Unfolding of the 30S Ribosomal Subunit of *Escherichia coli* and the Conformation of the Unfolded Subunit. Parallel Sedimentation and Optical Rotatory Dispersion Studies*

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ABSTRACT: 30S ribosomal subunits of *Escherichia coli* have been examined under various conditions. The opening up of the ribosomal tertiary structure (unfolding) was followed by sedimentation analysis, and changes in the base-paired secondary structure of the RNA moiety by optical rotatory dispersion measurements. When native subunits were equilibrated against media of varying Mg^{2+} content and ionic strength, two distinct structural transitions were seen. The first occurred when Mg^{2+} ions were removed with EDTA at a constant ionic strength of about 0.1. The sedimentation constant fell from 30 to 17–20 S and there was a small but significant disruption of base pairing. This conformation remained stable as the ionic strength was lowered in the absence of Mg^{2+} until a very low ionic strength was reached, at which there was a second drop in the sedimentation constant (to 5–8 S) accompanied by an extensive disruption of base pairs. Several speculative interpretations were considered.

One is that the second transition may involve the destruction of the base-paired loops commonly thought to be formed by adjacent complementary nucleotide sequences, while the first transition might involve the disruption of less stable base-paired regions formed by complementary stretches which are not adjacent to each other in the overall RNA sequence and help to determine the specific tertiary structure of the ribosome. When Mg^{2+} and salt were restored in the cold to their initial levels, the optical rotatory dispersion spectrum of ribosomes at either stage of unfolding rapidly reverted to that of native ribosomes, with partial restoration of biological activity. Incubation at 40° raised the biological activity considerably, with no additional effect on the optical rotatory dispersion spectrum. At low salt in the absence of Mg^{2+}, the optical rotatory dispersion spectrum of fully unfolded ribosomes was highly sensitive to changes in temperature. Its behavior was similar to but not identical with that of free rRNA. The observed differences indicate that the ribosomal proteins prevent the RNA moiety from attaining maximal base pairing, particularly at low temperatures.

The proteins of the ribosome are attached, apparently largely by electrostatic forces (Spitnik-Elson and Atsmon, 1969), to an RNA chain that is believed to be arranged in a series of double-stranded base-paired regions connected by single-stranded stretches (Doty *et al.*, 1959; Spirin, 1964). This nucleoprotein complex is folded in a unique way to form the compact specific structure of the intact ribosome. This structure is stable over a wide range of conditions but is disrupted under other conditions. A number of techniques have been employed to study ribosomal structure and its disruption. This communication presents results obtained with two of these techniques: sedimentation analysis, which yields information on the compactness of the ribosome, and optical rotatory dispersion and circular dichroism, which yield information on the amount of base pairing and base stacking in the rRNA moiety.

Sedimentation studies on *Escherichia coli* ribosomes have shown that the removal of Mg^{+} ions and salt causes the sedimentation constant to fall, on prolonged treatment reaching values as low as 5 S (Spirin *et al.*, 1963; Gesteland, 1966; Weller *et al.*, 1968; Spitnik-Elson and Atsmon, 1969). This is due to the unfolding of the nucleoprotein complex, converting the compact native ribosome into an open, more extended structure. However, this type of analysis does not show whether the base-paired conformation of the RNA moiety has changed.

The optical rotatory dispersion studies of Tinoco and his coworkers have made it possible to discern various features of RNA conformation (Cantor and Tinoco, 1965; Warshaw and Tinoco, 1966; Cantor *et al.*, 1966). The separation of paired bases causes a red shift in the position of the Cotton effect but not a large change in its magnitude, while a decrease in magnitude has been attributed largely to the disruption of base stacking. This technique and the companion one of circular dichroism have been applied to conformational studies of several types of RNA, including ribosomal RNA, both free and in the ribosome (Blake and Peacocke, 1965; McPhie and Gratzer, 1966; Bush and Scheraga, 1967; Sarkar and Yang, 1967; Miall and Walker, 1968; Wolfe *et al.*, 1968a,b; Wolfe and Kay, 1969; Cox, 1969; Adler *et al.*, 1970). Among others, the following observations have been reported. The rRNA in *E. coli* ribosomes (in 0.1–10 mM Mg^{2+}) appeared to be highly base paired, the pairs being predominantly G-C_1 (Bush and Scheraga, 1967). In other experiments, the base-paired conformation of free wheat embryo rRNA was found to be disrupted when the ionic strength was reduced (Wolfe *et al.*, 1968a,b) but that of complete ribosomes from the same source was not (Wolfe and Kay, 1969), suggesting that the ribosomal proteins stabilize the double-helical conformation of rRNA. Similarly, the dialysis of yeast ribosomes against EDTA caused no change in their optical rotatory dispersion spectrum (McPhie and Gratzer, 1966).

Taken together, the sedimentation and optical studies cited

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[1] Abbreviations used are: G-C, guanine-cytosine; OAc, acetate.

