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Electrophoresis of RNA in Formamide†

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ABSTRACT: The technique of acrylamide gel electrophoresis of nucleic acids in formamide is described. The method affords high resolution, and, within error, electrophoretic mobilities are independent of the base composition over the range of the materials studied. Fractionated poly(riboadenylic acid) and poly(ribouridylic acid) samples show mobilities lying on the same molecular weight–mobility curve as RNAs. It thus appears that polynucleotide chains in general are all effectively homologous in formamide, and this provides the basis for a reliable and convenient empirical method for molecular weight determination on unknown nucleic acids alone or in mixtures. The technique is analogous in scope to sodium dodecyl sulfate gel electrophoresis for proteins. Limitations have been explored and are discussed. Mobility–molecular weight relationships are shown for a series of acrylamide concentrations. Possibilities for the use of other nonaqueous solvent systems have been explored.

Early work in this laboratory (Richards *et al.,* 1965; McPhie *et al.,* 1966) showed that for a series of RNAs the electrophoretic mobility in polyacrylamide gel electrophoresis bore a smooth relation to the molecular weight within reasonable limits, and could therefore be used to provide estimates of the molecular weights of unknown species, singly or in a mixture. The molecular weight range of the method could be extended both upwards (Hadjiolov *et al.,* 1966; Tsanev *et al.,* 1969; Loening, 1967; Bishop *et al.,* 1967) and downwards (Philippsen and Zachau, 1972) by changing the gel concentration, or using agarose or agarose–acrylamide mixtures at the high-molecular weight end. Subsequently, it became apparent that a mobility–molecular weight calibration based on the readily available bacterial and cytoplasmic eukaryotic rRNA species did not necessarily hold good for all other RNAs (Loening, 1969; Groot *et al.,* 1970; Grivell *et al.,* 1971), and the latter authors showed that by changing the temperature the apparent molecular weights of mitochondrial rRNA based on

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such a calibration could be made to vary substantially with temperature. Thus even aside from the extreme case of fully base-paired two-stranded RNA, the mobility has an appreciable dependence (as might be expected) on the secondary structure. To escape from this limitation on the validity of the electrophoretic method of molecular weight determination we sought solution conditions in which secondary structure would be destroyed, and all RNA species rendered conformationally homologous, but in which gel electrophoresis experiments could still be carried out, and which would also not be prohibitively expensive. Such a situation would allow sufficiently accurate determination of molecular weights of nucleic acids in a manner analogous to the use of SDS gels to determine protein molecular weights, though more soundly based in hydrodynamic terms. We reported the development of such a method (Staynov *et al.*, 1972) based on the use of formamide as the electrophoretic solvent. We now describe a detailed exploration of this system, as well as a number of major improvements on the earlier procedure, and we give details to permit the wider exploitation of the method.

Materials and Methods

Escherichia coli rRNA was prepared from packed cells by the method of Robinson and Wade (1968), and rabbit reticulocyte rRNA by the phenol–sodium dodecyl sulfate method (Kirby and Parrish, 1966). Unfractionated yeast tRNA was obtained from General Biochemicals. Bromgrass mosaic virus RNA was given to us by Dr. J. Semal, *Penicillium cyaneofulvum* double-stranded RNA by Dr. K. Buck, *E. coli* 5S rRNA by Dr. E. G. Richards, and tobacco mosaic virus RNA by Dr. M. Spencer. Synthetic polyribonucleotides were brought from various commercial sources, mainly Miles Chemical Co. Poly(riboadenylic acid) was fractionated into narrow molecular weight cuts by salt precipitation, as described by Eisenberg and Felsenfeld (1967), and poly(ribouridylic acid) after the procedure of Inners and Felsenfeld (1970). Molecular-weight determination by sedimentation velocity of these fractions and their electrophoretic properties have been described previously (Pinder and Gratzer, 1974). Reaction of RNA with formaldehyde was carried out at 63° as described by Boedtker (1968).

