Interaction of Kanchanomycin with Nucleic Acids. I. Physical Properties of the Complex*

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ABSTRACT: Kanchanomycin, a highly cytotoxic antibiotic, which disrupts cellular synthetic processes, including inhibition of deoxyribonucleic acid dependent ribonucleic acid and deoxyribonucleic acid polymerases, forms stable complexes with various polynucleotides. Changes in the visible absorption spectrum of kanchanomycin indicate that the antibiotic interacts with polynucleotides only in the presence of Mg^{2+}. An initial complex (I) forms immediately and changes with time to a second complex (II) with different spectral properties. pH titration studies suggest that Mg^{2+} interacts with the first dissociable group of kanchanomycin and complex II formation involves a second dissociable group. There appears to be no base specificity or requirement for a given secondary structure in the polynucleotide for the initial complex formation.

Of all the polynucleotides studied, only polyadenylic acid forms a stable initial complex, which does not convert into complex II. Both complex forms are retained on Millipore filters. Complex formation is completely inhibited by 0.1 M NaCl. Complex I, but not complex II, can be partially dissociated by NaCl. Complex I is readily dissociated by EDTA; complex II is only slowly dissociated by EDTA. Kanchanomycin (in the second complex) decreases the buoyant density of $d(A\text{-}T)_n$, increases the sedimentation rate of $d(A\text{-}T)_n$, and increases the viscosity of deoxyribonucleic acid. Kanchanomycin does not increase the temperature of thermal transition of $d(A\text{-}T)_n$. Although the spectral changes require a Mg^{2+} concentration equal to the kanchanomycin concentration, the retention of the kanchanomycin–Mg^{2+}–polynucleotide complex on Millipore filters and the changes in viscosity of deoxyribonucleic acid require a Mg^{2+} concentration equal to the nucleotide concentration.

In recent years several antibiotics have been reported which inhibit DNA-dependent RNA synthesis by forming a complex with the DNA template (Reich and Goldberg, 1964; Ward *et al.*, 1965; Kersten *et al.*, 1966; Ueno *et al.*, 1967). For a given antibiotic there may be specific structural prerequisites in the polynucleotide for binding to occur. For example, actinomycin, chromomycin, mithramycin, and olivomycin require the 2-amino group of guanine of native helical DNA for complex formation (Reich and Goldberg, 1964; Cerami *et al.*, 1967; Ward *et al.*, 1965). On the other hand, it has been suggested that luteoskyrin, a hepatotoxin, binds to purine bases in denatured regions on the DNA molecule (Ohba and Fromageot, 1967). Luteoskyrin (Ueno *et al.*, 1967) as well as chromomycin, mithramycin, and olivomycin (Ward *et al.*, 1965), has been shown to require a divalent cation for binding to DNA.

Kanchanomycin, a yellow antibiotic of unknown structure produced by a *Streptomyces* species, is bactericidal and tumoricidal at extremely low concentrations (Liu *et al.*, 1963; Bateman *et al.*, 1965). This antibiotic has been found to inhibit both enzymatic RNA and DNA synthesis, but by different mechanisms (P. B. Joel, P. A. Friedman, and I. H. Goldberg, in preparation). While kanchanomycin inhibits DNA synthesis by interference with the template function of the DNA, it can inhibit RNA synthesis by inactivating the RNA polymerase itself. In this paper we present a study on the nature of the interaction of kanchanomycin with polynucleotides. In the presence of divalent cation the spectral properties of the antibiotic are altered and many of the physical properties of the polynucleotides are changed.

Materials and Methods

Kanchanomycin was a gift of Charles Pfizer, Maynard, N. J. A stock solution of the antibiotic was prepared at a concentration of 500 μg/ml in 37.5% dimethylformamide or 600 μg/ml in 100% dimethylformamide. The stock solution was stored in the dark at $-20°$; at this temperature kanchanomycin precipitates from 37.5% dimethylformamide but warming to room temperature redissolves the antibiotic. There is no change in the spectrum with repeated redissolving over a period of several weeks. In 0.01 M Tris (pH 7.5) containing 10% dimethylformamide, the molar extinction coefficient of kanchanomycin at wavelength 373 mμ is 25,000 based on a molecular weight of 600 (Liu *et al.*, 1963).

Calf thymus DNA was purchased from Sigma Chemical Co. The stock solution contained 4.5 mM DNA-P in 0.01 M Tris (pH 7.5). The molar extinction coefficient

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for nucleotide phosphate at 260 mμ was 6800. Heat-denatured DNA was prepared by heating a solution of native DNA in 0.01 M Tris (pH 7.5) in a boiling-water bath for 10 min and then immediately cooling the solution in a water bath at 0°.

Poly A, poly U, poly C, and poly I were purchased from Miles Laboratories, Elkhart, Ind. Unlabeled and tritiated (methyl-labeled thymidine) d(A-T)$_n$[1] were prepared by unprimed synthesis using *Escherichia coli* DNA polymerase (Schachman *et al.*, 1960). The d(A-T)$_n$ product was deproteinized with phenol and exhaustively dialyzed against 1 mM EDTA, 0.5 M NaCl, and 0.01 M Tris (pH 7.5) and finally against 0.01 M Tris (pH 7.5). The specific activity of the [^{3}H]d(A-T)$_n$ was approximately 2500 cpm/mμmole. The DNA polymerase and d($\overline{\text{DAP}}$-T)$_n$ were gifts from Dr. A. Cerami. Tritiated T7-DNA was prepared according to the method of Richardson *et al.* (1964).

Absorption spectra were recorded with a Cary 14 recording spectrophotometer. Thermal transition curves were determined with a Gilford Model 2000 multiple-sample absorbance recorder equipped with linear thermosensor control and a temperature-programming device.

To avoid artificially inducing cooperative binding of kanchanomycin to polynucleotides, the antibiotic was added to a polynucleotide solution without $MgCl_2$ to obtain uniform distribution of the dye in the solution prior to binding. $MgCl_2$ at twice final concentration in one-half final volume was then added slowly with gentle mixing. Solutions containing kanchanomycin were kept in the dark or a dim light at all times. In experiments requiring incubation, the samples were placed in covered test tubes and kept in the dark in a constant-temperature water bath at the temperature indicated.

In the experiments in which samples were filtered through Millipore filters, the filters were 24 mm in diameter with a pore size of 0.22 μ. After incubation, samples were diluted with approximately 5.5 volumes of cold 0.05 M NaCl–0.01 M Tris (pH 7.5) and immediately poured onto filters. The filters were then washed with approximately 15 ml of the same buffer. No air was allowed to pass through the filters until completion of the washing. The filters were dried under a heat lamp, placed in vials with 10 ml of scintillator solution (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of toluene), and counted in a Packard scintillation spectrometer.

Results

Spectral Changes upon Interaction of Kanchanomycin with DNA and Mg^{2+}. Addition of ethanol (final concentration 66%) to a solution containing 440 μM calf thymus DNA, 33 μM kanchanomycin, and 80 μM Mg^{2+} leads to the precipitation of yellow fibrous strands. This color is not extractable into either aqueous solution or 100% dimethylformamide. If ethanol is added to a similar solution of DNA and kanchanomycin in the absence of Mg^{2+}, white fibers are precipitated. To further elucidate the nature of the apparent binding of the antibiotic to DNA in the presence of Mg^{2+}, visible absorption spectra were studied under various conditions.

