both may be involved. Since thrombin adsorbs readily to fibrinogen, additional non-polar groups on the fibrinogen molecule may be expected to increase inactive bonding and thus reduce the free thrombin available for active complex formation. However, at $\mu = 0.075$ and pH 8.6, inactive binding of thrombin by modified fibrinogen appears to be compensated since the activation rates are the same under these conditions.

Ferry[23] has suggested that the structure of fibrin is built up by a lateral association of oriented molecules and that the orientation may be due to long-range electrostatic forces between charged groups on the activated fibrinogen molecule. If this is the case, once activation is accomplished, a low ionic strength medium may be expected to promote orientation and consequently polymerization, provided that the ionic strength is not so low that the molecules are prevented from approaching one another at all. This last condition is encountered during activation at ionic strength $\mu < 0.01$.

With normal activated fibrinogen, the expected results are observed; *i.e.*, the onset of polymerization at $\mu = 0.05$ is immediate, but at $\mu = 0.15$ it is delayed. The behavior of p-tolylazofibrinogen, however, is distinctly different since non-oriented bonding which is insensitive to ionic strength here plays an important role in the aggregation process.

CHARLOTTESVILLE, VA.

(23) J. D. Ferry, *Physiol. Rev.*, **34**, 753 (1954).

[CONTRIBUTION FROM THE NATIONAL HEART INSTITUTE, NATIONAL INSTITUTE OF MENTAL HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE]

Some Physical and Chemical Properties of the Ribonucleic Acid Contaminant of Rabbit Muscle Myosin Preparations

BY ELEMER MIHALYI, DAN F. BRADLEY AND M. IRENE KNOLLER

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Myosin B isolated from rabbit muscle by standard methods contains up to 1% ribonucleic acid (RNA) by weight as an impurity. This paper describes procedures for separating this RNA from the protein as well as some of its physical and chemical properties. Methods involving selective denaturation of the myosin yield a product with a protein/RNA ratio of 1.3–1.4, similar in RNA content to fish nucleo-tropomyosin. The protein is not myosin B but rather a heat stable protein with a low aromatic amino acid content, similar to tropomyosin. The RNA is not tightly bound to this protein, however, as they can be further separated by electrophoresis or by chromatography on ECTEOLA columns under mild conditions, yielding a product with a protein/RNA ratio of less than 0.1. The mean nucleotide composition of the RNA is 18.3% adenylic acid, 31.9% guanylic acid, 30.9% cytidylic acid and 18.9% uridylic acid. All samples analyzed were of this same composition despite wide variations in the extent and methods of fractionation of the RNA. The RNAs were electrophoretically homogeneous but highly heterogeneous in the ultracentrifuge, having a concentration independent, mean sedimentation coefficient of 6.6 S. The preparations showed a typical RNA ultraviolet absorption spectrum with a mean $\epsilon_{(P)}$ value of 7528 at 260 mμ. This $\epsilon_{(P)}$ value increased to 10,782 upon base hydrolysis to the nucleotides, and the spectrum of the hydrolyzate coincided with the sum of the absorptions of the component nucleotides as calculated from the measured nucleotide composition. The spectrum of the hypochromic effect is shown to have two maxima, which nearly coincide with those of guanylic and cytidylic acids.

Introduction

In a previous paper[1] it was shown that myosin prepared from rabbit skeletal muscle contains ribonucleic acid (RNA). In the present study we have isolated this RNA by a variety of methods and have made physical and chemical studies on the isolated material. The physicochemical measurements have been used to compare the extent to which the various isolation procedures tend to degrade, denature and fractionate RNA, and therefrom to estimate the properties of the "native" RNA. Because the isolated RNA possessed both a high sedimentation coefficient and a low $\epsilon_{(P)}$ value and therefore appeared to be one of the better RNA preparations, studies were made of the changes in its physicochemical properties under controlled conditions. Certain conclusions were drawn as to the structure and spectrum of this RNA.

Experimental

Myosin Preparation.—Myosin B was isolated from rabbit muscle as described in detail in the previous paper.[1] The back and hind leg muscles were minced, suspended in twice their weight of 0.9 M KCl, and extracted in the cold (0–4°) for 6 to 10 hours. The suspension was diluted with two liters of ice-cold deionized water and strained through cotton gauze. The filtrate was diluted with 12 liters of ice-cold deionized water to precipitate the myosin and allowed to settle overnight at about 4°. After decanting the supernate, the myosin precipitate was packed by centrifugation and dissolved by adding enough 3 M KCl to bring the final concentration to 0.6 M KCl. Insoluble impurities were removed by centrifugation for 10 minutes at 3000 × g. The myosin was reprecipitated by diluting the supernatant with 4 liters of ice-cold deionized water (to a final ionic strength of 0.05). It was then separated from the solution by centrifugation and redissolved in sufficient 3 M KCl to give a final concentration of 0.6 M KCl. Myosin solutions prepared in this way contained approximately 0.15 mg. of nucleotide material/ml. and about 150 times as much protein.

