COMPARISON OF ACTIVITY AND PROTEIN CONTENT OF RIBOSOMAL SUBUNITS PREPARED BY FOUR DIFFERENT METHODS FROM RABBIT RETICULOCYTES

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1. Introduction

Two functionally different methods have been commonly used to prepare ribosomal subunits for structural and functional studies. In one method polyribosomes are converted into single ribosomes by the 'natural process' of polypeptide chain elongation and termination, followed by dissociation into subunits by high salt treatment [1]. In the other method polyribosomes are incubated at high ionic strength with puromycin to cause premature polypeptide chain release, detachment of the ribosomes from the mRNA and dissociation into subunits [2]. These two methods and modifications of them are used interchangeably by various investigators; thus, we thought it important to compare the resultant subunits.

We also report here a comparison of the subunits obtained by dissociation of purified single (80 S) ribosomes and the free 40 S subunits which exist in cell lysates. These free 40 S subunits isolated under low ionic conditions from different types of cells are associated with proteins having initiation factor activity [3]. These proteins can be selectively removed by treating the subunits with high salt. However, no investigation of the resultant washed free 40 S subunits has been reported.

In this study we isolated polyribosomes, single ribosomes and free 40 S ribosomes from rabbit reticulocytes using low ionic conditions. Subunits were prepared as listed above and then compared in terms of recovery, specific protein content and activity in a fractionated cell-free system for globin synthesis.

2. Experimental

2.1. *Preparation of rabbit reticulocytes and their ribosomes*

Rabbit reticulocytes were prepared as previously described [4], except that cell lysis was stopped by restoring isotonicity with sucrose (0.27 M final) in the presence of 4 mM $MgCl_2$ and absence of KCl. Polyribosomes were purified from a postmitochondrial supernatant by centrifugation through sucrose cushions [1, 4], containing Buffer A (0.03 M KCl, 0.002 M $MgCl_2$, 0.02 M Tris–HCl, pH 7.6, and 0.001 M dithiothreitol); omitting the deoxycholate treatment. Analytical sucrose gradients show that the polyribosome preparation is devoid of free subunits and contains only 10% single ribosomes (fig. 1A).

The single ribosomes and free subunits in the supernatant above the polyribosome pellet were sedimented by centrifugation, resuspended in Buffer A, then separated in 35 ml 0.35 M to 1.05 M convex ex-

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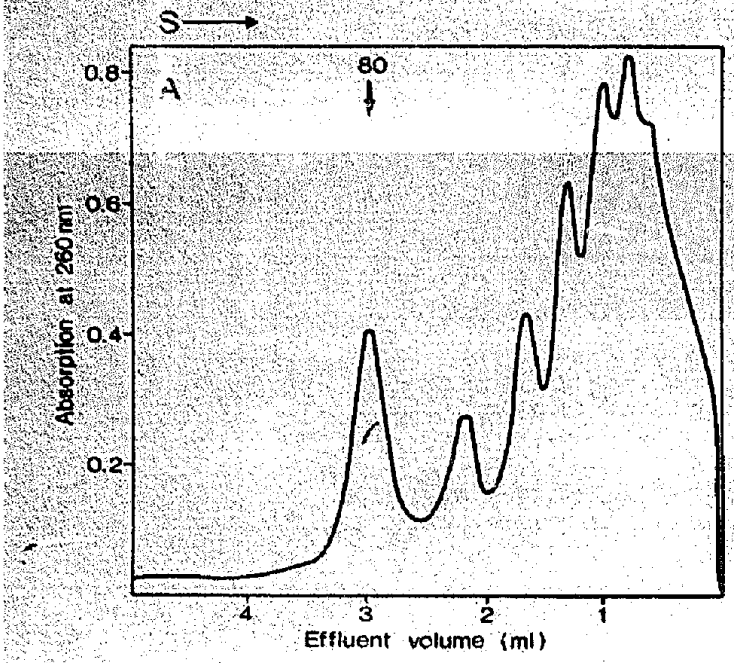

