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Thermal Denaturation of Ribosomes*

Moshe Tal

ABSTRACT: In dilute buffer of Tris-acetate (0.001 M, pH 7.2) where the nuclease-free ribosomes from *Escherichia coli* MRE 600 can be heated without aggregation, reversible ~23% hyperchromicity can be observed with ultraviolet spectrophotometry or by optical rotation (α_D line). The T_m is 62°. Mg^{2+} elevates the T_m whereas EDTA, urea, salt, and various organic reagents lower it. Analysis of ribosomal ash by emission spectrography revealed zinc and nickel in significant amounts and magnesium, calcium, and iron in smaller amounts. The sedimentation constants of natural ribosomes in Tris-acetate (0.001 M) are 24 S + 42 S. However, after heating to 65° and cooling their S values dropped to 17 S + 24 S ("heated particles"). Viscometric analysis showed that the conformation of heated particles is between that of natural ribosomes and an open ribosomal structure which exists at 65°. Reversible interconversion between the "open-molecule" and "heated-particle" conformations was found in repeated cycle of heating and cooling, as was measured by spectrophotometric, viscometric and optical rotation methods. Examination of the composition of "heated particles" showed that the ribosomal ribonucleic acid remained intact and that the particles retain virtually all their proteins. On the basis of these findings a model of thermal denaturation of ribosomes is proposed.

The function of ribosomes demands a high degree of internal order. This well-defined structure enables them to bind mRNA and aminoacyl-tRNA effectively and to form peptide bonds, by means of interaction with cytoplasmic factors. In order to clarify the dependence of the biological activities of the ribosomes on their structure, a system is needed which lends itself to the study of the secondary structure of ribosomes.

By means of several physical methods, ribosomes were found to have a high degree of internal order (Hall and Doty, 1959; Blake and Peacocke, 1965; McPhie and Gratzer, 1966; Bush and Scheraga, 1967).

Thermal denaturation studies can contribute to our understanding of the ribosomal structure. However,

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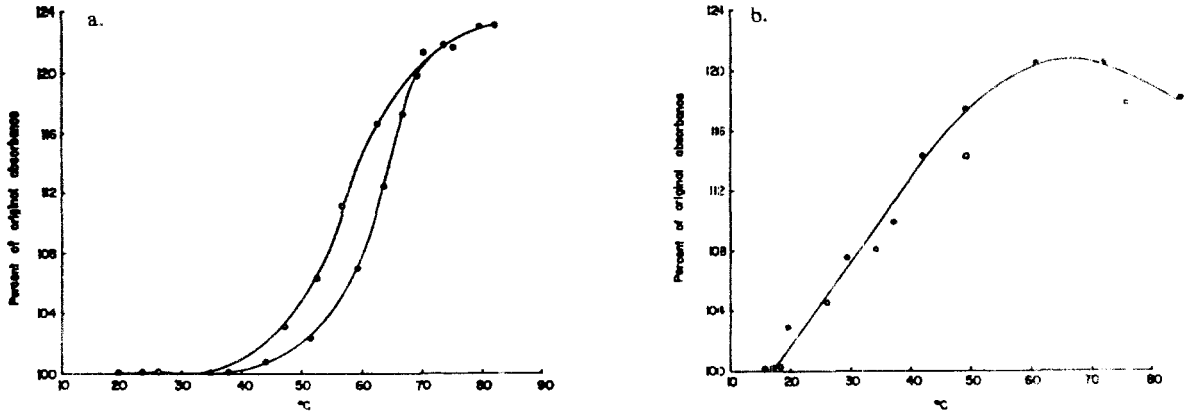

FIGURE 1: Thermal denaturation and renaturation studies. (a) Of ribosomes. Ribosomes dissolved in Tris-Ac (0.001 M, pH 7.2), concentration 0.500 optical density unit/ml. T_m was determined as described under Methods. (●) Heating curve; (○) cooling curve. (b) Of rRNA. rRNA dissolved in Tris-Ac (0.001 M, pH 7.2), concentration of 0.500 optical density unit/ml. (●) Heating curve; (○) cooling curve.

spectrophotometric measurements of thermal denaturation of ribosomes in various systems encountered technical difficulties due to turbidity which appeared upon heating (Zubay and Wilkins, 1960; Mangiantini *et al.*, 1965; Tamaoki and Miyazawa, 1966; Wolfe and Kay, 1967; Leon and Brock, 1967; Stenesh and Yang, 1967; McLaughlin *et al.*, 1968; Wolfe, 1968).

In the present study a ribosomal system from *Escherichia coli* is described which overcomes the difficulties mentioned above and permits spectrophotometrical studies of the thermal denaturation–renaturation process. This system has proved to be advantageous since it preserves the integrity of rRNA and the association of ribosomal proteins to rRNA.

In this study we have applied various reagents to the ribosomal system in order to study the relative contribution of the different types of the intraribosomal bonds (hydrogen, electrostatic, and van der Waals) to the thermal stability of the particles measured as a function of melting temperature, T_m. A study of this kind is relevant to a better understanding of the structure of ribosomes.

Materials and Methods

Strain. E. coli MRE 600 (RNase⁻) (Cammack and Wade, 1965) was selected for very low RNase activity.

Growing Conditions. The bacteria were grown at 37° in a medium containing per liter of deionized water: NaCl (5 g), KCl (2 g), NH_4Cl (1 g), $MgCl_2 \cdot 6H_2O$ (0.2 g), $MgSO_4$ (0.02 g), $CaCl_2 \cdot 2H_2O$ (0.0147 g), NaH_2PO_4 (0.032 g), gelatine (0.010 g), Tris (1.21 g), Casamino Acid (5.0 g), and glucose (10.0 g). The pH was adjusted to 7.2. The bacteria were grown up to the middle of the logarithmic phase, under vigorous aeration. Deionized water was found preferable to quartz-distilled water, because of the improved rate of growth. The harvested cells were washed in Tris-acetate (0.01 M) containing KCl (0.06 M), $MgAc_2$ (0.01 M), and mercaptoethanol (0.006 M) (pH 7.4) (standard buffer), and frozen until use. All materials were of analytical grade. The water for preparation of ribosomes and for all other experiments was quartz distilled.

Preparation of Ribosomes. Ribosomes were prepared by three alternate low- and high-speed centrifugations (Tissieres *et al.*, 1959). The ribosomal solution was dialyzed against several changes of Tris-acetate (0.001 M, pH 7.2). Another high-speed centrifugation was carried out and the pellet was dissolved in Tris-acetate (0.001 M, pH 7.2).

Preparation of Ribosomal Proteins. Ribosomal proteins were prepared according to Spitnik-Elson (1965).

