Physicochemical Properties of Complexes between Deoxyribonucleic Acid and Antibiotics Which Affect Ribonucleic Acid Synthesis (Actinomycin, Daunomycin, Cinerubin, Nogalamycin, Chromomycin, Mithramycin, and Olivomycin)*

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ABSTRACT: Three groups of antibiotics which form stable complexes with deoxyribonucleic acid (DNA) and inhibit DNA-dependent ribonucleic acid (RNA) synthesis were compared in their mode of interaction with DNA. Anthracyclines (cinerubin, daunomycin, nogalamycin) behave similarly to the acridine dyes, increasing viscosity and decreasing sedimentation of native DNA, but with the binding persisting at high ionic strength, unlike acridine dyes. Binding to DNA but without such "intercalation symptoms" was observed for actinomycins C and D and for the chromomycinlike antibiotics (chromomycin A_3, olivomycin, mithramycin). All of these agents sedimented with DNA and caused a progressive decrease in the buoyant density of DNA in cesium chloride and sulfate density gradients at increasing antibiotic concentration and with higher guanine and cytosine content of the DNA. Actinomycin caused a greater density shift with denatured than with native DNA, while the other antibiotics did not distinguish between these two forms of DNA or reacted preferentially with the native DNA. Significant decreases in the buoyant density of deoxyadenylate–deoxythymidylate copolymer were observed only with the anthracycline group of antibiotics. Actinomycins and anthracyclines caused stabilization of native DNA to thermal denaturation, whereas the chromomycinlike antibiotics had no such effect.

In addition to the already extensively studied actinomycins (see Reich and Goldberg, 1964, for a review) several other antibiotic dyes, including daunomycin, cinerubin, and chromomycin A_3, were recently reported to inhibit DNA-dependent RNA synthesis preferentially (Hartmann *et al.*, 1964; Kersten and Kersten, 1965; Kaziro and Kamiyama, 1965; Ward *et al.*, 1965). If the latter antibiotics are to become important tools in probing cellular synthetic processes the mode of physicochemical interaction of these and similar inhibitors with DNA should be evaluated. Such a study might throw light on the similarities and differences in their molecular action mechanisms as related to the chemical structure outlined in Figure 1 (A–C). As far as the structure of their chromophores is known, all could be derived from the three-ring anthracenelike system, either planar (Murty, 1960) and aromatic as in the case of the anthraquinone-derived anthracyclines (containing, however, a fourth nonaromatic ring; *cf.* Figure 1C) and heterocyclic actinomycins (Figure 1A), or in partially saturated nonplanar form (Figure 1B). In actinomycins, two lactone-cyclized polypeptides are attached to the phenoxazone chromophore, whereas sugars are the ring substituents in the two other antibiotic groups. On the basis of the chemical features and the physicochemical behavior all the presently studied antibiotics could be divided into three classes: (A) actinomycins (Figure 1A; Brockmann, 1960), (B) chromomycins (Figure 1B), mithramycin, and olivomycin (Gause, 1965), with the structure of the latter two still largely unknown, and (C) anthracyclines (Figure 1C; Brockmann, 1963), including daunomycin, cinerubin, and most probably nogalamycin, the latter of as yet undetermined structure (P. F. Wiley, personal communication). Many other antibiotics belonging to class B, including aburamycin (Nishimura *et al.*, 1957), antibiotic M5-18903 (Gale *et al.*, 1958), and to class C, including rubidomycin, most probably identical with daunomycin (Dubost *et al.*, 1964), rhodomycins, isorhodomycins, pyrromycin, aklavin, rutilantin (Brockmann, 1963), galirubin (Eckardt and Bradler, 1965), and ruticulomycin (Mitscher *et al.*, 1964), have been described, but were not included in the present study. The term anthra-

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TABLE I: Minimal Inhibitory Concentrations of the Antibiotic and Acridine Dyes.

Inhibitor	Inhibitory Concentration (μg/ml)[a]	
	B. subtilis W23	*E. coli* B
Actinomycin D (C_1)	0.02	>50
Chromomycin A_3	0.01	>100
Mithramycin	0.01	>20
Olivomycin	0.5	>20
Cinerubin	2	>50
Daunomycin	5	15
Nogalamycin	1	50[b]
Proflavine	2	>50
Acridine orange	>100	>100

[a] Determined by the gradient-plate technique (Szybalski, 1952) using Difco nutrient agar in both 10-ml wedge layers, the lower layer containing the inhibitor. One-day incubation at 37°. [b] Transient inhibition.

cyclines, as proposed by Brockmann (1963), will be used as a short trivial name for the antibiotics of class C, all containing the tetrahydrotetracenequinone chromophore. Acridine dyes (Figure 1D), reported to intercalate into native DNA (Lerman, 1964), were also included in this study. The common features and differences in the mode of binding of these three groups of antibiotics and of acridines to deoxyadenylate–deoxythymidylate copolymer (poly dAT[1]) and to native and denatured DNA of various guanine and cytosine contents are the subject of this communication. The minimal antibacterial concentrations of these agents are listed in Table I.