The spectra of kanchanomycin between 400 and 340 mμ in 10% dimethylformamide and in 1.5% dimethylformamide at pH 7.5 are shown in Figure 1A,B. In 10% dimethylformamide kanchanomycin shows a complex spectrum with peaks at 373 and 359 mμ, while in 1.5% dimethylformamide there is a broad curve with a peak at 373 mμ. Addition of excess native calf thymus DNA to kanchanomycin causes no alteration of the spectrum; but if excess Mg^{2+} (or Co^{2+} or Fe^{2+}) is subsequently added, there is an immediate hypochromic spectral shift to shorter wavelengths. The new spectrum has a single peak at 368 mμ in both 10 and 1.5% dimethylformamide. An 8–10-fold excess of DNA-P to antibiotic is required to avoid precipitation of the DNA–kanchanomycin complex. Increasing the DNA-P concentration serially showed no further hypochromic spectral shift, suggesting that all the antibiotic had interacted with DNA at the 8–10-fold DNA-P excess. Thus, no isosbestic point was obtainable at the ratios of DNA to kanchanomycin used for these studies.

A 600-fold excess of Na^+ to kanchanomycin has no effect on the spectrum of the antibiotic. On the other

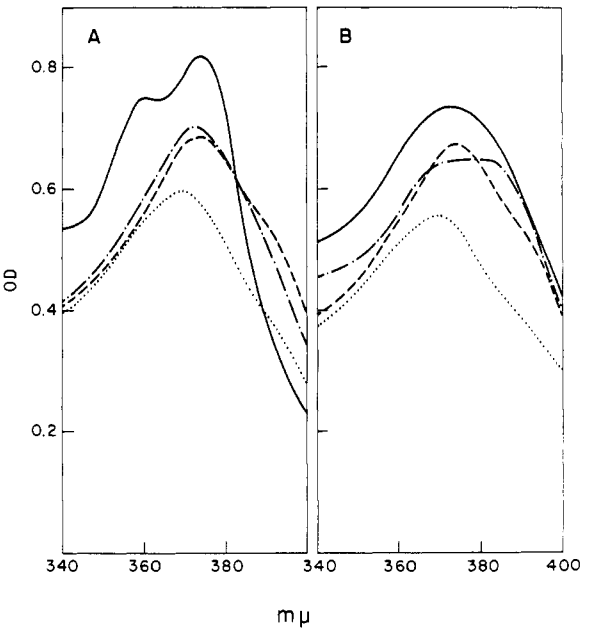

FIGURE 1: Effect of DNA and Mg^{2+} on the spectrum of kanchanomycin. All samples contained 33 μM kanchanomycin, 0.01 M Tris (pH 7.5), and either 10% dimethylformamide (A) or 1.5% dimethylformamide (B). The following additional components were present: (—) no additional component or 0.44 mM calf thymus DNA; (·-·-) 80 μM $MgCl_2$; (····) 0.44 mM calf thymus DNA and 80 μM $MgCl_2$ with the spectrum taken immediately after mixing; (---) 0.44 mM calf thymus DNA and 80 μM $MgCl_2$ after incubation for 20 hr at 37°.

[1] Abbreviations used: d(A-T)$_n$, the strictly alternating deoxyadenylate-deoxythymidylate copolymer; d($\overline{\text{DAP}}$-T)$_n$, same as d(A-T)$_n$ except that 2,6-diaminopurine replaces adenine.

TABLE I: Resolubilization of Precipitated Kanchanomycin.[a]

1. Kanchanomycin	0.72	1. Kanchanomycin	0.72
2. Kanchanomycin + Mg^{2+}, immediately	0.65	2. Kanchanomycin + DNA, immediately	0.72
3. Kanchanomycin + Mg^{2+} after 24 hr	0.11	3. Kanchanomycin + DNA after 24 hr	0.44
4. DNA added to sample 3, immediately	0.11	4. Mg^{2+} added to sample 3, immediately	0.33
5. Sample 4 after 24 hr	0.41	5. Sample 4 after 24 hr	0.61
6. Sample 4 after 72 hr	0.46	6. Sample 4 after 72 hr	0.64

[a] Two samples of 1.0 ml containing 33 μM kanchanomycin, 0.01 M Tris (pH 7.5), and 1.5% dimethylformamide were prepared and the $OD_{373m\mu}$ recorded. To sample 1 was added 80 mμmoles of $MgCl_2$; to sample 2 was added 440 mμmoles of calf thymus DNA. The $OD_{373m\mu}$ for each was recorded immediately and after 24-hr incubation at 37°. After the incubation, to sample 1 was added 440 mμmoles of calf thymus DNA and to sample 2 was added 80 mμmoles of $MgCl_2$. The $OD_{373m\mu}$ for each was recorded after 0, 24, and 72 hr of further incubation. All values have been corrected for optical density changes due to dilution.

hand, kanchanomycin can interact with Mg^{2+} alone. In 10% dimethylformamide Mg^{2+} causes a loss of the peak at 359 mμ and hypochromicity of the peak at 373 mμ. In 1.5% dimethylformamide the curve shows a striking hypochromic shift to a broad band of absorption between 383 and 370 mμ. These data suggest that the antibiotic first interacts with Mg^{2+}, and this complex then interacts with DNA. In fact, if Mg^{2+} is added first to the antibiotic and the characteristic spectral shift is obtained, subsequent addition of DNA causes a further spectral shift identical with that obtained if Mg^{2+} is added to a solution containing DNA and kanchanomycin.

With incubation in the dark there is a time-dependent shift in the spectrum of the kanchanomycin–Mg^{2+}–DNA complex. The shift, the same in 10 and 1.5% dimethylformamide, is hyperchromic with a return of the peak to 373 mμ and the appearance of a shoulder centered at about 388 mμ (Figure 1). A time-course study of this shift shows that it is complete by about 5 hr (Figure 2). The new spectrum resembles the kanchanomycin–Mg^{2+} spectrum in 10% dimethylformamide, except that the latter lacks the inflection at 388 mμ. In 1.5% dimethylformamide the new spectrum is clearly different from the corresponding kanchanomycin–Mg^{2+} curve (Figure 1B). Further, with time the kanchanomycin–Mg^{2+} complex precipitates in the absence of DNA. Thus, it seems likely that during incubation there is a shift to a new kind of kanchanomycin–Mg^{2+}–DNA complex as opposed to the dissociation of kanchanomycin–Mg^{2+} from DNA. Heat-denatured DNA causes alterations in the kanchanomycin spectrum identical with those caused by native DNA. The complexes formed with the two DNAs, however, can be distinguished by optical rotatory dispersion or circular dichroism measurements (Friedman *et al.*, 1969).

