Base Specificity in the Interaction of Polynucleotides with Antibiotic Drugs

Abstract. *Echinomycin, daunomycin, ethidium bromide, nogalamycin, chromomycin, mithramycin, and olivomycin inhibit RNA synthesis by RNA polymerase by interacting with the DNA template. Chromomycin and olivomycin form complexes with DNA, preferably in the helical form, but not with RNA. These complexes require guanine in DNA and the addition of a stoichiometric amount of bivalent cation. None of the other antibiotics requires the presence of any single base in the template for its action.*

The structure of helical polynucleotides is known in considerable detail. Therefore, if base specificity is observed in the interaction of small molecules with helical nucleic acids, the decision as to which functional groups of the polynucleotide are indispensable components of the binding site is greatly simplified, because the number of alternatives is small. Since the binding of actinomycins to DNA is absolutely dependent on guanine residues, a survey of other selected agents might provide additional examples of drugs with analogous specificities. We describe now some results with six antibiotics and with the trypanocidal agent, ethidium bromide (Fig. 1). Of these, chromomycin A (as well as its close relatives mithramycin and olivomycin) and nogalamycin show clear-cut specificity which is probably related to their fundamental biological action. Some preferential reactivity for individual bases has also been found in the interaction of polynucleotides with daunomycin and ethidium; however, the experimental conditions which reveal these specificities leave some doubt concerning their physiological significance.

We have studied the interaction of natural and synthetic polyribo- and poly-deoxyribonucleotides with each compound (i) spectrophotometrically with a Cary Model 14 spectrophotometer (because of the shortage of materials, no spectrophotometric measurements were made with synthetic DNA polymers), and (ii) by testing each combination for template activity in RNA synthesis catalyzed by RNA polymerase from *Escherichia coli*. The enzyme was purified and assayed in DNA-directed reactions according to Chamberlin and Berg (*1*); the experimental conditions for RNA-directed synthesis with the same enzyme were those of Fox *et al.* (*2*); all other experimental procedures have been described (*3*).

Curves for inhibition of enzymic RNA synthesis were obtained for each compound tested. These resembled closely that shown in Fig. 2 for echinomycin, and differed only in molar concentration and the ratio of antibiotic to DNA nucleotide required to obtain a given effect (Table 1).

Table 1. Ratio of drug to DNA nucleotide yielding 50-percent inhibition of RNA synthesis directed by calf-thymus DNA.

Drug	Drug concentration (mμmole/ml)	Calf-thymus DNA (mμmole/ml)	10^3 (Drug/DNA)
Ethidium	8.9	128	70
Daunomycin	3.56	128	28
Echinomycin	0.37	128	2.9
Nogalamycin	1.17	184	6.4
Chromomycin	0.10	128	0.78

Echinomycin, an antibiotic of unusual structure, was discovered by Corbaz *et al.* (*4*) and is probably identical with levomycin (*5*), antibiotic X948 (*6*), actinoleukin (*7*), and quinomycin *a* (*8*). It is highly toxic to mammals and inhibits the growth of Gram-positive and Gram-negative bacteria (*4, 6*). Echinomycin inhibits DNA and RNA synthesis of bacterial and mammalian cells in culture (*9*) and, at low concentrations, inhibits DNA-directed enzymic RNA synthesis. The degree of inhibition observed is a function of the echinomycin : DNA ratio and not simply of antibiotic concentration alone.

The effect of echinomycin is seen with each of the synthetic DNA polymers tested (Table 2), but no inhibition is observed when polyribonucleotides serve as templates for RNA polymerase (Table 3). When DNA is added to echinomycin solutions, changes in the ultraviolet absorption spectrum are produced (Fig. 3); these are reduced by prior denaturation of DNA. No change in spectrum was detected when natural or synthetic polyribonucleotides were used instead of DNA. From these findings we conclude that echinomycin, like actinomycin, inhibits enzymic synthesis of RNA by binding to the template polynucleotides; however, conclusions concerning the nature of the binding site must be deferred. We assume, provisionally, that the spatial organization of structures which interact with echinomycin is characteristic of helical DNA, is common to all base pair combinations, and does not exist with detectable frequency in RNA.

