itself. So that this method unlike chemical methods, can be used to detect changes in end-group frequencies due to enzyme attack or other structural change.

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Structure of Nucleic Acid–Poly Base Complexes*

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ABSTRACT: Complexes of DNA, RNA, and the synthetic polynucleotides, poly (A + U) and poly (I + C), with poly-L-lysine, prepared at high salt concentrations, have been studied.

The complexes containing DNA and the synthetic polynucleotides show anomalous circular dichroism, with greatly enhanced rotational strength, and in DNA and poly (A + U) an inversion of sign. Only relatively minor changes are found in the RNA complex. X-Ray diffraction studies of the complexes indicate that DNA remains in the B form, and appear to exclude any large degree of distortion of the helices, although poly (A + U) in the complex is evidently in the three-stranded form. No indication of periodicities in the range 30–150 Å were observed by low-angle X-ray diffraction. Reasons are given for believing that the anomalous optical activity may result from a liquid-crystal type of structure in the complexes. Electron microscopy shows that the salmon sperm DNA–polylysine complex is predominantly in the form of hollow doughnut-shaped particles, with a diameter of the order of 0.3 μ, and evidence of internal periodicity. With poly (A + U), cigar-shaped particles are found and, with poly (I + C) complexes, characteristic filaments. RNA in its complexes forms bundles of thin filaments, as well as long thick fibers, with a remarkably uniform diameter. They show no birefringence. The similarity of the DNA–polylysine particles to bodies found in chromatin of some kinds is noted.

The complexes formed between nucleic acids and the poly base, poly-L-lysine, have been widely studied as prototypic systems for nucleic acid–protein interactions. It has been established by Felsenfeld and his colleagues (Leng and Felsenfeld, 1966; Shapiro *et al.*, 1969) that if DNA and poly-L-lysine are allowed to react at high ionic strengths, where the interaction energy is diminished and the most stable conformations can form by "annealing," a high degree of specificity is manifested. The complexes formed under these conditions differ from those produced at low ionic strength in possessing a much higher thermal stability (Leng and Felsenfeld, 1966; Inoue and Ando, 1966), radically changed optical rotatory dispersion (Cohen and Kidson, 1968; Shapiro *et al.*, 1969) and circular dichroism (Shapiro *et al.*, 1969), and in manifesting a strong selectivity for DNA composition, with a strong preference for (A + T)-rich species. The high salt complexes are moreover particulate in nature, with apparently a rather narrow size distribution (Shapiro *et al.*, 1969). It is clear that it is these specific complexes which should be considered if such a simple system is to give information of general relevance to the specificity of the nucleic acid–protein recognition process.

Our object has been to examine the structural basis of the specific interaction between poly-L-lysine and helical poly-

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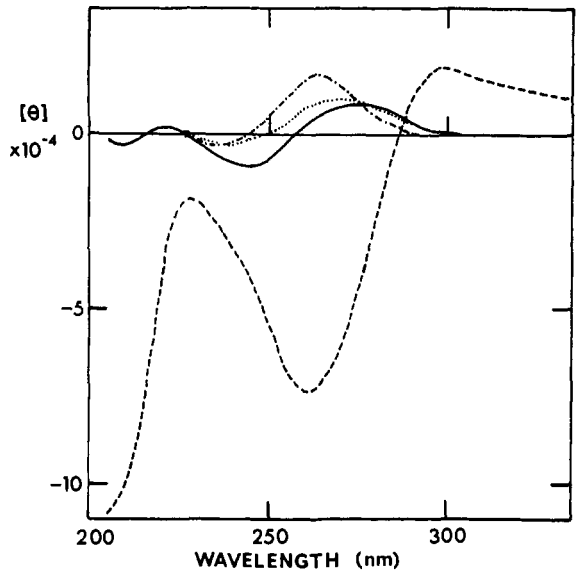

FIGURE 1: Typical circular dichroism curves of DNA and ribosomal RNA–polylysine complexes: (—) free DNA; (---) DNA–polylysine complex (phosphate–ε-amino groups, 2:1); (-·-·-) free RNA; (····) RNA–polylysine complex (2:1).

