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Separation of ribosomal subunits of Escherichia coli by Sepharose chromatography using reverse salt gradient

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ABSTRACT

A mixture of 30 S and 50 S subunits quantitatively adsorbs on a column of Sepharose–4B from the buffer: 0.02 M Tris–HCl, pH 7.5, containing 1.5 M $(NH_4)_2SO_4$. During elution by reverse gradient of ammonium sulphate (1.5 – 0.05 M) the subunits are eluted at different salt concentrations. Complete separation of subunits is attained in the absence of Mg^{2+} ions.

The 30 S subunits prepared from 70 S ribosomes according to this procedure are fully active in the codon–dependent binding of a specific aminoacyl–tRNA. After their reassociation with 50 S subunits isolated by zonal centrifugation, the resulting 70 S ribosomes are active in polypeptide synthesis at the same degree as control 70 S ribosomes in which both types of subunits were prepared by zonal centrifugation. The initial 70 S ribosomes for the chromatographic separation into subunits can be obtained by their pelleting from a crude extract with subsequent washing with concentrated solutions of NH_4Cl in the ultracentrifuge, or by salt fractionation of the crude extract according to a slightly modified procedure of Kurland [1].

INTRODUCTION

The only existing method of separation of ribosomal subunits is zonal centrifugation in a sucrose gradients. This procedure is painstaking and the devices are very expensive. At the same time the study of structure and functions of individual ribosomal components requires preparation of considerable amounts of ribosomal subunits. Therefore it is important to develop new simple and practical methods of their separation.

In this paper we describe a method of separation of ribosomal subunits from E.coli by means of adsorption chromatography on Sepharose–4B. The ribosomes are dissociated at low Mg^{2+} concentration and on the verge of precipitation at the high concentration of ammonium sulphate strongly adsorbed on Sepharose–4B. Then ribosomal subunits are eluted by a decreasing gradient of ammonium sulphate. The method is based on the difference in hydrophobic interaction of subunits with the agarose gel in the very low concentrations (or in the absence) of Mg^{2+} ions.

MATERIALS AND METHODS

^{14}C–phenylalanine (ČSSR) had a specific activity 318 mCi/mmole. Nitrocellulose filters with a pore diameter 0.4 μm were from "Synpore" (ČSSR). Poly (U) was from "Reanal" (Hungary).

Preparation and purification of 70 S ribosomes. Method I. Cells of strain E.coli MRE–600 stored in liquid N_2 were resuspended in two volumes of buffer I (0.02 M Tris–HCl, pH 7.1; 0.02 M $MgCl_2$; 0.2 M NH_4Cl; 0.001 M EDTA) and disrupted by a two–fold passage through a French press. The suspension was centrifuged 30 min at 30,000 × g and the ribosomes were precipitated from the crude extract by centrifugation during 6 h at 100,000 × g. The pellet was

resuspended in buffer I and the ribosomes were purified by centrifugation through sucrose cushion (I.I M) containing 10 mM $MgCl_2$ and 0.5 M NH_4Cl according to Staehelin et al [2].

Method II. 70 S ribosomes were obtained from the crude extract by three successive precipitations with ammonium sulphate according to Kurland [1], but the final stage of ribosome precipitation by high-speed centrifugation was omitted. As the first step, 210 g of ammonium sulphate was added slowly to the I 1 of crude extract containing 130,000 A_{260} units. After 30min stirring at 4°C the suspension was centrifuged 45 min at 5,000 × g. To the supernatant a new portion of 210 g of ammonium sulphate was added and the suspension was centrifuged again 45 min at 5,000 × g. The pellet of ribosomes was resuspended in buffer III (0.02 M Tris-HCl, pH 7.5; 0.05 M $(NH_4)_2SO_4$; 0.01 M $MgCl_2$; 0.002 M 2-mercaptoethanol) to a final volume I 1. The second and the third salt precipitations were similar to the first one with the only difference that the amount of ammonium sulphate added was decreased. In the second cycle we added at first 210 g of $(NH_4)_2SO_4$, then 175 g to the resulting supernatant, and in the third cycle – 210 g and 140 g correspondingly. The final pellet of ribosomes was resuspended in 130 ml of buffer III with concentration 535 A_{260} units/ml; it corresponds to the final yield of 4.6 g of ribosomes from 130,000 A_{260} units of initial crude extract.