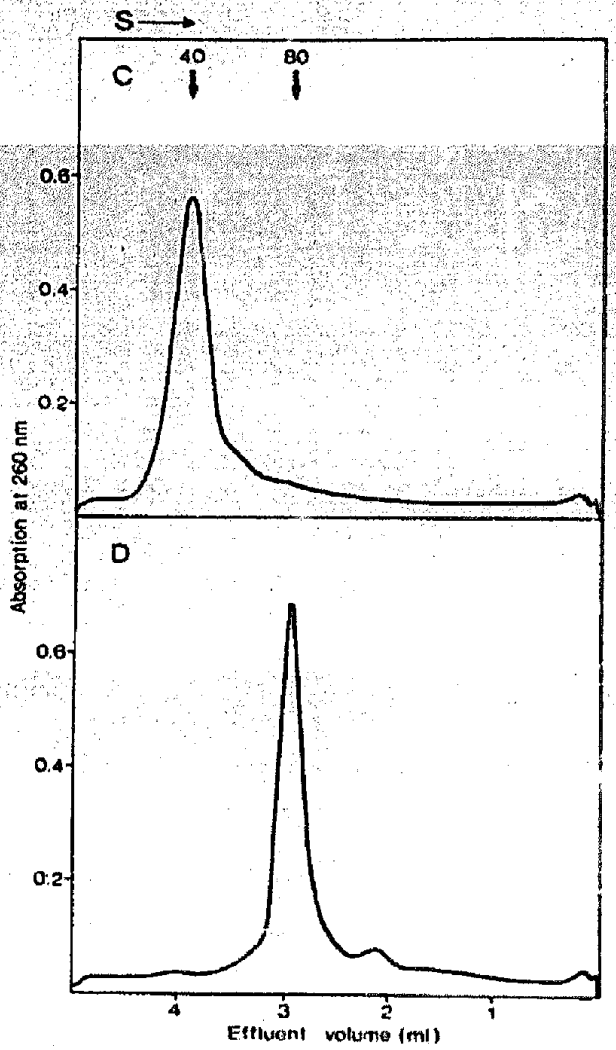

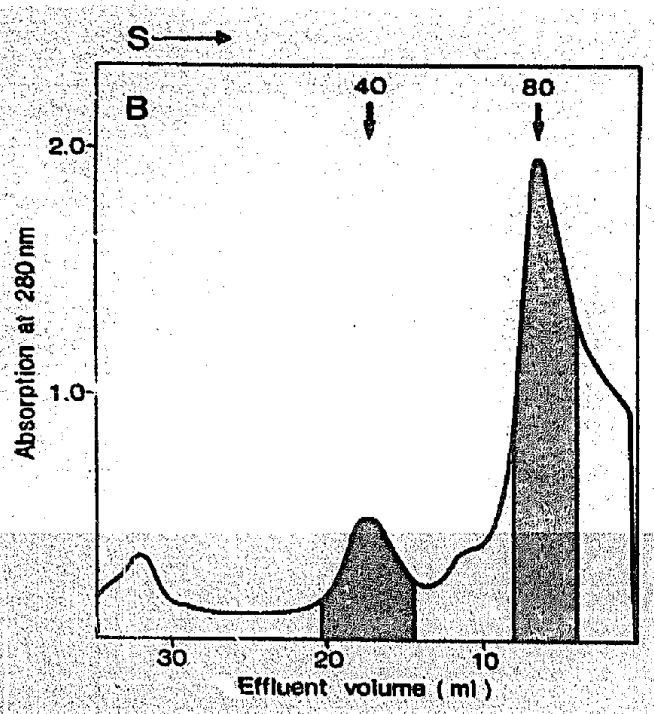

Fig. 1(A) Sucrose density gradient sedimentation pattern of a polyribosome preparation. Analysis was done on 5 ml 0.3 M to 1.5 M concave exponential sucrose gradients in 0.1 M KCl, 0.005 M $MgCl_2$, 0.02 mM Tris–HCl, pH 7.6, as previously described [4]. (B) Preparative sucrose gradient pattern of preparation of free 40 S subunits and single (80 S) ribosomes. The method as described in the text. (C) and (D) Analysis of the purity of the free 40 S subunits and the single (80 S) ribosomes; using the same type of gradient as described in (A).

ponential sucrose gradients in Buffer A [4]. The 40 S and 80 S ribosome fractions were collected as indicated by the shaded areas in fig. 1B, pelleted by centrifugation and resuspended in Buffer A. Analytical sucrose gradients show the purity of the fractions pooled (fig. 1C, D). The ribosome pattern shown in fig. 1B appears deficient in 60 S subunits compared to 40 S subunits. However, if the ribosomes are prepared using higher ionic conditions (e.g., 0.1 M KCl and 0.005 M $MgCl_2$) this is not the case (data not shown). 60 S sub-

units seem to be predominantly aggregated under the low ionic conditions used in the experiment related to fig. 1B; thus, no further attempt was made to isolate them.

