in Fig. 1. It is very apparent that the generated field depends linearly on the applied load. Piezoelectricity was difficult to measure for the β-polymorph since it is very soft and usually crumbled under the applied loads. The δ-polymorph crystals were obtained by heating the β-polymorph to about 250°C and then cooling to room temperature. The data shows that very large electric fields are generated within these crystals even with the application of a relatively small load; for example a field of 10 volts per centimeter was generated by a load of 400 g on a crystal whose area was 0.42 cm^2.

Since HMX is a secondary explosive it cannot be detonated without either a primary detonation of the explosive or one of a mechanical nature. If the piezoelectric voltage still depends linearly on pressure, in an average primary detonation of 500 kbar the generated field due to a detonation can be of the order of 10^7 volts/cm. This electric field is high enough to cause electrical breakdown (electron avalanche) within the crystal and hence to generate localized "hot spots" that finally result in explosion. By inspection of the crystal structures of RDX, TNT, PETN, NH_4NO_3, NH_4ClO_4 one finds that all these secondary explosives have crystal structures which should exhibit piezoelectricity. This same phenomenon could explain some of the strange properties of the azides. It is well known that lead azide can explode while being grown in crystal form from solution. It is well established that crystals can be extremely strained while growing. Thus one could envisage that the strains cause a piezoelectric voltage to be generated with subsequent explosion.

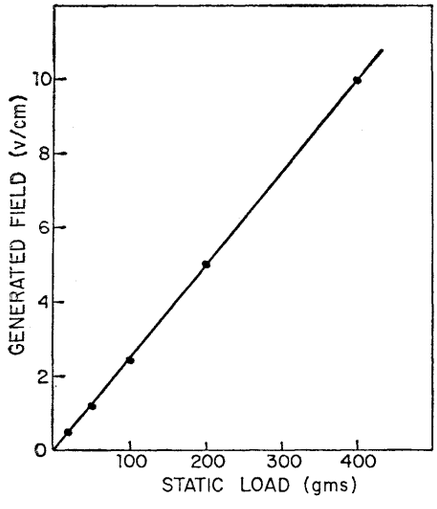

Fig. 1. Generated piezoelectric field as a function of applied load on a single crystal of δ-HMX at room temperature.

Thus some of the hot spots necessary for explosion of solid explosives might be produced by electrical breakdown of the crystal owing to a generated piezoelectric field.

J. N. MAYCOCK
D. E. GRABENSTEIN
Research Institute for Advanced Studies, 1450 South Rolling Road, Baltimore, Maryland

References and Notes

1. F. P. Bowden and A. D. Yoffe, *Fast Reactions in Solids* (Butterworths, London, 1958).
2. The 98-percent-pure HMX was supplied by the Explosives Laboratory, Picatinny Arsenal, Dover, New Jersey.

1 March 1966

Isopycnic Centrifugation for the Isolation of DNA Strands Coding for Ribosomal RNA

Abstract. *Denatured DNA preparations from* Escherichia coli *were centrifuged to equilibrium in cesium chloride solutions. Hybridizing experiments with radioactively labeled ribosomal RNA showed that the DNA strands complementary to ribosomal RNA were distributed on the heavy side of the DNA band. By fractionating this band the DNA strands coding for ribosomal RNA may be enriched 5- to 20-fold.*

When a DNA preparation is centrifuged to equilibrium in a cesium chloride solution of appropriate density, the CsCl is redistributed in the liquid column and forms a density gradient in which the macromolecules are concentrated in a zone, or band, at a height in the gradient corresponding to their buoyant density (*1*). Native DNA exhibits a buoyant density which is in general linearly related to the mole percentage of guanine and cytosine in the molecule (*2*). When DNA is denatured the separated strands of the double helix usually have a buoyant density higher than that of the native molecules by approximately 0.015 g/ml.

