Circular Dichroism Spectral Properties of Covalent Complexes of Deoxyribonucleic Acid and *n*-Butylamine†

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ABSTRACT: The addition of *n*-butylamine at a mole ratio of 0.4–11 (amine/nucleotide) to a solution of deoxyribonucleic acid (DNA) (approximately 0.2–2.7 mM) in 2% CH_2O and 20 mM NaCl, pH 7, results in significant changes in the circular dichroism (CD) spectrum before any marked decreases in the hypochromism occur. The character of these CD changes above 260 nm is similar to that previously observed in concentrated electrolyte solutions [Hanlon, S., Brudno, S., Wu, T. T., & Wolf, B. (1975) *Biochemistry 14*, 1648–1660]. At this concentration of CH_2O, the extent of the CD transformation is dependent on both the DNA and the amine concentration and is not seen in controls in which either the CH_2O or the *n*-butylamine is omitted from the reaction mixture. This transformation is only minimally affected by the removal of unreacted CH_2O or *n*-butylamine by exhaustive dialysis or by isolation on a concentrated NaCl gradient but can be significantly reversed by increasing the pH above 9. When the reaction is conducted in $^{14}CH_2O$, excess label is retained upon exhaustive dialysis (compared to the DNA control containing no *n*-butylamine), and the magnitude of ^{14}C label retained parallels the effect on the CD spectrum. The T_m's of these dialyzed products are significantly increased over that of the DNA control, and this elevation also parallels the amount of ^{14}C label retained. This suggests that the CD changes are caused by the covalent attachment of a positively charged *n*-butylamine to DNA bases via a CH_2 cross-link contributed by CH_2O. Despite the changes in the CD spectrum, the absorption spectrum and extinction coefficients of the dialyzed products are very little altered. Electron micrographs and hydrodynamic characteristics are also very similar to the DNA controls and show no evidence of tertiary folding or superstructure. We therefore conclude that the observed changes in the CD spectrum are not caused by interactions of the DNA duplex in an organized tertiary structure but rather reflect a change in DNA secondary structure, similar to the transformation effected by high electrolyte concentration.

The nature and concentration of simple inorganic ions are known to have a profound effect on the circular dichroism (CD)[1] spectrum of DNA, transforming the conservative spectrum observed for DNAs of random bases sequences in low or modest concentrations of salt to one in which the positive band above 260 nm is markedly depressed and, at the highest concentrations of LiCl and NH_4Cl, actually becomes negative. This transformation has been variously interpreted as due to (1) a change in secondary structure from either a more "B-like" state to a mixture of C (predominantly) and A forms (Hanlon et al., 1975) or a less dramatic transformation of the B structure (Baase & Johnson, 1979) or (2) the contribution of a negative ψ spectral component arising from interhelical interactions in a micellar or aggregated state of DNA in these solvents (Maniatis et al., 1974; Gray et al., 1978).

Our original interpretation of the CD spectral changes in concentrated electrolyte solution (as a B → (C + A) transformation) rested on the spectral assignments made for films of DNA by Tunis-Schneider & Maestre (1970). Recently, Zimmerman & Pheiffer (1980) have examined the fiber diffraction patterns of oriented fibers of salmon sperm DNA immersed in some of the solvents which generate "C" CD spectra in solution and have observed only B form X-ray patterns up to concentrations of LiCl of ~9 M (11 *m*). These authors suggest that the spectral assignments of Tunis-Schneider and Maestre for the C form may have been incorrect. Baase & Johnson (1979) concur that the transformation does not generate a C structure, since their determination of the winding angle change for the transfer of covalently closed PM 2 DNA from a medium of low ionic strength (0.05 M NaCl) to electrolyte solutions in which the C spectrum is observed (5.4 M NH_4Cl and 6 M LiCl) yields a value of only ~0.8°. This value is inconsistent with a B to C transformation.

The resolution of this controversy is difficult since the transformed spectrum of protein-free DNA is only produced at high concentrations of salt or mixed H_2O/alcohol solvents, media in which it is difficult to interpret physical data *in solution* without ambiguity. It would be useful to have a system in which the transformed CD spectrum is stable in aqueous solvents of modest electrolyte content. Since the transformation has been demonstrated to be roughly correlated with the reduction of the electrostatic repulsive interactions in the DNA molecule (Hanlon et al., 1978), one would expect to observe a similar transformation if cations were covalently attached to DNA in such a way as to not interfere with the base-stacked hydrogen-bonded interactions of the helical duplex. A wealth of data in the literature (Brutlag et al., 1969; Feldman, 1973) suggests that amino groups of proteins and amines can be covalently cross-linked to DNA bases by CH_2O. Thus, it should be possible to create stable complexes of DNA with simple amines in which the exocyclic amino groups of the DNA bases are cross-linked via a CH_2 to the amino groups of the amine. Under appropriate reaction conditions, the amine should retain its positive charge and the bulk of the Watson–Crick hydrogen bonds should remain intact, thus retaining the base-stacked duplex character of native DNA. Using this approach, we have prepared and partially characterized such

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[1] Abbreviations used: CD, circular dichroism; BuA, *n*-butylamine; NaEDTA, the sodium salt of ethylenediaminetetraacetic acid; DNA, deoxyribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

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complexes of calf thymus DNA and *n*-butylamine (BuA).[1] This paper reports the results of this study.

Experimental Procedures

The calf thymus DNA preparation employed in these experiments was a product of Calbiochem (Lot 900007) whose weight-average molecular weight had been previously estimated as 8.6×10^6 (Hanlon et al., 1976). Stock solutions were prepared at ~2.7 mM (in nucleotide concentration) in 20 mM NaCl, adjusted to pH 7. For most of the experiments reported herein, a lower molecular weight sample was prepared by sonicating portions of this stock to a molecular weight of 0.45×10^6 with a sonicator cell disruptor, Model W185D, (Heat Systems, Ultrasonics Inc.). This molecular weight was estimated from the value of the median sedimentation coefficient, $(s^0_{20,w})_{50}$, and the data of Eigner & Doty (1965). Unless otherwise noted in the text or legends, it can be assumed that the results described refer to this lower molecular weight preparation.

The DNA/CH_2O/BuA complexes were prepared at room temperature (~25 °C) by mixing solutions of DNA (0.2–2.7 mM) in 16–20 mM NaCl, buffered at pH 7 by either 10 mM NaEDTA[1] or 0.7 mM NaH_2PO_4/Na_2HPO_4, with 37% CH_2O at pH 7 to a final concentration of 2% CH_2O. Within 5 min, the reaction with the amine was initiated by an appropriate addition from a stock solution of amine at pH 7 and followed by CD and absorption spectroscopy. At various times after the amine addition, aliquots were removed from the reaction vessel and dialyzed in the cold (~10 °C) against three separate changes of 1 L of 20 mM NaCl, adjusted to pH 7 with NaOH or buffered with 0.7 mM NaH_2PO_4/Na_2HPO_4, for 18, 6, and 18 h (for each change) in order to remove unreacted BuA and CH_2O. The dialyzand/dialyzate ratio was generally 1:100 except for the ^{14}C-labeled samples for which it was 1:1000. Two controls, one containing DNA and CH_2O but no amine and the other containing CH_2O and BuA but no DNA, were also prepared and dialyzed in the same manner. The latter control (CH_2O/BuA) was prepared only for the ^{14}C labeling experiments.

