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The Effect of Reaction with Formaldehyde on the Sedimentation Rates of Ribonucleic Acids

By M. L. FENWICK
Sir William Dunn School of Pathology, University of Oxford

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It has been reported that the RNA of several bacteriophages and that of the larger ribosomal sub-units of mammalian cells sediment faster in the presence of 0·1 M-sodium chloride than is expected from their estimated molecular weights. The effect of blocking the hydrogen-bonding amino groups of these and other types of RNA was studied. The RNA of phage R17 no longer sedimented anomalously fast after treatment with formaldehyde. In contrast, the larger ribosomal RNA of HeLa cells appeared more aberrant than before, sedimenting faster than tobacco-mosaic-virus RNA (mol.wt. 2×10^6) in the presence of formaldehyde. The rapidly labelled nuclear 45s RNA of HeLa cells still sedimented faster than the larger ribosomal RNA after reaction with formaldehyde, showing no evidence of disaggregation. It is suggested that both the large ribosomal RNA and the 45s RNA of HeLa cells may have a non-linear structure.

The folding of RNA chains is affected by the surrounding cations (Spirin, 1963) and different species of RNA are influenced differently (Montagnier & Sanders, 1963). Consequently it is not surprising that there is no rigid and widely applicable formula relating the sedimentation rate of a molecule of RNA to its molecular weight, although several empirical relationships have been derived (Gierer, 1958; Kurland, 1960; Spirin, 1961), each applying to a particular set of conditions and based on a limited number of types of RNA.

Apparent discrepancies between molecular weight and sedimentation coefficient have been noticed in at least two instances. First, the RNA of a group of coliphages sediments appreciably faster than the larger ribosomal RNA of *Escherichia coli* under conditions designed to allow maximum intramolecular hydrogen-bonding and folding, although the molecular weights of both have been estimated to be about $1{\cdot}1 \times 10^6$ (Strauss & Sinsheimer, 1963; Gesteland & Boedtker, 1964; Sinha, Fujimara & Kaesberg, 1965; Stanley & Bock, 1965). The viral RNA appears to assume a more compact shape than the ribosomal. Another discrepancy concerns the larger ribosomal RNA of animal cells. This RNA, with an estimated molecular weight of $1{\cdot}6 \times 10^6$ (Petermann & Pavlovec, 1966; Hamilton, 1967), normally sediments at about the same rate as tobacco-mosaic-virus RNA, of molecular weight $2{\cdot}0 \times 10^6$ (Boedtker, 1960).

If these anomalies are a consequence of the different hydrogen-bonding potentials of different sequences of nucleotides it might be expected that masking the amino groups involved in bonding would produce chains with a similar, if diminished, tendency to fold, and so with sedimentation rates related to their lengths. Therefore I have studied the sedimentation of these and other species of RNA (including the rapidly labelled nuclear RNA of HeLa cells) after treatment with formaldehyde, which reacts with the amino groups of adenine, guanine and cytosine, probably forming Schiff bases ($\cdot N{:}CH_2$) (Fraenkel-Conrat, 1954; Sarkar & Dounce, 1961; Haselkorn & Doty, 1961).

MATERIALS AND METHODS

Ultracentrifugation

Density gradients. Small sucrose gradients of the exponential concave type (Bock & Ling, 1954) were centrifuged in the Spinco rotor SW 50. Three gradients can be made simultaneously within 10 min. with the device shown in Fig. 1. In each lower chamber is placed 3·6 ml. of 25% (w/v) sucrose and a small Teflon-coated magnet. The upper chambers are screwed on to make an air-tight seal and 4·8 ml. of water is added to each. The three magnets are conveniently rotated by a single magnetic stirrer placed beside the mixing chambers. With the delivery tubes touching the insides of the tops of three centrifuge tubes the taps are opened to allow a slow flow of liquid through the mixing chambers. If the exit holes in the upper chambers are of sufficiently fine bore the last drop will hang and stop the flow so that the apparatus need not be watched nor turned off when the 4·8 ml. gradients are completed.

The construction of larger sucrose gradients may be

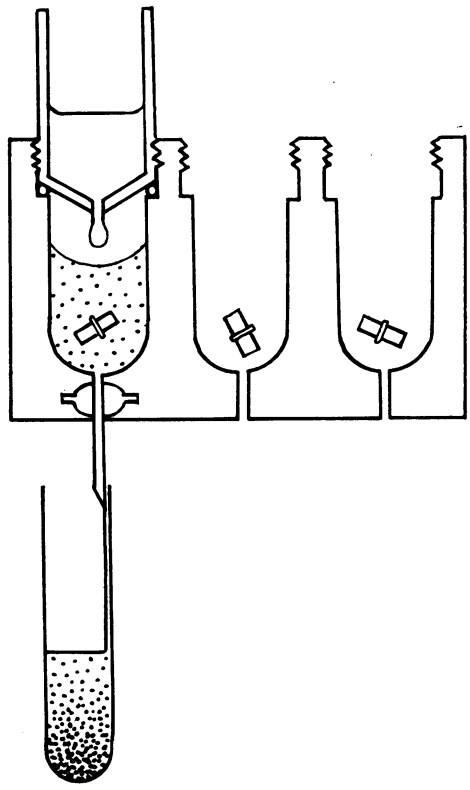

Fig. 1. Apparatus for making three exponential sucrose gradients simultaneously.

accelerated by using a multiple-tube peristaltic pump in conjunction with a linear density-gradient apparatus. The Delta flow inducer (Watson–Marlow Ltd., Marlow, Bucks.) pumps liquid from a single mixing chamber simultaneously through six or more delivery tubes. In this way three 28 ml. gradients can be made within 30 min. after 41 ml. of 25% sucrose is placed in the mixing chamber and 43 ml. of 5% sucrose in the parallel chamber. These gradients were centrifuged in the Spinco rotor SW 25.1. Similarly, six 16 ml. gradients for the rotor SW 25.3 (not used in the present work) can be made within about 20 min.

