STUDIES OF ESCHERICHIA COLI RNA-DNA HYBRID AND OF METHYLATION OF RNA IN T-4 INFECTED CELLS

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This is to certify that the

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John A Boezi
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ABSTRACT

STUDIES OF ESCHERICHIA COLI RNA-DNA HYBRID AND OF METHYLATION OF RNA IN T-4 INFECTED CELLS

by Robert Lee Armstrong

The reaction between Escherichia coli (^{3}H) RNA and denatured DNA to form ribonuclease stable hybrid was studied. The reaction rate is optimal at 78° when 0.50 M KCl, 0.01 M Tris (pH 7.3) is used as the solvent. Hybridization is dependent on homologous, denatured DNA. Native or denatured DNA from Bacillus subtilis or Pseudomonas fluorescens will not react with (^{3}H) RNA from E. coli. RNA-DNA hybrid formation is dependent on both the RNA and DNA concentrations as well as incubation at an elevated temperature (65-87°). The RNA-DNA hybrid melts 4-5° below the melting point of native DNA (94-95° and 99°, respectively) in 0.50 M KCl, 0.01 M Tris (pH 7.3). The stability of the RNA-DNA hybrid in the presence of ribonuclease is dependent upon the salt concentration. A sharp transition from ribonuclease resistant to ribonuclease sensitive occurs when the salt concentration is decreased. The midpoint of this transition occurs at 0.135 M KCl.

Hybridization was used to show that there is less messenger RNA present in glucose starved cells than is present in cells grown on minimal media, enriched media, or treated with chloramphenicol. Hybridization and nitrocellulose chromatography were used to isolate messenger RNA

from the rest of the cellular RNA. The specific activity of the isolated messenger RNA was higher than the pulse labeled RNA from which it was isolated. The molecular weight of the isolated messenger RNA was shown to be low by both methylated albumin-kieselguhr chromatography and sucrose gradient centrifugation. Hybridization does not occur with low molecular weight RNA more readily than with high molecular weight RNA. Most of the degradation of the isolated RNA apparently occurred during the denaturation of the hybrid. Isolated messenger RNA hybridized more readily with denatured DNA than did pulse labeled RNA.

Methylation of RNA occurs in T-4 infected E. coli K12 W6. About 60-70% of the RNA molecules which are methylated sediment with soluble RNA. The other 30-40% are a mixture of RNA molecules of diverse size which sediment faster than 4 S and which elute from a methylated albumin-kieselguhr column at salt concentrations greater than that required to elute soluble RNA. The methylation pattern of soluble RNA extracted from infected cells is different from the pattern of RNA from uninfected cells. Little or no methylation of ribosomal RNA occurs in infected cells. (^{3}H) methyl labeled RNA from infected cells hybridizes with T-4 DNA but not with E. coli DNA. Some soluble RNA synthesis may occur in infected cells since some of the (^{3}H) uracil labeled RNA extracted from T-4 infected cells elutes from the methylated albumin-kieselguhr column with soluble RNA.

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By

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INTRODUCTION

PART I. STUDIES OF E. coli RNA-DNA HYBRID.

Jacob and Monod postulated the existence of messenger RNA in 1961 (1). Gros, et. al., (2) and Brenner, et. al., (3) verified the existence of messenger RNA in bacterial systems. The existence of messenger RNA in plants and animal cells has been well established (4, 5). Messenger RNA is synthesized in the nucleus by RNA polymerase using DNA as the template. The resulting RNA is complementary to the strand of DNA which served as its template and is able, therefore, to carry the biological information from the DNA to the sites of protein synthesis. At these sites of protein synthesis, information is translated from a sequence of nucleotides into a sequence of amino acids in a protein.

In E. coli, growing logarithmically, about 1% of the total RNA is messenger RNA, 20% is soluble RNA, and the remaining 79% is ribosomal or ribosomal precursor RNA (6). If such cells are given a pulse of radioactive uracil for a short time (5% of the generation time in the presence of (^{3}H) uracil) then much of the radioactivity will be found in the messenger RNA fraction (2). This relatively selective labeling of messenger RNA can be used to follow the course of purification of messenger RNA.

Hall and Spiegelman (7) observed the formation of complex (hybrid) between T-2 denatured DNA and T-2 specific RNA. Hybrid formation is the reaction of RNA and denatured DNA to

form a RNA-DNA hybrid. The two complementary strands of

$$\text{RNA} + \text{dDNA} \longrightarrow \text{RNA-DNA}$$

denatured DNA can also react to form the renatured DNA

$$\text{dDNA} + \text{dDNA} \longrightarrow \text{DNA-DNA}$$

duplex. In each of the above reactions there is a hydrogen bonded, double stranded structure formed similar to the Watson-Crick (8) structure of native DNA.

In order for hybrid formation to occur there must be sufficient ionic strength in the reaction mixture to shield the negatively charged phosphate groups and allow the two separate strands of nucleic acids to approach each other closely. The reaction mixture must be heated, apparently to dissociate any non-specific hydrogen-bonding which may occur in a given nucleic acid molecule and to allow the complementary strands to form a hybrid. Denatured DNA is required and native DNA will not form any hybrid with RNA. Denatured DNA must be homologous to the RNA if hybridization is to occur. E. coli denatured DNA will not hybridize with pulse labeled RNA from T-2 infected cells but this RNA does hybridize with T-2 denatured DNA (7).

RNA present in the RNA-DNA hybrid is insensitive to pancreatic ribonuclease in comparison to free RNA which is rapidly degraded by ribonuclease. The RNA-DNA hybrid has a density intermediate between the densities of free RNA and denatured DNA (9). The RNA-DNA hybrid has a rather sharp melting profile similar to that of double stranded, hydrogen bonded DNA (10). The RNA-DNA hybrid is adsorbed on nitrocellulose in the presence of high salt concentrations while

free RNA is not adsorbed.

Several techniques have been used to detect the presence of RNA-DNA hybrid. Hall and Spiegelman (7) used CsCl density centrifugation (9). Free RNA has a density of 1.9 gm/ml and DNA has a density of about 1.7 gm/ml. The RNA-DNA hybrid will have a density intermediate between the density for DNA and RNA. CsCl equilibrium centrifugation can, therefore, separate the RNA from RNA-DNA hybrid from DNA but is expensive, time consuming, and applicable only to small samples. Bautz and Hall (11) developed a DNA cellulose column in which the glucosylated DNA of certain phages was chemically bound to cellulose. RNA was then heated with the column containing DNA and the unreacted RNA was washed from the column while RNA-DNA hybrid was retained. The technique is limited to those few DNA's containing glucosyl groups. Bolton and McCarthy (12) used agar to immobilize denatured DNA. The agar was cut into pieces and packed into a column. RNA was then heated on the column and the unreacted RNA was removed by washing. The agar column presents the problem of manipulating nucleic acids in agar and also prevents the isolation of RNA-DNA hybrid.

Nygaard and Hall (13) introduced nitrocellulose membrane filtration as a technique for the detection of RNA-DNA hybrids. By some unknown mechanism, nitrocellulose is able to selectively retain denatured DNA or RNA-DNA hybrid but not free RNA if the nucleic acids are placed in contact with the nitrocellulose in the presence of high salt concentrations. The method allows fast and simple analysis for the presence of RNA-DNA hybrid. Gillespie and Spiegelman (14)

reported that denatured DNA could be irreversibly bound to the nitrocellulose filters by filtering the DNA onto the filter, air drying the filter, and heating at 80° for a short time. With denatured DNA bound to the membrane filters DNA-DNA interactions cannot occur. Therefore, it is possible to detect, quantitatively, the RNA able to hybridize with only a limited portion of the genome (for example, soluble and ribosomal RNA homologous to 0.025% (15, 16) and 0.2% (17, 18) of the genome, respectively) because DNA-DNA interactions are prevented. Nitrocellulose column chromatography (10, 19, 20) can be used for the isolation of larger amounts of RNA-DNA hybrid. It works in the same manner as the membrane filters but readily allows the isolation of larger amounts of hybrid.

Since Hall and Spiegelman (7) observed the formation of a specific T-2 RNA-DNA hybrid, the reaction has been widely used to measure complementarity between RNA and DNA nucleotide sequences. Hybrid formation has been used to show that pulse labeled RNA is complementary to homologous DNA (21, 22, 23) and to demonstrate that DNA contains nucleotide sequences complementary to ribosomal RNA (17, 18, 24, 25, 26) and soluble RNA (15, 16). Hybrid formation has indicated the portion of the bacterial genome complementary to soluble (15, 16) and ribosomal (18) RNA. The hybridization reaction has been used to show that with most systems RNA synthesized *in vitro* is complementary to both strands of DNA whereas RNA synthesized *in vivo* appears to be complementary to only one of the DNA strands (27-30). Hybrid formation has shown that when intact phage DNA is transcribed *in vitro*, only one of

the two strands is transcribed but both strands are transcribed if the DNA was previously sheared (31). RNA-DNA hybrid formation has shown that pulse labeled RNA synthesized in ØX 174 infected cells is not able to hybridize with vegetative ØX 174 DNA. The RNA is able to hybridize with denatured, replicative ØX 174 DNA indicating that the RNA is complementary to the replicating strand of the replicating form of ØX 174 DNA and not to the vegetative DNA.

More recently, RNA-DNA hybrid formation has been used to map the location of genes directing the synthesis of ribosomal (32) and soluble (33) RNA in Bacillus subtilis. The nucleolus organizer region of the sex chromosome of Drosophila melanogaster has been shown to contain loci corresponding to the ribosomal RNA by using RNA-DNA hybrid formation (24, 25). The similarity of RNA has been compared by measuring the ability of the RNA to compete with a known RNA for hybridization sites on DNA (5, 10, 26, 34). Hall, et. al., (34) showed by the competition technique that messenger RNA for early phage enzymes was also transcribed during the late portion of infection. RNA-DNA hybrid formation has been used to show that there is specific messenger RNA made after induction of the galactose and lactose operons (34, 35). Imamoto, et. al., (37) used hybridization to show that, for the tryptophan synthetase operon, repression occurs at the level of transcription and derepression caused the synthesis of messenger RNA for the tryptophan operon. Bautz, et. al., (38) used hybridization and nitrocellulose columns to isolate messenger RNA for the rII region of T-4 phage.

The objectives of the work to be reported in this thesis were: 1. To study hybrid formation between E. coli nucleic acids. 2. To study the properties of the hybrid. 3. To use hybrid formation to isolate messenger RNA. 4. To characterize the messenger RNA isolated.

Previous studies of hybrid formation (7) had used phage nucleic acids and such a system is a great deal simpler than the bacterial system studied here. In order to obtain the most hybrid possible, the conditions required for hybridization were studied. A careful study of the properties of the hybrid may indicate new uses and/or means of detecting and isolating the hybrid. The central role of messenger RNA in information transfer makes the study of messenger RNA desirable. However, messenger RNA comprises only about 1% of the cellular RNA and therefore messenger RNA must be separated from the other 99% of the RNA in the cell. Hybridization is able to accomplish such a separation since 99% of the DNA genome codes for messenger RNA synthesis and 1% codes for the synthesis of soluble and ribosomal RNA (the latter two comprise 99% of cellular RNA). Thus, most of the RNA which hybridizes with denatured DNA will be messenger RNA. Isolation of the RNA-DNA hybrid from unhybridized RNA by nitrocellulose chromatography will give a mixture of denatured DNA and RNA-DNA hybrid. Denaturation of the hybrid and passage over a second nitrocellulose column will allow the isolation of messenger RNA. Such isolated messenger RNA can be characterized by a number of techniques, some of which will be described in this thesis.

PART II. STUDIES OF METHYLATION OF RNA IN T-4 INFECTED CELLS

The presence of methylated bases has been reported in Escherichia coli DNA (39), soluble (40) and ribosomal RNA (41). The existence of methylated bases in messenger RNA has not been described. However, none have been reported in viral RNA (42, 43) which serves as messenger RNA for viral specific proteins.

Methylation of nucleic acids occurs at the polynucleotide level (44). Methylases which catalyze the transfer of methyl groups from S-adenosylmethionine to DNA (45), soluble (46) and ribosomal (47) RNA have been described. Although the mechanism of biosynthesis is understood, the biological function of the methylated bases is still obscure.

Little is known of the methylation of nucleic acids in T-even infected cells. Several reports deal with in vitro methylation. Gold, et. al., (48) reported that extracts from T-2 infected cells are 100-fold more active in methylating methyl deficient E. coli DNA than extracts prepared from uninfected cells. These authors were unable to detect any increase in RNA methylation activity of extracts following infection. However, Wainfan, et. al., (49) did observe altered RNA methylase activity (using methyl deficient RNA as substrate and measuring the ratios of methylated bases formed) in extracts prepared from T-2 infected cells. The in vivo methylation of RNA in infected cells has not been reported previously. Part II of this thesis deals with such methylation in T-4 infected cells.

MATERIALS AND METHODS

PART I. STUDIES OF *E. coli* RNA-DNA HYBRID.

Growth of Bacteria. *E. coli* BB was grown at 32° on a gyrorotary shaker or in a fermentor in C medium (50) with 0.4% glucose (doubling time = 60 minutes). In one experiment, RNA extracted from cells growing exponentially in the above medium was compared to RNA extracted from cells: (a) growing exponentially in C medium with 1.0% vitamin free casamino acids and 0.5% yeast extract, (b) starved of an energy source by limiting the glucose to 0.04% and incubating 150 minutes after growth stopped, (c) treated with 56 μg/ml of chloramphenicol (Parke, Davis and Company) for 90 minutes.

Preparation of DNA. DNA was purified by the phenol extraction method of Saito and Muira (51). After the isopropanol precipitation DNA was dissolved in 0.06 M KCl, 0.01 M Tris (pH 7.3) and dialyzed in the cold against the same buffer for 14-16 hours. For the experiments on isolation of messenger RNA, the ribonuclease digestion was omitted from the above purification of DNA because traces of ribonuclease were found in certain of the purified DNA preparations. In the preparations of DNA not treated with ribonuclease, DNA was denatured and separated from the RNA by nitrocellulose column chromatography. DNA was denatured by heating to 100° for 10 minutes in 0.06 M KCl, 0.01 M Tris (pH 7.3) and then rapidly cooling to 0° by plunging into an ice bath. Denatured DNA could be stored in the frozen state without loss of activity provided the solution was not more concentrated than

200 μg/ml. If solutions more concentrated than 200 μg/ml were frozen, it was necessary to denature the DNA after each freezing and thawing in order to maintain 100% activity in RNA-DNA hybrid formation.

