port device, and argues that the new histone is associated with the chromosome *before* it is phosphorylated. It will obviously be of great importance to try to resolve the question of whether there is a delay in phosphorylation after the histone is deposited on the chromosome, or whether the lag period represents a mean time during which the histone is passing through a pool of newly synthesized material and that the phosphorylation event occurs fairly promptly after deposition upon the newly synthesized DNA. Such an analysis is currently under way. However, at this time the available evidence points toward a phosphorylation event involving essentially all histone F_1 molecules, possibly in the region of the DNA replication point. We do not know what the function of such an activity might be, but it seems unavoidable that it must play an important and integral part in chromosome replication, and as such would represent the first clearly identifiable role for a histone molecule.

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Effects of Agents That Influence Hydrogen Bonding on the Structure of Rat Liver Ribosomes†

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ABSTRACT: Rat liver ribosomes were exposed to a number of agents that affect hydrogen bonding. Ethanol, which strengthens hydrogen bonding in water, aggregated the ribosomes at 5°, and prevented dissociation to subunits at 35°. Ethylene glycol had similar but weaker effects. Urea, which weakens hydrogen bonding, first dissociated the ribosomes to active 59S and 40S subunits, with molecular weights of 3.0×10^6 and 1.5×10^6. At higher urea concentrations the subunits were converted to slowly sedimenting forms. The 39S large subunit had lost its 5S RNA, but its large RNA was still 28 S, and its molecular weight showed little change. The 27S small subunit still contained 18S RNA, but had lost 35% of its protein; its molecular weight was only 1.14×10^6. Formamide, another agent that weakens hydrogen bonding, also dissociated ribosomes. The first large subunits, 55 S, were active, but additional formamide converted most of them to 41S particles. The 5S RNA and its associated protein were detached but the residual particles showed no further loss of protein, and still contained 28S RNA. The small subunit, 27 S, still contained 18S RNA, but had lost 23% of its protein, and had a molecular weight of 1.27×10^6. The effects of both urea and formamide increased with time, and were enhanced by other factors that tend to dissociate ribosomes, such as increased temperature, decreased magnesium binding, or high pH. The effect of every agent could be attributed to its action on hydrogen bonds. Since liver ribosomes are highly hydrated, the sensitive hydrogen bonds may include those of ribosome-associated water as well as those of RNA and protein.

Since animal ribosomes can dissociate to subunits on warming, and reassociate on cooling, with no change in their total magnesium content (Petermann and Pavlovec, 1967), bonds other than salt linkages must also play an important part in subunit association. One cohesive force appears to be hydrogen bonding between the RNA bases, since ribosomal subunits do not reassociate when their amino groups are masked by formaldehyde treatment (Moore, 1966; Petermann and Pavlovec, 1969). The present study concerns the effects on ribosomal structure of agents that affect hydrogen bonding. The behavior of the ribosomal subunits was followed by analytical ultracentrifugation, and slowly sedimenting forms were isolated and characterized. Ethanol, which enhances hydrogen bonding in water (Franks, 1965), prevented dissociation on warming; ethylene glycol had a similar effect. Two agents that weaken hydrogen bonding, urea (Frank, 1965)

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and formamide (Helmkamp and Ts'o, 1961), first dissociated the ribosomes to active subunits. At higher concentrations of both agents the subunits unfolded, the 5S RNA was detached from the large subunit, and a portion of the protein was removed from the small one.

Materials and Methods

Liver ribosomes from normal adult male rates (N[1] ribosomes) were isolated as described by Petermann and Pavlovec (1967) and stored in 2% sucrose at −20° (Petermann, 1971a). In some experiments the rats were given 25 mg of phenobarbital/day, in the drinking water, for 7 days before sacrifice, to decrease the ribonuclease content of the liver (Louis-Ferdinand and Fuller, 1970), and the preparative procedure was modified by adding 1 mM dithiothreitol to all the solvents and clarifying the ribosome solution by centrifugation at 20,000*g* for 15 min before the magnesium precipitation step (Petermann and Pavlovec, 1971). These ribosomes are referred to as P ribosomes.

rRNA was isolated as described by Petermann and Pavlovec (1963), except that citrate was omitted. For sedimentation analysis in urea or formamide the RNA was equilibrated with buffer K containing 0.1 mM $MgCl_2$ on a column of G-100 Sephadex. To analyze for hidden breaks it was freed of magnesium by dialysis against 10 mM sodium phosphate–10 mM sodium citrate (pH 6.8) and heated to 90° for 2 min.

Equilibration with Buffers. For experiments in buffer K (30 mM KCl–1 mM potassium phosphate, pH 7.3) containing 0.5 mM $MgCl_2$ the solvent was adjusted by dilution. For example, 11 mg of ribosomes in 1 ml of 1 mM phosphate–0.2 mM $MgCl_2$, with $r = 0.55$, contained 4.8 μg of free magnesium and 114 μg of bound magnesium. When 1.5 ml of 50 mM KCl–1 mM phosphate (pH 7.2) was added the resulting solution was 30 mM in KCl, and contained 4.4 mg of ribosomes and 47.5 μg of magnesium per ml, about the same amount as ribosomes equilibrated with buffer containing 0.5 mM $MgCl_2$ ($r = 0.4$; bound magnesium = 33 μg/ml and free magnesium = 12 μg/ml). These calculations were based on the magnesium-binding curves of Petermann and Pavlovec (1967). When accurate sedimentation coefficients were required the sucrose was removed by dialysis against the final buffer for 2 or 3 hr in the apparatus of Englander and Crowe (1965) or in a Schleicher–Schuell collodion bag containing a glass rod 8 mm in diameter (Petermann and Pavlovec, 1969). Some solutions were concentrated by dialysis under reduced pressure.

To decrease the bound magnesium to r of less than 0.4 simple dilution did not suffice. The ribosomes were freed of sucrose and equilibrated with buffer K containing 0.1 mM $MgCl_2$ by rapid dialysis, as described above, or by gel filtration on G-100 Sephadex (Petermann and Pavlovec, 1967). For experiments in 300 mM KCl–0.5 mM $MgCl_2$–1 mM phosphate (pH 7.1), where the bound magnesium was very low ($r = 0.09$), the ribosomes were diluted with five volumes of 350 mM KCl–0.5 mM $MgCl_2$–1 mM phosphate, pelleted by centrifugation for 1 hr at 150,000*g*, resuspended in the 300 mM KCl buffer, and passed through a G-100 Sephadex column in that buffer.

