Hedamycin and Rubiflavin Complexes with Deoxyribonucleic Acid and Other Polynucleotides*

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ABSTRACT: The related antibiotics rubiflavin and hedamycin interact similarly with deoxyribonucleic acid *in vitro*. Formation of strong deoxyribonucleic acid–antibiotic complexes brings about a number of changes in the physical and chemical properties of the deoxyribonucleic acid. As a result of complex formation the visible absorption spectra of the antibiotics are red shifted and depressed, with development of shoulders suggesting that the antibiotic chromophores are in a hydrophobic environment. The temperature of the deoxyribonucleic acid melting transition is increased in the presence of the antibiotics, and enzymatic digestion of native deoxyribonucleic acid by snake venom phosphodiesterase is strongly inhibited. Exhaustive dialysis of *Escherichia coli* deoxyribonucleic acid–rubiflavin complexes showed that at low ionic strength the ratio of tightly bound rubiflavin molecules per deoxyribonucleic acid nucleotide is 0.53, while for hedamycin the ratio is 0.20. Denaturation of deoxyribonucleic acid did not greatly affect the tight binding of rubiflavin (ratio 0.57), but high ionic strength reduced it (ratio 0.23). Raising the percentage guanosine plus cytosine by using *Micrococcus lysodeikticus* deoxyribonucleic acid had little effect on the amount of tight binding (ratio 0.57), while for poly dAT, binding was decreased significantly (ratio 0.35).

Complexes of DNA with rubiflavin and hedamycin band in CsCl gradients at positions of considerably lower density than the uncomplexed DNA. Mixtures of the complexes with the uncomplexed DNA in CsCl solution form separate bands upon centrifugation, indicating that equilibration of antibiotic molecules among individual DNA molecules is not extensive. The binding is probably noncovalent, since rubiflavin can be extracted with benzene from an aqueous solution of the complex at high ionic strength. At least three kinds of binding to polynucleotides were recognized: (a) a strong ionic type prominent in complexes of the antibiotics with the homopolynucleotides poly U, poly C, and poly A; this binding was reversible to extensive dialysis at high ionic strength; (b) an effectively irreversible type found in all DNA–antibiotic complexes studied; this type was partially reversed by dialysis at high ionic strength, and was characterized by the appearance of shoulders in the visible spectrum of the antibiotics; (c) an interaction of antibiotic molecules with one another to cause their aggregation on the surface of DNA. Hedamycin, which is more potent than rubiflavin as a bactericidal agent, causes a much greater change in the properties of DNA per molecule bound than does rubiflavin, although the maximum number of hedamycin molecules that can bind per DNA nucleotide is only about half that of rubiflavin. The strong affinity of these antibiotics for DNA suggests an antibacterial mechanism in which rubiflavin and hedamycin become intracellularly bound to DNA and prevent DNA synthesis by inhibiting strand separation.

Rubiflavin, $C_{23}H_{29-31}NO_5$, is an antitumor and bactericidal antibiotic isolated from *Streptomyces* (SC 3728) (Aszalos *et al.*, 1965). We have previously reported (White and White, 1967) that rubiflavin causes a specific inhibition of DNA synthesis in *Escherichia coli* and that a portion of the DNA isolated from a rubiflavin-treated culture by isopycnic centrifugation bands at a lower density than native DNA. Various physical–chemical methods show that rubiflavin binds strongly to DNA *in vitro*. Rubiflavin lowers the buoyant density of DNA in CsCl gradients roughly in proportion to the molar ratio of rubiflavin to DNA nucleotide present in the complex. We therefore suggested that the antibacterial properties of rubiflavin appear to be a consequence of its tendency to bind to DNA. Hedamycin, $C_{41}H_{52}N_2O_{11}$, isolated from *Streptomyces griseoruber* (Schmitz *et al.*, 1967), has been reported to be similar to, but more potent than, rubiflavin in biological properties (Bradner *et al.*, 1967; Heinemann and Howard, 1966). Its bactericidal action has been studied and appears to be entirely analogous to that of rubiflavin (H. L. White and J. R. White, to be published). In this paper complexes of the two antibiotics with DNA and other polynucleotides are compared.

Materials and Methods

The following were generous gifts: rubiflavin from Dr. A. Aszalos of the Squibb Institute for Medical

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Research; hedamycin from Dr. W. T. Bradner of Bristol Laboratories; daunomycin hydrochloride from Dr. F. Arcamone of Farmitalia, Milan, Italy; nogalamycin from the Upjohn Co.

Calf thymus and *Clostridium perfringens* DNA were purchased from Worthington Biochemical Corp. and salmon sperm DNA (highly polymerized) from Calbiochem. Commercial DNA samples were dissolved at 2–5 mg/ml in PE6[1] buffer or in 10^{-3} M saline with gentle stirring at 4° for 3 days. Solutions were then dialyzed exhaustively at 4° against PE6 buffer. *Escherichia coli* and *Micrococcus lysodeikticus* DNA were isolated from bacterial cultures using the method of Marmur (1961). Tritium-labeled DNA (1.1×10^7 $\mu Ci/M_p$) was prepared from *E. coli* strain 15 T^-Phe^- grown in the presence of [methyl-^{3}H]thymine. Likewise [^{14}C]DNA (1.4×10^5 $\mu Ci/M_p$) was prepared by growing cells with [2-^{14}C]thymine, and [purine-^{14}C] DNA (1.4×10^5 $\mu Ci/M_p$) by growing cells in the presence of [8-^{14}C]guanine. Immediately after isolation these radioactive samples were exhaustively dialyzed against standard saline citrate (0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0) and stored frozen in 1-ml batches at concentrations of from 0.5 to 1.0 mg per ml. Before use these DNA samples were exhaustively dialyzed against PE6 or other appropriate buffer and assayed by ultraviolet absorbance.

Poly U, poly C, and poly A were purchased from Miles Chemical Co.; poly dAT from Biopolymers, Inc.; yeast RNA from Pabst Laboratories; deoxynucleoside monophosphates from Calbiochem; bovine plasma albumin from Armour Pharmaceutical Co.; snake venom phosphodiesterase and ribonuclease from Worthington Biochemical Corp. [^{3}H]Poly U (ammonium salt, 27.33 mCi/mmol) was purchased from Miles Laboratories, Inc.

Buoyant Densities. Centrifugations of DNA–antibiotic complexes were performed in a Beckman–Spinco Model E analytical ultracentrifuge, using an AN-D rotor and the 4°-sector cell with Kel-F centerpiece. Techniques were substantially as described by Mandel *et al.* (1968).

Absorption Spectroscopy. Spectra of antibiotics were determined using a Cary Model 14 recording spectrophotometer. Stability of antibiotics in various solvents was studied by following changes in spectra with time and by comparing biological activity against *E. coli* with that of a freshly prepared solution.

Spectral studies of interactions of rubiflavin or hedamycin with DNA were done in the following way (outlined for rubiflavin) in order to minimize errors caused by the instability of rubiflavin spectra and the strong tendency of both of these antibiotics to form fibrous precipitates with DNA. Salmon sperm DNA (Calbiochem), after exhaustive dialysis against PE6 buffer, was diluted to twice the highest DNA concentration to be used. Immediately prior to mixing with DNA, a fresh rubiflavin solution was prepared at twice the desired final concentration by adding microliter amounts of 1000 μg/ml of cold ethanolic stock solution to cold buffer. Rubiflavin and DNA solutions were mixed, always adding rubiflavin to DNA, slowly and with stirring, in order to achieve uniform mixing. For the reference cuvet, equal volumes of DNA and buffer were mixed in the same way. Usually, final concentrations were 1000 μg/ml of DNA and 10 μg/ml of rubiflavin (or 5 μg/ml of hedamycin). The spectrum was run on the Cary 14 spectrophotometer using a sensitive slide-wire (full-scale absorbance 0.1). This, at maximum DNA concentration, represented the spectrum of fully bound rubiflavin. Samples containing higher proportions of rubiflavin were prepared by diluting the first DNA–rubiflavin solution, slowly and with mixing, with a fresh aqueous rubiflavin solution (10 μg/ml). At the same time the DNA reference solution was diluted with an amount of buffer equal to the volume of rubiflavin solution added to the sample. In this way a series of spectra was prepared at constant rubiflavin concentration, but with decreasing DNA concentration.

