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CHROMATOGRAPHIC METHOD FOR THE ISOLATION OF ACTIVE 40S AND 60S SUBUNITS FROM RAT LIVER POLYRIBOSOMES

TERESA KOPACZ-JODCZYK, ANNA PASZKIEWICZ-GADEK, WŁODZIMIERZ ŁOPACZYŃSKI and WŁADYSŁAW GAŁASIŃSKI*

Department of Organic Chemistry, Institute of Chemistry and Biophysics, Medical School, 15-230 Bialystok 8 (Poland)

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SUMMARY

A rapid and simple procedure for isolation of 40S and 60S ribosomal subunits by ion-exchange column chromatography is described. The dissociated ribosomes can be separated and non-ribosomal proteins and low-molecular-weight substances removed. An assessment by physicochemical and functional criteria showed that the ribosomal subunits obtained are active and sufficiently homogeneous.

INTRODUCTION

The first isolation of eukaryotic, active ribosome subunits was described by Martin and Wool[1]. Since then many methods for the isolation of ribosomal subunits have been published. With a few exceptions[2–4], these methods are based on zonal ultracentrifugation in a sucrose gradient. A critical evaluation of the sedimentation methods has revealed the disadvantage that artefacts sometimes appear in the separation procedure[5].

We have therefore developed an alternative chromatographic method in which, as described in the preliminary report of Hamilton and Sandals[6], DEAE-Sephadex A-25 was used for the separation of eukaryotic ribosomal subunits. Under the conditions previously suggested[6], 40S ribosomal subunits are contaminated by puromycin, which prevents them being employed for activity determination after their reasociation with 60S subunits. In our modification, puromycin is completely discarded together with non-ribosomal proteins (ferritin, nucleotide pyrophosphatases) in the fraction eluted with the same buffer used for column equilibration. A relatively simple concentration of the fractions of separated ribosomal subunits using polyethylene glycol does not result in losses of their activities.

EXPERIMENTAL

Chemicals

DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals (Upp-

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sala, Sweden), polyethylene glycol 6000, tris(hydroxymethyl)aminomethane(tris) and 2-mercaptoethanol from Fluka (Buchs, Switzerland). Puromycin dihydrochloride was obtained from Nutritional Biochemicals (Cleveland, OH, U.S.A.), sucrose (RNA-ase-free) from BDH (U.K.) and GTP trilithium salt dihydrate from Calbiochem (San Diego, CA, U.S.A.). Poly (U) potassium salt was from Boehringer (Mannheim, F.R.G.), dithiothreitol from Loba-Chemie (Wien-Fischamend, Austria). [^{14}C]Phenylalanyl-tRNA was a gift from the Department of Molecular Biology UMCS, Lublin, Poland. All the other chemicals were of the highest grade commercially available.

Preparation of ribosomes

The polyribosomes were isolated from rat liver according to Moldave and Skogerson[7], then purified twice by ultracentrifugation (44,000 *g* for 20 h) through two layers of 1 *M* and 0.5 *M* sucrose, respectively, which contained 0.1 *M* Tris–HCl (pH 7.6), 0.01 *M* $MgCl_2$ and 0.5 *M* NH_4Cl. The purity of the ribosomes was estimated according to Peterman[8]. The ribosomal particle concentration was evaluated from the absorbance at 260 nm.

The dissociation of the ribosomes was performed according to Blobel and Sabatani[9].

Chromatographic separation of ribosomal subunits

For routine preparation of the subunits, 10 mg of dissociated ribosomes in a volume of 0.5 ml of buffered solution[9] (20 m*M* Tris–HCl pH 7.6, 5 m*M* $MgCl_2$, 0.5 *M* KCl and 1 m*M* puromycin) were applied to the DEAE-Sephadex A-25 column (1.5 × 28 cm), previously equilibrated with 20 m*M* Tris–HCl buffer (pH 7.6) containing 0.3 *M* KCl and 5 m*M* $MgCl_2$. The elution was carried out with the same buffer and a discontinuous gradient of KCl (0.3, 0.5, 0.7 *M*), at a flow-rate of *ca.* 0.7 ml/min and 2.2-ml fractions were collected. The whole chromatographic procedure was performed at room temperature.

Samples (0.2 ml) of each column fraction were diluted ten-fold in an elution buffer and their absorbances at 260, 280 and 230 nm were measured. The fractions from the peak of the 40S or of the 60S subunits were pooled and concentrated with polyethylene glycol 6000, then stored at −20°C.

Purity of the ribosomal subunits

The nature and purity of the ribosomal components separated by column chromatography were assessed by sucrose gradient analysis as described by Dickman and Bruenger[10].