Deproteinization.—The RNA was routinely separated from the bulk of the myosin B by coagulating the protein in hot 10% NaCl solution.[2] The myosin solution was slowly mixed with half its volume of 30% NaCl and heated in a steam-bath for 8 minutes after coagulation began (at 45°), for a total of about 10 minutes (final temperature about 97°). The suspension was blended for 20 seconds in a Waring Blendor, stirred for 30 minutes at 0°, and then centrifuged at 3000 × g for 10 minutes. The slightly turbid supernate was either filtered through a medium porosity sintered glass filter or centrifuged at 40,000 × g for 20 minutes to remove suspended, denatured protein which otherwise strongly adsorbed RNA in the following ethanol precipitation step.

The 10% NaCl extract was treated with two volumes of ice-cold ethanol and allowed to stand overnight at 4°. The

(1) E. Mihalyi, K. Laki and M. I. Knoller, *Arch. Biochem. Biophys.*, **68**, 130 (1957).

(2) J. N. Davidson and C. Waymouth, *Biochem. J.*, **38**, 375 (1944).

RNA–protein precipitate was collected by centrifugation and dissolved in about 20 ml. of 0.05 *M* KCl. The RNA in such preparations represented on the average of 0.006% of the wet muscle weight. If the colloidal, denatured protein had been removed in the previous step by filtration or centrifugation, 88–95% of the RNA could be dissolved in the 0.05 *M* KCl. If the protein had not been removed, however, the solution of the RNA was less complete and more alkaline conditions were required: 20–28% RNA dissolved in 0.1 *M* KCl at 35°, 39–64% in 0.1 *M* phosphate buffer at *p*H 7.2, and 74% in 0.1 *M* borax at *p*H 9.

As an alternative method for separating RNA from the bulk of the myosin B, the protein was coagulated by treatment with sodium dodecyl sulfate rather than hot 10% NaCl.[3] Five hundred ml. of myosin solution containing 10 mg. protein/ml. was prepared in 0.6 *M* NaCl rather than KCl to prevent precipitation of the potassium soap. This was mixed with 50 ml. of 5% sodium dodecyl sulfate in 45% ethanol, and stirred at room temperature for one hour. The mixture was blended in a Waring blendor to break up the elastic coagulated mass, made up to 1 *M* NaCl, and stirred for 30 minutes. After centrifugation, the RNA-protein solution was precipitated with ethanol as described above.

The ethanol-precipitated material contained about twice as much protein as RNA. To remove this residual protein further deproteinization procedures were investigated. A solution of the ethanol-precipitated material was shaken 7 times with $^1/_3$ volumes of 3:1 chloroform–isopropyl alcohol,[4] separating the phases between extractions by centrifugation. A protein/RNA ratio of 1.4 was obtained. No additional protein was precipitated from the ethanol-precipitated material when it was treated with sodium dodecyl sulfate. Extraction with water-saturated phenol[5] proved unsuccessful as both protein and RNA were taken into the phenol-rich phase.

ECTEOLA-cellulose Fractionation.—RNA-protein preparations were chromatographed on columns of ECTEOLA-cellulose following the procedure of Bradley and Rich.[6] The preparations in dilute salt (less than 0.05 *M*) were adsorbed on 1 cm. diameter columns containing 1.0 g. ECTEOLA with a nitrogen content of 0.16 meq./g. The columns were operated under pressure to flow at about 0.7 ml./min. The RNA and protein were eluted with NaCl solutions of increasing concentration. Fractions of 10 ml. volume were collected and analyzed for protein and RNA.

Sedimentation Studies.—RNA solutions were diluted to standard conditions of 0.25 *M* NaCl, 0.01 *M* potassium phosphate buffer (*p*H 6.8) and an optical density at 260 mμ of 0.5 (approximately 0.0025% RNA by weight). These diluted solutions were run at 59780 r.p.m. and 23–26° in a Spinco Model E ultracentrifuge.[6] The cell was photographed in ultraviolet light which was partially absorbed by the RNA solution. Optical densities of the developed negative were measured to within 0.01 along the image of the sedimentation boundary at 0.01 cm. intervals, using a photographic enlarger and photoelectric densitometer arrangement. The range of optical density between solutions and pure solvent was divided into 10 equal parts, and the distances from the liquid–air interface to the positions along the sedimentation boundary corresponding to these 10 optical densities were determined by interpolation from the graphically plotted density *versus* distance measurements. From the rate of change of these distances with time, as measured in successive photographs, the sedimentation coefficients were calculated in Svedberg units (cm./sec./dyne centrifugal force × 10^{13}) at 10% intervals, from the slowest 10% to the fastest 10%. These coefficients were adjusted to 20° by multiplying them by the ratio of the solvent viscosity at the rotor temperature to the solvent viscosity at 20°, using the tables of Svedberg and Pedersen.[7] These adjusted coefficients have been plotted as the cumulative per cent. (optical) of RNA which sediments at a rate less than given *S*-value.

A few studies also were carried out at higher RNA concentrations using the schlieren optical system in the same centrifuge.

Electrophoresis.—RNA preparations were dialyzed against 0.1 *M* potassium phosphate buffer at *p*H 7.0 for five hours at 4°. The dialysates were electrophoresed in an 11-ml. analytical cell in the Spinco Model H instrument at 0.8° using a current intensity of 8 to 24 mamp. In preparative runs, increased resolution was achieved by reverse compensation which was continued until the separation of the major component peaks appeared to be greater than the cell height. The cell compartments were then isolated by shearing and the contents analyzed.

