Binding of Hedamycin to Deoxyribonucleic Acid and Chromatin of Testis and Liver†

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ABSTRACT: The binding of the antibiotic hedamycin to DNA was evaluated by density gradient centrifugation in CsCl to determine the type I binding, which is essentially irreversible at high and low ionic strength. Exhaustive dialysis at low ionic strength was used to determine the sum of type I and type II binding (irreversible at low ionic strength but reversible at high ionic strength). The maximum ratio of hedamycin to DNA nucleotides (r_f) is 0.1 for type I and 0.1 for type II binding to free DNA, but these ratios decrease to 0.07–0.08 in chromatin of rat liver and a testis fraction (spermatogonia plus primary spermatocytes). The r_f for type I binding of hedamycin to monomeric nucleosomes of this testis fraction is considerably less than maximum binding to polymeric nucleosomes or chromatin, suggesting that hedamycin binds more effectively to "linker" DNA than to DNA attached to the core of the nucleosomes. Hedamycin binding to the chromatin of the spermatid fraction of testis is greatly decreased in comparison with chromatin of early stages; this correlates with the change from nucleohistones to nucleoprotamines at the middle to late spermatid stages of spermiogenesis.

Studies on the binding of various dyes, drugs, and antibiotics to DNA and chromatin have contributed to an understanding of the structure of these macromolecules and have suggested possible mechanisms of the biological activities of some of the drugs and antibiotics (for example: Irvin et al., 1949; Parker & Irvin, 1952; Waring, 1966; White & White, 1966, 1969).

Hedamycin is an antibiotic isolated in crystalline form from *Streptomyces griseoruber* (Schmitz et al., 1967). According to the structure of hedamycin that has been proposed by Bedford et al. (1975), it is an anthraquinone derivative, one side chain of which contains two epoxide groups (Figure 1). This antibiotic is inhibitory to a variety of microorganisms, HeLa cells, and some transplanted tumors (Bradner et al., 1967). White & White (1969) reported that hedamycin binds to DNA and inhibits DNA synthesis in bacterial cells—a property which could account for its bactericidal activity. Qualitative data indicated to these authors that the binding of hedamycin to DNA is of three types: type I, which is irreversible by exhaustive dialysis against 1 M NaCl or by passage through a CsCl gradient; type II, which is reversible in 1 M NaCl but irreversible by dialysis at low ionic strength; and type III, which consists of an aggregation of hedamycin molecules on the surface of the nucleic acid and is reversible by dialysis against salt solutions of either low or high ionic strength.

In the present paper we are presenting an evaluation of the binding of hedamycin to the DNA and chromatin of liver and testis of the rat. The kinetics and maximum extent of type I binding are determined by buoyant density gradient centrifugation, and the maximum extent of type I plus type II binding is determined by dialysis and spectrophotometry.

Materials and Methods

Hedamycin, a gift from Dr. W. T. Bradner of Bristol Laboratories, was dissolved at a concentration of 50 μg/mL in 1 mM sodium phosphate, 0.1 mM EDTA, pH 6 (PE6 buffer). The stock solutions of hedamycin in PE6 buffer were protected from light and kept in a refrigerator for use within 2 days. Hedamycin concentrations were determined by measuring absorbance at 428 nm, a wavelength at which the molar extinction coefficient for free hedamycin is 10 000 M^{-1} cm^{-1} for concentrations below 10 μg/mL. Beer's law is not followed for higher concentrations (White & White, 1969). Spectrophotometric data were obtained with a Cary 14 recording spectrophotometer.

Calf thymus and *Clostridium perfringens* DNA were purchased from Worthington Biochemical Corp. They were dissolved by gentle stirring in 1 mM sodium phosphate, pH 8, and were dialyzed exhaustively against both 10 mM EDTA and PE6 buffer. *Micrococcus lysodeikticus* DNA and the DNA of rat liver and testis were isolated by the method of Marmur (1961). The concentration of DNA solutions was determined by measuring absorbance at 260 nm. Published values for the molar extinction coefficients (ϵ_p) of various DNAs vary slightly (Mahler et al., 1964), but we have used an average value of 6700 M^{-1} cm^{-1} at 260 nm.

Isolation of Chromatin and Nucleosomes. Chromatin was isolated from liver and testes of Sprague-Dawley rats (150–200 g) by a method similar to that of Kumaroo (1968). Nuclei of liver or the seminiferous epithelial cells of testis were isolated by a modification (Holbrook et al., 1962) of the method of Chauveau et al. (1956). The nuclei of testis were subfractionated as described previously (Kumaroo et al., 1975) to yield a fraction highly enriched in nuclei of spermatids and a fraction enriched in nuclei of spermatogonia and primary spermatocytes. For isolation of chromatin the nuclei were suspended in 20 mL of 1.5 M sucrose containing 2 mM $MgCl_2$ and were disrupted in a French pressure cell (American Instrument Co., Inc.). The disrupted nuclei were layered over 1.7 M sucrose–10 mM Tris-HCl, pH 8, in three tubes of a Spinco SW 25.1 rotor. A rough gradient was formed by gently stirring the solution in the top two-thirds of the tube. The gradients were centrifuged for 3 h at 45 000*g*. The pellets were resuspended in 20 mL of 10 mM Tris-HCl, pH 8, and dialyzed at 0 °C for 10 h against 100 volumes of 10 mM Tris-HCl–0.2 mM EDTA at pH 8, and then twice for 6 h each against the buffer appropriate for subsequent experiments. After resuspension by 8–10

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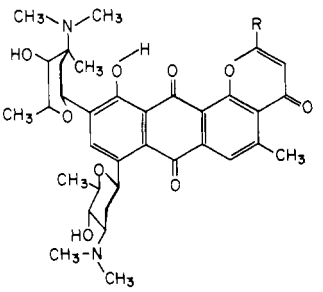

FIGURE 1: Structure of hedamycin proposed by Bedford et al. (1975). The group denoted by R- was suggested to be a *sec*-2,3;4,5-diepoxy-*n*-hexyl group. Chemical evidence (Sequin et al., 1977) has confirmed the presence of at least one epoxide moiety in the hedamycin molecule.

strokes of a hand-held homogenizer, the insoluble material was removed by centrifugation at 10 000*g* for 30 min. The supernate contained 250–450 μg of DNA/mL and was called chromatin solution. The chromatin of the spermatid fraction of rat testis was subfractionated into soluble and condensed fractions by the method which was used by Marushige & Dixon (1969) and Marushige & Marushige (1975) to fractionate whole testis chromatin.