Preparation of rRNA. rRNA was isolated from our ribosomal preparation by the method of Littauer and Eisenberg (1959), treated with bentonite according to Singer and Fraenkel-Conrat (1961), and dialyzed overnight against potassium acetate (0.01 M) in Tris-acetate (0.001 M, pH 7.2).

Protein Concentration. Protein was determined by Folin–Ciocalteu method as modified by Lowry *et al.* (1951), using a bovine serum albumin fraction V standard.

RNA Concentration. RNA was determined by ultraviolet absorption according to Elson (1959).

Determination of Hyperchromicity and T_m. The absorbance values were recorded on a Gilford recording spectrophotometer Model 2000. The regular concentration of ribosomes in the cuvette was 0.500 optical density unit/ml. However, in order to enable three cells to be recorded simultaneously, concentrations were set in small intervals, for example, 0.480, 0.500, and 0.520 optical density unit/ml. The rate of heating and cooling was approximately 0.5°/min. (Slower rates of changing temperature did not affect the results.) The buffer used for T_m measurements was boiled briefly in order to expel soluble gases. All cuvets were closed with Teflon stoppers. No correction was made for heat expansion of the solution. The T_m was defined as the midpoint between the maximum and minimum absorbance values at 260 mμ.

Sucrose Gradient. Separation of ribosomes was carried out on a 5–20% sucrose gradient in a Spinco SW 25.1 rotor at 21,000 rpm for 19 hr (Britten and Roberts, 1960).

Optical Rotation. A Hilger Standard polarimeter was

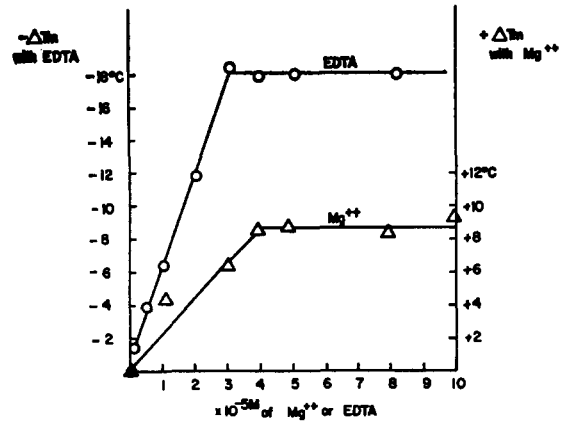

FIGURE 2: Influence of Mg^{2+} (right-hand scale) and EDTA (left-hand scale) on T_m of ribosomes. Abscissas final concentration of Mg^{2+} or EDTA; ordinates ΔT_m, representing the difference in T_m values between ribosomes in either Mg^{2+} or EDTA solutions and that of the control (63°). Experiment with magnesium entirely independent of that with EDTA.

used to measure the optical rotation, the light path being 40 cm and the light source being a sodium lamp. The tube was heated by means of a water jacket. The specific rotation, $[\alpha]_D$, was calculated according to the equation $[\alpha]_D = \alpha$ observed/Lc, L being in decimeters and c in grams per milliliter.

Analysis of Metals in Ribosomes. Dry ashing was carried out in a silica crucible and metals determined in the ash by emission spectrography (Hilger Watts spectrograph equipped with source unit FS 131) in the 2200–2700- and 2700–4900-mμ ranges, and by X-ray fluorescence (full-wave rectified Philips Eindhoven power supply; LiF crystal, 2d spacing = 4.0276 Å).

Analytical Ultracentrifugation. A Beckman Model E analytical ultracentrifuge equipped with the schlieren optics, electronic speed control, and photoelectric scanner for ultraviolet optics was used for determination of the sedimentation constants.

It should be emphasized that all sedimentation analysis on the heated particles were carried out at 20°. After exposure to an elevated temperature, the test tube containing the ribosomal solution was cooled at room temperature. All sedimentation constants are expressed as $s_{20,w}$.

Integrity of rRNA. The sedimentation profile of rRNA was examined according to Kurland (1960).

Measurement of Viscosity. Viscosity measurements were carried out in an Ostwald-Fenske viscometer No. 50, using a 10-l. water bath equipped with a Braun thermoregulator and a thermometer graduated to 0.1°. The efflux time of the buffer was 180 sec at 65° and 480 sec at 15°. The volume of solution examined was 5 ml. Reduced viscosity, η_{sp}/c, was determined with c taken in grams per milliliter.

Results

The Effect of Various Conditions on Thermal Stability of Ribosomes. Thermal denaturation studies with ribosomes were confined to experimental conditions permitting full reversibility, with absorbance resuming its original value (±1%) after heating and cooling. Ribosomes dissolved in Tris-acetate (0.001 M, pH 7.2) have a T_m of 62–63° (Figure 1a).

TABLE I: Ribosomes or rRNA Dissolved in Tris-Ac (0.001 M, pH 7.2).[a]

Urea (M)	T_m (°C)	KAc (M)	T_m (°C)
Melting Temperature of Natural Ribosomes			
0	62.0	0	62.0
0.2	61.5	0.01	57.0
0.5	56.5	0.02	50.5
1.0	53.5	0.06	48.0
2.0	43.0		
4.0	35.5		
$MgAc_2$ (M)			
Melting Temperature of rRNA			
0	37.5	0	36.5
1×10^{-4}	64.0	0.01	45.5
2×10^{-4}	68.0	0.02	48.5
5×10^{-4}	69.0	0.05	48.5
1×10^{-3}	69.5	0.06	49.5
2×10^{-3}	69.0	0.10	50.0

[a] Values of urea, KAc, and $MgAc_2$ are given in their final concentrations.

This denaturation–renaturation process can be characterized as follows: (1) hyperchromicity increase of 20–26% above the original absorbance; (2) narrow range of melting as compared with rRNA; (3) complete reversibility of ultraviolet absorbance; (4) absorption curve in the cooling process shifts toward lower temperature; and (5) in a repeated cycle of heating and cooling, the new curves coincided with the original cooling curve, regardless of whether the ribosomes were heated to maximum temperature of 80, 70, or 65°.

Examination of the pure particles, after separation in sucrose gradient, showed that their T_m values were almost identical. The T_m of the 30S particle was 1° lower than that of the 50S particle. The thermal absorption curve of rRNA is shown in Figure 1b. There is a significant difference between the denaturation profile of rRNA and that of the ribosomes.

The influence of Mg^{2+} ions and EDTA on the T_m of ribosomes is shown in Figure 2. Addition of Mg^{2+} elevates the T_m, whereas EDTA lowers it. Heating of ribosomes in presence of Mg^{2+} concentrations higher than 1×10^{-4} M causes turbidity which is reflected in absorption values higher than the original one.

