The Control of Hemoglobin Synthesis

A COMPARISON OF THE ROLE OF HEME IN RABBIT BONE MARROW AND RETICULOCYTES *

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SUMMARY

The incorporation of L-[U-14C]lysine into the α and β chains of hemoglobin has been compared in acetylphenylhydrazine-induced rabbit reticulocytes and normal and acetylphenylhydrazine-induced rabbit marrow. Evidence for a pool of α chains and αβ dimers in both reticulocytes and marrow cells was found in (a) an α:β specific activity ratio of less than 1.0 in purified hemoglobin, (b) the relative losses of α and β chain radioactivity on purification of the hemoglobin, (c) the radioactivity elution profile observed on gel filtration of the ribosome-free hemolysate, and (d) the effects of hemin added to the post incubation hemolysate. However, the data also indicated that the pool of α chains is smaller in the marrow cell than in the reticulocyte. In both groups of cells, addition of hemin to the incubation medium was followed by greater synthesis of α and β chains than was observed in the control cells. In addition, in marrow cells it was associated with virtually complete disappearance of the α chain and αβ pools, as evidenced by an increase in the α:β specific activity ratio in purified hemoglobin to almost 1.0 and by the disappearance of the high specific activity minor peak from the gel elution profile. On the basis of pulse labeling and chase experiments it was confirmed that both the pool of α chains and αβ dimers exist as intermediates in the pathway of hemoglobin biosynthesis. However, in the reticulocyte the radioactive α chain pool could be chased into hemoglobin to only a limited extent, in contrast to the marrow where almost complete transfer of radioactivity into hemoglobin occurred during the chase period. It is suggested that in the developing erythroid cell heme serves to promote hemoglobin synthesis by (a) promoting the synthesis of α and β chains and (b) promoting the conversion of αβ dimers to the hemoglobin tetramer. In addition it also serves to coordinate the synthesis and assembly of α and β chains.

Earlier studies in this laboratory on the biosynthesis of hemoglobin have demonstrated the presence in rabbit reticulocytes of a pool of α chains and of globin (αβ dimers). Addition of hemin (0.1 mM) to the incubation medium was associated with increased synthesis of α and β chains, enhanced combination of these newly synthesized chains, and conversion of αβ dimers to the hemoglobin tetramer; the α pool, however, was only slightly diminished (1).

In normal erythroid cell development reticulocytes contribute only a small fraction of the total hemoglobin production, they are unable to synthesize RNA, and their content of preformed RNA undergoes progressive decline (2–4). It was desirable therefore to study the synthesis of hemoglobin and the effects of added hemin at earlier stages of erythroid cell development, when the capacity to synthesize hemoglobin (5, 6) and the content of RNA are higher. In the present study the synthesis of hemoglobin and of its constituent α and β chains, and the effects of added hemin are compared in rabbit bone marrow and reticulocytes.

METHODS

The techniques for APH, 1 treatment of the rabbits, preparation of reticulocytes for incubation, preparation of reticulocyte hemolysates, and purification of hemoglobin by Sephadex G-100 gel filtration have been described in detail in a previous publication (1). The standard saline solution, incubation media, and hemin solution are identical with those used in the earlier study. Variations of the techniques as applied to bone marrow will be described.

Source and Preparation of Bone Marrow and Reticulocytes

Bone marrow was obtained from (a) normal, young (6 to 13 weeks old) rabbits and (b) APH-treated rabbits. Reticulocytes were obtained from APH-treated rabbits.

Sufficient marrow for one incubation was obtained from the two humeri, two femora, and two tibiae of the exsanguinated rabbit. The bones were opened longitudinally and the marrow scooped directly into about 20 ml of the rabbit's own heparinized plasma. The cells were dispersed by shaking the suspensions and then sieving them through a fine nylon mesh (Macalaster-Bicknell Corporation, New Haven, Conn., No. 9331). Surface fat and plasma were removed after centrifugation at 1000 × g. This procedure yielded 2.0 to 2.5 ml of a red marrow pellet to which 40 ml of a 25% suspension of normal rabbit erythrocytes in standard saline solution were added. This suspension was

1 The abbreviations used are: APH, acetylphenylhydrazine; CM-cellulose, carboxymethylcellulose; α:β ratio, specific activity of α chains (counts per min per mg)/specific activity of β chains (counts per min per mg).

