Biochemistry of Actinomycins*

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SUMMARY

The toxicity and biological activity of actinomycins at all levels of biological organization investigated to date are based on their ability to form tight, reversible complexes with DNA. The presence of guanine in a DNA preparation, preferably in the helical configuration, is necessary and sufficient for the formation of the characteristic complexes.

Bound actinomycin produces alterations in the physical properties of DNA including increased viscosity, failure to assume the A configuration under appropriate conditions, and marked increases in “melting temperature. These changes have their counterpart in the morphological distortions seen in isolated preparations or in salivary gland cells of Drosophila.

At the enzymatic and cellular level DNA-directed RNA synthesis is very sensitive to actinomycin, whereas the replication of DNA itself is relatively resistant. The formation of all species of RNA is blocked by actinomycin in all uninfected, susceptible organisms, but the growth of most RNA viruses is unaffected by the antibiotic.

The use of actinomycin has permitted some investigation of the physiology of template RNA, the functioning of which has shown considerable stability in a number of systems.

The biological activity of actinomycin is abolished by changes involving any of the major structural features of the molecule.

Actinomycins are colored peptide-containing antibiotics produced by Streptomyces species. These antibiotics were the first to be isolated from actinomycetes and were reported by Waksman and Woodruff (71) in 1940. They are, on a molar basis, the most potent antitumor substances known and have been successfully used in the treatment of several human neoplasms. The actinomycins are also bacteriostatic to many gram-positive organisms. Owing to the work of Brockmann (4) and Johnson (30) and their collaborators, the chemistry of actinomycins is known in great detail, and the complete synthesis of a naturally occurring actinomycin has been achieved (6).

This review will deal briefly with the following subjects:
1. Structural properties of actinomycins which affect their biological activity.
2. Binding of actinomycins to DNA.
3. Effect of actinomycins on DNA-dependent RNA-polymerase.
4. Correlation of the reactivity of actinomycins with DNA and their biological activity in various test systems.
5. Effects of actinomycin on mammalian cell lines in culture and on growth of animal viruses.
6. Effect of actinomycin on susceptible microorganisms.
8. Miscellaneous biological systems affected by actinomycin.

* Abbreviations used in this paper:
A, T, U, G, C—adenine, thymine, uracil, guanine, and cytosine, respectively.
poly A, poly U, poly G, poly C—3′ → 5′, polymeric ribonucleoside monophosphates of adenine, uracil, guanine, and cytosine, respectively.
ATP, UTP, GTP, CTP—ribonucleoside 5′-triphosphates of adenine, uracil, guanine, and cytosine, respectively.
NTP—ribonucleoside-5′-triphosphate.
RNA—ribonucleic acid.
DNA—deoxyribonucleic acid.

1 Literature reviewed to December, 1962.
STRUCTURAL FEATURES OF ACTINOMYCINS AFFECTING BIOLOGICAL ACTIVITY

Most actinomycin-producing cultures form mixtures of closely related substances, as has been found for other polypeptide antibiotics. These mixtures have been resolved by counter-current distribution and partition chromatography on columns and paper (4). The differences found among naturally produced actinomycins have been restricted to the peptide side-chains, and the variations have concerned the structure but never the number or the configuration of the a carbon atom of the constituent amino acids (4, 5). By supplementing the culture medium with a variety of amino acids it has been possible to influence the proportions of different actinomycins synthesized (38, 58). Many analogs of the naturally occurring amino acids are acceptable substrates for actinomycin biosynthesis (38), so that the number of potentially different actinomycins is great. Certain amino acid substitutions occur in either or both peptide chains, a few have so far been demonstrated only in one chain, and some substitutions have not been precisely characterized. The data in Table 1 list the known alternatives at each position in the chain and, where characterized, the particular polypeptide affected.

The availability for testing in various systems of many characterized and crystalline actinomycin has made it possible to deduce that the amino acid composition of both polypeptide chains can influence the biological activity of the molecule. If actinomycin C1 is taken as a reference standard, the substitution of L-γ-oxoproline for L-proline in the A-chain yields actinomycin X2 (Chart 1), with 50–100 per cent enhanced activity in all tests (7, 56). X2 is the major active component of cultures which synthesize mixtures of the X-type actinomycins (7). A minor component is actinomycin X1a, in which 1 mole each of sarcosine and L-γ-oxoproline replace the two proline residues of C1 (7). Since the oxoproline residue in X2 occurs only in the A chain (49), it may tentatively be assumed that the same holds for X1a. The biological activity of X1a is one-half that of X2. Thus the increased

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>AMINO ACID SUBSTITUTIONS IN PEPTIDE CHAINS OF ACTINOMYCINS</strong></td>
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<table>
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<tr>
<th>Position</th>
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<th>Both</th>
<th>A</th>
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<tr>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<td>L-pro</td>
<td>L-γ-oxopro</td>
<td>L-γ-oxopro</td>
</tr>
<tr>
<td>4</td>
<td>D-val</td>
<td>D-val</td>
<td>D-val</td>
<td>D-val</td>
</tr>
<tr>
<td>5</td>
<td>L-thr</td>
<td>L-thr</td>
<td>L-thr</td>
<td>L-thr</td>
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* Derivatives with single amino acid substitutions definitely assigned only to chain B have not been identified.

† And their acylated derivatives.

Data from Brockmann (4) and Katz (33).

Abbreviations: ala, alanine; a-hypro, allohydroxyproline; hypro, hydroxyproline; a-ileu, alloisoleucine; ileu, isoleucine; pro, proline; oxopro, γ-oxoproline; sar, sarcosine; val, valine; thr, threonine; Me, methyl.

CHART 1

Actinomycin C1

Actinomycin X2
activity obtained by substituting oxoproline for proline in the A-chain is eliminated when sarcosine replaces proline in the B-chain.

