Proc. Natl. Acad. Sci. USA
Vol. 73, No. 10, pp. 3506–3510, October 1976
Biochemistry

On the mechanism of delayed inhibition of protein synthesis in heme-deficient rabbit reticulocyte lysates

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

LUCY CHERBAS* AND IRVING M. LONDON

Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139; Department of Medicine, Harvard Medical School, Boston, Mass. 02115; and Harvard-MIT Program in Health Sciences and Technology, 77 Massachusetts Ave., Cambridge, Mass. 02139

Contributed by Irving M. London, August 2, 1976

ABSTRACT **In the absence of added hemin, protein synthesis in a rabbit reticulocyte lysate declines abruptly (shuts off) after about 5 min at 30°. In these studies we have examined the basis for the lag period preceding shut-off. The initiation factor that binds Met-tRNA$_f$, previously shown to be rate-limiting in inhibited, heme-deficient lysates, is found to be used stoichiometrically in the presence of excess inhibitor. We suggest that a principal effect of the inhibitor is to impair the recycling of the Met-tRNA$_f$-binding factor; the lag period is attributable largely to the presence of a pool of excess Met-tRNA$_f$-binding factor, which, once used in initiation, cannot be recycled because of the action of the inhibitor.**

When a rabbit reticulocyte lysate is incubated at 30° in the presence of added hemin, protein synthesis proceeds at a constant rate for a prolonged period, often more than an hour. If, instead, hemin is omitted from the reaction mixture, the synthetic rate declines abruptly about 5 min after the start of the incubation. During the 5 min period preceding shut-off, there is no detectable difference between the rates of synthesis in heme-containing (+H) and heme-deficient (−H) lysates (refs. 1 and 2; for review, see ref. 3).

The decrease in protein synthesis is immediately preceded by a decline in the amount of Met-tRNA$_f$·40S subunit complexes (4). Since the failure of protein synthesis can be remedied by supplying the initiation factor (IF)† required for the formation of such complexes (11), it is reasonable to infer that −H lysates become deficient in IF.

It is known that −H lysates contain one or more inhibitors of protein synthesis (12, 13); one such inhibitor which is stable and not reversible by hemin has been purified (14, 15). Partially purified fractions of this inhibitor have protein kinase activity (16) which may be specific for IF (ref. 17; P. Farrell, K. Balkow, T. Hunt, and R. J. Jackson, personal communication). Therefore, it appears that heme deficiency somehow generates an inhibitor which leads to the depletion of functional IF, possibly by phosphorylation, and thus inhibits further protein synthesis.

The kinetics of the inhibition (see Fig. 1) are much less understood. The period prior to the decline in protein synthesis (lag period) cannot be ascribed to the gradual depletion of a pool of endogenous heme (2) or to the completion of preformed nascent polypeptide chains (2, 4, 18). Here we present data suggesting that this lag period is not eliminated in a fresh lysate by a saturating concentration of endogenous inhibitor. In addition, we show that in the presence of excess inhibitor the number of peptide chains synthesized is related to the number of IF molecules initially present. These results, together with the sharp discontinuity in the rate of protein synthesis in −H lysates, form the basis of a model in which the action of the inhibitor results in impaired recycling of IF.

Abbreviations: +H, heme-containing; −H, heme-deficient; IF, initiation factor that binds Met-tRNA$_f$; T_L, time before shut-off of protein synthesis; S_L, synthesis before shut-off; S_N, synthesis attributable to completion of preformed nascent chains.

* Present address: The Biological Laboratories, Harvard University, 16 Divinity Ave., Cambridge, Mass. 02138.

† This initiation factor is known in the literature by the various names IF-MP (5), IF-L3 (6), IF-1 (7), IF-I (8), IF-E$_2$ (9), and EIF-3 (10); we shall refer to it simply as IF.

