The Determination of the Molecular Weight of Ribonucleic Acid by Polyacrylamide-Gel Electrophoresis

THE EFFECTS OF CHANGES IN CONFORMATION

By U. E. LOENING

Department of Botany, University of Edinburgh

1. The effects of changes in experimental conditions on the mobility of RNA in polyacrylamide-gel electrophoresis were investigated. 2. The linear relation between log(molecular weight) and electrophoretic mobility was shown to be independent within limits of salt or gel concentration. 3. The relative mobility of RNA with low content of guanylic acid and cytidylic acid residues was decreased in low-ionic-strength buffer. This was related to a small relative decrease in sedimentation coefficient. 4. However, Mg^{2+} ion caused almost no increase in mobility although it was associated with large increases in sedimentation coefficient. This suggested opposing actions of Mg^{2+} ion on the size and effective charge of the RNA. 5. It is concluded that the method provides a satisfactory measurement of molecular weight, which is almost independent of the nucleotide composition of RNA at moderate salt concentrations.

The analysis of high-molecular-weight RNA by polyacrylamide-gel electrophoresis has become widely used in the last 2 years. To interpret the results in terms of the molecular weight or other properties of the RNA it is necessary to know the extent to which changes in the conditions of electrophoresis and in the ionic environment affect the mobility and the apparent molecular weight of the RNA. This paper examines such effects in more detail and describes some improvements to the original method of Loening (1967).

It has been shown that the mobility of low-molecular-weight RNA in polyacrylamide gels is inversely related to the sedimentation coefficient (Richards, Coll & Gratzer, 1965), and the same was found to be true for ribosomal RNA (Loening & Ingle, 1967). It follows that the relative mobility should be inversely related to the log(molecular weight). This was found to be the case for a range of virus RNA species (Bishop, Claybrook & Spiegelman, 1967).

Fractionation by gel electrophoresis depends on molecular filtration. The electrophoretic mobility of RNA depends on the effective diameter of the molecule. When a compact RNA molecule is unfolded in a buffer of low ionic strength, its gel-electrophoretic mobility should decrease, since the effective diameter of the molecule becomes larger. A direct demonstration of this effect may be difficult to obtain, since the electrophoretic conditions (current and voltage) also vary with salt concentration. Experiments were therefore designed to compare the mobilities of r-RNA* species of similar weight but different base compositions in different ionic environments. RNA with a lower content of guanylic acid and cytidylic acid ('low-G+C RNA') unfolds more than 'high-G+C RNA' in low salt concentrations and its relative mobility should be decreased. In order that the electrophoretic conditions should be as similar as possible, two similar buffers were used, which differed only in Mg^{2+} ion concentration. A low-ionic-strength tris buffer at relatively high pH was used to obtain the maximum unfolding of the RNA. Mg^{2+} ion was added to the same buffer to obtain the most compact form of the RNA. The electrophoretic conditions were thus similar and the mobilities depended largely on the effects of Mg^{2+} ion on the RNA.

METHODS

Preparation of RNA. The method described by Parish & Kirby (1966) was used, as follows: *Xenopus* tadpoles, pea-seedling root tips, TMV or *Escherichia coli* (strain M.R.E. 600) were homogenized in medium containing 1% (w/v) of tri-isopropylnaphthalenesulphonate, 6% (w/v) of sodium 4-aminosalicylate, 1% (w/v) of NaCl and 6% (v/v) of phenol–cresol at 0–5°. The phenol–cresol contained A.R.

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* Abbreviations: r-RNA, ribosomal RNA; SDS, sodium dodecyl sulphate; TMV, tobacco mosaic virus; BMV, Brome mosaic virus.

phenol (500 ml.), redistilled *m*-cresol (70 ml.), water to saturate (more than 150 ml.) and 8-hydroxyquinoline (0·5 g.). The homogenates were shaken with 1 vol. of phenol–cresol and centrifuged at 2000*g* at 5° for 10 min. The phenol layer was removed, and 0·15 vol. of 3 M-NaCl was added to the supernatants (together with interface precipitates) to increase the NaCl concentration to about 0·5 M. The mixture was again shaken with phenol–cresol, sometimes at room temperature, and centrifuged. Nucleic acids were precipitated from the final supernatants with 2 vol. of ethanol at 0°.

Drosophila flies were immobilized with a small amount of solid CO_2 and immediately homogenized in a VirTis blender at half speed in a medium containing 30 mM-tris–HCl buffer, pH 7·5 at 0°, 0·15 M-NaCl, 0·5 % sodium naphthalene-1,5-disulphonate and an equal volume of phenol–cresol (Hastings & Kirby, 1967). The homogenate was centrifuged at 2000*g* for 10 min. at 0°. Sodium tri-isopropylnaphthalene-sulphonate and sodium 4-aminosalicylate were then added (final concn. 1 % and 6 % respectively) to the supernatants. Two further extractions with phenol–cresol then followed, as described above. Some samples of pea-seedling RNA were also prepared by the method for *Drosophila*, which has the advantage that it extracts little DNA. A sample of TMV RNA was also kindly given by Dr S. Sarkar.

