ficity of any kind involved in the determination of what kind of DNA binds with what kind of histone is an interesting question but is not considered in this paper.

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THE ROLE OF DEOXYRIBONUCLEIC ACID IN RIBONUCLEIC ACID SYNTHESIS, III. THE INHIBITION OF THE ENZYMATIC SYNTHESIS OF RIBONUCLEIC ACID AND DEOXYRIBONUCLEIC ACID BY ACTINOMYCIN D AND PROFLAVIN*

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COMMUNICATED BY B. L. HORECKER, MAY 22, 1962

Actinomycin D is one of a number of polypeptide antibiotics isolated in Waksman's laboratory.1, 2 Bacteriostatic effects, particularly on gram positive bacteria, and antitumor activity have been attributed to this compound.3, 4 Kirk5 has demonstrated that the addition of actinomycin D (0.2 to 0.5 µM) to exponentially growing cultures of Staphylococcus aureus stops RNA synthesis immediately. This effect is rapidly followed by an inhibition of protein synthesis, and later by a partial inhibition of DNA synthesis. The action of this compound is not related directly to energy production since both respiration and glycolysis of inhibited cells are unaffected by concentrations up to 0.1 mM.4 Kirk also demonstrated that the combination of DNA and actinomycin D results in a spectral change of the latter compound. These observations suggest the formation of a complex between these two compounds since Kawamata and Imanishi6 found no interaction of actinomycin and RNA and the reaction appears to be relatively specific for DNA. Although Rauen et al.5 have reported complex formation between actinomycin and RNA, 100 times more RNA than DNA is required.

Two recent reports have suggested that actinomycin acts by inhibiting the synthesis of "messenger" RNA. Nakata et al.7 have shown that reproduction of T2
phage and phage protein synthesis are markedly inhibited by actinomycin S while phage DNA synthesis is unaffected. Reich and co-workers have demonstrated that actinomycin D at concentrations of 0.1 \( \mu \text{M} \) inhibits the synthesis of RNA in L-cells in tissue culture and decreases the yield of the DNA containing vaccinia virus but does not inhibit cellular DNA synthesis or the multiplication of the RNA containing Mengo virus.

The present report is concerned with the effects of actinomycin on RNA polymerase and DNA polymerase, both of which require DNA. Both syntheses are inhibited by actinomycin, the RNA polymerase reaction being somewhat more sensitive.

Another compound which binds to DNA is proflavin. This dye has been shown by a number of investigators to be mutagenic. Proflavin exerts an action similar to that of actinomycin D and inhibits both enzymatic reactions leading to RNA and DNA synthesis. In this case, however, DNA synthesis is more sensitive than RNA formation.

Materials and Methods.—Actinomycin D was a gift from Merck Sharp and Dohme Research Laboratories, West Point, Pennsylvania, and for this gift we are indebted to E. Alpert. Proflavin sulfate was a commercial preparation of the Mann Research Laboratories. Mitomycin C was a gift of Y. Takagi, Department of Biochemistry, Kanazawa, Ishikawa, Japan.

RNA polymerase and DNA polymerase were purified and assayed as previously described. The nucleoside triphosphates were obtained as described in a previous publication.

Most of the DNA preparations tested were prepared by published procedures or were obtained as previously described.

Results.—Effects of actinomycin D on RNA polymerase and DNA polymerase: It was suggested to us by R. M. Franklin of the Rockefeller Institute that the effects of actinomycin D might be due to the inhibition of RNA synthesis by the DNA-linked RNA polymerase. As shown in Figure 1, actinomycin D is indeed a potent inhibitor of RNA polymerase. The extent of inhibition can be reduced by increasing the DNA concentration but is unaltered by the addition of the other components required for the synthesis of RNA.

A double reciprocal plot of the velocity versus the substrate concentration (DNA) in the presence of a constant amount of actinomycin D is shown in the inset to Figure 1. Inhibition by actinomycin D appears to be competitive with DNA. From this plot, \( K_I \) for actinomycin D was calculated to be \( 2.76 \times 10^{-9} \text{M} \). The \( K_I \) value reported here is not a measure of the association of the enzyme and the inhibitor but a measure of the association between the enzyme and the inhibitor complexed with DNA.