Treatment of Formamide. The formamide, which is obtainable from any laboratory chemicals suppliers, varies greatly in purity as received. Colored samples were rejected. The solvent is purified as follows: 2 g of mixed-bed ion-exchange resin, of coarse mesh (in our procedure Amberlite, monobed MB-1, bought from British Drug Houses Ltd) is added to 50 ml of formamide, and vigorously stirred for 2 hr. It is useful to follow the conductivity change during this period and to ensure that it has dropped to about 5 μmho (from a typical starting value of the order of 400 μmho). The extraction of ionic impurities is also accompanied by a change in apparent pH, which may likewise be used to follow the process. The resin should then be removed by filtration: if the formamide is left to stand over the resin for long periods, impurities are progressively leached out. The formamide is best used within 3–4 days and should be stored in the dark.

Preparation of Solutions and Gels. We have used cylindrical gels, made in glass tubes, 7 × 0.7 cm (i.d.) throughout the present work, though slab gels can equally well be adapted to the purpose (Staynov *et al.*, 1972). With an apparatus of the usual type (Davis, 1964), holding eight tubes, the following quantities were found convenient for setting up a single run. Acrylamide (4%) in 0.02 M diethylbarbituric acid (barbital) was prepared by dissolving in the deionized formamide 0.85 g of acrylamide monomer, 0.15 g of *N,N'*-methylenebisacrylamide, 0.092 g of diethylbarbituric acid, 0.06 ml of *N,N,N',N'*-tetramethylethylenediamine, adjusting to an apparent pH (measured with a standard glass electrode, and calomel reference, containing saturated aqueous potassium chloride) of 9.0, with small volumes of 1 N hydrochloric acid, with magnetic stirring to ensure rapid mixing, and making up to 25 ml. To this solution is added 0.2 ml of 18% w/v freshly prepared aqueous ammonium persulfate. The solution is poured (without the necessity of degassing) into the tubes to the marked height. The liquid columns are then carefully layered with 70% (v/v) formamide–water, using a hypodermic syringe, and left to set (usually about 30 min). The liquid is then removed and after blotting replaced by buffered formamide, prepared again by dissolving 0.092 g of diethylbarbituric acid in deionized formamide, adjusting with a small volume of 1 N sodium hydroxide, with magnetic stirring, to an apparent pH of 9.0, and making up to 25 ml.

Samples of RNA dissolve readily from the ethanol-precipitated state, lyophilized samples rather more slowly, in the buffered formamide. The same formamide solution containing 0.02 M barbital is used as solvent, with in addition 5% sucrose and a trace of Bromophenol Blue as tracker dye. In general 10 μl of sample, 1 mg/ml in RNA, was applied, and for the run a 1-cm column of buffered formamide was left over the sample with the aqueous reservoir solution above this. No disadvantages were found to result if aqueous reservoir solutions were used, and the one selected and used throughout was 0.02 M sodium chloride. For reasons which we are unable to explain, the use of either aqueous or formamide barbital buffer in the reservoirs led to zone disturbances. Naturally contamination with electrode products could cause large pH changes in the reservoirs, but this can be readily obviated by circulating the reservoir contents between the cationic and anionic chambers, or by using, as we have done, external electrodes. Reversible electrodes are easily prepared and their use is strongly recommended. It prevents any detectable change in pH regardless of how long the run or how small the electrode compartments.[2] The gels are run for about 1 hr or until the tracker dye has tra-

[1] Abbreviations used are: poly(rU), poly(ribouridylic acid); poly(dA), poly(riboadenylic acid).