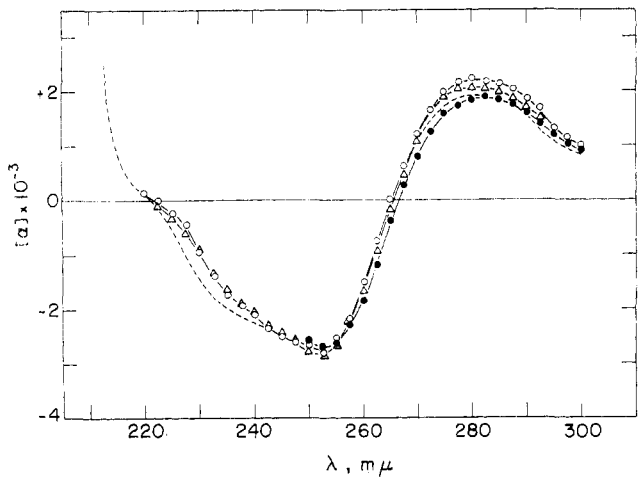

FIGURE 1: Optical rotatory dispersion of unfolded and refolded 30S ribosomes. Ribosomes (5.5 mg/ml) were dialyzed against 10 mM EDTA–NH_4^+ (pH 7) and 6 mM mercaptoethanol at 2° for 6 hr. The sedimentation constant, $s_{20,w}$, of the unfolded particles obtained was 16 S (measured at 14°). The unfolded ribosomes were then refolded according to Traub and Nomura (1969): the ribosomes were dialyzed for 20 hr against a buffer containing 5 mM H_3PO_4 neutralized to pH 7.4 with KOH, 20 mM $MgCl_2$, 0.3 M KCl, and 6 mM mercaptoethanol. Part of the ribosomes was incubated at 40° for 25 min, and a part was kept at 0°. The particles were recovered by centrifugation at 150,000*g* for 5 hr in the cold and the pellets resuspended in buffer 1 (10 mM Tris (pH 7.8), 10 mM $MgCl_2$, 30 mM NH_4Cl, and 6 mM mercaptoethanol). All the solutions were diluted for optical rotatory dispersion measurements with buffer without mercaptoethanol. Optical rotatory dispersion was determined at 28–30°. The results are expressed as units per milligram of ribosomes. (a) ---, native 30S ribosomes in buffer A. (b) ●—●, unfolded ribosomes (16 S) in 10 mM EDTA–NH_4^+. Owing to the absorption of EDTA at low wavelengths, optical rotatory dispersion could not be measured below 250 mμ. (c) △—△, refolded ribosomes, incubated at 40°; in buffer 1. (d) ○——○, refolded ribosomes, not incubated at 40°; in buffer 1.

above suggest that under conditions where at least some unfolding of the ribosome would be expected to occur, the ordered structure of its RNA moiety appears to be unaffected. However, these experiments were performed under various conditions, and some with bacterial ribosomes of the 70S class and others with nonbacterial ribosomes of the 80S class. We have therefore thought it worthwhile to study a single type of ribosome with both techniques. We have done this by unfolding the 30S ribosomal subunit of *E. coli* under several different sets of conditions, following the unfolding by sedimentation analysis and the effect on rRNA conformation by parallel optical rotatory dispersion measurements. Under our conditions we find two distinct steps in the unfolding process, each step involving both a drop in the sedimentation constant and the disruption of base pairing in the rRNA. We have also compared the optical rotatory dispersion spectra of unfolded ribosomes and free rRNA at different temperatures and find that the ribosomal proteins appear to prevent the attainment of maximal base pairing.

Methods

Ribosomes and rRNA. The source of ribosomes was *E. coli* MRE 600, a strain deficient in ribonuclease I (Cammack and Wade, 1965). Subunits (30 S) were prepared as described by Spitnik-Elson (1970), dialyzed against buffer A (1 mM $Mg(OA)_2$–10 mM Tris (pH 7.4)–0.1 M NH_4Cl), and stored at −180°. Ribosomal RNA was prepared from such 30S subunit preparations. Sodium dodecyl sulfate was added to a final concentration of 0.5% and the ribosomes were incubated 1 min at 37° and added to an equal volume of distilled phenol. After 30 min at room temperature 0.2 volume of chloroform were added and the mixture was left 15 min at room temperature and 15 min at 0°. The aqueous phase was separated and again extracted with phenol as before. RNA was precipitated with 2.5 volumes of cold (−20°) 95% ethanol–0.2% KOAc, left at least 1 hr at −20°, centrifuged down, dried, dissolved in buffer A, and dialyzed against the same buffer. Extensive unfolding was achieved by dialyzing ribosomes or rRNA at about 2 mg/ml against 1 mM EDTA–1 mM Tris (pH 7.4) at 2° for 24 hr and then against 1 mM Tris for 4–6 additional hr (Spitnik-Elson and Atsmon, 1969). The unfolded ribosomes sedimented at 5–8 S and the unfolded rRNA at about 2 S in the final solvent.

Determination of Concentration. Samples of RNA and ribosomes were hydrolyzed in 0.3 M KOH at 37° for 17 hr. The solutions were neutralized and the concentration of nucleotides calculated using the extinction coefficient $\epsilon^{1\%}(OD_{260\ m\mu}-OD_{290\ m\mu}) = 284.1$ (Elson, 1959). The contribution of the protein to this value is negligible and the concentration of the ribosomes was calculated from their RNA content, which is 60% according to numerous analyses of this type of preparation.

