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Irreversible Thermal Denaturation of *Escherichia coli* Ribosomes*

James W. Bodley

ABSTRACT: Irreversible thermal denaturation of high salt-washed *Escherichia coli* (B and Q13) ribosomes has been followed in terms of biological activity, sedimentation properties, and turbidity, as a function of solvent composition. In a buffer composed of 0.01 M Tris–0.01 M $MgAc_2$ (pH 7.4) ribosomes lose 50% of their ability to form polyphenylalanine from polyuridylic acid in 5 min at 57° ($T_{d,5}$). In this temperature range 70S ribosomes are destroyed, presumably by aggregation. The sharpness of this thermal transition is illustrated by the fact that in 0.01 M Tris–0.01 M $MgAc_2$ several hours are required for 50% inactivation at 50°, while at 60° 50% inactivation occurs within 1 min. Reduction of the magnesium ion concentration to 10^{-4} M reduces the $T_{d,5}$ to 53.5° and under these conditions the normal 50S and 30S subunits are converted into slower sedimenting particles (~35–40 and 25S). Monovalent cations ($K \geqslant NH_4 > Na$) in the presence of 0.01 M $MgAc_2$ (but not 10^{-4} M $MgAc_2$) protect ribosomes from thermal inactivation presumably by preventing aggregation. In 0.01 M Tris–0.01 M $MgAc_2$–0.1 M KCl (pH 7.4), ribosomes exhibit a $T_{d,5}$ of 60.5° and the loss in biological activity is closely paralleled by the destruction of the 30S subunit. The effect of heating on ribosome activity is characterized by an initial increase in activity (variable with the preparation) followed by a rapid but decreasing rate of inactivation. No explanation is provided for this activation, but it apparently results from an increase in the number of active ribosomes and is not due to a relaxation of the ribosomal structure which would also lead to translational ambiguity. Evidence is presented which suggests that the observed denaturation does not result from enzymatic degradation. It is also shown that ribosomes can be lyophilized from a variety of buffers with little or no alteration of physical or functional properties.

The ribosomes of *Escherichia coli* are not only structurally complex but they have a complex role in protein synthesis. They are known to contain 3 RNA molecules and probably as many as 40 different protein molecules. Very little information, however, is available on the way in which these individual components interact to produce the structural and biological features of the ribosomal particle.

Magnesium ion is essential to the maintenance of both the structural and functional integrity of the ribosome and several investigators have shown that ribosomes undergo a characteristic and partially reversible unfolding upon the removal of magnesium ion (see Gavrilova *et al.*, 1966; Gesteland, 1966). Kaji *et al.* (1966) have shown that mild proteolysis of ribosomes progressively destroys their biological activity in the order, protein synthesis, aminoacyl-tRNA binding, mRNA binding, and ultimately causes irreversible dissociation into apparently normal 50S and 30S subunits without the release of appreciable soluble material. On the basis of chemical modification of the ribosome, Moore (1966) has concluded that amino groups of rRNA are also involved in the binding of mRNA and in the interaction of the subunits. Protein sulfhydryl groups have also been implicated in ribosomal structure and function (Wang and Matheson, 1966; Tamaoki and Miyazawa, 1967; Traut and Haenni, 1967).

In addition to their role in binding mRNA and amino-

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acyl-tRNA it has recently been suggested that ribosomal protein is specifically responsible for peptide-bond formation (Monro, 1967). Recent experiments with reassembled, protein-deficient ribosomes (see Traub and Nomura, 1968) have also suggested unique functions for the various proteins.

The objective of the present investigation was to examine the irreversible structural and functional changes accompanying thermal denaturation of *E. coli* ribosomes in the hope of gaining further insight into the form of the particle required for activity and the nature of the forces which maintain its structure and how they are effected by environment. The thermal denaturation of ribosomes has in the past received only limited attention (Saunders and Campbell, 1966; Tamaoki and Miyazawa, 1966; Miyazawa and Tamaoki, 1967) and no attempt has been made to correlate alterations in structural and functional parameters. Furthermore, cognizance should be taken of the activation of RNase I as a possible cause of thermal denaturation of ribosomes as suggested by Schell (1965). Recently, McLaughlin *et al.* (1968) have examined the thermal stability of mRNA–tRNA–ribosome complexes and shown that such complexes can undergo a thermally induced reversible dissociation and that among other factors the intrinsic stability of the ribosome under some conditions establishes an upper limit to the thermal stability of these complexes.

Materials and Methods

Materials. E. coli cell paste (B and Q13, late log) was purchased from General Biochemicals, Inc. ^{14}C-Labeled amino acids were obtained from New England Nuclear Corp. The polynucleotides were purchased from Miles Laboratories and ATP, GTP, and phosphoenolpyruvate were purchased from Sigma. Pyruvate kinase was purchased from C. F. Boehringer und Soehne.

Preparation of Ribosomes and S-100. All operations were carried out between 0 and 4° unless otherwise specified and all pH measurements were made at ambient temperature, *ca.* 22°. Cell paste was thawed and washed twice with a buffer containing 0.01 M Tris-Cl–0.01 M $MgAc_2$ (pH 7.4) (TM buffer) and once with 0.01 M Tris-Cl–0.01 M $MgAc_2$–0.05 M KCl–0.01 M β-mercaptoethanol (pH 7.4) (TMKS buffer). The resultant cell paste was suspended in an equal volume of TMKS buffer, 4 μg/ml of DNase (Worthington) was added, and the suspension was sonicated with a Branson sonifier. The suspension was clarified by at least two 30-min centrifugations at 30,000*g* and the final supernatant was centrifuged for 8 hr at 30,000 rpm in the No. 30 rotor of the Spinco Model L preparative ultracentrifuge. The upper two-thirds of the supernatant (S-100) was aspirated, dialyzed against four changes of TMKS buffer, frozen, and stored at either −20 or −70°. The ribosomal pellet was suspended in a buffer containing 0.01 M Tris-Cl–0.002 M $MgAc_2$–0.5 M NH_4Cl (pH 7.4) (Salas *et al.*, 1965) with a glass–Teflon homogenizer. After standing for approximately 8 hr the suspension was centrifuged for 30 min at 30,000*g* (pellet discarded) and the ribosomes were pelleted by further centrifugation for 3 hr at 50,000 rpm in the Spinco No. 50 Ti rotor. The supernatant was discarded and the dark brown upper layer of the pellet was scraped away and discarded prior to resuspending the ribosomes. This washing procedure was repeated twice and the ribosomes were finally suspended in a small volume of TM buffer. The suspension was dialyzed against four changes of TM buffer, centrifuged at low speed, adjusted to a concentration of 50 mg/ml, and stored at either −20 or −70°. In some cases these ribosomes were further purified as described by Kurland (1966), *i.e.*, by differential ammonium sulfate precipitation followed by further high-salt washing, or by chromatography on DEAE-cellulose (Stanley and Wahba, 1967). Except as is noted below, all of the preparations exhibited identical behavior with respect to the effect of heat. Ribosome concentrations were calculated assuming $E^{1\%}_{260}$ 14.5 (Tissières *et al.*, 1959). Although no functional alteration of ribosomes was detected as a result of repeated freezing and thawing, all data reported were derived with once frozen ribosomes.