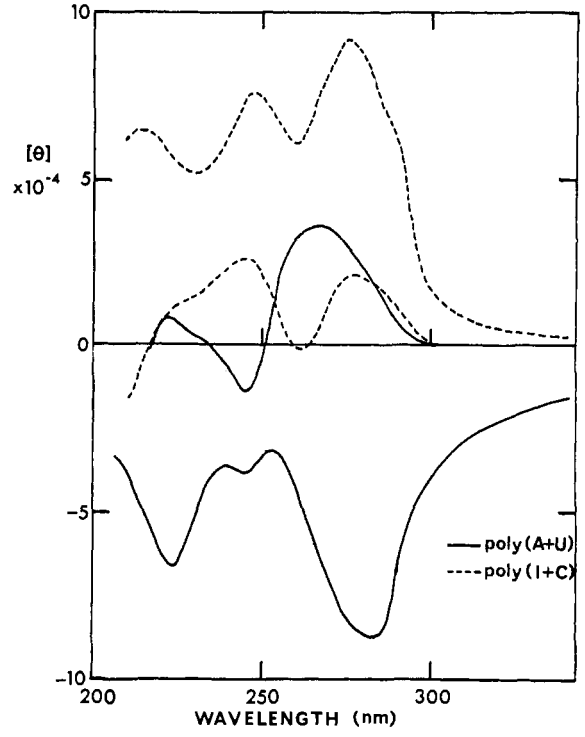

FIGURE 2: Typical circular dichroism curves of complexes between synthetic helical polynucleotides and poly-L-lysine. The curves of low amplitude are the circular dichroism of the free double-stranded polynucleotides; the other two curves correspond to polylysine complexes (2:1). (—) Poly (A + U) (which is almost certainly three stranded in the complex) (see Figure 3C); (---) poly (I + C).

nucleotides and to determine whether the physical characteristics of the complexes can be correlated with structural changes in the polynucleotide.

Experimental Section

High molecular weight DNA from salmon sperm and *Escherichia coli* were obtained from Sigma Chemical Co. The sedimentation coefficients ($s^0_{20,w}$) were, respectively, 17 and 19 S corresponding to molecular weights of the 4–5 million range (Eigner and Doty, 1965). *E. coli* rRNA was prepared from cells of nuclease-deficient strain, MRE 600 by the phenol detergent method of Nirenberg and Matthaei (1961). Synthetic polynucleotide samples from various commercial sources were screened by sedimentation and selected for high molecular weight. Two-stranded polynucleotide complexes were prepared under the conditions defined by Blake *et al.* (1967) and sedimented at about 10 S. A sample of poly (I + C) was also given to us by Dr. S. Arnott. Literature absorptivity values were used for polynucleotide concentrations. Poly-L-lysine samples of about 100,000 and 30,000 molecular weight from Pilot Chemical Corp. and Sigma were used. The concentrations were determined by weight, water content being initially estimated by measuring the primary amino group concentration of solutions by the dinitrofluorobenzene method (Dubin, 1960).

Complexes were prepared as indicated by Shapiro *et al.* (1969), by diluting solutions in sodium chloride; dilutions were made from 2 M sodium chloride. Most experiments were performed with 2:1 mixtures (phosphate–amino groups) since these were more manageable with respect to solubility. A detectable opalescence set in for poly (I + C) at 1.1 to 1.5 M NaCl, for rRNA at 1.3 to 1.7 M NaCl, and for poly (A + U) at about 1.2 M NaCl. Nucleotide concentration was always less than about 0.4 mg/ml before dilution.

Circular dichroism measurements were performed with a Jouan 185 dicrograph, with a scanning rate of 0.0625 nm/sec and a time constant of 4 sec. Peak absorbances were about 1 or below. Electron micrographs were taken with a Siemens Elmiskop 1 at approximately 40,000 magnification. Solutions containing 0.1–0.4 mg/ml of complex were deposited on carbon-coated grids and were positively or negatively stained with 2% aqueous uranyl acetate. The poly (A + U) and RNA–poly-L-lysine complexes did not stain positively. For X-ray diffraction, the complexes were pelleted by brief centrifugation at 70,000*g*. The gels were oriented as far as possible by stroking and examined at 92 and 98% relative humidity in an Elliott toroidal camera (Elliott, 1965), Franks camera (Franks, 1955), and in pin-hole collimating cameras (Langridge *et al.*, 1960), using calcite reflections for calibration. The samples diffracted X-rays only weakly, because of their high salt content. No differences in the low-angle X-ray patterns were detected when the salt was partially removed by washing the pellet with distilled water. The gels, unlike for example nucleohistone, were very stiff, possibly because of strong intermolecular interactions, and little orientation of the polynucleotide could be attained.