The reagent grade *n*-butylamine used in these reactions with CH_2O and DNA was a product of Aldrich. The concentrations of aqueous stock solutions were determined by titrating to pH 7 with 1.000 M HCl, with a Corning pH meter (Model 125) and a combination glass electrode. The concentrations of other amines used were estimated by weighing out quantities of the solid. This latter group included NH_4Cl (Mallinckrodt), spermidine trihydrochloride (Sigma), and Lys-Lys·2HCl·$0.5H_2O$ (Miles-Yeda Ltd).

The $^{12}CH_2O$ used in the bulk of these experiments was Fisher Certified Formalin which is 37% CH_2O in CH_3OH and H_2O. This stock 37% CH_2O was adjusted to pH 7 with 0.2 M NaOH just prior to use. The $^{14}CH_2O$ stock solution employed in the labeling experiments was prepared by diluting a 0.015-mL aliquot of 1% aqueous solution of specific activity 10 mCi/mmol (New England Nuclear) to 1.00 mL with the cold 37% CH_2O adjusted to pH 7 as described above. This gave a stock solution of ~36% CH_2O with a specific activity of 3.75 mCi/mol. This stock solution was employed in the reactions in a manner similar to that for the cold 37% CH_2O. All samples were counted with a Beckman LS 9000 scintillation counter and corrected for efficiency of counting and background. Dialysis fluid from the final dialysis was used for the latter count.

The experiments employing [1-^{14}C]ethylamine hydrochloride used a product of New England Nuclear at 20.8 mCi/mmol. It was diluted into a 0.24 M solution of [^{12}C]-ethylamine hydrochloride whose concentration had been estimated by ascertaining the amount of base required to titrate the aqueous solution at pH 6 to pH 10.6, the half-equivalence point, as well as by dry weight. The specific activity of the stock amine solution and that of the reaction mixture was 8.55 mCi/mol.

Absorption spectra were obtained with a Cary Model 14 CMR by using either 0.100- or 1.000-cm quartz cells. Except for the melting profiles, all spectra were measured at 25 °C. Concentrations of DNA solutions were estimated by using the measured absorbance at 259 nm at 25 °C, prior to the addition of reagents, the dilution factor caused by CH_2O and BuA addition, and the appropriate extinction coefficients at 259 nm determined for reagent free products. The phosphate determinations required for the latter determinations were performed by the method of Ames & Dubin (1960).

The thermal melting profiles were obtained in the manner previously described (Chan et a., 1979) on solutions which had been exhaustively dialyzed against 1 mM NaCl, pH7. Dialysis fluid was used as the reference solvent, and both sample and reference compartments were heated to the same temperature. Temperature of the sample cell contents was measured by a Yellow Springs Bridge and thermistor assembly. Values of the absorbance, A^T, at a given temperature (T) were corrected for thermal expansion effects by multiplying by the factor $\rho_{w,25}/\rho_{w,T}$, where ρ_w represents the density of water at 25 °C and temperature T, respectively.

Circular dichroism spectra were obtained at 27 °C with a Cary 60 spectropolarimeter equipped with a 6001 CD unit, in the manner previously described (Hanlon et al., 1975). Path lengths were either 0.100 or 1.000 cm. Concentrations of DNA ranged between 0.1 and 2.7 mM with no concentration dependency noted in this range. All spectral data are reported in mean residue ellipticity, $[\theta]_\lambda$, in deg cm^2/dmol at wavelength λ (in nanometers).

Reduced viscosities were measured with an Ostwald viscometer of average shear, 300 s^{-1} (for H_2O) at 25.00 °C at a concentration of 0.35 mM DNA or 0.011 g/dL. Sedimentation coefficients were obtained at DNA concentrations of ~0.1 mM DNA in 100 mM NaCl and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7, with a Spinco Model E ultracentrifuge equipped with absorption optics, using top-loading 12-mm cells. Under the experimental conditions of operation, there is no concentration dependence. The median sedimentation coefficients were corrected to the conventional standard conditions $(s^0_{20,w})_{50}$ as previously described (Hanlon et al., 1976), omitting, however, the buoyancy correction, which was negligible. The sedimentation coefficient distributions were calculated by the method of Shumacher & Schachman (1957). Separate distributions were calculated for three different times for each experiment. These three distributions were then averaged to provide the characteristic distribution of a given sample in a given ultracentrifuge experiment.

Electron micrographs were taken with a JEM 100 CX (Jeolco Instruments). Dialyzed samples were diluted to 2 μM DNA (nucleotide concentration) in 10 mM Tris at pH 8 and were immediately adsorbed onto glowed carbon/collodion-coated 400-mesh grids, positively stained with 0.02% uranyl acetate, and rotary shadowed with Pt. Pictures were taken at a magnification of 33 000×. Polystyrene latex beads of 109-nm diameter were sprayed on the back of the grids before sample deposition in order to provide a magnification marker.

Results and Discussion

Upon the addition of BuA to a solution of calf thymus DNA in 2% CH_2O at pH 7, the conservative CD spectrum normally

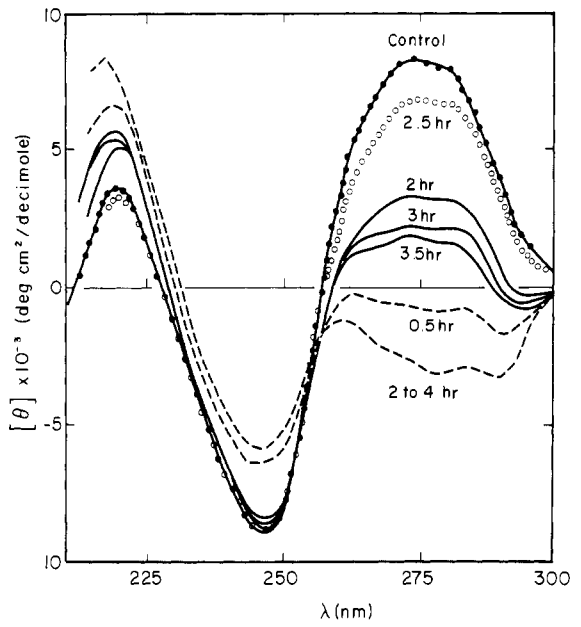


FIGURE 1: Transformation of the circular dichroism spectrum of the calf thymus DNA in 2% CH_2O upon addition of *n*-butylamine. Spectra of DNA controls (0.2–2.0 mM DNA), 0.5–2.0 h in 2% CH_2O and 16–20 mM NaCl, pH 7 (●); 0.20 mM DNA (8.6×10^6 daltons) reacted with 0.08 mM BuA and 2% CH_2O for 2.5 h (○); 0.19 mM DNA (8.6×10^6 daltons) reacted with 0.56 mM BuA and 2% CH_2O for 2, 3, and 3.5 h (—) in 20 mM NaCl and 0.7 mM NaH_2PO_4/Na_2HPO_4, pH 7; 2.37 mM DNA (0.45×10^6 daltons) reacted with 26.4 mM BuA and 2% CH_2O for 0.5 and 2–4 h in 16 mM NaCl and 10 mM NaEDTA, pH 7 (---).

