Differential Effects of Various Double-Stranded RNAs on Protein Synthesis in Rabbit Reticulocyte Lysates†

Jean Content, Bernard Lebleu, and Erik De Clercq*,‡

ABSTRACT: There exist remarkable differences in the ability of various double-stranded RNAs (ds RNAs) to inhibit protein synthesis in rabbit reticulocyte lysates. Natural ds RNAs (either mycophage, bacteriophage, or reovirus ds RNA) are extremely potent inhibitors of protein synthesis, effecting nearly 90% inhibition at a concentration as low as 10 ng/mL. A similar inhibitory potency is exhibited by the alternating copolymer $(A\text{-}U)_n\cdot(A\text{-}U)_n$. However, homopolynucleotide pairs such as $(A)_n\cdot(U)_n$ and $(I)_n\cdot(C)_n$ are much less efficient inhibitors. To achieve an inhibitory effect with these homopolymer pairs, 10- to 100-fold higher concentrations were required than for the natural ds RNAs and the extent of inhibition never exceeded 50–60%. In some reticulocyte lysates $(I)_n\cdot(C)_n$ was totally inactive in inhibiting protein synthesis. Two analogues of $(I)_n\cdot(C)_n$, $(I)_n\cdot(br^5C)_n$, and $(I)_n\cdot(s^2C)_n$ were also inactive in inhibiting protein synthesis even at concentrations up to 10 μg/mL. In some aspects, e.g., molecular size, the interferon inducing capacity of ds RNAs and their inhibitory effect on protein synthesis appear to be governed by the same structural parameters. However, the observation that the natural ds RNAs, while much more efficient as inhibitors of protein synthesis (in rabbit reticulocyte lysates), proved considerably less effective in inducing interferon (in primary rabbit kidney cell cultures) than their homopolymer counterparts [$(I)_n\cdot(C)_n$, $(I)_n\cdot(br^5C)_n$, $(I)_n\cdot(s^2C)_n$] clearly indicates that the fine structural requirements underlying the interferon inducing and protein synthesis inhibiting properties of ds RNAs are not identical.

Double-stranded ribonucleic acids (ds RNAs)[1] exhibit a variety of biological activities, the most characteristic of which are interferon induction and inhibition of protein synthesis (Carter and De Clercq, 1974). As interferon production itself requires de novo protein synthesis, it may seem paradoxal that ds RNAs, while blocking protein synthesis in general, allow one particular protein, interferon, to be produced. One may indeed expect that, if the intracellular concentration of ds RNA rises too quickly, the protein machinery would be operationally closed before it could accommodate the incoming interferon mRNA. The outcome of the "race" between interferon production and inhibition of protein synthesis, and the interferon levels that are finally achieved, may be determined by compartmental differences (location of interferon trigger receptor relative to the polysomal machinery), kinetic differences (time to trigger interferon production vs. time needed to inhibit protein synthesis), as well as structure–function differences (structural requirements involved in interferon induction as opposed to structural requirements involved in the inhibition of protein synthesis).

The interferon inducing capacity of ds RNA depends on a number of rather well-defined determinants such as a sufficiently long base-paired structure (sedimentation value: ≥4 S) with sufficiently high thermal stability (T_m: ≥60 °C; 0.15 M NaCl) and resistance to degradation by nucleases (De Clercq, 1974; Torrence and De Clercq, 1977). To act as an interferon inducer, the ds RNA should be of the classical Watson–Crick base-pairing type with the two helices in antiparallel position. There seems to be little restriction of base composition or sequence, since both natural ds RNAs, e.g., mycophage, bacteriophage and reovirus ds RNA, and synthetic ds RNAs, whether homopolymer pairs such as $(I)_n\cdot(C)_n$ and $(A)_n\cdot(U)_n$ or alternating copolymers such as $(A\text{-}U)_n\cdot(A\text{-}U)_n$, have been shown to induce interferon (Torrence and De Clercq, 1977). The same stringent specificity for a properly matched base-paired structure and lack of specificity for nucleotide sequence have been noted for the inhibition of protein synthesis by ds RNA in rabbit reticulocyte lysates (Hunter et al., 1975). Hunter et al. (1975) found natural ds RNAs, homopolyribonucleotide pairs, and alternating copolyribonucleotides equally effective in inhibiting protein synthesis. The minimum effective size for inhibition of protein synthesis appeared to be about 50 base pairs or 4 S.

In designing the experiments reported herein we were wondering whether the interferon inducing properties of ds RNAs and their inhibitory effects on protein synthesis are governed by the same structural determinants. This question is of obvious relevance not only to the mechanism of interferon induction and inhibition of protein synthesis by ds RNA but also to the biological role of ds RNA molecules in general.

Materials and Methods

Polynucleotides. f_2 ds RNA was extracted from *E. coli* infected with f_2 phage (Doskočil et al., 1971); it was purified as described by Táborský et al. (1974) and provided by L. Bo-

† From the Pasteur Institute, Brussels, Belgium (J.C.), the Department of Molecular Biology, University of Brussels, Rhode St. Genese, Belgium (B.L.), and the Rega Institute for Medical Research, University of Leuven, Leuven, Belgium (E.D.C.). *Received May 31, 1977.* The work was supported by grants from the Belgian FNRS (Fonds National de la Recherche Scientifique), FGWO (Fonds voor Geneeskundig Wetenschappelijk Onderzoek), and the Fonds Derde Cyclus (Katholieke Universiteit Leuven).

‡ Author to whom correspondence should be addressed at the Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium.

[1] Abbreviations used: ds RNA, double-stranded RNA; PC, *Penicillium chrysogenum*; $(I)_n$, poly(inosinic acid); $(C)_n$, poly(cytidylic acid); $(A)_n$, poly(adenylic acid); $(U)_n$, poly(uridylic acid); $(I)_n\cdot(C)_n$, poly(inosinic acid)·poly(cytidylic acid); $(A)_n\cdot(U)_n$, poly(adenylic acid)·poly(uridylic acid); $(I)_n\cdot(s^2C)_n$, poly(inosinic acid)·poly(2-thiocytidylic acid); $(I)_n\cdot(br^5C)_n$, poly(inosinic acid)·poly(5-bromocytidylic acid); $(A\text{-}U)_n\cdot(A\text{-}U)_n$, alternating copolymer of adenylic acid and uridylic acid; $(I\text{-}C)_n\cdot(I\text{-}C)_n$, alternating copolymer of inosinic acid and cytidylic acid; $(G\text{-}C)_n\cdot(G\text{-}C)_n$, alternating copolymer of guanylic acid and cytidylic acid; NPL, nuclease-pretreated lysate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*-*N*′-tetraacetic acid.

0006-2960/78/0417-0088$01.00/0

recký (Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia). The sedimentation value of $f_2$ds RNA in 0.1 M NaCl–0.05 M trisodium citrate was 8.1 S.

