The Characteristics of Inhibition of Protein Synthesis by Double-Stranded Ribonucleic Acid in Reticulocyte Lysates*

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TONY HUNTER,* TIM HUNT, and RICHARD J. JACKSON
From the Department of Biochemistry, University of Cambridge, Cambridge, England
HUGH D. ROBERTSON‡
From the Medical Research Council Laboratory of Molecular Biology, Cambridge, England

SUMMARY

All types of double-stranded RNA (dsRNA) tested inhibit protein synthesis in rabbit reticulocyte lysates. The inhibition is characterized by its strongly biphasic kinetics, and can be enhanced by preincubation of the lysate with dsRNA in the absence of protein synthesis. Only properly and extensively matched dsRNA (greater than about 50 base pairs) has this property: no form of DNA, single-stranded RNA or even RNA-DNA hybrids act as inhibitors in this way. The cause of the inhibition appears to be a failure of initiator tRNA to associate with native ribosomal subunits in the initiation process (DARNBROUGH, C., HUNT, T., and JACKSON, R. J. (1973) Biochem. Biophys. Res. Commun. 48, 1556-1564). We have shown that this block is not accompanied by stable association of dsRNA with the ribosomes. There are several reasons to believe that the mechanism of action of dsRNA may be complex with the possible involvement of at least one catalytic step. First, the lysate is inhibited by levels of dsRNA at which ribosomes are present in 100-fold excess over base pairs of dsRNA present. Second, high concentrations of dsRNA (greater than 10 μg per ml) are not inhibitory, but can in some, but not all experiments, reverse the inhibition caused by lower levels of dsRNA. Third, a lysate which has been inhibited by dsRNA, when mixed with a fresh lysate will inhibit synthesis in the mixture much more severely than would be expected from the concentration of dsRNA now present. These results indicate that low levels of dsRNA promote the formation of an inhibitor which may exist in two forms: one that is reversible by high levels of dsRNA and one that is irreversible.

Double-stranded RNA is an extremely potent inhibitor of the initiation of protein synthesis in reticulocyte lysates (1, 2). This effect is not a peculiarity of cell-free systems from reticulocytes, since other mammalian cell-free extracts are also inhibited by dsRNA,1 albeit at somewhat higher concentrations (3, 4). This property of dsRNA is interesting not only for its obvious relevance in the mechanism of initiation of protein synthesis in mammalian cells, but also for its possible relevance to the numerous other biological effects of dsRNA: the induction of interferon (5, 6), enhancement of the immune response (7), the causation of tumor regression (8), and general cytotoxicity (9-11).

In this paper we describe experiments designed to elucidate the mechanism of action of dsRNA on the reticulocyte lysate. We have already shown that the inhibition of protein synthesis is characterized by loss of 40 S/Met-tRNA\textsubscript{Met} initiation complexes (12) and that the inhibition can be reversed by 3':5'-cAMP and a variety of adenine derivatives (13). We now present evidence that although this form of inhibition is highly specific for dsRNA (although without regard to its composition or sequence of bases), dsRNA is not the proximate cause of the inhibition. We suggest that dsRNA acts by promoting the formation of an agent which probably inhibits protein synthesis by a catalytic mechanism. This agent may be closely related to that which inhibits globin synthesis in the absence of added hemin (14, 15).

MATERIALS AND METHODS

Preparation of Reticulocytes
Rabbit reticulocytes were obtained as described by Darnbrough et al. (16).

Preparation of Lysates
Lysates were prepared from washed reticulocytes as described by Hunt et al. (17) and stored frozen as lysates from individual rabbits in liquid nitrogen.

Incubation Conditions
Lysates were incubated at 30° together with a one-fifth volume of a master mix containing the components necessary for protein synthesis as previously described (16). dL-[\textsuperscript{-14}C]Phenylalanine

1 The abbreviations used are: dsRNA, double-stranded RNA; dsDNA, double-stranded DNA; ssRNA, single-stranded RNA; ssDNA, single-stranded DNA; RE, replicative ensemble.
was used at a final concentration of 1.0 μCi per ml, while [3S]-methionine was used at 75 μCi per ml. Samples withdrawn from incubations were treated as described (17) for estimation of acid-precipitable alkali-stable radioactivity. In cases where preincubation was carried out, everything except the hemin was omitted from the incubation. All incubations were carried out with [3H]phenylalanine unless otherwise stated.