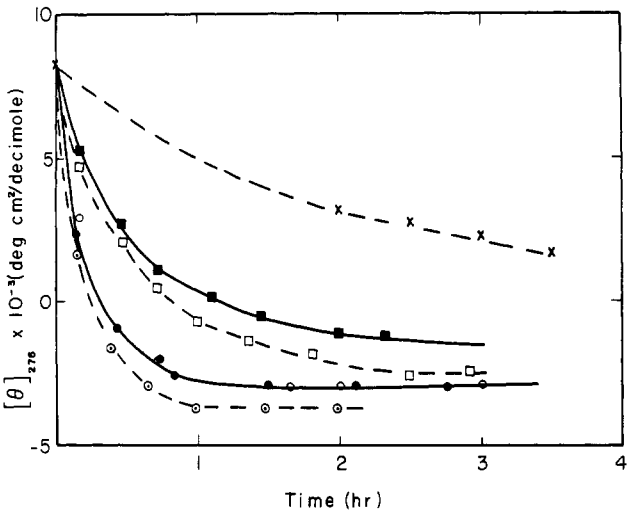


FIGURE 2: Effects of molecular weight of DNA and concentration of DNA and *n*-butylamine on the kinetics of the DNA reaction in 2% CH_2O. The values of $[\theta]_{275}$ are plotted against time for the unsonicated DNA sample of 8.6×10^6 daltons (---) and the sonicated sample of 0.45×10^6 daltons (—). Concentrations were 0.20 mM DNA and 0.57 mM BuA in 20 mM NaCl and 0.7 mM NaH_2PO_4/Na_2HPO_4, pH 7 (X), 2.74 mM DNA (□) and 2.45 mM DNA (■) with 7.3 mM BuA in 16 mM NaCl and 10 mM NaEDTA, pH 7, and 2.63 mM DNA (⊙) and 2.37 mM DNA (○–●) with 26.4 mM BuA in 16 mM NaCl and 10 mM NaEDTA, pH 7. The open (○) and solid (●) symbols in the latter represent the results from two separate experiments.

found at this electrolyte concentration (16–20 m M NaCl and 0.7 mM NaH_2PO_4/Na_2HPO_4 or 10 mM NaEDTA) transforms in the manner shown in Figures 1 and 2. As a function of time, there is a marked loss in the intensity of the positive band above 260 nm accompanied by less dramatic changes in the negative band and the small positive band below 260 nm. These transformations did not occur if either BuA or CH_2O was omitted from the reaction mixture. After 16 h, 2% CH_2O alone, under these electrolyte conditions, reduced the $[\theta]_{275}$ by only 1×10^3 deg cm^2/dmol without affecting the intensities of either the negative or the positive band below 260 nm in DNA's CD spectrum. Within the time frame of our reaction conditions (2–4 h), however, this reduction by CH_2O alone amounted to only 0.2×10^3 deg cm^2/dmol.

As might be expected, the rate of spectral transformation at a fixed CH_2O concentration (2% or 0.67 M) is a function of both the BuA and the DNA concentration. As Figure 2 shows, however, there is also a dependence of both the rate and the apparent extent of the transformation on the molecular weight of the DNA samples employed in the reaction. The transformation effected by BuA and CH_2O appears to be somewhat more rapid and proceed further for the higher molecular weight (8.6×10^6) preparation compared to the lower molecular weight (0.45×10^6) sample. At the present time we have no explanation to offer for this molecular weight dependency.

The absorption spectral properties were also followed for the reactions whose CD properties are shown in Figures 1 and 2. There was little change (approximately 2–5%) in the absorbance at the maximum for any of these solutions up to 2–4 h, although shifts in the wavelength position of the maxima from 258.5 nm in the unreacted control to 261 nm were observed at the highest BuA concentration. Thus, up to that time, the CD changes could not be attributed to base unstacking or denaturation.

After 4 h, however, the extinction coefficients of solutions at the highest BuA concentration (26.4 mM) begin to rise more significantly such that after 16 h at room temperature the absorbance of a sonicated sample of DNA at the maximum had risen by a factor of 1.30 and the wavelength position of the maximum had shifted to 264 nm. The CD spectrum of this latter solution was totally different from that displayed in Figure 1 and did not even belong to the same family. It exhibited a spectrum similar in shape to that of CH_2O-denatured DNA, with a positive band above 260 nm with a mean residue ellipticity of 3.4×10^3 deg cm^2/dmol at the maximum at 280 nm, a crossover at 267 nm, and a negative band with a mean residue ellipticity of -4.4×10^3 deg cm^2/dmol at the minimum at 250 nm. This denaturation phenomenon was not observed at lower DNA or BuA concentrations in the same time period. It is probably a function of the extent of the reaction, as reflected in the depression of the positive band of the CD spectrum.

None of these spectral transformations, both those occurring before the 4-h period as well as the denaturation effects after that time, were observed in control solutions consisting of only DNA maintained for the same time period in 2% CH_2O in the reaction solvent. Although the wavelength position of the maximum in the absorption spectrum of control DNA shifted from 258.5 to 260 nm in a 16-h period, the CD spectrum was very little affected. At the most, the value of $[\theta]_{275}$, the maximum of the positive band, was reduced by no more than 1×10^3 deg cm^2/dmol.

Since it was clear from the above results that significant denaturation occurred after 4 h or when the mean residue ellipticity at 275 nm had been reduced to -3×10^3 to -4×10^3 deg cm^2/dmol in the reaction mixtures, we restricted the preparation of complexes to that reaction time. Subsequent dialysis of these reaction products returned the absorbance maximum to 259 nm, with values of ϵ_{259nm} within 1–2% of that of underivatized DNA. The dialysis procedure, however, failed to result in full reversal of the changes in the CD spectra.

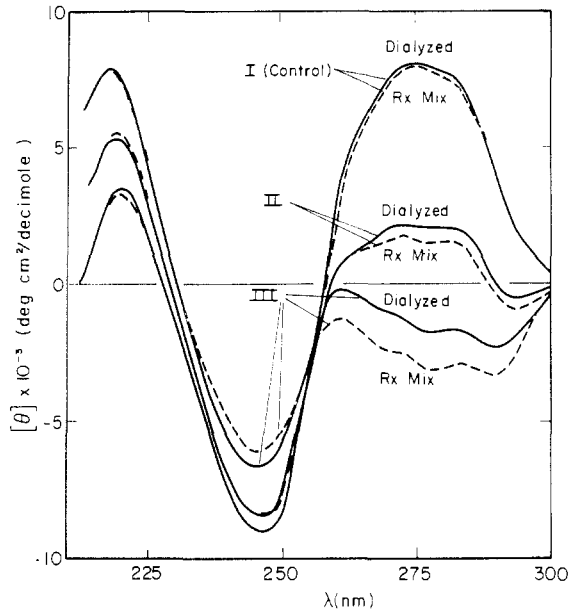


FIGURE 3: Effects of exhaustive dialysis on circular dichroism spectra of the products of the DNA–*n*-butylamine reaction in 2% CH_2O. Curve set I represents the DNA control between 0.2 and 2.5 mM DNA (CH_2O only, no BuA). Curve set II was obtained with 0.18 mM DNA (8.6×10^6 daltons) and 0.56 mM BuA. Curve set III was obtained with 2.37 mM DNA (0.45×10^6 daltons) and 26.4 mM BuA. The reaction solvents were either 20 mM NaCl and 0.7 mM NaH_2PO_4/Na_2HPO_4, pH 7 (I at 0.2 mM DNA and II), or 16 mM NaCl and 10 mM NaEDTA, pH 7 (I at 2.5 mM DNA and III). Solvents for the dialyzed products were either the same as that for the reaction (I at 0.2 mM DNA and II) or 20 mM NaCl, pH 7.