Pulse labeling and purification of RNA. (^{3}H) RNA was labeled by feeding (^{3}H) uracil (Nuclear Research Chemicals, Inc., Orlando, Florida, 4700 mc/mM) at 0.2 μg/ml to cells growing exponentially in glucose-C medium. After 3 minutes the culture was quickly cooled to 0°, centrifuged, and frozen in a dry ice-acetone bath. The frozen cells were transferred to 0.01 M acetate (pH 5.0) containing 1-2% sodium dodecyl sulfate (SDS). After lysis the nucleic acids were purified by the phenol method (52). DNA was removed by deoxyribonuclease treatment or by heating to 100° for 10 minutes and then quickly cooling. The KCl concentration was raised to 0.5 M and the mixture was passed through nitrocellulose membrane filters (13) or a nitrocellulose column (10 x 1 cm). Denatured DNA is retained on the nitrocellulose but RNA is not held on the nitrocellulose. The purified RNA was dialyzed overnight in the cold (3°) against 0.50 M KCl, 0.01 M Tris (pH 7.3). The denatured DNA content of certain (^{3}H) RNA's was determined by incubating an aliquot under conditions known to favor RNA-DNA hybrid formation (0.50 M KCl 0.01 M Tris (pH 7.3), 78°, 240 minutes) and testing for the presence of an (^{3}H) RNA-DNA hybrid which would result from the presence of any denatured DNA in the sample. If any hybrid was formed, the RNA sample was passed through another nitrocellulose column and retested. One passage over a nitrocellulose column was usually sufficient to remove all denatured DNA. Using these procedures, (^{3}H) RNA's were prepared having

specific activities of 3740-7125 counts/min/μg. The majority of the pulse labeled RNA prepared by these methods sediments between 8 and 14S but there is a portion which sediments more rapidly than 14S. The presence of any denatured DNA in purified nonradioactive RNA samples was determined, after incubation of the unlabeled RNA with (^{3}H) RNA previously shown to contain no denatured DNA, by testing for (^{3}H) RNA-DNA hybrid.

The concentration of RNA and DNA was calculated from the absorbancy at 260 mμ, using an extinction coefficient of 20 cm^2/mg. For analysis of radioactivity, 5-10% trichloroacetic acid (TCA) insoluble material was collected on a nitrocellulose membrane filter. Commercial salmon sperm DNA was added as "carrier". The membrane filters were dried and counted in a liquid scintillation spectrometer.

RNA-DNA hybrid formation. RNA-DNA hybrid was synthesized by incubating RNA and DNA in 0.50 M KCl, 0.01 M Tris (pH 7.3) at 78° unless otherwise specified. To assay for hybrid formation, 0.1 ml samples were removed at various times and added to 0.5 ml of 0.06 M KCl, 0.01 M Tris (pH 7.3) containing 10 μg of ribonuclease. After incubation at 37° for 10 minutes, the sample was diluted with 15 ml of 0.50 M KCl, 0.01 M Tris (pH 7.3). The presence of ribonuclease resistant (^{3}H) RNA hybridized with DNA was determined by the nitrocellulose membrane filtration technique of Nygaard and Hall (13). The ribonuclease digestion lowered the amount of unreacted RNA trapped on the filters to less than 0.1% of the total and gave less scatter in the data. For the later experiments the salt concentration during the ribonuclease

treatment was raised to 0.50 M with only a slight increase in background and a doubling of the amount of hybrid detected.

Nitrocellulose Chromatography. Nitrocellulose (type RS), a gift from Hercules Powder Co., Wilmington, Del., was thoroughly washed with water, homogenized in a Waring blender, and incubated overnight in 0.06 M KCl, 0.01 M Tris (pH 7.3) at 65°. Used nitrocellulose was washed with water and then 0.06 M KCl, 0.01 M Tris (pH 7.3), and incubated overnight at 65°. After this treatment, it was indistinguishable from unused material. The material was slurried, poured into a column, and packed to about one third the unpacked volume by tamping with a glass rod. The packed column was washed with several cycles of water followed by 0.50 M KCl, 0.01 M Tris (pH 7.3). Finally, the column was washed with several column volumes of the salt solution. The washing of the column is necessary to remove fragments of nitrocellulose from the column. This process can readily be followed by observing the absorbancy of the eluant at 230 mμ. The sample was applied to the washed column in 0.50 M KCl, 0.01 M Tris (pH 7.3). However, lower or higher salt concentrations may be used. Denatured DNA or RNA-DNA hybrid was eluted from the column by 0.01 M Tris (pH 7.3) or by water. Two or three column volumes were usually sufficient to remove most of the adsorbed material.

Methylated albumin-kieselguhr chromatography. Methylated albumin-kieselguhr (MAK) column chromatography was similar to that used by Sueoka and Cheng (53) except that both linear gradients and stepwise elution procedures were used. It was found that bits of methylated albumin eluted from the

column at higher salt concentrations and interferred with hybrid formation. Washing the column with about 5 column volumes of high salt (1.3 M NaCl in 0.05 M phosphate (pH 6.8)) was usually sufficient to remove all fragments of methylated albumin. Before applying the sample the column was washed with 5 volumes of 0.3 M NaCl, 0.05 M phosphate (pH 6.8).

Sucrose gradient centrifugation. A 0.2 ml sample of RNA was layered on 4.8 ml of 20%-5% sucrose dissolved in 0.1 M NaCl, 0.01 M Tris (pH 7.3). This sucrose gradient was centrifuged in the cold (3°) for $5\frac{1}{2}$ hours at 39,000 rpm. Three drop fractions were collected from the bottom of the tube. If optical densities were desired, 0.5 ml of water was added to each tube and the absorbancy at 260 mμ was determined. Radioactivity was determined by collecting the TCA precipitable material on a membrane filter and counting in a scintillation counter.

PART II. STUDIES OF METHYLATION OF RNA IN T-4 INFECTED CELLS.

The following description of materials and methods applies to the experiments involved in the studies of methylation of RNA. Except for the changes listed below procedures were the same as those given above.

Growth of Bacteria and Labeling. *E. coli* K12 W6, kindly supplied by Dr. E. Borek, was grown at 32° on a gyrorotary shaker in C medium (50) supplemented with 12.5 μg/l of biotin, 30 mg/l of L-methionine, and 0.4% glucose. The doubling time was about 90 minutes. When the cell density of a exponential culture reached 5×10^8 cells/ml, 20 mg/l of L-tryptophan and

wild type T-4 phage, at a multiplicity of 5-10, were added. Approximately 10 minutes after infection the cells were collected by centrifugation. The pellet of infected cells was resuspended in 0.4 volumes of C medium and repelleted. This pellet was suspended in 1 volume of C medium containing 12.5 μg/l of biotin and 0.4% glucose. The cells were "starved" for methionine for 4-5 minutes. The culture was split into 2 parts and to the one part 1 μg/ml of (^{3}H methyl) L-methionine (Nuclear Chicago, 150 mc/mM) was added. To the other part of the culture 1 μg/ml of unlabeled L-methionine and 4 μc/ml of ^{3}H uracil (Nuclear Research Chemicals, Orlando, Florida, 4700 mc/mM) was added. Labeling was allowed to proceed for 7 minutes (27-34 minutes after infection) and then the culture was rapidly cooled on ice and centrifuged in the cold. The pellets were quickly frozen by dipping into a bath at -25°. Control uninfected cultures were starved of methionine and labeled in a similar manner. In one experiment chloramphenicol (10 μg/ml) was added with the (^{3}H methyl) methionine. In this case, labeling was allowed to proceed for 60 minutes.

Purification of RNA: The frozen cells were lysed by stirring with 1% SDS in 0.01 M acetate buffer (pH 5.0). An equal volume of phenol was added and the mixture stirred 10 minutes. The aqueous layer was removed and then the phenol and interphase material were extracted with 1/2 volume of SDS. The aqueous layers were combined and precipitated with 2 volumes of cold ethanol. After 1 hour in ice the precipitate was collected and dissolved in 0.1 M Trizma (pH 8.0) 0.01 M $MgCl_2$, 0.1 mM EDTA. 25 μg/ml of pancreatic

deoxyribonuclease (Sigma) was added and the solution was incubated at 37° for 10 minutes. Two more phenol purifications were performed. The aqueous phase was dialyzed extensively against 0.50 M KCl, 0.01 M Tris (pH 7.3) to remove traces of phenol. The specific activity of RNA obtained from ^{3}H methionine fed cells was 15-50 counts/min/μg. The specific activity of uracil labeled RNA was 1000 counts/min/μg.

Hybridization. The assay for RNA-DNA hybrid using DNA fixed to nitrocellulose filters was according to the method of Gillespie and Spiegelman (14). The DNA was fixed to the filters by heating in an oven at 90° for 30 minutes. Hybrid formation was carried out at 79° for 2 hours in 0.50 M KCl, 0.01 M Tris (pH 7.3). After hybrid formation the filter was washed on both sides with 15 ml of 0.50 M KCl, 0.01 M Tris (pH 7.3) and then incubated at 37° for 10 minutes in 3.3 ml of 0.50 M KCl, 0.01 M Tris (pH 7.3) containing 10 μg/ml of pancreatic ribonuclease. Finally, each side of the filter was washed with 15 ml of 0.50 M KCl, 0.01 M Tris (pH 7.3), dried, and counted in a scintillation counter.

Paper electrophoresis. To test for the presence of methylated bases in (^{3}H) methionine labeled RNA, the RNA was hydrolyzed by incubating 18 hours at 37° in 0.3 M KOH. The solution was neutralized by adding $HClO_4$ and then concentrated to a small volume by lyophilization. The tube was cooled in ice and centrifuged to remove $KClO_4$ and then a portion of the clear supernatant was spotted on Whatman 3 MM paper. After the paper was wetted with 0.05 M ammonium formate (pH 2.8), electrophoresis was conducted for 2 hours at 2500 volts in a Gilson model D using Varsol as a coolant.

The major nucleotides were located by ultraviolet light and marked. Squares 1.9 x 1.9 cm were cut from the paper and placed in scintillation bottles and counted. The pattern of radioactivity obtained was compared with the published electrophoretic mobilities for the methylated bases (49).

RESULTS AND DISCUSSION

PART I. STUDIES OF *E. coli* RNA-DNA HYBRID.

Kinetics of (^{3}H) RNA-DNA hybrid formation. Figure 1 illustrates the time course for the reaction of pulse labeled (^{3}H) RNA and denatured DNA to form ribonuclease stable RNA-DNA hybrid. No reaction is observed if denatured DNA is omitted or if native DNA is substituted for denatured DNA (Fig. 1). Incubation of denatured DNA from either *Bacillus subtilis* or *Pseudomonas fluorescens* with pulse labeled (^{3}H) RNA from *E. coli* results in no measurable hybrid formation.

Effect of RNA and DNA concentration on hybrid formation. The formation of RNA-DNA hybrid is dependent upon the concentration of both denatured DNA and RNA (Fig. 2). Figure 2a illustrates that the amount of hybrid formed increases linearly with increasing RNA concentrations for both DNA concentrations tested (5 and 50 µg/ml.) Figure 2b shows that the amount of hybrid formed depends on the amount of added denatured DNA. The lack of linearity between hybrid synthesized and denatured DNA added at the higher concentrations is presumably due to the removal of denatured DNA from the reaction by DNA-DNA interactions which result in the formation of material not reactive with (^{3}H) RNA. For example, denatured DNA preincubated at 60 µg/ml for 60 minutes at 65° then assayed at 48 µg/ml, shows a 40% decrease in the initial rate of reaction with (^{3}H) RNA. If the DNA was preincubated as above but assayed at 5 µg/ml there was no loss in reactivity with

Figure 1. Kinetics of hybrid formation. Each reaction mixture contained 48 μg/ml of (^{3}H) RNA (3740 counts/min/μg). DNA, if present, was present in a concentration of 50 μg/ml. The upper line, ● —— ●, indicates hybrid formed when denatured DNA is used. The lower line, O —— O and △ ——△, indicates the lack of hybrid formation when no DNA is added or if native DNA is added.

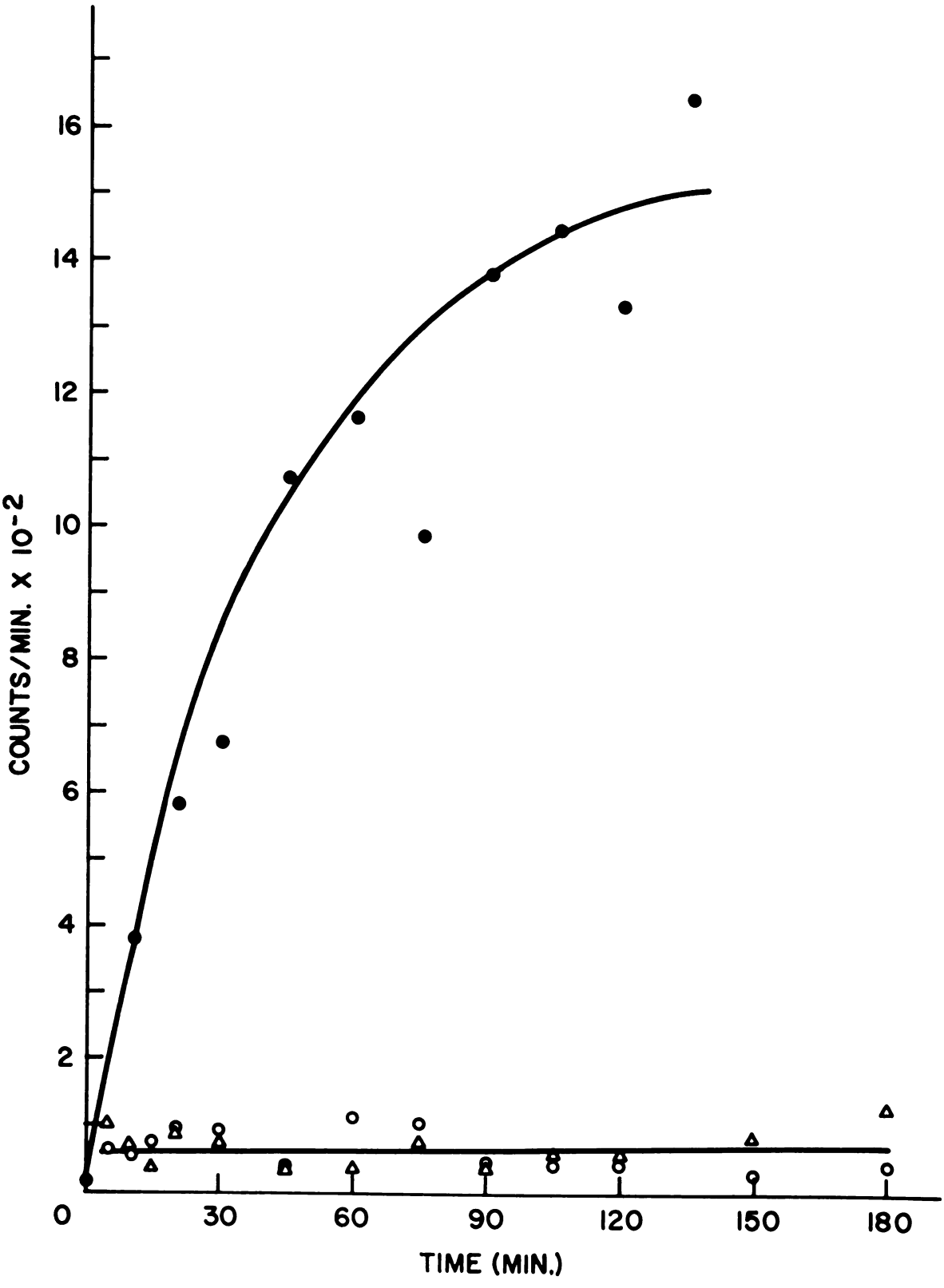

Figure 1

Figure 2. The effect of nucleic acid concentration on hybrid formation.

