CHARACTERIZATION, AND IDENTIFICATION OF TERMINAL NUCLEOTIDES OF AVIAN MYELOBLASTOSIS VIRUS RIBONUCLEIC ACID AND OF CHICKEN LEUKEMIC MYELOBLASTS RIBOSOMAL RIBONUCLEIC ACID

by

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INTRODUCTION

Nucleic acids have a particular significance in biology. Deoxyribonucleic acid (DNA) is considered to be the sole bearer of heredity, through evidence which has come mainly from the study of transformation, transduction, bacterial conjugation and protein metabolism[1]. On the DNA template are transcribed, messenger ribonucleic acid (mRNA), ribosomal ribonucleic acid (rRNA)[2] and transfer ribonucleic acid (tRNA)[3], that have base composition complementary to the specific stretches of DNA. Ribosomal RNA binds with specific proteins to constitute ribosome particles[4]. The mRNA and tRNA interact with ribosomes to form polyribosomes where translation occurs; that is, the message inscribed in the nucleotide sequences in form of triplet condons are decoded in the form of an assemblage of amino acids into a particular protein with specific amino acid sequences[5]. There are many species of tRNA, one or more for each of the twenty amino acids present in proteins. Each tRNA is coupled to its specific amino acid by a specific tRNA synthetase. Transfer RNA serves an adaptor between the mRNA and growing nascent polypeptide chain[6]. In RNA viruses the genetic RNA acts also as mRNA.

A base change in the DNA molecule causes a change in the RNA molecule transcribed on that particular stretch of DNA, and in turn will alter the structure of protein or impairs the physiological function of the organism[7]. Some stretches of DNA are not transcribed, but these are recognized by specific protein molecules and thus regulate the transcription of other stretches of DNA. The promotor part of the cistron, where RNA polymerase binds[8], and also operator genes of the galactose operon[9] and coliphage 434[10] where specific repressors bind, are the known examples of such stretches of DNA.

The nucleotide sequences of DNA and RNA molecules will help to understand the process of transcription and translation and subsequently lay the rational basis of genetic engineering. Holley was the first to demonstrate the nucleotide sequences of yeast alanine tRNA[11], that paved the way for discussing interaction between nucleic acid molecules and proteins. Since then much progress has been made and many more RNA molecules have been sequenced[12]. A particular rapid technique, requiring small amount of RNA, where ^{32}P radioisotope is used to prepare highly radioactive RNA has been devised by Sanger and associates[13]. With this new technique many workers are pursuing to sequence the total nucleotide composition of genetic RNA of bacteriophage: MS2[14], f2[15], R17[16], Qβ[17]. However the determination of nucleotide sequences of genetic RNA in animal viruses, responsible for tumor formation or polio needs attention. The elucidation of nucleotide sequences in animal viruses may eventually help in understanding several aspects about infection, development of viral genome and tumor formation in the host tissue at molecular genetic level.

Specific ribonucleases are used to break down the ribonucleic acid into oligonucleotides. One such ribonuclease T_1, from Takadiastase, is utilized in the determination of nucleotide sequences of ribonucleic acids. A T_1-like ribonuclease has been isolated from Aspergillus fumigatus[18] in Dr. Glitz's laboratory. It may be logical to compare the amino acid sequences in the two ribonucleases from different sources. Such studies on the structure of ribonucleases are required to reveal the mechanism of action of ribonucleases on ribonucleic acid.

Originally a tentative programme of research, discussed in the first report of September-October, 1970, was outlined. 1) Installation of high voltage electrophoresis apparatus for nucleotides fractionation: A two-dimensional procedure for

fractionating ribonuclease digest of ^{32}P labelled RNA is well known. The procedure requires the use of electrophoresis on cellulose acetate strip in the first dimension and on DEAE cellulose paper in the second dimension. The electrophoresis tank and high voltage power supply could be purchased commercially. Highly radioactive ^{32}P labelled RNA of E.coli A17 or phage f2, which may be readily prepared, could be used to test the working of the apparatus and also it can be used to learn nucleotide fractionation methods. The position of different nucleotides on the two-dimensional fingerprints could be compared to those reported by Sanger and his associates. 2) Nucleotides sequences of avian myeloblastosis virus ribonucleic acid: Avian myeloblastosis virus ribonucleic acid (AMV RNA) may be prepared in labelled radioactive form. The radioactive AMV RNA may be digested with specific ribonucleases, fragments separated on acrylamide gel by electrophoresis, and studied for nucleotides sequences. 3) Some structural aspects of ribonucleases: Ribonuclease T_1 from Takadiastase and ribonuclease F from Aspergillus fumigatus, though obtained from different sources, show similar specificity of action towards RNA in splitting of bonds involving guanosine residues. It could be interesting to probe the structure of two ribonucleases by succinylation[19], using ^{14}C succinic anhydride. Some studies on these enzymes could also be made to the techniques used in determination of amino acid sequences.

Previously it had been established that AMV RNA consists of a fast sedimenting 65S RNA component and a slow sedimenting 4S RNAs component, and also, it is considered that 65S RNA has a homogeneous structure with molecular weight 12×10^6 daltons[20]. Experiments, in our hand, showed that it was difficult to prepare ^{32}P labelled AMV RNA, with high specific radioactivity, suitable to study nucleotides sequences in AMV RNA in detail by the methods described by Sanger and associates. Diagonal electrophoresis of ^{32}P labelled AMV RNA hydrolysate, obtained by ribonuclease T_1 digestion, indicated

that 65S RNA may be composed of subunits, which aggregate to form 65S RNA. An aggregate structure for 65S RNA, during the progress of this work, was also reported from other laboratories[21], and it was maintained that 65S RNA is made of subunit RNA species held together by hydrogen bonds and can be disassociated by heat treatment into 35S subunits. Since it was not practicable to separate the constituent 35S subunits of 65S AMV RNA, therefore, it became impossible to study AMV RNA nucleotides sequences in detail. In view of lack of research funds available, and also short stay of the author in U.S.A., the study programme on structural aspects of ribonucleases was dropped. However, the studies on the nucleotide sequences were extended to the structure of ribosomal RNA of leukemic myeloblasts from infected chicken.

In this work the nucleic acid of avian myeloblastosis virus has been characterized. Chromatographic analysis of nucleosides formed upon alkaline hydrolysis of ^{3}H AMV RNA indicated, after making correction for non-equivalent incorporation of precursors, the 3′-terminal nucleoside of AMV RNA is adenosine. Alkaline hydrolysate of myeloblast ribosomal 18S RNA showed adenosine as the 3′-terminal nucleoside. The 3′-terminal nucleoside of ribosomal 28S RNA is primarily uridine. Fractionation of ^{32}P labelled 18S RNA, digested with RNase T_1, by diagonal electrophoresis gave a 3′-terminal fragment indicating the sequence-GpCp(2ApUp,2Cp,2Up)A_{OH}. The 5′-terminal major nucleoside diphosphate in alkaline hydrolysate of ^{32}P 18S RNA was pCp. Alkaline hydrolysates of ribosomal 28S RNA contained 5′-terminal pGp nucleoside diphosphate. Diagonal electrophoresis analysis of 28S RNA digest of pancreatic RNase A gave the 3′-terminal sequence -PypApGpGpU$_{OH}$.

INSTALLATION OF HIGH VOLTAGE IONOPHORESIS APPARATUS FOR NUCLEOTIDES FRACTIONATION

A two-dimensional fractionation procedure for radioactive nucleotides has been described by Sanger and coworkers.[13,22] High voltage ionophoresis is used for both dimensions. In the first dimension a small amount of ribonuclease digest of highly radioactive ^{32}P labelled RNA is applied on cellulose acetate strip and electrophoresed at pH 3.5 in a specially constructed electrophoresis tube. This tube was made of plexiglas, instead of glass tube, described by Sanger and Brownlee.[23] The second dimension electrophoresis was carried on DEAE cellulose paper at an acid pH. High voltage supply Model HV-5000 A and electrophoresis tank Model LT 48 A tank, were purchased from Savant Instruments, Inc. 221 Park Ave./Hicksville. The 3'-terminal oligonucleotides can be isolated by two-dimensional diagonal electrophoresis. Highly radioactive ^{32}P labelled Escherichia coli RNA and ^{32}P labelled phage f2 RNA have been used to test the working of electrophoresis apparatus. The results were compared with those published in literature.

Isolation of ^{32}P labelled Escherichia coli RNA

The cell of E. coli A19 were grown in low phosphate medium of Garen and Levinthal:[24] 8×10^{-2} M NaCl, 2×10^{-6} M $FeCl_3.6H_2O$, 2×10^{-2} M KCl, 1.2×10^{-1} M tris HCl (pH 7.5), 2×10^{-2}M NH_4Cl, 1×10^{-3} M $MgCl_2.6H_2O$, 1.4×10^{-4} M sodium β-glycerophosphate, $4H_2O$, 2×10^{-4} M $CaCl_2$, 5×10^{-4} M Na_2SO_4; 0.04% Bacto-peptone (Difco), containing methionine 20 μg/ml, 0.2% casamino acids (vitamin free) and 0.2% glucose which were autoclaved separately. The sterilization was at 15 lb for 10 min. The cells were harvested when O.D. 0.22 at 540 mu reached. The cells grew with doubling time of 74 min without casamino acids. The doubling time with casamino acid was 37 min. For labelling the cells were grown at 37°C in 100 ml

medium from an overnight culture slant without casamino acids. The medium was shaken in 500 ml Erlenmeyer flask with side tube on a gyrotory water bath incubator. Immediately after inoculation, carrier free ^{32}P at the 100 µCi/ml was added. The cells were harvested after 4 or 5 hr growth at O.D. 0.22. The cells were centrifuged on Sorvall RC2-B, washed with 0.01 M tris (pH 7.4), 0.001 M $MgCl_2$, and kept frozen. To the frozen cells 1.8 ml of 0.01 M tris (pH 7.4), 0.001 M $MgCl_2$, and 0.5% sodium dodecyl sulphate and 0.2 ml of bentonite (3 mg) in 0.01 M sodium acetate, pH 6.0 were added. (Bentonite, 2 g, was taken into 40 ml of water centrifuged at 3,000 x g for 20 min the supernatant centrifuged at 10,000 x g for 20 min and then the pellet was resuspended and held at room temperature with constant stirring in 0.1 M EDTA, pH 7.0, for two days and sedimented at 10,000 x g. The centrifugation repeated once more from 0.01 M sodium acetate, pH 6.0, and then taken into the medium at concentration of 2-6% determined by dry weight). The cells were shaken with an equal vol of water saturated phenol for 30 min and the aqueous layer recovered and treated once again with phenol. To the aqueous layer was added 0.1 vol of 2 M sodium acetate, pH 5.5, and the nucleic acids precipitated with 2 vol of 95% ethanol. The precipitate washed with cold 70% ethanol and freeze dried, and dissolved in water and kept frozen. Fractionation of RNA was carried out on polyacrylamide agarose composite gel by the method of Peacock and Dingman[25], using a vertical gel electrophoresis apparatus. The gel was prepared by mixing (a) 800 mg agarose in 113 ml of water refluxed for 15 min with magnetic stirring and cooled to 45°C, (b) 3.2 g cyanogum 41 in 16 ml buffer; tris (108 g), disodium EDTA (9.3 g), boric acid (55 g) in 1,000 ml, pH 8.3, and warmed to 45°C, (c) 5 ml TEMED (N,N,N',N'-tetramethylethylenediamine) solution, 0.067 ml TEMED/5 ml water and (d) 80 mg ammonium persulphate in 5 ml water. First agarose and cyanogum 41 were mixed and then TEMED and ammonium persulphate were added respectively. The mixture was poured into the

apparatus gel cell and a sample well former introduced. After 1 hr gelation the well former taken out under buffer and RNA sample applied in a drop of 20% sugar and bromophenol blue indicator. The running buffer was diluted 1:10 and circulated during electrophoresis. Also cold water was circulated around the gel cell and the electrophoresis was carried out at 4°C in a cold room. The current was passed at 200 V and after 2 hr the gel was removed. The gel was fixed in 1 M acetic acid for 15 min and then stained with methylene blue (0.2 g methylene blue /100 ml 0.4 M sodium acetate, pH 4.5). The stain was differentiated by washing overnight in 0.01 M acetic acid. The gel was wrapped in Saran wrap and placed on a X-ray film Kodak Blue Brand (BB-54) for radioautography in the dark for 10-15 min and the film developed. The 5S RNA band from the gel was cut out by the help of radioautography, and it was broken into small pieces and placed into a cylindrical tube (10 x 1 cm) having one end covered with DEAE cellulose paper. The gel was subjected to electrophoresis between two reservoirs containing 0.04 M tris acetate, pH 8.3. The 5S RNA migrated out of the gel and trapped on the DEAE cellulose paper. It was eluted by 30% triethylamine bicarbonate, pH 9.7, from the DEAE cellulose paper and dried. The residue is dissolved in water which is evaporated repeatedly to remove the triethylamine bicarbonate.