Penicillium chrysogenum (PC) ds RNA was prepared from mycelial macerates (Lemke and Ness, 1970); it was kindly supplied by R. J. Douthart (Lilly Research Laboratories, Indianapolis, Ind.).

Reovirus ds RNA was extracted from purified reovirus type 3 (Dearing strain) virions. The virus stock was a gift from J. Wérenne (International Institute of Cellular and Molecular Pathology, Brussels, Belgium). The ds RNA was extracted with phenol (+ 1% sodium dodecyl sulfate), precipitated with ethanol and finally dissolved in H_2O at 1 mg/mL.

$(I)_n$·$(C)_n$ and $(A)_n$·$(U)_n$ were constituted from homopolymers which were all obtained from P-L Biochemicals (Milwaukee, Wis.). Sedimentation values ($s_{20,w}$) (according to the manufacturer) were as follows: 9.4 S for $(I)_n$, 10.0 S for $(C)_n$, 9.8 S for $(A)_n$, and 7 S for $(U)_n$.

$(I)_n$·[^3H]$(C)_n$ was constituted from homopolymers which had been obtained from Miles Laboratories (Elkhart, Ind.). The sedimentation values were 4.8 S and 6.5 S, respectively. The specific activity of [^{3}H]$(C)_n$ was 65.6 μCi/μmol of P or 1 μCi per 6.1 μg of polymer.

$(I)_{12.5}$·$(C)_{13.2}$, *$(I)_{12.5}$·$(C)_{3.1}$*, *$(I)_{2.5}$·$(C)_{13.2}$*, and *$(I)_{2.5}$·$(C)_{3.1}$* refer to complexes of homopolymers with different sedimentation values (as indicated by the manufacturer, P-L Biochemicals, Milwaukee, Wis.). The individual sedimentation values are designated by the subscripts.

$(I)_n$·$(br^5C)_n$ was prepared as described previously (De Clercq et al., 1976); $s_{20,w}$ value of $(br^5C)_n$, 10.3 S. It was generously provided by P. F. Torrence (Laboratory of Chemistry, NIAMDD, National Institutes of Health, Bethesda, Md.).

$(I)_n$·$(s^2C)_n$ was kindly provided by K. Reuss (E. Merck, Darmstadt, Germany). The physicochemical and biological properties of $(I)_n$·$(s^2C)_n$ have been described recently (Reuss et al., 1976). The alternating copolymers *$(A\text{-}U)_n$·$(A\text{-}U)_n$*, *$(I\text{-}C)_n$·$(I\text{-}C)_n$*, and *$(G\text{-}C)_n$·$(G\text{-}C)_n$* were purchased from Biogenics Research Corp. (Chagrin Falls, Ohio) (Lot 377, 247, and 391, respectively).

The 9S globin mRNA was isolated by phenol extraction from rabbit reticulocyte polysomes; it was further purified by oligo(dT)-cellulose chromatography and sucrose gradient centrifugation as described previously (Soreq et al., 1974).

Other Materials. E. coli tRNA was purchased from Schwarz/Mann (Orangeburg, N.Y.); EGTA and hemin were from Sigma Chemical Co. (St. Louis, Mo.); micrococcal nuclease (EC 3.1.4.7; 29 000 U/mg) was from P-L Biochemicals (Milwaukee, Wis.); creatine phosphate and creatine phosphokinase were from Boehringer (Mannheim, Germany); and the radiolabeled amino acids were from the radiochemical Centre (Amersham, United Kingdom).

Preparation of Reticulocyte Lysates. Reticulocytes were obtained from phenylhydrazine-treated rabbits according to the procedure described by Housman et al. (1970). The lysates were prepared from individual rabbits (Pelham and Jackson, 1976) and stored in liquid nitrogen. For those experiments where the translation of exogenous mRNA was investigated, the lysates were first depleted from their endogenous mRNA activity by incubation with micrococcal nuclease (EC 3.1.4.7) in the presence of Ca^{2+}. The nuclease treatment was stopped with EGTA [ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid] and the nuclease-preincubated lysates (NPL) were stored in liquid nitrogen. For the preparation of NPL, the procedure described by Pelham and Jackson (1976) was slightly modified (Content et al., 1977).

Endogenous Protein Synthesis in Reticulocyte Lysates. To obtain maximal inhibition of protein synthesis by ds RNAs the procedure of Hunter et al. (1975) was applied. Briefly, the lysates were preincubated for 30 min at 30 °C in the presence of various ds RNA concentrations, 0.4 mM $MgCl_2$, 95 mM KCl, 10 mM creatine phosphate, 50 μg/mL of creatine phosphokinase, 25 μM hemin, and 110 μM of a mixture of all essential amino acids except the radiolabeled amino acids which were added after the preincubation period. Protein synthesis was started upon addition of these missing labeled amino acids. To monitor protein synthesis, we used either [4,5-^{3}H]leucine (230 μCi/mL; final specific activity, 23 Ci/mmol) or [^{35}S]-methionine (250 μCi/mL; final specific activity, 100 Ci/mmol) or a mixture of 14 ^{14}C-labeled amino acids from algal hydrolysate (5 μCi/mL; final specific activity, 54 Ci/carbon atom). The assay mixtures were then incubated at 30 °C for another 30 min (except when indicated otherwise) at which time an aliquot of the reaction mix was spotted onto 3 MM Whatman paper disks to measure hot Cl_3CCOOH insoluble radioactivity (Roberts and Paterson, 1973). Controls without ds RNA and with or without hemin were included in all experiments.

Exogenous Protein Synthesis in Reticulocyte Lysates. NPL were preincubated for 20 min at 30 °C in the presence of various ds RNA concentrations as described above for endogenous protein synthesis. Protein synthesis was started upon addition of the appropriate labeled amino acids (at the concentrations indicated above) and 30 μg/mL of purified rabbit globin mRNA. The assay mixtures were then incubated at 30 °C for another 30 min at which time they were processed for Cl_3CCOOH-precipitable radioactivity.

Sucrose Gradient Ultracentrifugation. To monitor the size of $(I)_n$·[^{3}H]$(C)_n$, 100-μL samples (containing 10 μg of polynucleotide) were layered onto 4 mL of linear 10–25% w/v sucrose gradients in 10 mM Tris-HCl, pH 7.4, 0.1 M LiCl, 1 mM EDTA, 1% sodium dodecyl sulfate in polyallomer tubes and centrifuged at 60 000 rpm for 210 min at 20 °C in a Beckman SW 60 Ti rotor. *E. coli* tRNA was used as a marker. A total of 500 μg of tRNA (in 100 μL of 10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM EDTA) was layered onto the sucrose gradient. At the end of the centrifugation run, the gradients were collected from the bottoms of the tubes. After 200 μL of water was added to each fraction, absorbance was measured in a Gilford spectrophotometer. Then 5 mL of Bray's scintillation fluid was added and the total radioactivity was determined in a Packard 3375 liquid scintillation spectrometer.

