The Purification and Properties of Ribonucleic Acid from Wheat Germ*

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Commercially available wheat germ has been used as a source of gram quantities of ribonucleic acid using ordinary laboratory equipment and a relatively simple procedure. By an extension of the method of Lane and Allen (1961), two fractions of RNA have been obtained, each showing negligible contamination. The lower molecular weight fraction shows a single boundary in the ultracentrifuge with a sedimentation coefficient of 4.1 S. The chain length, high content of trace nucleotide components, end groups, and biological activity all serve to characterize this fraction as s-RNA (or amino acid acceptor RNA). The higher molecular weight fraction contains two major components with sedimentation coefficients of 18 and 24 S, indicating ribosomal origin. Some degradation of the high molecular weight components is indicated by the presence of small quantities of material showing lower sedimentation coefficients. It has been shown that a slight residual nuclease activity is associated with this higher molecular weight fraction.

A relatively simple and inexpensive procedure for the preparation of minimally degraded ribonucleic acid (RNA)[1] in gram amounts could be valuable both in the study of the structure and properties of RNA itself and in the study of enzymes which act on RNA. While a large scale method exists for the preparation of s-RNA from yeast (Holley, 1963), another convenient method for the preparation of both s-RNA and high molecular weight RNA seemed possible using only ordinary apparatus and a readily available commercial source of material, wheat germ. The isolation and fractionation procedure of Lane and Allen (1961) was used as a basis for further purification. This report will describe the isolation procedure which has been developed as well as some properties of the two fractions of RNA which have been obtained.

MATERIALS AND METHODS

Method of Preparation of RNA.—Five lb of wheat germ (Fischer's, not toasted, purchased from a local grocery) was extracted in convenient portions using five volumes of ethanol-ether (1:1). The extract was discarded and the extraction was repeated using three volumes of ethanol-ether. Again the solvent was discarded and the wheat germ was spread in a thin layer to dry overnight at room temperature.

Fifty-gram portions of defatted wheat germ were suspended in 300 ml of 10^{-4} M EDTA (ethylenediaminetetraacetate), *p*H 8.0, in the bowl of a Waring Blendor. In one experiment sodium dodecyl sulfate (SDS) was added to the EDTA buffer at a level of 0.5%. Three hundred ml of 90% phenol solution (reagent grade, not redistilled) was added and the mixture was homogenized for 30 seconds at low speed and 30 seconds at high speed. The homogenate was then transferred to a large beaker and mixed slowly for 1 hour at room temperature using a magnetic stirrer.

The homogenate was next transferred to 250-ml polyethylene centrifuge bottles and spun at 1,500 × *g* for 15 minutes using a Servall centrifuge refrigerated to a temperature of 0°. All subsequent operations were carried out in the cold unless otherwise stated. After centrifugation the aqueous upper layer was removed by pipet, leaving behind as much of the white interfacial material as possible. The phenol layer and debris were then suspended in 150 ml of EDTA buffer, and the mixture was vigorously shaken and centrifuged as above. The aqueous layer was removed and combined with the aqueous layer from the previous centrifugation. The combined aqueous fractions were then centrifuged for 1 hour at 1,500 × *g* to remove the remaining phenol, and the aqueous layer was very carefully separated.

The supernatant aqueous layer was made 3.0 M with respect to sodium chloride and immediately centrifuged at 1,500 × *g* to remove phenol crystals. The aqueous portion was then rapidly filtered through a small plug of cotton and allowed to stand at 0° for 3 days. The high molecular weight RNA precipitate was collected by centrifugation and washed twice with 67% ethanol, twice with 95% ethanol, twice with absolute ethanol, and three times with ether. The product was further dried under vacuum and stored at 0°. No further purification of this fraction was attempted.

Crude s-RNA was precipitated from the 3 M NaCl supernatant solution by the addition of two volumes of ethanol. After allowing 2 days to complete precipitation, the product was collected by centrifugation and washed twice with 67% ethanol.

Combined crude s-RNA preparations from the entire 5 lb of wheat germ were suspended in 1 liter of distilled water. Portions of 250 ml were mixed with 250 ml of 2.5 M K_2HPO_4 and 12.5 ml of 33% H_3PO_4, and were then extracted with 250 ml of methyl Cellosolve (2-methoxyethanol, practical grade, Eastman) at room temperature. The interfacial material and lower layer were removed and re-extracted first with 50 ml and then with 35 ml of pre-equilibrated upper layer. The combined upper layers from the entire preparation were carefully transferred to dialysis tubing and dialyzed overnight against two 15-liter portions of distilled water at *ca.* 0°. To the material within the dialysis bag, NaCl was added to a concentration of 1 M, and the RNA was precipitated by the addition of two volumes of cold ethanol.

After allowing 2 days in the cold to complete precipitation, the s-RNA was collected by centrifugation and dissolved in 1 liter of 0.1 M Tris buffer [tris(hydroxy-

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[1] Abbreviations used in this work: RNA, ribonucleic acid; TMV-RNA, tobacco mosaic virus ribonucleic acid; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DEAE-cellulose, diethylaminoethyl-cellulose; DNA, deoxyribonucleic acid; CTP, cytidine triphosphate; CMP, cytidine monophosphate; ATP, adenosine triphosphate; AMP, adenosine monophosphate.