Isolation of control samples of 30 S and 50 S subunits. 70 S ribosomes, purified by method I, were dialysed 12 h against buffer II (0.02 M Tris-HCl, pH 7.5; 0.001 M $MgCl_2$; 0.5 M NH_4Cl). The separation of subunits was performed in the zonal rotor at 100,000 × g, using hyperbolic sucrose gradient 7.4 – 38% [3] in buffer II. In the fractions containing pure 30 S and 50 S subunits the concentration of Mg^{2+} was increased till 10 mM and the subunits were precipitated by the addition of 0.65 volumes of 95% ethanol. The subunits were then resuspended in buffer I and stored, as well as other preparations of ribosomes, in liquid N_2.

Assay for the activity of 30 S subunits in poly (U) – dependent binding of ^{14}C-Phe-tRNA^{Phe}. The isolation of ^{14}C-Phe-tRNA^{Phe} with a content of ^{14}C-phenylalanine 1500 pmole/A_{260} unit, as well as the preparation of poly (U) with an average molecular weight 30,000 daltons, were described earlier [4,5]. Estimation of activity of 30 S subunits included the measurement of two parameters: the fraction of 30 S subunits M able to bind specific aminoacyl-tRNA, and the binding constant K_a of ^{14}C-Phe-tRNA^{Phe} with the complex 30 S · · poly (U). As we showed earlier [5], the equilibrium fraction of subunits $\bar{\nu}$, containing bound tRNA, is a function of concentration of tRNA and conforms to Langmuir's isotherm: $\bar{\nu} = M \cdot K_a \cdot C/(1 + K_a \cdot C)$. In reverse coordinates $1/\bar{\nu}$ versus $1/C$ this equation yields a straight line. Its intersection with the ordinate gives M, and the slope $-1/MK_a$ value, from here K_a is easily computed. The incubation mixtures contained in 200 μl of buffer I: 5 pmole 30 S subunits, 10 μg poly (U) and 2 – 40 pmole ^{14}C-Phe-tRNA^{Phe}. After 45 min incubation at 0° the samples were filtered through nitrocellulose filters and $\bar{\nu}$ was calculated as the ratio of ^{14}C radioactivity adsorbed on the filter (in pmoles) to the amount of 30 S subunits in the probe (in pmoles). Before the experiments the 30 S subunits were reactivated 20 min at 40° in buffer I.

Assay for polyphenylalanine synthesis. The incubation mixtures contained in 570 μl of buffer I: 20 pmoles 30 S subunits, 30 pmoles 50 S subunits, 50 μg poly (U), 860 pmoles ^{14}C-Phe-tRNA^{Phe}, 25 μg EF-G factor, 20 μg EF-T_u-T_s factors and GTP with a final concentration I mM. For measurements of the kinetics of peptide synthesis the tempera-

ture of mixtures was rised till 30° and aliquots of 57 μl were withdrawn at indicated times. Then TCA with a final concentration 5% was added, and samples were heated 15 min at 95° with the following cooling and filtration through nitrocellulose filters. The level of polyphenylalanine synthesis was estimated as the average number of phenylalanine residues (n) incorporated into hot TCA insoluble precipitate per one ribosome. The factors of translation EF–G and EF–T_u–T_s were isolated according to Arai et al [6].