Interferon Induction Measurements. Interferon production was measured in primary rabbit kidney cells "superinduced" with cycloheximide and actinomycin D according to well-established procedures (De Clercq et al., 1976).

Results

Inhibition of Endogenous Protein Synthesis in Reticulocyte Lysates by Various ds RNAs. Dose-Response Effect. The inhibitory effects of ds RNAs on protein synthesis in reticulocyte lysates are particularly pronounced if the polynucleotides are preincubated with the lysate before protein synthesis can occur (Hunter et al., 1975). Even under these optimal conditions, the homopolymers $(U)_n$, $(A)_n$, $(I)_n$, and $(C)_n$ did not display an inhibitory effect, unless fairly high concentrations were employed (10^4 ng/mL) (Figure 1A). Unlike the single homopolymers, the homopolymer pairs $(A)_n$·$(U)_n$ and $(I)_n$·$(C)_n$ exerted an inhibitory effect at 100 and 10 ng/mL, respectively (Figure 1B). The maximal inhibition achieved by $(A)_n$·$(U)_n$ and $(I)_n$·$(C)_n$ did not exceed 60%. Under the same experimental conditions, f_2 ds RNA inhibited protein synthesis

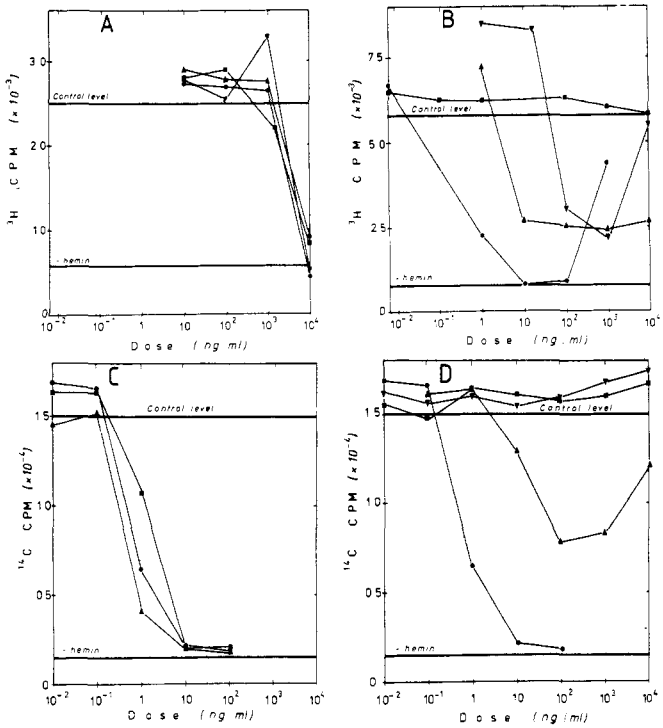

FIGURE 1: Inhibition of endogenous protein synthesis in reticulocyte lysates by different concentrations of synthetic homopolynucleotides (panel A), synthetic homopolynucleotide pairs (panel B), natural ds RNAs (panel C), and $(I)_n \cdot (C)_n$ analogues (panel D). The reticulocyte lysates were preincubated for 30 min at 30 °C in the presence of different polynucleotide concentrations (as indicated in the abscissa) and in the absence of either leucine (panels A and B) or a total of 14 amino acids (panels C and D). Protein synthesis was started upon addition of either [^{3}H]leucine (panels A and B) or the mixture of 14 ^{14}C-labeled amino acids (panels C and D). The reaction was stopped after an additional incubation of 30 min at 30 °C. Final volume of the reaction mixtures was 15 μL. The results are presented as ^{3}H cpm per 3 μL (panel A), ^{3}H cpm per 5 μL (panel B), or ^{14}C cpm per 13 μL (panels C and D). The levels of endogenous protein synthesis obtained in the presence and absence of hemin are indicated for each individual set of experiments. The data shown in panels A and B were obtained with a reticulocyte lysate (batch 191) that differed from the batch (241) used in the experiments presented in panels C and D. (Panel A) (●) $(I)_n$; (▲) $(C)_n$; (■) $(U)_n$; (▼) $(A)_n$. (Panel B) (●) f_2 ds RNA; (▲) $(I)_n \cdot (C)_n$; (■) $(I)_n \cdot (br^5C)_n$; (▼) $(A)_n \cdot (U)_n$. (Panel C) (●) f_2 ds RNA; (▲) reovirus ds RNA; (■) PC ds RNA. (Panel D) (●) f_2 ds RNA; (▲) $(I)_n \cdot [^3H](C)_n$; (■) $(I)_n \cdot (br^5C)_n$; (▼) $(I)_n \cdot (s^2C)_n$.

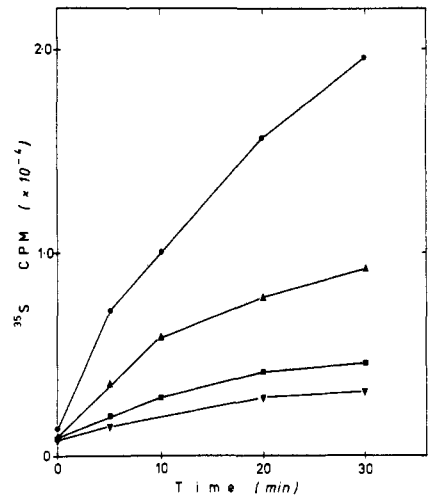

FIGURE 2: Kinetics of endogenous protein synthesis in reticulocyte lysates in the presence of $(I)_n \cdot (C)_n$ or f_2 ds RNA, both at 100 ng/mL. The reticulocyte lysates were preincubated in the presence of f_2 ds RNA or $(I)_n \cdot (C)_n$ but in the absence of methionine. Protein synthesis was started upon addition of [^{35}S]methionine (time zero). It was stopped after different times (as indicated in the abscissa). The results are presented as ^{35}S cpm per 2 μL. (●) Control; (▲) $(I)_n \cdot (C)_n$; (■) minus hemin; (▼) f_2 ds RNA.