TABLE I
PURIFICATION OF WHEAT GERM RNA

Step in Preparation	Property							
	Yield[a] OD_{260} Units	Extinction (1% soln)	ϵ_p	I_2 Test	RNA (%) (Orcinol)	P (%)	Protein (%)	DNA (%)
s-RNA fraction								
Crude preparation	84,000	35.95	1500	++++	122	7.4	1.12	0.7
Cellosolve extract	81,000	90.7	3900	+	87	7.2	2.62	0.8
DEAE-cellulose effluent	58,000 (2.74 g)	210	7500	−	97	8.7	1.64	1.5
DEAE-cellulose effluent (elution with 1 M NaCl in Tris)[b]		114	2400	−	48	14.7	1.30	1.4
High molecular weight RNA	152,000 (6.4 g)	237	7200	+	96	10.2	2.24	1.4

[a] The yield shown is for 5 lb of starting material. [b] Elution with 1 M salt in Tris buffer was not used as a purification step The data are presented to indicate the degree of purity attained if such a procedure were used.

methyl)aminomethane, primary standard grade Sigma 121, Sigma Chemical Company], *p*H 7.5, and applied to a column of DEAE-cellulose (Brown Company) of dimensions 115 cm length by 4.5 cm diameter. The column was washed with 7 liters of 0.1 M Tris buffer and the eluent was discarded. This was followed by a gradient of 0.0–1.0 M sodium chloride in Tris buffer using a total volume of 6 liters. Fractions of 18 ml were collected, the optical density at 260 mμ was measured using a Beckman DU spectrophotometer, and the peak fractions having an optical density greater than 2.0 were pooled. Sodium chloride was added to a final concentration of about 1 M and two volumes of ethanol were added to precipitate the RNA. After 2 days in the cold the product was collected and washed and dried as in the case of the high molecular weight RNA. Data on the yield and properties of each fraction are presented in Table I.

Tobacco Mosaic Virus RNA.—TMV-RNA was a gift of Dr. C. A. Knight. It was prepared by the method of Fraenkel-Conrat (1957), and had been stored in the dry state at room temperature for at least 3 years prior to use in these experiments.

Experimental Methods.—Protein was determined by the method of Lowry *et al.* (1951) using a bovine plasma albumin (Armour) standard. Phosphate was measured using the Fiske-Subbarow procedure as outlined in Cowgill and Pardee (1957). The orcinol determination of RNA and the diphenylamine determination of DNA have been described by Dische (1955). Optical density measurements were made using a Beckman DU spectrophotometer in the ultraviolet range of the spectrum and a Beckman B spectrophotometer in the visible range.

Sedimentation velocity was measured using a Spinco Model L preparative ultracentrifuge equipped with an SW 25.1 swinging-bucket rotor. Sucrose density gradients were prepared and fractionated using the apparatus and techniques described by Martin and Ames (1961). In one case sedimentation velocity was measured in a Spinco Model E analytical ultracentrifuge equipped with an ultraviolet optical system. Methylated albumin–kieselguhr was prepared using bovine plasma albumin and Hyflo Supercel according to the method of Sueoka and Cheng (1962). Columns of 2.8 cm length by 2.3 cm diameter were prepared using 25 ml of methylated albumin–kieselguhr suspension and small sintered glass funnels. Two mg of RNA was adsorbed on such a column, and elution was carried out with 600 ml of 0.05 M NaH_2PO_4 buffer employing a linear gradient of NaCl ranging from 0.05 to 1.95 M. A flow rate of 2.5 ml/minute was used.

RNA was hydrolyzed for nucleotide analysis using 1 M KOH at 30° for 24 hours. The hydrolysate from 50 mg of wheat germ RNA (or 13.5 mg in the case of tobacco mosaic virus RNA) was neutralized with solid Dowex 50-H^+, the *p*H was adjusted to 10 using NH_3 solution, and the suspension was filtered to remove the resin. The hydrolysate was then taken up on a column of Dowex-1-formate, x-8, 200–400 mesh, of dimensions 28 cm length by 1.5 cm diameter. Nucleosides were eluted using 300 ml of 0.01 M ammonium formate, *p*H 3.7. Nucleotides were then eluted using a parabolic gradient of 0.035–0.6 M ammonium formate, *p*H 3.7, in a volume of 3 liters, followed by a linear gradient over 700 ml of 0.6–3.0 M formate buffer. The method is essentially that described by Ofengand *et al.* (1961). Components of the digest were identified by means of their ultraviolet spectra measured with a Cary Model 14 spectrophotometer. Trace components were identified by comparison of the spectral data with those

TABLE II
THE COMPOSITION OF WHEAT GERM RNA AS DETERMINED BY ION-EXCHANGE CHROMATOGRAPHY[a]

Component	s-RNA	High Molecular Weight RNA	TMV-RNA	
			Found	Literature[b]
Nucleosides	1.3	0.2	0.1	
Adenosine	1.0			
Cytidine	0.3			
Nucleotides	98.7	99.8	99.9	100
Adenylic acid	21.8	21.5	26.6	29.5
Guanylic acid	29.7	31.7	26.9	25.25
Cytidylic acid	23.6	23.3	18.2	18.5
Uridylic acid	19.2	21.6	28.2	26.75
Pseudouridylic acid	2.6	1.9	0.0	
Guanosine diphosphate[c]	1.9			

[a] Expressed as mole per cent nucleoside plus nucleotide. [b] The literature values given were obtained by Black and Knight (1953). [c] The guanosine diphosphate content has been included in the total quantity of nucleotides. It has not been corrected for interference by other components of the postmononucleotide fraction (not included in the calculations of composition) and is certainly high. A better estimate of the actual quantity of guanosine diphosphate present in the hydrolysate would be closer to 1%.