Materials and Methods

Antibiotics and Chemicals. The antibiotic preparations were used as supplied, without any further purification. These were kindly supplied by Dr. G. Schmidt-Kastner, Bayer A.G., Leverkusen, Germany (actinomycin C), Dr. S. A. Waksman, Rutgers University, New Brunswick, N. J. (actinomycin D, Merck & Co., 1957 product), Drs. M. Kusumoto and K. Tanaka, Takeda Chemical Industries Ltd., Osaka, Japan (chromomycin A_3), Dr. T. J. McBride, Chas. Pfizer & Co., Maywood, N. J. (mithramycin), Dr. G. F. Gause, Institute of New Antibiotics, Moscow (olivomycin), Dr. H. Zähner, Institut für Mikrobiologie, Tübingen, Germany (cinerubin), Drs. F. Arcamone and A. Di Marco, Farmitalia, Milan, Italy (daunomycin), Dr. G. M. Savage and C. G. Smith, Upjohn Co., Kalamazoo, Mich. (nogalamycin). Acridine orange and proflavin were purchased from Fisher Scientific Co., Chicago, Ill. Stock solutions were prepared by dissolving 1 mg of these compounds per ml of sterile PE buffer, containing 10^{-3} M Na_2HPO_4 and 10^{-4} M EDTA, pH adjusted to 7.6. Some of the antibiotics dissolved only slowly and required prior pulverization in a glass homogenizer. Actinomycin was dissolved at 0°. Cinerubin (base) was dissolved in 10^{-3} M HCl and neutralized. Precautions were taken never to expose these solutions to light and to store at 4°. Other chemicals used were of analytical grade.

DNA. DNA was prepared from four different species of bacteria with widely varying base composition: *Sarcina lutea* (71% G + C, mol wt 28 × 10^6), *Escherichia coli* B (50% G + C), *Bacillus subtilis* strain ATCC 6051 (43% G + C), and *Cytophaga johnsonii* (33% G + C, mol wt 18 × 10^6). DNA was extracted and purified according to the simplified procedure of Marmur (1961) followed by additional deproteinization

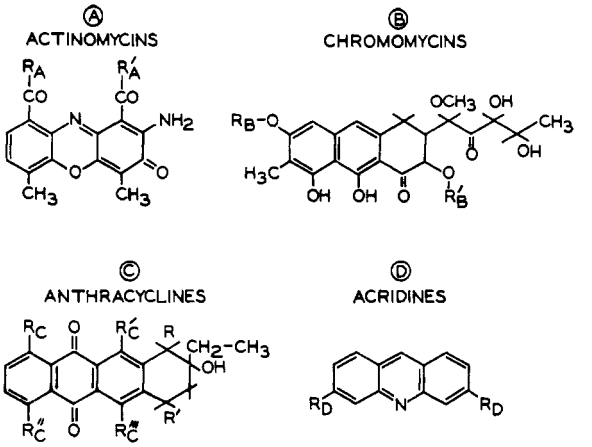

FIGURE 1: Structure of actinomycins (A) [including actinomycin D (= C_1) (R_A = R_A' = cyclical lactone of L-*N*-methylvaline, sarcosine, L-proline, D-valine, and L-threonine peptide; *cf.* Brockmann (1960)) and C (complex of C_1, C_2, and C_3)], chromomycinlike antibiotics (B) [including chromomycin A_3 (R_B = chromose D–chromose C–chromose B, R_B' = chromose A; Miyamoto *et al.* (1964) and K. Nakanishi, personal communication), olivomycin, and mithramycin (exact structures unknown, but most probably containing the same chromophore and similar sugars arranged in different sequence; K. Nakanishi, personal communication)], anthracyclines (C) [including daunomycin (R_C = R = H, R' = R_C' = R_C''' = OH, R_C'' = OCH_3, position of the amino sugar, daunosamine, unknown; Arcamone *et al.* (1964)), nogalamycin (exact structure unknown), and cinnerubin (R' = R_C = R_C'' = R_C''' = OH, R_C' = H, R = $COOCH_3$, position of amino sugars unknown; Ettlinger *et al.* (1959); Brockmann (1963)], and acridine dyes (D) [including proflavine (R_D = NH_2) and acridine orange (R_D = $N(CH_3)_2$; $N^+ \cdot HCl$)].

[1] Abbreviations used in this work: A, adenine; C, cytosine; G, guanine; T, thymine; poly dAT, deoxyadenylate–deoxythymidylate copolymer; SSC, 0.15 M sodium chloride, 0.02 M sodium citrate, pH 7.8; PE buffer, 10^{-3} M Na_2HPO_4 10^{-4} M EDTA, pH 7.6.

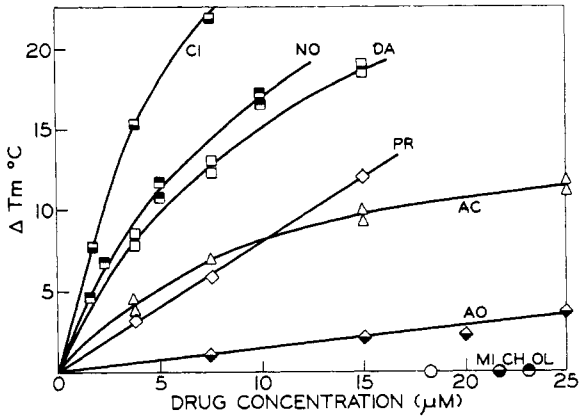

FIGURE 2: Effect of antibiotics and acridine dyes on the shift in the melting temperature (ΔT_m) of *C. johnsonii* DNA (40 μg of DNA/ml of PE solvent). The following symbols, abbreviations of drug names, and molecular weights were used for calculating the molar concentrations or ratios: actinomycin D (C_1), AC (△), 1200 (Brockmann, 1963); chromomycin A_3, CH (◓), 1052 (Miyamoto *et al.*, 1964); mithramycin, MI (◒), 1100 (Rao *et al.*, 1962, and K. V. Rao, personal communication); olivomycin, OL (○), 960 (Brazhnikova *et al.*, 1964); cinerubin, CI (▪), 875 (Ettlinger *et al.*, 1959); daunomycin, DA (□), 560 (Arcamone *et al.*, 1964); nogalamycin, NO (▫), 793 (P. F. Wiley, personal communication); acridine orange, AO (◊), 301; and proflavine sulfate (·H_2O), PR (◊) 324; 1μM = 1μg/ml for each 1000 molecular weight values.