In an attempt to examine the nature of the shift between the heating and cooling curves (Figure 1a) two cycles of denaturation–renaturation were performed. With 5×10^{-5} M $MgAc_2$ the T_m of the first heating curve was 70.5° and of the second 63°. On the other hand, with EDTA at low concentration (*e.g.*, 1×10^{-5} M) the T_m values were 56.5 and 53.0. A high concentration of

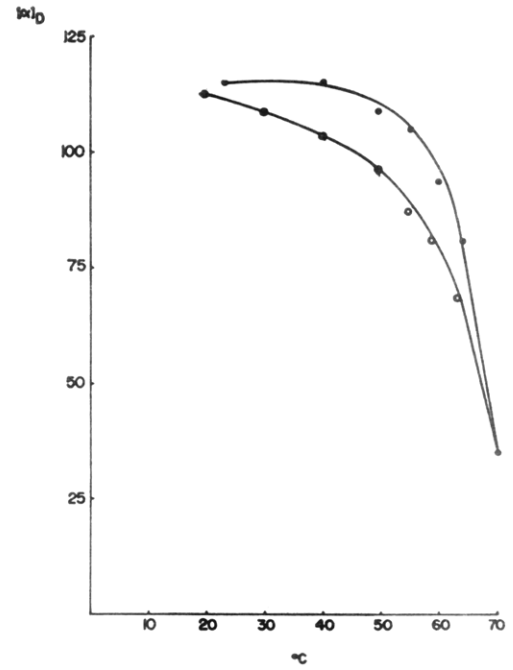

FIGURE 3: Specific optical rotation of ribosomes as a function of temperature. Ribosomes (concentration 2 mg/ml) dissolved in Tris-Ac (0.001 M, pH 7.2). Optical rotation was determined as described under Methods. (●) Heating curve; (○) cooling curve.

EDTA (4×10^{-5} M in the plateau region of Figure 2) yielded 44.0° in both cycles, with the shift eliminated.

Contrary to what was expected, salt lowered the T_m (Table I). Heating ribosomes in a solution containing more than 0.06 M KAc induced turbidity which resulted in a 50–70% increment in the absorption values, which interfered with the measurement of the hyperchromicity due to the conformational change. Such turbidity was observed when *E. coli* W ribosomes which contain RNase were heated at 68° for 10 min, even at low ionic strength.

The effect of various organic materials capable of interaction with the lypophilic regions in the ribosomes was examined. Both 0.004% *n*-propyl alcohol and 0.02% isoamyl alcohol brought the T_m down to 57°. Dioxane up to 1% had no influence on the T_m, but did cause an increase of a few per cent in hyperchromicity. Sodium dodecyl sulfate had a marked influence on the thermal stability of ribosomes. At a concentration of 0.04% the T_m was found to be similar to that of rRNA.

In order to study the forces maintaining structure in the ribosome, we examined the contribution of rRNA and ribosomal proteins to reversible denaturation. Addition of Mg^{2+} or KAc to an rRNA solution elevated the T_m (Table I) (in agreement with Boedtker (1960), Rodgers (1966), and others). When comparison of thermal stability of ribosomes and rRNA was made three significant differences were noticed: (1) without magnesium, the T_m of rRNA was 37.5° as against 62° for ribosomes (Figures 1a,b); (2) the concentration of Mg^{2+} producing the maximum effect in rRNA was 5×10^{-4} M, whereas in ribosomes it was much lower, 4×10^{-5} M (Table 1, Figure 2); and (3) the maximum Mg^{2+} concentration which the rRNA solution could stand without becoming turbid on heating is 2×10^{-3} M, whereas with ribosomes the limit was 1×10^{-4} M.

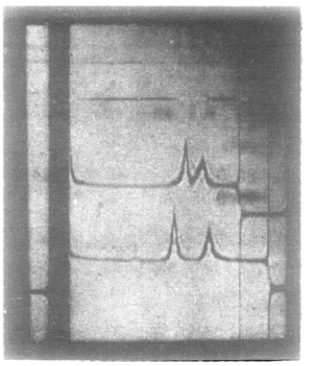

FIGURE 4: Sedimentation profile of ribosomes dissolved in Tris-Ac (0.001 M, pH 7.2), concentration 2.2 mg/ml. Upper profile: ribosomes heated to 65° for 5 min and then cooled to 20° showing 17S and 23S values; lower profile: unheated ribosomes showing 24S and 42S values.

The remarkable difference in T_m values between the rRNA and ribosomes is apparent in low ionic strength buffer (point 1). Closer T_m values were obtained in buffers of higher ionic strength (Friedman *et al.*, 1967; Cotter *et al.*, 1967).

The contribution of ribosomal proteins to hyperchromicity under similar experimental conditions was very difficult to assess because of their insolubility at neutral pH and low ionic strength (Spitnik-Elson, 1962, 1965). No hyperchromic effect was observed at 280 mμ when a solution of ribosomal protein in 0.01 N HCl was heated. This is probably because the protein molecules were already in a denatured state as a result of the method of preparation.

Another method for studying changes in internal organization in ribosomes during heating is the measurement of the rotation of polarized light as a function of temperature. The resulting plot obtained (Figure 3) corresponds to the absorbance curves. There is a sharp decline in $[\alpha]_D$ above 50°. This is in agreement with experiments conducted with poly A or hybrid poly A:poly U as regards distortion of the secondary structure (Fresco, 1959; Doty *et al.*, 1959; Samejima and Yang, 1965). On cooling, the rotation values approached the original value of $[\alpha]_D$ although there was a shift toward lower temperatures. A second cycle of heating and cooling yielded two curves which were indistinguishable from the cooling curve of the first cycle. This is in agreement with our spectrophotometrical experiments (Figure 1a).

Metals in Ribosomes. Neutral pH was found to be optimal for the effect of EDTA on the thermal stability of ribosomes. At neutral pH, Ni^{2+}, Zn^{2+}, and Fe^{3+} preferentially form complexes with EDTA (Ringbom, 1959). We have, therefore, determined the metals present in ribosomal ash by emission spectrography and X-ray fluorescence. Preliminary results showed that significant amounts of zinc and nickel and smaller amounts of magnesium, iron, and calcium were present. Traces of manganese, lead, cobalt, chromium, vanadium, barium, and strontium were also identified.

