The Effect of Temperature on the Magnesium Binding and Ultracentrifugal Properties of Rat Liver Ribosomes*

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ABSTRACT: Rat liver ribosomes were equilibrated with various buffers by passage through Sephadex columns at 5 or 37°. The amount of magnesium bound per mole of ribonucleic acid (RNA) phosphate (*r*) was determined, and analytical ultracentrifugation was carried out at the two temperatures. In 0.001 M potassium phosphate at pH 7.3, containing 0–0.3 M KCl, *r* was unaffected by temperature except in 0.1 mM $MgCl_2$, 0.03 M KCl, where it was 26% lower at 37°. Even when *r* was the same, however, the ribosomes showed more dissociation at 37° than at 5°. Although some of this dissociation appeared to result from RNase action, a reversible temperature effect was also noted, particularly in 0.3 M KCl or NH_4Cl. In 0.05 M Tris–0.025 M KCl (pH 7.6 at 25°), at magnesium concentrations below 1 mM, *r* was lower at 37° (pH 7.3) than at 5° (pH 8.3). The cause of this reduction was not the shift in pH, but rather an increased competition by Tris ion, since the change was greater at lower magnesium or higher Tris concentration. Dissociation of the ribosomes was correspondingly greater than in phosphate buffer. In the Tris buffer containing 5 mM $MgCl_2$, a medium often used for amino acid incorporation or polysome studies, the ribosomes formed dimers or larger aggregates, particularly at 5°.

The physical state of ribosomes or polysomes is frequently examined by sedimentation in sucrose gradients. Since these substances may be unstable at higher temperature, and the experiment takes several hours, it is usually carried out in the cold. The resulting particle distribution is then considered to represent the state of the ribosomes or polysomes at physiological temperature. In the analytical ultracentrifuge, however, ribosomes can be examined either cold or warm, and an increased dissociation at higher temperature has been observed (Petermann and Pavlovec, 1963a). A systematic study of this phenomenon has now been undertaken. Since ribosomal dissociation or association is usually correlated with the amount of magnesium bound (Petermann, 1964), bound magnesium has also been determined. To avoid prolonged dialysis and to reduce degradation by nucleases, the material was equilibrated with various buffers by rapid passage through a Sephadex column. In phosphate buffer, although magnesium binding was affected very little by temperature, the ribosomes dissociated on warming. In Tris buffer magnesium binding was reduced at 37°, and ribosomal dissociation was correspondingly increased.

Methods

Ribosomes. Ribosomes were isolated from rat liver cytoplasm in the presence of bentonite (Petermann and Pavlovec, 1963b), with the following modifications. The washed ribosomes were suspended in 0.001 M potassium phosphate–0.5 mM $MgCl_2$ (pH 6.8) and precipitated by the addition of one-tenth volume of 0.5 M $MgCl_2$. The precipitate was washed with 0.001 M phosphate–0.05 M $MgCl_2$ (pH 6.8), suspended in 0.001 M phosphate (pH 7.4), and dialyzed against 0.001 M phosphate–0.2 mM $MgCl_2$ (pH 7.4) overnight on a rocker. The preparation was then dialyzed against 0.001 M phosphate–0.3 or 0.2 mM $MgCl_2$ (pH 7.4) for another day, and clarified by centrifugation for 15 min at 20,000*g*. The solution was diluted to 9–12 mg/ml, one-twentieth volume of 20% sucrose was added, and the material was frozen rapidly in a Dry Ice–alcohol mixture and stored at −20°.

In one experiment the ribosomes were dialyzed for 1 day as usual, then for 1 day against 0.001 M potassium phosphate–0.3 M KCl–2.5 mM $MgCl_2$ (pH 7.2). The preparation was clarified in the usual way and used the same day.

Before equilibration in solvents in which magnesium binding was low, some of the ribosomes were washed, in order to reduce their magnesium content. The sample was diluted with four volumes of 0.001 M potassium phosphate–0.35 M NH_4Cl (pH 7.3) and sedimented at 150,000*g* for 90 min. The pellets were suspended in magnesium-free solvent and used immediately. In a few cases the ribosomes were both washed and suspended in the buffer in which they were to be equilibrated. All these samples have been called washed ribosomes.

RNA. rRNA was isolated and freed of magnesium as described previously (Petermann and Pavlovec, 1963b),

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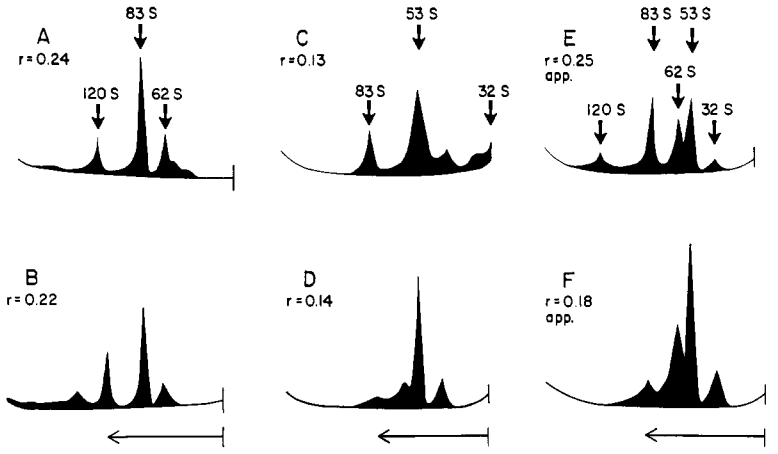

FIGURE 1: Magnesium binding and ultracentrifugal patterns of ribosomes in buffers containing 0.1 mM $MgCl_2$ at 5°. (A and B) In 0.001 M potassium phosphate–0.03 M KCl; (A) dialyzed; (B) equilibrated on Sephadex; (C–F) in 0.05 M Tris–0.025 M KCl; (C) dialyzed; (D–F) equilibrated on Sephadex; (D) washed with NH_4Cl; (E) no pretreatment; (F) dialyzed against 0.3 M KCl (see text). r = equivalents of magnesium per mole of RNA phosphorus. Since E and F were not in equilibrium with the eluting buffer the figures for r are only apparent values.

except that citrate was not added until the ethanol precipitate was dissolved. Samples were heated to 90° for 2 min, chilled quickly, and examined in the ultracentrifuge.

