Effects of Potassium Chloride Concentration on Protein Content and Polyphenylalanine Synthesizing Capability of 40S Ribosomal Subunits from Canine Pancreas†

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ABSTRACT: Ribosomal small subunits from canine pancreas were used to survey the effects of potassium chloride in the concentration range from 0.4 to 1.25 M. When combined with 60S particles, the treated 40S subunits showed no significant change in phenylalanine incorporating activity until exposure to 0.95 M KCl. Decreases in protein content of the subunits were observed at high ionic stengths. Attempts to separate dissociated ribosomal protein and the remaining core particles treated with 1.25 M KCl by centrifugation of the salt-treated particles though a 40% sucrose cushion led to the observation that ribosomal subparticles isolated in this manner retained full phenylalanine incorporating activity, whereas centrifugation through other solutions resulted in inactive or less active particles. Experiments were performed to elucidate the mechanism by which the 40% sucrose cushion was stabilizing the high salt treated 40S subunits. Two-dimensional gel electrophoresis of the proteins of the various particles isolated in the study was performed. The active 40S particles contained 23–31 protein spots. The isolation of fully active 40S subunits with fewer proteins than previously reported should simplify elucidating their role in the function of the small subunit.

The 80S cytoplasmic ribosome of eukaryotes is composed of two subunits. The work reported in this paper was carried out on the smaller (40S) subunit. The small ribosomal subparticle of rat liver has a molecular weight of $\sim 1.5 \times 10^6$ (Hamilton et al., 1971), of which ~780 000 is protein (Wool & Stoffler, 1974). The small subunit contains one molecule of 18S RNA and between 26 (Terao & Ogata, 1975) and 32 (Howard et al., 1975; Martini & Gould, 1975) proteins.

Understanding the detailed mechanism of protein synthesis requires a description of ribosomal proteins and their role in the process. With bacterial ribosomes, for example, Nomura & Held (1974) have been able to strip all the proteins from the RNA of both subunits and then add back individual proteins in a stepwise fashion with complete recovery of protein synthesizing capability. Successful manipulations of this type have not yet been possible with eukaryotic ribosomes. The objective of this work was to investigate the effects of exposure of the 40S ribosomal subunit from dog pancreas to increasing concentrations of KCl and to characterize changes in the subunit with regard to protein synthesizing ability, protein content, and protein patterns on two-dimensional gels.

During the course of this work an unexpected observation was made. The small subunit retains its protein synthesizing capability, even at high ionic strength, when it is centrifuged through a sucrose cushion.

Materials and Methods

Reagents. PMSF,[1] puro·2HCl, PCK, and lactoperoxidase were obtained from Sigma Chemical Co. Sodium [^{125}I]iodide was from New England Nuclear. [^{3}H]Phenylalanine, either 880 or 1000 mCi/mmol, was purchased from Amersham/Searle. Ultrapure urea was a product of Schwarz/Mann. ME was distilled from the commercially available preparation and stored at 4 °C. All other reagents were of the highest quality obtainable.

Buffers. All buffers were made in deionized water with a resistance of 10 MΩ or greater and were stored at 2 °C. Buffer for 80S monomers (80S-10) was 0.003 M $MgCl_2$, 0.10 M KCl, and 0.01 M Trizma base, pH 7.4. Buffer for ribosome dissociation (40-60-40) contained 0.003 M $MgCl_2$, 0.4 M KCl, and 0.01 M Trizma base, pH 7.4. Subunit buffer (80S-10 ME), in which isolated subunits were suspended and stored, was 80S-10 buffer which was adjusted to 0.01 M ME. Buffer for high salt treatment of subunits (1.25) contained 1.25 M KCl, 0.003 M $MgCl_2$, 0.01 M Trizma base, pH 7.4, and 0.02 M ME. PCK was made to a concentration of 16.67 mg/mL in 0.01 M glycine, pH 9.0.

Preparation of 80S Ribosomal Monomers. All operations were performed at 2 °C. The fresh tissue for this study was obtained by excision from dogs. It was immediately chilled on ice and stored at −20 °C. Seventy to eighty grams of pancreas thawed in 0.25 M sucrose and 10^{-3} M PMSF was pressed through a stainless screen (1-mm holes) to remove connective tissue. The tissue pulp in the sucrose–PMSF solution was homogenized in a Potter-Elvehjem-type glass homogenizer fitted with a Teflon pestle, and the homogenate was centrifuged for 10 min at 13000*g* in the Sorvall SS-34 rotor (Dickman & Bruenger, 1969). The supernatant solution was adjusted to pH 8.5 with 1 N NH_4OH and then brought to concentrations of 0.10 M KCl and 0.003 M $MgCl_2$. The solution was centrifuged for 35 min at 27000*g* in the Sorvall SS-34 rotor. The supernatant solution from this centrifugation was centrifuged for 90 min at 362000*g* in the Beckman Ti-60 rotor. The transparent portion of the supernatant was removed and stored at −80 °C to be used as the high-speed supernatant (HSS) in the phenylalanine incorporation assay. The pellets

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[1] Abbreviations used: PMSF, phenylmethanesulfonyl fluoride; puro·2HCl, puromycin dihydrochloride; PCK, phosphocreatine kinase; ME, β-mercaptoethanol; rpm, revolutions per minute; Bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Phe, phenylalanine; RNP, ribonucleoprotein; HSS, high-speed supernatant; Cl_3AcOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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from the high-speed centrifugation were suspended by homogenization in 40 mL of 80S-10 buffer which contained 10^{-4} M PMSF. The homogenate was centrifuged for 15 min at 27000*g* in the Sorvall SS-34. After the supernatant solution had been diluted to a final volume of 1.6 mL/g of starting tissue with 80S-10 buffer, 0.1 volume of fresh 5% sodium deoxycholate was added. The solution was then centrifuged for 75 min at 362000*g* in the Beckman Ti-60 rotor. The pellets contained 80S ribosomes.

