# THE ROLE OF MESSENGER RNA IN NASCENT PEPTIDE CHAIN ACCUMULATIONS

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1979

ABSTRACT

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An investigation of the origin of the nonuniform size distribution of rabbit globin nascent peptide chains was performed. Uniformly labeled globin nascent chains were isolated from a rabbit reticulocyte lysate cell-free protein synthesizing system. Analysis of the sizes of the nascent chains by gel exclusion chromatography under denaturing conditions was performed. Accumulations of nascent chains of discrete sizes were observed, as reported previously for nascent chains isolated from whole reticulocyte incubations (Protzel and Morris, 1972). Alteration of the relative concentrations of the components of the lysate protein biosynthetic machinery did not change the gel elution profile.

A wheat embryo-derived cell-free messenger RNA-dependent protein synthesizing system was prepared. Globin mRNA was isolated from rabbit reticulocytes and added to the cell-free system. Gel chromatographic analysis of the labeled nascent chains purified from the rabbit globin mRNA-directed wheat embryo-derived cell-free protein synthesizing system demonstrated nascent chain accumulations of the same sizes as observed in nascent chains isolated from rabbit reticulocytes.

The origin of globin nascent chain accumulations was seen to reside, therefore, in some property of the globin mRNA itself, and not in the

protein biosynthetic machinery within which the globin mRNA is undergoing translation.

Infection of rifampicin treated E. coli with the RNA bacteriophage, MS2, was found to yield, fifty minutes after infection, predominantly MS2 coat protein biosynthesis. Nascent chains purified from MS2 infected, rifampicin treated E. coli fifty minutes after infection will contain predominantly MS2 coat protein nascent chains. Gel chromatographic analysis of nascent chains purified from MS2 infected, rifampicin treated E. coli gave evidence for the existence of nascent chain accumulations during MS2 coat protein biosynthesis, in vivo. A comparison was made between a model of the MS2 RNA coat protein genome secondary structure and the positions on the genome where ribosomes would reside in a population of mRNA molecules for greater relative lengths of time to generate the observed nascent chain accumulations.

The data presented in this thesis support the hypothesis that the nascent chain accumulations are due to regions of mRNA secondary structure impeding the rate of ribosome movement during the process of peptide chain elongation.

## ACKNOWLEDGMENTS

I would like to thank Dr. Allan Morris for his guidance and help during the course of my graduate study, and Drs. F. Rottman, R. Patterson, and W. Smith for serving on my graduate advisory committee.

Special thanks go to my wife, Linda, for many hours of nimble-fingered clerical assistance and moral support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# INTRODUCTION

## Protein Biosynthesis

Messenger RNA (mRNA) contains information in its nucleotide sequence for the amino acid sequence of the protein to be assembled. The amino acids are initially bound to a transfer RNA (tRNA) molecule capable of interpreting the genetic code within an mRNA sequence and synthesis takes place upon a cellular organelle known as a ribosome. The assembly of amino acids into proteins takes place in a step-wise manner from the N-terminal to the C-terminal end of the protein (Bishop et al., 1960; Dintzis, 1961). The ribosome moves along the mRNA in a 5' to 3' direction (Ochoa, 1965) and each translating ribosome contains only one growing peptide chain (Warner and Rich, 1964). The rate at which a protein is produced by a mRNA molecule may be affected by the rate of initiation of protein synthesis, the rate of peptide bond formation, the rate at which the completed chains are released from the ribosomes, and the amount of mRNA present. The stability of the mRNA undergoing translation and the rate at which a protein is degraded by intracellular proteases are factors which will affect the amount of protein present in a cell (Schimke, 1973; Nomura et al., 1974; Weissbach and Ochoa, 1976; Weissbach and Pestka, 1977).

Hemoglobin Synthesis in the Rabbit Reticulocyte

Reticulocytes are immature erythrocytes which are still synthesizing protein. In mammals the nuclei of the reticulocytes have been extruded from the cell. Greater than 95% of the protein synthesis in rabbit reticulocytes is hemoglobin synthesis (Woodward et al., 1972). Rabbit hemoglobin contains two different proteins, α- and β-globin, which are present in a tetramer ($\alpha_2\beta_2$). Alpha globin has been reported to be synthesized at a slightly higher rate than β-globin in reticulocyte incubations (Tavill et al., 1968) and the ribosomes of rabbit reticulocytes have been shown to contain approximately equal numbers of α- and β-globin nascent chains (Hunt et al., 1968, Lodish, 1971, Protzel and Morris, 1973). Beta globin is synthesized on larger polysomes than α-globin (Hunt et al., 1968), and the amount of α-globin mRNA in a reticulocyte is larger than the amount of β-globin mRNA in a reticulocyte (Lodish, 1971). The average translation rates of both α- and β-globin have been determined to be equal, about 200 seconds per chain synthesized at 25° (Lodish and Jacobson, 1972; Hunt, 1974).

A model for hemoglobin biosynthesis, which incorporates the observations described above, has been described by Lodish (1976). Beta-globin mRNA, having a higher affinity for ribosomes than α-globin mRNA, undergoes initiation of protein synthesis at a higher rate than the initiation rate for α-globin mRNA. The higher rate of β-globin initiation would explain the increased sizes observed for polysomes synthesizing β-globin (Hunt et al., 1968). The higher concentration of α-globin mRNA relative to β-globin mRNA, reported to be present in the rabbit reticulocyte, has been proposed to balance the higher rate of

initiation of β-globin synthesis in order to yield approximately equal numbers of α- and β-globin nascent peptides (Lodish, 1971, 1976).

Control of Hemoglobin Synthesis by Hemin

Early studies of protein synthesis in isolated rabbit reticulocytes have demonstrated a requirement for added ferrous ion (Kruh and Borsook, 1956). Without added $Fe^{++}$ the rate of incorporation of radioactive amino acids into protein greatly decreases after a few minutes. Each globin has a heme group bound to it which contains iron, suggesting the existence of a mechanism of coordinate control of globin and heme biosynthesis. The requirement for $Fe^{++}$ has been found to be replaceable by hemin (Waxman and Rabinovitz, 1966; Bruns and London, 1965), supporting the conclusion that the stimulatory effect of iron is through increased heme biosynthesis. However, binding of heme to nascent chains could not be demonstrated, providing evidence that heme binds to globin after globin biosynthesis upon reticulocyte ribosomes (Morris and Liang, 1968).

Cell-free protein synthesizing systems prepared from the reticulocyte lysate have also been shown to have a requirement for added hemin. If hemin is not present in reticulocyte lysate incubations, an inhibitor of the initiation step of protein synthesis rapidly forms at 37° (Zucker and Schulman, 1968; Adamson et al., 1968). The formation of a protein synthesis inhibitor in vitro is thought to occur by the same mechanism that yields the inhibition of protein synthesis in iron deprived cells in whole cell incubations. This hemin controlled repressor (HCR) is a protein with a molecular weight of about 400,000 and is formed from a precursor of the same molecular weight (Adamson et al., 1972).

Inhibition of protein synthesis by HCR is thought to be due to phosphorylation of a protein synthesis initiation factor, eIF-2 (Levin et al., 1975, 1976; Balkow et al., 1975; Farrell et al., 1977). Addition of purified eIF-2 to hemin deprived reticulocyte lysate incubation mixtures has been shown to relieve the incubation mixtures from the inhibition of protein synthesis initiation (Kaempfer et al., 1974; Clemens et al., 1977). The phosphorylation of eIF-2 has been reported to be performed by a cyclic AMP independent protein kinase which, in turn, may be activated by phosphorylation by a cyclic AMP dependent protein kinase (Levin et al., 1976; Farrell et al., 1977; Kramer et al., 1976). Hemin has been demonstrated to prevent the binding of cyclic AMP to the cyclic AMP dependent protein kinase in a manner which is thought to control the activity of the cyclic AMP dependent protein kinase (Datta et al., 1978). The inhibition of protein synthesis initiation by HCR has also been shown to affect the initiation of non-globin protein synthesis in rabbit reticulocyte lysate incubation mixtures (Lodish and Desalu, 1973). Hemin dependent mechanisms of control of the initiation of protein synthesis have also been reported to exist in HeLa cells and in Krebs II ascites tumor cells (Beuzard et al., 1973; Weber et al., 1975).

## Protein Biosynthesis in RNA Bacteriophage Infected E. coli

A class of bacteriophages containing RNA as their genome has been described by Loeb and Zinder (1961). This RNA is capable of acting as a mRNA molecule in vivo and in vitro. Two classes of RNA phages have been found. One class consists of the closely related phages, MS2, R17, f2, M12, μ2 and the other class, the serologically distinct

phage Qβ. All these phages are specific for "male" E. coli since they enter the cell by adsorbing to the F pili during infection. A collection of reviews summarizes much of the knowledge available about these bacteriophages (Zinder, 1975).

The genome of the MS2 bacteriophage contains 3569 nucleotides which include the genetic information for three viral coded proteins synthesized by infected E. coli (Fiers et al., 1976). They are, in order from the 5' end of the viral genome, the maturation protein, the coat protein, and the replicase protein. The replicase protein has been shown to form an RNA-dependent RNA polymerase with three native E. coli proteins, the elongation factors Tu and Ts, and the S1 protein of the small ribosomal subunit (Federoff, 1975). The RNA-dependent RNA polymerase is responsible for replication of the bacteriophage genome.

Synthesis of replicase protein is thought to require prior translation of the coat protein RNA sequence. Bacteriophage mutants containing nonsense mutations (which cause premature termination of protein synthesis) near the beginning of the coat protein messenger RNA sequence have been shown to produce only a small amount of replicase protein when the bacteriophage RNA is added to an E. coli-derived cell-free protein synthesizing system. Bacteriophage RNA containing nonsense mutations in the last half of the coat protein RNA have been found to synthesize normal amounts of replicase protein under the same in vitro conditions (Tooze and Weber, 1967; Capecchi, 1967; Engelhardt, 1967).

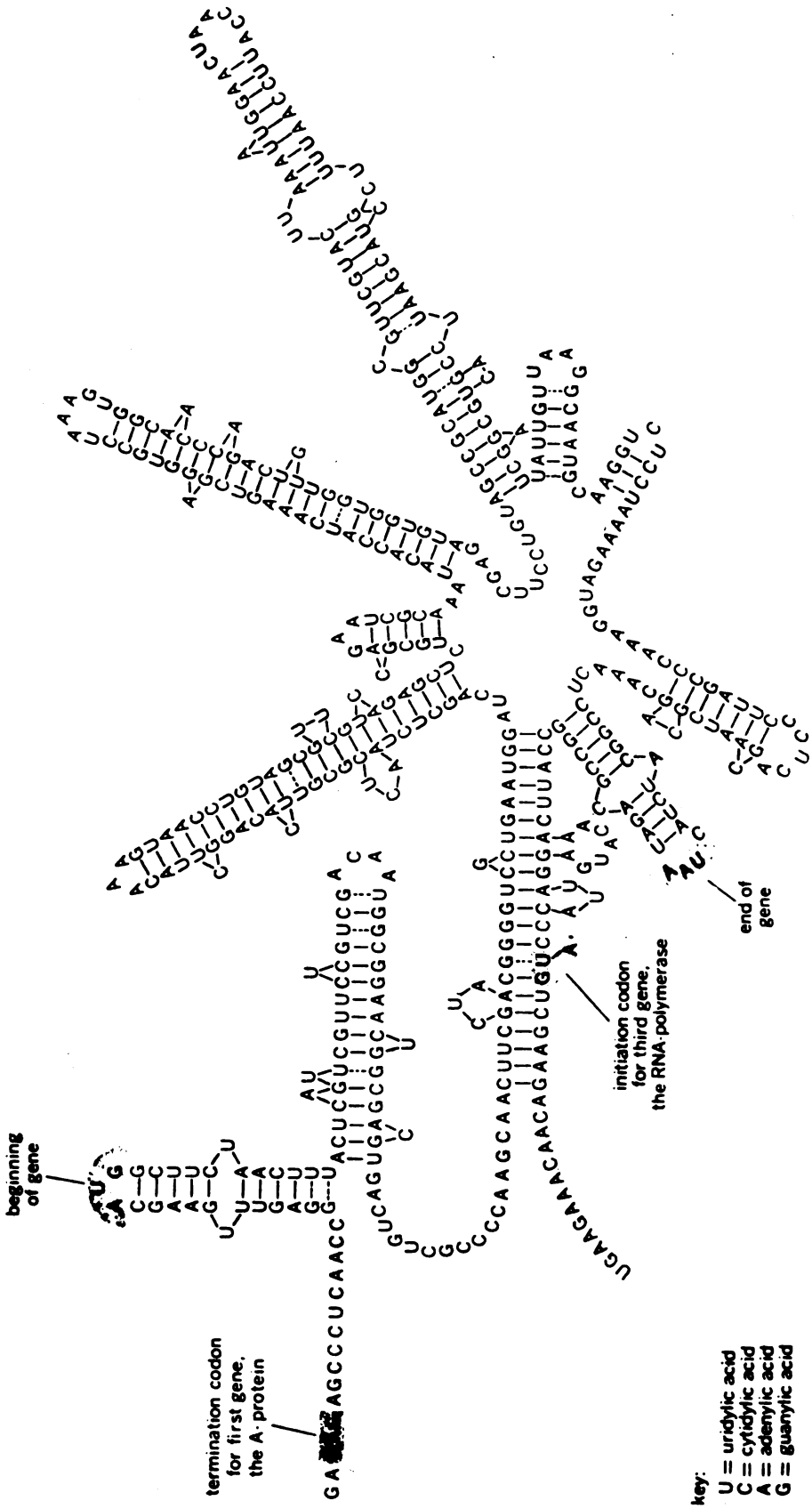
Production of intact, functional coat protein does not seem to be required for replicase synthesis, only translation of the mRNA sequence

which contains the genetic information for the N-terminal half of the coat protein. A change of conformation has been proposed to occur when a ribosome traverses the mRNA sequence which codes for the N-terminal half of the coat protein. The change of conformation is thought to expose the initiation region of the MS2 replicase protein, which has been concluded to exist in intact, untranslated MS2 RNA associated with part of the 5' half of the MS2 coat protein cistron in a structure which prevents initiation of replicase protein synthesis at this site (Lodish and Robertson, 1969).

Studies of the nucleotide sequence of the MS2 genome have demonstrated a region in the coat protein cistron which could undergo intramolecular base pairing with the regions at the start of the replicase subunit cistron (Min Jou et al., 1972). A proposed structure of the coat protein genome is shown in Figure 1. The structure which may be formed between the MS2 coat protein cistron RNA and the region bearing the initiation codon of the replicase cistron would be forced apart during translation of the MS2 coat protein mRNA region by a ribosome. The replicase protein synthesis initiation region would then be exposed. Figure 1, therefore, provides a structural explanation for the dependence of replicase protein synthesis upon translation of the 5' end of the MS2 coat protein mRNA sequence in vitro.

The ratio of coat protein synthesis to replicase protein synthesis changes during the course of infection of E. coli with an RNA bacteriophage. Early in infection the molar ratio of coat protein synthesis to replicase protein synthesis is 6:1. Late in the phage reproductive cycle the ratio of coat protein synthesis to replicase protein synthesis becomes greater than 30:1 (Kozak and Nathans, 1972). An explanation

Figure 1. The nucleotide sequence and proposed secondary structure of the bacteriophage MS2 coat protein cistron.


beginning of gene
termination codon for first gene, the A protein
initiation codon for third gene, the RNA polymerase
end of gene
key:
U = uridylic acid
C = cytidylic acid
A = adenylic acid
G = guanylic acid

for the change in coat protein to replicase protein synthetic ratios may lie in the observation that coat protein is capable of binding to bacteriophage RNA and of causing the repression of the *in vitro* translation of the replicase cistron RNA in *E. coli*-derived cell-free protein synthesizing systems (Sugiyama and Nakada, 1968; Ward *et al.*, 1968; Lodish, 1968; Eggens and Nathans, 1969). Coat protein has been found to bind to bacteriophage RNA in a ratio of 1-6 coat protein molecules per bacteriophage RNA molecule. Coat protein bound to bacteriophage RNA has been observed to protect a specific RNA fragment from nucleolytic digestion by Ribonuclease $T_1$. The nucleotide sequence of the RNA fragment protected from Ribonuclease $T_1$ digestion by bound coat protein, 59 nucleotides in length, has been determined and includes the region of the bacteriophage RNA which codes for the initiation of synthesis of the replicase protein (Bernardi and Spahr, 1972).

The requirement for translation of the coat protein mRNA region before the replicase protein can be synthesized may provide a means of timing the start of phage RNA reproduction, since the replicase protein is required for the assembly of the functional RNA-dependent RNA polymerase complex. Synthesis of the replicase subunit thus would be closely related to the synthesis of bacteriophage coat protein, thereby providing a system for the control of phage growth.

## Structure of Nucleic Acid Polymers

Deoxyribonucleic acid (DNA) is normally thought to occur in double helical configuration in both eukaryotes and prokaryotes (except for single-stranded DNA bacteriophages and eukaryotic satellite DNA). The helical structure of DNA forms as a result of the base pairing of

anti-parallel complementary strands. The structure that an RNA transcript of DNA possessed would be expected to depend in large part upon the nucleotide sequence of that particular RNA molecule since a complementary strand is not present, and may be expected to vary between RNA molecules of different sequences.

Studies of RNA structure have been performed and much information has been reported about the primary, secondary, and tertiary structure of a number of cellular RNA molecules. The combination of nucleotide sequence analysis of single purified RNA species with determination of their physical characteristics in solution have allowed structures to be proposed for a few RNA molecules. The advent of methods which allow the analysis of the base sequence of DNA will bring a great expansion of knowledge of primary nucleotide sequences (Sanger and Coulson, 1975; Gilbert and Maxam, 1977; Sanger et al., 1977).

## Transfer RNA

Transfer RNA serves an important and unique role during protein biosynthesis as the interface between nucleic acid and protein sequences. The term tRNA identifies a class of molecules in a cell, not a single molecular species of RNA. A review of tRNA sequence, structure, and role in protein biosynthesis has been published (Rich and Raj Bhandary, 1976). A bacterium contains about 60 different species of tRNA and a mammalian cell contains as many as 110 tRNA molecules. Transfer RNA molecules are 73-93 nucleotides long. Although different species of tRNA molecules have different primary base sequences and are of varying lengths, they all serve a similar purpose in protein biosynthesis.

The similarity in function of different tRNA molecules is reflected in a similarity in structure of the tRNA molecules whose nucleotide sequences have been determined. All may be arranged in the "cloverleaf" configuration involving the formation of a number of intramolecular base pairs (Holley et al., 1965). The "cloverleaf" structure has been confirmed by X-ray crystallographic studies which have shown that the tRNA molecule undergoes folding into an L-shaped molecule (Kim et al., 1973).

Transfer RNA secondary structure is an important determinant in the processing of tRNA precursor transcripts into mature tRNA molecules. Enzymatic cleavage of nucleotides from both the 5' and 3' end of a tRNA precursor involves endonucleases which act at the same place on tRNA molecules of different primary sequences (Schedl et al., 1974). Mutations at sites distant from the enzymatic cleavage point, which would affect potential regions of intramolecular base pairing, have been shown to alter the cleavage of precursor tRNA to mature tRNA (Smith, 1974; Altman et al., 1974). The effects of mutations at sites distant from the enzymatic cleavage point have been interpreted as demonstrating that the recognition sites of the ribonucleases involved in the processing of tRNA precursors require structural features of the whole precursor tRNA molecule other than the base sequence at the cleavage sites. The processing enzymes are thought to interact with the tRNA secondary structure in the maturation process as well as the primary nucleotide sequence of the precursor tRNA molecule.

Ribosomal RNA

Ribosomes are composed of approximately half RNA and half protein. Prokaryotic ribosomes contain 5S, 16S, and 23S ribosomal RNA (rRNA) molecules. Eukaryotic ribosomes contain 5S, 5-8S, 18S, and 28S rRNA molecules. The 16S and 18S RNA molecules are components of the small subunits of prokaryotic and eukaryotic ribosomes, respectively.

Synthesis of some rRNA molecules in both prokaryotes and eukaryotes occurs as a large RNA precursor which is subsequently cleaved into the individual RNA molecules found in the ribosomes. Mutants of E. coli deficient in Ribonuclease III have been shown to accumulate rRNA precursor molecules, which has been interpreted to indicate that this enzyme plays a role in rRNA maturation (Nikolaev et al., 1974). Electron microscopic analysis of RNA precursor molecules from mouse L-cell cultures and Xenopus laevis has demonstrated the presence of secondary structure regions along the length of these molecules (Wellauer and Dawid, 1974; Wellauer et al., 1974). Studies measuring hypochromicity during heat denaturation (Cox, 1966) and optical rotary dispersion (McPhie and Gratzer, 1966) have indicated that about 60-70% of the nucleotides of isolated bacterial rRNA participate in regions of secondary structure under physiological conditions. RNA secondary structure, therefore, has been identified in rRNA by a variety of methods and has been reported to be important in biological function as a recognition site for Ribonuclease III in cleavage of precursor E. coli RNA molecules to mature rRNA.

Measurements of RNA structure performed upon purified RNA molecules in solution have been proposed to be accurate measurements of the RNA structures that exist in the ribosome since the hypochromicity of

E. coli rRNA in solution and in the intact ribosome has been found to be nearly identical (Schlessinger, 1960). Most experiments upon rRNA structure have been performed upon prokaryotic RNA molecules. The following sections will summarize the experiments which have been performed upon different types of rRNA molecules.

5S Ribosomal RNA

Eukaryotic and prokaryotic organisms contain one 5S RNA molecule as a part of each large ribosomal subunit. Sedimentation studies have indicated that a high degree of asymmetry exists in 5S RNA molecules in solution (Boedtker and Kelling, 1967). Measurements of the degree of hypochromicity of 5S RNA have also been interpreted to indicate that secondary structure (about 60% of the bases) exists in this molecule under physiological conditions (Boedtker and Kelling, 1967).

Elucidation of the primary structure of a 5S RNA from prokaryotic and eukaryotic organisms has been accomplished (Monier, 1974). All of the 5S RNA molecules examined have been found to be 120 nucleotides in length. Base sequence homologies have been found within the prokaryotic classes and the eukaryotic classes of 5S RNA sequences which have been determined (Monier, 1974). A homology between E. coli 5S RNA and KB cell 5S RNA base sequences has also been reported (Sankoff and Cedergreen, 1973).

Several secondary structure models from 5S RNA have been proposed (Monier, 1974; Boedtker and Kelling, 1967). All involve base pairing of the 5' and 3' ends of the 5S RNA in a "stem" with the rest of the molecule taking on various configurations containing other regions of intramolecular base pairing. While no evidence exists to conclusively

distinguish between the proposed models, the hypochromicity of intact 5S RNA plus the different reactivity of individual residues in the molecule with chemical reagents and single strand specific endoribonucleases has been interpreted to indicate that such regions of secondary structure exist in solution (Monier, 1974).

16S and 18S Ribosomal RNA

The small subunits of both prokaryotic and eukaryotic ribosomes contain one molecule of RNA. The RNA molecules in the small ribosome subunits have a sedimentation rate of 16S in prokaryotes and 18S in eukaryotes. The primary sequence of E. coli 16S rRNA has been largely determined and may be arranged into structures involving regions of intramolecular base pairing within "hairpin loops" (Ehresmann et al., 1975; Fellner, 1974). Physical and enzymatic studies of rRNA are in agreement with the model of 16S rRNA structure proposed by Fellner and coworkers (Fellner, 1974; Ehresmann et al., 1975). Increases in both absorbance of rRNA solutions at 260 nm and reactivity of rRNA with formaldehyde at elevated temperatures have been observed, as expected for molecules containing significant regions of secondary structure (Doty et al., 1959; Cox, 1966; Cox et al., 1973). Treatment of rRNA with Ribonucleases $T_1$ and $T_2$ has demonstrated regions where these single strand speciric nucleases are not able to act (Ehresmann et al., 1972). Regions of intramolecular base pairing were proposed to be responsible for blocking the $T_1$ and $T_2$ action.

The binding of ribosomal proteins to the intact 16S molecule and to fragments of the 16S molecule has been investigated by Zimmermann and coworkers (Zimmermann, 1974). Binding sites for ribosome derived

proteins on the 16S RNA molecule have been found to be present in RNA fragments generated by limited nuclease digestion (Zimmermann, 1974). Ribosomal subunit proteins S4 and S8 have been shown to bind to separate regions of the 16S RNA molecule. A third region of the 16S molecule is protected from nuclease attack by ribosomal protein S7. Ribosomal RNA secondary structure is thought to be important for ribosomal protein binding since 16S RNA has been shown not to bind proteins S4 and S8 under conditions which disrupt RNA base pairs (Schulte et al., 1974). Binding sites for ribosomal proteins are thought to be localized at specific sites along the linear sequence of the 16S rRNA molecule and to contain regions of RNA secondary structure which are required for protein binding (Zimmermann, 1974).

A comparison of the resistance of E. coli 16S RNA and the resistance of a random sequence polynucleotide to Ribonuclease $T_1$ digestion has shown that the 16S RNA molecule has the greater stability (Ricard and Salser, 1973). The increased resistance of 16S RNA to Ribonuclease $T_1$ digestion has been interpreted to demonstrate that the structure of the 16S RNA is composed of longer, more stable regions of intramolecular base pairs than those which have been predicted for random polynucleotide sequences (Ricard and Salser, 1973; Gralla and DeLisa, 1974).

Shine and Dalgarno (1975) have proposed that 16S RNA in prokaryote ribosomes plays a role in the initiation of translation by forming intermolecular base pairs with region of the mRNA immediately preceding the initiation codon. Experimental evidence interpreted to support this hypothesis has been obtained by Steitz and Jakes (1975). They have reported the formation of a base paired fragment of E. coli 16S RNA

and an RNA fragment from bacteriophage R17 carrying the initiation region of the maturation protein (Steitz and Jakes, 1975). A 70S ribosomal complex has been formed with E. coli ribosomal subunits and the RNA fragment from R17. Formation of the ribosomal complex has been followed by colicin E3 treatment which cleaves the 16S RNA of the ribosome at a position about 50 nucleotides from the 3' end. Following disassociation of the ribosome complex, an RNA complex consisting of the 3' end of the 16S RNA and the R17 RNA fragment has been identified by polyacrylamide gel electrophoretic analysis. The RNA complex prepared in this manner could be disassociated with heat treatment at 65°. This behavior is expected for the non-covalent interaction between the RNA molecules by the base pairing predicted by Shine and Dalgarno (1975). The conclusion reached by Steitz and Jakes (1975) was that initiation of protein synthesis in E. coli involves the formation of a base paired region between 16S RNA and an RNA region preceding the initiation codon of protein synthesis.

The applicability of the model of Shine and Dalgarno (1975) to eukaryotic protein biosynthesis initiation is uncertain. Studies of the nucleotide sequence of eukaryotic mRNA have shown that the formation of such base paired regions with 18S rRNA is not possible for all mRNA species whose sequences in the region of the mRNA immediately preceding the initiation codon have been determined (Kozak, 1977; Steitz, 1978). Initiation of eukaryotic protein biosynthesis may not require the formation of such a complex. More studies of the initiation of eukaryotic protein biosynthesis are needed to clarify this point.

Bacteriophage RNA

Studies of the primary sequence of the single stranded RNA bacteriophage genomes have been greatly aided by the ability to purify large quantities of highly radioactively labeled RNA from the virion. Addition of purified bacteriophage RNA to E. coli-derived cell-free protein synthesizing systems has provided evidence supporting the proposal that control of the synthesis of bacteriophage specific proteins may lie, in part, in the structure of the bacteriophage genome.

Heat treatment of purified bacteriophage MS2 RNA results in an increase in absorbance at 260 nm, which has been interpreted to indicate the presence of intramolecular base pairing in these molecules (Strauss and Sinsheimer, 1963). Digestion of Qβ RNA with low concentrations of pancreatic ribonuclease at low temperatures yields a limited number of fragments (Bassel and Spiegelman, 1967). Ribonuclease $T_1$ digestion of MS2 RNA at 0° also yields a limited number of cleavage points (Min Jou et al., 1972). The limited generation of RNA fragments has been attributed to the inhibition of ribonuclease action by the formation of double stranded regions which are resistant to the nuclease attack.

Determination of the complete nucleotide sequence of the MS2 genome (3569 nucleotides long) has been accomplished by Fiers and coworkers (Min Jou et al., 1972; Fiers et al., 1975; Vandenberghe et al., 1975; Fiers et al., 1976). A structure has been proposed for the MS2 genome which involves extensive regions of secondary structure. Both hairpin turns and interactions involving nucleotide sequences in the RNA molecule distant from each other may be drawn (Fiers et al., 1976). The stability of these structures may be estimated by the procedures described by Tinoco and coworkers (Tinoco et al., 1971, 1973).

Bacteriophage T7 RNA

Regions of secondary structure have been reported to be involved in T7 early mRNA processing. Twenty percent of the T7 genome is transcribed as a single RNA molecule to form the T7 early mRNA class. Ribonuclease III has been reported to cleave the T7 early mRNA precursor molecule into the mRNA pieces found in infected E. coli (Dunn and Studier, 1975; Rosenberg et al., 1975). An E. coli strain deficient in Ribonuclease III has been shown to accumulate large RNA transcripts of the early T7 precursor mRNA containing all of the early T7 mRNA molecules (Dunn and Studier, 1973). The site for mRNA processing by Ribonuclease III is thought to be a double stranded hairpin structure in the T7 early mRNA precursor. Studies of the nucleotide sequences of T7 early mRNA isolated from T7 infected E. coli have shown that the different early mRNA species have identical base sequences at the 3' end and at the 5' end of the RNA molecules (Kramer et al., 1974). In vitro transcription of T7 DNA with E. coli RNA polymerase followed by treatment of the RNA which has been synthesized with purified Ribonuclease III also has yielded RNA pieces corresponding in size and in 5' and 3' terminal nucleotide sequences to the T7 early mRNA isolated from bacteriophage T7 infected E. coli (Rosenberg et al., 1974). Production of monocistronic T7 early mRNA from the polycistronic precursor RNA is therefore thought to be the result of Ribonuclease III acting at specific double stranded regions of the precursor RNA. Cleavage of the T7 early mRNA precursor is not required for growth of the bacteriophage since T7 grows quite well in Ribonuclease III deficient E. coli which synthesize only early mRNA precursor molecules (Dunn and Studier, 1975). The nucleotide sequence surrounding a

Ribonuclease III cleavage site in a single intercistronic region of T7 early mRNA has been determined (Rosenberg and Kramer, 1977) and, as predicted for a Ribonuclease III cleavage site, the base sequence of the cleavage site can be arranged into a potentially stable double stranded structure.

Eukaryotic Messenger RNA

Sequence analyses of portions of eukaryotic virion genomes have been performed. The nucleotide sequences derived from the analyses performed also show the potential for secondary structure in the form of hairpin loops. Although the nucleotide sequences which have been determined are becoming too numerous to describe, some examples which contain regions that may be folded into hairpin loops are described below.

The RNA tumor viruses contain two single stranded RNA molecules as their genome whose size is approximately 35S. The RNA molecules in a tumor virus genome are identical to each other. Analysis of the 5' sequences of the 35S RNA of Rous Sarcoma Virus has been performed (Haseltine et al., 1977). The sequence for the 110 nucleotides at the 5' end of Rous Sarcoma Virus may be arranged in a stable hairpin loop structure. Intermolecular base pairing of two RNA tumor virus RNA molecules and the single host tRNA molecule which seems to be tightly associated with the tumor virus genome may be at least partially responsible for the "head-to-head" structure reported for a number of these virus RNA molecules (Bender and Davison, 1976; Kung et al., 1976; Haseltine, 1977). The nucleotide sequences of mRNA molecules of the double stranded DNA virus, SV40, may be arranged in double stranded

hairpin loops (Bubramanian et al., 1977; Fiers et al., 1978; Reddy et al., 1978). The SV40 mRNA thus has the potential of forming hairpin structures in solution. Whether intracellular mRNA of Rous Sarcoma Virus or SV40 actually contain the double stranded regions has not been investigated.

Physical studies on ovalbumin mRNA have been performed in an attempt to characterize the secondary structure present in these molecules. The presence of regions of intramolecular base pairing in ovalbumin mRNA has been reported to be present based upon increases in absorbance when purified ovalbumin mRNA is heated or hydrolyzed by base (Holder et al., 1976).

Globin Messenger RNA

Globin mRNA has been reported to undergo thermal denaturation with an increase in the absorbance at 260 nm in a manner similar to that of rRNA (Lingrel et al., 1971; Holder and Lingrel, 1975; Vournakis et al., 1976). Although a copolymer of a random nucleotide sequence also shows thermal denaturation, globin mRNA shows greater thermal denaturation and the transition phase is sharper (Holder and Lingrel, 1975). The greater magnitude and sharper transition phase shown by globin mRNA during thermal denaturation has been interpreted to demonstrate that the regions which are denatured are present in greater amounts and are of greater stability in the globin mRNA than in the random copolymer (Holder and Lingrel, 1975).

Enzymatic digestion of globin mRNA with a single strand specific nuclease from Aspergillus orzyae, $S_1$, has yielded results which have been interpreted to show that about 70% of globin mRNA is resistant to

digestion with $S_1$ nuclease (Vournakis et al., 1976; Flashner and Vournakis, 1977). This insensitivity to nuclease digestion has been found to be unchanged over a four-fold range of enzyme concentration. Single stranded polyuridylate has been reported to be completely digested under identical reaction conditions (Flashner and Vournakis, 1977). Reaction of globin mRNA with formaldehyde to disrupt putative regions of RNA secondary structure has been found to decrease the resistance of mRNA to $S_1$ nuclease digestion from 70% to 40% (Vournakis et al., 1976). Analysis of the products of $S_1$ nuclease digestion of globin mRNA by polyacrylamide gel electrophoresis in 7 M urea has shown evidence for the presence of RNA fragments of discrete lengths. The RNA fragments obtained by $S_1$ nuclease digestion of globin mRNA have been found to range in length from 7 to 78 nucleotides. The globin mRNA has been proposed to contain hairpin loops which are resistant to $S_1$ nuclease digestion (Vournakis et al., 1976; Flashner and Vournakis, 1977). However, the positions of the proposed regions of secondary structure within the globin mRNA nucleotide sequence have not been investigated.

Another approach to the investigation of globin mRNA secondary structure has been taken by Scherrer and coworkers. Duck globin mRNA has been examined with dark field electron microscopy in an attempt to visualize regions of secondary structure. Both duck globin messenger ribonucleoprotein particles (obtained by EDTA disassociation of polysomes) and protein free mRNA molecules have been found to contain regions which bind uranyl acetate. The regions binding uranyl acetate have been interpreted to be regions of intramolecular secondary structure (Dubochet et al., 1973; Scherrer, 1973). Treatment of duck globin mRNA with formaldehyde has been shown to prevent subsequent

uranyl acetate binding, which has been interpreted to be due to the denaturation of regions of secondary structure present in the mRNA molecule (Dubochet et al., 1973).

The nucleotide sequence of rabbit β-globin mRNA has been determined (Efstratiadis et al., 1977). While under investigation, the secondary and tertiary structure of β-globin mRNA is not known at this time.

Nascent Chain Nonuniformities in Rabbit Reticulocytes

The rate of incorporation of radioactive amino acids into proteins during α- and β-globin biosynthesis has been measured. The average rate of chain elongation for both α- and β-globin has been found to be 200 seconds per globin chain at 25° (Lodish and Jacobson, 1972). This value, however, is an average of all the successive chain elongation steps required for the synthesis of one globin chain. Studies by Protzel and Morris (1974) have demonstrated, by means of analysis of the size distribution of nascent globin peptides, that there are relatively slow steps in the chain elongation process of rabbit α- and β-globin biosynthesis in reticulocyte whole cell incubations.

A uniform rate of chain elongation for all steps during translation of an mRNA would predict that every nascent chain size for a protein (from one amino acid long to a complete protein still attached to the tRNA) would be represented in equal numbers in the cellular nascent chain population (see Protzel and Morris, 1974, for a discussion of the expected results of the nascent chain profile analysis under the conditions of uniform nascent chain sizes). Chromatographic analysis of the size distribution of uniformly labeled nascent α- and β-globin

peptide chains from rabbit reticulocytes under denaturing conditions has revealed that there are accumulations of nascent chains of discrete sizes (Protzel and Morris, 1974). Reticulocytes have been incubated with radioactive amino acids and the nascent peptide chains purified from these cells by the method of Slabaugh and Morris (1970). Conditions of incubation have been used such that the nascent chain population is uniformly labeled at the time of its purification of peptidyl-tRNA (Protzel and Morris, 1973). Analysis of the nascent chain sizes has been performed on a Bio-Gel A 0.5 m chromatography column using 6.0 M guanidine-HCl, 0.1 M 2-mercaptoethanol (pH 6.5) as the solvent. Elution of proteins from Bio-Gel agarose columns under denaturing conditions has been shown to yield a linear relationship between the cube root of the distribution coefficient ($K_d^{1/3}$) and (molecular weight)$^{0.555}$ (Fish et al., 1969; Protzel and Morris, 1974).

Since there is one nascent chain per ribosome, the length of a nascent peptide chain is a measure of the position of a ribosome along the mRNA that it is translating. The accumulation of nascent peptide chains of discrete sizes on rabbit reticulocytes, therefore, would indicate that in a population of mRNA molecules ribosomes are residing longer at certain regions of the mRNA nucleotide sequence than at other regions during chain elongation. Because nascent chains have been isolated from a population of ribosomes which have been actively synthesizing protein, the nonuniform size distribution has been concluded to be the result of unequal rates of polypeptide chain elongation during protein biosynthesis for different regions of the same mRNA molecule. The presence of accumulations of nascent chains has been found to be independent of the radioactive amino acid used for

labeling of the nascent chains during the cellular incubations (Protzel and Morris, 1974).

An analysis of the origins of nascent chain size accumulations during protein biosynthesis is the subject of this thesis.

## MATERIALS

Cyclohexamide, chloramphenicol, guanidine-HCl (practical grade), bovine hemin (twice recrystallized), phosphocreatine, 2,4-dinitrophenyl alanine, ammonium persulfate, Triton X-114, N,N,N',N'-tetramethyl-ethylenediamine, and creatine phosphokinase (E.C. No. 2.7.3.2) were purchased from Sigma Chemical Company, St. Louis, Missouri. Phenyl-hydrazine-HCl, naphthalene (scintillation grade), and diethylpyrocarbon-ate were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. Rifampicin was obtained from Schwartz/Mann, Orangeburg, New York. Edeine B was purchased from Calbiochem Company, La Jolla, California. Bio-Rad Company, Richmond, California, was the source of Bio-Gel A 0.5m (10% agarose) and Bio-Gel P-10. Kerosene (APCO #476) was purchased from Carrier-Stephens Company, Lansing, Michigan. Nembutal (sodium pentobarbital) was obtained from Abbot Laboratories, North Chicago, Illinois. Research Products International was the source of 2,5-diphenyl-oxazole (PPO, scintillation grade), 1,4-bis 2-(4-methyl-5-phenyloxazoly) -benzene (POPOP, scintillation grade), and Triton X-100. Blue dextran 2000 was purchased from Pharmacia Company, Uppsala, Sweden. Micro-granular (preswollen) diethylaminoethyl cellulose (DE52) and carboxy-methyl cellulose (CM32) were obtained from Whatman Biochemicals, Ltd., Maidstone, Kent, Great Britain, as were GF/C glass fiber filters. Sodium heparin was purchased from Fisher Scientific Company, Fair

Lawn, New Jersey. Sparsomycin was generously donated by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland.

Radioactive amino acids were purchased from New England Nuclear, Boston, Massachusetts ($^{3}$H-tryptophan, 7.9 Ci/mmole, $^{14}$C-tryptophan, 50.7 mCi/mmole) and Amersham/Searle Company, Arlington Heights, Illinois ($^{3}$H-leucine, 1.0 Ci/mmole and 60 Ci/mmole, and $^{3}$H-aspartic acid, 178 mCi/mmole).

All other chemicals used were reagent grade.