[2] The following is a convenient design for reversible external electrodes. A 250-ml wide-mouthed reagent bottle may be used. A rubber stopper is drilled to receive a 1.5-cm glass U-tube to make the salt bridge to the reservoir, a 0.6-cm glass tube reaching to the bottom of the bottle, and a vent for escape of electrode gases. The narrow glass tube (soda glass) has a platinum wire sealed into the bottom, so as to make contact between mercury in the tube and a mercury calomel paste in the bottle. This may be 2 cm or so deep, and is prepared by thorough mixing with a little saturated potassium chloride solution using a pestle and mortar. The bottle is then filled with saturated potassium chloride. The bridge is made of 5% acrylamide (5% of this methylenebisacrylamide) in 1 M potassium chloride, set in the tube, and filling it completely. The bottle should be greased around the stopper on the outside to prevent "creep" of the salt solution within. Wires from the power supply terminals are inserted into the mercury in the bottom of the narrow tube. It is important that the platinum wire be fully immersed in the mercury calomel paste. After each use the electrodes are reversed and will then maintain themselves indefinitely. The bridges, the ends of which should be sealed with parafilm when not in use, must be replaced occasionally. Reversible silver silver chloride electrodes can also be used (Smithies, 1955).

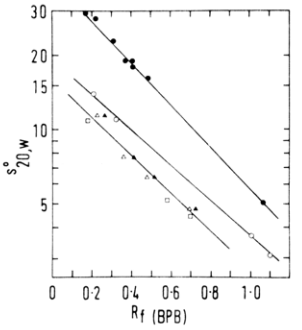

FIGURE 1: Relation between sedimentation coefficient and R_F (relative to Bromophenol Blue) in 2.2% aqueous polyacrylamide gels (0.05 M barbital (pH 8.5)), for native RNA species (●), RNA treated with formaldehyde, which is also present in the buffer (○), poly(riboadenylic acid) fractions without formaldehyde (▲), and in formaldehyde (△), and poly(ribouridylic acid) fractions in formaldehyde (□).

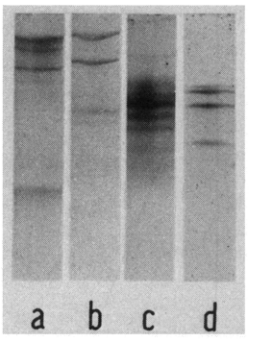

FIGURE 2: Typical electrophoresis patterns of RNA in formamide gels (experimental details as in text): (a) mixture of tobacco mosaic virus, *E. coli* 23S, 16S, and 5S rRNA; (b) rabbit reticulocyte rRNA, showing hemoglobin messenger bands (Gould and Hamlyn, 1973); (c) slightly degraded rabbit reticulocyte rRNA showing the appearance of "hidden" breaks in the formamide; (d) bromegrass mosaic virus RNA. (a), (b) and (c) are 4% gels and (d) a 3% gel.

versed most of the gel, at 5 mA/tube (constant current).

After electrophoresis the gels are removed from the tubes in the usual way, and stained overnight. The usual staining solutions are satisfactory. We have routinely used 0.1% pyronine Y in 0.5% acetic acid and 1 mM citric acid. After immersion in this stain overnight the gels are washed in 10% acetic acid to clear the background. It is also possible to carry out direct ultraviolet densitometry at 260 nm (Gilford scanner Unicam SP500 monochromator) on the unstained gels, equilibrated with 1% lanthanum acetate in 5% aqueous acetic acid. The background absorbance after this treatment is about 0.4.

Results

Our first attempts at electrophoresis of RNA in standard conformational states involved the reaction of the RNA with formaldehyde. This has been briefly alluded to in an earlier publication (Staynov *et al.*, 1972) and its use has been developed by Boedtker (1971). Some results obtained with formaldehyde-treated RNAs in aqueous buffer containing formaldehyde are shown in Figure 1. It is apparent that although the method works to an extent, the RNA is by no means homologous with the synthetic polynucleotides, and we have also found the reproducibility to be poor, presumably because of an irreproducible degree of reaction. Possible explanations are the introduction of cross-links by the formaldehyde (Feldman, 1967) with an attendant decrease in coil dimensions; incomplete reaction of G-C rich helices, or rather partial reversal of the formaldehyde reaction on cooling; or the persistence of single-stranded stacking interactions in the formaldehyde-treated RNA (Fasman *et al.*, 1965; Boedtker, 1967). It does not appear to have been established whether the cross-linking action of formaldehyde extends to the higher homologs. We have tried substituting propionaldehyde for formaldehyde as a denaturant, using the same experimental conditions. As judged by optical properties propionaldehyde reacted with the bases in much the same way as formaldehyde, but the electrophoretic results were certainly no better, and the differences between a series of RNA species and a set of poly(rA) fractions were equally marked. It is possible that reaction is less complete with this reagent.