Figure 2a. The effect of RNA concentration. The concentration of denatured DNA in reaction mixtures was 50, O —— O, and 5, ● —— ●, μg/ml. Each point represents the average of 3 samples assayed after 210 min incubation.

Figure 2b. The effect of DNA concentration. All reaction mixtures contained 48 μg/ml of (^{3}H) RNA (3740 counts/min/μg). Each point is the average of 3 samples assayed after 210 min incubation.

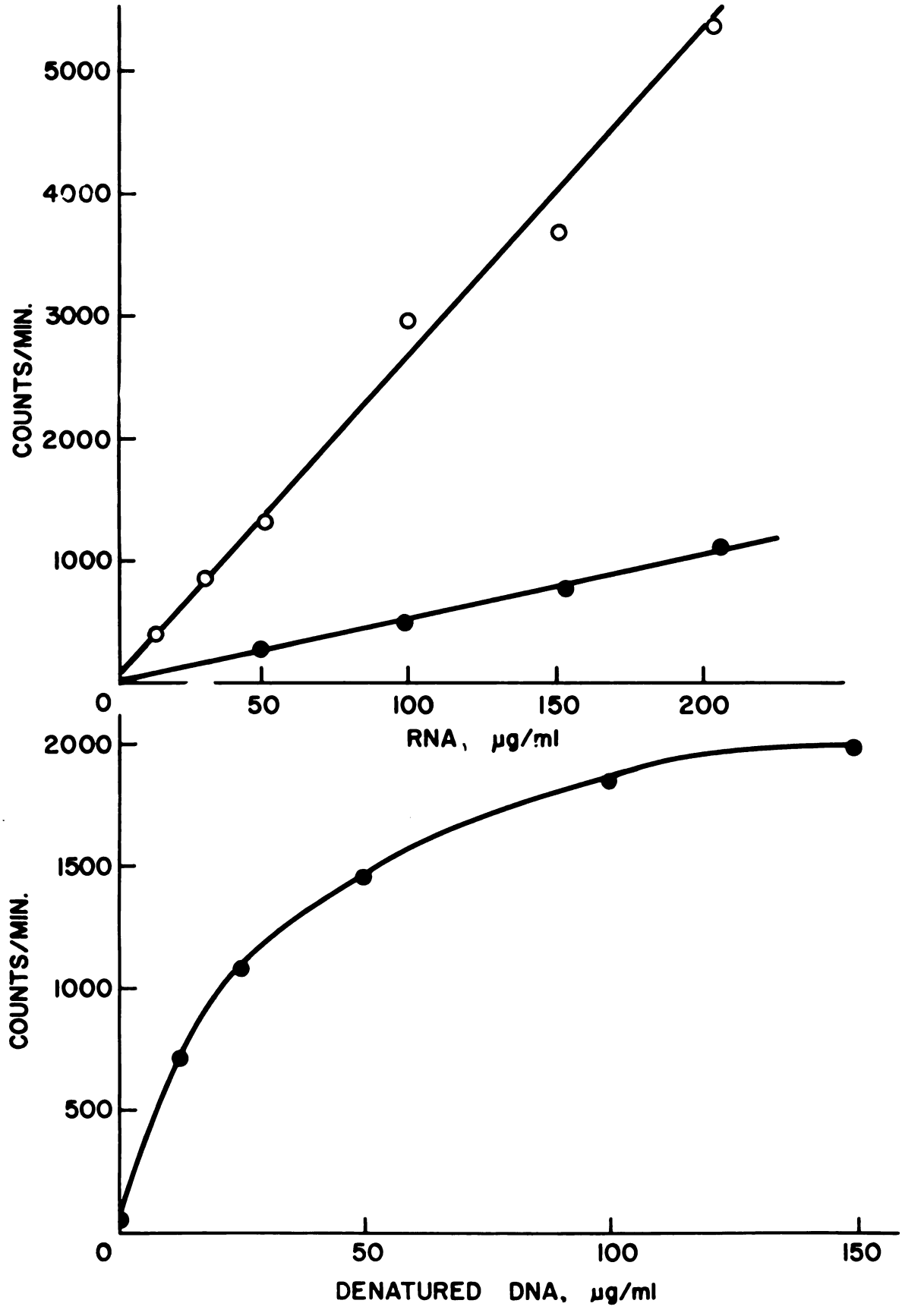
5000
4000
3000
2000
1000
0
COUNTS/MIN.
50
100
150
200
RNA, µg/ml
2000
1500
1000
500
0
COUNTS/MIN.
50
100
150
DENATURED DNA, µg/ml

Figure 2

(^{3}H) RNA. The loss in reactivity observed thus appears to be due to some type of aggregation and not renaturation because renatured DNA would be stable to dilution. The lack of linearity between denatured DNA added and RNA-DNA hybrid formed could also be due to self absorption of the radioactivity by increasing amounts of denatured DNA on each filter. It was shown, however, that no correction for self absorption is necessary for the DNA concentrations used in this experiment.

Effect of temperature on (^{3}H) RNA-DNA hybrid formation. Figure 3 gives the kinetics of hybrid formation at 3 different temperatures. Reaction mixtures for experiments shown in Figure 3a contained 48 μg/ml of denatured DNA and 40 μg/ml (^{3}H) RNA (7125 counts/min/μg). Reaction mixtures for experiments shown in Figure 3b contained 5 μg/ml of denatured DNA and 212 μg/ml of (^{3}H) RNA (4100 counts/min/μg). In both cases, 78° is the optimum temperature. At 65° hybrid formation ceases after 90 minutes in the reaction mixture containing 48 μg/ml denatured DNA and 40 μg/ml (^{3}H) RNA. Under these conditions, DNA-DNA interaction rather than RNA-DNA reaction is favored. At higher temperatures or lower concentrations of denatured DNA these DNA interactions don't remove DNA from reaction with (^{3}H) RNA and hybrid formation occurs for longer times.

Thermal denaturation of (^{3}H) RNA-DNA hybrid. (^{3}H) RNA-DNA hybrid, isolated free of unreacted RNA by nitrocellulose column chromatography, was heated at various temperatures in 0.50 M KCl, 0.01 M Tris (pH 7.3) for 10 minutes. After heating the samples were assayed for ribonuclease resistant (^{3}H)

Figure 3. The effect of temperature on hybrid formation.

Figure 3a. Effect of temperature on hybrid formation at high DNA concentration. The concentration of denatured DNA and (^{3}H) RNA (7125 counts/min/μg) was 48 and 40 μg/ml, respectively. The upper line, ● —— ●, represents 78°; the middle one, △ ——△, 87°; and the bottom one, 0 —— 0, 65° incubation.

Figure 3b. Effect of temperature on complex formation at low DNA concentration. The concentration of denatured DNA and (^{3}H) RNA (4100 counts/min/μg) was 5 and 212 μg/ml, respectively. The upper line, ● —— ●, represents 78°; the middle one, 0 —— 0, 65°; and the lower one, △——△, 87° incubation.

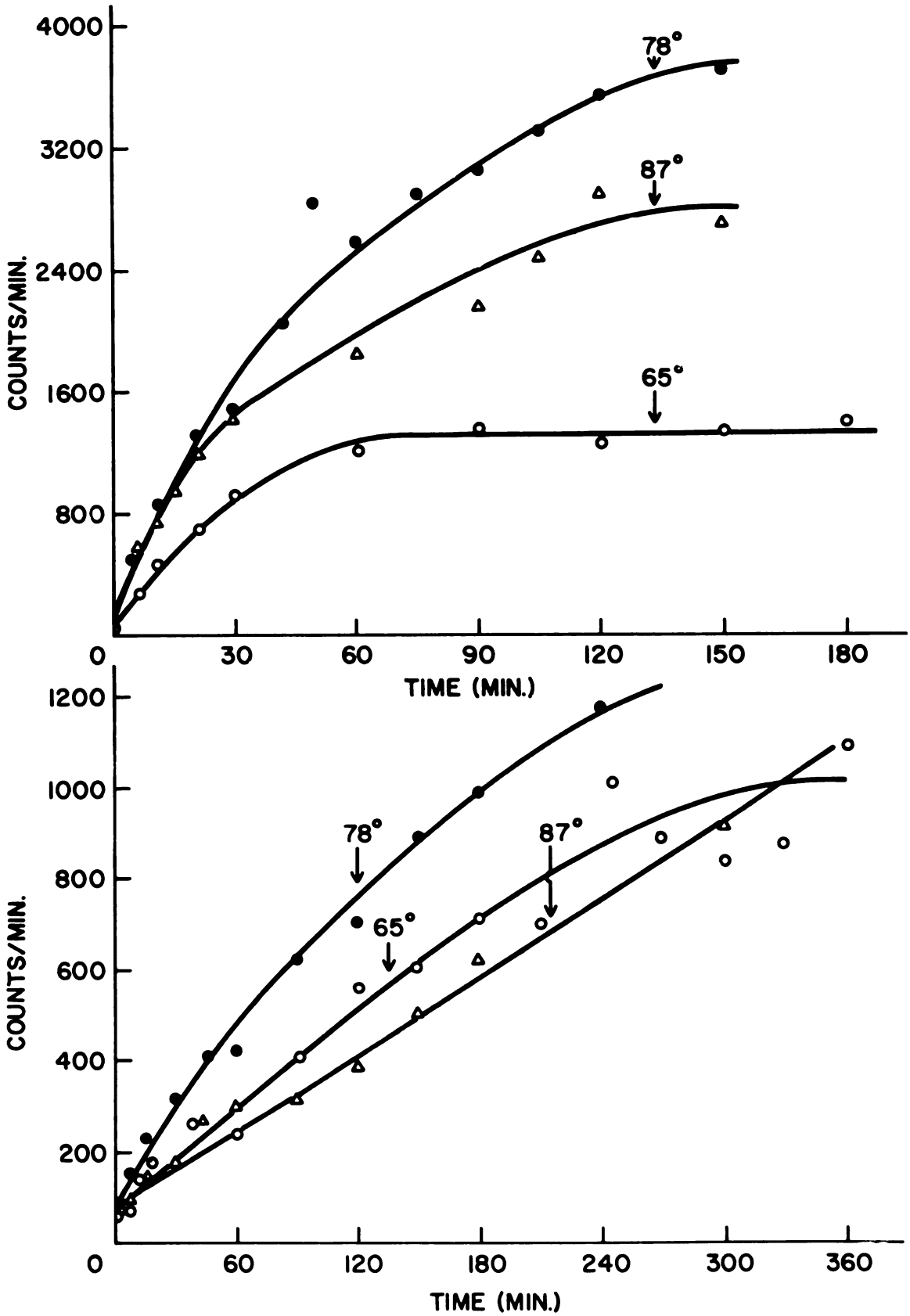
4000
3200
2400
1600
800
COUNTS/MIN.
78°
87°
65°
0
30
60
90
120
150
180
TIME (MIN.)
1200
1000
800
600
400
200
COUNTS/MIN.
78°
87°
65°
0
60
120
180
240
300
360
TIME (MIN.)

Figure 3

RNA-DNA hybrid. The results are shown in Figure 4a. The observed t_m is 94-95°. The t_m is defined as the temperature at which one half of the complex remains after treatment at that temperature for 10 minutes.

Thermal denaturation of DNA was determined by measuring the reactivity of the resulting denatured DNA with (^{3}H) RNA. Samples of DNA at a concentration of 60 μg/ml in 0.50 M KCl, 0.01 M Tris (pH 7.3) were heated for 10 minutes at various temperatures. (^{3}H) RNA was added and the reaction mixture was incubated at 78° for 150 minutes then assayed for ribonuclease resistant hybrid. The results are plotted on Figure 4a. The t_m for DNA denaturation is 99°. In this case, the t_m refers to the temperature at which the DNA is 50% denatured and thus one half as reactive in hybridization as fully denatured DNA. The DNA duplex is more stable than the RNA-DNA hybrid as shown by the difference in the t_m's of 4-5°. This greater stability of the DNA duplex relative to the RNA-DNA hybrid was also observed by Chamberlin and Berg (54). They noted the ØX 174 RNA-DNA hybrid melts 4-5° below the double strand derivative of ØX 174 DNA. Also, Bolton and McCarthy (55) observed that RNA elutes from a DNA agar column at a temperature 4° below that necessary to elute the corresponding DNA.

Effect of salt concentration on the stability of (^{3}H) RNA-DNA hybrid in the presence of ribonuclease.* In the presence of sufficient ionic strength, RNA in the RNA-DNA

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*This experiment was first performed by James C. Johnson.

Figure 4. Action of heat or low salt on hybrid.

Figure 4a. Thermal denaturation of (^{3}H) RNA-DNA hybrid. The hybrid was formed by incubating (^{3}H) RNA (3740 counts/min/μg) and denatured DNA at 212 and 5 μg/ml, respectively, for 4 hours. ● —— ●, ribonuclease stable hybrid remaining after 10 min incubation. O —— O, ribonuclease resistant (^{3}H) RNA-DNA hybrid formed after 150 min incubation using native DNA treated for 10 min at each temperature before incubating with (^{3}H) RNA at 78°.

Figure 4b. Sensitivity of hybrid to low salt concentration in the presence of ribonuclease. (^{3}H) RNA-DNA hybrid was treated with 16.7 μg/ml of ribonuclease for 10 min at 37° in a series of different salt concentrations. Samples were diluted with 15 ml of 0.50 M KCl, 0.01 M Tris (pH 7.3) and filtered through nitrocellulose membrane filters.