Amino acid incorporation experiments were typically performed in a final volume of 100 μl and contained the following components: 100 mM Tris-Cl (pH 7.8), 50 mM KCl, 10 mM β-mercaptoethanol, 5 mM phosphoenolpyruvate, 1 mM ATP, 0.05 mM GTP, 30 μg/ml of pyruvate kinase, approximately 1 mg/ml of S-100 protein (typical S-100 preparations had protein concentrations, determined by the method of Lowry *et al.* (1951), of 10 mg/ml), 0.5 μCi/ml of [^{14}C]amino acid (specific activities ranged from 248 to 366 mCi/mmole), 100 μg/ml of polynucleotide, up to 250 μg/ml of ribosomes, and optimal levels of $MgAc_2$. Magnesium optima were determined for each preparation and were generally 15 mM for poly U directed incorporation and 14 mM for poly A. Slight differences, however, were noted in the optima for different preparations and all experiments were performed under optimal conditions. Usually a solution was made which contained all of the above components except ribosomes and the polynucleotide. Ribosomes, generally in a volume of 5 μl, were added and the reaction was initiated by the addition of 5 μl of an appropriate polynucleotide solution. Incubations were for 30–60 min at 37°.

Quantitation of the polypeptide product with poly U as the template was performed essentially by the method of Bollum (1966). Following incubation at 37°, a portion of the reaction mixture (generally 75 μl) was removed and placed on a 2-cm square of Whatman No. 31ET paper. The paper was then immersed in 400–500 ml of 10% trichloroacetic acid. After all papers were collected (as many as 150) they were washed twice with 400–500-ml portions of 5% trichloroacetic acid and then heated at 90° for 15 min in 5% trichloroacetic acid. The heating was followed by another wash in 5% trichloroacetic acid and then two washes each in ethanol–ether (1:1, v/v) and ether, respectively. All washes were for 5 min (except the one at 90°) with gentle stirring while the papers were suspended in a wire basket. With poly A as the template the wash was the same except the papers were collected and washed twice in a solution of 5% trichloroacetic acid–0.25% sodium tungstate (pH 2.0) prior to heating at 90° in 5% trichloroacetic acid. After the final ether wash the papers were air dried and placed in

counting vials containing a toluene-based liquid scintillator (Packard Instrument Co.) and counted in a Beckman LS-100 scintillation counter. Counting efficiency under these conditions was approximately 70%.

Incorporation assays were linear for approximately 30 min and continued at a diminishing rate for 60–90 min. The assays were linear with respect to ribosome concentration up to 250 μg/ml for the *E. coli* B system (somewhat less for the Q13 system), the maximal level employed. Maximal incorporating activity (see below) for the B system was approximately 1.0 and 0.5 $\mu\mu$mole of amino acid per μg of ribosomes per 30 min for phenylalanine (poly U) and lysine (poly A), respectively. The Q13 system was approximately three to four times as active, while the substitution of Q13 S-100 for B S-100 approximately doubled the activity of B ribosomes. Radioactivity in the absence of either ribosomes or polynucleotide or with blank papers carried through the washing procedure generally did not exceed 100–200 cpm, and all of the data presented are corrected by such blank values. All incorporation data points, with the exception of the rate experiments, represent the average of at least duplicate determinations. The standard deviation of such replicates averaged $< \pm 6\%$.

Sedimentation Velocity Analysis. Sedimentation analyses were performed with a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. All runs were made with an AN-D rotor at a speed of 29,500 rpm using a 12-mm, 4° sector cell at a temperature of 22°. The rotor was accelerated at a constant rate (12 A) and timing was begun upon reaching a speed of 20,000 rpm (which required ~2.6 min). The rotor was near speed approximately 1.2 min later.

Unless otherwise specified, the sedimentation profiles were made from photographs taken at either 24 or 40 min with a schlieren bar angle of 60°. All sedimentation analyses were performed at a ribosome concentration of 5 mg/ml in a volume of 0.7 ml. The graphically depicted sedimentation coefficients are uncorrected for ribosome concentration or buffer salts and assume an average miniscus distance from the center of rotation of 5.98 cm. With the particular enlargement employed, the distance between the two outer boundaries of the sedimentation profile figures is proportional to the distance between the counter weight reference holes (1.6 cm) in the rotor. The relative proportion of the various ribosomal components was determined by measuring the areas of the schlieren peaks.

Thermal denaturation was conducted in a rapidly circulating water bath maintained to within $\pm 0.1°$ of the nominal temperature. Most of the experiments were conducted under conditions where the sample temperature (as measured with a thermistor probe) was within 1° of the bath temperature within 20 sec and was indiscernably different within 30 sec. Heating was terminated by either immersing the tube in an ice bath or removing an aliquot and adding it to an ice-cold assay solution. The two methods yielded identical results in terms of the loss of biological activity. Unless otherwise stated all heating experiments were performed at a ribosome concentration of 5 mg/ml.

As indicated above, ribosomes were stored at a concentration of 50 mg/ml in TM buffer. In order to add salt, the ribosomes were simply diluted into the appropriate buffer. In order to reduce the magnesium concentration the ribosomes were first diluted with low magnesium buffer and then dialyzed 12–24 hr against this buffer.

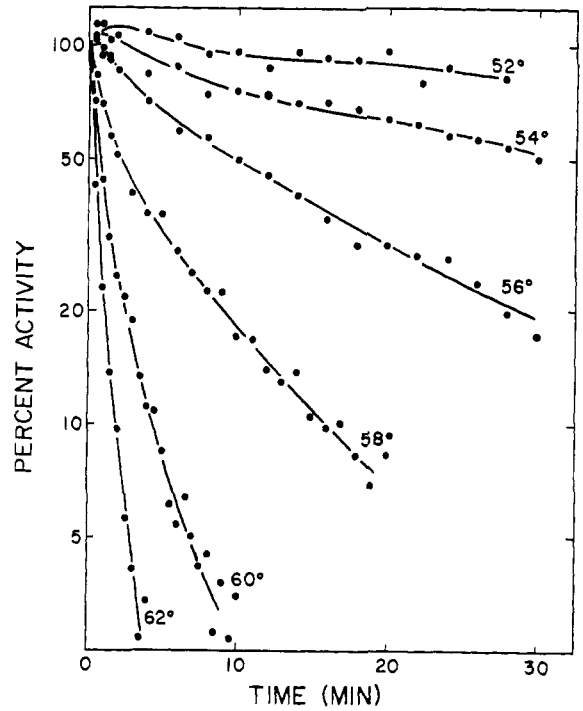

FIGURE 1: Rate of thermal inactivation of ribosomes in TM buffer. Separate but identical solutions of *E. coli* B ribosomes were heated at the indicated temperatures. Aliquots were periodically withdrawn and added to an assay solution and subsequently assayed for their ability to incorporate phenylalanine in response to poly U. Activity is expressed as per cent of that exhibited by unheated ribosomes. Procedural details in this and subsequent figures are as outlined in Materials and Methods.