Results

Figure 1 shows the circular dichroism of a DNA–poly-L-lysine complex (2:1). This is qualitatively very similar to the data reported by Shapiro *et al.* (1969), showing greatly enhanced optical activity compared to DNA and inversion

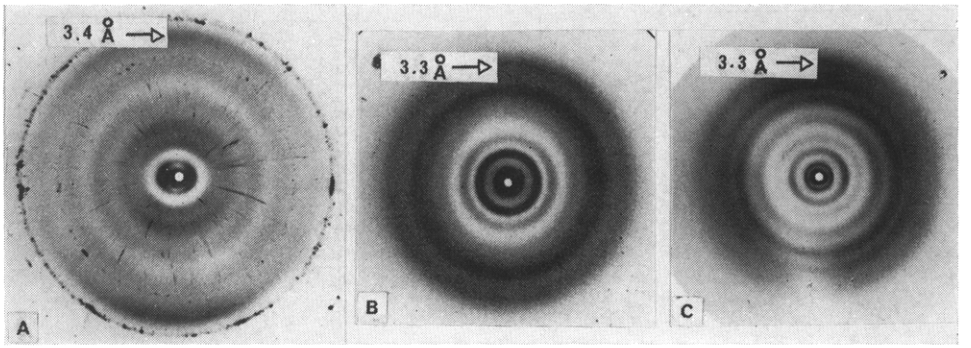

FIGURE 3: High-angle X-ray diffraction patterns of nucleic acid–poly-L-lysine equimolar complexes: (A) salmon sperm DNA complex, showing slight orientation, and spacings corresponding to the B conformation (see text). The sharp outer ring is the 3.0-Å calcite reflection used for indexing; (B) poly (I + C) complex; (C) poly (A-U) complex.

of sign of the principal longest wavelength Cotton effect. With RNA complexes, this effect is not observed. Instead there is a small red shift of the peak, with some diminution in amplitude (Figure 1). Complexes of poly (I + C) and poly (A + U) and polylysine were also examined, and showed anomalous circular dichroism (Figure 2). As with DNA there is substantial enhancement of the Cotton effects with considerable redistribution of relative intensities. With poly (A + U) the sign is reversed, but the Cotton effect at 246 nm appeared to retain its negative sense. In the poly (I + C) complex, the Cotton effects are not reversed, but that at longest wavelength is now the most prominent, and displays a distinct shoulder at its long-wavelength edge. In addition the shoulder in the region of 215 nm clearly emerges as a new maximum in the complex.

The X-ray diffraction diagrams of the complexes are poorly defined, partly because of overlay of polylysine diffraction, but more particularly by the striking failure of the gels to give any significant orientation. Slight orientation of the DNA–poly-L-lysine gels could sometimes be achieved, as shown in Figure 3A. The following spacings were determined: 3.33 Å (strong, sharp), 4.2 Å (weak, very diffuse), 5.6 Å (weak, diffuse), 8.5 Å (medium, diffuse), and 12.5 Å (strong, diffuse). In addition the equatorial reflection could be placed at 25 Å. These data are sufficient to identify the DNA conformation as the B form (Langridge *et al.*, 1960; Fuller, 1961) and exclude the likelihood of any major degree of distortion of the helix.

The pattern of the poly (I + C) complex (Figure 3B) is quite compatible with the double-helical conformation, but is not adequate to distinguish between the 11- or 12-fold forms (Arnott *et al.*, 1968). In the poly (A + U) complexes, the spacings indicate that the polynucleotide has entered the three-stranded state (Figure 3C; Arnott *et al.*, 1968).