For the reassociation experiments the unfractionated subunits were exposed to urea or formamide as described below, then dialyzed in a collodion bag with a glass rod. After 1-hr dialysis against buffer K containing 3 mM $MgCl_2$ the free Mg inside the bag had risen to 1.5 mM, and most of the urea or formamide had dialyzed out. If necessary the solution was concentrated by dialysis under reduced pressure against buffer K containing 1.5 mM $MgCl_2$. The pH of Tris buffers was measured at 5°.

Addition of Solvents. Cold absolute ethanol or ethylene glycol was added dropwise, with thorough mixing, to a ribosome sample in an ice bath. Urea (8 or 10 M), dissolved in the appropriate buffer and freshly treated with charcoal (Hamilton and Ruth, 1967), was added dropwise to ribosomes or RNA at 0°. Formamide was freshly titrated with 0.1 N KOH or 0.2 N HCl until a sample had a pH of 7.3 after dilution to 8.9 M with buffer K. It was added dropwise at 0°.

Isolation of Subunits. For the urea experiments 60 mg of ribosomes was mixed with one-tenth volume of 1.1 M KCl and dialyzed against buffer K containing 0.1 mM $MgCl_2$ in a small Zeineh dialyzer (Bio-Med Instruments, Chicago), for 2 hr. Urea was added to a final concentration of 2, 3, or 5 M. After standing for 30 min in ice the solution was dialyzed against the pH 7.8 buffer (10 mM KCl–0.05 mM $MgCl_2$–1 mM potassium phosphate, pH 7.8) for 45–75 min, in the Zeineh dialyzer, to remove most of the urea, and the subunits were fractionated by sedimentation in a 10–20% sucrose gradient in the pH 7.8 buffer, in a Spinco BXIV zonal rotor, for 19 hr at 26,000 rpm at 5° (Petermann, 1971a). For equilibrium centrifugation samples from the peak regions were fixed by dialysis against 2 M formaldehyde–0.2 mM $MgCl_2$–3 mM potassium phosphate (pH 7.3) at 5°, for 1 day. They were then dialyzed against 0.1 M formaldehyde–30 mM KCl–0.2 mM $MgCl_2$–0.01 M triethanolamine-HCl (pH 7.3) for 2 days. Other samples were stored at −20°. Prior to ultracentrifugal analysis the unfixed samples were dialyzed against the pH 7.8 buffer in collodion bags containing rods for 4 hr to remove the sucrose. To examine the RNA 0.36 volume of 1 M NaCl and 0.14 volume of 10% sodium dodecyl sulfate were added (Kurland, 1960; Gesteland, 1966). After standing at room temperature for 10 min the solutions were centrifuged for 10 min at 1000*g*, at 5°, and the supernatants were examined in the analytical ultracentrifuge.

To isolate the formamide subunits 80 mg of P ribosomes was mixed with 0.15 volume of 0.7 M KCl and dialyzed in the Zeineh dialyzer against 30 mM KCl–0.1 mM $MgCl_2$–3 mM potassium phosphate (pH 7.3) for 2.5 hr. After standing for 30 min with 8.9 M formamide the solution was dialyzed against the pH 7.8 buffer for 2 hr and centrifuged in the zonal rotor for 19 hr at 26,000 rpm. Samples from the three peak regions were fixed or stored at −20° until they could be dialyzed free of sucrose and treated as described above. Since the RNA tended to aggregate after the sodium dodecyl sulfate treatment, the NaCl concentration was reduced to 0.1 M.

Ultracentrifugal Analysis. Samples containing 1–3 mg of ribosomes or subunits per ml were examined in analytical ultracentrifuges in 30-mm double-sector cells, with the appropriate solvents in the reference compartments, at 44,000 rpm at 5°, or occasionally at 25 or 35° (Petermann and Pavlovec, 1967), with schlieren optics. More concentrated solutions were analyzed in 12- or 6-mm cells; when the sedimentation coefficients were very concentration dependent, for example, in 5 M urea, speeds of 40,000 or 36,000 rpm were used, to avoid excessive boundary sharpening. Samples containing isolated RNA were examined in 30-mm cells at 44,000 rpm

[1] Abbreviations used are: N ribosomes, material from untreated rats; P ribosomes, material from rats pretreated with phenobarbital; buffer K, 30 mM KCl–1 mM potassium phosphate (pH 7.3); pH 7.8 buffer, 10 mM KCl–0.05 mM $MgCl_2$–1 mM potassium phosphate (pH 7.8); r, milliequivalents of magnesium bound per mole of RNA phosphate.

TABLE I: Properties of Ribosomal Subunits and Particles Derived from Them.

	Dissociating Agent	Mol Wt ($\times 10^{-6}$)	Buoyant Density in CsCl (g/cm^3)	Protein Content (%)
Small subunit	2 M urea	1.50[a]	1.551[a,b] (0.002, 10)	55[a]
	5 M urea	1.14	1.589[b] (0.001, 4)	47
	8.9 M formamide	1.27	1.577 ± 0.001[c]	50
Large subunit	2.7 M urea	3.00[a]	1.614[a,b] (0.002, 12)	43[a]
	5 M urea	3.0	1.607 ± 0.001[c]	44
	8.9 M formamide (54 S)	3.0	1.598 ± 0.001[c]	45
	8.9 M formamide (41 S)	(3.0)[d]	1.617 ± 0.001[c]	42

[a] Hamilton *et al.* (1971). [b] Figures in parentheses show the standard error of the mean and the number of determinations. [c] Duplicate determinations. [d] Assumed value (see text).

at 5°. The concentrations of the various ultracentrifugal components were computed from the areas under the schlieren boundaries (Petermann, 1964). No correction was made for Johnston–Ogston effects. Since these effects would vary with the positions of the boundaries, all concentrations were calculated from pictures in which the principal boundaries were near the middle of the cell. The effects of urea and formamide on the refractive index increment of the ribosomes were measured in the Spinco Model H electrophoresis apparatus with interference optics. Urea (5 M) had no effect. In 8.9 M formamide the refractive index of the ribosomes was reduced 22%; all the concentrations measured in formamide were corrected proportionately. When a boundary did not clearly represent either subunit, the amount of each was estimated by assuming that large subunits made up 67% of the total, and small subunits 33%. These estimates were confirmed by analysis of fractions isolated by sucrose gradient centrifugation.

The viscosities and densities of solutions containing 15% (3.26 M) ethanol, 20% (3.22 M) ethylene glycol, 1–5 M urea, and 4.4, 6.6, and 8.9 M formamide, plus the appropriate buffer salts, were measured at 5° and, when needed, at 25 or 35°. For extrapolation to other urea and formamide concentrations the specific viscosity and the density were assumed to vary linearly with concentration. In correcting the sedimentation coefficients to $s_{20,w}$ the anhydrous partial specific volumes ($\bar{v}$) were used: whole ribosomes, 0.63 (Haga *et al.*, 1970); large subunit, 0.63; 40S small subunit, 0.645 (Hamilton *et al.*, 1971); slower forms of small subunit, 0.63; and RNA, 0.53 (Petermann and Pavlovec, 1966). For the small subunit $s^0_{20,w}$ was obtained by extrapolation of plots of s *vs.* concentration; for the large subunit, regression lines were calculated for $1/s$ *vs.* the concentration of that component plus all the slower material.

