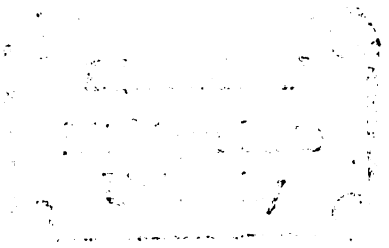

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thesis entitled

The Relationship Between Messenger RNA and Nascent Peptide Size Distribution: The Role of Messenger RNA Integrity

presented by

Howard Paul Hershey

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Biochemistry

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Date August 12, 1981

THE RELATIONSHIP BETWEEN MESSENGER RNA AND NASCENT PEPTIDE SIZE DISTRIBUTION: THE ROLE OF MESSENGER RNA INTEGRITY

By

Howard Paul Hershey

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1981

ABSTRACT

THE RELATIONSHIP BETWEEN MESSENGER RNA AND NASCENT PEPTIDE SIZE DISTRIBUTION: THE ROLE OF MESSENGER RNA INTEGRITY

By

Howard Paul Hershey

An investigation into the origion of the nonuniform rabbit globin nascent peptide sizes observed during translation of globin mRNA in different mRNA dependent cell-free protein synthesizing systems was performed. Accumulations of discrete size nascent peptides similar to those seen during globin synthesis in reticulocyte lysates were observed during translation of globin mRNA in the wheat germ cell-free protein synthesizing system. Globin mRNA translated in the mRNA dependent reticulocyte lysate gave rise to a distorted nascent peptide size distribution.

Analysis of the mRNA dependent reticulocyte lysate for the presence of nascent peptides in the absence of added mRNA showed that a reproducible size distribution of nascent peptides was present in this protein synthesizing system. These nascent peptides were postulated to arise from initiation of protein synthesis on globin mRNA fragments remaining in the mRNA dependent lysate following micrococcal nuclease digestion. Pactamycin inhibited the initiation of protein synthesis on these fragments and revealed an apparent inability to terminate protein synthesis normally on mRNA fragments.

No nascent peptides were found in the wheat germ cell-free protein synthesizing system in the absence of added mRNA. Pactamycin treatment

of the globin mRNA directed wheat germ cell-free system removed the nascent peptide population from polysomes synthesizing globin. Translation of globin mRNA fragmented by S_1 nuclease gave rise to a population of nascent peptides which remained following pactamycin addition to the cell-free protein synthesizing system.

Analysis of 3' end labeled globin mRNA showed that fragments were not present in the globin mRNA preparations used to direct globin synthesis in the wheat germ cell-free protein synthesizing system. Translation of 3' end labeled globin mRNA demonstrated that no fragments of globin mRNA were generated during protein synthesis in this system.

The data in this thesis document the presence of globin mRNA fragments capable of normal initiation, but not termination of protein synthesis by the normal sequence of events. Data are also presented showing that nascent peptides observed during translation of globin mRNA in the wheat germ cell-free protein synthesizing system are not due to globin mRNA fragments present in the protein synthesizing system.

ACKNOWLEDGEMENTS

I would like to thank Drs. H. J. Kung, A. Revzin, R. Patterson, and W. Wells for serving on my graduate advisory committee.

Special thanks are given to Dr. Allan J. Morris for his guidance, help, patience, and good humor during my graduate studies.

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INTRODUCTION

Protein Biosynthesis

Messenger RNA (mRNA) contains the necessary information within its nucleotide sequence to specify a polypeptide sequence. Ribosomes read the coding sequence of the mRNA from the 5' end to the 3' end (1), polymerizing amino acids sequentially in a stepwise fashion. Polypeptides are synthesized from the N terminal end to the C terminal end of the protein (2,3). Each ribosome traversing the mRNA contains a single nascent chain that is growing at the rate of one amino acid per triplet codon encountered by the ribosome (4). The aminoacyl-tRNA molecules read each codon in the mRNA sequence and the growing polypeptide is transferred to the new tRNA.

The rate of protein biosynthesis may be regulated by any of the steps involved in protein synthesis (initiation, elongation and termination steps) or by the amount of mRNA available for the synthesis of a given protein. The level of a given protein in a cell therefore is governed by a balance between the stability of each mRNA in the cytoplasm, the rate at which protein is being made from that mRNA, and the rate at which the protein product is being degraded (5,6,7).

Globin Biosynthesis

The synthesis of α- and β-globin in mammalian reticulocytes has been extensively studied. Mammalian reticulocytes are non-nucleated cells that are synthesizing α- and β-globin from residual mRNA and ribosomes.

These polysomes will soon be eliminated along with the remaining mitochondria in the cell, marking the transition of the reticulocyte to the mature erythrocyte.

Greater than 95% of the protein synthesis in the reticulocyte is dedicated to the production of α- and β-globin (8). Alpha globin is produced in a slight excess in rabbit reticulocytes (9). The observed translation rate of α- and β-globin are both equal, each protein requiring 200 seconds for completion (10,11).

The size of polysomes involved in β-globin synthesis has been shown to be larger than the observed size of polysomes synthesizing α-globin (12). Lodish found α-globin mRNA to be present in 40% greater quantities than β-globin mRNA in the rabbit reticulocyte (11). Lodish also found that the initiation of protein synthesis by ribosomes on β-globin mRNA was more efficient than the initiation rate observed for the α-globin mRNA. The rate of initiation observed for α-globin mRNA was 65% as frequent as that observed for β-globin messenger RNA. This lowered initiation rate for α-globin synthesis coupled with the approximate 40% excess of α-globin mRNA as compared with β-globin mRNA in the reticulocyte gives a final ratio of α- to β-globin synthesis of approximately 1:1. This faster initiation rate for β-globin mRNA was also proposed to account for the increased polysome size observed during β-globin synthesis.

Primary Structure of RNA

Ribonucleic acids are linear polymers of nucleoside monophosphates. Naturally occurring RNA is synthesized from the four ribonucleotide triphosphate by joining of the corresponding nucleoside monophosphates through 3'$\longrightarrow$5' phosphodiester bonds. In addition to the four major bases found in RNA, some minor bases are also found in RNA as are some

modifications of the ribose moiety (such as 2'-O-methyl derivatives). The primary structure of RNA may contain obviously available information as is the case with mRNA where the coding sequence specifies the order of amino acids for a specific protein. The information in RNA may also be expressed in a more subtle manner. The order of arrangement of bases in a polyribonucleotide also defines a series of intramolecular structural features which result in a conformation that the molecule may adopt. A knowledge of conformations adopted by different RNA molecules may well be necessary before an understanding of the functional aspects of these molecules can be accomplished (14).

RNA Secondary Structure

Elucidation of the secondary structure of large polyribonucleotides is an essential step in the understanding of RNA function. Secondary structure components in RNA species would be expected to be different for each RNA species analyzed due to the different nucleotide sequence present in each RNA.

Initial studies involving elucidation of RNA secondary structure were based on the known hypochromic shift that occurs when a polynucleotide adopts a more helical conformation from a more random conformation. This shift to lower absorbance is due primarily to base stacking interactions which help in the stabilization of the helices formed. The use of thermal denaturation to disrupt regions of helical structure has been widely used to determine the extent of secondary structure in RNA. The hyperchromic shift resulting from heat denaturation of RNA has been used to estimate the helical content of total ribosomal RNA (15), 16S and 18S ribosomal RNA (16), ovalbumin mRNA (17), globin mRNA (18) and 5S RNA (19). Using the method of hypochromic shift measurement following

thermal denaturation estimates of the helical content within RNA have ranged from 55 to 75%. The method, however, does not allow prediction of the location of the regions of putative helical structure.

The decrease in susceptibility of regions involved in base pairing to nuclease degradation has also been used to study RNA secondary structure. Digestion of 16S and 18S rRNA with ribonuclease T_1 and T_2 revealed regions of these molecules resistant to these structure specific nucleases (20). The resistance of 16S RNA to ribonuclease T_1 action lead Ricard and Sales to predict long regions of base paired structures (21). Fiers et al. used limited ribonuclease T_1 digestion to generate defined length fragments of the MS2 coat protein genome (22). Later work by Fiers et al. employed the single strand specificity of ribonuclease T_1 to complete the determination of the base sequencing the entire MS2 genome (23). The sensitivity of certain regions to digestion with nuclease in conjunction with prediction of secondary structure by theoretical calculations of free energies of proposed loops was used to predict the secondary structure map of the complete MS2 genome. The secondary structure of the potato spindle tuber virion has also been predicted by the use of sequence data to predict regions of secondary structure supplemented with nuclease sensitivity data (24).

S_1 nuclease, a single strand specific endonuclease, has been extensively used to map regions of secondary structure within RNA molecules. The digestion products resulting from S_1 digestion of 16S ribosomal RNA have been used to determine the interacting sequences within the molecule (25). Flashner and Vournakis subjected globin mRNA to limited digestion with S_1 nuclease under non-denaturing conditions and found that up to 75% of the globin mRNA sequences are resistant to

the action of the nuclease (26). The data from these experiments with globin mRNA were interpreted to support a structural model of globin mRNA in which a high degree of helical structure was present within the primary sequence. Wurst et al. have developed a method which localizes regions of secondary structure within an RNA sequence up to 150 nucleotides long. The data obtained from the specific cleavage of 5' [^{32}P]-labeled RNA using base specific nucleases in conjunction with analysis of the fragments resulting from RNA limited S_1 cleavage of the labeled RNA allows accurate prediction of secondary structure (27). This method was used to correctly predict the secondary structure of two different transfer RNA molecules whose secondary structures were previously known. Pavlakis et al. used a modification of the method of Wurst to predict the secondary structure of the 5' end of both the α- and β-globin mRNAs (28).

Branlant et al. have developed a method using Naja oxiana nuclease in the presence of high concentrations of magnesium to map secondary structure in RNA (29). The Naja oxiana nuclease under conditions of high magnesium concentration degrades regions of RNA-RNA interaction specifically. The method has been used to determine the secondary structure of U4 nuclear RNA. The RNA was labeled on the 3' end with ^{32}P and partially digested with T_1 and S_1 nucleases to ascertain the locations of single stranded regions of the RNA. Naja oxiana nuclease was then used to cleave the RNA specifically at the base paired regions. The digestion products were fractionated on polyacrylamide gels and identified by comparison with ribonuclease T_1 digestions performed under denaturing conditions along with alkali digested RNA data. The

secondary structure data was then used to assemble a secondary structure map of U4 RNA (30).

The recent advent of base sequencing methods for both DNA (31,32,33) and RNA (34,35) is increasing the number of RNA molecules for which a primary sequence is known. The primary sequence of an RNA can be used to generate a map of secondary structure within the nucleotide sequence. The relative stabilities proposed structures can be analyzed by procedures developed by Tinoco et al. (33,34). These procedures predict the free energy of formation of possible helical structures arising from a known primary sequence using a set of experimentally developed parameters. Another method of predicting base pairing has been developed by Salser (38). The method calculates the most stable combinations of compatable regions of a long RNA sequence by calculations of the free energy of formation for a given sequence of bases. Pavlakis et al. (28) have pointed out that proposed secondary structure models generated by computer using only primary sequence data do not always fully agree with structure maps assembled from structure specific nuclease cleavages. Future methods for predicting RNA secondary structure by computer analysis of sequence data may soon include secondary structure data from nuclease mapping studies along with primary sequence data to generate more accurate secondary structure maps.

Secondary Structure and RNA Function

Lodish was among the first investigators to observe a relationship between RNA structure and function (39). By denaturation of the structure of the bacteriophage f2 genome using formaldehyde, Lodish was able to increase the rate of f2 RNA directed protein synthesis by up to 20 fold for selected proteins coded for in the f2 genome. In addition,

several protein products were synthesized that did not correspond to any known authentic f2 gene products. These results were interpreted to demonstrate the importance of the secondary structure of f2 RNA to its proper function.

Translation of the replicase gene in MS2 bacteriophage is known to require synthesis of at least the N-terminal half of the coat protein cistron (40,41). The translation of the N-terminal half of the coat protein gene has been postulated to alter the conformation of the MS2 genome. This change exposes the replicase initiator which is proposed to be based paired to the 5' half of the coat protein cistron in the absence of translation. (42).

Ahlquist et al. have studied the 3' terminal secondary structure in a variety of viruses of the bromovirus family (43). Their results showed that the secondary structures generated for the 3' ends of all three RNA classes have been retained among all the bromoviruses. The structures generated not only showed conservation of the general shape of the 3' region, but also conservation of specific sequences within these regions. These results were confirmed by ribonuclease T_1 digestions. This conservation of the 3' end was postulated to be necessary for retaining the ability to be aminoacylated in vivo.

Recently, structural analysis of the ribosome binding sites for three bacteriophage T7 class III genes was performed by Rosa (44). All three binding sites contained a sequence of bases complimentary to the 3' end of 16S RNA, in keeping with the hypothesis of Shine and Dalgarno (45). The three binding sites studied appeared to have initiator regions which were buried in secondary structure when analyzed by the method of Wurst et al. (27). The secondary structure proposed for these binding

sites was postulated to aid in the binding of ribosomes to the correct initiation site for protein synthesis, and possibly protect the 5' end of the transcript from exonucleases attack.

Robertson et al. studied the binding of ribosomes to ^{125}I-labeled globin mRNA (46). Following nuclease digestion of the globin mRNA-initiator complex with pancreatic ribonuclease these investigators found a set of specific fragments of globin mRNA which were protected by ribosome binding. The nucleotide sequences of the fragments were then determined. The results showed that binding of the 40S ribosomal subunit to globin mRNA protected an 80 base sequence of the message, while assembly of the complete ribosome protected only half as much of the same region. These data suggested a conformational change in the globin message structure during assembly of the 80S initiation complex.

Eukaryotic mRNA Structure and Function

Messenger RNA can be subdivided structurally into four distinct regions of primary structure. These are a 5' untranslated region, a coding region, a 3' non-translated region and a Poly(A) region which is present on most but not all eukaryotic messages.

The 5' untranslated region consists of the base sequence from the 5' end of the mRNA to the initiator codon. The 5' end of this region usually begins with one of the three cap structures (see 47 for review). Messenger RNA molecules that have been uncapped have been shown to display a greatly reduced template activity when translated in the wheat germ cell-free system (48). In addition, 7-methyl guanylic acid, a cap analog, inhibits the translation of capped, but not uncapped mRNA (49). These data led to the suggestion that the cap may function by stimulation of the rate of initiation of protein synthesis (50). Beyond the cap is

the 5' untranslated region or leader sequence which continues to the initiator codon. This region has been observed to be variable in length in the mRNAs thus far sequenced. The suggestion has been made that a nucleotide sequence in the 5' non-coding region may serve as a binding site for the 3' terminus of the 18S RNA in the 40S ribosomal subunit. This would be analogous to the situation put forth for mRNA and 16S rRNA interactions during initiation of protein synthesis in procaryotes by Shine and Dalgarno (45). This hypothesis usually correlates well with the observed interaction of 16S RNA of prokaryotic ribosomes with the initiator region in prokaryotic mRNA. The experimental evidence does not appear to support a similar type of interaction for eukaryotic mRNA. Studies of the 5' noncoding region of eukaryotic mRNA thus far sequenced have not generally shown a sequence capable of base pairing with 18S RNA and initiation of protein synthesis may proceed by a mechanism that is not analogous to that seen in prokaryotes (51).

The coding region of mRNA consists of the sequence of bases that specifies the amino acid sequence of the protein to be biosynthesized. Interestingly, specific mRNA molecules display a non-random use of triplets when the degeneracy of the genetic code provides a choice. This preference for the use of specific triplets in a series of synonomous codons seems to be unique for each specific mRNA since no patterns emerge when the choice of codons is compared among different mRNA. Beta globin mRNA shows a weak preference for the base G in the third position of codons while α-globin mRNA shows a marked preference for codons ending in C (52,53). Codon choices used most frequently in the bacteriophage MS2 RNA favor those codons that end in U. While the significance of this bias for specific codons remains to be determined, some investigators

have suggested that the non-random use of codons represent the selection of a specific codon within a synonomous pool such that secondary structure within the mRNA can be optimized (54).

Following the coding region of the mRNA is the 3' untranslated region. This region continues to the start of the poly(A). The length of this 3' region varies considerably among different mRNAs whose nucleotide sequences are known. The function of the 3' untranslated region is not yet understood. Studies on the sequences of the duplicated human α- and β-globin genes provide few clues to the function of this region. These genes are thought to have arisen from a gene duplication and have developed little heterogeneity between the duplicated genes over time with the exception of the 3' non-coding regions which are unexpectedly divergent (55). In the Bromoviruses, however, the sequence homology of the 3' untranslated regions of the three classes of RNA found in the virions has been highly conserved (43). Proposed secondary structures of these viral RNA molecules also show a striking similarity indicating the sequence homology may have been retained so that secondary structure could be conserved.

The poly(A) region which follows the 3' untranslated region is present in approximately 70% of all eukaryotic mRNA (56). The poly(A) at one time had been postulated to function in the transport of mRNA from the nucleus to the cytoplasm, however, the transport and translation of non-poly(A) containing mRNA molecules has since been shown (57,58). The proposal has also been made that the poly(A) may function in the determination of the functional half-life of mRNA. Deadenylated globin mRNA has been shown to display no alteration in the initial rate of protein synthesis in cell-free protein synthesizing systems when compared with

poly(A) containing globin mRNA (59). At longer incubation times, however, the template ability of the deadenylated globin mRNA was lost more rapidly than the rate of loss observed for adenylated mRNA (59,60). The stability of the deadenylated globin mRNA could be restored by readenylation of the messenger RNA (61). Although it seems that poly(A) enhances mRNA stability *in vitro*, a defined function on this region has yet to be documented.

Structural Features of Globin mRNA

Globin mRNA is known to undergo an increase in absorbance at 260 nm following thermal denaturation. This increase in absorbance was shown to be more than could be accounted for by a random copolymer structure. Further studies on the thermal deaturation of globin mRNA by Holder and Lingrel indicated that up to 68% of the bases in the mRNA could be found involved in secondary structure (18).

Purified globin mRNA has also been subjected to nuclease digestion with S_1 nuclease. Flashner *et al.* subjected globin mRNA to digestion with S_1 nuclease under conditions where secondary structure would be stabilized (26). They found up to 75% of the globin sequence was resistant to the single strand specific nuclease. The S_1 digestion products of globin mRNA were found to be oligonucleotides ranging in size up to 80 nucleotides in length. Pretreatment of globin mRNA by methods known to disrupt helical structures such as heat or formaldehyde treatment greatly increased the S_1 susceptibility of the mRNA, while high salt concentrations lowered nuclease susceptibility. From these data and the previous work of Vournakis *et al.* (62), globin mRNAs were postulated to contain an extensive degree of helical structures within the α- and β-globin mRNA molecules.

Dubochet et al. (63) used dark field electron microscopy to study the structure of both duck and rabbit globin mRNA and globin messenger ribonucleoprotein particles. Following staining with uranyl acetate, dark blobs along the mRNA were seen under the electron microscope. These were interpreted to represent regions of complex secondary structure. Treatment of the mRNA with denaturing reagents prevented the binding of the stain to the message indicating the loss of secondary structure.

Favre et al. (64) examined the conformation of duck and rabbit globin mRNA by optical methods and by employing ethidium bromide to study intercalation of the dye in double stranded regions of the mRNA. These investigators also observed the hyperchromic shift at 260 nm when globin mRNA was heat denatured. In addition, the stimulation of ethidium bromide flourescence following intercalation of the dye into double stranded regions of globin mRNA was measured. The ethidium bromide titration profiles showed that up to 60% of the nucleotides of both duck and rabbit globin mRNA are involved in base pairing interactions, a number greater than predicted for a random copolymer conformation.

The nucleotide sequences of rabbit α- and β-globin mRNAs were determined during the time that these secondary structure studies were taking place (52,53). The α-globin messenger RNA is 552 nucleotides long and consists of a 50 nucleotide long 5' untranslated region, a 423 nucleotide coding region and a 89 nucleotide long 3' non-coding region. Beta globin mRNA is 590 nucleotides long and is distributed among a 57 nucleotide 5' non-coding region, a 438 nucleotide long coding region and a 95 nucleotide 3' untranslated region. A possible secondary structure map for α-globin mRNA was proposed by Heindell et al. (53) using the rules for the calculation of interaction energies between bases developed

by Tinoco et al. (37). This secondary structure is based solely on thermodynamic consideration and may not reflect the actual conformation adopted by the molecule in solution.

The previous data on the structure of globin mRNA using physical techniques provided only general information on structural features of globin mRNA. The method of Wurst et al. was modified and has been applied to the 5' end of both the α- and β-globin mRNA molecules by Pavlakis et al. (28). The results of secondary structure mapping were combined with the empirical rules of Tinoco to generate structure maps of the 5' ends of both the α-globin and β-globin messenger RNAs, providing the first detailed structure maps for the 5' ends of both messages. The results of their studies showed considerable base pairing in both molecules. The β-globin mRNA initiator codon is not present in a base paired structure and is both exposed and highly susceptible to nuclease attack. The α initiator codon, however, is present in a region of secondary structure and totally inaccessible to nuclease attack by either S_1 nuclease or ribonuclease T_1. The inaccessibility of the α initiator codon due to its involvement in secondary structure was postulated to be the reason for the slower initiation rate observed for α-globin synthesis.

Nascent Globin Peptide Nonuniformity

The rate of synthesis of globins in the reticulocyte has been determined by Lodish and Jacobson to be 200 seconds per chain at 25° (10). This value represents the average time required to synthesize a single polypeptide chain without regard to possible heterogenieties in the local translation rate along the mRNA during assembly of the protein. Studies performed by Protzel and Morris showed that the rate of elongation during

the synthesis of α- and β-globin is nonuniform (65). This nonuniformity was demonstrated by analysis of the size distribution of nascent peptides isolated from polysomes synthesizing α- and β-globin in rabbit reticulocytes.

As a ribosome traverses a mRNA during the process of translation, it carries with it one growing nascent peptide chain. The length of this nascent peptide is an exact measure of the location of a ribosome along the coding sequence of the message. If elongation were a process occuring at a uniform rate during protien synthesis, all possible nascent peptide sizes would be represented equally in the nascent chains of polysomes synthesizing that protein. Protzel and Morris showed that analysis of the size distribution of globin nascent peptides by gel filtration under denaturing conditions revealed a nonuniform distribution of sizes (65). The observed accumulations of nascent peptides along the mRNA represent areas of the mRNA where relatively high ribosome density are found on a time-average basis as compared with other areas of the coding sequence.

Chaney and Morris investigated the origins of this nonuniform translation rate (66). These workers attempted to find some component of the reticulocyte lysate cell-free protein synthesizing system whose presence in limiting quantities was responsible for the nascent peptide accumulations. Their results failed to find any limitation of components in the reticulocyte lysate cell-free system that could affect the nascent peptide size distribution. Translation of purified globin mRNA from rabbit reticulocyte was performed in the wheat embryo derived cell-free protein synthesizing system. Nascent peptides purified from the globin mRNA directed wheat embryo cell-free system displayed a similar distribution

of globin nascent peptides to that observed in the reticulocyte lysate (66). This led Chaney and Morris to conclude that the origin of the nascent peptide accumulations lies in the mRNA itself.

Chaney and Morris also studied the distribution of nascent peptides on polysomes synthesizing MS2 coat protein in rifampicin treated MS2 infected E. coli (67). Analysis of these nascent peptides by gel filtration under denaturing conditions yielded a nonuniform nascent peptide distribution, but a different distribution than that observed for globin nascent peptides. A correlation was made between the regions of the MS2 coat protein genome where ribosomes were accumulating and the locations of secondary structure in that same genome proposed by Fiers et al. (22, 23). This led Chaney and Morris to propose that regions of secondary structure in mRNA modulate the rate of ribosome translocation along the mRNA, giving rise to the nonuniform nascent peptide population.

Vary and Morris continued the studies into the origin of nascent peptide nonuniformity. This was accomplished by subjecting the fractions isolated from the gel chromatographic analysis of total globin nascent peptides to digestion with trypsin (68). The proportion of radioactivity in the α and β tryptic peptides was used to construct the individual nascent peptide size distributions. The α- and β-globin nascent peptide distributions were found to be different from one another. These investigators also made a correlation between the single stranded regions of S_1 nuclease susceptibility in the β-globin message performed by Pavlakis et al. (28) and the distribution of β-globin nascent peptides. The regions of S_1 susceptibility in the β-globin mRNA correlated with positions of minima in the β-globin nascent peptide distribution. This was in keeping with the original proposal of Chaney and Morris that

accumulation of nascent peptides represent areas of secondary structure in mRNA, while minima in the profile could now be correlated with single stranded regions of the mRNA.

MATERIALS

Cycloheximide, ethyleneglycol-bis-(β-aminoethylether)N,N'-tetra-acetic acid (EGTA), guanidinium chloride, (practical grade), bovine hemin, 2,4-dinitrophenyl alanine, dithiothreitol (DTT), creatine phosphokinase (EC 2.7.3.2), phosphocreatine (diTris salt), diethyl-pyrocarbonate, phenylhydrazine-HCl, Triton X-114 and TRIZMA base were purchased from Sigma Chemical Company, St. Louis, Missouri. Scintillation grade napthalene was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Bio-Rad Company, of Richmond, California was the source of Bio-Gel A-0.5M, Bio-Gel P-10, acrylamide, N,N'-methylene-bis-acrylamide, sodium dodecyl sulfate (SDS) and N,N,N',N' tetramethylene-diamine (TEMED). Nembutal (sodium pentobarbital) was obtained from Abbott Laboratories, North Chicago, Illinios. Phosphocreatine (dipotassium salt) was purchased from Calbiochem Company, La Jolla, California. Research Products International was the source of 2,5-diphenyloxazole (PPO) and 1,4 bis-2-(4-methyl-5-phenyloxazoyl)-benzene. Adenosine triphosphate, guanosine triphosphate and oligo (dT) cellulose (Type 7) were purchased from P-L Biochemicals, Milwaukee, Wisconsin. Blue Dextran 2000 and Sephadex G series gel filtration media were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Whatman Biochemicals Ltd., Maidstone, Kent, Great Britian was the source of microgranular preswollen diethylaminoethyl cellulose (DE52), carboxymethyl cellulose (CM32) and GF/C glass fiber filters. Sodium

heparin was purchased from Fisher Scientific Company, Fair Lawn, New Jersey.

Micrococcal nuclease (EC 3.1.31.1) and calf intestine alkaline phosphatase (EC 3.1.31.1) were obtained from Boeringer-Mannheim Biochemicals, Indianapolis, Indiana. New England Biolabs, Beverly, Massachusetts was the source of T_4 RNA Ligase (EC 6.5.1.3). Nuclease S_1 (EC 3.1.4.-) was obtained from Miles Laboratories, Elkhart, Indiana. Pactamycin was a gift of the Upjohn Company, Kalamazoo, Michigan. Sparsomycin was generously donated by Drug and Research Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland. General Mills, Inc., Minneapolis, Minnesota provided the gift of untoasted wheat germ. Tobacco acid pyrophosphatase was a gift from Dr. C. Vary, Syracuse University, Syracuse, New York. Radiolabeled compounds were purchased from Amersham/Searle Company, Arlington Heights, Illinois (L-[^{3}H]-Leucine, 60 Ci/mmol, L-[^{14}C]leucine, 300 mCi/mmole, L-[^{3}H]isoleucine, 15 Ci/mmole, Adenosine 5'-[^{32}P] triphosphate, 2000 Ci/mmole, and cytidine 3',5'[5'-^{32}P]-bisphosphate, 2000-3000 Ci/mmole) and New England Nuclear, Boston, Massachusetts (L-[^{3}H]-tryptophan, 125 Ci/mmole, and L-[^{14}C]-tryptophan, 58 Ci/mmole). T_4 polynucleotide kinase (EC 2.7.1.78) and NEF 963 aqueous counting cocktail were also obtained from New England Nuclear. Ultra pure sucrose and urea were obtained from Bethesda Research Labs., Bethesda, Maryland. X-Omat (XAR-5) films were from Eastman Kodak Inc., Rochester, New York as was "Stains-All". All other chemicals were of reagent grade or higher quality.

METHODS

Preparation of Rabbit Reticulocytes

New Zealand white male rabbits weighing 6-8 lbs. were made anemic with four daily injections of 2.5% phenylhydrazine-HCl (pH 7.3) prepared in NKM salts (69). On the seventh day after the start of phenylhydrazine treatment, rabbits were injected intravenously via the marginal ear vein with 100 mg of sodium nembutal and 2000 units of sodium heparin. Blood was removed by cardiac puncture using a 100 ml syringe fitted with an 18 gauge needle. The blood obtained was quickly cooled to 0°. The hematocrits of these preparations ranged from 10-17%. All subsequent steps were performed at 0°.

The blood was filtered through a double layer of glass wool to remove tissue pieces and the filtrate centrifuged at 4000 x g for 10 minutes. Plasma was removed by aspiration with care taken to remove the buffy coat from the surface of the red cell pellets. The cells were resuspended in a volume of Ringers saline approximately equal to the volume of plasma removed. Cells were then reisolated by centrifugation at 4000 x g x 10 minutes followed by removal of the supernatant layer. The cells were washed by this procedure two additional times.

Preparation of the Reticulocyte Lysate

Washed rabbit reticulocytes were lysed by swirling the cells with an equal volume of ice cold sterile water for ten minutes. The lysate was centrifuged at 25,000 x g x 20 minutes and the supernatant layer gently

decanted. Aliquots of the lysate were immediately frozen by injection into sterile glass vials maintained at -40° on dry ice. The lysate aliquots were capped and stored in liquid nitrogen until use. The conditions for protein synthesis in the reticulocyte lysate are shown in Table I.

Table I. Final concentration of the components of the reticulocyte lysate cell-free system. These components were added to 800 µl of lysate yielding a final volume of 950 µl.

$MgCl_2$	1.4 - 1.6 mM
KOAC	75 - 80 mM
Hemin	3.2 mM
ATP	1.0 mM
GTP	0.2 mM
Creatine phosphate (dipotassium)	11 mM
Amino acids	0.1 times the final concentration of Hunt (70)
Creatine phosphokinase	100 µg/ml

The potassium and magnesium concentrations chosen for use in each lysate were determined on the basis of the levels required to obtain the optimal rate of incorporation of [^{3}H]-leucine into trichloroacetic acid insoluble material. Aliquots of the reticulocyte lysate system were incubated at various concentrations of the ion to be optimized (K^+ or Mg^{2+}) in a reaction mixture supplemented with 50 µCi/ml L-[^{3}H]-leucine at 37°. At 10 minute intervals 5 µl aliquots of the incubations were removed and added to 1.0 ml of ice cold water. One ml of 1.0 N NaOH, 10 mM L-leucine was added to each sample. The samples were

incubated at 37° for 20 minutes. Four ml of 15% TCA, 10 mM L-leucine, 1.0% H_2O_2 were added and the samples incubated at 0° for 45 minutes. The precipitated protein was collected on GF/C filters by suction, washed with 25 ml of 10% TCA, 10 mM L-leucine and dried in scintillation vials at 100° for 45 minutes. After the vials had cooled, 5 ml of a Triton-xylene based liquid scintillation cocktail were added and incorporation of [^{3}H]-leucine into TCA precipitable material was determined.

Preparation of the Wheat Germ Cell-Free Protein Synthesizing System

A wheat germ cell-free protein synthesis system was prepared by a modification of the method of Gallis et al. (71). Untoasted wheat germ was obtained from General Mills, Inc., Minneapolis, Minnesota and contained approximately 70% wheat germ and 30% chaff. The wheat germ was ground very gently in order to break it into smaller pieces. The pieces which passed through a #14 Tyler sieve but retained by a #28 Tyler sieve were saved. The contaminating chaff was removed by floating 20 g of sieved wheat germ in 400 ml of chloroform:cyclohexane (15:1) in a 500 ml graduated cylinder. After mild swirling, the chaff settled to the bottom and the embryos were poured off into a coarse glass sintered funnel. The solvent was removed by aspirator suction, after which embryos were air dried overnight and stored at -20°.

Preparation of a wheat embryo S-23 fraction involved suspending 5 grams of embryos in 8 ml of grinding buffer (1 mM $Mg(OAc)_2$, 2 mM $CaCl_2$, 50 mM KCl and 1 mM dithiothreitol) with an equal weight of acid washed glass beads in a chilled mortar. The mixture was ground hard in the cold for 2 minutes. The embryos were ground with 8 ml additional grinding buffer for 2 more minutes in the cold, and the pasty mixture transferred to a sterile centrifuge tube. The material adhering to the

mortar was rinsed into the centrifuge tube with 9 ml of grinding buffer and the material in the tube thoroughly mixed with a sterile scoop spatula. The cell lysate was centrifuged for 10 minutes at 23,000 x g and the supernatant fluid decanted into a prechilled sterile centrifuge tube. The volume of the extract was estimated by adjacent tube volume, 0.01 volume of 100X buffer (1 M Tris-HCl pH 7.5, 100 mM $Mg(OAc)_2$) added and the extract centrifuged for 10 minutes at 23,000 x g. The supernatant layer was removed and passed over a 2.5 x 40 cm Sephadex G-25 (fine) column equilibrated with 20 mM HEPES pH 7.5, 40 mM KCl, 2.0 mM $Mg(OAc)_2$ and 1 mM DTT. The void volume fractions with the highest absorbance at 260 nm were pooled and the absorbance of this pooled S-23 fraction was measured at 260 nm. If the A_{260} were greater than 75 A_{260}/ml, the S-23 fraction was diluted with column buffer to an absorbance of 75 A_{260}/ml and frozen in a dry ice-acetone bath in 400 µl aliquots, or frozen directly if the A_{260} were less than 75/ml.

Determination of the Rate of mRNA Directed Protein Synthesis in the Wheat Germ Cell-Free System

Incubations for the determination of protein synthesis in the wheat germ system were performed in a volume of 20 µl or multiples thereof. The conditions employed for protein synthesis in the wheat germ system are shown in Table II. Each 50 µl reaction mixture contained 30 µl of the S-23 fraction. Incubations were performed at 28°C. At specified time intervals, 2 ml of 0.5 N NaOH, 0.25 mg/ml BSA and 10 mM L-leucine were added to each tube in order to terminate protein synthesis. Radioactivity incorporated into TCA precipitable material was determined as described above for reticulocyte lysate incubations but with H_2O_2 omitted in the 15% TCA used for protein precipitation.

Table II. Final concentrations of components added to the wheat germ cell-free system.