at a concentration as low as 1 ng/mL and the maximal inhibition it achieved nearly reached 90% (Figure 1B). In fact, all natural ds RNAs, whether originating from fungal, bacterial, or mammalian viruses, proved to be significantly more effective in inhibiting protein synthesis than the synthetic homopolymer pairs. This conclusion was based on both the extent of inhibition and the concentration required to obtain this inhibition. The doses needed for 50% inhibition of protein synthesis amounted to 0.5 ng/mL (reovirus ds RNA), 1 ng/mL (f_2 ds RNA), and 3 ng/mL (PC ds RNA) (Figure 1C). For all these natural ds RNAs, the extent of inhibition reached 90%. In marked contrast herewith, $(I)_n \cdot (s^2C)_n$ and $(I)_n \cdot (br^5C)_n$ did not cause any inhibition of protein synthesis, even at concentrations up to 10^4 ng/mL (Figure 1D). When assayed under the same conditions, $(I)_n \cdot (C)_n$ caused a partial reduction in protein synthesis (not exceeding 50%) and this only at 100–1000 ng/mL (Figure 1D). It should be emphasized that $(I)_n \cdot (C)_n$ exhibited a variable inhibitory effect on protein synthesis, when the same $(I)_n \cdot (C)_n$ preparation was assayed in different reticulocyte lysates (originating from different rabbits) or when different $(I)_n \cdot (C)_n$ preparations were compared in the same lysate system: for example, Figures 1B and 1D represent the effects of two different $(I)_n \cdot (C)_n$ preparations in two different lysate systems (batches 191 and 241, respectively). It is clear that in lysate 191 $(I)_n \cdot (C)_n$ exerted a greater inhibition of protein synthesis than in lysate 241. Yet, the $(I)_n \cdot [^3H](C)_n$ preparation referred to in Figure 1D was a more active preparation than the $(I)_n \cdot (C)_n$ preparation referred to in Figure 1B. If the latter preparation was used with lysate 241, no inhibition of protein synthesis was observed even at concentrations up to 10^3 ng/mL. $(I)_n \cdot (br^5C)_n$ did not show any hint of inhibitory activity, whether it was assayed with lysate 191 (Figure 1B) or lysate 241 (Figure 1D). The inhibitory activity of the natural ds RNAs was not influenced by the lysate batch number. Perfectly reproducible patterns of protein synthesis inhibition (similar to those depicted in Figure 1C) were obtained if the natural ds RNAs were assayed with different lysate batches.

Kinetics of Protein Synthesis. If the ds RNA is added to the reticulocyte lysate at the start of the normal incubation (period of protein synthesis), there is a latent phase during which protein synthesis remains unaffected (Hunter et al., 1975). The lag before onset of inhibition disappears if the ds RNA is present during the preincubation period. As shown in Figure 2, addition of either f_2 ds RNA or $(I)_n \cdot (C)_n$ to the lysate during the preincubation period resulted in a reduced rate of protein synthesis. Inhibition of protein synthesis was apparent as soon as it could be measured (after 5 min). f_2 ds RNA had a significantly greater inhibitory effect on the rate of protein synthesis than $(I)_n \cdot (C)_n$.

Inhibition of Exogenous Protein Synthesis in Reticulocyte Lysates by Various ds RNAs. Dose–Response Effect. Since ds RNAs are assumed to specifically inhibit the initiation of protein synthesis in reticulocyte lysates, we determined their inhibitory effects on protein synthesis programmed by globin mRNA that was added exogenously to the reticulocyte lysates. Therefore, the endogenous mRNA activity of the lysates was eliminated by pretreatment of the lysates with micrococcal nuclease. The nuclease-pretreated lysates (NPL) efficiently translated exogenous globin mRNA (Figure 3). Reovirus ds RNA, f_2 ds RNA, and PC ds RNA caused a dramatic inhibition of exogenous globin synthesis (Figure 3A). The doses required for 50% inhibition of exogenous protein synthesis

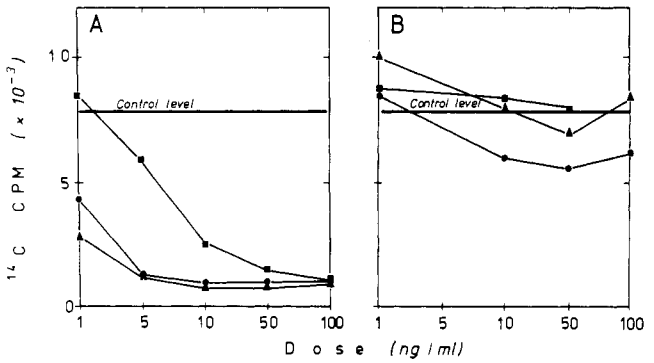

FIGURE 3: Inhibition of exogenous protein synthesis in reticulocyte lysates by different concentrations of natural ds RNAs (panel A) and synthetic homopolynucleotide pairs (panel B). The NPL (reticulocyte lysates which had been pretreated with micrococcal nuclease) were preincubated for 20 min at 30 °C in the presence of different polynucleotide concentrations (as indicated in the abscissa) and in the absence of a total of 14 amino acids. Protein synthesis was initiated upon additon of purified rabbit globin mRNA (at a final concentration of 30 μg/mL) and the appropriate 14 ^{14}C-labeled amino acids. The reaction was stopped after an additional incubation of 30 min at 30 °C. The results are presented as ^{14}C cpm per 12 μL. The endogenous activity in these assay mixtures amounted to 112 cpm. This value represents only 1.3% of the total activity and was not subtracted from the data shown. (Panel A) (●) f_2 ds RNA; (▲) reovirus ds RNA; (■) PC ds RNA. (Panel B) (●) $(I)_n \cdot (C)_n$; (▲) $(I)_n \cdot (br^5C)_n$; (■) $(I)_n \cdot (s^2C)_n$.

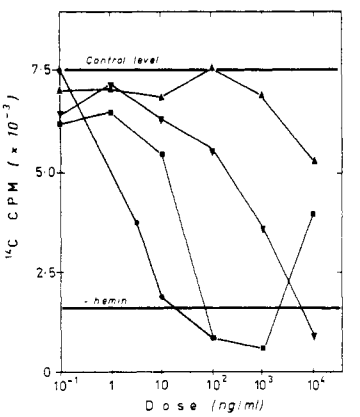

FIGURE 4: Inhibition of endogenous protein synthesis in reticulocyte lysates by different concentrations of alternating copolynucleotides. The assay conditions were the same as described for Figure 1 (panels C and D). The results are presented as ^{14}C cpm per 13 μL. (●) f_2 ds RNA; (▲) $(I\text{-}C)_n \cdot (I\text{-}C)_n$; (■) $(A\text{-}U)_n \cdot (A\text{-}U)_n$; (▼) $(G\text{-}C)_n \cdot (G\text{-}C)_n$.