Temp (°C)	$s_{20,w}$ (S)				
80.0		(24.80)	(18.95)	7.10	
75.0			16.10	8.30	
70.0			20.75	13.20	
67.5			20.20	13.80	
65.0			20.50	14.00	
62.5			20.30	15.13	
60.0		28.75	22.80	18.83	
57.5	40.20	29.58	24.65	19.75	
55.0	39.50	31.30	21.25		
20.0	43.75	29.95			

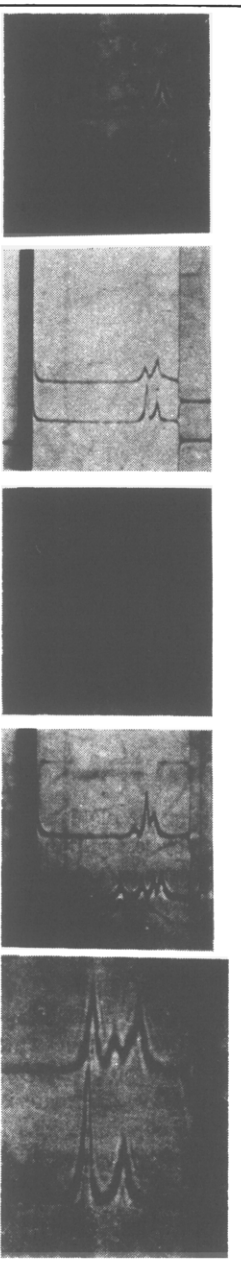

FIGURE 5: Sedimentation profile of ribosomes (2.5 mg/ml) dissolved in Tris-Ac (0.001 M, pH 7.2) after exposure to elevated temperatures for 10 min and cooling to 20°.

Sedimentation Analysis of Heated Ribosomes. Examination of the sedimentation pattern of ribosomes heated to 65° for 10 min showed a drastic decrease in the sedimentation constants from 24 and 42 S for natural particles to 17 and 23 S (Figure 4).

Our preliminary experiments were aimed at studying the conditions under which the slow particles, 17 S + 23 S, were formed. The changes in the sedimentation profile of the ribosomes were studied as a function of the temperature to which they had been heated (Figure 5). The heated particles were gradually derived from the natural ribosomes through intermediate forms. In the 62.5–67.5° range, the sedimentation constants and proportions of the different components remained unchanged. At 70.0° the sedimentation constants remained stable while the proportion of the faster component dropped. At higher temperatures an 8S component appeared.

Heating of ribosomes at 65° for periods ranging from 5 to 60 min yielded the slow-moving particles of 17 and 23 S. After heating for 120 min at 65° in addition to the 17S and 23S particles a small amount of a 9S particle appeared.

Special attention was paid to the determination of

TABLE II: Dependence of Sedimentation Constants upon Concentration and Ionic Strength.[a]

Concn (mg/ml)	Buffer (M)	Normal Ribosomes		Heated Particles		rRNA	
2.5	Tris (0.001) (pH 7.2)	26.5	40.3	14.0	22.9	2.4	6.8
	KAc (0.005)	29.4	47.8	17.7	30.3	9.7	10.9
	KAc (0.010)	32.4	50.8	18.1	30.1	8.5	9.8
	KAc (0.050)	31.1	50.2	21.1	34.1	15.3	18.9
	KAc (0.100)	32.4	52.1	21.4	33.4	15.2	19.0
	ratio of S values, 0.1 M KAc/0.001 M Tris	1.2	1.3	1.5	1.5	6.3	2.8
1.25	Tris (0.001)	27.5	44.0	14.1	26.6	3.0	7.5
	KAc (0.005)	30.5	49.3	18.5	32.1	11.2	13.2
	KAc (0.010)	31.8	51.7	18.7	31.6	13.3	16.7
	KAc (0.050)	31.8	52.2	20.2	34.7	16.2	20.2
	KAc (0.100)	33.5	51.2	22.3	34.5	16.8	21.8
	Ratio of S values, 0.1 M KAc/0.001 M Tris	1.2	1.2	1.6	1.3	5.6	2.9
0.05	Tris (0.001)	27.3	48.8	16.8	31.5	11.5	13.2
	KAc (0.005)	30.7	49.1	17.8	32.3	14.4	19.1
	KAc (0.010)	31.4	49.8	19.5	33.3	15.8	20.0
	KAc (0.050)			19.3	33.9	17.1	23.4
	KAc (0.100)			18.7	30.2	17.3	24.5

[a] Normal and heated ribosomes and rRNA were dissolved in Tris-Ac (0.001 M, pH 7.2). To these solutions KAc was added to the concentration indicated. In all solutions the pH was 7.2. The heated ribosomes were exposed to 65° for 20 min.

the composition and shape of these heated particles. As shown in Table II, there is a slight increase in the sedimentation constants of the heated particles with increasing ionic strength. The ratio of the S values obtained in KAc (0.1 M) and Tris-acetate (0.001 M), respectively, reflects the ability of the molecule to become more compact and move faster in the centrifugal field. The slight increase in S values referred to above is due to compactness of the structure. By contrast, similar experiments with rRNA showed a pronounced increase in S values due to the random-coil configuration. At low concentrations dependence of the S values on ionic strength is weaker than at high concentrations.

The low sedimentation constants of rRNA in concentrated solutions (at low ionic strength) reflect strong mutual interference by the stretched molecules. Much less interference was observed in the ribosomes and heated particles. It should be pointed out that the rRNA molecules retain their integrity, and that the same preparation had normal S values when the ionic strength was elevated. Similar results were obtained by Cox and Littauer (1962).

The dependence of S values of the heated particles upon ionic strength lies between that of the ribosomal spheres (Huxley and Zubay, 1960; Beer *et al.*, 1960) and that of the elongated rRNA molecules (Littauer and Eisenberg, 1959; Spirin, 1964). This observation suggested that the heated particles acquired different configurations. From the spectrophotometric experiment, in which the initial and the final values of absorbance were identical, we learned that the degree of internal order of the previously heated and of the unheated ribosomes might be similar. However, they may differ in shape. The hyperchromic effect (20–26%) (Figure 1a) can, therefore, be attributed to a different molecular configuration, namely, an open molecule present at elevated temperatures only and lacking secondary structure.

Viscometric Analysis. Upon heating, the specific viscosity, η_{sp}, of a ribosomal solution began to increase at 45° and reached a high level at 65°; upon cooling, it dropped to an intermediate level. A second cycle of heating and cooling yielded a plot coincident with the previous cooling curve (Figure 6), in agreement with our spectrophotometric and rotation of polarized light determinations.

Examination of η_{sp} of the extracted rRNA showed an increase in viscosity with increasing temperature. The cooling curve is close to the heating curve, and the final value of η_{sp} is almost identical with the value of the starting point (Figure 7). This observation indicated that rRNA, in contrast to ribosomes, did not acquire a different shape in solution after heating and cooling (in agreement with Cox and Littauer, 1962, and Spirin, 1964).

