A METHOD POTENTIALLY USEFUL FOR ESTABLISHING BASE SEQUENCES IN CODE WORDS

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CHAPTER I

INTRODUCTION

DNA is the genetic material of most organisms, including some viruses. Disregarding the small amount of amino acids that can not be removed by extensive purification (1, 2), DNA is composed of a backbone of deoxyribose molecules, held together by phosphodiester linkages. Purines and pyrimidines are attached to C'-1 of the deoxyribose molecules through a glycosidic bond. DNA usually occurs in a double stranded form stabilized by specific hydrogen bonding between the complementary bases. Since the genetic message is contained in DNA, it is involved in two different processes, namely,

1) replication

2) protein synthesis

Protein synthesis starts with transcription, that is, with the formation

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The following abbreviations are used:

DNA, deoxyribonucleic acid; RNA, ribonucleic acid; m-RNA, messenger RNA; s-RNA, soluble RNA; TMV, tobacco masaic virus; DNase, deoxyribonuclease; RNase, ribonuclease; AA, amino acid; phe, phenylalanine; lys, lysine; pro, proline; leu, leucine; E, enzyme; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; A, adenine; G, guanine; C, cytosine; T, thymine; U, uracil; pT, thymidine 5'-phosphate; Tp, thymidine 3'-phosphate; pTpT, thymidylyl(3'-5')thymidine 5'-phosphate; pTpTpT, thymidylyl (3'-5')thymidylyl(3'-5')thymidine 5'-phosphate; Tr, trityl-; Tr-T, 5'-O-trityl thymidine; pT-OAc, 3'-O acetyl thymidine 5'-phosphate; DCC, dicyclohexylcarbodiimide; DEAE cellulose, diethylaminoethyl cellulose; Ecteola cellulose, epichlorohydrin triethanolamine cellulose; poly U, polyuridylic acid; pT, pdA in figures indicate oligonucleotide-celluloses.

of a mono- or polycistronic m-RNA molecule. The base sequence of m-RNA is complementary to the base sequence of plus (+) or information carrying strand of DNA. The presence of m-RNA was at first postulated by Jacob and Monod (3). Studies of phage infection of E. coli by Brenner, Jacob and Meselson (4) constituted the first evidence in support of this postulate. Using the pulse labeling technique it was shown that RNA formed after phage infection has a higher density than that of ribosomal RNA. This indicates the polysome formation between the messenger and ribosomes. Shortly thereafter Hall and Spiegelman (5) demonstrated that RNA could be annealed to the strand of DNA from which it was copied. Nearest neighbor analyses performed on m-RNA, synthesized in vitro by a DNA dependent RNA polymerase, showed that the molecule formed was a complement of the primer DNA strand used (6). Bolton and McCarthy (7) were able to trap RNA by preparing phosphocellulose to which one strand of DNA was attached covalently. They also prepared agar in which a DNA strand was physically trapped. This agar preparation also retained a RNA molecule of complementary base sequence. Studies on phage infection have also shown that phage induced RNA, which was acting as messenger, was bound to ribosomes of the host, indicating that ribosomes were the site for protein synthesis (4). The discovery, that an in vitro system for protein synthesis could be reconstructed by combining ribosomes with supernatant fractions has established the validity of this idea (8-11). Although the binding mechanism of m-RNA to ribosomes is not known, the work of Okamoto and Takanami (12) and of Cannon, Krug and Gilbert (13) have demonstrated that m-RNA is attached to the 30 S portion whereas s-RNA is attached to the 50 S portion of the ribosome. Binding of m-RNA results in an aggregation of the ribosomes to form

polysomes which may contain twenty or more units (14, 15).

The amino acids which are incorporated into protein must first enter the cell. This transport is mediated by the "permeases" (16) that are located in the cell membrane. Once inside the cell amino acids are activated by amino acid-RNA ligases (amino acyl synthetases) (17) to form enzyme bound amino-acyl-adenylates with the release of an equivalent amount of inorganic pyrophosphate (17-19).

$$AA + ATP + E \rightleftharpoons AA\text{-}AMP\text{-}E + PP_i \qquad (1)$$

More than one amino acyl synthetase was found for some amino acids. The above reaction can be followed by the amino acid dependent incorporation of $^{32}PP_i$ into ATP and by the formation of amino acyl hydroxamates when high concentration of hydroxylamine is used. The amino acid moiety of the complex is transferred to s-RNA (20).

$$AA\text{-}AMP\text{-}E + s\text{-}RNA \rightleftharpoons AA\text{-}sRNA + AMP + E \qquad (2)$$

The reversibility of these reactions and the high energy nature of the AA-sRNA bond has been demonstrated (21, 22). The isolation of ^{14}C leu-adenosine after RNase digestion of ^{14}C leu-sRNA has indicated the involvement of the ...pCpCpA end of the s-RNA molecule (23, 24). Later both Feldmann and Zachau (25) and Wolfenden, Rammler and Lipmann (26) presented evidence that the amino acid is linked to the 3'-OH of adenosine. Experiments performed to purify individual s-RNA species, corresponding to particular amino acids, demonstrate that the code is degenerate; that is, there are more than one s-RNA species for most amino acids (27-30). Each s-RNA molecule has two sites of recognition; one for the proper amino acid, the other for the recognition of the appropriate position on the m-RNA. Modification of s-RNA using chemical (31-33) and enzymatic (34, 35) methods have provided the most

direct information on this problem. These two sites do not coincide and it is the latter site, "the anticodon" that is responsible for the proper placement of the amino acid on the m-RNA (36). Since only a short segment of m-RNA can be in contact with one ribosome at any moment, messenger and ribosomes must be in constant movement relative to each other. Ribosomes active in protein synthesis were shown to have two s-RNA molecules bound to them; the one had the nascent polypeptide chain attached to it and the second, the amino acid that will be added next to the chain (37). The next step is the formation of the peptide bonds, starting from the N-terminus (38, 39). This reaction is mediated by transfer enzymes that have very low specificity for s-RNA species and require GTP. The last step is the release of finished polypeptide. The fact that "nascent," enzymatically synthesized proteins can not be removed from the ribosomes by simple washing techniques (40, 41), and the finding of soluble enzyme fractions which increase the formation of soluble protein without increasing the total incorporation greatly (42) were taken as an evidence for the presence of "releasing enzyme(s)." On the other hand, until the systems are developed where incorporation into ribosomes is not occurring while "release" is being measured, the problem of whether or not "releasing enzyme(s)" exist remains open.

Both DNA and RNA contain four major bases, A, G, C and T in DNA and A, G, C and U in RNA. In addition to these, certain species of RNA have small amounts of unusual bases, e. g. pseudouridine, dimethylguanine, etc. More than twenty amino acids have been found in proteins. The "coding problem" is to determine how the limited variety of bases in nucleic acids control the sequence of the many amino acids found in

protein. Before going into a brief discussion of these, a glossary of terms that are used in this area will be given.

The codon: A group of bases on m-RNA that code for one amino acid. There are three consecutive bases in the "triplet code."

The anticodon: Three bases complementary to a codon, found on a s-RNA molecule which react with the codon.

Coding ratio: The ratio of the number of bases that code for a particular polypeptide, to the number of amino acid residues found in that polypeptide.

Overlapping code: A code where one base forms part of more than one codon. In partially overlapping triplet code, one base belongs to two codons, while a fully overlapping triplet code has two bases common to two codons.

Degenerate code: A code where one amino acid has more than one code word, hence more than one s-RNA species.

Ambiguous code: A code where more than one amino acid is coded by one codon.

Unique code: A code where all codons are unambiguous.

Congruent code: A code where all the codons have the same size and shape.

Sense word: Any code word that corresponds to an amino acid in the "wild type" gene.

Nonsense word: A triplet that does not code for any amino acid.

Universality: A condition which requires all organisms to have the same code words in the code word "dictionary."

Colinearity: The parallelism between the sequence of bases on m-RNA and the sequence of amino acids in the polypeptide

coded by it (43). It was also called "sequence hypothesis."

Transition: A change of one purine into another purine or of one pyrimidine into another pyrimidine in a codon.

Transversion: A change from a purine into a pyrimidine and vice versa, in a codon.

Reading frame: Any device used to differentiate between the sense and nonsense sequences during the reading of the message.

The unavailability of any correlation between an unusual base and any particular amino acid and all of the unusual bases being capable to form one of the two standard base pairs puts a limitation for the consideration of these in the coding problem. Some amino acids are found only in certain tissues and it has been shown that the hydroxylation of proline and lysine takes place after these amino acids are incorporated into protein (44). The problem then reduces to finding a correlation between four major bases and twenty amino acids. Early attempts to find a relation between these parameters were based on theoretical considerations. Due to the great difference between four and twenty the possibility of one base codings for one amino acid was dismissed. Assumption of involvement of two bases for one amino acid yields $4^2=16$ possibilities. This idea, which was called "the doublet code" (45, 46) is unlikely, since there are not enough words to code for even the twenty amino acids, let alone to account for the degeneracy (27-30). If the involvement of three bases in a code word is assumed, one has $4^3=64$ permutation possibilities, hence 64 code words. Although the availability of more code words than the total number of amino acids could not be explained for some time, recent evidence makes the triplet code very likely (47). Based on the consideration that free

amino acids could be absorbed from the intracellular fluid into the lattice openings of the DNA double helix and that the inter-nucleotide distances on DNA were similar to distances between amino acids on a stretched polypeptide Gamow (48) proposed a fully overlapping code. Shortly thereafter Brenner (49) showed by theoretical considerations that an overlapping code is impossible. The experiments carried out by Tsugita and Fraenkel-Conrat (50) and by Wittmann (51) on nitrous acid mutants of TMV have shown that most single mutations on the TMV-RNA resulted in only a single amino acid change in the coat protein. In the few cases where two amino acids were changed, they occurred on widely separated portions of the protein. Were the code overlapping at least two amino acids should have been changed by a single mutation.

The discovery by Nirenberg and Matthaei (52) that the cell-free incorporation of phenylalanine into polyphenylalanine was stimulated by enzymatically synthesized poly U has opened the way to an experimental approach to coding problem. The system was dependent on ribosomes, activating enzymes, s-RNA, ATP, ATP regenerating system and GTP. Poly U stimulated only the phenylalanine whereas poly A, poly C and poly I did not. In two laboratories a number of presumptive code words shown in Table I were obtained, by the use of synthetic messengers containing two and three nucleotides (53, 55). The synthetic messengers presumably act catalytically (53). The early synthetic messengers used contained a high proportion of U, in order to stimulate amino acids other than phenylalanine. Since natural m-RNA does not have such a high U content, the results obtained were questioned as to their biological validity. The code words (triplets) were obtained by matching the frequency of each triplet with random sequence in the messenger

TABLE I

AMINO ACID CODE WORDS OBTAINED IN CELL FREE SYSTEM (1962)

Amino Acid	Code Word	
	Nirenberg's Group	Ochoa's Group
Alanine	CCG	CUG GAG CCG
Arginine	CGC	GUC GAA GCC
Asparagine	ACA	UAA CUA CAA
Aspartic acid	ACA	GUA GCA
Cysteine	UUG OR UGG	GUU
Glutamic acid	ACA AGA AUG	AAG AUG
Glutamine	ACA	AGG ACA
Glycine	UGG	GUG AGG GCC
Histidine	ACC	AUC ACC
Isoleucine	UUA	UUA AAU
Leucine	GUU CUU AUU (UUU)	UAU UUC UGU
Lysine	AAA AAC AAG AAU	AUA AAA
Methionine	UGA	UGA
Phenylalanine	UUU	UUU UUC
Proline	CCC CCU CCA CCG	CUC CCC CAC
Serine	UCG UUC UCC	CUU CCU ACG
Threonine	CAC CAA	UCA ACA CGC
Tryptophan	UGG	UGG
Tyrosine	UAU	AUU
Valine	UGU	UUG

Sequences of bases are arbitrary.

used, with the frequency of each amino acid incorporated by that particular messenger. However, the frequences did not match for each amino acid, due to certain reasons including the degeneracy of the code. Due to differences in the stimulation efficiency of various polymers, which was influenced by secondary structure and size of the messenger, solubility of the products formed, etc., incorporation of a given amino acid (X) had to be normalized by reference to the incorporation of phenylalanine, corrected for background, and measured in a parallel experiment (I^{o}_{x} = normalized incorporation ratio) as suggested by Lanni (56a). The frequency of various triplets in a polymer that actively stimulated the incorporation of amino acids, calculated for (56) random sequence, should also be normalized by reference to the frequency of UUU ($f^{o}_{(x)}$ = normalized frequency ratio). Then a fitting index could be defined as F^{o}_{t}/I^{o}_{x}. In general, the "unordered" triplet whose f^{o} gave the best match with I^{o}_{x} should be taken to be the "unordered" code for amino acid X. Actually, this statistical approach has not been used by any investigator.

Since poly U stimulates the incorporation of leucine usually to 5-10% of the level of phenylalanine incorporation, in the presence of phenylalanine, whereas in its absence this level increases to 50% of the level of phenylalanine incorporation, small amounts of stimulation obtained should be mistrusted. The biggest shortcoming of this method has been its inability to provide an insight into the base sequences of the code words.

Another approach has been the amino acid replacement studies carried on human hemoglobins (57, 58), E. coli L-serine hydrolyase (adding indole) (E. C. No. 4.2.1.20) (59), TMV (60, 61) and on

proflavin mutants in the rII region of E. coli phage T4 (62). A further source of evidence has been the DNA-protein compositional correlations. The great range of DNA base compositions observed, especially for micro-organisms where the content of G + C goes from 72% to 25%, suggested the possibility of finding corresponding compositional changes in the over-all protein content of these microorganisms. Although the results have not been very satisfactory in every case, Sueoka (63, 64) has been able to show that there was usually about twice as much alanine in an organism with a DNA having 72% G + C compared with one having only 25% G + C. Fitch (64) has shown a certain correlation between doublet nucleotide sequences in DNA of various microorganisms and the amino acid content, on a more mathematical basis; also he has proposed base sequences for certain code words based on this correlation. All the above methods had the disadvantage of not being able to yield the base sequence of the code words. The cell free system which was the most direct approach, had two additional shortcomings. First, in order to stimulate amino acid incorporation, the synthetic messengers had to have a chain length which was many times larger than the size of a codon. Then, in conjunction with the "ribosome binding" (66) method, it made use of ribosomes. How the messenger is bound to ribosome is still not known. The presence of various enzymes in the ribosomes have also been shown (67, 68). A simple physiochemical system, where the conditions can be controlled at will, was deemed necessary for a better understanding of the coding problem and especially of the base sequences within the codons. Consideration of the minimum requirements for the interaction of m- and s-RNAs suggests that:

1) the anticodon of s-RNA ought to be especially reactive and

most probably in an exposed position relative to other nucleotide residues.

2) this interaction occurs through complementary base pairing between the bases on codon and anticodon, due to specific hydrogen bonding.

Thus it seemed likely that a selective chromatographic material could be prepared covalently by attaching the proper base sequence to an inert material like cellulose. This approach has been used for the study of antigen-antibody reactions (69), for the separation of various nucleosides (70), of ribonucleotides (71), of deoxyribonucleotides (72) and for purification of enzymes (73).

CHAPTER II

MATERIALS AND METHODS

Preparation of Oligonucleotides

The purity of the commercial nucleotides (Schwartz Bio-Research, Inc., Mountain View Ave., Orangeburg, N. Y.) was checked by paper chromatography in solvent I and VI as given under characterization of oligonucleotides by applying 1-5 μmoles (10-50 A_{260} units) on a 2.5 cm band. Solvent I can detect the presence of ribonucleotides in deoxyribonucleotides (74). All the samples used were found to be free of contamination within about 2% of the spotted material.

Oligothymidylic Acid (75)

Thymidine 5'-phosphate (1 mmole, calcium salt) was converted to pyridine salt by treatment with a slurry of pyridinium-Dowex 50 and repeated evaporation to dryness after addition of anhydrous pyridine, and was dried by six evaporations in the presence of anhydrous pyridine (7 ml each time). The gum was then dissolved in 8 ml of dry pyridine (stored over calcium hydride) and 1.50 g (7.5 mmoles) DCC added and the sealed reaction mixture was shaken rapidly. The clear mixture became turbid within five minutes and deposited semi-crystalline material. The reaction mixture was kept at room temperature for six days on a wrist shaker, then diluted with 10 ml of water. After three hours at room temperature, the mixture was extracted six times with

ether (25 ml each time) and the aqueous layer then concentrated at 30° in vacuo to a syrup. The syrup was dissolved in 13 ml of water, pH adjusted to 8.0 with lithium hydroxide and the solution evaporated. This process was repeated to insure complete removal of pyridine. The final aqueous solution was filtered from small amount of dicyclohexylurea and made up to 25 ml.

Oligodeoxyadenylic acid (74)

Finely divided deoxyadenosine 5'-phosphate (1 mmole, pyridine salt) obtained from the ammonium salt by passing through a column of Dowex 50 ion exchange resin in pyridinium form and lyophilization of the eulate, was suspended in 20 ml of dry pyridine and 2.5 ml (20 mmoles) of benzoyl chloride (Eastman Organic Chemicals, Rochester, N. Y.), from a newly opened vial, was added. The mixture which had an orange color and some precipitate was kept in the dark with exclusion of moisture. After one hour at room temperature, it was poured into a mixture of 50 ml of chloroform and 50 ml of water, in the cold. After 15 minutes the chloroform layer was separated and the aqueous layer extracted again with chloroform (2 x 25 ml). The combined chloroform extracts, which contained virtually all of the nucleotidic material, were evaporated in vacuo to a gum with a water bath temperature below 20°. The mixture was dissolved in 200 ml of 50% aqueous pyridine. To this solution was added 200 ml of 0.5 N sodium hydroxide in an ice bath. Amberlite IR-120 resin (75 ml) in pyridinium form was added rapidly after 23 minutes to remove the alkali. Sodium ions were removed completely by passing the solution through a column of the same resin (50 ml). The resin was washed thoroughly with water and the combined aqueous pyridine

solution of the product was evaporated in vacuo to about 20 ml from which most of the benzoic acid was crystallized. The mixture, which was kept cold in an ice bath, was repeatedly extracted with cold ether to remove benzoic acid. About 5 ml of pyridine was added and the solution was lyophilized. Treatment of the benzoylated nucleotide under the conditions given yielded directly a mixture of N-benzoyl-deoxyadenosine 5'-phosphate and N,3'-O-dibenzoyldeoxyadenosine 5'-phosphate which could immediately be polymerized. The lyophilized powder was dissolved in 5 ml of dry pyridine and was evaporated to dryness several times after addition of dry pyridine, admitting only dry air into the system after each cycle. The residual gum was dissolved in 1.0 ml of dry pyridine and 500 mg (2.45 mmoles) of DCC was added. The sealed reaction mixture was shaken mechanically in the dark and after 24 hours a further 500 mg of DCC was added and the sealed reaction mixture was shaken for six days. At the end of the six days the aqueous sodium hydroxide (5 ml of water + 3 ml of 1 N sodium hydroxide) was added and the mixture kept at room temperature for one hour. After three extractions with ether and filtration to remove dicyclohexylurea, the clear aqueous solution was converted to ammonium form by passing through an Amberlite IR-120 resin column. The total eluate and washings were evaporated to dryness in vacuo and the residue kept in 20 ml of concentrated ammonia for 48 hours, at room temperature, to remove the benzoyl groups. The solution was evaporated to dryness and the residue dissolved in 3 ml of water. The insoluble benzamide was removed by centrifugation and the sediment was washed twice with water. The combined aqueous solution was adjusted to pH 8-9 with ammonia for column chromatography.