Resolubilization of Precipitated Kanchanomycin by DNA and Mg^{2+}, and of Precipitated Kanchanomycin–Mg^{2+} Complex by DNA. While it seemed unlikely that the initial hypochromic spectral shift of kanchanomycin in the presence of Mg^{2+} and excess DNA resulted from precipitation of the complex, it was necessary to eliminate this possibility. This was done by demonstrating that DNA can actually resolubilize precipitated kanchanomycin and kanchanomycin–Mg^{2+} complex (Table I). The kanchanomycin–Mg^{2+} complex precipitates rapidly in the absence of DNA. Addition of DNA, with further incubation for 72 hr, leads to resolubilization of a large portion of the antibiotic. Kanchanomycin in the presence of DNA, but lacking Mg^{2+}, precipitates more slowly. Addition of Mg^{2+} to this mixture

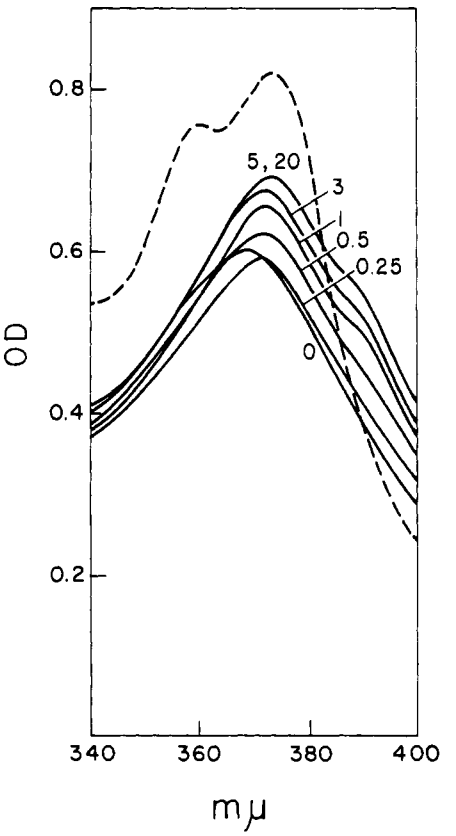

FIGURE 2: Time-dependent changes in the spectrum of kanchanomycin in the presence of DNA and Mg^{2+}. (---) Spectrum of a 1.0-ml sample containing 33 μM kanchanomycin, 0.44 mM calf thymus DNA, 0.01 M Tris (pH 7.5), and 10% dimethylformamide; (—) to the sample was added 80 mμmoles of $MgCl_2$ (in 5μl) and spectra were recorded after 0-, 0.25-, 0.50-, 1-, 3-, 5-, and 20-hr incubation at 37°.

resolubilizes almost all the precipitated kanchanomycin after 72-hr incubation. In the presence of Mg^{2+}, therefore, excess DNA actually retains the antibiotic in solution.

Absorption Spectra of Kanchanomycin at Varying pH. Since kanchanomycin interacts with a divalent cation, it was decided to determine if titratable protons are present on the molecule which can be displaced by Mg^{2+}. The spectrum of kanchanomycin in 10% dimethylformamide was recorded at different pH values (Figure 3). There is a loss of the peak at 359 mμ between pH 8.6 and 9.0; between pH 9.0 and 10.0 there is an obvious bathochromic, hyperchromic spectral shift to a new peak at 388 mμ. Since at pH 7.5 the addition of Mg^{2+} to kanchanomycin also leads to a loss of the 359-mμ peak, dissociation of the proton of the first dissociable group is probably involved. As was noted in Figure 1, the peak at 359 mμ also disappears as the concentration of dimethylformamide is lowered. A second dissociation likely occurs between pH 9.0 and 10.0; the inflection at 388 mμ in the spectrum of kanchanomycin bound to DNA present after incubation probably reflects partial dissociation of a second proton.

Interaction of Kanchanomycin with Ribohomopolymers. Kanchanomycin binds to synthetic polynucleotides in the presence of Mg^{2+} (Figure 4). Poly C, poly U, poly I, and poly A all interact with kanchanomycin–Mg^{2+} as evidenced by an immediate spectral shift identical with that which occurs in the presence of DNA and Mg^{2+}. There are differences, however, among the various ribohomopolymers with regard to the time-dependent spectral shift. Poly C induces spectral changes identical with those caused by DNA. The hyperchromic effect and bathochromic shift in the spectrum of kanchanomycin bound to poly U is smaller in magnitude after incubation for 20 hr than that with DNA or poly C. These changes are still less with poly I and absent with poly A.

Attempts to bind kanchanomycin to nucleotides leads to rapid precipitation of an apparent complex and precludes spectral measurements; 300-fold excesses of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine cause no shift in the spectrum of the antibiotic.

Magnesium Titration. In an effort to quantify the interaction of kanchanomycin with Mg^{2+}, a titration was attempted in which Mg^{2+} was added by increments to a constant concentration of kanchanomycin. It was not possible to reach an end point because of precipitation of the antibiotic. The quantity of Mg^{2+} required for complete interaction of kanchanomycin with polynucleotide, however, could be determined, as could the quantity needed for a complete time-dependent spectral shift (Figure 5). Since there is no time-dependent spectral shift with poly A, it was used for titrating the Mg^{2+} concentration required for complex I formation. It is apparent that equimolar quantities of kanchanomycin and Mg^{2+} are required for complete interaction with poly A. To determine the Mg^{2+} requirement for completion of the time-dependent spectral shift, increments of Mg^{2+} were added to constant concentrations of kanchanomycin and DNA. After an incubation period sufficient to allow for completion of complex II formation, the spectra were recorded. As in the initial reaction, Mg^{2+} must be present at a concentration equal to that of the antibiotic for the reaction to go to completion.

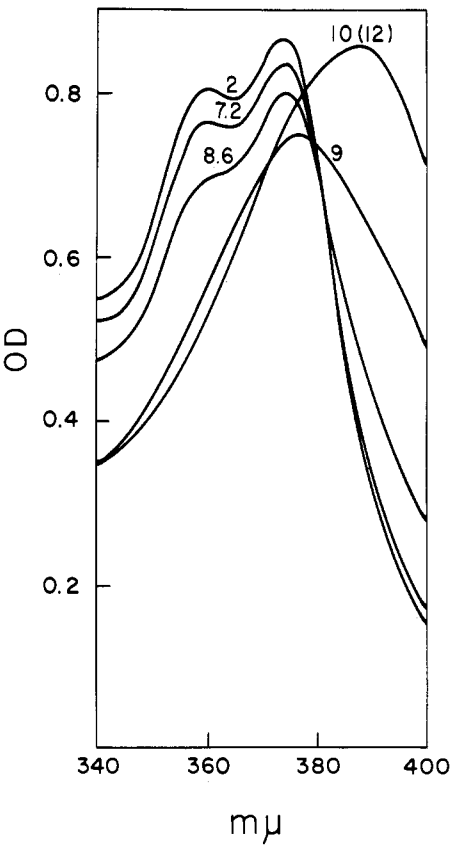

FIGURE 3: Effect of pH on the spectrum of kanchanomycin. The spectrum of 33 μM kanchanomycin was recorded in each of the following solutions: 0.01 M HCl (pH 2), 0.01 M Tris (pH 7.2 and pH 8.6), 0.01 M sodium carbonate (pH 9.0 and 10.0), and 0.01 M NaOH (pH 12.0). All solutions contained 10% dimethylformamide.

