A Study of the Thermal Stability of Ribosomes and Biologically Active Subribosomal Particles

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1. The ability of *Escherichia coli* ribosomes to function in poly(U)-directed protein synthesis was measured at elevated temperatures by using thermostable supernatant factors from *Bacillus stearothermophilus*. The amount of polyphenylalanine synthesized at 55°C was about the same as at 37°C, but the rate of synthesis was increased approximately fivefold. At 60°C the activity of the ribosomes was halved. 2. *E. coli* ribosomes can sustain the loss of approx. 10% of the double-helical secondary structure of RNA without losing activity. 3. Within the active ribosome the double-helical secondary structure of the rRNA moiety is stabilized compared with isolated rRNA, as judged by enzymic hydrolysis and by measurements of E_{260}. 4. The main products, over the range 0–55°C, of ribonuclease T_1 digestion of the smaller subribosomal particle of *E. coli* were two fragments ($s_{20,w}^0$ 15S and 25.3S) of approximately one-quarter and three-quarters of the size of the intact molecule, revealing the presence of a 'weak spot' where intramolecular bonds appear insufficient to hold the fragments together. 5. Subribosomal particles of *B. stearothermophilus* were more stable to heating, by approx. 10°C, than those of *E. coli*, and the stabilization of double-helical secondary structure of the RNA moiety was more striking. 6. Rabbit reticulocyte ribosomes were active in poly(U)-directed protein synthesis at 45°C, and half the activity was lost after heating to 53°C. Active subribosomal particles of rabbit reticulocytes and of oocytes of *Xenopus laevis*, like the bacterial subribosomal particles, underwent a conformational change to a slower-sedimenting form on heating. The temperature range of the transition depended on the species. 7. Slower-sedimenting particles, whether produced by EDTA treatment or by heating, had different 'melting' profiles compared with active subribosomal particles, providing another indication of conformational differences. 8. Comparison of the properties of the various subribosomal particles revealed greater variation in the secondary structure of the protein moieties (judged by measurement of circular dichroism) than in the secondary structure of the RNA moieties, which appeared to have features in common.

Ribosomes are complex multisubunit particles that play a central role in protein biosynthesis. Information about ribosome structure and function has been sought from thermal-denaturation studies. Tal (1969) showed that heating ribosomes from *Escherichia coli* led to a conformational change from the compact 'active' particle to a form having a larger hydrodynamic volume. The irreversible effects of heating on biological activity, sedimentation properties and turbidity of *E. coli* ribosomes were studied by Bodley (1969). The biological activity of ribosomes from psychrophilic and mesophilic bacteria were compared (Krajewska & Szer, 1967) and the activity of ribosomes from thermophilic bacteria was examined (Friedman & Weinstein, 1966).

Early thermal-denaturation studies were based largely on measurements of E_{260} as a function of increasing temperature with the aim of examining the thermal stability of the RNA moiety. Technical problems from turbidity that appeared on heating, and hydrolysis of the RNA moiety owing to RNAase* contaminants, made the results difficult to interpret (for review, see Tal, 1969), i.e. the changes in extinction could not be related to well-defined conformational changes.

The importance of identifying the conformational changes being monitored arises because subribosomal particles may exist in conformations ranging from forms that are active in protein biosynthesis to inactive forms that sediment at much slower rates, and it is not certain that the interactions between the rRNA and protein moieties are the same in all forms.

To avoid this ambiguity and to ensure that our results apply to functional particles we chose to

*Abbreviations: RNAase, ribonuclease; c.d., circular dichroism.

study the thermal stability of preparations of ribosomes and subribosomal particles that are active in cell-free protein synthesis.

We sought to correlate the conformation of ribosomes with their ability to function at temperatures above the normal for growth, to monitor any changes in the normal conformation (e.g. 'melting' of RNA double-helical secondary structure) that can be tolerated in a functional ribosome. The thermal stabilities of the secondary structure of the RNA and protein moieties within the ribosome and after isolation were examined by means of nuclease digestion, c.d. and u.v.-absorbance measurements, because they might yield information about RNA–protein interactions. Cotter *et al.* (1967) concluded that the proteins are not associated with the double-helical parts of RNA, on the basis of their observation that the 'melting' profiles of yeast subribosomal particles (conformation unspecified), in 0.1 M-NaCl, and of rRNA were congruent. The congruency of the 'melting' rRNA and ribosomes is not always seen (e.g. Tal, 1969) and merits further study.

To provide a broad frame of reference, active subribosomal particles from species that live at different temperatures were studied. In addition to subribosomal particles of *E. coli* M.R.E. 600, ribosomes from *Bacillus stearothermophilus*, from oocytes of the South African clawed toad *Xenopus laevis* and from reticulocytes of the rabbit were examined. The results allow us to relate the thermal stability of the quaternary structure of the ribosome with the temperature at which the cells normally develop. The secondary structure of the various rRNA species as judged by 'melting' profiles was studied *inter alia*.

Methods

General methods

U.v. spectrophotometry. A Unicam SP. 700 spectrophotometer was used for u.v. measurements. The sample cell-holder was a copper block that could be heated electrically to 95°C and maintained at a temperature within this region to better than 0.1°C. Values of extinction were corrected for changes in solute concentration due to thermal expansion of water.

Circular dichroism. The c.d. of RNA and subribosomal particles was measured in 1 mm pathlengths with a Rousset Jouan Dichrograph model CD 185 fitted with a thermostat, consisting of a hollow copper jacket through which water was circulated. The results are expressed as c.d. = $(E_L - E_R)$, when E_L and E_R are respectively the values of E per g-atom of P for left and right circularly polarized light, and as residue ellipticity θ_M where $\theta_M = 3300 \times$ c.d.

Ultracentrifugation. A Spinco model E ultra-

centrifuge fitted with u.v. optics was used for analytical studies. The camera was replaced by a photoelectric cell that scanned the slit, and the movement of the sedimenting species was followed by means of an $x-y$ recorder. Cells of 12 mm path-length were used in all cases. Measurements were made at temperatures in the range 15–20°C. RNA and subribosomal particle solutions were dilute (25–50 mg/l).

Isolation of subribosomal particles

Reticulocyte subparticles. The polyribosome fraction of reticulocytes was isolated by the method of Arnstein *et al.* (1964), suspended (10 mg/ml) in 0.25 M-sucrose – 20 mM - 2 - mercaptoethanol – 80 mM - KCl – 10 mM-MgCl$_2$ – 50 mM-Tris–HCl, pH 7.6. The suspension was made 1 M in KCl by the addition of 2.5 M-KCl–10 mM-MgCl$_2$, kept at 37°C for 4 min and then cooled in ice. Separation into subribosomal particles was performed by zonal centrifugation in a titanium B XIV rotor (MSE Superspeed 65 ultracentrifuge). A 15–40% sucrose gradient in 0.5 M-KCl–10 mM-MgCl$_2$ – 50 mM-Tris – HCl (pH 7.6) – 10 mM-mercaptoethanol was used. The sample and overlay displaced 250 ml of 40% sucrose from the rotor. Centrifuging was for 3 h at 43 000 rev./min (thermostat 7: approx. 10°C). Fractions of 15 ml were collected. The peak fractions were combined and subribosomal particles were precipitated by the addition of an equal volume of 95% (v/v) ethanol. The precipitate was collected by centrifuging and resuspended in 0.1 M-NH$_4$Cl–2 mM-MgCl$_2$–1 mM-dithiothreitol–0.02 M-Tris–HCl, pH 7.6.

E. coli subparticles. Frozen cells (*E. coli* M.R.E. 600) were obtained from the Microbiological Research Establishment, Porton, Wilts., U.K. Cells were disrupted by grinding with alumina, the ribosome fraction was obtained as described by Godson & Cox (1970) and subparticles were separated by zonal centrifugation. A portion (10 ml) of the ribosome solution (approx. 20 mg/ml) was loaded on a gradient of 15–40% sucrose containing 0.06 M-KCl–0.1 mM-MgCl$_2$–0.01 M-Tris–HCl, pH 7.6. The sample and overlay displaced 250 ml of sucrose from the rotor. After centrifuging at 43 000 rev./min for 3.5 h at approx. 5°C (a titanium MSE B XIV rotor and MSE Superspeed 65 ultracentrifuge were used) fractions (4×50 ml and then 35×15 ml) were collected. To the fractions containing subribosomal particles ethanol (2 vol.) was added and the precipitate obtained after 30 min at −12°C was separated by centrifuging and was resuspended in 0.1 mM-magnesium acetate–0.01 M-Tris–HCl, pH 7.6. The smaller subparticles were further purified by centrifuging for 3.5 h at 43 000 rev./min (MSE B XIV rotor) through a second gradient (15–40% sucrose–0.01 M-MgCl$_2$–0.05 M-KCl–0.025 M-Tris–HCl, pH 7.6). Fractions

were collected and the subparticles were recovered as described above.

B. stearothermophilus subparticles. Frozen cells were obtained from the Microbiological Research Establishment. The same methods were used to disrupt the cells, isolate the ribosome fraction and to separate the subparticles as for *E. coli.*

X. laevis oocyte subparticles. The isolation of subribosomal particles from oocytes of *X. laevis* was described in detail by Pratt & Cox (1971). Subparticles were obtained by heparin treatment or by centrifuging through 0.15 M-KCl-0.1 mM-MgCl$_2$-10 mM-mercaptoethanol-50 mM-Tris-HCl, pH 7.6.

Isolation of rRNA and proteins

rRNA. rRNA was isolated from subribosomal particles by precipitation as the guanidinium salt as described by Cox (1968a).

Proteins. A suspension of subribosomal particles was made 2 M in LiCl and 4 M in urea, and then kept overnight at 4°C (see, e.g., Nomura *et al.*, 1968). The precipitate of rRNA was separated by centrifuging. The supernatant was shown to be free of rRNA by the negligible value of c.d. at 265 nm.

Incubation of whole reticulocytes

The method of Lingrel & Borsook (1963) was followed. Reticulocytes from anaemic rabbits were separated by low-speed centrifuging and washed twice with Borsook's saline solution (0.13 M-NaCl-5 mM-KCl-7.4 mM-MgCl$_2$,6H$_2$O). The plasma was dialysed for 2 h at 0.4°C against 10 vol. of Borsook's solution. An amino acid mixture was prepared in Borsook's solution to reflect the amino acid composition of rabbit haemoglobin. The mixture contained 2 mM-L-alanine, 0.5 mM-L-arginine, 2.85 mM-L-aspartic acid, 5.3 mM-glycine, 2.4 mM-L-histidine, 0.3 mM-L-isoleucine, 0.3 mM-L-methionine, 2 mM-leucine, 1.8 mM-L-lysine, 1.4 mM-L-proline, 1.65 mM-L-serine, 1.7 mM-L-threonine, 0.3 mM-L-tryptophan, 0.8 mM-L-tyrosine, 3.2 mM-L-valine, 1.1 mM-L-hydroxyproline, 0.4 mM-L-cysteine and 8.0 mM-L-glutamine. A reagent mixture was prepared that contained 5.4 ml of this amino acid mixture, 6.4 ml of dialysed plasma, 2.7 ml of 0.164 M-Tris-HCl, pH 7.8, and 0.27 ml of Borsook's solution made 0.25 M in MgCl$_2$ and 10% in sucrose. The incubation mixture was prepared by adding, in the following order, 1.0 ml of washed cells, 2.64 ml of reagent mix, 0.15 ml of FeSO$_4$(NH$_4$)$_2$SO$_4$,6H$_2$O (10.5 mg/10 ml of Borsook's solution), [^{14}C]phenylalanine (0.01 ml) and 1.0 ml of unlabelled phenylalanine (2 μmol/ml of Borsook's solution). An unincubated control was kept in ice throughout the experiment. Incubated samples were kept at the required temperature for 1 h and then cooled in ice. Samples (0.1 ml) were removed and diluted with an equal volume of Borsook's solution

and then lysed with 1 M-NaOH (0.2 ml) containing phenylalanine (25 μmol/ml). After 1 h at room temperature the protein was precipitated by the addition of 10% (w/v) trichloroacetic acid (2.5 ml), filtered with Oxoid membranes and washed with hot and cold trichloroacetic acid etc. as described (Fig. 10) for samples incubated in a cell-free system.