With the isolated subunits, samples containing 60 μg/ml were examined in 12-mm cells, at 44,000 rpm and 5°, with ultraviolet optics. To check homogeneity the zonal fractions were also analyzed in the presence of 2 mM EDTA, which converted the large subunits to 35S particles and the small ones to 25 S (Petermann and Pavlovec, 1971). The sodium dodecyl sulfate treated samples were centrifuged at 60,000 rpm at 5°; the sedimentation coefficients, s_{20}, were corrected only for changes in the viscosity of water.

The molecular weights of the formaldehyde-fixed subunits were determined by the meniscus depletion method of equilibrium centrifugation (Hamilton *et al.*, 1971). Samples containing 0.6 A_{260} unit/ml were centrifuged for 3 days in multichannel cells, in an An-J rotor, at 5°, at 4800 rpm (small subunit) or 3000 rpm (large subunit), in an ultracentrifuge with a photoelectric scanner. Other samples, containing 3–4 A_{260} units/ml, were examined with interference optics.

For isodensity equilibrium centrifugation 0.2 A_{260} unit of fixed subunit was adjusted to a final volume of 1.0 ml and a density of 1.58 (small subunit) or 1.60 g per cm^3 (large subunit) by the addition of saturated CsCl. The solutions were centrifuged overnight at 52,000 (small subunit) or 44,000 rpm (large subunit), and the percentage of RNA was calculated from the reciprocal of the buoyant density by means of the calibration curve of Hamilton (1971).

The frictional ratios (f/f_0) were calculated (Edsall, 1953) from s^0, $\bar{v}$ as given above, and the molecular weights given in Table I. The measured molecular weights for large subunits were close to 3.0×10^6, but since the 55–60S particles still contained the 5S RNA and its associated protein, their molecular weights have been assumed to be 3.1×10^6.

Characterization of Detached RNAs. P ribosomes were treated with 5 M urea or 8.9 M formamide, dialyzed against the pH 7.8 buffer, and centrifuged in 2-ml tubes at 150,000*g* for 1.5 hr. The urea pellets were combined and washed by resedimenting once in the buffer and once in 0.01 M EDTA, and the supernatant and both wash solutions were analyzed by polyacrylamide gel electrophoresis (Petermann *et al.*, 1972). The formamide supernatant was examined by gel electrophoresis and by fractionation on G-100 Sephadex (Galibert *et al.*, 1965).

Chemical Analyses. Approximate ribosome concentrations were calculated from the absorbance at 260 nm, with an extinction coefficient, $E^{1\,cm}_{1\%}$, of 140. When an accurate measurement was needed RNA was determined by the orcinol method (Petermann, 1964). Bound magnesium was determined as described by Petermann and Pavlovec (1967).

Results

Agents That Decrease Dissociation. The effects of ethanol and ethylene glycol were studied with N ribosomes in a solvent in which the ribosomes dissociate to subunits on warming (Petermann and Pavlovec, 1967). Although r was low, 0.09, only 30% of the ribosomes dissociated at 5°; in addition to the 82S boundary, dimers and larger aggregates were still present (Figure 1). At 35° dissociation was extensive. After ethanol had been added to the cold ribosomes to a final concentration of 15% (3.26 M), on the other hand, no subunit boundaries

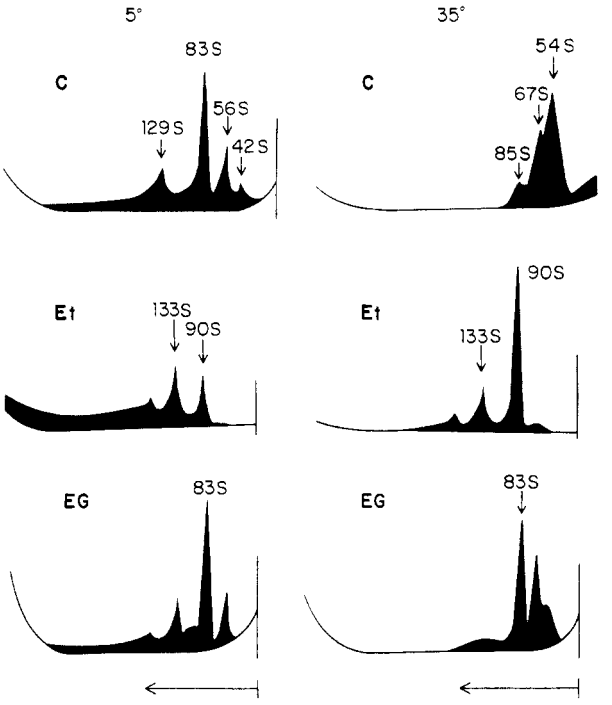

FIGURE 1: The effects of temperature, ethanol, and ethylene glycol on the sedimentation behavior of N ribosomes in 300 mM KCl–0.5 mM $MgCl_2$–1 mM potassium phosphate (pH 7.2); r = 0.09. C, controls; Et, in 3.26 M ethanol; EG, in 3.22 M ethylene glycol. Left, centrifuged at 5°; right, centrifuged at 35°. All at a concentration of 2 mg/ml.

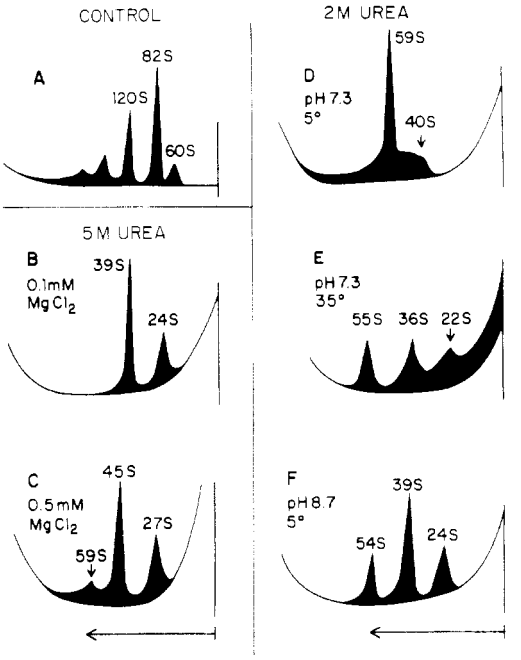

FIGURE 2: The effect of urea on the sedimentation behavior of N ribosomes and subunits, at a concentration of 2 mg/ml. (A) Control ribosomes in buffer K (30 mM KCl–1 mM potassium phosphate, pH 7.3), with 0.1 mM $MgCl_2$ (r = 0.22), at 5°. (B and C) Subunits produced in 5 M urea, at 5°. (B) Same buffer as in part A; (C) buffer K with 0.5 mM $MgCl_2$ (r = 0.4); both at 5°. (D, E, and F) Subunits produced in 2 M urea. (D and E) Same buffer as in part A, at two temperatures; (F) in 30 mM KCl–0.1 mM $MgCl_2$–1 mM potassium phosphate at pH 8.7.