HEPES*	12 mM
$Mg(OAc)_2$	3.0 mM
KOAC	140 mM
ATP	1.6 mM
GTP	0.16 mM
Phosphocreatine (diTris)	12.8 mM
Amino acids	32 μM
mRNA	0-5 μg/ml

*present in S-23 preparation

Preparation of the mRNA Dependent Reticulocyte Lysate Cell-Free Protein Synthesizing System

A mRNA dependent reticulocyte lysate was prepared by a modification of the procedure of Pelham and Jackson (72). A complete lysate cell-free protein synthesizing system was assembled as described earlier. The lysate was made 1 mM in $CaCl_2$ and 20 μg/ml in micrococcal nuclease, mixed thorougly and incubated at 20°C for 15 minutes. Sufficient 400 mM EGTA was then added to give a final EGTA concentration of 2 mM. After mixing, the mRNA dependent lysate was frozen in glass vials on dry ice in 400 μl aliquots and stored in liquid nitrogen. In some cases, the amino acid component was lyophilized before the lysate system was assembled in order to allow larger volumes of mRNA to be added without changing the final concentrations of the components of the system. In order to determine the ability of the mRNA dependent lysate to synthesize protein in response to exogenous mRNA, aliquots of these lysates were thawed at

4°. The lysate was either supplemented with increasing amounts of mRNA up to a final concentration of 5 μg/ml or H_2O if a measurement of the background synthetic rate was to be made. The mRNA dependent lysates were incubated at 37° and assayed in the same manner as described above for the reticulocyte lysate.

Preparation of Rabbit Globin mRNA

Rabbit globin mRNA was prepared by a modification of the method of Krystosek et al. (73). Washed rabbit reticulocytes were prepared as described above, but with 1 mM cycloheximide added to the Ringers saline. The cells were lysed by the addition of four volumes of RSB (10 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl pH 7.5) followed by incubation with gentle swirling at 0° for 5 minutes. The lysate was centrifuged at 25,000 x g for 15 minutes. The supernatant fraction was carefully transferred by pipet into ultracentrifuge tubes and centrifuged for 2.5 hours at 252,000 x g in a Beckman 60 Ti rotor. The supernatant fluid was discarded and the ribosome pellets used to prepare globin mRNA either immediately or the pellets were frozen at -80° for preparation of mRNA at a later time. The pellets were resuspended in HSB (High Salt Buffer, 1 mM EDTA, 150 mM NaCl and 10 mM Tris-HCl pH 7.5) and the suspension centrifuged at 16,000 x g x 10 minutes. The supernatant fraction was adjusted to 50 A_{260}/ml with HSB. The solution was warmed to room temperature and made 0.5% in SDS. After 5 minutes of standing, the RNA solution was added to a 30 ml Corex tube containing 1 gram of oligo (dT) cellulose which had been equilibrated with HSB. This slurry was mixed gently on a rocking shaker for one hour to allow the poly(A) containing fraction to hybridize to the oligo (dT). The slurry was poured into a sterile 1.0 x 10 cm glass column. The oligo (dT) cellulose column was washed with HSB

containing 0.5% SDS until A_{260} of the eluate fell to less than 0.05. The column was then washed with 2 volumes of HSB to remove SDS and the poly(A) containing RNA was then eluted from the column with H_2O. The fractions containing the RNA were pooled, made 1 mM in EDTA and heated for 10 minutes at 65°C. The solution was made 150 mM in NaCl and re-applied slowly to the oligo (dT) cellulose column. The column was then washed with HSB until the A_{260} of the eluate fell to 0.02. The mRNA was eluted with H_2O, pooled, and made 2% in KOAc. Two volumes of ethanol were added and the mixture allowed to stand at -20°C overnight to precipitate the messenger RNA. The precipitated mRNA was collected by centrifugation at 9000 x g x 20 minutes at 0°. The pellet was dissolved in a minimal volume of H_2O and the absorbance of the solution at 260 nm determined. The RNA solution was adjusted to a concentration of 1 mg/ml (assuming 20 A_{260} units/ml = 1 mg/ml RNA) and stored at -80° in small aliquots.

Identification of Products from Cell-Free Protein Biosynthesis: Separation of α- and β-Globin

The identification of the products synthesized by the reticulocyte lysate cell-free system or by either of the mRNA dependent cell-free systems was done by ion exchange chromatography using CM cellulose according to a modification of the procedure of Dintzis (74). Aliquots of postribosomal supernatant fractions of [^{3}H]-labeled lysates which had been centrifuged at 105,000 x g x 150 minutes to remove ribosomes were added slowly to 30 volumes of 0.6 N HCl in acetone maintained at -40° in a dry ice-acetone bath. Following 45 minutes of stirring, the globin was recovered by centrifugation at 13,000 x g x 30 minutes. The pellet was dissolved in Dintzis buffer (0.02 M pyridine, 0.2 M formic

acid, 0.05 M 2-mercaptoethanol) and dialyzed overnight against two 1 liter portions of the same buffer. The dialyzed solution was centrifuged at 30,000 x g x 20 minutes to remove insoluble material. A solution containing approximately 100,000 cpm was loaded onto a 1 x 25 cm CM32 cellulose column previously equilibrated with Dintzis buffer. The column was washed with 10 column volumes of Dintzis buffer and then eluted with a pyridinium formate gradient as previously described (75). Three ml eluate fractions were collected directly into scintillation vials and counted after addition of 10 ml of Formula 963.

Polysome Labeling for Preparation of Peptidyl-tRNA

Labeling of peptidyl-tRNA was performed in 950 µl incubations in the case of reticulocyte lysates, 400 µl for mRNA dependent reticulocyte lysates and 500 µl for wheat germ lysates. Incubations for wheat germ lysates were performed at 28° for 20 minutes while the reticulocyte lysates were incubated for 10 minutes at 37°. The labeling reactions were terminated by addition of two volumes of Medium B (75) containing 210 µM sparsomycin and 59 µM cycloheximide (medium B and antibiotics). Polyribosomes were isolated by centrifugation at 105,000 x g x 150 minutes at 2-4°.

Peptidyl-tRNA Purification

The polysomal pellet was resuspended in 0.75 ml of 0.25 M sucrose, 210 µM sparsomycin, 59 µM cycloheximide. The suspension was made 3.0 M in LiCl, 4.0 M in urea, 50 mM in 2-mercaptoethanol and 50 mM in NaOAc, pH 5.6 in a final volume of 2.0 ml. The mixture was incubated at 0° for 16 hours after which time it was centrifuged at 25,000 x g x 20 minutes. The supernatant fraction was applied to a 1.5 x 45 cm Bio-Gel P-10 column equilibrated with Buffer I. The column was eluted with Buffer I and the

radioactive material eluting in the void volume applied to a 1.0 x 2 cm DE52 cellulose column equilibrated with Buffer I. The DE52 column was washed with 100 volumes of Buffer I following which peptidyl-tRNA was recovered by stepwise elution of the column with Buffer II. The fractions eluted with Buffer II containing radioactivity were pooled and concentrated by ultrafiltration in an Amicon 8MC ultrafiltration cell (Amicon Corp., Lexington, Massachusetts) using an Amicon UM-2 membrane. When the sample had been concentrated to a volume of 0.3-0.5 ml, 3 ml of 6 M guanidinum chloride, 100 mM 2-mercaptoethanol pH 6.5 were added and the solution again concentrated to 0.5 ml. The concentrated peptidyl-tRNA was made 0.3 M in NaOH and incubated at 37° for 4 hours in order to cleave aminoacyl and peptidyl-tRNA bonds. The solution was then adjusted to approximately pH 5.6 with 6 N HCl as determined by pH paper and stored at -20° until analyzed.

Preparation of the Bio-Gel A 0.5m Column and Analysis of Nascent Peptide Size Distribution

The size distribution of nascent peptides was analyzed by gel filtration chromatography as described by Protzel and Morris (65). A slurry of 225 ml of Bio-Gel A 0.5m was brought to a final volume of 1 liter with deionized water in a graduated cylinder. The gel was allowed to settle to 40% of the original column height followed by removal of the upper layer by aspiration. This sequence was repeated twice. The slurry was then allowed to settle completely and the supernatant fluid was removed. The slurry was decanted into a 1 liter flask and sufficient solid guanidinum chloride slowly dissolved in the slurry to make 500 ml of 6 M guanidinum chloride solution. The slurry was then made 100 mM in 2-mercaptoethanol, titrated to pH 6.5 with 1 N HCl and brought to a

volume of 500 ml in a graduated cylinder. The slurry was de-gassed and poured in 1.5 x 100 cm Pharmacia analytical column to which sufficient 6 M guanidinum chloride had been added to fill the lower 20 cm of the column. Following 10 minutes of standing, the column was allowed to pack under a constant hydrostatic head of 50 cm. When the bed height had reached 90 cm, packing was stopped and the column washed with 2 column volumes of guanidinum chloride. The column was run for a minimum of 24 hours before application of a sample.

A sample to be analyzed was made 50 mM in dithiothreitol and 8% in sucrose. After two hours at room temperature, 60 μl of 3.6% Blue Dextran in 6 M guanidinum chloride and 40 μl of 0.2% DNP-alanine in 6 M guanidinum chloride were added to act as markers for the excluded and included volumes. The sample was applied to the column through a column of running buffer by layering the sample onto the top of the gel bed with a pasteur pipet. The sample was analyzed by gel filtration chromatography at a flow rate of 6 ml/hr and 0.9 ml fractions collected directly into scintillation vials for determination of radioactivity. Nascent peptide distributions were constructed by counting of 120 fractions which eluted between K_d=0 and K_d=1.

Cyanogen Bromide Cleavage of α- and β-Globin

Cyanogen bromide cleavage of globin was performed in a fume hood. [^{14}C]-labeled rabbit globin was dissolved in 70% formic acid at a concentration of 5 mg/ml. Cyanogen bromide was added to a 400 fold molar excess with regard to the methionine present. The solution was incubated in the dark for 48 hours at room temperature. After 48 hours of reaction the mixture was diluted with 10 volumes of water and lyophilized.

Determination of Distribution Coefficients

Elution data obtained from the elution of products during Bio-Gel A 0.5 m gel filtration chromatography in 6.0 M guanidium chloride were treated as described by Fish and Tanford (76).

The distribution coefficient, K_d, was calculated from the formula:

$$K_d = (V_e - V_o)/(V_1 - V_o)$$

where V_e = mass of solvent corresponding to the peak concentration of eluting solvent.

V_o = column void volume in mass of solvent.

V_1 = mass of solvent contained within the column and the gel matrix.

In this work, parameters were determined using the volume rather than the mass of the solvent.

Blue Dextran 2000 was used as a marker for the void volume and DNP-alanine employed as a marker for the volume of column which was accessible to solvent. The eluate fractions containing Blue Dextran and DNP-alanine were identified by visual inspection of the fractions. K_d values were then assigned to intermediate fractions by their relative positions between these markers for K_d 0 and K_d 1.0, respectively.

Recrystallization of Guanidinium Chloride

Guanidinium chloride was recrystallized by a modification of the method of Nozaki and Tanford (77). One kilogram of practical grade guanidinium chloride was dissolved in sufficient absolute ethanol at 75° with stirring to yield 3.5 l of solution. One gram of activated charcoal was added to the solution followed 5 minutes later by 1 gram of celite. The solution was then filtered hot through Whatman No. 1 filter paper in

an 18.5 cm Buchner funnel using a gentle vacuum. The filtrate was heated to redissolve any crystals which may have formed during filtering and sufficient toluene was added to induce crystal formation. The solution was allowed to cool to room temperature and then placed at 4° overnight.

The crystals were harvested by filtration in an 18.5 cm Buchner funnel, washed with -20° ethanol and air dried. The dried crystals were dissolved in a minimum volume of absolute methanol at 70°. When complete dissolution was achieved, the solution was allowed to cool to room temperature and then placed at -20° overnight. The crystals which formed were harvested as before and dried to constant weight in vacuo.

Preparation of Stock Urea, Buffer I, Buffer II, and DE52

Stock urea was prepared by deionizing a solution of 9.0 M urea by stirring with Amberlite MB-3 for at least 4 hours. The Amberlite was removed by filtration through a medium grade sintered glass funnel and the volume of the resulting filtrate adjusted to yield a solution with a final urea concentration of 8.54 M. This stock urea was used to make Buffers I and II for peptidyl t-RNA purification as described by Chaney and Morris (66).

DEAE cellulose (DE52) was suspended in 0.5 N HOAc (10.5 g DE52/100 ml 0.5 N HOAc). CO_2 was removed by agitating the suspension under reduced pressure. The pH of the slurry was adjusted to 5.6 with saturated NaOH and the suspension was allowed to settle to one half its original column height. "Fines" were removed by aspirating off the supernatant layer. The cellulose was resuspended in Buffer II and fines removed as before followed by two more washings in Buffer I.

Separation of Rabbit α- and β-Globin mRNAs

Rabbit globin mRNA was separated into its α and β components by preparative polyacrylamide gel electrophoresis using a Bio Rad agarose gel bridge. The gel was composed of 2.6% acrylamide wih a bisacrylamide to acrylamide ratio of 1:20 in 7 M urea. To prepare a gel for the separation of mRNA the legs of the gel bridge were filled with a 4.5% acrylamide gel in 7 M urea. The gel legs were prepared by mixing 22.5 ml of 40% acrylamide (acrylamide:bis of 20:1), 20 ml of 10 x TBE (1 X TBE = 90 mM Tris, 80 mM Boric acid and 2.5 mM Na_2EDTA) and 155.5 ml 9 M deionized urea. The solution was de-gassed, made 0.08% in ammonium persulfate, and 0.08% in TEMED and poured into the legs of the gel. The horizontal slab gel for the separation of mRNA was prepared by mixing 243 ml 9 M urea, 25 ml 31.5% acrylamide and 30 ml 10X TBE. Following removal of dissolved gasses, the acrylamide solution was supplemented with ammonium persulfate and TEMED to 0.08% and poured into the gel bridge. The horizontal polyacrylamide slab gel measured 15 x 21 x 1 cm.

After the gel had polymerized for one hour it was placed at 4° to cool until use. The RNA sample was made 7 M in urea by addition of RNAase free ultra pure urea. The sample was heated to 65° for 5 minutes and quick cooled; 0.1 volume of 10X TBE and 0.05 volume of 0.1% bromophenol blue in 50% glycerol were then added. The sample was loaded into the gel as a single band in a 1 mM x 1 cm x 10 cm sample slot made in the gel during casting. Electrophoresis was performed using a Bio-Rad model 1405 horizontal electrophoresis system, maintained at 20°, at a constant 100 V. Electrophoresis was stopped when the dye front had migrated the full 15 cm to the edge of the gel. The gel was removed from the bridge and stained with 2 μg/ml ethidium bromide in 0.2 M KCl until

the α- and β-globin mRNA bands could be seen under long wave UV light using a UVSL-25 mineralite lamp (Ultraviolet Products, Inc., San Gabriel, California). The bands were excised with a razor blade. The RNA was recovered by electrophoretic elution using a modification of the procedure of Weinand et al. (78). A horizontal 1% agarose gel in TBE was prepared in a 12 x 20 x 1.2 cm plexiglass tray. Two rectangular shaped holes of twice the width of the gel slice to be eluted were cut out of the gel. These slots were lined with sterile dialysis tubes which had been slit open at one side and knotted at both ends making sure the dialysis bags contacted all sides of the holes. A gel slice was placed in each bag, positioning the gel slice closest to the cathode. The remainder of the hole was filled with TBE to the upper edge of the gel slice. The agarose gel was connected by 3 MM paper wicks to the buffer chambers of the Model 1405 electrophoresis unit and electrophoresis performed overnight at 200 V. After electrophoresis the buffer in each of the gel slots was thoroughly mixed making sure that the portion of the dialysis bag nearest the anode was well agitated. The buffer was then removed, placed in a sterile Corex centrifuge tube and extracted with n-butanol to insure removal of all ethidium bromide. The buffer was then made 150 mM in NaCl and the mRNA bound to a 0.50 ml oligo (dT) cellulose column in a pasteur pipet. The column was washed and the RNA eluted as described above.

Translational Purity of Rabbit α- and β-Globin mRNAs

The translational purity of the α- and β-globin mRNAs was determined by the ratio of α- and β-globin synthesized when each mRNA was translated in either the nuclease inactivated lysate or the wheat germ lysate. An aliquot of the appropriate lysate was made 5 μg/ml in either α- or

β-globin mRNA and 100 μCi/ml in a radiolabeled amino acid. The incubation was allowed to proceed for 30 minutes followed by centrifugation of the incubation mixture for 2 hours at 105,000 x g at 4°. The supernatant fractions were analyzed for the amount of α and β globin synthesized by chromatography on CM cellulose as described above.

Limited Nuclease S_1 Digestion of Globin mRNA

Lyophilized nuclease S_1 was dissolved in S_1 Buffer (50 mM NaOAc pH 4.5, 200 mM KCl and 1 mM $ZnCl_2$) to a final concentration of 10^6 units/ml and stored at 4°. This solution was used as a working stock. Globin mRNA was made 1X in S_1 Buffer by addition of one tenth volume 10X S_1 buffer and the solution adjusted to an RNA concentration of 250 μg/ml. Nuclease S_1 was added to a final concentration of 250 units/ml and the reaction mixture incubated at 37°. At 5 minute intervals, aliquots of the mixture were withdrawn and the reaction terminated by addition of 0.1 volume of 0.5 M Tris-HCl pH 7.5, 0.02 M EDTA. Samples were stored at -80° until used.

3' End Labeling of Globin mRNA

The reaction conditions for the 3' labeling of globin mRNA are a modification of those used by 3' end labeling of RNA by England et al. (79). The components of the 3'labeling mixture are shown in Table III.

Table III. Final concentration of Components in the 3' Terminal Labeling of Globin mRNA.

HEPES pH 8.3	50 mM
$MgCl_2$	10 mM
Dithiothreitol	3.3 mM
ATP	6 μmM
Bovine Serum Albumin	10 μg/ml
Dimethyl Sulfoxide	10% (v/v)
5'[^{32}P]-pCp (2000-3000 Ci/mmole)	1.0 μM
mRNA	200 μg/ml

The concentration of mRNA in the reaction was approximately 1 μM. The reaction was allowed to proceed at 4° for 16 hours. The reaction was terminated by the addition of SDS and NaCl to final concentrations of 0.5% and 150 mM respectively. Unreacted pCp and any other low molecular weight impurities were removed by application of the reaction mixture to a small oligo (dT) cellulose column made in a pasteur pipet. The column was washed with HSB until no ^{32}P was detected in the column effluent. Labeled globin mRNA was then eluted from the column with H_2O, made 2% in KOAc pH 5.5 and precipitated with 2 volumes of ethanol. The mRNA was collected by centrifugation for 15 minutes in an Eppendorf 5412 table top centrifuge. The pelleted RNA was dissolved in water to a final concentration of 250 μg/ml as determined spectrophotometrically at 260 nm.

5' End Labeling of Globin mRNA

The 5' end of globin mRNA was labeled in a three step reaction based on a modification of a previous scheme (80). The 7-methyl guanosine of

the cap structure was removed with tobacco acid pyrophosphatase (TAP), the 5' end dephosphorylated with calf alkaline phosphatase, and the γ-[^{32}P]phosphate transferred from ATP to the 5' terminus of globin mRNA using T_4 polynucleotide kinase. For removal of the m^7G, 10 µg of globin mRNA were ethanol precipitated and redissolved in 20 µl of 50 mM NaOAc pH 6.0 freshly made 10 mM in 2-mercaptoethanol. One unit TAP was added and the mixture incubated at 37° for 30 minutes. Three µl of 0.5 M Tris-HCl pH 8.3 (at 37°), 1 µl of 5 units/ml calf alkaline phosphatase and sufficient water to bring the volume to 30 µl were added to the mixture. Dephosphorylation was carried out for 15 minutes at 37°. Two µl of 200 mM potassium phosphate pH 9.5 were added, followed by thorough stirring. The mixture was transferred to a tube containing dry γ-[^{32}P]-ATP. To this were added 2 µl of 0.2 M $MgCl_2$, 4 µl of 40 mM dithiothreitol, and 2 µl of 2000 units/ml T_4 polynucleotide kinase. The mixture was incubated for 30 minutes at 37°. The labeled mRNA was precipitated by addition of 100 µl 2 M NH_4OAc and 500 µl of ethanol; a procedure which precipitates RNA without precipitation of the proteins present in the mixture (33). The 5' end labeled mRNA was redissolved in HSB and repurified by chromatography on a Sephadex G-25 column to remove unincorporated label.

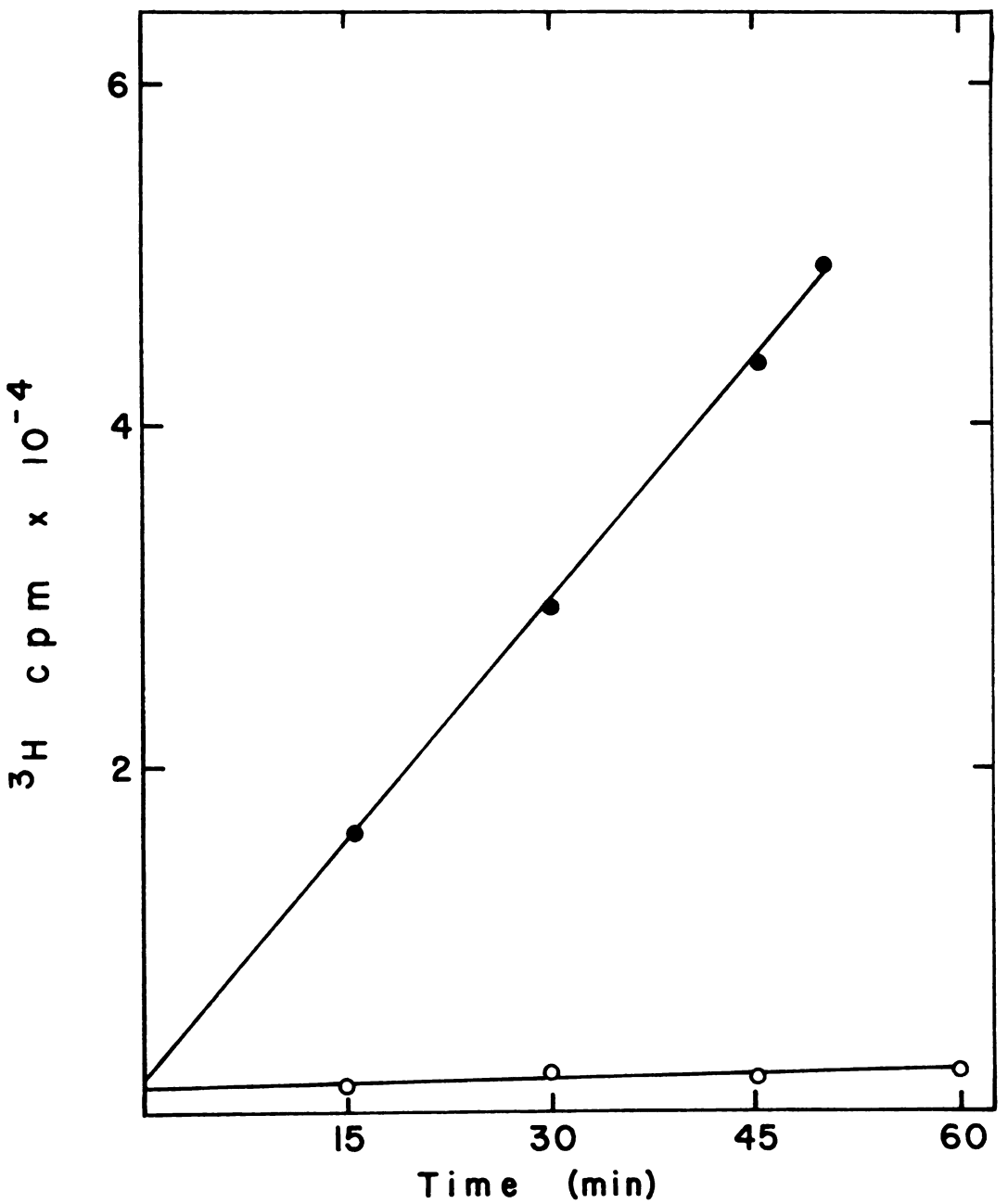
RESULTS

Wheat Germ Cell-Free System

In order to study the nonuniform accumulation of nascent globin peptides, globin mRNA purified from rabbit reticulocytes was translated in the wheat germ cell-free protein synthesizing system. In this manner, a determination could be made of whether or not the observed accumulations of nascent chain populations of discreet size classes were a function of the reticulocyte lysate biosynthetic system or some unique function associated with the globin messenger RNA itself. The wheat germ cell-free protein synthesizing system was chosen for the translation of purified globin mRNA for three reasons. First, this system was known to contain an extremely low level of endogenous messenger RNA activity and would respond well to exogenous mRNA. Second, the products synthesized in the wheat germ cell-free system could be labeled to a high specific radioactivity due to the ability to remove endogenous amino acids. Third, the wheat germ system was sufficiently phylogenetically distant from the rabbit reticulocyte system to provide a good comparative system for the study of the origion of the nascent peptide accumulations.

The wheat germ cell-free system was prepared by the method of Gallis et al. (71). Figure 1 shows the incorporation of [^{3}H]-leucine into TCA precipitable material in response to added globin mRNA. These data show that the system responded to added mRNA by incorporating the labeled amino acid into TCA precipitable material in a linear fashion for one

FIGURE 1. The incorporation of [^{3}H]-leucine into trichloracetic acid precipitable material by the wheat germ cell-free protein synthesizing system in the absence of added mRNA (○) or with 5 μg/ml added rabbit globin mRNA (●).


6
4
2
^3H cpm x 10^{-4}
15
30
45
60
Time (min)

hour. The rate of protein synthesis displayed an optimum at 3.0 mM $Mg(OAc)_2$ (Figure 2A) and 140 mM KOAC (Figure 2B). The rate of incorporation of labeled amino acid was dependent on the quantity of added mRNA until the system became saturated at a mRNA concentration of 10 µg/ml (Figure 3). All wheat germ incubations were performed at a concentration of 5 µg/ml of added mRNA in order to insure that the mRNA concentration was limiting.

Purification of Rabbit Globin mRNA

The mRNA used in these preliminary experiments was prepared by the method of Aviv and Leder (81). The method was found to yield low amounts of globin mRNA which were still highly contaminated with both 18S and 28S ribosomal RNA. Therefore, globin mRNA was purified by a modification of the "quick" method of Krystosek et al. as described in Methods. Figure 4 compares the rate of incorporation of globin mRNA purified by the two different methods. The mRNA prepared by the quick method of Krystosek directed incorporation of amino acids into polypeptides at twice the rate observed with mRNA purified by the method of Aviv and Leder. The doubling of the rate of synthesis was due to removal of rRNA found to contaminate mRNA prepared by the method of Aviv and Leder. All experiments were performed with globin mRNA purified by the method of Krystosek.

Characterization of the Product Synthesized in Response to Globin mRNA

A wheat germ lysate containing [^{3}H]-tryptophan was allowed to synthesize protein in response to added globin mRNA for 1 hour. Authentic [^{14}C]-globins were added to the incubation and polysomes were removed by centrifugation. Globins were prepared by the acid-acetone method and analyzed by CM cellulose chromatography as described in Methods. The elution profile shown in Figure 5 demonstrates that the

FIGURE 2. The dependence of the globin synthetic rate in the wheat germ cell-free system on the magnesium acetate concentration (Figure 2A) and the potassium acetate concentration (Figure 2B).

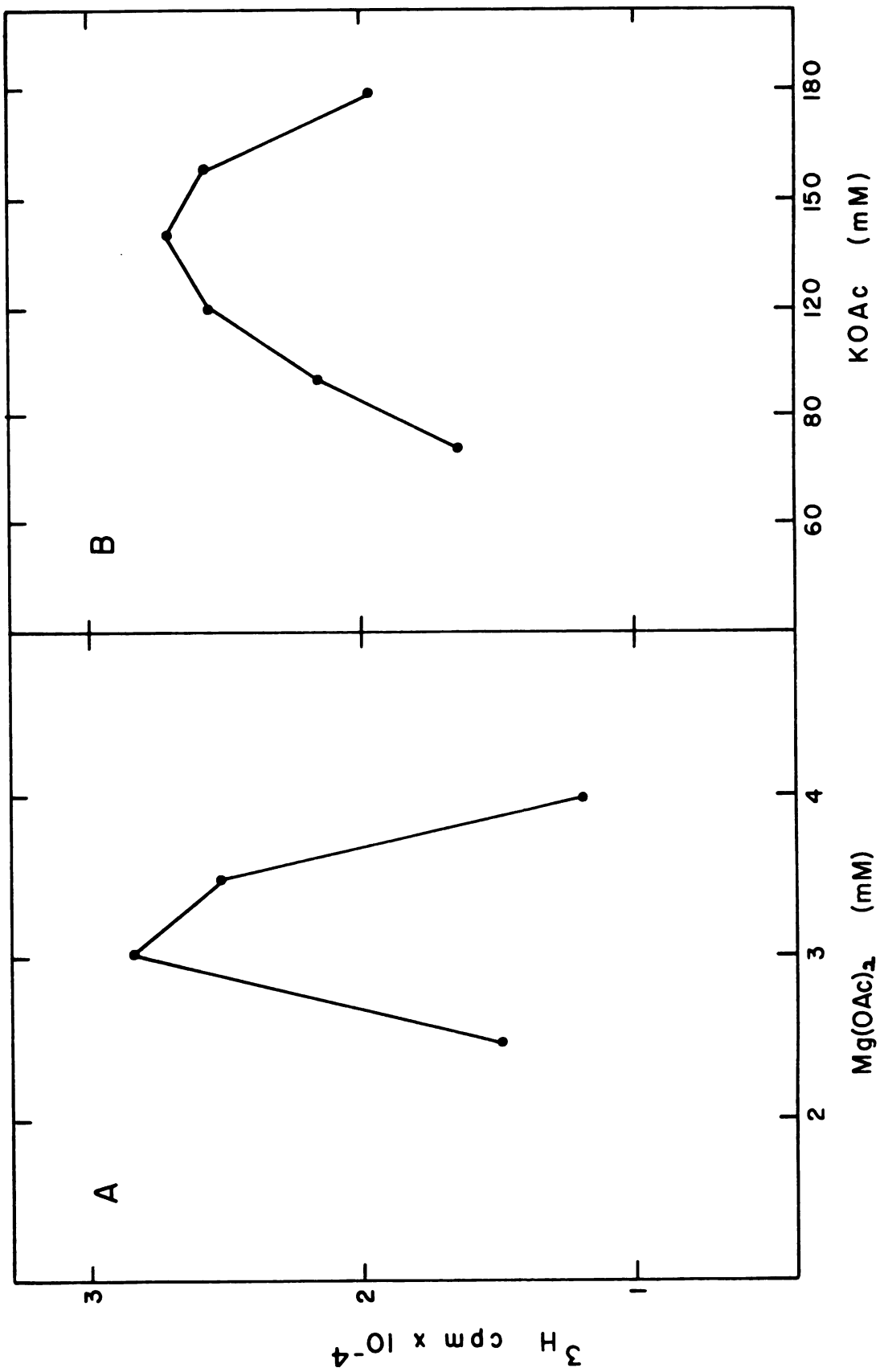

A
B
^3H cpm x 10^{-4}
1
2
3
$Mg(OAc)_2$ (mM)
2
3
4
KOAc (mM)
60
80
120
150
180

FIGURE 3. The effect of globin mRNA concentration on the rate of protein synthesis in the wheat germ cell-free system.

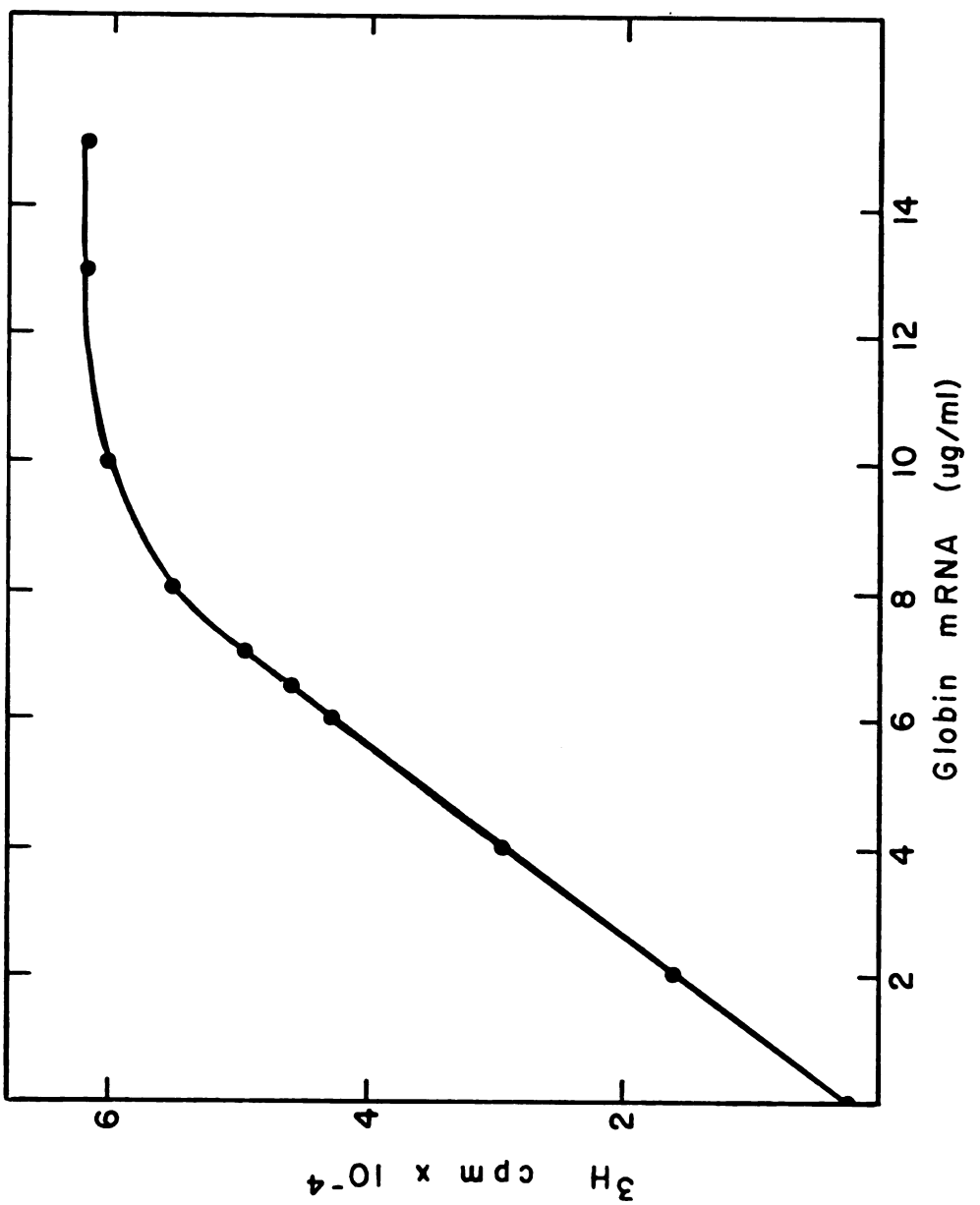

^3H cpm x 10^{-4}
6
4
2
2
4
6
8
10
12
14
Globin mRNA (ug/ml)

FIGURE 4. The incorporation of [^{3}H]-leucine into TCA precipitable material in the wheat germ cell-free system directed by globin mRNA purified by the method of Aviv and Leder (●), or Krystocek *et al.* (○).

