Interaction of Ribonucleic Acid Polymerase from *Escherichia coli* with Deoxyribonucleic Acid. Inhibition of Ribonucleic Acid Synthesis by Interaction of Luteoskyrin with the Transcription Complex†

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ABSTRACT: Luteoskyrin is a potent inhibitor of RNA synthesis by *Escherichia coli* RNA polymerase *in vitro*. In view of the known preferential binding of the drug to single-stranded nucleic acids, experiments were carried out to understand the inhibition of RNA synthesis using a native template, T7 DNA. Evidence that luteoskyrin exerts its effect by interacting with the transcription complex is the following. Added at the start of transcription, luteoskyrin reduces the number of chains initiated while their growth rate remains unaffected. Addition of the inhibitor during the course of transcription blocks chain elongation but does not bring about the dissociation of the transcription complex. Inhibition is independent of the kind of native template used and of its concentration. However, it is overcome by increasing the number of RNA polymerase molecules engaged in transcription. This behavior is quite unlike that of other inhibitors which act either on the template or on the enzyme alone. On the basis of our results it is suggested that luteoskyrin acts by first binding loosely to the DNA-bound enzyme followed, after initiation of RNA synthesis, by irreversible binding of the drug to exposed single-stranded segments of DNA immediately in front of the enzyme, thus preventing chain elongation.

The yellow pigment, luteoskyrin, initially isolated from rice which was contaminated by the mold *Penicillium islandicum*, has been the subject of extensive chemical and biological investigations (Uraguchi *et al.*, 1961; Shibata *et al.*, 1968; Kobayashi *et al.*, 1970).

The interest in luteoskyrin derives from its cytotoxicity for animal cells (Umeda, 1964). Luteoskyrin induces chromosome aberrations in Ehrlich ascites tumor cells (Schachtschabel *et al.*, 1969) and chromosome breakage in human leukocytes in culture (Keutel and Möckel, 1969). In addition, luteoskyrin inhibits post-ultraviolet (uv) irradiation growth of *Tetrahymena pyriformis*, suggesting that the pigment interferes with DNA repair (Mouton and Fromageot, 1971). Finally, in agreement with the above findings, luteoskyrin has been reported to be oncogenic. After long term ingestion of the pigment, laboratory animals develop hepatomas (Uraguchi, 1962).

The ability of luteoskyrin to cause tumors and chromosomal aberrations led to the investigation of interactions of the pigment with nucleic acids. Luteoskyrin was found to bind to DNA (Ueno *et al.*, 1967). The binding has an absolute requirement for magnesium ions. Two types of complexes can be observed. With single-stranded DNA luteoskyrin rapidly complexes with purine residues in a pigment:purine:magnesium ratio of 1:1:1 (complex I) (Ohba and Fromageot, 1967). With double-stranded nucleic acids, luteoskyrin forms complex II, in which large dye polymers are bound to only a few places on the DNA. This complex is formed slowly and shows no specificity with respect to base composition. In this case the phosphate groups of the backbone are thought to be the relevant functional groups, whose proper spacing allows dye–dye interaction (Ohba and Fromageot, 1967, 1968) and association of large dye polymers with the nucleic acid.

As expected, luteoskyrin interferes with the template properties of single-stranded DNA by formation of complex I, as shown by inhibition of RNA and DNA polymerase *in vitro* (Sentenac *et al.*, 1967; Mouton and Fromageot, 1971). Despite the preferential binding of the drug to unpaired templates, luteoskyrin is also a potent inhibitor of RNA synthesis when double-stranded DNA is used as a template (Sentenac *et al.*, 1967). This surprising observation suggested that luteoskyrin may not inhibit transcription by mere binding to the native template.

The present work was undertaken to explore the mechanism by which luteoskyrin inhibits transcription of native DNA. The results presented show that luteoskyrin inhibits DNA-bound RNA polymerase and blocks RNA chain initiation and elongation when a native template is transcribed. Inhibition is probably due to the binding of the dye to the transcription complex, jointly to the core enzyme, and to unpaired DNA in front of the transcribing unit.

Materials and Methods

RNA Polymerase. The most purified fraction of RNA polymerase from *E. coli* (hydroxylapatite fraction) was used in these studies. The enzyme was separated into core enzyme and σ components and assayed as previously described (Darlix *et al.*, 1969).

Nucleic Acids and Nucleotides. T7 DNA was prepared by phenol extraction of T7 bacteriophages as described elsewhere (Dausse *et al.*, 1972). Poly[d(A–T)] was obtained from Biopolymers, Inc. Calf thymus DNA was obtained from Sigma. [γ-^{32}P]ATP, [γ-^{32}P]GTP (2000 cpm per pmol), and [^{3}H]UTP were obtained from C.E.N. (Saclay). Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals.

