*STIMULATION OF GLOBIN-CHAIN INITIATION BY HEMIN IN THE RETICULOCYTE CELL-FREE SYSTEM**

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Recent experiments have shown that hemin or heme precursors stimulate globin synthesis in reticulocytes[1, 2] and in embryonic tissues,[3, 4] and that hemin increases the size and amount of polysomes in reticulocytes from iron-deficient rabbits.[2] It has been proposed that heme causes the release of completed nascent globin chains from the site of their synthesis.[5, 6] That this may not be the mechanism is suggested by experiments which show that the main product of a cell-free system from rabbit reticulocytes is a soluble globin dimer which can, in part, be converted to a tetramer by hemin.[7]

Since the original report by Kruh and Borsook[8] showing parallel rates of heme and globin synthesis in rabbit reticulocytes, interest has focused on the mechanism of regulation of hemoglobin synthesis. The idea has been advanced that selective release of globin chains from polysomes provides at least one point for regulating the synthesis of the globin portion of hemoglobin.[9] According to this scheme, α chains are released from polysomes only by β chains and $\alpha\beta$ dimers would constitute a first soluble intermediate, which would then be converted to hemoglobin.[10] Heme plays no role in this proposal.

We here report experimental results with an unfractionated cell-free system from rabbit reticulocytes which suggest that the function of hemin in globin synthesis is not solely in the terminal release of completed nascent chains. The results lead to the hypothesis that heme is specifically involved with an initiation process resulting in continued synthesis of new nascent chains from the amino-terminal valine, and that polysomal integrity is dependent on this function. This suggests a model for the translational control of globin synthesis by heme.

Methods.—Reticulocytes were obtained from rabbits made anemic with phenylhydrazine The cell-free system, prepared according to the proceedure of Lamfrom and Knopf,[11] has already been described.[7] Radioactivity was determined in a liquid scintillation counter with 65% efficiency for C^{14} and 21% for H^3. Total incorporation was determined with globin purified from the unfractionated system. Incorporation into nascent chains was determined with washed material which sedimented at 133,573 $\times$ g for 2.5 hr. Incorporation into soluble protein was determined with the remaining supernatant. Polysome profiles were obtained by automatic monitoring of 10–25% linear sucrose gradients which had been centrifuged at 78,700 $\times$ g for 3.5 hr in the Spinco SW25.3 rotor. Amino-terminal amino acid determinations were carried out using the three-cycle form of the Edman method[12] and the proceedure described by Blombäck *et al.*[13]

Results.— (1) *The effect of hemin on cell-free protein synthesis:* Figure 1 shows that 6.4×10^{-5} M hemin extends the period during which protein is synthesized. The effect of hemin is concentration-dependent with maximal stimulation from 3.2×10^{-5} M to 6.4×10^{-5} M. Higher concentrations were not tested. Hemin causes an initial reduction in the rate of synthesis, varying in different experiments, from 80 to 90 per cent of the rate of synthesis in the absence of hemin.

The amount of increased synthesis in the presence of hemin is variable and ranges from about 100 to 300 per cent.

It has been found that during storage at 0°C lysates lose their potential for stimulation by hemin much more rapidly than their protein-synthesizing ability, a fact which may account for the variability, since hemin may be interacting with a very labile component of the cell-free system.

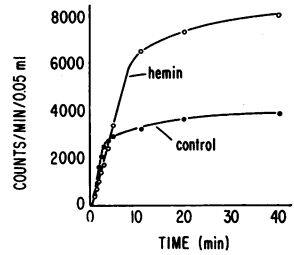

FIG. 1.—Effect of hemin on cell-free protein synthesis.

The specificity of hemin stimulation has been tested in various ways. Table 1 compares the effects of iron, protoporphyrin, and hemin in various combinations on the initial rate of amino acid incorporation and the total amount of incorporation at 20 minutes. In this experiment the initial rate lasted for three minutes in the absence of hemin and for ten minutes in its presence. Protoporphyrin (6.4×10^{-5} *M*) had little effect with or without iron present. Iron was initially inhibitory[14] and strongly antagonized the stimulation by hemin. Hemin at various concentrations, including that used in the reticulocyte cell-free system, did not stimulate protein synthesis in cell-free systems derived from rabbit liver and regenerating rat liver. Thus it appears that hemin, and not protoporphyrin or iron, specifically stimulated protein synthesis only in a system whose major product was globin chains.

