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A New Method for Preparing Ribosomal Ribonucleic Acid*

D. L. Weller and R. S. Morgan

ABSTRACT: The reaction of a soluble sulfonated polystyrene (molecular weight approximately 100,000) with *Escherichia coli* ribosomal subunits has been studied. Concentrations of the reagent in the range 0.1–0.8% lead to essentially complete dissociation of protein from nucleic acid. By varying the concentration of alcohol the ribonucleic acid (RNA) can be almost quantitatively separated from the protein. The nucleic acid isolated by this method is contaminated with 1–5% protein. Yields of the nucleic acid are usually about 90%. Characterization of the nucleic acid prepared from unfractionated subunits by this new method showed 16S (18.4S) and 23S (24.6S) RNA components in the usual mass ratio (*i.e.*, 1:2, slow:fast). From purified 50S subunits a main 23S component was obtained. The approximate molecular weights of these components are estimated to be 0.65 (16 S) and 1.1 $\times$ 10^6 (23 S).

Most methods used today for preparation of ribosomal RNA make use of phenol or a combination of sodium lauryl sulfate and phenol (Petermann, 1964). In this paper we report a new and simple method for the isolation of high molecular weight ribosomal RNA that employs a soluble sulfonated polystyrene (molecular weight approximately 100,000). Earlier studies had shown this polymer was a good RNase inhibitor (Danner, 1964), and that it had the ability to dissociate some of the protein from ribosomes (Morgan, 1966).

Our experiments show that concentrations of the polyanion in the range 0.1–0.8% lead to essentially complete dissociation of protein from *Escherichia coli* ribosomal subunits. The nucleic acid could be separated from the ribosomal protein by a combination of differential centrifugation and sucrose density gradient centrifugation. Yields by this method, however, were very low. The new procedure employs differential precipitation of RNA and of protein. The nucleic acid is quantitatively precipitated from solution by the addition of NaCl (0.01 M) and alcohol (52%). Under these conditions essentially all the protein remains soluble. RNA isolated by this method has chemical and physical properties similar to phenol-prepared nucleic acid.

Methods and Materials

The ribosomes used in these experiments were prepared from early log phase *E. coli B* (Grain Processing, Muscatine, Iowa) either by a method employing bentonite (D. L. Weller, manuscript in preparation) or by the $(NH_4)_2SO_4$ method (Elson, 1958; Kurland, 1966). Purified 50S ribosomes were isolated by differential centrifugation of mixtures of ribosomal subunits. The sulfonated polystyrene used was obtained as a free sample from Hagen Chemicals, Pittsburgh, Pa. This was the same batch employed in the previous study (Morgan, 1966). A sulfonated polystyrene (ET-409) of higher molecular weight can now be obtained from Dow Chemical, Midland, Mich. We have not yet investigated the properties of this compound.

Sedimentation analyses were done with a Spinco Model E ultracentrifuge at room temperature. The sedimentation coefficients were corrected to the viscosity and density of water at 20°.

The viscosity of solutions was measured with a constant-volume Ubbelohde viscometer (Cannon Instruments, State College, Pa.) with a solvent flow time of 305 sec at 20°. Flow-time measurements were made at 20°. Dust and large aggregates were removed from solutions, before the measurement of flow time, either by filtering through an HA Millipore filter (Millipore Filter Corp., Bedford, Mass.) or by centrifugation.

Absorption measurements were made either with a Beckman Model DB or Cary 15 recording spectrophotometer. Light path for all measurements was 1 cm.

Concentrations of ribosomes and RNA were estimated from A_{260} using an $E_{1\text{ cm}}^{0.1\%}$ of 16 and 23, respectively. Alternatively RNA concentrations were estimated by the orcinol method (Mejbaum, 1939) with yeast RNA (Schwartz BioResearch, Inc., Orangeburg, N. Y.) as a standard.

