# The Isolation and Characterization of Soluble Ribonucleic Acid Obtained by Solvent Extraction of Bacteria*

D. H. Rammler, T. Okabayashi,† and A. Delk

ABSTRACT: A procedure for obtaining soluble ribonucleic acid (s-RNA) from *Escherichia coli* is described. The method involves extraction of bacteria with pyrophosphate buffer (0.02 M, pH 7.4) containing isoamyl alcohol (10% v/v), bentonite (1 mg/ml), polyvinyl sulfate (1 mg/ml), and ethylenediaminetetraacetate (EDTA) (0.001 M). The principal product of this extraction is s-RNA. This has been demonstrated by a variety of methods both chemical and biochemical. The crude extract contains essentially no other ribonucleic acid (RNA) component except s-RNA as demonstrated by chromatography on methylated serum albumin. The s-RNA is homogeneous as evidenced by the elution pattern obtained by chromatography on diethylaminoethyl-cellulose in 7 M urea and by its sedimentation pattern in a sucrose density gradient. Its amino acid acceptor activity is comparable to s-RNA purified by two independent methods. The method is simple and rapid, and the extract contains on a dry weight basis less than 0.2% deoxyribonucleic acid (DNA) and 3.5% protein. In addition, the rate of nucleic acid extraction can be followed spectrophotometrically because the solvent has little ultraviolet absorption at 260 mμ.

A variety of methods for the isolation of soluble ribonucleic acids (s-RNA) from bacteria have been investigated (for summarizing references see Kirby,1964). Most procedures involve gross disruption of the bacteria, and subsequent purification requires removal of protein, polysaccharides, and extraneous nucleic acid (see Brown, 1963, for a recent review). As a preliminary to a study on the biochemistry of s-RNA, it was necessary to find a direct and rapid method for obtaining this substance from bacteria grown under a variety of conditions. The direct disruption of cells by phenol has been described (Monier, 1962; Holley, 1963; Brubaker and McCorquodale, 1963); however, this method is somewhat limited because the ultraviolet absorbancy of phenol prevents the immediate perusal of the extracted ribonucleic acid (RNA) by spectrophotometric methods. In this communication, we wish to report on a method for the isolation of s-RNA from bacteria. The method involves an alteration in the permeability of the bacterial cell surface by organic solvents (Deere, 1939; Lederberg, 1950; Rotman, 1958). This treatment, initially, allows the rapid leakage of small soluble nucleotides from the cells in addition to s-RNA. The release of the s-RNA requires only a few minutes and, because the solvent has no ultraviolet absorbancy, the released nucleic acid can be assayed spectrophotometrically. The solvent extract is relatively homogenous, less than 0.2% deoxyribonucleic acid (DNA) and 3.5% protein/unit dry weight of bacteria being extracted. The extracted RNA has been characterized and is principally s-RNA, as evidenced by its amino acid acceptor activity and nucleotide composition. In addition to its utility in biochemical studies, the ease of this method indicates its possible usefulness in isolating large amounts of s-RNA for chemical studies.

## Results and Discussion

*Release of RNA by Organic Solvents and Detergents.* In Table I the effects of several solvents and detergents on the release of RNA are shown. Of the various solvents tested, the organic alcohols, especially *n*-butyl alcohol and isoamyl alcohol, appear to be most effective while the aromatic hydrocarbons are less effective. Using low concentrations, the anionic detergent sodium lauryl sulfate and the neutral detergent Tween 80 were relatively ineffective, as was phenol.

*Nuclease Activity in the Solvent Extract.* The presence of nuclease activity in extracts of *Escherichia coli* has been indicated by several investigators (Neu and Heppel, 1964; Anraku and Mizuno, 1965). In the present investigation we have added bentonite (Singer and Fraenkel-Conrat, 1961), polyvinyl sulfate, pyrophosphate, and ethylenediaminetetraacetate (EDTA) to inhibit nuclease activity (Sela, 1962; Huppert and Pelmont, 1962). Pyrophosphate and EDTA were included in the extraction mixture to minimize possible phosphodiesterase activity, these enzymes generally requiring divalent cations (Razzell, 1963) for maximum activity. This mixture including isoamyl alcohol is termed the standard extracting solvent (see Experimental Section for exact composition). Phosphodiesterase activity in the solvent extract was assayed using *p*-nitrophenylthymidine 3′-phosphate, *p*-nitrophenylthymidine 5′-phosphate, and bis-*p*-nitrophenyl phos-

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† Syntex Postdoctoral Fellow, 1964–1965.

TABLE I: Effect of Solvents on the Release of *E. coli* RNA.[a]

| Addition to Extraction Solution (% v/v) | Specific Absorbancy | $\frac{A(280)}{A(260)}$ | Pentose (μg/OD 650 mμ) |
|---|---|---|---|
| *n*-Butyl alcohol (10%) | 1.74 | 0.44 | 28.5 |
| *sec*-Butyl alcohol (10%) | 1.04 | 0.5 | 19.3 |
| Isoamyl alcohol (10%) | 1.71 | 0.40 | 27.8 |
| Toluene (10%) | ..[b] | .. | 21.1 |
| Benzene (10%) | ..[b] | .. | 19.7 |
| Phenol (5%) | ..[b] | .. | 11.4 |
| Tween 80 (0.5%) | 0.57 | 0.60 | 3 |
| Sodium lauryl sulfate (0.01 M) | 0.43 | 0.52 | 3 |
| Sodium lauryl sulfate (0.01 M) + isoamyl alcohol (10%) | 1.68 | 0.46 | 32.5 |
| No additions | 0.18 | 0.41 | 3 |