Most viruses contain single molecules of DNA. Such DNA preparations show narrow bands in CsCl gradients, the sharpness reflecting the lack of molecular heterogeneity. In the cases of a few viruses, such as phage α (*3*, *4*), it has been found that on denaturation each of the complementary strands of DNA gives rise to a separate band in CsCl instead of one being super-imposed on another. It has been suggested that pyrimidine-rich strands are denser than purine-rich strands and that in these bacteriophage DNA's the composition of each of the paired strands is so disparate as to endow the strands with differing densities (*5*), whereas in most DNA preparations, presumably fortuitously, the ratio of purine to pyridine of all strands is similar and the molecules in the denatured preparation have not been resolved up to the present.

Analyses of these separated strands in the case of bacteriophage α show that the ratios of purine to pyrimidine bases in the strands are 0.78 and 1.18 (*4*). The difference between these ratios is less than that which must exist between the strand of DNA coding for ribosomal RNA and its complementary DNA strand [purine:pyrimidine = 0.773 and 1.227, and 0.725 and 1.275, for 16*S* and 23*S* RNA from *E. coli* (*6*)]. Experiments were therefore set up to test the possibility that the strands of DNA coding for the ribosomal RNA might be found situated eccentrically from the bulk of the denatured bacterial DNA after equilibrium centrifugation in a CsCl gradient. In this manner a preparation of DNA enriched in molecules coding for the ribosomal RNA might be obtained.

Preparations of H^3-labeled DNA were made from *Escherichia coli* B3 grown in a tris-glucose-casamino acid medium supplemented with H^3-thymine. Cells (5×10^8 per milliliter) were centrifuged and resuspended in 0.01*M* acetate buffer, *p*H 5.4, and lyzed by addition of sodium dodecyl sulfate (SDS) solution (to a final concentration of 2.0 percent SDS) together with washed bentonite (*7*) (to 0.5 mg/ml). An equal volume of neutral redistilled phenol was added to the mixture of bacteria and SDS, and after vigorous shaking the two phases were separated by centrifugation. The upper phase was removed and shaken a second time with fresh phenol; after centrifugation the nucleic acids were precipitated from the upper phase by addition of NaCl (final concentration 0.1 mole/liter) followed by two volumes of ethanol. The nucleic acids, redissolved in 0.01*M* EDTA ethylenediaminetetraacetate, *p*H 7.5, were treated with