MATERIALS AND METHODS

Materials. Salt-washed 40S ribosomal subunits from rabbit reticulocytes (19) and rabbit reticulocyte IF were gifts of D. H. Levin. The subunits were stored as described (19), at a concentration of 175 A_{260} units/ml, and diluted with nine parts distilled water just before addition to the reaction mixture. (One A_{260} unit is that amount giving an A_{260} of 1 when dissolved in one ml and the light path is 1 cm.) The IF preparation contained 0.3 mg/ml of protein, and was stored at −80° in a buffer consisting of glycerol (10% vol/vol), KCl (80 mM), dithiothreitol (1 mM), and Tris·HCl (25 mM, pH 7.5). On the basis of sodium dodecyl sulfate–acrylamide gel electrophoretic patterns, Dr. Levin estimated it to be 40–50% pure, and its specific activity, measured as its ability to cause the binding of [^{35}S]Met-tRNA$_f$ to salt-washed 40S subunits, was 1–2 pmol of Met-tRNA$_f$ bound per μg of protein. Hemin-irreversible inhibitor, 250-fold purified (15), was a gift of R. S. Ranu.

Lysates and Protein Synthesis Assays. Rabbit reticulocyte lysates were prepared and used as described previously (ref. 2, as modified in ref. 11). The optimal hemin concentration, determined for each lysate, was used in +H reaction mixtures; this was always approximately 20 μM. Leucine concentrations in the reaction mixture were generally 20–30 μM (including endogenous leucine and 10 μCi/ml of [^{14}C]leucine). At this concentration, leucine became limiting after approximately 20 min; therefore, the concentration was raised to 50–60 μM by the addition of unlabeled leucine in those experiments for which it was desirable to prolong protein synthesis. Reaction mixtures were prepared at 0°. In one experiment, which required mixing the contents of tubes that had been incubated at 30° for different periods of time, reaction mixtures were stored on ice for periods up to 30 min before being brought to 30°. Once the incubation had begun, tubes were not rechilled. The initial period of storage in the cold had no apparent effect upon the subsequent behavior of the lysates.

Nomenclature. For describing experiments involving the mixing of preincubated lysates, we shall designate preparations as +H and −H, followed by a number to indicate the number of minutes of incubation at 30° before the preparations were mixed: e.g., −H10 indicates a −H reaction mixture preincubated for 10 min at 30°.

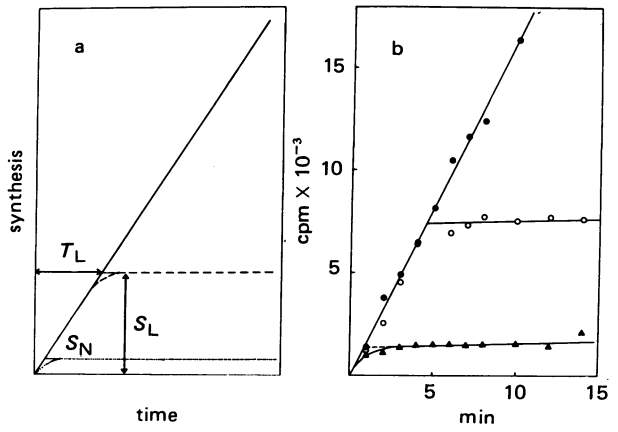

FIG. 1. Definition of T_L, S_L, and S_N. (a) Schematic time courses of protein synthesis in +H (—) and −H (- - -) lysates and in the presence of an inhibitor of initiation (·····). T_L, the lag period for inhibition; S_L, the amount of synthesis that takes place before shut-off; and S_N, the amount of synthesis occuring in the absence of initiation and hence attributable to the completion of preformed nascent chains, are indicated. (b) Experimental determination of T_L, S_L, and S_N. Reaction mixtures contained no additions (○), hemin (●), or hemin + edeine (5 μM final concentration) (▲). cpm refers to [^{14}C]leucine incorporated into material precipitable by trichloroacetic acid.