Luteoskyrin was prepared by J. C. Bouhet and P. Pham Van in our laboratory according to a method derived from the pro-

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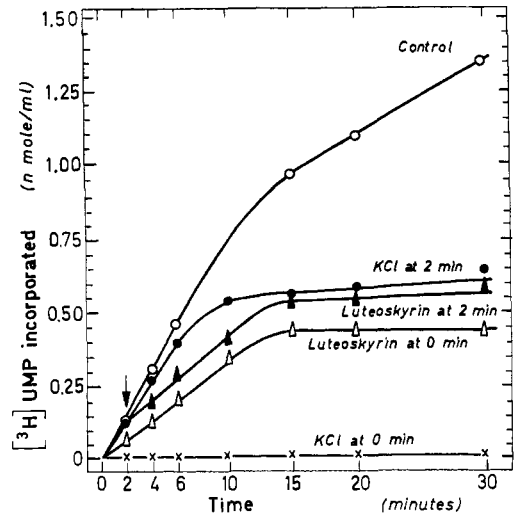

FIGURE 1: Inhibition of T7 DNA transcription by luteoskyrin. RNA synthesis was carried out in 1-ml incubation mixtures containing standard buffer (0.04 M Tris-HCl (pH 7.9)–0.01 M $MgCl_2$–0.1 mM EDTA–6 mM 2-mercaptoethanol–0.15 M KCl), T7 DNA (10 μg/ml), RNA polymerase containing σ (8.1 μg/ml), and the four nucleoside triphosphates (0.1 mM each) with [^{3}H]UTP (6000 cpm $nmol^{-1}$). Reaction was started by addition of the nucleotides, 150-μl aliquots were taken at different times, and synthesized RNA was recovered by acid precipitation. Luteoskyrin (6.7 μM) was added before addition of the nucleotides (△) or after 2 min of incubation (▲). For the purpose of comparison KCl (0.4 M) was added in separate incubation mixtures at 0 min (×) or after 2 min of incubation (●).

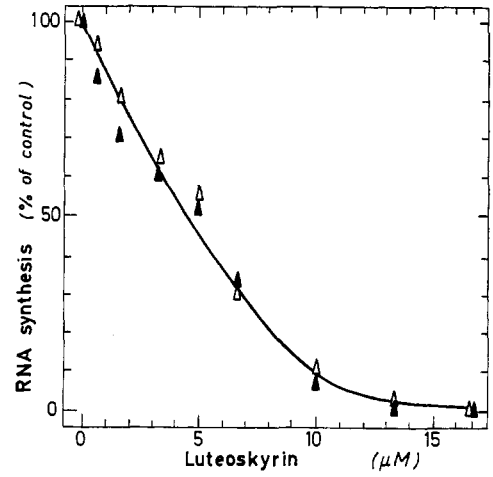

FIGURE 2: Inhibition of RNA synthesis by various concentrations of luteoskyrin added before or after the start of transcription. Incubation mixtures are described in Figure 1 except that the total volume was 0.3 ml. Various concentrations of luteoskyrin were added before addition of the nucleotides and RNA synthesis was measured after 20 min of incubation at 37° (△). In separate mixtures luteoskyrin was added after 2 min of incubation (▲). In this case control synthesis refers to the RNA made between 2 and 20 min of incubation. Control [^{3}H]UMP incorporation at 2 min, 0.18 nmol; at 20 min, 2.4 nmol.

cedure of Platel *et al.* (1968). The purity of crystalline luteoskyrin was checked by thin layer chromatography and by infrared, visible, and nuclear magnetic resonance (nmr) spectroscopy. Stock solutions were prepared by dissolving the dye in a minimal amount of 0.1 N NaOH and stored at 4° in 1 mM potassium phosphate buffer at pH 7.7. Luteoskyrin concentration was estimated spectrophotometrically using a molar absorption coefficient at 451 nm of 21,500.

Results

Effect of Luteoskyrin on the Kinetics of RNA Synthesis. The kinetics of RNA synthesis in the presence of luteoskyrin are shown in Figure 1. The drug was added either at zero time or 2 min after the start of the reaction, in order to allow most chain initiation to be completed. The effect on RNA synthesis of 0.4 M KCl, a concentration which prevents binding of RNA polymerase to the template but not the growth of previously initiated chains (Richardson, 1966), is shown in Figure 1 for the purpose of comparison. It is apparent that the extent of inhibition by luteoskyrin is independent of the time of drug addition. This is shown more clearly in the experiments summarized in Figure 2, carried out at different luteoskyrin concentrations. No significant difference in inhibition at any concentration is found whether the drug was added at 0 or 2 min. The results indicate that, unlike proflavine and high salt (Richardson, 1966; Sentenac and Fromageot, 1970), Congo Red (Krakow, 1965), or rifampicin (Sippel and Hartmann, 1968), which do not inhibit the growth of initiated chains, luteoskyrin blocks the transcription complex.

Effect of Luteoskyrin at the Onset of Transcription. When luteoskyrin is added at the start of transcription the inhibition of RNA synthesis can be largely accounted for by a decrease in the number of chains initiated. Figure 3 shows that initiations by ATP and by GTP are equally inhibited. The mean chain length of the remaining growing RNA chains is not affected (around 3000 nucleotides). The drug does not affect to an equal extent the entire population of enzyme molecules since one observes a reduction in the number of chains of RNA without a corresponding reduction in the mean chain length. Thus, an all or none effect on the transcription complex is displayed by the drug.