Chemical Analysis.—Analyses of the preparations were carried out by methods described in greater detail in the previous paper.[1] Total phosphorus, after digestion according to Jones, *et al.*,[8] and easily hydrolyzable phosphorus were estimated with the Fiske–SubbaRow method[9]; total nitrogen by digestion with a selenium–copper catalyst followed by liberation of the NH_3, diffusion of the NH_3 into acid in Conway dishes, and determination in acid with Koch–McMeekin's modified Nessler reagent[10]; ribose with a modified Bial reaction[11]; deoxyribose with the two methods of Dische[12]; and base composition with a combination of the spectrophotometric methods of Loring, *et al.*, and Kerr, *et al.*[13] Mono- and oligonucleotides were removed either by dialysis or ethanol precipitation prior to base determination.

The sum of the RNA and protein concentrations (mg./ml.) was calculated from the total nitrogen analysis, assuming an average nitrogen content of 16.5% for both. The RNA concentration was calculated either from the sum of the concentrations of the individual bases determined spectrophotometrically, or from the total phosphorus analysis. The protein concentration either was calculated by difference or determined directly by the Lowry procedure.[14]

Some difficulty was encountered with the estimation of easily hydrolyzable phosphorus due to precipitation of protein with the molybdate reagent. This difficulty was avoided by first precipitating the protein with trichloroacetic acid, incubating the resulting suspension at 100° for ten minutes to solubilize the RNA, and removing the remaining precipitate by centrifugation. The amount of phosphate liberated after 1 hour at 100° in 1 *N* H_2SO_4 was determined and adjusted to account for the simultaneous hydrolysis of pyrimidine nucleotides, as recommended by Kerr, *et al.*,[13] *i.e.*, the increase in phosphate after an additional 30 minutes hydrolysis was subtracted from the one hour value.

The total phosphorus and total base values agreed with each other with a mean deviation of 2%, while the easily hydrolyzable phosphorus values were an average of 8% lower than the total purine values. With ECTEOLA fractions these deviations were 6 and 17%, respectively.

For spectral studies stock solutions of nucleotides and of the purine bases, obtained from the Nutritional Biochemicals Corporation, were prepared by drying the materials to constant weight and dissolving 0.2–0.6 g. samples in distilled water. The stock solutions were diluted appropriately and made 0.1 *M* in potassium phosphate, *p*H 7.1. Guanine, in the free base form, because of its insolubility in water, was dissolved in 0.01 *M* HCl and then diluted with phosphate buffer.

Results

Chemical Composition.—Analyses of a number of the RNA preparations for protein content are presented in Table I. Although the standard method (heat, centrifugation, ethanol precipitation) resulted

(3) E. R. M. Kay and A. L. Dounce, THIS JOURNAL, **75**, 4041 (1953).

(4) M. G. Sevag, D. B. Lackman and J. Smolens, *J. Biol. Chem.*, **124**, 425 (1938).

(5) A. Gierer and G. Schramm, *Nature*, **177**, 702 (1956).

(6) D. F. Bradley and A. Rich, THIS JOURNAL, **78**, 5898 (1956).

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

(8) A. S. Jones, W. A. Lee and A. R. Peacocke, *J. Chem. Soc.*, 623 (1951).

(9) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

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(11) H. G. Albaum and W. W. Umbreit, *J. Biol. Chem.*, **167**, 369 (1947).

(12) Z. Dische, *Mikrochem.*, **8**, 4 (1930); *Proc. Soc. Exptl. Biol. Med.*, **55**, 217 (1944).

(13) H. S. Loring, J. L. Fairley and H. L. Seagran, *J. Biol. Chem.*, **197**, 823 (1952); S. E. Kerr, K. Seraidarian and M. Wargon, *ibid.*, **181**, 761 (1949).

(14) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *ibid.*, **193**, 265 (1951).

TABLE I

CHEMICAL COMPOSITION OF RNA

No.	Total bases: Total phosphorus	Easily hydrolyzable P: Total phosphorus	Purines: Total phosphorus	Protein / RNA	Preparative method[a]
II	1.00	0.46	0.49	4.82	2HDFL
III	0.98			3.00	2HFE
IV, VII, VIII	1.02	.50	.51	1.52	HFE
VI	0.95			1.37	HFES
IX, X				2.09	DSE
XIII, XIV, XVIII, XX	1.00	.47	.51	1.66	HCE
XX	0.97		.50	0.17	HCE ECT 0.05 *M* NaCl
XX	1.01	.46	.54	0.07	HCE ECT 0.5 *M* NaCl

[a] H, extraction of myosin for 10 minutes in hot 10% NaCl; 2H, two successive extractions in hot 10% NaCl; D, dialysis overnight against running 0.05 *M* KCl at 4°; F, filtration through sintered glass filter; L, lyophilization; E, ethanol precipitation; S, Sevag protein denaturation; DS, extraction of myosin for 30 min. at 23° in 0.5% sodium dodecyl sulfate C, centrifugation for 20 minutes at 40,000 × *g*; ECT, fractionation on ECTEOLA-cellulose columns; *M* NaCl, NaCl concentration at which fraction was eluted from ECTEOLA. The values are averages of all samples prepared by a particular procedure.

in 70 to 100-fold purification of RNA from the original myosin preparation (with a protein/RNA ratio of about 150) all of such samples contained appreciable amounts of protein (column 5).

