MOLECULAR PHARMACOLOGY, 9, 81-92

The Inhibition of Ribonucleic Acid Polymerase from *Escherichia coli* by 6-Chloro-8-aza-9-cyclopentylpurine

JOSEPH W. CRANSTON AND RAYMOND W. RUDDON[1]

Departments of Pharmacology and Oral Biology, The University of Michigan Medical and Dental Schools, Ann Arbor, Michigan 48104

(Received August 7, 1972)

SUMMARY

CRANSTON, JOSEPH W., AND RUDDON, RAYMOND W.: The inhibition of ribonucleic acid polymerase from *Escherichia coli* by 6-chloro-8-aza-9-cyclopentylpurine. *Mol. Pharmacol.* **9**, 81-92 (1973).

The inhibition of RNA synthesis *in vitro* by the purine nucleoside analogue 6-chloro-8-aza-9-cyclopentylpurine has been characterized. This compound was found to be a noncompetitive inhibitor of the incorporation of nucleoside triphosphates into RNA by binding to DNA-dependent RNA polymerase (ribonucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) isolated from *Escherichia coli*. The DNA-binding and initiation functions of the enzyme were blocked, but the compound did not affect RNA chain elongation. Studies of the interactions of 6-chloro-8-aza-9-cyclopentylpurine with amino acids, *N*-acetylamino acid analogues, and β-mercaptoethanol indicated that the drug reacts primarily with sulfhydryl groups of RNA polymerase. The inhibition could be partially reversed by dithiothreitol. The data suggest that 6-chloro-8-aza-9-cyclopentylpurine acts by inhibiting RNA polymerase function in a manner similar to the reversible sulfhydryl inhibitor *p*-chloromercuribenzoate.

INTRODUCTION

The purine nucleoside analogue 6-chloro-8-aza-9-cyclopentylpurine (Fig. 1) has been shown to inhibit cellular growth in two microorganisms, *Escherichia coli* and *Pseudomonas testosteroni* (1-3), and in an established line of Burkitt lymphoma cells grown in tissue culture.[2] This agent was found to inhibit DNA, RNA, and protein synthesis in *E. coli* by independent biochemical mechanisms (3, 4). Studies *in vitro* by Johnson *et al.* (4) showed 689[3] to inhibit RNA synthesis by attacking DNA-dependent RNA polymerase partially purified from *E. coli* B. Further studies revealed that significant inhibition of RNA polymerase was obtained only if all three alterations of the purine nucleoside structure, i.e., 6-halo-, 8-aza-, and 9-cyclopentyl groups, were present (5), and it was suggested that 689 inhibited the activity of enzymes involved in nucleic acid metabolism and synthesis by a site-directed alkylation (4, 5). In the present paper a detailed investigation of the effects of 689 on

This study was supported by Grant DE-02731 from the National Institutes of Health.

[1] To whom requests for reprints should be addressed: Department of Pharmacology, M6322 Medical Science I, Ann Arbor, Michigan 48104.

[2] L. M. Weisenthal and R. W. Ruddon, unpublished observations.

[3] The abbreviations used are: 689, 6-chloro-8-aza-9-cyclopentylpurine; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide.

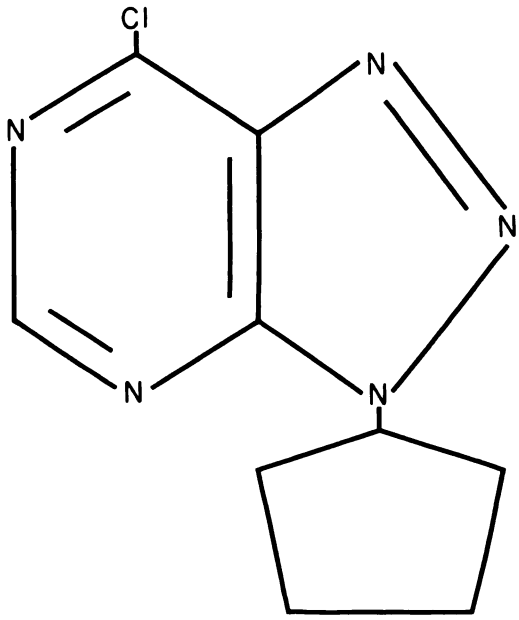

FIG. 1. *Structural formula of 6-chloro-8-aza-9-cyclopentylpurine*

RNA synthesis *in vitro* was undertaken to determine how the agent interacts with DNA-dependent RNA polymerase of *E. coli* B and what function of this enzyme is inhibited. The purine nucleoside analogue was found to bind tightly to RNA polymerase, primarily to enzyme sulfhydryl groups, and was observed to inhibit both the initiation and DNA-binding functions of the enzyme. Although its major inhibitory effect was upon the RNA polymerase core enzyme, 689 also inhibited the activity of σ factor to a lesser extent.

MATERIALS AND METHODS

Materials. Unlabeled ribonucleoside triphosphates were obtained from P-L Biochemicals. Uniformly labeled [^{14}C]adenosine 5′-triphosphate (515 μCi/μmole), generally labeled [^{3}H]adenosine 5′-triphosphate (9.6 mCi/μmole), adenosine [γ-^{32}P]5′-triphosphate (4.6 mCi/μmole), and [^{32}P]PP$_i$ as $Na_4{}^{32}P_2O_7$ (1.76 mCi/μmole) were purchased from New England Nuclear Corporation. Calf thymus DNA was obtained from Worthington Biochemical Corporation. Phage T4 DNA[4] was isolated according to the method described by Thomas and Abelson (6). T7 bacteriophages were grown and purified essentially as described by C. C. Richardson (7), and the DNA was isolated according to Thomas and Abelson (6). T7 [^{3}H]DNA was isolated from phage stocks grown in the presence of [^{3}H]thymine. L-Amino acids were obtained from Mann Research Laboratories. *N*-Acetyl-L-cysteine and *N*-acetyl-L-phenylalanine were products of Sigma Chemical Company. Dithiothreitol was obtained from Calbiochem. Norit was activated according to the procedure of Kamen (8). 689 was a gift from Dr. M. S. Zedeck of the Sloan-Kettering Institute for Cancer Research, New York. Additional 689 was synthesized in our laboratories, according to the method of Chang *et al.* (9), from the immediate precursor, 4-chloro-5-amino-6-cyclopentylaminopyrimidine, which was a gift from Dr. J. A. Montgomery of the Southern Research Institute, Birmingham, Ala.