# METHODS

## Preparation of Rabbit Reticulocytes

New Zealand white male rabbits weighing 6-12 pounds were made anemic by four daily injections of 2.5% acetylphenylhydrazine. The acetylphenylhydrazine was prepared in NKM salts (Allen and Schweet, 1961) and adjusted to pH 7.2-7.5. Upon the eighth day after the first injection 2000 units of sodium heparin and 100 mg of Nembutal were injected intravenously through the ear. The blood was removed by heart puncture with an 18 gauge needle and immediately cooled to 0°. The hematocrit was generally 15-25. All subsequent steps were performed at 0°.

The blood was poured through a layer of glass wool to remove pieces of tissue and centrifuged at 4000 x g for 20 minutes. The plasma was removed by aspiration and the cells gently suspended with a rubber policeman in a volume of reticulocyte saline solution (RS) equal to the plasma volume (Lingrel and Borsook, 1963). The cells in RS solution were isolated by centrifugation and aspiration of the upper layer, and then washed another time with the same volume of RS as used before. The washed reticulocytes were isolated again by centrifugation and aspiration of the upper layer of RS.

Preparation of Reticulocyte Lysate

Washed rabbit reticulocytes were lysed with an equal volume of ice cold deionized water for ten minutes with occasional mixing. The cell lysate was then centrifuged at 25,000 x g for 30 minutes. The supernatant fraction was immediately frozen in aliquots with dry ice and then placed in liquid nitrogen. Each aliquot was thawed immediately before use. The conditions for a lysate incubation mixture are shown in Table 1 (Darnbraugh, et al., 1973).

Table 1. Final concentrations of components added to the reticulocyte lysate incubation mixture.

| | |
|---|---|
| KCl | 42.0 mM |
| Hemin | 3.2 mM |
| ATP | 0.95 mM |
| GTP | 0.21 mM |
| Creatine Phosphate | 11.6 mM |
| Creatine Phosphokinase | 106 µg/ml |
| Amino Acids | One tenth the final concentration of Lingrel & Borsook (1961) as modified by Hunt (1968) |
| $MgCl_2$ | 1.0 - 1.6 mM |

Preparation of Wheat Embryo Cell-Free Protein Synthesizing System

Ionia variety wheat was obtained from Dr. Everett Everson of the Michigan State University's Department of Crop and Soil Science. Embryos were prepared from the wheat by the method of Marcus et al. (1975). Two hundred grams of wheat were treated at high speed for five

seconds in a Waring blender. The pieces which would pass through a #16 mesh sieve but not a #18 mesh were saved. The chaff was removed from this fraction by passing it twice through a blower. Contaminating endosperm fragments were removed by adding the embryo preparation to 300 ml of cyclohexane: carbon tetrachloride, 1:2, and allowing the endosperm fragments to settle from the embryo fragments, which float. The embryo fragments were then poured into a sintered glass filter and collected. The embryo fragments were then allowed to air dry and stored at -20°.

The preparation of the wheat embryo cell-free system was carried out as described by Marcus et al. (1975), except that dialysis of the S23 was carried out for two to three hours immediately before use. Incubations for protein synthesis were of a final volume of 150 µl or multiples of that amount. The temperature of the incubations was 30°. The conditions used for labeling of protein in the wheat embryo cell-free system are described in Table 2. The incubation conditions were the same as those reported by Marcus et al. (1975), except the optimum concentration of $MgCl_2$ for protein synthesis was determined to be 3.6 mM.

Determination of the Rate of Protein Synthesis

Forty µl samples were taken from an incubation mixture and added immediately to 1.0 ml of a 0.5 mg/ml BSA, 0.01 M leucine solution at 0°. One ml of 1.0 N NaOH, 0.01 M leucine was added to each fraction. After the samples had then incubated for 20 minutes at 40° and cooled on ice, 4.0 ml of 15% TCA, 0.01 M leucine were added. Following an incubation at 0° for at least one hour, the precipitated protein was collected by

Table 2. Final concentrations of components added to the wheat embryo cell-free incubations.

| | |
|---|---|
| $Mg(Ac)_2$ | 3.6 mM |
| KCl | 60.3 mM |
| GTP | 0.04 mM |
| DTT | 2.97 mM |
| Unlabeled Amino Acids<br>$^3$H-leucine (or $^3$H-tryptophan) | 0.05 mM<br>0.03 μCi/ml (5 μCi/150 μl incubation mixture) |
| mRNA | 1.6 to 8 μg/ml |

suction on a GF/C filter which had been washed with 10 ml of 10% TCA, 0.01 M leucine at 0°. The collected protein was washed with four 5 ml aliquots of 10% TCA, 0.01 M leucine and two 3 ml washes with ethanol-diethyl ether (1:1). The filters were placed in the bottom of a glass scintillation vial and dried at 90° for 45 minutes. After the vials had cooled to room temperature, 5 ml of a Triton X-114-xylene based liquid scintillation cocktail were added and the incorporation of $^3$H-leucine into TCA insoluble material determined. The liquid scintillation cocktail contained, per liter, 600 ml xylenes, 333 ml Triton X-114, 60 g naphthalene, 6 g PPO, and 0.5 g dimethyl POPOP.

Growth Condition of E. coli

Escherichia coli strain H1008 (Hfr+) was a gift of L. Snyder of Michigan State University's Department of Microbiology and Public Health. Escherichia coli strain Hfr H was a gift of David Friedman, Department of Microbiology, University of Michigan. Cells were grown

in a modified synthetic medium (MTPA) of Vinuela et al. (1967). The contents of MTPA medium are listed in Table 3.

Table 3. Contents of MTPA media.

| | |
|---|---|
| Tris-HCl (pH 7.5) | 0.1 M |
| NaCl | 8.5 mM |
| KCl | 0.1 M |
| $NH_4Cl$ | 0.02 M |
| $KH_2PO_4$ | 0.34 mM |
| $Na_2SO_4$ | 0.16 mM |
| $CaCl_2$ | 2.5 mM |
| $MgCl_2$ | 2.5 mM |
| Glucose | 0.01 M |
| 19 Unlabeled Amino Acids | 0.10 mM |
| $^3H$-Labeled Amino Acid | 0.03 mM |
| Thiamine hydrochloride | 10 µg/ml |

An overnight growth culture of E. coli in MTPA at 37° was used to inoculate flasks for growth of cells for MS2 infection. In a typical experiment, 100 ml of sterile MTPA in a 500 ml flask received a 5 ml inoculum. The inoculated MTPA media was then placed on a rotary shaker at 37° until the cell suspension had an absorbance at 540 nm of 0.25 (2-3 x $10^8$ cells/ml). The cells were growing in log phase with a doubling time of about 45 minutes at this point. The incubation mixture was then made 10 mM with $CaCl_2$. Five minutes later the incubation medium was inoculated with MS2 at a multiplicity of 100 MS2 virus per

cell. Ten minutes after viral infection rifampicin was added to a concentration of 1 mg/ml. Rifampicin had been shown to block host RNA synthesis and subsequently host protein synthesis (Hartmann et al., 1967). Bacteriophage protein synthesis had been shown to be unaffected by rifampicin (Fromageot and Zinder, 1968). Twenty minutes after rifampicin addition 200 μCi of a tritiated amino acid were added to the incubation mixture. Following further incubation for 15 minutes the solution was poured into 100 ml of Stopping Buffer (Table 4) at 0° in an ice water bath (Hothman-Iglewski and Franklin, 1967).

Table 4. Composition of Stopping Buffer.

| | |
|---|---|
| Tris-HCl (pH 7.5) | 0.1 M |
| NaCl | 8.5 mM |
| KCl | 0.1 M |
| $NH_4Cl$ | 0.02 M |
| $KH_2PO_4$ | 0.34 mM |
| $Na_2SO_4$ | 0.16 mM |
| $CaCl_2$ | 2.5 mM |
| $MgCl_2$ | 2.5 mM |
| Sodium Azide | 5.0 mM |
| Diethylpyrocarbonate | 0.01 % |
| Chloramphenicol | 200.0 μg/ml |

The cells were harvested by centrifugation at 12,000 x g for ten minutes and resuspended in 7 ml of Stopping Buffer. The cells were then lysed by six 15 second treatments with a Biosonik sonicator

(Braunwill Scientific, Rochester, New York) at 4°. Unlysed cells, membranes and cellular debris were pelleted by centrifugation at 25,000 x g for 20 minutes. Ribosomes were isolated from the supernatant fraction by centrifugation at 150,000 x g for two hours at 4°. These ribosomes were then used for the preparation of peptidyl-tRNA.

Preparation of MS2 Stock

One hundred ml of MTPA media were infected with E. coli and incubated in a rotary shaker until an absorbance of 0.25 was obtained. The culture was made 10 mM $CaCl_2$ and, five minutes later, infected with MS2. Incubation was continued until the solution began to clear and lysed bacteria were visible as clumps in the media. Two ml of chloroform then were added and the flask stored at 0° overnight. The next day bacterial debris were removed by centrifugation at 10,000 x g for ten minutes and the supernatant fraction stored over $CHCl_3$. Bacteriophage preparations had titers of around $2 \times 10^{12}$ phage per ml.

Purification of Peptidyl-tRNA

A stock urea solution was deionized by stirring an 8.54 M urea solution for 1.5 hours with Amberlite MB-3. The Amberlite was removed by filtration of the solution through a sintered glass funnel and the resultant urea solution used to prepare the buffers for peptidyl-tRNA purification, Buffer I and Buffer II. The urea concentration in Buffers I and II was reduced to 7.6 M to avoid occasional recrystallization of urea at 4°.

Buffer I contained 7.6 M urea, 0.05 M 2-mercaptoethanol, 0.1 M sodium acetate (pH 5.6). Buffer II was identical to Buffer I except the sodium acetate concentration was 0.75 M.

Eukaryotic ribosome pellets were suspended in 0.75 ml of 0.25 M sucrose, 0.059 mM cyclohexamide, 0.21 mM sparsomycin. Prokaryotic ribosomes were suspended in 0.75 ml of 0.25 M sucrose, 200 µg/ml chloramphenicol. Peptidyl-tRNA was isolated from ribosome suspensions as described by Slabaugh and Morris (1970). All subsequent steps were performed at 0°.

Ribosome suspensions were initially brought to 3.0 M LiCl, 4.0 M urea, 0.05 M 2-mercaptoethanol, 0.05 M sodium acetate (pH 5.6); incubated for 16 hours at 0°, and centrifuged at 10,000 x g for 20 minutes. The precipitate was discarded. The supernatant fraction containing the peptidyl-tRNA was used for the next step.

Lithium chloride was removed from the peptidyl-tRNA by desalting on a Bio-Gel P-10 column (1.9 x 32 cm). The column was eluted with Buffer I as shown in Figure 2. The radioactively labeled material which eluted in the void volume of the P-10 colume was pooled and adsorbed to a DEAE-cellulose (DE52) column prepared in Buffer I as described below.

The DEAE-cellulose (in microgranular preswollen form) was dissolved in about 60 ml of 0.5 M acetic acid and deaereated. The pH was adjusted to 5.6 with saturated NaOH. The "fines" were removed by allowing the DEAE-cellulose mixture to settle twice (in minutes) the height of the mixture (in cm) before removing the upper layer. The DEAE-cellulose was then washed once with Buffer II and twice with Buffer I. Approximately two grams of DEAE-cellulose were used for each peptidyl-tRNA isolation in the experiments reported in this thesis. The DEAE-cellulose was prepared in a chromatography column 0.9 cm in diameter and 3 cm high.

Figure 2. Desalting step of peptidyl-tRNA purification.

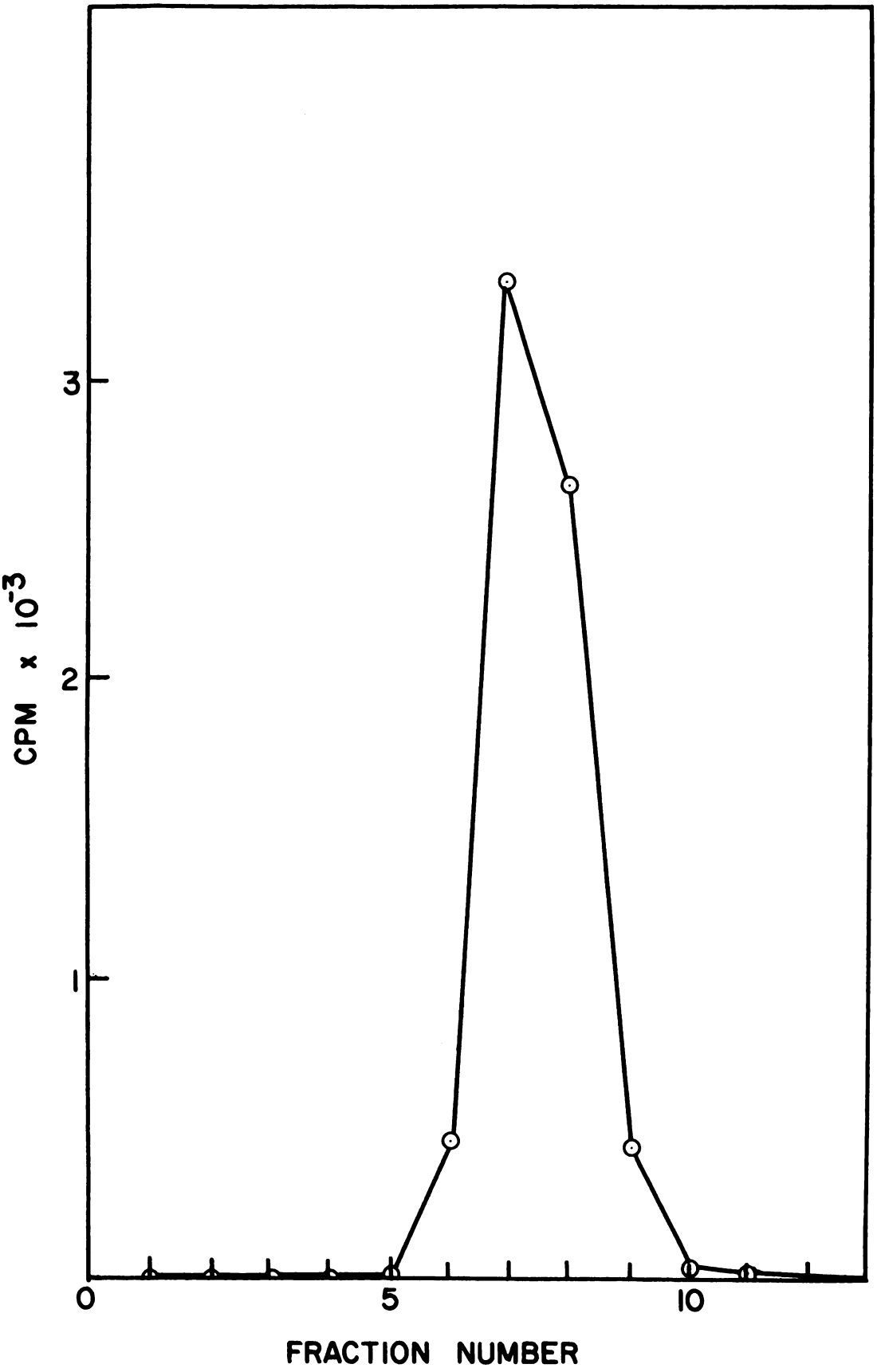



Figure 2

After the peptidyl-tRNA had been adsorbed to the DEAE-cellulose, the column was washed with about 300 ml of Buffer I. The peptidyl-tRNA was eluted from the DEAE-cellulose with Buffer II. A typical elution profile is seen in Figure 3.

The pooled fractions containing peptidyl-tRNA were concentrated to 0.3 ml on an Amicon model 8 MC microultrafiltration system (Amicon Corp., Lexington, Mass.) by pressure filtration with $N_2$ gas through an Amicon UM-2 filter. One ml of 6.0 M guanidine-HCl, 0.1 M 2-mercapto-ethanol (pH 6.5) was then added and the solution concentrated to 0.4 ml.

Twenty µl of 6.0 N NaOH were added to the concentrated peptidyl-tRNA solution followed by incubation at 37° for four hours in order to hydrolyze the peptide-tRNA ester bond. The solution was then neutralized with 20 µl of 6.0 N HCl and made 0.05 M with dithiothreitol. After two hours at room temperature 80 µl of 3.6% Blue dextran and 70 µl of 0.1% DNP-alanine, 60% sucrose were added before chromatographic analysis to serve as markers for $K_d = 0$ and $K_d = 1$, respectively.

Recrystallization of Guanidine-HCl

Practical grade guanidine-HCl was twice recrystallized by the method of Nozaki and Tanford (1967). Guanidine was dissolved in three liters of 100% ethanol at 70° to near saturation. Two g of activated charcoal were added, followed 5 minutes later with 5 g of Celite. The solution was filtered through a Whatman #1 filter paper in a steam heated, jacketed, Buchner funnel by suction. About 1.5 liters of benzene was added to the ethanol. After overnight storage at 0° the guanidine was collected in a Buchner funnel and dried under vacuum.

Figure 3. Elution of peptidyl-tRNA from DEAE-cellulose.

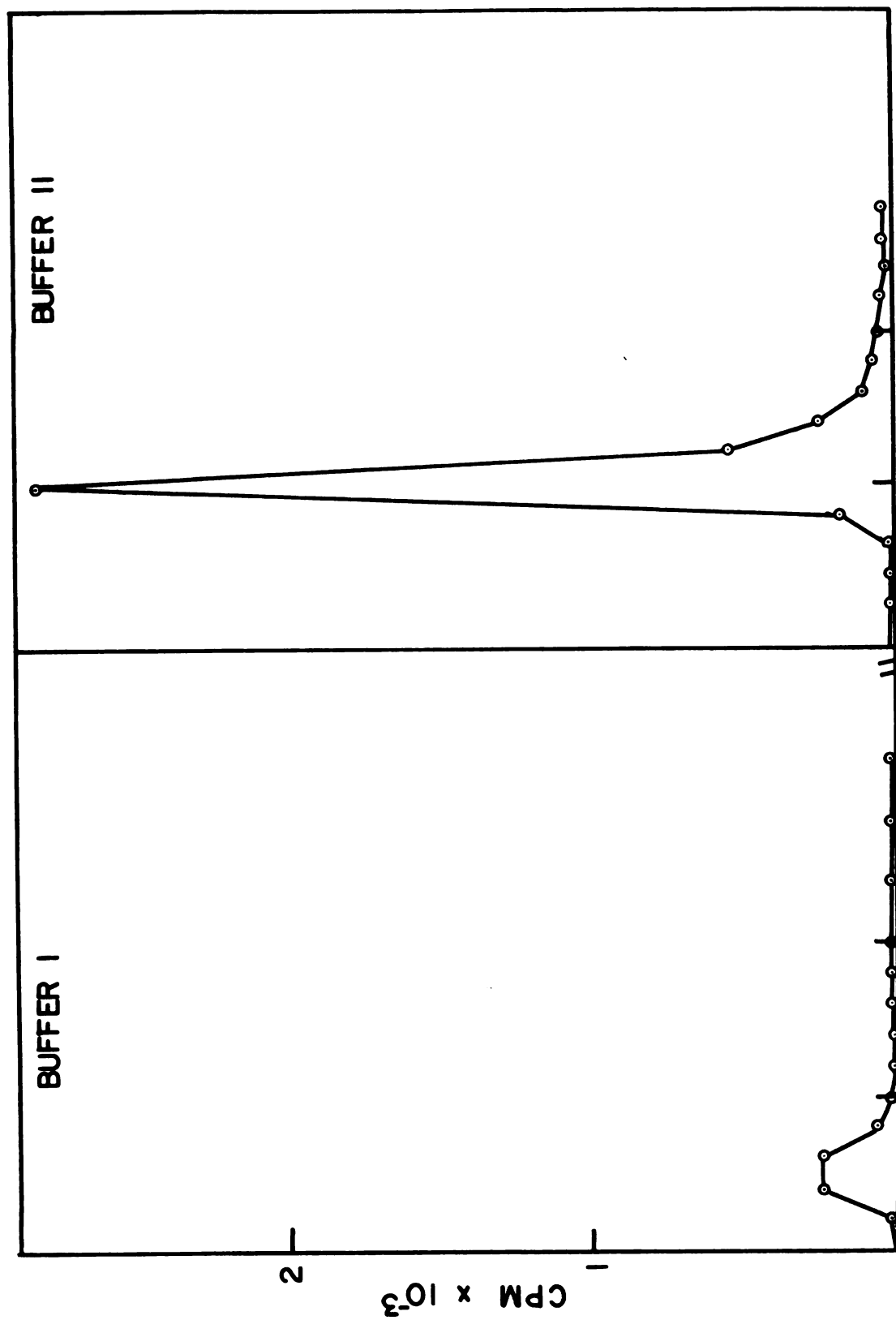



Figure 3

A second recrystallization was performed by dissolving the guanidine in a minimal amount of boiling anhydrous methanol. The guanidine-HCl was then precipitated by cooling the solution in a dry ice-acetone bath, collected in a Buchner funnel, and dried under vacuum.

## Analysis of Peptide Sizes

The size distribution of nascent peptide chains was analyzed by the gel chromatographic method described by Fish et al. (1969) and by Protzel and Morris (1974). Radioactively labeled nascent chains were subjected to chromatographic analysis under denaturing conditions (6.0 M guanidine-HCl, 0.1 M 2-mercaptoethanol, pH 6.5) on Bio-Gel A 0.5 m agarose.

Two hundred thirty ml of the Bio-Gel A 0.5 m slurry were added to 800 ml of water and allowed to settle. When the final volume of the gel plus water was 500 ml, twice recrystallized guanidine was slowly added with mixing by gently swirling the solution in a 1500 ml beaker (Fish et al., 1969). When the solution had been brought to 6.0 M guanidine-HCl, the pH was adjusted to 6.5 with 1.0 N NaOH.

A Pharmacia analytical column 100 cm x 1.6 cm was filled with the Bio-Gel A 0.5 m mixture to a bed height of 90 cm, washed with 6.0 M guanidine-HCl, 0.1 M 2-mercaptoethanol (pH 6.5) for 1.5 gel volumes and used for the nascent peptide chain analyses reported in this thesis. A pressure differential of 80 cm of solvent was maintained. The column eluate was collected directly into scintillation vials for determination of radioactivity.

Isolation of Rabbit Globin mRNA

Washed rabbit reticulocytes were prepared as described above. The cells were lysed by the addition of an equal volume of water followed by incubation at 0° for 10 minutes. Membranes were precipitated by centrifugation at 25,000 x g for 20 minutes. The supernatant fraction was removed and a ribosomal pellet prepared by centrifugation at 100,000 x g for two hours at 0°. Globin mRNA was prepared from the ribosome pellet by the oligo(dT)-cellulose chromatography method of Aviv and Leder (1972).

The reticulocyte ribosomal pellet was suspended in 0.1 M Tris-HCl (pH 9.0), 0.1 M NaCl, 1.0 mM EDTA to a concentration of 20 $A_{260}$ units per ml. One tenth volume of 10% SDS was then added. The ribosome containing solution was shaken with an equal volume of phenol-chloroform-isoamyl alcohol (50:50:1) for 10 minutes at room temperature. The phases were cooled to 4° and separated by centrifugation at 12,000 x g for 10 minutes. The aqueous (upper) phase was extracted with an equal volume of phenol-chloroform-isoamyl alcohol as described above. Following the second extraction, 0.1 volume of 20% potassium acetate (pH 5.5) was added to the upper phase. Two volumes of 95% ethanol were added and the mixture allowed to stand overnight at -20° to precipitate the RNA.

Precipitated RNA was collected by centrifugation at 12,000 x g for 20 minutes at -20°. The RNA pellet was dissolved in a minimal volume of 0.01 M Tris-HCl (pH 7.5), 0.5 M NaCl (binding buffer) and adsorbed to 0.5 g of oligo(dT)-cellulose prepared in binding buffer in a sterile 2 ml Pasteur pipet at room temperature. The adsorption was performed by allowing one column volume of the RNA containing material

to enter the column. The flow was then halted for five minutes to allow the poly(A) of the mRNA to hybridize to the oligo(dT). This step was then repeated until all the RNA containing buffer had entered the column. The column was washed extensively with binding buffer until the absorbance of the eluate at 260 nm returned to 0 (Figure 4). The RNA which was not retained by the column was called poly(A) minus RNA.

Elution of RNA which had hybridized to the column was achieved by washing the column with 0.01 M Tris-HCl (pH 7.5) as shown in Figure 4. The RNA containing fractions were determined by their absorbance at 260 nm and pooled. The RNA pool was divided into small aliquots and immediately frozen in liquid nitrogen. The RNA concentration in the pooled fractions was 0.12 mg/ml (20 $A_{260}$ units = 1 mg/ml).

Preparation of Aminoacyl-tRNA Synthetases from Rabbit Reticulocytes

A preparation containing aminoacyl-tRNA synthetases was prepared by a modification of the method of Smith and McNamara (1972). A sample of a rabbit reticulocyte lysate prepared as described above was centrifuged at 100,000 x g for two hours at 4°. Aminoacyl-tRNA synthetases were precipitated by the slow addition of $(NH_4)_2SO_4$ to 70% saturation. The solution was kept at 0° by placing the container in an ice bath. One-half hour after the addition of the last amount of $(NH_4)_2SO_4$ the mixture was centrifuged at 10,000 x g for 15 minutes at 4°. The precipitate was then washed with a volume of 70% saturated $(NH_4)_2SO_4$ equal to the starting volume. The precipitate was isolated by centrifugation at 10,000 x g for 15 minutes at 4°. The precipitate was then

Figure 4. Oligo(dT)-cellulose chromatography of globin mRNA.

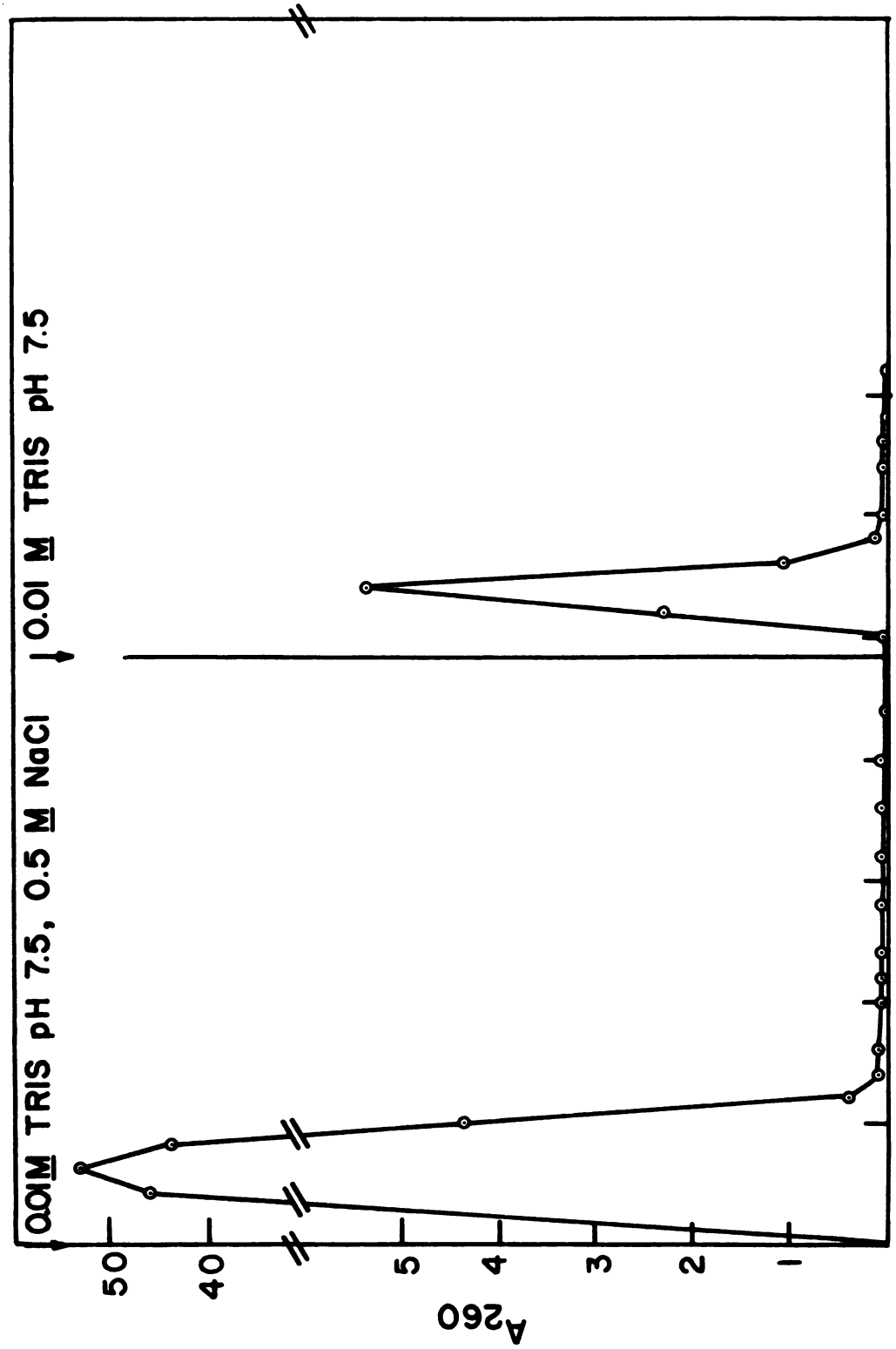

0.01M TRIS pH 7.5, 0.5 M NaCl
0.01 M TRIS pH 7.5
A260
FRACTION NUMBER
0
10
20
30
1
2
3
4
5
40
50

Figure 4

dissolved in 0.02 M Tris-HCl (pH 7.5), 25 mM KCl, 4 mM $MgCl_2$, 20% (v/v) glycerol and stored at -20°.

Preparation of a Salt Wash of Reticulocyte Ribosomes

Ribosomes from 1.6 ml of a rabbit reticulocyte lysate were isolated by centrifugation at 100,000 x g for two hours at 4°. The ribosomes were suspended in two ml of a solution containing 10 mM Tris-HCl (pH 7.4), 0.5 M KCl. The mixture was then centrifuged at 100,000 x g for two hours at 4°. The supernatant fraction was dialyzed overnight against 500 ml of 10 mM Tris-HCl (pH 7.5), 1.0 mM dithiothretiol, and 0.35 M KCl. The preparation was concentrated to 0.1 ml by pressure filtration through an Amicon UM-2 membrane and stored at -20°.

Ammonium Sulfate Fractionation of a Reticulocyte Postribosomal Supernatant Fraction

Following centrifugation of 1.6 ml of rabbit reticulocyte lysate at 100,000 x g for two hours the supernatant solution was diluted to 5 ml with a buffer consisting of 0.2 M Tris-HCl (pH 7.5), 25 mM KCl, 4 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20% glycerol. To this mixture was added powdered $(NH_4)_2SO_4$ to a final concentration of 70% of saturation. After gentle stirring for 30 minutes the precipitate which formed was removed by sedimentation at 30,000 x g for 30 minutes. The precipitate was washed by resuspension in the above buffer containing 70% $(NH_4)_2SO_4$ and sedimented as before. The final precipitate was dissolved in 2 ml of the above buffer and concentrated to a small volume, replacing the original solution with 10 mM Tris-HCl (pH 7.5), 1.0 mM dithiothreitol, 0.35 M KCl by ultrafiltration with an Amicon UM-2 membrane filter. The final solution (0.1 ml) was then stored at -20° until used.

Isolation of Rabbit Reticulocyte tRNA

A sample of a reticulocyte lysate prepared from an anemic rabbit was treated with an equal volume of $H_2O$-saturated phenol at room temperature for five minutes. The phases were separated by centrifugation in a clinical centrifuge and the phenol phase was extracted with one-half volume of 2% potassium acetate (pH 5.5). One-tenth of the original volume of 20% potassium acetate (pH 5.5) was added to the combined aqueous phases to bring the combined phases to a final concentration of 2% potassium acetate. Two volumes of 95% ethanol were added to the RNA containing solution and the sample stored overnight at -20°. The RNA precipitate was collected by centrifugation at 20,000 x g for 20 minutes at -20° and was then dissolved in a volume of 0.1 M Tris-HCl (pH 9.0) equal to the original volume of the lysate used. This sample was incubated at 37° for 30 minutes. The pH was adjusted with HCl to 7.5 at room temperature and the tRNA isolated by the method of Holley et al. (1961) as described below.

The RNA sample was adsorbed to a DEAE-cellulose column (1.4 x 6.0 cm) equilibrated with 0.1 M Tris-HCl (pH 7.5). Following extensive washing of the column with 0.1 M Tris-HCl (pH 7.5), the tRNA fraction was eluted with 1.0 M NaCl, 0.1 M Tris-HCl (pH 7.5). The elution profile of such an isolation procedure is shown in Figure 5.

After pooling the tRNA fractions, two volumes of 95% ethanol were added and the sample stored overnight at -20°. The RNA was isolated by centrifugation at 20,000 x g for 20 minutes at -20°. The RNA precipitate was resuspended in a small volume of $H_2O$, frozen, lyophylized, and stored at -20° until use.

Figure 5. Elution profile of rabbit reticulocyte tRNA from DEAE-cellulose.

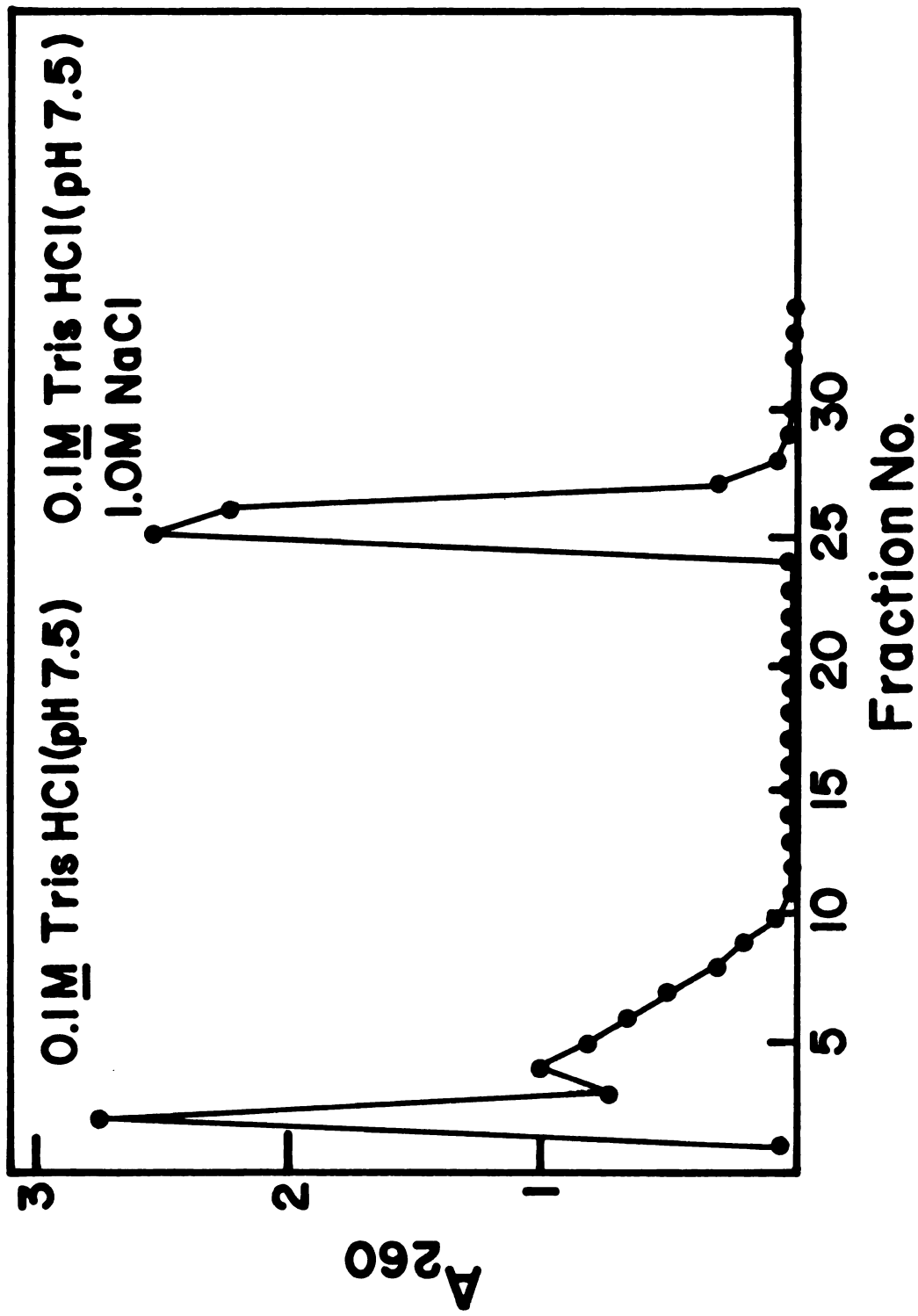

0.1M Tris HCl(pH 7.5)
0.1M Tris HCl(pH 7.5)
1.0M NaCl
A260
3
2
1
5
10
15
20
25
30
Fraction No.

Figure 5

Isolation of Rabbit Liver tRNA

Rabbit liver tRNA was isolated by the method described by Bose et al. (1971). Three hundred fifty grams of liver were obtained from healthy New Zealand white rabbits, cut into small pieces, suspended in 350 ml of 0.1 M Tris-HCl (pH 7.5), 0.35 M sucrose, 3.0 mM $MgCl_2$, 30 mM KCl, and mixed in a Waring blender for four minutes at 0°. The resultant mixture was centrifuged at 25,000 x g for 15 minutes and the precipitate discarded. The supernatant was then centrifuged at 125,000 x g for two hours at 4°. Acetic acid (1.0 M) was slowly added to the supernatant solution in an ice water bath until the pH was 5.0 and the sample mixed by stirring at 0° for 15 minutes. This procedure was followed by centrifugation at 20,000 x g for 15 minutes. The precipitate was resuspended in 200 ml of 20 mM Tris-HCl (pH 7.5). An equal volume of redistilled $H_2O$-saturated phenol was added and the solution stirred at room temperature for one hour. The phases were separated by centrifugation at 20,000 x g for 15 minutes at 0°. The phenol phase was extracted with 100 ml of 20 mM Tris-HCl (pH 7.5). Thirty ml (0.1 volumes) of 20% potassium acetate (pH 5.5) were added to the combined aqueous phases. Two volumes of 95% ethanol were then added and the RNA was allowed to precipitate overnight at -20°.

The RNA was collected the following morning by centrifugation at 25,000 x g for 20 minutes at -20°. Twenty ml of 1.0 M NaCl were added to the precipitate at 0°. The solution was centrifuged at 25,000 x g for 20 minutes at 0° and the precipitate extracted with 15 ml of 1.0 M NaCl. Following centrifugation the combined extracts were mixed with 40 ml of 1.0 M Tris-HCl (pH 9.0). The solution was incubated at 37° for 45 minutes to cleave the aminoacyl-tRNA bonds. This procedure

was followed by dialysis for 24 hours against two 4 liter volumes of $H_2O$ at 0°, changing the dialysis solution after 12 hours.

Dialysis was followed by allowing the sample to attain room temperature and adsorbing the sample to a DEAE-cellulose chromatography column (1.5 x 9.0 cm) which had been equilibrated with 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl. The column was washed with 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl until the absorbance at 260 nm of the eluate was zero. The tRNA was eluted with 0.01 M Tris-HCl (pH 7.5), 1.0 M NaCl. Two volumes of 95% ethanol were added to the tRNA pool, and after three hours at -20° the tRNA precipitate was isolated by centrifugation at 20,000 x g for 15 minutes at -20°.

The tRNA pellet was dissolved in 10 ml of $H_2O$, dialyzed overnight against 4 liters of $H_2O$ at 0°, lyophylized and stored at -20° for later use.

Determination of Leucine Accepting Capability of tRNA

The ability of tRNA to bind to amino acids in the formation of an aminoacyl-tRNA molecule was determined by incubating isolated tRNA, $^3$H-leucine, and the crude aminoacyl-tRNA synthetase preparation together to form a $^3$H-leucine-tRNA molecule (which was insoluable in 9% trichloroacetic acid). The final incubation mixture (0.5 ml) is shown in Table 5.