Preparation of Polynucleotides

**RNAs from RNA Bacteriophages**

f2—RNA was prepared by phenol extractions from f2 phage grown and purified as described by Engelhardt et al. (18). Pure 28 S phage RNA was prepared by fractionation of total f2 RNA on sucrose density gradients (39) and is termed f2 ssRNA. Alternatively, preparations which were essentially identical with f2 ssRNA, could be prepared by treatment of f2 RNA with RNase III (see below). f2 dsRNA is defined as f2 replicative ensemble (or RE) (18) from which ssRNA tails have been removed by mild pancreatic RNase digestion as described below.

High specific activity 32P-labeled f2 bacteriophage were grown according to Engelhardt et al. (18). Unlabeled f2 bacteriophage were the gift of Dr. R. E. Webster, The Rockefeller University, New York. The receptor from Escherichia coli cells infected with f2 was grown and purified according to Engelhardt et al. (18) and Robertson and Lodish (21). This material is a mixture of all structures which contain dsRNA, whether or not they are single-stranded tails. These nascent viral strands formed during viral replication (20, 23). Enzymatic and chromatographic analysis of the RE preparation used in these experiments performed as described (18) revealed that this unlabeled RE fraction contained about 60% dsRNA, the remainder consisting of single-stranded tails.

f2 dsRNA labeled with 32P was prepared by an adaptation of the annealing method described by Robertson and Zinder (23). 32P-Labeled f2 ssRNA (specific activity 1.6 × 10^6 cpm/μg) (20 μg) was mixed with 60 μg of unlabeled f2 RE and boiled for 3 min in 1 ml of 0.1 M NaCl, 0.001 M EDTA (pH 7.4) and heated for another 15 min. The material was made 90% in ethanol and layered onto a cushion of CsCl (1.3 g/ml) (20). On such a column, DNA and small molecular weight material eluted in Buffer A containing 35% ethanol; ssRNA, in 15% ethanol; and dsRNA (plus any stably attached ssRNA), in Buffer A alone. Whereas, greater than 99% of the 32P-labeled f2 RNA eluted from the column in 15% ethanol before annealing, 25% eluted in 15% ethanol and 75% in Buffer A alone after annealing. In other words, 75% of the single strands had supplanted the unlabeled "plus" strands in the double-stranded complex.

Since some of the 32P-labeled material would be expected to have unlabeled "tails," the material chromatographing as dsRNA was treated with pancreatic RNase to remove remaining single strands as follows: 1 μg of Worthington pancreatic RNase was added to 1 ml of 32P-labeled material in Buffer A, and incubated at 37°C for 5 min. After 5 min, 50% of the RNA was solubilized, and only an additional 3.7% in the next 10 min. dsRNA shows complete resistance to pancreatic RNase digestion under these salt conditions (23). Digestion was terminated with 0.7 ml of Buffer A saturated phenol. After extraction and centrifugation to separate the layers, the process was repeated twice more; between the second and the third extraction, the aqueous layer was made 0.1% in diethyl pyrocarbonate and incubated for 15 min at 25°C. Following the final phenol extraction, the aqueous layer was made 35% in ethanol and layered onto another cushion of CsCl (20). Fifty-three per cent of the RNA was solubilized in Buffer A, and 25% eluted in 15% ethanol and 75% in Buffer A alone after annealing. In other words, 75% of the single strands had supplanted the unlabeled "plus" strands in the double-stranded complex.