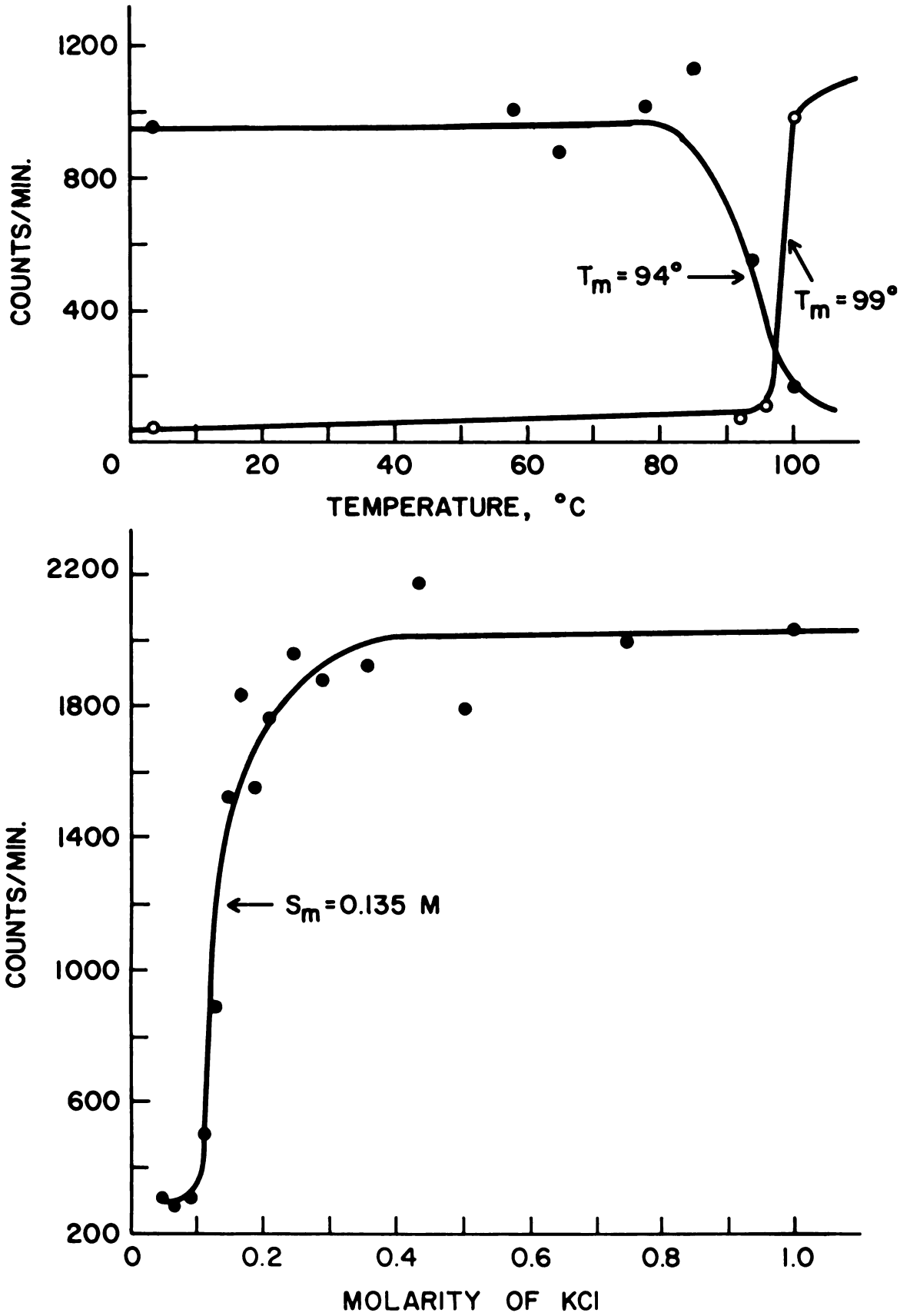
1200
800
400
0
COUNTS/MIN.
T_m = 94°
T_m = 99°
20
40
60
80
100
TEMPERATURE, °C
2200
1800
1400
1000
600
200
COUNTS/MIN.
S_m = 0.135 M
0
0.2
0.4
0.6
0.8
1.0
MOLARITY OF KCl

Figure 4

hybrid is not degraded by pancreatic ribonuclease. If, however, the salt concentration is too low, the RNA in the hybrid is degraded by ribonuclease. Figure 4b shows the transition of the RNA in the hybrid from ribonuclease sensitive at low salt concentrations to ribonuclease stable material at higher salt concentrations. The S_m is defined as the salt concentration at which one half of the RNA in the RNA-DNA hybrid is stable to ribonuclease treatment at 37°.

(^{3}H) RNA-DNA hybrid was formed by incubating 40 µg/ml of (^{3}H) RNA and 50 µg/ml of denatured DNA in 0.50 M KCl, 0.01 M Tris (pH 7.3) for 4 hours. The hybrid was isolated from unreacted RNA by nitrocellulose column chromatography and treated for 10 minutes at 37° with 16.7 µg/ml of pancreatic ribonuclease at several different salt concentrations. Samples were diluted with 15 ml of 0.50 M KCl, 0.01 M Tris (pH 7.3) and filtered through a nitrocellulose membrane. The S_m obtained was 0.135 M KCl. All experiments previously described were conducted with salt concentrations during the ribonuclease treatment of 0.135 M and therefore only one half of the hybrid formed was actually detected.

Comparison of RNA's from E. coli cultured under different conditions. RNA's extracted from cells cultured under different conditions may be compared by measuring the degree to which they compete for reaction with denatured DNA (34). One of the RNA's used for comparison is labeled. The other is used without label. If the two RNA's are identical, the addition of increasing concentrations of unlabeled RNA to a reaction mixture containing labeled RNA and denatured DNA will result in a decrease in the amount of label found in the hybrid. With

a large excess of unlabeled RNA, no labeled hybrid will be formed. If the two RNA's are totally different, no competition between the two will occur. Consequently, equal amounts of labeled hybrid will be synthesized in the absence and in the presence of a large excess of unlabeled RNA.

Using this technique, unlabeled RNA's extracted from cells, (a) grown on glucose-C medium, (b) grown on C medium plus casamino acids and yeast extract, (c) treated with chloramphenicol, (d) starved of an energy source, were used to compete in hybrid formation with pulse labeled (^{3}H) RNA extracted from cells grown on glucose-C medium. The results are shown in Figure 5. The lower line shows the competition between identical RNA's, i.e., unlabeled RNA extracted from cells grown on glucose-C medium and pulse labeled (^{3}H) RNA from cells grown on the same medium. The data for the RNA's from cells grown on enriched medium and from those treated with chloramphenicol fit the same line. Therefore, within the sensitivity of the technique, these cells contain RNA homologous to pulse labeled (^{3}H) RNA obtained from cells grown on glucose-C medium.

The upper line describes the competition between the pulse labeled (^{3}H) RNA and the RNA extracted from glucose starved cells. Little or no competition is observed. Thus, glucose starved cells contain little or no RNA homologous to pulse labeled RNA from cells grown on a glucose-C medium.

Cells grown on C medium supplemented with casamino acids and yeast extract and those treated with chloramphenicol contain RNA homologous to and in the same proportion as the RNA which is pulse labeled in cells growing on glucose-C

Figure 5. Comparison of RNA's from E. coli grown under different conditions. Each reaction mixture contained 5 μg/ml of denatured DNA and 212 μg/ml of (^{3}H) RNA. The ordinate shows the amount of ribonuclease resistant (^{3}H) hybrid formed after 210 min incubation (average of duplicate samples). ● —— ●, competing RNA from cells grown on glucose-C medium; △—— △ from enriched medium; ▲—— ▲, chloramphenicol treated; O —— O, glucose starved cells.

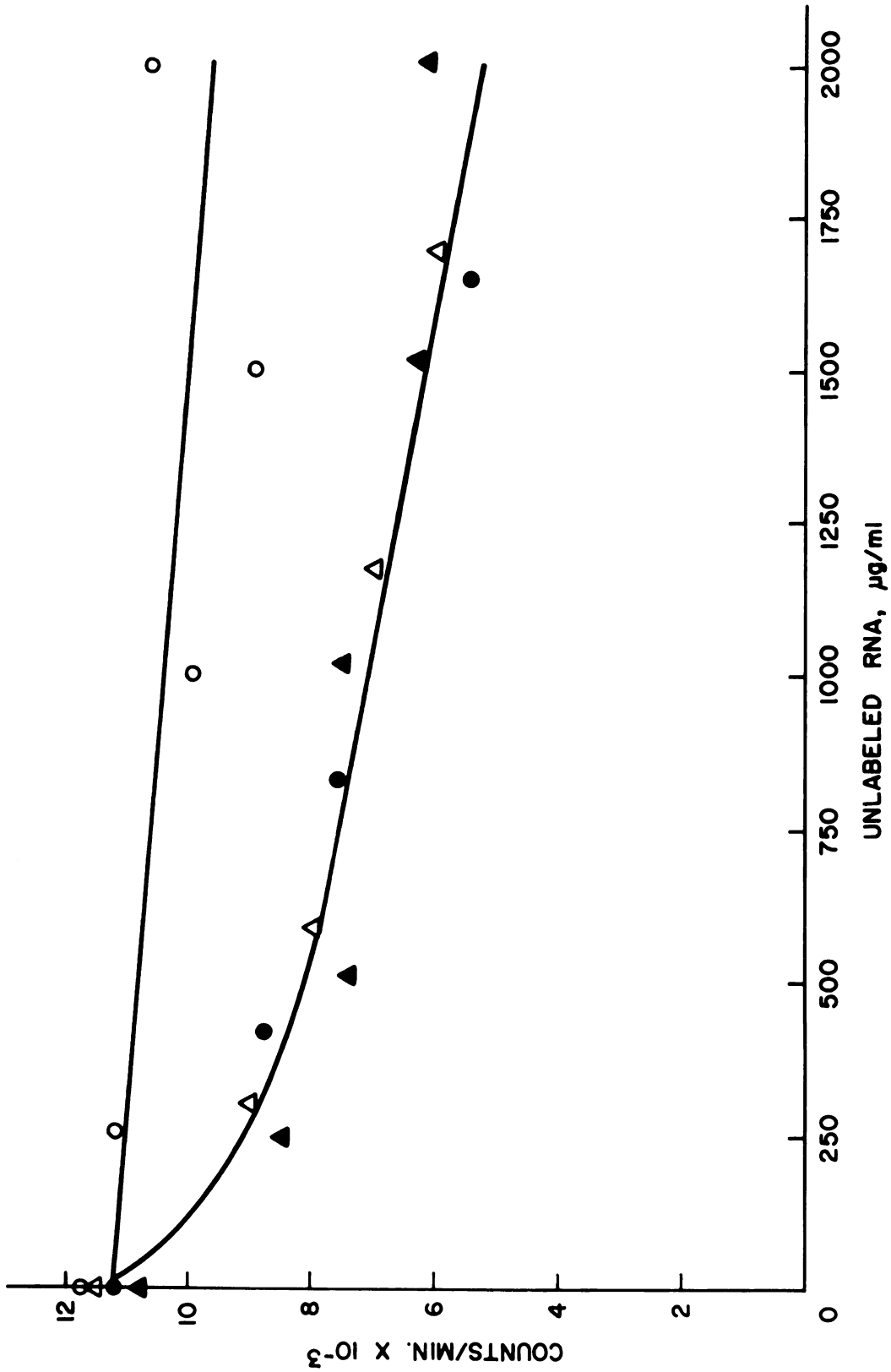
COUNTS/MIN. X 10⁻³
12
10
8
6
4
2
0
250
500
750
1000
1250
1500
1750
2000
UNLABELED RNA, µg/ml

Figure 5

medium. If cellular regulation occurs at transcription (DNA $\longrightarrow$ RNA), it may be expected that cells grown on enriched medium, where the synthesis of many enzyme systems is repressed, would not contain all of the messenger RNA molecules found in cells grown on glucose-C medium where these enzymes would be present. However, the messenger RNA's which direct the synthesis of these repressed enzymes probably constitute a small proportion of the total messenger RNA present in the cell. The competition technique might not detect such small differences. Alternatively, cellular regulation may occur at translation (RNA $\longrightarrow$ protein).

Following treatment of a culture with chloramphenicol for 90 minutes, a condition stopping growth by inhibiting protein synthesis, the messenger RNA content of the cells remains the same as before treatment. However, if growth is stopped by depletion of the energy source, the messenger RNA content of the cells is greatly decreased. This is understandable in view of the fact that chloramphenicol blocks protein synthesis but allows RNA synthesis (56). However, depletion of the energy source would result in the end of synthesis of all macromolecules followed by degradation of the unstable messenger RNA.

The lack of messenger RNA in glucose starved cells may explain the unusual sedimentation pattern observed for ribosomes extracted from such cells. McCarthy (57) observed that these ribosomes, when examined at 10^{-2} M Mg^{++}, sediment as 100-S particles. At this Mg^{++} concentration, however, ribosomes from exponentially growing cells sediment at 70 S and 85 S. These ribosomes would be expected to contain fragments

of messenger RNA as a result of degradation of polysomes during isolation. The attached fragments of messenger RNA may hinder aggregation of the particles in 10^{-2} M Mg^{++}. Ribosomes extracted from glucose starved cells, a condition which depletes their messenger RNA content, should not have these fragments attached. Accordingly, they aggregate to 100-S particles at 10^{-2} M Mg^{++}.

Behavior of DNA and RNA-DNA hybrid on nitrocellulose columns. A study of native, denatured, and renatured DNA and of RNA-DNA hybrid on nitrocellulose columns was conducted. In 0.50 M KCl, 0.01 M Tris (pH 7.3) denatured DNA and RNA-DNA hybrid is retained by the column but RNA, native, and fully renatured DNA are not retained.

A firmly packed column of nitrocellulose 1 cm in diameter by 10 cm in height was able to retain 500 μg of denatured DNA in the presence of 0.50 M KCl, 0.01 M Tris (pH 7.3). 80-100% of this DNA could be washed from the nitrocellulose column by 4-5 column volumes of water. Capacity was determined by pouring excess denatured DNA over the column and measuring the amount of denatured DNA which was retained on the column.

RNA-DNA hybrid is retained by the nitrocellulose column in 0.50 M KCl, 0.01 M Tris (pH 7.3) and is eluted from the column with 4-5 column volumes of water. The yield from the column is 90-100%. The first 1-2 column volumes will elute 70% of the material. Free RNA is not retained by the nitrocellulose column in either water or salt solution.

All of the native DNA from Ps. fluorescens and from Ps. fluorescens phage gh-1 (58) but only 85% of the E. coli DNA passes through a nitrocellulose column. The 15% of the

E. coli which is retained by the nitrocellulose column in salt can be washed from the column by water. The nature of this 15% of *E. coli* DNA which behaves abnormally on nitrocellulose is unknown but it is thought to contain denatured regions. *E. coli* DNA fractioned in the 2 phase system of Albertsson (59) as if it contained native and denatured DNA. The 2 phase system is able to separate native from denatured DNA (59). The phage gh-1 DNA fractionated as expected for native DNA. This evidence also indicates that the *E. coli* DNA as prepared by the method of Saito, *et. al.*, (51) may contain some denatured regions.

In order to study the behavior of "renatured" DNA on a nitrocellulose column, denatured DNA was "renatured" by heating at 78° for 4 hours in 0.50 M KCl, 0.01 M Tris (pH 7.3). When the "renatured" DNA was applied to a nitrocellulose column, 60-80% of the DNA was retained by the column. This probably represents denatured DNA and "renatured" DNA which still has large regions of DNA retaining the denatured character. It could be eluted from the nitrocellulose column by water. Rechromatography of the 20-40% of the "renatured" DNA which passed through the first column results in only a small portion being retained by the second column.

Double stranded nucleic acid molecules will exhibit an increase in absorbancy at 260 mμ after being heated to 100° and quickly cooled. The amount of hyperchromicity is proportional to the degree of double stranded structure. The portion of the "renatured" DNA which was retained by the nitrocellulose column was heated to 100° for 3 minutes and cooled by dipping in ice water. The increase in absorbancy at 260 mμ

was 8% after heating. The "renatured" DNA which passed through the nitrocellulose column gave a hyperchromicity of 15%. Native E. coli DNA heated and quickly cooled gave an increase in absorbancy of 22%. Therefore, the nitrocellulose column is able to distinguish the degree of secondary structure present in a DNA preparation and to separate highly structured molecules from less structured molecules.