Turbulence is noticeable in the larger tubes near the surface of the growing gradient when the sucrose solution is delivered at the top and runs freely down the inside of the centrifuge tube. To minimize this the three (or six) centrifuge tubes are placed in a rack in a water bath with the ends of the delivery tubes touching the insides about 1 cm. above the bottom. As sucrose solution is delivered its increasing weight causes the centrifuge tubes to sink gradually in the water bath. The surface of the gradient remains almost stationary, a short distance below the delivery tube (Fig. 2).

Monitoring. After centrifugation, the gradients were analysed with a model 180 Density Gradient Fractionator (Instrumentation Specialities Corp. Inc., Lincoln, Nebr., U.S.A.). The contents of the centrifuge tube were displaced

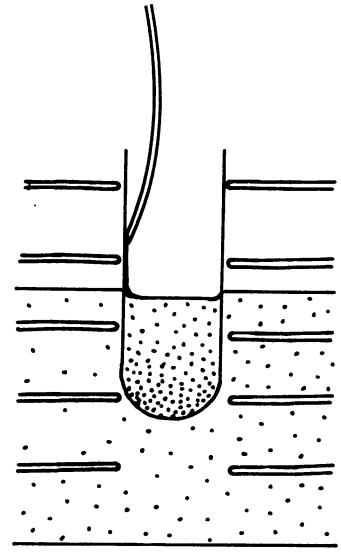

Fig. 2. Collection of larger sucrose gradients. The delivery tube is stationary and the centrifuge tube floats in the water bath.

slowly upwards through a flow cell (2 mm. light-path for SW 50 gradients, 5 mm. for SW 25.1 gradients) and E_{254} was recorded automatically on a moving chart. In all the Figures the top of the gradient is at the left of the recording. The displacing solution of 40% (w/v) sucrose contained 0·1 mg. of potassium dichromate/ml. so that the end of the gradient was marked by an abrupt rise in extinction. All the gradients shown in the Figures were centrifuged in the SW 50 rotor.

Analytical. Sedimentation coefficients were determined in a Spinco model E ultracentrifuge by using ultraviolet optics at 20°. The RNA concentration was 20–40 μg./ml. in a Kel-F centrepiece with 12 mm. light-path. Photographs were scanned with the Beckman Analytrol instrument.

Preparation of RNA

E. coli *ribosomal RNA.* To minimize the chances of interaction between the two species of ribosomal RNA, the ribosomal sub-units were separated before extraction of RNA. A 200 ml. culture of *E. coli* K12 strain Hfr_1 grown to a density of 4–5 $\times 10^8$ cells/ml. in TCG medium (Erikson, Fenwick & Franklin, 1964) was converted into spheroplasts with lysozyme and EDTA (Fenwick, 1968) and lysed in 4·5 ml. of 0·5 mM-magnesium acetate–0·01 M-tris, pH 7·2, with deoxyribonuclease (5 μg./ml.). After a convenient fall in the viscosity the lysate was centrifuged for 5 min. at 2500*g*, and the supernatant was layered on to three 28 ml. sucrose gradients containing 0·5 mM-magnesium acetate–0·01 M-tris and centrifuged at 20000 rev./min. for 15 hr. at 5° in the Spinco rotor SW 25.1. The contents of the tubes were fractionated and the 30 s and 50 s peaks pooled separately.

EDTA (0·01 M), NaCl (0·1 M) and sodium dodecyl sulphate

(0·5%) were added and the mixture was shaken vigorously for 2min. with half its volume of water-saturated phenol. The phases were separated by centrifugation and the RNA was precipitated from the aqueous layer with 2vol. of ethanol in the cold. The aqueous layer is usually below the phenol with the 50s sub-units because of the density of the sucrose solution in that part of the gradient. The RNA precipitate was centrifuged, redissolved in 2ml. of 0·1M-NaCl–0·01M-EDTA, precipitated a second time with ethanol and finally dissolved in water and adjusted to an extinction of 10 at 260mμ (about 1ml. from the 30s sub-units and about 2ml. from the 50s). The RNA solution, like all other types of RNA used, was stored at −10° and was stable for several months.

Phage R17 RNA. A culture (1l.) of growing *E. coli* Hfr_1 in TCG medium at about 10^8cells/ml. was infected with phage R17 at a multiplicity of 0·1 plaque-forming unit/cell, and incubation was continued for 5hr. or until prior lysis. Cells and debris were removed by centrifuging for 15min. at 10000*g*. Then $(NH_4)_2SO_4$ (350g.) was dissolved in the supernatant, which was left overnight in the cold. The sediment was collected after a further 15min. at 10000*g* and dissolved in 30ml. of 0·01M-tris, pH7·2. Further purification was unnecessary since there was no detectable contamination with ribosomes. RNA was extracted as described above and stored in solution in water at −10°.