RNA polymerase purification. RNA polymerase holoenzyme (enzyme containing the σ factor) was purified by either hydroxylapatite chromatography according to the method of Richardson (10) or glycerol gradient centrifugation according to the procedure of Burgess (11). These preparations contained minor contaminants, as evidenced on 0.1% SDS–5% polyacrylamide gels (12), and varied in specific activity from 250 to 450 units/mg of protein, with a unit of activity defined according to Burgess (11). The enzyme was routinely stored at −60° and was stable for at least 6 months.

RNA polymerase core enzyme (enzyme lacking the σ factor) was purified by phosphocellulose chromatography as described by Burgess (11). The core enzyme had a specific activity of 200 units/mg of protein and was also stable for at least 6 months when stored at −60°. Calf thymus DNA had a 10-fold greater template activity than T4 DNA with respect to core enzyme. This

[4] Initial stocks of T4 and T7 bacteriophages were kindly supplied by Drs. G. R. Greenberg and J. Hurwitz, respectively.

was in contrast to the holoenzyme, which was 1.5-fold more active with T4 DNA.

Assay of RNA polymerase. The basic RNA polymerase assay mixture *in vitro* contained, in addition to enzyme, 32.5 μmoles of Tris-HCl (pH 8.0), 3.3 μmoles of $MgCl_2$, 16.8 μmoles of KCl, 6.7 nmoles of dithiothreitol, 40 nmoles of EDTA, 10 μmoles of glycerol, 100 nmoles each of GTP, CTP, and UTP, 25 nmoles of [^{14}C]ATP (specific activity, 6×10^3 cpm/nmole), and either 30 μg of calf thymus DNA or 10 μg of T4 DNA in 0.4 ml. Reactions were routinely conducted for 10 min at 37° in a Dubnoff metabolic shaking incubator. Reactions were terminated by the addition of cold 5% trichloracetic acid; the labeled, acid-precipitable RNA was collected on nitrocellulose filters (Millipore, type HA, 0.45-μ pore size) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Under the reaction conditions employed enzyme concentration was limiting, and the rate of incorporation of labeled triphosphate was linear for at least 20 min. Salt concentrations which permitted optimal incorporation of nucleoside triphosphates into RNA were employed.

Preliminary incubation of 689. 689 had virtually no inhibitory effect on RNA polymerase activity if added directly to an assay mixture at zero time. Enzyme activity was inhibited when drug and enzyme were incubated together prior to the addition of DNA template and nucleoside triphosphates. The amount of inhibition obtained with a given concentration of 689 increased with increasing prior incubation time (4). Hence 689 was routinely incubated initially with RNA polymerase (10–15 μg) for 60 min at 37° in a 0.1-ml reaction mixture containing 5 μmoles of Tris-HCl (pH 8.0), 0.5 μmole of $MgCl_2$, 2.5 μmoles of KCl, 1 nmole of dithiothreitol, 5 nmoles of EDTA, 10 μmoles of glycerol, and 12.5% DMSO. DMSO was required to solubilize 689, and the drug was dissolved in this solvent and diluted to 25% DMSO with H_2O prior to each experiment. Control reactions (without drug) were conducted simultaneously and under the same experimental conditions as for 689-treated samples.

Purification and assay of σ factor. σ factor was isolated by phosphocellulose chromatography of the "high-salt" glycerol gradient holoenzyme according to the method of Burgess and Travers (13). The bulk of the σ factor activity was eluted from the column immediately following the initial protein peak. SDS–polyacrylamide gels (12) showed one major band of 90,000 daltons and a minor contaminant of lower molecular weight. The preparation lacked RNA polymerase activity. σ factor was assayed for stimulatory activity according to Burgess and Travers (13). σ factor (2 μg) stimulated core enzyme (10 μg) approximately 15-fold when 10 μg of T4 DNA were employed as template. To examine the effects of 689 on the components of RNA polymerase, either σ factor or core enzyme was first incubated with 689 for 60 min at 37° as described above. The other, unincubated protein component, i.e., core or σ factor, was then added to the incubation at zero time along with T4 DNA (10 μg) and nucleoside triphosphates. σ factor lost between 35% and 50% of its stimulatory activity during a 60-min preliminary incubation at 37° without drug. This was in accord with a previous report that the factor is somewhat labile under these conditions (13).

Assay of [γ-^{32}P]ATP incorporation. The amount of [γ-^{32}P]ATP incorporated into RNA chains is a measure of the initiation reaction (14). Assay conditions for measuring the incorporation of [γ-^{32}P]ATP (specific activity, 5.7×10^5 cpm/nmole) into RNA were the same as those described above for RNA polymerase; however, determination of acid-precipitable radioactivity was performed according to the method of Richardson (15).

Assay of PP_i exchange reaction. RNA polymerase catalyzes a DNA-dependent exchange reaction between [^{32}P]PP_i and nucleoside triphosphates. This exchange reaction can also be used to measure the initiation function (16, 17). The pyrophosphate exchange reaction was assayed by determining the amount of [^{32}P]PP_i incorporated into a form adsorbable by charcoal (Norit). Reaction conditions were the

same as described above, except that 0.4 μmole of [^{32}P]PP_i (specific activity, 2.5×10^6 cpm/μmole) was included in each incubation mixture. The reactions were terminated, and the Norit-adsorbable material was collected and counted as described by Krakow and Fronk (17).

Assay of RNA chain elongation rate. The size distribution of the RNA made in a 10-min RNA polymerase assay, which reflects the rate of elongation of RNA chains, was measured using a modification of the procedure of Richardson (18). Preliminary incubations and RNA polymerase assays were performed as described above. Control reactions were conducted with [^{14}C]ATP (specific activity, 3.0×10^4 cpm/nmole), and 689 reactions contained [^{3}H]ATP (specific activity, 9.6×10^4 cpm/nmole). Reactions were terminated by the addition of 0.1 ml of 0.4% SDS at 37°. The samples were then chilled to 0°, and the detergent was removed by centrifugation. Equal volumes of control and 689 reactions were mixed, and a 0.2-ml aliquot was layered on a 4.8-ml linear sucrose gradient (5–20%, w/v) containing 0.01 M Tris-HCl, pH 7.9, and 0.1 M KCl. The gradients were centrifuged for 4 hr at 36,500 rpm in a Spinco SW 50.1 rotor at 4°. The gradient tubes were punctured; 0.2-ml fractions were collected dropwise, precipitated with cold 5% trichloracetic acid, filtered, dried, and counted as described above.