Oligodeoxycytidylic Acid

Preparation of N^6,3'-O-Diacetyldeoxycytidine 5'-phosphate (76)

One mmole of free deoxycytidine 5'-phosphate was dissolved in a mixture of 2 ml pyridine and 20 ml of water and the solution lyophilized. The finely divided material thus obtained was suspended in 10 ml of dry pyridine and 3 ml of acetic anhydride was added. The stoppered flask was kept in the dark at room temperature and shaken frequently. In a few hours a clear solution resulted. After 18 hours 40 ml of water was added to the light cream colored solution in an ice bath. The solution was kept at room temperature for 1.5 hours and then concentrated to a syrup in vacuo at low temperature (water bath temperature below 20°) using a rotary evaporator. Water was added to the syrupy concentrate and the solution evaporated as above three more times to remove most of the pyridinium acetate. Finally 100 ml water was added and the solution was lyophilized to give a fine white powder.

Preparation of N^6-Anisyldeoxycytidine 5'-Phosphate (77)

To 1 mmole of pyridinium deoxycytidine 5'-phosphate, prepared as above, 30 ml of dry pyridine was added followed by 3 ml of freshly distilled anisyl chloride. The resulting solution was kept one hour in the dark at room temperature, 100 ml of water was added in an ice bath and the mixture rapidly extracted three times with 150 ml portions of chloroform and the total chloroform extracts were washed twice with 50 ml portions of water. The chloroform solution, which contained the nucleotidic material, was then evaporated under reduced pressure and the gum was taken up in 20 ml of pyridine. To the solution 10 ml of water was added quickly under agitation. Then 30 ml of 2 N sodium

hydroxide was poured in immediately thereafter and the flask cooled momentarily in an ice bath. The solution turned orange. After 18 minutes at room temperature 70 ml of IR-120 (H^+) was added and the total aqueous pyridine solution and washings were passed slowly through a 3 x 20 cm column of pyridinium IR-120 to ensure removal of all sodium ions. The total effluent and washings, with 5% pyridine, were concentrated to about 40 ml. The anisic acid, which separated, was removed by filtration and the solid was washed with water. The total filtrate was concentrated again at a low temperature to about 40 ml and any anisic acid that separated was again filtered. The aqueous solution was extracted three times with 50 ml portions of ether and then diluted with a little more than equal volume of pyridine before evaporation and then was lyophilized.

Polymerization of a Mixture of N^6,3'-O-Diacetyl-deoxycytidine 5'-Phosphate and N^6-Anisyl-deoxycytidine 5'-Phosphate (77)

A pyridine solution of 0.25 mmoles of deoxycytidine 5'-phosphate was mixed with a pyridine solution of 0.75 mmole of N^6-anisyldeoxycytidine 5'-phosphate and the solution was evaporated in vacuo. Dry pyridine (10 ml) was added and the solution was evaporated four times. After each evaporation, air dried over a Drierite column was admitted into the system. The frothy residue was dissolved in 1 ml of dry pyridine and 412 mg (2 mmole) of DCC was added. The mixture was agitated vigorously for 10 minutes during which a gum was formed in the flask. The contents were shaken mechanically for seven days in the dark at room temperature. A solution of 3 ml in sodium hydroxide +

5 ml water was added and the mixture was left for 40 minutes at room temperature. Then the mixture was extracted twice with ether and the insoluble dicyclohexylurea removed by filtration. The total alkaline solution was then adjusted to pH 9.0 by careful addition of IR-120 (H^+) resin and the resin removed by filtration and thoroughly washed with water. The total aqueous solution was evaporated to a syrup and to it was added 25 ml of concentrated ammonia. The stoppered mixture was left at room temperature for 60 hours before evaporation. The insoluble anisamide was removed by ether extraction and the aqueous solution was adjusted to pH 9.0 with alkali.

Fractionation of Oligonucleotides

Ecteola Cellulose - Lithium Chloride System (75)

Oligothymidylic acid (0.2 mmole) was placed on top of an Ecteola cellulose (Sigma Chemical Co., St. Louis, Mo.) column (4 x 23 cm). After a water wash (50 ml) the nucleotide material was eluted by lithium chloride solution, the concentration of which was increased stepwise from 0.05 M to 0.25 M. In this method linear oligonucleotides are eluted ahead of the corresponding cyclic nucleotides (Fig. 1).

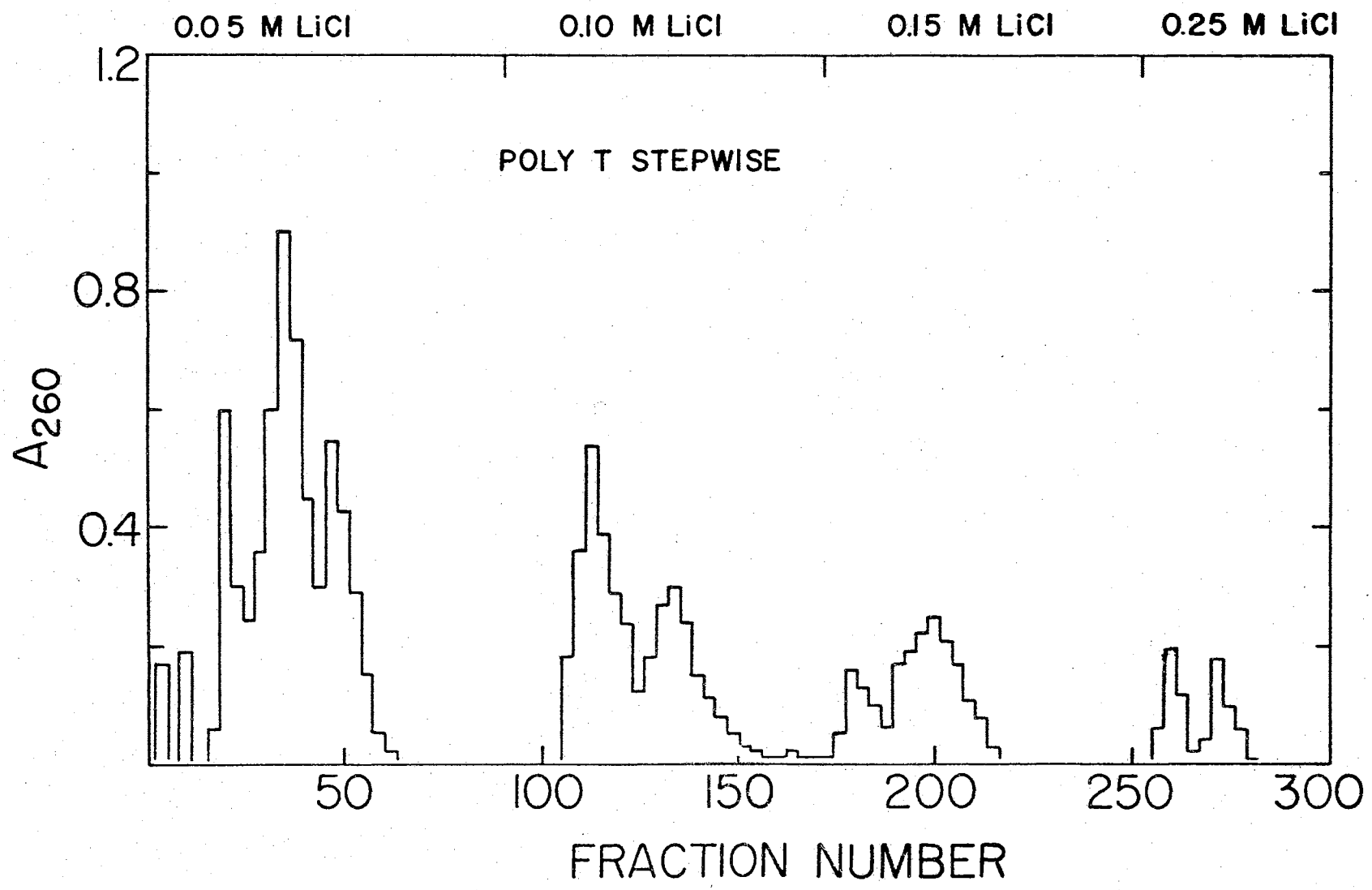
DEAE Cellulose - Triethylammonium Bicarbonate System (76)

Oligothymidylic acid 1 mmole, at pH 8-9, was applied to the top of a DEAE (Sigma Chemical Co., St. Louis, Mo.)-cellulose column (carbonate form, 4 x 30 cm) and carefully washed in with 300 ml water. Elution was made by gradient elution technique. The mixing vessel contained initially 4 liters of water and the reservoir 4 liters of 0.25 M triethylammonium bicarbonate (pH 7.5). When this eluant had passed through the column, 4 liters of 0.25 M triethylammonium

Figure 1

Chromatography of Oligothymidylic Acid on Ecteola-Cellulose Using Lithium Chloride

Oligothymidylate (0.2 mmole) was chromatographed on Ecteola-cellulose column (4 x 23 cm). Lithium chloride solution added stepwise in 0.5 liter amounts from 0.05 M to 0.25 M was used as eluant, 6 ml fractions being collected.


0.05 M LiCl
0.10 M LiCl
0.15 M LiCl
0.25 M LiCl
POLY T STEPWISE
1.2
0.8
0.4
A260
50
100
150
200
250
300
FRACTION NUMBER

bicarbonate was introduced to mixing vessel and 4 liters of 0.5 M of the same buffer into the reservoir. Flow rate was about 3 ml/minute and 18 ml fractions were collected. In this method cyclic oligonucleotides were eluted ahead of the corresponding linear nucleotides (Fig. 2).

DEAE-Cellulose-Ammonium Bicarbonate-Urea System (78)

Oligothymidylic acid (1 mmole) was applied to the top of a DEAE-cellulose column (bicarbonate form, 4 x 45 cm) which was packed in 7 M urea solution, and washed in with 200 ml urea solution. The elution was carried out by gradient elution technique using 3 liters of 7 M urea solution in the mixing vessel and 3 liters of 0.3 M ammonium bicarbonate solution (pH 6.7) in 7 M urea in the reservoir. In this system oligonucleotides are separated according to their chain length (charge) (Fig. 3).

The fractionation of oligodeoxyadenylic (74, 77) and oligodeoxycytidylic acids were performed using DEAE-cellulose-triethylammonium bicarbonate system. There are slight differences in conditions as seen in the references.

Characterization of Oligonucleotides

Paper Chromatography

Descending paper chromatography on Whatman No. 1 and 3 MM chromatographic paper, which was washed with 0.1 N hydrochloric acid, was used and 0.2-1.0 μmole (2-10 A_{260} units) of nucleotidic material was spotted.

Solvent I: isopropanol:concentrated ammonium hydroxide:water = 7:1:2 (79)

Solvent II: n-butanol:acetic acid:water = 5:2:3 (79)

Figure 2

Chromatography of Oligothymidylic Acid on DEAE-Cellulose Using Triethylammonium Bicarbonate

Oligothymidylic acid (1 mmole, pH 8-9) was chromatographed on DEAE-cellulose column (4 x 30 cm, carbonate form). A linear gradient of triethylammonium bicarbonate (0-0.5 M) was used as eluant, 18 ml fractions being collected.

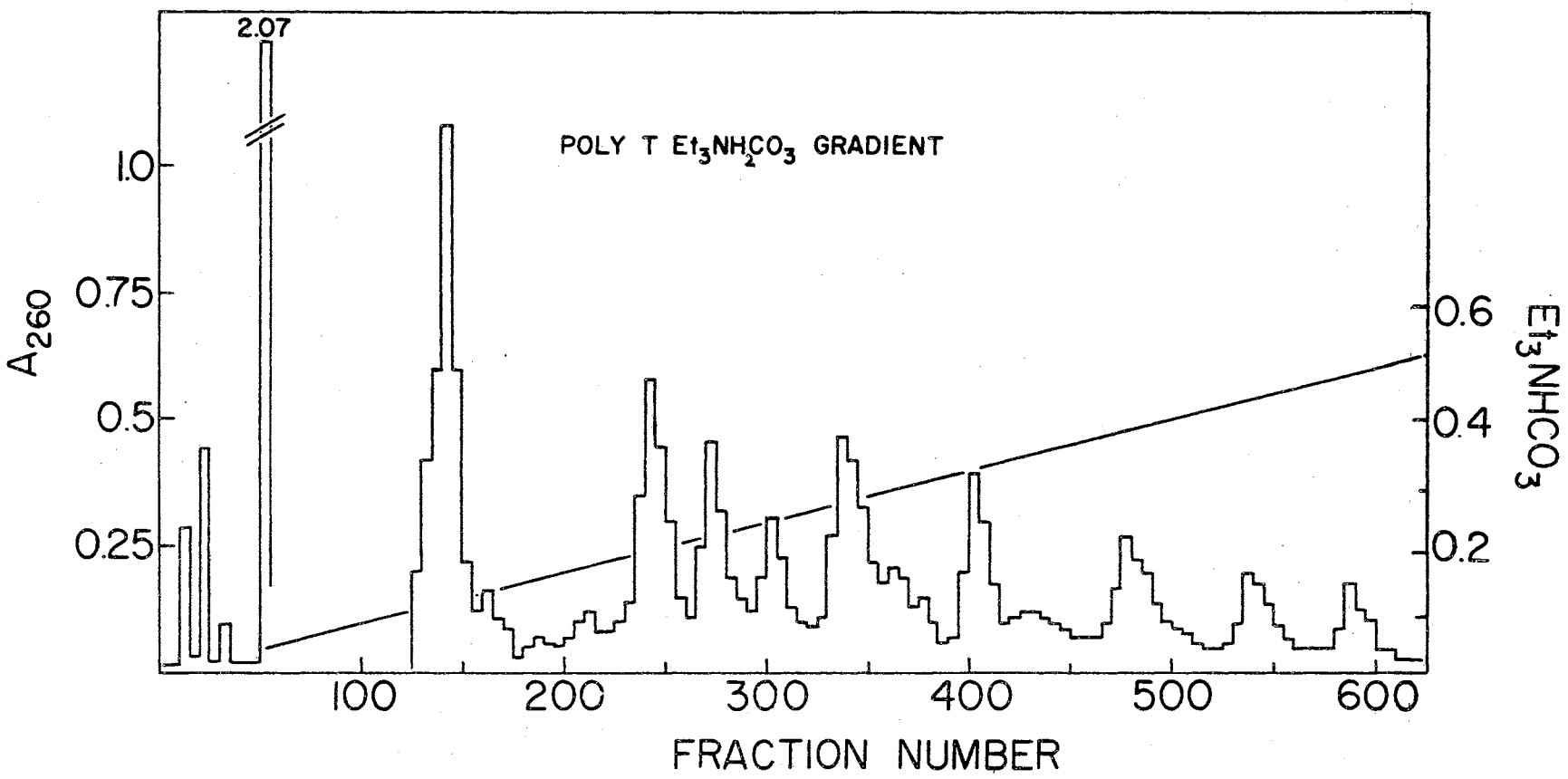
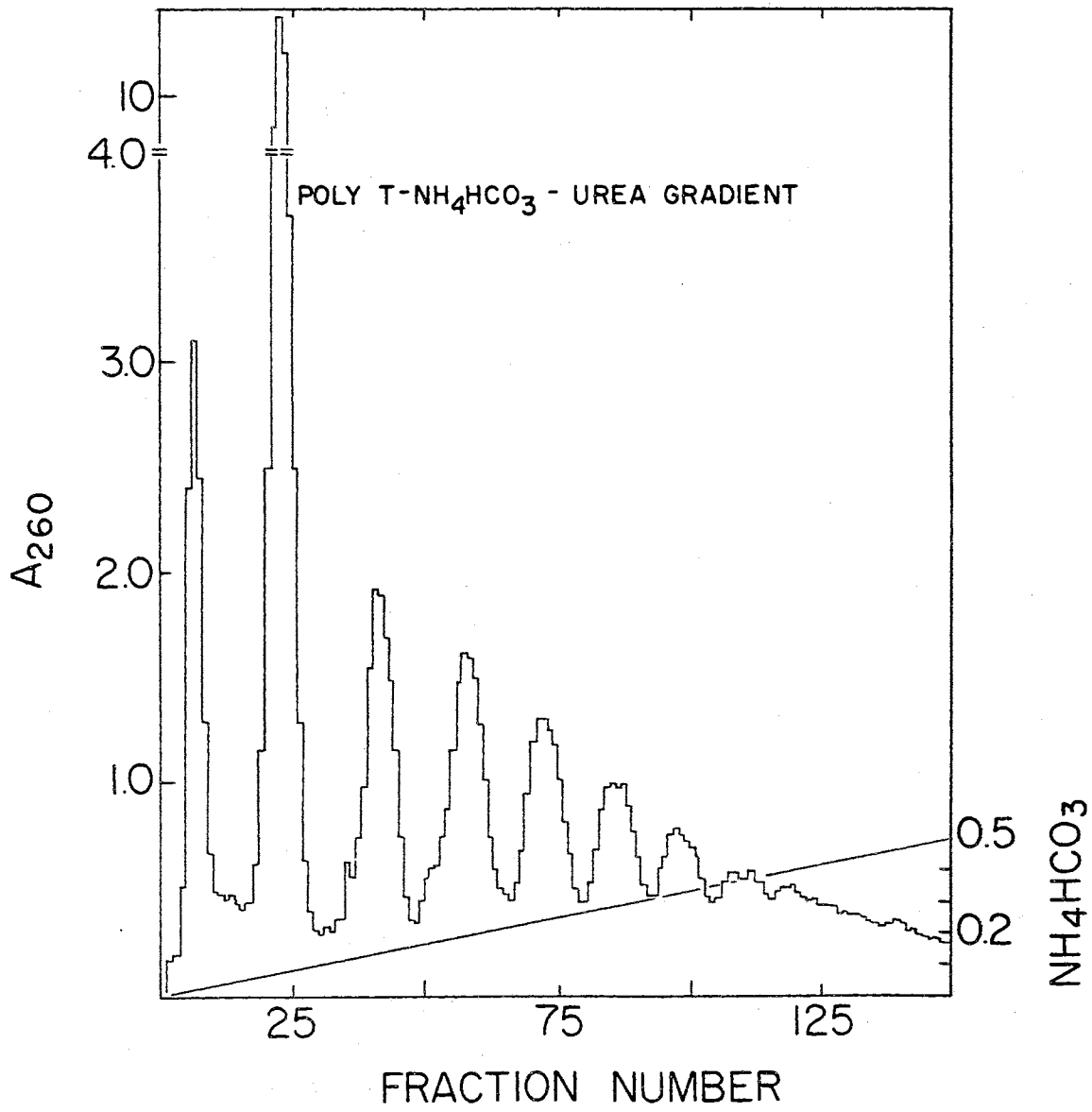

2.07
POLY T $Et_3NH_2CO_3$ GRADIENT
1.0
0.75
0.5
0.25
A_{260}
0.6
0.4
0.2
Et_3NHCO_3
100
200
300
400
500
600
FRACTION NUMBER

Figure 3

Chromatography of Oligothymidylic Acid on DEAE-Cellulose Using Ammonium Bicarbonate-Urea

Oligothymidylic acid (0.1 mmole, pH 8-9) was chromatographed on DEAE-cellulose column (4 x 45 cm, bicarbonate form) which was packed in 7 M urea solution. A linear gradient of ammonium bicarbonate (0-0.5 M, pH 6.7) in 7 M urea solution was used as eluant, 5 ml fractions being collected.


POLY T-NH4HCO3 - UREA GRADIENT
A260
10
4.0
3.0
2.0
1.0
0.5
0.2
NH4HCO3
25
75
125
FRACTION NUMBER

Solvent III: isopropanol:concentrated ammonium hydroxide: acetic acid:water = 4:1:2:2 (75)

Solvent IV: n-propanol:concentrated ammonium hydroxide: water = 55:10:35 (76)

Solvent V: ethanol:1 M ammonium acetate (pH 7.5) = 5:2 (76)

Solvent VI: isobutyric acid:1 M ammonium hydroxide:ethylenediamine tetra acetate (disodium, 0.1 M) = 100:60:1.6

Enzymatic Degradations

The terminal phosphate groups were removed by orthophosphoric-monoester phosphohydrolase (E. C. No. 3.1.3.1) (Worthington Biochemicals Corp., Freehold, N. J.). About 0.1 µmole (1.0 A_{260} unit) of nucleotide was contained in 0.05 ml of water. To it 0.002 ml of 1 M glycine buffer (pH 8.0) and 4 µg of enzyme (about 2 units) were added and the mixture was incubated for two hours at 37° (76).