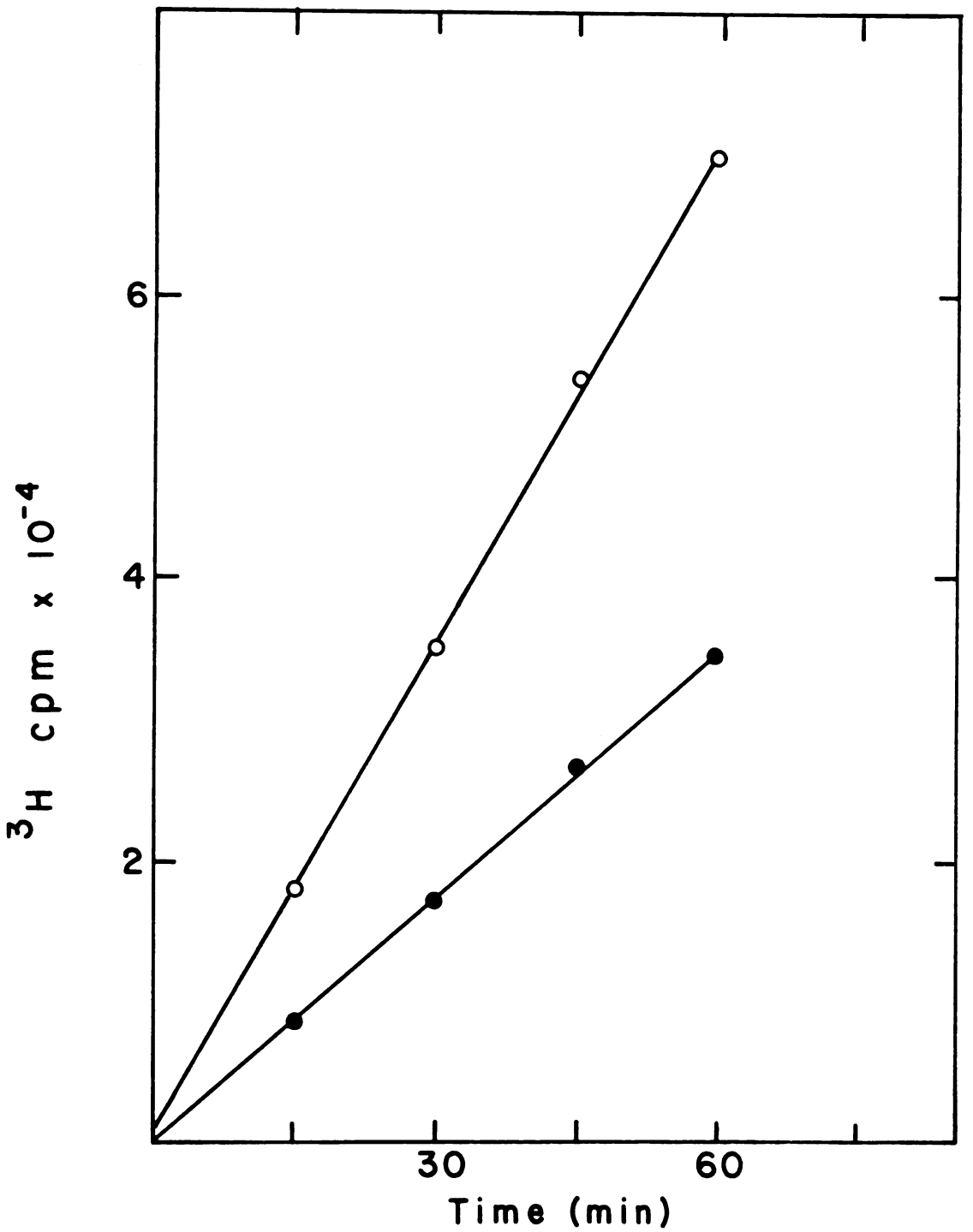
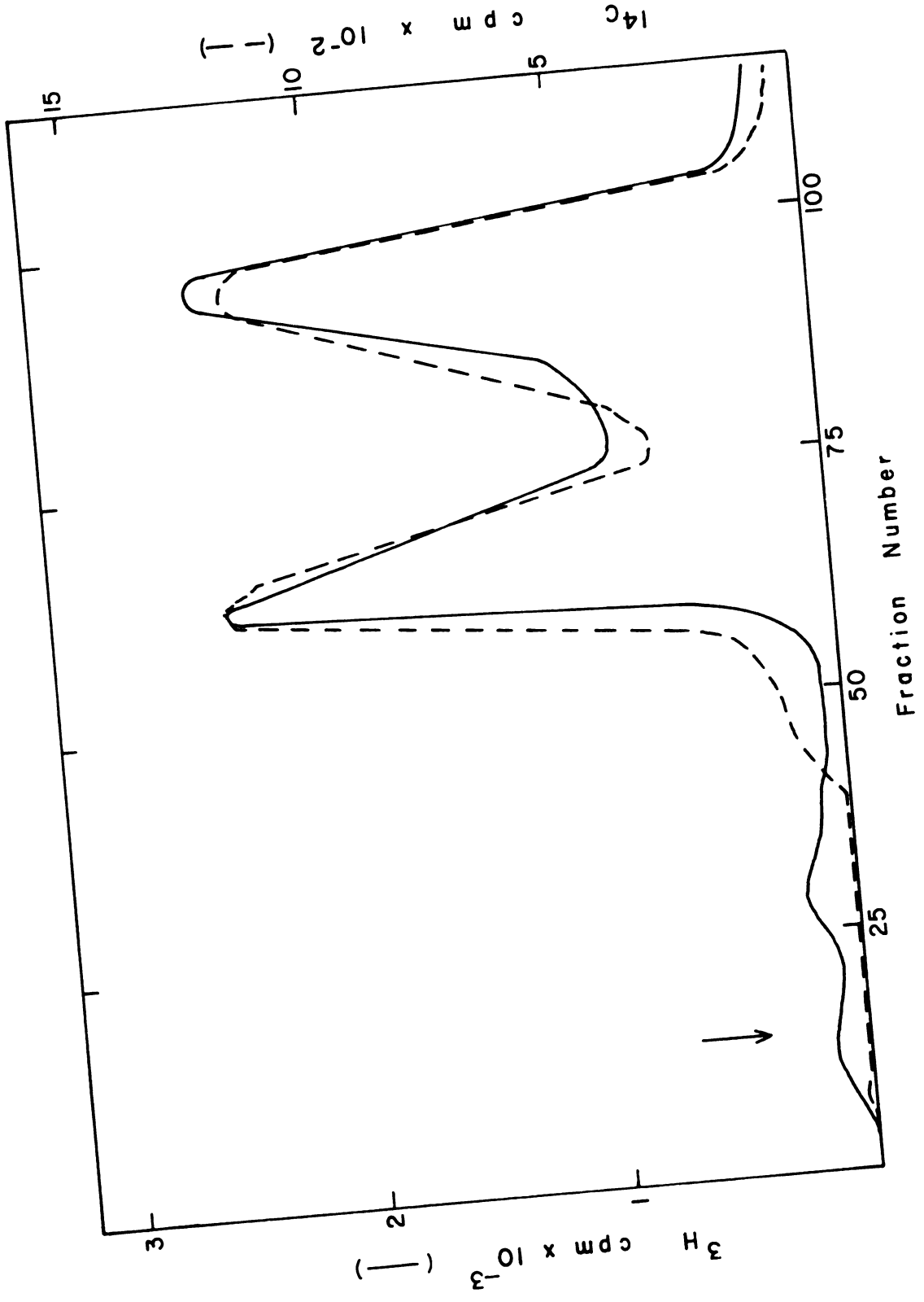

6
4
2
^3H cpm x 10^{-4}
30
60
Time (min)

FIGURE 5. CM-cellulose chromatography of the [^{3}H]-leucine labeled products synthesized in the wheat germ cell-free system in response to added globin mRNA. [^{14}C]-leucine labeled authentic α- and β-globins are included as internal standards. ^{3}H(——), ^{14}C (— —).


^{14}C cpm x 10^{-2} (— —)
15
10
5
^{3}H cpm x 10^{-3} (——)
3
2
1
100
75
50
25
Fraction Number

products of the wheat germ cell-free system co-migrated with authentic α- and β-globin during ion exchange chromatography. The α- and β-globin peaks were individually pooled and the solvent removed by lyophilization. The dried fractions were redissolved in 6 M guanidinum chloride, 100 mM 2-mercaptoethanol, pH 6.5 and chromatographed on a Bio-Gel A 0.5m column in the same solvent. Figure 6 shows the result of this procedure. It can be seen in Figure 6 that the product synthesized by the wheat germ system comigrated with the authentic globin molecules and are full length translation products.

Alpha and β-globin nascent chains were labeled with [^{3}H]-tryptophan for 20 minutes in a 0.5 ml wheat germ incubation mixture. Protein synthesis was terminated as described in Methods and the wheat germ incubation mixture combined with a reticulocyte lysate in which the nascent chains had been labeled with [^{14}C]-tryptophan. The polysomes were isolated by ultracentrifugation and the combined nascent chains purified. The size distributions of these nascent peptides were analyzed by Bio-Gel A 0.5m chromatography. The results of this analysis are seen in Figure 7. It can easily be seen that the nascent chain distribution which arises during the translation of globin mRNA in the heterologous wheat germ system are found at the same K_d values as those observed during globin synthesis in the reticulocyte lysate.

The occurrence of nascent chain accumulations of discrete sizes during globin synthesis in the wheat germ system indicates that the rate at which ribosomes traverse the mRNA is nonuniform in this system. In addition, the changes in the rate of translocation are the same as observed in both the intact reticulocyte and the reticulocyte lysate. While some slight variation in the magnitude of the peak heights may be

FIGURE 6. Bio-Gel A 0.5m gel filtration chromatography of α-globin and β-globin synthesized by the globin mRNA directed wheat germ cell-free system. Alpha and β-globins were separated by CM cellulose column chromatography and analyzed by Bio-Gel A 0.5m chromatography as described in the text. Authentic [^{14}C]-globins were included as internal standards. Figure 5A, analysis of α-globin; Figure 5B, analysis of β-globin, [^{3}H](——), [^{14}C](— —).

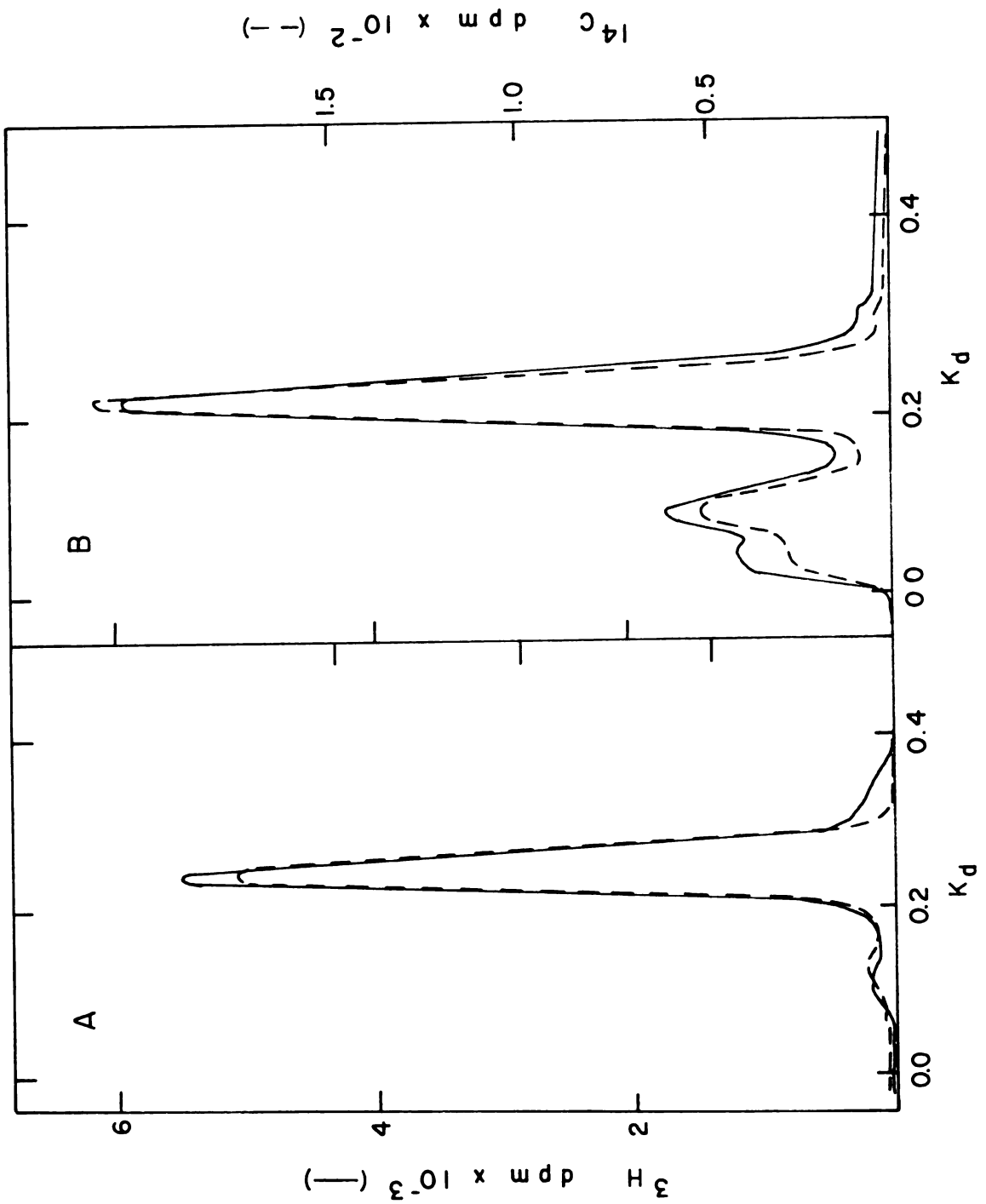
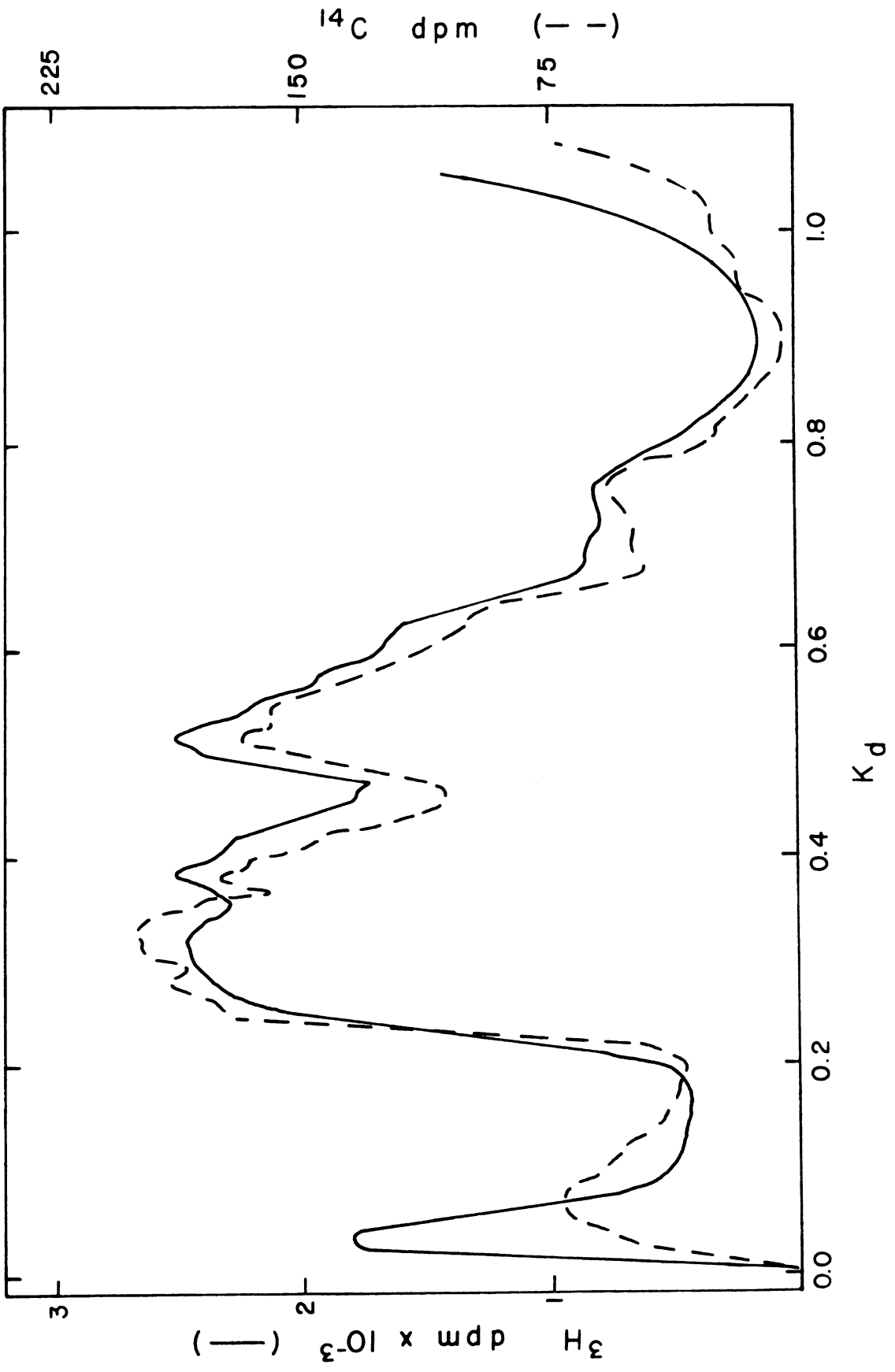

A
B
^3H dpm x 10^{-3} (——)
^{14}C dpm x 10^{-2} (– –)
K_d
K_d
0.0
0.2
0.4
0.0
0.2
0.4
2
4
6
0.5
1.0
1.5

FIGURE 7. Analysis of the size distribution of [^{3}H]-tryptophan labeled nascent peptides from the globin mRNA directed wheat germ cell-free system.


^{14}C dpm (— —)
225
150
75
^{3}H dpm x 10^{-3} (——)
3
2
1
0.0
0.2
0.4
0.6
0.8
1.0
K_d

observed, it is the position of the peak within the profile rather than its absolute magnitude which maps ribosome position on the message.

Calibration of the Bio-Gel A 0.5m Column

Fish and Tanford described a linear relationship between the $K_d^{1/3}$ and (molecular weight)$^{\cdot 555}$ for the elution of proteins from agarose based gel filtration columns using 6 M guanidinium chloride, 0.1 M 2-mercaptoethanol, pH 6.5 as a solvent (76). This relationship was verified for the elution of proteins from Bio-Gel A 0.5m by Protzel and Morris. The [^{14}C]-labeled cyanogen bromide generated fragments of rabbit globins (αCNBR1, βCNBR1) were used to calibrate the Bio-Gel A 0.5m column. Cleavage of [^{14}C]-tryptophan labeled rabbit α- and β- globin with cyanogen bromide generates only 2 labeled fragments.

Calibration of the Bio-Gel A 0.5m column was accomplished using a mixture of [^{14}C] β-globin, αCNBR1 and βCNBR2 supplemented with Blue Dextran and DNP-alanine as molecular weight markers.

Figure 8 shows the elution of these molecular weight markers from the column. Upon analysis of the data, the relationship of $K_d^{1/3}$ vs. MW $^{\cdot 555}$ was obeyed by the molecular weight markers. The data for the elution of the markers were fitted to a line by linear least squares regression and yielded the equation:

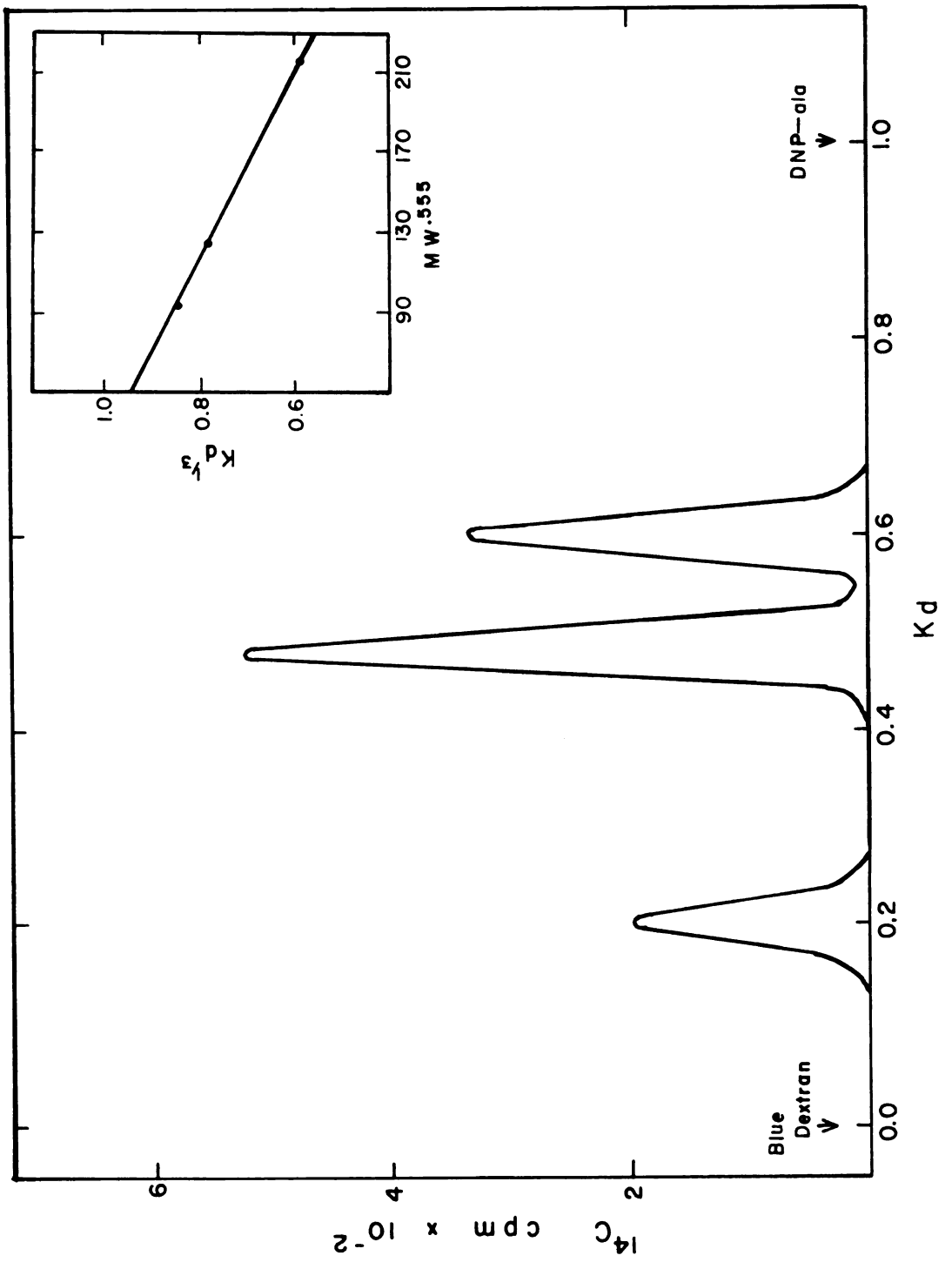
$$K_d^{1/3} = (-2 \times 10^{-3})MW^{\cdot 555} + 1.03$$

This relationship was used to calculate the average molecular weight of the peptides represented by the peaks and valleys in the nascent peptide distribution.

Methylmercuric Hydroxide Treatment of Globin mRNA

Methylmercuric hydroxide has been shown to be an effective denaturant for both DNA and RNA (82). Concentrations of 2.5 mM or

FIGURE 8. Calibration of the Bio-Gel A 0.5m analytical gel filtration column with [^{14}C]-tryptophan labeled cyanogen bromide fragments of α- and β-globin. The elution volumes of Blue Dextran and DNP-alanine are marked with arrows. The inset shows the plot of $K_d^{1/3}$ vs. $(MW)^{.555}$ for the data shown.


14C cpm x 10-2
Kd
Blue Dextran
DNP-ala
MW.555
Kd1/3

greater have been shown to denature nucleic acids as evidenced by alteration of electrophoretic mobility and by changes in optical properties of the polymers.

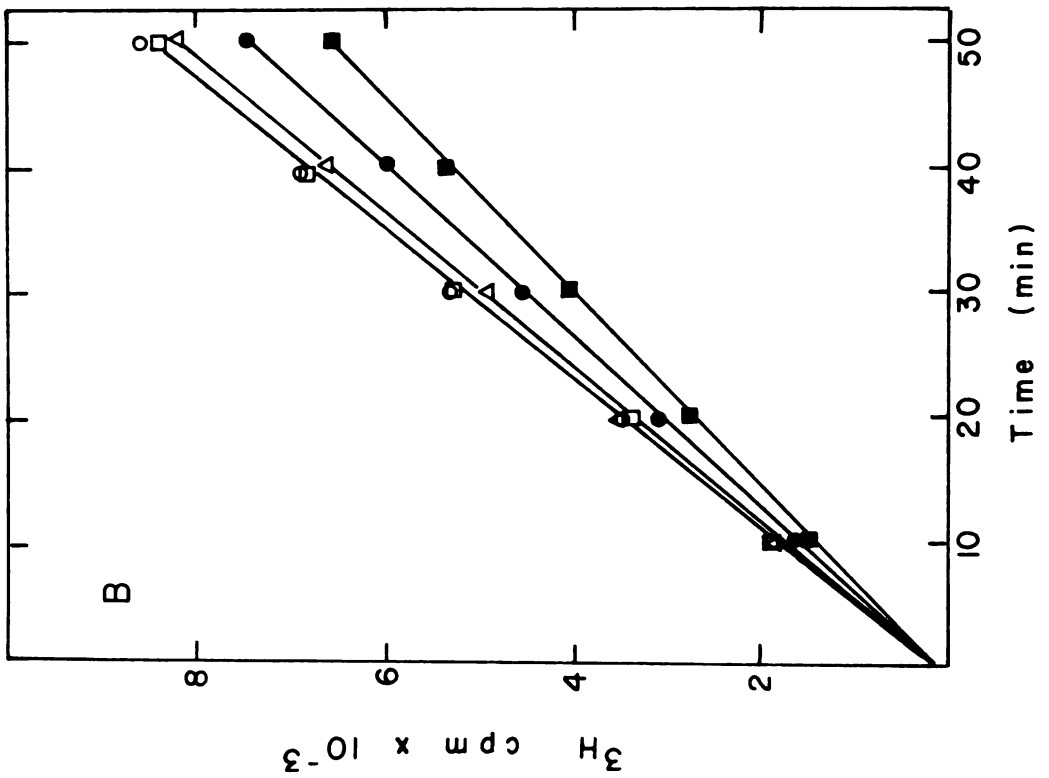
Payvar and Schimke have reported the enhancement of the translation of both ovalbumin and conalbumin mRNA in the mRNA dependent lysate following treatment of the mRNA with 2.5 mM methylmercuric hydroxide (83). Denaturation in this manner also changed the rate of synthesis of cDNA by reverse transcriptase as well as the length of cDNA transcript synthesized. These effects were attributed to secondary structure alteration of the mRNA upon denaturation.

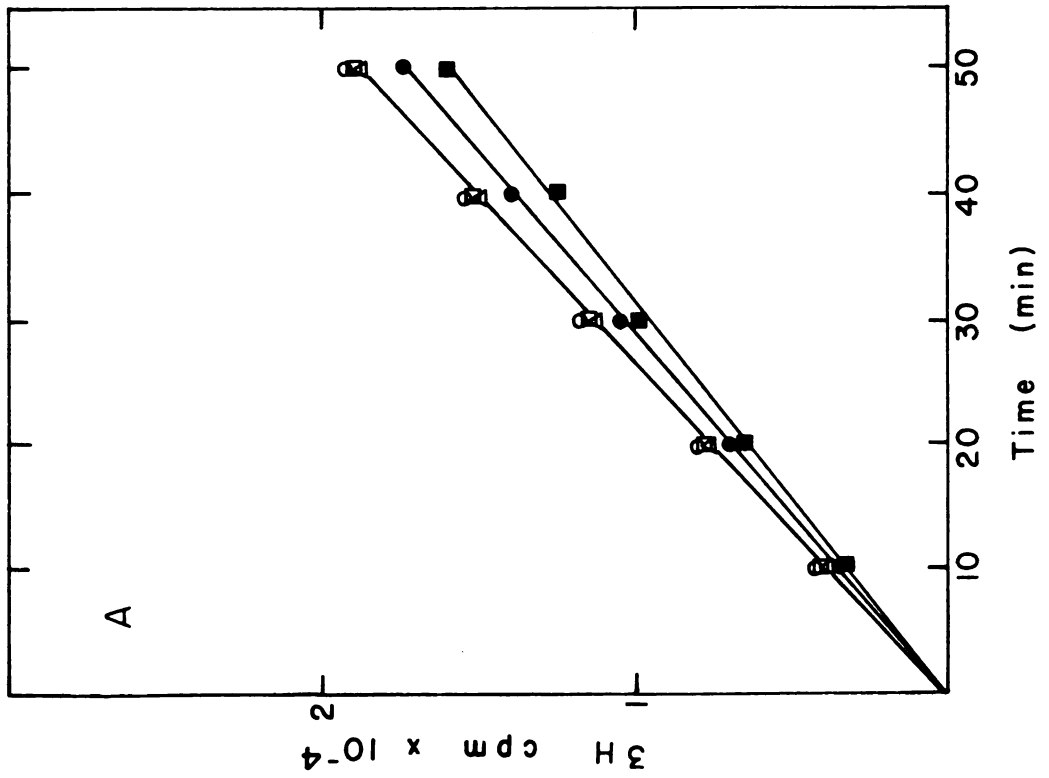
Experiments involving the translation of methylmercuric hydroxide treated globin mRNA in either the wheat germ cell-free protein synthesizing system or the mRNA dependent reticulocyte lysate were performed. These experiments were undertaken in order to determine if methylmercuric hydroxide denaturation of the globin mRNA would generate an altered nascent peptide distribution upon its translation.

The following procedure was performed in a fume hood. Methylmercuric hydroxide was added to globin mRNA to a final concentration of 0 to 5 mM. Control mRNA was treated with equivalent volumes of H_2O. The mRNA samples were incubated at room temperature for 5 minutes after which time the mRNA was immediately added to the appropriate protein synthesizing system.

The ability of methylmercuric hydroxide denatured globin mRNA to direct the synthesis of protein in either the mRNA dependent lysate or wheat germ cell-free system supplement with [^{3}H]-leucine is shown in Figure 9. Treatment of globin mRNA with concentrations of methylmercuric hydroxide up to 2.5 mM has no effect on the incorporation of

FIGURE 9. The effect of denaturation of globin mRNA with methylmercuric hydroxide on the incorporation of [^{3}H]-leucine into TCA precipitable material in the mRNA dependent reticulocyte lysate (Figure 9A) or the wheat germ cell-free system (Figure 9B). Pretreatment of globin mRNA at methylmercuric hydroxide concentrations of: ○, 0 mM (control): □ ,1.25 mM: △ ,2.5 mM: ○ ,3.75 mM: ■ ,5.0 mM.


B
^{3}H cpm x 10^{-3}
2
4
6
8
10
20
30
40
50
Time (min)


A
^{3}H cpm x 10^{-4}
1
2
10
20
30
40
50
Time (min)

[^{3}H]-leucine into TCA precipitable material. Treatment of RNA with concentrations greater than 2.5 mM showed lowered rates of protein synthesis. This reduction of synthesis was attributed to the inhibition of the cell-free systems by unreacted methylmercuric hydroxide at the higher concentrations of the denaturant used.

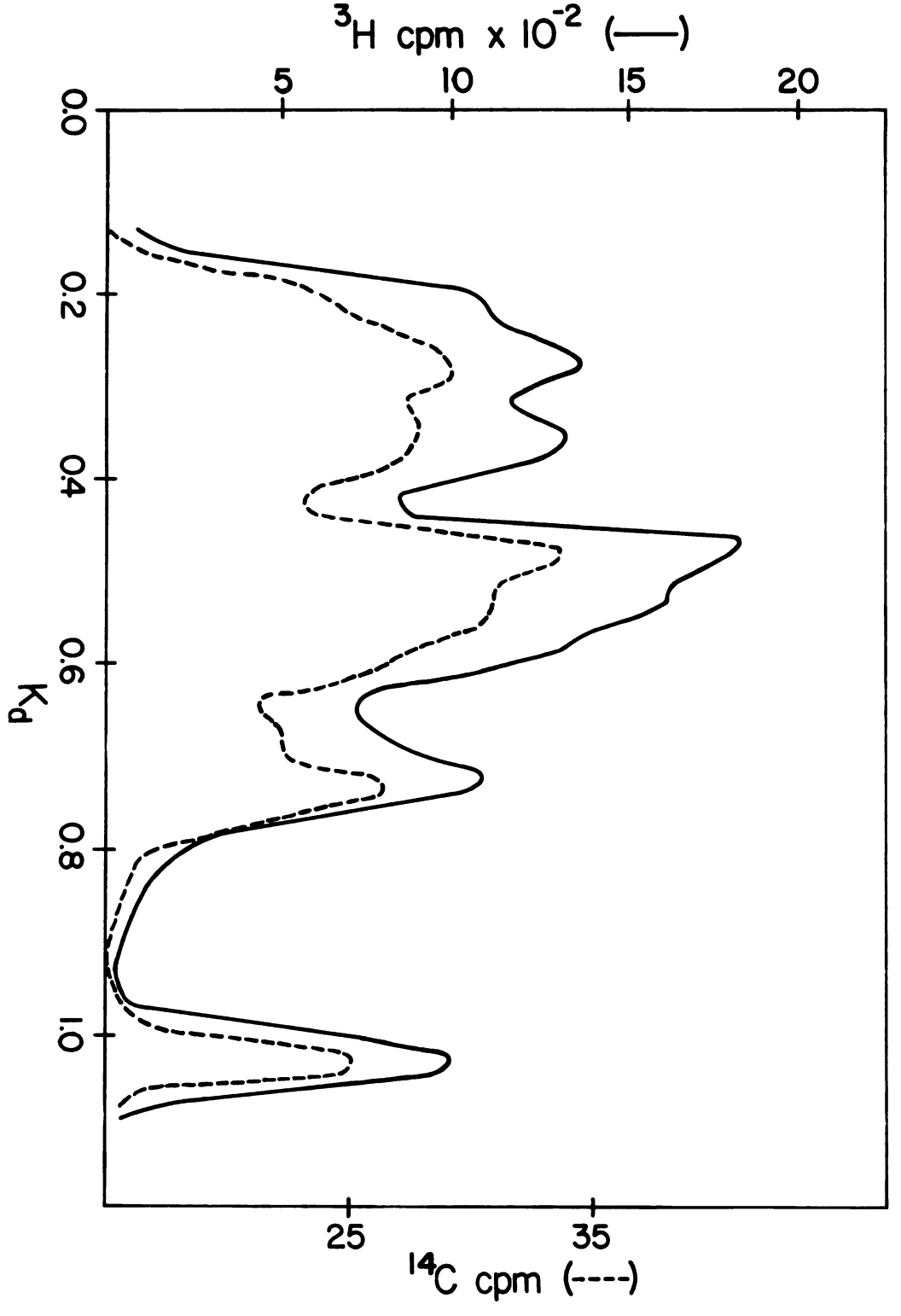
Globin mRNA treated with 2.5 mM methylmercuric hydroxide was incubated in both the cell-free protein synthesizing systems with [^{3}H]-tryptophan. The labeled nascent peptides were then mixed with [^{14}C]-tryptophan nascent peptides resulting from the translation of untreated globin mRNA.