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mixed, centrifuged, and washed once more in standard saline.
The addition of carrier red cells from normal peripheral blood
has been shown to protect marrow cells during the washing
procedure; the added cells contribute no more than 2 to 3% of
the radioactive recovery in globin, and this amount can be
ascribed to the presence of a normal number of reticulocytes
(5).

**Incubation**

**Standard Incubation**—An equal volume of lysine-poor medium
was added to the marrow-erythrocyte mixture, and this sus-
pension was divided into two equal portions. To one flask
hemin was added (0.1 mM), and to the other an equal volume of
Tris-HCl (0.05 M, pH 7.7) was added. Both flasks were
incubated for 30 min at 37° before addition of L-[U-14C]lysine
(50 to 80 μCi). The incubation was continued for an additional
60 min. The cells were washed with standard saline solution
and then lysed by addition of an equal volume of 0.0015 M
MgCl₂ and by freezing and thawing three times in solid CO²-
ethanol. The stroma-free, ribosome-free hemolysate was
obtained in the usual manner. Similar times of incubation
were used in the reticulocyte experiments.

**Pulse-Chase Incubations**—Two experiments were performed,
one with APH-induced reticulocytes, the other with normal
marrow. The packed reticulocytes (17 ml) were mixed with 17
ml of lysine-poor standard incubation medium, and the sus-
pension was divided equally between two flasks. The marrow cells
(5 ml) were mixed with 15 ml of normal, washed rabbit red
cells, suspended in an equal volume of standard, lysine-poor
medium, and divided equally between two incubation flasks.
Standard hemin solution (2 mg per ml; 0.0325 ml per ml of sus-
pension = 0.1 mM) was added to one marrow and one reticulocyte
incubation. An equal volume of buffer (Tris-HCl, 0.05 M,
pH 7.7) was added to the other flask of each pair. The sus-
pensions were maintained at 37° for 30 min and were then pulse-
labeled for 10 min with L-[U-14C]lysine (50 μCi). The incubation
was stopped by pouring the contents of each flask into 25 ml of
ice-cold standard saline containing added L-lysine (0.01 M).
After centrifugation, at 1000 × g, the cells were resuspended in 40
ml of the same solution; the suspensions were divided in half
and then were made up to 40 ml and recentrifuged at 1000 ×
g. One control and one hemin-treated sample of marrow and
reticulocytes were further incubated at 37° for 90 min with an
equal volume of medium which had been enriched with non-
istopic L-lysine (0.25 mM). The incubation medium for the
samples previously pulse-labeled in the presence of hemin also
contained added hemin (0.1 mM).

**Effects of Addition of Hemin to Hemolysate**—Duplicate samples
of APH-induced rabbit reticulocytes were incubated at 37°
in standard medium for 15 min; L-[U-14C]lysine (50 μCi) was then
added, and the incubation was continued for 60 min. Ribosome-
free hemolysates were prepared from the cells, and hemolysin
solution (2 mg per ml) was added to one sample to give a final con-
centration of 0.1 mM. After 15 min at room temperature both
samples were treated with carbon monoxide, and 3 ml of each
was applied to the Sephadex G-100 column.

**Hemoglobin Purification, Globin Preparation, and αß Chain Separation**

These were carried out by Sephadex G-100 gel filtration, acid-
acetone precipitation, and CM-cellulose chromatography,
respectively (1).

