A Comparison of Amino Acid Incorporation into the Hemoglobin and Ribosomes of Marrow Erythroid Cells and Circulating Reticulocytes of Severely Anemic Rabbits*

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The rates of hemoglobin synthesis were compared in rabbit marrow erythroid cells and in circulating reticulocytes. The marrow cells in a 60-minute incubation were about 2.5 times as active as reticulocytes. In shorter or longer periods there was less difference. Marrow cells contain 5–10 times the quantity of ribosomes. In marrow cells the ribosomes incorporated leucine in "fixed" and "transient" forms; the "fixed" form represents synthesis of structural ribosomal protein, the "transient" form represents intermediates in the synthesis of soluble protein. The amount of "transient" leucine per marrow cell ribosome increased during incubation to a maximum and then declined. At first, the "transient" radioactivity greatly exceeded the "fixed" radioactivity, but after 60 minutes of incubation the relation was reversed. In circulating reticulocytes both the "transient" and the "fixed" leucine residues attained their maximum values during the first 5 minutes of incubation, and these values were maintained throughout the incubation. The transfer rate per ribosome of leucine residues to hemoglobin was the same in different batches of reticulocytes, even though their steady-state values of incorporated "transient" leucine residues varied over a four-fold range. The time required to synthesize a hemoglobin molecule varies with the conditions within the cell. In an estimate of such time it must be specified whether two or four ribosomes are involved in the synthesis of one molecule of hemoglobin. In reticulocytes, on the assumption of four participating ribosomes, the time to synthesize a hemoglobin molecule was estimated as 0.95 minutes. The maximum rate of synthesis in marrow erythroid cells was 16.6×10^{-14} μmoles of hemoglobin per cell per minute; the comparable value for circulating reticulocytes in the severely anemic animal was 8.2×10^{-14} and in the normal animal 1.8×10^{-14}.

In the current theory of protein synthesis the specific amino acid sequence is determined by a messenger RNA whose base sequence is complementary to a portion of DNA on which it is synthesized. Messenger RNA operates as a template for amino acids while attached to a ribosome. In bacteria it appears that messenger RNA is destroyed quickly and is replenished by its DNA. Reticulocytes, which are about 30% of the erythroid cells of the marrow and which may comprise 80–90% of the red cells in the blood of a severely anemic rabbit, synthesize hemoglobin rapidly, and yet they are non-nucleated cells. Reticulocytes may retain a small amount of DNA[1] (Holloway and Ripley, 1952) and synthesize some RNA, but too little for the rate of hemoglobin synthesis observed in reticulocytes (Kruh and Borsook, 1955; Lingrel and Borsook, 1961, unpublished observation). Hemoglobin synthesis persists in reticulocytes for at least 3 days (Astaldi, 1960). It appears that the messenger RNA, with which they were endowed when they were nucleated cells in the marrow, must be more stable than in bacteria.

Over 40% of the marrow erythroid cells in a severely anemic rabbit have functionally active nuclei. Another 25–30% have nuclei which are probably inactive, and the remainder are the non-nucleated reticulocytes (Borsook *et al.*, 1962). Amino acid incorporation into the hemoglobin and ribosomes of marrow erythroid cells and of circulating reticulocytes has been compared. The two groups of cells were always obtained from the same animal or group of animals. It seemed that some insight might be gained into the biochemical changes during maturation of erythroid cells and possibly some further insight into the mechanism of protein synthesis in general, and of hemoglobin in particular.

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[1] Most of the DNA, if not all, was in the white cells which were not removed in the preparation of the reticulocytes.

EXPERIMENTAL PROCEDURES

Both the bone marrow cells and the circulating reticulocytes were obtained from rabbits made anemic by the daily subcutaneous injection for 7 days of 0.25 ml per kg of body weight of 2.5% phenylhydrazine acetate (neutralized). The blood hemoglobin fell to about 4 g per 100 ml, with a reticulocyte count of about 85%.

Marrow Cells.—After the animal was exsanguinated by heart puncture the long bones of the front and hind limbs were removed, the ends were sawed off, and the marrow was blown out through one end into a 30-ml beaker containing 5 ml of the reaction mixture (see below) minus the labeled amino acid. The cells were separated from connective tissue and fat by smearing the marrow under fluid around the side of the beaker, and the suspension was passed through two layers of gauze and then centrifuged in a graduated centrifuge tube. The supernatant solution was discarded and the fat wiped off the sides of the tube.