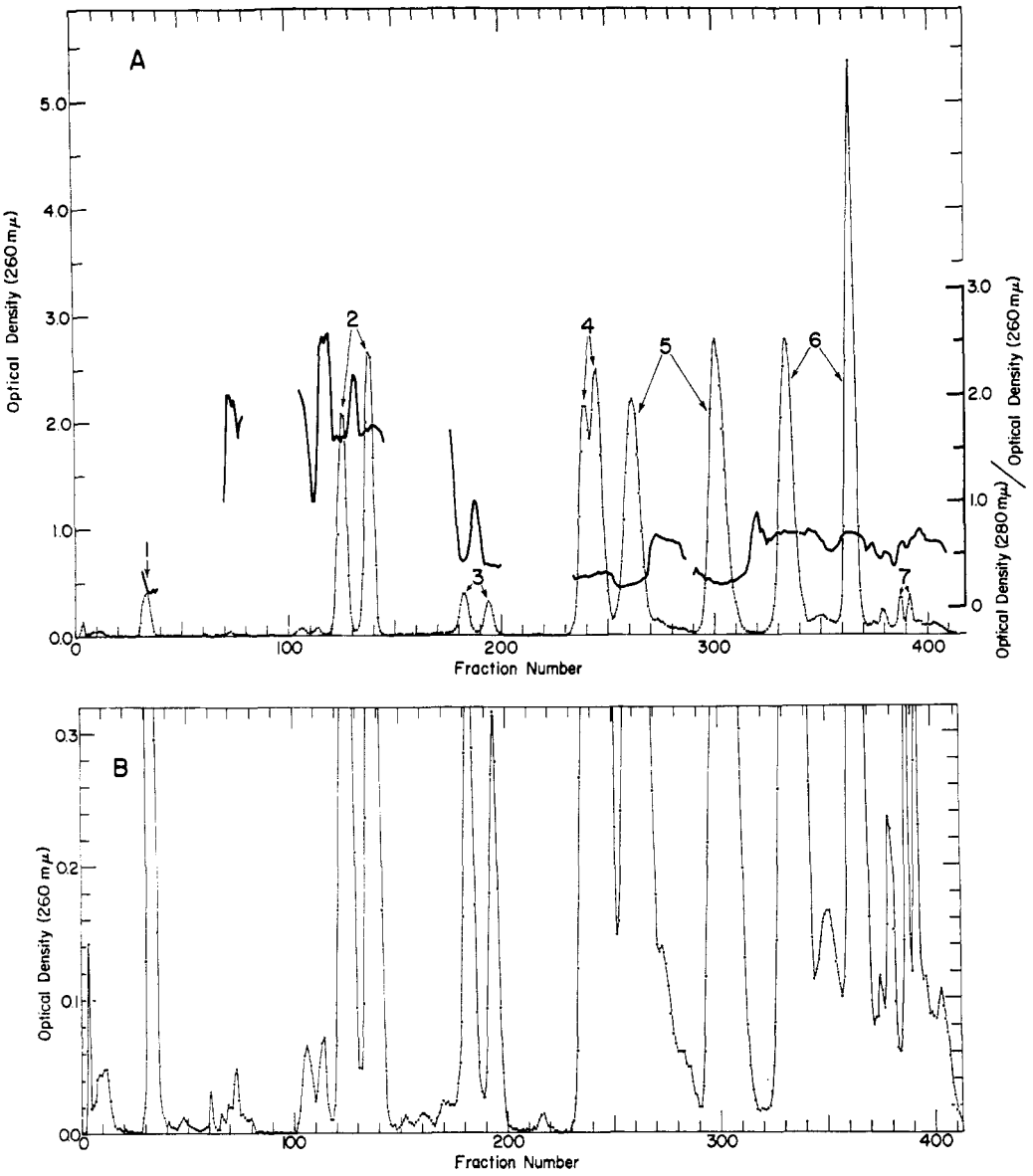

FIG. 1.—Nucleotide analysis of wheat germ s-RNA. Fifty mg of s-RNA was hydrolyzed in 1 M KOH at 30° for 24 hours, and the digest was neutralized with solid Dowex-50 H^+, made alkaline with NH_3, and applied to a column of Dowex-1 formate, x-8, 200–400 mesh, of dimensions 28 cm length by 1.8 cm diameter. Elution was with *p*H 3.7 ammonium formate buffer as follows: 0.01 M, 300 ml; 0.035—0.6 M in parabolic gradient, 3,000 ml; 0.6—3.0 M in linear gradient, 700 ml. The flow rate was 0.5 ml/minute, and fractions of 10 ml were collected. *Pattern A:* over-all scheme. Peaks 1 through 7 have been identified as follows: adenosine, cytidylic acids, pseudouridylic acids, uridylic acids, adenylic acids, guanylic acids, and guanosine diphosphates. *Pattern B:* expanded scale of the same diagram, showing base line detail.

published by Littlefield and Dunn (1958) and Smith and Dunn (1959).

RESULTS

Method of Preparation of RNA.—Qualitative and quantitative analytical data characterizing the two RNA fractions at various stages of purity are presented in Table I. The high molecular weight RNA and crude s-RNA preparations are essentially the same as those reported by Lane and Allen (1961), but on a large scale. At this stage of purification the latter fraction contains only about 15% s-RNA, as judged by the extinction at 260 mμ. Since s-RNA is a well-characterized class of ribonucleic acid, it was decided to concentrate effort on further purification of this material. In addition, the high molecular weight fraction appears to be more nearly pure at this stage.

