RIBOSOME SHIFTS IN THE COLD CELL-FREE LYSATE DURING ANALYSIS OF MATURING RETICULOCYTE RIBOSOMES

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The progressive loss of the capacity for protein synthesis during maturation of mammalian reticulocytes is associated with a reduction in ribosomal and total RNA. As the total ribosome content falls, the proportion of hexasomes, pentasomes and quadrisomes is reduced, while that of single ribosomes and diplosomes increases (Marks et al. 1963, Rifkind et al. 1964, Danon et al. 1965, Rowley, 1965). Somewhat contradictory results claiming that there is no increased proportion of single ribosomes were also reported (Glowacki and Millette 1965, Burka and De Bellis 1967).

In the present experiments young reticulocytes from donor rabbits were labelled with P^{32} and transfused to host rabbits in which they matured and from which blood samples were taken for analysis. The results of this study, showing a reduced proportion of polyribosomes in mature reticulocytes and displacement of ribosomes from one cluster to another in cell lysate during the experimental procedure, is presented here.

Blood containing about 40% reticulocytes was obtained by daily bleedings. This procedure avoided Heinz body formation and damage to the cell membranes that follows phenyl hydrazine treatment (Rifkind and Danon 1965, Rifkind 1965). The increasing proportion of osmotically more resistant population of reticulocytes was followed using a Fragiligraph Model D2 (Elron, Electronic Industries Ltd., Haifa, Israel). Ribosomes in the reticulocytes were labelled by injecting the donor rabbit intravenously with 2-3mc of P^{32} labelled phosphate (specific activity 50 to 90 Curies per mg phosphate) 24 to 40 hours before withdrawal of blood. Iron (50mg Imferon) was injected simultaneously. About 10 to 15ml of packed cells from blood rich in labelled reticulocytes resuspended in saline were transfused into a normal host rabbit that was either splenectomized to minimize sequestration of transfused cells, or irradiated

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with 550_r, to prevent erythropoiesis, 24 to 48 hours before transfusion. In some experiments, about 20 to 30ml of blood were withdrawn from the host rabbit before transfusing the reticulocyte-rich packed cells. Ribosome suspensions were prepared as previously described (Marks et al. 1963) and usually stored overnight in plastic centrifuge tubes in ice buckets placed in a cold room. Sucrose gradient analysis of the ribosomes was carried out as previously described (Marks et al. 1963). In order to compare the sedimentation pattern of ribosomes before and after reticulocyte maturation, ribosomes were prepared and characterized from two blood samples. The first sample of blood, taken from the host rabbit 40 to 60 minutes after transfusion was designated "young reticulocytes blood". The second blood sample taken 20 to 24 hours after transfusion was designated "matured reticulocytes blood".

In all the experiments, the proportion of slowly sedimenting ribosomes, i.e. single and double ribosomes, increased in reticulocytes that matured in the circulation of the host rabbit. The total ribosome content per volume of cells decreased to about 10-15% of the initial amount within 24 hours of transfusion to a splenectomized host rabbit, indicating remarkable maturation of the transfused reticulocytes. The specific activity of the ribosomes (CPM/OD) was reduced after 24 hours to approximately one third of the value noted about an hour after transfusion. However, radioactivity distribution along the gradient was similar to the optical density pattern. A representative experiment is depicted in Fig.1. The reduction in specific activity to about one third of its initial value over the same time interval, indicated dilution of the labelled ribosomes by the ones newly formed in the host rabbit, as confirmed by analysis of the sedimentation patterns of ribosomes derived from reticulocytes transfused to irradiated rabbits (Fig.2). In general these sedimentation patterns were similar to those obtained when normal splenectomized rabbits were transfused. However, with the irradiated hosts, the specific activity of the ribosomes showed no significant difference between early and matured samples. This, incidentally, indicates successful depression of marrow activity by the relatively low radiation dose.

The unexpected finding of uniform labelling along the gradient, following maturation in vivo, rather than the anticipated low activity in the polyribosome region and much higher activity in the slowly sedimenting region is not sufficiently explained by admixture of newly formed unlabelled reticulocytes. Considering that new reticulocytes are formed in the non-irradiated animals, the presence of practically uniform radioactivity could be due to liberation of P^{32} from components of the blood other than RNA, or through destruction of labelled cells and liberation of the P^{32}, followed by reincorporation of the isotope by newly formed reticulocytes in the host animal.

