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Preparative Two-Dimensional Polyacrylamide Gel Electrophoresis of ^{32}P-Labeled RNA

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The technique of polyacrylamide gel electrophoresis (GE) has been successfully applied to the fractionation of mixtures of transfer RNA and ribosomal RNA (1,2), viral RNA (3), and fragments obtained by partial hydrolysis of ribosomal (4,5) and viral (6) RNA molecules with ribonucleases. In particular, preparative electrophoresis on gel slabs has made possible the isolation and subsequent sequence analysis of important RNA fragments obtained by partial digestion of bacteriophage RNAs (7,8) and of 16S ribosomal RNA of *Escherichia coli* (9).

Fractionation of such partial digests at pH 8 results in a pattern of bands, the mobility of which decreases with the logarithm of the chain length of the fragments they contain (10). In the case of bacteriophage RNA digests, however, each band is a rather complex mixture of a few fragments of similar size, derived from different parts of the molecule, plus a number of smaller fragments moving as complexes held together by base-pairing interactions. These mixtures can be further resolved by gel electrophoresis at low pH in the presence of high urea concentrations (11). Under these conditions complexes between separate chains dissociate, and the mobility is a function not only of molecular weight but also of base composition. But even after purification on this type of gel, RNA fragments are often not entirely free of contaminants. We therefore worked out a two-dimensional method combining the fractionation at pH 8 with the one at pH 3.5 in the presence of urea.

Two-dimensional polyacrylamide gel electrophoresis was originally proposed by Raymond and Aurell (12) and recently applied by Kaltschmidt and Wittmann (13) for the fractionation of ribosomal proteins. The procedure reported here is suitable for the fractionation of complex RNA mixtures, and involves only the simple apparatus described previously (11). The fact that the RNA is concentrated in small spots after the separation has allowed the development of a modified extraction method.

MATERIALS AND METHODS

Apparatus

This has been described in detail elsewhere (11) and only the modifications necessary for two-dimensional separations are mentioned here *in extenso*. The electrophoresis cell is the one proposed by Akroyd (14), with adapted dimensions. It consists essentially of two glass plates forming the front and back walls, kept at 2 mm distance by two Perspex strips which form the side walls of the cell (Figs. 1 and 2). It is kept together by steel paper clips and provided with a provisional Plasticine bottom during the preparation of the gel. The dimensions of the cell are 40 × 20 cm for the separation in the first dimension and 30 × 25 cm for the second dimension.

For electrophoresis the cell with the prepared gel slab is put in the lower buffer reservoir and connected with the upper one by means of a wick consisting of two layers of Whatman 3MM paper. In the second dimension three separations may be carried out simultaneously in the arrangement illustrated in Fig. 2b. Since no buffer circulation system is used, the total capacity of the buffer reservoirs should be adapted to

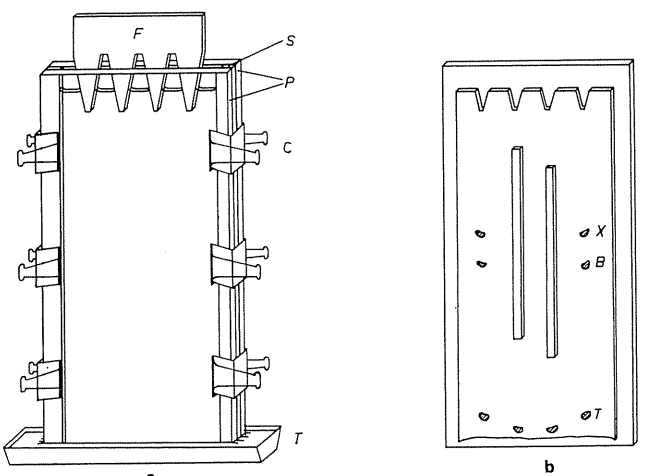

FIG. 1. Electrophoresis cell for separation in the first dimension: (a) During preparation of slab. P, glass plates, 20 × 40 × 0.4 cm; S, Perspex strips, 1.5 × 40 × 0.2 cm; C, steel clips; F, Perspex slot former; T, trough with Plasticine. (b) Dismantled after separation. The front glass plate and Perspex strips have been removed. Gel strips, 1 × 15 to 1 × 20 cm, which contain the radioactivity as detected by autoradiography, are cut out. These strips contain the dye markers xylene cyanol FF (X) and bromophenol blue (B), while trypan red (T) has run to the end of the slab.