Two–Dimensional electrophoresis. The proteins from 30 S subunits were extracted by 66% acetic acid in the presence of 0.1 M $MgCl_2$ as was described by Hardy et al [7]. After two–fold precipitation by 5 volumes of acetone [8], the total protein was dissolved in 6 M urea with a final concentration 2 mg/ml. For the two–dimensional electrophoresis a slightly modified procedure of Welfle [9] was used. For separation in the first dimension, 80 μg of total protein was applied to a gel rod (13 × 0.3 cm; 5% acrylamide and 0.27% bisacrylamide), and electrophoresis was continued for 4 h at pH 8.3 with a current 5 ma/tube. After the run, gel rods were removed from tubes and placed about 1 cm from the top of plates in apparatus. Then the space between plates was filled with the solution for the second dimension (18% acrylamide and 0.25% bisacrylamide) and, after polymerization, electrophoresis was continued for 18 h at pH 4.5 with the current 60 ma/slab. Finally the slabs (13 × 13 × 0.3 cm) were stained overnight with 0.002% Coumassie Brilliant Blue G–250 in 3% TCA.

RESULTS

Chromatography of isolated 30 S and 50 S subunits on Sepharose–4B in different ionic media. All experiments in this paper were performed in two stages, both at 5°C. In the first one subunits were transfered, by gelfiltration through a column of Sephadex G–50, in a buffer: 0.02M Tris–HCl, pH 7.5 with desired concentrations of Mg^{2+} and $(NH_4)_2SO_4$. The second stage was the adsorption chromatography on Sepharose–4B. The concentration of Mg^{2+} in this stage was the same as in first one while the concentration of $(NH_4)_2SO_4$ was changed as described below.

In the first stage 30 mg of subunits in 2 ml were applied to the columns of Sephadex G–50, (60 × 1.2 cm). To the fractions eluted from the columns without retention volume and containing ribosomes, ammonium sulphate was added to a final concentration 1.5 M. After that ribosomes were applied to the columns of Sepharose–4B equilibrated with the buffer: 0.02 M Tris–HCl, pH 7.5, containing 1.5 M $(NH_4)_2SO_4$ and Mg^{2+} in concentration chosen for this particular experiment. Elution was effected by 3 volumes of the same buffer but with a linear reverse gradient of ammonium sulphate 1.5 – 0.05 M. For the reason of conviniency we plotted on the abscissa not the number of fractions eluted from the columns but the refractometric indexes corresponding to the concentrations of salt in each fraction. In fig. 1A the data are presented for the elution of 30 S subunits alternatively in buffers with 0; 0.05; 0.5; 5 mM Mg^{2+}. The concentration of ammonium sulphate in the first stage of these experiments was the same – 0.6 M. We see that in all cases the subunits are quantitatively adsorbed on Sepharose–4B at 1.5 M $(NH_4)_2SO_4$ and eluted at a reduced salt concentration, always at n_D^{20} = 1.3610 (this corresponds to 1.2 M ammonium sulphate).

The elution of 50 S subunits (fig. 1B) proceeds in a different way: its elution profiles depend strongly on the concentration of Mg^{2+} ions. Three discrete forms of 50 S subunits can be distinguished: the form 1 with n_D^{20} = 1.3580, the form 2 with n_D^{20} = 1.3510 and form

3 with $n_D^{20} = 1.3410$.

In fig.2 the data are presented where the Mg^{2+} concentration was 0.05 mM through all experiments, but the concentrations of ammonium sulphate were different in the first stages. When the concentration of ammonium sulphate during the gelfiltration is reduced up to 0.2 M, the peak of 30 S subunits is spread but its elution proceeds at the same n_D^{20} (fig.2A). For the 50 S subunits (fig.2B) we observe the same three forms as in fig.1B. We see that the decreasing of salt concentration acts in the same direction as the decreasing of concentration of Mg^{2+}: it promotes the transition of form 1 into forms 2 and 3. Analysing these results we can conclude that a full separation of 30 S from 50 S subunits is possible when the latter exist in the forms 2 or 3. For this purpose a concentration of Mg^{2+} not exceeding 0.05 mM, and $(NH_4)_2SO_4$