Optical Measurements. All optical measurements were performed at ambient temperature in a Beckman DU spectrophotometer equipped with 10-mm microcells. Turbidity measurements of both native and denatured ribosomes (5 mg/ml) followed the relationship of Bayzer and Schauenstein (1955) over the range 400–700 mμ, namely, that a plot of log OD *vs.* log λ was linear, indicating the contribution of light scattering rather than chromophore adsorption. By extrapolation of such plots it was possible to show that with an A_{400} of 1.0 the contribution of turbidity to absorbancy at 260 mμ would be less than 10%.

Results

Effect of Heating on Biological Activity. Figure 1 illustrates a typical experiment in which the rate of loss of the biological activity of ribosomes (the ability to participate in the formation of polyphenylalanine) was followed as a function of time of incubation at various temperatures in TM buffer. The first effect observed

upon heating, although not marked with this ribosome preparation (see below), was an initial increase in the ability to support phenylalanine incorporation. Activity is then lost at a rapid but decreasing rate. Ribosomes did not exhibit first-order denaturation kinetics under any of the conditions examined. Perhaps the most striking feature of these data is the narrow range of temperature over which denaturation occurs. In fact, experiments have shown that at 50° in TM buffer approximately 6.5 hr is required for a 50% decrease in polyphenylalanine-synthesizing ability. The abruptness of this transition is more clearly demonstrated in Figure 2 (middle curve). These data were generated by heating separate but identical solutions of ribosomes for 5 min at various temperatures and, after cooling, measuring their ability to translate poly U. For convenience, the temperature at which 50% inactivation occurred was designated as the $T_{d,5}$. The average $T_{d,5}$ for ribosomes in TM buffer was 57°. Raising the level of magnesium in this buffer did not enhance the stability of the particles. Denaturation profiles are also shown in Figure 2 for ribosomes dissolved in two other commonly employed buffers, $TM10^{-4}$ buffer (0.01 M Tris-Cl–10^{-4} M $MgAc_2$, pH 7.4) and TMK buffer (0.01 M Tris-Cl–0.01 M $MgAc_2$–0.1 M KCl, pH 7.4). Ribosomes in $TM10^{-4}$ buffer and TMK buffer exhibited a $T_{d,5}$ of 53.5 and 60.5°, respectively.

The values of $T_{d,5}$ obtained by graphic interpolation were surprisingly constant with a maximum deviation generally less than ±0.5° and the behavior of the ribosomes was not altered by the further purification outlined in Materials and Methods. Out of six ribosome preparations (three *E. coli* B and three Q13) only one showed significantly different thermal stability with values of $T_{d,5}$ averaging approximately 1–2° lower in the buffers tested. Perhaps coincidentally, this same preparation also exhibited the maximum increase in activity on heating (Figures 4 and 5). The $T_{d,10}$ and $T_{d,30}$ in TM and TMK buffers are 1 and 3° lower than the respective $T_{d,5}$'s (data not shown). Experiments have shown the $T_{d,5}$ to be essentially independent of ribosome concentration (during heating) between 0.5 and 50 mg per ml in TM buffer. Experiments have also shown that the denaturation rate in TM buffer is essentially independent of pH over the range pH 7–8 and only slightly greater at pH 9. While it is known (Good *et al.*, 1966) that the pH of Tris-Cl buffer is strongly temperature dependent, it is unlikely that the present observations reflect a pH effect as such. In addition, β-mercaptoethanol (1–10 mM) had no detectable effect on thermal stability at least upon short-term incubation.

It should also be pointed out that the rate of thermal destruction of ribosomal activity was not noticeably affected by the composition of the subsequent assay mixture (at least in relation to the source of the S-100, B or Q13, or the concentration of amino acid, polynucleotide, or tRNA, or the length of the incorporation incubation). Clearly, however, the absolute activities varied widely under these conditions.

It is quite clear from the data in Figure 2 that KCl serves to protect the ribosome from thermal inactivation. This protection by salt is further explored in Figure 3. It is evident that maximum stability is achieved in the presence of 0.1 M KCl while NH_4Cl and NaCl are, in that order, less effective in protecting the ribosome against thermal inactivation. With one preparation of ribosomes NH_4Cl at a somewhat higher concentration was approximately as protective as KCl.

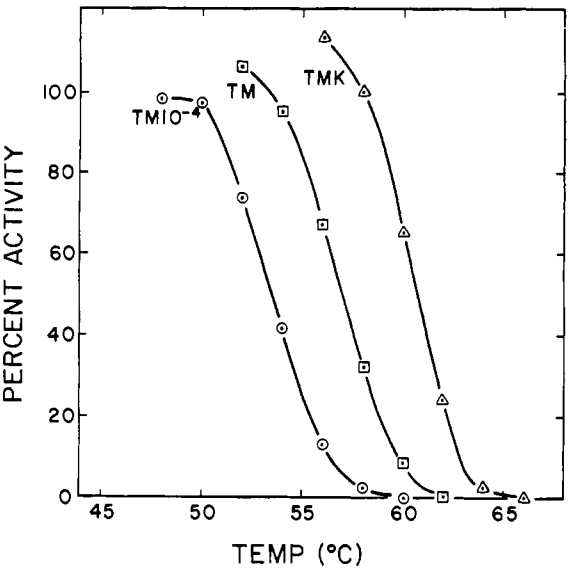

FIGURE 2: Thermal destruction of biological activity as a function of buffer composition. Separate solutions of ribosomes in the indicated buffers were heated for 5 min at various temperatures, chilled, and assayed for polyphenylalanine-synthesizing ability.

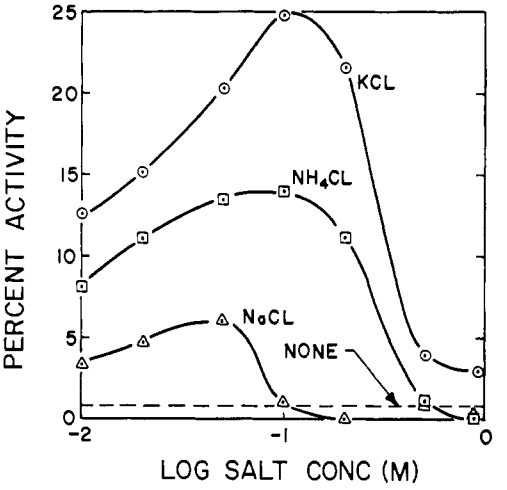

FIGURE 3: Protection from thermal inactivation by monovalent cations. Ribosomes in TM buffer supplemented with the indicated salt concentration were heated for 20 min at 60°, chilled, and assayed. The activity (polyphenylalanine synthesis) is expressed as per cent of the unheated ribosomes. The level of activity remaining after heating in TM buffer alone (0.8%) is indicated by the broken line.