At appropriate times 0.1 ml aliquots were transferred to 3.0 ml of 9% TCA, 3% $H_2O_2$, 10 mM leucine at 0°. The precipitate was isolated by suction filtration through GF/C filters and washed with three 10 ml aliquots of 10% TCA, 10 mM leucine at 0°. The filters were then placed in glass scintillation vials and heated at 100° for 45 minutes. After

the vials had cooled, 5 ml of Formula C were added and the radioactivity determined by liquid scintillation spectrometry.

Table 5. Incubation mixture for the determination of leucine accepting capability of tRNA.

| | |
|---|---|
| Tris-HCl (pH 8.0) | 50 mM |
| $MgCl_2$ | 15 mM |
| ATP | 5 mM |
| 2-Mercaptoethanol | 20 mM |
| Aminoacyl-tRNA Synthetase Prep. | 1:50 dilution |
| Transfer RNA Preparation | 1.0 mg/ml |
| $^3$H-leucine | 10 μM |

Treatment of tRNA with Periodate

The periodate treatment of Folk and Berg (1970) was used to create a preparation of rabbit liver tRNA which was not capable of binding amino acids. Thirty $A_{260}$ units (1.5 mg) of rabbit liver tRNA, isolated as described above, were dissolved in 6.0 ml of 0.1 M sodium acetate (pH 4.6). To one-half of the tRNA sample 0.5 ml of 0.1 M sodium acetate (pH 4.6) was added; 0.5 ml of 0.01 M sodium periodate (freshly prepared in the above acetate buffer) was added to the remaining half of the tRNA sample. Both samples were incubated at room temperature for 30 minutes in the dark. Following the incubation 1.0 ml of 5.0 M NaCl was added to each sample and the samples were cooled to 4°. Then two volumes (9 ml) of 95% ethanol, which had been cooled to -20°, were added and the samples were centrifuged immediately at 30,000 x g for 20 minutes at -20°.

The supernatant fractions of the above samples were removed and the presence of excess periodate in the sample to which the sodium periodate had been added was determined as described by Folk and Berg (1970). The presence of excess periodate was determined by setting a spectrophotometer to read an absorbance of approximately 2.5 at a wavelength of 232 nm with one ml of this sample in the cuvette. A small volume of 0.1 M ethylene glycol, 0.1 M sodium acetate (pH 4.6) was added. The rapid decrease in absorbance following the addition of the ethylene glycol was a qualitative indication that the reaction mixture contained excess periodate.

Immediately following their isolation, the tRNA precipitates were each dissolved in 2.0 ml of 0.1 M ethylene glycol, 0.1 M sodium acetate (pH 4.6) and allowed to incubate at room temperature in the dark for 10 minutes to remove any periodate. The samples were then put into an ice bath and a 0.5 ml volume of 5.0 M NaCl was added to each tube. Two volumes (5.0 ml) of 95% ethanol at -20° were added to each tube and the samples were centrifuged immediately at 30,000 x g for 20 minutes at -20°. The precipitates were dissolved in $H_2O$ and the absorbance at a wavelength of 260 nm was determined. The recovery of the tRNA was greater than 90%. Aliquots of both samples were lyophylized in test tubes and stored at -20° for later use.

Formaldehyde Treatment of Globin mRNA

Fifty µl of globin mRNA (0.12 mg/ml) were brought to 0.2 M NaCl. Two volumes of 95% ethanol were added and the samples incubated at -20° for two hours to precipitate the RNA. Precipitated RNA was collected by centrifugation at 20,000 x g for 20 minutes at -20°. After

discarding the supernatant solution, 0.5 ml of $H_2O$ was added, the samples were frozen with solid $CO_2$, and the frozen mixture was lyophylized.

The lyophylized RNA samples were dissolved in 0.1 ml of 0.2 M NaCl, 0.09 M $Na_2HPO_4$, 0.01 M $NaH_2PO_4$, and either 0.02 M or 0.03 M formaldehyde (Boedtker, 1967). The RNA samples were then incubated at 68° for 10 minutes. The RNA was precipitated by the addition of 95% ethanol at -20°. After a 30 minute incubation period at -20°, the samples were centrifuged at 20,000 x g for 20 minutes at -20°. The supernatant solution was discarded, 0.5 ml of $H_2O$ was added, the samples were frozen and lyophylized. Each sample was then dissolved in 50 µl of $H_2O$ and stored in liquid nitrogen.

Cyanogen Bromide Treatment of MS2 Coat Protein

Treatment of proteins with cyanogen bromide has been shown to cleave peptide bonds at methionine residues (Gross and Witkop, 1962). A solution of MS2 coat protein in 7.6 M urea, 0.15 M sodium acetate, 0.05 M 2-mercaptoethanol (pH 6.5) was dialyzed for two hours against one liter of 2% ammonium bicarbonate. The solution was then frozen and lyophylized. The sample was dissolved in 1.0 ml of 70% formic acid, 1 mg/ml cyanogen bromide. The reaction mixture was kept in the dark for 24-48 hours at room temperature. Nine ml of $H_2O$ were then added and the sample was frozen and lyophylized.

Separation of Alpha and Beta-Globin

A sample of $^{14}$C-tyrosine labeled rabbit globin prepared by Dr. A. Morris and H.-C. Hsung was used for the preparation of α- and β-globin chains. The sample was dissolved in 0.2 M formic acid, 0.02 M pyridine,

0.005 M 2-mercaptoethanol and adsorbed to carboxymethyl cellulose (CM32) in a chromatography column 1.0 x 25 cm. The globin was eluted by a nonlinear gradient containing 1,3,5,7,1,7, and 9 fold the concentration of 0.2 M formic acid, 0.02 M pyridine, 0.05 M 2-mercaptoethanol in separate chambers (Rabinovitz et al., 1964). The formic acid puridine gradient was prepared by using a 10 chamber Varigrad gradient maker (Buchler Instruments, Inc., Fort Lee, New Jersey). Each chamber contained 50 ml.

An elution profile demonstrating the separation of α- and β-globin is shown in Figure 6. Fractions 14-22 were pooled as the α-chain sample and fractions 27-33 were saved as the β-chain sample. Each sample was separately dialyzed against two successive one liter volumes of $H_2O$, frozen and lyophylized.

Cyanogen Bromide Treatment of Rabbit Alpha and Beta-Globin

Rabbit globin or β-globin was dissolved in 70% formic acid at a concentration of 5.0 mg/ml. A 400-fold molar excess of cyanogen bromide was added. The solution was kept at room temperature in the dark for 24-48 hours. Following the incubation at room temperature nine times the sample volume of $H_2O$ was added. The samples were frozen and lyophylized.

Determination of Distribution Coefficients

Elution data from the Bio-Gel chromatography experiments in 6.0 M guanidine-HCl were treated as described by Fish et al. (1969). The distribution coefficient ($K_d$) was calculated from the formula:

$$K_d = \frac{V_e - V_o}{V_i - V_o}$$

Figure 6. Elution of globin from CM-cellulose.

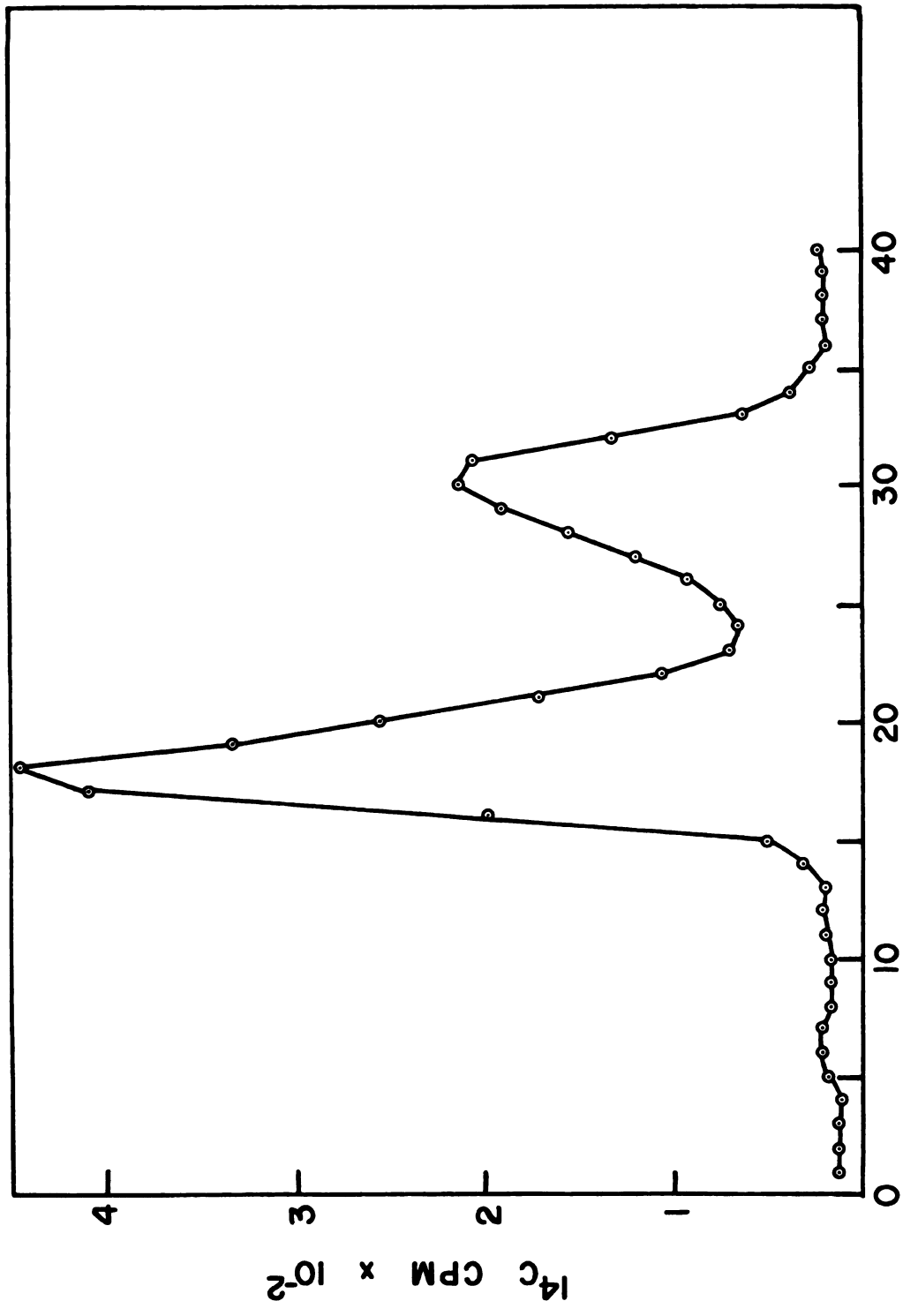



Figure 6

where $V_e$ is the elution weight of solvent which corresponds to the peak concentration of eluting solvent,

$V_o$ is the void volume (in weight of solvent),

$V_i$ is the weight of solvent contained in the gel matrix and column.

Blue dextran 2000 was used as a marker for the void volume and DNP-alanine was a marker for the total volume of the column which was accessible to solvent. In the experiments described in this thesis the peak fraction of Blue dextran and DNP-alanine were determined by visual inspection of the eluate fractions and the $K_d$ values of peaks of radioactively labeled peptides calculated from the marker positions and the fraction numbers of the peaks.

Preparation of Proteins for SDS Gel Electrophoresis

Analysis of the products of rifampicin treated, MS2 infected E. coli was carried out by the method of Vinuela et al. (1967). A ten ml solution of MTPA medium was inoculated with E. coli and grown to an absorbance of 0.25 at a wavelength of 540 nm (2-3 x $10^8$ cells/ml). The E. coli solution was infected with bacteriophage MS2 as described above. Forty-five minutes after infection 50 μCi of $^3$H-leucine were added. Following fifteen minutes of incubation at 37° the solution was cooled on ice. A four ml sample was brought to 0.1 M Tris-HCl (pH 8.4), 0.01 M EDTA, 0.14 M 2-mercaptoethanol in a final volume of 6 ml. All of the remaining operations were performed at room temperature.

The solution was then shaken with 2 ml of redistilled phenol saturated with 0.1 M Tris-HCl (pH 8.4), 0.01 M EDTA, 0.14 M 2-mercaptoethanol for three minutes. The phases were separated by centrifugation in a clinical centrifuge and the aqueous phase re-extracted once more

with 2 ml of redistilled phenol. The phenol phases were pooled and dialyzed against 300 ml of 0.1 M acetic acid, 0.14 M 2-mercaptoethanol. After two hours the dialysis fluid was changed and the dialysis continued until approximately one ml of the phenol phase remained in the dialysis bag. At this point the dialysis bag was carefully opened and the aqueous (upper) phase removed from the solution in the bag. The dialysis bag was then closed and dialysis was carried out overnight against 500 ml of 9.0 M urea, 0.05 M acetic acid, 0.14 M 2-mercaptoethanol. Dialysis was then performed for two hours against 500 ml of 8.6 M urea, 0.01 M EDTA, 0.14 M 2-mercaptoethanol, 0.1 M Tris-HCl (pH 8.4) for two hours. A stream of nitrogen was vigorously bubbled through the dialysis solution. A final dialysis was carried out for 24 hours against two 500 ml portions of 0.01 M sodium phosphate (pH 7.2), 0.1% SDS, 0.14 M 2-mercaptoethanol. The sample from the final dialysis was then used for polyacrylamide gel electrophoretic analysis as described below.

Polyacrylamide Gel Electrophoresis of Proteins

Polyacrylamide gel electrophoresis in 10% acrylamide gels and 0.1% SDS was performed as described by Fromageot and Zinder (1968). Cylindrical gels 9 cm in length and 0.5 cm in diameter were prepared from the following aqueous solutions mixed in a 1:1:2 ratio: 40% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bisacrylamide; 0.4 M sodium phosphate (pH 7.2); 0.14% ammonium persulfate. One-half ml of water was layered on top of the gels during the polymerization process to ensure a flat surface on the gel. The electrophoresis buffer was 0.1 M sodium phosphate, 0.1% (w/v) SDS.

A sample of protein (0.1 ml) prepared as described above was mixed with an equal volume of 0.01 M sodium phosphate (pH 7.2), 0.1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 50% (v/v) glycerol. Three µl of a 0.1% bromophenol blue solution in water were added and the solution carefully layered onto the top of a gel which was submerged in electrophoresis buffer. The electrophoresis was first carried out at 8 ma/gel for 20 minutes and then at 12 ma/gel for four hours. The gels were cut into 2 mm slices and placed into glass scintillation vials. Following the addition of 0.9 ml of 1 N NaOH, 0.1% SDS, the vials were tightly capped and incubated overnight at 80°. After cooling to room temperature the samples received 0.1 ml of 1 N HCl and 5 ml of Formula C scintillation fluid. The radioactivity present was determined in a Beckman LS-230 by liquid scintillation spectrometry.

# RESULTS

## Reticulocyte Lysate Cell-Free System

The experiments of Protzel and Morris (1974), in which the existence of size accumulations in uniformly labeled α- and β-globin nascent chains had been reported, were performed on nascent chains radioactively labeled in whole cell incubations. Although important in eliminating the use of an *in vitro* protein synthesizing system as a possible source of artifacts, the cellular incubation mixture was difficult (if not impossible) to manipulate in attempts to determine the origin of these nascent chain accumulations. For this reason it was decided to study the size distribution of nascent globin chains isolated from incubations of a cell-free protein synthesis system. Use of a cell-free system would enable one to investigate the nascent chain size accumulations by adding directly to the incubation mixture preparations which one desires to test for an effect upon the nascent chain elution profile.

The *in vitro* system chosen for the initial experiments described in this thesis was the rabbit reticulocyte lysate cell-free protein synthesizing system. Because reticulocyte lysate incubation mixtures utilize endogenous synthetic activity, it was not necessary to fractionate the system further or to purify and add endogenous mRNA. Since the observations of Protzel and Morris (1974) were made using whole reticulocyte incubation mixtures, preparation of the reticulocyte

lysate was a logical first step for the analysis of nascent α- and β-globin size distributions made in cell-free incubation mixtures.

A reticulocyte lysate cell-free system, originally described by Zucker and Schulmann (1968) and by Adamson et al. (1968), was prepared from washed rabbit reticulocytes as described in Methods. The incubation conditions used were similar to those described by Darnbraugh et al. (1973). In preliminary experiments, the optimum concentrations of each reagent for incorporation of $^{3}$H-leucine into TCA precipitable material were determined. The final concentrations in a typical lysate incubation mixture are described in Methods.

The concentration of $MgCl_2$ which gave the optimum incorporation of $^{3}$H-leucine into protein was determined for every lysate prepared. The optimum $MgCl_2$ concentration was always found to be between 1.0 and 1.6 mM. Only lysate preparations which gave linear rates of protein synthesis for at least 20 minutes at 37° were used for subsequent experiments.

## Steady State Labeling

The incubation conditions necessary to obtain uniformly labeled α- and β-globin nascent chains were determined for the reticulocyte lysate. Nascent chains were uniformly labeled so the elution profile of the gel chromatographic analysis in disintegrations per minute would be an accurate measure of the amounts of nascent chains of discrete sizes present in the eluate fractions.

Steady state labeling conditions were determined for the reticulocyte lysate in the following manner. From a lysate incubation mixture containing $^{3}$H-leucine, to label protein during protein biosynthesis,

0.5 ml aliquots were removed at time intervals. One ml of Medium B, containing 0.059 mM cyclohexamide and 0.21 mM sparsomycin, at 0° was immediately added to each sample. After all samples had been collected, they were centrifuged at 125,000 x g for 90 minutes. The supernatant solution was saved for further analysis and the ribosomal pellet was resuspended in 5 ml of Medium B. The ribosomes were again pelleted by centrifugation at 125,000 x g for 90 minutes and then dissolved in 1.0 ml of Medium B.

The $^{3}$H-leucine present in TCA precipitable material was determined for the resuspended ribosomes and for the supernatant from the first centrifugation. The concentration of ribosomes in the resuspended ribosome pellet was determined spectrophotometrically (11.3 $A_{260}$ units = 1 mg/ml, T'so and Vinograd, 1961). The concentration of hemoglobin in the supernatant solution was determined by conversion to CN-Hb with KCN and measuring the absorbance at 540 nm (0.718 $A_{540}$ units = 1 mg/ml, Austin and Drabkin, 1935). The results of the preceding experiment are shown in Figure 7. Disintegrations per minute per mg of protein were plotted as a function of time for the washed ribosomal pellet and for the post-ribosomal supernatant fraction. As shown in Figure 7, the ribosomes reached equilibrium with respect to $^{3}$H-leucine incorporation after seven minutes of incubation at 37°. Since the incorporation of radioactivity into soluble hemoglobin was increasing linearly as the amount of radioactivity in ribosomal material remained constant, it was concluded that the ribosomes were releasing labeled protein at the same rate that $^{3}$H-leucine was being incorporated into protein. Thus, from seven to twenty minutes after the start of the incubation, the nascent chains on the ribosomes were uniformly labeled and the

Figure 7. Steady state labeling of ribosomes.

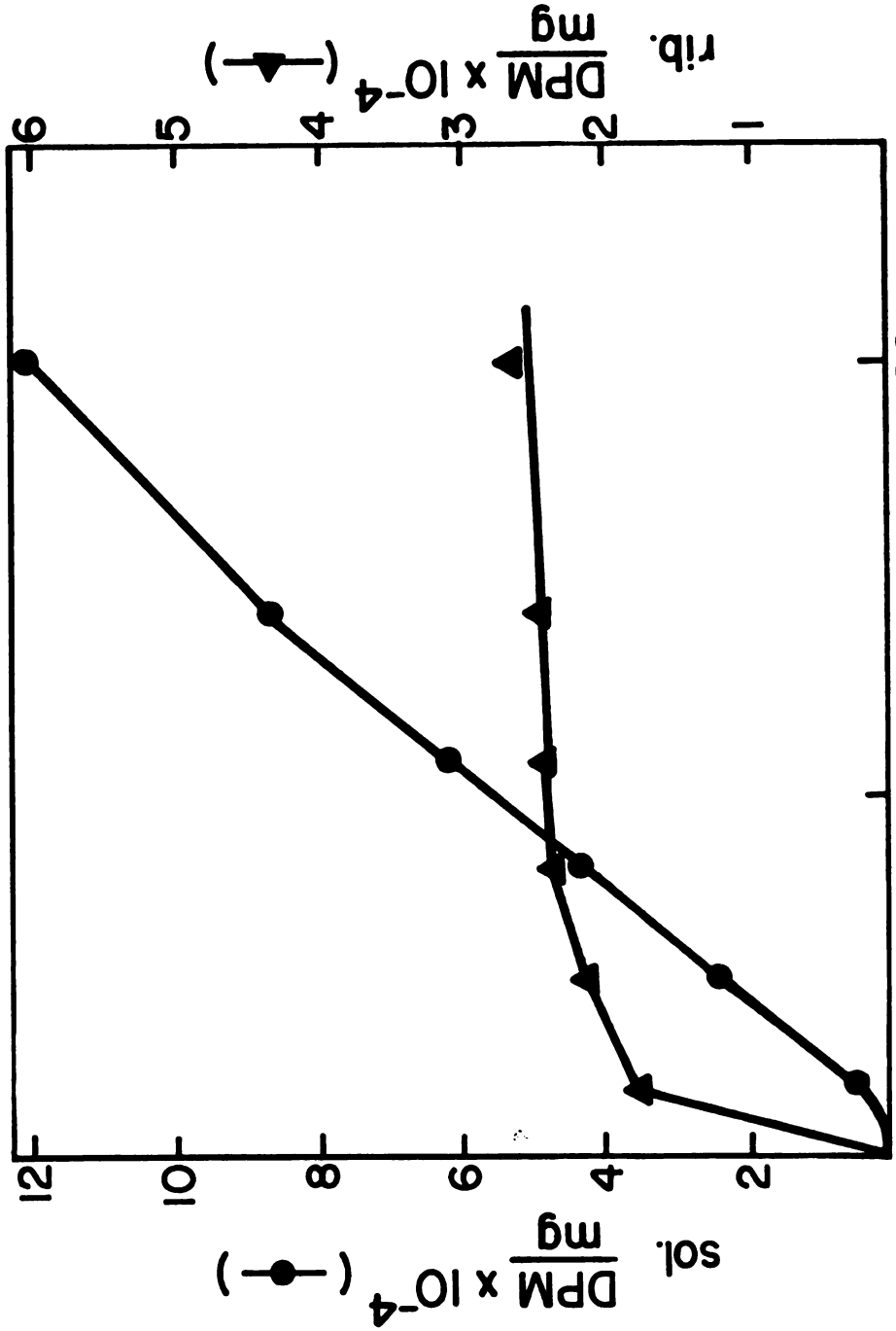



Figure 7

distribution of radioactivity was an accurate measure of the nascent chains. The results described above, which demonstrated that the labeled ribosomal material reached a plateau of radioactivity, were used as a basis for the selection of ten minutes as the incubation time for subsequent experiments. All of the experiments involving reticulocyte lysate cell-free protein synthesis were performed using frozen aliquots of the same lysate preparation to prepare the lysate incubation mixtures.

Addition of Reticulocyte tRNA to the Reticulocyte Lysate

The nascent chain accumulations observed by Protzel and Morris (1974) were also found to be present in nascent chains purified from the reticulocyte lysate cell-free system. The availability of a reticulocyte derived cell-free system, which also demonstrated nascent peptide accumulations, enabled experiments to be performed which were designed to determine if the addition of compounds to the lysate cell-free system could affect the nascent chain distributions. The hypothesis that the relatively slow steps of protein synthesis were due to limiting concentrations of some factor or factors involved in peptide chain elongation could be tested by attempting to purify selected compounds of the reticulocyte protein synthetic system and adding them back to a lysate incubation mixture. Increasing the concentration of such a hypothetically limiting component would greatly decrease or eliminate the accumulations of globin nascent chains if nascent chain accumulations were due to a putative factor being present in a stoichiometrically limiting quantity.

Reticulocyte ribosomes have been found to contain a tRNA population whose amino acid acceptance capability does not correspond to the codons present in α- and β-globin (Smith and McNamara, 1974), thus providing indirect confirmation of the presence of a nonuniform distribution of nascent globin peptides upon rabbit reticulocyte ribosomes. The possibility that one or more species of tRNA might create the observed accumulation of nascent globin chains of discrete sizes by means of their limited availability was considered since the interaction of a tRNA molecule with the mRNA occurs at specific sites on the mRNA due to the codon-anticodon interaction. Thus, if one or more isoacceptor species of tRNA were present in the reticulocyte at a relatively low concentration, ribosomes in the process of nascent peptide chain elongation would be delayed at the points where the codons for the tRNA species present in low concentrations resided. A delay in chain elongation at certain codons along the mRNA would create a build-up of ribosomes at the codon preceding the codon which interacts with the limiting tRNA. A corresponding accumulation of nascent peptide chains of discrete sizes would then be expected to occur.

Unfractionated tRNA was isolated from the reticulocyte lysate as described in Methods. A reticulocyte lysate incubation mixture was supplemented with unfractionated tRNA and nascent chains were labeled with $^{3}$H-tryptophan. As a control, a lysate incubation mixture which did not receive additional unfractionated tRNA was labeled with $^{14}$C-tryptophan at the same time. The incubation conditions and ribosome isolation for the lysate have been described previously (Methods). After the ribosome pellets from the experimental and control incubations were isolated by centrifugation, the ribosomes were combined and

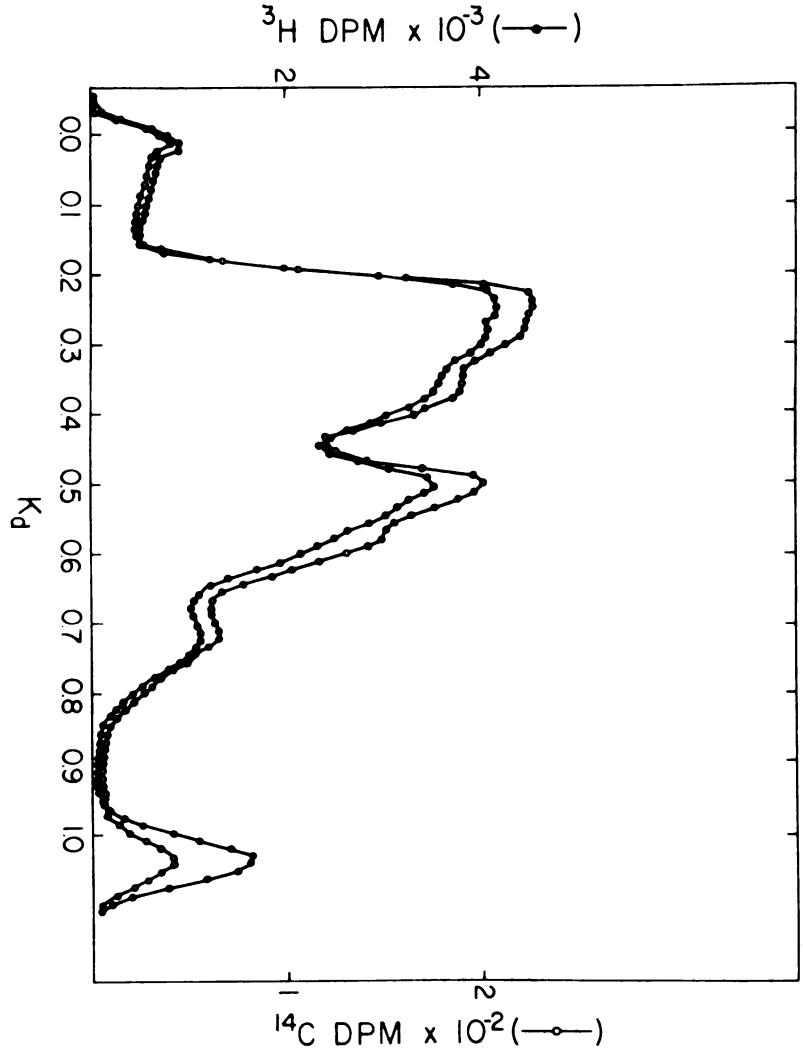
a peptidyl-tRNA fraction purified from this mixed sample. The radioactively labeled nascent peptides were analyzed for chain accumulations by Bio-Gel chromatography. Separate $^{3}H$ and $^{14}C$ disintegrations per minute were determined by simultaneous liquid scintillation spectrometry using the external standard channels ratio method. Calculations were made by using a program prepared by Mr. Howard Hershey and the Michigan State University CDC 6500.

The elution patterns for the $^{3}H$-tryptophan labeled nascent chains from the incubation mixture supplemented with tRNA and the $^{14}C$-tryptophan labeled nascent chains from the control incubation are shown in Figure 8. Both elution profiles were identical despite the increase in tRNA concentration to five times its original level in the incubation labeled with $^{3}H$-tryptophan. Had the nascent chain accumulations been due to the limitation of a tRNA species during nascent peptide chain elongation, the accumulations would have been expected to be decreased in the $^{3}H$-tryptophan labeled nascent chain profile. The preceding experiment, therefore, provided evidence that the limitation of tRNA isoacceptor species was not involved in the origin of the nascent chain accumulations observed upon gel chromatographic analysis under denaturing conditions.

## Characterization of Rabbit Liver tRNA

A tRNA sample was isolated from rabbit liver as described in Methods. The capability of the rabbit liver tRNA fraction to covalently bind radioactive leucine was used as a criterion for the tRNA population retaining its biological function during the isolation procedures. The assay conditions used to measure the ability of the

Figure 8. The effect of reticulocyte tRNA upon nascent chain accumulations.


$^{3}H$ DPM x $10^{-3}$ (—•—)
2
4
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
$K_d$
1
2
$^{14}C$ DPM x $10^{-2}$ (—o—)

rabbit liver tRNA preparation to covalently bind leucine were described in Methods. The disintegrations per minute were determined upon the material precipitated by 9% TCA.

Incubation of complete assay mixtures for 0, 5, and 50 minutes at 37° was seen to yield a maximum of 33.6 pmoles of leucine incorporated into TCA insoluble material per $A_{260}$ unit (Table 6). Incorporation was nearly complete by 5 minutes, as shown in lines 2 and 3 of Table 6, and was prevented if the sample was incubated at 0° instead of 37° (line 4). Treatment of a sample which had been incubated at 37°

Table 6. Treatment of rabbit liver tRNA to determine leucine acceptance capability.

| Line | Incubation Condition | DPM / 0.1 ml | pmoles leucine / $A_{260}$ unit |
|---|---|---|---|
| 1 | Complete, 0 min at 37° | 647 | 0.0 |
| 2 | Complete, 5 min at 37° | 4425 | 30.0 |
| 3 | Complete, 30 min at 37° | 4851 | 33.6 |
| 4 | Complete, 30 min at 0° | 492 | 0.0 |
| 5 | Complete, 30 min at 37°, followed by 0.87 N NaOH for 20 min at 37° | 376 | 0.0 |
| 6 | Minus tRNA, 0 min at 37° | 429 | 0.0 |
| 7 | Minus tRNA, 30 min at 37° | 582 | 0.0 |
| 8 | Minus synthetase, 0 min at 37° | 174 | 0.0 |
| 9 | Minus synthetase, 30 min at 37° | 230 | 0.4 |

for 30 minutes and subsequently with 0.87 N NaOH for 20 minutes at 37° before the addition of TCA prevented any of the radioactively labeled leucine from being precipitated (compare line 5 with line 3 of Table 6). Base treatment was expected to remove the TCA insoluble radioactivity since the aminoacyl-tRNA bond is not stable under basic conditions. Omission of either the tRNA or the crude synthetase preparations from the incubation mixture also prevented incorporation of radioactive leucine into TCA insoluble material (lines 6 and 7, and 8 and 9 of Table 6, respectively).

Comparison of the leucine acceptance capability determined here with the values derived by other investigators is difficult; there is a disagreement in the values reported in the literature with respect to the actual leucine acceptance capability of rabbit liver tRNA. Butler et al. (1975) reported this value to be 6.9 pmoles per $A_{260}$ unit. Smith and McNamara (1971) placed the figure at 53 pmoles per $A_{260}$ unit, and Gilbert and Anderson (1970) determined it to be 107 pmoles per $A_{260}$ unit. Since the value determined in this thesis (33.6 pmoles of leucine per $A_{260}$ unit) was within the range of the previously determined values, the method of preparation of tRNA described in Methods was considered to yield a tRNA preparation which retained its biological activity.

Treatment of rabbit liver tRNA with sodium periodate as described in Methods provided further characterization of this tRNA preparation. Periodate will react with the 2' and 3' hydroxyls of the ribose of tRNA to yield a dialdehyde structure. The modified tRNA structure was unable to covalently bind leucine under the conditions described in Methods. As can be seen in Table 7, rabbit liver tRNA treated with

buffer containing no periodate retained its capability to bind leucine (lines 1 and 2). The amount of leucine bound was not changed by the buffer treatment (33.0 pmoles bound per $A_{260}$ unit compared with an original value of 33.6 pmoles per $A_{260}$ unit as reported in Table 6). The addition of sodium periodate to the tRNA prevented the incorporation of leucine into TCA insoluble material (lines 3 and 4 of Table 7) as would be expected if the leucine were being bound to the tRNA preparation at the 3' end of the RNA molecules. Thus, on the basis of the data presented in Tables 6 and 7, it was concluded that the rabbit liver tRNA had been isolated in a biologically active form.

Table 7. Leucine acceptance capability of sodium periodate treated rabbit liver tRNA.

| Line | | DPM / 0.1 ml | pmoles of leucine / $A_{260}$ unit |
|---|---|---|---|
| 1 | Buffer treated tRNA, 0 min at 37° | 575 | 0.0 |
| 2 | Buffer treated tRNA, 30 min at 37° | 4702 | 33.0 |
| 3 | Periodate treated tRNA, 0 min at 37° | 639 | 0.0 |
| 4 | Periodate treated tRNA, 30 min at 37° | 603 | 0.0 |

Addition of Rabbit Liver tRNA to the Reticulocyte Lysate

Although the addition of rabbit reticulocyte tRNA did not alter the gel elution profile of the rabbit reticulocyte lysate nascent chains (Figure 8), it was felt that the use of another source of tRNA might change the elution profile of nascent chains. The possibility existed that the rabbit reticulocyte contained a limiting tRNA species in a very low concentration. Addition of a reticulocyte tRNA

preparation to a lysate incubation mixture might not raise the concentration of such a limiting tRNA to a high enough level to alter the nascent chain distribution profile. Therefore, a tRNA preparation from rabbit liver was examined for an effect upon nascent chain accumulations in the reticulocyte lysate.

Transfer RNA was isolated from nonanemic rabbit liver to determine if the addition of rabbit liver tRNA to the reticulocyte lysate cell-free system would affect nascent chain accumulation patterns. The isolation and characterization of the rabbit liver tRNA preparation was described above and in Methods. Ten $A_{260}$ units of rabbit liver tRNA were added to a reticulocyte lysate cell-free protein synthesizing system of one ml. The rabbit liver tRNA preparation was treated with 0.1 M sodium acetate (pH 4.6) (and not reated with periodate) as described in Methods. The tRNA sample retained its ability to bind leucine as described above. The lysate incubation conditions and procedures for the isolation and chromatographic analysis of nascent peptide chains were described in Methods.

An elution profile of the nascent chains labeled with $^{3}$H-tryptophan from a lysate incubation mixture which had received the addition of the rabbit liver tRNA is shown in Figure 9. An internal control of $^{14}$C-tryptophan labeled nascent chains from a lysate incubation mixture without additional tRNA was not used. However, comparison of Figure 9 with the $^{14}$C-tryptophan labeled nascent chain profile of Figure 8, which was derived from chromatographic analysis of nascent chains from a lysate incubation mixture which contained no additions, showed that the nascent chain accumulations occurred at the same $K_d$ values in both the incubation supplemented with rabbit liver tRNA and in the experiment

Figure 9. The effect of rabbit liver tRNA upon nascent chain accumulations

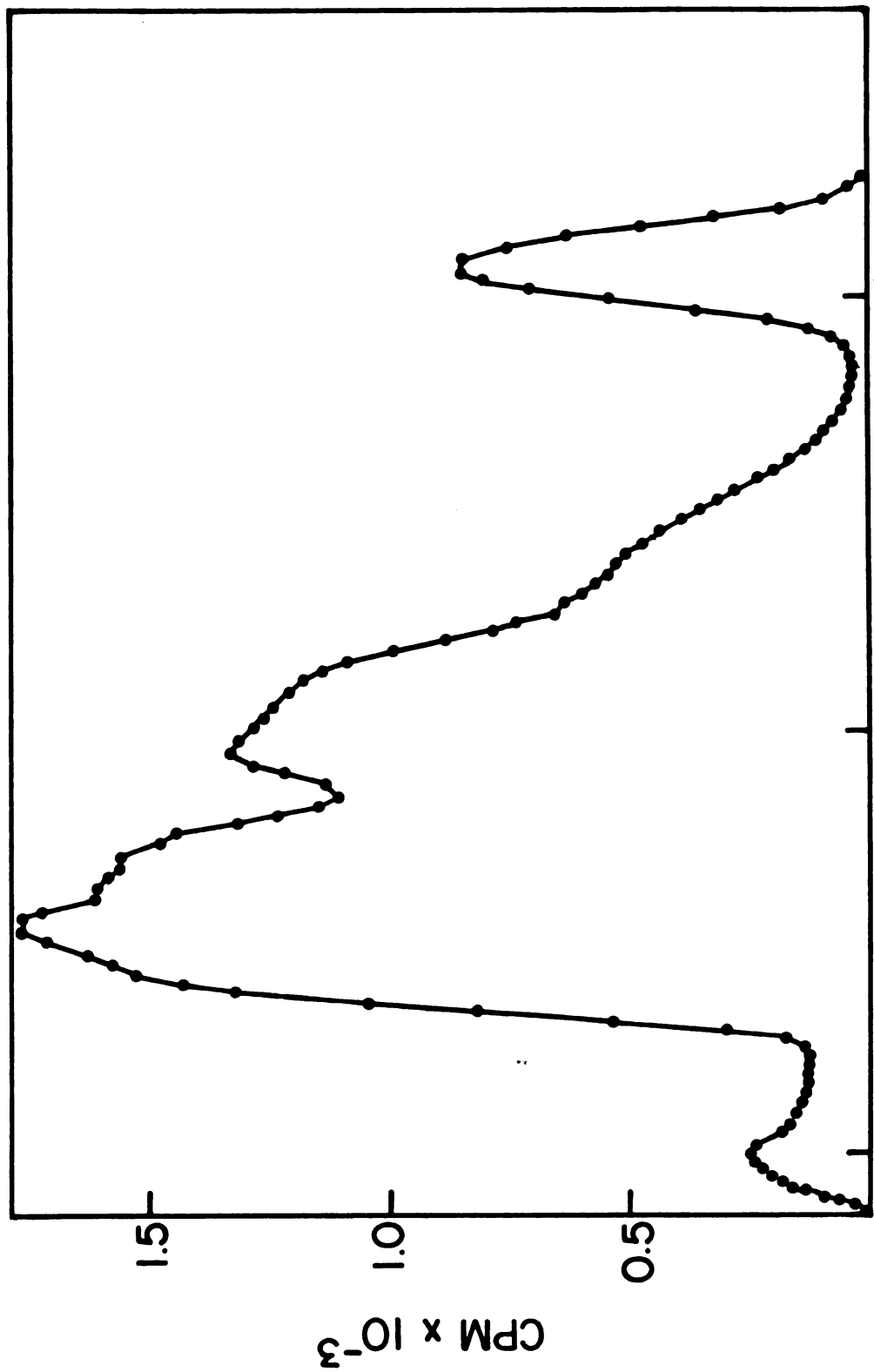



Figure 9

which received no additional tRNA. Therefore, rabbit liver tRNA as well as rabbit reticulocyte tRNA were concluded to have no effect upon the nascent globin peptide accumulations.