Isolation of Ribosomal Subunits. 80S ribosomes were suspended in 16 mL of 40-60-40 dissociation buffer and centrifuged for 20 min at 2500*g* in the Sorvall SS-34 rotor. At this stage, the A_{260}/A_{280} ratio was 1.8 or greater (Martin et al., 1971).

The Beckman Ti-14 zonal rotor was loaded at 2500 rpm according to the method of Sypherd & Wireman (1974). The rotor was filled with 40-60-40 buffer, and the sample solution (containing 5000–8000 A_{260} units of ribosomal particles) was layered "beneath" the buffer as a 32-mL inverse linear gradient (16-mL sample solution plus 16 mL of 7.4% sucrose). Next, a 7.4–38% hyperbolic sucrose gradient in 40-60-40 buffer similar to that described by Eikenberry et al. (1970), but scaled down to fit the smaller Ti-14 rotor, was introduced. Finally, a cushion of 45% sucrose was pumped into the rotor. The final contents of the rotor, from the center, were 271 mL of 40-60-40 buffer as an overlay, 32 mL of sample, 317 mL of 7.4–38% sucrose gradient, and 45 mL of 45% sucrose cushion. The rotor was spun at 30000 rpm for 11 h at 2 °C.

The contents of the rotor were displaced with 65% sucrose pumped to the edge of the rotor, 4-mL fractions were collected, and the A_{260} of each fraction was measured. Fractions containing the individual subunits were pooled and centrifuged for 18 h at 300000*g* in the Beckman Ti-60 rotor. Pellets containing the subunits were suspended in small volumes of 80S-10 ME and stored at −80 °C.

Purification of Large Ribosomal Subunits. 60S subunits were further purified by treatment with puromycin (Sherton et al., 1974) followed by a repetition of the hyperbolic sucrose gradient procedure. Before being pelleted, they were dialyzed against 4 L of 80S-10 ME buffer with two changes of buffer. Pelleted 60S subunits were stored at −80 °C in 80S-10 ME.

High Salt Treatment of Ribosomal 40S Subunits. Freshly prepared subunits were treated by slowly adding solid KCl until the desired concentration had been reached and then stirring the mixture for 1 h at 2 °C. The treated particles were then centrifuged through a 40% sucrose cushion as follows. A 13–15-mL aliquot of the treated subunits in a Ti-60 polyallomer tube was underlayered with 22–24 mL of 40% sucrose in 1.25 buffer. Centrifugation was for 18 h at 300000*g*. The subunits were suspended and stored in 80S-10 ME.

Phenylalanine Incorporation Assay. The ability of ribosomal monomers and/or subunits to synthesize polyphenylalanine was measured by incubation in 200 μL total volume with the following: 1 mM ATP; 5 mM creatine phosphate; 0.2 mM GTP; 0.1 mM NH_4Cl; 7 mM $MgCl_2$; 5 mM ME; 50 mM Tris, pH 7.5 at 25 °C; 20 μg of poly(U); 16.6 μg of PCK (1.9–2.7 units); 1 μCi of L-[^{3}H]phenylalanine; 40 μL of HSS; ribosomes (0.5–0.6 mg) or subunits (0.18–0.21 mg of 40S and 0.38–0.45 mg of 60S).

The mixtures were incubated at 37 °C for 30 min. At the end of incubation, a 100-μL aliquot of the assay mixture was applied to a Whatman 3-mm filter circle. The filters were soaked for 15 min in cold 10% Cl_3AcOH and then for 15 min in hot (90 °C) 10% Cl_3AcOH. Finally, the pads were soaked for 15 min in cold 5% Cl_3AcOH which contained 10 μg/mL DL-phenylalanine and washed with cold ether–ethanol and then ether. The pads were dried and then counted in 10 mL of scintillation fluid.

Analytical Ultracentrifugation. Sedimentation velocity runs were made on a Beckman Model E analytical ultracentrifuge using either Schlieren or ultraviolet optics.

Extraction of Ribosomal Protein. The extraction procedure was similar to that of Hardy et al. (1969). Extracted protein was dialyzed against 1 L of 8 M urea and 0.014 M ME at 5 °C with one change of dialysis medium. The protein solutions were then made 0.01 M in dithiothreitol and 0.01 M in Bistris, pH 4.0, and stored at −20 °C.

Two-Dimensional Gel Electrophoresis. The procedure used for electrophoresis was that of Mets & Bogorad (1974) as modified by Subramanian (1974) which uses low pH in the first dimension and an $NaDodSO_4$ separation in the second dimension.