BMV (kindly given by Dr J. Bancroft) was extracted with phenol–cresol from suspension in 0·1 M-sodium acetate buffer, pH 5, at 0°.

HeLa-cell r-RNA was kindly given by Dr S. Penman. It had been prepared by extraction of ribosomes in 1 % (w/v) SDS–0·5 M-NaCl–tris buffer at 55° with phenol and chloroform (Penman, 1965).

In all cases the RNA was freed from detergents and phenol by dissolving it in 0·5 % (w/v) SDS–0·15 M-sodium acetate buffer, pH 6, at room temperature and reprecipitating it with 2 vol. of ethanol at 0°. The r-RNA was prepared by extraction of the DNA and transfer RNA with 3 M-sodium acetate buffer, pH 6, at 0° overnight. The r-RNA precipitate was washed with 3 M-sodium acetate buffer, pH 6, and then dissolved in and reprecipitated from the SDS–acetate solution. RNA was stored at −20° as a suspension in the ethanolic SDS–acetate solution. For use in electrophoresis the suspension was centrifuged and the precipitate washed in aq. 75 % ethanol containing 0·5 % SDS and partially dried *in vacuo* for about 1 min. to remove the ethanol. It was then dissolved in electrophoresis buffer to a concentration of 1–4 mg./ml. The buffer also contained SDS (0·2 %) and sucrose (6 %).

Properties of the RNA. The molecular weights of r-RNA have been poorly documented. The molecular weights of RNA that have been assumed for the present work are given in Table 1.

The assumed weights of the HeLa-cell r-RNA are the highest of the range given by Hamilton (1967) for mammalian RNA, and are somewhat higher than the values quoted by Petermann & Pavlovec (1966). The values are consistent with electrophoretic mobility as described below. It was shown previously that HeLa-cell RNA prepared by the hot-phenol method is closely similar to other mammalian RNA prepared by extraction in the cold (Loening 1968*b*). The hot-phenol extraction results in the loss of a '7 s' piece of RNA from the '28 s' component, and probably causes a slight unfolding of the latter (Pene, Knight & Darnell, 1968). The effects of loss of weight and unfolding

Table 1. *Molecular weights of RNA*

Species	$10^{-6} \times$ Mol.wt.	Reference
E. coli	1·08 and 0·56	Stanley & Bock (1965)
HeLa cells	1·75 and 0·70	Hamilton (1967)
TMV	2·0	Boedtker (1960)
BMV	1·07, 0·76 and 0·32	Brockstahler & Kaesberg (1965)
Pea	1·28 and 0·71	} This paper, and Loening (1968*b*)
Drosophila	1·41 and 0·73	
Xenopus	1·52 and 0·70	

are opposed in gel electrophoresis, so that the net effect on mobility is small. It is possible that the small difference between the molecular weights of HeLa-cell RNA ($1·75 \times 10^6$) and other mammalian RNA ($1·72 \times 10^6$) is reproducible and is due to this effect (Loening, 1968*b*).

It is possible that the assumed molecular weights are not the true values. The present paper, however, examines changes in the apparent values due to differences in the electrophoretic conditions. The argument does not depend on exact knowledge of the absolute molecular weights; evidence is presented below that the relative values are correct.

To avoid confusion in the nomenclature of the r-RNA components of different species, they are referred to by their assumed molecular weights.

Sedimentation coefficients of some of the RNA species were determined by using a Spinco model E analytical ultracentrifuge. The r-RNA was dissolved to a concentration of approx. 40 μg./ml. (E_{260} 1·0) in the same buffers as used for electrophoresis (without sucrose). Centrifugation was at 44720 rev./min. at 24°; pictures were taken at 4 min. intervals. No corrections for viscosity or density were applied, since the buffers were closely similar and only comparative values were required. The sedimentation coefficients obtained are given in Table 2. Duplicate determinations suggested that the reproducibility was approx. ±0·5 s.

The base compositions of some of the RNA species are given in Table 3.

Electrophoresis. Polyacrylamide gels containing 2·0–2·6 % of acrylamide (recrystallized from chloroform) were prepared as previously described by Loening (1967, 1968*a*). The concentration of bisacrylamide was 5 % of that of the acrylamide throughout.