Isolation and characterization of messenger RNA. In order to obtain enough isolated messenger RNA for characterization, 20 mg of RNA and 5 mg of denatured DNA were dissolved in 100 ml of 0.50 M KCl, 0.01 M Tris (pH 7.3) and incubated for 4 hours at 78°. Nitrocellulose columns (4.5 x 20 cm) were used to separate the resulting hybrid from the unreacted RNA. After eluting the RNA-DNA hybrid from the nitrocellulose column, the hybrid was denatured by heating at 100° for 3 minutes. The salt concentration was raised to 0.50 M KCl and a second nitrocellulose column was used to separate the denatured DNA from the messenger RNA. The messenger RNA was extensively dialyzed against distilled water in the cold and concentrated by lyophilization. Approximately 300 μg of messenger RNA was isolated by this technique. Isolated messenger RNA was then characterized by several methods which are described below.

Isolated messenger RNA was chromatographed on a MAK column. The messenger RNA was eluted from the MAK column by low salt concentrations. Experiments to be described below will show that material eluted from a MAK column by low salt concentrations is low molecular weight material. Therefore, isolated messenger RNA is low molecular weight material.

Figure 6 shows the comparison of the MAK column elution profile for pulse labeled RNA and purified messenger RNA. The isolated messenger RNA could be low molecular weight material either because of degradation during isolation or because the hybridization reaction is selective against high molecular weight RNA and selective for low molecular weight RNA. To test which of these two alternatives is correct, (^{3}H) pulse labeled RNA was fractionated on a MAK column. An aliquot of each fraction was assayed for TCA precipitable radioactivity. Denatured DNA was added to selected fractions and the solution was incubated at 78^o for 7 hours to allow hybridization to occur. The percentage of radioactivity found in the hybrid was determined by filtering on a nitrocellulose membrane filter and counting in a scintillation counter. The results are plotted in Figure 7. The 16 and 23 S ribosomal RNA probably peaks in tubes 55 and 60 (Fig. 7) and hybridization of radioactive RNA is inhibited by the competition with unlabeled ribosomal RNA. Those fractions which eluted from the MAK column with a low salt concentration hybridized to a lesser extent than did fractions eluted from the column by higher salt concentrations. Hence, the hybridization reaction is not selecting only the lower molecular weight RNA.

Sucrose gradient centrifugation of fractions 18 and 60 (Fig. 7) was performed to check the correlation between the salt concentration necessary to elute the RNA from the MAK column and the molecular weight. Figure 8 illustrates the result of this centrifugation. RNA which was eluted from the MAK column by low salt (fraction 18) is low molecular

Figure 6. Behavior of pulse labeled and messenger RNA on a MAK column.

Figure 6a. MAK chromatography of pulse labeled RNA. The open circles represent the NaCl concentrations as obtained from the refractive index of selected fractions. The smooth curve without points represents the radioactivity of the eluted material. Each fraction contained 5 ml.

Figure 6b. MAK chromatography of messenger RNA. The smooth curve indicates the radioactivity of the messenger RNA eluting from the column. The dashed line represents the behavior of (^{14}C) labeled ribosomal RNA used as a marker. The eluting gradient was similiar to that in Fig. 6a.

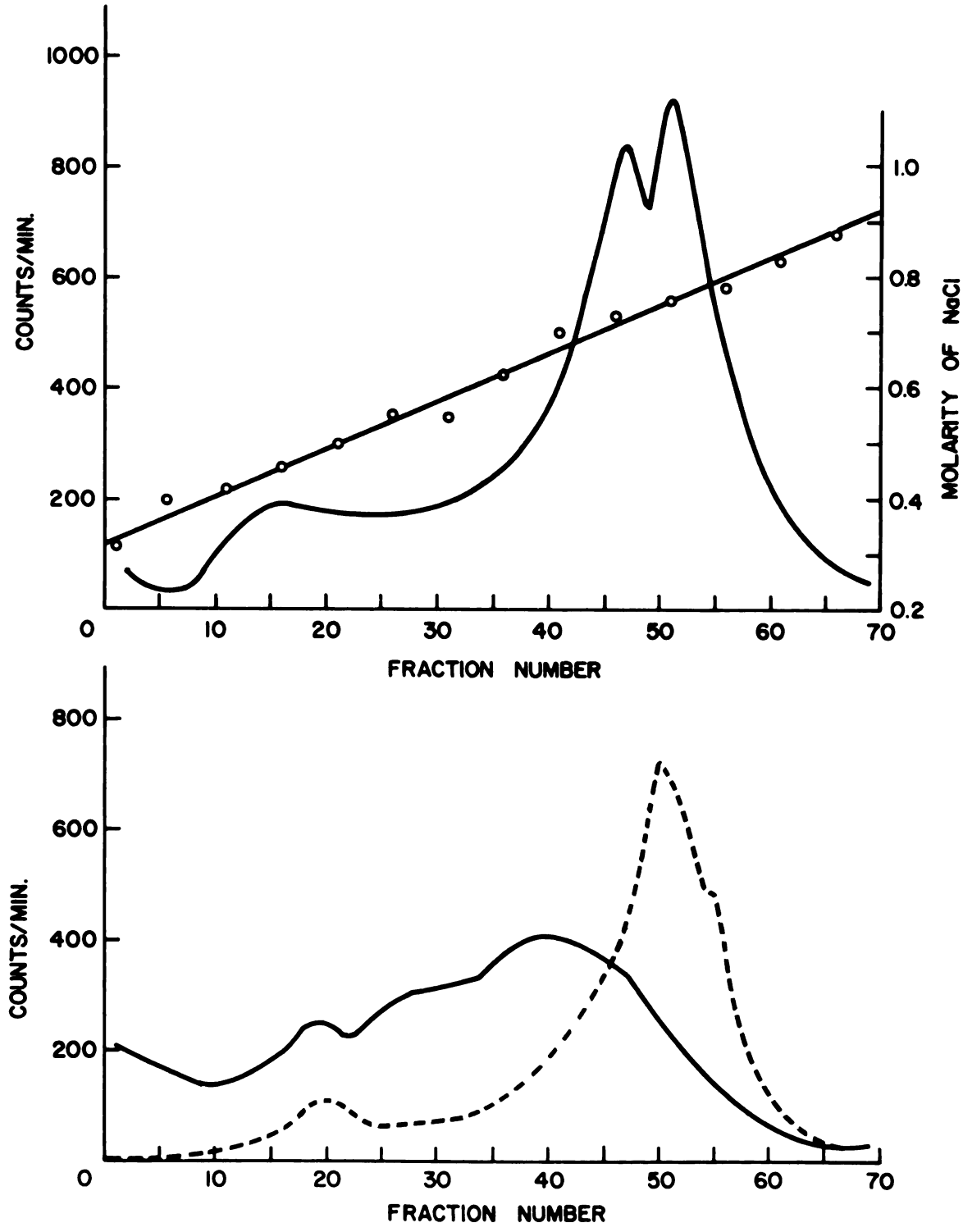
1000
800
600
400
200
0
COUNTS/MIN.
10
20
30
40
50
60
70
FRACTION NUMBER
1.0
0.8
0.6
0.4
0.2
MOLARITY OF NaCl
800
600
400
200
0
COUNTS/MIN.
10
20
30
40
50
60
70
FRACTION NUMBER

Figure 6

Figure 7. Fractionation of pulse labeled RNA on a MAK column. The solid line indicates the distribution of radioactivity which eluted from the MAK column. The dotted line, the part of the pulse labeled RNA hybridizing with denatured DNA. The dashed line indicates the percentage of radioactivity in selected fractions which is able to hybridize with denatured DNA. Each point represents the average of samples taken after 5 and 7 hours of incubation with denatured DNA at 78°. Elution was accomplished by a linear gradient of NaCl from 0.3 M to 1.0 M. Each sample contained 5 ml.

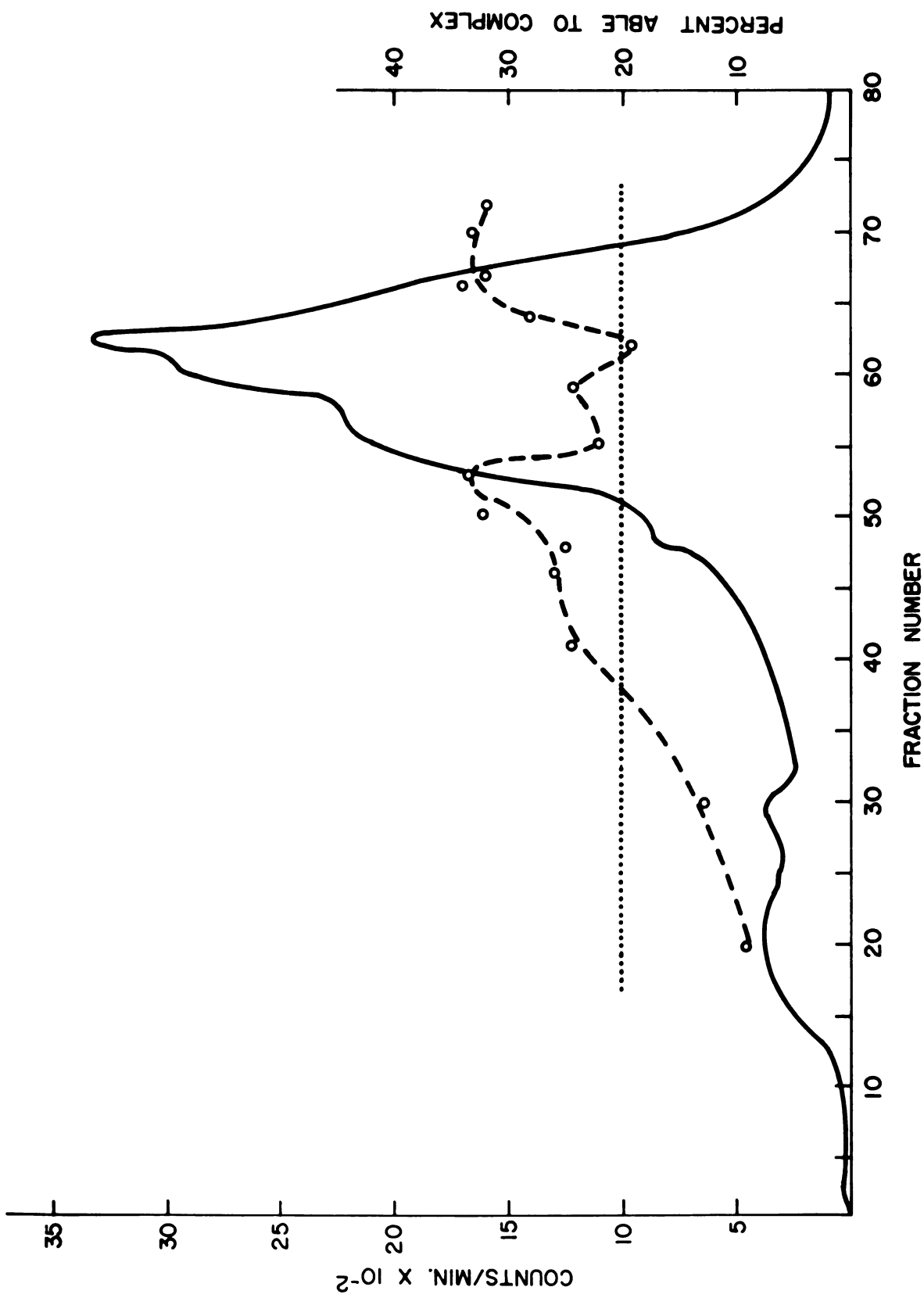
PERCENT ABLE TO COMPLEX
40
30
20
10
80
70
60
50
40
30
20
10
FRACTION NUMBER
COUNTS/MIN. X 10-2
35
30
25
20
15
10
5

Figure 7

Figure 8. Sucrose gradient centrifugation of 2 different fractions from a MAK column.

Figure 8a. Sucrose gradient centrifugation profile of fraction 60 in Fig. 7. Material was centrifuged at 39,000 rpm for 5½ hours. The markers were present as (^{14}C) labeled ribosomal RNA mixed with the sample before centrifugation.

Figure 8b. Sucrose gradient centrifugation profile of fraction 18 in Fig. 7. The sample was treated the same as Fig. 8a.

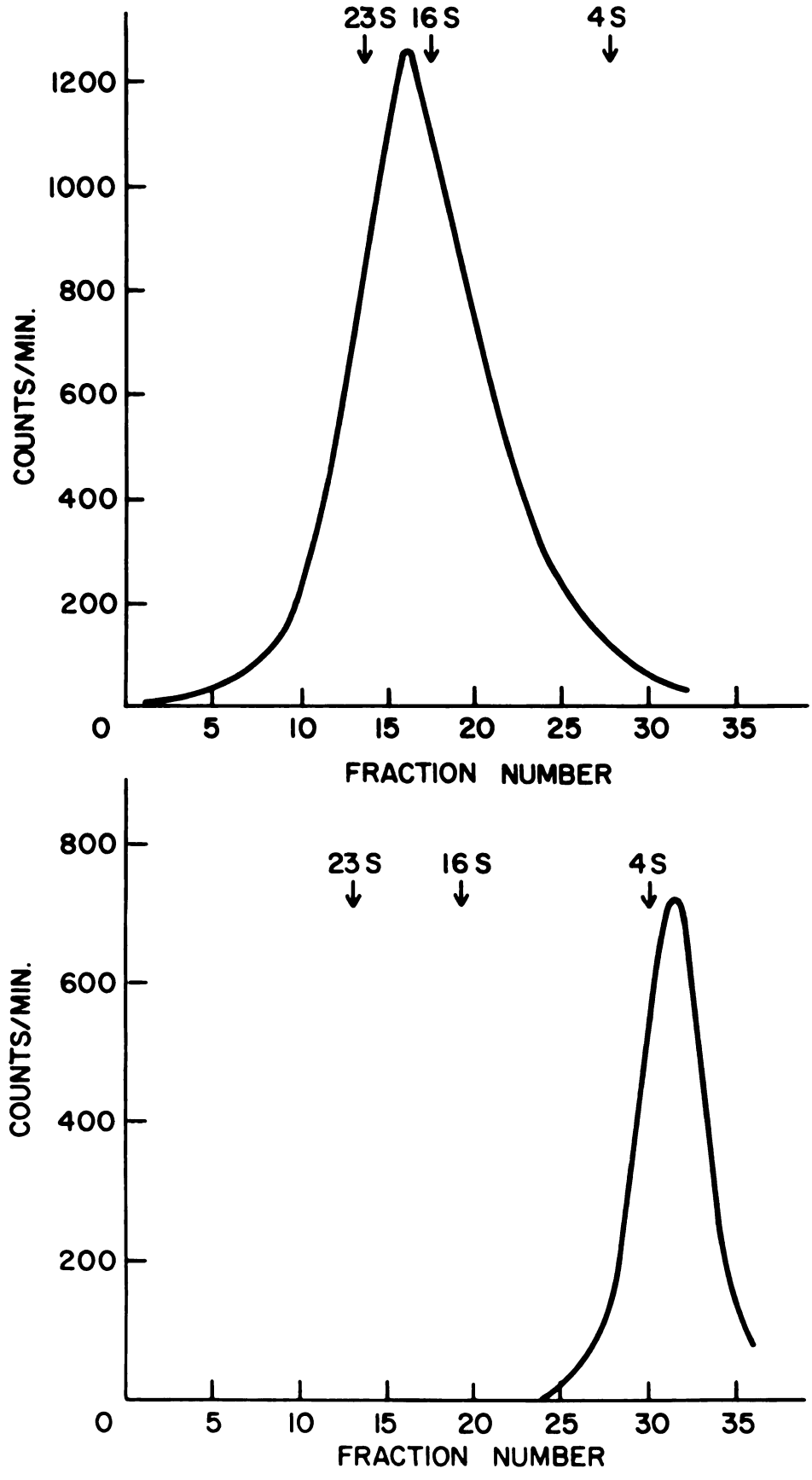
23S
16S
4S
1200
1000
800
600
400
200
0
COUNTS/MIN.
5
10
15
20
25
30
35
FRACTION NUMBER
23S
16S
4S
800
600
400
200
0
COUNTS/MIN.
5
10
15
20
25
30
35
FRACTION NUMBER

Figure 8

weight RNA while RNA requiring higher salt for elution (fraction 60) is of higher molecular weight. Thus the MAK column can be used to obtain an estimate of the molecular weight of RNA.