The replacement of one or both n-valine residues of C₁ by D-alloisoleucine yields actinomycins C₂ and C₃ (4) with only minor changes in activity (8, 56). The further substitution of one or both N-methylvalines of C₃ by N-methylisoleucine (as in actinomycins E) moderately increases the inhibitory potency in a bacterial RNA-polymerase assay (22). No comparison with the activity of other compounds has been reported for actinomycins Z (2) which have N-methyl-L-alanine in place of proline at position 3. It is of interest that only four of the ten amino acids in C₁ can furnish hydrogen atoms for hydrogen bonding. This number is

4. Replacement of the aromatic amino group by chlorine or hydroxyl (4, 45, 56, 62).
5. Replacement of one of the hydrogens of the nuclear amino group by β-aminoethyl or p-amino phenyl radicals (8, 56).
6. Bridging the quinoidal oxygen and amino group with a nitrogen atom, yielding a five membered ring (8, 56).
7. Replacement of both hydrogens of the chromophore amino group by alkyl residues.

The following changes may reduce biological activity to a greater or lesser extent:
1. Alkylation of the amino group by: methyl, β-diethylaminolucyl or β-hydroxyethyl groups (4, 5, 8, 45, 56).
2. Acylation of hydroxyl groups of the hydroxy-

TABLE 2

<table>
<thead>
<tr>
<th>ACTINOMYCIN</th>
<th>RELATIVE ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁</td>
<td>100 *</td>
</tr>
<tr>
<td>X₂</td>
<td>215 ✷</td>
</tr>
<tr>
<td>X₂₅</td>
<td>57 50</td>
</tr>
<tr>
<td>X₂₅ acetate</td>
<td>2.5 5</td>
</tr>
<tr>
<td>X₂₅ acetate</td>
<td>100 1.5</td>
</tr>
</tbody>
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* Data from Brockmann and Manegold (7).
† Data from Reich, Goldberg and Rabinowitz (56).
Nomenclature is that of Brockmann (4).

not altered in any of the naturally occurring modifications of the peptide chains.

Substitution of different proline derivatives at position 3 of the A-chain may alter the minimal inhibitory concentration in bacterial or tissue culture assays by a factor of 50 (7, 56). Acylation of the hydroxyl groups of hydroxyproline decreases activity further (Table 2). Although actinomycin X₂₅ acetate is less than 1 per cent as active as X₂, it remains a very toxic substance—inhibiting the growth of HeLa cells at 10⁻⁷ M (56).

The following alterations of actinomycin totally abolish activity in all assay systems tested:
1. Hydrolysis of both lactones, producing a dicarboxylic acid (37). The possibility that the preservation of one lactone may be compatible with partial retention of activity has not been excluded but appears remote.
2. Reduction of the aromatic ring system (4).
3. Reductive acetylation of the functional groups of the chromophore (4).

3. Substitution of hydrogen at position 7 of the chromophore of NH₂, OH, NO₂, etc., may moderately diminish some of the biological properties of actinomycins (45).

BINDING OF ACTINOMYCIN TO DNA

From the work of several investigators (34, 38, 39) it is clear that actinomycins may react with DNA (but not with RNA) to form complexes, of which at least a fraction do not dissociate in the course of electrophoresis (34), dialysis (38), ultracentrifugation (50), or chromatography on Sephadex (22). It is unlikely that any of the complexes are the result of covalent bond formation between actinomycin and DNA since (a) all spectrophotometrically demonstrable actinomycin may be removed from DNA by phenol or ethanol-ether and ether 2 or by high concentrations of urea (22), and (b) the

* E. Reich, unpublished observations.
antibiotic effect of actinomycin on at least one strain of a susceptible organism, *Bacillus subtilis*, is fully and rapidly reversible if actinomycin is removed by washing\(^1\) (41), or by the subsequent addition of DNA or deoxyguanosine.\(^2\) An organism whose genetic material had formed covalent links with a molecule such as actinomycin would not be expected fully to retain its colony-forming capacity.

When actinomycin reacts with DNA (but not RNA) the spectrum of the antibiotic is changed (34, 37, 50), its peak extinction in the visible region being reduced and shifted to longer wavelengths. Qualitatively similar spectral alterations may be produced by alkylation of the chromophore amino group or by addition of strong acid to actinomycin solutions. Several of the components of nucleic acids, of which deoxyguanosine was the most effective, have been found capable of inducing spectral changes in actinomycin solutions resembling those produced by DNA (35, 74). However, free deoxyguanosine is at least ten- to twentyfold less active in this respect than the same amount in native DNA.

The metachromatic behavior of actinomycin in the presence of DNA can be readily characterized by difference spectrophotometry. By varying the concentration of either DNA or actinomycins the difference spectra can serve as a measure of the maximal complex-forming capacity of various DNAs and of different actinomycins. By means of this method the following has been established (15):

a) the presence of guanine in a DNA preparation is necessary and sufficient for complex formation with actinomycin, since apurinic DNA and synthetic deoxyadenylc-thymidylic copolymer (57) (dAT) do not react with the antibiotic, whereas pyrimidinic DNA, synthetic deoxyguanylic-deoxycytidylic polymer (31) (dGC) and naturally occurring DNAs do. A minute amount of guanine suffices to promote some complexing since "crab dAT" (84), which contains approximately 2 per cent guanine (65), reacts with actinomycin.

b) The amount of actinomycin bound by DNA preparations parallels but is not directly proportional to their guanine content (15).

c) The maximum actinomycin-binding capacity of single-stranded (from bacteriophage \(\phi X-174\)) or heated DNAs is moderately less than that of equivalent unheated DNAs or native DNAs of similar base composition (15).

