(6) The role of pH gradients in the cell shifts of early wound healing should be examined in this light. From our results, one could anticipate that in internal lesions (e.g., gastric ulcers), any deviation from an optimal pH in either the acid or the alkaline direction would impair wound coverage by cell migration. Similar considerations may apply to the positively or negatively "chemotactic" reaction of cells toward infectious foci or tumors.

(7) The external asymmetries determining the direction of cellular advance along a given track have, in the cases tested thus far, turned out to be attributable to rear inhibition, rather than to front stimulation, of cells endowed with endogenous motility. In conjunction with earlier results,[14] this strengthens the conclusion that, contrary to physical "attractions," any observed acceleration of cell advance, for a given metabolic state, is due to the reduction of physical restraints rather than to active physical promotion.

* These investigations were supported in part by grants from the American Cancer Society, the National Science Foundation, and the National Cancer Institute (National Institutes of Health of the U.S. Public Health Service).

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*POLYRIBOSOMES AND PROTEIN SYNTHESIS DURING RETICULOCYTE MATURATION IN VITRO**

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Communicated by David Shemin, June 19, 1963

Erythroid cells provide a particularly suitable model for studying the factors regulating mammalian cell maturation. A variety of structural and biochemical changes are known to occur as erythroid cells mature, such as the onset of hemoglobin formation, the loss of the ability to synthesize DNA and to divide, the extrusion of the nucleus, and, eventually, as the reticulocyte matures to the erythrocyte, the

complete cessation of protein synthesis.[1-4] In reticulocytes, which synthesize protein, there is no detectable synthesis of ribosomal or messenger RNA.[5] In these cells, protein synthesis proceeds primarily on polyribosome structures which are clusters of 2 or more 78S ribosomes.[5-7] The present experiments are designed to determine if the decrease in protein formation which occurs as reticulocytes mature reflects a change in the clustered ribosomes. It has been found that 80–90 per cent of the ribosomes in a population of reticulocytes at the outset of maturation are arranged in clusters. As these cells mature *in vitro* to erythrocytes, the decrease in ability to synthesize protein is associated with a loss in the content of polyribosomes.

Methods.—Conditions for the maturation of reticulocytes in vitro: Reticulocytes were prepared from phenylhydrazine-treated rabbits according to the procedures of Borsook *et al.*[8] The cells were recovered from the whole blood by centrifugation and suspended in 2.0 volumes of a medium of the following composition: a mixture of L-amino acids,[8] 0.12 M NaCl, 4.75×10^{-3} M KCl, 1.18×10^{-3} M $MgSO_4$, 1.18×10^{-3} M KH_2PO_4, 2.5×10^{-2} M $NaHCO_3$, 2.4×10^{-5} M $FeCl_2$, 3.6×10^{-3} M glucose, and 6×10^{-4} M inosine, plus 2.0 volumes of plasma and 100,000 units of penicillin and 0.25 gm streptomycin per 100 ml of mixture. Both plasma and cells were obtained from the same rabbit. This suspension, generally in a final volume of 40 ml, was placed in a siliconized, water-jacketed incubation flask fitted with a cap, through which passed a pH electrode, stirring rod, burette tip, and gas inflow and outflow tubes. The pH of the incubation mixture was maintained constant at 7.37 by means of a pH-Stat (Radiometer Titrator, model TTTlc), which continuously monitored the pH of the incubation mixture and controlled the delivery of a solution of 0.3 M $NaHCO_3$ and 0.08 M glucose. Temperature was maintained at 37°, the mixture was continuously stirred, and the atmosphere in the chamber flushed with a 95% O_2–5% CO_2 mixture. At the outset and at various times during the incubation procedure, aliquots of the cell suspension were removed for the determinations indicated below.

Electron microscopy: The cell suspension was added to 20 volumes of cold 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, and allowed to fix for 4–6 hr. The cells were then washed with this buffer, and further fixed in 1% osmium tetroxide in 0.1 M phosphate buffer pH 7.3, dehydrated, and embedded in epoxy resin. Sections were stained with 1% uranyl acetate in 50% ethyl alcohol and examined in an RCA EMU-2 electron microscope. The total number and arrangement of ribosomes within an arbitrary but standard area inscribed upon micrographs of 50 randomly selected cells was determined. The proportion of cells which appeared devoid of any ribosomes was ascertained. The results of enumeration of ribosomes in duplicate samples differed by less than 5%. The thickness of the sections examined in the electron microscope varied between 400 and 600 Å. A certain fraction of polyribosome structures will be transected by the plane of sectioning and will appear to contain fewer than the actual number of ribosomes. As a consequence of this type of error, the number and size of polyribosomes will be underestimated while the number of single ribosomes will be overestimated. The magnitude of this error was evaluated by an analysis of electron micrographs inscribed with lines simulating adjacent planes of section. This procedure indicated that, on the average, 15% of the ribosomes appear as single particles in these micrographs, but are actually a part of polyribosomes in the cell.