HeLa-cell ribosomal RNA. Ribosomes were dissociated into sub-units as described by Fenwick (1968). About 3×10^8 cells were homogenized in 4·5ml. of 0·01M-tris and centrifuged for 10min. at 10000*g*. The supernatant, with 0·4M-NaCl added, was placed on three 28ml. gradients of 30–5% sucrose in 0·1M-NaCl–0·01M-tris and centrifuged for 15hr. at 18000rev./min. in the SW25.1 rotor at about 5°. The two clearly separated peaks were collected and RNA was extracted as described above, yielding about 1ml. of solution of E_{260} 10 from the 30s peak and about 2·5ml. from the 50s peak.

Tobacco-mosaic-virus RNA. Tobacco mosaic virus was sedimented by ultracentrifugation of a crude suspension of infected leaves supplied by Dr T. W. Tinsley, and RNA was extracted from the resuspended pellet in the presence of NaCl, EDTA, sodium dodecyl sulphate and phenol as described above.

HeLa-cell pulse-labelled RNA. About 6×10^7 growing HeLa cells were resuspended in 10ml. of Eagle's medium and incubated for 30min. at 37° before the addition of [^{3}H]uridine (2μc/ml.) (The Radiochemical Centre, Amersham, Bucks.). After 20min. the cells were chilled, resuspended in 5ml. of 0·1M-NaCl–0·01M-EDTA and sodium dodecyl sulphate was added to a final concn. of 1%. The mixture was shaken with 5ml. of water-saturated phenol for 5min. at 60° (Scherrer & Darnell, 1962). The phases were separated by centrifugation at 10000*g* for 10min. and the RNA was precipitated twice as described above and dissolved in 1ml. of water. It has been reported (Wagner, Katz & Penman, 1967) that extraction at this temperature can result in aggregation of ribosomal RNA, although not of rapidly labelled RNA. The concentrations used here were low enough to avoid this hazard.

Formaldehyde solution. This was obtained from British Drug Houses Ltd. (Poole, Dorset) (A.R. grade) at an assumed concentration of 36% (w/v). It was brought to pH6·8–7·2 with N-NaOH; this resulted in a final concentration of added Na^+ ions of approx. 5mM.

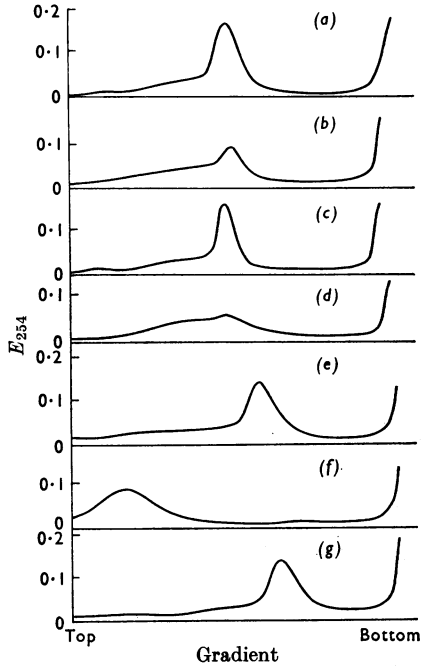

Fig. 3. Stability of *E. coli* lr-RNA: (*a*) untreated; (*b*) heated for 5min. at 98° in water; (*c*) heated for 90min. at 37° in 0·05M-glycine buffer, pH9; (*d*) heated for 60min. at 37° in 0·05M-glycine buffer, pH10; (*e*) heated for 4min. at 90° in 0·1M-NaCl–0·01M-EDTA; (*f*) heated for 4min. at 90° in mM-magnesium acetate; (*g*) unheated, in mM-magnesium acetate. Gradients (*a*)–(*d*) were centrifuged for 3hr. at 45000rev./min. in the SW50 rotor, at 10° in 0·1M-NaCl–0·01M-tris. Gradients (*e*)–(*g*) were centrifuged for 15hr. at 22000rev./min. in the SW50 rotor, at 7° in 0·1M-NaCl–0·01M-EDTA (*e*) or mM-magnesium acetate–0·01M-tris [(*f*) and (*g*)].

RESULTS

The larger and smaller species of ribosomal RNA obtained respectively from the larger (50s) and smaller (30s) ribosomal sub-units are designated lr-RNA and sr-RNA.

Stability of E. coli *lr-RNA.* The results of various preliminary tests of the stability of *E. coli* lr-RNA are shown in Fig. 3. The RNA was gradually degraded at 98° (Fig. 3*b*) but was stable at 37° at pH9 (Fig. 3*c*). At pH10 at 37° it was degraded: the density-gradient pattern (Fig. 3*d*) suggests a random breakdown. The sedimentation rate in the presence of 0·1M-sodium chloride was unaffected by preincubation in 5mM-EDTA–tris (Rogers, 1966) for 90min. at 10° or for 5min. at 60°. Thus the opening of the chains that occurs in low concentrations of EDTA (described below) was reversed on adding 0·1M-sodium chloride.

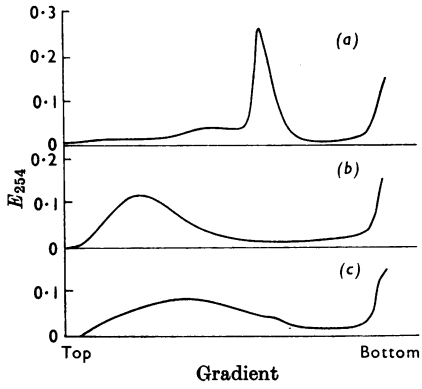

Fig. 4. Stability of phage R17 RNA: heated for 4 min. at 90° in (*a*) mM-magnesium acetate–0·01 M-EDTA; (*b*) mM-magnesium acetate; (*c*) mM-magnesium acetate–0·1 M-NaCl. Gradients were centrifuged for 2½ hr. at 48000 rev./min. in the SW 50 rotor at 10° in 0·1 M-NaCl–0·01 M-EDTA.