The following experimental result further shows that luteoskyrin does not affect the RNA chain growth rate. After a short synthesis, ^{32}P-labeled RNA made in the presence of luteoskyrin was cochromatographed on methylated albumin Kieselguhr column with control ^{3}H-labeled RNA. Figure 4 shows the chromatographic profiles of the ^{3}H- and ^{32}P-labeled RNA. In both cases RNA chains are eluted in a single

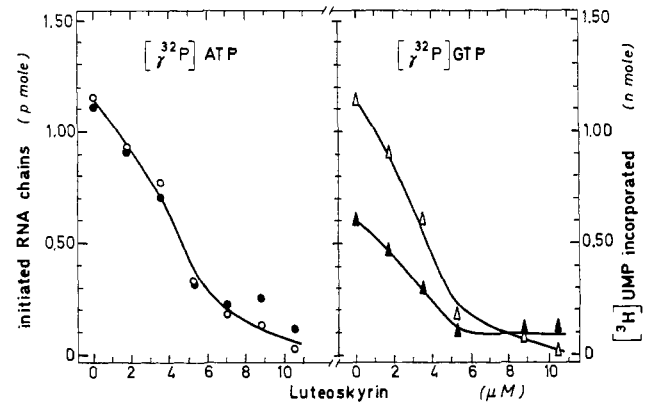

FIGURE 3: Effect of luteoskyrin on chain initiation by ATP and GTP. Incubation mixtures (0.3 ml) described in Figure 1 contained RNA polymerase (4.6 μg/ml) and [γ-^{32}P]ATP or [γ-^{32}P]GTP together with [^{3}H]UTP. Luteoskyrin was added before the nucleotides, and after 20 min of incubation at 37° RNA was recovered by acid precipitation and Millipore filtration (Sentenac *et al.*, 1968b). (○ and △) [^{3}H]UMP incorporation; (●) [γ-^{32}P] ATP incorporation; (▲) [γ-^{32}P]GTP incorporation.

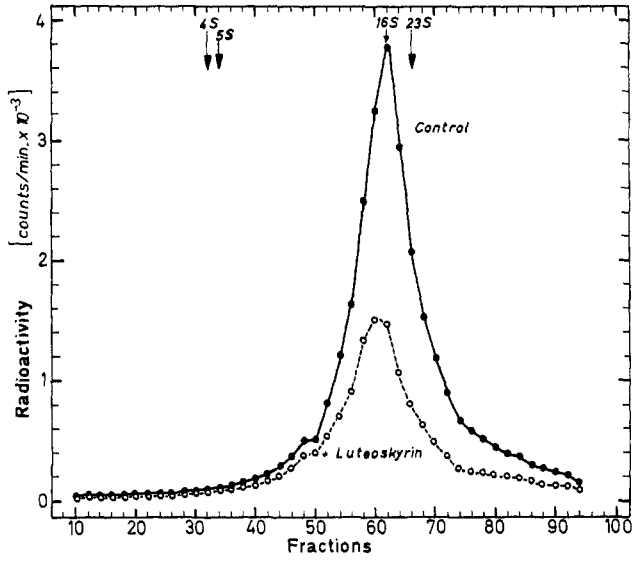

FIGURE 4: Cochromatography on an MAK column of the RNA synthesized in the presence or the absence of luteoskyrin. Two incubation mixtures (1 ml) contained standard buffer described in Figure 1 with 0.05 M KCl, T7 DNA (45 μg), and the four nucleoside triphosphates. The mixtures were equilibrated for 1 min at 37° and then the reaction was started by the addition of RNA polymerase (20 μg). In one mixture luteoskyrin (4.5 nmol) was added together with the enzyme. Incubation was carried out for 1 min at 37° and the reaction was stopped by addition of 0.2% sodium dodecyl sulfate final concentration. The detergent was precipitated in the cold with 0.1 M KCl. The two supernatants were mixed and the RNA was chromatographed on an MAK column as previously described (Darlix *et al.*, 1968). [^{3}H]UTP (50,000 cpm per nmol) was used as a marker in control incubation and [α-^{32}P]ATP was used in the presence of luteoskyrin. Inhibition of RNA synthesis with luteoskyrin was 50%. (●) control ^{3}H-labeled RNA; (○) ^{32}P-labeled RNA made in the presence of luteoskyrin. The elution of marker nucleic acids from *E. coli* under the same conditions is indicated by the arrows.

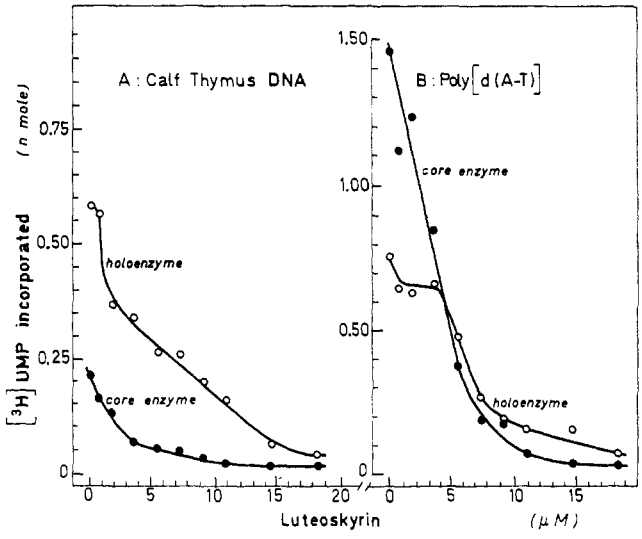

FIGURE 5: Effect of luteoskyrin on RNA synthesis by core enzyme and holoenzyme. (A) Incubation mixtures (0.3 ml) contained standard buffer described in Figure 1, calf thymus DNA (33 μg/ml), and either core enzyme (8 μg/ml) or the same amount of core enzyme saturated with purified σ factor (holoenzyme). Various concentrations of luteoskyrin were added and the reaction was started by addition of the four nucleotides. Incubation was carried out for 20 min at 37°. (B) Poly[r(A-U)] synthesis was performed in a similar manner except that poly[d(A-T] (22 μg/ml) replaced calf thymus DNA and only ATP and [^{3}H]UTP were added. (●) RNA synthesis with core enzyme; (○) RNA synthesis with holoenzyme.