Isolation of ^{32}P labelled phage f2 RNA.

E. coli ATCC 15766 was grown overnight in the medium containing tryptone, 10 g; NaCl, 8 g; yeast extract, 1 g; and water to 1,000 ml, and 1 g of glucose, 0.29 g calcium chloride (anhydrous), 10 mg of thiamine added separately. Overnight growth of 1 ml culture is added in 150 ml fresh culture medium and the 500 ml Erlenmeyer flask incubated at 37°C on a gyrotory water bath incubator. When the growth reached to O.D. 0.22 at mμ 540, one ml of lysate or about 5×10^9 purified phage particles are added in the flask, immediately after phage

infection carrier free ^{32}P at 100 µCi/ml medium is added and the incubation is continued for 1-3 hr. To the lysate was added 42 g of solid ammonium sulphate slowly with stirring. The stirring is continued at least 1 hr or overnight. The precipitate is collected by centrifugation at 10,000 x g for 45 min. The pellet is resuspended in 4 ml of NTE buffer (0.1 M NaCl, 0.05 M tris HCl, pH 7.6, 0.01 M EDTA). The suspension is shaken with 0.6 vol of 1,1,2-trichlorotrifluoroethane and centrifuged at 10,000 x g for 15 min and the aqueous layer is removed. The interface is shaken with small volume of NTE buffer and the aqueous layers combined. To the aqueous layer is added $CsCl_2$ at 0.5 gm/ml and centrifuged in Beckman 65 rotor at 50,000 rpm for 18 hr. The virus band is recovered and dialyzed against NTE to remove the $CsCl_2$. The purified phage is shaken with an equal volume of water saturated phenol and the RNA from the aqueous layer is precipitated with 2 vol of ethanol. The precipitate is washed with 70% ethanol. The RNA is further purified, if desired, by passing through a Sephadex G-50 (fine) column (1.2 x 18 cm) equilibrated with water.

Enzymic digestion of ^{32}P labelled RNA and the fractionation of oligonucleotides by two-dimensional ionophoresis.

Ribonuclease T_1 digest of ^{32}P labelled 5S RNA of *E. coli* fractionated by two dimensional methods of Sanger and associates. The RNA was dissolved in 0.02 M tris HCl, pH 7.4, containing 0.002 M EDTA (neutralized). The substrate to enzyme ratio was 20:1. About 5-10 µg RNA dissolved in 10 µl of enzyme solution. The incubation was carried out at 37°C for 30 min. The digest was applied to cellulose acetate strip (3 x 60 cm) and subjected to ionophoresis in a pH 3.5 buffer system, 0.5% pyridine-5% acetic acid (v/v) containing 0.001 M EDTA, at 3,000 V for 2 hr. The oligonucleotides transferred from the cellulose acetate strip to DEAE cellulose paper as it has been described. Ionophoresis in the second

dimension on DEAE cellulose paper (46 x 85 cm) was in 7% formic acid and at 800 V.

^{32}P labelled phage f2 RNA digested with T_1 ribonuclease, and the 3'-terminal oligonucleotides isolated by two dimensional diogonal electrophoresis.[26] The phage RNA (5-30 µg) was digested at 37°C for 30 min in 10 µl of 0.1 M NaCl, 0.01 M tris HCl, pH 7.2, 0.05 M EDTA with T_1 ribonuclease. (A solution of the enzyme, 1 mg/1 ml, in 0.01 M tris, pH 7.2, 0.02 M EDTA neutralized; was acidified by the addition of 0.05 vol of N HCl and incubated for 10 min at 22°C; then it was neutralized by the addition of 0.1 vol of 1 M tris, pH 7.2, plus 0.05 vol of IN NaOH). The substrate to enzyme ratio was 20:1. The digest was applied to a DEAE cellulose paper, Whatman DE 81, and subjected to ionophoresis in 7% formic acid at 800 V for 14 hr. The position of oligonucleotides were located by radioautography and this strip was cut out. The paper strip was placed on to a curved glass surface and held there with cellophane tape and alkaline phosphatase (BAP-F) from Worthington Biochemicals, Inc. dissolved in 0.5 M (NH_4) HCO_3, 0.025 M $MgSo_4$, 0.001 M $ZnSO_4$ at concentration of 20 units/ml, was applied to the strip. About 1 ml of the enzyme solution on a 1 x 30 cm strip of paper was enough to dephosphorylate the nuclectides. The paper strip was placed inside the chromatographic tank, which had water at the bottom to keep the atmosphere humid, and incubated for 3-4 hr at 37°C. After dephosphorylation of oligonucleotides the paper strip was washed to remove salts by gentle streaming of distilled water, dried and sewn onto a DEAE cellulose paper (48 x 85 cm) and the ionophoresis was performed at 800 V, in second dimension, for 14 hr. In radioautograph the 3'-terminal end remained unaffected by alkaline phosphatase action, appeared at the diagonal.

Radioautography

Radioautographs were made on Kodak Blue Brand (BB-54) X-ray film. The ionophoretogram was placed against the X-ray film in a standard X-ray cassette lined with lead sheets. After 1 to 3 days or two weeks, depending upon specific radioactivity of RNA, the X-ray film is developed to observe nucleotide fingerprints.

Results and discussion.

The two-dimensional ionophoresis of ^{32}P labelled E.coli 5S RNA digest of ribonuclease T_1 resolved into many spots, Fig. 1. A ribonuclease T_1 digest of ^{32}P labelled f2 RNA was subjected to ionophoresis on DEAE cellulose paper. The oligonucleotides were then treated with alkaline phosphatase and run at right angle to the first dimension. The 3'-terminal oligonucleotide appeared on the digonal, Fig.2. The fingerprints on radioautograms were similar to the results published by Sanger and coworkers. These experiments helped to learn the techniques of ionophoresis and also it became possible to use the ionophoresis apparatus with confidence.

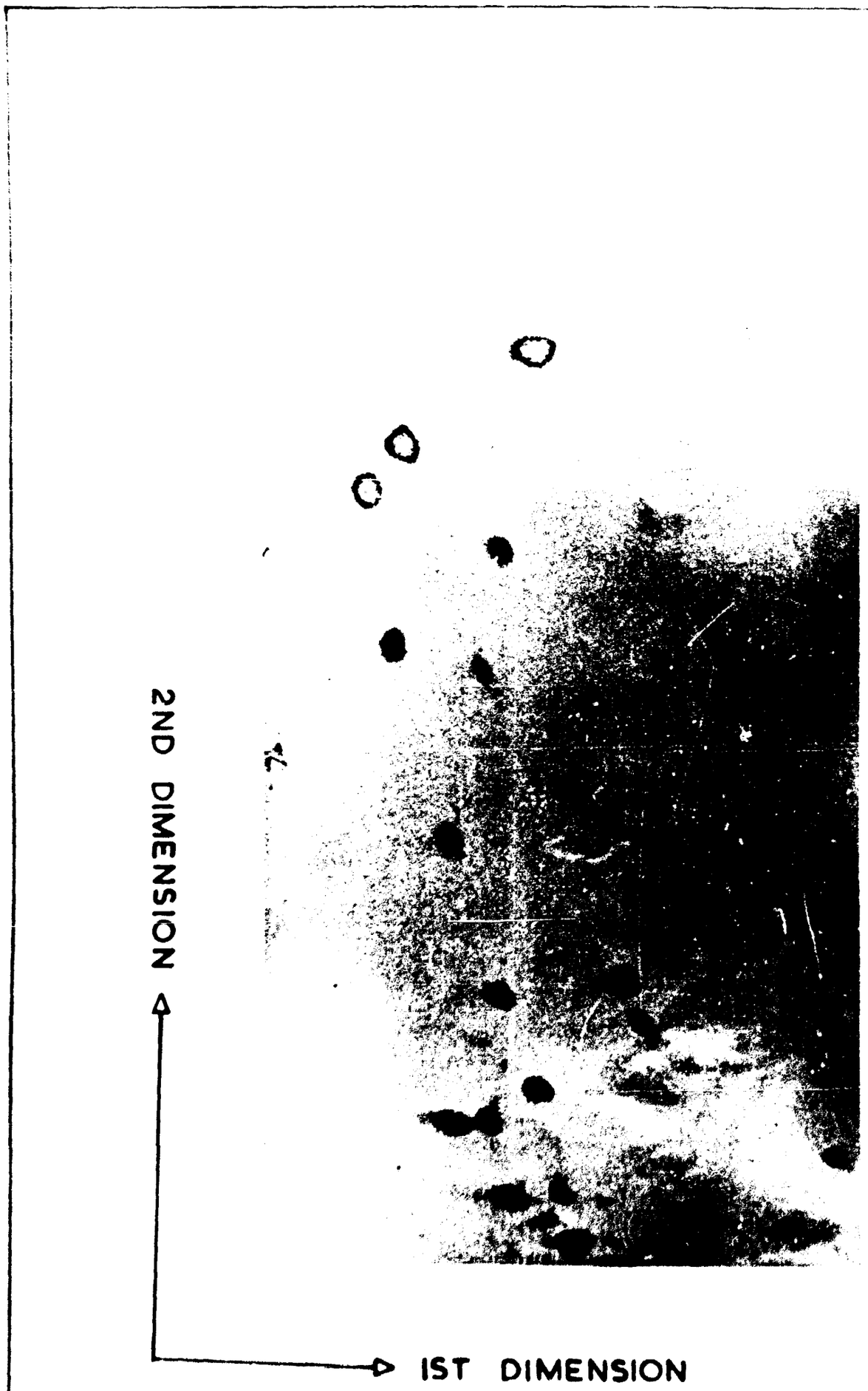

2ND DIMENSION
1ST DIMENSION

Fig. 1. A two dimensional fractionation of a ribonuclease T_1 digest of 5S RNA of Escherichia coli (After ref. 22).