The chain length of oligonucleotides was determined from the ratio of total phosphate to phosphate that was removed by alkaline phosphatase.

Phosphorus determinations were made according to Chen, Toribara and Warner (80).

Protein was determined according to Lowry, Rosenbrough, Farr and Randall (81).

Preparation of Selective Chromatographic Material

Nucleotidic material (2 mmoles) was taken to dryness, in vacuo, using a rotary evaporator with a water bath temperature below 15°. Dry air was admitted to the system followed by addition of about 10 ml dry pyridine and drying was repeated four more times. The residue was

dissolved in 50 ml dry pyridine and 6 g of cellulose, from which the fines were removed by suspension in water, and then dried in vacuo, over phosphorous pentoxide at 110° for two days (Whatman Powdered Standard Grade), 2.5 g DCC and a few small glass beads (that were dried in a drying oven) were added and the mixture was shaken mechanically for five days. Cellulose was filtered over a glass fritted funnel and washed with pyridine. It was allowed to stand over night in 50% aqueous pyridine and then washed with warm ethanol and water. The combined washings and filtrate were extracted three times with ether and were brought to dryness six times from water in a rotary evaporator. The residue was dissolved in distilled water and the amount of nucleotide material that did not react with cellulose was determined at 260 mμ in this solution.

Preparation of s-RNA

E. coli (Crookes strain grown in a medium consisting of 1% yeast extract, 1% bactotryptone, 0.5% glucose, 0.5% potassium monohydrogen phosphate) s-RNA was prepared according to the method described by Zubay (82). The commercial s-RNA (E. coli strain B) (General Biochemicals Incorporated) that was later used was stripped of the bound endogenous amino acids according to the same procedure. s-RNA (1 g) was suspended in 40 ml of 0.2 M glycine buffer (pH 10.3) and incubated for three hours at 36°. Solid sodium chloride was added to a concentration of 0.4 M and s-RNA was precipitated by the addition of two volumes of ethanol. The precipitate was dissolved in water and dialyzed over night against two changes of distilled water (400 volumes) in the cold room. Stripped s-RNA was obtained by lyophilization.

Preparation of Charged s-RNA

Modified Zubay Procedure (82)

A reaction mixture containing 500 μmoles of tris buffer (pH 7.5), 5 μmoles of magnesium chloride, 250 μmoles of KCl, 5 μmoles of β mercaptoethanol, 15 μmoles of ATP (disodium), 1 μmole of GTP, 4.2 μμmoles of ^{14}C-lysine or 2.7 μμmoles of ^{14}C-phenylalanine or 5.0 μμmoles of ^{14}C-proline (New England Nuclear, uniformly labeled L-isomer), 10 mg E. coli s-RNA (either prepared according to Zubay or purchased from General Biochemicals Incorporated) and 3 mg of S-100 fraction (prepared as described by Nirenberg (83)) in a total volume of 5 ml was incubated for 30 minutes at 37°. The reaction was stopped by addition of 2.5 ml of water and 19 ml of 88% aqueous, redistilled phenol. The mixture was shaken vigorously for six minutes and then centrifuged at 30,000 x g for 20 minutes. The upper layer which contained the s-RNA was removed by pipette and to it was added 0.1 volume of 1 M sodium chloride and two volumes of ethanol. After standing in the cold for about 20 minutes the precipitate was collected by centrifugation at 16,000 x g and either dialyzed over night against three changes of 60 volumes of distilled water or washed three times by successively suspending in 1 M sodium acetate (pH 5.0) and precipitating with ethanol. The final precipitate was either dissolved in 0.05 M sodium acetate (pH 5.0) and the solution stored at -20° or dissolved in distilled water and lyophilized and kept at -20°.

General Chromatographic Procedure

The nucleotide-cellulose was poured into a 1.2 x 2.0 cm chroma-

tography column and washed thoroughly with "concentrated buffer" (1 M sodium chloride in 0.01 M sodium phosphate buffer, pH 7.0). Charged s-RNA (15 A_{260} units) was introduced into the column in 1 ml concentrated buffer. During the first experiments the column was left for two hours for equilibration. Later, the same results were obtained, without equilibration. The column was eluted with 25 ml concentrated buffer, 1 ml fractions being collected. This was sufficient to elute the A_{260} peak. Then elution was continued with 25 ml of a "dilute buffer" (0.01 M sodium chloride in 0.0001 M sodium phosphate buffer, pH 7.0), to elute the retained material. After the chromatography, the column was regenerated by washing with "dilute buffer" followed by "concentrated buffer."

Incorporation Into Trichloroacetic Acid-Tungstate Insoluble Material

^{14}C-Phe-sRNA and a dialyzed, lyophilized "second peak" eluted from the oligothymidylate column were incubated separately in a total volume of 0.25 ml, that contained 25 μmoles of tris buffer (pH 7.8), 40 μmoles ammonium chloride, 2.5 μmoles of magnesium acetate, 0.2 μmoles of GTP, 1 μmole of β-mercaptoethanol, 10 μg polyuridylate (synthesized in our laboratory by T. F. Dunn) and 600 μg of protein from an E. coli 30,000 x g supernatant solution, for 15 minutes at 36° (84). A portion of the mixture was absorbed on a paper disc, washed with ice cold 5% trichloroacetic acid, 0.25% sodium tungstate solution for 15 minutes twice (about 8 ml of solution being used per disc) and once with the same solution at 90° for 15 minutes (85). Then the discs were washed with 95% ethanol, dried and counted with Bray's solution (86) in a Packard Tricarb liquid scintillation counter

(Model 3003, counting efficiency 85%, for ^{14}C internal standard in Bray's solution).

CHAPTER III

RESULTS

Fractionation of Oligonucleotides

Fractionation of the Oligonucleotides

Ecteola-Cellulose-Lithium Chloride System

This system yielded pure fractions of oligothymidylic acid, in two runs, as judged by paper chromatography in solvents I and II, using the descending technique. An elution pattern of 0.2 mmoles of oligothymidylate reaction mixture from such a column is shown in Fig. 1. "Peak 191," tetrathymidylate used in chromatography of Phe-sRNA, was obtained in this fractionation. However, this system lacked the convenience of the linear gradient elution system using triethylammonium bicarbonate (pH 7.5) and removal of lithium chloride was rather difficult. The resolution of this system was also limited, as the number of fractions obtained in this system from the same batch of oligothymidylate was considerably smaller than that obtained by the gradient elution system.

DEAE-Cellulose-Triethylammonium Bicarbonate System

This system was reproducible as observed in eight runs and the removal of the eluant was simple, due to the volatility of triethylamine. An elution pattern of 1 mmole oligothymidylate reaction mixture from such a column is shown in Fig. 2. However, fractionation of

1 mmole of oligonucleotide mixture required 12-16 liters of buffer and concentration of the pooled fractions was a time consuming operation. The buffer was prepared by passing carbon dioxide gas through an ice cooled suspension of triethylamine (141 ml) in water, until pH dropped to 7.5. Then the solution was made up to one liter with ice cold water. During chromatography, at room temperature, carbon dioxide was released which interrupted the fluid flow through the gradient bridge between reservoir and mixing vessel. In addition to this difficulty the fractions obtained contained invariably some of the higher and lower members of oligonucleotides as judged by paper chromatography in solvents I and III, and required either chromatography on a second DEAE cellulose column with different buffer compositions, or paper chromatographic purification.

DEAE-Cellulose-Ammonium Bicarbonate-Urea System

In this system (Fig. 3) the fractions of oligothymidylate emerge in the order of their chain length, that is, according to the charge of the molecule. Each fraction can be contaminated by the cyclic oligonucleotide having one more nucleotide residue. Paper chromatography of individual peaks reveals only two spots up to the tetranucleotide fraction, the R_f values of which correspond to those given for linear and cyclic oligonucleotides. Since in cyclic oligonucleotides all phosphate groups are diesterified, they presumably cannot react with cellulose. This method was found to be the most convenient for fractionating oligonucleotides for the preparation of selective chromatographic material. Prior to charging to cellulose, each fraction was pooled and urea was removed according to a modification of the desalting procedure of Rushizky and Sober (87). The pooled

fractions were diluted to have an ammonium bicarbonate concentration of about 0.01 M, adsorbed on DEAE-cellulose (20 g of DEAE for 200 A_{260} units of nucleotide) and washed with five bed volumes of 0.01 M ammonium bicarbonate (pH 8.6). Then the nucleotides were eluted by 0.7 M ammonium bicarbonate (pH 8.6).

Characterization of the Oligonucleotides

Paper Chromatography

Considerable variation of R_f values occurred between successive runs. The same variation was also observed among the R_f values given by Khorana (75, 79). The use of markers besides pT was found necessary. Fractions obtained by DEAE-ammonium bicarbonate-urea system were used as markers. The results of paper chromatography in three different solvents, of fractions obtained with second system, are given in Table II. A corresponding experiment found in reference (76) is given in Table III.

Enzymatic Degradation

The chain length of the fractions obtained by column chromatography was determined from the ratio of total phosphate to the number of phosphate groups that could be dephosphorylated by alkaline phosphatase. Most references in the literature recommend the use of Tris buffer (pH 8.6) during alkaline phosphatase treatment (76, 88). However, Tris is phosphorylated by the enzyme causing lower results (89) and alkaline phosphatase is inhibited by citrate buffer (90). As a consequence glycine buffer (pH 8.6) was used during alkaline phosphatase treatment. To about 0.1 μmole of nucleotide, dissolved in 0.05 ml water, was added 0.002 ml of 1 M glycine buffer (pH 8.6) and 4 μg of enzyme and the mixture was incubated for two hours at 37°, then the reagents for

TABLE II

R_f AND R_x VALUES OF FRACTIONS OF OLIGOTHYMIDYLIC ACID MIXTURE, OBTAINED BY DEAE-CELLULOSE-TRIETHYLAMMONIUM BICARBONATE SYSTEM, SIMILAR TO THAT SHOWN IN FIG. 2

Fraction	R_f in Solvents			R_x in Solvents[a]			Nature of the fraction, based on R_f and R_x values[c]
	I	II	III	I	II	III	
A	0.81	-	-	7.40	-	-	Pyridinium-pT
B	0.261	0.765	-	2.381	1.085	-	3'-5' TMP
C	0.033[b]	0.732	-	0.298[b]	1.039	-	$(pT)_3$[b], pT
D	-	-	-	-	-	-	-
E	0.11	0.610	0.369	1.00	0.865	0.921	pT, $c(pT)_2$, $(pT)_2$
F	0.052	0.597	0.432[b]	0.235	0.849	1.078[b]	$c(pT)_4$, $c(pT)_2$, pT[b]
G	0.018	-	0.416	0.136	-	1.037	$(pT)_4$
H	0.052	0.296	0.192	0.235	0.421	0.437	$c(pT)_4$ $(pT)_6$
I	-	-	0.22	-	-	0.511	$c(pT)_5$
K	-	-	0.192	-	-	0.437	$(pT)_6$
L	0.017	-	0.195	0.162	-	0.444	$(pT)_6$
M	-	-	0.146	-	-	0.332	$(pT)_7$
N	-	-	0.115	-	-	0.250	$(pT)_8$
O	-	-	0.106	-	-	0.226	$(pT)_9$
P	-	-	0.065	-	-	0.139	$(pT)_{10}$
R	-	-	0.025	-	-	0.053	$(pT)_{11}$
S	-	-	-	-	-	-	-

[a] $R_x = \frac{R_f \text{ of fraction}}{R_f \text{ of pT}}$ in the same solvent

[b] trace amounts, probably due to degradation during lyophilization of the peaks or paper chromatography, except in peak C

[c] the order of R_f and R_x values corresponds to the order of oligonucleotide given; $(pT)_2$; pTpT; $c(pT)_2$ = cyclic pTpT; etc.

TABLE III

R_f AND R_x VALUES OF THE FRACTIONS OF OLIGOTHYMIDYLIC ACID MIXTURE, OBTAINED BY DEAE-CELLULOSE-TRIETHYLAMMONIUM BICARBONATE SYSTEM, GIVEN IN REFERENCE (75)

Fraction	R_f in Solvents		R_x in Solvents[a]		Nature of the fractions
	I	III	I	III	
1	0.55	-	3.11	-	Pyridinium-pT
2	0.47	-	2.65	-	3'-5' TMP
3	0.177	0.43	1.00	-	pT
4	0.24	-	1.40	-	$c(pT)_2$
5	-	0.37	0.54	0.86	$(pT)_2$
6	-	-	0.53	-	$c(pT)_3$
7	-	0.31	0.28	-	$(pT)_3$
8	-	-	0.24	-	$c(pT)_4$
9	-	0.27	0.125	-	$(pT)_4$
10	-	-	-	-	$c(pT)_5$ + unidentified
11	-	0.22	-	-	$(pT)_5$
12	-	0.17	-	-	$(pT)_6$
13	-	-	-	0.34	$(pT)_7$
14	-	-	-	0.27	$(pT)_8$
15	-	-	-	0.20	$(pT)_9$
16	-	-	-	0.14	$(pT)_{10}$
17	-	-	-	0.09	$(pT)_{11}$
18	-	-	-	-	$(pT)_{12}$

[a] $R_x = \frac{R_f \text{ of fraction}}{R_f \text{ of pT}}$ in the same solvent

phosphate determination were added directly into the incubation mixture.

Total phosphate was determined, after hydrolysis of an aliquot of the nucleotidic material with two volumes of 70% perchloric acid at 100° for 15 minutes (Table IV).

Preparation of Selective Chromatographic Material

Nucleotide material was covalently attached to cellulose according to the following schematic reaction.

$$\text{Cellulose-OH} + \text{pT(pT)}_n\text{pT} \xrightarrow{\text{DCC}} \text{Cellulose-O-pT(pT)}_n\text{pT}$$

The extent of reaction, when unfractionated oligonucleotide mixture is used as the starting material, is about 50% as can be seen from Table V. Two separate batches of oligothymidylic acid were reacted with cellulose. The first on a 0.1 mmole scale was used for most of the experiments reported with the small columns (1 x 10 cm, filling) while the second on a 4 mmole scale was used for the large column (2.5 x 10 cm, filling) described on page 54. There were two small oligothymidylate-cellulose columns. With oligodeoxyadenylic acid and oligodeoxycytidylic acid the preparation of the selective chromatographic material was performed only once.

Chromatography of Phe-sRNA

Most of the chromatographic experiments were performed with an oligonucleotide-cellulose that was prepared from an unfractionated condensation mixture.

Chromatography of ^{14}C-Phe-sRNA on pure cellulose yielded a single peak around fraction 8, which contained all of the 260 mμ absorbing material as well as radioactivity (Fig. 4). When ^{14}C-Phe-sRNA was chromatographed on oligothymidylate-cellulose (Fig. 5), a second peak

TABLE IV

DETERMINATION OF CHAIN LENGTH OF OLIGOTHYMIDYLATE FRACTIONS OBTAINED BY DEAE-CELLULOSE-TRIETHYLAMMONIUM BICARBONATE SYSTEM GIVEN IN TABLE II

Fraction	Alkaline phosphatase splittable phosphate[1] A_{820} units I	Total phosphate[2] A_{820} units II	Average chain length of fractions II/I
E	0.203	0.298	1.5
F	0.068	0.216	3.2
G	0.064	0.289	4.5
H	0.032	0.180	5.0
I	0.100	0.530	5.3
K	0.074	0.524	7.0
AMP	0.107	0.118	1.1

[1]To about 0.1 μmole of nucleotide, dissolved in 0.05 ml water, was added 0.002 ml of 1 M glycine buffer (pH 8.6) and 4 μg of enzyme and the mixture was incubated for two hours at 37°.

[2]Total phosphate was determined on an aliquot of nucleotide after hydrolysis with two volumes of 70% perchloric acid at 100° for 15 minutes.

TABLE V

REACTION OF VARIOUS OLIGODEOXYRIBONUCLEOTIDES WITH CELLULOSE

Polymer	Wave length mμ	Amount before reaction A_{260} units	Amount unreacted A_{260} units	Extent of reaction %
Oligothymidylate	267	1400	625	55
Oligodeoxyadenylate	260	1036	450	57
Oligodeoxycytidylate	272	465	250	48

The extent of reaction was determined by measuring the A units, at the designated wave lengths, before and after the reaction.

Pyridine was removed by evaporating the filtrate six times after the dicyclohexylcarbodiimide was extracted into ether.

Figure 4

Chromatography of Phe-sRNA on Cellulose

Phe-sRNA (15 A units at 260 mμ) was chromatographed at room temperature. After introduction of the labeled s-RNA the column equilibrated for two hours and was eluted first with 25 ml concentrated buffer (1 $\underline{\underline{M}}$ sodium chloride + 10^{-2} $\underline{\underline{M}}$ sodium phosphate, pH 7.0) then with 25 ml dilute buffer (10^{-2} $\underline{\underline{M}}$ sodium chloride + 10^{-4} $\underline{\underline{M}}$ sodium phosphate, pH 7.0), 1 ml fractions being collected. Open circles represent A_{260} and closed circles, counts per minute. The arrow indicates the change of the buffer.