As was previously noted, one of the distinguishing results of heating ribosome solutions was an initial increase in activity at moderate temperatures. Figure 4

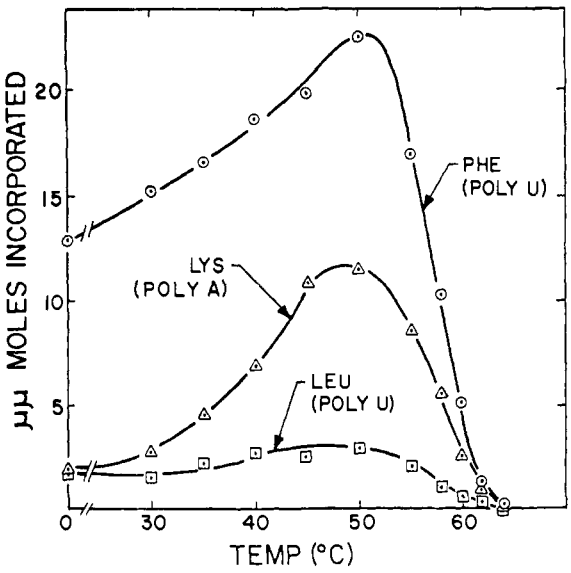

FIGURE 4: The effect of 5 min incubation in TMK buffer at various temperatures on the ability of ribosomes to translate poly U and poly A. After heating at the indicated temperatures the solutions were chilled and added to the assay solutions optimized for the three activities. The assay incubation was for 60 min at 37°. The results are expressed in $\mu\mu$moles of amino acid incorporated per 100 μl of assay (25 μg of ribosomes).

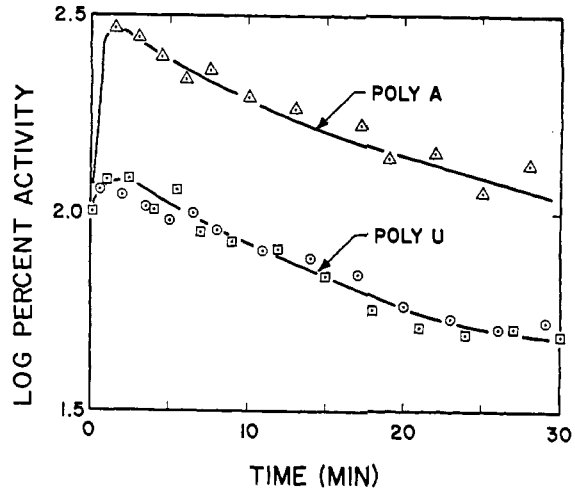

FIGURE 5: Rate of loss of poly U and poly A translating ability upon incubation of ribosomes in TMK buffer at 58°. A ribosome solution was placed in a bath at 58° and samples were periodically withdrawn and added to cold assay solutions for subsequent measurements of incorporating ability. Activity is expressed as per cent of the unheated controls. (△) Lysine incorporation with poly A as template; (⊙) phenylalanine; and (⊡) leucine incorporation with poly U as template.

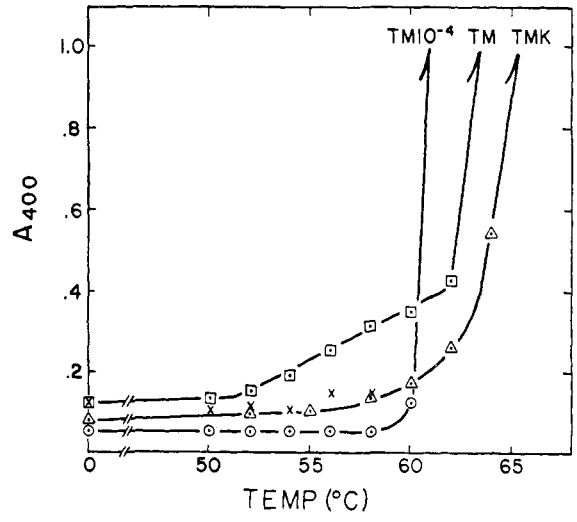

FIGURE 6: Effect of heating on ribosomal turbidity in various buffers. Ribosomes in the indicated buffers were heated 5 min at the indicated temperature and chilled, and their A_{400} was determined at ambient temperature. The x represents the turbidity of ribosome solutions which have been made 0.01 M in $MgAc_2$ after heating in $TM10^{-4}$ buffer.

illustrates this phenomenon with a ribosome preparation which showed a more pronounced increase. With this preparation heating for 5 min at 50° doubled the ability of the ribosomes to support polyphenylalanine synthesis and increased their ability to translate poly A by sixfold. This thermal "activation" varied with different ribosome preparations from 0 to 100% for poly U translation and 0–500% for poly A translation, but no correlation was evident between thermally induced increases in the ability to translate these two polynucleotides. It was evident, however, that the thermally "activated" ribosomes possessed activities which were more comparable than ribosomes which had not been heated. Figures 4 and 5 also illustrate that the leucine ambiguity (see Bodley and Davie, 1966) increased in parallel with phenylalanine incorporation. Figure 5 also shows that the rate of loss of activity with poly U (both phenylalanine and leucine incorporation) or poly A as messenger was identical.

Thermally Induced Physical Changes. Figure 6 illustrates the turbidity of ribosome solutions at ambient temperature after heating for 5 min at various tempertures in the three buffers. The turbidity of unheated ribosome solutions at this concentration (5 mg/ml, A_{260} 72.5) is low and ribosomes in $TM10^{-4}$ buffer routinely exhibited one-third to one-half the turbidity of ribosomes in TM buffer. Heating for 5 min in this latter buffer resulted in a nearly linear A_{400} increase over the temperature range 52–62° and then rose sharply. This sharp rise in turbidity presumably reflects either aggregation of ribosomes or coagulation of ribosomal protein. In low magnesium ($TM10^{-4}$ buffer) there was no significant change in turbidity up to 58°. Readdition of magnesium to ribosomes heated in $TM10^{-4}$ buffer increased the turbidity by the difference between unheated ribosomes in TM and $TM10^{-4}$ buffers, respectively. The turbidity in TMK buffer rose gradually till approximately 62°.