Buffers. In the first series of experiments the buffer was 0.001 M potassium phosphate. Ribosomes were dialyzed, or equilibrated on Sephadex, with buffers containing 0.1, 0.2, or 0.3 mM $MgCl_2$ at pH 7.3. Magnesium binding was also studied in buffers containing 0.03 M KCl and 0.1, 0.5, or 2.5 mM $MgCl_2$ at pH 7.3, or 0.3 M KCl and 2.5 mM $MgCl_2$ at pH 7.1–7.2. One experiment was carried out in 0.3 M NH_4Cl–2.5 mM $MgCl_2$ (pH 7.3). In the second series 0.05 M Tris buffers (0.0115 M Tris–0.0385 M Tris-HCl), containing 0.025 M KCl and 0.1–5 mM $MgCl_2$ were used. These buffers were pH 8.3 at 5°, pH 7.6 at 25°, and pH 7.3 at 37°. The effect of varying Tris concentration was also examined.

Equilibration. Samples containing 35–60 mg of ribosomes were equilibrated with the various buffers by passage through a 2 × 95 cm column of G-100 Sephadex. The cold experiments were carried out in a cold room at 5°, and no column was used more than three times. For the warm experiments the temperature of the column was kept at 37° by a water jacket, and no column was used for more than 2 days.

In the experiments in phosphate buffer the KCl concentration of the sample was adjusted before it was put on the column. Samples of 1.2 ml were collected, and their ribosome concentrations were estimated from the absorbance at 260 mμ, with an extinction coefficient ($E^{1\%}_{1\,cm}$) of 125 (Petermann, 1964). The ribosome peak emerged at the void volume (130 ml) in about 2 hr. When the column was at 37° the collecting tubes were kept at that temperature until a sample was removed for ultracentrifugal analysis, which was performed immediately at 35–37°. In some cases a second sample was chilled and examined at 5°. In a few experiments where the equilibration was carried out at 5° half the ultracentrifugal sample was analyzed promptly at 37 and 5°, and half was kept at 37° for 2 hr before analysis. Some ribosome samples were equilibrated by dialysis for 2 or more days, with stirring or rocking, at 5°.

Ultracentrifugal Analysis. Samples containing about 2 mg of ribosomes/ml were examined in analytical ultracentrifuges in 30-mm double-sector cells, with schlieren optics, at 5° and 44,000 rpm, or at 37° and 36,000 rpm. For analyses at 37° the rotor and cell were warmed in an incubator, and kept under an infrared lamp while the cell was inserted and aligned. The temperature control (RTIC) was set at 37° as soon as the rotor was put into the vacuum chamber. Two minutes after the oil diffusion pump was turned on the refrigeration was started. It was turned off after 5 min (just before acceleration), turned on again after the last picture had been taken, and kept on for 8 min before deceleration. This procedure prevented the condensation of oil on the lower collimating lens. With a black-anodized rotor the actual temperature during the run was about 35°. For analyses with ultraviolet absorption optics solutions containing 80 μg of ribosomes/ml were examined in a 12-mm cell at 5° and 44,000 rpm.

The sedimentation coefficients of the ribosomes and of the large and small subunits varied with the solvent and with nucleoprotein concentration (Petermann, 1964). For convenience the dimer has been called 120 S, the single ribosome, or monomer, 83 S, the large subunit 53 S, and the small subunit 32 S. A 62S boundary represented a compact form of the large subunit, dimers of the small subunit, or a mixture of these.

The amounts of the various components were calculated from the areas under the curves, with correction for radial dilution. At the low concentrations used the Johnston–Ogston effect was negligible. Samples containing 2 mg/ml of RNA were analyzed in a 30-mm cell,

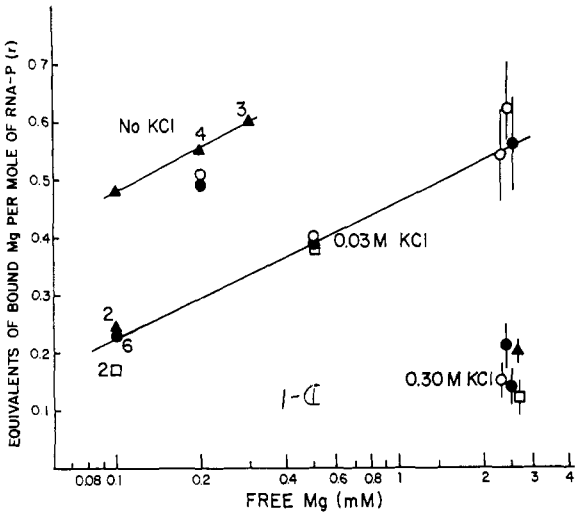

FIGURE 2: Magnesium binding in 0.001 M potassium phosphate, with no KCl or 0.03 M KCl at pH 7.3 or with 0.3 M KCl at pH 7.2. At 5 (solid symbols) or 37° (open symbols). (▲) Dialyzed; (●, □) equilibrated on Sephadex; (□) washed ribosomes (see text). For points in 2.5 mM $MgCl_2$ the errors resulting from 2% errors in total and free magnesium are indicated by vertical lines. Where several experiments have been averaged the number is given. With no KCl the values are 0.58, 0.57, 0.52, and 0.52 in 0.2 mM $MgCl_2$, and 0.66, 0.58, and 0.57 in 0.3 mM $MgCl_2$. With 0.03 M KCl, 0.1 mM $MgCl_2$, the values are 0.24 and 0.24 for the dialyzed samples; 0.26, 0.26, 0.23, 0.23, 0.23, and 0.22 for the samples equilibrated on Sephadex at 5°; and 0.17 and 0.17 for the samples equilibrated at 37°.