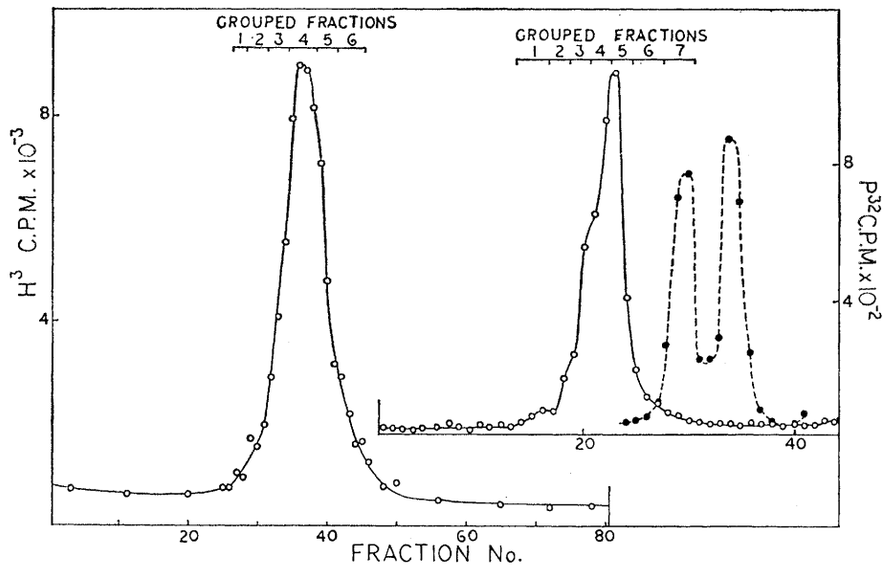

Fig. 1. Profiles of denatured DNA from *Escherichia coli* centrifuged to equilibrium in CsCl gradients. In order to minimize the centrifugation time required to reach equilibrium there were layered above and below the DNA (in 1 to 1.5 ml of CsCl of appropriate density) CsCl solutions of 10-percent lower and 10-percent higher density. The tubes were centrifuged for 20 hours at 30,000 rev/min and then for 48 hours at 25,000 rev/min (Spinco Model L, rotor SW 39). The dashed curve (right) shows the distribution of denatured DNA (P^{32} label) from *Bacillus megaterium* G phage run in a separate tube but at the same time as the DNA from *E. coli*. Thus the widths of the bands but not their relative positions are significant.

previously boiled pancreatic ribonuclease (Nutritional Biochemical Corp.) to destroy RNA and then treated twice more with SDS-phenol before final precipitation with saline-ethanol. The DNA was redissolved in 0.01*M* NaCl to a concentration of 0.01 to 0.03 percent and denatured by heating to 100°C for 10 minutes (or by treatment with sodium hydroxide to *p*H 12.5, followed by neutralization). Cesium chloride was then added and the solution was centrifuged for at least 68 hours. The contents of the tube were collected (50 to 80 fractions) from the bottom of the pierced tube, and the DNA distribution was determined by radioactivity measurements (Nuclear Chicago scintillation counter). The DNA fractions through the gradient were then grouped for the subsequent experiments (Fig. 1). A sample of sheared, denatured T2 coliphage DNA was similarly fractionated.

The shape of the DNA distributions generally resembled that shown in Fig. 1 (right), that is, pronouncedly skewed on the dense side of the peak. Some differences between the band profiles in different experiments were observed (Fig. 1, left and right); these differences could reflect a failure to reach an equilibrium distribution, or disturbance of the zones during manipulations, but the properties of the DNA fractions obtained in this way were consistent for each of seven preparations so treated.

Ribsosomal RNA was labeled by growing *E. coli* B for five or six generations in P^{32} (specific activity, 1 to 2 $\mu c/\mu g$), and then in excess unlabeled phosphate for one generation. The cells were concentrated and lyzed as for the DNA preparations but the deproteinized nucleic acids obtained from the phenol-SDS treatment were digested then with electrophoretically purified deoxyribonuclease (Worthington Biochemicals Corporation). The RNA was precipitated by sodium chloride–ethanol, then redissolved and centrifuged at 4°C through a 5 to 15 percent sucrose gradient (*8*), and the 23*S*, 16*S*, and 4*S* RNA peaks identified by the P^{32} activity were grouped. The RNA was again precipitated by sodium chloride–ethanol (Fig. 2).

To detect the presence of DNA strands which, it may be presumed, originally coded for each of the labeled RNA species, samples of the labeled RNA were incubated with each of the denatured DNA fractions from the CsCl gradients under conditions where complementary base pairing can occur with the formation of double-strand hybrids of RNA and DNA. These so-called "hybridizing" experiments were made initially by the procedure of Nygaard and Hall (*9*). Subsequent experiments were made by the membrane filter technique (*10*); both methods gave similar results.

The ability of P^{32}-labeled 23*S*- and 16*S*-ribosomal and 4*S*-"transfer" RNA to hybridize with successive DNA fractions from the banding experiments is indicated in Fig. 3. Such binding was not observed when the mixtures were not heated; moreover the ribonuclease resistance of the bound RNA suggests that specific aggregation (presumably through hydrogen bonding) has occurred during incubation. To test further the significance of the P^{32}-RNA binding, ribosomal RNA was incubated with five successive fractions obtained from equilibrium CsCl centrifugation of sheared denatured bacteriophage T2 DNA. No trace of bound P^{32} RNA was observed with any fraction.

It is evident that the DNA hybridizing with the ribosomal RNA is distributed eccentrically in the bond formed by the total DNA; this phenomenon is less marked in the case of 4*S* (transfer) RNA, and its significance is uncertain since this fraction may be contaminated with fragments of ribosomal RNA.

