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# Regulation of protein synthesis in rabbit reticulocyte lysates: Characteristics of inhibition of protein synthesis by a translational inhibitor from heme-deficient lysates and its relationship to the initiation factor which binds Met-tRNA$_f$

(translational regulation/heme-deficiency inhibitor/Met-tRNA$_f$ binding initiation factor)

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ABSTRACT In heme-deficient reticulocyte lysates a translational inhibitor which regulates protein synthesis is formed or activated. To define the mechanism of action of the translational inhibitor (RI), RI was partially purified. We have utilized the isolated RI to examine its relationship to the translational inhibitor formed *in situ* in heme-deficiency, some quantitative aspects of inhibition of protein synthesis, and the relationship of RI concentration to the initiation factor (IF-MP) which forms a ternary complex with Met-tRNA$_f$ and GTP (IF-MP·Met-tRNA$_f$·GTP). The results demonstrate that the activity of isolated RI is related to the *in situ* heme-deficiency inhibitor by several criteria: (*a*) the biphasic kinetics of inhibition manifested by RI in lysates containing optimal levels of hemin are very similar to those observed in heme-deficiency, i.e., an initial period in which several rounds of protein synthesis proceed at the control rate followed by an abrupt decline in the rate of protein synthesis. (*b*) Both inhibitions are accompanied by the disaggregation of polyribosomes with a concomitant increase in 80S ribosomes. (*c*) Both inhibitions are reversed by IF-MP.

The isolated RI blocked protein synthesis in lysates at temperatures ranging from 15° to 30°. Although the rate of protein synthesis was a function of the temperature of incubation, the number of rounds of protein synthesis prior to shut-off was essentially the same at various temperatures. When RI was added to lysates, at increasing intervals after the start of incubation, the period of synthesis before shut-off (lag) progressively decreased.

The inhibition of protein synthesis by RI was immediately reversed by the addition of IF-MP. The extent of reversal increased with increasing concentrations of IF-MP; at low levels of RI almost complete reversal of inhibition by IF-MP was obtained. However, at high levels of RI which did not appreciably increase the degree of inhibition of protein synthesis, equivalent amounts of IF-MP were less effective in reversing inhibition. These results suggest that the inhibition of protein synthesis by the isolated inhibitor involves the initiation factor IF-MP.

Protein synthesis in reticulocytes and reticulocyte lysates is regulated by heme (1–3). In heme-deficiency, there is rapid formation or activation of a translational inhibitor (4–6) which blocks protein chain initiation (2, 3, 5, 7–9). The formation or activation of the inhibitor does not require protein synthesis (4). Three different forms of the inhibitor have been described: hemin-reversible, intermediate, and hemin-irreversible (10, 11). In most of these studies, however, reticulocyte lysates, ribosome-free supernates, or relatively crude inhibitor preparations were used as the source of inhibitor. In seeking to define the molecular mechanism involved in the regulation of protein synthesis by the inhibitor, we have studied the effects of a more purified inhibitor preparation. In this paper, we describe some quantitative aspects of inhibition of protein synthesis by a hemin-irreversible inhibitor preparation (RI) which we partially purified and the relationship of this inhibition to the initiation factor IF-MP which binds Met-tRNA$_f$. Data are presented which support the hypothesis that the heme-deficiency inhibitor is related to the physiological inhibitor which is formed or activated in heme-deficiency. A preliminary account of a part of these studies has been reported previously (12).

Abbreviations: RI, translational inhibitor of heme-deficient reticulocyte lysates; buffer A, 5 mM [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (pH 7.2), and 100 mM KCl; IF, initiation factor.

## EXPERIMENTAL PROCEDURES

**Materials.** The materials were obtained from the following sources: [$^{14}$C]leucine (303 Ci/mol) from New England Nuclear Corp.; DEAE-Sephadex A50 from Pharmacia Fine Chemicals; ATP, GTP, creatine phosphate, and creatine phosphokinase from Sigma Chemical Corp.; bovine serum albumin from Miles Research Laboratories, and ultra pure ammonium sulfate from Schwarz/Mann Biologicals.