Addition of Periodate Treated tRNA to the Reticulocyte Lysate

A lysate incubation was also performed with the addition of 10.0 $A_{260}$ units of sodium periodate treated rabbit liver tRNA added to one ml of the lysate incubation mixture. The periodate treated tRNA sample had been shown to have no leucine accepting capability, as reported above. Nascent chains were labeled with $^{3}$H-tryptophan, purified from the incubation mixture, and analyzed by Bio-Gel chromatography as described in Methods. Comparison of the elution profile of Figure 10 with the $^{14}$C-tryptophan labeled profile of Figure 8 showed no significant alteration in the nascent chain elution profile. Addition of a tRNA population incapable of participating in protein synthesis also did not affect the nascent chain accumulation pattern.

Addition of Salt Wash Factors to the Reticulocyte Lysate

The inability of tRNA preparations from rabbit reticulocytes or rabbit liver to alter the nascent chain size distribution elution profile led to attempts to determine if the nascent chain size accumulations were related to the limitation of other components of the reticulocyte lysate cell-free protein biosynthetic machinery. Treatment of rabbit reticulocyte ribosomes with high salt concentrations and of rabbit reticulocyte postribosomal supernatants with 70% $(NH_4)_2SO_4$ have been reported to yield preparations containing protein synthesis initiation and elongation factors (Woodley et al., 1974; Hardesty et al., 1971). Different fractions of the reticulocyte lysate were

Figure 10. The effect of periodate-treated rabbit liver tRNA upon nascent chain accumulations.

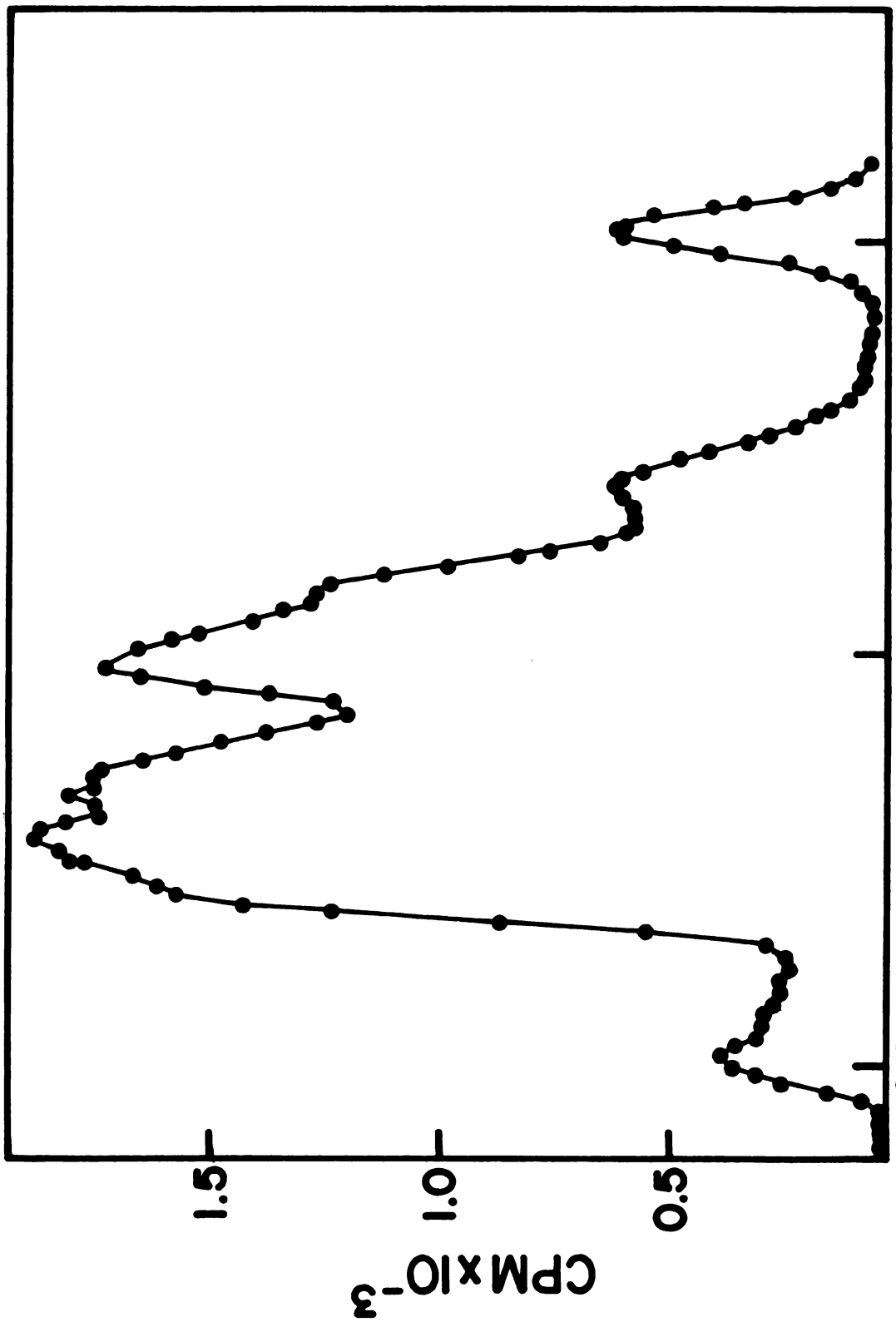


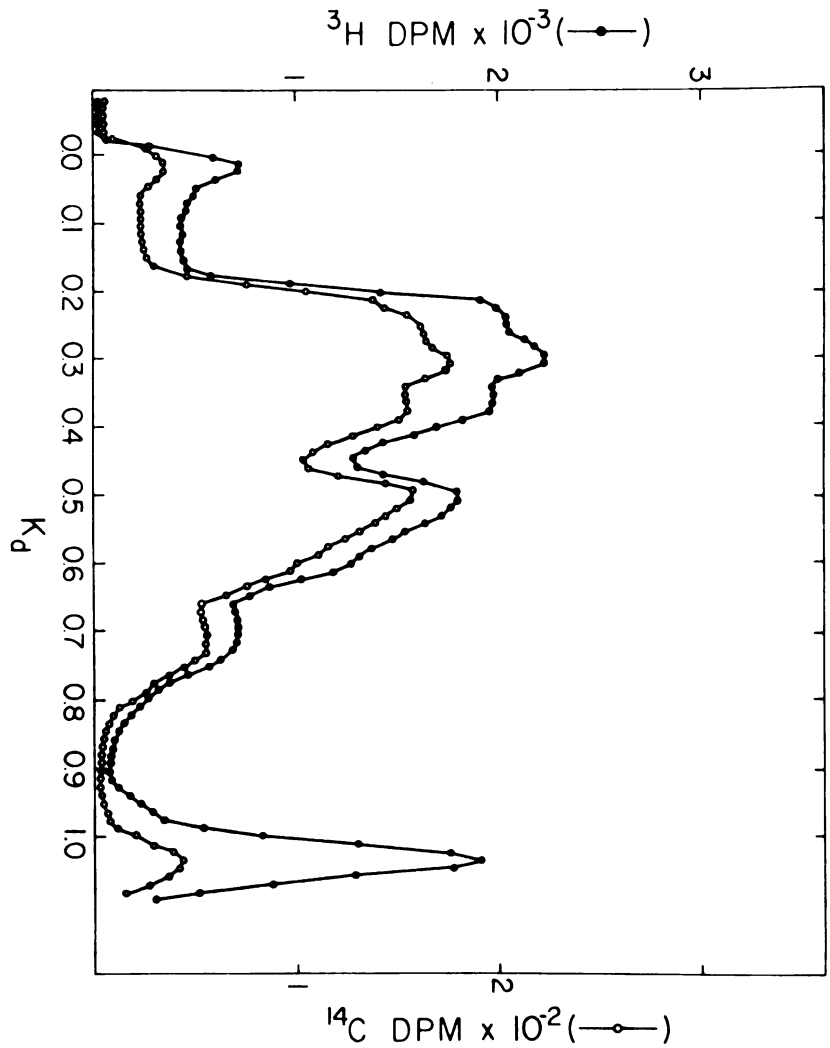

Figure 10

prepared for use in an analogous manner to the experiments involving the addition of tRNA to the reticulocyte lysate incubation mixture. If the nascent chain size accumulations were due to the limitation of a component present in such a preparation, addition of the preparation to the lysate incubation mixture would be expected to reduce the heights of the peaks of the accumulations in the Bio-Gel elution profile.

Treatment of polysomes with 0.5 M KCl had been reported to release protein factors involved in the initiation of protein biosynthesis (Woodley et al., 1974). Acting upon the premise that the 0.5 M KCl wash preparations might contain factors associated with nascent chain accumulations, reticulocyte lysate polysomes were washed with 0.5 M KCl as described in Methods. The material eluting from the polysomes was concentrated and added to the lysate cell-free system to give a concentration in the incubation mixture three times that present originally. Nascent chains were labeled with $^{3}$H-tryptophan, purified, and analyzed upon Bio-Gel A 0.5 m under denaturing conditions with an internal standard consisting of $^{14}$C-tryptophan labeled nascent chains that had been co-purified from a lysate which received no additions.

As shown in Figure 11, the results of the experiment described above failed to demonstrate any change in the nascent globin peptide elution profile upon addition to the lysate cell-free system of the factors eluted from the lysate polysomes by 0.5 M KCl. If such a limiting factor did exist in the 0.5 M KCl wash of reticulocyte lysate ribosomes, the elution profile of the $^{3}$H-labeled nascent chains would have shown a decrease in the heights of the nascent chain size accumulations.

Figure 11. The effect of salt wash factors upon nascent chain accumulations.


$^{3}H$ DPM x $10^{-3}$(—●—)
1
2
3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
$K_d$
1
2
$^{14}C$ DPM x $10^{-2}$(—○—)

## Addition of Ammonium Sulfate Precipitated Factors to the Reticulocyte Lysate

Adjustment of a reticulocyte lysate postribosomal supernatant to 70% $(NH_4)_2SO_4$ had been reported to precipitate protein factors involved in the elongation steps of protein synthesis (Hardesty et al., 1971). A reticulocyte lysate postribosomal supernatant was adjusted to 70% $(NH_4)_2SO_4$ at 0° by the slow addition of powdered $(NH_4)_2SO_4$ at 0° as described in Methods. The material precipitated by 70% $(NH_4)_2SO_4$ was added to a lysate incubation mixture to bring the final concentration of the prepared factors to three times that normally present. Nascent chains were uniformly labeled with $^3$H-tryptophan. A control incubation with $^{14}$C-tryptophan was prepared for use as an internal standard. Ribosomes from both incubations were mixed and the nascent chains purified as described previously in Methods.

Gel chromatographic analysis of the purified nascent chain populations yielded elution profiles for the experimental $^3$H-labeled nascent chains and the control $^{14}$C-labeled nascent chains. No alterations in the nascent chain accumulation profile were induced by the addition of the factors precipitated by 70% $(NH_4)_2SO_4$ (Figure 12). The discontinuity of the elution profile was due to the failure of a fraction collector. From the graphs in Figure 12, no evidence for the presence of a factor or factors in the material precipitated by 70% $(NH_4)_2SO_4$ which could be related to the origin of the nascent globin chain accumulations could be found.

## Inhibition of Protein Biosynthesis by Edeine

The preceding experiments failed to provide evidence for the involvement of a factor or factors of the reticulocyte lysate cell-free

Figure 12. The effect of $(NH_4)_2SO_4$ precipitated proteins upon nascent chain accumulations.

Figure 12

protein biosynthetic system in the accumulation of nascent α- and β-globin peptides of discrete sizes during protein biosynthesis. However, these results did not definitively disprove the hypothesis that such factors were associated with the accumulations observed in the nascent globin chains of rabbit reticulocytes. Such components of the reticulocyte lysate could exist and be either not purified by the isolation procedures used or destroyed by these isolation procedures. Therefore, a separate and entirely independent approach was taken to examine the possibility that the nascent chain accumulations observed in rabbit reticulocytes were due to a stoichiometrically limiting factor or factors of peptide chain elongation.

The antibiotic, edeine, had been reported to inhibit protein biosynthesis specifically at the step of initiation (Obrig et al., 1971). The effects of increased concentrations of edeine upon the rate of protein biosynthesis in the reticulocyte lysate incubation mixture were determined. The results of this experiment are shown in Figure 13.

An edeine concentration of 19 μM was found to inhibit protein biosynthesis to about 25% of its original rate. Since edeine reportedly inhibits the initiation step of protein biosynthesis, the rate of nascent chain elongation should be unchanged. The number of ribosomes involved in peptide chain elongation in an incubation with added edeine would, therefore, be expected to be one-fourth of the number of active ribosomes found in an uninhibited incubation mixture. The reduction in number of actively translating ribosomes would have the effect of increasing by four times the concentrations of the individual components which were involved in nascent peptide chain elongation (elongation factors, tRNA, etc.) relative to the concentration of the

Figure 13. Inhibition of protein biosynthesis by edeine.

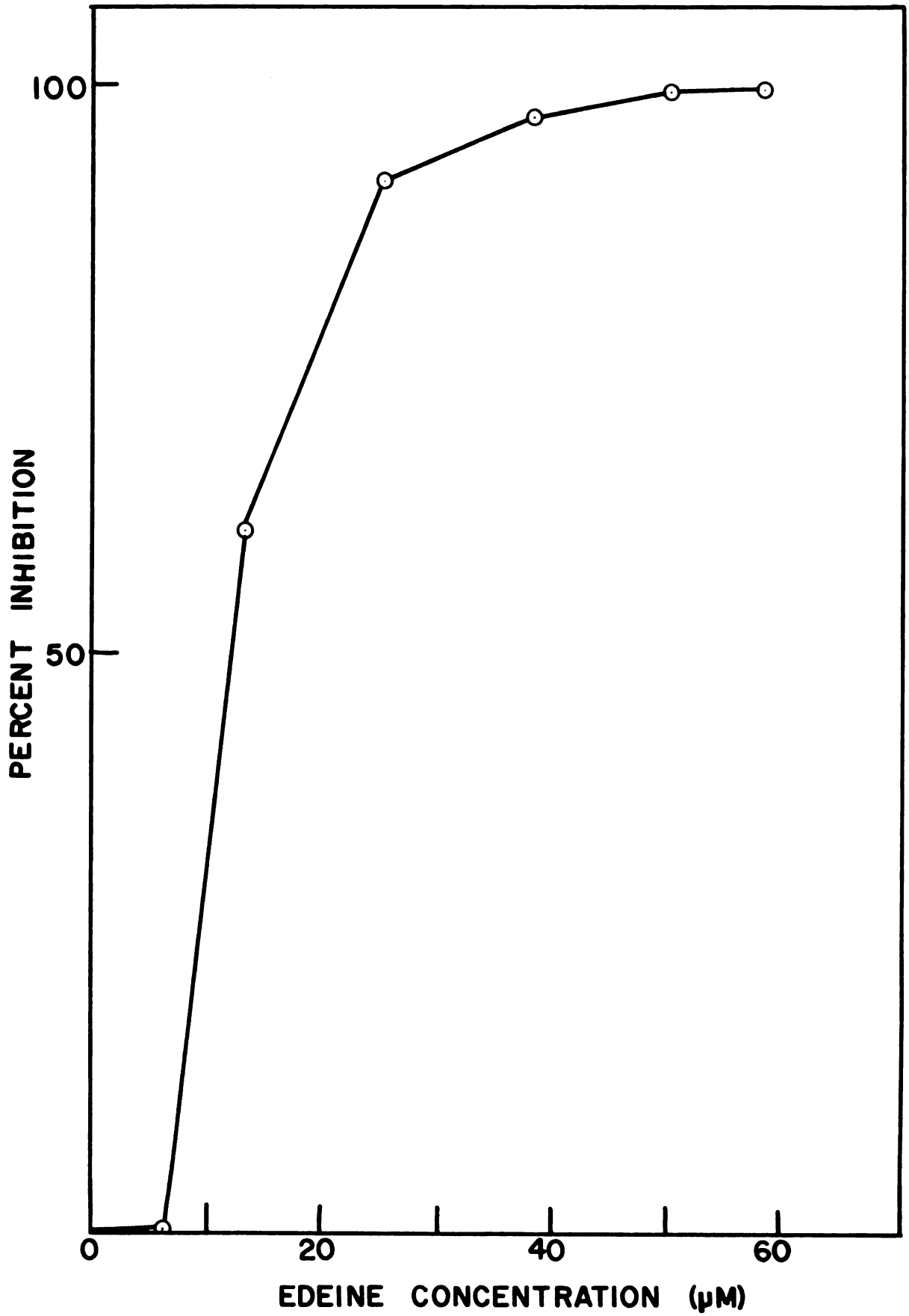

100
50
PERCENT INHIBITION
0
20
40
60
EDEINE CONCENTRATION (μM)

Figure 13

active ribosomes. The nascent chain accumulations which had been observed would be expected to be decreased or eliminated if they were indeed due to a limiting concentration of a factor or factors involved in peptide chain elongation.

Nascent globin chains were labeled with $^{3}$H-tryptophan in a reticulocyte lysate incubation mixture which contained 19 μM edeine. Ribosomes isolated from the incubation containing edeine were mixed with ribosomes from a lysate incubation mixture to which no edeine was added. In the control incubation nascent globin chains were labeled with $^{14}$C-tryptophan. The nascent chains were isolated and Bio-Gel A 0.5 m chromatographic analysis performed. The results from two separate experiments performed in such a manner, shown in Figure 14 and 15, indicated that no alteration was induced in the nascent globin chain accumulation pattern from the lysate incubation mixture in which protein synthesis was inhibited by the addition of 19 μM edeine.

The results obtained by the addition of edeine to the cell-free lysate incubation mixtures were in agreement with the results obtained in the attempts to purify a putative factor or factors from the reticulocyte lysate cell-free protein synthesizing cell-free system. Neither approach was able to provide evidence for the existence of such a factor. It was, therefore, concluded that such a factor did not exist and that the origin of the nascent globin chain accumulations originated in some other aspect of α- and β-globin biosynthesis.

## Wheat Embryo Cell-Free System

The inability of the experiments described in the preceding section to demonstrate a factor or factors in the reticulocyte lysate, which

Figure 14. The effects of edeine upon nascent chain accumulations.

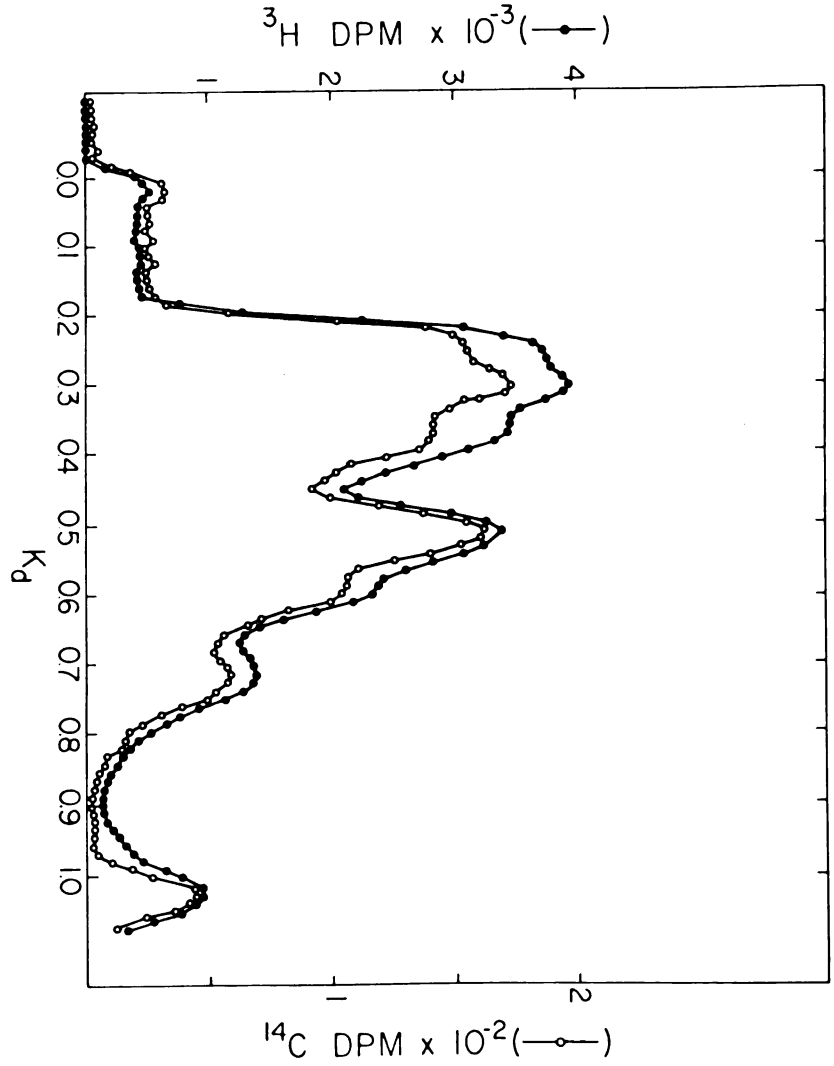

$^{3}H$ DPM x $10^{-3}$(—●—)
1
2
3
4
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
$K_d$
1
2
$^{14}C$ DPM x $10^{-2}$(—○—)

Figure 15. Elution profile of nascent chains from a lysate containing edeine.

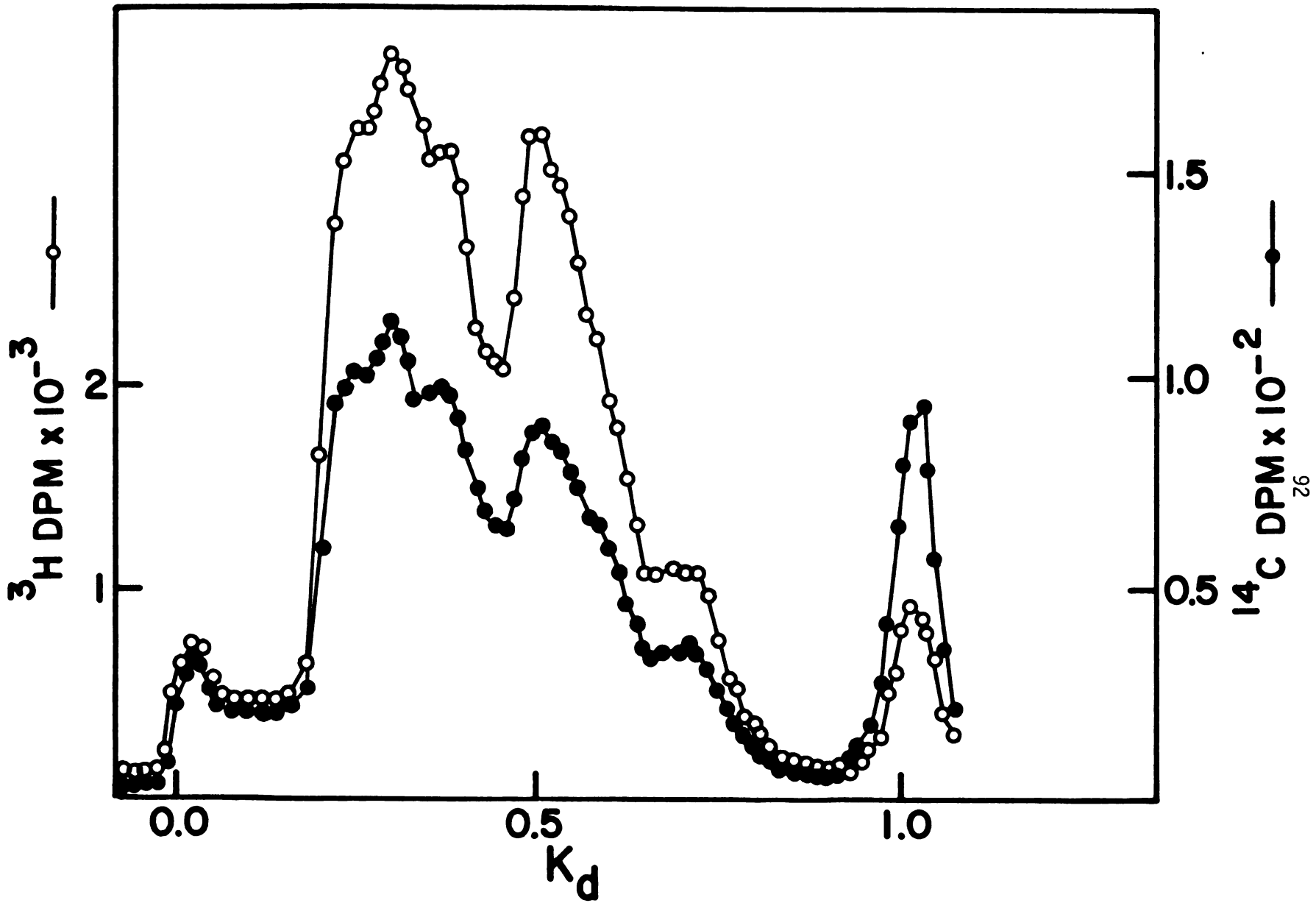



Figure 15

could be the origin of the nascent globin peptide accumulations, led to an attempt to determine if these accumulations were associated with translation of globin mRNA by the protein biosynthetic machinery of the reticulocyte lysate, or if similar size accumulations were present in nascent chains derived from globin mRNA directed protein biosynthesis in an entirely different cell-free system. The wheat embryo cell-free system was chosen for these experiments because of its dependence upon added mRNA for protein synthesis and its phylogenetic distance from rabbits.

Globin mRNA was prepared from rabbit reticulocytes by the method of Aviv and Leder (1972). The wheat embryo cell-free system was prepared by the method of Marcus et al. (1975). The incorporation of $^{3}$H-leucine into protein was dependent upon added globin mRNA (Figure 16). The rate of protein synthesis showed an optimum at a concentration of 3.6 mM $Mg(Ac)_2$ in Figure 17. An incubation of globin mRNA at a concentration of 8 µg/ml gave a linear rate of incorporation of $^{3}$H-leucine into protein (Figure 18).

Nascent chains were labeled with $^{3}$H-tryptophan for 20 minutes in an incubation mixture of 5.6 ml containing 6.5 µg globin mRNA per ml. Protein synthesis was halted and ribosomes isolated as described in Methods. The ribosomes were then mixed with ribosomes isolated by centrifugation from a reticulocyte lysate in which the nascent chains had been uniformly labeled with $^{14}$C-tryptophan. The combined nascent chains were purified and Bio-Gel A 0.5 m chromatographic analysis was performed.

The analysis of the nascent chains purified from the wheat embryo incubations indicated that nascent chain accumulations were also present

Figure 16. Effect of mRNA concentration upon protein synthesis in the wheat embryo cell-free system.

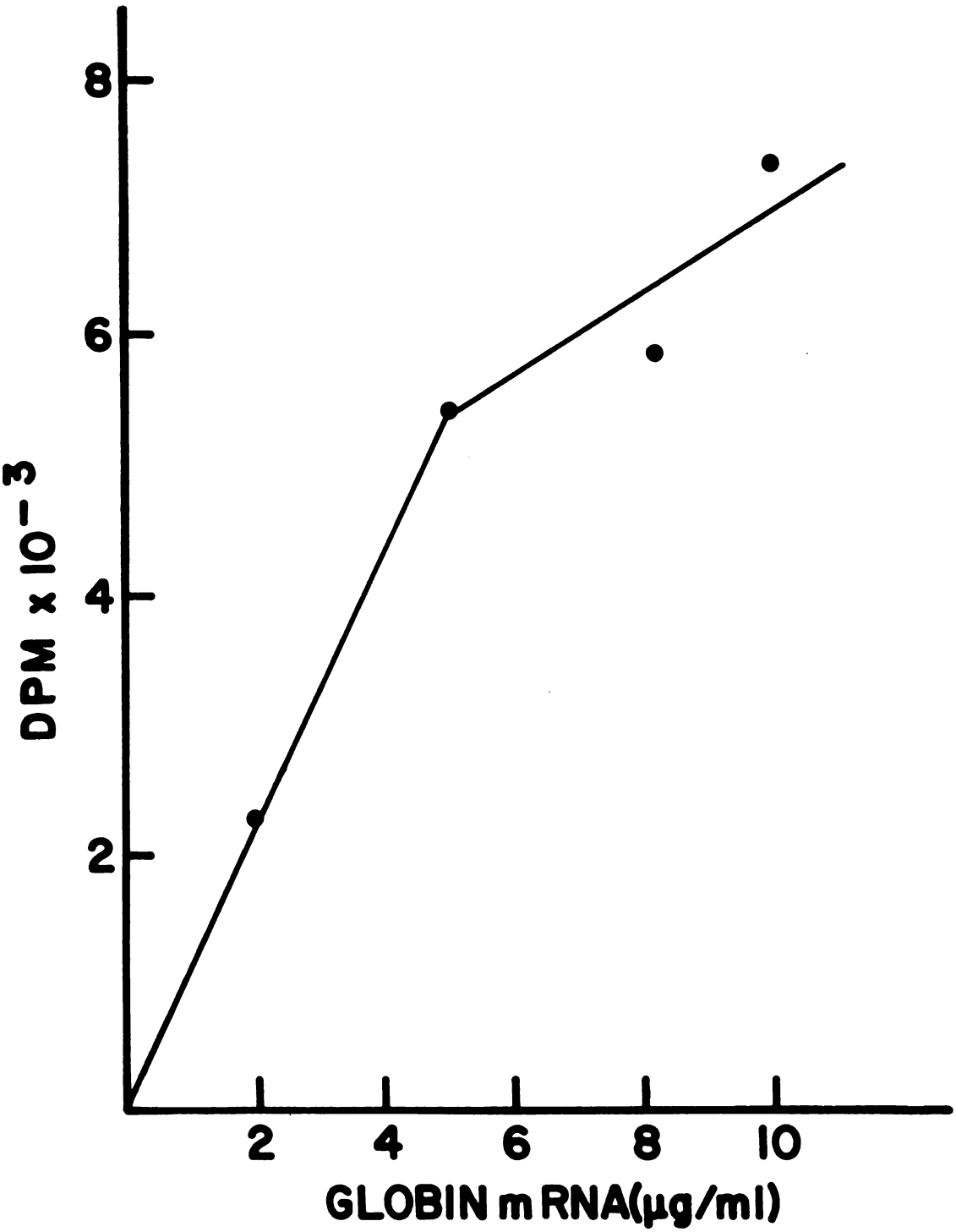



Figure 16

Figure 17. Protein synthesis as a function of $Mg(Ac)_2$ concentration in the wheat embryo cell-free system.

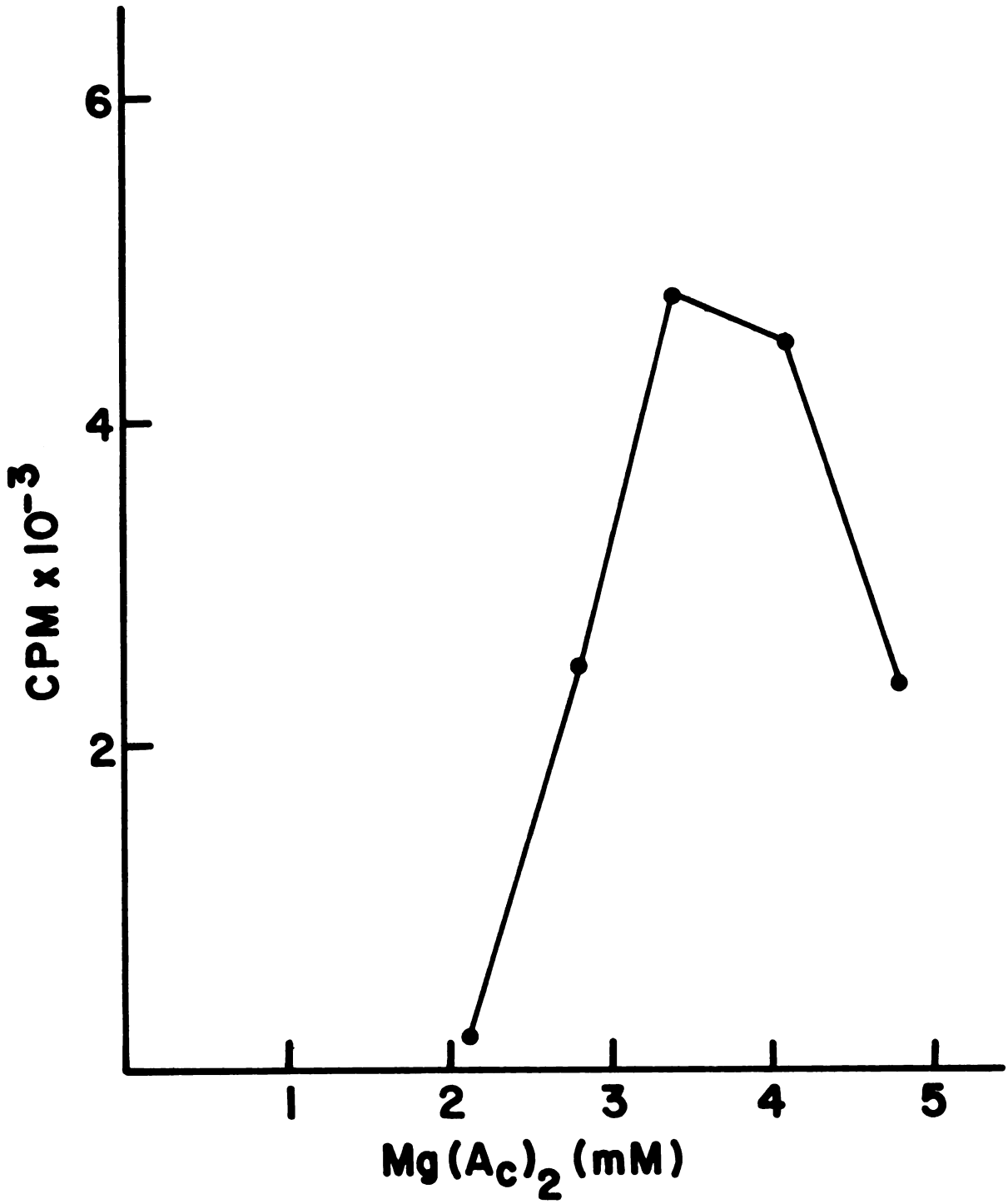



Figure 17

Figure 18. Incorporation of $^{3}H$-leucine into protein as a function of time in the wheat embryo cell-free system.

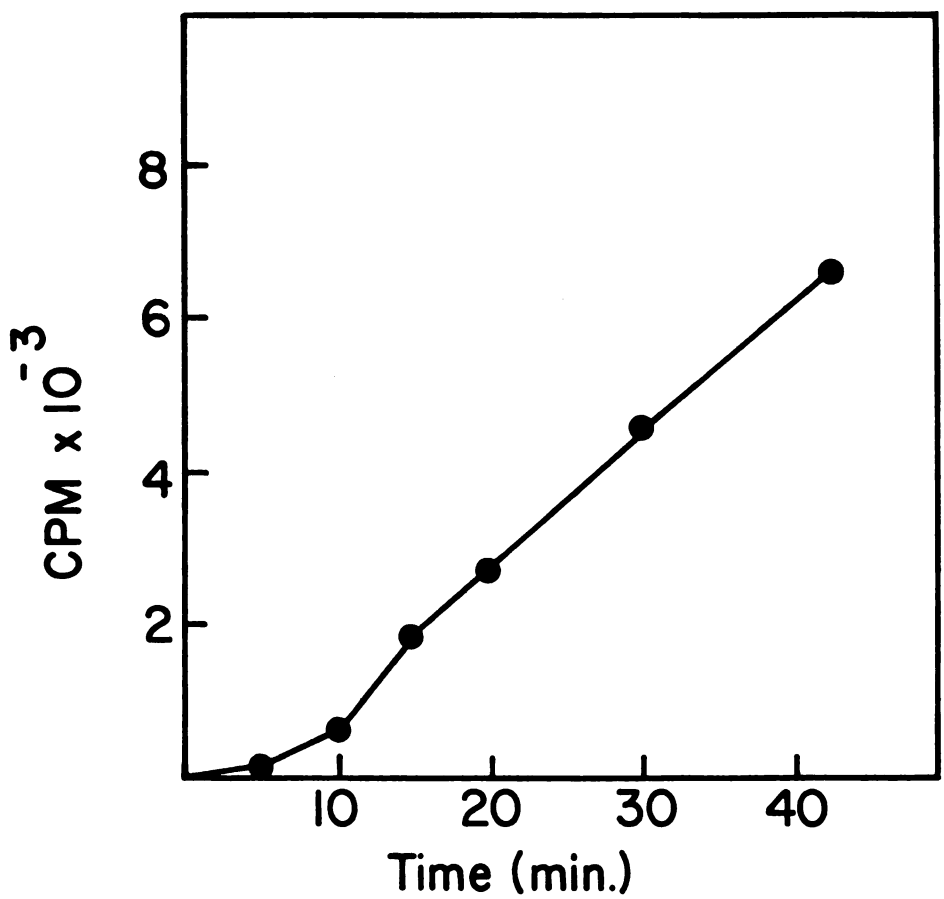



Figure 18

in the wheat embryo cell-free system during globin mRNA dependent protein synthesis (Figure 19). Accumulations of nascent chains at a $K_d$ greater than 0.4 (> 80 amino acids in length) were correlated between the lysate and wheat embryo systems. The relative magnitude of the nascent chains at distribution coefficients less than 0.4 was decreased, probably due to premature termination in the wheat embryo cell-free system similar to that described by Roberts et al. (1973).

Previous experiments with nascent chains purified from the reticulocyte lysate cell-free system had shown that the relative heights of the peaks in the gel elution profiles could vary between two elution profiles derived from two separate Bio-Gel chromatographic analyses of the same nascent globin chain sample. Hence, addition of $^{14}$C-tryptophan labeled polysomes as an internal standard was employed to correct for any technical difficulties during the nascent chain purification and column chromatography. Most experiments involving the wheat embryo cell-free system were performed using only a single $^{3}$H-amino acid as the radioactive label. In all of these analyses the presence of nascent globin chain accumulations of the same sizes as those observed in the reticulocyte lysate and whole cell incubations was quite evident. Not until the experiment shown in Figure 19 using $^{14}$C-tryptophan labeled nascent chains from the rabbit reticulocyte lysate as an internal control was performed was the presence of the decrease in the nascent chain profiles at $K_d$ values less than 0.4 adequately demonstrated. The decrease had been observed in the single label experiments, but evaluation of the differences in magnitude was felt to be unreliable.

Figure 19. Elution profile of nascent chains from the globin mRNA directed wheat embryo cell-free system.

$^{3}H$ DPM x $10^{-3}$ (—●—)
$^{14}C$ DPM x $10^{-2}$ (—○—)
1
2
3
4
5
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
$K_d$

After the completion of the experiments described in this thesis involving globin mRNA-directed protein biosynthesis in the wheat embryo-derived cell-free system, an incubation was performed in this laboratory by Mr. Howard Hershey using a similar wheat germ derived cell-free system prepared by a different procedure (Gallis et al., 1975). Nascent chains were labeled with $^{3}$H-tryptophan in the wheat germ system incubations in a manner identical to the experiment using the wheat embryo cell-free system. The reticulocyte lysate cell-free system used earlier in this thesis was incubated with $^{14}$C-tryptophan to label nascent chains. The ribosomes isolated by centrifugation were mixed. Nascent chains were purified and analyzed by Bio-Gel A 0.5 m chromatography in 6.0 M guanidine-HCl, 0.1 M 2-mercaptoethanol (pH 6.5). The results of the experiment utilizing the system prepared by the different procedure are shown in Figure 20. A comparison of the $^{3}$H-elution profile from the wheat germ cell-free system with the profile from the reticulocyte lysate system indicated that the same nascent chain accumulations observed in reticulocyte lysate derived nascent chains were present throughout the entire wheat germ-derived nascent chain profile.

Analysis of the soluble products of a 45-minute incubation of globin mRNA in the wheat germ cell-free protein synthesizing system has been performed. An unlabeled reticulocyte lysate post-ribosomal supernatant was added to the $^{3}$H-tryptophan labeled wheat germ soluble protein and the mixture treated as described in Methods. The elution profile shown in Figure 21 (also performed by Mr. Hershey) demonstrated the co-migration of the radioactivity with the protein as measured by the absorbance at 280 nm.