The choice of denaturing media suitable for the present purpose is not wide. Concentrated aqueous urea, for example, is a poor denaturant (Cox and Kanagalingam, 1967). Among nonaqueous solvents the choice is limited to those with good solvent properties for nucleic acids, and which are also ionizing solvents. Formamide as a solvent for nucleic acids has been the subject of intensive study by Ts'o and his coworkers (Helmkamp and Ts'o, 1961; Ts'o *et al.*, 1962, 1963) and was shown to be an effective denaturant. Strauss *et al.* (1968), however, reported that dimethyl sulfoxide was more potent yet in this regard, and we therefore explored the possibilities of using this solvent for electrophoresis. It may be deionized in the same manner as formamide, with some decrease in conductivity. The resulting material then gives a neutral reaction. It did not in our hands support the polymerization of the acrylamide mixture; a gel prepared from aqueous solution could, however, be equilibrated with the solvent, containing a supporting electrolyte. Flat-gel electrophoresis could be performed in this medium, and RNA migrated in the gel, though with marked trailing. Because of the manipulative advantages of formamide-based gels, we have not, however, examined this system in greater detail. Two other, and very polar, solvents, *N,N*-dimethylformamide and *N,N*-dimethylacetamide, which, like other alkyl-substituted amides, are more powerful denaturants than formamide (Herskovits, 1962; Levine *et al.*, 1963), did not support the polymerization reaction, and caused rapid dehydration of preformed gels, with formation of a fibrous solid.

In earlier experiments, an unbuffered system was used, and gave satisfactory results (Staynov *et al.*, 1972). It should be noted that untreated formamide, as received, gives an alkaline reaction, does not support the polymerization of acrylamide, and, although it can be introduced into gels by soaking, gives poor results. With external electrodes the apparent pH remains stable, but it has nevertheless appeared to us that it is desirable to control the hydrogen ion activity by buffering the solution. We have pointed out (Pinder *et al.*, 1974) that it is necessary to ascertain by titration whether a buffer ion titrates in the appropriate range, before using it in the nonaqueous medium. Several buffers were for various reasons unsatisfactory, and diethylbarbiturate was therefore adopted. At the apparent pH of 9.0 (0.520 V glass electrode potential, uncorrected for junction potentials as discussed in Pinder *et al.*, 1974), the nucleic acid bases are un-ionized. In general we have found that separations are better and zones sharper in the buffered than in the unbuffered system, and, in fact, the results are considerably better than in aqueous systems. Some typical separations are shown in Figure 2.