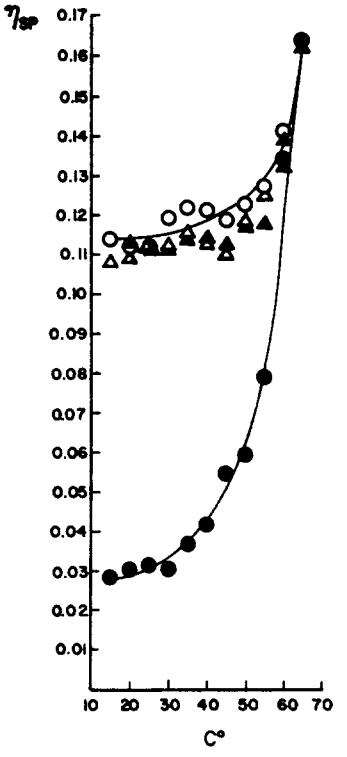

FIGURE 6: Specific viscosity of ribosomes as a function of temperature in two consecutive cycles of heating and cooling. Ribosomes (concentration 2 mg/ml) dissolved in Tris-Ac (0.001 M, pH 7.2). (●) First heating curve; (○) first cooling curve; (▲) second heating curve; (△) second cooling curve.

The reduced viscosity, η_{sp}/c, was found to be independent of concentration at 20° for both unheated and heated particles. There is a slight deviation from linearity at 65°. The extrapolated value for the heated and the open ribosomes is, respectively, 2.5 and 5.3 times that of the natural ribosomes (Figure 8). For natural ribosomes similar values of η_{sp} were obtained by Ts'o *et al.* (1958), Hall and Doty (1959), Tissieres *et al.* (1959), and Schachman (1963).

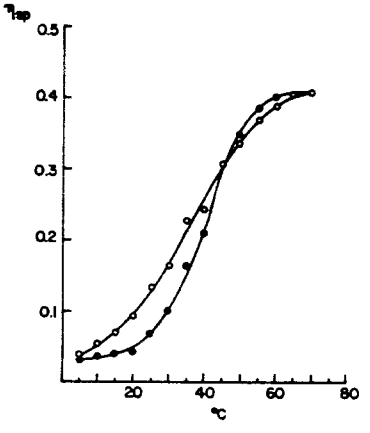

FIGURE 7. Specific viscosity of rRNA as a function of temperature, rRNA (concentration 0.25 mg/ml) dissolved in Tris-Ac (0.001 M, pH 7.2). (●) Heating curve; (○) cooling curve.

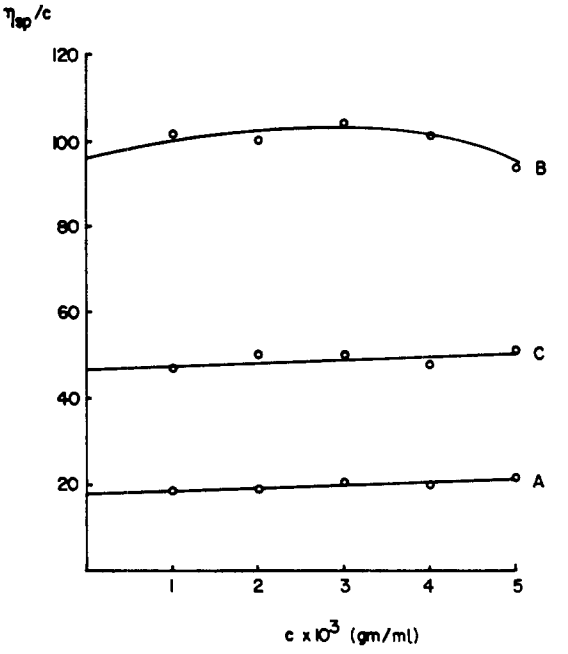

FIGURE 8: Reduced viscosity as a function of concentration. Ribosomes dissolved in Tris-Ac (0.001 M, pH 7.2). (A) Natural ribosomes at 20°; (B) particles heated to 65° and viscosity determined at this temperature; (C) particles heated to 65° and cooled to 20° before determination of viscosity.

In studies of the influence of ionic strength on viscosity, η_{sp}/c was found to increase with decreasing salt concentration (Figure 9). The ratio between η_{sp}/c values at low and high ionic strength is similar for all three components.

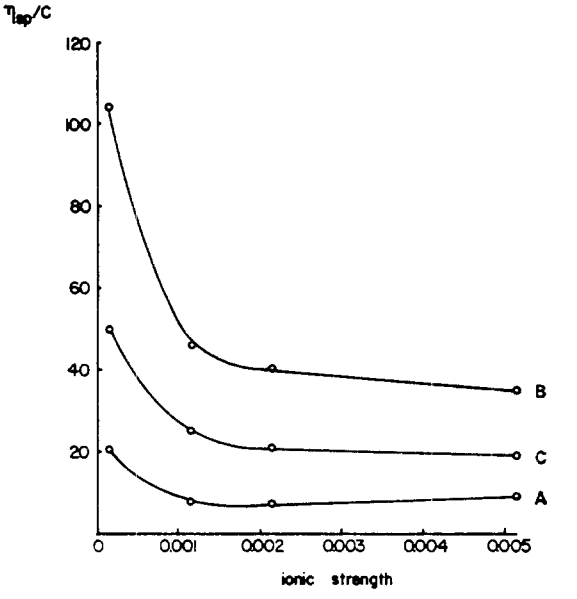

FIGURE 9: Reduced viscosity of ribosomes as function of ionic strength. The ribosomes were dissolved in Tris-Ac (0.001 M, pH 7.2) (ionic strength 1.37 × 10^{-4}) with NaCl added to final ionic strength as indicated. (A) Natural ribosomes at 20°; (B) particles heated to 65° and viscosity determined in this temperature; (C) particles heated to 65° and cooled to 20° before determination of viscosity.

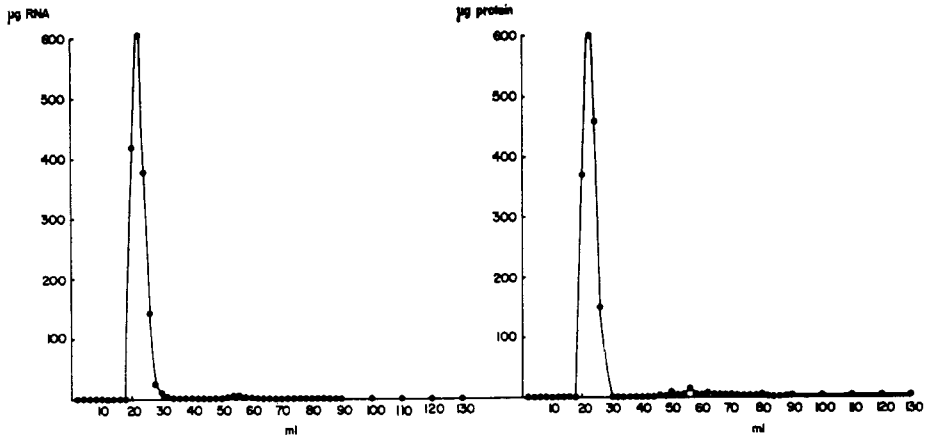

FIGURE 10: Chromatography of ribosomes dissolved in Tris-Ac (0.001 M, pH 7.2) heated to 65° for 10 min on heated Sephadex G-200 column (diameter, 1.2 cm; length, 45 cm). Temperature maintained by means of water jacket. Fractions of 2 ml were collected; eluent was the same solution as above.