Interspecies hybridization could also be demonstrated. Ribosomal RNA labeled with P^{32}, from *Bacillus subtilis* and *B. megaterium* showed a similar preference for the dense strands of denatured DNA from *E. coli* (Fig. 4).

This skewed distribution of ribosomal RNA binding was not observed when native DNA was centrifuged in CsCl, fractionated, denatured, and hybridized. This result is not unexpected. The complementary DNA strands of phage α differ in density by 0.009 g/ml, and the ribosomal-coding DNA strand and its complement would be expected from their purine-pyrimidine ratios to be more disparate. On the other hand, native DNA carrying these sequences should have a guanine-cytosine content close to 54 percent and differ from the mean density of *E. coli* DNA by only 0.003 g/ml (*2*).

Since undenatured DNA (which cannot hybridize under these experimental conditions) would appear on the light side of a mixture of native and denatured DNA, it was necessary to show that all the DNA was denatured (that is, capable of showing its hybridizing

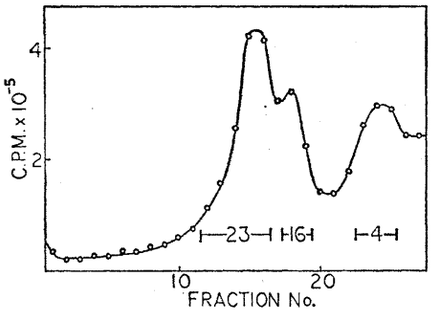

Fig. 2. Sedimentation profile of P^{32}-labeled RNA extracted from *E. coli* B grown in P^{32} tris-glucose minimum medium followed by a "chase" with excess unlabeled phosphate. RNA was dissolved in 0.5 ml of 0.1*M* NaCl, 0.01*M* EDTA *p*H 7.5 and centrifuged at 4°C for 16 hours at 23,000 rev/min (Spinco SW 25 rotor) through a 5 to 15 percent sucrose gradient in 0.01*M* tris *p*H 7.4. The 23*S*, 16*S*, and 4*S* fractions are indicated.

potential). Some DNA samples were therefore heated to boiling before being adsorbed to the B6 filters. No increase in the ability to hybridize could be detected after such treatment.

In order to assess to what degree the spread of denatured *E. coli* DNA through the band is due to density heterogeneity, the denatured strands of *B. megaterium* G phage (*11*) were similarly centrifuged to equilibrium in CsCl (Fig. 1, right). The sedimentation coefficients of these denatured strands and those of the *E. coli* DNA were compared by zone sedimentation. The median sedimentation coefficients of the two preparations of polynucleotide strands were the same; hence it may be concluded that homogeneous molecules of denatured *E. coli* DNA should be distributed in a band in CsCl not much wider than either of the bands representing the two strands of the *B. megaterium* G phage DNA. Thus the width of the *E. coli* denatured DNA band is compatible with considerable density heterogeneity among the molecules, and the fact that the strands coding for ribosomal RNA are distributed across only half of the band is consistent with their occurring in a population of molecules of uniform density.

The approximate specific activities of the RNA and DNA preparations were measured by relating the radioactivity to the optical density of the solutions. Saturating hybridizing experiments were performed with ribosomal RNA and unfractionated DNA (*10*). It was found that 0.35 percent of the DNA could be saturated with 16*S* and 23*S* RNA combined. The first and second banded fractions together amount to approximately 3 to 5 percent of the total DNA, and in these fractions a large proportion of the strands hybridizing with the ribosomal RNA are concentrated. Thus the ribosomal-RNA-coding strands may be enriched up to 20-fold to make up 8 percent of the molecules of DNA in the densest fractions.