**Preparation of Rabbit Reticulocyte Lysates and Protein Synthesis Assay.** Reticulocyte lysates were prepared from rabbits (White New Zealand, 2–3 kg) made anemic by the injection of acetylphenylhydrazine as described previously (6). The procedures for the preparation of incubation mixture and for measurement of protein synthesis have been described (6, 13). Protein synthesis was assayed by the incorporation of [$^{14}$C]leucine into proteins in 50 $\mu$l aliquots at 30° (exceptions are noted in the *text*); 0.4 $\mu$Ci of [$^{14}$C]leucine (specific activity 150 Ci/mol) was added to a standard 50 $\mu$l reaction mixture. Incubation mixtures contained hemin (10–20 $\mu$M) unless indicated otherwise in the *text*.

The activity of the translational inhibitor in fractions from various stages of purification was assayed by its inhibitory effect in a standard incubation mixture (50 $\mu$l) for protein synthesis and in the presence of optimal concentrations of hemin. One unit of inhibitor protein is defined as the $\mu$g of inhibitor protein that reduced protein synthesis to the level observed in control lysates without added hemin at 30° after 60 min of incubation.

**Preparation of Translational Inhibitor from Rabbit Reticulocyte Lysates.** The translational inhibitor was formed or activated by the incubation of a ribosome free supernate (supernatant fluid obtained after centrifugation of lysates at 150,000 × *g* for 3 hr) in the absence of hemin at 36° for 3 hr. All subsequent procedures were carried out at 4°. The sample was diluted with an equal volume (30 ml) of buffer A containing 5 mm [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (Hepes) at pH 7.2 and 100 mM KCl. The inhibitor was precipitated from the supernate by the slow addition of solid ammonium sulfate to 40% saturation and left overnight in an

ice bath. The precipitate was collected by centrifugation (40 min at 24,000 × *g*) and dissolved in buffer A at a final concentration of 20–30 mg of protein per ml. The preparation was dialyzed for 6 hr against 500 ml of buffer A, with one change of buffer after 3 hr, and finally for 16 hr against 2 liters of the buffer. The protein preparation was clarified by centrifugation (30,000 × *g* for 20 min).

The protein sample (180 mg) was applied to a DEAE-Sephadex A50 column (1.6 × 50 cm) pre-equilibrated with buffer A [details of the preparation of DEAE-Sephadex A50 have been described, (14)]. The protein was eluted by a stepwise KCl gradient in buffer A; the material eluting between 0.3 and 0.4 M KCl contained most of the inhibitory activity. This fraction was pooled and then concentrated (in an Amicon membrane filtration apparatus) and dialyzed against buffer A. The inhibitor protein preparation was stored at −70°. A 250-fold purification was achieved. The translational inhibitor has since been purified to apparent homogeneity; details of the purification will be described elsewhere (R. S. Ranu and I. M. London, manuscript in preparation). An alternative procedure for the partial purification of the inhibitor has been reported by Gross and Rabinovitz (15).

**Preparation of Initiation Factor Which Forms a Ternary Complex with Met-tRNA$_f$ and GTP (Met-tRNA$_f$-GTP-Factor).** The initiation factor [variously described in the literature as IF-1 (16, 17); L3 (18); IF-E$_2$ (19); EIF-3 (20) and IF-MP (21, 22)] was isolated by chromatography of a 0.5 M KCl salt wash from reticulocyte ribosomes on DEAE-cellulose and phosphocellulose. The procedure is a modification of a previously described method (18). One microgram of the initiation factor preparation bound 0.44 pmol of Met-tRNA$_f$ in ternary complex with GTP.

**Determination of the Concentration of Protein.** Protein concentrations were determined by the method of Lowry *et al.* (23), using bovine serum albumin as a standard.

## RESULTS

### Kinetics of protein synthesis in the presence of RI: Influence of RI protein concentration

The effect of various concentrations of RI on the kinetics of protein synthesis in lysates supplemented with optimal concentrations of hemin is presented in Fig. 1. The degree of inhibition of protein synthesis increased progressively with increasing concentrations of RI. The kinetics of inhibition observed with the addition of 2.2 μg of RI protein are closely parallel to those observed in heme-deficiency (Fig. 1). These kinetics are characterized by an initial period of several minutes during which several rounds of synthesis proceed at the control rate (lag period) followed by an abrupt decline in the rate of protein synthesis ("shut off"). A further increase in RI concentration by a factor of five decreased the lag period from 7 to 8 min to 2 min (results not shown).