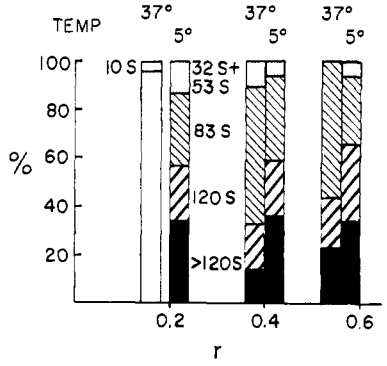

FIGURE 3: The relative amounts of the various ultracentrifugal components of ribosomes in 0.001 M potassium phosphate, 0.03 M KCl, and 0.1, 0.5, or 2.5 mM $MgCl_2$. Ribosomes were equilibrated at 37° and analyzed at 35° or equilibrated and analyzed at 5°.

with buffer in a reference cell, at 25° and 48,000 rpm, with schlieren optics.

Chemical Analyses. Samples for magnesium analysis were stored at −20° until extracted with 2.5% trichloroacetic acid. Magnesium was determined by the titan yellow method (Petermann, 1960) or by atomic absorption spectrophotometry. The same result was obtained by either method. The difference between total and buffer magnesium was taken to represent bound magnesium. It was expressed as equivalents per mole of RNA phosphate (*r*). For experiments in 2.5 or 5 mM $MgCl_2$ the errors resulting from 2% errors in free and total Mg were calculated. RNA concentration was measured by a modified orcinol method (Petermann, 1964).

Hyperchromicity. The effect of temperature on absorbance at 260 mμ was measured as described by Cavalieri *et al.* (1962), on solutions containing 40 μg of ribosomes/ml, in 0.03 M KCl–0.001 M potassium phosphate–0.1 mM $MgCl_2$ (pH 7.3).

Results

Ribosomes. These ribosomes, isolated in the presence of bentonite, contained large amounts of dimers, trimers, and larger particles. Preparations that had been dialyzed against 0.001 M phosphate–0.3 mM $MgCl_2$ had an *r* of 0.6, and contained 75% of particles larger than monomers; when the phosphate buffer contained 0.2 mM $MgCl_2$ the ribosomes had an *r* of 0.55 and contained 70% of larger particles.

The proportion of particles greater than monomers was unaffected by further dialysis or by equilibration at 5° but was reduced to about 50% by sonication for 10 sec at 20 kcycles. This brief sonication caused no apparent damage to the rRNA; after it had been isolated, freed of magnesium, and heated to dissociate any hydrogen-bonded fragments, RNA from sonicated ribosomes showed the same ultracentrifugal pattern as RNA from unsonicated material. The ribosomes washed with NH_4Cl had an *r* of about 0.1 and were 45% dissociated to subunits.

Equilibration in Sephadex. The attainment of equilibrium in the Sephadex column was checked under conditions where removal of magnesium would be extensive and errors of analysis would be minimal. Equilibration in the cold was compared with thorough dialysis. Thus ribosomes put through the column at 5°, in 0.001 M phosphate–0.03 M KCl–0.1 mM $MgCl_2$, seemed to be in equilibrium with the solvent; they contained the same amount of magnesium, and showed the same degree of dissociation, as ribosomes dialyzed for 2 (Figure 1) or 6 days. Donnan effects are small in these buffers (Petermann, 1960; Goldberg, 1966) and have been ignored. Thus under the conditions of these experiments, with 60 mg of ribosomes in 0.03 M KCl, this volume of Sephadex was sufficient for the reduction of *r* from 0.55 to 0.23.

For experiments at 37° comparison with dialysis was impractical, since the ribosomes were not stable for the long time required for equilibrium dialysis. Instead, the magnesium content of the sample was adjusted to a level close enough to the expected final value so that the change on equilibration was well within the limits given above. For final *r* values of 0.3 or less *r* was reduced to 0.1 by the NH_4Cl wash. For *r* of 0.3–0.4 a preliminary