d) At a fixed level of DNA, the observed spectral changes are exactly proportional, over a wide range of actinomycin concentrations, to the amount of actinomycin present (56). Therefore, at equilibrium, no more than a few per cent of the available actinomycin can remain uncomplexed to DNA, and the dissociation of complexes must be very small.

e) In a given DNA preparation the sites available for binding a reactive actinomycin—e.g., actinomycin X\(_2\), may differ in number (and perhaps in binding properties) from those available to a less readily complexed actinomycin such as X\(_{68}\) acetate (56). However, it appears likely that the apparent differences in reactivity of various actinomycins will be correlated with differences in the dissociation of their respective DNA-complexes.

f) The difference spectra of all biologically active actinomycins are qualitatively similar in the position, direction, and relative magnitudes of the spectral changes (56). Quantitative differences in spectral changes are observed among various actinomycins and derivatives and have been correlated with changes in biological activity (45, 56).

It might be expected that the binding of actinomycin to DNA would affect the physical properties of the nucleic acid, and increases in viscosity of DNA solutions have been observed on addition of actinomycin.\(^3\) \(^4\) This alteration could result from greater length or decreased flexibility of the polymer. The viscosity change seen with actinomycin is much smaller than that produced by acridines,\(^3\) whose effects on DNA have been attributed to intercalation of the dye between adjacent base pairs (40). For another physical consequence of actinomycin binding to DNA, please see note at the end of this paper.

**EFFECT OF ACTINOMYCINS ON DNA-DEPENDENT RNA-POLYMERASE**

Slotnick (62, 63), working with cultures of *Bacillus subtilis*, first reported that actinomycin rapidly inhibited protein and RNA synthesis, but not DNA synthesis. A similar observation was later recorded for growing *Staphylococcus aureus* (37). In these experiments with bacteria the well established coupling of protein and RNA synthesis was maintained when the culture was inhibited by actinomycin. A clear-cut differentiation of the susceptibility of these syntheses to actinomycin could be demonstrated in L-cells (54, 55); following exposure to actinomycin, the initial synthetic rates for protein and DNA were unchanged, whereas the formation of RNA could be rapidly and almost quantitatively suppressed. The growth of RNA viruses and virus-directed RNA synthesis proceeded at normal rates in cells in-

\(^1\) W. Müller, personal communication.

\(^2\) L. S. Lerman and J. M. Kirk, personal communication.
hibited by actinomycin (54, 55), suggesting that RNA synthesis in mammalian cells falls into two categories: (a) that of the uninfected host cell (whose genetic material consists of DNA) which are presumably entirely DNA-dependent and completely actinomycin-sensitive and (b) that of the infecting virus (having RNA as its genome), which is RNA-dependent and completely actinomycin-insensitive. This formulation was supported by the fact that actinomycin was known to bind to DNA but not to RNA and could be supposed on this basis to distinguish between the two potential templates for RNA formation. The assumed inhibition of DNA-dependent RNA formation was shortly thereafter demonstrated in crude extracts of HeLa cells (14), and then in purified enzymes derived from E. coli (21, 27, 56) which were capable of catalyzing such a synthesis. This synthetic reaction, which is catalyzed by RNA-polymerase, is known to require DNA (26), bivalent cations, and four ribonucleoside triphosphates (72); and the RNA produced has a base composition and nearest neighbor frequency complementary to the DNA primer (72). The enzymic synthesis of DNA by DNA-polymerase, which had previously been shown to be sensitive to high concentrations of actinomycin (38), was found to be less susceptible to the antibiotic than RNA synthesis by DNA-dependent RNA-polymerase (27), paralleling the behavior of intact cellular systems.

The inhibition of RNA synthesis by actinomycin observed in whole cells and enzyme preparations is not due to an effect on the enzyme protein directly, or to competition with any of the required cofactors or precursor nucleotide triphosphates; it is entirely dependent on binding of actinomycin to primer DNA. This conclusion is supported by the following evidence: (a) The inhibition of RNA-polymerase by actinomycin cannot be overcome by increasing the concentration of enzyme, cofactors, or precursors; it can be overcome by increasing amounts of DNA (22, 27, 56). (b) The inhibitory effectiveness against RNA-polymerase of actinomycins and their derivatives parallels their capacity to complex with DNA (56). The inactive derivatives have been found not to compete with the parent compounds in the enzymatic reaction.

(c) The susceptibility to actinomycin of a DNA-directed RNA synthetic reaction is completely dependent on the capacity of the primer to bind the drug. The formation of RNA with synthetic dAT as primer is absolutely resistant to actinomycin (15, 16) (although sensitive to proflavine (15), thereby differentiating the effect of the two agents); the same is probably also true for oligothymidylicate-directed poly A synthesis (27). RNA synthesis primed by crab dAT, which contains 2.5 per cent guanine, is measurably but only modestly actinomycin-sensitive (15). In general, the actinomycin susceptibility of reactions catalyzed by RNA-polymerase reflects the binding capacity of the priming DNA (15, 16).

The DNA-dependent RNA-polymerase of Micrococcus lysodeikticus and Azotobacter vinelandi have been isolated by Weiss and collaborators and Krakow and Ochoa, respectively. The highly purified enzymes can use DNA for priming RNA synthesis in the usual manner (72). In the absence of DNA, some RNA preparations may serve as primers for the synthesis of complementary RNA by this enzyme (73). The DNA-directed reaction is very actinomycin-sensitive (39), as is the case for RNA-polymerases from other sources, but the RNA-primed reaction is actinomycin-insensitive (39). This experiment shows dramatically that the effect of actinomycin is related to the nature of the primer, rather than the enzyme protein. The antibiotic can bind only to DNA primers, whereas the enzyme is the same in both reactions. Other enzymes involved in nonDNA-dependent RNA metabolism are not inhibited by actinomycin.