TABLE 1. *A comparison of the effects on protein synthesis of iron, protoporphyrin IX, and hemin in various combinations.*

	Per Cent Minus Hemin	
	Relative specific activity at 2 min	Relative amount protein synthesized in 20 min
− Hemin	100	100
+ Hemin	88	325
− Hemin + Fe^{++}	70	124
+ Hemin + Fe^{++}	90	142
+ Protoporphyrin IX	108	136
+ Protoporphyrin IX + Fe^{++}	80	116
+ Hemin + protoporphyrin IX	88	349

Four ml of the complete cell-free system containing 3 μc of the uniformly labeled C^{14}-amino acid mixture (sp. act. ~1 mc/mg) was incubated in the presence of the above additions for various times. Aliquots of 0.05 ml were removed and radioactivity in total protein determined as described in *Methods*. All concentrations were 6.4×10^{-5} *M*.

(2) *The effect of hemin on polysomes:* Figure 2 shows the effect of hemin in the cell-free system on the accumulation and disappearance of polysomes. It is apparent that the presence of hemin results in an increase in the proportion of polysomes to 80*S* material and stabilizes the aggregates. In the absence of hemin, complete polysome disaggregation occurs between 5 and 7 minutes, while in the presence of hemin, the polysomes are stabilized for more than 45 minutes. Hemin is not acting as a nuclease inhibitor because polysomes from a cell-free system containing hemin are as sensitive to small amounts of ribonuclease (1 μg/ml) as are those from a control lysate.

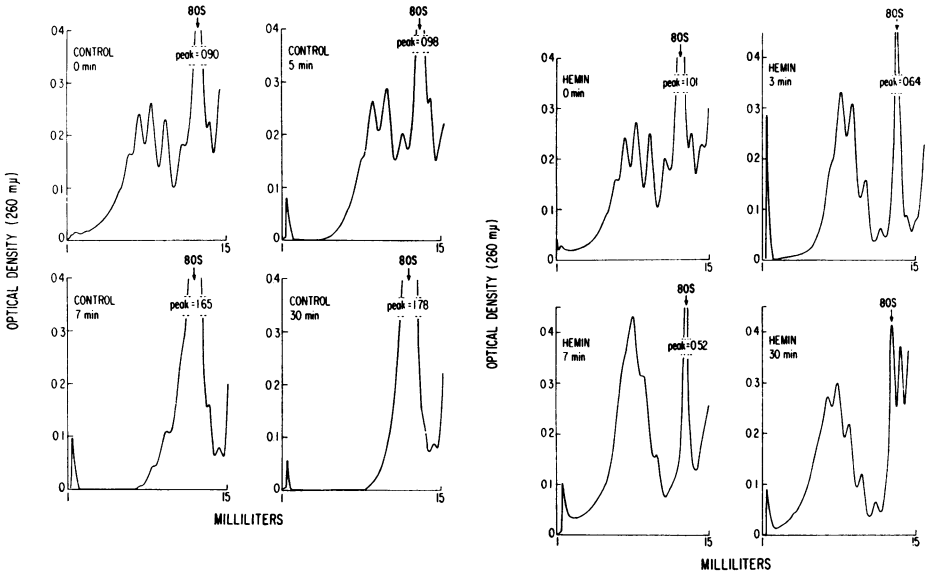

FIG. 2.—Effect of hemin on polysome distribution in the cell-free system.

(3) *The effect of hemin on the incorporation of radioactive amino acid into nascent and soluble protein:* To determine where hemin affected protein synthesis, the kinetics of incorporation of a radioactive amino acid into nascent and soluble protein was measured. A comparison of the specific activities of the nascent and soluble fractions in the presence and absence of hemin is shown in Figure 3.