It will be clear that the effective pore size of polyacryl-

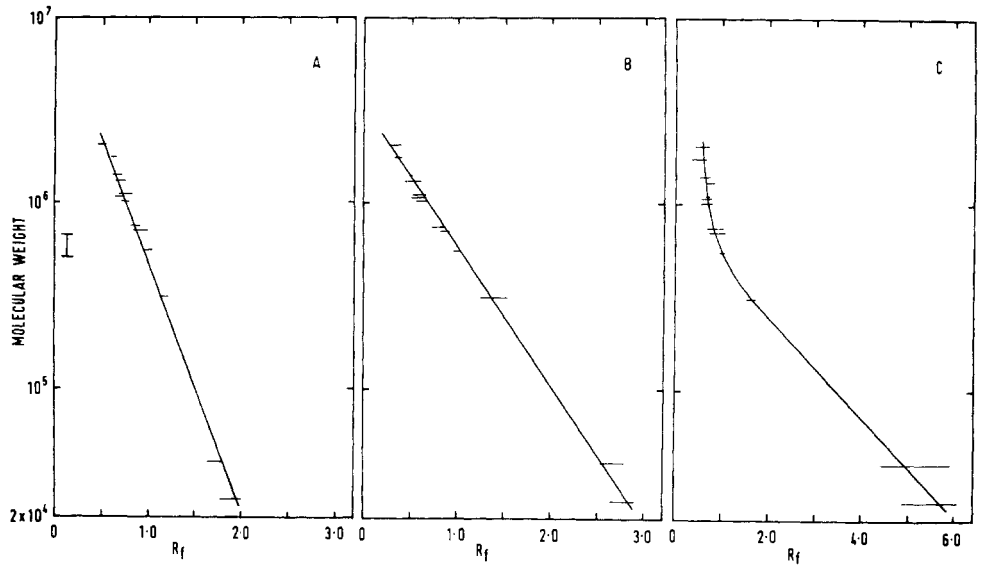

FIGURE 3: Calibrations relating molecular weight of RNA with R_F (relative to 16S *E. coli* rRNA) in formamide polyacrylamide gels. The bars show the scatter from series of 4–5 experiments. The vertical bar at the left-hand ordinate indicates the spread of published values for the molecular weight of one extensively characterized species, 16S *E. coli* rRNA. Polyacrylamide concentrations are (A) 3%, (B) 4%, and (C) 5%. The RNA species used are, reading from the high-molecular weight end: TMV, rabbit reticulocyte large ribosomal subunit, yeast large ribosomal subunit, μ2 phage, *E. coli* large ribosomal subunit, bromegrass mosaic virus components 1, 2 and 3, yeast and rabbit reticulocyte small ribosomal subunit (identical), *E. coli* small ribosomal subunit, bromegrass mosaic virus component 4, *E. coli* 5S, and unfractionated yeast tRNA.

amide gels is by no means the same in formamide as in water. In a theory of gel electrophoresis (see Chrambach and Rodbard 1971, for a review), based on the formulation of gel structure of Ogston (1958), this would mean that the effective diameter of the molecules of the gel matrix depends on their solvation. Thus a 4% formamide gel behaves like a 2.5% aqueous gel in terms of its molecular filtration properties, but mechanically it resembles an aqueous gel of similar or higher acrylamide concentration. This results in considerable manipulative advantages. For high-molecular weight RNA 3 or 4% gels are in fact generally optimal.

A disadvantage of the formamide system, which we have been unable to overcome, is a relatively poor reproducibility of mobility from run to run, compared with aqueous gels, and even to an extent from tube to tube within an experiment. The latter is not a serious limitation, but for optimal results a molecular weight determination should ideally be based on molecular-weight standards in the same tube (or a set of parallel tracks in a flat gel). The molecular weight–mobility relations shown in Figure 3 are *not* based on a single experiment, and therefore represent a lower limit of precision [*cf.* plot given in our earlier paper (Staynov *et al.*, 1972) derived from direct comparison in single flat gels]. Deviations from the log-linear plot occur at a critical molecular weight, which depends on the gel concentration, exactly as observed in aqueous systems (see, *e.g.*, Richards *et al.*, 1965; Dingman and Peacock, 1968). Within error, all species clearly obey the same relation, and this applies also to the synthetic polynucleotides, poly(riboadenylic acid), and poly(ribouridylic acid). This is reiterated in Figure 4 which we give here to demonstrate also that the original unbuffered system described by us (Staynov *et al.*, 1972) likewise gives serviceable results. For practical purposes therefore the RNA species are devoid of significant structure in the formamide and are all effectively homologous with each other and with the synthetic homopolymers. This is consistent with the results of optical studies shown in the accompanying paper (Pinder *et al.*, 1974). The small amount of structure, or whatever features of the polynucleotide chains in formamide the small residual circular dichroism and melting effects reflect, obviously exerts no significant effect on the electrophoretic mobilities. The addition of small amounts of water to the formamide, up to about 5%, also has no systematic effect on relative mobilities, as would be expected from the observed insensitivity of ionic conductivities in a similar solvent, dimethylformamide, to low concentrations of water (Juillard, 1970). Two-stranded RNA, as noted in the accompanying paper, melts in formamide on warming, and remains in the melted state after cooling. It then migrates in the same manner as all other RNA species. As also noted by Bevan *et al.* (1973), the melted RNA stains a different color with Toluidine Blue from the undenatured, with which it can coexist in the formamide. We have not here explored the low molecular weight range, for which more concentrated gels would be required. The formamide method has, however, been shown to work well in this range, using 12% acrylamide gels for tRNA and its fragments (Staynov *et al.*, 1974), and in 20% gels with low molecular weight poly(riboadenylic acid) samples (Brownlee *et al.*, 1973).