peak corresponding to the same chain length of about 1500 nucleotides (Darlix *et al.*, 1968). The possibility that luteoskyrin acts similarly to proflavine by interfering only with the binding of RNA polymerase to DNA and that all initiated chains elongate normally (Richardson, 1966) is made unlikely by the above finding that the dye inhibits effectively after most initiation is completed.

Transcription of poly[d(A-T)] or calf thymus DNA was inhibited to approximately the same extent, whether core enzyme or holoenzyme was used (Figure 5). Thus, the σ factor is not involved in inhibition by luteoskyrin in agreement with the hypothesis that it is the transcription complex (*i.e.*, core enzyme) which is sensitive to the drug. Moreover, the inhibition is independent of the template used since luteoskyrin inhibits equally well whether T7 DNA, T4 DNA, calf thymus DNA, or poly[d(A-T)] was transcribed.

Effect of Luteoskyrin on the Transcription Complex. It has already been pointed out that drugs which act mainly on the template lead to a reduction in the RNA chain length, an effect not seen with luteoskyrin. Further evidence that the action of this drug on RNA synthesis using a native DNA template cannot be explained by its binding to the template alone is shown in Figure 6. It can be seen that a 65-fold increase in T7 DNA does not decrease the extent of inhibition by luteoskyrin. Although luteoskyrin binding to native DNA has been demonstrated (complex II), this complex is not responsible for inhibition. This is further supported by the finding that preincubation of template and drug leads, in fact, to a substantial *decrease* of inhibition, probably by removal of dye from solution by formation of complex II, in which large dye polymers are bound to only a few places on the DNA (results not shown).

In contrast, when transcription of denatured template is inhibited by luteoskyrin (Sentenac *et al.*, 1967) the inhibition can be overcome by increasing the concentration of denatured template. This finding is consistent with the suggestion that the binding of luteoskyrin to the purines of denatured templates (complex I) is responsible for its inhibition of transcription of unpaired templates.

The hypothesis that in the case of transcription of native templates luteoskyrin acts on the transcription complex suggests that increasing the number of enzyme molecules engaged in transcription should reduce inhibition. As can be seen in Figure 7, inhibition by luteoskyrin is indeed dependent on the amount of enzyme added. Inhibition curves follow a characteristic pattern. At low enzyme:DNA ratios, where a high proportion but low absolute number of enzyme molecules is actively engaged in transcription, the dye is highly inhibitory. As the amount of enzyme is increased inhibition is greatly reduced, and there exist thresholds of strong inhibition the lengths of which are a function of the amount of drug added. Levels of enzyme above this threshold reduce inhibition and at low drug concentrations overcome it completely (per increment of enzyme above threshold). Above 50 RNA polymerase molecules per T7 DNA, RNA synthesis is not any more proportional to the amount of enzyme added, and the same amount of enzyme in the presence of luteoskyrin saturates the DNA. This observation suggests that luteoskyrin binds to transcribing RNA polymerase molecules.

The thresholds observed above seem to correspond to titration of the transcribing complexes by luteoskyrin, where 2.5 nmol of dye inhibits 1 μg of enzyme. Experiments shown in Figure 8 indicate in fact that inhibition depends on drug con-

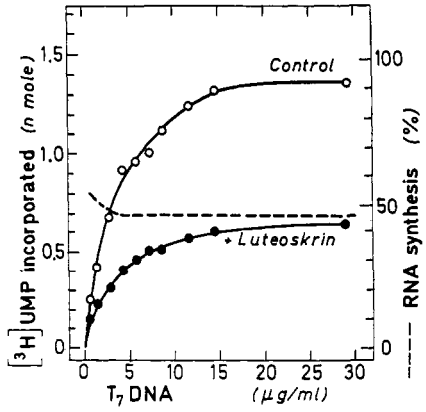

FIGURE 6: Effect of increasing DNA template concentration on inhibition of transcription by luteoskyrin. Incubation mixtures (0.3 ml) described in Figure 1 contained RNA polymerase (4.6 μg/ml) and various concentrations of T7 DNA; luteoskyrin (6 μM) was added before the nucleotides. Incubation was carried out for 20 min at 37°: (- - -) RNA synthesis in the presence of luteoskyrin expressed as a per cent of control.