Assay for binding of enzyme to DNA. Binding of RNA polymerase holoenzyme to DNA was measured using the nitrocellulose membrane method (19). A modification of the procedure used by Hinkle and Chamberlin was employed (20). RNA polymerase was diluted in "binding" buffer containing 0.01 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, 0.05 M KCl, 0.02 mM dithiothreitol, and 0.1 mM EDTA and initially incubated for 60 min at 37° with or without 689. The concentration of enzyme in each preliminary incubation was 100 μg/ml. Binding reactions contained 50 μg of BSA, 5 μg of T7 [^{3}H]DNA (approximately 11,000 cpm), and a limiting concentration of enzyme in 0.1 ml of binding buffer. Reactions were initiated by the addition of enzyme and incubated for 10 min at 37°. The reaction mixtures were then diluted to 1.0 ml with binding buffer and immediately filtered through nitrocellulose filters (Millipore, type HA, 0.45-μ pore size), which had been thoroughly soaked in binding buffer for at least 60 min. Very gentle suction was used in the filtration step. Filters were dried and counted as above. RNA polymerase assays were performed simultaneously with the binding reactions. The methods used in these assays differed from those previously described in that the reaction volume was 0.2 ml, 5 μg of unlabeled T7 DNA replaced either calf thymus or T4 DNA as template, the ATP concentration was doubled, and 50 μg of BSA were added to each reaction mixture.

Determination of binding between 689 and RNA polymerase. RNA polymerase holoenzyme (100 μg/ml) was subjected to a preliminary incubation in the presence or absence of 689 as previously described. Control and drug-treated enzymes were then dialyzed against 1000 volumes of a buffer containing 0.05 M Tris-HCl (pH 8.0), 0.005 M $MgCl_2$, 0.025 M KCl, 0.05 mM EDTA, and 12.5% DMSO for 13.5 hr at 4° with a single buffer change. The difference spectrum between 689-treated and control enzymes was then determined with a Beckman Acta III dual-beam spectrophotometer.

Determination of binding of 689 with L-amino acids, N-acetyl-L-amino acid analogues, and β-mercaptoethanol. Various L-amino acids, *N*-acetyl-L-amino acid analogues, or β-mercaptoethanol were dissolved at a concentration of 4 mM in buffer containing 0.1 M Tris-HCl (pH 8.0 or 7.2), 0.01 M $MgCl_2$, 0.05 M KCl, and 0.1 mM EDTA. Each was immediately mixed with an equimolar concentration of 689 and incubated for 1 hr at 37°. After 1 hr the samples incubated with or without 689 were diluted with buffer containing 0.05 M Tris-HCl (pH 8.0 or 7.2), 0.005 M $MgCl_2$, 0.025 M KCl, 0.05 mM EDTA, and 12.5% DMSO such that the concentrations of the amino acids, *N*-acetyl analogues, β-mercaptoethanol, and 689 were 50 μM. The difference spectra between amino acid–689 mixtures and the respective amino acid, *N*-acetyl analogue, or β-mercaptoethanol controls were determined with a Beckman DB-G dual-beam spectrophotometer and

compared with the ultraviolet spectrum of the 689 control.

RESULTS

Effect of 689 on RNA Synthesis in Vitro

Kinetic studies. The purine nucleoside analogue 689 was found to inhibit RNA synthesis *in vitro* in a dose-dependent manner when the compound was initially incubated with RNA polymerase holoenzyme for 60 min. Figure 2 represents the composite dose-inhibition curve for a number of holoenzyme preparations. A 50% inhibition occurred at about 0.16 mM 689, and greater than 95% inhibition was achieved at a concentration of 1 mM. Similar dose-inhibition curves were observed regardless of the DNA template employed. However, when DNA, rather than enzyme, was first incubated with drug, no inhibition of RNA synthesis was observed (4).

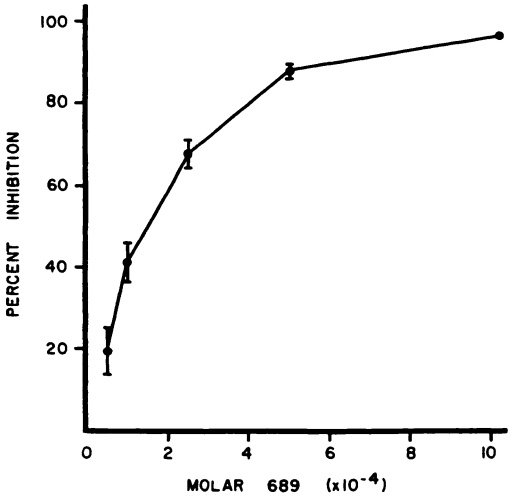

FIG. 2. *Inhibition of RNA synthesis by various concentrations of 689*

RNA polymerase holoenzyme (10–15 μg) was incubated with 689 for 60 min and then assayed as described under MATERIALS AND METHODS. Calf thymus DNA (30 μg) was employed as the template in these experiments, and control enzyme activity ranged between 2.5 and 4.5 nmoles of AMP incorporated in 10 min for various enzyme preparations. Results are presented as the mean percentage inhibition ± standard error as compared to controls where n (number of experiments for a given concentration of 689) exceeds 2. The value of n is between 4 and 8 for all points except 1 mM 689, where $n = 2$.