Nucleosomes (chromatin subunits) were prepared from the nuclei of the spermatogonia plus primary spermatocyte fraction of rat testis by digestion of the nuclei with micrococcal nuclease by the method of Noll (1974) as applied to trout testis (Honda et al., 1974). Nuclei were digested with 100 A_{260} units/mL of micrococcal nuclease for 3 min (in other experiments the digestion was 6 min with 600 A_{260} units/mL) at 37 °C in TMKS buffer (Honda et al., 1974) containing 1 mM $CaCl_2$. Digestion was terminated by chilling on ice and addition of 0.02 mL of 0.1 M EDTA at pH 7. The nuclei were centrifuged for 10 min at 3000*g*, lysed by suspension in 1 mL of 1 mM EDTA, pH 7, and then centrifuged for 10 min at 12 000*g*. The supernatant fraction containing nucleosomes was dialyzed at 5 °C for 2 h against PE6 buffer containing 1 mM EDTA and then for 2 h against PE6 buffer for use in the hedamycin binding experiments. Other portions of the nucleosome fractions were used for polyacrylamide gel electrophoresis of the DNA by the method of Hewish & Burgoyne (1973). Samples of the nucleosome fractions were used for electrophoretic analysis of histones by procedures applied previously to chromatin (Branson et al., 1975) involving extraction with 0.4 N H_2SO_4, precipitation with ethanol, and electrophoresis in polyacrylamide gels by the acid–urea method of Panyim & Chalkley (1969) followed by staining with amido black, destaining, and densitometric scanning with a Gilford Model 2000 recording spectrophotometer.

The concentrations of the various preparations of chromatin and nucleosomes were expressed as mM DNA nucleotide, and the DNA in each preparation was determined by the method of Burton (1956) with calf thymus DNA as a standard.

Equilibrium Gradient Centrifugation. Cesium chloride equilibrium density gradient centrifugation and the density calculations were performed as described by Mandel et al. (1968) and White & White (1969) with the use of a Beckman-Spinco Model E analytical ultracentrifuge and an AN-D rotor with Kel-F centerpiece. *Micrococcus lysodeikticus* DNA, which has a buoyant density of 1.7245 g/cm^3 in CsCl (Szybalski, 1968), was used as a density marker. In many experiments marker DNA was present both in the same rotor cell with the DNA–hedamycin complexes and in a separate reference cell.

Competition Experiments. Hedamycin was diluted to 16.7 μM, and calf thymus DNA (or chromatin) to 360 μM nucleotide, in PE6 buffer. Over a period of 2 min, 2 mL of dilute hedamycin was added dropwise with stirring to 1 mL of the DNA (or chromatin) solution and maintained at the appropriate temperature. Immediately after addition of the hedamycin solution to the calf thymus DNA (or chromatin) and at various time intervals thereafter, 0.5-mL aliquots of the reaction mixture containing 60 nmol of DNA were each rapidly mixed with individual 0.1-mL portions of 600 μM *Micrococcus* DNA in small test tubes. Then, each sample was wrapped in foil and allowed to react at 25 °C for at least 18 h to assure that all the hedamycin present was irreversibly bound to either the calf thymus DNA (or chromatin) or the *Micrococcus* DNA. After the reaction had continued to completion, the density of each DNA–hedamycin complex was determined by performing CsCl equilibrium density gradient centrifugation on 25 μL of each sample.

Determination of Maximum Type I Binding Ratio and Kinetics of Binding of Hedamycin to DNA and Chromatin. Rat liver DNA (2 mL of 335 μM DNA-nucleotide in PE6 buffer), or similar concentrations of the chromatins of rat liver of testis, was mixed with 2 mL of hedamycin (67 μM) in the same buffer. The temperature was maintained at 2 °C in a controlled water bath, and at intervals of 1 to 18 h thereafter 0.4-mL samples of the solution were removed, and 200 nmol of *Micrococcus* DNA in PE6 buffer was added to quench any further reaction of the hedamycin with the rat liver DNA or chromatins. After quenching, a 25-μL sample of each solution was used for determination of the density of the rat DNA–hedamycin complex by cesium chloride equilibrium density gradient centrifugation. The value of r_f for each complex was calculated from the data of Figure 5.

Determination of Binding of Hedamycin to DNA and Chromatin by Exhaustive Dialysis. Hedamycin in PE6 buffer was added dropwise, with stirring, to solutions of DNA or chromatin in PE6 buffer (30–120 μM DNA nucleotide). After 10 h at 25 °C in the dark, the solutions were transferred to cellophane bags and dialyzed for 5 days at 4 °C against 50 volumes of PE6 buffer with three changes of buffer the first day and one change each day thereafter. The final concentration of hedamycin remaining in the dialysis bag was determined spectrophotometrically from the absorbance at 440 nm and the molar extinction coefficient of 6880 cm^{-1} M^{-1} for hedamycin bound to DNA (White & White, 1969). It should be noted that at the end of the exhaustive dialysis there is no unbound hedamycin remaining, and there is only the DNA–hedamycin complex present for spectrophotometric determination. The concentration of DNA was determined by the method of Burton (1956) applied after precipitation of DNA and proteins with cold 1 M perchloric acid and extraction of DNA nucleotides from the precipitate by heating at 90 °C for 30 min with 0.5 M perchloric acid as described previously (Kumaroo et al., 1975). Hedamycin does not interfere with the determination of DNA by this method. The data for concentrations of DNA and bound hedamycin after dialysis were used for calculation of r_f values.