Three buffers were used: (1) E buffer contained 36 mM-tris, 30 mM-NaH_2PO_4 and 1 mM-EDTA (disodium salt), pH 7·7–7·8 at room temperature. The running buffer in the buffer compartments also contained SDS (0·2 %) ('specially purified' grade; British Drug Houses Ltd., Poole, Dorset). This buffer has a greater buffering capacity and a lower u.v. absorption than the tris–acetate buffer described by Loening (1967), and has been used as a routine for gel electrophoresis of RNA. The use of SDS minimizes nuclease action; the electrophoresis can then be carried out at room temperature without degradation of the RNA. Details of stock solutions and a table of volumes for the simple preparation of a range of gel concentrations were published by Loening (1968*a*). (2) Low-salt buffer contained 30 mM-tris, 16 mM-HCl and 0·1 mM-EDTA (disodium salt), pH 8·1 at 25°. SDS (0·1 %) was added to the running buffer. (3) Mg

Table 2. *Sedimentation coefficients of RNA*

For details of buffers, see under 'Electrophoresis'.

		S (s)	
Species	In low-salt buffer	In E buffer	In Mg buffer
E. coli	—	19·4	—
	—	15·2	—
Pea	21·0	22·0	28·6
	14·8	15·9	19·6
Drosophila	20·8	24·6	31·4
	14·1	16·2	21·0
Xenopus	24·4	26·6	34·4
	15·2	16·1	20·0
HeLa cells	27·4	29·3	36·4
	15·0	16·0	19·6
TMV (approx. value)	24	32	34

Table 3. *Base compositions of RNA*

	G+C content (moles/100 moles)		
Species	'28s'	'18s'	Reference
HeLa cells	66·8	55·7	Willems, Wagner, Laing & Penman (1968)
Xenopus	63·5	52·8	U. E. Loening (unpublished work)
Pea	55	49·0	U. E. Loening (unpublished work)
Drosophila	42·1	43·8	Hastings & Kirby (1966)

buffer was the same as the low-salt buffer but with magnesium acetate (2 mM) added.

In the preparation of gels with the last two buffers, a solution containing the acrylamide and catalysts in the low-salt buffer was prepared, and 12 μl. of 0·25 M-magnesium acetate was added to a 15 ml. portion of the solution. In this way gels made with the two buffers were closely comparable and variations between batches were avoided. The small amount of EDTA added to these buffers was to chelate heavy-metal ions, which can cause the RNA to streak during electrophoresis. The Mg^{2+} ion was present in a 20-fold excess over the EDTA, so that chelation of the Mg^{2+} ion can be neglected. The Na^+ ion concentration was kept at a minimum to show the greatest effect of Mg^{2+} ion on the RNA (Boedtker, 1960).

In all cases the gels were pre-run for at least 30 min. before the RNA sample was applied, to remove some of the polymerization catalysts and to allow the SDS to enter the gels. Gels in E buffer were run at room temperature (20–25°) at a potential gradient of 7 v/cm. and 5·1 mA/gel of 0·25 in. diam. Gels in low-salt and Mg buffers were run at 25° in an incubator, at 7 v/cm. and 3·2 mA/gel. Magnesium dodecyl sulphate crystallized out at lower temperatures.

In most cases the RNA was dissolved in the buffer in which it was to be fractionated, but also containing 6% of

sucrose; however, the results depended only on the running buffer and were independent of the buffer in the small volume of applied RNA solution. A 25 μl. volume or less of RNA solution was layered on the gels. Mixtures of several species of RNA were prepared beforehand and layered as a single solution; two species could be compared, however, by layering them sequentially in 10 μl. each.

Electrophoresis was continued for the times shown in the individual figures. The gels were then scanned at 265 nm. in a Joyce–Loebl Chromoscan fitted with a medium-pressure mercury lamp (ST 75), a 265 nm. interference filter and a liquid filter containing *p*-dimethylaminobenzaldehyde (15 mg./100 ml. of A.R. methanol). This filter removes the strong 365 nm. emission from the lamp; it is unstable in daylight, but stable to u.v. light. The slit width used was 2 mm. × 0·1 mm. Since this work was completed it has been found that a limiting factor in the resolution of adjacent peaks was the focusing of the image of the slit in u.v. light. The adjustment required owing to shortening of the focal length of the lens is much greater than had been supposed. When proper correction for this is made, the resolution between close peaks is at least doubled.

A broad band of high background density was frequently found near the top of the gels, particularly when the gels had been stored for a day or more without pre-running or when the ammonium persulphate catalyst was impure. This density was removed after electrophoresis by washing in water or in buffer for $\frac{1}{2}$–3 hr. There was almost no diffusion of high-molecular-weight RNA even after washing for 12 hr., but transfer RNA was largely eluted in this time.

RESULTS

Resolution of the method. To determine the resolution and reproducibility of the electrophoretic separations, the three possible mixtures of RNA components of two of the three species, *Xenopus*, *Drosophila* and pea, were fractionated as shown in Fig. 1. The larger r-RNA components of the three species were readily resolved from each other. Their apparent molecular weights were obtained by comparison of their mobilities with those of HeLa-cell RNA and *E. coli* RNA as in Fig. 3, and are given in Table 1. Clearly differences in molecular weight of less than $0·1 \times 10^6$, or 7%, can be resolved.

