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Fate of Reticulocyte Ribosomes During *in Vivo* Maturation*

Robert H. DeBellis

ABSTRACT: Changes in the distribution of ribosomes in rabbit reticulocytes have been studied during maturation *in vivo*. ^{32}P was given intravenously to label the ribonucleic acid of these cells and the distribution of the isotope in ribosomes was followed with time. Both the percentage of ribosomes existing as polysomes and the aggregate size distribution of polysomes remained constant during the maturation of the reticulocytes. These findings are particularly noteworthy in view of the observed loss in the total content of ribosomes. The results of this study, employing an autologous system for the maturation of cells *in vivo*, are in agreement with the results of previous studies from this laboratory in which a heterologous system was employed. These investigations suggest that, once formed, each individual polysome aggregate continues to function unchanged until its ultimate destruction during the process of reticulocyte maturation. If 80S monomers are produced during the destruction of polysomes they in turn are short lived since they cannot be detected as an increased percentage of monosomes with increased cell age.

During the course of maturation of the mammalian reticulocyte to the circulating erythrocyte a number of biochemical and morphological changes occur among which are a loss of the capacity to synthesize protein and a loss of ribosomal material. Previous *in vitro* (Marks *et al.*, 1963b; Rifkind *et al.*, 1964) and *in vivo* (Rifkind *et al.*, 1964; Glowacki and Millette, 1965; Rowley, 1965; Danon *et al.*, 1965; Burka and DeBellis, 1967; Danon and Cividalli, 1968) studies have established that reticulocyte maturation involves a progressive loss of polysomes as well as a loss of total cellular ribosomal content. However, studies from different laboratories have suggested three patterns of reticulocyte maturation. The *in vitro* studies demonstrated a preferential loss of polysomes and an ordered shift in the size of the remaining polysome clusters toward single ribosomes (Marks *et al.*, 1963b; Rifkind *et al.*, 1964; Danon *et al.*, 1965). In the second group of studies, there was an increase in the per cent of monoribosomes but the size distribution of the remaining polysomes remained unaltered (Glowacki and Millette, 1965; Rowley, 1965). Finally, in the third study all size classes of ribosomes were lost at proportional rates (Burka and DeBellis, 1967).

The present investigation was designed to further study the fate of ribosomes during cell maturation using a technique

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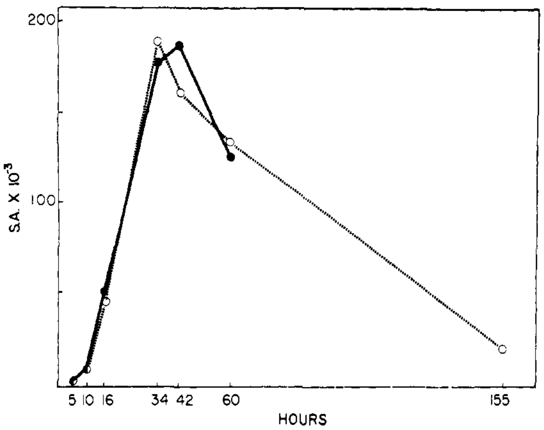

FIGURE 1: Specific radioactivity of reticulocyte ribosomes obtained at varying times following intravenous ^{32}P administration. All samples were obtained from the same animal. Closed circles represent the specific activity of pelleted ribosomes. Open circles represent the average specific activity of all classes of ribosomes as observed by sucrose density gradient analysis. Specific activity is expressed as counts per minute per milligram of ribosomes in all figures.

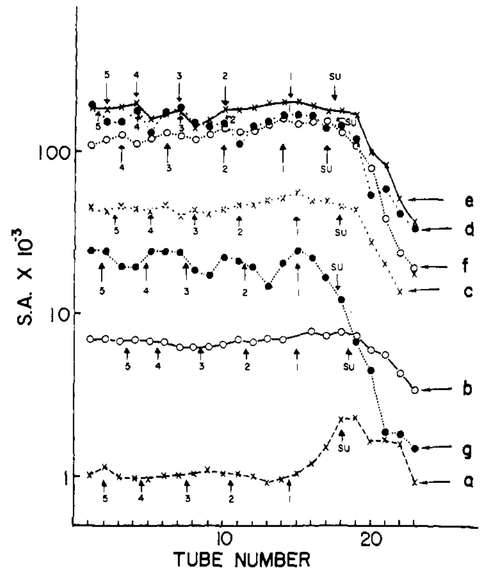

FIGURE 2: Semilogarithmic plot of the specific radioactivity of ribosomes separated by sucrose density gradient centrifugation. Reticulocyte ribosomes were obtained from the same animal at 5, 10, 16, 34, 42, 60, and 155 hr following ^{32}P administration. The sucrose density gradient analysis at each of these times is represented in the graph by the letter a to g, respectively. The numbers in parentheses represent the ribosome aggregate sizes in different portions of each gradient. SU represents the region of the gradients containing subunits.