Figure 20. Elution profile of nascent chains from the globin mRNA directed wheat germ cell-free system.

$^{14}C$ DPM x $10^{-2}$ (—o—)
2
1
$^{3}H$ DPM x $10^{-3}$ (—●—)
3
2
1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
$K_d$

Figure 21. Analysis of the soluble products of the globin mRNA directed wheat germ cell-free system.

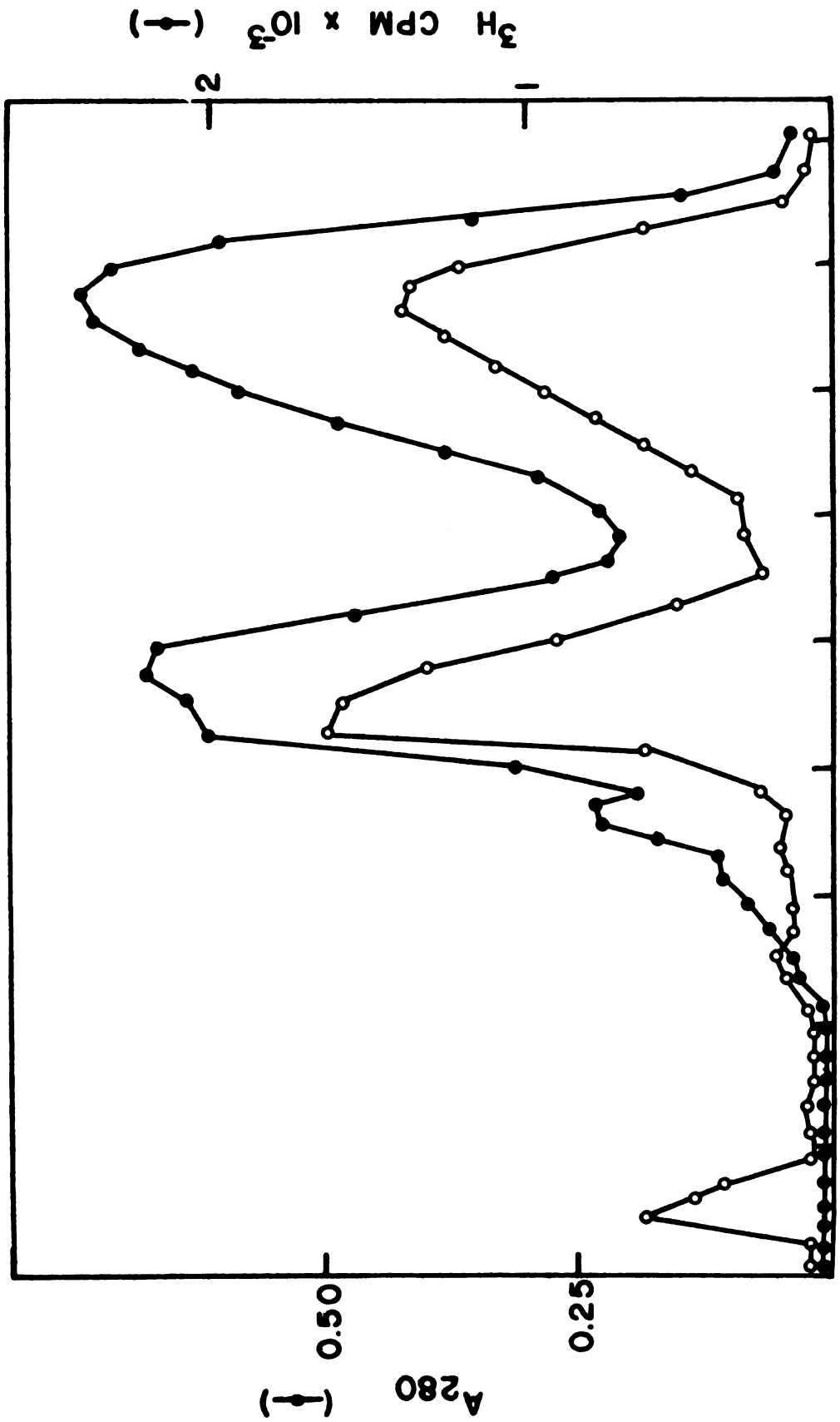



Figure 21

The only part of the reticulocyte which was added to the wheat embryo-derived cell-free system was the purified globin mRNA fraction. The discrete size accumulations observed upon gel chromatographic analysis of nascent globin chains in the rabbit reticulocyte were thus associated with the globin mRNA. The ability to cause the nascent chain size accumulations was retained during the mRNA isolation procedure involving phenol extraction of SDS treated polysomes. Therefore, the globin mRNA itself (and not mRNA bound protein factors) must have been responsible for the nascent chain accumulation profile observed for nascent chains isolated from a globin mRNA programmed wheat embryo cell-free protein synthesis system.

Attempts to Modify Globin mRNA In Vitro

Globin mRNA had been reported to exist in solution in a conformation which contained intramolecular base pairing (Holder and Lingrel, 1975; Favre et al., 1975; Dubochet et al., 1973; Vournakis et al., 1976; Flashner and Vournakis, 1977). The reports of globin mRNA secondary structure led to experiments which were designed to attempt to modify the globin mRNA secondary structure without destroying its ability to be translated in the wheat embryo cell-free system. If the origin of the nascent chain accumulations was associated with some structural aspect of globin mRNA, and the structure could be altered without preventing the translation of the globin mRNA, then changes in the globin mRNA secondary structure might lead to changes in the nascent chain elution profile.

The experimental protocol used to analyze the elution profiles of nascent chains from the wheat embryo cell-free system was changed

for the following set of experiments. An internal $^{14}C$-labeled standard was not used. Instead, comparisons were made between an elution profile obtained for $^{3}H$-tryptophan labeled nascent chains for a control incubation, and an elution profile obtained for $^{3}H$-tryptophan labeled nascent chains from an incubation in which synthesis was programmed by the experimentally treated globin mRNA. Comparison of two different profiles could be used to detect significant differences in the positions of nascent chain accumulations in the elution profiles even though, as stated earlier, the significance of differences in the heights of the nascent chain accumulations between two chromatographic analyses of the same sample could not be assessed.

A second alteration in the experimental protocol was to omit the purification of the nascent chains. Instead, ribosomes isolated by centrifugation were dissolved directly in guanidine column solution and prepared for column chromatographic analysis as described in Methods. The modified analytical procedure had the advantage of omitting the 3-4 days required to purify the peptidyl-tRNA.

A 0.6 ml wheat embryo incubation mixture containing 2.4 μg of globin mRNA was incubated for 20 minutes at 30°; 1.0 ml of ice-cold Medium B, 0.059 mM cyclohexamide and 0.21 mM sparsomycin was then added. Ribosomes were isolated by centrifugation and prepared for column chromatographic analysis by base treatment as described in Methods. The elution profile of the ribosomal bound material from the incubation described above is shown in Figure 22. A comparison of this elution profile with the purified nascent chain elution profile from the wheat embryo system (the $^{3}H$-labeled curve of Figure 19) indicates the presence of only two differences in the elution profiles. The elution profile

Figure 22. Elution profile of polysomal material from the globin mRNA directed wheat embryo cell-free system.

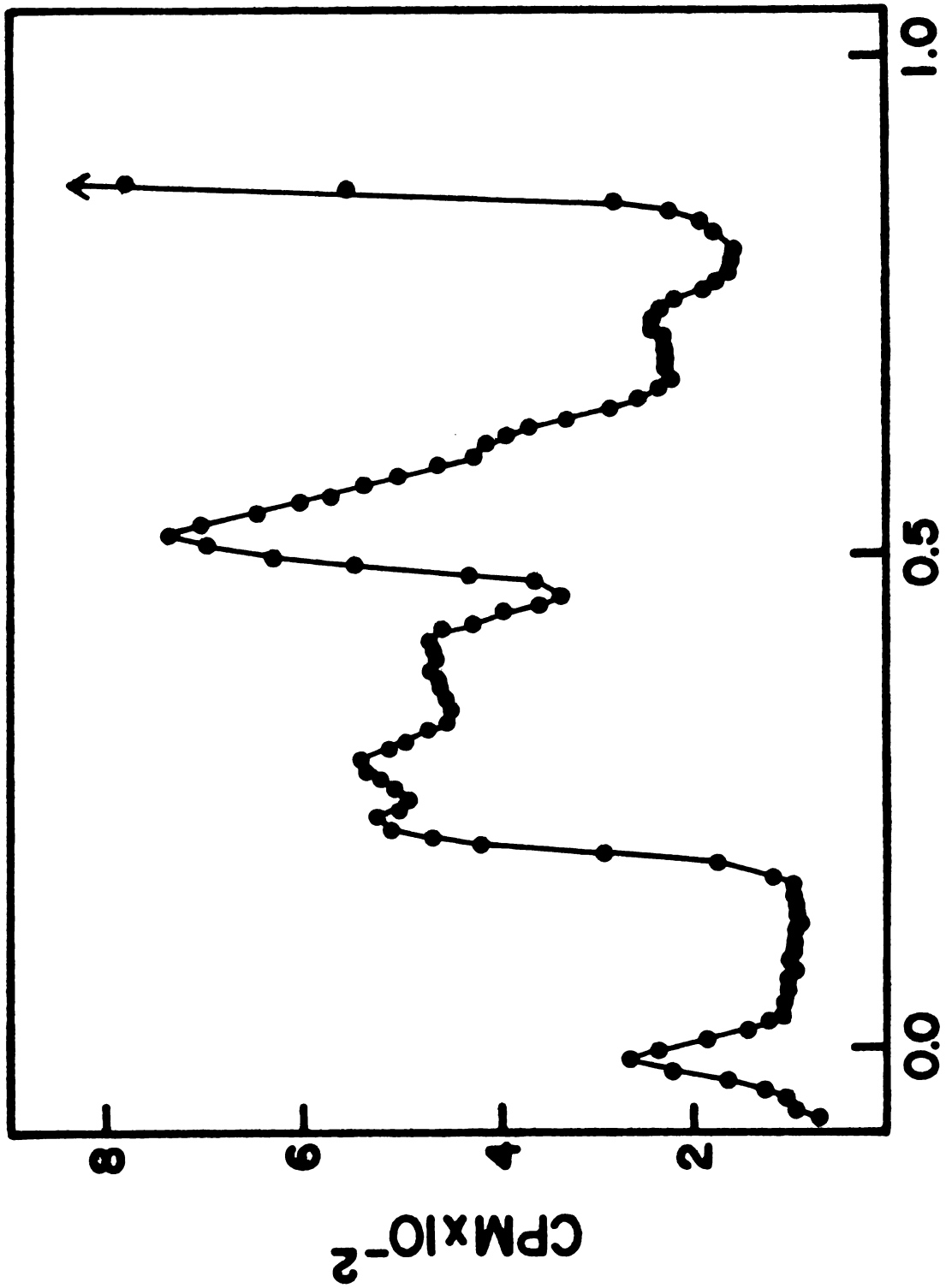



Figure 22

obtained from the unlabeled ribosomes has a large peak at $K_d$ = 1.0 which is due to contamination of the ribosomes with radioactive amino acids and aminoacyl-tRNA. A small peak is also seen at $K_d$ = 0.22 in the elution profile obtained from chromatography of the ribosomal fraction. The peak at $K_d$ = 0.22 was thought to be due to the presence of α- and β-globin which had been synthesized and released from the TRNA, but was contaminating the ribosomal preparation.

A similar contamination by completed globin chains was observed when ribosomes from the reticulocyte lysate were prepared for gel chromatographic analysis without nascent chain purification in a manner identical to that described in Methods (Figure 23). A peak in the elution profile at $K_d$ = 0.22 was seen which was not present in elution profiles of purified reticulocyte lysate nascent chains (Figures 8, 11, and 14). Since the $K_d$ value for purified β-globin was 0.22 (Protzel, 1974, and this thesis), it was concluded that the added accumulation observed in chromatographic analysis of ribosomes was due to contaminating globin.

## The Effect of Spermidine Upon the Nascent Chain Size Distribution

The addition of naturally occurring polyamines (spermine, spermidine) to a cell-free protein biosynthetic system had been shown to increase synthetic activity (Atkins et al., 1975). Addition of spermidine to the wheat embryo-derived cell-free system was performed to determine if any effect could be observed upon the activation of nascent chains of discrete sizes during globin mRNA directed protein biosynthesis. A concentration of 80 μM spermidine was used and the optimum concentration of $Mg^{++}$ was reduced to 2.1 mM The amount of

Figure 23. Elution profile of polysomal material from the reticulocyte lysate.

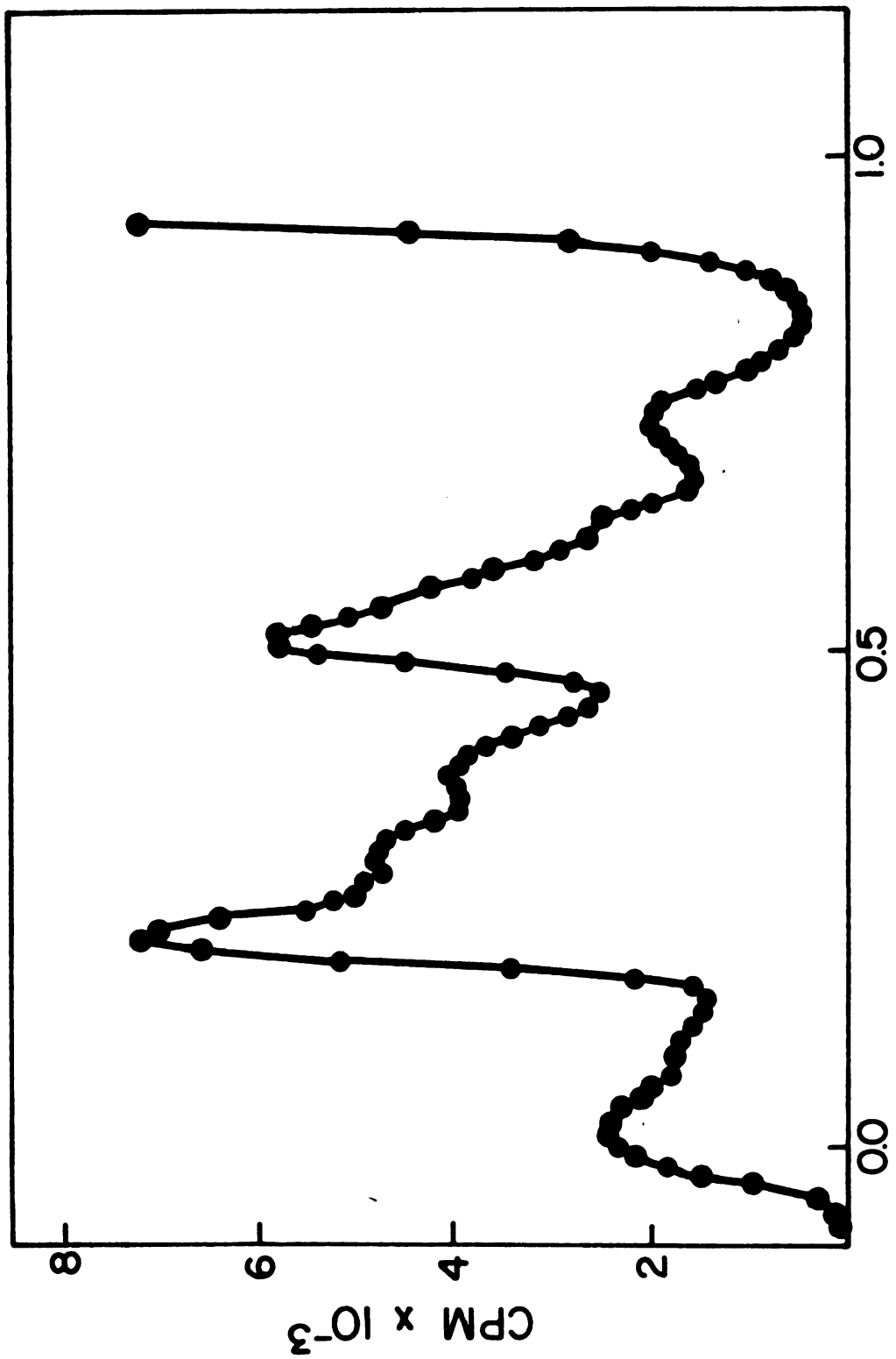



Figure 23

incorporation of $^{3}$H-leucine into TCA insoluble material was unchanged in the wheat embryo derived incubations containing 80 μM spermidine during a 45-minute incubation period at 30°

The incubation mixture was prepared in a manner identical to that described for an incubation mixture to which no spermidine had been added (except as described above for the $Mg^{++}$ concentration). Ribosomes were isolated by centrifugation and prepared for Bio-Gel A 0.5 m column chromatography without further purification of the nascent chain fraction. The results of the addition of 80 μM spermidine to the incubation mixture are shown in Figure 24. The nonuniform elution pattern was not altered by the addition of 80 μM spermidine to the incubation mixture as shown by comparing Figure 24 with Figure 22.

Heat Treatment at 65° of Globin mRNA

Evidence had been presented that *E. coli* galactose operon mRNA (gal mRNA) existed in different conformations which affected the relative rates of synthesis of the three gene products of this mRNA and the *in vitro* mRNA half-life (Schumacher and Ehring, 1975). Gal mRNA was heated to 65° for two minutes followed by cooling quickly in an ice water bath. Heat treatment of gal mRNA changed the ratios and amounts of the products synthesized in an *in vitro* cell-free protein synthesizing system when gal mRNA was added. Ethanol precipitation of gal mRNA changed the biosynthetic properties of the mRNA back to those of the original, unheated fraction.

The observations of Schumacher and Ehring (1975) led us to attempt to determine if alterations in the pattern of nascent chain accumulations could be induced in the wheat embryo cell-free system by similar

Figure 24. Effect of spermidine upon the nascent chain elution pattern in the globin mRNA-directed wheat embryo cell-free system.

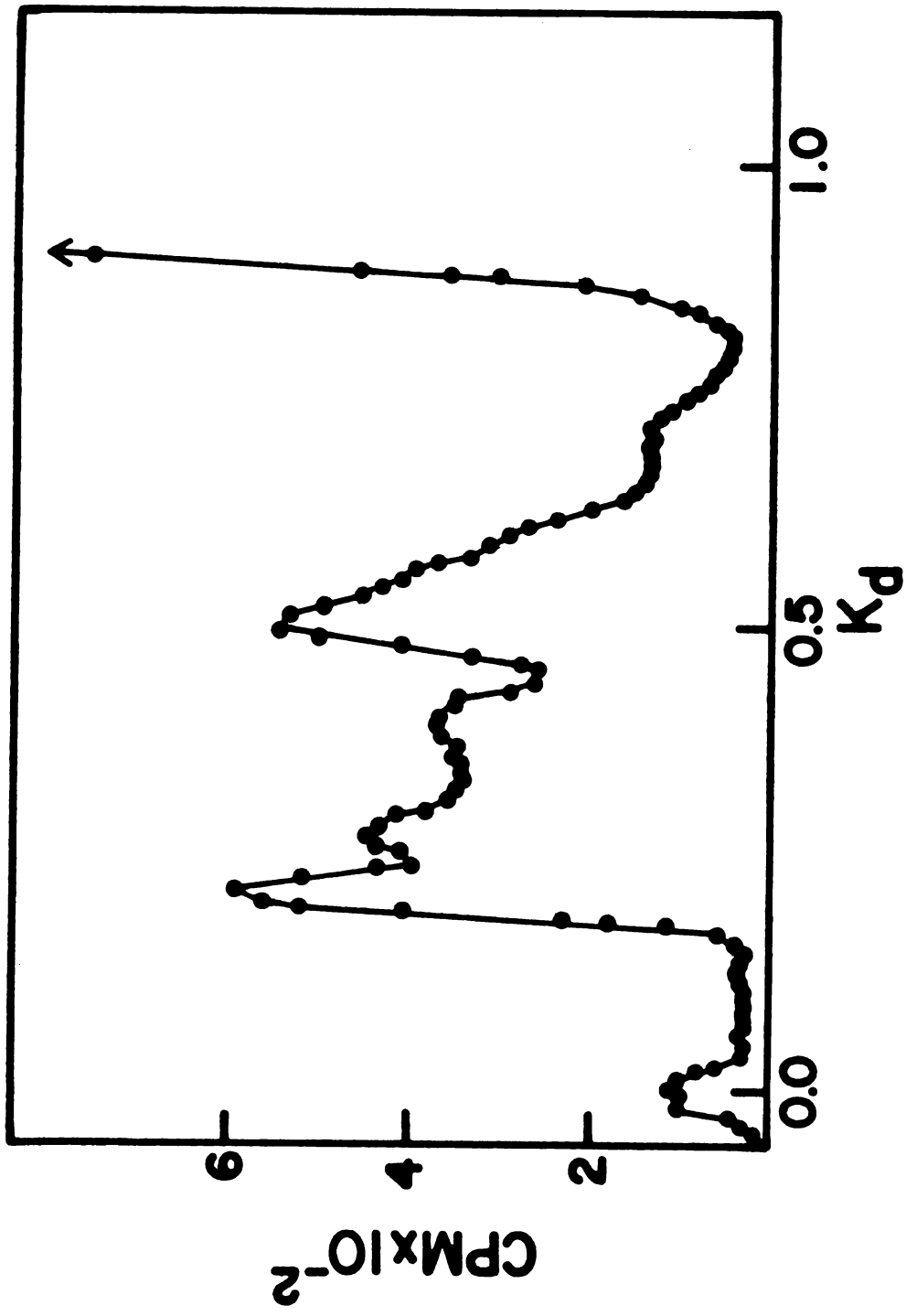



Figure 24

treatment of the globin mRNA prior to the incubation. A tube containing 2.4 μg of globin mRNA in 20 μl was heated in a water bath to 65° for five minutes. The solution was then quickly cooled by placing the tube in an ice water bath. After the solution was cooled, 0.58 ml of a complete wheat embryo cell-free system (minus mRNA) was added to the mRNA containing tube. The incubation mixture was incubated at 30° for 20 minutes with $^{3}$H-tryptophan added to label nascent chains. Ribosomes were isolated by centrifugation, prepared for column chromatography and subjected to Bio-Gel A 0.5 m chromatographic analysis as described in Methods. The elution profile obtained from the Bio-Gel chromatographic analysis is shown in Figure 25. A comparison of Figure 25 with the elution profile for untreated mRNA (Figure 22) showed that no alterations were induced in the nascent chain elution profile when globin mRNA was heated to 65° for five minutes and then quickly cooled prior to translation. Thus, heat treatment of globin mRNA was unable to induce a change in the nascent chain elution profile.

DMSO Treatment of Globin mRNA

Dimethyl sulfoxide was known to denature regions of intramolecular base pairing in DNA and RNA. Treatment of mRNA with DMSO would be expected to disrupt regions of secondary and tertiary structure. If such regions were associated with the accumulation of nascent chains of discrete sizes, it was expected that DMSO treatment might alter the nascent chain elution profile.

Protein synthesis, however, was inhibited by DMSO. Preliminary experiments found that the rate of incorporation of $^{3}$H-leucine into protein was inhibited to 50% of its original level when the incubation

Figure 25. Elution profile from the heat treated globin mRNA-directed wheat embryo cell-free system.

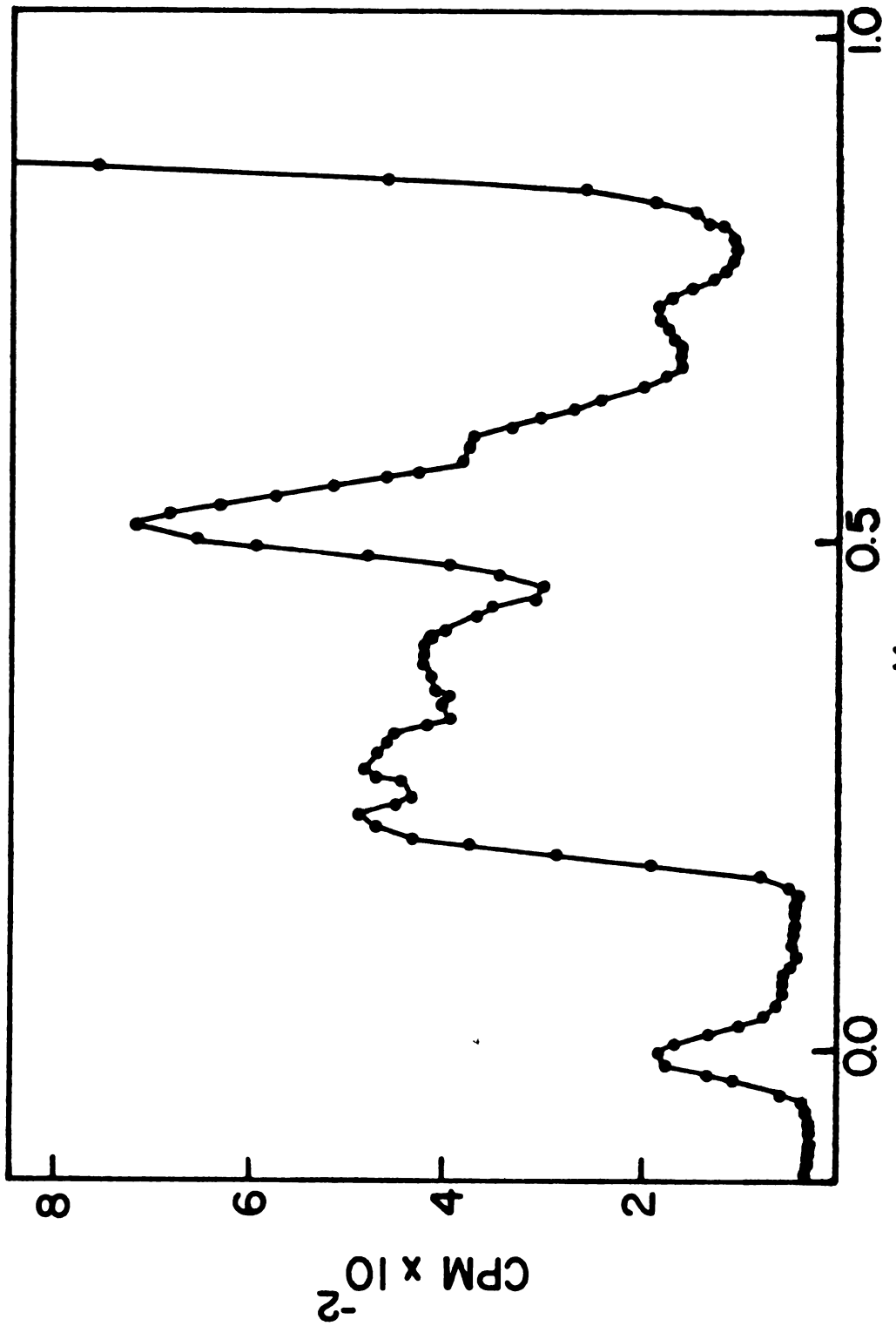



Figure 25

mixture contained 3% DMSO. Dimethyl sulfoxide (20 µl) was placed in a tube with 20 µl of globin mRNA (0.12 mg/ml). The 50% DMSO solution containing the globin mRNA was heated in a water bath to 70° for ten minutes, cooled in an ice bath and added to the components of a wheat embryo cell-free system. The final concentration of DMSO in the incubation mixture was 3%.

The incubation mixture was incubated at 30° for 20 minutes with $^{3}$H-tryptophan to label nascent chains. Ribosomes were isolated by centrifugation, treated with base to cleave the peptide-tRNA bond, and subjected to gel chromatographic analysis. The elution profile from the incubation containing DMSO treated mRNA is shown in Figure 26. Again, a comparison of the elution profile in Figure 26 with that in Figure 22 (a control incubation) failed to demonstrate significant alterations in the observed accumulations of nascent chains.

Thus, pre-treatment of globin mRNA with 50% DMSO at 70° for ten minutes failed to alter the mRNA in a manner which affected the nascent chain accumulation patterns. Since the final concentration of DMSO in the wheat embryo cell-free incubation mixture was only 3%, it was possible that the DMSO concentration in the final mixture was too low to affect mRNA secondary or tertiary structure. The denaturing effect of 50% DMSO at 70° on globin mRNA could have been reversed due to the cooling of RNA, the reduction of DMSO to 3%, and the association of mRNA with ribosomes during translation.

Formaldehyde Treatment of Globin mRNA

The reaction of RNA with formaldehyde had been shown to cause alterations in the secondary structure of that RNA (Boedtker, 1967).

Figure 26. Elution profile from the DMSO treated globin mRNA-directed wheat embryo cell-free system.

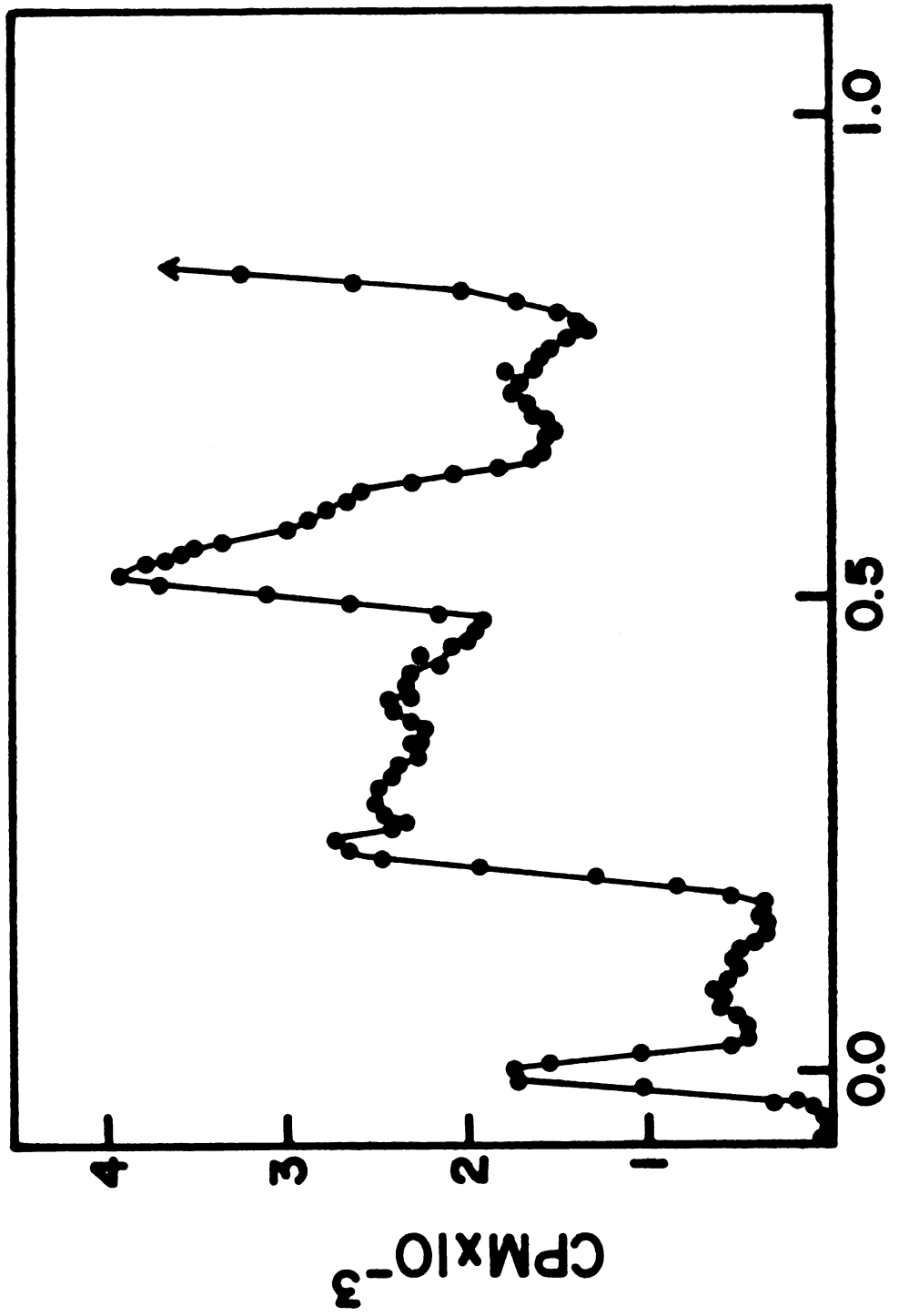



Figure 26

Reaction of formaldehyde with f2 RNA caused synthesis of the phage coded replicase subunit to be dependent no longer upon the translation of the coat protein cistron, presumably due to a disruption in the secondary structure of the RNA molecule (Lodish, 1970). Experiments, involving the addition of formaldehyde treated globin mRNA to the wheat embryo cell-free protein synthesizing system, were designed to determine if the elution profile of nascent chains isolated from the wheat embryo cell-free system could be altered by treatment of globin mRNA with formaldehyde.

Globin mRNA was treated with formaldehyde as described in Methods. Wheat embryo-derived incubation mixtures (1.2 ml each) which contained 4.8 μg of formaldehyde treated mRNA per incubation mixture were prepared. One incubation contained mRNA, which had been treated with 0.02 M formaldehyde at 68° for 10 minutes, and a second incubation contained mRNA treated with 0.03 M formaldehyde at 68° for 10 minutes. Ribosomes were isolated by centrifugation and analyzed by Bio-Gel column chromatography. The elution profile for ribosomes obtained from the incubation mixture which contained 0.02 M formaldehyde treated globin mRNA is shown in Figure 27. Figure 28 shows the elution profile for ribosomes obtained from the incubation which contained globin mRNA treated with 0.03 M formaldehyde.

The graph in Figure 27 (0.02 M formaldehyde treated globin mRNA) showed a nascent chain elution profile which had accumulations at the same $K_d$ values as the control elution profile (Figure 22). However, the heights of the larger nascent chain peaks ($K_d < 0.4$) were decreased significantly relative to the large peak at $K_d = 0.5$. The heights of these regions in Figure 28 (0.03 M formaldehyde treated mRNA) showed

Figure 27. Elution profile from the formaldehyde treated globin mRNA-directed wheat embryo cell-free system.

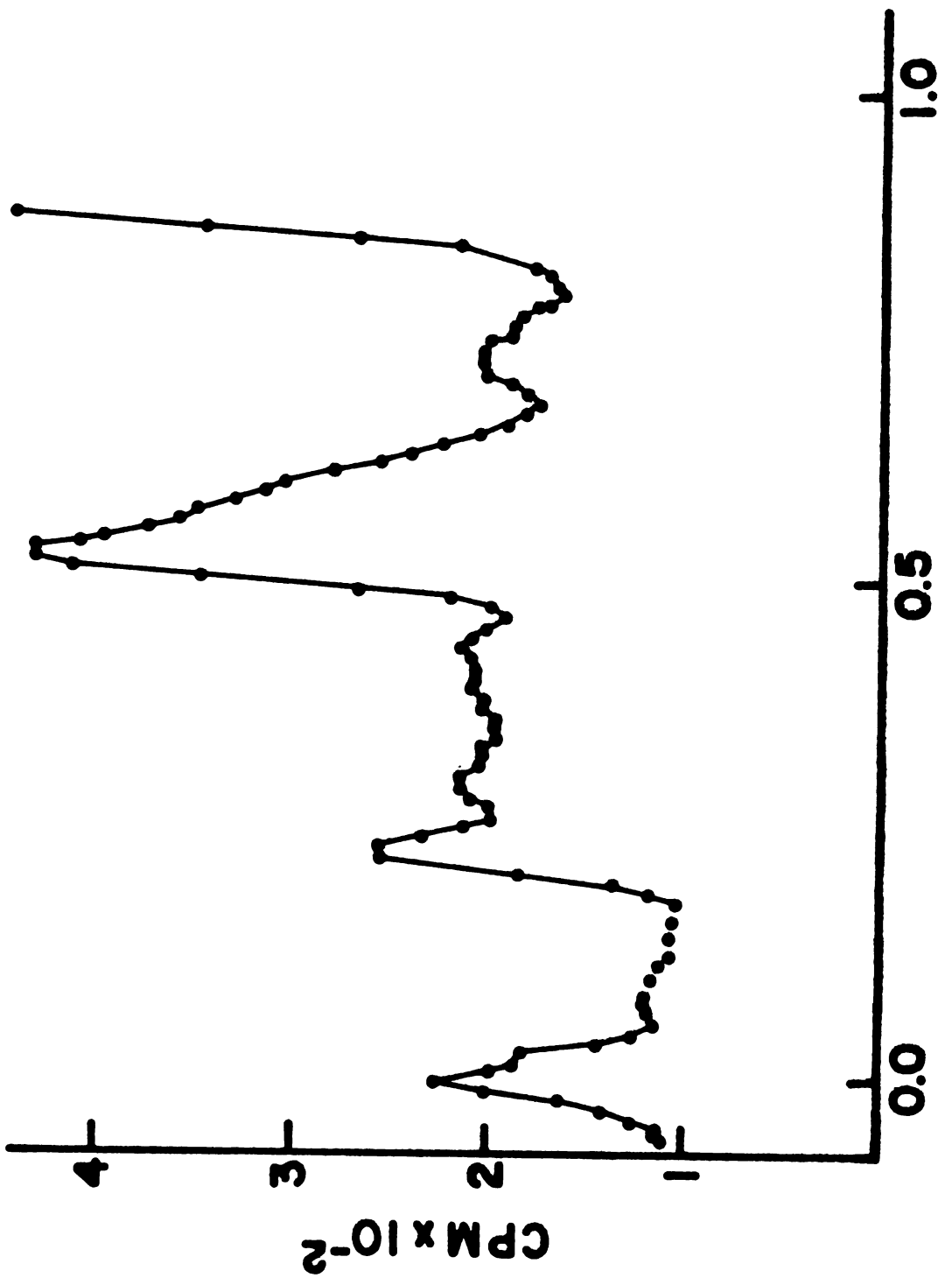



Figure 27

Figure 28. Effect of formaldehyde treatment of globin mRNA upon the elution profile of nascent chains from the wheat embryo cell-free system.

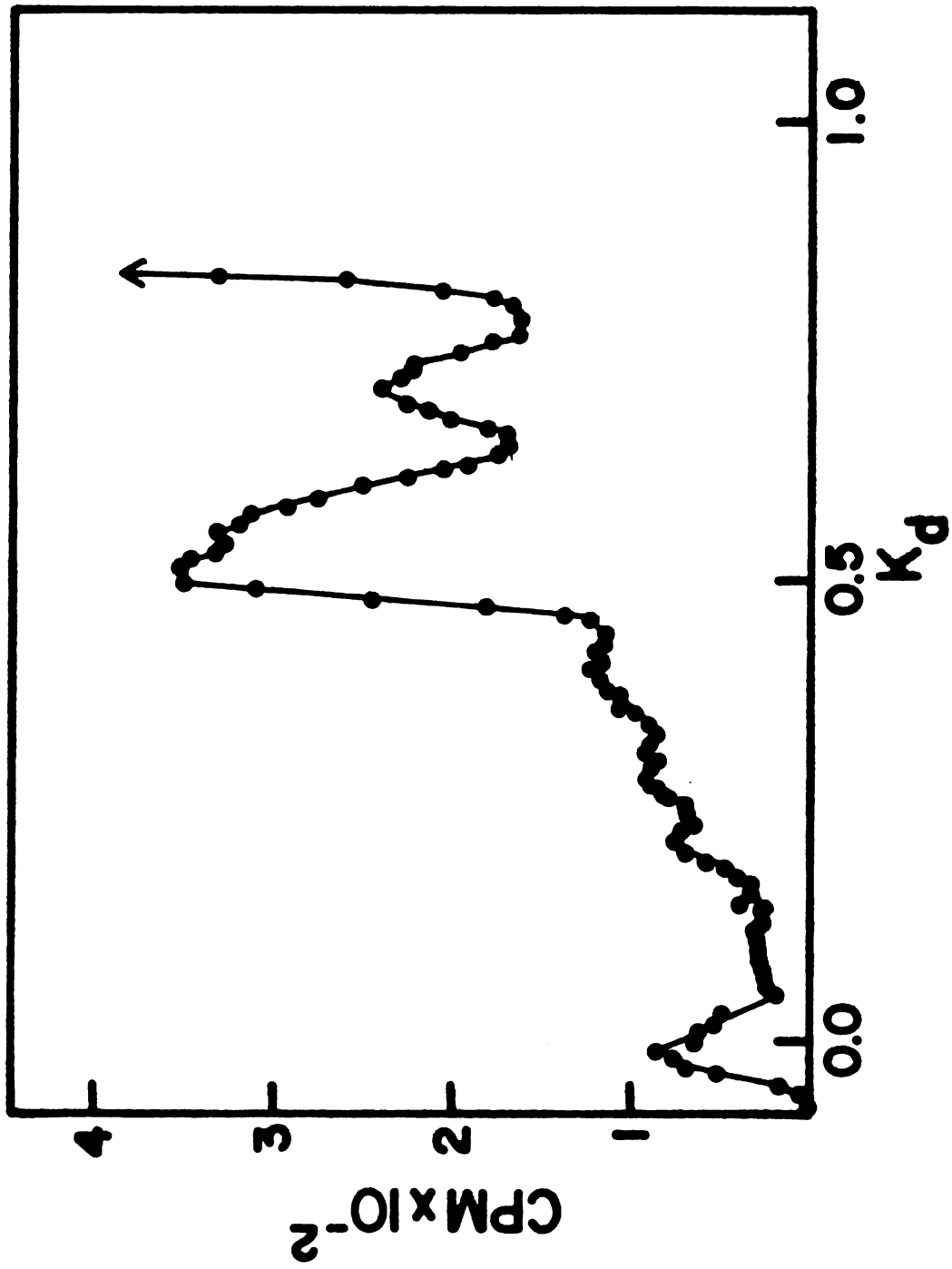



Figure 28

an even greater relative decrease in the nascent chain accumulations of $K_d < 0.4$. However, the positions of nascent chain accumulations remained at the same $K_d$ values as in the control. The decrease in magnitude of nascent chains of larger sizes ($K_d < 0.4$) prevented the use of higher concentrations of formaldehyde.