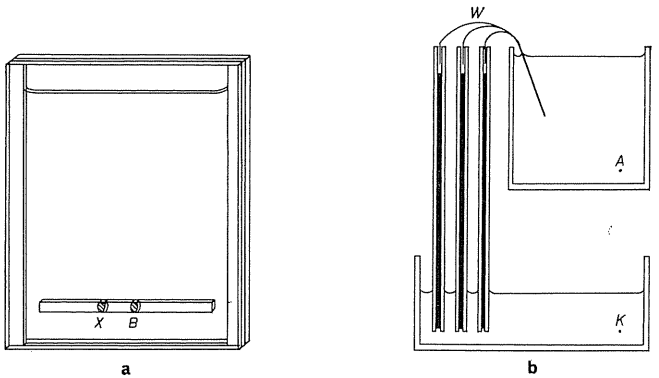

FIG. 2. Electrophoresis cell for separation in the second dimension: (a) During preparation of slab. A strip cut out of the first-dimensional, acid gel (Fig. 1b) is aligned parallel to, and at 4 cm from, a short edge of a 25 × 30 cm glass plate, which is assembled to a new cell. The latter is filled with buffer and monomer solution to 3 cm from the top. Plasticine bottom and steel clips are omitted from the drawing. X and B are dye markers as in Fig. 1b. (b) During electrophoresis (transverse section): Three cells are put in the lower buffer compartment containing the cathode K. Paper wicks W form electrical contact with the upper buffer compartment containing anode A. Other details are described in the text and in reference 11.

the number of separations carried out in order to avoid important pH fluctuations. Satisfactory results were obtained with a total buffer volume of about 3 liters per gel slab, which resulted in a pH deviation of less than 0.5 unit on either side of the initial value.

For safety the entire assembly is put under a Perspex cover. Lifting this cover acts upon a switch interrupting the tension on the electrodes.

Composition of the Gels

Since the net charge of an RNA molecule is appreciably lower at pH 3.5 than at pH 8, the acrylamide concentration of the acid gel must be lower than that of the neutral gel if the molecule is to have approximately the same mobility in both directions. It is then advantageous to choose the acid gel as the first dimension and the neutral gel as the second. At the start of the second separation the pH difference between the two gels rapidly disappears. The RNA moves from a more diluted into a more concentrated gel, which results in a sudden drop in mobility and a concomitant zone sharpening. Apart from these considerations it is desirable to separate first under the most denaturing conditions, i.e., at acid pH and in the presence of urea. This applies particularly to mixtures of RNA fragments such as those present in the bands obtained

after one-dimensional gel electrophoresis of partial hydrolyzates of bacteriophage RNA. These mixtures contain complexes kept together by intermolecular base-pairing, which must be dissociated and resolved before a refractionation according to chain length is attempted.

The acrylamide concentrations chosen for the first dimensional, acid gel, are 10% for fractionation of RNA mixtures with a maximum chain length of about 80 nucleotides, and 8% when the maximum chain length is higher. The concentration of the second, neutral gel, is twice that of the acid gel. Acrylamide concentrations are expressed as per cent weight/volume and do not include the cross-linker *N,N'*-methylenebisacrylamide added in the weight ratio 1:30. Both reagents, of the grade purissimum, are obtained from Serva (Heidelberg, Germany), and are used without further recrystallization.

The buffer in the reservoirs and in the gel has the same composition, viz., 0.025 *M* citric acid, 6 *M* urea for the separation in the first dimension, and 0.04 *M* Tris-citric acid, pH 8, for the second dimension. Table 1 summarizes the concentrations and amounts of stock solutions needed

TABLE 1
Composition of Gels

The concentration of the gels is expressed in per cent (weight/volume) acrylamide, not including the cross-linker. Solutions of ammonium persulfate, ferrous sulfate, and ascorbic acid must be freshly prepared at regular intervals, as they deteriorate on storage. Other stock solutions are kept frozen until use.