**Polynucleotides**

DNA-RNA hybrids—An aliquot of the RNA polymerase reaction was mixed with phenol and the aqueous layer was separated by CsCl equilibrium density gradient as described by Colby et al. (27). Double-stranded RNA—An aliquot of the RNA polymerase reaction was diluted to 0.1 ml in 0.01 M Tris-HCl (pH 7.4), 0.001 M sodium acetate, 0.1 M NaCl, and treated with 25 μg/ml of pancreatic DNase (Worthington DNFF) for 15 min at 37°C; then the mixture was made twice in Buffer A (by the addition of 0.02 ml of 10 times Buffer A) and incubated 25 min more in the presence of 5 μg/ml of pancreatic DNase. Then 1 ml of diethyl pyrocarbonate was added and the reaction was diluted to 0.5 ml and extracted with 0.2 ml of phenol. The aqueous layer was made 35% in ethanol and chromatographed on a cellulose CM11 column. The vast majority of the material flowed through the column in 35% ethanol, indicating that it had been solubilized by pancreatic RNase and was therefore either free ssRNA or RNA released from the DNA-RNA hybrids by pancreatic DNase. However, 2 to 3% of the RNA chromatographed in the position of dsRNA (eluting in Buffer A alone). This RNA was pooled and its double-stranded properties were confirmed by tests with pancreatic RNase and E. coli RNase III. The final specific activity was 1.2 × 10^6 cpm/μg. The 32P dsRNA was extensively dialyzed against water and lyophilized.

**Synthetic Polynucleotides**

Poly(U) was obtained from British Drug Houses. Poly(A), poly(U) (which was always boiled in solution for 2 min before use), poly(A)·(U) and poly(A)·(C) were all obtained from Miles Laboratories. Poly(C) was obtained from Boehringer and Soehne. Poly(A)·U and poly(C)·(C) were prepared as described by Robertson et al. (24).

**Other Polynucleotides**

dsRNA obtained from a virus grown in *Penicillium chrysogenum* was the kind gift of Dr. D. Planterose, Beecham Research Laboratories, Betchworth, Surrey, England. Reovirus was a gift of Dr. J. J. Schech, National Institute for Medical Research, Mill Hill, London. TMV virus was the kind gift of Dr. R. N. Perham, Department of Biochemistry, University of Cambridge. Calf thymus DNA and total yeast RNA were obtained from British Drug Houses. *Escherichia coli* ribosomal RNA was prepared from ribosomes purified from E. coli MRE 600 by phenol extraction. Sendai RNA was a gift of Dr. D. Mahy, Department of Virology, University of Cambridge.

**Enzymes and Enzyme Assays**

**RNome**

RNome III was prepared as described by Robertson et al. (24) with the modifications described by Robertson and Hunter (19).
The conditions for digestion of nucleic acids with RNase III have been reported previously (21).

**DNase**

DNase was obtained from Worthington Biochemical Corp. and was DPFF grade. Conditions used for DNase digestion are described above.

**Sucrose Gradients**

Sucrose gradients were run as described in the appropriate figure legends and were fractionated through a flow cell which was monitored at 200 nm. Gradient fractions were precipitated with cold 8% trichloroacetic acid together with the addition of 200 μg of carrier serum albumin for estimation of radioactivity.

**Materials**

ATP, GTP, creatine phosphate and creatine phosphokinase were all obtained from Boehringer and Soehne, Mannheim. He-

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*ND, Not done.

<sup>a</sup> See “Preparation of Polynucleotides.”

Fig. 1. Kinetics of protein synthesis in the presence of f<sub>2</sub> dsRNA or poly(I). Samples (15 μl) were taken at the times indicated from 100-μl reactions set up and assayed as described under “Incubation Conditions.” All polynucleotides and the sodium fluoride were added at the start of the incubation. △—△, control; ○—○, + 50 ng per ml of f<sub>2</sub> dsRNA; ▲—▲, + 10<sup>4</sup> ng per ml of poly(I); ○—○, + 3 X 10<sup>4</sup> ng per ml of poly(I); ◆—◆, + 10 mM sodium fluoride.
were such preincubated lysates significantly more sensitive to capacity of the lysates to synthesize protein (see Fig. 3C), nor these conditions without dsRNA only marginally reduced the hemin and dsRNA in the absence of the other components necessary for protein synthesis. Preincubation for up to 1 hour under these conditions without dsRNA only marginally reduced the capacity of the lysates to synthesize protein (see Fig. 3C), nor were such preincubated lysates significantly more sensitive to

different types of dsRNA we found the same shape of curve in each case.