Results

Analyses of Chromatin and Nucleosome Preparations. Nucleosomes obtained by brief digestion with micrococcal nuclease of the nuclei of the spermatogonia + primary spermatocytes of rat testis yielded DNA which had been hydrolyzed to fragments which were multiples of a unit length (Figure 2a), viz., the DNA was derived from nucleosomes in which a majority (approximately 75%) of the nucleosomes were present in "strings" of 2 or more units. Digestion of the nuclei for a longer period of time yielded preparations in which

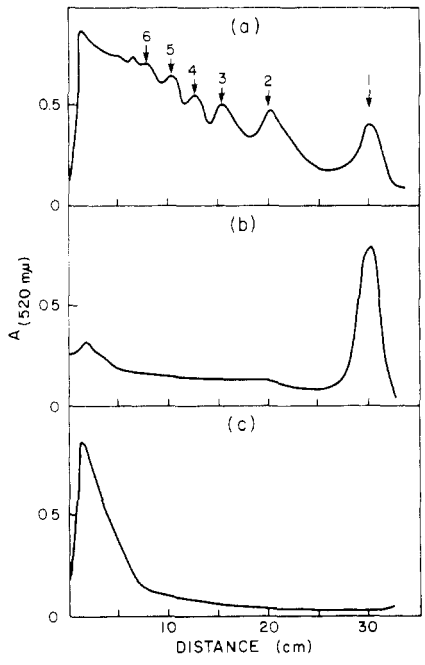

FIGURE 2: Densitometer scans of polyacrylamide gel (2.5%) electrophretic patterns of DNA (stained with ethidium bromide) from (a) nuclei of spermatogonia + primary spermatocytes digested with 100 A_{260} units/mL of micrococcal nuclease for 3 min, (b) the same nuclei as in a but digested with 600 units/mL of micrococcal nuclease for 6 min, and (c) nuclei of late stage spermatids digested with 600 units/mL of micrococcal nuclease for 6 min.

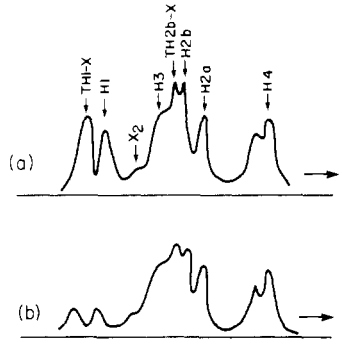

FIGURE 3: Densitometric tracings of polyacrylamide gel electrophoretic patterns of histones from (a) chromatin and (b) mononucleosome fraction obtained from the nuclei of the spermatogonia + primary spermatocytes of seminiferous epithelial cells of the testis.

approximately 90% of the nucleosomes were present as mononucleosomes as indicated by the fact that approximately 90% of the DNA obtained from these nucleosomes had unit length (Figure 2b) indicating that the DNA linker regions between nucleosomes had been hydrolyzed. Micrococcal nuclease digestion of nuclei of the middle to late stage spermatids did not yield DNA fragments (Figure 2c).

Comparison of the gel electrophoretic pattern of the histones extracted from testis chromatin (Figure 3a) with the pattern for histones from the mononucleosomes of nuclei of spermatogonia + primary spermatocytes (Figure 3b) shows in the latter a decreased proportion of the lysine-rich histone fractions H1 and TH1-x (previously designated as X_1; Branson et al., 1975) relative to histone fractions H2b, X_2, TH2b-x (previously designated as X_3), H3, and H4. Data have been presented previously (Morris, 1976; Noll, 1976) tending to show that histone H1 is attached to the linker DNA of polynucleosomes. The data presented here (Figure 3) tend to indicate that the unusual H1 histone of testis, which we have designated as TH1-x, also is associated with linker DNA.

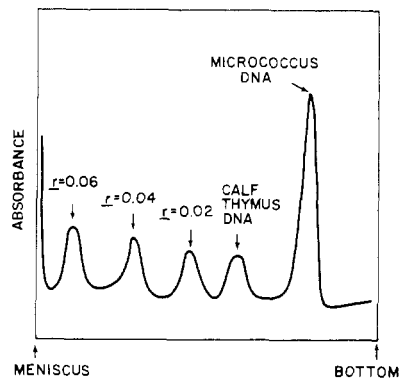

FIGURE 4: Microdensitometer tracing resulting from a CsCl gradient (initial density = 1.680 g/cm^3) containing *Micrococcus lysodeikticus* DNA, calf thymus DNA, and calf thymus DNA-hedamycin complexes with hedamycin/DNA molar ratios (r) of 0.02, 0.04, 0.06, and 0.08. The last complex is visible only as a rising curve at the meniscus. The complexes were incubated at 25 °C for 18 h before mixing; then a mixture of the DNAs and complexes was added to CsCl and centrifuged as described in Materials and Methods.

TABLE I: The Reaction of Hedamycin with DNA in the Presence of 5.5–6 M CsCl.[a]

sample	density (g/cm^3)	hedamycin/DNA[b] (molar ratio) r_f
experiment A		
calf thymus DNA	1.6657	0.025
Micrococcus DNA	1.7065	0.016
experiment B		
Calf thymus DNA	1.6551	0.035
Micrococcus DNA	1.7103	0.013

[a] Details of the experiment are described in the text. [b] From Figure 5.

Stability of the Type I DNA–Hedamycin Complex. The remarkable stability of the type I complex of hedamycin with DNA can be illustrated in several ways. One of the most striking examples is shown in the microdensitometer tracing, Figure 4, which illustrates that complexes having various hedamycin/DNA molar ratios (r) can be mixed together and then separated by banding in CsCl. There was no significant difference between the microdensitometer tracing of a freshly prepared mixture of complexes and a sample of the same mixture kept at 4 °C for 8 days before being placed in CsCl. Even at rather low exchange rates, the free DNA bands would have been shifted to lower density—especially after 8 days. In the experiment shown, however, the density of the calf thymus DNA band was 1.6917 g/cm^3 when calculated from a *Micrococcus* DNA band centrifuged separately and 1.6925 g/cm^3 when calculated from the *Micrococcus* band in the mixture as shown. For comparison, the density of calf thymus DNA in cesium chloride has been reported to be 1.6925 g/cm^3 (Szybalski, 1968). Thus the buoyant densities of the two free DNA species were affected only very slightly, if at all, by the presence of hedamycin-DNA complexes in the solution.