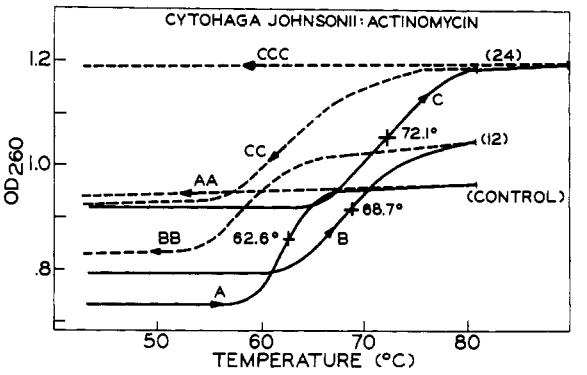

FIGURE 3: Melting profiles (OD_{260} *vs.* °C) of *C. johnsonii* DNA (40 μg/ml of PE buffer) in the absence (curve A) or presence of actinomycin (12 mμM/ml, curve B; 24 mμM/ml, curve C) as traced by the recording thermospectrophotometer (Szybalski and Mennigmann, 1962). Solid lines indicate the OD_{260} changes upon heating (1°/100 sec), and dashed or dotted lines upon cooling (1°/20 sec). As indicated by the vertical bars, the cooling cycle was started either at 81° (broken lines AA, BB, CC) or at 90° (dotted line CCC). A curve similar to AA was obtained when the cooling cycle of control DNA was started at 69°.

with water-saturated, freshly distilled phenol. Exhaustively dialyzed stock solutions of DNA were stored at 4° in SSC (0.15 M sodium chloride, 0.02 M sodium citrate, pH 7.8) or in PE buffer at 0.5 to 1 mg/ml. A synthetic poly dAT copolymer was kindly contributed by Drs. R. B. Wells and H. G. Khorana of the University of Wisconsin, Madison, Wis.

Temperature of Thermal Transition. Thermal transition curves were recorded automatically, using a "recording thermospectrophotometer" described by Szybalski and Mennigmann (1962). Solutions, degassed just before use, were contained in 0.8-ml stoppered cuvets of 10-mm light path.

Viscosity Measurements. The rotating cylinder viscometer of Zimm and Crothers (1962) was used, which permits viscosity measurements under conditions practically independent of shear (shear rates below 0.1 sec^{-1}). To allow comparison with the thermal transition data, *C. johnsonii* DNA dissolved in PE buffer was employed. Many repetitive measurements were made in a darkened room after adjusting the DNA–dye solutions (3 ml) to 25°.

Analytical Ultracentrifugation. Sedimentation and buoyant density determinations on DNA and its complexes were carried out under conditions employed in this laboratory (Erikson and Szybalski, 1964), using Spinco Model E centrifuges equipped with ultraviolet optics. The Joyce-Loebl Mark IIIC recording microdensitometer was employed for tracing the photographs. Further details are in the individual figure legends and in the text.

Results

Effect on the Thermal Transition ("Melting") of DNA. As previously shown, actinomycins (Haselkorn, 1964) and anthracyclines (Kersten and Kersten, 1965) increase the "melting" temperature of DNA. DNA of low G + C content (*Cytophaga johnsonii* DNA, 35% G + C) and a PE solvent of low ionic strength (*cf.* Materials and Methods) were principally employed to ensure thermal transition at temperatures practically attainable with available equipment. The present comparative study on the effect of antibiotic concentrations on DNA "melting" behavior revealed that anthracyclines were more effective in raising the melting temperature than actinomycin, whereas the chromomycin-like antibiotics displayed no effect under the conditions used (Figure 2). Two acridine dyes, proflavine and acridine orange, which were included in the study for reference purposes, also shifted the "melting" of DNA toward higher temperatures, but were progressively less effective than actinomycin at comparable molar concentrations (Figure 2, curves AO and PR).

Actinomycin was found not only to increase the melting temperature of DNA but also to stabilize the residual links between the complementary strands; DNA heated only slightly above the temperature of

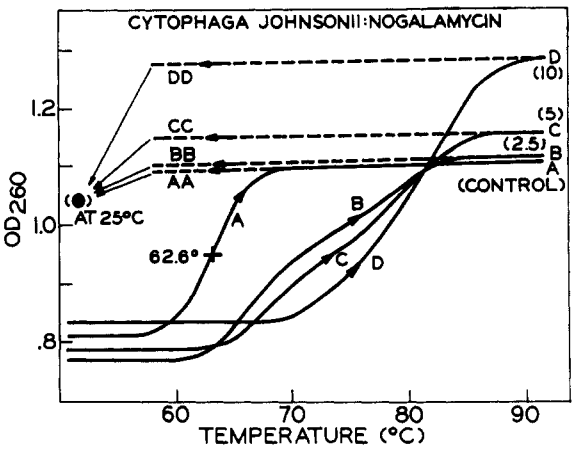

FIGURE 4: Melting profile of *C. johnsonii* DNA (40 μg/ml of PE buffer) in the absence (curve A) or in the presence of nogalamycin (2.5 mμM/ml, curve B; 5 mμM/ml, curve C; 10 mμM/ml, curve D). Upon cooling to 25° all the broken curves converge to the indicated point (●). For other details see the legend to Figure 3.

