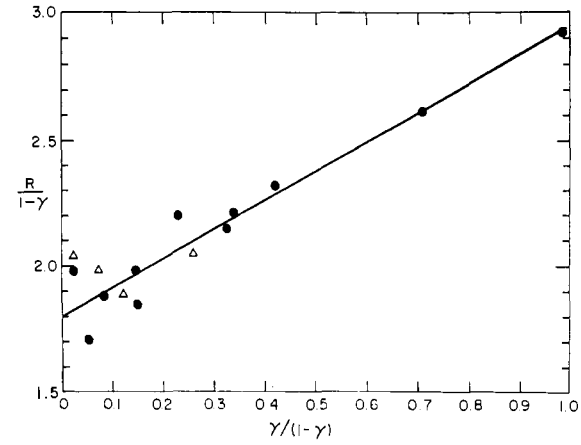

FIGURE 2: $R/1 - \gamma$ *vs.* $\gamma/1 - \gamma$ for the competition of ML and PM DNAs for spermine at 4°. (●) 0.143 M TMA-Cl; (△) 0.167 M TMA-Cl.

aware of, and will discuss below, the difficulties attendant upon a detailed analysis of binding data of this kind. The values of n and K determined by binding are used in this paper simply to compute the fraction of bound Ca^{2+} or spermine, in order to correct the competition data. It is therefore not necessary to invoke any model of binding; n and K may be viewed as empirical parameters that summarize the experimental data. The values of these parameters under a variety of conditions are shown in Table II. There is some uncertainty in determining these values, as can be seen from Figure 1 and from the two sets of results obtained in two separate measurements of the binding of spermine to PM DNA at 4°. Great accuracy is not as important in this case as in most binding experiments, since the values of n and K are required only for correction of the competition data for the presence of unbound ion, and the experiments are designed to reduce the dependence of the final result upon errors in n and K. The correction of the competition data is straightforward. Since the concentration of DNA and the total concentration of Ca^{2+} or spermine are known, the concentration of these ions bound to the DNA on each side of the membrane can be calculated. The number we wish to calculate is R, the ratio of spermine or Ca^{2+} bound to one DNA relative to that bound to the DNA on the opposite side of the membrane (Appendix 1).

It is apparent that the value of R is dependent upon the concentration of spermine or Ca^{2+}. This is because, as sites are saturated with one of these cations, the number of available sites is reduced, but since there are more cations bound to the G·C-rich DNA, and in addition the intrinsic binding parameters of the two DNAs differ, the ratio R changes with increasing $\bar{\nu}$. For this reason, it would be useful to calculate the value of R at infinite dilution of bound cation. The value of this parameter, which we shall call R_0, could be obtained by plotting R values against spermine or Ca^{2+} concentration and extrapolating to zero concentration. We chose instead a method based upon the same kind of semiempirical model used for the binding studies. Since the competing DNAs both have linear binding curves of the kind shown in Figure 1, it is easy to show (Appendix 1) that $R_0 = (R - n_1 \cdot \gamma/n_2)/(1 - \gamma)$, where R is the calculated ratio of radioactive cation bound to DNA_1, compared with that bound to DNA_2, n_1 and n_2 are the corresponding values of n for these DNAs defined by the separate binding curves, and $\gamma = \bar{\nu}_1/n_1$. A plot of $R/(1 - \gamma)$ *vs.* $\gamma/(1 - \gamma)$ therefore intersects the y axis at R_0. Figure 2 shows the result of plotting the competition data in this manner for spermine distributed between ML and PM DNAs in the presence of TMA^+; the value of $R/(1 - \gamma)$ all fall within the range 1.7–2.9, and extrapolate to $R_0 \sim 1.8$. It should be noted that there is no theoretical reason to expect the slopes of the curves corresponding to different TMA^+ concentrations to be identical. We have analyzed the data from all the other competition experiments in a similar way. The results are summarized in Table III, where R_0 is given for various pairs of DNAs competing for spermine or Ca^{2+}. Considering the scatter of the data, R_0 is probably reliable to within ±10%. The values are reasonably self-consistent: The products R_0(ML/SM) × R_0(SM/PM) and R_0(ML/EC) × R_0(EC/PM) have values of 1.85 and 1.81, as compared with the value of 1.80 for R_0(ML/PM). The laws of equilibrium of course require that the two products have the value of R_0(ML/PM). Note that the value of R_0(ML/PM) is considerably greater than the ratio of the values of nK for ML and PM given in Table II. This is probably attributable to errors in the values of nK arising from the uncertainty in extrapolation to $\bar{\nu} = 0$ of graphs such as those in Figure 1. The error has very little effect upon calculation of R_0, as noted above, but demonstrates the unsuitability of separate binding measurements with individual DNAs for the measurement of small differences in binding.