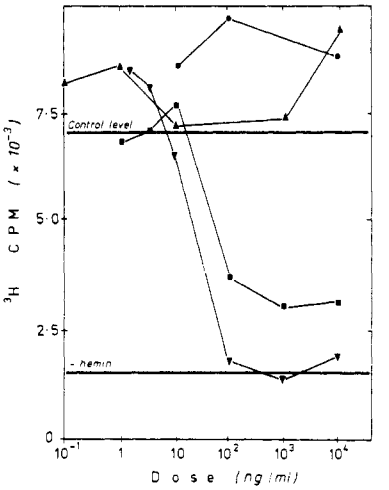

FIGURE 5: Inhibition of endogenous protein synthesis in reticulocyte lysates by different concentrations of $(I)_n \cdot (C)_n$ preparations of varying molecular size. The assay conditions were the same as described for Figure 1 (panel B) (reticulocyte lysate batch 191). The results are presented as ^{3}H cpm per 5 μL. (●) $(I)_{2.5} \cdot (C)_{3.1}$; (▲) $(I)_{2.5} \cdot (C)_{13.2}$; (■) $(I)_{12.5} \cdot (C)_{3.1}$; (▼) $(I)_{12.5} \cdot (C)_{13.2}$.

corresponded well with those required for 50% inhibition of endogenous protein synthesis (Figure 1C). Again, the maximal inhibition achieved by the natural ds RNAs reached 90% (Figure 3A). On the contrary, the synthetic homopolymer pairs $(I)_n \cdot (C)_n$, $(I)_n \cdot (br^5C)_n$, and $(I)_n \cdot (s^2C)_n$ exerted little, if any, inhibitory effect on exogenous protein synthesis (Figure 3B). The maximal inhibitory effect attained by $(I)_n \cdot (C)_n$ did not exceed 30% (Figure 3B).

Alternating Copolynucleotides. Three commercially available alternating copolymers, $(A\text{-}U)_n \cdot (A\text{-}U)_n$, $(I\text{-}C)_n \cdot (I\text{-}C)_n$, and $(G\text{-}C)_n \cdot (G\text{-}C)_n$ were compared with f_2 ds RNA for their inhibitory activity on endogenous protein synthesis in reticulocyte lysates. In keeping with previous data (Hunter et al., 1975), $(A\text{-}U)_n \cdot (A\text{-}U)_n$ proved nearly as effective in inhibiting protein synthesis as f_2 ds RNA (Figure 4). Fifty percent inhibition of protein synthesis was obtained at an $(A\text{-}U)_n \cdot (A\text{-}U)_n$ concentration of 3 ng/mL. To obtain a similar inhibition with $(G\text{-}C)_n \cdot (G\text{-}C)_n$, 100 ng/mL was required (Figure 4). No inhibitory effect was observed for $(I\text{-}C)_n \cdot (I\text{-}C)_n$ unless the concentration was increased up to 1 μg/mL.

$(I)_n \cdot (C)_n$ Preparations of Varying Molecular Size. Next, we examined the influence of molecular size. $(I)_n$ and $(C)_n$ preparations of different molecular size [$s_{20,w}$: 2.5 and 12.5 S for $(I)_n$; 3.1 and 12.2 S for $(C)_n$] were annealed at a 1:1 mol P stoichiometry. The $(I)_{2.5} \cdot (C)_{3.1}$, $(I)_{2.5} \cdot (C)_{13.2}$, $(I)_{12.5} \cdot (C)_{3.1}$, and $(I)_{12.5} \cdot (C)_{13.2}$ complexes were then assayed for inhibition of endogenous protein synthesis in reticulocyte lysates. The complexes composed of a high molecular size $(I)_n$ (12.5 S) were both active in inhibiting protein synthesis. The 50% inhibitory doses of these complexes fell in the range of 50 to 100 ng/mL (Figure 5). The low molecular size $(I)_n$ (2.5 S), whether annealed to a low or high molecular size $(C)_n$ (3.1 or 13.2 S), did not exhibit an inhibitory activity in the reticulocyte cell-free system (Figure 5).

Integrity of $(I)_n \cdot (C)_n$ upon Incubation with the Reticulocyte Lysates. To account for the variable sensitivity of different lysate systems (obtained from different rabbits) to the inhibitory activity of $(I)_n \cdot (C)_n$, one might assume the presence in some lysates of a nuclease that could specifically degrade ds RNAs such as $(I)_n \cdot [^3H](C)_n$ (Figure 6), suggesting that no degradation of $(I)_n \cdot [^3H](C)_n$ occurred during its incubation with the reticulocyte lysates.

Inhibition of Protein Synthesis as Compared with Interferon Induction. To a certain extent the inhibitory effects of ds RNAs on protein synthesis (in reticulocyte lysate systems) and their ability to induce interferon (in cultured cells) appear to be determined by similar structural parameters. For a series of $(I)_n \cdot (C)_n$ complexes of various molecular size, both the interferon inducing ability and the inhibition of protein synthesis depended on maintaining a high molecular size of $(I)_n$. The size of $(C)_n$ was of little or no consequence to either of these effects (Table I). However, when natural ds RNAs and synthetic homopolynucleotide pairs were compared for their interferon inducing activities and protein synthesis inhibiting properties, an inverse correlation was noted. Natural ds RNAs inhibited protein synthesis at concentrations which were several orders of magnitude lower than those at which homopolymer pairs such as $(I)_n \cdot (C)_n$, $(I)_n \cdot (br^5C)_n$, and $(I)_n \cdot (s^2C)_n$ showed inhibition. Yet, the homopolymer pairs proved far more effective than the natural ds RNAs in inducing interferon in

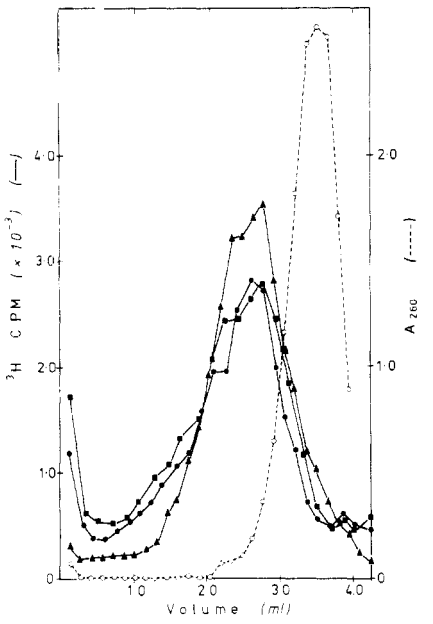

FIGURE 6: Sucrose gradient analysis of $(I)_n \cdot [^3H](C)_n$ after incubation with the reticulocyte lysates. $(I)_n \cdot [^3H](C)_n$ (10 μg/10 μL; 1500 ^{3}H cpm per μg) was first incubated with 100 μL of either reticulocyte lysate batch 191 or batch 241 for 30 min at 30 °C, and then layered onto the sucrose gradients (see Materials and Methods for further details). The lysate batches 191 and 241 originated from different rabbits (see also legend to Figure 1). *E. coli* tRNA was included as a marker (O-----O). (▲—▲) Control $(I)_n \cdot [^3H](C)_n$. (■—■) $(I)_n \cdot [^3H](C)_n$ incubated with reticulocyte lysate 191. (●—●) $(I)_n \cdot [^3H](C)_n$ incubated with reticulocyte lysate 241.

cultured cells (Table II). The alternating copolymers $(A\text{-}U)_n \cdot (A\text{-}U)_n$, $(I\text{-}C)_n \cdot (I\text{-}C)_n$, and $(G\text{-}C)_n \cdot (G\text{-}C)_n$ displayed an intermediate behavior. On the one hand, $(A\text{-}U)_n \cdot (A\text{-}U)_n$ inhibited protein synthesis at a concentration which was only tenfold higher than the concentration at which f_2 ds RNA showed inhibition. On the other hand, $(A\text{-}U)_n \cdot (A\text{-}U)_n$ was only ten times less effective than $(I)_n \cdot (C)_n$ in stimulating interferon production (Table II).