[a] *E. coli* (strain $K_{12}$) was grown on Davis medium (Davis and Mingioli, 1950) for 4 hr. The cells were collected by centrifugation, washed with pyrophosphate buffer, and resuspended (1.2 OD/ml at 650 mμ) in a solvent similar to the standard solvent except that it did not contain isoamyl alcohol. The solvents noted in this table were added and the suspensions were shaken for 30 min at 24°. The bacterial debris was removed by centrifugation, and the supernatant was assayed as indicated. Specific absorbancy is OD (260 mμ)/OD (650 mμ) of the bacteria. Pentose is expressed as micrograms of orcinol-positive material with adenosine 5′-phosphate as the standard. [b] The ultraviolet absorbancy of the solvent prevented accurate direct OD determination.

phate (Razzell, 1963). Ribonuclease activity was assayed using the method described by Anraku and Mizuno (1965). The time of assay was 30 min at 37° and under these conditions, in the standard extracting solvent, no significant enzymatic activity could be detected. Thus, the acid-soluble nucleotide present in 1 ml of a 10-min extract (see below) contained 0.58 OD[1] units after 30 min, while the control contained 0.58 OD.

In order to test the generality of this extraction process on *E. coli*, bacteria grown under two different conditions were extracted. These conditions were growth to stationary phase on enriched medium and growth to exponential phase on minimal medium. In an effort to follow the extraction process in an analytical fashion, the nucleic acid, measured as orcinol-positive pentose or as OD (260 mμ)/OD (650 mμ), is used as a measure of the efficiency of extraction. This is termed specific absorbancy and, from the bacterial stoichiometric data in the Experimental Section, this figure may be related directly to per cent dry weight of bacteria or per cent total perchloric acid extractable nucleic acid.

*Rate of Nucleic Acid Released from Solvent-Treated Bacteria.* The rate of release of nucleic acids from bacteria grown under both conditions and treated with the standard solvent is shown in Figure 1. As indicated, there is an immediate increase in specific absorbancy upon the addition of isoamyl alcohol. After the initial release of optical density, there is a lag followed by a rise which remains relatively constant after 2 hr. The release of RNA from bacteria grown on Davis medium (●) is somewhat different from the release observed with bacteria grown on enriched medium (○). Although the release is extremely rapid in both cases, the release of RNA is faster with bacteria grown on Davis medium. Kinetic results similar to those shown here were obtained for bacteria grown on minimal medium and extracted under similar conditions but with tris(hydroxymethyl)aminomethane-(Tris) HCl (0.02 M, pH 7.0), potassium phosphate (0.02 M, pH 7.0), or water in place of pyrophosphate buffer. The rate of release did not vary from experiment to experiment, but the total optical density units released varied somewhat. With

TABLE II: Rate of Protein and DNA Released from Solvent-Treated Bacteria.[a]

| Time of Extraction (min) | Protein (mg/OD of bacteria) A | Protein (mg/OD of bacteria) B | DNA (mg/OD of bacteria) A | DNA (mg/OD of bacteria) B |
|---|---|---|---|---|
| 0 | 0.009 | 0.004 | 0.000[b] | 0.000 |
| 10 | 0.015 | 0.014 | 0.000 | 0.000 |
| 30 | 0.018 | 0.015 | 0.0002 | 0.000 |
| 60 | 0.019 | 0.019 | 0.0006 | 0.0001 |
| 90 | ... | 0.020 | 0.0008 | 0.0002 |
| 120 | 0.022 | 0.021 | ... | 0.0002 |
| 240 | ... | 0.033 | ... | 0.00026 |
| 240[c] | ... | 0.013 | ... | ... |

[a] Bacteria (5 ml) suspended at a concentration of 20 OD/ml in the standard extracting solution minus isoamyl alcohol were placed in 12-ml centrifuge tubes. Isoamyl alcohol (0.5 ml) was added and the suspensions were agitated rapidly on a reciprocal shaker for the intervals indicated. One sample was immediately centrifuged after the addition of isoamyl alcohol and termed the zero-time (0) sample. The method of Lowry *et al.* (1951) was used to analyze protein and DNA is reported as mg of deoxyadenosine 5′-phosphate as determined by the diphenylamine method of Dische (1953). The bacteria used for analysis indicated in column A were grown on enriched medium while those in column B were obtained from minimal medium. [b] Below the sensitivity of the assay. [c] Extraction mixture minus isoamyl alcohol.

[1] One OD unit is defined as that amount of material per milliliter of solution which produces an absorbancy of 1 in a 1-cm light path cell at the desired wavelength.

