Physicochemical characterization of the ribosomal RNA species of the Mollusca

MOLECULAR WEIGHT, INTEGRITY AND SECONDARY-STRUCTURE FEATURES OF THE RNA OF THE LARGE AND SMALL RIBOSOMAL SUBUNITS

Piero CAMMARANO, Paola LONDEI and Filomena MAZZEI

Laboratorio di Dosimetria e Biofisica delle Radiazioni C.N.E.N., C.S.N. Casaccia, Rome, Italy, and
Istituto di Biologia Generale (II Cattedra) Università di Roma, Rome, Italy

and Armando FELSANI

Laboratorio di Embriologia Molecolare, C.N.R., Naples, Italy

Received 3 March 1980)

1. The rRNA species of the Cephalopoda *Octopus vulgaris* and *Loligo vulgaris* were found to have unexpectedly high sedimentation coefficients and molecular weights. In 0.1 M-NaCl the L-rRNA (RNA from large ribosomal subunit) has the same s_{20} value as the L-rRNA of the mammals (30.7S), whereas the S-rRNA (RNA from small ribosomal subunit) sediments at a faster rate (20.1S) than the S-rRNA of both the mammals and the fungi (*Neurospora crassa*) (17.5S). The molecular weights of the L-rRNA were determined by gel electrophoresis in formamide and found to be 1.66×10^6 (*Octopus*) and 1.89×10^6 (*Loligo*); the mol.wt. of the S-rRNA of both species is 0.96×10^6 , i.e. much larger than that of the mammals (0.65×10^6) and almost coincident with that of the '23S' RNA of the prokaryotes. 2. By contrast, the less evolved Gastropoda and Lamellibranchiata (*Murex trunculus* and *Macrocallista chione*) have S-rRNA and L-rRNA species with mol.wts. of 0.65×10^6 and approx. 1.40×10^6 . 3. All the mature L-rRNA molecules of the Cephalopoda are composed of two unequal fragments held together by regions of hydrogen-bonding having a similar, low, thermal stability in the two species; the molecular weights of the two fragments composing the L-rRNA are estimated to be 0.96×10^6 and 0.88×10^6 (*Loligo*) and 0.96×10^6 and 0.65×10^6 (*Octopus*). The S-rRNA of both species is a continuous chain with exactly the same molecular weight (0.96×10^6) as the heavier of the two fragments of the L-rRNA. 4. The secondary-structure features of the L-rRNA and S-rRNA species of the Cephalopoda were investigated by thermal 'melting' analysis in 4.0 M-guanidinium chloride; 60–70% of the residues are estimated to form short, independently 'melting' bihelical segments not more than 10 base-pairs in length. 5. Bases are unevenly distributed between non-helical and bihelical portions of the rRNA molecules, G and C residues being preferentially concentrated in bihelical domains. 6. The secondary-structure regions of the L-rRNA species of *Octopus* and *Loligo* are heterogeneous, including two discrete fractions of independently 'melting' species that give rise to biphasic 'melting' profiles: a fraction consisting of shorter (G + C)-poorer segments (60–68% G + C, not more than 5 base-pairs in length) and a fraction consisting of longer (G + C)-richer segments (80–88% G + C, 5–10 base-pairs in length). No evidence for heterogeneity has been detected in the S-rRNA.

We have reported (Cammarano *et al.*, 1975) that the molecular weight of the S-rRNA of all the Deuterostomes, from sea urchins to man, has been

Abbreviations used: S-rRNA and L-rRNA, RNA obtained from the large and the small ribosomal subunit, respectively; SDS, sodium dodecyl sulphate.

stringently conserved at 0.65×10^6 (see also Loening, 1968), whereas the molecular weight of the L-rRNA has been conserved at 1.35×10^6 – 1.38×10^6 in the lower members of the group, but has increased to 1.65×10^6 in the warm-blooded animals. Both rRNA species of the Deuterostomes

appear to be truly continuous polynucleotide chains, except for the presence of the ubiquitous 5.8S RNA fragment, which is associated by limited regions of hydrogen-bonding with the major portion of the L-rRNA (Pene *et al.*, 1968; King & Gould, 1970; Sy & McCarthy, 1970).

In the present study, the principal features of the L-rRNA and S-rRNA of the Cephalopoda (Octopoda and Decapoda) have been investigated and compared with those of the Lamellibranchiata and the Gastropoda.

The two major rRNA components of the Di-branchiata appear to have diverged extensively from the corresponding rRNA species of the less evolved Gastropoda and Lamellibranchiata, the L-rRNA of the Decapoda being even larger than that of the warm-blooded animals and the S-rRNA being much larger than that of some Protozoa with unusually large S-rRNA, such as *Euglena gracilis* (Rawson & Stutz, 1968; Loening, 1968; Cammarano *et al.*, 1975) and *Acanthamoeba castellanii* (Loening, 1968; Stevens & Pachler, 1972; Cammarano *et al.*, 1975). The L-rRNA of the Cephalopoda is a discontinuous molecule composed of two principal segments held together by a heat-labile structure and appears to comprise two distinct fractions of bihelical segments differing in both length and G + C content. The most interesting aspect of the results is the evidence that the S-rRNA has exactly the same molecular weight as the heavier of the two strands composing the L-rRNA.

Experimental

Media

The buffer compositions were as follows: RNA-extraction buffer, 35 mM-sodium acetate/15 mM-acetic acid (pH 5.1)/10 mM-MgCl₂/200 mM-NaCl/1.0 mM-EDTA/SDS (20 g/litre); high-salt buffer, 10 mM-Tris/HCl (pH 7.6)/100 mM-NaCl/1.0 mM-EDTA (disodium salt) (used for sucrose-density-gradient fractionations); buffer E (Loening, 1968), 30 mM-Tris/36 mM-NaH₂PO₄ (pH 7.8)/1.0 mM-EDTA (disodium salt) (used for sucrose-density-gradient fractionations and gel electrophoresis under non-denaturing conditions); 4.0 M-guanidinium chloride/5 mM-NaH₂PO₄/5 mM-Na₂HPO₄ (pH 7.0) (used for thermal-'melting' experiments); buffered formamide, deionized formamide (Pinder *et al.*, 1974) containing 20 mM-diethylbarbituric acid, adjusted to pH 9.0 (glass electrode) with 1.0 M-NaOH.

RNA extraction

RNA was extracted from the hepatopancreas of *Octopus vulgaris*, *Loligo vulgaris* (squid), *Murex trunculus* (sea snail) and *Macrocallista chione* (clam) and from the liver of male albino rats (Sprague-Dawley). The organs were excised as

rapidly as possible, frozen in a solid-CO₂/acetone bath, and stored frozen at -80°C until used. To extract RNA, the solid-frozen organs were supplemented with 5 vol. of RNA-extraction buffer; after the further addition of 5 vol of aq. 85% (w/v) phenol containing all the components of the RNA-extraction buffer at the specified final concentrations, plus 0.1% (w/v) 8-hydroxyquinoline, the tissues were homogenized for 15–20 s at 0–4°C in a Waring Blendor homogenizer. The homogenates in phenol were centrifuged at 10 000 g for 10 min; the aqueous phases were re-extracted with 1 vol. of phenol containing all the components of the RNA-extraction medium except MgCl₂, and 1 vol. of a 3-methylbutan-1-ol/chloroform mixture (1:99, v/v). The aqueous phases were separated by centrifugation and extracted a second time with 1 vol. of aq. 85% (v/v) phenol in extraction medium lacking MgCl₂, and 1 vol. of the 3-methylbutan-1-ol/chloroform mixture. The RNA was precipitated from the final aqueous phases by the addition of 2.5 vol. of 95% (v/v) ethanol at -20°C.

RNA preparations obtained from the Lamellibranchiata and the Gastropoda were heavily contaminated by polysaccharides; the latter were removed by centrifuging the RNA through a preformed CsCl gradient ($\rho = 1.55\text{--}1.73\text{ g/cm}^3$) for 20 h in a Spinco SW 39 rotor operated at 38 000 rev./min and 20°C. The RNA ($\rho = 1.90\text{ g/cm}^3$) sedimented at the bottom of the tube, whereas polysaccharides formed a broad whitish band with a buoyant density of 1.69 g/cm³. *Escherichia coli* rRNA was extracted from crude ribosomes as described by Nirenberg & Matthaei (1961); *Neurospora crassa* (wild strain T-187) was grown, and RNA was extracted, as described by Cammarano *et al.* (1973); tRNA (rat liver) was isolated by the method of Brungraber (1962).

The RNA was freed from contaminating DNA by precipitation in 2.5 M-NaCl/50 mM-Tris/HCl (pH 7.5)/1.0 mM-EDTA; the solutions were left at 0°C for at least 3 h and RNA was sedimented by centrifugation at 15 000 g for 30 min.

Isolation of L-rRNA and S-rRNA

About 12 mg of unfractionated RNA dissolved in high-salt buffer was loaded on six preparative (37.5 ml) linear 5–40% (w/v) sucrose density gradients made in the same solvent. The tubes were centrifuged for 16 h in a Spinco SW 27 rotor operated at 23 000 rev./min and 4°C. Fractions corresponding to the L-rRNA and S-rRNA components (respectively 30S and 20S in high-salt buffer) were separately pooled and the RNA species were collected by precipitation with 2.5 vol of 95% (v/v) ethanol. As convenient, the RNA pellets were dissolved in high-salt buffer, in buffer E or in buffered formamide, pH 9.0; in this latter case the

pellet was first evaporated to dryness in a vacuum oven at 30°C and then dissolved in buffered deionized formamide containing 1.0 mM-EDTA (disodium salt); the RNA was precipitated again with 2.5 vol of 95% (v/v) ethanol and lastly redissolved in buffered formamide, pH 9.0, containing 6% (w/v) sucrose, at a concentration of approx. 2.0 mg of RNA/ml. RNA concentrations were determined by using an absorption coefficient, $A_{260}^{1\text{mg/ml}}(1\text{ cm}) = 24$.