Discussion

Globin synthesis in rabbit reticulocyte lysates is exquisitely sensitive to inhibition by ds RNA. In fact, Robertson and Hunter (1975) considered inhibition of globin synthesis as the most sensitive method to detect double-helical RNA, since it responded to ds RNA concentrations as low as 0.1 ng per mL. Various ds RNAs have been shown to inhibit protein synthesis in reticulocyte lysates: e.g., $(I)_n \cdot (C)_n$ (Shenk and Stollar, 1972; Hunter et al., 1975; Grill et al., 1976), $(A)_n \cdot (U)_n$ (Hunter et al., 1975), $(A\text{-}U)_n \cdot (A\text{-}U)_n$ (Hunter et al., 1975), reovirus ds RNA (Hunter et al., 1975; Grill et al., 1976), *Penicillium chrysogenum* ds RNA (Hunter et al., 1975), ds RNA extracted from nuclei of HeLa cells (Bases and Kaplan, 1973) and human leukemic blast cells (Torelli et al., 1975), and the replicative ds RNA forms of poliovirus (Hunt and Ehrenfeld, 1971; Ehrenfeld and Hunt, 1971), sindbis virus (Shenk and Stollar, 1972), cowpea mosaic virus (Reijnders et al., 1975) and f_2 bacteriophage (Hunter et al., 1975).

Penicillium chrysogenum ds RNA, reovirus ds RNA, and f_2 (replicative) ds RNA have also been reported to inhibit the translation of encephalomyocarditis viral RNA in Krebs II ascites cell extracts (Robertson and Mathews, 1973), although the ds RNA concentrations at which inhibition of protein synthesis in Krebs cell extracts was achieved were considerably higher than those required to inhibit globin synthesis in reticulocyte lysates. Robertson and Mathews (1973) ascribed the lowered sensitivity of the Krebs II ascites cell extract to the presence in the extract of a nuclease that readily digested both natural and synthetic ds RNAs. Apparently, rabbit reticulocyte lysates do not contain such nuclease (Figure 6).

Other cell-free extracts which have been employed to demonstrate the inhibition of protein synthesis by ds RNA include mouse L cell extracts (Graziadei and Lengyel, 1972), Hela cell extracts (Celma and Ehrenfeld, 1974) as well as extracts prepared from interferon-treated L cells (Kerr et al., 1974, 1976; Roberts et al., 1976a,b) and interferon-treated Ehrlich ascites cells (Brown et al., 1976; Sen et al., 1976; Lebleu et al., 1976). Not all cell-free protein synthesis systems respond to the translation-inhibiting properties of ds RNA: e.g., Reijnders et al. (1975) and Grill et al. (1976) were unable to demonstrate an inhibitory effect of ds RNA on protein synthesis in wheat germ extracts.

While Hunter et al. (1975) found natural ds RNAs, synthetic homopolynucleotide pairs, and alternating copolynucleotides equally effective in inhibiting protein synthesis in rabbit reticulocyte lysates, we clearly established that there

TABLE I: Molecular Size Requirements for Inhibition of Protein Synthesis and Interferon Induction by $(I)_n \cdot (C)_n$.

Polymer	Rel interferon inducing potency[a]	Rel protein synthesis inhibiting potency[b]
$(I)_{12.5} \cdot (C)_{13.2}$	1	1
$(I)_{12.5} \cdot (C)_{3.1}$	1	0.5
$(I)_{2.5} \cdot (C)_{13.2}$	0.02	<0.005
$(I)_{2.5} \cdot (C)_{3.1}$	0.02	<0.005

[a] Ratio of interferon titer obtained for each particular $(I)_n \cdot (C)_n$ sample to interferon titer obtained for $(I)_{12.5} \cdot (C)_{13.2}$. All polymers were tested at 10 μg/mL (2×10^{-5} M in P) in primary rabbit kidney cells superinduced with cycloheximide and actinomycin D (De Clercq et al., 1976). [b] Ratio of ID_{50} (dose inhibiting protein synthesis by 50%) of $(I)_{12.5} \cdot (C)_{13.2}$ to ID_{50} of the other $(I)_n \cdot (C)_n$ samples. The ID_{50} values were calculated from the data presented in Figure 5.

TABLE II: Relative Interferon Inducing Capacity and Protein Synthesis Inhibiting Capacity of Different Classes of Double-Stranded RNAs.

Double-stranded RNA	Rel interferon inducing potency[a]	Rel protein synthesis inhibiting potency[b]
Class I: natural ds RNAs		
PC ds RNA	0.01-0.02	0.3
Reovirus ds RNA	0.01-0.02	2
f_2 ds RNA	0.01-0.02	1
Class II: homopolymer pairs		
$(I)_n \cdot (C)_n$	1	<0.01-0.0001
$(I)_n \cdot (br^5C)_n$	1	<0.0001
$(I)_n \cdot (s^2C)_n$	1	<0.0001
Class III: alternating copolymers		
$(A\text{-}U)_n \cdot (A\text{-}U)_n$	0.1	0.1
$(I\text{-}C)_n \cdot (I\text{-}C)_n$	0.01	<0.0003
$(G\text{-}C)_n \cdot (G\text{-}C)_n$	0.005	0.003

[a] Ratio of interferon titer obtained for each particular ds RNA to interferon titer obtained for $(I)_n \cdot (C)_n$. All polymers were tested at 10 μg/mL (2×10^{-5} M in P) in primary rabbit kidney cells superinduced with cycloheximide and actinomycin D (De Clercq et al., 1976). [b] Ratio of ID_{50} (dose inhibiting protein synthesis by 50%) of f_2 ds RNA to ID_{50} of the other ds RNAs. The ID_{50} values were calculated from the data presented in Figures 1 (C, D) and 4.

exist pronounced differences in the protein synthesis inhibiting properties of these three classes of ds RNAs. The natural ds RNAs of either mycophage, bacteriophage, or reovirus suppressed the translation of both endogenous and exogenous globin mRNA at concentrations which were at least four orders of magnitude lower than those at which the homopolymer pairs $(I)_n \cdot (C)_n$, $(I)_n \cdot (br^5C)_n$, and $(I)_n \cdot (s^2C)_n$ showed inhibition. The alternating copolymers $(A\text{-}U)_n \cdot (A\text{-}U)_n$, $(I\text{-}C)_n \cdot (I\text{-}C)_n$, and $(G\text{-}C)_n \cdot (G\text{-}C)_n$ exhibited an intermediate behavior. Of these alternating copolymers, $(A\text{-}U)_n \cdot (A\text{-}U)_n$ most closely approached the natural ds RNAs in inhibitory activity.