Heating of the ribosomal solution at 65° for up to 40 min did not result in a decrease of viscosity. In a typical viscometric experiment the ribosomes were exposed to 65° for shorter periods of time. Longer heating caused a steady decrease in viscosity. This is in agreement with our previous observations which revealed a slower sedimenting component after 2-hr heating at 65°.

Integrity of the Heated Particles. Experiments were carried out on heated particles to determine if the ribosomal proteins remained attached to rRNA and whether the rRNA stayed intact. (1) A solution of heated particles was centrifuged in an analytical ultracentrifuge until the observed peaks passed the division in the partition cell. Examination of the supernatant showed almost no release of RNA and protein in preparations previously heated to 70°. (2) In a profile obtained directly on a Sephadex G-200 column, heated by a water jacket to 65°, the amount of protein and RNA in the included peak, which represents the released materials, is less than 1% of the material in the excluded peak (Figure 10). Moreover, no release of protein could be detected when 200 optical density units (260 mμ) of ribosomes in 1 ml of Tris-Ac (0.001 M, pH 7.2) containing 1.6×10^{-2} M EDTA (pH 7.2) were chromatographed on a Sephadex G-200 column at 45° equilibrated with 0.001 M Tris-Ac (pH 7.2) and 1.6×10^{-2} M EDTA (pH 7.2) (protein determination was preceded by dialysis for elimination of the EDTA).

In addition, it should be recalled that it was previously shown that the first cooling curve (Figure 1a) and subsequent heating curve are typical of a ribonucleoprotein complex (T_m = 54°) and not of rRNA (T_m = 37°).

The integrity of the rRNA from the heated particles was determined by examination of the sedimentation pattern after treatment with sodium dodecyl sulfate. The sedimentation profiles of rRNA showed that heating of ribosomes to 60, 65, and 67.5° does not damage the rRNA, while heating to 70 and 72.5° enhanced the decomposition of the faster component (Figure 11). There is a remarkable correlation between the sedimentation profile of the heated ribosomes (Figure 5) and the rRNA extracted from them (Figure 11). Up to 67.5° the relative amount of the components is constant in both. Identical results with regard to the integrity of rRNA in ribosomes after exposure to heat treatment were obtained on examination of phenol-extracted rRNA. In both cases the rRNA was examined in low ionic strength (0.01) in order to reveal breaks.

Influence of High Ionic Strength and of Mg^{2+} on Heated Particles. Dialysis of the heated particles against KAc (0.05 M)–$MgAc_2$ (0.0001 M) in Tris-Ac (0.01 M, pH 7.4) elevated the sedimentation constants from 15 to 23 S and from 22 to 32 S, respectively. These values were considerably lower than the 28 and 42 S of natural ribosomes observed in Tris (0.001 M, pH 7.2). The 22S and 32S particles were not homogeneous as judged from their sedimentation profile. Similar results were also obtained with unfolded ribosomes by Gavrilova *et al.* (1966) and Gesteland (1966).

The heated particles were also studied for their ability to associate and form an analog of the 70S particle in Tris (0.01 M, pH 7.2) and $MgAc_2$ (0.01 M) (Tissieres and Watson, 1958; Tissieres *et al.*, 1959). Under these conditions the heated particles formed aggregates of over 100 S, rather than the 70S particles obtained from natural ribosomes. Weller and Horowitz (1964) reported similar results with unfolded particles obtained from EDTA-treated 50S ribosomes.

Discussion

On the basis of the available information, it can be concluded that, upon heating, natural ribosomes undergo conversion into heated particles *via* an intermediate stage of open molecules (Figure 12). The reversibility of the conversion of open molecules to heated particles was demonstrated by spectrophotometric, viscometric, and rotation of polarized light determinations. These open structures consist of rRNA to which ribosomal proteins are attached.

Studies of ribosomes during heating were performed to determine the integrity of the rRNA which serves as a skeleton for the ribosome, and the retention of ribosomal proteins. The integrity of the rRNA extracted