Results

Bacterial ribosomes

Activity of subribosomal particles in vitro at 37–65°C. The ability of *E. coli* subribosomal particles to function in the poly(U)-directed synthesis of polyphenylalanine was studied at 37–65°C by using
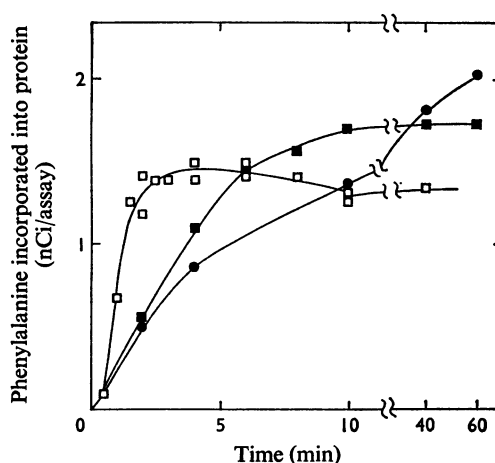

Fig. 1. *Time-course of polyphenylalanine synthesis at different temperatures*

Each assay contained, in 0.5 ml, 35 μmol of KCl, 8 μmol of MgCl$_2$, 12.5 μmol of Tris–HCl, pH 7.6, 5 μmol of GSH, 1 μmol of ATP, 80 μg of smaller subribosomal particles, 160 μg of larger subparticles, 20 μg of poly(U), supernatant factors (1 mg of protein), unlabelled amino acids (the mixture contained 2 μmol of each of the 20 amino acids except phenylalanine/ml), 50 μg of pyruvate kinase, 5 μmol of phosphoenolpyruvate, ATP and GTP (0.05 and 0.0125 μmol/μmol of phosphoenolpyruvate respectively) and [^{14}C]phenylalanine (specific radioactivity 50 nCi/nmol). There was negligible protein synthesis in the absence of energy or of ribosomes. The pool of non-radioactive phenylalanine amounted to approx. 2 nmol/assay; the effective specific radioactivity of the radiotracer was approx. 10 nCi/nmol. ●, *E. coli* subparticles with *E. coli* supernatant factors at 37°C; ■, *E. coli* subparticles with *B. stearothermophilus* supernatant factors at 37°C; □, *E. coli* subparticles with *B. stearothermophilus* factors at 55°C.
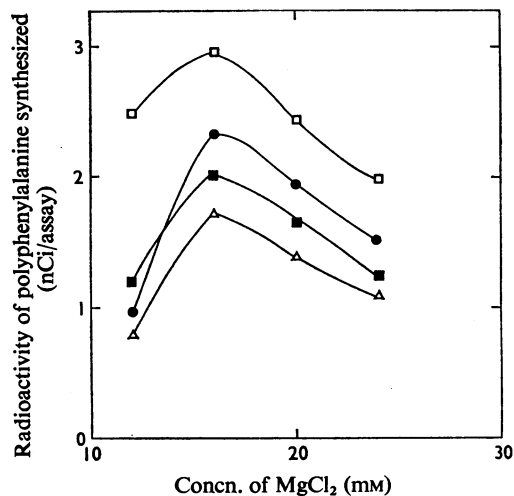
Fig. 2. *Dependence of cell-free protein synthesis on MgCl₂ concentration*

Incubation was for 1 h and, except for the concentration of MgCl₂, the composition of each assay mixture is given in the legend to Fig. 1. □, *E. coli* ribosomes with *E. coli* supernatant factors at 37°C. *E. coli* ribosomes with *B. stearothermophilus* supernatant factors at: ■, 37°C; ●, 45°C; △, 55°C.

Fig. 3. *Activity of E. coli subribosomal particles in cell-free protein synthesis at different temperatures*

The activity of *E. coli* ribosomes in poly(U)-directed synthesis was measured at the temperature indicated. ○, *E. coli* subribosomal particles with *B. stearothermophilus* supernatant factors (see Figs. 1 and 2); □, data of Krajewska & Szer (1967) for *E. coli* B ribosomes and either *E. coli* or *Pseudomonas* sp. 412 supernatant factors. Heating ----, irreversible inactivation of *E. coli*. ribosomes measured by heating in 0.1 mM-MgCl₂-0.01 M-Tris–HCl, pH 7.6, to the temperature indicated for 5 min and then assaying at 37°C for activity in poly(U)-directed synthesis (Bodley, 1969).

thermostable *B. stearothermophilus* supernatant factors. The efficiency of *B. stearothermophilus* factors was compared with that of *E. coli* supernatant factors at 37°C. With homologous supernatant factors the synthesis of acid-insoluble protein was rapid during the first 10 min and continued for as long as 60 min at a lower rate. In contrast protein synthesis was complete after 10 min when *B. stearothermophilus* supernatant factors were used (Fig. 1). The optimum concentration of MgCl₂ was the same for both sets of factors (Fig. 2) and (at all four concentrations of MgCl₂) more protein was synthesized after 60 min, when homologous factors were used.

The amounts of protein synthesized in the cell-free system incubated at 45–65°C are given in Fig. 3. As at 37°C, 16 mM-MgCl₂ was optimum (Fig. 3). More polyphenylalanine was synthesized at 45°C than at 37°C, and the incorporation at 50°C ranged from 85 to 105% of that at 37°C in four independent experiments. At 55°C protein synthesis was still 80–100% of that found at 37°C, although synthesis was completed within 2 min (Fig. 1).

These results complement the studies of Krajewska & Szer (1967), Bodley (1969) (see Fig. 3) and Kikuchi & Monier (1971). Lodish & Robertson (1969) showed that *E. coli* cell-free systems translate bacteriophage f₂ RNA as readily at 49°C as at 37°C. The plot of
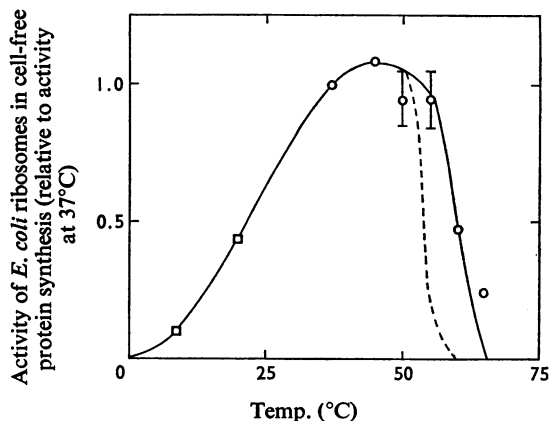
activity in a cell-free system measured at the elevated temperature relative to activity at 37°C (Fig. 3) was very similar to that obtained by plotting the relative activity at 37°C (after heat treatment of ribosomes alone) against the temperature of the heat treatment (Bodley 1969). This correlation suggests that, in our experiments, the ribosome fraction alone limits protein synthesis at elevated temperatures; this was expected, since we chose to use enzymes and supernatant factors that were thermostable.

The major result is that *E. coli* ribosomes function at temperatures well above the optimum for growth, and within the range in which temperate thermophiles fluorish. At 55°C, in the comparable conditions *E. coli* rRNA has lost one-third or more of its double-helical secondary structure. This poses the question of conformation of the RNA moiety in functional ribosomes at elevated temperatures. The possibilities include (i) that the RNA moiety of a fully functional ribosome has, like isolated RNA, lost one-third or more of its double-helical secondary structure at elevated temperatures, and (ii) that the ribosome possesses a precise quaternary structure that is retained on heating so that the conformation of the rRNA moiety is scarcely altered.

These possibilities were studied by using RNAase T_1 to probe the secondary structure of the smaller subribosomal particle, and also by the use of 'melting' profiles. The sedimentation rate at approx. 20°C was also measured after heat treatment.

Choice of solvent for conformational studies. Ionic conditions that are suitable for bacterial cell-free protein synthesis at 37°C are not suitable for thermal-denaturation studies because of aggregation of the ribosome fraction at temperatures above about 60°C. We chose to study active subribosomal particles in a solvent (0.1mM-MgCl₂-0.01M-Tris-HCl, pH7.6) with ionic strength sufficiently low to avoid precipitation at elevated temperatures but sufficient $MgCl_2$ to maintain the native conformation at 25°C and also to swamp the bivalent cations from extraneous sources.
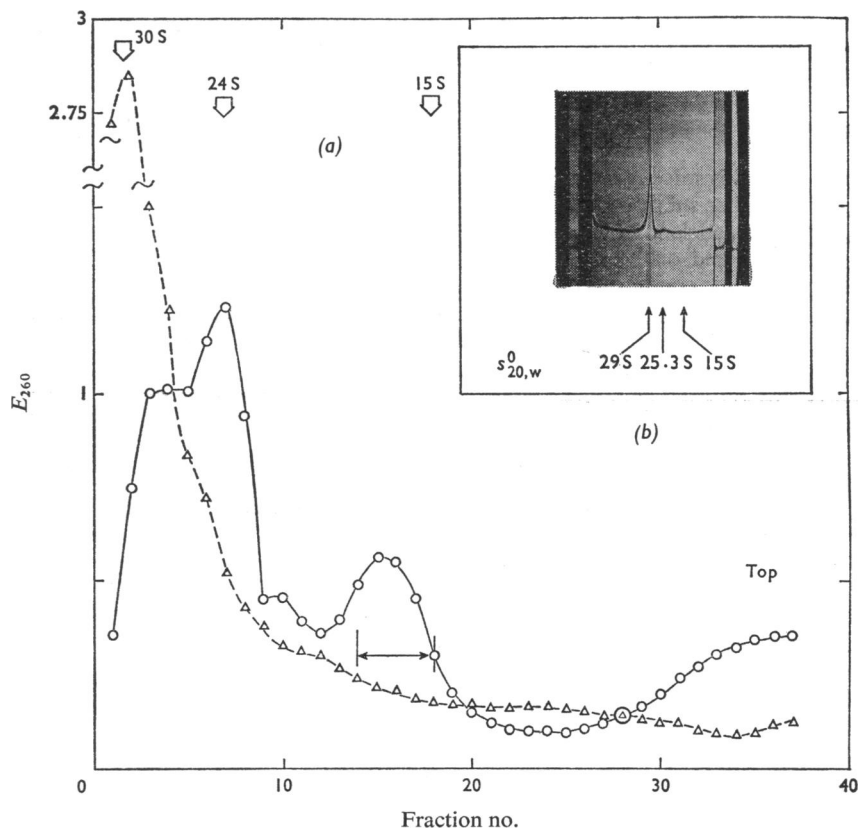

Fig. 4. *Sedimentation profile of the smaller subribosomal particles of E. coli after hydrolysis with RNAase T_1 at 35°C*

(a) The smaller subribosomal particles of *E. coli* (2.5mg/ml in 0.1mM-MgCl₂-0.01M-Tris-HCl, pH7.6) were treated with RNAase T_1 (200 units) at 35°C for 15min, cooled and kept overnight at 4°C and then layered on a 5-20% sucrose gradient containing 0.1mM-MgCl₂-0.01M-Tris-HCl, pH7.6, and centrifuged at 27000rev./min (Beckman SW-27 rotor) for 16h at 4°C. Fractions (approx. 1ml) were collected by means of a siphon and E_{260} was measured in cells of 5mm path-length. The vertical arrows indicate the values of $s_{20,w}$ calculated on the basis of the assumption that the distance travelled is directly proportional to s, and that the concentration does not influence the relative values of $s_{20,w}$. Fractions 14–18 (↔) were pooled, concentrated by pressure dialysis and the protein components fractionated by polyacrylamide-gel electrophoresis. △, Control untreated sample; ○, hydrolysed sample. (b) Analytical centrifuge pattern of the subribosomal particles (6.8mg of *E. coli* smaller subparticles/ml of 0.1mM-MgCl₂-0.01M-Tris-HCl, pH7.6) hydrolysed with RNAase T_1 at 0°C. Centrifugation was at 44770rev./min (Beckman An DB rotor) at 8.9°C. The picture was taken 40min after full speed had been reached. The values of $s_{20,w}$ were calculated from the dependence of $s_{20,w}$ on concentration of the smaller subparticles given by Tissières *et al.* (1959).