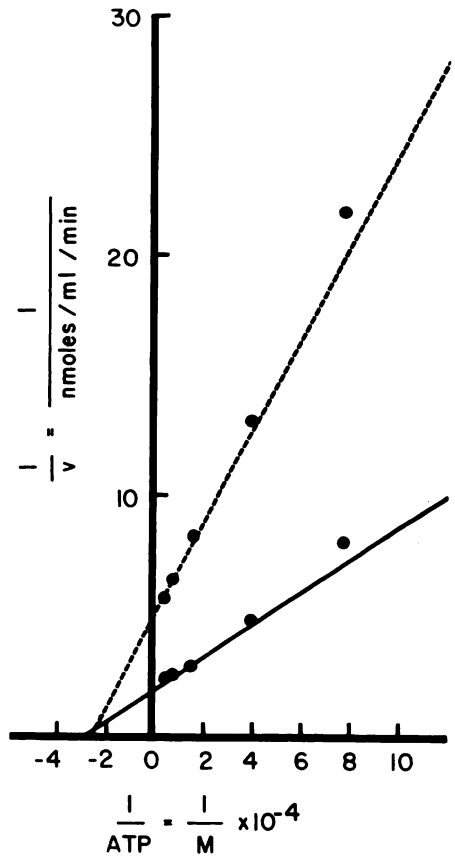

FIG. 3. *Double-reciprocal analysis of RNA polymerase activity at various ATP concentrations in the presence and absence of 689*

689 (0.1 mM) was incubated with RNA polymerase holoenzyme (15 μg), and assays were performed at ATP concentrations from 13 to 250 μM, according to the conditions outlined under MATERIALS AND METHODS. The data were analyzed by regression analysis according to the method of Wilkinson (21), and the difference in V_{max} between the control (●——●) and 689 (●- - -●) ATP saturation curves was found to be highly significant ($p < 0.0005$). In contrast, the difference in apparent K_m was insignificant ($p > 0.1$).

The apparent K_m value was determined for each nucleoside triphosphate with RNA polymerase holoenzyme: ATP, 42 μM; UTP, 27 μM; GTP, 20 μM; and CTP, 17 μM. 689 was found to be a noncompetitive inhibitor of the incorporation of each nucleoside triphosphate into RNA, in that the V_{max} was decreased in the presence of the drug while the apparent K_m remained unchanged.

Figure 3 shows a representative Lineweaver-Burk double-reciprocal plot for ATP incorporation in the presence of 0.1 mM 689. It should be pointed out that when more than one triphosphate was varied, the substrate saturation curves became sigmoidal. This may reflect a rate-limiting event during the initiation step in RNA synthesis, as originally proposed by Anthony *et al.* (22).

Effect of 689 on core RNA polymerase and σ factor. When RNA polymerase holoenzyme is subjected to phosphocellulose chromatography, the enzyme subunit σ factor is removed and the resulting core enzyme, although retaining the ability to synthesize RNA, lacks specificity for initiation and has very low efficiency for transcription of certain templates (e.g., T4 and T7 DNA) (23). Highly specific antibiotic inhibitors of the core RNA polymerase, most notably the rifamycins and streptolydigin, have been extensively investigated (24–26). However, an inhibitor possessing specificity for the σ factor has not yet been discovered. Therefore it was of interest to examine the effects of 689 on both the core enzyme and the σ factor. Core enzyme and σ factor were isolated and characterized as described under MATERIALS AND METHODS. When T4 DNA was employed as template, 689 inhibited both the core enzyme and the σ factor (Table 1). In addition, the effects of 689 on the core enzyme (without the addition of σ factor) were compared with the effects on the holoenzyme using calf thymus DNA as template. This comparison could be made when calf thymus DNA was employed as the template, since the core enzyme can efficiently transcribe this template in the absence of σ factor (23). The results showed that the holoenzyme was inhibited 15–20% more than the core enzyme for a given concentration of 689 (Table 1). Hence the data from these experiments indicate that 689 inhibits both the core enzyme and the σ factor, but has a much greater inhibitory effect on the core enzyme.

Effect of 689 on individual steps in RNA synthesis. The synthesis of RNA by DNA-dependent RNA polymerase is a complex process which can be separated into the following consecutive steps: (a) binding of the enzyme to sites on the DNA template, (b) initiation of RNA synthesis leading to the formation of the first phosphodiester bond, (c) elongation of RNA chains by the stepwise addition of ribonucleoside monophosphates to the 3′-hydroxyl end of growing RNA chains, and (d) termination of synthesis and release of RNA product. The effects of 689 on the DNA-binding, initiation, and elongation steps in RNA synthesis were investigated to see which RNA polymerase function was inhibited by this compound.

The effect of 689 on the initiation of RNA synthesis *in vitro* was determined by two procedures, [γ-^{32}P]ATP incorporation and pyrophosphate exchange, as described under

TABLE 1

Effect of 689 on core RNA polymerase and σ factor

RNA polymerase holoenzyme (10 μg), core enzyme (10 μg), or σ factor (2 μg) was incubated with 689 for 60 min and then assayed as described under MATERIALS AND METHODS. Core enzyme or σ factor was added to the assay at zero time where indicated. T4 DNA (10 μg) or calf thymus DNA (30 μg) was employed as template. Results are presented as the mean percentage inhibition ± standard error as compared to controls where n (number ot experiments enclosed in parentheses) exceeds 2, and as ± range where $n = 2$.

Component initially incubated	Component added at zero time	DNA template	Inhibition	
			0.25 mM 689	0.5 mM 689
			%	%
σ factor	Core enzyme	T4	31.2 ± 13.6 (2)	64.1 ± 6.0 (2)
Core enzyme	σ factor	T4	73.0.± 3.0 (2)	92.5 ± 1.2 (2)
Holoenzyme	None	T4	72.5 ± 4.0 (7)	89.6 ± 2.6 (6)
Core enzyme	None	Calf thymus	47.8 ± 3.9 (5)	73.3 ± 2.2 (6)
Holoenzyme	None	Calf thymus	67.7 ± 3.6 (7)	87.9 ± 1.9 (8)

TABLE 2

Effect of 689 on incorporation of [γ-^{32}P]ATP

RNA polymerase holoenzyme (10 μg) was incubated with 689 (0.25 mM) for 60 min and then assayed for [γ-^{32}P] incorporation (initiation) and [^{14}C]ATP incorporation (RNA synthesis) as described under MATERIALS AND METHODS. T_4 DNA (10 μg) was employed as the template. The numbers in parentheses represent percentage inhibition.