As we will argue in the Discussion, this unequal distribution of Ca^{2+} or spermine between DNAs of different base composition is the result of the intrinsically greater binding strength of TMA^+ to A·T-rich DNA, which results in displacement of spermine or Ca^{2+} to the DNA richer in G·C pairs. In Appendix 2 we present a simple model to explain the binding

TABLE I[a]

Electrolyte (M)	Test Ion (M), °C	DNA Concn (M)	Ratio
	Selective ML/PM		
0.143 TMA-Cl	Spermine (1.51×10^{-5}), 4	6.9×10^{-4}	1.41
0.143 TMA-Cl	Spermine (2.28×10^{-5}), 4	6.9×10^{-4}	1.33
0.143 TMA-Cl	Spermine (2.90×10^{-5}), 4	6.9×10^{-4}	1.28
0.143 TMA-Cl	Spermine (1.53×10^{-5}), 4	9.2×10^{-4}	1.45
0.143 TMA-Cl	Spermine (0.76×10^{-5}), 4	9.2×10^{-4}	1.54
0.143 TMA-Cl	Spermine (0.46×10^{-5}), 4	9.2×10^{-4}	1.55
0.143 TMA-Cl	Spermine (0.15×10^{-5}), 4	9.2×10^{-4}	1.69
0.143 TMA-Cl	Spermine (8.7×10^{-6}), 4	6.6×10^{-4}	1.51
0.143 TMA-Cl	Spermine (2.99×10^{-6}), 4	0.86×10^{-3}	1.46
0.143 TMA-Cl	Spermine (7.48×10^{-6}), 4	0.86×10^{-3}	1.45
0.143 TMA-Cl	Spermine (1.49×10^{-5}), 4	0.86×10^{-3}	1.46
0.143 TMA-Cl	Spermine (3.64×10^{-5}), 4	0.86×10^{-3}	1.34
0.143 TMA-Cl	Spermine (5.02×10^{-5}), 4	0.86×10^{-3}	1.25
0.077 TMA-Cl	Spermine (9.4×10^{-6}), 4	7.1×10^{-4}	1.50
0.167 TMA-Cl	Spermine (2.4×10^{-5}), 23	5.4×10^{-4}	1.38
0.143 TMA-Cl	Spermine (1.40×10^{-5}), 23	7.5×10^{-4}	1.48
0.143 TMA-Cl	Spermine (2.85×10^{-5}), 23	7.5×10^{-4}	1.38
0.143 TMA-Cl	Spermine (4.27×10^{-5}), 23	7.5×10^{-4}	1.23
0.167 TMA-Cl	Spermine (1.63×10^{-5}), 4	9.8×10^{-4}	1.42
0.167 TMA-Cl	Spermine (8.15×10^{-6}), 4	9.8×10^{-4}	1.47
0.167 TMA-Cl	Spermine (4.9×10^{-6}), 4	9.8×10^{-4}	1.57
0.167 TMA-Cl	Spermine (1.6×10^{-6}), 4	9.8×10^{-4}	1.66
0.048 TMA-Cl	Ca^{2+} (3.79×10^{-5}), 23	1.95×10^{-3}	1.19
0.048 TMA-Cl	Ca^{2+} (1.71×10^{-5}), 23	2.38×10^{-3}	1.26
0.048 TMA-Cl	Ca^{2+} (3.43×10^{-5}), 23	2.38×10^{-3}	1.25
0.048 TMA-Cl	Ca^{2+} (6.52×10^{-5}), 23	2.38×10^{-3}	1.24
0.143 TEA-Cl	Spermine (8.7×10^{-6}), 4	6.6×10^{-4}	1.51
0.048 TEA-Cl	Ca^{2+} (3.7×10^{-5}), 23	2.5×10^{-3}	1.30
0.048 TEA-Cl	Ca^{1+} (3×10^{-5}), 23	2.3×10^{-3}	1.22
0.025 MTEA-Cl	Ca^{2+} (3.7×10^{-5}), 23	2.3×10^{-3}	1.30
0.055 PTMA-Cl	Ca^{2+} (3.6×10^{-5}), 23	2.05×10^{-3}	1.16
	ML/SM		
0.143 TMA-Cl	Spermine (2.82×10^{-6}), 4	7.5×10^{-4}	1.44
0.143 TMA-Cl	Spermine (7.48×10^{-6}), 4	7.5×10^{-4}	1.31
0.143 TMA-Cl	Spermine (1.46×10^{-5}), 4	7.5×10^{-4}	1.27
0.143 TMA-Cl	Spermine (3.70×10^{-5}), 4	7.5×10^{-4}	1.15
0.143 TMA-Cl	Spermine (2.94×10^{-5}), 4	6.2×10^{-4}	1.15
0.143 TMA-Cl	Spermine (4.38×10^{-5}), 4	6.2×10^{-4}	1.10
	ML/EC		
0.143 TMA-Cl	Spermine (2.78×10^{-6}), 4	8.7×10^{-4}	1.35
0.143 TMA-Cl	Spermine (1.47×10^{-5}), 4	8.7×10^{-4}	1.35
0.143 TMA-Cl	Spermine (3.70×10^{-5}), 4	8.7×10^{-4}	1.22
0.143 TMA-Cl	Spermine (5.19×10^{-5}), 4	8.7×10^{-4}	1.17
0.143 TMA-Cl	Spermine (1.43×10^{-5}), 23	7.6×10^{-4}	1.38
	SM/PM		
0.143 TMA-Cl	Spermine (2.87×10^{-6}), 4	6.6×10^{-4}	1.18
0.143 TMA-Cl	Spermine (7.61×10^{-6}), 4	6.6×10^{-4}	1.12
0.143 TMA-Cl	Spermine (1.48×10^{-5}), 4	6.6×10^{-4}	1.15
0.143 TMA-Cl	Spermine (3.70×10^{-5}), 4	6.6×10^{-4}	1.10
	EC/PM		
0.143 TMA-Cl	Spermine (1.42×10^{-5}), 23	7.1×10^{-4}	1.09
0.143 TMA-Cl	Spermine (1.50×10^{-5}), 23	7.6×10^{-4}	1.14
0.143 TMA-Cl	Spermine (3.0×10^{-5}), 23	7.6×10^{-4}	1.06
0.143 TMA-Cl	Spermine (4.54×10^{-5}), 23	7.6×10^{-4}	1.10
0.143 TMA-Cl	Spermine (3.3×10^{-6}), 4	1.09×10^{-3}	1.17
0.143 TMA-Cl	Spermine (4.9×10^{-6}), 4	1.09×10^{-3}	1.10
0.143 TMA-Cl	Spermine (6.5×10^{-6}), 4	1.09×10^{-3}	1.14
0.143 TMA-Cl	Spermine (8.2×10^{-6}), 4	1.09×10^{-3}	1.13