An apparent minimum protein/RNA ratio of 1.3–1.4 was obtained by methods employing selective denaturation of the protein. This value corresponds to the RNA content of fish nucleo-tropomyosin, according to Hamoir.[15] Higher ratios were obtained with preparations subjected to a second heat denaturation in 10% NaCl (II and III) or to dialysis (II). The first heating extracts of 80–90% of the RNA and the second merely solubilizes additional protein, while dialysis results in a selective loss of RNA.

When ECTEOLA-cellulose columns are charged with RNA preparations and eluted with salt solutions of increasing concentration, the protein is released at lower and the RNA at higher concentrations (Fig. 1). At the peak in the RNA elution

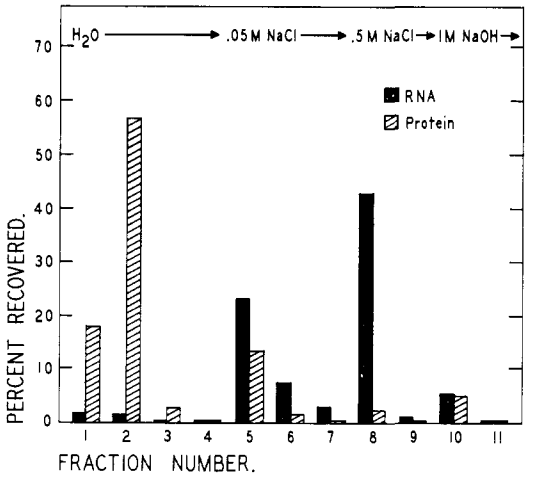

Fig. 1.—Separation of RNA and protein by ion-exchange chromatography on ECTEOLA-cellulose columns. The initial charge was RNA XVIII containing 1.75 mg. RNA and 3.60 mg. protein.

profile the protein/RNA ratio (in this case, 0.11) is considerably lower than in the original material, and has been made as low as 0.02 in some experiments. It seems clear that at least part of the RNA may be prepared protein-free by this procedure.

The electrophoretic mobility of the RNA was about twice that of the protein component (Fig. 2). A preparative run was carried out in which the two

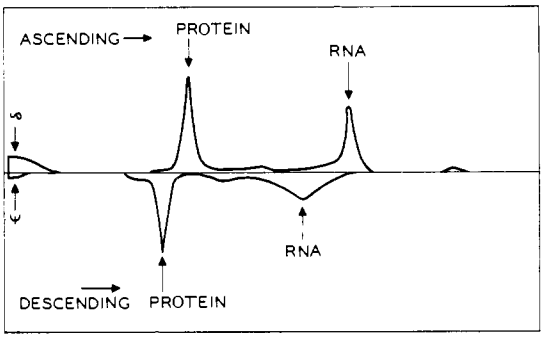

Fig. 2.—Separation of RNA and protein by electrophoresis. The figure is a tracing of photographs of the ascending and descending limbs of the cell taken with the schlieren optical system. The sample was RNA XV containing 1.13 mg./ml. RNA and 2.05 mg./ml. protein. Current, 24 mamp.; time, 138 min.; bar angle, 45°. Protein/RNA ratio initially, 1.84; as calculated from the number of interference fringes of the two major peaks in the ascending limb assuming equal specific refractive increments[16] is 1.53. Under these conditions (*p*H 7) both protein and RNA move toward the anode.

major peaks were completely separated by reverse compensation. The analyses of the cell compartments appear in Table II. These data show that the RNA migrates essentially independent of the protein components and therefore does not exist as a tightly bound nucleoprotein. These data do not exclude, however, the possibility that the RNA is tightly bound to a small amount (*ca.* 20%) of protein in the final preparations.

Nucleotide Composition.—The data in Table III show that all RNA preparations analyzed have nearly identical nucleotide compositions. These RNAs were prepared by a variety of methods which involved removing part of the RNA from the original sample, either by dialysis (50% loss), by ethanol precipitation (26–30%), by electrophoresis

(15) G. Hamoir, *Biochem. J.*, **50**, 140 (1952).

(45%), or by chromatography (up to 75%). The greater scattering of values in the ECTEOLA fractions is to be expected, because of the relatively low RNA and high NaCl concentrations in the samples.[16]

TABLE II

ELECTROPHORETIC PURIFICATION OF RNA

Compartment	Total N	RNA N, mg./ml.	Protein N	Protein/RNA
Original	0.799	0.334	0.465	1.39
Anode	.222	.183	.039	0.21
Center	.800	.355	.445	1.25
Cathode	.446	.126	.320	2.53

The mean nucleotide composition of our RNAs, which are of the high guanine–cytosine type, show the regularities observed by Elson and Chargaff,[17] namely, nearly equal amounts of adenylic and uridylic acids, guanylic and cytidylic acids, purines and pyrimidines, and 6-amino and 6-keto-nucleotides.