Optical rotatory dispersion was measured from 210 to 310 mμ with a Cary Model 60 recording spectropolarimeter. The cell path length was 2 or 10 mm and the optical density was not allowed to exceed 1.7 units. In the experiments of Figures 1 and 2 the concentrations of mercaptoethanol in the optical cell were 0.13 and 0.4 mM, respectively. It did not interfere with the optical rotatory dispersion measurements at these concentrations. Mercaptoethanol was not present in the other experiments. In some experiments a water-jacketed cell was used and the temperature was controlled between 5 and 40°. In other experiments the temperature varied between 28 and 30°. Rotation is given in units of specific rotation: $[\alpha] = 100\alpha/cl$, where α is rotation in degrees; l, path length in decimeters; and c, concentration in g/100 ml.

Circular dichroism was measured with a Cary Model 6001 circular dichroism attachment to the same spectropolarimeter. The cell path length was 10 mm. The temperature varied between 28 and 30°. The results are reported in the form of specific ellipticity, $[\theta] = 100\theta/cl$, where θ is the observed ellipticity in degrees; c, concentration in g/100 ml; and l, path length in decimeters. It is related to the mean residue ellipticity, by the following relationship: mean residue ellipticity $= [\theta] \times m/100$, where m is the mean residue weight.

Results

Effect of Unfolding the Ribosome. Traub and Nomura (1969) have shown that the partial unfolding of 30S ribosomes to biologically inactive 16S particles can be fully reversed under conditions elucidated by them. When the particles were incubated in the cold in the high salt–Mg^{2+} medium employed by these authors, they attained only a low ability to incorporate amino acids, but regained full biological activity if they were also heated at 40°. In an approximate repetition of their experiment, we dialyzed a solution of 30S ribosomes (5.5 mg/ml) against 10 mM EDTA for 6 hr in the cold. The unfolded ribosomes, which sedimented at 16 S, were then refolded according to Traub and Nomura, one portion entirely in the cold, while another portion was

TABLE I: Incorporation Activity of Refolded 30S Ribosomes.

	$s_{20,w}$ of Unfolded Ribosomes (S)			
	16 S[a]		8 S[b]	
	cpm[c]	%	cpm[c]	%
Native ribosomes, control	5200	100	5084	100
Unfolded and refolded				
Incubated at 40°	4135	80	2860	56
Not incubated at 40°	1963	38	1580	31
Unfolded, not refolded			87	1.7
0 time			72	1.4

[a] See Figure 1 for details of unfolding and refolding. [b] See Figure 2 for details of unfolding and refolding. [c] Incorporation of [^{14}C]Phe-tRNA. The reaction mixture contained in a final volume of 50 μl; 50 mM Tris-HCl (pH 7.4); 15 mM Mg $(OAc)_2$; 2 mM GTP; 100 μg/ml of poly(U); 6000 cpm [^{14}C]Phe-tRNA (250,000 counts/mg of tRNA); supernatant, 0.1 mg of protein; 100 μg of 50S untreated ribosomes; and 50 μg of 30S ribosomes. After 30 min at 35° the reaction was stopped by adding 3 ml of 5% trichloroacetic acid. The precipitates were heated in a boiling-water bath for 15 min, brought to room temperature, and collected on membrane filters. The filters were dried and radioactivity measured in toluene-based scintillation fluid. The tRNA was prepared according to Avital and Elson (1968) and charged with [^{14}C]-phenylalanine (specific activity 512 mCi/mmole) according to Vogel *et al.* (1968).

heated at 40° for 25 min. These refolded ribosomes showed about 40 and 80% of the original biological activity, respectively (Table I). Figure 1 shows optical rotatory dispersion spectra of the original ribosomes and the unfolded and refolded samples. The curves are all quite similar, although the unfolded ribosomes showed a slight red shift, indicating that most of the ordered structure of the rRNA was maintained during the unfolding achieved in this experiment.

We then employed conditions known to cause a higher degree of unfolding (Spitnik-Elson and Atsmon, 1969). Ribosomes were dialyzed at a lower concentration (1.8 mg/ml), for a longer time, and against a medium of lower ionic strength (see legend of Figure 2 for details). After this treatment they sedimented as a single peak at about 8 S. As before, portions were refolded according to Traub and Nomura (1969), without and with a 25-min incubation at 40°; and incorporation assays showed a restoration of about 30 and 60% of the original biological activity, respectively (Table I). Figure 2 shows that the optical rotatory dispersion spectra of the refolded ribosomes are very similar to that of the original subunits. The unfolded ribosomes, however, show a distinct red shift in the position of the Cotton effect. The values for λ_{max}, $\lambda_{crossing}$ (zero rotation), and λ_{min} changed from 280, 265.5–266, 252 mμ to 285, 270–271, 255, an approach toward the values measured for rRNA in water (288, 272, 258 mμ) and calculated for fully non-base-paired rRNA (288, 276, 262 mμ; see Table I in Bush and Scheraga (1967). The amplitude of the Cotton effect peak decreased by about 30%. The changes may be taken to indicate that the conditions employed in this experiment caused consider-