The reactions of actinomycin with synthetic dGC are of interest. This double-stranded polymer has been shown to consist of a mixture of polydG and polydC homopolymers (31). dGC directs the synthesis of an RNA containing only guanine and cytosine (10), and each strand of this DNA can prime the formation of complementary RNA independently of concurrent replication of the other strand (15). All the guanine residues, and therefore all the actinomycin binding sites, are present in only one—the polydG strand. Nevertheless, actinomycin inhibits RNA synthesis primed by both strands of dGC (15), although poly G formation, directed by polydCytidyllic acid, is somewhat less sensitive than is poly C synthesis, which is primed by the polydG strand (15).

It is noteworthy that dGC-dependent reactions are much less actinomycin-sensitive than would have been expected on the basis of the guanine content of the primer. Although not reflecting the high guanine content the actinomycin-sensitivity in this, as in other instances, parallels the actinomycin-binding capacity of the DNA (15). dGC binds actinomycin poorly, suggesting that the properties of a binding site may be influenced by the nucleotide sequences or DNA structure in the

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5 I. H. Goldberg, E. Reich, and M. Rabinowits, unpublished results.

4 J. H. Goldberg, E. Reich, and M. Rabinowits, unpublished results.

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5 1. H. Goldberg, E. Reich, and M. Rabinowits, unpublished results.
immediate environment of the guanine residues. Long sequences of deoxyguanylic acid appear not to present the most effective sites for complexing actinomycin, and this may in part provide an explanation for the fact that the maximal binding capacity of different DNAs runs parallel with, but is not directly proportional to, their guanine content.

In addition to catalyzing RNA synthesis, RNA-polymerase catalyzes a DNA-dependent exchange between inorganic pyrophosphate and the terminal pyrophosphate residues of ribonucleoside triphosphates. This reaction, which can proceed with a single ribonucleoside triphosphate in the absence of concurrent polynucleotide synthesis, is much less sensitive to actinomycin than the synthetic reaction with the same DNA preparations (16, 27). Nevertheless, the difference in actinomycin-sensitivity of synthetic reactions primed by DNAs of different binding capacity has its counterpart in the exchange reactions directed by the same DNAs (16). For example, dAT-primed exchange reactions are absolutely actinomycin-resistant (16); and exchanges primed by M. lysodeikticus DNA (G content, 37 per cent) are more sensitive to actinomycin than those primed by T. pyriformis DNA (G content, 18 per cent) (16).

A working hypothesis concerning the basis of actinomycin effects which is consistent with most of the facts might be formulated as follows: actinomycins possessing the appropriate structural features (e. supra) bind tightly to DNA at a site which certainly involves a guanine moiety, preferably in a helical configuration. The bound actinomycin physically obstructs polynucleotide chain growth, thereby arresting RNA synthesis. Since the ribonucleoside triphosphate-PP exchange reaction can proceed with single ribonucleoside triphosphate independently of polynucleotide synthesis, it is probable that short segments of DNA containing the requisite complementary bases can serve as primers of exchange. The relative actinomycin resistance of the exchange reaction can therefore be visualized as the product of segments of DNA left free to direct exchange in the presence of a concentration of bound actinomycin molecules sufficient to prevent the formation of acid-insoluble polynucleotides. Acid-soluble oligonucleotides may be formed under these conditions.

The above hypothesis does not account for the following observations:

1. The synthesis of DNA is much less sensitive to actinomycin than that of RNA, although the primer for both reactions is the same. In the enzymatic synthesis of DNA this fact might be due to the necessary presence in the reaction mixture of deoxy-GTP which could be expected to bind actinomycin and prevent its complexing with the DNA primer. However, this explanation cannot hold for the in vivo situation. Alternative possibilities are that the configuration of the primer, or its steric relationship to the enzyme and precursors in the act of replication (in which it is single-stranded), may differ sufficiently in the two nucleic acid syntheses to account for the change in actinomycin-susceptibility.

2. RNA-polymerase can catalyze homopolymer production (e.g., poly A and poly U) formation when primed with certain DNAs (10) (high in AT content). Poly A and poly U are not produced when all four ribonucleoside triphosphates are present, being synthesized only when the appropriate precursor is present singly (10). This synthesis, which may reflect the presence of complementary homopolymeric segments in the primer (cf. discussion of dGC above), is resistant to actinomycin (16, 27), as might be anticipated from any reaction directed by portions of DNA containing little or no guanine. Since heteropolymer formation is sensitive to actinomycin, the presence of the antibiotic would be expected to shift the enzymatic reaction in favor of homopolymer production. However, this does not occur, suggesting that the enzyme is immobilized at regions of the DNA which cannot prime homopolymer synthesis. The results of several experiments undertaken to test this possibility are not consistent with the hypothesis that RNA-polymerase may be bound to DNA in the presence of actinomycin and NTP. The data suggest instead that the enzyme is displaced from the DNA primer by the binding of the antibiotic.