At high concentrations of dsRNA, when no inhibition was evident, equal amounts of normal α and β chains were being synthesized. During the latent phase, with inhibitory concentrations of dsRNA, the ratio of α and β chains synthesized was also unity. However, during the inhibitory phase, when the synthesis of both chains was markedly reduced, the residual synthesis represented an approximately 2-fold excess of β chain synthesis over α chains (data not shown). This finding may reflect the higher affinity of the β chain mRNA for the few remaining active ribosomes (28).

Effect of Preincubation of Lysates with dsRNA—The inhibition described in the previous section was observed at all temperatures between 20 and 37°, but the level of incorporation reached before onset of inhibition decreased as the temperature was lowered. This observation prompted us to test whether the length of the latent phase was changed if the lysates were preincubated with hemin and dsRNA in the absence of the other components necessary for protein synthesis. Preincubation for up to 1 hour under these conditions without dsRNA only marginally reduced the capacity of the lysates to synthesize protein (see Fig. 3C), nor were such preincubated lysates significantly more sensitive to

dsRNA when this was added after the preincubation (data not shown). In contrast, when the dsRNA was present in the preincubation, the lag before onset of inhibition was greatly reduced by comparison with the lag observed if the same concentration of dsRNA was added at the start of the incubation. In all cases (15 μl) were taken at the times indicated for assay of radioactivity. 

Effect of Preincubation of Lysates with dsRNA

Evidence for Formation of Inhibitor in Lysates Incubated with dsRNA

Further experiments were designed to distinguish between the two distinct ways in which dsRNA could cause inhibition: (a) some component in the lysate essential for initiation may be effectively removed from the system by binding to dsRNA or by

