mentioned that the variation in outer chain size as a function of molecular weight has not as yet been established. If this were the case, it might directly bear upon the results. It is hoped that a more complete understanding of our observations will follow from a continuation of these studies.

Our results indicate that the high molecular weight glycogen species are enzymatically more readily attacked when incubated with the phosphorylase. It has been shown recently by Stetten and Stetten[21] that the turnover of liver and muscle glycogen *in vivo* follows the pattern proposed by previous enzymatic studies *in vitro*,[22] the outer chains being metabolically most active. The work reported here would suggest that if the present enzymatic studies may be similarly extended to the whole animal, it is the outer chains of the high molecular weight species which are subject to most rapid turnover, so far as phosphorylase action is concerned.

We would point out that the present experiments do not rule out the possibility of a splitting of a weak linkage in these polysaccharides by either an enzymatic or non-enzymatic reaction.

Acknowledgments.—The authors wish to express their grateful appreciation to Mrs. Jean Cicirelli who carried out the centrifugal experiments and accompanying calculations on a number of the samples and to Mr. R. E. Post who performed a number of the diffusion experiments. One of us (J.L.) should like to acknowledge the financial support, by Grant 1270i of the National Science Foundation for a portion of this work.

URBANA, ILLINOIS

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(22) J. Larner, *ibid.*, **202**, 491 (1953).

[CONTRIBUTION FROM THE SECTION ON PHYSICAL CHEMISTRY, NATIONAL INSTITUTE OF MENTAL HEALTH]

The Fractionation of Ribonucleic Acids on ECTEOLA-Cellulose Anion Exchangers[1]

BY D. F. BRADLEY AND A. RICH

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Cellulose treated with epichlorohydrin and triethanolamine has been investigated as an anion exchanger for fractionating ribonucleic acid (RNA). RNA is adsorbed from 0.01 *M* neutral phosphate buffer and eluted with sodium chloride solutions of increasing concentration. Elution profiles differ significantly for RNA's from different sources as well as from the same source isolated by different methods. RNA's with higher mean sedimentation coefficients are eluted at higher salt concentrations and the fractions eluted at these higher salt concentrations have higher mean sedimentation coefficients. However, the fractions are nearly as heterogeneous with respect to sedimentation coefficient as the starting material indicating that sedimentation velocity of the adsorbed RNA is not the only factor determining the elution process.

Introduction

Many lines of evidence have accumulated which point to an intimate association between ribonucleic acid (RNA) and protein synthesis. If such a relationship exists, it is most probable that there exists an inherent heterogeneity in intracellular RNA which reflects the large variety of proteins which are synthesized in a single cell. This study was prompted by a desire to resolve some of this heterogeneity by developing methods for fractionating RNA.

In addition to this inherent heterogeneity, there is also a superimposed artifact heterogeneity which arises during the isolation of the RNA from the living organism. At the present time, we cannot clearly separate these two sources of heterogeneity.

For this study, we have adopted an ion-exchange adsorbent of cellulose treated with epichlorohydrin and triethanolamine (ECTEOLA). Bendich, Fresco, Rosencranz and Beiser[2] have used this material to fractionate desoxyribose nucleic acid (DNA) and this has stimulated us to try various forms of ECTEOLA on RNA. In this study, we have fractionated several RNA's from different sources prepared by different methods, and have measured the heterogeneity of the resultant fractions.

(1) Presented in part at the 128th National Meeting of the American Chemical Society at Minneapolis, Minn., Sept. 11, 1955.
(2) A. Bendich, J. R. Fresco, H. S. Rosencranz and S. M. Beiser, THIS JOURNAL, **77**, 3671 (1955).

Experimental

Samples of ECTEOLA were generously contributed to this study by Drs. Peterson and Sober of the National Cancer Institute.[3] The ECTEOLA was treated with 1.0 *M* NaOH, 1.0 *M* KH_2PO_4 and 0.01 *M* neutral phosphate buffer in succession before every run.