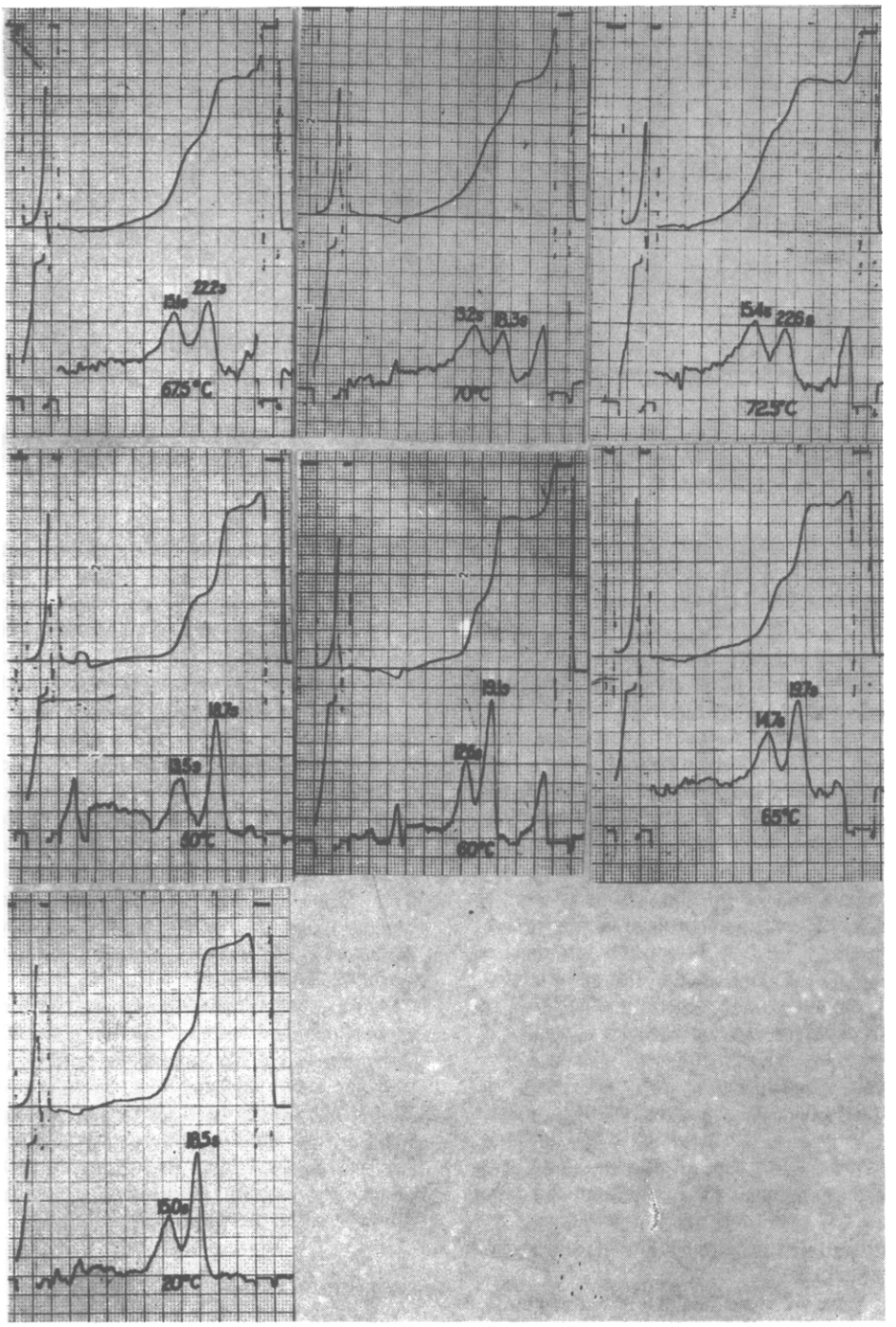

FIGURE 11: Sedimentation profile of rRNA extracted from heated ribosomes, dissolved in Tris-Ac (0.001 M, pH 7.2), concentration 2 optical density units/ml. The solution (1 ml) was heated to the temperature indicated for each profile for 10 min. After cooling to room temperature, 1 ml of 0.2% sodium dodecyl sulfate plus 0.20 M NaCl (pH 7.2) was added, and the solution was incubated at 37° for 15 min. Curves were recorded on an analytical ultracentrifuge Spinco Model E equipped with scanner and monochromator. The upper profile in each shows the absorption at 260 mμ; the lower curve is the derivative.

from heated particles (Figure 11) parallel the thermal stability of heated ribosomes as determined by their sedimentation profile (Figure 5). This indicates that the integrity of the rRNA determines the sedimentation pattern of the ribosomes after their exposure to elevated temperatures.

The conditions essential for a study of reversible denaturation of ribosomes are low salt concentrations and the absence of ribosomal RNase. When these conditions are not fulfilled, ribosomal proteins are released and form aggregates, which create turbidity and prevent accurate measurement of the hyperchromic effect due to confor-

mational change. Moreover, if RNase is one of the released proteins, degradation of the rRNA takes place. Such difficulties were encountered by several investigators (Hall and Doty, 1959; Zubay and Wilkins, 1960; Schlessinger, 1960; Ohtaka and Uchida, 1963; Tamaoki and Miazawa, 1966; Pace and Campbell, 1967; Wolfe and Kay, 1967; Leon and Brock, 1967; Stenesh and Yang, 1967; Wolfe, 1968, and others). In contrast, our system is free of these disadvantages as the *E. coli* MRE 600 used was selected for its very low RNase activity, and the experiments were confined to low ionic strength.

In comparative studies of the thermal denaturation of ribosomes and rRNA, differences were found in the T_m, Mg^{2+} dependence, and slopes of denaturation curves. The higher T_m of ribosomes as compared with that of rRNA indicates the existence of cohesive forces and less repulsion between negative charges due to the associated proteins. This is also reflected in the different concentration of Mg^{2+} required to obtain a maximal increase in T_m in ribosomes as compared with rRNA (Table I, Figure 2). The significant difference between the slopes of the heating curves (Figure 1a,b), shows that as soon as the ribosomes begin to unfold the denaturation process is rapidly completed, whereas in rRNA there are probably regions with different melting temperatures, as is reflected in the gradual increase in absorbance (Felsenfeld and Sandeen, 1962; Fresco *et al.*, 1963; Cox, 1966).

Reagents affecting different types of bonds alter the thermal stability of ribosomes. As expected, Mg^{2+} elevates the T_m. Other types of reagents such as KAc, urea, and *n*-propyl alcohol are capable of interacting with different regions of the ribosome. This interaction results in a weakening of intraribosomal cohesive forces and is reflected in the lower thermal stability of the particles. KAc accelerates the melting process of ribosomes by masking of the electrostatic bonds which become accessible at elevated temperature.

The very pronounced effect of sodium dodecyl sulfate and EDTA on the thermal stability of ribosomes suggests that proteins and metal ions, in combination or separately, are essential for ribosome stability. Shin and Eichhorn (1968) reported that the presence of Zn^{2+} permits reversible thermal denaturation of DNA, without a change in the ionic environment. They suggested that the specific location of Zn^{2+} may enable DNA molecules to undergo reversible thermal denaturation. It is possible that the Zn^{2+} which we found in ribosomes play similar role. Gesteland (1966) found that upon prolonged treatment with EDTA, slower sedimenting ribosomes were obtained, and that the rate of sedimentation was not completely restored when Mg^{2+} was added. A possible explanation may be that EDTA removes, in addition to Mg^{2+}, other cations which play essential roles in stabilization of ribosomal structure and biological activity.

In considering the reversibility in hyperchromicity after heating and cooling of ribosomes, a distinction should be made between two phenomena. The first is the identity of initial and final absorbance values, the second is the shift between two consecutive heating and cooling cycles. The identity of the absorbance values may indicate a similar internal order in the original and heated particles, whereas the shift indicates that less energy is required for melting the preheated particles than the natural ribosomes. After the first melting, ribosomes do not resume their original structure. In subsequent cycles the modified particles melt and re-form reversibly, as is indicated by the overlapping of the heating and cooling curves. Metal ions in the ribosomes are probably involved in the formation of the heated particles. This is based on the observation that high EDTA concentrations eliminate the shift.

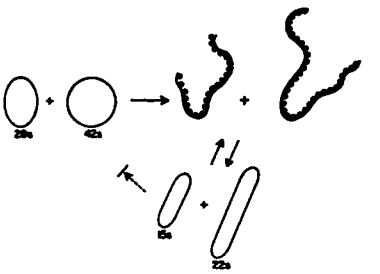

FIGURE 12: Schematic representation of the thermal denaturation of ribosomes. 28 and 42 S represent natural ribosomes. The circles in the open structures represent ribosomal protein; 15S and 22S particles represent heated particles, shown as ellipsoids. The broken arrow denotes the incomplete change in S values due to increase in ionic strength.