Table 1. *Sedimentation coefficients of* E. coli *lr-RNA in various media*

Samples were centrifuged at 20° except in one case, mM-EDTA at 35°, in which a viscosity correction was applied.

Medium	S_{20}
0·1 M-NaCl–0·01 M-EDTA	22·4
10 mM-EDTA	16·1
mM-EDTA	7·6
mM-EDTA at 35°	5·9
0·25 mM-EDTA	4·8
0·1 mM-EDTA	8·7
0·01 mM-EDTA	15·6

The lr-RNA was unaffected by heating for 4 min. at 90° in 0·1 M-sodium chloride–0·01 M-EDTA (Fig. 3*e*) but was much altered by heating in mM-magnesium acetate (Fig. 3*f*). This effect probably resulted from degradation and not merely from a change of shape of the molecule, since it could not be reversed by subsequent treatment with EDTA.

Stability of phage R17 RNA. The stability of phage R17 RNA was also examined, since it has been reported (Bishop, 1966) that the RNA of phage ZIK/1 assumes a more slowly sedimenting form after being heated. As shown in Fig. 4, heating in the presence of Mg^{2+} ions resulted in much degradation (Fig. 4*b*), but this effect was decreased if 0·1 M-sodium chloride was present during the heating to compete with the Mg^{2+} ions (Fig. 4*c*), or abolished if excess of EDTA was present (Fig. 4*a*). The resulting sedimentation pattern depended on the relative concentrations of Na^+ and Mg^{2+} ions during heating.

Sedimentation rates and melting curves of E. coli *lr-RNA.* Before treatment with formaldehyde was attempted it was important to know the conditions under which the molecules became fully extended, revealing the hydrogen-bonded amino groups, since it has been shown that the rate and extent of reaction of formaldehyde with RNA depends upon the degree of secondary structure (Staehelin, 1958; Haselkorn & Doty, 1961; Boedtker, 1967). Consequently *E. coli* lr-RNA was further characterized by measurements of sedimentation coefficient and increase in extinction on heating in various media. The *S* values in Table 1 show that the chain was in its slowest-moving, most loosely coiled (Spirin, 1963) form in 0·25 mM-EDTA. Lower concentrations allowed some contraction and increase of sedimentation rate, and RNA dissolved in water alone was far from fully extended at 20°. The optimum concentration of EDTA is probably that which is just enough to bind contaminating traces of bivalent cations.

The loss of hydrogen-bonded secondary structure of the chains as the ionic strength is lowered is reflected in a decrease in the hyperchromic effect observed on subsequent heating (Boedtker, 1960). The melting curves in Fig. 5 may be compared with the sedimentation coefficients of Table 1. The T_m fell to about 8° in the sample in 0·1 mM-EDTA but rose again at lower EDTA concentration. Both in 0·01 mM- and in 10 mM-EDTA at 20° the secondary structure (as indicated by extinction) was largely intact although the sedimentation coefficient decreased to 16 S. Presumably the change was due to a loosening of tertiary structure. The curves obtained on cooling the specimens closely resembled the heating curves except in the presence of mM-Mg^{2+} (Fig. 5*g*). This may well have been due to degradation on heating with Mg^{2+} ions (cf. Fig. 3*e*–3*g*).

Reaction of E. coli *lr-RNA with formaldehyde.* The reaction of lr-RNA with formaldehyde was followed by measuring the increase in E_{260} resulting largely from disorganization of the hydrogen-bonded structure. Fig. 6 shows that at 37° the reaction was somewhat faster in 0·01 M-EDTA alone than in 0·1 M-sodium chloride–0·01 M-EDTA. Reference to Fig. 5 reveals that the T_m was about 40° in 0·01 M-EDTA, whereas in 0·1 M-sodium chloride–0·01 M-EDTA the T_m was about 55° and most of the secondary structure was intact at 37°. The reaction with formaldehyde was also followed at 37° in mM-EDTA (T_m about 20°) but was little, if at all, faster than in 10 mM-EDTA. Fig. 6 shows that the reaction was virtually complete within 1 hr. in 0·01 M-EDTA, and that only a small and reversible hyperchromic effect was observed on subsequently raising the temperature to 70°.

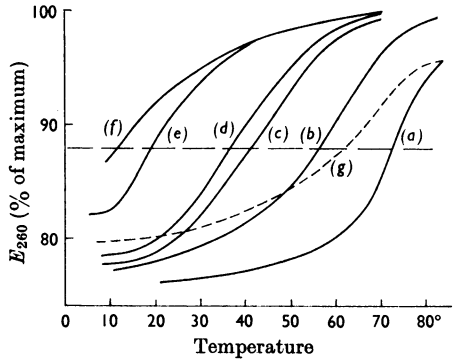

Fig. 5. Melting curves of *E. coli* lr-RNA in various media: (*a*) mM-magnesium acetate; (*b*) 0·15M-NaCl; (*c*) 0·01mM-EDTA; (*d*) 10mM-EDTA; (*e*) mM-EDTA; (*f*) 0·1mM-EDTA. The horizontal broken line crosses the curves in the region of the T_m. Curve (*g*) is the cooling curve of sample (*a*), in mM-magnesium acetate.