Effect of Preincubation upon Inhibition by f2 dsRNA—The inhibition of [3H]phenylalanine incorporation. The values for these incorporations are plotted against the length of preincubation. O—O, control; □—□, + 50 ng per ml of dsRNA; ○—○, + 5 ng per ml of dsRNA; •—•, + 0.5 ng per ml of dsRNA. C, 120 μl of a different lysate were preincubated at 30° with hemin and with the concentrations of f2 dsRNA indicated. At the times shown 20-μl samples from the preincubations were mixed with 5 μl of master mix and incubated for a further 30 min at 30° to determine [3H]phenylalanine incorporation. The values for these incorporations are plotted against the length of preincubation. O—O, control; □—□, + 50 ng per ml of dsRNA; ○—○, + 5 ng per ml of dsRNA; •—•, + 0.5 ng per ml of dsRNA. C, 120 μl of a different lysate were preincubated at 30° with hemin and with the concentrations of f2 dsRNA indicated. At the times shown 20-μl samples from the preincubations were mixed with 5 μl of master mix and incubated for a further 30 min at 30° to determine [3H]phenylalanine incorporation. The values for these incorporations are plotted against the length of preincubation. O—O, control; □—□, + 50 ng per ml of dsRNA; ○—○, + 5 ng per ml of dsRNA; •—•, + 0.5 ng per ml of dsRNA. C, 120 μl of a different lysate were preincubated at 30° with hemin and with the concentrations of f2 dsRNA indicated. At the times shown 20-μl samples from the preincubations were mixed with 5 μl of master mix and incubated for a further 30 min at 30° to determine [3H]phenylalanine incorporation. The values for these incorporations are plotted against the length of preincubation. O—O, control; □—□, + 50 ng per ml of dsRNA; ○—○, + 5 ng per ml of dsRNA; •—•, + 0.5 ng per ml of dsRNA. C, 120 μl of a different lysate were preincubated at 30° with hemin and with the concentrations of f2 dsRNA indicated. At the times shown 20-μl samples from the preincubations were mixed with 5 μl of master mix and incubated for a further 30 min at 30° to determine [3H]phenylalanine incorporation. The values for these incorporations are plotted against the length of preincubation. O—O, control; □—□, + 50 ng per ml of dsRNA; ○—○, + 5 ng per ml of dsRNA; •—•, + 0.5 ng per ml of dsRNA. C, 120 μl of a different lysate were preincubated at 30° with hemin and with the concentrations of f2 dsRNA indicated. At the times shown 20-μl samples from the preincubations were mixed with 5 μl of master mix and incubated for a further 30 min at 30° to determine [3H]phenylalanine incorporation. The values for these incorporations are plotted against the length of preincubation. O—O, control; □—□, + 50 ng per ml of dsRNA; ○—○, + 5 ng per ml of dsRNA; •—•, + 0.5 ng per ml of dsRNA.
FIG. 4. Detection of an inhibitor in lysates incubated with dsRNA. A, 10-μl aliquots were withdrawn at the times shown from 100-μl incubations set up as described under “Incubation Conditions” with the following concentrations of *Penicillium chrysogenum* dsRNA: 100, 30, 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 ng per ml. Kinetics of amino acid incorporation into protein (cpm) are shown only for a control and the following concentrations of dsRNA: X—X, 10 ng per ml of dsRNA; •—•, 3 ng per ml of dsRNA; □—□, 1 ng per ml of dsRNA; ○—○, 0.3 ng per ml of dsRNA. B, after 16 min, the incubations described in A were cooled in ice and 1 volume (30 μl) was mixed with 2 volumes of fresh incubation mixture containing all the components described under “Incubation Conditions,” including hemin and radioactive amino acid, thus generating 3-fold diluted incubations containing final concentrations of dsRNA as 33 ng per ml etc. These mixtures were then incubated and 10-μl samples were withdrawn at the times shown for assay of radioactivity. Final dsRNA concentrations were as follows: —, control (no dsRNA); X—X, 3.3 ng per ml of dsRNA; •—•, 1 ng per ml of dsRNA; □—□, 0.33 ng per ml of dsRNA; ○—○, 0.1 ng per ml of dsRNA. C, after 16 min the incubations described in B were cooled in ice and a 3-fold dilution was carried out as described in B. These new mixtures were then incubated and 10-μl samples were withdrawn at the times shown for assay of radioactivity. Final dsRNA concentrations were as follows: —, control (no dsRNA); X—X, 1.1 ng per ml of dsRNA; •—•, 0.33 ng per ml of dsRNA; □—□, 0.11 ng per ml of dsRNA; ○—○, 0.033 ng per ml of dsRNA. D, the results of A, B, and C together with the parallel incubations containing the other dsRNA concentrations described in A but not illustrated, are replotted to show the net synthesis occurring in each of the three 16-min incubation periods against the actual concentrations of dsRNA in the incubations on a logarithmic scale. •—•, data from the first incubation (A); ○—○, data from the second incubation (B); X—X, data from the third incubation (C). ———, mean value of the three control incubations (i.e. those lacking dsRNA) which differed by less than 10%.

being inactivated in the presence of dsRNA, or (b) the presence of dsRNA in the incubation may lead to the formation of an initiation inhibitor, such as occurs when lysates are incubated without hemin (14, 29). This type of inhibitor may be detected by mixing an inhibited lysate (i.e. one that has been incubated with dsRNA under standard conditions for protein synthesis assays) with a portion of fresh lysate and testing whether the protein synthesis activity of this mixture is more severely restricted than would be expected from a consideration of the amount of dsRNA present. An experiment of this type is illustrated in Fig. 4. First, standard protein synthesis assays were carried out with various concentrations of dsRNA (Fig. 4A). After 16-min incubation, 1 volume of each of these assays was mixed with 2 volumes of fresh incubation mix containing unincubated lysate and all the usual components for protein synthesis assays, but no dsRNA. Protein synthesis in these mixtures was then monitored over a 16-min incubation period (Fig. 4B), and then each assay was diluted again 3-fold in fresh incubation mix and incorporation assayed for another 16 min (Fig. 4C). The total synthesis occurring in the 16 min incubation period is plotted against the actual concentration of dsRNA in each incubation (Fig. 4D). The synthesis occurring in the second incubation (Fig. 4B) (where the dsRNA concentration had been reduced 3-fold) is much less than would be expected by comparison with the synthesis occurring in the first incubation (Fig. 4A) even allowing for the possibility that the ribosomes in the portion of the lysate previously incubated with dsRNA might be irreversibly inactivated. We also verified that the characteristics of inhibition were the same in the first, second and third incubations, namely loss of polysomes and loss of Met-tRNA<sub>Met</sub> from the 40 S ribosomal subunit.