Glutaraldehyde-fixed and washed erythrocytes were also examined in suspension by phase-contrast microscopy and the relative number of mature, bidiscoidal cells estimated.

Conditions for determination of C^{14}-amino acid incorporation by the cells: The cells were recovered from an aliquot of the incubation mixture by centrifugation and resuspended in a medium containing either C^{14}-L-valine or C^{14}-L-leucine as described elsewhere.[5] Incubation was for 15 min at 37°. Following this labeling procedure, the cells were recovered by centrifugation and lysed by a short exposure to a hypotonic solution (1.5×10^{-3} M $MgCl_2$, 10^{-3} M Tris-chloride buffer, pH 7.5). This technique preserves leukocytes and tends to disrupt only the red cells.[5] Ribosomes were prepared from the cell lysate by centrifugation at 105,000 × g for 120 min. The supernatant was removed and saved (105,000 × g supernatant). The pellet of ribosomes was rinsed three times with a solution of 10^{-3} M Tris-chloride buffer, pH 7.5, 1.5×10^{-3} M $MgCl_2$ and 5×10^{-2} M KCl (solution A). The ribosomes were suspended in solution A, and the absorbancy, measured at 260 mμ and 280 mμ, and the radioactivity of the solution were determined.[5] The extinction coefficient of the ribosomes in solution A at 260 mμ was 11.1 optical density units per mg ribosome.

Ribosomes were characterized by their sedimentation properties in sucrose density gradients.[5, 9] The conditions of centrifugation are indicated in the legend for Figure 1. The 105,000 × *g* supernatant was centrifuged for a second time at 105,000 × *g* for 120 min. The supernatant of this second centrifugation was recovered, and aliquots were taken for determination of hemoglobin and protein concentrations, as well as radioactivity incorporated into the protein.[5]

Other determinations: At each time when aliquots were removed for electron microscopy and for incubation with the C^{14}-amino acid, the following determinations were also made: enumeration of erythrocytes, reticulocytes, nucleated red blood cells and leukocytes,[10] hematocrit,[10] and osmotic fragility of the cells.[11]

Results and Discussion.—Distribution of ribosomes in reticulocytes: An estimate of the proportion of ribosomes arranged in polyribosome structures in the intact cell was obtained from examination of electron micrographs of sectioned reticulocytes. Such an analysis revealed that 80–90 per cent of the ribosomes were polyribosomes within the population of reticulocytes at the outset of *in vitro* maturation (Table 1). This value was somewhat higher than the proportion of ribosomes sedimenting with a sedimentation coefficient greater than 78S as estimated by the sucrose density gradient method. Among the possible explanations for finding a lower proportion of ribosomes sedimenting with a sedimentation coefficient greater than 78S, as compared with the ribosomes appearing as polyribosomes in sectioned cells, are (1) that a fraction of the polyribosomes are disrupted in the preparation of ribosomes for analysis by sucrose density gradient technique, and (2) that the procedure of lysis of the reticulocytes does not recover the polyribosomes as completely as the 78S ribosomes. Previous studies have demonstrated that polyribosomes may be broken down to 78S particles by such procedures as homogenization during resuspension of the ribosome pellet, repeated centrifugation, or exposure to high salt concentrations.[5, 6, 12] The limited manipulations involved in the preparation of the electron micrographs suggests that the results obtained by this type of analysis more accurately reflect the proportion of the ribosomes which are polyribosomes in the intact reticulocyte.

TABLE 1

PROPORTION OF RETICULOCYTE RIBOSOMES THAT ARE POLYRIBOSOMES

	Method	
Rabbit* No.	Electron microscopy % polyribosomes†	Sucrose density gradient % > 78S‡
I	91	75
II	83	76
III	81	65
IV	90	75
V	84	—
VI	—	66

* All rabbits had 55–80% reticulocytes in the red cell population.