Treatment of isolated globin mRNA with formaldehyde failed to affect the nascent peptide chain accumulation positions which were observed when ribosomes from a wheat embryo cell-free system utilizing formaldehyde treated mRNA were analyzed by gel chromatography upon a Bio-Gel A 0.5 m column under denaturing conditions. The relative magnitudes of the larger nascent chain sizes were decreased in the elution profile obtained from the formaldehyde treated mRNA. The decrease in the relative amounts of larger nascent chains may be due to either premature termination of protein synthesis or to the induction of chain cleavage of some of the mRNA molecules by formaldehyde modification of the mRNA.

All of the experiments described above, which attempted to modify globin mRNA structure, were characterized by the requirement that the conditions used be mild enough to permit the protein biosynthetic incubation medium to function. The nascent chain size distribution analysis involves the isolation of nascent chains from polysomes containing mRNA molecules which have undergone translation for some time. Structures within the mRNA molecules which affect translation would be those which could reform after ribosome passage through a mRNA region. Therefore, the failure to observe an effect of the various treatments could have been due to the continued stability of regions of secondary and tertiary structure in the modified mRNA.

Bacteriophage MS2 Protein Synthesis

The experiments described in this thesis, using the reticulocyte lysate cell-free protein synthesis system and the wheat embryo-derived cell-free system to analyze the nascent peptide chain size accumulations, indicated that the origin of the nascent chain nonuniformities residues in some aspect of the globin mRNA itself. Since, as mentioned above, globin mRNA had been shown to contain regions which have some form of secondary structure involving intramolecular base pairing (hairpin loops and possibly more complicated structures), it was thought that such regions might be associated with the lowered relative rates of peptide chain elongation observed for specific regions of globin mRNA (as analyzed by the nascent globin chain accumulations). A lowered rate of chain elongation was envisioned to involve the ribosome having to slow its rate of movement along the mRNA as it entered a region of secondary structure which would have to be removed (by melting out the base pairs) before the ribosome could translate that region.

At the time the experiments in this thesis were performed, the only mRNA whose primary structure had been determined was that of the RNA bacteriophage, MS2. Furthermore, as described in the Introduction, a structure for the RNA coat protein gene had been proposed which was in quite good agreement with known physical and biological properties of the bacteriophage genome (Min Jou et al., 1972). Experiments were, therefore, designed in which E. coli would be infected with the RNA bacteriophage, MS2, under conditions in which nearly all of the protein synthesis would be for the MS2 coat protein. A nascent chain population, which was essentially a MS2 coast protein nascent chain population, could then be isolated. The results of Bio-Gel A 0.5 m

chromatographic analysis of the nascent chain population under denaturing conditions could be used to examine the hypothesis that lowered rates of chain elongation are associated with translation of regions of mRNA secondary structure.

Infection of E. coli by any of the closely related RNA bacteriophages (MS2, R17, F2, etc.) had been shown to result in synthesis of bacteriophage RNA and the three phase coded proteins. Fromageot and Zinder (1968) found that if infection of E. coli is accompanied by treatment with the antibiotic rifampicin, nearly all of the protein synthesis occurring in the cells would be synthesis of protein coded for by the MS2 genome. Rifampicin had been shown to act upon the E. coli DNA-dependent RNA polymerase to block RNA synthesis (Hartmann et al., 1967). MS2 RNA synthesis was not affected because it had been demonstrated to be synthesized by a different enzyme, an RNA-dependent RNA replicase as described in the Introduction. Synthesis of the phage specific proteins proceded unaffected by the rifampicin, and the titer of infective phase particules produced by infected, rifampicin treated cells was nearly the same as that of cells which had received no rifampicin (Fromageot and Zinder, 1968).

Growth and infection of E. coli strains H 1008 and Hfr H have been described in Methods. Both strains gave similar results in the experiments described below. Initial experiments were performed to repeat the observations of Fromageot and Zinder (1968). Cells (Hfr H) were grown to an absorbance at 540 nm of 0.25 (2-3 x $10^8$ cells/ml), $CaCl_2$ added, and phage and rifampicin added as described in Methods. Twenty minutes after infection 5.0 μCi of $^3$H-leucine were added to 5 ml of cell suspension. One ml aliquots were taken and cooled on ice

immediately. Incorporation of the $^{3}$H-leucine into TCA precipitable material was analyzed as described in Methods.

The results of such an experiment performed by infection of E. coli Hfr H with MS2 is shown in Figure 29. The rate of protein synthesis was stimulated by MS2 infection twelve times over the rate in uninfected cells when measured at times greater than 40 minutes after infection. Protein synthesis under the incubation conditions described above was thus dependent upon bacteriophage MS2 infection.

A partial characterization of the $^{3}$H-leucine labeled products of MS2 infected E. coli was performed to determine the products of MS2 directed protein synthesis. Growth and infection of E. coli H1008 was described in Methods. Fifty μCi of $^{3}$H-leucine were added 45 minutes after infection and the incorporation continued for 15 minutes at 37°. The solution was then cooled to 0° and the protein extracted for analysis as described in Methods. Ten percent polyacrylamide, 0.1% SDS cylindrical gels (0.5 x 10 cm) were prepared and the sample analyzed by SDS gel electrophoresis as described in Methods.

The results of an electrophoretic analysis of the proteins isolated from MS2 infected, rifampicin treated E. coli are shown in Figure 30. The arrow marks the migration of bromophenol blue. The large peak of radioactivity at fractions 34-35 corresponded to MS2 coat protein. Each fraction represented a 2 mm slice. The in vivo protein synthesis during the time period of labeling was greater than 80% MS2 coat protein synthesis. Therefore, the nascent protein chains present on polysomes of MS2 infected E. coli at this time may be expected to be greater than 80% MS2 coat protein nascent chains.

Figure 29. Bacteriophage MS2 induced protein synthesis in rifampicin treated E. coli.

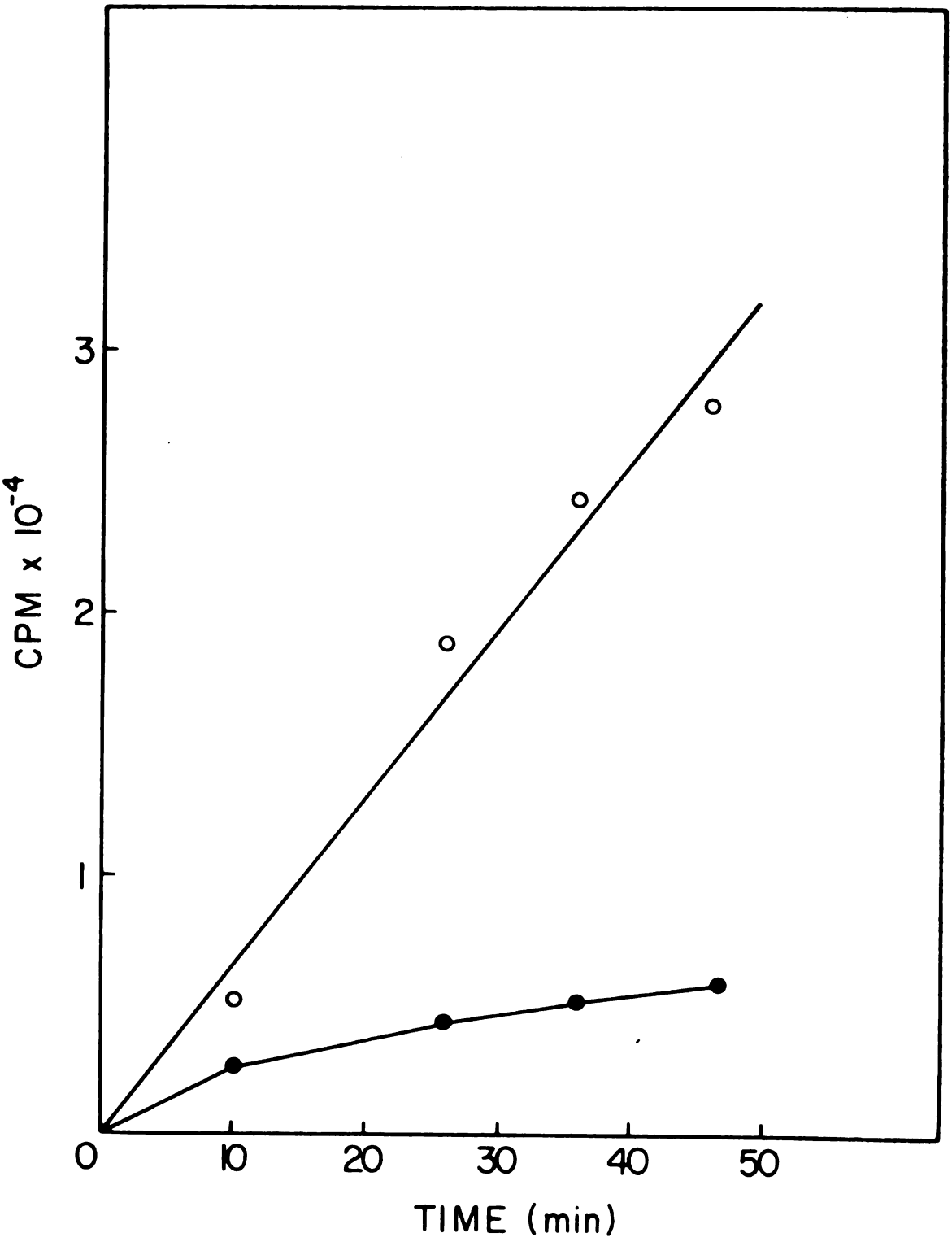
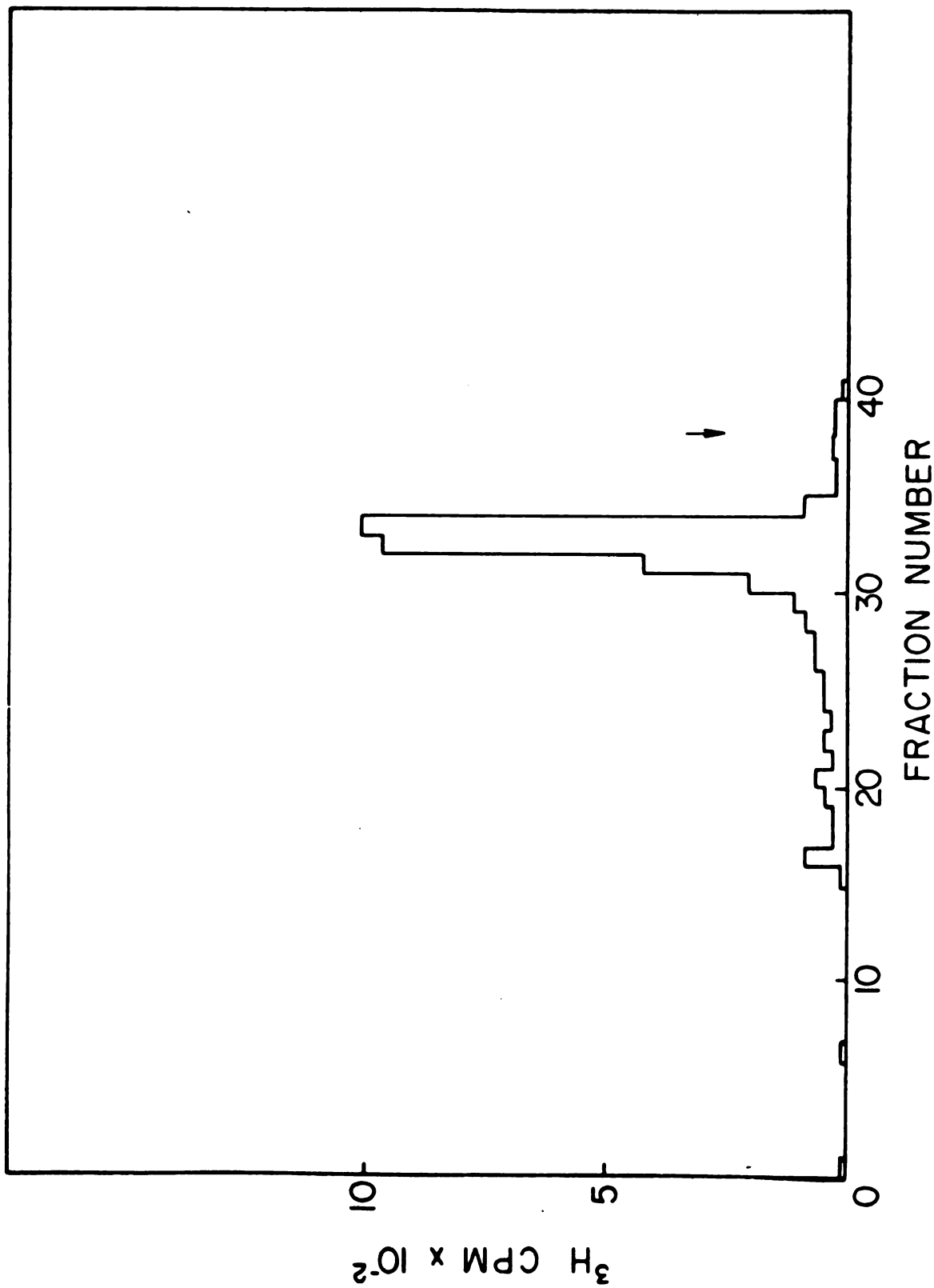

CPM x $10^{-4}$
3
2
1
0
10
20
30
40
50
TIME (min)

Figure 30. A SDS polyacrylamide gel analysis of the products of MS2 infected, rifampicin treated E. coli.


$^3H$ CPM x $10^{-2}$
0
5
10
FRACTION NUMBER
10
20
30
40

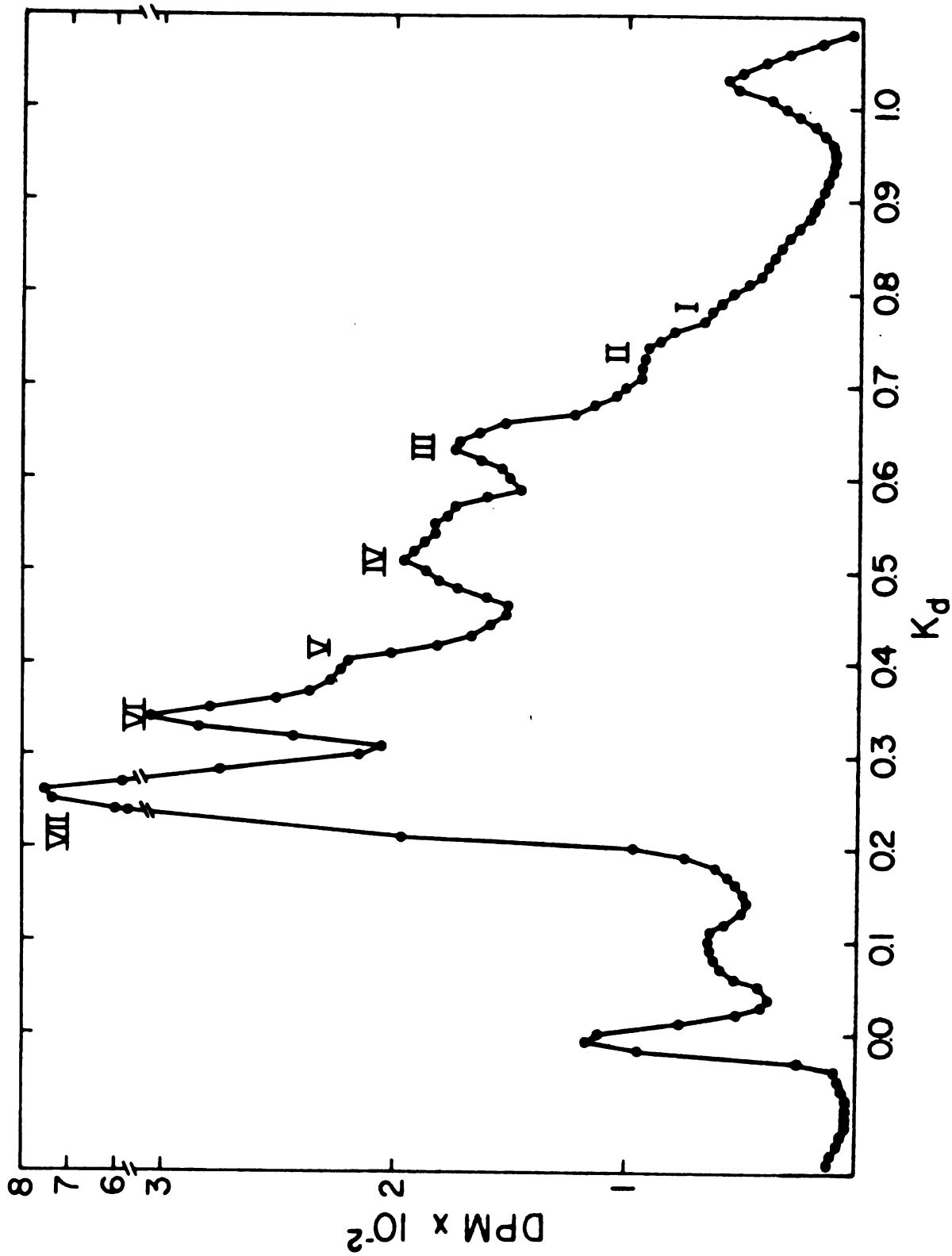
Analysis of Nascent Chain Sizes from MS2 Infected E. coli

Nascent chains labeled with $^{3}$H-leucine were isolated from MS2 infected, rifampicin treated E. coli H1008 as described in Methods. Bacteriophage MS2 infected, rifampicin treated E. coli were shown above to be maintaining linear protein synthesis at the time of isolation. The products of synthesis were also shown above to be greater than 90% MS2 coat protein at the time of nascent chain isolation. Chromatographic analysis of the nascent chains upon Bio-Gel A 0.5 m in 6 M guanidine-HCl, 0.1 M 2-mercaptoethanol (pH 6.5) was performed.

The elution profile for $^{3}$H-leucine labeled MS2 coat protein nascent chains is shown in Figure 31. A nonuniform size distribution of the MS2 coat protein nascent peptides was present. However, the elution profiles of the nascent chain population isolated from the E. coli and rabbit reticulocyte synthetic systems were quite different, as seen in a comparison of Figure 31 with Figure 8. Thus, the nascent chain accumulations of discrete sizes were not the same for the synthesis of different proteins. Each mRNA being translated produced nascent chain accumulations whose sizes were unique for that mRNA.

Nascent chains were also purified from E. coli H1008 at a time earlier in infection. A 100 ml culture of E. coli H1008 was infected with MS2 and treated with rifampicin as described in Methods. The radioactive label (200 μCi of $^{3}$H-leucine) was added 15 minutes after infection and the cells were poured into stopping buffer 30 minutes after infection. MS2 coat protein was the predominant product at this time after infection. Nascent chains were purified and analyzed as described in Methods.

Figure 31. Elution profile of $^{3}$H-leucine labeled nascent chains from MS2 infected, rifampicin treated E. coli.


DPM x 10⁻²
8
7
6
3
2
1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
$K_d$
I
II
III
IV
V
VI
VII

The results of Bio-Gel A 0.5m chromatographic analysis of the nascent chain preparation purified from MS2 infected, rifampicin treated E. coli 30 minutes after infection are shown in Figure 32. Nascent chain accumulations were present at the same $K_d$ values observed in Figure 31. Therefore, the accumulations observed in MS2 infected E. coli were not unique to the nascent chain population present 50 minutes after infection. The accumulation of MS2 coat protein nascent chains in the Bio-Gel elution profile was not dependent upon the use of $^3$H-leucine for the in vivo labeling procedure. Figure 33 shows the results of an incubation identical to that described for Figure 31, in which the nascent chains were labeled with $^3$H-aspartic acid. As can be seen by comparison of the two figures, there were accumulations of nascent chains of similar $K_d$ values. (The origin of the radioactivity of $K_d$ = 0.96 is unknown and does not correspond to any MS2 coat protein nascent chain and does not appear in other MS2 coat protein profiles.) The relative heights of the points of accumulations were seen to vary. However, the $K_d$ values of the nascent chain accumulations remained relatively constant.

An incubation utilizing $^3$H-asparagine as the radioactive label yielded results similar to the incubations using $^3$H-leucine and $^3$H-aspartic acid as the radioactive label. Data from nine different experiments utilizing these three different amino acids to label nascent chains is presented in Table 8. The $K_d$ values in Table 8 will be discussed later in a comparison of the MS2 coat protein RNA with the positions along the RNA where ribosomes bearing nascent chains of those sizes reside.

Figure 32. Elution profile of nascent chains isolated from MS2 infected rifampicin treated *E. coli* 30 minutes after infection.

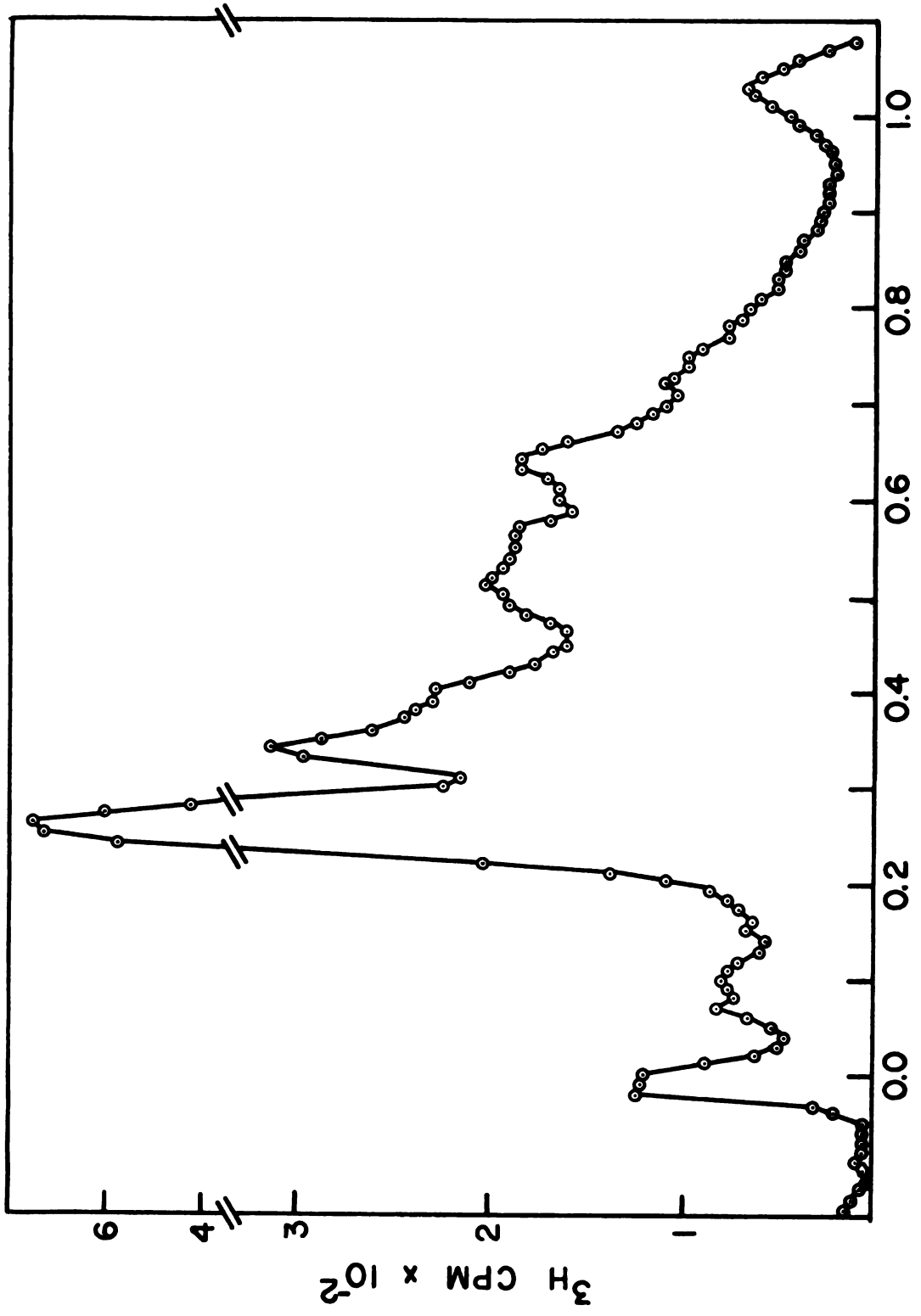



Figure 32

Figure 33. Elution profiles of $^{3}H$-aspartic acid labeled nascent chains from MS2 infected, rifampicin treated E. coli.

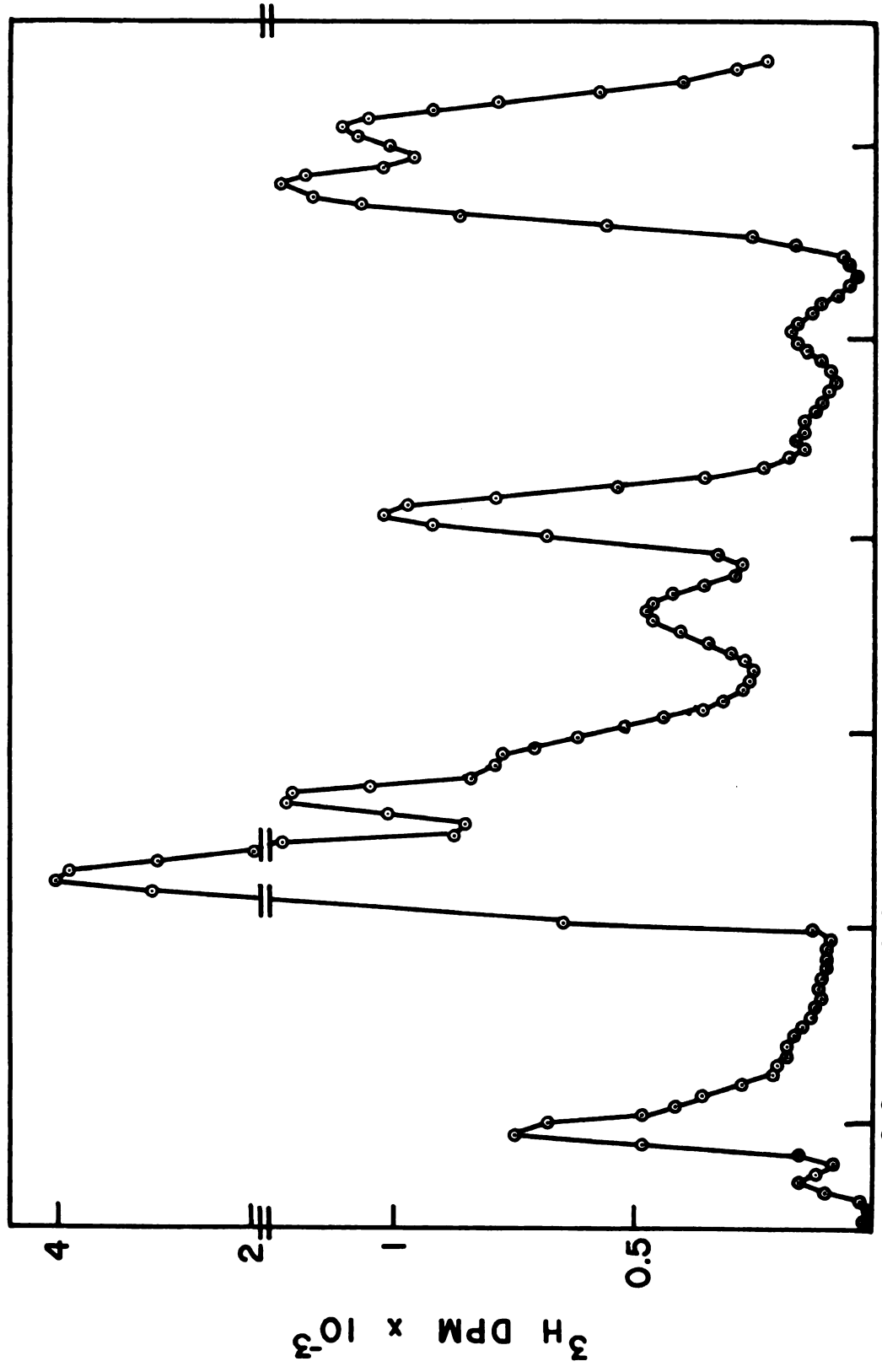



Figure 33

Table 8. Distribution coefficients of nascent MS2 coat protein chains from nine separate experiments using leucine, aspartic acid, and asparagine to radioactively label nascent chains.

| Peak Number | $K_d$[a] + S.D. |
|---|---|
| I | 0.80 ± 0.01[b] |
| II | 0.72 ± 0.01 |
| III | 0.63 ± 0.005 |
| IV | 0.52 ± 0.01 |
| V | 0.39 ± 0.01 |
| VI | 0.33 ± 0.005 |
| VII | 0.25 ± 0.005 |

[a]Data presented here summarize the results of nine analyses of the size distribution of nascent coat protein peptides. The distribution coefficient ($K_d$) of each maximum in the Bio-Gel A 0.5 m elution profile is indicated (see, for example, Figure 5).

[b]Detected as a minor peak in five of the nine experiments.

Elution Profile of Nascent Chains From Uninfected E. coli

One hundred ml of MTPA media were inoculated with 2 ml of a culture of E. coli Hfr H grown overnight in MTPA media. The culture was incubated at 37° until the absorbance at 540 nm was 0.25, a cell concentration of 2-3 x $10^8$ cells/ml. Calcium chloride was added as usual for the infection procedure with bacteriophage MS2 as described in Methods. Infection and rifampicin treatment were omitted. Nascent chains were labeled with 100 μCi of $^3$H-asparagine in the same manner as described in Methods for labeling of MS2 infected E. coli. The nascent peptide chains were purified from these cells and subjected to Bio-Gel

A 0.5 m chromatographic analysis under the denaturing conditions as described for infected cells in Methods. The elution profile obtained from the chromatographic analysis is shown in Figure 34. The profile for nascent chains purified from uninfected E. coli shows a steadily ascending curve of radioactivity as chain length increases with very few points at which there are nascent chain accumulations. The accumulations which were seen may be due to the presence of nascent peptides of an E. coli protein made in relatively large amounts.

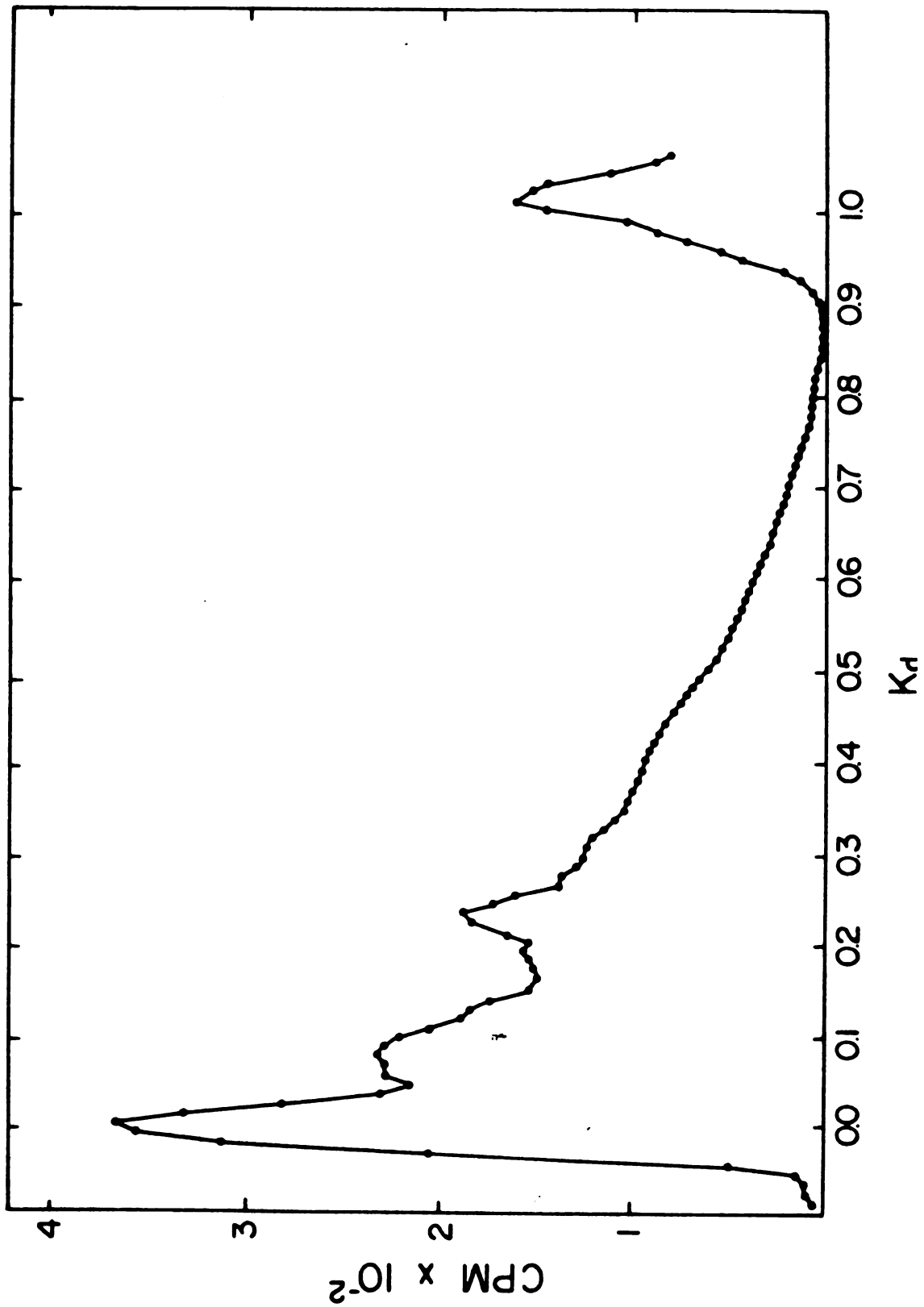
The increase in the radioactivity at $K_d$ = 0 was expected since all nascent chains greater than about 25,000 daltons will elute together in this region. The radioactivity at $K_d$ = 1.0 was due to contaminating aminoacyl-tRNA which purified with peptidyl-tRNA. In the region in which nascent MS2 coat protein chains elute ($K_d$ = 1.0 to $K_d$ = 0.25), no chain accumulations were observed.

Base Lability of the E. coli Nascent Chains

A sample of $^3$H-leucine labeled nascent chains was prepared from MS2 infected, rifampicin treated E. coli H1008 as described in Methods. Prior to the adsorption to DEAE-cellulose, a portion of the sample was brought to pH 10 and incubated for four hours at 37°. The pH of the sample was then adjusted to pH 5.6 with 6 N HCl and made 0.10 M 2-mercaptoethanol. After a two hour incubation at room temperature the sample was passed through a DEAE-cellulose chromatography column equilibrated with Buffer I and treated in a manner identical to nascent chain preparations.

While a portion of the nascent chain preparation which did not receive base treatment was found to contain radioactive material which

Figure 34. Elution profile of nascent chains from uninfected E. coli.


CPM x 10⁻²
4
3
2
1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Kd

bound to the DEAE-cellulose in Buffer I and eluted with Buffer II, the sample which received the previous base treatment passed entirely through the column. No material in the base treated sample was bound to the DEAE-cellulose, as was predicted since the peptidyl-tRNA purification procedure was based on the ability of DEAE-cellulose to bind the tRNA part of the peptidyl-tRNA.

Two conclusions may be drawn from the inability of the base treated material to adsorb to DEAE-cellulose: 1) the material which binds to DEAE-cellulose did so by its attachment to RNA via an alkali labile bond, and 2) there were no labeled proteins present in this sample which have a net negative charge sufficient to bind to the DEAE-cellulose and contaminate the nascent chain fraction with completed protein.

Test of the Bio-Gel Column with Alpha- and Beta-Globin Derived Peptides

A linear relationship between $K_d^{1/3}$ and (molecular weight)$^{0.555}$ had been demonstrated for the elution of proteins from Bio-Gel agarose chromatography columns under denaturing conditions (6 M guanidine-HCl, 0.1 M 2-mercaptoethanol, pH 6.5) by Fish et al. (1969) and had been extended to agarose beads of smaller dimensions (Bio-Gel A 0.5 m, 10% agarose) for analysis of proteins of smaller sizes (Protzel and Morris, 1974). Although the column buffer and column gel used in the experiments reported in this thesis were identical to those used by Protzel and Morris (1974), a test of the Bio-Gel column used in this thesis was performed to determine if the behavior of standard globin derived peptides upon it was similar to that observed previously.

Alpha- and β-globin were separated as described in Methods. Treatment of proteins with cyanogen bromide under aqueous, acidic conditions at room temperature had been shown to break the peptide bond of protein at methionine residues (Gross and Witkop, 1962). The peptides which may be derived from cyanogen bromide treatment of α- and β-globin (each protein contains a single methionine in its structure) are illustrated in Figure 35.

A sample to be used for the test of the Bio-Gel column was dissolved in 0.4 ml of 6.0 M guanidine, 0.1 M 2-mercaptoethanol (pH 6.5) column buffer and brought to 0.05 M dithiothreitol. Following a two hour incubation at room temperature, the sample was treated in an identical manner to that described in Methods for chromatographic analysis of peptidyl-tRNA preparations.

The elution profile for isolated β-globin treated with cyanogen bromide is shown in Figure 36. Three peaks are seen. One ($K_d$ = 0.22) corresponded to unreacted β-globin. The peaks at $K_d$ = 0.52 and $K_d$ = 0.33 corresponded to cyanogen bromide cleavage products: β-CB-1 and β-CB-2, respectively.

A second experiment was performed in which a mixture of α- and β-globin peptides generated by cyanogen bromide treatment of globin were analyzed by Bio-Gel chromatography. The elution profile is seen in Figure 37. A peak which corresponded to β-CB-1 was again seen at $K_d$ = 0.52. The peak which was at $K_d$ = 0.64 corresponded to α-CB-1. The broad peak at $K_d$ = 0.30 was derived from overlapping peaks of α-CB-2, β-CB-2, and β-globin.