The detection of the induced inhibitor in the assays described
information of radioactivity incorporated into protein. In this case another set of incubations with a different lysate were set up per ml of Penicillium chrysogenum dsRNA solution, before con- was raised to 10 pg per ml by addition of 100 volume of a 1 mg after the start of the incubation and the concentration of dsRNA was raised to 10 pg per ml of Penicillium chrysogenum dsRNA solution, before continuing the incubation. The time courses for the incubations containing 10 ng per ml of dsRNA at 1, 2, 3, 4, 5, 7, and 10 min radioactivity. Aliquots (15 ~1) were taken from the incubation and lysates previously incubated with dsRNA was moderately served and expected protein synthesis was observed only if the concentration of dsRNA present. This marked difference between the ob- considerably less than would be expected from the concentration with a low level of dsRNA. This experiment produced two types of result; the variability appeared to depend on the particular reticulocyte lysate used. One type of response was that the inhibitory effects of the low level of dsRNA became completely dominant over the noninhibitory effects of 10 pg per ml of dsRNA after a few minutes, and several minutes before the inhibition of protein synthesis in the assays containing low concentrations of dsRNA was manifest (Fig. 5A). The other re- one was that if high enough concentrations were used, inhibition of a small dsRNA molecules of random size was fractionated on a sucrose density gradient centrifugation (19). Purified 28 S viral RNA did not exhibit inhibition of a dsRNA type, and instead, if high enough concentrations were used, inhibition of a ssRNA type was seen, as other workers have noted (30). In Class 6 the lowest concentration to give detectable inhibition is listed. All these nucleic acids were tested under conditions of preincubation, but none of them showed enhancement of inhibi- For Class c the highest concentration tested is listed, although a whole range of concentrations was tested to ensure that an inhibition of the dsRNA type was not being masked by concentration dependence. All the nucleic acids in Class c were tested in the preincubation assay, but still showed no inhibition. In the case of the f1 RNA-DNA hybrids, an inhibition of the dsRNA type was observed at high concentrations. However, this inhibitory activity was not susceptible to pancreatic DNase digestion and we feel that this inhibition was due to a contamination of the hybrid preparation with dsRNA generated during the synthesis of the hybrid with E. coli RNA polymerase (see "Preparation of Polynucleotides"). Note that despite the common sequences shared by all the polynucleotides derived from f1 phage, only f1 dsRNA is inhibitory.

Size Requirement—To determine the minimum size of dsRNA effective in inhibition, a preparation of Penicillium chrysogenum dsRNA containing a major 14 S species and a minor fraction of smaller dsRNA molecules of random size was fractionated on a sucrose gradient (Fig. 9). Each fraction was tested for inhibitory activity at the same final RNA concentration (10 ng per ml) and the percentage inhibition caused by each fraction is plotted in Fig. 6. When RNAs from selected regions of the original gradient were rerun, they sedimented with approximately their original S value as judged both by optical density and by inhibitory ac- activity. This indicates that the distribution of dsRNA on the
the use of dsRNA prepared from E. coli infected with the sus3 mutant of f2. This RNA is about 30 to 50 base pairs in size, whereas the inhibition observed with poly(U) was unaffected by RNase III. Even if preincubation was 10^7 cpm per µg) nor with f2 [32P]dsRNA (2 ng per ml; specific activity 1 × 10^7 cpm per µg) were we able to detect any stable association of dsRNA with any of the ribosomal constituents by sucrose gradient analysis either during the latent or the inhibitory phase. In these experiments we also confirmed that at the onset of the inhibitory phase there was a dramatic breakdown of the polysomes together with loss of nascent chains (1) and an increase in the amount of ribosomal subunits (12). Although negative, these experiments were sensitive enough to have detected a stable interaction of dsRNA with the ribosomes at a level of 0.1 ng per ml, the lowest inhibitory concentration of dsRNA. They cannot rule out, however, a transient or unstable association of dsRNA with the ribosomes.