Determination of sedimentation coefficients

Sedimentation coefficients, s_{20} , of the rRNA species investigated, in high-salt buffer and in buffer E, were assigned by zone-velocity centrifugation in linear sucrose gradients calibrated with a reference RNA of known s_{20} value. The preliminary calibration was accomplished by the method of Stutz & Noll (1967), by measuring the distance d travelled by the reference RNA as a function of centrifugation time t , by using sufficient time intervals to cover the entire length of the tube; a calibration curve was made by plotting the distance travelled against the product $s_{20} \times t$ obtained by multiplying the sedimentation coefficient of the reference RNA by the experimentally chosen centrifugation time. Sedimentation coefficients of the unknown were determined from the graph by finding the $s_{20} \times t$ value corresponding to its path length (Stutz & Noll, 1967).

The L-rRNA component of rat liver was chosen as the reference, and assigned a value of 30.7S (in high-salt buffer), on the basis of analytical-ultra-centrifuge data (P. Londei, unpublished work). For calibrating the gradients, samples of purified L-rRNA (1.0 A_{260} unit in 0.5 ml of high-salt buffer) were applied to six linear 5–40% (w/v) sucrose gradients (11.5 ml) made in high-salt buffer; the gradients were centrifuged in a Spinco SW41 rotor operated at 38000 rev./min and $20 \pm 1^\circ\text{C}$; after chosen time intervals, individual tubes were withdrawn, and the distances travelled by the L-rRNA were measured by using an ISCO (Instrumentation Specialties, Lincoln, NE, U.S.A.) density-gradient fractionator. Once the gradients had been calibrated, sedimentation coefficients of unknowns were routinely determined by centrifuging the RNA samples for 4.0 h at 38000 rev./min and 20°C . The linear interpolation method of Martin & Ames (1961) was also used; this was accomplished by centrifuging the rRNA being investigated with a variety of reference rRNA species; s_{20} values assigned by the latter procedure coincided almost exactly with those established with the aid of a calibration curve.

Gel electrophoresis

Gel electrophoresis of rRNA under non-denaturing conditions was carried out for 3.0 h in 2.4%

polyacrylamide gels made in buffer E (Loening, 1968) at a constant current of 5 mA/tube. Gel electrophoresis in formamide was performed as described in detail by Pinder *et al.* (1974), by using 4.2% acrylamide gels; electrophoreses were carried out for 90–100 min at 5 mA/tube. The distribution of the RNA was monitored by scanning the u.v. absorbance at 260 nm after washing the gels thoroughly with water to remove formamide, and using the linear transport accessory of a Gilford spectrophotometer. Molecular weights were estimated from the relative gel-electrophoretic mobilities, by using a linear relationship between distance travelled and logarithm of molecular weight (Gould & Hamlyn, 1973). The molecular weights of the reference L-rRNA and S-rRNA of *E. coli* were assumed to be 1.10×10^6 (Kurland, 1960; Granboulan & Scherrer, 1969) and 0.55×10^6 (Ehresmann *et al.*, 1975), and those of the mammalian L-rRNA and S-rRNA species were taken as 1.65×10^6 and 0.65×10^6 respectively (Petermann & Pavlovic, 1966; Hamilton, 1967; Cammarano *et al.*, 1975). The molecular weight of unfractionated tRNA (rat liver) was taken as 0.028×10^6 (Dawid & Chase, 1972; Gould & Hamlyn, 1973).

Heating studies

Thermal-dissociation experiments. Samples of purified L-rRNA or S-rRNA in buffer E were incubated in buffer E containing 0.2% SDS as described in the legend to Fig. 4; the samples were then electrophoresed at 20 – 25°C in 2.4% acrylamide gels made in buffer E; the relative proportions of intact molecules and thermal-dissociation product were quantified gravimetrically, or by the product of peak height and half width, by using the u.v.-absorption tracings obtained by scanning the gels at 260 nm.

Thermal-'melting' experiments. Increases in A_{260} and A_{280} of the RNA samples on raising the temperature were recorded automatically by using a Gilford 2400 spectrophotometer equipped with an automatic dual-wavelength scanning programmer, a reference compensator and a thermosensor for continuously monitoring the temperature in the cell housing. The rate of heating was 1.5 – $1.7^\circ\text{C}/\text{min}$; A_{260} values were corrected for the dilution of the RNA owing to thermal expansion of the solvent. Data points were recorded every $\frac{1}{3}^\circ\text{C}$.

Analysis of thermal-'melting' spectra

The theoretical basis of the method has been described in complete detail by Cox (1970). The following parameters were deduced from the absorbance/temperature curves at 260 and 280 nm: (a) the mole fractions, $f_{(\text{G}\cdot\text{C})}$ and $1 - f_{(\text{G}\cdot\text{C})}$, of G·C and A·U base-pairs in independently 'melting' bihelical segments, (b) the average number, N , of base-pairs

in bihelical segments and (c) the fraction, f_{dh} , of nucleotide residues that participate in secondary-structure formation.

Calculation of $f_{(G \cdot C)}$. When RNA is heated, the increment ΔA_{260} in absorption arises from the denaturation of both A·U and G·C base-pairs, whereas the increment ΔA_{280} reflects solely the 'melting' of G·C base-pairs (Fresco *et al.*, 1963; Cox, 1970). The mole fractions, $\delta f_{(G \cdot C)}$, and $\delta f_{(A \cdot U)}$, of G·C and A·U base-pairs in bihelical segments 'melting' to an unfolded form over any temperature interval ΔT is governed by the equation (Cox, 1970):

$$\frac{\delta f_{(A \cdot U)}}{\delta f_{(G \cdot C)}} = \frac{\Delta \epsilon_{280(G \cdot C)}}{\Delta \epsilon_{260(A \cdot U)}} \cdot \frac{\Delta \epsilon_{260}}{\Delta \epsilon_{280}} - \frac{\Delta \epsilon_{260(G \cdot C)}}{\Delta \epsilon_{260(A \cdot U)}} \quad (1)$$

and

$$\frac{\Delta \epsilon_{260}}{\Delta \epsilon_{280}} = \frac{\Delta A_{260}}{\Delta A_{280}}$$

where $\Delta A_{\lambda} = A_{\lambda}^{T_2} - A_{\lambda}^{T_1}$, T_2 and T_1 being the upper and the lower limits of the temperature interval under study; the terms $\Delta \epsilon_{\lambda(G \cdot C)}$ and $\Delta \epsilon_{\lambda(A \cdot U)}$ are the molar increments in absorption corresponding to the 'melting' of A·U and G·C base-pairs. If phosphate buffers are used as the solvent, $\Delta \epsilon_{280(G \cdot C)} = 3070$, $\Delta \epsilon_{260(A \cdot U)} = 3900$, $\Delta \epsilon_{260(G \cdot C)} = 1800$; if 4.0 M-guanidinium chloride is used, $\Delta \epsilon_{280(G \cdot C)} = 3070$, $\Delta \epsilon_{260(A \cdot U)} = 3650$ and $\Delta \epsilon_{260(G \cdot C)} = 1700$ (Cox, 1970).

Depending on the solvent used, the explicit forms of eqn. (1) to give $\delta f_{(G \cdot C)}$ are:

$$\delta f_{(G \cdot C)} = \frac{1}{1 + \left[\left(0.79 \frac{\Delta A_{260}}{\Delta A_{280}} \right) - 0.46 \right]} \quad (1a) \text{ (solvent 10–100 mM-phosphate buffers, pH 7.0)}$$

and

$$\delta f_{(G \cdot C)} = \frac{1}{1 + \left[\left(0.84 \frac{\Delta A_{260}}{\Delta A_{280}} \right) - 0.46 \right]} \quad (1b) \text{ (solvent 4.0 M-guanidinium chloride/10 mM-phosphate buffer, pH 7.0)}$$

Calculation of N . The 'melting' temperature, T_m , of a bihelix is governed by the mole fraction, $f_{(G \cdot C)}$, of G·C base-pairs and by the length, N , of the bihelix, so that N can be calculated approximately provided that both $f_{(G \cdot C)}$ and T_m are known. The number of base-pairs in bihelical segments 'melting' independently over any temperature range ΔT was

calculated by the equation (Kallenbach, 1968; Cox, 1970):

$$N = \frac{1.5 \ln b + \ln \tau - 1.5 \ln 2}{\left(\frac{1}{T_m^\infty} - \frac{1}{T_m} \right) \cdot \frac{\Delta H}{R}} \quad (2)$$

where b is the number of unpaired residues within a loop, τ is the equilibrium constant for stacking two adjacent base-pairs on each other, taken as 100; $\Delta H(-33.5 \text{ kJ} \cdot \text{mol}^{-1})$ is the molar enthalpy, per mol of nucleotide pairs, for increasing the length of the helix by one base-pair: $R = 8.31 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$; T_m^∞ is the 'melting' temperature of a bihelix of infinite length having the same nucleotide composition as the bihelix under study, calculated by the equation (Cox, 1970):

$$\frac{1}{T_m^\infty} = (1 - f_{(G \cdot C)}) \cdot \frac{1}{T_{m(A \cdot U)}} + f_{(G \cdot C)} \cdot \frac{1}{T_{m(G \cdot C)}} \quad (3)$$

where $T_{m(A \cdot U)}$ and $T_{m(G \cdot C)}$ are 345 K and 376 K respectively (Cox, 1970). Values of N in eqn. (2) were calculated by allowing an error of ± 10 K in $T_m^\infty - T_m$.

Calculation of f_{dh} . The fraction, f_{dh} , of the total residues that are located in bihelical domains was calculated by the equation (Boetdker, 1967; Cox, 1970):

$$f_{dh} = \frac{(\epsilon_s - \epsilon)}{(\epsilon_s - \epsilon_{dh})} \quad (4) \text{ (solvent 10–100 mM-phosphate buffer, pH 7.0)}$$

where ϵ is the molar-residue absorption coefficient of the RNA at 25°C (i.e. the absorption coefficient of RNA per g-atom of RNA phosphorus), ϵ_s is the molar-residue absorption coefficient of a single-stranded but stacked conformation of the polynucleotide, estimated as described by Boetdker (1967) and Cox (1970); ϵ_{dh} is the calculated

molar-residue absorption coefficient of an entirely double-helical polynucleotide having the same base composition as the RNA under study, calculated by eqn. (7).