Methyl Cellosolve extraction according to the method of Kirby (1956) is aimed primarily at the removal of polysaccharide contaminants. After this step the preparation no longer gives a deep blue color with iodine, and the high value in the orcinol determination of RNA is considerably reduced. Chromatography according to the method of Holley *et al.* (1961) using DEAE-cellulose and elution from the column with 1 M NaCl was attempted as the next step in the purification, but without success. The resulting s-RNA contained abnormally large amounts of phosphate, and the apparent inorganic phosphate value was quite variable, ranging as high as 10%. The apparent inorganic phosphate showed acid stability properties similar to those of the polyphosphates characterized by Wiame (1949). When the same type of column was used with a salt gradient in the elution buffer, the s-RNA preparation was found to be free of inorganic phosphatelike material. The values obtained in the orcinol determination, extinc-

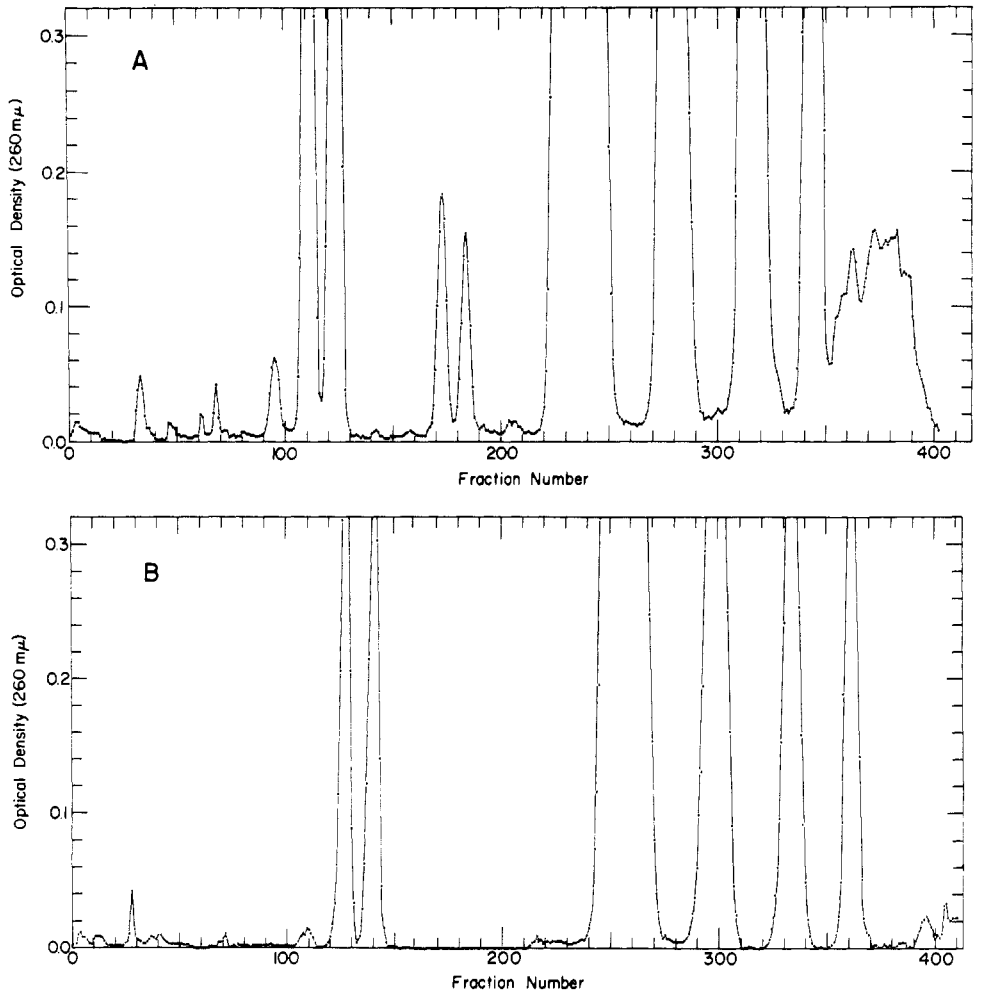

FIG. 2.—Nucleotide analysis of RNA digests. *Pattern A:* 50 mg of wheat germ high molecular weight RNA digested and chromatographed as in Figure 1. *Pattern B:* 13.5 mg of tobacco mosaic virus RNA digested and chromatographed as in Figure 1.

tion measurement, and phosphorus determination all agree with expected values for RNA (See Table I).

Properties of RNA from Wheat Germ.—A number of properties of both the s-RNA and high molecular weight RNA fractions of the wheat germ preparation have been examined. The analytical data presented in Table I indicate that both fractions are relatively free of gross contamination, and in fact indicate a rather high degree of purity. The extinction coefficients and ϵ_p values are typical of values obtained for RNA. Polysaccharide appears absent from the s-RNA fraction, although a small amount may still be present in the high molecular weight RNA. The protein values presented have not been corrected for a slight background color reaction due to nucleotides, as demonstrated by Ramachandran and Fraenkel-Conrat (1958). A possible interference by residual phenol must also be considered. The values obtained in the diphenylamine determination of DNA are maximal. Similar apparent DNA levels have been found in all RNA samples analyzed.

Alkaline hydrolysates of wheat germ RNA have been analyzed for nucleotide composition by chromatography on Dowex-1 ion-exchange resin. The results are presented in Table II along with the results of a similar analysis of a smaller quantity of RNA from tobacco mosaic virus (TMV-RNA) intended as a control.