were noted, and most of the material had aggregated to particles greater than dimers. When this solution was warmed to 35° the aggregation was reversed, but, unlike the control, the pattern showed mainly single ribosomes and dimers, with only a trace of subunits. At both temperatures $s_{20,w}$ increased to 90 S for the monomers and 133 S for the dimers. Ethylene glycol (20%, 3.22 M) also decreased dissociation at both temperatures, but to a lesser degree (Figure 1).

Agents That Increase Dissociation. Urea and formamide caused the ribosomes to dissociate, and the subunits to assume conformations with lower values of $s^0_{20,w}$. For convenience, these forms have been named according to approximate sedimentation coefficients; the precise values are given below. The rRNAs in buffers containing sodium dodecyl sulfate, with s_{20} ranging from 17 to 20 S and from 30 to 35 S, have been called 18 and 28 S.

Urea. In buffer K containing 0.1 mM $MgCl_2$, although r was only 0.22, most of the ribosomes were still monomers or dimers (Figure 2A). At 5° 2 M urea dissociated 80% of them to subunits (Figure 2D). At 35° dissociation was complete, and most of the subunits were converted to slowly sedimenting forms (Figure 2E); in contrast to the experiments at 5°, degraded material appeared at the miniscus. Electrical charge also affected subunit behavior. At pH 8.7 (Figure 2F) dissociation was complete at 5°, and most of the subunits sedimented slowly. The effect of bound magnesium is described below.

In the pH 7.8 buffer the subunits showed an ultracentrifugal pattern like that in Figure 2D. The zonal pattern (Figure 3A) showed 59S and 40S peaks plus a little 27 S and some slow material. When various fractions were examined in the presence of EDTA, however, large subunits were found in the 40S region, and small subunits in the 59S region.

The 3 M urea-treated subunits showed less 59 S and more 39–40 S and 27 S, both in the analytical ultracentrifuge (not shown) and in the zonal pattern (Figure 3B). In the presence of EDTA the 27S peak proved to be only small subunits, and its RNA gave an 18S boundary plus 20% of slowly sedimenting material. The 39–40S region contained both large and small subunits, and its RNA showed both 28 and 18 S. The 59S peak was almost entirely large subunits.

With 5 M urea and 0.1 mM magnesium chloride (Figure 2B) conversion to the slow forms was complete. Both boundaries were sharp, even at this low concentration, and no slowly sedimenting material was evident. When more magnesium was present (Figure 2C) the changes were less extreme. The P ribosomes behaved like N ribosomes. In the zonal fractionation (Figure 3C) 31% of the ultraviolet-absorbing material sedimented in the small-subunit region; these particles gave a sharp boundary in the analytical ultracentrifuge (Figure 4A), and their RNA (Figure 4B) showed only 18 S. After formaldehyde fixation the particles had a molecular weight of 1.14×10^6 and a buoyant density of 1.589 g/cm^3, corresponding to a protein content of 47% (Table I). The large-subunit zone (Figure 3C) contained 62% of the ultraviolet-absorbing material. In the analytical ultracentrifuge fraction 42 gave a sharp boundary (Figure 4C), and its RNA was still 28 S (Figure 4D). The fixed particles had a molecular weight of 3.0×10^6 and a buoyant density of 1.607 g/cm^3, corresponding to 44% protein (Table I).

Interpreting the schlieren patterns in Figure 2 in the light of the zonal experiments, we conclude that while a 24–27S boundary represents only small subunits, the faster boundaries may contain either subunit.

Reversibility of Dissociation. In buffer K containing 1.5

TABLE II: Sedimentation of the Large Subunit, Measured on Unfractionated Mixtures, in Buffer Alone or Buffer Containing Urea or Formamide, at 5°.[a]

s^0 (S)	k	f/f_0	Urea or Formamide (M)	$MgCl_2$ (mM)	KCl (mM)	pH
Buffer alone						
60		1.88[b]	0	0.1–1.5	30	7.3
58.7		1.92[b]	0	0.5	30	8.7
59.1	0.027	1.92[b]	0[c]	0.2	30	7.3
58.5	0.049		0[c]	0.1	10	7.8
+Urea						
58.7	0.059	1.92[b]	0.5–3	0.1	30	7.3
53.8	0.053	2.05[d]	4	0.1	30	7.3
44.7	0.065	2.47[d]	4, 5	0.5	30	7.3
39.7	0.056	2.78[d]	3–5	0.1	30	7.3
+Formamide						
54.9	0.031	2.06[b]	4.4–8.9	0.1	30	7.3
55.7	0.047	2.03[b]	8.2	0.1	30	7.3
40.5	0.052	2.73[d]	8.2	0.1	30	7.3
35.1	0.032		8.9	0[e]	20[f]	8.7

[a] In phosphate buffer except where indicated. k is the constant in the equation $1/s = 1/s^0(1 + kc)$, computed from each regression line, with c = mg/ml. f/f_0 is the frictional ratio. [b] Molecular weight 3.1×10^6 (see Methods). [c] 2 M urea dialyzed out (see text). [d] Molecular weight 3.0×10^6. [e] EDTA present. [f] 10 mM Tris buffer.

mM $MgCl_2$ the subunits obtained in 2 M urea reassociated almost completely to 81 S or larger particles. The 5 M urea-treated subunits sedimented more rapidly, at 34 and 54 S, but did not reassociate.

Effect of Time. Since about 50 min had elapsed between the addition of urea and the photograph used for concentration measurements, the effect of additional time was examined. When N ribosomes were kept in buffer K containing 0.1 mM $MgCl_2$, in 3 M urea, for an additional 2 hr before analysis the 39 S rose from 54 to 85%. With P ribosomes the amount of 39 S doubled in 2 hr, but the RNA (extracted with sodium dodecyl sulfate) still showed normal amounts of 28 and 18 S, and only 9% of slowly sedimenting material.