Samples of RNA were generously contributed to this study by Drs. E. Volkin of the Oak Ridge National Laboratory (calf liver),[4] S. A. Morell of the Pabst Laboratories (yeast-A), F. W. Allen of the University of California (yeast-C),[5] C. A. Knight of the University of California (tobacco mosaic virus (TMV) RNA),[6] E. Mihalyi of the National Heart Institute (muscle)[7] and E. L. Grinnan and W. A. Mosher of the University of Delaware (rat liver).[8] A commercially available preparation from Nutritional Biochemicals was also used (yeast-B). Solutions of the RNA's in 0.01 *M* neutral phosphate buffer were analyzed for RNA concentration from their optical absorption at 260 mμ, assuming O.D. (1 cm. cell, 1% solution) = 200. The fractionations were all performed at room temperature, 22–24°, unless otherwise indicated.

In the kinetic experiments various volumes of ECTEOLA suspension and RNA solution were mixed, shaken and centrifuged after a measured contact time. Adsorption from salt solution was performed by adding solid NaCl to the RNA solution prior to mixing with the ECTEOLA. Desorption into salt solution was performed by mixing the ECTEOLA and RNA solutions, shaking for 20 minutes, adding solid NaCl to the mixture, shaking and centrifuging

(3) E. A. Peterson and H. A. Sober, *ibid.*, **78**, 751 (1956).
(4) E. Volkin and C. E. Carter, *ibid.*, **73**, 1516 (1951).
(5) A. Crestfield, K. C. Smith and F. W. Allen, *J. Biol. Chem.*, **216**, 185 (1955).
(6) C. A. Knight, *ibid.*, **197**, 241 (1952).
(7) E. Mihalyi and D. F. Bradley, in preparation.
(8) E. L. Grinnan and W. A. Mosher, *J. Biol. Chem.*, **191**, 711 (1951).

after a measured desorption time. The amount of RNA adsorbed to the ECTEOLA was calculated as the initial amount minus the amount remaining in the supernate.

The column fractionations were carried out in 1-cm. diameter columns containing 0.5 g. of ECTEOLA with a nitrogen content of 0.16 meq./g. The column was charged and eluted under pressure, at a flow rate of about 0.7 ml./min. The RNA was eluted by NaCl solutions of successively increasing concentration (in 0.01 *M* neutral phosphate) with 10.0 ml. fractions collected at each concentration. A number of column fractions were also carried out at 4°, at a flow rate of about 0.05 ml./min. with 1 to 2 ml. fractions. In contrast to the stepwise procedure above, in these experiments the RNA was eluted by salt solutions of continuously increasing concentration.

Sedimentation studies of fractionated and unfractionated RNA were carried out in 0.4 *M* NaCl and 0.01 *M* neutral phosphate buffer in the concentration range 0.001 to 0.004% RNA. Photographs of the sedimentation cell were taken in ultraviolet light (254 mμ) in an appropriately modified Spinco Model E Ultracentrifuge.[9] The variation in concentration of RNA within the sedimentation cell was determined from optical density and distance measurements on the photographic negative. From these data the sedimentation coefficient of the weight average sedimenting molecule was calculated using the fundamental definition of sedimentation coefficient. This observed sedimentation coefficient was adjusted to 20° by multiplying by the ratio of the viscosity of the solvent at the observed mean rotor temperature to that at 20°. The boundary half-width was defined as the distance in cm. which would include half of the RNA moving slower and half of the RNA moving faster than the mean sedimenting molecule, *i.e.*, the distance which includes the mid-50% of the sedimenting RNA.

Results

Kinetics.—Figure 1 shows the results of a typical kinetic experiment, in which TMV RNA is adsorbed to and desorbed from ECTEOLA. Under

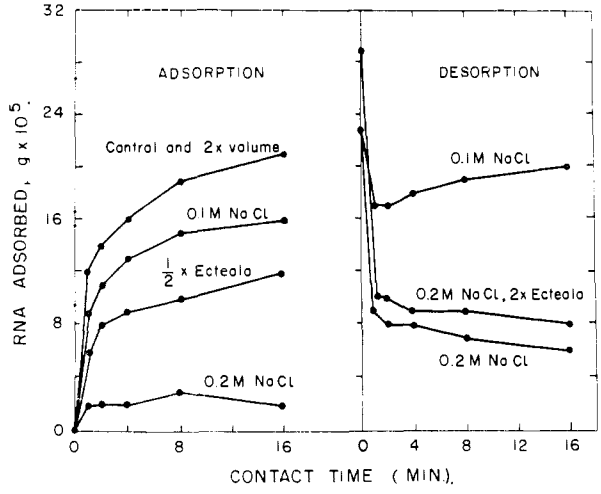

Fig. 1.—Adsorption and desorption of TMV RNA from ECTEOLA: control, 31 × 10^{-6} g. of RNA, 19 mg. ECTEOLA, 15 ml. liquid volume.