TABLE I (*Continued*)

Electrolyte (M)	Test Ion (M), °C	DNA Concn (M)	Ratio
	Nonselective ML/PM		
0.143 NaCl	Spermine (2.24×10^{-5}), 4	6.9×10^{-4}	0.97
0.143 NaCl	Spermine (2.78×10^{-5}), 23	7.5×10^{-4}	1.00
0.143 LiCl	Spermine 1.37×10^{-5}), 23	7.5×10^{-4}	1.01
0.143 LiCl	Spermine (2.77×10^{-5}), 23	7.5×10^{-4}	1.01
0.143 KCl	Spermine (1.44×10^{-5}), 23	7.5×10^{-4}	1.02
0.091 NaCl	Spermine (1.34×10^{-5}), 23	6.2×10^{-4}	0.98
0.091 NaCl	Spermine (2.2×10^{-5}), 23	6.2×10^{-4}	1.02
0.091 CsCl	Spermine (1.45×10^{-5}), 23	6.2×10^{-4}	1.06
0.091 CsCl	Spermine (2.91×10^{-5}), 23	6.2×10^{-4}	1.05
0.048 KCl	Ca^{2+} (1.8×10^{-5}), 23	2.4×10^{-3}	1.04
0.091 NaCl	Spermine (6.7×10^{-5}), 4	1.68×10^{-3}	0.96
0.091 LiCl	Spermine (6.7×10^{-5}), 4	1.68×10^{-3}	0.96
0.091 CsCl	Spermine (6.7×10^{-5}), 4	1.68×10^{-3}	1.02
0.091 KCl	Spermine (6.7×10^{-5}), 4	1.68×10^{-3}	1.00
0.031 CsCl	Ca^{2+} (3×10^{-5}), 23	2.3×10^{-3}	1.03
0.143 TBA-Cl	Spermine (3.0×10^{-6}), 4	7.0×10^{-4}	0.97
0.143 TBA-Cl	Spermine (6.1×10^{-6}), 4	7.0×10^{-4}	1.01
0.046 TBA-Cl	Ca^{2+} (3.0×10^{-5}), 23	2.3×10^{-3}	1.05
0.031 TBA-Cl	Ca^{2+} (3.0×10^{-5}), 23	2.3×10^{-3}	1.07
	SM/PM		
0.143 NaCl	Spermine (8.55×10^{-5}), 4	6.6×10^{-4}	0.98
	ML/EC		
0.143 NaCl	Spermine (2.74×10^{-5}), 23	7.6×10^{-4}	0.99
	EC/PM		
0.143 NaCl	Spermine (4.0×10^{-5}), 23	7.6×10^{-4}	1.00

[a] ML, *Micrococcus lysodeikticus;* PM, *Proteus mirabilis;* SM, *Serratia marcescens;* EC, *Escherichia coli;* TMA-Cl, trimethylammonium chloride; TEA-Cl, triethylammonium chloride; MTEA-Cl, methyltriethylammonium chloride; PTMA-Cl, phenyltrimethylammonium chloride; TBA-Cl, tetrabutylammonium chloride. The notation ML/PM means that DNAs from these two sources were used in the competition. The base compositions (per cent A·T) of the DNAs used are: ML = 28, PM = 62, SM = 42, EC = 50; 0.001 M sodium cacodylate–cacodylic acid (pH 7) present in all solutions.