different from those previously employed. The results reported here suggest that during maturation of the reticulocyte all classes of ribosomes are lost at rates proportional to their initial concentration.

Materials and Methods

^{32}P Reticulocytes. A reticulocytosis was induced in rabbits by the administration of phenylhydrazine (DeBellis *et al.*, 1964); 2 days after the last injection of phenylhydrazine, 5 mCi of carrier-free ^{32}P was injected into the marginal vein of the ear. At times thereafter, serial samples of blood were obtained by cardiac puncture for the preparation of ribosomes.

Ribosome Preparation. The reticulocyte-rich blood was washed with isotonic saline. The washed, packed cells were shock lysed (DeBellis *et al.*, 1964) with four volumes of 1.5×10^{-3} M $MgSO_4$–10^{-3} M Tris-Cl buffer (pH 7.6; Tris-Mg) at 0°. Isotonicity was restored with 10% NaCl and stroma was removed by centrifugation at 12,000*g* for 10 min. The ribosomes were sedimented by centrifugation at 150,000*g* for 60 min and then resuspended in Tris-Mg for sucrose density gradient analysis.

Sucrose Density Gradient Centrifugation. Linear gradients were made using 5–20% sucrose dissolved in Tris-Mg. Ultracentrifugation was performed for 2 hr at 25,000 rpm utilizing an SW25.1 head in a Model L Spinco ultracentrifuge. Following centrifugation the bottom of the centrifuge tube was punctured and serial fractions were collected for measurement of optical density and radioactivity (DeBellis *et al.*, 1964).

Measurement of Ribosome Content. Optical density was measured at 260 mμ after suitable dilution of samples with Tris-Mg. Absorbance was converted into milligrams of ribosomes using an extinction coefficient of 11.0 optical density units/mg of ribosomes when read at 260 mμ.

Measurement of ^{32}P-Labeled Ribosomes. The ^{32}P-labeled ribosomes were precipitated with 10% cold trichloroacetic acid; the precipitates were collected on Millipore filters and washed with 5% trichloroacetic acid at 0°. The dried disks were counted in a Nuclear-Chicago low-background gas-flow counter. ^{32}P measurements were corrected for decay. Specific activity is expressed as counts per minute per milligram of ribosomes.

Determination of Proportion of Individual Ribosome Classes. The per cent of the total ribosomes in each class of ribosomes, *i.e.*, per cent monomers, dimers, trimers, etc., was determined by fitting individual symmetrical curves by eye under each of the peaks observed on the total radioactive ribosome pattern. The areas under the experimentally obtained and final idealized curves were then integrated. The sums of the areas under the individual curves were within 6% of the areas of the radioactive patterns.

Results

Specific Activity of Ribosomes. Following administration of ^{32}P there is a 9-hr period during which few reticulocytes containing labeled ribosomes appear in the circulation (Figure 1). Following the 9-hr lag period there is a rapid rise in the specific activity to a peak value at approximately 40 hr. This is followed by a subsequent fall over the next 110 hr to a specific activity of 10% of the peak value. In one experiment the specific activity of ribosomes obtained 17 days following ^{32}P administration was approximately 3% of the peak specific activity observed at 43 hr. At each time measured, the specific activity of pelleted ribosomes agreed well with the average specific activity of ribosomes obtained from sucrose gradients. Figure 2 illustrates the specific activity of serial samples of reticulocyte ribosomes obtained from the same animal and separated by sucrose density gradient centrifugation. The specific activity of all classes of ribosomes remained