fully developed hyperchromicity (82°) was returned upon cooling to its native structure whenever actinomycin was present at sufficient concentration (Figure 3, curves BB and CC), thus mimicing the behavior of cross-linked DNA (*e.g.*, Szybalski and Iyer, 1964). Only when the temperature was increased much further (90°) did the denaturation become fully irreversible (curve CCC, Figure 3). This "overheating" was not necessary for irreversible melting of the control DNA (Figure 3, curve AA). To stabilize the DNA structure, actinomycin had to be present during the thermal

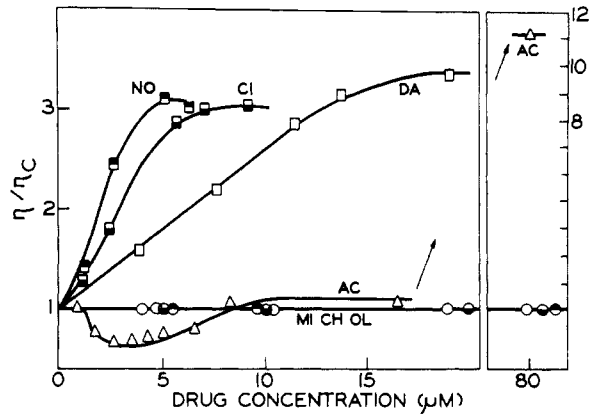

FIGURE 5: Effect of several antibiotics at various concentrations on the relative increase in the reduced specific viscosity (η/η_c) of *C. johnsonii* DNA (20 μg/ml of PE solvent), measured at 25° and at 0.01–0.05 sec^{-1} shear rate. Reduced specific viscosity of control DNA solution η_c = 160 dl/g. Viscosity of DNA + antibiotic designated as η.

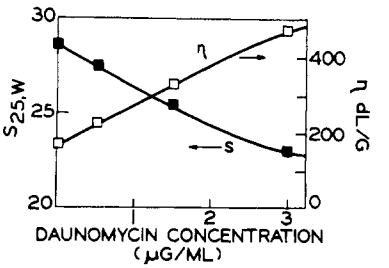

FIGURE 6: Effect of daunomycin on the reduced specific viscosity (η) and sedimentation coefficient ($s_{25,w}$) of *C. johnsonii* DNA (10 μg/ml PE of solvent) measured at 25°. Viscosity measured at 0.01–0.05 sec^{-1} shear rate.

transition; adding actinomycin at 70° followed by immediate cooling in an experiment otherwise similar to that represented by curves C and CC (Figure 3) did not result in spontaneous renaturation of the DNA. These results are in agreement with the data of Haselkorn (1964), who has shown that the temperature of irreversible denaturation is increased more by actinomycin than that of the reversible transition. No such pronounced stabilization of residual interstrand links was observed with the other two groups of antibiotics or with acridine dyes.

Among the anthracyclines tested, cinerubin has the highest effect on the melting temperature, followed by nogalamycin and daunomycin (Figure 2); the molecular weight and number of sugar residues in each of these antibiotics decrease in the same order. The melting profile of DNA in the presence of intermediate concentrations of all three antibiotics exhibits a characteristic double-wave pattern, as exemplified by curves B and C, Figure 4. Since the DNA–antibiotic complex exhibits some hypochromicity at 260 mμ (Kersten and Kersten, 1965) at room temperature, the second wave of the hyperchromic shift might be caused by the release of the antibiotic at temperatures in the neighborhood of 80°. Further shift of the melting curve at still higher concentrations of nogalamycin eliminates the inflection, most probably by aligning both steps in the hyperchromic shift (curve D, Figure 4). Upon cooling, the hypochromicity seemed to become reestablished, as evidenced by the sharper drop in the absorbance of the antibiotic-containing DNA samples (Figure 4). No double-wave pattern within the DNA melting region was observed with either acridines or other groups of antibiotics tested.

Some other DNA solvents were evaluated because of some of their specific characteristics (Szybalski and Mennigmann, 1962). In a solvent composed of one volume of methanol and one volume of PE buffer the melting temperature of *C. johnsonii* DNA (49°) is affected less by daunomycin (ΔT_m = 5° at 4.5 μg of daunomycin/ml) than in PE buffer (ΔT_m = 12.5°). This methanol–PE solvent can be used for some water-insoluble compounds as, *e.g.*, a carcinogen dimethyl-

TABLE II: Effect of Antibiotics on Sedimentation Coefficient ($s_{25,w}$) and Viscosity (η) of *S. lutea* DNA.[a]

	Control	DA	CI	NO	CH
Antibiotic concn (mμM/ml)	—	5	5	6.2	5
$s_{25,w}$[b]	31.0	28.8	28.0	26.5	31.1
η(dl/g)[c]	140	165	190	225	140

[a] 20 μg of DNA (60 mμM nucleotide) per ml of SSC. [b] 35,600 rpm, 25°, 30-mm cell, sedimented in SSC corrected for water. [c] Reduced specific viscosity measured at 25° and at 0.01–0.05 sec^{-1} shear rate.