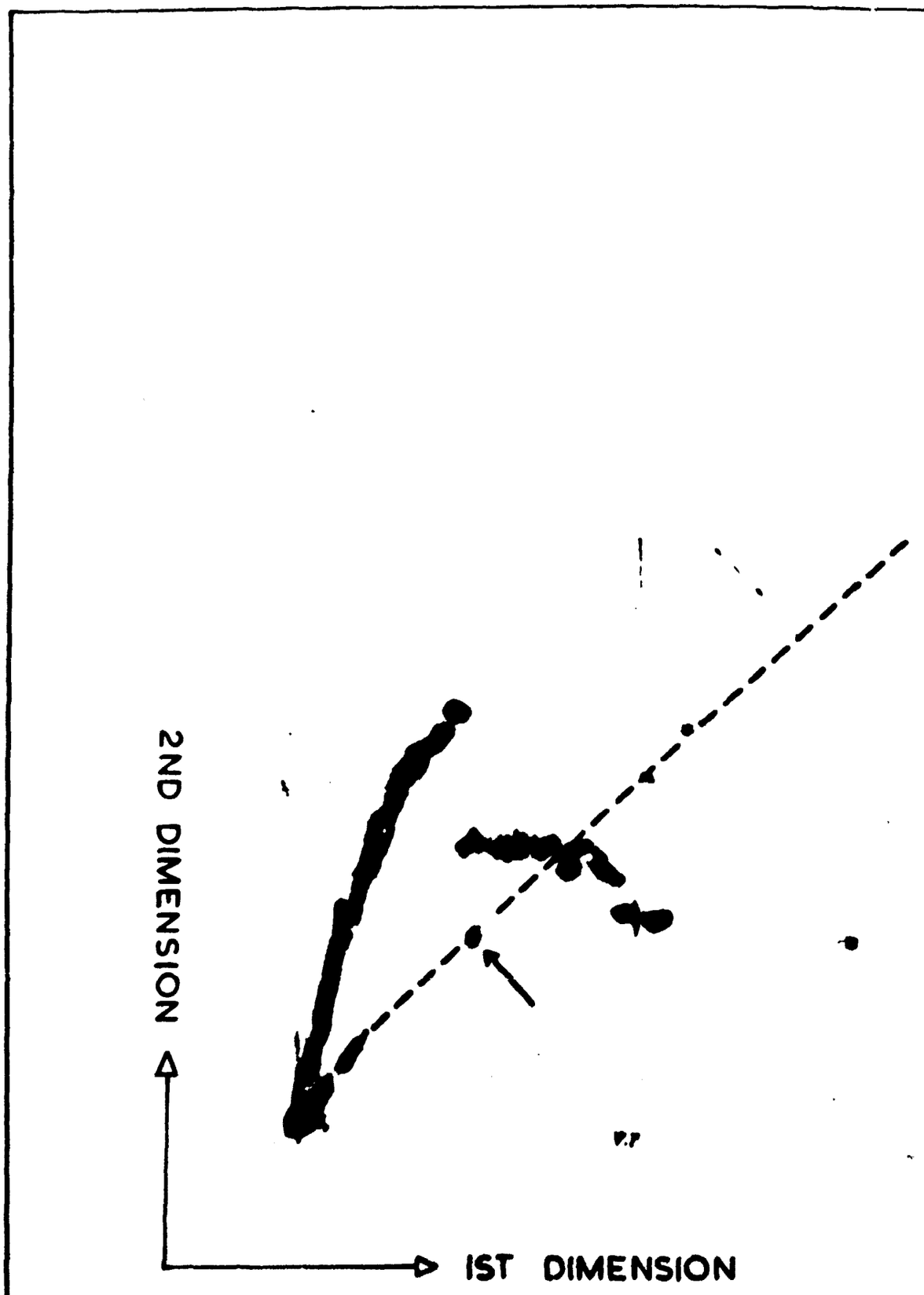

2ND DIMENSION
1ST DIMENSION

Fig. 2. Phosphatase diagonal of a ribonuclease T_1 digest of f2 RNA (After ref. 26).

CHARACTERIZATION OF AVIAN MYELOBLASTOSIS VIRUS RIBONUCLEIC ACID AND IDENTIFICATION OF 3-TERMINAL NUCLEOSIDE

Avian myeloblastosis virus is reported to contain ribonucleic acid. The purified RNA from avian myeloblastosis virus is shown to have two components; a fast sedimenting 65S RNA component and a slow sedimenting 4S RNAs component. It is considered that 65S RNA has a homogeneous structure of molecular, weight $10\text{-}12^{6}$ daltons.[20] Erikson[27] reported that 65S RNA when heated above $70^{\circ}C$, it dissociates to 35S RNA component(s) which is the only detectable break down product in certain experiments. Some studies, made by Duesberg and Vogt, suggested an aggregate structure for 65S RNA that dissociates with heat or dimethyl sulfoxide treatment into several major subunits of 35S RNA and variable amount of smaller heterogeneous pieces. Recently, Erikson and associates[28] claimed that 65S RNA dissociates to release mainly 35S RNA form and a small homogeneous 4S RNA species; and it is demonstrated that AMV RNA contains almost exclusively an unphosphorylated residue of uridine at the 3′-terminus.

This study shows that subunits, smaller than 35S, released upon heat dissociation from 65S RNA; arise to a greater extent depending on stay of virus in culture, presumably through nuclease degradation of AMV-RNA. Chromatographic analysis of nucleosides formed upon alkaline hydrolysis of ^{3}H AMV RNA indicated, after correction for non-equivalent incorporation of precursors, that the major 3′-terminal nucleoside of AMV RNA is adenosine.

Growth of avian myeloblastosis virus

The BAI strain A of avian myeloblastosis virus, B group was used. The leukemic chickens (white leghorns Kimber Farm K-137) were identified by use of peripheral blood smears. The birds, at 10-15 day of age, were bled using a heparin

treated syringe by cardiac puncture. The blood was centrifuged at low speed in a clinical centrifuge. The upper plasma layer rich in virus was saved to inoculate newly hatched chickens which were injected intra-peritoneally. The middle layer of myeloblasts was collected and resuspended at 2-3 x 10^7 myeloblasts per ml of medium (Appendix 1). The bottom layer of red blood cells was discarded.

The myeloblasts suspension, 10 ml portions, was poured in 100 mm plastic tissue culture dish, incubated at 38°C in humidified atmosphere constantly flushed by CO_2-air mixture to keep pH around 7.4, Robinson and Baluda (1965). The cells were harvested at 2-3 hr interval by centrifugation and resuspended into fresh medium; the virus rich supernatant saved over ice. The virus was harvested, three times, and the three supernatant fractions combined, total volume 2000 ml, to recover the virus particles.

For preparation of ^{32}P labelled viral RNA, myeloblasts were cultured in medium deprived of $NaH_2PO_4.H_2O$ that contained $H_3{}^{32}PO_4$, (carrier free) at 100 µCi/ml of medium. To label viral RNA with tritium, myeloblasts were cultured in medium; deprived of adenosine; supplemented with a combination of tritiated uridine (36µCi/ml, specific activity 36 Ci/mmole), cytidine and adenosine (25 µCi/ml, specific activity 25 Ci/mmole); in spinner culture flasks. Additional quantities of $NaHCO_3$ are added in the medium to keep pH around 7.4. The first virus harvest, prelabelled virus collected after 6 hr growth, was discarded. Then the medium was changed at 2-3 hr intervals and the virus supernatant combined total volume about 800 ml, and virus purification started immediately.

Purification of virus

The virus rich supernatant clarified of cell debris, by centrifugation in Sorvall GSA rotor at 8,5000 rpm for 30 min, and 1 M tris HCl buffer, pH 8.5, is added at 1.25 ml/100 ml of

the supernatant. The virus is concentrated by precipitation with ammonium sulphate at 35.3 g/100 ml of the combined supernatant and tris buffer. Ammonium sulphate was added slowly and the mixture was stirred for 45 min, over ice. The precipitated virus collected by centrifugation in Sorvall GSA rotor at 8,500 rpm for 30 min, and the precipitate is resuspended in TE buffer (0.01 M tris, pH 7.4, 0.001 M EDTA), total volume 140 ml, and spun at 8,500 rpm for 10 min to clear debris and bubbles. Then the virus suspension layered on top of sucrose cushion 3 ml 65% sucrose in D_2O at bottom and 6 ml 20% sucrose in NTE buffer (0.1 M NaCl, 0.01 M tris, pH 7.4, 0.001 M EDTA) on top, and spun in Beckman SW 25.2 rotor at 24,000 rpm for 1 hr. The viral band was collected on top of heavy sucrose and diluted, 1 ml to 4.5 ml with NTE buffer, and subjected to 15-20% sucrose density gradient, 6 ml 15% sucrose in NTE and 6 ml 60% sucrose in NTE; in Beckman SW 25.3 rotor at 24,000 rpm for 4 hr, with brakes off. The viral bands were collected into tubes in ice. All the operations are carried out at 5°C or over ice.

The hot virus, precipitated with ammonium sulphate, is resuspended in TE buffer, total volume 36 ml, and spun at 8,500 rpm for 10 min to clear debris and bubbles. The virus suspension was layered on sucrose cushion, 1 ml of 65% sucrose in D_2O and 3 ml of 20% sucrose in NTE buffer, spun at 24,000 rpm in a Beckman SW 25.3 rotor for 1 hr. The viral band collected on top of heavy sucrose, 1 ml virus diluted with NTE buffer to 4.5 ml, and purified further by isopycnic banding in 15-20% sucrose linear density gradient, and centrifugation was done in Beckman SW 65 rotor at 64,000 rpm for 1½ hr. The purified viral band was collected.

Isolation of viral RNA

The virus particles were diluted with NTE buffer and then both sodium dodecyl sulphate (SDS) and β-mercaptoethanol added to 1% concentration. The solution was extracted three

times, with equal volume of buffer saturated phenol (Appendix II). The RNA was precipitated from aqueous layer by addition of 0.1 vol of 2 M sodium acetate, pH 5.5; and 2 vol of ethanol and allowed to stand overnight at -20°C and collected by centrifugation in Beckman SW 25.1 rotor at 24,000 rpm for 1 hr.

Gel electrophoresis

Gel electrophoresis of RNA was performed on acrylamide-agarose composite gel, Peacock and Dingman (1968).[25] Agarose, 800 mg, in 113 ml of water was refluxed for 15 min and cooled to 45°C. Cyanogum 41, 3.2 gm was dissolved in 16 ml of X10 buffer (tris, 108 gm; disodium salt of EDTA, 9.3 gm; boric acid, 55 gm, pH 8.3) and heated to 45°C. Ammonium persulphate, 50 gm, freshly dissolved in 5 ml water is added to agarose solution and 0.075 ml of TEMED (N,N, N^1, N^1-tetramethylethylene-diamine) is added to cyanogum solution. The agarose and cyanogum solutions were mixed quickly and thoroughly and then allowed to gel for 1 hr. After electrophoresis the RNA is stained with methylene blue.

Isolation of 3′-terminal oligonucleotide

The 3′-terminal oligonucleotide can be isolated by two-dimensional diagonal electrophoresis on DEAE cellulose paper with phosphomonoesterase treatment between electrophoresis steps. Dephosphorylation of oligonucleotides at 3′-end permits a rapid migration of these components in the second dimension, and 3′-terminal oligonucleotide never carried a terminal phosphomonoester, and so it is not affected by enzyme and moves identically in both dimensions and is found on the diagonal of electrophoretogram. ^{32}P labelled AMV RNA, 20-30µg RNA, specific activity 5-6 x 10^4 cpm/µg RNA was digested in 20µl of 0.1 M NaCl, 0.5 M EDTA and 0.01 M tris HCl, pH 7.2, at 37°C for 30 min with ribonuclease T_1; substrate to enzyme ratio, 20:1. A solution of T_1 enzyme, 1mg/ml in 0.01 M tris HCl, pH 7.2, 0.02 M EDTA was acifified by the addition of 0.05 vol

of N HCl and incubated for 10 min at 22°C, to inactivate any contaminating phosphatase, if present, and the solution was then neutralized by addition of 0.1 vol of 1 M tris HCl, pH 7.2, plus 0.05 vol of 1 N NaOH. The digest was applied to as a spot on 15 x 85 cm strip of DEAE cellulose paper and ionophoresed in 7% formic acid at 800 V for 14 hr. The position of oligonucleotides traced by radioautography and a strip of paper about 2 cm wide cut out. The strip of paper was placed on a glass rack and alkaline phosphatase solution, freshly prepared in 0.5 M $(NH_4)HCO_3$, 0.025 M $MgSO_4$, 0.001 M $ZnSO_4$, at a concentration of 20 units/ml, was applied to it, 2 ml of phosphatase solution was required for 2 x 30 cm strip of paper, and incubated at 37°C in humidified atmosphere. After dephosphorylation, the salts were washed off the paper with distilled water, and the paper dried. The paper was stitched to DEAE cellulose paper 85 cm long and wide enough to hold the entire first dimension. The ionophoresis was performed at right angle to the first dimension, at 800 V for 18 hr. The paper was dried and nucleotides were located by radioautography.