The nature of the physical change of ribosomes following heating in TM buffer is recorded in Figure 7a. There was no major change in the qualitative nature of the sedimentation profile but simply a loss of material sedimenting as 100 S or less. This material appeared

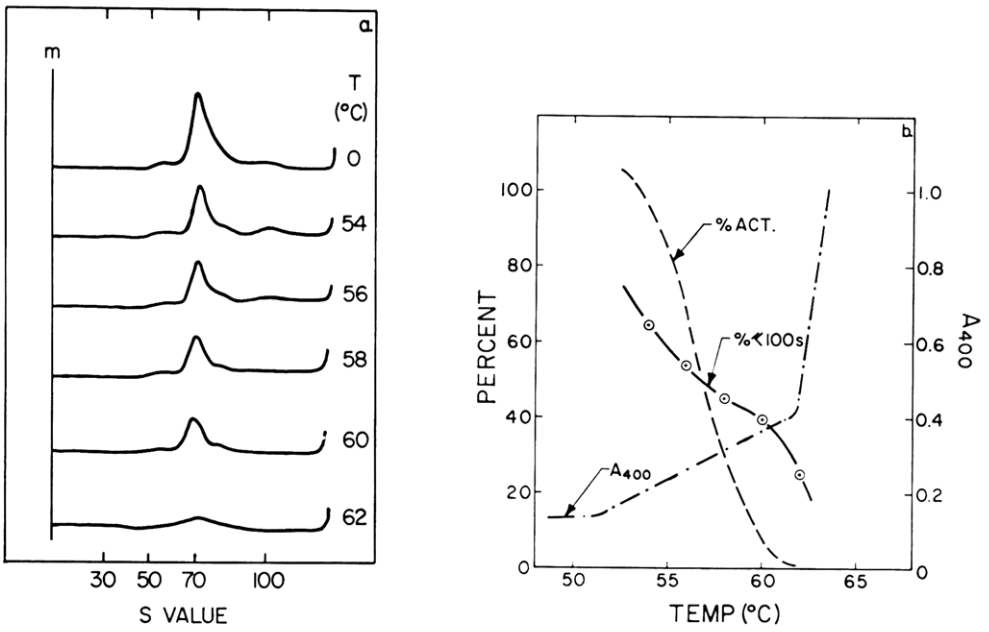

FIGURE 7: Sedimentation behavior of ribosomes heated in TM buffer. (a) Sedimentation profiles of ribosomes after heating for 5 min at the indicated temperatures. The m in this and subsequent figures marks the positions of the menisci. (b) The data points represent the per cent (relative to the unheated control) particles sedimenting $\leqslant$ 100 S. The broken lines are schematic representations of the activity (Figure 2) and turbidity (Figure 5) under these conditions.

to be lost as higher, heterogeneous, aggregates which are no longer visible in this frame. Although there was no detectable increase in material sedimenting slower than 70S, after heating for 5 min at 54° there was a modest increase in 100S dimers. Prolonged incubation at lower temperatures (4 hr at 37°; Figure 8) accentuated this effect, yielding approximately 50% 100S particles. There was at the same time no effect on biological activity or subsequent thermal stability at higher temperatures.

Figure 7b demonstrates the relationship between thermally induced physical and functional changes of ribosomes in TM buffer. Although the ribosomes appeared to aggregate over a somewhat broader range of temperature, physical and functional destruction were closely associated.

Ribosomes are not only more functionally resistant to thermal denaturation in TMK buffer (Figure 2) but the nature of the physical change is different than in TM buffer. Over the temperature range 58–64° there is a progressive decrease in 70S particles with a concomitant increase in 50S subunits (Figure 9a). That this is not due entirely to simple irreversible dissociation to 50S and 30S subunits is demonstrated by the absence of the latter particle (particularly noticeable at 64°). From the data in Figure 9b it would appear that the loss in biological activity can be almost totally accounted for by the destruction of the 30S subunit. While this correlation exists with a 5-min heating interval, Figure 10 shows that with continued heating biological activity is lost much more rapidly than is the 70S ribosome.

As would be predicted on the basis of turbidity measurements, heating in TM10^{-4} buffer does not lead to aggregation of ribosomes or coagulation of ribosomal protein over the range of temperature associated with biological inactivation (Figure 11a,b). In fact, a new slow sedimenting peak appears between the 30S and 50S positions and there seems to be a small but definite shift in the position (decrease in its sedimentation velocity) of the slowest moving component. Based on the behavior of the unheated subunits in this system these new particles would have corrected sedimentation coefficients of approximately 35–40 and 25S, respectively.

The data in Figure 9b suggest that these physical changes may slightly precede the loss in biological activity but it is clear that activity is lost more rapidly than is material sedimenting $\leqslant$50S.

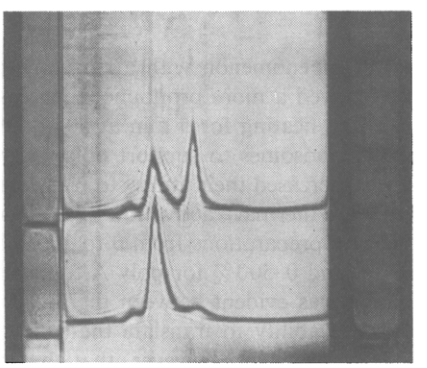

FIGURE 8: Formation of 100S dimers by incubation of ribosomes at 37° in TM buffer. A solution of ribosomes in TM buffer was divided in half; a portion (upper pattern) was incubated for 4 hr at 37° while the remainder (lower pattern) was kept on ice. The two major peaks in the upper pattern correspond to 70S and 100S particles. The photograph was taken 16 min after reaching two-thirds speed.

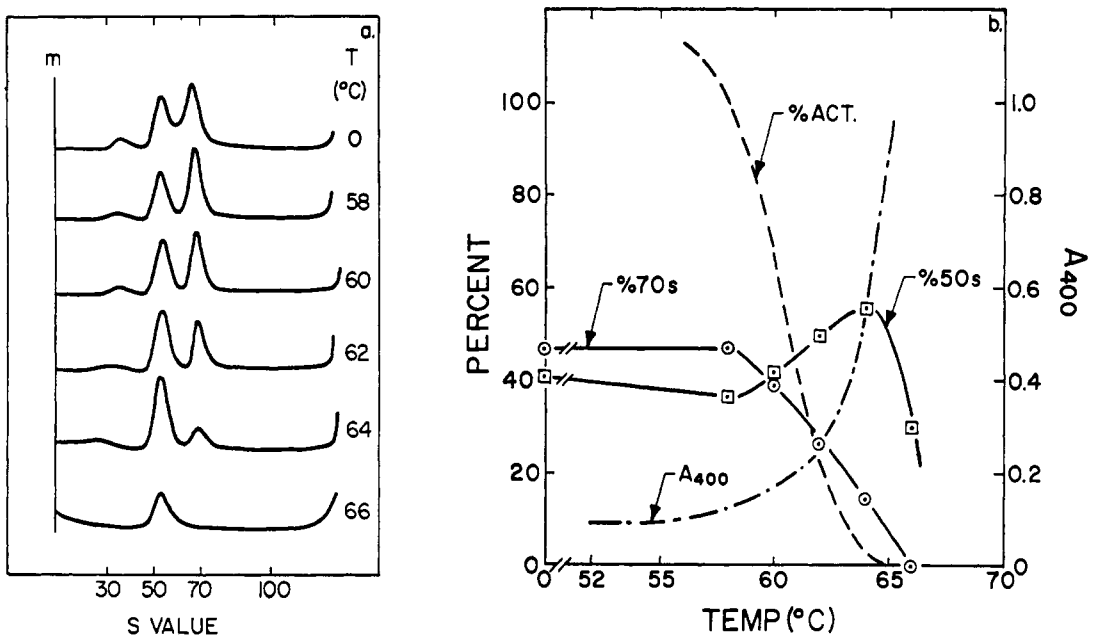

FIGURE 9: Sedimentation behavior of ribosomes heated in TMK buffer. (a) Sedimentation profiles of ribosomes after heating for 5 min at the indicated temperatures. (b) The data points represent the per cent (relative to the total sedimenting particles in the unheated control) of the 50S (⊡) and 70S (⊙) particles. The broken lines are schematic representations of the activity (Figure 2) and turbidity (Figure 5) under these conditions.