Daunomycin was first described and

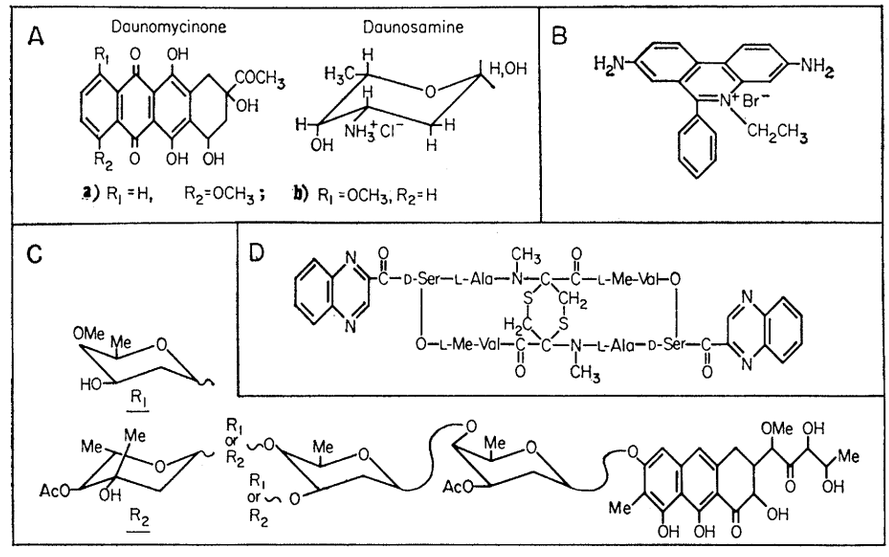
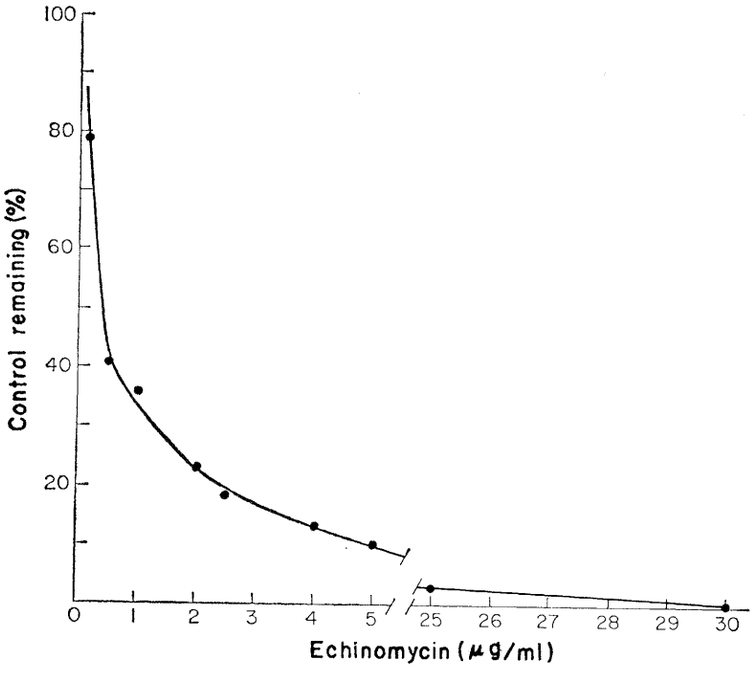


Fig. 1. Structures of *A*, daunomycin (*35*) (R_1 and R_2 may be as in *a* or *b*); *B*, ethidium bromide (*36*); *C*, chromomycin A_3 (*37*); and *D*, echinomycin (*38*). Nogalamycin is similar in structure to daunomycin, possessing a tetracycline-like chromophore linked to an amino sugar. Olivomycin and mithramycin are closely related to chromomycin, differing by minor variations in their chromophores and sugar components (*32*).