Identification of 3′-terminal nucleoside

Identification of 3′-terminal nucleoside was made by chromatographic analysis of nucleosides formed upon alkaline hydrolysis of ^{3}H AMV RNA. About 20µg RNA was dissolved in 0.33 N KOH, sealed inside a capillary tube, and incubated at 38°C for 18 hr. Alkaline hydrolysate was mixed with 20µg of each of adenosine, cytidine, guanosine and uridine and the mixture was neutralized by Bio Rex-70 ammonium form. Nucleotide in alkaline hydrolysate were determined by electrophoresis on Whatman No.1 paper in 0.5% pyridine - 5% acetic acid, pH 3.5 containing 0.001 M EDTA. The four nucleosides were separated on DEAE paper, using water as solvent, by chromatography[30]. The true RNA base composition was determined from the alkaline hydrolysate of ^{32}P labelled AMV RNA. The entire electrophoretogram or chromatogram was sectioned in 1 cm strips and

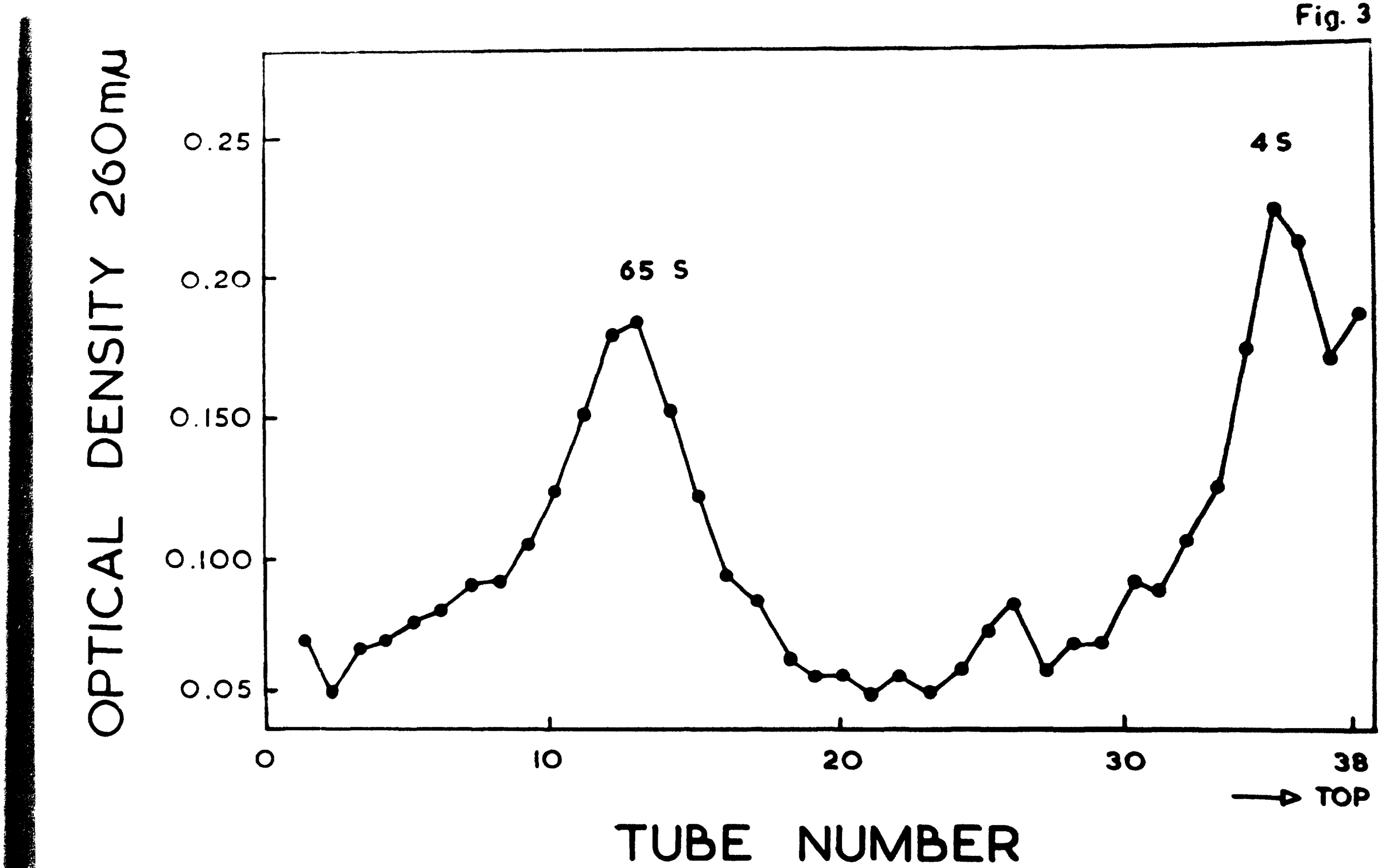
radioactivity counted in liquid scitillation spectrophotometer. The total amount of nucleoside or nucleotide was determined from the percentage in each of the four peaks. The calculation, after correction for non-equivalent incorporation of precursors, indicated the 3'-terminal nucleoside.

Results and discussion

The RNA isolated from avian myeloblastosis virus, grown in cold medium, by phenol sodium dodecyl sulphate method, was layered on 5-20% sucrose density gradient and centrifuged. The sedimentation pattern revealed that AMV RNA consists of two major components, a heavy 65S fast sedimenting component and a light 4S sedimenting component, Fig. 3. Robinson and Baluda[20] estimated that 65S RNA component has a molecular weight of 12×10^6 daltons and it represents the intact form of AMV RNA. The 65S AMV RNA when heated, above 70°C, it dissociates to 35S RNA subunits. It is shown that 4S RNA species are methylated[27] and it can be aminoacylated with different individual amino acids by aminoacyl tRNA synthetases prepared from chicken embryo.

On electrophoresis, AMV RNA in polyacrylamide agarose gel, is resolved into a slow migrating 65S component and a fast migrating 4S component, Fig. 4A. Additional slight bands for 28S, 18S and 11S RNA could be observed in some AMV RNA preparations. However the additional bands do not appear in RNA preparations made from virus of cultures harvested at shorter intervals and also when due precautions are taken against degradation of RNA by ribonuclease action. The fast sedimenting 65S RNA component, collected from sucrose gradient, just enters inside the gel, Fig. 4B, and slow sedimenting RNA moves with *E. coli* 4S RNA, Figs. 4C and 4E. The AMV 65S RNA when heated at 70°C for 3 min in buffer, it disociates into subunits which band at 35S position in polyacrylamide agarose gel, Fig. 4D. However, two slight 4S bands become visible in

Fig. 3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38
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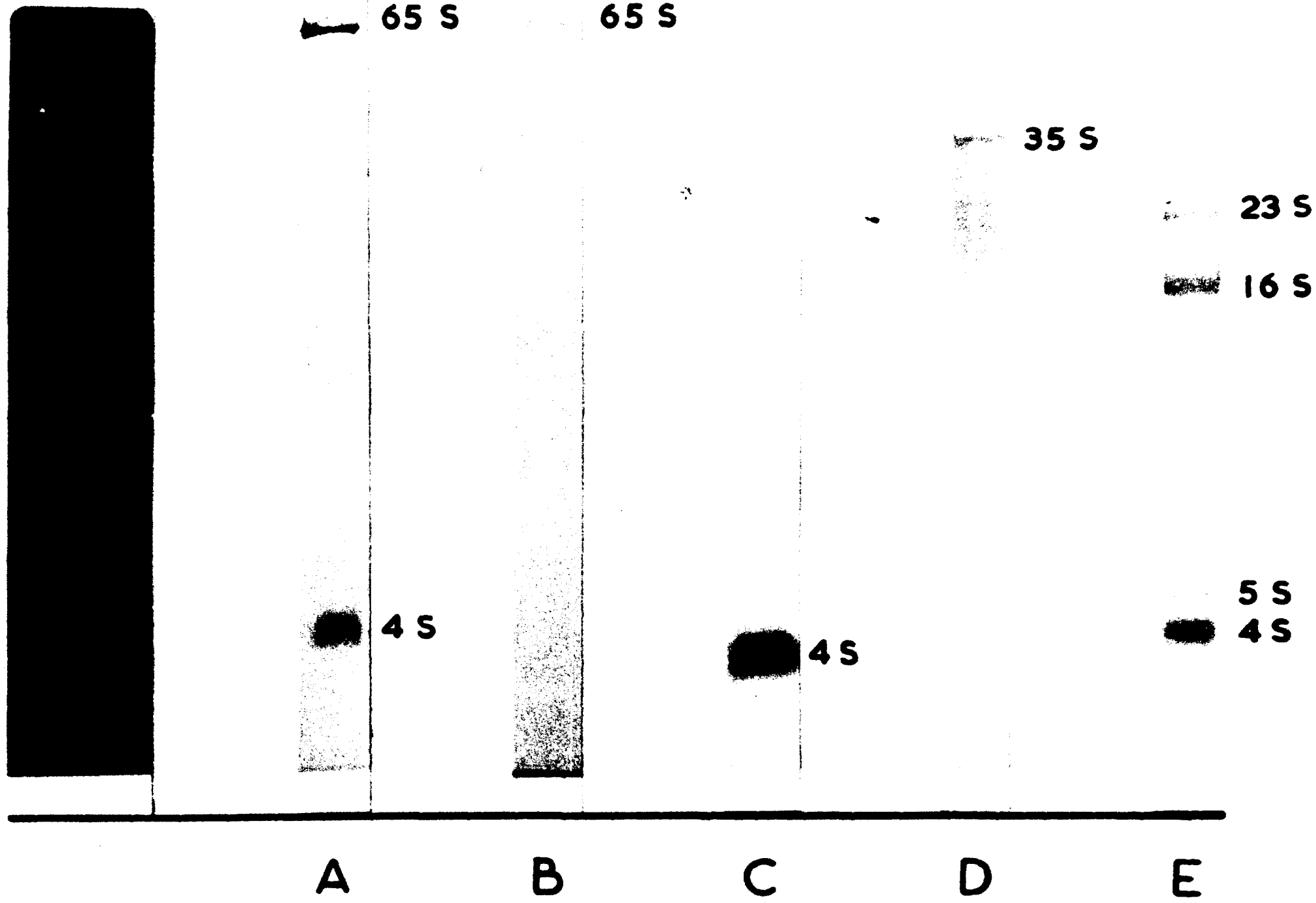
TUBE NUMBER

Fig. 3. Sedimentation of ribonucleic acid extracted from avian myeloblastosis virsus isolated by phenol-sodium dodecyl sulphate method. The RNA was dissolved in 0.2 ml of 0.1 M NaCl, 0.01 M tris pH, 7.4, 0.001 M EDTA buffer and layered on top of 12 ml 5-20% sucrose density gradient. Centrifugation was at 39,000 rpm for 6 hr in Beckman rotor SW 40 at 5°C.


65 S
65 S
35 S
23 S
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5 S
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D
E

Fig. 4. Relative electrophoretic mobilities of RNA samples in agarose acrylamide composite gels. A) AMV RNA isolated by phenol-sodium dodecyl sulphate method. B) fast sedimenting 65S RNA precipitated from sucrose density gradient. C) slow sedimenting 4S RNA precipitated from sucrose density gradient. D) heat treated 65S RNA, the 65SsRNA from sucrose gradient was precipitated with ethanol, redissolved in 0.01 M tris, pH 7.4, 0.001 M EDTA buffer, heated at 70°C for 3 min and then cooled rapidly in ice cold water, E) the reference Escherichia coli, RNAs, 23S, 16S, 5S and 4S. The RNA was electrophoresed in 2.4% acrylamide-0.6% agarose composite gels in tris-EDTA-borate buffer (pH 8.3) for 2 h at 200 V and 0°C and stained with 0.2% methylene blue in 0.4 M sodium acetate, pH 4.5, and differentiated with several washings of 0.02 M acetic acid.