The effect of heating ribosomes in TM10^{-4} buffer on their capacity to re-form 70S particles upon the readdition of magnesium was explored in the experiment presented in Figure 12a,b. It is evident that the majority of the slow-moving components aggregate to a form sedimenting in the general region of the 70S particle but there is a definite appearance of a particle sedimenting at ~80 S as well as a small increase in material sedimenting slower than 70 S. While the total material sedimenting at 70–80 S exceeds the functional capacity of the ribosome (Figure 12b) there may be a direct relationship between activity and the ability to re-form 70S particles. It should be pointed out that it makes no difference in the biological activity of ribosomes heated in TM10^{-4} buffer following cooling whether they are simply added to an assay solution (containing high magnesium), if magnesium is added to the ribosome solution prior to the assay or if the ribosomes are dialyzed into high magnesium (TM buffer).

In the course of this investigation the somewhat surprising observation was made that ribosomes can be lyophilized from solution without significant alteration of their properties following resolution. Figure 13 compares the sedimentation behavior of lyophilized and unlyophilized ribosomes in three buffers. In each case the ribosomes were lyophilized in the indicated buffer and redissolved by simply adding water. The resulting sedimentation profiles in TM and TMK buffers were virtually identical with the unlyophilized controls. In TM-10^{-4} buffer the subunit profiles appeared to be slightly broadened as a result of lyophilization. In all buffers the recovery of activity, measured as the ability to translate poly U, was essentially complete, ranging from 80 to 100%.

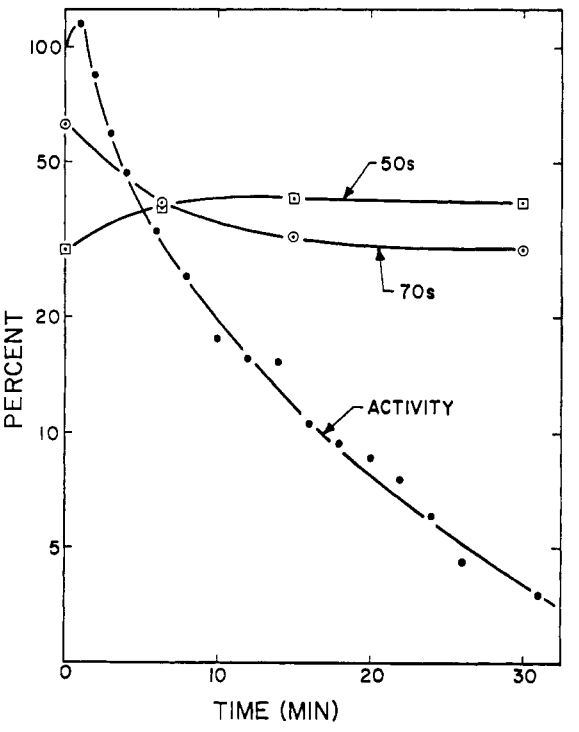

FIGURE 10: Rate of physical and function denaturation upon heating at 62° in TMK buffer. Aliquots of a ribosome solution at 62° were withdrawn and either chilled for subsequent sedimentation analysis or added to an ice-cold assay solution for the assay of polphenylalanine-synthesizing activity.

Normally, ribosome solutions are stored by quick-freezing concentrated solutions which are thawed only once and any unused portion is discarded. Lyophilized ribosomes do not appear to be significantly hydroscopic and are quite stable to storage; in a typical experiment less than 20% of the activity was lost as a result of storage of the lyophilized powder for 10 days at room temperature. It is suggested that lyophilization may under some conditions offer a convenient method of storage of ribosomes.

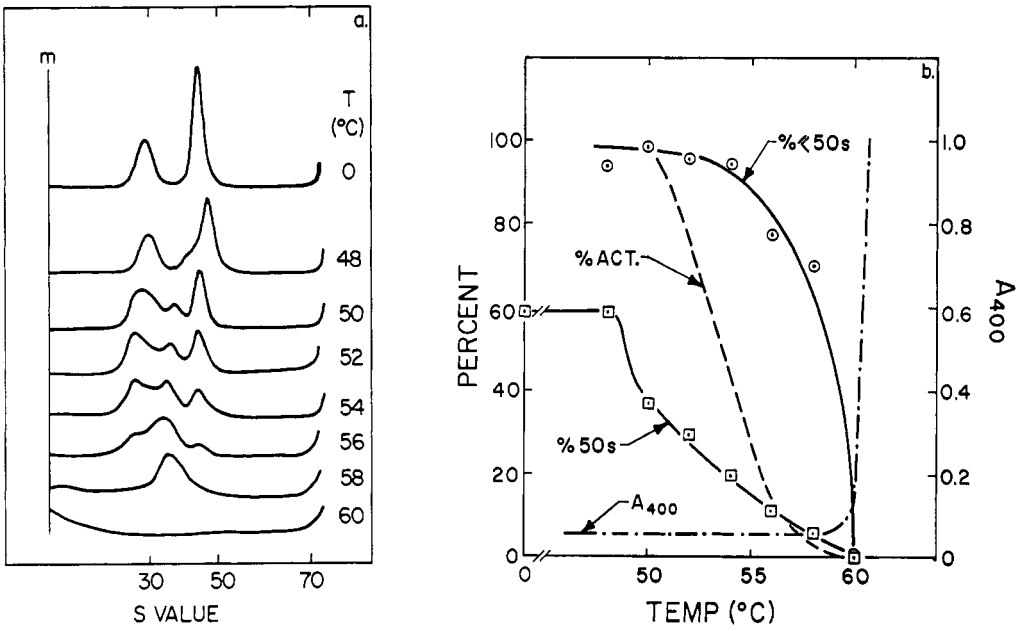

FIGURE 11: Sedimentation behavior of ribosomes heated in $TM10^{-4}$ buffer. (a) Sedimentation profiles of ribosomes after heating for 5 min at the indicated temperatures. (b) (⊙) Per cent (relative to the total sedimenting particles of the unheated control) particles sedimenting ≪ 50 S. (⊡) Per cent sedimenting as 50S particles. The broken lines are schematic representations of the activity (Figure 2) and the turbidity (Figure 5) under these conditions.