Retention of the Polynucleotide–Kanchanomycin Complex on a Millipore Filter. When a solution of kanchanomycin (40 mM in 8% dimethylformamide and 0.01 M Tris, pH 7.5) with an optical density at 373 mμ of 0.90 was passed through a Millipore filter (0.22-μ pore), the optical density of the filtrate at 373 mμ was negligible, indicating complete retention of the kanchanomycin on the filter. This observation prompted an investigation of the possible retention of the polynucleotide–kanchanomycin complex on Millipore filters. Figure 6 shows that $d(A\text{-}T)_n$, $d(\overline{DAP}\text{-}T)_n$, and T7-DNA are retained on Millipore filters (0.22-μ pore) in the presence of kanchanomycin and Mg^{2+}. The per cent of the polymer retained on the filters increases as the kanchanomycin concentration is increased, but does not reach 100%. Because kanchanomycin may bind cooperatively, some polymer molecules probably remain entirely antibiotic free at the concentrations employed in these studies and are not retained by the filters. In the samples containing no kanchanomycin 20% of the $d(A\text{-}T)_n$ and of the $d(\overline{DAP}\text{-}T)_n$ are retained on the

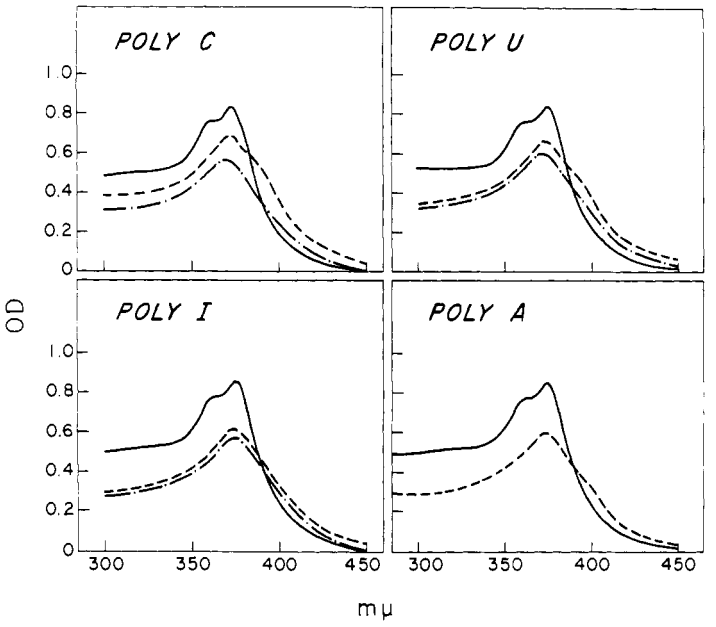

FIGURE 4: Effect of ribohomopolymers and Mg^{2+} on the spectrum of kanchanomycin. (—) Samples contained 33 μM kanchanomycin, 0.01 M Tris (pH 7.5), 10% dimethylformamide, and 0.44 mM poly C, poly U, poly I or poly A as indicated in 1.0 ml. (·····) 80 mμmoles of $MgCl_2$ (in 5 μl) was added to the above samples and the spectra were read immediately. (---) Samples were incubated 20 hr at 37° after addition of the $MgCl_2$ and the spectra were reread. (The spectrum of kanchanomycin incubated 20 hr with poly A and $MgCl_2$ is identical with the curve obtained immediately after mixing.)

filters. This fraction may represent aggregates of the respective polymers (see Figure 9 and Discussion).

The per cent of $d(A\text{-}T)_n$ retained in the presence of a given concentration of kanchanomycin is greater on filters of 0.22-μ pore size than either 0.45 or 0.1 μ. The lower retention on 0.1-μ filters is presumably due to the increased suction necessary for filtration, since the complex will leach off of a 0.22-μ filter if air is permitted to flow through the filter between washings.

In all experiments reported, the incubated samples are diluted with and washed on the filter with an ice-cold solution of 0.01 M Tris (pH 7.5) and 0.05 N NaCl. When NaCl is omitted from the wash fluid, the per cent of $d(A\text{-}T)_n$ (in the presence of kanchanomycin) retained on the filter decreases to about one-half of that retained when NaCl is present. $MgCl_2$ at 5 mM can replace NaCl, giving comparable retention of the $d(A\text{-}T)_n$–kanchanomycin complex.

Several variables which might influence the extent of polynucleotide retention were studied. Figure 7A shows the per cent of $d(A\text{-}T)_n$ (at three concentrations) retained on filters as the concentration of $MgCl_2$ is varied in the incubation of $d(A\text{-}T)_n$ with kanchanomycin. The results indicate that the Mg^{2+} concentration must be equal to or greater than that of $d(A\text{-}T)_n$ (as nucleotide concentration) for maximal retention of $d(A\text{-}T)_n$. This is in contrast to the spectral shift (Figure 5) where the Mg^{2+} concentration need be equal only to that of the kanchanomycin for maximal effect.

The quantity of $d(A\text{-}T)_n$ retained is decreased as the concentration of NaCl in the incubation mixture is increased (Figure 7B). The NaCl was present with the $d(A\text{-}T)_n$ prior to addition of kanchanomycin and $MgCl_2$. No complex which could be retained on Millipore filters is formed in the presence of 0.1 M NaCl.

The retention of $d(A\text{-}T)_n$ increases as the per cent of dimethylformamide in the incubation of $d(A\text{-}T)_n$ and kanchanomycin is increased, with a leveling off at about

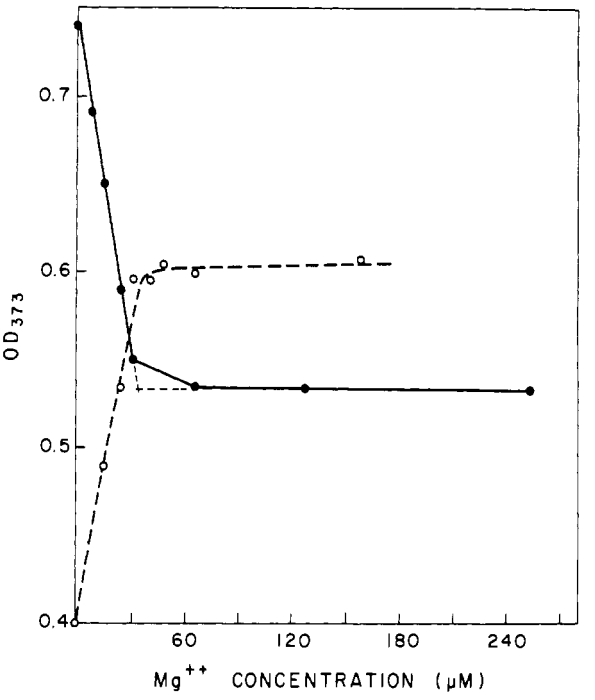

FIGURE 5: Determination of the Mg^{2+} concentration necessary for complete interaction of kanchanomycin with polynucleotides. (●—●) Each sample contained 30 μM kanchanomycin, 0.44 mM poly A, 0.01 M Tris (pH 7.5), 10% dimethylformamide, and the concentration of $MgCl_2$ indicated. The OD_{373} was read immediately after mixing. (○---○) Each sample contained 30 μM kanchanomycin, 0.44 mM calf thymus DNA, 0.01 M Tris (pH 7.5), 10% dimethylformamide, and the concentration of $MgCl_2$ indicated on the graph. The OD_{373} was read after samples had incubated 20 hr at 37°.