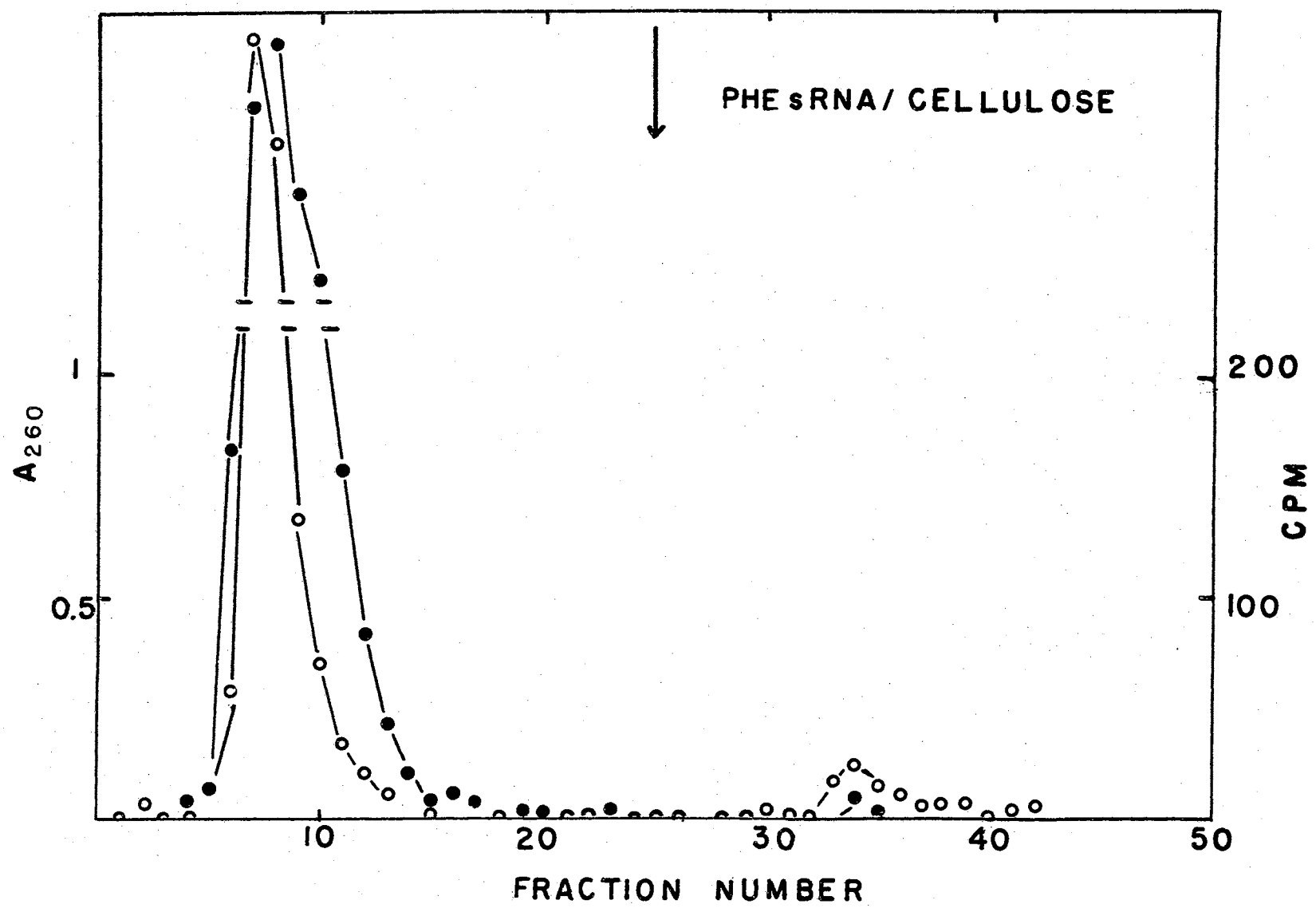
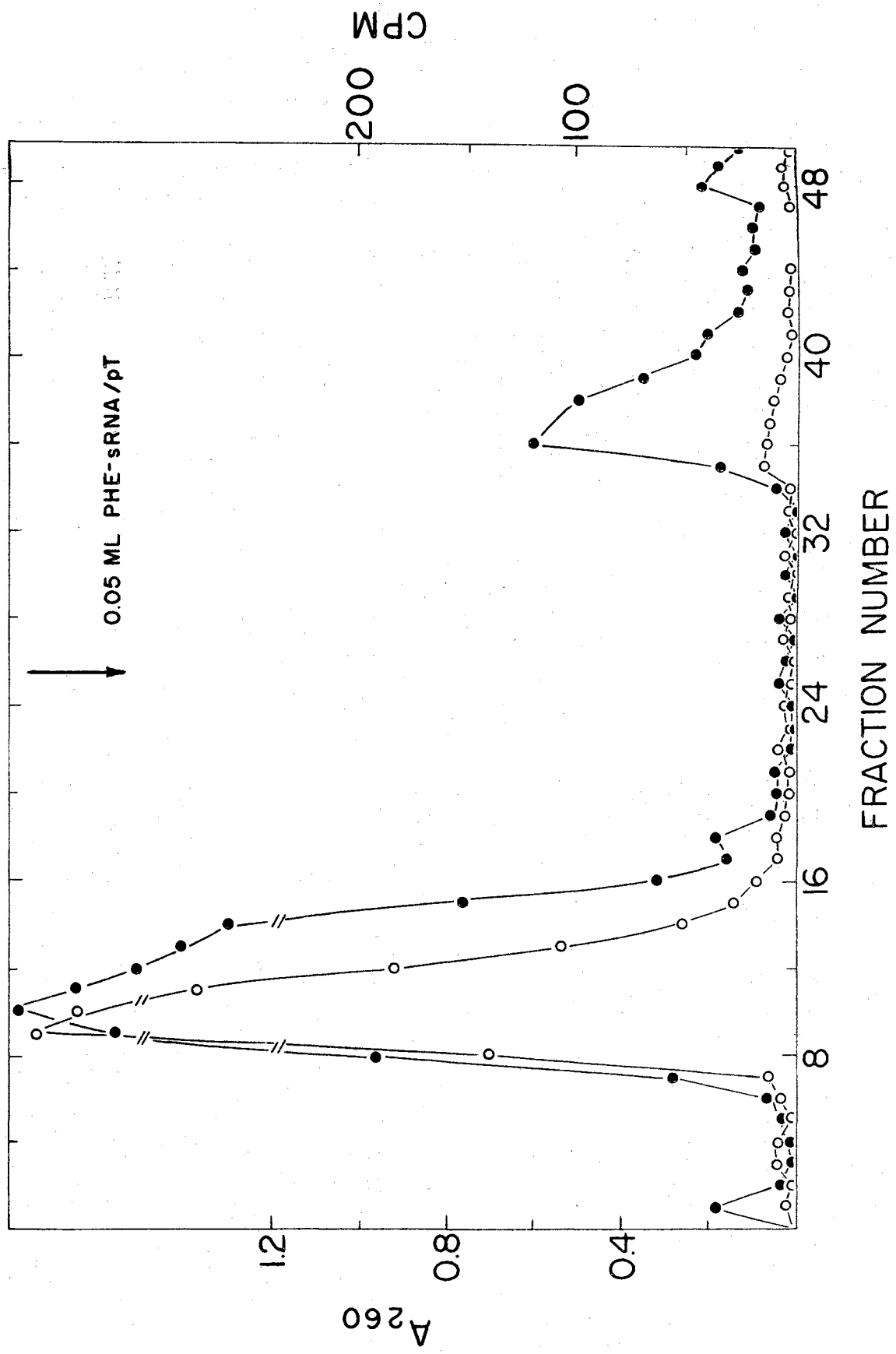

PHE sRNA/ CELLULOSE
A260
1
0.5
200
100
CPM
10
20
30
40
50
FRACTION NUMBER

Figure 5

Chromatography of Phe-sRNA on Oligothymidylate-Cellulose

Phe-sRNA (15 A units at 260 mμ) was chromatographed at room temperature. After introduction of the labeled s-RNA, the column equilibrated for two hours and was eluted first with 25 ml of concentrated buffer (1 M sodium chloride + 10^{-2} M sodium phosphate, pH 7.0) then with 25 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 7.0), 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.


CPM
200
100
0.05 ML PHE-sRNA/pT
A260
1.2
0.8
0.4
8
16
24
32
40
48
FRACTION NUMBER

appeared around fraction 36 with dilute buffer, after the first peak containing 260 mμ absorbing and radioactive material had been eluted with concentrated buffer. The specific activity of the ^{14}C-Phe-sRNA in the second peak was increased 3.5 fold. This result represents purification of a particular s-RNA species (Fig. 5). When enzymatically synthesized polyuridylic acid was covalently attached to cellulose, chromatography resulted in the retention of a second peak of Phe-sRNA (Fig. 6). The polyuridylic acid used contained about 300 uridylic acid residues per molecule.

When ^{14}C-Phe-sRNA was chromatographed on polyadenylate-cellulose column, there was no retention of a second peak (Fig. 7). This result demonstrates the selectivity of the method.

Peak 191, which contains tetrathymidylate obtained by Ecteola-cellulose chromatography using lithium chloride as the eluant, when attached to cellulose resulted in the retention of Phe-sRNA (Fig. 8 top). Peak G, tetrathymidylate obtained by DEAE-cellulose chromatography, using triethylammonium bicarbonate as the eluant, when attached to cellulose also retains Phe-sRNA (Fig. 8 bottom). These results demonstrate that oligonucleotides of comparable size to codons can be used to retain a particular s-RNA species.

Chromatography of Lys-sRNA

Chromatography of ^{14}C-Lys-sRNA on cellulose did not result in the retention of a second peak, as was the case with Phe-sRNA (Fig. 9). ^{14}C-Lys-sRNA was retained on oligodeoxyadenylate-cellulose column and the 260 mμ absorbing and radioactive material of the second peak could be eluted by the dilute buffer (Fig. 10). Enzymatically synthesized polyadenylic acid, when attached to cellulose covalently, also caused

Figure 6

Chromatography of Phe-sRNA on Polyuridylate-Cellulose

Phe-sRNA (15 A units at 260 mμ) was chromatographed at room temperature. After introduction of the labeled s-RNA, the column equilibrated for two hours and was eluted first with 25 ml of concentrated buffer (1 $\underline{\underline{M}}$ sodium chloride + 10^{-2} $\underline{\underline{M}}$ sodium phosphate, pH 7.0) then with 25 ml of dilute buffer (10^{-2} $\underline{\underline{M}}$ sodium chloride + 10^{-4} $\underline{\underline{M}}$ sodium phosphate, pH 7.0), 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.

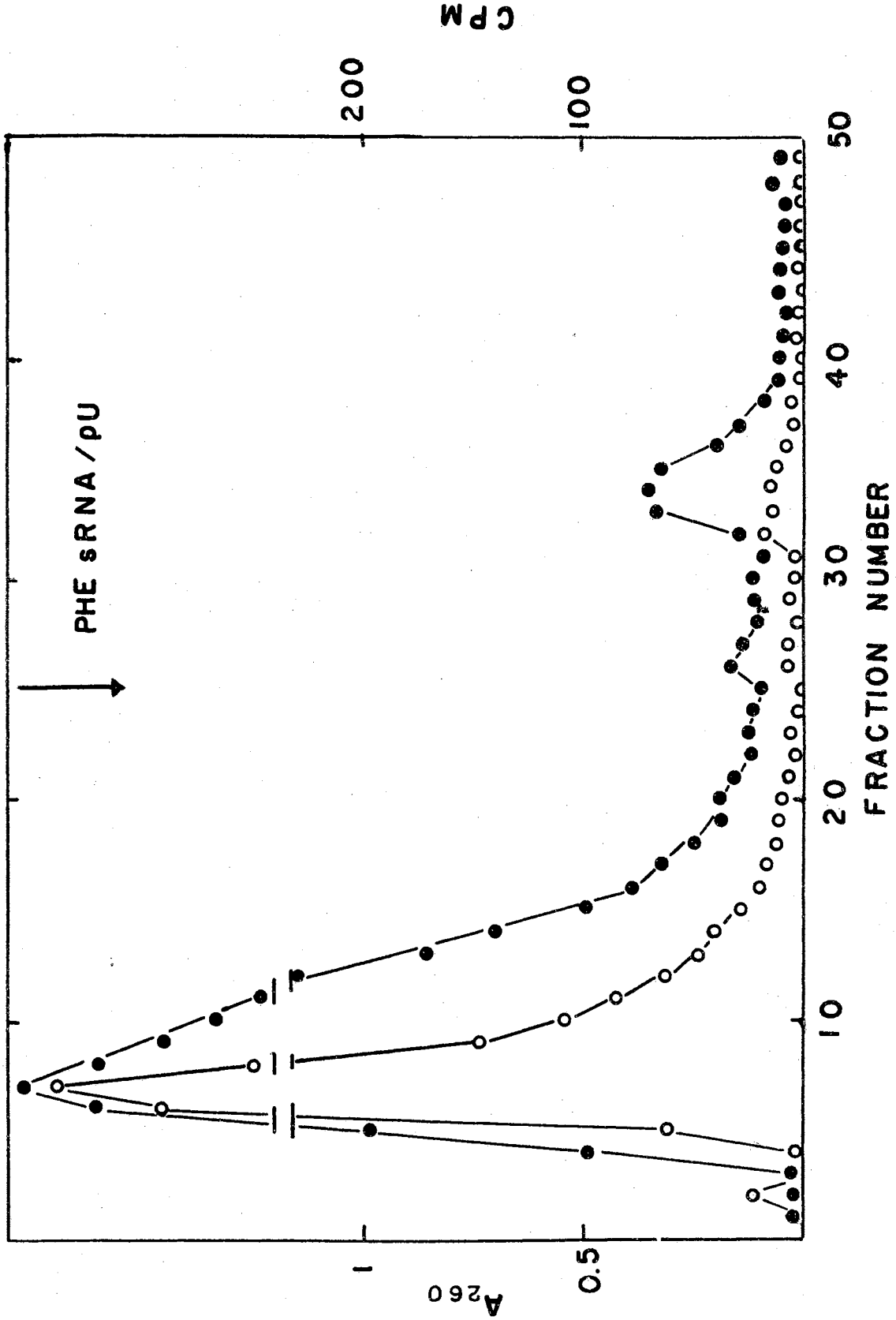

CPM
200
100
50
40
30
20
10
FRACTION NUMBER
PHE sRNA / pU
A260
1
0.5

Figure 7

Chromatography of Phe-sRNA on Polyadenylate-Cellulose

Phe-sRNA (15 A units at 260 mμ) was chromatographed at room temperature. After introduction of the labeled s-RNA, the column equilibrated for two hours and was eluted first with 25 ml of concentrated buffer (1 $\underline{\underline{M}}$ sodium chloride + 10^{-2} $\underline{\underline{M}}$ sodium phosphate, pH 7.0) then with 25 ml of dilute buffer (10^{-2} $\underline{\underline{M}}$ sodium chloride + 10^{-4} $\underline{\underline{M}}$ sodium phosphate, pH 7.0), 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.

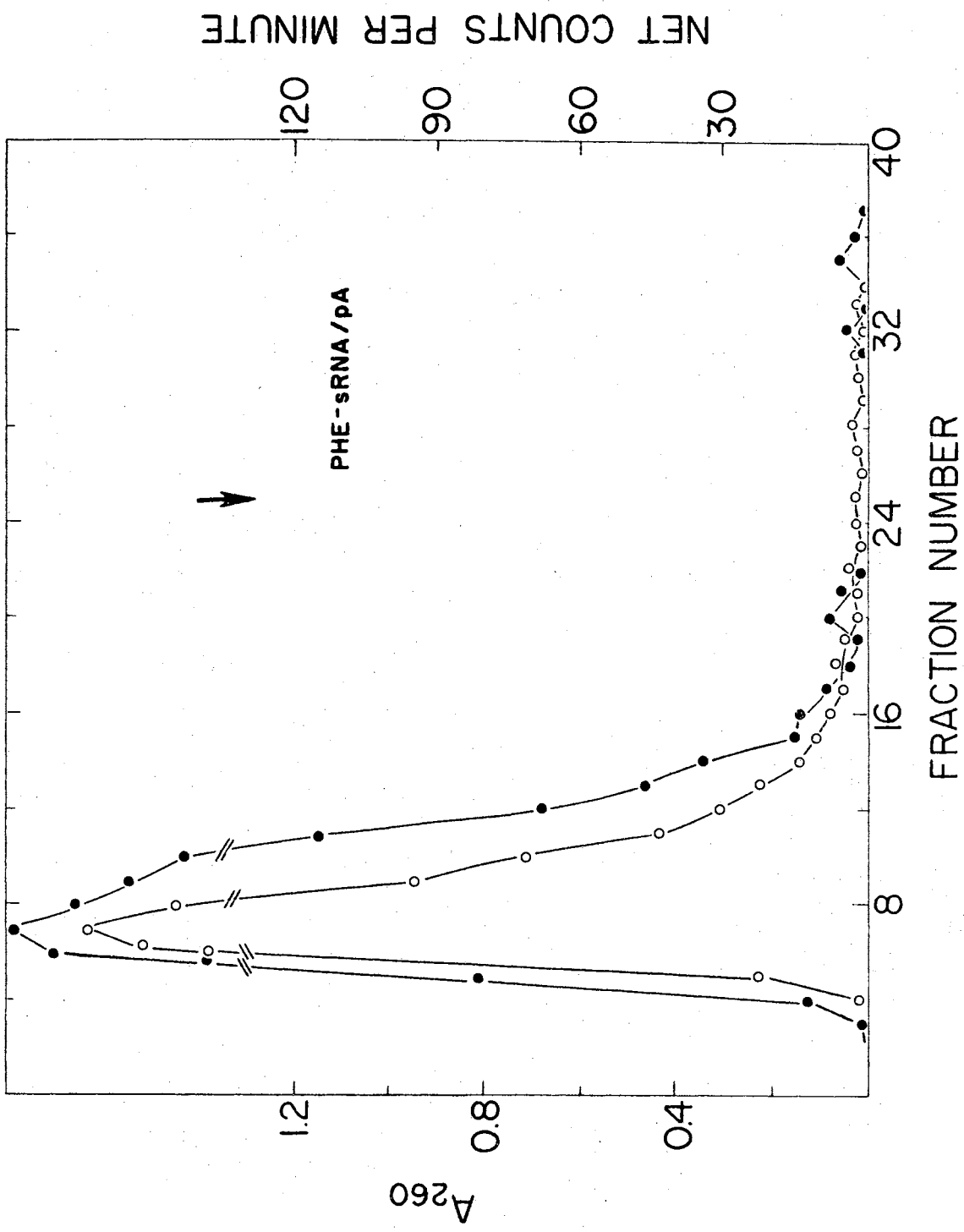

NET COUNTS PER MINUTE
120
90
60
30
PHE-sRNA/pA
A260
1.2
0.8
0.4
8
16
24
32
40
FRACTION NUMBER

Figure 8 Top

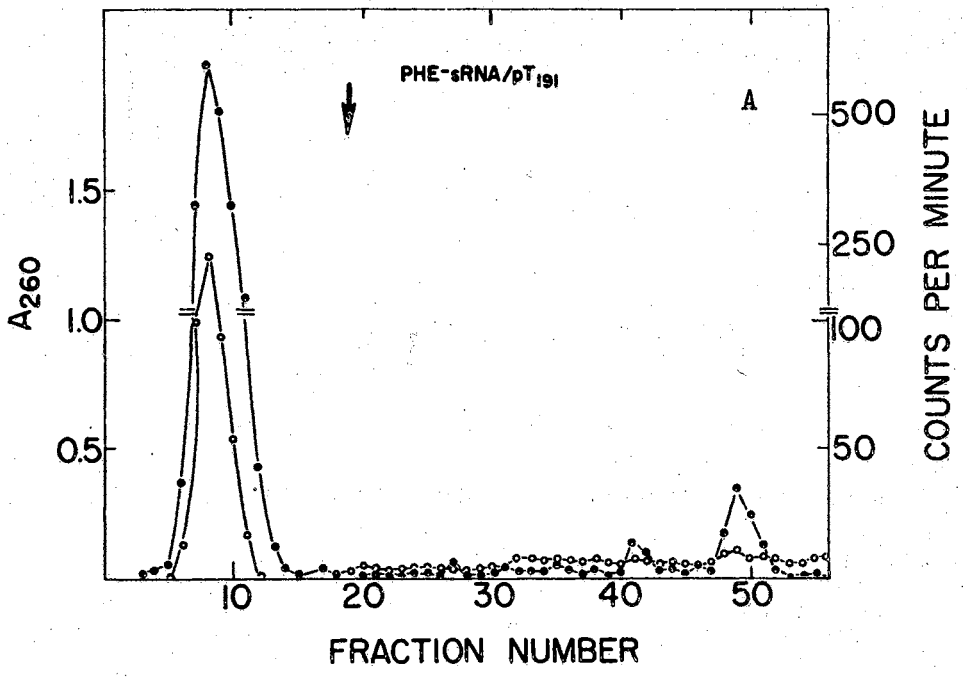
Chromatography of Phe-sRNA on Peak 191 of Fig. 1

Phe-sRNA (8 A units at 260 mμ) was chromatographed at room temperature, on tetrathymidylate-cellulose, peak 191 of Fig. 1. Elution was started immediately after the introduction of labeled s-RNA with 18 ml of concentrated buffer (1 $\underline{M}$ sodium chloride + 10^{-2} $\underline{M}$ sodium phosphate, pH 7.0) then with 37 ml of dilute buffer (10^{-2} $\underline{M}$ sodium chloride + 10^{-4} $\underline{M}$ sodium phosphate, pH 7.0), 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute

Figure 8 Bottom

Chromatography of Phe-sRNA on Peak G of Table II

Phe-sRNA (15 A units at 260 mμ) was chromatographed at 2^{o}, on nucleotide-cellulose containing peak G of Table II. Table IV shows that this peak corresponds to tetrathymidylate. Elution was started immediately after the introduction of labeled s-RNA with 40 ml of concentrated buffer (2 $\underline{M}$ sodium chloride + 10^{-2} $\underline{M}$ sodium phosphate, pH 5.6) then with 40 ml of dilute buffer (10^{-2} $\underline{M}$ sodium chloride + 10^{-4} $\underline{M}$ sodium phosphate, pH 5.6), 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.