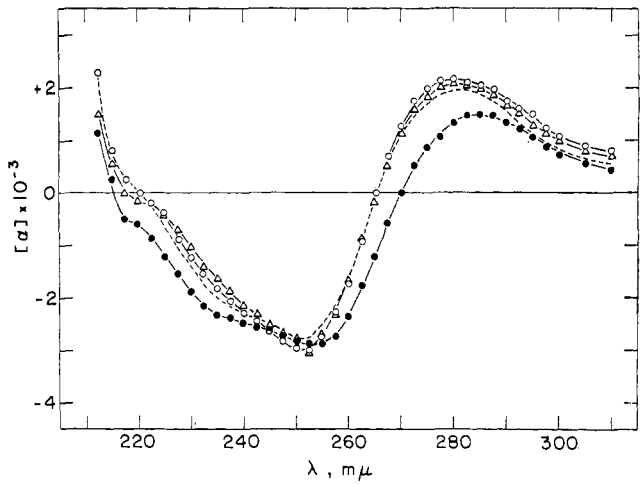

FIGURE 2: Optical rotatory dispersion of unfolded and refolded 30S ribosomes. Ribosomes (1.8 mg/ml) were dialyzed against 1 mM EDTA–NH_4^+, 1 mM Tris (pH 7.4), and 6 mM mercaptoethanol for 24 hr, and against 1 mM Tris and 6 mM mercaptoethanol for 6 additional hr. The sedimentation constant of the unfolded ribosomes was 8 S (measured at 23°). The ribosomes were refolded as in Figure 1. Optical rotatory dispersion was determined at 28–30°. The results are expressed as units per mg of ribosomes. (a) ---, native 30S ribosomes as in Figure 1. (b) ●—●, unfolded ribosomes (8 S) in 1 mM Tris (pH 7.4). (c) △—△ and (d) ○—○, refolded ribosomes as in Figure 1.

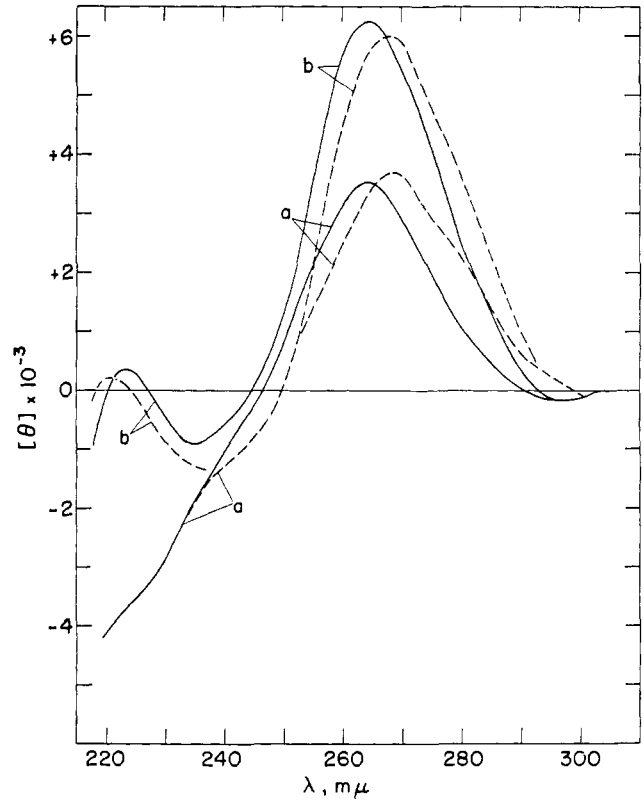

FIGURE 3: Circular dichroism curves of unfolded 30S ribosomes and 16S rRNA. 30S ribosomes and rRNA prepared from 30S ribosomes were each dialyzed at 2° against 1 mM EDTA and 1 mM Tris (pH 7.4) for 24 hr and against 1 mM Tris for 6 additional hr. The sedimentation constants of the unfolded ribosomes and rRNA were 8 and 2 S in 1 mM Tris. Circular dichroism was determined at 28–30°. Results are expressed as units per milligram of ribosomes or units per milligram of RNA. (a) Ribosomes, (b) RNA. (—) in buffer A, (----) in 1 mM Tris.

TABLE II: 30S Ribosomes: Effect of Different Media on Sedimentation and Optical Rotatory Dispersion Spectrum.

Sample	Concn (mM) $Mg(OAc)_2$	NH_4Cl	Tris (pH 7.4)	EDTA (pH 7.4)	Ionic Strength	$s_{20,w}$ (S)	$\lambda_{crossing}$ (mμ)
1	1	100	10		0.111	29.8	265.6
2		100	1	1	0.107	20.3	266.7
3 [a]		50	1	1	0.057	19.0	267.0
4		20	1	1	0.027	17.2	267.0
5			1	1	0.007	10.8	270.0
6 [b]			1		0.0008	8.5	270.0

[a] Samples 1–5: ribosomes (2.8 mg in 4 ml of buffer A, which is the medium of sample 1) were dialyzed for 28 hr at 2° against 500-ml volumes (changed three times) of the media shown above. [b] As above, except that the ribosomes were dialyzed against the medium of sample 5 for 24 hr and then against that of sample 6 (changed once) for an additional 4 hr. A portion of each sample was taken for sedimentation analysis in a cell of 30-mm light path in a Beckman-Spinco Model E analytical ultracentrifuge, and a portion was taken for the determination of the optical rotatory dispersion spectrum in a cell of 2-mm light path. Both determinations were carried out at 25° with undiluted samples.

able destruction of the base-paired structure of the rRNA within the ribosome, while base stacking was largely unaffected at the temperature employed (*cf.* Cantor *et al.*, 1966). As the circular dichroism curves of Figure 3 show, the disruption of base pairs is about the same as occurs in pure rRNA under the same conditions.