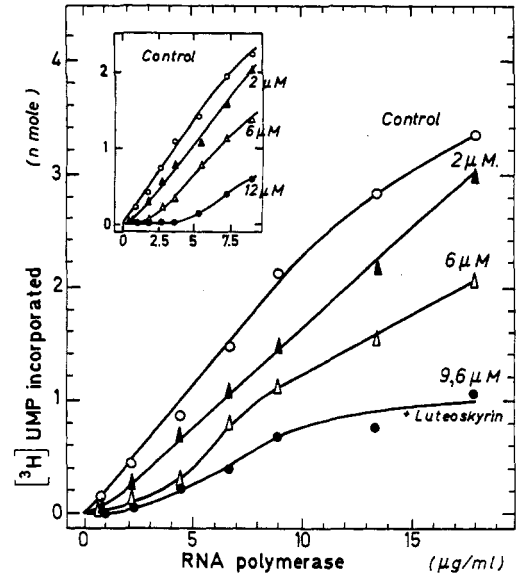

FIGURE 7: Inhibition of RNA synthesis at various enzyme concentrations. Incubation mixtures (0.3 ml) described in Figure 1 contained various enzyme concentrations. Luteoskyrin was added before the nucleotides and incubation was carried out for 20 min at 37°. Different luteoskyrin concentrations used were: 2, 6, and 9.6 μM. The same type of experiment carried out at very low enzyme concentrations is shown in the insert. Luteoskyrin concentrations used were 2, 6, and 12 μM.

centration and not only on the enzyme:luteoskyrin molar ratio. Inhibition depends on the final volume of the incubation mixture, except when luteoskyrin is first preincubated with the enzyme–DNA complex in the presence of magnesium. These results again suggest that luteoskyrin interacts with RNA polymerase bound to DNA and that the enzyme–DNA–inhibitor complex is not freely reversible.

The possibility that binding of inhibitor to the transcribing RNA polymerase molecule causes its dissociation from the DNA template was investigated. After a short incubation, RNA synthesis was stopped by addition of enough luteoskyrin to provide complete inhibition of transcription. If luteoskyrin dissociates the transcription complex the growing RNA chains should be released from the DNA template like after treatment with the protein denaturing agent sodium dodecyl sulfate. Figure 9 shows the sedimentation pattern of the RNA after luteoskyrin or sodium dodecyl sulfate treatment compared to an untreated control. The results clearly indicate that luteoskyrin does not dissociate the transcribing RNA polymerase molecule. Therefore, it appears that binding of luteoskyrin prevents chain propagation by freezing the transcription complex.

Interaction of Luteoskyrin and RNA Polymerase. Complex formation between luteoskyrin and RNA polymerase was studied by zone sedimentation. Enough luteoskyrin was added to produce 95% inhibition of transcription. Under these conditions (Figure 10) polymerase activity cannot be detected in the glycerol gradient. Some trailing of residual activity near the bottom of the tube and the presence of a yellow pellet indicated that the enzyme and the dye had sedimented. Since luteoskyrin alone interacts with Mg^{2+} ions in a complex similar to complex II (Pham Van and Bouhet, 1973[1]), it was of interest to determine whether Mg^{2+} plays a role in the enzyme–dye interaction. For that purpose RNA polymerase, usually stored in buffers containing Mg^{2+}, was desalted on a Sephadex column, mixed with luteoskyrin, and sedimented as above. Under these conditions RNA polymerase activity is fully recovered (actually more than expected from control) as seen from Figure 10. Similar results were obtained by adding EDTA to the enzyme to chelate the Mg^{2+}.

Experiments were also carried out with gel electrophoresis to try to visualize RNA polymerase–luteoskyrin interaction. One cannot expect by this technique to detect binding of a few dye molecules to the large RNA polymerase molecule but only drastic changes such as aggregation or dissociation of enzyme molecules. Luteoskyrin migrates faster than RNA polymerase and was easily detectable by its yellow color even after staining the enzyme with Coomassie Blue. In the absence of Mg^{2+} no binding of drug to enzyme was seen. RNA

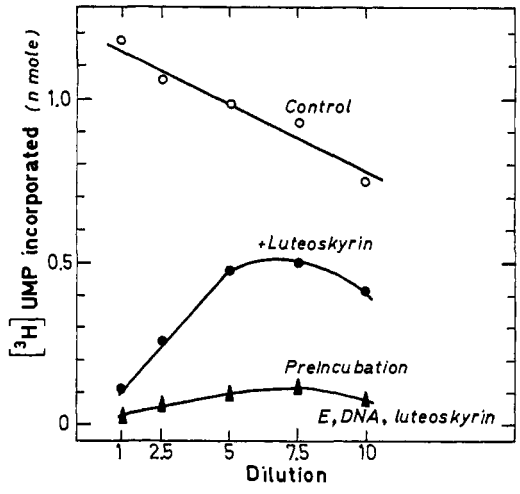

FIGURE 8: Inhibition as a function of luteoskyrin concentration. Incubation mixtures contained RNA polymerase (1.4 μg), T7 DNA (3 μg), luteoskyrin (1.8 nmol), nucleotides, and standard buffer (see Figure 1). The final volume of incubation mixture varied from 0.1 to 1 ml as indicated in the figure: (○) [^{3}H]UMP incorporation (20 min at 37°) in the absence of luteoskyrin; (●) [^{3}H]UMP incorporation in the presence of luteoskyrin. Alternatively, RNA polymerase, T7 DNA, and luteoskyrin were first preincubated (2 min at 0° in 0.1 ml) before dilution (▲).