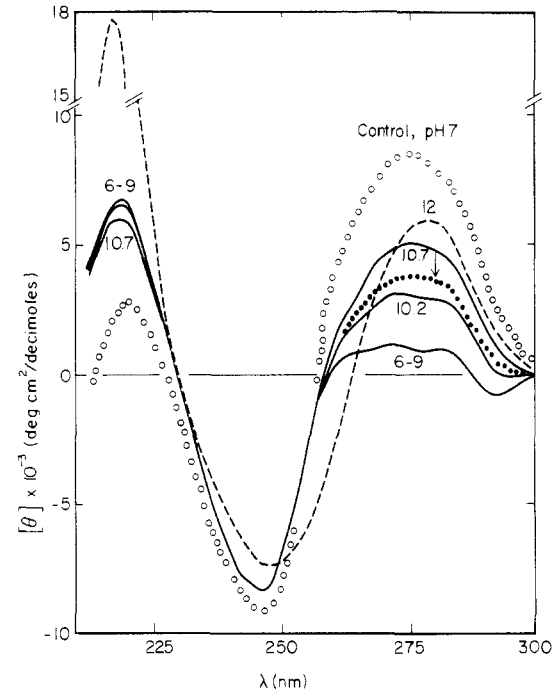


FIGURE 4: Transformation of the circular dichroism spectrum of the DNA–*n*-butylamine complex as a function of pH. The curves coded as (—) represent the spectra of a dialyzed complex at 0.16 mM DNA (8.6×10^6 daltons) in 20 mM NaCl as the pH is increased from 6 to 10.7 by the addition of NaOH. (The value of ϵ_{259} at the latter pH was only 5% higher than that at pH 6.) The curve coded as (●) demonstrates the effects of rapidly dropping the pH from 10.7 to 8.9. The curve coded as (---) represents the spectrum obtained on the latter solution after increasing the pH to 12. (○) represents the spectrum of the control DNA solution (0.15–2.5 mM DNA, no BuA).

Figure 3 demonstrates this point since it compares the CD spectra of the dialyzed products with those obtained in the reaction mixtures just before dialysis was initiated.

The magnitude of the partial reversal of the CD spectral transformation was dependent on the variables of the dialysis procedure such as temperature, volume ratios of dialyzand/dialyzate, volume and number of changes of dialyzate, and time allotted for each change. The conditions of the initial dialysis period seemed to be especially crucial since subsequent variations in the time and number of dialysis changes had relatively little effect on the CD properties of the product. In order to obtain consistent spectra of dialyzed products formed under specific reaction conditions, it was necessary to maintain the standard dialysis procedure and schedule given under Experimental Procedures.

One of these dialyzed products whose $[\theta]_{275}$ was -0.8×10^3 deg cm^2/dmol was centrifuged on a 2–4 M NaCl gradient. Upon elution, a single peak was observed which accounted for 99% of the DNA loaded onto the gradient. The CD spectrum of the fraction taken from the maximum of the peak after dialysis to remove excess NaCl (whose concentration was estimated at 2.3 M) was essentially identical with that obtained on the sample before centrifugation. (Its value of $[\theta]_{275}$ was -0.2×10^3 deg cm^2/dmol). These results coupled with those described in the preceding paragraph indicate that the product of the DNA/CH_2O/BuA reaction is a covalent one of reasonable stability at pH 6–7 in the cold (~10 °C).

Using the CD spectrum as an indicator, we also found that the dialyzed product was stable for at least 24 h at room temperature as long as the pH was maintained at 7 in NaCl solvents ranging from 1 to 100 mM. Furthermore, the CD spectrum exhibited no marked dependency on either the NaCl concentration within this range or on the DNA concentration in the range between 0.16 and 2.7 mM. At pH 8.5, however, in the presence of 40 mM Tris, there was a small but measurable reversal of the CD spectral properties over the same time period (24 h) at room temperature.

Increasing the pH above 9 to 10.7 resulted in marked and rapid increases in the intensity of the positive band prior to any hyperchromic increase in the absorption spectrum due to the onset of alkaline denaturation. These changes are shown in Figure 4. Fifty percent of the return to the original intensity of the unreacted control was achieved by pH 10.6. A subsequent decrease in pH from 10.7 to 8.8, in order to avoid the onset of alkaline denaturation, lowered the positive band but failed to return the CD spectrum to what had been previously observed at this latter pH, indicating that some – but not all – of the increase in the intensity of the positive band is attributable to the loss of reaction products from the DNA molecule under the alkaline conditions described. The other factor is presumably the loss of positive charge on an amine attached to the DNA.

This latter point was confirmed by repeating the same experiment at a single pH below 10.7, the point at which significant alkaline denaturation begins in this solvent. The rate of amine loss due to OH^- catalysis at the lower pHs is considerably reduced, and if the spectral measurements are confined to only one wavelength (275 nm) or a rapid scan of the positive band, the increase of the rotational strength of the positive band due to an increase in pH is almost totally reversed by returning the pH to 7. For instance, when a derivative at pH 6.3 with a value of $[\theta]_{275}$ of -1.76×10^3 deg cm^2/dmol is brought to pH 10.4, $[\theta]_{275}$ increases to 2.12×10^3 deg cm^2/dmol, with little concomitant increase (~4%) in the extinction coefficient at 259 nm. If the pH is lowered *immediately* (within 5–15 min) to 6.5, the value of $[\theta]_{275}$ returns to -0.76×10^3 deg cm^2/dmol. The control (underivatized) DNA sample in the same pH range has a value of $[\theta]_{275}$ of

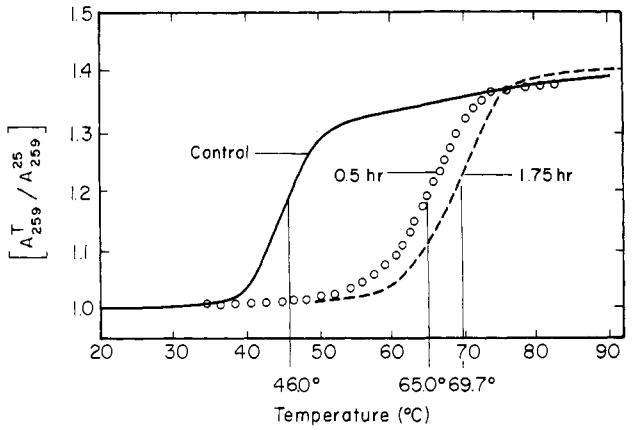


FIGURE 5: Melting profiles of a DNA control and the DNA–*n*-butylamine complexes. Dialyzed complexes isolated after 0.5 h (O) and 1.75 h (---) from a reaction mixture which contained 2.37 mM DNA, 2% CH_2O, and 26.4 mM BuA and a control sample (—) whose reaction mixture contained no BuA were redialyzed against 1 mM NaCl, pH 7. Melting profiles were obtained in this solvent at 0.13–0.14 mM DNA and are plotted as the ratio of the absorbance at 259 nm at temperature T to that at 25 °C ($A_{259}{}^T/A_{259}{}^{25}$). Values of the T_m's are indicated on the abscissa for each curve.

8.2×10^3. If one assumes that the fractional increase in the value of $[\theta]_{275}$ at pH 10.4 represents a conversion of positively charged amine residues to the unprotonated conjugate base form, then these data cited above yield a pK_a of 10.8–10.9 for the amine residue, a value consistent with what one would expect for a secondary amine electrostatically perturbed by the proximity of negative charges.