Nascent peptides were co-purified and analyzed by chromatography on Bio-Gel A 0.5m. The result of methylmercuric hydroxide treatment of globin mRNA on the nascent peptide distribution in the wheat germ cell-free system is shown in Figure 10. The analogous experiment performed in the mRNA dependent lysate gave identical results. These experiments failed to show any change in the distribution of nascent peptides following methylmercuric hydroxide denaturation of globin mRNA. These data agree with results obtained by Chaney and Morris who demonstrated that denaturation of globin mRNA by a variety of methods had no effect on the observed size distribution of nascent peptides (84).

Limited Digestion of Globin mRNA with S_1 Nuclease

The presence of regions of base pairing within mRNA has been demonstrated for several mRNAs (17,18,22,28). Using S_1 nuclease, Wurst *et al*. reported a method for mapping regions of secondary structure (27). The method determines the location of paired and unpaired bases in a sequence of RNA and has been applied to the 5' region of mouse and rabbit globin mRNA (28). These reports led to the design of experiments which

FIGURE 10. The effect of methylmercuric hydroxide denaturation of globin mRNA on the size distribution of globin nascent peptides in the wheat germ cell-free system.

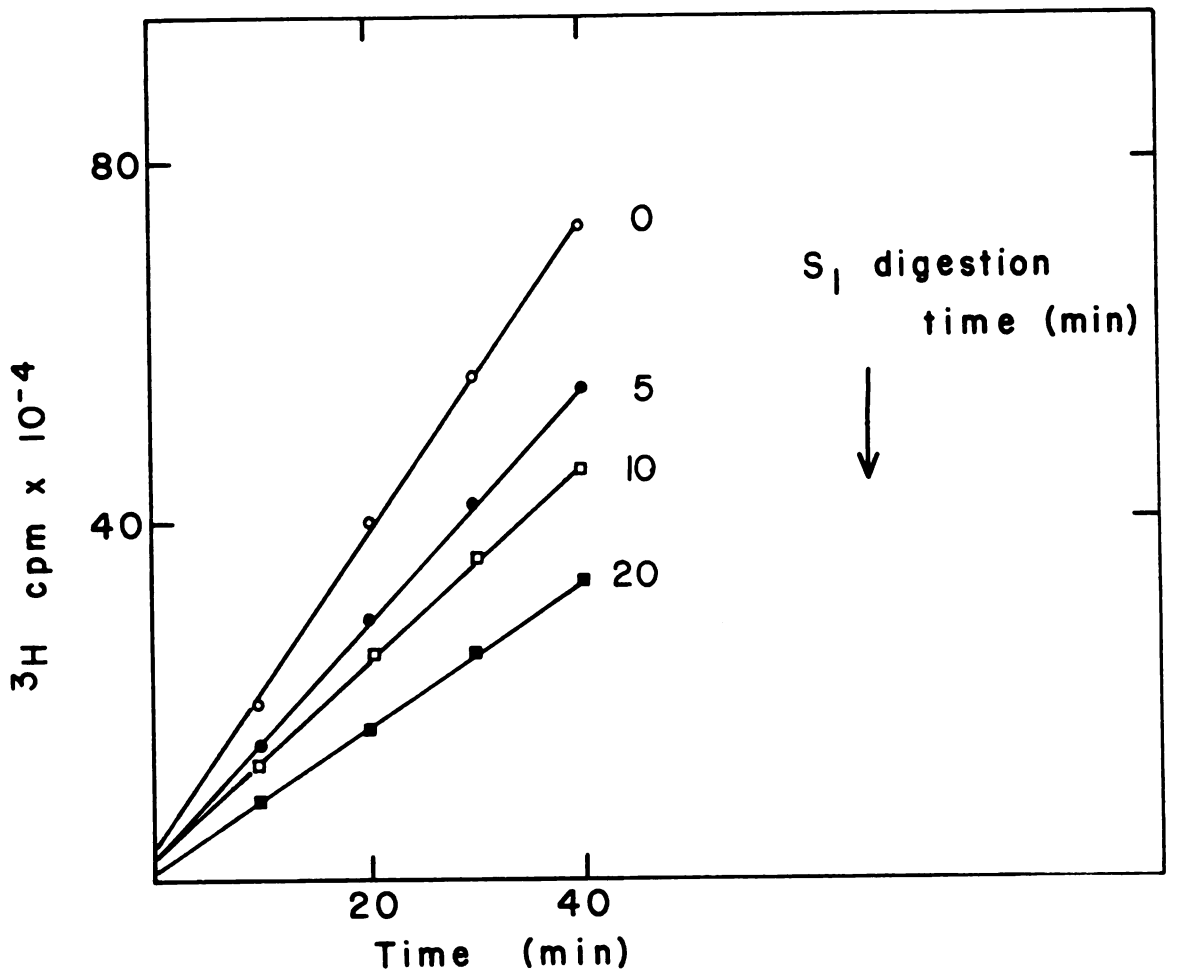

^{3}H cpm x 10^{-2} (——)
5
10
15
20
0.0
0.2
0.4
0.6
0.8
1.0
K_d
25
35
^{14}C cpm (----)

attempted to introduce cleavage points in the single stranded regions of globin mRNA. If secondary structure of the mRNA were somehow associated with the accumulation of nascent peptides, then translation of mRNA which had been cleaved at areas containing specific structural features might lead to specific changes within the distribution of nascent chains.

S_1 nuclease, a structure specific endonuclease, was used to introduce cleavages in globin mRNA at single stranded regions of the molecule. This cleaved mRNA was then translated in the wheat germ cell-free system to observe if changes in the nascent chain profile could be induced. Comparisons were made between a control nascent chain profile prepared from undigested mRNA with nascent chain profiles obtained from the translation of mRNA which had been subjected to increasing degrees of nuclease digestion. Differences in the positions of nascent chain accumulations within the profile as well as possible gross changes in peak heights could be used as evidence of a significant change in the distribution of nascent peptides.

Globin mRNA was subjected to digestion with S_1 nuclease under non-denaturing conditions. The conditions were chosen so as to obtain a 50% loss of the translational ability of mRNA after twenty minutes of digestion. Messenger RNA was digested for various times as described in Methods. Aliquots of the mRNA from each digestion time were translated in the wheat germ cell-free system to test for the ability to direct incorporation of amino acids into TCA precipitable material. Figure 11 shows the result of one such digestion and translation. The mRNA subjected to a 20 minute nuclease digestion directed the synthesis of globin at a 50% reduced rate when compared with control levels. Interestingly, a 25% loss of translational ability of the mRNA was

FIGURE 11. The rate of globin mRNA directed protein synthesis in the wheat germ cell-free system following mRNA digestion with S_1 nuclease.


80
40
^3H cpm x 10^{-4}
0
5
10
20
S_1 digestion
time (min)
20
40
Time (min)

observed within the first 5 minutes of digestion while an additional 15 minutes of digestion was required to reach a 50% loss. This may well indicate a rapid cleavage of the most exposed structures within the mRNA, followed by cleavages at less accessible regions.

Effects of S_1 Digestion of Globin mRNA on the Nascent Chain Size Distribution

The digestion of S_1 nuclease has been shown to specifically cleave globin mRNA in single stranded regions of the molecule (26,28). If such regions correlate with the slow and fast regions of ribosome movement along the messenger RNA, then translation of mRNA following partial nuclease digestion could alter the distribution of nascent peptides produced during translation of S_1 nuclease digested globin mRNA in a predictable fashion.

Nuclease treated mRNA was incubated in the wheat germ cell-free system with [^{3}H]-tryptophan for 20 minutes and nascent chains purified as described earlier. The results of the analyses of the nascent peptides by gel filtration are presented in Figure 12A-D. A control nascent chain profile is shown in Figure 13. The size distribution of nascent peptides isolated from the zero digestion time profile is identical to the distribution in the control profile. This indicates that the method used to stop S_1 nuclease digestion was valid and that the enzyme had been completely inactivated, and in itself, the presence of nuclease caused no perturbation of the nascent chain size distribution. A five minute digestion of mRNA has visible effects on the nascent chain profile. The shoulder in the control at K_d 0.53 shows the beginning of development of a peak while the shoulder at K_d 0.58 is also enlarged. The large peak at K_d 0.47 is reduced in magnitude while a peak at K_d 0.67 has

FIGURE 12. The effect of S_1 digestion of globin mRNA on the globin nascent peptide size distribution observed in the wheat-germ cell-free system. A - no mRNA digestion, B - 5 minute S_1 digestion of mRNA, C - 10 minute S_1 digestion of mRNA, D - 20 minute S_1 digestion of mRNA.

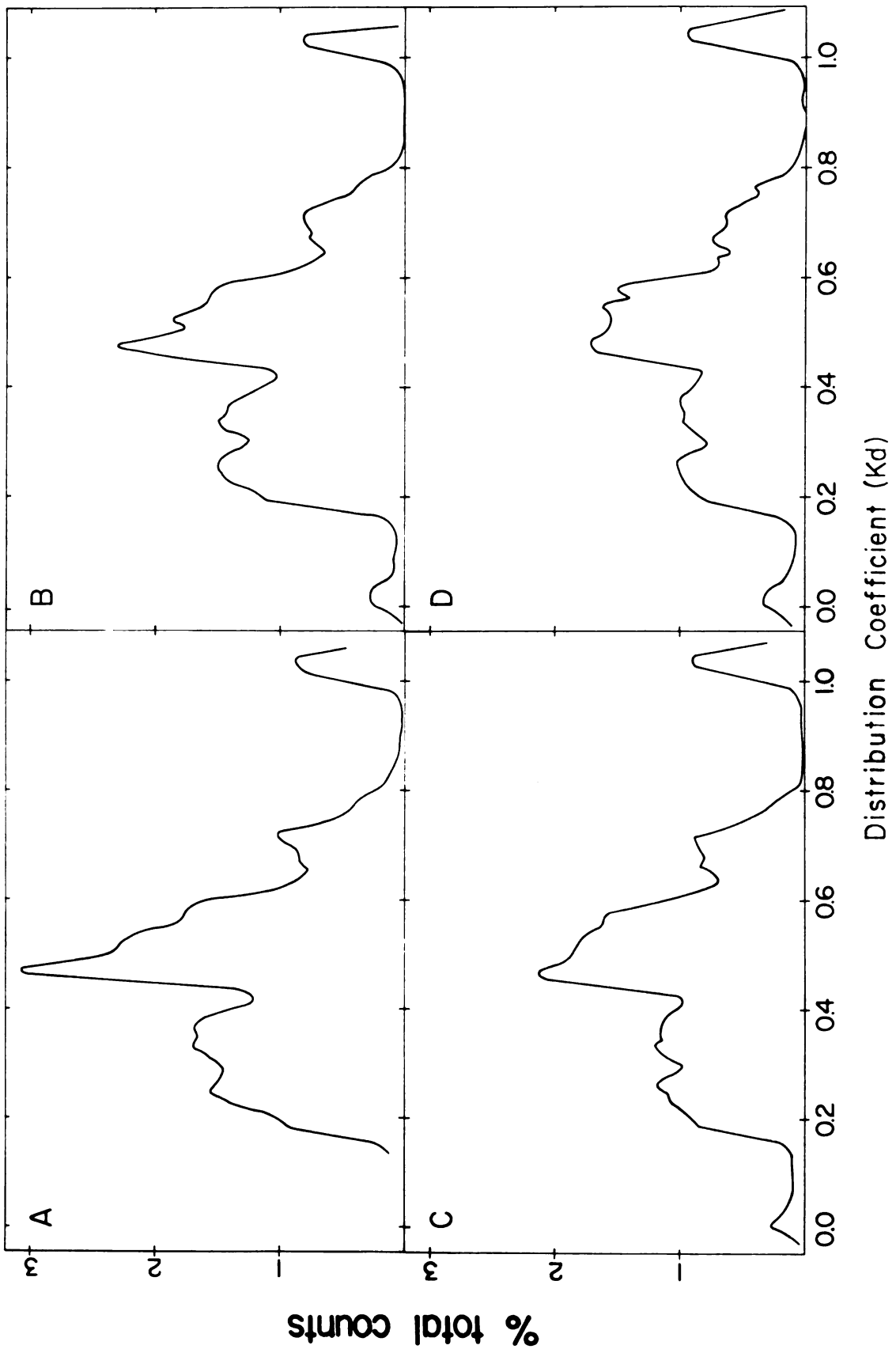
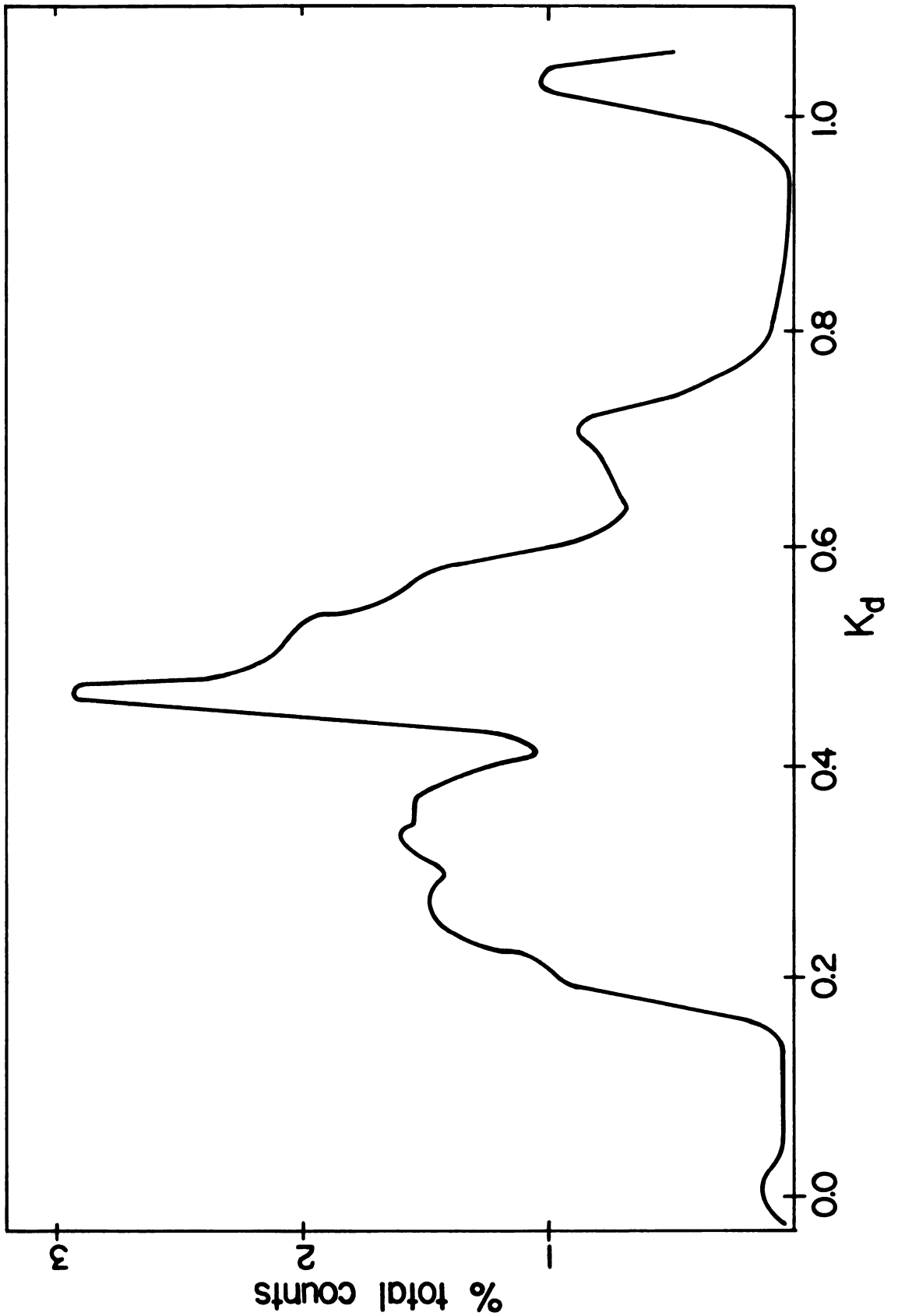

A
B
C
D
% total counts
Distribution Coefficient (Kd)
0.0
0.2
0.4
0.6
0.8
1.0
1
2
3

FIGURE 13. The size distribution of nascent peptides in the globin mRNA directed wheat germ cell-free system before S_1 nuclease digestion of globin mRNA.


% total counts
1
2
3
0.0
0.2
0.4
0.6
0.8
1.0
K_d

begun to form. At 10 minutes of digestion, the peaks of K_d 0.53 and 0.58 continue to develop as does the peak at 0.67. The area under the curve in the region from K_d 0.4 to 0.6 is greatly increased. By 20 minutes of nuclease digestion, the elution profile of nascent chains accumulating on the message is strikingly altered. Most noticeable is the downward shift of the major peak in the control profile at K_d 0.47 whose amplitude has been reduced as compared with other peaks. The peaks which appear at K_d 0.53 and 0.58 are now equal in magnitude and are prominant features. The minimum at K_d 0.43 has been reduced and the peak at K_d 0.67 is now larger than the peak at K_d 0.71. A small shoulder has developed at K_d 0.76. It is obvious then that selected cleavage of globin mRNA with S_1 nuclease does indeed alter the distribution of nascent chains in the elution profile.

The loss of nascent peptides isolated from the translation of mRNA of the wheat germ cell-free system did not follow the loss of mRNA translational ability. While a 50% decrease in the rate of globin synthesis was observed after a 20 minute digestion of mRNA only 28% of the nascent peptide pool had been eliminated. The significance of this result will be discussed in later sections.

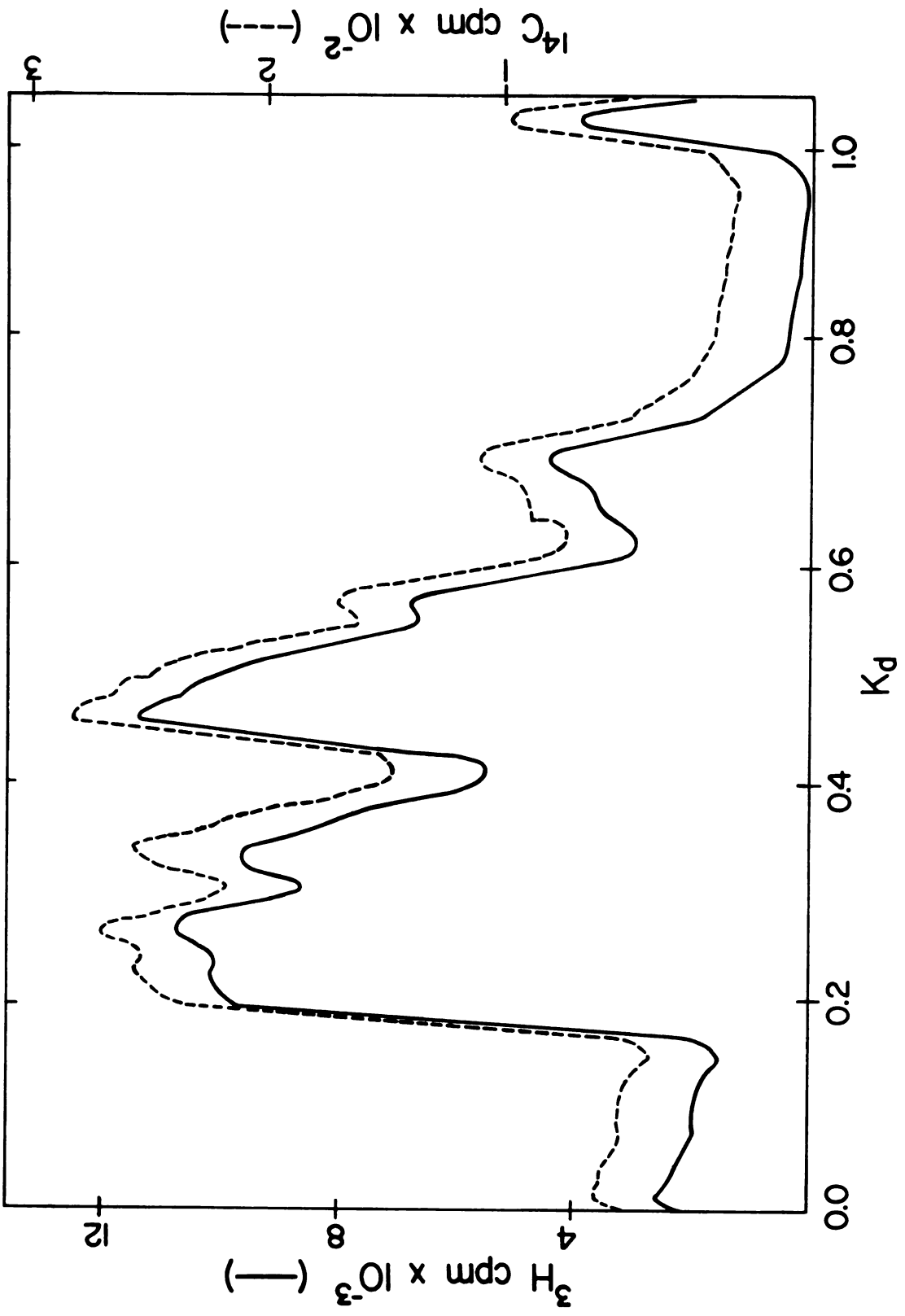
Preparation of a mRNA Dependent Rabbit Reticulocyte Lysate

The wheat germ cell-free system displayed considerable variation in the ability to synthesize protein in response to added mRNA when different batches of wheat germ were used. This variation was attributed to differences in wheat samples obtained from General Mills, Inc. since the differences were not observed between S-23 fractions prepared at different times from a single batch of wheat. This situation often required the procuring and testing of several batches of wheat before one

suitable for use was found. An attempt was therefore made to develop a translation system which displayed less batch to batch variation in the ability to synthesize protein. Pelham and Jackson reported the development of an efficient mRNA dependent reticulocyte lysate (72). This was accomplished by removal of the endogenous globin mRNA in the lysates with micrococcal nuclease, a calcium requiring nuclease isolated from Staphlococcus aureus. Initial experiments were performed to test the feasibility of this system in the study of the origin of the nascent peptide non-uniformity.

Since micrococcal nuclease was used to degrade the endogenous mRNA activity, it was necessary to show that no protease or Ca^{2+} independent nucleases were present in the micrococcal nuclease preparations used in these studies. A complete rabbit reticulocyte cell-free incubation was assembled and supplemented with $CaCl_2$ to 1 mM and EDTA to 2 mM. Micrococcal nuclease was added to a final concentration of 20 μg/ml and the mixture incubated at 20° for 15 minutes to simulate the digestion conditions to be used for endogenous mRNA degradation. A control lysate was incubated in parallel without $CaCl_2$, EGTA or nuclease. After 15 minutes at 20°, the nuclease containing lysate was supplemented with [^{3}H]-tryptophan, the control was supplemented with [^{14}C]-tryptophan and polysomes labeled by incubation at 37° for 10 minutes. Protein synthesis was terminated with medium B and antibiotics, the lysates were mixed together and the nascent peptides co-purified and analyzed. The results of such an experiment are shown in Figure 14. It can be seen that the two profiles are superimposible throughout the entire K_d range. Therefore there was no calcium independent nuclease or protease

FIGURE 14. The elution profile of nascent peptides from the reticulocyte lysate in the absence (——) or presence (— —) of inactive micrococcal nuclease.


^{3}H cpm x 10^{-3} (——)
4
8
12
^{14}C cpm x 10^{-2} (----)
1
2
3
0.0
0.2
0.4
0.6
0.8
1.0
K_d

activity associated with the micrococcal nuclease preparation that could alter the nascent chain distribution.

Destruction of Endogenous mRNA in the Reticulocyte Lysate

To determine the optimum concentration of nuclease required for removal of endogenous mRNA activity the following experiment was performed. A complete reticulocyte lysate was incubated at 20°. Aliquots of 100 µl of lysate were removed at specified time intervals, made 2 mM in EGTA and 100 µCi/ml in [^{3}H]-leucine and transferred to a 37° bath. The rate of protein synthesis for each digestion time was determined. Figure 15 shows that by 3 minutes of digestion 50% of the endogenous protein synthesizing activity was destroyed, and by 15 minutes >98% reduction of synthesis was attained. It is interesting to note that the rate of inactivation of the lysate was non-linear and followed a time course similar to that observed for the S_1 nuclease digestion of purified globin mRNA.

When supplemented with globin messenger RNA, the mRNA dependent reticulocyte lysate incorporated labeled amino acid into TCA precipitable material for greater than 40 minutes at 37°. There was minimal incorporation of label into TCA precipitable material in the absence of added mRNA (Figure 16). The rate of protein synthesis was dependent on the quantity of added messenger RNA until saturation of the system was achieved at 12 µg/ml of mRNA.

Nascent Peptide Analysis from the mRNA Directed Synthesis of Globin in the mRNA Dependent Reticulocyte Lysate

A 400 µl aliquot of the mRNA dependent reticulocyte lysate was made 5 µg/ml in globin mRNA and nascent peptides were labeled with [^{3}H]-tryptophan for 10 minutes at 37°. Protein synthesis was halted and the

FIGURE 15. The time course of micrococcal nuclease inactivation of endogenous protein biosynthetic activity in the reticulocyte lysate.

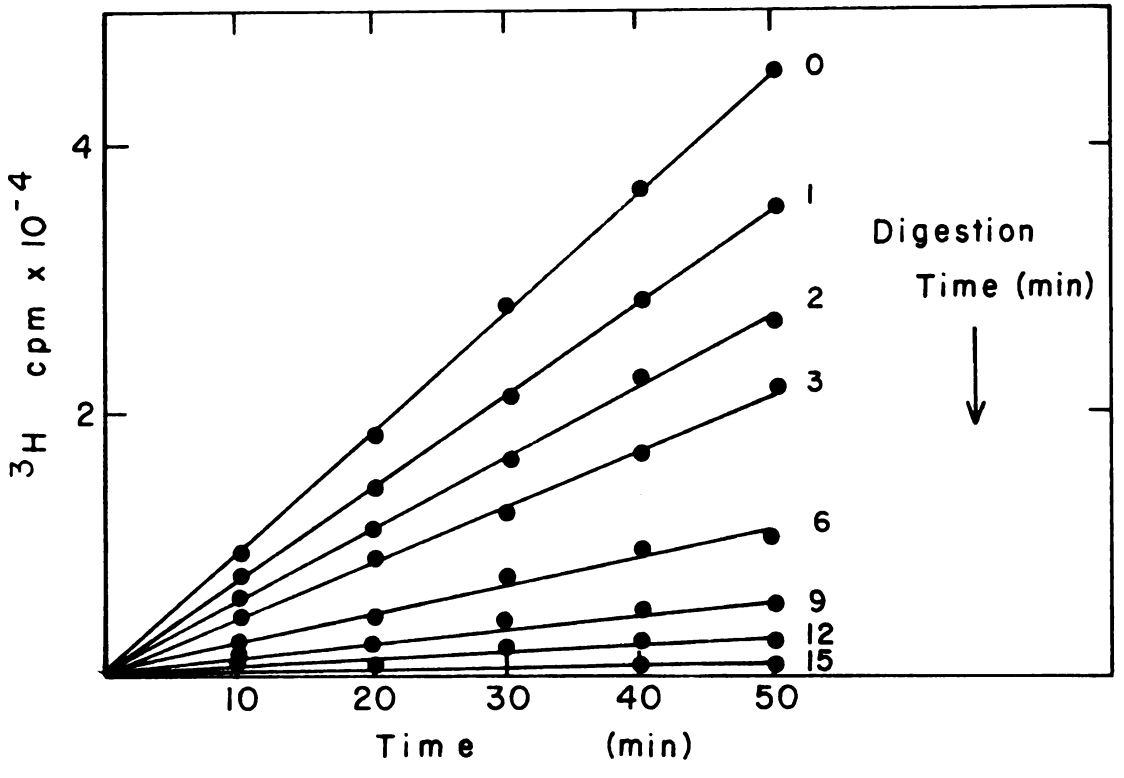
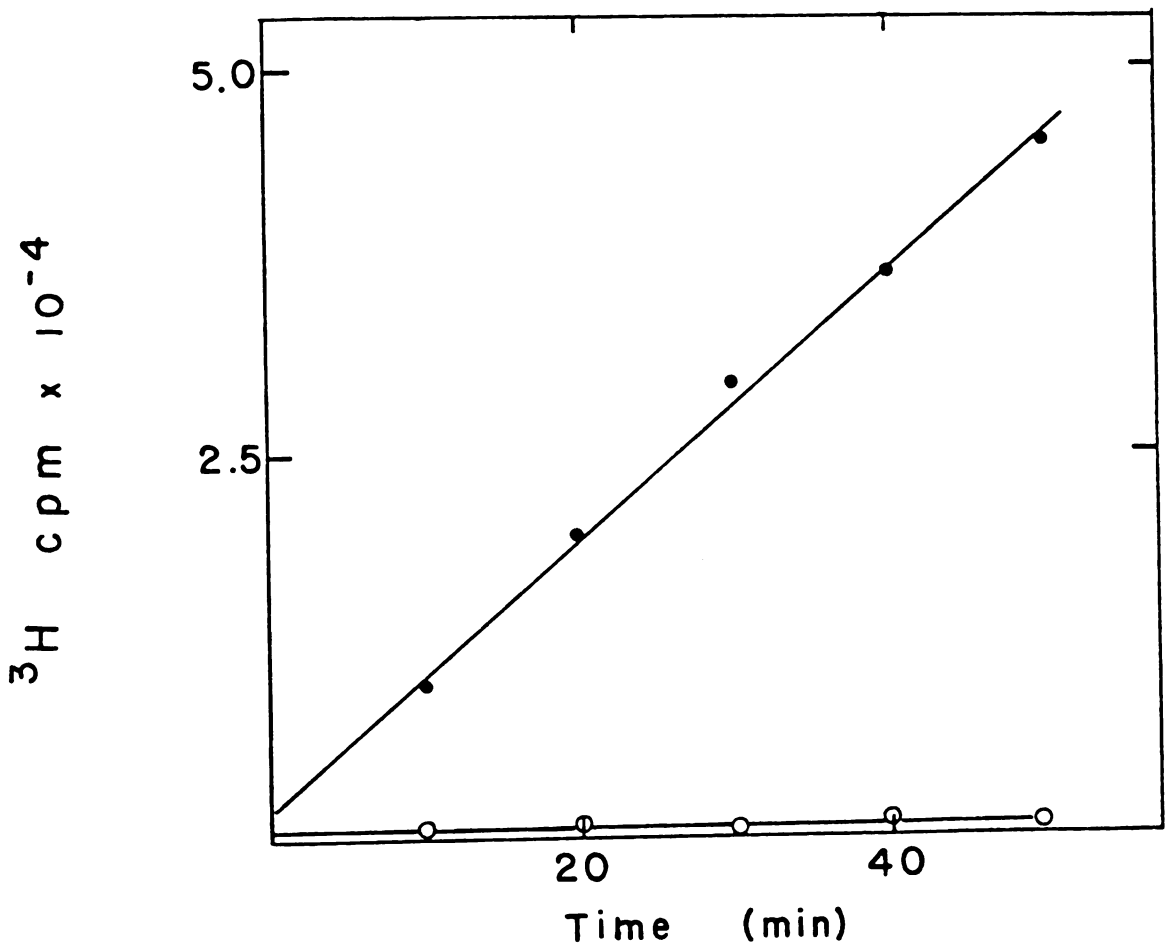

^3H cpm x 10^{-4}
4
2
10
20
30
40
50
Time (min)
0
1
2
3
6
9
12
15
Digestion
Time (min)

FIGURE 16. The globin mRNA directed incorporation of [^{3}H]-leucine into TCA precipitable material in the mRNA dependent lysate. No added mRNA (O), 5 µg/ml globin mRNA (●).


5.0
2.5
^3H cpm x 10^{-4}
20
40
Time (min)

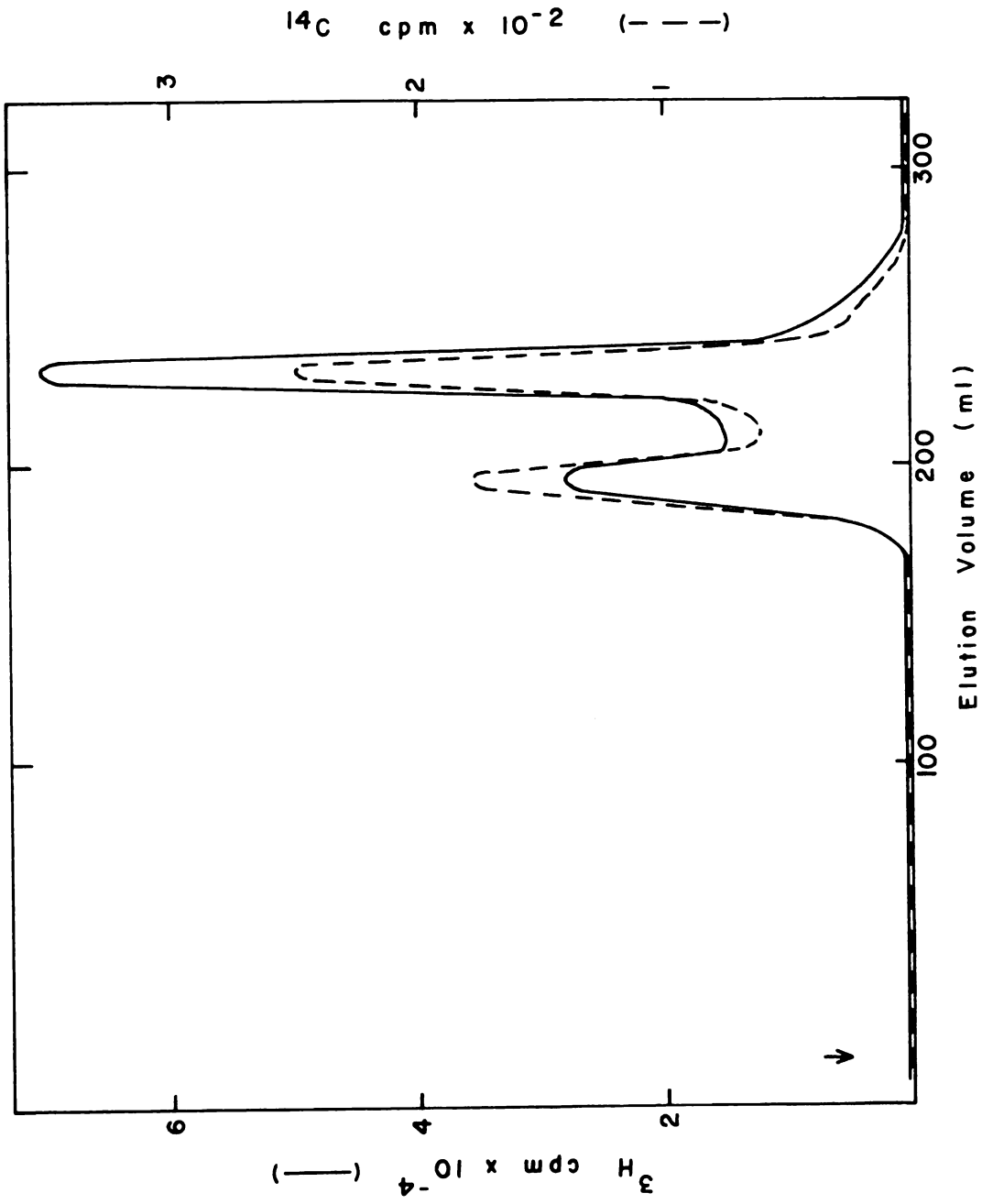
incubation mixture combined with an intact reticulocyte lysate whose nascent peptides had been labeled with [^{14}C]-tryptophan. Nascent peptides were co-purified and chromatographed on a Bio-Gel A 0.5m column. Analysis of the combined nascent peptides is shown in Figure 17. The size distribution of nascent peptides shows a surprising difference when compared to that observed in the intact lysate. The shoulder seen in the intact lysate at K_d 0.58 is now a major peak in the profile and the peak at K_d 0.71-0.72 has also become enlarged. The remainder of the distribution shows a similar distribution of components although the high molecular weight region has been reduced in relative amplitude.

