Control of Protein Degradation in Reticulocytes and Reticulocyte Extracts by Hemin*

Joseph D. Etlinger‡ and Alfred L. Goldberg§
From the Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115

Received for publication, March 14, 1979

Previous studies have established that hemin stimulates production of globin and other proteins in reticulocytes and nonerythroid cells. Such studies generally equated increased production with more rapid synthesis and ignored the additional possibility that hemin decreases protein degradation. Addition of hemin to cell-free extracts of rabbit reticulocytes consistently inhibited the rapid ATP-dependent degradation of abnormal globins containing puromycin or an amino acid analog, of apohemoglobin, and of apomyoglobin. In addition hemin decreased the slower degradation of normal hemoglobin. Hemin reduced to a similar extent the degradation of nonheme proteins, including lysozyme, α-casein, and mixed Escherichia coli proteins. Therefore, the primary action of hemin probably involves an inhibition of the ATP-dependent proteolytic system rather than a specific stabilization of globin resulting from heme binding. Accordingly, the degradation of globin was much more sensitive to exogenous hemin in the presence of ATP than in its absence.

Addition of hemin to intact reticulocytes also inhibited the degradation of abnormal and normal hemoglobin while stimulating protein synthesis. These actions of hemin on synthesis and degradation appear additive in promoting net protein accumulation and thus preventing an imbalance between the production of globin and heme. However, the relative importance of the effects of hemin on synthesis and proteolysis varied in different cell preparations. Thus, in certain preparations, the enhanced accumulation of globin induced by hemin resulted primarily from decreased degradation, while in others enhanced synthesis appeared to be the more important effect. The basis for this variability in the responses of whole cells to hemin is unclear.

Globin production in reticulocytes (1), in reticulocyte extracts (2), and in frog oocytes injected with hemoglobin mRNA (3) depends on the supply of heme. In iron-deficient animals or humans, hemoglobin levels are less than normal and supplying hemin stimulates globin synthesis (4). Hemin has also been shown to increase the production of a nonheme protein in reticulocytes (5) and average cell protein in ascites tumor cells (6). Many studies have indicated that heme stimulates protein synthesis at the translational level by enhancing the rate of polypeptide initiation (6, 7). In cell lysates, heme appears to promote this process by preventing the inactivation of a specific initiation factor (eif-2) (6–12).

The accumulation of any cell protein depends upon both its rates of synthesis and degradation (13–15). The possibility that hemin also promotes net production of globin by decreasing the degradation of this protein has not been investigated. In erythroid cells hemoglobin is an unusually stable protein, and it has generally been assumed that this polypeptide, once synthesized, is stable for the lifespan of the cell. However, certain abnormal hemoglobins, including several human variants, are known to be rapidly degraded within reticulocytes (15, 16). The rate of degradation of these proteins and even the resistance of normal hemoglobin to intracellular degradation may depend on the availability of heme.

Recently, we have been studying the rapid hydrolysis of abnormal globins that result from the incorporation of amino acid analogs or puromycin into hemoglobin (17, 18). This degradative process like protein breakdown in other cells (15) requires metabolic energy (17, 18). We have described in reticulocyte extracts a nonlysosomal, ATP-dependent proteolytic system that appears responsible for this process (17–19) in intact cells. Several human hemoglobin variants are also selectively degraded by this system (20). Analogous ATP-stimulated proteolytic activities also have been found in other mammalian cells and in bacteria (21, 22). The present report demonstrates that hemin apparently by inhibiting this nonlysosomal system can markedly decrease the intracellular degradation of abnormal polypeptides as well as of normal globin. Furthermore, this inhibition of proteolysis may be an important factor contributing to the stimulation of globin production by hemin in intact cells.