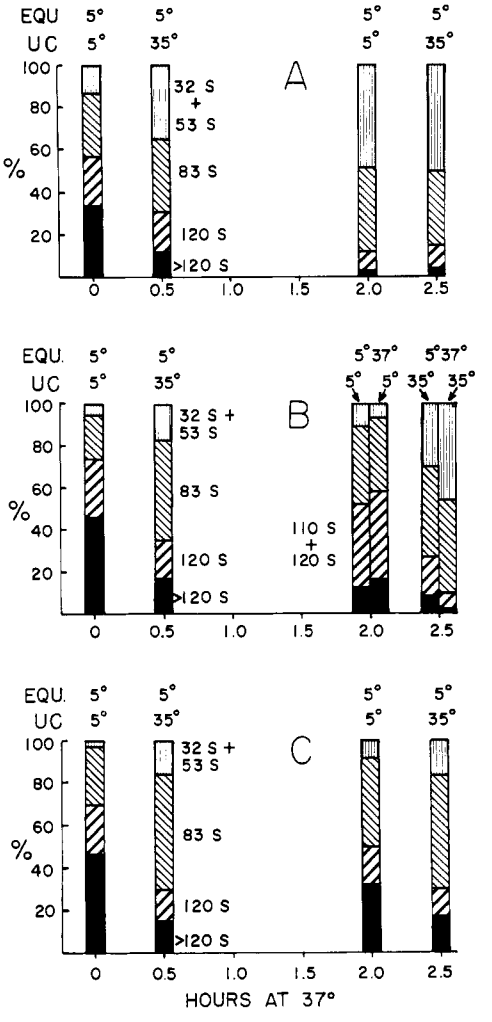

FIGURE 4: The effect of standing at 35–37° on the relative amounts of ribosomal components. The samples equilibrated on Sephadex at 5° (equ 5) were analyzed at 5 (UC 5) or 35° (UC 35) immediately or after 2 hr at 37°. The samples equilibrated for 2 hr at 37° (equ 37) were analyzed at 5 or 35° immediately. All samples contained 0.001 M potassium phosphate (pH 7.3). (A) In 0.03 M KCl–0.1 mM $MgCl_2$; r = 0.22; (B) in 0.3 M KCl–2.5 mM $MgCl_2$; r = 0.20; (C) in 0.3 M NH_4Cl, 2.5 mM $MgCl_2$; r = 0.15.

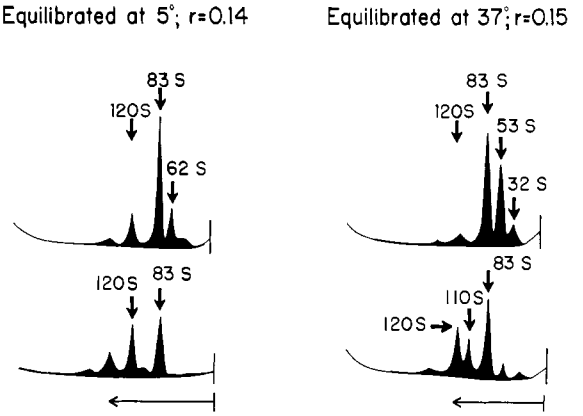

FIGURE 5: The effect of temperature on ultracentrifugal patterns of ribosomes in 0.3 M KCl, 0.001 mM potassium phosphate, and 2.5 mM $MgCl_2$ (pH 7.3). The left column shows samples equilibrated at 5° and centrifuged at 35 (upper pattern) and 5° (lower pattern). The right column shows samples equilibrated at 37° and centrifuged at 35 and 5°.

wash was carried out in the equilibration buffer; since the changes in r were less than 0.3 and the final r values were the same for washed and unwashed ribosomes, equilibration appeared to be complete.

Magnesium Binding and Ultracentrifugal Behavior

Phosphate Buffers. In 0.001 M potassium phosphate at pH 7.3 magnesium was tightly bound, and r remained high even in 0.1 mM $MgCl_2$ (Figure 2). In 0.2 mM $MgCl_2$ equilibration on Sephadex at 5° gave material with slightly lower r, but the same ultracentrifugal pattern as the dialyzed material. Equilibration at 37° gave a similar value of r but reduced the proportion of particles greater than monomers from 70 to 50%.

Phosphate-Chloride Buffers. The addition of 0.03 M KCl greatly reduced the binding of magnesium (Figure 2); ten times as much free magnesium was needed to reach the same value of r. At 5° equilibration was complete even in 0.1 mM $MgCl_2$, as described above. At 37°, however, equilibration down from the original magnesium-rich preparation gave an apparent r of 0.24. When r had first been reduced to 0.1 by the NH_4Cl wash, on the other hand, the value attained on the Sephadex column was 0.17. Since this change was only a fraction of the known equilibrating capacity of the column, 0.17 was accepted as the equilibrium value. In 0.5 mM $MgCl_2$ equilibration appeared to be complete at 37°, since a preliminary wash in the equilibration buffer did not change the r attained in Sephadex. The decrease in magnesium binding at the higher temperature was surprisingly small, only 26% in 0.1 mM $MgCl_2$ and negligible at the two higher magnesium concentrations.

Although temperature had little affect on their total magnesium binding, it did change the ultracentrifugal behavior of the ribosomes (Figure 3). In these experiments each ultracentrifugal analysis was made at a temperature near that of the Sephadex column. In 2.5 or 0.5 mM magnesium (r = 0.55 or 0.40) the cold ribosomes were still mainly dimers and larger particles, whereas the warm samples showed chiefly monomers. In 0.1 mM $MgCl_2$ the cold sample (r = 0.22) showed little dissociation. When it was warmed, however, the proportion of subunits increased to 35% (Figure 4A). The sample equilibrated at 37°, with r = 0.17, was completely dissociated (Figure 3) and contained a small amount of 10S material.

This effect of temperature on ribosomal dissociation suggested that some melting of RNA hydrogen bonds might be taking place; but when two samples were tested

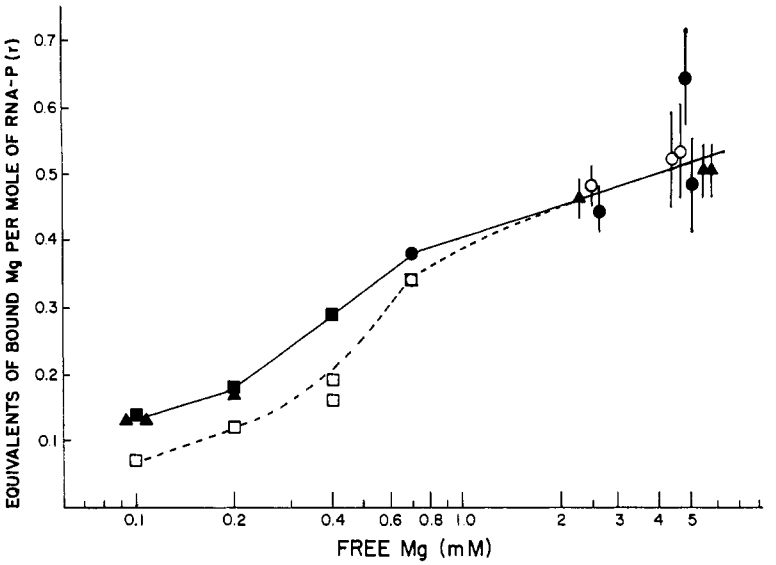

FIGURE 6: Magnesium binding in 0.05 M Tris–0.025 M KCl. (solid symbols) At 5°, pH 8.3; (open symbols) at 37°, pH 7.3. (▲) Dialyzed; (●, ■) equilibrated on Sephadex; (■) washed ribosomes (see text). For points in 2.5 or 5 mM $MgCl_2$ the errors resulting from 2% errors in total and free magnesium are indicated by vertical lines.

for hyperchromicity on heating in this buffer no increase in optical density was noted between 12 and 51°.

