

THE PREPARATION AND CHARACTERIZATION OF RIBONUCLEIC ACIDS FROM YEAST*

BY ARTHUR M. CRESTFIELD, KENDRIC C. SMITH,† AND
FRANK WORTHINGTON ALLEN

(From the Department of Physiological Chemistry, University of California
School of Medicine, Berkeley, California)

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Considerable variation in composition occurs among samples of ribonucleic acids from yeast. These variations are due in part to the procedures that are employed for isolation and subsequent purification. A recent study by Loring, Fairley, and Seagran (1) notes that variations may be due to (a) the occurrence of different ribonucleic acids in the particulate components of cells, (b) partial enzymatic degradation, and (c) partial chemical degradation. All methods recently published for isolation have sought to avoid partial chemical degradation by complete abandonment of earlier drastic procedures which incorporated the use of extremes in pH and prolonged treatment by heat. However, in discarding drastic methods and accepting milder methods which involve lengthy extractions at physiological pH, the way has been opened for enzymatic degradation to occur in a considerable degree.

That enzymatic degradation may occur during procedures to isolate ribonucleic acids from tissues was first demonstrated by Bacher and Allen (2). Volkin and Carter (3), by the use of guanidine hydrochloride, and Kay and Dounce (4), by the use of sodium dodecyl sulfate, have developed procedures to minimize enzymatic degradation. However, neither of the procedures effects the complete inhibition of crystalline ribonuclease from beef pancreas.¹ The rapidity of destruction of ribonucleic acids in yeast is shown by a recent report of Bourdet and Mandel (5) wherein autolysis for 10 minutes at 50° destroys 93 per cent of the nucleic acids originally present.

The procedure to be described was designed specifically to eliminate enzymatic degradation as a factor during isolation. The ribonucleic acids that have been isolated by the use of this procedure differ significantly in certain properties from samples described heretofore.

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Preparation

Materials and Reagents—Bakers' yeast (*Saccharomyces cerevisiae*) was supplied directly from the filter press by the Consumers Yeast Company, Oakland, California.

Sodium dodecyl sulfate was prepared by the extraction of 300 gm. batches of Duponol C with 7 liters of boiling ethanol. The extract was filtered while hot through a Celite pad on a Büchner funnel. Crystallization from the filtrate at 0° yielded 178 gm. of ether-dried product. The sulfur content of the product was 9.8 per cent as compared with 11.1 per cent for sodium dodecyl sulfate. Difficulty was encountered in the attempted use of several products sold as sodium dodecyl sulfate.

Step 1. Extraction of Nucleic Acids—A 4 liter beaker is fitted with a top plate that contains a narrow slot through which a motor-driven stirring rod and a thermometer can be introduced. 500 ml. of a solution which contains 2 per cent sodium dodecyl sulfate, 4.5 per cent ethanol, 0.0125 M NaH_2PO_4 , and 0.0125 M Na_2HPO_4 are placed in the beaker. A Fisher burner is used to bring the solution to boiling with constant stirring. The top plate is removed, and, with constant stirring, 150 gm. of yeast cut into very fine pieces are added as one batch to the boiling solution. The top plate is immediately replaced to reduce loss of water by evaporation. The temperature after the addition of yeast ranges from 83–87°. Heating is continued with stirring for 1 minute, after which the beaker and contents are transferred to a boiling water bath for 2 minutes in order to avoid excessive foaming. At the time of transfer to the water bath the temperature of the solution ranges from 92–94°. After 2 minutes in the water bath the contents of the beaker are poured into a 2 liter beaker which is immersed in a solid carbon dioxide-Cellosolve freezing mixture. The contents of the beaker are stirred manually for 4 to 8 minutes until the temperature drops to approximately 4°. The mixture is centrifuged at 2000 r.p.m. for 30 minutes at 0°.

Comments—Experiments were conducted to study the relationship between the concentration of detergent and time of heating necessary to effect maximal extraction of ribonucleic acids from the yeast. Ribonucleic acids were determined by modifications of the method of Scott and Fraccastoro (6). Sodium dodecyl sulfate concentrations were varied from 0.5 to 5.0 per cent. The heating period was varied from 2 to 10 minutes. With 2 per cent concentration of sodium dodecyl sulfate and a 3 minute heating period, 90 per cent of the ribose nucleic acids present in the yeast were found to be present in the supernatant liquid. No advantage was found by increasing either the time of heating or the concentration of sodium dodecyl sulfate. The addition of the phosphate buffer maintained the pH of the supernatant fluid between 6.5 and 6.7.