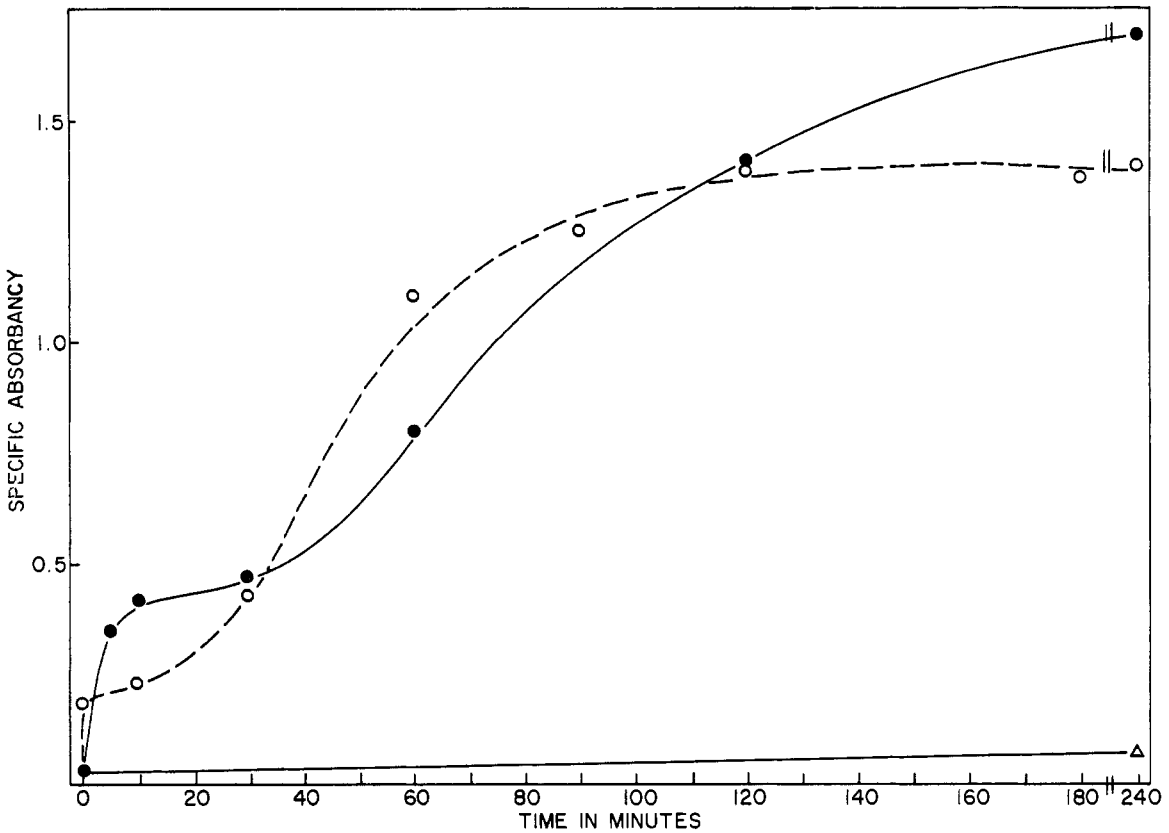


FIGURE 1: The rate of nucleic acid released from solvent-treated bacteria. *E. coli* grown on enriched medium (○) for 18 hr and grown on minimal medium (●) for 4 hr were suspended (20 OD/ml) in the standard extracting solvent (5 ml) and vigorously shaken on a reciprocal shaker for the intervals indicated. The extracted bacteria were removed by centrifugation (8000 rpm, 10 min, 5°), and the supernatant was assayed for absorbancy at 260 mμ. The control (△) contained the standard solvent, minus isoamyl alcohol. Specific absorbancy is the ratio of the OD at 260 and at 650 mμ.

TABLE III: The Nucleic Acid Composition of the Solvent Extract.[a]

| Chromatographic Analysis | Nucleic Acid Chromatographed (OD) | OD Recovered | OD Eluted with Initial Column Wash | | OD Eluted in Peaks I and II | | | OD Eluted in Peak III | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | OD | % Total Recovered | OD | % | 280/260 | OD | % | 280/260 |
| Column A[b] | 19.0 | 20.1 | 2.03 | 10 | 6.79 | 34 | 0.7 | 11.98 | 56 | 0.56 |
| Column B | 27.0 | 24.15 | 1.28 | 5.25 | 7.89 | 32.4 | 0.73 | 14.98 | 61.7 | 0.54 |
| Column C | 29.7 | 28.24 | 7.2 | 25.5 | 6.74 | 23.8 | 0.73 | 14.3 | 50.5 | 0.47 |

[a] The bacteria extracted were similar to those used in the experiments shown in Figure 1. The columns and methods used for chromatography are described in Figure 2. [b] See Figure 2.

higher concentrations of cells in the standard solvent the specific absorbancy was less; with cells which had been frozen prior to solvent extraction, the amount of protein, RNA, and DNA increased, presumably because of the cell lysis which occurred during freezing and thawing.

*Rate of Protein and DNA Released from Solvent-Treated Bacteria.* The values for protein and DNA released from solvent-treated bacteria as a function of time are indicated in Table II. Each assay was made on a portion of the supernatant obtained from the kinetic experiment indicated in Figure 1.