T_m Determinations. DNA–rubiflavin and DNA–hedamycin melting transitions were determined in PE6 buffer or in PE6 buffer with NaCl added up to 0.10 M to give increased ionic strengths. Solutions for T_m work with rubiflavin and hedamycin were prepared as described above for spectrophotometry, using DNA which had been dialyzed against the appropriate PE6 buffer. Melting transitions were determined with a Gilford Model 2000 spectrophotometer. In a typical run the four cuvets contained: (1) buffer, (2) DNA solution, (3) DNA–antibiotic solution, and (4) antibiotic alone. Usually 40 μg/ml of DNA was present in 2 and 3. To study the influence of per cent guanine plus cytosine (% G + C) of DNA on melting transitions obtained with antibiotics, experiments were done with the following DNA samples: *Cl. perfringens* (30% G + C), salmon sperm (42%), calf thymus (44%), *E. coli* (50%), and *Micrococcus lysodeikticus* (72%) Sueoka (1961). Before a run each cuvet was bubbled with helium for 5 min and immediately covered so that a water seal was made around the ground-glass stopper. After initial absorbances at 260 mμ were recorded, the full-scale absorbance of the instrument was set to give maximum sensitivity (0.3 full-scale absorbance for 40 μg/ml of DNA), and the offset control was adjusted in order to zero all samples. Temperature was raised at 0.5°/min, while the instrument automatically recorded absorbance changes, ΔA, in each cuvet at intervals of 5 sec/cuvet. A fifth channel recorded temperature within the cuvet chamber. Transition midpoints, T_m, were calculated from replots of ΔA *vs.* temperature.

When absorbance no longer changed with temperature the circulating pump was shut off, and the instrument continued to record changes during slow cooling. Final absorbance of slowly cooled samples was read after about 15 hr to give per cent renaturation.

Optical rotatory dispersion spectra of salmon sperm DNA in the presence of rubiflavin were determined

[1] Abbreviations used: PE6 buffer, 10^{-3} M sodium phosphate 10^{-4} M sodium EDTA brought to pH 6.0; poly dAT, the double-stranded, alternating copolymer of deoxyadenylate and deoxythymidylate; PEI, polyethylenimine.

with a Cary Model 60 spectropolarimeter. Spectra were scanned from 550 to 200 mμ with full scale = 20 mdeg, scan speed = 1 Å/sec, and time constant = 10. All curves were referred to a buffer base line.

Viscosity. Viscosities of DNA and DNA–antibiotic complexes were determined at 36.20° with a Zimm–Crothers low shear viscometer (Beckman Instruments, Inc.), adjusted to give a shear stress of 1.33×10^{-3} dyn/cm^2 as calculated by the equations of Zimm (1962). Flow time for solvent alone was 182.3 sec for one turn of the rotor. At least three measurements at each concentration were averaged for specific viscosity calculations.

Enzymatic Digestion of DNA. Hydrolysis of radioactive DNA was accomplished in 0.10 M Tris-Cl buffer (pH 8.8) at 36–37° using 50 μg/ml of snake venom phosphodiesterase (Williams, 1961). Samples were removed at intervals with capillary pipets and spotted at the origin of PEI-cellulose thin-layer plates. Hydrolysis of native DNA was complete in 6 hr. Digestion products were located by autoradiography, after which portions of thin-layer plates were scraped off and counted. Details of chromatography and scintillation counting have been described (White and White, 1968).

Rates of digestion of DNA–antibiotic complexes by snake venom phosphodiesterase were studied in the above Tris buffer using native or denatured [^{3}H]DNA (40 μg/ml) at molar ratios of 0.05 and 0.10 for rubiflavin and 0.05 for hedamycin. During incubation at 36–37°, 20-μl samples were spread on Whatman No. 1 filter paper disks and assayed for acid-precipitable counts per minute using a batch process (Bollum, 1966).

Solvent Extraction. DNA–rubiflavin solution (5 ml) and organic solvent (5 ml) were shaken vigorously on a Burrell wrist-action shaker for 30 min and then centrifuged for 5 min at 2500 rpm. Ionic strength of the aqueous buffer was increased by adding either NaCl, CsCl, or $NaClO_4$.

Exhaustive Dialysis. Since the binding of rubiflavin and hedamycin to DNA was very strong and not of an equilibrium type, an exhaustive dialysis method was devised for studying the nature of this binding. DNA for these experiments was first dialyzed exhaustively against PE6 buffer at 4°. This buffer was used in all these experiments except where it was desired to increase ionic strength by adding NaCl to PE6 buffer. A series of DNA–rubiflavin or DNA–hedamycin solutions was prepared at selected molar ratios of antibiotic to nucleotide (r values). Usually DNA concentration was kept constant at 4×10^{-5} M and antibiotic concentration was varied. Similar results were obtained when DNA was varied and antibiotic concentration was held constant. Solutions were allowed to remain at 4° overnight, protected from light; 1 ml of each was then placed in a washed dialysis bag (Union Carbide 8/100) in a scintillation-type vial and dialyzed against 20 ml of buffer. A small stirring flea was placed in the bottom of each vial, and as many as 20 such vials were placed on one magnetic stirrer. These were covered with aluminum foil and kept in a cold room at about 4°. The outside buffer was changed after 1, 3, and 5 days. In experiments with 1 M NaCl present in the buffer, solutions were first prepared in PE6, then dialyzed against PE6 containing 1 M NaCl for 6 days with three changes, and finally dialyzed against PE6 alone overnight. On day 7 the contents of each dialysis bag were assayed for antibiotic in the visible region using the Cary 14 spectrophotometer with sensitive slide-wire. DNA controls were used for spectral DNA assay and also as reference when DNA–antibiotic solutions were assayed in the visible region. *E. coli*, salmon sperm, *M. lysodeikticus*, and *Cl. perfringens* DNAs were studied in the above experiments. *E. coli* DNA was labeled either with [^{3}H]thymine or [^{14}C]thymine. From spectral assays (and radioactivity measurements when *E. coli* DNA was used), final r values, r_f, were calculated. These were plotted against initial r values, r_i, to give an indication of the strength and extent of strong binding.

The homopolynucleotides poly U, poly C, and poly A were dialyzed against PE6 buffer and assayed spectrally before use in the above experiments. Molar extinctions employed, based on nucleotide phosphate, were 8000 for poly A, 8600 for poly C, and 9500 for poly U (Shugar, 1960; Michelson and Monny, 1966). Poly (rA):poly (rU) was prepared by mixing equimolar amounts of poly A and poly U in PE6 buffer containing 0.1 M NaCl and allowing the mixture to remain at room temperature for 20 hr. At this time a spectral assay showed the expected hypochromicity (E_p = 7000) (Peacocke and Blake, 1967).

Results

Solubility and Stability. Rubiflavin was stable for at least 6 weeks in absolute ethanol if kept refrigerated and protected from light. Biological activity and spectra were relatively unchanged during this time. However, as was reported to us (A. Aszalos, 1964, personal communication), rubiflavin was only slightly soluble and was very unstable in aqueous solutions: a decrease in the absorption spectrum occurred within minutes without a shift of the absorption maxima, and this decrease was accelerated by such procedures as bubbling nitrogen or air through the solution or centrifugation. Therefore aqueous solutions were prepared from an ethanolic stock immediately before use.

Sodium borate, sodium phosphate, and Tris-HCl, each at concentrations of 10^{-3} and 10^{-1} M and at pH values of 6, 8, and 9.2, were not suitable as buffers. Sodium phosphate–EDTA buffer at 10^{-3} M phosphate and 10^{-4} M EDTA was studied at the same pH values and was suitable, since the rubiflavin spectrum remained stable for at least 10 min in this buffer at the three pH values. At 1 hr the absorbance was only slightly less, but at 20 hr, the visible spectrum was about 20% lower, although the absorption maxima were not shifted. Raising the ionic strength to 0.1 M with NaCl or raising phosphate and EDTA concentrations by a factor of 10 did not change these results significantly. Therefore, phosphate–EDTA buffer (pH 6) was chosen as a suitable solvent. The spectrum of rubiflavin was strongly dependent upon pH. A pH titra-

tion indicated a pK_a value of 8.1 or 8.2. Another transition near pH 10 caused a color change from amber to rosy red. Since the species present below pH 8 exhibited greater aqueous stability and biological potency, pH 6 was chosen as an optimum pH for *in vitro* studies. In phosphate-EDTA buffer at pH 6.0 (PE6 buffer), stored in the dark at 4°, the bactericidal activity of rubiflavin decreased gradually with time, being only 16% of its original value after 5 weeks. During this time the rubiflavin spectrum decreased, but no changes in wavelengths of maximum absorbance were observed.