When the fraction of residues that are stacked in non-helical domains is small [solvent 4.0M-guanidinium chloride/10mM-phosphate buffer (pH 7.0) (Cox & Kanagalingam, 1967)], ϵ_s tends to ϵ_{am} , where ϵ_{am} is the molar-residues absorption coefficient of the entirely single-stranded, 'unstacked' conformation of the polynucleotide obtained on heating the RNA to 95°C; in this latter condition the fraction, f_{dh} , of the residues participating in secondary structure is calculated by the equation (Cox, 1970):

$$f_{dh} = \frac{(\epsilon_{am} - \epsilon)}{(\epsilon_{am} - \epsilon_{dh})} \quad (5) \text{ (solvent 4.0M-guanidinium chloride/10mM-phosphate buffer, pH 7.0)}$$

Molar-residue absorption coefficients of the partly double-helical and of the entirely amorphous forms of RNA (ϵ and ϵ_{am} respectively), were calculated from the A_{260} of the intact polynucleotide at 25°C (for ϵ) and at 95°C (for ϵ_{am}), and from the absorption, $A_{hydr.}$, of the polynucleotide after 12h incubation at 37°C in 0.3M-KOH:

$$\epsilon = \frac{A_{260}}{A_{hydr.}} \cdot \epsilon_{nucl.} \quad (6)$$

where $\epsilon_{nucl.}$ is the molar-residue absorption coefficient of the RNA computed from the molar absorption coefficients of the nucleotides in alkali and from the mole fractions of individual nucleotides in the RNA being investigated.

Values of ϵ_{dh} that are required in eqns. (4) and (5) were calculated by the equation (Cox, 1970):

$$\epsilon_{dh} = \epsilon_{am} - (\Delta\epsilon_{(G \cdot C)} \cdot f_{(G \cdot C)}) + (\Delta\epsilon_{(A \cdot U)} \cdot f_{(A \cdot U)}) \quad (7)$$

where $\Delta\epsilon_{(G \cdot C)}$ and $\Delta\epsilon_{(A \cdot U)}$ are the molar increments in absorption at 260nm owing to the 'melting' of A·U and G·C base-pairs (see eqn. 1).

Calculation of n_{dh} . The number, n_{dh} , of the independently 'melting' bihelical segments per molecule was calculated by the equation:

$$n_{dh} = \frac{f_{dh} \cdot n}{2N} \quad (8)$$

where n is the total number of base residues per molecule calculated from the molecular weight of the

RNA, and $2N$ is the average number of nucleotides in the independently 'melting' bihelical segments.

Results

Sedimentation behaviour of Octopus and Loligo rRNA

Zone-velocity centrifugation of Mg^{2+} -free RNA from the hepatopancreas of *Octopus vulgaris* and *Loligo vulgaris* in sucrose gradients made in high salt (100mM- Na^+) consistently provided a u.v.-absorption profile typical of other eukaryotic rRNA species. From a calibration curve (see the Experimental section), sedimentation coefficients of 31S

and 20S were calculated for the two major rRNA components of *Octopus* and *Loligo*; the s_{20} value of the S-rRNA of rat liver as estimated from the calibration was 17.7S, thereby suggesting the existence in the Cephalopoda of a S-rRNA somewhat larger in size than that of the mammals. A calibration error could be ruled out on the basis of the experiments in Fig. 1, in which the rRNA molecules of the Cephalopoda were centrifuged in the same gradient, mixed with those of *E. coli*, *Neurospora crassa* and rat liver in several possible combinations. As Fig. 1 shows, sedimentation analysis of the rRNA species in high-salt buffer was capable of resolving all of the larger, but none of the smaller, components of the reference rRNA species. The latter sedimented as a single boundary broader than that observed when each of the three marker rRNA species was centrifuged individually. However, the S-rRNA of *Octopus* sedimented at a considerably faster rate than those of both *Neurospora* and rat liver, very close to the L-rRNA ('23S') of *E. coli*. The L-rRNA sedimented at exactly the same position as the L-rRNA of the rodents used as the calibration reference. The sedimentation coefficients, s_{20} ($\pm$ S.E.M.; eight determinations), of the L-rRNA species of *E. coli*, *Neurospora crassa* and rat liver were, respectively, 23.5 ± 0.22 , 25.8 ± 0.02 and 30.4 ± 0.14 S; the s_{20} value of the unresolved S-rRNA band of the above species was 17.3 ± 0.11 S. The L-rRNA of *Octopus* and *Loligo* coincided with that of rat liver of 30.4S and the S-rRNA of the Cephalopoda had an average s_{20} of 20.3 ± 0.22 S, similar to that of the unicellular flagellate *Euglena gracilis* and of the dipterian species *Ceratitis capitata* (Rawson & Stutz, 1968; P. Cammarano, unpublished work).

Fig. 1. Sedimentation patterns of various combinations of *Octopus* rRNA with *Neurospora crassa* rRNA, *E. coli* rRNA and rat liver rRNA

The rRNA species (0.5 A_{260} unit of each) were centrifuged for 4.0h in a 5–40% (w/v) linear sucrose gradient (11.5 ml) in a Spinco SW 41 rotor operated at 20°C and 38000 rev./min. (a) *Octopus* rRNA mixed with *E. coli* rRNA. (b) *Octopus* rRNA mixed with *Neurospora crassa* and *E. coli* rRNA. (c) *E. coli* rRNA. (d) *Octopus* rRNA mixed with rat liver rRNA. (e) *Octopus* rRNA mixed with rat liver and *E. coli* rRNA. (f) Rat liver rRNA. Key: O, *Octopus* L-rRNA, o, *Octopus* S-rRNA; C, *E. coli* L-rRNA, c, *E. coli* S-rRNA; R, rat liver L-rRNA, r, rat liver S-rRNA; N, *Neurospora* L-rRNA; n, *Neurospora* S-rRNA.

When buffer E was used, all the rRNA species sedimented at a 15–20% slower rate than in the high-salt buffer; the overall amount of hypochromic change observed on heating the rRNA in buffer E was found to be no smaller than that obtained in high-salt buffer, the $A_{260}^{90^{\circ}\text{C}}/A_{260}^{25^{\circ}\text{C}}$ ratios of both *Octopus* and *Loligo* L-rRNA being 1.24–1.25 regardless of the solvent. From this, it is inferred that the lower *s* values of these RNA species in buffer E (containing 36 mM- NaH_2PO_4) reflect solely a change in asymmetry, as might result from increased repulsion between negatively charged phosphate groups, without accompanying loss of secondary-structure regions.

Gel-electrophoretic comparisons in buffer E

When the rRNA species of *Octopus* and *Loligo* were electrophoresed on gels made in buffer E, with *E. coli* and rat liver marker rRNA species, a different pattern was obtained (Fig. 2). The L-rRNA of the Cephalopoda still co-migrated with the L-rRNA of the rodents, but the S-rRNA had exactly the same gel-electrophoretic mobility as the '23S'

RNA of *E. coli*. As Fig. 2 shows, mixtures of *Octopus* and *E. coli* rRNA gave only three peaks of u.v. absorption instead of four, as seen in the sucrose-gradient patterns of these rRNA mixtures in high-salt buffer (Fig. 1): the '16S' of *E. coli*, the L-rRNA of the Cephalopoda and a third peak arising from the overlapping of the S-rRNA of the Cephalopoda with the '23S' RNA of the prokaryote.

The combined evidence obtained from both sedimentation-velocity and gel-electrophoretic comparisons (Fig. 1a and Fig. 2) indicates that: (i) the Cephalopoda have unusually large rRNA components, the L-rRNA being similar to, and the S-rRNA being larger than, the corresponding RNA components of mammalian ribosomes; (ii) these rRNA species have a higher frictional coefficient than those of *E. coli* and rat liver; therefore, relative to the *E. coli* reference rRNA species, the S-rRNA of the Cephalopoda moves less fast in the gels and sediments more slowly in the sucrose gradients than is accounted for by the molecular weight. This would render molecular-weight estimates higher than real

Fig. 2. Gel-electrophoretic fractionations of *Octopus vulgaris*, *E. coli* and rat liver rRNA species on 2.4% polyacrylamide gels in buffer E

(a) *Octopus* rRNA electrophoresed with *E. coli* unfractionated rRNA; (b) *Octopus* rRNA, *E. coli* rRNA and rat liver rRNA. Symbols have the same meaning as in Fig. 1. Arrows indicate peaks arising from the overlapping of *E. coli* L-rRNA with S-rRNA of Cephalopoda.

on the basis of the relative gel-electrophoretic mobility, but lower than real on the basis of the sedimentation rate in the sucrose gradients.

Thermal 'lability' of the L-rRNA in the Dibranchiata

When whole-cell RNA from *Octopus* or *Loligo* was incubated at 60°C for 200s and then subjected to electrophoresis in buffer E, the pattern shown in Fig. 3 was obtained; most of the L-rRNA dis-

Fig. 3. Effect of heating on the integrity of unfractionated *Octopus* rRNA

(a) Unheated *Octopus* rRNA. (b) *Octopus* RNA incubated for 200s at 60°C in buffer E. Gel electrophoresis was performed as described in the Experimental section.

appeared, with a concomitant increase of u.v. absorption in the zone of the gel where the S-rRNA migrated; additionally, a new sharply delineated band, moving faster than the S-rRNA, became apparent. This result suggested that: (i) most L-rRNA molecules have a latent scission at one specific point lying near the middle of the polynucleotide chain; (ii) conditions that disrupt hydrogen bonds also cause the dissociation of the L-rRNA into two components, one the size of the S-rRNA and the other smaller than the S-rRNA.