Striking similarities exist in the structural features of ds RNAs which are recognized by anti-ds RNA antibody and those which are required for interferon induction (Johnston et al., 1975). These similarities suggest that the cellular receptor site involved in the interferon induction process is protein in nature. The stringent specificity for ds RNA coupled with the lack of specificity for a particular base sequence suggests that the shutoff by double-stranded polyribonucleotides of the initiation of protein synthesis in reticulocyte lysates also depends on an interaction of the ds RNA with protein rather than nucleic acid (Hunter et al., 1975).

Some structural features which determine the interferon inducing capacity of ds RNAs seem also applicable to their inhibitory activity on protein synthesis: e.g., the necessity of a threshold molecular size of 4 S or approximately 50 base pairs (De Clercq, 1974; Hunter et al., 1975). For $(I)_n \cdot (C)_n$, the interferon inducing ability is more dependent upon maintaining a high molecular size of $(I)_n$ than of $(C)_n$ (Tytell et al., 1970; Stewart and De Clercq, 1974). This appears also to be the case for the inhibitory effect of $(I)_n \cdot (C)_n$ on protein synthesis (Figure 5). The fact that $(A)_n \cdot (U)_n$ was about ten times less active than $(I)_n \cdot (C)_n$ both in the inhibition of protein synthesis (Figure 1B) and the induction of interferon (De Clercq et al., 1974) also points to some convergence in the structural requirements for interferon induction and inhibition of protein synthesis.

There are, however, remarkable differences in the interferon inducing and protein synthesis inhibiting properties of natural (mycophage, bacteriophage, and reovirus) ds RNAs and synthetic homopolynucleotide pairs [$(I)_n \cdot (C)_n$, $(I)_n \cdot (br^5C)_n$, and $(I)_n \cdot (s^2C)_n$]. The former are extremely potent inhibitors of protein synthesis, yet relatively weak inducers of interferon (Table II). The latter are less efficient, if not inefficient, as protein synthesis inhibitors, and yet behave as superior interferon inducers (Table II). One might postulate, therefore, that the inferior interferon inducing behavior of natural ds RNAs is somehow related to their pronounced inhibitory effect on cellular protein synthesis. However, our attempts to directly demonstrate an inhibitory effect of the natural ds RNAs on protein synthesis in intact cells (primary rabbit kidney cells, human skin fibroblasts) have so far been unsuccessful. This failure contrasts with the findings of Cordell-Stewart and Taylor (1971, 1973a,b) who reported that addition of bovine enterovirus (replicative) ds RNA to intact cell cultures (e.g., mouse L cells and L 1210 cells) led to a cessation of cellular protein synthesis.

Acknowledgments

We thank L. De Wit and Anita Van Lierde for expert technical assistance.

References

Bases, R., and Kaplan, B. H. (1973), *Biochim. Biophys. Acta 312,* 574–580.

Brown, G. E., Lebleu, B., Kawakita, M., Shaila, S., Sen, G. C., and Lengyel, P. (1976), *Biochem. Biophys. Res. Commun. 69,* 114–122.

Carter, W. A., and De Clercq, E. (1974), *Science 186,* 1172–1178.

Celma, M. L., and Ehrenfeld, E. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2440–2444.

Content, J., De Wit, L., and Horisberger, M. (1977), *J. Virol. 22,* 247–255.

Cordell-Stewart, B., and Taylor, M. W. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1326–1330.

Cordell-Stewart, B., and Taylor, M. W. (1973a), *J. Virol. 11,* 232–237.

Cordell-Stewart, B., and Taylor, M. W. (1973b), *J. Virol. 12,* 360–366.

De Clercq, E. (1974), *Top. Curr. Chem. 52,* 173–208.

De Clercq, E., Edy, V. G., Torrence, P. F., Waters, J. A., and Witkop, B. (1976), *Mol. Pharmacol. 12,* 1045–1051.

De Clercq, E., Torrence, P. F., and Witkop, B. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 182–186.

Doskočil, J., Fuchsberger, N., Vetrák, J., Lackovič, V., and Borecký, L. (1971), *Acta Virol. 15,* 523.

Ehrenfeld, E., and Hunt, T. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1075–1078.

Graziadei, W. D., III, and Lengyel, P. (1972), *Biochem. Biophys. Res. Commun. 46,* 1816–1823.

Grill, L. K., Sun, J. D., and Kandel, J. (1976), *Biochem. Biophys. Res. Commun. 73,* 149–151.

Housman, D., Jacobs-Lorena, M., Rajbhandary, U. L., and Lodish, H. F. (1970), *Nature (London), 227,* 913–918.

Hunt, T., and Ehrenfeld, E. (1971), *Nature (London) 230,* 91–94.

Hunter, T., Hunt, T., Jackson, R. J., and Robertson, H. D. (1975), *J. Biol. Chem. 250,* 409–417.

Johnston, M. I., Stollar, B. D., Torrence, P. F., and Witkop, B. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4564–4568.

Kerr, I. M., Brown, R. E., and Ball, L. A. (1974), *Nature (London) 250,* 57–59.

Kerr, I. M., Brown, R. E., Clemens, M. J., and Gilbert, C. S. (1976), *Eur. J. Biochem. 69,* 551–561.

Lebleu, B., Sen, G. C., Shaila, S., Cabrer, B., and Lengyel, P. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3107–3111.

Lemke, P. A., and Ness, T. M. (1970), *J. Virol. 6,* 813–819.

Pelham, H. R. B., and Jackson, R. J. (1976), *Eur. J. Biochem. 67,* 247–256.

Reijnders, L., Aalbers, A. M. J., Van Kammer, A., and Berns, A. J. M. (1975), *Biochim. Biophys. Acta 390,* 69–77.

Reuss, K., Scheit, K.-H., and Saiko, O. (1976), *Nucleic Acids Res. 3,* 2861–2875.

Roberts, B. E., and Paterson, B. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2330–2334.

Roberts, W. K., Clemens, M. J., and Kerr, I. M. (1976a), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3136–3140.

Roberts, W. K., Hovanessian, A., Brown, R. E., Clemens, M. J., and Kerr, I. M. (1976b), *Nature (London) 264,* 477–480.