Actinomycin D also inhibits the enzymatic synthesis of DNA catalyzed by DNA polymerase (Fig. 2). This inhibition is also reduced by increasing the DNA concentration. \( K_I \) for actinomycin D in the DNA polymerase reaction is \( 2.7 \times 10^{-7} \text{M} \). It appears that the synthesis of DNA is approximately 10-fold less sensitive to actinomycin D than the corresponding synthesis of RNA.

The effects of actinomycin D with various DNA primers: As shown in Table 1, the inhibitory effect of actinomycin D is not limited to calf thymus DNA. RNA polymerase-catalyzed reactions which are primed with human bone marrow DNA
FIG. 1.—The inhibition of RNA polymerase by actinomycin D. The reaction mixture (0.5 ml) contained: 90 µM each of ATP, GTP, and CTP, 80 µM α-P32 UTP containing 1.42 × 10⁶ cpm per µmole, 4 mM MnCl₂, 8 mM MgCl₂, 2 mM mercaptoethanol, 50 mM Tris buffer, pH 7.5, calf thymus DNA (38 µmolecules as deoxynucleotide), 1.84 µg of RNA polymerase (ammonium sulfate IIb), and varying amounts of actinomycin D as indicated. This was incubated for 20 min at 38° after which the activity was measured as previously described.11

The determination of the Kₑ of actinomycin D was carried out with 0.1 µM actinomycin D and varying amounts of DNA. The reciprocal velocity versus the reciprocal substrate concentration plot is presented in the inset figure.

FIG. 2.—The inhibition of DNA polymerase by actinomycin D. The reaction mixture (0.5 ml) contained: 14 µM (each) dATP, dGTP, and dTTP, 8 µM α-P32 dCTP containing 0.87 × 10⁶ cpm per µmole, 4 mM MgCl₂, 2 mM mercaptoethanol, 50 mM Tris buffer, pH 8.0, calf thymus DNA (24 µmolecules as deoxynucleotide), 0.14 µg of DNA polymerase (ammonium sulfate 50%-40% fraction), and actinomycin D as indicated. The reaction mixture was incubated and assayed as described in Figure 1.

The reciprocal velocity versus reciprocal substrate concentration plot was carried out with 8 µM actinomycin D.
or Micrococcus lysodeikticus DNA are also inhibited. The degree of inhibition is independent of the nature of the ribonucleotide which is used to measure the reaction.

The degree of inhibition is also similar with φX 174 DNA, suggesting that double-stranded DNA, as such, is not required for the observed effects. Reaction mixtures containing heat-denatured DNA as primer are also inhibited by actinomycin D.

On the other hand, reaction mixtures primed with polydeoxythymidylicate are not affected by actinomycin D nor is the DNA-dependent production of polyribadenylate or polyribouridylicate sensitive to actinomycin D. Recently, Chamberlin and Berg\(^{16}\) have reported the formation of polyribadenylate (poly A) by RNA polymerase preparations. The production of poly A has already been noted by us\(^{16}\) as well as by Stevens.\(^{17}\) In agreement with the results obtained by Chamberlin and Berg, the reaction leading to poly A is dependent on DNA, Mn\(^{++}\), and ATP. However, in contrast to their results, our partially purified enzyme fractions catalyze a very limited poly A production. The ratio of AMP incorporation with ATP as the only nucleoside triphosphate to AMP incorporation in the presence of the other three ribonucleoside triphosphates has varied in our hands from 0.07 to 0.3. This is in contrast to the value of 10 reported by Chamberlin and Berg\(^{15}\) for their purified enzyme preparations. The marked differences between our results and those of Chamberlin and Berg appear to be related to different assay conditions used to measure the above reaction. In addition to the formation of poly A, a smaller amount of polyuridylicate is also produced. This DNA-dependent poly A and polyuridylicate synthesis is also insensitive to actinomycin.