[1] Pham Van, P., and Bouhet, J. C. (1973), manuscript in preparation.

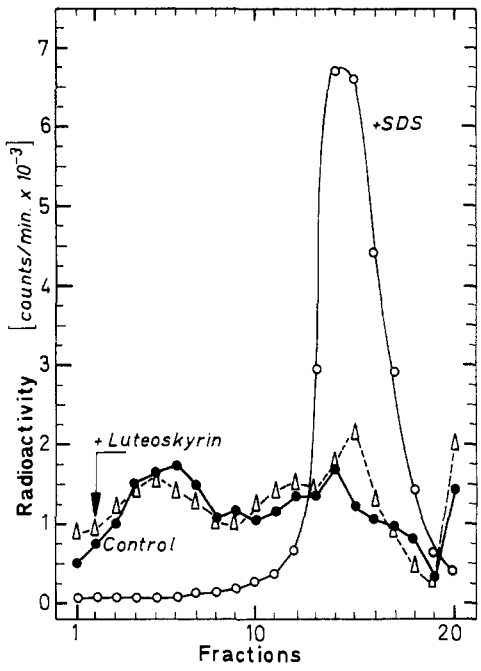

FIGURE 9: Sedimentation profile of the transcription complex after luteoskyrin treatment. Three identical mixtures (0.3 ml), A, B, and C, containing standard buffer described in Figure 1 with 0.05 M KCl, T7 DNA (16 μg/ml), four nucleotides with [^{3}H]UTP (24,000 cpm per nmol) and RNA polymerase (9 μg/ml) were incubated for 5 min at 37°. The reaction was stopped by addition of 0.03 M EDTA final concentration in mixture A, 0.2% sodium dodecyl sulfate final concentration in mixture B, and luteoskyrin (5 nmol) in C. After 1 min at 37° 0.03 M EDTA was added in mixtures B and C. Then the different mixtures were layered on top of 4.8 ml of a glycerol gradient (10–30%) in 0.01 M Tris-HCl (pH 7.9), 0.1 M KCl, and 0.1 mM EDTA and centrifuged for 75 min at 65,000 rpm in a SW-65 rotor. Fractions (0.25 ml) were collected and the RNA was precipitated with 5% trichloroacetic acid and counted: (●) control incubation A; (○) sodium dodecyl sulfate treatment, incubation B; (△) luteoskyrin, incubation C.

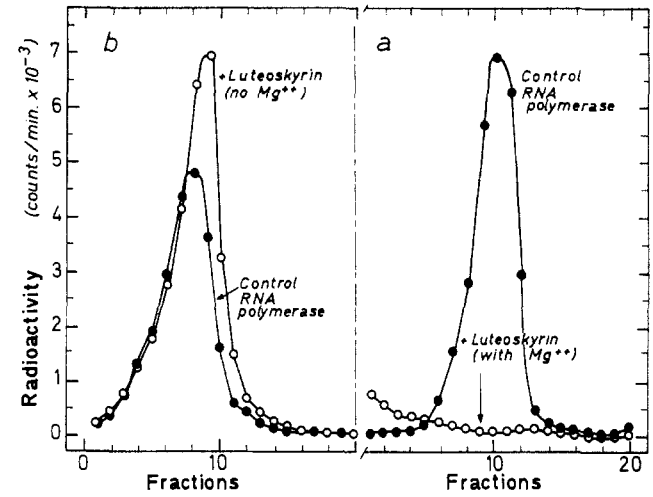

FIGURE 10: Interaction between RNA polymerase and luteoskyrin in the presence of Mg^{2+}. (A) RNA polymerase (24 μg) and luteoskyrin (18 nmol) were mixed in 10 mM Tris-HCl (pH 7.9)–1 mM $MgCl_2$–0.1 mM 2-mercaptoethanol–0.01 mM EDTA in 0.25 ml and layered on top of 4.8 ml of a glycerol gradient (10–30%) in 0.02 M Tris-HCl (pH 7.9), 0.5 mM 2-mercaptoethanol, and 0.05 M KCl, and centrifuged for 4 hr at 65,000 rpm in an SW-65 rotor. Fractions (0.25 ml) were assayed for enzymatic activity as described in Figure 1: (●) RNA polymerase without luteoskyrin; (○) RNA polymerase plus luteoskyrin. (B) The same experiment was carried out with RNA polymerase previously desalted on a Sephadex G-50 column equilibrated with 0.02 M Tris-HCl (pH 7.9)–1 mM 2-mercaptoethanol–0.05 M KCl–10% glycerol.

polymerase migrated as usual as a protomer (Sentenac *et al.*, 1968a). Variable results were obtained in the presence of Mg^{2+}. Some enzyme coprecipitated with luteoskyrin and Mg^{2+} ions and did not enter the gel. Sometimes a hazy yellow band of protein was observed in addition to the normal RNA polymerase band.