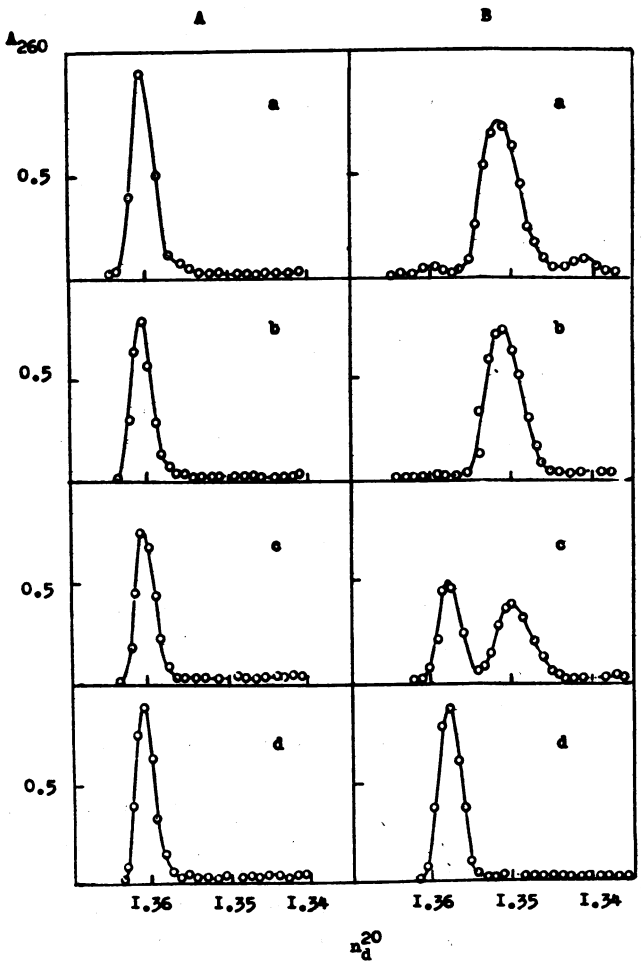

Fig.1. Chromatography of isolated 30 S and 50 S subunits on Sepharose—4B at different concentrations of Mg^{2+}. (For details see the text). a – 0 mM^{2+}; b – 0.05 mM; c – 0.5 mM; d – 5 mM. A – experiments with 30 S subunits, B – with 50 S subunits.

concentration in the range of 0.6 – 0.8 M are needed at the first stage of separation procedure.

Separation of 70 S ribosomes into subunits by chromatography on Sepharose–4B. This experiment was performed as follows. The concentration of ammonium sulphate was 0.6 M during gelfiltration, and Mg^{2+} was absent in both stages.

As can be seen from fig.3, during elution by a reverse salt gradient the ribosomal subunits are fractionated into three peaks . Peak 1 (n_D^{20} = 1.3610) contains pure 30 S subunits – it was proved by both the measurement of their sedimentation constant (fig.4B, a) and analysis for the content of 16 S ribosomal RNA (fig.4A, a). Two–dimensional electrophoresis of proteins