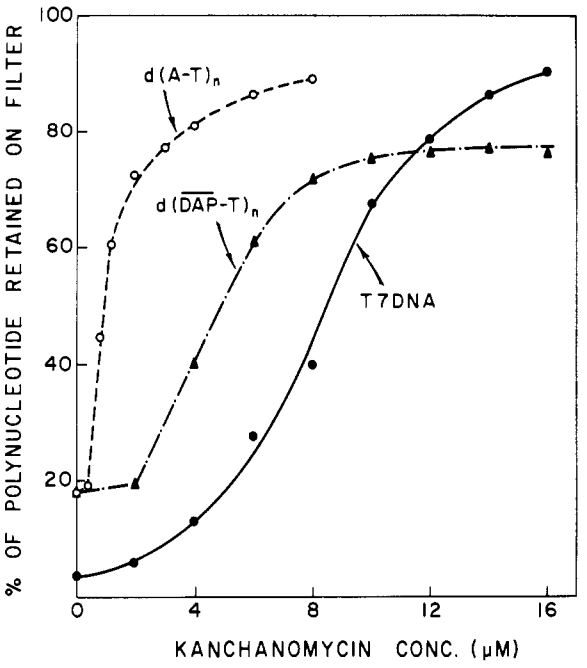

FIGURE 6: Retention of polynucleotides on Millipore filters as a function of kanchanomycin concentration. Each sample contained [^{3}H]d(A-T)$_n$, [^{3}H]d($\overline{\text{DAP}}$-T)$_n$, or [^{3}H]T7-DNA at 80 μM in 0.01 M Tris (pH 7.5), 0.16 mM $MgCl_2$, 8% dimethylformamide, and kanchanomycin as indicated. Total volume was 50 μl. Samples with d(A-T)$_n$ and d($\overline{\text{DAP}}$-T)$_n$ were incubated 30 min at room temperature; samples with T7-DNA for 3.5 hr. The filtration technique is described in Methods.

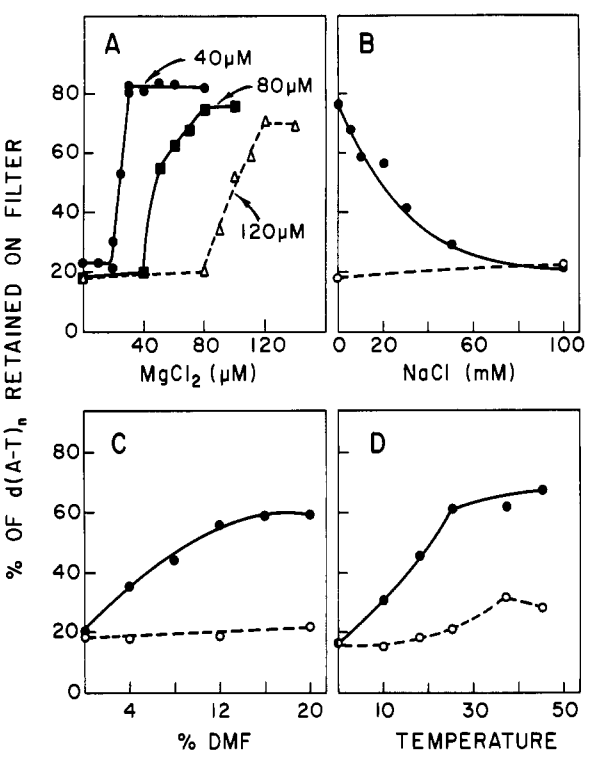

FIGURE 7: Variables affecting the formation of a d(A-T)$_n$–kanchanomycin complex retainable on Millipore filter. (A) Each sample contained 40, 80, or 120 μM [^{3}H]d(A-T)$_n$ as indicated, 3 μM kanchanomycin (except at 0 M Mg^{2+} which contained no kanchanomycin), 0.01 M Tris (pH 7.5), 8% dimethylformamide, and $MgCl_2$ as indicated. Total volume was 50 μl. Samples were incubated 30 min at room temperature. (B) Each sample (●) contained 80 μM [^{3}H]d(A-T)$_n$, 1.6 μM kanchanomycin, 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), 8% dimethylformamide, and a final NaCl concentration as indicated. Control samples (○) contained no kanchanomycin. Total volume was 50 μl. The d(A-T)$_n$ and NaCl were mixed in 30 μl prior to addition of kanchanomycin and $MgCl_2$. Samples were incubated 1 hr at 25°. Each value represents the average of five experiments. (C) Each sample (●) contained 80 μM [^{3}H]d(A-T)$_n$, 1 μM kanchanomycin, 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and a final dimethylformamide concentration as indicated. Control samples (○) contained no kanchanomycin. Total volume was 50 μl. Samples were incubated 1 hr at 25°. (D) Each sample (●) contained 80 μM [^{3}H]d(A-T)$_n$, 1.6 μM kanchanomycin, 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 8% dimethylformamide. Control samples (○) contained no kanchanomycin. Total volume was 50 μl. Samples were incubated for 30 min at the temperature indicated.

16% dimethylformamide (Figure 7C). The temperature at which the mixture of d(A-T)$_n$ and kanchanomycin is incubated also affects retention of d(A-T)$_n$ (Figure 7D). Between 0 and 25° there is an approximately linear increase in the per cent of d(A-T)$_n$ retained on filters. Longer incubations at the temperatures studied do not increase the extent of complex formation.

Spectral studies indicate that all kanchanomycin is likely bound under conditions in which maximal retention of d(A-T)$_n$ does not occur; *i.e.*, low Mg^{2+} concentration. It may be that increasing Mg^{2+} concentration, increasing dimethylformamide concentration, or increasing temperature alter the complexed d(A-T)$_n$ in some way so as to increase its retention on the filters rather than to increase the amount of antibiotic bound to d(A-T)$_n$.

Results similar to those with Millipore filters have been obtained with G-25 Sephadex columns. Both free kanchanomycin and the polynucleotide–kanchanomycin complex are retained on top of such columns.

Dissociability of the d(A-T)$_n$–Kanchanomycin Complex. Figure 8 shows studies testing the reversibility of the d(A-T)$_n$–kanchanomycin complex. In the experiment represented in Figure 8A, [^{3}H]d(A-T)$_n$ is incubated with kanchanomycin under the usual conditions. At varying intervals (5 min to 6 hr) aliquots are removed and either filtered immediately or incubated with a fivefold excess of unlabeled d(A-T)$_n$, with 2 M NaCl (final concentration), or with EDTA (50 times the Mg^{2+} concentration) for 30 min and then filtered.