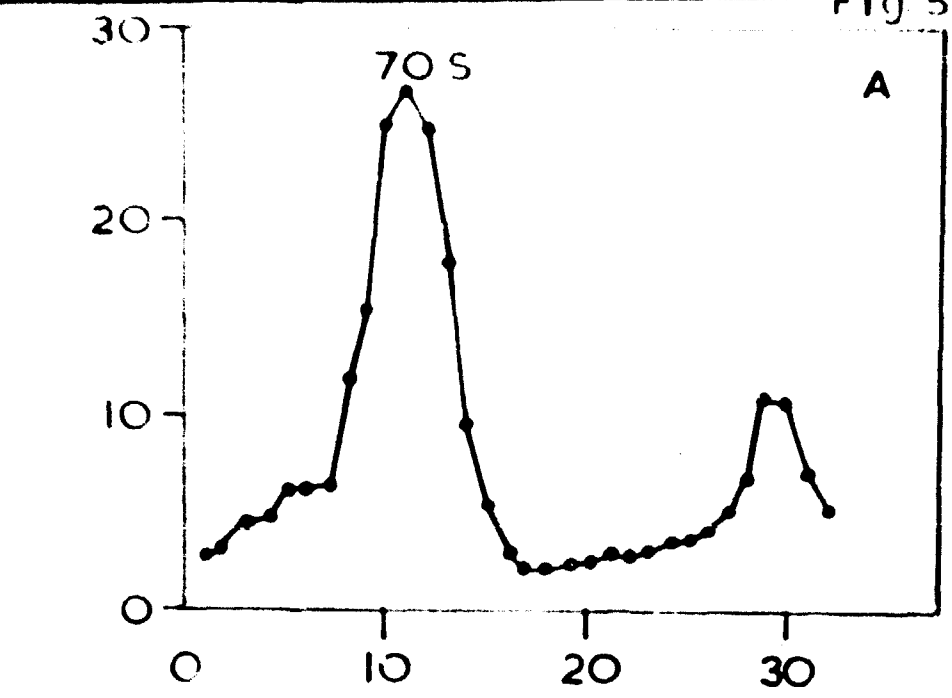
addition to 35S subunits in heat dissociated preparations of AMV 65S RNA if AMV virus or AMV RNA is handled without due care.

The two-dimensional electrophoresis of ribonuclease T_1 digest of ^{32}P labelled 65S AMV RNA showed a series of spots on the diagonal. This suggested either AMV 65S RNA is made of discontinuous heterogeneous polynucleotides held by hydrogen bonds or AMV 65S RNA is degraded under the existing experimental conditions.

Duesberg and Vogt[21] showed that AMV 65S RNA heat dissociates into 35S subunits and various amounts of heterogeneous smaller pieces of RNAs. Recently it is claimed[28] that AMV 65S RNA heat dissociates into 35S RNA subunits and a homogeneous 4S species. In this study, it is shown that quality of AMV 65S RNA depends on the time interval between harvests of virus made for isolation of AMV RNA. The sedimentation of ^{3}H AMV RNA, prepared from virus harvested at 2 hr intervals, in 5-20% sucrose gradient is shown in Fig. 5A. A similar sedimentation profile for AMV RNA is obtained if the harvests are made at 6 hr intervals. The distribution of components of heat dissociated AMV 65S RNA prepared from the virus harvested at 6 hr intervals is shown in Fig. 5B, and that from the virus harvested in Fig. 5C. It seems that components smaller than 35S RNA arise to a greater extent during prolonged culture, presumably through ribonuclease degradation of AMV RNA.

The chromatographic analyses of KOH hydrolysate of ^{3}H AMV 65S RNA identify the major 3′-terminal nucleoside Adenosine. This is true if either 65S or 35S RNA is analysed from virus harvested at either 2 hr or 6 hr intervals, Table 1. Therefore the smaller RNA products seem into Fig. 5B, probably have 3′-phosphorylated end which could not appear as nucleoside in alkaline hydrolysates. This result does not agree with that reported by Erikson and associates[28], who found uridine at 3′-end. Also data are presented that 3′-terminal uridine in

Fig 3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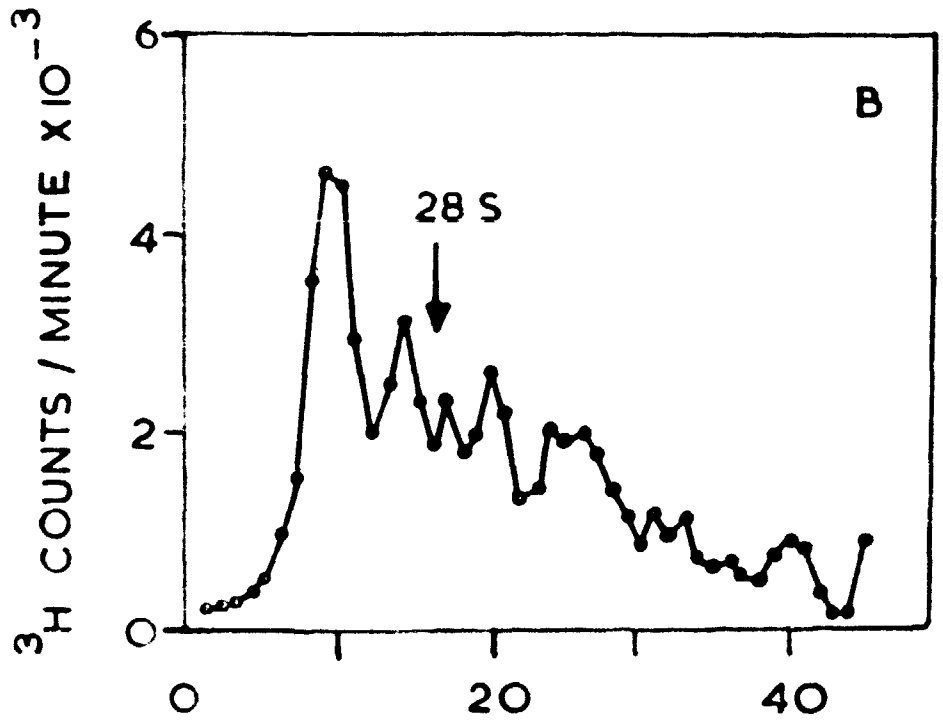
^{3}H COUNTS / MINUTE X 10^{-3}
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28 S
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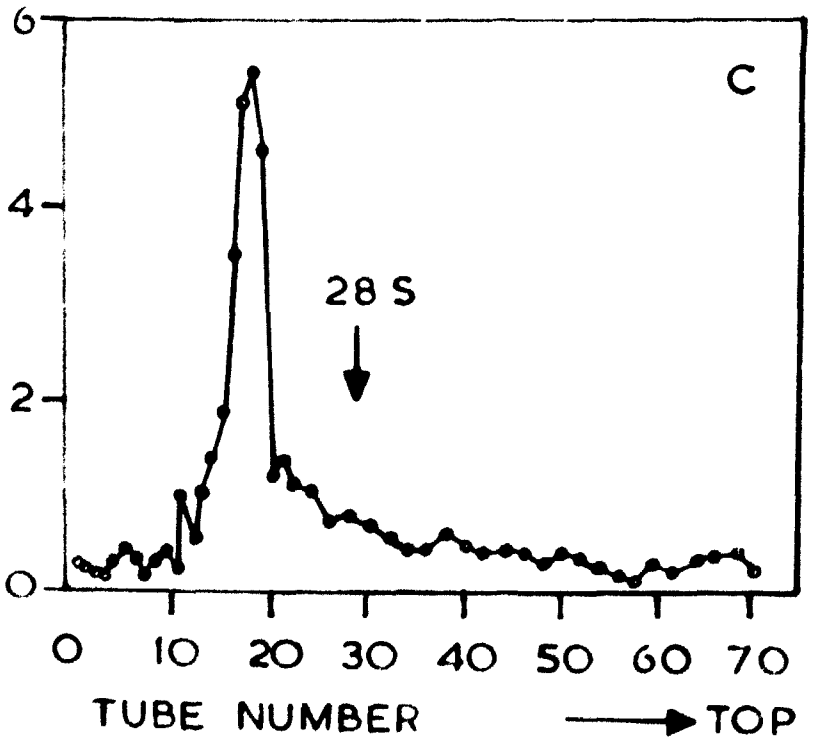
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TUBE NUMBER
TOP

Fig. 5. Sedimentation of AMV RNA. A) AMV RNA layered on top of 4.4 ml 5-20% sucrose gradient, in NTE buffer pH 7.4. Sedimentation was in Beckman rator SW 50 at 36,000 rpm at 5°C for 2 hr. B) Sedimentation of heat disscciated 65S AMV RNA isolated from virus harvested at 6 hr intervals. The RNA was dissolved in 0.2 ml of 0.01 M tris pH 7.4 + 0.001 M EDTA, heated at 70°C for 3 min and chilled quickly, layered on top of 13 ml 5-20% sucfose gradient. Sedimentation was in Beckman rotor SW 40 at 39,000 rpm at 5°C for 6 hr. The arrow indicates the position of 28S avian myeloblast ribosomal RNA. C) Sedimentation of heat dissociated 65S AMV RNA isolated from virus harvested at 2 hr intervals. The conditions were the same as described for 65S RNA isolated for virus harvested at 6 hr intervals.

Table 1.

Nucleotides and nucleosides found in KOH hydrolysate of AMV RNA

RNA preparation	Components analyzed	KOH hydrolysate products			
		A	C	G	U
1. ^{32}P AMV RNA	^{32}P nucleotides (%)	25.6	23.5	30.1	20.8
2. ^{3}H AMV RNA	^{3}H nucleotides (%)	5.4	47.5	8.0	39.1
6 hr harvest	^{3}H nucleosides (cpm)	52	89	-	76
70S RNA	corrected ^{3}H nucleosides(cpm)	247	44	-	40
	percentage nucleosides	75	13	-	12
3. ^{3}H AMV RNA	^{3}H nucleotides (%)	7.6	56.0	1.8	34.6
2 hr harvest	^{3}H nucleosides (cpm)	46	100	-	77
70S RNA	corrected ^{3}H nucleosides (cpm)	155	42	-	46
	percentage nucleosides	64	17	-	19
4. ^{3}H AMV RNA	^{3}H nucleotides (%)	8.9	47.7	1.8	41.6
2 hr harvest	^{3}H nucleosides (cpm)	28	86	-	61
35S RNA	corrected ^{3}H nucleosides (cpm)	81	42	-	31
	percentage nucleosides	53	27	-	20

About 5-10µg RNA ($1-10x10^{6}$ cpm) was hydrolysed in 0.33 N KOH at 38°C for 18 hr, then 20µg of each of adenosine, cytidine, guanosine and uridine were added as carrier. The resulting mixture was neutralized by Bio Rex-70, ammonium form. Nucleotides were separated on whatman No.1. paper in pyridine-acetate, pH 3.5. Nucleotides and four nucleosides were separated on DEAE-paper by chromatography and water was used as solvent. The counts obtained for (^{3}H) nucleosides were corrected and expressed as percent of the total nucleoside counts present.

case of C-type viruses.[31] The only disagreement, with Erikson coworkers, lies in the methods of virus and RNA purification, which involves an isopycinic banding of virus in sucrose in this work. It may be noted that myeloblast 28S RNA has 3′-terminal uridine. Further support for an adenosine at 3′-terminal in the viral RNA is found in the presence of polyadenylate sequences in many viruses including tumor viruses,[32] and the probable 3′-terminal location of poly A in the case of vaccinia viral RNA.[33] Baluda & Datta, with whom the author has associated in certain experiments, believe that polyadenylate sequences are similarly located at 3′-terminal end of AMV RNA.

TERMINAL NUCLEOTIDES OF RIBOSOMAL RIBONUCLEIC ACID FROM CHICKEN LEUKEMIC MYELOBLASTS

The studies on the nucleotide sequences that may be confined originally to the structure of AMV RNA, were extended to the structure of ribosomal RNA of leukemic myeloblasts from infected chicken. Also it could be useful to compare the terminal nucleotide structure of high molecular weight ribosomal RNA of the host cell to the AMV RNA.

Myeloblast culture

The leukemic myeloblast cells were grown as it has been described earlier. The myeloblast culture was prelabelled with ^{32}P labelled H_3PO_4 or ^{3}H labelled nucleosides, for 10-12 hr, and the radioactive medium changed four times at 5 hr intervals. The labelled myeloblasts collected by centrifugation and used for RNA extraction immediately.