MATERIALS AND METHODS

Reticulocytes were isolated as described previously (17) from phenylhydrazine-treated rabbits maintained on a normal diet (Purina Rabbit Chow). For measurements of protein degradation, the cells were preincubated at 37°C for 90 min in 3 to 4 volumes of Krebs-Ringer phosphate buffer containing (2 mg/ml) glucose and plasma levels of the amino acids (17) with or without heme (50 μM). Hemin was dissolved in 0.05 M Tris base (pH 9.0), and an equal volume of this buffer was added to the control incubations. The cells were then incubated in a similar medium that contained either the normal amino acids or the valine analog, 2-amino-3-chlorobutyric acid (1 mM), in place of valine. ACB was kindly provided to us by Dr. W. F. Prouty (Eli Lilly Co., Indianapolis). To produce puromycin-containing preparation.

2. G. DeMartino, N. Strnad, and A. L. Goldberg, manuscript in preparation.
3. The abbreviation used is: ACB, 2-amino-3-chlorobutyric acid.
polypeptides, the cells were incubated with 10 µg/ml of puromycin in the presence of all the plasma amino acids. After incubation for 5 min, 10 µCi/ml of [3H]leucine (6 Ci/nmol) were added for an additional 2 min. The cells were then washed 3 times and resuspended in the Krebs-Ringer phosphate buffer containing glucose, normal plasma amino acids, and 10 mM nonradioactive leucine. Cells were incubated at 37°C, and at various times aliquots were removed and treated with trichloroacetic acid, as described previously (17). Protein breakdown was expressed as the amount of acid-soluble radioactivity released relative to that initially incorporated into protein (i.e. acid-precipitable material) (17).

For measurement of net production of new proteins, the reticulocyte extracts were incubated with [3H]leucine for prolonged periods in the presence or absence of hemin (50 to 500 µM) without any preincubation. For measurements of protein synthesis, in the absence of significant protein degradation, cells were preincubated with or without hemin for varying periods of time and then were exposed to [3H]leucine for 2 min. Net production and synthesis are expressed as disintegrations per min incorporated into acid-precipitable protein (17).

The soluble cell-free proteolytic system was prepared from rabbit reticulocytes as described previously (17). To follow degradation of ACB- and valine-containing protein or puromycyl polypeptides, cell-free extracts were prepared from cells containing these labeled substrates (17). Alternatively, [3H]-labeled apohemoglobin, apomyoglobin, lysozyme, and α-casein were added exogenously to extracts prepared from cells which contained no labeled proteins. To label these substrates, equine myoglobin, lysozyme, and α-casein obtained from Sigma Chemical Co. (St. Louis) were methylated using [3H]formaldehyde. Extraction of the hemebinding moiety from labeled hemoglobin and myoglobin was carried out to obtain the apoproteins (24). Approximately 0.1 mg (10^6 dpm) of these exogenous substrates was added to each milliliter of extract. [3H]-Labeled proteins from Escherichia coli were being grown in a K12 strain (A333) in the presence of limiting amounts of [3H]methionine, followed by addition of trichloroacetic acid to precipitate protein and solubilization with NaOH, as described elsewhere (25). Degradation in the presence or absence of hemin (15 to 75 µM) was measured after incubation of cell-free extracts containing ATP (1 mM) for 30 min, as described previously (17).

**RESULTS**

**Inhibition of Protein Degradation by Hemin in Reticulocyte Extracts**—We have previously described a soluble ATP-dependent proteolytic system which selectively hydrolyzes various abnormal globins (17, 18). For the present cell-free assays, two procedures were used. In one type of experiment, the cell lysates were prepared from reticulocytes which had previously been allowed to incorporate [3H]leucine into proteins. Reticulocyte proteins containing the valine analog 2-amino-5-chlorobutyric acid in place of valine were degraded approximately five times faster than normal (i.e. valine-containing) protein in accord with earlier results. Hemin (75 µM) consistently reduced the degradation of both valine- and ACB-containing proteins by over 70% (Table I). Incorporation of puromycin into growing polypeptides leads to the premature release from ribosomes of the incomplete proteins which are then rapidly hydrolyzed. The addition of hemin to the cell-free extracts inhibited the rapid breakdown of these puromycyl polypeptides by at least 50% (Table I).