**Duplicate incubation of equal aliquots of normal rabbit marrow**

The cells were incubated in standard lysine-poor medium with-
out added hemin for 30 min at 37°, and then labeled for a further
60 min with L-[U-14C]lysine (50 μCi).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source of Chain</th>
<th>Specific Activity</th>
<th>α:β Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL MARROW</td>
<td>HEMOLYSATE</td>
<td>2752</td>
<td>1920</td>
</tr>
<tr>
<td></td>
<td>HEMOGLOBIN</td>
<td>1246</td>
<td>940</td>
</tr>
<tr>
<td>MARROW</td>
<td>HEMOLYSATE</td>
<td>2656</td>
<td>1898</td>
</tr>
<tr>
<td></td>
<td>HEMOGLOBIN</td>
<td>1304</td>
<td>1012</td>
</tr>
</tbody>
</table>

**Measurements of Radioactivity**

Radioactivity measurements were made on trichloroacetic
acid precipitates of fractions eluted from the Sephadex G-100
columns and on solutions of globin and of α and β chains pre-
pared from hemolysates and purified hemoglobin, as described
previously (1). Since there are 12 lysine residues in both the α
and β chains of rabbit globin (7) a direct comparison of the specific
activities of the two chain types could be made.

**RESULTS**

**Standard Incubation**—Table I shows the results of a duplicate
incubation of normal bone marrow in the absence of added hemin.
Two equal aliquots of the marrow erythrocyte suspension were
incubated for 60 min with L-[U-14C]lysine (50 μCi), and the
procedures for hemoglobin purification, globin preparation, and
αß chain separation were carried out in parallel on the two
ribosome-free hemolysates. The specific activities of the globin
and of the α and β chains from the duplicate incubations showed
good agreement.

Table II presents the specific activities of globin and of α and
β chains prepared from hemolysates and from purified hemoglobin
of both bone marrow and reticulocytes. In Experiments 4 and
5 in which marrow hyperplasia was produced as a result of
APH-induced hemolysis, marrow and peripheral reticulocytes
were obtained from the same rabbits. In purified hemoglobin
from control incubations of normal marrow the α:β ratio was
0.67 (mean value), in APH marrow 0.58, and in APH-induced
reticulocytes it was 0.36. It is evident that with similar times of
incubation the α:β ratio in marrow was considerably higher
than in reticulocytes.

Table III presents the fall in specific activities of globin and of α and
β chains on purification by Sephadex G-100 gel filtration. The
percentage of decrease in specific activity of the α chains
column (10) in bone marrow (45 to 62%) was always less than
the decrease in specific activity of the α chains in reticulocytes
(73 to 11%).

The Sephadex G-100 elution profiles of absorbance at 540 nm
and radioactivity for ribosome-free hemolysates of APH marrow
and reticulocytes are shown in Fig. 1. For both marrow and
reticulocytes the main hemoglobin peak of radioactivity was
followed by a minor peak of radioactivity which had little
540 nm or 280 nm absorbance and represented trichloroacetic
c precipitable material of high specific activity. Purified hemo-
Table II

Effect of added hemin on relative specific activities of α and β chains of globin prepared from unpurified hemolysates of bone marrow and reticulocytes and hemoglobin purified by Sephadex G-100 chromatography

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source of Globin</th>
<th>Specific Activity</th>
<th>Control</th>
<th>Hemin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Globin</td>
<td>α</td>
<td>β</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cpm/mg</td>
<td></td>
<td>cpm/mg</td>
</tr>
<tr>
<td>NORMAL</td>
<td>Hemolysate</td>
<td>1712</td>
<td>1137</td>
<td>1476</td>
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<tr>
<td>MARROW</td>
<td>Hemoglobin</td>
<td>844</td>
<td>590</td>
<td>902</td>
</tr>
<tr>
<td>2</td>
<td>Hemolysate</td>
<td>651</td>
<td>375</td>
<td>457</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>277</td>
<td>208</td>
<td>308</td>
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<tr>
<td>APH-MARROW</td>
<td>Hemolysate</td>
<td>7066</td>
<td>1204</td>
<td>1648</td>
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<td>4</td>
<td>Hemoglobin</td>
<td>744</td>
<td>504</td>
<td>892</td>
</tr>
<tr>
<td>5</td>
<td>Hemolysate</td>
<td>1830</td>
<td>1029</td>
<td>1426</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>641</td>
<td>429</td>
<td>734</td>
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<tr>
<td>APH-</td>
<td>Hemolysate</td>
<td>7876</td>
<td>8582</td>
<td>8366</td>
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<tr>
<td>RETICULOCYTES</td>
<td>Hemoglobin</td>
<td>4506</td>
<td>1992</td>
<td>5862</td>
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<tr>
<td>5</td>
<td>Hemolysate</td>
<td>9467</td>
<td>9669</td>
<td>9469</td>
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<tr>
<td></td>
<td>Hemoglobin</td>
<td>5212</td>
<td>2548</td>
<td>6725</td>
</tr>
</tbody>
</table>