	Vol. (ml) required to prepare 150 ml gel			
	1st dimension		2nd dimension	
Stock solution	8% acid	10% acid	16%, pH 8	20%, pH 8
Monomer/buffer:				
Acrylamide, 400 gm/liter, + *N,N'*-methylenebisacrylamide, 13 gm/liter	30	37.5	60	75
9 *M* urea	100	100	—	—
1 *M* citric acid	3.75	3.75	—	—
1 *M* Tris adjusted to pH 8 with citric acid	—	—	6	6
Catalyst:				
$FeSO_4 \cdot 7H_2O$, 2.5 gm/liter	0.6	0.6	—	—
Ascorbic acid, 100 gm/liter	0.6	0.6	—	—
H_2O_2, 300 gm/liter	0.06	0.06	—	—
N,N,N',N'-Tetramethyl-ethylenediamine	—	—	0.06	0.06
$(NH_4)_2S_2O_8$, 100 gm/liter	—	—	0.6	0.6

for the preparation of each gel, as well as the catalyst to be added immediately before the mixture is poured into the electrophoresis cell for polymerization.

Separation in the First Dimension

Cleaning of the glass plates, assembly of the cell, pouring of the slabs, loading of the sample and autoradiography have been described in detail (11). These parts of the procedure are carried out at room temperature. The slabs, 17 by 35 cm in size, are provided with 4 to 6 slots of 5 × 2 mm by insertion of a suitable Perspex slot former before polymerization (Fig. 1a), depending on the number of separations to be carried out. The slabs are subjected to a prerun of about 1 hr before being loaded.

The ^{32}P-labeled RNA mixture to be fractionated is precipitated with ethanol in the presence of 100 μg carrier yeast RNA (BDH) and the precipitate collected in the tip of a disposable 1.5 ml conical plastic centrifuge tube (Eppendorf, Hamburg, Germany). The precipitate is carefully dried *in vacuo* and dissolved in 6 μl 6 *M* urea solution. The density is increased by addition of 3 μl of an aqueous solution containing 500 gm sucrose, 300 gm urea, 5 gm trypan red, 2 gm xylene cyanol FF, and 2 gm bromophenol blue per liter. After loading, the sample forms a layer of approximately 1 mm in the slot. The conditions of electrophoresis and the distances moved by the dye markers are given in Table 2. The separations are carried out in the cold room at 4°C but the temperature in the gel slabs is actually higher and depends on the electrical resistance and the applied tension.

The run is stopped when the fastest marker, trypan red, reaches the

TABLE 2
Conditions of Electrophoresis and Distances Migrated

	Gel			
	1st dimension		2nd dimension	
	8% acid	10% acid	16%, pH 8	20%, pH 8
Conditions:				
Voltage (V)	900	900	350	350
Current (mA)	57	51	50	43
Time (hr)	4	6	15	15
Distance migrated (cm)				
Xylene cyanol FF	16	17	10.5	7
Bromophenol blue	19	20	20	13.5
Trypan red	35	35	—	—