A second protocol utilized reticulocyte extracts from unlabelled cells to which [3H]- or [3S]-labeled proteins were added. It is noteworthy that purified hemoglobin and myoglobin are relatively poor substrates for this degradative system even after labeling with [14C]formaldehyde. However, extraction of their heme moieties (24) makes these polypeptides appreciably more sensitive to proteolysis. In the presence of hemin, the hydrolysis of these apoproteins was reduced by up to 70% (Table I and Fig. 1). A marked inhibition of globin hydrolysis was also found with much lower concentrations of hemin, ranging between 5 and 30 µM (Fig. 1).

A simple explanation of such results would be that the binding of heme to the apoprotein and the abnormal globins reduces their susceptibility to proteolytic enzymes. However, the inhibition of proteolysis by hemin was not restricted to heme-containing proteins. As shown in Tables I and II, hemin also decreased the breakdown of [3H]-labeled α-casein and mixed [3S]-labeled proteins from E. coli. Furthermore, the magnitude of hemin's effect on the degradation of the non-heme proteins was similar to the inhibition of globin degradation. In addition, the degradation of globin and that of casein were inhibited to a similar extent over a wide range of hemin concentration (Table II).

The similar reduction of hydrolysis of apohemoglobin, apomyoglobin, and proteins (e.g. casein) not containing a heme prosthetic group indicates that the inhibition of proteolysis cannot be simply explained by stabilization of protein substrates by the binding of heme. Instead, this effect must involve primarily an inhibitory action of hemin on the proteolytic system. Additional evidence in support of this conclusion comes from the observation that hemin consistently inhibited ATP-dependent proteolysis to a greater extent than the residual, ATP-independent degradation occurring in such extracts (Fig. 1).
Inhibition by hemin of the breakdown of α-casein and globin

\( ^{14} \text{C}-\text{CH}_3\text{-}a\)-casein or \( ^{14} \text{C}-\text{CH}_3\text{-}(\text{apo})\text{hemoglobin} \) were added to cell-free extracts as in Table I. Degradation was measured after incubation in the presence of ATP (1 mM) for 30 min, as previously described (17).

<table>
<thead>
<tr>
<th>Hemin (µM)</th>
<th>α-Casein</th>
<th>Globin</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>38</td>
<td>35</td>
<td>31</td>
</tr>
<tr>
<td>76</td>
<td>75</td>
<td>59</td>
</tr>
</tbody>
</table>

**Effect of Hemin on Proteolysis in Whole Cells**—To test whether the supply of hemin might influence protein degradation in intact cells, reticulocytes were initially incubated with or without hemin (500 µM) for varying periods of time and then were exposed to a pulse of [\( ^{1} \text{H} \)]leucine in the presence of valine or ACB. This concentration of hemin was shown previously to promote globin production by reticulocytes (5). Incorporation of ACB in place of valine resulted in proteins which were degraded approximately 15 times faster than normal (i.e. valine-containing) protein (Fig. 2) in accord with earlier results (17). After 90 min of exposure to hemin, the degradation of ACB-containing protein was inhibited by 40 to 50% and the breakdown of valine-containing protein by about 20 to 25% (Fig. 2). Treatment with hemin for 30 min caused a slightly smaller inhibition of proteolysis, and treatment for 4 h produced a slightly larger effect (data not shown). Analogous experiments tested whether hemin also reduced the rapid intracellular degradation of incomplete proteins (14, 15) produced by incorporation of puromycin. As shown in Fig. 3, incubation of reticulocytes with hemin caused a marked inhibition of the degradation of puromycyl polypeptides. This marked inhibition of proteolysis did not result from some nonspecific toxic effect of heme treatment (e.g. as might result from inhibition of energy production), since the rate of protein synthesis in such cells was similar to or even greater than that seen in the absence of hemin (Fig. 4, Table III).

Although such results were obtained many times and although hemin’s inhibitory effects after 45 min were highly significant (\( p < 0.02, N = 15 \)), proteolysis in some preparations of reticulocytes showed little or no decrease upon addition of hemin. However, proteolysis in dialyzed extracts prepared from such unresponsive cells was always inhibited by hemin. These observations thus suggest that some dialyzable factor, for example, endogenous hemin levels, may vary in the different preparations. For example, if hemin stores were high within reticulocytes from certain rabbits, the hemin added in vitro may not have a significant effect in the intact cells (see below).