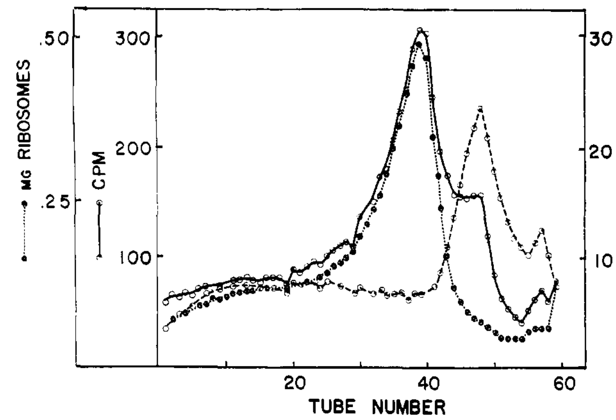

FIGURE 3: Sucrose density gradient analysis of the ribosomes of reticulocytes obtained 90 min following ^{32}P administration. The polysomes (tubes 1–32) represent 42% of the total ribosomes; the 80S monomers (tubes 33–42) represent 48% of the total ribosomes. (The subunits are in tubes 45–49.)

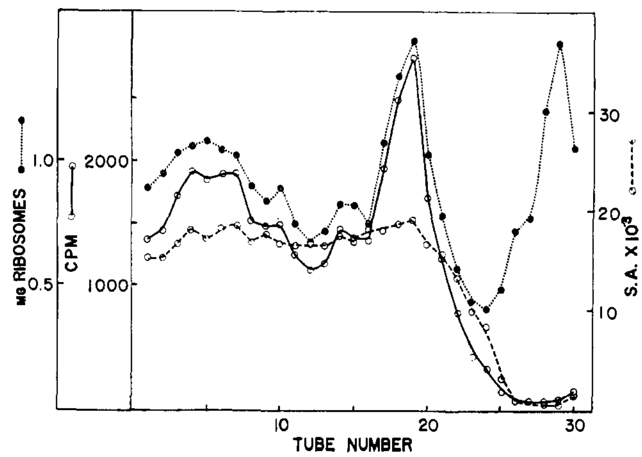

FIGURE 4: Sucrose density gradient analysis of the ribosomes of reticulocytes obtained 139 hr following ^{32}P administration. The polysomes (tubes 1–15) represent 58% of the total ribosomes; the 80S monomers (tubes 16–20) represent 33% of the total ribosomes.

constant for each of the time intervals tested except for the 5- and 155-hr samples. As reported previously (DeBellis, 1964), the specific activity of subunits (40S plus 60S particles) was found to be greater than that of the remainder of the ribosomes at the early times tested. This is illustrated in Figure 2a, where there is a twofold difference in the specific activity at 5 hr following ^{32}P administration, and in Figure 3 where the difference is greater than threefold at 90 min following ^{32}P administration. In contrast, as shown in Figures 2g and 4, the specific activity of subunits at later times following ^{32}P administration tended to be lower than the average specific activity of the remaining ribosomes. This latter finding is probably due to the presence of contaminating low molecular weight, nonradioactive ultraviolet-absorbing material. When the ribosome content per cell is high (young cells), this material has little significance, however when the ribosome content per cell is low (old cells), it assumes a much greater significance in lowering the true specific activity of subunits.

Size Distribution of ^{32}P Ribosomes. The per cent of the total ribosome population existing as polysomes was quite variable from rabbit to rabbit, ranging from 40 to 70%, but was relatively constant in serial samples from the same animal. This variation from animal to animal is illustrated in Figures 3 and 4. In Figure 3, the polysomes represent 42% of the total ribosome population in one animal whereas in Figure 4 they represent 58% of the total ribosomes of another animal. Subunits usually represented approximately 10% of the total ribosomal content, a figure in agreement with the findings of Joklik and Becker (1965). The size distribution of ribosomes was examined in serial samples taken between 10 and 60 hr from the same animal. The per cent distribution of the different ribosome classes as a function of time is shown in Figure 5. There was no significant change in the proportion of each class of polyribosome nor was there a significant difference in the per cent of single ribosomes as a function of time.

Discussion

The present study has demonstrated that during the maturation of reticulocytes *in vivo*, the profile of ribosomes remains constant. An analysis of the results is facilitated by a consideration of the events that occur following the intravenous administration of ^{32}P; 1 to 5 hr after injection, the first cells containing ^{32}P-labeled rRNA appear in the circulation (DeBellis *et al.*, 1964). Since the circulating reticulocyte is incapable of RNA synthesis (Burka *et al.*, 1963; Marks *et al.*, 1963a), it follows that reticulocytes containing ^{32}P-labeled ribosomes at short intervals following the *in vivo* administration of ^{32}P represent those cells most recently matured from reticulocyte precursor cells and most recently released from the bone marrow into the peripheral circulation. In the present study, the specific activity of polysomes and 80S ribosomes were equal at all times studied and the size distribution of ribosomes remained constant as a function of time.