The protein content of the RNA was determined by direct analysis of the nucleic acid samples (Rama-

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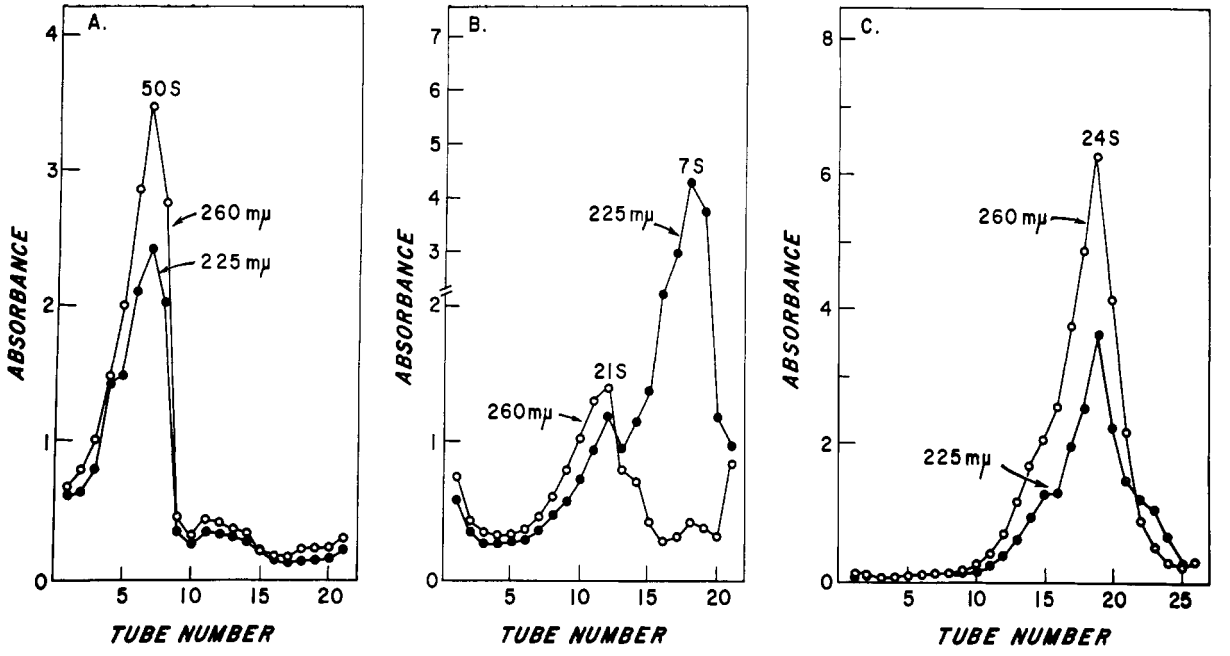

FIGURE 1: Sucrose gradient centrifugation profiles. (A) Purified 50S ribosomes. (B) Material like that in A was treated with sulfonated polystyrene (0.13%) for 16 hr at 4–8°. (C) Pellet obtained from material like that in B after centrifuging at 50,000 rpm for 6 hr. Solvent, 10 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$. A and C were centrifuged for 8 hr and B for 11.5 hr at 25,000 rpm. Sedimentation is from right to left.

chandran and Frankel-Conrat, 1958) using the method of Lowry *et al.* (1951), with bovine albumin (Pentex, Inc., Kankakee, Ill.) as a standard. Pancreatic DNase and RNase were obtained from Worthington Biochemical Corp., Freehold, N. J.