Activity of the subunits and reassociated ribosomes

The biological activity of the ribosomal subunits was assayed in a poly (U)-dependent Phe-tRNA binding system.

The incubation mixture, total volume 0.5 ml, contained: 50 m*M* Tris–HCl, pH 7.5; 50 m*M* KCl; 6 m*M* $MgCl_2$; 200 μg poly (U); 0.2 m*M* GTP; 2 m*M* dithiothreitol; 17 μg EF-1 purified on hydroxyapatite; 3.6 nmol (9700 cpm) [^{14}C]Phe-tRNA; isolated ribosomal subunits or polyribosomes. The incubation was carried out at 37°C for 15 min. The incubation mixture was then chilled in an ice–water bath and diluted in

2.5 ml of cold buffer, 33 m*M* Tris–HCl (pH 7.5), 67 m*M* KCl and 6.6 m*M* $MgCl_2$. The ribosomes were adsorbed on HA 0.45-μm filters (Millipore) then washed twice with 3 ml of the buffer and dried. The radioactivity retained on the filters was measured in 5 ml of scintillation liquid, toluene–0.4% 2,5-diphenyloxazole(PPO)–0.01% 1,4-bis(5-phenyloxazolyl-2)benzene(POPOP), in a liquid scintillation counter Isocap-300 (Nuclear Chicago).

The activity of the reassociated ribosomes in polyphenylalanine synthesis was investigated in samples of 1 ml which contained: 50 m*M* Tris–HCl, pH 7.6; 6 m*M* $MgCl_2$; 80 m*M* NH_2Cl; 0.2 m*M* GTP; 2 m*M* dithiothreitol; purified elongation factors; 22 μg EF-1 + 28 μg EF-2; 3.6 nmol (9700 cpm) [^{14}C]Phe-tRNA and ribosomes. For the reassociation, subunit fractions were combined in a 40S/60S ratio of *ca.* 1/2.5

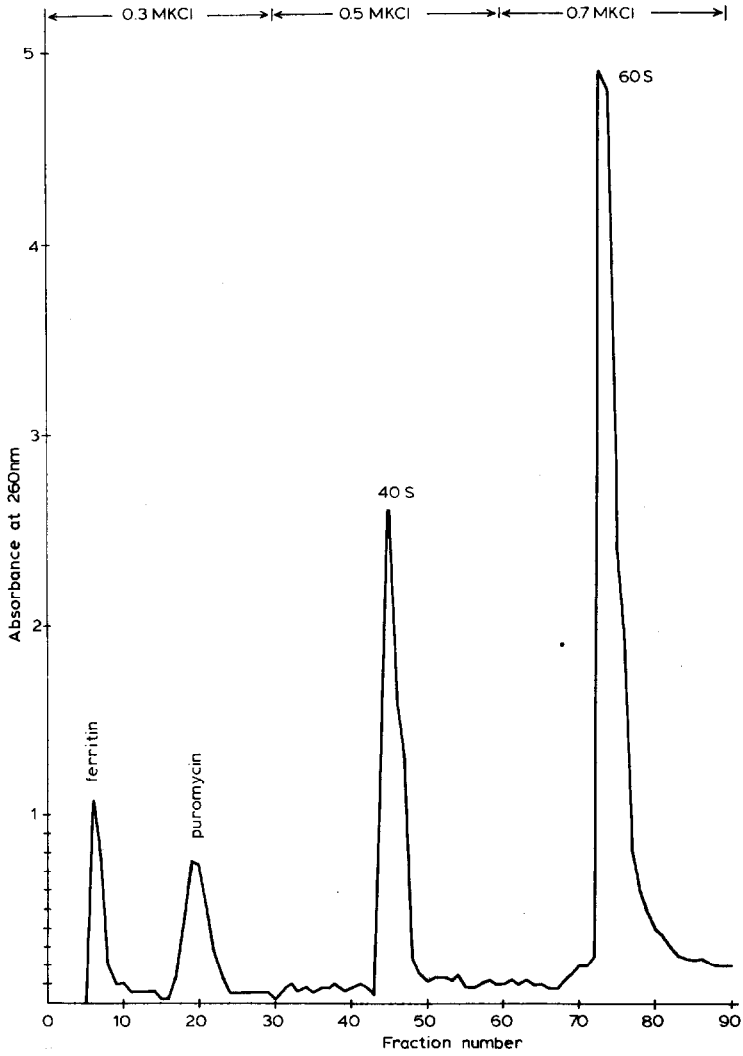

Fig. 1. The separation of ribosomal subunits on a DEAE-Sephadex A-25 column. About 120 A_{260} units of dissociated ribosomes were applied and eluted as described under Experimental.