### Inhibition of protein synthesis by RI: Effect of temperature

Fig. 2 shows the effect of temperature on protein synthesis in the presence of inhibitor. In each instance, the rate of protein synthesis declined abruptly after the incorporation of about 3700 cpm of [$^{14}$C]leucine. However, the time taken to reach this point varied considerably with temperature: at 30°, 6 min; 25°, 12 min; 20°, 24 min; and at 15°, about 60 min. The extent of protein synthesis prior to shut-off may vary from lysate to lysate, but in a given lysate the amount of protein synthesized during the lag period at various temperatures is strikingly constant with a given concentration of RI.

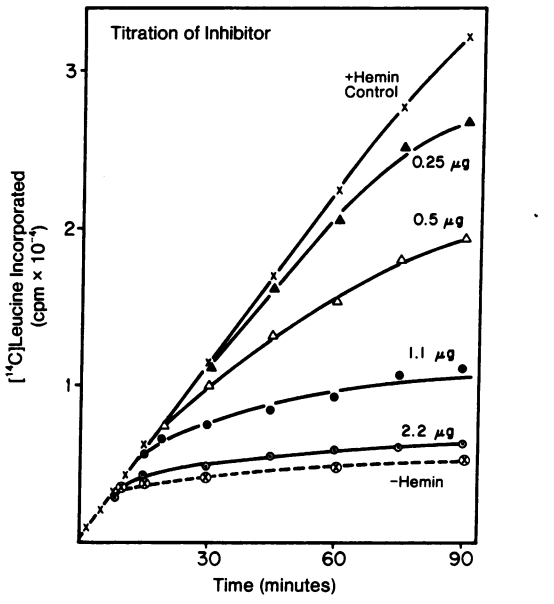


FIG. 1. Kinetics of protein synthesis: effect of RI protein concentration. Protein synthesis reaction mixtures (50 μl) containing 10 μM hemin were incubated at 30° with indicated amount of RI protein. A minus hemin control was also included. At intervals, aliquots were removed and assayed for protein synthesis. The values of [$^{14}$C]leucine incorporated given in the figure are for 5 μl aliquots.

### Effect of delayed addition of RI on kinetics of protein synthesis

Because the lag period (time of linear protein synthesis until shut-off) is characteristic of inhibition of protein synthesis in heme-deficient lysates as well as in lysates containing optimal amounts of hemin but with added RI (Fig. 1), it was of interest to determine whether the delayed addition of RI would influence the lag period. The delayed addition of RI decreased the lag time (Fig. 3). When RI was added 8 min after the start of incubation, protein synthesis ceased within the succeeding minute. A lag time of about 1 min is consistent with the time required for the completion of preexisting polypeptide chains on polyribosomes at 35°. This is in sharp contrast to a shut off time of 4 min when RI was added at the start of incubation. Similar results were observed when incubations were performed at 30° (results not shown); lag periods of 7.5 min, 6 min, 5 min,

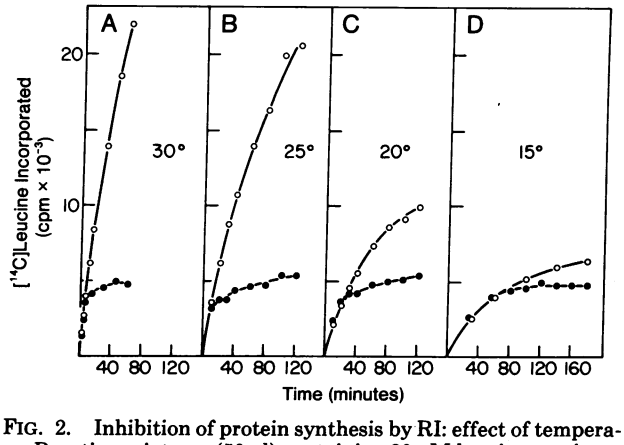


FIG. 2. Inhibition of protein synthesis by RI: effect of temperature. Reaction mixtures (50 μl) containing 20 μM hemin were incubated with 2.2 μg of RI, at 30° (A), 25° (B), 20° (C), and 15° (D). At intervals, 5 μl aliquots were taken out and assayed for protein synthesis. O—O, + hemin control; ●—●, + RI.