An analysis of the nature of the products synthesized by the mRNA dependent lysate was performed on the labeled globin present in the post-ribosomal supernatant fraction from this incubation. Authentic [^{14}C]-globins resulting from the synthesis of globin in the [^{14}C]-labeled control reticulocyte lysate were already present in the postribosomal supernatant fraction and were used as markers.

Globin was prepared from the postribosomal supernatant fraction by acid-acetone precipitation and analyzed by CM cellulose chromatography as described in Methods. The results are shown in Figure 18. The α:β ratio of 0.96 calculated from the areas under the α- and β-globin synthesized in the reticulocyte lysate. The α:β ratio observed for the translation of globin mRNA in the mRNA dependent lysate was calculated to be 0.79. This imbalanced globin synthesis was reproducibly observed in several different mRNA dependent lysate preparations as well as with different preparations of globin mRNA. These same globin mRNA preparations synthesized α- and β-globin in the wheat germ system at a ratio of 0.92-0.95. The ratio of α- to β-globin mRNA in intact reticulocytes is

FIGURE 17. Elution profile of [^{3}H]-tryptophan labeled nascent peptides from the globin mRNA directed micrococcal nuclease inactivated reticulocyte lysate.

FIGURE 18. CM cellulose chromatography of the [^{3}H]-tryptophan labeled products synthesized by the mRNA dependent lysate in response to globin mRNA. The [^{14}C]-globins represent the products synthesized in the reticulocyte lysate.


^{14}C cpm x 10^{-2} (— — —)
3
2
1
300
200
100
Elution Volume (ml)
6
4
2
^{3}H cpm x 10^{-4} (——)

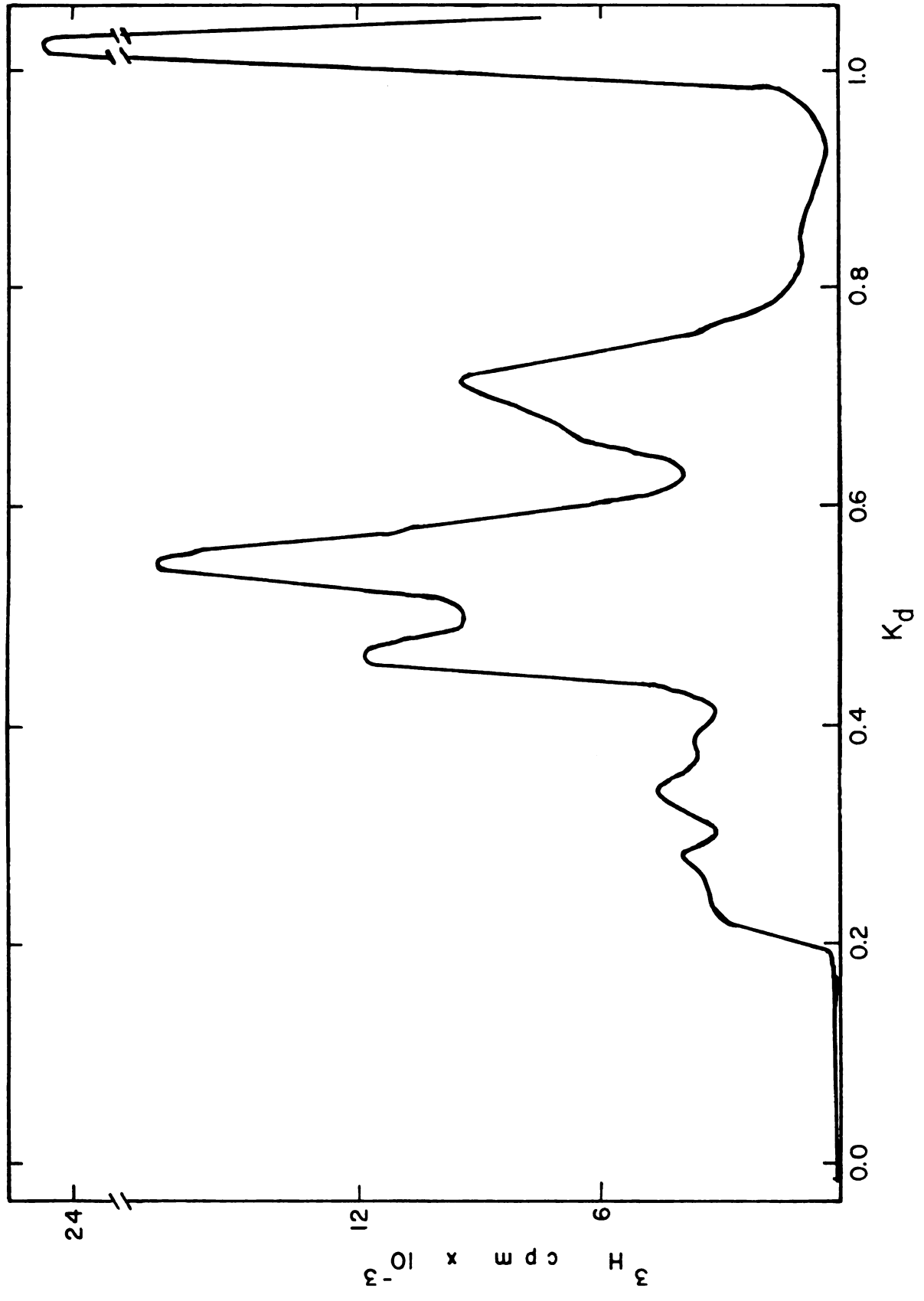
approximately 1.4:1 (10). Beta globin mRNA, however, is known to initiate protein synthesis at a rate that is 30-40% greater than for the α-globin mRNA (10). The higher rate of β mRNA initiation results in an approximate 1:1 ratio of α- and β-globin synthesis observed in intact reticulocytes. The digestion of endogenous globin mRNA with micrococcal nuclease has somehow altered the mechanisms in the reticulocyte by which the α:β ratio is maintained.

Analysis of Background Nascent Peptides in the mRNA Dependent Reticulocyte Lysate in the Absence of Added mRNA

It can be seen from Figure 16 that the mRNA dependent lysate shows no detectable incorporation of amino acids into TCA precipitable material in the absence of added mRNA.

The mRNA dependent lysate was, therefore, analyzed for the presence of a nascent peptide fraction in the absence of added mRNA. These experiments determined if changes observed in the size distribution of nascent chains isolated following the translation of globin mRNA in the mRNA dependent lysate was attributable to the added mRNA or was some function of the system used to translate the mRNA. An aliquot of the mRNA dependent lysate was supplemented with [^{3}H]-tryptophan but no mRNA, and incubated for 10 minutes at 37°. Protein synthesis was terminated, ribosomes were isolated by centrifugation, and nascent chains were prepared. The result of one such analysis is shown in Figure 19. The presence of two peaks of accumulation at K_d 0.58 and 0.71 are seen in the size distribution of background nascent peptides. These peaks, as well as other minor accumulations, reveal the source of the gross enlargement of peaks within the nascent chain profile obtained when translating globin mRNA in the mRNA dependent lysates. It is interesting

FIGURE 19. The elution profile of nascent peptides synthesized in the absence of added mRNA by the mRNA dependent reticulocyte lysate.


24
12
9
0.0
0.2
0.4
0.6
0.8
1.0
K_d
^3H cpm x 10^{-3}

to note that these two accumulations which are the two major components of the background are normally seen as minor peaks in nascent peptide profiles observed in either the intact reticulocyte lysate or nascent peptides purified from globin mRNA directed protein synthesis in wheat germ lysates.

Effect of Micrococcal Nuclease Digestion of Endogenous Globin mRNA on the Nascent Peptide Size Distribution

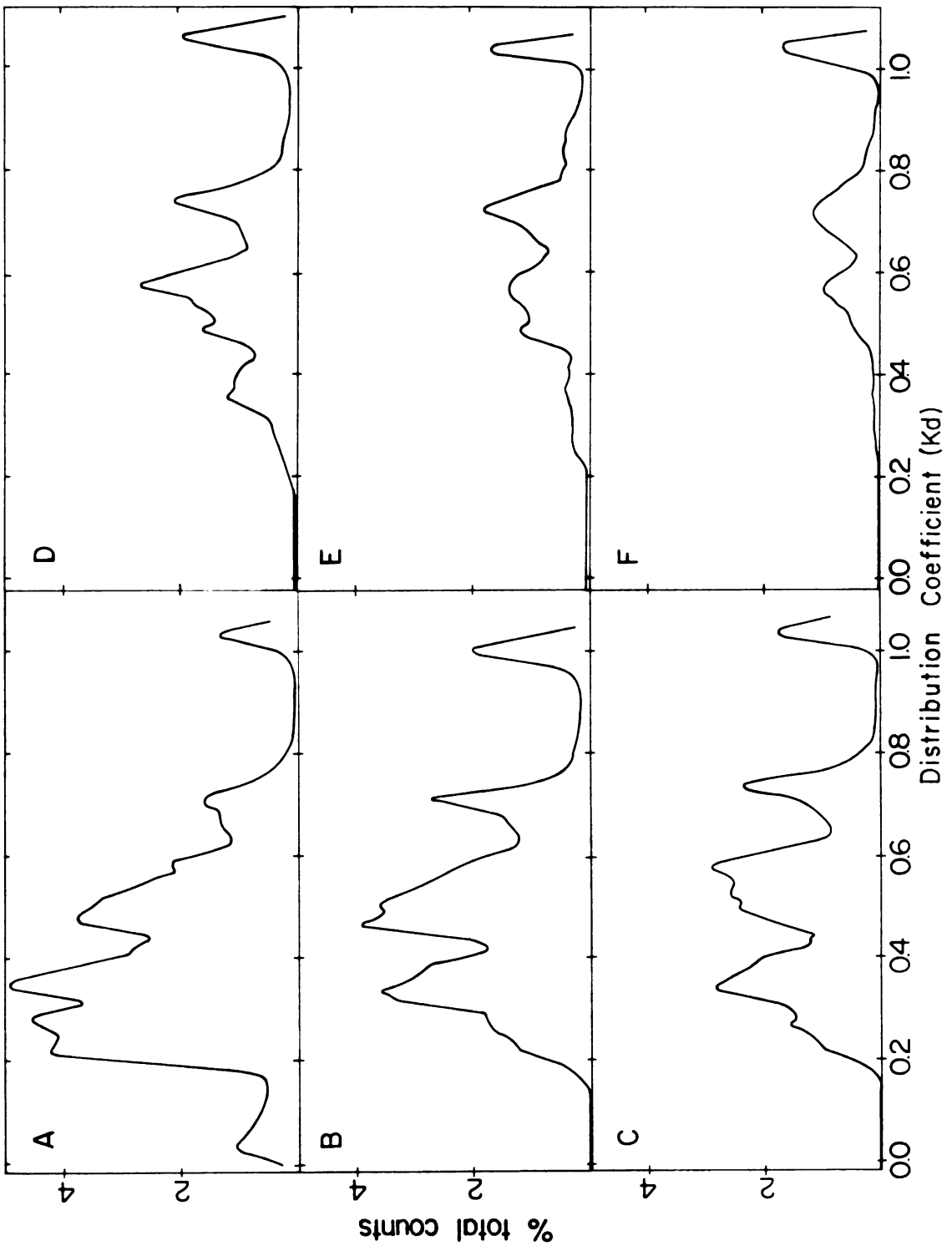
The observation that a discrete population of nascent peptides were present in the mRNA dependent lysate after destruction of the endogenous mRNA with micrococcal nuclease was unexpected. This discovery of a background containing a significant number of nascent peptides in the nuclease inactivated lysate while displaying minimal synthesis of globin led to further studies on the fate of the nascent chain population as the endogenous globin mRNA of the reticulocyte lysate was being degraded. These experiments examined the changes in nascent peptide populations from its known distribution in the intact reticulocyte lysate to the ultimate distribution obtained by the nascent peptide population when no apparent protein biosynthetic activity was observed. If micrococcal nuclease first attacked the endogenous mRNA at specific sites of high susceptibility rather than cleaving in a random fashion, then useful information as to the conformation of the message during translation in the reticulocyte lysate could be obtained.

A 2.8 ml reticulocyte lysate incubation was assembled and supplemented with 1 mM $CaCl_2$ and 10 µg/ml micrococcal nuclease. At specified time intervals, 500 µl aliquots of the mixture were removed and the digestion stopped by addition of EGTA to a concentration of 2 mM. Aliquots of 100 µl from each lysate were supplemented with [^{3}H]-leucine

and the rate of protein synthesis was determined. The remainder of each lysate was added to a tube containing 100 μCi of [^{3}H]-tryptophan and incubated at 37° for 10 minutes. Two volumes of medium B and antibiotics were added to each lysate and nascent chains were prepared. Figure 20A represents the nascent chain profile at zero time. The results of nuclease digestion on the nascent peptide population are shown in Figures 20B-F.

Following only two minutes of digestion, the high molecular weight peaks at K_d 0.22 and 0.28 have been drastically reduced. The peak at K_d 0.35 appears to be intact, but a new peak at K_d 0.38 has appeared. The shoulder that appears in the control nascent chain profile at K_d 0.53 has developed into a major peak. The small peak at K_d 0.71 is also greatly enhanced. At four minutes of digestion the peak which appeared at K_d 0.38 following two minutes of digestion has now begun to be destroyed. The peak at K_d 0.47 in the control nascent chain distribution is showing a reduction in amplitude while the peak at K_d 0.53 has apparently remained unchanged. A peak has persisted at K_d 0.58 where a shoulder existed before nuclease digestion. The peak located at K_d 0.71 has remained unchanged. Eight minutes of digestion reveals the loss of the accumulations at K_d 0.35 and 0.38. The K_d 0.47 peak has been degraded considerably as has the K_d 0.53 peak. The accumulation at K_d 0.71 is still a major feature of the nascent chain distribution. After 12 minutes of micrococcal nuclease digestion, all peaks below K_d 0.5 are virtually destroyed while the rate of protein synthesis has been reduced 98%. The peak at K_d 0.58 is now reduced in magnitude, leaving the peak at K_d 0.71 as the major feature of the nascent chain profile with a new peak at K_d 0.78 appearing. At 15 minutes of digestion, the

FIGURE 20. The effect of the time of micrococcal nuclease digestion on the nascent peptide size distribution in the reticulocyte lysate: A, control lysate, no digestion: B, 2 minute digestion: C, 4 minute digestion: D, 8 minute digestion: E, 12 minute digestion: F, 15 minute digestion.


A
B
C
D
E
F
2
4
0.0
0.2
0.4
0.6
0.8
1.0
% total counts
Distribution Coefficient (Kd)

accumulations are similar to those observed in the profile seen in Figure 19 for the nascent peptide background in the mRNA dependent lysate. During the course of micrococcal nuclease digestion, the rate of protein synthesis was reduced to 1% of control levels, however, after 15 minutes of digestion, the size of the nascent chain fraction was still 22% of the size of the nascent peptide fraction observed without nuclease digestion. Three results of significance should be noted from this series of experiments. First, the degradation of the endogenous messenger RNA appears to be non-random, as shown by the selective loss of peaks in the nascent peptide distribution. The second fact to be noted is the apparent transient appearance of peaks such as those observed at K_d 0.38, 0.53 and 0.58. These may be intermediate products that result from the selective degradation of regions within the endogenous mRNA. These accumulations may also be true peaks of nascent chain accumulations which were unobserved until partial degradation of larger peaks allowed their rise to prominence in the profile. Finally, a strong correlation exists between the data obtained in these experiments for short micrococcal nuclease digestion times in reticulocyte lysates and the results of studies on the effect of S_1 nuclease digestion of globin message for long time periods on the nascent peptide size distribution.

Confirmation of Background Nascent Peptides in a Commercially Available mRNA Dependent Reticulocyte Lysate

Experiments were performed to analyze the possible presence of nascent peptides in other nuclease inactivated lysates in the absence of added mRNA. A complete kit for the synthesis of protein using the micrococcal nuclease inactivated reticulocyte lysate was obtained from Bethesda Research Laboratories. When supplemented with [^{3}H]-leucine

and 5 μg/ml globin mRNA labeled amino acid was incorporated into TCA precipitable material for 40 minutes (Figure 21).

A 0.5 ml incubation mixture was supplemented with [^{3}H]-leucine and mRNA and the nascent peptides resulting from the translation of globin mRNA were co-purified with [^{14}C]-leucine labeled nascent peptides from an untreated reticulocyte lysate. Leucine was used to label nascent peptides to incorporate sufficient radioactivity into the nascent peptide fraction to permit the double label analysis of the nascent peptides as shown in Figure 22. The commercially available lysate appeared to contain a population of nascent peptides strikingly similar to those observed following S_1 nuclease globin mRNA in the wheat germ cell-free system. The size distribution of nascent peptides observed in the absence of mRNA is shown in Figure 23. The nascent peptide fraction found in the absence of mRNA amounted to 14% of the total observed in the incubation with mRNA. This similar size distribution of nascent peptides in the absence of added mRNA in the commercially available mRNA dependent reticulocyte lysate and mRNA dependent lysates prepared for this study by the author shows that the nascent peptides present in these inactivated lysates appear to be a result of micrococcal nuclease digestion of th rabbit reticulocyte lysate.

Effect of Pactamycin on the Background Nascent Peptide Size Distribution in the mRNA Dependent Reticulocyte Lysate

The antibiotic pactamycin at low concentrations has been shown to selectively inhibit the initiation of reticulocyte protein synthesis (85,86). Since neither the elongation nor the termination steps of protein synthesis are affected, analysis of the fate of nascent peptides present in the mRNA dependent lysate in the presence of pactamycin should

FIGURE 21. The rate of mRNA directed protein synthesis in a commercially available mRNA dependent lysate.

rcially

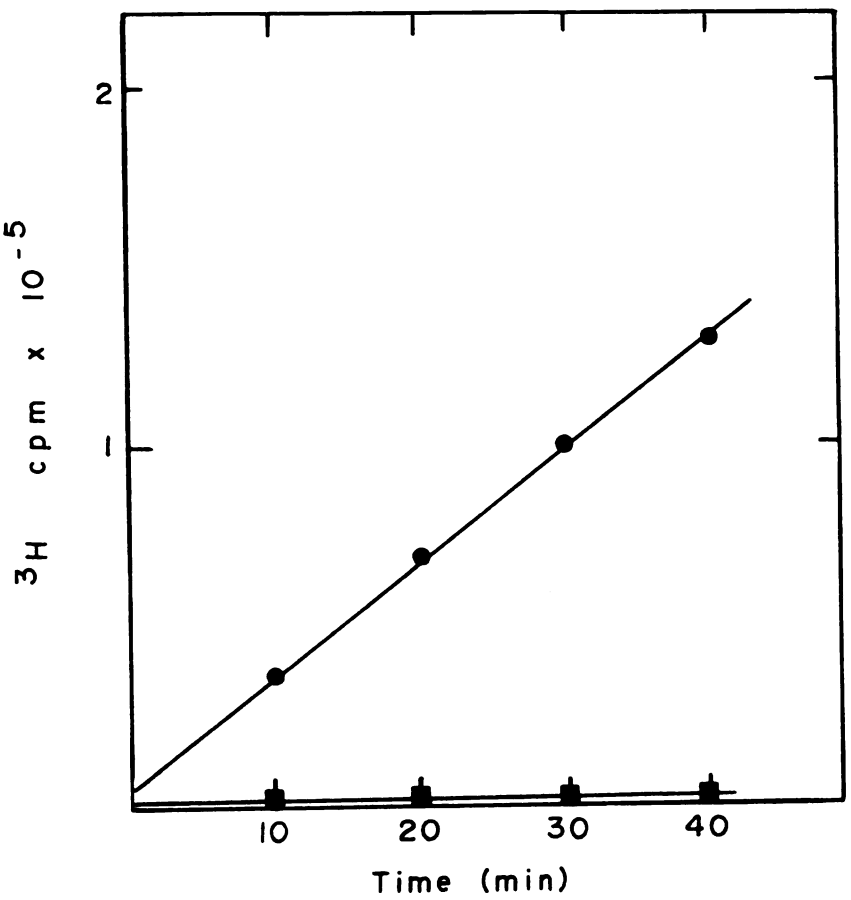


FIGURE 22. The elution profile of leucine labeled nascent peptides from the commercially available globin mRNA directed mRNA dependent reticulocyte lysate.

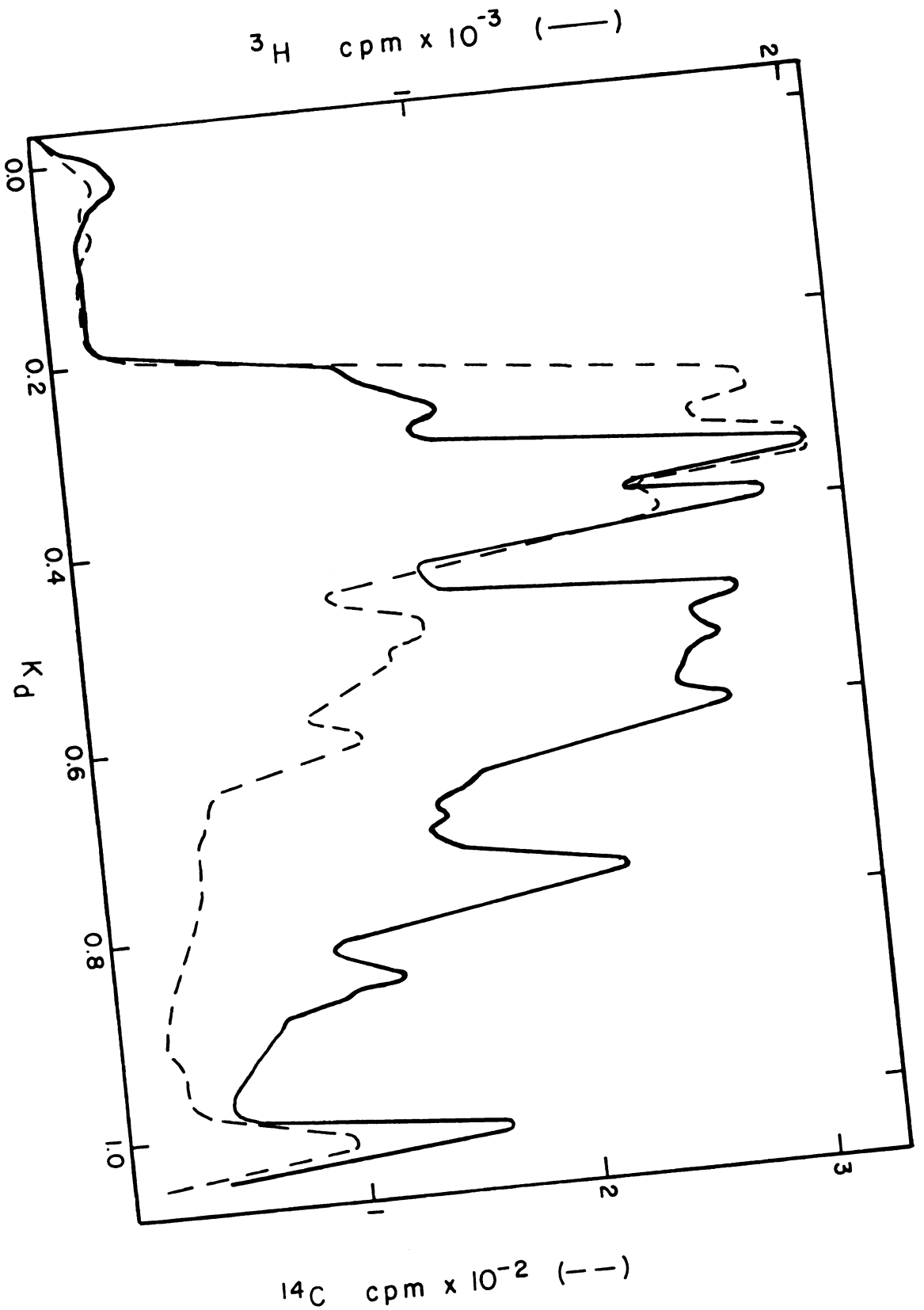
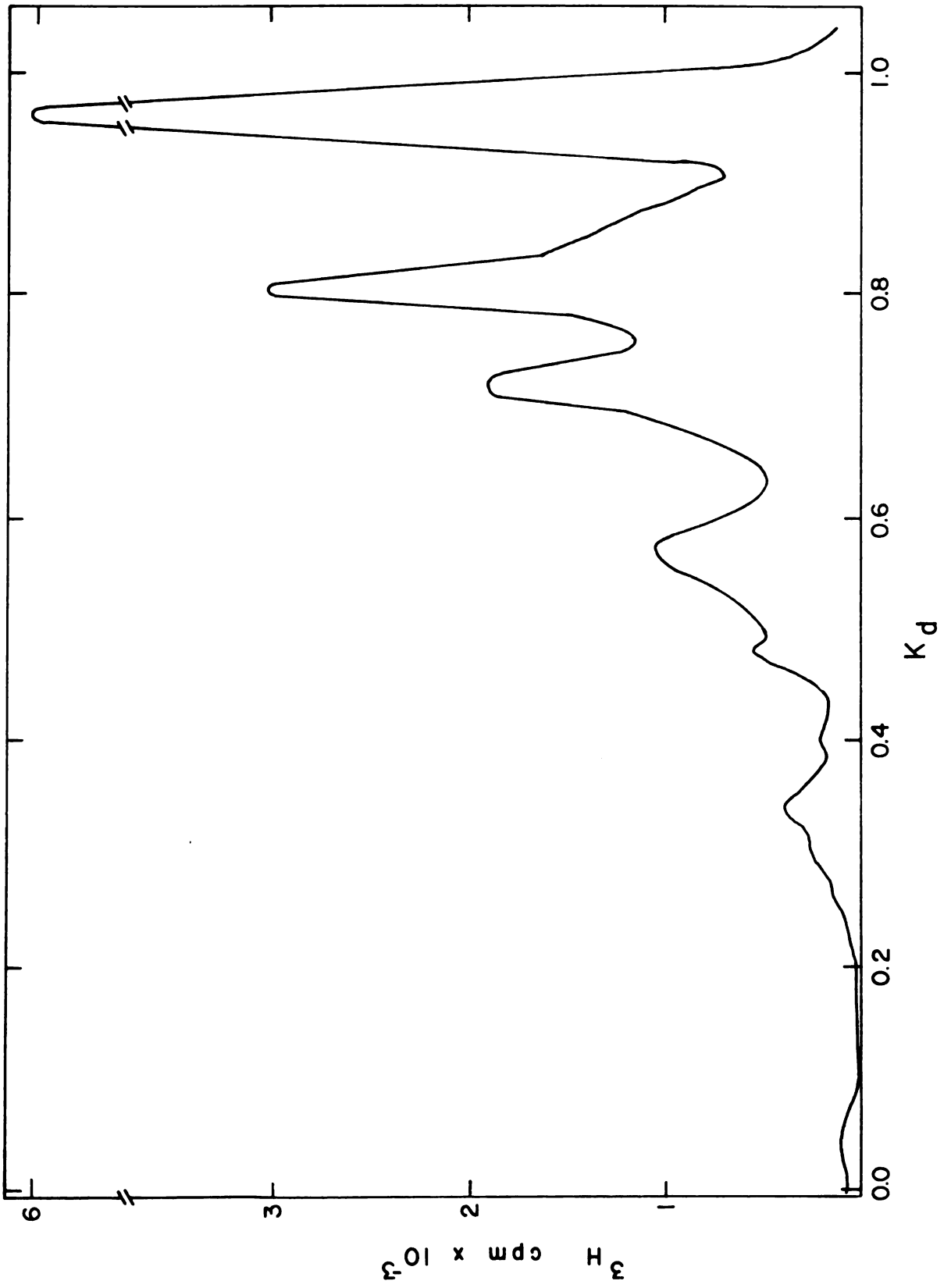

^3H cpm x 10^{-3} (——)
2
0.0
0.2
0.4
K_d
0.6
0.8
1.0
2
3
^{14}C cpm x 10^{-2} (– –)

FIGURE 23. The size distribution of nascent peptides present in the commercial mRNA dependent lysate in the absence of added mRNA.


^3H cpm x 10^{-3}
6
3
2
1
0.0
0.2
0.4
0.6
0.8
1.0
K_d

yield information as to whether the nascent peptides observed in the absence of added mRNA represent a fraction of fragmented mRNA still elongating and terminating.

The effects of pactamycin on protein synthesis in the mRNA dependent reticulocyte lysate were first investigated in order to show that pactamycin was functioning by inhibition of mRNA directed protein synthesis following micrococcal nuclease digestion. A mRNA dependent lysate was supplemented with [^{3}H]-leucine and incubated with globin mRNA for 20 minutes. At that time, half of the mixture was made 1 μM in pactamycin while the remaining half was supplemented with water. The incubation was continued for 40 additional minutes with time points taken at 5 minute intervals. The results are shown in Figure 24. At 10 minutes after pactamycin addition, no further radioactivity was incorporated into TCA precipitable material.

The effect of pactamycin on the nascent peptides present in the micrococcal nuclease inactivated lysate in the absence of added mRNA then was determined. One ml of micrococcal nuclease treated lysate was supplemented with 200 μCi of [^{3}H]-tryptophan and incubated without mRNA for 10 minutes at 37°. Half of the incubation mixture was removed and was made 1 μM in pactamycin and 100 μCi/ml in [^{14}C]-tryptophan. Both fractions were then incubated at 37° for an additional 10 minutes and protein synthesis was terminated with 2 volumes medium B and antibiotics. Nascent peptides were prepared from both mRNA dependent lysates. These nascent peptides were analyzed by chromatography on Bio-Gel A 0.5m. Figure 25 shows the nascent peptide distribution present in the mRNA dependent lysate in the absence of added mRNA. The result of a 10 minute pactamycin chase is shown in Figure 26. The chase period did not

FIGURE 24. The effect of pactamycin on protein synthesis in the mRNA directed micrococcal nuclease inactivated lysate.

o - control
▲ - pactamycin added to 1.0 μm at 20 minutes

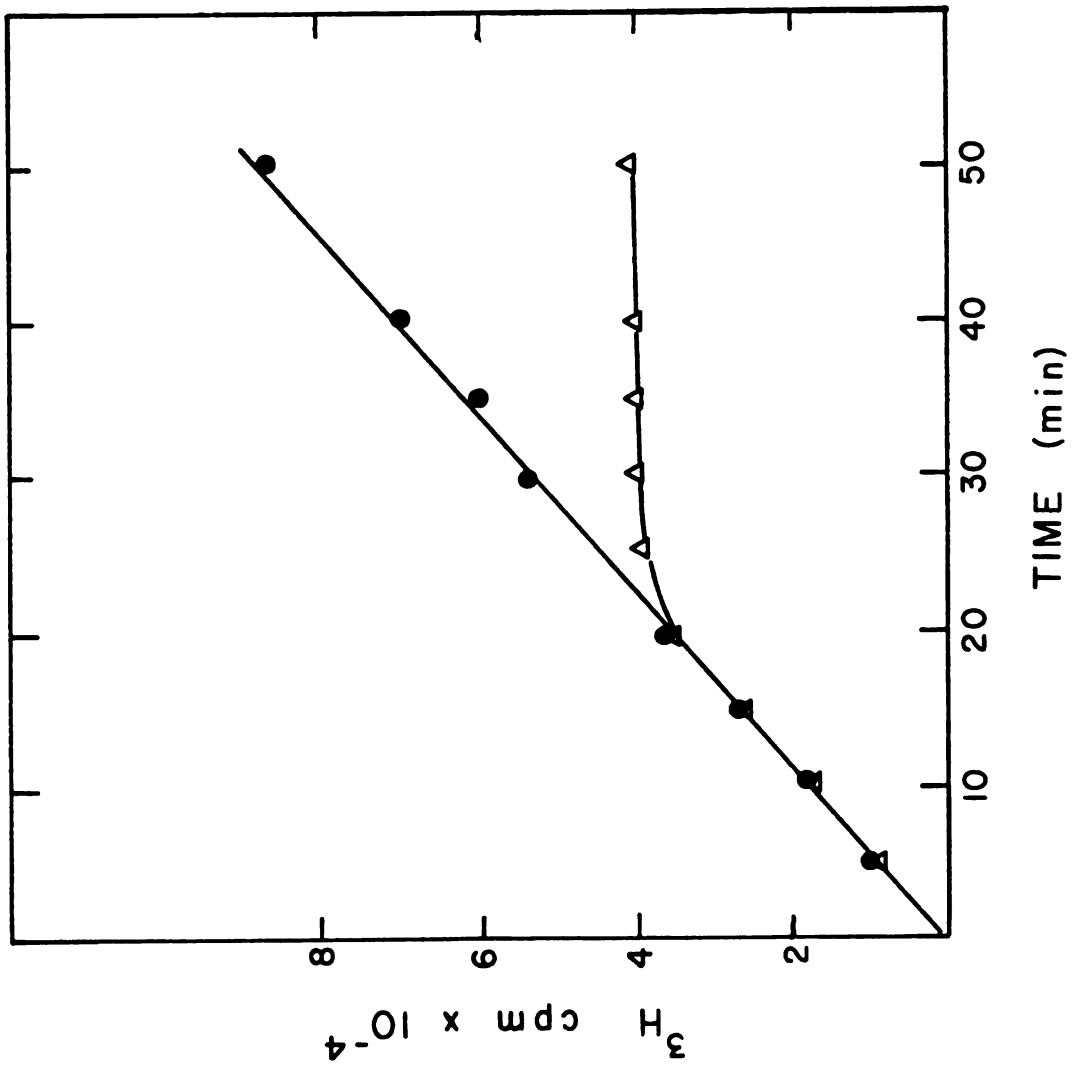

^3H cpm x 10^{-4}
2
4
6
8
10
20
30
40
50
TIME (min)

FIGURE 25. The size distribution of background nascent peptides of the mRNA dependent lysate before pactamycin addition.