Reaction mixture	Incorporation	
	[γ-^{32}P]ATP	[^{14}C]ATP
	pmoles	
Control	2.7	2303
689-treated	0.9 (66.7)	674 (70.7)

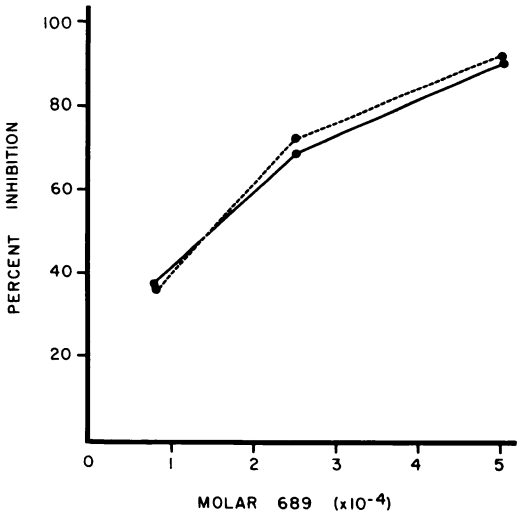

FIG. 4. *Effect of 689 on pyrophosphate exchange*
RNA polymerase holoenzyme (10 μg) was incubated with 689 for 60 min and then assayed for PP_i exchange (initiation) and [^{14}C]ATP incorporation (RNA synthesis) as described under MATERIALS AND METHODS. T4 DNA (10 μg) was employed as the template. ●——●, PP_i exchange; ●- - -●, [^{14}C]ATP incorporation.

MATERIALS AND METHODS. Using either method, it was observed that 689 inhibited the initiation reaction to the same extent as RNA synthesis (Table 2 and Fig. 4).

In contrast to its effect on the initiation reaction, 689 was shown not to inhibit the rate of elongation of RNA chains. When the size distributions of RNA chains made in the presence and absence of 689 (0.1 mM) were compared, there was no apparent difference (Fig. 5). Hence it was concluded that this compound inhibited the number of RNA chains initiated but had no effect on the length of successfully initiated chains. The heterogeneity of the RNA made *in vitro* by DNA-dependent RNA polymerase (Fig. 5) has been reported by other laboratories (18, 27, 28) and appears to be due to the absence of the transcription factor, ρ, which presumably provides specificity for correct chain termination *in vivo* (28).

The effect of 689 on the binding reaction between template DNA and RNA polymerase was determined by a modification of the nitrocellulose filter assay technique (19, 20). This technique is based on the fact that RNA polymerase binds to nitrocellulose filters but certain DNAs do not. However, if the DNA has complexed to the enzyme, it will be retained by the filter. Thus, if the DNA contains a known amount of radioactive label, the amount of DNA bound to RNA polymerase can be determined. Figure 6 shows the 689 dose-inhibition curves for the binding of RNA polymerase holoenzyme to T7 [^{3}H]DNA and for RNA synthesis using unlabeled T7 DNA as the template. Inhibition of the binding reaction closely approximated the corresponding inhibition of RNA synthesis for the concentrations of 689 employed. Hence it appears that a significant part, if not all, of the effects of 689 on RNA polymerase activity can be explained by inhibition of the binding reaction between enzyme and template DNA.

Binding of 689 to RNA polymerase. 689, because of its purine nucleoside-like structure, has an ultraviolet absorption spectrum with a single absorption peak at 264–265 nm (pH 8.0) and a millimolar extinction coefficient of 9.5 (see Fig. 8). This property of the compound was used to detect 689 bound to RNA polymerase. RNA polymerase holoenzyme was incubated in the presence or absence of 250 μM 689 and dialyzed to remove free 689 as described under MATERIALS AND METHODS. It has been shown that 689 inhibition of RNA polymerase cannot be reversed by dialysis (4). The ultraviolet difference spectrum (between 689-treated and control RNA polymerase) that was obtained repre-

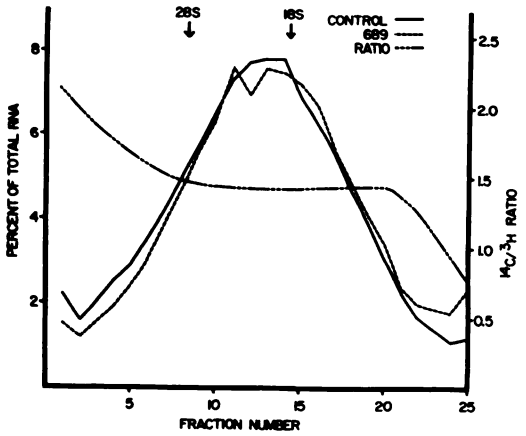

FIG. 5. *Effect of 689 on rate of RNA chain elongation*

RNA polymerase holoenzyme (15 μg) was incubated with 689 (0.1 mM) for 60 min, assays were performed, and the RNA product was collected as described under MATERIALS AND METHODS. Calf thymus DNA (30 μg) was employed as the template. 689 inhibited total RNA synthesis by 63.8% in this experiment. The RNA in each fraction is expressed here as a percentage of the total RNA on the gradient. In addition, the ratio of [^{14}C]RNA (control) to [^{3}H]RNA (689) in each fraction was determined and was essentially the same over most of the gradient. [^{3}H]RNA from rat liver cytoplasm was used as a marker in these experiments, and the locations of the 18 S and 28 S peaks are indicated.

sented the ultraviolet absorption of 689 bound to RNA polymerase. The difference spectrum showed a single absorption peak at 292–295 nm (Fig. 7) and confirmed the belief that 689 does bind to RNA polymerase at concentrations which inhibit transcription *in vitro*.

Interaction of 689 with L-Amino Acids, N-Acetyl-L-amino Acid Analogues, and β-Mercaptoethanol

Preferential binding to sulfhydryl groups. The difference spectrum observed in Fig. 7 does not indicate the nature of the binding between 689 and RNA polymerase. In order to determine the potential binding sites of the drug, a representative group of α-amino acids, including L-alanine, L-valine, L-cysteine, L-cystine, L-phenylalanine, L-tyrosine, L-tryptophan, L-aspartic acid, L-serine, L-threonine, L-histidine, L-lysine, and L-arginine, were allowed to interact with equimolar 689 at pH 8.0, and the difference spectra between treated and untreated amino acids were compared with the ultraviolet spectrum of 689 as outlined under MATERIALS AND METHODS. A partial summary of the results can be observed in Fig. 8A–C. L-Cysteine produced a complete shift in the 689 ultraviolet spectrum, with a single absorption maximum at 290 nm and a millimolar extinction coefficient of 13.8 (Fig. 8A). Most α-amino acids, including L-phenylalanine, L-valine, L-cystine, L-tyrosine, L-tryptophan, L-serine, L-threonine, L-histidine, L-lysine, and L-arginine, produced a subtle broadening of the 689 spectrum at pH 8.0, but the peak absorption remained at 264–265 nm. The ultraviolet spectral shift produced by