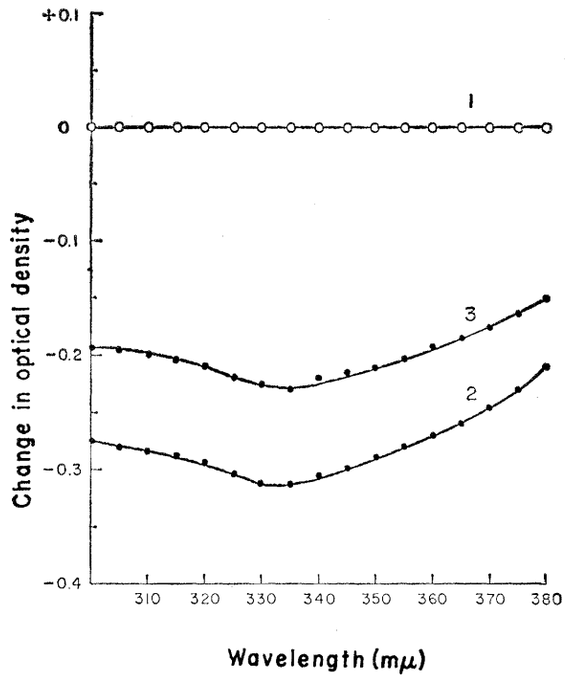


Fig. 2 (left). Effect of echinomycin on RNA synthesis primed by calf-thymus DNA. The reaction mixture contained 32 mμmole of DNA-phosphorus and 8 units of enzyme protein in a final volume of 0.25 ml. Tritiated-CTP (5.5×10^6 count min^{-1} $\mu mole^{-1}$) was the radioactive precursor. Incubation was for 30 min, 37°C. Fig. 3 (right). Difference spectra of echinomycin solutions read against the same solutions containing ribohomopolymers (curve *1*), native DNA (curve *2*), and heat-denatured DNA (curve *3*). All measurements were made in 0.01*M* tris-HCl, *p*H 7.4, containing 0.01*M* NaCl. The concentrations of reactants were: echinomycin, 50 μg/ml—that is, about 47 mμmole/ml (optical density at 328 mμ was 0.92, 1-cm light path); native and heat-denatured calf-thymus DNA-phosphorus, 170 mμmole/ml; polyA, polyI, polyU, and polyC, 400 μg/ml.

studied by DiMarco and collaborators (*10, 11*). They reported that daunomycin preferentially inhibits cellular RNA synthesis, a finding subsequently confirmed with isolated enzymes (*12*). The template activity for RNA synthesis of all deoxynucleotide polymers is inhibited by daunomycin (Table 2). In experiments with higher ratios of daunomycin to DNA nucleotide, RNA synthesis could be depressed still further.

With the ribohomopolymers, daunomycin inhibited the template activity of polyA (*13*) and polyI but not that of polyU or polyC. The addition of daunomycin to the enzyme incubation medium resulted in the formation of a colored precipitate which included the polynucleotide template. This reaction of daunomycin with the purine nucleotide polymers could account for the observed inhibitory effect of the antibiotic on RNA-directed RNA synthesis, since polymers which did not so precipitate functioned normally as templates. Under conditions where the polymers were not precipitated (that is, in the absence of Mn^{++} ions), difference spectra could be obtained for the interactions of polyA, polyI, and polyU with daunomycin (*14*). Addition of purine nucleoside monophosphates, but not pyrimidine nucleotides, caused slight alterations in the spectra of daunomycin solutions. However, no reaction was observed with nucleosides, thereby confirming a previous report (*10*).