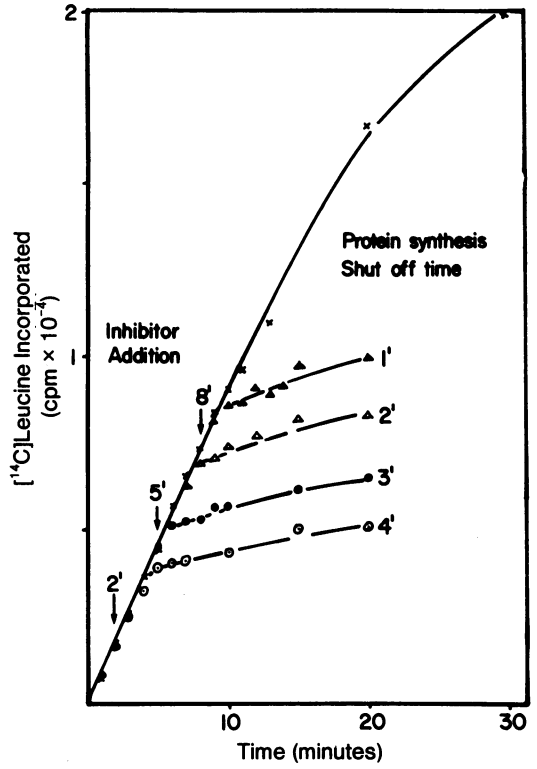


FIG. 3. Kinetics of protein synthesis: effect of delayed addition of RI. Reaction mixtures (50 μl) containing 10 μM hemin were incubated at 35°. RI (2.2 μg) was added at the start of incubation or after 2, 5, or 8 min of incubation. At intervals, samples (5 μl) were removed for protein synthesis assay. ×—×, plus hemin control; ⊙—⊙, RI was added at the start of incubation; ●—●, at 2 min; △—△, at 5 min; and ▲—▲, at 8 min after the start of incubation.

and 4 min were observed when RI was added to the incubation mixture at the start, or after 4 min, 8 min, or 12 min of incubation, respectively.

### Relationship between RI concentration and the reversal of inhibition of protein synthesis by IF-MP

It has previously been shown that the addition of the initiation factor IF-MP which forms a ternary complex with Met-tRNA$_f$ and GTP (IF-MP·GTP·Met-tRNA$_f$) prevents the onset of inhibition in heme-deficiency or in the presence of RI (13). However, little is known about the quantitative relationship between RI and this initiation factor. The relationship between RI concentration and the degree of reversal of inhibition of protein synthesis by IF-MP was examined as shown in Fig. 4. The following aspects should be noted: (*a*) the inhibition of protein synthesis was immediately reversed by the addition of IF-MP. (*b*) The degree of reversal increased with increasing concentrations of IF-MP. (*c*) At low levels of RI (1.1 μg) almost complete reversal was obtained with 0.9 and 1.5 μg of IF-MP, (Fig. 4A). (*d*) Although the degree of inhibition produced by 4.4 μg of RI (Fig. 4C) was not appreciably different from that caused by 2.2 μg of RI (Fig. 4B), the reversal of inhibition by equivalent amounts of IF-MP was not as pronounced in the presence of higher RI concentrations.

Data in Figs. 5 and 6 show that as in heme-deficiency, the inhibition of protein synthesis by RI was accompanied by the disaggregation of polyribosomes with a concomitant increase in 80S ribosomes. These results are in agreement with those of Mizuno *et al.* (24). Following the addition of IF-MP, protein synthesis is resumed (Fig. 5) and polyribosomes are reformed (Fig. 6). This synthesis can be blocked by edeine [an inhibitor of initiation, Obrig *et al.* (25)] (Fig. 5) with a resultant disaggregation of polyribosomes into 80S monomers.

## DISCUSSION

The experimental data presented in this study provide evidence that the mechanism of action of RI by several criteria, is very similar to that of the inhibitor which is formed or activated during prolonged heme-deficiency (6, 10, 26): (*a*) the biphasic kinetics of inhibition are similar to those observed in heme-deficiency; after several rounds of synthesis at the control rate, there is an abrupt decline in protein synthesis at about 7–8 min after the start of incubation; (*b*) inhibition of protein synthesis is accompanied by disaggregation of polyribosomes into 80S ribosomes; (*c*) inhibition is reversed by the initiation factor IF-MP. These results indicate that, as in the case of heme-deficiency (27), RI blocks polypeptide chain initiation. The similarity of RI to the physiological inhibitor is further supported