Experiments which were conducted to test the effect of heating and sodium dodecyl sulfate concentration on the activity of crystalline ribonuclease from beef pancreas showed complete inactivation by 0.5 per cent sodium dodecyl sulfate at pH 7.0 and 3 minutes heating.

Step 2. Preparation of Crude Ribonucleic Acids—The supernatant fluid from Step 1 is poured with stirring into 2 volumes of cold ethanol. The precipitate is collected by centrifugation at 2000 r.p.m. in the cold for approximately 15 minutes. The collected precipitate is then washed with two 150 ml. portions of cold 67 per cent ethanol. In washing it is advisable to homogenize the precipitate with a glass rod and add the wash liquid slowly. 5 to 10 drops of a 2 N solution of sodium chloride are added to insure flocculation and recovery. 15 to 20 minutes centrifugation at 2000 r.p.m. are sufficient for each step in the washing. The crude nucleic acids are suspended in 80 per cent ethanol and permitted to stand overnight in the cold room to aid in the removal of protein.

Comments—The precipitate, if collected and analyzed at this point, contains over 80 per cent of the ribonucleic acids that were present in the original yeast. The precipitate itself is 60 to 65 per cent ribonucleic acids. Analysis of the precipitate for ribonuclease activity yielded negative results.

Step 3. Purification of Ribonucleic Acids—The crude nucleic acids from Step 2 are collected by centrifugation and dissolved in 130 to 180 ml. of water. The solution is turbid, with a pH of approximately 8. The solution is immediately neutralized (pH 7.0) by the addition of 1 N acetic acid. Turbidity is removed by centrifugation for 30 minutes at 20,000 r.p.m. at 0° in a Spinco preparative centrifuge. A certain amount of floating material is not removable at this point. Solid sodium chloride is added to the supernatant fluid to bring the final concentration to 1 M. Upon standing at 0°, the turbidity increases slightly and the viscosity increases markedly. In about 30 minutes a gel separates. The supernatant fluid, which is yellow and contains a small amount of floating material, is separated from the gel by centrifugation at 2000 r.p.m. for 1 hour. The supernatant liquid is discarded. The gel is washed successively with three 150 ml. portions of 67 per cent ethanol, to each of which has been added 1 ml. of a 2 M solution of sodium chloride. The washed precipitate is dissolved by the slow addition of water with continuous stirring. The pH of the solution is 7. It is dialyzed for 36 hours at 4° against frequent changes of distilled water. Turbidity is removed, first by filtration through Celite and finally through a D-7 Steriflo asbestos pad (F. R. Hormann and Company, Newark, New Jersey). The clear, colorless solution is lyophilized to yield a white solid. The yield is 1.4 gm., which is equivalent to 60 to 70 per cent of that originally present in the yeast.

Comments—Many procedures for the preparation of deoxyribonucleic acids (7-10) and for the preparation of ribonucleic acids (4, 11, 12) employ 1 M sodium chloride for the separation of denatured proteins from nucleic acids. The proteins are expected to sediment, while the nucleic acids remain in solution. Much to our surprise it was found that 1 M sodium chloride as employed in Step 3 brought about the precipitation of 90 per cent of the ribonucleic acid present in the solution. According to our knowledge the precipitation of ribonucleic acids at 0° by addition of sodium chloride to 1 M concentration has not been reported previously. A study of the effect of sodium chloride concentration from 0.15 to 2.15 M showed that maximal precipitation of ribonucleic acids occurred in the range from approximately 1 to 2 M sodium chloride. No trend in the amounts of non-nucleic acid substances that are precipitated could be demonstrated. Contaminating proteins and deoxyribonucleic acids are mainly soluble in 1 M sodium chloride. Deoxyribonucleic acids as contaminants were measured by the method of Stumpf (13). The ribonucleic acids from Step 3 contain less than 0.5 per cent deoxyribonucleic acids. Semiquantitative estimation of contamination by proteins was performed by the use of the bromophenol blue staining technique as described by Kunkel and Tiselius (14). Less than 2 per cent protein contamination was found. Since further reduction of the protein contamination involves the use of time-consuming procedures (15) in which phosphodiester bonds may be broken, it was felt advisable to characterize the sample without further purification.