The smaller ribosomal components of the three species were clearly similar, with a molecular weight of approx. $0·7 \times 10^6$. No resolution was obtained between pea RNA and *Xenopus* RNA or between pea RNA and *Drosophila* RNA, but in the mixture of *Xenopus* RNA and *Drosophila* RNA two components could just be distinguished. The apparent difference in molecular weight between them was $0·03 \times 10^6$. Their relative quantities, and separate gels run with the RNA of each species mixed with *E. coli* RNA, showed that the component of lower mobility was the *Drosophila* RNA. It thus appears that the pea RNA of mol.wt. $0·7 \times 10^6$ is intermediate in mobility between the *Drosophila* RNA and *Xenopus* RNA, and that this difference in molecular weight, approx. $0·015 \times 10^6$, is below the
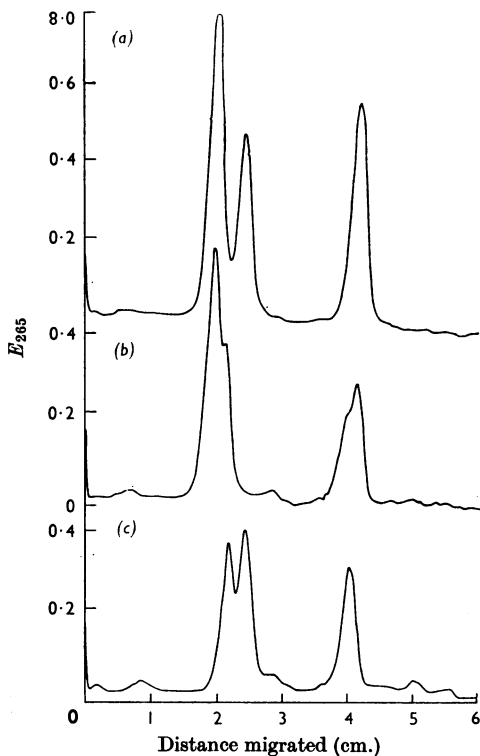
Fig. 1. Electrophoresis of mixtures of similar r-RNA species. Samples (10 μl.) of pea r-RNA (1 mg./ml.), *Drosophila* r-RNA (1 mg./ml.) and *Xenopus* r-RNA (2 mg./ml.) were used. Two RNA species were applied to each of three gels, as shown. (a) *Xenopus* and pea; (b) *Xenopus* and *Drosophila*; (c) pea and *Drosophila*. The samples were applied sequentially; electrophoresis was in 2·4% gels in E buffer for 3 hr. at 24°. Further details are given under 'Electrophoresis'. The gels were scanned by a Chromoscan at 265 nm.

limits of resolution. Since this work was completed it has been found that the resolution between adjacent peaks was limited by the optical scanning, as described in the Methods section.

Fig. 1 also illustrates the reproducibility between separate gels run at the same time in the same tank. The reproducibility between different batches of gels, or gels of different ages from the same batch, was very poor in comparison. This is because the effective gel concentration or pore size varies with the polymerization conditions and with the age of the gel.

Effects of changes in gel concentration. The relative electrophoretic mobility of RNA was shown to be related to the sedimentation coefficient by Richards *et al.* (1965) and to log (molecular weight) by Bishop *et al.* (1967). It is clear, however, that the relative

mobility of two RNA molecules varies with the concentration of the gel and perhaps with other conditions (Loening, 1967). It is therefore essential to show that an apparent molecular weight can be obtained that is independent of the conditions of electrophoresis.

It has been found that the effective gel concentration for the same nominal concentration varies in different laboratories. In the present experiment the nominal concentrations were varied, as a model for other differences.

A separation of a mixture of TMV RNA, HeLa-cell r-RNA, pea r-RNA and *E. coli* r-RNA is shown in Fig. 2. This separation was repeated with gels of different concentrations, all run in the same tank at the same time. The relation between electrophoretic mobility and the logarithm of the assumed molecular weight of the RNA species is shown in Fig. 3. The absolute mobilities all increased with decreasing gel concentration. In addition, the relative mobilities varied with different gel concentrations; for example, the mobility of the HeLa-cell RNA component of mol.wt. $1·75 \times 10^6$ was 0·53 and 0·19 times that of the *E. coli* RNA component of mol.wt. $0·56 \times 10^6$ in the 2·0% gel and the 2·6% gel respectively. Nevertheless at all gel concentrations a linear relation was obtained between mobility and log (molecular weight) of r-RNA. Thus for the determination of apparent molecular weight a single known marker is insufficient; at least two markers are required. A similar result was obtained with mixed agar-polyacrylamide gels, over a larger range of molecular weights but with less precision, by Peacock & Dingman (1968).