Thermal treatment of ribosomes results in the formation of slower sedimenting particles. The sedimentation profile obtained after heating to 57.5° for 10 min (Figure 5) indicates that the natural ribosomes convert into 17S and 23S particles through intermediates. On the basis of the proportions of the components and their homogeneity, it is reasonable to assume that the 17S particle is derived from the original 24S particle and the 23S from the 42S particle. The integrity of rRNA confirms this conclusion. Heated particles with identical sedimentation profiles are obtained upon exposure to various temperatures within the T_m region: 62.5, 65.0, and 67.5° (Figure 5). Apparently, all ribosomes are unfolded to the same extent over the entire melting range.

Fluctuations in the S values of ribosomes and heated particles in different experiments are attributable to differences in the ribosomal preparations used. In most of our preparations, the reduction in S value upon heating was 40 ± 8%. Nevertheless, the main characteristics of the system, namely, the ratio of the amounts of heated particles, and their homogeneity are common to all preparations.

A conformational change occured in ribosomes after heating. This conclusion is based on the observed lowering of S values, the dependence of sedimentation upon ionic strength, and results of viscometric analyses. The shape of particles exposed to elevated temperatures may be intermediate between the compact ribosomes and elongated rRNA. There are a few possible molecular conformations which might account for the altered properties of heated particles: an ellipsoid, a swollen sphere, a ribosomal core with dangling polymer chains or

a flat disk. Further experiments are necessary to determine the nature of the modified conformation.

The viscometric experiments confirmed our proposed scheme (Figure 12) of thermal denaturation of ribosomes according to which three types of conformation are involved. The newly acquired conformation of heated particles is typical of a ribonucleoprotein complex (Figure 6). Conversely, rRNA reverts to its original conformation upon heating and cooling (Figure 7). Spirin (1964) noted three reversible configurational changes in TMV–RNA under varying conditions of ionic strength and temperature. By comparing these two systems it can be concluded that ribosomal structure is less flexible than that of RNA.

The dependence of reduced viscosity on ionic strength is considerable at low salt concentrations. The η_{sp}/c ratio at ionic strengths of 1.37×10^{-4} and 5×10^{-3} is similar for ribosomes, open molecules, and heated particles (Figure 9) A, B, and C, respectively. This may be attributed to a similarity among these molecules with respect to their electric charge distribution. In other words, these three ribosomal conformations are probably similarly saturated with proteins. This hypothesis is supported by our experimental findings which show that ribosomal proteins remain strongly bound to the rRNA and do not dissociate at elevated temperatures (Figure 10). In addition, on the basis of this hypothesis the increase in S value with increasing ionic strength of natural and heated ribosomes may also be attributed to conformational change.

Inman and Baldwin (1962) examined the dependence of the reduced viscosity on ionic strength in the helix–coil transition of d(AT):d(AT) (a hydrogen bond form of the alternating copolymer deoxyadenylic and thymidylic acids). They found that over a 1000-fold range of salt concentrations η_{sp}/c of the helix changed only by a factor of 2. This small change was similarly obtained in our experiments with ribosomes, and in both systems can be attributed to the rigidity of the molecules. However, in the same range of ionic strength η_{sp}/c for d(AT) single strand changed by a factor of 100, whereas in our system η_{sp}/c for the open molecules change only by a factor of 2. This difference may be explained by the wider range of ionic strength used by Inman and Baldwin (1962), and more significantly, by the fact that the d(AT) is fully charged, whereas in the open molecules in our system in which the rRNA is saturated with proteins considerably fewer charges are accessible.

Acknowledgment

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Deformylation and Protein Biosynthesis*

David M. Livingston and Philip Leder

ABSTRACT: An enzyme which removes formyl groups from *N*-formylmethionylaminoacyl-transfer ribonucleic acid and its chemically synthesized analog, *N*-formylmethionylpuromycin, has been prepared from extracts of *Escherichia coli*. The specificity of the enzyme is that expected of a deformylase active during protein biosynthesis. It fails to act upon *N*-formylmethione or *N*-formylmethionyl-transfer ribonucleic acid until the blocked amino acid is transferred into an initial intermediate of protein synthesis, *N*-formylmethionylaminoacyl-transfer ribonucleic acid. The critical role of the formyl blocking group prior to the formation of the initial peptide bond and its presumed redundancy thereafter are consistent with this specificity. As previously demonstrated and confirmed in these studies, the enzyme also deformylates free formyl peptides. In addition, the differences observed in N terminals of *in vitro* and *in vivo* synthesized *E. coli* and certain bacteriophage proteins are likely accounted for by the marked lability of the enzyme in crude and purified extracts. Both SO_4^{2-} and SO_3^{2-} increase the initial reaction rate of the enzyme, but neither of these anions nor a variety of other compounds prevent the enzyme's rapid *in vitro* inactivation. Following the removal of the formyl blocking group, N-terminal methionine is removed by an aminopeptidase present in these preparations, a reaction required by the nature of the N-terminal residues of many bacterial and ribonucleic acid viral proteins.

The amino blocking group of F-Met-tRNA[1] is required for efficient formation of the initial ribosome binding complex and first peptide bond during protein synthesis (Bretscher and Marcker, 1966; Eisenstadt and Lengyel, 1966; Nakamoto and Kolakofsky, 1966; Zamir *et al.*, 1966; Leder and Nau, 1967; Salas *et al.*, 1967). Studies using synthetic RNA messengers, however, suggest that such blocking groups are not involved in subsequent steps in the elaboration of the polypeptide chain (Nakamoto and Kolakfsky, 1966), although they may serve to protect the nascent peptide from aminopeptidase degradation (Bretscher and Marcker, 1966). The ultimate redundancy of the formyl group is indicated by its absence in completed *Escherichia coli* and RNA bacteriophage proteins (Waller, 1963; Konigsberg *et al.*, 1966), despite its universal occurrence in those proteins synthesized *in vitro* (Adams and Capecchi, 1966; Capecchi, 1966; Webster *et al.*, 1966). An enzymatic activity which catalyzes the removal of formyl groups from free formyl peptides has, in fact, been detected in extracts of both *E. coli* B and *Bacillus stearothermophilus* (Gussin *et al.*, 1966; Fry and Lamborg, 1967; Adams, 1968). If, however, the formyl group is not involved in the elaboration of the polypeptide chain, it is reasonable to expect F-Met to serve as a substrate for such an enzyme *only after* its incorporation into the initial F-Met-aminoacyl-tRNA intermediate during protein synthesis. These studies present an additional test of the redundancy of the formyl group during protein assembly by using F-

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[1] Abbreviations: F-Met, *N*-formylmethionine; F-Met-Puro, *N*-formylmethionylpuromycin; $AUGU_3$ and $AUGU_6$, the oligoribonucleotides corresponding to the trinucleoside diphosphate ApUpG followed by a sequence of three and six uridylic acid residues, respectively.