The differential counts of the marrow cells were made on smears fixed with absolute MeOH and stained with Giemsa solution, whose *p*H was adjusted to 5.75 with a buffer consisting of 1 M citric acid–2 M sodium diphosphate. One thousand cells were classified into basophilic, polychromatic, and orthochromatic and non-nucleated erythroid cells; the last were taken to be all reticulocytes (Belcher *et al.*, 1954).

Reticulocytes.—About 50 ml of blood was drawn by cardiac puncture into a 50-ml syringe whose walls had been moistened with 1% heparin in saline. The blood was transferred to a beaker containing 0.75 ml of the heparin solution. The animal's chest was then opened, 1.5 ml of heparin solution was put into the thoracic cavity, the ventricles and descending aorta were cut, and the blood was collected with a syringe. All the blood collected was strained through four layers of gauze and spun in a refrigerated centrifuge in 50-ml plastic cups for 5 minutes at 4000 × *g*; the supernatant solution was removed by suction without any of the

cells being taken off. The cells were suspended in nine volumes of ice-cold saline solution and centrifuged again, and the supernatant solution was discarded. The paste of cells was mixed thoroughly with a rubber-tipped stirring rod. The percentage of reticulocytes was determined from smears stained with Brecher's New Methylene Blue (Brecher, 1949).

Saline Solution.—A saline solution has been developed experimentally for rabbit blood cells which is superior to usual saline solutions (Borsook *et al.*, 1957). It contains 0.13 M NaCl, 0.005 M KCl, and 0.0074 M $MgCl_2 \cdot 6H_2O$.

Amino Acid Mixture.—The amino acids, L-form, were made up in the above saline solution, with the following concentrations ($M \times 10^{-3}$): alanine 2.0, arginine 0.5, aspartic acid 2.85, glycine 5.3, histidine 2.4, isoleucine 0.3, lysine 1.8, methionine 0.3, phenylalanine 1.6, proline 1.4, serine 1.65, threonine 1.7, tryptophan 0.3, tyrosine 0.8, valine 3.2, hydroxyproline 1.1, cysteine 0.4, glutamine 8.0. The amino acids were dissolved by bringing their suspension to 100°. The *p*H after cooling was adjusted to 7.75 with NaOH. The solution was divided into small portions and kept frozen until used. It was not autoclaved, as this leads to destruction of the phenylalanine.

Dialyzed Plasma. The plasma of anemic rabbits was dialyzed at 4° against three changes of 10 volumes each of the saline solution.

C^{14}-Labeled Leucine.—Either L-leucine-1-C^{14} or uniformly labeled leucine was used, the results being the same with either form. In either case the amino acid was purified by chromatography (Moore *et al.*, 1958). The commercial preparations obtained had about 2% of other labeled amino acids. The radioactive leucine was 0.01 M with respect to the saline solution and had a specific activity of 100,000 to 125,000 cpm per μmole.

Reagent Mixture.—The composition of the reagent mixture was: amino acid mixture, 54.0 ml; a solution of $MgCl_2$ (0.25 M) plus glucose (10%) in the saline solution, 2.7 ml; Tris-HCl (0.164 M, *p*H 7.75), 27.0 ml; trisodium citrate (10^{-2} M) in dialyzed plasma, 21.6 ml; and sodium bicarbonate (10^{-2} M) in dialyzed plasma, 32.4 ml. The plasma was used because it facilitates amino acid incorporation in reticulocytes (Borsook *et al.*, 1957) and in marrow cells (Morell *et al.*, 1958), and because it reduces lysis during incubation and subsequent washing of the cells.

Reaction Mixture.—The components of the reaction mixture were added in the following proportions and order: cell suspension (packed marrow cells or reticulocytes), 5.0 ml; reagent mixture, 13.2 ml; $KFe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (10.5 mg/10 ml, saline), 0.75 ml; penicillin and streptomycin (each 60 mg/ml in saline), 0.1 ml; leucine-1-C^{14}, 1.0 ml. The final *p*H was 7.5.