In order to determine whether any longer-range order, such as a supercoil, is present in the complexes, all of these were examined in the low-angle region. The results are shown in Figure 4 in comparison to a typical native nucleohistone pattern which shows the familiar low-angle reflections, including the semimeridional 110-Å reflection, thought to be associated with a regular super-coiled structure (Pardon *et al.*, 1967). The DNA–poly-L-lysine complex generated no detectable reflections between 25 and 150 Å (Figure 4B); the poly (A + U) complex low-angle pattern was similar except for a weak diffuse ring at about 35 Å which is probably an interhelix packing reflection. However, both the poly (I + C) (Figure 4D) and the rRNA complexes (Figure 4C) produced continuous low-angle scatter; the former generated weak scatter 28 and 150 Å and no discrete reflection, whereas the latter produced intense continuous scatter between 30 and 120 Å. There is thus no good evidence from this source of any regular order in the complexes, of periodicity up to about 150 Å, but for RNA it is conceivable that the central scatter is generated by large periodicities in the structure.

The electron microscope study was undertaken initially to look for evidence of ordered structures of large periodicity, Shapiro *et al.* (1969) having already shown that DNA–polylysine complexes were particulate in nature, with a rather narrow size distribution. In our complexes of polylysine with salmon sperm DNA, particulate bodies of various kinds were in fact seen, predominant among which were toroidal,

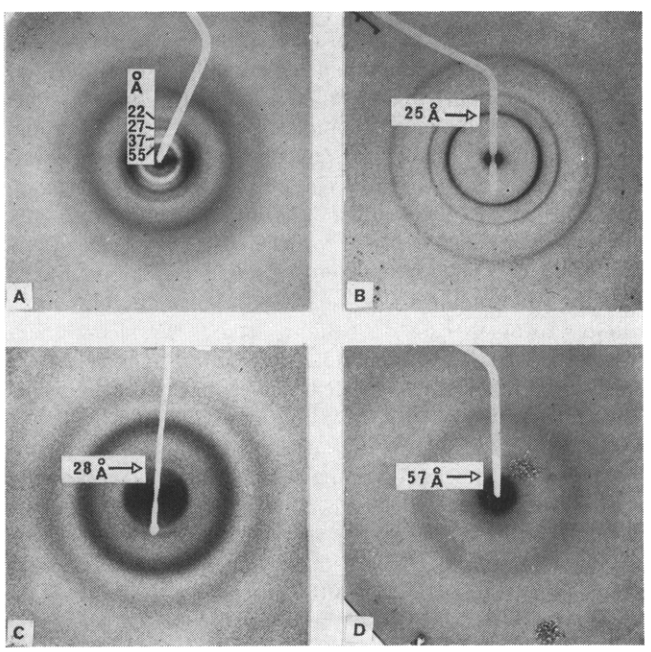

FIGURE 4: Low-angle X-ray diffraction patterns of complexes. (A) Native calf thymus nucleohistone, showing the characteristic series of low-angle reflections; (B) DNA–polylysine equimolar complex showing no reflections below 25 Å; (C) poly (I + C)–polylysine complex, showing diffuse central scatter; (D) rRNA–polylysine complex.

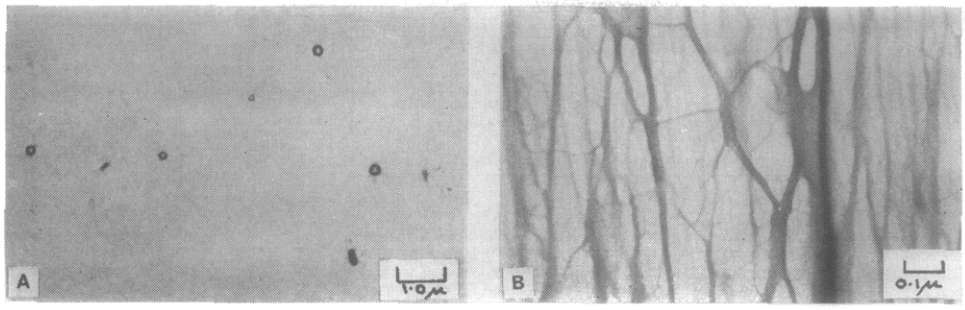

FIGURE 5: Electron micrographs of salmon sperm DNA–poly-L-lysine complexes: (A) complex prepared at high salt concentration (Shapiro *et al.*, 1969) (×7500); (B) complex prepared at low salt concentration (0.01 ionic strength) (×60,000).

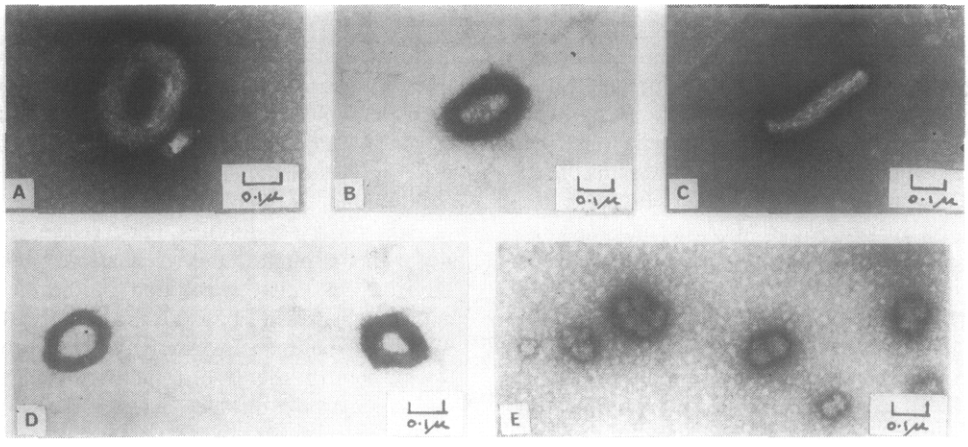

FIGURE 6: Electron micrographs of typical DNA–poly-L-lysine complex particles (×60,000). Parts A and C negatively stained salmon sperm DNA complexes (note apparent internal structure); parts B and D the same, positively stained (note fibers spreading from circumferences); part E particles abundantly found in complexes with *E. coli* DNA (all ×60,000).

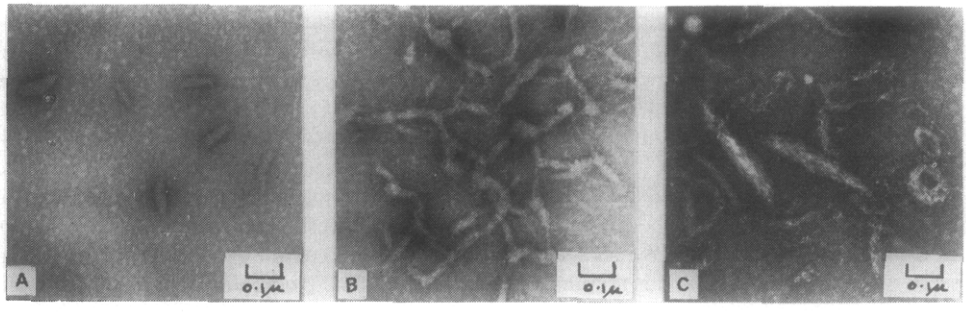

FIGURE 7: Complexes of helical synthetic polynucleotides with poly-L-lysine. Parts A and B poly (A + U) complex (polynucleotide probably three stranded); the formations seen in part B appear on the same grids as the isolated particles in part A; part C poly (I + C) complex (all ×60,000).

doughnut-shaped objects, with an external diameter in the region of 0.3 μ (Figure 5A). These particles increased in number with time after formation of the complex, and show in many cases what appear to be filaments of DNA separating from the circumference. There are also rod-like particles, which may be collapsed doughnuts, and various intermediate states. In negatively stained particles a substructure appears to be discernible (see Figure 6A) with a period of some 500 Å; this could however be a staining artefact. If the complex is prepared by mixing DNA and polylysine at low ionic strength (0.1), specific complexes are not formed (Leng and Felsenfeld, 1966) and the electron micrographs indeed show irregular threads, though a few doughnuts are sometimes to be seen. We must note that in our complexes of *E. coli* DNA the doughnuts, though present were less well defined, and we see many round particles apparently consisting of clusters of smaller particles with a hollow center (Figure 6D).

The poly (A + U) complexes show predominantly cigar-shaped objects (Figure 7A), which in places link up to form nets (Figure 7B). Poly (I + C) complexes, on the other hand, give rise to long thick fibers, of variable diameter, which in places curl to give formations that may be related to the

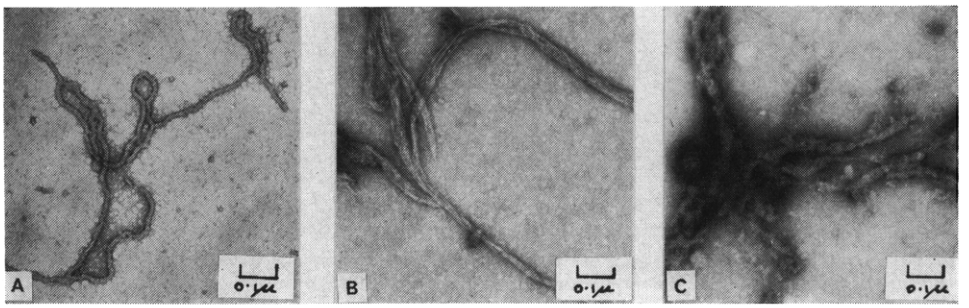

FIGURE 8: Complexes of rRNA with poly-L-lysine. Parts A and B two types of structure coexisting in complexes prepared at high ionic strengths. The thick isolated filamants of very uniform thickness shown in (A) predominate; (B) shows bundles of thin filaments; (C) complex prepared at low salt concentration (ionic strength, 0.01) (all ×60,000).

doughnuts (Figure 7C). Some structure (though we can again not exclude a staining artefact) appears to be present within these formations.

With rRNA a quite different phenomenon is observed. Whereas when the complex is prepared at low salt concentrations long irregular filaments occur, high-salt complexes show many long threads with remarkably constant thickness of about 160 Å, which associate into intricate nets (Figure 8A). There are also present in smaller quantities, structures of a different kind (Figure 8B) presenting the appearance of twisted bundles of thin filaments. In the polarizing microscope gels prepared from these complexes showed no birefringence, even after smearing between the microscope slide and cover slip. (We are indebted to Dr. M. Spencer for this determination.)

Discussion

The specific complexes which were shown by the seminal, work of Felsenfeld and his coworkers (Leng and Felsenfeld, 1966; Shapiro *et al.*, 1969) to be formed if the salt concentration is properly controlled have certain distinctive properties. In particular their stability as judged by melting profiles is very high, and they are formed preferentially with (A + T)-rich species. Another attribute is evidently the greatly enhanced and inverted optical activity (Cohen and Kidson, 1968; Shapiro *et al.*, 1969) which is illustrated in Figure 1. This is not present in our complexes of polylysine with rRNA. The solution, as in the case of the other complexes, shows considerable light scattering, and the red shift and diminution in amplitude are most probably explicable simply in terms of this. Circular dichroism, depending of course on the mode of measurement, is subject to the same aberrations as absorbance measurements in inhomogeneous media. Both the scattering and the "sieve" effect lead to distortion of the spectrum; flattening of the bands and red shifts are predicted (Urry and Ji, 1969) and observed (Ji and Urry, 1969). At all events, it is clear that the structural factors which lead to the anomalous circular dichroism of the DNA complexes are absent in those of RNA.

Complexes of polylysine with the fully helical polynucleotides, poly (A + U) and poly (I + C) show the anomalous circular dichroism (Figure 2), with large increases in rotational strength, and inversion of at least the two most prominent Cotton effects of poly (A + U) but not those of poly (I + C). The redistribution of relative intensities indicates that different transition moments are differently affected by the formation of the complexes. One may surmize that the large changes in optical activity accompany the formation of specific complexes, and that the required structures are only formed by fully helical polynucleotides.

The origins of the changes in optical activity are not easy to determine, as Shapiro *et al.* (1969) have noted. One may consider three possibilities. (1) A large change in base tilt and/or helix pitch. It is clear from well-founded theory (Tinoco, 1968) that a rotation of the electronic transition dipoles of successive bases in the helix relative to each other can bring about an inversion in the sense, magnitude and distribution of the optical activity and any considerable diminution in the distance between the chromophores could give rise to a large increase in the interaction potential and hence in the optical activity. (2) An appropriate disposition of the charge of the ϵ-amino groups in relation to the bases could supply a large asymmetric perturbation, with for example consequent mixing of the π–π^* transition with out-of-place transitions. (3) An ordered anisotropic arrangement of the molecules within the particles of complex, similar to that of cholesteric liquid crystals, could generate the large optical activity observed in such systems (Robinson, 1961; De Vries, 1962).

The interpretation of the X-ray data which we have described in terms of the conformation of the polynucleotide in the complexes presents considerable difficulties. The apparent transition of the poly (A + U) to a three-stranded form in the complex is not relevant to these considerations; three-stranded complexes are always formed under conditions of high electrostatic shielding, whether by sodium or magnesium ions (Stevens and Felsenfeld, 1964; Blake *et al.*, 1967), and their circular dichroism does not differ greatly from that of the two-stranded form (Brahms, 1965). In the DNA complexes, the X-ray diffraction patterns seem to leave little doubt that the double helix is in the normal high-humidity B form, which one supposes to obtain in aqueous solution (Brahms and Mommaerts, 1964; Maestre and Schneider, 1969), and is not distorted in any gross way, such as would greatly alter the interaction between transition moments. It may be noted that in the less-defined complexes formed at low ionic strengths, the DNA evidently also remains in the B form (Wilkins, 1956; Zubay *et al.*, 1962). We must emphasize at the same time, that we cannot completely exclude that a change in the geometry of the complex occurs when it is pelleted and partially dried, although this seems relatively unlikely.

The second explanation of the optical activities cannot be altogether eliminated, but one must suppose that as with other poly acid–poly base complexes (see, *e.g.*, Katchalsky, 1964) association involves direct pairing of charges, and indeed this is strongly borne out by the work of Miller and Inbar (1969), who show that there is progressive displacement of counterions in DNA–polylysine complexes as the salt concentration is decreased. Any strong interaction of the charged ϵ-amino group with a base is therefore very improbable.

There remains the third explanation, that the anomalous rotatory strength arises from the kind of phenomenon associated with some liquid crystals. Very large rotations in such media have long been known, and were theoretically treated for the cholesteric type of structure by De Vries (1951). Robinson and his coworkers (Robinson *et al.*, 1958; Robinson, 1961) have found cholesteric liquid crystals in polybenzyl-L-glutamate, and also in DNA, and striking example of these and other forms were also described in gels of helical RNA (Spencer *et al.*, 1962). Robinson and coworkers found that the De Vries theory satisfactorily explains the optical activity of liquid crystalline polybenzyl-L-glutamate, the rotation increasing with the periodicity of the structure. Our complexes are not a single phase, and if the particles are to be regarded as having liquid crystalline character, the solution must then be seen as a liquid crystal phase dispersed in the aqueous medium. If the rotational strength arises entirely from the particles, we can extrapolate to the hypothetical pure liquid-crystalline phase. From the partial specific volume of the complex which we derive by adding the contributions for DNA and polylysine, one finds that a molar solution (referred to phosphorus) corresponds to some 25% by volume of complex. It is then possible to work out the ellipticity or optical rotation of the pure complex in the absence of the aqueous phase. Thus, for example, taking the value of Shapiro *et al.* (1969) for the molar residue rotation of the complex at 350 nm, it transpires that a 1-mm layer of pure complex phase would have a rotation of $-350°$. De Vries' treatment for cholesteric liquid crystals of periodicity small compared to the wavelength, reduces as Robinson (1961) has noted to $\phi = -4.5 \times 10^4 \Delta n^2 P \lambda^{-2}$, where P is the periodicity and λ the wavelength, both in microns, and Δn the intrinsic birefringence of the substance. Since we are concerned here only with the orders of magnitude we put $\Delta n = 0.1$, which is the highest birefringence commonly observed in DNA fibers, and with $\phi = -350°$ and $\lambda = 0.35$ μ, P emerges as about 0.07 μ or 700 Å. It can be argued that this type of calculation has too little basis in physical reality, and that there is too much flexibility in the physical and numerical assumptions that have to be made to make it quantitatively useful. We give it here merely to show that a liquid crystalline type of structure *could* in principle quantitatively account for the anomalous optical activity. As with polybenzylglutamate (Robinson, 1961) the rotations will be expected to depend on concentration, structure, molecular weight, etc., and indeed Shapiro *et al.* (1969) have noted the wide variability of the magnitude of optical activity in DNA–polylysine complexes.

At all events the observed optical activities are in no way incompatible with a periodicity of the order of 500 Å, which appears to be present in the electron micrograph of Figure 5A (and also of Figure 7C). Such a large periodicity would not in fact be expected to appear in our low-angle X-ray diffraction patterns.

Light-scattering and sedimentation data led Shapiro *et al.* (1969) to the conclusion that the DNA–polylysine complexes had the form of particles of rather uniform size and high solvation (another factor arguing for liquid crystals) which if spherical would have a diameter of some 0.34 μ. This agrees well with the size range of our doughnut particles. Each body must contain many DNA molecules and in many cases some of these can be seen separating from the circumference. The particles produced by the helical synthetic polynucleotides evidently do not have the same tendency to bend and form rings. One may conjecture that they too have liquid crystalline types of structure, presumably differing in periodicity from those of DNA.

The RNA complexes are striking because of the very uniform thickness of the fibers. rRNA is known to contain short double-helical segments separated by single-stranded loops, and there have been reports (see Spirin, 1963) of positive birefringence in such molecules, which would suggest an alignment of the short helices perpendicularly to the long axis. If these short helices were of uniform length this would suggest an explanation for the uniformity of the complexes in the electron microscope. Unfortunately, we are compelled to exclude this explanation because of the absence of any measurable birefringence in the polarizing microscope.

Elongated particulate structures in the electron microscope were also previously reported by Matsuo *et al.* (1969) for complexes particularly of two-stranded RNA with poly-L-lysine prepared at the intermediate salt concentration of 0.5 M.

We cannot at this stage reach any conclusions about the disposition of the polynucleotide helix and the polylysine within any of the various particles. The DNA complexes are curiously reminiscent of ring chromosomes (White, 1948) and indeed all shapes seen in the electron micrographs have at least superficially similar counterparts in chromosomes (White, 1948) of various kinds. It may be noted that chromosomes show little birefringence and the DNA is therefore evidently not arranged in any simple linear uniform manner. The possibility may be considered that DNA has a general propensity to form circular structures (*cf.* the tubular formations recently described in electron micrographs of interphase nuclei of chick erythrocytes (Davies and Small, 1969)), and that it is this property which determines the morphology of chromatin.

Acknowledgments

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Added in Proof

Examination of the electron microscope images of the DNA–polylysine particles, such as that shown in Figure 6A, by optical diffraction reveals that internal periodicities are indeed present. These observations, by Dr. E. J. O'Brien, are being further pursued.

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Uracil–Thymine Adduct from a Mixture of Uracil and Thymine Irradiated with Ultraviolet Light*

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ABSTRACT: A uracil–thymine adduct isolated from a uracil and thymine mixture irradiated with ultraviolet (254 nm) light in frozen aqueous solution is characterized as 5-hydroxy-6-(4′-pyrimidin-2′-one)dihydrothymine. This assignment was made on the basis of mass, ultraviolet, infrared, and nuclear magnetic resonance spectra. The compound readily undergoes dehydration to yield P_2B (6-(4′-pyrimidin-2′-one)thymine) which has been identified as a product from the acid hydrolysates of DNA irradiated with ultraviolet light. A mechanism for its formation is presented and its possible importance in the photochemistry and photobiology of nucleic acids is discussed.

A new product has recently been isolated from acid hydrolysates of DNA irradiated with ultraviolet light (254 nm) both *in vivo* and *in vitro* (Varghese and Wang, 1967). This product, P_2B, which has been characterized as 6-(4′-pyrimidin-2′-one)thymine (I) (Wang and Varghese, 1967), is derived from a cytosine–thymine adduct (II) or (III). It can also be isolated from acid hydrolysates of thymidine–cytidine mixture irradiated in frozen aqueous solution (Varghese and Patrick, 1969) and from thymine–uracil mixture irradiated in frozen aqueous solution (M. N Khattak and S. Y. Wang, unpublished results).

We now wish to report the isolation and characterization of a uracil–thymine adduct (U–T adduct, III), a precursor of P_2B (I). This compound may be analogous to, indeed may even be identical with, the pyrimidine moiety of the ultraviolet-induced lesions in DNA responsible for the isolation of P_2B in acid hydrolysates. In addition, this U–T adduct readily undergoes dehydration to give P_2B.

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