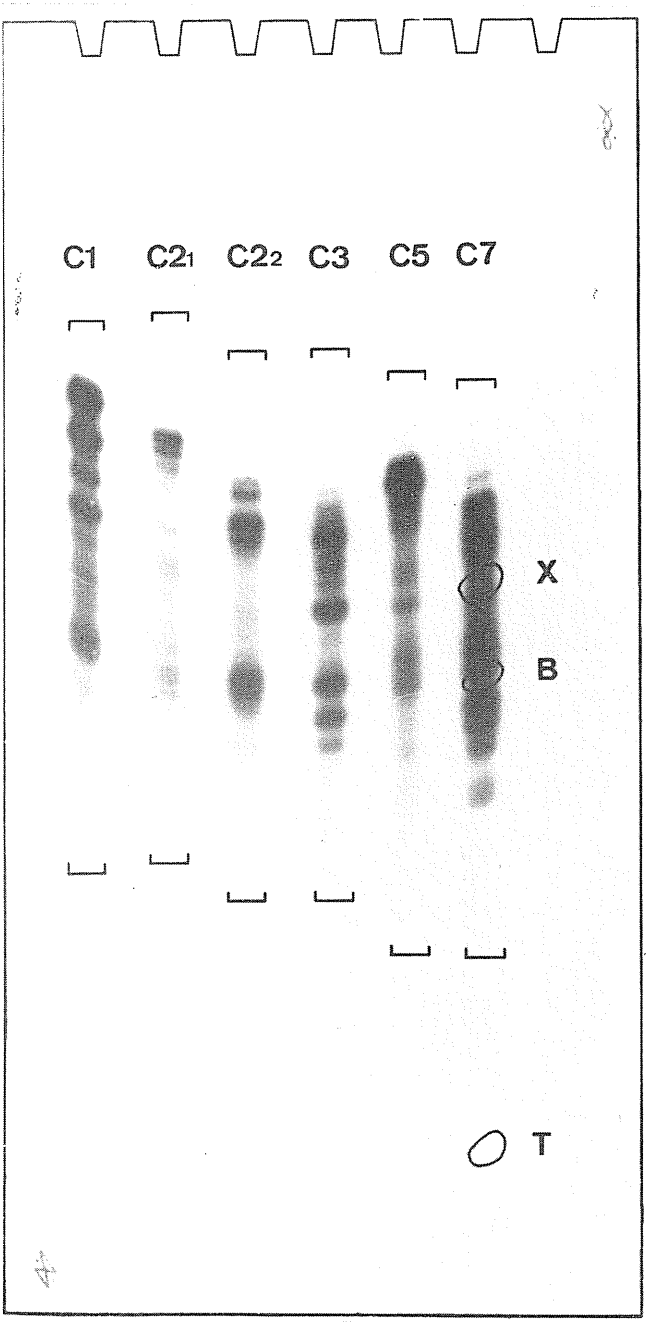

FIG. 3. Fractionation of RNA fragments, first dimension. Material extracted from bands C1, C2-1, C2-2, C3, C5, and C7 of an MS2 RNA partial digest separated on a 12% pH 8 gel (see reference 10 for numbering system) was subjected to electrophoresis on a 10% gel containing 0.025 *M* citric acid and 6 *M* urea, under

end of the slab. The cell is dismantled and an autoradiograph is prepared. Examples are shown under "Results" (Fig. 3) below. A rectangular piece of gel encompassing all the radioactivity, 1 cm wide and not more than 20 cm long, is cut out as indicated in Fig. 1b. A smooth and straight edge is obtained by cutting the gel slowly with a scalpel or razor blade along a ruler. The strip usually contains the blue xylene cyanol marker and the bromophenol blue spot, which is green at pH 3.5. Trypan red runs ahead of the fastest nucleotide material at this pH.

Separation in the Second Dimension

The strip excised from the acid gel is transferred with two pairs of tweezers onto a glass plate, 25 × 30 cm, which will form the back wall of the electrophoresis cell for the second dimension. As illustrated in Fig. 2a, the strip is aligned 4 cm from the bottom edge of the plate, and neatly parallel to it. The cell is then assembled, put upright into a trough with Plasticine, and filled with the proper monomer/buffer solution (Table 1) to 3 cm from the upper edge. Before polymerization occurs, some urea diffuses out of the gel strip into the surrounding solution. This results in threads of denser urea solution sinking to the bottom and causes a somewhat irregular polymerization below the strip. Since the RNA migrates from bottom to top in the second separation, this phenomenon does not disturb the pattern of spots.

Electrophoresis is started immediately after polymerization is complete, and is carried out overnight under the conditions mentioned in Table 2. Soon after the potential is applied, the color of the bromophenol blue in the strip changes from green to blue, indicating that the pH is rising to the value in the surrounding gel. When passing upward and leaving the strip, the dye spots are flattened into bands of some 2 mm thickness, due to their lower mobility in the upper, concentrated gel. Similarly the RNA, which occurs in the strip in small bands parallel to the direction of movement, are compressed to round spots of some 5 mm diameter.

After electrophoresis the cell is dismantled, the gel slab is covered with plastic film, provided with radioactive ink labels, and autoradiographed as described earlier (11). Spots of interest are cut out with a scalpel in small discs of about 5 mm diameter. The dye spots are useful references for locating particular components when separations have to be reproduced.

Recovery of RNA from the Gel

Since the RNA spots are concentrated in small discs of gel not exceeding 50 μl in volume, a simplified extraction procedure can be fol-

lowed, different from the one previously described (11) for larger pieces of gel.

The plunger is removed from a 2 ml disposable plastic syringe (Braun, Melsungen, Germany) and a disc of Whatman 52 paper, 10 mm in diameter, is pressed against the bottom. The disc, which may be cut with a sharp cork-borer, must be perfectly circular and fit snugly in the syringe. The gel disk is then introduced and crushed between the paper disc and the plunger by turning the latter around under firm pressure. This is conveniently done by inverting the syringe and pressing the head of the plunger against the center of a rubber-covered slowly rotating disc mounted on the shaft of an electric motor. The plunger can be carefully withdrawn, while being turned in the opposite direction, so that the gel cake and the paper disc stick to the bottom of the syringe.