Table III

Effect of added hemin on decline in α and β chain specific activity produced by purification of hemoglobin by Sephadex G-100 chromatography of hemolysates derived from bone marrow cells and reticulocytes

The decrease in the specific activity is the difference observed between the specific activities of the globin, α chains and β chains prepared from the hemolysate, and the specific activities of these proteins prepared from hemoglobin purified from the same hemolysate (see Table II).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source of Globin</th>
<th>DECREASE IN SPECIFIC ACTIVITY (cpm/mg)</th>
<th>PERCENTAGE DECREASE IN SPECIFIC ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control (2)</td>
<td>Hemin (3)</td>
</tr>
<tr>
<td>NORMAL</td>
<td>Hemolysate</td>
<td>868</td>
<td>733</td>
</tr>
<tr>
<td>MARROW</td>
<td>Hemoglobin</td>
<td>374</td>
<td>329</td>
</tr>
<tr>
<td>2</td>
<td>Hemolysate</td>
<td>1130</td>
<td>1216</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>1189</td>
<td>970</td>
</tr>
<tr>
<td>APH-MARROW</td>
<td>Hemolysate</td>
<td>3170</td>
<td>3086</td>
</tr>
<tr>
<td>5</td>
<td>Hemoglobin</td>
<td>4227</td>
<td>2014</td>
</tr>
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</table>

globin was isolated from the main 540 nm absorbance peak, thereby excluding the minor peak, which has been previously identified as a mixture of α chains and probable αβ dimers by means of ion exchange chromatography, peptide mapping, and spectrophotometry (1).

The findings of an α:β ratio of less than 1.0, the losses of α chain radioactivity on purification of hemoglobin, and the presence of a minor peak on Sephadex G-100 chromatography are interpreted as evidence for a pool of α chains in both marrow cells and reticulocytes. This pool appears to be smaller, however, in the marrow cell than in the reticulocyte.

The addition of hemin to the incubation of marrow cells had several effects. (a) There was a stimulation of α and β chain synthesis as shown by an increase in the specific activity of α.
chains and β chains prepared from the unpurified hemolysate (Table II, Experiments 1, 2, 4, and 5); the increase in specific activity of the chains prepared from purified hemoglobin was even greater, e.g. a nearly 4-fold rise in α chain specific activity (Experiment 1). (b) The α:β specific activity ratio in purified hemoglobin rose markedly, approaching 1.0 in the case of normal marrow (0.94 and 0.97), and approaching 0.9 in the case of APH-induced marrow (0.86 and 0.88); this finding is in sharp contrast to those in APH-induced reticulocytes in which the ratios attained were only 0.32 and 0.63 (Experiments 4 and 5, Table II). (c) The loss of α chain specific activity on purification was greatly diminished in marrow cells as compared to reticulocytes, in which a considerable loss persisted (Table III, columns 10 and 11). (d) The minor peak of radioactivity in the Sephadex G-100 elution profile almost disappeared in the marrow cells incubated with hemin (Fig. 1); in all experiments with bone marrow, only a small trail of radioactivity following the major hemoglobin peak persisted, in distinction to the elution profile observed with reticulocytes in which the minor peak became somewhat smaller but in no instance disappeared following incubation with hemin. These effects of hemin in marrow cells indicate that in addition to promoting α and β chain synthesis, the addition of hemin largely eliminates the pool of α chains and coordinates the production of new α and β chains. The fact that there is also loss of β chain specific activity on purification in the control experiments (Table III, columns 6 and 12) suggests that the minor peak also contains excess β chains, which disappear with elimination of the major peak on incubation with hemin.