Melting profiles of the derivatized samples also suggested that there was indeed a positively charged group—presumably butylamine—attached to the DNA product obtained from the reaction mixtures containing BuA. Samples for these and the hydrodynamic and electron microscope experiments described below were prepared by removing aliquots after 0.5 and 1.75 h from reaction mixtures containing 2.4 mM DNA (0.45×10^6 daltons) and 26.4 mM BuA in 2% CH_2O, 16 mM NaCl, and 10 mM NaEDTA, pH 7. A control sample with no BuA was simultaneously prepared and removed after 2 h. These reaction products were then dialyzed against 20 mM NaCl, pH 7, and examined by CD and absorption spectroscopy. Values of $[\theta]_{275}$ and ϵ_{259} are given in Table I. Their extinction coefficients differ insignificantly from one another and from that of an unreacted DNA sample determined in the same solvent (6680 ± 150 M^{-1} cm^{-1}).

These samples were then redialyzed against three changes of 1 mM NaCl, at dialyzand/dialyzate ratios of 1:100, and the thermal melting profiles shown in Figure 5 were obtained. The control DNA solution melted at 46 °C whereas the 0.5- and 1.75-h complexes exhibited T_m's of 65.0 and 69.7 °C, respectively. These data are recorded in Table I together with the hyperchromic increase exhibited by the three solutions upon melting. These latter values differ little (2–5%) from that which we have previously found for solutions of unreacted calf thymus DNA in this solvent (1 mM NaCl) (Chan et al., 1979). Table I also includes the values of $[\theta]_{275}$ measured in 1 mM NaCl, which are similar to the values obtained in 20 mM NaCl. It should be noted that the depression of the intensity of the positive band follows the increase in T_m.

The increase in T_m of the amine complexes is consistent with a reduction of the negative charges of the phosphate groups of both helix and coil forms by the attached positively charged amine groups coupled with a concomitant contraction of the derivatized coil form compared to a underivatized coil form of the control at the same ionic strength. The application of

Table I: Properties of Covalently Linked Complexes of DNA and *n*-Butylamine after Dialysis

sample reaction time (h)	$\epsilon_{259} \pm 154$ (M^{-1} cm^{-1}) in 20 mM NaCl	$[\theta]_{275} \pm 0.2] \times 10^{-3}$ (deg cm^2/dmol) in mM NaCl (mM), pH 7			melting properties in 1 mM NaCl		moles of ^{14}C bound/mol of nucleotide ± 0.01	hydrodynamic properties in 100 mM NaCl, pH 7[a]	
		1	20	100[a]	$T_m \pm 0.2$ (°C)	$A^{90}/A^{25} \pm 0.02$		$(s^0_{20,w})_{50\%} \pm 0.1$ (S)	$(\eta_{sp}/C)_{0.011\%} \pm 0.1$ (dL/g)
0 (control)	6670	7.95	8.17	8.20	46.0	1.39	0.027	7.7	3.7
0.5	6760	0.80	0.80	0.64	65.0	1.39	0.124	7.7	3.9
1.75	6610	−1.46	−1.61	−1.79	69.7	1.41	0.159	7.5	3.8

[a] Buffered by 10 mM NaH_2PO_4/Na_2HPO_4.

Manning's theory of polyelectrolyte behavior (Manning, 1969, 1978) as subsequently modified by Record (Record, 1975; Record et al., 1978) to DNA melting properties in NaCl indicates that the function, $dT_m/d \log a_{Na^+}$, is proportional to the number of Na^+ counterions released in the transition. The reduction of the negative charge per nucleotide in both helix and coil occasioned by the attachment of the positively charged amine, coupled with the anticipated change in the coil geometry caused by the partial neutralization of the phosphate negative charges (which has been seen in other experimental systems; Record et al., 1976), will result in fewer counterions released and, correspondingly, a lower value of $dT_m/d \log a_{Na^+}$. Since this polyelectrolyte effect is considerably diminished at high concentrations of NaCl, T_m's for both the amine derivatives and the control are approximately equal at ~1 M NaCl. Thus, a lower value of $dT_m/d \log a_{Na^+}$ means a higher T_m when control and amine complex are compared at a fixed NaCl concentration which falls within the range in which the theory is applicable. As shown in the Appendix (see paragraph at end of paper regarding supplementary material), application of these concepts and the experimental data of Record et al. (1976) for coil contraction as a function of neutralization results in a predicted T_m of 64 °C for the 0.5-h sample (whose amine content as described in the next section was approximately 0.11–0.12 mol/nucleotide). Considering the number of approximations and assumptions involved in the calculation, this is embarrassingly close to the value of 65.0 °C observed experimentally. The agreement supports the fact that the covalently bound amine residues must be positively charged at pH 7.

Since it seemed reasonable to assume that the butylamine was being attached to DNA bases via a CH_2 contributed by CH_2O, we conducted the reaction described in the paragraph above in $^{14}CH_2O$ in order to assess the extent of amine bound. The amount of excess label retained, upon exhaustive dialysis by the DNA in the absence and presence of BuA for reaction times of 0.5 and 1.75 h, is reported in Table I. After dialysis, the control solution retained only 0.03 mol of ^{14}C/mol of nucleotide, in contrast to the two experimental solutions which retained 0.12 and 0.16 mol of ^{14}C/mol of nucleotide for the 0.5- and the 1.75-h samples, respectively. It could be demonstrated that the excess retention in these samples was not attributable to the buildup of large cross-linked polymers of BuA and CH_2O by the fact than an additional control consisting of only 2% $^{14}CH_2O$ and 26.4 mM BuA exhibited no differential in the counts found inside and outside the dialysis bag. It is reasonable to conclude that the values quoted for the experimental solutions reflect the covalent attachment of BuA to DNA and presumably give the *maximum* amount of BuA bound under the reaction conditions described.

The CD spectra of these dialyzed ^{14}C-labeled solutions are shown in Figure 6. The values of $[\theta]_{275}$ are very close to those given in Table I for the samples prepared with cold CH_2O under identical reaction conditions. As this figure and the data in Table I demonstrate, the decrease in the intensity of the positive band and the increase in T_m of the experimental samples parallel the increase in the amount of ^{14}C bound to DNA.

In order to further document the covalent attachment of amines under our reaction conditions, we repeated the above experiments with a ^{14}C-labeled amine and cold CH_2O. Since labeled *n*-butylamine was not commercially available, we used [1-^{14}C]ethylamine which had essentially the same effect on the CD spectrum of DNA. Equivalent reaction conditions produced products which retained 0.11 and 0.15 mol of

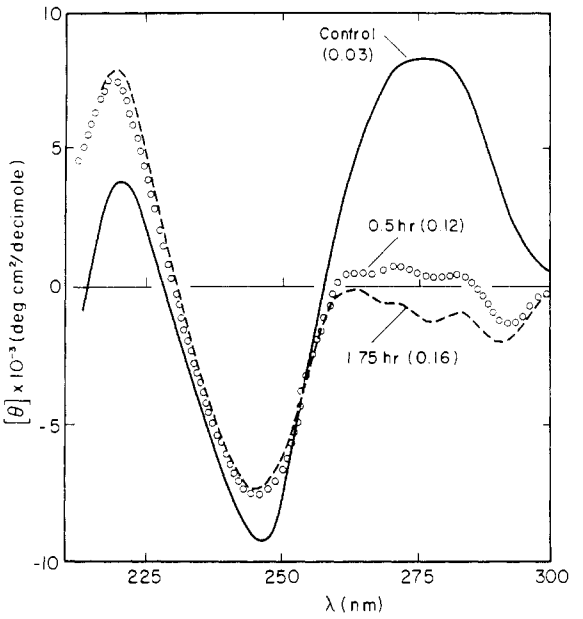


FIGURE 6: Circular dichroism spectra of the dialyzed products isolated from reaction mixtures containing [^{14}C]formaldehyde. the numbers in parentheses give the moles of ^{14}C retained per mole of nucleotide upon exhaustive dialysis of the aliquots removed from the reaction mixture at 2 h for the 2.37 mM DNA control (—) (no BuA) and at 0.5 h (O) and 1.75 h (---) for the mixture containing 26.4 mM BuA. The reaction solvent was 16 mM NaCl, 10 mM NaEDTA, and 2% $^{14}CH_2O$ in all cases.