These suppositions are corroborated by the results in Fig. 4, showing the effect of heat-treatment on the molecular integrity of purified rRNA species of *Octopus* and *Loligo*. Samples of L-rRNA were incubated for 200s at several temperatures in buffer E containing 0.2% SDS, and the percentage fraction of L-rRNA molecules converted into thermal-dissociation products was quantified by gel electrophoresis in the same solvent. As the tracings in Fig. 4 show, gentle heating (38–50°C) resulted in the direct conversion of the L-rRNA into two molecular species which were released in approximately similar amounts, the amount of surviving L-rRNA molecules depending on temperature. Under similar conditions the S-rRNA (of both *Octopus* and *Loligo*) remained fully stable (result not shown). The temperature required to attain 50% dissociation of the L-rRNA species during a 200s heating period in buffer E (termed temperature of strand separation) was estimated to be 42–43°C for both species (Fig. 5). It should be noted that at all temperatures tested no equilibrium was reached during a 200s incubation period, the amount of intact L-rRNA molecules decreasing progressively with increasing length of heat-treatment (Londei *et al.*, 1977). Because of the non-equilibrium character of the thermal-dissociation kinetics, the temperature of strand separation, as defined above, depends primarily on the arbitrarily chosen length of exposure to heat and is not obviously related to the 'melting' temperature, T_m , of the base-paired region involved in keeping together the two pieces composing the L-rRNA.

The results of heating experiments also revealed that the L-rRNA species of *Octopus* and *Loligo* generate somewhat different dissociation products. When the purified L-rRNA molecules of the Octopoda and the Decapoda were dissociated by heat and then electrophoresed on parallel gels at the same time in the same tank, the larger fragments of the two species coincided in mobility, whereas the smaller fragment of *Loligo* L-rRNA had a slower mobility than that obtained from *Octopus* L-rRNA. This result indicated that the L-rRNA components of the Octopoda and the Decapoda, although not distinguishable in the native state, might have different molecular weights, the L-rRNA of *Loligo* being heavier than that of *Octopus*.

Fig. 4. Thermal-dissociation patterns of purified L-rRNA (*Loligo vulgaris*)
Samples (3–5 μ l) of purified L-rRNA (30–40 μ g) in buffer E containing 0.2% (w/v) SDS were added to solutions of buffer E (95–97 μ l) containing 0.2% SDS, preheated at the desired incubation temperature in thin-walled glass tubes; incubations were terminated after 200 s by chilling the tubes in crushed ice. Then 100 μ l samples containing 0.8–1.0 A_{260} unit were electrophoresed for 3 h on 2.4% acrylamide gels in buffer E at 5 mA/gel column (0.6 cm $\times$ 7.0 cm).

Fig. 5. Plots of dissociation of *Octopus* (a) and *Loligo* (b) L-rRNA versus temperature

The percentage fraction of L-rRNA dissociated at each temperature during a 200s heating period in buffer E was quantified from the u.v.-absorption tracings (e.g. Fig. 4) and plotted against temperature. The fraction of L-rRNA molecules dissociated on heating was calculated as the ratio of the dissociation products over the total of intact molecules plus dissociation products.

Gel electrophoresis under denaturing conditions

The u.v.-absorption profiles of *Loligo* and *Octopus* unfractionated rRNA run on 'formamide gels' reproduced almost exactly that of heat-denatured rRNA electrophoresed on 'aqueous gels' (see Fig. 3b). Figs. 6 and 7 illustrate experiments in which purified L-rRNA and S-rRNA species of *Octopus* (Fig. 6) and *Loligo* (Fig. 7) were analysed on 'formamide gels' both individually and in combination with the marker (*E. coli* and rat liver) rRNA species.

The purified S-rRNA of both species yielded only one symmetrically shaped narrow band, with a relative mobility indicating a mol.wt. ($\pm$ s.e.m., 10 determinations) of $0.96 (\pm 0.007) \times 10^6$. By contrast, the L-rRNA yielded two components present with an apparent mass ratio of approx. 1:1. The two components of *Octopus* L-rRNA had mol.wts. ($\pm$ s.e.m., 10 determinations) of $0.96 (\pm 0.006) \times 10^6$ and $0.65 (\pm 0.005) \times 10^6$, whereas those of *Loligo* had mol.wts. of $0.96 (\pm 0.005) \times 10^6$ and $0.88 (\pm 0.009) \times 10^6$ (8 determinations); the larger of the L-rRNA components of *Loligo* was therefore identical with that of *Octopus*, where the smaller one was about 0.25×10^6 daltons heavier than that of *Octopus*.

These results allow the following conclusions: (i)

the L-rRNA species of *Octopus* and *Loligo* have mol.wts. of 1.66×10^6 and 1.89×10^6 respectively; (ii) essentially all of the L-rRNA molecules have a hidden break positioned 0.96×10^6 daltons from one end of the L-rRNA chain; (iii) the S-rRNA of both species is an uninterrupted polynucleotide having exactly the same weight as the heavier of the two fragments composing the L-rRNA. This latter conclusion is also consistent with the observation that the two components of denatured rRNA are present with an apparent mass ratio of heavy to light species of approx. 2:1 in the gel-electrophoretic profiles of unfractionated rRNA (Fig. 3b), but appear with a ratio of about 1:1 in the gel patterns of purified L-rRNA (Figs. 6 and 7). Evidently, when the L-rRNA is denatured its constituent fragments are released in equimolar amounts, and the 'excess' 0.96×10^6 daltons component seen in the 'gel patterns' of denatured unfractionated rRNA arises from the overlapping of the heavier fragment of the L-rRNA with the intact S-rRNA.

These results show conclusively that *Octopus* ribosomes are composed of a large subunit with a 'mammalian-type' L-rRNA (mol.wt. 1.65×10^6) (Cammarano *et al.*, 1975) and a small subunit with a S-rRNA heavier than that of *Euglena* (mol.wt. 0.85×10^6 – 0.87×10^6) (Loening, 1968; Rawson & Stutz, 1968; Cammarano *et al.*, 1975).

Fig. 6. Electrophoresis of isolated rRNA components of *Octopus vulgaris* (hepatopancreas) in acrylamide gels in formamide

(a) *Octopus* L-rRNA electrophoresed individually; (b) *Octopus* L-rRNA run in combination with *E. coli* rRNA, rat liver rRNA and rat liver tRNA; (c) *Octopus* S-rRNA electrophoresed individually; (d) *Octopus* S-rRNA run in combination with tRNA, *E. coli* rRNA and rat liver rRNA. The *Octopus* L-rRNA and S-rRNA components in the electrophoretic patterns of the mixtures are indicated by their extrapolated mol.wts. ($\times 10^{-6}$); marker rRNA species are indicated by the symbols defined in Fig. 1. The absorption scale in panels (b) and (d) is as for (a) and (c). The straight line plots indicate the linear relationship between the logarithms of the molecular weights of the marker mRNA species and the distance migrated in the gel.

Fig. 7. Electrophoresis of isolated *Loligo* L-rRNA and S-rRNA components in acrylamide gels in formamide (a) *Loligo* L-rRNA run individually; (b) *Loligo* L-rRNA run mixed with *E. coli* rRNA, rat liver rRNA and tRNA; (c) *Loligo* S-rRNA run individually; (d) *Loligo* S-rRNA mixed with *E. coli* rRNA, rat liver rRNA and tRNA. *Loligo* L-rRNA and S-rRNA are indicated by their extrapolated mol.wts. ($\times 10^{-6}$). Reference RNA species are denoted by the symbols defined in Fig. 1. The absorbance scale in panels (b) and (d) is as for (a) and (c). The meaning of the straight-line plots is described in the legend to Fig. 6.

rRNA species of Gastropoda and Lamellibranchiata

When whole-cell rRNA of *Octopus* was fractionated on conventional gels (buffer E) mixed with rRNA from either *Murex trunculus* (Fig. 8a) or *Macrocallista chione* (Fig. 8b), both rRNA components of the two latter species migrated at a faster rate than the corresponding rRNA components of *Octopus*. The rRNA species of *Murex* and *Macrocallista* co-migrated on electrophoresis on the same gel, and exhibited identical apparent mol.wts. (L-rRNA, 1.40×10^6 ; S-rRNA 0.70×10^6) relative to marker *E. coli* rRNA species (Fig. 8c). Gel electrophoresis of purified L-rRNA components of *Murex* and *Macrocallista* in formamide as solvent led to the appearance of multiple u.v.-absorption bands (results not shown), presumably arising from

artificial breakage during the lengthy procedure required to isolate the hepatopancreas and to remove polysaccharides (see the Experimental section). By contrast, the S-rRNA of both species was fully stable and could be analysed in the 'formamide gels'. Relative to *E. coli* reference rRNA species, the S-rRNA of both the Gastropoda and the Lamellibranchiata had a mol.wt. ($\pm$ s.e.m., 6 determinations) of $0.65 (\pm 0.007) \times 10^6$ (result not shown). The result allow the conclusion that the molecular weight of the S-rRNA of the Lamellibranchiata and the Gastropoda is similar to that of the Deuterostomes, the green plants and most fungi (Cammarano *et al.*, 1975); the L-rRNA is probably similar to the 1.35×10^6 – 1.38×10^6 daltons L-rRNA of most lower vertebrates and the sea urchins (Cammarano *et al.*, 1975).

Table 1. Parameters required for the interpretation of the thermal-'melting' profiles and calculation of amount of secondary structure (f_{dh})

The nucleotide compositions (%) required to calculate ϵ values by eqn. (6) were obtained from Lava-Sanchez *et al.* (1972), as follows. *Octopus* L-rRNA: C = 30.8, A = 18.3, U = 17.0, G = 33.9. *Octopus* S-rRNA: C = 30.2, A = 18.3, U = 17.9, G = 33.6. *Loligo* L-rRNA: C = 29.8, A = 19.1, U = 19.6, G = 31.5. *Loligo* S-rRNA: C = 29.0, A = 18.9, U = 20.8, G = 31.3.