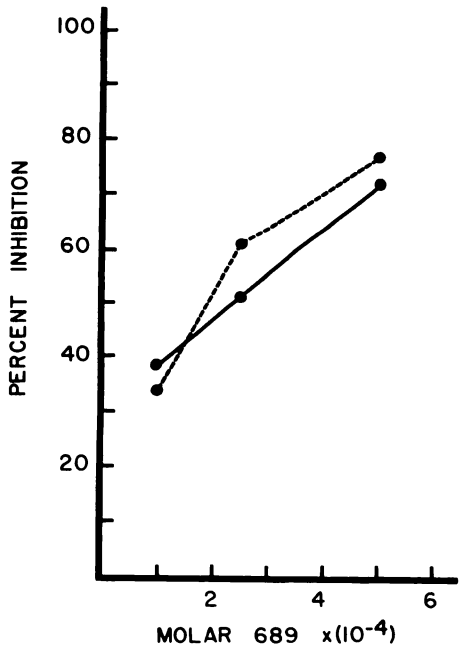

FIG. 6. *Effect of 689 on binding reaction between T7 [^{3}H]DNA and RNA polymerase*

RNA polymerase holoenzyme (100 μg/ml) was incubated with 689 for 60 min and then assayed for T7 [^{3}H]DNA binding to RNA polymerase (template binding) and [^{14}C]ATP incorporation (RNA synthesis) as described under MATERIALS AND METHODS. The concentration of RNA polymerase in the binding assay was 1.5 μg/ml, and in the assay for RNA synthesis, 10 μg/ml. ●——●, T7 [^{3}H]DNA binding; ●- - -●, [^{14}C]ATP incorporation.

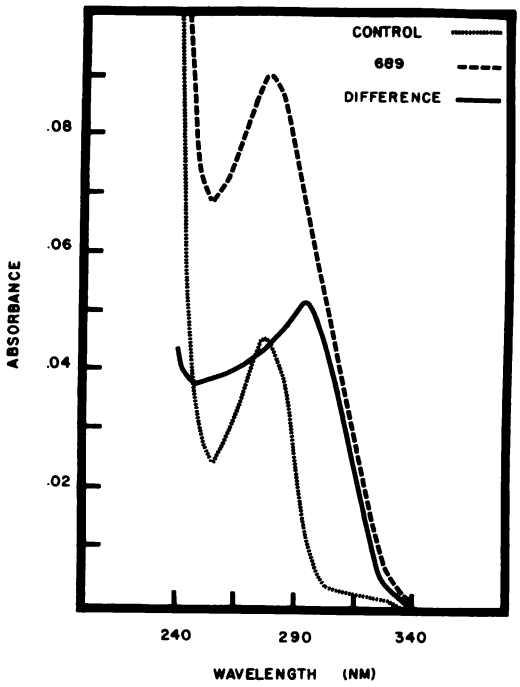

FIG. 7. *Binding of 689 to RNA polymerase*

RNA polymerase holoenzyme was incubated in the presence and absence of 689 (0.25 mM) and dialyzed. Ultraviolet spectrophotometry was performed as described under MATERIALS AND METHODS. RNA synthesis was inhibited by 70.3% prior to dialysis, and by 78.6% after dialysis under these conditions. Wavelength scans for the ultraviolet spectrum of untreated RNA polymerase (control), 689-treated RNA polymerase (689), and the difference spectrum between treated and untreated RNA polymerase (difference) are shown.

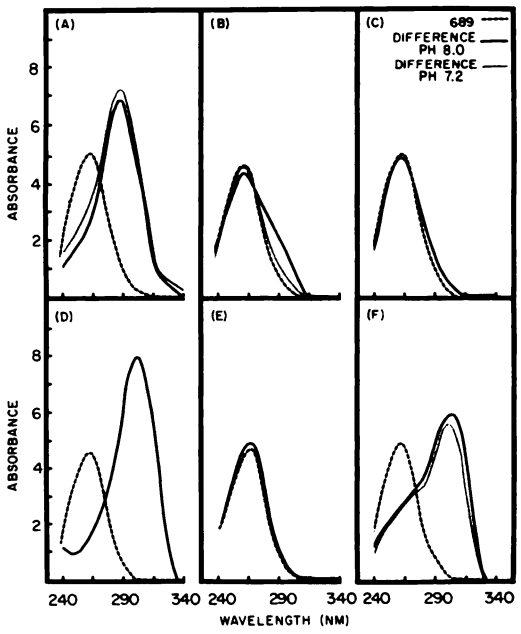

FIG. 8. *Binding of 689 to L-amino acids, N-acetyl-L-amino acid analogues, and β-mercaptoethanol*

L-Cysteine (A), L-phenylalanine (B), L-alanine (C), *N*-acetyl-L-cysteine (D), *N*-acetyl-L-phenylalanine (E), and β-mercaptoethanol (F) were incubated with equimolar 689 for 60 min, and ultraviolet spectrophotometry was performed as described under MATERIALS AND METHODS. Wavelength scans representing the ultraviolet spectrum of 689 and difference spectra between treated and untreated reactions at pH 8.0 or pH 7.2 are shown.

L-phenylalanine, an amino acid producing one of the more pronounced shifts, is shown in Fig. 8B. L-Alanine (Fig. 8C) and L-aspartic acid had essentially no effect on the 689 ultraviolet spectrum at pH 8.0. This observation confirmed that L-cysteine was the most reactive amino acid with 689 and suggested that the sulfhydryl group was the reactive moiety. It was believed that the diverse group of amino acids which weakly interacted with 689 at pH 8.0, e.g., phenylalanine, were reacting primarily by their α-amino group.