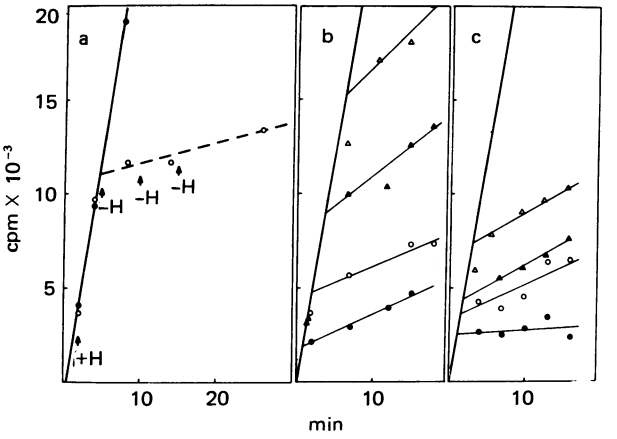

FIG. 2. Protein synthesis in mixtures of +H and −H lysates. Incubations were carried out as described in the *text*. Incorporated radioactivity was measured in 5 μl aliquots at the times indicated. (a) Control tubes +H (●) and −H (○). Arrows indicate the times of incubation at which tubes were taken for mixing. (b) Time course of incorporation in mixtures of +H2 and −H5 tubes. Abscissa: time after mixing; ordinate: incorporation after mixing. Ratios of +H to −H components in the mixtures were 1:2 (●), 1:1 (○); 2:1 (▲), and 3:1 (△). Heavy solid line represents the rate of synthesis in the control +H tubes shown in part (a). (c) Same as (b), with −H15 in place of −H5.

RESULTS

Lag period and completion of nascent chains

Fig. 1a shows schematic time courses of protein synthesis by +H and −H lysates and by a lysate in which initiation has been blocked. The figure is intended to define graphically the time before shut-off (T_L), synthesis before shut-off (S_L), and synthesis attributable to completion of preformed nascent chains (S_N).

Protein synthesis is not completely inhibited in −H lysates; the rate of synthesis after shut-off is typically about 5% of the initial rate. We shall not concern ourselves here with the "leakiness" of the inhibition, but shall attempt to explain the lag period and the discontinuity at shut-off.

Some typical experimental time courses are shown in Fig. 1b. Here, following a technique described previously (18), we have measured the amount of incorporation required to complete the preformed nascent chains by adding the inhibitor edeine, which prevents the initiation of new peptide chains. In this experiment, T_L is 4.5 min; in the lysates used for the experiments described in this paper, the value varied from 4 to 7 min. S_L was much more variable between lysates, because of a large variation in the initial rate of protein synthesis for the various lysates. Nonetheless, S_N was always the amount of incorporation which occurred in 0.75 min of normal synthesis; this value was obtained for several lysates, using edeine at concentrations of 0.5–5 μM, and corresponds to 1.5 min for synthesis of a complete globin chain (cf. refs. 20 and 21).

Effect of saturating concentrations of inhibitor on the lag period

In order to determine whether the lag period reflects the initial absence or low concentration of the inhibitor, we examined the kinetics of synthesis in the presence of saturating concentrations of the inhibitor. We chose to use the inhibitor present in −H lysates at the time of shut-off rather than a purified preparation of hemin-irreversible inhibitor in order more nearly to approach physiological conditions.

Using a lysate for which 10 and 20 μM hemin were equally effective, we brought aliquots of a reaction mixture containing 20 μM hemin and of a similar −H reaction mixture to 30°. The incubations were begun at different times, in order that +H2 tubes (for explanation of nomenclature, see *Materials and Methods*) could be mixed with −H5, −H10, and −H15 tubes. Each of these combinations was mixed in the ratios 3:1, 2:1, 1:1, and 1:2. Protein synthesis was monitored in each mixture and in unmixed controls (Fig. 2). In Fig. 2b and c, we have subtracted from each experimental value the amount of radioactivity already incorporated at the time of mixing, as calculated from the control time courses of Fig. 2a. The figures thus show incorporation after mixing as a function of time after mixing.

In numerous experiments we have found that synthesis in such mixtures immediately resumes the control (+H) rate, and then slows abruptly after a lag period. This finding is illustrated by the fact that the early time points for the various mixtures in Fig. 2b fall on the line representing synthesis in the +H control. Hence, we drew each experimental curve as two intersecting straight lines, an ascending line identical to the +H control time course, and a more nearly horizontal line defined by the later experimental time points (see Fig 2b and c). The intersection of the two lines was taken to be S_L, the amount of synthesis that took place between mixing and shut-off. Fig. 3 contains a summary of these values for S_L.