Properties

All data are calculated on the basis of the dry weight obtained by drying to constant weight in a vacuum oven at 110°.

Phosphorus—Total phosphorus was determined by a modification of the procedure by Griswold *et al.* (16).

Mononucleotide Composition—Alkaline hydrolysis and chromatography were carried out as described by Smith and Allen (17). Mononucleotide areas were eluted by the use of the method of Hotchkiss (18). Spectrophotometric data were converted to moles of nucleotide by use of the extinction coefficients of Cohn (19). Recoveries of mononucleotides accounted for 95 per cent of the total phosphorus and 95 per cent of the total ultraviolet absorption of the alkaline hydrolysate at 260 m μ . The polynucleotides which were present in the alkaline hydrolysate and which were evident on chromatograms were estimated to be of the same order of magnitude (approximately 3 per cent) as those found by Smith and Allen (17).

Mononucleotides Liberated by Ribonuclease Action—Hydrolysis by ribonuclease was accomplished by the serial addition of 10 μ l. portions of a 10 per cent solution of ribonuclease (Armour, lot 38959) at 0, 2, and 6 hours to 500 μ l. of a 4.7 per cent aqueous solution of the ribonucleate. Hydrol-

ysis was complete in 8 hours. Chromatography, elution, and spectrophotometry were carried out as described in the foregoing paragraph.

TABLE I

Chemical and Physical Properties of Ribonucleate from Yeast in Comparison with Data of Other Investigators

The figures in parentheses are bibliographic citations.

	Present study	Comparative data
Phosphorus, % dry weight.....	8.2	
Adenylic acid, moles per 100 moles.....	25	26 (21), 22 (21), 29 (22), 27 (23), 21 (24)
Guanylic " " " 100 ".....	28	29 (21), 34 (21), 24 (22), 26 (23), 32 (24)
Cytidylic " " " 100 ".....	20	21 (21), 23 (21), 20 (22), 21 (23), 25 (24)
Uridylic " " " 100 ".....	27	24 (21), 22 (21), 28 (22), 24 (23), 23 (24)
Polynucleotides, % of total.....	3	
Cytidylic acid liberated by ribonuclease, % of total.....	48	29 (22), 57 (24)
Uridylic acid liberated by ribonuclease, % of total.....	50	31 (22), 57 (24)
Buffer capacity pH 6.0-8.0, mole OH ⁻ per mole P.....	0.032	0.085* (20), 0.03 (25)
Acidic groups liberated by ribonuclease		
(a) mole per mole P.....	0.45	0.28 (26), 0.34* (20)
(b) " " " total mononucleotide.....	0.48	
(c) " " " pyrimidine.....	1.0	0.72-0.92 (27)
(d) moles per mole liberated pyrimidine mononucleotide.....	2.1	1.6 (27)
$E_{1\text{ cm}}^{1\%}$ 260 m μ , 0.01 M HCl.....	208	
" after alkaline hydrolysis, 260 m μ , 0.01 M HCl.....	284	
$E_{1\text{ cm}}^{1\%}$ after ribonuclease action, 260 m μ , 0.01 M HC.....	269	
Sedimentation, $s_{20,w}$	2.8-4.0	1.9* (28), 1.1-2.3* (29), 2.1* (30), 1.6-4.4 (31)
" ".....	7.0-9.5	
Intrinsic viscosity, $[\eta]_p$	70	7.8*† (30), 13*† (32), 17-30 (33)

* Commercial ribonucleic acid.

† Reduced viscosities.

Titrimetric Data—The proportion of secondary phosphoryl dissociations was determined by titration of a 0.38 per cent solution of the ribonucleate in 0.10 N sodium chloride with 0.01 N solution of sodium hydroxide. Precautions for exclusion of carbon dioxide were observed. The titer between

pH 6.0 and 8.0 was doubled and considered to represent the maximal amount of secondary phosphoryl groups (20).

Acidic groups which were liberated as a result of ribonuclease action were determined by titration to pH 7.5 of a suitable control and the enzymatic digest in 0.10 N sodium chloride.

Sedimentation and Viscosity—Sedimentation measurements at 25° were made in a Spinco ultracentrifuge. Viscosity measurements at 30° were made in Ostwald viscosimeters. All sedimentation studies were performed on a 1 per cent solution of nucleate in 0.2 M sodium chloride which contained 0.05 M sodium phosphate buffer at pH 6.8. Viscosity measurements were made in the same solvent.