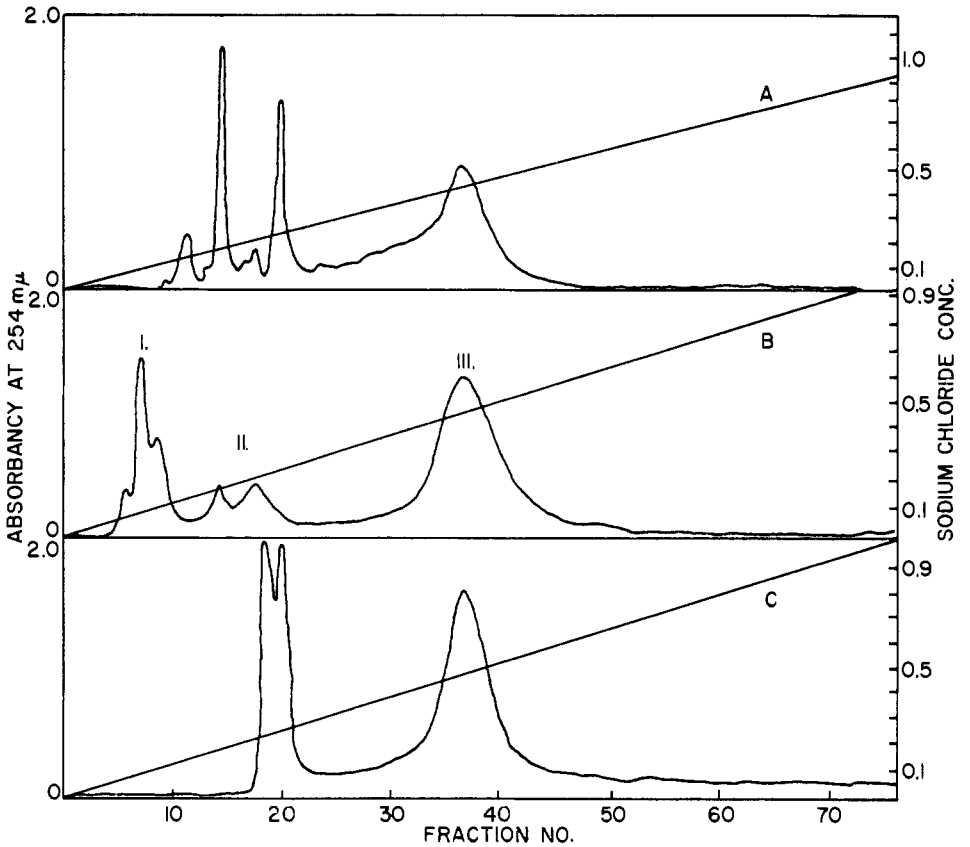


FIGURE 2: DEAE-cellulose chromatography of extracts containing nuclease inhibitors. The chromatographed RNA was obtained by extracting bacteria for 30 min at 23° in extracting solvents which contained different nuclease inhibitors. Pattern A was obtained from RNA extracted from bacteria with the standard solvent minus polyvinyl sulfate and bentonite; pattern B contained both polyvinyl sulfate (1 mg/ml) and bentonite (1 mg/ml); pattern C contained bentonite (1 mg/ml). Prior to chromatography, the extract was dialyzed for 16 hr against water and then lyophilized. The lyophilized powder was dissolved in water and a small amount of insoluble material was removed by centrifugation. The columns were uniform (1 × 14 cm) in size and the flow rates were 1 ml/min. Elution was carried out using a linear salt gradient (250 ml of 7 M urea containing 0.01 M Tris-HCl, pH 7.8, in the mixing vessel, and 250 ml of 7 M urea in 0.01 M Tris-HCl, pH 7.8, containing 1 M NaCl in the reservoir).

*Diethylaminoethyl-cellulose (DEAE-cellulose) Chromatography of Solvent Extracts Obtained in the Presence of Different Nuclease Inhibitors.* The effect of two nuclease inhibitors (Sela, 1962) included in the standard extracting solvent on the chromatographic pattern of the RNA released by extracting bacteria for 30 min is shown in Figure 2. The bacteria were grown on minimal medium. Chromatography of the extract was carried out in 7 M urea (Tomlinson and Tener, 1963) in an effort to detect any large breakdown products of s-RNA. The elution pattern of the extract which did not contain nuclease inhibitors is different from the two patterns which contained inhibitors, indicating possible enzymatic hydrolysis of the s-RNA. For this reason, these inhibitors were included in the standard extracting solution. Information regarding recovery yields from these analyses is shown in Table III. The elution pattern, shown in Figure 2, demonstrates that the principal RNA present in the extract is approximately the size of s-RNA. The variation in the early peaks are most likely the result of contamination by polyvinyl sulfate, bentonite, and protein, in addition to different oligonucleotides. Paper chromatography of peak I, pattern B (*n*-propyl alcohol–ammonia–$H_2O$, 55:15:30), indicated at least seven components, the major ultraviolet absorbing component remaining at the origin. In the same system, peak II of pattern B contained at least three nucleotidic components in addition to a component remaining at the origin.

*Isolation and Characterization of RNA from E. coli Grown on Enriched and Minimal Medium.* Prior to the extraction process, bacteria were isolated from the growth medium by centrifugation, followed by a washing with ice-cold pyrophosphate buffer. The cells were then resuspended in pyrophosphate buffer containing the added nuclease inhibitors and upon the addition of isoamyl alcohol were shaken vigorously for 10 min. The cell debris was removed by centrifugation and the supernatant either was dialyzed against water, concentrated by lyophilization, or added directly to a