Results

Treatment of Purified 50S Subunits with the Sulfonated Polystyrene. The treatment of purified 50S ribosomes (2.5 mg/ml) (Figure 1A) with sulfonated polystyrene (0.13%) led to the appearance of a nucleic acid containing component sedimenting at about 20S (Figure 1B). Ribosomes and the reagent in 10 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$ were mixed and held overnight (16 hr) at cold room temperature (4–8°). The mixture (1 ml) was layered on a 25-ml linear 5–20% sucrose gradient prepared in Tris-Mg^{2+}. Following centrifugation in the SW 25.1 swinging-bucket rotor of the Spinco Model L, the tubes were punctured and 13-drop aliquots (about 1 ml) were collected. The optical density of the aliquots was determined at 260 and 225 mμ. These data are plotted against tube number in Figure 1. The sulfonated polystyrene has a characteristic absorption maximum at 225 mμ with an $E^{0.1\%}_{1\,cm}$ of 30 (Figure 3A, curve 2). In the region 240–280 mμ it has, however, a much lower extinction (*i.e.*, 1–2). Therefore, the A_{260} curve represents mainly the RNA component, for the nucleic acid has an extinction coefficient at this wavelength some eight to ten times greater than the polymer. Thus, treatment of purified 50S subunits with the sulfonated polystyrene has clearly produced a new nucleic acid containing component that sedimented at about 20 S. Sedimentation rates are estimated by ratios of linear distances sedimented.

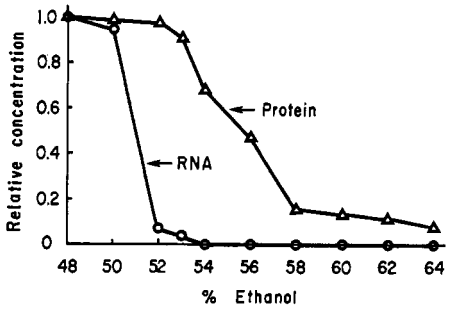

FIGURE 2: Solubility of RNA and protein in the ribosome-sulfonated polystyrene mixture in the presence of 0.01 M NaCl and varying amounts of ethanol.

When the A_{260} reading is low (*i.e.*, tubes 15–21, Figure 1B), the A_{225} profile represents mainly the reagent. Under these conditions the reagent sedimented at about 7 S (Figure 1B).

The nucleic acid containing component was purified by differential centrifugation of the reaction products. Analytical ultracentrifugation of the purified material showed a main component that sedimented at 14 S

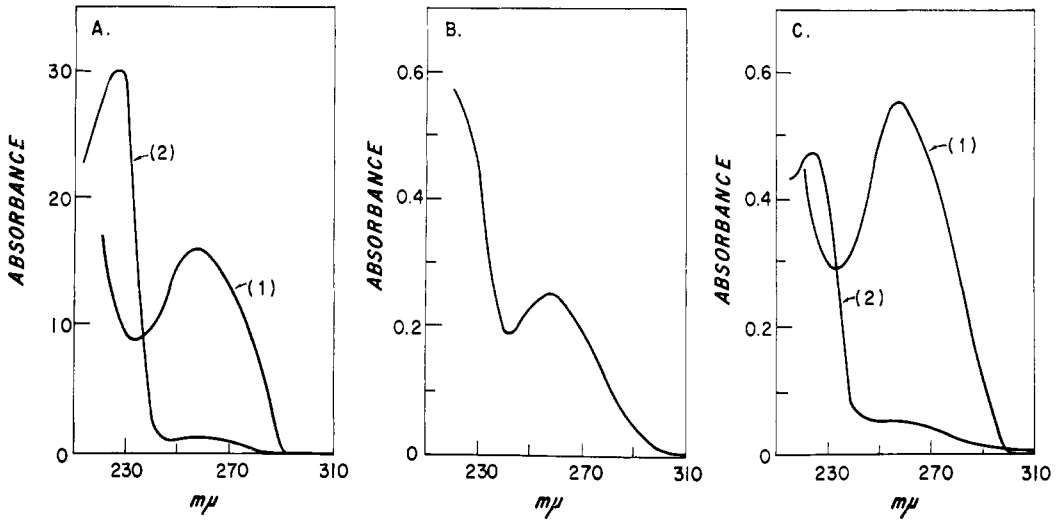

FIGURE 3: Absorption spectra. (A) Curve 1, ribosomes (1 mg/ml); solvent 5 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$. Curve 2, sulfonated polystyrene (1 mg/ml); solvent 10 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$. (B) Mixture of the two, mass ratio of ribosomes:sulfonated polystyrene is 0.96; solvent 5 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$. (C) Fractions isolated by differential precipitation. Curve 1, RNA. Curve 2, protein fraction; solvent 5 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$.