Incubation Procedure and Subsequent Preparation of Cells for Analysis.—The reaction mixture was transferred to an Erlenmeyer flask of ten times the volume, clamped in a water bath at 37.5°, and rocked (flask open to the air) at 100 cycles per minute. At the end of the incubation the reaction mixture was transferred to 9 volumes of an ice-cold washing solution consisting half of dialyzed plasma and half of saline containing 10^{-2} M nonradioactive leucine. The suspension was quickly cooled to 5° and centrifuged in a refrigerated centrifuge at 4000 × *g* for 5 minutes. The supernatant solution was removed by suction, the packed cells were resuspended in 10 volumes of the ice-cold washing solution and again centrifuged, and the supernatant solution was discarded.

Preparations of Extracts for Measurement of Incorporated C^{14} Amino Acids.—The washed marrow and blood cells, which had been incubated with C^{14}-leucine, were both lysed at 0° by the addition of four volumes of a 0.005 M $MgCl_2$ solution followed 30 seconds later by one volume of 1.5 M sucrose containing 0.15 M KCl. Although 30–45% of the marrow cells were white, only the erythroid cells were lysed by this procedure. The white cells and the ribosomes of the erythroid cells remained intact. White cells, red cell stroma, mitochondria, and ghosts were removed by centrifugation at 12,000 × *g* for 10 minutes. The intact ribosomes and soluble proteins, including hemoglobin, were in the supernatant solution. The ribosomes were sedimented by centrifugation at 105,000 × *g* for 90 minutes. The soluble protein in the supernatant was over 90% hemoglobin.

For measurement of specific activity the soluble protein, mainly hemoglobin, was precipitated with 7% trichloroacetic acid. After standing for several hours (at times overnight) the precipitate was redissolved with 1 N NaOH and then reprecipitated with a large volume of 7% trichloroacetic acid and centrifuged; the supernatant was discarded. The precipitate was washed twice with 7% trichloroacetic acid. It was then washed with approximately 20 volumes of 0.5% HCl in acetone or until no more color was extracted. The precipitates were then washed twice with acetone and twice with ether and dried at 90° for an hour. A weighed sample was placed on a planchet and spread by addition of several drops of benzene. The radioactivity was determined by a Geiger-Müller end-window low background counter.

The preparation of the ribosomes for weighing and counting was similar to that for the soluble proteins except that the treatment with trichloroacetic acid was at 4° and the dissolution in NaOH was omitted.

Procedure in "Chase" Experiments.—In some experiments, after the cells were incubated with labeled amino acid they were washed and reincubated with unlabeled amino acid. The procedure was as follows: After the designated period of incubation with labeled amino acid, the reaction was stopped by addition of four volumes of ice-cold dialyzed plasma diluted with an equal volume of saline and chilled rapidly in an ice-salt bath to 5°. The cells were separated out in a refrigerated centrifuge, resuspended in nine volumes of ice-cold plasma-saline containing 10^{-2} M nonradioactive leucine, and again centrifuged out. There were three such washings. One aliquot of cells was lysed and analyzed. The other aliquot was resuspended in the same as the initial reaction mixture except that unlabeled leucine was used. At the end of the "chase" incubation period the cells were centrifuged out, washed once with nine volumes of saline, lysed, and analyzed.

Preparation of Ribosomes.—The washing solution for the ribosomes consisted of 0.025 M KCl, 0.004 M $MgCl_2$, and 0.03 M Tris at *p*H 8.0. The ribosomes were redissolved and centrifuged twice in the washing solution. All visible hemoglobin was removed.

Results

Figure 1 shows a typical time course of leucine incorporation into marrow erythroid cells and circulating reticulocytes. In the reticulocytes the rate was constant throughout the 60-minute incubation. In the marrow cells the rate in the first 10 minutes was a little less than that in the reticulocytes, after which it increased, until in the 30–60 minute interval it was three times that in the reticulocytes. In longer incubations (Table IV) it was observed that the rate of incorporation into the marrow cells began to decline after 60 minutes of incubation.

The differences between marrow cells and reticulo-

cytes shown in Figure 1 were found to be related to the difference in their ribosome concentration and in the times required for maximum incorporation into their ribosomes. The number of ribosomes per cell was higher in marrow cells than in reticulocytes. The experiment of Figure 1 was that with animal No. 2 in Table I. The number of ribosomes per cell in the marrow was 5.4 times that in the reticulocytes.