The elution pattern obtained with the hydrolysate of wheat germ s-RNA is shown in Figure 1-A. Peak 1 has been identified as adenosine, while peaks 2 through 6 are the 2′ and 3′ isomers of cytidylic acid, pseudouridylic acid, uridylic acid, adenylic acid, and guanylic acid, respectively. The peaks numbered 7 consist primarily of the 2′,5′ and 3′,5′ isomers of guanosine diphosphate. An expanded version of the same elution pattern is shown in Figure 1-B. A large number of trace components are apparent, some of which have been tentatively identified. The material eluted before peak 1 is cytidine. The two small peaks eluted before cytidylic acid have spectral properties similar to cytidylic acid. They are probably not 5-methyl cytidylic acid, since material with the spectral properties of 5-methyl cytidylate (see Figure 1-A) is found in the early portions of the main cytidylic acid peaks. The compounds eluted between the two adenylic acid peaks and between the guanylic acid peaks have been tentatively identified as methylated derivatives of adenylic and guanylic acids, respectively.

The detailed pattern of a chromatographic analysis of a hydrolysate of wheat germ high molecular weight RNA is shown in Figure 2-A. A number of qualitative and quantitative differences from the s-RNA pattern are apparent. The total nucleoside content is much lower in the case of the high molecular weight RNA hydrolysate. Pseudouridylic acid is present, but in lower quantities than were found present in the s-RNA hydrolysate. Neither the methylated adenylic and

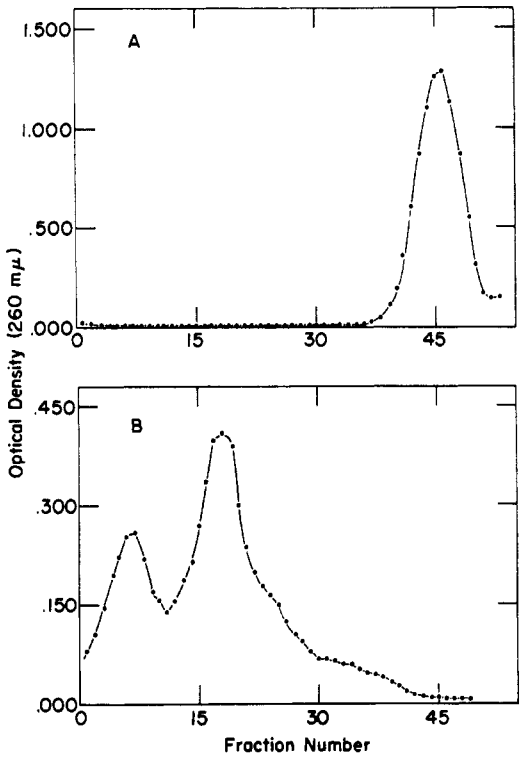

FIG. 3.—Sedimentation of wheat germ RNA in sucrose density gradients. RNA (1.5 mg) dissolved in 0.5 ml of 0.1 M imidazole buffer, pH 7.0, layered on top of 25 ml of sucrose gradient (5–20% sucrose in 0.1 M imidazole), and centrifuged at 24,000 rpm for 24 hours at 0° using a Spinco Model L centrifuge equipped with an SW-25.1 swinging-bucket rotor. Fractionation: 30 drops/tube. *Pattern A:* wheat germ s-RNA. *Pattern B:* wheat germ high molecular weight RNA.

guanylic acid isomers nor the nucleoside diphosphates are apparent in the elution pattern of Figure 2-A. In the latter case, however, the small amount expected may be masked by the much larger quantity of alkali-stable oligonucleotides. A small amount of the cytidylic acid-like component is present in this digest, as well as in the digest of TMV-RNA shown in Figure 2-B (note that the quantity of TMV-RNA hydrolyzed is about 25% of the amount of wheat germ RNA hydrolyzed in each case). Since TMV-RNA is believed to be free of minor base components (see Littlefield and Dunn, 1958), it is possible that this component is an artifact. Quantitative estimates of the composition of each RNA hydrolysate are presented in Table II.

Sedimentation velocity in sucrose density gradients has been examined using each wheat germ RNA preparation. The patterns obtained using 1.5 mg of RNA are shown in Figure 3. Wheat germ s-RNA was also sedimented in the analytical ultracentrifuge and found to form a single sharp boundary with a sedimentation coefficient of 4.1 S. Using the position of the s-RNA peak in the sucrose density gradient as a reference, the sedimentation coefficients of the two major components of the high molecular weight RNA preparation have been estimated to be 18 and 24 S.

In one preparation sodium dodecyl sulfate was added to the extraction buffer. The sedimentation pattern obtained with the high molecular weight fraction of this preparation is shown in Figure 4-A. Sedimentation of the same preparation in the presence of polyvinyl sulfate, an inhibitor of ribonuclease (Bernfeld *et al.*, 1960), resulted in the pattern shown in Figure 4-B. Marker s-RNA was also present in the

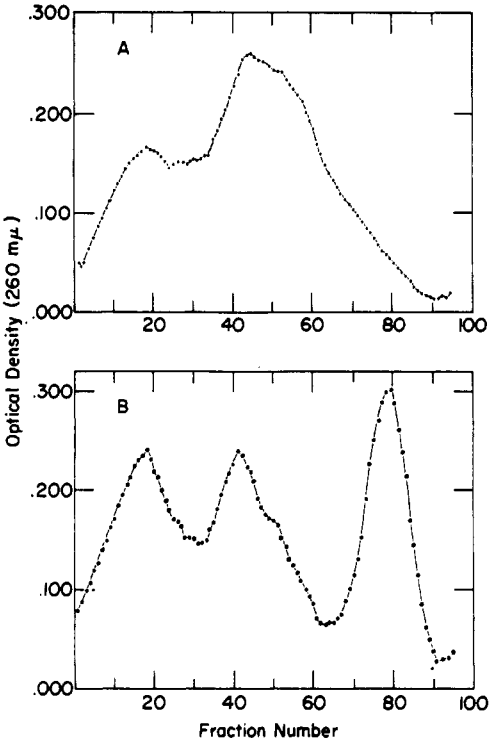

FIG. 4.—Sedimentation of wheat germ high molecular weight RNA prepared in the presence of sodium dodecyl sulfate. Experimental conditions as in Figure 3. Fractionation: 16 drops/tube. *Pattern A:* no inhibitor added to sucrose buffer. *Pattern B:* 0.2 mg/ml of polyvinyl sulfate added to sucrose gradient; 0.5 mg s-RNA present as marker.