PHE-sRNA/pT191
A
A260
1.5
1.0
0.5
500
250
100
50
COUNTS PER MINUTE
10
20
30
40
50
FRACTION NUMBER

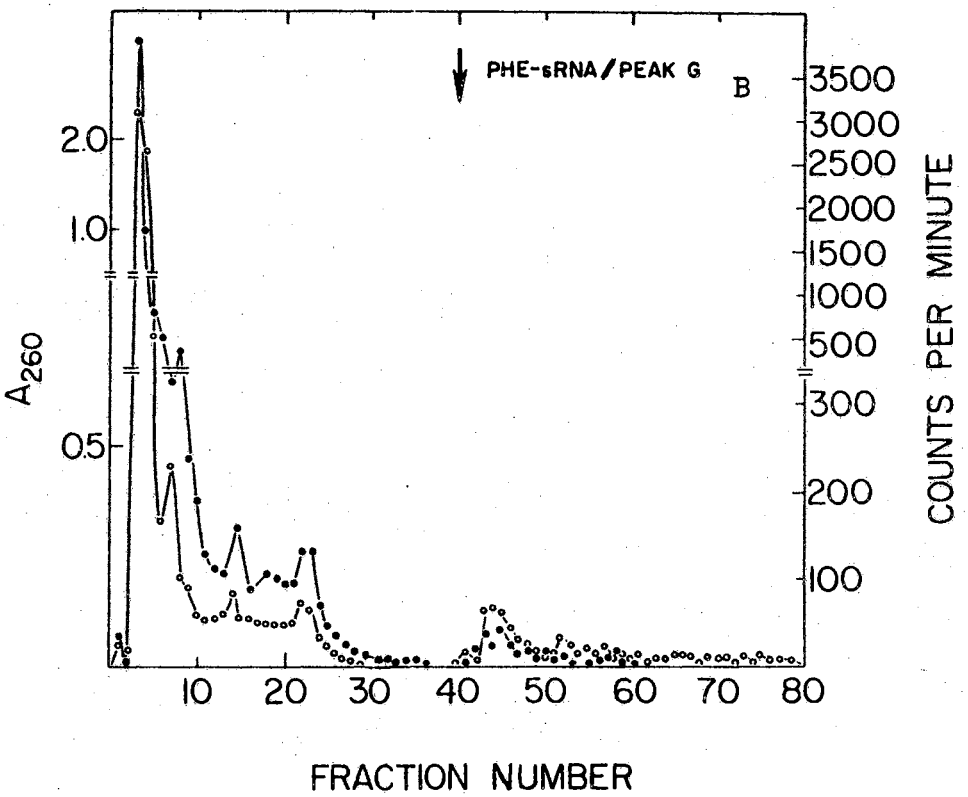

PHE-sRNA/PEAK G
B
A260
2.0
1.0
0.5
3500
3000
2500
2000
1500
1000
500
300
200
100
COUNTS PER MINUTE
10
20
30
40
50
60
70
80
FRACTION NUMBER

Figure 9

Chromatography of Lys-sRNA on Cellulose

Lys-sRNA (15 A units at 260 mμ) was chromatographed at 2°, without equilibration. Elution was started with 25 ml of concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 5.6) then with 25 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 5.6), 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.

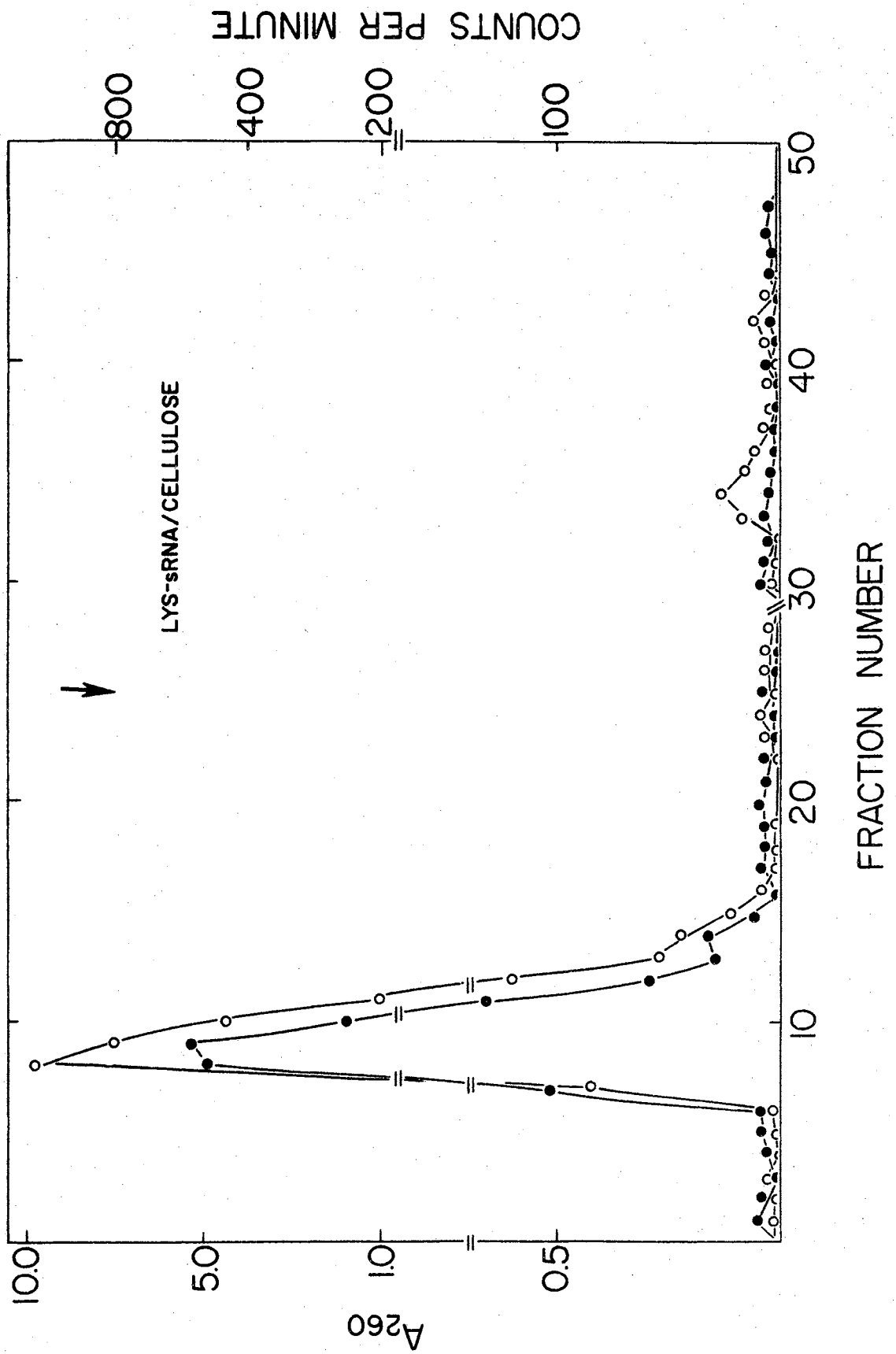
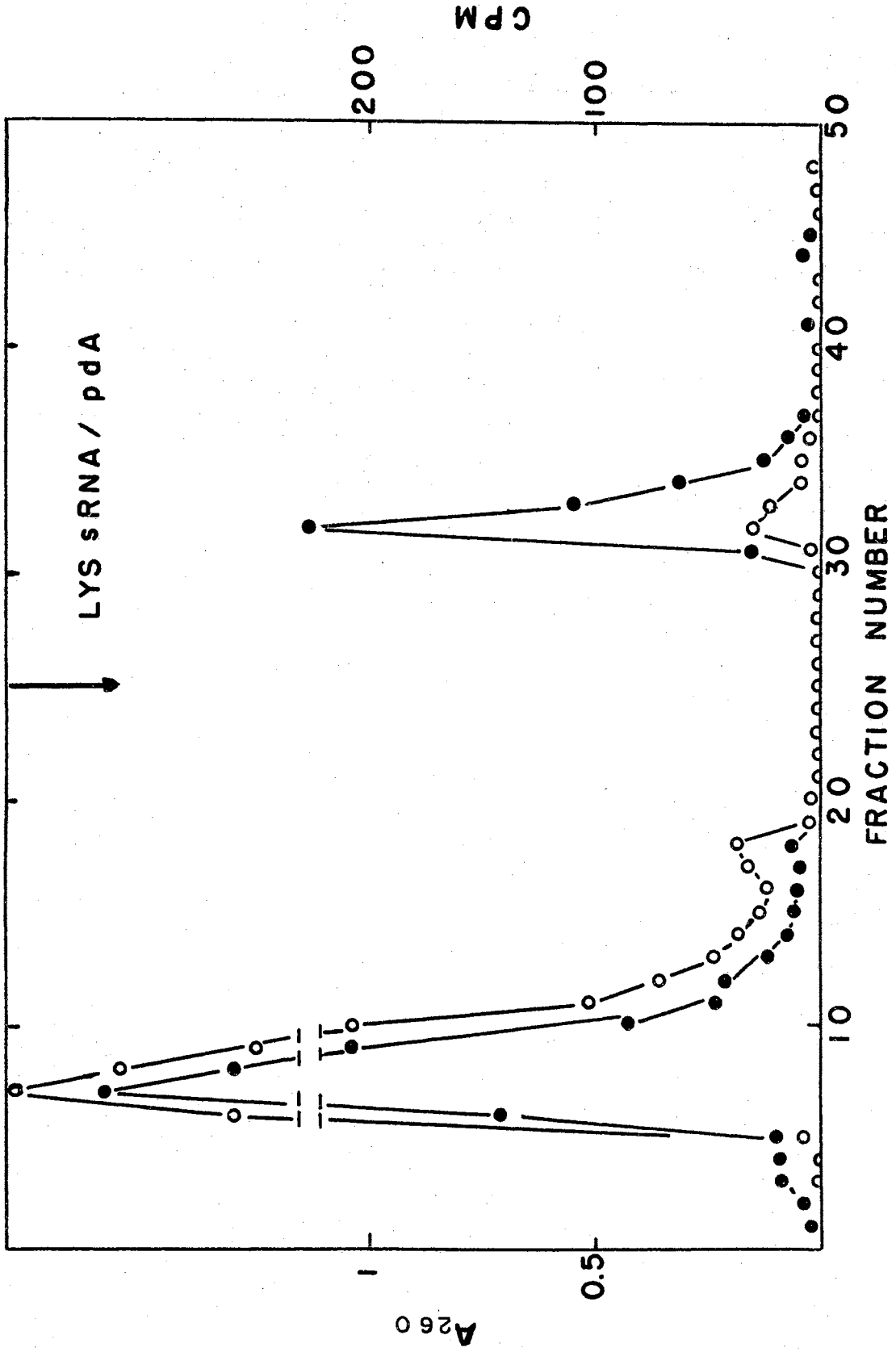

COUNTS PER MINUTE
800
400
200
100
LYS-sRNA/CELLULOSE
A260
10.0
5.0
1.0
0.5
10
20
30
40
50
FRACTION NUMBER

Figure 10

Chromatography of Lys-sRNA on Oligodeoxyadenylate-Cellulose

Lys-sRNA (45 A units at 260 mμ) was chromatographed at 50°, without equilibration. Elution was started with 25 ml of concentrated buffer (1 M sodium chloride + 10^{-2} M sodium phosphate, pH 7.0) then with 25 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 7.0); 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.


CPM
200
100
50
40
30
20
10
FRACTION NUMBER
LYS sRNA / pdA
A260
1
0.5

retention of ^{14}C-Lys-sRNA (Fig. 11). In control experiments with oligothymidylate-cellulose column, ^{14}C-Lys-sRNA was not retained, showing the selectivity of the method (Fig. 12). These experiments demonstrate that the results are not unique for Phe-sRNA and the retention of various s-RNA species is not incidental.

The appearance of the major portion of radioactivity in the first peak was not expected at the beginning of the investigations. This radioactivity could result from three causes:

1) overloading of the column

2) lability of the amino acyl-sRNA bond

3) degeneracy

Another possibility was the inability to find the right conditions for the system; so, before designing experiments to answer the above possibilities, the effect of various parameters on the retention of s-RNA was investigated.

Characterization of the System

Effect of Buffer Composition

Since the molecular weight of s-RNA molecule is about 25-30,000, an increase of ionic strength by raising the concentration of sodium chloride from 1 M to 2 M was hoped to improve the retention of s-RNA molecules. Fig. 13 shows that the elution patterns corresponds to each other favorably, hence the higher ionic strength was used for many experiments.

Effect of Buffer pH

To help increase the stability of amino acyl-sRNA bond, the effect of a change of buffer pH from 7.0 to 5.6 was investigated. This was

Figure 11

Chromatography of Lys-sRNA on Polyadenylate-Cellulose

Lys-sRNA (15 A units at 260 mμ) was chromatographed at 2°, after two hours of equilibration. Elution was started with 25 ml of concentrated buffer (1 $\underline{\underline{M}}$ sodium chloride + 10^{-2} $\underline{\underline{M}}$ sodium phosphate, pH 7.0) then with 25 ml of dilute buffer (10^{-2} $\underline{\underline{M}}$ sodium chloride + 10^{-4} $\underline{\underline{M}}$ sodium phosphate, pH 7.0); 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.

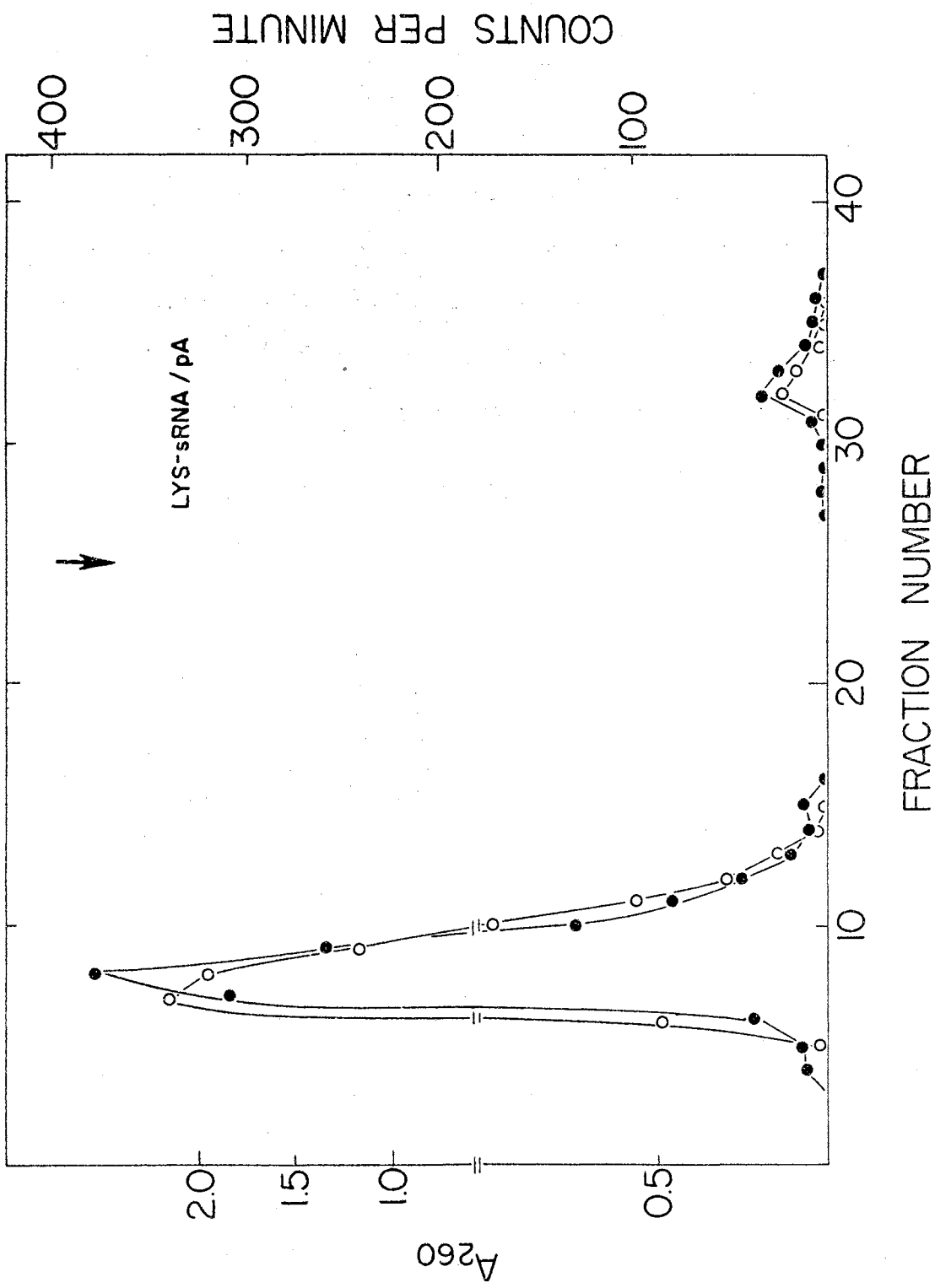

COUNTS PER MINUTE
400
300
200
100
LYS-sRNA / pA
A260
2.0
1.5
1.0
0.5
10
20
30
40
FRACTION NUMBER

Figure 12

Chromatography of Lys-sRNA on Oligothymidylate-Cellulose

Lys-sRNA (15 A units at 260 mμ) was chromatographed at 2° after two hours of equilibration. Elution was started with 25 ml of concentrated buffer (1 M sodium chloride + 10^{-2} M sodium phosphate, pH 7.0) then with 25 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 7.0); 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.

