SEPARATION OF THE MESSENGER RNA OF RETICULOCYTE POLYRIBOSOMES.

G. MARBAIX (1) and A. BURNY.
Laboratory of Biological Chemistry,
Faculty of Sciences,
University of Brussels, Belgium.

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Attempts at isolating reticulocyte messenger RNA have been briefly reported before : Arnstein & Cox (1963) found that a RNA fraction which strongly stimulates amino acid incorporation sediments with ribosomal RNA. Cohn (1964) detected two RNA fractions which are more rapidly labeled than the average RNA of the cells.

Hemoglobin synthesis in reticulocytes occurs in polyribosomes, structures made of several 80 S ribosomes attached to a threadlike molecule of RNA which is most probably the informational or messenger RNA (Warner et al. 1963, Gierer, 1963, Hardesty et al. 1963). The present communication describes the labeling and complete separation of this RNA from the other RNA fractions of the cells.

METHODS.

I.- Isolation of polyribosomes from reticulocytes.

Rabbits received four injections of phenylhydrazine (0,3 ml of 2,5 % solution per kg of body weight)at one day intervals. Two days after the last injection the rabbits were killed by bleeding under nembutal anesthesia. The blood was heparinized, chilled and centrifuged in the cold at 2800 g for 15 min. The cells were washed with an isotonic solution (0.14 M NaCl, $5X10^{-3}$M KCl and $5X10^{-3}$ M Mg acetate). The thin upper layer which contains the bulk of the leucocytes was pipetted off and discarded. In order to lyse the red cells without breaking the remaining leucocytes, the cell pellet was rapidly dispersed in 4 volumes of hypotonic solution (Tris-HCl 10^{-2}M, $1.5X10^{-2}$ M KCl, 10^{-3} M Mg acetate)at 4°C; isotonicity was restored 30 seconds later by the addition of 84.8 mg of sucrose per ml of lysate (Morell et al. 1958). Intact cells and cells debris were

(1) Aspirant du Fonds National de la Recherche Scientifique.

removed by centrifugation (15 min at 12,000 g). A fraction enriched in polyribosomes was obtained by centrifuging the extract for 1 hour at 105,000 g.

The polyribosomes were observed by centrifuging for 2 hours at 32,000 rpm in the Spinco rotor SW 39, on a 18-36 % linear sucrose gradient (in a medium containing $5X10^{-2}$ M Tris-HCl pH 7.4, $2.5X10^{-2}$ M KCl and $5X10^{-3}$ M Mg acetate). The distribution of the particles was recorded by the method of Edward and Mathias (1963) with a Cary 15 Spectrophotometer.

II.- Extraction of RNA.

The RNA of polyribosomes was extracted in 0.5 % sodium dodecylsulfate, following the procedure of Kurland (1960) and Staehelin (1964). It was then fractionated by sucrose gradient centrifugation : 200 µl of the solution containing 1.5 mg RNA per ml were layered on a 5-20 % sucrose gradient (in $5\ X10^{-3}$ M Tris HCl, pH 7.4) and centrifuged for 7 hours at 37,500 rpm in the SW39 rotor of a Spinco centrifuge. The tubes were punctured and alternate drops were collected for measuring the absorbancy at 260 mµ, or for determining radioactivity, using the liquid scintillator of Bray (1960).

RESULTS.

A rabbit made anemic with phenylhydrazine (see methods) received one intravenous injection of 5 mC orthophosphate-^{32}P 15 hours before sacrifice. The sedimentation pattern of the reticulocyte extract in the SW39 rotor is shown in fig. I. At least 6 peaks of polyribosomes can be seen. The sedimentation constants shown were obtained in a parallel run of an aliquot of the same preparation in the analytical ultracentrifuge (Spinco Model E); the figures obtained agree perfectly with those reported by Gierer (1963). RNA was extracted from the 105,000 g pellet by a 0.5 % solution of sodium dodecylsulfate, and centrifuged on a sucrose gradient after adding 50 µg of yeast transfer RNA (to serve as an internal marker of the 4S RNA fraction) (fig. 2). The optical density distribution shows essentially two peaks 26S and 16S besides the 4S peak of the added S-RNA. The distribution of radioactivity is clearly different; in addition to the 26, 16 and 4S peaks, a fairly broad band appears in the 9-10S region. The specific activity of this fraction is at least 8 times higher than that of the 26S or 16S peaks, indicating that a much larger part of it was made after the injection of radioactive phosphate. High labeling of the 4S fraction must be due to the rapid turnover of its terminal sequence (Burny and Chantrenne, 1964). The labeled 9-10S fraction was observed