Care was taken to check by ultracentrifuging that the RNA moiety was not hydrolysed during the heating experiments.

This solvent was used for the routine preparation of active subparticles and it is suitable for spectrophotometric measurements. A study of the effect of hydrolysis of the smaller subparticle by RNAase T_1 in the solvent at approx. 0°C has been reported (Brimacombe et al., 1971a). The irreversible inactivation of ribosomes in this solvent is known (Bodley, 1969) and the thermal inactivation of ribosomes in this solvent takes place at about 7°C less than is given in Fig. 3. The 'melting' profile of rRNA in this solvent is within 5°C of that found in buffers suitable for protein synthesis in vitro. The same relation probably applies to subribosomal particles (see Fig. 8).

The limitation in using this solvent is that the biological activity of the smaller subribosomal particle is diminished in solutions deficient in K^+, although full activity may be recovered on heating to 37°C in buffers resembling the ionic conditions of the cell-free system (Zamir et al., 1969; Kikuchi & Monier, 1970; Kagawa et al., 1971; Naslund & Hultin, 1971). When the smaller subribosomal particle of E. coli isolated by our standard procedure was preincubated in 0.1 M-KCl–10 mM-$MgCl_2$ at 37°C for 10 min an increase in polyphenylalanine synthesis of 40% was found. Thus conformational changes might occur in 0.1 mM-$MgCl_2$–0.01 M-Tris–HCl, pH7.6, that are reversed in the conditions of the cell-free system.

Hydrolysis of the smaller subribosomal particles of E. coli by RNAase T_1 at elevated temperatures (0–65°C). The conformation of the ribosome strongly influences the course of hydrolysis by nucleases (see, e.g., Cox, 1969). Conversely a study of the products of enzymic hydrolysis offers a means of probing conformation. Earlier studies showed that the smaller subribosomal particle of E. coli in 0.1 mM-$MgCl_2$–0.01 M-Tris–HCl, pH7.6, yields two nucleoprotein fragments on hydrolysis with RNAase T_1 (EC 2.7.7.26) at 0°C (Brimacombe et al., 1971a,b).

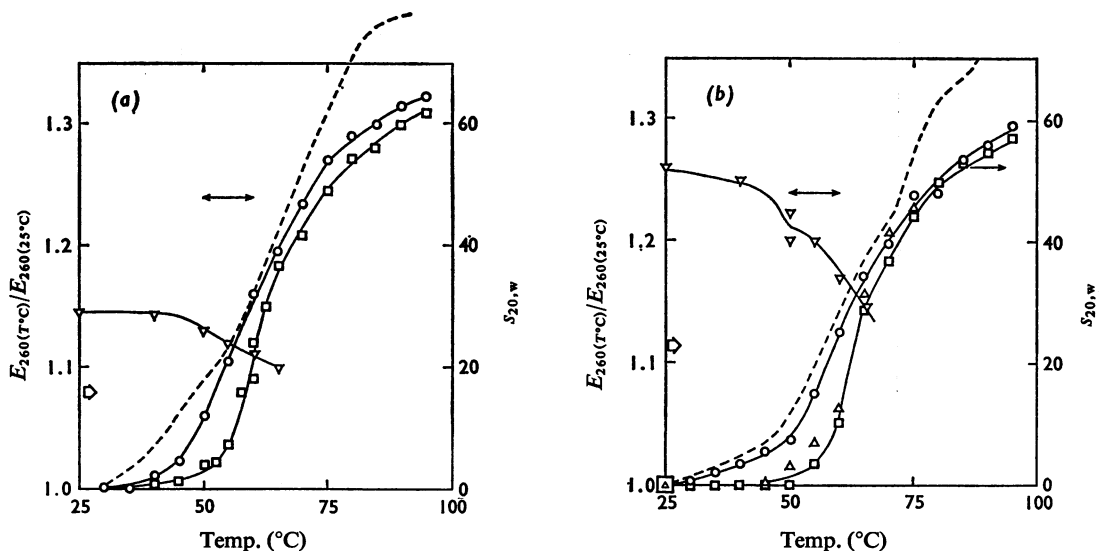This particular reaction was studied at temperatures in the range 0–65°C and the products were

Fig. 5. *'Melting' profile and $s_{20,w}$ values of E. coli subribosomal particles and rRNA in 0.1 mM-$MgCl_2$–0.01 M-Tris–HCl, pH7.6*

(a) Smaller subribosomal particles; (b) larger subribosomal particles. ∇, $s_{20,w}$ measured at 10–20°C after heating to the temperature shown (for details of the experimental procedure see the legend to Fig. 7). Two boundaries were found on sedimenting the larger subparticles after heating to 50°C; otherwise a single boundary was observed. $\square$, Relative E_{260} of subparticles; $\bigcirc$, relative E_{260} of rRNA; $\triangle$, relative E_{260} of subparticles when lysozyme was present in the ratio ribosome/lysozyme of 4.7:1, by wt. (cf. Daya & Gratzer, 1969); –––––, 'melting' profile of rRNA of rabbit reticulocytes (Fig. 15), in (a) $(E_{260(T°C)}/E_{260(25°C)}) \times 1.4$ and in (b) $(E_{260(T°C)}/E_{260(25°C)}) \times 1.55$ (i.e. $\times$ mol.wt. of reticulocyte rRNA/mol.wt. of E. coli rRNA) so that the two profiles can be compared on a molar basis; $\leftrightarrow$, temperature range over which subparticles in 0.1 mM-$MgCl_2$–0.01 M-Tris–HCl, pH7.6, are heated and a diminution of biological activity at 37°C is observed. $\Rightarrow$, $s_{20,w}$ of subparticles in 0.2 mM-EDTA–0.01 M-Tris–HCl, pH7.6.
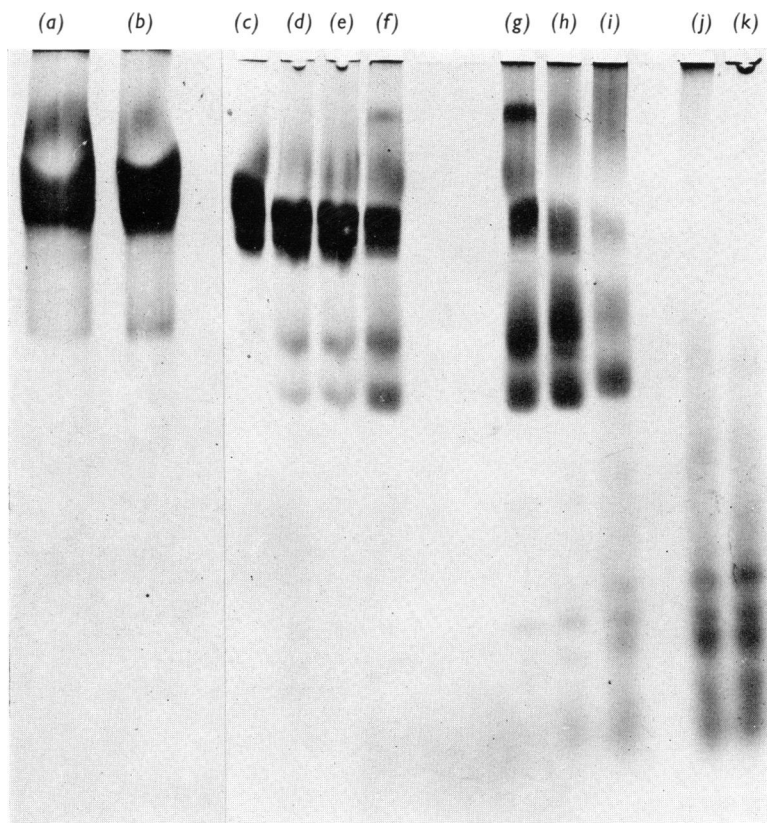
EXPLANATION OF PLATE I

Electrophoretograms of the smaller subparticles of E. coli ribosomes after hydrolysis with RNAase T_1 at 0–65°C

To subparticles (5 mg/ml in 0.1 mM-$MgCl_2$–0.01 M-Tris–HCl, pH 7.6) RNAase T_1 (1000 units/10 mg of subparticles) was added. Portions (0.4 ml) were heated to temperatures in the range 0–65°C, cooled in ice and samples (40 µl) were loaded on 3.5% polyacrylamide–0.5% agarose gels (Brimacombe *et al.*, 1971*a*). Both the gel and reservoir buffers were 1 mM-magnesium acetate–10 mM-potassium phosphate buffer, pH 7.0. Electrophoresis was for 17 h at 100 V and 15 mA. Gels were stained with 0.5% Methylene Blue in 0.2 M-acetic acid–0.2 M-sodium acetate and destained in running tap water. Gels: (*a*) control heated to 50°C for 3 min; (*b*) control heated to 40°C for 15 min; (*c*) control at 0°C; (*d*) subparticles+RNAase T_1 at 0°C; (*e*) subparticles+RNAase exposed to 25°C for 15 min; (*f*) enzyme+subparticles kept at 35°C for 15 min; (*g*) subparticles+enzyme kept at 40°C for 15 min; (*h*) subparticles+enzyme kept at 50°C for 3 min; (*i*) subparticles+enzyme kept at 55°C for 3 min; (*j*) subparticles+enzyme kept at 60°C for 3 min; (*k*) subparticles+enzyme kept at 65°C for 3 min.

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separated by electrophoresis as described earlier (Brimacombe et al., 1971a,b). In some cases a sample was also fractionated by zonal centrifugation. Heating up to 50°C diminished the amount of material in the region of the gel where the intact particle is found and increased the yield of the two fragments (Plate 1). Faster-moving bands that were very faint after hydrolysis at 50°C and below were more pronounced in the sample heated to 55°C and were the only easily visible bands in the electrophoretograms of the samples heated to 60° and 65°C. We infer that there is a major conformational change between 55° and 60°C that alters the course of enzymic hydrolysis.