Chemical Analyses. RNA was determined with the orcinol reaction, and protein was determined by a modification of the Lowry method (Campbell & Sargent, 1967). Bovine serum albumin and lyophilized 80S protein were used as standards in the Lowry determination.

Iodination of Ribosomal Protein. 40S subunits from a zonal separation were first dialyzed for 20 h vs. 6 L of 0.4 M NaOAc, pH 5.6, with one change of dialysis medium. After the precipitated subunits were concentrated by centrifugation for 30 min at 12000*g* in the Sorvall SS-34 rotor, they were suspended in 5 mL of 0.4 M NaOAc, pH 5.6. To this solution was added 5 μg of lactoperoxidase, 1 mCi of $Na^{125}I$, and 50 μL of a 1:600 dilution of 30% hydrogen peroxide (Miyachi et al., 1972). After incubation at 24 °C for 30 min, the treatment was repeated. The reaction mixture was then dialyzed overnight at 4 °C against 500 mL of 25% sucrose in 40-60-40 buffer with one change of dialysis medium. The solution was diluted to 38 mL with 40-60-40 buffer–25% sucrose and then made 1.25 M in KCl, and treatment of the subunits proceeded as previously described.

Bio-Gel P100 Gel Filtration. Bio-Gel P100 was swelled for 24 h at room temperature in 1.25 buffer which contained 25% sucrose. The gel suspension was cooled to 4 °C and packed in a 2.5 (i.d.) × 60 cm column (final bed height = 45 cm). 40S subunits in 1.25 buffer–25% sucrose were applied to the column and eluted with the same buffer. The flow rate was 0.5 mL/min; 5-mL fractions were collected. The solution was continually monitored at A_{254} and A_{280}.

Results

Purity of the 40S Subunit Preparation. The 40S small subunit was found to be relatively uncontaminated by large subunits. The small subunit preparation showed only 10% of its full activity in the phenylalanine incorporation assay unless 60S subunits were added. Figure 1 shows a Schlieren pattern of a sedimentation velocity run of the isolated small subunit. The slower sedimenting peak represents the small ribosomal subunit with a sedimentation coefficient of 37 S. The faster sedimenting material is a dimer of the small subunit with a sedimentation coefficient of 54.5 S (Vanduffel et al., 1975; Wettenhall et al., 1973). Decreasing amounts of monomer were observed as the salt concentration increased.

Survey of Salt Concentrations. A batch of small subunits from a single preparation was used to survey the effects of the entire range of potassium chloride concentrations. Immediately upon isolation from the zonal rotor, the pool of 40S subunits was divided into aliquots and KCl was added to each aliquot to the desired concentration. The solutions were stirred for 1 h at 4 °C, and then the subunits were isolated by cen-

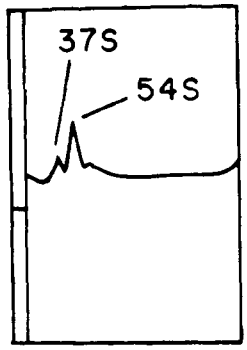

FIGURE 1: Sedimentation velocity profile of the small ribosomal subunit. Conditions were those described under Materials and Methods. The concentration of the particles was 4.9 mg/mL in 80S-10 ME buffer, rotor speed was 44 770 rpm, and temperature was 4 °C. Sedimentation was from left to right.

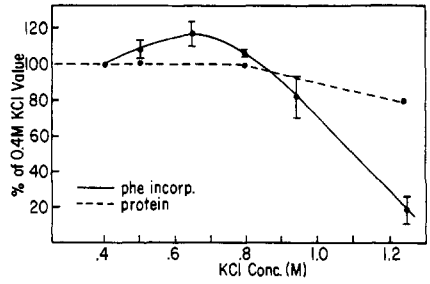

FIGURE 2: Influence of potassium chloride concentration on the phenylalanine incorporating activity of the small ribosomal subunit. The data are shown as the percentage of the 0.4 M KCl value (i.e., no salt treatment) in phenylalanine incorporation and protein content plotted against the KCl concentration in the salt treatment. The salt treatment and protein and incorporation assays were conducted as described under Materials and Methods. 100% incorporation = 12000 cpm of [^{3}H]Phe per mg of RNP. 100% protein = 0.47 mg of protein per mg of RNP. The error bars show the range of phenylalanine incorporation of two experiments run in duplicate.

trifugation. The pellets were each resuspended in a small volume of 80S-10 ME buffer. Measurements were made of phenylalanine incorporating activity and protein content.

As shown in Figure 2, there was no significant loss in the phenylalanine incorporating activity of the 40S subunits until exposure to 0.95 M KCl and above. At 0.95 M KCl, the subunits retained 80% of the phenylalanine incorporating activity of the 0.40 M KCl subunits. However, at 1.25 M salt the subunits had only 15–25% of the phenylalanine incorporating activity of the 0.40 M KCl particles.

Lowry protein determinations of the salt-treated 40S particles showed that the 0.4, 0.5, and 0.8 M KCl treated subunits had the same protein content (47%), while the 1.25 M KCl treated subunits had less protein (38%). Increasingly large amounts of protein were found in the supernatant solution from which these particles were centrifuged as the salt concentration increased (data not shown).