Parameter	<i>Octopus</i> L-rRNA		<i>Loligo</i> L-rRNA		
	10mm-phosphate	4 M-guanidinium chloride	10mm-phosphate	4 M-guanidinium chloride	
ϵ (25°C)	7400	8100	7100	8100	(eqn. 6, $T = 25^\circ\text{C}$)
ϵ_{am} (90°C)	9600	9600	9600	9600	(eqn. 6, $T = 90^\circ\text{C}$)
ϵ (25°C)	8400	—	8500	—	
ϵ_{dh}^0	7050	7200	7000	7150	(eqn. 7)
$\epsilon_{nucl.}$	10600	10600	10600	10600	(from base composition)

	<i>Octopus</i> S-rRNA		<i>Loligo</i> S-rRNA		
	10mm-phosphate	4 M-guanidinium chloride	10mm-phosphate	4 M-guanidinium chloride	
ϵ (25°C)	6900	7300	6700	7050	(eqn. 6)
ϵ_{am} (90°C)	8700	8700	9650	8650	(eqn. 6)
ϵ_s (25°C)	7900	—	8000	—	
ϵ_{dh}^0	6150	6300	6000	6150	(eqn. 7)
$\epsilon_{nucl.}$	10600	10600	10560	10560	(from base composition)

f_{dh}	<i>Octopus</i> L-rRNA		<i>Loligo</i> L-rRNA		
	10mm-phosphate*	4 M-guanidinium chloride†	10mm-phosphate	4 M-guanidinium chloride	
	0.74	0.63	0.66	0.67	

f_{dh}	<i>Octopus</i> S-rRNA		<i>Loligo</i> S-rRNA		
	10mm-phosphate	4 M-guanidinium chloride	10mm-phosphate	4 M-guanidinium chloride	
	0.57	0.60	0.65	0.64	

* From 10mm-phosphate buffer, pH 7.0, containing 1% (v/v) formaldehyde on cooling (eqn. 4).
† From spectra in 4 M-guanidinium chloride (eqn. 5).

Fig. 8. Gel-electrophoretic comparisons of unfractionated rRNA from *Murex trunculus*, *Macrocallista chione* and *Octopus vulgaris* in non-denaturing gels containing buffer E. (a) Fractionation of a mixture of *Octopus* rRNA and *Murex* rRNA mixed with *E. coli* rRNA. Inset shows a semilog plot of molecular weight versus mobility; (b) *Macrocallista* L-rRNA and S-rRNA mixed with *E. coli* rRNA; (c) *Macrocallista* L-rRNA and S-rRNA mixed with *E. coli* rRNA. Electrophoresis on 2.4% acrylamide gels in buffer E was performed as described in the Experimental section. Key: O, o, *Octopus* L-rRNA and S-rRNA; Mu, mu, *Murex trunculus* L-rRNA and S-rRNA; Ma, ma, *Macrocallista chione* L-rRNA and S-rRNA.

Both rRNA components of the Cephalopoda are considerably richer in GMP and CMP ($G + C = 63\text{--}65\%$) than those of both the Gastropoda and the Lamellibranchiata ($G + C = 55\text{--}58\%$), and very similar to the large-sized L-rRNA of the mammals, with 67% $G + C$ (see Lava-Sanchez *et al.*, 1972); the

latter has been shown to contain tracts of bihelical secondary structure that are extremely rich in GMP and CMP ($G + C$ approx. 78%) (Cox, 1970). For this reason the principal features of the bihelical secondary structure of the 'giant' rRNA species of the Cephalopoda were investigated in detail.

Fig. 9. Thermal-'melting' profiles of *Octopus* L-rRNA and S-rRNA species in 100mM-phosphate buffer and in 4.0M-guanidinium chloride

(a) *Octopus* L-rRNA in 100mM-phosphate buffer (pH 7.0); (b) *Octopus* L-rRNA in 4.0M-guanidinium chloride/10mM-phosphate buffer (pH 7.0); (c) S-rRNA in 100mM-phosphate buffer (pH 7.0); (d) S-rRNA in 4.0M-guanidinium chloride/10mM-phosphate buffer (pH 7.0). ●, ΔA_{260} ; ○, ΔA_{280} . ΔA values are normalized to $A_{260} = 1$ at 25°C.

Total helix content of rRNA

The numerical values of the parameters that are required to calculate the fraction, f_{dh} , of nucleotide residues that participate in bihelical secondary structure (eqns. 3 and 4) are listed in Table 1. Molar residue absorption coefficients of the rRNA species investigated were determined in sodium phosphate buffer (pH 7.0) and 4.0M-guanidinium chloride/10mM-sodium phosphate buffer (pH 7.0).

As Table 1 shows, in 4.0M-guanidinium chloride the ϵ values (25°C) are generally higher than in phosphate buffer, as expected if in the former solvent the hypochromism arising from the fraction, f_{ss} , of residues that are stacked in single-stranded parts is largely diminished at room temperature (Cox & Kanagalingam, 1967; Cox, 1970). Thus, when 4.0M-guanidinium chloride is used, changes in absorption on heating are mostly accounted for by the 'unstacking' of bases that are located in bihelical portions of rRNA; since f_{ss} is small, ϵ_s tends to ϵ_{am} and eqn. (5) could be used to calculate f_{dh} (Cox, 1970).

When phosphate buffer is used as the solvent, the disruption of base-stacking in non-helical parts of the molecule makes a significant contribution to the overall amount of hypochromic change, thus leading to overestimated values of f_{dh} ; i.e. $\Delta\epsilon_{260}$ arises from the sum of the fractions $f_{dh} + f_{ss}$ of the residues that unstack on heating in both bihelical and non-helical parts. Since $\epsilon_s < \epsilon_{am}$, eqn. (4) was used for estimating f_{dh} (Boetdker, 1967; Cox, 1970).

Values of ϵ_s that are required for calculating f_{dh} by eqn. (4) were determined by the treatment of Boetdker (1967) and Cox (1970), by measuring the hypochromism attained when the formaldehyde-treated rRNA molecules were cooled from 90°C to 25°C. For this purpose, purified L-rRNA and S-rRNA species were heated to 90°C in 10mM-phosphate buffer (pH 7.0), containing 1% (v/v) formaldehyde in order to complete hydroxymethylation of the bases and thus prevent the re-formation of base-pairing on cooling. When the formaldehyde-treated RNA molecules were cooled again to 25°C, part of the original hypochromism was resumed owing to the restoration of stacking interactions alone.

Values of f_{dh} calculated by eqn. (4) (solvent 10mM-phosphate buffer pH 7.0) and by eqn. (5) (solvent 4.0M-guanidinium chloride/10mM-phosphate buffer, pH 7.0) were basically similar (Table 1). In both *Octopus* and *Loligo* a fraction, $f_{dh} = 0.65$ – 0.75 , of the residues of L-rRNA and a fraction, 0.58 – 0.65 , of the residues of S-rRNA appear to be involved in the formation of bihelical structures

Base composition of bihelical and single-stranded regions

Thermal-'melting' spectra of the rRNA species in

phosphate buffer and in 4.0M-guanidinium chloride are illustrated in Figs. 9 and 10. The main parameters of the 'melting' curves (T_m and inter-quartile range of the transition) are listed in Table 2. The rRNA molecules display similar 'melting' properties, except that the breadth of the 'melting' range of *Loligo* L-rRNA was 5°C smaller than that of *Octopus* L-rRNA, indicating a greater homogeneity of the secondary structure regions in the former species.

The average G + C content, $f_{(G+C)}$, of the bihelical species 'melted' after heating the rRNA at 95°C was deduced from the ratios of the increments in A_{260} (reflecting the denaturation of both A·U and G·C base-pairs) and A_{280} (reflecting the denaturation of G·C base-pairs only) (eqns. 1a and 1b). As Table 2 shows, the ratios $\Delta A_{260}/\Delta A_{280}$ obtained in 4.0M-guanidinium chloride were consistently smaller than those obtained in phosphate buffers, owing chiefly to a smaller amount of hypochromic change at 260 nm (as indicated in Figs. 9 and 10). The mole fractions, $f_{(G+C)}$, of GMP and CMP residues in bihelical regions reflected closely the overall G + C content, $f_{(G+C)}$, of the rRNA species when phosphate buffers were used, but were marked higher than the average G + C content [$f_{(G+C)}/f_{(G+C)} = 1.15$] when guanidinium chloride was used as the solvent.

This divergence in the results obtained with two solvents is readily explained in terms of contribution to ΔA_{260} arising from bases that are stacked in single-stranded regions: in phosphate buffers the 'non-helical' nucleotides make a significant contribution to changes in absorption on heating, so that the ratios $\Delta A_{260}/\Delta A_{280}$ approach the values predicted from the average base composition; in 4.0M-guanidinium chloride $f_{ss} \rightarrow 0$, and thus the ratios of the hypochromicities reflect principally the base composition of the fraction, f_{dh} , of the residues that 'unstack' in helical domains.

The results obtained in guanidinium chloride therefore reveal that particular bases are unevenly distributed between the double-helical and the single-stranded parts of the rRNA chains, GMP and CMP residues being preferentially concentrated in bihelical domains. The approximate distribution of individual bases in helical and single-stranded parts of the rRNA species investigated was then calculated from the number, n_{dh} , of residues in helical regions and from the mole fractions, $f_{(G+C)}$ and $1 - f_{(G+C)}$, of G·C and A·U base-pairs, respectively, neglecting imperfections and G·U base-pairings. The results (Table 3) indicate that single-stranded regions, which constitute only approximately one-third of the L-rRNA mass, include about 50% of all adenine residues and 50% of the uridine residues, but less than 20% of the cytosine residues. Similar evidence for clustering of GMP and CMP in bihelical domains is also observed in the S-rRNA,

Fig. 10. Thermal-'melting' spectra of *Loligo* L-RNA and S-rRNA in 100mM-phosphate buffer and in 4.0M-guanidinium chloride

(a) *Loligo* L-rRNA in 100mM-phosphate buffer (pH 7.0); (b) *Loligo* L-rRNA in 4.0M-guanidinium chloride/10mM-phosphate buffer (pH 7.0); (c) S-rRNA in 100mM-phosphate buffer (pH 7.0); (d) S-rRNA in 4.0M-guanidinium chloride/10mM-phosphate buffer (pH 7.0). $\bullet$, ΔA_{260} ; $\circ$, ΔA_{280} . ΔA values are normalized to $A_{260} = 1$ at 25°C .

although the phenomenon is less pronounced than for L-rRNA. Overall, bihelical regions of *Octopus* and *Loligo* L-rRNA are estimated to contain 75% and 73% G·C base-pairs respectively; helical regions of S-rRNA contain 72% G·C base-pairs (*Octopus*) and 65% G·C base-pairs (*Loligo*).