A_{260}, that is the proportion in which they form 80S ribosomes[11]. The mixtures were preincubated at 37°C for 10 min, then to 200 μg of poly (U) were added the samples and the incubation was continued for 30 min. The incubation was stopped by adding 1 ml of 10% trichloroacetic acid, and boiling on a water-bath for 15 min. After cooling, the samples were filtered through glass fibre filters, washed four times with 5% trichloroacetic acid and dried. The radioactivity retained on the filters was measured as described above.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of rat liver ribosomes, previously dissociated into subunits in buffer, which contained 0.5 *M* KCl and 1 m*M* puromycin. The same buffer without puromycin, but containing 0.3 *M* KCl, was used for the column equilibration and for elution of the first peak, non-ribosomal, brown protein-ferritin. Ferritin was identified by its A_{260nm}/A_{320nm} ratio of 1.51[12]. The second peak, which was eluted with the same concentration of KCl, was identified spectrophotometrically as the puromycin previously used for the ribosome dissociation. The next peak, eluted at 0.5 *M* KCl, contained only 40S subunits whilst 60S subunits were eluted as a sharp peak with 0.7 *M* KCl. The buffer containing 0.6 *M* KCl did not completely elute 60S subunits and the resulting peak was very flat.

It seems that in order to obtain a good separation of the subunits it is essential that the appropriate concentration of KCl be maintained in the elution buffer. Other important factors are the length of time the samples are kept on the column and the time of elution with the various solutions of the gradient. When the elution with 0.3 *M* KCl was protracted to give 40 fractions, *ca.* 88 ml, the A_{260} ratio of the material eluted in the 40S and 60S peaks was 1/4. Further protraction of the elution with this solution to give over 50 fractions, *ca.* 110 ml of eluate, resulted in no separation of the subunits and the total ribosomal materials was eluted in one peak only when 0.7 *M* KCl was applied. It seems probable that if the subunits are kept too long in 0.3 *M* KCl they may be reassociated. This was confirmed by an experiment in which the non-dissociated ribosomes were eluted with the gradient usually applied; most of the investigated material was eluted at 0.7 *M* KCl as a peak shifted about 6–8 fractions later with respect to that containing 60S subunits.

Our experimental data show that subunits obtained from 10 mg of ribosomes can be separated on a column of 1.5 × 28 cm, with a recovery of about 60%, as measured by $A_{260\ nm}$. The ratio of the 40S and 60S subunits eluted from the column is 1/2.2–1/2.8 A_{260}. Rechromatography of both 40S and 60S peaks revealed insignificant mutual contamination. The purity of the polyribosomes was assessed by determination of the absorbances at 235, 260 and 280 nm[8]. The ribosomal preparations having absorbance ratios, A_{260}/A_{235} and A_{260}/A_{280}, of 1.16 and 1.51, respectively, were also used for isolation of the subunits; lower ratios generally indicate the presence of impurities, but these did not interfere with the separation of the subunits on the DEAE-Sephadex A-25 column. In order to estimate the degree of contamination of the separated subunits, a sedimentation analysis on a sucrose density gradient was performed. The results presented in Fig. 2 show that the fractions of 40S and 60S subunits have insignificant or no mutual contamination. This was also confirmed by other experiments, in which the activity of separated and isolated ribosomal subunits was assayed.

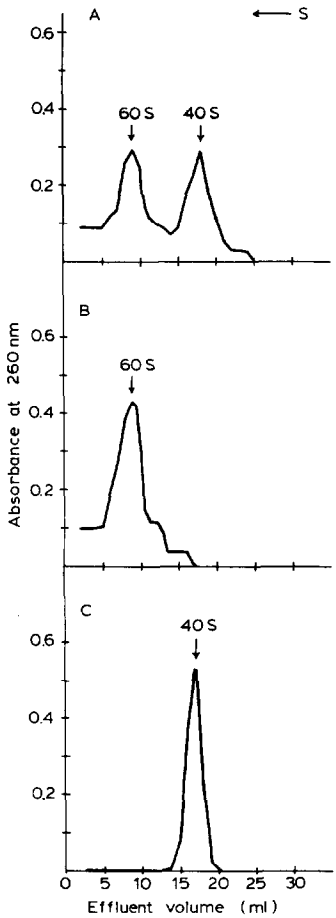

Fig. 2. Sucrose density gradient analysis of 40S and 60S ribosomal subunits. The peak fractions presented in Fig. 1 were collected and samples of 2 ml were layered on top of the linear sucrose density gradient, 10–30% (30 ml), which contained 10 m*M* Tris–HCl (pH 7.4), 3 m*M* $MgCl_2$, 0.5 *M* KCl and 10 m*M* 2-mercaptoethanol. Centrifugation was carried out at 25,000 rpm in a VAC 601 ultracentrifuge for 14 h at 4°C, using a SW 25 rotor. Fractions of 1 ml were collected and their absorbance at 260 nm was measured. Measurements were carried out against nine blank gradient solutions that were parallelly ultracentrifuged without ribosomal subunits. A, Mixture of 3 A_{260} units of 40S fraction and 5 A_{260} units of 60S fraction. B, 5 A_{260} units of 60S fraction; C, 5 A_{260} units of 40S fraction.