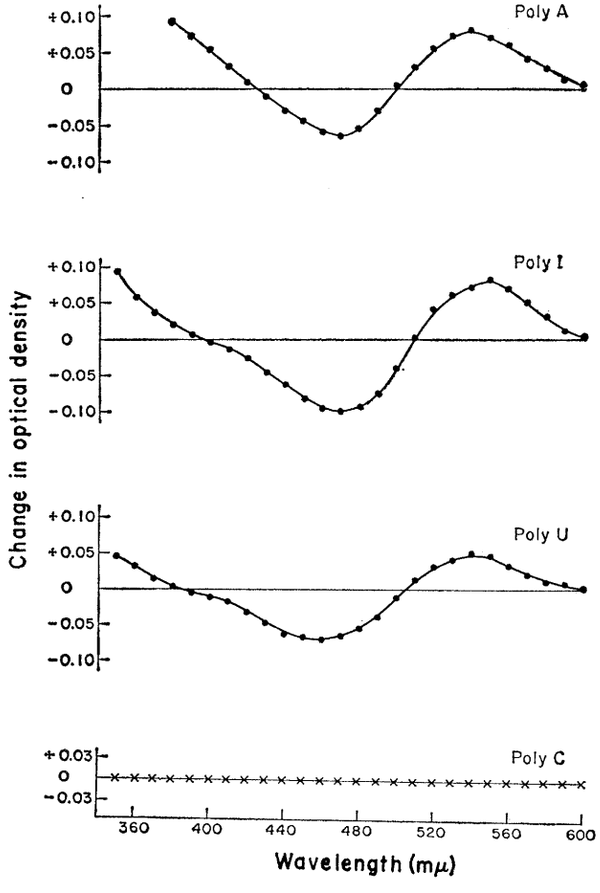
Daunomycin increases the viscosity of DNA (*15*), decreases the sedimentation coefficient of DNA (*16*), and stabilizes DNA against denaturation by heat (*15*). The binding of daunomycin to DNA decreases with increasing ionic strength, and DNA is precipitated by high concentrations of the antibiotic (*16, 17*). Our results show that daunomycin inhibits RNA synthesis regardless of base composition of the DNA templates tested and reacts preferentially with purine nucleotides. The aforementioned properties resemble those of proflavine (*18*) and are consistent with the suggestion that daunomycin, or at least its chromophore, intercalates between adjacent base pairs in helical DNA. Proflavine, which inhibits DNA-dependent RNA synthesis to a lesser extent than it inhibits DNA synthesis (*19*), consists of a planar ring system without side-chains; daunomycin, which, like actinomycin, preferentially suppresses RNA synthesis (*10, 12*), contains a ring system somewhat larger than that of proflavine, and also has an amino-sugar side chain. Thus, the selective action of daunomycin on RNA synthesis may arise from the projection of portions of the antibiotic molecule into one of the grooves of DNA. If the nucleic acid polymerases differ in their specificity for the grooves of DNA (*20, 21*), their differential susceptibility to daunomycin might result from the steric obstruction of one groove by the nonintercalated portion of the antibiotic. Since RNA synthesis is the more sensitive process, it will be of interest to establish whether the obstructed groove in this instance, as in that of actinomycin, is the minor groove (*22*).

Ethidium bromide is a synthetic antimicrobial agent which inhibits DNA and RNA synthesis of intact organisms (*23*) and enzyme preparations (*24*). Ethidium forms complexes with DNA (*24*), its absorption spectrum is changed in the presence of DNA (*24*), and complex formation probably