The TMV RNA did not quite fit the above relation. At all gel concentrations its molecular weight was apparently higher than the published value. This could be because the molecule is relatively unfolded compared with r-RNA and has a relatively low sedimentation coefficient (Boedtker, 1960). Its apparent fit in line for the 2·6% gel was due to the closeness of its band to the origin on the gel. The apparent molecular weight was also not independent of gel concentration, but varied between $2·2 \times 10^6$ and $2·5 \times 10^6$. Further, some TMV RNA samples also contained a second heavier component (not shown).

The distance between the peaks is approximately the same at all gel concentrations, so that the lines in Fig. 3 are parallel; the resolution between peaks thus depends on the time of separation and not much on gel concentration. To increase the resolution it is necessary to increase the time of electrophoresis; the gel concentration can be increased to keep the separation within a convenient total length of gel and to minimize diffusion.
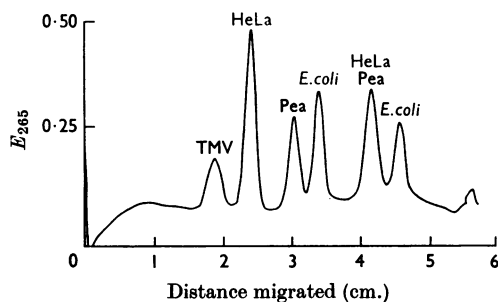
The true polymer concentration of a gel can be

Fig. 2. Electrophoresis of a mixture of distinct RNA species. A mixture was prepared containing 50–80 μg. of each of HeLa-cell r-RNA, pea r-RNA, *E. coli* r-RNA and TMV RNA in 200 μl. of E buffer (with SDS and sucrose). A 20 μl. sample of the mixture was layered on the gel. Electrophoresis was in a 2·0% gel in E buffer, for 2·5 hr. The gel was scanned by a Chromoscan at 265 nm. immediately after electrophoresis; the background density, seen particularly as a broad shoulder over the first 1·5 cm. of the gel, could be greatly decreased by washing the gel in buffer or water. Further details are given under 'Electrophoresis'.
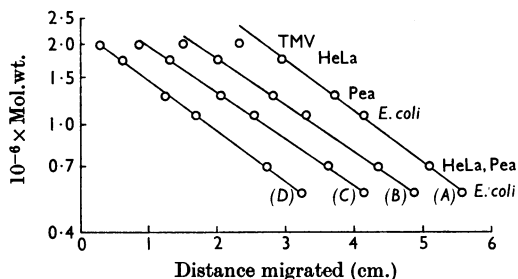

Fig. 3. Relation between molecular weight and gel electrophoretic mobility at different gel concentrations. The mixture of RNA as in Fig. 2 was fractionated in 2·0% (A), 2·2% (B), 2·4% (C) and 2·6% (D) gels as indicated. Electrophoresis of the four gels in E buffer was done together in one tank for 2½ hr. at 24°. The gels were washed for several hours before being scanned; the apparent mobilities in the 2·0% gel are therefore higher than in the experiment of Fig. 2 because of the increase in length of the gel during washing. The molecular weights of the RNA are plotted on a logarithmic scale against distance migrated in each gel.

decreased by washing it in buffer for 1–2 days before electrophoresis. The gels then swell to about 1½ times their former volume. It was found that the mobility of RNA then increased only slightly; if the gels were run in their original-diameter tubes, in which they increased disproportionally in length, and were then scanned in the open quartz cuvette, in which the length returned approximately to that for the free swollen condition, the increase in mobility was exactly compensated by the decrease in length of the gel in the cuvette. It was also found that Semliki Forest virus RNA (kindly given by Dr E. Martin, National Institute for Medical Research, Mill Hill, London N.W.7) did not enter either a fresh or a pre-swollen 2·4% gel, but had a high mobility in a 2·0% gel. It is concluded that the pre-swelling does not increase the pore size of the gels appreciably. The linear relationship of mobility to log (molecular weight) was maintained in the swollen gels.

The first 2 mm. of most gels has a lower effective gel concentration. The mobility of components near the origin is therefore overestimated, as seen with TMV RNA in the 2·6% gel (Fig. 3). This effect can be avoided either by continuing electrophoresis for much longer times or by cutting off the first few millimetres of the gel with a razor blade.