To confirm these hypotheses, two experiments were performed. First, a series of α-amino acids, including L-cysteine, L-phenylalanine, L-valine, L-tyrosine, L-tryptophan, L-serine, and L-lysine, were allowed to interact with 689 under the conditions described previously, but at pH 7.2. At this lower pH more of the α-amino group of each amino acid would be in the ionized (NH_3^+), presumably less reactive state (29). Hence, at pH 7.2 only L-cysteine was expected to cause a significant shift of the 689 ultraviolet spectrum. This was confirmed, and the results for L-cysteine and L-phenylalanine can be observed in Fig. 8A and B. To confirm directly that the sulfhydryl group of L-cysteine was reacting with 689, the *N*-acetyl analogue of L-cysteine was incubated with 689 at pH 8.0 as described previously. *N*-Acetyl-L-cysteine caused a complete shift in the 689 ultraviolet spectrum, with a single absorption peak at 300–302 nm (Fig. 8D),

verifying 689 interaction with the sulfhydryl group. *N*-Acetyl analogues of other amino acids, including *N*-acetyl-L-phenylalanine (Fig. 8E), produced no change in the 689 ultraviolet spectrum. Finally, the sulfhydryl reagent β-mercaptoethanol was shown to produce a marked shift in the 689 ultraviolet spectrum at both pH 8.0 and 7.2 (Fig. 8F). The conclusion that can be drawn from these data is that 689 preferentially and actively interacts with the sulfhydryl group of L-cysteine over other amino acid functional groups. These data have also been confirmed by thin-layer chromatography. A complex between [^{35}S]cysteine and 689 has been observed using this method.[5]

Correlation of the above data on individual amino acids, *N*-acetyl amino acid analogues, and β-mercaptoethanol with the ultraviolet difference spectrum obtained between 689-treated and untreated RNA polymerase (Fig. 7) indicates that 689 binds to RNA polymerase primarily, if not entirely, through the sulfhydryl groups of cysteinyl moieties. Furthermore, we have observed that dialysis of 689-treated RNA polymerase in the presence of excess dithiothreitol leads to a marked reversal of the 689 inhibition. When the dialysis was performed in the absence of dithiothreitol, 689 (0.25 mM) inhibited RNA polymerase (10 μg) activity by 67.5% when assayed prior to dialysis and by 72.5% when assayed after dialysis. The addition of dithiothreitol (10 mM) to the dialysis buffer decreased the inhibition to 22.0% after dialysis.

DISCUSSION

The purine nucleoside analogue 6-chloro-8-aza-9-cyclopentylpurine has been shown to inhibit RNA synthesis *in vitro* by attacking the DNA-dependent RNA polymerase of *E. coli*. A rather complex series of events occurs during the transcription reaction catalyzed by RNA polymerase. The effects of 689 on these various functions were determined, and the compound was found to inhibit the DNA-binding and initiation functions of the enzyme but to have no effect on the rate of elongation of successfully initiated chains. 689 inhibited the incorporation of each nucleoside triphosphate into RNA in a noncompetitive manner. This observation, in conjunction with the data showing that 689 inhibited enzyme-template binding, suggested that the compound does not compete for triphosphate-binding sites. However, we have not investigated the effect on substrate binding directly.

Structurally, RNA polymerase is a complex molecule consisting of a number of subunits. The complete enzyme (holoenzyme) has a molecular weight of approximately 490,000 and the subunit composition $\beta'\beta\alpha_2\sigma$. Core enzyme has a molecular weight of approximately 400,000 and has the subunit composition $\beta'\beta\alpha_2$ (30, 31). Unlike the antibiotic inhibitors rifampicin and streptolydigin, which specifically inhibit the β subunit of the core enzyme (32), 689 inhibits both the core enzyme and σ factor, the latter to a much lesser extent. Ultraviolet spectral evidence obtained with individual amino acids, *N*-acetylamino acid analogues, and the sulfhydryl reagent β-mercaptoethanol showed 689 to be highly reactive with sulfhydryl groups. The ultraviolet difference spectrum obtained between drug-treated and untreated RNA polymerase (Fig. 7) correlated well with the 689 spectral shift produced by free cysteine (Fig. 8A). Therefore it appears that 689 binds to RNA polymerase primarily through enzyme sulfhydryl groups.

Ishihama and Hurwitz (16) have investigated the effects of various chemical treatments upon the different functions of RNA polymerase. They observed that agents which interacted with sulfhydryl groups, including *p*-chloromercuribenzoate, $HgCl_2$, cyanuric fluoride, iodoacetate, *N*-ethylmaleimide, and tetrathionate, led to the inactivation of DNA-binding and initiation functions of the enzyme. Hence the evidence obtained in this study suggests that 689 inhibits RNA polymerase by attacking enzyme sulfhydryl groups in a manner similar to these known sulfhydryl group inhibitors. Other mechanisms of inhibition, such as attack on other amino acid functional groups essential to RNA polymerase function, may be involved in 689 inhibition, but these would appear to be of less significance.

[5] J. W. Cranston and R. W. Ruddon, unpublished observations.

Presumably the inhibitory effects of 689 result from the interaction of the drug with numerous sulfhydryl groups on the enzyme. Various indirect evidence leads us to this conclusion. First, despite the fact that a precise titration of the enzyme sulfhydryl groups with 689 was not performed, the observed difference spectrum between treated and untreated RNA polymerase (Fig. 7) suggests that a substantial number of sulfhydryl groups are bound. An estimation based on the extinction coefficient of the cysteinyl-689 complex indicates that approximately 18 enzyme sulfhydryl groups are bound. Second, the compound was shown to have a high degree of reactivity with free L-cysteine, *N*-acetyl-L-cysteine, and β-mercaptoethanol. It apparently can bind to both the core enzyme and the σ factor of RNA polymerase. Also, we have recently obtained evidence that 689 will bind to the sulfhydryl groups of bovine serum albumin.[5] Hence 689 appears to have the capacity to bind very readily to available sulfhydryl groups. Finally, Sümegi *et al.* (33), investigating the inhibition of purified core RNA polymerase of *E. coli* K-12 by the weakly electrophilic agent cystamine (which specifically interacts with highly reactive sulfhydryl groups), observed that no inhibition of RNA synthesis occurred until four sulfhydryl groups were bound and that inhibition increased as the number of bound sulfhydryl groups also increased. Complete inhibition was obtained when 12 sulfhydryl groups were bound. This latter evidence indicates that a number of sulfhydryl groups of RNA polymerase are involved in the function of the enzyme.

Ishihama and Hurwitz (16) reported the loss of a 2 S subunit from RNA polymerase after treatment with the reversible sulfhydryl inhibitor *p*-chloromercuribenzoate, and recently Ishihama (34) has identified this subunit as the α subunit of the enzyme. Hence treatment of RNA polymerase with sulfhydryl inhibitors appears to disrupt the quaternary structure of the enzyme and suggests that sulfhydryl groups play a role in holding the enzyme subunits together in a native configuration. It is interesting that the prior incubation of DNA template with RNA polymerase partially protected the enzyme from inhibition by cystamine (33) and 689 (35). These data suggest that binding of the enzyme to DNA induces conformational changes in the enzyme which reduce the reactivity of its sulfhydryl groups and prevent the dissociation of subunits. Evidence for a DNA-induced conformational change in RNA polymerase has also been indicated from studies with rifampicin, in which the formation of a binary complex between DNA and enzyme has been shown to render the enzyme partially resistant to this inhibitor (36, 37).