Finally, no large change in the sedimentation coefficient of the subunit dimer was observed between the 0.4 M KCl treated particles (55 S) and the 0.8 M KCl treated particles (51 S), but the 0.95 M KCl treated subunits showed a pronounced drop in *s* value (42 S) and a further drop was noted with 1.25 M KCl particles (38 S).

Centrifugation through Sucrose Cushion. For the purpose of concentrating the proteins removed from the 1.25 M KCl treated subunits, a method was needed to separate the salt-dissociated proteins from the remaining core particles since the previously used procedure (simply centrifuging the core particles from solution via differential centrifugation) gave contamination of the pellet of core particles with proteins. The

Table I: Effects of Sucrose Concentration during Centrifugation on Polyphenylalanine Synthesizing Activity of 40S Ribosomal Subunits[a]

treatment of small subunit	incorpn (cpm/mg of RNP)	% of 0.40 M KCl value
(a) Effect of Centrifugation through a Sucrose Cushion		
0.4-40[b]	10 831	100
1.25-40[c]	3 749	35
1.25-40 cush[d]	11 728	108
(b) Reactivation of 40S Subunits by Centrifugation through a Sucrose Cushion		
1.25-40[c]	3 761	34
1.25-40 recovd[e]	7 592	68
1.25-40 cush[d]	11 123	100
(c) Reactivation of 40S Subunits		
1.25-40 cush[d]	8 041	100
1.25-40[c]	1 264	16
1.25-40 recovd[e]	2 430	30
1.25-40-25 suc[f]	2 879	36

[a] For phenylalanine incorporation determinations, 0.18–0.21 mg of 40S subunits was combined with 0.41–0.44 mg of purified 60S subunits to attain the indicated incorporation. The assay is detailed under Materials and Methods. [b] 40S subunits exposed to 0.4 M KCl and centrifuged. [c] 40S subunits exposed to 1.25 M KCl and centrifuged. [d] 40S subunits exposed to 1.25 M KCl and centrifuged through 40% sucrose. [e] 40S subunits exposed to 1.25 M KCl, centrifuged, suspended in their own supernatant solution, and centrifuged through 40% sucrose. [f] 40S subunits exposed to 1.25 M KCl, centrifuged, suspended in 25% sucrose–1.25 M KCl, and centrifuged through 40% sucrose.

method chosen was that of layering the salt-treated mixture (which contained ~25% sucrose as residual from the zonal separation) onto a 40% sucrose cushion. The 40% sucrose was made in 1.25 M KCl buffer so that the ionic environment of the 1.25 M KCl treated 40S subunits would not change. The subunits were then centrifuged through the cushion. The average buoyant density of the ribosomal proteins (Hamilton et al., 1971) was found to be close to the density of the 40% sucrose of 2 °C (Newburn, 1975) so it was reasoned that the RNA-containing core particles would readily sediment through the sucrose cushion while the lighter, separated proteins would proceed through the sucrose cushion more slowly. Separation of the protein-deficient core particles from the removed proteins would thus be enhanced.

When the high salt treated particles (treated with 1.25 M KCl) were isolated in this manner, they were found to have retained full phenylalanine incorporating activity. The following experiment was performed to substantiate this finding. Small subunits from one preparation were divided into two aliquots. One of the aliquots was treated by merely isolating the 40S subunits from solution via differential centrifugation and resuspending them in 80S-10 ME buffer (these particles, exposed only to 0.40 M KCl, were designated 0.4-40). The other aliquot was treated with 1.25 M KCl. Upon completion of the incubation in high salt, the mixture was further subdivided into two portions. One portion was centrifuged directly, and the pellet was resuspended in 80S-10 ME buffer (these 40S subunits had been exposed to 1.25 M KCl and so were designated 1.25-40); this procedure had previously produced inactive subunits. The other high salt treated portion was layered over and then centrifuged through a 40% sucrose cushion as before, and the pellet was resuspended in 80S-10 ME buffer (these particles, which had been treated with 1.25 M KCl and had been centrifuged through a 40% sucrose cushion, were called 1.25-40 cush). When these three subunit preparations were tested in a phenylalanine incorporating system with purified 60S subunits, the data in Table Ia were

obtained. The 1.25 M KCl treatment had apparently caused the 1.25-40 subunits to retain only 35% of the activity of the 0.4-40 particles and yet the 1.25-40 cush particles were fully active. The only difference between the 1.25-40 and the 1.25-40 cush preparations was the method of concentration. Clearly the sucrose cushion had an activating or, at least, a stabilizing effect on the high salt treated 40S subunits. Experiments were designed to clarify the action of the sucrose cushion.

The first such experiment attempted to determine if 1.25-40 particles which were suspended in their own supernatants were active if the suspension was recentrifuged through a 40% sucrose cushion. As shown in Table Ib, the 1.25-40 cush subunits were quite active while the 1.25-40 subunits had greatly reduced activity. By comparison, the 1.25-40 subunits which had been suspended in their own supernatant and then recovered by centrifugation through a 40% sucrose cushion (subunits now referred to as 1.25-40 recovd) were twice as active as the 1.25-40 particles.