Sedimentation Coefficient of the Subunits. The most rapidly sedimenting form of the large subunit, 60 S, was found in small amounts with undissociated ribosomes (Figure 2A, Table II). Slightly slower forms were seen when ribosomes were partially dissociated at pH 8.7 or after 2 M urea had been dialyzed out.

When the anhydrous partial specific volumes of the subunits were used to calculate the density corrections for sedimentation coefficients measured in urea solutions, very consistent results were obtained over a wide range of urea concentrations. Thus for large subunits in solvents containing 0.5–3 M urea 8 measurements fell close to the regression line, $1/s = 1/s^0(1 + kc)$; the standard deviation of the slope was only 6%. The intercept, s^0, was 58.7 S, close to the values obtained in urea-free solution (Table II). For the slowest form, 39.7 S, 13 measurements, in 2–5 M urea, also fell close to one line. As s^0 decreased k remained about the same (Table II), so that the slope of the line increased about 40% relative to its intercept.

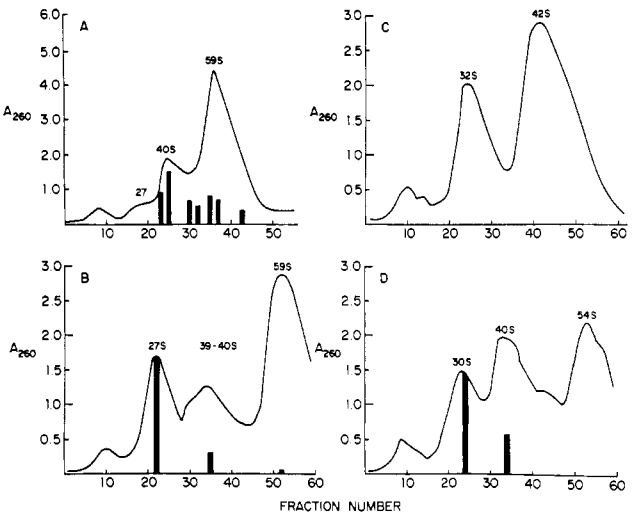

FIGURE 3: The fractionation of subunits in the zonal rotor, in the pH 7.8 buffer. (A) N ribosomes, dissociated in 2 M urea. (B, C, and D) P ribosomes; (B) dissociated in 3 M urea, (C) 5 M urea; (D) 8.9 M formamide. The sedimentation coefficients were measured in the analytical ultracentrifuge, in the gradient buffer. The vertical bars show the relative amounts of small subunit, measured in the presence of EDTA.

After the urea had been removed the 5 M urea-treated subunits showed sedimentation coefficients ranging from 42 to 54 S, as described above.

Free small subunits were not found in control preparations, but a 40S boundary was seen at pH 8.7; in dilute urea; and after 2 M urea had been dialyzed out (Table III). Both the 27S and 24S forms were observed over a range of urea con-

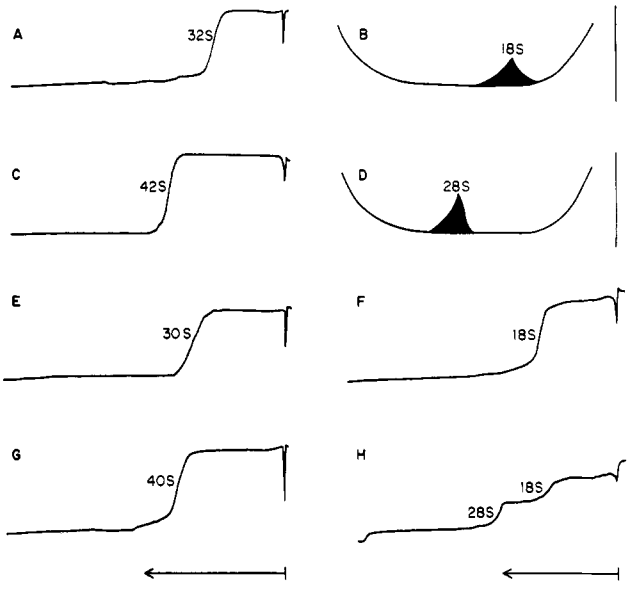

FIGURE 4: Ultracentrifugal patterns of isolated subunits from P ribosomes, in the pH 7.8 buffer, and of the RNAs released from them by sodium dodecyl sulfate. (A) 5 M urea-treated small subunit and (B) its RNA. (C) 5 M urea-treated large subunit and (D) its RNA. (E) 8.9 M formamide-treated small subunit, and (F) its RNA. (G) Mixture of 8.9 M formamide-treated subunits (fraction 34 in Figure 3D) and (H) their RNAs. (B and D) Schlieren optics; other patterns, ultraviolet optics; all at 5°.

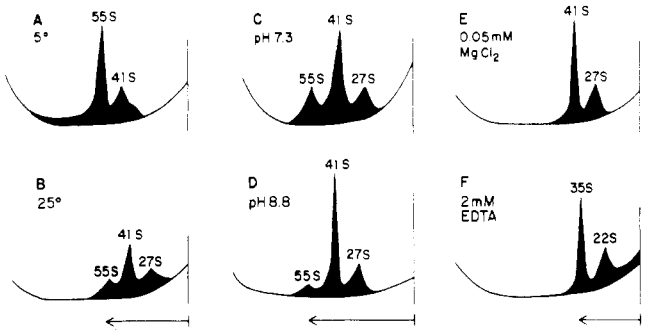

FIGURE 5: The effect of formamide on the sedimentation behavior of N ribosomal subunits. (A and B) In buffer K (30 mM KCl–1 mM potassium phosphate, pH 7.3)–0.1 mM $MgCl_2$ (r = 0.22)–6.6 M formamide, at two temperatures. (C and D) In 30 mM KCl, 0.1 mM $MgCl_2$–1 mM potassium phosphate–8.9 M formamide, at 5°, at two pH values. (E and F) In 20 mM KCl–10 mM Tris (pH 8.6)–8.9 M formamide, at 5°, with and without a trace of free Mg^{2+}.

centrations. The concentration of small subunits was too low for a reliable determination of k. On removeal of the urea the 27S form changed to 32 to 34 S, depending on the magnesium concentration of the buffer.

Release of Small RNAs. When either N or P ribosomes had been exposed to 5 M urea 7% of the total ultraviolet-absorbing material was found near the top of the sucrose gradient (Figure 3C). To obtain this material in more concentrated form, P subunits were pelleted and washed in the pH 7.8 buffer. Of the total A_{260} in the original ribosomes, 3.4% was recovered in the first supernatant and 1% in the wash solution; on gel electrophoresis each sample showed a band in the tRNA region, a 5S RNA band, and a third band moving 35% as fast as the tRNA, but no degraded rRNA. The second wash, in EDTA, released only 0.3% of the total A_{260}.