Evidence that the hydrolysis at 35°C yields the same smaller fragment as hydrolysis at 0°C was obtained as follows. The products of hydrolysis at 35°C were separated by zonal centrifugation (Fig. 4). The fractions of the peak in the 15S region were pooled and concentrated and the protein composition was determined by electrophoresis as described previously (Brimacombe et al., 1971a,b). The same four protein bands were found as reported for the smaller fragment obtained by hydrolysis at 0°C.

We further conclude from their sedimentation properties that the fragments retain a compact conformation after their release from the subparticle, because the molecular mass of the fragments was the same whether determined chemically or by sedimentation analysis. The smaller fragment is about one-quarter of the subparticle (approx. 200000 daltons mass), since the RNA moiety is about 330–380 nucleotides long and there are four protein subunits. The larger fragment amounts to about three-quarters of the subribosomal particle. The main products of digestion at 0°C were characterized (Fig. 4) by values of $s_{20,w}$ of 13.2S and 20.3S, compared with 25.3S for the intact subparticle (concn. 6.8mg of subparticles/ml). The values of $s_{20,w}$ were calculated to be 15.0S, 23.2S and 29S by using the relation between $s_{20,w}$ and concentration given by Tissières et al. (1959). The molecular mass of the smaller fragment was estimated as approx. 260000 daltons (cf. approx. 200000 daltons calculated from the chemical composition) on the basis of the assumption that the conformation of the fragments is homologous with that of the intact subparticles and the intact ribosome. The agreement between the two estimates of molecular mass indicates that this assumption is valid.

Irreversible changes in the sedimentation properties of subribosomal particles brought about by heating. The value of $s_{20,w}$ of the smaller subribosomal particle of E. coli decreased from approx. 28.5S at 10–20°C to 26S after exposure to 50°C and to 24S after exposure to 55°C (Fig. 5a), whereas the susceptibility of the subparticle to RNAase T_1, judged by the electrophoretograms of the fragments (Plate 1), was altered between 55° and 60°C. Fig. 5(a) shows that there is a further irreversible 'unfolding' of the subparticle over

the range 55–60°C and also 'melting' of approx. 33% of double-helical secondary structure. It appears that the subparticle can 'unfold' to a limited extent apparently without altering the pattern of nucleoprotein fragments obtained by RNAase T_1 hydrolysis.

Two boundaries of 40S and 44S (approx. 70% of the material) were found on sedimenting the larger subribosomal particle of E. coli after heating to 50°C. The products were not characterized further. A single slower-sedimenting species was found after exposure to still higher temperatures.

The changes in $s_{20,w}$ of the subribosomal particles found on heating to 50°C are probably reversed on heating in the cell-free system (see, e.g., Zamir et al., 1969; Kikuchi & Monier, 1970).

'Melting' profiles. The difference in the spectrum over the range 220–330nm of the heated rRNA or subparticle and a sample kept at 25°C was measured.
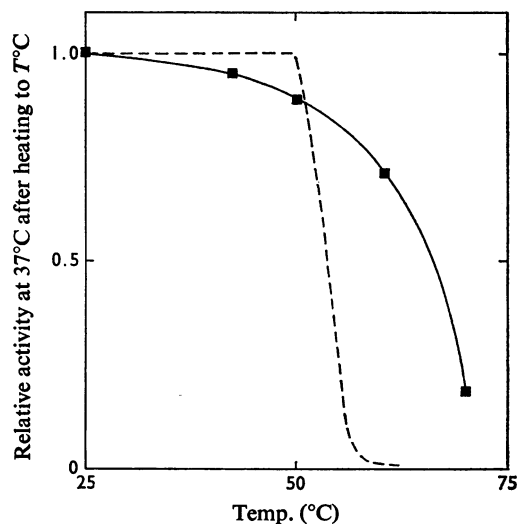

Fig. 6. *Irreversible thermal denaturation of the smaller subribosomal particles of B. stearothermophilus*

The smaller subribosomal particles of B. stearothermophilus (1mg/ml of 0.1mM-$MgCl_2$–0.01M-Tris–HCl, pH7.6) were heated to T°C for 3min, chilled in ice, and then assayed in the cell-free system at 37°C (■—■). The conditions were the same as described in the legend to Fig. 1 except that the smaller subparticles were from B. stearothermophilus and not E. coli. The amount of protein synthesized in the control unheated sample was slightly less than that found for the E. coli smaller subribosomal particles. ----, Irreversible denaturation of E. coli smaller subparticles in the same solvent (see Fig. 3).
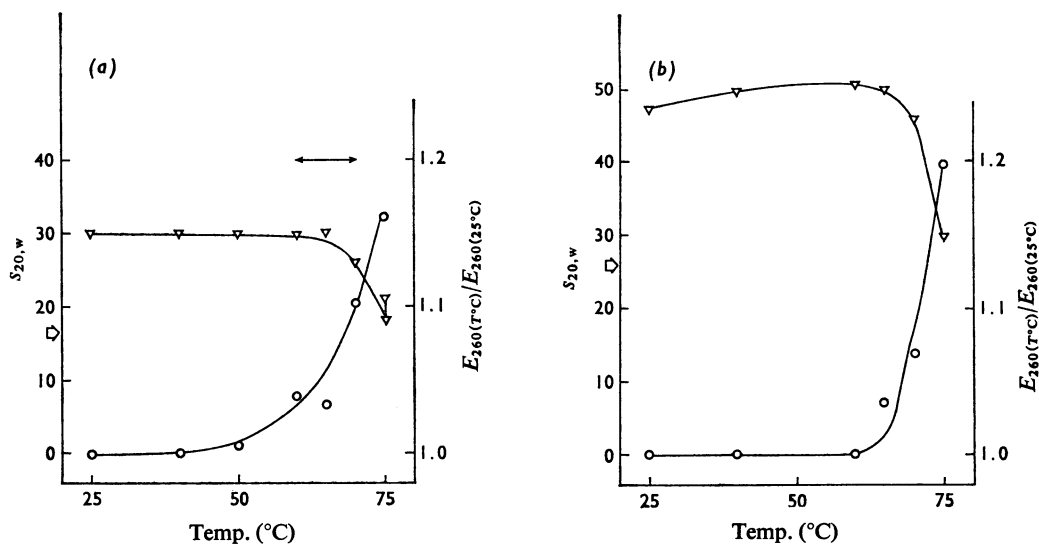
Fig. 7. *Effect of heating on* $s_{20,w}$ *of B. stearothermophilus subparticles*

Samples (3 ml) of a solution of subparticles (50 μg/ml of 0.1 mM-MgCl$_2$-0.01 M-Tris–HCl, pH 7.6) were transferred to a sample cell of a spectrophotometer kept at the desired temperature and E_{260} was monitored continuously for 3 min. The solution was then cooled in ice and the sedimentation profile was measured at 10–20°C. ○, ΔE_{260} {i.e. $(E_{T°C} - E_{25°C})/(E_{25°C})$} at the temperature indicated. In these experiments and in Figs. 5, 14 and 15 (except where shown) the sedimenting boundaries were characteristic of a single species, i.e. 80% of the mass sedimented as a single boundary with a range of no more than 2S from the mean. ▽, $s_{20,w}$ measured at 10–20°C after heating to the temperature indicated. ⇒, $s_{20,w}$ of subparticles in 0.2 mM-EDTA–0.01 M-Tris–HCl, pH 7.6, at 10–20°C. (*a*) Smaller subribosomal particles; (*b*) larger subribosomal particles.

The observed differences (cf. Cox, 1970, 1971) could be reconstructed by mixing the reference difference spectra for 'melting' rA·rU and rG·rC base pairs in the appropriate proportions of base pairs (Cox, 1971). Except where indicated corrections for scattered light were not necessary.

The 'melting' profiles of the subparticles (Fig. 5) show that, up to 55°C, when full biological activity is retained there is little change in E_{260}, confirming that loss of double-helical secondary structure was not extensive and was much less than that found for rRNA alone. It is possible that in the ionic conditions of the cell-free system the increments in E might be slightly less than those in Fig. 5 (cf. Fig. 8), which represent maximum values. At 55°C, E_{260} of the smaller subparticle had increased from 1.0 at 25°C to 1.035, corresponding to a loss of approx. 10% at most of double-helical secondary structure. The E_{260} of the larger subparticle increased from 1.0 at 25°C to 1.02 at 55°C, corresponding to a loss of 6%, at most, of double-helical secondary structure. At 60°C approx. 50% of the activity in the cell-free system was found, and the increments in E_{260} were

10% of the maximum for the smaller subparticle, corresponding to a loss of one-third of double-helical secondary structure, and 5% at most for the larger subparticle, corresponding to a loss of approx. 17% of double-helical secondary structure. Thus although the double-helical secondary structure of the rRNA moiety is more stable to thermal denaturation than is rRNA alone, some loss (17–33%) of double-helical secondary structure can be tolerated with 50% of activity in poly(U)-directed protein synthesis still retained.

The 'melting' profile also reflects the transition from a compact 'active' conformation to an unfolded form, since the profile of the subparticles is sharper than that of rRNA over the range 55–60°C. The relative sharpness of the transition is characteristic of a co-operative transition, i.e. one that arises from the simultaneous rupture of a number of non-covalent bonds.

Thermal denaturation of B. stearothermophilus subribosomal particles. B. stearothermophilus is a thermophile that can grow at 50–70°C, a temperature range over which rRNA 'melts'. Thus any stabilization of
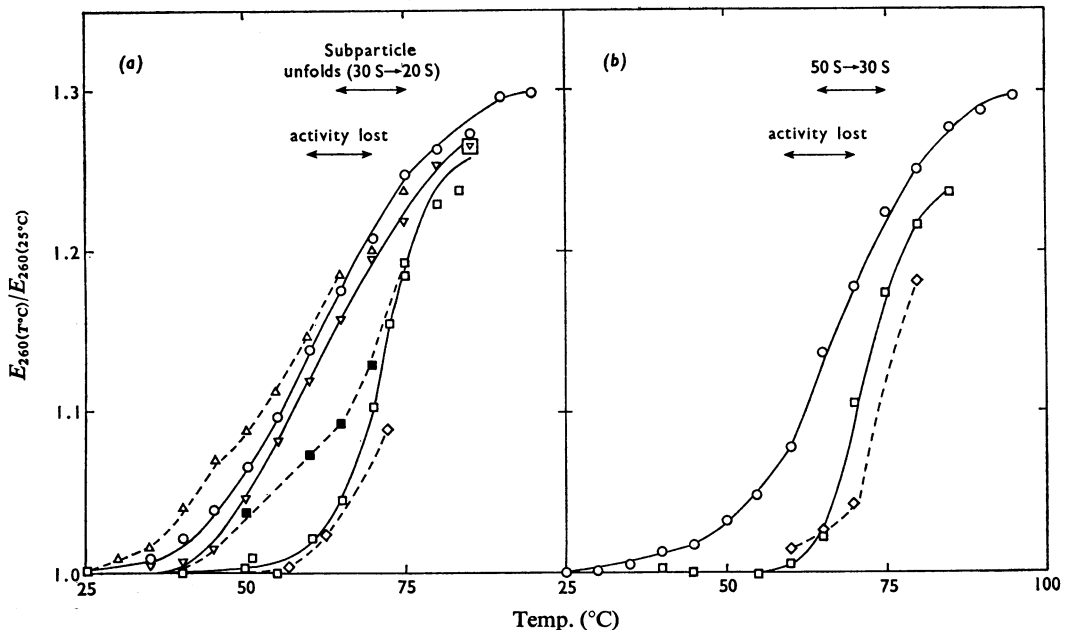
Fig. 8. *'Melting' profiles of B. stearothermophilus subribosomal particles and rRNA*