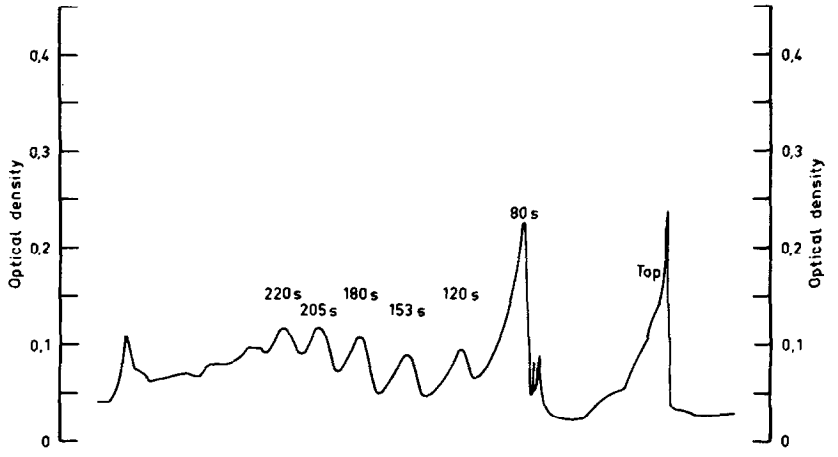

Fig. 1 : Sedimentation pattern of reticulocyte polyribosomes in 18 % - 36 % sucrose gradient (for details, see text).

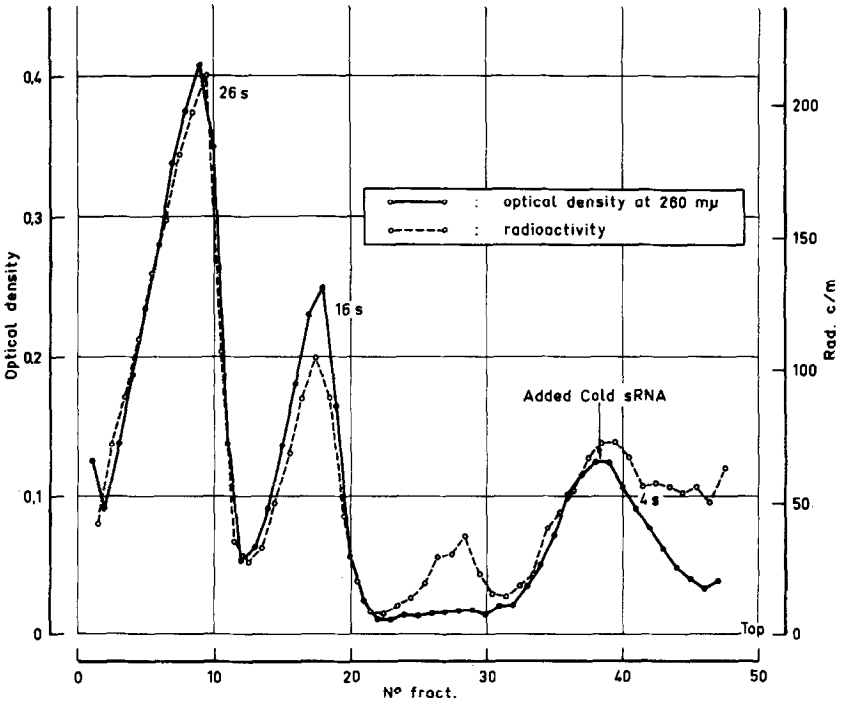

Fig. 2 : Sedimentation pattern of SDS extracted polyribosomal RNA in 5 % - 20 % sucrose gradient.

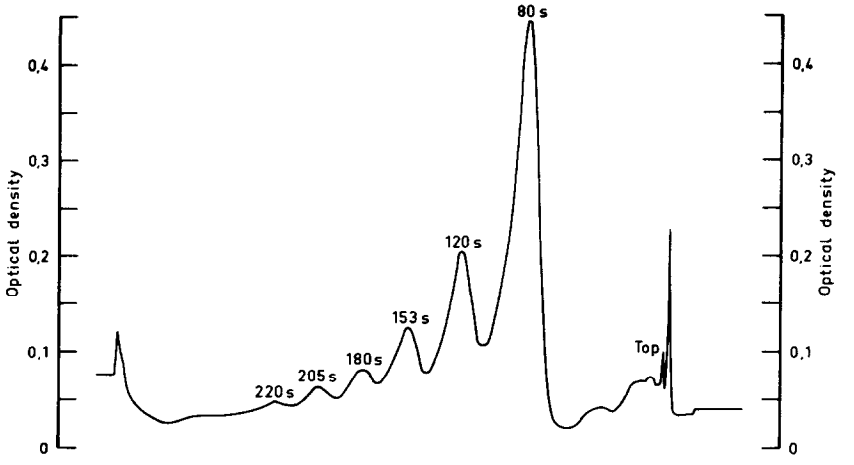

Fig. 3 : Sedimentation pattern of polyribosomes, after treatment with RNAse.