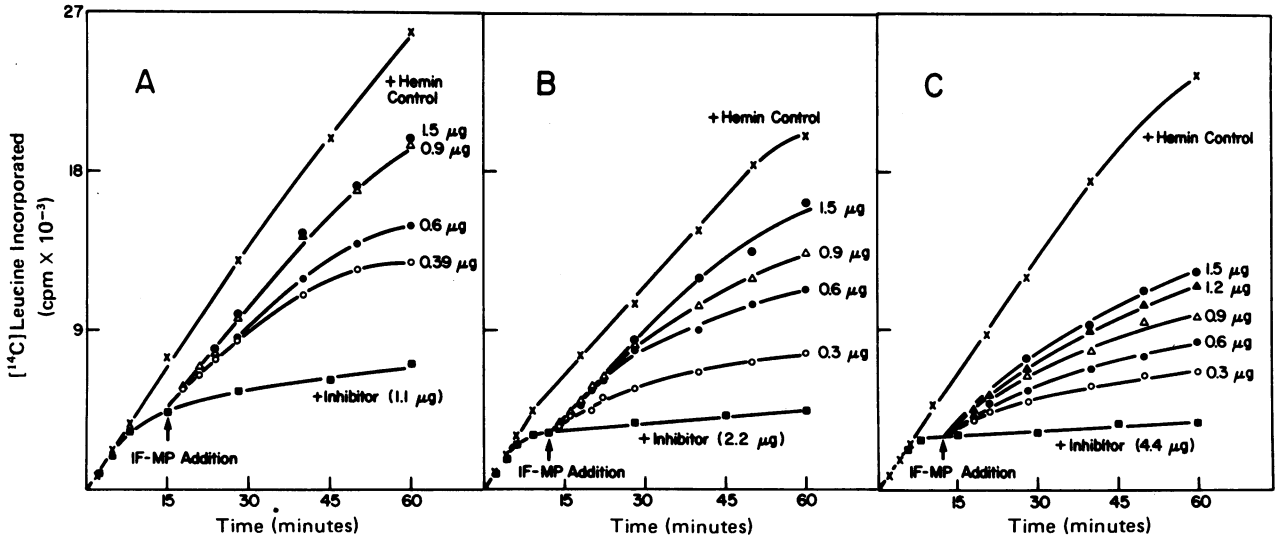


FIG. 4. Reversal of inhibition of protein synthesis by the initiation factor IF-MP: effect of RI concentration. Reaction mixtures (50 μl) containing 10 μM hemin were incubated at 30° with 1.1 μg (panel A) or 2.2 μg (panel B) or 4.4 μg (panel C) of RI. After 15 min (panel A) and 12 min (panel B and C) of incubation, IF-MP (amount as indicated) was added. At intervals, protein synthesis in 5 μl aliquots was assayed.

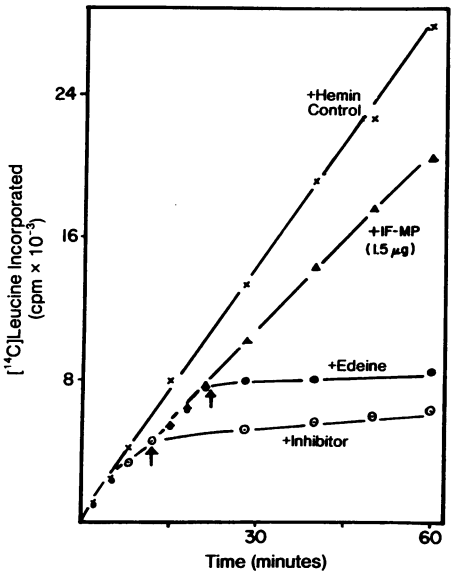


FIG. 5. Protein synthesis in the presence of RI, RI + IF-MP, and RI + IF-MP + edeine. Reaction mixtures (100 μl) containing 10 μM hemin were incubated at 30° with 2.2 μg of RI. After 12 min of incubation 1.5 μg of IF-MP was added to two of the samples. Edeine (0.5 μM) was added to one of these samples after 22 min of incubation. At intervals, aliquots (5 μl) were taken out and assayed for protein synthesis. ×—×, plus hemin control; ⊙—⊙, plus RI control; ▲—▲, plus RI + IF-MP; ●—●, plus RI + IF-MP + edeine.