Formamide. The effects of formamide were similar to those of urea; 6.6 M formamide dissociated most of the N ribosomes at 5°, and the effects were more marked at 25°, where a 27S boundary appeared (Figure 5A,B). In 8.9 M formamide dissociation was complete at 5° (Figure 5C); at the higher pH (Figure 5D) less 55 S remained. In Tris buffer at pH 8.6 conversion to 41 and 27 S was complete (Figure 5E), and when the last trace of Mg^{2+} was chelated by EDTA (Figure 5F) both subunits sedimented even more slowly.

Properties of Isolated Fractions. P ribosomes dissociated with 8.9 M formamide, then fractionated in a sucrose gradient, showed three main peaks (Figure 3D). The 30S fraction gave single sharp boundaries in the analytical ultracentrifuge, both in the pH 7.8 buffer (Figure 4E) and with EDTA. Its RNA also gave a sharp boundary (Figure 4F), with very little material slower than 18 S. The fixed subunit had a molecular weight of 1.27×10^6 and a buoyant density of 1.577 g/cm³, corresponding to 50% protein (Table I). The 54S fraction gave single sharp boundaries, both in the pH 7.8 buffer and in EDTA. Its RNA was mainly 28 S. The fixed subunit had a molecular weight of 3.0×10^6 and a buoyant density of 1.598 g/cm³, corresponding to a protein content of 45%. The middle fraction (Figure 3D) was mainly large subunits in the 41S form (Figure 4G), and most of its RNA was still 28 S (Figure 4H). Since it was a mixture the molecular weight was not measured. In CsCl the major band had a density of 1.617 g/cm³, corresponding to 42% protein, so a molecular weight of 3.0×10^6 could be assumed (Table I). Interpreting the schlieren patterns (Figure 5), we again find that, while 27 S represents only small subunits, the faster boundaries may include both.

TABLE III: Sedimentation of the Small Subunit in Solvents Containing Urea or Formamide at 5°.[a]

s^0 (S)	f/f_0	Urea or Formamide (M)	KCl (mM)	$MgCl_2$ (mM)	Buffer	pH
Buffer alone						
40	1.61[b]	0	30	0.1	Phosphate	8.7
		[c]	10	0.1	Phosphate	7.8
+Urea						
40	1.61[b]	1.0	20	0.1	10 mM Tris	8.6
		1.8	30	0.1	Phosphate	8.3
27	2.08[d]	2	20	0.1	10 mM Tris	8.6
		4, 5	30	0.5	Phosphate	7.3
		4	30	1.3	Phosphate	7.3
24		2	30	0.1	Phosphate	8.7
		3–5	30	0.1	Phosphate	7.3
+Formamide						
27	2.24[e]	8.9	30	0.1	Phosphate	7.3
		8.9	30	0.1	Phosphate	8.8
		8.9	20	0.05	10 mM Tris	8.6
22		8.9	20	0[f]	10 mM Tris	8.6

[a] f/f_0 is the frictional ratio. [b] Molecular weight 1.50×10^6. [c] 2 M urea dialyzed out (see text). [d] Molecular weight 1.14×10^6. [e] Molecular weight 1.27×10^6. [f] EDTA present.

Reversibility of Dissociation. When N subunits obtained in 8.9 M formamide (Figure 5C) were equilibrated with buffer K containing 1.5 mM $MgCl_2$ 80% of them associated to 81 S or larger particles. In a second experiment, ribosomes in buffer K containing 0.2 mM $MgCl_2$ were treated with 6.6 M formamide, then dialyzed against the pH 7.8 buffer used in the zonal fractionations; they were 54% dissociated. In the 1.5 mM Mg buffer only 5% of this material failed to reassociate.

Effect of Time. The effect of time was examined with N ribosomes in 20 mM KCl–0.5 mM $MgCl_2$–10 mM Tris (pH 8.2)–7.8 M formamide, containing 100 μg of fine bentonite/ml (Petermann and Pavlovec, 1963). After 50 min at 5° the sample contained equal amounts of 55 and 35 S, but 5 hr later the material had been completely converted to 35 and 22 S. The RNA was isolated by the phenol method, freed of magnesium, and heated to reveal hidden breaks. Material from the 50-min sample showed the large 16–18S boundary seen in RNA from fresh N ribosomes (Petermann and Pavlovec, 1963), whereas the RNA of the 5-hr sample had been converted to 10 S.

A second experiment was carried out on P ribosomes. The control, in buffer alone, contained only 10% of subunits (Figure 6, top). After 50 min in 6.6 M formamide most of the ribosomes had dissociated. During the next 5 hr there was a progressive change to the slow forms of both subunits, but the boundaries were still sharp, the last pattern resembling Figure 5C. The RNA extracted with sodium dodecyl sulfate tended to aggregate, so that some of it sedimented at about 40 S (Figure 6, bottom). Unlike the subunits, it showed little change with time.

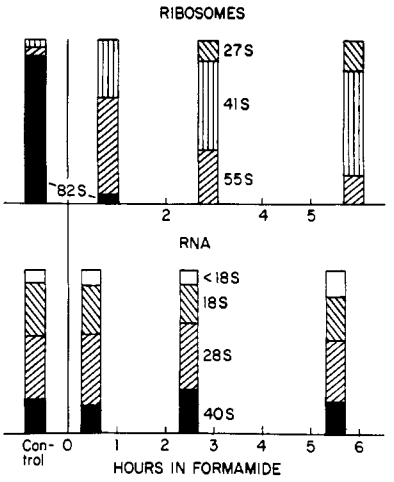

FIGURE 6: The effect of time on the sedimentation behavior of formamide-treated P ribosomes and their RNAs. Top, ribosomes and subunits; control, in 30 mM KCl–0.1 mM $MgCl_2$–3 mM potassium phosphate (pH 7.3). Formamide was added, to 6.6 M, at time zero, and the solution was kept at 5°. Samples for ultracentrifugal analysis were taken immediately and after 2 and 5 hr; the photographs were made at the times shown. (■) 82S and larger particles. Bottom, sodium dodecyl sulfate treated RNA. The control sample was taken from the control ribosomes. The other samples were taken from the formamide solution and treated with sodium dodecyl sulfate at the indicated times.