The ultraviolet absorption spectra of marrow and reticulocyte ribosomes over the range 230–420 mμ were the same. This may be taken to indicate that the composition of the ribosomes remains constant during the maturation of reticulocytes.

TABLE I

CONCENTRATION PER CELL OF RIBOSOMES IN MARROW CELLS AND CIRCULATING RETICULOCYTES

The molar concentration of ribosomes was computed on the basis of 4×10^6 as the "molecular weight" of ribosomes (Dintzis *et al.*, 1958; Ts'o, 1958).

Animal No.	Marrow Cells mg × 10^9	Marrow Cells μmoles × 10^{12}	Reticulocytes mg × 10^9	Reticulocytes μmoles × 10^{12}
1	3.9	1.0	0.4	0.1
2	4.9	1.2	0.9	0.22
3	4.8	1.2	0.7	0.17
4	4.0	1.0	0.6	0.15

However, "chase" experiments revealed functional differences between the ribosomes in marrow cells and in reticulocytes. Figure 2 depicts the time course of incorporation of labeled amino acids into the ribosomes of reticulocytes and the change in labeling in "chase" experiments. It is seen in Figure 2 that the reticulocyte ribosomes attained their maximum labeling within 5 minutes' incubation. They lost 90% of their radioactivity in the "chase" incubation as quickly as they had originally incorporated it. The radioactivity remaining after the "chase" is designated "fixed" radioactivity. The "transient" radioactivity is total minus "fixed" radioactivity. In some preparations of reticulocytes the "fixed" radioactivity was as high as 30% of the total.

A quite different picture of "transient" and "fixed" radioactivity was observed in the marrow cell ribosomes (Table II). Their specific activity increased throughout the incubation, and the "fixed" radioactivity was an increasingly large proportion of the total. Approximate values of the "fixed" and "transient" radioactivity were calculated as follows. Let A, T, and F represent respectively the total, "transient," and "fixed" radioactivity incorporated during the incubation with labeled leucine, B the total radioactivity at the end of the "chase," and C the radioactivity incorporated during the "chase," that is, radioactivity which presumably came from labeled precursors not yet assembled into finished ribosomes and assembled during the 45-minute "chase" period. The warrant for this conclusion is the finding after 120 minutes of incubation of more radioactivity in the ribosomes after than before the "chase."

$$T + F = A \quad (1)$$

$$F + C = B \quad (2)$$

$$T = A - (B - C) \quad (3)$$

C was calculated as follows. Take the rate of "fixed" labeling as x cpm/mg, and the average labeling rate during the "chase" as y cpm/mg. Then, substituting

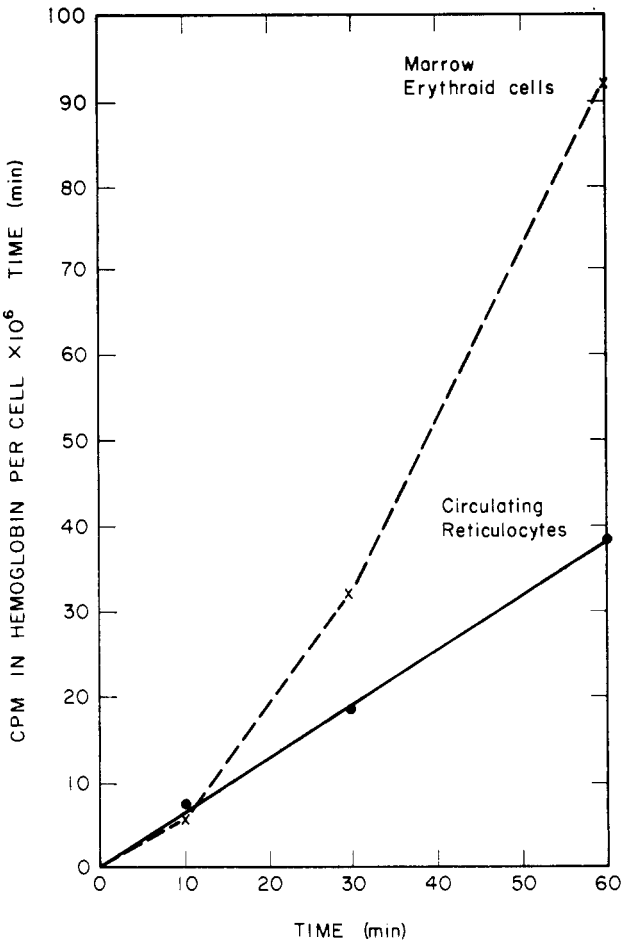

FIG. 1.—Incorporation of leucine into the hemoglobin of marrow erythroid cells and circulating reticulocytes.