**Pulse-Chase Experiments**—The results of these experiments are shown in Tables IV and V and Figs. 2 and 3. On labeling for 10 min, the effect of added hemin on the synthesis of globin is not observed in marrow cells or reticulocytes (Table IV); it is clearly observed with longer periods of labeling (Table I).

With pulse labeling for 10 min followed by a chase of 90 min, the following effects were observed in marrow cells. (a) The minor peak of radioactivity in control cells declined during the chase and a similar amount of radioactivity appeared in the main peak of hemoglobin; in hemin-treated cells, a distinct minor peak was not observed, but with the chase there was a shift of eluted radioactivity to the main hemoglobin peak (Fig. 2) and an increase in the specific activity of purified hemoglobin (Table IV). (b) These shifts of radioactivity were associated with increases of α:β specific activity ratios in purified hemoglobin from 0.48 to 0.77 in control cells and from 0.78 to 0.93 in hemin-treated cells. (c) There was a considerable reduction in

**Table IV**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source of Globin</th>
<th>Control</th>
<th>Hemin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α</td>
<td>β</td>
</tr>
<tr>
<td><strong>NORMAL MARROW</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 MIN PULSE-LABEL</td>
<td>Hemolysate</td>
<td>328</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>112</td>
<td>64</td>
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<tr>
<td>90 MIN CHASE</td>
<td>Hemolysate</td>
<td>316</td>
<td>206</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>190</td>
<td>162</td>
</tr>
<tr>
<td><strong>APH-RETICULOCYTES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Hemolysate</td>
<td>1900</td>
<td>2128</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>438</td>
<td>130</td>
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<td>90 MIN CHASE</td>
<td>Hemolysate</td>
<td>1852</td>
<td>2164</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>1268</td>
<td>844</td>
</tr>
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</table>
TABLE V
Comparison of decline in $\alpha$ and $\beta$ chain specific activity produced by purification of hemoglobin from hemolysates of both pulse-labeled and pulse-labeled and chased marrow cells and reticulocytes. (See Table IV.)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>DECREASE IN SPECIFIC ACTIVITY (cpm/mg)</th>
<th>PERCENTAGE DECREASE IN SPECIFIC ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (2)</td>
<td>Hemin (3)</td>
</tr>
<tr>
<td>NORMAL MARROW</td>
<td>216</td>
<td>146</td>
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<tr>
<td>10 MIN PULSE-LABEL</td>
<td>126</td>
<td>94</td>
</tr>
<tr>
<td>90 MIN CHASE</td>
<td>1462</td>
<td>878</td>
</tr>
<tr>
<td>APH-INDUCED MARROW</td>
<td>584</td>
<td>330</td>
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<td>10 MIN PULSE-LABEL</td>
<td></td>
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</tr>
<tr>
<td>90 MIN CHASE</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The losses of both $\alpha$ and $\beta$ chain specific activity on purification in control cells as a result of the chase (Table V, columns 10 and 12). These findings provide evidence for a precursor-product relationship between the proteins of the minor peak and hemoglobin.

In the APH-induced reticulocytes, there are several findings which differ from those in marrow cells. (a) The $\alpha/\beta$ ratio in purified hemoglobin after a 10-min label was extremely low (0.20 in control, 0.35 in hemin-treated cells). This rose to only 0.58 and 0.76, respectively, following the 90-min chase period (Table IV). (b) The minor peak (Fig. 3) represented the major fraction of the total radioactivity (74%) in control cells and fell to only 37% after the chase. (c) In the hemin-treated cells the postchase minor peak was still quite prominent (17% of the total). (d) There was considerable persistent loss of $\alpha$ chain specific activity on purification (Table V, columns 5 and 11) in the hemin-treated cells; in contrast the fall in $\beta$-chain specific activity was much reduced (Table V, columns 7 and 13).

These observations suggest that in reticulocytes the pool of...


α chains and αβ dimers is much larger than in marrow erythroid precursors, that it is the pool of αβ dimers which is largely chased into hemoglobin, and that there is a residual α chain pool after the chase even in the presence of added hemin. In the marrow, however, both pools can be almost completely chased into hemoglobin.