Effects of changes in conformation. The similarity in apparent molecular weight between *Drosophila* RNA and *Xenopus* RNA (Fig. 1) and the large difference in base composition (Table 3) suggested that these two species would be suitable examples for the experiments on the effects of conformational changes. The fractionation shown in Fig. 1(b) in E buffer was repeated with the low-salt and Mg buffers. Fig. 4(a) shows that in the Mg buffer the two RNA components of mol.wt. $0·7 \times 10^6$ were exactly coincident; there was no evidence of the small separation obtained in E buffer. The RNA components of mol.wt. $1·41 \times 10^6$ and $1·52 \times 10^6$ were separated according to weight as before. When the mixture was fractionated in the low-salt buffer, the *Drosophila* RNA components would be expected to unfold more than the *Xenopus* RNA components, so that their relative mobilities should fall. Fig. 4(b) shows that this is the case; the RNA components of mol.wt. $0·7 \times 10^6$ separated into two peaks, of which the slower was the *Drosophila* RNA (as determined on separate gels). The *Drosophila* RNA component of mol.wt. $1·41 \times 10^6$ also had a decreased mobility so that its peak became almost coincident with the peak of the *Xenopus* RNA component of mol.wt. $1·52 \times 10^6$.

Fig. 4(b) shows some breakdown products of the *Drosophila* RNA in low-salt buffer. It is assumed that these are the results of the release of broken pieces of the polynucleotide chain when the molecule is sufficiently unfolded. Such breakdown products were not found with the *Xenopus* RNA (run alone in separate gels). The *Drosophila* RNA showed few breakdown products in E buffer and none in the Mg buffer. Fig. 4(a) shows that in the Mg buffer there was a peak of high-molecular-weight RNA, due presumably to aggregation. The components of this have not been identified.

The separations shown in Figs. 4(a) and 4(b) were run at the same time in adjacent tanks at the
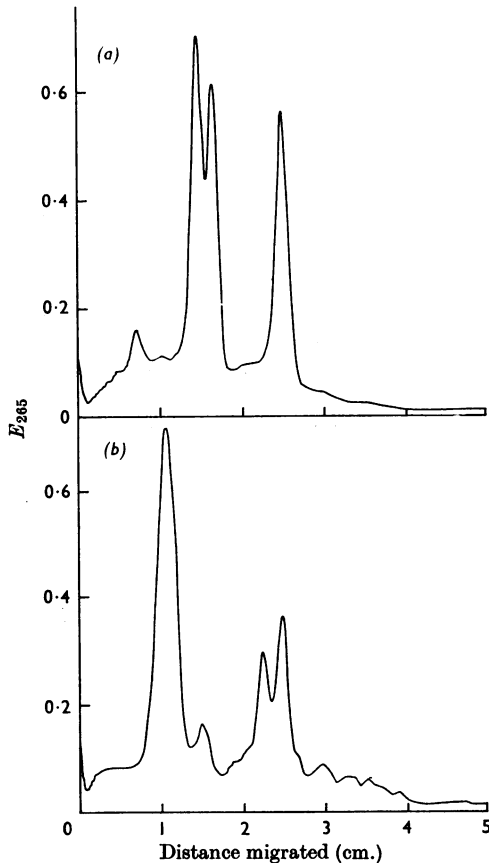
Fig. 4. Effects of low-salt and Mg buffers on the mobilities of RNA species of different base compositions. The electrophoresis of the mixture of *Xenopus* RNA and *Drosophila* RNA shown in Fig. 1(*b*) was repeated in (*a*) the Mg buffer and (*b*) the low-salt buffer. Electrophoresis was in 2·4% gels for 2½ hr. at 25° (approx. 3·5 mA/tube at 50 v). The gels were scanned by a Chromoscan at 265 nm.
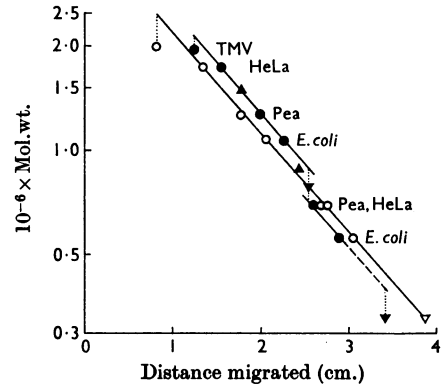

Fig. 5. Relation between molecular weight and electrophoretic mobility in low-salt and Mg buffers. Electrophoresis of the mixture of TMV RNA, HeLa-cell r-RNA, pea r-RNA and *E. coli* r-RNA shown in Figs. 2 and 3 was repeated in the low-salt (○) and in the Mg (●) buffers. Electrophoresis was in 2·2% gels for about 2 hr. as in Fig. 4. The mobilities of *Amoeba* RNA and BMV RNA were determined relative to those of HeLa-cell r-RNA and *E. coli* r-RNA in separate experiments and are normalized to fit on the same scale. ○ and ▽, Low-salt buffer; ●, ▼ and ▲, Mg buffer. ○ and ●, Components of the mixture as in Figs. 2 and 3; ▽ and ▼, BMV RNA; ▲, *Amoeba* RNA. Scans of *Amoeba* RNA have been published by Loening (1968*b*). In most cases the mobilities of RNA in the Mg buffer were slightly greater relative to those in the low-salt buffer than is shown here. The components of mol.wt. 0·56 × 10⁶ and 0·7 × 10⁶ then had the same mobility in Mg buffer as in low-salt buffer, as shown in the example of Fig. 4.