In the next experiment, pellets of 1.25-40 subunits were suspended in different solutions. One pellet was suspended, as above, in its own supernatant while another pellet was suspended in 25% sucrose in 1.25 buffer. Both were then centrifuged through 40% sucrose cushions, and then, as usual, the pellets were resuspended in small volumes of 80S-10 ME. As Table Ic shows, the 1.25-40 subunits had a greatly reduced phenylalanine incorporating activity when compared to the 1.25-40 cush particles. The subunits which had been suspended in 25% sucrose in 1.25 buffer and centrifuged (called 1.25-40-25 suc) had about the same activity as the 1.25-40 recovd subunits; their activity was twice the level of the 1.25-40 subunits but only one-third of the level of the 1.25-40 cush subunits.

In a further attempt to clarify the role of the 40% sucrose cushion in the stabilization of the 40S subunits, 40S ribosomal proteins were labeled with radioactive iodine. The followng procedure was used in the iodination experiments. For the purpose of obtaining iodinated ribosomal proteins, 40S subunits in 40-60-40 buffer were dialyzed vs. 0.4 M sodium acetate, pH 4.5. After concentration, the subunits were iodinated by using a modified procedure of Miyachi et al. (1972). The subunits were then dialyzed against 40-60-40 buffer which contained 25% sucrose. In addition to changing buffers, the dialysis also removed unbound radioactive iodine. The subunits were then treated with 1.25 M KCl in the usual manner. The supernatant and the pellet from the subsequent high-speed centrifugation contained approximately the same amounts of radioactivity (0.22% of the total input radioactivity was found in the supernatant and 0.19% of the total input was in the pellet). Next, the supernatant which contained radioactive ribosomal protein was removed by the high salt treatment and was mixed with unlabeled 40S subunits which had been treated with 1.25 M KCl. The mixture was centrifuged through a 40% sucrose cushion, and a suspension of the pellet was examined for the presence of radioactivity. A radioactive pellet would have indicated that ribosomal proteins were being removed during the incubation in 1.25 M KCl and that a reassociation of the dissociated proteins was occurring during the incubation or the centrifugation through the 40% sucrose cushion. In this case, a radioactive pellet would have been expected since the radioactive ribosomal proteins should have competed with the cold proteins during any reassociation process. Since the labeled proteins were added in a roughly 1:1 ratio of protein to subunits in the dissociation mixture, about half of the particles would be expected to contain radioactive proteins if reassociation were occurring. As shown in Table II, the pellet contained only 1.5% of the input radioactivity, while 99% of the radioactivity was in the supernatant (1.25-40 cush supernatant). Since the 1.25-40 cush pellet was active in phenylalaine incorporation and control 1.25-40 subunits from the same preparation were inactive (see Table II), the absence of radioactivity in the 1.25-40 cush pellet provides evidence that the 40% sucrose cushion is not mediating a reconstitution-type process. The small amounts of radioactivity in the pellet could represent nonspecific binding of the ^{125}I-labeled proteins.

Table II: Incorporation of Labeled Proteins into Subunits and the Subunits' Phenylalanine Activity[a]

sample	cpm of ^{125}I	% of input	cpm of [^{3}H]-Phe/mg of RNP	Phe incorpn (% of 1.25-40 cush value)
^{125}I-labeled proteins	4.34×10^5	100		
1.25-40 cush supernatant	4.30×10^5	99		
1.25-40 cush pellet in 80S-10 ME	6.5×10^3	1.5	9090	100
1.25-40			846	9

[a] Details of the isolation of the sample and counting of the ^{125}I are given in the text. Details of the phenylalanine incorporation assay are given under Materials and Methods.

In a final attempt to define the role of the 40% sucrose cushion in the stabilization of the 40S ribosomal subunits, experiments were conducted to more clearly define the composition of the 1.25 M KCl dissociation mixture (the 40S subunits in 1.25 M salt). An assumption had originally been made that ribosomal proteins were dissociated from the 40S subunits during the incubation of the ribosomal particles in the 1.25 M salt. 40S subunits from a zonal separation were incubated as usual in 1.25 M KCl and then applied to a 2.5 × 45 cm Bio-Gel P100 column to test this assumption. Bio-Gel P100 retards peptides under M_r 100 000, so ribosomal core particles would be excluded from the gel while dissociated ribosomal proteins would be retarded by the P100 since they would have molecular weights under 100 000 (Wool & Stoffler, 1974). The column was kept under "dissociating" conditions, i.e., 25% sucrose in 1.25 buffer, during sample migration through the column. Figure 3 shows the absorbance profile of the column effluent. The vast majority of 280-nm-absorbing material eluted from the column at the void volume; only a small trailing portion immediately after the main peak and an extremely small "peak" ($A_{280} < 0.08$) centered on fraction 40 indicated the presence of further 280-nm-absorbing material.