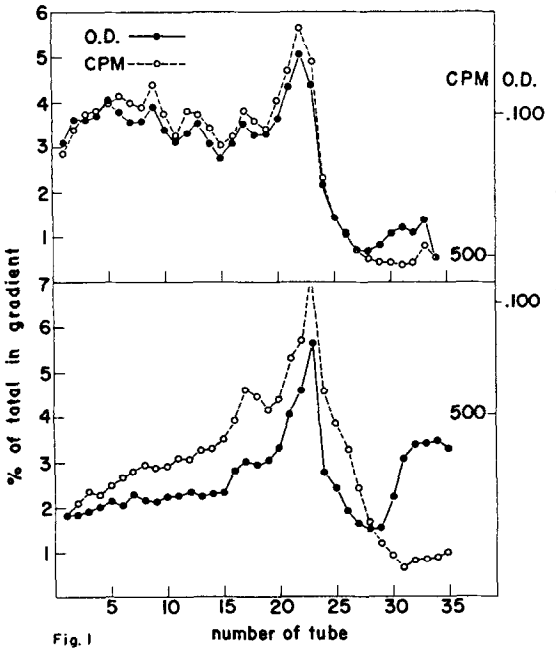

Fig. 1

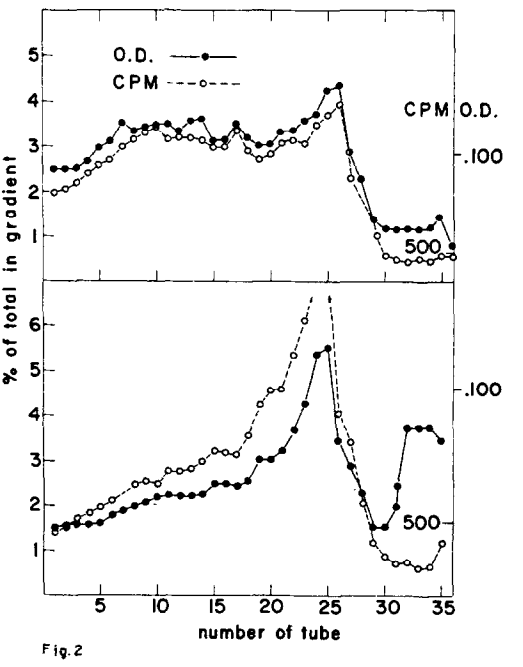

Fig. 2

Fig. 1 - Sedimentation patterns of ribosomes from reticulocytes of a host splenectomized rabbit to which P^{32} labelled reticulocytes were transfused, taken 40 minutes after transfusion (top) and 24 hours after transfusion (bottom). Note the reduced proportion of polyribosomes after 24 hours and the close similarity of the radioactivity profile with that of the optical density pattern both before and after maturation.

Fig. 2 - Sedimentation patterns of ribosomes from reticulocytes taken from an irradiated host rabbit after transfusion. Top - 40 minutes after transfusion, bottom - 24 hours after transfusion. Note that in this experiment, in which the host rabbit was irradiated, the radioactivity per O.D. unit remained practically unchanged after maturation; in the experiment described in Fig.1, in which the host rabbit was splenectomized but not irradiated, the radioactivity per O.D. unit was reduced after maturation by a factor of three.

Another possibility is that the even distribution of activity is due to displacement of ribosomes from one polysome to another during the preparation of ribosomes, when unlabelled and labelled ribosomes of different cellular origin could come into direct contact. An *in vitro* experiment aimed at clarifying whether the first or the second hypothetical explanation is correct, was designed. "Labelled young reticulocyte-rich blood" was mixed *in vitro* with "unlabelled mature reticulocyte blood" in a volume ratio of 1:9 washed packed cells, so that the O.D. units of labelled and unlabelled ribosomes were similar. The mixed cells were lysed together. The sedimentation pattern obtained in a representative experiment is depicted in Fig.3. It shows that radioactivity is

distributed almost uniformly along the gradient. The general specific activity of the ribosomes derived from the mixed cells lysed together was practically that which would be expected in a dilution of the radioactive ribosomes with "cold" ones. However, the anticipated radioactivity distribution curve i.e. activity following the same pattern as that of the labelled ribosomes only, did not appear. Similar results were obtained with "labelled matured reticulocyte blood" mixed with "unlabelled young reticulocyte-rich blood".

It was concluded that ribosomes are displaced from one cluster to another. Migration of ribosomes between polyribosomes and the monosome pool may occur if protein synthesis takes place in the hemolysate. Unlabelled ribosomes could then interchange places, resulting in a practically uniform distribution of radioactivity.

Protein synthesis at low temperatures in a cell-free system has been reported (Goodman and Rich 1963). However, in the present experiments the temperature was kept very low, and no measures were taken to activate the system by adding "optimal" concentrations of enzymes, ions and substrate. Experiments were performed to determine whether incorporation of amino acids takes place between the time cells are lysed and the time the gradient fractions are precipitated with TCA. Puromycin was added to the hemolysate in two experiments in order to ascertain whether cessation of protein synthesis will affect the distribution of radioactivity along the gradient. The results of this experiment are given in Fig. 4, and show that amino acid incorporation does occur.