To the gel cake is added 500 μl 1 *M* NaCl and the syringe is shaken on a vortex, rapidly enough to bring the gel particles in suspension but gently enough to leave the paper disc undisturbed. After about 10 sec, liquid starts running through the disc and by that time the syringe is placed on top of a disposable 1.5 ml plastic centrifuge tube for collection of the filtrate. After a few minutes standing, the last drops are pressed out of the syringe by introduction of the plunger.

A suitable amount of carrier yeast RNA, usually 20 to 100 μg, is added to the extract. After thorough mixing with 500 μl isopropanol, the RNA precipitate is allowed to form in the cold and collected by centrifugation. The yield of the extraction procedure is over 80%. No dependence on chain length was observed with RNA fragments up to 200 nucleotides long. Of course, incomplete precipitation is to be expected if small oligonucleotides, e.g., chain length 5 or less, are handled.

RESULTS AND DISCUSSION

Purification of RNA Fragments Obtained by Partial Hydrolysis of Bacteriophage RNA

Hydrolysis of large RNA molecules from ribosomes or bacteriophages with T_1 RNase at low temperature and fractionation by one-dimensional gel electrophoresis give rise to reproducible patterns of bands (7–9). The details of the technique used in the case of MS2 RNA (11) and the patterns obtained by separation of partial digests on 12% and 6% gels

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the conditions given in Table 2. ^{32}P activity in C1 to C7 ranged from 3×10^7 to 8×10^7 dpm and autoradiography took 5 min. The average maximum chain length of the RNA fragments is 80 in C1, 74 in C2-1, 68 in C2-2, 64 in C3, 57 in C5, and 51 in C7. Spots of the dye markers xylene cyanol FF (X), bromophenol blue (B), and trypan red (T) are circled. Strips 1 cm wide were cut out between the marks for separation in the second dimension.

(10) have been published and it has been pointed out that each band so obtained is in fact a mixture of different RNA fragments. These may stem from regions which are far apart in the primary structure of the RNA molecule.

Fragments moving together in a band are heterogeneous with respect to chain length. One or more of them have a length close to a value N, which we shall call the average maximum chain length of the fragments in the band, and which may be estimated from the mobility (10). The band contains additional shorter chains, probably moving as complexes kept together by base-pairing between complementary sequences, where the sum of chain lengths approximately equals N. This has been verified by sequence determination in a few instances (15).

The two-dimensional method described above is routinely applied to the purification of RNA fragments for sequence analysis. Figure 3 illustrates the first dimension of the fractionation of bands C1 to C7 from a partial digest of MS2 RNA separated on a 12% gel (see reference 10 for the numbering system on 12% and 6% gels). The average maximum chain length ranges from 80 nucleotides in band C1 to 51 in C7. The result obtained in the case of band C3 after the second dimension is shown in Fig. 4, with an indication of the chain length of several fragments as determined by sequence analysis.

Fractionation of Partial Hydrolyzates of Purified RNA Fragments

Sequence analysis of a purified RNA or RNA fragment usually involves partial digestion, e.g., with T_1 RNase. The products so obtained mostly show the overlaps necessary for reconstruction of the complete sequence of oligonucleotides. Figure 5a shows the two-dimensional separation of a partial T_1 RNase digest of an RNA fragment itself isolated by the same method from band B12 of a 12% gel and from band γ 8 of a 6% gel (10). This fragment originates from positions 20 to 104 from the 3′-OH terminus of MS2 RNA (16).

Factors Governing Distribution of Spots in the Two-Dimensional Pattern

At pH 8, i.e., under the conditions of the second dimension, the net charge of an RNA molecule increases as a function of chain length. The buffer used for the first dimension gives a pH reading of 3.5 and contains 6 *M* urea. Under these conditions net charge is a function both of chain length and of base composition. Moreover, protonation of the cytosine and adenine residues and the presence of urea should result in a dissociation of chains kept together by base-pairing between complementary segments.