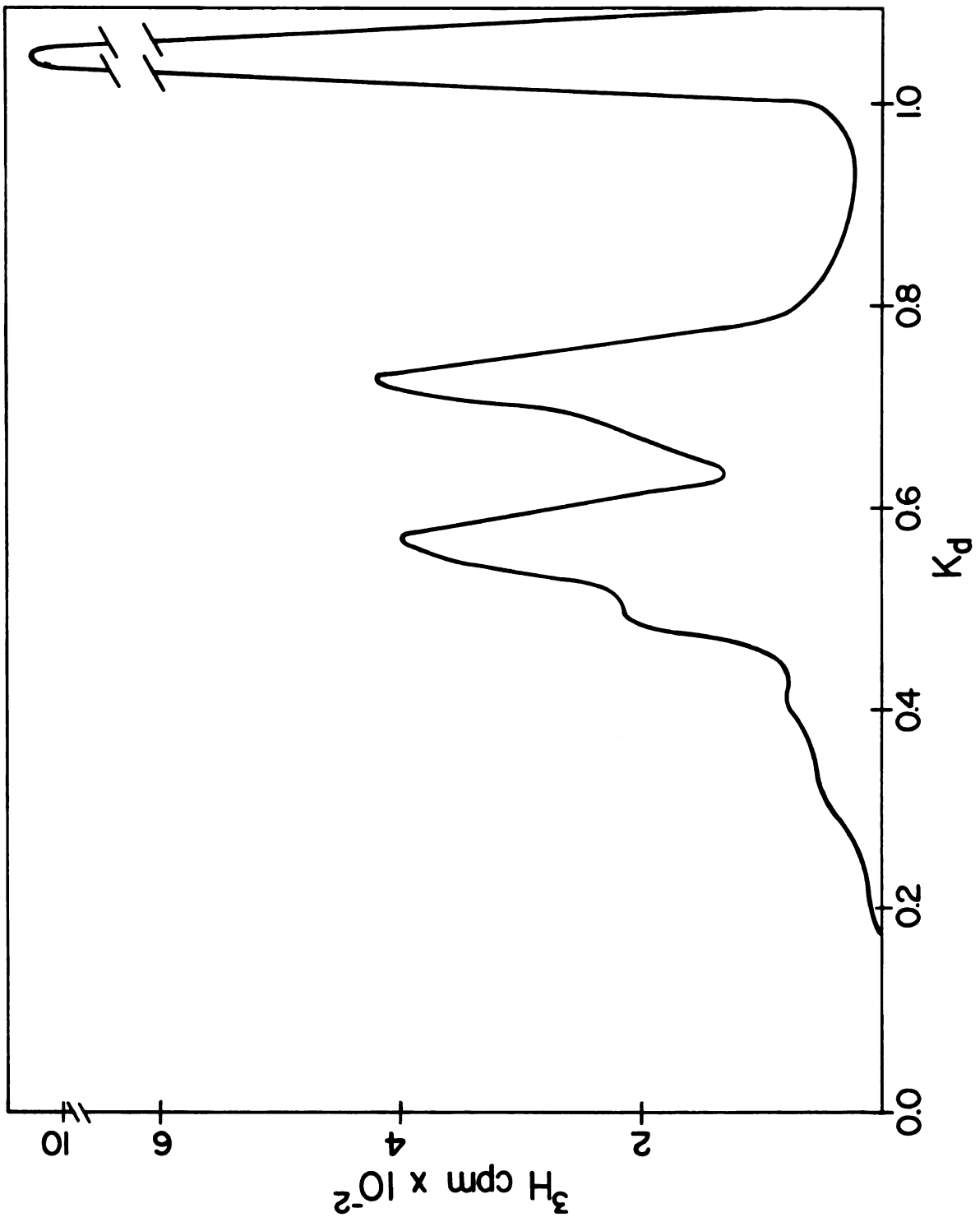

^{3}H cpm x 10^{-2}
2
4
6
10
0.0
0.2
0.4
0.6
0.8
1.0
K_d

FIGURE 26. The effect of a 10 minute pactamycin chase on the background of nascent peptides of the mRNA dependent lysate.

^{14}C cpm x 10^{-2} (---)
2
1.0
0.8
0.6
K_d
0.4
0.2
0.0
^{3}H cpm x 10^{-2} (—)
8
2
1

effectively alter the size distribution of nascent peptides and the total size of the nascent peptide pool was reduced by only half. If, however, the incubation time in the presence of pactamycin was increased to 25 minutes much of the remaining nascent peptides were chased from the nascent chain fraction (Figure 27). In the absence of pactamycin these peaks remain after 25 minutes. No [^{14}C]-labeled peptides appeared in the nascent peptide fraction during the chase period demonstrating that no initiation of new peptides has occurred. These data indicate that the process which formed the background nascent peptides is a dynamic one involving synthesis of protein and re-initiation of synthesis on a mRNA or fragments of the endogenous message that were refractory to micrococcal nuclease degradation. A substantial portion of the nascent chains in the mRNA dependent lysate could be eliminated by increasing the micrococcal nuclease concentration to 35 μg/ml as seen in Figure 28. Lysates digested at 35 μg/ml of micrococcal nuclease showed greatly decreased abilities to synthesize protein in response to added mRNA and were not considered usable.

Analysis of the Background Nascent Peptides in the Wheat Germ System

The wheat germ cell-free system was analyzed for the presence of an endogenous population of nascent peptides in the absence of added mRNA. This analysis was performed to determine if any background from non-globin nascent chains were present in the globin nascent peptide distribution observed in the wheat germ system.

One milliliter of a wheat germ mixture supplemented with [^{3}H]-tryptophan, but no mRNA, was incubated for 20 minutes and protein synthesis was stopped with medium B and antibiotics. Nascent peptides were purified as before and subjected to gel filtration on Bio-Gel A 0.5m.

FIGURE 27. The effect of a 25 minute pactamycin chase on the size and amount of nascent peptides present in the mRNA dependent reticulocyte lysate.

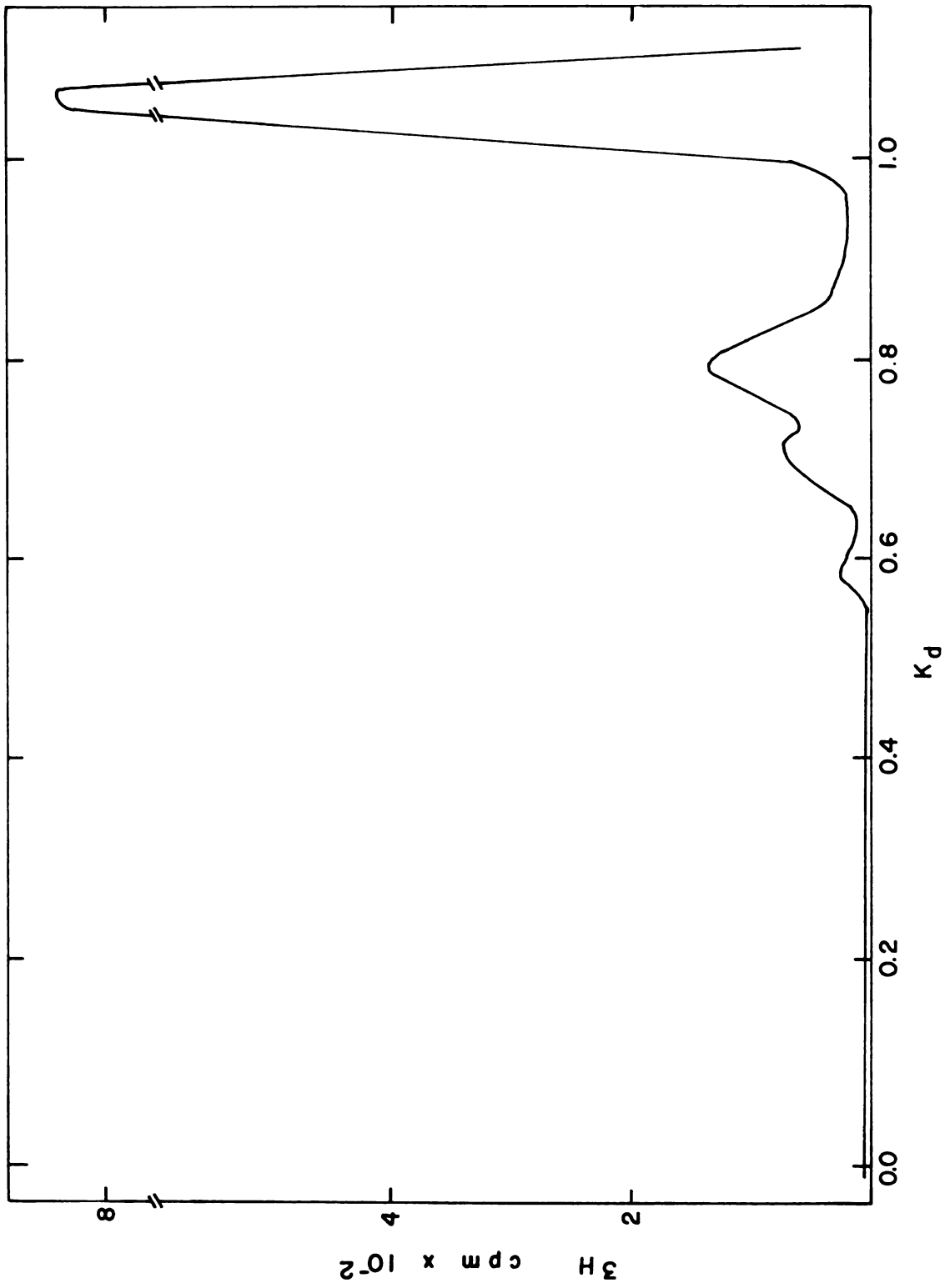
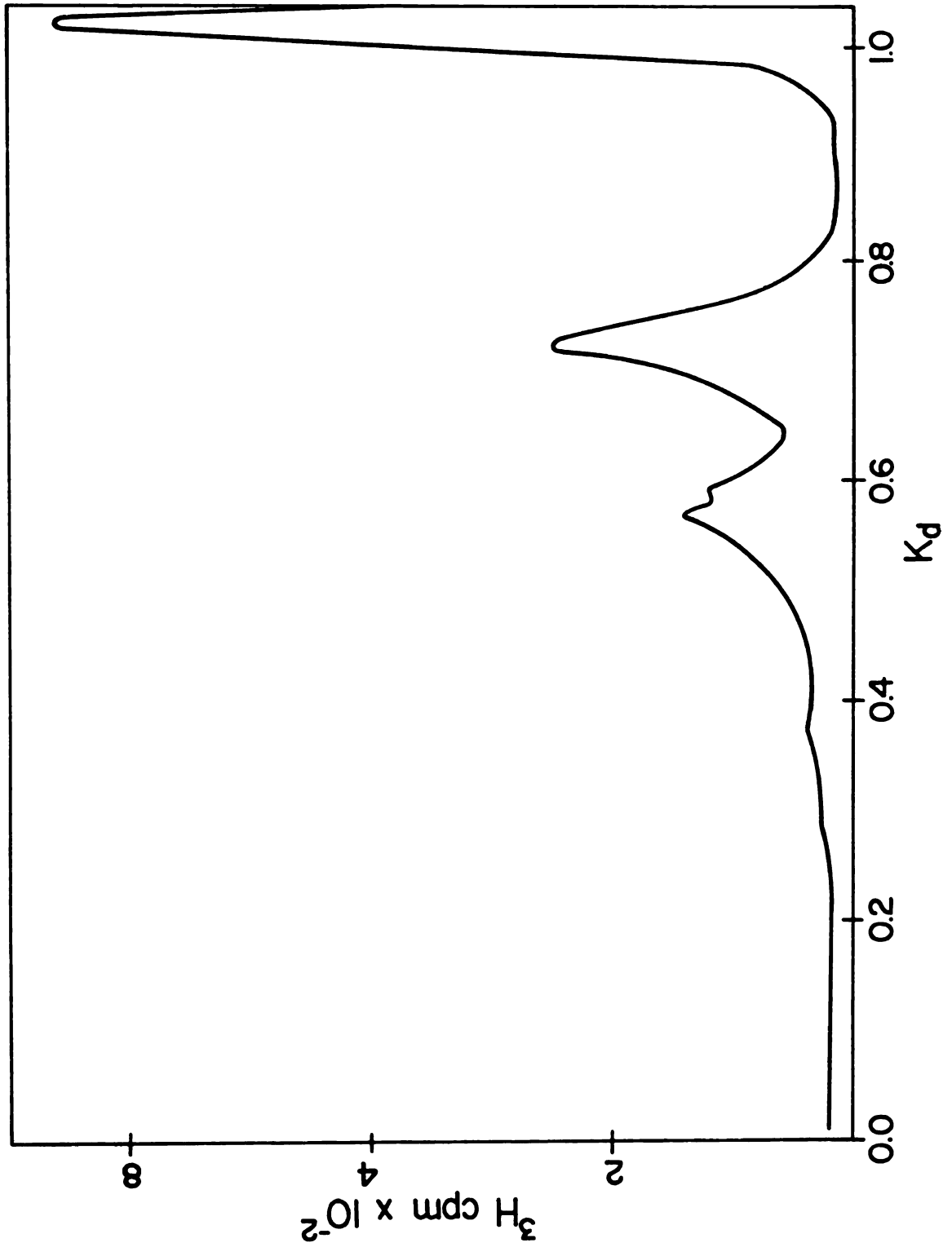

0.0
0.2
0.4
0.6
0.8
1.0
K_d
2
4
8
^3H cpm x 10^{-2}

FIGURE 28. The effect of digestion with high micrococcal nuclease concentration on the background size distribution of nascent peptides of the mRNA dependent lysate.


0.0
0.2
0.4
0.6
0.8
1.0
K_d
2
4
8
^3H cpm x 10^{-2}

The profile of background nascent peptides in the wheat germ system is seen in Figure 29. The elution profile shows that there are no nascent peptides present in the wheat germ system in the absence of an added mRNA. Therefore, all of the nascent peptide accumulations seen during the translation of globin mRNA in the wheat germ are attributable to nascent peptides residing on ribosomes involved in the synthesis of α- and β-globin.

The Effect of Pactamycin on the Nascent Peptide Size Distribution in the Wheat Germ Cell-Free System

Pactamycin has been shown to effectively inhibit the initiation of protein synthesis in the wheat germ cell-free system (87). The effect of pactamycin on the translation of globin mRNA in the wheat germ cell free system first was examined. Aliquots of wheat germ cell-free lysates supplemented with 5 μg/ml globin mRNA and 100 μCi/ml [^{3}H]-leucine were incubated with increasing concentrations of pactamycin. The time course of incorporation of labeled leucine into TCA precipitable material was determined as a function of pactamycin concentration. Figure 30 shows the effect of increasing concentrations of pactamycin on the rate of globin synthesis in the wheat germ cell-free system. A concentration of 1.0 μM pactamycin was found to inhibit protein synthesis by 99% in the wheat germ system as compared with the rate of protein synthesis in the absence of the antibiotic.

Pactamycin at concentrations less than 10 μM has been reported to selectively inhibit only the initation step of protein synthesis (86). Addition of pactamycin to a wheat germ cell-free system synthesizing protein from globin mRNA should result in the rapid loss of the nascent chain population as nascent globin peptides are completed and released

FIGURE 29. The size distribution of nascent peptides isolated from the wheat germ cell-free system in the absence of added mRNA.

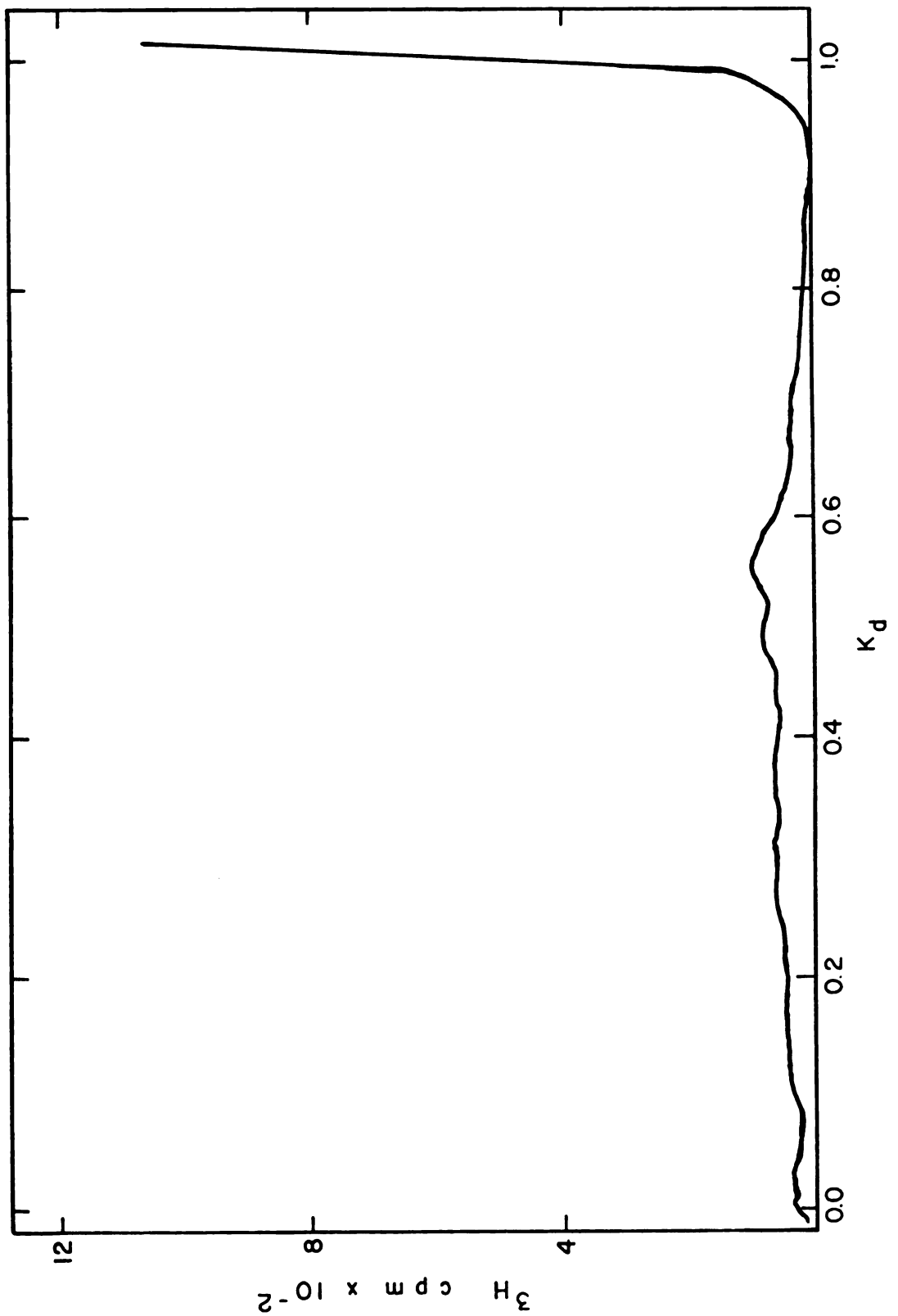
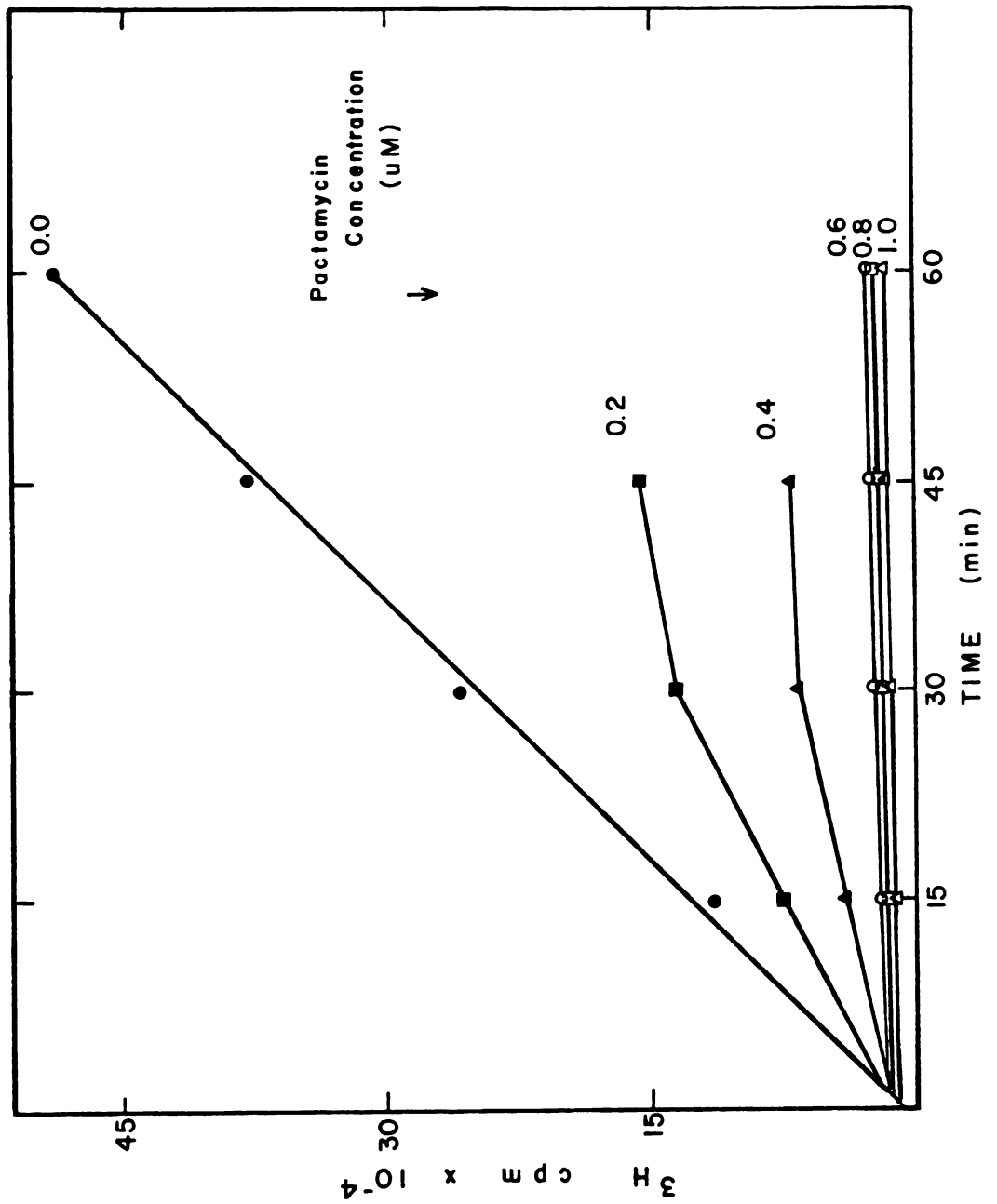

12
8
4
0.0
0.2
0.4
0.6
0.8
1.0
K_d
^3H cpm x 10^{-2}

FIGURE 30. The effect of pactamycin on the rate of globin mRNA directed protein synthesis in the wheat germ cell-free system.


Pactamycin
Concentration
(uM)
0.0
0.2
0.4
0.6
0.8
1.0
45
30
15
60
45
30
15
TIME (min)
^3H cpm x 10^{-4}

while no new synthesis is initiated. A 1.5 ml wheat germ cell-free mixture was supplemented with [^{3}H]-tryptophan and globin mRNA and incubated at 28° for 20 minutes. At that time, half of the mixture was made 1.0 μM in pactamycin. Protein synthesis in the other half of the mixture was stopped with medium B and antibiotics. The incubation supplemented with pactamycin was continued at 28° for an additional 20 minutes. Protein synthesis was stopped in the pactamycin treated incubation by addition of medium B and antibiotics and nascent chains were prepared from both samples. The control nascent peptide size distribution is shown in Figure 31. Addition of pactamycin reduced the size of the nascent chain population found on ribosomes synthesizing globin in the wheat germ lysate to 3% of the level found in the control incubation. The size profile of the material in the nascent chain fraction remaining following a 20 minute pactamycin chase is shown in Figure 32. The bulk of the counts in the remaining 3% are shown to be in aminoacyl-tRNA which co-purifies with peptidyl-tRNA in the purification used to obtain nascent peptides.

An identical experiment to the one described above using pactamycin to chase nascent peptides resulting from the translation intact globin mRNA in the wheat germ cell-free system was performed, but with S_1 nuclease digested globin mRNA used to direct protein synthesis in the wheat germ cell-free system.

A wheat germ cell-free incubation was supplemented with 5 μg/ml globin mRNA subjected to 10 minutes of S_1 nuclease digestion. After 20 minutes of incubation half of the reaction mixture was made 1 μM in pactamycin and incubated for an additional 20 minutes before protein synthesis was stopped. Protein synthesis was stopped in the other half of the mixture immediately. Figure 12C shows the nascent peptide size

FIGURE 31. The nascent peptide size distribution from the globin mRNA directed wheat germ system prior to addition of pactamycin.

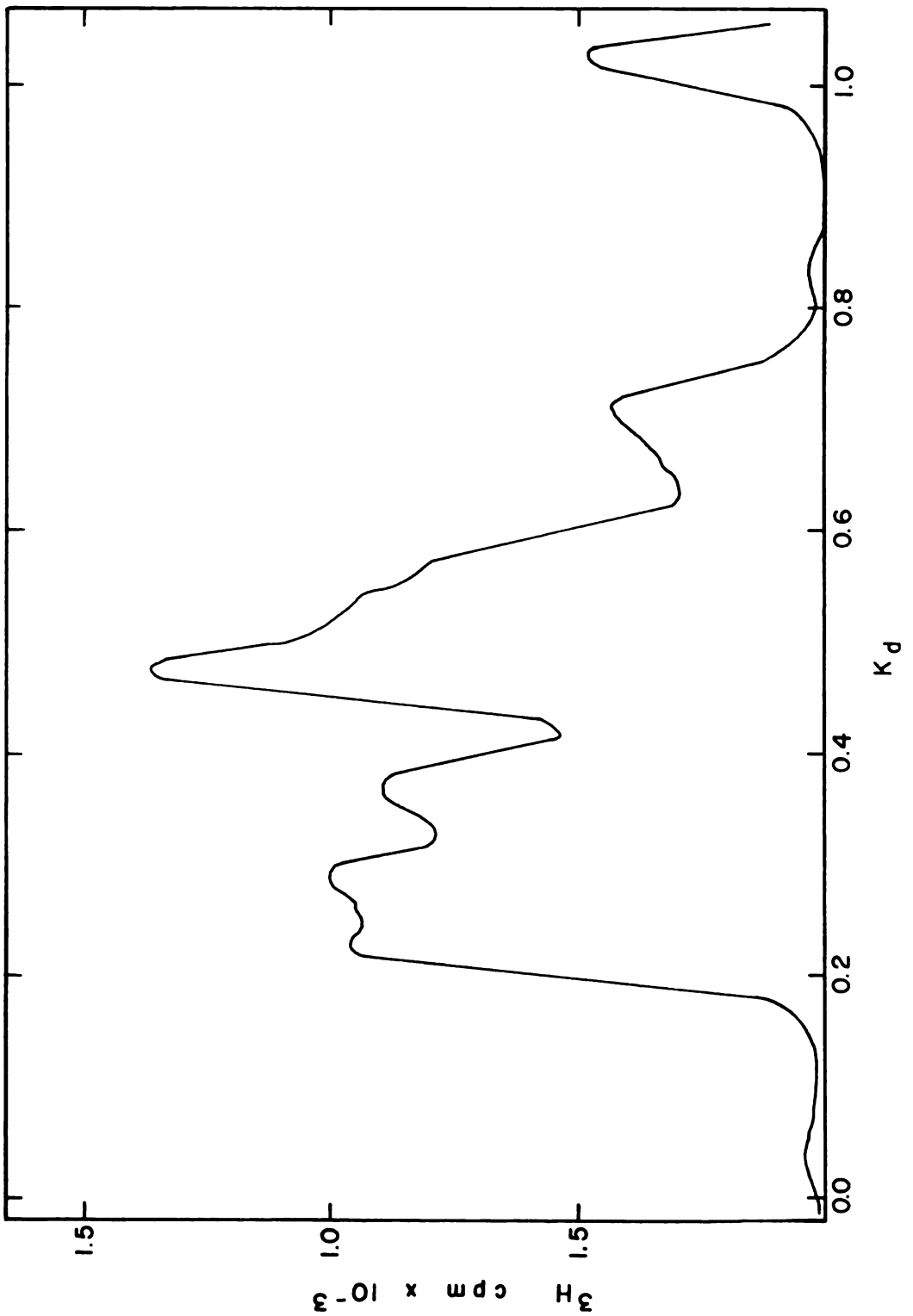
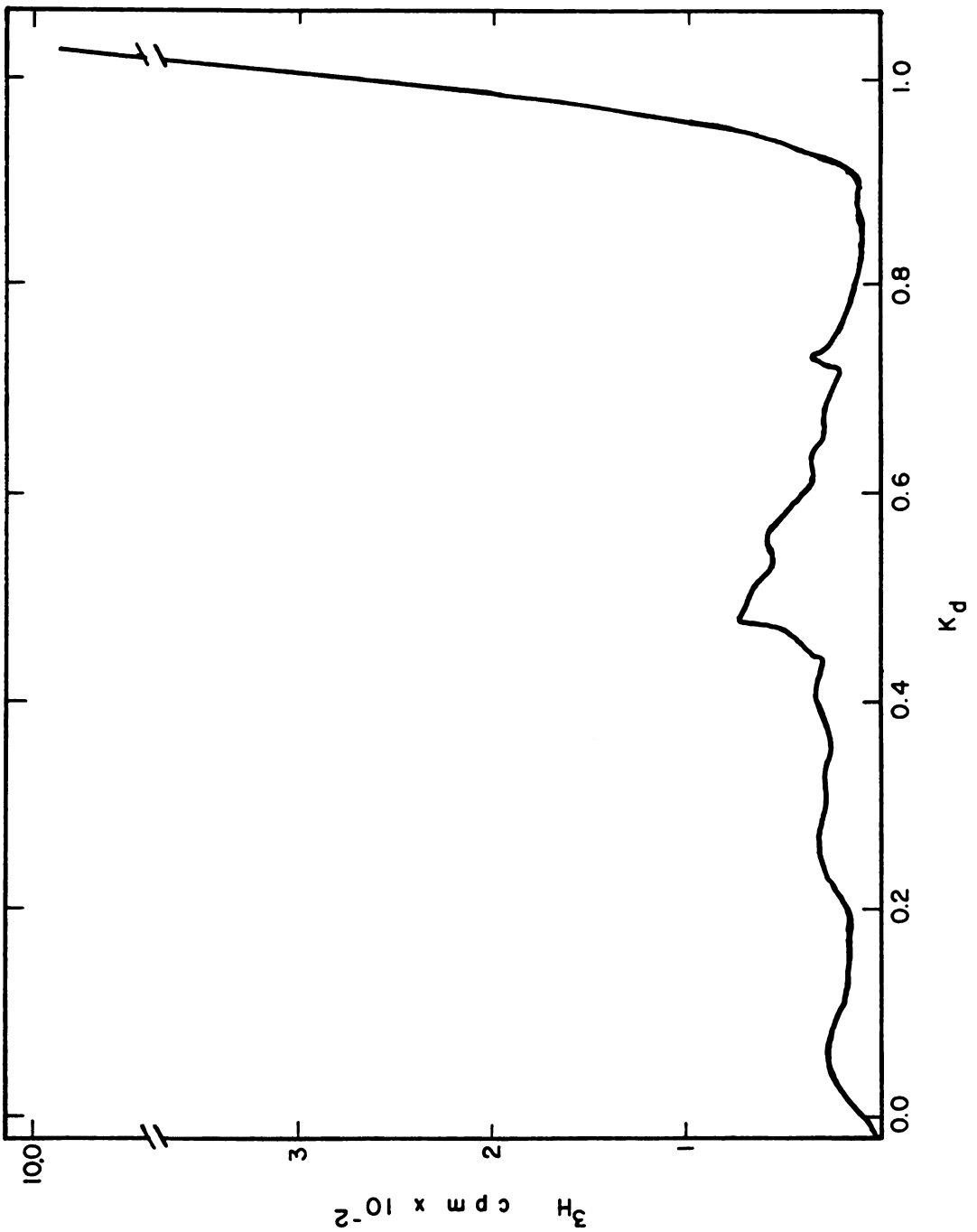

1.5
1.0
1.5
^3H cpm x 10^{-3}
0.0
0.2
0.4
0.6
0.8
1.0
K_d

FIGURE 32. The effect of a 20 minute pactamycin chase on the nascent peptide size distribution from the globin mRNA directed wheat germ cell-free system.


10.0
3
2
1
0.0
0.2
0.4
0.6
0.8
1.0
K_d
^3H cpm x 10^{-2}

distribution resulting from the translation of 10 minute S_1 digested globin mRNA. The result of a 20 minute pactamycin chase on the nascent peptide size distribution is shown in Figure 33.