A second possible effect of temperature was on RNase activity. To study this, ribosomes equilibrated with buffer containing 0.1 mM magnesium at 5° (r = 0.22) were examined in the ultracentrifuge at 5 and 35° immediately, and again after incubation for 2 hr at 37°. The distribution of ultracentrifugal components is plotted against the total time at 35–37° in Figure 4A. The control sample, kept at 5°, still showed 57% of particles larger than monomers, a decrease of only 10% from the starting material, and there was little dissociation to subunits. The ultracentrifugal analysis at 35° took about 0.5 hr, by which time an extensive shift to more slowly sedimenting components had taken place. When the ribosomes were kept for 2 hr at 37° before analysis even more extensive disaggregation and dissociation were noted, and the temperature of analysis now made little difference. In all these experiments the boundaries were sharp, and only traces of degraded material appeared; nevertheless, as discussed below, some damage from RNase action seemed to have occurred.

The effect of time was also studied in 0.001 M potassium phosphate–0.3 M KCl–2.5 mM $MgCl_2$ at pH 7.2 (Figure 4B). The ribosomes were equilibrated with the buffer at both temperatures. Since the bound magnesium was low, and the free magnesium was high, the error in measuring bound magnesium was large. Although r was quite low, equilibration seemed to be complete, since values of r for material dialyzed or equilibrated at 5°, and for washed or unwashed ribosomes equilibrated at 37°, all agreed within experimental error (Figure 2). No effect of temperature on magnesium binding could be seen; r appeared to be 0.15–0.20 at both temperatures. In spite of the low magnesium content of these ribosomes, material equilibrated and sedimented at 5° showed mainly dimers and larger particles; on warming to 35° most of these were converted to monomers (Figures 4B and 5). Some of this material was kept at 37° for 2 hr. The sample that was analyzed warm now showed 30% dissociation, but the subunits reassociated to 83S, 110S, and 120S particles on cooling (Figure 4B). Ribosomes equilibrated and analyzed warm were 45% dissociated and underwent a similar reassociation on cooling (Figures 4B and 5, right). Both cooled samples showed unusually large 110S boundaries. Some damage by RNase had apparently occurred here also.

Ribosomes equilibrated with 0.001 M phosphate–0.3 M NH_4Cl–2.5 mM $MgCl_2$ (pH 7.3) at 5° also had a low r (0.15) and showed little dissociation at 5°; 70% of the material was still dimers or larger particles (Figure 4C). On warming to 35° most of these rapidly sedimenting components were converted to monomers, and the pattern resembled the upper left diagram in Figure 5. Incubation for 2 hr at 37° caused little additional change, and chilling this solution again caused aggregation to 110–120S and faster polydisperse material. RNase action seemed to be minimal. At 37° the Sephadex column developed gas bubbles, and equilibration was not attempted.

From these experiments it appeared that warming the ribosomes to 37° increased dissociation by two different mechanisms. One effect increased with time and was irreversible. It was less in 0.3 M than in 0.03 M KCl, and was further inhibited in 0.3 M NH_4Cl; this effect was probably due to the action of residual RNase. The second effect was independent of time, was reversible, and was most marked in 0.3 M KCl; this seemed to be a true temperature effect.

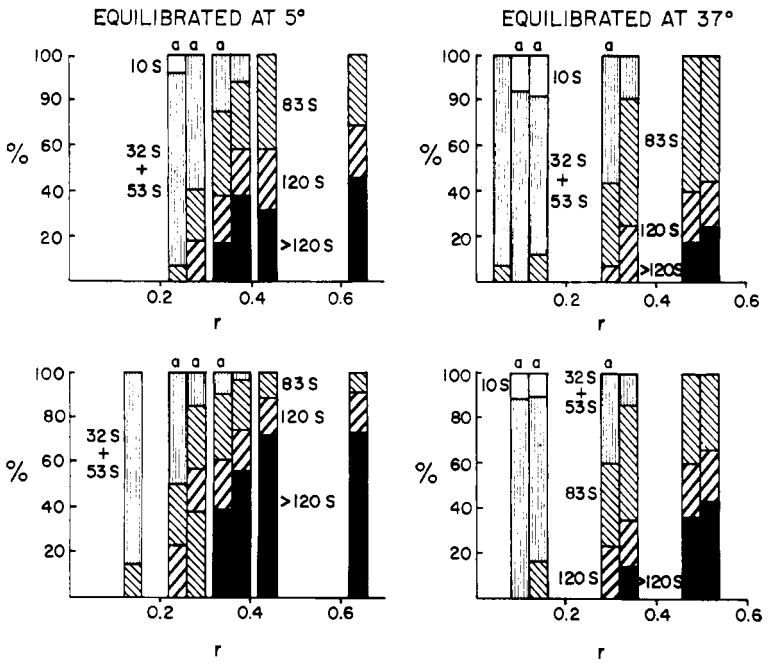

FIGURE 7: The relative amounts of the various ultracentrifugal components of ribosomes equilibrated at 5 (pH 8.3) or 37° (pH 7.3), in 0.025 M KCl, 0.05 M Tris, and 0.1–5 mM $MgCl_2$. Upper row: analyzed at 35°; lower row: analyzed at 5°. *a* indicates apparent *r*, from experiments where equilibration was incomplete; true *r* was about 18% lower (see text).