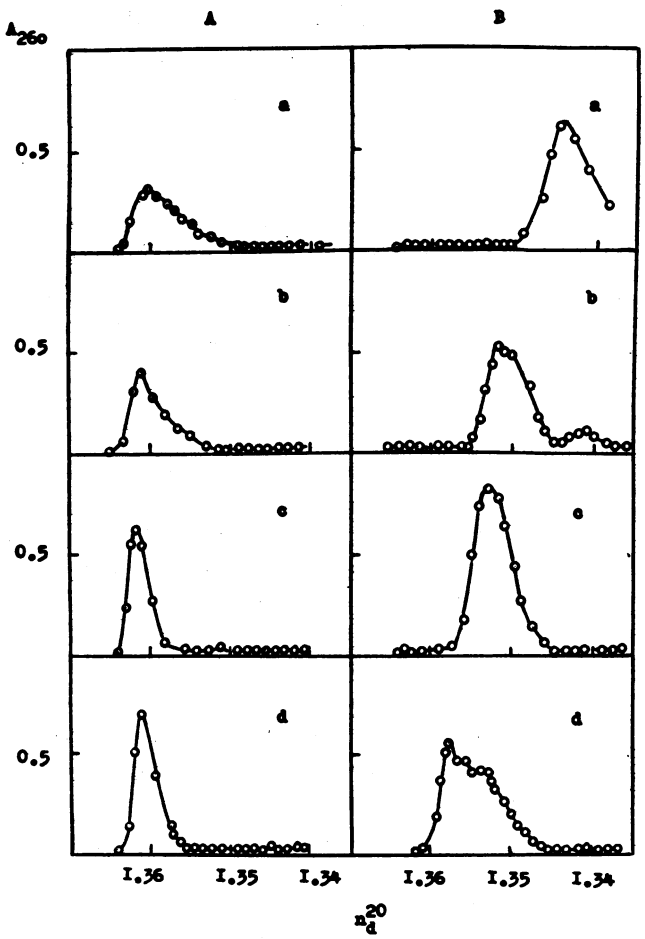

Fig.2. Chromatography of isolated 30 S and 50 S subunits on Sepharose–4B at 0.05 mM magnesium ions. The concentration of ammonium sulphate during the first stages (gelfiltration through Sephadex G–50 columns) of experiments was: a – 0.2 M; b – 0.4 M; c – 0.6 M; d – 0.8 M (in the experiment with 50 S subunits – 1.2 M). A – experiments with 30 S subunits. B – with 50 S subunits.

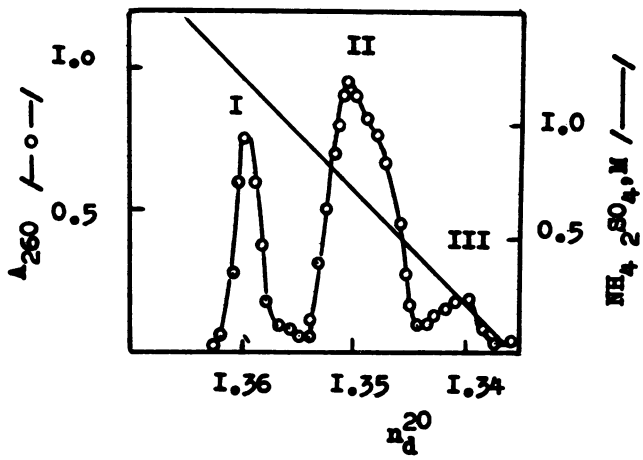

Fig.3. Chromatography of ribosomes on Sepharose–4B in the absence of magnesium ions. 880 mg of 70 S ribosomes isolated by method I (see "Materials and Methods") were passed through a column of Sephadex G–50 (60 x 4.2 cm) with eluting buffer: 0.02 M Tris – HCl pH 7.5; 0.6 M $(NH_4)_2SO_4$. In fractions eluted without retention volume the concentration of salt was increased to 1.5 M, and the ribosomes were then applied to a column with 440 ml of Sepharose–4B (33 x 4.2 cm) equilibrated with the same buffer. Elution of ribosomal subunits was affected by 3 volumes of buffer with a linear salt gradient 1.5 – 0.05 M.

extracted from these subunits revealed that their protein composition is identical with that of the control 30 S subunits isolated by zonal centrifugation (fig.5).

The subunits from peaks II and III are characterized by sedimentation constants 47 S and 33 S correspondingly (fig.4B b, c) and contain the ribosomal 23 S RNA only (fig.4A b, c) . Obviously, the peaks II and III are two partially unfolded forms of 50 S subunits with a good separation on Sepharose–4B. Comparing refractometric indexes in figures 1, 2 and 3 we see, that material from peak II corresponds to 50 S subunits of form 2 and material from peak III – to 50 S subunits of form 3. The study of subunits of form I showed that they preserved their original sedimentation constant 50 S. Hence, the form I is an intact 50 S subunits which were stabilized by high ammonium sulphate concentration at low magnesium (fig. 2B), or by a sufficient concentration of Mg^{2+} at moderate (0.6 M) concentration of $(NH_4)_2SO_4$ (fig.1B).