The above experiments indicated that while ribosomes can be unfolded quite extensively without large conformational changes in their RNA moiety (Figure 1), a higher degree of unfolding may be accompanied by a significant disordering of the RNA (Figure 2). This was examined in greater detail in the experiment illustrated by Table II and Figures 4, 5, and 6. In this experiment ribosomes originally dissolved in buffer A were equilibrated with solvents of different ionic strength lacking, except for the original sample, Mg^{2+} ions. Each sample was then divided into two portions, of which one was taken for sedimentation analysis and the other for the determination of the optical rotatory dispersion spectrum. Both measurements were performed at 25° without changing either the solvent or the ribosome concentration. The experimental details and results are given in Table II. Figure 4 shows the optical rotatory dispersion spectra. In Figure 5 both $s_{20,w}$ and $\lambda_{crossing}$ are plotted as a function of the ionic strength and Mg^{2+} ion content of the medium.

It is seen that the ribosome underwent two changes. The first took place when Mg^{2+} was removed from the medium with no appreciable change in ionic strength, and the second occurred in the absence of Mg^{2+} when the ionic strength was reduced. In both cases the change was expressed as a decrease in the sedimentation constant and a red shift in the optical

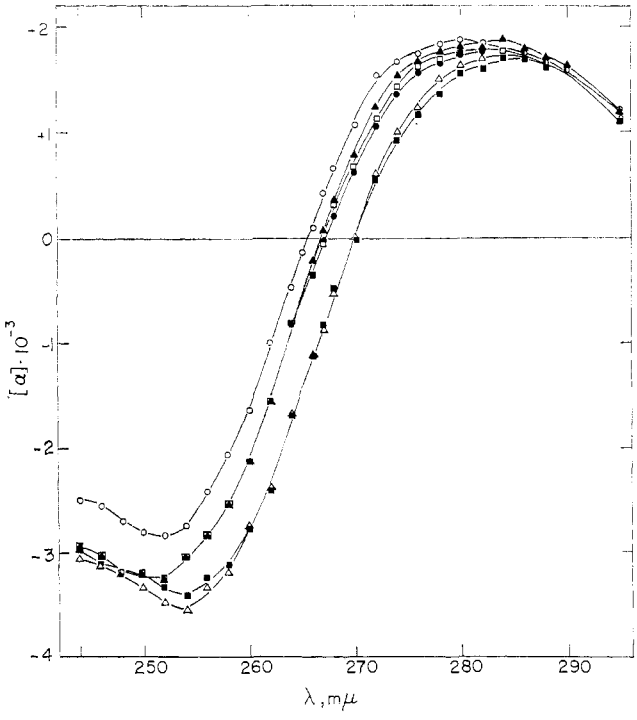

FIGURE 4: Optical rotatory dispersion spectra of 30S ribosomes in media of different ionic composition. See Table II for details. Results are expressed as units per milligram of ribosomes. Medium (see Table II): ○—○, 1; ▲—▲ 2; □—□, 3; ●—●, 4; △—△, 5; ■—■, 6.

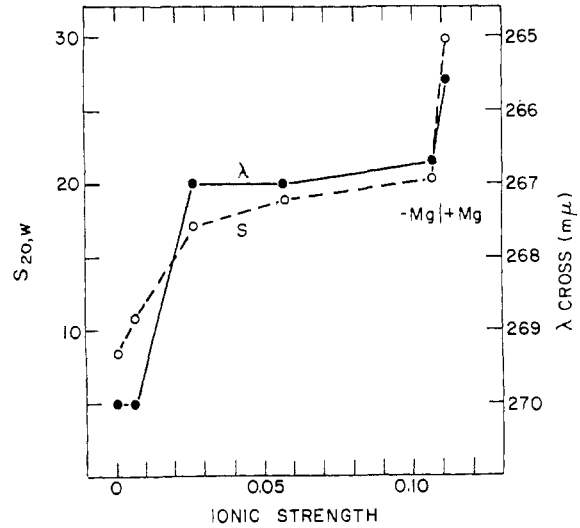

FIGURE 5: 30S ribosomes in different media: sedimentation constant and wavelength of zero rotation ($\lambda_{crossing}$) as functions of ionic strength. See Table II for details. λ = λ crossing; $s = s_{20,w}$.