Over 97% of the nucleotides in these preparations were found to be ribonucleotides, the remaining being desoxyribonucleotides.

Sedimentation Studies.—The sedimentation profile of the 10% NaCl extract is shown in Fig. 3A. This curve shows the RNA to be highly heterogeneous: 10% does not leave the meniscus, 50% has an S greater than 6.6 and 20%, an S greater than 17. The material which does not leave the meniscus probably consists of mono- and oligonucleotides. Assuming an unhydrated spherical molecule with a partial specific volume of 0.55, the mean sedimentation coefficient of 6.6 corresponds to a molecular weight of 31,700. In view of the high degree of heterogeneity, the mean sedimentation coefficient of 6.6 is not a particularly significant quantity.

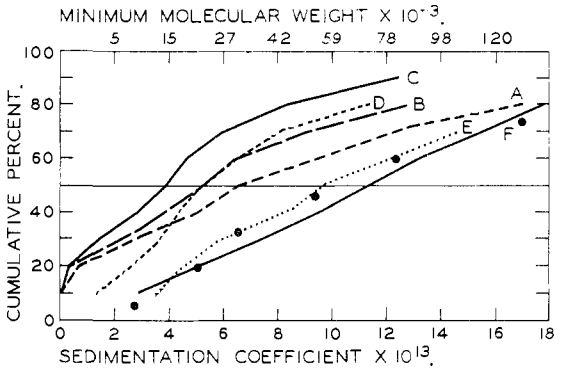

Fig. 3.—Sedimentation profiles of RNA preparations. A, hot 10% NaCl extract of myosin B; B, 10% NaCl extract after 24 hours at 4°; C, 10% NaCl extract after one day at 23°; D, 10% NaCl extract after dialysis for one day against 0.05 M KCl at 4°; E, ethanol precipitable material in 10% NaCl extract; F, ethanol precipitable material in sodium dodecyl sulfate extract of myosin.

Some variation was observed in the sedimentation profiles of 10% NaCl extracts from different myosin preparations. At least part of this variation may be attributed to the lability of the RNA as demonstrated by the decrease in high-S components when the extract is maintained at room temperature (Fig. 3C) or even at 4° (Fig. 3B) for 24 hours. The profile shift may be interpreted as a degradative fragmentation of the RNA, a type of process which may occur at any time during the preparation.

Dialysis of the extract against 0.05 M KCl at 4° results in a dialysate profile (3D) which is steeper (less heterogeneous) than the starting material (3A). This shift may be interpreted as a loss of small, low-S molecules through the membrane and of large, high-S molecules by degradation. Considerable degradation must have occurred as one-half of the RNA passed through the membrane in an overnight dialysis.

The sedimentation profile of the ethanol-precipitable RNA (3E) in the 10% NaCl extracts is shifted toward higher-S components. A significant percentage (26–30%) of the RNA in the 10% NaCl extract consists of small, low-S molecules which are not precipitated by ethanol, and the shift in profile is just what would be expected to follow the removal of this low-S material from the original sample. The results of a calculation of this profile shift appear as solid circles in Fig. 3 and were obtained by adjusting the cumulative per cent. values (P) of the original material (3A) according to the formula

$$P_{adj} = 1.35P - 35.1$$

to account for the loss of 26% of the lowest-S material. The coincidence of these values with the profile of the ethanol-precipitable RNA (3E) shows that fractionation on the basis of sedimentation coefficient is a sufficient explanation of the profile shift and that RNA degradation and denaturation probably do not occur during the ethanol precipitation step.

The ethanol-precipitable RNA which has been prepared by sodium dodecyl sulfate (SDS) denaturation of the myosin has a profile (3F) shifted toward higher-S values as compared with that prepared by heat denaturation (3E). Although the curves are not strictly comparable since the percentage of the RNA recovered after ethanol precipitation was less (57% as compared to 74%) in the case of the SDS-treated material the higher-S character of this material suggests that less RNA degradation occurs during room temperature SDS denaturation of the protein than during heat denaturation.

RNA eluted from ECTEOLA-cellulose columns at 0.2 M NaCl also exhibits sedimentation heterogeneity but has a profile shifted toward higher-S values as compared to unfractionated material. The low-S RNAs are eluted at lower salt concentrations, while some of the very high-S RNAs are bound very tightly to the column and are not eluted with NaCl under these conditions.[6] This fractionation method has therefore a somewhat different effect on the profile than ethanol precipitation. Because of its low absorption, the protein does not contribute to the RNA sedimentation profile as determined with the ultraviolet optical sys-

(16) S. W. Englander and H. T. Epstein, *Arch. Biochem. Biophys.*, **68**, 144 (1957).

(17) D. Elson and E. Chargaff, *Biochim. Biophys. Acta*, **17**, 367 (1955).