Functional activity of 30 S subunits isolated by chromatography on Sepharose–4B. In fig. 6 are presented the results of titration of the complex 30 S · poly (U) by the specific ^{14}C–Phe–$tRNA^{Phe}$. We see that 30 S subunits, prepared chromatographically, are fully active in the codon–dependent binding of aminoacyl–tRNA (in reverse coordinates $1/\bar{\nu} \rightarrow 1$ when $1/C \rightarrow 0$), as well as the control subunits separated by centrifugation. A slight difference is observed in the values of binding constants K_a: they equal $3 \cdot 10^7$ M^{-1} and $2 \cdot 10^7$ M^{-1} correspondingly. To test the subunits in polypeptide synthesis, two identical incubation mixtures were composed. The only difference was that in the first one the 70 S ribosomes were obtained by reassociation of chromatographically prepared 30 S subunits with 50 S subunits isolated by zonal

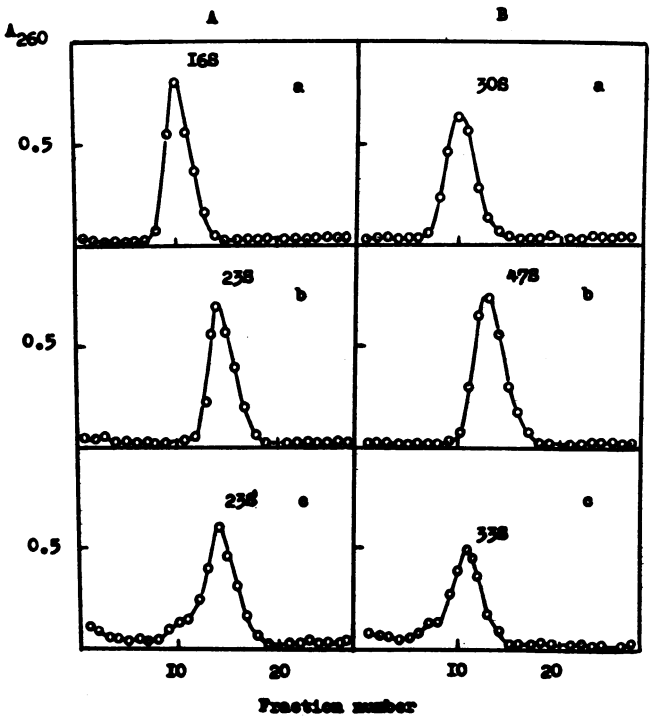

Fig.4. Sedimentation analysis of material from peaks I, II and III in fig.3. A: 5 A_{260} units of material from each peak were deproteinized 30 min at 20° in the buffer 0.02 M Tris–HCl, pH 7.1; 0.1 M LiCl; 0.1% SDS. Then these mixtures were layered on the sucrose gradient in the same buffer. Sedimentation proceeded 2h at 50,000 rev/min and 20°. a – material from peak I, b – from peak II and c – from peak III. B: 5 A_{260} units from each peak were layered on the sucrose gradients 5 – 20% in buffer 0.02 M Tris–HCl, pH 7.1; 0.001 M $MgCl_2$; 0.2 M NH_4Cl. Sedimentation proceeded 2 h at 40,000 rev/min and 5°. a – material from peak I, b – from peak II and c - from peak III.

centrifugation; in the control mixture both types of subunits were originated from zonal centrifugation. As seen from fig.7, in both cases the initial rates, as well as the final levels of polyphenylalanine synthesis, are identical.