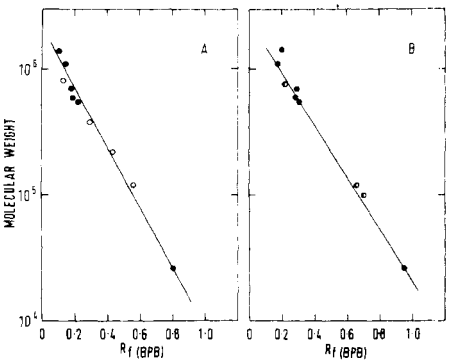

FIGURE 4: Common calibrations in formamide polyacrylamide gels (unbuffered) for (A) RNA (●) and poly(riboadenylic acid) fractions (○); (B) RNA (●); and poly(ribouridylic acid) fractions (◐).

The use of aqueous reservoir solutions appears to have lit-

tle effect on the electrophoresis. We have determined the extent of water penetration into the gel during a run by equilibrating slices of the gels after their removal from the tubes with dry formamide and analyzing the solvent in the near-infrared. Only in the bottom 1 cm of the gels is there an appreciable increase in water content.

Experimental precision aside, the accuracy of the method depends on the accuracy with which the molecular weights of standard RNA species are known, and here unfortunately the literature generally gives a wide choice of values. These are reflected by error bars in Figure 3.

Formamide gels can also be satisfactorily used for the separation of proteins. We have in addition attempted to extract the RNA from bacterial cells directly into formamide. Evidently this solvent, despite its very high dielectric constant, is not wholly effective in dissociating nucleoprotein complexes. Protein contaminants might give rise to problems of aggregation in formamide therefore. We have, however, met with moderate success by adding chloroform to cells treated with the cationic detergent, cetyldimethylbenzylammonium chloride in formamide, and collecting the formamide phase. The detergent salt of the nucleic acids (Duval and Ebel, 1965) is soluble in formamide, as in many nonaqueous solvents, and this solution can be used directly for electrophoresis in formamide gels. We have not, however, found conditions for extraction of all or most of the RNA, and such a strategy for direct extraction of cells into the gel solvent might repay further exploration.

Discussion

The method which we have described, though no doubt capable of many variations, is convenient and gives good results. We stress the importance of pH control in these experiments, and the desirability of using buffer circulation or better, external electrodes, to prevent contamination of the reservoir solution, and ultimately the gels, with electrode products. We have shown that barbital offers satisfactory buffering in formamide, but it must not be assumed that the buffering range of any ion is the same in this solvent as in water, and the chemical instability of two other buffers has been noted. Phosphate has been used in formamide (Reijnders *et al.*, 1973; Duesberg and Vogt, 1973) but there is no indication that it functions as a buffer in the conditions used by these authors.