CORRELATION OF REACTIVITY OF ACTINOMYCINS WITH DNA AND THEIR BIOLOGICAL ACTIVITY IN VARIOUS TEST SYSTEMS

Several years ago a series of actinomycins and derivatives was assayed for the capacity to prolong the lives of mice infected with a transplantable mouse leukemia (8). The minimum effective concentration for the different compounds extended over a concentration range of approximately 1000-fold. The antibiotic activity against a strain of Bacillus subtilis of some of the derivatives paralleled the findings obtained with mice (45). Subsequently (56), sixteen actinomycins and derivatives were compared for their capacity to (a) bind to DNA, (b) inhibit RNA-polymerase of E. coli and HeLa cells, (c) inhibit the growth of B. subtilis and HeLa cells. The relative activity of any compound in one test system correlated well with its effectiveness in all other systems. Compounds
which failed to bind to DNA were ineffective against RNA-polymerase preparations, HeLa cells, and B. subtilis. The good correlation of the activities of many compounds at different levels of biological organization—the intact animal, cultured mammalian cell, bacterial culture, purified enzyme, and DNA molecule—provides strong evidence for a unitary basic action mechanism for actinomycins. This action is presumed to depend on their capacity to bind to DNA, as a consequence of which RNA synthesis and the expression of cellular genetic potentialities are inhibited. Since the multiplication of some RNA viruses can proceed normally in cells exposed to high concentrations of antibiotic for 24 hours prior to infection, all the complex structural, enzymatic, and energy-yielding cellular components required for virus growth must be unaffected by actinomycins. Therefore, it appears possible that actinomycins may be completely innocuous to all cellular processes not primarily involved in DNA metabolism.

EFFECTS OF ACTINOMYCINS ON MAMMALIAN CELL LINES IN CULTURE AND ON GROWTH OF ANIMAL VIRUSES

The division of mammalian cell lines is sensitive to very low levels of actinomycin. Threshold concentrations of antibiotic permit some cell division for up to two doubling times, following which the mitotic activity of the culture stops (55). L-cells or HeLa cells which have been incubated in the presence of growth-suppressing concentrations of actinomycin lose much of their histochemically demonstrable RNA (17, 60). The nucleoli are affected first—their disappearance may be complete by 12–24 hours. Cytoplasmic basophilia and characteristic acridine-orange fluorescence decrease slowly thereafter. Concentrations of actinomycin which do not inhibit initial rates of DNA synthesis permit nuclear enlargement and moderate increases in cellular DNA content.

Since actinomycin binds to DNA (and is concentrated in the cell nucleus) the effect of any quantity of antibiotic is dependent on the amount of DNA present—i.e., the cell population density. Concentrations twenty- to 50-fold greater than those required to inhibit cell division lower RNA synthesis to less than 25 per cent of normal without affecting protein and DNA synthesis; further increases in actinomycin concentration may completely (>99 per cent) suppress incorporation of uridine into RNA, whereas initial rates of protein and DNA synthesis are only slightly impaired. L-cells maintained in concentrations of actinomycin which completely (>99 per cent) inhibit RNA formation retain the capacity to synthesize protein (measured by incorporation of radioactive amino acids) for at least 48 hours. The rate of protein synthesis after 24 hours in actinomycin is 5–10 per cent, and at 48 hours it is approximately 4–5 per cent that of logarithmically growing cells. This rate of protein synthesis compares favorably with estimates of the rate of protein production by highly efficient, differentiated, non-dividing mammalian cells such as mature plasma cells and pancreas. The actinomycin-treated cells have a lower RNA content than the rapidly dividing culture and resemble also in this respect differentiated, resting cells.

The synthesis of all cellular fractions of RNA—"soluble" ribosomal, and nuclear—may be suppressed by actinomycin (51, 55, 68), that of the soluble (4S) RNA being slightly less sensitive than the polymers of high molecular weight. Therefore, it seems possible that all species of RNA of uninfected cells are produced in the nucleus, in a DNA-dependent reaction under genetic control. The slightly greater actinomycin resistance of soluble RNA synthesis may be due to (a) terminal labeling, occurring in part in the cytoplasm, and (b) the possibility of a more rapid flow of radioactive precursors into cytoplasmic soluble RNA than into cytoplasmic ribosomal RNA due to the presence of a relatively large nuclear and/or nucleolar pool of high molecular weight RNA. The small fraction (0.1 per cent) of actinomycin-resistant RNA "synthesis" is restricted to 4S material,9 where it is located in terminal or near-terminal positions (44).

The effect of actinomycin on the induction of liver enzymes in intact rats has been examined. It was found that actinomycin could inhibit the appearance of the enzymes with hormone but not with substrate as inducer (19).

Actinomycin and animal virus growth.—The growth of animal viruses is classified as "indifferent" to actinomycin, if high concentrations (>2 \(\mu g/ml\)) of the antibiotic (which totally suppress cellular RNA synthesis), added at the time of infection, do not reduce virus yield in single-cycle growth experiments; as "sensitive" if low concentrations (<0.1 \(\mu g/ml\)) do significantly inhibit virus yield; as "intermediate" if the system does not appear to fit into either of the other categories. Several of the viruses listed below have been tested in experiments other than the single-cycle type, but the behavior of known reference viruses in the

8 E. Reich and G. Acs, unpublished observations.
9 G. Acs, personal communication.
10 R. M. Franklin, personal communication.
same systems has made this tentative classification possible:

DNA viruses: Vaccinia (54, 55), Herpes simplex III—sensitive.

RNA viruses: Mengovirus (54, 55)—indifferent; Poliovirus (61), Coxsackie A-9—indifferent; Newcastle disease (37)—indifferent; Influenza (S)—intermediate; Fowl plague—sensitive; Chikungunya (group A arborvirus)—indifferent; Reovirus (18)—intermediate; Rous sarcoma virus (23)—indefinable (see below).

It is not surprising that growth of DNA viruses should be actinomycin-sensitive, since the synthesis of RNA in a DNA-dependent, actinomycin-sensitive reaction would presumably be the obligatory first step in the expression of the viral genes. For reasons cited above, the indifference to the antibiotic of most of the RNA viruses tested is also not surprising. The intermediate position of Reovirus (90 per cent inhibition in single-cycle growth) might be explained by its relatively long latent period, during which cellular enzyme systems ultimately required for some phase of virus growth could be deteriorating and their replacement rendered impossible in the presence of actinomycin. The susceptibility of fowl plague and, to some extent, influenza virus is noteworthy, since the growth cycle of these viruses is known to include a nuclear phase. Actinomycin inhibition of the multiplication of these viruses might operate at a primary level (interference with functions of cellular or perhaps newly formed DNA required for the infectious process); or it could be secondary to structural changes in the nucleus resulting from the physical distortions in DNA produced by complexing of the antibiotic. There is no evidence to suggest whether the replication of the viral RNA is inhibited by actinomycin, or whether another phase of the virus life cycle is affected.