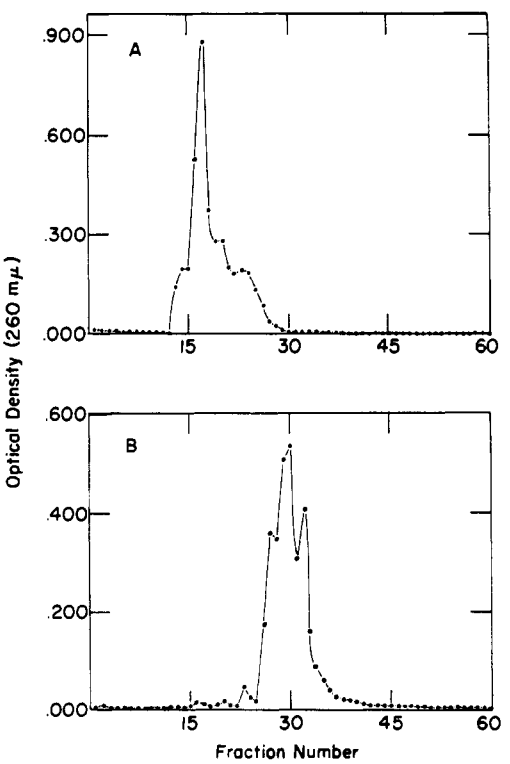

FIG. 5.—Chromatography of wheat germ RNA on methylated albumin—kieselguhr. RNA (2.0 mg) was adsorbed on a column of 2.8 cm length by 2.3 cm diameter and eluted using 600 ml of 0.05 M NaH_2PO_4 with a linear gradient of 0.05—1.95 M NaCl. Fractions of 10 ml were collected using a flow rate of 2.5 ml/minute. *Pattern A:* wheat germ s-RNA. *Pattern B:* wheat germ high molecular weight RNA.

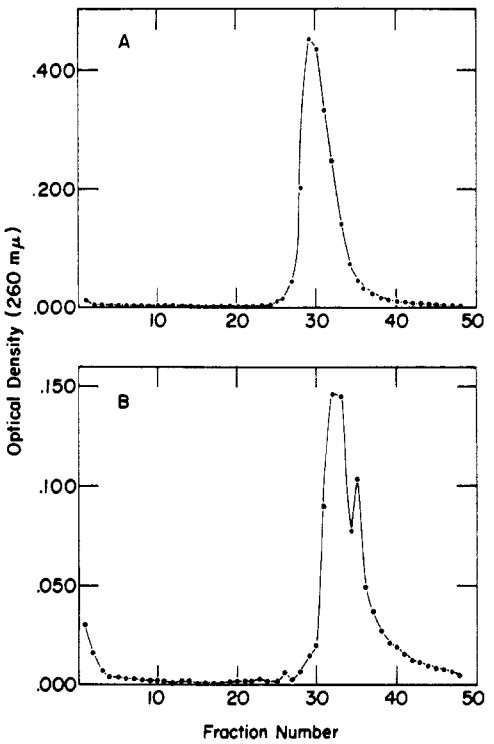

FIG. 6.—Methylated albumin–kieselguhr chromatography of high molecular weight RNA components isolated by sucrose density gradient chromatography. Sedimentation as in Figure 3, with 0.2 mg/ml of polyvinyl sulfate added to the sucrose gradient. Chromatography as in Figure 5. *Pattern A:* 18 S component. *Pattern B:* 24 S component.

latter case. Sedimentation of high molecular weight RNA prepared in the absence of sodium dodecyl sulfate resulted in patterns which were indistinguishable in the presence and absence of polyvinyl sulfate.

The behavior of each RNA fraction upon chromatography on methylated albumin–kieselguhr columns has been examined. The elution patterns are shown in Figure 5. Chromatography of high molecular weight materials which had been separated into 18 and 24 S components by sucrose density gradient centrifugation resulted in the elution patterns shown in Figure 6. The 24 S component is eluted from the column slightly after the 18 S component, but the overlap of the two species is considerable. Polyvinyl sulfate was added to the sucrose density gradient to protect the RNA from nuclease degradation. If the inhibitor was not added, the 24 S component was eluted over the entire range of the gradient used in methylated albumin–kieselguhr chromatography. In contrast, the s-RNA pattern (Figure 5-A) was unaltered after incubation at 28° for periods up to 36 hours in the absence of polyvinyl sulfate.

The biological activity of the s-RNA preparation described herein has been examined in two laboratories. Dr. P. Berg found the wheat germ s-RNA inactive as an acceptor for either leucine or valine using the incorporation system from *E. coli* (Bergmann *et al.*, 1961). Dr. V. Daniel, using the rat liver system of Littauer and Daniel (1961), found the wheat germ s-RNA preparation able to incorporate the CMP and AMP moieties of CTP and ATP. The single amino acid tested, leucine, was also incorporated. These results are presented in Table III.