accounts for the inhibition of nucleic acid synthesis (*24*). Ethidium inhibits the function of RNA polymerase with all DNA preparations that we tested, whether native or denatured, naturally occurring or synthetic (Tables 1 and 2). We have also observed that complex formation (measured spectrophotometrically) is inhibited by elevated ionic strength, and it is much lower with the unstructured apurinic and apyrimidinic acids than with native DNA. These findings are in accord with the proposal of Fuller and Waring (*25*) that ethidium, like proflavine, forms complexes by intercalation between adjacent base pairs of the DNA helix. It was therefore not surprising that, as in the case of daunomycin, the template function of some ribohomopolymers was sensitive to ethidium (Table 3). Further study of this phenomenon under conditions of the enzyme assay disclosed the following: (i) ethidium interacts with polyA, polyI, and polyU, but not with polyC; (ii) the spectrum of ethidium is altered as a consequence of the interaction (Fig. 4), but the degree of interaction differs in magnitude and heat stability from that characteristic of helical DNA (*26*); (iii) the formation of complexes (measured spectrophotometrically) is inhibited by Mn^{++}; and (iv) similar spectral changes were produced by adenylic and guanylic acids, but not by uridylic or cytidylic acids. The properties of these complexes of ethidium with mono- and polyribonucleotides differ from those previously described (*24*) and are probably unrelated to the biological properties of the drug (*27*).

Nogalamycin has been studied by Bhuyan and Smith (*28*), who observed that it binds to DNA, selectively inhibits DNA-dependent RNA synthesis, and markedly raises the T_m (transition temperature) of dAT but not of dGdC. Nogalamycin does not impair the template function either of dGdC, dIdC, or of ribohomopolymers we have tested (Tables 2 and 3).

Chromomycin A_3, mithramycin, and olivomycin were discovered in Japan (*29*), the United States (*30*), and the U.S.S.R. (*31*), respectively; are closely related to each other; and show minor differences in the structure of their chromophores and sugar components (*32*). Their tumor-inhibiting properties and their toxicities differ somewhat from those of the actinomycins (*32*). Chromomycin A_3 has been reported to inhibit selectively cellular (*33*) and enzymic (*12*) RNA synthesis, and to



