Proflavine Binding to Transfer Ribonucleic Acid, Synthetic Ribonucleic Acids, and Deoxyribonucleic Acid*

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SUMMARY

The ability of proflavine to directly inhibit protein synthesis has led to an investigation of complex formation between the drug and ribonucleic acid. Evidence has been obtained from absorption and fluorescence spectra, as well as heat denaturation curves, that proflavine forms a complex with transfer ribonucleic acid which is qualitatively similar to the deoxyribonucleic acid-proflavine complex. Additional studies with polyadenylate, polyuridylate, and polycytidylate emphasize the importance of nucleic acid secondary structure, the ionic environment, and the molar ratio of nucleotide to proflavine in determining the type of drug-nucleic acid complex which will be formed.

Although the interaction between proflavine and deoxyribonucleic acid has been the subject of extensive investigation (1-8), complex formation between proflavine and ribonucleic acid has received relatively scant attention. Earlier studies in our laboratory which demonstrated that proflavine causes a direct inhibition of protein synthesis (9) have led us to compare the physical interaction of proflavine with deoxyribonucleic acid to its interaction with synthetic and natural ribonucleic acids. These studies were done in the very same medium as that used for protein synthesis, as well as in media of lower ionic strength and without divalent cations. The results support our conclusion (9) that proflavine inhibition of protein synthesis results primarily from an ability of the drug to form a stable complex with transfer ribonucleic acid. These studies also indicate that the ability of proflavine to interact with a given type of nucleic acid, as well as the nature of the complex, is strongly influenced by: (a) the ratio of nucleotide concentration to proflavine concentration; (b) the base composition and secondary structure of the nucleic acid; and (c) the ionic composition of the medium. The importance of these same factors has also been emphasized by previous investigators in studies on the interaction between acridine orange (and related basic dyes) and nucleic acids (10-12).

EXPERIMENTAL PROCEDURE

Materials—Interaction between proflavine and polynucleotides were studied in three media: (a) Standard buffer (Tris-HCl, 0.01 M, pH 7.8; magnesium acetate, 0.005 M; potassium chloride, 0.06 M); (b) Buffer 1 (sodium phosphate, 0.01 M, pH 6.8; disodium EDTA, 1 X 10⁻⁴ M; NaCl, 3 X 10⁻³ M); (c) Buffer 2 (the same as the previous buffer but has 0.1 the concentration of phosphate). The first buffer was chosen since it is the same as that used for our amino acid incorporation experiments (9, 13). Buffers 1 and 2 were included to determine the effects of solvents lacking divalent cations and with a lower ionic concentration and are similar to a buffer used in previous studies on the binding of drugs to nucleic acids (14).

Proflavine sulfate (mol. wt., 325.3) was obtained from Allied Chemical Corporation. Stock solutions were prepared daily in each of the above buffers at a concentration of 0.5 mg per ml. The sodium salt of highly polymerized calf thymus DNA (guanine + cytidine = 43% (15)) was purchased from Worthington. Escherichia coli tRNA was purchased from General Biochemicals. Poly U (s = 8.36), poly C (s = 6.05), and poly A (s = 11.3) were purchased from Miles Chemical Company. The concentrations of nucleic acid stock solutions were determined spectrophotometrically in distilled water with the use of the following conversion values: calf thymus DNA, 20.2 A₂₅₀ units = 1 mg per ml (16); E. coli tRNA, 24.0 A₂₅₀ units = 1 mg per ml (17); poly U 27.9 A₂₅₀ units = 1 mg per ml; poly C, 18.4 A₂₅₀ units = 1 mg per ml; poly A, 34.6 A₂₅₀ units = 1 mg per ml. The conversion values for poly U, poly A, and poly C are based on molar extinction coefficients kindly supplied by R. F. Beers, Jr.²

1 The abbreviations used are: poly A, polyadenylate; poly U, polyuridylate; poly C, polycytidylate; tRNA, transfer ribonucleic acid.