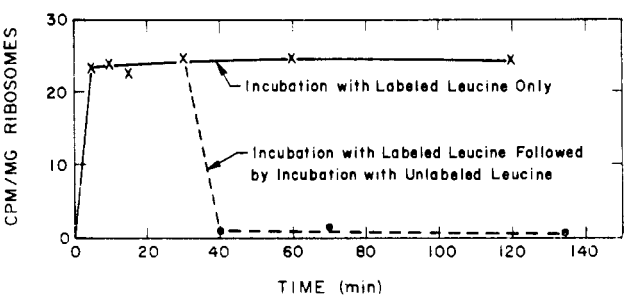

FIG. 2.—Incorporation of leucine into ribosomes of circulating reticulocytes.

data from Table II in equation (2), one obtains for 60 and 120 minutes respectively:

$$60x + 45y = 157.0 \quad \text{and}$$

$$120x + 45y = 281.7,$$

from which $x = 2.08$, $y = 0.72$, and $y\,x = 0.346$.

At 60 and 120 minutes the system was nearly saturated with labeled precursors. Under these conditions the value of C for a 45-minute "chase" incubation is 31.4. The system was not saturated during the first part of the incubation. By use of the ratio $y\,x = 0.346$, approximate values of F and C throughout the incubation may be obtained by the equation $Tx + 45\,(0.346x) = B$, where T is time in minutes. There is a small error in assuming x to be constant through time T. Up to 30 minutes' incubation x increased, after which it became constant. The calculated

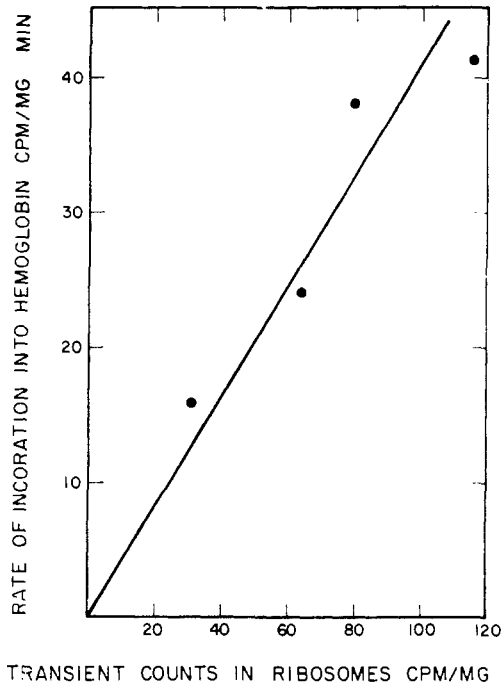

FIG. 3.—Correlation between "transient" radioactivity in ribosomes and rate of hemoglobin synthesis.

TABLE II

INCORPORATION OF LABELED LEUCINE INTO MARROW ERYTHROID CELL RIBOSOMES BEFORE AND AFTER "CHASE" TREATMENT

Incubation Time (min.)	Incorporation (cpm/mg) "Unchased" A	"Chased" B	$A - B$
10	27.0	10.2	16.8
30	94.0	55.9	38.1
60	186.5	157.0	29.5
120	255.0	281.7	−26.7

values of C in the early periods are too low, but the error does not appear to be large. Values for F having been obtained by the foregoing procedure, T was computed from experimental values for A by means of equation (1). The data of this experiment are given in Table II and the calculated values are collected in Table III.

When the rates of incorporation into the hemoglobin were plotted against the values of T, "transient" radioactivity in the ribosomes, Figure 3 was obtained.