Hedamycin was also reported to be light-sensitive and unstable in aqueous solutions, losing 90% of its potency at room temperature in 2 hr (W. T. Bradner, 1966, personal communication). Hedamycin was only slightly soluble in ethanol and exhibited complete loss of antibacterial potency when stored (at 4° and dark) in dimethylformamide for 2 months. In PE6 buffer, however, we found no significant change in spectrum or potency against *E. coli* over a period of 3 months if the solution was kept refrigerated and protected from light. Hedamycin could be dissolved directly in PE6 buffer at a maximum of 50 μg/ml, and this was used as a stock solution. Clearly hedamycin is much more stable in aqueous solutions than rubiflavin.

Absorption Spectra. The aqueous absorption spectra, at pH 6, of rubiflavin and hedamycin are very similar. Both compounds have broad maxima at 428 mμ and sharp peaks at 245 mμ with shoulders near 270 mμ. Hedamycin has an extra peak at 212 mμ. Molar extinctions are quite different, being for rubiflavin at 10 μg/ml at 245 mμ, 20,000, and at 428 mμ, 3700; and for hedamycin at 245 mμ, 46,000, at 428 mμ, 10,000, and at 212 mμ, 48,000. Molecular weights of rubiflavin and hedamycin are 412 and 748, respectively (Aszalos *et al.*, 1964; Schmitz *et al.*, 1966). The absorbance of neither compound follows Beer's law, but in the concentration range used in this work (0–10 μg/ml), the extinctions cited above were applicable.

Chromatography and Electrophoresis of Rubiflavin and Hedamycin. Chromatography of both compounds on Eastman preformed silica gel thin layers was achieved using mixtures of alcohol–organic acid–water in various ratios. In isoamyl alcohol–formic acid–water (3:2:1, v/v), rubiflavin gave a single distinct spot with R_F 0.43, while hedamycin gave two distinct spots, of nearly equal ultraviolet intensity, having R_F values of 0.41 and 0.32. Hedamycin may have been degraded by the acidic solvent into two components. Electrophoresis of rubiflavin and hedamycin on Whatman No. 1 paper strips in a Gelman chamber indicated that both compounds were positively charged at pH 6 and 7.4.

Reducibility. Both rubiflavin and hedamycin were reduced irreversibly by sodium borohydride, rubiflavin going from an amber to a colorless solution and hedamycin from yellow to colorless. The visible maximum of rubiflavin was shifted from 428 to 306 mμ. When sodium hydrosulfite was the reducing agent, the 428-mμ maximum of both compounds was red shifted to near 500 mμ in the visible region. Hydrosulfite reduction was largely reversible, and after standing in air for a few minutes, the solutions turned from red back to their original colors.

TABLE I: Half-Wave Potentials *vs.* Saturated Calomel Electrode.[a]

Compound	$E_{1/2}$ (V)	Molar Diffusion Current (μA)
Rubiflavin	−0.36	1.0×10^3 [b]
Hedamycin	−0.42	5.0×10^3 [c]
	−1.5	1.1×10^4
Nogalamycin	−0.42	3×10^3 (uncertain)
Daunomycin	−0.60	7.9×10^3

[a] Tris buffer, 0.10 M, pH 7.4. [b] Solutions contained 5% ethanol. [c] Solutions contained 5% dimethylformamide.

Both compounds were reducible at the dropping mercury electrode. Half-wave potentials measured with a saturated calomel reference electrode at pH 7.4 are given in Table I. Hedamycin was distinguished from rubiflavin by a much higher diffusion current and two distinct waves. In order to achieve practical concentrations for polarography, rubiflavin was diluted from an absolute ethanol solution into Tris-HCl buffer, and hedamycin from a freshly prepared concentrated dimethylformamide solution. The anthraquinone antibiotics, daunomycin and nogalamycin, were included for comparison. The latter, being very insoluble, was of uncertain concentration. Its similarity to hedamycin in polarographic behavior is of interest.

Electron spin resonance spectrometry has shown that rubiflavin causes a free-radical signal to appear in cultures of *E. coli* strain B (White and Dearman, 1965), indicating that rubiflavin can be intracellularly reduced. The fact that the electron paramagnetic resonance signal lacks hyperfine structure suggests that rubiflavin free radicals may be bound to large molecules. M. T. Huang (unpublished data) has observed a similar signal lacking hyperfine structure from hedamycin in cultures of *E. coli*.

In Vitro Interaction with DNA

Equilibrium Gradient Centrifugation. When solutions of [^{3}H]DNA from *E. coli* were mixed with rubiflavin to give various *r* values and these solutions were centrifuged separately in a CsCl gradient in the Spinco Model L preparative ultracentrifuge, decreases in buoyant density were readily observed (White and White, 1967) for *r* values of 0.1 or more.

In the Beckman–Spinco Model E analytical ultracentrifuge decreases in buoyant density were observed in CsCl gradients for complexes of both rubiflavin and hedamycin with native or denatured DNA. It was found that if DNA was placed in the same gradient with DNA–antibiotic complex, two distinct bands were obtained, corresponding to DNA alone and DNA–antibiotic complex. For salmon sperm DNA–

rubiflavin at $r = 0.10$, the change in buoyant density due to complex formation was $\Delta\rho = -0.014$ g/cm^3; for DNA–hedamycin at $r = 0.05$, $\Delta\rho = -0.077$ g/cm^3.

The remarkable stability of bound complexes in CsCl gradients was demonstrated as follows. A series of *E. coli* DNA–rubiflavin solutions was prepared having r values of 0.10, 0.20, 0.30, and 0.40. Samples of these solutions, containing 1 μg of DNA each were mixed with CsCl along with 1 μg of *E. coli* marker DNA. Following centrifugation of such a mixture, distinct bands were observed for each of the complexes as can be seen in Figure 1. The position of the lowest band does not differ from that of uncomplexed DNA banded separately, indicating that DNA in this band has not become complexed with a detectable amount of antibiotic. Equivalent amounts of DNA were originally present in each complex. Thus it appears that a portion of each complex remains undissociated. When any of these DNA–antibiotic complexes was centrifuged by itself, one band at the position of lowered density appeared, and there was no indication that any unbound DNA was present. When the experiment of Figure 1 was carried out with freshly prepared samples, that is, with complexes prepared within 0.5 hr of addition to CsCl, the equilibrium position of the native marker was raised, and all samples banded diffusely in the density region between the expected positions of free DNA and of the most highly complexed DNA. Thus in this case the complexes were rather unstable, and antibiotic molecules in some cases became free of DNA and later bound to another DNA molecule. Complexes of greater stability were formed when a solution of the complex was incubated for 2 hr at 40° prior to addition of CsCl. Observations similar to those described above for DNA–rubiflavin complexes were also made after centrifugation of DNA–hedamycin complexes.

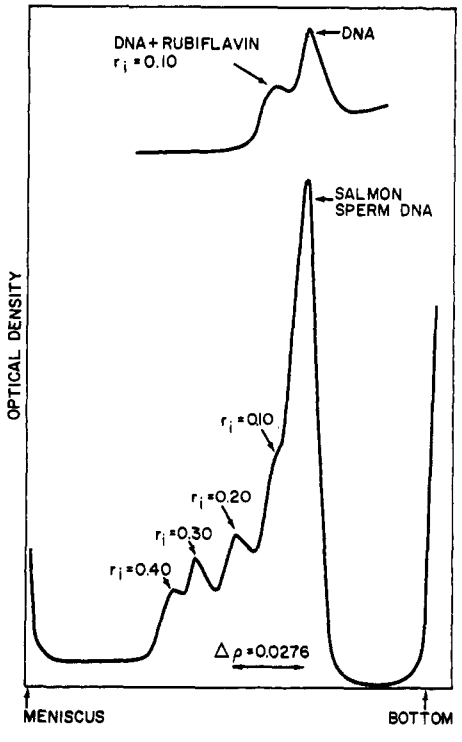

FIGURE 1: Microdensitometer tracing resulting from a CsCl gradient containing salmon sperm DNA and four DNA–rubiflavin complexes. Complexes having the values of r_i indicated were prepared separately and held at 4° for 3 days before mixing with DNA in CsCl solution. Centrifugation in the Spinco Model E analytical ultracentrifuge was for 16 hr at 44,770 rpm. The experiment, which illustrates the great stability of the complexes, is described in the text. The upper tracing shows that somewhat better definition of the bands is obtained when DNA and only one complex are present in the gradient.