Tris-Cl Buffers. In 0.05 M Tris–0.025 M KCl with 0.1 mM $MgCl_2$ ribosomes dialyzed for 2 days at 5° had an *r* of only 0.13 and were 90% dissociated. They were also degraded, as shown by spreading in the ultracentrifugal pattern and the presence of slowly sedimenting material (Figure 1C). In contrast, the sample passed through a Sephadex column containing this buffer had an apparent *r* of 0.25 and was only 60% dissociated (Figure 1E). Adjusting the KCl concentration before the sample was put on the column had no effect.

Before equilibration, one sample was dialyzed against a buffer containing 0.3 M KCl. Although *r* was reduced to 0.20, the free magnesium was so high (2.5 mM) that the total amount of magnesium applied to the Sephadex column was 0.6 mg, as much as in the usual sample, and the final *r* was still high (Figure 1F). Reducing just the bound magnesium before the sample was put on the column did not ensure equilibration.

To reduce the total magnesium and avoid degradation, the best procedure was the preliminary NH_4Cl wash. After passage through Sephadex in the Tris buffer at 5° the washed ribosomes had $r = 0.14$, close to that of the dialyzed sample; although 85% of the material was dissociated, the ultracentrifugal boundaries remained sharp, and no degradation was noted (Figure 1D). The equilibrations in 0.1, 0.2, and 0.4 mM $MgCl_2$ were carried out with both unwashed and NH_4Cl-washed ribosomes, at both temperatures. In 0.2 mM $MgCl_2$ at 5° the washed ribosomes reached the same *r* as a dialyzed sample (Figure 6). Every value obtained on unwashed ribosomes ("apparent *r*") was too high. In order to obtain an estimate of the relative amounts of free and bound magnesium in these samples, the total (bound plus free) values for washed, equilibrated ribosomes, at a concentration of 2 mg/ml, were plotted against free magnesium at each temperature. For the total magnesium content of each unwashed, partially equilibrated ribosome sample the corresponding free magnesium was read from the curve, and bound magnesium was calculated by difference. The apparent *r* values obtained on the unwashed ribosomes were about 18% too high. In 0.7 mM $MgCl_2$ at 37°, on the other hand, since unwashed ribosomes and ribosomes washed in this buffer reached the same value of *r* (Figure 6), equilibration seemed to be complete. At 5° the magnesium binding curve (Figure 6) had about the same slope as the phosphate curve (Figure 2), but was moved to the right by a factor of 2.4; thus *r* was 0.46 at 1 mM magnesium in the phosphate-chloride buffer (total monobasic cation, 0.032 M) and at 2.4 mM magnesium in the Tris-Cl buffer (total monobasic cation, 0.064 M). The binding was not affected by temperature at high magnesium concentrations, but was markedly reduced at 37° when the free magnesium was low.

The distribution of ultracentrifugal components is shown in Figure 7. These ribosome preparations, which initially contained about 70% of dimers, trimers, etc., aggregated still further when equilibrated in 5 mM $MgCl_2$ at 5° ($r = 0.6$). One sample was adjusted to 80 μg/ml, kept at 37° for 2 min, chilled in ice, and examined in the ultracentrifuge at 5° with ultraviolet optics; even at this high dilution, it still showed 79% of particles faster than

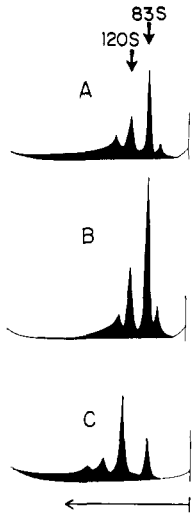

FIGURE 8: Effects of sonication and Tris–KCl buffer on ultracentrifugal patterns of ribosomes. (A and B) In 0.001 M potassium phosphate–0.2 mM $MgCl_2$ (pH 7.3); r = 0.57; (A) control; (B) after sonication for 10 sec; (C) sonicated ribosomes after dialysis against 0.025 M KCl–0.05 M Tris–2.5 mM $MgCl_2$ (pH 8.3 at 5°); r = 0.50.

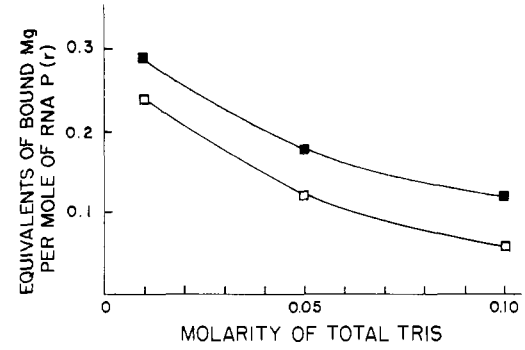

FIGURE 9: The effect of Tris concentration on the magnesium binding of NH_4Cl-washed ribosomes in 0.025 M KCl–0.2 mM $MgCl_2$. The buffers contained 23% Tris and 77% Tris-HCl on a molar basis. (■) Binding at 5° (pH 8.3); (□) binding at 37° (pH 7.3).

monomers. As magnesium was removed by equilibration at 5°, the ribosomes analyzed at 5° showed disaggregation and dissociation, but no degradation. Warming to 35° caused further disaggregation of the fast-sedimenting components, but led to extensive dissociation only when r was 0.3 or less.