The formamide method affords high resolution, better indeed than in aqueous systems, and is analogous in scope to sodium dodecyl sulfate gel electrophoresis in the study of proteins, though more firmly based on conformational homology. The limitation of reproducibility between absolute mobilities in different gels may, despite the absence of systematic dependence of relative mobilities on small amounts of added water, be due to variability of water content in this rather hygroscopic solvent. As the calibration curves show, as well as the internal reproducibility, this is evidently not related to water-dependent conformational effects on the RNA, but rather water-dependent electrostatic effects, leading to some variability in mobility. This effect could perhaps be eliminated by operating in a strictly anhydrous system, but this would be experimentally too demanding, and in any case we have not found it to be a serious limitation. The problem of molecular weight standards is of considerable concern. At present one can only take one's choice from reported values, which sometimes have a spread of as much as 30%. One may hope that with the advent of ultraviolet scanner optics, and perhaps more importantly, improved methods of measuring partial specific volumes, reliable data may increasingly become available.

In addition to molecular weight determination, gel electrophoresis in formamide can serve for detection of "hidden" breaks in the chain, (Figure 2c), and it could doubtless be used to give increased resolution in complex mixtures as a part of a two-dimensional system (DeWachter and Fiers, 1972), with an aqueous buffer in the first dimension. Possibilities of extracting cells with formamide (which should also effectively inhibit nuclease degradation), followed by direct analysis on gels, may also be worth pursuing.

Since our first report concerning the use of formamide gels (Staynov *et al.*, 1972), the method has been successfully applied in a number of laboratories. Gould and Hamlyn (1973) described the separation of α- and β-chain hemoglobin messengers, which appears to be impossible in aqueous gels, and a number of applications to viral RNA, to DNA fragments (Maniatis and Ptashne, 1973), poly(riboadenylic acid) tracts from mRNA (Brownlee *et al.*, 1973), etc., have been reported. In several of these cases the procedure described here, with the use of buffered gels, has already been used.

It appears that dimethyl sulfoxide might also be put to use as an electrophoretic solvent for nucleic acids, should any serious doubts arise about the complete denaturation of, for example, a very G:C-rich double-stranded RNA in formamide. Optimal conditions would first have to be established. Conductivities of electrolytes are much lower in this solvent, and buffering ranges are greatly affected. Barbital for instance does not appear to provide significant buffering in the relevant range.

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Length Heterogeneity in the Poly(adenylic acid) Region of Yeast Messenger Ribonucleic Acid†

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ABSTRACT: The population of poly(A) chains obtained from yeast spheroplast RNA contains molecules ranging from about 20 to 60 nucleotides in length. Poly(A) containing RNA obtained from spheroplasts which have been exposed to a short pulse of [^{3}H]adenine contain label predominantly in the longest poly(A) sequences (~60 nucleotides in length). Shorter poly(A) molecules are found in cytoplasmic polyribosomes and are labeled when spheroplasts are exposed to [^{3}H]adenine for a considerably longer time. This strongly suggests that, like in human cells (Sheiness, D., and Darnell, J. E., (1973), *Nature (London), New Biol. 241,* 265) the poly(A) sequence in yeast messenger RNA becomes shorter with time. We also find that there are rapidly labeled very large poly(A) containing RNA species which are obtained from total cell extracts. The size distribution of these molecules is larger than messenger RNA obtained from polyribosomes.

Although poly(A) has been found in RNA species in many eucaryotic cells (Edmonds *et al.,* 1971; Darnell *et al.,* 1971a,b; Lee *et al.,* 1971; McLaughlin *et al.,* 1973) its metabolic role still remains to be elucidated. A function related to the production or utilization of genetic message is suggested by the presence of poly(A) in messenger RNA. Experimental evidence is available from cultured mammalian cells which indicates a nuclear function for poly(A) (see, for example, Adesnik *et al.,* 1972). In addition, recent evidence shows that poly(A) shortens with time in the polyribosomes of Hela cells (Sheiness and Darnell, 1973).

We wish to characterize the mRNA of the simple eucaryote *Saccharomyces cerevisiae,* both with respect to its possible passage through precursor forms prior to entrance into polyribosomes, and with respect to its turnover in polyribosomes. This, we hope, will provide the background information necessary to begin to analyze mutants which are defective in RNA and protein synthesis. We report here the isolation and characterization of poly(A) containing RNA from yeast spheroplasts.

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