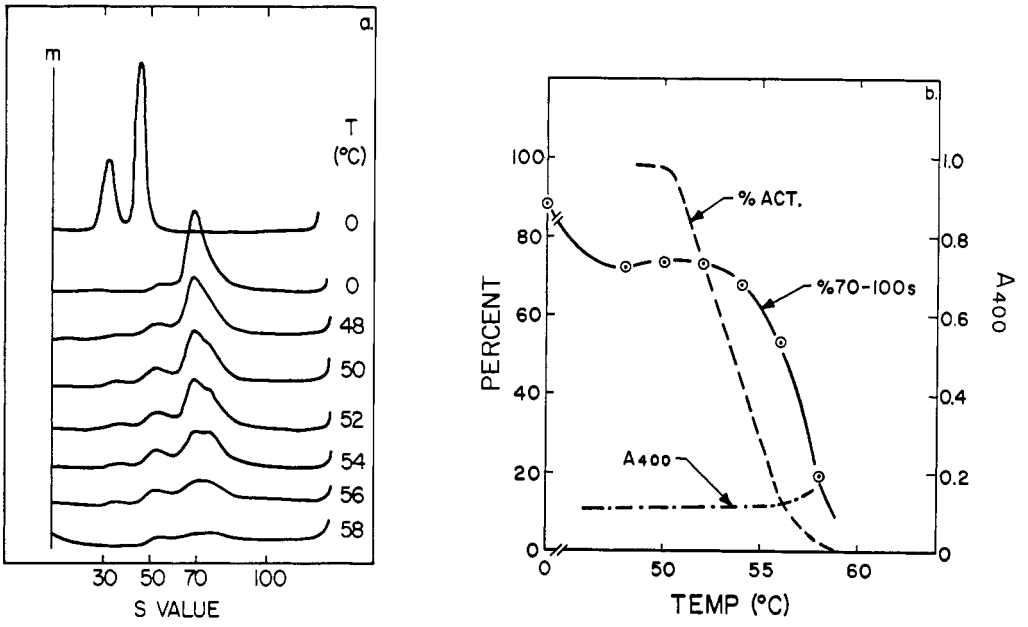

FIGURE 12: Sedimentation behavior of ribosomes heated in $TM10^{-4}$ buffer after the readdition of magnesium ion. (a) The top pattern is of unheated ribosomes in $TM10^{-4}$ buffer after the addition of 10 μl of H_2O/ml of solution. The subsequent patterns are of ribosomes heated in $TM10^{-4}$ buffer for 5 min at the indicated temperatures followed, after cooling, by the addition of 10 μl of 1 M $MgAc_2$/ml of solution. (b) The data points represent the per cent (relative to the total sedimenting particles of the unheated control to which $MgAc_2$ was added) particles sedimenting ~70–100 S. The broken lines are schematic representations of the activity (Figure 2) and turbidity (Figure 5) under these conditions.

Discussion

In all respects *E. coli* B and Q13 ribosomes exhibited identical behavior with regard to thermal denaturation. The absence of RNase I (the "latent ribosomal RNase") in *E. coli* Q13 appears to rule out this enzyme as the cause of the thermal denaturation described in this report. Similarly, RNase II is probably not involved because chromatography on DEAE-cellulose, which is known to remove this enzyme (Stanley and Wahba, 1967), did not alter the stability of ribosomes. This taken together with the uniform behavior of various preparations (with the one previously noted exception) would suggest that the denaturation phenomena reported here reflect effects on intrinsic ribosomal structure rather than the activation of a degradative enzyme.

It was originally anticipated that, because of the structural and functional complexity of the ribosome, heating might lead to subtle conformational changes and loss of the ability to support over-all protein synthesis in the absence of major structural change. Clearly, however, gross and irreversible (at least under the present conditions) structural changes are closely associated with biological inactivation, although the nature of physical changes is dependent upon the buffer employed. Departures from this generality are as follows. Ribosomes in TM buffer show a marked proclivity for aggregation either forming 100S dimers on prolonged incubation at moderate temperatures (*e.g.*, 2–12 hr at 37°) and some dimers but mostly larger heterogeneous aggregates upon heating for shorter intervals at higher temperatures (*e.g.*, 5 min at 54°). In the first case there is no effect on biological activity or subsequent thermal stability and in the second case aggregation appears to slightly precede the loss in biological activity. Upon heating for short intervals (*e.g.*, 5 min) in TMK buffer gross structural change (destruction of the 30S subunit) closely parallels the loss in activity. Prolonged heating in this buffer, however, produces apparently normal 70S particles which retain only a small fraction of their original activity. It would appear from the present data that the 50S subunit has a $T_{d,5}$ in TMK buffer approximately 4° higher than the 30S subunit. This was the only buffer in which a distinct difference was noted in the thermal stability (structural) of the two subunits and of course there is at present no evidence whether the 50S particle retains biological activity under these conditions. In $TM10^{-4}$ buffer there was a suggestion that irreversible unfolding (irreversible at least in low magnesium) of the 50S subunit may slightly precede the loss of biological activity (in high magnesium). Upon restoring magnesium, however, to ribosomes heated in $TM10^{-4}$ buffer, particles sedimenting at 70–80S are generated in excess of their ability to translate poly U.

It is perhaps somewhat surprising in view of the importance of magnesium ion in the maintenance of the structure and function of the ribosome that a reduction by two orders of magnitude in the free magnesium concentration should have only a moderate effect on the thermal stability of these particles. It should be pointed out, however, that this reduction in free magnesium only reduces the level of bound magnesium by 30–35% (Choi and Carr, 1967; Sheard *et al.*, 1967). While this magnesium is essential for biological activity and for the binding together of the two subunits it apparently plays only a relatively modest role in stabilizing ribosomal structure, at least in relation to thermal denaturation. It is apparent, however, that thermally induced changes (presumably unfolding) occur at low magnesium that are prevented by the presence of high magnesium concentrations.

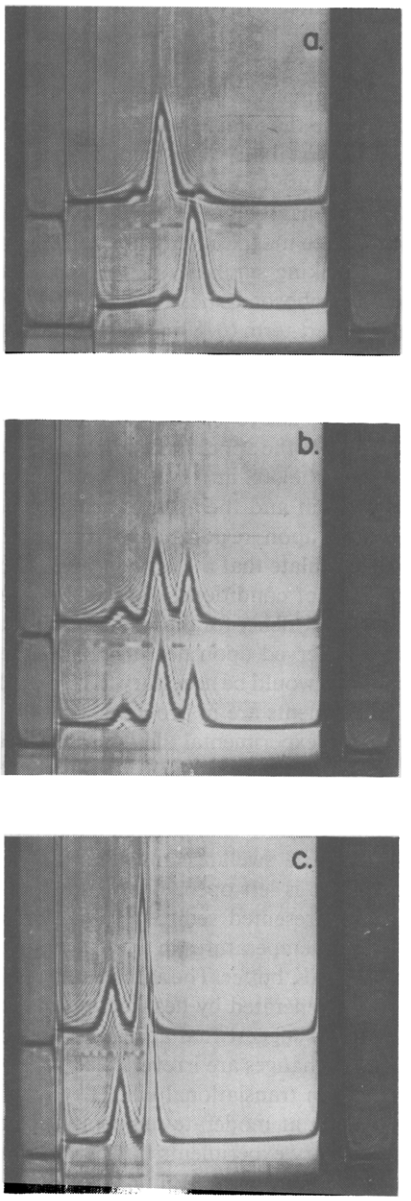

FIGURE 13: Sedimentation velocity behavior of ribosomes following lyophilization. Ribosomes at a concentration of 5 mg/ml were shell-frozen in ethanol–Dry Ice and then lyophylized for 4–5 hr against an ethanol–Dry Ice trap at a pressure of 5 μ. The resulting ribosome powder was redissolved by adding a volume of water equal to that originally present. In each case the lower pattern represents the control which was held at 0° while the lyophilization (upper pattern) was being conducted. The buffers employed were (a) TM, (b) TMK, and (c) $TM10^{-4}$.