The column fractions containing the peak of 280-nm-absorbing material were pooled and then divided into two aliquots. One aliquot was layered over 40% sucrose in 1.25 buffer and centrifuged; the pellet was resuspended, as usual, in 80S-10 ME. The other aliquot was dialyzed against 80S-10 ME overnight and then centrifuged, and the pellet was resuspended in 80S-10 ME. The resuspended material from the first aliquot was called 1.25-40 col, cush (col, cush = column, cushion) while the resuspended 40S subunits from the second aliquot were referred to as 1.25-40 col, dial (column, dialysis). The two samples were tested for phenylalanine incorporating activity with the results shown in Table III. It is quite evident that the material centrifuged through the sucrose cushion was fully active while the other aliquot was almost completely

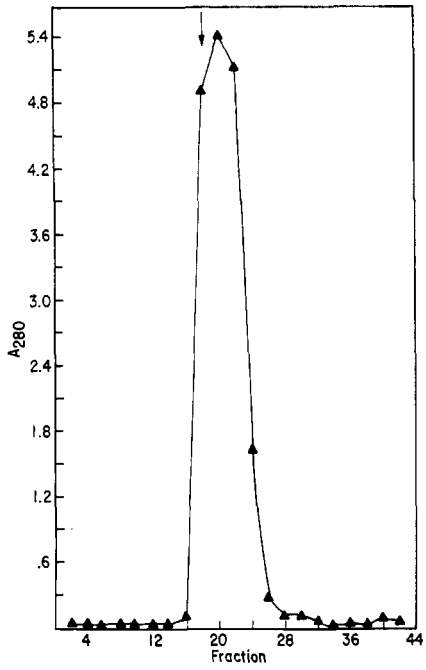

FIGURE 3: Bio-Gel P100 gel filtration column of 1.25 M KCl treated 40S subunits. 40S subunits (17.2 mg) in 1.25 buffer–25% sucrose were applied to a 2.5 × 45 cm Bio-Gel P100 column which had been equilibrated with 1.25 buffer–25% sucrose. The same buffer was used as elution buffer. 5-mL fractions were collected, and the absorbance at 280 nm was read as shown. The arrow indicates the position where Blue Dextran eluted from the column. The peak fractions were processed as described in the text.

Table III: Phenylalanine Incorporation Activity of High Salt Treated 40S Subunits Passed through a P100 Column[a]

small subunit	incorpn (cpm/mg of RNP)	% of 1.25-40 cush value
1.25-40 cush	9083	100
1.25-40 col, cush	9434	104
1.25-40 col, dial	876	10

[a] For the phenylalanine incorporation determination, 0.18–0.21 mg of 40S subunits was combined with 0.44–0.45 mg of purified 60S subunits to attain the indicated incorporation. The assay is detailed under Materials and Methods. The isolation of the various particles is outlined in the text.

inactive. We made no attempt to determine if the inactivating event was the dialysis or the centrifugation without a sucrose cushion.

Gel Data. Two-dimensional gel electrophoresis was used throughout the study to check the protein content of various 40S subunit preparations. Gels of 0.4-40 subunits, those subunits which had undergone no extra salt treatment and which were the subunits routinely isolated from the zonal separations, showed 31 possible spots, of which 24 were darkly staining spots (Figure 4). The general pattern of the protein spots in these gels is similar to that of Hanson et al. (1974), who used the same electrophoresis sytem.

The subunits which were treated with 1.25 M KCl and then centrifuged through a 40% sucrose cushion, 1.25-40 cush subunits, were shown by two-dimensional gel electrophoresis to contain 29 possible protein spots with 25 of these spots staining darkly. The only protein spots present on the 0.4-40 subunit gels that were not present on the 1.25-40 cush subunit gels were 6, a lightly staining spot, and 20, a dark spot. All other protein spots on the 0.4-40 gels were present on the 1.25-40 cush gels. Proteins 15 and 29 stained more intensely on the 1.25-40 cush gels than on the 0.4-40 gels.

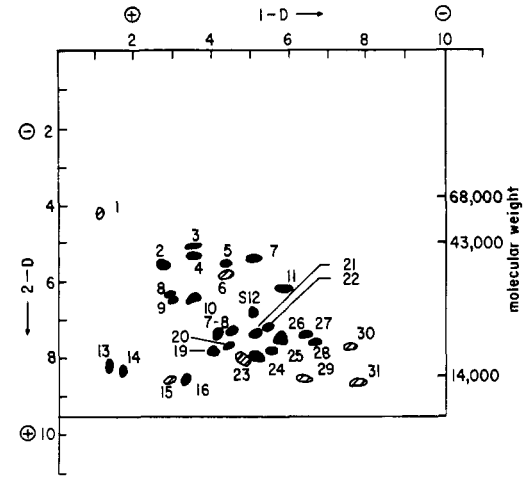

FIGURE 4: Schematic representation of a two-dimensional electropherogram of 0.4-40 small ribosomal subunit proteins. The solid spots represent the darkly stained spots; the crosshatched spots were lightly staining protein spots or spots not always seen. The conditions of electrophoresis are described under Materials and Methods. Briefly, the first dimension gel contained 8 M urea and 4% acrylamide and was run at low pH and the second dimension was a 10% gel run with 3.5 mM $NaDodSO_4$. All illustrated gels contain 0.15–0.20 A_{280} unit of protein. The anode was on the left in the first dimension and on the bottom in the second dimension. The right-hand ordinate shows the migration of three proteins of known molecular weight: lysozyme (14 000), ovalbumin (43 000), and bovine serum albumin (68 000).

