Labilizing Action of Intercalating Drugs and Dyes on Bacterial Ribosomes†

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ABSTRACT: Twelve compounds which are known or assumed to bind to duplex DNA by intercalation, accelerated the degradation of *Escherichia coli* ribosomes at 52°, while ethidium bromide at 10^{-4} M caused disassembly of ribosomes centrifuged to equilibrium in cesium chloride at 10^{-2} M Mg^{2+}. Among the disassembly products were free rRNA, free proteins, and two ribonucleoprotein particles with sedimentation coefficients of 37 and 43 S. A fluorometric titration of ribosomes with ethidium indicated that 66% of potential intercalation sites in rRNA became occupied. The acceleration of thermal degradation of ribosomes by five known intercalators correlated directly with the fraction of bound drug estimated to intercalate into supercoiled DNA at its equivalence point.

Acridine Orange, a member of a group of compounds which bind strongly to double-helical DNA by intercalation, has been employed as a molecular probe of the accessibility of RNA ribosomes (Morgan and Rhoads, 1965; Furano *et al.*, 1966). Subsequently it was shown, however, that this aminoacridine unfolds (Hultin, 1969) or disassembles (Permogorov and Sladkova, 1968) ribosomes unless high concentrations of Mg^{2+} are used to stabilize the particles, which, in turn, weakens the affinity of the rRNA binding sites for Acridine Orange (Cotter *et al.*, 1967). Additionally, the DNA intercalators ethidium bromide, quinacrine, and chloroquine, in direct proportion to their total planar area, labilize *Escherichia coli* ribosomes to heat (Wolfe, 1971). We therefore propose, and have tested in this study, the hypothesis that substances which are DNA intercalators also possess the group property of degrading or disassembling ribosomes when these particles are subjected to thermal or ionic stress.

Acridine Orange binds to rRNAs but not to ribosomal proteins (Cotter *et al.*, 1967). We assume that the facilitation of ribosome degradation and disassembly by intercalating compounds is accompanied and, perhaps, caused by intercalative binding of these compounds to duplex segments of ribosome RNAs *in situ*.

Materials and Methods

Ethidium bromide, propidium iodide, and spermine were purchased from Calbiochem, Los Angeles, Calif.; chloroquine from Winthrop Laboratories, New York, N. Y.; quinine was purchased from The Vitarine Co., New York, N. Y.; *E. coli* A19 16S and 23S RNA was obtained from the Miles Laboratories, Kankakee, Ill.; [^{14}C]phenylalanine and [^{3}H]-uridine were purchased from the New England Nuclear Corp., Boston, Mass,; optical grade cesium chloride was obtained from the Harshaw Chemical Co., Solon, Ohio. Other drugs and chemicals were obtained from the internal supply of the Walter Reed Army Institute of Research.

Thermal degradation of *E. coli* C-2 ribosomes obtained conventionally was carried out as previously described (Wolfe, 1968; Wolfe and Hahn, 1968). Ribosomes were maintained at 52° in suspensions comprised of: 1×10^{-3} M magnesium acetate, 6×10^{-2} M KCl, 3×10^{-2} M Tris-HCl (pH 7.2), and 1.0 A_{260} unit of ribosomes. Drug was added before heating, while ribosomes were micropipetted after the experimental medium had reached 52°. Absorbancy was measured at 260 nm in a Gilford Model 2000 recording spectrophotometer equipped with a Haake circulating flow heater.

Equilibrium density gradient centrifugation was carried out by an adaptation of the technique of Meselson *et al.* (1964). To a volume of 4.7 ml of CsCl (n_D^{23} 1.375), containing 2×10^{-2} M Tris-HCl (pH 7.2) and 1×10^{-2} M Mg^{2+}, was added 0.05 ml of a solution of 1×10^{-2} M ethidium bromide, the solution was shaken, and 0.25 ml of a suspension containing 20 A_{260} units of *E. coli* ribosomes was added, gently shaken, and centrifuged for 40 hr at 4° in a Beckman L2-65B centrifuge using a SW 50.1 rotor at 34,500 rpm. Fractions were obtained by drop collection. A_{260} determinations were carried out after dilution of fractions with 1.0 ml of a buffer containing 2×10^{-2} M Tris-HCl (pH 7.2) and 1×10^{-2} M Mg^{2+}. Pooled or individual fractions were dialyzed two or more times for 8–12 hr at 4° against 100 volumes of buffer. [^{14}C]Phenylalanine and [^{3}H]uridine disintegrations per minute were determined in a Nuclear-Chicago Mark II liquid scintillation counter after precipitation of fractions with 2 ml of cold 5% trichloroacetic acid, filtration utilizing type HA filters (Millipore Filter Corp., Bedford, Mass.), and dissolution in a dioxan-based liquid scintillation solution (Wolfe *et al.*, 1971).