same voltage. The electrophoretic conditions were therefore closely comparable. The *Xenopus* RNA component of mol.wt. $0·7 × 10^6$ had approximately the same mobility in both buffers. The mobilities of the *Xenopus* and *Drosophila* RNA components of mol.wt. $1·52 × 10^6$ and $1·41 × 10^6$ were only slightly greater in the Mg buffer than in the low-salt buffer. Thus the absolute mobilities of the components are not much affected by a structural change of the RNA that results in very large changes in sedimentation coefficients (Table 2). The relative decrease of mobility of the *Drosophila* RNA in low-salt buffer is correlated with small differences in sedimentation coefficients between *Drosophila* RNA and *Xenopus* RNA. This result shows that, although unfolding of a 'low-G + C' RNA in low-salt

buffer results in a decrease in mobility, there is some factor that opposes the expected increase of mobility of all types of RNA when the molecules are compact in Mg buffer.

The small difference in the effect of Mg buffer on the larger and smaller ribosomal components (Fig. 4) is also seen when the mixture of Fig. 2 is fractionated in Mg buffer and low-salt buffer. Fig. 5 shows that there is a discontinuity in the relation between log (molecular weight) and mobility in Mg buffer. All the larger ribosomal components appear to have mobilities slightly higher in relation to their weight than have the smaller components, suggesting either some structural difference between the two components or some change in electrophoretic properties that occurs at mol.wt. about $0·8 × 10^6$. The difference between the two ribosomal components is not due to their different base compositions (Table 3), since the *Drosophila* and *Xenopus* components behave similarly.

To investigate the discontinuity in the relation in Mg buffer further, the mobilities of RNA species intermediate in molecular weight between the *E.*

coli RNA of 1.07×10^6 and the animal or plant RNA of 0.7×10^6 were determined. The smaller ribosomal component of *Amoeba* provides one example. This has a molecular weight determined in E buffer of 0.89×10^6 (Loening, 1968b). Fig. 5 shows that its electrophoretic mobility in Mg buffer bridges the discontinuity in the linear relation. In low-salt buffer this RNA has a lowered mobility, an effect similar to that with *Drosophila* RNA. A second example is the middle one of the three components of BMV RNA. Electrophoresis in E buffer indicated that the molecular weights of BMV RNA relative to HeLa-cell RNA and *E. coli* RNA were 1.07×10^6, 0.78×10^6–0.79×10^6 and 0.33×10^6. These are in reasonable agreement with the published molecular weights (Table 1). In low-salt buffer the component of mol.wt. 0.78×10^6 was slightly unfolded, so that its apparent molecular weight was 0.81×10^6. In Mg buffer, in which the distance between the *E. coli* RNA of mol.wt. 1.07×10^6 and the HeLa-cell RNA of mol.wt. 0.7×10^6 is much decreased, the position of the BMV RNA of mol.wt. 0.78×10^6 was almost coincident with that of the HeLa-cell RNA of mol.wt. 0.7×10^6 (Fig. 5). The apparent weight of the BMV RNA was then 0.73×10^6 relative to the RNA components of mol.wt. 0.7×10^6 and 0.56×10^6, and 0.92×10^6 relative to the larger ribosomal components. Thus the BMV component also bridges the discontinuity in the linear relation.

The smallest of the BMV components showed a decreased mobility in the Mg buffer, with an apparent molecular weight of 0.4×10^6 by extrapolation, as shown in Fig. 5.

The mobilities of TMV RNA are also indicated in Fig. 5. In Mg buffer the molecule is clearly more compact than in E buffer, but its mobility still did not lie on the line of those of the larger ribosomal components. In low-salt buffer, TMV RNA expands, and it had a much lower relative mobility than r-RNA. Its apparent molecular weight is then about 2.5×10^6.

In the low-salt buffer the mobilities of most of the RNA species were linearly related to their log (molecular weight) values, as in E buffer (Fig. 5). The decreased mobility due to low-salt buffer was found only with 'low-G + C' RNA such as *Drosophila* RNA, and could just be observed with the pea RNA component of mol.wt. 0.7×10^6 as shown in Fig. 5.

DISCUSSION

The results confirm the expectation that the electrophoretic mobility of a 'low-G + C' RNA falls relative to that of a 'high-G + C' RNA when the salt concentration in the gel is lowered to that of the low salt buffer. This relative fall is correlated with a small relative fall in sedimentation coefficient, showing that the 'low-G + C' RNA unfolds more than the 'high-G + C' RNA.