TABLE I: Relative Amounts of RNA and Protein in Fractions Isolated by Differential Centrifugation.

Expt	Component (S)	RNA (μg)	Protein (μg)	Total	% RNA	% Protein
I	Purified 50	653	386	1039	63	37
	21	950	117	1067	89	11
II[a]	Purified 50	252	102	354	71	29
	24	506	19	525	97	3

[a] Components were further purified by sucrose density gradient centrifugation.

(concentration, 1.3 mg/ml). Addition of cold TCA[1] (7%) led to essentially complete precipitation of the nucleic acid (*i.e.*, orcinol analysis of the TCA-soluble supernatant was negative). Analysis of the precipitate for RNA and protein showed this component was rich in RNA; that is, it contained 11% protein (Table I, expt I). Further purification of this component by sucrose gradient centrifugation (Figure 1C) indicated that the majority of this protein could be washed away (Table I, expt II). The final product was contaminated with only 3% protein. Therefore, the reaction of sulfonated polystyrene with purified 50S ribosomes yielded essentially pure RNA. The original 50S subunits were also apparently contaminated with a small amount of soluble protein for the relative amount of RNA was increased from 63 to 71% by sucrose gradient purification (Table I). These analyses were done on material from tubes 15–19 (Figure 1C) and 3–8 (Figure 1A), which were pooled and precipitated with TCA.

Separation of RNA and Protein by Differential Precipitation. Following the reaction of ribosomes and sulfonated polystyrene, the RNA and the protein exhibited different solubility characteristics which allowed essentially complete separation of nucleic acid from protein (Figure 2). In this experiment, ribosomes (2.7 mg/ml) were mixed with sulfonated polystyrene (0.8%) in 5 mM Tris-HCl (pH 7.4)–0.1 mM Mg^{2+} and stored for 16 hr at 4–8°. At this time aliquots were removed. The final NaCl concentration of each of these was brought to 0.01 M and varying amounts of absolute alcohol were added. After mixing, the samples were stored at 4–8° for 30–45 min. Reagents (*i.e.*, NaCl solution and alcohol) generally were at room temperature. Following this chilling samples were centrifuged for 15 min at 4–8° in the clinical centrifuge at 4000 rpm. Supernatants were carefully removed and analyzed for RNA and protein. The relative concentrations of nucleic acid and protein

[1] Abbreviation used: TCA, trichloroacetic acid.