The profiles of isolated messenger RNA and of pulse labeled RNA after sucrose gradient centrifugation are given in Figure 9. The results again indicate that isolated messenger RNA is low molecular weight material. Most of the radioactivity in the pulse labeled RNA sediments faster than 8 S material while all of the messenger RNA sediments slower than 8 S material.

Sucrose gradient centrifugation and MAK chromatography indicated that the incubation at 78° was causing some degradation of the labeled RNA. MAK chromatography of RNA incubated at 65° showed little, if any, degradation of pulse labeled RNA. Therefore, hybridization at 65° was tried. The yield of RNA in the hybrid decreased by 20-30% without any increase in the molecular weight of the isolated messenger RNA. Pulse labeled RNA was not degraded during nitrocellulose chromatography. To check if degradation of RNA occurred during the denaturation of the hybrid, pulse labeled RNA dissolved in 0.01 M Tris (pH 7.3) was heated to 100° for 3 minutes and quickly cooled. After the heat treatment both MAK chromatography and sucrose gradient centrifugation indicated degradation of RNA. Therefore, another method of denaturing the RNA-DNA hybrid was sought. An attempt to denature the hybrid by lowering the pH of the solution was made. The RNA-DNA hybrid was placed in 0.05 M phosphate buffer (pH 6.8) and when one equivalent of HCl was added the

Figure 9. Sucrose gradient centrifugation of pulse labeled and messenger RNA.

Figure 9a. Sucrose gradient centrifugation profile of pulse labeled RNA. Sample was layered on 5-20% sucrose dissolved in 0.1 M NaCl and centrifuged at 39,000 rpm for 5½ hours. The three markers were obtained from (^{14}C) labeled RNA mixed with the sample before centrifugation.

Figure 9b. Sucrose gradient centrifugation profile of messenger RNA. Treated as pulse labeled RNA above (Fig. 9a).

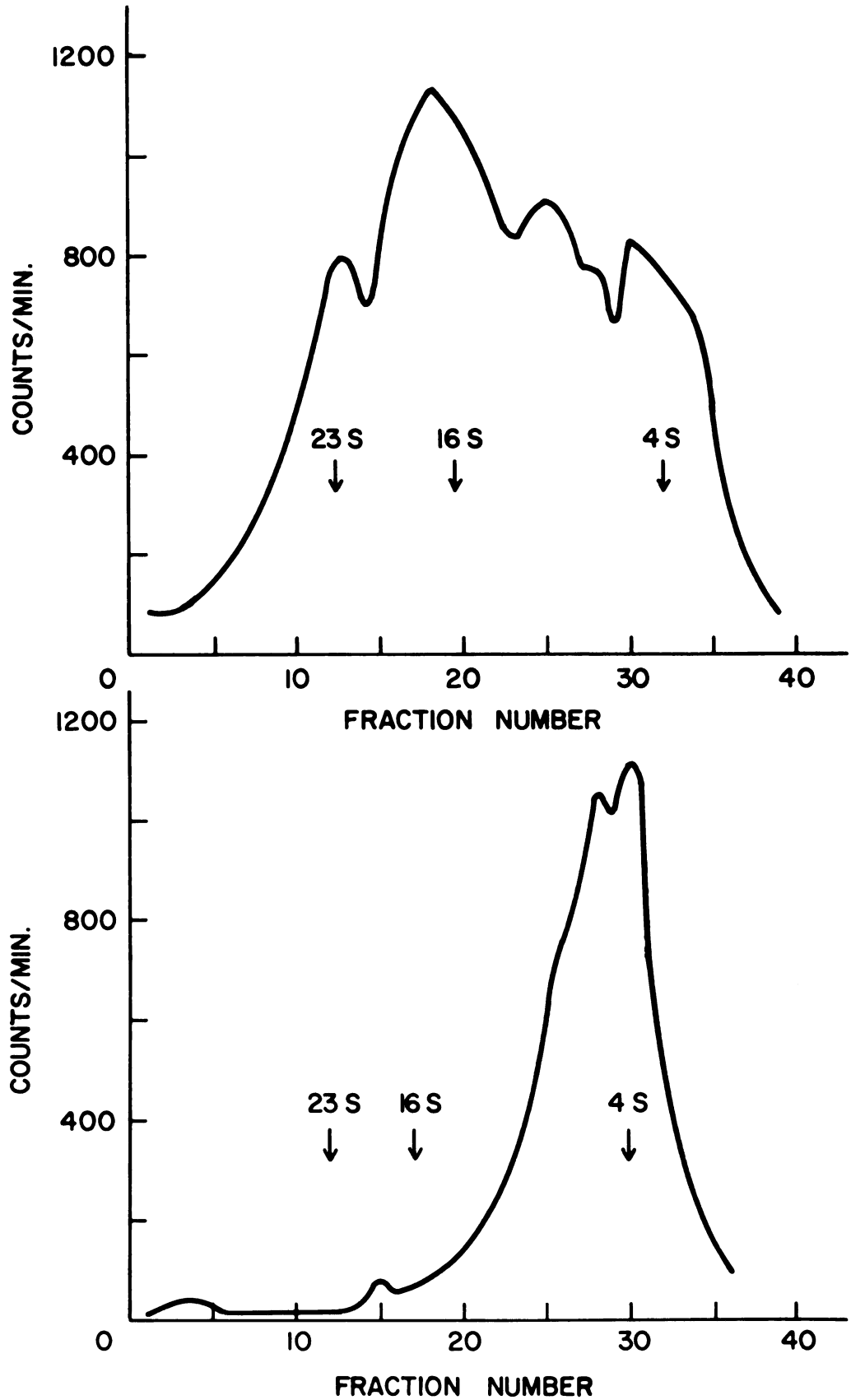
1200
800
400
0
COUNTS/MIN.
23 S
16 S
4 S
10
20
30
40
FRACTION NUMBER
1200
800
400
0
COUNTS/MIN.
23 S
16 S
4 S
10
20
30
40
FRACTION NUMBER

Figure 9

resulting pH was 2. After a 5 minute incubation in an ice bath, one equivalent of NaOH was added to raise the pH to 6.8. All of the (^{3}H) RNA formerly in the RNA-DNA hybrid became sensitive to ribonuclease indicating the low pH denatured the hybrid. However, both MAK chromatography and sucrose gradient centrifugation indicated that the isolated messenger RNA had a low molecular weight. Pulse labeled RNA was degraded by the low pH so the method was not used.

One preparation of isolated messenger RNA exhibited 4.1 times as great a specific activity as the starting material. The optimum increase in specific activity that could be expected is 30-100 fold. This low increase in specific activity obtained may have been caused by several different factors. During the dialysis step after isolation of messenger RNA from denatured DNA, about one half of the TCA precipitable radioactivity was lost. This loss in radioactivity is apparently caused by some of the degraded messenger RNA passing through the pores of the dialysis membrane and into the dialysis medium. Secondly, the ultraviolet spectrum of the isolated messenger RNA indicated there were bits of nitrocellulose present since the relative value of the absorbancy at 230 mμ was high as compared to the absorbancy at 260 mμ. Thirdly, the preparation of messenger RNA may have contained bits of denatured DNA which were too small to be retained by the nitrocellulose column and these bits of DNA would decrease the specific activity of the product. Since the molecular weight of the isolated messenger RNA was very low, no attempt was made to achieve a greater fold purification.

Purified messenger RNA should be able to hybridize more readily than can pulse labeled RNA because the latter contains some labeled ribosomal RNA precursors (to 60% of the radioactivity, reference 12) which must compete with a large amount of unlabeled ribosomal RNA for limited hybridization sites on denatured DNA. However, it was discovered that the isolated messenger RNA formed very little hybrid. The ultraviolet spectrum of the isolated messenger RNA indicated the presence of nitrocellulose fragments. It was thought that the nitrocellulose might inhibit hybrid formation, and therefore, a method to remove these fragments was sought. When the isolated messenger RNA was placed on a MAK column and eluted in a stepwise manner the ultraviolet spectrum indicated the removal of the nitrocellulose fragments. This RNA was also able to readily hybridize with denatured DNA. Since the MAK column was able to remove the nitrocellulose fragments, all messenger RNA was placed on a MAK column and eluted in a stepwise manner before characterization.

The ability of isolated messenger RNA to hybridize with denatured DNA was tested as follows: Denatured DNA (final concentration, 50 μg/ml) was added to isolated messenger RNA and the solution was incubated at 78° for 4 hours to allow hybrid formation. An aliquot was removed and tested for ribonuclease stable RNA-DNA hybrid and for TCA precipitable radioactivity. RNA-DNA hybrid and denatured DNA were removed from unreacted RNA by filtration through a nitrocellulose membrane filter. DNA was added to the filtrate to give a final concentration of 50 μg/ml. The process of incubation, sampling, filtration, and addition of denatured DNA was

repeated 5 times. The results are summarized in Table I.

As a comparison, the ability of pulse labeled RNA to form a hybrid was determined. The pulse labeled RNA was placed on a MAK column and eluted in a stepwise manner as used for the messenger RNA purification above. Denatured DNA (final concentration, 50 μg/ml) was added and the solution was incubated at 78° for 4 hours. An aliquot was removed and tested for ribonuclease stable RNA-DNA hybrid and for TCA precipitable radioactivity. The RNA-DNA hybrid and denatured DNA were separated from the unreacted RNA by filtering through a nitrocellulose membrane filter. Denatured DNA was added to the filtrate to give a final concentration of 50 μg/ml. The process of incubation, sampling, filtration, and the addition of denatured DNA was repeated 4 times. The results are summarized in Table II.

When the data of Table I is compared with that in Table II, it can be seen that the isolated messenger RNA hybridizes with denatured DNA more readily than the unfractionated pulse labeled RNA. After five incubations, a total of 83% of messenger RNA has hybridized with denatured DNA while only 48.8% of the pulse labeled RNA has hybridized. The greater ease with which messenger RNA hybridizes with denatured DNA is probably due to presence labeled ribosomal RNA which is present in the pulse labeled material (12) and which competes with the cellular pool of ribosomal RNA for hybridization with DNA.

TABLE I. HYBRIDIZATION OF MESSENGER RNA

Incubation	(^{3}H) RNA in Reaction Mixture	(^{3}H) RNA-DNA Hybrid Counts/min/ml		% Radioactivity in Hybrid*
	TCA Insoluble Counts/min/ml	0 hr	4 hr	
1	20,820	0	5,190	20.1
2	11,240	180	2,670	17.8
3	7,000	80	1,435	12.7
4	3,850	85	935	12.7
5	2,410	125	505	15.9

TABLE II. HYBRIDIZATION OF PULSE LABELED RNA

Incubation	(^{3}H) RNA in Reaction Mixture	(^{3}H) RNA-DNA Hybrid Counts/min/ml		% Radioactivity in Hybrid*
	TCA Insoluble Counts/min/ml	0 hr	4 hr	
1	16,760	200	2,790	12.5
2	9,030	120	1,070	8.5
3	6,110	290	585	7.7
4	4,100	95	590	9.8
5	2,620	50	383	10.3

*Zero time values were substracted from the 4 hour values before calculating the percentage of radioactivity in the hybrid. The net count/min in the hybrid was multiplied by 0.803 (To correct for greater efficiency in counting (^{3}H) on nitrocellulose membranes as compared to TCA precipitable counts) before dividing by the input TCA precipitable counts.

PART II. STUDIES OF METHYLATION OF RNA IN T-4 INFECTED CELLS.

Characterization of (^{3}H) methyl labeled RNA from T-4 infected cells. The specific activity of (^{3}H) methyl labeled RNA from T-4 infected cells was 15,000 cpm/mg. The specific activity of (^{3}H) methyl labeled RNA from T-4 infected cells treated with chloramphenicol was 53,000 cpm/mg. 93-95% of the radioactive TCA insoluble material was alkali labile (0.3 M KOH, 37°C, 18 hrs) and was also degraded by ribonuclease. The 5-7% of the material which is alkali stable is not degraded by a mixture of deoxyribonuclease and venom phosphodiesterase. This radioactive material is assumed to be a protein contaminant.

To demonstrate that the radioactivity is present in the methyl groups of RNA and not due to a labeling of the purine and pyrimidine bases themselves, a sample of RNA from the T-4 infected, chloramphenicol treated cells was hydrolyzed by alkali and analyzed by paper electrophoresis at pH 2.8. The RNA from the chloramphenicol treated cells was used in this experiment because its specific activity is greater than that of RNA prepared from infected cells not treated with chloramphenicol. The two RNA's are considered to be equivalent since their MAK elution profiles are identical (see below).

The results are presented in Figure 10. Some radioactive material is observed at the origin. No appreciable radioactivity is found in the cytidylic acid and adenylic acid region. A second radioactive compound moves somewhat faster than adenylic acid. Additional radioactive compounds are

Figure 10. Electrophoresis of nucleotides. Sample was spotted on Whatman 3 mm paper. Electrophoresis was performed at 2500 volts for 2 hrs at pH 2.8. Squares 1.9 x 1.9 cm were cut from the dried paper and counted in a scintillation counter. The circles represent the location of the spots seen under ultraviolet light and correspond to the 4 major nucleotides.