2 R. F. Beers, Jr., personal communication.
Methods—Spectrophotometric measurements were performed on a Zeiss spectrophotometer, model PMQ II. Heat denaturation studies were done on a Beckman model DUR in conjunction with a Gilford model 2000 multiple sample absorbance recorder. These studies were carried out in standard quartz cuvettes with a light path of 1 cm. Fluorescence studies were carried out in an Amino-Bohman spectrophotofluorometer equipped with a Sola rectifier power supply for a 150 watt Zenon arc lamp, model No. 281188. Precipitation of complexes did not occur within the concentration ranges of polymer (0 to 200 µg per ml) and dye (6 x 10⁻⁷ to 5 x 10⁻⁵ M) used in these experiments. Spectral studies were carried out in a Cary recording spectrophotometer model 14. Heat denaturation studies were performed essentially by the method of Marmur and Doty (18). The temperature at which 50% of the total hyperchromicity was observed is designated as Tm.

RESULTS

Effect of Nucleic Acids on Absorption Spectrum of Proflavine in Standard Buffer—The absorption spectrum of proflavine (5 x 10⁻⁵ M), when measured in Standard buffer and in the absence of nucleic acid, revealed a maximum at 444 µm. Based on the absorption coefficients given under “Methods” and assuming a mean molecular weight for nucleotides of 334, 17 µg per ml of nucleic acid represents a nucleotide concentration equivalent to 5 x 10⁻⁵ M proflavine. When tested at nucleotide concentrations approximately 6-fold greater (on a molar basis) than the proflavine concentration, tRNA caused a "bathochromic shift" (to a higher wave length) in the absorption maximum of the drug; poly A produced quenching of proflavine absorption as well as a "hypsochromic shift" (to a lower wave length) in the absorption maximum of the drug; and poly U had a negligible effect (Fig. 1). The bathochromic effect of tRNA is qualitatively similar to that obtained when DNA (1, 8) or unfractionated RNA (1) interacts with proflavine.

A more quantitative comparison of the effect of nucleic acid type and concentration on the absorption spectrum of proflavine was obtained by adding increasing amounts (5, 10, 25, 50, 100, and 200 µg per ml) of different nucleic acids to a solution containing a fixed concentration of proflavine (5 x 10⁻⁵ M) and measuring the absolute absorption at 430, 444, and 460 µm. The order of effectiveness in "quenching" ([444 proflavine + nucleic acid]/[444 proflavine]) was poly A > DNA > tRNA > poly U (Fig. 2). Poly C had a negligible effect on quenching.

Shifts in the absorption maximum of proflavine to a higher or a lower wave length were expressed by calculating the A444: A460 ratios obtained at each concentration of nucleic acid. Increasing concentrations of DNA and tRNA produced a progressive decrease in the A444: A460 ratio (Fig. 3). At equivalent concentrations DNA was more effective in this respect than tRNA. Poly A, poly U, and poly C had a slight or no effect on the A444: A460 ratio. Increasing concentrations of poly A produced a shift in the absorption maximum of proflavine to 430 µm, with a maximum effect at 25 to 50 µg per ml of poly A (Fig. 4). DNA and tRNA caused an increase in the A444: A460 ratio, which reflects their bathochromic effect, whereas poly U and poly C had a negligible effect on this ratio (Fig. 4).

Effect of Nucleic Acids on Absorption Spectrum of Proflavine in Buffer 1—The above results provided evidence for nucleic acid specificity in the physical interaction between proflavine and polynucleotides. Since previous studies indicated an effect of ionic strength and divalent cations on the interaction between basic dyes and nucleic acids (5, 10-12), it was of interest to repeat these studies in buffers of low ionic strength and lacking divalent cations. When the interaction between nucleic acids and proflavine was studied in Buffer 1, the effects of nucleic acids on quenching of proflavine absorption at 444 µm (Fig. 5) were more complex than the results obtained in standard buffer. Low concentrations (5 to 25 µg per ml) of poly A and also poly U produced marked quenching of proflavine absorption.