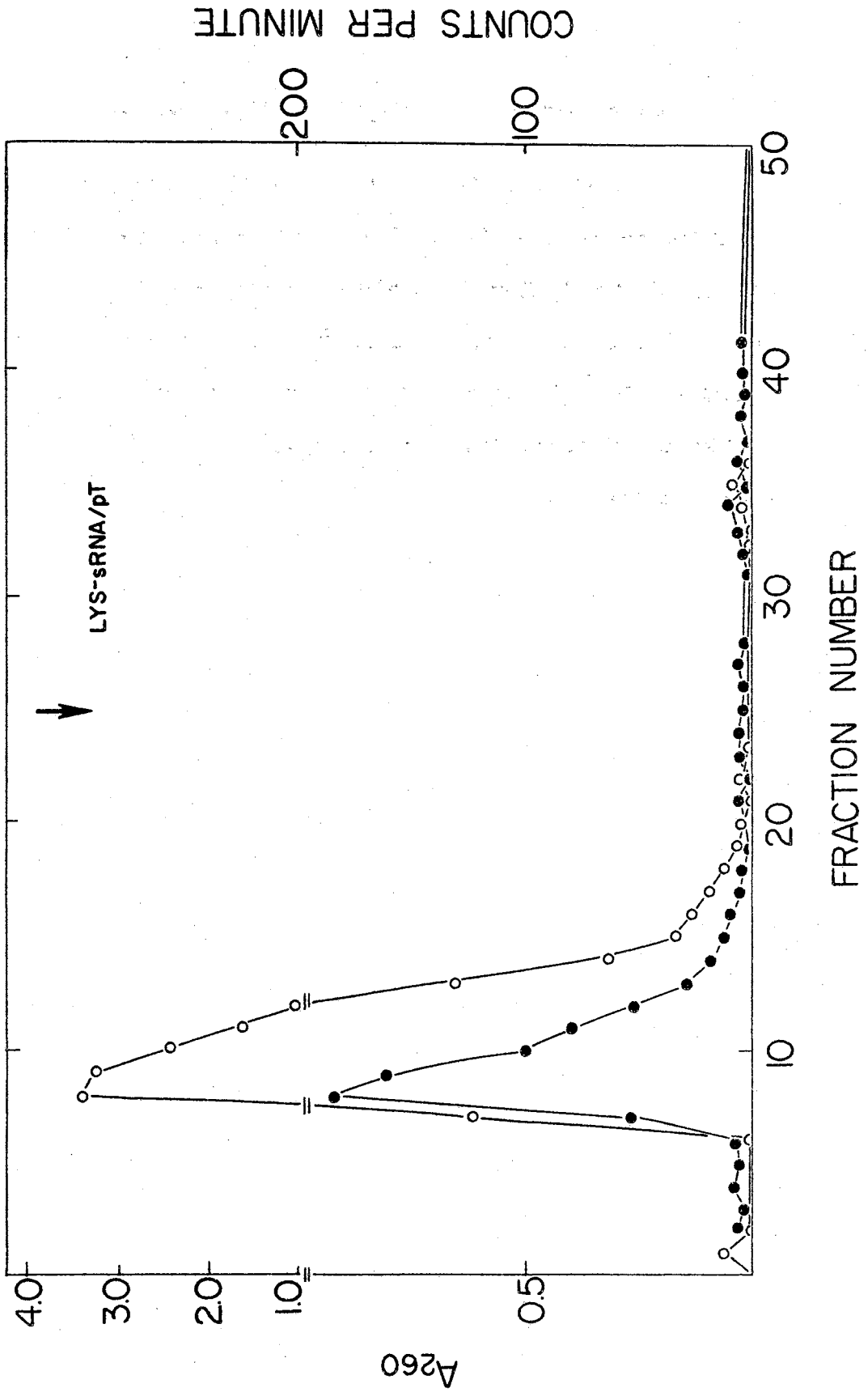
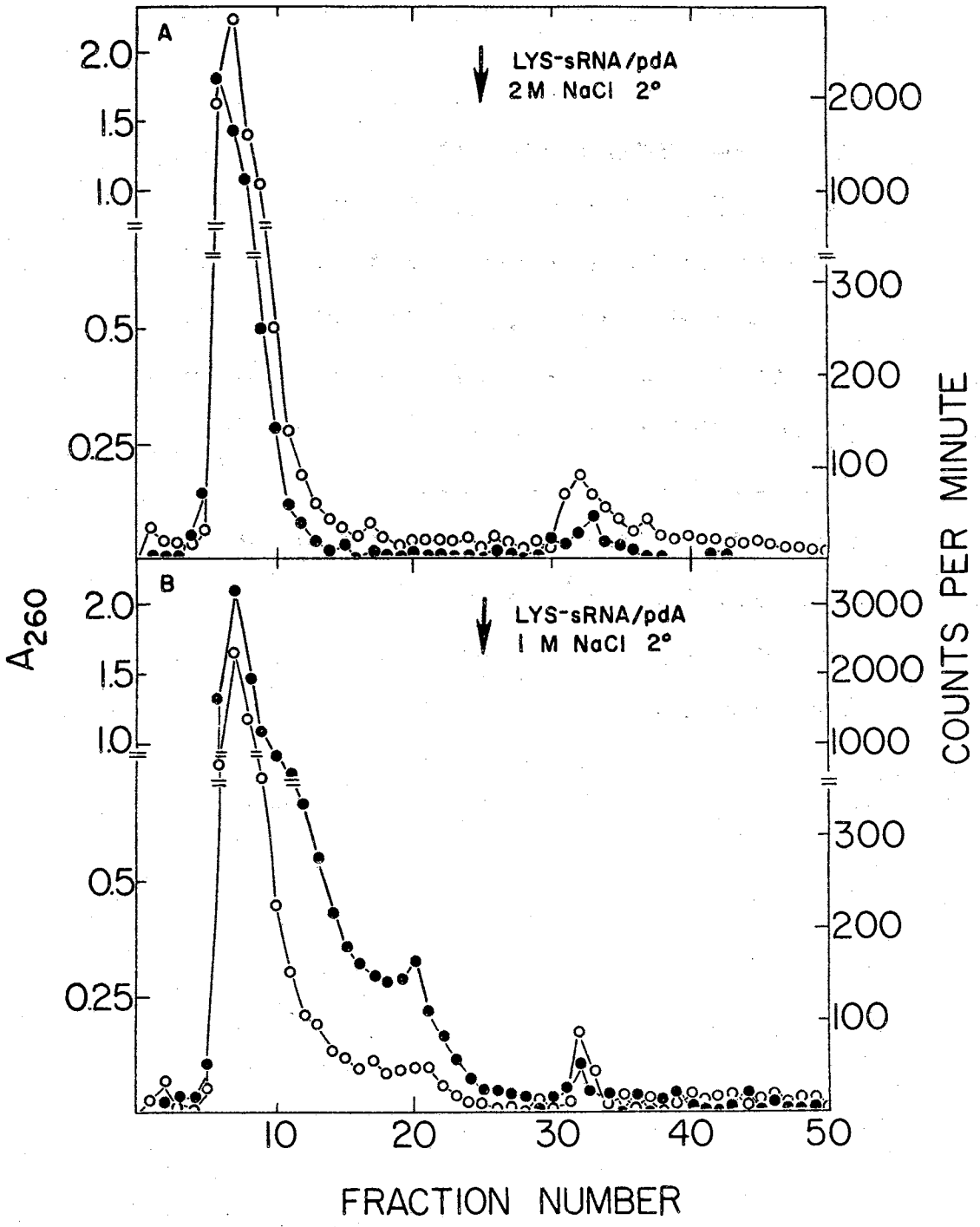

COUNTS PER MINUTE
200
100
50
40
30
20
10
FRACTION NUMBER
LYS-sRNA/pT
A260
4.0
3.0
2.0
1.0
0.5

Figure 13

Effect of Buffer Composition on the Retention of Charged s-RNA

A) Lys-sRNA (15 A units at 260 mμ) was chromatographed at 2°, without equilibration. Elution was started with 25 ml of concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 7.0) then with 25 ml of dilute buffer ($2 \cdot 10^{-2}$ M sodium chloride + 10^{-4} M sodium phosphate, pH 7.0); 1 ml fractions were collected.

B) Lys-sRNA (15 A units at 260 mμ) was chromatographed at 2° C, without equilibration. Elution was started with 25 ml of concentrated buffer (1 M sodium chloride + 10^{-2} M sodium phosphate, pH 7.0) then continued with 25 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 7.0), and fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.


A
LYS-sRNA/pdA
2 M NaCl 2°
B
LYS-sRNA/pdA
1 M NaCl 2°
A260
2.0
1.5
1.0
0.5
0.25
2000
1000
300
200
100
3000
2000
1000
300
200
100
COUNTS PER MINUTE
10
20
30
40
50
FRACTION NUMBER

deemed desirable because of the lability of the bond at alkaline pH, and it was felt that slightly acidic conditions might improve the stability. As will be seen later (Fig. 20), the lability of the bond is still pronounced even at pH 5.6; nevertheless, since the results of chromatography at pH 5.6 did compare favorably with those at pH 7.0 (Fig. 14), pH 5.6 was used in some experiments.

Effect of Elution Scheme

Continued elution of the column with concentrated buffer up to twice the normal volume (50 ml) could not remove the retained s-RNA species, which was then eluted with the dilute buffer (Fig. 15). This result is consistent with the expectation that the retention is due to hydrogen bond formation in solutions of high ionic strength. As soon as the ionic strength of the eluant is reduced to 1:100 to 1:200 of the original, elution of the retained material starts.

Effect of Column Dimensions

Change of height:diameter ratio from 10:1 to 1:2 did not result in a strikingly different elution pattern (Fig. 16). Repeated runs on the large column, that will be described below where height:diameter ratio is 2.5:1, have also substantiated this result.

Effect of Temperature

Chromatography of charged s-RNA was performed at 2°, room temperature (22°) and at 50°. As seen in Fig. 17, there are no gross effects attributable to temperature variation. However, as will be seen below, the higher the temperature the more labile the amino acyl-sRNA bond becomes. As a consequence, most of the experiments were performed at 2°.

Figure 14

Effect of pH on the Chromatography of Charged s-RNA

A) Phe-sRNA (15 A units at 260 mμ) was chromatographed at 2°, without equilibration. Elution was started with 25 ml of concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 7.0) then continued with 25 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 7.0); 1 ml fractions were collected.

B) Phe-sRNA (15 A units at 260 mμ) was chromatographed at 2°, without equilibration. Elution was started with 25 ml of concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 5.6) then continued with dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 5.6); 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.

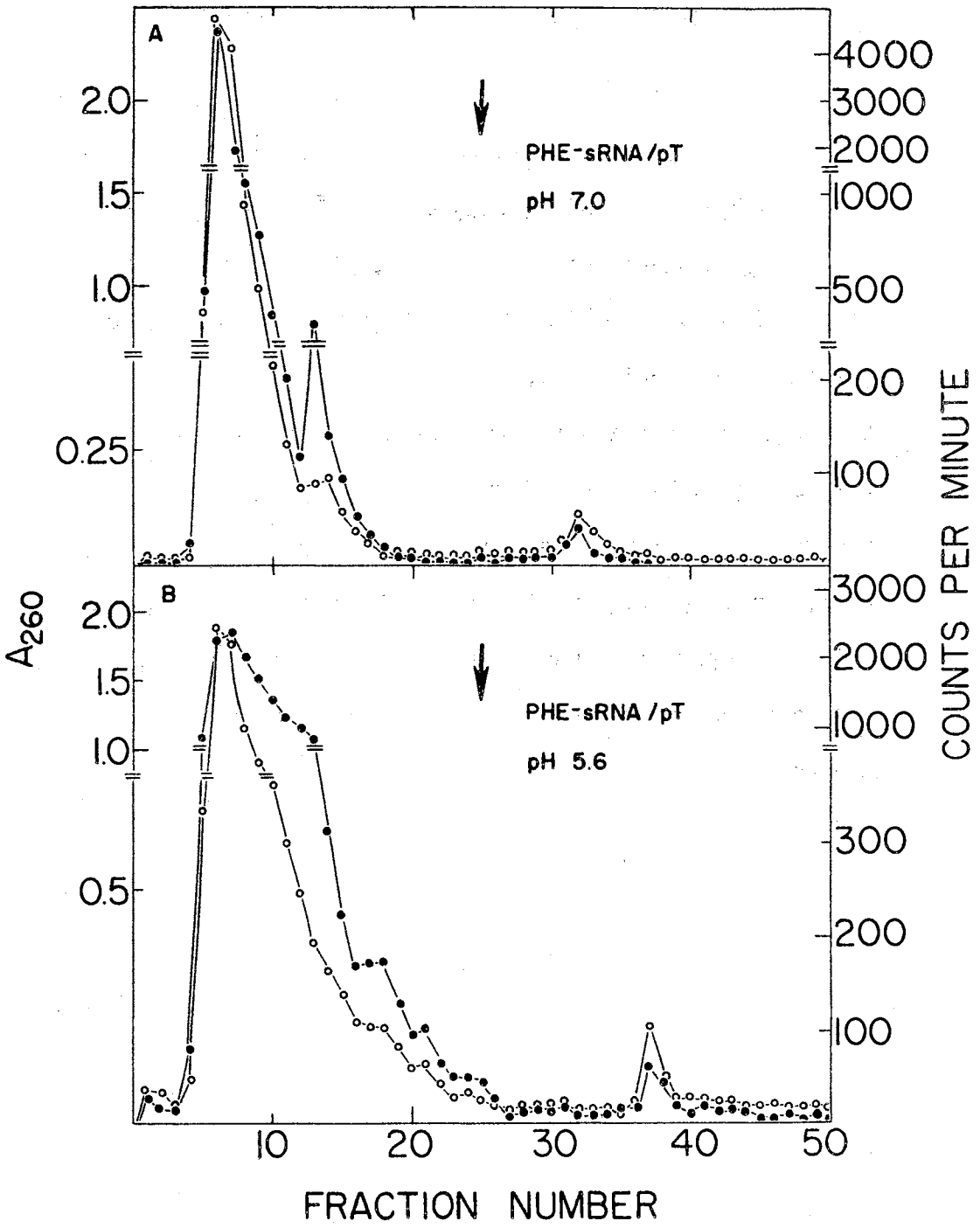

A
PHE-sRNA/pT
pH 7.0
B
PHE-sRNA/pT
pH 5.6
A260
2.0
1.5
1.0
0.25
0.5
4000
3000
2000
1000
500
200
100
300
10
20
30
40
50
FRACTION NUMBER
COUNTS PER MINUTE

Figure 15

Effect of Elution Schedule on the Chromatography of Charged s-RNA

A) Phe-sRNA (15 A units at 260 mμ) was chromatographed at 2°, without equilibration. Elution was started with 25 ml of concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 5.6) then continued with 25 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 5.6); 1 ml fractions were collected.
B) Phe-sRNA (15 A units at 260 mμ) was chromatographed at 2°, without equilibration. Elution was started with 50 ml of concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 5.6) then continued with 25 ml dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 5.6); 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.

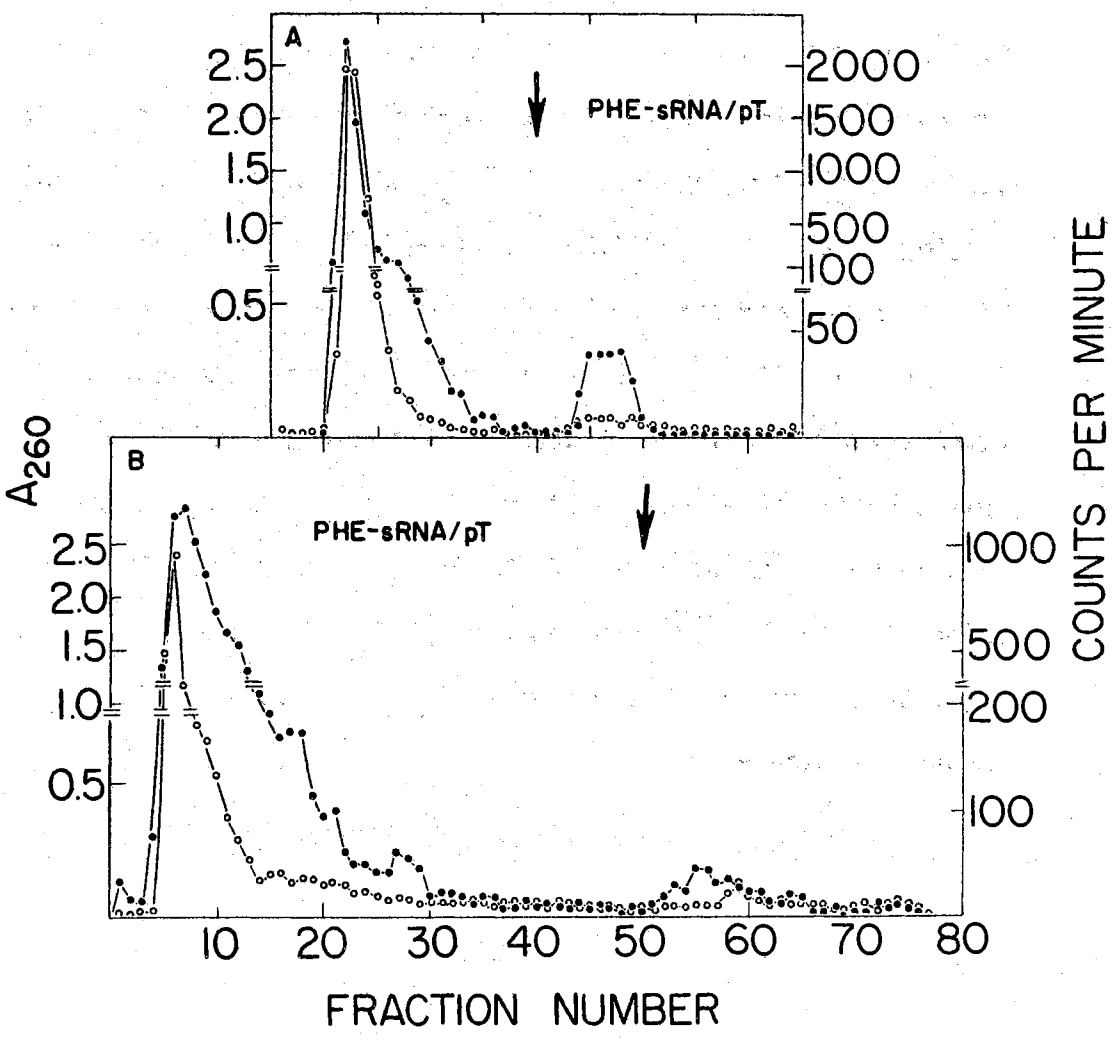

A
PHE-sRNA/pT
2.5
2.0
1.5
1.0
0.5
2000
1500
1000
500
100
50
B
PHE-sRNA/pT
A260
2.5
2.0
1.5
1.0
0.5
1000
500
200
100
COUNTS PER MINUTE
10
20
30
40
50
60
70
80
FRACTION NUMBER

Figure 16

Effect of Column Dimensions on the Chromatography of Charged s-RNA

A) Phe-sRNA (15 A units at 260 mμ) was chromatographed at 2°, without equilibration, on a oligothymidylate-cellulose column (height: diameter = 10:1). Elution was started with 25 ml of concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 5.6) then continued with 25 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 5.6); 1 ml fractions were collected.

B) Phe-sRNA (15 A units at 260 mμ) was chromatographed at 2°, without equilibration, on a oligothymidylate-cellulose column (height: diameter = 1:2). Elution was started with 25 ml of concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 5.6) then continued with 25 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 5.6); 1 ml fractions were collected.

Open circles represent A_{260} and closed circles, counts per minute.

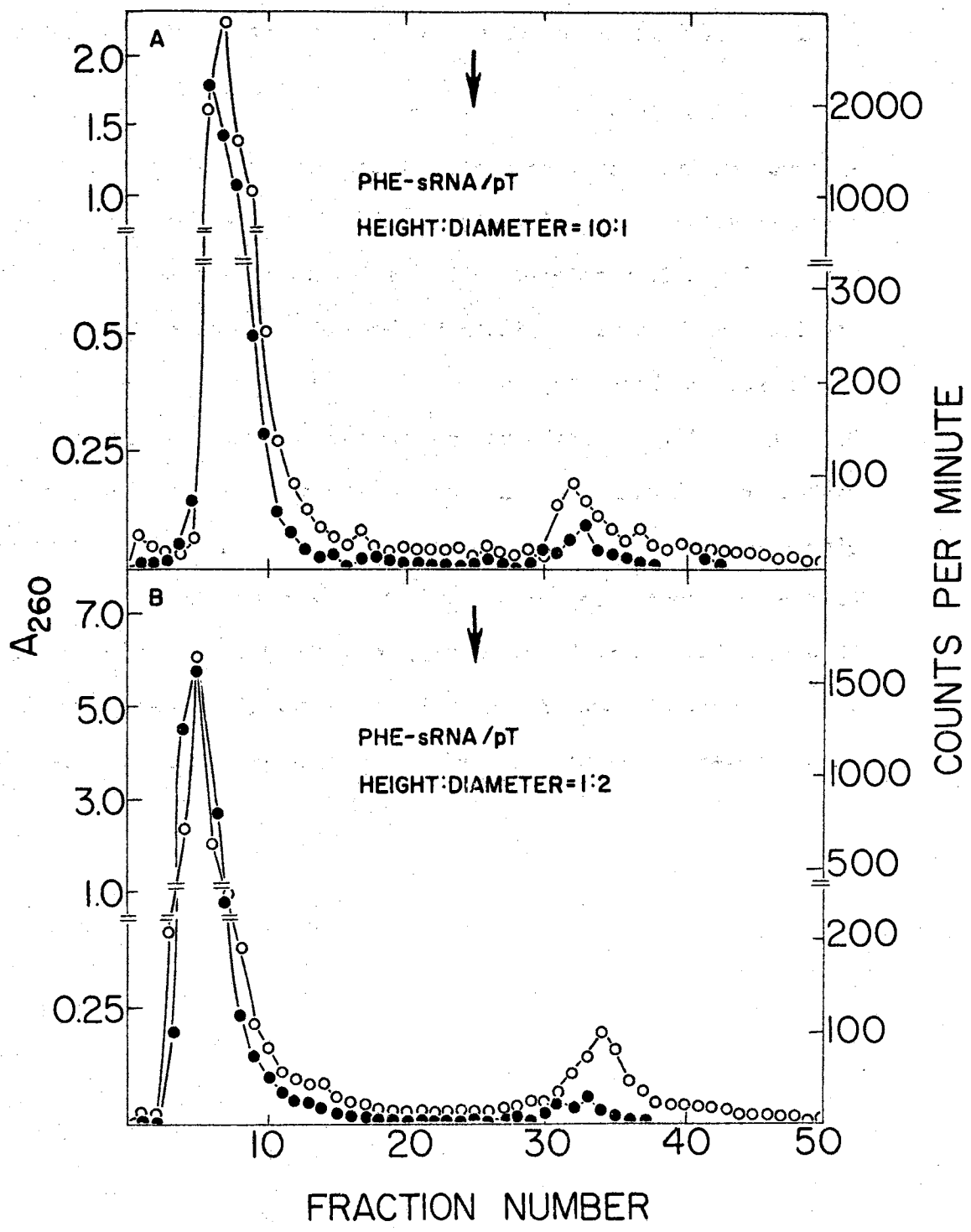

A
PHE-sRNA/pT
HEIGHT:DIAMETER=10:1
B
PHE-sRNA/pT
HEIGHT:DIAMETER=1:2
A260
COUNTS PER MINUTE
FRACTION NUMBER
2.0
1.5
1.0
0.5
0.25
7.0
5.0
3.0
1.0
0.25
2000
1000
300
200
100
1500
1000
500
200
100
10
20
30
40
50