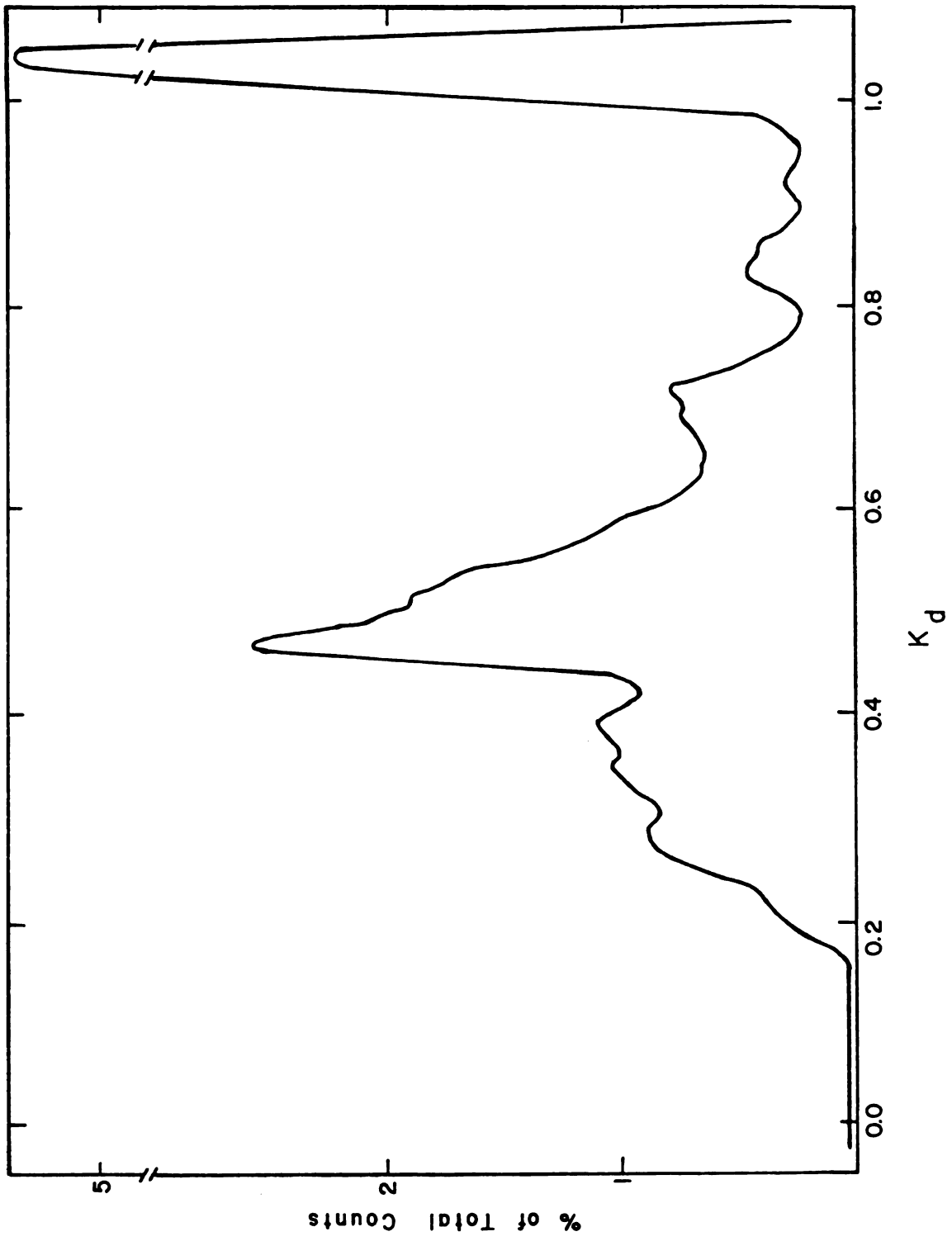
The quantity of nascent peptides present in the pactamycin chased incubation was 18% of that found in the absence of treatment with the inhibitor. This is in contrast with the 3% found remaining in the nascent peptide fraction following pactamycin chase of nascent peptides resulting from the translation of intact globin mRNA. The nascent peptide size distribution following a pactamycin chase looks strikingly similar to a nascent peptide size distribution obtained from the translation of intact globin message. The fact that the nascent chain population following a pactamycin chase is reduced over 5 fold when compared with the non-pactamycin chased incubation should be remembered.

Separation of α- and β-Globin mRNA

The distribution of nascent chains observed during synthesis of globin in the reticulocyte is a composite profile representing a mixture of α- and β-globin nascent peptides. Interpretation of the data thus far obtained using structure specific degradation of messenger RNA could be made more meaningful if the data for the translation of single mRNA species were available.

In order to resolve the nascent peptide size distribution into its α and β component profiles, globin mRNA was separated into α- and β-globin mRNA fractions. By translation of the separated α- and β-globin mRNAs, the elution profiles of α-globin and a β-globin nascent chains could be determined and information relating to the conformation of each mRNA could be deduced. The locations of peaks in the resolved patterns could aid in the interpretation of data from the S_1 nuclease

FIGURE 33. The size distribution of globin nascent peptides from 10 minute S_1 nuclease digested globin mRNA directed protein synthesis in the wheat germ cell-free system following a 20 minute pactamycin chase.


% of Total Counts
5
2
1
0.0
0.2
0.4
0.6
0.8
1.0
K_d

digestion of globin mRNA as well as data obtained from the degradation patterns observed in reticulocyte lysates subjected to micrococcal nuclease digestion.

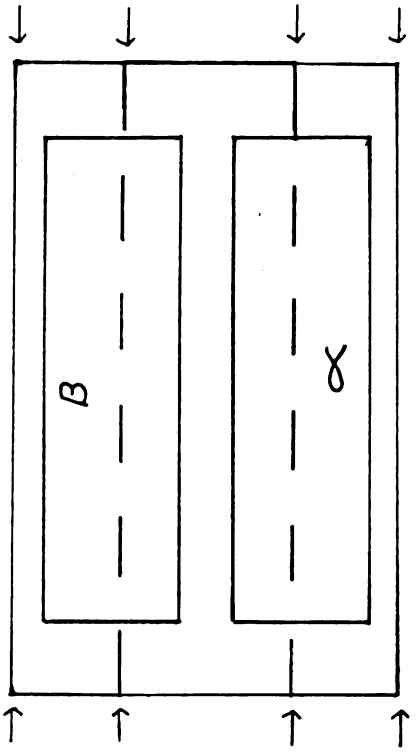
Alpha globin mRNA is 552 nucleotides long, while β-globin mRNA is 590 nucleotides long. These lengths exclude the contribution of the poly(A) to the total length of these mRNAs. The difference in the molecular weights of the two mRNA species allowed their separation in polyacrylamide gels under denaturing conditions.

Following separation and staining of preparative polyacrylamide gels, bands of mRNA were excised with a sterile razor blade. RNA in each band was recovered by electrophoretic elution as described in Methods. It was found that the exact location chosen to slice the polyacrylamide gel greatly affected the purity of the recovered mRNA as well as the yield. Figure 34 is an exact duplication of the appearance of the preparative gel following electrophoresis. It was found that by slicing the gel according to the scheme in "A" up to 60% of the total mRNA could be recovered, but the cross contamination of one mRNA species with the other was approximately 20%. However, if the gel was cut according to scheme "B", total recovery of the electroeluted RNA fell to 20% but mRNA purity of greater than 90% was achieved.

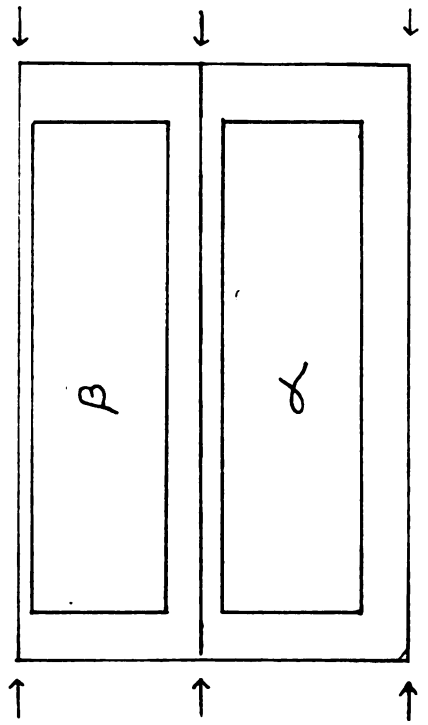
The contamination of α-globin mRNA with β-globin mRNA, if a single cut was made between the two bands, as shown in Figure 34A may be explained by the heterogeneity of the poly(A) on each mRNA species. The α-globin mRNA contamination was probably due to β-globin mRNA molecules with short poly(A) tracts co-migrating with α-globin mRNA with long poly(A) tracts. Similarily, long poly(A) tracts α-globin mRNA contaminates the short poly(A) tract β-globin mRNA. By discarding this

FIGURE 34. Slicing of preparative polyacrylamide gels for optimum purity of mRNA fractions.

B



A

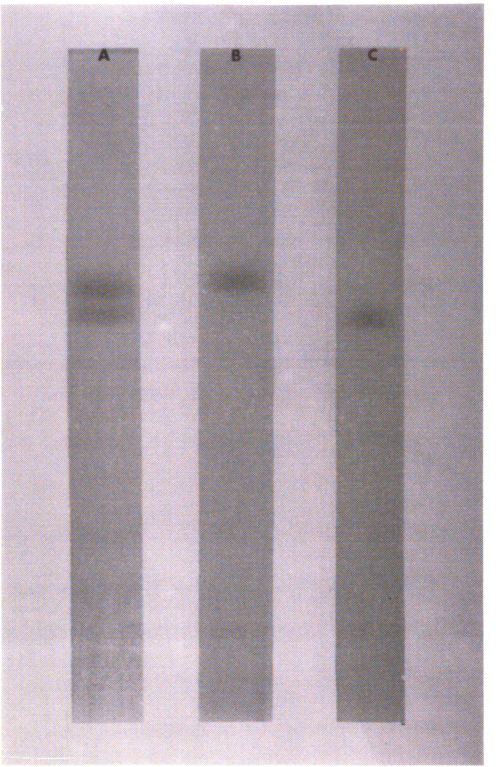


region of overlap between mRNAs in the gel, individual messenger RNAs of high purity were obtained. It was decided that the sacrifice of yield in RNA was justified in order to obtain high purity α- and β-globin mRNA globin mRNA fractions in a single electrophoretic step. Vournakis et al. (28) have since reported a complex two dimensional electrophoresis system for separation of α- and β-globin mRNA. However, the feasibility of this method for large scale preparation is not known.

Assessment of the Translational Purity of α- and β-Globin mRNA

Figure 35 shows the electrophoretic separation of α- and β-globin mRNA together with unfractionated total globin mRNA. Each can be seen as a narrow migrating band with no apparent contaminating bands. The translational purity of each message was determined by its translation in the wheat germ cell-free system followed by analysis of the products synthesized by chromatography on CM cellulose to determine the relative amounts of α- and β-globin synthesized. A 0.5 μg aliquot of α-globin mRNA was incubated in a 100 μl wheat germ cell free incubation mixture supplemented with [^{3}H]-isoleucine for 60 minutes and centrifuged to remove polysomes. Following ultracentrifugation, the supernatant fraction was analyzed for the amount of α- and β-globin that was present. Figure 36 shows the result of one such separation. From the peak areas and correcting the peak areas for the number of isoleucines present in α- and β-globin, it was determined that the α-globin mRNA fraction directed the synthesis of >95% α-globin. Uniformly labeled [^{14}C]-globins were included to verify the positions of authentic α- and β-globin. The result of an analysis of the products synthesized by the β-globin mRNA fraction in the wheat germ system supplemented with [^{3}H]-tryptophan is shown in Figure 37. For this fraction, 93% of the counts appear in the

FIGURE 35. Gel electrophoresis of globin mRNA: A, total globin mRNA: B, α-globin mRNA: C, β globin mRNA.

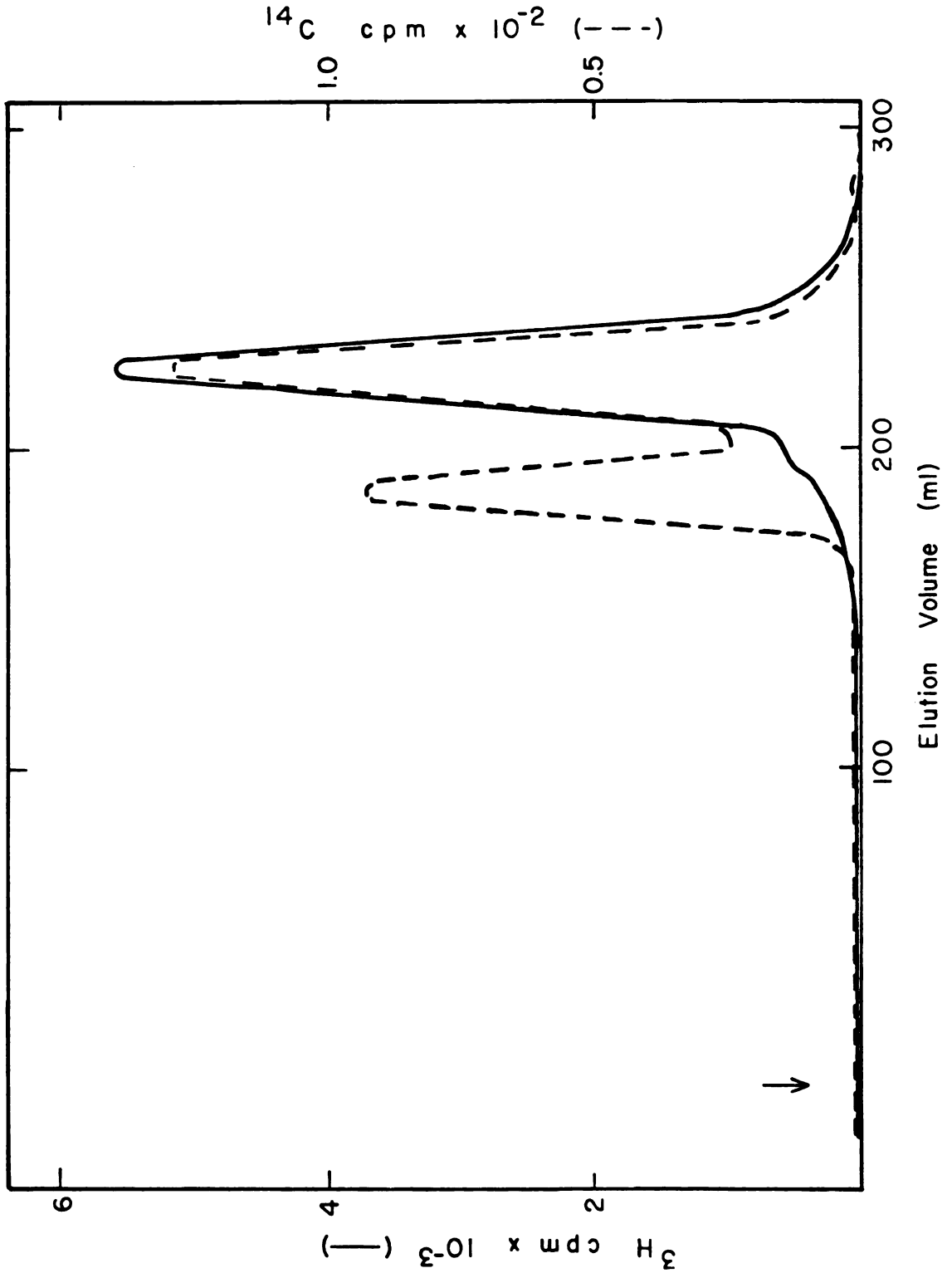


A: B,

FIGURE 36. CM cellulose chromatography of the [^{3}H]-isoleucine labeled products synthesized by the α-globin mRNA directed wheat germ system. The arrow indicates the start of gradient elution of the column. [^{14}C]-tryptophan α- and β-globins are included as internal standards. [^{3}H](——), [^{14}C](— —).

14C cpm x 10-2 (– –)
3
2
1
300
200
100
Elution Volume (ml)
12
8
4
3H cpm x 10-3 (——)

FIGURE 37. CM-cellulose chromatography of the [^{3}H]-tryptophan labeled products synthesized by the wheat germ cell-free system in response to purified β-globin mRNA. [^{14}C]-tryptophan globins were included as internal standards.


^{14}C cpm x 10^{-2} (- - -)
1.0
0.5
300
200
100
Elution Volume (ml)
^{3}H cpm x 10^{-3} (——)
6
4
2

β-globin peak. Therefore, it can be assumed that analysis of the nascent chains present on ribosomes during translation of the α-globin mRNA should be greater than 95% α-globin nascent chains and similarly, analysis of nascent chains isolated during translation of β-globin mRNA should be greater than 93% β-globin nascent peptides.

Determination of the α- and β-Globin Nascent Peptide Distribution

Purified α-globin mRNA was incubated in a 0.50 ml wheat germ cell-free incubation supplemented with [^{3}H]-isoleucine to label the nascent peptides. After twenty minutes, the incubation reaction was terminated with medium B and antibiotics and globin nascent peptides isolated. These nascent peptides were then analyzed by chromatography on a Bio-Gel A 0.5m column. The elution profile for [^{3}H]-isoleucine labeled α-globin nascent peptide is shown in Figure 38.

It can be seen that the size distribution of nascent peptide resulting from the translation of purified α-globin mRNA is quite dissimilar to those accumulations resulting from the translation of total globin mRNA. The α-globin nascent peptide distribution determined here is in good agreement with the α-globin nascent peptide profile seen following labeling of the reticulocyte lysates from homozygous B_{112} Val/Val rabbits with [^{3}H]-isoleucine. These rabbits have a valine substituted for the only isoleucine in the β chain at positions 112. Labeling of reticulocyte lysates prepared from these rabbits with [^{3}H]-isoleucine labels only α-globin nascent peptides. Although there was some peak height variance, these two patterns displayed accumulations at the same K_d values. Labeling of α-globin nascent peptides with [^{3}H]-tryptophan resulted in the pattern shown in Figure 39. Although the peak heights vary slightly between tryptophan and isoleucine patterns due to

FIGURE 38. The elution profile of [^{3}H]-isoleucine α-globin nascent peptides from the α-globin mRNA directed wheat germ cell-free system.

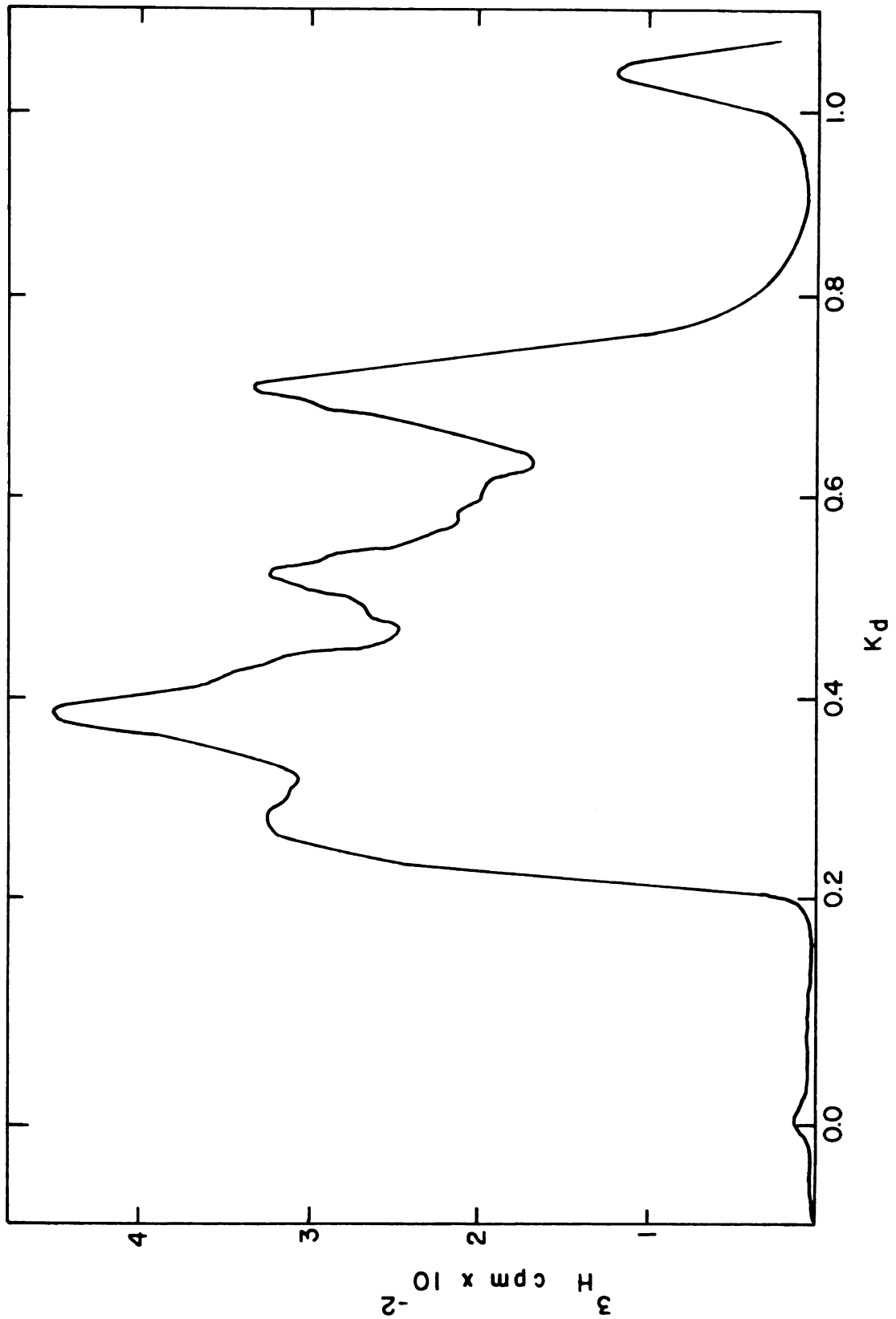
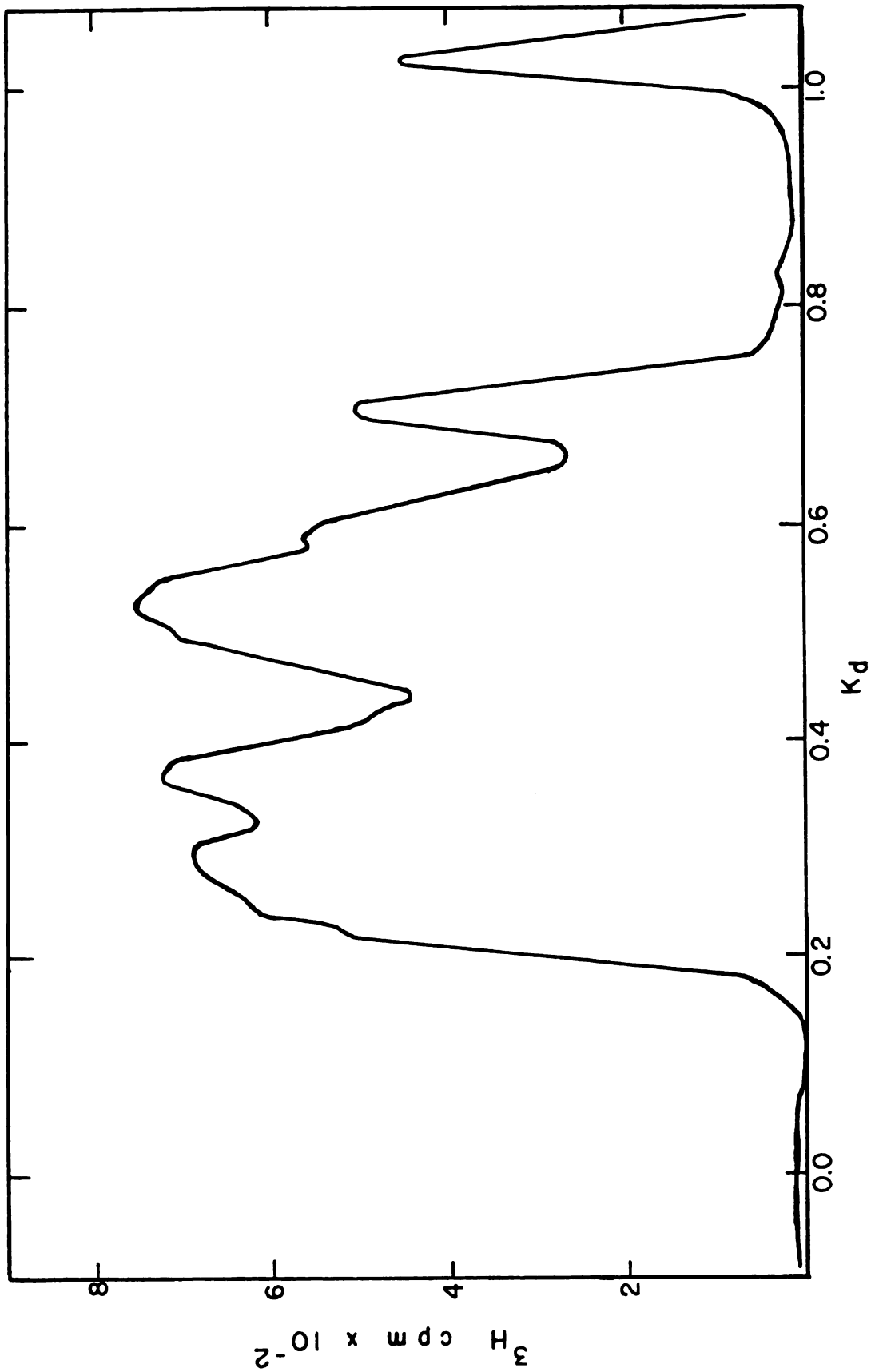

^3H cpm x 10^{-2}
4
3
2
1
0.0
0.2
0.4
0.6
0.8
1.0
K_d

FIGURE 39. The size distribution of α-globin nascent peptides labeled with [^{3}H]-tryptophan from the α-globin mRNA directed wheat-germ cell-free system.


^3H cpm x 10^{-2}
2
4
6
8
0.0
0.2
0.4
0.6
0.8
1.0
K_d

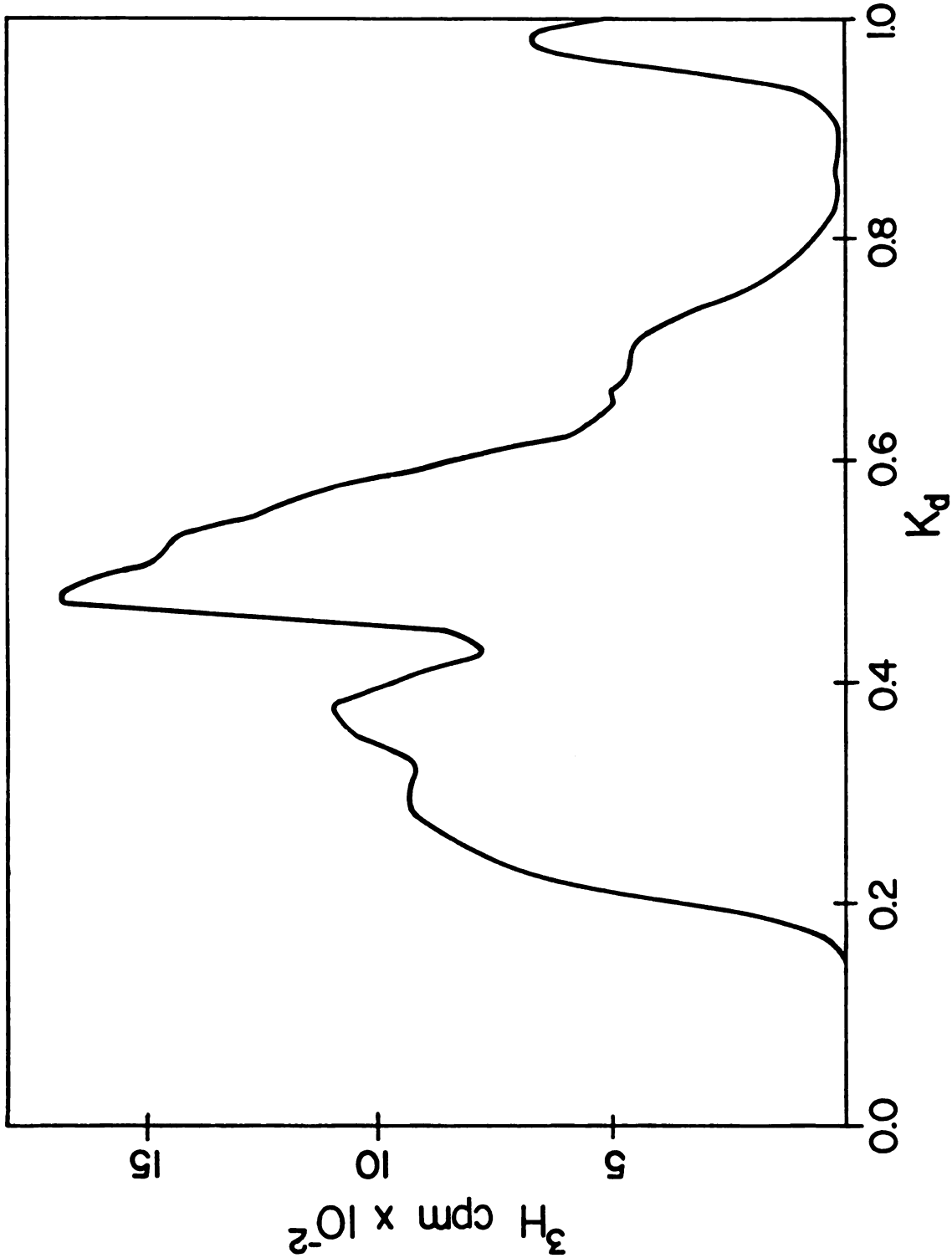
the entry of the two amino acids into the growing nascent peptides at different locations, accumulations of nascent peptides occur at the same K_d values regardless of the amino acid employed in the labeling.

The β-globin nascent peptide distribution was determined by labeling of polysomes with [^{3}H]-tryptophan in a 0.5 ml wheat germ incubation. Following 20 minutes of labeling, the incubation was terminated and nascent peptides purified as before. The nascent peptides were analyzed by Bio-Gel A 0.5m chromatography. The results of one such analysis is shown in Figure 40. The β-globin nascent chain profile is significantly different from both the α-globin profile, and the total globin nascent peptide profile. The region of nascent peptide accumulations from K_d 0.45 to 0.55 are highly populated in the β profile with no peaks of nascent peptide accumulation at K_d values greater than 0.28. A large peak seen in this β-globin profile at K_d 0.47 also appears prominently in the mixed nascent peptide size distribution.

Comparison of the α- and β-Globin Nascent Peptide Chain Size Distribution

The α- and β-globin nascent peptide size distributions are quite different when compared to one another. The high molecular weight region of the mixed globin nascent peptides from K_d 0.20 to 0.28 represents mainly α- globin peptides. This agrees with the observations of Protzel and Morris that completed α-globyl tRNA accumulates on polysomes in large amounts compared to completed β-globyl tRNA (75). The main peaks of accumulation in the nascent peptide profile in the K_d region of 0.45-0.55 represent a major contribution of β-globin peptides to the mixed profile of α- and β-globin nascent chains. These nascent peptide profiles are in basic agreement with the α- and β-globin nascent peptide profiles determined by Vary using tryptic digestion analysis of a mixed

FIGURE 40. The size distribution of β-globin nascent peptides from the β-globin mRNA directed wheat germ cell-free system.

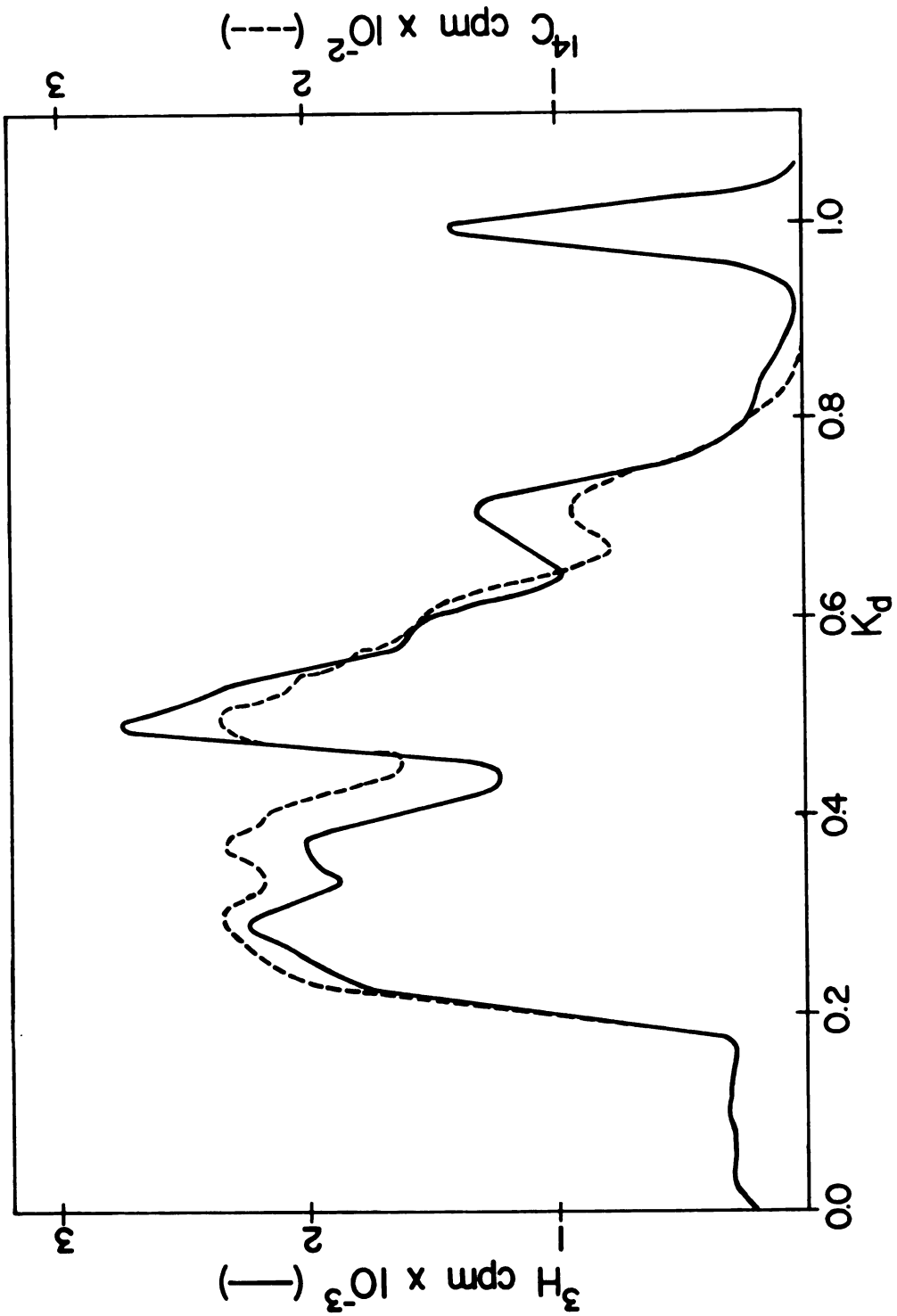

0.0
0.2
0.4
0.6
0.8
1.0
K_d
5
10
15
^3H cpm x 10^{-2}

nascent peptide distribution in order to separate the α and β components of the mixed profile.

The Elution Profile of Nascent Peptides Resulting from the Translation of Recombined Mixture of Purified α- and β-Globin mRNA

In order to insure the validity of the α- and β-globin nascent chain profiles, purified α- and β-globin mRNA were recombined and translated in a wheat germ cell-free incubation. The ratio of α- to β-globin mRNA in the reconstituted mixture was 1.4:1, the same proportion of these messenger RNAs found in intact reticulocytes. Following a 20 minute labeling of nascent peptides with [^{3}H]-tryptophan, the reaction was stopped with medium B and antibiotics and the mixture combined with [^{14}C]-tryptophan labeled nascent peptides from polysomes of a reticulocyte lysate. Nascent peptides were purified and the size distribution on Bio-Gel A 0.5m determined. Figure 41 shows the results of this experiment. The reconstituted α- and β-mRNA mixture yields a size distribution of nascent peptides which is indistinguishable from nascent peptides isolated from an intact reticulocyte lysate. The fact that the α- and β-globin mRNAs can be separated by gel electrophoresis, recombined and generate a size distribution profile of nascent peptides nearly indistinguishable from that observed in the reticulocyte lysate is significant. This experiment demonstrates that the nascent peptide size distribution profiles derived from the translation of the separated messages are the true α and β nascent peptide profiles since recombination of the messenger RNAs from which the individual profiles were derived gives back the original mixed globin nascent chain distribution observed in the reticulocyte lysate.