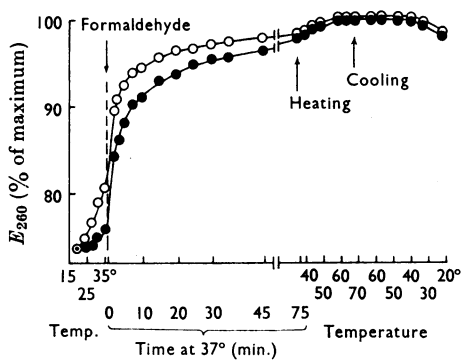

Fig. 6. Reaction of *E. coli* lr-RNA with formaldehyde. Two samples of RNA were warmed in the spectrophotometer to 37°. At zero time formaldehyde was added to each, and E_{260} was followed over the next 75min. The final concentrations in the two samples were: (●) 0·1M-NaCl–0·01M-EDTA with 6% (w/v) formaldehyde; (○) 0·01M-EDTA with 6% formaldehyde. The temperature was then raised to 70° and finally lowered to 20°.

Effect of formaldehyde and salt on the sedimentation rate of E. coli *lr-RNA.* The sedimentation of formaldehyde-treated RNA is shown in Fig. 7. In 0·01M-EDTA with 6% formaldehyde (Fig. 7*a*) it formed a band in the 7s region of the gradient (cf. about 16s in 0·01M-EDTA without formaldehyde treatment, Table 1). In the additional presence of 0·1M-sodium chloride the RNA band appeared at about 12s (Fig. 7*b*). Thus although the presence of 0·1M-sodium chloride did not affect the final increase in E_{260} after treatment with formaldehyde (Fig. 6), it allowed a certain amount of coiling

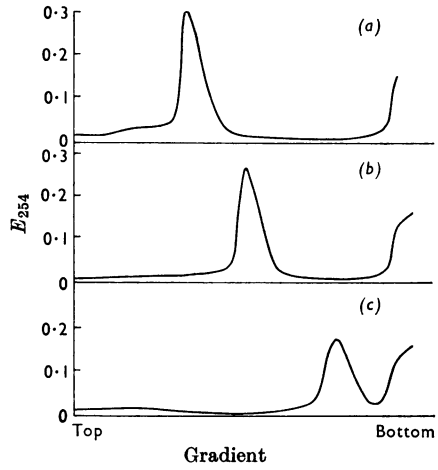

Fig. 7. Effect of formaldehyde on the sedimentation of *E. coli* lr-RNA. The RNA was treated with 6% formaldehyde in 0·01M-EDTA for 1hr. at 37° and then centrifuged for 15hr. at 28000rev./min. in the SW50 rotor in gradients containing: (*a*) 0·01M-EDTA with 6% formaldehyde; (*b*) 0·1M-NaCl–0·01M-EDTA with 6% formaldehyde; (*c*) 0·1M-NaCl–0·01M-EDTA.

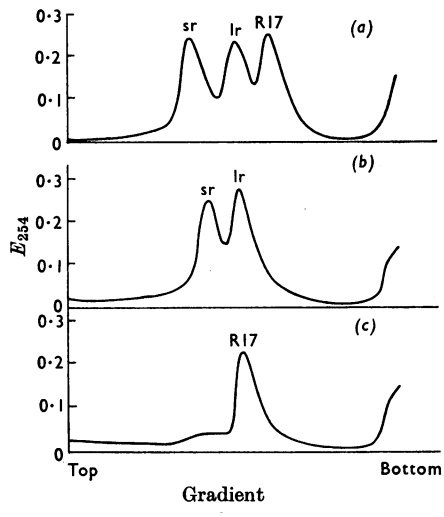

Fig. 8. Comparison of phage R17 RNA with *E. coli* r-RNA: (*a*) *E. coli* lr- and sr-RNA and phage R17 RNA in 0·1M-NaCl–0·01M-EDTA after 2½hr. at 48000rev./min. in the SW50 rotor, at 10°; (*b*) *E. coli* lr- and sr-RNA treated with formaldehyde for 1hr. at 37° in 0·01M-EDTA, and then centrifuged for 15hr. at 28000rev./min. in the SW50 rotor, at 10° in 0·1M-NaCl–0·01M-EDTA with 6% formaldehyde (*c*) phage R17 RNA treated as in (*b*).

of the molecules. If formaldehyde was omitted from the gradient the RNA reached the 20s region (Fig. 7*c*); this increase in sedimentation speed is