**Physiological Importance of Hemin’s Effects on Degradation**—Since hemin can inhibit proteolysis substantially in intact reticulocytes, we attempted to determine whether this effect might contribute to the stimulation of net globin production induced by hemin (1, 2, 6, 7). These experiments involved simultaneous measurements of protein accumulation, synthesis, and degradation. To study the net production of valine or ACB-containing proteins in the presence or absence of hemin, reticulocytes were incubated with [\( ^{1} \text{H} \)]leucine, and accumulation of radioactive proteins was measured after varying periods of time (Fig. 4). By 30 min, the hemin-treated preparations showed consistently greater accumulation of ACB-containing protein than control cells, and the magnitude of this effect increased with time. Hemin had smaller stimulatory effects on the accumulation of labeled valine-containing proteins. The magnitude of this stimulation by hemin of globin production varied considerably in the 15 to 20 different preparations studied. To illustrate this variability, data from two rabbits are shown in Fig. 4.

Although data on labeled amino acid incorporation have been used as a measure of rates of protein synthesis, this interpretation is not valid, since it ignores the loss of newly synthesized proteins by degradation. In order to determine the actual effects of hemin on synthesis of valine or ACB-containing proteins, cells were exposed to labeled leucine for periods too short to allow substantial protein degradation (Table III). For these experiments, reticulocytes were preincubated with or without hemin for 30 min to 4 h. They were then exposed to [\( ^{1} \text{H} \)]leucine for 2 min, and the radioactivity incorporated into protein was measured. Interestingly, in

---

**Fig. 2. Effect of hemin (500 µM) on the degradation of proteins in reticulocytes.** Upper, normal (valine-containing) protein in the presence (□) and absence (○) of hemin. Lower, proteins containing the valine analog, t-α-amino-β-chlorobutyric acid in the presence (□) and absence (○) of hemin. Cells were preincubated in the presence or absence of hemin for 90 min. Labeled proteins were synthesized and their hydrolysis measured as described in the text.

---

**Fig. 3. Effect of hemin (500 µM) on the degradation of puromycyl polypeptides in reticulocytes.** The cells were preincubated with (□) or without (○) hemin for 90 min prior to exposure to a low concentration of puromycin and [\( ^{1} \text{H} \)]leucine. The subsequent degradation of proteins synthesized in the presence of puromycin was measured in the presence or absence of hemin, as described in the text.

---

* Also unpublished data.
many experiments (e.g. Fig. 4, Table III), the presence of hemin stimulated the accumulation of new protein by the reticulocytes but failed to significantly increase the rates of protein synthesis. In fact, in these experiments, hemin had an inhibitory effect on the synthesis of normal proteins at 30 min, although at 4 h a slight stimulation of this process might have occurred. Since proteolysis in the same cells showed a substantial inhibition by hemin (Fig. 2), the stimulatory effect of hemin on protein accumulation in this reticulocyte preparation (Fig. 4A) must have been due almost entirely to decreased degradation.

This conclusion is consistent with several other observations. For example, the delay in hemin's stimulation of globin production (Refs. 1 and 4, Fig. 4A) was interpreted by previous workers as evidence that added hemin promotes globin syn-

![Figure 4: Effect of hemin on accumulation of newly synthesized proteins in two different preparations of reticulocytes (A and B). These figures illustrate the variable responses of different preparations of reticulocytes to hemin. A, reticulocytes were prepared in the usual fashion. The cells were incubated with (+) or without (○) hemin (500 μM) for the time indicated in the presence of [14C]leucine (10 μCi/ml). Reticulocytes were allowed to synthesize proteins containing valine or ACB as described in the text. At the times indicated, accumulation of labeled protein was measured. B, similar measurements were carried out on reticulocytes obtained from rabbits maintained on a "low iron" diet (Nutritional Biochemical Co.) for 5 weeks. In addition, to stimulate erythropoiesis, the animals were bled three times over this period and then treated in the usual way with phenylhydrazine. The observed large response to hemin, however, was not observed reproducibly after such treatments. The basis for this variability in the presence to hemin has not yet been eluci-