FIGURE 41. The nascent peptide size distribution of globin nascent peptides resulting from the translation of a reconstituted mixtures of α- and β-globin mRNA in the wheat germ cell-free system.

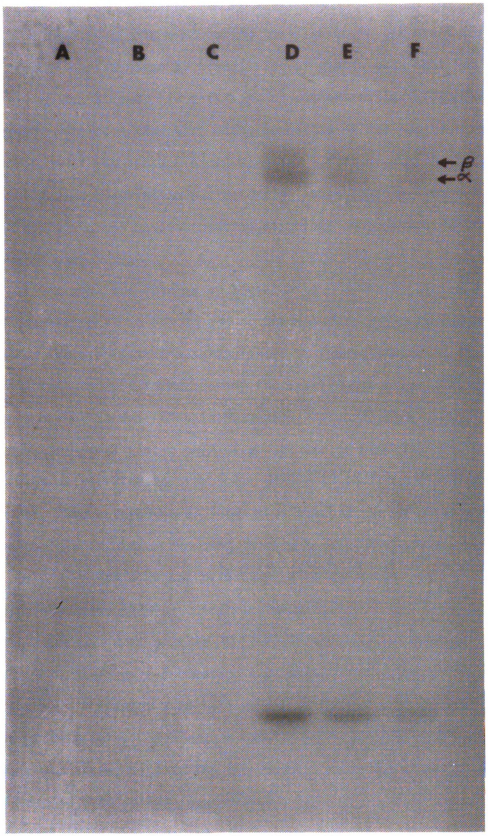

^{14}C cpm x 10^{-2} (----)
1
2
3
0.0
0.2
0.4
0.6
0.8
1.0
K_d
^{3}H cpm x 10^{-3} (——)
1
2
3

5' End Labeling of Globin mRNA

The 5' end of globin mRNA contains a "cap" structure. This cap structure prevents the addition of a 5' [^{32}P]-label into globin mRNA. Hence, 5' end labeling of globin mRNA should not incorporate ^{32}P into the mRNA. The incorporation of a [^{32}P]-label into the globin mRNA can be assumed to occur only at the 5' end of fragments of globin mRNA present in the mRNA preparations. Since previous data have shown that fragments of mRNA can contribute to the accumulations in the nascent peptide fraction, it was essential to know if a proportion of the mRNA in the preparations used in these studies were fragmented during isolation.

Globin mRNA was dephosphorylated with calf alkaline phosphatase and 5' labeled with γ-[^{32}P]-ATP using T_4 induced polynucleotide kinase as described in Methods. Following removal of unreacted ATP by passage of the mixture over a Sephadex G-25 column, it was observed that no ^{32}P had been incorporated into the mRNA. However, when the capping nucleotide was removed from globin mRNA with tobacco acid pyrophosphatase (TAP) extensive incorporation of ^{32}P into mRNA was observed. The [^{32}P]-labeled mRNA was subjected to gel electrophoresis in a 7 M urea, 2.6% acylamide vertical slab gel along with control globin mRNA in adjacent lanes. Following autoradiography of the gel to determine the migration of [^{32}P]-labeled RNA, the gel was stained in 0.05% "Stain All" in 50% formamide to visualize the location of intact globin mRNA. Figure 42 is an autoradiogram of one such gel. Arrows mark the migration of the α- and β-globin mRNAs in parallel lanes. These results show the lack of incorporation of ^{32}P into RNA in the absence of pretreatment with TAP was not due to a fault of the labeling system, but rather was due to the nature of the mRNA which contained only intact 5' ends.

FIGURE 42. Autoradiogram of 5' [^{32}P]-labeled globin mRNA. Lanes A, B, and C, 5' [^{32}P]-labeling without TAP treatment; Lanes D, E, F, treatment of globin mRNA with TAP and 5' labeling.


A
B
C
D
E
F
2β

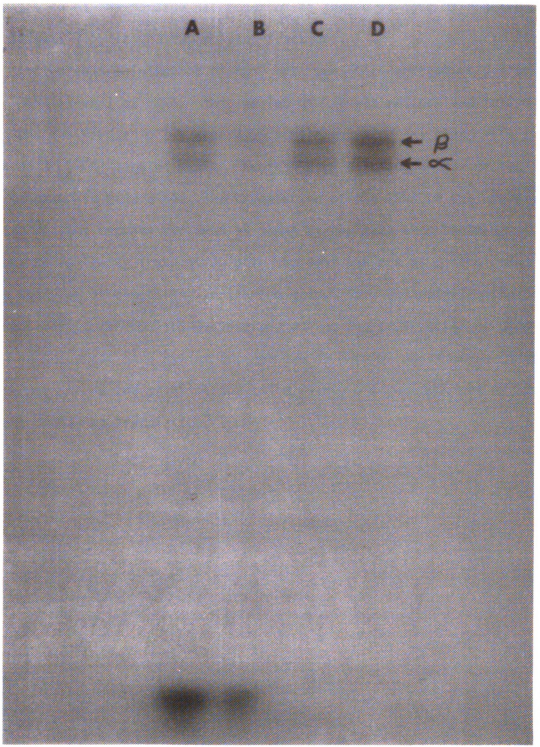
3' End Labeling and Translation of Globin mRNA

The fate of globin mRNA during translation in the wheat germ cell-free system was assessed by translation of globin mRNA that had been labeled at the 3' end. In this way, the determination of whether or not fragments of globin mRNA were being generated by nuclease during the translation of the message could be made. If fragmentation of the message during translation was occurring, this could be another factor contributing to the nascent peptide distribution observed during translation of globin mRNA.

Globin mRNA was labeled by ligation of 5'-[^{32}P]-cytidine,3,5' bisphosphate (pCp) to the 3' end of the molecule using T_4 induced RNA ligase. The globin mRNA was repurified to remove unreacted pCp and any degradation products formed during the labeling by oligo (dT) cellulose chromatography as described in Methods. The products resulting from the 3' end labeling of globin mRNA were analyzed by electrophoresis in a 7 M urea-2.6% polyacrylamide slab gel. Figure 43 is an autoradiogram of one such gel. The autoradiogram shows that the labeled mRNA co-migrated with authentic globin mRNA. The mRNA labeled by this method averaged 3-5 x 10^5 cpm ^{32}P/µg RNA with approximately 3% of the input label incorporated into poly(A) containing material.

A 2.5 µg aliquot of this 3' [^{32}P]-labeled globin mRNA was added to a 0.5 ml wheat germ incubation supplemented with 100 µCi of [^{3}H]-tryptophan. Polysomes were labeled for 20 minutes and nascent peptides prepared as before. Upon resuspension of the polysome pellet in 0.25 M sucrose and antibiotics during the initial step of nascent peptide purification, 94% of the input ^{32}P was recovered. No ^{32}P was detected in the postribosomal supernatant. This indicated that all of

FIGURE 43. Autoradiogram of 3' [^{32}P]-labeled globin mRNA. Lanes A and B, mRNA prior to oligo (dT) cellulose chromatography; C and D, after purification of mRNA by oligo (dT) cellulose column chromatography.

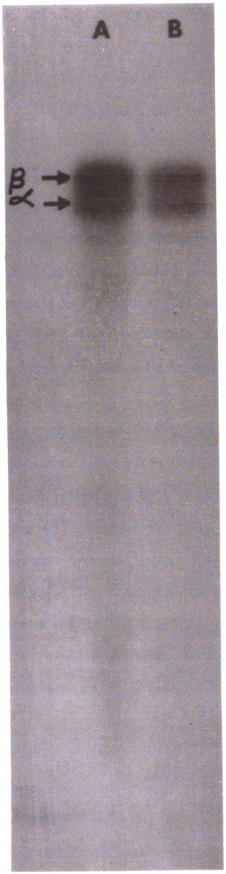


A
after
.

the labeled mRNA was used during protein synthesis. In addition, no degradation of the message had occurred as judged by lack of appearance of radioactivity in the supernatant fractions.

LiCl-urea precipitation of rRNA was found to quantitatively precipitate mRNA as well as rRNA. This pellet of RNA was redissolved in 7 M urea and analyzed on a 7 M urea-2.6% acrylamide slab gel along with control 3' [^{32}P]-labeled mRNA. The result of autoradiography of the gel is shown in Figure 44. The translation of the mRNA in the wheat germ system did not degrade the mRNA as shown by the fact that the mRNA recovered following translation gave the same pattern as control mRNA when analyzed by denaturing acrylamide gels. This demonstrates that the nascent peptide accumulations observed during the translation of globin mRNA in the wheat germ system are not due to partial degradation of the messenger RNA during translation, since nearly all the mRNA was recovered intact following translation.

FIGURE 44. Autoradiogram of 3' [^{32}P]-labeled globin mRNA recovered after translation of the mRNA in the wheat germ cell-free system.


A
B
β
α

DISCUSSION

Rabbits release large quantities of reticulocytes into the circulation in response to phenylhydrazine induced anemia. The reticulocyte represents the final precursor cell in the erythropoietic sequence. These non-nucleated cells synthesize hemoglobin on residual polysomes remaining from the orthochromic erythroblast stage of development. Reticulocytes continue to synthesize protein for 24-48 hours before the loss of the remaining ribosomes and mitochondria marks its transition to the mature erythrocyte.

The major proteins synthesized in the rabbit reticulocyte are the α and β subunits of hemoglobin. These two proteins represent approximately 95% of the total protein synthesized by the reticulocyte.

Protzel and Morris (65) analyzed the size distribution of nascent peptides found on ribosomes translating α- and β-globin mRNA in the intact rabbit reticulocyte. Their studies demonstrated that a nonuniform size distribution existed within the nascent peptides of α- and β-globin. Selected size classes of nascent peptides were found to be present in either larger or smaller amounts than would be predicted by a uniform rate of translation.

The elongation step of protein synthesis consists of codon recognition, peptide bond formation and translocation. During the translocation phase, the ribosome advances from the codon occupied during peptide bond formation to the next codon along the mRNA. The peptidyl-tRNA which

occupies the P site immediately following translocation represents a unique marker of defined length. Since each ribosome carries only one growing nascent peptide, the length of that peptide chain allows the placement of that ribosome at an exact position along the coding sequence of the messenger RNA. The nonuniform distribution of molecular weights observed within the nascent peptides found during synthesis of globin represents regions of high and low ribosome densities on the messenger RNA rather than a random spacing of ribosomes advancing at a constant rate.

The mechanism which gives rise to these accumulations of ribosomes at specific sequences along the globin mRNA in rabbit reticulocyte lysates was studied by Chaney and Morris (66). Their work demonstrated that the origin of the nascent peptide nonuniformity was not a result of a limiting component of the protein synthesizing system of the reticulocyte and was apparently associated with some functional aspect of the mRNA. Secondary structure within the globin mRNA was postulated to be responsible for the accumulation of nascent peptides during elongation. Chaney and Morris (67) went on to show that coat protein nascent peptides purified from MS2 infected rifampicin treated E. coli also display a nonuniform nascent peptide distribution. A comparison was made between the proposed regions of secondary structure in the coat protein cistron and the observed accumulations of nascent peptides. Their data supported the hypothesis that secondary structure in the mRNA was responsible for the nonuniform elongation rate observed during protein synthesis.

Vary and Morris (68) expanded this hypothesis by proposing that secondary structure components present in the globin mRNA molecules were responsible for the modulation of ribosome movement across the mRNA. The

positions of the β-globin nascent peptide accumulations were correlated with regions of secondary structure in a proposed secondary structure map of β-globin mRNA proposed by Pavlakis et al. (28). A strong positive correlation was noted between single stranded regions found by Pavlakis in the β-globin mRNA and minima in the nascent chain profile. These studies indicated that the minima found in the nascent peptide size profiles represent single stranded regions in the mRNA.

The studies presented here began with the preparation of highy purified globin mRNA. The method of Krystosek (73), as modified in this work, was chosen as the method of choice for preparation of globin mRNA. Translation of mRNA prepared by this method in a wheat germ cell-free system resulted in a 100% increase in the rate of incorporation of radiolabeled amino acid into protein when compared to globin mRNA used in previous studies. This increase in the rate of protein synthesis was attributed to removal of contaminating rRNA. The globin nascent peptide size distribution obtained from the translation of these mRNA preparations in the wheat germ cell-free system showed no size differences in nascent chain accumulations when compared with patterns obtained by Chaney and Morris using globin mRNA of lower purity prepared by the procedure of Aviv and Leder (81).

At the time that studies on the origin of the nascent peptide nonuniformity were being carried out, Payvar and Schimke (83) reported the enhancement in the translation rate of mRNA denatured with methylmercuric hydroxide. This increase in the efficiency of translation was reported to be due to the shortening of transit times of ribosomes translating the mRNA species studied. Treatment of globin mRNA with methylmercuric hydroxide showed no detectable enhancement of the translation rate of the

mRNA. These results indicated that if transit times were shortened, it was done at the expense of increased times of other phases of protein synthesis. No alteration was observed in the distribution of nascent peptides isolated following translation of methylmercuric hydroxide denatured globin mRNA in the wheat germ cell-free system. If methylmercuric hydroxide does indeed shorten the transit time of ribosomes along the mRNA it does so by a mechanism in which the translocation of ribosomes at points of large hinderance (maxima in the profile) are shortened to the same degree as areas where little hinderance occurs (minima). Stated more simply, transit times were not reduced at any specific structure rich region of the mRNA. The lack of an effect on the nascent peptide distribution following denaturation of globin mRNA is in agreement with the results of Chaney and Morris (84) who failed to perturb the nascent peptide size distribution using a variety of denaturing agents.

Techniques employing manipulation of the globin mRNA by denaturation of possible secondary structure were abandoned in favor of a new approach to the study of this problem. Structure specific cleavage of globin mRNA was used to perturb the nascent chain distribution that resulted from translation of these modified messenger RNAs. Changes in the distribution of nascent peptides then could be correlated with proposed structural features of the globin mRNA molecules. The first approach chosen to study the effect of secondary structure on the distribution of nascent peptides was to assess the effect of alteration of the mRNA template at points of single stranded sequences. S_1 nuclease preferentially degrades single stranded regions in DNA and RNA (89). Several investigators have previously shown that up to 75% of the globin mRNA molecule is resistant to attack by this nuclease, indicating a much

higher percentage of the globin mRNA molecule is involved in secondary structure interactions than would be predicted for a random sequence copolymer (27,64).

Globin mRNA was subjected to limited digestion by S_1 nuclease. The digestion products of RNA with S_1 nuclease under these conditions have been shown to be sequences of RNA of defined length generated by attack of the RNA substrate in loop and hairpin structures (26,28). The digestion of globin mRNA was expected to produce a series of mRNA fragments of discrete lengths which varied in their distances from the 5' end of the messenger RNA to the location of a structure specific area where the mRNA would be broken. Translation of this fragmented RNA in the wheat germ system should then result in large increases in the population of nascent peptides residing at an established region of structure immediately before cleavage points, or changes in position of selected peaks in the nascent chain profile if new conformations of the messenger RNA were adopted.

Two unexpected results were observed in the analysis of the data from the S_1 nuclease digestion of globin mRNA. First, the rate of loss of translational activity resulting from mRNA digestion with S_1 nuclease did not parallel the loss of material from the nascent peptide fraction. At the time that 50% reduction of the translational activity of the mRNA had been achieved as a result of nuclease action on the mRNA greater than 70% of the nascent peptide population had been retained. Later experiments with micrococcal nuclease helped to clarify this anomaly which shall be discussed with the micrococcal nuclease data.

Second, the nascent peptide size distribution did not show the generation of new peaks of accumulation when nascent peptides resulting from

the translation of S_1 digested globin mRNA was analyzed. The distribution of material among the peaks of accumulation was altered. Peaks of lesser significance in control nascent peptide patterns developed into major accumulations following S_1 treatment of globin mRNA. These results may support the hypothesis that regions of double strandedness are responsible for the accumulation of nascent peptides. Attack of S_1 nuclease in limiting concentrations at single stranded regions of the mRNA has been shown to generate globin mRNA fragments rich in secondary structure (26,28). The cleavage of mRNA in this fashion should tend to accentuate minor accumulations in the nascent peptide size distribution. A leveling of the peak heights that remained in the nascent peptide size distribution was also observed as a result of S_1 nuclease digestion of globin mRNA. This leveling effect could be proposed to occur as long digestion times of mRNA with S_1 nuclease eventually cleaved at points of single-stranded sequence whose nuclease susceptibilities were lower, creating shorter length mRNA fragments. This would tend to lessen the proportion of material seen in higher molecular weight nascent peptides. The initiation of protein synthesis at internal initiators on mRNA fragments without an intact 5' end created by S_1 nuclease digestion was not considered. Evidence has shown that initiation at internal initiation sites following cleavage of the capped mRNA is minimal (90,91,92).

During the time these studies were in progress, Pelham and Jackson (72) described a method for the preparation of a message dependent protein synthesizing system from the reticulocyte lysate. Endogenous mRNA was destroyed in the lysate by incubation with micrococcal nuclease in the presence of Ca^{2+}. Chelation of Ca^{2+} with EGTA inactivated the nuclease following the digestion and a lysate dependent upon

addition of exogenous mRNA was created. This mRNA dependent lysate was reported to have a low background of incorporation while maintaining the capacity to synthesize high molecular weight products at a high efficiency.

The possibilities for using this mRNA dependent lysate for the studies on the secondary structure of actively translating globin mRNA were assessed. Globin mRNA could be modified and then returned to the protein biosynthesizing system from which it was isolated, thereby studying only the effects of mRNA modification and eliminating differences between protein synthetic systems. The mRNA dependent reticulocyte lysate cell-free system was prepared by the method of Pelham and Jackson (72). The incubation conditions which yielded a 99% reduction in the level of reticulocyte lysate endogenous protein synthesis were determined for lysates prepared for this work and were found to be within the ranges determined by Pelham and Jackson. These mRNA dependent lysates responded to added mRNA in the expected manner and would synthesize protein products for greater than 40 minutes at 37°. The 37° temperature was used for these experiments rather than 30° as used by the developers of the system so that data obtained with the mRNA dependent reticulocyte lysate would be comparable to incubation conditions used for the nascent peptide preparations from the intact reticulocyte lysate.

Nascent peptides were prepared from the translation of globin mRNA in the messenger RNA dependent reticulocyte lsyate and analyzed by Bio-Gel A 0.5m gel filtration chromatography. A comparison of [^{3}H]-tryptophan labeled nascent peptides from a mRNA dependent lysate supplemented with globin mRNA with [^{14}C]-tryptophan labeled nascent peptides isolated from a control reticulocyte lysate incubation gave unexpected

differences between the two profiles. All peaks of nascent chain accumulation observed between K_d 0.5 and 1.0 were greatly exaggerated in the mRNA dependent lysate system. These enhanced peaks of accumulation were shown to be due to some factor other than the mRNA preparation used, since analysis of nascent peptides prepared from translation of the same mRNA in the wheat germ system showed a normal distribution of nascent peptides. The origion of these enhanced peaks of accumulation was therefore sought in the inactivated lysate preparation.

Incubation of the mRNA dependent lysate with [^{3}H]-tryptophan in the absence of added mRNA revealed the presence of a substantial amount of nascent peptides, even though no significant amount of product was synthesized as determined by the incorporation of radiolabel into TCA precipitable material. This pool of nascent peptides could represent as much as 22% of the labeled nascent peptide pool size observed in the reticulocyte lysate prior to nuclease inactivation. The observed peak accumulations of nascent peptides in the absence of globin synthesis corresponded to peaks of nascent peptide accumulations normally observed during the synthesis of globin in the reticulocyte, reticulocyte lysate, or the wheat germ cell-free system programmed with globin mRNA. The major peaks of nascent chain accumulations occurred at K_d 0.58 and 0.71 with minor accumulation at K_d 0.28, 0.34, 0.47, 0.60 and 0.78. Digestion of lysates with levels of nuclease in excess of that needed to destroy endogenous protein biosynthetic activity could eliminate all of these accumulations with the exceptions of those seen at K_d 0.58 and 0.71 whose presence were invariant. The lysates subjected to these extensive nuclease digestion conditions, however, were severely deficient in their ability to respond to added mRNA and were considered unusable.

Analysis of the change in the distribution of the nascent peptide fraction remaining with increasing degrees of nuclease digestion of the endogenous globin mRNA in the reticulocyte lysate was performed. The results of these investigations showed that the nuclease digestion of the endogenous globin mRNA of the reticulocyte lysate does not occur by attack of the message in random locations. Random attack of the message would result in the loss of material from all areas of the nascent peptide distribution in a uniform fashion since the probability of nuclease attack at any position along the mRNA sequence would be equal in this digestion scheme.

The observed change in the distribution of nascent peptides does not fit this model. The loss of nascent peptides during micrococcal nuclease digestion of globin mRNA suggests a non-random attack upon the globin mRNA. Regions of the nascent peptide size distribution where minima exist appear to be early points of nuclease attack. These include early loss of peaks at K_d 0.22, and K_d 0.28, followed by slow loss of peaks at K_d 0.38, K_d 0.47, and K_d 0.53, while peaks at K_d 0.35, K_d 0.58 and K_d 0.71 appeared most resistant.

Labeling of nascent peptides following nuclease digestion with [^{3}H]-leucine allowed observation of possible accumulations which occur before entry of the first tryptophan. This labeling revealed additional accumulations of nascent peptides at K_d 0.74, K_d 0.85 and 0.90 corresponding to nascent chain accumulations 14 amino acids, 7 amino acids and 4 amino acids long. These heretofore unobserved accumulations correspond to accumulations observed by Vary in the globin nascent peptides from reticulocyte lysates N-terminally labeled with formyl methione (68). These peaks were only observed in the studies performed

here following extensive nuclease digestion of mRNA and were not observed in control nascent peptide size distributions.

The data support a proposal where the micrococcal nuclease degradation of globin mRNA is a multi-step process. First, there is an initial attack at the exposed single stranded regions of the message. This is supported by the sequential loss of peaks of nascent peptide accumulations immediately following the major minima in the profile. Second, there is attack by micrococcal nuclease at less susceptible regions of the mRNA represented by the observed loss of selected peaks of nascent peptide accumulation at longer nuclease digestion times. Finally, a population of nuclease resistant mRNA fragments is reached represented by the persistent nascent peptide accumulations at K_d 0.58 and K_d 0.71. Leucine labeling also shows peaks of nascent peptides at K_d 0.74, 0.85 and 0.90 which remain after nuclease digestion. It should be noted that K_d 0.90 may represent the entry of leucine into either the α-globin molecule at position number 3 or the β-chain at amino acid number 2.

Further investigations into the nature of the nascent peptides found in the background of the nuclease inactivated lysate were performed with pactamycin, a selective inhibitor of initiation in both the reticulocyte lysate and the wheat germ cell-free systems. Pactamycin has been shown to selectively inhibit the initiation of new protein synthesis, while having little effect on either elongation or termination (85,86). The results obtained in this study support this mode of action for pactamycin. By inhibiting initiation of new globin synthesis, greater than 97% of the nascent peptide pool could be chased off of polysomes during incubation of globin mRNA in the wheat germ system in the presence of pactamycin. However, when pactamycin was added to wheat germ incubations

directing globin synthesis from S_1 nuclease treated globin mRNA, a significant fraction of the nascent peptides remained following the chase period. These nascent peptides represent a fraction of the total nascent peptides turning over much more slowly than observed for nascent peptides arising from the translation of intact globin mRNA. The quantity of nascent peptides in the pactamycin resistant fraction is of the proper size to account for the discrepency noted between unequal loss of mRNA translational activity following S_1 digestion of a globin mRNA and the excess of labeled nascent chains found. Those nascent peptides observed in the presence of pactamycin may well represent ribosomes reaching the end of a mRNA fragment lacking a terminator codon at the 3' end. Thus these nascent peptides persist, artificially increasing the size of the active nascent peptide pool.

The size distribution of the nascent peptides which remain following translation of cleaved globin mRNA and a chase of actively synthesizing polysomes with pactamycin was also examined. The profiles of these nascent chains were quite similar to patterns observed when nascent peptides from an uninhibited system translating intact mRNA were analyzed. The fraction of nascent peptides remaining following the translation of S_1 digested globin mRNA in the wheat germ cell-free system was always less than 15% of the amount of the nascent peptides found in the absence of the inhibitor.

This can be compared with the proportion of pactamycin resistant nascent peptides resulting from the translation of inact globin mRNA, which was less than 3%. This indicates that the majority of nascent peptides analyzed from the translation of intact mRNA are indeed due to structural components within the messenger RNA and not a result of some

degradation process. This result was supported by experiments with 3' labeled globin mRNA. No fragments of mRNA were generated during the translation of the mRNA in the cell-free translation system. The fact that the length of these shorter nascent peptides which persist in the presence of pactamycin following nuclease cleavage of mRNA are the same length and display a comparable size distribution as nascent peptides observed from the translation of intact mRNA is significant. This observation fits well with the hypothesis correlating the accumulation of nascent peptides of discrete size classes with structural features of the messenger RNA.

The formation of nascent peptides resistant to runoff in the presence of pactamycin following translation of only S_1 digested globin mRNA also strongly supports the proposal that the nascent peptides observed in the inactivated reticulocyte lysate are fragments of globin mRNA surviving nuclease treatment. This persistence of nascent peptides was only observed in the cases where globin mRNA was subjected to the action of nuclease before translation. There also appears to be broadening of the peaks seen in the size distribution of nascent peptides remaining following a pactamycin chase. This spreading could represent the distance from the 5' end of a region of secondary structure where Chaney and Morris predicted the slowing of elongation to occur to an S_1 cleavage point on 3' side of the structural feature where the termination of protein synthesis is slowed. This postulation would be in agreement with the concept of ribosomes reaching the end of a coding sequence without encountering a terminator sequence terminating at a reduced rate.

Several conclusions may be made from these experiments and their relationship to the proposal that nascent peptide accumulations are the

result of the modulation of ribosome translocation rates along the mRNA by regions of secondary structure.

First, micrococcal nuclease cleavage of globin mRNA displays a non-random pattern of attack. There is precedent for the nonrandom nature of micrococcal nuclease action in the work of LaFond et al. (93) who showed that the digestion of chicken erythrocyte chromatin yielded a non-random pattern of cleavage products. Laskey et al. have also recently reported a highly sequence specific nature in the action of micrococcal nuclease on DNA (94). Their results showed that micrococcal nuclease digestion of end labeled linear DNA molecules of known sequence produced only a limited number of cleavage sites in the DNA. The micrococcal nuclease cleavages generated a highly specific pattern of fragments. Micrococcal nuclease also appears to degrade globin mRNA in a non-random fashion. The micrococcal nuclease cleavage of globin mRNA seems to occur in selected regions of the messenger RNA which may be highly exposed. This is evidenced in the nascent peptide size distribution by the loss of peaks of nascent peptide accumulations in a specific order during the nuclease digestion.

Second, there appears to be a core of residual mRNA fragments which are resistant to micrococcal nuclease digestion present in the mRNA dependent reticulocyte lysate. This is reflected in a nascent peptide fraction found in the reticulocyte lysate following nuclease inactivation. The observation correlates well with the results of Pavlakis et al. (28) who have reported that the initiator region of the α-globin mRNA is inaccessible to either S_1 or T_1 nuclease attack due to its involvement in secondary and tertiary structure. If indeed a portion of the 5' end of either globin mRNA containing an initiator sequence were

resistant to micrococcal nuclease, these fragments could well give rise to the observed nascent peptides seen in the absence of added mRNA. The appearance of radiolabel in nascent peptides indicates that initiation on some residual template has occurred. Pactamycin inhibition of this initiation also supports the suggestion that this is a dynamic process.

The appearance of a nascent peptide fraction terminating protein synthesis at a reduced rate following mRNA cleavage when compared with the normal nascent peptide pool was noted. The appearance of these peptides at the exact molecular weights observed for accumulation of nascent peptides from the translation of intact mRNA is significant. This result is also in agreement with the working hypothesis of Chaney and Morris stated earlier. S_1 nuclease is known to cleave specifically at single stranded regions, while micrococcal nuclease has been shown to cleave preferentially at the 5' side of A or T residues (89,93,94). The products of such limited cleavage are mixtures of molecules ranging from intact mRNA to mononucleotides. Initiation of protein synthesis on capped mRNA in eukayotic systems is known to be heavily favored on the cap containing fragment only, even after extensive mRNA cleavage (90,91,92). Translation of messenger RNA fragments generated in these experiments that contain intact initiator sequences should then give rise to nascent peptide accumulations that reflect the length of the translating fragment. These nascent peptides resulting from protein synthesis on globin mRNA fragments were observed in the presence of pactamycin. The observation that initiation does take place on fragmented RNA and leads to the development of nascent peptides of similar lengths but in different proportions than observed for the accumulations of nascent peptides

using intact mRNA supports the concept of initiation on mRNA fragments at the proper initiation site.

The slow turnover of a nascent peptide fraction when using fragmented mRNA is an interesting result. The fate of a translating ribosome reaching the end of a mRNA without a terminator codon is unknown. From the data presented here, temptation leads one to suggest that a form of termination does occur, but at a rate lower than the normal termination process at the correct terminator codon.

The additional interpretation of data obtained required a precise knowledge of the nascent peptide distribution arising from the translation of pure α- and β-globin mRNA. The globin nascent peptide distribution analyzed thus far had been a composite profile of both α- and β-globin nascent peptides superimposed upon one another. During the course of these studies, the nucleotide sequences of both α- and β-globin mRNA have been elucidated by various laboratories (52,53). In addition, several models predicting the secondary structure for these molecules have become available (53,58). Elucidation of α and β nascent peptide distributions by translation of purified α and β globin mRNA was undertaken so that the unequivocal α and β nascent peptide size distributions could be determined. These profiles would represent the true α- and β-globin nascent peptide distributions, free of any possible interactions which may coordinate the synthesis of α- and β-globin.

Alpha and β-globin messenger mRNAs were separated in a single step electrophoetic separation in a polyacrylamide slab. The separated RNA species were recovered by electrophoretic elution and the α- and β-globin nascent peptide distributions determined. Alpha and β-globin nascent peptide size distributions were shown here to be distinct and

individually recognizable patterns when separated from one another. While the β pattern is characterized by large accumulations at K_d 0.285, 0.375 and 0.47, the α pattern shows major accumulations at K_d 0.22, 0.28, 0.35, 0.50, 0.58, and 0.70.

The α pattern displays a large peak at K_d 0.22 corresponding to completed α-globyl tRNA. This large accumulation of α-globyl tRNA but not β-globyl tRNA is in agreement with the data of Protzel and Morris who reported the same observation from analysis of the labeling of nascent peptides of intact reticulocytes (75). At the time the present work was progressing, C. Vary developed a high speed liquid chromatography system to analyze the proportion of [^{3}H]-tryptophan labeled α- and β-globin tryptic peptides present in the mixed nascent peptide distribution (68). By analysis of the fractions eluting from the Bio-Gel A 0.5m column he was able to construct α- and β-globin nascent peptide distributions from the products of synthesis of α- and β-globin mRNA in the reticulocyte lysate. Although there is some difference in the magnitudes of peaks between the distributions obtained by the methods of Vary and the method used here, patterns obtained by translation of purified globin mRNAs agree with the data of Vary. The re-establishment of the globin nascent peptide size distribution following the mixing of α- and β-globin mRNA in the same proportion found in the whole reticulocyte must be considered convincing evidence that the distributions obtained by translation of the mRNAs individually represent the true α- and β-globin nascent peptide distributions.

The nascent peptide accumulations obtained from the translation of either S_1 cleaved globin mRNA in the wheat germ cell-free system or from micrococcal nuclease digested reticulocyte lysates can be

interpreted more accurately with the known α and β accumulations. The rise to prominence of selected nascent peptide accumulations following S_1 digestion of globin mRNA appears to represent structural features already present in the α- and β-globin nascent peptide size distributions. The appearance of peaks at K_d 0.35, 0.58, and 0.71 may be stable structures that persist and can be observed once peaks representing less stable structures are lost due to nuclease action on the mRNA. After the most accessible regions are cleaved, the more stable regions are then apparently degraded until a highly stable core of mRNA fragments are left, represented by resistant nascent peptides. This digestion procedure may form the foundation of a useful approach for the prediction of the relative stability of regions of secondary structure within a mRNA molecule.

The last results of this work to be noted relate to the use of the mRNA dependent reticulocyte lysate. The micrococcal nuclease inactivated lysate responds to globin mRNA at the in vivo ratio of 1.4:1 (α:β) by synthesizing α- and β-globin at a ratio of 0.7:1. This compares poorly with the ratio of 1:1 observed in intact reticulocytes, reticulocyte lysates and mRNA directed wheat germ lysates. This altered α:β ratio has also been noted by Stewart et al. (95). They speculated that a fragment of mRNA could survive nuclease treatment and effectively compete with added mRNA for initiation. The data presented in this work document the presence of fragments of endogenous mRNA capable of initiating protein synthesis in the mRNA dependent lysate. These fragments (possibly α initiator codon containing sequences) could compete for initiation of protein synthesis with exogenous mRNA added to this mRNA dependent lysate.

Others have also noted a preferential translation of mRNAs in the mRNA dependent reticulocyte lysate (95). The results presented here as well as those of others should raise questions as to the validity of using the mRNA dependent lysate for the study of the simultaneous translation of multiple mRNA species. Ratios of mRNA present in a mixture used to direct protein synthesis are not reflected in the ratio of products synthesized by this system. Indeed, the system cannot even synthesize α- and β-globin into the proper ratios following nuclease digestion in response to the mRNA endogenous to the system.

The data presented in this thesis supports a model of protein synthesis in which mRNA secondary structure modulates the rate of ribosomal movement along the mRNA. The previous data that led to that model and data from the work presented here are in agreement with one another and consistent with the proposed model stated above.

The nascent peptide size distribution observed from the translation of globin mRNA has been shown to be sensitive to perturbation through the action of specific nuclease cleavage of areas of the globin mRNA sequence. The changes observed in the nascent peptide size profile resulting from the translation of these modified mRNAs was correlated wih areas of the mRNA where nuclease cleavage was thought to occur. The results obtained from these studies continue to support the hypothesis that regions of secondary structure present in mRNA modulate the rate of the elongation step during protein biosynthesis.

The presence of fragments of mRNA capable of at least the initiation of protein synthesis in the micrococcal nuclease inactivated reticulocyte lysate has been documented in this work. The presence of these fragments in this cell-free translation system adds an unknown factor to the work

of those investigators studying the competition among mixtures of mRNA species for initiation and translation in the inactivated reticulocyte lysate. The data in this work recommends to these people that wheat germ cell-free systems may be better suited to these types of studies.

The study of the termination of protein synthesis on fragmented mRNA poses questions that have yet to be answered. The results from work in this thesis suggest that ribosomes reaching the end of a mRNA without encountering a terminator codon will terminate protein synthesis by some mechanism yet to be elucidated but at a rate less than seen for the normal termination sequence. The significance of these findings must wait until a better understanding of these events can be established.

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