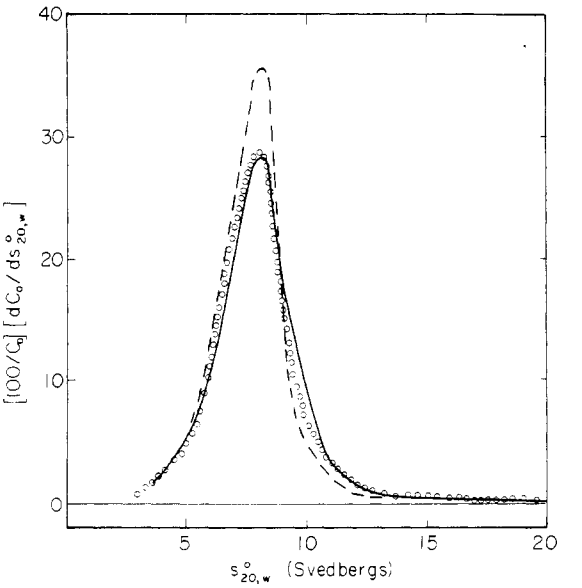


FIGURE 7: Sedimentation distribution profiles of the DNA control and DNA–*n*-butylamine complexes. The complexes and control preparations described in the legend of Figure 5 were sedimented at 0.1 mM DNA in 100 mM NaCl and 10 mM NaH_2PO_4–Na_2HPO_4, pH 7. (—) represents the control, and (O) and (---) represent the complexes isolated after 0.5 and 1.75 h, respectively.

^{14}C/mol of nucleotide for samples whose CD spectra were those of the 0.5- and 1.75-h reaction products. No ^{14}C label was retained by the control in this instance. The difference between the values quoted above and those given in Table I, although within experimental error, probably reflects a small amount (~0.01 mol of CH_2O/nucleotide) of cross-linked DNA bases in the complexes.

The same samples prepared for the melting experiments were also examined by hydrodynamic and electron microscopic techniques in order to ascertain whether the change in the CD properties could be attributed to the formation of tertiary

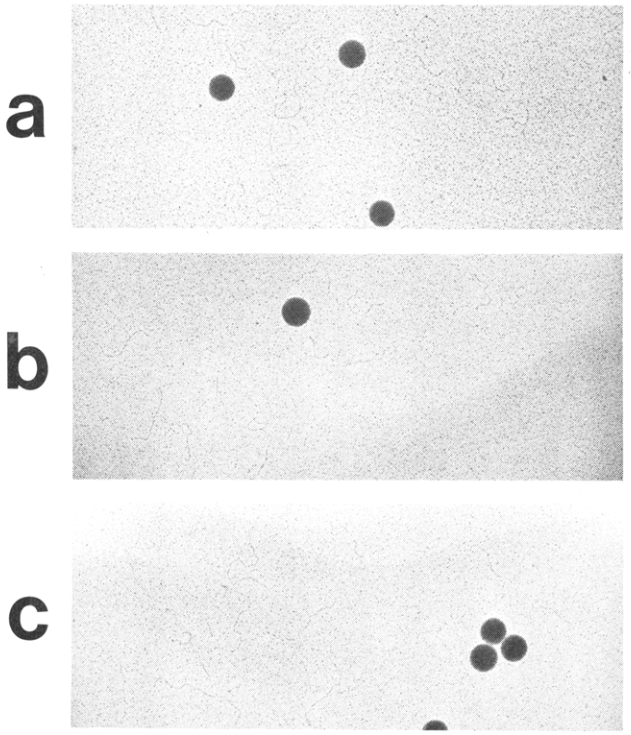


FIGURE 8: Electron micrographs of the DNA control and DNA–*n*-butylamine complexes. (a) DNA control; (b) DNA–BuA, 0.5-h complex; (c) DNA–BuA, 1.75-h complex. Magnification of final prints is 33 150×. The large spheres are polystyrene latex markers of 109-nm diameter. The samples shown are those preparations described in the legend of Figure 5.

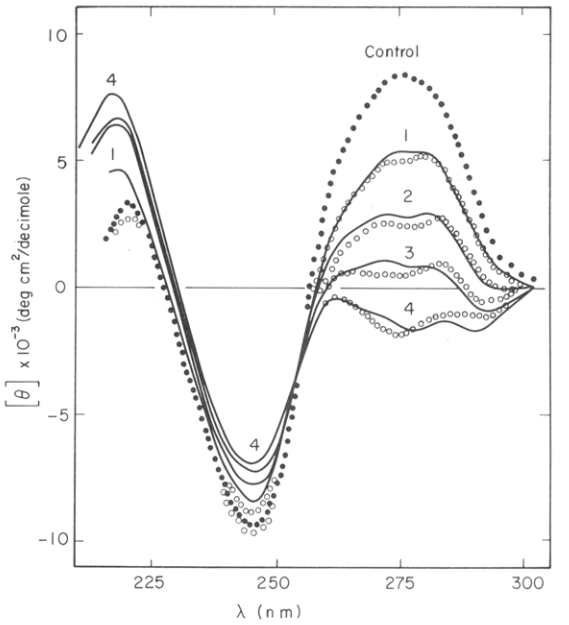


FIGURE 9: Comparison of the circular dichroism spectral changes induced by *n*-butylamine complexion with those found in concentrated electrolyte solutions. Spectrum of the DNA control (●) in 20 mM NaCl, pH 7. Spectra of the DNA–BuA complexes (—) isolated from reaction mixtures containing 2% CH_2O at various times and at variable DNA and amine concentrations. Curve 1 represents a spectrum in a reaction mixture containing 2.5 mM DNA and 7.3 mM BuA after 10 min. Curves 2–4 represent the spectra of dialyzed products from reaction mixtures containing 0.2 mM DNA (8.6×10^6 daltons) and 0.57 mM BuA, 1.36 mM DNA (8.6×10^6 daltons) and 5.4 mM BuA, and 2.37 mM DNA (0.45×10^6 daltons) and 26.4 mM BuA, respectively. The spectra of DNA (8.6×10^6 daltons) in various concentrated electrolytes are represented by (○). These salt concentrations are (1) 2.93 *m* NaCl (which has a spectrum identical with that in 1.14 m LiCl), (2) 3.56 *m* LiCl, (3) 6.41 *m* LiCl, and (4) 11.03 *m* LiCl. These four salt spectra were taken from Hanlon et al. (1975) and Wolf et al. (1977).

structures, perhaps collapsed or aggregated forms of DNA. The results of these experiments are shown in Figures 7 and 8 and in Table I. There is no marked difference in the hydrodynamic properties of the DNA–BuA complexes compared to the control. There is no evidence in the sedimentation profiles shown in Figure 7 of a fast moving component. If anything, the distribution of the experimental solutions falls at somewhat lower $s^0_{20,w}$ values compared to the control. This effect may be due to a slightly higher partial specific volume of the complexes (which more than compensates for the small increase in their molecular weights) rather than an increase in the persistence length or frictional coefficient of the DNA.