TABLE II: Apparent Binding Constants of Spermine and Ca^{2+} to DNA.

DNA	Electrolyte (M)	Ion, °C	*nK*	*n*
ML	TMA-Cl (0.143)	Spermine, 23	7,000	0.081
ML	TMA-Cl (0.143)	Spermine, 4	6,200	0.065
PM	TMA-Cl (0.143)	Spermine, 4	4,750	0.070
PM	TMA-Cl (0.143)	Spermine, 4	5,800	0.082
ML	TMA-Cl (0.0475)	Ca^{2+}, 23	1,800	0.12
ML	TEA-Cl (0.0475)	Ca^{2+}, 23	3,100	0.14
ML	TBA-Cl (0.0475)	Ca^{2+}, 23	3,100	0.14
ML	CsCl (0.15)	Spermine, 23	1,000	0.11
ML	LiCl (0.091)	Spermine, 4	>3,500	
ML	NaCl (0.091)	Spermine, 4	>3,500	
ML	KCl (0.091)	Spermine, 4	>3,500	
PM	CsCl (0.047)	Spermine, 4	>15,000	
ML	LiCl (0.047)	Spermine, 4	>20,000	
ML	NaCl (0.047)	Spermine, 4	>20,000	
ML	KCl (0.047)	Spermine, 4	>20,000	
PM, SM[a]	NaCl (0.15)	Spermine, 4	780	0.057

[a] Taken from Hirschman *et al.* (1967).

TABLE III

DNA Pair[a]	R_0 (Obsd)[b]	R_0 (Calcd)
ML/PM (4°)	1.80 ± 0.04	1.76
ML/PM (23°)	2.00 ± 0.20	
ML/PM$_{Ca^{2+}}$ (23°)	1.32 ± 0.11	1.32
ML/EC (4°)	1.51 ± 0.04	1.43
EC/PM (4°)	1.20 ± 0.07	1.23
EC/PM (23°)	1.14 ± 0.09	
ML/SM (4°)	1.49 ± 0.01	1.25
SM/PM (4°)	1.24 ± 0.03	1.41

[a] Except where indicated, the bound ion is spermine. [b] The error shown is the standard deviation of the extrapolated value of R_0 obtained using a plot such as that shown in Figure 2. It does not include error arising from inaccuracy in the values of the binding parameters, nK and $\bar{\nu}$.

data, based upon the supposition that binding of spermine or Ca^{2+} requires displacement of TMA^+, that it requires more energy to displace TMA^+ from the neighborhood of an A·T pair than from a G·C pair, and that the amount of energy is independent of whether or not TMA^+ has been displaced from a neighboring pair. The comparison between predictions of R_0 based upon this model and the observed values are given in Table III. The agreement is reasonably good for competition experiments involving ML, PM, and EC DNAs. Considering that only a single parameter, α, describes both Ca^{2+} and spermine binding, it is interesting that the results for Ca^{2+} binding to the ML/PM pair are consistent with the results for spermine binding to the same pair, *i.e.*, that the value of R_0 for Ca^{2+} is approximately the square root of the value of R_0 found for spermine (see Appendix 2). However, predictions for competition between ML and SM are in serious disagreement with experiment; the affinity of SM DNA for spermine is not as great as the simple model requires for a DNA of this base composition and random sequence. It is not surprising that the simple model is not satisfactory in detail, but use of a more complicated one, with more adjustable parameters, does not seem justified by the data available. (Note that a change of the A·T content of SM DNA from 0.42 to 0.48 would be sufficient to bring the calculated values of R_0 for ML/SM and SM/PM into agreement with experiment.) The results strongly suggest that the binding data are consistent with some model in which TMA^+ is preferentially bound to A·T pairs.

We have studied the competitive binding of spermine and calcium by two DNAs, usually ML DNA and PM DNA, in supporting electrolytes of the amino acids lysine, arginine, and histidine. The amino acid concentrations shown in Table IV are those in which moderately strong binding of spermine to DNA occurs as determined by separate binding experiments. The pH of histidine solutions was adjusted to 6.0, so that about half of the amino acid molecules carried a net charge. In all other solutions, the pH was about 7.0. Table IV shows that in all these electrolyte solutions no redistribution of radioactive cation occurs. In accordance with our interpretation, this implies that there is no selective binding of the amino acids themselves.

Competitive binding studies were also done in solutions of the amino acid derivatives, *N*-α-acetyllysine methyl ester hydrochloride, and histidine methyl ester to eliminate possible interference of charged α-amino and carboxyl groups in amino acid selectivity. At the concentrations of these derivatives that gave moderately strong binding of spermine, there was again no redistribution of the radioactive cation, as shown in Table IV. This appears to confirm that the monomer units are not able to bind to DNA selectively.