Subunits which had been treated with 1.25 M KCl and then centrifuged from solution, 1.25-40 particles, displayed fewer protein spots on two-dimensional gels than either the 0.4-40 or the 1.25-40 cush subunits. These inactive particles form gel patterns with only 24–26 possible protein spots and with only 16–20 of the spots appearing as darkly staining spots. The most notable difference on the gels of the 1.25-40 proteins was the complete absence of protein spots 3, 22, 27, and 30. Other proteins were diminished in amount on the 1.25-40 protein gels compared to the 1.25-40 cush protein gels; these proteins included 4, 9, 10, 11, 12, 28, and 29.

The particles which had been through the P100 column and were concentrated by centrifugation through a sucrose cushion, 1.25-40 col, cush subunits, showed 23 protein spots on 2-D gels with 18 of the spots staining darkly. When compared to the gels of 1.25-40 cush proteins, the 1.25-40 col, cush protein gels lacked protein spots 3, 9, 10, 19, 29, and 31. Protein spots 17 and 28 were present in diminished proportions compared to the 1.25-40 cush protein gels, and the 1.25-40 col, cush protein gels also had three additional protein spots, possibly derivatives of other protein. Figure 5 shows a 1.25-40 col, cush gel. 1.25-40 col, cush particles were fully active in phenylalanine incorporation. 40S subunits which had been through the P100 column and were concentrated by differential centrifugation, 1.25-40 col, dial subunits, which were inactive in the phenylalanine incorporation assay, had the same protein pattern on two-dimensional gels as 1.25-40 col, cush particles with only minor exceptions. These exceptions included the following: 1 was absent; 17, 18, and 22 were present in reduced amounts; 19 was present (Figure 6). Table IV summarizes the gel data and compares the various particles studied.

Discussion

Effects of Increasing KCl Concentrations. One of the original aims of this study was to monitor the removal of ribosomal proteins from the small subunits as the potassium chloride concentration was increased. Previous investigations by other workers on this question have reported varying results. Clegg & Arnstein (1970) found that increasing concentrations

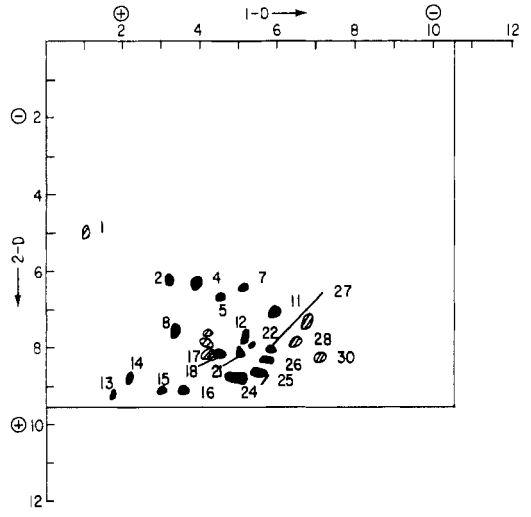

FIGURE 5: Schematic representation of a two-dimensional electropherogram of 1.25-40 col, cush small ribosomal subunit proteins. See legend to Figure 4 for details of the procedure and an explanation of the schematic.

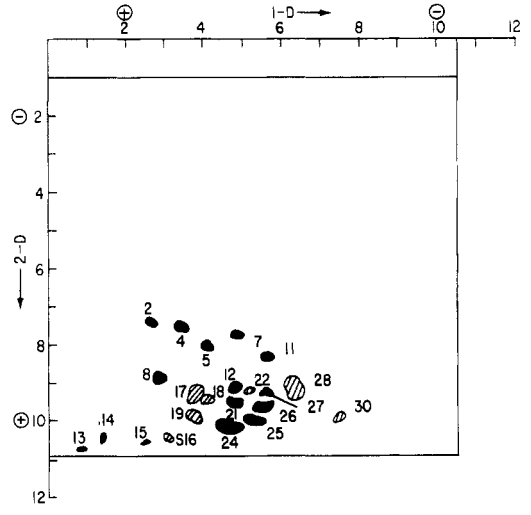

FIGURE 6: Schematic representation of a two-dimensional electropherogram of 1.25-40 col, dial small ribosomal subunit proteins. See legend to Figure 4 for details of the procedure and an explanation of the schematic.

of KCl removed increasing amounts of protein from rat liver ribosomes. Their studies suggested that discrete, discontinuous transformations were occurring in the ribosomes. Ford (1971) reported that increases in KCl concentration resulted in decreases in protein content for both ribosomal subunits of *Xenopus* ovary, but his data indicated that no discrete protein-deficient particles were produced. Neither Clegg & Arnstein nor Ford studied ribosomal polypeptide synthesis activity in relation to the protein loss of the ribosomes. However, Naora & Pritchard (1971) and Mechler & Mach (1971) observed decreases in phenylalanine incorporation activity as salt concentration in ribosome treatment increased (ranges 0.5–2.0 M KCl for Naora & Pritchard and 0.3–0.9 M KCl for Mechler & Mach). However, both of these groups attempted to examine salt effects on both subunits simultaneously.