Solvent Extraction. The tenacious nature of the binding of rubiflavin and hedamycin to DNA suggested that the binding might be covalent. This is apparently not the case, as shown for rubiflavin. The antibiotic could be extracted from an aqueous layer by organic solvents such as benzene, chloroform, or chloroform–isoamyl alcohol mixtures. This extraction did not take place with DNA–rubiflavin complexes at $r = 0.50$ at either pH 6.0 or 8.0. However, when a monovalent salt at concentration of 1 M was present in the aqueous layer, the rubiflavin was extractable. Sodium perchlorate, CsCl, or NaCl served equally well. Precipitation of some of the DNA at the interface was apparent at these high salt concentrations.

Absorption Spectroscopy. In the presence of DNA, the visible absorption spectra of rubiflavin and hedamycin were depressed, red shifted, and characterized by shoulders. Spectra of DNA-bound rubiflavin or hedamycin were dependent upon the manner of solution preparation, that is, interactions were not reversible, and therefore equilibrium binding theory could not be applied. Moreover, no isosbestic points were found. For spectral studies solutions were prepared in PE6 buffer as described in Methods in order to prevent precipitation of complex. It was found that when the molar ratio, r, of rubiflavin to DNA nucleotide was less than unity, the spectrum remained stable for hours or days.

Figure 2 shows that, for salmon sperm DNA–rubiflavin, at the lowest r value, the 428-mμ peak was shifted to 440 mμ. An identical shifted spectrum was obtained for $r < 0.01$. As fresh rubiflavin was added in order to decrease DNA concentration while keeping rubiflavin at constant concentration, the height of the spectrum increased until $r = 0.04$. Then further dilutions depressed but did not shift the spectrum until r approached 1.0, when it began to shift to the blue. At $r = 1.0$ the peak was at 433 mμ, and the shoulders were lost. At larger r values the peak returned to 428 mμ but remained depressed. If the solutions having $r > 1$ were allowed to sit for a few hours, their spectra became more intense without a shift in the maximum.

Spectra from a similar experiment for DNA–hedamycin solutions are shown in Figure 3. As a solution having an initial r value of 0.006 was raised to 0.20, the spectrum remained the same, with maximum at 440 mμ and shoulders similar to, but somewhat stronger than, those of bound rubiflavin. The spectrum began to show a blue shift at $r = 0.40$, which continued until $r = 0.75$, when the maximum was at 428 mμ and

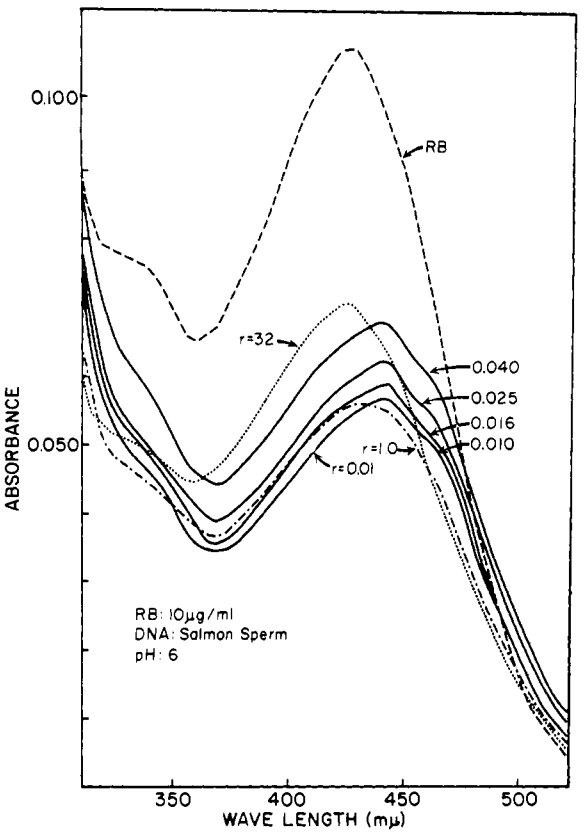

FIGURE 2: Absorption spectra of DNA–rubiflavin complexes. The broken line labeled rubiflavin is the spectrum of free rubiflavin. Other curves, denoted by the molar ratio, *r*, of rubiflavin to DNA nucleotide, are the spectra of solutions prepared as described in Materials and Methods.

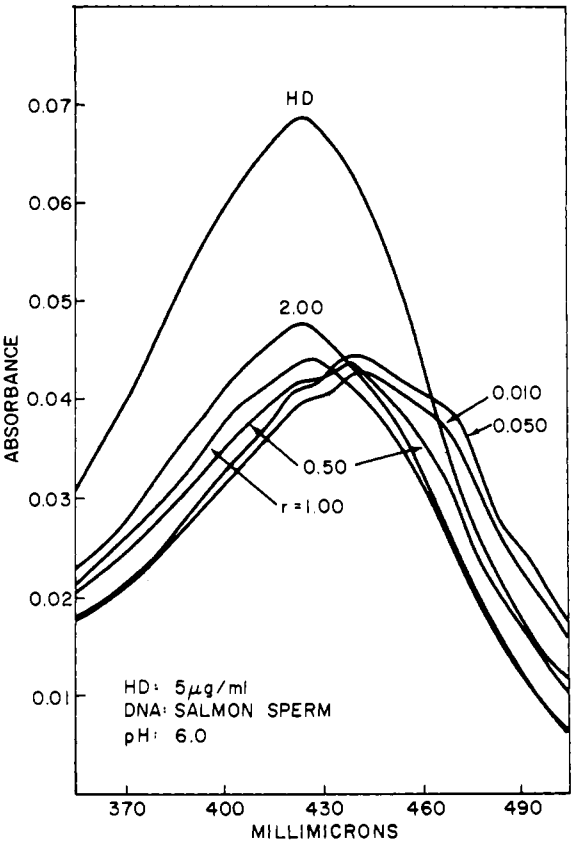

FIGURE 3: Absorption spectra of DNA–hedamycin complexes. The curve labeled hedamycin is the spectrum of free hedamycin. Other curves, denoted by the molar ratio, *r*, of hedamycin to DNA nucleotide, are the spectra of solutions prepared as described in Materials and Methods.

shoulders had disappeared. A further increase in *r* did not change the spectrum until $r = 2.0$, when absorption at 428 mμ increased.

DNA from *E. coli*, *M. lysodeikticus*, and *Cl. perfringens* gave results similar to salmon sperm DNA with both rubiflavin and hedamycin. The above spectral changes were strongly inhibited by 0.01 M magnesium ion in all solutions. Although spectra still showed evidence of an interaction, the peak did not shift to 440 mμ, and no shoulders were generated. The fact that PE6 buffer tends to stabilize the rubiflavin spectrum suggests that magnesium (and other divalent cations) may interact with the antibiotic directly. However, magnesium bound to DNA phosphates might also modify the nature of rubiflavin binding to DNA. Rubiflavin which had been refrigerated in PE6 buffer for 5 weeks and which retained only about 16% of its biological activity was still capable of binding to DNA *in vitro*, giving a spectrum with a maximum at 440 mμ and shoulders typical of bound rubiflavin, although of higher absorbance than that obtained with fresh rubiflavin. The spectrum of DNA-bound rubiflavin was similar at pH 6.0, 7.4, and 9.1, although spectra of fresh rubiflavin solutions varied with pH. At pH 9.1 the complex tended to settle out of solution in a yellow gelatinous form which, when shaken, gave a reproducible bound-rubiflavin spectrum.

Heat-denatured DNA at pH 6.0 bound both rubiflavin and hedamycin, and the spectra of the bound antibiotics were similar to those found with native DNA. However at pH 12.5, where DNA is denatured and rubiflavin is in its red alkaline form, no binding was observed. Reduction of rubiflavin by borohydride in the presence of DNA tended to reduce binding.

The four 5′-deoxyribonucleotides were used in binding studies with rubiflavin at initial *r* values of 0.01. None gave spectral shifts or generation of shoulders. However some depression of absorbance was observed for 5′-dAMP and 5′-dTMP. No interaction at pH 6.0 between rubiflavin and *E. coli* ribosomes was detected. Likewise no binding with bovine plasma albumin was observed.

With yeast RNA at $r = 0.07$, the spectrum of the rubiflavin complex was lower than that of free rubiflavin. The maximum was red shifted to 437 mμ and exhibited shoulders similar to the spectrum of DNA–rubiflavin. Interactions with the homopolymers, poly A, poly C, poly U, and poly (rA):poly (rU), also gave depressions and shifts to 437 mμ, but no definite shoulders were observed. On the other hand, the alternating

copolymer, poly d(AT), gave a bound rubiflavin spectrum similar to that of DNA–rubiflavin.