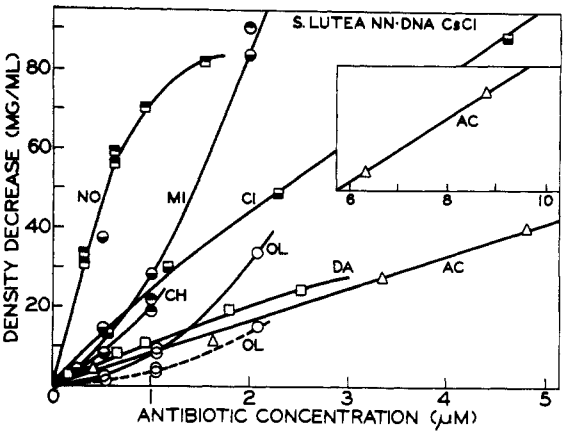

FIGURE 7: Effect of various antibiotics (mμM/ml of CsCl solution) on the decrease in buoyant density (control density minus density in presence of antibiotics) of native (NN) *S. lutea* DNA in CsCl gradient. DNA (2 μg) was well mixed with the indicated amounts (mμM) of antibiotics in 0.2 ml of PE buffer and 2–10 min later supplemented with 0.8 ml of saturated CsCl solution. The density of the mixture was adjusted to approximately 1.7 g/ml. Centrifugation was carried out for 22–24 hr at 44,770 rpm, 25°. The density determinations are based either on the micropicnometric measurements with an accuracy of 0.1 mg/ml (anthracyclines) or on the band position with respect to the poly dAT reference band, the density of the latter found not to be affected by the other antibiotics (MI, CH, OL, AC) tested under the present experimental conditions. Since addition of olivomycin causes splitting of the DNA band in addition to a density decrease, the solid line indicates the shift in the more affected component and the broken line in the less affected one. The insert at the upper right corner provides density shift data for the higher actinomycin concentration. For curve designation see legend to Figure 2.

benzanthracene, which, however, was found not to influence the melting behavior of DNA at a concentration of 5 μg/ml (unpublished results). The melting temperatures are also quite low in 7.2 M sodium perchlorate, but at this high ionic strength actinomycin (up to 40 μg/ml) or daunomycin (up to 4 μg/ml) had hardly any effect on the thermal transition of *C. johnsonii* DNA (34% G + C) occurring at 37.5°; a very small shift, from 59.5 to 62°, was observed for *S. lutea* DNA (71% G + C) at 40 μg of actinomycin/ml. Actinomycin (40 μg/ml) produced a small shift (from 91 to 91.7°) in the melting temperature of *C. johnsonii* DNA dissolved in 57.5% CsCl (w/w), *i.e.*, at the concentration used for determination of the buoyant density of DNA and its complexes with the dyes.

Effect on the Viscosity and Sedimentation Coefficient of DNA. Each group of antibiotics tested affects in a different manner the viscosity and sedimentation coefficient of native DNA. The chromomycinlike antibiotics have no influence on either the viscosity or the sedimentation coefficient of DNA (Table II, Figure 5), although they cosediment with DNA as shown by Kersten and Kersten (1965) for chromomycin A_3. The anthracyclines raise sharply the viscosity of DNA and at the same time lower the sedimentation coefficient. In both cases nogalamycin is most active, followed by cinerubin and daunomycin (Table II, Figures 5 and 6). These effects are quite analogous to those observed for the acridine dyes, indicating stiffening and/or elongation of the DNA molecule, and are interpreted by Lerman (1964) as intercalation of the dye molecules between adjacent nucleotide-pair layers.

The actinomycin effect on the hydrodynamic properties of DNA is of a more complex nature. At low actinomycin concentrations the viscosity of DNA slightly decreases (Figure 5), whereas the sedimentation coefficient increases (Rauen *et al.*, 1960), both indicative of tighter coiling of the DNA molecules. At somewhat higher concentrations of actinomycin the viscosity and sedimentation return to almost normal values, whereas at still higher actinomycin concentrations the viscosity of DNA reaches a very high value (Figure 5). Similar results were recently reported by W. Müller (personal communication) and interpreted as indicative of intramolecular cross-linking of DNA by actinomycin dimers in its low concentration range and intermolecular linking (aggregation) at high actinomycin to DNA ratios.

Effect on Buoyant Density of DNA. When sedimented in CsCl or Cs_2SO_4 gradients, DNA exhibits a characteristic buoyant density depending on its conformation, base composition (Meselson *et al.*, 1957; Sueoka *et al.*, 1959; Schildkraut *et al.*, 1962; Erikson and Szybalski, 1964), and presence of various ions, *e.g.*, silver or mercury (Davidson *et al.*, 1965). In the presence of the antibiotics tested, the DNA density becomes drastically depressed, indicating formation of DNA–antibiotic complexes, stable at high ionic strength. As

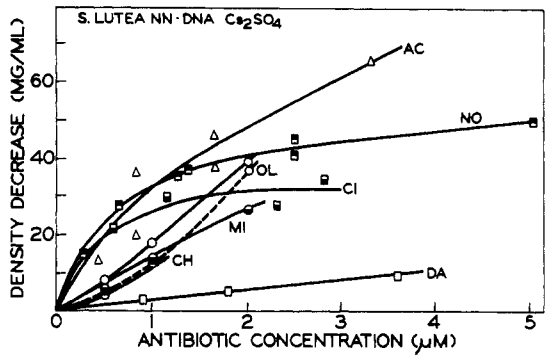

FIGURE 8: Effect of various antibiotics (mμM/ml of Cs_2SO_4 solution) on the decrease in buoyant density of native *S. lutea* DNA in the Cs_2SO_4 gradient. DNA (2 μg) was well mixed with the indicated amounts (mμM) of antibiotics in 0.4 ml of PE buffer and 2–10 min later supplemented with 0.6 ml of saturated Cs_2SO_4 solution. The density of the mixture was adjusted to approximately 1.4 g/ml, and centrifugation was carried out at 31,410 rpm for 44–48 hr at 25°. All densities were determined by micropicnometric measurements. For curve designation, including two OL curves, see the legends for Figures 2 and 7.