![Fig. 1](https://example.com/fig1.png)  
Effect of nucleic acids on the absorption spectrum of proflavine (5 x 10⁻⁵ M) in Standard buffer. Nucleic acids were tested at 100 µg per ml. — free proflavine; ——, proflavine + poly A; ——, proflavine + tRNA; ——, proflavine + poly U.

![Fig. 2](https://example.com/fig2.png)  
Fig. 2. Effect of nucleic acids on proflavine (5 x 10⁻⁵ M) absorption at 444 µm studied in Standard buffer. "Quenching" = ([A444 proflavine + nucleic acid]/[A444 proflavine]). ▲—▲, tRNA; ○—○, DNA; △—△, poly A; O—O, poly U; O—O, poly C.

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at 444 μM, but higher concentrations of these two nucleic acids tended to reverse this quenching effect. With DNA, tRNA, and poly C, however, quenching was maximum at high concentrations (50 to 200 μg per ml) of nucleic acids. As in Standard buffer, DNA and tRNA produced an appreciable bathochromic shift in the absorption of proflavine. At a given concentration of nucleic acid, DNA was again more effective in this respect than tRNA. Poly A, poly U, and poly C exerted only a slight effect on the A_{444}:A_{460} ratio. As in the previous studies, low concentrations of poly A produced a hypsochromic shift in the absorption of proflavine; high concentrations of poly C produced a slight hypsochromic effect; poly U had no appreciable effect; and both DNA and tRNA caused an increase in the A_{444}:A_{430} ratio.

![Fig. 3. Effect of nucleic acids on the relative absorption of proflavine (5 × 10^{-5} M) at 444 and 460 μM studied in Standard buffer.](image)

![Fig. 4. Effect of nucleic acids on the relative absorption of proflavine (5 × 10^{-5} M) at 444 and 430 μM studied in Standard buffer.](image)

The above studies were repeated in a buffer which contained 0.1 of the phosphate concentration (Buffer 2). At 5 to 25 μg per ml, the order of effectiveness of nucleic acids in quenching proflavine absorption at 444 μM was the following: poly A > poly U > tRNA > poly C > DNA. Maximum quenching occurred with 25 to 50 μg of nucleic acid, and higher concentrations of all of these nucleic acids tended to reverse the quenching of proflavine absorption. As in the previous studies, DNA and tRNA were the most effective nucleic acids in producing a bathochromic shift in proflavine absorption. Low concentrations of poly A again produced a hypsochromic shift in proflavine absorption, and in this buffer poly U and poly C had a similar but less marked effect.

**Effect of Nucleic Acids on Fluorescence of Proflavine—**The fluorescence spectrum of free proflavine in Standard buffer or in Buffer 1 indicated an excitation maximum at 450 μM and an emission maximum at 515 μM. Because of its extreme fluorescence only a very low concentration of proflavine (6 × 10^{-7} M) could be used in these studies. The effects of the addition of increasing amounts of various nucleic acids to a fixed concentration of drug were monitored in the range of 450 to 515 μM. When studied in Standard buffer, or in Buffer 1, DNA (3 μg per ml) and tRNA (10 μg per ml) caused a slight shift in the excitation maximum of proflavine from 450 to 460 μM but no appreciable shift in the wave length at which maximum emission occurred (515 μM). DNA and tRNA caused marked quenching of the intensity of proflavine emission at 515 μM (Figs. 6 and 7). DNA was more effective in this respect than tRNA when studied in Standard buffer (Fig. 6), but tRNA was as effective as DNA when studied in Buffer 1 (Fig. 7). In contrast to DNA and tRNA, poly C, poly U, and poly A did not cause a shift in the excitation maximum of proflavine, nor did they quench proflavine absorption at concentrations as high as 200 μg per ml (Figs. 6 and 7).