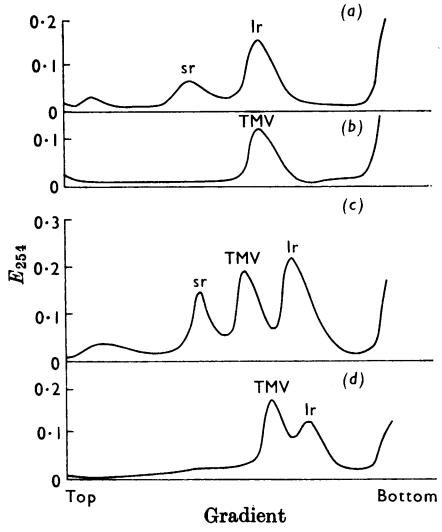

Fig. 9. Comparison of tobacco-mosaic-virus (TMV) RNA with HeLa-cell r-RNA: samples of whole HeLa-cell RNA (*a*) and TMV RNA (*b*) were centrifuged for 2½hr. at 48000rev./min. in the SW50 rotor, at 10° in 0·1M-NaCl–0·01M-EDTA. Samples of the same types of RNA were mixed, and incubated with 6% (w/v) formaldehyde in 0·01M-EDTA for 1hr. at 37°; NaCl was added to a final concn. of 0·1M and the mixture was centrifuged (*c*) for 15hr. at 28000rev./min. in the SW50 rotor, at 7° in a gradient containing 0·1M-NaCl–0·01M-EDTA with 6% formaldehyde. Samples of TMV RNA and HeLa-cell lr-RNA were mixed and incubated with 6% formaldehyde in 0·5mM-EDTA for 1hr. at 65° (*d*) before the addition of NaCl and centrifugation as in (*c*) but at 10°.

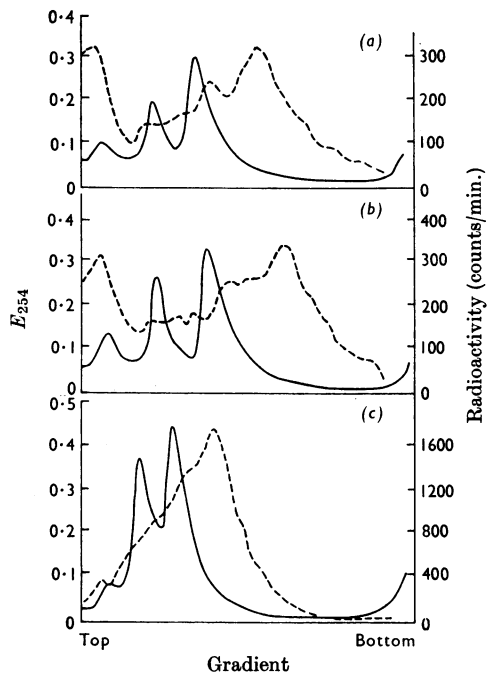

Fig. 10. HeLa-cell rapidly-labelled RNA. (*a*) RNA was extracted from HeLa cells labelled for 20min. with [^{3}H]-uridine and centrifuged for 75min. at 48000rev./min. in the SW50 rotor, at 10° in 0·1M-NaCl–0·01M-EDTA. (*b*) A sample of the same RNA was incubated with 6% formaldehyde for 1hr. at 37° in 0·01M-EDTA and centrifuged for 2½hr. at 48000rev./min. in the SW50 rotor, at 10° in 0·1M-NaCl–0·01M-EDTA with 6% formaldehyde. (*c*) A sample of another similar RNA preparation was treated with 6% formaldehyde in 0·01M-EDTA for 2min. at 90° followed by 30min. at 60° and then centrifuged as in (*b*) but for 2hr. ——, E_{254}; ----, ^{3}H counts/min.

presumed to reflect reversal of the formaldehyde reaction (Haselkorn & Doty, 1961; Eyring & Ofengand, 1967) as the RNA sedimented into the formaldehyde-free gradient. Subsequently sedimentation rates were measured in the presence of 0·1M-sodium chloride–0·01M-EDTA with 6% formaldehyde.

Comparison of formaldehyde-treated RNA samples in sucrose gradients. Phage R17 RNA is clearly distinguished from *E. coli* lr- and sr-RNA in 0·1M-sodium chloride–0·01M-EDTA (Fig. 8*a*). However, after treatment with formaldehyde, although *E. coli* lr- and sr-RNA were still quite distinct, as shown in Fig. 8(*b*) (their sedimentation pattern was the same whether they were treated together or separately), the band of phage R17 RNA was no longer distinguishable from that of *E. coli* lr-RNA (Fig. 8*c*).

Fig. 9 shows first that in 0·1M-sodium chloride–0·01M-EDTA, tobacco-mosaic-virus RNA (*b*) is not easily distinguished from HeLa-cell lr-RNA (*a*). It was surprising to find that after treatment with formaldehyde tobacco-mosaic-virus RNA sedimented more slowly than HeLa-cell lr-RNA, midway between it and HeLa sr-RNA (Fig. 9*c*). The peaks were readily identified by comparison with single species sedimented in separate gradients. The reaction was repeated in 0·5mM-EDTA at 65°, i.e. in conditions of complete disruption of secondary structure, in case HeLa-cell lr-RNA should be exceptionally resistant to reaction with formaldehyde. However, the relative positions of the lr-RNA and tobacco-mosaic-virus RNA peaks were confirmed (Fig. 9*d*).

The rapidly sedimenting RNA of HeLa cells (Scherrer & Darnell, 1962) was labelled by a pulse of [^{3}H]uridine, as described in the Materials and Methods section. Fig. 10 shows that the relative positions of the peaks of extinction and radioactivity were little affected, even by treatment with formaldehyde at high temperature (Fig. 10*c*) to ensure that no interchain links involving hydrogen

Table 2. *Sedimentation coefficients of various types of RNA before and after reaction with formaldehyde*

All measurements were made at 20° in 0·1 M-NaCl–0·01 M-EDTA, with or without 6% (w/v) formaldehyde. The standard treatment was for 1 hr. at 37° with 6% formaldehyde in 0·01 M-EDTA before the addition of 0·1 vol. of 1 M-NaCl. The extra treatment was for 1 hr. at 65° with 6% formaldehyde in 0·5 mM-EDTA before the addition of 0·1 vol. of 1 M-NaCl–0·1 M-EDTA.