Differences in S_L for mixtures of −H5, −H10, and −H15 with +H2 in a given ratio may reasonably be attributed to differences among the −H lysates in the effective concentration of the inhibitor. The −H component in all cases is nearly devoid of active IF, since protein synthesis by such a lysate is entirely dependent upon the addition of exogenous IF (18). The +H component, being identical in all cases, contributes the same IF titer. Therefore, the concentration of active IF in a mixture of +H and −H lysates is fixed by the ratio of the two lysates. The other components required for protein synthesis are probably not depleted sufficiently to affect the rate of synthesis in any of the −H lysates, because addition of IF, even after 15 min of −H incubation, can restore synthesis temporarily to virtually the initial rate (P. Farrell, personal communication). Differences in behavior between mixtures of +H2 with −H5

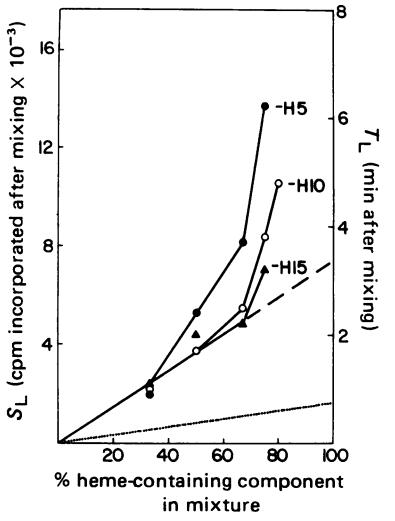

FIG. 3. Summary of S_L in the experiment of Fig. 2. Mixtures contained −H5 (●), −H10 (○), or −H15 (▲). ·····, calculated values for S_N; – –, calculation of minimum value for S_L (for explanation of calculations, see *text*).

and −H15 in a given ratio are, therefore, due to differences in the concentration of the inhibitor.

In general, when the proportion of the −H component in a mixture was less than 50%, S_L in the mixture decreased with the length of preincubation of the −H component (Fig. 3). Therefore, the −H5, −H10, and −H15 lysates may be considered as sources of progressively increased amounts of inhibitory activity. When the proportion of the −H component in the mixture was high, e.g., one part +H to two parts −H, all three −H lysates produced the same S_L. Thus, even though the amount of inhibitory activity is increased (i.e., by the substitution of −H15 or −H10 for −H5), the lag time is not shortened. In other words, in these mixtures, the inhibitor concentration was saturating.

In order to determine whether initiation of peptide chains continues in the presence of saturating concentrations of the inhibitor, we must compare S_L in these mixtures with S_N, the synthesis attributable to the completion of preformed nascent chains. S_N can be calculated. Since the −H component is depleted of polysomes (1), the synthesis attributable to the completion of preformed nascent chains may be obtained by multiplying the value of S_N for the +H lysate by the fraction of the mixture contributed by the +H lysate. This function is indicated by the dotted line in Fig. 3. Clearly, even in the presence of a saturating amount of inhibitory activity, there is continued initiation of protein synthesis. A similar result has been reported using the inhibitor present after prolonged incubation of a heme-deficient post-ribosomal supernatant (22).

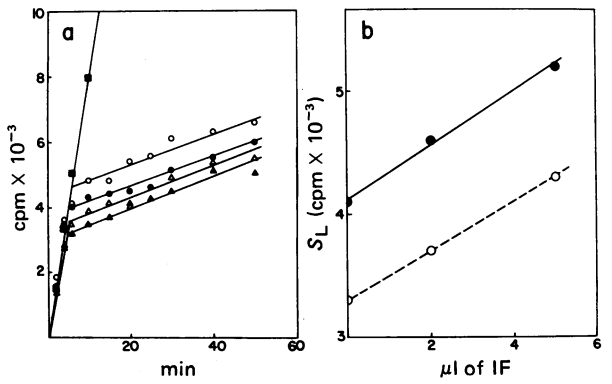

FIG. 4. Relief of inhibition by an IF preparation. IF (protein concentration, 0.3 mg/ml) and 40S subunits (17.5 A_{260} units/ml) were included in 60 µl reaction mixtures. Incorporated radioactivity was determined in 4 µl samples throughout the incubation. (a) Representative incorporation curves: ■, +H; ●, −H ○, −H + 2 µl of IF; ▲, −H + 6 µl of subunits; △, −H + 6 µl of subunits + 2µl of IF. (b) Summary of S_L determined graphically as in part (a): ●, without added subunits; ○, with 6 µl of subunits.