TABLE III

NUCLEOTIDE COMPOSITION OF RNA

No.	Mole % Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Mole ratios A/U	G/C	Pu/Py	6-Am/6-K
Unfractionated material								
II	18.0	31.0	31.5	19.5	0.92	0.98	0.96	0.98
IV	19.0	32.3	29.6	19.1	.99	1.09	1.05	.95
VII	17.2	31.1	32.7	19.0	.91	0.95	0.93	.99
XIII	17.5	31.0	32.2	19.3	.91	0.96	0.94	.99
XIV	17.6	33.0	31.1	18.3	.96	1.06	1.02	.95
XVIII	19.2	33.5	28.8	18.5	1.04	1.16	1.11	.92
XX	19.6	31.8	30.2	18.4	1.06	1.05	1.06	.99
Mean	18.3	31.9	30.9	18.9	0.97	1.03	1.00	0.97
Material fractionated on ECTEOLA column								
XVIII Ect. 0.05	18.4	27.5	32.8	21.3	0.86	0.84	0.85	1.05
XVIII Ect. 0.5	16.4	31.3	32.7	19.6	.84	.96	.91	0.96
XIX Ect. 0.05	19.2	29.3	30.5	21.0	.91	.96	.94	.99
XX Ect. 0.05	20.0	31.5	28.4	20.1	.99	1.11	1.06	.94
XX Ect. 0.5	17.9	35.4	29.8	16.9	1.06	1.19	1.14	.91
Mean	18.3	31.0	30.8	19.7	0.93	1.01	0.98	0.97
Material fractionated by electrophoresis								
XVI Electro.	18.2	30.5	30.5	20.8	0.88	1.00	0.95	0.95

TABLE IV

SPECTROSCOPIC DATA ON RNA PREPARATIONS[a]

No.	Native RNA $\epsilon_{(P)max}$	$\epsilon_{(P)min}$	$\epsilon_{260}/\epsilon_{280}$	Base hydrolyzed RNA $\epsilon_{(P)max}$	$\epsilon_{(P)min}$	$\epsilon_{260}/\epsilon_{280}$	Hypochromic effect at 260 mμ %	$\Delta\epsilon_{(P)}$
III	7001258	4734238	1.914					
IV	7297260	4597238	1.961					
V	6766258	4462238	2.010					
VI	6795258	4260238	1.854					
XIII	7716258	4691236	2.039					
XVII	7856258	4449234	2.064	10548238	5666230	1.825	33.3	2692
XVIII	8199258	5454238	1.962	10447258	6725236	1.775	27.0	2201
XIX	7984258	4197232	2.088	11025258	.....	...	39.4	3117
XX	8136258	4838236	2.023	1110258	6514232	1.807	35.9	2974
Mean	7528 ± 500	.....	1.990	10782 ± 285	.....	1.802	33.9	2746 ± 299
XIII ECT.	8829258	4571232	2.078	10713258	5368230	1.806	20.2	1884
XIV ECT.	8107258	3691230	2.121	10872258	4619228	1.895	33.4	2765
XVIII ECT. 0.5 *M*	7967258	3426230	2.122	10683258	4338226	1.845	33.7	2659
XX ECT. 0.05 *M*	8584	.....	2.133	.....	.....	...	..	..
XX ECT. 0.5 *M*	8179	.....	2.100	.....	.....	...	..	..

[a] RNA was base hydrolyzed for one hour at 40° in 0.5 *M* NaOH to nucleotides. The hypochromic effect is defined as the difference in $\epsilon_{(P)}$ between the base hydrolyzed and native RNA. $\epsilon_{(P)}$ values of the component nucleotides calculated from their individual absorptions and the average composition of the RNA (*cf.* Table IV) are $\epsilon_{(P)}$ (max, 258 mμ) = 10,790; $\epsilon_{(P)}$ (min, 226 mμ) = 4691; $\epsilon_{(P)}(260)/\epsilon_{(P)}(280)$ = 1.799. All spectra were measured at *p*H 7.1 in 1 *M* potassium phosphate buffer. Subscripts indicate the wave lengths at which $\epsilon_{(P)max}$ and $\epsilon_{(P)min}$ occur.

tem, even though it makes an appreciable contribution to the profile determined with the schlieren optical system. Schlieren photographs taken during a centrifuge run at high concentration (0.42%) show that despite the high concentrations of RNA and protein, the RNA sediments faster than and independently of the protein component. The *S*-values corresponding to the three observed RNA peaks are 3.8, 6.4 and 8.5 *S* while that of the protein peak is 2.8. The weighed mean *S* of the three RNA peaks is about 5.8 *S*. The peaks vary in number and area among the preparations, and become broader and lower upon standing.

Ultraviolet Absorption Spectrum.—The absorption spectrum of a typical RNA preparation appears in Fig. 4A. Following the suggestion of Chargaff and Zamenhof,[18] the absorption is expressed in $\epsilon_{(P)}$ values, the optical density of a solution containing one gram atom of RNA phosphorus per liter. Although these preparations contained appreciable amounts of protein, the

(18) E. Chargaff and S. Zamenhof, *J. Biol. Chem.*, **173**, 327 (1948)