In organic solvents the visible absorption maxima of both rubiflavin and hedamycin were red shifted, the largest shift being obtained with benzene. These spectra are shown for benzene and ethanol in Figure 4 in the case of rubiflavin. Besides inducing red shifts, the more hydrophobic solvents caused the appearance of several shoulders, which were similar for both antibiotics. Also shown in the figure for comparison is an aqueous spectrum of rubiflavin in the presence of excess salmon sperm DNA. The DNA-bound antibiotic spectra are similar to those of the free compounds in a hydrophobic environment.

Melting Transition of DNA. Rubiflavin and hedamycin both caused a large increase in T_m of DNA, while absorbances of the antibiotics alone are not significantly changed on heating. The T_m of DNA–rubiflavin complexes was studied as a function of r value, ionic strength, and per cent guanosine plus cytosine of DNA. For DNA–antibiotic complexes the T_m was considered to be the temperature at which the absorbance was the same as that of the control DNA sample at its midpoint. In the presence of rubiflavin the transition occurred over a broader temperature range, and achievement of full hyperchromicity was gradual. On cooling, DNA–rubiflavin samples tended to renature much more readily than controls. The effects on

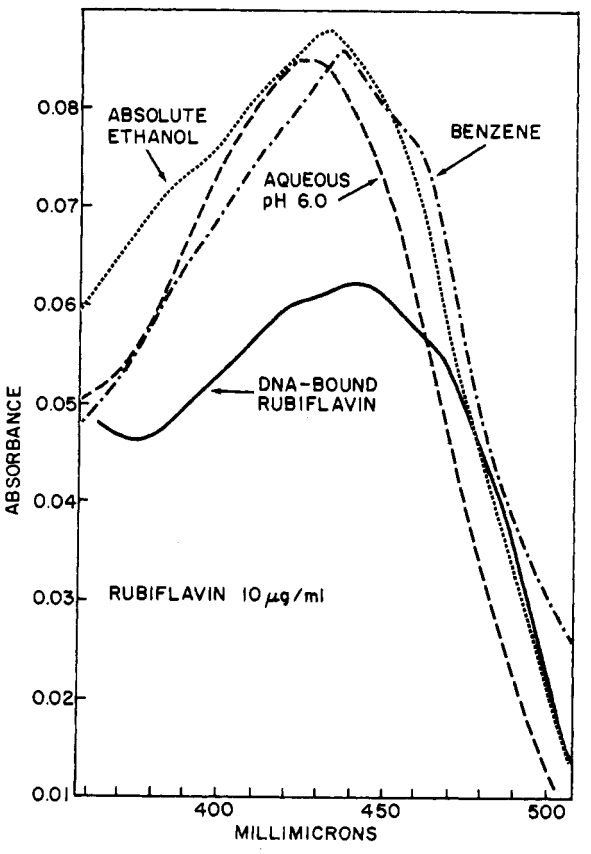

FIGURE 4: Absorption spectra of rubiflavin in aqueous and organic solvents.

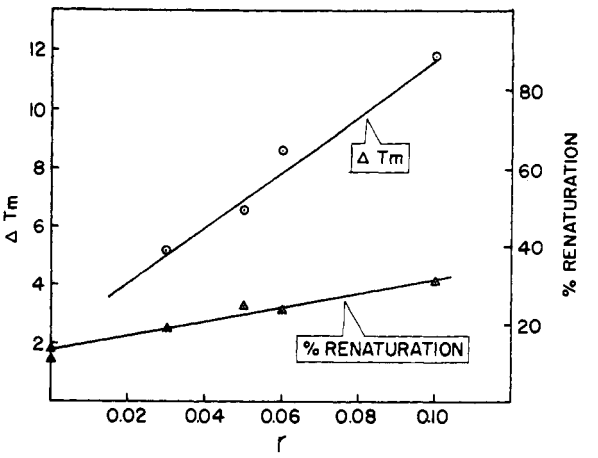

FIGURE 5: Increase in T_m of DNA and per cent renaturation after slow cooling as a function of molar ratio of rubiflavin to DNA nucleotide. The melting transitions of salmon sperm DNA–rubiflavin complexes were studied in PE6 buffer as described in Materials and Methods. The T_m of uncomplexed DNA was 50.3°.

T_m and per cent renaturation are illustrated as a function of r in Figure 5 for salmon sperm DNA. There was no clear dependence upon per cent guanosine plus cytosine. Variation of the ionic strength from 0.001 to 0.01 M did not significantly affect the increase in T_m obtained at a particular r value. At an ionic strength of 0.10 M, the increase in T_m was reduced by at least half, but in this case transitions were occurring in a higher temperature range, and the effect of temperature on the nature of the complex is uncertain. At low ionic strength (0.001 M) the effect of rubiflavin on renaturation upon slow cooling was much more pronounced, since DNA alone lost only 4% of its hyperchromicity, compared with 44% for rubiflavin at $r = 0.10$.

At r values higher than 0.2, melting curves were multiphasic, but no plateau could be reached at the highest temperature readily attainable with a water bath (94°).

With hedamycin the increase in T_m of DNA was much more pronounced than with rubiflavin. At $r = 0.025$ with salmon sperm DNA at an ionic strength of 0.003 M, the increase in T_m was at least 20°, but a plateau could not be reached.

Since the antibiotic molecules might have become dislodged from their DNA binding sites during the heating process, visible spectra were recorded before, during, and after T_m determinations. For salmon sperm DNA–rubiflavin at $r = 0.17$ and ionic strength 0.003 M, the spectrum obtained at the plateau of the DNA–rubiflavin melting transition was similar to that of free rubiflavin, suggesting that at that temperature the DNA–rubiflavin complex was largely dissociated. On slow cooling the DNA–rubiflavin spectrum shifted about halfway back. The manner in which degradation of the rubiflavin molecule may have contributed to these observations is not known.

In the case of DNA–hedamycin, at $r = 0.025$ and an ionic strength of 0.003 M, a plateau had not been reached at 92° where a visible absorption spectrum was

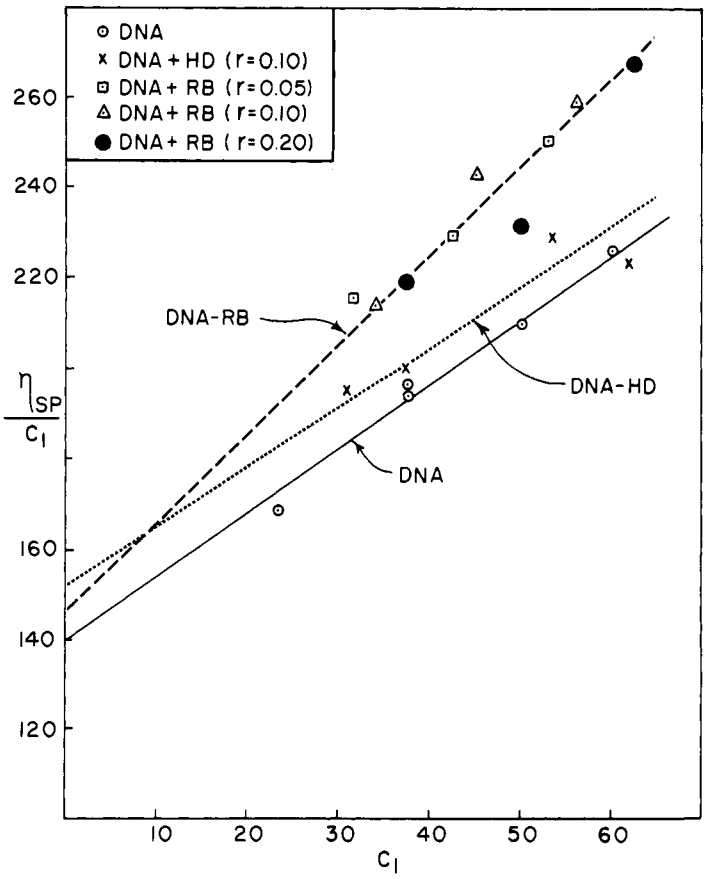

FIGURE 6: Determination of apparent intrinsic viscosities of DNA–rubiflavin and DNA–hedamycin complexes. Molar ratio of antibiotic to salmon sperm DNA nucleotide was kept constant by dilution of more concentrated solutions of DNA–antibiotic complex with PE6 buffer. C_1 = concentration of complex in micrograms per milliliter.

taken. The DNA–hedamycin spectrum was blue-shifted toward that of free hedamycin on heating, but it did not lose its fine structure, and on cooling it was shifted back to its original position.