Table III
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Protein containing</th>
<th>Duration of hemin pretreatment</th>
<th>Protein synthesis</th>
<th>Stimulation by hemin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Valine</td>
<td>0.5</td>
<td>4.4 × 10^5</td>
<td>-24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>5.9</td>
<td>-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
<td>6.2</td>
<td>-5</td>
</tr>
<tr>
<td></td>
<td>ACB</td>
<td>0.5</td>
<td>5.9</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>3.1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
<td>2.1</td>
<td>10</td>
</tr>
<tr>
<td>II</td>
<td>Valine</td>
<td>0.5</td>
<td>3.5</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>4.9</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
<td>1.9</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>ACB</td>
<td>0.5</td>
<td>2.8</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>4.4</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
<td>1.6</td>
<td>12</td>
</tr>
</tbody>
</table>

thesis only after endogenous heme has been depleted. This explanation cannot easily account for the absence of a time lag in hemin's effects on net production of analog-containing globin production in these cells primarily by inhibiting protein breakdown. A reduction in proteolysis should lead to greater protein accumulation only after a period of time long enough for there to be significant degradation of labeled protein in the control cells. Since the abnormal proteins are hydrolyzed much more rapidly (Figs. 2 and 3), an inhibition of the degradative process by hemin should affect their accumulation at short times more dramatically than that of normal cell proteins, as observed in Fig. 4A.

In other preparations (e.g. Fig. 4B) where hemin appeared to cause a greater stimulation of net protein production, there was a marked stimulation of protein synthesis (Table III) as well as a large inhibition of proteolysis (similar to that shown in Fig. 2). The greater effect on net globin production shown in Fig. 4, therefore, must result from the stimulation of synthesis as well as an inhibition of degradation. Finally, in certain other preparations of reticulocytes, hemin stimulated synthesis of normal and ACB-containing proteins several-fold, while degradation was inhibited only slightly, if at all.

The basis for this variability in the responses of different preparations of reticulocytes to hemin is still unclear, especially since hemin consistently inhibited proteolysis in cell-free systems, as discussed above. The dependence of the stimulation of synthesis and the reduction of proteolysis on hemin concentration differed also within the same preparation of reticulocytes. For example, in a preparation where hemin influenced both processes significantly, hemin stimulated synthesis over a wide range of concentrations, although this effect fell off at high hemin concentrations (Table IV). (Thus, in experiments where no effect on synthesis was observed, the concentration of hemin may have been too high to demonstrate such an effect.) Hemin also inhibited degradation significantly (30%), but the magnitude of this effect was similar at all concentrations tested.
Hemin Inhibits Protein Degradation in Reticulocytes

**Influence of hemin on synthesis and degradation of analog-containing proteins in a single preparation of reticulocytes**

The effects of various hemin concentrations on synthesis and degradation of ACB-containing proteins were measured as described in Fig. 2 and Table III on a single preparation of reticulocytes. Very similar data on the effects of hemin were obtained when protein degradation was measured after 45 min.

<table>
<thead>
<tr>
<th>Hemin concentration (µM)</th>
<th>Protein synthesis (dpm × 10⁻¹)</th>
<th>Stimulation</th>
<th>Protein degradation (%)</th>
<th>inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.4</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>19.6</td>
<td>110</td>
<td>41</td>
<td>36</td>
</tr>
<tr>
<td>250</td>
<td>17.6</td>
<td>87</td>
<td>49</td>
<td>23</td>
</tr>
<tr>
<td>500</td>
<td>13.3</td>
<td>43</td>
<td>42</td>
<td>32</td>
</tr>
</tbody>
</table>