The data of Table I demonstrate that free hedamycin will bind to DNA in the presence of 5.5–6 M CsCl at 25 °C, although the rate of binding is much slower than the rate at low ionic strength. In experiment A, 0.208 μg hedamycin in a volume of 0.025 mL was added to 0.9 mL of 6.1 M CsCl followed by 1 μg of calf thymus DNA in 0.025 mL. (If all the

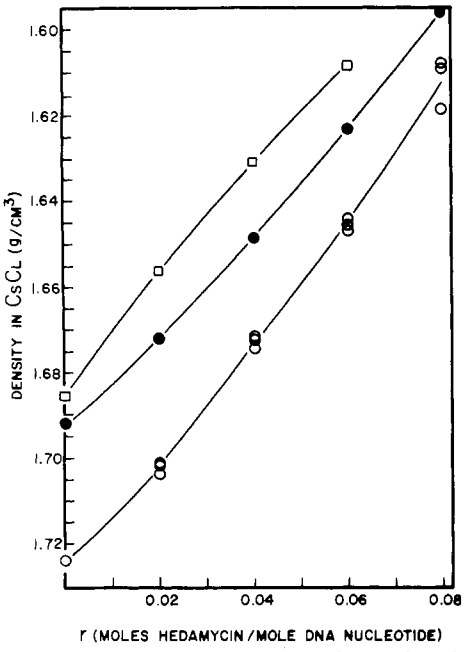

FIGURE 5: Buoyant density of DNA-hedamycin complexes in CsCl. The complexes were incubated for at least 18 h at 25 °C before centrifugation as described in Materials and Methods. Identification of curves: (□) *Clostridium perfringens* DNA-hedamycin; (●) calf thymus DNA-hedamycin; (○) *Micrococcus lysodeikticus* DNA-hedamycin.

TABLE II: Competition Experiment.[a]

conditions	band	density (ρ) (g/cm³)	hedamycin/DNA[b] (molar ratio) r_f
time (t) = 3 h	calf thymus DNA	at meniscus ($\rho \leq$ 1.6028)	≥0.076
temp = 25 °C	*Micrococcus* DNA	1.7040	0.018
r_i = 0.093[c]	chromatin DNA	1.6196	0.063
	Micrococcus DNA	1.6943	0.026
time (t) = 21 h	chromatin DNA	at meniscus ($\rho \leq$ 1.6077)	≥0.072
temp = 25 °C	*Micrococcus* DNA	1.7128	0.019
r_i = 0.090[c]			
time (t) = 21 h	chromatin DNA	1.6254	0.058
temp = 0 °C	*Micrococcus* DNA	1.6762	0.038
r_i = 0.090[c]			
time (t) = 48 h	chromatin DNA	1.6229	0.060
temp = 0 °C	*Micrococcus* DNA	1.6899	0.028
r_i = 0.090[c]			

[a] Details of this experiment are presented in Materials and Methods and discussed in the text. The chromatin used in these experiments was isolated from normal rat liver. [b] Based on Figure 5. [c] r_i = initial moles of hedamycin/moles of DNA nucleotides.

hedamycin reacted with the DNA, the r_f would be 0.095.) After 36 h at 0 °C, 1 μg of *Micrococcus* DNA in 0.025 mL was added, the density was adjusted with water to 1.680 g/cm³, and the densities of the DNA-hedamycin complexes were determined in the Model E ultracentrifuge by a 20-h run at 25 °C. Experiment B was similar to experiment A, except that, during the 36-h incubation period, a 6-h period was at 25 °C. Experiment A indicates that calf thymus DNA interacted with hedamycin in 6 M CaCl at 0 °C, but the reaction was incomplete even after 31 h since some free hedamycin remained to interact with micrococcal DNA which was added prior to centrifugation. The binding of hedamycin to calf thymus DNA was greater in experiment B in which a 6-h period at 25 °C was included as a part of the total incubation period. Thus, hedamycin does bind to DNA in 6 M CsCl at a sufficient rate to disclose significant dissociation had it occurred after adding the mixture of complexes to the CsCl (Figure 4), although at 0 °C type I binding is quite slow at such high ionic strength.

The stability of the DNA-hedamycin complex is further demonstrated by the fact that in many of the experiments described below, DNA, which was added as a density marker in the same rotor cell as the DNA-hedamycin complexes in question, banded at the same position as DNA in a separate cell. Also, marker DNA added in two portions to a solution of complexes in CsCl—before and after an incubation period at 25 °C—formed a single band (data not shown).

When sufficient time is permitted for completion of reaction of hedamycin with DNA under conditions in which the ratio of hedamycin/DNA is <0.1, then no free hedamycin can be detected, and the initial hedamycin/DNA ratios, r_i, are equal to the final ratios, r_f, for the hedamycin-DNA complexes in the CsCl gradient. Thus, it is possible to plot a standard line for buoyant density of complex ρ vs. r (Figure 5) which can be used to determine r for any DNA-hedamycin complex in the range of r where binding is irreversible at high ionic strength.

Competition Experiments. As explained above, the dependence of density of a DNA-hedamycin complex upon the amount of hedamycin bound can be used not only to determine the amount of hedamycin bound when the reaction has gone to completion, but also to detect free hedamycin in solution. The "competition" experiments, described in Materials and Methods, were designed to follow the progress of the binding reaction with time. *Micrococcus* DNA, added at various times (t) after addition of hedamycin to calf thymus DNA, competes for the free or reversibly bound hedamycin. The amount of hedamycin bound to each of the two DNA species after the reaction had run to completion was determined by measuring the buoyant densities of the complexes. In this way, it was possible to follow the progress of type I (irreversible) binding, by measuring the decrease in density of the calf thymus DNA and the increase in density of the *Micrococcus* DNA.