What will happen to a mixture of RNA fragments such as those present in a band from a partial digest of bacteriophage RNA separated

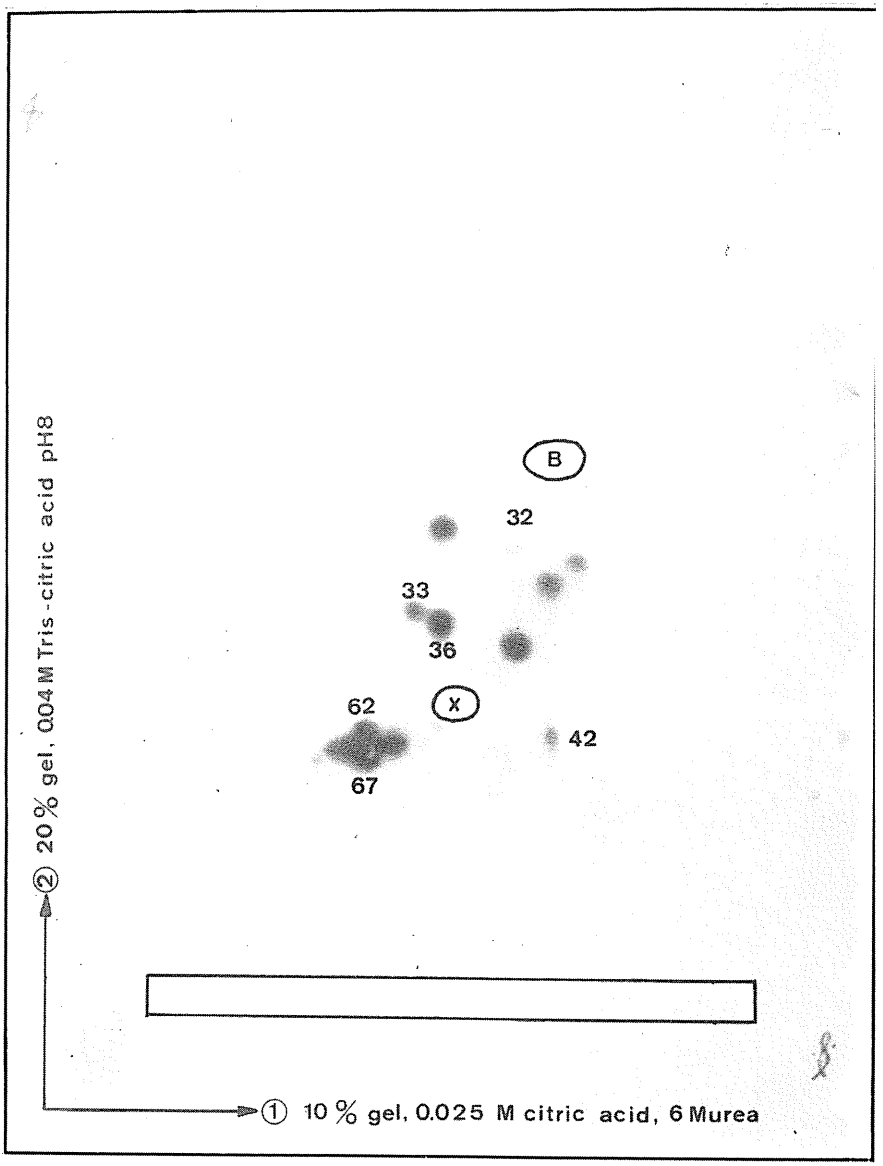


FIG. 4. Two-dimensional electropherogram of RNA fragments. A gel strip of 15 × 1 cm containing the mixture of RNA fragments marked C3 in Fig. 3 was cut out of the 10% acid gel used for the first dimension and enclosed in a 20% pH 8 gel at the position indicated by the rectangle. Electrophoresis in the second dimension was carried out according to the conditions given in Table 2 and the gel was autoradiographed for 5 min. Spots of known composition are marked by a number indicating their chain length as determined by sequence analysis. Spots of the dye markers xylene cyanol FF (X) and bromophenol blue (B) are circled.