Heterogeneity of bihelical regions

If the distribution of nucleotide sequences in bihelical regions were near to random, the ratios $\Delta A_{260}/\Delta A_{280}$ would be predicted to be the same over the whole 'melting' range; deviations from this

Table 2. Main parameters of the thermal-'melting' curves of purified rRNA components of *Octopus* and *Loligo* in different solvents

IQR is the interquartile range of the 'melting' transition, i.e. the temperature range over which ϵ_{260} changes from 25 to 75% of the total increment. The ratio $f_{(G\cdot C)}/f_{(G+C)}$ indicates the extent to which the average G·C content of the double-helical parts differs from the overall G + C content ($f_{(G+C)}$) estimated by chemical methods. $f_{(G+C)}$ values of *Octopus* and *Loligo* L-rRNA species are 0.647 and 0.613 respectively; $f_{(G+C)}$ values of *Octopus* and *Loligo* S-rRNA species are 0.638 and 0.603 respectively.

Solvent	Octopus L-rRNA					Octopus S-rRNA				
	IQR	T_m (°C)	$\Delta A_{260}/\Delta A_{280}$	$f_{(G\cdot C)}$	$f_{(G\cdot C)}/f_{(G+C)}$	IQR	T_m (°C)	$\Delta A_{260}/\Delta A_{280}$	$f_{(G\cdot C)}$	$f_{(G\cdot C)}/f_{(G+C)}$
10mM-phosphate	24.0	53	1.05	0.66	1.02	25	56	1.31	0.615	0.965
100mM-phosphate	21.0	64.5	1.23	0.64	0.99	25	71.5	1.23	0.64	1.00
4 M-guanidinium/ 10mM-phosphate	24.0	61.0	0.86	0.75	1.15	24.5	61	0.91	0.72	1.13

Solvent	Loligo L-rRNA					Loligo S-rRNA				
	IQR	T_m (°C)	$\Delta A_{260}/\Delta A_{280}$	$f_{(G\cdot C)}$	$f_{(G\cdot C)}/f_{(G+C)}$	IQR	T_m (°C)	$\Delta A_{260}/\Delta A_{280}$	$f_{(G\cdot C)}$	$f_{(G\cdot C)}/f_{(G+C)}$
10mM-phosphate	14.5	50	1.19	0.65	0.90	21	56.5	1.40	0.59	0.99
100mM-phosphate	17.0	62.5	1.23	0.63	1.03	22.5	68.0	1.42	0.59	0.99
4 M-guanidinium/ 10mM-phosphate	19.0	60.0	0.93	0.71	1.19	23.0	63.0	1.12	0.64	1.07

Table 3. Estimated distribution of bases between non-helical and helical parts of *Octopus vulgaris* and *Loligo vulgaris* rRNA species

Numbers of helical residues/molecule are calculated from the total number of residues n and the nucleotide mole fractions reported by Lava-Sanchez *et al.* (1972) (see Table 1). n is calculated by assuming 330 as the average molecular weight of a nucleotide. Numbers of non-helical residues/molecule are calculated as $n_{A(U)}^{hel} = n \cdot f_{dh} \cdot \frac{1}{2}(1 - f_{(G\cdot C)})/2$ and $n_{G(C)}^{hel} = n \cdot f_{dh} \cdot f_{(G\cdot C)}/2$; values of $f_{(G\cdot C)}$ and f_{dh} are taken from Tables 2 and 1 respectively. The following values were used for the calculations: *Octopus* L-rRNA, $n = 5000$, $f_{dh} = 0.68$; *Loligo* L-rRNA, $n = 5730$, $f_{dh} = 0.66$; *Octopus* S-rRNA, $n = 2910$, $f_{dh} = 0.58$; *Loligo* S-rRNA, $n = 2910$, $f_{dh} = 0.64$.

	Octopus L-rRNA				G + C mole fraction	Loligo L-rRNA				G + C mole fraction
	A	U	G	C		A	U	G	C	
Residues/molecule	915	850	1695	1540	(0.647)	1094	1123	1805	1707	(0.613)
Helical residues/ molecule	425	425	1275	1275	(0.755)	511	511	1380	1380	(0.733)
Non-helical residues/ molecule	490	425	420	265	(0.429)	583	612	425	327	(0.386)

	Octopus S-rRNA				G + C mole fraction	Loligo S-rRNA				G + C mole fraction
	A	U	G	C		A	U	G	C	
Residues/molecule	532	521	978	879	(0.638)	550	605	911	845	(0.603)
Helical residues/ molecules	236	236	608	608	(0.720)	326	326	605	605	(0.650)
Non-helical residues/ molecule	296	285	370	271	(0.525)	224	279	306	240	(0.520)

predicted behaviour indicate clusters of either G·C or A·U base-pairs (Cox *et al.*, 1976). The principal feature of the thermal-'melting' spectra of the L-rRNA in Fig. 10 (solvent 4.0M-guanidinium chloride) is the evidence that $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ is much

larger in the lower phase of the 'melting' transition than in the upper range; for instance, for *Octopus* L-rRNA $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ was 1.35 over the range 25–50°C, but 0.70 above 50°C. Biphasic 'melting' profiles such as those of *Octopus* indicate the

Table 4. Properties of *Octopus* and *Loligo* L-rRNA species deduced from spectrophotometric data (solvent 4.0M-guanidinium chloride/10 mM-sodium phosphate buffer, pH 7.0)

Values of Δn_{dh} are based on mean values of N , $b = 20$. $f_{(G \cdot C)} = \Sigma (\Delta f_{(G \cdot C)} \times \Delta f_{dh}) / f_{dh}$. RNA mass (daltons) = $(\Delta f_{dh} / f_{dh}) \cdot (\text{mol.wt.})$.

Octopus L-RNA									
'Melting' range (°C)	T_m (K)	G·C content (%)	$10^{-3} \times \Delta(1/T)$	$N \pm \Delta N$		Δf_{dh}	Δn_{dh}	RNA mass (daltons)	Average $f_{(G \cdot C)}$ and N
				$b = 3$	$b = 20$				
20-30	298	58.3	0.601	2	3	0.024	20	0.48×10^6	$\bar{f}_{(G \cdot C)} = 0.60$, $\bar{N} = 3-5$
30-40	308	59.5	0.496	3	4	0.064	40		
40-50	318	60.2	0.395	3	5 ± 1	0.094	47		
50-60	328	76.8	0.341	4 ± 1	6 ± 1	0.132	45	1.17×10^6	$\bar{f}_{(G \cdot C)} = 0.88$, $\bar{N} = 6-12$
60-70	338	86.8	0.276	5 ± 1	8 ± 2	0.145	55		
70-90	353	97.1	0.176	8 ± 3	12 ± 5	0.166	34		
$\bar{f}_{(G \cdot C)} = 0.74$				$f_{dh} = 0.625$			$n_{dh} = 241$		
Loligo L-rRNA									
'Melting' range (°C)	T_m (K)	G·C content (%)	$10^{-3} \times \Delta(1/T)$	$N \pm \Delta N$		Δf_{dh}	Δn_{dh}	RNA mass (daltons)	Average $f_{(G \cdot C)}$ and N
				$b = 3$	$b = 20$				
20-30	298	72.4	0.637	2	3	0.020	19	0.86×10^6	$\bar{f}_{(G \cdot C)} = 0.68$, $\bar{N} = 3-7$
30-40	308	68.9	0.519	3	4 ± 1	0.043	31		
40-50	318	69.1	0.417	3 ± 1	5 ± 1	0.089	51		
50-60	328	66.5	0.315	4 ± 1	7 ± 2	0.155	63	1.03×10^6	$\bar{f}_{(G \cdot C)} = 0.80$, $\bar{N} = 8-17$
60-70	338	79.7	0.258	5 ± 2	8 ± 3	0.189	68		
70-90	353	80.0	0.133	10 ± 6	17 ± 9	0.175	29		
$\bar{f}_{(G \cdot C)} = 0.74$				$f_{dh} = 0.671$			$n_{dh} = 261$		

existence in the secondary structure of bihelical regions having largely different contents of G·C base-pairs.

The degree of heterogeneity of the secondary-structure regions of the rRNA species investigated is analysed in detail in Tables 4 and 5, listing the main parameters (G + C content and length) of the bihelical segments 'melting' independently over 10°C temperature intervals. The mole fractions, $f_{(G \cdot C)}$, of G·C base-pairs, and the number, N , of base pairs of the 'melting' species were deduced from the spectra in 4.0M-guanidinium hydrochloride by respectively eqn. (1b) (from $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ over the interval ΔT), and by eqn. (2) (from $\delta f_{(G \cdot C)}$ and the T_m of the partial transition).

Octopus L-rRNA. Table 4 shows that the G·C content of the independently 'melting' bihelical segments of *Octopus* L-rRNA ranges from 58% G·C base-pairs to approx. 95% G·C base-pairs, and the helix length varies from 3–5 base-pairs to no more than 12 base-pairs (limiting values are for $b = 3$ and $b = 20$), there being approx. 240 independently 'melting' species per molecule. Longer segments of about 8–12 base-pairs are also richer in G·C than the shorter segments [i.e. no long (A·U)-rich species appear to be present].

To generalize, the results reveal that the secondary structure of *Octopus* L-rRNA is composed of two major fractions of helical segments having largely different thermal stability: (i) a fraction, approx. 30% of the total residues, consisting of short (G·C)-poorer segments ($f_{(G \cdot C)} = 0.60$, $N = 5$) that 'melts' over the range 25–50°C, and (ii) a fraction, approx. 70% of the residues, consisting of longer (G·C)-rich segments ($f_{(G \cdot C)} = 0.88$, $5 \leq N \leq 10$) 'melting' above 50°C. Short segments of about 60% G·C base-pairs constitute about 0.50×10^6 daltons mass, whereas longer segments of approx. 88% G·C base-pairs are included in 1.10×10^6 daltons mass.