In one experiment in which virus yield was measured 24 hours after initial infection, the growth of Rous sarcoma virus in chick embryo monolayers was sensitive to low concentrations of actinomycin (0.1 μg/ml) only if the latter was added to the culture during the first 10 hours following virus inoculation. In another experiment the effect of the same low concentrations of actinomycin on the rate of virus production was measured (23). When fully transformed, infected chick embryo monolayers which were excreting virus at maximum rates were treated with this concentration of actinomycin (known to suppress RNA synthesis by 98 per cent within 5 hours), virus production after 20 hours was only slightly lower than that of an equal control population. These findings may be interpreted tentatively to suggest that the formation of the mature cell-virus complex involves an actinomycin-sensitive step. Once the infected complex has been stabilized, virus growth appears independent of DNA. If this is correct, DNA would function in some way other than that of replicating viral nucleic acid.

From their resistance to actinomycin (54), the growth cycle of simple RNA viruses appears to require no involvement of DNA (53, 61)—either of the host cell, or virus-specific—or any cellular RNA synthesis. The possible role of DNA in the life cycles of influenza, fowl plague, and Rous sarcoma viruses (all containing lipide) is obviously of great interest and requires clarification. A parallel susceptibility of influenza and vaccinia growth to a purine analog (5,6-dibromobenzimidazole riboside) has been previously observed (67).

Heller (13) found that low concentrations of actinomycin enhanced (by increased plaque size and number) the growth of several RNA viruses in chick embryo monolayers. He suggested that this effect might be ascribed to a concurrent, 50-fold inhibition of interferon production, the synthesis of which is presumed to be ultimately under control of the host cellular genome.

By suppressing completely cellular RNA synthesis actinomycin has permitted the demonstration of virus RNA formation in poliovirus (59), Newcastle disease (37) and Mengovirus infection (25, 44). The properties of the nucleic acid produced in the virus-directed synthesis suggest that all the RNA formed in the actinomycin-resistant process is copied from the RNA as it exists in the infectious virus (24). Only a minor fraction of the total virus-specific RNA formed intracellularly is ultimately incorporated into new mature particles (59).

**EFFECT OF ACTINOMYCIN ON SUSCEPTIBLE MICROORGANISMS**

Gram-positive, but not gram-negative, bacteria are sensitive to actinomycin (71). As in the case of mammalian cells, the effect of a given level depends on the population density. Appropriate concentrations of actinomycin arrest RNA synthesis more or less immediately, and low levels of protein synthesis may continue for one-tenth to one-half of a generation time (27, 38, 41, 55). In bacteria DNA formation is more resistant to actinomycin than protein synthesis, (52, 62)
whereas in mammalian cells the reverse is true (55). Presumably this results from the coupling of RNA and protein metabolism in bacteria, while mammalian cells may lack the refined control mechanisms (repression and feedback inhibition) responsible for this synchronization of synthetic activities, and their templates may be more stable.

The effect of actinomycin is basically bacteriostatic, although some strains of *B. subtilis* lyse rapidly on addition of actinomycin in the manner frequently characteristic of bacilli in unfavorable environments. Threshold concentrations of the antibiotic may permit slow growth without cell division, as a result of which very long filamentous forms are produced (97, 38). The ratios of various macromolecular components are altered in these filaments.

Since the synthesis of all RNA species in bacteria can be suppressed by actinomycin (1, 41, 52) it is assumed that, as in the case of mammalian cells, the entire cellular complement of RNA is produced in a DNA-dependent reaction under genetic control, none being autonomously replicated. The synthesis of sRNA (measured by the incorporation of uracil-C14 into RNA-pseudouridine) in cultures only partially inhibited by actinomycin is suppressed to the same extent as total RNA synthesis (1).

Actinomycin has been used to stop RNA synthesis rapidly with the aim of demonstrating breakdown of the fraction of RNA which is rapidly labeled when bacteria are exposed to appropriate radioactive precursors for very short periods (41). This fraction is considered by some to constitute a special category of RNA and has been named "messenger-RNA." When actinomycin is added to cultures of *B. subtilis* following a 5-minute pulse of radioactive uracil, the breakdown of a portion of the previously synthesized RNA is observed (41, 52). This has been interpreted to mean that the RNA which breaks down does so even in the absence of actinomycin and that the depolymerization is related to the assumed template function. It remains possible that the observed degradation might be caused by actinomycin. When actinomycin is added to cultures which have accumulated RNA in the presence of chloramphenicol, the newly formed RNA breaks down rapidly (1, 52). This latter breakdown is independent of protein synthesis, as is the breakdown of the pulse-labeled fraction, and its initial rate is approximately equal to the preceding synthetic rate. Appropriate control experiments show that the depolymerization of chloramphenicol RNA seen in the presence of actinomycin cannot be due simply to the inhibition of synthesis of a portion of the RNA which is being continually degraded and resynthesized.