TABLE III

"TRANSIENT" AND "FIXED" RADIOACTIVITY INCORPORATED INTO MARROW ERYTHROID CELL RIBOSOMES

	Incubation Time (min.) 10	30	60	120
"Fixed" radioactivity (F)	7.8	74.4	125.6	250.3
Radioactivity incorporated during "chase" (C)	12.6	37.4	62.8	62.8
"Transient" radioactivity (T)	46.1	113.6	120.0	8.8
Average value of "transient" radioactivity in the interval $\frac{(\text{initial value} + \text{final value})}{2}$ (cpm/mg)	31.2	79.8	116.8	64.4
Observed incorporation rate into hemoglobin in the interval (cpm/mg min.)	16.2	37.8	41.2	24.2

There was a good correlation throughout the course of the incubation between "transient" radioactivity in the ribosomes and the rate of hemoglobin synthesis, even though, as Table III shows, the "transient" radioactivity in the ribosomes rose to a maximum and then declined. Table III shows also that the attainment of a constant rate of "fixed" radioactivity was slower than the attainment of the maximum "transient" radioactivity.

The incorporation of "fixed" radioactivity may be taken to represent synthesis of structural ribosomal protein. The rate was constant in the 60–120 minute interval at 4 cpm/minute per mg of ribosomes. The specific activity of the leucine was 105,250 cpm/μmole. Rabbit ribonucleoprotein consists of 8.7% leucine (Ts'o *et al.*, 1958). From these data the rate of synthesis of marrow erythroid cell ribosomal protein is 5.7×10^{-3}% per minute of the original ribosomal protein, or 8.2% in 24 hours. This very low rate supports the conclusion based on other considerations (Borsook *et al.*, 1962) that there is no significant increase of apparatus for synthesizing hemoglobin in most of the erythroid cells of the marrow. The increase occurs in the very small fraction of early erythroblasts.

The lag in the attainment of the maximum specific activity of the ribosomes in the marrow cells may be the result of a number of factors: slower diffusion of labeled leucine into the marrow erythroid cells than into reticulocytes; a larger quantity of unlabeled leucine in the marrow cells, which is dissipated by incorporation into hemoglobin and by equilibration with labeled leucine in the external medium; a slower attainment of the steady state between loading the ribosomes and unloading completed peptides. The maximum specific activity observed in marrow cells is lower than the maximum would have been if there had not been an inactivating process in operation.

Tables IV and V collect the data on the relation between the labeling of the hemoglobin and of the ribosomes in the marrow cells and circulating reticulocytes. Columns 7 and 8 give the rates of ribosome transfer and of turnover of ribosomal radioactivity respectively. These are defined as μmoles leucine incorporated into hemoglobin/μmoles ribosomes $\times$ time (minutes) and μmoles leucine incorporated into hemoglobin/total "transient" radioactivity in ribosomes $\times$ time (minutes) respectively. The "molecular weight" of the ribosomes was taken as 4×10^6 (Dintzis *et al.*, 1958; Ts'o, 1958).

Column 8 in Table IV shows that in marrow erythroid cells the rate of hemoglobin synthesis was proportional to the "transient" radioactivity in the ribosomes (Turnover of Ribosomal Radioactivity) and not to the ribosome concentration (Ribosome Transfer Rate). This was to be expected; the lack of proportionality with the ribosome concentration in the later periods of incubation may have been due to rapid inactivation of many ribosomes in these cells prior to their disappearance in the course of their maturation (Table I).

In reticulocytes the relation between ribosomes and hemoglobin synthesis was reversed. Hemoglobin synthesis was proportional to the number of ribosomes and not to the "transient" radioactivity. We have no explanation for this unexpected finding nor for the difference in this respect between marrow erythroid cells and reticulocytes.

The transfer rate of reticulocyte ribosomes was 20. The maximum rate observed in marrow cells was 15.6. These are averages for all the ribosomes in a batch of cells. Autoradiographic observations (Borsook *et al.*, 1962) disclosed that a large fraction of both marrow cells and reticulocytes were inactive. Representative figures are 48% of the marrow erythroid cells and 60%