these conditions the adsorption is independent of RNA concentration but proportional to the weight of ECTEOLA. The adsorption also depends on the salt concentration, dropping off sharply between 0.1 and 0.2 *M* NaCl. The amount of RNA retained by ECTEOLA during desorption also drops off sharply between 0.1 and 0.2 *M* NaCl, and is roughly proportional to the weight of ECTEOLA at a particular salt concentration. At 0.1 *M* NaCl the rapid initial desorption is followed by a gradual readsorption which may result from a slow change in the *pK* of the exchanger in the presence of NaCl.[10] At higher salt concentrations this effect is offset by the increased eluting power of the higher salt concentration. The amount of RNA adsorbed in 16 minutes in salt solution is less than that retained after 16 minutes of desorption in the same salt concentrations, indicating that this contact time is not sufficient to establish adsorption-desorption equilibrium from both sides of that equilibrium.

Column Fractionation.—In Fig. 2 are presented elution profiles of several RNA's together with the mean sedimentation coefficients of the unfractionated RNA's. As the sedimentation coefficient increases the elution profile shifts toward higher salt concentrations and increasing amounts of RNA are released from the column only by 1.0 *M* NaOH. This tightly bound material is not released by 1.0 *M* KH_2PO_4, 1.0 *M* K_2HPO_4, saturated salt solution, 1% sodium polyphosphate or 0.01 *M* neu-

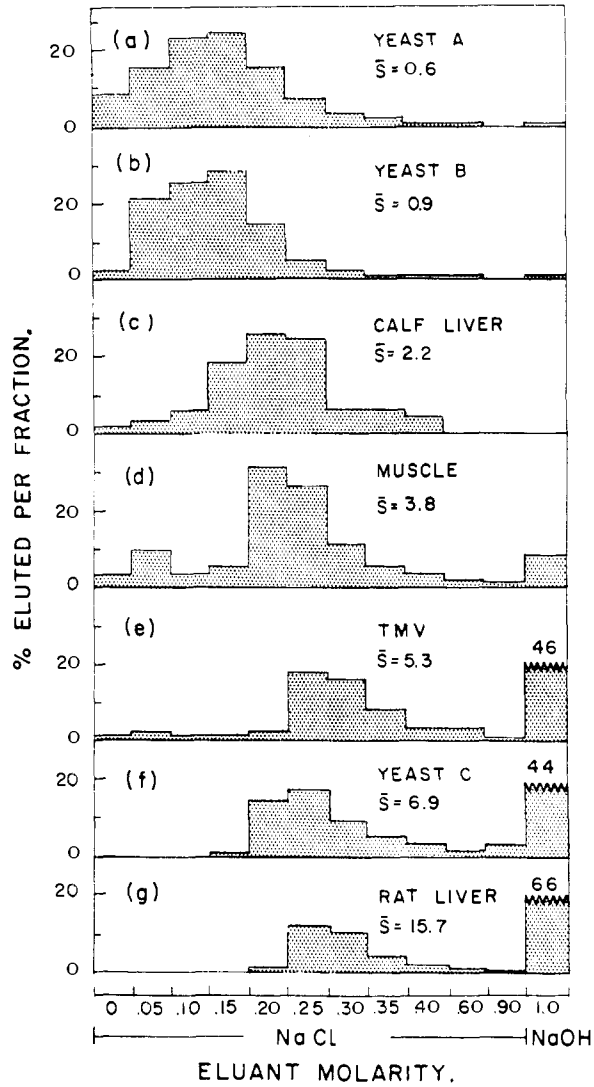

Fig. 2.—Fractionation of different RNA's on ECTEOLA: 0.5–0.7 mg. of RNA per fractionation.

(9) K. V. Shooter and J. A. V. Butler, *Nature*, **175**, 500 (1955).

(10) E. A. Peterson and H. A. Sober, personal communication.

tral phosphate buffer at 95°, although it is released by 1.0 *M* NaCl at 95° as well as the 1.0 *M* NaOH at room temperature.