A possible explanation for these findings is that when ^{32}P-labeled ribosomes are isolated in the presence of unlabeled ribosomes, there may be an equilibration of the two populations as has been suggested by Danon and Cividalli (1968).

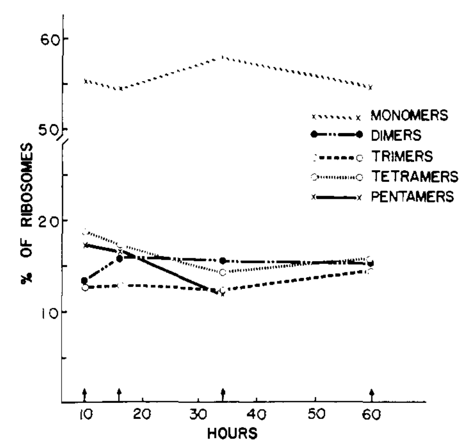

FIGURE 5: Per cent distribution of ^{32}P-labeled ribosomes of different size clusters at varying times following ^{32}P administration. The results are expressed as % of the sum of all ribosomes of aggregate sizes 1–4 (*i.e.*, monomers to tetramers), since pentomers were pelleted in the 60-hr sample and could not be quantitated (see Figure 2f).

This would appear to be highly unlikely since deliberate attempts to attach ^{32}P-labeled 80S ribosomes to unlabeled polysomes during protein synthesis in a cell-free system have been completely unsuccessful (R. H. DeBellis, manuscript in preparation). An alternative explanation is that young (^{32}P labeled) and old (unlabeled) reticulocytes have similar polysome distributions. Consequently, mixing of labeled and unlabeled ribosomes at any time following ^{32}P administration would result in an equal specific activity for all classes of ribosomes (Figures 2–4). Furthermore the proportion of each class of ribosome would remain constant with time (Figure 5).

The results of this study contrast with those of previous *in vitro* (Marks *et al.*, 1963b; Rifkind *et al.*, 1964) and *in vivo* (Rifkind *et al.*, 1964; Glowacki and Millette, 1965; Rowley, 1965; Danon *et al.*, 1965) studies. It is felt that the methods used in the present study are closer to physiological conditions than those used in the other studies.

Since it has been shown that agents that interfere with energy utilization cause a reversible disaggregation of ribosomes in the intact cell (Conconi *et al.*, 1965; Marks *et al.*, 1965; Godchaux and Herbert, 1965), the preferential loss of polysomes found with *in vitro* maturation may be related in part to a sufficient utilizable energy source.

The results of Glowacki and Millette (1965) and of Rowley (1965) are basically in agreement with the findings in the present study. Their studies showed differences in the per cent monoribosomes only when the youngest and oldest cells were compared. Although this minor difference remains unexplained, a possible explanation may rest in the experimental design used by Glowacki and Millette and by Rowley. These workers separated reticulocytes by differential centrifugation, relying on the observation that cell density is primarily a function of cell age (Watson and Clark, 1937; Pritchard, 1949; Allison and Burn, 1955; Borun *et al.*, 1957; Borsook *et al.*, 1962; Leif and Vinograd, 1964; Millette and Glowacki, 1964; Danon and Marikovsky, 1964). However, it should be noted that cell density is also correlated with cell origin, *i.e.*, a reticulocyte derived directly from a basophilic normoblast is less dense than one derived from an orthochromic normoblast (Glowacki and Millette, 1965). Consequently, studies based on cell density separations do not take into account the varied origin of reticulocytes produced under anemic stress (Borsook *et al.*, 1962; Brecher and Stohlman, 1962; Stohlman, 1962).

The present findings agree with the results of other studies (Burka and DeBellis, 1967; Bishop, 1966) in which reticulocyte maturation was studied by unrelated *in vivo* techniques. Together, the studies lend support to the concept that reticulocytes are formed containing a given size profile of ribosomes. This profile remains constant despite a continual loss of ribosomes during cell maturation.

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