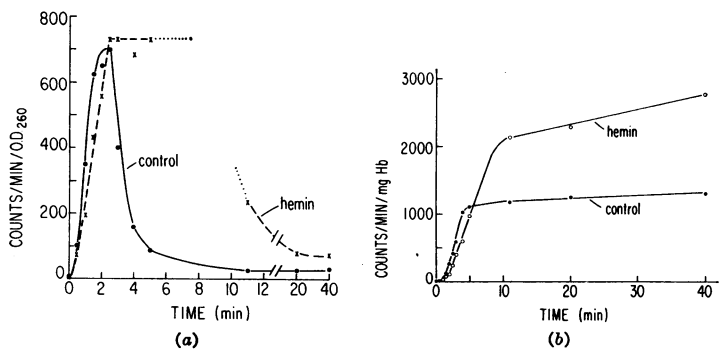

FIG. 3.—Effect of hemin on the incorporation of C^{14}-amino acids into nascent chains (*a*) and soluble protein (*b*).

With or without hemin, polysomes, after a short lag, became almost equally saturated with radioactivity, but in the presence of hemin the polysomes remained saturated for a much longer time. In the absence of hemin, nascent chain release was rapid and almost complete, with 3–10 per cent of the nascent chains remaining associated with the ribosomal material (in the presence of hemin as much as 15% remained after 40 min). The remaining nascent radioactivity

TABLE 2. *The specific activities of the soluble protein and the amino-terminal valine in the presence and absence of hemin.*

	Minus Hemin		Plus Hemin			
	Cpm/mg hemoglobin	Cpm/μmole amino-term. amino acid	Cpm/mg hemoglobin	Cpm/μmole amino-term. amino acid	Sp. act. of protein (+ H/− H)	Sp. act. of amino-terminal amino acid (+ H/− H)
Globin	26,002	8,890	76,531	25,421	2.99	2.87
α-Chains	13,725	—	34,220	—	2.49	—
β-Chains	29,076	—	65,077	—	2.24	—

One ml of the complete cell-free system was incubated for 60 min in the presence and absence of hemin with 25 μc of [C^{14}] uniformly labeled amino acid mixture (see below). Globin was purified from each and split into α and β chains. The specific activities were determined as described in *Methods*. Protein concentration was determined by a ninhydrin assay after hydrolysis of the protein for 24 hr in 6 *N* HCl at 105°.

Amino acid mixture (in a total of 1000 μc): L-Ala, 80 μc; L-Arg, 70 μc; L-Asp, 80 μc; L-Glu, 125 μc; Gly, 40 μc; L-His, 15 μc; L-Ileu, 140 μc; L-lys, 60 μc; L-Phe, 80 μc; L-Pro, 50 μc; L-Ser, 40 μc; L-Thr, 50 μc; L-Tyr, 50 μc; L-Val, 80 μc.

was not bound, charged sRNA because it was not solubilized by hot trichloroacetic acid (TCA). The results show that hemin stimulated continued synthesis of nascent chains.

It appears that hemin has an effect on the incorporation of amino acids into total and soluble protein which may be attributed to a reduced rate of nascent chain release.

(4) *Evidence for increased globin-chain initiation in the presence of hemin: De novo* synthesis of α and β chains has been previously demonstrated in this cell-free system.[15] To determine whether hemin stimulates initiation of new globin chains, from the amino-terminal valine of both α and β chains as opposed to random initiation along the peptide chain, the radioactive-soluble product of the cell-free system incubated in the presence and absence of hemin was subjected to amino-terminal analysis by the Edman procedure.[12, 13] Table 2 summarizes the results from an experiment in which a mixture of C^{14}- amino acids was used as the radioactive tracer. In the amino acid mixture, valine accounted for 8 per cent of the total radioactivity (see Table 2). In this experiment, the α and β chains were separated and their specific activities determined (Fig. 4). The effect of hemin on both the specific activities of the purified globin, α and β chains, and

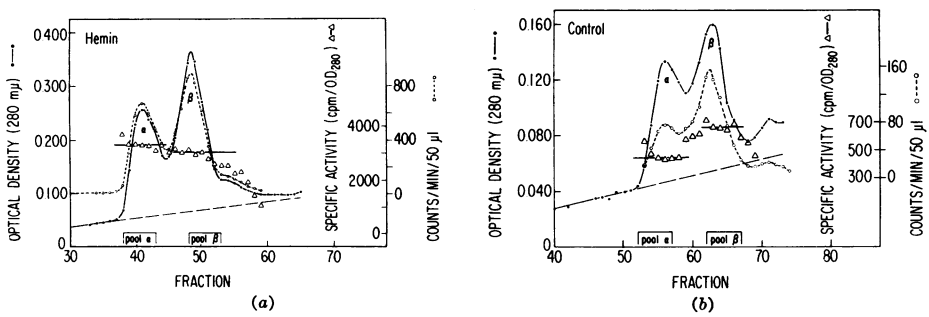

FIG. 4.—Carboxymethylcellulose chromatography[25] of globin from the cell-free system incubated in the presence (*a*) and absence (*b*) of hemin.