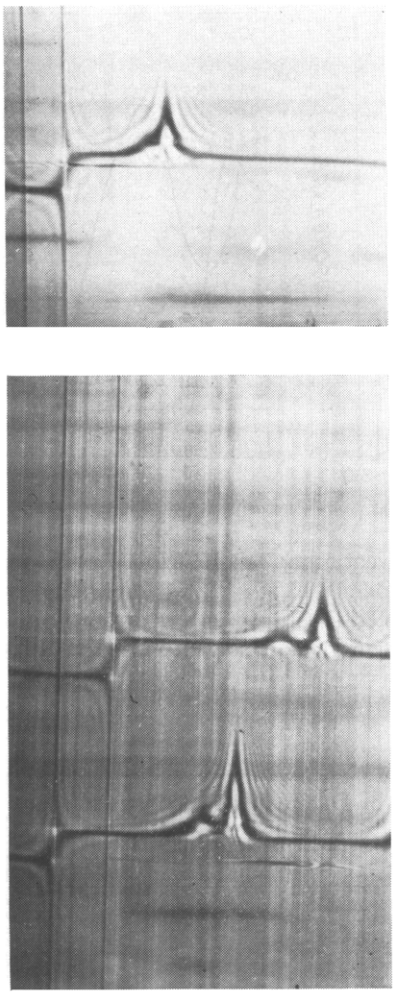

FIGURE 4: Sedimentation pattern of RNA. (A) Material isolated from purified 50S solvent 5 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$. The concentration is 1.0 mg/ml. $s_{20,w} = 18.1$. The picture was taken 42 min after reaching 31,410 rpm. The bar angle is 50°. (B) Material isolated from unfractionated subunits, solvent 0.01 M acetate (pH 4.6)–0.1 M NaCl. The concentration is 1.9 mg/ml in the lower pattern and 1.25 mg/ml in the upper pattern; $s_{20,w} = 16.0$ and 18.7 for the lower pattern and 16.4 and 19.9 for the upper pattern. The picture was taken 74 min after reaching 31,410 rpm. The bar angle is 55°.

found in these supernatants are plotted as a function of the alcohol concentration (per cent by volume) in Figure 2. The data clearly demonstrate that the nucleic acid is less soluble in alcohol than the protein. This difference in solubility can be used to separate the RNA and protein, for at 52% alcohol almost all the ribosomal protein is soluble while all the RNA is insoluble. It should be noted that the alcohol concentration is critical, for an increase of 2% from 52 to 54% leads to the precipitation of about 30% of ribosomal protein.

The protein content of the RNA can be reduced by redissolving the precipitate and reprecipitation with alcohol. We have used 56% alcohol for reprecipitation to ensure complete recovery of RNA. After three such washes, direct analysis of the nucleic acid for protein showed it was contaminated with 5% protein.

The spectrum of RNA isolated by this procedure (Tris buffer, pH 7.4) is shown in Figure 3C, curve 1. Sulfonated polystyrene has a characteristic absorption maximum at 225 mμ (Figure 3A, curve 2), so that contamination of the nucleic acid by the reagent can be easily recognized by determining the ratio $A_{260}:A_{235}$. Typically, for RNA essentially free of the reagent, this ratio is close to 2 in Tris-Mg^{2+}. A 1:1 mixture by weight of ribosomes and sulfonated polystyrene gave a ratio of less than 1 (Figure 3B). It appeared that the spectrum of the mixture was equal to the sum of the sulfonated polystyrene and ribosome spectra (compare Figures 3A and B). However, small differences between the two cannot be excluded by these data.

Ribosomal protein can be precipitated from the solution void of RNA almost quantitatively by increasing the alcohol concentration to 64% (Figure 2). This precipitate is readily soluble in Tris-Mg^{2+}. Inspection of Figure 3C, curve 2, shows the protein so obtained is heavily contaminated by the sulfonated polystyrene reagent.

Characterization of the RNA. The nucleic acid prepared with the sulfonated polystyrene reagent and isolated by differential precipitation was characterized as follows. From the unfractionated ribosomal subunits, two RNA components were seen (Figure 4B), while a single main component was obtained from purified 50S subunits (Figures 1C and 4A). The two components obtained from unfractionated subunits were in a mass ratio of about 1:2 (slow:fast). Extrapolation of the sedimentation rate of these components in 0.01 M acetate buffer (pH 4.6)–0.1 M NaCl to infinite dilution gave values of 18.4 and 24.6 S (Figure 5). The purified 50 S yielded mainly the faster component which had a sedimentation constant of 23.4 S in 5 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$.

RNA isolated from unfractionated subunits exhibited a reduced viscosity in the concentration range 0.2–0.3 g/dl in 0.01 M acetate buffer (pH 4.6)–0.1 M NaCl that was essentially independent of concentration. Extrapolation of the reduced viscosity to infinite dilution gave a value of 0.42 dl/g.

Molecular weight estimates from sedimentation velocity alone were made using the following relationship: $M = (102 \times s)^{1.78}$ (Kurland, 1960), where M is the molecular weight and s is the sedimentation coefficient (in Svedbergs) extrapolated to infinite dilution. A value of 1.1×10^6 was calculated for the faster component (24.6 S) and a value of 0.65×10^6 for the slow component (18.4 S).