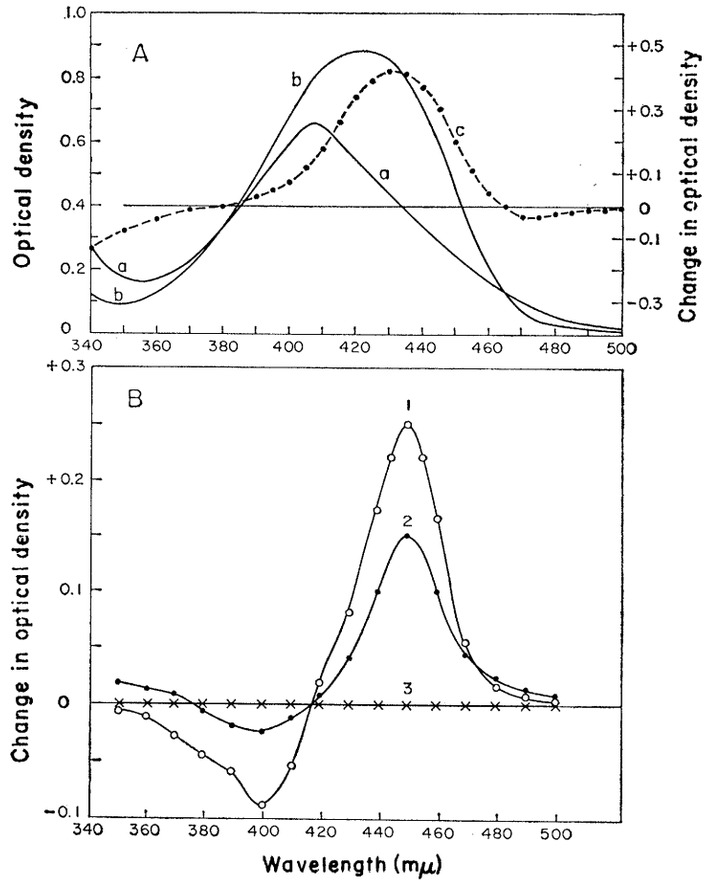


Fig. 4 (left). Difference spectra of ethidium solutions read against the same solutions containing polyA, polyI, polyU, and polyC. All measurements were made in 0.1*M* tris-HCl, *p*H 7.9. The concentrations of reactants were: ethidium, 40 μg/ml (100 mμmole/ml) (optical density at 480 mμ = 0.54 1-cm light path); polyA, polyI, polyU, and polyC, 400 μg/ml. Fig. 5 (right). *A*, Effect of Mg^{++} on the spectrum of chromomycin A_3. Curve *a*, chromomycin (100 mμmole/ml) in 0.01*M* tris-HCl, *p*H 7.4, containing 0.01*M* NaCl. Curve *b*, the same, with added $MgCl_2$ (0.05*M*). Curve *c*, the difference spectrum resulting from the addition of $MgCl_2$. *B*, Effect of Mg^{++} on the interaction of chromomycin with DNA. Difference spectra of chromomycin solutions (100 mμmole/ml) read against the same solutions containing DNA (425 mμmole/ml). Curve *1*, native calf-thymus DNA. Curve *2*, heat-denatured calf-thymus DNA in 0.01*M* tris-HCl, *p*H 7.4-0.01*M* NaCl containing 0.03*M* $MgCl_2$. Curve *3*, same as curve *1*, but without $MgCl_2$.

Table 2. Inhibition of DNA-primed RNA synthesis by various drugs. RNA synthesis was measured (*1*) for 30 minutes at 37°C; eight units of enzyme protein were used. The concentrations of DNA-phosphorus used were: calf thymus, 120 μM; crab dAT (0.018 O.D./ml at 260 mμ), 3.0 μM; synthetic dAT, 6.0 μM; dAdT, 7.2 μM; dGdC, 53 μM; and dIdC, 16 μM (*13*). The specific activity of the tritiated nucleoside triphosphates used was 5.5×10^6 count min^{-1} $\mu mole^{-1}$. The labeled nucleoside triphosphate incorporated in the control reactions with each DNA preparation was ($\mu\mu$mole): calf thymus (H^3-CTP), 1740; crab dAT (H^3-ATP), 580; synthetic dAT (H^3-ATP), 6830; dAdT (H^3-ATP), [?]; dGdC (H^3-GTP), 410; dGdC (H^3-CTP), 57; dIdC (H^3-GTP), 3460; dIdC (H^3-CTP), 1180.

Drug		Inhibition of various DNA primers (%)								
		Calf-thymus DNA		Crab dAT	dAT	dAdT	dGdC		dIdC	
Sample	Amount (mμmole/ml)	Drug/DNA	Inhibition (%)				GTP*	CTP†	GTP*	CTP†
Ethidium	100	0.78	100	100	100		75.8	26.2	36.2	25.6
Daunomycin	71	.55	95	100	97.3		17.9	17.9	18.2	30.9
Echinomycin	27.5	.21	100	98.0	97.2		41.8	29.8	13.7	25.4
Nogalamycin	26.3	.20	100	100	100	0	3.0	3.5	0	0
Chromomycin	100	.78	96.5	1.5	4.0		53.4	70.2	5.0	0

* GTP refers to percentage inhibition of incorporation of labeled GTP as primed by dC strand of both dGdC and dIdC polymers. † CTP refers to percentage inhibition of incorporation of labeled CTP as primed by the dG strand of the dGdC primer and the dI strand of the dIdC primer.

alter its spectrum in the presence of DNA (*12*).

Chromomycin A_3 inhibits RNA synthesis, but only when the governing template is a DNA preparation which contains guanine (Tables 1, 2, and 3). Spectrophotometric analysis of the chromomycin-polynucleotide interaction has so far revealed the following:

1) The spectrum of chromomycin is altered by Mg^{++} (also by Ca^{++}, Cu^{++}, Mn^{++}, Co^{++}, and Zn^{++}) (Fig. 5*A*) and by polyamines, but not by Na^+, Li^+, K^+, or Cs^+ ions. The changes evoked by the divalent cations differ somewhat qualitatively and quantitatively.