TABLE I: Effect of Increasing Concentrations of Nonpolymerase Proteins on Inhibition of Transcription.[a]

Aspartate transcarbamylase (μg/ml)	0	16.5	41	82	124	165	250	330
Inhibition (%)	76	68	58	46	45	44	38	36
Serum albumin (μg/ml)	0	16.5	50	82	130	165	250	330
Inhibition (%)	77	70	65	59	53	52	53	53

[a] Incubation mixtures (0.3 ml) described in Figure 1 contained RNA polymerase (6 μg/ml), luteoskyrin (6.7 μM), and various concentrations of serum albumin or aspartate transcarbamylase. Incubation was for 10 min at 37°. RNA synthesis was measured by incorporation of [α-^{32}P]ATP. At the highest concentration, serum albumin slightly stimulated [α-^{32}P]AMP incorporation (by 20%) while aspartate transcarbamylase lowered RNA synthesis by 15%.

As shown in Table I the addition of large quantities of unrelated proteins, such as aspartate transcarbamylase or serum albumin, to the reaction mixture partially reversed inhibition by luteoskyrin. However, in the presence of 6 μg/ml of RNA polymerase inhibition was reduced only twofold by the addition of 250 μg/ml of aspartate transcarbamylase. Thus, luteoskyrin appears to bind nonspecifically to various proteins but the observed inhibition of RNA polymerase and the reversal of inhibition by excess polymerase presented in Figure 6 cannot be the result of such nonspecific dye–protein interactions.

Discussion

The cytotoxic pigment luteoskyrin has the interesting property of binding preferentially to single-stranded nucleic acids in the presence of Mg^{2+}. Luteoskyrin, not unexpectedly, inhibits transcription of denatured templates. This inhibition is most likely the result of the dye–template interaction since inhibition is inversely related to template concentration and is independent of enzyme concentration (Sentenac *et al.*, 1967).

The interaction of luteoskyrin with double-stranded DNA results in the complexing of large dye–dye polymers to the DNA in only a few places (complex II). Transcription of native templates is, however, inhibited by luteoskyrin and the experiments described in this paper were carried out to try to explain this inhibition. Evidence is presented that in contrast to most other drugs, which inhibit RNA polymerase by reacting with either the template or the enzyme alone, luteoskyrin acts on the transcription complex: (1) addition of luteoskyrin at zero time leads to a reduction in the number of chains initiated while their growth rate remains unaffected; (2) addition of the drug during the course of the reaction blocks chain elongation without dissociating the transcription complex; (3) increasing amounts of RNA polymerase ac-

tively engaged in the process of transcription overcome the inhibition; (4) inhibition is independent of the template used and of DNA concentration. Preincubation of luteoskyrin and DNA, in fact, lowers the inhibition, probably by the formation of the noninhibitory complex II.

The possibility remains that, in addition to its well-known affinity for unpaired DNA, luteoskyrin could also bind to RNA polymerase itself like Congo Red (Krakov, 1965), heparin (Walter *et al.*, 1967), and rifampicin and its derivatives (Wehrli *et al.*, 1968; Sippel and Hartmann, 1970). However, these compounds tend to be much more active against free enzyme molecules than against DNA-bound enzyme and they are poorly effective or ineffective on the transcription complex.

Streptolydigin was shown to inhibit chain initiation and elongation (Siddhikol *et al.*, 1969) by interacting with RNA polymerase (Schleif, 1969). Inhibition results in this case from stabilization of the enzyme–template complexes by the drug (Von der Helm and Krakow, 1972) leading to a reduction in the rate of chain growth (Cassani *et al.*, 1971). Inhibition of chain initiation observed at high concentrations of streptolydigin is a consequence of the drastic inhibition of the elongation process. In contrast, luteoskyrin displays an all or none effect on the transcription complex: at intermediate doses, the dye blocks initiation (or elongation, when it is added after the start of transcription), but unaffected RNA polymerase molecules can synthesize normal RNA chains.

Experiments were carried out to determine a possible interaction between RNA polymerase and luteoskyrin. Indeed, complex formation was observed in the presence of Mg^{2+} by zone sedimentation. Luteoskyrin–RNA polymerase interaction does not seem to be related to the tightly bound metal ions in RNA polymerase (Scrutton *et al.*, 1971) since the dye does not bind to the enzyme after desalting it by filtration through Sephadex G-25. Highly purified RNA polymerase (core enzyme) contains two tightly bound zinc atoms per molecule which may be involved in chain initiation. As a chelating agent, luteoskyrin could have an effect on transcription similar to that of 1,10-phenanthroline. This, however, is not the case since luteoskyrin inhibits RNA synthesis at any time, whereas 1,10-phenanthroline has no effect when added after the start of polymerization (Scrutton *et al.*, 1971). Furthermore, addition of a large excess of other proteins like serum albumin or aspartate transcarbamylase, which is also a zinc metalloprotein (Rosenbusch and Weber, 1970), does not overcome the inhibition, unlike augmentation of RNA polymerase. The observed interaction between luteoskyrin and RNA polymerase in the presence of Mg^{2+} probably reflects coprecipitation of the enzyme with the Mg^{2+}–luteoskyrin complex. This would not occur during transcription since added DNA prevents the precipitation of luteoskyrin with Mg^{2+} (Ueno *et al.*, 1966).