(a) Smaller subribosomal particles in □, 0.1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH7.6, or ◇ Nirenberg's standard buffer (10 mM-$MgCl_2$-50 mM-KCl-0.025 M-Tris-HCl, pH7.6). A correction was made for scattered light about 62°C by extrapolating from scatter between 300 and 330nm. Turbidity was excessive at 72°C and above (see Fig. 9). ■, Second heating cycle, subparticles which were first heated to 75°C and cooled to 25°C and had regained the initial E_{260} value (solvent 0.1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH7.6). △, A sample of the solution of smaller subparticles in 0.1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH7.6, was made 0.05% in sodium dodecyl sulphate by the addition of 25 μl of a 5% solution to 2.5ml. ○, RNA isolated from the smaller subparticles by precipitation as the guanidinium salt (solvent 0.1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH7.6); ▽, RNA as above (solvent 1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH7.6). When subparticles in 0.1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH7.6, are previously exposed to the temperature range indicated (↔) the activity in a cell-free system at 37°C is diminished (cf. Fig. 6) and the subparticle unfolds, as shown by a decrease in $s_{20,w}$ (Fig. 7). (b) Larger subribosomal particles in 0.1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH7.6 (□). ○, RNA from the larger subparticles isolated by precipitation as the guanidinium salt heated in the same buffer as the subparticles; ◇ subparticles in 1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH7.6, at 60°C and above: an allowance for scattered light (see Fig. 9) was made as in (a).

double-helical secondary structure within the subribosomal particle should be clearly seen. This proves to be the case.

The activity of the smaller subparticle in cell-free protein synthesis was measured at 37°C after heating to elevated temperatures for 2min (solvent 0.1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH7.6). The stability of the subparticle was greater by approx. 10°C than that of E. coli subribosomal particles (Fig. 6). No irreversible change in $s_{20,w}$ measured at 20°C was found until the subparticles were heated to about 65°C (Fig. 7). The increase in E_{260} at this temperature (Fig.

8) was no more than 5% compared with approx. 16% for isolated rRNA.

As with rRNA (Fig. 8a), increasing the $MgCl_2$ concentration from 0.1 to 1 mM led to an increase of no more than 5°C in T_m of the subparticles. Allowance was made for the contribution of scattered light because the solutions became turbid; the effect was small but detectable at 60°C (Fig. 8), but considerable at higher temperatures where the rRNA moiety was seen to 'melt'. The plot of turbidity against temperature (Fig. 9) appears to offer an approximate method for following the 'unfolding' of subribosomal
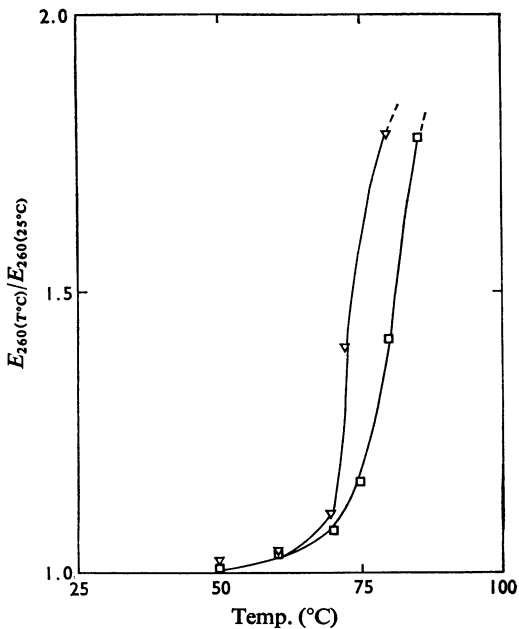
Fig. 9. 'Melting' profiles for B. stearothermophilus subparticles at higher ionic strengths

No allowance for scattered light was made and this was appreciable above 60°C owing to turbidity, which made the major contribution to E_{260}. ∇, Small subribosomal particles in 10mM-MgCl$_2$–50mM-KCl–25mM-Tris–HCl, pH7.6; □, larger subparticles in 1mM-MgCl$_2$–10mM-Tris–HCl, pH7.6.

particles. On this basis our observations agree with earlier reports of the stability of B. stearothermophilus ribosomes (Mangiantini et al., 1965; Friedman & Weinstein, 1966; Stenesh & Holazo, 1967; Saunders & Campbell, 1966).

Reticulocyte ribosomes

Thermal denaturation of ribosomes from eukaryotes. The upper limit at which rabbit reticulocyte ribosomes can function was sought, first by incubating reticulocytes at elevated temperatures, secondly by means of a cell-free system, and thirdly by measuring the irreversible effects of heat treatment on the ability of ribosomes to function in cell-free protein synthesis at 37°C.

Effect of temperatures on the reticulocyte cell-free system. Temperatures above the normal body temperature of the rabbit adversely affect the ability of reticulocytes to synthesize protein. It appears that the limiting factor is not the thermal stability of the ribosome fraction. Reticulocytes were kept at 0°C
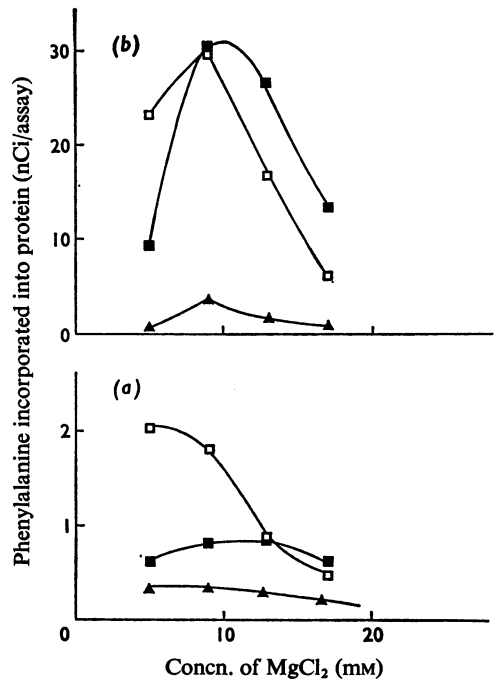

Fig. 10. *Dependence of amino acid incorporation into acid-insoluble protein by reticulocyte ribosomes on the concentration of MgCl$_2$*

(a) Endogenous activity [no poly(U)]; (b) poly(U)-stimulated incorporation at □, 37°C, ■, 45°C and ▲, 50°C. The cell-free system used by Schweet et al. (1958) was used with several modifications. Each assay tube contained, in 0.5ml: 0.25mg of ribosomes, 15μmol of KCl, 3.5μmol of MgCl$_2$, 12.5μmol of Tris–HCl, pH7.6, 5μmol of GSH (neutralized with M-KOH), approx. 2mg of pH5 enzymes, 50μg of pyruvate kinase, 5μmol of phosphoenolpyruvate kinase, ATP and GTP in the proportion of 0.05 and 0.0125μmol/μmol of phosphoenolpyruvate, and [^{14}C]phenylalanine (approx. 0.01μmol of specific radioactivity 50mCi/mmol) in a mixture of unlabelled amino acids (0.05ml). The unlabelled amino acids were an equimolar mixture (2μmol/ml) of each of the 20 amino acids except phenylalanine. When required 20μg of poly(U) was added. After incubation 0.2ml of M-NaOH containing 5μmol of unlabelled DL-phenylalanine was added and the incubation was continued for 5min at 37°C and 15min at room temperature and was terminated by the addition of 2.5ml of 10% (w/v) trichloroacetic acid. The protein was isolated by filtration on 3cm diameter Oxoid membrane filters (Oxo Ltd., London E.C.4, U.K.), washed once with 2.5ml of cold and twice with 5.0ml of hot (90°C) 5% (w/v) trichloroacetic acid and finally twice with 5.0ml of water. The samples were counted at infinite thinness with a low-background

and samples were also incubated by the method of Lingrel & Borsook (1963) for 1h at 37°C (38.7°C is normal body temperature of the rabbit), 45° and 50°C.

Incorporation of radioactive phenylalanine into protein at 0°, 37°, 45° and 50°C was respectively 9.4, 488, 90 and 21.4pmol of phenylalanine/assay (containing approx. 80μg of ribosomes). Reticulocytes survived as intact cells after incubation at 45°C, but about half were lysed after incubation at 50°C. In contrast, in poly(U)-directed protein synthesis in the cell-free system the ribosome fraction was as active at 45° as at 37°C.

The performance of the cell-free system at elevated temperatures was studied by preparing the assay mixtures at 0°C and then plunging them into a water bath at the elevated temperature. The assay mixture reached the temperature of the bath within 35s. The ability of the cell-free system to function at 45°C (Cox et al., 1970) was confirmed. The optimum concentration of MgCl₂ for the poly(U)-directed synthesis of polyphenylalanine was higher at 45° than at 37°C (Fig. 10) and the same incorporation occurred at both temperatures. The endogenous activity of the polyribosome fraction was less at 45°C.

The kinetics of protein biosynthesis in the standard cell-free system in which the concentration of MgCl₂ was 8mM was studied at 37°C and at 45°C (Fig. 11). The endogenous activity (mainly the synthesis of haemoglobin) continued for about 40min at 37°C. When the temperature was increased to 45°C the rate of haemoglobin synthesis was roughly the same for the first 10min as at 37°C but it then decreased (Fig. 11). Evidently ribosomes are functional at 45°C and the cut-off in endogenous synthesis after 10min suggests that a factor or factors are present in limiting amounts; possibly ribosomes recycle to a much lesser extent at 45° than at 37°C (cf. Nair & Arnstein, 1965).

The poly(U)-directed synthesis of polyphenylalanine was more extensive at 45° than at 37°C, and at both temperatures synthesis was complete after 20min. Raising the temperature decreased the lag before protein synthesis was detected. Half the polyphenylalanine was synthesized after 9min at 37°C and after 6min at 45°C. At 45°C polyphenylalanine continued to be synthesized after haemoglobin synthesis had almost ceased. The initiation of poly(U)-directed synthesis does not require initiation factors at the MgCl₂ concentration used in the assay. Much decreased incorporation of phenylalanine into acid-
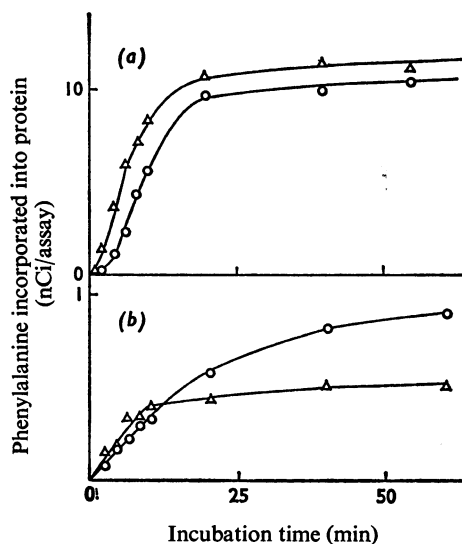

Fig. 11. *Kinetics of the stimulation of phenylalanine incorporation into protein by reticulocyte ribosomes at 37°C and 45°C*

Each assay tube contained the usual components for the standard cell-free system (see Fig. 10) and the concentration of MgCl₂ was 8mM. (a) Poly(U)-directed synthesis; (b) endogenous activity [no poly(U)]. ○, 37°C; △, 45°C.

insoluble protein was obtained when the cell-free system was incubated at 50°C (Fig. 10). The extent of incorporation was not affected by changing the pH (measured at approx. 20°C) of the Tris–HCl buffer from 7.6 to 8.0. At 55°C the enzyme fraction precipitated. The decrease in activity at 50°C and above was not necessarily due to inactivation of the ribosome fraction, because the activity of enzymes and factors is not known.