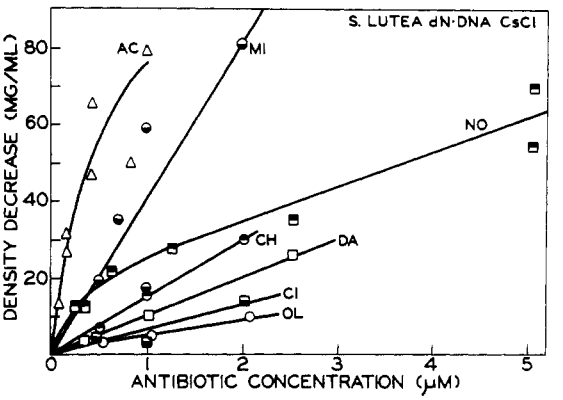

FIGURE 9: Effect of various antibiotics on the decrease in buoyant density of denatured (dN) *S. lutea* DNA (heated for 5 min at 100° in PE buffer followed by rapid chilling at 0°) in the CsCl gradient, under the conditions described in the legend for Figure 7.

can be seen in Figure 7, nogalamycin and mithramycin are most effective on a molar basis in decreasing the buoyant density of DNA, followed by cinerubin, chromomycin, olivomycin, daunomycin, and actinomycin. As expected, the acridine dyes, which are known to be displaced from DNA at high ionic strengths, have no effect on the buoyant densities of DNA in cesium salt gradients. The tetracycline antibiotics, their four-carbon ring skeleton superficially resembling the anthracycline chromophore structure, were also without effect on the buoyant density of DNA.

The interaction between olivomycin and several preparations of *S. lutea* DNA was somewhat different from other observed interactions. Although the control DNA formed a single narrow band in the CsCl gradient, this band was dissociated into two, a lighter and a heavier band, when exposed to olivomycin. The density–concentration relationships both for the lighter (solid line) and for the heavier (broken line) component of DNA are included in Figure 7. The upward curvatures of the mithramycin, olivomycin, and chromomycin curves indicate that binding of these antibiotics to DNA seems to be either a cooperative phenomenon or to be inhibited by some impurities which react with the antibiotics. The latter explanation seems to be more probable, since the density shift caused by the chromomycinlike antibiotics tended to be somewhat irreproducible when different batches of CsCl were used. Indeed, in one early series of experiments very little of the upward concavity was observed for the mithramycin and chromomycin curves, with the density shifts rapidly overtaking those observed at similar molar concentrations of nogalamycin. Although it was suspected that contamination with divalent ions might be responsible for the variability in the buoyant density shifts, this seems not to be the case, since only a very small effect was found with addition of 10^{-4} M $MgCl_2$; density decreases are listed in parentheses for the following solvents: PE (38 mg/ml), PE + Mg^{2+} (34 mg/ml), H_2O (two bands: 20 and 27 mg/ml), H_2O + Mg^{2+} (31 mg/ml), all containing 0.5 mμM mithramycin and 2 μg of *S. lutea* DNA per 0.2 ml of solvent per ml of final CsCl solution. It might be interesting to note that mixing mithramycin and *S. lutea* DNA in pure water solvent (H_2O) results in the appearance of two DNA bands during CsCl gradient centrifugation, a phenomenon hitherto observed by us only with olivomycin. At chromomycin concentrations higher than 0.5 μM, the ultraviolet (260 mμ) absorbance of the DNA band in CsCl decreases, reaching approximately half at 1 μM; the DNA band disappears at a chromomycin concentration of 2 μM, most probably forming a precipitate, since a hypersharp band was observed at a density approximately 90 mg/ml lower than that of DNA (Figure 7).

Since these antibiotics contain various amounts of sugar residues, and since DNA-bound sugars were found to decrease the DNA buoyant density in CsCl while increasing it in the Cs_2SO_4 gradient (Erikson and Szybalski, 1964), the density of the DNA–antibiotic complexes was determined also in the Cs_2SO_4 gradient. As expected, all the sugar-containing antibiotics affect the DNA density in Cs_2SO_4 (Figure 8) less than in CsCl (Figure 7), whereas peptide-containing actinomycins have more effect in Cs_2SO_4 than in CsCl. Separation of *S. lutea* DNA into two bands by olivomycin in the Cs_2SO_4 gradient (Figure 8) is somewhat less pronounced than in CsCl (Figure 7).