An attempt has been made to evaluate the degree of secondary structure of each RNA fraction by means

TABLE III

THE BIOLOGICAL ACTIVITY OF WHEAT GERM S-RNA[a]

Substrate	Nucleotide Added	Incorporation (mμmoles/mg RNA)
ATP-C^{14}	—	4.8_5
ATP-C^{14}	CTP	4.0
CTP-C^{14}	—	1.9_5
L-Leucine-C^{14}	—	0.9_2
L-Leucine-C^{14}	CTP	0.9_5

[a] Activity was measured in a rat liver system by Dr. V. Daniel.

of measurement of the optical density–temperature transition curves shown in Figure 7. The s-RNA preparation shows a 29% increase in optical density and a T_m of 58°, while the high molecular weight fraction has a T_m of 55° and an increase in optical density of 33%.

DISCUSSION

The s-RNA fraction isolated from wheat germ appears to be typical of amino acid acceptor RNA preparations. Analysis of the composition of alkaline hydrolysates shows an apparently large number of trace components absent from other forms of RNA (see Figures 1 and 2). Hydrolysis indicates a molecular weight of 25–30,000, assuming that the nucleosides adenosine and cytidine arise from chain ends bearing free 2′ and 3′ hydroxyl groups. This is confirmed by the presence of an approximately equimolar quantity of guanosine diphosphate, presumably arising from the nonacceptor end of the molecule. The end-group analysis is in agreement with the results of Lane and Allen (1961), as well as generally agreeing with analyses of s-RNA (see, e.g., Ralph *et al.*, 1962). The complexity of the elution diagram in the nucleoside diphosphate region (see Figure 1) is such that other end groups, presumably in lesser quantities than guanosine diphosphate if present at all, could not be identified. In addition the value presented for guanosine diphosphate is undoubtedly too high, since it has not been corrected for contaminating alkali-stable materials eluted in the same region (see Lane and Butler, 1959). The small amount of cytidine detected is indicative of some acceptor-end degradation, as is the incorporation of AMP using the rat liver enzyme system (see Table III).

The sedimentation pattern of Figure 3 is in agreement with the chemical evidence for the chain length of the s-RNA fraction. It is also clear from Figure 3 that no material larger than s-RNA is present in this fraction, since nothing is seen to sediment more rapidly than the main peak. Chromatography on methylated albumin–kieselguhr (Figure 5) confirms the lack of larger polynucleotide materials, since nothing appears to be eluted after the complex s-RNA peak. The absence of smaller components which would be eluted before the main peak is more clearly apparent. The degree of secondary structure indicated by the melting-out curve shown in Figure 7 is similar to results which have been reported for other preparations of s-RNA (e.g., Ofengand *et al.*, 1961). Finally, the biological activity measured in the rat liver system of Littauer and Daniel (1961) clearly indicates that this fraction of the wheat germ preparation is s-RNA.

The high molecular weight fraction of wheat germ RNA is somewhat less well characterized than the s-RNA. The analytical data of Table I indicate that

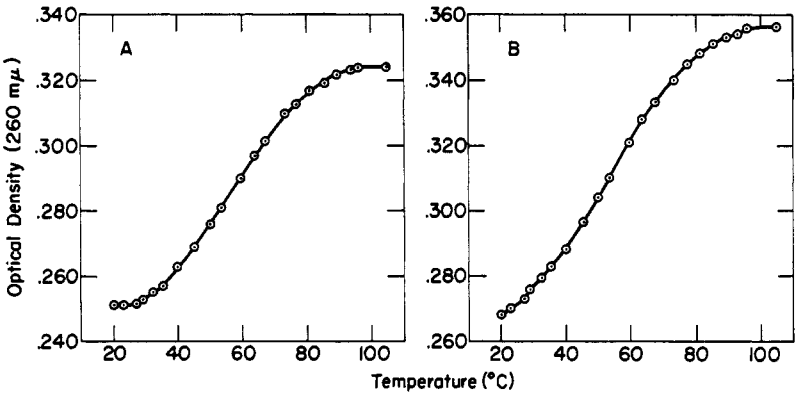

FIG. 7.—Temperature transition curves of wheat germ RNA. RNA samples dissolved in 0.1 M cacodylate buffer, *p*H 7.0. The optical densities were measured at the temperatures indicated using a Beckman DU spectrophotometer equipped with thermospacers. The values obtained were corrected for thermal expansion of water to a standard of 20°. *Pattern A:* wheat germ s-RNA. *Pattern B:* wheat germ high molecular weight RNA.

the preparation is of a high degree of purity, although some polysaccharide contamination still is likely. Nucleoside analysis is in accord with the high molecular weight designation, since only about 1 mole of nucleoside per 500 moles of nucleotide could be detected. This is indicative of an average chain length of about 500, assuming all the material eluted prior to fraction 50 arises from nonphosphorylated chain ends and is uncontaminated with nonnucleoside ultraviolet-absorbing material. However, the approximate sedimentation coefficients obtained from the sucrose density gradient sedimentations indicate that the bulk of the high molecular weight material has a chain length greater than 500. Lane and Allen (1961), using chromatography and charcoal adsorption in the purification of nucleosides from alkaline hydrolysates of high molecular weight RNA, found an average chain length of 1,200, a result more in accord with the accepted molecular weights of ribosomal RNA. Evaluation of the chain length of the TMV-RNA on the basis of nucleoside recovery is unwarranted in view of the previous history of the sample.

The alkaline hydrolysate of wheat germ high molecular weight RNA contains considerable material eluted after the mononucleotides, although not as much as was found in the case of s-RNA. This postmononucleotide fraction is almost entirely absent in the hydrolysate of TMV-RNA (Figure 2). Its presence in the hydrolysate of wheat germ high molecular weight RNA may indicate a contamination of this fraction with s-RNA, or it may be a true characteristic of the high molecular weight fraction. The second alternative is favored since contamination by s-RNA could only involve such species as had negligible quantities of *N*-methylated adenylic and guanylic acids. Moreover, sedimentation and chromatographic studies indicate minor contamination of the high molecular weight fraction with s-RNA.