A comparison of the $K_d$ values determined for these peptides by Protzel (1973) and the $K_d$'s determined in Figures 36 and 37 is shown

Figure 35. The possible peptides derived from α- and β-globin by cyanogen bromide treatment.

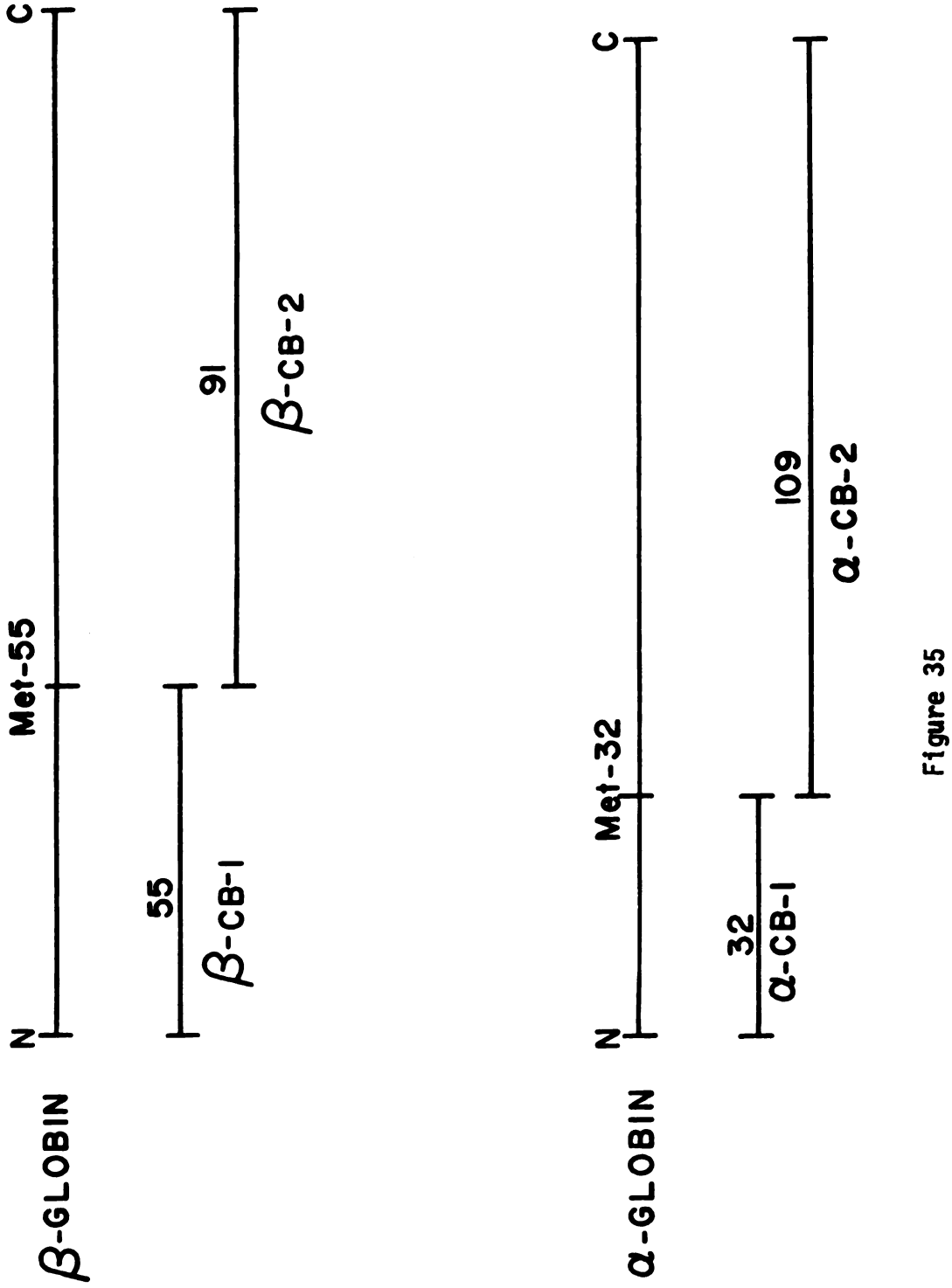

β-GLOBIN
N
Met-55
C
β-CB-1
55
β-CB-2
91
α-GLOBIN
N
Met-32
C
α-CB-1
32
α-CB-2
109

Figure 35

Figure 36. Elution profile of cyanogen bromide treated β-globin.

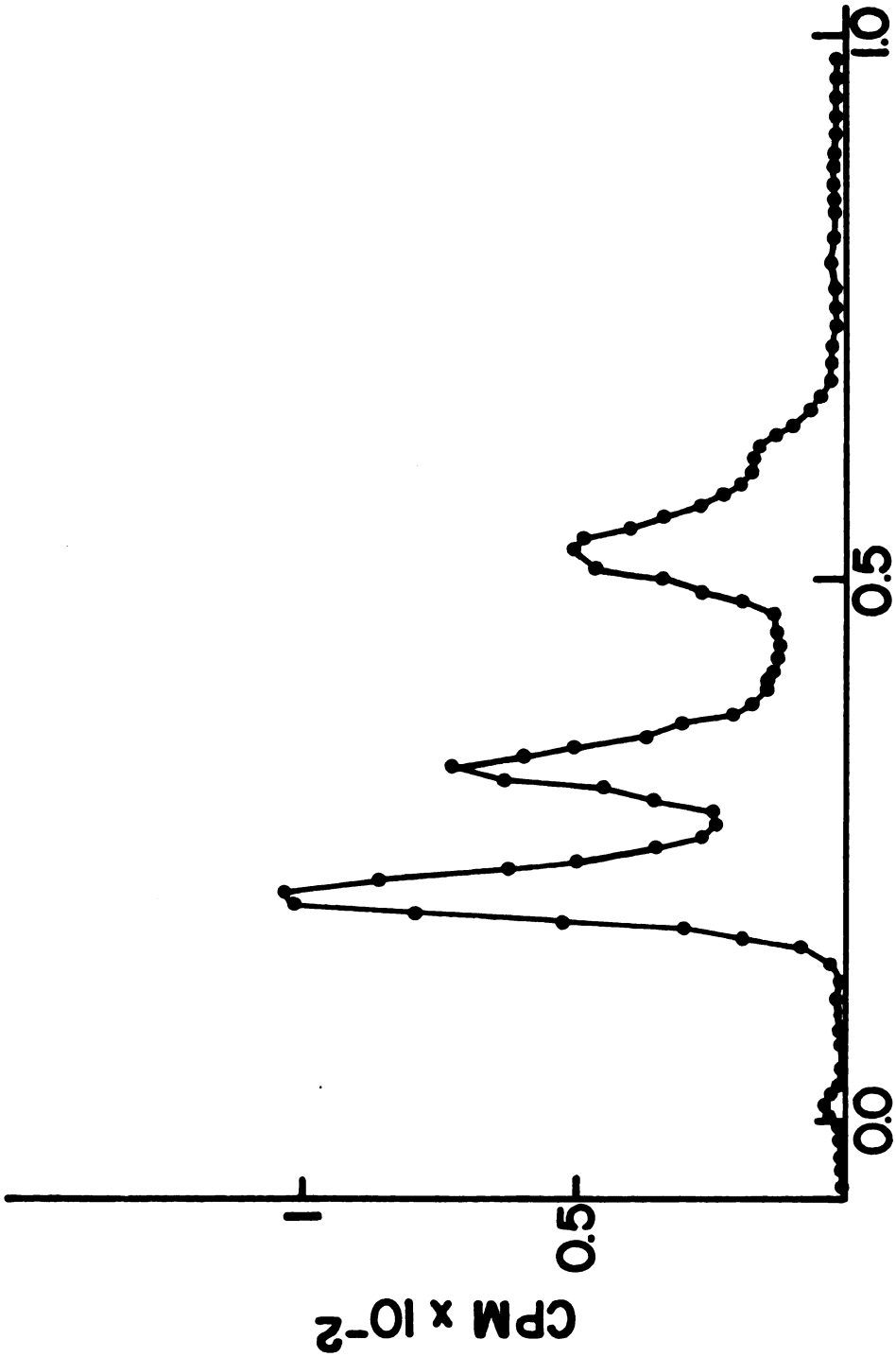



Figure 36

Figure 37. Elution profile of cyanogen bromide treated α- and β-globin.

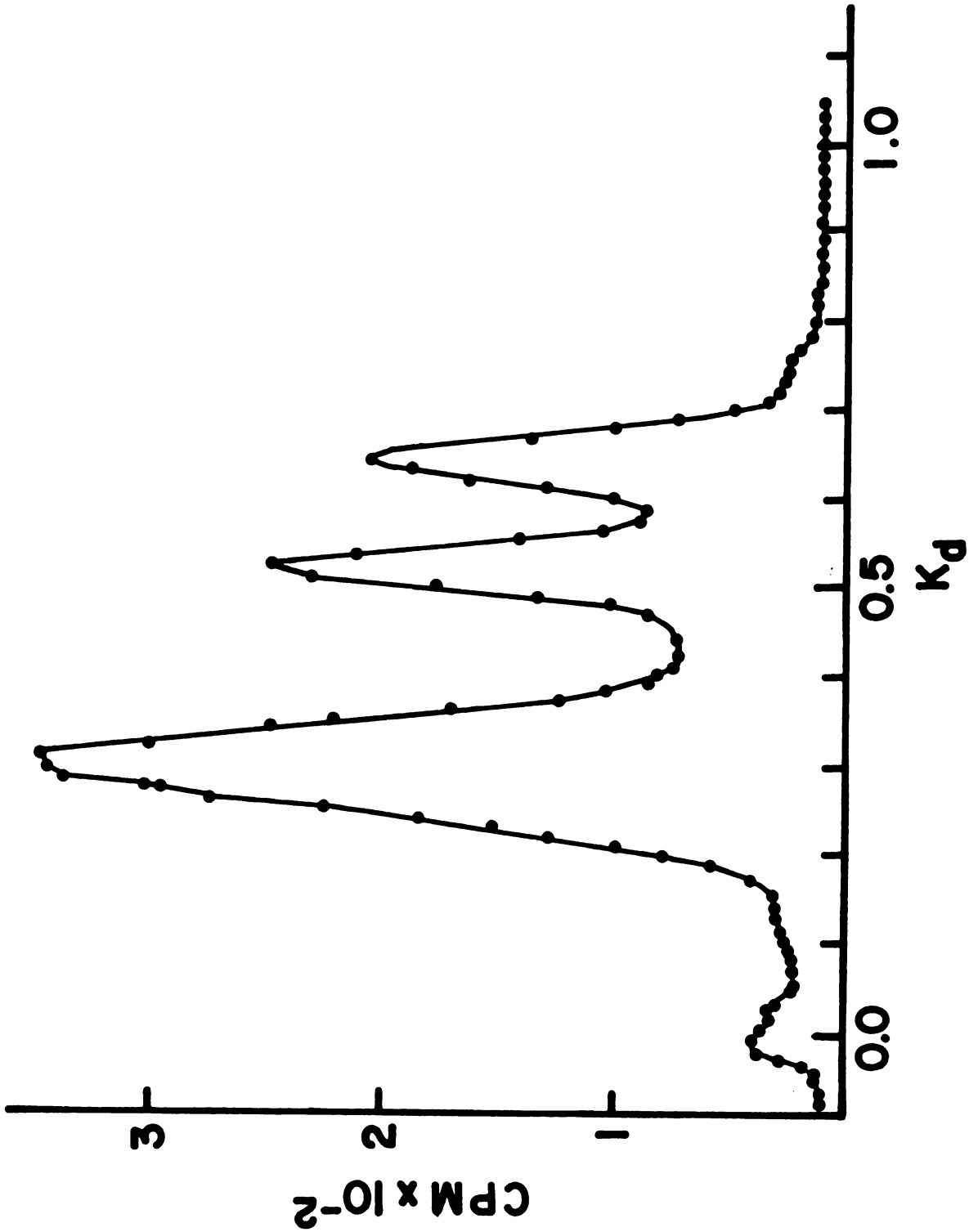



Figure 37

in Table 9. Four peptides, whole β-globin, β-CB-1, β-CB-2, and α-CB-1 all had experimentally determined $K_d$ values which were within 0.01 $K_d$ units of the $K_d$ values determined for these peptides by Protzel (1973). The close agreement of the experimentally determined $K_d$ values was evidence that the linear relationship determined by Protzel and Morris (1974) and Fish et al. (1969) between $K_d^{1/3}$ and (molecular weight)$^{0.555}$ was indeed valid for the chromatographic analyses performed in this thesis.

Table 9. A comparison of the distribution coefficients of α- and β-globin peptide markers obtained by Protzel (1973) and in Figures 36 and 37.

| | Protzel Figure 11 | Figure 36 | Figure 37 |
|---|---|---|---|
| β-globin (146) | 0.22 | 0.21 | |
| β-CB-2 (91) | 0.33 | 0.33 | |
| β-CB-1 (55) | 0.52 | 0.53 | 0.52 |
| α-CB-2 | 0.30 | | (0.30) |
| α-CB-1 (32) | 0.63 | | 0.64 |

## Isolation of MS2 Coat Protein Derived Peptides

The Bio-Gel A 0.5 m column, used for the analysis of nascent chain accumulation patterns, was calibrated using radioactively labeled MS2 coat protein and peptides derived from MS2 coat protein by treatment of a radioactively labeled, coat protein fraction with cyanogen bromide. The calibration with coat protein peptides was performed for the purpose of determining the molecular weights of MS2 coat protein

nascent peptides. The possible peptide fragments which may be derived from cyanogen bromide treatment of MS2 coat protein are outlined in Figure 38. The fraction, which did not bind to DEAE-cellulose during the purification of $^{3}$H-leucine nascent peptide chains from MS2 infected E. coli, was used as a source of radioactively labeled MS2 coat protein since MS2 coat protein had been shown to be the major (> 80%) radioactively labeled protein product of the E. coli incubations. Analysis of the MS2 coat protein sample as described above by Bio-Gel A 0.5 m chromatography under denaturing conditions demonstrated a single peak of radioactivity with a $K_d$ of 0.25 (Figure 39).

The MS2 coat protein sample was used for preparation of radioactively labeled peptides by cyanogen bromide treatment since MS2 coat protein was the only protein which was radioactively labeled as shown above. The products of cyanogen bromide treated MS2 coat protein were analyzed by Bio-Gel A 0.5 m chromatography. The elution profile obtained from chromatographic analysis of a preparation of cyanogen bromide treated coat protein is shown in Figure 40. Three peaks of radioactivity were seen. All were of a $K_d$ value higher than than that of unreacted coat protein (smaller molecular weights). All the coat protein in this sample had, therefore, reacted with cyanogen bromide in at least one of the two methionines. The peak at $K_d$ = 0.557 clearly corresponded to a peptide 41 amino acids long. The accumulations at $K_d$ = 0.716 were from peptides 20 and 21 amino acids long. The peak of radioactivity at $K_d$ = 0.345 was assigned a length of 88 amino acids for two reasons: 1) the size of a peptide 88 amino acids long corresponded to the molecular weight calculated for this $K_d$ value from the data of Protzel and Morris (1974) using α- and β-globin

Figure 38. The possible peptides derived from MS2 coat protein by cyanogen bromide treatment.

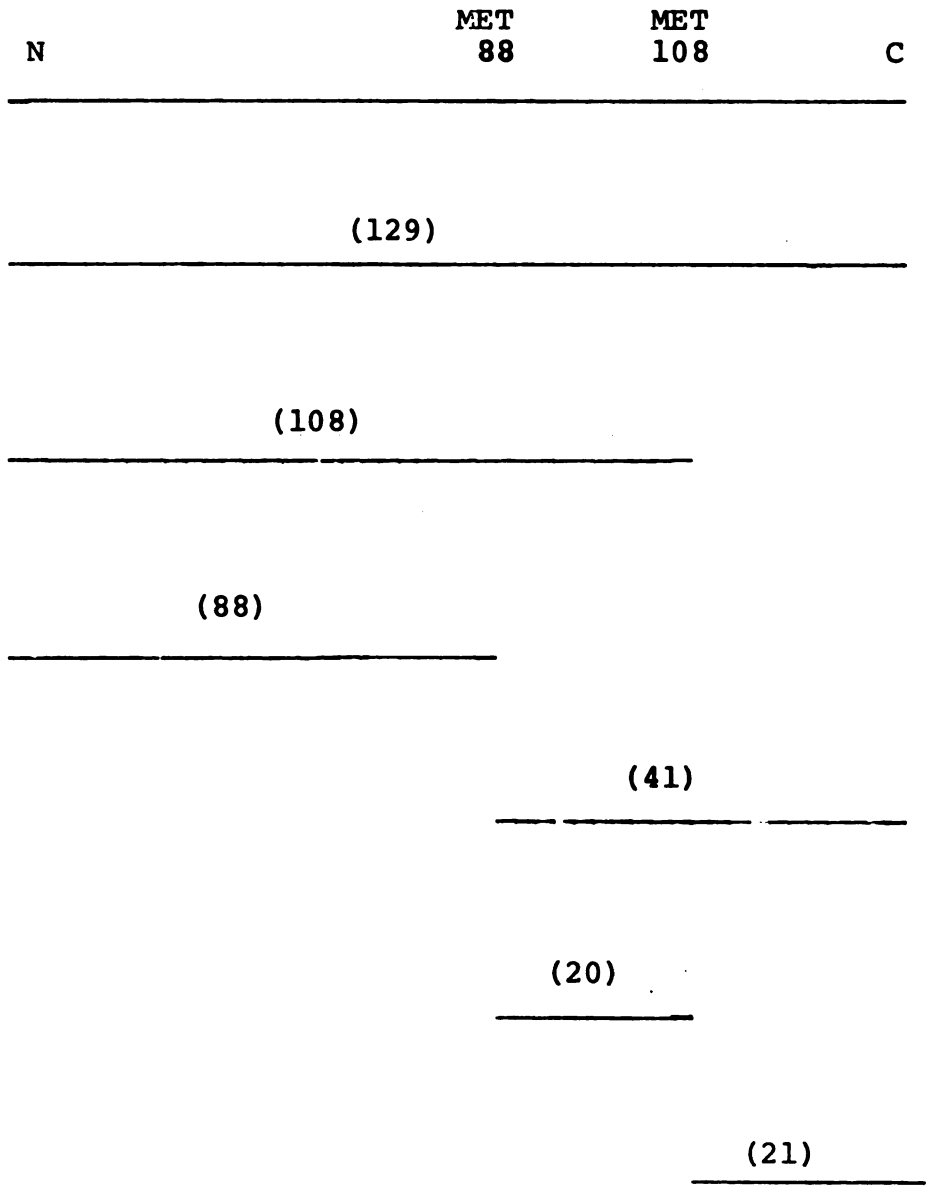



Figure 38

Figure 39. Elution profile of MS2 coat protein.

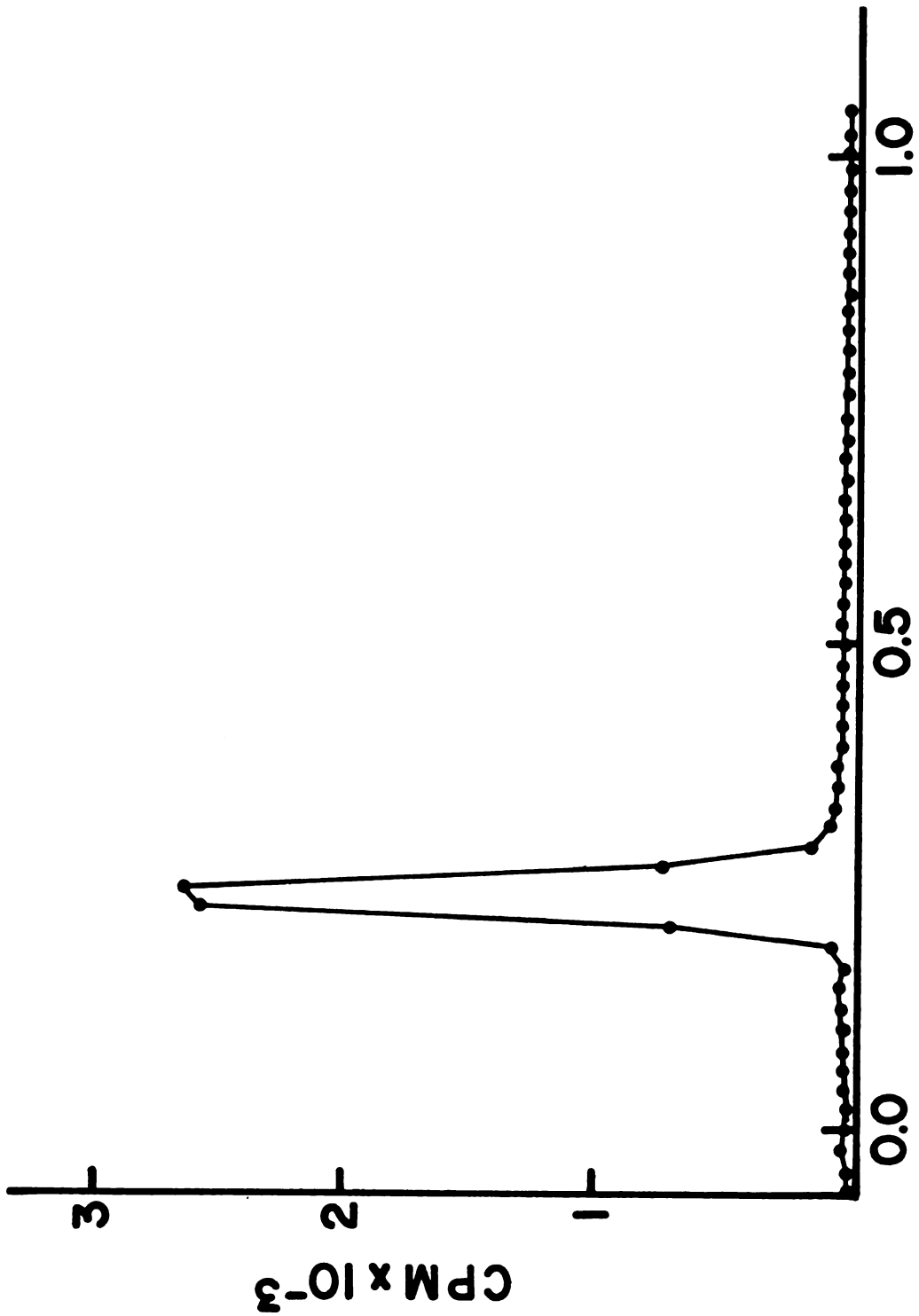



Figure 39

Figure 40. Chromatographic analysis of the peptides derived by treatment of MS2 coat protein with cyanogen bromide.

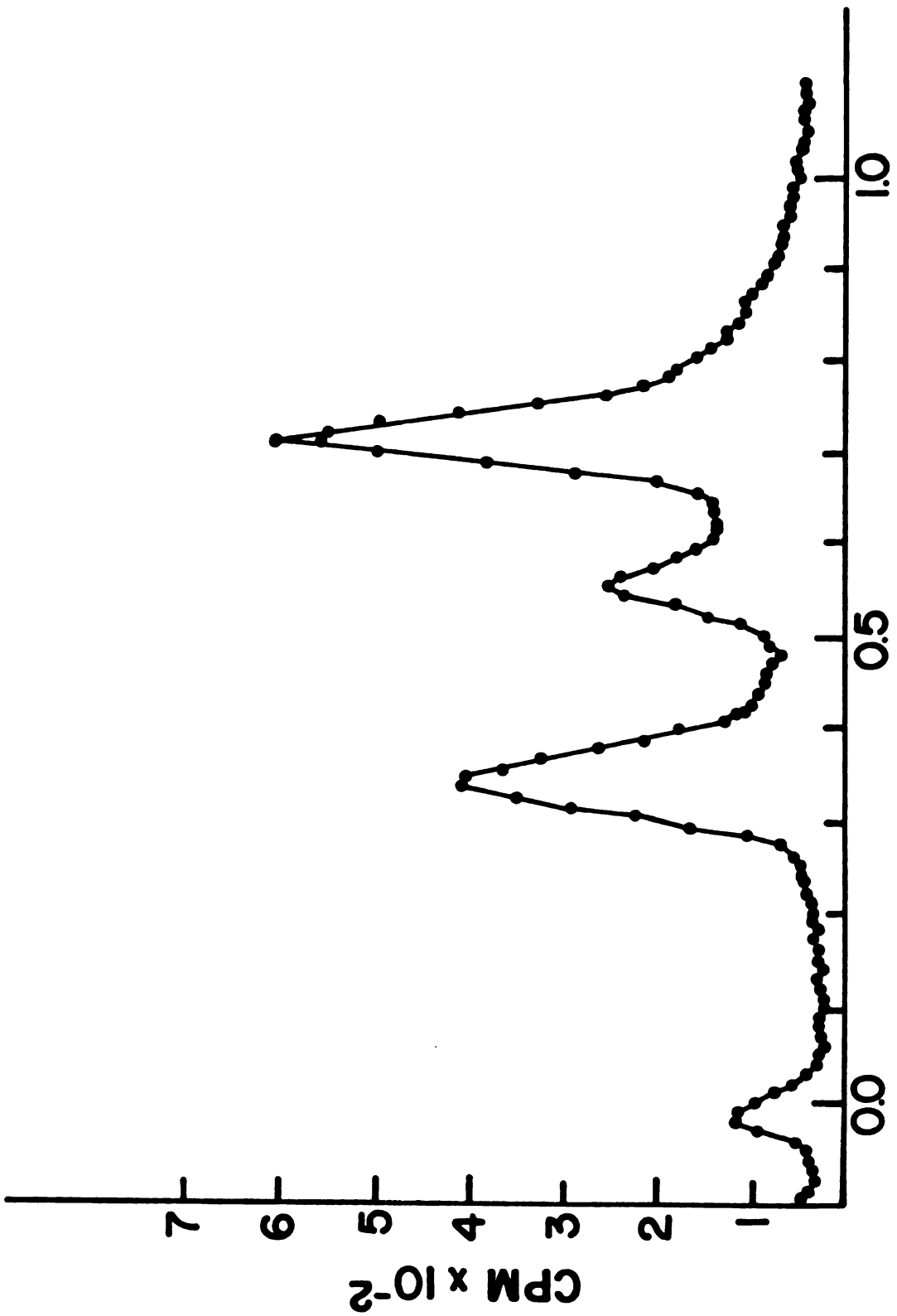



Figure 40

peptide standards, and 2) in order to generate peptides of 41 amino acids long ($K_d$ = 0.557), the MS2 coat protein must be cleaved by cyanogen bromide only at amino acid position 88 and not 108. Because a single peak appeared at $K_d$ = 0.345, only one of the two peptides (88 or 108 amino acids long) was possible. Since the labeled peptide which corresponded to 41 amino acids in length was present, the accumulation at $K_d$ = 0.345 was concluded to be of peptides 88 amino acids long.

Calibration of the Bio-Gel a Column with MS2 Coat Protein Derived Peptide Markers

Peptide markers were prepared by cyanogen bromide treatment of $^3$H-leucine labeled MS2 coat protein as described in Methods. Labeled MS2 coat protein (not treated with cyanogen bromide) was added to the cyanogen bromide treated MS2 coat protein preparation and the peptides subjected to Bio-Gel A 0.5 m column chromatographic analysis after being dissolved in 0.4 ml of guanidine solution. Two separate analyses were performed and are shown in Figure 41 and 42. The $K_d$ values were determined for each peak in Figures 41 and 42. The molecular weights were calculated from the amino acid composition of the peptides. The values obtained by such calculations are shown in Table 10.

The values expressed in Table 10 were used to calculate the linear relationship between $K_d^{1/3}$ and $(MW)^{0.555}$ for the MS2 derived peptides using a Hewlett-Packard HP-65 calculator and the program for the solution of the linear regression equation. The equation for the line calculated in such a manner was:

$$(MW)^{0.555} = 495.07 - (474.21)K_d^{1/3}$$

Figure 41. Chromatographic analysis of MS2 coat protein and cyanogen bromide derived peptides.

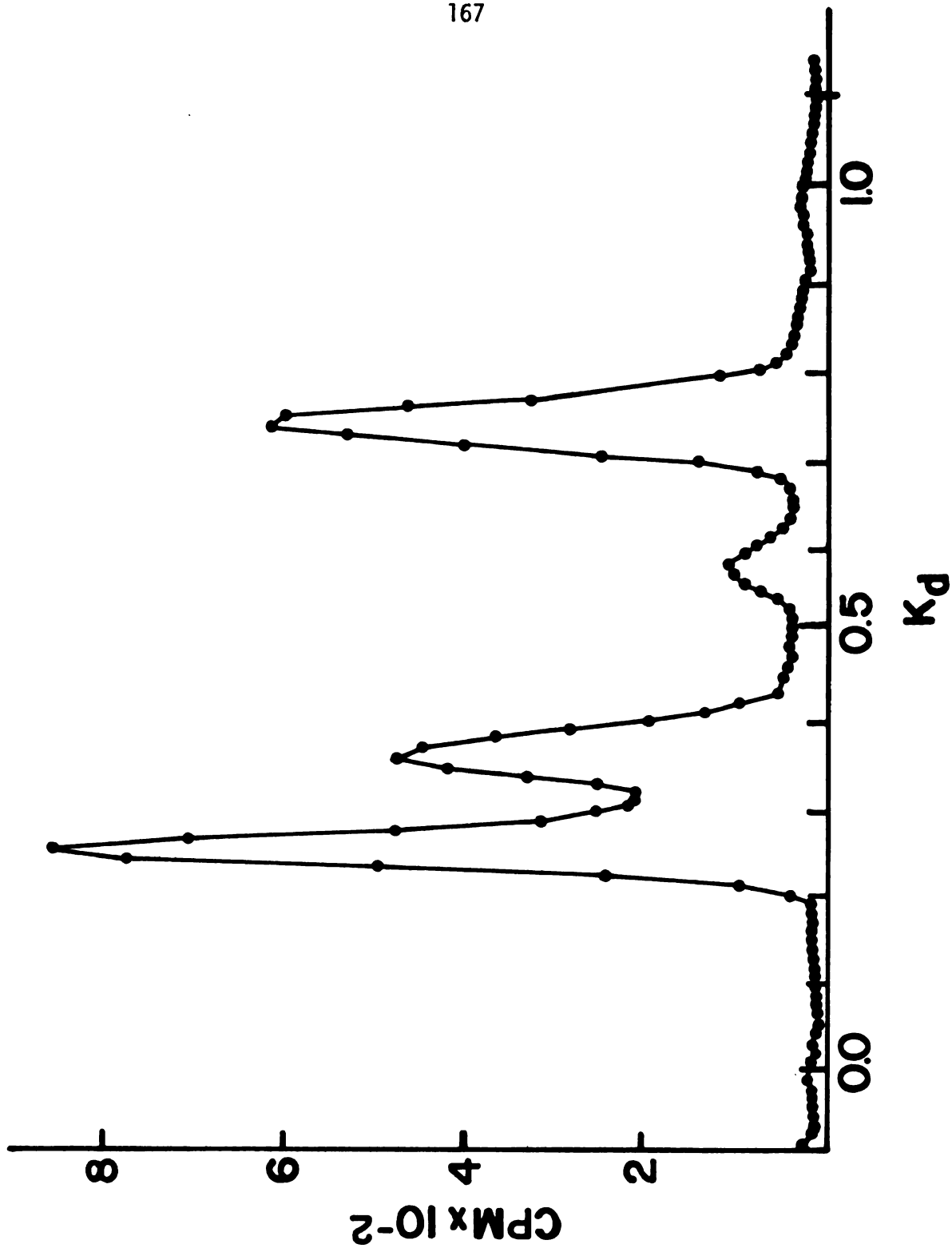



Figure 41

Figure 42. Bio-Gel chromatography of MS2 coat protein and coat protein cyanogen bromide derived peptide markers.

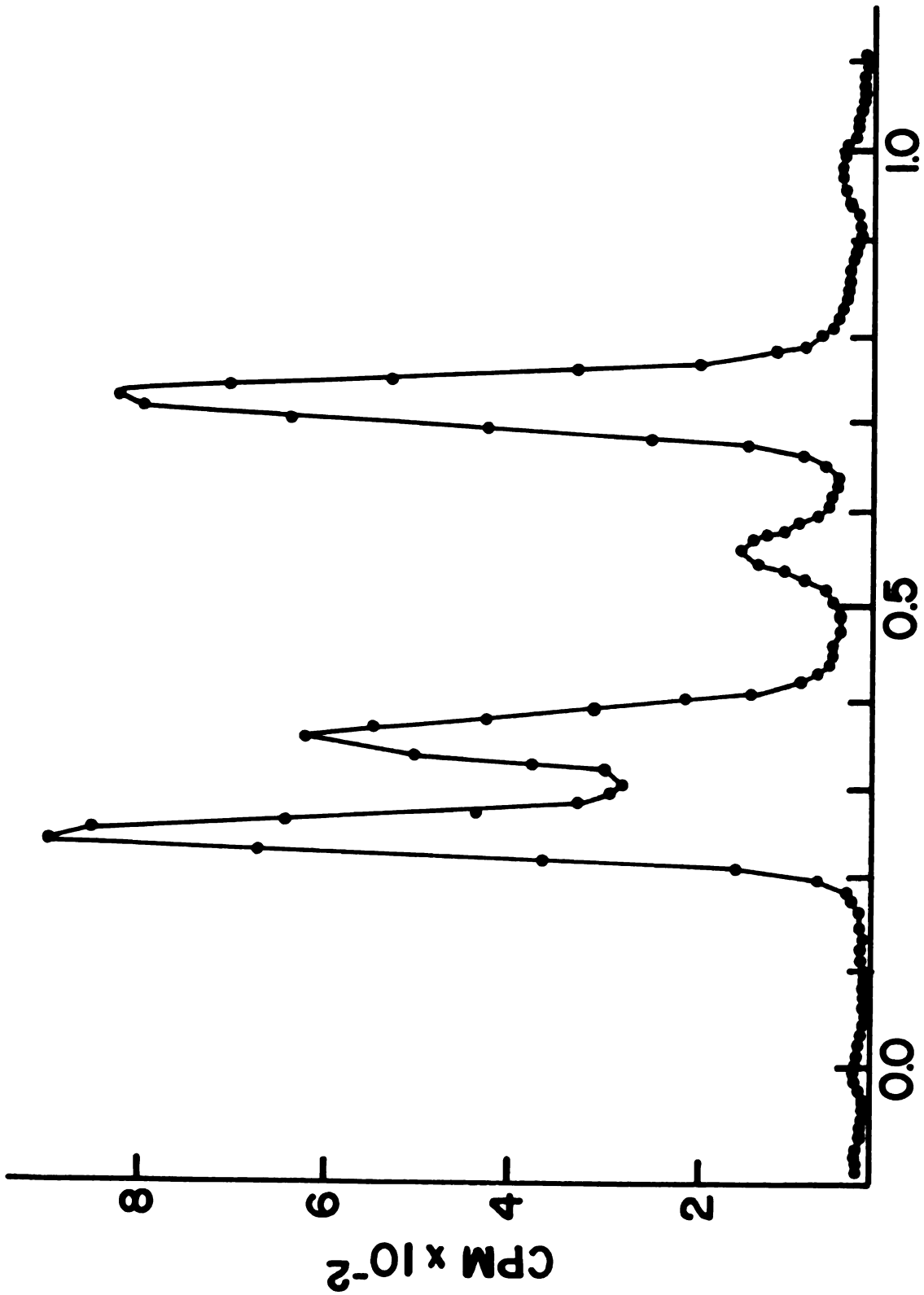



Figure 42

Table 10. Data derived from Figure 41 and 42 which have been used to calibrate the Bio-Gel A 0.5 column for MS2 coat protein derived peptides.

| $K_d$ | | $(K_d)^{1/3}$ | | Molecular Weight | $(MW)^{0.555}$ |
|---|---|---|---|---|---|
| Figure 41 | Figure 42 | Figure 41 | Figure 42 | | |
| 0.249 | 0.247 | 0.629 | 0.627 | 13,713 | 197.75 |
| 0.352 | 0.351 | 0.706 | 0.705 | 9,415 | 160.50 |
| 0.565 | 0.562 | 0.820 | 0.825 | 4,267 | 103.45 |
| 0.730 | 0.722 | 0.900 | 0.897 | 2,118 | 70.13 |

The equation on page 165 is expressed graphically in Figure 43. The line was a "good fit" for the data ($r^2$ = 0.9936).

The molecular weight ranges of the MS2 coat protein mascent chain size accumulations was calculated from the $K_d$ values and standard deviations expressed in Table 10 for MS2 coat protein nascent chain size accumulations using this linear relationship. The molecular weight ranges in Table 11, therefore, represented the sizes of the nascent chain accumulations in the Bio-Gel elution profiles of MS2 coat protein nascent chains and will be used in the Discussion section in a comparison of the regions of nascent MS2 coat protein size accumulations and the proposed structure of the MS2 genone.

Figure 43. A graph of the relationship between $K_d^{1/3}$ and $(MW)^{0.555}$ for MS2 derived peptides.

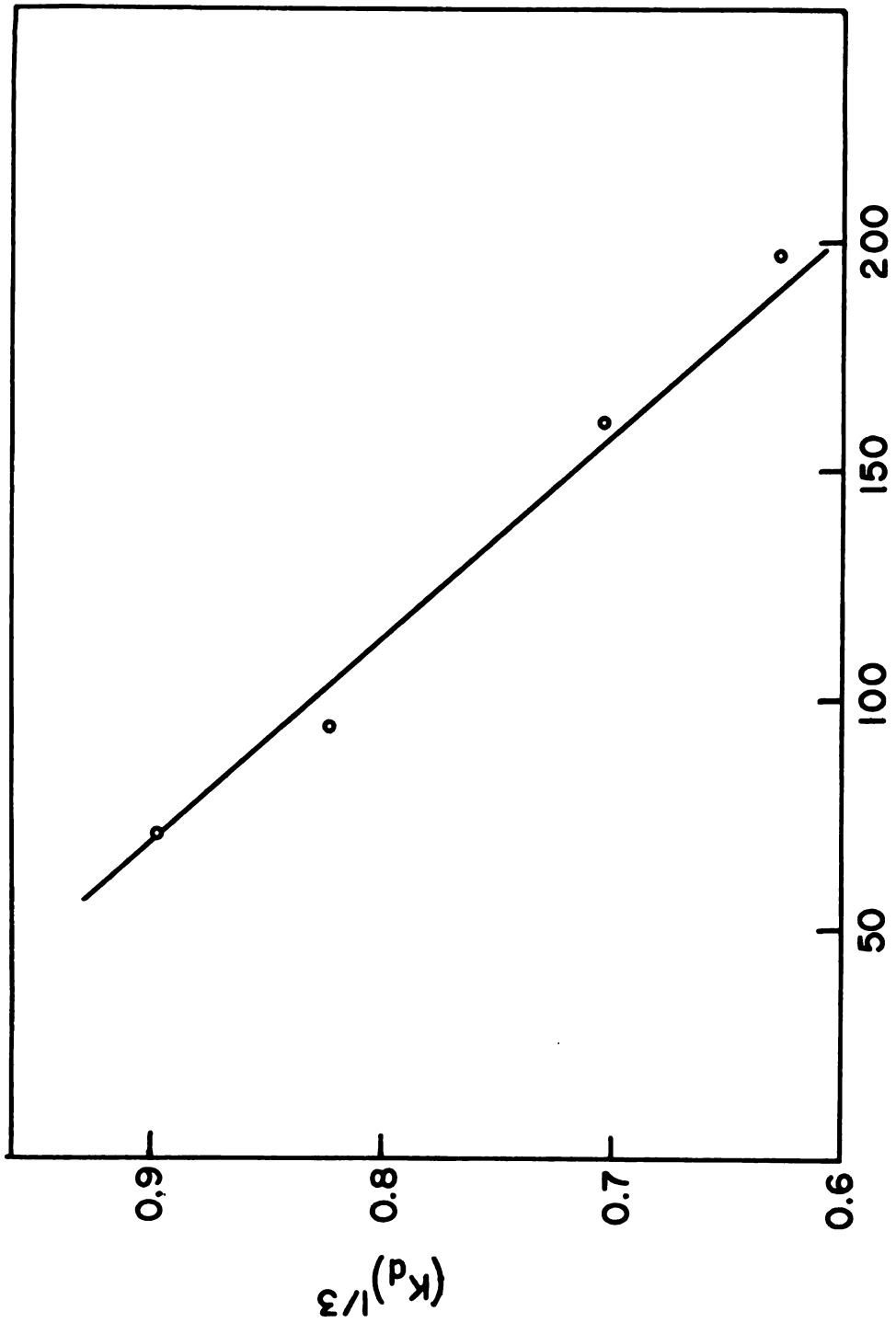



Figure 43

Table 11. Molecular weights calculated for MS2 coat protein nascent chain accumulations of Table 9 using the relationship expressed in Figure 43.

| Peak Number | $K_d$[a] + S.D. | Molecular Weight[c] |
|---|---|---|
| I | 0.80 ± 0.01[b] | 1,280-1,444 |
| II | 0.72 ± 0.01 | 2,005-2,223 |
| III | 0.63 ± 0.005 | 3,155-3,296 |
| IV | 0.52 ± 0.01 | 4,869-5,261 |
| V | 0.39 ± 0.01 | 7,907-8,495 |
| VI | 0.33 ± 0.005 | 9,973-10,337 |
| VII | 0.25 ± 0.005 | 13,293-13,789 |

[a]Data presented here summarize the results of nine analyses of the size distribution of nascent coat protein peptides. The distribution coefficient ($K_d$) of each maximum in the Bio-Gel A 0.5 m elution profile is indicated (see, for example, Figure 5).