Since the secondary DNA structure might strongly affect the formation of complexes, the effect of antibiotics on the buoyant density of *denatured* DNA in the

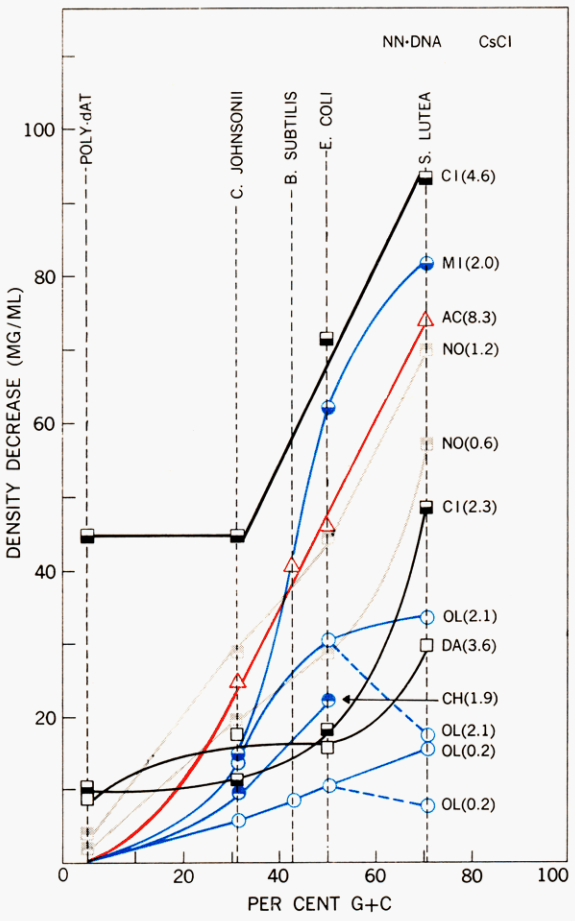

FIGURE 10: Effect of base composition of DNA (poly dAT, 0% G + C; *Cytophaga johnsonii*, 34% G + C; *B. subtilis*, 43% G + C; *E. coli*, 50% G + C; *S. lutea*, 71% G + C) on the decrease in buoyant density of native DNA's in the CsCl gradient. The experimental conditions were similar to those outlined in the legend for Figure 7, with 2 μg of DNA and the following amounts of antibiotics per 0.2 ml of PE buffer and per 1 ml of CsCl solution: 10 μg of actinomycin, 2 and 4 μg of cinerubin, 2 μg of daunomycin, 0.5 and 1 μg of nogalamycin, 2 μg of chromomycin A_3, 2 μg of mithramycin, 0.2 and 2 μg of olivomycin (6 μg of DNA was used together with 0.2 μg of olivomycin). The figures in parentheses indicate μmolar concentrations of antibiotics; for symbols and abbreviations of the antibiotic names consult the legend to Figure 2. The broken lines connect to points representing the *S. lutea* DNA component exhibiting the low olivomycin-effected shift.

CsCl gradient was also evaluated (Figure 9). Whereas there is either some decrease (nogalamycin) or little difference in the density shift of the native *vs.* denatured DNA in the presence of the anthracyclines and chromomycinlike antibiotics, only actinomycin seems to have a more pronounced effect on denatured than on native DNA as measured by the increase in the buoyancy shift toward lower density values. No separation into two bands was observed for denatured *S. lutea* DNA in the presence of olivomycin or any other antibiotics.

To determine the relationship between the antibiotic-effected buoyant density shift of the DNA and its base composition, the DNA of various bacterial species varying from 34 to 71% G + C and synthetic poly dAT were used. As can be seen in Figure 10, the density shift in the presence of the antibiotics tends to increase at higher G + C content of DNA, with the relationships varying from approximately linear to highly irregular ones. No density shift of poly dAT was observed with actinomycin- and chromomycinlike antibiotics. Only anthracyclines effected the density decrease of poly dAT; the most pronounced density shift was observed with cinerubin. As previously mentioned, *S. lutea* DNA (71% G + C) precipitated in the presence of 1.9 μM chromomycin; no useful experimental point therefore could be obtained for this DNA (Figure 10). Forking out of the olivomycin curves is caused by splitting of the *S. lutea* DNA band into two components, as discussed earlier.

Discussion

Tenacious binding to DNA and resulting inhibition of DNA-dependent RNA synthesis are common properties of the antibiotics included in this study. The mechanism of binding, however, seems to be different for each class of agents.

Anthracyclines. Interaction between this group of antibiotics and DNA seems to affect profoundly the conformation of DNA and has several features in common with that reported for the acridine dyes and similar planar compounds, which were postulated by Lerman (1964) to intercalate between adjoining nucleotide pairs (see also Drummond *et al.*, 1965, Tubbs *et al.*, 1964, Ohnishi and McConnell, 1965, for chlorpromazine; Fuller and Waring, 1964, for ethidium bromide; Weinstein *et al.* (1965), for miracil D). Anthracycline-caused increases in viscosity and decreases in sedimentation of native DNA are consistent with the intercalation hypothesis. However, binding of the anthracyclines (and ethidium bromide) to DNA is little affected by the high ionic strength of the medium, whereas the acridines are fully displaced at increasing salt concentrations. Amino sugar residues must be responsible for this stabilization of the antibiotic–DNA complex, since it was very recently shown by Calendi *et al.* (1965) that *N*-acetylation of the sugar residue in daunomycin results in a profound decrease in its affinity to DNA. The anthracycline–DNA complex retains its stability even at elevated temperatures, increasing considerably the melting temperature of DNA, and at very high ionic strengths. The stability of the complex under the latter conditions contributes to the progressive decrease in the buoyant density of the DNA complexes sedimented in CsCl or Cs_2SO_4 density gradients (Figures 7 and 8).