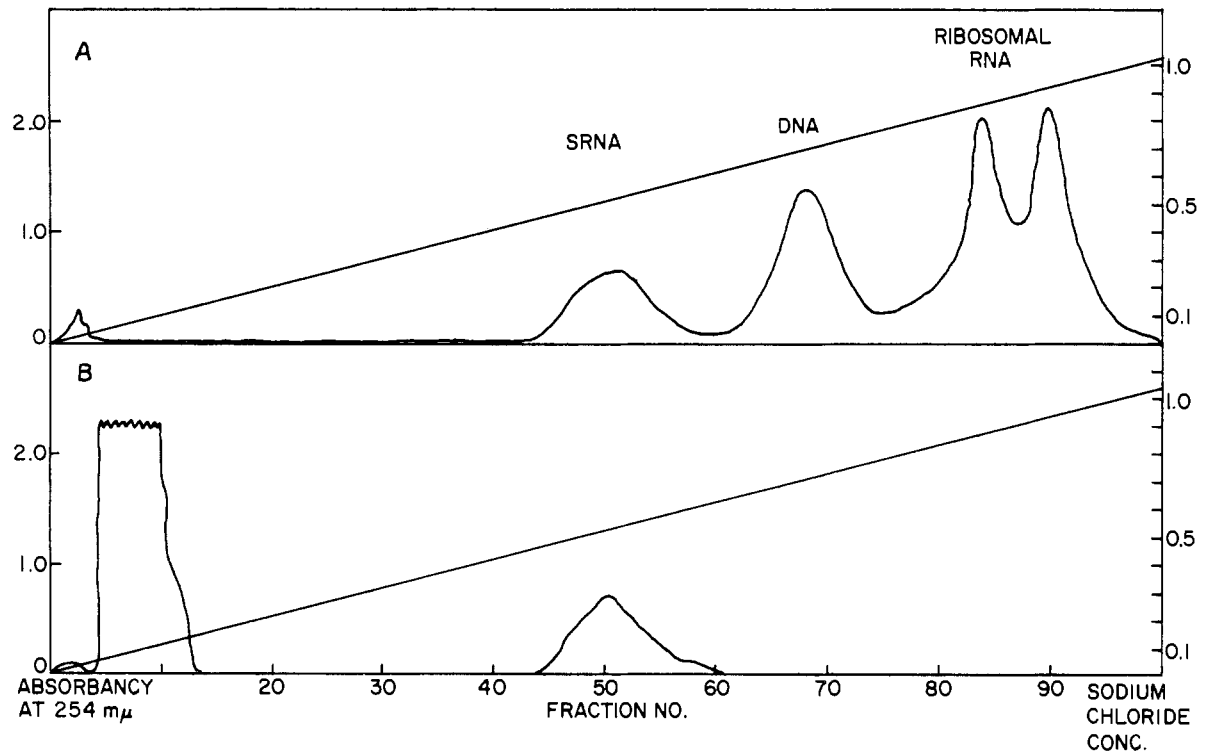


FIGURE 3: The elution pattern of extracted RNA chromatographed on methylated serum albumin columns. The elution diagram A was obtained by chromatography of the nucleic acid obtained from nonsolvent-treated cells. The nucleic acid was isolated as described by Midgley (1962). Diagram B was obtained by chromatography of the solvent extract without any prior purification. The column (2 × 16 cm) was packed as described by Mandell and Hershey (1960) and was loaded with approximately 15 OD of extract. It was developed with a linear gradient of NaCl (200 ml of 0.1 M NaCl in the mixing vessel and 200 ml of 1.1 M NaCl in the reservoir) solution containing 0.05 M phosphate buffer, pH 6.7. The flow rate was 0.75 ml/min. The elution patterns were obtained using an ISCO recording ultraviolet analyzer (Instrument Specialties Co., Lincoln, Nebr.).

DEAE-cellulose column with subsequent gradient elution. In an attempt to establish standard conditions, the bacteria were always washed and resuspended to a similar absorbancy at 650 mμ prior to extraction; however, the utility of direct extraction of the bacteria in the growth medium after the addition of nuclease inhibitors and isoamyl alcohol was not excluded. The extraction process is fully described in the Experimental Section.

*Nucleic Acid Composition of the Extract.* A portion of the crude extract was chromatographed on a column of methylated serum albumin. This type of chromatography has been shown to differentiate between s-RNA, ribosomal RNA, and DNA (Ishihama *et al.*, 1962). In Figure 3 the elution pattern of the RNA obtained from the extract is compared with the nucleic acid composition of the nonextracted cells. A comparison of the two patterns demonstrates that no significant DNA or ribosomal RNA is present in the extract. The pattern indicates that the extract contains two major fractions; the first presumably contains small molecular weight substances while the second is eluted at a salt concentration similar to that which elutes s-RNA.

*Chemical Characterization of Extracted RNA.* (1) CHROMATOGRAPHY ON DEAE-CELLULOSE IN 7 M UREA. Rechromatography of the s-RNA obtained from chromatography of the crude extract on DEAE-cellulose in 7 M urea, using the same linear gradient already described, yielded a single symmetrical peak which eluted from the column at 0.49 M NaCl.

(2) SUCROSE DENSITY CENTRIFUGATION. The sedimentation pattern of the DEAE-cellulose chromatographed RNA in sucrose (Martin and Ames, 1961) is shown in Figure 4. The s-RNA yielded a single symmetrical peak whose sedimentation rate is essentially that of purified commercial *E. coli* s-RNA.

(3) NUCLEOTIDE COMPOSITION. The nucleotide composition derived from alkaline hydrolysates of the extracted s-RNA is shown in Table IV. The high proportion of cytidylic acid obtained from chromatography of the hydrolysate on Dowex-2 formate is indicative of s-RNA. In each chromatographic analysis, a small peak was obtained prior to cytidylic acid. This substance was shown to be adenosine by its paper chromatographic and ultraviolet spectral properties. Although pseudouridylic acid was not isolated, a small peak was observed in the region of the elution pattern where this substance is normally obtained. Further

TABLE IV: Nucleotide Composition of Extracted RNA.[a]

| Nucleotide | Moles/100 Moles of Recovered Nucleotide | |
|---|---|---|
| | Column A | Column B |
| Adenylic acid | 19.9 | 22.2 |
| Guanylic acid | 32.2 | 30 |
| Cytidylic acid | 28.6 | 29 |
| Uridylic acid | 19.7 | 19.8 |

[a] The extracted RNA (80–100 OD at 260 mμ) was hydrolyzed in 0.5 N KOH at 37° for 18 hr. The KOH was removed as its insoluble perchlorate and the hydrolysis product was placed on a Dowex-2 formate (×8, 200–400 mesh) column (1 × 20 cm). The column was washed with 80 ml of water and eluted with an exponential gradient which contained 50 ml of water in the mixing vessel and 30 ml of 1 N formic acid in the reservoir. When the 30 ml of 1 N formic acid had passed into the mixing vessel, the column was developed further with the addition of 300 ml of 4 N formic acid to the reservoir. Each peak was isolated and the concentration of nucleotide was determined using the extinction values given by Beaven *et al.* (1955). Column A is the nucleotide composition of s-RNA obtained from bacteria grown on enriched medium while B is that obtained from bacteria grown on minimal medium.