Fluorescence of ethidium was determined in a Aminco-Bowman spectrophotofluorometer set at an excitation wavelength of 545 nm and an emission wavelength of 590 nm. Titrations were carried out by adding microliter aliquots of 3.3×10^{-3} M ethidium bromide to 3.0-ml aliquots of ribosomes, or repetitively to a single 3.0-ml aliquot. Ribosome concentrations were estimated from the A_{260} of triplicate samples of dilute suspensions, using an absorbance of 150 for 10 mg of 70S ribosomes, and assumed to be 64% RNA. The hypothetical number of intercalation sites was estimated by dividing the number of component nucleotides in the RNA of ribosomes by 4.2, the minimum ratio of nucleotide to drug observed for intercalation into DNA.

Sedimentation coefficients were determined in a Beckman Model E ultracentrifuge with a uv scanner. Analysis of dialyzed fractions was done by boundary sedimentation at

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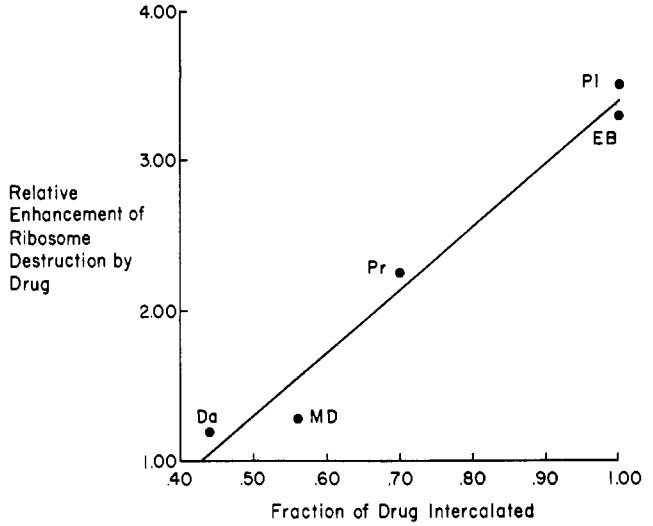

FIGURE 1: Correlation between the acceleration of ribosome degradation by intercalators (on the ordinate) and the fractional concentrations of these substances (on the abscissa) at their equivalence points (Waring, 1970). PI = propidium iodide; EB = ethidium bromide; Pr = proflavine; MD = Miracil D; Da = daunomycin.

34,140 rpm results corrected to 20°. Band sedimentation was performed at 20° on undialyzed fractions by appropriate dilution and layering over CsCl (n_D^{23} 1.359) containing 2×10^{-2} M Tris-HCl (pH 7.2) and 1×10^{-2} M Mg^{2+}.

Results

Table I compares a variety of DNA intercalants and other compounds with respect to their ability to accelerate thermal degradation of ribosomes. Propidium iodide induced the most rapid hyperchromicity, while ethidium bromide ranked next in effectiveness. Compounds of lesser total planar area proved less effective, with the general order of ranking: phenanthridines > acridines > quinolines. Most of these substances are known or presumed to intercalate into double-helical DNA with the exception of spermine, Crystal Violet, irehdiamine A, and primaquine. Quinine, which intercalates into DNA with low stoichiometry (Estensen *et al.*, 1969), had no effect while irehdiamine A resembled intercalators in its effects on both DNA (Waring, 1970) and ribosomes. These data show that strong DNA intercalators, as a group, reduce the stability of ribosomes.