The characteristics of the inhibitory effects of 689 and the fact that dithiothreitol can partially reverse these effects suggest that 689 acts like *p*-chloromercuribenzoate in its interaction with RNA polymerase and not like irreversible sulfhydryl inhibitors such as iodoacetate. However, the precise nature of the 689 interaction with sulfhydryl groups has not yet been elucidated. Johnson *et al.* (4) suggested that 689 was directed to the active sites of certain sensitive enzymes involved in nucleic acid metabolism because of its purine nucleoside-like structure. However, since 689 will bind sulfhydryl groups of bovine serum albumin as well as RNA polymerase, it appears that the compound can bind to a number of proteins with available sulfhydryls. Whether enzyme activity is altered would then depend on the role of the bound sulfhydryl groups in the function of the enzyme. Previous structure-activity relationship studies (5) would seem to indicate that the unique purine nucleoside-like structure of 689 may play a role in directing the drug to sulfhydryl groups which are crucially involved in maintenance of enzyme function, but this remains to be determined.

ACKNOWLEDGMENT

The authors wish to thank Mrs. Donna Lundeen for her help in the preparation of the manuscript.

REFERENCES

1. M. S. Zedeck, A. C. Sartorelli, P. K. Chang, K. Raška, Jr., R. K. Robins, and A. D. Welch, *Mol. Pharmacol.* **3**, 386–395 (1967).
2. M. S. Zedeck, A. C. Sartorelli, J. M. Johnson, and R. W. Ruddon, *Bacteriol. Proc.* Abstr. G78, 32 (1968).

3. M. S. Zedeck, A. C. Sartorelli, J. M. Johnson, and R. W. Ruddon, *Mol. Pharmacol.* **5**, 263–270 (1969).
4. J. M. Johnson, R. W. Ruddon, M. S. Zedeck, and A. C. Sartorelli, *Mol. Pharmacol.* **5**, 271–285 (1969).
5. R. W. Ruddon, C. H. Rainey, and M. S. Zedeck, *FEBS Lett.* **7**, 119–124 (1970).
6. C. A. Thomas and J. Abelson, *in* "Procedures in Nucleic Acid Research" (G. L. Cantoni and D. R. Davies, eds.), pp. 553–561. Harper and Row, New York, 1966.
7. C. C. Richardson, *J. Mol. Biol.* **15**, 49–61 (1966).
8. M. D. Kamen, *Methods Enzymol.* **6**, 313–318 (1963).
9. P. K. Chang, L. J. Sciarini, A. C. Sartorelli, and M. S. Zedeck, *J. Med. Chem.* **11**, 513–515 (1968).
10. J. P. Richardson, *Proc. Nat. Acad. Sci. U. S. A.* **55**, 1616–1623 (1966).
11. R. R. Burgess, *J. Biol. Chem.* **244**, 6160–6167 (1969).
12. A. L. Shaprio, E. Vinuela, and J. Maizel, *Biochem. Biophys. Res. Commun.* **28**, 815-820 (1967).
13. R. R. Burgess and A. A. Travers, *Methods Enzymol.* **21**, 500–506 (1971).
14. U. Maitra and J. Hurwitz, *Proc. Nat. Acad. Sci. U. S. A.* **54**, 815–822 (1965).
15. J. Richardson, *Nature* **225**, 1109–1112 (1970).
16. A. Ishihama and J. Hurwitz, *J. Biol. Chem.* **244**, 6680-6689 (1969).
17. J. S. Krakow and E. Fronk, *J. Biol. Chem.* **244**, 5988–5993 (1969).
18. J. P. Richardson, *J. Mol. Biol.* **21**, 115–127 (1966).
19. O. W. Jones and P. Berg, *J. Mol. Biol.* **22**, 199–209 (1966).
20. D. C. Hinkle and M. Chamberlin, *Cold Spring Harbor Symp. Quant. Biol.* **35**, 65–72 (1970).
21. G. N. Wilkinson, *Biochem. J.* **80**, 324–332 (1961).
22. D. D. Anthony, E. Zeszotek, and D. A. Goldthwait, *Proc. Nat. Acad. Sci. U. S. A.* **56**, 1026–1033 (1966).
23. R. R. Burgess, A. A. Travers, J. J. Dunn, and E. K. F. Bautz, *Nature* **221**, 43–46 (1969).
24. G. Hartmann, K. O. Honikel, F. Knüsel, and J. Nüesch, *Biochim. Biophys. Acta* **145**, 843–844 (1967).
25. C. Siddhikol, J. W. Erbstoeszer, and B. Weisblum, *J. Bacteriol.* **99**, 151–155 (1969).
26. W. Wehrli and M. Staehelin, *Bacteriol. Rev.* **35**, 290–309 (1971).
27. H. Bremer and M. W. Konrad, *Proc. Nat. Acad. Sci. U. S. A.* **51**, 801–808 (1964).
28. J. Roberts, *Nature* **224**, 1168–1174 (1969).
29. W. C. J. Ross, "Biological Alkylating Agents," pp. 33–39. Butterworths, London, 1962.
30. R. R. Burgess, *J. Biol. Chem.* **244**, 6168–6176 (1969).
31. D. Berg and M. Chamberlin, *Biochemistry* **9**, 5055–5064 (1970).
32. A. Heil and W. Zillig, *FEBS Lett.* **11**, 165–168 (1970).
33. J. Sümegi, T. Sanner, and A. Pihl, *FEBS Lett.* **16**, 125–127 (1971).
34. A. Ishihama, *Biochemistry* **11**, 1250–1258 (1972).
35. J. W. Cranston and R. W. Ruddon, *Pharmacologist* **12**, 537 (1970).
36. A. E. Sippel and G. R. Hartmann, *Eur. J. Biochem.* **16**, 152–157 (1970).
37. E. K. F. Bautz and F. A. Bautz, *Nature* **226**, 1219–1222 (1970).