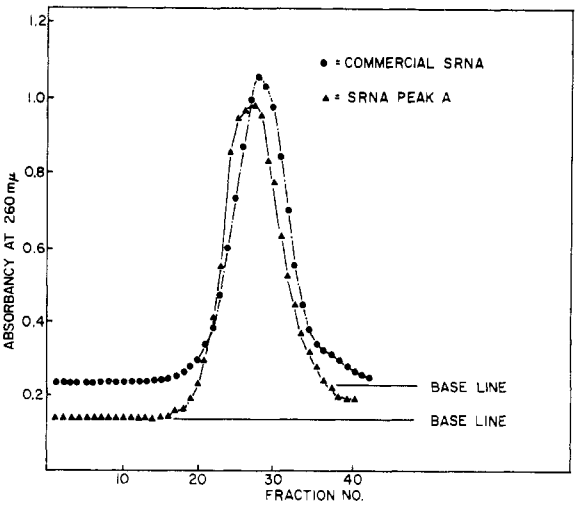


FIGURE 4: The sedimentation pattern of extracted RNA in sucrose. The sedimentation of s-RNA (5 OD) was performed using a Spinco Model L centrifuge (S.W. 39 rotor) at 35,000 rpm for 20.5 hr. The sucrose density gradient (5–20%) contained Tris-HCl (0.01 M, pH 7.8) in NaCl (0.2 M) and was prepared as described by Martin and Ames (1961). Fractions (0.1 ml) were obtained from a small aperture punctured at the bottom of the tube and were diluted and read at 260 mμ.

elution of the column using the method described by Ingram and Pierce (1962) yielded two peaks which eluted in the nucleoside diphosphate region. The initial peak was obtained in small amount and was not investigated further. On paper chromatography the second peak had an $R_F$ value similar to an authentic guanosine diphosphate marker. The ultraviolet spectrum in acid and base was essentially the same as guanylic acid.

The nucleotide analysis of the extracted RNA is thus consistent with the composition of s-RNA obtained by several methods by many investigators (Brown, 1963).

*Biochemical Characterization of Extracted RNA.* The final characterization of s-RNA as amino acid transfer ribonucleic acid (t-RNA) (Brown, 1963) resides in its ability to accept amino acid when added to an appropriate enzymatic system. The amino acid acceptor activity of the s-RNA prepared by solvent extraction of bacteria is compared with s-RNA purified by two other methods and is shown in Table V. The results indicate that the s-RNA obtained by solvent extraction has biological properties which are similar to purified s-RNA obtained by other methods.

*Yield of Extracted Amino Acid t-RNA.* Although the amount of extracted RNA is known both in substance and activity, the total amount remaining in the extracted cells depends again upon the efficiency of the method of extraction. Assuming that all of the s-RNA which remains in the cell debris after solvent extraction can be isolated by disruption of this material in a French press followed by a phenol treatment as described by Midgley (1962), an approximate yield based on recovered amino acid acceptor activity may be calculated. When the RNA obtained in this fashion is chromatographed on DEAE-cellulose, as described, the s-RNA peak eluting between 0.5 and 0.6 M NaCl contains $5.9 \times 10^4$ cpm of amino acid acceptor activity/g dry weight of bacteria. Peak A (see Experimental Section) which is the s-RNA in the solvent extract contains $1.5 \times 10^5$ cpm of amino acid acceptor activity/g dry weight of bacteria; thus the yield of extracted RNA is 72% of the total recoverable amino acid acceptor activity.

Assuming that the pyrimidine content of *E. coli* is 42% of the total RNA (Midgley, 1962) and using an average value of 347 as the molecular weight for both pyrimidine and purine nucleotides, the purine content being determined as orcinol-positive pentose (see Experimental Section), the following yields based on dry weight of bacteria were obtained: total RNA, 11.5%; solvent-extracted RNA, 1.5%; RNA remaining in cell debris, 8.3%; s-RNA obtained by DEAE-cellulose chromatography of the solvent extract, 0.2%.

The yield of purified s-RNA obtained from *E. coli* by solvent extraction varies somewhat from the values reported by other investigators and does not represent complete extraction of s-RNA. Ofengand *et al.* (1961) reported that s-RNA comprises about 1% of the dry weight of *E. coli;* Tissières (1959) found 0.3 g of s-RNA/300 g wet weight and Zubay (1962) found 5 g of s-RNA/2000 g wet weight of *E. coli.*

## Conclusions

Organic solvents have been used to alter the permeability of bacterial cell surfaces for a number of years; however, the exact nature of the alteration remains obscure. In addition to organic solvents, EDTA–lysozyme treated *E. coli* have been shown to preferentially leak or secrete certain enzymes (Neu and Heppel, 1964). It has been demonstrated that the rapid release of RNA is dependent upon the addition of isoamyl alcohol to the extracting solvent. The possibility exists that the function of isoamyl alcohol on the cell surface is to create small apertures which act as sieves for the soluble cytoplasmic constituents of the cell. The size of the nucleic acid found in the extract would, therefore, in some way, be a function of the dimensions of such solvent-induced apertures. Such a mechanism is in keeping with the observation that the rapidly extracted RNA contains no significant DNA or ribosomal RNA and is principally composed of nucleotides, presumably some oligonucleotides and s-RNA. The kinetic data indicate that the extraction is biphasic. One explanation for this type of curve is that the nucleic acid obtained during approximately the first 10 min of extraction is principally derived from the soluble constituents of the cell, while the nucleic acid obtained subsequently is larger and derived by solvent-induced alterations of the particulate nucleic acid containing components and/or by a time-dependent increase in the cell surface aperture. Such a suggestion is strengthened not only by the time-dependent nucleic acid composition of the extract (see Table II), but also by the similarity in the specific absorbancy (1.7 and 1.4) found in the kinetic studies (Figure 1) with the specific absorbancy (1.82 and 1.86) found for perchloric acid extraction of the cells (see Experimental Section). In the presence of bentonite and polyvinyl sulfate no significant ribonuclease or phosphodiesterase activity was found in the cell extract; however, the possibility that the nucleic acid released from within the cell is the product of enzymatic hydrolysis cannot be excluded.