Equilibration at 37° converted much of the rapidly sedimenting material to monomers even at high magnesium concentrations, and led to dissociation and some degradation in low magnesium buffers. These effects must have resulted from a number of factors; the RNase action and the real temperature effect noted in the phosphate experiments, and in addition the change from pH 8.3 to 7.3 and the decreased magnesium binding at 37°. When the solutions were chilled to 5° some reassociation of subunits occurred at r = 0.3, and at r = 0.5 there was aggregation to trimers and larger particles.

The effect of high magnesium in Tris buffer was reexamined on ribosome preparations that had been sonicated to beak up the aggregates. In phosphate buffer, with r = 0.57, 70% of the original preparation sedimented faster than the 83S boundary (Figure 8A); the sonicated material contained 43% of 83 S, 27% of 120 S, and 16% of larger particles (Figure 8B). When these sonicated ribosomes were dialyzed overnight against 0.025 M KCl–0.05 M Tris–5 mM magnesium, r remained about the same (0.50) but the concentration of 83S particles fell to 11%, the 120S peak increased to 31%, and the rest of the material formed larger aggregates (Figure 8C). In this buffer unsonicated ribosomes showed 70% of material larger than monomers (Figure 7, lower left).

To test whether the decrease in magnesium binding at 37° was due to increased competition by the basic Tris ion, the effect of varying Tris concentration was studied (Figure 9). At each temperature r varied inversely with Tris concentration, and the decrease in r on warming ranged from 17% in 0.01 M Tris to 50% in 0.1 M Tris.

Discussion

When rat liver extracts were treated with bentonite to adsorb RNase, the purified ribosomes were found mainly in the form of dimers, trimers, and larger aggregates. Some of these aggregates may have been polysomes, either whole or fragmented by mechanical manipulation, since they were stable at 5° on removal of magnesium down to r = 0.2, but were converted to monomers by two treatments that tend to break mRNA, warming to 35° or brief sonication that did not damage rRNA. When the charges on these monomers were largely neutralized by magnesium or potassium, in the cold, they associated to form random aggregates.

Equilibration of ribosomes on Sephadex was preferable to dialysis, since it could be carried out quickly, and RNase damage was reduced; but the capacity of the column to remove magnesium was limited. Use of a larger column was impractical because it would have increased the time the ribosomes had to be kept at 37°. The attainment of equilibrium was checked in low magnesium buffers, where the shifts in bound magnesium were extensive. In phosphate buffer containing 0.03 M KCl at 5° equilibration appeared to be complete. Reduction of r from 0.55 to 0.23 in this buffer, or to 0.35 in Tris-KCl, however, represented the limit of the method; for lower levels of r much of the magnesium had to be removed by a preliminary wash, or equilibration was incomplete.

In 0.3 M KCl equilibration seemed to be complete. The efficiency of the column depends on the KCl concentration of the solvent, and in magnesium-free buffer 0.1 M KCl suffices for complete removal of

magnesium (Petermann and Pavlovec, 1966). The process of equilibration seems to consist in a gradual substitution of potassium for part of the magnesium, since the magnesium is eluted gradually from the column. By contrast, when the magnesium is removed from the ribosomes by chelation with citrate, it emerges from a Sephadex column in a wide peak (Petermann and Pavlovec, 1963b).

Since the ultracentrifugal pattern of ribosomes often varies with temperature (Petermann and Pavlovec, 1963a) and dissociation is usually correlated with decreased magnesium binding, the small temperature effect on r in phosphate buffer was unexpected. The reason for this result is obscure. One important factor in determining the extent of magnesium binding is competition by potassium (Petermann, 1960), and the ratio of potassium to magnesium was high. In 0.001 M potassium phosphate–0.1 mM $MgCl_2$, the molar ratio of potassium to magnesium is 18; in phosphate plus 0.03 M KCl–2.5 mM $MgCl_2$, it is 12.7; and in the other buffers it is much higher. Wiberg and Neuman (1957), who studied the binding of calcium and magnesium to DNA and RNA in barbital buffer, found that "varying the equilibration temperature between 10 and 40° did not measurably change the observed binding values."

Although temperature had little effect on the total amount of magnesium bound in phosphate buffer, the ribosomes showed less aggregation, and more dissociation to subunits, at 35° than at 5°. Some of these effects are probably due to RNase that had not been removed by the bentonite. The rapid disaggregation was probably due to breakage of mRNA in the polysome fragments, while the gradual increase in subunits and the appearance of degraded material could be ascribed to breakage of rRNA. Since the ultracentrifugal boundaries were sharp and the amount of degraded material was small, damage by nucleases could not have been extensive.

In 0.3 M KCl the RNase activity appeared to be reduced. The high salt concentration may have decreased ribosomal binding of RNase (Salas *et al.* 1965); free RNase would have been retarded on Sephadex G-100 (Lansink, 1964). After 2 hr at 37° the ribosomes were partially dissociated, but, unlike those in the low-salt samples, these subunits reassociated on cooling. The concentration of 110S particles was unusually high; what combination of subunits these represent is not known. The high degree of association of these ribosomes, at low total magnesium, suggests either that the fraction of magnesium that holds the subunits together has been preferentially retained, or that neutralization of electrical charge contributes to ribosomal stability. In 0.3 M NH_4Cl RNase action seemed to be even less than in 0.3 M KCl; even after 2 hr at 37°, dissociation had increased only a few per cent.

The reasons for the reversible dissociation at higher temperature, without loss of magnesium, are not known. One possibility is redistribution of the magnesium. An r of 0.15 corresponds to about 450 atoms of magnesium/ribosome, and only a small fraction of these may be connecting the two subunits together. A second possibility is the melting of RNA hydrogen bonds, which appear to influence the stability of *Escherichia coli* ribosomes (Watson, 1964; Moore, 1966). The dissociation of rat liver rRNA with increasing temperature was paralleled by hyperchromicity (Petermann and Pavlovec, 1963b); but the ribosomes, like those of calf liver (Hall and Doty, 1959) and *E. coli* (Gesteland, 1966) showed no hyperchromicity in this temperature range. A third factor may be decreased hydrogen bonding in the water associated with the ribosomes. Liver ribosomes contain about 3 g of water/g of dry substance (Petermann, 1964), and the melting of water structures (Berendsen, 1966) may decrease ribosomal stability. Ethanol, which increases the hydrogen bonding of water, prevents ribosomal dissociation, whereas bond breakers such as urea and formamide enhance it (M. L. Petermann and A. Pavlovec, unpublished data).