Selected intercalants (Waring, 1970) among those listed in Table I convert supercoiled DNA to a form having a minimum sedimentation coefficient (equivalence point). Different concentrations of intercalants are required for such conversions and an estimate (Waring, 1970) has been made of the fraction of compound intercalated at the equivalence point. Figure 1 compares the effect of selected compounds on the conversion of supercoiled DNA with the effects of these drugs on ribosome breakdown. Propidium iodide and ethidium bromide acted strongly on DNA as well as on ribosomes, proflavine had an intermediate effect and Miracil D and daunomycin had the least effect. The degree of intercalation into supercoiled DNA paralleled the acceleration of hyperchromicity and breakdown of ribosomes, and suggests that drug intercalation reduces ribosome stability.

Equilibrium Density Gradient Centrifugation of Ribosome in the Presence of Ethidium. Centrifugation to equilibrium in CsCl results in the Mg^{2+}-dependent banding of ribosomes into core particles, intact ribosomes and meniscus proteins. Reduction in the Mg^{2+} concentration causes degradation of ribosomes which results in accumulation of RNA in the more dense region of the gradient (Meselson *et al.*, 1964). Figure 2 illustrates the effect of ethidium bromide on ribosomes in such gradients containing the normally stabilizing Mg^{2+} concentration of 1×10^{-2} M. In the absence of ethidium bromide the A_{260} profile showed the typical bimodal distribution characteristic of intact ribosomes and core particles while the presence of 1×10^{-4} M ethidium bromide resulted in formation of a large peak (I) in the more dense region of the gradient as well as a broad, composite maximum displaced from peaks of the control. This composite area was resolvable by curve analysis into two major peaks (II, III). Increase in ethidium bromide concentration resulted in decrease in peaks II and III, and increase in peak I, while an increase in Mg^{2-} to 4×10^{-2} M induced distribution similar to ethidium bromide free systems.

Figure 3 illustrates the distributions of acid-insoluble [^{14}C]phenylalanine and [^{3}H]uridine obtained when a mixture of ribosomes labeled with single isotopes was centrifuged to equilibrium in the presence of ethidium bromide. [^{14}C]Phenylalanine was not detected in peak I, but two maxima occurred congruent with peaks II and III. These experiments demonstrated that the uv-absorbing material comprising peak I was free RNA rather than ribonucleoprotein and, in addition, indicated the presence of two major ribonucleoprotein fractions in the gradient.

The two ribonucleoprotein peaks observed after equilibrium centrifugation with ethidium bromide were analyzed

TABLE I: Effect of Selected Compounds on Disassembly of Ribosomes at 52°.[a]

Compounds (2×10^{-5} M)	Disassembly Time (End Point) (min)	Ratio of End Points (Control Sample/Exptl Sample)
Propidium iodide	9.5	3.52
Ethidium bromide	10.1	3.31
Methylene Blue	14.8	2.26
Proflavine	14.8	2.26
Quinacrine	21.2	1.58
Nitroakridin 3582	21.2	1.58
Irehdiamine A	26.2	1.28
Miracil D	26.2	1.28
Acridine Orange	26.8	1.25
Daunomycin	28.1	1.19
Berberine	29.1	1.15
Chloroquine	29.9	1.12
Crystal Violet	31.9	1.05
Control	33.5	1.00
Primaquine	34.5	0.97
Quinine	35.3	0.95
Spermine	67.0	0.50

[a] Experimental mixtures contained approximately 1.00 A_{260} unit of *E. coli* C-2 ribosomes, 1×10^{-3} M Mg^{2+}, 3×10^{-2} M Tris-HCl (pH 7.2), and 6×10^{-2} M KCl. Total volume, 1.0 ml.