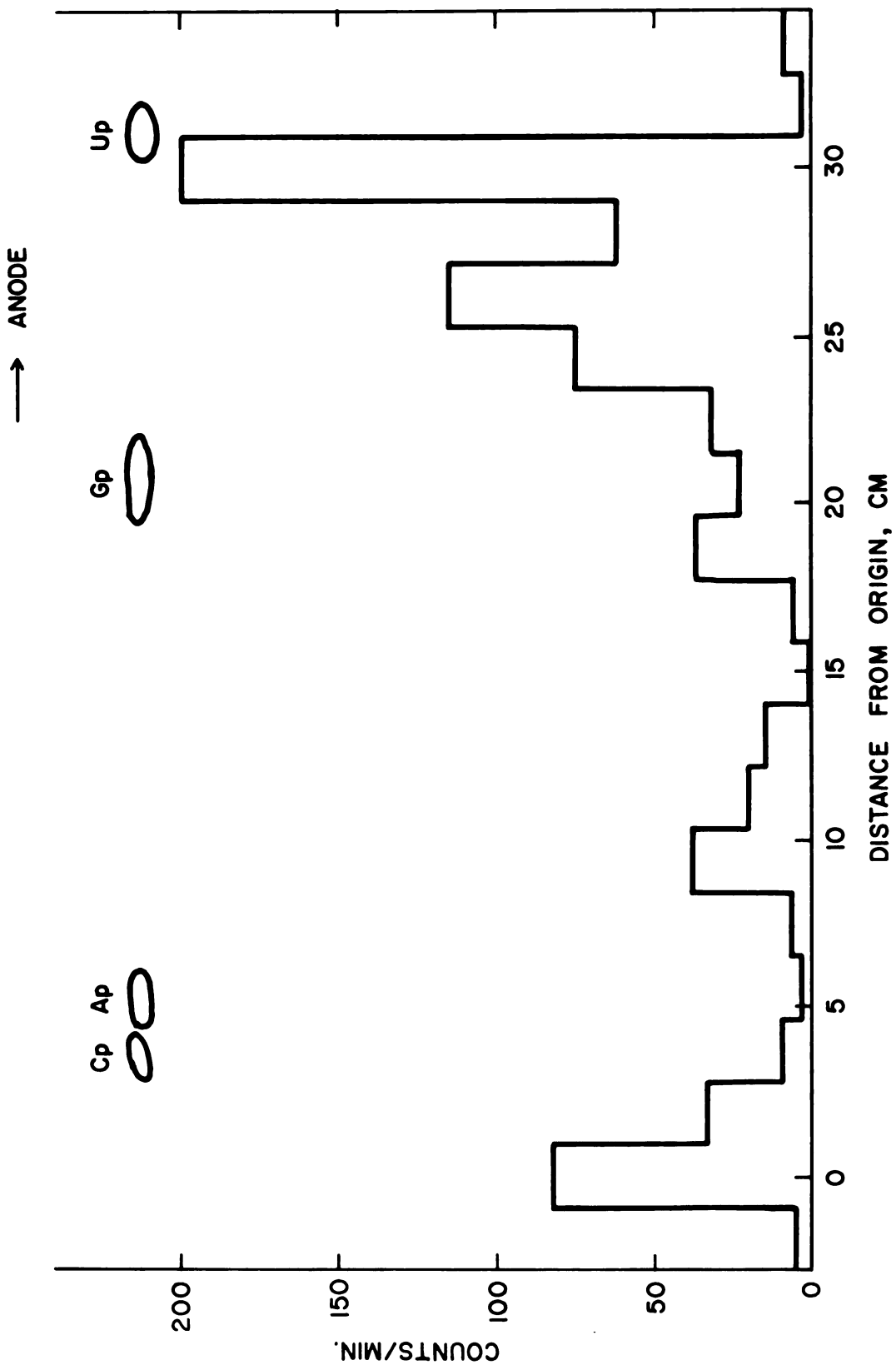
COUNTS/MIN.
200
150
100
50
0
0
5
10
15
20
25
30
DISTANCE FROM ORIGIN, CM
Cp Ap
Gp
Up
ANODE

Figure 10

observed in the guanylic acid and uridylic acid regions. Electrophoresis followed by solvent chromatography in the second dimension (49) suggests that the radioactive compounds in the Gp and Up regions are the methylated derivatives of guanylic acid, and thymidylic acid.

Sucrose gradient sedimentation analysis of (^{3}H) methyl labeled RNA from T-4 infected cells. The sucrose gradient sedimentation profile of (^{3}H) methyl labeled RNA from T-4 infected cells is given in Figure 11a. Approximately 70% of the labeled RNA sediments at 4S. The remaining labeled RNA sediments faster--exhibiting a wide range of sedimentation velocities. For comparison, the sedimentation profile of T-4 specific RNA labeled with (^{3}H) uracil is presented in Figure 11b.

MAK chromatography of (^{3}H) methyl labeled RNA from T-4 infected cells. The MAK elution profile of (^{3}H) methyl labeled RNA is presented in Figure 12. About 60% of (^{3}H) methyl labeled RNA elutes in the soluble RNA region. The remaining 40% of the radioactive RNA elutes at higher salt concentrations. In the soluble RNA region, the radioactive profile, a measure of soluble RNA methylated during the labeling period, and absorbancy profile, a measure of total soluble RNA, are different--with the specific activities in this region differing by greater than 100-fold. The elution profile given in Figure 12 has been reproduced more than 6 times on two different RNA preparations. The MAK elution profile for (^{3}H) methyl labeled RNA from T-4 infected cells treated with chloramphenicol (not shown) is identical to that presented in Figure 12.

Figure 11. Sucrose gradient centrifugation of labeled RNA from T-4 infected cells.

Figure 11a. Sucrose gradient centrifugation pattern of (^{3}H) methyl labeled RNA from T-4 infected cells. The dashed line indicated absorbancy at 260 mμ and the solid line, radioactivity. The three peaks in the absorbancy profile correspond to 23 S and 16 S ribosomal RNA and to soluble RNA (left to right). Centrifugation was for 5½ hours at 39,000 rpm.

Figure 11b. Sucrose gradient centrifugation pattern of (^{3}H) uracil labeled RNA from T-4 infected cells. Conditions were as in Fig. 11a.

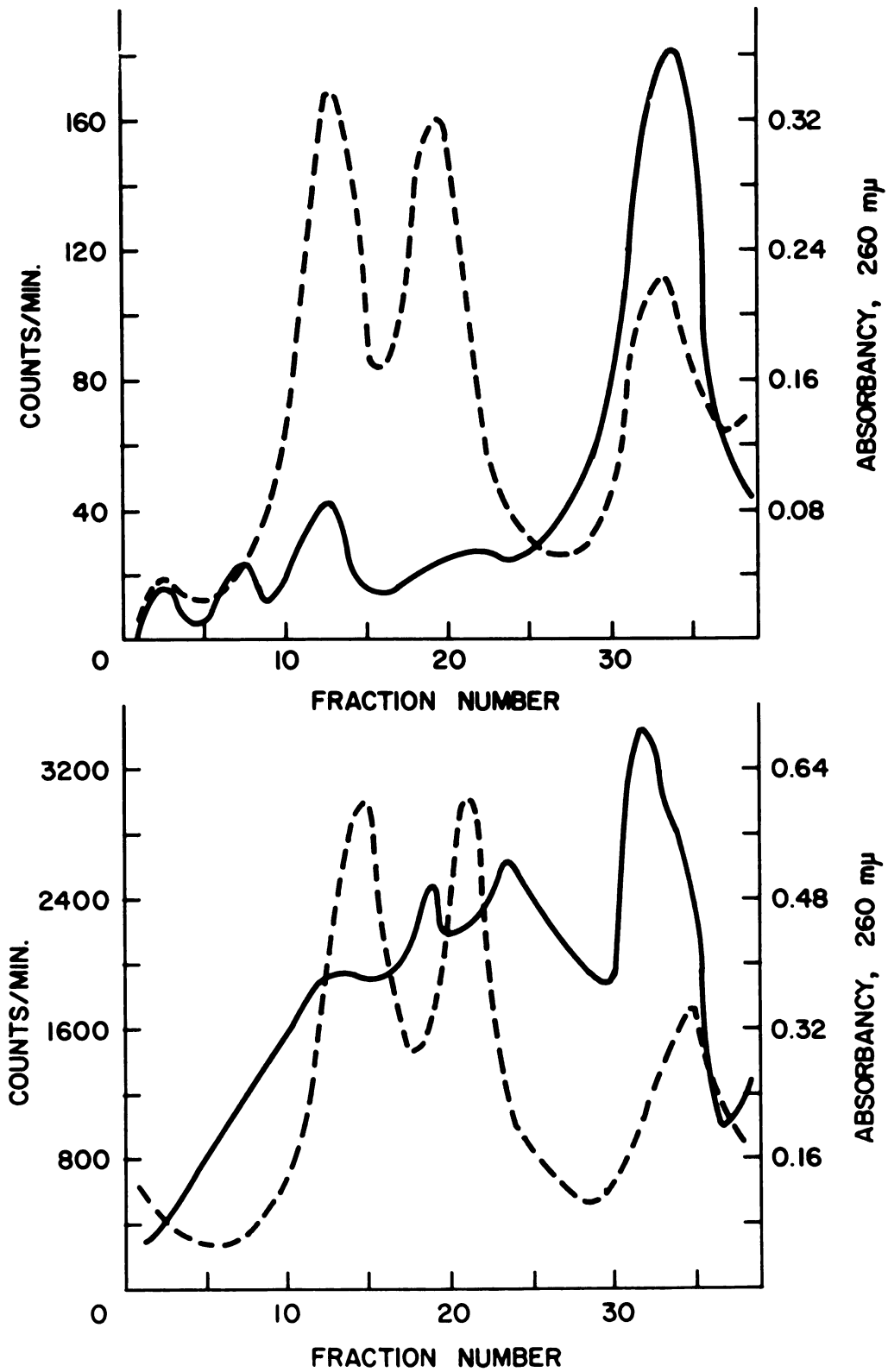
160
120
80
40
0
COUNTS/MIN.
0.32
0.24
0.16
0.08
ABSORBANCY, 260 mμ
10
20
30
FRACTION NUMBER
3200
2400
1600
800
0
COUNTS/MIN.
0.64
0.48
0.32
0.16
ABSORBANCY, 260 mμ
10
20
30
FRACTION NUMBER

Figure 11

Figure 12. Behavior of (^{3}H) methyl labeled RNA from T-4 infected cells on a MAK column. The solid line indicates radioactivity and the dashed line, absorbancy at 260 mμ. The soluble RNA elutes first, followed by 16 S and 23 S ribosomal RNA. Each fraction contained 2 ml. Gradient was 0.3 NaCl to 1.0 M NaCl, all dissolved in 0.05 M phosphate buffer (pH 6.8). 200 ml of each salt was used.

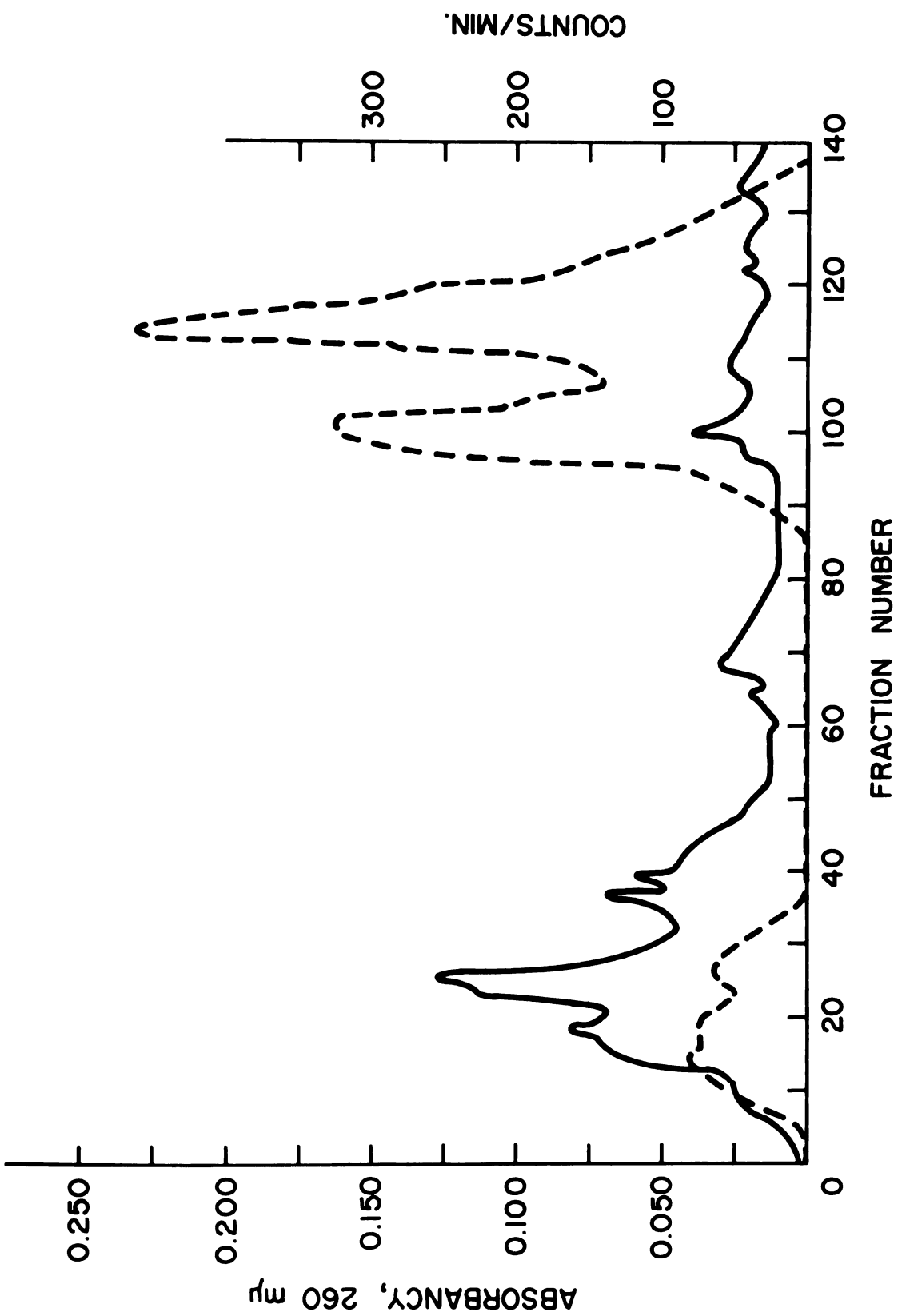
ABSORBANCY, 260 mμ
0.250
0.200
0.150
0.100
0.050
0
20
40
60
80
100
120
140
FRACTION NUMBER
300
200
100
COUNTS/MIN.

Figure 12

Any (^{3}H methyl) methionyl-soluble-RNA or (^{3}H methyl) methionine containing peptidyl-soluble-RNA present in the RNA preparations would contribute to the radioactive profile in the soluble RNA region. However, these compounds have not been detected in the RNA preparations. Following treatment of the RNA under conditions which would cleave either acyl bond to soluble RNA (pH 8.8, 45 minutes, 35° and pH 10.0, 60 minutes, 37°), the TCA insoluble radioactive content is unchanged and the elution pattern in the soluble region is essentially unaltered.

MAK chromatography of (^{3}H) methyl labeled RNA from uninfected cells. The MAK elution profile of (^{3}H) methyl labeled RNA from uninfected cells is presented in Figure 13. Radioactivity is observed in both the soluble and ribosomal RNA region. The radioactive profile and the absorbancy profile in the soluble RNA region are different. A comparison with the elution profile of (^{3}H) methyl labeled RNA from infected cells (Fig. 12) reveals that the methylation pattern of the two RNA's in the soluble RNA region are distinctly different. Further, little or no methylation of ribosomal RNA occurs in T-4 infected cells.