Perhaps a better comparison of the relative contri-

bution to thermal stability of the bound magnesium removed on lowering the concentration from 10^{-2} to 10^{-4} M, however, would be to compare the approximately 7° difference in $T_{d,5}$ in TMK buffer as compared with TM-10^{-4} buffer, since the addition of KCl to the latter buffer does not improve stability. This comparison, however, is also tenuous because monovalent cations are known to displace bound magnesium particularly at lower concentrations of free magnesium (Choi and Carr, 1967).

There is a striking similarity between the sedimentation patterns of ribosomes heated in TM10^{-4} buffer and generated by short-term (6–8 hr) dialysis against EDTA (Gesteland, 1966). This similarity also holds true following the readdition of magnesium after heating in TM10^{-4} buffer or dialysis back to TM buffer from EDTA. These similarities include the appearance of particles sedimenting slower than the 30S and 50S subunits, respectively, in low magnesium and the appearance of a new component (~80S) upon restoration of magnesium. It is tempting to speculate that the same process is operative under both sets of conditions, although at the present time we have no evidence for the reversibility of the physical changes observed upon heating. Experiments with isolated subunits would be necessary to rule on this point and such experiments are in progress.

By virtue of experimental design only irreversible structural and functional changes would have been observed in the present study. The extent to which gross but reversible structural change (*e.g.*, dissociation of the subunits or particle swelling) may have preceded the observed changes is left open. Leon and Brock (1967), however, have presented sedimentation profiles of ribosomes at high temperatures in a buffer approximately equivalent to TMK buffer. The agreement between these data and those generated by heating and quick cooling (Figures 7 and 8) suggest that at least in this buffer all gross structural changes are irreversible.

The increase in translational efficiency of ribosomes following heating at moderate temperatures presents a puzzle. Preliminary experiments in this laboratory have shown, in agreement with the findings of McLaughlin *et al.* (1968), that incubation at moderate temperatures (*ca.* 50°) leads to a parallel increase in the ability of ribosomes to bind specific aminoacyl-tRNAs in response to mRNA (Nirenberg and Leder, 1964). Presumably, therefore, the increase in translational efficiency of ribosomes results from an increase in the proportion of active ribosomes in the preparation. It is not evident however, why the ability to translate one polynucleotide should be effected more than another. We were unable to correlate "activation" with any detectable changes in sedimentation properties of the ribosomes. It is possible that the observed differences in the extent of thermal "activation" from one ribosome preparation to the next reflect a difference in the metabolic state of the organism from which the ribosomes were derived (all of these preparations were from "late-log" cells) rather than a minor difference in preparative technique. We are in the process of testing this possibility and further exploring the nature of this activity increase. It is worthy of mention that Gesteland (1966) observed a similar variable activation upon dialysis against EDTA.

The practical temperature extremes to which ribosomes can be subjected without detectable loss of structural or functional integrity depends on the particular buffer employed, and decreases in the order: TMK buffer, TM buffer, TM10^{-4} buffer. Fifty per cent of the biological activity of ribosomes is lost in 5 min of heating in these buffers at 60.5, 57, and 53.5°, respectively ($T_{d,5}$'s). Ribosomes will withstand short-term incubations (up to 5 min) at temperatures up to 5° below these $T_{d,5}$'s and most preparations for more prolonged periods (several hours) at temperatures up to 10° below these $T_{d,5}$'s. This of course is excepting the previously noted increase in activity upon heating of some preparations and the tendency of ribosomes to aggregate in TM buffer. At least in TM buffer, this thermal stability is relatively concentration (0.5–50 mg/ml) and pH independent over the range 7–8 and appears to be independent of the presence or absence of a reducing compound (β-mercaptoethanol). It was further demonstrated that ribosomes can be lyophilized from the above three buffers with little or no effect on their physical or biological properties.

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Hydrogen-Exchange Measurements on *Escherichia coli* Transfer Ribonucleic Acid before, after, and during Its Aminoacylation*

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ASBTRACT: The conformation of transfer ribonucleic acid has been compared with aminoacyl transfer ribonucleic acid by using the Sephadex hydrogen–tritium-exchange technique. The exchange-out kinetics of the acylated and unacylated transfer ribonucleic acid are almost identical, suggesting that secondary structures are closely similar. However, a small but consistent difference was observed. The aminoacyl transfer ribonucleic acid seems to possess three or four more stabilized hydrogens than the unacylated transfer ribonucleic acid, representing approximately 4% the measurable structure. Thus, the aminoacyl transfer ribonucleic acid has at least as much structure involving hydrogen bonds as has unacylated transfer ribonucleic acid and perhaps slightly more.

In addition, a new application of hydrogen exchange is described. It was possible to carry out measurements that should detect changes in transfer ribonucleic acid structure that might occur during the transient interval in which it binds to the amino acid–adenosine monophosphate–synthetase complex, undergoes acylation, and then dissociates.

In the last few years evidence has accumulated suggesting that tRNA may have a regulatory function in addition to its adaptor function. For example, the appearance of a new tRNA upon phage or virus infection (Kano-Sueoka and Sueoka, 1966; Hay *et al.*, 1967), the appearance in bacterial spores of a new tRNA component not found in vegetative cells (Doi and Kaneko, 1966), the formation of aminoacyl-tRNA as a required step for the possible control of RNA synthesis (Böck *et al.*, 1966), and the repression of enzymes involved in amino acid biosynthetic pathways, *e.g.*, those involved in the histidine, valine, and isoleucine pathways (Schlesinger and Magasanik, 1964; Eidlic and Neidhardt, 1965; Freundlich, 1967), all implicate tRNA in a role other than that of simply an adaptor molecule.

The latter type of control, that is, by a tRNA–aminoacyl-tRNA interconversion, could be effected by induction of a change in the three-dimensional structure of the tRNA upon aminoacylation or deacylation. Such a change in conformation could also explain the preferential binding to ribosomes of the aminoacylated form of tRNA (Seeds *et al.*, 1967). That tRNA can assume one or more conformational states (which affect the acceptor activity of the molecule) has been shown by Gartland and Sueoka (1966) and Lindahl *et al.* (1966). Here, we ask the question: "Does the conformation of tRNA differ from the conformation of aminoacyl-tRNA?" The answer to this question is sought using the method of hydrogen exchange (Hvidt and Nielsen, 1966; Englander, 1968) to compare the structure of tRNA before and after the attachment of the amino acid.

Hydrogen atoms covalently bound to nitrogen or oxygen exchange rapidly with those of water. However, when these hydrogens are hydrogen bonded, the

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