**DISCUSSION**

The initiation of protein synthesis in reticulocyte lysates is inhibited by several types of polynucleotides: dsRNA (2), single-stranded f2 RNA (30) and homopolynucleotides such as poly(1) (33). Our results show that the following criteria can unambiguously distinguish the inhibition caused by dsRNA from the effects of other polynucleotides: (a) the much lower concentrations of dsRNA required for inhibition; (b) the failure of high concentrations of dsRNA to inhibit; (c) the biphasic kinetics of protein synthesis; (d) the disappearance of 40 S/Met-tRNA^met complexes shortly before the abrupt cessation of synthesis (12) (in contrast poly(1) causes a large increase in the level of these complexes (16)); (e) the formation of a potent inhibitor of initiation in lysates incubated with dsRNA; (f) the loss of inhibition observed upon pretreatment of the dsRNA with E. coli RNase III (1) (a nuclease specific for double-helical RNA (24)).

These differences indicate that dsRNA inhibits initiation by a different mechanism than do other polynucleotides. The stringent specificity for dsRNA coupled with the lack of specificity for nucleotide sequence suggests that the inhibition involves interaction of the dsRNA with protein rather than nucleic acid. The requirements for inhibition are double-stranded RNA segments of at least 50 base pairs in length, but since the specific inhibitory activity does not vary much with the size of the dsRNA above this minimum length, the size of the binding site of the protein(s) is likely to be shorter than this. On the other hand the binding site seems likely to be greater than a single base pair, so that we can infer that a level of 0.6 ng per ml of dsRNA (10^8 M in base pairs), which is close to the minimum inhibitory concentration, would represent a concentration of protein binding sites of not more than 5 × 10^{-13} M, and possibly as little as 10^{-15} M. In contrast our lysates typically contain ribosomes at about 2.5 × 10^{-14} M (16) and native subunits at about 10^{-8} M (5%) of the total ribosome population. If initiation factor proteins are present in approximately the same concentrations as the native protein synthesis at various times during a complete incubation of the lysate. The dsRNA was completely stable, while the ssRNA was slowly converted to acid solubility. Addition of RNase III at the end of the incubation, however, caused 90% loss of acid-insoluble radioactivity, showing that it was still in the form of dsRNA. We also tried to see if any replication of the added dsRNA occurred, by adding, in addition to the normal components, cTP and [α-32P]UTP to the incubation. There was no incorporation of label into acid insoluble material, thereby ruling out this possibility. (Lower limit of detection at 50 ng per ml of dsRNA was 1 ng per ml of RNA synthesized.)

[32P]-labeled dsRNA was also used in an attempt to demonstrate an association between dsRNA and a ribosomal component. Neither with f2 [32P]dsRNA (50 ng per ml; specific activity 2 × 10^8 cpm per µg) nor with f1 [32P]dsRNA (2 ng per ml; specific activity 1 × 10^7 cpm per µg) were we able to detect any stable association of dsRNA with any of the ribosomal constituents by sucrose gradient analysis either during the latent or the inhibitory phase. In these experiments we also confirmed that at the onset of the inhibitory phase there was a dramatic breakdown of the polysomes together with loss of nascent chains (1) and an increase in the amount of ribosomal subunits (12). Although negative, these experiments were sensitive enough to have detected a stable interaction of dsRNA with the ribosomes at a level of 0.1 ng per ml, the lowest inhibitory concentration of dsRNA. They cannot rule out, however, a transient or unstable association of dsRNA with the ribosomes.
subunits, which seems likely especially with factors that have anti-association factor activity, these considerations eliminate any models in which dsRNA initiates inhibition by irreversibly binding an initiation factor and effectively removing it from the system. Hypotheses in which the initiation factor binds to the dsRNA but is later released in an inactive form are still tenable, but such models would seem to suggest that addition of dsRNA at 100 to 1000 times the minimum inhibitory level (i.e. at levels where there would be enough binding sites to accommodate all the initiation factor present in the lysate) should cause almost instantaneous inhibition of initiation, rather than the slight reduction in the lag period which is in fact observed.