Evidence for the partial degradation of the wheat germ high molecular weight RNA is seen in the sucrose density gradient centrifugation pattern of Figure 3 and the methylated albumin–kieselguhr chromatography elution diagram shown in Figure 5. In the former the RNA does not sediment as two separate sharp components, but as two rather broad and overlapping peaks. A considerable amount of material with lower S values, ranging as low as that of s-RNA, is present in the high molecular weight fraction. Chromatography on methylated albumin–kieselguhr also indicates the presence of material of lower molecular weight than the main components, since some minor peaks appear in the elution diagram before elution of the major fraction. Intact ribosomal RNA would be expected to behave as the main fraction does both in sedimentation and chromatography; however, the small materials would not be expected, and the two major species would be more homogeneous.

Degradation during the process of centrifugation, as was observed in the case of high molecular weight RNA prepared in the presence of sodium dodecyl sulfate, is indicative of the presence of a ribonuclease in the preparation. Nuclease activity was established by showing that the degradation could be reduced by polyvinyl sulfate, a ribonuclease inhibitor. Inclusion of inhibitor in sucrose density gradients resulted in sharper peaks and a more normal distribution of components. Chromatography of the individual peaks separated by sucrose density gradient centrifugation indicated that the nuclease is apparently associated with the 24 S component, since this component was most affected by omission of the inhibitor. A good separation of 18 and 24 S components by methylated albumin–kieselguhr chromatography has not been attained, even with the aid of polyvinyl sulfate inhibitor. This is probably due to the mildly degraded state of the RNA and to the limitations of the chromatographic method as it was used. The nuclease activity although present, is slight. It appears that the presence of sodium dodecyl sulfate in the initial phenol extraction results in greater nuclease contamination of the high molecular weight RNA. Rushizky *et al.* (1963) have shown that low molecular weight nucleases are readily extracted from aqueous solution by phenol, and we find the extraction greatly reduced by the presence of detergent. Micelle formation by added SDS leads to a distribution of nuclease between the phenol and aqueous phases; however, the nuclease activity present in the aqueous phase is not manifest until the SDS is removed. Thus, the SDS-prepared RNA, when sedimented in the presence of a nuclease inhibitor, appears less degraded than high molecular weight RNA prepared in the absence of detergent, while SDS-prepared RNA sedimented in the absence of nuclease inhibitor appears somewhat more degraded. Since the SDS method of extraction gives a more labile product and no improvement in yield, the detergent has not been included in the standard method of preparation. It is concluded that the high molecular weight fraction of the wheat germ RNA preparation is of ribosomal origin, but has been slightly degraded

during the processing of the wheat germ and/or the preparation of the RNA.

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The Chemistry of Pseudouridine. Synthesis of Pseudouridine-5′-Diphosphate*

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The synthesis of 5-β-D-ribofuranosyluracil-5′-diphosphate (pseudouridine-5′-diphosphate) has been achieved from 5-β-D-ribofuranosyluracil (pseudouridine) using the cyanoethylphosphate method to prepare the monophosphate and the nucleoside phosphoramidate method to make the diphosphate. During the synthesis of the monophosphate two side reactions occurred. One of these was an alkylation of the pyrimidine ring to give a compound tentatively assigned the structure of 1-*N*-cyanoethyl-5-β-D-ribofuranosyl-5′-phosphate. The other involved an anomerization of the C—C glycosyl bond to give 5-α-D-ribofuranosyluracil-5′-phosphate. This anomerization reaction also occurred in the preparation of 5-β-D-ribofuranosyluracil-5′-phosphoramidate and probably in the synthesis of 5-β-D-ribofuranosyluracil-5′-diphosphate. The ultraviolet spectra of all the 5′-substituted derivatives of pseudouridine resemble that of 5-α-D-ribofuranosyluracil (pseudouridine-B) *regardless of the configuration of the glycosyl bond.* It is postulated that the spectral difference at *p*H 12 between various isomers and derivatives of pseudouridine is due to the presence or absence of specific hydrogen bonds. The role of the allyl ether grouping in pseudouridine chemistry is discussed. Tentative structural assignments for the pseudouridine isomers (B, C, A_F, and A_S) are made on the basis of spectral data and a consideration of reaction mechanisms.

As an extension of our studies on the specificity of polynucleotide phosphorylase (Lengyel and Chambers, 1960) and on the chemistry of pseudouridine (Shapiro and Chambers, 1961) we have undertaken the synthesis of ψUDP.[1] The reaction sequence employed is outlined in Figure 1. These reactions are all well known and in fact the pathway is identical to that employed for the synthesis of 2-thiouridine-5′-diphosphate (Lengyel and Chambers, 1960). However, certain special problems were anticipated in applying these reactions to pseudouridine and others were encountered during the course of the work described in this paper.

The first problem was a source of starting material. The isolation procedures from ribonucleic acid (Yu and Allen, 1959; Cohn, 1960, 1961) are not easily adaptable to the preparation of gram quantities of pseudouridine, and the only published synthesis (Shapiro and Chambers, 1961) is not satisfactory as a preparative method because of the low yield. Reports by Adler and Gutman (1959) and by Adams *et al.* (1960) suggested that human urine was a reasonably good source. In collaboration with Dr. W. E. Cohn, a satisfactory

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[1] The following abbreviations are used: ψUMP, pseudouridine-5′-monophosphate; ψUMP-NH_2, pseudouridine-5′-phosphoramidate; ψUDP, pseudouridine-5′-diphosphate.