Optical Rotatory Dispersion of DNA–Rubiflavin (White, 1967). Rubiflavin alone exhibited multiple Cotton effects in the ultraviolet region and a broad negative Cotton effect centering near 440 mμ. At a higher concentration (50 μg/ml) a positive Cotton effect at 245 mμ became more prominent than at lower concentrations. In the presence of DNA, optical rotatory dispersion spectra were not additive, and no extrinsic Cotton effect appeared in the visible region. The optical rotatory dispersion spectrum of DNA is quenched by bound rubiflavin (r = 0.25) at wavelengths lower than 270 mμ. This effect is most striking near 200 mμ where native DNA exhibits a sharp peak.

Viscometry. Using the low-shear viscometer of Zimm and Crothers (1962) viscosities of DNA–rubiflavin and DNA–hedamycin complexes were studied at r values where the antibiotics could be assumed to be completely bound. By diluting with buffer, DNA concentration could be decreased while r remained constant. Plots of the reduced viscosity, η_{sp}/c, *vs.* concentration of DNA showed that, although the specific viscosity of DNA was increased by both antibiotics, the extrapolated "apparent intrinsic viscosities" were not significantly different at different r values. When calculations were made with respect to weight concentration of complex rather than of DNA, curves for three r values tended to be superimposed and to extrapolate to the same point (Figure 6). When DNA–rubiflavin complexes were diluted with rubiflavin, keeping the rubiflavin concentration constant, the extrapolated values for intrinsic viscosity were much greater, but in this case the composition of the complexes changed (that is, r increased) as the DNA concentration was lowered. Therefore no attempt was made to interpret such results quantitatively.

Enzymatic Digestion Rates and Products. Native DNA, when bound to either rubiflavin or hedamycin, was strongly resistant to digestion by snake venom phosphodiesterase. However, if DNA was denatured prior to antibiotic treatment, the complexes were much less resistant to digestion, as shown in Figure 7. Although the denatured DNA control was more readily hydrolyzed than the native DNA, this difference was even more pronounced in the presence of the antibiotics. In both instances DNA–rubiflavin is less resistant to digestion than DNA–hedamycin, even though hedamycin is present at only half the molar concentration of rubiflavin. Digestion products of native DNA–antibiotic complexes, in which DNA was labeled with both [^{3}H]thymine and [^{14}C]purine, were separated by thin-layer chromatography on PEI-cellulose. Autoradiography of the chromatograms revealed that products of low mobility, presumed to be oligonucleotides, were formed during the enzymatic

digestion in the presence, but not in the absence, of the antibiotics. Both rubiflavin and the hedamycin remained at the origins of these chromatograms.

Dialysis. Results of exhaustive dialysis experiments with DNA–rubiflavin and DNA–hedamycin are presented in Figures 8 and 9. With native and denatured DNA all curves of r_f (final r) *vs.* r_i (initial r) were composed of two linear regions, with a steep initial slope and a more gentle slope at higher r_i values. The r_f values at the intersection of the two linear regions probably represent irreversibly bound antibiotic. On a molar basis rubiflavin was bound more extensively than hedamycin by a factor of about 2, and this binding was not greatly different with native and denatured DNA. Neither was the binding significantly dependent upon % G + C. Dialysis against 1 M NaCl decreased the binding by a factor of about one-half for both antibiotics. Visible spectra after dialysis resembled those shown in Figures 2 and 3 for the completely bound antibiotics, with maxima at 440 mμ and distinct shoulders.

Results with the homopolymers, poly C, poly A, and poly U, showed less binding of an irreversible type. With hedamycin, none at all was found with poly A or poly C. The copolymer, poly (rA):poly (rU), prepared from poly A and poly U, was studied only with rubiflavin and gave results similar to those for poly A alone. On the other hand, the alternating copolymer, poly d(AT) gave an r_f *vs.* r_i curve quite similar to that obtained with DNA.

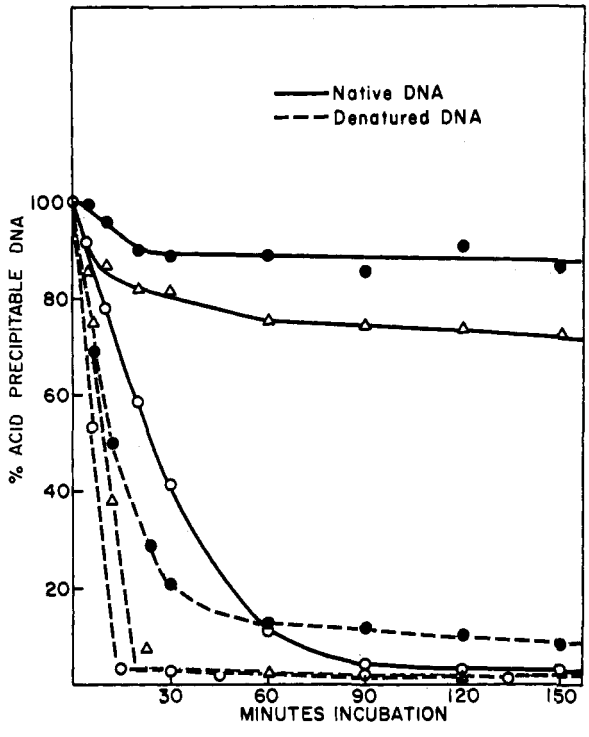

FIGURE 7: Rates of digestion of *E. coli* DNA–rubiflavin and DNA–hedamycin complexes. Native or heat-denatured [^{3}H]DNA was complexed with rubiflavin or hedamycin to give molar ratios of antibiotic to nucleotide as indicated below. Digestion by snake venom phosphodiesterase was followed by assaying acid-precipitable counts per minute in 25-μl samples. Controls contained 20,000 cpm/25-μl sample at time zero. Identification of curves: ●, control DNA; △, DNA + rubiflavin (r = 0.10); ○, DNA + hedamycin (r = 0.05); solid lines, native DNA; dashed lines, denatured DNA.

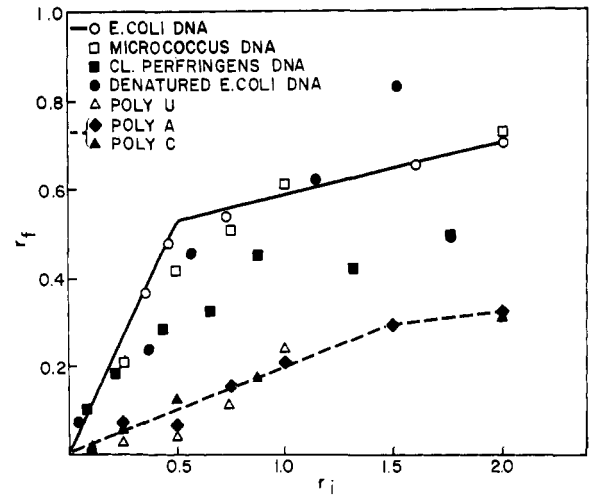

FIGURE 8: Exhaustive dialysis of DNA and other polynucleotides in the presence of rubiflavin. r_i = molar ratio of rubiflavin to nucleotide prior to dialysis against PE6 buffer; r_f = molar ratio after exhaustive dialysis as described in Materials and Methods. *E. coli* [^{14}C]DNA was assayed in each solution before and after dialysis by scintillation counting. Other polymers were assayed by ultraviolet absorbance in controls which contained no rubiflavin. The E_m of rubiflavin bound to DNA is 2340 at 440 mμ. This value was used as an approximation for rubiflavin bound to other polynucleotides.

With all these synthetic polymers except poly d(AT), spectra after dialysis were different in appearance from those found with DNA, in that they were broad smooth curves with no apparent shoulders. Maxima were at 437–440 mμ.

Because interactions between DNA and hedamycin or rubiflavin seemed somewhat similar to those found by others with nogalamycin and daunomycin (Kersten *et al.*, 1966; Calendi *et al.*, 1965), these two anthraquinone antibiotics were compared in exhaustive dialysis experiments with labeled *E. coli* DNA. Figure 10 shows that they give r_f *vs.* r_i curves which are similar in character to those obtained with rubiflavin and hedamycin.

The values of the initial slope, and the values at which the slope changed, r_{fx}, are listed in Table II for all of the above dialysis experiments. These values give a rough indication of strength of binding and extent of binding, respectively.