**Effect of actinomycin D on the pyrophosphate exchange reaction catalyzed by RNA polymerase:** RNA polymerase catalyzes an exchange reaction between PP\(_1\) and ribonucleoside triphosphates.\(^{11}\) This reaction is completely dependent on the presence of DNA but does not require the presence of all four ribonucleoside triphosphates. The exchange reaction is inhibited by DNase but is insensitive to RNase. In view of the different requirements for the exchange reaction compared with synthesis of RNA, it was of interest to determine the effect of actinomycin D on this reaction. As shown in Table 2, actinomycin D inhibits PP\(_1\) incorporation only slightly. Concentrations which markedly inhibit RNA synthesis produce only about 20 per cent inhibition of the exchange reaction.

### Table 1

**Effects of Actinomycin D on the RNA Polymerase Reaction Primed by Different DNA Preparations**

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Addition of actinomycin D</th>
<th>Nucleotide Incorporation—Nucleotide Incorporation—</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AMP (μ moles in 20 minutes)</td>
</tr>
<tr>
<td>1. Human bone marrow</td>
<td>−</td>
<td>2.41</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.34</td>
</tr>
<tr>
<td>2. <em>M. lysodeikticus</em></td>
<td>−</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.04</td>
</tr>
<tr>
<td>3. <em>φX 174</em></td>
<td>−</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.07</td>
</tr>
<tr>
<td>4. Thymus (heated at 100°C for 4 min)</td>
<td>−</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.16</td>
</tr>
<tr>
<td>5. Polydeoxythymidylicate</td>
<td>−</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.51</td>
</tr>
</tbody>
</table>

The activity of RNA polymerase was measured as described in Figure 1. The amount of DNA added was 50 μ moles of human bone marrow DNA, 100 μ moles of polydeoxythymidylicate, 38 μ moles of calf thymus DNA, 25 μ moles of *M. lysodeikticus* DNA, or 5.5 μ moles of *φX 174* DNA. One μM actinomycin D was added where indicated. The enzyme was ammonium sulfate 11B (2.04 μg of protein).
TABLE 2

THE EFFECT OF ACTINOMYCIN D ON THE PYROPHOSPHATE EXCHANGE REACTION CATALYZED BY RNA POLYMERASE

<table>
<thead>
<tr>
<th>Additions</th>
<th>PPI Incorporated (mmoles)</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>5.25</td>
<td>—</td>
</tr>
<tr>
<td>2. 0.2 μM actinomycin D</td>
<td>4.34</td>
<td>17</td>
</tr>
<tr>
<td>3. 0.5 μM actinomycin D</td>
<td>4.20</td>
<td>20</td>
</tr>
<tr>
<td>4. 1.0 μM actinomycin D</td>
<td>3.92</td>
<td>25</td>
</tr>
<tr>
<td>5. 5.0 μM actinomycin D</td>
<td>3.36</td>
<td>36</td>
</tr>
</tbody>
</table>

The reaction mixture (0.5 ml) contained 80 μM each of the 4 ribonucleoside triphosphates, 8 mM MgCl₂, 50 mM Tris buffer, pH 7.5, 4 mM mercaptoethanol, 1 mM PPy¹³ containing 2.22 × 10⁶ cpm per pmole, 2.2 msmoles of calf thymus DNA, and 2.4 M of RNA polymerase (ASIIB). This mixture was incubated for 30 min at 38°C, after which the extent of PPy¹³ incorporation was measured as previously described.¹¹

RNA polymerase preparations also catalyze a slow pyrophosphorolysis of the RNA produced.¹¹ This pyrophosphorolytic reaction is completely insensitive to actinomycin D.

Effect of proflavin on RNA polymerase and DNA polymerase: We have examined the effect of proflavin on DNA and RNA synthesis and have found this compound to inhibit both reactions (Figs. 3 and 4). Proflavin appears to inhibit RNA and DNA synthesis in a manner analogous to that observed with actinomycin D. It also acts as a competitive inhibitor, and the degree of inhibition is dependent on the DNA concentration. Kᵢ of proflavin in the RNA polymerase reaction is 4.05 × 10⁻⁵M; in the DNA polymerase reaction, the inhibition is more pronounced and the Kᵢ is 2.05 × 10⁻⁶M.