Our data indicate (Figure 2) that KCl treatment in the concentration range from 0.4 to 0.8 M seems to have no effect on the phenylalanine incorporating activity of the 40S ribosomal subunit from canine pancreas. The constancy of sedimentation behavior and protein content of the particles confirmed that the subunits were unaffected by salt treatment in this range. After exposure to 0.95 M KCl, the 40S subunits still retained ~80% of the phenylalanine incorporating activity of untreated particles. Exposure of 40S subunits to 1.25 M KCl, however, decreased the phenylalanine incorporating capacity to 15–25% of that of the reference subunits. These changes at high salt concentrations were also paralleled by changes in sedimentation behavior and protein content.

Table IV: Effect of Ionic Strength and Subsequent Treatment on Phenylalanine Incorporating Activity and Numbers of Proteins of Ribosomal Small Subunits Observed after Two-Dimensional Gel Electrophoresis[a]

	subunit treatment and Phe incorporating act.				
protein no.	active 0.4-40	inactive 1.25-40	active 1.25-40 cush	active 1.25-40 col, cush	inactive 1.25-40 col, dial
1	+	+	+	+	0
2	++	++	++	++	++
3	++	0	++	0	0
4	++	++	++	++	++
5	++	+	++	++	++
6	+	0	0	0	0
7	++	++	++	++	++
8	++	++	++	++	++
9	++	+	++	0	0
10	++	+	++	0	0
11	++	+	++	++	++
12	++	+	++	++	++
13	++	++	++	++	++
14	++	++	++	++	++
15	+	++	++	++	++
16	++	++	++	++	+
17	++	++	++	+	+
18	++	++	++	++	+
19	++	++	++	0	+
20	++	0	0	0	0
21	++	++	++	++	++
22	++	0	++	++	+
23	+	++	+	+	+
24	++	++	++	++	++
25	++	++	++	++	++
26	++	++	++	++	++
27	++	0	++	++	++
28	++	+	++	+	+
29	+	+	++	0	0
30	+	0	+	+	+
31	+	+	+	0	0
total	31	24–26	29	23	23
dark staining	24	16–20	25	18	15

[a] (++) Protein present, darkly staining spot; (0) protein undetected; (+) protein present, lightly staining spot.

Centrifugation through Sucrose Cushion. In extending the study to examine the changes in protein content in the 40S subunits treated with 1.25 M KCl, an attempt was made to remove supposedly dissociated ribosomal protein from the remaining core particles in 1.25 M KCl treatment mixtures. These mixtures were centrifuged through a 40% sucrose cushion. This treatment resulted in subunits which retained full phenylalanine incorporating activity (referred to as 1.25-40 cush particles). Prior to this, 1.25 M KCl treated subunits had been prepared by simply centrifuging the particles from the mixture. This method gave particles (called 1.25-40) that were inactive in phenylalanine incorporation. On two-dimensional gel electrophoresis, the untreated 0.4-40 subunits showed 31 protein spots. The inactive 1.25-40 subunits showed a gel pattern with 24–26 protein spots. (Gel patterns of the proteins extracted from active 1.25-40 cush particles more closely resembled those of the 0.4-40 subunits than those of the 1.25-40 subunits.) Gels of 1.25-40 cush proteins contained 29 protein spots. The only difference between the 1.25-40 and

the 1.25-40 cush particles was the method of isolation.

The experiments performed have not suggested a mechanism by which the sucrose cushion was acting, but some general conclusions can be drawn from the results. The data indicate that a reconstitution-type process can be excluded. Partial activation of inactivated subunits showed no dependence on the addition of dissociated proteins (Table Ic), and the failure of particles centrifuged through the sucrose cushion to incorporate iodine-labeled ribosomal proteins (Table II) also excludes a reconstitution-type process mediated by the 40% sucrose cushion.

Data from the P100 column (Figure 3 and Table III) and the 1.25-40 particles lack of proteins certainly suggest that proteins are being dissociated from the subunit. However, the possibility also exists that the protein dissociation may be a distinct and separate event from the inactivation of the subunit. These data also suggest that the 40% sucrose cushion could be affecting the conformational state of the 40S subunits. 1.25-40 col, cush and 1.25-40 col, dial particles showed almost the same protein pattern on two-dimensional gels (Figures 5 and 6 and Table IV); yet, the 1.25-40 col, cush particles were active in phenylalanine synthesis while the 1.25-40 col, dial subunits were inactive (Table III). These results raise the possibility of conformational transitions. Arpin et al. (1972) showed that large ribosomal subunits from rat liver were inactivated by a conformational change induced by specific salt treatment. The salt treatment was shown not to involve deproteinization of the subunit. Cox et al. (1976) found that the large subunit of rabbit reticulocyte ribosomes was inactivated at 0.5 M NH_4Cl without detectable loss of protein; a conformational change was concomitant with inactivation.

Reduced Protein Content of Active 40S Subunits. The active 1.25-40 col, cush subunits contained fewer proteins (23 protein spots) than the 0.4-40 subunits (31 protein spots). The number of proteins in the 1.25-40 col, cush subunit preparation seems significantly lower than that previously reported for active 40S subunits (Howard et al., 1975; Terao & Ogata, 1975; Hanson et al., 1974; Lin & Wool, 1974).

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