Discussion

Rubiflavin and hedamycin behave as distinctly different compounds as shown by their solubility characteristics, aqueous stability, chromatographic behavior, molar extinctions, and half-wave potentials. However, they are similar in absorption spectra in

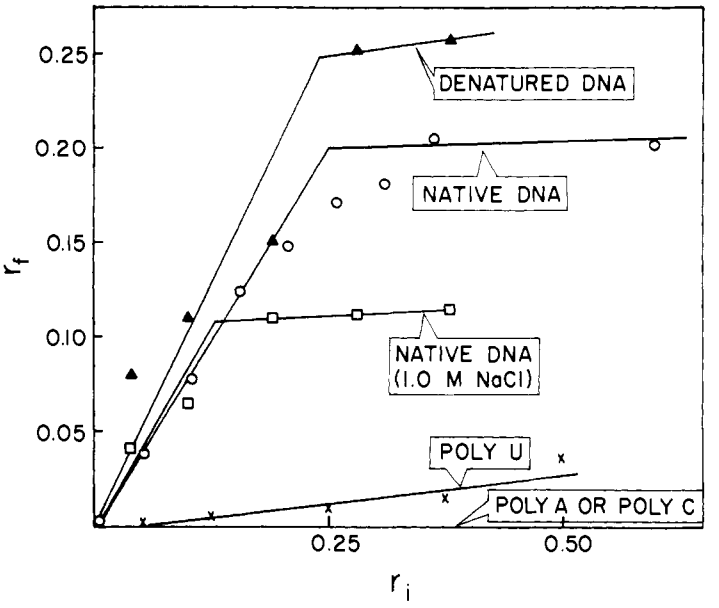

FIGURE 9: Exhaustive dialysis of DNA and other polynucleotides in the presence of hedamycin. Buffer was PE6, except where 1.0 M NaCl was added to PE6 as indicated. r_i = initial molar ratio of hedamycin to nucleotide; r_f = final molar ratio of hedamycin to nucleotide. *E. coli* [^{14}C]DNA was assayed in each solution before and after dialysis by scintillation counting. Other polymers were assayed by ultraviolet absorbance in controls containing no hedamycin. The concentrations of hedamycin were determined by visible spectra. The E_m of hedamycin bound to DNA is 6880 at 440 mμ. This value was used as an approximation for hedamycin bound to other polynucleotides.

aqueous and nonaqueous solvents, in their response to reducing agents, in ionic charge, and in antibacterial spectra. The structural differences between them seem to confer upon hedamycin greater aqueous stability and antibacterial potency.

Both hedamycin and rubiflavin show a strong tendency to bind to DNA. This binding probably comprises at least three general types: (a) an ionic interaction, probably with phosphate groups, which is diminished at high ionic strength; (b) a nonionic, essentially irreversible binding that is revealed by exhaustive dialysis experiments at high ionic strength and by cesium chloride gradient studies; and (c) an aggregation of antibiotic molecules through interactions among themselves, with the nucleic acid providing a surface for the interaction.

The irreversible type of binding is typical of DNA irrespective of per cent guanosine plus cytosine. It may be slightly greater with denatured DNA. The spectra of DNA–antibiotic solutions before or after exhaustive dialysis are always characterized by distinct shoulders, similar to those obtained by dissolving the antibiotics alone in a hydrophobic solvent (Figure 4). The electronic transition which gives rise to the 428-mμ band in the hedamycin or rubiflavin spectrum is assumed to be an $n \rightarrow \pi^*$ transition, since the 428-mμ band is blue shifted in going from benzene to hydrogen-bonding solvents (Sidman, 1958). The energy change involved in the wavelength shift from 442 mμ in benzene to 428 mμ in aqueous buffer is 2.24 kcal/mole. The shoulders in benzene are due to vibrational contributions and do not appear in aqueous antibiotic solutions because hydrogen-bonding interactions with water tend to eliminate them. The fact that these shoulders are present in aqueous DNA–antibiotic solutions and also in RNA–antibiotic solutions is therefore evidence that the chromophores in the bound antibiotic molecules are in a nonaqueous environment. Since 1 M sodium chloride causes the extent of DNA–antibiotic binding to be reduced to about half, it appears that both nonionic and ionic interactions contribute to the "irreversible" binding at low ionic strength.

A hint that nucleotide sequence may play an important part in the strong, nonionic type of binding comes from the studies with poly A, poly U, and poly C. The complexes of rubiflavin and hedamycin with these homopolymers, while showing red shifts and absorbance depression, give smooth, broad spectra without

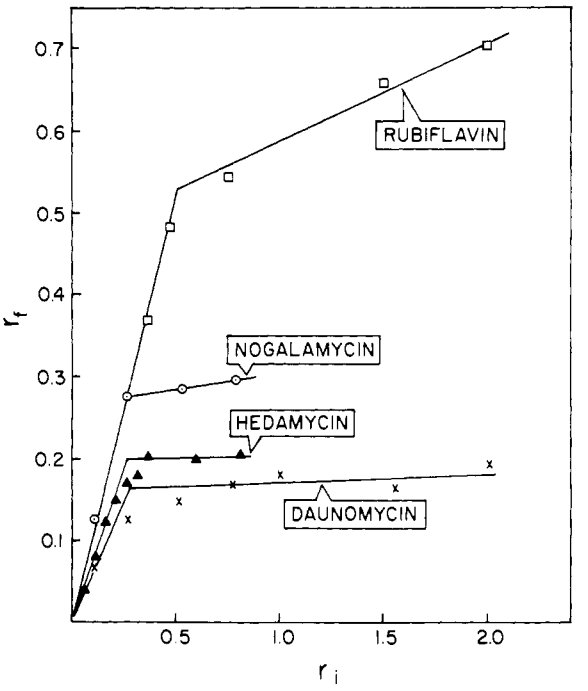

FIGURE 10: Exhaustive dialysis of DNA–nogalamycin and DNA–daunomycin complexes. *E. coli* [^{14}C]DNA was assayed in each solution by scintillation counting. Free and bound antibiotics were assayed by visible absorbance. DNA–hedamycin is included for comparison.

the obvious shoulders which characterize the nonionic binding. Also, homopolymer–antibiotic binding is greatly diminished during exhaustive dialysis. Binding of rubiflavin with poly (rA): poly (rU), which results in spectra similar to those for poly A–rubiflavin, disappeared after exhaustive dialysis against 0.1 M saline. The homopolymers, then, appear to interact with rubiflavin and hedamycin mainly by ionic mechanisms, which are reversible during exhaustive dialysis at sufficiently high ionic strength. Thus the nucleotide sequence in DNA, RNA, and poly d(AT), all of which give spectra with distinct shoulders, may play a role in the nonionic binding.

The series of visible spectra determined at constant rubiflavin or hedamycin concentration and decreasing DNA concentration apparently reflect all three kinds of binding mentioned above. At low r values spectra are red shifted and characterized by shoulders, suggesting the nonionic type of binding. In the case of DNA–rubiflavin (Figure 2), an initial increase in absorbance at 440 mμ occurs with increasing r up to $r = 0.04$, when one rubiflavin was present for every 25 nucleotides. At this point spectra begin to decrease as though stacking of rubiflavin along the helix might be causing interactions between adjacent rubiflavin molecules (Michaelis, 1947; Bradley and Wolf, 1959). This effect is not obvious in the case of the hedamycin molecule (Figure 3), which because of its larger size may not have the same tendency to stack upon itself. As r increases (to about 1 for rubiflavin or 0.5 for hedamycin complexes), spectra become smoother (disappearance of shoulders) and blue shifted toward the wavelengths of the antibiotics alone. At high r values the antibiotic molecules probably aggregate on the surface of the DNA molecules.

Since spectral shifts in the above experiments occur at r values near 1.0 for DNA–rubiflavin and near 0.5 for DNA–hedamycin, it may be concluded that, under the experimental conditions, one rubiflavin can bind to each nucleotide and one hedamycin to every pair of nucleotides, before aggregation begins to take place. Exhaustive dialysis experiments (Table II) indicated that the "irreversible" component of this binding in PE6 buffer is about half the above values for both antibiotics.

It is worth noting that hedamycin, although more potent than rubiflavin in biological action, binds to a lesser extent than rubiflavin. The data for r_f/r_i in Table II indicate that while binding by both antibiotics to DNA is effectively irreversible, the maximum r value achieved with hedamycin is less than half that for rubiflavin. However, one bound hedamycin molecule decreases the buoyant density of DNA in cesium chloride considerably more than one bound rubiflavin molecule.