Irreversible effects of brief exposure to elevated temperatures on the biological activity, extinction at 260nm and sedimentation profile of the polyribosome fraction of reticulocyte ribosomes. The standard cell-free system used in the present study contained 0.1M-sucrose, 0.03M-KCl, 5–10mM-MgCl₂ and 0.025M-Tris–HCl, pH7.6. A similar buffer (0.25M-sucrose – 0.025M-KCl – 1mM-MgCl₂ – 0.05M-Tris – HCl, pH7.6) was used to study the irreversible effects of preheating ribosomes to elevated temperatures. To avoid hydrolysis the polyribosome fraction was kept at the elevated temperature for 2min after allowing 2min for thermal equilibrium to be attained. The E_{260} attained a plateau value during this time, showing that ample time was allowed for changes in the conformation of the rRNA moiety. The kinetics of

(1.5c.p.m.) Geiger tube counter (model 181B; Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.), fitted with an ultra-thin Micromil window giving a counting efficiency of approx. 5%.
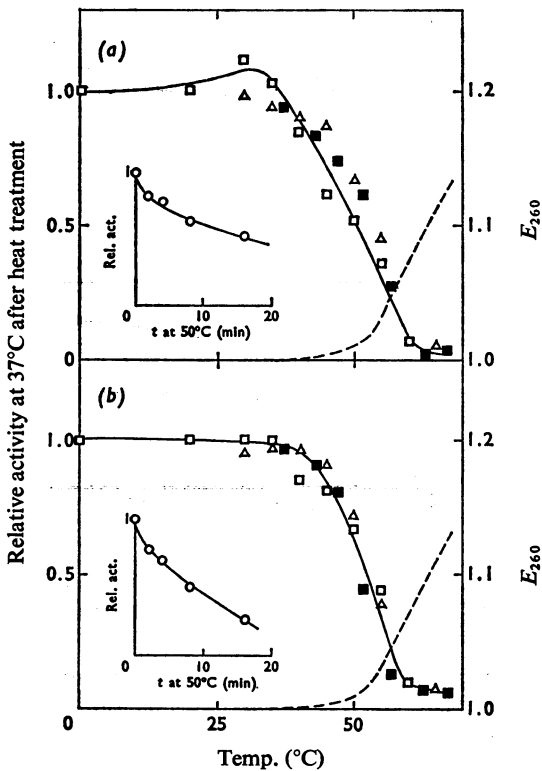
Fig. 12. *Irreversible effects of heating at elevated temperatures for 2 min on the ability of the polyribosome fraction of reticulocytes to synthesize protein at 37°C*

(*a*) Endogenous activity; (*b*) poly(U)-stimulated activity. □, ■, Independent experiments (solvent 0.25 M-sucrose – 0.025 M-KCl – 1 mM-MgCl$_2$ – 0.05 M-Tris–HCl, pH 7.6); △, polyribosome fraction (1–2.5 mg/ml) was heated in the same solvent as (*a*) and (*b*) except that concentration of MgCl$_2$ was increased to 10 mM. In each case the heated solution was allowed to cool slowly to room temperature; it was then kept in ice before being assayed in the cell-free system. In both (*a*) and (*b*) the inset shows the kinetics of inactivation at 50°C and the broken line denotes the relative increase in E_{260} measured at the elevated temperature. In Expt. 1 the control non-incubated sample incorporated 230 pmol of phenylalanine into protein/mg of polyribosome fraction and when poly(U) was present the amount of protein synthesized was 2260 pmol of polyphenylalanine/mg of ribosomes. In Expt. 2, in the control 208 pmol of phenylalanine was incorporated/mg of polyribosome fraction and when poly(U) was present the amount incorporated was 1270 pmol of phenylalanine/mg of polyribosome fraction. For the kinetics experiment the control incorporated 150 pmol of phenylalanine/
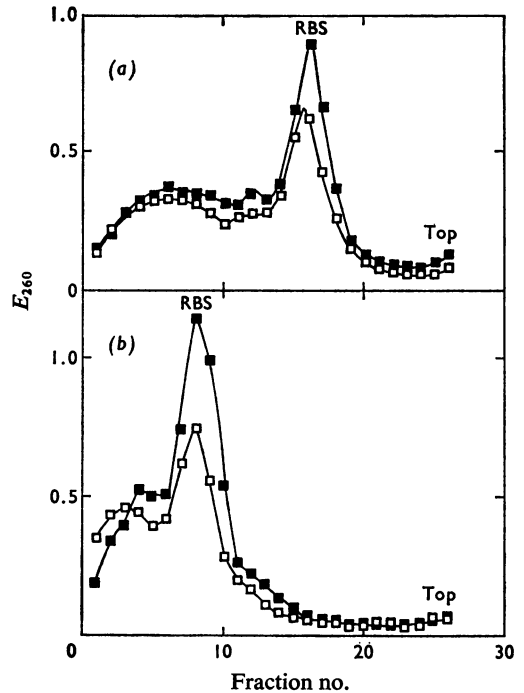

Fig. 13. *Irreversible effects of 2 min exposure to elevated temperatures on the sedimentation properties of the polyribosome fraction of reticulocytes*

Samples of the polyribosome fraction that had been heated were assayed for biological activity (Fig. 12) and sedimentation properties. (*a*) Polyribosome profile obtained by centrifuging for 3.5 h (Beckman SW-25 rotor at 25000 rev./min) through 15–30 % sucrose containing 0.01 M-KCl–1.5 mM-MgCl$_2$–0.01 M-Tris–HCl, pH 7.6. (*b*) Ribosome and sub-ribosome profile obtained by zone-centrifuging for 17 h at 17500 rev./min, otherwise conditions were as in (*a*). RBS indicates the ribosome peak. □, Control kept at 0°C; ■, sample heated to 50°C.

inactivation at 50°C revealed that the initial rapid decrease in activity was followed by a slower decrease (inset in Fig. 12). The amount of protein synthesized at 37°C was about the same after heating at 50°C for 2 min as the value obtained by extrapolating to zero time at 50°C. Heating for 2 min at 50°C appears sufficient to reveal the effects of changes in rRNA double-helical secondary structure on biological activity and short enough to avoid the slower in-

mg of polyribosome fraction and when poly(U) was present incorporation was 850 pmol of phenylalanine/ mg of polyribosome fraction.

activation arising from other causes. The extinction of the solution was measured at the elevated temperature as well as before heating and after cooling. The ability of the samples to incorporate amino acids into protein in a cell-free system was then measured (Fig. 12). The sedimentation profile of the heated samples was also obtained by zonal centrifugation at about 4°C through 15–30% sucrose to reveal the polyribosome pattern or through 15–45% sucrose to measure the proportion of ribosomes and of slower-sedimenting species such as subparticles (Fig. 13). The polyribosome pattern is a very sensitive index of nuclease activity and the persistence of most of the polyribosomes at 50°C is an indication that nuclease activity was not important at this temperature. The small proportion (approx. 10%) of extinction found trailing behind the ribosome fraction in the subparticle region of the gradient was not significantly increased by previous heat treatment (Fig. 13b), indicating that there was no major irreversible increase in the proportion of subparticles as a result of heating (cf. Petermann & Pavlovec, 1963).

The effect of preheating the ribosome fraction on the poly(U)-directed synthesis of protein was roughly parallel to the effect on endogenous activity (Fig. 12). About half the activity was lost on heating to approx. 53°C ($MgCl_2$ concentration was 1–10mM). After exposure to 60–67°C some 3–10% of activity was left. The residual activity at 67°C (approx. 100pmol of phenylalanine incorporated/mg of ribosomes) was still appreciable when poly(U) was present (cf. the effect of ribonuclease treatment on biological activity: Szer, 1969; Delihas, 1970; Cahn et al., 1970; Huvos et al., 1970).

The changes in E_{260} which are also given in Fig. 12 were independent of concentration over the range studied, i.e. 0.1–10mg of ribosomes/ml of buffer. The initial value of E_{260} was regained to within 1% after heating to 95°C and then cooling to 25°C. After exposure to 55°C, when about 40% of the biological activity was retained, the E_{260} of the polyribosome fraction had increased by 4%, corresponding to 'melting' of about 12% of the double-helical secondary structure. Thus the level of double-helical secondary structure can be decreased to a limited extent without inactivating the ribosome.

The 'melting' profile of the bacterial subribosomal particles studied can be divided into three regions: (1) the temperature range over which the compact conformation and activity are retained; (2) the range over which the transition to a slower-sedimenting species or expanded form takes place (this transition may not be reversed on cooling); (3) the range over which the RNA moiety of the expanded form 'melts'. Whereas within the active subribosomal particles the double-helical secondary structure of the RNA moiety is stabilized compared with isolated RNA this is not the case for the expanded form. Thus the shape
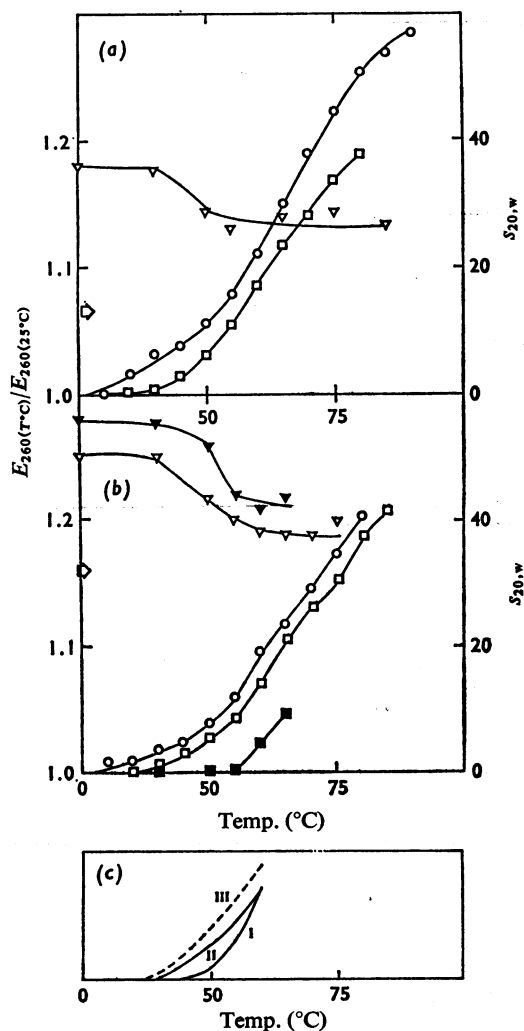
Fig. 14. 'Melting' and sedimentation properties of subribosomal particles and rRNA of rabbit reticulocytes