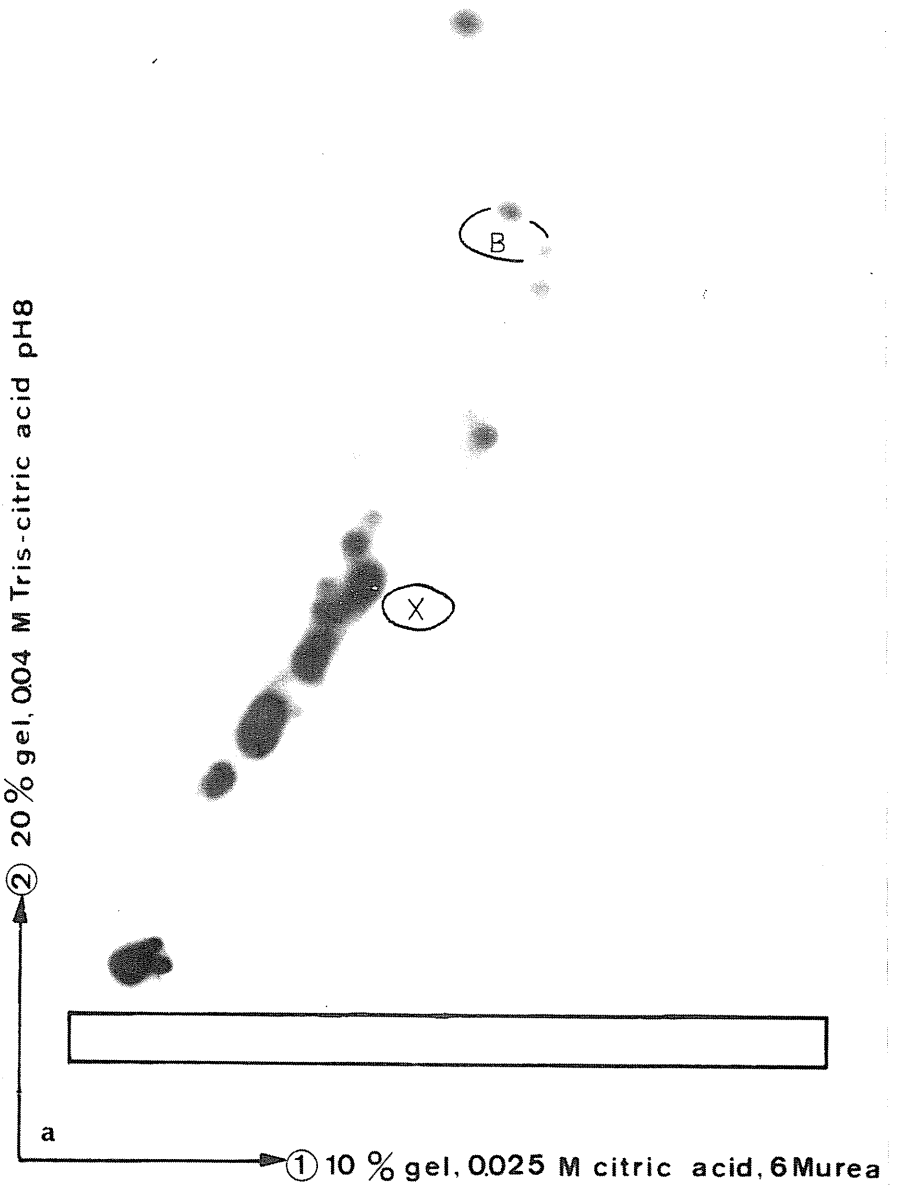
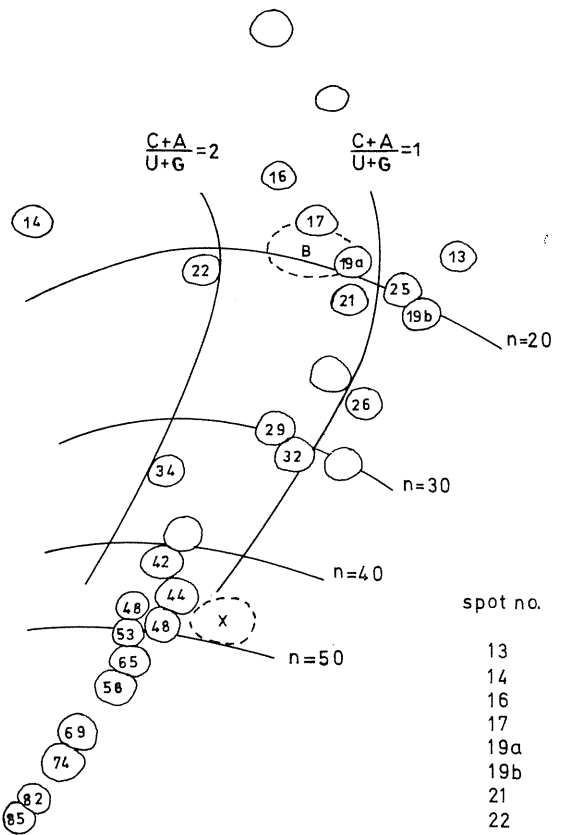
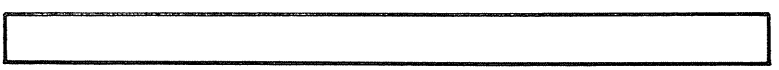