† The proportion of the total number of ribosomes which appeared in clusters of 2 or more. These values are corrected for the estimated number of polyribosomes transected by the plane of sectioning as described in *Methods*.

‡ The proportion of the ribosomes sedimenting with sedimentation coefficients greater than 78S was estimated by integration of the area under the peaks in the pattern of distribution of ribosomes after sedimentation in the sucrose density gradient.

Effect of in vitro maturation on ribosome clusters and protein synthesis: The occurrence of reticulocyte maturation in the *in vitro* system employed was evidenced by a decrease in the percentage of cells that stained as reticulocytes, an increase in the relative number of mature, bidiscoidal cells as evaluated in phase microscopy, and an increase in the osmotic fragility of the cells.

In each of six experiments, *in vitro* maturation was associated with a decrease

in the rate of amino acid incorporation into soluble protein and in the amount of labeled amino acids associated with ribosomes (Table 2). The kinetics of amino acid incorporation by cells at the outset and following *in vitro* maturation were examined. The incorporation of amino acids associated with the ribosomes reached a plateau level within two min which persisted for as long as one hr. During this time, amino acid incorporation into soluble protein continued to increase at a linear rate. In each study (Table 2) the decrease in the *rate* of amino acid incorporated into soluble protein was comparable to the fall in the level of newly incorporated amino acid associated with ribosomes. This finding is consistent with the concept that the decrease in protein synthesis is due to a decrease in the cell content of ribosomes active in protein formation.

TABLE 2

EFFECT OF RETICULOCYTE MATURATION *in vitro* ON PROTEIN SYNTHESIS AND RIBOSOME CONTENT

Experiment	*In vitro* maturation time (hr)	Amino Acid Incorporation (mμmoles/10^{10} cells)*		Ribosome Content†			
				Electron microscopy (no./unit area of 50 cells)		Sucrose density gradient (mgm)	
		Ribosomes	Soluble protein	Total	Clustered	Total	>78S
I	0	8.10	316	2,242	2,040	4.4	3.3
	24	0.76	28	1,485	1,100	3.0	1.4
II	0	5.01	212	685	570	2.6	2.0
	24	0.15	5	133	57	0.6	0.2
III	0	5.70	280	1,717	1,390	4.8	3.6
	22	1.62	76	702	619	1.4	1.0
IV	0	8.5	354	2,744	2,290	5.9	ND‡
	21	1.9	70	957	440	2.0	ND
V	0	6.4	391	ND	ND	4.2	ND
	24	0.6	35	ND	ND	2.1	ND
VI	0	9.6	428	ND	ND	4.2	2.7
	21	1.6	67	ND	ND	1.6	0.9

* During the 15 min incubation.
† Determined by enumeration of the ribosomes in electron micrographs of sectioned cells and as estimated from analysis of the patterns of sedimentation in sucrose density gradients.
‡ ND indicates determination not performed.

More direct evidence that the fall in protein synthesis as reticulocytes mature is related to a decrease in ribosome clusters is presented in Figures 1 and 2. Ribosomes isolated from reticulocytes before and after *in vitro* maturation were analyzed by centrifugation in a sucrose density gradient. With maturation the proportion of ribosomes which sediment with a sedimentation coefficient greater than 78S decreases, while the proportion of 78S ribosomes increases (Fig. 1). The decrease in the polyribosomes with reticulocyte maturation was further established by examination of electron micrographs of sectioned cells. At the outset of maturation, ribosomes are observed predominantly in clusters of 2 or more (Fig. 2*A*). With *in vitro* maturation, there is a fall in the total number of ribosomes and an even more striking decrease in the proportion of the ribosomes which are polyribosomes (Table 2, Fig. 2*B*). The polyribosomes which remain show an alteration in the pattern of distribution characterized by a shift toward smaller clusters, as well as an increase in the proportion of single ribosomes (Fig. 3). The decrease in the total ribosome content of cells following maturation is consistent with the observations of Bertles and Beck[13] that rabbit reticulocyte maturation *in vitro* is accompanied by a conversion of cellular ribonucleic acid to low molecular weight products, chiefly purines and pyrimidines.