In Figures 6a and 6b the reactions of hedamycin with calf thymus DNA and with liver chromatin are followed over a 3-h period. Rat liver DNA from the chromatin was found to have the same density as calf thymus DNA. Chromatin DNA is separated from chromatin proteins during centrifugation in CsCl. Table II presents the results of similar competition experiments involving longer reaction times (t) and/or higher temperatures. The reaction proceeds over a period of hours for both purified DNA and for chromatin, with the rate of reaction for the chromatin being much slower than for the DNA (Figures 6a and 6b).

Kinetics of Type I Binding of Hedamycin to DNA, Chromatin, and Nucleosomes and Determination of Maximum Binding Ratios. The experiments summarized in Figure 7 show most strikingly the differences in rates of type I binding of hedamycin with free rat liver DNA and with the DNA of the chromatins of rat liver and testis (both dispersed and condensed). There was no significant difference in rate of type I binding of hedamycin to DNA of rat liver or testis (not shown in Figure 7) in comparison with calf thymus DNA. However,

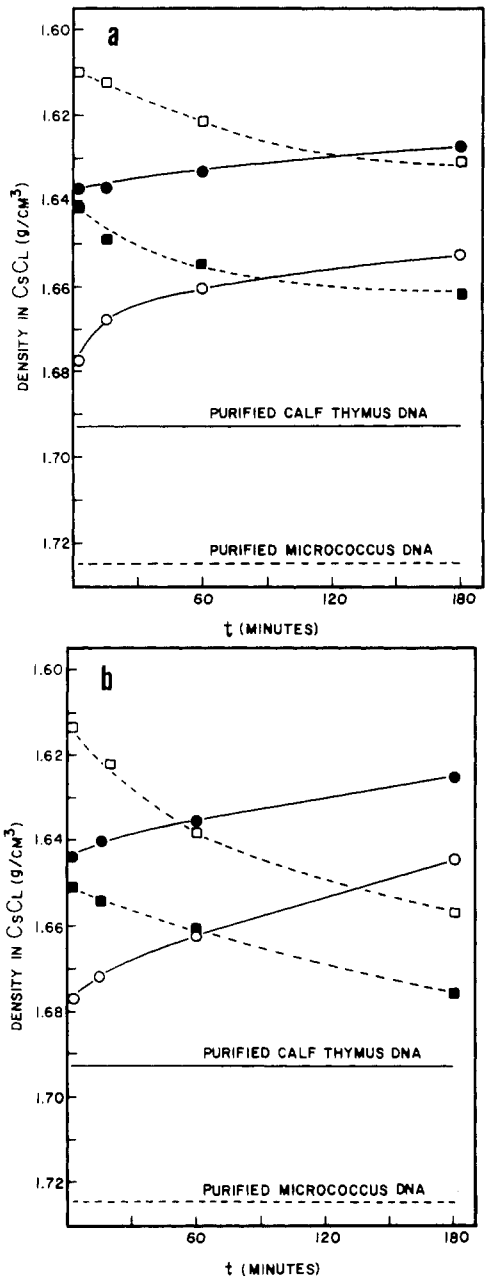

FIGURE 6: (a) Buoyant densities of DNA-hedamycin complexes resulting from a competition experiment at 0 °C. After hedamycin had been incubated with calf thymus DNA or liver chromatin for the indicated time, *Micrococcus* DNA was added to compete for the remaining hedamycin. When binding had proceeded to completion (Materials and Methods), the complexes were fractionated on a cesium chloride density gradient. Initial molar ratio, hedamycin/calf thymus DNA or chromatin DNA = 0.105. Identification of curves: (●) calf thymus DNA-hedamycin complexes; (■) *Micrococcus* DNA added at time t to compete with calf thymus DNA; (○) liver chromatin DNA-hedamycin complexes; (□) *Micrococcus* DNA added at time t to compete with chromatin. (b) Competition experiment at 8 °C. Details in a. Initial molar ratio, hedamycin/calf thymus DNA or liver chromatin DNA = 0.095. Identification of curves: (●) calf thymus DNA-hedamycin complexes; (■) *Micrococcus* DNA added at time t to compete with calf thymus DNA; (○) liver chromatin DNA-hedamycin complexes; (□) *Micrococcus* DNA added at time t to compete with chromatin.

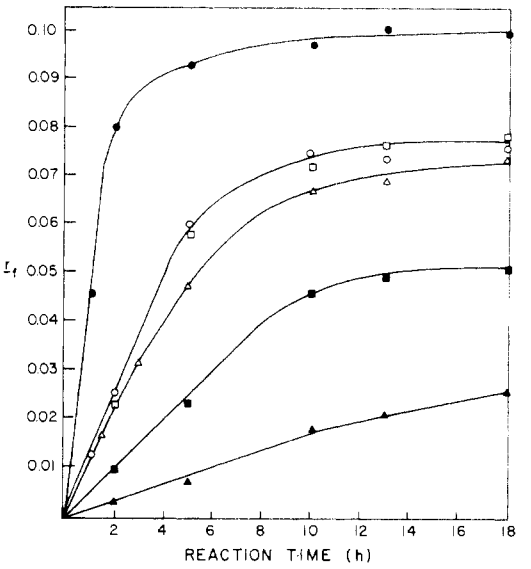

FIGURE 7: Kinetics of the type I binding of hedamycin to (●) rat liver DNA; (○) rat liver chromatin; (□) chromatin from spermatogonia and primary spermatocytes of rat testis; nucleosomes from spermatogonia and primary spermatocytes of rat testis prepared under conditions in which the nuclei were digested with 100 A_{260} units/mL of micrococcal nuclease for 3 min at 37 °C (△) and with 600 units/mL for 6 min (■); (▲) chromatin from middle to late stage spermatids of rat testis. Values of r_f were determined by the buoyant-density centrifugal method after reaction of hedamycin with DNA or chromatin in PE6 buffer at 25 °C for the specified time intervals. For these experiments, $r_i = 0.2$.