The data which were obtained by the foregoing analytical procedures together with certain of the data for specimens of ribonucleic acids from yeast from other investigations are given for comparison in Table I.

DISCUSSION

Partial enzymatic degradation during isolation has been eliminated as a factor in the present method. As far as is known, no one of the steps in the isolation could bring about the rupture of phosphodiester bonds. It seems reasonable then to assume the absence of polynucleotide fragments in the sample.

In the procedure 60 to 70 per cent of the ribonucleic acids which are present in the yeast are isolated. Accumulative losses of 30 to 40 per cent of the total are accounted for at each step of the procedure. Unless the losses should prove to contain a higher proportion of one or several species, the preparation can be considered as representative of the distribution of species of ribonucleic acids in yeast.

The composition of ribonucleic acids in terms of mononucleotide constituents can be assumed to be known only for mixtures of the nucleic acids and their degradation products. Until the mononucleotide composition is explicitly known for a given ribonucleic acid molecule, such information, while useful, is not definitive. Samples of ribonucleic acids which contain a high percentage of fragments as a result of partial enzymatic degradation by ribonuclease may contain a low proportion of cytidylic and uridylic acids. The proportion of uridylic acid in the present sample is high, but that of cytidylic acid shows little deviation from data of previous investigators.

Ribonuclease liberates part of the pyrimidine constituents of ribonucleic acids as free mononucleotides; the remainder of the pyrimidines appears as end-groups of purine-containing polynucleotides. These end-groups have thus far been identified as 3'-pyrimidine nucleotides which are esterified through their 5' positions to adjacent purine nucleotides (22, 27). The liberation of a pyrimidine nucleotide, whether as a free mononucleotide or

as the end-group of a polynucleotide, is accompanied by the liberation of a secondary phosphoryl group. If it is assumed that no pyrimidine nucleoside is linked in such a way that ribonuclease cannot release a secondary phosphoryl dissociation from at least one of the phosphate groups esterified with it, then the secondary phosphoryl groups liberated should equal the pyrimidine content. This result is obtained for the present sample. Less than an equivalent amount of secondary phosphoryl dissociations (24) would be liberated if (a) the terminal phosphate groups of the undegraded nucleic acid had been esterified at the 2' or 3' position of pyrimidine nucleoside residues, (b) enzymatic and chemical degradation during isolation of the sample had exposed an additional proportion of the pyrimidine content as chain endings of this same type, and (c) some pyrimidine constituents are linked within chains in such a manner that ribonuclease cannot act on either side.

The possibility that some of the acidic groups liberated by ribonuclease show primary phosphoryl dissociations (34) is not excluded. These groups would be completely titrated under the conditions employed and would be called secondary phosphoryl dissociations. The titration curves before and after ribonuclease action permit an estimate to be made of the number of the released acidic groups accounted for as secondary phosphoryl dissociations. Such estimations show that 9.6 per cent of the new acidic groups are not found as secondary phosphate dissociations of pK 6.0. However, this figure reduces to 4.5 per cent if a pK of 5.9 is taken.

An accurate interpretation of the buffer capacity of the isolated ribonucleic acids between pH 6.0 and 8.0 in terms of the proportion of secondary phosphoryl groups requires information concerning the ionization of other groups in the ribonucleic acids in this region. Such data are known only for mononucleotides. If all of the buffer capacity in this region is due to the dissociation of a secondary phosphoryl group with a pK of 6.0, then the sample contains one secondary phosphoryl group per 16 phosphorus atoms. However, it is possible that other dissociating groups are partially titrated in the region of pH 6 to 8. In this case there would be less than one secondary phosphoryl group per 16 phosphorus atoms. These data are comparable to those of Gulland *et al.* (35) for their preparation of deoxyribonucleic acids from thymus and mark the first time that such a similarity between a sample of deoxyribonucleic acids and ribonucleic acids from yeast has been noted in this pH region.

Experience in this laboratory with the neutral sodium salts of other preparations of ribonucleic acids had shown such samples to be readily soluble in water. The preparation that is obtained in this procedure is a white, tough, fibrous material. When placed in water, the fibrous mass imbibes water to form a gel which, regardless of agitation to promote solution, is only slowly soluble. In the studies on viscosity, the results were

The isolated sample has fewer secondary phosphoryl dissociations and a higher percentage of phosphodiester bonds which are susceptible to hydrolysis by ribonuclease than previous samples had. Other properties are described and discussed.

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