$\triangledown$, $\blacktriangledown$, $s_{20,w}$ measured at 10–20°C after heating to the temperature shown. A single sharp boundary was found in all cases even after heating to elevated temperatures. $\Rightarrow$, $s_{20,w}$ of subparticles in 0·2mM-EDTA–0.01 M-Tris–HCl, pH 7.6; $\square$, $\blacksquare$, E_{260} of subribosomal particles; $\circ$, E_{260} of rRNA. Solvents: $\blacktriangledown$, $\blacksquare$, 0.025 M-KCl–1 mM-$MgCl_2$–0.01 M-Tris–HCl, pH 7.6; $\triangledown$, $\square$, $\circ$, 0.1 mM-$MgCl_2$–0.01 M-Tris–HCl, pH 7.6. (a) Smaller subparticles. (b) Larger subparticles. (c) I, Polyribosome fraction (cf. Fig. 12), solvent 0.25 M-sucrose–0.025 M-KCl–1 mM-$MgCl_2$–0.05 M-Tris–HCl, pH 7.6; II, calculated from (a) and (b) for an equimolar mixture of subparticles; III, rRNA (equimolar mixture) in the same solvent as I.
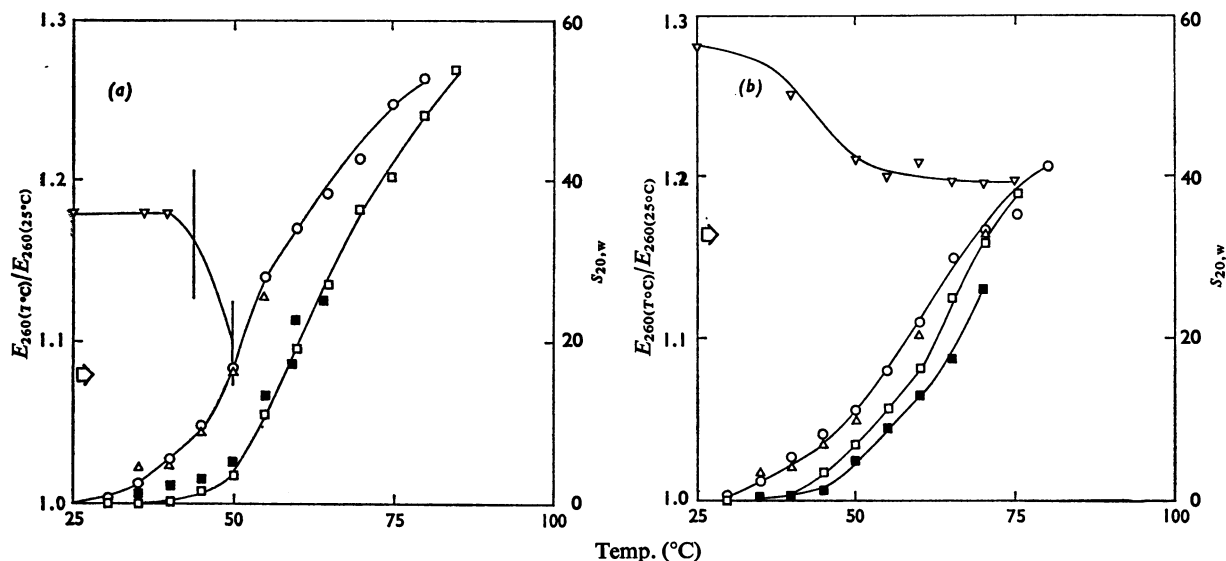
Fig. 15. *'Melting' profiles and sedimentation properties of subribosomal particles and rRNA of X. Laevis oocytes*

∇, $s_{20,w}$ measured at 10–20°C after heating to the temperature indicated. The vertical lines indicate the spread in $s_{20,w}$ found when single boundaries were not obtained. $\Rightarrow$, $s_{20,w}$ of subparticles in 0.2mM-EDTA–0.01 M-Tris–HCl, pH 7.6; $\square$, E_{260} of subparticles produced by heparin treatment (Pratt & Cox, 1971); $\circ$, E_{260} of rRNA; $\triangle$, E_{260} of subparticles produced by zone centrifuging through 1mM-EDTA, pH 7.6. Solvents: ∇, $\square$, 0.1mM-MgCl$_2$–0.01 M-Tris–HCl, pH 7.6; $\triangle$, $\blacksquare$, 0.25 M-sucrose–0.025 M-KCl–1 mM-MgCl$_2$–0.05 M-Tris–HCl, pH 7.6. (a) Smaller subribosomal particles; (b) larger subribosomal particles.

of the 'melting' profile will depend critically on the temperature range over which the conversion into a slower-sedimenting form takes place. If, e.g. by EDTA treatment at 0°C, the transition takes place before the RNA moiety begins to 'melt' there could be little difference in the 'melting' profiles of the RNA and subribosomal particle. Active reticulocyte and *X. laevis* oocyte subparticles (see Fig. 14*a,b* and Fig. 15*a,b* respectively) began unfolding at 40°C and the transition was complete at 55°C (T_m approx. 47°C). The 'melting' profile of a functional polyribosome fraction in KCl-containing buffer (Fig. 14*c*) clearly shows stabilization of the double-helical secondary structure of the RNA moiety.

'Unfolding' took place over the range 50–55°C (cf. 50–55°C for the thermal inactivation of rat liver subribosomal particles; Reboud *et al.*, 1972) when reticulocyte subparticles were heated in 0.025 M-KCl – 1mM-MgCl$_2$ – 0.01 M-Tris – HCl, pH 7.6 (see Fig. 15*a,b*). It is well known that Mg^{2+} affects the stability of the ribosome, but Naslund & Hultin (1971) have shown that K$^+$ is also important since ribosomes have decreased thermal stability in K$^+$-deficient buffers. Subparticles of *X. laevis* oocyte

ribosomes that were isolated by zonal centrifuging in EDTA and were then dissolved in buffer (0.25 M-sucrose – 0.025 M-KCl – 1 mM-MgCl$_2$ – 0.05 M-Tris – HCl, pH 7.6) closely resembled rRNA in their profile, whereas active subparticles in the same buffer were more stable (Fig. 15*a,b*).

The different 'melting' properties of the subparticles treated in different ways probably reflect the organization of RNA and protein, because the 'melting' profile of RNA alone is not so sensitive to K$^+$ concentration, e.g. the 'melting' profile of rRNA in 0.025 M-KCl–1 mM-MgCl$_2$–0.01 M-Tris–HCl, pH 7.6, was scarcely distinguishable from the profile in 0.1 mM-MgCl$_2$–0.01 M-Tris–HCl, pH 7.6, and was independent of previous treatment with EDTA.

Circular dichroism of subribosomal particles and their constituents

The conformation of both rRNA and ribosomal protein contribute to c.d. (Fig. 16) but their contributions differ according to wavelengths. The individual contributions of the rRNA and ribosomal protein moieties were deduced from $E_L - E_R$ at 265 and at
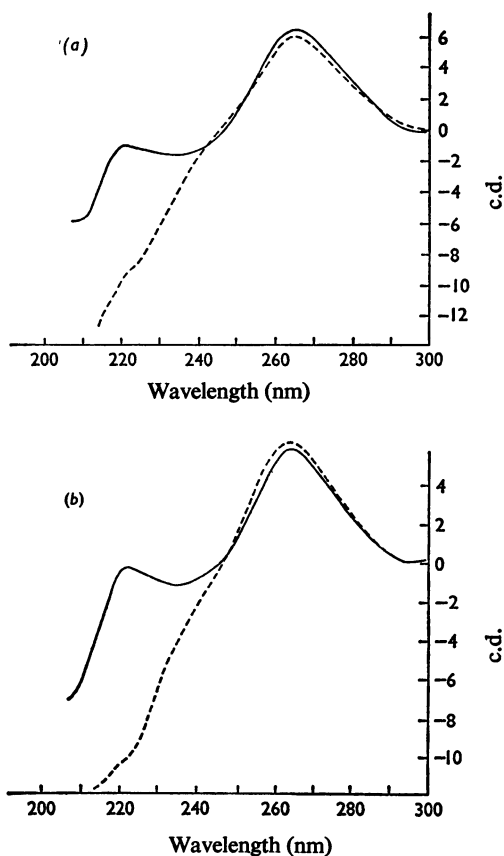
Fig. 16. *Circular dichroism of subribosomal particles and of rRNA*

(a) Smaller subribosomal particles of *X. laevis* oocytes ($E_{260} = 6.4$) and rRNA ($E_{260} = 6.4$). (b) Larger subparticle of *B. stearothermophilus* and rRNA ($E_{260} = 7.35$ in each case). The solvent was 0.1 mM-$MgCl_2$-2 mM-Tris-HCl, pH 7.6, and measurements were made in cells of 1 mm path-length. ——, Subparticles; ---, rRNA.
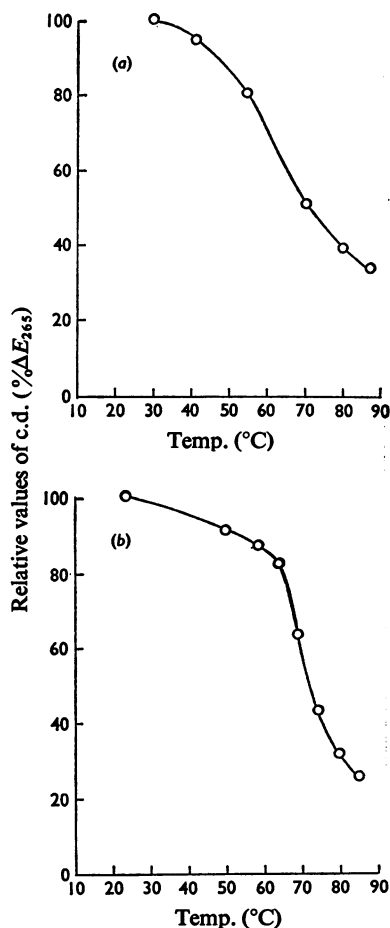

Fig. 17. *'Melting' profiles of subribosomal particles*

Changes in the c.d. at 265 nm measure alterations in the secondary structure of rRNA. (a) Larger subparticles of *X. laevis* oocytes; (b) larger subparticles of *B. stearothermophilus*. The subribosomal particles were in 1 mM-$MgCl_2$-0.01 M-Tris-HCl, pH 7.6.

225 nm respectively. The ribosomal protein moiety contributes little to $E_L - E_R$ at 265 nm, where there is a maximum in the c.d. of rRNA, and changes in the conformation of rRNA have little effect on $E_L - E_R$ at 225 nm (Cox *et al.*, 1971), where the contribution of ribosomal protein is appreciable. We followed heat-induced conformational changes in both the RNA and protein moieties by measuring the c.d. spectrum at different temperatures.

When subribosomal particles were heated it was apparent that ΔE_{265} decreased much more rapidly than ΔE_{225}, indicating that the double-helical secondary structure of rRNA was more labile than the secondary structure of the protein moiety (Figs. 17 and 18). The decrease in $E_L - E_R$ at 265 nm was found to take place over the same temperature range as changes in hypochromism (measured by the increment in E_{260}: e.g. Figs. 8 and 15).