**DISCUSSION**

These studies have demonstrated an ability of hemin to reduce the degradation of abnormal and normal proteins in both reticulocytes and reticulocyte extracts. These effects have been obtained with similar concentrations of hemin that have been shown previously to promote protein synthesis and net globin accumulation in these preparations. Theoretically, this inhibition of proteolysis may result from an inhibitory effect on the proteolytic system directly or from an interaction of hemin with the labeled proteins that makes them more resistant to proteolytic attack. Several observations suggest that the primary effect of hemin in the cell-free extracts is to inhibit the ATP-dependent degradative system. First, hemin reduced the hydrolysis of nonheme proteins (i.e., lysozyme, α-casein, and denatured E. coli proteins) as well as the breakdown of analog-containing and incomplete rabbit globins (Table I) that probably do not form a heme pocket. Furthermore, hemin had similar inhibitory effects on the breakdown of these proteins as on the degradation of apohemoglobin and apomyoglobin (Table I). In fact, over a wide variety of hemin concentrations, the degradation of apohemoglobin and α-casein was inhibited to similar extents (Table II). Finally, hemin preferentially inhibited the ATP-dependent degradation of ¹⁴C-H₂-globin in the extracts with little effect on the slower ATP-independent hydrolysis of this substrate (Fig. 1).

Since this ATP-stimulated system appears responsible for the selective degradation of abnormal proteins in reticulocytes (17–19), these latter findings probably account for the effects of hemin found with the intact cells (Figs. 2 and 3). An effect of hemin on the ATP-dependent degradative system appears reminiscent of hemin's effects on protein synthesis, which involve an inhibition by heme of an ATP-dependent inactivation of the initiation factor eif-2 (6–12). It is thus possible that the mechanism of hemin's effects on protein synthesis and degradation are in some way related. Perhaps this ATP-dependent proteolytic system may also play a role in inactivating the initiation factor, or it may involve the same heme-sensitive protein kinase (6–12) that regulates the initiation of protein synthesis. Evidence for these interesting possibilities is lacking and must await further characterization of this novel proteolytic system.

Hemin may also have additional effects on the protein substrates that contribute to its ability to reduce proteolysis in intact reticulocytes (Figs. 2 and 3). Our original hypothesis was simply that the binding of hemin stabilized the globin chains against intracellular proteolysis, and this possibility remains a very attractive one. Many examples are known where the binding of cofactors or substrates reduces the degradation of enzymes in mammalian cells. In fact, one of the best documented examples of this sort is the protective effect of heme on tryptophan oxygenase in liver (26, 27). The presence of heme is known to promote tetramer formation in hemoglobin (24, 28), and such oligomeric structures are generally more resistant to proteolytic attack than free monomers (14). Furthermore, binding of this ligand is known to stabilize and promote the solubility of hemoglobin (28, 29) and myoglobin (30, 31) and to decrease the proteolytic susceptibility of the latter protein. We have found that hemoglobin and myoglobin become approximately 10-fold more susceptible to the ATP-dependent proteolytic system after removal of the heme moiety.

It is possible that substrate stabilization by hemin may contribute to the inhibition of proteolysis in intact cells (Figs. 2 and 3), even though such effects may be of less importance in our cell-free studies (Tables I and II). In the latter studies, hemin was added rapidly to the extracts containing apoglobin or abnormal globins, while proteolysis was being measured. Although slow mixing of globin and heme through a matrix does lead to a quantitative reassembly of hemoglobin, the rapid approach that had to be used here may not result in appreciable reconstitution of hemoglobin (20). Thus, these cell-free studies should not be interpreted as excluding additional possible effects of hemin in stabilizing protein substrates. (In fact the complex kinetics of inhibition found with the intact cells (Figs. 1 and 2) may indicate multiple actions of hemin possibly on the substrate and on the proteolytic system.) It is noteworthy that a large number of the human hemoglobins for which there is evidence for rapid degradation in vivo (15) involve mutations in the heme pocket; these variants ("unstable hemoglobins") have a low affinity for heme and tend to dissociate in vitro (32). Recently, Klemes and Goldberg (18) have found that the ACB-containing globin also has an unusually low affinity for heme. Therefore, high exogenous levels of hemin may be necessary for stabilization of these abnormal proteins. Similarly, the slow degradation of normal (i.e., valine-containing) proteins observed in intact cells (Fig. 2) may have occurred in part because the incubated reticulocytes lacked adequate levels of hemin for maximal protection against degradation.