Since tetralysine binds tightly to DNA, it was possible to study its binding directly, rather than by its effect upon spermine binding. [^{3}H]Tetralysine was used in direct competitive binding studies between ML DNA and PM DNA. In this case, supporting electrolyte was NaCl at concentrations sufficiently low to assure that a large fraction of the radioactive tetralysine was bound. There does not appear to be any measurable preference of tetralysine for A·T-rich DNA (Table IV).

Discussion

The principal method employed in this investigation, dialysis competition, is a particularly sensitive technique for measuring small differences in binding affinity. Since the polyelectrolyte concentrations on opposite sides of the membrane are equal, any effects of the Donnan equilibrium are to a large extent cancelled. The method is not subject to the relatively large uncertainties connected with direct measurement of the binding constants of individual DNA samples by dialysis equilibrium. Although a direct measurement must still be made in order to correct the competition results for unbound ion, the effect of error in the direct measurement upon the final result is much reduced when the competition experiments are carried out under conditions where a large fraction of the total ion is bound.

The sensitivity of our measurements has also been increased by the use of tightly bound ions, Ca^{2+} and spermine, as "indicators" of the affinity of the more weakly bound univalent cations, such as Na^+ and TMA^+. The technique is a great deal more sensitive than that of direct measurement of univalent ion binding. A similar method was employed by Latt and Sober (1967) in their study of oligolysine–polyribonucleotide interactions; they used the oligolysine as the tightly bound ion, and interpreted the change in the oligolysine affinity with change in supporting electrolyte in terms of the binding of the supporting electrolyte. Our extension of their method has the advantage that the "indicator" ions, Ca^{2+} and spermine, do not appear to exhibit any variation in affinity for DNAs of varying base composition. As we have pointed out above, the analysis of data is simplified considerably by extrapolation of all results to zero concentration of bound ion. We do not attempt to account for the shape of the entire binding curve, but only compare the relative free energies of binding of the first spermine or Ca^{2+} with DNAs of varying base composition. It is therefore unnecessary to take into account electrostatic or statistical corrections which may be difficult to evaluate.

The disadvantage of the method is that it actually measures the *ratio* of relative affinities of the indicator ion and the univalent cation for DNA. The fact that spermine in the presence of NaCl is bound with equal strength to two DNAs of different base composition means that the relative affinity

TABLE IV

Electrolyte (M)	Test Ion (M)	DNA (M)	Ratio ML/PM
Lysine (0.15)	Spermine (7.2×10^{-5})	1.079×10^{-3}	1.02
Lysine (0.15)	Spermine (4.3×10^{-5})	1.079×10^{-3}	0.98
Lysine (0.15)	Spermine (1.4×10^{-5})	1.079×10^{-3}	0.98
Lysine (0.04)	Spermine (4.3×10^{-5})	1.69×10^{-3}	1.03
Lysine (0.04)	Spermine (2.7×10^{-5})	1.69×10^{-3}	0.98
Lysine (0.03)	Spermine (4.3×10^{-5})	1.69×10^{-3}	1.02
Lysine (0.03)	Spermine (2.7×10^{-5})	1.69×10^{-3}	1.04
Lysine (0.02)	Spermine (4.3×10^{-5})	1.69×10^{-3}	0.99
Lysine (0.02)	Spermine (2.7×10^{-5})	1.69×10^{-3}	0.99
NALM[a] (0.15)	Spermine (7.6×10^{-5})	1.13×10^{-3}	1.01
NALM (0.15)	Spermine (4.6×10^{-5})	1.13×10^{-3}	1.01
NALM (0.15)	Spermine (5.8×10^{-5})	1.05×10^{-3}	0.99
NALM (0.15)	Spermine (2.7×10^{-5})	1.05×10^{-3}	1.01
NALM (0.15)	Spermine (5.8×10^{-5})	1.05×10^{-3}	1.00
NALM (0.15)	Spermine (5.8×10^{-5})	1.05×10^{-3}	0.96
NALM (0.153)	Spermine (7.7×10^{-5})	6.5×10^{-4}	1.03
NALM (0.153)	Spermine (4.6×10^{-5})	6.5×10^{-4}	1.04
NALM (0.153)	Spermine (1.5×10^{-5})	6.5×10^{-4}	1.02
NALM (0.153)	Spermine (7.7×10^{-6})	6.5×10^{-4}	1.05
NALM (0.153)	Spermine (4.6×10^{-6})	6.5×10^{-4}	1.07
NALM (0.153)	Spermine (1.5×10^{-6})	6.5×10^{-4}	1.05
NALM (0.10)	Spermine (7.6×10^{-5})	1.13×10^{-3}	1.03
NALM (0.10)	Spermine (4.6×10^{-5})	1.13×10^{-3}	1.03
NALM (0.05)	Spermine (7.2×10^{-5})	1.82×10^{-3}	1.08
NALM (0.05)	Spermine (1.44×10^{-4})	1.82×10^{-3}	1.03
Histidine (0.10)	Spermine (2.7×10^{-5})	1.107×10^{-3}	0.93
Histidine (0.10)	Spermine (5.8×10^{-5})	1.107×10^{-3}	0.96
Histidine (0.10)	Ca^{2+} (4.7×10^{-5})	1.107×10^{-3}	0.95
Histidine (0.10)	Ca^{2+} (2.4×10^{-5})	1.107×10^{-3}	0.91
Histidine (0.05)	Ca^{2+} (4.7×10^{-4})	1.46×10^{-3}	0.95
Histidine (0.05)	Ca^{2+} (2.4×10^{-4})	1.46×10^{-3}	0.98
Histidine methyl ester (0.04)	Spermine (5.8×10^{-5})	1.46×10^{-3}	1.01
Histidine methyl ester (0.04)	Spermine (2.7×10^{-5})	1.46×10^{-3}	0.98
Histidine methyl ester (0.03)	Spermine (5.8×10^{-5})	1.46×10^{-3}	1.01
Histidine methyl ester (0.03)	Spermine (2.7×10^{-4})	1.46×10^{-3}	1.01
Histidine methyl ester (0.02)	Spermine (5.8×10^{-5})	1.46×10^{-3}	1.02
Histidine methyl ester (0.02)	Spermine (2.7×10^{-5})	1.46×10^{-3}	0.98
Arginine (0.088)	Spermine (3.4×10^{-5})	6.0×10^{-4}	1.00
Arginine (0.139)	Spermine (3.2×10^{-5})	6.0×10^{-4}	0.99
	[^{3}H]Tetralysine		
NaCl (0.14)	Tetralysine (1.176×10^{-3})	1.928×10^{-3}	1.00
NaCl (0.14)	Tetraylsine (1.176×10^{-3})	1.928×10^{-3}	1.01
NaCl (0.14)	Tetralysine (1.176×10^{-3})	1.928×10^{-3}	1.03
NaCl (0.12)	Tetralysine (1.176×10^{-3})	1.928×10^{-3}	1.01
NaCl (0.12)	Tetralysine (1.176×10^{-3})	1.928×10^{-3}	1.02
NaCl (0.10)	Tetralysine (1.176×10^{-3})	1.928×10^{-3}	1.06
NaCl (0.10)	Tetralysine (1.176×10^{-3})	1.928×10^{-3}	1.01
NaCl (0.10)	Tetralysine (2.94×10^{-4})	1.079×10^{-3}	1.06
NaCl (0.10)	Tetralysine (5.5×10^{-4})	1.079×10^{-3}	1.04
NaCl (0.05)	Tetralysine (6.0×10^{-4})	1.079×10^{-3}	1.07
NaCl (0.05)	Tetralysine (3.0×10^{-4})	1.079×10^{-3}	1.00