Isolation of ribosomal 30 S and 50 S subunits without application of the ultracentrifuge. Similar results were obtained when the initial 70 S ribosomes were purified by the modified procedure of Kurland (method 2 in "Materials and Methods"). As seen in fig.8, these ribosomes are also divided into 3 peaks during elution by the reverse salt gradient. Peak I corresponds, like that in fig.3, to pure 30 S subunits, peaks II and III – to 47 S and 33 S forms of 50 S subunits (more detailed data on this experiment were published earlier in our preliminary paper [13]).

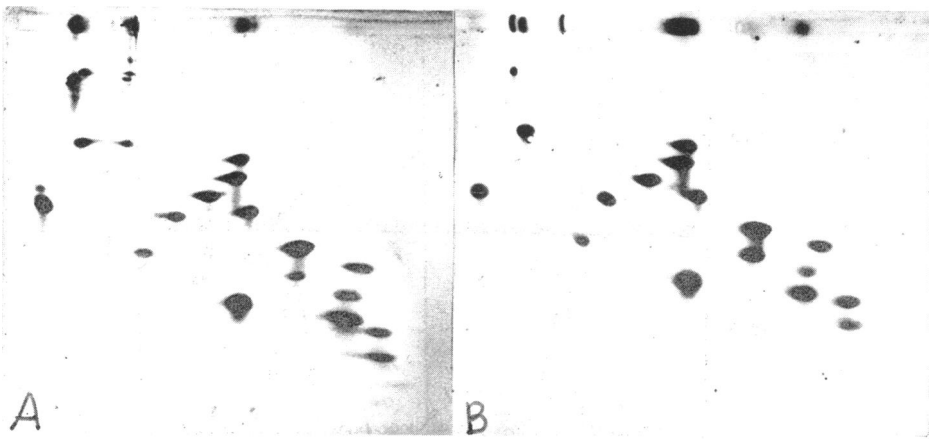

Fig.5. Two–dimensional electrophoresis of ribosomal proteins from 30 S subunits prepared by chromatography on Sepharose – 4B (A) or by zonal centrifugation (B).

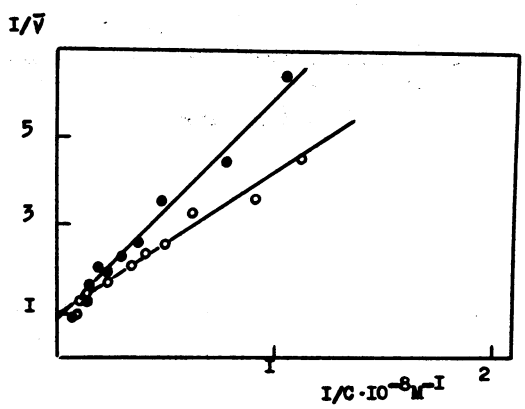

Fig.6. Measurement of the fraction of active 30 S subunits M and the binding constant K_a (see for detailes "Materials and Methods"). Subunits were isolated chromatographically (—o—) or by zonal centrifugation (—●—).

DISCUSSION

Chromatography in the reverse salt gradients was applied earlier for the separation of proteins [10] and transfer RNA's [11]. In this work it was used for the separation of ribonucleoprotein particles – ribosomal subunits from bacteria. The effect was attained due to the conformational alterations of 50 S subunits in a medium with extremely low magnesium and high salt concentration. In this state the hydrophobic interaction of 50 S subunits with agarose is changed and this results in their elution from the column with Sepharose–4B at a much lower ionic strength than that for the 30 S subunits.