2) The spectrum of chromomycin is not altered by polynucleotides in the absence of bivalent cations (Fig. 5*B*). Of the cations tested, only Mg^{++}, Mn^{++}, Zn^{++}, and, to a lesser extent, Co^{++}, can promote the DNA-chromomycin interaction.

3) The magnitude of the alterations in the spectrum of chromomycin produced by DNA is a function of Mg^{++} concentration (Fig. 6). The amount of Mg^{++} required to promote maximum interaction between DNA and antibiotic corresponds to a 1:1 molar equivalence with antibiotic concentration. Therefore, the metal probably exerts its effect primarily through a prior interaction with chromomycin (not by altering the structure of DNA), and it is probably the antibiotic-Mg^{++} complex which binds to DNA. The Mg^{++} requirement for chromomycin binding to DNA is also observed if DNA is precipitated by alcohol from chromomycin solutions, the precipitate being yellow in the presence of, and white in the absence of, Mg^{++}. The spectrophotometrically detectable interaction between DNA and chromomycin is abolished by a 100-fold excess of EDTA (in relation to Mg^{++}).

4) Heat-denatured DNA is quantitatively much less effective than native DNA in producing shifts in the spectrum (Fig. 5*B*).

5) No changes in spectrum are produced by TMV RNA, even in the presence of Mg^{++}, or by any of the usual ribonucleosides, ribonucleotides, deoxyribonucleosides, or deoxyribonucleotides. This is in contrast with actinomycin, which has been shown to react indiscriminately with purine nucleosides and analogs (*20, 34*).

6) The maximum binding capacity for chromomycin of DNA preparations (per unit of phosphorus) increases slightly as the guanine content increases.

7) The interaction between chromomycin and DNA is unaffected by elevated ionic strengths (1*M* NaCl and 5*M* CsCl) but is reduced by urea.

8) Mithramycin and olivomycin are qualitatively indistinguishable from chromomycin A_3 in the above tests. In addition, neither mithramycin nor olivomycin inhibits dAT-directed RNA synthesis at concentrations which completely inhibit the same reaction directed by calf-thymus DNA.

The reaction between chromomycin and Mg^{++} and the fact that Mg^{++} is required for the chromomycin-DNA interaction are noteworthy. Since Mg^{++} alters the visible and ultraviolet spectrum of chromomycin, the metal must form some kind of complex directly either with atoms forming part of, or with functional groups which can be conjugated with, the chromophore. One or more of the numerous, available, oxygen-containing groups attached to the chromophore seems likely to mediate Mg^{++} binding.

With the exception of the requirement for Mg^{++}, many properties of chromomycin (and of mithramycin and olivomycin) are similar to those of actinomycin. The DNA complexes of both compounds are unaffected by ionic strength, have similar hydrodynamic properties, and selectively inhibit RNA synthesis as compared with DNA synthesis; the proportion of drug to DNA that results in 50-percent inhibition is the same. Both compounds form complexes preferentially with native, rather than with denatured, DNA; neither interacts significantly with RNA; and both have absolute base specificity for guanine in DNA. Most evidence (*20, 21*) suggests that the spec-

Table 3. Inhibition of RNA-primed RNA synthesis by various drugs. RNA synthesis was measured (*2*) for 30 minutes at 30°C with 8 units of enzyme protein; polyI- and polyUG-primed syntheses were carried out under the conditions described (*2*) for polyA- and polyU-primed synthesis, respectively. All ribohomopolymers were used at a concentration of 20 μg/0.3 ml in each reaction vessel. The number of millimicromoles of labeled nucleoside triphosphate (5.5×10^6 count min^{-1} $\mu mole^{-1}$) incorporated in the control reactions were: polyA, 1.78; polyU, 3.54; polyC, 4.02; polyI, 0.05; polyUG, 0.07. PolyUG was constructed with 3 to 1 ratio (U : G).