Loligo L-rRNA. The mole fraction, $f_{(G \cdot C)}$, of G·C base-pairs and the average length, N , of the double-helical segments 'melting' to an amorphous form over 10°C temperature intervals are listed in Table 4. In general, the base composition of the bihelical fraction of *Loligo* L-rRNA appears to be more homogeneous ($f_{(G \cdot C)}$ ranges from 0.65 to 0.77) than that of *Octopus* L-rRNA, as expected from the smaller interquartile range of the 'melting' transition in all solvents used (see Table 3). The average length of the bihelical segments appears to lie in the range 3–13 base-pairs; as *Octopus* L-rRNA, the shorter segments are also poorer in GMP and CMP residues

Table 5. Properties of *Octopus* and *Loligo* S-rRNA species deduced from spectrophotometric data (solvent 4.0M-guanidinium chloride/10 mM-sodium phosphates buffer, pH 7.0)Values of Δn_{dh} are based on mean values of N , $b = 20$. $\bar{f}_{(G \cdot C)} = \Sigma (\Delta f_{(G \cdot C)} \times \Delta f_{dh}) / f_{dh}$.

Octopus S-rRNA							
'Melting' range (°C)	T_m (K)	G·C content (%)	$10^{-3} \times \Delta(1/T)$	$N \pm \Delta N$		Δf_{dh}	Δn_{dh}
				$b = 3$	$b = 20$		
20-40	303	67	0.568	2	4	0.060	22
40-50	318	69	0.416	3 ± 1	5 ± 1	0.088	26
50-60	328	71	0.327	4 ± 1	6 ± 2	0.131	32
60-70	338	74	0.242	6 ± 2	9 ± 3	0.131	21
70-80	348	81	0.175	8 ± 3	12 ± 5	0.137	17
80-90	358	98	0.137	10 ± 5	15 ± 8	0.058	6
$\bar{f}_{(G \cdot C)} = 75.2$						$f_{dh} = 0.61$	$n_{dh} = 124$
Loligo S-rRNA							
'Melting' range (°C)	T_m (K)	G·C content (%)	$10^{-3} \times \Delta(1/T)$	$N \pm \Delta N$		Δf_{dh}	Δn_{dh}
				$b = 3$	$b = 20$		
30-40	308	52	0.476	3	4 ± 1	0.028	10
40-50	318	65	0.408	3 ± 1	5 ± 1	0.113	33
50-60	328	61	0.302	5 ± 1	7 ± 2	0.136	28
60-70	338	76	0.248	6 ± 2	8 ± 3	0.138	25
70-80	348	69	0.147	9 ± 5	14 ± 8	0.209	22
$\bar{f}_{(G \cdot C)} = 69$						$\bar{f}_{dh} = 0.624$	$n_{dh} = 118$

than longer segments. There are about 260 independently 'melting' species per molecule, as expected from the larger size of *Loligo* L-rRNA. To generalize, the results indicate the existence of two main fractions of double-helical segments; (i) a fraction, approx. 45% of the total residues, consisting of shorter (G + C)-poorer segments ($\bar{f}_{(G \cdot C)} = 0.68$, $3 \leq N \leq 7$) that 'melts' over the range 20–60°C, and (ii) a fraction, approx. 55% of the residues, consisting of longer (G + C)-richer segments ($\bar{f}_{(G \cdot C)} = 0.80$, $7 \leq N \leq 17$) that 'melts' above 60°C. Shorter segments of about 68% G·C constitute approx. 0.85×10^6 daltons mass, whereas longer segments of approx. 80% G·C base-pairs are included in about 1×10^6 daltons mass.

Octopus and *Loligo* S-rRNA. Table 5 lists the secondary-structure parameters of *Octopus* and *Loligo* S-rRNA, i.e. length, G·C content and number of bihelical segments 'melting' independently over 10°C temperature intervals. The data indicate that the secondary structure of the S-rRNA is less heterogeneous than that of the L-rRNA, the values of $\bar{f}_{(G \cdot C)}$ averaging 65% (*Loligo*) and 70% (*Octopus*) over the whole 'melting' range; a sharp division into long (G·C)-rich and short (G·C)-poor bihelical segments is not apparent. However, by comparison with the L-rRNA, the S-rRNA of both

Octopus and *Loligo* appears to contain a larger proportions of shorter (4–6 base-pairs) helical segments; these constitute about 50% of the total helical segments of the S-rRNA, but only 25–30% of those of the L-rRNA. It is calculated that *Octopus* and *Loligo* S-rRNA species contain about 120 independently 'melting' bihelical segments per molecule.

Discussion

Giant size of the rRNA species in the Dibranchiata

The two rRNA components of such primitive animals as the Cephalopoda are the largest mature rRNA molecules detected until now; the L-rRNA is similar to (*Octopus*) or larger than (*Loligo*) that of the mammals; the S-rRNA is even larger than that of some unicellular eukaryotes that have unusually large S-rRNA, such as *Acanthamoeba castellanii* and *Euglena gracilis* (mol.wt. 0.83×10^6 – 0.88×10^6) (Loening, 1968; Stevens & Pachler, 1972; Cammarano *et al.*, 1975) and almost coincident with the '23S' RNA of the prokaryotes.

The occurrence in the eukaryotes of a S-rRNA of mol.wt. approx. 1×10^6 is not unexpected; in most animal and plant species the maturation of the '18S' RNA (mol.wt. 0.65×10^6) entails the loss of

0.30×10^6 – 0.35×10^6 daltons 'excess' polynucleotide from a '20S' precursor molecule of approx. 1×10^6 daltons (see Loening *et al.*, 1972); S-rRNA of 1×10^6 daltons mass therefore may have arisen by the omission of the final trimming steps in processing, so that the whole '20S' RNA became incorporated in the mature ribosome.

Of greater evolutionary significance is the evidence that the two principal rRNA species of the much evolved Cephalopoda are considerably larger than those of less evolved molluscs such as Gastropoda and Lamellibranchiata, the divergence in rRNA mass between squid and clams (or snails) being more extensive than that existing between the mammals and the Urochordata (Cammarano *et al.*, 1975). This result provides unequivocal evidence that increases in rRNA mass similar to, or greater than, that which occurred in the evolution of the more recently advanced Deuterostomes may have been experienced along the evolution of highly primitive animal phyla. In fact, a strong divergence in rRNA molecular weight is also found between the much evolved Diptera and other insects or lower crustaceans (Cammarano *et al.*, 1975). It is noteworthy that the most striking increases in rRNA mass have been incurred by species that evolved towards very high G+C content, such as the warm-blooded animals, or unusually low G+C content, such as the Diptera (Lava-Sanchez *et al.*, 1972). The results in the present paper suggest that the evolution of the 'giant' rRNA species in the mollusca may have arisen by the 'addition' of extremely (G+C)-rich RNA tracts to a smaller 'evolutionary core' of approx. 55% G+C (Lava-Sanchez *et al.*, 1972; Cox *et al.*, 1973).

Integrity of the rRNA chains

To our knowledge, this is the first report to show the occurrence of a discontinuous L-rRNA species in animals other than insects. As in the insects (see Shine & Dalgarno, 1973), nearly all of the L-rRNA molecules of the Cephalopoda appear to consist of two principal pieces held together by region(s) of hydrogen-bonding having a similar thermal stability in the Octopoda and in the Decapoda. By contrast, the S-rRNA is a heat-stable chain with exactly the same size as the heavier of the two segments composing the L-rRNA.

There are two possible explanations for the existence of a chain break in the L-rRNA. Firstly, the L-rRNA could consist of two sections held together by hydrogen-bonds *in vivo*. Alternatively, one might argue that during RNA extraction and handling the L-rRNA undergoes specific enzymic attack at a considerably higher frequency than the S-rRNA, but the hydrogen-bonds involved in secondary structure conceal the existence of the break. No definite argument exists to exclude this

latter possibility; however, the first alternative is favoured in the light of data showing that in some dipterian species cleavage of the L-rRNA into two main sections results from late 'processing' events occurring no earlier than 3 h after ribosome assembly, the newly synthesized RNA being a truly continuous, covalently linked polynucleotide chain (Lava-Sanchez & Puppo, 1975).

The principal secondary-structure features (base composition and length) of the bihelical region(s) involved in stabilizing the 'nicked' L-rRNA of the Cephalopoda could be deduced from the analysis of the thermal-'melting' spectra of the L-rRNA, provided that the 'melting' temperature of the 'stabilizing helix' were known approximately. We have shown elsewhere (Londei *et al.*, 1977) that in buffer E the 'melting' temperature of the bihelical region(s) holding together the two segments of discontinuous L-rRNA species is almost coincident with the temperature of strand separation, T'_m , when a 200 s period of heat-treatment is chosen as the standard condition for determining this latter parameter. The temperature of strand separation, T'_m (200s), of *Octopus* and *Loligo* L-rRNA has been found to be 42–43°C (Fig. 5), well below the 'melting' temperature, T_m , of these L-rRNA species in buffer E ($T_m = 58^\circ\text{C}$). From the thermal-'melting' spectra of the L-rRNA in buffer E, bihelical segments 'melting' over the range 35–45°C were estimated to be no longer than 5 base-pairs and to contain approx. 60% G·C base-pairs (P. Londei, unpublished work). Therefore the nicked L-rRNA of these species is stabilized either by one or by multiple such short bihelical segments having a G+C content generally lower than the average G+C content of the bihelical portion of the L-rRNA.