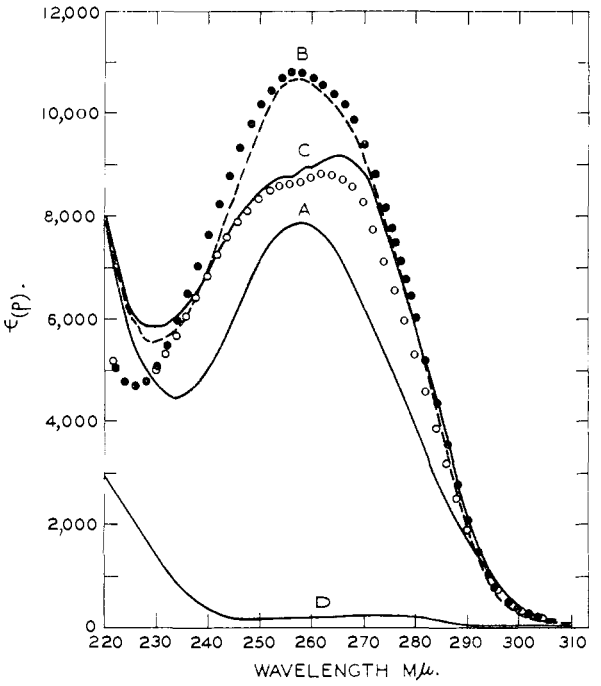

Fig. 4.—Ultraviolet absorption spectra of RNA preparations: A, ethanol precipitable material in a hot 10% NaCl extract (RNA XVII); B, A after hydrolysis for one hour at 40° in 0.5 *M* NaOH; C, A after hydrolysis for one hour at 100° in 1 *N* H_2SO_4; D, heat stable protein at a concentration of 322 mg./ml.; filled circles, calculated absorption of component nucleotides; open circles, calculated absorption of component pyrimidine nucleotides and free purines. Spectra A, B, C were determined in 1 *M* potassium phosphate, *p*H 7.1.

contribution of this protein to the absorption above 240 mμ is negligible. In Fig. 4D is given the absorption curve of the RNA-free protein, isolated by chromatography on ECTEOLA, in a hypothetical solution with a protein/RNA ratio of 1.0 and 1 *M* in RNA phosphorus. This heat-stable protein resembles tropomyosin in its low specific absorption (0.57 at 280 mμ for 1 mg./ml.) and must have a low aromatic amino acid content.

In Table IV are summarized spectral data of several preparations. Although the $\epsilon_{(P)}$ values show considerable individual variations, which seem to be common with RNA preparations, they are generally lower than those reported in the literature.[3,19,20]

The calculated absorption spectrum of the sum of the component nucleotides in the RNA appears as filled circles in Fig. 4. These values were calculated from the spectra of the individual nucleotides and the nucleotide composition of the RNA. The spectrum of the RNA (Fig. 4A) is considerably lower, between 230 and 290 mμ, than that of the component nucleotides. Magasanik and Chargaff[19] were first to point out that the absorption of the native RNA (and DNA) is lower than the sum of the absorptions of its nucleotides (the hypochromic effect). The variation in $\epsilon_{(P)}$ of the native RNA preparations may be interpreted in terms of variations in the degree of denaturation and degradation occurring during isolation.

When the native RNA is hydrolyzed for an hour at 40° in 0.5 *M* NaOH and the hydrolyzate neutralized, the resulting spectrum (Fig. 4B) corresponds to that of the nucleotides, except below 240 mμ where protein absorption becomes significant. The absorption of the alkali hydrolyzed ECTEOLA fractions (not shown), however, corresponds to that of the nucleotides, including the region below 240 mμ, since these fractions are protein-free. When RNA is hydrolyzed for an hour at 100° in 1 *N* H_2SO_4 and the hydrolyzate neutralized, the spectrum closely resembles the sum of the absorbances of the pyrimidine nucleotides and purine bases (Fig. 4C). The higher absorption of the hydrolyzate may be indicative of incomplete liberation of the free purine bases.

There is at present no definitive interpretation of the hypochromic effect in terms of molecular structure.[19,21] Several of our observations may prove relevant in this regard. The first of these observations is that when RNA is hydrolyzed under conditions which result in oligonucleotide formation,[19] namely, an hour at 35° in 0.5 *M* NaOH, the hypochromic effect of the hydrolyzate, while smaller than in the native RNA, still amounts to about 13% at 260 mμ. This observation is in agreement with that of Laskowski and Privat de Garilhe[22] who showed that isolated oligonucleotides have about a 10% hypochromic effect. Apparently the helical polynucleotide structure is not a requirement for at least part of the hypochromic effect.

The second observation is that the hypochromic spectrum (Fig. 5A), scaled up to match the sum of guanylic and cytidylic acids at 260 mμ, bears a striking resemblance in shape to the sum of guanylic and cytidylic acids (Fig. 5B) in the proportions existing in the RNA while markedly different from the sum of adenylic and uridylic acids (Fig. 5C). The hypochromic spectrum was calculated by subtracting at each wave length the $\epsilon_{(P)}$ value of the native RNA from that of the base hydrolyzed RNA (nucleotides). Magasanik and Chargaff[19] have already pointed out that the hypochromic effect is large in fragments which have high guanine content. We suggest that the hypochromic effect may reside mainly in the guanylic and cytidylic acid residues.