Stoichiometry of the reversal of inhibition by IF

Addition of purified IF to a −H lysate causes an increase in S_L (18). We have confirmed this observation (Fig. 4) and found that the increase in S_L is proportional to the amount of IF added (Fig. 4b; see also ref. 23). Assuming the IF molecular weight to be 140,000 (24), the purity of our preparation to be 50%, and the leucine concentration in the final reaction mixture to be 30 µM (confirmed by isotope dilution), one can calculate that, for each molecule of added IF, the equivalent of 0.5 additional globin chain is made before shut-off.‡

In order to inactivate some of the endogenous IF, isolated salt-washed 40S ribosomal subunits were added to the reaction mixture (Fig. 5). Two effects were observed. First, in both +H and −H lysates, the subunits caused a slightly delayed inhibition of protein synthesis, approximately in proportion to the concentration of added subunits; this inhibition was apparently not a result of IF depletion, since it was not prevented by the simultaneous addition of purified IF (data not shown). Second, in −H lysates S_L was reduced by an amount proportional to the concentration of exogenous subunits (Fig. 5); this effect was reversed by the addition of exogenous IF (Fig. 4a). For each 40S subunit added, S_L was decreased by an amount equivalent to 0.4 globin chain§; for each IF molecule added, there was 0.5 additional globin chain synthesized. Thus, the ratio of IF molecules to globin chains synthesized was maintained, even when the rate of protein synthesis was substantially reduced (30% in the experiment shown in Fig. 4), and this ratio was similar whether calculated from the addition of exogenous IF or from the removal of endogenous IF.

Presence of an excess of IF in +H lysates

When exogenous IF was added to a +H lysate, no significant effect was observed. The activity of the IF preparation was confirmed by its ability to cause a marked delay in shut-off in a lysate containing purified inhibitor. Hence, it is evident that IF is not limiting for protein synthesis in a +H lysate.

DISCUSSION

Inactivation of IF. In the presence of excess inhibitor in a −H lysate, IF can support protein synthesis only to the extent

‡ Calculating for a 4 µl aliquot of reaction mixture: The leucine concentration is 30 µM; its radiactivity is 4×10^4 cpm/4 µl. Therefore, 1 cpm = 1.8×10^9 molecules of leucine, and 1 cpm incorporated corresponds to 10^8 globin chains. When 1 µl of a 50% pure IF preparation containing 0.3 mg/ml of protein is added to a 60 µl reaction mixture, a 4 µl aliquot of the reaction mixture contains 10^{-8} g of IF, corresponding to 4.2×10^{10} molecules. 1 µl of IF in a 60 µl reaction mixture causes an increased incorporation of 220 cpm/4 µl aliquot (Fig. 4b). This gives 0.5 globin chain/IF molecule.

§ At 17.2 A_{260} units = 1 nmol of 40S subunits (25), 6 µl of a suspension at 17.5 A_{260} units/ml in 60 µl of reaction mixture gives 4×10^{10} exogenous subunits/4 µl aliquot. This leads to a decrease in S_L of 850 cpm/4 µl aliquot (Fig. 5b), or 8.5×10^{10} fewer globin chains (see calculation in previous footnote). Thus, the linear relation between S_L and the volume of added subunits (Fig. 5b) corresponds to a decrease in synthesis of 0.4 globin chain per subunit added.

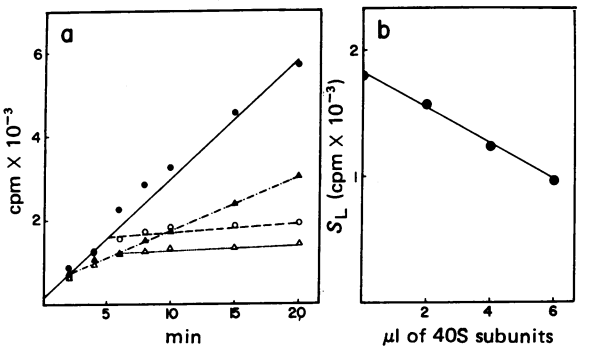

FIG. 5. Effect of salt-washed 40S subunits on incorporation. Hemin and/or various volumes of a suspension of salt-washed 40S subunits (approximately 17.5 A_{260} units/ml) were included in a reaction mixture (60 μl final volume). Incorporated radioactivity was measured in 4 μl samples from each tube at the indicated times. (a) ●, +H; ○, −H; ▲, +H + 4 μl of subunits; △, −H + 4 μl of subunits. (b) S_L, determined graphically as in part (a), as a function of the amount of added subunit suspension.

of 0.4–0.5 peptide chain per IF molecule; this amount of synthesis takes about 3 min (see below). In a +H lysate, protein synthesis continues at a constant rate for 30–60 min or even longer. Thus, each molecule of IF is used at least 10 times as frequently in the +H as in the −H lysate.