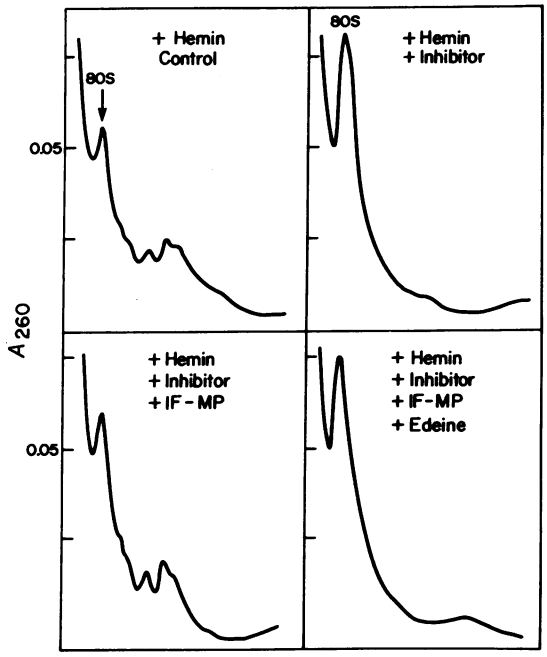


FIG. 6. Polyribosomal profile of incubation mixtures in Fig. 5. Aliquots of 60 μl from each reaction mixture, after 28 min of incubation, were removed and diluted with one volume of ice-cold buffer B [Tris·HCl (pH 7.6), 20 mM, KCl, 80 mM, and magnesium acetate, 2 mM]. Samples were layered on 5 ml of 15–50% (wt/vol) sucrose density gradients in buffer B and centrifuged in a Spinco SW 50.1 rotor, at 38,000 rpm for 1 hr. The temperature during the centrifugation run was maintained at 2°. The gradients were scanned for absorbance at 260 nm (13).

by the findings that the inhibition produced by RI is potentiated by ATP but blocked by high levels of GTP (2 mM) and cyclic AMP (10 mM) (28). Similar effects of these nucleotides were described earlier in heme-deficiency (29, 30). RI differs from the *in situ* heme-deficiency inhibitor in that the action of the latter at least in the early stages of inhibition, is reversed by hemin, whereas RI exerts its action in the presence of optimal concentrations of hemin.

The results on the effect of temperature on the action of RI show that RI was active even at temperatures as low as 15°. It is of particular interest to note that the level of protein synthesis prior to shut-off was constant at each temperature of incubation, an indication that the number of rounds of protein chain initiation before shut-off may be the same in each case. These results are similar to those previously observed in heme-deficiency (6) and provide further evidence for the close relationship of RI to the heme-deficiency inhibitor.

The lag period is observed in lysates in heme-deficiency and in lysates containing optimal levels of hemin to which RI has been added. The addition of RI, at intervals after the start of incubation, however, leads to a progressive decrease in the lag time. There are several possible explanations for this observation. It is possible that at the start of incubation IF-MP may be present in excess but as incubation proceeds the amount of active IF-MP may be progressively diminished; the remaining IF-MP may be recycled with great efficiency and may somehow be protected from inactivation so that protein synthesis can proceed (given enough amino acid mixture, particularly leucine) at the initial rate for extended periods as long as 90–120 min. But when RI is added, after excess IF-MP is no longer present, the remaining IF-MP may be inactivated and shut-off would occur very rapidly. The involvement of IF-MP in the mechanism of action of RI is discussed below. Another possibility is that during the early stages of incubation (the lag period) a component(s) (perhaps IF-MP) may exist in a form not readily susceptible to the action of RI. However, as protein synthesis proceeds, the component(s) may become more susceptible to inactivation by RI. Still another possibility is the accumulation during the early stages of incubation of a component which potentiates the action of RI. Of these possibilities there is some evidence for the first, i.e., the presence of excess initiation factor IF-MP (L. Cherbas and I. M. London, unpublished observation).

The inhibition of protein synthesis by RI is immediately reversed by the initiation factor IF-MP and the degree of reversal is increased with increasing concentrations of the initiation factor. However, at higher levels of inhibitor, at which inhibition of protein synthesis was not appreciably increased, there was significantly less reversal of inhibition by equivalent amounts of IF-MP. This finding suggests that one of the components inactivated by RI may be the initiation factor IF-MP. The association of protein kinase activity with RI (31) and the recent findings of Farrell *et al.* (P. Farrell, K. Balkow, T. Hunt, and R. J. Jackson, personal communication) and from this laboratory that RI phosphorylates initiation factor IF-MP suggest the involvement of phosphorylation in the mechanism of action of RI. These observations coupled with the data in the present study on the relationship of RI concentration to the reversal of inhibition of protein synthesis by IF-MP indicate that the regulation of protein synthesis in reticulocyte lysates by RI involves the initiation factor IF-MP.

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