the amino-terminal phenyl thiohydantoin (PTH) derivative from the purified globin was determined. It can be seen that the specific activity of the amino-terminal amino acid of the purified globin was increased in the presence of hemin and was about equal to the increased specific activity of the globin itself. When the α and β chains were analyzed separately, it was found that hemin affected their syntheses to about the same extent, although it is apparent that approximately twice as many β chains were synthesized as were α chains, assuming no dilution of the specific activity of the latter by a free pool of α chains.[16] There was a loss of some protein, in the step between purified globin and the purified α and β chains, since both the specific activities and the $^{+}H/-H$ ratio decreased significantly. Nevertheless, the radioactive protein synthesized in the presence of hemin was chromatographically identical to α and β chains.

Sufficient radioactive amino acids were added so that the amino-terminal amino acid of the purified globin could be identified by paper chromatography. The only radioactive amino-terminal amino acid detected was valine.

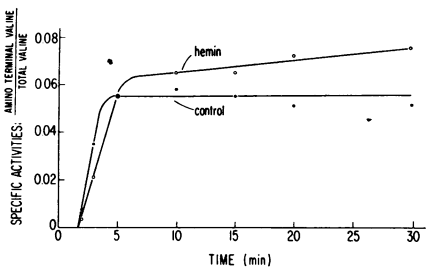

FIG. 5.—The ratio of amino-terminal C^{14}-valine to total C^{14}-valine incorporated into soluble protein in the presence and absence of hemin.

Since both the α and β chains of rabbit hemoglobin contain valine in the amino-terminal position, it is clear that hemin is not involved with random initiation of peptide chains, unless it be specifically at valine sites along the chain. Evidence that suggests this is not the case was obtained by calculating the ratios of the specific activities of amino-terminal valine to total valine incorporated in the presence and absence of hemin. Since Figure 5 shows that this ratio does not exceed 0.083 (the value obtained from rabbit hemoglobin uniformly labeled with valine) in the presence of hemin, it seems unlikely that hemin caused chain initiation at internal valine residues of the globin chains.

(5) *Effects of puromycin and NaF on the stabilization of polysomes by hemin:* The ability of hemin to prolong cell-free protein synthesis by allowing increased chain initiation from the amino-terminal valine of both α and β chains has been shown directly and is accompanied by a pronounced stabilization of polysomes. Since initiation of α and β chains is not dependent on the existence of polysomes,[15] it seemed reasonable to determine whether hemin-mediated polysome stabilization was a result of, or a cause of, the observed increased chain initiation. To determine this, the cell-free system was incubated in the presence of the two inhibitors of protein synthesis, puromycin and NaF. Puromycin is known to inhibit both *in vivo*[17] and *in vitro*[18] protein synthesis by causing premature release of nascent polypeptide chains. NaF, on the other hand, inhibits protein synthesis in the reticulocyte cell-free system by preventing chain initiation, resulting