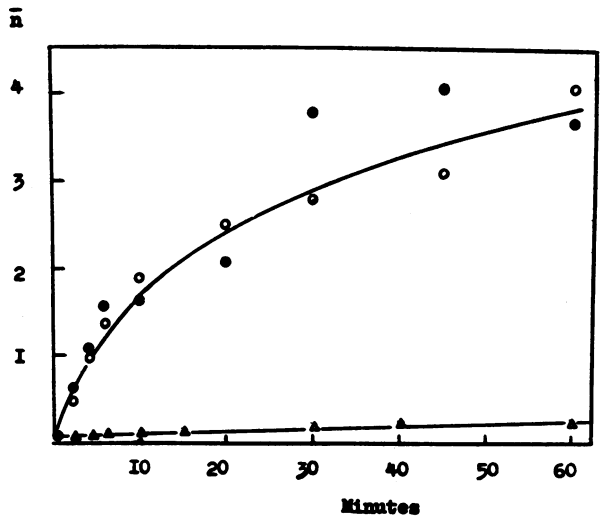

Fig.7. Kinetics of polyphenylalanine synthesis. (see for details "Materials and Methods"). —o—, 70 S ribosomes were obtained by reassociation, of 30 S subunits isolated chromatographically with 50 S subunits isolated by zonal centrifugation; —●—, both types of subunits in 70 S ribosomes were originally from zonal centrifugation; —Δ—, control without 30 S subunits.

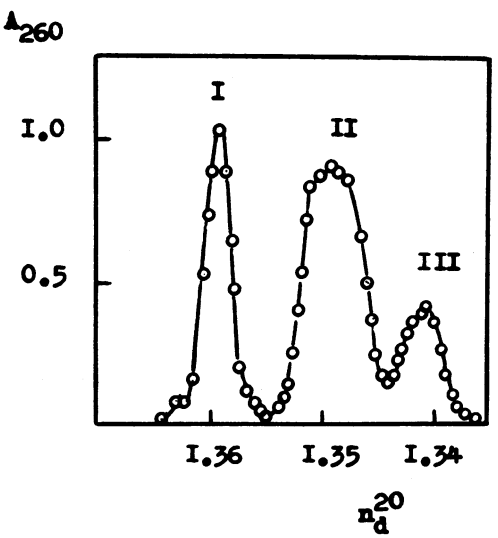

Fig.8. Chromatography on Sepharose—4B of 70 S ribosomes purified by ammonium sulphate fractionation (method 2, see "Materials and Methods"). Conditions for this experiment are identical to those in fig.3.

The study of 30 S subunits prepared by chromatography showed that they have an unchanged sedimentation constant, contain undegraded 16 S ribosomal RNA and the same set of S–proteins as the control subunits prepared by zonal centrifugation. The study of their functional capabilities revealed that they are fully active in poly (U)–dependent binding of ^{14}C–Phe – $tRNA^{Phe}$ and, if reassociated with control 50 S subunits, have the usual activity in polypeptide synthesis. These data enable us to assume that the 30 S subunits isolated by chromatography on Sepharose–4B can be used for both preparation of individual ribosomal components and all functional tests in protein biosynthesis.

50 S subunits were found, in low magnesium and high salt concentrations, in two specific states: a slightly unfolded 47 S form and a highly unfolded 33 S form. Similar results (unfolding of 50 S subunits into 46 S and 28 S forms) were observed earlier by Gavrilova et al [12]. Preliminary studies of both unfolded forms of 50 S subunits showed that they are inactive in reassociation with 30 S subunits. Hence we can recommend the chromatographycally isolated 50 S subunits only as a source of L–proteins and ribosomal RNA. Nevertheless, the easy preparation of all three forms of 50 S subunits by chromatography as well as very characteristic differences in the spectrum of L–proteins (data on their protein composition will be published elsewhere) may initiate new useful experiments for the study of relationship between the structure and functions of the larger ribosomal subunit.

In conclusion we would like to emphasize that the method described here is far less painstaking than the separation of subunits by zonal centrifugation and, at the same time, it has no limitations in respect to the quantity of subunits to be isolated in one run. Increasing the volume of the Sepharose–4B column till several hundreds milliliters and the charge of ribosomes (it can attain up to 15 mg per milliliter of the column) we can prepare in one run gram quantities of pure ribosomal subunits. If the initial 70 S ribosomes for the separation of subunits are obtained according to the simplified procedure of Kurland, the chromatographic preparation of ribosomal subunits can dispense (in some particular cases; see above) totally with the use of ultracentrifuges.

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