2.2. *Preparation of ribosomal subunits*

'Run-off' subunits were prepared from the polyribosome fraction (fig. 1A) essentially as described by Falvey and Staehelin [1]. The subunits were separated by centrifugation on sucrose gradients [4] in Buffer B (0.5 M KCl, 0.003 M $MgCl_2$, 0.02 M Tris–HCl, pH 7.6, 0.001 M dithiothreitol). The separated subunits were pelleted by centrifugation, then resuspended in 0.1 M KCl, 0.004 M $MgCl_2$, 0.02 M Tris–HCl, pH 7.6, 0.001 M dithiothreitol, at concentrations of 140 A_{260} units/ml and 50 A_{260} units/ml for the 60 S and 40 S subunits, respectively. These aliquots were quick-frozen in liquid N_2, then stored at −80°C.

'Puromycin' subunits were prepared from the polyribosome fraction (fig. 1A) using a modification of the method of Blobel and Sabatini [2]. Polyribosomes in Buffer B (50 A_{260}/ml) were incubated for 30 min at 37°C with 2 mM puromycin and 2 mM GTP; then isolated by sucrose gradient centrifugation and pelleting as described above.

'80 S-derived' subunits were prepared from the single ribosome preparation (fig. 1B) by incubation of the ribosomes (90 A_{260}/ml) for 10 min at 37°C in Buffer B; then isolated as described above.

'Washed free-40 S' subunits were prepared by incubation of free 40 S subunits (fig. 1B) (50 A_{260}/ml) for 15 min at 4°C under continuous stirring in a solution containing 0.5 M KCl, 0.008 M $MgCl_2$, 0.02 M Tris–HCl, pH 7.6, 0.001 M dithiothreitol, and 0.0002 M EDTA; then isolated as described above.

Both 'Run-off' and 'Puromycin' subunits obtained from polyribosomes, as well as '80 S-derived' subunits, all gave reproducible recoveries of about 60% of the indicated starting material. The A_{260} ratio between 60 S subunits and 40 S subunits is about 2.5. The sucrose gradient fractions were pooled in narrow regions to eliminate cross-contamination of subunits; hence the relatively small yield.

2.3. *Cell-free globin synthesis*

In vitro rabbit globin synthesis in a fractionated cell-free system was done according to Schreier and Staehelin [4], using an 'A-fraction' obtained from reticulocyte ribosomes as a source of initiation factors.

2.4. *Two-dimensional electrophoresis*

Proteins from the various subunits, prepared according to Spitnik-Elson [5], were separated via two-dimensional polyacrylamide gel electrophoresis as previously described [6, 7], with minor modifications [8]. See the legend to fig. 3 for details of the electrophoresis conditions.

3. Results

The activities of the differently prepared subunits were tested in a partially purified cell-free system for the synthesis of rabbit globin [4]. Fig. 2 shows the