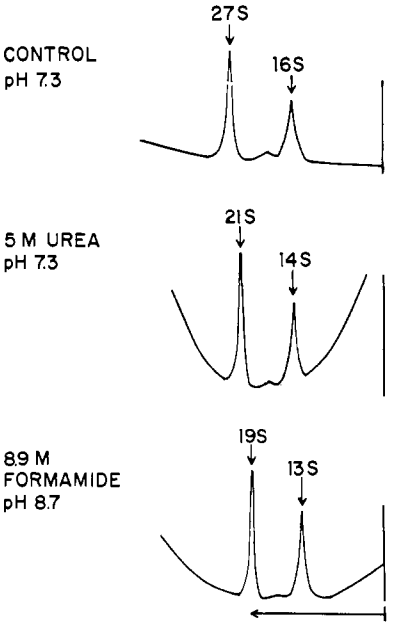

FIGURE 7: The effects of urea and formamide on ultracentrifugal patterns of rRNA in buffer K containing 0.1 mM $MgCl_2$. The sedimentation coefficients are $s_{20,w}$ at this concentration, 1.2 mg/ml.

Sedimentation Coefficients. The anhydrous partial specific volume was also used for the density corrections in formamide-containing solvents. For large subunits in 4.4 to 8.9 M formamide 20 points fell close to one regression line, with s^0 = 54.9 S (Table II). A second line, computed from six values obtained on one preparation in 8.2 M formamide, had a higher k but a similar intercept, 55.7 S. In this same experiment the slower form of large subunit also gave a good regression line, with s^0 = 40.5 S. For each line k was slightly lower than that of the comparable urea line. In other experiments the regression line was almost flat, or $1/s$ varied normally at concentrations above 2 mg/ml, then increased on further dilution; the boundaries remained sharp. This anomalous behavior may have been due to unfolding that increased on dilution. For the 35S line k is low, and the unfolding may also have varied here. The small subunits were 27 or 22 S in formamide (Table III) and 30 S after its removal (Figure 4E).

Release of Small RNAs. When the subunits obtained in 8.9 M formamide were fractionated or pelleted 5% of the A_{260} units remained in the supernatant. On gel electrophoresis this material showed a band in the tRNA region, but no degraded rRNA. The 5S RNA band was very faint, and the slow band, which represents a complex of 5S RNA and protein (Petermann *et al.*, 1972), was quite intense. When this supernatant was fractionated on Sephadex, 1.3% of the total RNA of the ribosomes was recovered in the complex, and 1% in the tRNA region of the elution pattern. On gel electrophoresis the complex fraction showed only the slow band plus free 5S RNA, and the tRNA fraction showed only a 5S band and a band in the tRNA region.

Effects of Urea and Formamide on RNA. The ultracentrifugal pattern of the rRNA (Figure 7) shows the usual major components and small intermediate boundary (Petermann and Pavlovec, 1963); at this concentration the sedimentation coefficients were a little less than 28 and 18 S. In urea or formamide $s_{20,w}$ decreased, but the relative amounts of the three components were not significantly changed.

Discussion

Hydrophobic bonding seems to be relatively unimportant in maintaining ribosomal structure, since it would be weakened by ethylene glycol (Kay and Oikawa, 1966) and strengthened by a rise in temperature (Nemethy and Scheraga, 1962), just the opposite of what actually occurred. Hydrogen bonding, on the other hand, appears to play an important role. Since liver ribosomes are highly hydrated (Haga *et al.*, 1970), bonding in their associated water may contribute to stability; the cohesive effect of ethanol and the disruptive effects of elevated temperature and urea on ribosomes parallel the effects of these agents on water (Franks, 1965; Frank, 1965). Ethanol caused nonspecific aggregation at 5°, and prevented dissociation to subunits at 35°. The high sedimentation coefficient of the monosome, 90 S, may indicate a decrease in bound water. Ethanol also stabilizes the association of *E. coli* ribosomal subunits (Spirin and Lishnevskaya, 1971).

Urea has a "structure-breaking" effect on water (Frank, 1965; Hammes and Schimmel, 1967), and loosens RNA structure (Singh and Keller, 1968; Sugo, 1968). A common denaturing agent for proteins, it may weaken hydrogen or hydrophobic bonds (Bull and Breese, 1970). Urea dissociates *E. coli* ribosomes (Spirin and Lishnevskaya, 1971), and unfolds the large subunit (Roberts and Walker, 1970). It releases a mRNA–protein complex from liver polysomes (Lee and Brawerman, 1971), and with liver ribosomes it enhances ribonuclease activity at 37° (Tashiro *et al.*, 1960).

In our experiments urea first dissociated the ribosomes to 59S and 40S subunits. The 59S subunit had a molecular weight of 3.0×10^6 and a protein content of 45% (Hamilton *et al.*, 1971), and contained the expected amount of 5S RNA (Petermann and Pavlovec, 1971). The 40S subunit had a molecular weight of 1.5×10^6 and a protein content of 55% (Hamilton *et al.*, 1971). These subunits could reassociate to active 81S ribosomes (Petermann *et al.*, 1969). An improved preparative procedure (Petermann, 1971a) and the amino acid incorporation studies (Petermann and Pavlovec, 1971) are described elsewhere.

With 5 M urea both subunits were converted to slowly sedimenting forms, and the 5S RNA was detached; some of it appeared in a nucleoprotein complex. The residual large subunit, 39 S, showed little change in molecular weight or protein content, and its RNA was still 28 S. Since its sedimentation coefficient was strongly concentration dependent, and its frictional ratio had increased 45% (Table II), the particle had probably unfolded in some way. Electron micrographs of 39S material showed highly asymmetrical, lobed forms (Dr. J. Y. Haga, personal communication).

The small subunit underwent greater changes. The 27S particle still contained 18S RNA, but 35% of its protein had been stripped off; the residual particle resembled the 30S subunit obtained by dissociation with EDTA (Hamilton and Ruth, 1969). The concentration dependence of s was not measured, but f/f_0 was high (Table III). If the small subunit is considered to be a prolate ellipsoid, the 40S particle has an axial ratio of 1.64 (Nonomura *et al.*, 1971), and the frictional ratio due to asymmetry, $(f/f_0)_A$ (Edsall, 1953), is 1.03. The frictional ratio due to hydration, $(f/f_0)_H$, is 1.61/1.03, or 1.56. If the 27S particle has a similar hydration $(f/f_0)_A$ would be 2.08/1.56, or 1.33, and its axial ratio would be about 6. This model, although approximate, suggests that, in addition to losing protein, the 27S particle has unfolded. The 24S particle may represent an even looser form of the same random coil, or may have lost additional protein.

Formamide is not a common protein denaturant, but it does weaken RNA hydrogen bonds (Helmkamp and Ts'o, 1961; Petermann and Pavlovec, 1963; McConaughy *et al.*, 1969). With ribosomes its initial effect was dissociation to subunits. The 55S particles obtained with 6.6 M formamide can combine with urea 40S subunits to give active ribosomes (M. L. Petermann and A. Pavlovec, unpublished data). With 8.9 M formamide half the large subunits were converted to 41 S. They had lost their 5S RNA and its associated protein (Petermann *et al.*, 1972), but little or no additional protein was detached, and the large RNA was still 28 S. Since s was highly concentration-dependent, and f/f_0 was markedly elevated (Table II), these particles had probably unfolded.