[a] NALM = *N*-α-acetyllysine methyl ester.

of spermine for the two DNAs is the same as the relative affinity of Na^+ for these DNAs. This would be true if, for example, both spermine and Na^+ were bound x times as tightly (where x is any number) to the A·T-rich DNA as to the G·C-rich DNA. It is for this reason that we have carried out measurements in LiCl, KCl, and CsCl, as well as in NaCl. If spermine had an intrinsic affinity (let us say) for A·T-rich DNA which was exactly balanced out by the affinity of Na^+ for A·T-rich DNA, it is exceedingly unlikely that Li^+, K^+, and Cs^+ would also have precisely the same affinity; if they did not have the same affinity, spermine would be distributed unequally on opposite sides of the membrane in the presence of these cations. The results given in Table I show that there is essentially no preferential binding of spermine or Ca^{2+} in the presence of Na^+, Li^+, K^+, or Cs^+. One of the measured ratios for ML/PM competition in CsCl is 1.06, and another 1.05. These are slightly too small to be considered a significant deviation from one, given our experimental error. However, if we take these values as correct, we can calculate an upper limit to R_0(ML/PM) in CsCl of about 1.18, which is much smaller than the value of R_0 in TMA^+, and must be considered as indicative of the maximum possible error. We conclude that in fact all of these ions (spermine, Li^+, Na^+, K^+, and Cs^+) have no preferential affinity, *i.e.*, within our limits of error the value of x discussed above is one. This conclusion is further reinforced by similar results obtained with Ca^{2+} as the indicator ion. Thus, any unequal distribution of the indicator ion is entirely the result of an intrinsic preferential binding of the weakly bound univalent cation.

On the basis of this argument, we conclude that the results given in Table I show that TMA^+ is preferentially bound to A·T-rich DNA, and displaces the indicator ions to the G·C-rich DNA for that reason. Similarly, tetraethyl- and phenyltrimethylammonium chlorides are also bound more tightly to A·T-rich DNA, while tetrabutylammonium ion is not.