The effect of actinomycin on formation of several enzymes has been investigated by Pollock, and his results are summarized as follows: with concentrations of actinomycin causing 60–70 per cent inhibition of growth (a) the formation of β-galactosidase (constitutive, cell-bound) and α-amylase (constitutive, extracellular) of *B. subtilis* were inhibited to the same extent as growth; (b) maltose-induced α-glucosidase production was more sensitive, being inhibited 90–95 per cent under the same conditions; (c) formation of penicillinase in a constitutive strain (*B. cereus*) was unaffected for 3 hours; (d) penicillinase production of an inducible strain (*B. cereus*) was inhibited to the same extent as growth if actinomycin was added with the inducer; (e) if inducer was present for 40 min. prior to actinomycin, the same concentration of antibiotic as in (d) and (a) above did not affect induced penicillinase production for at least 40 min.; (f) with "high" concentrations of actinomycin (growth inhibition > 70 per cent) penicillinase production stopped in all strains except constitutive *B. cereus*, which continues to elaborate enzyme for 30 min.; (g) very low concentrations of actinomycin (0.01–0.025 μg/ml) which cause negligible (0–920 per cent) slowing of growth do not affect penicillinase production in *B. cereus*. With *B. subtilis*, however, there results a marked (50 per cent) inhibition of induced α-glucosidase formation and an unexpected stimulation (up to 100 per cent over 4 hr.) of induced penicillinase synthesis. This stimulation was dependent on submaximal inducer concentrations and was not seen in one constitutive mutant. These findings were interpreted as possibly indicating (a) differential inhibition of RNA synthesis by different genes based on their GC content and actinomycin-binding sites; thus the failure to inhibit penicillinase synthesis might be due to a relatively lower GC content of the controlling gene. "The relative quantities of actinomycin and cells used are such that, even if all the drug were in combination with the bacterial DNA present, the number of molecules of drug pen gene would be about unity, assuming a mean molecular weight of 650,000 for a gene. Thus the conditions were optimal for demonstrating such a differential effect, if it exists." Experiments were designed to test the possibility that the stimulation of penicillinase production seen with low antibiotic concentrations might be due to interference with production of RNA which could be involved in the expression of hypothetical

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M. R. Pollock, personal communication.
regulatory (repressor) genes, but confirmatory evidence for this view could not be obtained.

An antibacterial effect of actinomycin which is difficult to relate to its other biological effects is that observed by Foley (11), who found the antibiotic to be a competitive inhibitor of pantothenate, in several pantothenate-requiring microorganisms. RNA synthesis appears to be inhibited by actinomycin even in these strains (74).

USE OF ACTINOMYCIN IN DEMONSTRATION OF STABILITY OF PROTEIN-FORMING TEMPLATES

Since all fractions of cellular RNA of susceptible bacterial and mammalian cells are produced in an actinomycin-sensitive and presumably DNA-dependent reaction, the use of the antibiotic might be expected to permit the demonstration of stable protein-forming units. Such a demonstration is of obvious importance in view of the widely accepted suggestion that RNA templates are short-lived and require renewal (29). Several impressive examples of stable protein-synthesizing systems are well known. These include (a) enucleate amoebae, (b) enucleate Acetabulariae, and (c) mammalian reticulocytes (also enucleate). In these cases protein synthesis proceeds for prolonged periods, and the absence of a nucleus eliminates any source of genetic information which might provide a continuous supply of unstable "messengers." Although it has been suggested that DNA-dependent RNA synthesis might occur in mammalian reticulocytes (42), this appears now to have been ruled out (43, 47). Furthermore, hemoglobin synthesis in rabbit reticulocytes is completely resistant to actinomycin (52). Therefore, in these instances the genetically determined protein-forming templates are probably embodied in the stable ribosomal RNA.

As noted above, actinomycin-treated L-cells, which are synthesizing no RNA, incorporate amino acids into protein at a rate which decreases for 8–12 hours, and thereafter plateaus at a level corresponding to 5–10 per cent that of the growing culture. The cells of a logarithmically growing culture synthesize the equivalent of 80 per cent of their dry weight as protein in 24 hours. On this basis the actinomycin-treated cell produces protein equal to 4–8 per cent of its dry weight per day. This value is close to the estimate of the synthetic capacity of mature plasma cells which are actively forming antibodies (48). Since a level of protein-forming capacity which approximates that of efficient differentiated cells can be preserved for at least 36 hours in the complete absence of RNA synthesis, it appears possible that the templates of mammalian cells under physiological conditions may be stable for indefinitely long periods—long, perhaps, even in relation to the life of the organism. If this were the case, the lifespan of templates would tend strongly to stabilize the differentiated character of cells, and loss of cellular differentiation would require the destruction of templates or their dilution, for example, by mitosis.

In bacteria, as in mammalian cells, the use of actinomycin made possible the demonstration of long-lived protein-forming units. Owing to the numerous interlocking sensitive control phenomena which characterize the integration of bacterial metabolism, such a demonstration requires unusual conditions. The arrest of the synthesis of a bacterial protein might be caused by the disintegration of a template; it could also be the product of a control mechanism.

When a very high concentration of actinomycin (which completed suppressed RNA synthesis) was added to B. cereus cultures following commitment to sporulation, there was no interference with the normal rate or extent of sporulation, and the viability and heat stability of the small spores produced was normal (70). This experiment shows clearly that the complex morphogenetic process of sporulation, requiring for its completion many hours of protein synthesis and involving an orderly, controlled, sequential induction of enzyme activities, could proceed normally in the complete absence of RNA synthesis. It is most interesting that the control and integration of the many biochemical activities which culminate in the appearance of a mature spore could occur at a nongenetic level, since RNA synthesis, which presumably functions as the communication medium linking the gene with the rest of the cell, was shown to have been eliminated by actinomycin.