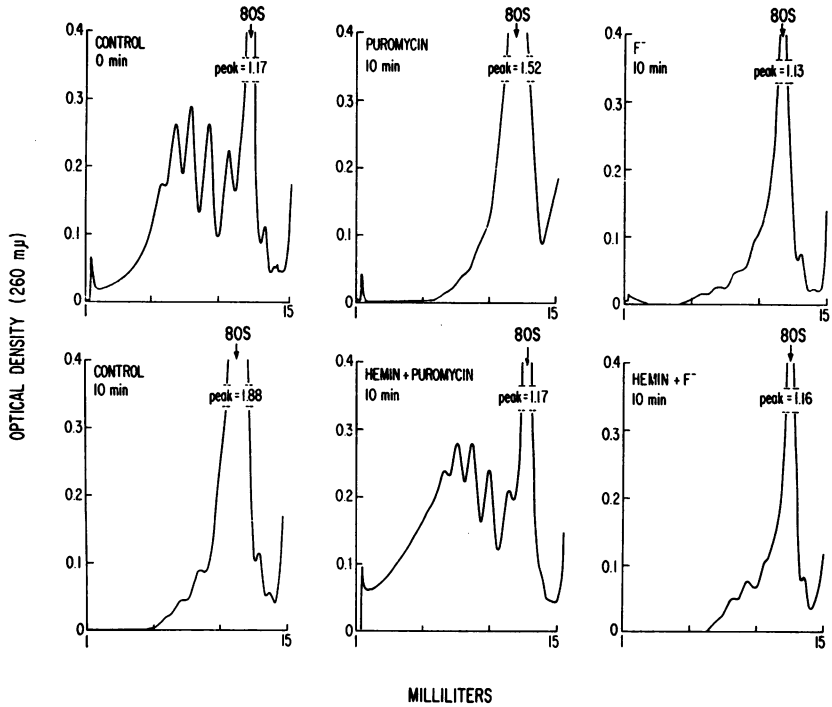

FIG. 6.—Effects of puromycin, NaF, and hemin on polysome distribution in the cell-free system.

in an inability to form the first peptide bond[19] but not the formation of internal peptide bonds.[20] It has also been postulated that NaF inhibits the binding of mRNA to ribosomes.[21]

Table 3 shows that 1.4×10^{-4} *M* puromycin completely inhibited protein

TABLE 3. *Effect of puromycin and NaF on cell-free protein synthesis in the presence and absence of hemin.*

Expt.		Specific Activity (cpm/mg hemoglobin) of Soluble Protein − Hemin	+ Hemin
1*	− Puromycin	1096	2493
	+ Puromycin (1.4×10^{-4} *M*)	<10	<10
2†	− NaF	2289	5030
	+ NaF (1×10^{-2} *M*)	363	356

* A cell-free system containing 0.7 μc/ml of C^{14} uniformly labeled amino acid mix was incubated for 15 min in the presence and absence of hemin as indicated.

† A cell-free system containing 5.5 μc/ml of C^{14}-valine (sp. act. 267 mc/mmole) was incubated for 15 min in the presence and absence of hemin as indicated. The specific activity of the soluble protein was determined as described in *Methods*.

synthesis, whether or not hemin was present. Nevertheless, as shown in Figure 6, polysomes were stabilized by hemin in the presence of puromycin. In contrast to puromycin, NaF partially inhibited protein synthesis and hemin was without effect on this inhibition or on polysome disaggregation.

These results suggest that the hemin effect probably involves the formation of the first peptide bond. Furthermore, if the postulated mode of action of puromycin is correct, these results also demonstrate that premature release of nascent chains does not affect polysome stability, and thus the effect of hemin on polysome stability cannot depend solely on nascent chain release.

Discussion.—The reason for using an unfractionated reticulocyte cell-free system to investigate the control of hemoglobin synthesis rests on the finding that, in this system, extensive *de novo* synthesis of both α- and β-globin chains occurs. This is not the case for the totally fractionated system.[11] When hemin is added to the unfractionated system, it produces effects similar to those observed with whole reticulocytes.

It has been demonstrated that the increased synthesis of protein in the presence of hemin is accompanied by a corresponding increase in the initiation of α- and β-globin chains from their amino-terminal valine residues, thus excluding the possibility that hemin acts nonspecifically.

In the absence of hemin, release of nascent chains is almost complete with a concomitant disaggregation of polysomes to 80*S* monomers. Hemin extends the period of nascent chain synthesis and polysome integrity. These two effects have been dissociated by the use of two different inhibitors of protein synthesis. When nascent chains are released prematurely from polysomes by the action of puromycin, hemin-mediated polysome stabilization is still observed. When protein synthesis is blocked with NaF, which prevents the formation of the first peptide bond, hemin is unable to stabilize the polysomes. These results are compatible with the idea that hemin is involved with a chain initiation process. In conjunction with the observed nascent chain release in the absence of added hemin, it strongly suggests that hemin does not act solely through a chain release mechanism.