**Effect of Proflavine on Heat Denaturation Profile of DNA,**
tRNA, and Poly A—The preceding studies indicated that proflavine readily forms complexes with DNA, tRNA, and poly A. The results also suggested that the poly A-proflavine complex is qualitatively different from that formed with DNA or tRNA. It was, therefore, of interest to determine the effect of proflavine on the secondary structure of DNA, tRNA, and poly A, as well as the effect of loss of secondary structure of the nucleic acid on the stability of the proflavine complex. The heat denaturation profile of these nucleic acids was measured in the absence and presence of proflavine while monitoring at 260 nm to follow the secondary structure of the nucleic acid, and at 444 nm to follow the proflavine component of the complex. These studies were done in Buffer 1 rather than Standard buffer, since Buffer 1 appeared to favor the formation of proflavine-nucleic acid complexes. Calf thymus DNA (20 µg per ml) had a \( T_m \) (50% of total hyperchromicity) of 70° (Fig. 8). Proflavine (6 \( \times \) 10\(^{-7}\) M) caused an increase in the \( T_m \) of this DNA to approximately 78° (Fig. 8). The ability of acridine dyes to protect DNA against heat denaturation has been previously described (4, 19). In the presence of proflavine, total hyperchromicity of DNA was also somewhat greater than in its absence. The explanation for the latter effect is not known, but spermine (20), as well as miracid D, (14) can also increase the total hyperchromicity obtained during heat denaturation of DNA. Measurement at 444 nm indicated a partial loss of proflavine quenching in the temperature range of 25-65° and a much more pronounced loss of quenching which paralleled the heat denaturation of the DNA (Fig. 8).
resulted in a 3° increase in the $T_m$. As with DNA, there was a slight loss of proflavine quenching at 444 m$\mu$ at low temperatures, but the major loss of proflavine quenching paralleled the loss of secondary structure of the nucleic acid.

Fig. 10 indicates the effect of proflavine on the heat denatur-

![Graph showing effect of proflavine on heat denaturation of DNA, tRNA, and poly A.](http://www.jbc.org/)
with intercalation, the evidence does not exclude other modes of interaction. Further studies are required, therefore, to establish the exact nature of the proflavine-tRNA complex.

It must be emphasized that although the results are compatible for intercalation of proflavine with tRNA is, of course, indirect. The nuclease action (40) and this release does not parallel the loss of secondary structure of poly A. The unique properties of the proflavine-poly A complex may relate to the fact that at neutral pH poly A exists not as a double stranded hydrogen-bonded structure, but as a molecule in which secondary structure is due to a stacking of the bases (23, 24). Our results suggest that whereas proflavine can form a stable complex with DNA and tRNA, by intercalating and thereby achieving a large area of overlap between drug and base pairs, the fit of the proflavine ring system between stacked bases in poly A may be less favorable, thus yielding an unstable complex. It is of interest that the calculations of Pullman (25) predict that, among the nucleotides, adenylc acid should be the strongest electron donor. If poly A were to serve as a strong electron donor and proflavine as an electron acceptor, this interaction would produce quenching of fluorescence, since this phenomenon is attributed to a charge transfer reaction (21).

The fact that magnesium ions, by forming complexes with nucleic acid phosphate residues, prevent proflavine stacking. Also consistent with proflavine stacking is the fact that minimal absorption at 444 mµ occurred when the molar ratio of nucleotides to proflavine was approximately equal and was partially reversed at higher concentrations of nucleic acid. It appears that this type of complex can also form with DNA, tRNA, and poly A, especially at low nucleic acid concentrations and in the low ionic strength buffer. Under the latter conditions, raising the concentrations of these nucleic acids also tended to reverse their quenching effect on proflavine absorption at 444 mµ. The fact that, in the heat denaturation studies, a small fraction of proflavine was released from DNA and tRNA prior to the loss of nucleic acid secondary structure, suggests that even in the case of DNA and tRNA a portion of bound proflavine is loosely attached to the nucleic acids by means of stacking, rather than by intercalation.

The ability of proflavine to form complexes with tRNA may provide a tool for examining aspects of tRNA structure required for the function of these molecules in protein synthesis. Along these lines it will be of interest to extend the results obtained with unfractionated tRNA to purified transfer ribonucleic acids specific for individual amino acids and codons.

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