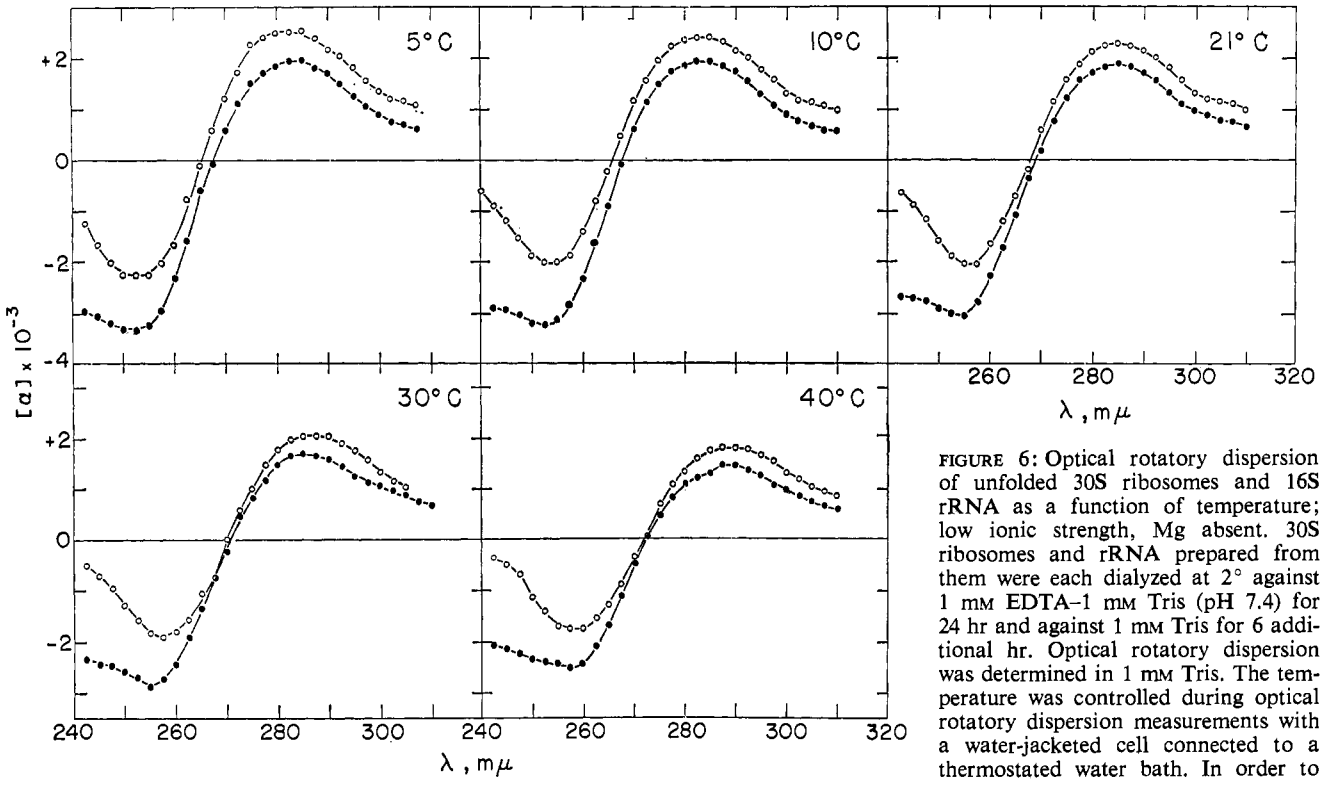

FIGURE 6: Optical rotatory dispersion of unfolded 30S ribosomes and 16S rRNA as a function of temperature; low ionic strength, Mg absent. 30S ribosomes and rRNA prepared from them were each dialyzed at 2° against 1 mM EDTA–1 mM Tris (pH 7.4) for 24 hr and against 1 mM Tris for 6 additional hr. Optical rotatory dispersion was determined in 1 mM Tris. The temperature was controlled during optical rotatory dispersion measurements with a water-jacketed cell connected to a thermostated water bath. In order to compare equal amounts of RNA in and out of the ribosome, all results are expressed as units per 0.6 mg of RNA. ●—●, ribosomes; O—O, RNA.

rotatory dispersion spectrum, *i.e.*, an unfolding of the ribosome accompanied by the disruption of base pairs. The magnitude of the optical rotatory dispersion maximum was not appreciably reduced, indicating that the degree of base stacking was essentially unchanged. The change from the most compact form (sample 1) to the most extended form (sample 6) was not continuous but involved two distinct conformational transitions, the first from a compact to a relatively stable partly unfolded form, and the second to a more highly unfolded form. Only the first transition appears to have occurred in the experiment of Figure 1, where the ionic strength of the unfolding medium was 0.056. In the experiment of Figure 2, both transitions occurred.

Rate of Refolding. An attempt was made to determine the time course of the re-formation of base pairs when unfolded 8S ribosomes were refolded. This was done by following the optical rotatory dispersion at the fixed wavelength of 267 mμ, which is sensitive to this type of interaction. The initial measurement was made with a solution of unfolded ribosomes in 1 mM Tris, pH 7.4 (8 S). The ionic strength of the solution in the cell was then raised to that of buffer A by the addition of a small volume of a concentrated salt solution, and the optical rotatory dispersion measurement was resumed within 2 min. The optical rotatory dispersion reading reverted to that characteristic of native 30S ribosomes within these 2 min, and no further change was observed.

Effect of Temperature on Pure rRNA and rRNA in the Ribosome. We have examined the effect of temperature on the optical rotatory dispersion spectra of 30S ribosomes and pure 16S rRNA under several different conditions. In one set of experiments, illustrated in Figure 6, the ribosomes were first extensively unfolded with EDTA–Tris and Tris as described above, and the optical rotatory dispersion spectra were determined at low ionic strength in the absence of Mg^{2+}. The final medium was 1 mM Tris, in which the unfolded ribosomes sedimented at about 5 S and the RNA at about 1.8 S. As the temperature was raised from 5 to 40° in this medium, both RNA and unfolded ribosomes underwent marked spectral changes, which appeared to be completely reversed when the temperature was returned to 5°.