The formation of a kanchanomycin–d(A-T)$_n$ complex (I) which is retained on Millipore filters is essentially complete in 5-min incubation. The addition of a fivefold excess of unlabeled d(A-T)$_n$ as early as 5 min after the initial mixing of [^{3}H]d(A-T)$_n$, kanchanomycin, and Mg^{2+}, fails to significantly reduce the amount of [^{3}H]d(A-T)$_n$ retained on the filters. Apparently the complex does not spontaneously dissociate and reassociate under the usual incubation conditions. In contrast, the addition of 2 M NaCl dissociates about 50% of the d(A-T)$_n$–kanchanomycin complex when added

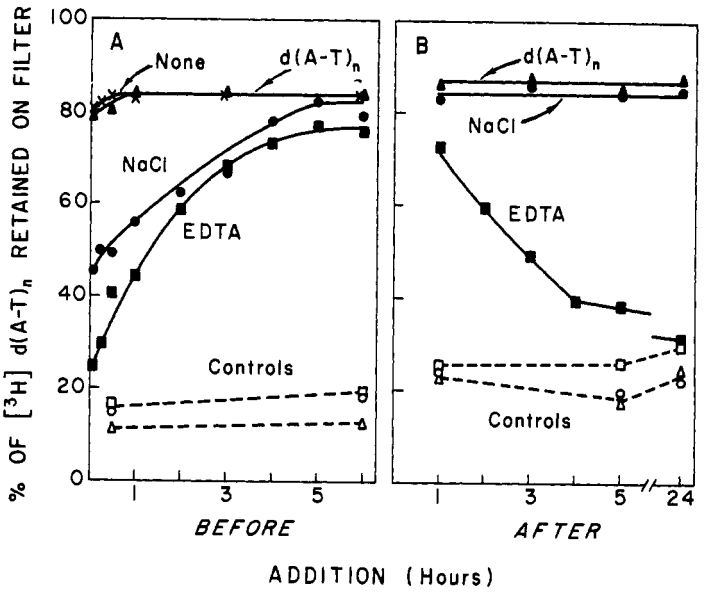

FIGURE 8: The dissociability of the d(A-T)$_n$–kanchanomycin complex with added d(A-T)$_n$, NaCl, or EDTA. (A) A solution containing 80 μM [^{3}H]d(A-T)$_n$, 1.6 μM kanchanomycin, 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 8% dimethylformamide was incubated at 25°. A control solution contained no kanchanomycin. At the times indicated 20-μl samples were withdrawn and incubated 30 min at 25° with 20 μl of the solutions indicated below and then filtered. (X—X) 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 8% dimethylformamide (except 5, 15, 30, and 60 min which were filtered immediately). (▲—▲) 400 μM d(A-T)$_n$ (unlabeled), 0.8 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 8% dimethylformamide. (●—●) 4 M NaCl, 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 8% dimethylformamide. (■—■) 16 mM EDTA, 0.01 M Tris (pH 7.5), and 8% dimethylformamide. The open symbols connected by dotted lines indicate the corresponding control values. (B) A solution containing 80 μM [^{3}H]d(A-T)$_n$, 1.6 μM kanchanomycin, 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 8% dimethylformamide was incubated 24 hr at 25°. A control solution contained no kanchanomycin. At 24 hr samples were withdrawn, mixed with an equal volume of the solutions indicated above, and incubated at 25°. At the times indicated 30-μl aliquots were withdrawn and filtered. The same symbols and solutions apply to B as used in A. At the end of the initial 24-hr incubation 83% of the [^{3}H]d(A-T)$_n$ was retainable on Millipore filters.

within 30 min after the complex is formed. As the complex assumes its second form (II) it can no longer be dissociated by 2 M NaCl. By 5 hr none of the complex is dissociated on incubation for 30 min with 2 M NaCl.

The addition of EDTA almost completely dissociates the d(A-T)$_n$–kanchanomycin complex when added 5 min after the initial complex is formed. As complex II forms, dissociation becomes much less sensitive to EDTA. By 5 hr after formation of the initial complex only slight dissociation occurs during a 30-min incubation with EDTA.

To determine whether longer incubations with these same agents might dissociate complex II, [^{3}H]d(A-T)$_n$ and kanchanomycin are first incubated for 24 hr to form complex II, and samples are removed and incubated for up to an additional 24 hr with a fivefold excess of unlabeled d(A-T)$_n$, 2 M NaCl, or EDTA at 50 times the Mg^{2+} concentration. Aliquots are removed and filtered at the indicated times during the second incubation (Figure 8B). Even after 24 hr with either unlabeled d(A-T)$_n$ or 2 M NaCl there is no decrease in the amount of [^{3}H]d(A-T)$_n$ complexed with kanchanomycin. In contrast, EDTA slowly dissociates complex II, giving complete dissociation within 24 hr.

Further evidence that the kanchanomycin–polynucleotide complex does not spontaneously dissociate and reassociate in solution was provided by equilibrium dialysis in which kanchanomycin bound to native DNA is placed on one side of the membrane while unbound native DNA is placed on the other side. Spectral measurements demonstrate that all the antibiotic is retained on its original side. Since free kanchanomycin in the presence of Mg^{2+} can be shown to pass through the dialysis membrane, it is concluded that dissociation of the complex does not occur under these conditions.

Effect of Kanchanomycin on the Sedimentation Rate of d(A-T)$_n$. Solutions of [^{3}H]d(A-T)$_n$ incubated with or without kanchanomycin were analyzed by zonal centrifugation on sucrose density gradients. Aliquots of fractions along the gradient were measured for total radioactivity (Figure 9). The sedimentation pattern of the d(A-T)$_n$ is heterodisperse with the main peak at tubes 13 and 14, a shoulder at tube 18, and tailing toward the bottom of the gradient (curve C). Kanchanomycin causes the radioactivity to sediment more rapidly (curve K). The total recoveries of radioactivity from the control and kanchanomycin-containing gradients are identical (96%). Hence the lowering of the d(A-T)$_n$ peak in the presence of kanchanomycin is due to broadening of the peak over the lower portion of the gradient, not to loss of material.