Some of these results differ from those for the synthetic helical polyribonucleotides. Latt and Sober (1967) found that oligolysine was bound more tightly to poly rI:rC than to poly rA:rU, and concluded that this was largely the result of an intrinsic preference of Na^+ for poly rA:rU. They found that the affinity of oligolysine for poly rI:rC was enhanced in the presence of TMA^+. It is not possible to make direct comparisons between these results and the results reported here; the two-strand polyribonucleotides differ considerably in secondary structure and charge density from DNA. Poly rA:rU and poly rI:rC probably also differ from each other in structure and charge density (Davies, 1967) so that some of the preferential interactions of these polyribonucleotides may be peculiar to them alone.

The binding of TMA^+ to DNA is weak, and the free energy of transfer from a site on G·C-rich DNA to one on A·T-rich DNA is not large. The simple model described in Appendix 2 is not correct in detail, since among other things binding of one spermine molecule to DNA probably displaces fewer than four TMA^+ ions. The order of magnitude of the effect is probably estimated correctly, however, and we conclude that the transfer of TMA^+ from G·C to A·T involves a free-energy change of the order of magnitude of 200 cal/mole.

What is the origin of the weak forces that cause TMA^+ to be more tightly bound to A·T-rich DNA? Strauss *et al.* (1967) have compared the intrinsic binding constants of several univalent cations for DNA and polyphosphate. They conclude that whereas Li^+, Na^+, K^+, and Cs^+ have about equal affinities for the two kinds of polyion, TMA^+ is bound more tightly to DNA than to polyphosphate. They suggest that hydrophobic interactions between TMA^+ and DNA are responsible for this increased affinity. The fact that tetraethyl and phenyltrimethylammonium ions behave like TMA^+, while tetrabutylammonium ion does not, suggests that a steric factor is involved: tetrabutylammonium ion is too large to fit into the large groove of the double helix; the others are not.

In the preceding paper (Shapiro *et al.*, 1969) we have discussed various models that could account for the selectivity of polylysine for A·T-rich DNA. Similar arguments can be applied to the binding of TMA^+. The preferential interaction of TMA^+ with A·T-rich DNA may reflect the existence of a specific preferred site of interaction, or it may arise from some special property of an A·T-rich region of the DNA molecule, such as relative ease of deformation. The experiments with PBS-2 DNA described in another paper show that TMA^+ reverses polylysine selectivity in the presence of DNA containing uracil rather than thymine residues; the possible role of the methyl group of thymine as a site of TMA^+ binding is thus eliminated, but as in the case of polylysine, other specific interactions cannot be ruled out. Whatever the mechanism, it is clear that small and relatively uncomplicated molecules are capable of responding to base composition differences in double-helical DNA far below the denaturation temperature.

Appendix 1

Calculation of R and R_0. The relationship used to analyze the binding data is $K = \bar{\nu}/(n - \bar{\nu})A_F$, where $\bar{\nu} = A_B/P_0$, n is the "saturation" value of $\bar{\nu}$, K is the equilibrium constant, A_F and A_B the concentrations of free and bound calcium or spermine, and P_0 the total monomolar concentration of DNA. For any total concentration of A and P_0, it is thus possible to calculate the concentration of free A by solving a quadratic equation. Note that this equation is empirical: it fits the data and provides a simple analytical means of expressing A_F and A_B as a function of the other variables.

In a dialysis competition experiment in which DNA concentrations on opposite sides of the membrane are equal, let D be the *observed* ratio of total A concentrations on opposite sides of the membrane, and let R be the ratio of bound A on one side of the membrane to bound A on the other. Then

$$D = \frac{A_{B_1} + A_{F_1}}{A_{B_2} + A_{F_2}}$$

where the numerical subscripts refer to side 1 and side 2 of the membrane. Since $A_{F_1} = A_{F_2}$ at equilibrium and $R = A_{B_1}/A_{B_2}$

$$D = \frac{R + A_{F_2}/A_{B_2}}{1 + A_{F_2}/A_{B_2}}$$

Since A_{F_2} and A_{B_2} can be calculated from n and K as described above, R can also be calculated.

To calculate the dependence of R upon the amount of bound A, we define $\bar{\nu}_1$, $\bar{\nu}_2$, n_1, and n_2 as the values of $\bar{\nu}$ and n on the two sides of the membrane. R_0 is the limiting value of R

as $\bar{\nu}_1$ and $\bar{\nu}_2$ approach zero. Then, since

$$K_1 = \frac{\bar{\nu}_1}{(n_1 - \bar{\nu}_1)A_F} \text{ and } K_2 = \frac{\bar{\nu}_2}{(n_2 - \bar{\nu}_2)A_F}$$

(note again that these are *empirical* statements), it follows that

$$R = \frac{\bar{\nu}_1}{\bar{\nu}_2} = \frac{K_1(n_1 - \bar{\nu}_1)}{K_2(n_2 - \bar{\nu}_2)}$$

As the total concentration of A approaches zero, R approaches the value $K_1n_1/K_2n_2 = R_0$. Therefore

$$R = R_0\left(\frac{1 - \bar{\nu}_1/n_1}{1 - \bar{\nu}_2/n_2}\right)$$

We define $\gamma = \bar{\nu}_1/n_1$ and $\zeta = n_1/n_2$. Then $\bar{\nu}_2/n_2 = \gamma\zeta/R$. Substituting in the preceding equation for R and solving for R_0, we find $R_0 = (R - \gamma\zeta)/(1 - \gamma)$. Therefore plotting $R/(1 - \gamma)$ as a function of $\gamma/(1 - \gamma)$ should give a straight line of slope ζ with a value of R_0 when $\gamma/(1 - \gamma) = 0$. Note that no special assumptions are made in this derivation about the relative values of n_1, n_2, K_1, or K_2. It is only necessary to know one pair of values of n and K for one of the DNA samples.