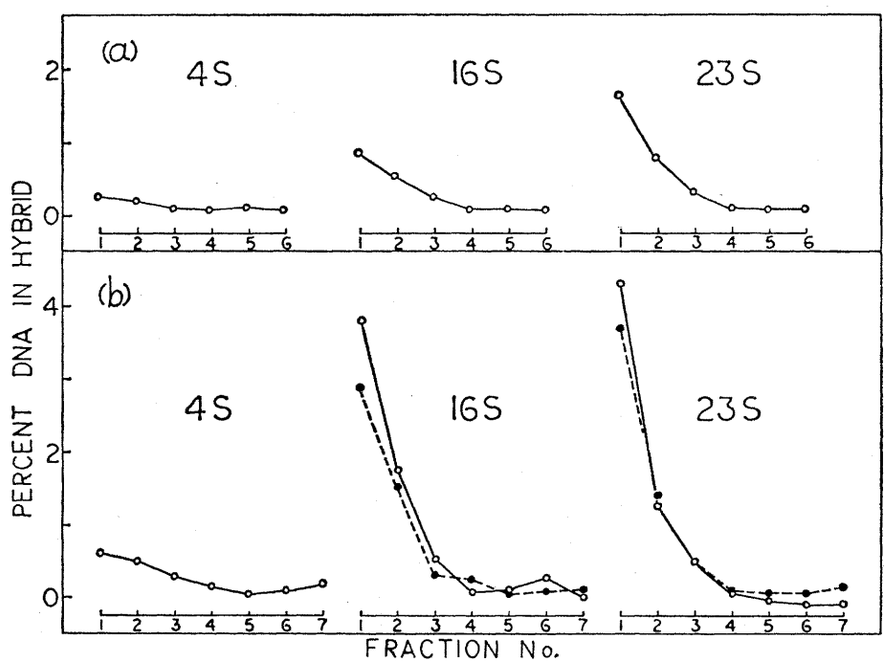

Fig. 3. The ability of the grouped samples shown in Fig. 1 as fractions 1 to 6 (*a*) and 1 to 7 (*b*), respectively, to hybridize with 4*S*, 16*S*, and 23*S* RNA. Between 0.2 and 5 μg DNA was adsorbed to each filter for the assays, and sufficient P^{32}-RNA was used to saturate the DNA. Experiments with two different RNA preparations are shown in (*b*) by the full and dashed lines.

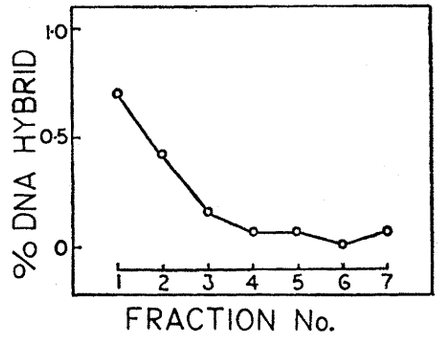

Fig. 4. Ability of 23*S* P^{32}-ribosomal RNA from *Bacillus megaterium* S to hybridize with successive fractions from a third banded preparation of denatured DNA from *Escherichia coli* (the profile is not shown but was similar to that in Fig. 1, right).

Quantitative experiments (*12*) have shown that about ten copies of the ribosomal RNA sequences occur in the genome of these bacteria. The eccentric banding of the appropriate high-molecular-weight DNA strands in these experiments suggests that these replicated sequences are adjacent since, if the sequences coding the ribosomal RNA were interspersed with sequences with less extreme disparity in bases, the density difference would be swamped. However, this fortunate chance permits the isolation of DNA in which the strands dictating the synthesis of ribosomal RNA are highly enriched. This RNA can be isolated undegraded from the bacterial cell. Thus it is feasible to hybridize intact RNA "messages" to the DNA strands from which they were transcribed, a circumstance which should permit a study of the sequences or structures in the DNA just preceding and following the region transcribed. It is believed that RNA synthesis is controlled by reactions occurring on such adjacent regions of the genome (that is, the "operator" sites); thus the findings reported show the feasibility of probing the structure of the operator.