As Figure 8 demonstrates, the electron microscopic appearance of the DNA–BuA complexes is indistinguishable from that of the control. No evidence of collapsed or toroidal forms—or aggregation—was evident on any portions of the grids containing the 0.5- and 1.75-h complexes.

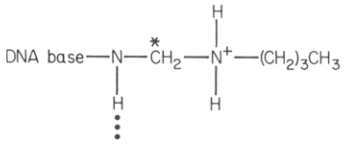
The changes observed in the positive band of the CD spectrum due to the attachment of BuA are reminiscent of the transformations seen in the presence of high concentrations of electrolyte (Hanlon et al., 1975, 1978). In order to facilitate comparison with the earlier results, we have shown in Figure 9 some spectra chosen from the earlier studies together with those of complexes whose positive bands showed comparable rotational strengths. When compared in this manner, it can be seen that the effects of BuA substitution on the CD spectrum above 260 nm are strikingly similar to the effects of electrolyte. The small differences which are observed are probably due to a change in the intrinsic spectral properties of those bases which are substituted with BuA and the effects of the higher refractive index of the more concentrated electrolyte solutions.

The differences in the two spectral sets become greater below 260 nm. There are blue shifts in both the minimum of the negative band and the maximum of the small positive band of the derivatized product compared to the unreacted DNA at any electrolyte concentration, including the control in 0.02 *m* NaCl. The origin of these differences is uncertain at the present time.

The transformation in the CD spectrum effected by CH_2O and BuA is not unique to BuA. As Figure 10 shows, reaction with a variety of amines, including NH_4^+, will give rise to CD spectra with depressed positive bands, although the reaction rates will vary. These results suggest that the transformation in structure caused by the reaction with the amine is not dependent on the nature of the organic portion of the amine.

Conclusion

On the basis of these experimental results as well as what is known of the chemistry of amine and aldehyde reactions, (Feldman, 1973; McGhee & von Hippel, 1975a,b, 1977a,b), it is clear that the transformation in the CD spectrum of DNA in the presence of BuA and CH_2O in those solutions exhibiting little or no change in the extinction coefficient of the maximum is directly attributable to the formation of a cross-linked product whose probable structure is



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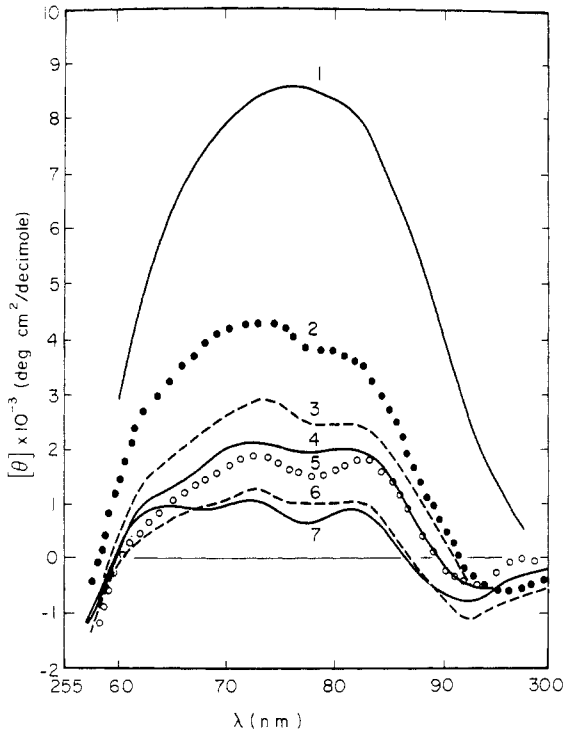

FIGURE 10: Comparison of circular dichroism spectra of the DNA–*n*-butylamine complexes with other amine complexes. The curves represent the spectra of the dialyzed products (in 20 mM NaCl at pH 7 or 20 mM NaCl and 0.7 mM $NaH_2PO_4 0/Na_2HPO_4$, at pH 7) removed from the reaction mixture containing 2% CH_2O and amine at various times. The DNA was the 8.6×10^6 dalton sample, and its concentration was 0.2 mM in all reactions except for curve 7. The amine concentrations were as follows: (1) 0 (DNA control); (2) 0.60 mM NH_4; (3) 0.04 mM spermidine; (4) 0.56 mM BuA; (5) 0.12 mM Lys–Lys; (6) 0.08 mM spermidine; (7) 2.37 mM DNA (0.45×10^6 daltons) and 26.4 mM BuA.

where (N–H...) indicates a reacted exocyclic amino group in which the Watson–Crick hydrogen bond is still intact. The central $*CH_2$ unit is provided by CH_2O. This structure is stable enough to resist exhaustive dialysis at pH 7 but will, as expected, disintegrate rapidly at pHs above 8.5–9.

The reaction product (I) is created at a relatively low degree of amine reaction, amounting to no more than 20% of nucleotide substitution. Since both amino and imino groups should be capable of reacting with the amine and CH_2O, the total number of available sites per nucleotide in a 42% GC content DNA should be minimally 1.21 (for monosubstitution of the exocyclic amino groups). If Watson–Crick hydrogen bonds are to be maintained, thus excluding imino reactions, then this value drops to 0.71. Thus, the product (I) is formed at anywhere from 16% to 28% of total saturation. If the reaction is allowed to proceed beyond this degree of substitution, the loss of hypochromicity of DNA indicates that the groups involved in Watson–Crick hydrogen bonds begin to react. The latter are probably a combination of the ring imino groups of guanine and thymine and the exocyclic amino groups of adenine, guanine, and cytosine which may take on a second CH_2·BuA group to become disubstituted. Alternatively, the presence of the basic amine group on the helix may catalyze the addition of CH_2O alone to base sites, thus opening up the Watson–Crick sites more rapidly than in the control.

A second conclusion which we may draw with some certainty from these experimental results is that the transformation in the CD properties *cannot* be attributed to interchain or interhelical interaction due to the formation of a tertiary or superstructure of DNA; there is no evidence of a collapsed or aggregated form of DNA in the hydrodynamic and electron microscopic experiments. By extension, we suggest that the transformed CD spectrum of DNA found at high concentrations of electrolytes is also not due to the presence of a condensed form of DNA.

We are thus lead to the conclusion that the changes observed in the CD spectrum upon attaching the positively charged amine are due to one or both of two possible causes: (1) an effect of the positive charge on the electronic properties of the bases themselves thus changing the intrinsic electronic and magnetic properties of the monomers or (2) a change in secondary structure of the polynucleotide.

In view of the relatively low degree of amine substitution required to effect rather dramatic changes in the CD spectrum of the positive band, possibility 1 seems an unlikely explanation, although it cannot be ruled out entirely. It seems more likely that secondary structural changes induced by the charge neutralization are occurring which markedly alter the CD properties. It is clear, for instance, that the winding angle of DNA changes in electrolyte solutions (Anderson & Bauer, 1978), and these changes parallel the changes in the rotational strength of the positive band of DNA at these same electrolyte concentrations (Chan et al., 1979; Baase & Johnson, 1979). It is reasonable to assume that the lowered rotational strength of the DNA–BuA complexes above 260 nm reflects similar increases in the duplex winding angle, although this is not the only interpretation. The CD spectrum of poly(dG-dC) in high salt (Pohl & Jovin, 1972), where it is thought to be in a Z form helix (Wang et al., 1979), exhibits a negative band at 290 nm and a positive band at 265 nm. It is possible that the addition of butylamine to calf thymus DNA induces Z-form helix in dG-dC enriched sequences, thus resulting in a lowering of both the positive band in the DNA spectrum above 260 nm and the negative band below 260 nm.[2]

Whether these CD changes reflect a conversion to a more C-like state, or to other types of B structures, perhaps mixed with Z forms, is uncertain at the present time. The stability of the DNA structural change in these complexes at modest electrolyte concentrations will permit these complexes to be examined by other techniques such as X-ray diffraction and Raman and NMR spectroscopy. The results of these future studies should thus illuminate the relationship between secondary structure and the CD characteristics of DNA.