The experiments reported here confirm the observation that as reticulocytes mature in the circulation, the degree of aggregation of the ribosomes in clusters decreases, while total content in ribosomes is markedly reduced. They indicate, however, on the one hand, that ribosomes can be displaced from one cluster to another, and that single ribosomes may participate again in the formation of polyribosomes even during the experimental procedure at low temperature. On the other hand, ribosomes originating from a "young" polyribosome can be attached in proximity to a single "matured" ribosome to form a diplosome or be, at the moment of fractionation on the gradient, a single particle, explaining the radioactivity found in the 80S and 110S region of the gradient after mixture with unlabelled matured ribosome population.

The profile of a sucrose gradient of reticulocyte ribosomes seems to represent an instant image of a dynamic process that continues during its preparation. Furthermore, a gradient obtained from reticulocytes pooled from several rabbits does not necessarily represent the arithmetic average of all the individual gradients.

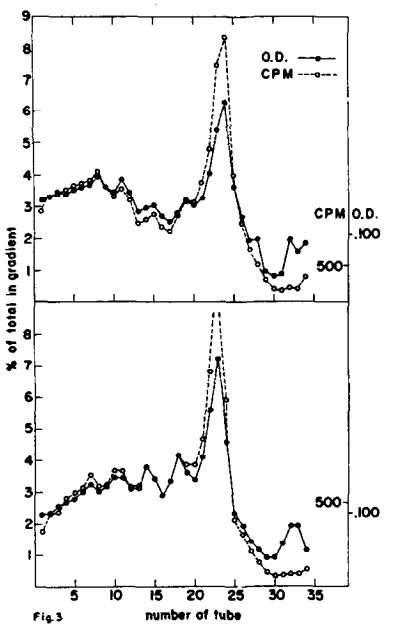

Fig. 3

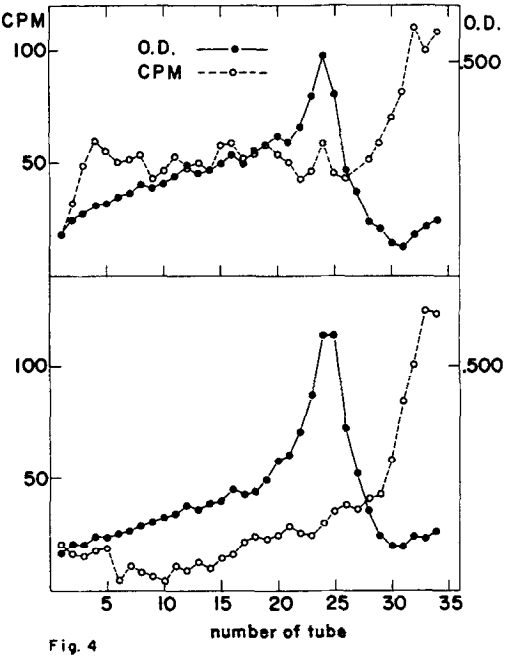

Fig. 4

Fig. 3 - Changes in sedimentation patterns of ribosomes after mixing in vitro P^{32} labelled reticulocyte-rich blood with unlabelled blood of a host rabbit in which unlabelled reticulocytes matured. Top - sedimentation pattern of labelled ribosomes from reticulocyte-rich blood. Bottom - sedimentation pattern of ribosomes prepared from a mixture of one volume of packed cells from the same blood as above and nine volumes of packed cells from the host blood in which unlabelled transfused reticulocytes matured for 24 hours, so that ribosomes of the labelled sample were of approximately equal amount to the unlabelled ones.

Fig. 4 - Amino acids incorporation during the preparation of ribosomes and their sedimentation in the sucrose gradient. Top - sedimentation pattern of ribosomes from reticulocyte-rich blood and the radioactivity pattern of C^{14} labelled amino acids in hot TCA precipitates. The labelled amino acids were introduced immediately after the osmotic hemolysis of the red cells. Bottom - a sample of the same hemolysate as above to which puromycin was added immediately after the addition of C^{14} labelled amino acids.

The reduction in degree of aggregation of ribosomes in maturing reticulocytes is apparently not due to dilution of ribosomes and the consequent reduced probability of a ribosome encountering a messenger RNA molecule since migration of ribosomes took place even in the diluted hemolysate. It may, therefore, be concluded that the reduced degree of aggregation of ribosomes is due to the reduced capacity of the messenger RNA molecule to keep ribosomes together or to a reduction in the length of the messenger RNA molecule as it reaches the end of its life span.

Statistical analysis of the phenomenon (Perl 1964) has indicated, that the reduction in the number of ribosomes per cluster very probably takes place by "dropping off" of single ribosomes. Whether all single ribosomes in a mature reticulocyte are capable of recycling or whether the very old ones lose this capacity is a subject for further study. It may offer another explanation for the degradation of polysomes.

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