TABLE IV

RELATION BETWEEN LABELED LEUCINE INCORPORATION INTO HEMOGLOBIN AND INTO RIBOSOMES IN MARROW ERYTHROID CELLS

1	2	3	4	5	6	7	8
Expt. No.	Incubation Time Interval (min.)	Leucine Incorporated into Hemoglobin (μmoles)	Quantities of Ribosomes (μmoles $\times$ 10^3)	Average Specific Activity of the Ribosomes (μmoles/ μmoles)	Total "Transient" Leucine in Ribosomes (μmoles $\times$ 10^3)	Ribosomal Transfer Rate[a] (μmoles per ribosome per min.)	Turnover of Ribosome Radioactivity[a] (per min.)
1	0–10	0.25	9 7	0.67	6.5	2.6	3.9
	10–30	0.53	9.7	1.21	11.7	2.7	2.3
2	0–10	0.41	11.0	1.08	11.9	3.7	3.4
	10–30	1.74	11.5	2.50	28.7	7.5	3.0
	30–60	4.22	9.6	4.39	42.1	14.6	3.3
3	0–10	0.36	5.1	1.36	6.9	7.0	5.2
	10–30	0.89	5.1	3.43	17.5	8.7	2.5
	30–60	2.07	4.4	4.81	21.1	15.7	3.3
	60–90	1.48	4.5	2.92	13.1	11.0	3.8
4	0–10	0.15	3.45	1.15	3.9	4.3	3.0
	10–30	0.72	3.45	3.03	10.4	10.4	3.4
	30–60	1.12	3.45	4.34	15.0	10.8	2.5
	60–120	1.39	3.45	2.44	8.4	6.7	2.8

[a] See text for definition.

TABLE V

RELATION BETWEEN LABELED LEUCINE INCORPORATION INTO HEMOGLOBIN AND INTO RIBOSOMES IN CIRCULATING RETICULOCYTES

1	2	3	4	5	6	7	8
Expt. No.	Incubation Time Interval (min.)	Leucine Incorporated into Hemoglobin (μmoles)	Quantities of Ribosomes (μmoles $\times$ 10^3)	Average Specific Activity of the Ribosomes (μmoles/ μmoles)	Total "Transient" Leucine in Ribosomes (μmoles $\times$ 10^3)	Ribosomal Transfer Rate[a] (μmoles per ribosome per min.)	Turnover of Ribosome Radioactivity[a] (per min.)
1	0–10	1.23	5.1	0.83	4.2	23.6	29.3
	10–30	1.63	4.3	0.97	4.2	18.6	19.4
2	0–10	1.77	6.2	1.26	7.8	28.5	22.7
	10–30	2.37	6.0	1.36	8.1	19.6	14.6
	30–60	4.34	6.1	1.64	10.0	23.1	14.5
3	0–20	33.0	61.0	3.73	227.5	18.0	7.2
4	0–30	8.1	13.6	2.40	32.6	19.8	11.9
	0–30	8.7	15.1	1.63	24.6	19.2	11.7
	0–30	8.6	14.5	1.89	27.4	19.7	10 4
	0–30	7 9	15.0	1.44	21.6	17.5	12.2
5	0–5	1.02	9.5	3.6(	34.2	21.3	6.0
	5–10	0.97	9.5	4.34	41.2	20.4	4 5
	10–30	3.46	9.7	4.22	41.1	17.6	4.2
6	0–5	1.41	9.4	3.24	30.6	29.3	7.7
	5–10	1 48	11.4	1.98	22.6	25.9	13.3
	10–15	0.73	12.5	2.07	25.9	11.6	6 1
	15–3(	3.14	13.1	1.54	20.2	15.9	10.4
	30–60	5.27	10.9	1.66	18.1	16.1	9.7

[a] See text for definition.

of the circulating reticulocytes which were inactive. Also, some cells were more active than others. In the reticulocytes of varying activity the activity of their ribosomes was potentially the same, but their actual activity was determined by soluble factors in their cytoplasm.[2]

An estimate of the ribosome transfer rate leads to a value for the time required to synthesize a hemoglobin molecule. A rabbit hemoglobin molecule consists of two α and two β peptides which have different amino acid sequences. Accordingly their synthesis is governed by different genes and hence different messenger RNA molecules on ribosomes. Any value given for the time required to synthesize a hemoglobin molecule is incomplete without a statement of the number of participating ribosomes. From the genetic evidence and the structure of hemoglobin one may say that either two or four ribosomes are involved in the synthesis of the four peptides. The following data bear on the question of the number of ribosomes involved. In marrow erythroid cells the maximum labeling of "transient" radioactivity was 4.8 μmoles of leucine per μmole of ribosome, and these were contained in the 52% of the active cells. If one assumes (see below)