MAK chromatography of (^{3}H) uracil labeled RNA from T-4 infected cells. Methylation of soluble RNA in infected cells could occur on pre-existing host soluble RNA or on soluble RNA synthesized de novo. Although soluble RNA synthesis in T-even infected cells supposedly does not occur, (60) an experiment was designed to determine if any soluble RNA synthesis could be detected under conditions where methylation of soluble RNA was observed.

Figure 13. Behavior of (^{3}H) methyl labeled RNA from uninfected cells on a MAK column. Designations and conditions are the same as for Fig. 12.

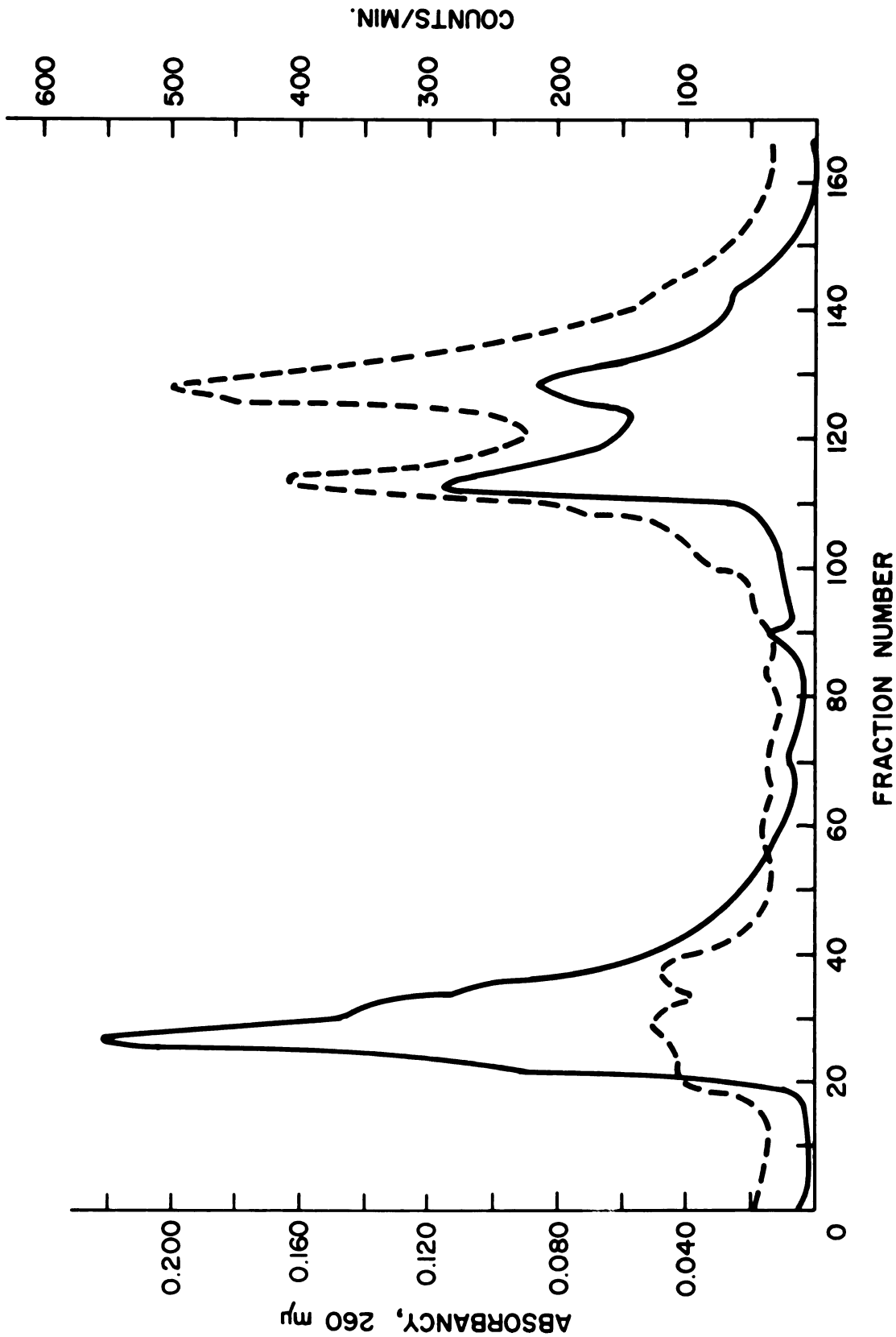
ABSORBANCY, 260 mμ
0.200
0.160
0.120
0.080
0.040
0
20
40
60
80
100
120
140
160
FRACTION NUMBER
COUNTS/MIN.
600
500
400
300
200
100

Figure 13

(^{3}H) uracil labeled RNA was prepared from infected cells and analyzed by MAK chromatography. The elution profile is shown in Figure 14. About 6% of the radioactive material elutes in the soluble RNA region suggesting that soluble RNA synthesis may occur in infected cells. A second fraction, amounting to about 6% of the radioactive material elutes immediately after the soluble RNA region. This material has not been identified. The radioactive RNA's eluting in the ribosomal RNA region probably correspond to T-4 specific messenger RNA II, III, and IV described by Ishihama, et. al. (61).

Hybridization of RNA from T-4 infected cells with denatured T-4 DNA. 4-5.5% of the (^{3}H) methyl labeled RNA purified from infected cells reacts with denatured T-4 DNA immobilized on a membrane filter to form ribonuclease resistant product (Table 3). About one half of the product is T-4 specific RNA-DNA hybrid. The remainder results from non-specific adsorption (noise) of radioactivity to the filter. The noise level was evaluated by treating a sample of RNA with ribonuclease before incubation in the hybridization test. About 2.2% of the radioactivity of the ribonuclease degraded RNA sample is still adsorbed on the filter in the hybridization test. This material is assumed to be radioactive protein contaminating the (^{3}H) methyl labeled RNA preparation. (^{3}H) methyl labeled RNA does not react with denatured E. coli DNA.

(^{3}H) uracil labeled RNA from infected cells reacts with denatured T-4 DNA in the hybridization test (Table 4). Pretreatment of the RNA with ribonuclease stops completely

Figure 14. Behavior of (^{3}H) uracil labeled RNA from T-4 infected cells on a MAK column. Designations and conditions are the same as for Fig. 12.

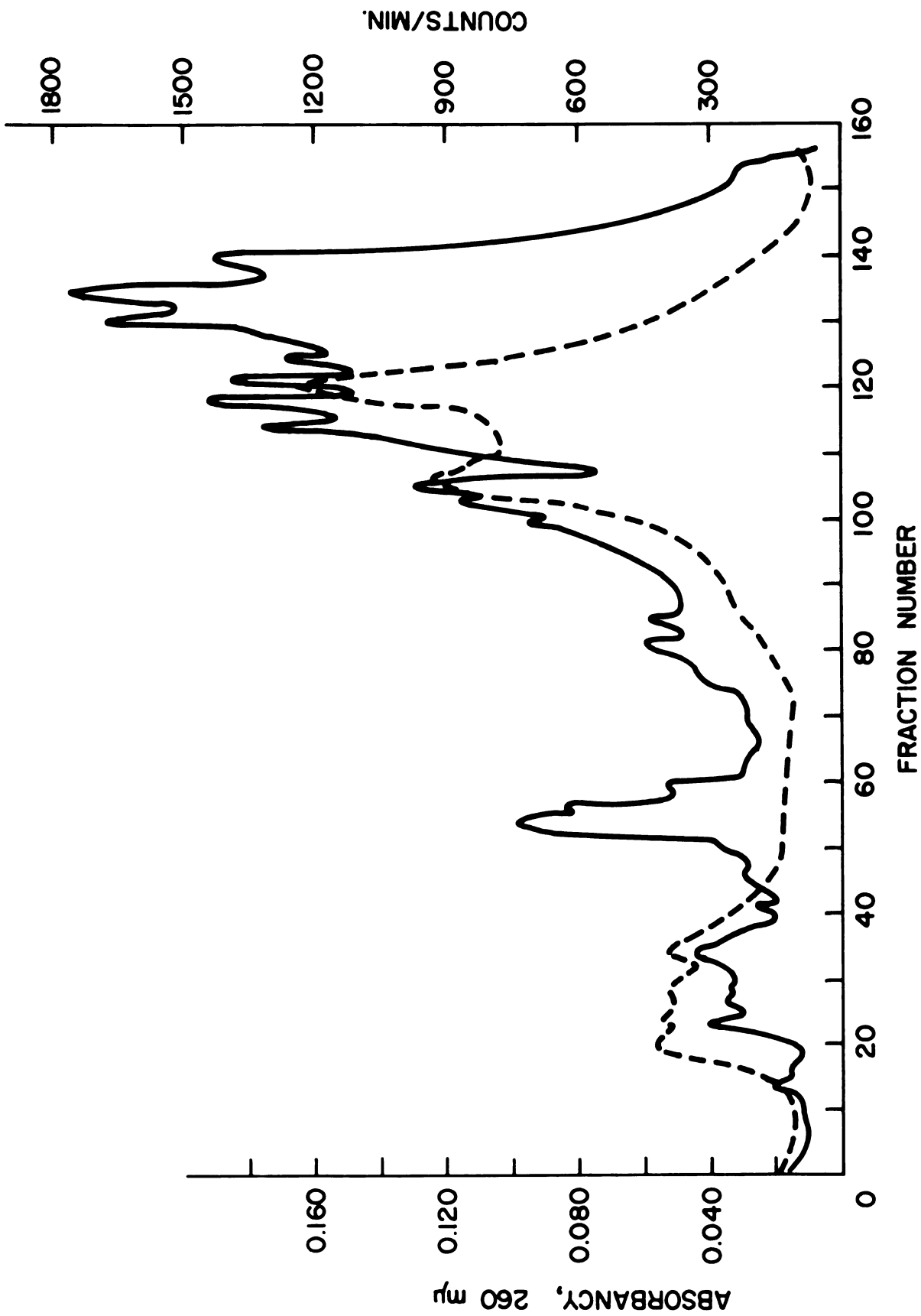
ABSORBANCY, 260 mμ
0.160
0.120
0.080
0.040
0
20
40
60
80
100
120
140
160
FRACTION NUMBER
COUNTS/MIN.
1800
1500
1200
900
600
300

Figure 14

TABLE III. HYBRIDIZATION OF (^{3}H) METHYL LABELED RNA

(^{3}H) Methyl Labeled RNA Counts/min/ Reaction Tube		Denatured DNA 125 µg/Filter	(^{3}H)RNA-DNA Hybrid Counts/min		%(^{3}H)RNA in Hybrid
			0 hr	2 hr	
Exp. 1*	3618	T-4	29	172	3.95
Exp. 2	3246	T-4	7	182	5.48
Ribonuclease Pretreated		T-4	6	73	2.06
Exp. 3	6492	T-4	24	302	4.56
Ribonuclease Pretreated		T-4	10	153	2.20
Exp. 4	3246	E. coli	8	47	1.20
Exp. 5	6492	E. coli	0	100	1.54

*Experiment 1 is the average of 5 trials with a single preparation of (^{3}H) labeled RNA.

Experiments 2-5 were performed on a second preparation of (^{3}H) methyl labeled RNA.

TABLE IV. HYBRIDIZATION OF (^{3}H) URACIL LABELED RNA

(^{3}H) Uracil Labeled RNA Counts/min/ Reaction Tube		Denatured DNA 125 µg/Filter	(^{3}H)RNA-DNA Hybrid Counts/min		%(^{3}H)RNA in Hybrid
			0 hr	2 hr	
Exp. 1	28,780	T-4	23	7,357	25.48
Ribonuclease Pretreated		T-4	29	34	0
Exp. 2	28,780	E. coli	6	73	0.23

subsequent hybrid formation. No significant reaction is observed between (^{3}H) uracil labeled RNA and denatured E. coli DNA.

Discussion

Methylation of RNA occurs in T-4 infected cells. About 60-70% of the RNA methylated is soluble RNA. The other 30-40% is RNA's of diverse size which sediment faster than 4S and which elute from a MAK column at salt concentrations greater than that required to elute soluble RNA. Little or no methylation of ribosomal RNA occurs in T-4 infected cells.

The methylation pattern of soluble RNA in infected cells is distinguishable from the pattern in uninfected cells. The MAK elution profile of soluble RNA methylated in infected cells is different from the elution profile of total soluble RNA (measured as the absorbancy profile) and is different from the elution pattern of soluble RNA methylated in uninfected cells.

Methylation of soluble RNA in infected cells may occur on soluble RNA made de novo and/or may occur on pre-existing cellular soluble RNA. Soluble RNA synthesis has not previously been observed in T-even infected cells (60), however, in an experiment described above, radioactive RNA which elutes from a MAK column in the soluble RNA region was extracted from T-4 infected cells labeled with (^{3}H) uracil. This radioactive RNA was not synthesized by uninfected cells present in the culture. Infection of the culture was complete as evidenced by the fact that none of the (^{3}H) uracil labeled RNA reacts with denatured E. coli DNA in the hybridization

test (Table IV). The radioactive RNA may represent soluble RNA synthesized de novo in the infected cell or, may not be soluble RNA at all, but rather fragments of T-4 specific messenger RNA produced by degradation of messenger RNA during the purification procedure. However, the presence of T-4 specific messenger RNA in the (^{3}H) uracil labeled RNA preparation whose sedimentation profile appears normal (with some RNA sedimenting faster than 23 S) and whose MAK elution profile at the higher salt concentration is similar to messenger RNA II, III, and IV fractions obtained by Ishihama, et. al. (61), argues against degradation of messenger RNA as the source of radioactive RNA eluting in the soluble RNA region. Experiments are in progress to characterize more completely this radioactive RNA. In the connection that this RNA may represent soluble RNA synthesized de novo in T-4 infected cells, it is interesting to note that Earhart and Neidhardt (62) recently observed the appearance of a valyl soluble RNA synthetase in infected cells.

Another possibility is that all or part of the methylation of soluble RNA in infected cells may occur on cellular soluble RNA. Modification of host soluble RNA by methylation may play some role in the control of protein synthesis at the translation level. However, modification of host soluble RNA might not effect its capacity to be charged by host amino acyl synthetases. Sueoka and Kano-Sueoka (63) observed that the amino acyl charging patterns of 16 soluble RNA's (leucyl soluble RNA was an exception) prepared from uninfected cells and T-2 infected cells were identical when charged with amino acyl synthetases from uninfected cells.

Hybridization studies have shown that RNA methylated in T-4 infected cells reacts with denatured T-4 DNA but not with denatured E. coli DNA. Preliminary experiments, using RNA fractionated by sucrose gradient centrifugation, indicates that the methyl labeled RNA which hybridizes with T-4 DNA sediments faster than 4 S. This hybridizable RNA may be methylated T-4 specific messenger RNA. Hybridization between any soluble RNA synthesized de novo and host soluble RNA modified by methylation with their respective template DNA's would not be observed because of the low specific activity of the labeled RNA's and the small number of the complementary sites on the DNA genome.

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