Grayzel *et al.*[2] reported that hemin has no effect on the disaggregation of polysomes by puromycin in iron-deficient reticulocytes; and Williamson and Schweet[22] reported an increased rate of polysome disaggregation in the totally fractionated system in the presence of puromycin. The contrary results presented here may be unique for the unfractionated cell-free system. Nevertheless, this positive result is in accord with a function for hemin in chain initiation.

The possibility is raised that heme may play a direct role in regulating the translation of globin messenger RNA. A mechanism which would require heme for the initiation of globin messenger RNA translation would explain why heme and globin synthesis are coupled in reticulocytes, since heme biosynthesis is feedback inhibited by free heme.[23]

Although it has been demonstrated that hemin stimulates chain initiation, it is nonetheless clear that chain initiation can occur in this cell-free system in the absence of exogenous hemin. Thus, an obligatory dependence of chain initiation on hemin has not been demonstrated. It is possible that the existence of very small pools of free heme, or the availability of heme from hemoglobin[24] may suffice to explain the *de novo* synthesis observed in the absence of exogenous hemin.

It is possible that the increased chain initiation and the preservation of the

polysomes caused by hemin are specifically related. Polysomes may be stabilized because increased initiation, involving heme, and ribosomal attachment to polysomes are mutually dependent.

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[1] Bruns, G. P., and I. M. London, *Biochem. Biophys. Res. Commun.*, **18**, 236 (1965).
[2] Grayzel, A. I., P. Hörchner, and I. M. London, these PROCEEDINGS, **55**, 650 (1966).
[3] Levere, R. D., and S. Granick, *J. Biol. Chem.*, **242**, 1903 (1967).
[4] Wainwright, S. D., and L. K. Wainwright, *Can. J. Biochem.*, **45**, 344 (1967).
[5] Cline, A. L., and R. M. Bock, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966), p. 321.
[6] Gribble, T. J., and H. C. Schwartz, *Biochim. Biophys. Acta*, **103**, 333 (1965).
[7] Zucker, W. V., and H. M. Schulman, *Biochim. Biophys. Acta*, **138**, 400 (1967).
[8] Kruh, J., and H. Borsook, *J. Biol. Chem.*, **220**, 905 (1956).
[9] Baglioni, C., and B. Colombo, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 29 (1964), p. 347.
[10] Felicetti, L., B. Colombo, and C. Baglioni, *Biochim. Biophys. Acta*, **129**, 380 (1966).
[11] Lamfrom, H., and P. M. Knopf, *J. Mol. Biol.*, **9**, 558 (1964).
[12] Edman, P., *Ann. N.Y. Acad. Sci.*, **88**, 602 (1960).
[13] Blombäck, B., M. Blombäck, P. Edman, and B. Hessel, *Biochim. Biophys. Acta*, **115**, 371 (1966).
[14] Van Stone, J., R. O. Coleman, and P. Heller, *Nature*, **210**, 843 (1966).
[15] Lamfrom, H., and P. M. Knopf, *J. Mol. Biol.*, **11**, 589 (1965).
[16] Shaeffer, J. R., *Biochem. Biophys. Res. Commun.*, **28**, 647 (1967).
[17] Allen, D. W., and D. C. Zamecnik, *Biochim. Biophys. Acta*, **55**, 865 (1962).
[18] Morris, A., R. Arlinghaus, S. Favelukes, and R. Schweet, *Biochemistry*, **2**, 1084 (1963).
[19] Lin, S., R. D. Mosteller, and B. Hardesty, *J. Mol. Biol.*, **21**, 51 (1966).
[20] Ravel, J. M., R. D. Mosteller, and B. Hardesty, these PROCEEDINGS, **56**, 701 (1966).
[21] Dreyfus, J. C., and G. Shapira, *Biochim. Biophys. Acta*, **129**, 601 (1966).
[22] Williamson, A. R., and R. Schweet, *J. Mol. Biol.*, **11**, 358 (1965).
[23] Karibian, D., and I. M. London, *Biochem. Biophys. Res. Commun.*, **18**, 243 (1965).
[24] Bunn, H. F., and J. H. Jandl, these PROCEEDINGS, **56**, 974 (1966).
[25] Beard, N. S., and S. A. Armentrout, these PROCEEDINGS, **58**, 750 (1967).