Hedamycin also has a greater influence than rubiflavin on the DNA melting transition. Both antibiotics cause a strong stabilization of the double helix, hedamycin increasing T_m by about six times as much as rubiflavin on a molar basis. Spectra determined at high temperature suggest that heat dissociates the DNA–rubiflavin complex and that the DNA strands do not completely separate until this dissociation occurs. The DNA–hedamycin complex appears to be the more heat stable, and perhaps this is why a plateau in the melting transition is not reached for DNA–hedamycin. The hyperchromicities obtained with both rubiflavin and hedamycin indicate that hydrogen bonds are broken, but strand separation is inhibited, resulting in a gradually decreasing slope in melting transitions as the temperature is raised (Ginoza and Zimm, 1961; Crothers and Zimm, 1964).

The rate and extent of digestion of the DNA–antibiotic complexes by snake venom phosphodiesterase further illustrate the ability of rubiflavin and hedamycin to stabilize the double helix. Digestion by this exonuclease proceeds from the 3′ end of DNA and produces 5′-deoxynucleotides (Williams, 1961). The hy-

TABLE II: Parameters from Exhaustive Dialysis Experiments.

Polymer	Rubiflavin		Hedamycin	
	r_f/r_i	r_{fx}	r_f/r_i	r/r_{fx}
DNA, *E. coli*, native	1.0	0.53	0.80	0.20
DNA, *E. coli*, native (1 M NaCl)	0.75	0.23	0.76	0.10
DNA, *E. coli*, denatured	0.80	0.57	0.88	0.25
DNA, *M. lysodeikticus*, native	0.84	0.57		
DNA, *Cl. perfringens*, native	0.92	0.42		
Poly U	0.09	See Figure 8	0.04	See Figure 9
Poly A	0.25	0.13	0	0
Poly C	0.25	0.13	0	0
Poly d(AT)	0.77	0.35		
Poly (rA):poly (rU)	0.24	0.30		
Poly (rA):poly (rU) (0.1 M NaCl)	0	0		
	Daunomycin		Nogalamycin	
DNA, *E. coli*, native	0.61	0.16	1.0	0.27

drolysis of native DNA was strongly inhibited when complexed with either rubiflavin or hedamycin. However, when DNA was denatured before antibiotic addition, much less inhibition was observed. This implies that the antibiotics had blocked base-pair separation required for hydrolysis of native DNA by this enzyme. The initial small amount of hydrolysis which occurred with native DNA–antibiotic complexes might then have resulted from digestion of each DNA molecule from the 3′ end up to the point where a rubiflavin or hedamycin molecule was found. These compounds may similarly block strand separation required during DNA synthesis.

Because the aqueous DNA–rubiflavin complexes are dissociated in high salt solutions by shaking with benzene, a covalent type of cross-linking is not likely. With regard to the possibility of intercalation of antibiotic between bases of DNA, increases in "apparent" intrinsic viscosity were of relatively small magnitude when compared with increases observed for proflavine and acridines, which are thought to bind by intercalation (Drummond *et al.*, 1966; Lerman, 1961). Therefore, on the basis of the viscosity results, intercalation does not appear to be a prominent mechanism for rubiflavin and hedamycin. The increase in molecular weight of DNA caused by bound antibiotic may be sufficient to cause the small increases in "apparent" intrinsic viscosity observed with these two compounds. However, the ligand actinomycin D has very recently been shown by Müller and Crothers (1968) to increase the intrinsic viscosity of DNA, provided that the latter is of low molecular weight. Accordingly these workers have reclassified actinomycin D as an intercalating antibiotic. Since the effect of molecular weight of DNA on the intrinsic viscosity of DNA–hedamycin and DNA–rubiflavin complexes has not been studied, it remains possible that intercalation contributes to the nonionic component of the binding. However, intercalation is not a necessary requirement for an effective noncovalent cross-linking of DNA chains if rubiflavin and hedamycin are able to bind in one of the grooves of the double helix by strong ionic and nonionic interactions.

Daunomycin and nogalamycin are anthracycline antibiotics which bind to DNA to cause T_m increases and buoyant density decreases in CsCl gradients (Kersten *et al.*, 1966). At low ionic strength they exhibit a kind of irreversible binding to DNA similar to that of rubiflavin and hedamycin, as revealed in exhaustive dialysis experiments (Figure 10). Nogalamycin is very similar to hedamycin in molecular weight, polarographic behavior, and nature of binding to DNA. However, the ultraviolet and visible spectra of both nogalamycin and daunomycin (Calendi *et al.*, 1965) are entirely different from those of rubiflavin and hedamycin. Also they are reported to be specific inhibitors of RNA synthesis rather than of DNA synthesis (Ward *et al.*, 1965; Bhuyan and Smith, 1965), and therefore might be bound along the minor groove of DNA, as is indicated in the case of actinomycin (Reich and Goldberg, 1964). Rubiflavin and hedamycin, on the other hand, because of their specific effect on DNA synthesis at low concentrations, are more likely situated in the large groove of DNA, which may be the location of DNA polymerase during active DNA synthesis (Reich, 1964).

The strong *in vitro* interactions between DNA and hedamycin and rubiflavin appear to have their counterpart *in vivo*. Bacterial DNA synthesis is specifically inhibited at low but lethal concentrations of the antibiotics, and DNA–antibiotic complexes can be isolated from *E. coli* cells which have been killed by the antibiotic (White, 1967; White and White, 1967, and in preparation). We suggest the following mechanism of action: the antibiotics bind strongly to intracellular DNA by forces having both ionic and nonionic components; this binding prevents DNA strand separation, which is a requirement for the semiconservative mechanism of DNA synthesis, and the cell, being unable to replicate its DNA, is no longer viable. The greater effectiveness of the hedamycin molecule, both *in vitro* and in bactericidal action, is probably related to its greater size, which would enable it to extend itself over a proportionately larger section of the helix, perhaps allowing it to interact with more sites on both DNA strands.

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Evidence for a Phosphoryl–Enzyme Intermediate in Alkaline Phosphatase Catalyzed Reactions*

Harold Barrett,† Roy Butler,‡ and Irwin B. Wilson

ABSTRACT: Kinetic evidence is presented which demonstrates the formation of a catalytic phosphoryl–enzyme intermediate during the hydrolysis of phosphate esters by alkaline phosphatase. Nine phosphate esters were hydrolyzed in the presence of 1 M Tris acting as a phosphate acceptor in competition with water and the ratios of the products were measured. Precisely 1.39 equiv of *O*-phosphoryl Tris was formed for every equivalent of P_i regardless of the particular ester that was hydrolyzed. The phosphoryl–enzyme theory proposes that a phosphoryl–enzyme intermediate is formed as a step in the hydrolysis of phosphate esters followed by reaction of this intermediate with water and with other phosphate acceptors to regenerate enzyme and produce P_i and transphosphorylation products of other acceptors. In this theory, the leaving group of the ester is no longer present when the reaction with Tris and with water takes place and therefore cannot influence the ratio of products. Under these circumstances, the ratio of products must be a constant, independent of the actual ester which is used as a substrate. It is also true that a different ratio of products must be obtained for each ester if these reagents react with an entity which still contains the different leaving groups.

Since a constant ratio of products was obtained, it may be concluded that a phosphoryl–enzyme occurs as an intermediate in the enzymic hydrolysis of phosphate esters.

The most important question concerning the mechanism of alkaline phosphatase is whether a catalytic phosphoryl–enzyme intermediate is formed during the hydrolysis of phosphate esters. Widespread interest in this question was greatly stimulated by the remarkable observations of Engstrom (1961, 1962a,b, 1964), Engstrom and Agren (1958), Schwartz and Lipmann (1963), Schwartz (1961), Pigretti and Milstein (1965), and Milstein (1964), who obtained a phosphoprotein containing a phosphorylated serine side chain by incubating the enzyme (and contaminating proteins) with low concentrations of ^{32}P-labeled P_i in acid pH. There is a tendency to assume that this result, the formation of a phosphoprotein, proves the formation of a transient phosphoryl–enzyme intermediate during enzymic catalysis. However, reflection indicates that this observation in itself does not give us any information on this question. First, we note that the phosphoprotein is thermodynamically very stable; it is 10^5 times more stable than *O*-phosphorylserine (Vladimirova *et al.*, 1961) and *O*-phosphorylethanolamine derivatives (Wilson and Dayan, 1965). Alkaline phosphatase, of course, catalyzes the synthesis as well as the hydrolysis of phosphate esters, the direction being

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