Type of RNA	Treatment with formaldehyde: None	Standard	Extra
E. coli sr	16·5	8·9	—
HeLa-cell sr	18·5	10·2	—
E. coli lr	22·4	11·7	—
Phage R17	26·5	11·8	11·8
HeLa-cell lr	29·6	17·9	16·3
Tobacco mosaic virus	30·8	14·8	14·5

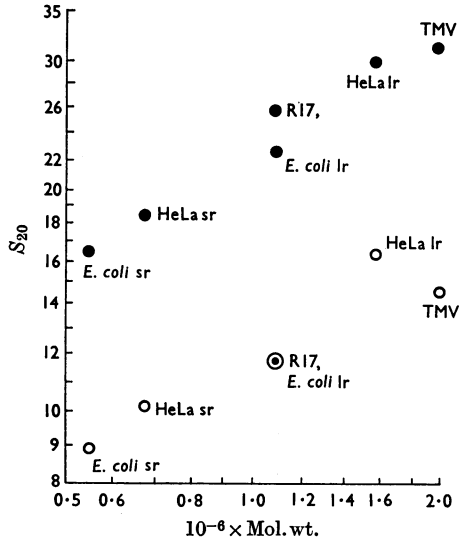

Fig. 11. Possible relationships between sedimentation coefficient and molecular weight. The following estimates of molecular weights have been used: *E. coli* sr-RNA: $0{\cdot}55 \times 10^6$ (Stanley & Bock, 1965); HeLa-cell sr-RNA: $0{\cdot}67 \times 10^6$ (Petermann & Pavlovec, 1966, with rat sarcoma cells); *E. coli* lr-RNA: $1{\cdot}07 \times 10^6$ (Stanley & Bock, 1965); phage R17 RNA: $1{\cdot}1 \times 10^6$ (Gesteland & Boedtker, 1964; Sinha *et al.* 1965); HeLa-cell lr-RNA: $1{\cdot}64 \times 10^6$ (Petermann & Pavlovec, 1966, with rat sarcoma cells); tobacco-mosaic-virus (TMV) RNA: $2{\cdot}0 \times 10^6$ (Boedtker, 1960). Sedimentation coefficients were measured in: (○) 0·1 M-NaCl–0·01 M-EDTA with 6% (w/v) formaldehyde; (●) 0·1 M-NaCl–0·01 M-EDTA.

bonding would survive. The pattern was essentially unchanged. Extrapolation from the known S values of the ribosomal RNA to the peaks of radioactivity gave coefficients of about 45 S without and about 27 S with formaldehyde.

Sedimentation coefficients in formaldehyde. Measurements were made in the analytical ultracentrifuge with various single species of RNA with the results given in Table 2. The high-temperature formaldehyde treatment caused a small decrease in the sedimentation coefficient of HeLa-cell lr-RNA and the lower value is among those plotted in Fig. 11, which shows the relationship between $\log S_{20}$ and log (estimated molecular weight).

DISCUSSION

Preliminary stability tests were carried out because, although it has been concluded (Stanley & Bock, 1965) that the lr-RNA and sr-RNA of *E. coli* are both intact covalently linked chains of quite different length, other reports have suggested the possibility of a weak link in the lr-RNA chain, breakage of which yields material sedimenting like sr-RNA (Midgley, 1965; Martin, 1966), or that the lr-RNA chain can be induced to change its shape so that it sediments like a molecule of sr-RNA (Rogers, 1966; Midgley & McIlreavy, 1967). The bacterial lr-RNA used in this work showed no tendency to change to material resembling sr-RNA; nor did the RNA of phage R17 assume a different form on heating. Both underwent a probably random thermal degradation, particularly in the presence of Mg^{2+} ions, which emphasized the importance of avoiding Mg^{2+} ions in handling RNA (Lindahl, 1967). All operations were therefore carried out in the presence of EDTA. The stability of the lr-RNA of *E. coli* under the conditions of formaldehyde treatment was confirmed by the increase in sedimentation rate on reversal of the reaction in gradients lacking formaldehyde (Fig. 7).

The sedimentation rates of a number of different species of RNA have been measured in the presence of formaldehyde and sodium chloride. The purpose of the formaldehyde was to prevent base-pairing by reacting with the amino groups involved in hydrogen-bond formation. The added Na^+ ions permitted the folding of the formylated chain by shielding the negative charges of phosphate groups. The degree of folding under these conditions might reasonably be expected to be less dependent on precise nucleotide sequence and to produce a moderately compact structure whose dimensions would be limited primarily by chain length. At the same time the overall base composition may exert some specific influence on shape through its effect on base stacking. The probable reaction of formaldehyde with available heterocyclic imino nitrogen atoms (Eyring & Ofengand, 1967) to form hydroxymethyl groups must also be considered, but its effect

is unlikely to be greatly influenced by small variations in base composition.