Possible origin of the giant-sized rRNA species

Both the giant size of the S-rRNA and the different sizes of the L-rRNA in the Octopoda and the Decapoda may be explained by a processing scheme that is in simple accord with known facts about processing pathways and general arrangement of gene and transcribed spacer regions of rDNA (i.e. DNA coding for rRNA) (see review by Rungger & Crippa, 1977). According to the scheme (Fig. 11), the rRNA components of the Dibranchiata arose in evolution by the omission of cleavage steps in 'processing', so that the transcripts of both the internal and the external spacer regions of a rDNA repeat unit, or the transcript of the external spacer region only, became conserved in the mature ribosome.

The pre-rRNA (precursor to the mature L-rRNA and S-rRNA) of both *Octopus* and *Loligo* is assumed to be 2.90×10^6 daltons in mass, similar to that of *Xenopus laevis* and of the most lower species (Rungger & Crippa, 1977) and to contain, in the

Fig. 11. Proposed processing pathway of pre-rRNA in (a) *Octopus* and (b) *Loligo*

The following abbreviations are used; pre-rRNA, primary precursor to rRNA; rDNA, ribosomal DNA repeat unit; Int.Sp., internal spacer region of rDNA; Ext.Sp., external spacer region of rDNA; NT, non-transcribed region of rDNA; Te.Sp., transcribed portion of external spacer region of pre-rRNA; Ti.Sp., transcribed internal spacer. The 0.65×10^6 daltons segment of pre-rRNA is assumed to be identical with the '18S' cistron of most eukaryotic organisms. F₁ and F₂ are the fragments of L-rRNA obtained on denaturation. RNA mass is indicated by daltons ($\times 10^{-6}$). Vertical arrows indicate cleavage sites.

5'–3' direction, (a) a segment of approx. 0.35×10^6 daltons corresponding to the transcribed portion of the external spacer region, (b) a 0.65×10^6 daltons stretch identical with the mature '18S' RNA of the lower molluscs, the Deuterostomes, the green plants and many fungi (see Cammarano *et al.*, 1975), (c) a segment of approx. 0.25×10^6 daltons corresponding to the internal spacer region transcript, and (d) a 1.65×10^6 daltons segment identical with the mature L-rRNA species of *Octopus*.

During 'processing' the pre-rRNA of the Decapoda would be cleaved at only one site in the middle; the transcripts of the external and internal spacer regions would be conserved in the S-rRNA and the L-rRNA respectively, thus generating mature molecules with mol.wts. of 1×10^6 (S-rRNA) and 1.90×10^6 (L-rRNA). The pre-rRNA of the Octopoda would be cleaved at two sites in the middle, as indicated; the external-spacer-region transcript would still be conserved in the S-rRNA, yielding again a molecule of approx. 1×10^6 daltons, but the internal-spacer-region transcript would be cleaved off, thus leaving aside a mature 1.65×10^6 daltons L-rRNA species.

Nicking of the L-rRNA species at a distance of 3000 nucleotides from the terminus opposite to the internal spacer transcript would then generate the 1×10^6 and 0.90×10^6 daltons fragments of *Loligo*, or the 1×10^6 and 0.65×10^6 daltons fragments of *Octopus*. In the proposed processing the conserved sequences corresponding to the external-spacer transcript are assumed to be accommodated at the 5'-end of the S-rRNA, thus leaving the 3'-end free to terminate with a pyrimidine-rich cluster involved in mRNA recognition (Shine & Dalgarno, 1975; Dasgupta *et al.*, 1975; Van de Voorde *et al.*, 1976; Legon, 1976; Baralle, 1977).

Secondary-structure features of rRNA

The following properties of the double-helical fraction of the rRNA of the Dibranchiata have been established: (i) a fraction, approx. 70%, of the residues of the L-rRNA, and a fraction, approx. 60%, of the residues of the S-rRNA, participate in secondary-structure formation; (ii) each of the bases is unevenly distributed between the non-helical and the bihelical portions of the rRNA chains, GMP and CMP residues being preferentially concentrated in bihelical domains to the extent that approx. 50% of the adenines and 50% of the uridines are clustered in single-stranded parts which constitute no more than one-third of the L-rRNA molecule; (iii) independently 'melting' bihelical segments appear to be no longer than 13 base-pairs; and (iv) the shapes of the 'melting' curves of the L-rRNA are distinctly biphasic, indicating the existence of two discrete populations of bihelical segments having largely different thermal stabilities, namely a fraction consisting of short (G + C)-poorer segments and a fraction consisting of longer (G + C)-richer segments. The second phase of the 'melting' transitions of the L-rRNA species, arising from the denaturation of the long (G + C)-rich segments, is not present in the 'melting' curves of the prokaryotic rRNA molecules and appears to constitute a distinctive evolutionary feature of the eukaryotes (Cox, 1970; Cox *et al.*, 1973). No evidence of the biphasicity has been observed with S-rRNA.

The validity of using thermal 'melting' in 4.0M-guanidinium chloride to analyse the secondary structure of partly double-helical molecules such as rRNA has been demonstrated by using purified tRNA species (Cammarano *et al.*, 1977). The possibility exists that guanidinium chloride at 4.0M

concentration leads to the disruption of short (A·U)-rich regions of base-pairing. However, this is contradicted by the finding that when the nicked L-rRNA of *Tetrahymena pyriformis*, which dissociates completely into two distinct fragments after a 200 s incubation at 37°C (in buffer E) (F. Mazzei, unpublished work), was dissolved in 4.0 M-guanidinium chloride/10 mM-phosphate buffer (pH 7.0), and then subjected to gel electrophoresis in buffer E, all the L-rRNA molecules remained intact, indicating that A·U-rich regions and/or short regions of base-pairing are not disrupted in this solvent.

References

- Baralle, F. E. (1977) *Cell* **10**, 549–558
- Boetdker, H. (1967) *Biochemistry* **6**, 2718–2727
- Brungaber, E. (1962) *Biochim. Biophys. Res. Commun.* **8**, 1–4
- Cammarano, P., Felsani, A., Romeo, A. & Alberghina, F. M. (1973) *Biochim. Biophys. Acta* **308**, 404–411
- Cammarano, P., Pons, S. & Londei, P. (1975) *Acta Biol. Med. Ger.* **34**, 1123–1135
- Cammarano, P., Mazzei, F., Londei, P., De Rosa, M., De Rosa, S. & Gambacorta, A. (1977) in *Proc. Symp. Translation of Natural and Synthetic Polynucleotides* (Legocki, A., ed.), pp. 367–379, University Department of Agriculture, Poznan
- Cox, R. A. (1970) *Biochem. J.* **117**, 101–118
- Cox, R. A. & Kanagalingam, K. (1967) *Biochem. J.* **103**, 749–758
- Cox, R. A., Hüvos, P. & Godwin, E. A. (1973) *Isr. J. Chem.* **11**, 407–422
- Cox, R. A., Godwin, E. A. & Hastings, J. R. B. (1976) *Biochem. J.* **155**, 465–475
- Dasgupta, R., Shih, D. S., Saris, C. & Kaesberg, P. (1975) *Nature (London)* **256**, 624–628
- Dawid, I. B. & Chase, J. W. (1972) *J. Mol. Biol.* **63**, 217–231
- Ehresmann, C., Stiegler, P., Mackie, G. A., Zimmermann, R. A., Ebel, J. P. & Fellner, P. (1975) *Nucleic Acids Res.* **2**, 265–278
- Fresco, J. R., Klotz, L. C. & Richards, E. G. (1963) *Cold Spring Harbor Symp. Quant. Biol.* **28**, 83–90
- Gould, H. J. & Hamlyn, P. H. (1973) *FEBS Lett.* **30**, 301–304
- Granboulan, N. & Scherrer, K. (1969) *Eur. J. Biochem.* **9**, 1–20
- Hamilton, M. G. (1967) *Biochim. Biophys. Acta* **134**, 473–475
- Kallenbach, N. R. (1968) *J. Mol. Biol.* **37**, 445–466
- King, H. W. S. & Gould, H. (1970) *J. Mol. Biol.* **51**, 687–702
- Kurland, C. G. (1960) *J. Mol. Biol.* **35**, 355–365
- Lava-Sanchez, P. A. & Puppo, S. (1975) *J. Mol. Biol.* **95**, 9–20
- Lava-Sanchez, P. A., Amaldi, F. & La Posta, A. (1972) *J. Mol. Evol.* **2**, 44–45
- Legon, S. (1976) *J. Mol. Biol.* **106**, 37–53
- Loening, U. E. (1968) *J. Mol. Biol.* **38**, 355–365
- Loening, U. E., Grierson, D., Rogers, M. E. & Sartirana, M. L. (1972) *FEBS Symp.* **23**, 395–405
- Londei, P., Biagini, R., Mazzei, F. & Cammarano, P. (1977) in *Proc. Symp. Translation of Natural and Synthetic Polynucleotides* (Legocki, A., ed.), pp. 361–366, University Department of Agriculture, Poznan
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
- Nirenberg, M. W. & Matthaei, J. H. (1961) *Proc. Natl. Acad. Sci. U.S.A.* **47**, 1588–1601
- Pene, J. I., Knight, E., Jr. & Darnell, J. E. (1968) *J. Mol. Biol.* **33**, 609–623
- Petermann, M. L. & Pavlovic, A. (1966) *Biochim. Biophys. Acta* **114**, 264–276
- Pinder, J. C., Staynov, D. Z. & Gratzer, W. B. (1974) *Biochemistry* **13**, 5373–5378
- Rawson, J. R. & Stutz, E. (1968) *J. Mol. Biol.* **33**, 309–314
- Rungger, D. & Crippa, M. (1977) *Prog. Biophys. Mol. Biol.* **31**, 247–269
- Shine, J. & Dalgarno, L. (1973) *J. Mol. Biol.* **75**, 57–72
- Shine, J. & Dalgarno, L. (1975) *Nature (London)* **254**, 34–38
- Stevens, A. R. & Pachler, P. F. (1972) *J. Mol. Biol.* **66**, 225–237
- Stutz, E. & Noll, H. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **57**, 774–781
- Sy, J. & McCarthy, K. S. (1970) *Biochim. Biophys. Acta* **199**, 86–94
- Van de Voorde, A., Contreras, R., Rogers, R. & Fiers, W. (1976) *Cell* **9**, 117–120