Appendix 2

Dependence of Ca^{2+} and Spermine Binding upon Base Compositions. We suppose that when an ion such as Ca^{2+} binds to DNA dissolved in TMA-Cl, a certain number of TMA^+ ions (probably less than two TMA^+ ions for each Ca^{2+}) are displaced from the neighborhood of the DNA. We assume that the binding of Ca^{2+} affects two neighboring base pairs on the DNA helix. If we do not distinguish A·T pairs from T·A pairs, or G·C pairs from C·G pairs, there are three kinds of sites. A·T followed by A·T, A·T followed by G·C, and G·C followed by G·C. Let k_1, k_2, and k_3 be the respective affinity constants for the binding of Ca^{2+} to these three sites, in the limit of large excess of DNA concentration relative to Ca^{2+} concentration. We assume that the three constants are unequal because it is more difficult to displace TMA^+ from A·T than from G·C. We also assume that each base pair acts independently of its neighbor, and that Ca^{2+} has no intrinsic preference for A·T relative to G·C. It follows that $k_1/k_2 = k_2/k_3 \equiv \alpha$. The parameter α reflects the difficulty in transferring TMA from A·T to G·C.

If there are two DNAs with mole fraction A·T equal to f_1 and f_2, respectively, competing for Ca^{2+}, then the limiting ratio R_0 of Ca^{2+} bound to DNA_1 compared with that bound to DNA_2 is given by

$$R_0 = \frac{k_1f_1^2 + 2k_2f_1(1 - f_1) + k_3(1 - f_1)^2}{k_1f_2^2 + 2k_2f_2(1 - f_2) + k_3(1 - f_2)^2}$$

The terms in this expression are obtained by multiplying each binding constant by the probability of finding the appropriate sequence of two base pairs, assuming random sequence. Using the relationship between k_1, k_2, and k_3

$$R_0 = \frac{\alpha^2f_1^2 + 2\alpha f_1(1 - f_1) + (1 - f_1)^2}{\alpha^2f_2^2 + 2\alpha f_2(1 - f_2) + (1 - f_2)^2} = \frac{(\alpha f_1 + 1 - f_1)^2}{(\alpha f_2 + 1 - f_2)^2}$$

If f_1 and f_2 are known, α can be evaluated.

An analogous expression can be derived for spermine binding; here there are four kinds of sites

$$R_0 = \frac{(\alpha f_1 + 1 - f_1)^4}{(\alpha f_2 + 1 - f_2)^4}$$

Since α reflects only the TMA^+ binding preference, it has the same value in this equation as in the one derived for Ca^{2+}.

To obtain the calculated results shown in Table III, we have systematically varied the single parameter α, and using the known values of f_1 and f_2 for each combination of competing DNAs, we have calculated R_0 from the two equations given above. The values in Table III are the best fit and correspond to $\alpha = 0.65$. It should be noted that the expression for R_0 using spermine as test ion is the square of the corresponding expression for Ca^{2+}. This arises from the nature of the model, in which it is assumed that the binding of a spermine molecule is formally equivalent to the successive binding of two calcium ions.

References

Berns, K. I., and Thomas, C. A., Jr. (1965), *J. Mol. Biol. 11*, 476.

Cohn, W. E. (1957), *Methods Enzymol. 3*, 724.

Davies, D. R. (1967), *Ann. Rev. Biochem. 36*, 321.

Felsenfeld, G., and Hirschman, S. Z. (1965), *J. Mol. Biol. 13*, 407.

Hirschman, S. Z., and Felsenfeld, G. (1966), *J. Mol. Biol. 16*, 347.

Hirschman, S. Z., Leng, M., and Felsenfeld, G. (1967), *Biopolymers 5*, 227.

Latt, S. A., and Sober, H. A. (1967), *Biochemistry 6*, 3293.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Marmur, J., and Doty, P. (1962), *J. Mol. Biol. 9*, 801.

Meselson, M., Stahl, F. W., and Vinograd, J. (1957), *Proc. Natl. Acad. Sci. U. S. 43*, 581.

Shapiro, J. T., Leng, M., and Felsenfeld, G. (1969), *Biochemistry 8*, 3219.

Strauss, U. P., Helfgott, C., and Pink, H. (1967), *J. Phys. Chem. 71*, 2550.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley.