# Interaction of Kanchanomycin with Nucleic Acids. II. Optical Rotatory Dispersion and Circular Dichroism*

Paul A. Friedman, Ting-Kai Li,† and Irving H. Goldberg

ABSTRACT: Optical rotatory dispersion and circular dichroism measurements have been employed to study the binding of kanchanomycin to deoxyribonucleic acid, ribonucleic acid, and synthetic polynucleotides. Kanchanomycin is optically active and exhibits an extrinsic Cotton effect in the presence of $Mg^{2+}$ and polynucleotides. In the presence of $Mg^{2+}$ the antibiotic forms two distinct complexes with deoxyribonucleic acid, ribonucleic acid, and certain other polynucleotides, an initial complex I which is converted with time into a final complex II, in agreement with spectral data. The rate and the extent of the time-dependent shift vary, depending upon the base composition of the polynucleotide examined. Complex I dissociates readily and complex II slowly upon removal of $Mg^{2+}$ with a chelating agent leading to restoration of the original optical properties of free kanchanomycin; thus, it is unlikely that covalent bonds are formed in either complex. Kanchanomycin binds quantitatively to polyadenylic acid when added to solutions containing equivalent amounts of polyadenylic acid and either native calf thymus deoxyribonucleic acid or polyguanylic acid. Titration of polyadenylic acid with kanchanomycin suggests that for complex I formation there are as many binding sites per polynucleotide as there are bases. The data suggest that formation of complex I involves in part electrostatic interaction of kanchanomycin–$Mg^{2+}$ with the negatively charged phosphate containing backbone of the polynucleotides. Formation of complex II appears to involve the nucleotide bases since bromination of polyuridylic acid, which destroys the aromaticity of the pyrimidine ring, allows formation of complex I but prevents conversion into complex II.

Kanchanomycin, a highly cytotoxic antibiotic of unknown structure, which causes rapid cessation of synthetic processes in mammalian and bacterial cells, inhibits isolated DNA-dependent RNA and DNA polymerases (P. B. Joel, P. A. Friedman, and I. H. Goldberg, in preparation) and interacts with polynucleotides in the presence of magnesium (Friedman *et al.*, 1969). Absorption spectrophotometric studies indicate that the antibiotic forms two distinct complexes with certain polynucleotides, an initial complex (I) which converts with time into a final complex (II). The two types of complexes can be differentiated by their spectral properties and their relative ease of dissociation upon removal of magnesium with a chelating agent.

Since the shifts in wavelength of maximal absorption upon complex formation are quantitatively small, further elucidation of the nature of these complexes has been sought by study of optical rotatory dispersion and circular dichroism. Optical rotatory dispersion has recently been employed to advantage in studying the interaction of dyes and other antibiotics with polynucleotides (Blake and Peacocke, 1965–1967a,b; Gardner and Mason, 1967; Yamaoka and Ziffer, 1968).

The data clearly indicate that complex I is formed with all the polynucleotides examined; the rate and extent of the time-dependent shift to complex II varies, depending upon the base composition of the polynucleotide. It is suggested that the initial binding of the antibiotic–$Mg^{2+}$ complex to polynucleotide involves in part the negatively charged phosphate ribose backbone of the polynucleotides. Complex I is readily dissociated by the removal of $Mg^{2+}$ with a chelating agent, while complex II is only slowly dissociated. Since such treatment results in the restoration of the original optical properties of the free antibiotic, it is not likely that covalent bonds are formed in these interactions.

## Materials and Methods

Stock solutions of kanchanomycin and of native and of heat-denatured calf thymus DNA were prepared as previously described (Friedman *et al.*, 1969). All solutions measured for optical activity contained standard buffer, 0.01 M Tris (pH 7.5) in 10% dimethylformamide unless otherwise indicated.

Poly A, poly C, poly U, poly G, poly I, and poly (A,G) were purchased from Miles Laboratories, Elkhart, Ind. Bromination of poly U was performed by the method of Yu and Zamecnik (1963). $d(A\text{-}T)_n$ was a gift of Dr. A. Cerami; sodium polyphosphate and sodium poly-L-glutamate were gifts of Dr. Bert L. Vallee.

Optical rotatory dispersion and circular dichroism spectra were measured from 450 to 300 mμ with a Cary

* From the Department of Medicine, Harvard Medical School, and the Beth Israel Hospital, and from the Biophysics Research Laboratory of the Department of Biological Chemistry, Harvard Medical School, and the Peter Bent Brigham Hospital, Boston, Massachusetts. *Received October 22, 1968.* This work was supported by grants from the National Institutes of Health, U. S. Public Health Service (GM 12573 and CA 10736) and the American Cancer Society.

† Markle Scholar in Academic Medicine.

Model 60 recording spectropolarimeter and circular dichroism attachment. The slit width of the instrument was programmed to yield constant light intensity at all wavelengths. Measurements were performed at 23° in a 1-cm path-length cell having fused-quartz end plates (Opticell). The instrument was calibrated to give zero rotation for the buffer blanks at all wavelengths. Based on a molecular weight of 600 for kanchanomycin, optical rotatory dispersion was expressed in molar rotation calculated from the equation, $[M] = \alpha_{obsd} \times MW/l \times C$, where $\alpha_{obsd}$ was rotation observed in degrees, $MW$ was the molecular weight of kanchanomycin, $l$ was the path length in decimeters, and $C$ was the concentration of antibiotic in g/100 ml. Circular dichroism measurements were expressed as molecular ellipticity calculated from the equation, $[\Theta] = \Theta_{obsd} \times MW/10 \times l \times C$, where $\Theta_{obsd}$ was observed ellipticity, $MW$ was the molecular weight of kanchanomycin, $l$ was the path length in centimeters, and $C$ was the concentration of antibiotic in grams per milliliter.

In one series of experiments optical rotatory dispersion was expressed as specific rotation calculated from the equation, $[\alpha] = \alpha_{obsd} \times 10^2/l \times C$, where path length, $l$, was in decimeters and concentration, $C$, was in g/100 ml. Optical activity of either the nucleic acid or the polynucleotide involved has been subtracted from all spectra described. Where indicated, the samples were incubated at 37° for the specified length of time.

## Results

*Optical Rotatory Dispersion and Circular Dichroism Spectra of Kanchanomycin, Kanchanomycin Plus Magnesium, and Kanchanomycin Complexed with Poly A or with Native Calf Thymus DNA.* Kanchanomycin is optically active, exhibiting a complex Cotton effect curve within its broad, visible absorption envelope centered at about 373 mμ (Figure 1). It consists of two shallow troughs at 378 and 345 mμ, a crossover point at 331mμ, and a peak at 308 mμ, suggesting two negative Cotton effects. The circular dichroism spectrum shows two corresponding negative extrema at approximately 362 and 331 mμ. The optical activity of kanchanomycin in 2 M NaCl, 8 M urea, or in 100% dimethylformamide is identical with that shown in Figure 1. Upon addition of $Mg^{2+}$ the optical rotatory dispersion trough at 378 mμ increases in amplitude, that at 345 mμ decreases, the crossover point shifts from 331 to 340 mμ, and a new peak appears at 316 mμ. The resulting circular dichroism curve shows corresponding shifts in the two negative extrema to 365 and 336 mμ and a positive extremum appears at approximately 403 mμ. In the presence of $Mg^{2+}$ and excess poly A, kanchanomycin undergoes dramatic and immediate optical rotatory dispersion and circular dichroism spectral shifts: the optical rotatory dispersion curve shows a deep trough at 378 mμ, a crossover point at 355 mμ, and a peak at 320 mμ while the circular dichroism curve shows a large negative extremum at 357 mμ (Figure 1). In the absence of $Mg^{2+}$ poly A does not alter the spectrum of kanchanomycin. As has already been observed by

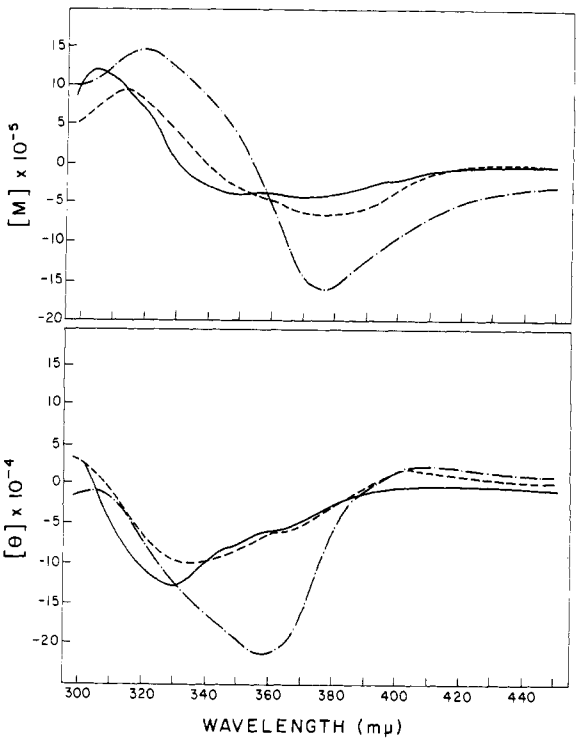


FIGURE 1: Optical rotatory dispersion and circular dichroism spectra of kanchanomycin, kanchanomycin plus $Mg^{2+}$, and kanchanomycin plus poly A in the presence of $Mg^{2+}$. (—) 33 μM kanchanomycin; (---) 33 μM kanchanomycin and 80 μM $Mg^{2+}$; (·-·-·) 33 μM kanchanomycin, 440 μM poly A, and 80 μM $Mg^{2+}$; the spectrum is unchanged after 20-hr incubation.

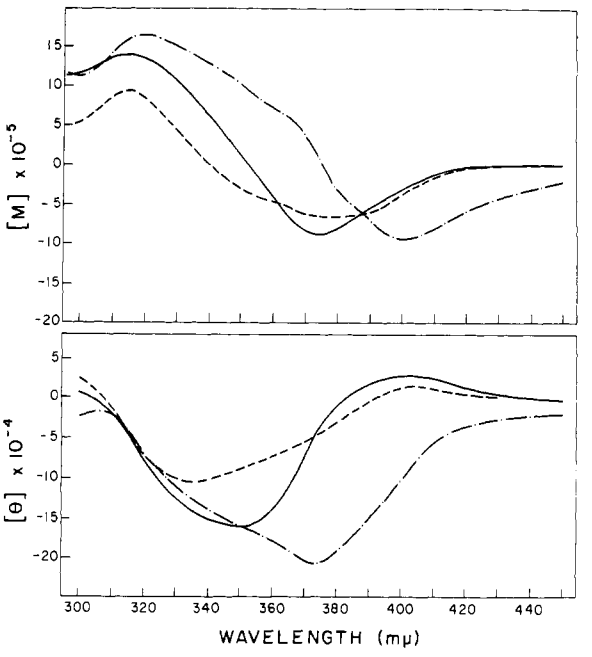


FIGURE 2: Optical rotatory dispersion and circular dichroism spectra of the interaction of kanchanomycin with native calf thymus DNA. (---) 33 μM kanchanomycin and 80 μM $Mg^{2+}$; (—) 33 μM kanchanomycin, 440 μM native calf thymus DNA, and 80 μM $Mg^{2+}$ without incubation; (·-·-·) same after 20-hr incubation.

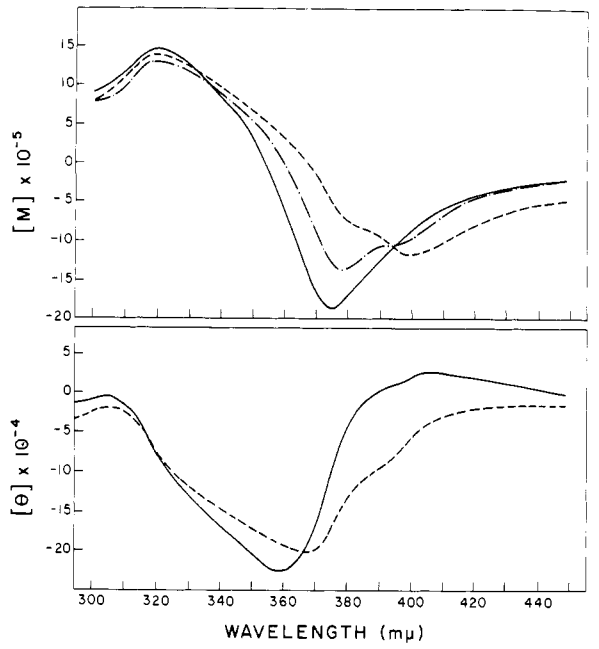


FIGURE 3: Optical rotatory dispersion and circular dichroism spectra of the interaction of kanchanomycin with poly U. (—) 33 μM kanchanomycin, 440 μM poly U, and $Mg^{2+}$ (80 μM or 1 mM) without incubation; (---) the solution with 80 μM $Mg^{2+}$ after 20-hr incubation; (-·-·) the solution with 1 mM $Mg^{2+}$ after 20-hr incubation.

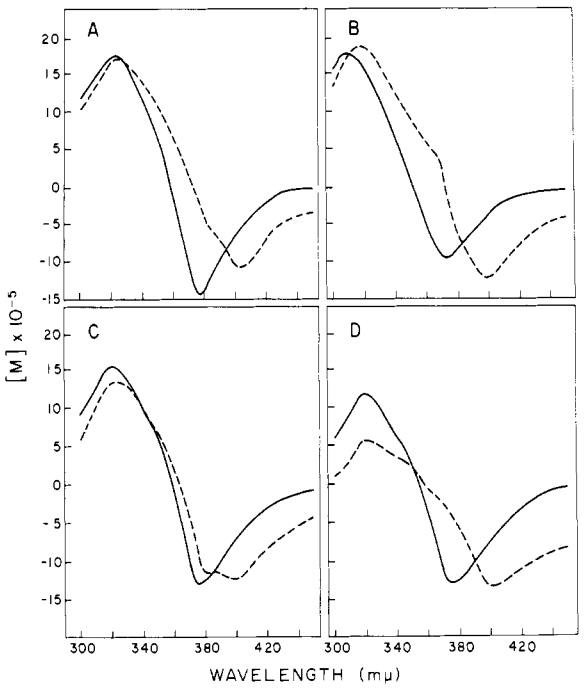


FIGURE 4: Optical rotatory dispersion of the interaction of kanchanomycin with various other polynucleotides. (A) *E. coli* tRNA, (B) poly G, (C) poly A:G (1:1), and (D) poly d(A-T)$_n$. All solutions contained 33 μM kanchanomycin, 80 μM $Mg^{2+}$, and 440 μM of the respective polynucleotide. (—) No incubation and (---) 20-hr incubation.

absorption spectrophotometry (Friedman *et al.*, 1969), kanchanomycin complexed to poly A is stable, since there is no alteration of the optical rotatory dispersion and circular dichroism curves on incubation of the sample at 37°.

The optical rotatory dispersion and circular dichroism spectra of kanchanomycin complexed with native calf thymus DNA in the presence of $Mg^{2+}$ differ from that with poly A (Figure 2). The immediate effect on the optical rotatory dispersion spectrum is the appearance of a discrete but less deep trough at 374 mμ, a 352-mμ crossover point, and a peak at 315 mμ while the circular dichroism curve shows a negative extremum at 351 mμ and a shoulder at 331 mμ; again there is a positive extremum at approximately 403 mμ. With incubation, however, there is a further spectral change, a gradual broadening of the Cotton effect with a shift of the trough to longer wavelengths. After 20-hr incubation this transformation is complete; there is a new trough at 400 mμ and a new crossover at 376 mμ. The circular dichroism spectrum shows a corresponding shift with a negative extremum at 375 mμ and loss of the positive extremum. These results are compatible with those from absorption spectra and indicate that kanchanomycin forms an initial complex (I) with calf thymus DNA which is converted with time into a second complex (II).

*Complexes of Kanchanomycin with Other Polynucleotides.* Since the complex formed with native DNA differs from that formed with poly A in terms of the time-dependent spectral shifts and the spectral location of the optically active bands, other polynucleotides were examined. The interaction of poly U with the antibiotic shows a number of unique features (Figure 3). While the immediate optical rotatory dispersion and circular dichroism spectra are virtually identical with those that kanchanomycin gives in the presence of poly A and $Mg^{2+}$, conversion into complex II does occur, but the shift is slower than with calf thymus DNA, *i.e.*, only part of complex I has converted into complex II after 20-hr incubation. Moreover, complex II formation can be further retarded by higher concentrations of $Mg^{2+}$($10^{-3}$ M). This stabilization of complex I by high $Mg^{2+}$ is observed only with poly U. Interaction of antibiotic with poly C is identical with that with poly U except that this effect of high $Mg^{2+}$ is lacking, and there is a more rapid conversion into complex II.

Interaction of kanchanomycin with some of the other polynucleotides and nucleic acids studied is shown in Figure 4. With *Escherichia coli* tRNA (Figure 4A) an initial complex forms which has an optical rotatory dispersion spectrum identical with that of poly U and undergoes complete transition to complex II after 20-hr incubation. The spectra of kanchanomycin with purified whole yeast RNA or heat-denatured calf thymus DNA are identical with that with *E. coli* tRNA except for slight differences in the rate of conversion into complex II. The initial optical rotatory dispersion spectrum of kanchanomycin complexed to denatured calf thymus DNA thus differs from that for native DNA and is similar to those of the ribohomopolymers in

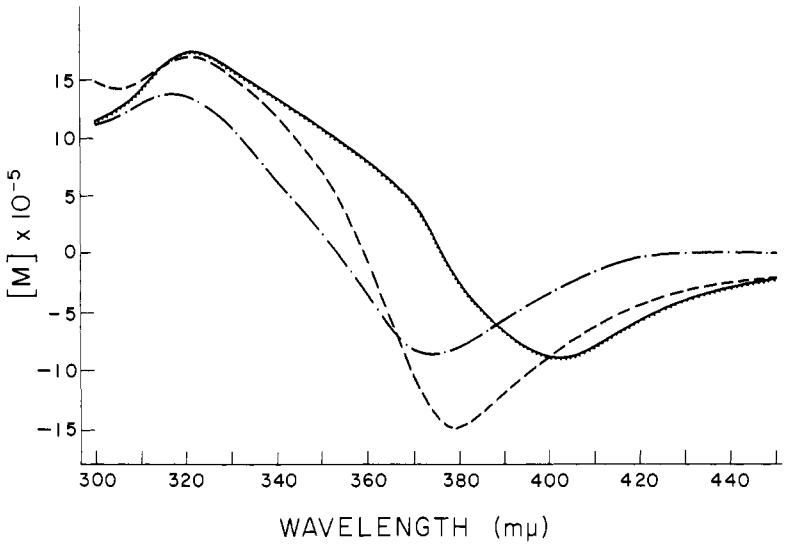


FIGURE 5: Competition between poly A and native calf thymus DNA for kanchanomycin (optical rotatory dispersion). (---) 33 $\mu$M kanchanomycin, 440 $\mu$M native calf thymus DNA, 80 $\mu$M $Mg^{2+}$, and 440 $\mu$M poly A (which was added last), zero time and 20-hr incubation; (-·-·-) 33 $\mu$M kanchanomycin, 440 $\mu$M native calf thymus DNA, and 80 $\mu$M $Mg^{2+}$, no incubation; (—) 33 $\mu$M kanchanomycin, 440 $\mu$M native calf thymus DNA, and 80 $\mu$M $Mg^{2+}$ after 20-hr incubation; (····) addition of 440 $\mu$M poly A to the solution containing 33 $\mu$M kanchanomycin, 440 $\mu$M native calf thymus DNA, and 80 $\mu$M $Mg^{2+}$, incubated 20 hr prior to poly A addition. The immediate pattern does not change even after 4 hr of further incubation.

having a deeper trough at 376 m$\mu$. The initial complex formed between the antibiotic and poly G shows an optical rotatory dispersion spectrum with a shallow trough at 374 m$\mu$, a crossover point at 352 m$\mu$, and a peak at 312 m$\mu$ (Figure 4B), closely resembling that of the kanchanomycin–$Mg^{2+}$–DNA initial complex. Since both native DNA and poly G contain ordered structures, this may account for the similarity in the optical properties of their antibiotic complex. With poly (A,G) kanchanomycin forms an initial complex, but the shift to complex II is only partial after incubation for 20 hr (Figure 4C). No further spectral changes occur even after another 28-hr incubation indicating that this transition has reached an end point. These data suggest that poly (A,G) has "runs" of adenine (regions structurally identical with poly A) with which the antibiotic may interact to form complex I and which do not convert into complex II. In contrast, the initial complex formed with the synthetic double-stranded copolymer $d(A\text{-}T)_n$ appears to convert completely into complex II (Figure 4D). The magnitude of the optical change representing the initial complex is slightly greater than that observed with native DNA. In $d(A\text{-}T)_n$ there are no "runs" of adenine but rather perfect alternation of adenine with thymine in each strand. It seems, therefore, that binding of antibiotic to single adenine residues, if this occurs with dAT, does not prevent conversion into complex II.

In all cases, kanchanomycin does not form a complex with the polynucleotides unless $Mg^{2+}$ is present. Previous studies indicate a requirement for $Mg^{2+}$ concentration equivalent to kanchanomycin concentration for complete interaction (Friedman *et al.*, 1969), thus, the 80 $\mu$M $Mg^{2+}$ used in the above studies was more than enough to allow all antibiotic present to interact with polynucleotide.

*Preference of the Antibiotic for Poly A.* Since complex I formed with poly A is stable, it was of interest to determine if this stability would be reflected by a tendency of the antibiotic to bind to poly A in a solution containing poly A and a second polynucleotide. Kanchanomycin (33 m$\mu$moles/ml) was added to 3-ml samples containing 440 m$\mu$moles/ml (as phosphate) each of poly A and either native calf thymus DNA or poly G in 0.01 M Tris (pH 7.5). $Mg^{2+}$ was added last to a final concentration of $8 \times 10^{-5}$ M. In both cases the observed optical rotatory dispersion spectrum of kanchanomycin was identical with that obtained under similar conditions but with only poly A present in solution. There was no alteration of the optical rotatory dispersion pattern with incubation indicating that all the antibiotic was bound to poly A. Further, if the antibiotic is first exposed to either poly G or calf thymus DNA in the presence of $Mg^{2+}$ and a complex I optical rotatory dispersion pattern is obtained, immediate addition of an equivalent amount of poly A to the solution results in a new optical rotatory dispersion pattern identical with that for interaction of kanchanomycin with poly A (Figure 5). Here too there is no change in the spectrum with incubation indicating that there has been a quantitative shift of all the antibiotic molecules to poly A. However, if complex II with either poly G or calf thymus DNA is first allowed to form and then poly A is added, the antibiotic remains complexed to the initial polynucleotide as demonstrated by retention of the typical complex II optical rotatory dispersion pattern seen immediately after poly A addition and with subsequent incubation. These data in-

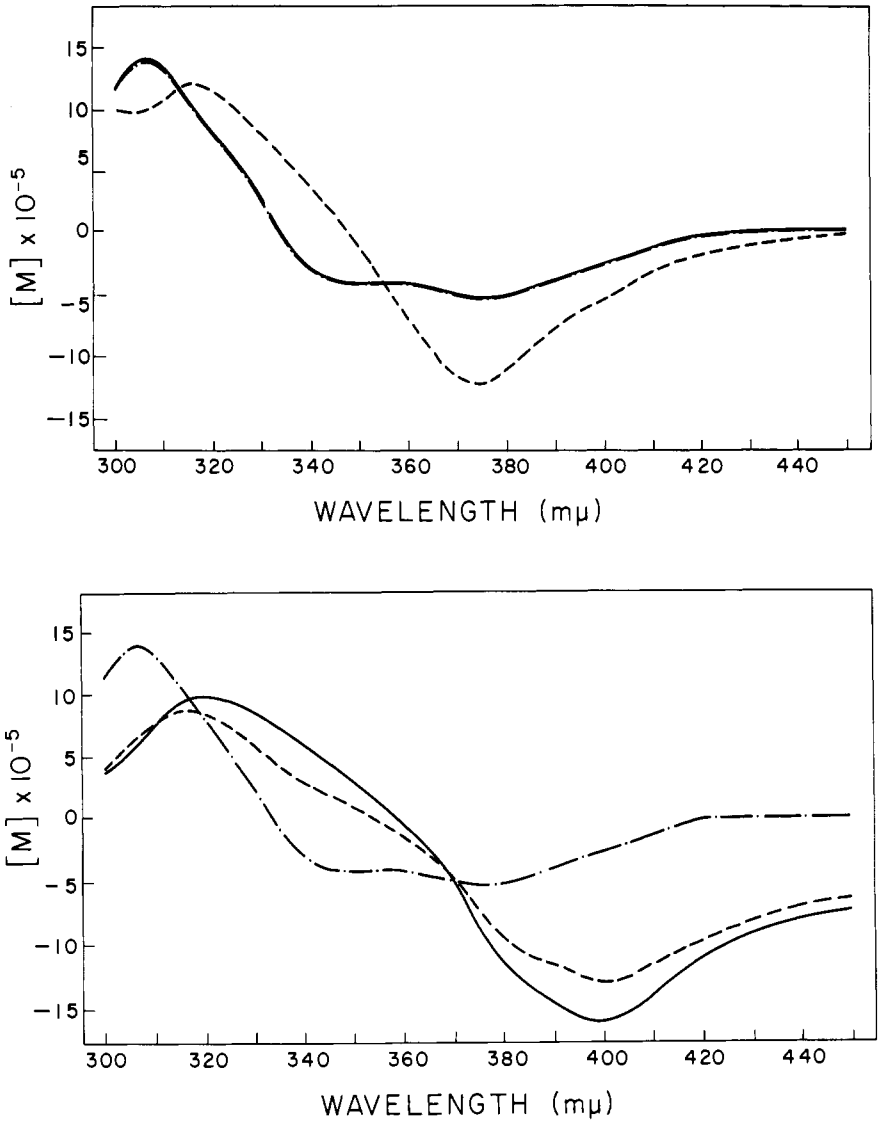


FIGURE 6: Reversibility of complex I and complex II by EDTA (optical rotatory dispersion). (A) (—) 33 μM kanchanomycin; (---) 33 μM kanchanomycin, 440 μM native calf thymus DNA, and 80 μM $Mg^{2+}$, no incubation; EDTA was then added to a final concentration of 8 mM and an immediate spectrum recorded (·---·). (B) (—) 33 μM kanchanomycin, 440 μM native calf thymus DNA, and 80 μM $Mg^{2+}$, 4-hr incubation. EDTA was added to a final concentration of 8 mM and spectra were recorded immediately (---) and after 2 (·---·) hr incubation.

dicate that some structural element of the poly A molecule allows the antibiotic special access to its binding sites and imparts to it a degree of stability such that even if kanchanomycin has formed a complex with native calf thymus DNA or poly G it will abandon that molecule to complex with poly A. Complex II, however, once formed between the antibiotic and that polynucleotide, is stable in the presence of poly A.

*Reversibility of Complex I and II.* The effects of a 100-fold molar excess of EDTA over $Mg^{2+}$ on complexes I and II are shown in Figure 6. Addition of EDTA to complex I leads to immediate dissociation of the complex and return of the optical rotatory dispersion spectrum to that of free kanchanomycin (Figure 6A). Addition of the chelating agent to complex II results in a much slower dissociation which becomes complete after 2-hr incubation at 37° (Figure 6B). Thus, it is evident that neither complex is the result of an irreversible reaction, though complex II is of such a nature that $Mg^{2+}$ is more tenaciously retained.

*Titration of Poly A with Kanchanomycin.* In an effort to determine the number of binding sites available for kanchanomycin on a polynucleotide molecule a titration was performed in which the poly A concentration was kept constant at 66 mμmoles phosphate/ml while the antibiotic concentration was varied over a range from one-tenth to one-half the concentration of the phosphate in the polymer (Figure 7). Poly A was the polynucleotide of choice for this study since only one type of complex (I) forms with kanchanomycin. Precipitation of the complex prevented accurate measurements above a ratio of 1:2. At ratios of 1:2 and 1:3.3,

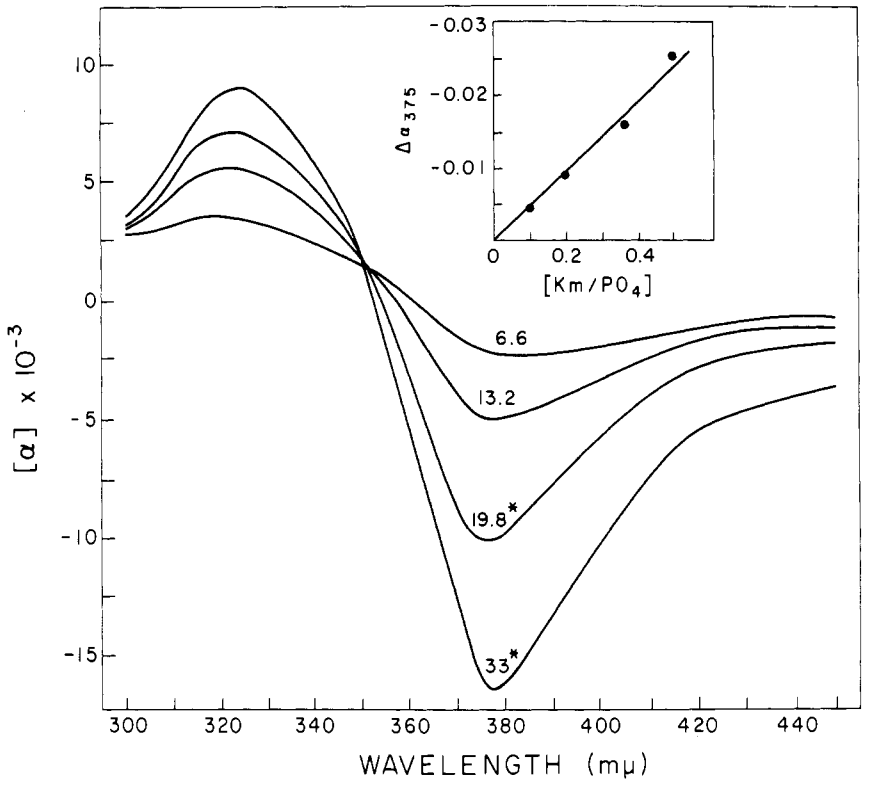


FIGURE 7: Titration of kanchanomycin *vs.* poly A. Optical rotatory dispersion spectra of individual 3-ml solutions containing 66 $\mu$M poly A and 80 $\mu$M $Mg^{2+}$ with varying concentrations of kanchanomycin (6.6, 13.2, 19.8, and 33 $\mu$M, respectively). The insert shows the $\Delta\alpha_{375}$ (observed $\alpha_{375}$ of each of the above solutions of kanchanomycin minus the $\alpha_{375}$ of free kanchanomycin at each of these concentrations) plotted *vs.* the ratio of kanchanomycin to poly A–phosphate. *These samples showed evidence of slight precipitation at the time the spectra were run.

precipitation also became evident at the end of the spectral measurements. The data suggest that kanchanomycin is fully bound at a ratio of antibiotic/nucleotide as high as 1:2 (Figure 7) and it is likely that for complex I formation there are as many binding sites per polymer as there are nucleotides.

It has been reported that the extrinsic Cotton effect of proflavine bound to calf thymus DNA is abolished by decreasing the ratio of dye to nucleotide (Blake and Peacocke, 1965). Such a result can be accounted for by dye–dye interactions. To see if dilution of kanchanomycin in the presence of a constant concentration of calf thymus DNA would affect the optical rotatory dispersion of the complex, the spectrum of a 1-ml solution containing 33 m$\mu$moles of kanchanomycin complexed to 440 m$\mu$moles of DNA-P (a ratio of 1:13) measured in a 1-cm path-length cell was compared with that of a 10-ml solution containing 3.3 m$\mu$moles/ml of kanchanomycin complexed to 440 m$\mu$moles/ml of DNA-P (a ratio of 1:130) in a 10-cm path-length cell. No difference in the optical rotatory dispersion pattern was observed. These results indicate that either dye–dye interactions do not contribute to the complex Cotton effect that bound kanchanomycin exhibits or such interactions occur to the same extent even at the lower ratio of antibiotic to DNA.

*Interaction of Kanchanomycin with Brominated Poly U and with Polyelectrolytes.* In an attempt to clarify further the nature of the binding of kanchanomycin to polynucleotides, the interaction of the antibiotic with a number of other macromolecules was studied. A comparison was made between the complex formed with poly U and that formed with brominated poly U, in which the pyrimidine moiety has been saturated at the C5, 6 position but which retains intact the phosphate–ribose backbone (Figure 8). It is apparent that the antibiotic can still form complex I with brominated poly U, but there is virtually no conversion into complex II. Only a slight spectral shift is observed which is attributable to unbrominated residues in the polymer with which the antibiotic may bind and still convert into complex II. Thus, by abolishing the aromaticity of the pyrimidine rings of poly U, conversion into complex II is prevented. It is not known, however, what prevents the formation of complex II with poly A.

Since complex I forms with brominated poly U, it was of interest to see if kanchanomycin would interact with other polyanions. Sodium polyphosphate and sodium polyglutamate in the presence of $Mg^{2+}$ cause a shift in the kanchanomycin optical rotatory dispersion spectrum similar to that caused by the ribohomopolymers (Figure 9A). No shift of the spectrum occurs with incubation. In addition, the circular dichroism spectra demonstrate that high ionic strength (2 M NaCl) partially diminishes the interaction with polyphosphate (Figure 9B). It was necessary to use a higher $Mg^{2+}$ concentration to obtain binding in all studies involving polyphosphate since this polymer competes with the

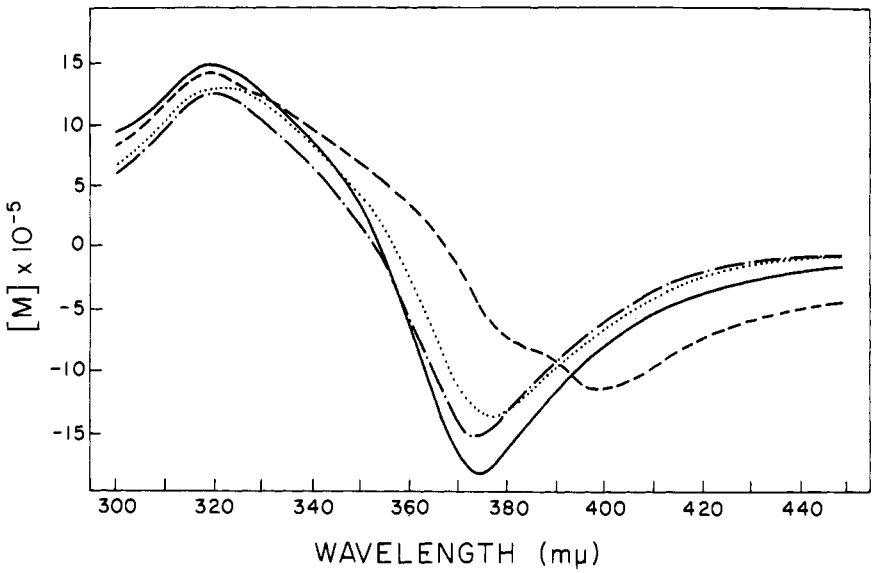


FIGURE 8: Optical rotatory dispersion of the interaction of kanchanomycin with brominated poly U. (—) 33 μM kanchanomycin, 440 μM poly U, and 80 μM $Mg^{2+}$, no incubation; (---) same solution after 20-hr incubation; (·-·-) 33 μM kanchanomycin, 440 μM brominated poly U, and 80 μM $Mg^{2+}$, no incubation; (····) same solution after 20-hr incubation.

antibiotic for the cation. As with poly U, a $Mg^{2+}$ concentration of 1 mM may prevent the formation of a second complex; however, the stability of complex I between kanchanomycin and brominated poly U at a $Mg^{2+}$ concentration of 80 μM suggests that this is highly unlikely.

Large excesses (500 μg/ml) of neither bovine serum albumin nor polylysine with or without $Mg^{2+}$ give any evidence of interaction with kanchanomycin (33 μM). The former is negatively charged at pH 7.5 while the latter is a polycation. The addition of kanchanomycin and $Mg^{2+}$ to AMP or ADP results in immediate precipitation thus precluding optical rotatory dispersion measurements.

## Discussion

The amino acridines, of which proflavine and acridine orange have been most extensively studied, are optically inactive molecules which become optically active when bound to nucleic acids possessing ordered structures (Neville and Bradley, 1961; Mason and McCaffery, 1964; Blake and Peacocke, 1966, 1967a,b; Gardner and Mason, 1967). Unlike these dyes, free kanchanomycin itself has optical activity which may arise in one of two manners: first, there may be an inherent dissymmetry in the molecule, as is the case for the glutarimide antibiotics (Johnson *et al.*, 1968) which are optically active. Second, polymerization of inherently symmetric dye molecules could lead to a species devoid of a rotation–reflection symmetry axis (Crabbé, 1965). For example, the monomer of pseudoisocyanine (1,1′-diethyl-2,2′-cyanine) is optically inactive but polymerizes in aqueous solution to form an optically active helix (Mason, 1964). Moreover, a molecule may possess optical activity both in monomeric and aggregated states, *e.g.*, actinomycin (T. K. Li, 1967, unpublished data; Ziffer *et al.*, 1968; Crothers *et al.*, 1968). Since the structure of kanchanomycin is currently unknown, it is difficult to ascertain from which source its dissymmetry derives. However, the large magnitude of the optical activity of free kanchanomycin suggests that it has inherent asym-

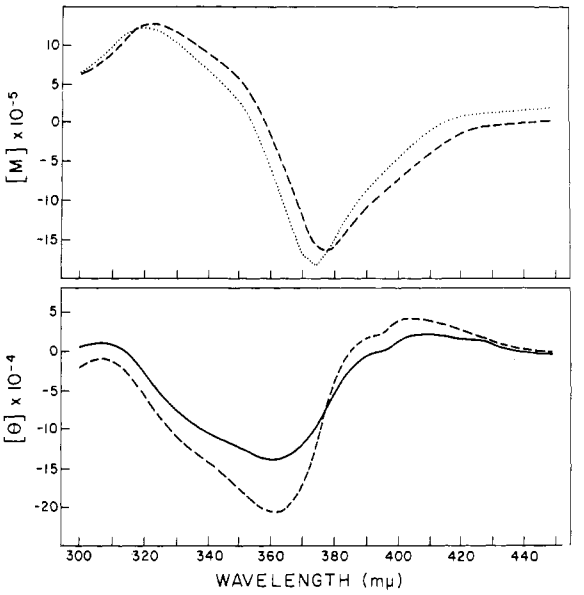


FIGURE 9: Top: (A) optical rotatory dispersion of the interaction of kanchanomycin with polyelectrolytes. (····) 33 μM kanchanomycin, sodium polyglutamate (500 μg/ml), and 80 μM $Mg^{2+}$ after 0- and 20-hr incubation; (---) 33 μM kanchanomycin, sodium polyphosphate (500 μg/ml), and 1 mM $Mg^{2+}$ after 0- and 20-hr incubation. Bottom: (B) circular dichroism of the interaction of kanchanomycin with polyphosphate in high salt. (---) 33 μM kanchanomycin, sodium polyphosphate (500 μg/ml), and 1 mM $Mg^{2+}$, no incubation; (—) similar solution also containing 2 M NaCl; the 1 mM $Mg^{2+}$ was added last.

metry since the molar rotation and ellipticity values for inherently dissymmetric molecules are generally higher when compared with inherently symmetric but asymmetrically perturbed chromophores (Crabbé, 1965). Further, the optical rotatory dispersion properties of kanchanomycin remain unchanged in 8 M urea, 2 M NaCl, or in 100% dimethylformamide suggesting that the antibiotic does not polymerize in aqueous solution at the concentrations employed in these studies. Finally, since $Mg^{2+}$ must be present in a concentration equal to kanchanomycin for interaction with polynucleotides to be complete, it is likely that the optically active monomer forms a complex with $Mg^{2+}$ which subsequently interacts with the polynucleotide.

Cooperative binding of dyes to macromolecules has been reported for acridine orange, proflavine, and methylene blue (Beers and Armilei, 1965; Blake and Peacocke, 1965; Stone, 1965). Optical activity decreases and disappears as the ratio of binding sites to dye increases causing decreasing aggregation of dye molecules. It has been calculated that the dimer of acridine orange (Gardner and Mason, 1967) is itself the repeat unit responsible for optical activity and that for proflavine aggregates as small as the dimer can yield optical activity (Cox and Peacocke, 1967). While cooperative binding of kanchanomycin to polynucleotides appears to occur (Friedman *et al.*, 1969), a tenfold increase in the ratio of phosphate to kanchanomycin causes no change in optical activity. Thus, it is likely that dye–dye interaction does not contribute in a major manner to spectral changes occurring when kanchanomycin binds to polynucleotides.

Recent optical rotatory dispersion and circular dichroism studies on the binding of amino acridines to nucleic acids support the concept that there are two types of binding: one, at high nucleotide/dye ratio, in which the acridine can interact both with the nucleotide bases and electrostatically with the negative phosphate groups on the polynucleotide chain; and a second, at low nucleotide/dye ratio, in which the acridine binds primarily to the negative phosphate groups and which is further stabilized by dye–dye interaction (Blake and Peacocke, 1966, 1967a; Pritchard *et al.*, 1966). For other compounds known to form complexes with DNA including Miracil D (Hirschberg *et al.*, 1968), quinacrine (O'Brien *et al.*, 1966), and chloroquine (Cohen and Yielding, 1965), it has been suggested that they bind by the first mechanism. Basic antibiotics of the amino glycoside type, however, appear to form complexes with DNA purely by electrostatic interaction (Zimmer *et al.*, 1967).

The rapidly formed initial complex between kanchanomycin and nucleic acids in the presence of $Mg^{2+}$ (complex I) appears to be in large measure electrostatic in nature involving the negatively charged phosphate groups. This conclusion is founded on the observations that kanchanomycin interacts with polyphosphate and polyglutamate yielding optical rotatory dispersion and circular dichroism spectra that are very similar to those of complex I formed with poly A and other polynucleotides as well as with denatured DNA. These are unlike the initial complex formed with nucleic acids by the hepatotoxin, luteoskyrine, which requires purine bases and denatured regions in the polynucleotide (Ohba and Fromageot, 1967). It is proposed that the kanchanomycin–$Mg^{2+}$ complex is a positively charged species which interacts electrostatically with the phosphates of the polynucleotide backbone like the aforementioned basic antibiotics. However, the smaller magnitude of the optical rotatory dispersion and circular dichroism spectra that are observed with native DNA, poly G, and d(A-T)$_n$ suggest that there is a dependence of optical activity upon the conformation of the polymer. Since native DNA and d(A-T)$_n$ are helical structures and since poly G has a very stable secondary structure at neutral pH, this would imply that the change in optical activity of kanchanomycin induced by these polynucleotides as compared with others may reside in the differences in orientation of the individual dye molecules to the polynucleotide as well as to each other. Despite the likely inherent dissymmetry of kanchanomycin, as a ligand it is still subject to environmental influences, and one must conclude that the smaller magnitude of optical activity (and, thus molecular dissymmetry) displayed by kanchanomycin bound to the above species reflects these environmental differences.