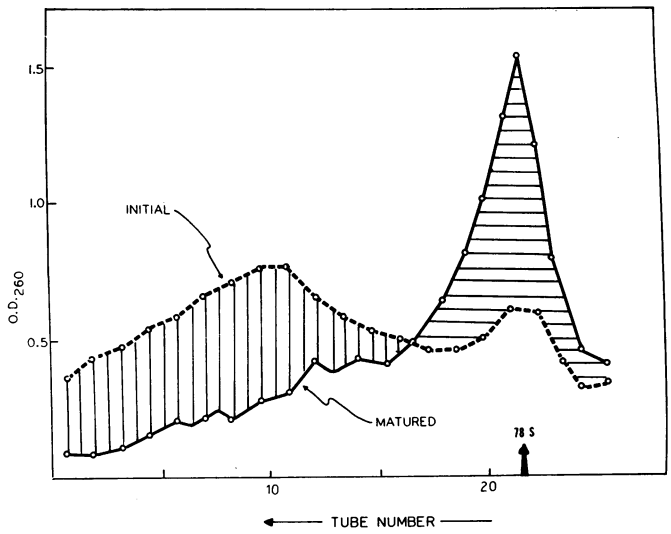

FIG. 1.—Effect of *in vitro* maturation of reticulocytes on the distribution of ribosomes of various sizes. Sucrose density gradient analysis of ribosomes isolated from reticulocytes before (broken line) and after (solid line) a 22-hr period of *in vitro* maturation. The ribosomes were suspended in a solution of 10^{-3} *M* Tris-HCl buffer, pH 7.5, 1.5×10^{-3} *M* $MgCl_2$, and 5×10^{-2} *M* KCl, and sedimented by centrifugation in a 5–20% sucrose density gradient prepared from the same solution. Centrifugation was for 2 hr at 25,000 rpm in a Spinco SW-25 rotor. The gradient was analyzed as previously described.[5]

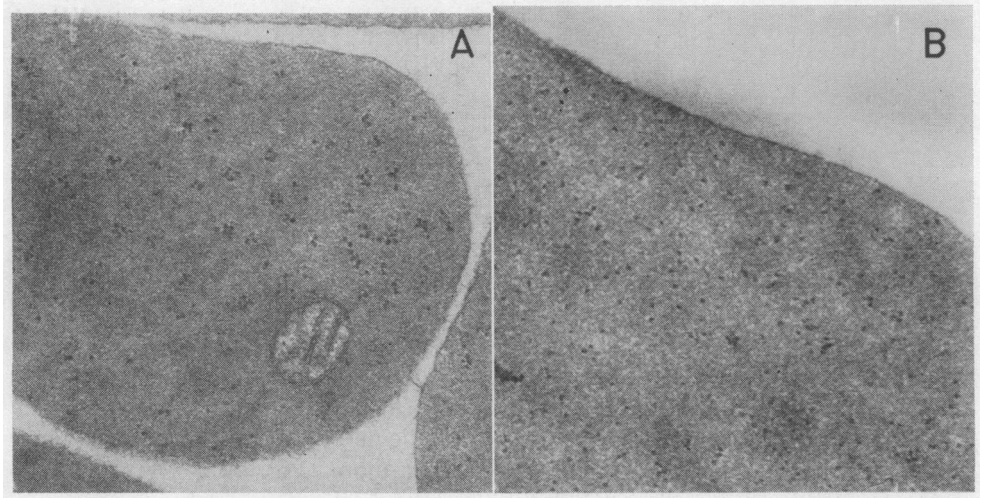

FIG. 2.—(*A*) A portion of two reticulocytes at the outset of *in vitro* maturation. Numerous ribosomal clusters are observed in a finely granular, dense material presumed to be hemoglobin. A mitochondrion is present in the cell. (*B*) Part of a recticulocyte following 24 hr of *in vitro* maturation. The cytoplasm contains many ribosomes, but these are virtually all single nonclustered particles. (Magnification 38,000 ×).

The ribosomes involved in protein synthesis were examined by determining the pattern of incorporation of C^{14}-leucine onto ribosome clusters of various sizes. At both the initial time and following *in vitro* maturation, the specific activity of the ribosomes in the region corresponding to sedimentation coefficients greater than

100S was markedly higher than that of 78S ribosomes (Fig. 4). These data demonstrate that, despite marked loss in the capacity to form protein, protein synthesis which continues to occur proceeds predominantly on the polyribosomes. In 3 of 4 experiments, a decrease in the specific activity of polyribosomes occurred during *in vitro* maturation (Table 1 and Fig. 4). Thus, as reticulocytes mature, there is a fall in the capacity of polyribosomes to incorporate amino acids into polypeptides, as well as a decrease in the amount of polyribosomes. This finding suggests that polyribosomes may become partially or completely inactive while remaining polyribosomes. One of the possible explanations for these findings is that the loss of polyribosomes occurs only after messenger RNA or another component of the protein synthetic mechanism is depleted during reticulocyte maturation.