The effect of actinomycin in vivo: In view of the selective effect of actinomycin D on the RNA polymerase system in vitro, it was of interest to determine the effect of this drug in intact bacteria. Initially, the effect of actinomycin D on Escherichia coli was examined but it was found not to affect either whole cells or spheroplasts.
It was evident that permeability barriers may prevent access of the drug to its sites of action. In contrast to *E. coli*, *Bacillus subtilis* is extremely sensitive to actinomycin and 0.2 μM actinomycin D completely blocks growth. With lower concentrations, there is observed a marked decrease in growth (as measured by optical density) and the formation of long, snake-like forms (Fig. 5). These observations were similar to those found with an inhibitor such as mitomycin C, or by other

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**Fig. 4.**—The inhibition of DNA polymerase by proflavin. The additions were as described in Figure 2 with the exception that proflavin was added as indicated in place of actinomycin D.

**Fig. 5.**—The effect of actinomycin D on intact *Bacillus subtilis*. Cells of *B. subtilis* were grown overnight on glucose-semi-synthetic media without (A) and with (B), 0.1 μM actinomycin D. Phase contrast microscopy; the magnification was identical for both fields as indicated by the reference line.
conditions causing unbalanced growth, and prompted us to examine the effect of actinomycin D on the synthesis of RNA, DNA, and protein by B. subtilis.

In agreement with the results of Kirk with S. aureus, RNA synthesis is markedly inhibited (90–95%), protein synthesis is also inhibited but to a lesser degree (50–75%), while DNA synthesis is least affected (25%) at a level of actinomycin D equal to 0.2 μM. The small amount of RNA synthesis that still occurs (2–8% of the normal) in the case of actinomycin D-intoxicated cells has been examined after pulse-labeling (30 sec) such cells with P³². The distribution of P³² of the RNA, measured after alkaline hydrolysis, in the 2'(3')-mononucleotides does not resemble the base composition found either for the RNA or for the DNA of B. subtilis.

We have also measured the effect of actinomycin D on a number of other enzymatic reactions involved in RNA metabolism. None of these enzymatic reactions are inhibited at concentrations which affect RNA polymerase and DNA polymerase. The systems examined included the RNA-dependent production of polyriboadenylate, an RNA-dependent polymerase obtained from E. coli and the enzyme which adds CMP and AMP to the terminal end of soluble RNA. These reactions, as well as both RNA and DNA polymerases, are not affected by mitomycin.

Both actinomycin D and proflavin inhibit the stimulation of amino acid incorporation into protein produced by the RNA polymerase system. These studies were carried out with the E. coli cell free preparation; the observed effects will be described in greater detail elsewhere.

Discussion.—The inhibitory effects of actinomycin D and proflavin appear to be related to their ability to bind to DNA. The DNA complex formed inhibits RNA synthesis and DNA synthesis in a qualitatively similar manner, but with significant quantitative differences. These quantitative differences are in accord with the in vivo action of these inhibitors: namely, there is very little effect of actinomycin D on DNA synthesis, while RNA synthesis is markedly inhibited. The DNA-dependent synthesis of RNA is exceedingly sensitive to this inhibitor. At 0.5 μM actinomycin D, RNA polymerase reaction is inhibited nearly 80 per cent; at this concentration, there is virtually no effect on the DNA polymerase system.

Proflavin has been shown to alter phage replication in E. coli. When added to bacteria infected with T2 or T4, lysis occurs but the yield of progeny liberated is limited to those phage particles already present at the time of proflavin addition. If the inhibitor is added early, during the latent period, no viable phage are produced but cell lysis can occur. The material lysed as above or prematurely with cyanide contains incomplete phage particles which have little or no nucleic acid but appreciable amounts of sulfur. Such observations suggest that “messenger” RNA production occurs to some extent while DNA synthesis has been blocked. The in vitro effects of proflavin are in accord with these observations. Proflavin concentrations (30 μM) which inhibit DNA synthesis 85 per cent inhibit RNA polymerase by only 30 per cent.