Other bacterial species have DNA molecules of a lower mean density, and it might be expected that their molecules coding for ribosomal RNA would form bands in CsCl even more eccen-

trically than in the case of *E. coli*. However, for some unknown reason, experiments with *B. subtilis* and *B. megaterium* have not borne out this expectation.

PETER F. DAVISON
Department of Biology, Massachusetts Institute of Technology, Cambridge

References and Notes

1. M. Meselson, F. W. Stahl, J. Vinograd, *Proc. Nat. Acad. Sci. U.S.* **43**, 681 (1957); J. Vinograd and J. E. Hearst, *Fortschr. Chem. Org. Naturstoffe* **20**, 372 (1962).
2. C. L. Schildkraut, J. Marmur, P. Doty, *J. Mol. Biol.* **4**, 430 (1962).
3. S. Aurisicchio, A. Coppo, P. Donini, C. Frontali, F. Graziosi, G. Toschi, *Res. Rep. Phys. Lab. Inst. Superiore di Sanita, Rome*, No. ISS 61/33 (1961).
4. J. Marmur and S. Cordes, in *Informational Macromolecules*, H. J. Vogel, V. Bryson, J. O. Lampen, Eds. (Academic Press, New York, 1963), p. 79.
5. M. Ageno, M. Area, E. Dove, C. Frontali, L. Frontali, G. Tecce, *Res. Rep. Phys. Lab. Inst. Superiore di Sanita, Rome*, No. ISS 64/47 (1964).
6. W. M. Stanley, Jr. and R. M. Bock, *Biochemistry* **4**, 1302 (1965); K. McQuillen, *Progr. Biophys. Biophys. Chem.* **12**, 67 (1962).
7. H. Fraenkel-Conrat, B. Singer, A. Tsugita, *Virology* **14**, 54 (1961).
8. M. Hayashi and S. Spiegelman, *Proc. Nat. Acad. Sci. U.S.* **47**, 1564 (1961).
9. A. P. Nygaard and B. D. Hall, *Biochem. Biophys. Res. Commun.* **12**, 98 (1963).
10. D. Gillespie and S. Spiegelman, *J. Mol. Biol.* **12**, 829 (1965).
11. J. S. Murphy, *Virology* **4**, 563 (1957). I thank Dr. J. Marmur for phage G and its host; Dr. Marmur pointed out the width of strand separation on banding.
12. S. A. Yankofsky and S. Spiegelman, *Proc. Nat. Acad. Sci. U.S.* **49**, 538 (1963).
13. I thank Mrs. Joan Moor for technical assistance. Supported by grant NB 00024 from NIH.

14 March 1966

Native and Renatured Transfer Ribonucleic Acid

Abstract. *Transfer ribonucleic acid, isolated under conditions in which the original macromolecular structure is never denatured, is indistinguishable from transfer ribonucleic acid prepared by conventional methods involving denaturing steps. This finding is consistent with the absence of direct genetic control of the formation of macromolecular structure of transfer ribonucleic acid.*

The reversibility of the denaturation of sRNA (*1*) in solution has been studied by a variety of methods. On cooling thermally denatured sRNA, complete recovery of hypochromicity and amino acid acceptor and transfer activities has been observed (*2, 3*). Solvent denaturation with 8*M* urea has also been found to be reversible (*3*).

In that a solution of sRNA in distilled water shows little or no hypochromicity (*4*) and a denaturing effect of phenol on polynucleotide helices has been noted (*5*), it is evident that conventional preparative methods for sRNA, involving phenol-water extractions, dialysis against distilled water, or exposure to elevated temperatures, lead at some stage to denaturation of the molecules. It has generally been presumed, however, that such denaturation is completely reversible. Thus, sRNA is not bound to proteins or any large molecules in cell-free extracts (*4*). Furthermore, heavy metals do not seem to be integral parts of the structure of sRNA (*6*). Nevertheless, it is apparent that studies on the reversible conformational transitions of sRNA have been made thus far with renatured, rather than truly "native" sRNA that has never undergone denaturation. This distinction would be of significance, were sRNA to be stabilized in vivo in a conformation that is not the lowest energy state in vitro.