The present findings provide further evidence that proteolysis should not be ignored in investigations of the control of intracellular protein concentrations (14, 15). Previous studies of the regulatory effects of hemin have generally failed to distinguish between the net production of new proteins and the actual rate of protein synthesis, even though this distinction is easy to make experimentally (Fig. 4 versus Table III). The present findings indicate that hemin directly inhibits the degradation of globin and that this process can contribute significantly to the stimulation of protein accumulation by hemin. It is unclear to what extent this inhibition of proteolysis may have affected previous studies of hemoglobin production. In cell-free experiments where little endogenous hemin is present, such effects may be substantial and may affect the stability of products of cell-free translation. Degradation of translational products may also contribute to the apparent rapid cessation of globin production in the absence of added hemin (1–7). Several observations also suggest that hemin may promote the accumulation of newly synthesized protein in intact cells by inhibiting proteolysis. In frog oocytes, the injection of hemin along with mRNA for hemoglobin causes a large enhancement of net globin production (9), and the time course of this effect is consistent with a large inhibition of proteolysis by hemin. Recently, the gene for hemoglobin has been cloned on a viral genome, and the globin synthesized in virally infected cells is rapidly degraded, probably probably.

because of the lack of sufficient hemin to retard this process (33).

The present studies on intact cells suggest that the inhibition of proteolysis by hemin can play an important role in promoting globin production in vivo, in addition to its well established ability to stimulate protein synthesis. The physiological importance of hemin's control of proteolysis is difficult to establish by simply adding exogenous hemin to reticulocytes, and it will be of appreciable interest to study globin production and breakdown in cells where endogenous hemin production is prevented (e.g. with Fe\(^{3+}\) chelators or inhibitors of heme synthesis).

Evaluation of the relative import of hemin's effect on protein degradation and synthesis was unfortunately complicated by the large variations that we have found in the magnitude of these two effects in different cell preparations. Thus, in reticulocytes from certain rabbits, we failed to observe a clear stimulatory effect of hemin on protein synthesis (Table III), even though hemin in these cells promoted the net accumulation of new protein (Fig. 4) and clearly decreased their catabolism (Figs. 2 and 3). Thus, the latter effect must be primarily responsible for the enhancement of globin production in these reticulocytes. Such observations also clearly indicate that hemin can retard globin degradation independently of an effect on protein synthesis. However, such results were not true of all reticulocyte preparations. In certain experiments, protein synthesis was stimulated dramatically by hemin with only modest (Table IV) or insignificant (not shown) effects of protein degradation, while in occasional experiments large changes were observed in both processes.

This variability was found only with intact reticulocytes; in contrast, in the dialyzed reticulocyte extracts, hemin had highly reproducible inhibitory effects on protein breakdown (Tables I and II). Despite appreciable effort on our part, we were unable to determine exactly what factors are responsible for the variations in the responses to hemin of protein synthesis and degradation in different cells. Previous studies of protein synthesis in intact reticulocytes have also noted large variations in the stimulation by exogenous hemin (1),\(^6\) but this variability has not received much attention. It has been assumed that differences in endogenous heme content are responsible for the differences in the effects of exogenous hemin. However, we have not yet been able to prove this explanation in extensive studies comparing iron-deficient and normal rabbits (e.g. Fig. 4). Possibly some other dialyzable factor varies from animal to animal and influences the response to the added hemin. Other possible explanations include genetic variations between rabbits (e.g. in hemin transport), differences in the maturational state of cells, and nutritional factors affecting the basal rates of protein turnover. All could be in part responsible for the variable effects of hemin on synthesis and breakdown in intact cells. Although in specific preparations, the effect of hemin in stimulating synthesis or in reducing proteolysis may be the predominant factor promoting protein accumulation, the physiological consequences of these two effects of hemin are similar and should be additive. Both help prevent an imbalance between the accumulation of globin and heme moieties in the cell.

\(^6\) Personal communication from both L. London and T. Hunt.

Acknowledgments—We are very grateful to Mary-Beth Tobacco, Nina Stied, Marc Gluckman, and Cathy Castiglia for their expert technical help in these experiments and to Yoel Klemes for preparing some of the labeled substrates. We also thank Elsa Fox and Lynn Abbott for valuable assistance in preparing this manuscript.

REFERENCES