for rat liver chromatin the initial rate of reaction was one-third the rate of reaction with free DNA, and the maximum value of r_f was 0.076 for chromatin and 0.10 for free DNA. The rate and maximum r_f of hedamycin binding to the chromatin of a fraction containing spermatogonia and primary spermatocytes were identical with the rate and maximum r_f of binding of hedamycin to chromatin of rat liver. However, the rate and maximum r_f of binding of hedamycin to nucleosomes prepared from the spermatogonia plus spermatocytes were significantly smaller than the corresponding values for the chromatin isolated from the same testicular fraction. This was particularly true when the nuclei (from spermatogonia plus spermatocytes) were digested with micrococcal nuclease under conditions (600 units/mL for 6 min) which yielded mononucleosomes. The decrease in r_f (in comparison with chromatin) was much less when the less drastic nuclease digestion (100 units/mL for 3 min) was used which yielded a high proportion of nucleosome polymers. The rate and maximum r_f of binding to the condensed chromatin of the spermatid nuclei were strikingly lower than the binding of hedamycin to any of the other chromatins examined.

Absorption Spectra of Hedamycin and Complexes of Hedamycin with DNA and Chromatin. The data of Figure 8 show that the absorption spectrum of free hedamycin, with an absorption maximum of 428 nm, is red-shifted to a maximum of 440 nm when hedamycin is bound to DNA or chromatin, and there also is a large decrease in the extinction coefficients and appearance of shoulders on the absorption peak. The absorption spectrum of hedamycin bound to chromatin is identical with that of hedamycin bound to DNA under conditions in which all hedamycin is bound, but a higher concentration of chromatin than DNA is required to attain complete binding. The absorption spectra of hedamycin bound to DNA by type I and type II binding appear to be identical. Consequently, we attempted to determine binding (sum of types I and II) of hedamycin to DNA and chromatin by a direct spectrophotometric absorption method by application of the equations:

$$A = \epsilon_f C_f + \epsilon_b C_b$$

$$C_t = C_f + C_b$$

in which A = total absorbance in a 1-cm cuvette of a solution containing both free hedamycin and DNA-bound hedamycin,

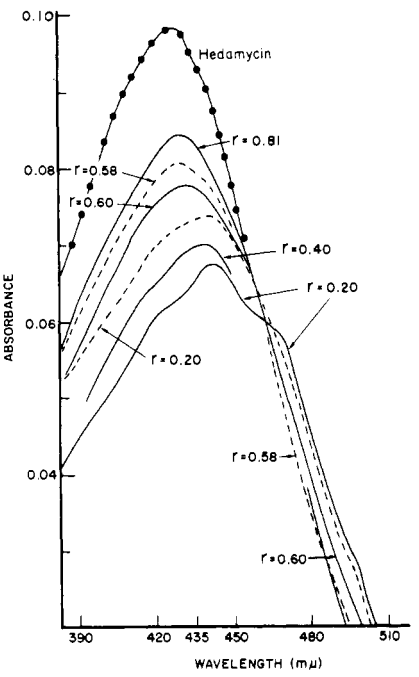

FIGURE 8: Absorption spectrum of free hedamycin (●—●) and the spectra of hedamycin bound to DNA (—) and to rat liver chromatin (- - -) in PE6 buffer at various values of r. The complexes were prepared with a constant total concentration of hedamycin by accurate addition of 0.2 mL of 60 μM hedamycin in PE6 buffer to 1.0 mL of various concentrations of calf thymus DNA or rat liver chromatin in PE6 buffer with constant stirring. The mixtures were allowed to stand at room temperature in the dark for 1 h before measurement of absorption (1-cm cuvette) in a Cary Model 14 recording spectrophotometer with a 0–0.1 absorbance slide-wire vs. PE6 buffer, DNA solution, or chromatin solution, respectively, in the reference curvette.

ϵ_f and ϵ_b are the molar extinction coefficients at a fixed wavelength of bound and free hedamycin, respectively, and C_f and C_b are the molar concentrations of the two species and C_t is the total molar concentration of hedamycin. Application of these equations to the data of Figure 8 yielded data for C_b from which r_f values were calculated by use of the known concentration of DNA. However, this procedure yielded values of r_f (averages of 0.31 and 0.21 for binding to DNA and rat liver chromatin, respectively) which were high in comparison with r_f values by exhaustive dialysis (below). This discrepancy appears to be due to the fact that, after the type I and II binding sites are filled, hedamycin exhibits a reversible and weak "stacking" interaction with DNA (type III binding) which is characterized by a decrease in the absorption spectrum of hedamycin without the red-shift which is characteristic of the spectra of hedamycin bound to DNA by types I and II binding.

Determination of Maximum Binding of Hedamycin to DNA, Chromatin, and Nucleosomes by Exhaustive Dialysis. When hedamycin was incubated with DNA, chromatin, or nucleosomes and then dialyzed exhaustively, all of the remaining hedamycin was in the bound form (types I and II), and no free hedamycin remained. Consequently, the concentration of bound hedamycin could be determined from the absorbance at 440 nm and the molar extinction coefficient for bound hedamycin at that wavelength. Since solutions of chromatin and nucleosomes are slightly turbid, the absorbance of the hedamycin–chromatin or hedamycin–nucleosome complexes after exhaustive dialysis was determined vs. a similarly dialyzed chromatin or nucleosome solution in the reference cuvette. The r_f values were calculated from the determined concentrations of bound hedamycin and DNA after the dialysis.