Nucleic acid extraction

Myeloblasts, 1 ml packed cells, were suspended in 10 ml of NTE buffer (0.1 M NaCl, 0.01 M tris, pH 7.4, 0.001 M EDTA) containing 1% of β-mercaptoethanol; and then sodium dodecyl sulphate (SDS) is added to 1% concentration. After the cells had lysed, an equal volume of buffer saturated phenol is added. The mixture was shaken for 30 min. After centrifugation the aqueous layer, containing nucleic acids, was treated with phenol, two times. The first precipitate of nucleic acid formed by addition of 0.1 vol of 2 M sodium acetate, pH 5.5, and 2 vol of ethanol to the aqueous solution, was removed by spooling on a glass rod. The aqueous-ethanol mixture was allowed to stand for 24 hr at $-20^{\circ}C$ and then the second precipitate removed by centrifugation.

Isolation of 28S and 18S RNA

The extracted total nucleic acids were treated with 3 M sodium acetate, pH 6.0, at 3 mg RNA/ml, three times, to remove

DNA and small molecular weight ^{34}RNA. The ribosomal RNA was washed with 70% ethanol containing sodium chloride (1%) and reprecipitated from NTE buffer. The precipitate was dissolved in 0.01 M tris, pH 7.4, 0.001 M EDTA, 0.01 M lithium chloride and 0.5% SDS, layered on top of 28 ml 5-20% sucrose gradient and spun in Beckman SW 25.1 rotor at 24,000 rpm at 8°C for 18 hr. The peak fractions of 28S and 18S RNA were collected and RNA precipitated and were rerun on the same gradient to ensure purification. The final precipitate was washed with 70% alcohol, dried in a desiccator, dissolved in water and kept frozen. The specific activity varied 5-8 x 10^5 cpm/µg for ^{32}P labelled RNA and 2-4 x 10^6 cpm/µg for ^{3}H labelled RNA.

Gel electrophoresis

Gel electrophoresis was performed on polyacrylamide-agarose composite gels as described by Peacock and Dingman (1963). Agarose solution, 1% in water, 15 ml was refluxed for 15 min and cooled to 48°C and cyanogum solution, 2.5% in (x2 concentrated) buffer, was warmed at 48°C. Just before the gels were poured, freshly prepared 10% ammonium persulphate solution, 150 µl was added to agarose and TEMED (NNN'N'-tetramethylethylenediamine), 12 µl, was added to cyanogum. Then agarose and cyanogum solutions were mixed quickly and thoroughly and poured immediately into plexiglas tubes (0.25 inch diameter and 12 cm long). After polymerization was complete, 1-2 hr, the gels were pushed out at top of tubes, the top 1-2 mm of the gel was sliced flate with razor blade. A rubber ring was used to hold the gels inside the tubes. The running buffer contained per 1000 ml tris, 10.8g; disodium salt of EDTA, 0.93g; boric acid, 5.5g; pH 8.3. Electrophoresis was carried out in cold at 0°C at constant current of 2mA per gel. The gels were prerun for one hour. RNA samples were loaded in 20-30% sucrose, containing 0.1% bromophenol blue as tracking marker. After electrophoresis the gel was sliced in 1 mm pieces with help of stainless steel razer blades stacked together in a stainless steel block.

Radio-activity was minitored in scintillation counter using 'Aquasol'. For staining the gels were fixed for 30 min in 1 M acetic acid and stained with methylene blue, 0.2% in 0.4 M sodium acetate, pH 4.5, for 30 min at room temperature and destained with several changes of 0.01 M acetic acid.

Isolation of 3′-terminal fragments

Pancreatic ribonuclease A, snake venom phosphodiesterase and bacterial alkaline monoesterase were obtained from Worthington Biochemical Corp. Ribonuclease T_1, was obtained from Calbiochem Corp. Ribonucleic acid 28S or 18S was digested either with RNase A or RNase T_1, and 3′-terminal fragment obtained by two dimensional diagonal electrophoresis in 7% formic acid. The 3′-terminal diagonal oligonucleotide was cut out, and purified on DEAE cellulose paper in pyridine-acetate, pH 3.5. The digestion of 3′-terminal fragment with enzyme or KOH, and analysis of the products was done as described by Sanger and coworkers.[13,22]

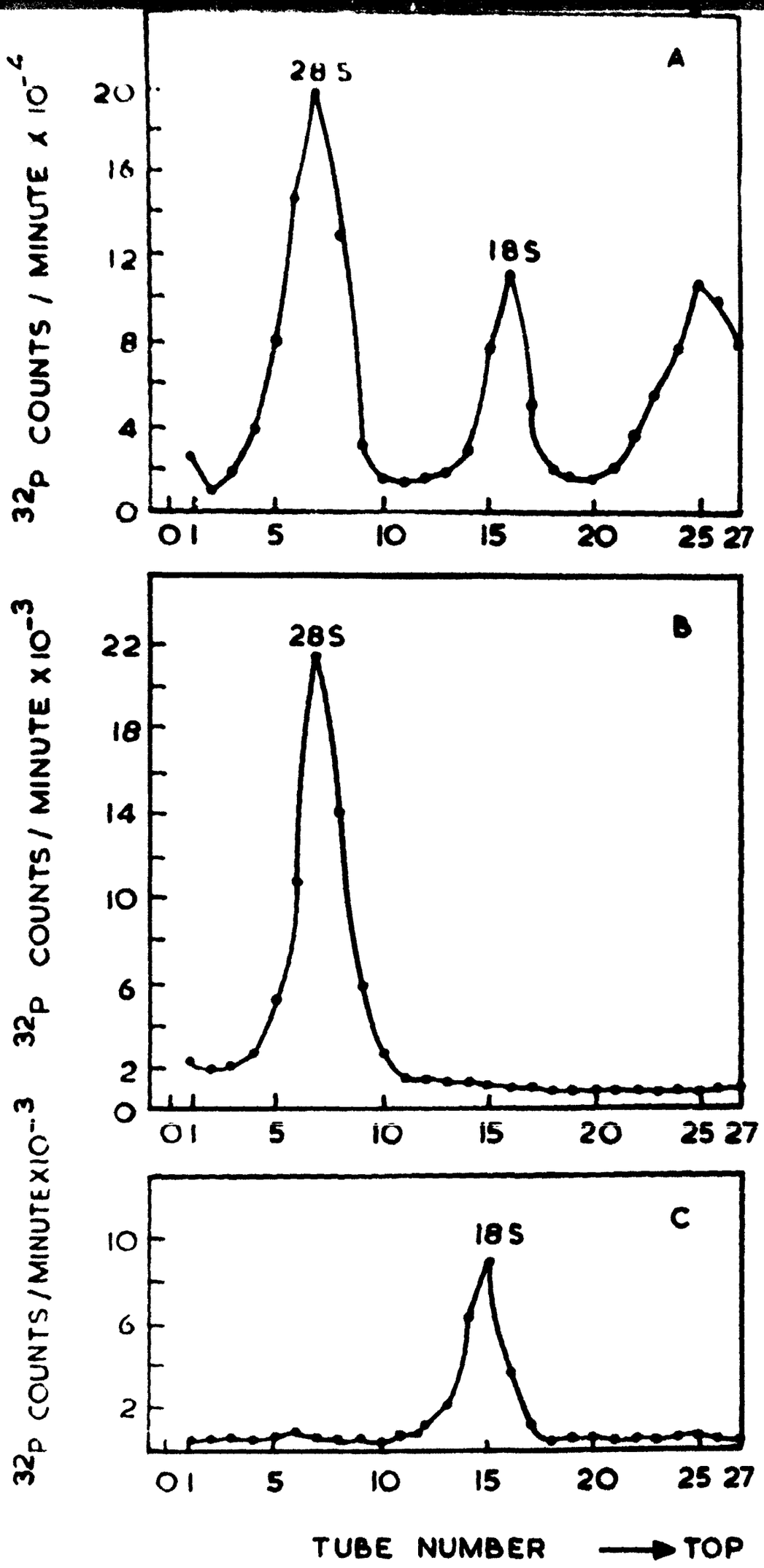
Identification of 3'(2') 5'-terminal nucleoside diphosphate

The ribonucleic acid, 28S RNA or 18S RNA, was hydrolysed in 0.33 M KOH at 38°C for 18 hr and the digest was electrophoresed in the first dimension on Whatman No. 540 paper and in the second dimension on DEAE ion-exchange paper, in pyridine-acetate, pH 3.5, containing 0.001 M EDTA. Alkaline hydrolysis released 3'(2') 5'-nucleoside diphosphate from a 5′-terminal end of a phosphorylated polynucleotide only. The 3'(2') 5′-nucleoside derivatives move more rapidly in the first dimension than mononucleotides and moves slowly on DEAE ion-exchange paper in the second dimension. It was possible to confirm the identification of 3'(2') 5′-nucleoside diphosphate derivatives by co-chromatography with known markers pAp, pCp, pGp, pUp and by their dephosphorylation with bacterial alkaline monoestrase.

Results and discussion

A typical distribution of sedimentation of RNA, on 28 ml 5-20% sucrose density gradient, after extraction of total nucleic acids with 3 M sodium acetate, pH 6.0, that removed DNA and low molecular weight RNA, is shown in Fig. 6 A. The peak fractions of 28S RNA and 18S RNA were collected and rerun on a similar sucrose density gradient. Cross contamination of 28S RNA or 18S RNA, with each other, as judged by profiles in sucrose density gradient centrifugation, was not detectable, Fig. 6 B & C. Either RNA species, 28S RNA or 18S RNA, collected from the peak fractions of the rerun profiles on sucrose density gradient, exhibited single band in polyacrylamide-agarose gels upon electrophoresis, Fig. 7. No DNA, transfer RNA, or other cellular contaminant RNA was present. The myeloblast 28S RNA or 18S RNA obtained for this study appeared intact and physically homogeneous.

Tritium labelled myeloblast 28S RNA or 18S RNA was hydrolysed in alkali. The hydrolysate was desalted by passage through Bio Rex-70, ammonium form. A part of the hydrolysate was chromatographed on DEAE cellulose paper with marker nucleosides. Each nucleoside was monitored for radioactivity. Since labelling was not uniform in all nucleosides, nucleotides from the second part of the hydrolysate were fractionated by paper electrophoresis, and tritium in each was estimated. The specific activity of each nucleotide in tritiated RNA was calculated from the base composition of RNA that was determined by electrophoretic analysis of ^{32}P labelled RNA. The measured tritium activity in each nucleoside was corrected to account for non-equivalent incorporation, Table, 2. The predominant nucleoside present in KOH digest of the 28S RNA is uridine and while that of the 18S RNA is adenosine. These nucleosides could arise only from the non-phosphorylated 3-end of the RNA molecules. Therefore the 28S RNA has shown 3-terminus to be uridine and the 18S RNA has shown 3-terminus to be andenosine.


A
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^{32}P COUNTS / MINUTE x 10^{-4}
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^{32}P COUNTS / MINUTE X 10^{-3}
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TUBE NUMBER → TOP

Fig. 6. Sedimentation of chicken leukemic myeloblast RNA. A) Total nucleic acids were extracted with phenol-sodium dodecyl sulphate method and precipitated with ethanol and treated three times with 3 M sodium acetate, pH 6.0, to remove DNA and the ^{32}P RNA reprecipitated and layered on top of 28 ml of 5-20% sucrose density gradient in 0.01 M $LiCl_2$, 0.01 M tris, 7.4, 0.001 M EDTA containing 0.5% sodium dodecyl sulphate. Centrifugation was in Beckman rotor SW 25.1, at 24,000 rpm at 8°C for 18 hr. B) ^{32}P RNA 28S peak fractions were collected, precipitated with ethanol, and rerun in sucrose density gradient under conditions described above. C) ^{32}P RNA 18S peak fractions were also collected, precipitated with ethanol, and rerun in sucrose density gradient as described above.

Table II.

Nucleotides and nucleosides found in KOH hydrolysate of myeloblast RNA.