A weight-average molecular weight of 10^6 was calculated using sedimentation and viscosity data (Scheraga and Mandelkern, 1953) for unfractionated ribosomal RNA in 0.01 M acetate buffer (pH 4.6)–0.1 M NaCl. In making this calculation the mass ratio of the RNA

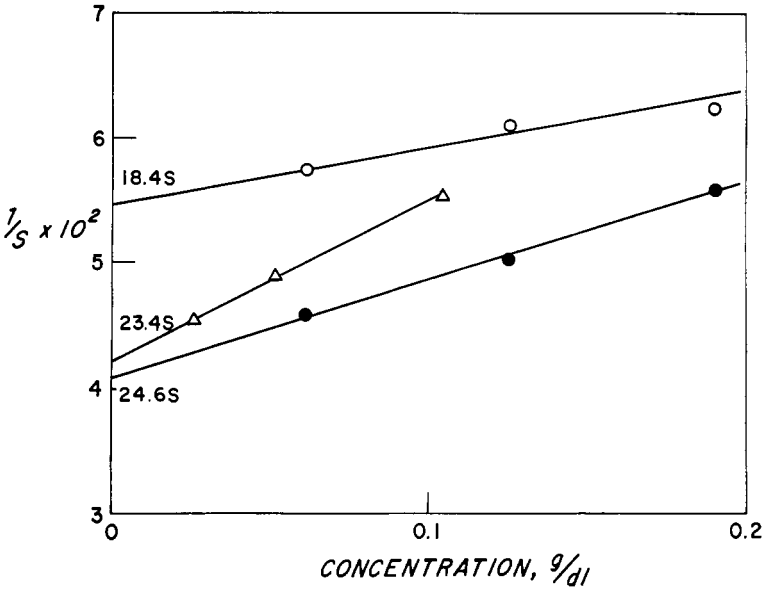

FIGURE 5: Dependence of sedimentation on total concentration for ribosomal RNA. Faster (●—●) and slower (○—○) components were isolated from unfractionated subunits. The solvent is 0.01 M acetate (pH 4.6)–0.1 M NaCl. The main component was isolated from purified 50S subunits (△—△). The solvent is 5 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$. Extrapolated values are shown on the graph.

components was taken as 1:2 (slow:fast), the value of $\bar{v}$ was taken as 0.55 ml/g, and a value of 2.25×10^6 was used for the hydrodynamic parameter, β (as determined for ribosomal RNA at 25° by Kurland, 1960).

Digestion of the ribosomal RNA by RNase (0.2–1 μg/ml) in 5 mM Tris-HCl (pH 7.4)–0.1 mM $MgAc_2$ resulted in an increase in the absorbance at 260 mμ of 28–36%. The lower value was usually observed on relatively short incubation (about 1 hr) with RNAase while the upper limit was observed after relatively long incubations (16–20 hr) at room temperature. Storage of the RNA at 4–8° in Tris-HCl buffer (pH 7.4)–0.1 mM $MgAc_2$ for about 2 weeks had no apparent effect on sedimentation pattern of the nucleic acid, indicating that the RNA prepared in this manner is quite stable.

Discussion

The reaction of a soluble sulfonated polystyrene (molecular weight approximately 100,000) with ribosomal subunits from *E. coli* has been studied. We have found that concentrations of the reagent in the range 0.1–0.8% dissociated almost all the protein from ribosomal RNA. Two methods were employed for purification of the RNA. The first used differential centrifugation followed by sucrose density gradient centrifugation. This procedure gave low yields and required much effort. A new, simple procedure was developed for isolation of RNA that employed differential precipitation. The yields of nucleic acid by the new procedure were usually about 90%. Both methods gave RNA that was contaminated with 1–5% protein. Earlier experiments with yeast ribosomes had indicated that the sulfonated polystyrene dissociated only some of the protein (Morgan, 1966). We have not yet explored the differences found with ribosomes from these two sources.