Drug		Inhibition of various RNA primers (%)				
Sample	Amount (mμmole/ml)	PolyA	PolyU	PolyC	PolyI	PolyUG
Ethidium	100	81.6	70.5	0	69.1	30.0
Daunomycin	71	90.3	0	10.0	92.6	5.0
Echinomycin	21	0	0	0	0	0
Nogalamycin	26.3	0	0	0	0	0
Chromomycin	100	0	0	0	0	0

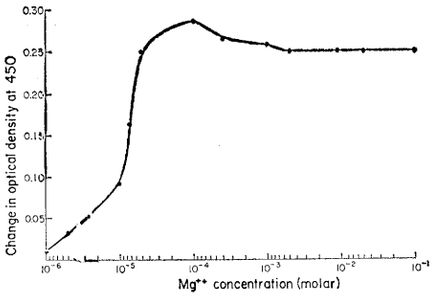


Fig. 6. The interaction of chromomycin with DNA; stoichiometry of the Mg^{++} requirement. The extent of chromomycin complex formation with DNA was measured by change in absorbance at 450 mμ as compared with a solution of chromomycin containing the appropriate concentration of Mg^{++}. All solutions contained chromomycin (100 mμmole/ml) and an excess of native calf-thymus DNA (850 mμmole/ml). The isosbestic point increases from 408 mμ to 421 mμ with increasing Mg^{++} concentration.

ificity of actinomycin binding is mediated by the amino group of guanine which is located in the minor groove of the DNA helix. That chromomycin fails to inhibit the template function of dIdC raises the possibility of the same being true for this antibiotic.

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13. Abreviations: dAT, deoxyadenylic-thymidylic copolymer; crab dAT, a deoxyadenylic-thymidylic copolymer containing 2 to 5 percent deoxyguanylic and deoxycytidylic acids, isolated from crabs of the genus *Cancer*; dAdT, deoxyadenylic-thymidylic homopolymer mixture; dGdC, deoxyguanylic-deoxycytidylic homopolymer mixture; dIdC, deoxyinosinic-deoxycytidylic homopolymer mixture; PolyA, polyadenylic acid; polyI, polyinosinic acid; polyU, polyuridylic acid; polyC, polycytidylic acid; polyUG, mixed polymer of uridylic and guanylic acids; AMP, adenosine monophosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; TMV, tobacco mosaic virus.
14. The interaction of daunomycin with ribohomopolymers, nucleosides, and nucleotides was studied in 0.1*M* tris, *p*H 7.9. The concentration of reactants used was: daunomycin, 90 mμmole/ml; polyI, polyA, polyU, and polyC, 200 μg/ml; all nucleosides and nucleotides, 10 μmole/ml.
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Chondroitin Sulfate: Inhibition of Synthesis by Puromycin

Abstract. *Puromycin reversibly inhibits synthesis of chondroitin sulfate by vertebral chondrocytes from 10-day-old chick embryos. Treatment of these cells with puromycin for 5 hours does not affect viability or capacity to multiply on subsequent release from their matrix. It is suggested that synthesis of this polysaccharide is coupled with that of protein; there may be a feedback control in its synthesis.*

Our purpose is to present data indicating that synthesis of chondroitin sulfuric acid (CSA) is intimately associated with protein synthesis, since puromycin, an inhibitor of protein synthesis (*1*), also inhibits the uptake of sulfate and glucose into this polysaccharide. A similar observation has recently been reported on the uptake of S^{35}-sulfate by bone rudiments of chick embryo (*2*).

We used the cartilagenous vertebral columns from 10-day-old chick embryos, dissected free of connective tissues and perichondrium, which we shall refer to as "trunks" (*3*). In each series of experiments duplicate trunks of approximately equal volume were individually incubated at 37°C in 2 ml of Eagle's minimum essential medium (MEM) containing 10 percent of horse serum and both penicillin and streptomycin, each at 50 unit/ml (*4*). In experiments designed to demonstrate the reversibility of the inhibition induced by puromycin, trunks were transferred to a puromycin-free medium, either MEM or an enriched medium