RNA preparation	Components analyzed	KOH hydrolysate products			
		A	C	G	U
18S RNA	^{32}P nucleotides (%)	23	25	29	23
	^{3}H nucleotides (%)	1.9	49.6	7.6	40.9
	^{3}H nucleosides (cpm)	66	482	-	399
	corrected ^{3}H nucleosides (cpm)	806	244	-	188
	percentage nucleosides	65	20	-	15
28S RNA	^{32}P nucleotides (%)	18.6	27.9	36.6	16.9
	^{3}H nucleotides (%)	1.9	6.0	7.4	30.7
	^{3}H nucleosides (cpm)	-	433	-	1297
	corrected ^{3}H nucleosides (cpm)	-	201	-	713
	percentage nucleoside	-	22	-	78

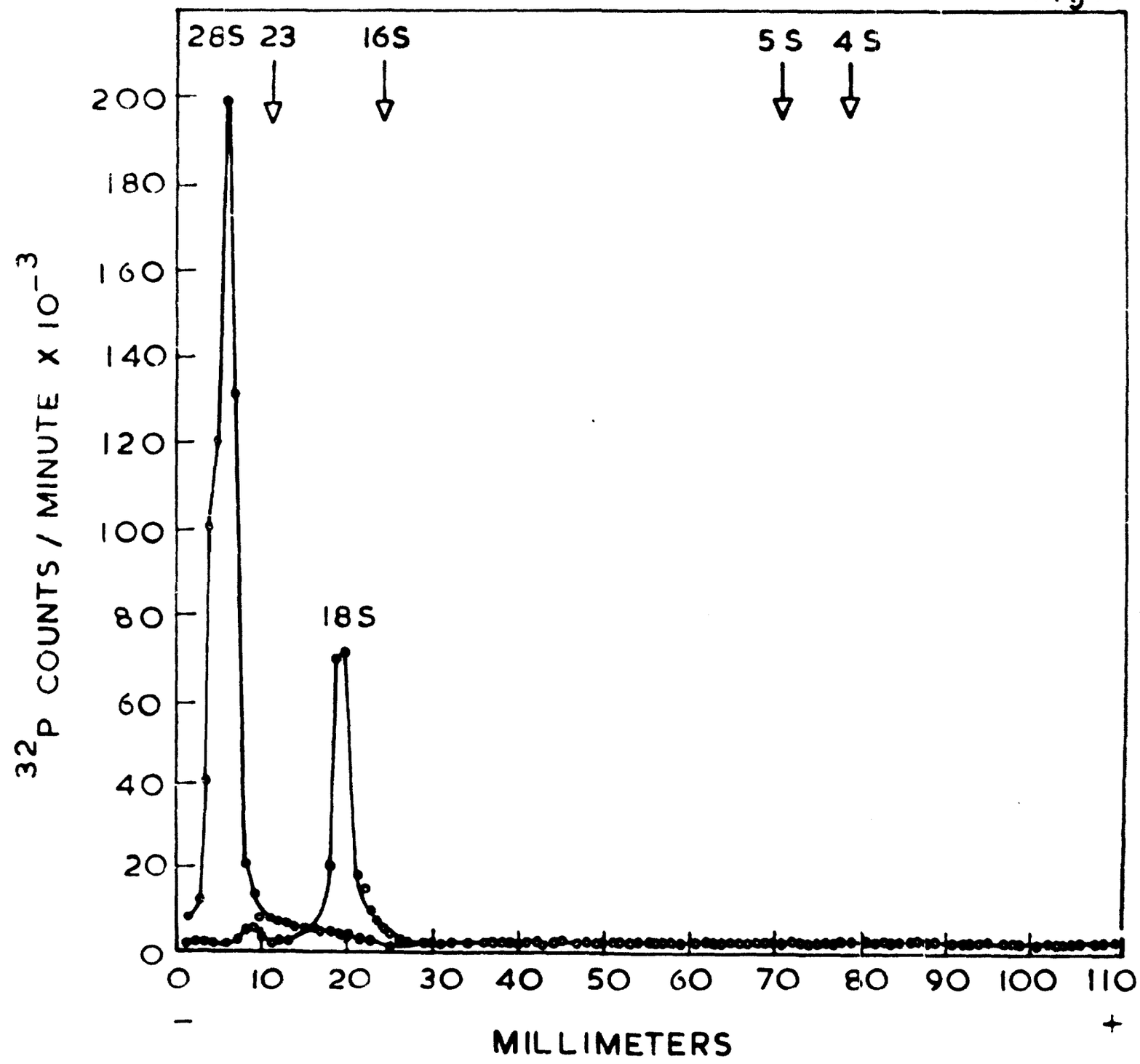
About 5-10µg RNA (1-2x10^{6}/cpm) was hydrolysed in 0.33 N KOH at 38°C for 18 hr, then 20 µg of each of adenosine, cytidine, guanosine and uridine were added. The mixture was neurtralized by Bio Rex-70, ammonium form. Nucleotides were separated on Whatman No.1. paper in pyridine-acetate, pH 3.5. Nucleotides and four nucleosides were separated on DEAE-cellulose paper by chromatography. Water was used as solvent. The counts obtained for tritiated nucleosides were corrected for base composition, determined using ^{32}P RNA, and expressed as percent of the total nucleoside counts present.

Myeloblast ribosomal ^{32}P labelled 28S RNA was digested with ribonuclease A and the products separated by diagonal two dimensional electrophoresis. A radioautogram of the fingerprint is shown, Fig. 8. The oligonucleotide, that appeared on the diagonal, eluted by triethylamine bicarbonate buffer and the radioactive material dried from small volumes of water repeatedly. The 3'-terminal oligonucleotide further purified on DEAE cellulose paper by electrophoresis in pyridine-acetate, pH 3.5. The mononucleotides released upon digestion of the oligonucleotide material in 0.2 N NaOH separated on Whatman No. 1 paper, located by radioautography and analysed by means of scintillation counter. A part of the oligonucleotide material digested with snake venom phosphodiesterase and the products separated by electrophoresis. The proportions of hydrolysis products, released upon NaOH or snake venom phosphodiesterase digestion, is shown in Table 3. The sequence of nucleotides in the 3'-terminal fragment could be written,-PypApGpGpU$_{OH}$. The digest of ribonuclease T_1 of ^{32}P labelled 28S RNA failed to show the presence of any oligomer that could migrate at the diagonal upon electrophoresis and this could be only true of 3'-terminal oligonucleotides with guanosine residue at penultimate position.

Myeloblast ribosomal ^{32}P labelled 18S RNA was digested with ribonuclease T_1 and the hydrolysis products separated by diagonal two dimensional electrophoresis. A radioautogram of the fingerprint is shown in Fig. 9. The 3'-terminal oligonucleotide was digested with alkali snake venom phosphodiesterase and pancreatic ribonuclease A; and the comparison of hydrolysis products of each reaction separated by electrophoresis, Table 3. This provided evidence for the sequence-GpCp(2Cp, 2ApUp, 2Up) A_{OH}. The 3'-terminal oligonucleotide lacked guanylic acid and this could only be true of 3'-terminal oligonucleotide.

Terminal 3'(2') 5'-nucleotide diphosphate from alkaline hydrolysate of myeloblast ribosomal RNA were separated by two dimensional electrophoresis first on Whatman No. 540 paper and

Fig. 7


28S
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^{32}P COUNTS / MINUTE X 10^{-3}
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MILLIMETERS

Fig. 7. Gel electrophoresis of ^{32}P RNA 28S and ^{32}P RNA 18S. The peak fractions of 28S and 18S RNA collected from sucrose density gradient run, and electrophoresed on agarose - acrylamide, 0.5% agarose and 2.5% cyanogum 41, gel columns, 11.1 cm long and ¼ inch in diameter. Electrophoresis was at 2mA per gel column. Escherichia coli RNAs, 23S, 16S 5S, 4S were used as markers, indicated by arrows. After electropboresis the gels were frozen in hexane, cooled in dry ice and cut into 1mm pieces on a slicer made of stainless steel blades stacked in stainless steel blocks. Each slice was treated with 0.1 ml of hydrogen peroxide at 50°C. and counted in 10 ml of 'Aquasol' scintillator. The gels, run with marker RNA, were stained with methylene blue 0.2% in 2.4 M sodium acetate, pH 4.5, and differentiated with several changes of 0.1 M acetic acid.

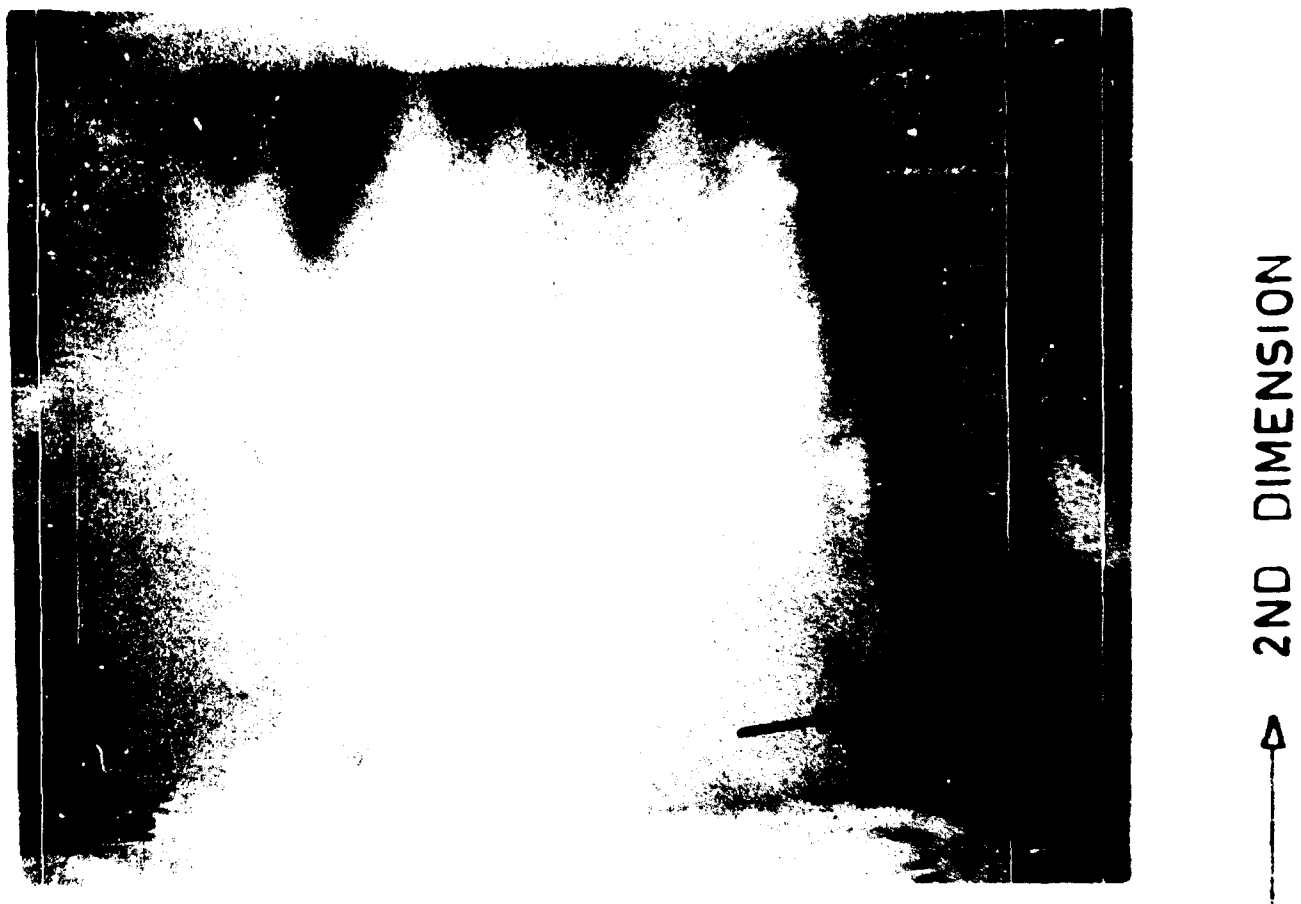
2ND DIMENSION
1ST DIMENSION

Fig. 8. Phosphatase diagonal of ribonuclease T_1 digest of ^{32}P RNA 18S. First dimension is ribonuclease T_1 digest ionophoresis and second dimension is after phosphatase treatment ionophoresis. About 10 ug RNA ($4-5 \times 10^6$ cpm) was digested in 0.1 M tris, pH 7.2, 0.02 M EDTA with acid treated ribonuclease T_1 (1 mg enzyme/ml in 0.01M tris, pH 7.2, 0.02 M EDTA was acidified by addition of 0.05 vol of 1N HCl and incubated for 10 min at 37°C and the solution was then neutralized by addition of 0.1 vol of 1M tris, pH 7.2, plus 0.05 vol of 1N NaOH). The substrate to enzyme ratio was, 10:1, incubated at 37°C for 30 min. The digest was subjected to ionsphoresis on 15 x 85 cm DEAE-cellulose paper in 7% formic acid at 800 V for 14 hr. The radioactivity was located by radioautography and the strip, about 2 cm wide, was cut out. The strip was placed on a glass chromatographic trough and soaked with alkaline phosphatase, 20 U/ml concentration in 0.5 M $(NH_4)HCO_3$, 0.025 M $MgSO_4$, 0.0001 M $ZnSO_4$, at 1 ml per 1 x 30 cm strip paper; and incubated at 37°C for 2 hr in water saturated atmosphere. After dephosphorylation the strip was washed off salts with water, dried and sewn onto a DEAE-cellulose paper, 46 x 80 cm, and ionophoresis was performed at right angle to the first dimension. The arrow indicates the diagonal 3′ OH terminal fragment.