The choice of protocol for isolating RNA from its biological source has a marked effect on both elution profile and sedimentation coefficient as can be seen from the data on the three samples of yeast RNA (Fig. 2a, b, f). Many factors undoubtedly contribute to denaturing and degrading the RNA during isolation. Merely allowing the RNA to stand in a 0.01*M* salt solution at room temperature, for example, results in a rapid shift of the elution profile to lower salt concentrations as well as a lowering of the sedimentation coefficient (Fig. 3).

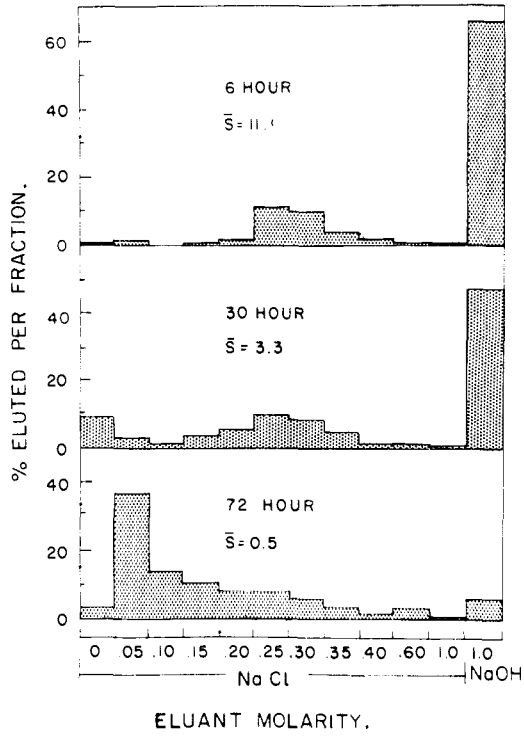

Fig. 3.—Alteration of rat liver RNA upon standing at room temperature (23°). At time zero lyophilized RNA was dissolved in 0.01 *M* neutral phosphate buffer. Samples for sedimentation and chromatographic analysis were removed from this stock solution at the indicated times. Sedimentation analysis at 0.003% RNA, 0.4 *M* NaCl, neutral phosphate buffer; 0.6 mg. RNA used per ECTEOLA fractionation.

The elution profiles examined usually have but a single peak. No new peaks appeared in these profiles when the resolution of the columns was increased by using a slower flow rate, continuously increasing salt concentration, and collecting smaller fractions (Fig. 4). The total fraction eluted with 1 *M* NaCl under these conditions was roughly the same as that obtained in the kinetic experiments with slurries.

Although the larger molecular weight RNA's with the higher sedimentation coefficients are more interesting since they are presumably less denatured and degraded, it is these RNA's which resist elution under mild conditions (Fig. 2). Kinetic experiments suggested that the amount of RNA re-

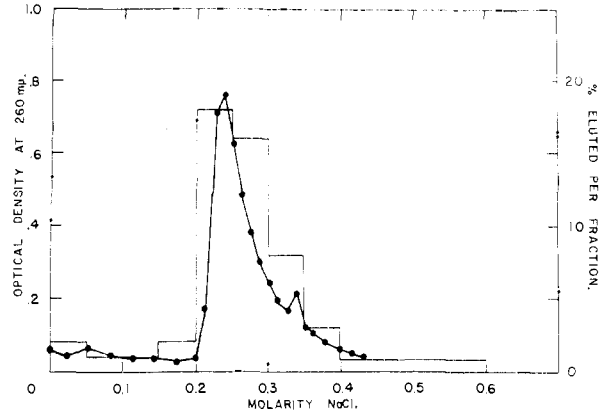

Fig. 4.—Fractionation of TMV RNA on ECTEOLA: - - -, continuous gradient elution; 1.7 mg. RNA on 1.0 g. of ECTEOLA; 2.8 ml./fraction; 0.1 ml./min. flow rate; 50% eluted with NaCl; —, discontinuous gradient elution; 0.5 mg. RNA on 0.5 g. ECTEOLA; 2 ml./min. flow rate, 10 ml./fraction; 52% eluted with NaCl.

tained by ECTEOLA at a given salt concentration increased roughly proportional to the weight of ECTEOLA. Accordingly, by decreasing the column size from 0.5 to 0.05 g. ECTEOLA the amount of TMV RNA retained at 0.6 *M* NaCl dropped from 0.23 to 0.08 mg. Decreasing the density of binding sites from 0.16 to 0.05 meq. N/g. using a 0.2 g. column further reduced this binding to 0.03, corresponding to 91% elution compared with 54% in the standard column. Smaller columns with these and slightly modified exchangers are currently being investigated.