The observed sedimentation rates in the presence of formaldehyde have resolved the first anomaly mentioned in the introduction. The RNA of phage R17 and that of the larger ribosomal sub-unit of *E. coli*, which are thought to have about the same molecular weight, sedimented in formaldehyde at very similar rates. This suggests that in the normal unformylated condition the viral RNA takes up a more compact (27s) form than the ribosomal (23s) because it has a higher proportion of complementary regions in its nucleotide sequence. It also provides additional evidence for the linearity of the viral RNA, which has previously been inferred from electron micrographs (Granboulan & Franklin, 1966). If phage R17 RNA was circular it would be expected to sediment faster than a linear molecule of the same chain length when partly or fully extended. The single-stranded DNA of phage ϕx 174 has been resolved into a faster (circular) and a more slowly (linear) sedimenting form by exposure to low ionic strength, high pH or formaldehyde (Fiers & Sinsheimer, 1962).

The most surprising result was the high sedimentation coefficient of formaldehyde-treated HeLa-cell lr-RNA relative to that of tobacco-mosaic-virus RNA. The RNA of tobacco mosaic virus is well characterized. Its molecular weight ($2{\cdot}0 \times 10^6$) is known, probably within less than $\pm 10\%$, from chemical as well as physical data, and the structure of the virus particle demands that its RNA is a single linear molecule. The molecular weight and structure of mammalian lr-RNA are rather less certain, but recent estimates from measurements of viscosity (Petermann & Pavlovec, 1966) and sedimentation equilibrium (Hamilton, 1967) of about $1{\cdot}65 \times 10^6$ have been obtained for the lr-RNA of a rat tumour. However, the sedimentation coefficient (30s in 0·1M-sodium chloride) would suggest a rather higher value (Click & Tint, 1967), close to that of the 31s RNA of tobacco mosaic virus, and after treatment with formaldehyde the discrepancy is even greater. Assumption of a similar degree of folding for the two RNA samples would then suggest a very high molecular weight of about $2{\cdot}7 \times 10^6$ (see below) for HeLa-cell lr-RNA. A more probable explanation, however, would seem to be that it has an unusually compact shape after reaction with formaldehyde so that it sediments faster than tobacco-mosaic-virus RNA, although the latter is believed to have a higher molecular weight. Various reasons for this can be imagined. Formaldehyde may fail to react with certain critical hydrogen-bonded amino groups of HeLa lr-RNA. This is improbable since the anomaly persisted when the reaction was carried out under fully 'melted' conditions. The formaldehyde treatment may, in certain species of RNA, cause some intra-chain cross-linking by the interaction of newly formed hydroxymethyl groups ($:N \cdot CH_2 \cdot OH$) with further amino groups, perhaps even involving protein that was not removed during the isolation of the RNA; or such cross-links may exist normally and their presence become more evident after treatment with formaldehyde. Another possibility is that HeLa-cell lr-RNA has an important difference in structure from the other types of RNA examined. An anomalous sedimentation behaviour might result if it were a circular, looped or branched molecule. The distinction between the linear and circular forms of the DNA of phage ϕx 174 has been mentioned above.

It is clear that no reliable estimate of molecular weight can be made from sedimentation data alone, either in the absence (as pointed out by Gesteland & Boedtker, 1964) or in the presence of formaldehyde, because of the uncertainty of structural influences. The results summarized in Fig. 11 are based on measurements of molecular weight and sedimentation rate, all of which are probably subject to an error of at least 10% and possibly much more. Nevertheless, as mentioned in the introduction, they can be used to derive empirical relationships of limited usefulness of the type $M = KS^{\alpha}$. With the omission of HeLa-cell lr-RNA, which seems to have unusual properties, the relationships obtained from Fig. 11 by the method of least squares are $M = 2400S^{1{\cdot}93}$ in 0·1M-sodium chloride–0·01M-EDTA, and $M = 1260S^{2{\cdot}75}$ in 0·1M-sodium chloride–0·01M-EDTA with (w/v) 6% formaldehyde.

If these two formulae are used to estimate the molecular weight of the rapidly labelled nuclear RNA of HeLa cells, values of $3{\cdot}6 \times 10^6$ and $1{\cdot}1 \times 10^7$ are obtained. The great discrepancy between these estimates shows that something is seriously wrong with one or both and is reminiscent of the case of HeLa-cell lr-RNA discussed above (the two formulae yield values of $1{\cdot}7 \times 10^6$ and $2{\cdot}7 \times 10^6$ for HeLa-cell lr-RNA). Again the high sedimentation rate in formaldehyde may indicate a cross-linked, looped or branched structure, and by analogy with HeLa-cell lr-RNA the true molecular weight of the 45s RNA is more likely to be in the region of the lower estimate. However, such an estimate must be regarded as speculative. Bramwell & Harris (1967) have shown that rapidly labelled RNA of HeLa cells, although it sediments faster than lr-RNA at intermediate ionic strength, closely resembles sr-RNA in low concentrations of EDTA. This resemblance is strengthened by the kinetics of exonuclease degradation (W. T. Riley, personal communication) and by end-group studies (Tamaoki & Lane, 1967), which indicate that rapidly labelled RNA and sr-RNA have similar mean chain lengths. If this is so, the sedimentation behaviour suggests

that the 45s RNA is either exceptionally compact or else aggregated in such a way that although the 5′-termini of the chains are exposed to exonuclease attack the interchain links are not broken by heating in formaldehyde.

Note added in proof. RNA extracted from encephalomyocarditis virus, purified and provided by Dr A. T. H. Burness, had sedimentation coefficients of 35s in 0·1 M-sodium chloride–0·01 M-EDTA and 15·6s after standard formaldehyde treatment (see Table 2). The latter value would suggest a molecular weight of about $2{\cdot}4 \times 10^6$ (cf. Burness, Vizoso & Clothier, 1963).

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