Fig. 9

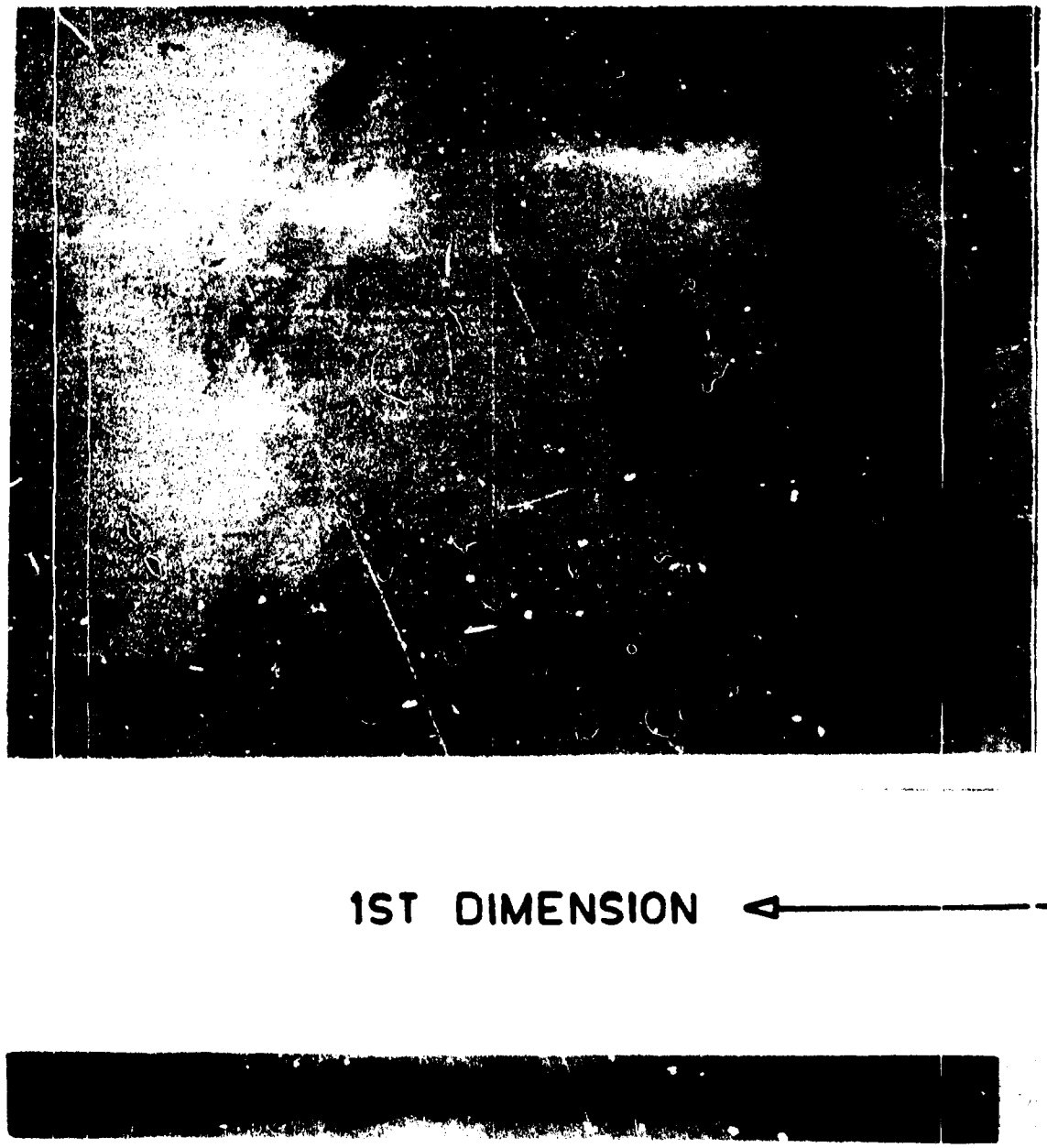

Fig. 9. Phosphatase diagonal of ribonuclease A digest of ^{32}P RNA 28S. First dimension is ribonuclease A digest ionophoresis and second dimension is after phosphatase treatment ionophoresis. About 10 µg RNA, 4-5 x 10^6 cpm, was digested with ribonuclease A, substrate to enzyme ratio, 10:1, for 3 hr at 37°C in 0.02 M tris, pH 7.4, 0.002 M EDTA. The remaining treatment was exactly the same as described for fig. 8. The arrow indicates the diagonal 3′ OH terminal fragment.

Table III.

Base composition of 3'-terminal oligonucleotide of 28S rRNA from a pancreatic ribonuclease A digest, and base composition of 3'-terminal oligonucleotide of 18S rRNA from a T_1 RNase digest.

RNA component	NaOH	VPDL	RNase A
28S	1Ap	2pG	
	2Gp	1pU	
18S	2Ap	3pA	2Up
	3Gp	2pC	2ApUp
	4Up	4pU	3Cp

Table IV

Terminal nucleotides of ribosomal RNA

Source	Component	5'-terminus	3'-terminus	Reference
Avian myeloblasts	18S	pC--	--GC $(AU)_2(C)_2(U)_2$ A_{OH}	
	28S	pG--	$--P_YAGGU_{OH}$	
L-Cells	18S	pU--	$--A_{OH}$	35
	28S	pC--	$--U_{OH}$	35
Rabbit reticulocytes	18S	-	$--GAUCAUUA_{OH}$	36
	28S	-	$--GUUUGU_{OH}$	36
E. coli.	16S	pAAAUG--		37
			--GAU (AC) $(C)_4(U)_3$ A_{OH}	38
	23S	pGGU--		37
			$--GCUUAACCUU_{OH}$	39

second on DEAE cellulose paper. Analysis of alkaline hydrolysates of ^{32}P labelled 28S RNA showed only one radioactive spot corresponding to a guanosine 3'(2') 5'-diphosphate. The identity of guanosine 3'(2') 5'-diphosphate was confirmed by treatment with bacterial alkaline monoestrase, which resulted in complete dephosphorylation of pGp. Analysis of alkaline hydrolysates of the ribosomal 18S RNA component showed only one spot corresponding to pGp. The recovery of the pGp in two experiments was calculated to be one mole per 1780 nucleotides and one more per 2241 nucleotides, corresponding to an approximate molecular weight of 18S RNA of 0.53 and 0.67 x 10^6 in each case.

The published ribosomal RNA terminal sequences for comparison are listed in Table, 4. The 5-end is phosphorylated in all RNA species, but no consistent terminal nucleotide is seen. Ribosomal 18S RNA all terminate at the 3-end predominantly in adensoine while ribosomal 28S RNA terminate in uridine. Only a few features are common and the homology beyond this point is difficult to see.

SUMMARY

It is shown that quality of AMV RNA depends on the time intervel for harvests of virus from tissue culture. AMV RNA isolated from the virus harvested at 6 hr intervel, on heat dissociation, show smaller fragments than 35S RNA prerumably due to ribonuclease action. 65S AMV RNA is an aggregate structure made of 35S RNA subunits. Alkaline Hydrolysis of 65S or 35S ^{3}H AMV RNA documents that 3′-terminal is predominately adenosine. Analyses of KOH hydrolysate of ribosomal RNA of chicken leukemic myeloblasts gave about 1 mole of cytidine 3′(2′), 5′-diphosphate and 1 mole of adensoine for 18S RNA and similar analyses for 28S RNA gave only guanosine 3′(2′)-diphosphate and uridine. Analyses of 3-terminal oligonucleotides indicated that nucleotide sequence of 18S rRNA is--GC $(AU)_2(C)_2(U)_2$ A_{OH} and nucleotide sequence of 28S rRNA is- $-P_yAGCUOH$.

APPENDIX I

Basal medium

NaCl	6.8 gm
KCl	0.4 gm
$CaCl_2$(anhydrous)	0.2 gm
$MgSO_4.7H_2O$	0.1 gm
$NaH_2PO_4.H_2O$	0.125gm
+$NaHCO_3$	0.2 gm
Glucose	2.0 gm
1-Serine	30 mg
1-Proline	40 mg
1-Hydroxylproline	10 mg
Glycine	50 mg
*Aspartic acid	30 mg
*Glutamic acid	60 mg
*Adenosine	20 mg
Phenol red	16 mg
Amino acid x 100 (EM)	40 ml
**Vitamin mixture	40 ml
***Antimycotic	1 ml
Streptomycin sulphate	0.5 gm
Penicillin G	250,000 units
Water (double distilled) to	1000 ml
Adjust pH with NaOH to 7.4	

+$NaHCO_3$: dissolve separately and add last.

*Hard to dissolve: dissolve separately in water at 45°C in 30ml of 1N NaOH.

**Vitamin mixture stock: thiamine hydrochloride, 200mg; riboflavin, 20mg; pyridoxine hydrochloride, 200mg; folic acid, 200mg; biotin, 20mg; Nicotinamide, 200mg; choline chloride, 200mg; inositol, 350mg; pantothenic acic, 200mg; and water (double distilled) to 1000ml.

***Antimycotic (N-butyl-P-hydroxybenzoate) stock: dissolve 200mg antimycotic per 1000ml and autoclave at 15 lb pressure for 10 min.

Sterilize the medium by passage through millipore filter.

Growth medium

To 88ml of basal medium is added 2ml of 10% glutamine (sterilized by passage through millipore filter), 5ml of inactivated foetal calf serum and 5ml of inactivated chicken serum.

APPENDIX II

Buffer saturated phenol

1. Melt phenol in 50°C water bath and pour 850ml into 2000ml cylinder with glass stopper.
2. Add an equal volume of double distilled water.
3. Add 90ml of saturated tris base to adjust pH8.0.
4. Add 3.6ml of 0.5M EDTA (final concentration 10^{-3}M).
5. Add 4ml β-mercaptoethanol.
6. Invert cylinder 10 minutes, allow to separate in the cold room, aspirate water layer from top.
7. Wash phenol with an equal volume of NTE buffer (0.1M NaCl, 0.01M tris-HCl, pH8.5, 0.001M EDTA), two time. Invert 5 minute each time, allow to separate in cold room and aspirate from top each time.
8. Add an equal volume for fresh buffer to the phenol and add 0.1% (of total volume) β-mercaptoethanol. Invert to mix and pour into marked 'Phenol' bottles. Store in refrigerator.

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