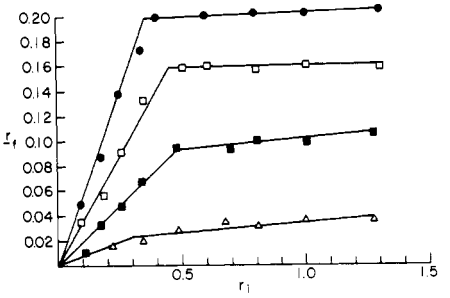

FIGURE 9: Evaluation of the extent of binding of hedamycin to free rat testis DNA (●), chromatin of the spermatogonia plus spermatocyte fraction of rat testis (□), monomeric nucleosomes from the spermatogonia plus spermatocyte fraction (■), and condensed chromatin of spermatids (△) by exhaustive dialysis. Values of r_f are the sum of type I and type II binding.

The data of Figure 9 show that the maximum binding of hedamycin to the chromatin of the spermatogonia plus spermatocyte fraction is only 75–80% of the binding to free DNA. Hedamycin binding to the mononucleosome fraction is even more restricted (43% of maximum binding to free DNA). Binding of hedamycin to the condensed chromatin fraction of spermatids is only 10–20% of binding to free DNA. The binding evaluated by the exhaustive dialysis method is the sum of types I and II, and the r_f values are approximately twice those found for type I binding by the CsCl density-gradient centrifugation method.

The binding of hedamycin to chromatin and nucleosomes appears to be entirely attributable to binding to DNA rather than to chromosomal proteins. By the method of exhaustive dialysis we were not able to detect any binding of hedamycin to histones of liver or testis or to nonhistone chromosomal proteins of liver.

Discussion

The data presented in this and the previous publication (White & White, 1969) indicate that hedamycin binds to DNA very strongly in a manner which cannot be reversed by exhaustive dialysis against buffers of low ionic strength such as PE6 buffer. The maximum r_f for binding of hedamycin to DNA at low ionic strength is 0.2 mol of hedamycin per mol of DNA nucleotide (identical values were obtained for calf thymus DNA and the DNA of rat liver and testis). A portion of the hedamycin which is bound at low ionic strength can be dissociated from the DNA complex at high ionic strength (1 or higher). Binding of this type is designated here as type II while the hedamycin which remains bound at high ionic strength is designated here as type I. The method of exhaustive dialysis at low ionic strength (PE6 buffer) yields values of r_f corresponding to the sum of type I plus type II binding while the CsCl density gradient centrifugation method gives values of r_f for type I binding. The r_f for type II binding is calculated by difference.

The type I linkage between hedamycin and DNA may be covalent. When a solution of the DNA–hedamycin complex (r = 0.1) in PE6 buffer containing 1.0 M NaCl was treated with an equal volume of chloroform, the hedamycin was not extracted but remained associated with the DNA at the interface. In contrast, a solution of hedamycin in PE6 buffer is completely extracted by chloroform.

It seems probable that hedamycin first binds to DNA by the type of binding which we have designated as type II. In view of the structure of hedamycin it seems likely that type II

binding may involve intercalation of hedamycin into DNA. This initial binding then may be followed by relatively slow (6–10 h at 25 °C) alkylation of the DNA bases by interaction of the epoxide groups of the hedamycin side chain to yield covalent binding of approximately half the hedamycin molecules—binding which would be irreversible even at high ionic strength, viz., type I binding. The fact that only half the initial reversibly bound hedamycin molecules become irreversibly bound suggests that there may be some specificity of interaction of hedamycin with the DNA bases. Although this explanation of the binding of hedamycin to DNA seems reasonable to us, it should be emphasized that at present we do not have evidence to support this hypothesis.

The effect of antibiotic binding on the density of DNA in CsCl or Cs_2SO_4 has been studied for a variety of antibiotics. The binding of mitomycin C to DNA is covalent but requires chemical or enzymatic activation of the antibiotic (Iyer & Szybalski, 1964). For many other antibiotics (Kersten et al., 1966; Radloff et al., 1967), the DNA–antibiotic complex in CsCl or Cs_2SO_4 is either in equilibrium with unbound antibiotic, or the reversibility is unknown. Rubiflavin–DNA complexes have properties in CsCl similar to those of hedamycin (White & White, 1969).

The effect of hedamycin binding upon the buoyant density of DNA was used to study the time-course of the binding reaction. The curves presented in Figures 6 and 7 and Table II show that type I binding of hedamycin to DNA or to the DNA of chromatin takes place over a period of hours, and the rate of binding shows some increase from 0 to 25 °C.

The fact that the type I binding of hedamycin to free DNA or to DNA of chromatin is essentially irreversible prevents the use of equilibrium equations for calculation of association constants by Scatchard plots, but the irreversibility of the binding is an advantage in determining the maximum r_f values for binding of hedamycin. With ligands which bind to DNA reversibly, extrapolations are necessary to determine the maximum number of ligands bound per DNA nucleotide, but such extrapolations are not necessary in the case of hedamycin.

The facts that the rates of binding of hedamycin to DNA of chromatin and the maximum binding ratios (r_f) by types I and II are smaller for the various chromatins than for the corresponding DNA suggest that the chromosomal proteins restrict the access of hedamycin to the binding sites on the DNA. However, it is important to note that the decrease in maximum r_f values for binding of hedamycin to DNA of the chromatin of the spermatogonia plus spermatocyte fraction of testis is not large (r_f = 0.1 for DNA vs. 0.07–0.08 for chromatin by type I binding). These data are more compatible with current views of the structure of chromatin as consisting of DNA coiled around a core of two molecules of each of the four major histone fractions H2a, H2b, H3, and H4 (reviewed by Kornberg, 1977) than with earlier hypotheses involving a coating of chromosomal proteins around segments of the DNA. In the currently accepted model of chromatin structure the DNA would be readily accessible to binding reactions with hedamycin, although some restrictions would be anticipated. It is of great interest that hedamycin binding to the polymeric nucleosomes (resulting from partial hydrolytic breakage of only a few of the DNA "linkers" between nucleosomes) is considerably more extensive than binding of hedamycin to monomeric nucleosomes (r_f maximum of type I binding of 0.07 vs. 0.05). This observation suggests that the DNA of the "linker" regions is more accessible to hedamycin (as well as nuclease) than the DNA which is more closely associated with the histone "core" of the nucleosome. However, the data suggest that hedamycin does bind to DNA of the core, although to a smaller extent than to the linkers.