From Table I it is seen that 40S subunits bind [^{14}C]Phe-tRNA, while 60S subunits do not.

The ability of reassociated subunits to synthesize polyphenylalanine was investigated. Table II shows that the combined fractions of 40S and 60S subunits, in the presence of poly (U), were very active in comparison with the subunits with no poly (U) added. Despite the higher activity of polysomes, in the presence of poly(U), it can be assumed that most of the chromatographically separated subunits participated in the polypeptide synthesis.

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TABLE I

ACTIVITY OF RIBOSOME SUBUNIT FRACTIONS IN [^{14}C] Phe-tRNA BINDING DIRECTED BY POLY(U)

Incubation and preparation of samples for radioactive analysis were carried out as described in Experimental.

Ribosome species	*Amount (nmol)*	*Radioactivity* (cpm per nmol ribosomes)*	*No. of [^{14}C]Phe-tRNA bound per ribosome unit*
40S fraction	0.035	3485	1.3
60S fraction	0.040	–	–
polysomes	0.100	160	0.05

* Each sample contained 3.6 nmol (9700 cpm) [^{14}C]Phe-tRNA. Values are corrected for the control reaction mixture without EF-1.

TABLE II

ACTIVITY OF RIBOSOME SUBUNIT FRACTIONS IN SYNTHESIS OF POLYPHENYLALANINE

Ribosomes were incubated in the cell-free system as described in Experimental.

Ribosome species	*Amount (nmol)*	*Radioactivity* (cpm per nmol of ribosomes)*	*No. of Phe polymerized per limiting ribosome unit*
Polysomes	0.09	3633	1.3
Polysomes + poly(U)	0.09	12,955	4.8
40S + 60S	0.07 + 0.1	443	0.2
40S + 60S + poly(U)	0.05 + 0.1	9140	3.4

* Corrected for the control samples without elongation factors.

scribed here the possibility of contamination of the 60S subunit peak by 80S ribosomes, which are also eluted with a gradient of 0.7 *M* KCl, was taken into concentration. In order to prevent such contamination, the ribosomes were preincubated with puromycin. However, it should be borne in mind that such preincubation may partly change the structure and function of the ribosomes[13]. In spite of this, we consider that the method can serve as an useful alternative to sucrose density gradient centrifugation for obtaining relatively pure and active ribosomal subunits.

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REFERENCES

1 T. E. Martin and I. G. Wool, *Proc. Nat. Acad. Sci. U.S.*, 60 (1968) 569.
2 S. Hjertén, S. Jersted and A. Tiselius, *Anal. Biochem.*, 11 (1965) 211.
3 S. V. Kirillov, V. I. Makhano, N. N. Peshin and Yu. P. Semenkov, *Nucleic Acids Res.*, 5 (1978) 4305.

4 K. L. Manchester and J. E. Manchester, *FEBS Lett.*, 30 (1980) 15.
5 A. S. Spirin, N. V. Belitsina and E. B. Lishevskaya, *FEBS Lett.*, 24 (1972) 219.
6 M. G. Hamilton and W. O. Sandals, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 38 (1979) 227.
7 K. Moldave and L. Skogerson, *Methods Enzymol.*, 12 (1979) 478.
8 M. L. Peterman, *The Physical and Chemical Properties of Ribosomes*, American Elsevier, New York, 1964.
9 G. Blobel and D. D. Sabatini, *Proc. Nat. Acad. Sci. U.S.*, 68 (1971) 390.
10 R. Dickman and E. Bruenger, *Biochemistry*, 8 (1969) 3295.
11 T. E. Martin, E. S. Rolleston, R. B. Low and I. G. Woll, *J. Mol. Biol.*, 43 (1969) 135.
12 R. J. Jackson, A. J. Munro and A. Korner, *Biochim. Biophys. Acta*, 91 (1964) 666.
13 T. E. Martin, I. G. Wool and J. J. Castels, *Methods Enzymol.*, 20 (1971) 417.