The presence of Tris reduced the binding of magnesium. In 0.05 M Tris at 5° the binding curve was shifted to the right, so that over twice as much free magnesium was required for the same level of bound magnesium. In 0.2 mM magnesium r was inversely proportional to the Tris concentration. These facts suggest competition between Tris, a primary amine, and Mg^{2+}. When the temperature was raised to 37°, the change in r was greater at higher Tris concentration; more Tris-Cl dissociated to Cl^- and the charged amine (Good *et al.*, 1966), and the competition with Mg^{2+} was proportionately greater.

The change in pH, from 8.3 at 5° to 7.3 at 37°, had a relatively small effect on magnesium binding; in 0.01 M Tris r was only 17% lower at 37°. The magnesium binding of *E. coli* ribosomes, in 0.005 M Tris buffer at 4°, was found to be less at pH 8.3 than at pH 7.4 (Goldberg, 1966); but if these pH values were measured at 25° they may have been substantially higher at 4°.

When the ribosomes were equilibrated in Tris buffers at 37° there was RNase action, especially at low magnesium concentrations, where degradation was noted. The reversible temperature effect was also present. The increase in pH at 5° probably favored association to dimers rather than to larger aggregates, as in the case of sarcoma ribosomes (Petermann, 1960).

The buffer containing 0.05 M Tris–0.025 M KCl–5 mM $MgCl_2$ (pH 7.6 at room temperat re) is of particular interest, since it is the original medium A of Littlefield and Keller (1957), which is still extensively used for rat liver systems in studies of *in vitro* amino acid incorporation (at 37°) and polysome structure (at 5°). The tendency of free ribosomes to dimerize, or aggregate, in this buffer at 5° may account for the high concentration of dimers found in preparations from both nuclei (Howell *et al.*, 1964; Wilson and Hoagland, 1965) and cytoplasm (Franklin and Godfrey, 1966; Howell *et al.*, 1964; Wettstein *et al.*, 1963) of rat liver. On the other hand, the numerous dimers found in cytoplasmic extracts of liver tumors and of

nonneoplastic liver from animals treated with CCl_4 or actinomycin D were converted irreversibly to monomers by brief warming to 37° (Webb and Potter, 1966). Furthermore, the dimers observed by Wunner *et al.* (1966) in livers of rats fed tryptophan-deficient amino acid mixtures were converted to monomers by a very low concentration of RNase.

Preliminary experiments (M. L. Petermann and A. Pavlovec, unpublished data) have suggested that extra ribosomes may also attach to polysomes in medium A in the cold, as suggested by Wettstein *et al.* (1963). Munro *et al.* (1964) found extra ribosomes associated with rat liver polysomes in a high-salt, high magnesium buffer. Thus while the examination of ribosomes and polysomes at low temperature and in high magnesium solvents has the great advantage of reducing nuclease action, it may sometimes give a misleading picture of the physical state of the active units.

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References

Berendsen, H. J. (1966), *Federation Proc. 25*, 971.
Cavalieri, L. F., Small, T., and Sarkar, N. (1962), *Biophys. J. 2*, 339.
Franklin, T. J., and Godfrey, A. (1966), *Biochem. J. 98*, 513.
Gesteland, R. F. (1966), *J. Mol. Biol. 16*, 67.
Goldberg, A. (1966), *J. Mol. Biol. 15*, 663.
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., and Singh, R. M. M. (1966), *Biochemistry 5*, 467.
Hall, B. D., and Doty, P. (1959), *J. Mol. Biol. 1*, 111.
Howell, R. R., Loeb, J. N., and Tomkins, G. M. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1241.
Lansink, A. G. W. J. (1964), Yeast Ribosomes and Magnesium Ions, Netherlands, Janssen.
Littlefield, J. W., and Keller, E. B. (1957), *J. Biol. Chem. 224*, 13.
Moore, P. B. (1966), *J. Mol. Biol. 22*, 145.
Munro, A. J., Jackson, R. J., and Kroner, A. (1964), *Biochem. J. 92*, 289.
Petermann, M. L. (1960), *J. Biol. Chem. 235*, 1998.
Petermann, M. L. (1964), The Physical and Chemical Properties of Ribosomes, Amsterdam, Elsevier.
Petermann, M. L., and Pavlovec, A. (1963a), *J. Biol. Chem. 238*, 318.
Petermann, M. L., and Pavlovec, A. (1963b), *J. Biol. Chem. 238*, 3717.
Petermann, M. L., and Pavlovec, A. (1966), *Biochim. Biophys. Acta 114*, 264.
Salas, M., Smith, M. A., Stanley, W. M. Jr., Wahba, A. J., and Ochoa, S. (1965), *J. Biol. Chem. 240*, 3988.
Watson, J. D. (1964), *Bull. Soc. Chim. Biol. 46*, 1399.
Webb, T. E., and Potter, V. R. (1966), *Cancer Res. 25*, 1022.
Wettstein, F. O., Staehelin, T., and Noll, H. (1963), *Nature 197*, 430.
Wiberg, J. S., and Neuman, W. F. (1957), *Arch. Biochem. Biophys. 72*, 66.
Wilson, S. H., and Hoagland, M. B. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 600.
Wunner, W. H., Bell, J., and Munro, H. N. (1966), *Biochem. J. 101*, 417.