Robertson, H. D., and Hunter, T. (1975), *J. Biol. Chem. 250,* 418–425.

Robertson, H. D., and Mathews, M. B. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 225–229.

Sen, G. C., Lebleu, B., Brown, G. E., Kawakita, M., Slattery, E., and Lengyel, P. (1976), *Nature (London) 264,* 370–373.

Shenk, T. E., and Stollar, V. (1972), *Biochim. Biophys. Acta 287,* 501–513.

Soreq, H., Nudel, U., Salomon, R., Revel, M., and Littauer,

U. Z. (1974), *J. Mol. Biol. 88,* 233-245.
Stewart II, W. E., and De Clercq, E. (1974), *J. Gen. Virol. 23,* 83-89.
Táborský, I., Doskočil, J., and Zajicová, V. (1974), *Acta Virol. 18,* 106-112.
Torelli, U., Cadossi, R., Torelli, G., Ferrari, St., and Ferrari, Se. (1975), *FEBS Lett. 56,* 96-100.
Torrence, P. F., and De Clercq, E. (1977), *Pharmacol. Ther.* (in press).
Tytell, A. A., Lampson, G. P., Field, A. K., Nemes, M. M., and Hilleman, M. R. (1970), *Proc. Soc. Exp. Biol. Med. 135,* 917-920.

Design of New Photoaffinity Labels for Ribosomal Peptidyltransferase†

K. Quiggle, M. L. Wejrowski, and S. Chládek*

ABSTRACT: The chemical syntheses of 6-azido-2′(3′)-*O*-L-phenylalanylpurine ribonucleoside (**4a**), 2′(3′)-*O*-(4-azido-L-phenylalanyl)adenosine (**4b**), and cytidylyl(3′→5′)-6-azido-2′(3′)-*O*-L-phenylalanylpurine ribonucleoside (**7**) are described. 6-Azidopurine ribonucleoside 5′-triphosphate (**10**) was also synthesized starting from 6-methylmercaptopurine ribonucleoside. All of these compounds (**4a, 4b, 7,** and **10**) are readily photolyzed by ultraviolet (UV) light. Compounds **4a, 4b,** and **7** are active in the ribosomal peptidyltransferase-catalyzed release of the Ac-Phe residue from the Ac-Phe-tRNA·70S ribosome·poly(U) complex. It follows that the 6-azidopurine moiety of compounds **4a** and **7,** as well as the 4-azido-L-phenylalanine moiety of **4b,** are recognized by the peptidyltransferase enzyme, and therefore these moieties are suggested for incorporation into tRNA as photoaffinity labeling reagents.

The affinity labeling method is a promising approach to the study of ribosomal structure. In principle, this method should yield information on the direct involvement of various elements (proteins and rRNAs) in ribosomal recognition sites. Thus, it should be possible to identify ribosomal components involved in the peptidyltransferase reaction, GTPase reaction, mRNA binding sites, etc. Until now most of the reagents used for affinity labeling of peptidyltransferase sites were analogues of *N*-acyl-AA-tRNA[1] or peptidyl-tRNA with an electrophilic or photoactive label attached to the *N*-acyl residue. Using various reagents of this type, several 50S proteins and 23S RNAs have been implicated as parts of the A and P sites of peptidyltransferase (for a review see Cooperman, 1977). To our knowledge, no labeling of peptidyltransferase sites with a functional analogue of AA-tRNA (possessing a free α-amino group) has as yet been described.

In our approach to the affinity labeling of peptidyltransferase recognition sites we sought to develop labeling reagents with photoactive groups placed specifically at various positions of the 3′ terminus of AA-tRNA or Ac-AA-tRNA (e.g., on the 3′-terminal adenosine residue, or the amino acid moiety). Previous work with simple models of the 3′ terminus of AA-tRNA as substrates for the peptidyltransferase reaction has indicated that these entities play important roles in the binding of substrates to the enzyme loci. In addition, it was observed that peptidyltransferase can apparently tolerate considerable modification at position 6 of the adenosine residue (Rychlík et al., 1969, 1970; Žemlička et al., 1975; Chládek et al., 1976). We therefore decided to use as probes modified derivatives of AA-tRNA which have the photolabile azido group placed on the 3′-terminal nucleoside residue or on the amino acid side chain. In this report we describe the initial results of our approach utilizing 2′(3′)-*O*-aminoacyl nucleosides and oligonucleotides that incorporate an azido group. The chemical syntheses of several compounds of this type, containing 6-azidopurine ribonucleoside and 4-azidophenylalanine moieties, their behavior in the peptidyltransferase reaction, and their photolytic properties are described. In addition, we describe the synthesis of 6-azidopurine ribonucleoside 5′-triphosphate. This compound is designed to be incorporated enzymatically into the 3′ terminus of tRNA to yield a photoactive tRNA.

Experimental Procedure

General Methods

Chromatography. Paper chromatography was performed by the descending technique on Whatman No. 1 paper using the following solvent systems: S_1, 2-propanol-concentrated ammonium hydroxide-water (7:1:2); S_2, 1-butanol-acetic acid-water (5:2:3); S_3, butanol saturated with 10% acetic acid; S_4, 1-propanol-concentrated ammonium hydroxide-water (6:3:1); S_5, isobutyric acid-water-concentrated ammonia-0.1 M EDTA (57:38:4:1); S_6, 2-propanol-3 M ammonium hydroxide-0.1 M boric acid (7:2:1); S_7, ethanol-1 M ammonium acetate, pH 3.8 (5:2). Thin-layer chromatography (TLC) was done on silica gel coated aluminum foils (silica gel 60 F-254, Brinkmann Instruments, Westbury, N.Y.) in systems S_8

† From the Michigan Cancer Foundation, Meyer L. Prentis Cancer Center, Detroit, Michigan 48201. *Received June 24, 1977.* This paper is No. 29 in the series Aminoacyl Derivatives of Nucleosides, Nucleotides, and Polynucleotides. For the preceding report in this series see Goldberg et al. (1977). This investigation was supported in part by U.S. Public Health Service Research Grant No. GM-19111-05 from the National Institutes of Health, and in part by an institutional grant to the Michigan Cancer Foundation from the United Foundation of Greater Detroit.

[1] Abbreviations used are: AA-tRNA, aminoacyl transfer ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid (tetrasodium salt); $Et_3NH_2CO_3$, triethylammonium bicarbonate; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; DCC, dicyclohexylcarbodiimide; DSS, 4,4-dimethyl-4-silapentane-5-sulfonate; Boc, *tert*-butyloxycarbonyl; A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; C-A-Phe, cytidylyl(3′→5′)-2′(3′)-*O*-L-phenylalanyladenosine; TLC, thin-layer chromatography; UV, ultraviolet; NMR, nuclear magnetic resonance.

0006-2960/78/0417-0094$01.00/0