Preliminary experiments have indicated that levels of the sulfonated polystyrene much below 0.1% dissociate only a small fraction of protein from the ribosomal RNA, but at these levels the reagent cannot protect the exposed nucleic acid from degradation (Danner, 1964). Therefore, for preparation of RNA from unfractionated subunits, the higher level of the reagent is recommended. In most experiments the sulfonated polystyrene and ribosomes were reacted together for 16–20 hr. However, a reaction time as short as 2.5 hr was sufficient for complete dissociation of the protein from ribosomal RNA. A combination of shorter times (*i.e.*, about 1 hr) and lower concentrations of the reagent (*i.e.*, less than 0.1%) produced from purified 50S subunits a component in addition to the RNA that may be an intermediate in the conversion of the subunit to free nucleic acid. Investigations are in progress to determine the nature of this intermediate. The fate of 30S subunits under similar conditions is not known, but the sulfonated polystyrene at these concentrations probably will not protect the exposed nucleic acid from degradation by the latent ribosomal ribonuclease which is bound to this subunit (Tal and Elson, 1963).

The nucleic acid prepared by using the sulfonated polystyrene and isolated by the differential precipitation

procedure has hydrodynamic properties and approximate molecular weights similar to RNA prepared by the phenol methods (Petermann, 1964). This new method, however, is much simpler than the phenol methods, and the yields of RNA are comparable.

Some exploratory experiments concerned with the mechanism of the sulfonated polystyrene and ribosome reaction have been conducted. *p*-Toluenesulfonate (sodium salt) at a concentration of 0.5–1% had no apparent effect on the sedimentation pattern of purified 50S subunits. Higher levels of the sulfonated toluene (*i.e.*, 2–4%) led to changes that were probably the result of the increase in the Na^+ concentration (Elson, 1961), rather than the toluenesulfonate anion. Although this compound is not the true monomer of the sulfonated polystyrene, the results suggest the reaction is due to the unique structure of the polymer. Clearly, the reaction is not solely due to the sulfonated aromatic residues.

The other major reaction product of sulfonated polystyrene and ribosomes (*i.e.*, in addition to RNA) is the ribosomal protein. Chemical methods of purifying the protein from the contaminating sulfonated polystyrene reagent are being explored. Hopefully, the protein can be isolated free of the reagent in a state suitable for physical and chemical studies.

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The Conformation of Ribonucleosides in Solution. The Effect of Structure on the Orientation of the Base*

Werner A. Klee and S. Harvey Mudd

ABSTRACT: The conformations of a number of adenosine derivatives in dilute aqueous solution have been investigated with the aid of measurements of the optical rotatory dispersion, infrared and ultraviolet spectra, and dissociation constants. The compounds studied include adenosine, N^1-methyladenosine, 2′-adenylic acid, 3′,5′-cyclic adenylic acid, 2′-*O*-methyladenosine, 2′,3′-isopropylideneadenosine, 5′-methylthioadenosine, *S*-adenosylmethionine, and *S*-adenosylhomocysteine, as well as other adenosine derivatives containing sulfur substituents on the 5′-carbon. The introduction of a sulfur atom, of either the thioether or sulfonium type, results in change in sign of the Cotton effect centered around 260 mμ. It is proposed that this change is due to an alteration in the conformation of the nucleoside. The relevance of these observations to the more general question of the conformation of nucleosides in solution is discussed.

The present studies were initiated as the result of calorimetric investigations of the enthalpy changes during methyl transfer from a number of sulfonium compounds to the common acceptor, homocysteine (Mudd *et al.*, 1966). It was found that transmethylation from (−)-*S*-adenosyl-L-methionine, the most important biological methyl donor, is accompanied by an unusually high enthalpy change, the basis of which was not clear. To gain some further understanding of this question, *S*-adenosylmethionine and the product formed from it during methyl transfer, *S*-adenosylhomocysteine, have now been studied by a variety of techniques which give some indication of the conformations assumed by these compounds in dilute aqueous solution.

The results of these studies enable us to exclude a number of sterically plausible conformations of *S*-

* From the Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, U. S. Department of Health, Education, and Welfare, U. S. Public Health Service, National Institutes of Health, Bethesda, Maryland 20014. *Received July 11, 1966.*