Addition of Hemin to Postincubation Ribosome-free Hemolysate of Acetylphenylhydrazine-induced Reticulocytes (Table VI, Fig. 4)—The minor peak from the control hemolysate was prominent, representing 45% of the total radioactivity eluted from the column and was associated with an α:β ratio in purified hemoglobin of 0.41. After addition of hemin to the hemolysate, chromatography on Sephadex G-100 revealed that much of the minor peak radioactivity had been transferred into purified hemoglobin, the residual minor peak representing only 24% of the total radioactivity. Since the total radioactivity eluted from each of the two columns was very similar, these changes represent conversion of precursor protein in the minor peak into hemoglobin. The α:β ratio in purified hemoglobin rose slightly from 0.41 to 0.50. If the mechanism of the shift is a conversion of αβ dimers to the hemoglobin tetramer, the α:β specific activity ratio within the αβ pool would have to be greater than 0.41. It can be calculated from the relative increases in α and β chain specific activities in purified hemoglobin following the addition of hemin that this ratio is 0.76. An α:β ratio of less than 1.0 in the αβ pool provides further evidence that the α chains entering the αβ pool have been diluted by a previously existing pool of unlabeled α chains.

### DISCUSSION

The data obtained indicate that there exists a pool of α chains and a pool of αβ dimers in normal rabbit bone marrow as well as in APH-induced reticulocytes. Evidence for these pools has been found in the α:β specific activity ratio of less than 1.0 in purified hemoglobin, the relative losses of α and β chain radioactivity on purification of the hemoglobin, and the elution profiles observed on Sephadex G-100 chromatography of the ribosome-free hemolysate. Quantitative comparisons of these three measurements in bone marrow and reticulocytes indicate that the combined size of both pools is smaller in marrow cells than in the reticulocyte and that the relative contribution of αβ dimers is greater in marrow cells. The previous existence of unlabeled α and αβ pools, therefore, is not confined to APH-induced reticulocytes and cannot be viewed as a phenomenon occurring only in cells which are nearing completion of maturation. The phenomenon is evident in the bone marrow of normal rabbits and in hyperplastic marrow of APH-treated rabbits, and therefore is not attributable to an effect of APH treatment.

The addition of hemin to the incubation of marrow cells serves to promote the synthesis of α and β chains and to eliminate both the α pool and αβ pool almost completely. In the reticulocyte, however, in which α and β chain synthesis is also promoted, the α chain pool largely persists. This difference is not attributable to an effect of APH, since both normal marrow and APH-stimulated marrow behaved similarly in this respect. It is possible that in marrow cells, hemin promotes the synthesis of β chains to a greater degree than α chains, thereby providing an excess of β chains which would become available for combination with the previously formed free α chains. This differential effect of added hemin might be mediated through feedback inhibition by previously existing excess α chains on the de novo synthesis of α chains (8). With disappearance of the α pool, this inhibitory effect would be removed and equal synthesis of α and β chains would be established. In these circumstances, hemin could be said to promote and to coordinate both the synthesis of α and β chains and the assembly of the hemoglobin tetramer in marrow cells. Restoration of coordinate α and β chain synthesis is thus initiated and then maintained by adequate concentrations of hemin. The observed effects of exogenous hemin in marrow erythroid cells of normal rabbits suggests that the synthesis or availability of endogenous heme may be rate limiting for overall hemoglobin synthesis. The mechanism of these heme effects is as yet unknown. The fact that the effect of added hemin has been demonstrated at the reticulocyte stage of maturation indicates that it occurs at the level of translation of the mRNA for globin and the assembly of the globin subunits. Evidence has

### Table VI

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific Activity</th>
<th>Ratio α/β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Globin</td>
<td>α</td>
</tr>
<tr>
<td>6 (a) CONTROL</td>
<td></td>
<td>10108</td>
</tr>
<tr>
<td>HEMOLYSATE</td>
<td></td>
<td>5208</td>
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<tr>
<td>HEMOGLOBIN</td>
<td></td>
<td>9816</td>
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<tr>
<td>6 (b) HEMIN ADDED TO HEMOLYSATE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEMOLYSATE</td>
<td></td>
<td>7150</td>
</tr>
<tr>
<td>HEMOGLOBIN</td>
<td></td>
<td>7150</td>
</tr>
</tbody>
</table>