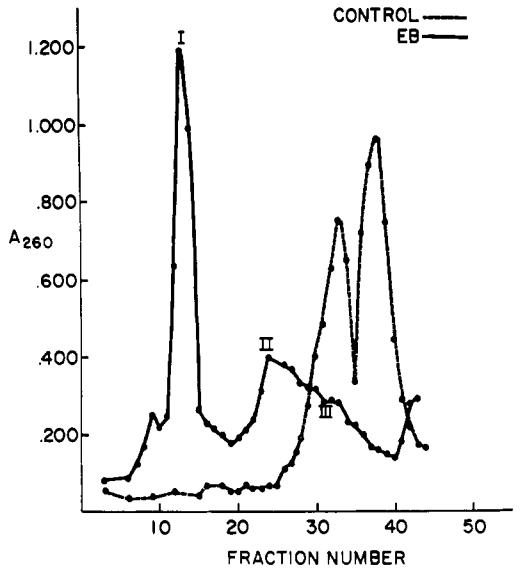

FIGURE 2: A_{260} profiles of ribosomes in CsCl equilibrium density gradients in the absence and presence of 1×10^{-4} M ethidium bromide.

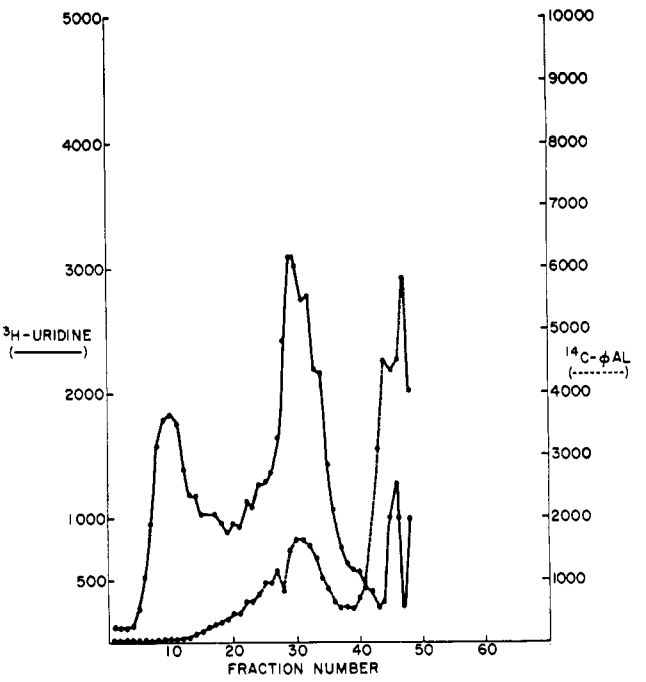

FIGURE 3: The distribution of ribosomal [^{3}H]uridine and [^{14}C]-phenylalanine (dpm) in a CsCl equilibrium density gradient containing 1×10^{-4} M ethidium bromide.

by ultracentrifugation. Particles of 37 S (II) and 43 S (III) were observed, in addition to heterogeneous, uv-absorbing material. It is possible that the 43 S particle was identical to the 42S core particle (Meselson *et al.*, 1964; Nomura and Erdmann, 1970) while the 37S particle may constitute a species of lower protein content in view of its greater buoyant density.

Ribosomal Enhancement of Ethidium Bromide Fluorescence. The fluorescence profiles of CsCl gradients containing ethidium bromide and ribosomes were congruent with the A_{260} and [^{3}H]uridine peaks. Since enhancement of ethidium bromide fluorescence in high salt has been shown to be specifically caused by intercalation into duplex regions of polyribonucleotides (LePecq and Paoletti, 1967), we have estimated the extent of intercalation into the RNA through measurement of the enhancement by ribosomes of ethidium bromide fluorescence.

E. coli 70S ribosomes were suspended in aliquots of concentrated CsCl, normally used for equilibrium gradient centrifugation, with 4×10^{-2} M Mg^{2+} and titrated with ethidium bromide. Figure 4 depicts the changes in fluorescence in a typical experiment in which ribosomes containing 2.86×10^{-7} mole of potential intercalation sites was titrated with ethidium bromide. Enhancement of fluorescence occurred until 1.98×10^{-7} mole of ethidium bromide was added. Approximately 66% of the potential intercalation sites were apparently occupied in the experiment depicted in Figure 4.

Discussion

The present work has shown that a number of drugs and dyes, known or presumed to bind to duplex DNA by intercalation, decreased the stability of *E. coli* ribosomes subjected to thermal or ionic stress. Quinine, which intercalates with a low stoichiometry (Estensen *et al.*, 1969) and primaquine, which binds to DNA in a nonintercalative manner (Morris *et al.*, 1970), did not labilize ribosomes.