Supplementary Material Available

Appendix discussing the application of Manning's theory of polyelectrolyte behavior as modified by Record (4 pages). Ordering information is given on any current masthead page.

References

Ames, B., & Dubin, D. (1960) *J. Biol. Chem. 235*, 770.
Anderson, P., & Bauer, W. (1978) *Biochemistry 17*, 594–601.
Baase, W. A., & Johnson, W. C., Jr. (1979) *Nucleic Acids Res. 6*, 797–814.
Brutlag, D., Schlehuber, C., & Bonner, J. (1969) *Biochemistry 8*, 3214–3218.
Chan, A., Kilkuskie, R., & Hanlon, S. (1979) *Biochemistry 18*, 84–91.
Crothers, D., & Zimm, B. (1965) *J. Mol. Biol. 12*, 525–536.
Eigner, J., & Doty, P. (1965) *J. Mol. Biol. 12*, 549–580.
Feldman, M. Y. (1973) *Prog. Nucleic Acid Res. Mol. Biol. 13*, 1–49.

[2] Since we do not have appropriate reference spectra for Z family helical segments in host Watson–Crick B family helices, it is impossible to test this hypothesis by a suitable spectral analysis at the present time.

Frank-Kamenetskii, M. D. (1973) *Biopolymers 10*, 2623–2624.
Gray, D., Taylor, T., & Lang, D. (1978) *Biopolymers 17*, 145–157.
Hanlon, S., Brudno, S., Wu, T. T., & Wolf, B. (1975) *Biochemistry 14*, 1648–1660.
Hanlon, S., Glonek, T., & Chan, A. (1976) *Biochemistry 15*, 3869–3875.
Hanlon, S., Chan, A., & Berman, S. (1978) *Biochim. Biophys. Acta 519*, 526–536.
Maniatis, T., Venable, J., Jr., & Lerman, L. (1974) *J. Mol. Biol. 84*, 37–64.
Manning, G. (1969) *J. Chem. Phys. 51*, 924–933.
Manning, G. (1978) *Q. Rev. Biophys. 11*, 179–246.
McGhee, J. D., & von Hippel, P. H. (1975a) *Biochemistry 14*, 1281–1296.
McGhee, J. D., & von Hippel, P. H. (1975b) *Biochemistry 14*, 1297–1303.
McGhee, J. D., & von Hippel, P. H. (1977a) *Biochemistry 16*, 3267–3275.
McGhee, J. D., & von Hippel, P. H. (1977b) *Biochemistry 16*, 3276–3293.
Pohl, F., & Jovin, T. (1972) *J. Mol. Biol. 67*, 375–396.
Record, M. T., Jr. (1975) *Biopolymers 14*, 2137–2158.
Record, M. T., Jr., Woodbury, C., & Lohman, T. (1976) *Biopolymers 15*, 893–915.
Record, M. T., Jr., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103–178.
Schumaker, V. N., & Schachman, H. K. (1957) *Biochim. Biophys. Acta 23*, 628–639.
Tunis-Schneider, M., & Maestre, M. (1970) *J. Mol. Biol. 52*, 521–541.
Wang, A., Quigley, G., Kolpak, F., Crawford, J., van Boom, J., van der Marel, G. & Rich, A. (1979) *Nature (London) 282*, 680–686.
Wolf, B., Berman, S., & Hanlon, S. (1977) *Biochemistry 16*, 3655–3662.
Zimmerman, S., & Pheiffer, B. (1980) *J. Mol. Biol. 142*, 315–330.

Isolation and Characterization of Rat Liver Nuclear Matrices Containing High Molecular Weight Deoxyribonucleic Acid†

Ronald Berezney* and Linda A. Buchholtz

ABSTRACT: Rat liver nuclear matrices isolated by a method which limits DNA degradation contain a major portion of the total nuclear DNA. A majority of the DNA sediments at ≥100 S on alkaline sucrose gradients, which represents an estimated single strand size of ≥500 kilobases. These DNA-rich matrices were virtually identical with previously isolated DNA-depleted matrices in recovery of total nuclear protein and overall polypeptide composition on sodium dodecyl sulfate–acrylamide gels. Thin-sectioning electron microscopy revealed a structure similar to the DNA-depleted matrices with the addition of a prominent meshwork of DNA fibrils extended throughout the matrix interior. In vivo labeling of regenerating livers showed a continuous association of newly replicated DNA with DNA-rich matrices (≥80% of total labeled DNA) which is independent of the pulse period (1 min to 4 h). Moreover, the matrix-associated DNA is highly enriched in replicating intermediates after a 1-min in vivo pulse including a small amount of the primary Okazaki fragments. The matrix-associated replicating intermediates (4–50 S) are effectively chased into DNA of replicon size and larger (100 S) following a 1-h pulse. DNA-rich nuclear matrices may therefore provide a useful in vitro system for studying DNA replication in correlation with the higher order, intranuclear arrangement of eukaryotic DNA.

Interest has focused, recently, on the nuclear matrix as a structural milieu for the organization and integration of nuclear processess (Berezney & Coffey, 1976; Comings, 1978; Wunderlich, 1978; Shaper et al., 1979; Berezney, 1979a, 1981). A number of properties of isolated nuclear matrices support this possibility, including association of newly replicated DNA (Berezney & Coffey, 1975, 1976; Berezney, 1979b; Pardoll et al., 1980), newly transcribed RNA (Faiferman & Pogo, 1975; Miller et al., 1978; Herman et al., 1978; Herlan et al., 1979; Long et al., 1979; van Eekelen & van Venrooij, 1981), steroid receptor binding sites (Barrack et al., 1977, 1979; Barrack & Coffey, 1980; Agutter & Birchall, 1979), viral precursor proteins (Hodge et al., 1977; Chin & Maizel, 1977; Deppert, 1978; Buckler-White et al., 1980), and carcinogen binding sites (Hemminki & Vainio, 1979; Blazsek et al., 1979). With regard to DNA replication, it has been observed that a small proportion of the total nuclear DNA remains tightly bound to isolated nuclear matrices (Berezney & Coffey, 1975; Berezney, 1979a). This tightly bound or matrix-attached DNA represents 1–2% of the total DNA when isolated after controlled endogenous digestion of rat liver nuclei (Berezney & Coffey, 1975, 1976, 1977; Berezney, 1979a). The matrix-attached DNA fragments have an average size of 1–2 kilobases (kb)[1] and are highly enriched in 1-min-pulsed, in vivo

† From the Division of Cell and Molecular Biology, Department of Biological Sciences, State University of New York, Buffalo, New York 14260. *Received February 3, 1981.* Supported by U.S. Public Health Service Grant GM 23922. Portions of these studies were presented in preliminary form at the 71st Annual Meeting of the American Society of Biological Chemists (Berezney & Buchholtz, 1980) and at the Second International Congress on Cell Biology (Berezney et al., 1980).

[1] Abbreviations used: kb, kilobases; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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