The curves are shown in Figure 6. There was little change between 5 and 10°. At higher temperatures the peak amplitudes diminished steadily and the wavelengths of maximum, zero, and minimum rotation moved to longer values, indicating an extensive disruption of hydrogen bonding and probably a less extensive disordering of base stacking. Both RNA and unfolded ribosomes showed the same response to temperature, but the changes were more marked with RNA. At the lowest temperatures the crossing point of RNA was about 2–2.5 mμ to the blue of that of unfolded ribosomes, and the RNA peak amplitude was higher. These results suggest that at low temperatures the free RNA is more highly base paired than the unfolded ribosomes. With rising temperature the differences between the two curves diminished, until at 40° the crossing points were the same and the difference in peak amplitude was less.

Figure 7 shows a similar comparison, this time of rRNA and native 30S ribosomes and in a medium of higher ionic strength which also contained 1 mM Mg^{2+} (buffer A). Here the position of the Cotton effects did not change with temperature from 5 to 40°, while the peak amplitude decreased only slightly for RNA and almost not at all for the ribosomes. The Cotton effect of the RNA was about 1 mμ to the blue of that of the ribosomes at all temperatures.

The data of Figures 6 and 7 are partially summarized in Figure 8, in which the wavelength of the crossing point is plotted against temperature. The effect of temperature on rRNA is similar to that found by Vournakis and Scheraga

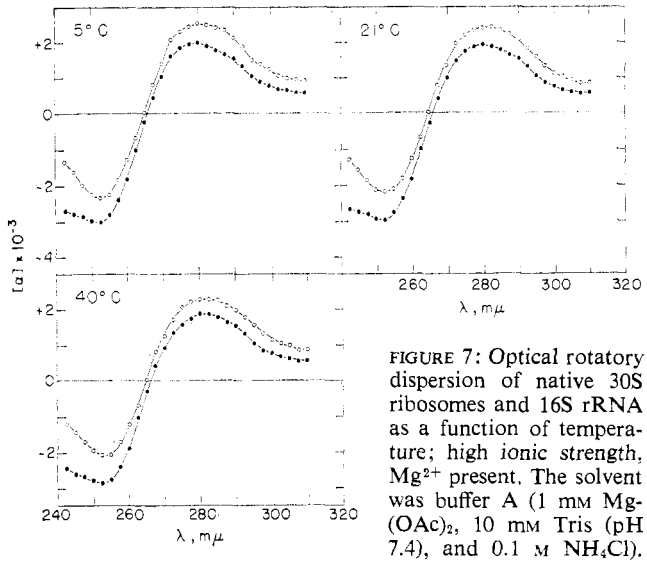

FIGURE 7: Optical rotatory dispersion of native 30S ribosomes and 16S rRNA as a function of temperature; high ionic strength, Mg^{2+} present. The solvent was buffer A (1 mM Mg-$(OAc)_2$, 10 mM Tris (pH 7.4), and 0.1 M NH_4Cl). Temperature was controlled thermostatically with a water-jacketed cell. Results are expressed as units per 0.6 mg of RNA, whether free or in the ribosome. ●—●, ribosomes; ○—○, RNA.

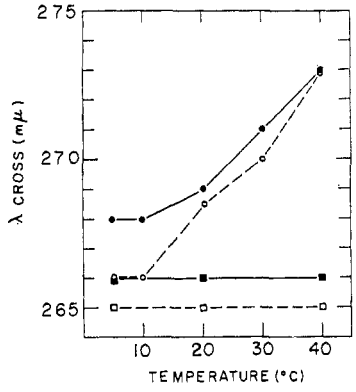

FIGURE 8: Effect of temperature on the crossing point of the Cotton effect of 30S ribosomes and 16S rRNA. The data are taken from the experiments of Figures 6 and 7. ●—●, unfolded ribosomes in 1 mM Tris (pH 7.4). ○----○, rRNA in 1 mM Tris. ■—■, native ribosomes in buffer A (1 mM $Mg(OAc)_2$, 10 mM Tris (pH 7.4), and 0.1 M NH_4Cl). □----□, rRNA in buffer A.

(1966) for yeast tyrosine and alanine tRNAs. Base pairing persisted at low temperatures (10° or less) both in the presence and absence of Mg^{2+}. At higher temperatures the base-paired conformation was stabilized if Mg^{2+} was present. Results of a similar nature on the circular dichroism of *E. coli* ribosomes and rRNA have been obtained by Adler *et al.* (1970), whose report appeared while this manuscript was being prepared.

In all experiments the magnitude of the 252-mμ trough was larger for ribosomes than for rRNA. Interpretation of this is made difficult by possible interference by the 233-mμ trough of ribosomal proteins.