TABLE V: Amino Acid Acceptor Activity of Extracted RNA.[a]

| s-RNA Sample | Amino Acid Acceptor Activity [cpm/OD (260 mμ)] |
|---|---|
| Peak A[b] | 2286 |
| *E. coli* RNA[c] | 2080 |
| Commercial s-RNA[d] | 2077 |

[a] Amino acid acceptor activity was determined using the method described by Ofengand *et al.* (1961). The enzyme preparation used was a crude 105,000*g* fraction from *E. coli*. Each analysis contained $5.5 \times 10^5$ cpm of radioactive *Chlorella* protein hydrolysate (0.5 mc/mg) and 1.3 mg of crude enzyme protein. The data are corrected for the amino acid acceptor activity associated with the enzyme preparation. The corrected values are approximately three times higher than the background value. [b] See Experimental Section for method of isolation (method A). [c] A gift of Dr. A. T. Norris of Stanford University, Palo Alto, Calif. This s-RNA was purified as described by Ofengand *et al.* (1961). [d] *E. coli* s-RNA obtained from General Biochemical Corp., Chagrin Falls, Ohio.

## Experimental Section

*Materials and Methods. Chlorella* protein hydrolysate ($^{14}C$, 0.5 mc/mg) was obtained from New England Nuclear Corp. Bentonite was a product of British Drug Houses, Ltd., Poole, England. Potassium polyvinyl sulfate and s-RNA were purchased from General Biochemical Corp., Chagrin Falls, Ohio. Isoamyl alcohol was a product of Allied Chemical Co., Morristown, N. J. All other chemicals were commercial reagent grade. Bentonite was suspended in distilled water (10 g/l.) for 2 days and centrifuged at 30,000*g*. The sediment was discarded and the supernatant was concentrated to dryness and redissolved in water for use.

Sucrose density gradients were performed as described by Martin and Ames (1961). Chromatography on methylated serum albumin columns was similar to that described by Mandell and Hershey (1960). The nucleotide composition was determined using the method described by Osawa *et al.* (1958). The biological acceptor activity was determined using the method described by Ofengand *et al.* (1961) with the radioactive counting carried out on a Nuclear-Chicago gas flow counter equipped with a micromil window. Deoxypentose was determined by the diphenylamine test (Dische, 1953). Pentose was determined by the orcinol method, using adenosine 5′-phosphate as the standard (Schneider, 1957). The optical density of bacteria was measured at 650 mμ using 1-ml cuvets in a Beckman DB spectrophotometer.

*Standard Extracting Solvent.* The standard extracting solvent consisted of: 10% isoamyl alcohol (v/v), 1.0 mg of bentonite/ml, 1.0 mg of polyvinyl sulfate/ml in pyrophosphate buffer (0.02 M, pH 7.4) containing EDTA (0.001 M).

*Growth Conditions. E. coli* (strain $K_{12}$) was grown on enriched medium (Difco, Penassay, Antibiotic Medium 3, 1.75%; 10 l.) with vigorous aeration at 37° for 18 hr. The bacteria were harvested by centrifugation (8000 rpm, 5°) and washed with pyrophosphate buffer (0.02 M, pH 7.4). The washed bacteria were resuspended and used as described in the text. The bacteria were grown on Davis medium (Davis and Mingioli, 1950) at 37°. After overnight adaptation to this medium, the bacteria were resuspended in fresh Davis medium and grown to

their exponential phase (4–5 hr), harvested, and washed as described above.

*Solvent Extraction of E. coli.* A suspension of bacteria grown on enriched medium (728 ml, 20 OD/ml) was shaken in the standard solvent for 10 min at 23°. The debris was removed by centrifugation (8000 rpm, 5°). The composition of the extract was as follows: OD (260 mμ), 6; $A(280)/A(260)$, 0.58; protein, 0.017 mg/OD of bacteria; DNA, 0.001 mg/OD of bacteria. No significant nuclease activity could be detected in the extract.

*Isolation of RNA from Extract.* METHOD A. A portion of the extract (200 ml; $A(280)/A(260)$, 0.58) was immediately lyophilized. The lyophilized residue was dissolved into 40 ml of water (1320 OD, $A(280)/A(260)$, 0.64) and this rather opaque solution was dialyzed against water (1 l., 6 hr). The dialyzed solution was centrifuged to remove a precipitate and the cloudy supernatant (36 ml; 360 OD; $A(280)/A(260)$, 0.53) was diluted with water (64 ml) and placed on a DEAE-cellulose column. The initial water wash contained ultraviolet absorbing material (105 OD; $A(280)/A(260)$, 0.44). The column was developed with a linear gradient of NaCl solution (500 ml of 0.01 M Tris-HCl, pH 7.5, in the mixing vessel and 500 ml of 1.1 M NaCl containing 0.01 M Tris-HCl, pH 7.5, in the reservoir). Several small peaks (50 OD) were obtained before (see Figure 2 for comparison) the major symmetrical peak (peak A; 102 OD; $A(280)/A(260)$, 0.47) which was eluted between 0.5 and 0.6 M NaCl. No further nucleotidic material could be eluted from the column with this gradient. The column was stripped with 2 N KOH containing 2 N NaCl. Approximately 32 OD [$A(280)/A(260)$, 0.52] was obtained in this fraction.