Binding of hedamycin to the condensed chromatin of the spermatid fraction of rat testis was greatly restricted (r_f maximum for type I binding of approximately 0.03) in comparison with the other chromatins and DNA. Honda et al. (1974) found that the chromatin of the late stages of spermiogenesis in the trout does not consist of the subunit (nucleosome) structure which is found in chromatin of early stages of spermatogenesis. At the spermatid stages in trout testis histones are replaced by protamines (Ling et al., 1969). Similarly, during late stages of spermiogenesis in testis of the rat the histones are replaced by cysteine-containing protamines (Kistler et al., 1973; Kumaroo et al., 1975). This drastic reorganization of the chromatin evidently renders the DNA much less accessible to binding of hedamycin than chromatin of early stages of spermatogenesis.

The data presented here reveal that hedamycin can be useful in studies on the structure of chromatin, and this antibiotic can be particularly useful in studies of changes in chromatin structure such as those occurring in spermatogenesis. In addition, these studies provide data of value in proposing mechanisms for the inhibition of DNA synthesis and cellular replication by this antibiotic.

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Mapping of Transcribed Regions of *Euglena gracilis* Chloroplast DNA†

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ABSTRACT: RNA transcription from defined regions of the *Euglena gracilis* chloroplast genome has been characterized by hybridization of total cell RNA to ^{3}H-labeled chloroplast DNA restriction endonuclease fragments. Chloroplast DNA was digested into five fragments of 53, 35, 25, 10, and 6.9 kilobase pairs (kbp) with *Pst*1. The 53-kbp DNA was also subfractionated by *Bam*HI digestion. The extent of transcription of the *Pst*1 fragments was found to be 30, 17, 15, 2.2, and 2.3 kb of RNA, respectively. The total amount of RNA transcription of 67 kb represents 26% of the double-strand information content of the genome. Transcribed regions are dispersed throughout the DNA. The RNA transcripts are present in two major abundance classes in the cell. High abundance transcripts of approximately 10^6 copies/cell were mapped in the rRNA gene region of the 53-kbp fragment and in the 35-kbp fragment. Low abundance transcripts of approximately 1000–4000 copies/cell were mapped in all five *Pst* fragments.

The chloroplast genome of *Euglena gracilis* is a covalently closed superhelical double-strand DNA of 130–140 kbp (Manning & Richards, 1972; Gray & Hallick, 1978a). This DNA is extensively transcribed in *Euglena* at all stages of chloroplast development (Chelm & Hallick, 1976; Rawson & Boerma, 1976). In rapidly growing cells containing fully developed chloroplasts 23% of the DNA or approximately 60 kb of RNA is transcribed (Chelm & Hallick, 1976). Two classes of RNA transcripts are known to be encoded on the chloroplast DNA. These are the 16S and 23S rRNA of chloroplast ribosomes (Scott & Smillie, 1967; Stutz & Rawson, 1970) and approximately 25 tRNAs (Schwartzbach et al., 1976; McCrea & Hershberger, 1976). A detailed restriction endonuclease map of *Euglena* chloroplast DNA has been described (Gray & Hallick, 1977, 1978a). The rRNA gene region was located on the genome as three tandemly repeated 5.6-kbp[1] segments, each coding for a 16S and 23S rRNA (Gray & Hallick, 1978a; Rawson et al., 1978). The repeated segment also contains a 5S ribosomal RNA gene (P. W. Gray & R. B. Hallick, manuscript in preparation). Transcription from the rRNA genes accounts for 14–15 kb of the 60-kb total RNA transcript. Twenty-five tRNAs, which have not been mapped, represent an RNA transcript totaling approximately 2.5 kb. The remaining 43 kb of RNAs are of unknown function and genome map position.

In an effort to better understand the function and organization of chloroplast DNA transcription units, we have physically mapped the transcribed regions of the chloroplast genome. Specific restriction fragments from chloroplast DNA were purified, radioactively labeled to high specific activity in vitro, and utilized as hybridization probes for defined regions of the chloroplast genome. This study has led to a quantitative transcription map of *Euglena* chloroplast DNA. We have measured both the fraction of each chloroplast DNA region transcribed and the abundance to which the RNA transcripts from each region accumulate in the cell.

Materials and Methods

Materials. The sources of all materials have been described (Chelm & Hallick, 1976; Gray & Hallick, 1978a).

Preparation of DNA and RNA. Covalently closed, superhelical *Euglena* chloroplast DNA was isolated by centrifugation of chloroplast lysates in CsCl–ethidium bromide as previously described (Chelm et al., 1977b). The recombinant DNA plasmid, pPG5, constructed from a ligation of *Eco*R1 digested pMB9 and an *Eco*R1 fragment of chloroplast DNA (P. W. Gray, R. J. Hall, & R. B. Hallick, manuscript in preparation) was isolated by the cleared lysate procedure (Guerry et al., 1973) and purified by equilibrium centrifugation in a CsCl–ethidium bromide gradient (Cohen & Miller, 1970). P2, EK1 recombinant DNA procedures were used. *Euglena* total cellular RNA was isolated from cells grown to 4 or 72 h of chloroplast development, as previously described (Chelm & Hallick, 1976).

Preparative Scale Restriction Endonuclease Digestion of DNA. Intact chloroplast DNA was digested with *Pst*I, or double digested with *Pst*I and *Bam*HI. The plasmid pPG5 was double digested with *Eco*R1 and *Sal*1. Limit restriction en-

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[1] Abbreviations used: kbp, kilobase pair; kb, kilobase; SSC, 0.15 M NaCl, 0.015 M sodium citrate; *Pst*A–E, BP 3–4, and *Eco*H, restriction endonuclease fragments of *Euglena* chloroplast DNA produced following digestion with *Pst*1, *Bam*H1–*Pst*1, or *Eco*R1, respectively. For nomenclature, see Gray & Hallick (1978b).

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