This effect of heme deficiency on the stoichiometry of IF function seems best explained by an inhibition of the recycling of IF; i.e., some step in the IF cycle between the formation of a 40S initiation complex and the reentry of IF into the pool of available factor is impaired by the action of the inhibitor. In an earlier study, the partially purified inhibitor decreased the binding of Met-tRNA$_f$ to 40S subunits in a highly purified system (11), but it was apparent that simple inhibition of binding failed to account for the delayed inhibition in the whole lysate (3, 11). An inhibition of recycling of IF, rather than inhibition of binding of Met-tRNA$_f$ to the 40S subunit, is compatible with (*a*) the undiminished formation of 40S subunit·Met-tRNA$_f$ complexes in whole lysates during the lag period (4), and (*b*) the finding that, in the whole lysate, the partially purified inhibitor did not diminish the amount of IF-dependent binding of methionine to 40S subunits when elongation was blocked (and recycling of IF would therefore not be expected to occur) (26). An inhibition of recycling also implies a close coupling between the inactivation of IF and its use in protein synthesis, and thereby accounts both for the constancy of the ratio between added IF and increased protein synthesis when the rate of protein synthesis is varied (Fig. 4b) and for the relative constancy of S_L at different rates of protein synthesis. The latter phenomenon has been reported both for −H lysates (2) and for +H lysates to which purified inhibitor has been added (15).

S_L in the Presence of Excess Inhibitor. Once the inhibitor has reached a saturating concentration, S_L will be determined by the amount of available IF. Using the data of Fig. 3, one can estimate S_L for a hypothetical −H lysate in which an excess of inhibitor is present from the start of the incubation.

Since an inhibited −H lysate is entirely dependent upon exogenous IF, we may assume that it contains no functional IF. In the experiment depicted in Figs. 2 and 3, therefore, each mixture contains an amount of IF proportional to the amount of +H lysate in the mixture and, in those mixtures in which the inhibitor is saturating, S_L is proportional to the amount of +H lysate. A straight line through those points in Fig. 3 that correspond to saturating inhibitor, extrapolated to 100% +H lysate, will thus give S_L for a lysate to which excess inhibitor is added at the start of the incubation. The broken line in Fig. 3 is our estimate of such a line; at 100% +H lysate, it gives a value of S_L = 7400 cpm, corresponding to T_L = approximately 3 min. Thus, even after the addition of saturating concentrations of inhibitor to a fresh lysate, a lag period of approximately 3 min may be expected.

Size of the IF Pool. If the reuse of IF is inhibited in a −H lysate, then the pool of available IF must decline steadily. Since no inhibition is detectable for the first 4–5 min of incubation, IF is not limiting in protein synthesis until it is almost exhausted. Two observations confirm the presence of an excess of IF. First, we have shown directly that IF is not initially limiting. Second, a 1:1 mixture of a +H lysate, which contains a normal concentration of IF, and an inhibited −H lysate, which contains little or no functional IF, resumes the control +H rate of protein synthesis (e.g., Fig. 2b); this implies that IF at half its normal concentration is not rate-limiting for protein synthesis.

Using the effect of known quantities of exogenous IF upon S_L (Fig. 4b) and our calculated value for S_L in the presence of excess inhibitor, we estimate that the original lysate must contain IF at a concentration of approximately 20 μg/ml. The amount actually extractable from the ribosomes of 1 ml of lysate in two different procedures is 1–5 μg (ref. 5; D. H. Levin, personal communication). Since a portion of the IF in the lysate is known to be in the post-ribosomal supernatant (refs. 23 and 27; W. C. Merrick, personal communication) and a substantial portion of the ribosome-bound factor is undoubtedly lost during purification, our rough estimate of 20 μg/ml does not seem unreasonable.

The IF Cycle. Our data suggest that there is a considerable excess of functional IF in a fresh lysate and that some step between the formation of a 40S initiation complex and the reentry of IF into that pool is inhibited in heme deficiency. Evidence for a multi-step cycle for IF has been reported (28). The inhibition probably involves phosphorylation of IF (ref. 17; Farrell *et al.*, personal communication), though additional modes of inhibition have not been excluded.