The ellipticity of the protein moiety was substantially higher for *B. stearothermophilus* ribosomal proteins than for *X. laevis* oocyte ribosomal proteins (Fig. 18), indicating a greater degree of secondary
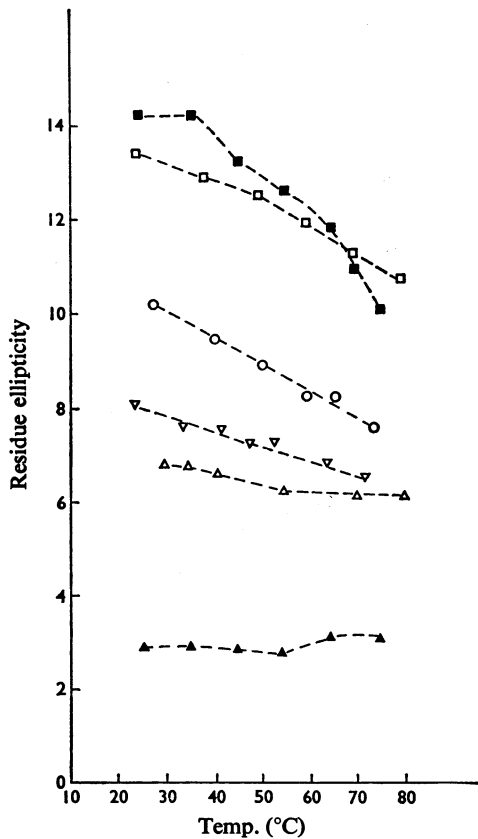
Fig. 18. *Dependence of the residue ellipticity at 225 nm of the protein moiety of subribosomal particles and of ribosomal proteins on temperatures*

▽, Smaller subribosomal particles of *X. laevis* oocytes; △, larger subribosomal particles of *X. laevis* oocytes; ▲, ribosomal proteins of *X. laevis* oocyte larger subparticles; ○, smaller subribosomal particles of *B. stearothermophilus*; □, larger subparticles of *B. stearothermophilus*; ■, ribosomal proteins of larger subparticles of *B. stearothermophilus*. For the subparticles the solvent was 0.1 mM-$MgCl_2$–0.01 M-Tris–HCl, pH 7.6; isolated ribosomal proteins were dissolved in 1 M-LiCl–2M-urea. Measurements were made in cells of 0.5 mm and 1 mm path-length. The c.d. over the range 210–310 nm was measured in each case.

structure. Moreover isolated *B. stearothermophilus* proteins had about the same ellipticity as within the subribosomal particle. In contrast ribosomal proteins of *X. laevis* oocytes had little structure after isolation (cf. Bielka *et al.*, 1972). In *X. laevis* oocyte ribosomes it appears that the proteins have a stable conformation when bound to rRNA (Fig. 18), sug-

gesting that rRNA stabilizes ribosomal protein conformation.

Discussion

The points that we wish to emphasize are as follows.

(1) Ribosomes function over a wide range of temperature, that spans the range over which the intact cell functions.

(2) Active subribosomal particles have a compact quaternary structure that undergoes a transition on heating to a slower-sedimenting species. The transition is not reversed on cooling, showing the existence of metastable states. The transition is characterized by a T_m (the temperature at which half the change in $s_{20,w}$ is found) that is related to the optimum temperature for the growth of the cell (Table 1).

(3) The thermal stability of the quaternary structure of the subribosomal particles studied ranged from approx. 45° to 70°C. This difference of approx. 25°C in thermal stability is attributed to small differences in the strength or number of non-covalent protein-protein or rRNA–ribosomal protein interactions such as hydrogen bonds or hydrophobic bonds. The co-operation of a number of non-covalent bonds acting together to preserve the quaternary structure of the subribosomal particle is most clearly shown by the steepness of the 'melting' profile of *B. stearothermophilus* subparticles.

(4) In the particular case of the smaller sub-ribosomal particle of *E. coli* the finding that the principal products of hydrolysis remain the same over the range 0–55°C provides further evidence about the quaternary structure of the active subparticle. To produce fragments at least two conditions are necessary: (*a*) the RNA moiety should be accessible to RNAase T_1 and cleaved at a point where the break in the chain is not 'hidden' by double-helical secondary structure holding the fragments together, and (*b*) other intramolecular interactions such as RNA-protein and protein–protein interactions between the fragments must be insufficient to hold the fragments together. It appears that there is one site where these conditions are fulfilled, and it is inferred that intramolecular interactions between the larger and smaller fragments (of about three-quarters and one-quarter of the intact subparticle respectively) are weaker than the forces maintaining the compact conformation.

(5) The stability of the quaternary structure of the active subribosomal particle is determined by neither rRNA nor ribosomal proteins but by the interaction of the two components. The influence of ribosomal proteins on rRNA double-helical secondary structure is most clearly seen in the case of *B. stearothermophilus*, although the same effect is apparent in the other species studied. The influence of rRNA on ribosomal protein secondary structure is best

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Table 1. *Summary of effects of temperature on properties of subribosomal particles*

The buffer in which ribosomes were heated (column 4) was 0.1 mM-MgCl$_2$–0.01 M-Tris–HCl, pH 7.6. Values for temperature range of transition are from Figs. 6, 8, 15 and 16. References: (a) Friedman & Weinstein (1966); (b) Fig. 11; (c) Cox et al. (1970); (d) Bodley (1969); (e) Fig. 8.

Species	Temperature range over which species functions (°C)	Approx. temperature range over which ribosomes function in protein synthesis (°C)	Temperature at which activity at 37°C decays to 50% after heating in buffer (°C)	Temperature range of transition to slower-sedimenting species (°C)	
				Smaller subparticles	Larger subparticles
E. coli	25–45	10–55	53.5 (d)	45–60	40–55
B. stearothermophilus	50–70	37–70 (a)	67 (e)	65–75	65–75
Rabbit reticulocytes	~37	15–50 (b)	52.5 (b)	40–50	40–55
X. laevis oocytes	15–21	15–50 (c)	—	40–50	40–50

illustrated in the *X. laevis* system, since isolated proteins retain approx. 20% of the secondary structure of the ribosomal protein moiety of the subparticle.

(6) Not all of the double-helical secondary structure of the RNA moiety is essential for the activity of the ribosome, since 10% can be 'melted', e.g. at 55°C when *E. coli* ribosomes remain fully functional. This value could be a minimum, since half the activity of *E. coli* ribosomes is retained at a temperature when about 17% of double-helical secondary structure is 'melted'.

(7) Stabilization of double-helical secondary structure within the active subparticle arises from the interaction with ribosomal proteins. Since ribosomal proteins are strongly basic stabilization might be expected if proteins interacted with the double-helical regions of the RNA moiety, thereby decreasing electrostatic repulsion between phosphate residues on the complementary segments of RNA. Other mechanisms might also be important. The different 'melting' properties of expanded subparticles suggests that RNA–protein interactions are altered as a result of the conformational change.

(8) Secondary structure of the ribosomal protein moiety varies less with temperature than does the secondary structure of the rRNA moiety.

(9) Comparison of the 'melting' properties of rRNA from the smaller subribosomal particles revealed that the secondary structure of all four species had much in common (Table 2), judged by the 'melting' range and by the E_{260}/E_{280} ratio, which is a measure of the proportion of rA·rU and rG·rC base pairs 'melting' over the temperature range studied (25–95°C).

The common features in the double-helical secondary structure of rRNA from *E. coli* and from *B. stearothermophilus* is perhaps related to the finding that active hybrid subparticles can be formed between *E. coli* rRNA and *B. stearothermophilus* ribosomal

proteins and between *B. stearothermophilus* rRNA and *E. coli* ribosomal proteins (Nomura *et al.*, 1968).

In contrast rRNA of the larger subribosomal particle showed a greater variation in secondary structure. The main difference lies in the rG·rC-rich double-helical secondary structure present in rRNA of the larger subribosomal particle of *X. laevis* oocytes and rabbit reticulocytes but not of *E. coli* or *B. stearothermophilus*.

It was reported earlier (Cox, 1966, 1970) that the nucleotide composition of the double-helical regions of the larger subribosomal particle of rabbit reticulocytes ranges from about 57% rG·rC to about 80% rG·rC. The regions of approx. 55% rG·rC correspond in their T_m values to the regions found in the bacterial rRNA species, and account for approx. 1.1×10^6 daltons mass, i.e. a mass equivalent to that of 23 S RNA of the larger subparticle of *E. coli*. The remaining approx. 0.6×10^6 daltons mass of rRNA of the larger subribosomal particle of the eukaryotes studied forms double-helical secondary structure of approx. 80% rG·rC base pairs that 'melt' at temperatures above the 'melting' range of *E. coli* rRNA. The similarities and differences are readily apparent (Fig. 5) when rRNA of reticulocytes and of *E. coli* is compared after an allowance is made for the difference in molecular weight (approx. 1.1×10^6 and 1.7×10^6 respectively). When the values of ΔE_{260} found for reticulocyte rRNA were increased by 1.55 (1.7/1.1), so that the values of ΔE_{260} can be compared with rRNA of *E. coli* on a molar basis, the profiles were very similar over the range 25–75°C but not at 75–95°C (Fig. 5).

It appears that there are quite extensive regions that are rG·rC-rich, since fragments several hundred nucleotides long and containing approx. 80% rG·rC have been isolated from rat or rabbit liver ribosomes by Hadjiolov & Milchev (1967) and by Delihas (1967).

Table 2. *Comparison of properties of rRNA derived from 'melting' profiles*

The nucleotide composition of rRNA of *B. stearothermophilus* was reported by Saunders & Campbell (1966). Data for other rRNA species were reviewed by Cox (1968b). G+C (%) of the double-helical part of rRNA was calculated (Cox, 1970) by means of the equation:

$$\delta f_{rA \cdot rU} / \delta f_{rG \cdot rC} = 0.73 \, (\Delta E_{260}/\Delta E_{280}) - 0.37$$

where $\delta f_{rA \cdot rU}$ and $\delta f_{rG \cdot rC}$ respectively are the mol fractions of $rA \cdot rU$ and $rG \cdot rC$ base pairs 'melting' over the temperature range (in this case 25–95°C) over which the increments in E_{260} and E_{280} are observed. The solvent was $0.1 \, \text{mM-MgCl}_2 - 0.01 \, \text{M-Tris} - \text{HCl}$, pH 7.6.

	G+C (chemical analysis) (%)	Approx. T_m (°C)	E_{260}/E_{280}	G+C of double-helical part (%)
rRNA of smaller subparticles				
E. coli	53.7	60	1.60	54.5 ± 1
B. stearothermophilus	61.4	62	1.44	57.5 ± 1
Rabbit reticulocyte	59.5	65	1.54	55.5 ± 1
X. laevis	60	58	1.64	53.8 ± 1
rRNA of larger subparticles				
E. coli	55	62	1.71	52.5 ± 1
B. stearothermophilus	56.2	67	1.63	54 ± 1
Rabbit reticulocyte	66.9	69	1.1	55–80 (mean 68.5 ± 1)
X. laevis	67	65	1.1	55–80 (mean 68.5 ± 1)

Apparently there are few such $rG \cdot rC$-rich regions in *E. coli* rRNA. rRNA of *Xenopus* and of mammals have about 60% of base sequences in common (Birnstiel & Grunstein, 1972), so that the close similarity in the secondary structure of rRNA of reticulocytes and of *X. laevis* might be expected. However, we emphasize that because the rRNA molecules of the four larger subparticles have features in common a high degree of homology in nucleotide sequence is not mandatory (cf. the 'clover-leaf' structure and nucleotide sequences of tRNA).

Evolutionary divergence from prokaryotes to eukaryotes appears to be more clearly expressed in rRNA of the larger subparticle than in rRNA of the smaller subparticle.

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