Figure 17

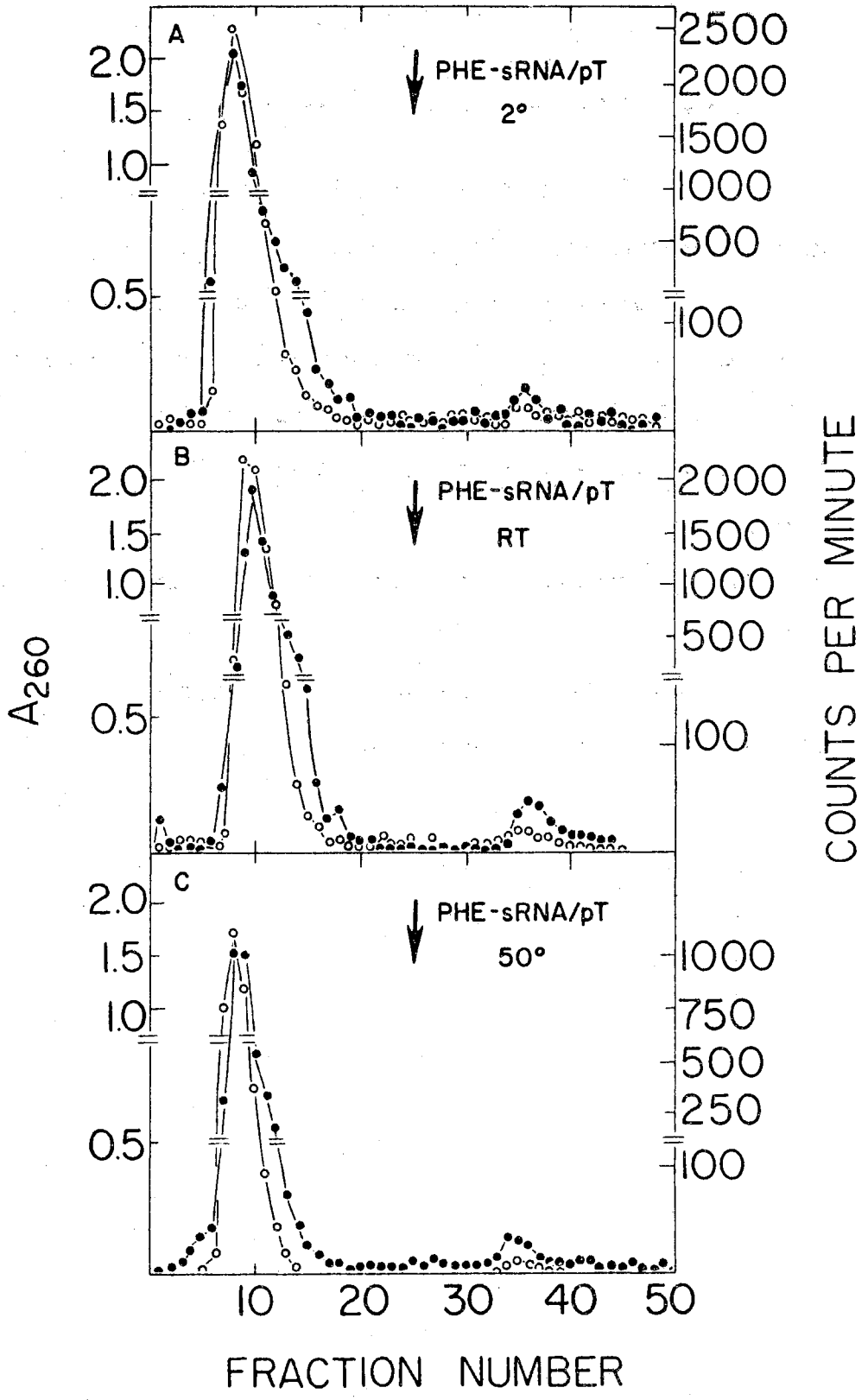
Effect of Temperature on the Chromatography of Charged s-RNA

A) Phe-sRNA (15 A units at 260 mμ) was chromatographed at 2°, without equilibration. Elution was started with 25 ml of concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 7.0) and then continued with 25 ml of dilute buffer (2.10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 7.0); 1 ml fractions were collected.

B) Same as above except the chromatography being performed at room temperature.

C) Same as above except the chromatography being performed at 50°.

In all the above figures open circles represent A_{260} and closed circles, counts per minute.


A
PHE-sRNA/pT
2°
B
PHE-sRNA/pT
RT
C
PHE-sRNA/pT
50°
2.0
1.5
1.0
0.5
2500
2000
1500
1000
500
100
750
250
10
20
30
40
50
A260
COUNTS PER MINUTE
FRACTION NUMBER

Rechromatography of s-RNA and the Capacity of Nucleotide Cellulose

In order to be able to work with larger quantities of s-RNA, oligothymidylic and oligodeoxyadenylic acids were synthesized, starting with 8 mmoles of nucleotide. The unfractionated mixture was attached to cellulose as described under Materials and Methods, resulting in an incorporation of about 4 mmoles (Table VI).

A large glass column was designed, with interchangeable parts held together by screw clamps (Fig. 18). Oligothymidylate- and oligodeoxyadenylate cellulose were filled in separate sections.

Unlabeled s-RNA (1-20 mg) was chromatographed on this column. Rechromatography of the first peak obtained by chromatography of up to 5 mg of s-RNA did not result in any discernible retention in either section. Rechromatography of the first peak obtained from 10 mg s-RNA results in the retention of appreciable quantities of 260 mμ absorbing material on both polythymidylate and deoxyadenylate-cellulose (Fig. 19). According to these experiments the capacity of nucleotide cellulose appears to be about 5 mg of s-RNA per 4 mmoles per chromatography.

Characterization of the Retained Peak

The material eluted by dilute buffer was characterized as charged s-RNA by the following experiments:

Radioactivity of the second peak was precipitated by two volumes of ethanol. Charged s-RNA also is precipitated under these conditions (Table VII).

Incubation of the second peak, as well as of original ^{14}C-Phe-sRNA, at pH 10, in glycine buffer, for 30 minutes at 37°, resulted in the

TABLE VI

REACTION OF OLIGODEOXYRIBONUCLEOTIDES WITH CELLULOSE FOR THE LARGE COLUMNS

Polymer	Wave length mμ	Amount before reaction A_{260} units	Amount unreacted A_{260} units	Extent of reaction %
Oligothymidylate	267	78,900	39,000	50
Oligodeoxyadenylate	260	75,900	37,300	49

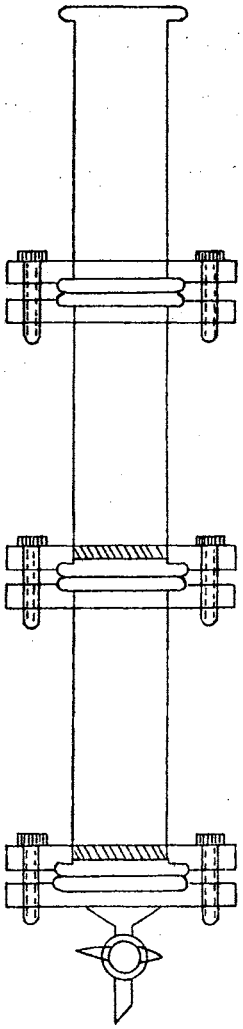
Extent of reaction was determined by measuring the A units, at the designated wave lengths, before and after the reaction.

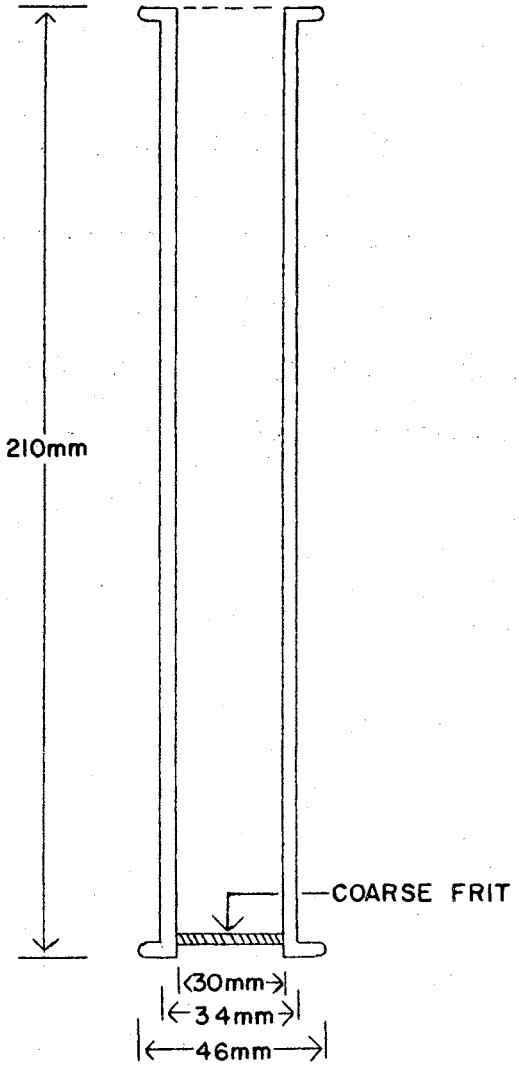
Pyridine was removed by evaporating the filtrate six times after the dicyclohexylcarbodiimide was extracted into ether.

Figure 18

Large, Glass Chromatography Column, with Interchangeable Parts

Two of the sections have frittes (coarse) fused into them, the other two are used to extend the height of fritted sections, when they are separated to elute the retained material. Three two-piece aluminum screw clamps are used to hold the sections together.




210mm
COARSE FRIT
30mm
34mm
46mm

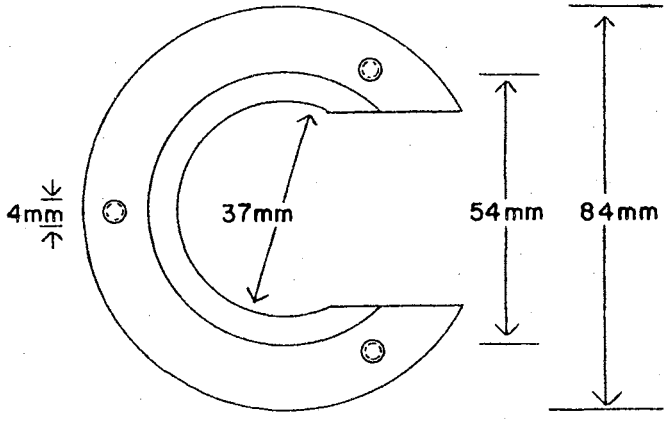

4mm
37mm
54mm
84mm

Figure 19

Chromatography of 10 mg Stripped s-RNA on the Large Column

s-RNA (*E. coli* B, 10 mg) was dissolved in 5 ml concentrated buffer (2 M sodium chloride + 10^{-2} M sodium phosphate, pH 5.6) and introduced into the large column, while both sections containing oligothymidylate-cellulose and oligodeoxyadenylate-cellulose were held together, as seen on the left side of Fig. 18. Elution was started immediately with the same buffer at room temperature; 5 ml fractions were collected with a flow rate of 5 ml per minute. Absorbancy was measured to insure that 260 mμ absorbing material was removed completely. Sections containing the different nucleotide celluloses were separated, stopcocks were attached to each and elution of each continued with dilute buffer ($2 \cdot 10^{-2}$ M sodium chloride + 10^{-4} M sodium phosphate, pH 5.6) separately. The first peak that was obtained with the concentrated buffer was dialyzed 24 hours against three changes of 200 volumes of water and the dialysate was lyophilized. The dry powder remaining after lyophilization was again dissolved in the same concentrated buffer and chromatographed over the regenerated column as before. Open circles represent A_{260} of the first chromatography and closed circles of the second chromatography.

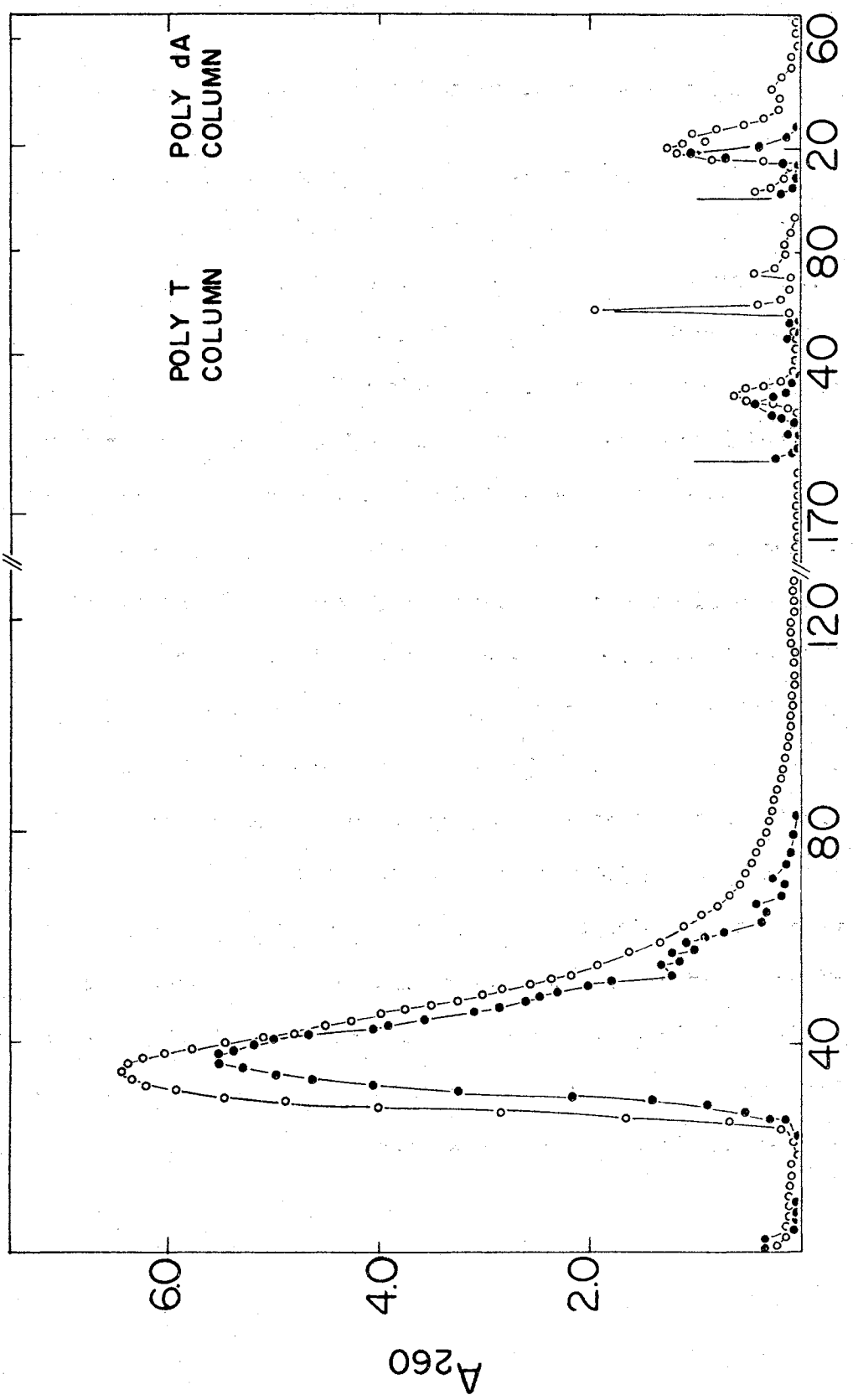

POLY dA
COLUMN
POLY T
COLUMN
A260
6.0
4.0
2.0
40
80
120
170
80
40
20
60
FRACTION NUMBER

TABLE VII

CHARACTERIZATION OF THE RETAINED PEAK

	Counts per minute per sample			
	Charged s-RNA		Chromatographed second peak	
	Precipitate	Supernatant Solution	Precipitate	Supernatant Solution
A) Stripping	23	2094	13	287
B) Precipitation	6454	40	136	1

Stripping was performed by incubation of control as well as of second peak at pH 10.0, in glycine buffer, for 30 minutes at 37°.

Precipitation was done by the addition of two volumes of ethanol, on second peak and also on control.

alcohol solubilization of radioactivity (Table VII).

The radioactivity of the second peak was incorporated into trichloroacetic acid-sodium tungstate insoluble material, after extensive dialysis of the pooled second peak material, in a polyuridylic acid stimulated amino acid incorporating system (Table VIII). The absence of salt in the second peak is imperative to obtain incorporation.

Experiments Designed to Investigate the Cause of Radioactivity in the First Peak

1) Overloading

Experiments performed with the large column described above have shown that overloading of the column may be one of the reasons of radioactivity of the first peak. In the small columns, where about 40 μmoles of nucleotidic material was attached to cellulose, 0.5 mg of charged s-RNA was chromatographed. Since with the large column the capacity of the chromatographic material was found to be approximately 5 mg per 4 mmoles of nucleotide-cellulose, the small columns were overloaded. Chromatography of 100 and 200 μg of s-RNA were performed to investigate this possibility. However, due to relatively low specific activities of the labeled amino acyl s-RNAs, the retained material had an insignificantly low radioactivity.

2) Stability of amino acyl-sRNA bond

During the early phases of the investigation, charged s-RNA was dissolved in 0.05 M sodium acetate (pH 5.0) and kept frozen at -20°. To check whether the stability of the bond and its time dependence was a possible cause of occurrence of radioactivity in the first peak, ^{14}C-Phe-sRNA and ^{14}C-Lys-sRNA solutions in the buffers used, were

kept at room temperature and at 2° up to five days and the s-RNA was precipitated after addition of about 300 µg of carrier RNA by the addition of ethanol. The mixture was kept in the cold for two hours and centrifuged at 8,000 x g for 15 minutes. After removal of the supernatant solution, the precipitate was dissolved in 1 ml distilled water. This solution and 1 ml aliquot of the supernatant solution were counted.

Freshly charged and lyophilized s-RNA, when dissolved in pH 5 buffer and immediately precipitated with ethanol, showed a loss of 10% of the counts incorporated (Fig. 20). These results indicate the lability of the amino acyl-sRNA bond. Since the rate of deacylation is much less at 2°, it is advisable to perform the chromatography at 2°.

Chromatography of a Mixture of ^{14}C-Phe and Uncharged s-RNA

When a mixture of ^{14}C-Phe and uncharged s-RNA was chromatographed on oligothymidylate-cellulose, the free amino acid emerged slightly ahead of the s-RNA peak which eluted around the eighth fraction, corresponding to the hold up volume of the column. There was considerable overlap of both peaks (Fig. 21). The nature of the small peak which was eluted by the concentrated buffer was not investigated.

3) Degeneracy

This possibility could not be investigated experimentally because of the failure in synthesizing TpTpC. However, when fractions of the first peak were precipitated with ethanol, substantial amount of radioactivity was precipitated. This can be taken as an indication of degeneracy or incomplete interaction between charged s-RNA and

TABLE VIII

INCORPORATION OF RADIOACTIVITY FROM CHARGED s-RNA INTO PROTEIN

	Counts per minute per sample			
	Charged s-RNA		Chromatographed second peak	
System	Input	Incorporated	Input	Incorporated
Experiment A	1944		1400	
Complete		1045		620
- messenger		157		127
Experiment B	2436			
Complete		941		
- GTP		228		
- Enzyme		276		
+ Puromycin		377		

Experiments A and B were performed with the same batch of charged s-RNA.