We propose that the effects of the active compounds used in these studies are related to their intercalation into the duplex regions of rRNA. Evidence for this assumption is as follows. (1) Destabilization of ribosomes was a group property of strong DNA intercalators. (2) A direct correlation between the ribosome-labilizing potency of compounds and the fraction of bound drug estimated to be intercalated into supercoiled DNA (Waring, 1970); and (3) the enhancement of the fluorescence of ethidium bromide in the presence of ribosomes.

The hypothesis of drug or dye intercalation into ribosomes rests upon the extent to which the RNA is arranged in the form of duplex stretches. rRNA has been estimated to consist to approximately 70% (Attardi and Amaldi, 1970)

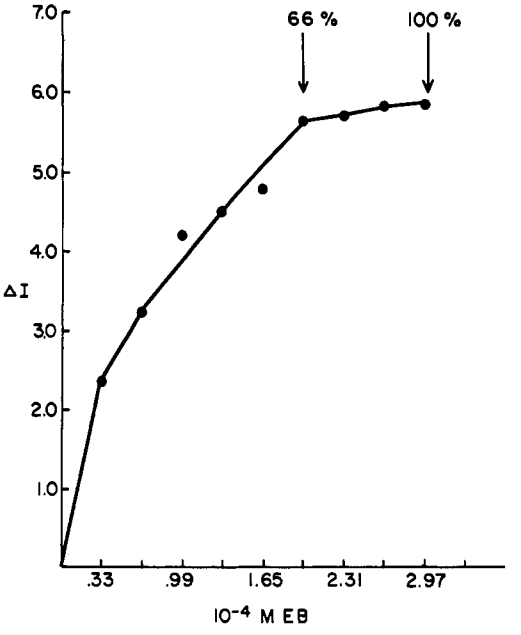

FIGURE 4: Titration of 629 μg of ribosomes with ethidium bromide in concentrated CsCl containing 4×10^{-2} M MgAc and 2×10^{-2} M Tris-HCl (pH 7.2). The diagram depicts the increase in fluorescence of ethidium bromide which occurred in the presence of ribosomes.

of double-stranded helical segments alternating with single-stranded RNA located principally in bends in the RNA chain (Cox, 1966; Spirin and Gavrilova, 1969). Our fluorometric titration of ribosomes is consistent with this estimate, and suggests that the secondary structure of RNA within the ribosomal particle is similar to that of purified RNA in solution (Spirin and Gavrilova, 1969). The function of the duplex regions in rRNA may be concerned with the binding and orientation of ribosomal proteins, especially of those which are constituents of "core particles" obtained in cesium chloride equilibrium centrifugation (Meselson *et al.*, 1964) or upon partial reassembly of ribosomes from their macromolecular constituents (Traub and Nomura, 1968; Nomura Erdmann, 1970). Reassembly experiments when carried out in the presence of ethidium bromide (Bollen *et al.*, 1970) also lead to the formation of incomplete ribonucleoprotein particles. This result as well as our disassembly experiments suggest that intercalation of ethidium bromide into rRNA either limits reassembly or causes partial disassembly of ribosomes because it interferes with protein–RNA interactions.

This interference may occur through dye- or drug-induced changes in the conformation of rRNA, or direct competition between intercalating compounds and proteins or Mg^{2+} for rRNA binding sites. Intercalation into DNA locally untwists, lengthens and stiffens the double helix (reviewed by Yang and Samejima, 1969), and intercalation into rRNA may well produce comparable configurational changes (Semmel and Daune, 1967; Popa and Repanovici, 1969).

The principal fact elicited from these studies is that a group of compounds which stabilize DNA to thermal hydrogen-bond breakage conversely labilize ribosomes to heat and, in the case of the phenanthridines, to ionic stress. That intercalants also interfere with DNA–protein interactions is borne out by observations that intercalants in general inhibit the enzymatic polymerization or hydrolysis of DNA and that proflavine prevents the association of protein and DNA to form bacteriophage (Demars, 1955).

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