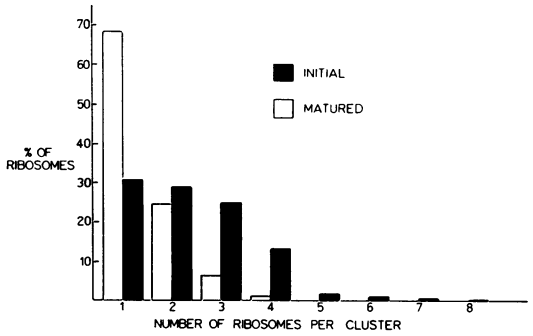

FIG. 3.—Effect of maturation on the distribution of ribosomes in various size clusters as determined by analysis of electron micrographs of sectioned cells. The values before (open bars) and after (closed bars) *in vitro* maturation are expressed as per cent of the total number of ribosomes per standard area of 50 cells. These values are not corrected for the error introduced by sectioning because the redistribution of ribosomes among each of the classes of polyribosomes cannot be made with accuracy.

It has been proposed that the polyribosomes are composed of a number of 78S ribosomes attached to a strand of messenger RNA.[6, 7, 14–16] Although evidence has been obtained consistent with this hypothesis,[5–7, 14–21] there has been no

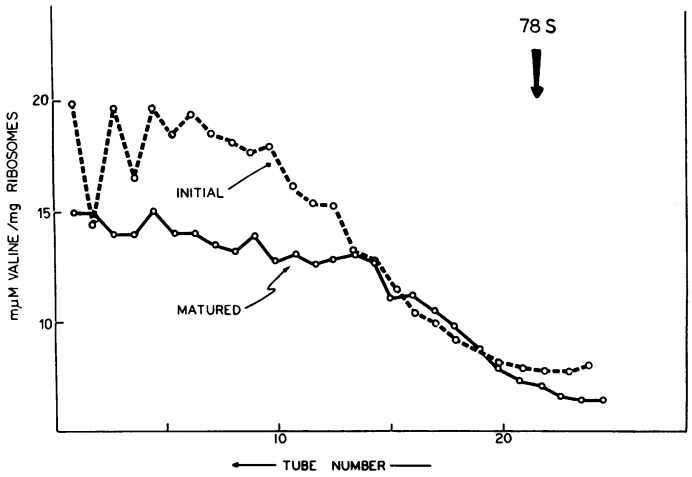

FIG. 4.—Effect of *in vitro* maturation of reticulocytes on the pattern of amino acid incorporation on ribosomes of various sizes. The reticulocytes were incubated with C^{14}-leucine under the conditions described in *Methods*. The preparation of ribosomes and the conditions for sucrose density analysis were identical to those indicated in the legend for Fig. 1. The pattern of incorporation of C^{14}-leucine on the ribosomes is expressed as mμmoles leucine incorporated per mg of ribosomes before (broken line) and after (solid line) *in vitro* maturation.

definitive demonstration that the substance essential for the maintenance of polyribosome structure is totally accounted for by a messenger RNA, i.e., a fraction of RNA which has the biological property of a template for protein synthesis. In reticulocytes, there is no detectable synthesis of any RNA component of the polyribosome.[5] The stability of these polyribosomes is therefore dependent on a factor or factors other than continued RNA synthesis. There is evidence that a portion of ribosomal RNA,[22] as well as transfer RNA,[21] may be important to the stability of the polyribosomes. The present observations suggest that the maintenance of the polyribosome structure is a fundamental aspect of the regulation of erythroid cell maturation.

Summary.—In a population of relatively immature rabbit reticulocytes, 80–90 per cent of the ribosomes appear as polyribosomes in electron micrographs of sectioned cells. Evidence has been obtained indicating that the maturation of reticulocytes to erythrocytes is associated with a decrease in the functional capacity, as well as in the number of polyribosomes which are the site of protein formation. It is likely that these alterations in polyribosomes account for the decrease in protein synthesis which occurs as reticulocytes mature.

The authors are indebted to the Department of Microbiology and, in particular, Dr. Councilman Morgan for making available the facilities of the electron microscope laboratory.

* This investigation was supported in part by grants from the U.S. Public Health Service and the National Science Foundation.

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