FIG. 5. Two-dimensional electropherogram of partial hydrolyzate of an RNA fragment. An RNA fragment extending from position 20 to 104 from the 3′-OH terminus of MS2 RNA, the sequence of which has recently been established (16), was isolated from band B12 on a 12% gel fractionation and band γ 8 on a 6% gel fractionation (10) of partial T_1 RNase hydrolyzates of MS2 RNA. The fragment, with an activity of 2×10^6 dpm ^{32}P, was hydrolyzed with 10 units T_1 RNase for 20 min at 0° in 20 μl 0.1 *M* Tris-HCl, pH 7.5, plus 0.001 *M* EDTA, in the presence of 200 μg carrier yeast RNA. Fractionation was on a 10% acid gel in the first dimension and on a 20% pH 8 gel in the second, under the conditions given in Table 2. (a) Autoradiogram of gel obtained after 3 hr exposure. The



spot no.	C+A / U+G
13	0.62
14	2.75
16	1.29
17	1.12
19a	1.11
19b	1.11
21	0.91
22	2.14
25	0.79
26	0.86
29	0.93
32	1.00
34	1.86

b



rectangle indicates position of strip cut out from first-dimensional gel. (b) Scheme of position of main spots. Those unambiguously identified by sequence analysis are indicated by a number corresponding with their chain length. The (C + A)/(U + G) ratios of the shorter components are listed at the right; those for the longer ones range from 1.07 to 1.62. Two sets of lines divide the gel surface into several areas according to the chain length (N) and the (C + A)/(U + G) ratio of the RNA fragments. There are minor deviations such as the positions of spots 19b and 25. Both move faster than expected—the former in the first, the latter in the second, dimension.

by one-dimensional gel electrophoresis (10)? In the first dimension, the complexes consisting of smaller fragments will dissociate and all the components, large and small ones, will run over a distance which depends on their chain length as well as on their base composition. In the second dimension, they will be arranged mainly according to chain length, and those that happened to occupy the same position after the first dimension are very likely to be separated.

The influence of base composition and chain length is apparent from Fig. 5b. The pattern of spots can be divided into sectors according to the chain length and the ratio (C + A)/(U + G) of the components. Partial digests of pure RNA fragments usually contain products ranging in size from oligonucleotides of chain length 10 or less to fragments nearly as long as the original molecule. The former may show extremes in base composition while the latter closely resemble each other in this respect. Hence a typical pattern is obtained in which the longer components more or less fall onto a diagonal while the short ones spread out into a fan.

For the same reason mixtures of RNA fragments of a high average chain length yield patterns in which spots are grouped more or less around a diagonal, whereas for mixtures of shorter fragments the spots are more randomly distributed, since the shorter the chains, the more likely are divergences from the mean base composition.

Finally it should be noted that not only at pH 3.5, but also at pH 8, some dependence of mobility on base composition seems to exist. Furthermore, some striking differences in mobility, such as between spots 32 and 42 (Fig. 4), cannot be explained by the factors mentioned above. It is suspected that secondary structure must be responsible for this. It is easy to imagine that a sequence showing internal base complementarity and folding back on itself to form a stem-and-loop structure would move differently from one having the same length and base composition but which, by virtue of its different sequence, remains as a random coil.

SUMMARY

Complex mixtures of RNA molecules may be separated by two-dimensional electrophoresis on polyacrylamide gel slabs. The first dimension of the separation is carried out on acid gels in the presence of a high urea concentration, the second on more concentrated gels buffered at pH 8. The method has been applied to the complete separation of RNA fractions obtained after a preliminary gel electrophoresis of partial enzymic digests of ^{32}P-labeled bacteriophage RNA. Another application is the fractionation of partial digests as obtained in sequence determination of RNA molecules. Spots are detected by autoradiography and

by a simple micro procedure which yields the material in a ed form suitable for sequence analysis by fingerprinting.

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