The tendency of kanchanomycin when added to a solution containing equivalent amounts of poly A and either native calf thymus DNA or poly G, to bind to poly A indicates that more than electrostatic interaction with phosphate is involved in complex I formation, when polynucleotides are involved. In addition, with poly A complex I is stable in contrast to those with other polynucleotides. A binding preference for poly A has also been noted for acridine orange (Beers and Armilei, 1965). The reason for the high affinity and stability is unclear. All of the double-stranded and other single-stranded polynucleotides examined form complex II. At neutral pH, about two-thirds of the bases in poly A are in a single-stranded helical conformation stabilized by base stacking (Brahms *et al.*, 1966; Van Holde *et al.*, 1965). A similar conformation has been described for poly C (Fasman *et al.*, 1964; Michelson *et al.*, 1967), but its complex I is also unstable. Hence, strandedness and helicity are not adequate explanations. In this regard, it is of interest that poly (A,G) converts only partially into complex II, and d(A-T)$_n$ converts completely into complex II. In the former, there are "runs" of poly A and poly G, while in the latter, adenine alternates with thymine. Hence, unless kanchanomycin exhibits even greater affinity for thymine than for adenine, these data suggest that the cooperative interaction of adjacent adenines with kanchanomycin–$Mg^{2+}$ stabilizes complex I.

On the other hand, complex II formation clearly implicates an interaction of the antibiotic with the polynucleotide bases. Bromination of poly U, which abolishes the aromaticity of the pyrimidine ring, prevents the formation of complex II. Polyphosphates and poly-L-glutamate, lacking the aromatic bases, do not form complex II. Since complex II of kanchanomycin–$Mg^{2+}$ with DNA can be dissociated by the chelating agent EDTA, covalent bonds are not involved, distinguishing this complex from those in which irrever-

sible chemical bonds are formed between dye and nucleic acid.

Another factor to be considered underlying the spectral changes in the conversion of complex I into complex II is a time-dependent change in polynucleotide conformation induced by the bound kanchanomycin. Kanchanomycin, interacting with native calf thymus DNA in the presence of a $Mg^{2+}$ concentration equivalent to the nucleotide concentration, induces a time-dependent increase in viscosity of the solution (Friedman *et al.*, 1969). Such an effect is not observed under the conditions employed for these spectral studies. Further, measurements in the 260-m$\mu$ range reveal no differences in optical rotatory dispersion between solutions of complexes I and II of kanchanomycin–$Mg^{2+}$ with DNA. However, since kanchanomycin itself also exhibits optical activity in this wavelength region, it cannot be completely ruled out that changes in optical rotation resulting from alternation of polynucleotide conformation may be masked by equal and opposite changes in optical activity of bound kanchanomycin.

Other studies with kanchanomycin have shown that the antibiotic inhibits DNA-dependent RNA and DNA polymerases (P. B. Joel, P. A. Friedman, and I. H. Goldberg, in preparation), uncouples oxidative phosphorylation by mitochondria (P. A. Friedman and I. H. Goldberg, 1967, unpublished results), and interacts with polynucleotides in the presence of $Mg^{2+}$ (Friedman *et al.*, 1969). The present study confirms and examines in greater detail the binding of kanchanomycin to polynucleotides, and shows, in addition that its interaction is not restricted to nucleic acids and polynucleotides. It also binds to other polyanionic molecules, such as polyphosphates and polyglutamic acid, indicating that it can interact with a variety of negatively charged molecules. It appears, therefore, that this highly cytotoxic antibiotic may interfere directly not only with nucleic acid synthesis, but also with other pathways of cellular metabolism. That its action in cell-free systems has some specificity, however, is indicated by experiments in which polypeptide synthesis by extracts from *E. coli* was not affected by high levels of the antibiotic (I. H. Goldberg, 1967, unpublished results).

## References


Beers, R. F., and Armilei, G. (1965), *Nature 208*, 466.

Blake, A., and Peacocke, A. R. (1965), *Nature 206*, 1009.

Blake, A., and Peacocke, A. R. (1966), *Biopolymers 4*, 1091.

Blake, A., and Peacocke, A. R. (1967a), *Biopolymers 5*, 383.

Blake, A., and Peacocke, A. R. (1967b), *Biopolymers 5*, 871.

Brahms, J. A., Michelson, A. M., and Van Holde, K. E. (1966), *J. Mol. Biol. 15*, 467.

Cohen, S. N., and Yielding, K. L. (1965), *J. Biol. Chem. 240*, 3123.

Cox, R. A., and Peacocke, A. R. (1967), *J. Polymer Science 23*, 765.

Crabbé, P. (1965), Optical Rotatory Dispersion and Circular Dichroism in Organic Chemistry, Holden-Day, San Francisco, Calif., pp 7–24.

Crothers, O. M., Sabol, S. L., Ratner, D. I., and Muller, W. (1968), *Biochemistry 7*, 1817.

Fasman, G. D., Lindblow, C., and Grossman, L. (1964), *Biochemistry 3*, 1015.

Friedman, P. A., Joel, P. B., and Goldberg, I. H. (1969), *Biochemistry 8*, 1535 (this issue; preceding paper).

Gardner, B. J., and Mason, S. F. (1967), *Biopolymers 5*, 79.

Hirschberg, E., Weinstein, I. B., Gersten, N., Merner, E., Finklestein, T., and Carchnon, R. (1968), *Cancer Res. 28*, 601.

Johnson, F., Duquette, L. G., and Hennis, H. E. (1968), *J. Org. Chem. 33*, 904.

Mason, S. F. (1964), *Proc. Chem. Soc.*, 119.

Mason, S. F., and McCaffery, A. J. (1964), *Nature 204*, 468.

Michelson, A. M., Massoulie, J., and Guschbauer, W. (1967), *Prog. Nucleic Acid Res. Mol. Biol. 6*, 83.

Neville, D. M., and Bradley, D. F. (1961), *Biochim. Biophys. Acta 50*, 397.

O'Brien, R. L., Olenick, J. G., and Hahn, F. E. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1511.

Ohba, T., and Fromageot, P. (1967), *European J. Biochem. 1*, 147.

Pritchard, N. J., Blake, A., and Peacocke, A. R. (1966), *Nature 212*, 1360.

Stone, A. L. (1965), *Biopolymers 3*, 617.

Van Holde, K. E., Brahms, J., and Michelson, A. M. (1965), *J. Mol. Biol. 12*, 726.

Yamaoka, K., and Ziffer, H. (1968), *Biochemistry 7*, 1001.

Yu, C.-T., and Zamecnik, P. C. (1963), *Biochim. Biophys. Acta 76*, 209.

Ziffer, H., Yamaoka, K., and Mauger, A. B. (1968), *Biochemistry 7*, 996.

Zimmer, C., Triebel, H., and Thrum, H. (1967), *Biochim. Biophys. Acta 145*, 742.