The observation that RNA synthesis can be inhibited in vivo with only partial inhibition of protein synthesis is of particular interest. This raises some questions concerning the nature of “messenger” RNA, which, according to Jacob and Monod, should possess a short half-life. A possible explanation is that not all “mes-
senger" RNA molecules are short-lived but that some may act catalytically for a long time after synthesis of new messenger is interrupted.

The finding that virtually all synthesis of RNA is blocked by actinomycin D suggests that the DNA-directed synthesis of RNA may be involved in the formation of ribosomal RNA and soluble-RNA. Gros and coworkers have shown that the components of "messenger RNA" ultimately appear in ribosomal RNA. The precise role of RNA produced by the RNA polymerase in the synthesis of the other RNA species remains to be elucidated.

Summary.—The inhibition of the enzymatic synthesis of DNA and RNA by actinomycin D and by proflavin has been reported. The inhibition is competitive in nature and can be reversed by increasing concentrations of DNA. It has been demonstrated that the DNA-dependent synthesis of RNA is more sensitive to this drug than DNA synthesis.

The inhibition of RNA polymerase by actinomycin D may depend on the type of DNA used to prime the reaction. The synthesis of RNA is sensitive to this inhibition when primers such as human marrow DNA, heated thymus DNA, φX 174 DNA, and M. lysodeikticus DNA are employed. Reaction mixtures primed with the synthetic oligonucleotide, polydeoxythymidylate, are not inhibited by actinomycin D.

By contrast, the DNA-dependent exchange reaction between inorganic pyrophosphate and the ribonucleoside triphosphates is only poorly inhibited, although the same enzyme appears to be involved. Concentrations of actinomycin D which completely inhibit the synthesis of RNA inhibit the exchange reaction only about 20 per cent.

The addition of actinomycin D to Bacillus subtilis results in the production of long "snake-like" cells. This appears to be related to unbalanced growth resulting from the preferential inhibition of RNA synthesis. The role of DNA-like RNA as an intermediate in protein and RNA synthesis has been discussed.

* These experiments were supported by grants from the National Institutes of Health and the Health Research Council of the City of New York.
† Senior Postdoctoral Fellow of the National Institutes of Health.
‡ Postdoctoral Fellow of the National Institutes of Health.
§ National Science Foundation Cooperative Fellow.
15 Chamberlin, M., and P. Berg, these PROCEEDINGS, 48, 81 (1962).
REACTIVATION AND HYBRIDIZATION OF REDUCED ALKALINE PHOSPHATASE*

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There is an abundance of evidence that the structural gene for a protein controls its primary amino acid sequence,1,2 and there is strong evidence that the folding of the polypeptide chain of ribonuclease is largely determined by the primary structure.3 However, for relatively large proteins, especially those composed of more than one polypeptide chain, it is not clear whether folding and polymerization are entirely determined by primary structure or require additional information available within the cell. It has been suggested, for example, that both α chains of hemoglobin are made on the same ribosome, are released as a dimer, and combine in the cytoplasm with a similarly made β-chain dimer to form the complete hemoglobin molecule.4

As part of a study of the synthesis and polymerization of the separate peptides of a protein molecule, experiments have been carried out on the dimerization and concurrent restoration of enzymatic activity of alkaline phosphatase inactivated by reduction of disulfide bridges. This enzyme,5 purified from Escherichia coli, has a molecular weight of 80,000 and in its native state appears to be a tightly folded globular molecule. It is resistant to proteolysis by trypsin and chymotrypsin and is stable at 85°C for at least 30 min in the presence of 10−3 M Mg++. Enzymatic activity persists even after incubation in 6 M urea, but a combined treatment with urea and thioglycollic acid leads to reduction of disulfide bonds and makes the resultant sulfhydryl groups on the molecule accessible to alkylation by iodoacetic

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8 Wood, W. B., and P. Berg, these PROCEEDINGS, 48, 94 (1962).
10 Tissières, A., D. Schlessinger, and F. Gros, these PROCEEDINGS, 46, 1450 (1960).
11 After this paper had been written, Goldberg and Rabinowicz20 reported that actinomycin D inhibits DNA-linked RNA synthesis in HeLa cells.