These considerations lead us to question the hypothesis proposed by Kaempfer and Kaufman (34) that initiation is inhibited because IF-3, an initiation factor with anti-association factor activity (35), is inactivated by direct complexing with dsRNA. This hypothesis is based on the observations that supplementation of the lysate with IF-3 prevented the inhibition caused by dsRNA and that IF-3 itself bound strongly to dsRNA. Although IF-3 bound to DNA less strongly (34), the discrimination between DNA and dsRNA in this binding reaction is nowhere near as great as in protein synthesis assays (Table 1).

Our results suggest that the mechanism of action of dsRNA is more complex than direct inactivation of IF-3. We have shown that lysates which have been incubated with dsRNA contain an inhibitor more potent than the dsRNA itself, and all our observations are consistent with the idea that the effect of dsRNA on protein synthesis is explicable in terms of the formation and action of this inhibitor. We do not as yet know the nature of this inhibitor other than that gel filtration studies have shown it to be macromolecular. It is not impossible that interaction of IF-3 with dsRNA is part of the inhibitor-forming process. It is also possible that the inhibitor inactivates IF-3, although we favor a catalytic rather than stoichiometric relationship between the two.

Since the addition of high levels of dsRNA to lysates incubated with inhibitory concentrations for various times produced two different types of response, depending on the particular lysate used, it seems necessary to postulate that the inhibitor may exist in two possible forms; one reversible by high levels of dsRNA and one irreversible. The results in Fig. 5 suggest that in some lysates the irreversible species is formed significantly more slowly than the reversible species, whereas in other cases the irreversible form is generated very rapidly. Although the existence of two forms of inhibitor and the variability between lysates are puzzling features, there are not unprecedented. There is a close parallel between lysates incubated with low levels of dsRNA and those incubated without hemin. In both situations the kinetics of protein synthesis are biphasic (17, 36) and the 40S/Met-tRNA* complexes disappear shortly before the abrupt cessation of protein synthesis (29). In addition we have shown elsewhere that a number of compounds can prevent and reverse the inhibition seen in both conditions (13), whereas Kaempfer and Kaufman have shown that addition of excess IF-3 prevents not only the inhibition caused by dsRNA (34) but also that caused by omission of hemin (35). The two types of response, no reactivation or delayed reactivation, noted in Fig. 5, are also seen when hemin is added to lysates incubated for various times without hemin (36, 37). Several lines of evidence have led to the conclusion that incubation without hemin leads to the formation of an inhibitor which may exist in two forms, reversible by hemin or irreversible (29, 37). The reversible species may be a precursor of the irreversible form (37), which has been partially characterized as a large molecule and which may act catalytically since one molecule can apparently prevent the functioning of several hundred ribosomes (38). A further similarity between lysates incubated with low levels of dsRNA or without hemin is that they are committed to stop protein synthesis several minutes (and therefore several rounds of synthesis) before inhibition actually occurs, since the addition of the appropriate reactivating substance after a brief incubation period does not prevent the onset of inhibition although it may render the shut-off transitory (Fig. 5 and Refs. 17, 36, and 37).

In spite of the close similarities described, we do not wish to imply that the mechanism of inhibition of protein synthesis is absolutely identical in the two cases. If this were so, we might expect that high levels of dsRNA could substitute for hemin in preventing inhibition, and we have so far failed to observe this. Also, preliminary gel filtration studies suggest that the inhibitor formed in the presence of dsRNA is larger than that formed in the absence of hemin. It seems clear that in both situations the inhibition involves a complex multi-step process. It is possible that the terminal stages of this process are identical in the two conditions, whereas the early steps are different.

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Unpublished observations.
The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates.

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