[b]Detected as a minor peak in five of the nine experiments.

[c]The range of molecular weights indicated by the standard deviation associated with each $K_d$ value was calculated using the values obtained from a Bio-Gel A 0.5 m colume standardized with MS2 coat protein and the peptides derived from MS2 coat protein by cyanogen bromide cleavage (12).

# DISCUSSION

The size distribution of nascent α- and β-globin peptides in whole cell incubations of rabbit reticulocytes has been shown to be nonuniform (Protzel and Morris, 1974). Accumulations of discrete sizes were observed upon gel chromatographic fractionation of uniformly labeled nascent chains under denaturing conditions. The presence of size accumulations in the α- and β-globin nascent chain population isolated from rabbit reticulocytes implies that the rate of peptide chain elongation along the globin mRNA is not uniform. Some regions of the mRNA sequence are concluded to have a slower rate of peptide chain elongation than others. Since each ribosome involved in translation has one nascent peptide chain attached to it, the accumulation of nascent chains of any length corresponds to the accumulation of ribosomes at a specific region along the mRNA being translated. The accumulation of ribosomes at specific regions along the mRNA would be, therefore, the result of the lowered rate of ribosome movement along the mRNA in these regions (relative to other regions of that same mRNA). The results of Protzel and Morris (1974) demonstrated, therefore, that the rate of peptide chain elongation in whole cell incubations of rabbit α- and β-globin is not uniform. There are regions of the mRNA population which are translated relatively slowly compared to other regions of the mRNA. However, the origin of the difference in the relative rate of chain elogation was not investigated. The

purpose of the experiments described in this thesis was to investigate the origin of the nonuniform size distribution of rabbit globin nascent chains.

Manipulation of the whole cell incubations in an attempt to analyze the origin of the nascent chain size accumulations would be quite difficult. The presence of the cellular plasma membrane prevents the easy addition of agents to the incubation mixture in attempts to affect the nascent chain size accumulations, as analyzed by elution of the nascent chain population from Bio-Gel A 0.5 m under denaturing conditions. Therefore, it was decided to isolate nascent chains from a cell-free protein synthesizing incubation mixture. Utilization of a cell-free protein synthesizing system has enabled experiments to be performed in which a component of the cell-free system involved in the process of nascent chain elongation was purified and then added to a cell-free incubation mixture in an attempt to determine if a stoichiometrically limiting amount of that component is the cause of the lowered relative chain elongation rates observed by gel chromatographic analysis of the nascent chain population for size accumulations.

The rabbit reticulocyte lysate cell-free protein synthesizing system was chosen for these experiments for several reasons. First, synthesis of the same proteins studied by Protzel and Morris (1974), rabbit $\alpha$- and $\beta$-globin, takes place in the reticulocyte lysate cell-free system. Second, since protein synthesis is programmed by endogenous mRNA, it is not necessary to purify globin mRNA and add it to the incubation mixture. Finally, the lysate system does not require fractionation of the reticulocytes beyond lysis and removal of the cell membranes in its preparation. When compared to whole cell

incubation mixtures, the reticulocyte lysate is "more physiological" than more highly fractionated cell-free systems.

After characterization of the reticulocyte lysate incubation mixture and determination of the incubation conditions which would yield maximum amounts of protein synthesis, a series of experiments was designed to determine if a limiting component of this system could be demonstrated as the origin of nascent globin peptide size accumulations. Preliminary experiments had determined that the nascent chain population from the lysate also demonstrated a nonuniform size distribution upon Bio-Gel A 0.5 m chromatographic analysis under denaturing conditions. Accumulations have been observed in nascent chain populations purified from rabbit reticulocyte incubation mixtures of the same sizes as described for the whole cell incubations of Protzel and Morris (1974).

The first component of the reticulocyte lysate cell-free protein synthesizing system to be investigated was tRNA. Transfer RNA was chosen initially because the interaction between tRNA isoacceptor species and mRNA occurs at specific regions of the mRNA determined by the codons along the mRNA nucleotide sequence. It is possible to envision a tRNA species being present at such a low concentration that the ribosome involved in translation of the mRNA would have to pause when the ribosome reached the codon which interacted with that scarce tRNA. A slow step, if it existed, could cause an accumulation of nascent chains of the size corresponding to the codon directly prior to that involved with the tRNA in a low concentration.

Reticulocyte tRNA was prepared by the method of Holley et al. (1961) from the frozen reticulocyte lysate as described in Methods.

Lysate incubation mixtures were prepared which had received the addition of the purified tRNA. As described in the Results section, raising the tRNA concentration of the reticulocyte lysate incubation mixture to as much as five times the original concentration did not alter the elution profile obtained upon chromatographic analysis of the size distribution of the nascent chain population under denaturing conditions. The identical result was obtained in experiments utilizing different lysate incubation mixtures and different tRNA preparations. No alterations in the nascent chain accumulation pattern were observed.

An experiment was also performed to examine the possibility that there did exist a limiting tRNA in reticulocytes at such low concentrations that even an increase to five times its original concentration would not be sufficient to reduce the size accumulations observed in the Bio-Gel elution profile of the rabbit α- and β-globin nascent chain population. Transfer RNA was purified from rabbit liver by the method of Bose et al. (1971) and added to the reticulocyte lysate cell-free protein synthesis system. Nascent chains were radioactively labeled, purified and analyzed by Bio-Gel A 0.5 m chromatography. Addition of rabbit liver tRNA to the lysate incubation mixture gave the same result as the addition of reticulocyte tRNA. The gel elution profile was not altered. The size accumulations were still present in the radioactively labeled nascent chain population.

Experiments involving tRNA from two different sources, the rabbit reticulocyte and rabbit liver, have therefore failed to alter the nascent globin chain size accumulations in the reticulocyte lysate incubation mixtures when the tRNA concentrations were brought to as much as five times the original level. The conclusion drawn from the

experiments involving the addition of tRNA preparations to the incubation mixtures is that the nascent globin chain size accumulations are not due to a limitation of one or more tRNA isoacceptor species in the rabbit reticulocyte.

After limiting amounts of tRNA had been eliminated as the possible origin of the globin nascent chain accumulations, an attempt was made to determine if other components of the reticulocyte lysate incubation were responsible for the size accumulations. Experiments were performed in an attempt to extract such a component from the reticulocyte lysate. The concentration of this component could then be increased to excess to a reticulocyte lysate incubation mixture.

Two procedures were used in the attempt to purify such a component. The first involved the preparation of a 0.5 M KCl wash of ribosomes isolated from the reticulocyte lysate by centrifugation. The 0.5 M KCl salt wash preparation was added to a lysate incubation mixture in an analogous fashion to the experiments involving reticulocyte tRNA. Adjusting the concentration of the reticulocyte lysate incubation mixture to three times the endogenous level of these factors failed to alter the nascent chain accumulation pattern. The salt wash preparation (a crude fraction containing many different proteins eluted from the ribosomes) did not contain a factor which was capable of altering the presence of nascent globin peptide size accumulations in the rabbit reticulocyte lysate incubation mixtures.

A second experiment was performed in which the proteins precipitated from a 70% $(NH_4)_2SO_4$ reticulocyte lysate postribosomal supernatant were added to a lysate incubation mixture in excess of their normal concentration. A lysate incubation mixture was adjusted to

three times the original concentration of the $(NH_4)_2SO_4$ precipitated factors. Chromatographic analysis of the nascent chain population purified from this incubation showed, as described in Results, that no alteration of the Bio-Gel elution profile was observed. The material precipitated from a rabbit reticulocyte postribosomal supernatant by 70% $(NH_4)_2SO_4$ did not contain a component which was capable of altering the presence of the nascent chain size accumulations in the rabbit reticulocyte lysate cell-free synthesizing system.

The attempts to identify a component of the reticulocyte lysate cell-free protein synthesizing incubation mixtures which is responsible for the nascent chain accumulations by being present in limiting amounts are all subject to the same reservation. That is, if such a factor does indeed exist, it may not have been purified during the procedures used in these experiments. Alternatively, the limiting factor could also have been inactivated by these purification procedures. If such a factor is either not purified or is inactivated, addition of the preparations of tRNA, ribosome salt wash factors, or postribosomal supernatant factors would not alter the concentration of the limiting component in the lysate incubation mixtures and would not be expected to affect the nascent chain size accumulations observed in the gel elution profiles. Due to the reservations described above with respect to the attempt to purify components of the reticulocyte lysate, an entirely separate and independent approach to the question has been devised. Instead of adding components of the lysate chain elongation system back to incubation mixtures, a method has been used which would increase the relative concentrations of all the chain elongation components relative to actively translating ribosomes.

The antibiotic, edeine, has been shown to specifically inhibit protein synthesis at the step of the initiation of mRNA translation (Obrig et al., 1971). Inhibition of protein synthesis by edeine (as measured by a decrease in the rate of incorporation of a radioactive amino acid into TCA precipitable material) to 50% of its original rate would be expected to result in a 50% reduction in the number of active ribosomes involved in mRNA translation since edeine specifically inhibits the initiation and not elongation step of polypeptide synthesis. By the same reasoning, inhibition of the protein synthetic rate to 25% of the original value would result in a 75% reduction in the number of ribosomes actively translating the mRNA population.

Reducing the amount of ribosomes involved in chain elongation by this method would have the effect of increasing the concentration of all the soluble components of the chain elongation system in relation to the active ribosomes. Although the absolute concentration of the components of peptide chain elongation in the lysate incubation mixtures would not be altered, the ratio between the elongation components and the ribosomes involved in chain elongation would be increased to four times its original value in incubations which have protein synthesis inhibited to 25% of its uninhibited rate by edeine. The experiment described above achieves the same effect as the experiments involving purification of lysate components and adding them back to lysate incubation mixtures. The concentration of a hypothetical factor of the chain elongation machinery which is postulated to be the origin of the nascent chain accumulations by being present in limiting amounts would be increased four times relative to the active ribosome concentration. The increase in relative concentration is accomplished

without purifying any components from the reticulocyte lysate. The possibility that a limiting component of the translation machinery has not been purified or that it has been inactivated is therefore eliminated.

Edeine was added to a reticuloycte lysate incubation mixture at a concentration which had been determined to reduce the protein biosynthetic rate to 25% of its initial uninhibited value. Nascent globin chains were labeled and purified as described in Methods. The results of Bio-Gel A 0.5 m chromatographic analysis under denaturing conditions of the nascent α- and β-globin peptides indicated that there was no effect upon the nascent chain size accumulations by inhibiting protein biosynthesis to 25% of its original value by edeine.

Two entirely separate approaches have, therefore, failed to demonstrate that the stoichiometrically limiting quantity of any component of the reticulocyte lysate cell-free protein synthesis system is the origin of the nascent chain size accumulations observed upon Bio-Gel chromatographic analysis of purified nascent chains under denaturing conditions. The conclusion has been reached that nascent chain size accumulations are associated with some other aspect of protein biosynthesis than limiting components of the protein biosynthetic machinery.

Since the origin of the nascent chain size accumulations did not seem to lie in the concentrations of the components of the protein biosynthetic machinery in the reticulocyte lysate, it was decided to determine if the presence of size accumulations in the nascent chain population was associated with the reticulocyte lysate incubation mixture itself, or was present in globin nascent chains isolated from another system in which globin mRNA was serving as the template for

protein biosynthesis. The effects of the translation system (the reticulocyte lysate) could, therefore, be separated from the effects of the template (the globin mRNA).

The protein biosynthetic system chosen was the wheat embryo-derived cell-free protein biosynthetic system (Marcus et al., 1975). The wheat embryo cell-free system had been shown previously to synthesize rabbit α- and β-globin from purified mRNA and had a low endogenous incorporation rate (Efron and Marcus, 1973). Furthermore, the relatively large phylogenetic distance between rabbits and wheat helps to reduce the possibility that a unique property of the reticulocyte lysate protein biosynthetic machinery would also be present in the wheat embryo cell-free system with respect to the origin of the observed nascent chain accumulations. A prokaryotic system was not chosen because eukaryotic mRNA will only be translated at a low rate (if at all) in prokaryotic cell-free systems (Davies and Kaesberg, 1973).

Globin mRNA was purified from rabbit reticulocytes as described in Methods using the oligo(dT)-cellulose chromatography procedure of Aviv and Leder (1972), and added to the wheat embryo-derived cell-free system as described in Results. Nascent chains were purified as described in Methods and analyzed by Bio-Gel A 0.5 m chromatography. The elution profile of nascent chains isolated from rabbit globin mRNA programmed protein biosynthesis in the wheat embryo-derived cell-free system is nearly identical to the profile obtained by chromatographic analysis of nascent chains isolated from the reticulocyte lysate cell-free system.

The globin mRNA purification procedure involves the treatment of polysomes with 1% SDS and phenol. Proteins bound to the nucleic acids

will be denatured and removed by this treatment. The oligo(dT)-cellulose chromatography will separate the globin mRNA from other types of nucleic acids since rRNA and tRNA do not contain poly(A) regions in their primary sequence and will not bind to oligo(dT)-cellulose. Only rabbit globin mRNA is the common factor present in both the reticulocyte lysate and wheat embryo cell-free incubation mixtures. (This statement, of course, regards reticulocyte and wheat embryo ribosomes, elongation factors, tRNA, etc. as being "different".) The conclusion drawn from this experiment is that the origin of the nascent α- and β-globin chain size accumulations lies in the globin mRNA itself and not in the protein biosynthetic system in which it is being translated.

Three separate approaches have been taken in the experiments described above: 1) it has not been possible to isolate a fraction from the rabbit reticulocyte lysate which is capable of affecting the nascent chain size distribution when added back to a lysate incubation mixture; 2) inhibition of the rate of initiation of protein biosynthesis in the reticulocyte lysate to 25% of its uninhibited rate (which will increase the concentration of protein synthesis elongation components by four times in relation to the number of actively translating ribosomes) does not cause an alteration in the elution profiles observed upon gel chromatographic analysis of nascent chains purified from the partially inhibited reticulocyte lysate incubation mixtures; 3) accumulations of identical sizes are observed in the nascent chain populations isolated from the globin mRNA-directed wheat embryo-derived cell-free protein synthesis system in which the rabbit globin mRNA is the only component present that has been purified from the rabbit reticulocyte. The three types of experiments all lead to one

conclusion concerning the origin of the globin nascent chain size distributions: the origin of the nascent chain size accumulations must lie in some aspect of the globin mRNA structure and not in the translation system being used or the availability of the components needed for peptide chain elongation.

As discussed in the Introduction, all RNA species which have been studied in both eukaryotes and prokaryotes contain regions of intramolecular base pairing. Regions of secondary structure have been deduced to exist from physical, enzymatic, and sequence studies. Since globin mRNA had been shown to contain such regions by a variety of physical and enzymatic methods, it was felt that the origin of the size distributions observed in the chromatographic analysis of the rabbit α- and β-globin nascent chain population might be related to the presence of such structures. Such regions of secondary structure must be removed before a ribosome involved in translation of a mRNA could traverse a region involved in the base pairs. A ribosome might be expected to slow its rate of movement when the ribosome reached a base paired region during protein biosynthesis. The slowed movement would be the result of the ribosome either pushing through the double stranded region or waiting until the region became single stranded through a normal "breathing" of the molecule.

In order to examine the hypothesis that mRNA secondary structure was the origin of the nascent chain size accumulations, it was necessary to be able to purify nascent peptide chains from the translation of a single mRNA. The nucleotide sequence of the mRNA being translated must have been determined and a structure for this RNA sequence proposed. Chromatographic analysis of the nascent chains upon a

Bio-Gel A 0.5 m column under denaturing conditions would enable a comparison of the peptide lengths of any nascent chain size accumulations observed in the elution profile and the proposed structure. If the hypothesis that the nascent globin peptide size accumulations are associated with regions of secondary structure is correct, the regions of RNA secondary structure should be associated with the positions on the mRNA at which the rate of ribosome movement is relatively slow (as analyzed by the accumulation of nascent chains of discrete sizes).

Only one mRNA met the requirements outlined above at the time the experiments described in this thesis were performed. The mRNA meeting the above requirements was the mRNA for bacteriophage MS2 coat protein, which is also the MS2 bacteriophage genome. It was possible, as shown in Results, to obtain 90% MS2 coat protein biosynthesis after infection of E. coli with MS2. The primary structure of the MS2 coat protein cistron (and the entire MS2 genome) had been determined and a structure proposed for the MS2 coat protein RNA nucleotide sequence involving extensive regions of intramolecular base pairs (Min Jou et al., 1972; Vandenberghe et al., 1975; Fiers et al., 1975, 1976). The proposed structure of the MS2 coat protein RNA (shown in Figure 1) involved the formation of a number of hairpin turns which could be expected to reform after translation of that region by a ribosome. Therefore, it was decided to determine the Bio-Gel elution profile for MS2 coat protein nascent chains purified from MS2 infected E. coli which were synthesizing essentially only MS2 coat protein. Bacteriophage MS2 coat protein, 129 amino acids long, was of a small enough size that analysis of the nascent chain

elution profile could be performed upon the same Bio-Gel A 0.5 m chromatography column used for nascent globin peptide size distribution analysis.

Previous reports have demonstrated that treatment of bacteriophage MS2 or f2 infected E. coli with either actinomycin D or rifampicin would result in cessation of host protein synthesis, but not affect bacteriophage RNA coded protein synthesis (Vinuela et al., 1967; Fromageot and Zinder, 1968). The bacteriophage RNA synthesis is not affected by rifampicin because its replication is catalyzed by a rifampicin-resistant RNA-dependent RNA polymerase distinct from the DNA dependent RNA polymerase used for E. coli RNA synthesis (Hartmann et al., 1967).

Using incubation conditions similar to those described by Fromageot and Zinder (1968), the synthesis of bacteriophage MS2 proteins was studied as described in Results. Protein synthesis in rifampicin treated E. coli was found to be increased to approximately twelve times the uninfected rate. Protein synthesis in rifampicin treated E. coli was dependent upon infection by bacteriophage MS2. The rate of incorporation of $^{3}$H-leucine in MS2 infected, rifampicin treated E. coli was linear from 20 to 65 minutes post-infection.

MS2 infected, rifampicin treated E. coli were labeled with $^{3}$H-leucine for a 15 minutes period starting 45 minutes after infection. The protein was isolated from the cells and analyzed by SDS-polyacrylamide gel electrophoresis. The products of protein biosynthesis were seen to be greater than 80% MS2 coat protein. Therefore, the nascent chain population present in MS2 infected, rifampicin treated E. coli

at 45 to 60 minutes after infection contained greater than 90% MS2 coat protein nascent chains.

Purification of nascent MS2 coat protein chains from infected E. coli was described in Methods. Analysis of the size distribution of the nascent chain population by Bio-Gel A 0.5 m chromatography under denaturing conditions demonstrated a nonuniform gel elution profile. Nascent chain accumulations of identical sizes were evident in profiles of nascent chains labeled with both $^{3}$H-leucine and $^{3}$H-aspartic acid, indicating that the size accumulations are independent of the radioactive label used, as is the case for nascent α- and β-globin nascent chains described earlier in this thesis. Translation of different mRNA yielded unique patterns of nascent chain size accumulations, as would be predicted if the origins of those accumulations resided in the mRNA itself.

In contrast to the observations of the elution profile obtained by chromatographic analysis of nascent chains purified from MS2 infected, rifampicin treated E. coli, the elution pattern of nascent chains isolated from an E. coli culture which had not been treated with either bacteriophage MS2 or rifampicin showed very few regions of nascent chain accumulations. There were no size accumulations in the region of MS2 nascent chain peptides ($K_d$ greater than 0.25). Those accumulations, which were present in the elution profile of nascent chains purified from uninfected E. coli, were thought to be due to either E. coli proteins made in relatively large amounts or to ribosomal proteins which adsorb to the DEAE-cellulose column under the isolation conditions. Because synthesis of E. coli proteins was inhibited by rifampicin addition, host nascent chains could not have

contributed to the MS2 coat protein nascent chain elution profile.

Since a population of nascent chains obtained from a protein synthesizing system is being analyzed, the lengths of the MS2 coat protein nascent chains which are found to accumulate in the population of nascent MS2 peptides may be estimated from their molecular weight ranges (Table 12) using the amino acid sequence of the MS2 coat protein (Dayhoff, 1972). The population of nascent chains of different sizes will all start at the N-terminal end of the coat protein amino acid sequence. The molecular weights of the possible 129 peptides which could be present in a MS2 coat protein nascent chain population have been determined by summing the molecular weights of the peptide residues starting with the N-terminal alanine. In such calculations the presence of an N-terminal formylmethionine in some of the peptides has not been included, although its presence is noted. The result of converting the molecular weight ranges of Table 11 to amino acid lengths is shown in Table 12.

As described previously, one consequence of the relationship between the $K_d$ and the molecular weight of the peptide in the elution profile is an increasing slope of the elution profile as the $K_d$ value decreases (Protzel and Morris, 1974). The increasing slope of the elution profile arises since more members of the nascent peptide population are being collected per unit of volume at low $K_d$ than at high $K_d$. A maximum in the elution profile will occur near the $K_d$ associated with the molecular weight of the completed protein ($K_d$ = 0.25, i.e., Peak VII in the case of coat protein.

Still another factor which affects the general elution pattern of labeled nascent peptides is the location of the particular amino

Table 12. Amino acid lengths of MS2 coat protein nascent chain accumulations calculated from the data in Table 11.

| Peak Number | $K_d$[a] + S.D. | Molecular Weight[c] | Length of Nascent Peptide[d] |
|---|---|---|---|
| I | 0.80 ± 0.01[b] | 1,280-1,444 | 11-14 |
| II | 0.72 ± 0.01 | 2,005-2,223 | 20-22 |
| III | 0.63 ± 0.005 | 3,155-3,296 | 31-32 |
| IV | 0.52 ± 0.01 | 4,869-5,261 | 47-50 |
| V | 0.39 ± 0.01 | 7,907-8,495 | 75-81 |
| VI | 0.33 ± 0.005 | 9,973-10,337 | 93-96 |
| VII | 0.25 ± 0.005 | 13,293-13,789 | 125-130 |

[a]Data presented here summarize the results of nine analyses of the size distribution of nascent coat protein peptides. The distribution coefficient ($K_d$) of each maximum in the Bio-Gel A 0.5 m elution profile is indicated (see, for example, Figure 5).

[b]Detected as a minor peak in five of the nine experiments.

[c]The range of molecular weights indicated by the standard deviation associated with each $K_d$ value was calculated using the values obtained from a Bio-Gel A 0.5 m column standardized with MS2 coat protein and the peptides derived from MS2 coat protein by cyanogen bromide cleavage (12).

[d]The length of nascent peptides corresponding to the calculated molecular weights was determined using the amino acid sequence of MS2 coat protein and the molecular weight of each amino acid residue involved, beginning with the N-terminal residue of the mature coat protein. An average molecular weight of 110 was used for residue 130 of peak VII.

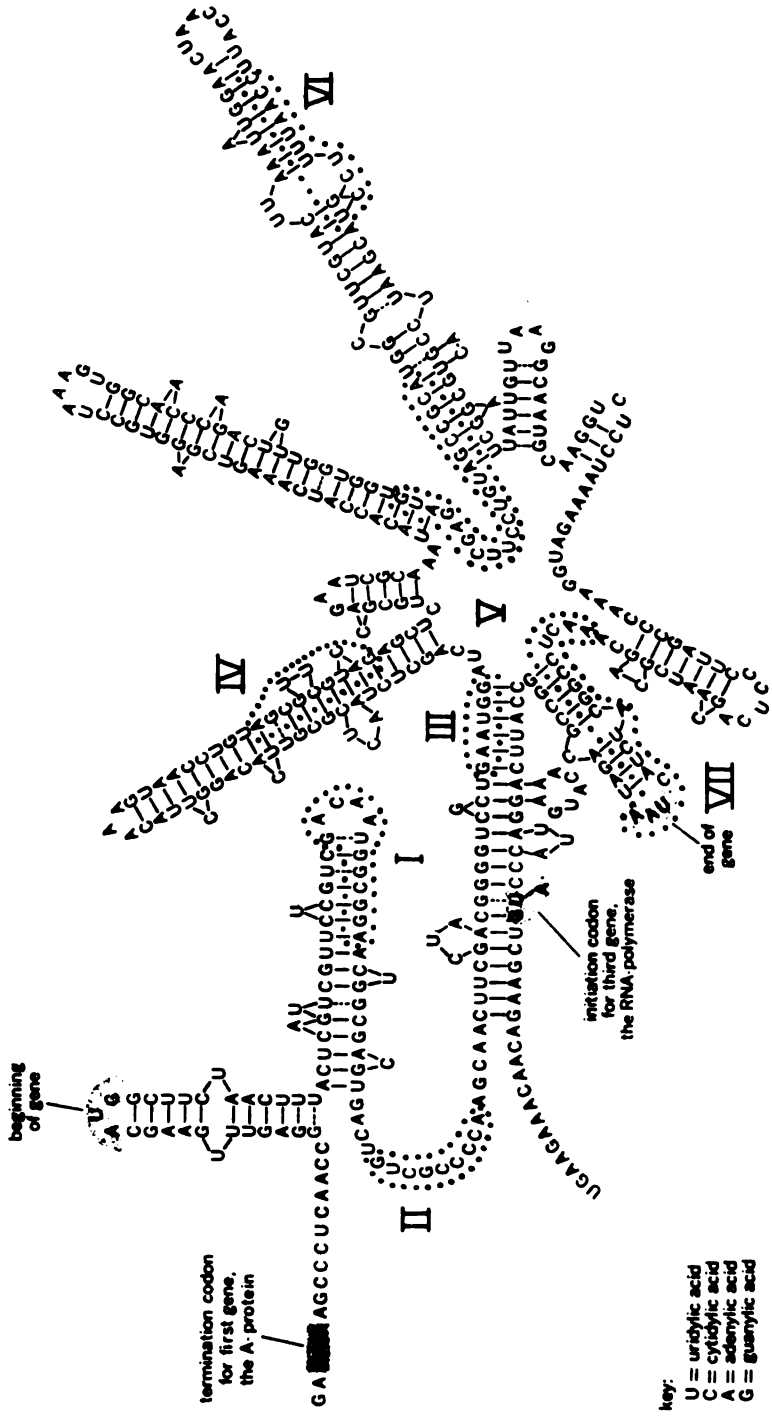
acid used as the source of radioactivity in the amino acid sequence of the nascent peptide. The grouping of seven leucine residues in the region of peptide lengths corresponding to $K_d$ values of 0.391 to 0.282 undoubtedly contributed to the amplitude of the peak seen at $K_d$ = 0.25 in Figure 31.

Nascent MS2 peptides of approximately 11-14, 20-22, 31-32, 47-50, 75-81, and 91-96 amino acid residues in length accumulate in the population of nascent MS2 peptides. The large peak of labeled nascent peptides seen at $K_d$ = 0.25 is thought to be a product of the properties of the Bio-Gel column and the particular labeled amino acid used as described above.

From the proposed "Flower Model" for the MS2 coat protein gene, one may determine the codons which direct the synthesis of peptides of the lengths indicated in Table 12 and also compare those locations along the base sequence of the MS2 coat protein genome with the proposed regions of secondary structure in the MS2 RNA. These data are summarized pictorially in Figure 44.

A consideration of the relative stability of the proposed regions of secondary structure of the "Flower Model" indicates that the two smaller "hairpin" loops between regions VI and VII are probably not stable at the temperature at which the incubations were conducted (Min Jou et al., 1972). However, the small hairpin loop immediately following region IV of Figure 44 would be expected to be stable if the structure were written in a slightly different manner. The stable arrangement would place four base pairs in the stem and six nucleotides in an unbounded loop. This arrangement has a calculated free energy value of -6.4 kcal per mole at 25° using the stability estimates

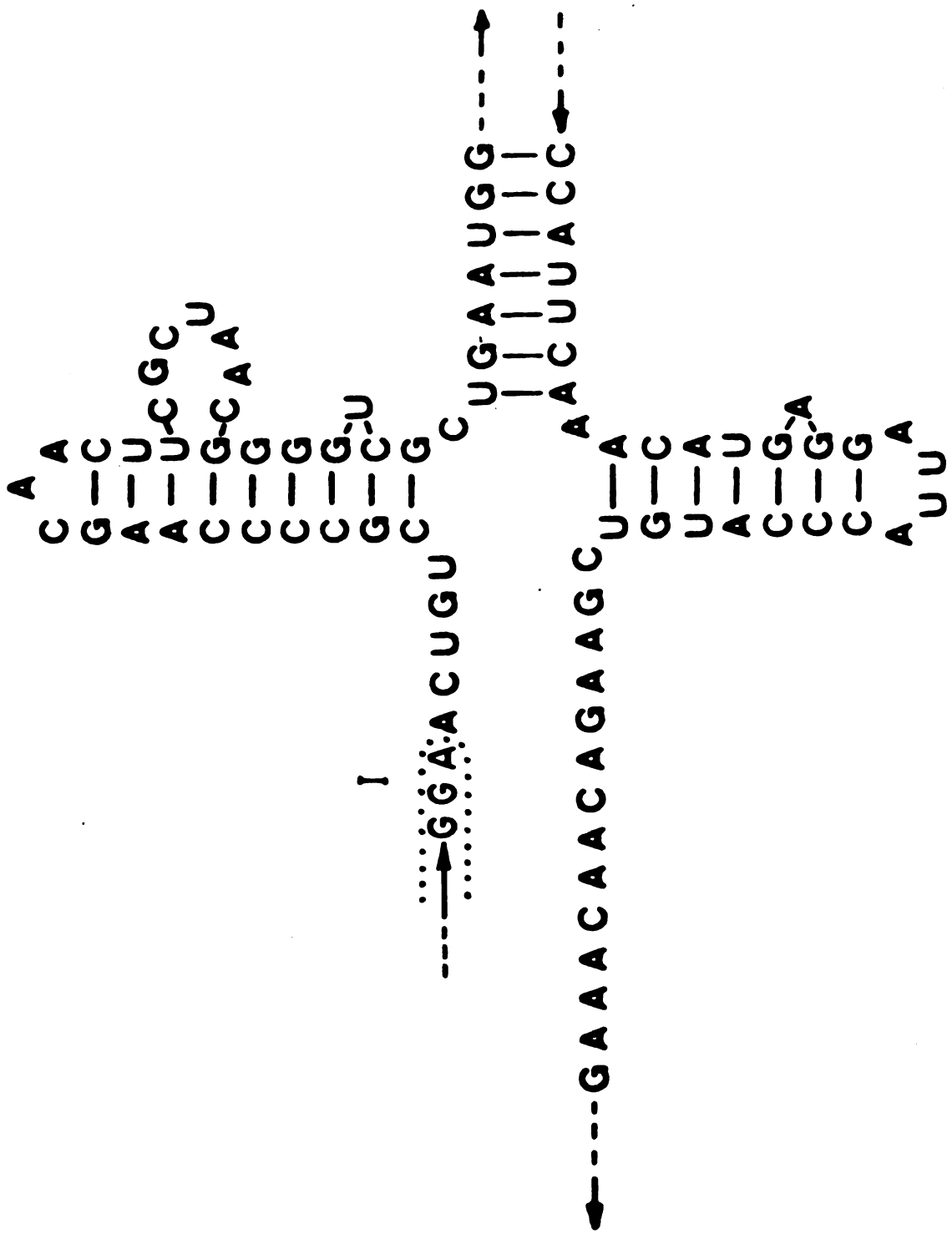
Figure 44. The positions to which nascent chain accumulations correspond on the MS2 coat protein cistron nucleotide sequence.


beginning of gene
termination codon for first gene, the A- protein
initiation codon for third gene, the RNA-polymerase
end of gene
I
II
III
IV
V
VI
key:
U = uridylic acid
C = cytidylic acid
A = adenylic acid
G = guanylic acid

of Tinoco et al. (1971, 1973). A structure in a yeast $tRNA^{ser}$ molecule containing a similar stem of four base pairs has been found to have a $T_m$ of 83° (Coutts, 1971). An alternate structure for the region between I and II has been proposed which would place an additional "hairpin" loop of moderate stability immediately following region I of Figure 44 (Min Jou et al., 1972). This alternate structure is shown in Figure 45. Correlation of the size of nascent peptides which accumulate during MS2 coat protein biosynthesis and the position of the ribosome carrying those lengths of nascent peptides in the flower model of the MS2 genome reveals that five of the six regions along the MS2 coat protein RNA where accumulations of nascent peptides occur during translation (i.e., I, II, III, IV, and V) occur at, or very near, a point where the ribosome involved in translation of the RNA would be entering a predicted region of RNA secondary structure in the MS2 genome.

Region VI of Figure 44 does not correlate with the beginning of a region of double stranded structure of MS2 RNA according to the model as shown. Region VI, however, is immediately 5' to a region of the MS2 structure for which alternative structures may exist, a region where secondary structures has been considered as "highly tentative" by the authors of the "Flower Model". This highly tentative region includes the potentially stable structure immediately preceding region VII. Since the "Flower Model" for the coat protein of MS2 has been predicted on theoretical grounds using the base sequence of only that region of MS2 and not the complete sequence of MS2, which is now known to contain some 3,569 nucleotide residues, the proposed secondary structure for the coat protein region may be oversimplified.

Figure 45. An alternate structure for a portion of the MS2 coat protein cistron.



Consequently, secondary structural effects of the other regions of MS2 RNA upon the rate of translation of the coat protein gene have not been considered here. Neither have attempts been made to take into account possible ribosome-ribosome interactions, or queuing, upon a given mRNA during the translation of the coat protein region since such interaction might be expected to hold open regions of an mRNA which might otherwise return to a double structure between each round of translation (von Heijne et al., 1977).

Lodish and Robertson (1969) have provided evidence that initiation of translation of the RNA polymerase gene of MS2 RNA may occur only when the initiation of codon for that gene is liberated from a region of secondary structure of MS2 RNA by translation of the coat protein gene (See Figure 1 and 45). The data presented in this thesis suggest that the effects of secondary structure are more general and have the ability to alter the rates of chain elongation as well by altering the rates of translation of specific regions of the mRNA.

The report that the in vitro rates of bacteriophage f2 coat protein synthesis, when measured between the 3rd and the 70th codons and the 3rd and 129 codons, are identical is not incompatible with the data reported here (Webster and Zinder, 1969). The observations presented in the present experiments involve the measurement of relative rates of nascent peptide chain elongation between different codons along the mRNA. The rates of chain elongation measured by Webster and Zinder are averages of the chain elongation rate for a number of successive elongation steps and may well include a relative nonuniformity of elongation rates within the regions studied.

Measurement of the *in vivo* nascent chain elongation rate of *E. coli* protein synthesis yields a value of about 1000 amino acids per minute at 37° (Forchhammer and Lindahl, 1971). *In vitro* studies have shown that the rate of T4 lysozyme synthesis is 180 amino acids per second at 31° (Wilhelm and Haselkorn, 1970), and the rate of f2 coat protein chain elongation at 33° is 30 amino acids per minute (Webster and Zinder, 1969). Thus, previously measured rates of chain elongation are seen to vary for different mRNA's with the RNA bacteriophage coat protein elongation rate being much lower than that of other proteins. The suggestion has been made that this lower elongation rate during f2 coat protein synthesis is due to the phase RNA secondary structure (Wilhelm and Haselkorn, 1970; Capecchi and Webster, 1975). However, the means by which this might occur was not clear. The first indication of the validity of this idea has been presented in this thesis.

The data presented in this thesis have led to the hypothsis that the origin of nascent chain size accumulations during chain elongation arises as a result of the slowing of the rate of ribosome movement along the mRNA by mRNA secondary structure. As ribosomes reside in certain regions (those preceding the areas of mRNA secondary structure) longer than other regions, the nascent chain population purified from these ribosomes will show size accumulations upon a chromatographic analysis by elution from Bio-Gel A 0.5 m under denaturing conditions.

Although the correlation between bacteriophage MS2 coat protein nascent chain accumulations and the proposed secondary structure of the MS2 coat protein cistron is quite good (five of six points of accumulation correspond to the beginning of a region of a potentially stable region of secondary structure), the extent to which this

hypothesis is applicable to other mRNA species must be determined. Since the nascent chain accumulations have been shown in this thesis to be associated with the mRNA itself, and not the translation system (for globin mRNA), it would be possible to determine the Bio-Gel elution pattern for any mRNA which has been purified by translation of that mRNA in the wheat germ cell-free system. Alternatively, the mRNA-dependent reticulocyte lysate cell-free system prepared by nuclease treatment could be used (Pelham and Jackson, 1976). Such data, coupled with the nucleotide sequences of the mRNA, could allow comparison of the potential regions of secondary structure of the mRNA with the regions at which ribosomes demonstrate slower rates of chain elongation as measured by nascent chain accumulations. The existence of more examples of such correlations of nascent chain size accumulations with regions of potential mRNA secondary structure would give further support to this hypothesis.

Direct analysis of the regions of mRNA which are involved in secondary structure is another useful approach. If regions of secondary structure can be shown to exist in defined areas of the mRNA by physical or enzymatic means, the correlation with the nascent chain size accumulations in a manner similar to that described in this thesis for MS2 coat protein would provide independent evidence in support of the hypothesis expressed here. Such analyses are now being undertaken for rabbit $\alpha$- and $\beta$-globin mRNA in the laboratory in which the work reported in this thesis was performed.

A third approach to the question of the effects of mRNA secondary structure upon chain elongation would be to perform *in vitro* experiments using a bacterial mRNA-dependent cell-free protein synthesis

system and bacteriophage RNA. In an analogous manner to the rabbit α- and β-globin size accumulation analysis, the MS2 coat protein nascent chain elution profile was first investigated in vivo. Analysis in a cell-free system would allow the closer study of the size accumulations in the coat protein nascent chain population.

The hypothesis proposed as a result of the experiments conducted in this thesis, that the origin of the nascent chain size accumulations observed upon Bio-Gel chromatographic analysis under denaturing conditions of a nascent chain population lies in the mRNA secondary structure, is the simplest explanation of the data presented. Other interactions such as limiting tRNA concentrations, mRNA binding proteins, effects of tRNA isoacceptor species upon the rate of the peptidyl transferase reaction, obstruction of the ribosome by a complex folding of the mRNA, etc., could be postulated to have similar effects in the translation of an mRNA. Some of the possibilities eliminated in this thesis for globin mRNA would have to be investigated for each mRNA examined in order to positively eliminate them. However, no other explanation of the nascent chain size accumulations fits the data reported here as well as the one presented, that the origin of the relatively slow steps in nascent chain elongation is a result of mRNA secondary structure impeding the movement of the ribosome during translation.

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