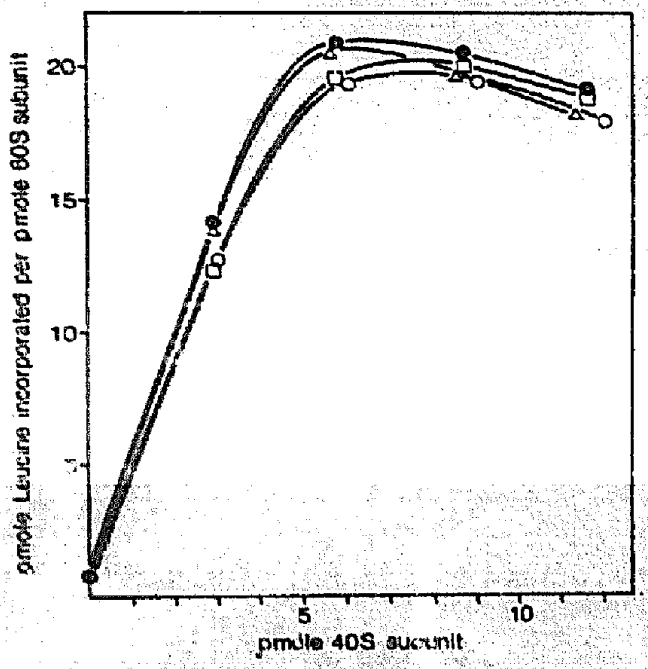

Fig. 2. Comparison of the activities of different 40 S subunits in cell-free globin synthesis. 40 S subunits were added in amounts indicated on the abscissa to 0.1 ml incubation mixtures, as previously described [4], containing 0.32 A_{260} units (8 pmoles) of 'run-off' 60 S subunits. One A_{260} unit of 60S subunits is assumed to be 25 pmoles, and 1.0 A_{260} unit of 40 S subunits is assumed to be 63 pmoles. (○ —○) 'Run-off' 40 S, (● —●) 'Puromycin' 40 S (▲ —▲) '80 S-derived' 40 S, and (□ —□) 'washed free' 40 S subunits.