The inhibition by luteoskyrin of the transcription unit could then occur in two steps. The loose binding of the drug to the DNA-bound enzyme, with Mg^{2+}, would lead to a locally high concentration of the drug; then unwinding of the DNA during chain initiation would allow the rapid and tight binding of the drug to the unpaired nucleic acid, preventing further RNA polymerase propagation (Kosaganov *et al.*, 1971; Saucier and Wang, 1972). Another antibiotic kanchanomycin resembles luteoskyrin very much as far as its interaction with nucleic acids is concerned (Friedman *et al.*, 1969a,b). Kanchanomycin was also found to inhibit RNA synthesis *in vitro* apparently in much the same way as luteoskyrin (Joel *et al.*, 1970). The mode of action of the two compounds is therefore very likely the same. These authors showed also that Kanchanomycin inhibits DNA synthesis by *E. coli* DNA polymerase I (Joel *et al.*, 1970). Luteoskyrin was also found to inhibit DNA repair *in vitro* (Mouton and Fromageot, 1971) which is consistent with its affinity for unpaired DNA.

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Characterization of the Poly(adenylic acid) Regions and the Adjacent Nucleotides in Heterogeneous Nuclear Ribonucleic Acid and Messenger Ribonucleic Acid from HeLa Cells†

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ABSTRACT: Poly(A) segments derived from either Hn-RNA or mRNA molecules with T1 RNase consisted of two uridylate and one–two cytidylate residues per 195 adenylate residues and were devoid of any GMP residues. Pancreatic RNase produced poly(A) segments approximately 195 nucleotides long which consisted exclusively of adenylic acid. These experiments indicated that at least 80–90% of the poly(A) in Hn-RNA and mRNA is located at the 3′-OH termini and that the nucleotides adjacent to the poly(A) in Hn-RNA and mRNA are the same. This result further supports the hypothesis that some Hn-RNA molecules are precursors to mRNA molecules.

The nucleus of mammalian cells contains very high molecular weight RNA molecules varying in size from 6,000 to at least 30,000 nucleotides in length (Hn-RNA, heterogeneous nuclear RNA) which is similar in base composition to the messenger RNA fraction (mRNA) recovered from the polyribosomes (Scherrer *et al.*, 1963; Scherrer and Marcaud, 1965; Houssais and Attardi, 1966; Soeiro *et al.*, 1966) in the cell cytoplasm. The speculation was advanced a number of years ago that the mRNA was a product of posttranscriptional modification of the higher molecular weight Hn-RNA (Darnell, 1968). Recent experiments have shown that Hn-RNA and mRNA contain adenylic acid rich regions, poly(A), about 200 nucleotides long which are located at the 3′-OH terminus of the RNA molecules (Kates, 1970; Edmonds *et al.*, 1971; Lee *et al.*, 1971; Darnell *et al.*, 1971b; Mendecki *et al.*, 1972; Molloy *et al.*, 1972a; and Sheldon *et al.*, 1972). The poly(A) appears to be initially added to Hn-RNA molecules following transcription and is thereafter transferred to the cytoplasm as part of mRNA molecules (Darnell *et al.*, 1971a; Jelinek *et al.*, 1973). It appears therefore that mRNA is derived from a high molecular weight nuclear precursor and that the nucleotide sequences involved in protein synthesis are located near the 3′ terminus of the Hn-RNA precursor molecules.

In the experiments reported here an examination was made of poly(A) segments derived by the separate or combined use of T1 RNase (specific for guanylate residues; Arina *et al.*, 1968) and pancreatic RNase (specific for pyrimidine residues; Markham and Smith, 1952). From these studies it was possible to determine that the poly(A) segments in Hn-RNA and mRNA molecules consisted entirely of adenylic acid residues and that the nucleotides adjacent to the nuclear poly(A) were the same as those adjacent to the mRNA poly(A), giving further support to the hypothesis that some Hn-RNA molecules are precursors to mRNA molecules. In addition, as explained in detail below, base composition analysis of the poly(A) segments labeled and derived in various ways indicated that the great majority, if not all, of the poly(A) was located immediately at the 3′-OH end of the Hn-RNA and mRNA chains.

Experimental Procedures

Hela cells were grown in suspension culture as described (Eagle, 1959) and 3×10^8 cells was labeled in 150 ml of medium with either 5 mCi of [^{3}H]adenosine (18.3 Ci/mmol), 20 mCi of [^{3}H]uridine (24.3 Ci/mmol), or 50 mCi of $^{32}PO_4$ (carrier free, Schwarz-Mann). ^{32}P labeling was carried out by washing cells twice and resuspending in PO_4 free Eagle's medium supplemented with 5% dialyzed fetal calf serum. Unless otherwise stated, cells were treated for 30 min with 0.05 μg/ml of actinomycin D to stop ribosomal RNA synthesis prior to labeling with isotope for 4 hr (Perry, 1963).

Labeled cells were fractionated as previously described (Molloy *et al.*, 1972b) and phenol-extracted mRNA and Hn-RNA isolated after sucrose gradient sedimentation (Soeiro and Darnell, 1969). Nuclease digestion of purified RNA samples was carried out at 37° for 60 min in a buffer designated NET (0.1 M NaCl–0.01 M EDTA–0.01 M Tris, at pH 7.4) with pancreatic RNase (Sigma recrystallized 5×) 2 μg/ml or T1 RNase (Sankyo) at 10 units/ml. Nuclear samples were digested and extracted twice to ensure cleavage at all susceptible bonds. After digestion, samples were phenol extracted and RNase-resistant material precipitated with ethanol and redissolved in buffer designated as NETS (the same as NET with 0.2% sodium dodecyl sulfate added).

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