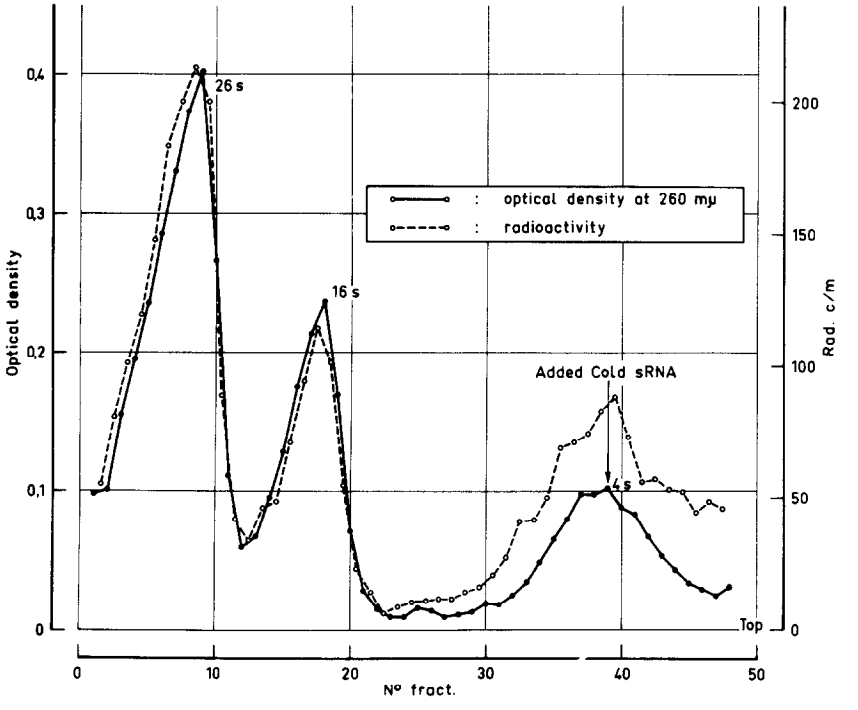

Fig. 4 : Sedimentation pattern of SDS extracted RNA from polyribosomes which have been treated with RNAse.

10 h, 15 h and 20 h after phosphate 32 injection. Figures 3 and 4 show respectively the sedimentation patterns of the cell extract and of the RNA, after treatment of the reticulocyte extract with a very small amount of pancreatic ribonuclease (0.01 µg/ml for 5 minutes at 37°). The heavy polyribosomes have almost completely disappeared whereas the amounts of 80S and 120S (dimers) have greatly increased showing that the RNA thread of the polyribosomes was broken by ribonuclease.

The sedimentation pattern of the ribosomal RNAs from this same preparation was not changed by the RNAse treatment, but the 9-10S radioactive band was eliminated, and more ^{32}P was found on the heavy side of the 4S peak.

It will be noticed that the method used for isolating the 9-10S RNA avoids any mechanical treatment likely to break the thin RNA thread of the polyribosomes. Another mild extraction procedure (Barlow et al. 1963) was compared with the present method; most of the highly labeled 9-10S RNA fraction remained in solution after Li Cl treatment, together with transfer RNA.

DISCUSSION AND CONCLUSION.

The 9-10S peak is made of RNA since it is destroyed by pancreatic RNAse. This RNA must be largely unprotected for it is extremely sensitive to the action of this nuclease; a short treatment by 0.01 µg/ml RNAse is sufficient to destroy it. This RNAse treatment also results in the destruction of the polyribosome structure, leaving free ribosomes (80S and 120S). The 9-10S RNA peak therefore must be made of the thread-like RNA to which the ribosomes were attached.

The higher specific activity of this fraction as compared to that of ribosomal RNA proves either that a larger fraction of it than of the ribosomal RNA was made after ^{32}P injection, or that it was turning over faster. This RNA fraction therefore has the properties ascribed to messenger RNA (Jacob et Monod, 1961). A sedimentation constant 9-10S corresponds to a molecular weight in the range of 160,000 for single stranded RNA (Gierer, 1958). Such an RNA would code for a 160 amino acids chain, assuming a coding ratio of 3 and is therefore approximatively the size needed for the synthesis of one hemoglobin chain.

Stimulation of amino acid incorporation by this RNA is presently being studied.

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