**Fig. 4.** Effects of hemin added to the post-incubation ribosome-free hemolysate of APH-induced reticulocytes on the Sephadex G 100 elution profile. ——, absorbance at 540 nm; ---, radioactivity (counts per min per 0.1 ml).
been presented to show that heme does not become associated with the nascent polypeptide chain (9) so that a role in propagation which is dependent upon insertion of the prosthetic group appears unlikely. Grayzel, Hörchner, and London (10) and Waxman and Rabinovits (11) have demonstrated heme-dependent polyribosome formation and stabilization in rabbit reticulocytes. This effect reflects a mechanism for increasing the number of ribosomes on the messenger RNA. A greater number of ribosomes on the messenger RNA of heme-treated cells than control cells could result from better maintenance of the rate of initiation or a true increase in the rate of initiation of globin chains. One need not invoke an increase in the rate of chain propagation or release. Evidence to support either of these mechanisms for the action of heme on initiation has been obtained by Zncker and Schulman (12) and Adamson, Herbert, and Kemp (13) in a cell-free system. Data favoring a mechanism dependent upon the preservation of synthesis have been obtained by Hunt, Vanderhoff, and London. Our findings that the effect of added heme on the synthesis of globin is not observed on labeling for 10 min but is observed with longer periods of labeling is direct evidence for a role of heme in the maintenance of synthesis. Recent studies have indicated that heme may prevent the formation of a soluble inhibitor of protein synthesis (14) or may remove an inhibitor already formed (15). The mechanism by which heme maintains the synthesis of globin is explored and discussed more fully in the studies of Hunt, Vanderhoff, and London.

The pulse-chase experiments in both marrow and reticulocytes provide evidence for the existence of α chains and αβ dimers as normal intermediates in the biosynthesis of hemoglobin, lending support to the earlier reticulocyte data of Baglioni and Campana (16). In reticulocytes, however, the persistence of the α pool after the incubation with added heme and the failure to chase the α chains completely into hemoglobin may be related to a change in the physical state of the α chains. Such a change might cause the α chains to combine less readily with β chains or to act, perhaps, less effectively as possible inhibitors of α chain synthesis. It is probable that αβ dimers in control cells might reflect a deficiency of heme necessary for the conversion of the αβ dimer to the hemoglobin tetramer. This concept is supported by the findings on addition of heme to the postincubation, ribosome-free hemolysate of control reticulocytes.

Examination of the data on the unpurified hemolysates of marrow (Tables II and III) reveals certain anomalies. (a) The specific activities of the globin are consistently much higher than those found in its constituent α and β chains, both in the absence and presence of added heme; (b) The α:β specific activity ratio is consistently less than 1.0 especially in control hemolysates. In contrast, reticulocytes for the most part show a ratio of 1.0 or slightly greater, which is consistent with the concept of near equality of over all α and β chain synthesis. These anomalies may have one of several possible explanations. (a) There may be excess β chain synthesis in early erythroid cells. If these β chains formed tetramers (β4) they would chromatograph at a similar elution volume as normal hemoglobin (α2β2) on Sephadex G-100. This possibility, however, would not explain the presence of the minor peak, the relative loss of α and β chain specific activities on purification nor the persistent loss of β chain specific activity on purification after incubation with added heme. (b) There may be a protein contaminant, unlabelled or of low specific activity, in globin prepared from the unpurified hemolysate, which chromatographs with α chains on CM-cellulose. In some experiments this would require more than a 20% contamination of the α chains, a phenomenon not observed by excessively high 280 nm absorbance in the α chain eluates from the chain separation columns. In addition, tryptic hydrolysis and peptide mapping of such α chains failed to reveal any anomalous peptides. (c) In early stages of rabbit erythroid cell development, the rate of amino acid incorporation may be greater in what appears to be a minor hemoglobin component than in the major component; in reticulocytes, the principal synthesis is of the major component (17). If the subunits of the minor component behave differently from those of the major component on a CM-cellulose chain separation column (e.g. an α chain variant in the minor component might not be eluted with the α chain of the major component) α:β ratios less than 1.0 could result. (d) There may be high specific activity contaminants in globin prepared from the unpurified hemolysate which chromatograph in part with β chains. This would result in an unduly high β chain specific activity, a falsely low α:β ratio, and an excessive loss of β chain specific activity on purification, which would be evident even after incubation with added heme (Table III, column 7). This latter explanation may be the correct one in the light of the findings by Braverman and Bank (18) in nonthalassemic human marrow and reticulocyte hemolysates of α:β ratios averaging about 1.3. These workers were able to separate the human globin chains by CM-cellulose chromatography in 8 M urea, a technique which yields α and β chains of high purity. The high specific activity contaminants derived mainly from the myeloid cells appeared in the early eluates, well before the fraction containing the globin chains. Further elucidation of the basis of this anomaly must await a more rigorous method for separation of the chains of rabbit globin.