The results of another experiment which suggested stability of bacterial templates have been published recently. The respiration-deficient mutants of Staphylococcus aureus are extremely sensitive to actinomycin. Low concentrations of the antibiotic permitted substantially normal DNA and protein synthesis without net increase in RNA. Experiments with radioactive precursors, which might have shown whether some RNA turnover was occurring, were not performed. The results of such experiments would obviously be of great interest. Finally, the actinomycin resistance of penicillinase production by B. cereus, which follows the latent period of induction, resembles that previously found also for the effect of ultraviolet light (69) and provides another instance...
suggestive of stability of template function in microorganisms.

The many lines of evidence pointing to relative stability of protein-synthetic templates in different organisms suggests that it is premature to discard the proposition that the stable ribosomal RNA is the carrier of genetic specificity governing the synthesis of proteins. The functions of ribosomes, which in many instances require no additional "messengers," could be subject to controls in addition to any which may be operating at the genetic level.

MISCELLANEOUS BIOLOGICAL SYSTEMS AFFECTED BY ACTINOMYCIN

The effect of actinomycin on the development of fertilized sea-urchin eggs has been investigated by Gross, whose interesting results have been summarized as follows: (a) very high concentrations of actinomycin (> 20 μg/ml) are required to suppress RNA synthesis in early embryos; these concentrations totally suppress incorporation of precursors into RNA by the eight-cell stage; (b) actinomycin-treated embryos undergo mitosis and cleavage normally, occasionally at a slightly reduced rate; late cleavage planes begin to deviate from normal. A multicellular mass results, which ultimately (94 hours) undergoes disintegration. There is no formation of cilia, hatching enzyme, or blastocoel. In all these respects the actinomycin-treated embryos resemble parthenogenetic mero-gones; (c) DNA synthesis proceeds at a rate somewhat lower than normal in actinomycin-treated embryos; (d) protein synthesis is unaffected by actinomycin for periods as long as 94 hours. These findings point to (a) stability of the protein-forming apparatus and (b) the fact that the sea-urchin egg is equipped with all the RNA required for the phenomena which precede hatching, including more or less orderly mitosis and cleavage.

Actinomycin affects the structure of giant chromosomes, such as the salivary chromosomes of Drosophila and lampbrush chromosome of Triturus. The salivary chromosomes lose their flexibility (as would be expected from the viscosity increase seen in DNA solutions), tend to adhere to one another, and the characteristic band pattern is no longer visible, the appearance of the chromatins having become relatively uniform throughout. When the lampbrush chromosomes are exposed to actinomycin (28), the lateral loops shrink rapidly (5 min.), whereas the length of the chromosome is preserved. The isolated chromosome is normally a flexible, gently undulating structure; the addition of actinomycin converts it to a straight, rigid rod. Uridine incorporation into acid-insoluble material in the loops, the chromosome, and the nucleolus is markedly decreased by actinomycin. None of these effects was observed when an inactive derivative of actinomycin was substituted for the parent compound.

An interesting effect of actinomycin (32) (and mitomycin C) has recently been recorded by Karakashian and Hastings. They found that concentrations of actinomycin which only partially inhibited growth ultimately abolished luminescence rhythm of the dinoflagellate Gonyaulax polyedra. This finding constitutes the first positive evidence concerning a biochemical pathway involved in a biological rhythm. The many antimetabolites and growth-inhibitors which had previously been tested in this system did not affect the photosynthetic and bioluminescent rhythms.

DISCUSSION

Many aspects of actinomycin action remain to be explored, particularly those related to its reaction with DNA. These include among others: (a) more rigorous definition of the physico-chemical parameters of complex formation with DNA; (b) characterization of the structural properties of binding sites in native DNA; (c) precise stereochemistry of bound actinomycin (12); (d) environmental factors which affect the complexing reaction; (e) intranuclear distribution of actinomycin; (f) potential mutagenicity of actinomycins—this has been examined in only two systems—Drosophila, where its action was interpreted as antimutagenic, and E. coli, which is not permeable to the antibiotic; (7) characterization of actinomycin resistance in bacterial and mammalian cells; (8) further investigation of the effect of actinomycin on specific protein synthesis in different organisms. In addition, there are many fundamental problems in cellular physiology, host-virus relationships, immunology, protozoology and other areas of biology in the analysis of which actinomycin might be of some use. Because of its toxicity, and because the biochemical reaction which it blocks is of fundamental importance for the survival of all cells, it appears unlikely, as foreseen by its discoverers (71), that the therapeutic usefulness of actinomycin will extend far beyond those conditions to which it has already successfully been applied.

P. Gross, personal communication.

A. Shafiq, E. Reich, and G. Acs, unpublished observations.
Note added in proof:

Recently, actinomycin has been found to increase markedly the resistance of the DNA molecule to heat denaturation, and to raise the "melting temperature" (Tm) of DNA preparations by as much as 14°C. This effect is observed only at rather high antibiotic concentrations (molar ratio actinomycin/DNA nucleotide = 0.025—0.5; this is precisely the concentration range which corresponds to the inhibition of DNA-polymerase by actinomycin, whereas RNA-polymerase is inhibited at much lower levels (actinomycin/DNA nucleotide = 0.0004—0.0075). Thus the effect of actinomycin on DNA-polymerase would appear to be an indirect one, mediated via physical changes which impair strand separation of the primer, while its effect on RNA-polymerase cannot be correlated with any observable physical alteration of the primer. Since dAT does not bind actinomycin, it is not surprising that neither its Tm nor its primer activity for DNA-polymerase is influenced by actinomycin. Haskelkorn has also observed the effect of actinomycin on the Tm of DNA and has made the additional important discovery that DNA-RNA hybrids to not bind actinomycin, showing that the mere presence of deoxyguanosine in a nucleic acid is not sufficient per se to guarantee reaction with actinomycin and that the stereochemical configuration of the polymer is also a determining factor in this reaction.

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Biochemistry of Actinomycins

Edward Reich