In order to determine whether a true fractionation had occurred, selected fractions were rechromatographed after dialysis at 4° against 0.01 *M* neutral phosphate buffer and the elution profiles compared (Fig. 5). Although the peak in the elution profile corresponds to the salt concentration at which the fraction was originally eluted, the fractions have nearly as broad profiles as the original RNA. The higher resolution elution techniques also produced fractions which gave broad profiles upon rechromatography.

Sedimentation Coefficient.—The fractions differ in mean sedimentation coefficient as well as chromatographic properties, as shown in Table I. In general the fractions eluted at higher salt concentrations have higher sedimentation coefficients. These data are consistent with the previous observations that RNA which has a higher mean sedimentation coefficient has an elution profile which is shifted toward higher salt concentrations as well as the fact that degrading and denaturing RNA lowers the sedimentation coefficient and shifts the elution profile toward lower salt concentrations. These data suggest that the sedimentation coefficient is a factor in determining the elution process. However, the wide range of sedimentation coefficients in fractions eluted at a given salt concentration from different RNA's suggests that it is not the only factor involved.

The sedimentation coefficient is a function of both molecular size and shape and without additional information it is impossible to separate these two

TABLE I

MEAN SEDIMENTATION COEFFICIENTS ($\bar{S} \times 10^{13}$) OF RNA'S

Eluant, *M* NaCl	Yeast A	Yeast B	Calf liver	Muscle	Yeast C[a]	Yeast C
	Unfractionated					
	0.60	0.92	2.24	3.89	6.18	6.91
	Fractionated					
0.05	0.06	0.11				
.10	.17	.29				
.15	.60	.95	0.86			
.20	1.42	1.76	2.39	2.97		4.41
.25			3.76	4.47	5.64	6.28
.30				4.21	6.15	5.80
.35						4.82

[a] Yeast-C solution chromatographed after standing 40 days at 4°. 2 mg. of yeast A and fresh yeast C chromatographed; 1 mg. all others.

factors. However, it is possible to set reasonable limits as to the molecular weight range in which these RNA's fall. The smallest molecular weight consistent with a sedimentation coefficient of 6×10^{-13} corresponds to a spherical RNA of molecular weight 27,500 (specific volume = 0.55) while the largest corresponds to a rod-like RNA molecule of molecular weight 31,000 (20 Å in diameter) or higher with a thinner rod. The rod calculation was made by assuming that the rod has the hydrodynamic properties of the inscribed prolate ellipsoid and evaluating the Svedberg frictional coefficient of the ellipsoid from the Perrin equation.[11] These calculations indicate that the samples of RNA used in this study are much smaller in molecular weight than most samples of DNA, which have molecular weights greater than 1,000,000. It is probable that these relatively low molecular weights reflect denaturation and degradation in isolation.

Sedimentation Boundary Spreading.—The spreading of a sedimentation boundary may reflect either sedimentation heterogeneity, diffusion, or both.[12] The boundaries examined in this study could be fitted with reasonable fit by a Gaussian distribution in (linear) distance along the sedimentation axis with a maximum in the distribution function at the position corresponding to the weight average sedimenting molecule. The fit is nearly within the limits of error in measuring the optical densities of the photographs, *i.e.*, 0.02. The boundary half-widths (Δ) may therefore be converted either into an apparent heterogeneity coefficient ($\sigma_s = \Delta/1.349w^2tx$) or an apparent diffusion coefficient ($D = \Delta^2/3.64t$).[12] As can be seen from Table II in the case of TMV RNA the apparent heterogeneity coefficient is a constant while the apparent diffusion coefficient is not. Furthermore, the apparent diffusion coefficients are too large for RNA, being nearly as large as those of the mononucleotides ($D = 4 \times 10^{-6}$).[13]

Hence, we must conclude that under these conditions, sedimentation coefficient heterogeneity appears to be the principal factor affecting the rate of

(11) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford, The Clarendon Press, 1940, Part IB.