Formamide treatment converted most of the small subunits to a 27S form that still contained 18S RNA but had lost 23% of its proteins. Its high frictional ratio (Table III) suggested that it too had unfolded; if $(f/f_0)_H$ was still 1.56, $(f/f_0)_A$ was 1.44 and the axial ratio of the equivalent ellipsoid had increased to 8. The isolated rRNAs had lower sedimentation coefficients in both urea and formamide, but no extensive change occurred.

The effects of urea increased with time, but this was not due to ribonuclease action, since the changes were the same for N and P ribosomes; they involved little change in RNA structure; and they were arrested by the removal of the urea. When N ribosomes were kept in formamide for 5 hr the subunits gradually changed to their slow forms, and the RNA was converted to 10 S, corresponding to a particle size of about 300,000. Since 28S RNA isolated from fresh N ribosomes and similarly treated has a particle size of about 550,000 (Petermann and Pavlovec, 1963), the change in formamide amounted to one additional break per chain. The effects of time appeared to be independent of ribonuclease action, however, since they were the same for P ribosomes, and their RNAs (extracted with sodium dodecyl sulfate) remained 18 and 28 S.

During the first hour at 5° in either urea or formamide degradation by ribonuclease appeared to be minimal. The particles gave sharp ultracentrifugal boundaries, even at low concentrations, and little slowly sedimenting material appeared. The released RNAs corresponded roughly to the expected amounts: 5S RNA 1.7% and tRNA (1.5 molecules/ribosome) 1.7% of the total rRNA. Fragments of mRNA, about 30,000 daltons/ribosome (Warner *et al.*, 1962) would contribute an additional 1.3%. Degraded rRNA, which gives distinct bands in gel patterns (Gould, 1966), was never observed.

With both urea and formamide the disruptive effects were enhanced at higher pH or with less Mg^{2+}, conditions that increase electrostatic repulsion. Ribosomal subunits appear to be springy structures, whose shapes depend on a delicate balance between cohesion (due to the hydrogen bonding in RNAs, proteins, and associated water) and the electrostatic repulsion of the RNA phosphates. Cations neutralize the charges, and active 60S and 40S subunits can be obtained by dissociating liver ribosomes in 880 mM KCl–12.5 mM $MgCl_2$ (Martin and Wool, 1969). An understanding of the balance between these cohesive and disruptive forces should help us to interpret the conformational changes that appear to accompany the peptide elongation cycle (Petermann, 1971b).

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Effect of Methanol on the Partial Reactions of Polypeptide Chain Elongation†

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ABSTRACT: The effect of methanol on a number of the partial reactions of protein synthesis with *Escherichia coli* ribosomes has been examined. The alcohol virtually abolished formation of *N*-Ac(Phe)$_2$-tRNA without producing a corresponding effect on *N*-acetylphenylalanylpuromycin formation or T-dependent binding of Phe-tRNA. T-dependent hydrolysis of GTP, on the other hand, was briskly stimulated. Similarly, methanol strongly inhibited translocation while stimulating the G-dependent reactions of [^{3}H]GTP binding to ribosomes and nonturnover hydrolysis of GTP. The effect of the alcohol on the G-catalyzed binding of [^{3}H]GDP and turnover hydrolysis of GTP was relatively minor. Ethanol, 2-propanol, and acetone were found to have effects qualitatively similar to those of methanol.

Methanol and ethanol are known to have a number of interesting effects on ribosomal structure and function. Monro and Marcker demonstrated that *Escherichia coli* ribosomes catalyzed an mRNA-independent formation of *N*-formylmethionylpuromycin from f-Met-tRNA or *N*-formylmethionyloligonucleotides in the presence of ethanol (Monro and Marcker, 1967) or methanol (Monro *et al.*, 1968). The reaction could be carried out by isolated 50S subunits (Monro, 1967a), and the replacement of puromycin by aminoacyl-tRNA resulted in the formation of dipeptidyl-tRNA and oligopeptidyl-tRNA (Monro, 1967b). Tompkins *et al.* (1970) observed a codon-independent termination reaction in the presence of ethanol, while Scolnick and Caskey (1969) found that the interaction of ribosome, terminator codon, and release factor was stabilized by ethanol. Tompkins (1970) was able to obtain a T-factor-dependent formation of dipeptide with codons as mRNA provided ethanol was present. We demonstrated that a highly specific extraction (the PI extraction) of the 50S subunit could be performed with ethanol and NH_4Cl under appropriate conditions (Hamel and Nakamoto, 1971; Hamel *et al.*, 1972). Ballesta *et al.* (1971) reported that the G-dependent guanosine triphosphatase activity of CsCl-extracted 50S particles could be reconstituted with methanol. These observations indicated that an examination of the effect of these alcohols on the G-dependent and T-dependent activities of *E. coli* ribosomes would be of interest. The results of our study with methanol are reported here.

Materials and Methods

Materials. [^{3}H]GDP of specific activity 1430 μCi/μmole was purchased from New England Nuclear. Fusidic acid was a gift of Dr. Josef Fried. Other materials were described previously (Hamel *et al.*, 1972).

Methods. E. coli NH_4Cl-washed ribosomes, protamine-treated supernatant, G and T factors, Phe-tRNA, and *N*-AcPhe-tRNA were prepared as described previously (Hamel *et al.*, 1972). The T factor used was the "peak 1T" factor repurified on hydroxylapatite (Hamel *et al.*, 1972). Protein concentrations were determined by the method of Lowry *et al.* (1951), with lysozyme as standard. All assays contained 0.01 M $MgCl_2$, 0.05 M imidazole-HCl (pH 7.4), 0.08 M NH_4Cl, and 0.012 M 2-mercaptoethanol, except as indicated. Polyphenylalanine synthesis assays were performed as described previously (Hamel *et al.*, 1972).

Preparation of the Ribosome–Poly(U)–N-AcPhe-tRNA Complex. The complex was prepared by a 1-hr incubation at 37° in a mixture containing 388 μg/ml of nonradioactive *N*-AcPhe-tRNA, 2 mg/ml of ribosomes, and 200 μg/ml of poly(U). After incubation the mixture was cooled to 0°.

Formation of N-Acetyl[^{14}C]phenylalanylpuromycin. Ribosome–poly(U)–*N*-AcPhe-tRNA complexes were formed as described above, except that 394 μg/ml of *N*-Ac[^{14}C]Phe-tRNA was used. Each assay tube contained, in a final volume of 0.250 ml, 0.125 ml of complex, 0.125 μmole of puromycin,

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