The most pronounced density shift was obtained with nogalamycin, which contains two sugar residues, with progressively lesser effects observed for cinerubin (three

sugar residues) and daunomycin (one sugar residue; Figure 7). Among "intercalating" agents which do not contain any amino sugars, the acriflavin dyes do not effect any buoyant density shift of DNA in the CsCl gradient, whereas ethidium bromide (2,7-diamino-9-phenylethylphenanthridinium bromide; Waring, 1965) decreases the buoyant density of DNA, as observed by Dr. E. Reich (personal communication) and in this laboratory. The present experiments indicate also that the DNA–anthracycline interaction increases with rising G + C content of the DNA (Figure 10), although even poly dAT shows some anthracycline binding at 25°, as inferred from the density shift. By the same criterion, denatured DNA's seem to bind anthracyclines not much less than native DNA. This result agrees with the unpublished observation of the senior author that the nogalamycin spectrum is affected to a similar extent by both native and denatured DNA. Viscosity augmentation and decrease in sedimentation constant were observed only for anthracycline complexes with native and not with denatured DNA. The effects of the anthracyclines on the X-ray diffraction pattern of DNA (*cf.* Lerman, 1964) or on the actual elongation of DNA molecules (*cf.* Cairns, 1962), were not included in the present study.

Actinomycins. As could be inferred from the published and present data, actinomycins D and C bind strongly to DNA, but do not show any symptoms of "intercalation." The viscosity of the DNA–actinomycin complexes varies only slightly at moderate concentrations of the antibiotic, first decreasing and then increasing (Figure 2). Only at very high concentrations of actinomycin (100 μg/ml) does the viscosity of both native and denatured DNA's rise to extreme values, indicating some aggregation process. Pronounced buoyant density decreases were observed in the presence of actinomycin, with denatured DNA being affected more than the native (Figures 7 and 9). Binding as measured by the buoyant density shift depended strongly on the G + C content of the DNA; no shift was observed for poly dAT.

Chromomycinlike Antibiotics. Although many features of the binding between these antibiotics and DNA were similar to that observed with actinomycin, the following major differences emerged: at the concentrations presently employed chromomycin, mithramycin, and olivomycin do not effect the melting temperature of DNA (Figure 2). However, chromomycin and mithramycin were found to increase the melting temperature of DNA at very high concentrations (100 μg/ml) and only in the presence of $MgCl_2$ (10^{-4} M), indicating that these ligands are potentially able to stabilize the secondary structure of DNA by forming thermostable bridges between the complementary DNA strands. Gradual loss of hypochromicity of the mithramycin–DNA complex observed at submelting temperatures could be accounted for almost entirely by the reversible increase of mithramycin absorbance under these conditions of heating. This result does not seem to be at variance with the preliminary results of Kaziro and Kamiyama (1965), who have seen a 10° increase in the melting temperature of DNA (13 μg/ml) in the presence of 50 μg/ml of chromomycin A_3, assuming that magnesium ions might have been inadvertently present in the solvent used by these authors. The buoyant density shift was similar for the native and denatured DNA, although it was difficult to obtain day-to-day reproducible values due to some unknown factors. Precipitation (?) or band splitting (Figures 7 and 8) were also observed with these antibiotics. The presence of magnesium ions was not necessary for observing the density shift, although it was recently shown by Ward *et al.* (1965) that the spectral shift of chromomycin–DNA complexes depends on the presence of 10^{-4} M $MgCl_2$. This result, however, is understandable since it was found by the present authors (W. K. and H. K.) that CsCl substitutes for $MgCl_2$ in enhancing the spectral shift of DNA complexes with chromomycin or mithramycin.

The present results lead to conclusions similar to those derived from the enzymatic study on the inhibition of the DNA-dependent RNA synthesis (Hartmann *et al.*, 1964; Ward *et al.*, 1965), although daunomycin or nogalamycin inhibition of this reaction seems to be less G + C dependent than the shift in the buoyant density of DNA by these antibiotics. Decreasing melting temperature shift by nogalamycin with increasing G + C content of DNA, as reported by Bhuyan and Smith (1965), seems to be at variance with the presently observed increase in density shift by this antibiotic with higher G + C content of the DNA (Figure 10). It is obvious, however, that the average G + C content is not the only criterion of antibiotic binding, since *S. lutea* DNA of homogeneous G + C content is split into two components in the presence of olivomycin (Figures 7, 8, 10). This phenomenon, which is quite reproducible, does not seem to be caused by any artifacts of an uneven mixing process, since it was observed only with *S. lutea* DNA and not with other DNA's studied, and since it cannot be simulated with the related antibiotic chromomycin, even if half of the DNA is mixed with the antibiotic before adding CsCl solution and half is added afterwards. These results might indicate some specificity of olivomycin for special features of the DNA structure (base sequences?), other than the gross base composition.

It is not known whether the presently studied natural microbial products are some pathological freaks of nature, or more probably exemplify some of the controlling molecules acting on the DNA transcription level. Understanding the relationship between the structure of these molecules and their mode of action might help in the search for the hitherto hypothetical repressor substances. Ancillary phenomena, as, *e.g.*, the omnipresence of the spontaneously renaturable DNA fraction (*cf.* review of Szybalski, 1964) or frequent reports on amino acids or sugars found in strong association with DNA, might also be caused by the presence of natural cell components with features similar to those of some presently studied antineoplastic antibiotics. One could also marvel about the ingenious chemical design of these naturally occurring compounds, combining several functional groups in one

molecule, each reacting with DNA by a different mechanism. If specific base sequences or DNA conformation are required for effective binding of some of these antibiotics (*e.g.*, olivomycin), these compounds may prove to be useful in probing the structure and function of nucleic acids.

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