(12) J. W. Williams, R. L. Baldwin, W. M. Saunders and P. Squire, THIS JOURNAL, **74**, 1542 (1952).

(13) G. Schramm, W. Albrecht and K. Munk, *Z. Naturforsch.*, **7b**, 10 (1952).

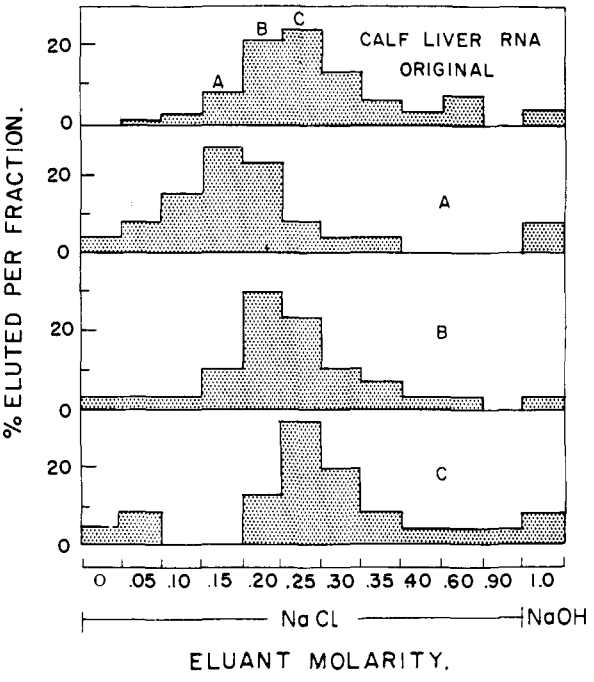

Fig. 5.—Rechromatography of calf liver RNA fractions: initial charge, 1.8 mg. of RNA on 0.5 g. of ECTEOLA.

boundary spreading. These RNA's are quite heterogeneous in this respect, about 67% of TMV RNA falling within a range of about ±3.6 Svedberg units.

Sedimentation boundary half-widths are presented in Table III for the same fractionated and unfractionated RNA's which appear in Table II. In general the fractions eluted at the higher salt concentrations have broader boundaries and therefore are more heterogeneous with respect to sedimentation coefficient. Although some of the boundaries of the fractions are narrower than the starting materials others have boundaries which are as broad or even broader, indicating that the fractionation process definitely does not result in fractions which are appreciably less heterogeneous with respect to sedimentation coefficient than the original RNA.

Discussion

Shape factors remaining constant, RNA's with higher sedimentation coefficients, have higher molecular weights and therefore more phosphate groups to bind with the basic nitrogen groups of ECTEOLA. Higher salt concentrations will be required to break the increased number of RNA–ECTEOLA bonds and elute the RNA. Evidence has been presented that, indeed, RNA's with higher mean sedimentation coefficients have elution profiles shifted toward higher salt concentrations and that fractions eluted at these higher salt concentrations have higher mean sedimentation coefficients.

However, RNA prepared from a different source by a different technique may have a different average shape factor so that a given sedimentation coefficient may correspond to a different molecular weight, tightness of binding, and elution profile. Evidence has been presented that, indeed, fractions eluted at a given salt concentration from different samples of RNA have different mean sedimentation coefficients.

TABLE II

SEDIMENTATION BOUNDARY SPREADING OF TMV RNA

TMV RNA in 0.2 *M* NaCl and 0.01 *M* neutral phosphate buffer at a concentration of 0.0037%. These values are the directly observed half-widths with no correction factors applied.

ω(rad./sec.) $\times$ 30/π	29,500	29,500	42,040	42,040	42,040	59,780	59,780	59,780
t(sec.) $\times$ 1/60	64	112	32	64	96	16	32	48
$\bar{x}$(cm.)	5.967	6.090	5.998	6.163	6.338	5.998	6.185	6.338
Δ(cm.)	0.109	0.175	0.108	0.200	0.321	0.124	0.222	0.321
σ_s($\times 10^{13}$)	3.7	3.4	3.6	3.2	3.4	4.1	3.6	3.3
D($\times 10^{6}$)	0.9	1.3	1.7	2.9	4.9	4.4	7.1	9.9
S($\times 10^{13}$)		6.96			6.85			6.99

TABLE III

SEDIMENTATION BOUNDARY HALF-WIDTHS (Δ, CM.) OF RNA'S

30 ω/π = 59,780 (r.p.m.), t/60 = 64 (minutes), $\bar{x}$ = 6–6.4 cm. These values are the directly observed half-widths.