We wish to thank Drs. P. Cherbas, V. Ernst, T. Hunt, R. J. Jackson, D. H. Levin, and R. S. Ranu for helpful discussion during the experimental work and the preparation of the manuscript. The work was supported by USPHS Grant 5R01 AM-16272 and by a postdoctoral fellowship to L.C. from the Health Sciences Fund of the Massachusetts Institute of Technology.

1. Zucker, W. F. & Schulman, H. M. (1968) *Proc. Natl. Acad. Sci. USA* **59**, 582–589.
2. Hunt, T., Vanderhoff, G. & London, I. M. (1972) *J. Mol. Biol.* **66**, 471–481.
3. London, I. M., Clemens, M. J., Ranu, R. S., Levin, D. H., Cherbas, L. F. & Ernst, V. (1976) *Fed. Proc.*, in press.
4. Legon, S., Jackson, R. J. & Hunt, T. (1973) *Nature New Biol.* **241** 150–152.
5. Safer, B., Anderson, W. F. & Merrick, W. C. (1975) *J. Biol. Chem.* **250**, 9067–9075.
6. Levin, D. H., Kyner, D. & Acs, G. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 41–45.
7. Gupta, N. K., Woodley, C. L., Chen, Y. C. & Bose, K. K. (1973) *J. Biol. Chem.* **248**, 4500–4511.
8. Cashion, L. M., Dettman, F. L. & Stanley, W. M., Jr. (1974) in *Methods in Enzymology*, eds. Moldave, K. & Grossman, L. (Academic Press, New York), Vol. 30, pp. 153–171.
9. Schreier, M. H. & Staehelin, T. (1973) *Nature New Biol.* **242**, 35–38.
10. Ranu, R. S. & Wool, I. G. (1975) *Nature* **257**, 616–618.
11. Clemens, M. J., Henshaw, E. C., Rahamimoff, H. & London, I.

M. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2946–2950.
12. Maxwell, C. R. & Rabinovitz, M. (1969) *Biochem. Biophys. Res. Commun.* **35**, 79–85.
13. Howard, G. A., Adamson, S. C. & Herbert, E. (1970) *Biochim. Biophys. Acta* **213**, 237–240.
14. Gross, M. & Rabinovitz, M. (1973) *Biochem. Biophys. Res. Commun.* **50**, 832–838.
15. Ranu, R. S., Levin, D. H., Delaunay, J., Ernst, V. & London, I. M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2720–2724.
16. Levin, D. H., Ranu, R. S., Ernst, V., Fifer, M. A. & London, I. M. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4849–4853.
17. Levin, D. H., Ranu, R. S., Ernst, V. & London, I. M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3112–3116.
18. Clemens, M. J., Safer, B., Merrick, W. C., Anderson, W. F. & London, I. M. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1286–1290.
19. Levin, D. H., Kyner, D. & Acs, G. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1234–1238.
20. Hunt, T., Hunter, T. & Munro, A. (1969) *J. Mol. Biol.* **43**, 123–133.
21. Lodish, H. F. & Jacobsen, M. (1972) *J. Biol. Chem.* **247**, 3622–3629.
22. Maxwell, C. R., Kamper, C. S. & Rabinovitz, M. (1971) *J. Mol. Biol.* **58**, 317–327.
23. Gross, M. (1975) *Biochem. Biophys. Res. Commun.* **67**, 1507–1515.
24. Ranu, R. S. & Wool, I. G. (1976) *J. Biol. Chem.* **251**, 1926–1935.
25. Darnbrough, C., Legon, S., Hunt, T. & Jackson, R. J. (1973) *J. Mol. Biol.* **76**, 379–403.
26. Balkow, K., Mizuno, S., Fisher, J. M. & Rabinovitz, M. (1973) *Biochim. Biophys. Acta* **324**, 379–409.
27. Fuhr, J. E., London, I. M. & Grayzel, A. I. (1969) *Proc. Natl. Acad. Sci. USA* **63**, 129–134.
28. Walton, G. M. & Gill, G. N. (1975) *Biochim. Biophys. Acta* **390**, 231–245.