The finding of excess α chain synthesis over β chain synthesis has been well described in β-thalassemia (19-23) and has been accounted for by a specific deficiency in the synthesis of β chains. This deficiency has been ascribed to inadequate transcription of the α mRNA for β chains (24), a very likely possibility. An abnormality of β chain initiation, however, has not been eliminated, despite recent evidence that there is no deficiency of initiation factors (25). An alternative explanation recently offered by Schwartz (26) is that there may be an increased rate of decay of the mRNA for β chains during erythroid cell maturation. This suggestion is based on the finding that globin chains synthesis in the marrow of patients heterozygous for β-thalassemia is balanced, in contrast to the situation in reticulocytes from the same patients in whom α chain synthesis predominates.

Huehns and Modell (23) and Bank (27) have shown that there are free α chains present in the lysate of erythrocytes from patients with β-thalassemia which are capable of combining with hemoglobin H (β4) to form hemoglobin A. The extent of the excess α chain synthesis is reflected in α:β specific activity ratios in the unpurified hemolysate which are greatly in excess of 1.0 (19, 21, 27). However, in these circumstances the presence of a large pool of α chains did not always have an equivalent dilutional effect on α chains incorporated into purified hemoglobin, so that the α:β ratio in purified hemoglobin was not invariably less than 1.0 (27). In our experiments, α:β ratios of less than 0.5 in purified hemoglobin have been found in rabbit reticulo-
cytes in which the ratio in the hemolysate has not been significantly greater than 1.0. This anomaly in thalassemic reticulocytes, of a large $\alpha$ chain pool with little dilutional effect, suggests that the pool is not homogeneous but rather that it consists of two principal compartments, a relatively large compartment which is not freely miscible with newly released $\alpha$ chains, and a smaller compartment of more recent origin which is miscible and could have a dilutional effect on freshly released, labeled $\alpha$ chains, and which also could be capable of combining in vitro with hemoglobin $\Pi$ ($\beta_4$) to form hemoglobin $\Lambda$ ($\alpha_2\beta_2$). Such a phenomenon may result from a gradual alteration in the physical state of the excess $\alpha$ chains in thalassemic reticulocytes. It would correlate with the increased tendency of $\alpha$ chains in high concentration to aggregate (28) and ultimately to form inclusions (29) or to become associated with the cell stroma (30). The greater the degree of aggregation and precipitation, the less likely are such chains to act as a soluble pool to dilute newly synthesized $\alpha$ chains. The result is a situation in which a smaller pool of free, soluble $\alpha$ chains (as is found in rabbit reticulocytes and marrow cells following incubation in the absence of added hemin) produces a greater dilution of the $\alpha$ chains in hemoglobin than does the much larger but predominantly precipitated pool of excess $\alpha$ chains formed during the maturation of human thalassemic reticulocytes.

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The Control of Hemoglobin Synthesis: A COMPARISON OF THE ROLE OF HEME IN RABBIT BONE MARROW AND RETICULOCYTES
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