Corresponding aliquots of the sucrose gradient fractions were filtered through Millipore filters to determine which contained antibiotic bound to the d(A-T)$_n$. In the kanchanomycin-containing incubation the d(A-T)$_n$ which is retained on the filter (curve KF) and hence

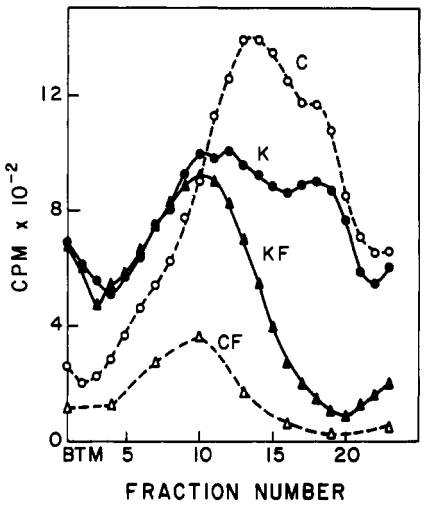

FIGURE 9: Effect of kanchanomycin on the sedimentation rate of d(A-T)$_n$. Solutions containing 92 μM [^{3}H]d(A-T)$_n$, with or without 1.84 μM kanchanomycin, 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 8% dimethylformamide were incubated 1 hr at room temperature. Aliquots of 175 μl were layered on separate 5.0-ml gradients of 5–20% sucrose in 0.01 M Tris (pH 7.5), 0.05 M NaCl, and 0.16 mM $MgCl_2$. Following centrifugation for 40 min at 65,000 rpm in an SW65 rotor in a Spinco Model L2-65 ultracentrifuge fractions along the gradient were collected. An aliquot of each fraction was counted in Bray's solution (Bray, 1960). Another aliquot was filtered through Millipore filters as described in Methods. The cpm for the aliquots in Bray's solution was adjusted to give the same efficiency as aliquots counted on filters as described in Materials and Methods. C represents the pattern of total radioactivity without added antibiotic and CF the radioactivity retained by Millipore filters. K and KF, respectively, are similar except that antibiotic was included in the incubation.

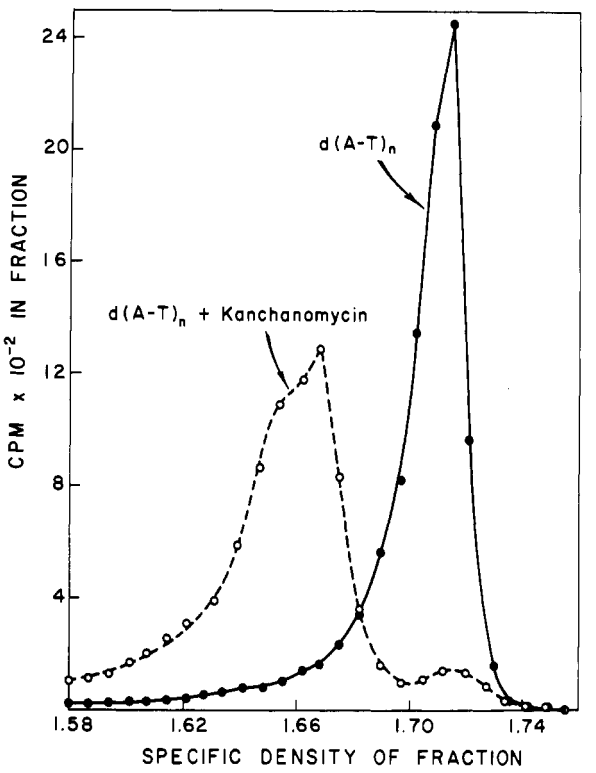

FIGURE 10: Effect of kanchanomycin on the buoyant density of d(A-T)$_n$ as determined by CsCl density gradient centrifugation. Solutions containing 80 μM [^{3}H]d(A-T)$_n$, 0 or 8 μM kanchanomycin, 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 8% dimethylformamide were incubated 24 hr at 25°; 0.25 ml of each solution was mixed with 4.75 ml of CsCl of specific density of 1.70 in 0.01 M Tris (pH 7.5) and 0.16 mM $MgCl_2$. The tubes were centrifuged in an SW39 rotor in the Spinco Model L2 ultracentrifuge for 120 hr at 33,000 rpm. The gradients were fractionated by the collection of drops. Aliquots were taken for specific density measurements. The remainder of each fraction was counted with Bray's scintillation fluid. Only those fractions over which there was a linear change in specific density were graphed. This included the middle 60% of the fractions.

bound to kanchanomycin coincides in magnitude and position with the rapidly sedimenting peak of radioactivity in curve K. It is of interest that in the control incubation the small peak of d(A-T)$_n$ which binds to the filter (curve CF) has the same sedimentation rate as the d(A-T)$_n$–kanchanomycin complex (curve KF). As was noted in Figure 7, approximately 20% of the d(A-T)$_n$ binds to Millipore filters in the absence of kanchanomycin, and from its sedimentation property this portion may represent aggregates of the d(A-T)$_n$.

Effect of Kanchanomycin on the Buoyant Density, Temperature of Thermal Transition, and Viscosity of Polynucleotides. To determine the effect of kanchanomycin on the buoyant density of DNA, solutions of [^{3}H]d(A-T)$_n$ incubated 24 hr with or without kanchanomycin were centrifuged to equilibrium in CsCl, and fractions were collected for radioactivity and density measurements (Figure 10). The d(A-T)$_n$–kanchanomycin complex has a distinctly lower and broader range of buoyant density than the free d(A-T)$_n$.

The effect of kanchanomycin on the temperature of thermal transition, T_m, of d(A-T)$_n$ was tested on solutions containing 80 μM d(A-T)$_n$, 0 or 8 μM kanchanomycin, 0.16 mM $MgCl_2$, 0.01 M Tris (pH 7.5), and 8% dimethylformamide, which were incubated 24 hr at 25°. The T_m was identical (49.2°) for both d(A-T)$_n$ and the d(A-T)$_n$–kanchanomycin complex. The total hyperchromicity, however, was 46% for d(A-T)$_n$ but only 33% for the d(A-T)$_n$–kanchanomycin complex. As the ratio of kanchanomycin to d(A-T)$_n$ was raised, the T_m remained unchanged but the extent of the hyperchromicity was further reduced and the transition occurred over an increasingly broad temperature range. While it may be that the reduction in extent of the hyperchromic shift at the higher ratios results from precipitation of the complex, no precipitate could be observed. An alternative explanation for the lack of shift in T_m as well as for the reduction in extent of hyperchromicity is based on the concept that cooperative binding of antibiotic to d(A-T)$_n$ results in regions of unmodified polymer which will melt at the usual temperature. Regions complexed with kanchanomycin might fail to melt leading to the reduction in extent of hyperchromicity. The significance of the increasing breadth of the melting curve with increasing ratios of antibiotic to d(A-T)$_n$ is unclear, but it could conceivably

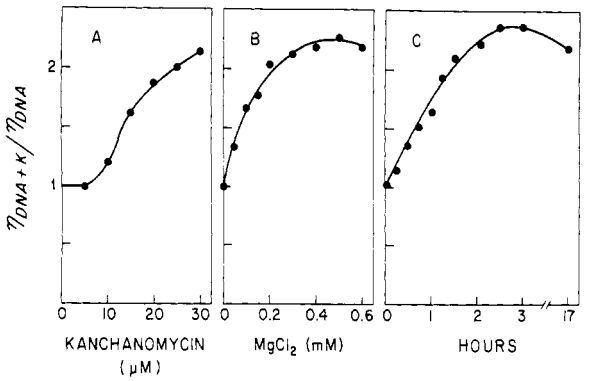

FIGURE 11: Effect of kanchanomycin on the viscosity of DNA. Samples contained 0.4 mM calf thymus DNA, 0.01 M Tris (pH 7.5), 10% dimethylformamide, and $MgCl_2$ and kanchanomycin in the concentrations indicated below. Incubations and viscosity measurements were at 25°. Viscosity was measured in an Ostwald viscosimeter. Results are expressed as the ratio of the specific viscosity of the solution containing both DNA and kanchanomycin to the comparable solution containing DNA but no kanchanomycin. (A) Samples contained 0.4 mM $MgCl_2$ and kanchanomycin as indicated and were incubated 2 hr prior to viscosity measurement. (B) Samples contained 0 or 20 μM kanchanomycin and $MgCl_2$ as indicated and were incubated 2.25 hr. (C) Samples contained 0.4 mM $MgCl_2$ and 0 or 20 μM kanchanomycin and were incubated for the period indicated prior to viscosity measurement.

result from the decreasing size of the unmodified regions in the polymer.