Discussion

Sedimentation data show the RNA preparations to be highly heterogeneous. It might be supposed that these different RNAs derived from a single "parent" RNA *in vivo*, which became degraded and fragmented during the isolation procedures. The data do not eliminate such a possibility. The constancy of the base distribution among different samples subjected to varying fractionation procedures would indicate either that the bases are distributed uniformly along the parent molecule, or

(19) B. Magasanik and E. Chargaff, *Biochim. Biophys. Acta*, **7**, 396 (1951).

(20) K. K. Tsuboi and R. E. Stowell, *ibid.*, **6**, 192 (1950); T. G. Northrop and R. L. Sinsheimer, *J. Chem. Phys.*, **22**, 703 (1954).

(21) R. Thomas, *Biochim. Biophys. Acta*, **14**, 231 (1954); R. Thomas, *Bull. soc. chim. biol.*, **35**, 609 (1953); P. D. Lawley, *Biochim. Biophys. Acta*, **21**, 481 (1956).

(22) M. Privat de Garilhe and M. Laskowski, *J. Biol. Chem.*, **223**, 661 (1956).

that the breakages occurred at random points along the chain. If, on the other hand, the RNA were initially heterogeneous then the constancy of base distribution would indicate either that none of the fractionation methods (dialysis, precipitation, electrophoresis, chromatography) were based on nucleotide composition, or that all of the molecules have approximately the same distribution.

In contrast to the high degree of heterogeneity displayed in the ultracentrifuge, the RNA traveled as a single peak in the electrophoresis apparatus. This, seemingly contradictory, behavior is frequently observed with polymers, which may have the same electrophoretic mobility within wide limits of the degree of polymerization, and also with some protein aggregates.[23] Apparently, the rate of electrophoretic migration in these instances is determined by the charge per unit mass and is largely independent of size.

The RNA preparations were surprisingly labile even in 10% NaCl indicating either the presence of a destabilizing material (*e.g.*, a heat stable ribonuclease) or that the RNA molecules are formed of sub-units held together by weak hydrogen bonds. The sedimentation profile was not altered, however, in concentrated urea solution which ought to have broken any such weak bonds. The RNAs were found to be more labile in the original myosin preparation than in the 10% NaCl extracts suggesting that some labilizing material was present in the myosin preparations which was only partially removed by treatment with hot 10% NaCl.

The sedimentation coefficients were practically independent of concentration over a hundred-fold range of concentration, from 0.001% using ultraviolet optics to 0.4% using schlieren optics. This behavior differs from that of DNA, in which case the sedimentation constant shows a marked concentration dependence.[24] This concentration independence is most easily explained by assuming that the RNAs are very nearly spherical in shape and therefore do not strongly interact with one another in solution.

The methods investigated were not able to isolate a completely protein-free RNA. It was shown unequivocably, however, that the apparently constant protein/RNA ratio of 1.4 in the standard preparations is only fortuitous, and that there is no strong interaction between RNA and protein in this system. However, the RNAs further fractionated by electrophoresis or chromatography still contained 2–21% protein which may possibly be tightly bound to the RNA. It may be mentioned that very few protein-free RNA preparations are described in the literature[17] and a small amount of proteinaceous material may be an integral part of the molecule. Since an initial step of the preparation was a heating in salt solution, any loose protein–RNA association would have been broken in this step, and remain undetectable. The fact that the RNA traveled more slowly into than out of the protein solution in the electrophoretic experiments may be an indication that RNA and the heat-stable protein do form some sort of loose interaction. However, this velocity difference may be explained in other ways such as differences in the viscosity, or field strength in the two solvents.

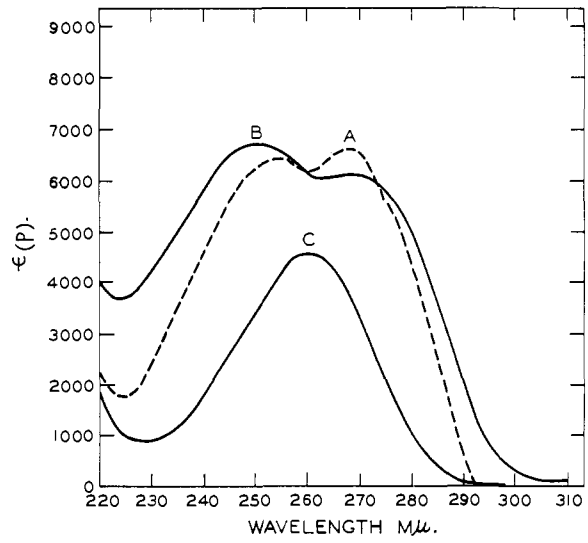

Fig. 5.—Ultraviolet absorption spectrum of the hypochromic effect: A, spectrum of the hypochromic effect × 2.28 (RNA XIV) ECTEOLA fraction 0.5 *M* NaCl; B, absorption of component cytidylic + guanylic acids; C, absorption of component adenylic + uridylic acids.

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(23) E. F. Fitzgerald and R. M. Fuoss, *J. Polymer Sci.*, **14**, 329 (1954); J. T. G. Overbeek and D. Stigter, *Rec. trav. chim.*, **75**, 543 (1956); K. O. Pedersen, *Nature*, **128**, 150 (1931); E. Mihalyi, *Acta Chem. Scand.*, **4**, 351 (1950).

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