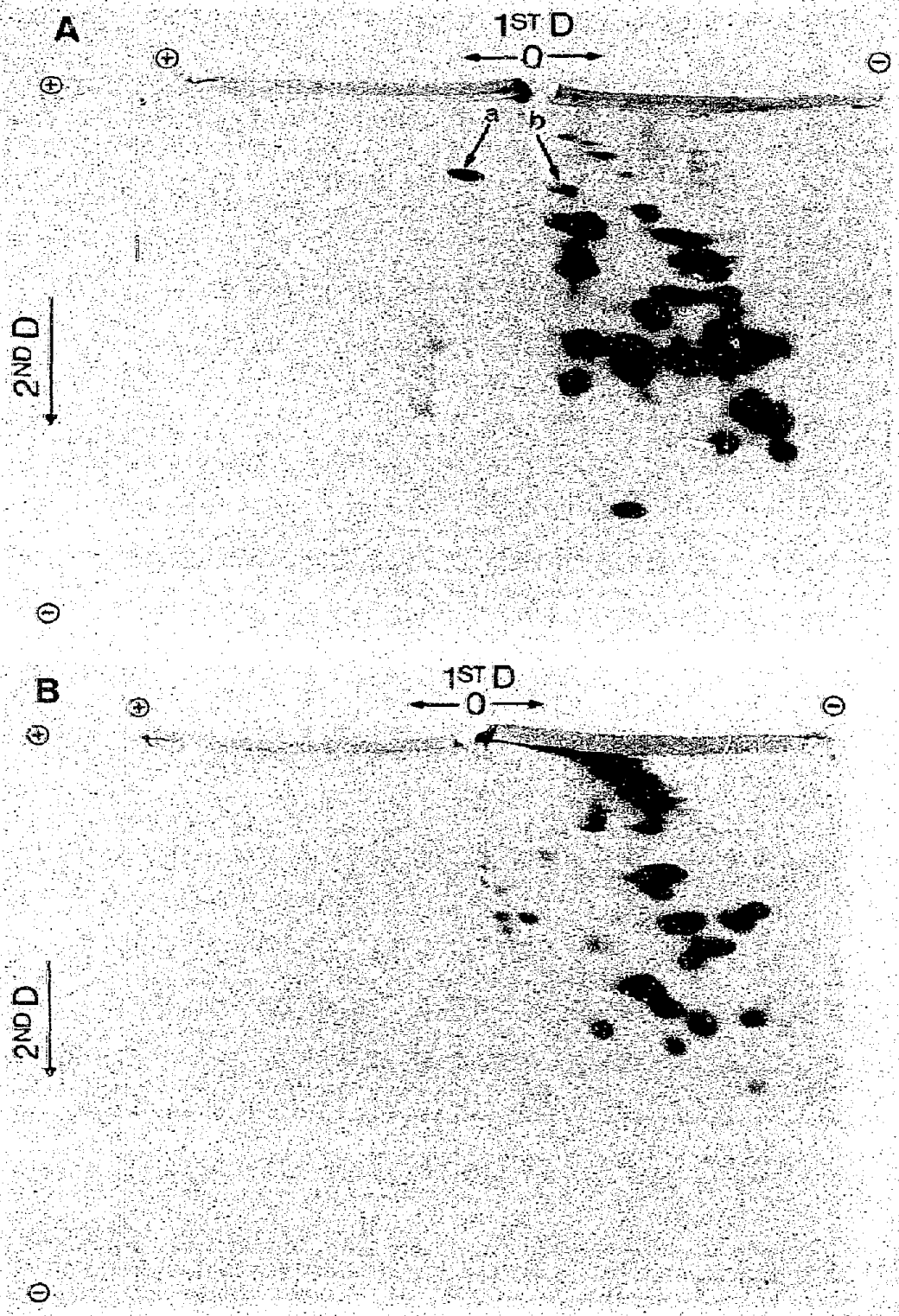

Fig. 3. Two-dimensional electropherograms of ribosomal proteins from (A) 40 S (washed-free) subunits and (B) 60 S (run-off) subunits of rabbit reticulocytes. Sample load was approximately 5 μg protein/stained spot. Electrophoresis time, 1st-D: 3.5 hr at 4 mA/gel; 2nd-D: 7 hr at 150 V/gel (room temperature in both cases). The arrows indicate protein differences found in the various 40 S subunit preparations.

synthetic activities of the different 40 S subunits when titrated into a system containing 'Run-off' 60 S subunits. No significant differences in the activities are obtained among the various 40 S subunits. The same experiments with 'Puromycin' or '80 S-derived' 60 S subunits gave similar results (data not shown), suggesting that the activities of the three preparations of 60 S subunits are not significantly different either.

Total proteins extracted from each of the different subunits were separated into their individual components on two dimensional acrylamide gels. Fig. 3A and B show the patterns obtained from separation of proteins from 40 S and 60 S subunits, respectively. The specific 40 S electropherogram shown (fig. 3A) was from a preparation of 'washed free 40 S' subunits.

Two protein spots were shown to be different between this preparation and the other methods of subunit preparation (i.e., puromycin treatment, natural run-off, or 80 S-derived, see Experimental Section). The spot marked 'a' was present in larger amounts in the preparation shown than in the other preparations, as judged by relative stain intensity; and the spot marked 'b' was present only on gels of 'washed-free 40 S' subunits as shown.

The pattern shown of the electropherogram of the 60 S subunits (fig. 3B) was obtained using a preparation of 'Run-off' 60 S subunits. The various other methods of preparing 60 S subunits (see Experimental) all yielded identical patterns to that shown.

4. Discussion

Efforts have been made to develop and optimize the preparation of active eucaryotic ribosomal subunits [1, 2, 4]. Active subunits can be obtained from all three populations of ribosomes in the cell: polysomes, single ribosomes and subunits. Each of the methods described here have their assets and liabilities. Preparation of derived subunits from single ribosomes, and especially free subunits, give very low yields, since they represent only a small part of the whole ribosome population [9]. For preparation of large amounts of subunits either the 'run-off' method or the 'puromycin' method can be used. The 'run-off' method is probably most similar to the in vivo situation, but requires addition of a rather undefined group of factors (pH 5 enzymes) to the system. The 'puromycin' method alleviates this problem, but introduces an artificial reaction in the seemingly natural sequence of reactions by causing premature peptide chain release and subsequent ribosome release from the mRNA. Both methods give structurally and functionally equivalent subunits. These results indicate for the first time that the proteins obtained from these two very different methods of preparation are apparently *bona fide* ribosomal proteins, and not artifacts of one method or another.

The 'free 40 S' subunits after treatment with 0.5 M KCl and high Mg^{2+} concentration are functionally equivalent to the other types of 40 S subunits if tested in this rather crude fractionated system. However, they show two proteins in significant amounts on the gel electropherogram that are either not present or reduced on the other types of subunits. Since the free 40 S subunits in the cell presumably are in a stage of polypeptide chain initiation [10, 11], it may be interesting to study the functional role of these two proteins in a more purified system.

Acknowledgements

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