For this work, a preparation of sRNA from yeast was made under conditions in which the original, macromolecular structure in vivo is presumably retained. Exposure to organic solvents, elevated temperature, or low ionic strength during the isolation process was avoided. The "native" sRNA so obtained has been compared on the basis of several physicochemical and biochemical criteria with "renatured" sRNA prepared by conventional procedures.

For the preparation of "native" sRNA, 170 g of bakers' yeast (harvested in the budding phase) were frozen, and ground with two parts of acid-washed glass beads (0.2 mm diameter) under liquid nitrogen. The material was then rapidly brought to 0°C and extracted for 30 minutes with 700 ml of a mixture of 0.01*M* sodium cacodylate, 0.004*M* $MgCl_2$, and 0.001*M* EDTA (Na), *p*H 6.2, in the presence of 4 mg of purified bentonite per milliliter (*7*). All subsequent purification steps were carried out at 2° to 4°C. After centrifugation at 13,000*g* for 30 minutes the extract was passed through a carboxymethylcellulose column (17 by 4.5 cm) equilibrated with the extracting solvent. The sRNA did not adsorb to the column. Solid NaCl was added to the eluate to a concentration of 0.05*M*, and the solution was immediately passed through a DEAE-cellulose column (19 by 2.2 cm). A linear NaCl gradient was used to elute the sRNA fraction, which appeared after 450 ml (Fig. 1). In order to concentrate the sRNA fraction, three volumes of a mixture of 0.01*M* sodium cacodylate and 0.002*M* $MgCl_2$, *p*H 6.2, were added, and the material was readsorbed onto a smaller DEAE-cellulose column (9 by 2.2 cm). The sRNA was then eluted with a mixture of 1.2*M* NaCl, 0.01*M* sodium cacodylate, and 0.002*M* $MgCl_2$, *p*H 6.2, in a volume of 50 ml. This solution was dialyzed against several changes of a mixture of 0.1*M* NaCl, 0.01*M* sodium cacodylate, 0.005*M* $MgCl_2$, and 0.0005*M* EDTA (Na), *p*H 6.85, for 18 hours, and frozen. For further studies dilutions were made with the last dialyzate. The yield was 100 to 110 mg of sRNA, an extinction coefficient at 22°C of A_{258} equal to 206 being used for 1 percent sRNA.

The preparation had a phosphorus content of 8.7 ± 0.2 percent and a protein content of < 2 percent. No loss of amino acid acceptor activity occurred after the preparation in solution was kept for 48 hours at room temperature, 22°C, an indication of the total absence of nucleases.

For comparison, sRNA isolated from commercial bakers' yeast by a procedure (*8*) involving denaturing steps such as phenol-water extractions and extensive dialysis against distilled water was used. This preparation, which has been extensively characterized and shown to be free from contaminants (*9*), was dissolved in a mixture of 0.1*M* NaCl, 0.01*M* sodium cacodylate, 0.005*M* $MgCl_2$, and 0.0005*M* EDTA (Na), *p*H 6.85 at room temperature and dialyzed against the same solvent at 2° to 4°C.

Absorption spectra of both preparations, recorded on a Cary 14 spectrophotometer at 22°C, were identical in the 220 to 300 $m\mu$ regions. Ratios for the absorption spectra were as follows: 220 $m\mu$/260 $m\mu$ = 0.67; 230 $m\mu$/260 $m\mu$ = 0.44; 280 $m\mu$/260 $m\mu$ = 0.47. Spectra in the interval from 310 to 370 $m\mu$ on more concentrated sRNA solutions (2 mg/ml) showed no absorption peaks, indicating the absence of significant amounts of thiopyrimidines in both preparations (*10*). Absorbance-temperature profiles at 260 $m\mu$ between 5° and 95°C, obtained in a thermostated Beckman DU spectrophotometer, also revealed no differences between the two sRNA preparations.

Sedimentation velocity experiments