METHOD B. To the remaining portion of the extract (550 ml; 3300 OD; $A(280)/A(260)$, 0.58) was added three volumes of cold ethanol, and the mixture was allowed to stand at −20° for 18 hr. The precipitate was collected by centrifugation (8000 rpm, 5°, 30 min.) and redissolved in water. This was recentrifuged to remove an insoluble substance. The supernatant contained 2200 OD [$A(280)/A(260)$, 0.6] of which 681 OD [$A(280)/A(260)$, 0.56] remained after dialysis and removal of insoluble material formed during dialysis. The dialyzed RNA was placed on a DEAE-cellulose column which was developed as described in method A. Approximately 116 OD [$A(280)/A(260)$, 0.62] was obtained in an initial water wash of the column. Minor peaks, similar to those obtained in the chromatography discussed in method A, contained approximately 110 OD [$A(280)/A(260)$, 0.53] while the major peak, eluting between 0.5 and 0.6 M NaCl, contained 260 OD [$A(280)/A(260)$, 0.48]. No other nucleotidic material was obtained during the elution. Stripping the column as described above afforded an additional 157 OD [$A(280)/A(260)$, 0.68].

*Bacterial Stoichiometry.* One optical density unit of bacteria grown on enriched medium contains $1.7 \times 10^{-3}$ g wet weight of bacteria. When this amount of bacteria is freeze-dried for 18 hr, the dry weight is $3.7 \times 10^{-4}$ g. Cold perchloric acid (10%) leaching (Schneider, 1957) provides $1.57 \times 10^{-6}$ g of orcinol-positive material, the specific absorbancy being 0.28. Subsequent treatment of the bacteria with perchloric acid (10%, 70°) for 30 min provides an additional $22.3 \times 10^{-6}$ g of orcinol-positive material per optical density unit of bacteria, specific absorbancy 1.86.

One optical density unit of bacteria grown on minimal medium weighs $2.8 \times 10^{-3}$ g wet weight. When this amount of bacteria is freeze-dried for 18 hr, the dry weight is $4.8 \times 10^{-4}$ g. After leaching with perchloric acid as described, $2.95 \times 10^{-6}$ g of orcinol-positive material was obtained (specific absorbancy 0.126, $A(280)/A(260)$, 0.46). Hot perchloric acid extraction afforded $20.5 \times 10^{-6}$ g of pentose (specific absorbancy 1.82).

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# The Stability of Polyuridylic Acid in Acid Solution*

D. H. Rammler,† A. Delk,† and C. W. Abell‡

ABSTRACT: The acid stability of the phosphodiester bond in polyuridylic acid (poly-U) was investigated in solutions of low and high ionic strength using loss in biological messenger activity as a measure of bond hydrolysis. It was found that treatment of poly-U in acetic acid (0.01 M, pH 3.45) solution at 37° caused a rapid loss in messenger activity. By physical methods this loss was shown to be the result of fragmentation of poly-U through acid-catalyzed hydrolysis of the phosphodiester linkage.

Little or no loss in messenger activity was observed when the hydrolysis was attempted with acetic acid (0.01 M, pH 3.45) containing sodium chloride (0.2 M). The stability of various sizes of poly-U as a function of pH, temperature, and ionic strength was studied.

The development of an assay system from bacteria which will measure a specific biological property, namely, messenger activity, has provided a method of studying the relationship between chemical structure and biological function for ribonucleic acids (RNA) [see Nirenberg *et al.* (1963) and Speyer *et al.* (1963) for recent reviews]. The ability of polyuridylic acid (poly-U) to direct phenylalanine incorporation into peptide before and after a variety of chemical modifications to the polymer has been investigated (Michelson and Grunberg-Manago, 1964; Szer and Ochoa, 1964). However, the finding that there is an optimum size of poly-U in terms of its ability to function as an efficient messenger ribonucleic acid (m-RNA) in the amino acid incorporating system (Martin and Ames, 1962; Jones *et al.* 1964) and the observation that this messenger efficiency may vary with the nature of the terminating nucleoside end (Coutsogeorgopoulos and Khorana, 1964; Michelson and Grunberg-Manago, 1964; Nirenberg and Leder, 1964) indicate that, as a prerequisite to a study on the chemical modification of polyribonucleotides, it is necessary to know their general chemical stability. Thus, the effects of solvents, of pH, of temperature, and of ionic strength must be established.

In this communication[1] we wish to report on the acid stability of the phosphodiester bond in poly-U in solutions of low and high ionic strength.

## Materials

Adenosine triphosphate (ATP)[2] and guanosine triphosphate (GTP) were purchased from Pabst Laboratories, Milwaukee, Wis. Phosphoenolpyruvate and pyruvic kinase were obtained from California Corp. for Biochemical Research, Los Angeles, Calif. Phenylalanine (U-$^{14}$C) was the product of Nuclear Chicago Corp., Des Plaines, Ill. Poly-U was purchased from Miles Chemical Co., Elkhardt, Ind., and was also synthesized using polynucleotide phosphorylase purified as described by Singer and Guss (1962); the uridine diphosphate used in the synthesis was prepared chemically (Moffatt and Khorana, 1961). Sephadex G-200 was a product of Pharmacia Co., Sweden, and was sieved to remove particles smaller than 200–400 mesh size. All other chemicals were the commercially available reagent grade.

## Methods

The cell-free incorporating system used was similar to that described by Nirenberg (1963). Protein was determined using the method of Lowry *et al.* (1951).

*Standard Assay System.* The reaction mixture (0.25

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‡ National Cancer Institute, National Institutes of Health, Bethesda, Md.

[1] Abbreviations used: ATP = adenosine triphosphate, GTP = guanosine triphosphate, TCA = trichloroacetic acid.
[2] A preliminary account of this work has been reported (Abell *et al.*, 1963).