Figure 20

Stability of the Aminoacyl s-RNA Bond

Phe-sRNA and Lys-sRNA (10 mg each) were dissolved in 20 ml concentrated buffer (2 $\underline{\underline{M}}$ sodium chloride + 10^{-2} $\underline{\underline{M}}$ sodium phosphate, pH 5.6) and 1 ml aliquots were introduced into 16 centrifuge tubes, making two 8 tube sets of each charged s-RNA. One set of each was kept at 2° and the other at room temperature. Starting at zero time (immediately after dissolution and addition of about 300 µg of carrier s-RNA) two duplicates were precipitated by the addition of 2 ml of cold ethanol on the first, second and fifth day. The mixtures were kept in the cold for two hours and centrifuged at 10,000 g for 30 minutes. Supernatant solutions were decanted and 1 ml was counted in Tricarb Model liquid scintillation spectrometer, after the addition of 10 ml of Bray's counting fluid. The precipitate was dissolved in 1 ml distilled water and counted similarly.

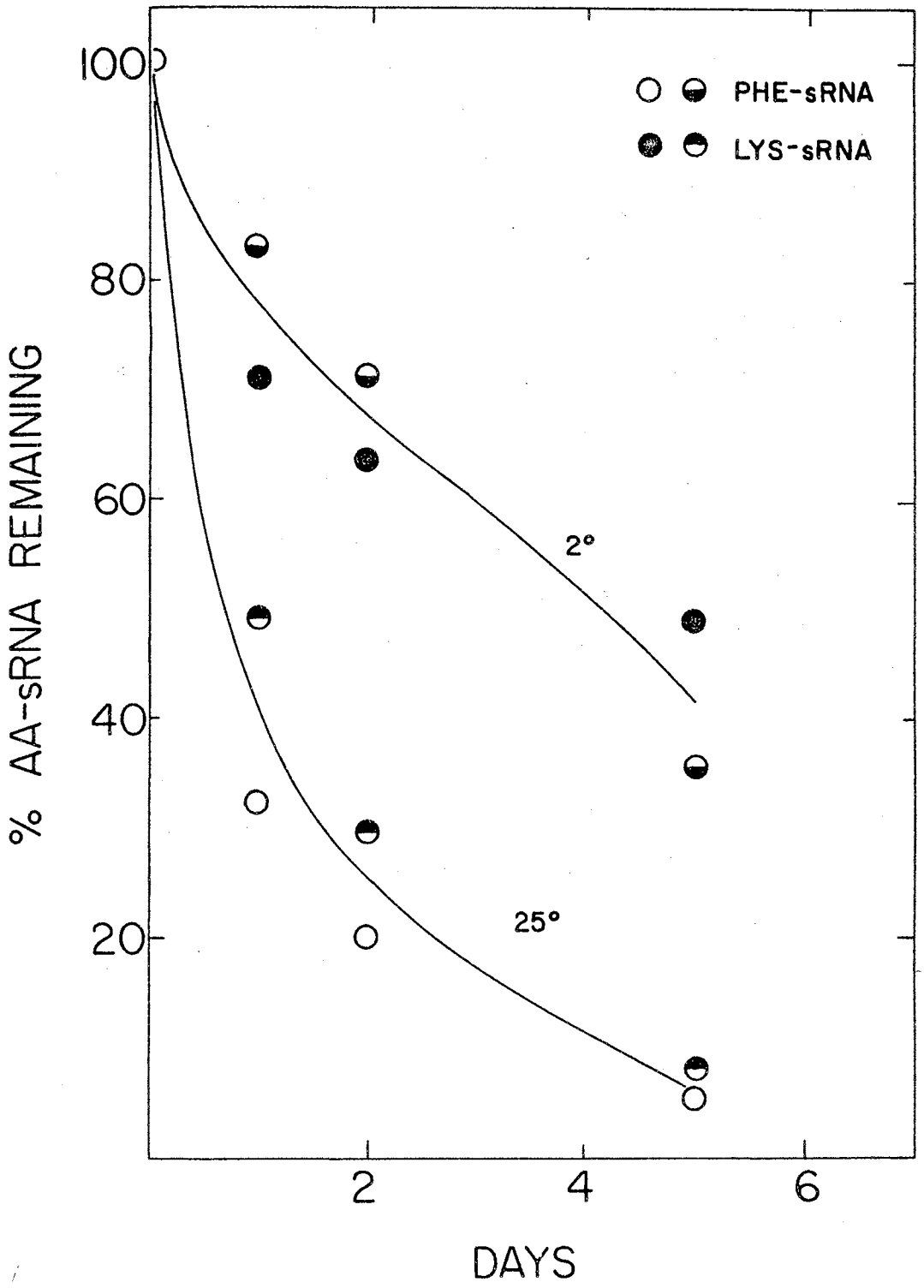
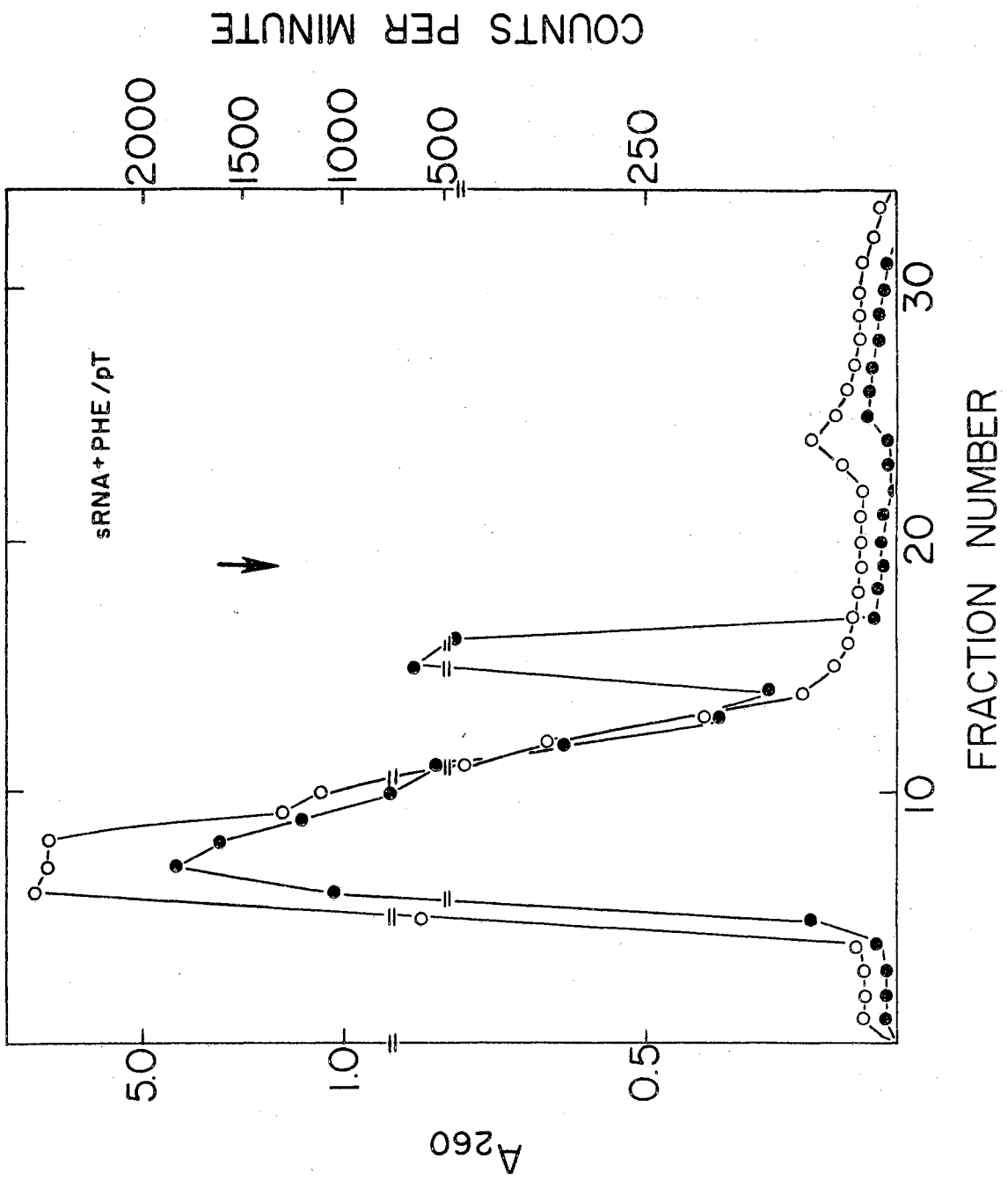

100
80
60
40
20
% AA-sRNA REMAINING
PHE-sRNA
LYS-sRNA
2°
25°
2
4
6
DAYS

Figure 21

Chromatography of a Mixture of s-RNA and Free Phe on Oligothymidylate-Cellulose Column

s-RNA (10 mg) and ^{14}C-Phe (0.01 µc) were dissolved in 1 ml concentrated buffer (1 M sodium chloride + 10^{-2} M sodium phosphate, pH 7.0) and chromatographed after two hours of equilibration, at room temperature. After the A_{260} peak was removed (19 ml of concentrated buffer) elution was continued with 16 ml of dilute buffer (10^{-2} M sodium chloride + 10^{-4} M sodium phosphate, pH 7.0), 1 ml fractions were collected. Open circles represent A_{260} and closed circles, counts per minute.


COUNTS PER MINUTE
2000
1500
1000
500
250
sRNA + PHE / pT
A260
5.0
1.0
0.5
10
20
30
FRACTION NUMBER

nucleotide-cellulose.

CHAPTER IV

DISCUSSION

At present the following methods are available for investigation of the coding problem:

1) The cell free amino acid incorporating system, using enzymatically synthesized messenger RNA containing random base sequences,

2) The same system using messengers synthesized by the block copolymerization method of Thach and Doty (91) where a certain control can be exerted on the base sequence of the product,

3) The same system using messengers of known repeating sequences,

4) Ribosome binding of charged s-RNA, using triplets of known sequence,

5) Chromatographic method described in the preceding pages.

While providing the first direct experimental approach to the coding problem, the cell free amino acid incorporating system was limited by the type of synthetic messengers available. The enzyme used in the synthesis of these messengers, nucleosidediphosphate: polynucleotide nucleotidyltransferase (E. C. No. 2.7.7.8) (polynucleotide phosphorylase), produced only long chains of polynucleotides and when mixtures of nucleotides were used, a random base sequence was obtained. The reasons for this were the ready reversibility of the reaction which resulted in the phosphorolysis of the primer and the much slower rate of chain initiation relative to chain growth.

Thach and Doty found that when the polymerization is performed in the presence of high concentrations of sodium chloride (1 mM to 1 M) it is possible to control not only the chain length of the polymers but also to add only a single base to a short oligonucleotide chain acting as primer (91). The "equilibrium state" after a short incubation period does not correspond to thermodynamic equilibrium and by extending the incubation period with the enzyme up to 24 hours it was possible to achieve the most probable distribution of the products. Under these circumstances the synthesis of tri- and tetranucleotides of known sequence becomes possible (92). Oligonucleotides obtained this way can be used in the amino acid incorporating system as well as in the ribosome binding system, which will be described below.

Chemical synthesis of hetero oligodeoxyribonucleotides and ribonucleotides constitutes the most direct approach to the messengers with known base sequence. The chain length of these oligonucleotides, however, is rather short and the longer chain lengths the lower the yield. Since longer chain length messengers have a greater stimulatory effect in the cell free system, Nishimura, Jacob and Khorana (93) have synthesized various deoxyribotrinucleotides and used them as primers for nucleoside triphosphate:RNA nucleotidyl transferase (E. C. No. 2.7.7.6) (RNA polymerase) as shown for d(TTC):

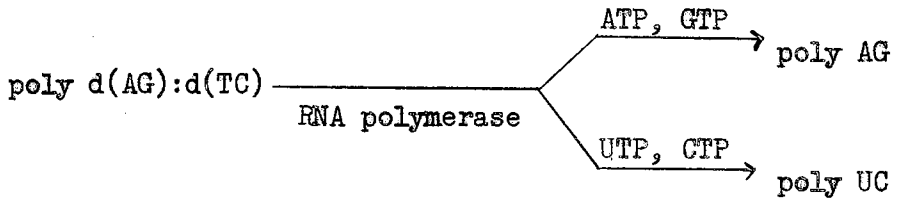
$$d(TTC)_3 \xrightarrow[\text{RNA polymerase}]{\text{ATP, GTP}} \text{poly AAG}$$

Then the high molecular weight poly AAG is used in the cell free amino acid incorporating system.

$$\text{poly AAG} \xrightarrow[\text{AA-incorporating system}]{} \text{poly lys + poly glu + poly arg}$$

This result indicates that there is no overlapping in the code and once the reading of the message starts, it goes on normally leading to a homopolypeptide.

In other experiments poly d(AG):d(TC) or poly d(AC):d(TG) were used as a primer for RNA polymerase:



Then any of the heteroribopolymers was used in the cell free amino acid incorporating system, for example:

$$\text{poly UC} \xrightarrow[\text{AA-incorporating system}]{} \text{poly (ser + leu)}$$

The incorporation of either of the amino acids required the presence of the other, even when the messenger was present, indicating that each of the two possible triplets were coding for two amino acids and in order for any peptide bond to form both of them had to be present simultaneously. The parallelism between the sequence of messenger and the heteropolypeptide was established by formation of dinitrophenyl-peptides followed by enzymatic degradation on the polypeptide and corresponding enzymatic degradations on the polyribonucleotides.

Since all of these methods are closely related, in as much as cell free system is concerned, the same critique applies to all of them. In order to stimulate polypeptide formation synthetic messengers must be bound to ribosomes. The nature of this binding, as well as

the interaction of s-RNA, messenger and enzyme(s) remain unclear. Various enzymes have been shown to be associated with ribosomes (94). None of these systems can actually use a triplet of known sequence.

Although the interaction of polyribonucleotides, ribosomes and s-RNA has recently been studied by ultracentrifugation (95) this method has some of the limitations of the above stated methods. Nirenberg and Leder (96) have found that when a mixture of charged s-RNA, ribosomes, containing triplets instead of long polymer messengers, was filtered through cellulose nitrate filter, ribosome-messenger-sRNA complex was retained only when the appropriate triplet was used. This method which requires an incubation period of only 20 minutes at 24° to give optimum binding, is rapid and sensitive enough to be used as an assay of binding of s-RNA to all possible triplet combinations and hence of code words. In this system doublets and deoxyribotrinucleotides are not functional; 5'-phosphorylated triplets have the highest stimulatory power for ribosomal binding followed by trinucleotidediphosphates. 3'-Phosphorylated triplets are almost inactive. Nirenberg and Leder believe that 5'-terminal, 3'-terminal and internal code words may differ from one another, even when the base sequence of all of them are the same, and may have different functions on the messenger.

Phosphorylation of a 3'-terminal code word, for example, might make a nonsense word out of a sense word and might thereby control the function of the messenger in a number of ways, by effecting the attachment or non attachment of ribosome, sensitivity of messenger to degradation by endogenous enzymes etc. The attractive feature of this suggestion is that only a modification of an existing code word,

not a modification of a base, is being postulated. Through the use of ribosome binding method a certain degeneracy pattern of the amino acid code has emerged (97). For most amino acids two pyrimidines at the 3-position of the triplet seem to be interchangeable while still coding for the same amino acid. For a few amino acids the interchangeability of two purines at the 3-position of the triplet has also been demonstrated. Leucine was found to be the only amino acid for which two purines at 1-position of the triplet can substitute each other. For two amino acids all four bases seem to be interchangeable at 3-position. These patterns are summarized in Table IX.

In addition some nonsense words, that is triplets that did not bind any aminoacyl-sRNA, have also been obtained experimentally.

Regardless of the uncertainties introduced to the method through the use of ribosomes this system is very elegant and it constitutes the most direct approach to the coding problem.

All the code words obtained or predicted by the use of this method are given in Table X (98).

Oligonucleotides attached to cellulose has been used in the fractionation of oligonucleotides mixtures (99). In the latter investigation use was made of stepwise increase in temperature to break hydrogen bonds between the complementary bases. Since the melting temperatures of complementary oligonucleotide pairs increase with the chain length, it was found possible to elute only some of the oligonucleotides by keeping the temperature of the column at a certain level (which corresponded to the proper melting temperature of the lowest melting point) than raising it so that it reached the melting temperature of the second pair etc.

TABLE IX

DEGENERACY PATTERNS OBSERVED IN THE RIBOSOME BINDING METHOD

m-RNA	-p-p U/C	-p-p G/A	-p-p G/A/U/C	U/C p-p-
Amino acid	Phe	Lys	Ser	Leu
	Tyr	Glu(NH_2)	Thr	
	Leu	Met	(Pro)	
	Asp	(Glu)	(Arg)	
	Asp(NH_2)	(Try)	(Gly)	
	His		(Ala)	
	(Cys)		(Val)	
			(Leu)	

Amino acids in parentheses are predicted to belong to the corresponding groups.

TABLE X

AMINO ACID CODE WORDS OBTAINED BY THE RIBOSOME BINDING METHOD (1965 REF. 99)

UUU	Phe	UCU	Ser	UGU	Cys	UAU	Tyr
UUC		UCC		(UGC)		UAC	
(UUA)	Leu	(UCA)	Ser	UGA	Nonsense*	UAA	Nonsense
UUG		UCG		(UGG)	or Try	UAG	
CUU	Leu or Nonsense*	CCU	Pro	CGU	Arg	CAU	His
CUC		CCC		CGC		CAC	
(CUA)	Leu	CCA	Pro	CGA	Arg	CAA	Glu(NH_2)
CUG		(CCG)		(CGG)		CAG	
AUU	Ileu	ACU	Thr	AGU	Ser	AAU	Asp(NH_2)
AUC		ACG		AGC		AAC	
(AUA)	Met	ACA	Thr	AGA	Arg or	AAA	Lys
AUG		ACG		(AGG)	Nonsense*	AAG	
GUU	Val	GCU	Ala	GGU	Gly	GAU	Asp
(GUC)		(GCC)		(GGC)		GAC	
(GUA)	Val	(GCA)	Ala	(GGA)	Gly	GAA	Glu
(GUG)		(GCG)		(GGG)		(GAG)	

*It is possible that these sequences are readable internal-, but non-readable terminal codons.

Sequences in parentheses are predicted.

The method described in this thesis has not been developed to the extent where triplets of known sequence are used because of difficulty of chemically synthesizing the triplets. Nevertheless as it stands it offers the following advantages over the other methods:

1) Deoxyribonucleotide polymers, which are much easier to synthesize chemically than ribonucleotide polymers, can be used. These compounds were found ineffective in the ribosome system.

2) The oligonucleotides are not destroyed during the experiment, so that the selective chromatographic material can be used repeatedly.

3) The system is free of any interference from contaminating enzymes that would be present in ribosomal preparations.

4) The selectivity of the technique does permit purification of a particular s-RNA species. Experiments with larger quantities of nucleotide cellulose have indicated the possibility of producing larger quantities of these s-RNA species for chemical studies.

SUMMARY

A method potentially useful for the determination of base sequences in code words has been developed. It consists of covalently attaching short polynucleotide chains to cellulose using dicyclohexylcarbodiimide and passing a charged, amino acid-labeled s-RNA preparation through a column prepared from the material. Retention of a particular species of s-RNA is presumably due to hydrogen binding between three bases attached to the column material and the anticodon triplet of the s-RNA since a reduction in ionic strength results in elution of the retained s-RNA. Thus columns containing oligothymidylate- or polyuridylate-cellulose retain Phe-sRNA while columns prepared from cellulose or polyadenylate-cellulose do not retain Phe-sRNA. Reciprocal experiments using Lys-sRNA demonstrate that oligodeoxyadenylate- and polyadenylate-cellulose columns retain Lys-sRNA. Tetrathymidylate attached to cellulose also retains Phe-sRNA, demonstrating the feasibility of the method for small sized messengers. The method has been scaled up to fractionate and purify large quantities of s-RNA.

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