The effect of kanchanomycin on the viscosity of calf thymus DNA was measured as described in Figure 11. The viscosity of DNA increases as the concentration of kanchanomycin is increased (Figure 11A) and is maximal at a Mg^{2+} concentration equal to that of the DNA (nucleotide concentration) (Figure 11B). At a Mg^{2+} concentration equal to the kanchanomycin concentration little or no effect on the viscosity of DNA is observed. The effect on viscosity develops slowly, reaching a maximum after about 2.5-hr incubation at 25° (Figure 11C).

Discussion

Kanchanomycin resembles luteoskyrin (a bispolyhydroxyanthraquinone) in several respects (Ueno *et al.*, 1967; Ohba and Fromageot, 1967). Both antibiotics react with Mg^{2+} and both require Mg^{2+} or another divalent cation for interaction with polynucleotides. Both antibiotics rapidly form one type of complex (I) which slowly converts into a second form (II). Both increase the sedimentation rate of polynucleotides. In contrast, however, kanchanomycin shows no base specificity or requirement for a particular secondary structure in the polynucleotide for formation of complex I. Luteoskyrin, on the other hand, is reported to form complex I with the purine bases of denatured DNA but not with native DNA. Finally, while kanchanomycin complexed with only poly A fails to convert into complex II, luteoskyrin complexed with only poly I fails to form the second complex.

Kanchanomycin also shares properties with chromomycin, olivomycin, and mithramycin. Like kanchanomycin, these antibiotics interact with divalent cations (Ward *et al.*, 1965), require a divalent cation for binding to polynucleotides (Ward *et al.*, 1965), and decrease the buoyant density, but do not affect the thermal transition temperature of polynucleotides (Kersten *et al.*, 1966). Kanchanomycin differs from the chromomycin-like antibiotics in that kanchanomycin shows no requirement for guanine in the polynucleotide for complex formation.

Complex I formation between kanchanomycin, Mg^{2+}, and polynucleotides is most likely electrostatic, involving the negatively charged sugar phosphate backbone of the polynucleotides. This is suggested by the absence of a specific base or secondary structural requirement in the polynucleotide for interaction and by the dissociation or the complete inhibition of formation of complex I by high salt concentration.

The nature of complex II, which forms slowly over a period of about 5 hr, is not known. Apparently its structure does not involve the formation of covalent bonds since it can be dissociated, although slowly, by EDTA. The inability of NaCl to dissociate complex II and the fact that complex II is much more slowly disrupted by EDTA than is complex I suggests that a change in structure has occurred which protects Mg^{2+} from the chelating agent. Similarly, a conformational change in the complex could account for the alteration in its sedimentation properties. Since formation of complex II occurs under conditions in which viscosity is unchanged; *i.e.*, a Mg^{2+} concentration equal to antibiotic, it is very unlikely that complex II represents a molecular aggregate of complex I.

From our studies it has not been possible to determine whether the interaction of kanchanomycin and polynucleotides involves intercalation of the dye between adjacent nucleotide pairs as observed with acridine dyes and similar planar compounds (Lerman, 1964; Drummond *et al.*, 1966; Tubbs *et al.*, 1964; Ohnishi and McConnell, 1965; Fuller and Waring, 1964; Weinstein *et al.*, 1965; Riva, 1966; Kersten *et al.*, 1966). Intercalation would be expected to decrease rather than increase the sedimentation velocity of the kanchanomycin–$d(A\text{-}T)_n$ complex and to increase the temperature of thermal transition (Kersten *et al.*, 1966). It may be, however, that aggregation of the kanchanomycin–$d(A\text{-}T)_n$ complexes occurs; the increase in sedimentation velocity caused by aggregation may mask any decrease expected from intercalation. In addition, with intercalation there should be an increase in the viscosity of the kanchanomycin–$d(A\text{-}T)_n$ complex, which is found. This increase, however, may reflect aggregation and not intercalation. The absence of an effect of kanchanomycin on the temperature of thermal transition makes unlikely other types of interstrand stabilization by the antibiotic such as seen with the actinomycins (Haselkorn, 1964; Reich, 1964) or with quinacrine (O'Brien *et al.*, 1966).

For maximal changes in the spectrum of kanchanomycin upon addition of polynucleotides there is a requirement for a concentration of Mg^{2+} at least equal

to that of kanchanomycin. In contrast, kanchanomycin increases the viscosity of DNA and causes retention of polynucleotides on Millipore filters only when the Mg^{2+} concentration is equal to the nucleotide concentration.

Apparently neutralization of all the negative charges on the polynucleotide by Mg^{2+} or the resultant tighter coiling of the helix permits some further interaction, possibly aggregation of polymers, which alters these properties of the complex.

The binding of kanchanomycin to polynucleotides appears to be cooperative; *i.e.*, there seems to be a greater attraction of a given kanchanomycin molecule to a DNA molecule already binding one or more kanchanomycin molecules than to a DNA molecule with no bound kanchanomycin. This is suggested by the broad range of kanchanomycin concentration over which an increasing per cent of the T7-DNA is retained on the Millipore filters (Figure 6). Further, the $d(A\text{-}T)_n$ complexed to kanchanomycin exhibits a broader range both of sedimentation coefficients and buoyant densities than the control $d(A\text{-}T)_n$ (Figures 9 and 10), suggesting that multiple species exist; *i.e.*, a progression of polynucleotide molecules having increasing quantities of bound kanchanomycin molecules. In both the sucrose and CsCl gradients there appear to be some $d(A\text{-}T)_n$ molecules with little or no bound kanchanomycin.

Cooperative binding to polyribonucleotides has been shown previously for acridine orange (Beers and Armilei, 1965).

Since kanchanomycin was distributed uniformly in the polynucleotide solutions prior to binding, the observed cooperative binding was not artificially induced by inhomogeneous addition of antibiotic. Furthermore, to minimize the effect of nonuniform distribution of divalent cation a dilute solution of magnesium chloride was always added while mixing to the solution containing polynucleotide and antibiotic. That distribution in solution of the divalent cation contributes little to the cooperative binding is suggested by the fact that addition of magnesium chloride in small volumes at high concentrations to solutions of kanchanomycin and polynucleotide has little effect on the parameters which indicate that the binding is cooperative.

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