	Unfractionated					
	Yeast-A	Yeast-B	Calf liver	Muscle	Yeast-C	Yeast-C
	0.155	0.104	0.224	0.353	0.474	0.604
Eluant, *M* NaCl	Fractionated					
0.05	0.040	0.090				
.10	.070	.094				
.15	.141	.148	0.166			
.20	.206	.134	.193	0.246		0.376
.25			.236	.385	0.418	.552
.30				.327	.504	.482
.35						.500

In an ECTEOLA of 0.16 meq. nitrogen/g. there are about 8000 Å.[3] per basic nitrogen group. An RNA of molecular weight 41,000 (specific volume = 0.55) occupies 37,000 Å.[3], sweeping out a volume equivalent to only about 5 nitrogen groups. Considering the variety of shapes possible for an RNA molecule, and the rough cellulose terrain to which the nitrogen groups are attached, it would not be surprising if such an RNA were to make contact with and bind to either considerably more or less than 5 nitrogen groups. Conversely, RNA's over a wide range of molecular weight may be expected to bind with 5 nitrogen groups and therefore elute together when the salt concentration of the eluant becomes sufficiently high to break an average of five bonds.

Further adding to the molecular weight and sedimentation heterogeneity at this salt concentration will be RNA's bound to ECTEOLA with 4 bonds, which are broken with more difficulty than the average because of a particularly favorable packing of the RNA on the cellulose surface or, conversely, those with six bonds that are weaker because of unfavorable packing. It is not surprising therefore that RNA fractions are indeed heterogeneous with respect to sedimentation coefficient although on the average the mean sedimentation coefficient increases with increasing salt concentration.

We are collaborating with Dr. Peterson and Dr. Sober to develop exchangers which will provide a greater number of RNA-exchanger bonds per RNA. As this number of bonds increases the average deviation from the mean number of bonds should decrease percentagewise and result in more homogeneous fractions.

An additional factor in the fractionation process may be the basicity of the RNA molecule. RNA's with higher cytosine and lower uracil contents will contain more basic amino groups and bind less tightly to the basic nitrogen groups of the ECTEOLA. This type of effect has been observed in the binding of DNA to basic protein. The DNA's which contain higher cytosine and lower 5-methyluracil contents are bound less tightly and are eluted at lower salt concentrations from DNA-protein complexes.[14] As long as molecular size or sedimentation coefficient is a significant factor in determining tightness of binding, the average molecule in a fraction will be larger in a more tightly bound fraction. As other factors such as cytosine content become increasingly important, however, the heterogeneity of the fractions will increase.

These fractionation experiments have demonstrated heterogeneity in RNA isolated from various sources. As pointed out in the introduction, we cannot at the present time differentiate between the intrinsic heterogeneity and the artifact heterogeneity arising from the isolation procedures. However, the marked differences between the fractionation of three yeast RNA's isolated by different methods strongly suggests that the artifact heterogeneity constitutes an important part of the observed distribution.

We included tobacco mosaic virus RNA in this study in the hope that it might show less heterogeneity than the material isolated from more complex organisms. This has not proved to be the case and as above, we believe this is an artifact of isolation.

It is anticipated that further improvements will be developed in the ECTEOLA adsorbent system so that we will be able to effectively separate RNA into fractions which are homogeneous with respect to molecular weight. At the present time, the severest limitation to resolving intrinsic heterogeneity is found in the inadequate methods used for isolating RNA. We hope that this work will stimulate others to try different and milder methods for preparing this material.

Acknowledgment.—We wish to acknowledge the assistance of Mr. J. Katsuranis in carrying out many of the column fractionations presented in this paper. We wish to express our appreciation to Drs. E. A. Peterson, H. A. Sober and A. Bendich for many suggestions during the course of this work and for previewing this manuscript.

BETHESDA, MARYLAND

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