When the RNA components of mol.wt. 0.7×10^6 of *Drosophila*, pea and *Xenopus* are compared, it appeared that the relative mobilities in both low-salt buffer and E buffer were correlated directly with the base compositions. In both cases the mobility of the pea RNA was intermediate between those of the *Drosophila* RNA and the *Xenopus* RNA, but the differences in mobility in E buffer were small and close to the limit of resolution of the method. In the presence of Mg^{2+} ions all three components had the same mobility. The simplest conclusion therefore is that the true molecular weights of the RNA components of mol.wt. 0.7×10^6 of the three species are exactly identical, and that the small separation obtained in E buffer is due to conformational differences. This conclusion is consistent with the sedimentation coefficients in E and low-salt buffers.

The RNA component of mol.wt. 0.7×10^6 of the pea r-RNA is therefore similar to that of the animals, and differs markedly from the smaller *E. coli* component in electrophoretic mobility and in sedimentation coefficient. This result is contrary to that obtained by Click & Tint (1967) and by Stutz & Noll (1967) by density-gradient centrifugation. The discrepancy has not been explained.

It is probable then that the mobility of r-RNA in E buffer is closely related to the true log (molecular weight). Small overestimates of molecular weight are obtained with RNA of very low G + C content. Thus the molecular weight of the *Drosophila* RNA of mol.wt. 1.41×10^6 has probably been slightly overestimated relative to that of the *Xenopus* RNA of mol.wt. 1.52×10^6.

The above results in low-salt buffer were as predicted and are correlated with small differences in sedimentation coefficients. A much more striking finding, however, is that the very large differences between the sedimentation coefficients of RNA components in the low-salt buffers and those in the Mg buffers appeared to result in almost no change in the absolute mobilities of the RNA. The mobilities of the larger r-RNA components were increased slightly in the Mg buffer compared with those in the low-salt buffer, but the smaller components had unchanged mobilities and the small BMV RNA component of mol.wt. 0.33×10^6 had a lower mobility in the Mg buffer than in the low-salt buffer. The experiments suggest that the increase in mobility due to the compactness of the molecules in Mg buffer was opposed by another factor such as a loss in negative charge. This could be brought about by the masking of internal charges in the molecule, possibly by binding Mg^{2+} ions. The proportion of masking would be relatively less for

the larger-molecular-weight RNA, which shows a slightly increased mobility in Mg buffer.

Some masking of charge is implicit in the common statement that RNA cannot be fractionated according to molecular weight by free electrophoresis because the ratio of charge to mass is constant (Richards & Gratzer, 1968). This statement assumes that all parts of the molecule have the same frictional resistance to flow. This is clearly not the case for a large folded molecule. The relative frictional resistance decreases per unit mass for the larger molecules, which is the basis of the fractionation of RNA by sedimentation. Since experimentally RNA cannot be fractionated by free electrophoresis, it follows that its effective charge becomes masked exactly in proportion to its lowered frictional resistance.

In Mg buffer, the masking of charge or other opposing property of RNA is slightly less effective in larger molecules than in smaller, as is shown by the discontinuities in the relation of mobility to molecular weight in Fig. 5. Electrophoresis in Mg buffer cannot therefore be used directly for the determination of molecular weights, but closely similar molecules can be compared and the technique is useful for the detection of the extent of folding relative to that in other buffers. It may be predicted that in Mg buffer free electrophoresis of RNA species should show small differences in mobility according to mass; the larger RNA molecules would have the higher mobility.

The comparison between r-RNA and viral RNA shows some discrepancies. The molecular weights of the BMV RNA were determined approximately correctly relative to r-RNA, but the molecular weight of TMV RNA was overestimated by 10–20% under all conditions. It was the only case in which the apparent molecular weight varied with gel concentration. Bishop *et al.* (1967) also found that the weight of TMV RNA was overestimated by about 12% relative to that of BMV RNA and *E. coli* RNA. The relation between the sedimentation coefficient and the mobility of TMV RNA is not simple (the S values in Table 2 are not sufficiently precise for exact comparisons). TMV RNA thus has to a small extent the properties of DNA, in which the mobility is almost independent of the molecular weight (Loening, 1967) and does not vary much with gel concentration (U. E. Loening, unpublished work). This result is to be expected if TMV RNA has long stretches of base-paired double-helical regions. Several properties of this molecule therefore require further study, such as the effects of the irreversible change in sedimentation coefficient after heat treatment of a fresh preparation (Boedtker, 1960).

This paper describes only relative values of molecular weights. The published values of the molecular weights of r-RNA were modified slightly (Table 1) to fit with the electrophoretic mobilities and some values were assumed only from electrophoretic determinations. Small adjustments of the values used in this paper may therefore be expected when further absolute determinations of molecular weights become available.

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