[2] This is based on the unpublished observation by R. L. Millette in this laboratory (in 1961) on reticulocytes fractionated in a bovine serum albumin gradient according to their activity (Borsook *et al.*, 1962).

that the active and inactive cells contained the same quantity of ribosomes, then in the active cells there were 9.6 leucine residues per active ribosome. In reticulocyte ribosomes the specific activity varied from 0.8 to 4.3, and 60% of the cells were inactive. Taking the highest specific activity and the per cent of active cells one obtains a value of 10.7 leucine residues per ribosome. These estimates of course are in no way decisive, but both the marrow and reticulocyte values are close to a "theoretical" value of 9.5 leucine residues per ribosome on the basis that one globin peptide is made on one ribosome. The "theoretical" value is also based on the assumptions of 19 leucine residues per globin peptide (Schroeder *et al.*, 1950) and on the average in the steady state of half a finished molecule per ribosome. On the assumption that two α or β peptides are made on one ribosome the estimated transfer rate of 20 gives 1.9 minutes as the time required for two ribosomes to synthesize a hemoglobin molecule, *i.e.*, the time required by one ribosome to transfer 38 residues to hemoglobin.

If four ribosomes are involved, one for each peptide, and if all four have a transfer rate of 20, they synthesize a hemoglobin molecule in 0.95 minutes.

In marrow cells the ribosome transfer rate varied throughout the incubation period; it rose to a maximum and then declined. Even the maximum was lower than the value in reticulocytes, which not only was constant throughout an incubation but was nearly the same in all reticulocyte preparations. As the time required to synthesize a hemoglobin molecule depends on the ribosome transfer rate, this time varied in marrow cells throughout the incubation. Certainly the varying activity in the same group of marrow cells is to be ascribed to changing conditions within the cells. This may also be the cause of the differences between marrow cells and reticulocytes, of the lower specific activity of some ribosomes, and of the inactivity of some cells (of which no indication could be seen in their staining characteristics). Accordingly the time required to synthesize a hemoglobin molecule is dependent on conditions within the cell.

In Table VI are compared rates of synthesis *in vitro* of hemoglobin in marrow erythroid cells and circulating reticulocytes. In a 60-minute incubation about 3.0 times as much hemoglobin was synthesized in a marrow cell as in a reticulocyte. The transfer rate per marrow cell ribosome over a 60-minute interval is about one half that of the reticulocyte ribosome, but this is more than compensated for by their having 5–10 times as many ribosomes per cell. It is also seen that the activity of the circulating reticulocytes was the same whether the anemia was produced by phenylhydrazine or by bleeding. On the other hand the activity of reticulocytes in the normal animal was 15–25% of that in the severely anemic animal. The reticulocytes in the blood of a normal animal are smaller and their immediate erythroblast precursors in the marrow are less active than the reticulocytes in the blood of a severely anemic rabbit (Borsook *et al.*, 1962).

Morell *et al.* (1958) reported nucleated marrow cells to be eight times as active as the circulating reticulocytes in a rabbit made anemic with phenylhydrazine. Under the conditions of our experiments marrow cells were more than twice as active and the reticulocytes four times as active as those reported by Morell *et al.* The latter authors do not mention adding iron to their reaction medium. Reticulocytes are deficient in available iron, and their rate of hemoglobin synthesis in a 2–4 hour incubation can be increased 6-fold by an iron salt (Borsook *et al.*, 1957).

TABLE VI

In vitro HEMOGLOBIN SYNTHESIS BY THE MARROW ERYTHROID CELLS AND CIRCULATING RETICULOCYTES OF A SEVERELY ANEMIC RABBIT

(μmoles per cell per minute $\times 10^{14}$)

Duration of Incubation (min.)	Marrow Erythroid Cells	Circulating Reticulocytes Produced by		
		Phenylhydrazine	Bleeding	Normal
10	3.2	3.4		
	6.2	8.6		
	6.6			
	6.0			
30	3.4	2.6		
	11.4	6.6	8.2	
			7.4	
				1.8
	10.0			
	11.6			
60	16.6	6.8	8.2	
			7.2	
				1.2
90	13.5			
120	11.0			
μmoles ribosomes per cell $\times 10^{14}$	97.5–122.5	10–22.5		

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