Discussion

Optical rotatory dispersion and circular dichroism measurements on ribosomes from various sources have indicated that rRNA in the ribosome is largely base paired (Bush and Scheraga, 1967) and that the association of the ribosomal proteins with the rRNA to form the ribosomes does not involve a major change in the conformation of the rRNA (Sarkar and Yang, 1967; McPhie and Gratzer, 1966; Wolfe and Kay, 1969; Blake and Peacocke, 1965; Bush and Scheraga, 1967). When our experiments were performed under conditions similar to those employed in the above-cited works, the results were comparable. In the presence of Mg^{2+} and salt the Cotton effect of the ribosomal optical rotatory dispersion spectrum was about 1 mμ to the red of that of pure rRNA at all temperatures tested, as previously shown by Bush and Scheraga (1967, see Table I). This difference is small and does not indicate any major conformational change in the rRNA when it is incorporated into the ribosomal structure. The amplitude of the Cotton effect peak was lower with ribosomes than with pure rRNA, as has also been observed in reticulocyte ribosomes (Cox, 1969; Figure 2). This difference was not found by Sarkar and Yang (1967) in their work on *E. coli* ribosomes, perhaps owing to a high extinction coefficient chosen for calculating the concentration of ribosomes.

The native structure of the ribosome is disrupted when Mg^{2+} and salt are removed, and if the treatment is carried far enough, the compact particle is converted to an extended chain still carrying all of the ribosomal proteins but closely resembling free rRNA in its hydrodynamic properties. This was first shown by Spirin *et al.* (1963) and was later studied by others (Cammack and Wade, 1965; Gesteland, 1966; Weller *et al.*, 1968; Spitnik-Elson and Atsmon, 1969). We have followed the unfolding of the 30S subunit by equilibrating the ribosome against media of different ionic composition, first removing Mg^{2+} without changing the ionic strength and then lowering the ionic strength in the absence of Mg^{2+}. This treatment stopped the process of unfolding at a number of stages along the way, and the sedimentation constant and optical rotatory dispersion spectrum of each stage were determined.

The unfolding process proceeded in two stages under these conditions. The removal of Mg^{2+} converted the 30S ribosome into a partly unfolded particle of about 17–20 S, with a relatively small but significant disruption of base pairing. This particle remained stable as the ionic strength was reduced from 0.11 to 0.027, but at lower ionic strengths it unfolded further with an extensive disruption of base pairs, apparently being converted into the fully extended form.

Discrete intermediate stages in the unfolding of both the 30S and 50S subunits have been observed by others (Spirin *et al.*, 1963; Cammack and Wade, 1965; Gavrilova *et al.*, 1966; Gesteland, 1966; Weller *et al.*, 1968) who followed changes in the sedimentation constant. In addition to confirming these observations, our results show that both conformational transitions involve the disruption of base pairs in the RNA moiety. In the second transition, the unfolding of the 17–20S particle at very low ionic strength, it seems likely that the base-paired regions denatured are or include the helical hairpin loops believed to make up a large part of the rRNA structure (Doty *et al.*, 1959). These proposed loops constitute the secondary structure of the rRNA. As for the nature of the less stable base-paired regions disrupted during the first conformational transition, the conversion of the 30S ribosome to a 17–20S particle, at least two possibilities may be considered. These regions may also be hairpin loops, either inherently less stable than the others or less highly stabilized by the ribosomal proteins; or they may be formed by complementary stretches which are not adjacent to each other in the linear RNA chain and which, in pairing, control the folding of the ribosome and cause the formation

of its specific tertiary structure (*cf.* Studier, 1969, on DNA conformation).

We have also compared the optical rotatory dispersion spectra of fully unfolded ribosomes to those of pure rRNA at different temperatures. These spectra showed the greatest differences at low temperature (5–10°), where the crossing point of the Cotton effect of unfolded ribosomes lay significantly to the red of that of pure rRNA and its peak amplitude was lower. These data suggest that the ribosomal proteins may prevent the attainment of maximum base pairing at low temperature. The effect is small but seems real.

At higher temperature (40°) the crossing points became the same and the difference in peak amplitude diminished. We have not ruled out the possibility that some protein may become detached from the unfolded ribosome at this temperature. However, it has been shown that in the same or comparable low salt media no protein is detached in the cold (Spirin *et al.*, 1963), and at 25° our sedimentation patterns have shown only a single nucleoprotein component with no sign of detached proteins (experiment of Table II). It therefore seems doubtful that significant detachment of protein would occur in this medium at 40°, or at least, that it would be extensive enough to contribute significantly to the observed covergence of the optical rotatory dispersion properties of the nucleoprotein and RNA at that temperature.

We conclude, therefore, that although the ribosomal proteins are known to stabilize the helical conformation of the rRNA within the ribosome (Wolfe and Kay, 1969), they do not prevent its disruption under all conditions. At very low ionic strength the opening up of the compact ribosomal structure may be accompanied by destruction of this helical conformation. When such opened up ribosomes are compared to free rRNA in a low salt medium, it can be seen that the ribosomal proteins seem to interfere, to a small but apparently real degree, with the formation of base pairs in the RNA. This is apparent at low temperatures (5–10°). As the temperature is raised, the base pairs in the free RNA seem to dissociate more readily, so that at temperatures of 30 and 40° both structures appear to have approximately the same extent of base pairing. In high salt media the difference between RNA in and out of the native ribosome is diminished to a degree where its significance is questionable.

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